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Assessment of Exon 2 and 3 Mutations in *NPM1* Gene and Studying the Gene Expression of *MDR1*Gene in some Acute Myeloid Leukemia Patients in Iraq

A Thesis

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Summary

Mutations in *NPM1* gene ranked within the most frequent known genetic marker in AML. In order to detect the frequency of *NPM1* mutations within the region of exon 2 and exon 3 in Iraqi AML patients using conventional polymerized chain reaction technique, the region from 2380 bp to 4140 bp of *NPM1* gene, which represents the region flanking both exon 2 and 3, was amplified by using two specific primers in a thermal cycler. Results showed that the concentration of genomic DNA after extraction was ranged between (52-93) µg/ml with purity of (1.8-1.92). On the other hand results of amplification showed that an amplified product of NPMX1 primer was 1150 bp while the second primer NPMX2 was 1337 bp, both were represented the complete fragment of *NPM1* gene within exon 2 and 3. To investigate the possible mutations in *NPM1* gene, the nucleotide sequence of the amplified product was determined. Results showed that three single nucleotide polymorphism SNP G/A792, G/A794 and G/A797 were detected within intron region in 90% of patients and 70% of healthy volunteers. During the analysis of *NPM1* gene sequencing, single nucleotide variant was identify in exon 3 only in 70% of AML patients A/G 1275 (rs 753788683). In addition SNP was observed in two sites G/A635 and G/A660 within intron region of 80% patients but not in healthy controls. No genetic variation observed in exon 2 during sequences analysis of amplified *NPM1* gene.

The correlation between *MDR1* gene over expression and resistance to chemotherapy treatment in acute leukemia was investigated. Blood samples were collected from 40 AML patients and 10 healthy volunteers. The RNA was extracted from all samples and used as template for c DNA synthesis. The result showed that the concentration was ranged from (2.02-2.13) ng/ul and the purity from (1.90-1.95), the extracted RNA was reverse transcripted into cDNA. The 40

AML patients were categorized dependent on their chemotherapy induction, newly diagnosed (No treatment started), first course chemotherapy induction, second chemotherapy induction and patients in consolidation. Their results showed that no significant differences in gene expression occurred for the newly diagnosed patients and first course induction patients as compared with controls. Nevertheless significant decreases in CT value were recorded for both the second induction AML group and AML consolidation group with *p* value of 0.0001and 0.0001, respectively, as compared with healthy controls, indicating the induction for higher expression of *MDR1* gene by increasing the challenge of AML patients with chemotherapy regime.

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Abbre.	Mean
ALL	Acute lymphocytic leukemia
AML	Acute myeloid (or myelogenous) leukemia
BM	Bone marrow
bp	Base pair
RUNX1	Runt-related transcription factor 1
CLL	Chronic lymphocytic leukemia
CML	Chronic myeloid (or myelogenous) leukemia
D.W.	Distell water
DNA	Deoxyribonucliec acid
CBFB	Core-binding factor subunit beta
EDTA	Ethylene diamine tetra acetic acid

EtBr	Ethidium bromide
MYH11	Myosin-11
HSCs	Hematopoietic stem cells
LSCs	leukemic stem cells
PML	promyelocytic leukemia
RARA	retinoic acid receptor alpha
MLL	KMT2A lysine methyltransferase 2A
NCBI	National Center for Biotechnology Information
NPM1	nucleophosmin 1
PCR	Polymerase chain reaction
Ph	Philadelphia
CEBPA	CCAAT/enhancer binding protein alpha
SNP	Single nucleotide polymorphism
TBE	Tris-Borate-EDTA
c-KIT	KIT proto-oncogene receptor tyrosine kinase
RT-qPCR	Real Time Quantification Polymerase Chain Reaction
MDR1	Multi-Drug Resistance Gene
GADPH	Glyceraldehydes 3 phosphate dehydrogenase
RT-PCR	Reverse transcription polymerase chain reaction

Chapter One

Introduction and Literature Review

1.1. Introduction

Acute myeloid leukemia (AML) is a cancer of the myeloid line of blood cells, characterized by the rapid growth of abnormal white blood cells that build up in the bone marrow and interfere with the production of normal blood cells. AML is the most common acute leukemia affecting adults, and its incidence increases with age (Ding *et al*., 2012).. AML is clinically, cytogenetically and molecularlya heterogeneous disease (Zou, 2007). High risk AML constitutes a biologically distinct subset of diseases Comprised sizeable percentage with adults (Estey,2012). Unfortunately, Resent study in Iraq indicate that genetic alteration with leukemia caused by polluted environment (AlFaisal et al., 2014).

In the population of Iraq, 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 (Ministry of Health, Iraqi cancer Board, 2014).

Nucleophosmin 1 (*NPM1*) mutations are the most frequent mutation (25% - 30%) in AML patients, with female predominance.*NPM1* mutations result in the aberrant expression of the NPM1 protein in the cytoplasm rather than the nucleus, stimulating myeloid proliferation and leukemia development (Falini *et al*., 2007). The *NPM1* gene contains 12 exons in human and located at chromosome 5q35. The NPM molecule contains distinct domains that account for its multiple biochemical functions. Regulation of NPM traffic between the nucleus and cytoplasm mostly occurs through the nuclear localization signal (NLS) and nuclear export signal (NES) motifs. The NLS signal drives NPM from the cytoplasm to the nucleoplasm, where it is translocated to the nucleolus through its nucleolar binding

domain, particularly tryptophan residues 288 and 290 (Hingorani *et al*., 2000). NPM remains in nucleoli, even though it contains highly conserved hydrophobic leucine-rich NES(nuclear export signal) was encode by exon 2 and exon 3 motifs within residues 94–102 and 42–49, which drive it out of the nucleus (Wang *et al*., 2005).

Most studied *NPM1* mutations were restricted to exon 12. Whatever the type of mutation, all variants cause alterations at the C-terminus of the NPM leukemic mutants (exon 12)and this responsible for dislocation into cytoplasm, that is, changes of tryptophan(s) 288 and 290 and creation of an additional nuclear export signal (NES), and this protein tends to be stable and accumulated in the cytoplasm (Suzuki *et al*., 2005). Limited studies were conducted in detecting mutation in*NPM1* gene within exon2 and exon 3 of AML patients. Both exons encoded for The N-terminal portion of *NPM1* protein which play a major role in protein oligomerization and chaperon activities also this region encode the nuclear export signals (NES) that proves protein accumulation in cytoplasm. (Herrera *et al*., 1996)

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 (Baguley, 2010). 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)

Amis of study:

- Detection of exon 2 and 3 mutations in *NPM1* gene by analyzing of genomic DNA from samples of Iraqi patients with acute myeloid leukemia.
- Studying the gene expression of *MDR1* gene from different acute myeloid leukemia patients which were categorized depending on their treatment challenge.

Accordingly, the following steps were deopted:

- Collection of blood samples from leukemic Iraqi patients and normal individuals.
- Extraction of DNA from leukemia Iraqi patients and normal individuals.
- Amplification of exon 2 and 3 in *NPM1* gene using specific primer in normal and patients.
- Detect the possible mutation in *NPM1*gene leads to acute leukemia.
- Monitoring the expression of MDR1 gene using Real Time-PCR, after RNA extraction and making cDNA and compare the expression before and after the exposure to chemotherapy.

1.2. Literature review

1.2.1. Leukemia

Leukemia was first documented approximately 150 years ago. In the years since, several advances have been made in understanding the mechanisms of leukemia. Medical advances have developed better ways of diagnosing, treating, tracking cases, and classifying leukemia in recent years. However being able to link the development of this disease to environmental, developmental, and physical factors still remains largely a mystery (Lackritz, 2001).

Leukemia is a type of cancer involving the blood forming cells; mostly white blood cells. When a person has leukemia, the white blood cells do not fully mature and the body rapidly produces immature white blood cells. These cells are different from normal cells and are unable to perform the functions mature cells do. Eventually these leukemic cells replace normal cells in the bone marrow of an individual. Often this uncontrolled proliferation can cause the leukemic cells to spill over into the blood stream and eventually enter vital organs. This overwhelms the amount of normal/mature blood cells like red blood cells, nonmalignant white cells, and platelets. There are several types of leukemia. Each type affects a different type of white blood cell and involves a different level the white blood cells maturity (Lackritz, 2001).

1.2.2. Types of Leukemia

There are four types of leukemia: Acute myeloid leukemia (AML), Chronic myeloid leukemia (CML), Acute lymphocytic leukemia (ALL), and Chronic lymphocytic leukemia (CLL) (Esteyand Appelbaum, 2012).

- ❖ AML is type of cancer that is fast growing and found in the blood and bone marrow of an individual. AML is the most common type of

leukemia. It develops from the body's bone marrow making blast cells that do not fully mature and then these immature cells develop into white blood cells. Due to these cells being immature they are unable to defend the body against infections. The bone marrow may also produce abnormal red blood cells and platelets. These abnormal cells will outnumber the normal cells in time due to their ability to rapidly produce

- CML is similar to AML. It starts in the bone marrow, where immature cells do not develop into mature cells and eventually outnumber the mature cells. CML can start out slow but its progression can become fast like that of AML and spread to any organ in the body.
- ALL develops after abnormal white blood cells accumulate in the bone marrow. ALL's progression is rapid. Healthy lymphocytes are replaced with immature cells that are unable to function. These immature cells eventually make their way to the blood stream were they are transported to other organs and tissues; including the brain, liver, lymph nodes. Once they reach other organs and tissues the cells continue to reproduce rapidly. ALL mainly affects the B and T cells; which play an active role in protecting the body from infection.
- CLL is very similar to ALL expect it is slow in progression. Mature, healthy lymphocytes are overgrown and crowded by immature cells that are able to function properly. These immature cells are transported to other parts of the body, brain, liver, lymph nodes, via the bloodstream. Like ALL once these cell types have reach their destination they begin to reproduce, just slower.

1.2.3. Acute Myeloid Leukemia AML

It's a heterogeneous disorder characterized by clonal expansion myeloid progenitors (blasts) in the bone marrow and peripheral blood. Previously incurable, AML is now cured in approximately 35% to 40% of improving but remains grim. Recent studies have revealed that the disorder arises from a series of recurrent hematopoietic stem cell genetic alterations accumulated with age. Using deep sequencing techniques on primary and relapsed tumors, a phenomenon called clonal evolution has been characterized with both founding clones and novel sub clones, impacting the therapeutic approach (Ding *et al*., 2012).

1.2.3.1. Morphology

Morphologically, AML blasts vary in size from slightly larger than lymphocytes to the size of monocytes or larger. The nuclei are large in size, varied in shape and usually contain several nucleoli. AML blasts express antigens found also on healthy immature myeloid cells, including common differentiation (CD) markers CD13, CD33 and CD34 (Campos *et al.,* 1989). Other cells markers are expressed depending on the morphological subtype of AML and stage of differentiation block such as monocytic differentiation markers (CD4, CD14 and CD11b), erythroid (CD36 and CD71) and mega-karyocytes markers(CD41a and CD61). On occasion, AML blasts also co-express antigens restricted to T or B cell lineages including Terminal deoxy nucleotidyl Transferase (TdT), Human leukocyte antigen-antigen D related(HLA-DR), CD7 and CD19. Rarely, the blasts can exhibit morphologic and immune-phenotypic features of both myeloid and lymphoid cells that make it difficult to classify them as either myeloid or lymphoid in origin. These cases considered as mixed phenotypic leukemia and usually portend a worse overall survival (Wolach and stone, 2015).

1.2.3.2. Epidemiology of AML

In children, the most frequently occurring hematological malignancies include acute leukemia, of which 80% are classified as acute lymphoblastic leukemia (ALL) and 15%–20% as acute myeloid leukemia (AML). The incidence rate of AML in infants is 1.5 per 100,000 individuals per year, the rate decreases to 0.9 per 100,000 individuals aged 1–4 and 0.4 per 100,000 individuals aged 5–9 years, after which it gradually increases into adulthood, up to an incidence of 16.2 per 100,000 individuals aged over 65 years (Howlader *et al.*, 2012). The underlying cause of AML is unknown, and childhood AML generally occurs *de novo*. In adult and elderly patients, AML is often preceded by Myelo-dysplastic syndrome (MDS). In children, the occurrence of AML preceded by clonal evolution of preleukemicmyelo-proliferative diseases, such as MDS or Juvenile Myelomonocytic Leukemia (JMML), is rare though. Germ-line affected individuals, such as those with Fanconi anemia or Bloom syndrome, have an increased risk for developing AML as a secondary malignancy (Seif, 2011; Tonnies *et al.*, 2002). Recently, germ-line mutations in several genes, such as *TP53*, *RUNX1*, *GATA2* and *CEBPA*, have been found in families with an unexplained high risk of AML, suggesting a familial predisposition to develop AML (Hahn *et al*., 2011; Link *et al.*, 2011; Owen *et al*., 2008).

1.2.3.3. Classification

Over the years there have been several different classification systems for AML based on etiology, morphology, immune-phenotype and genetics. In the 1970s, AML was classified according to the French-American-British classification system using mainly morphology and immune phenotype / cytochemical criteria to define eight major AML subtypes (FAB M0 to M7) (Bennett *et al*., 1976). The World Health Organization (WHO) classification of

AML, replaced the old French-American-British classification system to become the essential modality for AML classification today. The WHO classification was updated in 2008 and identifies seven AML subtypes(Vardiman *et al.*, 2009):

- ❖ AML with recurrent genetic abnormalities (RUNX1-RUNX1T1 t(8;21) (q22;q22), CBFB-MYH11 Inv(16) (p13.1q22), t(16;16) (p13.1;q22), PML-RARA t(15,17)(q22;q12), MLL 11q23 abnormalities, etc.) and with gene mutations (Nucleophosmin1 NPM1 and CEBPA mutated gene).
- ❖ AML with myelodysplasia-related changes.
- ❖ Therapy related myeloid neoplasms.
- ❖ AML not otherwise specified (NOS) (similar to FAB Classification M0-M7 with others such as acute mega-karyoblastic leukemia, acute panmyelosis with myelofibrosis and pure erythro leukemia.
- ❖ Myeloid sarcoma.
- ❖ Myeloid proliferations related to Down syndrome.
- ❖ Blastic plasmacytoid dendritic cell neoplasm.

Based on etiology alone, AML can also be subdivided into three distinct categories: (1) Secondary AML (s-AML) (associated with antecedent myelodysplastic syndrome MDS or other myeloid proliferative disorder MPD); (2) Therapy-related AML (t-AML) (associated with prior toxin/chemotherapy exposure) and (3) *de novo* AML (Lindsley *et al*., 2015).

1.2.3.4. Cytogenetics

Non-random chromosomal abnormalities (e.g., deletions, translocations) are identified in approximately 52% of all adult primary AML patients and have long been recognized as the genetic events that cause and promote this disease (Byrd *et*

al., 2002). Certain cytogenetic abnormalities, including the t(8;21)(q22;q22), t(15;17) (q22;q12) and inv(16)(p13.1;q22) are associated with longer remission and survival, while alterations of chromosomes 5, 7, complex karyotype and 11q23 are associated with poor response to therapy and shorter overall survival (Döhner, 2015).In contrast, about 40%–50% of all AML cases are cytogenetically normal when assessed using conventional banding analysis. Although, this group has an intermediate risk of relapse, a substantial heterogeneity is found in this population in terms of clinical outcome. Molecular screening of this AML category is critical for prognostic categorization and treatment strategy(Gaidzik *et al*., 2008).

1.2.4. Molecular Abnormalities

During the last decade, several studies have shown that the presence or absence of specific gene mutations and/or changes in gene expression can further classify AML cases and have an effect on the patients' prognosis (Lindsley *et al*., 2015;CancerGenome Atlas Research Network, 2013).

1.2.4.1. Fms-Like Tyrosine Kinase 3 (FLT3) Mutations

First described in 1991, *FLT3* was found to be strongly expressed in hematopoietic stem cells with important roles in cell survival and proliferation (Gilliland and Griffin, 2002). *FLT3* gene located on chromosome 13q12, encodes a membrane-bound receptor tyrosine kinase (*RTK*) that belongs to the RTK subclass III family, characterized by five immunoglobulin-like extracellular domains, a single trans-membrane domain, a juxta-membrane domain (*JMD*) and an intracellular domain consisting of two protein tyrosine kinase (*PTK*) domains linked by a kinase-insert domain. Other members of the RTK subclass III include macrophage colony-stimulating factor (M-CSF) receptor, c-KIT and the receptors for platelet-derived growth factors A and B (Maroc *et al*., 1993). *FLT3* is normally

expressed by myeloid and lymphoid progenitor cells and expression is lost as hematopoietic cells differentiate. *FLT3* plays an important role in the proliferation, differentiation and survival of multi-potent stem cells and is over expressed at the RNA and protein level in AML blasts. Internal tandem duplications (ITD) in the juxta-membrane (JM) domain or mutations in the second tyrosine kinase domain(TKD) of the *FLT3* gene have been found in 20% of all AML cases and 30% to 45% of CN-AML patients (Döhner et al,. 2015; Kelly *et al.*, 2002).

1.2.4.2. C-KIT.

The *Kit* proto-oncogene, located on chromosome 4 q12, encodes a trans-membrane glycoprotein, which is a member of the type III RTK family, and whose ligand is stem cell factor (SCF). SCF binding promotes c-KIT dimerization and trans-phosphorylation that leads to activation of downstream signaling pathways involved in proliferation, differentiation, migration and survival, particularly of hematopoietic stem cells (Okuda et al,. 2000). Ligand-independent activation of c-KIT can be caused by different types of mutations that have been reported in AML and also in other human malignancies including mast cell disorders, gastrointestinal stromal tumors, and testicular germ cell tumors (Blume, 2001).

1.2.4.3. Runt-Related Transcription Factor (RUNX1) Mutations

Runt-related transcription factor (*RUNX1*) has been shown to be essential in normal hematopoiesis .*RUNX1* mutations are found in 5%–13% of AML (Marcucci *et al.*, 2011). In general, studies have shown *RUNX1* mutations are associated with resistance to standard induction therapy with inferior overall survival for both younger and older patients (Mendler *et al.*, 2012).

1.2.4.4. DNA Methyltansferase 3A (*DNMT3A*) Mutations.

Mutations in the DNA methyltansferase 3A (DNMT3A) gene occurs in 18%–22% of all AML cases and in about 34% of CN-AMl (Ley *et al*., 2010). Missense mutations affecting arginine codon 882 are more common than those affecting other codons causing a defect in normal hematopoiesis and proper methylation (Marcucci *et al*., 2012). Recently, *DNMT3A* mutations have been identified as pre-leukemic mutations, arising early in AML evolution and persisting in times of remission (Shlush *et al*., 2014).

1.2.4.5. Ten–Eleven Translocation 2 (*TET2*) Mutations

The ten–eleven translocation oncogene family member 2 (*TET2*) is found mutated in about 9%–23% of AML patients. *TET1*gene product an enzyme involved in the conversion of 5-methylcytosine to 5-hydroxymethylcytosine in DNA, which is a process thought to play an important role in DNA de-methylation (Chou *et al*,. 2011).

1.2.4.6. Tumor Protein p53 (*TP53*) Mutation

The tumor suppressor gene TP53 is found in 8%–14% of AML cases. These mutations and deletions are primarily associated in AML with complex karyotype (69%) and are rare in patient without chromosomal deletions. In general, TP53 mutations confer a very adverse prognosis with documented chemo-resistance (Haferlach *et al*., 2008).

1.2.5. Nucleophosmin *(*NPM1*)* Gene and Protein

NPM1 is a multifunctional phosphor-protein that encodes for a number of functional domains through which the molecule is able to bind many partners in distinct cellular compartments.NPM displays nucleolar localization and constantly

shuttles between the nucleus and the cytoplasm. The shuttling activity of *NPM1* along with its capacity to interact with many partners involves the protein in multiple cellular functions (Grisendi *et al*,. 2006).

These functions include ribosome biogenesis and the transport of pre-ribosomal particles, maintenance of genomic stability through the control of cellular ploidy and centrosome duplication and the control of cellular proliferation. NPM1 is also a survival factor and participates in DNA-repair processes. In addition, NPM1 is involved in regulating the activity and stability of crucial tumor suppressors such as ARF and p53 (Maiguel *et al*., 2004). The NPM1 polypeptide chain has a modular structure containing distinct sequence motifs including a nuclear-export signal (NES) and a nucleolar localization signal (NuLS) that resides in the C-terminal region of the protein. In AML patients the NuLS is the target of the C-terminal mutation that determines its substitution into an extra NES, thus resulting in an aberrantly localized protein that resides in the cytoplasm of leukemic blasts. The shuttling activity of NPM1 and its proper sub cellular localization are thought to be crucial for normal cellular homeostasis and the identification of the NPM1 mutant counterpart in AML patients has emphasizedthis hypothesis. Thus, genetic alteration of NPM1 can contribute to oncogenesis by directly affecting NPM1 functions (Falini *et al*., 2005).

1.2.5.1. Structure of *NPM1*

The *NPM1* gene, mapping to chromosome 5q35 in humans, contains 12 exons (Figure 1-1). It encodes for 3 alternatively spliced nucleophosmin isoforms: B23.1, B23.2 and B23.3(Chang and Olson, 1990).

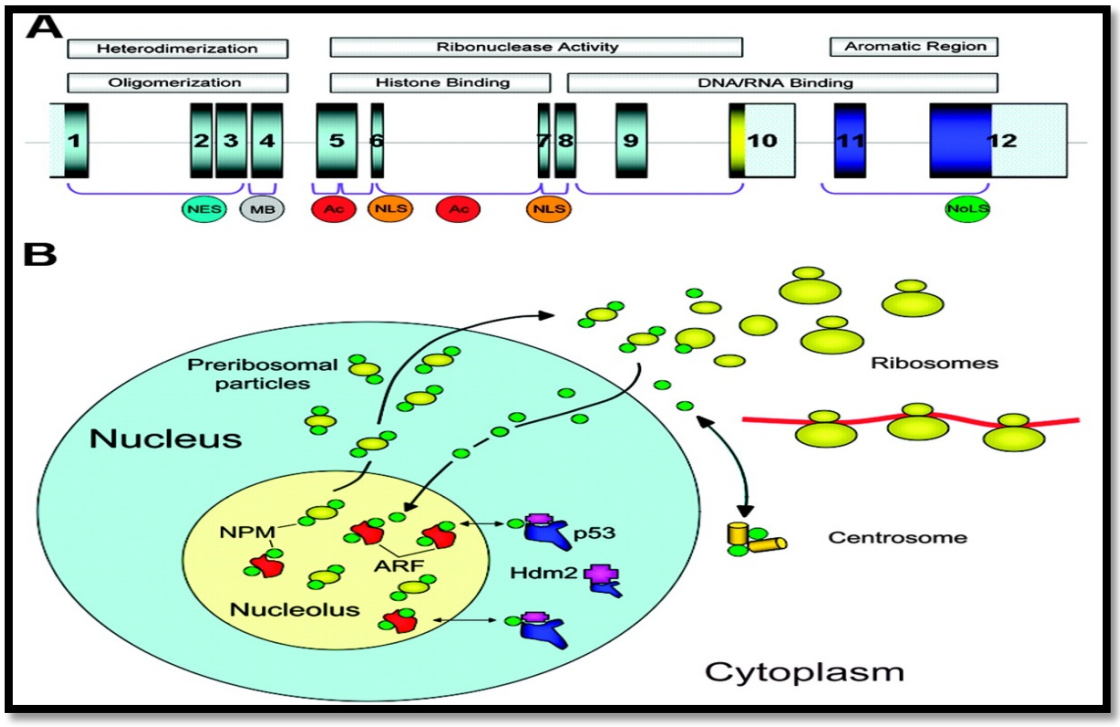

Figure (1-1): The NPM1 gene encodes for a protein involved in multiple functions. **(A)** The NPM1 gene contains 12 exons. **(B)** NPM is a nucleolar phospho protein that shuttles between the nucleus and cytoplasm. Shuttling plays a fundamental role in ribosome biogenesis, since NPM transports pre-ribosomal particles. In cytoplasm, NPM binds to the unduplicated centrosome and regulates its duplication during cell division. Furthermore, NPM interacts with p53 and its regulatory molecules (ARF, Hdm2/Mdm2) influencing the ARF-Hdm2/Mdm2-p53 onco-suppressive pathway (Chang and Olson, 1990),

B23.1, the prevalent isoform, is a 294–amino acid protein of about 37 kDa, which shares a conserved N-terminal region with the other nucleophosmin isoforms and has multiple functional domains (Wang *et al*., 1993). The N-terminus portion contains a hydrophobic region, regulating self-oligomerization and NPM chaperone activity toward proteins, nucleic acids and histones (Namboodiri *et al*., 2004; Hingorani *et al*.,2000).

In resting and proliferating cells, more than 95% of NPM is present as an oligomer; both the nonpolar N-terminus region and multimeric state of NPM appear to be crucial for proper assembly of maturing ribosomes in the

nucleolus. The middle portion of NPM contains 2 acidic stretches that are critical for histone binding; the segment between the acidic stretches exerts ribonuclease activity (Hingorani et al., 2000**)**. The C-terminal domain, with its basic regions involved in nucleic acid binding and ribonuclease activity, is followed by an aromatic short stretch encompassing tryptophan 288 and 290, which are critical for NPM binding to nucleolus (Namboodiri et al., 2004).

B23.1 is also equipped with a bipartite nuclear localization signal (NLS), leucine-rich nuclear export signal (NES) motifs, and several phosphorylation sites regulating its association with centrosomes and sub-nuclear compartments. B23.1 shows restricted nucleolar localization (Wang *et al*., 2005; Nishimura *et al*., 2002).

B23.2, a truncated isoform, lacks the last 35 C-terminal amino acids of B23.1 (containing the nucleolar-binding domain) and is found in tissues at very low levels. In immune-cytochemical analysis, B23.2 is located in nucleoplasm. Little information is available on the B23.3 isoform, which consists of 259 amino acids (Dalenc *et al*., 2002).

1.2.5.2. Expression and Functions of NPM Protein

Nucleophosmin is a highly conserved phosphoprotein that is ubiquitously expressed in tissues. It is one of the most abundant of the approximately 700 proteins identified to date in the nucleolus by proteomics. Although the bulk of NPM resides in the granular region of the nucleolus, it shuttles continuously between nucleus and cytoplasm, (Andersen *et al*., 2005; Cordell *et al*., 1999).

NPM nucleocytoplasmic traffic is strictly regulated, since important NPM functions, including transport of ribosome components to the cytoplasm and control of centrosome duplication, are closely related to its ability to actively mobilize into distinct sub cellular compartments (Wang *et al*., 2005).

The *NPM*1 gene contains 12 exons in human and located at chromosome 5q35. The NPM molecule contains distinct domains that account for its multiple biochemical functions (Hingorani *et al*., 2000). The N-terminal hydrophobic portion of *NPM1* contains the regions which are responsible for the self-oligomerization and chaperone activities of the NPM protein. The oligomerization domain of *NPM1* also contains a highly conserved motif that appears critical for mediating ADP-ribosylation factor (ARF) binding *in vivo* (Herrera *et al*., 1996;Enomoto*et al*., 2006).

Regulation of NPM traffic between the nucleus and cytoplasm mostly occurs through the nuclear localization signal(NLS) and nuclear export signal(NES) motifs as show in the Fig. (1-1). The NLS signal directs NPM molecule from the cytoplasm to the nucleoplasm, where it is translocated to the nucleolus through its nucleolar binding domain, particularly tryptophan residues 288 and 290 (Yun *et al*., 2003). NPM remains in nucleoli, even though it contains highly conserved hydrophobic leucine-rich NES(nuclear export signal)was encode by exon 2 and exon 3 motifs within residues 94–102 and 42–49, which drive it out of the nucleus (Wang *et al*., 2005;Yu*et al*., 2006).ee

NPM plays a key role in ribosome biogenesis providing the necessary export signals and chaperoning capabilities that are required to transport components of the ribosome from nucleus to cytoplasm (Yu *et al*., 2006). A major role of NPM is to mediate, through a Crm1-dependent mechanism, nuclear export of the ribosomal protein L5/5S rRNA subunit complex. Other elements that implicate NPM in the processing and/or assembly of ribosomes are its nucleocytoplasmic shuttling properties and intrinsic RNAse activity, and the ability to bind nucleic acids, to process pre-RNA molecules, and to act as a chaperone, thus preventing protein

aggregation in the nucleolus during ribosome assembly (Savkur and Olson, 1998; Wang *et al*., 1994).

NPM also maintains genomic stability, controlling DNA repair and centrosome duplication during mitosis. NPM associates with the unduplicated centrosome in resting cells and dissociates from it after CDK2-cyclinE–mediated phosphorylation on threonine, thus enabling proper chromosome duplication. NPM re-associates with centrosomes at the mitotic spindle during mitosis as a result of phosphorylation (Zhang *et al*., 2004; Okuda, 2002).

Nucleophosmin undergoes various post-translational modifications that modulate it's activity. It was first identified as a phosphor-protein, and contains numerous phosphorylation sites that are fundamental for specific functions, including the control of centrosome duplication and RNA binding. Phosphorylation of NPM by casein kinase 2 is important for nucleolar organization and integrity. Mutation of the CK2 phosphorylation site of NPM induces dissociation of protein complexes and subsequent nucleolar breakdown (Okuwaki,. 2008).

1.2.5.3. Interaction with p53

NPM regulates p53 levels and activity. A functional link exists between nucleolar integrity, NPM, and p53 stability, since stimuli inducing cellular stress (eg, UV radiation or drugs interfering with rRNA processing) lead to loss of nucleolar integrity, relocation of NPM from nucleolus to nucleoplasm, and p53 activation. Nucleoplasmic NPM increases p53 stability by binding to, and inhibiting, Hdm2/Mdm2 (a p53 E3-ubiquitin ligase), although "nucleolar stress" can also activate p53 in an Hdm2/Mdm2-independent fashion. Thus, under stress

conditions, the nucleolus functions as a “stress-sensor” with NPM playing a key role in potentiating p53-dependent cell-cycle arrest (Kurki *et al*., 2004;Maiguel *et al*., 2004).

1.2.5.4. Interaction with ARF(Cyclin Dependent Kinase Inhibitor 2A)

In cultured unstressed cells, most NPM and ARF co-localize in the nucleolus, in high-molecular-weight complexes containing other nucleolar proteins. The NPM-ARF interaction helps stabilize both proteins, since ARF influences NPM poly-ubiquitination and NPM protects ARF from degradation. The functional significance of NPM and ARF interactions is still in debate. There is evidence that ARF targets NPM into nucleoli for degradation, that the ARF-NPM complex sequesters Hdm2/Mdm2 into the nucleolus thereby activating p53 in the nucleoplasm, that ARF impedes NPM shuttling, thereby producing cell-cycle arrest in a p53-independent fashion, and that NPM sequesters ARF into the nucleolus, impairs ARF-Hdm2/Mdm2 association, and inhibits ARF's p53-dependent activation. Despite these uncertainties, NPM and ARF activities appear closely coordinated and regulate cell proliferation and apoptosis through precise sub-nuclear compartmentalization (Korgaonkar *et al*., 2005; Zhang 2004).

In response to cellular stress, NPM and ARF are redistributed to the nucleoplasm. Competition between Hdm2/Mdm2 and NPM for ARF binding promotes formation of NPM-Hdm2/Mdm2 and ARF-Hdm2/Mdm2 binary complexes and strongly activates the p53 pathway. Thus, through its nucleolar association with NPM, ARF may directly access ribosome function to inhibit cell growth, and via its nucleoplasmic interaction with Hdm2/Mdm2 and ARF-BP1, it may regulate the p53 cell-cycle pathway (Gjerset and Bandyopadhyay, 2005;Chen *et al*., 2005).

1.2.5.5. NPM1 and Cancer

NPM1 is clearly having both growth promoting and tumor suppressive functions. Over expression of NPM1 enhances cell division and cell growth presumably through stimulatory effects on rRNA transcription, ribosome subunit export and DNA replication during S-phase. Over expression of NPM1 could possibly promote oncogenesis by interfering with the activation of p53 by ARF(Feuerstein *et al*., 1988). The level of NPM1 is generally elevated in tumor cells compared with normal cells. Increased levels of NPM1 may at least in part reflect a higher growth rate and an increased demand for ribosome biogenesis in cancer cells. NPM1 is over expressed in many types of major human solid tumors including, but not limited to, tumors of the thyroid, brain, liver and prostate (Liu *et al*., 2007).

1.2.5.6. NPM Abnormalities in AML

Genetic abnormalities involving the NPM1 gene have been described in different types of hematologic malignancies (Falini *et al*., 2007). Chromosomal translocations involving NPM1 are frequent in anaplastic large-cell lymphomas expressing the anaplastic lymphoma kinase (ALK), and can be found in rare cases of AML. The t(2;5) translocation characteristic of anaplastic large-cell lymphomas involves the NPM1 and ALK genes (Falini, 2001). The t(5;17), was described in four cases of acute promyelocytic leukemia (APL) fuses NPM1 gene to the retinoic acid receptor-alpha gene. While the t(3;5) described in less than 1% of cases of AML fuses NPM1 and the myeloid leukemia factor (MLF)1 gene (Redner *et al*., 1996). These translocations result in expression of fusion protein, NPM-ALK, NPM-RAR alpha and NPM-MLF1, which are directly linked to the pathogenesis of the respective neoplasms.

1.2.5.7. The Pathogenesis of AML with Mutated NPM1

NPM is known to interact with crucial regulators of genome integrity, such as p53, HDM2 and ARF. p53 is tumor suppressor gene, frequently mutated in human cancers, that is activated in response to DNA damaged and oncogene expression, initiating a transcriptional response that ultimately leads to cell cycle arrest or apoptosis. The stability of the p53 protein is regulated by HDM2, an ubiquitin E3 ligase that favors p53 degradation, and by the nucleolar protein ARF, which antagonizes HDM2 activity. NPM sequesters ARF within the nucleolar compartment, where it acts as a stress sensor (Colombo *et al*., 2002).Upon activation by genotoxic stimuli, ARF is dislocated to the nucleoplasm where it stabilizes p53-dependent cell cycle arrest or apoptosis (Gjerset, 2006).

The nucleophosmin cytoplasmic mutant protein maintains the ability to interact with ARF and, sequesters it in the cytoplasm. The cytoplasmic delocalization of ARF leads to its partial degradation, destabilization of p53and, therefore, to impairment of its tumor-suppressor function(Colombo *et al*., 2006).

Overexpression of NPM enhances cell growth and inhibits apoptosis. Ribosome metabolism and protein synthesis are closely correlated with cell growth rate, and NPM exerts a pivotal role in regulating different steps of ribosome biogenesis, form transcription of rRNA genesto maturation of pre-rRNA ribosome export and polysome formation (Maggi et al,. 2008)

1.2.6. Multidrug Resistance Genes

Cells exposed to toxic compounds can develop resistance by a number of mechanisms including decreased uptake, increased detoxification, alteration of target proteins, or increased excretion. Several of these pathways can lead to multidrug resistance (MDR) in which the cell is resistant to several drugs in

addition to the initial compound. This is a particular limitation to cancer chemotherapy, and the MDR cell often displays other properties, such as genome instability and loss of checkpoint control, that complicate further therapy. ABC genes play an important role in MDR, and at least six genes are associated with drug transport. Three ABC genes appear to account for nearly all of the MDR tumor cells in both human and rodent cells. These are *ABCB1*/*PGP*/*MDR1*, *ABCC1*/*MRP1*, and *ABCG2*/*MXR/BCRP* (Allen *et al*., 1999).

1.2.7. ABCB1 Description (MDR1)

ATP binding cassette subfamily B member 1 (ABCB1) is one of many ubiquitous adenosine triphosphates (ATP)-binding cassette (ABC) genes present in all kingdoms of life that is responsible for cellular homeostasis (Jones and George, 2004).ABC genes encode transporter and channel proteins possessing multiple membrane spanning domains that form a pore, and intracellular nucleotide-binding domains for ATP dependent translocation of substrates or ions across the cell membrane (Borst and Elferink, 2002).Although bacterial ABC proteins function as both importers and exporters, all eukaryotic ABC proteins are efflux pumps. ABCB1 is one of 49 putative members in the super family of human ABC transporters within subfamily B (MDR/TAP), which is one of seven phylogenetically distinct sub-families with overlapping substrate specificity (Sharom,2008; Zhou, 2008).

1.2.7.1. Molecular and Protein Structure

ABCB1 was first cloned by Riordan *et al*., in 1985. The gene is less than 25kb from ABCB4 on chromosome 7q21.12. Analysis of human cell lines, liver tissue,

and lymphocytes consistently show ABCB1 to contain 29 exons in a genomic region spanning 209.6 kb (Bodor *et al*., 2005; Riordan *et al*., 1985).

Two 5′ exons are not translated. Two primary transcriptional start regions exist: a proximal promoter in exon 1 and intron 1 for constitutive expression, and a cryptic distal promoter active in drug-selected cell lines and cancer patient samples for over expression of the protein product. The ABCB1 promoter region contains a few low-frequency polymorphisms and is relatively invariant compared with other genes in the genome (Wang *et al*., 2006).

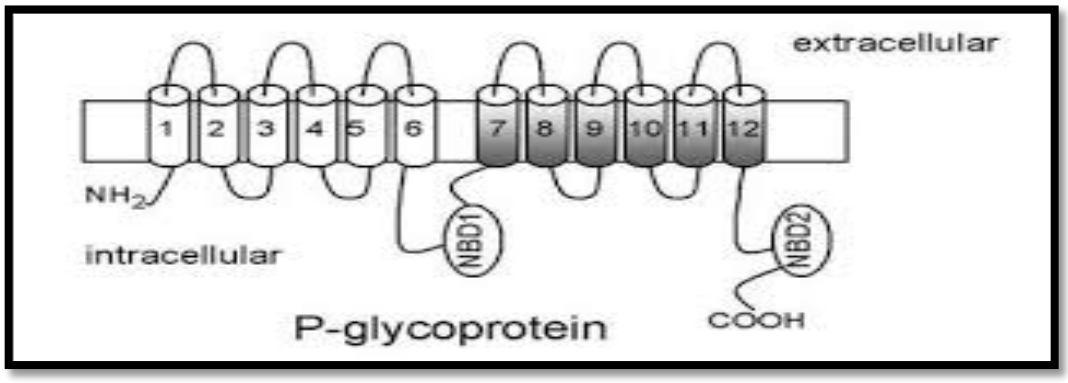

Fig. (1-2): Structure of P-glycoprotein (Bodor *et al*., 2005)

The mRNA is 4872 bp in length, including the 5′ non translated region, which gives rise to a protein that is 1280 amino acids in length, named P-glycoprotein (P-gp). The secondary structure of P-gp(Fig. 1-2) reveals two homologous halves to the protein, each containing six transmembrane domains and a nucleotide-binding domain (Bodor *et al*., 2005).

P-gp is post-translationally modified by phosphorylation and N-glycosylation. Differential phosphorylation of P-gp by kinases has been shown to influence P-gp activity (Lelong-Rebel and Cardarelli, 2005).The 12 transmembrane helices form a toroidal protein with an aqueous pore. Two

nucleotide-binding domains for the protein lie in the cytoplasm. The pore is lined with hydrophobic and aromatic amino acids at the extracellular-facing half of the pore, whereas the cytosolic-facing portion of the pore contains polar, charged residues. Structural analysis reveals two openings in the protein at the lipid bilayer to permit extraction of substrates directly from the membrane upon their passive diffusion into the cell (Aller *et al*., 2009; Rosenberg *et al*., 1997). Several highly conserved residues within the pore are able to recognize a diverse range of substrates. The protein exhibits high conformational flexibility to allow for structural rearrangements in binding and effluxing substrates (Aller *et al*., 2009).

1.2.7.2. Tissue Distribution and Function

P-gp is expressed in a polarized manner in the plasma membrane of cells in barrier and elimination organs, where it has protective and excretory functions (Brinkmann and Eichelbaum, 2001). It plays an important role in first-pass elimination of orally administered drugs to limit their bioavailability by effluxing drugs from the lumen-facing epithelia of the small intestine and colon, and from the bile-facing canaliculi of the liver. It eliminates substrates from the systemic circulation at the urine-facing side of the brush border membrane of proximal tubules in the kidney, and again through biliary excretion. It restricts the permeability of drugs into 'sanctuary' organs from the apical or serosal side of blood–tissue barriers (e.g. blood–brain, blood–cerebral spinal fluid, blood–placenta, blood–testis barriers) (Fromm, 2004). In lymphocytes and other immunological and blood components, P-gp putatively plays a role in viral resistance and in trafficking cytokines and enveloped viruses (Raviv *et al*., 2000).

P-gp is also thought to be important for steroid partitioning and lipid homeostasis in the periphery and central nervous system (Jeannesson *et al*., 2009).

Intracellular P-gp with unknown function has been detected in the endoplasmic reticulum, vesicles, and the nuclear envelope, and has been associated with cell trafficking machinery. Relevant to the clinical challenge of MDR, P-gp is over expressed in numerous tissues transformed by cancer (Miller *et al*., 2008).

1.2.7.3. Mechanism of *MDR1* and its Role in Drug Resistance.

Biochemical studies suggested that substrate transport by MDR1 is coupled to ATP hydrolysis. There is speculation that MDR1 may work as a flippase so that substrate initially interacts with the inner leaflet of the lipid bilayer and then MDR1 flips the compound to the outer leaflet. Nonetheless, the currently accepted model is the "hydrophobic vacuum pump", in which the substrate directly interacts with the protein's drug binding pocket and is pumped into the extracellular space, assisted by hydrolysis of ATP to ADP (Sharom *et al*., 1993; Raviv *et al*., 1990).

The function of MDR1 as a transporter is similar to other drug transporters. Within the long list of MDR1 substrates, it is evident that some of the substrates (drugs) are also substrates of drug metabolizing enzymes, especially CYP3A4. CYP3A4 is located at 7q22.1, very close to MDR1 on the chromosome, suggesting their inter-dependency during evolution to protect the host organism from toxins by detoxification and extrusion. Some MDR1 substrates, e.g. anticancer drugs, can be effluxed by other MDR transporters, suggesting redundancy, or "back up" in transporter function. For example, vincristine is a substrate of MDR1 and MRP1 while doxorubicin is a substrate of MDR1, MRP1 and BCRP (Renes *et al*., 1999; Doyle *et al*., 1998).

1.2.7.4. ABCB1 Genetics

Researches were focused on the function of MDR1 in order to determine the factors that affect the expression of MDR1,since it is a highly-conserved gene(Loo

et al., 2004). Mutation studies have confirmed that changes in crucial amino acid residues in the trans-membrane domains, ATP-binding domains, Walker-A motifs, Walker-B motifs, or the signature motif might affect MDR1 function. Mutations may also affect folding pathways or protein conformation.(Sauna *et al*., 2002).

There are 1279 SNPs in the ABCB1 gene region, 62 of which are coding 22 synonymous, 41 non-synonymous, and one in the start codon. The number and frequency of SNPs observed varies by ethnicity. Excluding SNPs below 5% allele frequency, there are approximately 124 SNPs observed in Caucasians, 134 in African–Americans, 153 in Chinese, and 166 in Japanese (Fung and Gottesman, 2009).

1.2.7.5. MDR1 Expression in AML

Physiologically M1/P-gp is expressed in many tissues in the human body, like liver, pancreatic ducts, intestine, kidney, adrenal cortex, placenta or the endothelium of brain capillaries and the choroid plexus epithelium(Rao *et al*., 1999).Furthermore, some populations of blood cells exhibit significant MDR1 levels. These are T-lymphocytes, NK-cells and immature CD34 positive cells (Chaudhary and Roninson, 1991). Approximately 50% of AML patients have leukemic blasts that express *ABCB1*, with a slightly greater rate of expression and at higher levels in elderly patients (Leith, 1997). Detection of the *ABCB1* gene product is more frequent in elderly patients and has been more strongly linked to inferior outcomes in this population. This age-dependent prognostic relevance of *ABCB1* expression could stem from the tendency of younger patients to receive more intensive chemotherapeutic regimens than older patients, a bias that could mask the relevance of *ABCB1* expression as a prognostic factor in younger patients(Pallis and russel., 2000).

1.2.7.6. Single Nucleotide Polymorphisms of the ABCB1 gene

Reports have addressed the impact of genetic polymorphisms on drug transporters. Among the 48 ABC transporters, ABCB1 is one of the most thoroughly studied and characterized, with more than 50 SNPs reported. Polymorphisms in ABCB1 could alter clinical outcomes via a number of mechanisms. The efficiency of transporter function could be increased via decreased binding to inhibitors or, on the other hand, dysfunctional transporters could lead to increased toxicity due to decreased export of chemotherapy drugs from normal tissues, particularly bone marrow cells (Fung and Gottesman, 2009).

Kimchi-Sarfaty *et al*., (2007) analyzed the role of synonymous mutations in protein folding and function. They reported that synonymous SNPs (3435C>T, 1236C>T and 2677G>T) in the ABCB1 gene sequence result in a protein with reduced drug and inhibitor interactions. One hypothesis to explain this result is that when frequent codons are changed to rare codons in a cluster of infrequently used codons, the timing of co-translational folding is affected and may result in altered function. Structural changes in mRNA caused by synonymous mutations could also alter the rate of co-translational folding.

1.2.7.7. The Role of Epigenetic Signals in ABC Transporter Expression

Methylation profiling studies had shown that aberrant DNA methylation is a dominant mechanism in the progression of AML. They have also demonstrated that methylation profiles allow the prediction of survival outcome. Although these studies did not focus on ABC transporters, they point to the important role of methylation in malignancy, and support future studies regarding epigenetics and ABC transporters (Gupta *et al*., 2009).

It is known that methylation-controlled J protein (MCJ) is silenced by hypermethylation, which in turn prevents proper degradation of c-Jun, causing increased expression of *ABCB1* (Hatle *et al*., 2007). In contrast, both hypomethylation and hyperacetylation at the 5' promoter of ABCB1 leads to increased expression of Pgp (Tabe *et al*., 2006; Nakayama *et al*., 1998). In a retrospective analysis using a small patient population, Nakayama and colleagues noted that 8 of 12 (66%) samples from patients who achieved first complete remission had hyper ethylated ABCB1 promoter versus none of 7 patients with chemo-refractory leukemia (Nakayama *et al*., 1998).

Additionally, two of six patients evaluated were found to have loss of methylation of the ABCB1 promoter regions in leukemic cells at the time of relapse. Whether loss of ABCB1 promoter methylation is causal versus circumstantial in initiating chemo-resistance is unclear. Thus, while small, these studies suggest that ABC transporter expression or function should be monitored closely in clinical trials evaluating agents designed to modulate epigenetic control of gene expression.

1.2.8.Real-time Polymerase Chain Reaction Validation

Reverse transcription (RT) followed by a polymerase chain reaction (PCR) represents the most powerful technology to amplify and detect trace amounts of mRNA (Heid *et al*., 1996; Lockey, 1998). Real-time qRT-PCR has advantages compared with conventionally performed ‘semi-quantitative end point’ RT-PCR, because of its high sensitivity, high specificity, good reproducibility, and wide dynamic quantification range (Schmittgen *et al*., 2000; Bustin, 2000).

SYBR® Green dye was used in RT-PCR quantitation, this dye was useful as a screening tool for quickly assess the relative expression levels of a variety of

genes in a variety of sample types (Rajeevan *et al*., 2001).The cycle at which the growth curve crosses a specified threshold is called the cycle threshold (Ct).The Ct value can be used for qualitative or quantitative analysis and it is used for making comparisons between samples. The point at which the product's detected fluorescence crosses the threshold is called the CT value. In a 100% efficient PCR, the amplified product will double at each cycle. This means that differences in CT between different samples correspond to differences in starting amount of the target sequence (Lee et al., 2004).

Chapter Two

Materials and Methods

2. Materials and Methods

2.1 Materials

2.1.1 Equipment and Apparatuses

The following equipment and apparatus were used throughout this study as show in the Table (2-1):

Table (2-1): Equipment and Apparatuses used in this study

Equipment	Company (Origin)
Automatic Micropipette	Eppendorff (Germany)
Gel Documentation System	Bio-Rad (USA)
Gel Electrophoresis Unit	Bio-Rad (Italy)
Laminar Flow Hood	Telestar (Spain)
Microwave Oven	Shownic (Thailand)
Nano-drop Spectrophotometer	Thermo Scientific (USA)
Refrigerator	Beko (Turkey)
Sensitive Balance	Sartorius (Germany)
Thermal Cycler 7500 Real-Time PCR System	Applied Biosystems (Germany)
Thermal Cycler TC-5000	Techne (England)
Vortex	Clay Adams (Germany)
Water Bath	Memaret (Germany)

2.1.2. Chemicals and Biological Materials

The chemicals and biological materials used in this study are presented in Table (2-2).

Table (2-2): chemicals and biological materials

Material	Company (Origin)
Free Nuclease Distell Water**,** Loading Dye, TBE Buffer 10X (Tris-Borat EDTA)**,** Ethidium Bromide**,** Agarose**,** B-mercaptoethanol, Bromophenol Blueloading dye. qPCR Master Mix	Promega (USA)
DNA Ladder Marker (100bp), DEPC-DW. Master Mix, , RT-PCR PreMix	BioNeer(Korea)
RNase and DNase- Free Distell Water	Geneaid (Korea)
Ethanol Absolute	BDH (England)

2.1.3. Kits and other materials

The kits and other materials were used in this study: are given in Table (2-3).

Table (2-3): kit and other materials

Kit	Company (Origin)
SYNC™DNA Extraction Kit**,** Total RNA Mini kit	Genead (Korea)

2.1.4. Primers.

Amplification of *NPM* gene and estimated The expression levels of *MDR1* transcripts was achieved by using specific primers indicated in Table (2-4). These primers were designed according to this study and supplied by BioNeer (Korea) company in a lyophilized form of different pmols concentrations. Lyophilized primers were dissolved in a free DNase/RNase water to give a final concentration of 100 pmol/µl as a stock solution. The stock solution was stored at -20˚Cuntil use.

A working solution of 10 pmol/µl of each primer was prepared by adding 10µl of primer stock solution to 90µl of deionized distilled water to get a final concentration of 10 pmol/µl. In addition a working solution of 50 pmol/µl of oligo (dT) primer was prepared by adding 50µl of primer stock solution to 50µl of deionized distilled water to get a final concentration of 50pmol/µl.

Table (2-4):Designed primers for *NPM1* gene amplification and *MDR1*Gene Expression

Primers	**Sequence (5'→3')**	Annealing	**Product size (bp)**
NPMX1 Forward	TGT GAA CTA AAG GCC GAC AA	60˚**C**	1159
NPMX1 Revers	CCA CAG GGC TAG GTT CTG AG	60˚**C**	1159
NPMX2 Forward	AGC CCT TGT AAA AGG CAT CG	60˚**C**	1337
NPMX2 Revers	CCC AAG GGA AAC CTA GAA GG	60˚**C**	1337
MDR1 Forward	GGA GGC CAA CAT ACA TGC CT	60˚**C**	205
MDR1 Revers	CAG GGC TTC TTG GAC AAC CT	60˚**C**	205
GAPDH Forword	AGA AGG CTG GGG CTC	60˚**C**	----
GAPDH Revers	AGG GGC CAT CCA CAC AG	60˚**C**	----
oligo(dT)	14 mer	----	----

2.2.Methods

2.2.1. Subjects

Blood samples were collected from 50 male and female AML patients (with either newly diagnosed or already treated patients) from Baghdad Teaching Hospital during the period from November 2015 to January 2016.A volume of 5 ml of blood samples were collected in sterile EDTA tubes.

Also fresh blood samples were collected from 40 AML patients from Hematology Unit of Baghdad Teaching Hospital through the period from February 2015 to May 2016, as well as 10 healthy donors for MDR1gene expression. 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.

2.2.2.Detection of *NPM1*Mutations in AML

Blood samples (5 ml) were collected from 50 patients with AML, and from 10 healthy control volunteers. The DNA was extracted from blood. The region from 2380 bp to 4140 bp of *NPM1* gene (30181bp) was amplified by using two specific primers in a thermal cycler. Amplification products of the *NPM1* gene were sent for sequencing and analyzed by Macrogen Company (korea).

2.2.3. DNA Extraction

Total genomic DNA was extracted from 50 blood samples collected from AML patients, and 10 blood samples collected from healthy volunteers DNA extraction was carried out by using SYNC™ DNA Extraction Kit (Geneaid). Table (2-6) showed the components and preparation of kit.

Table (2-6):Kit Components and Preparation

Component	Preparation
GSB Buffer (40ml)	Ready to use.
W1 Buffer (45ml)	Ready to use.
Wash Buffer (25ml)	It was prepared by mixing the wash buffer with 100ml absolute ethanol.
Proteinase k (11mg x 2 vial)	It was prepared by dissolving the contents of one in 1.1ml of D.D.W. It was stored at 4˚C**.**
Elution Buffer(30ml)	Ready to use.
GS Columns x 100	Ready to use.
Collection Tubes x 200	Ready to use.

❖ **Protocol procedure:**

❖ Blood Sample Preparation

A volume of 200µl of whole blood was transfer to a micro centrifuge tube (1.5ml volume), then 20µl of proteinase k was added , mixed by pipetting and incubated at 60ºC for 5 min.

❖ Cell Lysis

A volume of200µl of GSB buffer was added and mixed vigorously, then incubated at 60ºC for 5 min with inverting the tube every two minutes. After incubation,

❖ DNA Binding

A volume of 200µl of absolute ethanol was added to cell lysate and mixed by shaking vigorously for 10 seconds, then a GS column was placed in a 2 ml collection tube, and the mixture was transferred to the GS column, and then centrifuged at 16,000 rpm for 1 minute. After centrifugation, the 2ml collection tube containing the flow – through was discarded and the GS column was transferred to a new 2ml collection tube.

❖ Washing

A volume of 400µl of W1 Buffer was added to the GS column and centrifuged at 16,000 rpm for 30 second, then the flow -through was discarded and the GS column was placed back in the 2 ml collection tube. After that 600µl of wash buffer was added to the GS column and centrifuged at 16,000 rpm for 30 seconds. The flow- through was discarded and the GS column was placed back in the 2ml collection tube and centrifuged again for 3 minutes at 16,000 rpm to dry the column matrix.

❖ Elution

The dried GS column was transferred to a clean micro centrifuge tube, and then 100µl of pre-heated elution buffer was added into the center of the column matrix and centrifuge at 16,000 rpm for 30 second to elute purified DNA.

2.2.4. Quantitation of DNA Concentration

Purity and concentration of DNA solution extracted from blood samples were determined by measuring the absorbencies of DNA solutions at 260nm (A260) and 280nm (A280) by using Nano drop spectrophotometer.

2.2.5. Agarose Gel Electrophoresis

After genomic DNA extraction, agarose gel electrophoresis was adopted to confirm the presence and integrity of the extracted DNA.

2.2.5.1.Preparation of 1X TBE Buffer

The 1X TBE Buffer was prepared from 10X TBE buffer (as stock solution) by mixing 100ml of this stock solution with 900ml of distilled water.

2.2.5.2. Preparation of Agarose Gel (0.7%) (2%)

- A volume of 100ml of 1X TBE buffer solution were placed in 250 ml conical flasks.
- Agarose powder of 0.7 g, 2 g was added to TBE buffer to get a final concentration of 0.7%, 2%
- Contents were heated to boiling in microwave oven until the gel particles were completely dissolved.
- The solution was cooled to 55-60˚C after then aliquots of 2µl of ethidium bromide (10mg/ml) was added to the flask, and then the contents were mixed thoroughly.

2.2.5.3.Casting of the Horizontal Agarose Gel

Agarose gel solution was poured into the gel tray, and left to stand at room temperature for 30 minutes until complete solidification, then fixed comb was carefully removed and the tray was placed in the tank filled with 1X TBE buffer until it reached 2-3 mm over the surface of the gel.

2.2.5.4. Gel Electrophoresis

Aliquot of 5µl of pure DNA solution was mixed first with 2µl of bromophenol blue loading dye, then DNA sample was loaded in to the wells and the electrophoresis was carried out for 1hr at 5V/cm. DNA bands were analyzed using gel documentation system.

2.2.6.The PCR analysis of Nucleophasmin (*NPM1*)Gene.

The total size of *NPM1* gene is up to 30181bp with 12 exons. The gene starts from 5001bp to 28181bp DNA linear (Chang and Olsen 1990). Two primers were designed in this study (Table 2-4) and used to amplify the region from 2380 bp to 4140 bp of the gene *NPM1*. This region covered about two exons (exon 2 and exon 3) (Fig. 2-1).

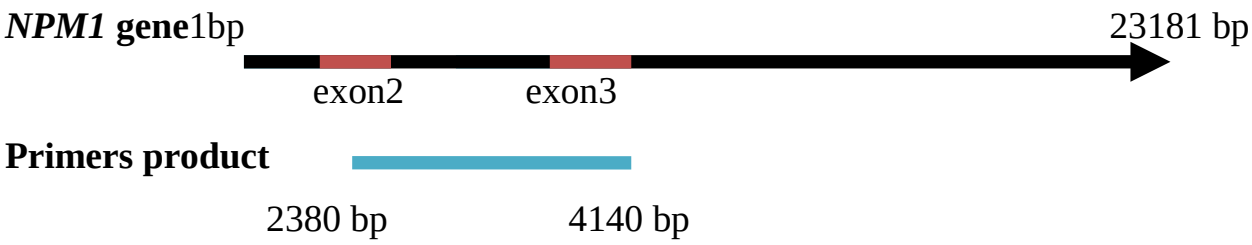

Figure (2-1): The product of two primers from *NPM1* gene

2.2.6.1 PCR amplification.

PCR was carried out in a total volume of 20µl. The reaction components are indicated in Table (2-7).

DNA extracted from blood samples were used for the amplification of the region flanking from 2380 to 4140 pb of NPM1 gene by using specific primers in a thermal cycler. Polymerase chain reaction was carried out according to the amplification program shown in Table (2-8), and then amplified products were analyzed on agarose gel (2%) in presence of 100bp DNA ladder marker.

- The template DNA 3 µl and the primers 1 µl were added to Accupower PCR tube.
- The distill water was added to PCR tube up to total volume of 20µl.
- The contents were mixed by vortexing.
- PCR of samples was performed.

Table (2-7): Components of reaction mixture for amplification of *NPM1*

Component	Volume (20µl)
Master Mix *Taq*DNA Polymerase (1U), dNTPs (250uM), MgCl2 (1.5 mM), KCl (30 mM) Tris-HCl (10 mM),	Lyophilized
Forward Primer	1
Reverse Primer	1
DNA Template	3
Nuclease free water.	15

Table (2-8): PCR amplification program of *NPM1*

Step	Temperature (˚C)	Time	No. of Cycles
Initial	**94**	**5 min**	**1 cycle**
Denaturation	**94**	**1 min.25 sec**	**35cycle**
Annealing	**60**	**1 min**	
Extension	**72**	**1 min .25 sec**	
Final Extension	**72**	**7 min**	**1 cycle**

2.2.7. Sequencing of Amplified *NPM1* Gene Product

Amplified products NPM1gene was sent for sequencing to Macrogen Company (korea). The sequencing for these products was compared with the information in gene bank of the National Center for Biotechnology Information (NCBI) for standard NPM1gene, using Bio edit .

2.2.8. Assessment of Multidrug Resistance Gene (*MDR1*) Expression in AML Patients using RT-qPCR

Fresh blood samples were collected from AML patients at different levels of chemotherapy treatment and healthy donors as well, in order to investigate the degree of *MDR1*expression in AML patients and compare results with normal healthy donors.

The real-time PCR experiment was designed in singleplex assay, the chemistry used in real-time qPCR was DNA-binding dyes SYBR Green1. The method used for quantification of gene expression in RT-qPCR was “two steps” RT-qPCR, in this method RNA was isolated from blood samples, after RNA extraction, cDNA was synthesized by use reverse transcriptase-PCR and used as template for real-time as illustrated in Fig. (2-2).

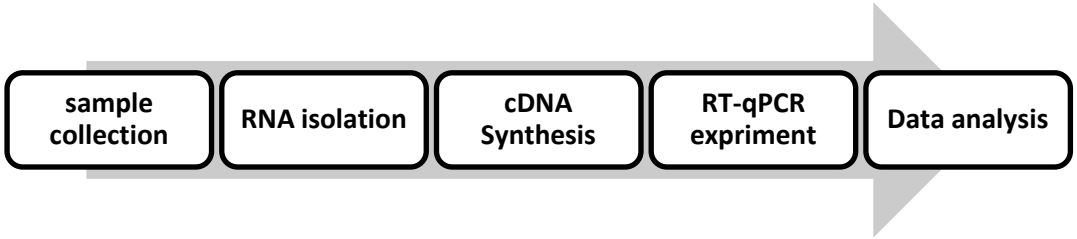

Figure (2-2): MDR1 gene expression analysis workflow

2.2.8.1. Assessment of Therapy

Response to treatment was categorized as complete remission (CR); preserving complete remission according to established conditions for >6 months: after induction chemotherapy and absence of leukemia in other sites; non-responder (NR) as 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).

Accordingly, patient samples were categorized into four groups, group (A) consisted of 10 newly diagnosed AML patients (No treatment started), group (B) included 10AML patients after first course induction of chemotherapy; group (C) comprised 10 AML patients after second induction and group (D) included 10 AML patients in consolidation.

2.2.8.2. RNA Isolation

RNA extraction was carried out by using Total RNA Mini Kit (Blood/Culture Cell) Protocol System supplied from Geneaid (Korea).

Kit Contents:

Component	Preparation
RBC Lysis B (200ML)	Ready to use.
RB Buffer (60ml)	Ready to use.
W1 Buffer (50ml)	Ready to use.
Wash Buffer (25ml)	It was prepared by mixing Wash Buffer 25ml with 100ml absolute ethanol
RNase-free water (6ml)	Ready to use.
RB column (100 column)	Ready to use
2 ml Collection Tubes (200 tube)	Ready to use

Protocol:

- RBC lysis/cell harvesting:

  Fresh human blood was collected in anti-coagulated tubes. A volume of1ml RBC lysis buffer was added to 1.5ml micro centrifuge tube, and then 300µl of whole human blood was added and mixed by inversion. The tube was incubated on ice for 10 minutes. After incubation, the tube was centrifuged at 3000x g for 10 min. The supernatant was removed completely. After centrifugation, cells were re-suspended in 100µl of RBC lysis buffer by pipetting the pellet.

- Cell lysis:

  Aliquots of 400µl of RB buffer and 4µl of B-mercaptoehanol were added to the re-suspended cells, and then the mixture was shaked vigorously and incubated at room temperature for 5 min. Then 500µl of 70% ethanol was added and mixed vigorously. The RB column was placed in 2ml collection tube, and then 500µl of mixture was transferred to RB column. The RB column was centrifuged at 16000 x g for 1 min, the flow-through was discarded and the remained mixture was transferred to the same RB column. The RB column was centrifuged again at 16000 x g for 1 min. The flow-through was discarded and the RB column was placed to new 2ml collection tube.

- Washing:

  A volume of 400µl of W1 buffer was added into RB column then centrifuged at 16000 x g for 30 seconds and discarded the flow-through. The RB column was placed in to 2ml collection tube and600µl of wash buffer was added into RB column, then centrifuged

at 16000 x g for 30 seconds and discarded the flow-through. After then, the RB column was placed back in the 2ml collection tube and 600 µl of wash buffer was added into RB column, then centrifuged at 16000 x g for 30 seconds and discarded the flow-through. The RB column was placed back in the 2ml collection tube and centrifuged at 16000 x g for 3 minutes.

- RNA Elution

The dried RB column was placed in new cleaned 1.5 ml micro centrifuge tube and 50µlof RNase-free water was added into center of column matrix, then let stand for a minute, then centrifuged at 16000 g for 1 min to eluted the purified RNA.

2.2.8.3 Quantitation of RNA Concentration

Purity and concentration of RNA solution extracted from blood samples were determined by measuring the absorbencies of RNA solutions at 260nm (A260) and 280nm (A280) by using Nano drop spectrophotometer.

2.2.8.4.cDNASynthesis

Reverse transcription of total RNA extracted from blood samples to cDNA was achieved using RT-PCR PreMix kit according to program shown in Table (2-9). cDNA synthesis was carried out in a total volume of 20 µl by following the below protocol:

- The RNA template 18 µl and the Oligo (dT) 2 µl primers were mixed in a sterile tube as indicated below:

- The mixture was incubated in 70˚C for 5 minute then placed it on ice.
- The incubated mixture was transferred to RT/PCR PreMix tube contains the reverse transcriptase enzymes and mixtures of nucleotides buffers.
- .PCR protocol was performed.

Table (2-9): Program for Reverse transcription.

Step	Temp. (˚C)	Time (min)
cDNA synthesis	50	60
RTase inactivation	95	5
Holding	4	5

2.2.8.5.Real Time Quantification Polymerase Chain Reaction (RT-qPCR)

The *MDR1* gene was determined by Real Time-qPCR by using SYBR Green dye assay. The synthesized cDNA was used as template for quantitative detection of *MDR1* gene expression mixed with the MDR primers. Also the endogenous control Glyceraldehydes 3 phosphate dehydrogenase (GADPH) was determined in each sample. GADPH gene was used as reference gene to calculate the *MDR1* gene expression. The mRNA levels of endogenous control gene GADPH were amplified and used to normalize the mRNA levels of the *MDR1* gene and correct synthesis of cDNA as well as the calculations descriptions. Two non-template controls were also included in each run.

The following PCR reagents were added into qPCR plate (20µl reaction) for GADPH and *MDR1* gene in separated tubes for each sample.

MDR1		GADPH	
Component	**20 µlRxn**	**Component**	**20 µl Rxn**
SYBR Green qPCR Master mix	10	SYBR Green qPCR Master mix	10
MDR forward	1	GADPH forward	1
MDR reveres	1	GADPH reveres	1
cDNA	3	cDNA	3
DEPC-distilled water	5	DEPC-distilled water	5

Real-Time qPCR was carried out according to the amplification program shown in Table (2-10).

Table (2-10): Real Time qPCR program for MDR1 gene and GAPDH endogenous control

step	Temp. (°C)	Time	Cycle
Pre-Denaturation	95	5 min	1
Denaturation	95	20 sec	40
Annealing/Extension	60	40 sec	
Detection(Scan)			
Melting	65-90	----	---

2.2.8.6.Data Analysis

Relative quantification method analysis was used to determine the results of MDR1 gene expression. In this method, multiple samples were compared, the normal sample was used as control sample, and the expression of the *MDR1* gene in the AML patient (four groups) was expressed as a relative increase or decrease to the control.

The relative expression of MDR1 gene in AML patient and healthy was calculated using the equation below (Livak, 2001):

- Ratio=2-(ΔΔ CT).
- Ct (threshold cycle) is the intersection between an amplification curve and a threshold line. It is a relative measure of the concentration of target in the PCR reaction.
- ΔΔCT= ΔCT (target) -ΔCT(untreated calibrator).
- Normalized by endogenous control.
- ΔCT target = CT (mean MDR1) - CT (mean GADPH).
- ΔCT untreated = CT (mean MDR1) - CT (mean GADPH).

2.3 Statistical Analysis:

A one way analysis of variance ANOVA (Duncan) was performed to test whether group variance was significant or not. Data were expressed as mean ±standard error and statistical significances were carried out using Graph Pad Prism version 6.

Chapter three

Results and Discussion

3. Results and Discussion

3.1 Patient Database

The study consisted of 90 Iraqi AML patients (41 male and 49 female), all patients were above 18 years, with median age of 33. Peripheral Blood samples (5ml) were collected from the patients during the period started from December 2015 to May 2016, which were admitted to Baghdad Teaching Hospital (Baghdad-Iraq). All patients were previously diagnosed for AML based on updated French-American-British (FAB) classification (Vardiman et al., 2009).

Samples were classified into two groups: the first constitute 50 samples which were inspected for *NPM1* mutations with 10 healthy control , while the other group (40 samples) was used for *MDR1* gene expression with 10 healthy control.

3.2.Detection of *NPM1*GeneMutation.

3.2.1.Genomic DNA Extraction.

In order to study the *NPM1* gene mutation in Iraqi patients with AML, total genomic DNA was extracted from blood samples of patients and healthy controls. Results showed a sharp DNA bands obtained after extraction and electrophoresis on 0.7% agarose gel (Fig. 3-1). The purity of extracted DNA from all samples was ranged between 1.8-1.92, while the concentration was ranged between 52-73 ng/µl. The purity and concentration of DNA were suitable and recommended for further genetic analysis by using PCR technique (Boesenberg-Smith *et al.*, 2012).

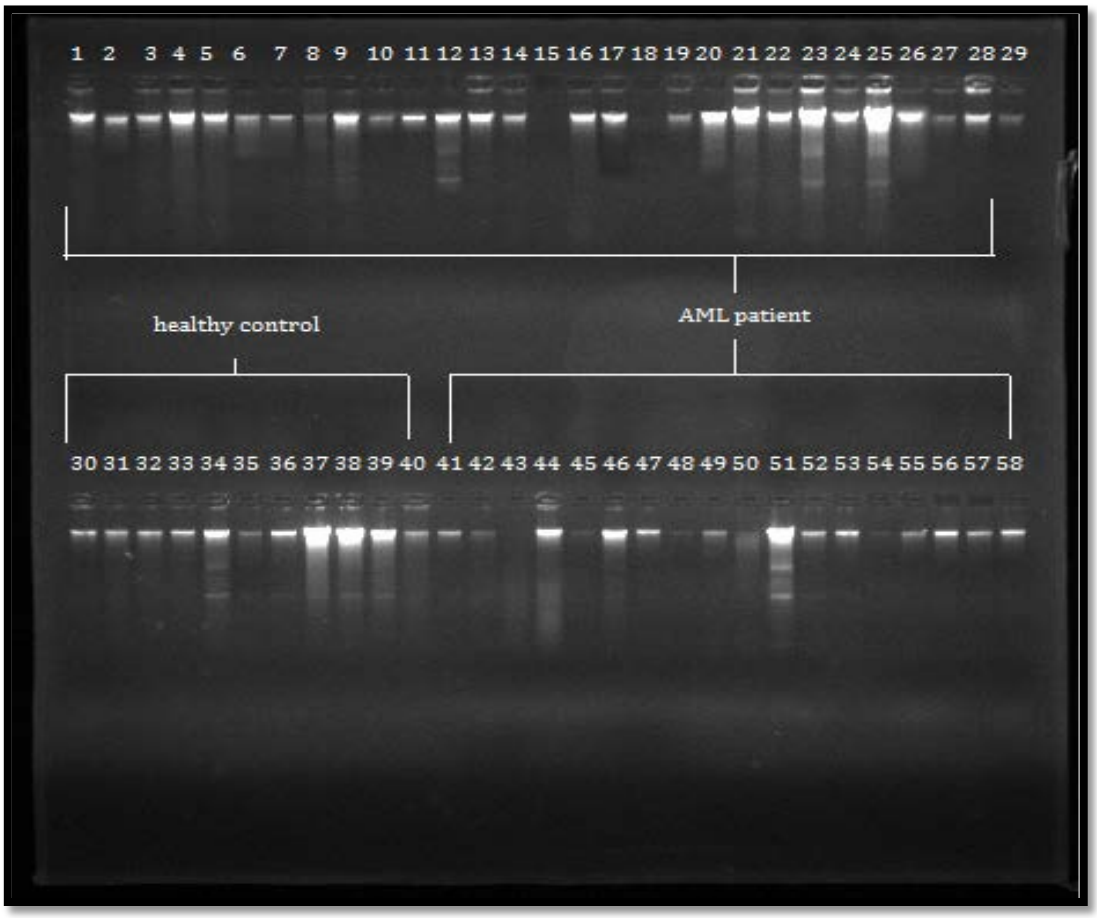

Figure (3-1): Gel electrophoresis of genomic DNA on agarose gel (0.7%) for 1 hour at 70V.

*** Lane (1-29) AML Patients.**

*** Lane (41-58) AML Patients.**

*** Lane (30-40) Healthy Controls.**

3.2.2.*NPM1* Gene Amplification

The region of *NPM1* was amplified from extracted DNA from each blood samples of patients with AML and healthy controls. Polymerase chain reaction was achieved under optimum amplification conditions by using specific primers. Results illustrated in Fig. (3-2) showed that the amplified products were appeared as clear bands after electrophoresis on agarose gel (2%). The product of NPMX primer (Fig. 3-2A) was single clear band with molecular size 1150 bp, this product

represent about 2380 bp to 3447bp of amplified region and cover exon 2 from *NPM1* gene, while the product of NPMX2 primer (Fig. 3-2B) also showed a single clear band with molecular size 1337 bp, this product represent about 2707 bp to 4140 bp of amplified region and covered exon 3 from*NPM1* gene.

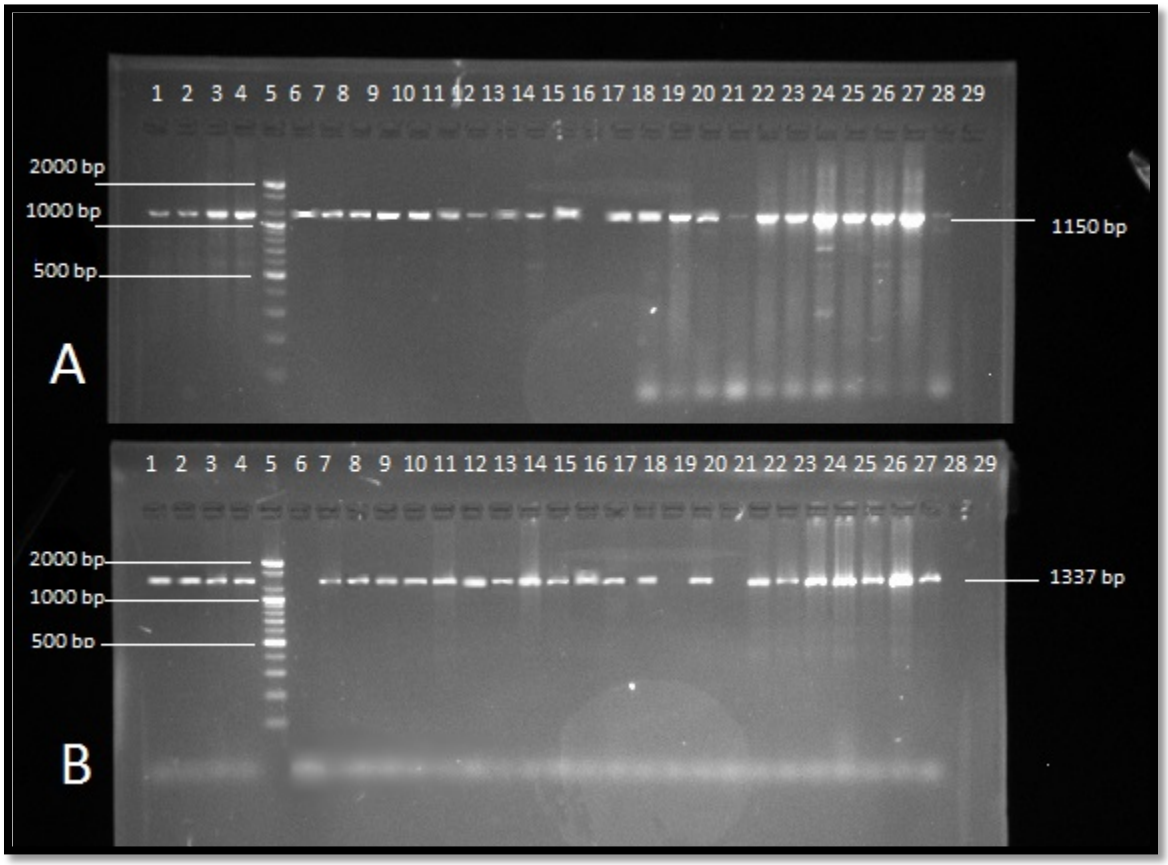

Figure (3-2): Gel electrophoresis for PCR products of *NPM1*gene on agarose gel (2%) for 1 hour at 70Vin the presence of 100bp DNA Ladder marker. (A): PCR product of NPMX1primer (B): PCR product of NPMX2 primer.
*** Lane (1-4) Healthy Control.**
*** Lane (5) DNA Ladder Marker.**
*** Lane (6-29) Patient Bands.**

3.2.3.Sequencing of Amplified of *NPM1* Gene

In order to examine genetic variation for *NPM1* gene in AML Iraqi patients, sequencing of exon 2 and exon 3 from *NPM1* gene that encode the nuclear export signal (NES) was performed to determine any genetic variation in this region that associated with AML as compared with healthy individuals. The complete nucleotide sequence of two PCR products (the region from 2380 bp to 4140 bp) was checked to determine any genetic changes in this region. Results of sequencing that illustrated in Table (3-1) shows the genetic variation associated with AML.

Table (3-1):Genetic variation in *NPM1* gene

Type	Substitution	Molecular Consequences	Position subjct	Healthy Control (10)	AML Patient (10)
SNP	G/A	Intron variation	792	70%	90%
SNP	G/A	Intron variation	794	70%	90%
SNP	G/A	Intron variant	797	70%	90%
SNP	A/G	Exon 3	1275	0%	70%
SNP	G/A	Intron variant	660	0%	80%
SNP	G/A	Intron variant	635	0%	80%

SNP: Single Nucleotide Polymorphism

3.2.3.1.*NPM1* Polymorphisms

Three different single nucleotide polymorphism (SNP) G/A792, G/A794 and G/A797 were detected in the intron (non coding region) region as shown in Fig.(3-3), which were existed in 90% of patient samples and 70% of healthy volunteers.

These SNPs were not before correlated with mutant NPM1 Iraqi AML patients and being first described.

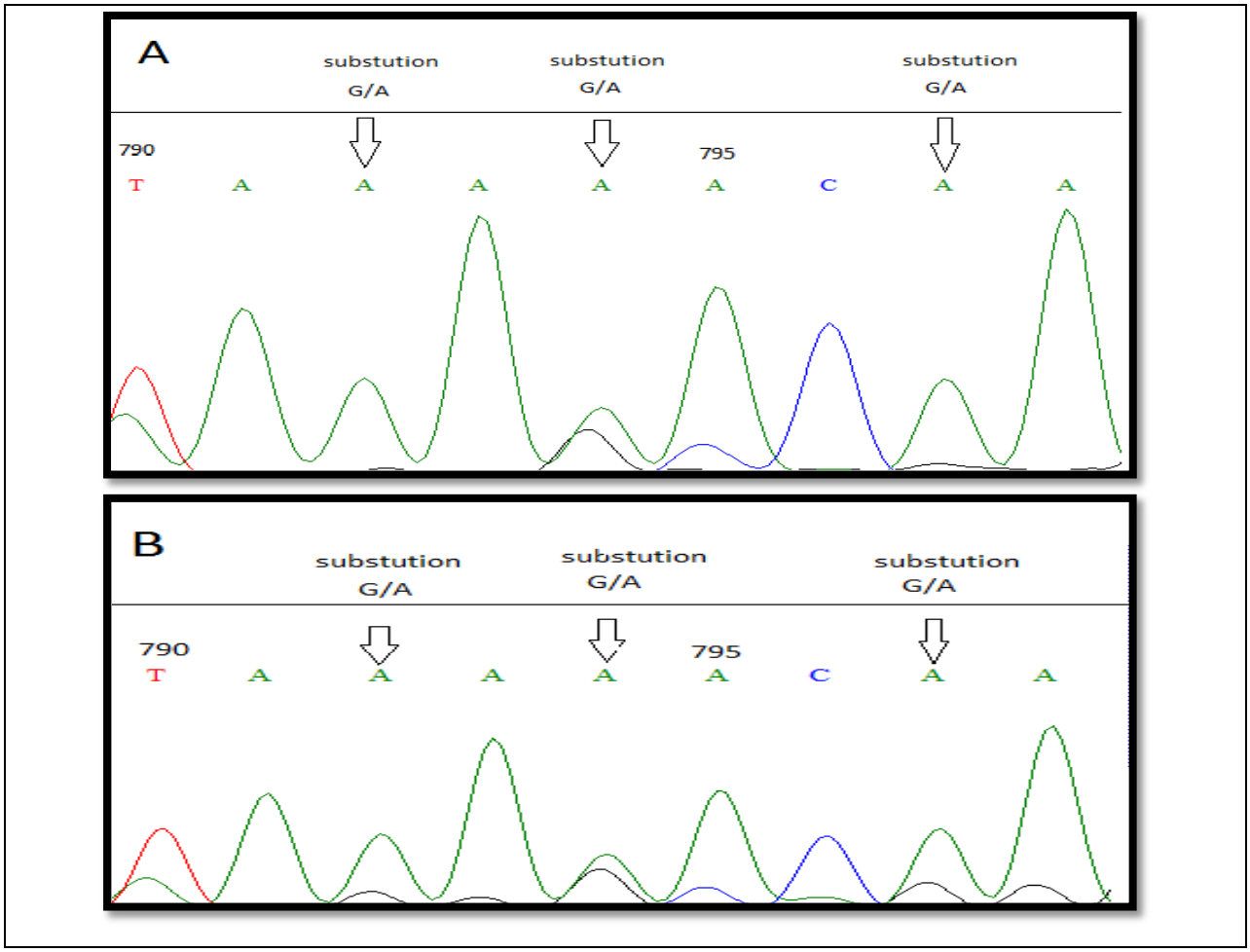

Figure (3-3): Sequencing of amplified PCR product indicates base substation in three positions of *NPM1*gene. (A) Healthy control (B) AML patient.

3.2.3.2.*NPM1* Mutations

During the analysis of *NPM1* gene sequencing, single nucleotide variant was identify in exon 3 that encode NES (Fig. 3-4) that was identified in 70% AML patients A/G 1276 (rs 753788683), this single nucleotide polymorphism was correlated with mutant *NPM1*.

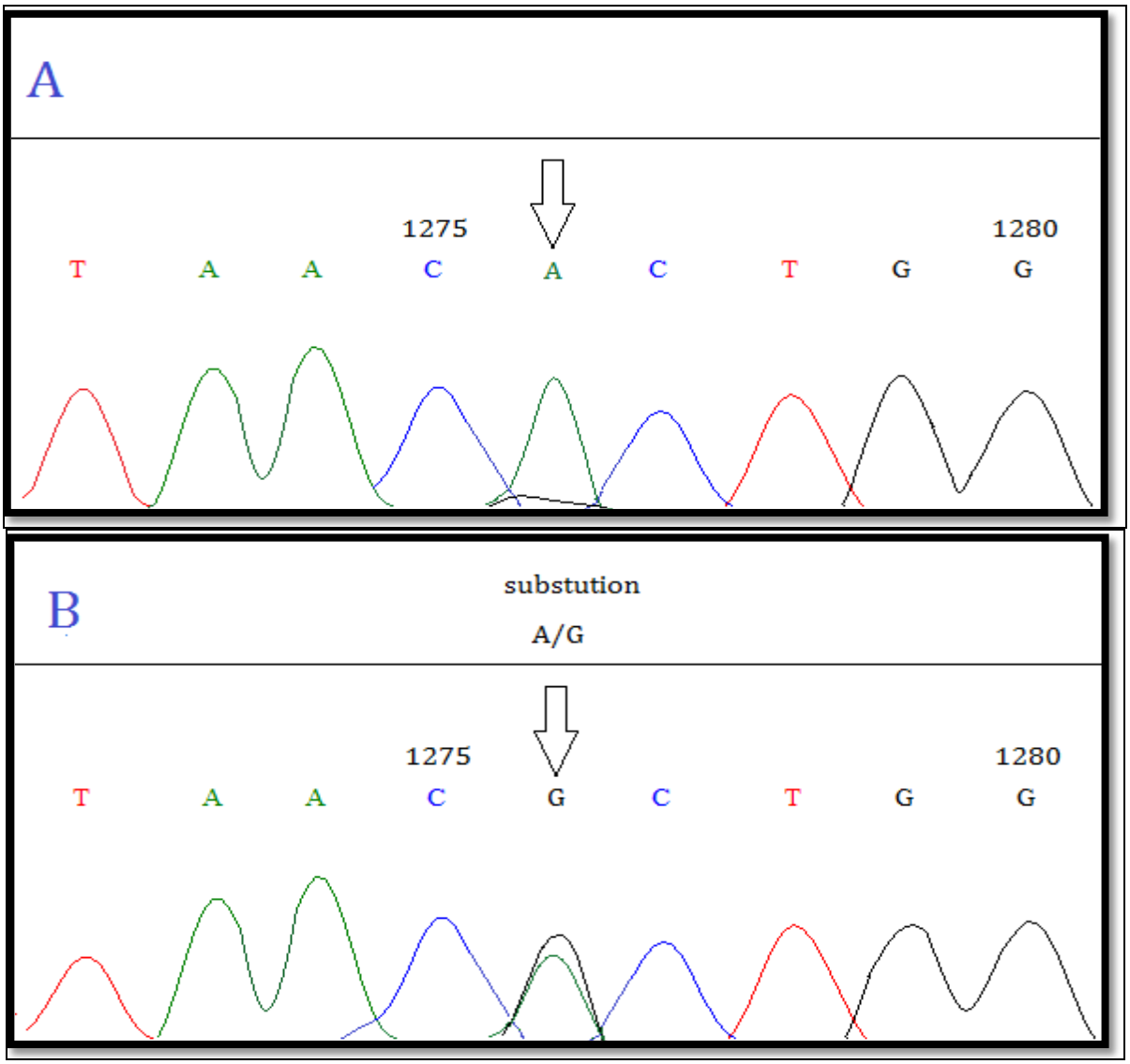

Figure (3-4): Sequencing of amplified PCR product indicates substation in position 1276 of *NPM1* gene in exon 3. (A) healthy control; (B) AML patient with base substation SNP.

Also single nucleotide variant was observed in two sites G/A635 and G/A660 within intron region that identify in 80% Iraqi AML patients(Fig. 3-5). These SNP were correlated with mutant *NPM1*. No genetic variation was observed in exon 2 during sequences analysis of amplified *NPM1* gene.

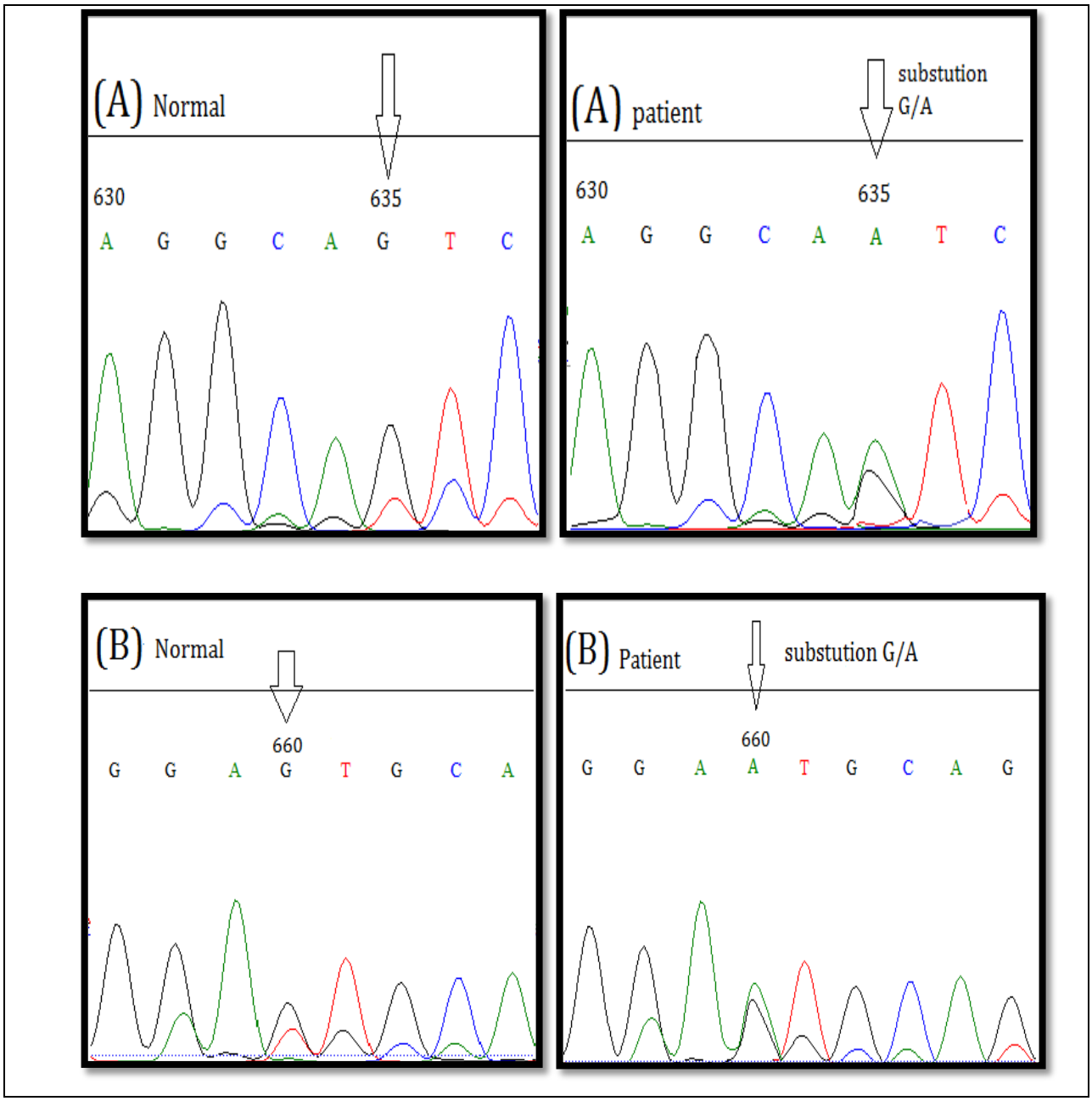

Figure (3-5):Sequencing of amplified *NPM1* gene. (A) Base substation in position 635 G/A, (B) Base substation in position 660 G/A.

NPM1 mutations are restricted to exon 12. Around 40 molecular variants of *NPM1* mutations have been described to date in AML patients. The most common mutation (so-called mutation A12) duplicates a TCTG tetra nucleotide. Mutations B and D are observed in about 10% and 5% of cases, respectively; other

mutations are very rare (Falini *et al*., 2007). Independently of their type, all mutation variants generate common alterations at the C-terminus of the NPM leukemic mutants which are responsible for their dislocation into cytoplasm, which leading to the creation of another nuclear export signal (NES) due to changes of tryptophan(s) 288 and 290 (Suzuki *et al*., 2005).

Most studied mutations were restricted in exon 12 and those mutations tend to modify NLS into a new NES and as a results inhibition of NLS, so the mutant protein tend to be more stable and more accumulated in the cytoplasm (Chou *et al*., 2006). 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 SNP were observed in intron and exon 3 that encode the NES signal. The mutation in the intron region not affecting on amino acids sequences but may be affect transcription factor, gene expression, and messenger RNA degradation. The mutation in the exon 3 was checked for its effect on amino acid sequences by aligning exon3 amino acid of Iraqi AML patients with reference sequence.

3.2.4.Nucleotide Sequence Alignment of the Amplified of *NPM1* Gene

The results of sequence process were analyzed using online blastn developed by National Center Biotechnology Information (NCBI) at (http://www.ncbi.nlm.nih.gov) and BioEdit program to detect the genetic changes of *NPM1* gene. Results were illustrated in Fig. (3-6) which showed that the nucleotide alignment and the position of the nucleotide of patient group (healthy control in appendix 1) was matched with nucleotide in reference sequence mentioned in NCBI that showed 99% similarity under sequence ID: NC-000005.10Length: 181538259 Range from 1: 171390116 to 171391642of *Homo sapiens* chromosome 5.

Score	Expect	Identities	Gaps	Strand
2787 bits(1509)	0.0	1521/1527(99%)	0/1527(0%)	Plus/Plus

Features:

Nucleophosmin isoform 1

```
Query  1          TTATCTTTAAGAACGGTACTTAAACTTTCAAAATAAACTACTTAACCCTACTTGATTTCA  60
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  171390116  TTATCTTTAAGAACGGTACTTAAACTTTCAAAATAAACTACTTAACCCTACTTGATTTCA  171390175

Query  61         GCCTTTTAGTTTCTATTCATGTGGCTTGAGACttttttttCCTTTGCTGACTGCTTATAAA  120
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  171390176  GCCTTTTAGTTTCTATTCATGTGGCTTGAGACTTTTTTTTCCTTTGCTGACTGCTTATAAA  171390235

Query  121        ATACTATTTCTTACACCTGGGTATTGTGTGTACCTCACTGTCTGTACATCTTTGAAGAAC  180
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  171390236  ATACTATTTCTTACACCTGGGTATTGTGTGTACCTCACTGTCTGTACATCTTTGAAGAAC  171390295

Query  181        TTGGCATCTATACCACTCATTTGGTAACCATGTAAATTCCAGTCACACTGTTCAACTTGC  240
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  171390296  TTGGCATCTATACCACTCATTTGGTAACCATGTAAATTCCAGTCACACTGTTCAACTTGC  171390355

Query  241        CTTATTTTCTTCAGTTGAATTACAAAGCCCTTGTAAAAGGCATCGATAATCTTTCATGTC  300
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Sbjct  171390356  CTTATTTTCTTCAGTTGAATTACAAAGCCCTTGTAAAAGGCATCGATAATCTTTCATGTC  171390415

Query  301        TACCAGAGACTAGCCTTCTGCTCAACTTAGAAGTTGCTCAATAAATATGTTAGGTGGTCT  360
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Sbjct  171390416  TACCAGAGACTAGCCTTCTGCTCAACTTAGAAGTTGCTCAATAAATATGTTAGGTGGTCT  171390475

Query  361        ATATTGTGTGTATGCATATTTATGCATTTCTTAAATTTTCTGAATATGAGAAACTGATTT  420
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Query  421        GCCAAGATCACAAAACCTGAGGACAACATTGCACAAATTTGTTTTCCAGGGTAATAGTGA  480
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Sbjct  171390536  GCCAAGATCACAAAACCTGAGGACAACATTGCACAAATTTGTTTTCCAGGGTAATAGTGA  171390595
```

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Query  481        GAGGTTTATACTTGTTTTTTaaaaaaatcacagtcatgtgcctcattgcaactgctgggt  540
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Sbjct  171390596  GAGGTTTATACTTGTTTTTTAAAAAAATCACAGTCATGTGCCTCATTGCAACTGCTGGGT  171390655

Query  541        caacagatggatcgcatatacaatggtggtcccataagattattataccttatttttaat  600
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Query 601         ataccttcctgttttttttttctttggaggcaatctcgctctgtcacccaggctggaa  660
                   |||||||||||||||||||||||||||||| ||||||||||||||||||||||||
Sbjct  171390716  ATACCTTTCCTGTTTTTTTTTTTCTTTGGAGGCAGTCTCGCTCTGTCACCCAGGCTGGAG  171390775

Query  661        tgcagtggtgtaatctcggctcactgcaacctctgatttccgagttcaagtaattccctg  720
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Sbjct  171390776  TGCAGTGGTGTAATCTCGGCTCACTGCAACCTCTGATTTCCGAGTTCAAGTAATTCCCTG  171390835

Query  721        cctcaccctcccgagtagctgggattacaggcatgtgccaccacacctggctaatttttg  780
                   ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  171390836  CCTCACCCTCCCGAGTAGCTGGGATTACAGGCATGTGCCACCACACCTGGCTAATTTTTG  171390895


Query  781        tgtttttagtaaaaacaaggttgttaccatgttgtccaggctggtctcaaactcctgacc  840
                   |||||||||||| | || ||||||||||||||||||||||||||||||||||||||||||||
Sbjct  171390896  TGTTTTTAGTAGAGACGAGGTTGTTACCATGTTGTCCAGGCTGGTCTCAAACTCCTGACC  171390955

Query  841        tcatgatctgcccgcctcagcctccctaagtgttgggattacaggcatgagccaccaagc  900
                   ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  171390956  TCATGATCTGCCCGCCTCAGCCTCCCTAAGTGTTGGGATTACAGGCATGAGCCACCAAGC  171391015

Query  901        ccggccctttcctatgcttagatgcacaaatactgtgtttcggttgcttacagtattcag  960
                   ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  171391016  CCGGCCCTTTCCTATGCTTAGATGCACAAATACTGTGTTTCGGTTGCTTACAGTATTCAG  171391075

Query  961        tacagtaacactgtacaggtttgtagcctaggtgtgtgtagtaggctataccatctaggt  1020
                   ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  171391076  TACAGTAACACTGTACAGGTTTGTAGCCTAGGTGTGTGTAGTAGGCTATACCATCTAGGT  171391135

Query  1021       ttgtataagcacattcttatgattgtacaaagatgaaattgtctaacaacacatttctca  1080
                   ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  171391136  TTGTATAAGCACATTCTTATGATTGTACAAAGATGAAATTGTCTAACAACACATTTCTCA  171391195
```

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Query  1081       gaacctagccctgtggttaagtgacgcatggctgCATATAACATTTAGTgggggggTGTA  1140
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  171391196  GAACCTAGCCCTGTGGTTAAGTGACGCATGGCTGCATATAACATTTAGTGGGGGGGTGTA  171391255

Query  1141       AAATAGGTGGAACTCAAAAGTTGAAGTAGTAttttttttttGTTCACAGGTCAGTTTAGG  1200
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  171391256  AAATAGGTGGAACTCAAAAGTTGAAGTAGTATTTTTTTTTTTGTTCACAGGTCAGTTTAGG  171391315

Query  1201       GGCTGGTGCAAAGGATGAGTTGCACATTGTTGAAGCAGAGGCAATGAATTACGAAGGCAG  1260
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  171391316  GGCTGGTGCAAAGGATGAGTTGCACATTGTTGAAGCAGAGGCAATGAATTACGAAGGCAG  171391375

Query  1261       TCCAATTAAAGTAACGCTGGCAACTTTGAAAATGTCTGTACAGCCAACGGTAAGGGCACT  1320
                  |||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||
Sbjct  171391376  TCCAATTAAAGTAACACTGGCAACTTTGAAAATGTCTGTACAGCCAACGGTAAGGGCACT  171391435

Query  1321       TACATACTTTGGATGTTGTGTCAAGGTTTAATTCTGTTTTAAGGTAGGTTTGGTGTCATT  1380
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  171391436  TACATACTTTGGATGTTGTGTCAAGGTTTAATTCTGTTTTAAGGTAGGTTTGGTGTCATT  171391495

Query  1381       TAGTTGTGCCAAGGAGATAGAAAGTGGTTCTTTATCTTCTGTCACTGGAGTTCGATGGTC  1440
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  171391496  TAGTTGTGCCAAGGAGATAGAAAGTGGTTCTTTATCTTCTGTCACTGGAGTTCGATGGTC  171391555

Query  1441       AACTCTTGAACATGGGGGCTTCTGCTGCTACTTTTATCAGAGGTGGAAAAACAGGTTCAC  1500
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  171391556  AACTCTTGAACATGGGGGCTTCTGCTGCTACTTTTATCAGAGGTGGAAAAACAGGTTCAC  171391615

Query  1501       TGGTTTGTTGATTTGGCTTATGTGTTT  1527
                  |||||||||||||||||||||||||||
Sbjct  171391616  TGGTTTGTTGATTTGGCTTATGTGTTT  171391642
```

Figure (3-6):Nucleotide sequence alignment of the amplified *NPM1* Gene

3.2.5.Alignment of Exon 3 Amino Acids

The amino acids sequence encoded by exon 3 of*NPM1*gene in healthy controls was indicated in the Appendix (1). The AML Iraqi patients were examined and compared with the reference sequence recorded in NCBI. Results (Table3-2)

explain the amino acids alignment between patients and reference sequence ID: NP_002511.1, Length: 294, Range 1: 47 to 87 nucleophosmin isoform 1 *Homo sapiens*.

Table (3-2): Alignment of exon 3 amino acids 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

Query	1189	VSLGAGAKDELHIVEAEAMNYEGSPIKVTLATLKMSVQPTV	1311
		VSLGAGAKDELHIVEAEAMNYEGSPIKVTLATLKMSVQPTV	
Sbjct	47	VSLGAGAKDELHIVEAEAMNYEGSPIKVTLATLKMSVQPTV	87

The position of amino acid (threonine, T) that related to the expected rs753788683 SNP occurred in this exon 3 at position 75 in the reference sequence. These results showed there was no change in the sequence of amino acids (100% similarity) between patient and reference sequence. The codon that encodes the threonine at position 75 was ACA. The SNP was a substitute on mutation in the changed A to G 1275 (rs753788683) causes transition in the codon from ACA to ACG, however this new codon encodes the same amino acid threonine, so the resulted mutation was silent and not affected the amino acid sequence.

The exon3 and exon2 encode the N-terminal region of NPM1 protein. Even with the mutation in exon3 (rs753788683),this mutation was not affected on amino acid sequences. 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, 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).In addition the N-

terminal region contain high conserved hydrophobic leucine-rich NES motifs (Wang *et al*., 2005;Yu*et al*., 2006), therefore it is very difficult that mutations to occur in this region.

3.3.Assessment of Multidrug Resistance Gene (*MDR1*)Expression.

One of the major problems in the treatment of AML is the progress of resistance against chemotherapeutic agents. To determine the degree of acquisition of resistance, quantitative gene analysis has been developed to distinguish *MDR1* gene expression in AML Iraqi patients.

3.3.1. RNA Extraction.

In order to study *MDR1* gene expression in Iraqi patients with AML, RNA was first extracted from fresh blood samples of patients and healthy controls. The concentration of RNA solution extracted from all samples was determine by nano-drope and were ranged between 2.02- 2.13 ng/µl, while the purity was ranged between 1.85-1.90.

3.3.2.cDNA Synthesis

Reverse transcription (RT) is the process by which RNA is used as a template to synthesize cDNA. This cDNA then serves as the template in the real-time reaction (Freeman *et al*., 1999).The method of reverse transcription was performed in two-step RT method, the two-step reaction requires that the reverse transcriptase reaction and PCR amplification be performed in separate tubes. The extracted RNA from blood samples were reverse transcriptase by used oligo (dT) as primer for cDNA synthesis. The cDNA reverse transcriptase from only RNA with poly A tails.

3.3.3.Real-time Polymerase Chain Reaction Validation

The Real Time - PCR run (first run), was shown several samples of DNA was appeared as a line over the threshold line (positive result), and the positive control (one curve) with the negative control (the line appear under the threshold line).

Specific primers were designed for *MDR1* gene expression in real-time PCR. The result of amplification reaction was summarized in the Fig. (3-8). The amplification reaction increase dependent on mRNA copy of MDR1 gene in each group that treated with chemotherapy. Also real-time PCR products were shown only a single melting temperature peak was observed for each reaction figure (3-9), thus suggesting that nonspecific amplification did not occur, like primer dimmer, DNA contamination and other non-specific binding.

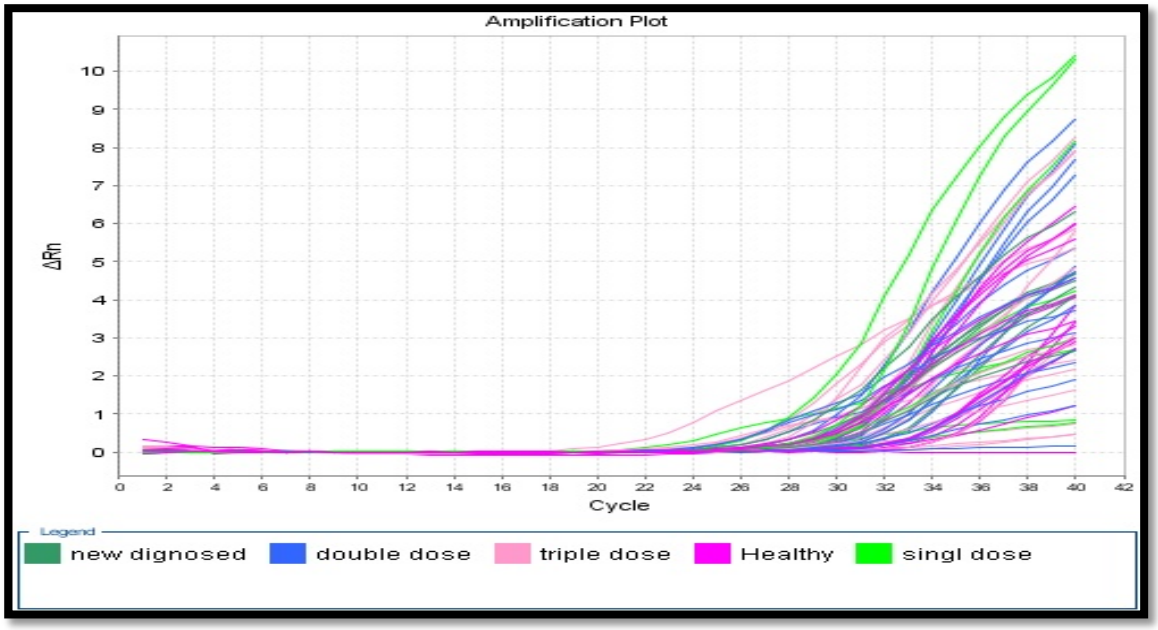

Figure (3-7): The result of Real Time qPCR amplification plot of several samples

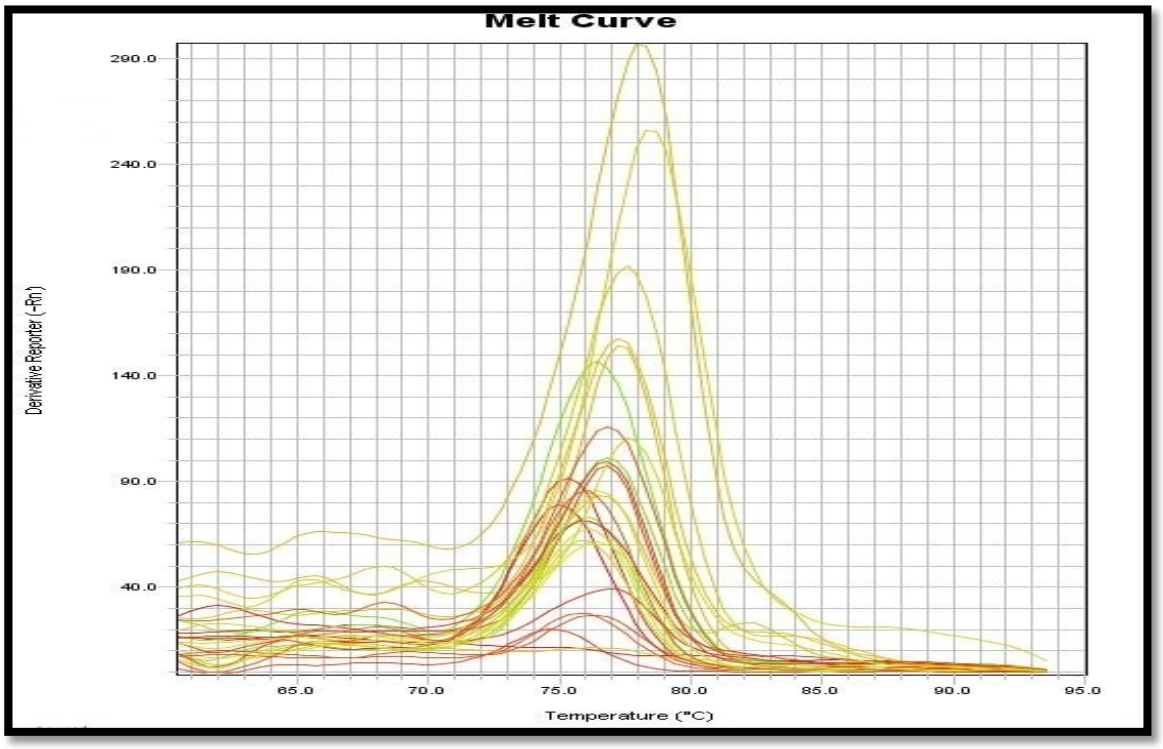

Figure (3-8):The result of Real Time qPCR melting curve of several samples .

3.3.4. Expressions of *MDR1*.

The expression of *MDR1* gene in AML patient with acute myeloid leukemia was achieved by RT-qPCR. The patients were categorized dependent on their chemotherapy induction, as newly diagnosed AML (No treatment started) consisted of 10 patients, AML patients after first course induction of chemotherapy, AML patients after second induction and AML patients in consolidation. The healthy control (untreated) used as calibrator. The results which summarized in table (3-3).

Table (3-3) show result of relative quantification of MDR1 gene between healthy calibrator and four AML group.

Group	Mean ct of MDR1 gene	Mean ct of GADPH	ΔCt	Ratio (2-ΔΔct)	P Value
Healthy control	32.01± 0.8138	27.66± 0.9781	4.35	1	---
New diagnosed	32.80± 2.178	28.28± 1.368	4.52	-0.34	0.1591
Single dose	30.31± 1.353	27.15 ± 1.954	3.42	2.22	0.2222
Double dose	29.45± 1.026	27.69 ±1.038	1.55	5.6	< 0.0001
Triple dose	27.76± 1.976	27.12± 2.042	0.56	7.58	< 0.0001

indicates the *MDR1* expression and the GADPH endogenous control, between the different groups. The Y axis represents the mean CT value of expression while X axis represents the five group of analysis. Result were shown in Fig.(3-9) of GADPH expression that no significant differences occurred in healthy control and the four groups of AML patients.

In addition, results also were indicated that no significant differences occurred for the newly diagnosed patients first course AML patients as compared with controls (Fig. 3-10). Nevertheless significant decreases in CT value were recorded for both the second induction AML group and AML consolidation group with *p* value of 0.0070 and <0.0001, respectively, as compared healthy controls, indicating the induction for higher expression of *MDR1* gene by increasing the challenge of AML patients with chemotherapy regime.

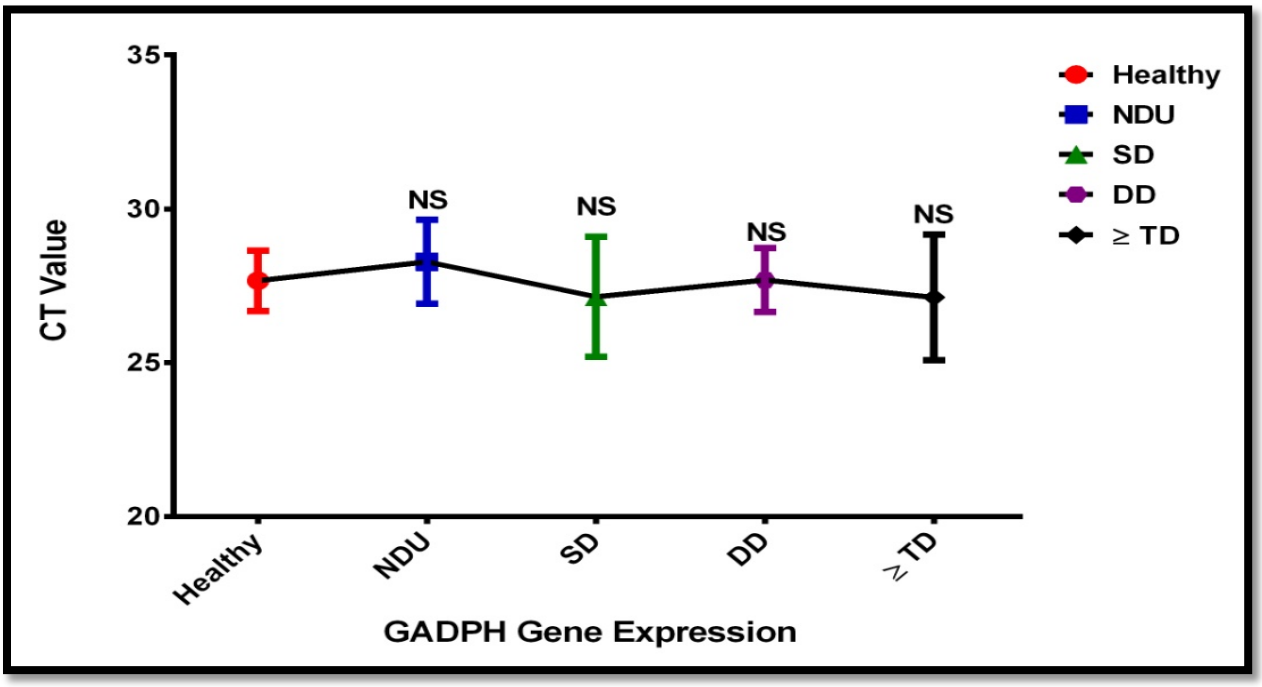

Figure (3-9): GADPH endogenous control gene expression in contol and AML patients.(NDU) New diagnose untreated, (SD) single dose, (DD) double dose, (TD) Triple dose, (NS) non-significant

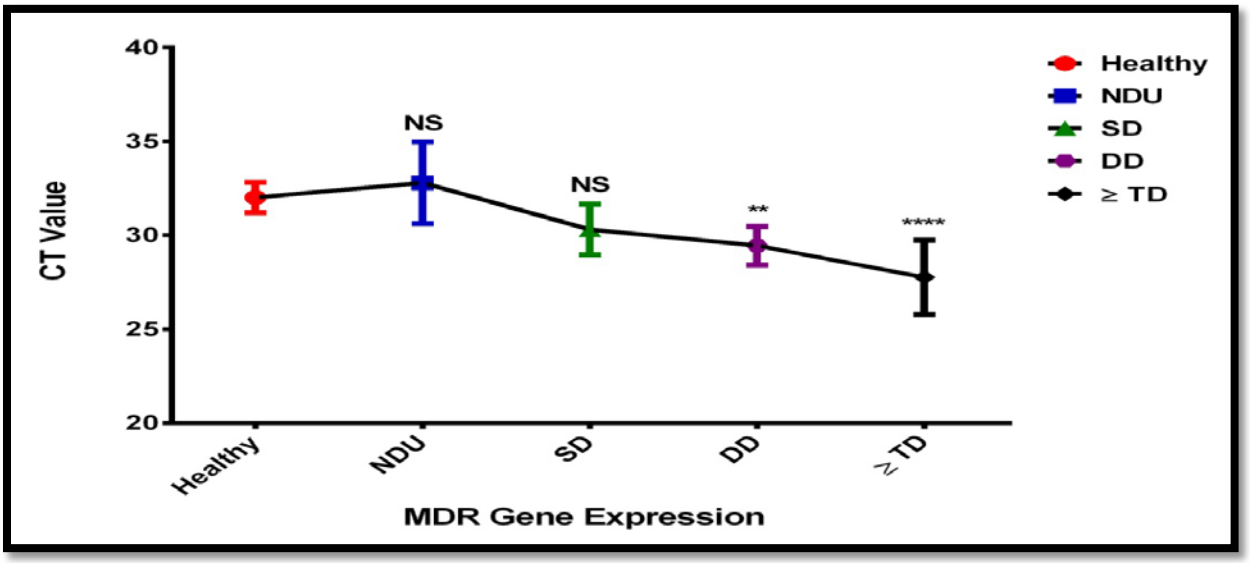

Figure (3-10): The expression of MDR1 gene in patient and control.(NDU) New diagnose untreated, (SD) single dose, (DD) double dose, (TD) Triple dose, (NS) non-significant.

The goal of chemotherapy treatment is to achieve a complete remission of AML 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 genetics 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 (Kern *et al*., 2000; Ismail and Hosny, 2011; Yanada and Naoe, 2012).

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. 3-11). Results showed no significant coloration between healthy and newly diagnosed, since the relative expression ratio was -0.34 fold p value 0.1591, therefore both groups have similar *MDR1* gene expression, this suggesting that no induction in gene expression. Also no significant differences between the health 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* value < 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 value < 0.0001.

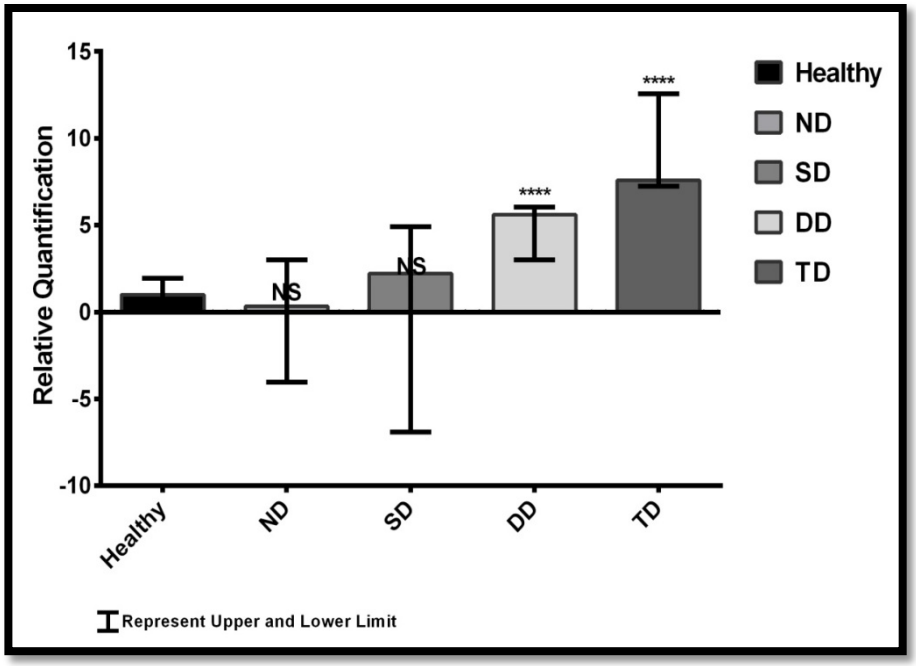

Figure (3-11): Relative quantification of different AML groups compared with healthy controls (calibrator). (ND) new diagnosed, (SD) single dose, (DD)Double dose, (TD) triple dose

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 MDR1was observed in group (2) 5.6fold and group (3) 7.58 fold, and in other hand the non-difference in *MDR1* gene expression between the calibrator and new 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 about7.58 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 of drug 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 somewhat more differentiated and do not have long-term self-renewal capacity. When therapy is discontinued, the CSCs repopulate the 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 (Fletche *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 cur patient and also increase the severity of AML disease.

chapter four

Conclusions and Recommendations

4.1. Conclusions:

1. Single nucleotide polymorphism was observed in the three positions (SNP) G/A792, G/A794 and G/A797 that not correlated with AML disease and being first described in Iraqi population.
2. Two mutations were observed in intron region at position G/A635 and G/A660 and identify in 80% Iraqi AML patients. These SNP were correlated with AML Iraqi patients.
3. Single nucleotide polymorphism was observed in exon 3 that was identified in 70% AML patients A/G 1276, the type of SNP was silent mutation and had no effect on amino acid sequence.
4. No genetic variations were detected in exon2 of AML patient.
5. *MDR1* gene expression was correlated with chemotherapy induction in AML patients.
6. High *MDR1* gene expression was detected in second chemotherapy induction and patients in consolidation AML groups; this suggested the chemotherapy resistant up-regulation could be acquired during induction treatment.
7. Increased *MDR1* expression after induction associated with early relapse and progression disease.

4.2. Recommendations:

1. Detecting mutations in other exon of *NPM1* gene with the incidence of AML in Iraqi patient.
2. Detecting the association of other SNPs in *NPM1* gene with the incidence of AML in Iraqi patients.
3. Studying different SNPs in different genes and their association with the incidence of AML in Iraqi patients.
4. Assessing the drug resistance capability of AML patients which in turn consider as an important aspect of treatment before induction of chemotherapy.
5. Detecting SNP (s) related to *MDR1* gene that has effect on the response to chemotherapy in AML patient.
6. Studying hypo-methylation and hyperacetylation at the 5' promoter of *MDR1*gene which may leads to increased expression in AML patient.
7. Studying other transport genes that associated with chemotherapy resistant.

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Appendix (1) Nucleotide sequence alignment of the amplified *NPM1* Gene for healthy control.

Score	Expect	Identities	Gaps	Strand
2723 bits(1474)	0.0	1492/1501(99%)	0/1501(0%)	Plus/Plus

Nucleophosmin isoform 1

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Query  1     TTAACTTTAGGAACGGTACTTAAACTTTCAAAATAAACTACTTAACCCTACTTGATTTCA  60
             ||| ||||| ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  7413  TTATCTTTAAGAACGGTACTTAAACTTTCAAAATAAACTACTTAACCCTACTTGATTTCA  7472

Query  61    GCCTTTTAGTTTCTATTCATGTGGCTTGAGACtttttttCCTTTGCTGACTGCTTATAAA  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  7473  GCCTTTTAGTTTCTATTCATGTGGCTTGAGACTTTTTTTCCTTTGCTGACTGCTTATAAA  7532

Query  121   ATACTATTTCTTACACCTGGGTATTGTGTGTACCTCACTGTCTGTACATCTTTGAAGAAC  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  7533  ATACTATTTCTTACACCTGGGTATTGTGTGTACCTCACTGTCTGTACATCTTTGAAGAAC  7592

Query  181   TTGGCATCTATACCACTCATTTGGTAACCATGTAAATTCCAGTCACACTGTTCAACTTGC  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  7593  TTGGCATCTATACCACTCATTTGGTAACCATGTAAATTCCAGTCACACTGTTCAACTTGC  7652

Query  241   CTTATTTTCTTCAGTTGAATTACAAAGCCCTTGTAAAAGGCATCGATAATCTTTCATGTC  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  7653  CTTATTTTCTTCAGTTGAATTACAAAGCCCTTGTAAAAGGCATCGATAATCTTTCATGTC  7712

Query  301   TACCAGAGACTAGCCTTCTGCTCAACTTAGAAGTTGCTCAATAAATATGTTAGGTGGTCT  360
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Query  361   ATATTGTGTGTATGCATATTTATGCATTTCTTAAATTTTCTGAATATGAGAAACTGATTT  420
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Sbjct  7773  ATATTGTGTGTATGCATATTTATGCATTTCTTAAATTTTCTGAATATGAGAAACTGATTT  7832

Query  421   GCCAAGATCACAAAACCTGAGGACAACATTGCACAAATTTGTTTTCCAGGGTAATAGTGA  480
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Sbjct  7833  GCCAAGATCACAAAACCTGAGGACAACATTGCACAAATTTGTTTTCCAGGGTAATAGTGA  7892
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Query  481   GAGGTTTATACTTGTTTTTTaaaaaaaTCACAGTCATGTGCCTCATTGCAACTGCTGGGT  540
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Sbjct  7893  GAGGTTTATACTTGTTTTTTAAAAAAATCACAGTCATGTGCCTCATTGCAACTGCTGGGT  7952

Query  541   CAACAGATGGATCGCATATACAATGGTGGTCCCATAAGATTATTATACCTTATTTTTAAT  600
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  7953  CAACAGATGGATCGCATATACAATGGTGGTCCCATAAGATTATTATACCTTATTTTTAAT  8012

Query  601   ATACCTTTCCTGttttttttttCTTTGGAGGCAGTCTCGCTCTGTCACCCAGGCTGGAG  660
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Sbjct  8013  ATACCTTTCCTGTTTTTTTTTTTCTTTGGAGGCAGTCTCGCTCTGTCACCCAGGCTGGAG  8072

Query  661   TGCAGTGGTGTAATCTCGGCTCACTGCAACCTCTGATTTCCGAGTTCAAGTAATTCCCTG  720
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Query  721   CCTCACCCTCCCGAGTAGCTGGGATTACAGGCATGTGCCACCACACCTGGCTAATTTTTG  780
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Sbjct  8133  CCTCACCCTCCCGAGTAGCTGGGATTACAGGCATGTGCCACCACACCTGGCTAATTTTTG  8192

Query  781   TGTTTTTAGTAAAAACAAGGTTGTTACCATGTTGTCCAGGCTGGTCTCAAACTCCTGACC  840
             |||||||||||  | || ||||||||||||||||||||||||||||||||||||||||||
Sbjct  8193  TGTTTTTAGTAGAGACGAGGTTGTTACCATGTTGTCCAGGCTGGTCTCAAACTCCTGACC  8252

Query  841   TCATGATCTGCCCGCCTCAGCCTCCCTAAGTGTTGGGATTACAGGCATGAGCCACCAAGC  900
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Query  901   CCGGCCCTTTCCTATGCTTAGATGCACAAATACTGTGTTTCGGTTGCTTACAGTATTCAG  960
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Sbjct  8313  CCGGCCCTTTCCTATGCTTAGATGCACAAATACTGTGTTTCGGTTGCTTACAGTATTCAG  8372

Query  961   TACAGTAACACTGTACAGGTTTGTAGCCTAGGTGTGTGTAGTAGGCTATACCATCTAGGT  1020
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Query  1021  TTGTATAAGCACATTCTTATGATTGTACAAAGATGAAATTGTCTAACAACACATTTCTCA  1080
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  8433  TTGTATAAGCACATTCTTATGATTGTACAAAGATGAAATTGTCTAACAACACATTTCTCA  8492

Query  1081  GAACCTAGCCCTGTGGTTAAGTGACGCATGGCTGCATATAACATTTAGTgggggggTGTA  1140
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Query  1141  AAATAGGTGGAACTCAAAAGTTGAAGTAGTAttttttttttGTTCACAGGTCAGTTTAGG  1200
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  8553  AAATAGGTGGAACTCAAAAGTTGAAGTAGTATTTTTTTTTTTGTTCACAGGTCAGTTTAGG  8612

Query  1201  GGCTGGTGCAAAGGATGAGTTGCACATTGTTGAAGCAGAGGCAATGAATTACGAAGGCAG  1260
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Query  1261  TCCAATTAAAGTAACACTGGCAACTTTGAAAATGTCTGTACAGCCAACGGTAAGGGCACT  1320
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Query  1381  TAGTTGTGCCAAGGAGATAGAAAGTGGTTCTTTATCTTCTGTCACTGGAGTTCGATGGTC  1440
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Query  1441  AACTCTTGAACATGGGGGCGTCTCCTGCTACTTTTATCATAGGTGAAAAAACAGGTTCAC  1500
             |||||||||||||||||| ||| ||||||||||||||| ||||| ||||||||||||||
Sbjct  8853  AACTCTTGAACATGGGGGCTTCTGCTGCTACTTTTATCAGAGGTGGAAAAACAGGTTCAC  8912

Query  1501  T  1501
             |
Sbjct  8913  T  8913
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الملخص

الطفرات في مورثة NPM1 تعتبر من اكثر الطفرات شيوعا في مرضى ابيضاض الدم النخاعي الحاد. تم استخدام تقنية PCR للكشف عن حدوث الطفرات في مورثة NPM1 في الاكسون ٢ والاكسون ٣. القطعة من bp ٢٣٨٠ الى bp ٤١٤٠ التي تمثل المنطقة المحيطة الاكسون ٢ و٣ من مورثة NPM1، تم تضخيم هذه القطعة باستخدام نوعين من البوادء. اظهرت النتائج التركيز الحامض النووي للعينات (٥٢ -٩٣) ng/ml بينما كانت النقاوة من (١.٨- ١.٩٢). من جهة اخرى كان ناتج البادئ الاول هو ١١٥٠ bp بينما كان ناتج البادئ الثاني هو ١٣٣٧ bp . ايضا اظهرات النتائج ثلاثة SNP (٧٩٢ G/A، ٧٩٤ G/A، ٧٩٧ G/A) تم الكشف عنها في في منطقة intronفي٩٠% في المرضى ٧٠% في الاصحاء. ايضا من خلال البحث ، تم الكشف عن SNP في الاكسون ٣ بنسبة ٧٠% من المرضى في منطقة ١٢٧٥ A/G . بل الاضافة تم الكشف عن اثنان SNP في منطقة النترون بنسبة ٨٠ % في المرضى لاكن ليس في الاصحاء في الموقع ٦٣٥ A/G ، ٦٣٦ A/G . لم يتم العثور على اي طفرة في منطقة الاكسون ٢.

تم دراسة زيادة التعبير الجيني لمورثة MDR1 وعلاقتها بالمقاومة للعلاج الكيميائي، تم جمع عينات من ٤٠ مرضى ابيضاض الدم النخاعي الحاد و١٠ متطوعين اصحاء. تم عزل RNA من جميع العينات وتحويلها الى Cdna . اظهرت النتائج ان تركيز RNA يتراوح بين (٢.٠٢-٢.١) ng/ml بينما النقاوة (١.٩٠-١.٩٥). تم تقسيم عينات المرضى الى اربعة مجاميع اعتمادا عل عدد جرعات المعطاة، اظهراالنتائج ان لا يوجد اي تغير في تعبير الجيني بين المجموعة الأولى والمجوعة الثانية مقارنتا مع الاصحاء . بينما اظهرت النتائج ان هناك نقصان في قيمة CT في المجموعة الثالثة والمجموعة الرابعة مقارنتا مع الاصحاء وسجل قيمة P value ٠.٠٠٠١ لكلا المجوعتان. هذا يدل على ان زيادة التعبير الجيني له علاقة في مقاومة العلاج الكيميائي لمرضى ابيضاض الدم النخاعي الحاد

الإهداء

لايطيب الليل إلا بشكرك ولايطيب النهار إلى بطاعتك.. ولا تطيب الآخرة إلا بعفوك .. ولا تطيب الجنة إلا برضاك

الله جل جلاله

إلى من بلغ الرسالة وأدى الأمانة .. ونصح الأمة .. إلى نبي الرحمة ونور العالمين

سيدنا محمد صلى الله عليه وآله وصحبه وسلم

إلى من كلله الله بالهيبة والوقار .. إلى من علمني العطاء بدون انتظار .. إلى من أحمل أسمه بكل افتخار

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إلى من كان دعائها سر نجاحي إلى ملاكي في الحياة .. إلى معنى الحب والحنان ..

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إلى من حبهم يجري في عروقي إلى القلوب الطاهرة الرقيقة والنفوس البريئة إلى رياحين حياتي

أخوتي

الى من يدعمني في كل خطوات حياتي .. الى الروح التي سكنت روحي

زوجيتي الحبيبة

إلى كل من بذل الجهد والعطاء

أساتذتي الكرام

جمهورية العراق
وزارة التعليم العلي و البحث العلمي
جامعة النهرين
كلية العلوم
قسم التقانة الاحيائية

تقييم الطفرات في الاكسون ٢ و ٣ لموروثة NPM1 و دراسة التعبير الجيني لموروثة MDR1 في بعض مرضى ابيضاض الدم النخاعي الحاد في العراق

رسالة
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من قبل
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