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Genetic Mutation Assessment of NPM1 Gene and Gene Expression of MDR1 in Iraqi Patients with Acute Myeloid Leukemia

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Abstract: Since its discovery, mutations in *NPM1* have been frequently associated with a large number of Acute Myeloid Leukemia (AML) patients. The vast majority of genetic changes were previously detected in exon 12, however little information report mutations in Nuclear Export Signal region (NES) of *NPM1*. Sequencing analysis included exon 2 and 3 for 75 Iraqi AML patients showed three SNPs, G/A792, G/A794 and G/A797 were detected within intron region of 90% AML and 70% healthy subjects. Other SNPs were only detected in AML subjects in which single nucleotide variant was identified in exon 3 of 70% AML subjects (A/G1275 rs753788683) in addition, two SNPs (G/A635 and G/A660) within intron region of 80% of AML subjects were detected. No genetic variation observed inexon 2 of amplified *NPM1* gene. The correlation between *MDR1* gene over expression and resistance to chemotherapy treatment showed no significant differences in gene expression between newly diagnosed and first-course induction subjects. Nevertheless, significant decreases in CT value were recorded for both the second induction AML and AML consolidation patients with *p* value of 0.0258 and 0.0007, respectively, as compared with healthy controls, indicating the induction of higher expression of *MDR1* gene by increasing the challenge of AML patients with chemotherapy regimen.

Keywords: AML, *NPM1*, Multidrug Resistance, Sequencing

Introduction

Acute Myeloid Leukemia (AML) is a type of heterogeneous disorder with a diversity of genetic abnormalities and cytogenetic alterations that provide a significant disease prognosis and determining therapy responsiveness (Liu *et al*., 2007). AML resulted from a series of frequent hematopoietic stem cell genetic aberration accumulated with age (Ding *et al*., 2012). Childhood AML generally occurs *de novo* (Hamed *et al*., 2011).

Nucleophosmin 1 (*NPM1*) mutations are most frequently identified (25-30%) in AML patients, with female predominance. These mutations lead to the abnormal expression of *NPM1*, releasing the protein in the cytoplasm rather than the nucleus and stimulating myeloid proliferation and leukemia development (Falini *et al*., 2007). *NPM1* gene consists of 12 exons and located at chromosome 5q35. The NP Mmolecule contains distinct domains responsible for its multiple biochemical functions (Hingorani *et al*., 2000). The traffic of NPM between the nucleus and cytoplasm is highly regulated and mostly the regulation comes through the Nuclear Localization Signal (NLS) and Nuclear Export Signal (NES) motifs. NLS directs NPM from the cytoplasm to the nucleoplasm, which then translocates to the nucleolus through its nucleolar binding domain, mainly tryptophan residues 288 and 290 (Yu *et al*., 2006). NPM remains in nucleoli, though it contains highly conserved hydrophobic leucine-rich NES was encoded by exon 2 and 3 motifs within

residues 94-102 and 42-49, which drive it out of the nucleus (Wang *et al*., 2005; Yu *et al*., 2006).

Most studied *NPM1* mutations were restricted to exon 12. All variants cause alterations at the C-terminus of the NPM leukemic mutants, within exon 12 region and this alteration is responsible for NPM dislocation into the cytoplasm, due to changes of tryptophan (s) 288 and 290 and creation of an additional NES. Thisprotein tends to be stable and accumulated in the cytoplasm (Suzuki *et al*., 2005). Limited studies were conducted in detecting mutations in *NPM1* gene within exon 2 and/or exon 3 of AML patients. Both exons encoded for the N-terminal portion of *NPM1* protein which plays a major role in protein oligomerization and chaperone activities (Herrera *et al*., 1996) also this region encode the Nuclear Export Signals (NES) that prove protein accumulation in the cytoplasm (Kazem *et al*., 2011).

Within Iraqi population, leukemia considered one of the most common types of cancer after breast cancer and lung cancer. The annual incidence of leukemia in Iraq is about 728 per 10,000 and it affects males 57% more than females 43%. As far as the prevalence is concerned, leukemia is the third most prevalent form of cancer after breast cancer in Iraqi population with annual significant increases each year (ICB, 2014).

On the other hand, treatment of leukemia considered as a challenge due to the development of resistance to chemotherapeutic agents. Assessing the drug resistance of leukemic cells is, therefore, an important aspect of treatment. One of the main mechanisms of resistance is rapid drug efflux mediated by various members of the ATP-binding cassette transporter super family, such as multidrug resistance gene 1 (*MDR1*), which encodes P-glycoprotein (Schaich *et al*., 2005). Therefore, the current study was aimed to detect possible mutation(s) within exon 2 and 3 in *NPM1* gene and monitoring the expression of *MDR1* gene by comparing the expression before and after the exposure to chemotherapy.

Materials and Methods

Patients and Samples

The study consisted of 75 Iraqi AML patients (45 male and 30 female), all patients were above 18 years, with median age of 33. Peripheral Blood samples (3-5 mL) were collected from the patients during the period starting from December 2015 to March 2016, which were admitted to Baghdad Teaching Hospital (Baghdad-Iraq). All patients were diagnosed with AML based on updated French-American-British (FAB) classification (Vardiman *et al*., 2009).

Most patients were treated with chemotherapy. The induction chemotherapy regimens were, combined cytarabine plus adriamycin or combined vincristine plus doxorubicin or daunorubicin and All-Trans Retinoic Acid (ATRA) plus induction chemotherapy, depended on the subtype of AML. Accordingly, patients were categorized into four groups, newly diagnosed (No treatment started) 15 patients; 20 AML patients after first-course induction of chemotherapy; 20 AML patients after second induction and 20 AML patients in consolidation. In addition, another 25 blood samples were collected from healthy volunteers and considered as controls. The current study was approved by the Board of Baghdad Teaching Hospital and the College of Biotechnology at Al-Nahrain University.

Screening for NPM1 Gene Mutations

DNA was extracted from both patients and health volunteers using SYNC™ DNA Extraction Kit (Genead, South Korea). Primers used for *NPM1* gene amplification were: *NPMX1*F *5′*-TGTGAACTAAAGGCCGACAA-*3′*, *NPMX1*R *5′*-CCACAGGGCTAGGTTCTGAG-*3′*, *NPMX2*F*5′*-AGCCCTTGTAAAAGGCATCG-*3′*, *NPMX2*R *5′*-CCCAAGGGAAACCCTAGAAGG-*3′*.

Gene mutations flanking the region start from 2380 to 4140 pb of *NPM1* gene (Fig. 1), which represent exons 2 and 3, were analyzed by amplifying the indicated region of the gene and analyzed on agarose gel (2%) in presence of 100 bp DNA ladder marker, followed by direct sequencing technique (Macrogen, South Korea). Sequencing products were compared with the information in gene bank of the National Center for Biotechnology Information (NCBI) for standard *NPM1* gene, using Mega software, version 6.

MDR1 Expression in AML Patients

The expression of *MDR1* transcripts was estimated by Real Time-qPCR. Primers were designed and supplied by BioNeer (South Korea) in a lyophilized form in the following sequence: *MDR1*F*5′*-GGAGGCCAACATACATGCCT-*3′*, *MDR1*R*5′*-CAG GGCTTCTTGGACAACCT-*3′*. Lyophilized primers were dissolved in a free DNase/RNase water to give a final concentration of 100 pmol μL^{-1}; it was stored at -20°C until use. In addition a working solution of 50 pmol μL^{-1} of oligo (dT) primer was prepared using deionized distilled water. The real-time PCR experiment was designed in singleplex assay, the chemistry used in real-time qPCR was DNA-binding dyes SYBR Green1.

RNA extraction was carried out by using Total RNA Mini Kit (Blood/Culture Cell) Protocol System (Geneaid, South Korea). The purity of RNA was measured at 260/280 nm absorbance using Nanodrop. While Reverse transcription of total RNA to cDNA was achieved using RT-PCR PreMix kit (Bioneer, South Korea). Real-Time qPCR was carried out according to the designed amplification program.

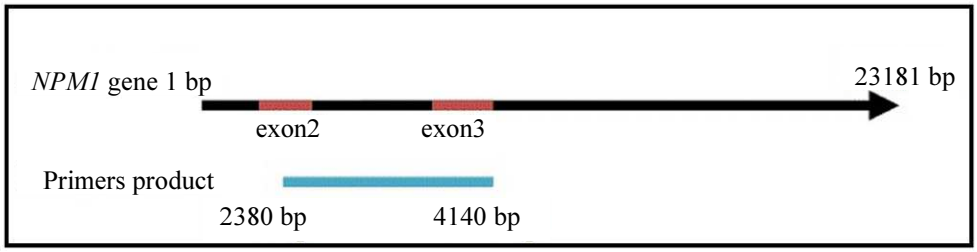

Fig. 1: The product of *NPM1* gene primers

Data and Statistical Analysis

Relative expression of *MDR1* gene in AML patient and healthy control was calculated using the equation: Ratio = 2-(ΔΔ CT) (Livak, 2001). Statistical analysis was carried out using Graph Pad Prism (version 6). One way analysis of variance ANOVA (Duncan) and Chi-square were performed to test whether group variance were significant or not. Data were expressed as mean ± standard error. *P*-values were considered to be statistically significant if $p \leq 0.01$.

Results

NPM1 Gene Amplification

The region of *NPM1* was amplified from the extracted DNA of both AML patients and healthy controls. Results illustrated in Fig. 2 shows the amplified products appeared as clear bands after electrophoresis on agarose gel (2%). The product of NPMX1 primer (Fig. 2A) was a single clear band with molecular size of 1150 bp, this product represents about 2380 to 3447 bp of amplified region and cover exon 2 from *NPM1* gene, while the product of NPMX2 primer (Fig. 2B) was also a single band with molecular size of 1337bp, which represent 2707 to 4140bp of amplified region that covered exon 3.

NPM1 Gene Sequencing and Mutations

The complete nucleotide sequence of PCR products (2380 to 4140bp) was analyzed to determine any genetic changes in this region. Results of sequencing that illustrated in Table 1 shows the genetic variations and frequencies of mutations associated with the studied AML cases.

Three different Single Nucleotide Polymorphism (SNP) G/A792, G/A794 and G/A797 were detected in the intron region. G/A792 was found in 68 of 75 AML patients (90.6%), G/A794 was found in 60 of 75 AML patients (80%), while G/A797 was found in all AML patients. All of the three were detected in 17 of 25 healthy controls (68%). These SNPs were not before correlated with mutant *NPM1* Iraqi AML patients and are first described. All statistical analysis that compares between AML cases and healthy controls were summarized in Table 2.

Single nucleotide variant was identified in exon 3 that encode NES, it was found in 53 of 75 AML patients (70.6%). This variant was not detected in healthy subjects. Based on statistical analysis, this *NPM1* mutation was significantly associated with AML ($p = 0.0022$). In addition, single nucleotide variant was observed in two sites G/A635 and G/A660 within intron region that were identified in 80% of AML patients. These SNPs were significantly correlated with mutant *NPM1* ($p = 0.0153$). No genetic variation was observed in exon 2 during sequences analysis of amplified *NPM1* gene.

Amino acids sequence encoded by exon 3 of *NPM1* gene in AML patients were examined and compared with the reference sequence recorded in NCBI. Results (Table 3) shows the alignment between patients and reference sequence ID: NP-002511.1, Length: 294, Range 1: 47 to 87 nucleophosmin isoform 1 *Homo sapiens*.

Query	1189	VSLGAGAKDELHIVEAEAMN	1311
		YEGSPIKVTLATLKMSVQPTV	
		VSLGAGAKDELHIVEAEAMN	
		YEGSPIKVTLATLKMSVQPTV	
Sbjct	47	VSLGAGAKDELHIVEAEAMN	87
		YEGSPIKVTLATLKMSVQPTV	

The position of amino acid (threonine, T) related to the expected rs753788683 SNP occurred in exon 3 at position 75 within the reference sequence. The results showed no changes in the sequence of amino acids (100% similarity) between AML sequence and the reference sequence. The codon that encodes the threonine at position 75 was ACA. The SNP was a base substitution mutation that changed A to G 1275 (rs753788683) causes transversion in the codon from ACA to ACG, however, this altered codon encoded the same amino acid threonine, so the resulted mutation was silent and not affected the amino acid sequence.

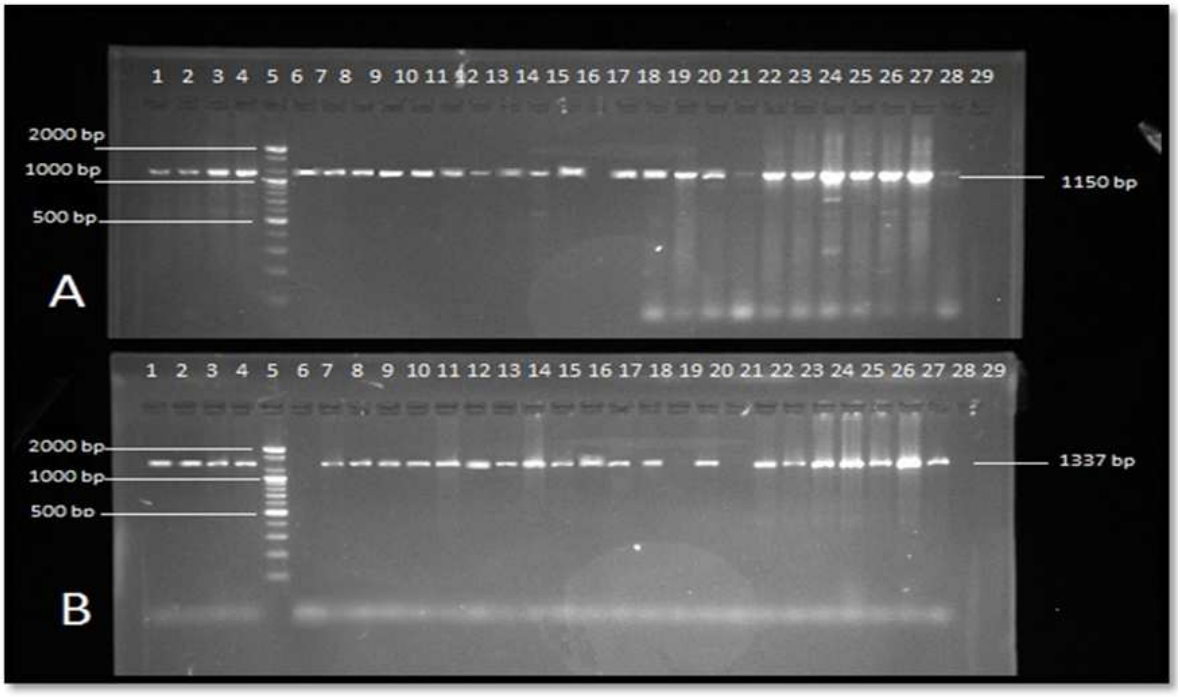

Fig. 2: PCR products of *NPM1* on agarose gel (2%) ran for 1 h at 70 V in the presence of 100 bp DNA Ladder marker. (A): PCR products of NPMX1 primer (B): PCR products of NPMX2 primer. Lane (1-4) Healthy Control. Lane (5) DNA Ladder Marker. Lane (6-29) Patient Bands

Table 1: Genetic variation in *NPM1* gene

Type	Substitution	Variation	Position	Healthy (25)	AML (75)
SNP	G/A	Intron	792	17	68
SNP	G/A	Intron	794	17	60
SNP	G/A	Intron	797	17	75
SNP	A/G	Exon 3	1275	0	53
SNP	G/A	Intron	660	0	60
SNP	G/A	Intron	635	0	60

Table 2: Statistical analysis of NPM1 mutations

Variant	*P* value	Sig.	OR	CI
G/A792	0.006	**	4.571	1.014-20.6
G/A794	0.216	NS	1.882	0.683-5.185
G/A797	<0.0001	****	73.34	4.036-1333
A/G1275	0.0022	**	0.0466	0.001-1.955
G/A660	0.0153	*	0.076	0.0017-3.26
G/A635	0.0153	*	0.076	0.0017-3.26
Sig: Significance.	OR: Odd Ratio.	CI: Confidence of Interval (95%)		

Table 3: Alignment of *NPM1* exon 3 amino acids sequence between AML sample and reference sequence

Score	Expect	Method	Identities	Positives	Gaps	Frame
84.3 bits(207)	3e-17	Compositional matrix adjust.	41/41(100%)	41/41(100%)	0/41(0%)	+1

MDR1 Gene Expression Assessment in AML Patients

The expression of *MDR1* gene in AML patients was achieved by RT-qPCR. The patients were categorized depending on their chemotherapy induction, as newly diagnosed (No treatment started), first-course induction of chemotherapy, second induction and AML patients in consolidation. The healthy control (untreated) used as calibrator.

The result of amplification reaction was summarized in the Fig. 3. The amplification reaction increase dependent on mRNA copy of *MDR1* gene in each group that treated with chemotherapy. Also, real-time PCR

products showed only a single melting temperature peak was observed for each reaction Fig. 4, thus suggesting nonspecific amplification occurred, like primer dimmer, DNA contamination and other non-specific bindings.

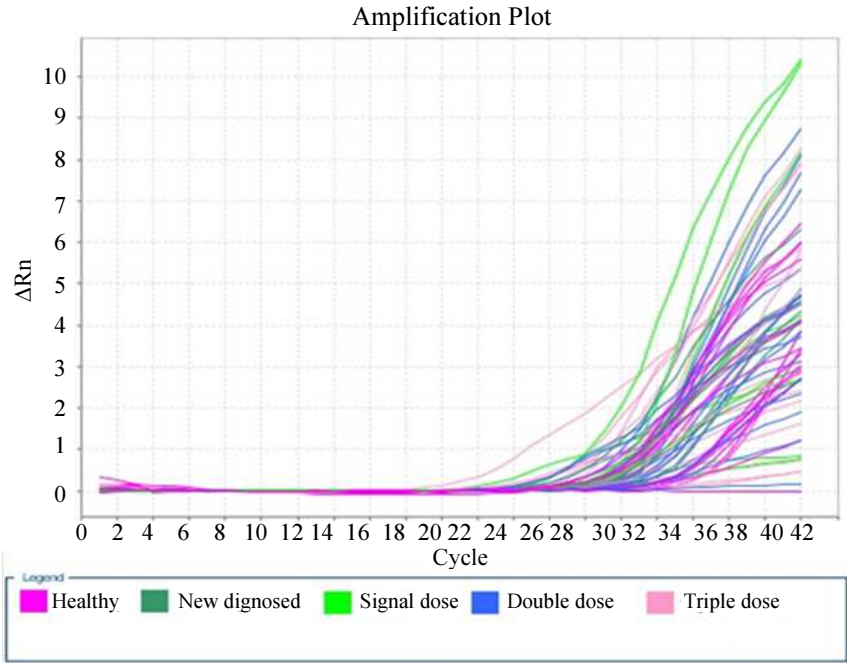

Fig. 3: Real-Time qPCR amplification plot of *MDR1* gene

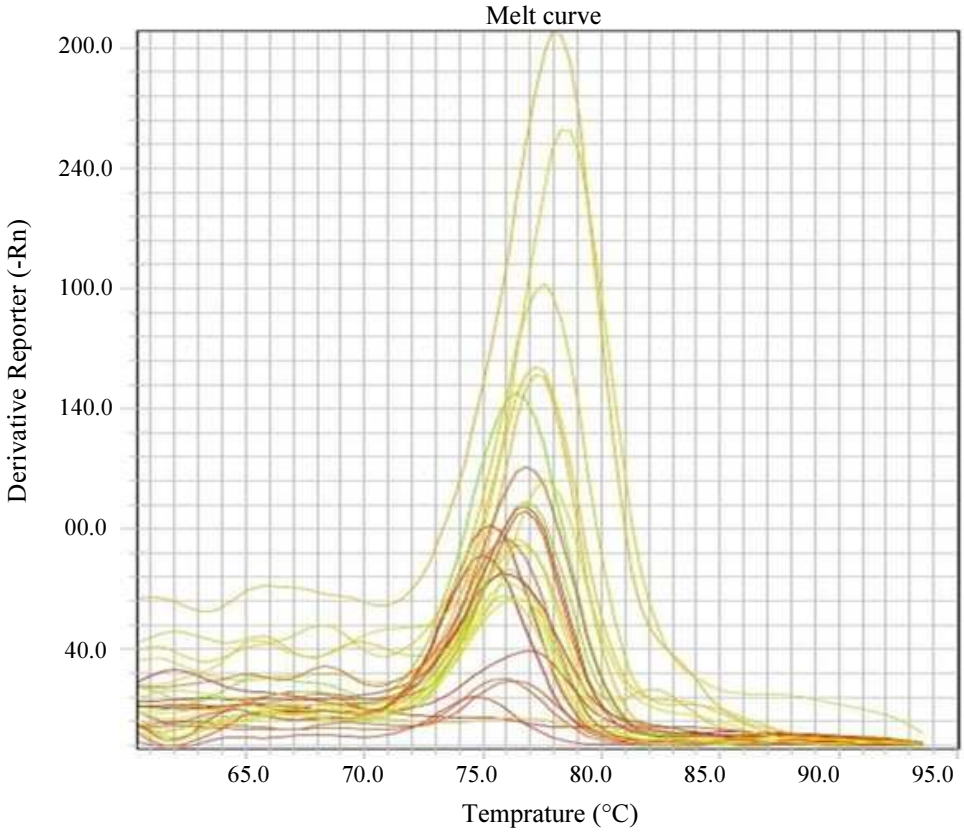

Fig. 4: Real-Time qPCRmelting curve of tested samples

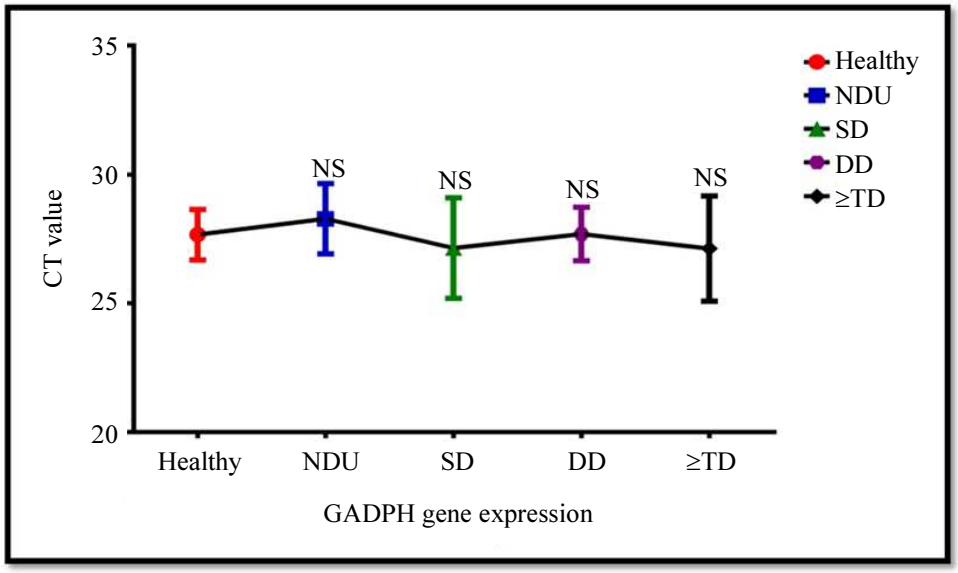

Fig. 5: GADPH endogenous control gene expression in healthy controls and AML patients. New Diagnoses Untreated (NDU), Single Dose (SD), Double Dose (DD), Triple Dose (TD), Non-Significant (NS)

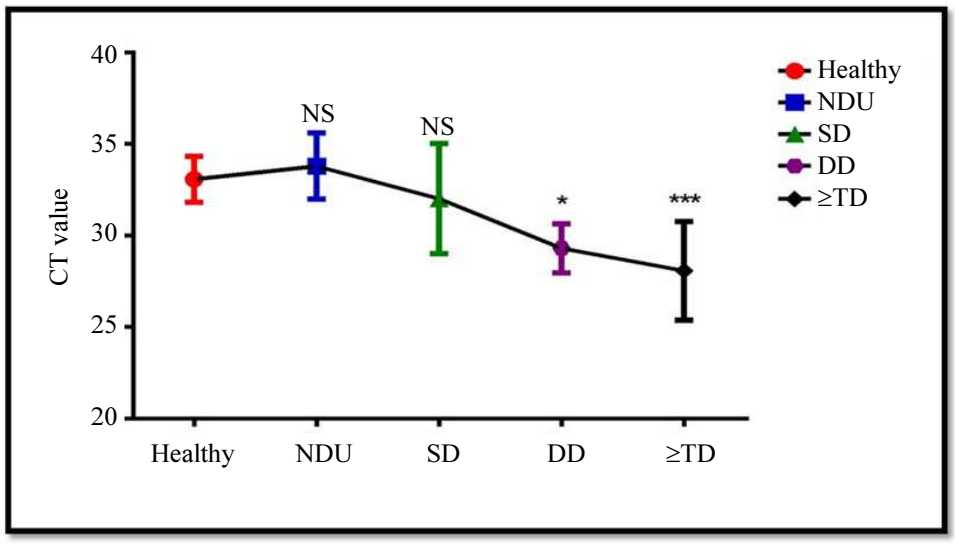

Fig. 6: The expression of *MDR1* gene in AML patients and healthy controls. New Diagnoses Untreated (NDU), Single Dose (SD), Double Dose (DD), Triple Dose (TD), Non-Significant (NS)

The expression *MDR1* gene was compared between the different groups (Fig. 5 and 6). The Y-axis represents the mean CT value of expression while X-axis represents the five group of analysis. Result (Fig. 5) showed that the expression of the standard GADPH gene had no significant differences between healthy control and the four groups of AML patients. No significant differences in *MDR1* expression between the newly diagnosed AML group and first course AML patients as compared with controls (Fig. 6). Nevertheless significant decreases in CT value were recorded for both the second induction AML group and AML consolidation group with p value of 0.0258 and 0.0007, respectively, as compared with healthy controls, indicating the induction of higher expression of *MDR1* gene by increasing the challenge of AML patients with chemotherapy regimen.

Further assessment of different *MDR1* gene expression in AML patients was done by calculating the relative ratio for testing patient groups as compared with healthy controls (Fig. 7). Results showed no significant coloration between healthy and newly diagnosed since the relative expression ratio was -0.34 fold (p = 0.1591), therefore both groups have similar *MDR1* gene expression.

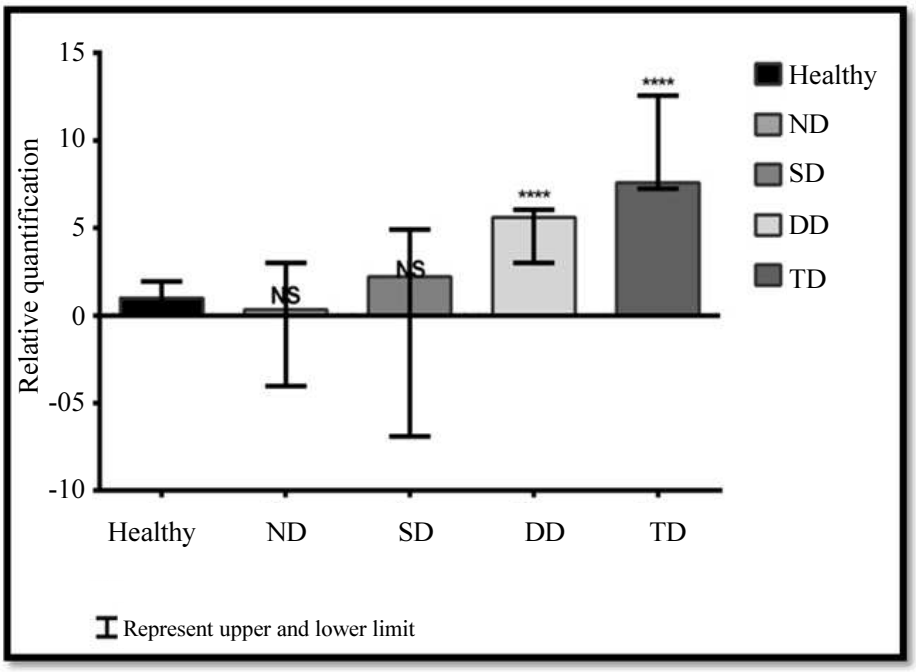

Fig. 7: Relative quantification of different AML groups compared with healthy controls (calibrator). New Diagnosed (ND), Single Dose (SD), Double Dose (DD), Triple Dose (TD), Non-Significant (NS)

This suggested no induction in gene expression. Also no significant differences between healthy calibrator and single chemotherapy AML induction group with relative expression ratio of 2.22 fold, *p* value 0.2222. Up-regulation of *MDR1* gene was clearly observed in AML patients with second induction by 5.6 fold increase with significant differences as compared with calibrator ($p<0.0001$), in addition *MDR1* high gene expression, was also observed in AML consolidation group with a relative ratio of 7.58 fold increase and significant differences comparing with control group ($p<0.0001$).

Discussion

The exon 3 and exon 2 encode the N-terminal region of NPM1 protein. In this study a mutation in exon 3 (rs753788683) was recorded, even though this mutation was not affected on amino acid sequences. Mutations in intron region not affecting on amino acids sequences but may affect gene expression. The N-terminal portion of NPM1 protein was important for oligomerization and chaperon activities (Herrera *et al*., 1996) and any mutation in this region cause defect in portion function. *NPM1* mutations are restricted to exon 12 of AML patients (Kazem *et al*., 2011) and all mutations tend to defect NLS signal and modify it into new NES signal in exon 12, the NES signal was important in mutant NPM1 protein to accumulated the protein in the cytoplasm (Chou *et al*., 2006). More than 40 molecular variants of *NPM1* mutations have been described in AML patients (Tan *et al*., 2008). The most common mutation (so-called mutation A12) duplicates a TCTG tetra nucleotide (Szankasi *et al*., 2008). Mutations B and D are observed in about 10 and 5% of cases, respectively; other mutations are very rare (Falini *et al*., 2007). In addition the N-terminal region contains high conserved hydrophobic leucine-rich NES motifs (Wang *et al*., 2005; Falini *et al*., 2009), therefore it is very difficult that mutations to occur in this region. The region that encodes for NES was important for protein function and any genetic variation in NES signal may attribute in increasing or decreasing protein function.

The goal of chemotherapy treatment is to achieve a complete remission of AML and then to prevent relapse in post-remission therapy. In the last few years, there have developing changes 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 (Almeida and Ramos, 2016; Webber *et al*., 2008).

Longley and Johnston (2005), stated that such findings correlate with the concept of AML being an intrinsically resistant disease and that such up-regulation could be acquired during induction treatment. This support our study that higher expression of *MDR1* significantly observed in group two 5.6 fold and group three 7.58 fold and in another hand the non-difference in

MDR1 gene expression between the calibrator and newly diagnosed ratio -0.34 fold. Up-regulation of MDR1 after induction has been reported by others. 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 (Baran *et al*., 2007).

The existence of Cancer Stem Cells (CSCs) has been proposed as a potential mechanism ofdrug resistance and a potential therapeutic target (Gupta *et al*., 2009). CSCs are a putative population of malignant cells within a tumor that possess the characteristics associated with normal stem cells. In this theory's most elaborate conception, CSCs can self-renew and divide by asymmetric division, reconstituting the heterogeneity of the original tumor. CSCs are also considered to be highly resistant to chemotherapeutic agents (Honoki, 2010). In this model, chemotherapy kills most of the cancer cells that are some what more differentiated and do not have long-term self-renewal capacity. When therapy is discontinued, the CSCs repopulate cancer, there by mediating disease relapse. In contrast, molecular remission after consolidation has important prognostic value. This implies that despite CR, in these patients a number of cancer cells survive treatment and can grow out to cause a relapse. 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.

In addition to the involvement of the ATP-Binding Cassette (ABC) family in the efflux and reduction of intracellular drug concentration in malignant conditions, it has recently been shown that these proteins have a role in transportation of prostaglandins, leukotrienes, Sphingosine-1-Phosphate (S1P) and Platelet-Activating Factor (PAF) out of the cells. These lipids cause activation of different signaling pathways, which results in cell proliferation, migration, survival and activation of angiogenesis, metastases and the escape of leukemic cells from immune surveillance (Fletcher *et al*., 2010). Various ABC transporters are specific to lipids. MDR1 is involved in the efflux of PAF; a PAF molecule activates its G-protein-coupled receptor, PAER and induces up regulation of the anti-apoptotic proteins Bcl-2 and Bcl-XL. These molecules prevent programmed cell death and cause expansion of resistant cells (Raggers *et al*., 2001). Therefore MDR1 protein has another function that important in AML proliferation. When the AML patients take chemotherapy the expression of *MDR1* gene will increase, so the function of *MDR1* gene will increase cell proliferation of leukemia cells. This suggested that when patient taken chemotherapy then relapsed occurred after consolidation, the chemotherapy will be ineffective in our patient and also increase the severity of AML disease.

Conclusion

In conclusion, reported mutations detected within *NPM1* of exon 3 showed significant correlation with AML as compared with healthy controls, however such mutations did not affect NES amino acid sequence. No genetic variations were detected in exon 2 of AML patient. In addition, MDR1 gene expression was significantly correlated with chemotherapy induction in AML patients.

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Author's Contributions

Ahmed Abduljabbar Suleiman, Tamadher Abbas Rafaa and Hasan Abdulwahab Jwad: Participated in all experiments designed the research plan and organized the study.

Ali Zaid Al-Saffar: Coordinated the data-analysis and contributed to the writing of the manuscript.

Conflict of Interest

The contribution of the authors was equal 100% and the authors declare that there is no competing interest. The project was completely funded by Al-Nahrain University-College of Science.

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