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Genetic and Biochemical Study of Myelodysplastic Syndrome patients in Iraq

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بِسْمِ اللَّهِ الرَّحْمَٰنِ الرَّحِيمِ

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Dedication

*To the smile of life, the source of endless support and all love ....**My Mother***

*To the person who suffered from the hardships and struggled to bring me to this stage…… To the person who supported , encouraged me to science…….. **My father***

*To those who surrounded me and encouraged me to reach my dreams… **My brother and sisters***

Wid

Summary

The myelodysplastic syndrome (MDS) is a heterogeneous group of clonal hematopoietic disorders characterized by various clinical presentations, risks for leukemia transformation and reduced survival. This makes the accurate diagnosis and risk stratification of MDS patients clinically relevant.

The present study was done in the laboratory of DNA, department of Biology, Faculty of Science, University of Babylon. The samples collection and practical work of the present study extended through the period from June 2019 to the November 2020. Samples of myelodysplastic syndrome collected from different regions of Iraq.

The samples were females and males with age range (20-83) years old. These include (75) blood samples which grouped as following: control group (25) samples (15 males and 10 females), and patients group including (50) blood samples (29 males and 21 females).

The present study is divided in four parts: demographic hematological, physiobiochemical and molecular studies. The first part include studying the correlation of gender age, and body mass index with the disease. The second part include estimation of hematological parameters. While the third part include determination of adiponectin, leptin, resistin hormones, ferritin, oxidative stress or reactive oxygen species, and antioxidants. The fourth part includes studying the genetic effect of candidates genes: Adiponectin leptin (*LEP*), resistin (*RETN*), splicing factor 3 B1 (*SF3B1*) and serine arginine-rich splicing factor 2 (*SRSF2*) genes in patients with myelodysplastic syndrome.

The results of the current study was showed that the male percentage value of myelodysplastic syndrome was more than female percentage value (58% and 42%, respectively). Results indicate that the age risk of disease incidence was begin in 60 years old and increased between 60-75 years old for both sexes, while body mass index was decreased significantly in patients with MDS patients.

The results of hematological parameters exhibited that were decreased significantly ($P \leq 0.05$) in most parameters except MCH which increased significantly compared to control and non-significant differences in basophil MCV and MCHC. Biochemical study showed significant increase ($P \leq 0.05$) in concentration of adiponectin and resistin hormones, while leptin hormone showed no significant differences at ($P \leq 0.05$) between patient and control. Serum ferritin level was significantly ($P \leq 0.05$) higher in patients with MDS.

Oxidative stress or reactive oxygen species showed higher significant ($P < 0.05$) concentration in patients with MDS as compared with control. Antioxidants activity superoxide dismutase (SOD) and glutathione peroxidase (GPx), exhibited higher significant at ($P \leq 0.05$),between patients and control, While GSH showed lower significantly ($P \leq 0.05$), activity. Also correlation results indicated negative correlation between adiponectin and resistin with BMI, Hb, and platelets, while positive correlation with ferritin. Also, there were negative correlation (r=-247) among resistin and leptin, on the other hand adiponectin has positive correlation (r=624) with resistin.

In the molecular study, for adiponectin gene showed a high percentage of the adiponectin rs1501299 mutated genotype GG and alleles G in MDS patients (54% and 73% respectively) compared with the healthy individuals in control group (36% and 62% respectively), and this was associated with significant differences ($P \leq 0.05$), while leptin gene identified a clear relationship between the genetic heterogeneity of *LEP* gene with MDS where the genotype GG and mutant allele G appeared in a high percentage (59.1% and 70% respectively) among MDS patients. This means that the *LEP* has become an etiological factor for this syndrome. Resistin gene (*RETN)* showed that no significant variation between patient with myelodysplastic syndrome and control group using PCR-SSCP and sequencing techniques that indicated a single nucleotide polymorphism (SNPs) located in intron 2.

Sequencing of splicing factor 3B1 (*SF3B1*) gene there was single nucleotide polymorphism (SNPs) located in intron 11(rs3217350), and the rate of mutant II genotype and I allele were significantly higher among cases with MDS (92.5% and 92% respectively) compared to healthy individuals in control group (60% for each one of them), on other hand serum level of ferritin increased in cases with mutant II genotype (1003.41ng/ml) compared with wild DD genotype (355.04 ng/ml) so that reflected statistical difference (P=0.0019). Gene expression of serine arginine-rich splicing factor 2 (*SRSF2*), there was a significant variation between patients and control derived from folding changes of melting curve and amplification of real-time PCR products.

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List of Abbreviations

Symbol	Definition
MDS	Myelodysplastic syndrome
AML	Acute Myeloid Leukemia
SNP	single nucleotide polymorphism
NCBI	National Center for Biotechnology Information
FISH	Fluorescence In Situ Hybridization
TNF-α	Tumor Necrosis Factor-alpha
ARMS	Amplification Refractory Mutation System
SSCP	Single Strand Conformation Polymorphism
BM	Bone marrow
SF	Serum Ferritin
NGS	Next Generation Sequence
PCR	Polymerase Chain Reaction
ZRSR2	Zinc Finger CCCH-Type
U2AF1	U2 Small Nuclear Auxiliary Factor 2
PRPF40	Pre-mRNA Processing Factor 40 homology B
PAGE	PolyAcrylamide Gel Electrophoresis
bp	Base pair
CBL	Casitas B-lineage
JAK2	Janus kinase 2
SETBP1	SET binding protein 1
IDH1	Isocitrate dehydrogenase 1
ETV6	Ets-leukemia virus
STAG2	Stromal antigen 2
IO	Iron overload
C/EBP	CAAT/Enhancer binding protein

NARS	Asparaginyl-tRNA synthetase
hnRNA A1	Heterogenous nuclear ribonucleoprotein A1
SS	Splicing site
CMML	Chronic mylomonocytic leukemia
MCV	Mean corpuscular volume
MCH	Mean corpuscular hemoglobin
MCHC	Mean corpuscular hemoglobin concentration
HCT	Hematocrit
PM	Pico mole
RCMD	Refractory cytopenia with multilineage dysplasia
RA	Refractory anemia
MDS/MPN	Myelodysplastic syndrome/ Myeloproliferative neoplasm
RUNX1	Runt related transcription factor 1

1. Introduction

Myelodysplastic syndromes (MDS) are clonal stem cell malignancies characterized by cytopenias, inefficient hematopoiesis, dysplasia in one or more myeloid cell lineages and increased risk of development of acute myeloid leukemia (AML). It is sub-classified based on percent of bone marrow (BM) and peripheral blood blasts, type/degree and number of dysplastic cell lineages, presence/absence of ring sideroblasts (RS) and presence of specific chromosomal abnormalities. The median age at the diagnosis of MDS is 71 years (Visconte *et al.*,2014).

Anemia is represent in most patients, the mean corpuscular volume (MCV) is often increased and an increased erythrocyte distribution width (RDW) which the erythropoiesis disturbances. A dimorphic red blood cell (RBC) population (macrocytes and microcytes), anisocytosis, poikilocytosis, nucleated red blood cells, basophilic stippling and Howell-Jolly bodies are also indications that the erythrocyte has undergone abnormal development (Greebreg *et al.*,2013). Peripheral blood may reveal very abnormal nuclei such as Pelger-Huet anomalies and hypo-or hypersegmentation and ring forms nuclei also occur in neutrophils are important morphological features in MDS/MPN peripheral blood when diagnosing and distinguishing MDS/MPN is important to understand the similarities and differences in pathologic mechanism from similar diseases (AML, infectious diseases and other causes of cytopenia). The platelet morphological changes include giant platelets and platelets hypogranulation or agranulation. Some platelets may possess large fused granules. Circulating micromegakaryocytes (dwarf cells), multiple small nuclei separated by strands of nuclear material, and large mononuclear cells with dysmorphic nuclear features have been described in peripheral blood from patients with MDS (Vallesp *et al*.,1998)

Adipocyte secretes active biological molecules, mainly including leptin, resistin, and adiponectin (Ouchi *et al.*,2011). Adipokines are cytokines secreted predominately by the adipose tissue. They exert a variety of distinct metabolic,

endocrine, and immune functions, both locally and systemically. Adiponectin is an adipocyte-derived secretory protein, which is an important regulator of inflammatory responses (Zacharioudaki *et al.*,2009). In many inflammatory states, adiponectin levels are inversely correlated with pro-inflammatory markers (Behnes *et al.*,2012). Overall, adiponectin exerts predominantly anti-inflammatory effects and suppresses the proliferation of myelomonocytic progenitor cells (Mandal *et al.*,2011).

Leptin exerts actions through its specific receptor which is localized to the cell membrane and present in a variety of hematopoietic cells, such as hematopoietic progenitor cells (Mouzaki *et al.*,2009). Leptin, produced by adipocytes and stromal cells, represents a pleiotropic adipokine, regulating energy homeostasis as well as metabolic, reproductive, neuroendocrine, and immune functions. Leptin's major physiological role is to signal inadequate rather than excess energy stores. Indeed, hypoleptinemia found in a small but significant percentage of obese humans is associated with hyperinsulinemia and impaired T-cell function. Furthermore, accumulating experimental evidence suggests that leptin through its receptor may stimulate various signal transduction pathways leading to cell growth, apoptosis inhibition, migration and invasion. Particularly, leptin *in vitro* induces proliferation and apoptosis inhibition in cytokine-dependent leukemia cell lines. However, in contrast to several *in vitro* studies, epidemiological study have reported inconsistent associations between leptinemia and risk of malignancies. Our group, which has focused on adipokines and cancer has shown that hypoleptinemia and not hyperleptinemia is linked to risk of several malignancies (Dalamaga *et al.*,2013). Adiponectin has been shown previously to be inversely related to risk of AML, MDS, myeloproliferative disorders and multiple myeloma (Petridou and Mantzoros, 2006).

Another adipokine, resistin, forms an important link between obesity, insulin resistance, and diabetes (Stofkova *et al.*,2010). In humans, increased levels of resistin have been found in mononuclear leukocytes and macrophages (Jamaluddin

et al.,2012). Resistin has further been associated with inflammation in systemic autoimmune diseases (Tanaka *et al*.,2013).

Ferritin is a main iron storage protein found in all cell types thus it can be used as a reliable indicator of its overload. Hence, the continuous screening and serum ferritin and iron levels assessment associated with other clinical and laboratory indicators can provide a substantial insights of diagnosis and treatment in MDS patients (Dehghani and Sanei, 2020). Previous study showed an important role of oxidative stress in the pathogenesis of myelodysplasia. An increased level of DNA oxidative damage has been observed in MDS CD34+ bone marrow cells, suggesting their involvement in ineffective haematopoiesis by inducing apoptosis in bone marrow precursors (Low-risk MDS patients also exhibit oxidized nucleotides in more differentiated CD34- bone marrow cell populations, which contributes to genomic instability and disease progression (Novotna *et al*.,2009).DNA damage caused by oxidative stress plays an important role in the initiation and carcinogenic process of mutation. A recent study has suggested that oxidative stress leads to increased mutation frequency in a murine model of myelodysplastic syndrome (MDS) (Chung *et al.*, 2014), and that additional mutations lead to the progression of MDS. Furthermore, a correlation has been reported between the serum ferritin level and serum reactive oxygen species (ROS) levels in MDS patients (Gonçalves *et al.*,2015).

A wide array of recurring genetic mutations involved in RNA splicing, histone manipulation, DNA methylation, transcription factors, kinase signaling, DNA repair, cohesion proteins, and other signal transduction elements has been identified as important substrates for the development of MDS. Cytogenetic abnormalities, namely those characterized by loss of genetic material (including 5q- and 7q-), have also been strongly implicated and may be influence the clonal architecture which predicts such mutations and may provoke an inflammatory bone

marrow microenvironment as the substrate for clonal expansion (Shallis *et al.*,2018).

Alternative splicing occurs in > 90% of human protein-coding genes resulting in the production of multiple mRNA isoforms, and is a major source of protein diversity (Saez *et al.,* 2017). Alternatively and aberrantly spliced mRNA isoforms are oftenfound in human cancers and play a role in tumorigenesis (Scotti and Swanson,2016). The most frequently mutated splicing factors in MDS form part of a complex that coordinates 3′splice site recognition duringpre-mRNA splicing. It has been demonstrated that the presence of splicing factor mutations leads to aberrant 3′splice site recognition (Pellagatti and Boultwood, 2017) resulting in the generation of aberrantly spliced mRNA transcripts in the bone marrow cells of patients with myeloid malignancies and in mouse models expressing these mutations (Shiozawa *et al.*, 2018).

1.1.Aim of the study

Study the risk factors associated with myelodysplastic syndrome patients by

1. Demoghrapgic study of myelodysplastic syndrome and their relationship with age and gender.
2. Determination of hematological parameters.
3. Estimation of serum adiponectin, leptin and resistin also, genetically and there correlation with other parameters.
4. Measurement of serum ferritin with reactive oxygen species and antioxidants.
5. Detection of gene polymorphism of adiponectin , leptin and resistin genes by tetra amplification refractory mutation, sequencing and single strand conformational polymorphism respectively.
6. Sequencing of splicing factor 3B1 (*SF3B1*) gene.
7. Gene expression of serin arginine-rich factor 2 (*SRSF2*) gene.

2. Review of Literature

2.1. Definition of Myelodysplastic Syndrome

Myelodysplastic syndromes (MDS) commonly known as bone marrow (BM) failure, defined as heterogeneous group of myeloid-clonal disorders, due to the failure of blood-cells-maturation. The co-morbidities are caused by a varying degree of cytopenia and clonal instability with a tendency to progress mostly in case called acute myeloid leukemia (AML) but it is less common into acute lymphoblastic leukemia (Steensma, 2015).

Generally, MDS caused in 5/100,000 of human beings, on the other hand, the patients in 70 years and older than this age, this rate rises between 22 and 45 per 100,000 and keep increasing by age. The treatment of MDS is considered complicate in general way by advanced age of the patients (median ages, 65–70 years), resulting in nonhematologic-comorbidities, in addition to relative inability to tolerate certain intensive forms of therapy among older patients. Also, when the disease develops to AML, the patients will response in low levels to the standard-therapy than patients with *de novo* AML (Chen *et al.*,2019).

2.2. Classification of Myelodysplastic Syndrome

2.2.1. French-American-British (FAB) Classification

Many developed classifications were performed for prediction of MDS which transformed to acute myeloid leukemia. One of the most common types of classification was French-American-British system in 1982, table (2-1), this system was designed according to the blasts-percentage also according to the characteristics of the blood and bone marrow, namely medullary and peripheral blast cell count, in addition to ringed sideroblasts, number of monocytes in peripheral blood, and Auer

rods. According to this classification, patients are diagnosed with MDS when dysplastic-changes in bone marrow are present and/or myeloblast-cells are between 5 and 30% of all bone-marrow-cells. Five subgroups with significantly different prognoses were established: refractory anemia (RA) with blasts <5% in BM, refractory anemia with ringed sideroblasts (RARS) with blasts <5% and ring sideroblasts > 15%, refractory anemia with excess of blasts between 5 and 20% (RAEB), RAEB in transformation to acute leukemia and blast cells ranged between 20 and 30% (RAEB-T) and chronic myelomonocytic leukemia characterized by increase of peripheral blood monocytes (CMML). (Bennett *et al*., 2005; Vardiman *et al*., 2009).

Table (2-1) : Myelodysplastic syndrome Types According to FAB Classification

Type	Blasts in blood	Blasts in bone marrow
Refractory anemia (RA)	<1%	Blasts <5%, ring sideroblastic <15%
Refractory anemia with ring sideroblastic (RARS)	<1%	Blasts <5%, ring sideroblasts >15%
Refractory anemia with excess of blast (RAEB)	<5%	Blasts 5–20%
Refractory anemia with excess blast in transformation (RAEB-t)	<30%	Blasts 20–30%
Chronic myelomonocytic leukemia (CMMoL)	<5% with increase Monocytes	Blasts 0–20%

CMML, chronic myelomonocytic leukemia blast cells <20% and monocytes ≥1000/µl); RA, refractory anemia <1% in PB and <5% blasts in BM; RAEB, RA with excess blasts in PB <5% and 5–20% blasts in BM; RAEB-t, RAEB with excess blasts in transformation between 20 and 30%; RARS, RA with ringed sideroblasts >15% (Bennett *et al.*, 2005).

2.2.2. The World Health Organization (WHO) Classification

The World Health Organization (WHO) Classification consider a last-updated system in 2008 (Swerdlow *et al*., 2008; Vardiman *et al*., 2009). The molecular features identified by the next generation sequencing (NGS) displayed important knowledge to understand the pathophysiology of MDS; this lead to yield other markers for indication of diagnosis and prognosis (Greenberg *et al.*, 2012, Arber *et al.*, 2016). In addition to clinical studies, the pathological proved WHO is a hypothesis for this complete program and the molecular genetics, hematologic, cytologic, and morphologic (Jaiswal *et al*., 2014). According to a lot of information and experience about MDS, the new classification table (2-2), which revises; produced improvements in the cytopenia and morphological changes, additionally, the influence of genetic information in MDS diagnosis and Classification (Genovese *et al.*, 2014).

Table (2-2): 2016 WHO Criteria of classifications of myelodysplastic syndromes.

Type	Dysplastic Lineage	Cytopenia [1]	Ring Sideroblasts in erythroid elements of Bone Marrow	Blasts	Cytogenetics
MDS-SLD	1	1 or 2	RS < 15 % (or < 5 %) [2]	PB < 1 % BM < 5% No Auer rods	Any, unless fulfills criteria for isolated del(5q)
MDS-MLD	2 or 3	1-3	RS<15% (or<5%2)	PB < 1% BM < 5% No Auer rods	Any, unless fulfills criteria for isolated del(5q)
MDS-RS MDS-RS-SLD	1	1 or 2	RS ≧ 15% (or ≧ 5%2)	PB < 1% BM < 5% No Auer rods	Any, unless fulfills criteria for isolated del(5q)
MDS-RS-MLD	2 or 3	1-3	RS ≧ 15% (or ≧ 5%2)	PB < 1% BM < 5% No Auer rods	Any, unless fulfills criteria for isolated del(5q)
MDS with isolated del(5q)	1-3	1-2	None or any	PB <1% BM <5% No Auer rods	del(5q) alone or with 1 additional abnormality except -7 or del(7q)
MDS-EB MDS-EB-1	0-3	1-3	None or any	PB 2~4% or BM 5~9%, no Auer rods	Any
MDS-EB-2	0-3	1-3	None or any	PB 5~19% or BM 10%~19% or Auer	Any
MDS-U With 1% PB blast	1-3	1-3	None or any	PB=1% [3], BM<5%, Auer rods	Any
with SLD and pancytopenia	1	3	<15% [4]	PB <1% BM <5% No Auer rods	MDS defining abnormality
RCC	1-3	1-3	None	PB <2% BM <5% No Auer rods	Any

WHO: World Health Organization; MDS: myelodysplastic syndromes; PB: peripheral blood; BM: bone marrow; RS: ring sideroblasts; MDS-SLD: MDS with single lineage dysplasia; MDS-MLD: MDS with multilineage dysplasia; MDS-EB: MDS with excess blasts; MDS-U: MSD, unclassifiable; RCC: refractory cytopenia of childhood. [1] Cytopenias MDS-defining: Hb<100g/L, PLT<100×109/L, ANC<1.8×109/L; absolute monocytes count<1.0×109/L; [2] with *SF3B1* mutation; [3] 1% PB blasts must record on at least two separate observations; [4] If with ≥15% ring sideroblasts and significant erythroid dysplasia, and are classified as MDS-RS-SLD (Hong and He, 2017).

2.3. Prognostic scoring system : International prognostic scoring system and revised (IPSS/IPSS-R)

Owing to the variability in outcome of MDS subtypes and to help in treatment selection, the International MDS Risk Analysis Workshop (IMRWS) developed a scoring system named International Prognostic Scoring System (IPSS). Firstly, published in 1997 and then revised in 2012 (IPSS-R)(Greenberg *et al.*, 1997; Greenberg *et al.*,2012) (Tables 2-3 and 2-4).

Table (2-3): Survival and AML evaluation based on International prognostic scoring system and revised (IPSS)

International Prognostic Scoring System (IPSS)					
Prognostic variable	**0.0**	**0.5**	**1.0**	**1.5**	**2.0**
Blasts in BM	**<5**	**5-10**	**-**	**11-20**	**21-30**
Karyotype	**Good**	**Intermediate**	**Poor**	**-**	**-**
Cytopenia	**0/1**	**2/3**	**-**	**-**	**-**

Risk category	Total score	Median survival (y) without treatment	25% AML prognostic (y) without treatment
Low	**0**	**5.7**	**9.4**
Intermedaite-1	**0.5-1.0**	**3.5**	**3.3**
Intermediate-2	**1.5-2**	**1.1**	**1.1**
High	**≥2.5**	**0.4**	**0.2**

Cytogenetics: good; normal, -Y alone, del(5q) alone, del(20q) alone; poor: complex (≥abnormalities) or chromosome 7 abnormality; intermediate: other abnormalities. Presence of karyotype t (8:21), t (15,17), and inversion 16, denote AML rather than MDS. Cytopenia; neutrophil <1800/ mcl, platelets<100,000/mcl, hemoglobin <10/mgm/dl. Adapted from NCCN guidelines version 1 (2019).

Table (2-4): Survival and AML evaluation based on the Revised International Prognostic Scoring System (IPSS-R)

Revised International Prognostic Scoring System (IPSS)							
Prognostic variable	**0.0**	**0.5**	**1.0**	**1.5**	**2**	**3**	**4**
Cytogenetics	**Very good**		**Good**		**Intermediate**	**Poor**	**Very good**
Blasts in BM	**≤2**	**-**	**>2-<5**	**-**	**5-10**	**>10**	**=**
Platelets	**≥100**	**50-<100**	**<50**	**-**	**-**	**-**	**-**
ANC	**≥0.8**	**<0.8**	**<50**	**-**	**-**		

Risk category	Total score	Median survival (y) without treatment	25% AML prognostic (y) without treatment
Very low	**≤1.5**	**8.8**	**Not reached**
Low	**>1.5-≤3**	**5.3**	**10.8**
Intermediate	**>3-≤4.5**	**3.0**	**3.2**
High	**>4.5-≤6**	**1.5**	**1.4**
Very high	**>6.0**	**0.8**	**0.7**

Cytogenetics: very good; del(11q), -Y alone; Good; normal, del(12p), double including del(5q), del(20q); poor: -7, inv(3)/t(3q)/ del (3q), double inducing -7/ del(7q), complex = abnormalities: very poor> 3 abnormalities; intermediate: del(7q), +8, +19, (i17p), and any other single or double independent clones. Presence of karyotype t (8:21), t (15,17), and inversion 16, denote AML rather than MDS. Adapted from NCCN guidelines version 1 (2019).

Based on the International Prognostic Scoring System (IPSS), MDS patients were also categorized as low risk MDS (47.5%) by virtue of their infrequent transformation to AML and good prognosis, intermediate-1 risk MDS (5%), intermediate-2 risk MDS (16.8%) and high risk MDS (30.7%). The score involved the following independent predictor factors: age ≥60 years, platelets count ≤200x10^9/L, Hb <10gm/dl, unfavourable cytogenetics, and BM blast ≥4% (Bejar *et al.*,2011).

Cytogenetic analysis by FISH is also typically done to identify chromosomal abnormalities as this can influence both prognosis and treatment. It also helps

determine clonality. While a normal karyotype does not rule out MDS, around half of patients will have some type of cytogenetic abnormality. MDS is typically associated with aneuploidy, while translocations are less common. The most frequently observed alterations include del(5q), monosomy 7 or del(7q), trisomy 8, and del(20q). Deleting the long arm of 5, or del(5q), is associated with a better prognosis than others and responsiveness to lenalidomide, one of the treatments for MDS. The WHO MDS classifications list MDS with isolated 5q as one of the categories. This category is defined as isolated del(5q) and can include one other cytogenetic abnormality except for monosomy 7 or del(7q) (Greenberg *et al.,* 2017). Some cytogenetic abnormalities are associated with prior exposure to chemotherapeutic agents. Deletion of all or part of chromosomes 5 and 7 associates with prior use of alkylating chemotherapeutic agents such as cyclophosphamide. Translocation of 11q23 is usually seen in patients with prior exposure to topoisomerase II inhibitors such as doxorubicin and is commonly associated with p53 mutations (Pedersen-Bjergaard *et al.,* 2008).

2.4. Epidemiology of Myelodysplastic Syndrome

The exact number of people with MDS is not known because it cannot be diagnosed and the syndrome does not need to be traced. Some estimates are for 10,000 to 20,000 new cases each year in the United States alone. The number of new cases is likely to increase each year as the population ages, and some authors suggest that the number of new cases in cases greater than 70 years may reach 15/ 100,000 per year. The typical age for diagnosing multiple-syndrome syndrome and infection is between 60 and 75 years; Few people are younger than 50, and diagnosis is rare in children. Males are affected slightly more than females (Aul *et al.,* 2001). The median age of the qualified MDS patients at diagnosis was 76 years (interquartile range, 66-83 years). Female patients were not significantly older than male patients at the time of diagnosis. The numbers of male and female patients increased gradually with age. Patients aged ≥80

years had the highest prevalence of MDS (Wang *et al.*,2019). Aging is the most important risk factor for the development of MDS. Owing to errors in DNA replication and spontaneous mutations from normal metabolic by products (eg, conversation of cytosine to thymidine by oxidative deamination from reactive oxygen species), coding mutation accumulation in hematopoietic stem cells at a mean ± SD rate of 0.13±0.02 exonic mutation per year of life (Xie *et al.*,2014). When an acquired DNA mutation or combination of mutations promotes growth or generates a survival advantage to a hematopoietic stem or progenitor cell, clonal hematopoiesis emerges. Approximately 10%of individuals older than 70 years have clonal mutations in genes associated with myeloid neoplasia, such as DNA methytransferase 3 alpha (*DNMT3A*), ten eleven tanslocation 2 (*TET2*), and splicing factor 3 B1 (*SF3B1*), and these persons have a 0.5% to 1 chance per year of acquiring additional mutation that lead to progression to MDS or another hematological neoplasm, similar in magnitude to the risk of monoclonal gammopathy of undetermined significance (Carraway and Saygin, 2020).

Epidemiology seeks to describe patterns of disease according to demographic factors and other exposures, thereby elucidating etiologic factors (causes of disease) and predictors of prognosis (such as survival). Epidemiologic research of MDS has been fairly limited in comparison to other hematopoietic cancers (such as acute myeloid leukemia (AML), no doubt due to difficulty in case- finding from a historical lack of reporting of MDS in cancer registries. The International Classification of Diseases for Oncology listed MDS as malignant for the first time in its 3rd edition in 2000 (ICD-O-3), thereby spurring registration of MDS in cancer registries worldwide (Deeg *et al.*, 2013).

The incidence rate of MDS in the United State for the years 2003-2007 has been estimated at 4.3 per 100, 000 people which accounts for 15,000 new cases every year (Ma *et al.*,2007 ; Sekeres, 2011).

2.5. Etiology of Myelodysplastic Syndrome

Congenital disease, such as Fanconi anemia, are known to increase the risk of myelodysplastic syndrome (Deschler and Lübbert, 2006). In addition, ionizing radition and chemotherapy for a previous malignancy, and occupational exposure to benzene are also established risk factors (Strom *et al.*,2008). Patients with MDS who have a history of cancer treatment are considered to have secondary or therapy-related MDS and they tend to have a much poorer prognosis (Tefferi and Vardiman, 2009).To date, ideas regarding the role of ionizing radiation in the induction of various forms of MDS are based on clinical observations, in complex therapy that has been applied to a high dose of radiation. The main random effects of ionizing radiation are indicated by the possibility of various forms of leukemia (Iwanaga *et al*., 2011).

The latent period for developing AML, acute leukemia, and chronic myelogenous leukemia dependent on the nature and dose of radiation ranges between 2-5 years. In MDS, the latent period of disease progression is significantly longer. There are major biological differences between MDS and AML that indicate potential target cells exposed to radiation, the nature of damage, and cellular and molecular genetic abnormalities. (Iwanaga *et al.,* 2011; Tsushima *et al.,* 2012). Many case reports, case studies, and epidemiological studies show that MDS is the result of benzene exposure when gasoline exposure levels are less than 10 ppm. It has been suggested that benzene-induced MDS is an early or predisposing event in causing benzene-induced blood diseases (Irons *et al.*, 2005).

Gasoline has a toxic effect on hematology, reproductive diseases and the neuroscience system. The most likely mechanism for benzene-induced leukemia is by phenolic metabolites that work in harmony to produce DNA damage. This leads to mitotic recombination, chromosome transmission, and chromosome imbalance, and these genetic toxic events in turn lead to activation of proton genes, loss of

heterozygousness, and disruption of tumor suppressor genes. In recent years, the relationship between benzene and smoking-forming malignancies has been established. Benzene is known as a blood poison, and benzene has an effect on all blood-forming cells, and this occurs by residing in the bone marrow, which leads to the production of abnormal cells. (Ifeanyi, 2018).

2.6. Diagnosis of Myelodysplastic Syndrome

Generally, MDS a first as non-responsive anemia with diagnosed according to the presence of the abnormal cells in complete blood cells, also the diagnosis of the disease then confirmed by bone marrow-aspiration and -biopsy performance. Both procedures provide different information. The bone marrow-aspirate allows for detailed evaluation of cellular morphology and evaluation of percent of blasts. The bone marrow biopsy allows for determination of bone marrow cellularity and architecture. Diagnosis is established by the presence of dysplasia. There are a lot of types (morphological) are which found to classify patients with MDS (Arber *et al*., 2016). There is little problems about the benefits of bone marrow biopsy, many researchers believe that biopsy of bone marrow play a role in the diagnosis and potentially in addition to selecting therapy. Cellularity is better assessed by bone marrow biopsy. A number of additional tests are needed to complete the laboratory evaluation of a patient with MDS. Most important of which is the analysis of bone marrow cytogenetics. It is well established that cytogenetic patterns are very heterogeneous in MDS (Haase *et al*., 2007). Cytogenetics are of importance to calculate prognosis of patients and in some subsets of patients to select the most effective form of therapy(Schanz *et al*., 2012). Findings of dysplastic features from peripheral blood are limited. Large or abnormally granulated platelets, pseudo–Pelger-Huët anomaly, hypogranular cytoplasm in neutrophils, basophilic stippling, and poikilocytosis in red blood cells (RBCs) are features that can be observed in a

peripheral blood smear using light microscopy. However, these features are not unique to MDS and observe frequently. Bone marrow examination must, therefore, the performer to diagnose MDS (Jaffe *et al.*,2017).

A number of other assays can be used to help in the diagnosis of MDS. These include the use of:

- Peripheral blood (PB) and bone marrow (BM) aspirate: The diagnosis of MDS is based on the quantitative in addition to the qualitative evaluation of the cytological composition of the PB and BM, this achieved by use of basic important hematological techniques, examples of these techniques are hemocytometry, fixed and stained with panoptical stains, optical microscopy on PB and BM films, and cytochemistry for the detection of iron in the erythroblast (Greenberg *et al.,* 2017).
- Cytomorphology of dysplasia: On PB examination, the observation of the presence of morphological abnormalities in the red blood cells (RBC) is quite usual, including the occurrence of circulating nucleated RBC (NRBC) with dimorphic stigmata of dyserythropoiesis, which is not rare. On the other hand, characteristic results in the detection of two RBC populations and their diagnosis, one of these populations considerably normal, but the second one, being a direct expression of the anomalous neoplastic clone, is microcytic or macrocytic. Dysgranulopoiesis in neutrophils is variably observed, from absent to severe, and can involve both the nucleus and cytoplasm, and/or abnormalities in size (Goasguen *et al*., 2014).
- Count and identification of blast cell: The upper-blast-cell-threshold for the diagnosis of MDS is <20% in the PB and/or BM, on a PB differential count performed on 200 nucleated cells, and/or on a myelogram performed on 500 nucleated cells (Mufti *et al.,* 2008).

- Cellularity of the bone marrow: BM cellularity is most often increased in cases of MDS, at diagnosis, with hyperplasia of the erythroid or granulocytic series, or both (Bennett *et al*., 2009).
- Quantification of dysplasia: Precise morphological criteria, both quantitative and qualitative, have been identified for each lineage for the definition of morphological dysplasia, to recognize dysplasia within a specified lineage in the BM, it is necessary that dysplastic features are present in at least 10% of the erythroid precursors (not taking into account mature erythrocytes) and/or 10% of the granulocytic cells (in this case, also including mature cells) out of a count of at least 200 cells of each lineage, and/or in a minimum of 10% of megakaryocytes out of at least 30 cells of the megakaryocyte lineage (Goasguen *et al*., 2016).
- Histopathology: the biopsy of the bone marrow should be combined with the aim of excluding reactive and secondary myelodysplasia, and this will provides a precise evaluation of cellularity, bone-marrow-architecture, distribution and localization of various cellular components, degree of fibrosis, the presence of anomalous localization of granulocyte precursors (ALIP) in intertrabecular areas and/or in the central zones of hemopoietic-tissue, the presence of clusters of megakaryocytes, and the presence of micro-megakaryocytes (Orazi, 2007).
- Flow cytometry (FC): this method used in the studying of the characteristics of the maturation of the precursors, looking for the anomalous expression of immunophenotypic markers as possible indicators of dysplasia of a particular lineage (Van De Loosdrecht *et al*., 2013).
- Genetics: With the availability of new diagnostic platforms, such as gene expression profiling (GEP), single nucleotide polymorphism (SNP)-array, and next-generation sequencing (NGS), genetic and/or molecular lesions are reported in more than 90% of MDS patients (Haferlach *et al*., 2014). Some

clonal cytogenetic abnormalities are associated with specific morphological anomalies affecting the megakaryocyte and erythroid series (Gupta *et al.*, 2007).

2.6.1. Morphological Features of Blood Parameters

The prognosis of myelodysplastic syndrome is mainly based on the morphological findings of peripheral blood and bone marrow. Morphological examination has many advantages: it is a simple, technically easy and inexpensive method, which gives quick results; Moreover, it has predictive significance and must be supplemented, but not replaceable, by other tests. Morphological examination requires peripheral blood smear, bone marrow aspiration, and bone marrow trephine biopsy (Giagounidis and Haase, 2013; Invernizzi *et al*., 2015).

Some features are best seen in mature cells in the peripheral blood, such as large or abnormally granulated platelets, basophilic stippling and poikilocytosis in red blood cells, and pseudo–Pelger-Huët anomaly and hypogranular cytoplasm in neutrophils. Others occur in immature precursors and are best seen in the bone marrow. Immature hematopoietic precursors (nucleated red blood cells, immature granulocytes, megakaryocyte nuclei, and mononuclear megakaryocytes) may circulate in the peripheral blood of MDS patients and can show the same anomalies as seen in the marrow(Jaffe *et al*., 2017).

Peripheral blood may reveal very abnormal nuclei such as Pelger-Huet anomalies and hypo-or hypersegmentation and ring forms nuclei also occur in neutrophils are important morphological features in MDS/MPN peripheral blood when diagnosing and distinguishing MDS/MPN is important to understand the similarities and differences in pathologic mechanism from similar diseases AML, infectious diseases and other causes of cytopenia (Invernizzi *et al*., 2015).

The most striking abnormalities are hypogranulated neutrophils. The defect in granulation may be seen in myelocytes early in the course of disease. Very abnormal nuclei, such as Pelger-Huet anomalies and hypo- or hypergranulation, and ring-shaped nuclei in neutrophils. The platelet morphological changes include giant platelets and platelets hypogranulation or agranulation. Some platelets may possess large fused granules. Circulating micromegakaryocytes (dwarf cells), multiple small nuclei separated by strands of nuclear material, and large mononuclear cells with dysmorphic nuclear features have been described in peripheral blood from patients with MDS. Monocytic hyperplasia is a common finding in dysplastic marrows and can be the dominant manifestation of the hematopoietic abnormalities of CMML for months or years. Cytoplasmic changes may include uneven staining such as a dense ring of basophilia around the periphery with a clear unstained area around the nucleus (Fuchs, 2019).

According to International Working Group on Morphology of MDS (IWGM-MDS), the different maturing-stages of monocytic cells, a promonocyte differs from a monoblast for the irregular nuclear outline but has similar immature chromatin pattern; it is a blast equivalent and should be counted as such. Atypical/immature monocytis which are characterized by a more condensed-chromatin-pattern and less-evident-nucleoli, but its distinction from a promonocyte can be very difficult. Monocytic cells can be better identified with the nonspecific esterase-reaction. Monoblasts and promonocytes, however, are rare in MDS, and their presence is rather indicative of CMML or AML with monocytic differentiation (Invernizzi *et al*., 2015).

2.6.2. Bone Marrow Assessment

It is very important to do the performing of an accurate blast count on both the peripheral smear and the bone marrow in every MDS case. In optimum way, at least 200 cells in the blood smear and at least 500 cells in the aspirate smear should be counted to ensure a precise blast percentage. By blast-threshold MDS separated from AML always about 20% in the bone marrow and/or blood. In the previous 2008 WHO Classification, a rule has been excluded, include the erythroid precursors from the blast percentage calculation when erythroid precursors exceeded 50% of marrow cells to diagnose acute erythroid leukemia has been eliminated. So that, the percentage of the myeloblast of BM is now always derived from all nucleated cells in all MDS and AML cases, irrespective of the percentage of erythroid cells (Arber *et al*., 2016).

The marrow biopsy often showed the hematopoiesis-disorganization, this occur when the erythroid and myeloid elements are intimately admixed rather than forming discrete clusters in the marrow space. Although not pathognomonic for MDS, dysplastic-morphology is a stringent feature in establishing the diagnosis. Some of important features in the peripheral smear also consider good seen, the pseudo-Pelger-Huët anomaly and hypogranular cytoplasm in neutrophils, while others are more apparent in the bone marrow. Bone marrow blasts are increased in some MDS cases and may circulate in the peripheral blood, but are always <20% of the bone marrow and peripheral blood nucleated cells. Auer rods are uncommonly seen in MDS and, if present, indicate high-grade disease (MDS with excess blasts) (Willis *et al.,* 2005).

Although experienced observers can generally agree on the presence of significant dysplasia (Della Porta *et al.*, 2015a), there is some subjectivity to interpreting and quantifying the degree of dysplasia (Senent *et al.,* 2013). The 2016 WHO Classification recommends that at least 10% of cells in a lineage demonstrate dysplastic features to be considered significant (Brunning *et al.*, 2008). However,

dysplastic features involving >10% of a hematopoietic lineage may be encountered in normal individuals and are even more frequently seen in patients with reactive cytopenias, (Parmentier *et al*., ; 2012, Steensma, 2012 ; Della Porta *et al*., 2015b).

2.7.3. Symptoms and Signs

Regarding on the MDS diagnosis, there no specific history, except that is related to bone marrow-failure in form of ecchymoses, petechiae, and bleeding from nose and gum are the general manifestations of thrombocytopenia. In addition to, the fever, also recurrent infection, and up to shock may be a manifestation of neutropenia (Valent *et al*., 2007).

The patients with MDS are usually presenting with non-specific-symptoms. Some of them may not be in symptomatic stage when a cytopenia is detected on routine blood tests. Many, particularly older individuals, also carry other co-morbidities, therefore symptoms may be attributed to other conditions at the beginning. The common symptoms of anemia for example shortness of breath, malaise, exercise intolerance, fatigue, and dizziness are common. Fatigue and cognitive dysfunction have also been attributed to cytokine effect in some patients. Bruising in addition to bleeding is less commonly a prominent problem. Infection, although the principal cause of death in MDS, is not a prevalent initial presenting feature. Most common infections in the course of MDS include bacterial pneumonia and skin abscesses (Sayar, 2013).

Symptoms of advanced malignancy such as excessive night sweats or significant weight loss are uncommon. Autoimmune phenomena including arthritis, fever, skin rash or ulceration, peripheral edema, pleural effusion, pericarditis and neuropathy have been reported in 10% of patients with MDS. Hepatomegaly or splenomegaly if found,

would suggest overlap diseases such as chronic myelomonocytic leukemia (CMML) or leukemic progression (Sayar, 2013).

2.7. Pathophysiology of Myelodysplastic Syndrome

The complicated mechanism due to MDS development caused by many genetic and chromosomal abnormalities, which may occur de novo or secondary to one of the etiologic factors. Cytogenetic abnormalities are seen in more than 80% of patients and include translocations or more commonly, aneuploidy (loss or gain of a chromosome) (Greenberg *et al*., 2017).

According to pathogenesis, the common two types of MDS are: primary or *de novo* and secondary. In pediatric cases, in addition to extensive studies, the causes of origin and progression of primary MDS in these ages are not yet fully understood. On the other hand, pathogenesis of adult and pediatric MDS is multifactorial (Wu *et al*., 2015), for many reasons, the ineffective hematopoiesis may be caused by heterogenous defects of BM stem cells. It is postulated, that genetic defects in the pluripotent progenitor cells lead to genetic instability with consequent numerous molecular and cellular abnormalities. Also, excessive BM apoptosis plays role in pathogenesis (Niemeyer and Kratz, 2008; Hasle and Niemeyer, 2011).

According to genetics, changes in cytogenetics have an important role in the International prognostic scoring system (IPSS). One of these changes, the deletion that occur in the long arm of chromosome 5 (5q) which is consider the most common abnormal karyotype and may be subdivided into 2 categories: first one treatment-related MDS with 5q deletion, usually with exposure to alkylating agents, and in turn the second de novo isolated 5q deletion. Patients who have these mutation (5q deletion) are in most time related to prior chemotherapeutic agents, on the other hand, also have other cytogenetic abnormalities and/or TP53 mutations and usually portends

a poor prognosis. Isolated 5q deletion without other cytogenetic abnormalities has a significantly better prognosis. Other cytogenetic abnormalities commonly studied include normal karyotype, deletion 7q (-7), trisomy 8 and –Y (Zahid *et al*., 2017).

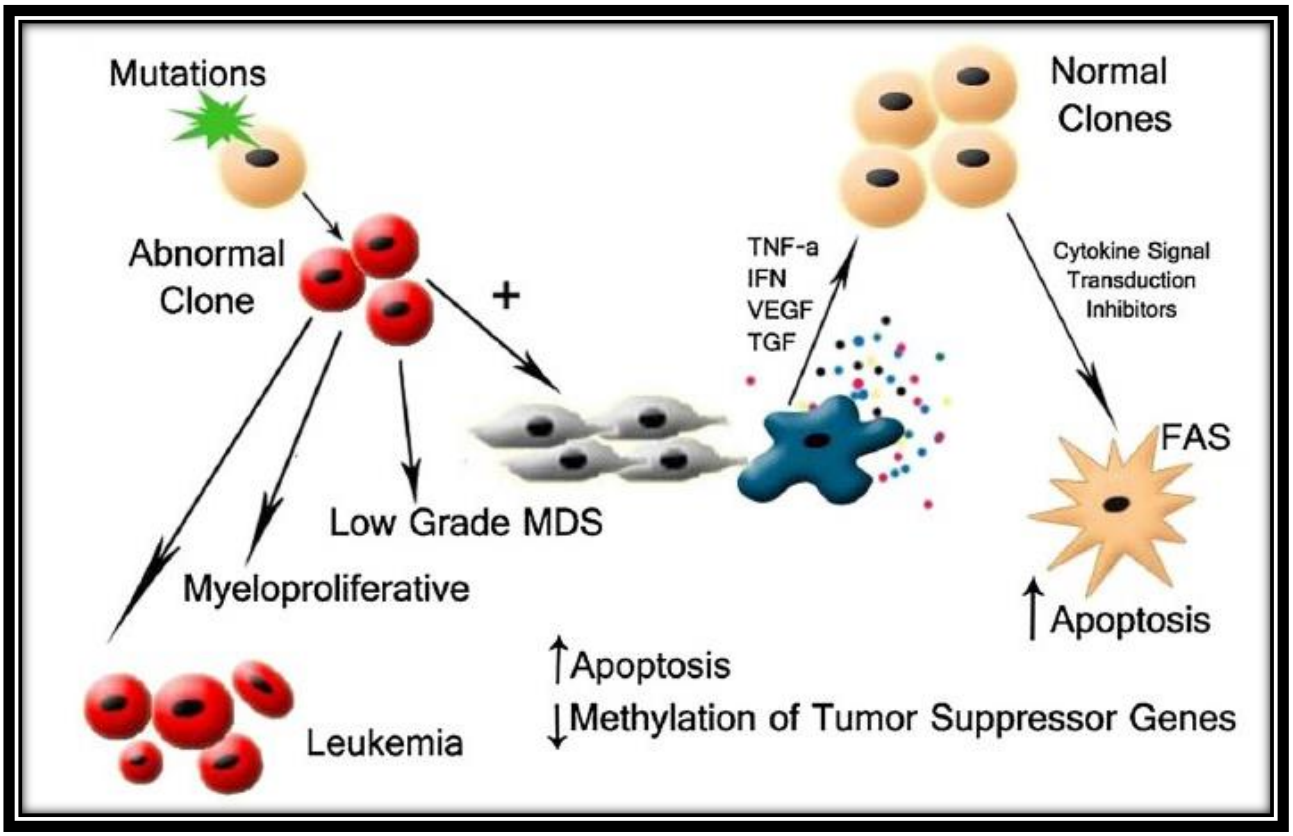


Figure (2-1): Model for pathogenesis of MDS. A mutation or epigenetic alteration in hematopoietic stem cells (HSC), leads to generation of pro-inflammatory milieu in marrow microenvironment that can result in apoptotic cell death of normal HSCs. Inhibition of myelo-suppressive cytokine signaling cascades can stimulate hematopoietic activity in HSCs (Bachegowda *et al*., 2013).

Over 100 somatic point mutations have been implicated in MDS, and there is some overlap with AML. The most common somatic alterations include mutations in *TET2*, *SF3B1*, *ASXL1*, *DNMT3A*, *SRSF2*, *RUNX1*, *TP53*, *U2AF1*, *EZH2*, *ZRSR2*, *ST AG2*, *CBL*, *NRAS*, *JAK2*, *SETBP1*, *IDH1*, *IDH2*, and *ETV6* genes. These mutations have been shown to correlate with various features. *TP53* mutations are associated

with complex cytogenetics and poor overall survival. *RUNX1* and *TP53* tend to correlate with worse thrombocytopenia. *TET2* mutations have a better response to hypomethylating agents (Greenberg *et al.*, 2017).

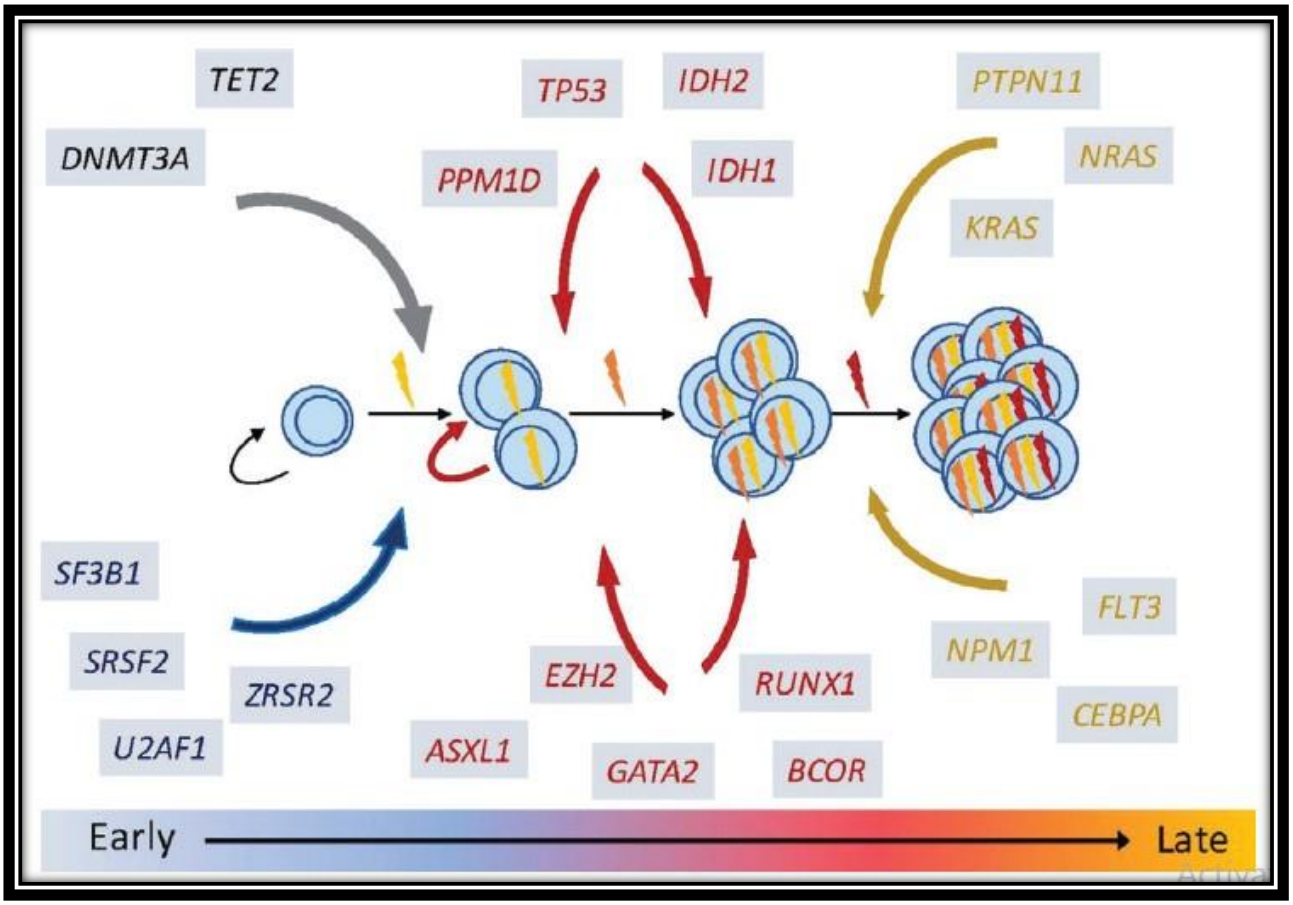


Figure (2-2) : Gene mutations have stereotyped positions in the MDS clonal hierarchy. Recent knowledge suggests that individual mutations occurs in highly stereotyped order and strong patterns of co-mutation association and exclusivity (Mutations affecting epigenetic modifier genes (DNMT3A, TET2, ASXL1, EZH2, etc.) or RNA spliceosome components (SF3B1, SRSF2, and U2AF1) tend to arise in the initiation and early progression phase of MDS and rarely occur at the time of transformation. Mutations in growth factor signaling pathways (NRAS, KRAS, PTPN11, FLT3, etc.) are frequently acquired and expanded in subclones at time of progression to high-grade MDS or secondary AML (Fuchs, 2019).

2.8. Hematopoiesis

Hematopoietic stem cells (HSCs) are multipotent cells, which possess self-renewal capacity and are responsible for the life-long production of all mature blood and immune cell types (Fig 2-4). Under homeostatic conditions, HSCs reside in a quiescent state in the bone marrow but can exit dormancy to contribute to blood production in response to infection, inflammation, or blood loss (Laurenti and Gottgen, 2018). Development of normal hematopoietic cells is an ordered multi-step process, tightly regulated by a complex network of intrinsic factors and microenvironmental cues that control cell fate decisions within the bone marrow (BM) (Pelayo *et al*., 2012; Purizaca *et al*., 2012; Boulais and Frenette, 2015). During malignant hematological disorders, including acute leukemias (AL), the uncontrolled differentiation of precursors of the lymphoid or myeloid series sustains tumor growth at the expense of normal blood cell production. Moreover, selection and dominance among leukemic clones occur while competing for niche resources and creating abnormal BM microenvironments that co-participate in the pathobiology of the disease (Purizaca *et al.*, 2012; Kim *et al*., 2015; Vilchis-Ordoñez *et al*., 2015). Thus, due to the complexity and health impact of ALL (Gupta *et al.*, 2014).

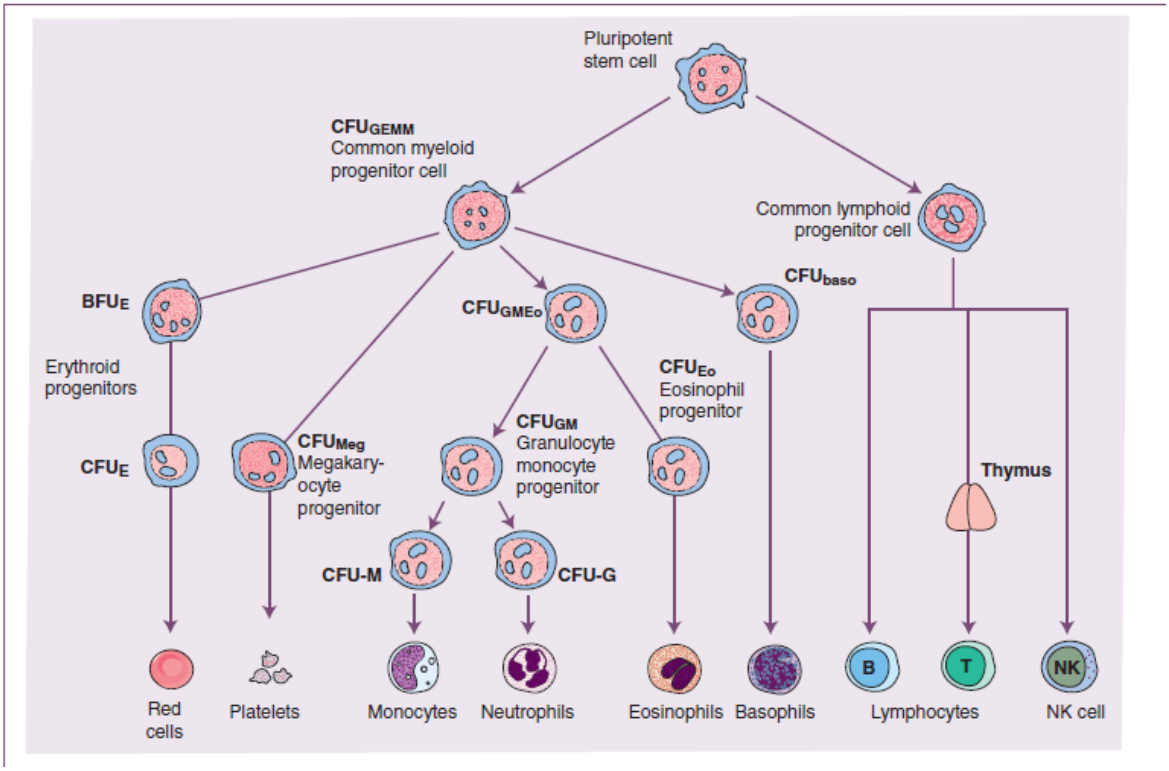


Figure (2-3): Diagrammatic representation of the bone marrow pluripotent stem cell and the cell lines that arise from it. Various progenitor cells can be identified by culture in semi-solid medium by the type of colony they form. It is possible that an erythroid/megakaryocytic progenitor may be formed before the common lymphoid progenitor diverges from the mixed granulocytic/monocyte/eosinophil myeloid progenitor. Baso, basophil; BFU, burst-forming unit; CFU, colony-forming unit; E, erythroid; Eo, eosinophil; GEMM, granulocyte, erythroid, monocyte and megakaryocyte; GM, granulocyte, monocyte; Meg, megakaryocyte; NK, natural killer (Jagannathan-Bogdan and Zon, 2013).

2.9. Some hormones and its relation with myelodysplastic syndrome

2.9.1. Adiponectin hormone

In the mid of nineties (in 1995), the discover that explains adipose tissue as an important endocrine organ that secretes a number of biologically active adipokines into the bloodstream, this discover was a big step forward in understanding human metabolic mechanisms. Adiponectin is one of the most important cytokines produced by adipose tissue. Since adiponectin acts on peripheral target tissues through specific receptors, so that it can be classified as a hormone (Di Zazzo *et al.*,2019).

Adiponectin is a cytokine produced by adipocytes that acts on specific receptors of several tissues through autocrine, paracrine, and endocrine signaling mechanisms. The involvement of adiponectin occurs in the regulation of cell survival, cell growth, and apoptosis (Rasouli and Kern, 2008; Orlando *et al.*, 2019).

Adiponectin have a vital role inside the human-body, like the pathophysiological role in metabolic activities by acting on peripheral tissues involved in glucose and lipid metabolism such as skeletal muscle, also in organs, the liver. Low levels of Adiponectin are associated with the development of cardiovascular complications of obesity in adulthood because adiponectin has anti-inflammatory, anti-atherogenic, and insulin-sensitizing effects (Orlando *et al.*, 2019). Furthermore, it inhibits the classical pro-inflammatory function of macrophages, promoting an M2 macrophage phenotype (Mandal *et al.*,2011*)* and diminishing phagocytosis and cytokine production upon lipopolysaccharide-stimulation by interfering with nuclear factor kappa-B activation. Moreover, adiponectin reduces T-lymphocyte recruitment via the reduction of interferon-beta production (Okamoto *et al.*,2008). Adiponectin shows previously to be inversely related to the risk of AML, MDS, myeloproliferative disorders, and multiple myeloma (Petridou *et al.*, 2006).

It was found that there is a correlation of adiponectin with lipoprotein metabolism; especially, the association of the hormone with the metabolism of high-density lipoprotein (HDL) and triglyceride (TG). On the other hand, appearance of adiponectin in increasing of HDL and TG decreasing. Adiponectin also increases ATP-binding cassette transporter A1 and lipoprotein-lipase (LPL) and decreases hepatic lipase, which may elevate HDL. Increased LPL mass/activity and very low-density-lipoprotein (VLDL) receptor and reduced apo-CIII may increase VLDL catabolism and result in the reduction of serum TG. Further, adiponectin hormones have a different property in molecular anti-atherosclerotic, such as reduction of scavenger receptors in macrophages in addition to the increase of cholesterol efflux. The

suggestion of that findings is explain that high levels of circulating adiponectin can protect against atherosclerosis. Weight loss, exercise, nutritional factors, anti-diabetic drugs, lipid-lowering drugs, and anti-hypertensive drugs have been associated with an increase of serum adiponectin level (Yanai and Yoshida, 2019).

The gene encoding Adiponectin spans approximately 15.8 kb and is structured in three exons on chromosome 3q27; this region has been linked to a susceptibility locus for metabolic syndrome, type two diabetes, and cardiovascular disease (Takahashi *et al.,* 2000). The full-length form of Adiponectin is a 247 amino acid protein with four domains: an amino terminal signal sequence, a variable region, a collagenous domain, and a carboxyterminal globular domain (Gunter *et al*., 2011).

2.9.2. Leptin hormone

Leptin which contains 167 amino acids, Leptin (LEP) is a 16 kDa protein that is synthesized and secreted by white adipose tissue (Saxena and Modi, 2015), was discovered in 1994. It is a hormone secreted by adipocytes and has been found to regulate the intake of food (Minikoshi *et al.,* 2002). Particularly, leptin in vitro induces proliferation and apoptosis inhibition in cytokine-dependent leukemia cell lines (Konopleva *et al.,*1999). A different important functions and specific roles have related to leptin, for example in controlling of body mass, in addition to remodeling of bone, and a role in hypothalamic regulation of food intake and body weight (Ye and Lu, 2013; Saxena and Modi, 2015). Leptin have a role in the regulation of eating behavior through central neuroendocrine mechanisms. It is structurally same function to cytokines, on the other hand, leptin contains an intrachain disulphide bond which has functional significance (Ahima and Flier, 2000). This circulating leptin correlates in positive way with leptin mRNA and protein levels in adipose tissue. This hormone also, regulates neuroendocrine function and energy expenditure, and has since provided significant insights in obesity (Facey *et al.*, 2017). Leptin has been postulated

as an angiogenic factor based on its angiogenic effect in rodent models and in vitro systems (Cao and Hegele, 2001; Park *et al*.,2001). In rat leukemia model the administration of an anti-leptin receptor monoclonal antibody halved the bone marrow content of leukemic cells and substantially decreased angiogenesis (Iveresn *et al*.,2002).

Leptin is a product of obesity gene *ob* gene or *LEP* gene, expressed by the corresponding of this gene, this gene is located on chromosome 7 (Ye and Lu, 2013). Main channel of the leptin gene is JAK/STAT. It is the chief transduction signaling channel that leptin uses to perform its effects or functions. This channel takes signal from exterior of the cell, during DNA transcription and cell activity, signals transfer to the cell nucleus by passing through cell membrane, activate the promoters and these promoters interact with the DNA (Frühbeck, 2006). Several single-nucleotide polymorphisms (SNPs) found in the *LEP* gene may be associated with serum leptin concentration or body mass index (BMI), for example the G2548A polymorphism of the *LEP* gene (Hassanzadeh *et al*., 2013).

2.9.3. Resistin hormone

Resistin is hormone protein with molecular weight 12 kDa and rich of cysteine polypeptide, secreted by macrophages in human. It is the founding member of the resistin-like molecule (RELM) hormone family, and it consists of 108 amino acid peptides; in human blood, it circulates as a dimeric protein consisting of two 92-amino acid polypeptides (Tiaka *et al*., 2011, Sood and Shore, 2013).

Resistin hormones can suppress the ability of insulin to stimulate cellular glucose uptake and also have important plays a role in obesity, insulin resistance, diabetes (Jamaluddin *et al*., 2012), rheumatoid arthritis (RA), and osteoid arthritis (OA) (Boström *et al.,* 2011; Su *et al*., 2015). Some authors believe that in humans, resistin plays a big necessary role in inflammatory processes than in insulin resistance, as

serum resistin levels correlate better with subclinical inflammation than with insulin resistance. In addition, it has been proven in studies that human resistin alone can promote inflammation (Jiang *et al*., 2014), while other studies have also shown that human resistin may exert anti-inflammation in response to a fatal endotoxin challenge (Jang *et al*., 2017).

These conflicting findings point to the notion the proinflammatory or anti-inflammatory function of resistin is context related and disease specific. Generally, as total, resistin in metabolism plays a pathologic role in promoting insulin resistance, atherosclerosis, and hypertension. In humans, positive association of the resistin levels with central/visceral obesity (but not BMI) and play a role in proinflammatory processes (Kim *et al.*, 2013; He *et al*., 2017; Jiang *et al*., 2016).

The gene encoding RETN is located on chromosome 19p13 and a high heritability of serum resistin levels has been evaluated. Several single-nucleotide polymorphisms (SNPs) described in the resistin gene have been associated with RETN levels, the most common SNPs of Retin gene: 420C/G; 44G/A; 62G/A;394C/G and 299 G/A that association with resistin levels (Kumar *et al.*, 2014; 2015; Zayani *et al.*, 2017).

2.10. Iron overload (Ferritin Levels)

Iron overload in patients with myelodysplastic syndrome may be a risk factor that leads to poor outcomes after a fictitious stem cell transplant (Jacobi and Herich, 2016; Wermke *et al.*, 2012). Current methods of assessing IO range from simple and readily available assessments of plasma markers such as serum ferritin (SF) to the more complex approaches that involve magnetic resonance imaging (Wood, 2015).

Some of the principal molecules involved in iron homeostasis include transferrin, hepcidin, and ferroportin. Ferritin is the principal protein involved in the storage of intracellular iron (Shah *et al.*, 2012; Merkel and Nagler, 2014). Serum ferritin level is a

well-known indicator of iron burdenand inflammation. Serum ferritin can be affected by acute infection, inflammation, and malignancy as an acute phase reactant which should be taken into consideration when it is used to demonstrate iron loading (> 1000 ng/mL) (Alessandrino *et al*., 2010; Anderson, 2011 ; Jacobi and Herich, 2016). (Fig. 2-3).

High serum ferritin levels are likely in patients with myelodysplastic syndrome due to frequent red blood cell transfusions. However, elevated iron load is often present in MDS patients, even during diagnosis, before RBCs. This may be caused by ineffective erythrocytes and / or increased intestinal iron absorption (Cortelezzi *et al.*, 2000, Fleming and Ponka, 2012).

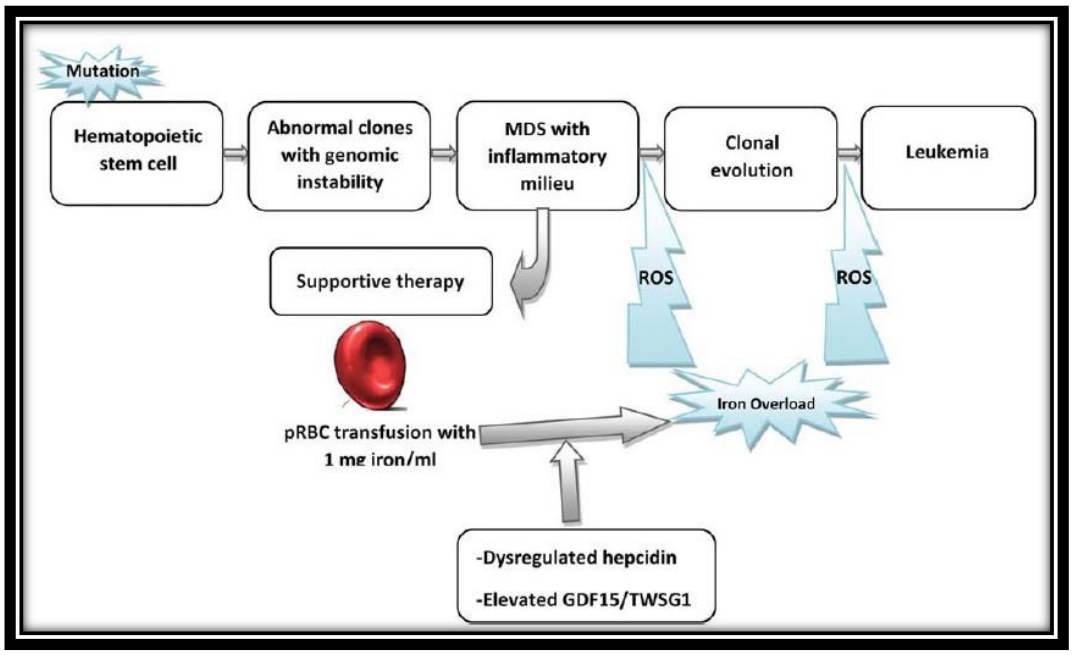


Figure (2-4) : Iron overload in myelodysplastic syndromes (MDS). GDF15 indicates growth differentiation factor-15; pRBC, packed red blood cell; ROS, reactive oxygen species; TWSG1, twisted gastrulation protein 1(Moukalled *et al*., 2018).

2.11. Oxidative Stress or Reactive Oxygen Species (ROS)

As a term, the oxidative stress explains the amplifying of the free-radicals by human body, these reactions due to damage of biomolecules, the consumption of

oxygen occur in one to two percent that occur by mitochondria, due to formation of reactive-oxygen-species normally, this chain of reactions will cause dysfunction to the mitochondria (Fig. 2-5), because of the increase in the reactive molecules that found in the mitochondria (Mantzarlis *et al.*, 2017).

Additionally, many studies linked between the increasing in the oxidative stress and its contribution in the damage of the DNA, the reactive oxygen species has been produced in all the aerobic cells, on the other hand, will be removed by antioxidant. Interestingly, the oxidative stress appears with disabled balance reactive oxygen production and antioxidant system (Novotna *et al.*, 2009, Chung *et al.*, 2014).

ROS production seems to be paly role in contribution to the proliferation and migration of hematopoietic cells that express a variety of neoplastic tyrosine kinases. Thus, increased oxidative stress in leukemia cells may be a potential therapeutic target, although there are different opinions about whether it should aim therapeutic strategies to antagonize or promote oxidative stress in leukemia cells (Reddy, 2011).

Reactive oxygen species may also be involved in mediating the suppression of hepcidin in MDS patients with iron overload (Frank *et al.*,2020), because they can repress the hepcidin gene by preventing C/EBPalpha and STAT-3 binding to the hepcidin promoter. Therefore, it was not surprising that serum hepcidin levels increased after amelioration of oxidative stress parameters by deferasirox treatment (Ghoti *et al.*,2009).

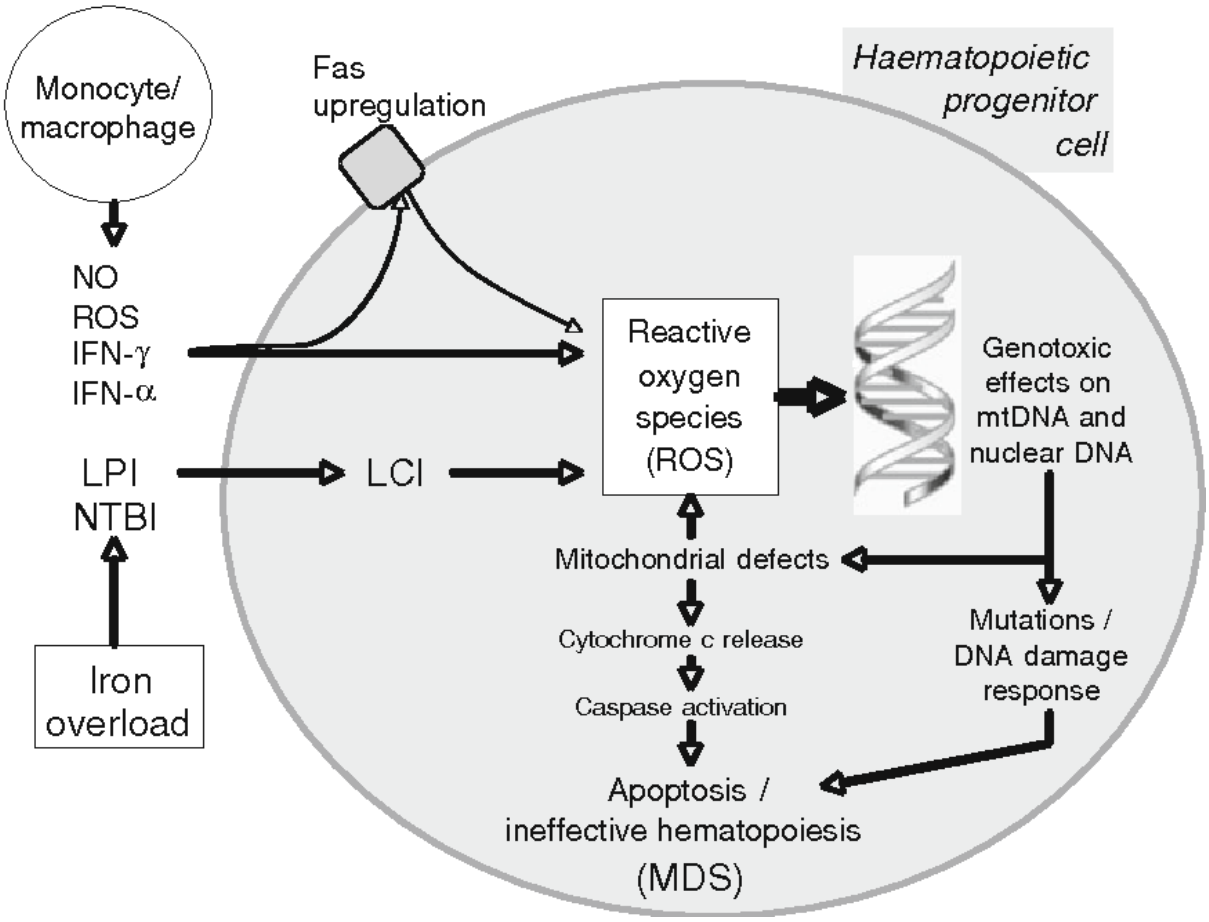


Figure (2-5): The role of oxidative stress (Reactive oxygen species) in myelodysplastic syndrome, IFN, interferon; LCI, labile cell iron; LPI, labile plasma iron; MDS, myelodysplastic syndromes; NO, nitric oxide; NTBI, non-transferrin-bound iron; ROS, reactive oxygen species (Gattermann and Rachmilewitz, 2011).

2.11.1. Antioxidants

Antioxidants are found in many natural food sources such as vegetables, fruits and drinks, and nutritional antioxidants such as flavonoids may help reduce the risk of death from coronary heart disease and myocardial infarction (Waltenberger *et al.*, 2016). Moreover, epidemiological studies and meta-analyzes indicate that long-term

consumption of vegetable polyphenols can protect us from a range of diseases, such as cancer, cardiovascular disease, and diabetes, Osteoporosis, and neurodegenerative diseases (such as Alzheimer's) (Deis *et al*., 2021). With an aging population and only a small proportion of the population consuming the recommended amount of fruits and vegetables per day, there are great opportunities to improve overall health and against degenerative diseases of aging by improving diet (Cătană *et al*., 2018).

In a previous study, the level of reactive oxygen species (ROS) was measured in subsets of bone marrow cells as well as the level of expression of 28 text encoding major enzymes involved in the antioxidant cellular response. Moreover, a specific signature of antioxidants has been identified, called "antioxidants" for different sub-groups of MDS or secondary acute myeloid leukemia (SAML) that made regression of expression levels of antioxidants that could cause disease progression (Picou *et al.*, 2019).

2.11.1.1. Superoxide Dismutase

Defined as catalysis enzyme that alternately has catalysis function to the dismutation of super-oxide-radical (O_2^-) due to the conversion to ordinary molecular oxygen (O_2) and hydrogen peroxide (H_2O_2). Superoxide production occurred as a by-product of oxygen metabolism and, if not regulated, causes many types of cell damage. Hydrogen peroxide is also damaging and is degraded by other enzymes such as catalase. Thus, SOD is an important antioxidant defense in nearly all living cells exposed to oxygen (Hayyan *et al*., 2016).

The isoforms of SOD are SOD1, SOD2 and SOD3, the dismutation of superoxide, for example, can occur spontaneously (particularly at a low pH) but is predominantly regulated enzymatically via a mitochondrial specific superoxide dismutase SOD2 to hydrogen peroxide. On the other hand, SOD isoforms SOD1 and

SOD3 perform the same function in the cytoplasm and extracellular space respectively. (Magder, 2006).

In hematopoietic stem cells (HSC), consequences of ROS include decreased glycolysis, shifting the cell from anaerobic to aerobic metabolism and causing HSC to exit the quiescent state, leading to HSC exhaustion or senescence. ROS oxidizes DNA bases, resulting in accumulation of mutations. Membrane oxidation alters fluidity and permeability, ROS alters cellular signaling pathways resulting in toxicity to organs and hematopoietic cells, in keeping with adverse clinical outcomes in MDS (Kim and Leitch, 2021), on the other hand, some researchers compared the blood levels of oxidative stress markers and antioxidant levels in patients with acute lymphoblastic leukemia (ALL) and AML. Interestingly, they also showed reduced levels of SOD, glutathione, and catalase, additionally, lower levels of SOD expression would lead to increased superoxide levels and lead to intracellular oxidative stress (Sallir *et al*., 2019).

2.11.1.2. Glutathione

Glutathione is a peptide that contains sulfhydryl and is a non-protein thiol dominant in eukaryotic cells. The sulfhydryl (SH) group contains glutathione containing electrons that can interact with the poor biochemical structures of electrons, such as fats, in the cell and thus limit damage to fats, DNA and protein. The compounds (glutathione complexes) formed by this reaction accumulate inside the cells and have the ability to become toxic molecules. These chemicals must be transported outside the cell, and this is accomplished by ATP-dependent plasma membrane vectors. The extracellular compounds are then metabolized by cell surface enzymes and excreted in the kidneys as Mercapturic acid (Meda *et al.*, 2019).

2.11.1.2.1.Glutathione Peroxidase (GPX)

Generally, these compounds belong to the enzyme family, and have peroxidase activity, the functional role of these types occur in protection the cells from oxidative-damage. Biologically, the specific function of GPX is to reduce lipid-hydro-peroxidase to alcohols, also reduce the free hydrogen peroxidase to water (Kasiri *et al.*,2020).

2.11.1.2.2.Glutathione Reductase (GSH)

The chemical structure of glutathione reductase (GSH) consists of three constituents of amino acids named as γ-glutamyl-cysteinyl-glycine, GSH consider ubiquitous substrate specific antioxidant enzyme, have a many function inside the cell, because glutathione metabolism is playing prominent role in sulfur metabolic regulation in all living cells throughout the system. Glutathione reductase (GR) expression suggests that increase or decrease of glutathione level causes oxidative stress in inter-intracellularsurfaces (Dwivedi *et al.*,2020).

In previous studies, role of glutathione in MDS and AML has been linked with oxidative stress, first demonstrated that increased levels of oxidised glutathione in CD34+ AML cells of bone marrow (Pei *et al*., 2013). The other studis showed that glutathione reduced in addition to super oxide dismutase (SOD) levels with increase in ROS levels also with an expected increase in malondialdehyde, a well-defined marker of oxidative stress in patients with AML (Ghoti *et al*., 2007; Sallir *et al*., 2019).

2.12. Some genes that related with myelodysplastic syndrome

2.12.1. Splicing factors

RNA splicing is a mechanism by which a pre-messenger RNA is processed, removing non-coding introns and fusing exons to form a mature, protein encoding mRNA. The 5’splice site of the upstream exon is fused to the 3’splice site of the

downstream exon in spliceosomes that consist of small ribonucleoprotein (snRNP) complexes. Subtle changes to the spliceosome can influence the specificity of splicing, allowing the inclusion or exclusion of alternative exons into the final mRNA, thereby altering the peptide sequence of the translated protein and potentially changing its function. Alternative splicing has long been observed in cancer although the precise mechanisms whereby cancer cells create different splice forms remain unclear (Wood *et al.*,2021).

Mutations in genes encoding proteins that are part of the splicing complex were identified in patients with MDS, including the *SF3B1, U2AF1, ZRSR2,* and *SRSF2* genes, and less frequently in the *SF3A1, PRPF40B, U2AF2*, and *SF1* genes (Graubert *et al.*,2012). Remarkably, all these proteins are involved in precise recognition of the 3' splice site66 (Figure 2-6). The splicing process is regulated through cis-elements that recruit trans-acting splicing factors to affect use of nearby splice sites. Many splicing factors have modular organization, with separate sequence-specific RNA binding modules and splicing effector domains. For example, serine/arginine-rich (SR) proteins contain N-terminal RNA recognition motifs (RRMs) that bind to exonic splicing enhancers (ESEs) in pre-mRNAs and C-terminal arginine-serine (RS) rich domains that promote exon inclusion . Analogously, the hnRNP A1 binds to exonic splicing silencers (ESSs) through its RRM domains and inhibits exon inclusion through a C-terminal Glycine-rich domain (Wang *et al*.,2009).

Mutations in splicing factors and epigenetic regulators constitute the vast majority of abnormalities with mutations in transcription factors, kinase signaling, DNA repair, and cohesin proteins being less commonly, yet recurrently observed in MDS Mutations in the splicing factor 3b subunit 1 (SF3B1) gene, which encodes a key subunit of the spliceosome, are found to be most incident somatic mutation in MDS with 20%-28%

of MDS patients possessing such and in fact was the first spliceosomal mutation discovered in this population (Papaemmanuil *et al.*,2011)..

Splicing factor mutations define clinical phenotypes in MDS to some extent (Papaemmanuil *et al*., 2011; Yoshida *et al*., 2011), and have differing prognostic impacts (Saez *et al*., 2017; Ogawa, 2019). *SF3B1* mutations (frequency 20–28% in MDS) are strongly associated with the presence of ring sideroblasts in the bone marrow (Malcovati, 2011) and are predictive of a favorable prognosis in MDS patients (Ogawa, 2019). It has been noted by several groups that MDS patients with *SF3B1* mutations rarely progress to sAML (Pellagatti and Boultwood, 2017; Armstrong *et al.*, 2018), and interestingly SF3B1 mutations have been shown to be mutually exclusive with mutations in other genes that are associated with leukemic transformation (Makishima *et al*., 2017).

Also, *SRSF2* mutations occur in ~15% of MDS patients, with a higher frequency (40–50% of cases) in patients with chronic myelomonocytic leukemia (Haferlach *et al*., 2014; Patel *et al*., 2017). The frequency of *U2AF*1 mutations is ~7–11% in MDS patients (Thol *et al.*, 2012; Papaemmanuil *et al*., 2013; Haferlach *et al*., 2014; Ogawa, 2019). In contrast to the favorable prognosis associated with SF3B1 mutations, MDS patients with *SRSF2 o*r *U2AF1* mutations typically have a poor overall survival and an increased risk of AML transformation (Wu *et al*., 2012, 2013, 2016; Patnaik *et al*., 2013; Ogawa, 2019).

Pre-mRNA splicing is a fundamental cellular process performed by the spliceosome in which introns are excised from pre-mRNA transcripts to form mature mRNAs. Alternative splicing occurs in > 90% of human protein-coding genes resulting in the production of multiple mRNA isoforms, and is a major source of protein diversity (Pellagatti and Boultwood, 2017; Saez *et al*., 2017). Alternatively and aberrantly spliced mRNA isoforms are often found in human cancers and play a

role in tumorigenesis (Chabot and Shkreta, 2016; Scotti and Swanson, 2016). The most frequently mutated splicing factors in MDS form part of a complex that coordinates 3′ splice site recognition during pre-mRNA splicing. It has been demonstrated that the presence of splicing factor mutations leads to aberrant 3′ splice site recognition (Yoshida *et al*., 2011; Pellagatti and Boultwood, 2017) resulting in the generation of aberrantly spliced mRNA transcripts in the bone marrow cells of patients with myeloid malignancies and in mouse models expressing these mutations (Colla *et al*., 2015; Shirai *et al.*, 2015; Obeng *et al*., 2016; Joshi *et al*., 2017; Mupo *et al*., 2017; Yip *et al*., 2017; Fei *et al*., 2018; Kon *et al*., 2018; Shiozawa *et al*., 2018; Pellagatti and Boultwood, 2021).

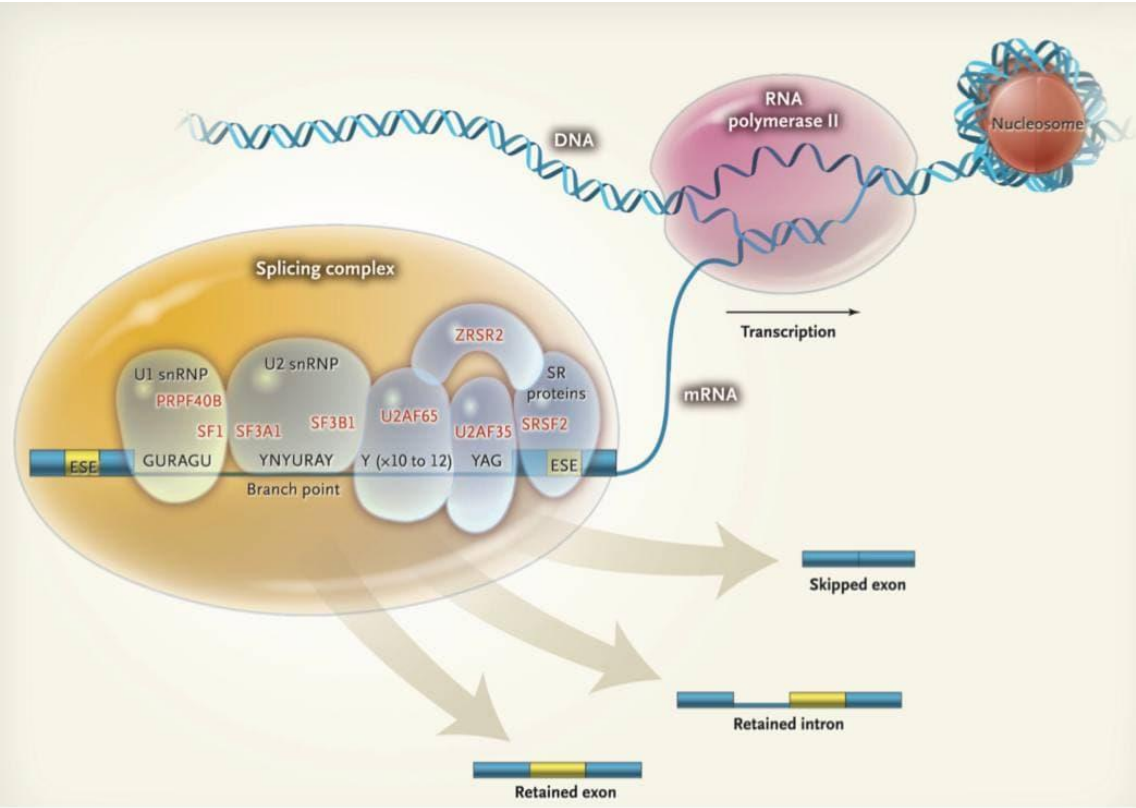


Figure (2-6): Pre-messenger RNA (pre-mRNA) is the primary transcript generated by RNA polymerase II. The spliceosome consists of multiple small nuclear ribonucleoprotein (snRNP) complexes and is responsible for processing pre-mRNA to messenger RNA (mRNA). Due to differential splicing, the resulting mRNA can vary in the inclusion or exclusion of specific exons. In MDS, mutations have been identified in genes encoding SF3B1, SF1, SF3A1, SRSF2, U2AF35, U2AF65, ZRSR2, and PRPF40B (shown in red). These mutations may result in the skipping of exons, retention of introns, or retention of exons thereby altering the function of the translated proteins (Ebert and Bernard, 2011).

2.12.1. 1. Splicing Factor 3b Subunit gene

Splicing Factor 3b Subunit gene (*SF3B1*) is an essential component of the U2 snRNP that binds the intron branch point sequence (BPS) during spliceosome assembly and helps to identify SSs that are used in catalysis. SF3B1 mutation are generally associated with disease, and the most common point mutant found in subtypes of MDS is SF3B1 K700E (Kielkopt, 2018). Mutation found in MDS promote the recognition of non-consense BPS and activate cryptic 3′ SSs (Carrocci *et al.*,2017), however the mechanism for how altered splice selection translate to hematological

malignancy is still under debate. In $CD34^{+}$ cells from patients with SF3B1 mutations, genes involved in cell cycle regulation, iron homeostasis, DNA damage, and RNA processing are deregulated (Dolatshad *et al.*,2015). In order to explain the link between aberrant splicing and gene expression, one study examined RNA-seq data from mutant SF3B1 patient samples (Alsafadi *et al.*,2016).

The most common splicing event was use of a cryptic 3′ SS, via recognition of an alternate BPS upstream of the canonical BPS and about half of the affected transcripts were targets for NMD (Darman *et al.*,2015). In *Sf3b1* k700E knok-in mice, alternate 3′ SS usage was observed, as was inefficient hematopoiesis (Mupo *et al.*,2017). However, these mice did not develop other MDS phenotypes such as ringed sideroblasts as seen in humans, and this was potentially due to differences in disease mechanisms between human and mouse. Additionally, aberrantly spliced transcripts in these mouse models showed little overlap with changes observed in human patients, perhaps owing to the decreased conservation of intron sequences between species. Several investigations have also looked into the role of SF3B1 outside of splicing. *SF3B1* was found to associated with mononucleosomes in HeLa cells, preferentially over exons, and SF3B1 association with chromatin influenced splicing (Kfir *et al.*,2015). However, Murthy *et al,* (2018), also found an association of *SF3B1* with exons in chromatin, but this association did not predict splicing outcomes (Murthy *et al.*,2018). These results point to the role of *SF3B1* as a chromatin modifier.

2.12.1.2. Serine and Arginine Rich Splicing Factor 2 gene

Serine/arginine-rich splicing factor 2 (*SRSF2*) gene is one of the representative candidates which is located on chromosome 17q25. Encoded SRSF2 protein belongs to the SR-protein family and the function of this protein is binding to RNA with a recognition motif to eliminate introns (Phelan *et al.*,2012). Somatic *SRSF2* mutations have been identified more frequently in CMML and less in MDS patients. Many

studies indicated the prognostic value of *SRSF2* mutations in MDS and CMML cases (Ouyang *et al.*,2017). However, with the high prevalence of *SRSF2* mutations, the distinct role of *SRSF2* in clinical impact has been controversial. *SRSF2* mutations in 24 out of 193 individuals with MDS and found that these mutations are an unfavorable prognostic factor in MDS patients (Thol *et al.*,2011). Kang *et al*.,(2015) observed mutations in 13 out of 129 patients with MDS and demonstrated that *SRSF2* mutations had no effect on MDS patients . Ouyang *et al.(*2017) detected *SRSF2* mutations in 14 patients out of 56 individuals with CMML and reported that the presence of *SRSF2* mutations was correlated with shorter overall survival (OS). Patnaik *et al.,(*2013), suggested that *SRSF2* was frequently mutated in CMML cases and had no prognostic impact on patient.

3. Materials and Methods

3.1. Materials

The listed chemicals, instrument, and biological materials were used to perform the experiments of this study as shown in Tables (3-1), (3-2) and (3-3).

Table (3-1): Chemicals that were used during this study.

Chemical name	Supplying company
Agaros	Pronadisa/ Spain
Ammonium presulfate APS	Sigma/ German
Acrylamide	Himedid/ India
Bis acrylamide	Scr/ Chine
Bromophenol blue stain	Sigma – USA
DNTB	Germany
Ethanol	Biosolve company/ USA
Ethedium bromide	Promega
Ferrous ammonium sulfate	Germany
Glycerol	Merck- England
GSH	Germany
H_2SO_4	Germany
Hydrogen peroxide	Germany
Leishman′s stain	Daryagani- India
Mgcl2	Cyntol/ Russia
Nuclease free water	Cyntol/ Russia
Na2HPO4	BDH-England
NaN_3	Germany
Nacl	Germany
O- dianisidine dehydrocloride	Germany
Pyragallol	Fluka- India
Sodium nitrate	Germany
Syper green	Germany
TEMED	Sigma/ German
Tris Borate EDTA	Thomas BAKer/India
Tetra-butyhydroperoxide	Germany
Xylenol organ	Germany

Table (3-2) Kits and Enzyme used in this study

Kit and Enzyme	Manufacturer Com. and Origin
DNA Extraction Kits From Blood	Favorgen- Taiwan
DNA Ladder(100bp)	Cyntol /Russian
DNA loading dye	Promega_ USA
GENEzol TriRNA Pure Kit	Geneaid
Human ADP/Acrp30(Adiponectin) ELISA Kit	Elabscinece
Human Leptin ELISA Kit	Demeditec Diagnostics GmbH (Germany)
Human Resistin ELISA Kit	Demeditec Diagnostics GmbH (Germany)
PCR Master Mix	Cyntol/ Russia
Primer pair	Macrogen- Korea
Proteinase K	Biolabs- England

Table (3-3): Instruments that were used during this study.

Instruments	Manufacturer
Autoclave	Haramaya (Japan)
Centrifuges	Hettich (Germany)
Cooling centrifuges	Hettich (Germany)
Deep Freeze	GFL (Germany)
Deionizer	Sartorius (Germany)
Digital camera	Genex (Germany)
Distillater	GFL(Germany)
EDTA tube (5ml) , Test tube with Separating gel	AFCO (Jordan)
Eliza reader & washer	Biotek (USA)
Gel electrophoresis unit	Cleaver scientific (Japan)
GTC SeriesThermocycler	Cleaver scientific (UK)
Hood	Fisher Scientific (Germany)
Hot plate with magnetic stirrer	Heidolph (Germany)
Incubator	Memmert (Germany)
Micropipette(100-1000) µl,(10-100) µl ,(5-50)µl,(0.5-10)µl	Dragon med.(Germany)
Number channel pipette (20-200) µl	Huawei (Germany)
Photoducomentation	Cleaver scientific (UK)
Refrigerator (4°C and -20°C)	Philips (Japan)
Sensitive balance	Sartorius (Germany)
Shaker water bath	Memmert (Germany)
Spectrophotometer	Shemadzu (Japan)
UV transilluminator	Cleaver scientific (Japan)
Vortex	Bioneer (Korea)

3.2. Design of the study

The experimental design of this study as shown in figure (3-1).

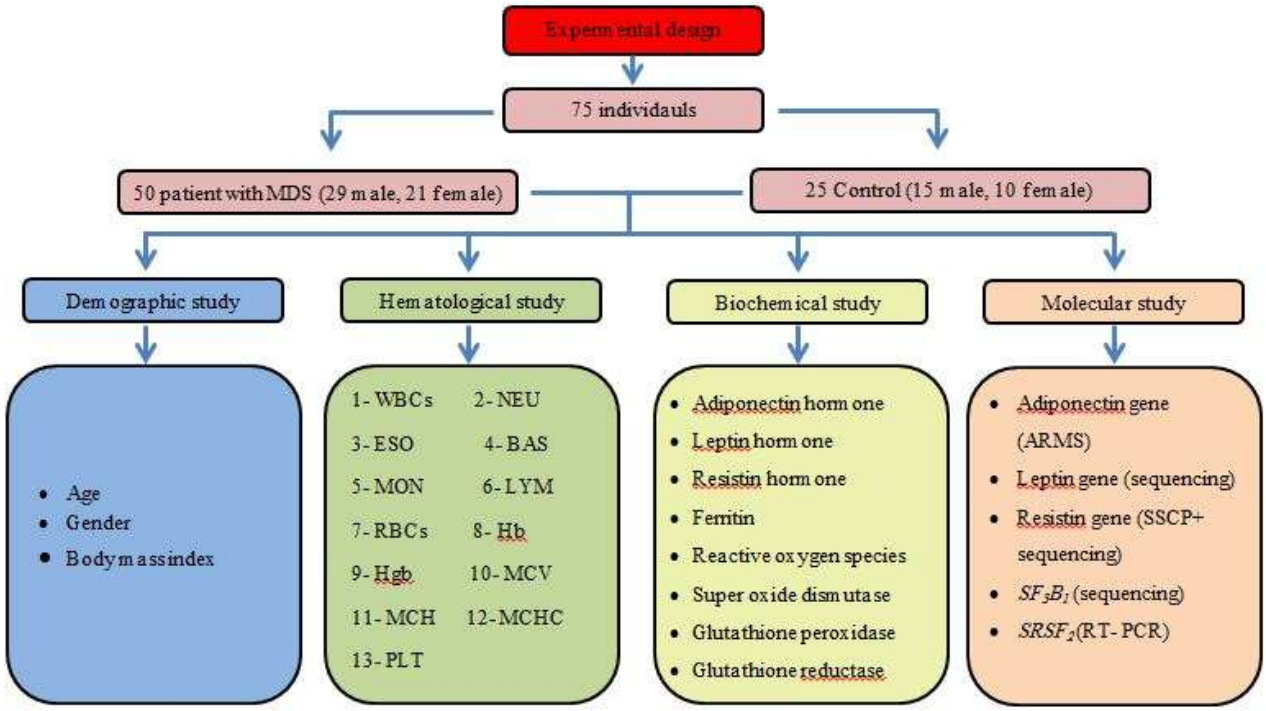


Figure (3-1): Scheme of study design.

3.3. Methods

3.3.1. Setting and Data Collection Time

The present study was done in the laboratory of DNA. In the department of Biology, College of Sciences, University of Babylon.

The samples collection and practical work of the present study extended through the period from June 2019 to November 2020 . Samples of myelodysplastic syndrome (MDS) were obtained of several location from patients who attended to the Blood Disease Center/ Medical City/, National Center for Research and Treatment of Hematology/ Al-Mustaniriyah University, Baghdad province, Merjan Teaching Hospital in Babylon province, AL-Hussein Center for Oncology Treatment and Blood Disease / Al-Hussein Medical City/ Karbala province, and AL-Furat AL-Awset for Tumors and Blood Disease, Najaf province, Iraq. The patients were males and females. Samples include bone marrow aspirate and blood were obtained from each subject by vein puncture, was put in the

Ethylenediaminetetraacetic acid (EDTA) tubes and gel tube for separation of serum by centrifugation and stored at -20°C until used in measurement of hormones and oxidative stress. Blood in the EDTA tubes was stored in -40°C (deep freeze) in order to be used later in molecular study.

3.3.2. Patients and conditions of study

The study comprised from 50 patients with Myelodysplastic syndrome. Patients were (30 male and 20 female) as Myelodysplastic Syndrome patients group with age average (20-83) years old. In contrast, the study included (25) people apparently healthy that included 15 male and female 10 with age average (20-85) years as the control group matched as patient group.

3.3.3. Survey Questionnaires

Questionnaire has been taken from the patient and case sheet included: age, gender, and Body Mass Index (BMI).

3.4. Anthropometric assessments

The anthropometric techniques used to measure weight and height were recommended by Lohman *et al*., (1988). All anthropometric measurements were taken with stress on body height and weight that were measured in light clothes using a portable stadiometer.

Height was determined using a wall mounted, non-extendable measuring tape with subjects standing in an erect barefoot position, arms by side, and feet together.

Weight measurements were taken with each subject standing at the center of the weighing scale in light clothing without shoes and socks.

Body mass index (BMI) was calculated using the formula

BMI = weight (kg)/ height^2 (m)^2 and classifying under weight (BMI<18), normal (BMI 18 - 24.9), overweight (BMI 25 - 29.9), obesity (BMI 30-39.9) and morbid obesity (BMI > 40) (Sturm,2007).

3.5. Measurement of blood parameters

The blood parameters include estimation Complete Blood Count (CBC), by blood analyzer device and bone marrow film from bone marrow aspirate.

3.6. Biochemical Analysis

To determine the serum Adiponectin, Resistin, and Leptin, the quantitative sandwich enzyme immunoassay technique was used. Also, measurement of ferritin, oxidative stress (Reactive oxygen species) and antioxidants.

3.6.1. Measurement of Serum Adiponectin

Include determination human ADP/Acrp30. (According to Elabscience company, USA).

3.6.1.1. Assay Procedure

Reagents were allowed to reach the room temperature all the reagents should be mixed thoroughly by gently swirling before pipetting.

1. The standard working solution was added to the first two columns: each concentration of the solution was added in duplicate, to one well each, side by side (100 µL for each well). The plate was covered with sealer provided in the kit and incubated for 90 min at 37 ̊C.

2. The liquid was removed out each wall, don′t washed. Immediately, 100µL of Biotinylated Detection Ab working solution to each well. The plate was covered with sealer. Gently mixed up and was incubated for 1 hour at 37◦C.

3. The solution was aspirated from each well, and added 350 µL of wash buffer to each well. The solution was soaked for 1- 2 minutes and the solution was aspirated from each well. It was dried against clean absorbed paper. This wash step was repeated 3 times in total.

4. HRP Conjugate working solution (100 µL), was added to each well and covered the plate with sealer. Incubation for 30 minute at 37◦C.

5. The solution was aspirated from each well, the washing process was repeated for five times.

6. Substrate Reagent 90 µL was added to each well and covered with new plate sealer. Incubation for about 15 minute at 37°C. The plate was protected from light.

7. Stop solution 50 µL was added to each well.

8. The optical density (OD value) of each well at once, using a micro-plate reader set to 450 nm.

9. The OD value is proportional to the concentration of Adiponectin.

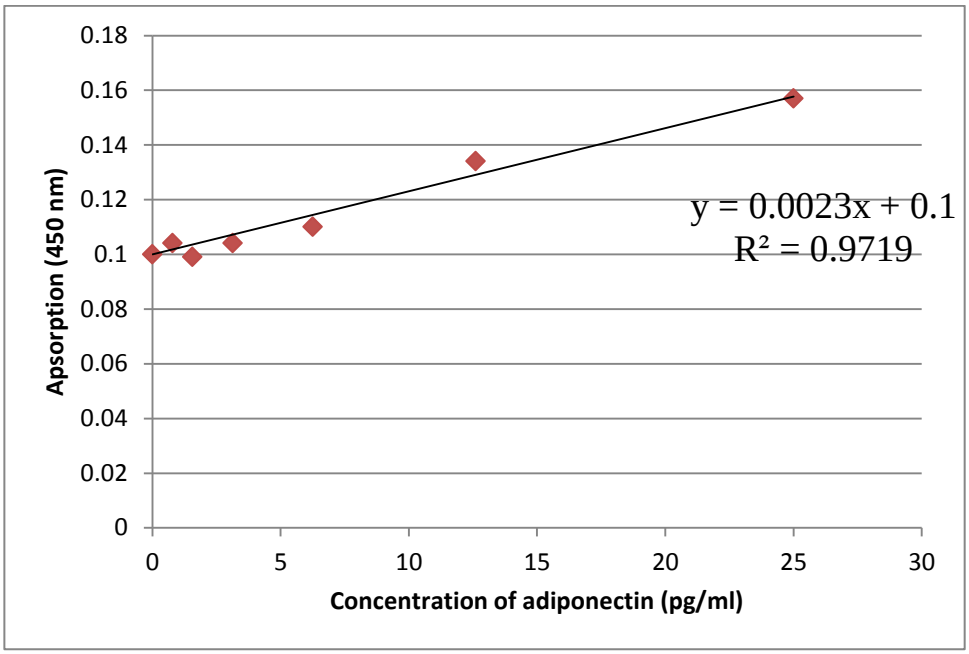


Figure (3-2): The standard curve for determination of adiponectin.

3.6.2. Measurement of serum Leptin

According to Demeditics Diagnostics GmbH, Germany.

3.6.2.1. Assay procedure

Preparation of reagents		Reconstitution	Dilution
A-E	Standards	In 750 µl dilution buffer VP	-
KS1 and KS2	Control sera	IN 500 UL dilution buffer VP	-
WP	Washing buffer	-	1:20 with Aqua dest
Sample dilution is in general not necessary use 20 µl undiluted per determination			
Before assay procedure bring all reagent to room temperature 20-25 C			
Assay procedure in double determination			
Pipette	Regents		Position
100 µl	Dilution buffer VP		Pipette in all required wells
20 µl	Dilution buffer VP (Blank)		A1/A2
20 µl	(1ng/ml) A Standard		B1/B2
20 µl	Standard B (10ng/ml)		C1/C2
20 µl	Standard C (25ng/ml)		D1/D2
20 µl	Standard D (50ng/ml)		E1/E2
20 µl	Standard E (100ng/ml)		F1/F2
20 µl	Control serum KS1(undiluted)		G1/G2
20 µl	Control serum KS2(undiluted)		H1/H2
20 µl	sample (undiluted)		In the rest of the wells according the requirements
	The well was covered with the sealing tape		
Sample was incubated for 1 h at 20-25 c 200 -350 rpm			
5 * 300 µl	The contents of the wells was aspirated and washed 5* with 300 µl each washing buffer wp/well		In each well
100 µl	Antibody – pod – conjugate AK		In each well
The well was covered with the sealing tape			
Sample was incubated for 1 h at 20-25 c 200 -350 rpm			
5*300 UL	The contents of the wells was aspirated and washed 5* with 300 µl each washing buffer wp/well		In each well
100 UL	Substrate solution		In each well
Substrate solution was added and incubated for15 minutes in the dark at 20 – 25 c			
100 µl	Stopping solution was added		In each well
	The absorbance was measured within 30 min at 450 nm with > 590 nm as reference wavelength.		

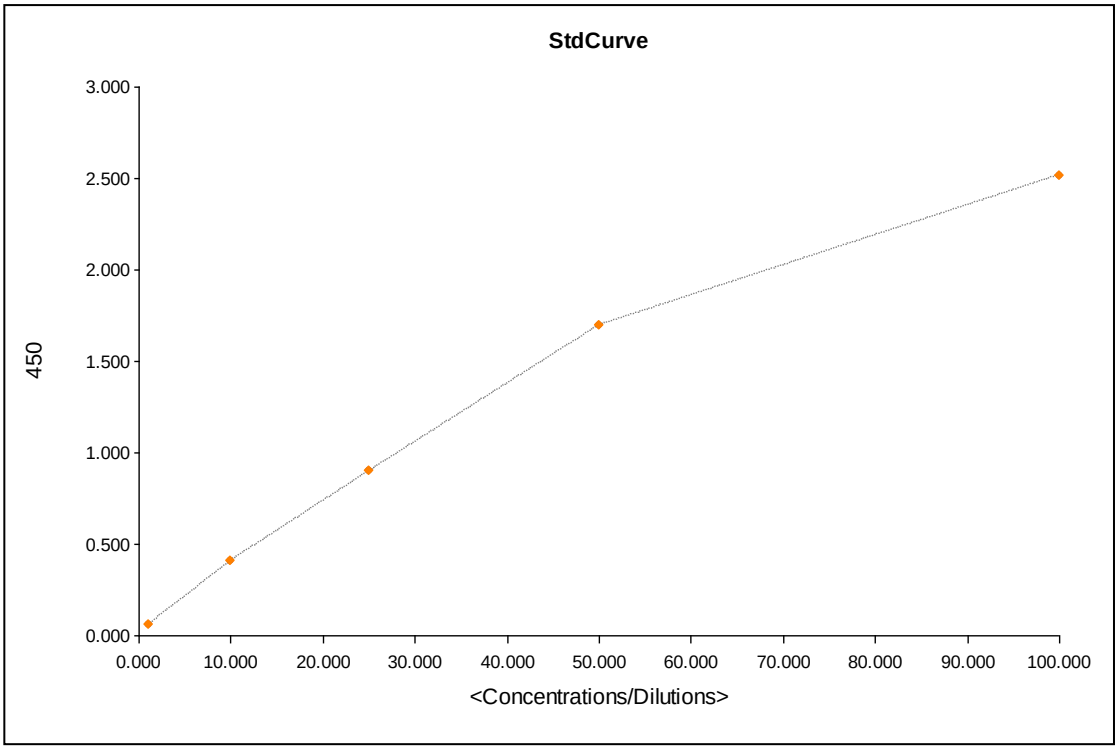


Figure (3-3): Standard curve of for determination of leptin concentration pg/ml.

3.6.3. Measurement of serum Resistin

According to Demeditics Diagnostics GmbH, Germany.

3.6.3.1. Assay Procedure

1- Sample Buffer PP 100 µL was added in wells A1/A2 (blank) and

2- Pipette in positions B1/2 100 µl of the Standard A (0.02 ng/m،

pipette in positions C1/2 100 µl of the Standard B (0.1 ng/ml)،

pipette in positions D1/2 100 µl of the Standard C (0.3 ng/ml)،

pipette in positions E1/2 100 µl of the Standard D (0.6 ng/ml)،

pipette in positions F1/2 100 µl of the Standard E (1 ng/ml).

3- To control the correct accomplishment 100 µl of the 1:21 (or in respective dilution rate of the sample) in Sample Buffer PP diluted Control Sera KS1 and KS2 can be pipetted in positions G1/2 and H1/2.

4-Sample buffer 100 µl was pipetted to each of the diluted sample in the rest of the wells, according to requirements.

5- The wells was covered with sealing tape and incubated the plate for 2 hours at room temperature (shake at 350 rpm). After incubation the contents was aspirated of the wells and washed the wells 5 times with 300 µl Washing buffer WP / well.

6-Following the last washing step, 100 µl was pipetted of the 1:100 with Dilution Buffer VP diluted. Antibody Conjugate AK was added in each well. The wells was covered with the sealing tape and incubated for 1hour at room temperature (shake at 350 rpm.

7- After incubation the wells washed 5 times with Washing Buffer WP as described in step 5.

8-Following the last washing step, 100 µl was pipetted of the 1:100 with Dilution Buffer VP diluted Enzyme Conjugate EK in each well. The wells covered with the sealing tape and incubated for 30 minutes at room temperature (shake at 350 rpm.

9- After incubation the wells washed 5 times with Washing Buffer WP as described in the step 5.

10- The TMB-substrate solution 100 µl was pipetted in each well.

11-The plate was incubated for 30 minutes in the dark at room temperature.

12- The reaction was stopped by adding 100 µl of Stopping Solution SL to all wells.

13-The absorbance was measured within 30 minutes at 450 nm (reference filter: ≥ 590 nm).

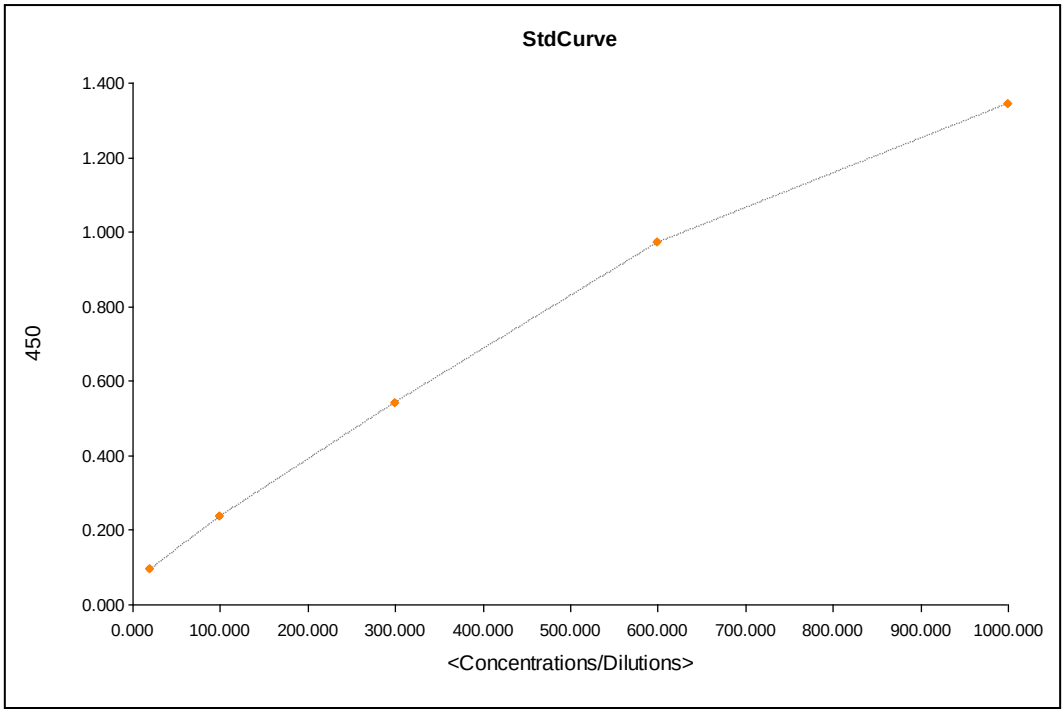


Figure (3-4): Standard curve for determination of resistin concentration.

3.7. Determination of serum ferritin

Serum ferritin was measured by using Mini Vidas automated immunoassay system based on Enzyme Linked Fluorescent Assay (ELFA).

3.8. Determination of oxidative stress (Total reactive oxygen species)

3.8.1. Principle:

The TOS of serum was determined using a novel method, developed by Erel, (2005). Oxidants found in the sample oxidize the ferrous ion–o-dianisidine complex to ferric ion. By glycerol molecules the oxidation reaction is enhanced, which are richly found in the reaction medium. The ferric ion creates a colored complex with xylenol orange in an acidic medium. The color intensity, which can be measured spectrophotometrically, is related to the total amount of oxidant molecules found in the sample. The test is calibrated with hydrogen peroxide and the outcome are expressed in terms of micromolar hydrogen peroxide equivalent per liter (µmol H_2O_2Eq/L).

3.8.2. Assay reagents

Reagent 1: Reagent 2 was prepared by dissolving 3.17 g of O-dianisidine dihydrochloride and 1.96 g of ferrous ammonium sulfate in 1000 mL of H_2SO_4 solution, 25 mM. The ending reagent was composed of 10 mM O-dianisidine dihydrochloride and 5 mM ferrous ammonium sulfate. This reagent is stable for as a minimum 6 months at 4°C.

Reagent 2: Reagent 1 was prepared by dissolving 8.18 g of NaCl and 114 mg of xylenol orange in 900 mL of H_2SO_4 solution, 25 mM. One hundred milliliters of glycerol was given to the solution. The ending reagent was composed of 140 mM NaCl , 150µM xylenol orange and 1.35 M glycerol. The pH value of the reagent was 1.75. This reagent is stable for as a minimum 6 months at 4°C.

Hydrogen peroxide: (100 µmol/L) was freshly diluted and standardized daily using a molar extinction coefficient of 43.6 $M^{-}1\ cm^{-}1$ at 240 nm.

3.8.3. Procedure

	Blank	Standard	Sample
Distilled water	50 µl	-----	------
Sample	------	-----	50 µl
Hydrogen peroxide	------	50 µl	------
R1	1 ml	1 ml	1 ml
Test tubes were mixed by vortex, and then add:			
R2	250 µl	250 µl	250 µl

Quietly mix the content of each tube after addition, allow standing at room temperature for 5 minute, read spectrophotometrically at 560 nm.

$$\text{Total oxidants status} = \frac{\text{A.test}}{\text{A.STD}} * Conc.of \quad STD$$

3.9. Determination of antioxidants

3.9.1. Super-oxide dismutase activity determination

3.9.1.1. Reagent

1-Tris buffer (pH 8.0): was prepared by dissolving 0.258 gm of tris and 0.111 gm of Ethylenediaminetetraacetic acid (EDTA) in dH2O and completing the volume to 100 ml.

2-Pyragallol solution (0.2 mM): was prepared by dissolving 0.0252 gm of pyragallol with 10 ml of HCl and completing the volume to 100 ml with dH2O.

3.9.1.2. Procedure

According to Marklund and Marklund (1974),

1-Reaction mix is consisting of 50 µl crude enzyme extract with 2 ml of tris buffer and 0.5 ml of pyragallol (0.2 mM) which absorbs light at 420 nm.

2-Control solution contains the same materials except for the enzyme extract that was replaced by dH2O. As a blank, dH2O was used.

3-Single unit of enzyme is defined as the amount of enzyme that is capable of inhibiting 50% of pyragallol oxidation. SOD activity was calculated using the following equation:

$$SOD\ Activity\ (unit) = \frac{\frac{\%P}{50\%} \times R}{T}$$

Where:

• *%P:* percentage of the inhibition of pyragallol reduction

*Note: %P of every sample is calculated by comparing Δabs of the sample (X%) with Δabs of control (100%)

• *R:* Total reaction volume (2.55 ml)

• *T:* Time of reaction in minutes (2 minutes)

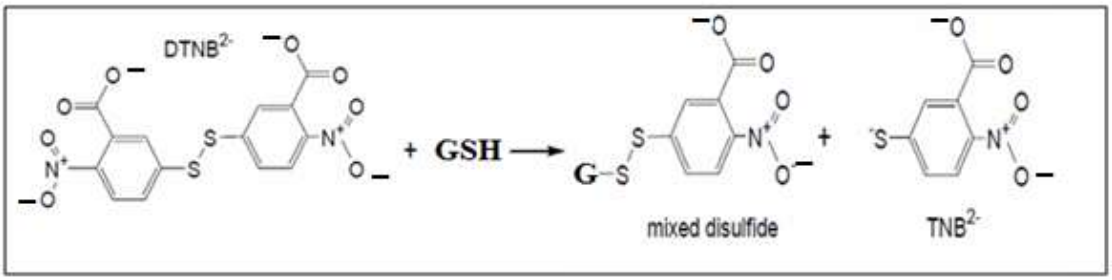
3.9.2. Assay of Glutathione Peroxidase (GPx) Activity

3.9.2.1. Principle:(Rotruck, *et al*.,1973)

Glutathione peroxidase catalyzes the following reaction:

$$2GSH + LOOH \xrightarrow{GPX} GSSG + LOH + H2O$$

The decrement of reduced glutathione concentration can be monitored by Ellman's reagent [5,5'-Dithio-bis-(2-nitrobenzoic acid) (DTNB)].



3.9.2.2. Reagents

1. Solution A: (0.4 M NaH_2PO_4) 55.6 g of NaH_2PO_4 was dissolved in 1L of water.
2. Solution B: (0.1 M Na_2HPO_4) 107.12 g of Na_2HPO_4 was dissolved in 1L of water.
3. Sodium phosphate buffer (pH 7.0) (0.4 M): prepared by mixing 39 of solution A and 61 ml of solution B and dilute to 200 ml with D.W. which contain 0.0744 g EDTA.
4. Sodium azide (10mM): 0.06501g of NaN_3 was dissolved in 100ml of D.W.
5. Reduced glutathione (4 mM): prepared by dissolving 0.1228 gm of a GSH in a final volume of 100 ml of 0.4M EDTA solution.
6. Tert- butylhydroperoxide (2.5mM)
7. Na_2HPO_4 (0.4 M): 5.68 g of Na_2HPO_4 was dissolved in 100ml of D.W.
8. Sodium nitrate (0.1%)
9. DTNB {19.8 mg in 100 ml 0.1% sodium nitrate}

3.9.2.3. Procedure

Reagents	Test	STD	Blank
Sodium phosphate buffer	400µL	400µL	400µL
Sodium azide	100µL	100µL	100µL
Reduced glutathione	200 µL	200 µL	------
D.W.	200 µL	250 µL	450 µL
Sample	50 µL	-------	------
Tert- butylhydroperoxide	200 µL	200 µL	200 µL
Mix by vortex and incubate for 3 minutes at 37°C, after that, the reaction was terminated with 0.5 ml of 10% TCA and Centrifuge for 15 minutes at 300 xg, then remove 2 ml of supernatant in a clean tube , and add			
Na_2HPO_4	3ml	3ml	3ml
DTNB	1ml	1ml	1ml

The color developed was read at 412 nm trough 3 min.

3.9.1.4. Calculation:

$$\text{The residue reduced GSH in test} \quad \text{tube} = \frac{\text{A.test}}{\text{A.STD}} * Conc.of \quad STD$$

Se-dependent glutathione peroxidase activity (µmol of glutathione utilized/min) = Conc. of GSH in STD - Conc. of GSH in test * D.F.

$$\text{Se - GPX activity (µmol of GSH utilized/min)} = \frac{\text{Conc. of GSH in STD - Conc. of GSH in test}}{\text{time(3min)}} * D.F.$$

3.9.3. Glutathione reductase

Reduced glutathione was measured following the method of Sedlak and Lindsay, (1968).

3.9.3.1. Procedure

1-First, 3.0 mL precipitating solution containing metaphosphoric acid, Na2EDTA and NaCl was added to 2.0 mL of the sample.

2-The mixture was centrifuged at 4500 × g for 10 min. 1.0 mL of supernatant was added to 4.0 mL of 0.3 M Na2HPO4 solution and 0.5 mM DTNB (5,5 -dithiobis-2-nitrobenzoic acid) was then added to this solution.

3-Reduced glutathione was measured as the difference in the absorbance values of samples in the presence and the absence of DTNB at 412 nm.

4-Glutathione (GSH) value was calculated as nmol GSH/mg protein in the tissues and mmol GSH/g Hb in whole blood using the reduced glutathione as a reference (hemoglobin levels were estimated in whole blood using the Drabkins' solution).

3.10. Molecular analysis

3.10.1. DNA extraction

Principle

The manufacturer protocol (FavorGen/ Taiwan) was followed for extraction the DNA from frozen blood sample and this protocol could be summarized as follows:

Step 1-Sample Preparation

1. A mount 200µl of blood was transferred up to a 1.5ml microcentrifuge tube (not provided). If the sample volume is less than 200µl, we added the appropriate volume of PBS.

2. A mount 30µl Proteinase K (10 mg/ml) was added to the sample and briefly mix. Then incubate for 15 minutes at 60ºC.

Step 2 –RBCs Lysis

3. A mount 200µl FABG Buffer was added to the sample and mix by vortex.

4. The sample was incubated in a 70ºC water bath for 15 minutes to lyse the sample. During incubation, the sample was inverted every 3 minutes.

5. Elution Buffer required preheat (for Step 5 DNA Elution) in a 70ºC water bath.

Step 3 – DNA Binding

7. A mount 200µl ethanol (96~100%) was added to the sample and vortex for 10 seconds. (Pipetting if there is any precipitate.)

8. A FABG Column was placed to a 2ml collection tube. The sample mixture was transferred (including any precipitate) carefully to FABG Column, and centrifuged for 5 minute at full speed (14,000 rpm or 10,000 x g) then discard the 2ml collection tube. The FABG Column was placed in a new 2ml Collection tube.

Step 4 – Washing

9. FABG Column was washed with 400µl W1 Buffer. The sample was centrifuged for 30 seconds at full speed (14,000 rpm or 10,000 x g) and discard the flow-through.

10. The FABG Column was placed back in the 2ml Collection tube. FABG Column was washed with 600µl Wash Buffer (ethanol added). The sample was centrifuge for 30 seconds at full speed (14,000 rpm or 10,000 x g) and discard the flow-through. --Make sure that ethanol has been added into Wash Buffer when first open.

11. The FABG Column was placed back in the 2ml Collection tube and centrifuged for an additional 3 min at full speed (14,000 rpm or 10,000 x g) to dry the column. --Important Step! This step will avoid the residual liquid to inhibit subsequent enzymatic reactions.

Step 5 – Elution

12. The dry FABG Column was placedto a new 1.5ml microcentrifuge tube.

13. A mount 100µl of Preheated Elution Buffer or TE was added to the membrane center of FABG Column and standed FAGB Column for 3~5 min or until the buffer is absorbed by the membrane. --Important Step! For effective elution, make sure that the elution solution is dispensed onto the membrane center and absorbed completely.

14. The samples was centrifuge for 30 seconds at full speed (14,000 rpm or 10,000 x g) to elute the DNA . --Standard volume for elution is 100 µl. If sample has low number of cells, reduce the elution volume (30 µl - 50 µl) to increase DNA concentration. If higher DNA yield is required, repeat the DNA Elution step to increase DNA recovery and the total volume could be 200 µl.

Step Final - Pure DNA 19. The DNA fragment was stored at 4ºC or -20ºC

3.10.1.1. Evaluation of the Extracted DNA

Amount and purity of DNA: The amount and purity of the extracted DNA were evaluated by spectrophotometer NanoDrop (Thermo Scientific NanoDrop 2000, USA). The DNA concentration (ng/µl) and the ratio of optical density OD260nm and OD280nm (R 260/280) were measured. DNA is considered pure when the ratio 260/280 is around 1.8.

3.10.1. 2. Polymerase chain reaction

3.10.1.2.1. Primer design

The design of PCR primers according to the protocol of (Hashim *et al.*, 2015) briefly as follow: The primers were designed by the aid of NCBI-primer BLAST online software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome) , at the same time the produced primers was checked for specificity for their target sequences by performing the BLAST against the human genome , then the primers pair was selected according to the demand criteria such as : product length , the similarity of melting temperature , primers length , specificity , etc. Then the mutations was interred according to the design demands .

The primer ability to form secondary structure was checked by the aid of Oligo Calc online software (http://www.basic.northwestern.edu/biotools/oligocalc.html) , the primer would be rejected if it had 5 bases or more able to form self-dimerization and/or it had 4 bases able to form hairpin .

Each primers pair was checked for dimer formation by the aid of "Multiple Primer Analyzer" online software from Thermo Fisher Scientific Inc.© , the sensitivity of the software was adjusted to the value 2 , the primer pair would be rejected if it made any dimers in this degree of sensitivity .

3.10.1.2.2. Reconstituting and diluting primers

Primers were commonly shipped in a lyophilized state. The units of a lyophilized primer are given as a mass, in Pico moles. To create a stock of primers, one would reconstitute the primer in sterile, nuclease free H,O. The company supplies the amount of sterile, nuclease-free H,O to be added to each primer to obtain master stock (100mol/µl) that will be used again to obtain working stock. As following:

1-The tube was spin down before opening the cap, then the desired amount of water was added according to the oligos manufacturer to obtain a 100 pmol/ul (Master Stock).

2-Vortex properly for re-suspend the primers evenly. Then 10ul of the master stock was transfered to a 0.2ml Eppendorf tube that contains 90ul of sterile, nuclease-free H.O (Working Stock).

3-The master stock is stored at -20 C' and the working stock is stored at-20 C Finally, the working stock was thawed on ice and vortex before using in PCR and then stored at-20 C. Sequences of primers used for PCR amplification this study were shown in Table (3-4).

Table (3-4): Primer Sequences the studied genes and their locations

Gene	Primer Sequences ′5_′3	Size Product (bp)	T_m (°C)	Ref.
Adepo-out-rs266729	F: 5′-GGACTG TGGAGATGATATCTGGGG GGC A -3′ (outer) R: 5′-TGGCCTAGAAGCAGCCTGGAGAAC TGG A -3′	299	73.1 73.1	(Hashemi *et al.*,2013)
C_intr_F G_allel_intr_R	5′-CTTGCAAGAACCGGCTCAGATCCCCC -3′ (inner) 5′-GAGCTGTTCTACTGCTATTAGCTCTGC-3′	155 201	72.8 68.3	
Adiponectin_rs1501299	F:5′-GAGCTGTTCTACTGCTATTAGCTC TGC -3′ (outer) R:5′-GAATATGAATGTACTGGGAATAGG GATG-3′	476	68.3 65.7	
G_allel_F T_allel_R	5′-CCTCCTACACTGATATAAACTATATGA GGG -3′ (inner) 5′-TGTGTCTAGGCCTTAGTTAATAATGAA CGA -3′	244 299	68.0 66.6	
Lep	F: 5′-GGTGGTGGATCTGTCCAAGG-3′ R: 5′-CAAAGTGCAAGCAGGGTTCC-3′	309	62.5 60.5	Designed in the present study
RETN	F: 5′-TGAGAGGATCCAGGAGGTCG-3′ R: 5′-GGACCCTGTCTTGAGTTGGG-3′	285	62.5 62.5	Designed in the present study
SF3B1 exon-13	F:5′-GTACATGAGCATTTCATCAGTA-3′ R:5′-CAACCATTTCTTTCCATAATCA-3′	223	50.6 54.7	(Rujiracchaivej *et al.*,2018)

Tm: Annealing Temperature.

3.10.1.2.3. PCR experiments

Conventional PCR was used to amplify a target DNA using specific primer pairs. PCR typically consists of three consecutive steps (denaturation, annealing, and elongation) of repeated cycles to get PCR product (amplicon). The PCR thermal cycling conditions are mentioned in the Table (3-5). The size of PCR products (5 µL) were analyzed in 2% (w/v) agarose gel by electrophoresis using 1X TBE buffer

and visualized by staining with simply safe dye. Product size was determined by comparison with size 100 bp DNA ladder (Intronbio/Korea).

Table (3-5): Recommended volumes and concentrations for applying PCR into AccuPower® PCR tubes.

Component	Volume (µl)	Concentration
PCR master mix	8	2.5X
Forward primer	1	DNA 10 pmol/ µl
Reverse primer	1	DNA 10 pmol/ µl
Template DNA	2	50 ng
Free water	7.5	
Mgcl2	0.5	25 mM
Final volume (dH_2O)	20	

The genes studied were amplified using specific primers according to mention conditions in table (3-6).

Table (3-6): Amplification conditions of the study genes.

Genes	Initial Denaturant. °C	Denaturation. °C	Anneal. °C	Extension °C	Final extension °C, Cycle	Techniques used
ADIPO	94 (5 min)	94 (30 sec)	60 (30sec)	72 (30sec)	72 (5 min)	**ARMA**
	1 cycle	35 cycle			1 cycle	
LEP	94 (5 min)	94 (30 sec)	61 (30 sec)	72 (30 sec)	72 (5 min)	**PCR, Sequence**
	1 cycle	30 cycle			1 cycle	
RETN	94 (5 min)	94 (30 sec)	63 (30 sec)	72 (30 sec)	72 (5 min)	**SSCP, Sequence**
	1 cycle	35 cycle			1 cycle	
Sf3b1	94 (5 min)	94 (30 sec)	55 (30 sec)	72 (45 sec)	72 (5 min)	**PCR, Sequence**
	1 cycle	35 cycle			1 cycle	

Extension: Extension temperature ; Denaturant.: Denaturation temperature ; Anneal.: Annealing temperature.

3.10.2. Prepration of solution

A- Ethidium Bromide Staining Solution

Ethidium bromide 10µm of 10 mg/ml concentration in TBE buffer 100 ml 0.5x. The solution stored in a dark bottle at room temperature (Robinson and Lafleche, 2000).

B- Ammonium Persulfate (10%) APC

Twenty milligrams of ammonium persulfate were dissolved in 200 µl distilled water.

C- Tris Borate EDTA (TBE) buffer

About 500 ml of TBE (1X) prepared by adding 50 ml of TBE (10X) stock solution to a final volume of 500 ml of d H_2O.

E- SSCP gel loading buffer

The gel loading buffer composed of (0.25% Bromophenol blue, 0.25% Xylene cyanol, 95% Formamide and 10 mM Sodium hydroxide) was used to load PCR product during single strand conformation polymorphism DNA electrophoresis.

3.10.3. Agarose Gel Electrophoresis

Electrophoresis through agarose is the standard method used to separate, identify, and purify DNA fragments. The technique is simple, rapid to perform, and capable of resolving fragments of DNA that cannot be separated adequately by other procedures. Furthermore, the location of DNA within the gel can be determined directly by staining with low concentrations of the fluorescent intercalating dye ethidium bromide; bands containing as little as 1–10 mg of DNA can have detected by direct examination of the gel in ultraviolet light. If necessary, these DNA bands can be recovered from the gel and used for various cloning purposes (Sam Brook and Russell, 2001).

This method, whereby charged molecules in solution, chiefly proteins and nucleic acids, migrate in response to an electric field, is allied electrophoresis. Their rate of migration, or mobility, through an electric

field depends on the strength of the field, the net charge, size, and shape of the molecules, and the ionic strength, viscosity, and temperature of the medium the molecules are moving. The DNA movement in the gel depends on its molecular weight , conformation, and concentration of the agarose, the voltage applied, and strength of the electrophoresis buffer, and the following procedure was used (Harisha, 2007).

3.10.3.1. Gel Electrophoresis Protocol

1- The gel–casting tray places in the plastic tray, checked that the comb's teeth are approximately 0.5 cm above the gel bottom.

2- The gel 0.8% was prepared by dissolving 0.8 gm of agarose respictivelly in 40 ml of 0.5x TBE for DNA extraction. For PCR product (1.6%) was prepared by dissolving 1.6 gm of agarose in 80 ml of 0.5X TBE buffer and heated by a microwave oven for 2 minutes and this is horizontal gel electrophoresis

3-The agarose solution was cooled to approximately 55°C and added 10µm of ethidium bromide to the gel and mixed by swirling.

4- The gel then was poured to the gel try and let to polymerize for 30 minutes

5-- The polymerized gel then was transferred to the electrophoresis devise and submerged with 0.5 TBE running buffer.

6- Five microliters of PCR product were mixed with 2µl of loading dye and carefully loaded by mechanical pipet to the gel wells.

7- The electrophoresis was carried out by setting the device 100 volts for 60 minutes.

8- The gel then was imaged and the image was analyzed to determine the extracted DNA and PCR product molecular weight.

3.10.4. Amplification Refractory Mutation System (ARMS) for adiponectin gene

Amplification refractory mutation system (ARMS) PCR: Allele-specific amplification (AS-PCR) or ARMS-PCR is a general technique for the detection of any point mutation or small deletion (Newton and Graham, 1989). The genotype (normal, heterozygous and homozygous states) of a sample could be determined using two complementary reactions: one containing a specific primer for the amplification of normal DNA sequence at a given locus and the other one containing a mutant specific primer for amplification of mutant DNA. The composition of this reaction explained in table (3-7).

Table (3-7): Component of PCR-ARMS reaction for *rs266729* genotyping .

Component	Volume (µl)	Concentration
PCR master mix	8	2.5X
F outer primer	1	DNA 10 pmol/ µl for each
R outer primer	1	
C allele primer	1	
G_allele_primer	1	
Template DNA	2	50 ng
Free water	5.5	
Mgcl2	0.5	25 mM
Final volume (dH_2O)	20	

3.10.5. Single-Strand Conformation Polymorphism (SSCP)-Principle

The accurate analysis of genetic variation has major implications in many areas of biomedical research, including the identification of infectious agents, the diagnosis of infections, and the detection of unknown or known disease-causing mutations. Mutation scanning methods, including PCR-coupled single-strand conformation polymorphism (SSCP), have significant advantages over many other nucleic acid techniques for the accurate analysis of allelic and mutational sequence variation. The SSCP protocol can readily detect point mutations for amplicon sizes of up to 450-500 bp, and usually takes 1 days to carry out. This user-friendly, low-cost, potentially high-throughput platform has demonstrated the utility to study a wide

range of pathogens and diseases, and has the potential to be applied to any gene of any organism (Gasser *et al* ., 2006)

3.10 .5. A. Procedure of SSCP

1-The glass plates were cleaned thoroughly with warm tap water, and then rinsed with tap water, deionized water, and finally ethanol. They were dried either by wiping or by air.

2- The long plate was put down first on a clean surface, and then the left and right spacers were placed along the sides of the long plate. The short plate was put on top of the spacers so that it was even with the bottom edge of the long plate.

3-The single screw of each sandwich clamp was loosened, and each clamp was placed by the appropriate side of the gel sandwich with the locating arrows facing up and toward the glass sandwich.

4-The gel sandwich was held firmly and fit it into the left and right clamps. The screws were tightened enough to hold the plates in place. It was checked whether the plates and spacers were even at the bottom. If this was not the case, the plates and spacers were realigned to obtain a good seal. Failure to do so could have resulted in a gel leakage when casting, as well as buffer leakage during the run.

5-The gray sponge was placed onto the front casting slot. The sandwich assembly was placed on the sponge with the short glass plate facing forward. The sandwich was pressed down, and the handles of the camshaft were held down as well to lock the sandwich in place.

6-The gel solution was poured into the sandwich.

7-A comb was inserted into the top of the sandwich to form the sample wells and was left to polymerize at room temperature for at least 1 h

8-After polymerization, the comb was removed by pulling it straight up slowly and gently. The gel sandwich was released from the casting stand and attached to the

zcore with the short glass plate facing the core. The core was turned to its other side and the second gel sandwich was attached.

9-The upper and lower chambers were filled with 0.6 X TBE buffer and the wells were rinsed out thoroughly with running buffer using a syringe and needle.

10-The core and the attached gel sandwiches were placed into the electrophoresis tank; allowing the core to lock in place. The lid was put on and the system was connected to an external water chiller. The temperature was set and pre-run for 20 min to reach the desired temperature.(Menounos & Patrinos, 2010)

3.10. 5. B. Sample Preparation

1-2x SSCP Gel Loading dye was prepared of 95% Formamide, 20 µm EDTA pH8.0 , 0.05 bromophenol blue.

2- SSCP Gel was prepared

In SSCP, a non-denaturing polyacrylamide gel is used to separate single stranded DNA. Altered conformation due to a mutation in the sequence can cause the mutant single-stranded DNA to migrate differently than control this migration can be seen as a band shift between the mutant and control DNA and samples as follow:

1. 2.5 µl of PCR product was added with 2.5 µl of 2X SSCP gel loading dye to a microfuge tube DNA. Genty mixed.

2. The tubes were placed into a 95°C water bath for 7-10 min and then on ice for about 5 min (denaturation step).

3. Load 5 µl of the samples and control into wells of an 15% acrylamide/bis gel (29:3:0.7), and 5X TBE buffer.as shown in table (3-6).

Table (3.8): Gel electrophoresis compenant of SSCP.

Subject	Volume
30% acrylamide/bis (29:1)	5 ml
5X TBE	1 ml
TEMED	10 µl
10% ammonium persulfate	200 µl
dH_2O	4 ml

3. 10. 5. C. Electrophoresis

1-When the running buffer had reached the desired temperature, the pre-run was stopped, the wells were rinsed with running buffer again, and 10 µL of the samples was loaded into the wells using "long" tips.

2-The gel was run at a constant power of 100 v for 6–12 h at 5–15؛C.

3-After the electrophoresis were completed, the power supply and water chiller system off were turned off, the electrodes were disconnected, and the core out of the electrophoresis tank was pulled out carefully.

3.10.5. D. Silver Staining

1- The core and gel sandwich were laid on a padded surface to absorb buffer spills. The gel sandwich was removed from the core. The gel was removed carefully from the plates, and it was rinsed briefly in deionized water.

2- The gel was immersed in a tray containing solution 1 (45 ml deionized water, 5ml ethanol, 0.1 gram silver nitrate and 250 µl acetic acid) and the tray was placed on top of a shaker to be mixed for at least 30 min.

3- Solution 1 was poured off and the gel was briefly rinsed with deionized water.

4- Solution 2 was put in a water bath at 55 C. As soon as solution 1 was removed, solution 2 (1.5 g NaOH, 75 µL formaldehyde, 50 ml deionized water) was added for 20 min.

5- When the bands were clearly visible, the second solution was thrown and the third solution (5 ml ethanol, 45 ml deionized water and 250 µl acetic acid) was added for 5 min.

6- The gel was placed on top of the LED light, then the bands were read and the results on the gel were recorded and a photo was taken afterwards.

3.10.6. DNA Sequencing of PCR amplicons

The resolved PCR amplicons were commercially sequenced from both forward and reverse termini according to instruction manuals of the sequencing company (Macrogen Inc. Geumchen, Seoul, South Korea). Only clear chromatographs obtained from ABI sequence files were further analyzed, ensuring that the annotation and variations are not because of PCR or sequencing artifacts. By comparing the observed DNA sequences of the investigated samples with the retrieved neighboring DNA sequences of the NCBI Blastn engine, the virtual positions and other details of the retrieved PCR fragments were identified.

3.10.6.1. Interpretation of sequencing data

The sequencing results of the PCR products of different samples were edited, aligned, and analyzed as long as with the respective sequences in the reference database using BioEdit Sequence Alignment Editor Software Version 7.1 (DNASTAR, Madison, WI, USA). The observed variations in each sequenced sample were numbered in PCR amplicons as well as in its corresponding position within the referring genome. The highlighted SNPs were visualized to the dbSNP database to check their originality. Each particular SNP was positioned according to its place in the reference genome.

3.10.6.2. Checking the novelty of SNPs

The determination of the previous deposition of the observed SNP was performed by viewing its corresponding dbSNP position. Then, the dbSNP position for the detected SNP was documented. The observed SNPs were submitted to the dbSNP

database to check their novelty. Each particular SNP position was checked in its corresponding reference genome to assess whether it was previously deposited in the dbSNP server.

3.10.6.3. Gene Accession number

Genes studies are accessible to gene bank as the following:

- Leptin *(LEP gene)*:sequence 1 (MZ544161) and end in sequence 24 (MZ544184).
- Resistin *(RETN gene)*: sequence 1 (MZ604892), and end in sequence 6 (MZ604897).
- *SF3B1gene:* sequence 1 (MZ488300) to sequence 25 (MZ6048324).

3.10.7. Real-Time PCR

Real-Time PCR were investigated and analysis in the laboratory of College of Science/ Unversity of Babylon according to Livak and Schmittgen, (2001).

3.10.7.1. Real-Time PCR Primer

The primers used in the study are shown in table (3-9).

Table (3-9): Primers used for gene expression.

Primer sequence for gene expression (′5-′3)		Product size bp	Tm	Ref.
House - keeping gene *(hagpdhr)*	F:5′-TCGCTCAGACACCATGGGGAAG-3′ R:5′-GCCTTGACGGTGCCATGGAATTTG-3′	198	60.5 56.4	Komeno *et al.*, 2015)
***HSEx* gene**	F: 5′-CTACAGCCGCTCGAAGTCTC-3′ R: 5′-TTGGATTCCCTCTTGGACAC-3′	174	62.5 58.4	

3.10.7.2. Total RNA Extraction (GENEzol™ TriRNA pure kit/Korea)

1. Sample Homogenization and Lysis Sample preparation: Samples was performed at room temperature.

1. Liquid sample 200µl was transferred up to a 1.5 ml of microcentrifuge tube (RNase-free).

2.3 volumes of GENEzol™ Reagent was added per 1 volume of sample (3:1) then mixed well by vortex.

3.The sample mixture was incubated for 5 minutes at room temperature.

2. **RNA Binding**

1. The sample was centrifuged at 12-16,000 x g for 1 minute to remove cell debris then the clear supernatant was transfered to a new 1.5 ml microcentrifuge tube (RNase-free).

2. Amount 1 volume of absolute ethanol was added directly to 1 volume of sample mixture (1:1) in GENEzol™ Reagent.

3. Then mixed well by vortex then RB Column was placed in a 2 ml Collection Tube.

4. A mount 700 µl of the sample mixture was transferred to the RB Column. Centrifuge at 14-16,000 x g for 1 minute then discard the flow-through.

5. The RNA Binding Step was repeated by transferring the remaining sample mixture to the RB Column.

6. The mixture was centrifuged at 14-16,000 x g for 1 minute then discarded the flow-through. The RB Column was placedin a new 2 ml Collection Tube.

3. **RNA Wash**

1. A mount 400 µl of Pre-Wash Buffer (make sure ethanol was added) was added to the RB Column then centrifuge at 14-16,000 x g for 30 seconds.

2. The flow-through was discarded then the RB Column was placed back in the 2 ml Collection Tube.

3. A mount 600 µl of Wash Buffer (make sure ethanol was added) was added to the RB Column.

4. Centrifuge at 14-16,000 x g for 30 seconds then the flow-through was discarded . The RB Column was placed back in the 2 ml Collection Tube.

5. A mount 600 µl of Wash Buffer (make sure ethanol was added)was added to the RB Column.

6. Centrifuge at 14-16,000 x g for 30 seconds then the flow-through was discarded.

7. The RB Column was placed back in the 2 ml Collection Tube. NOTE: For blood samples only, wash the RB Column again with 600 µl of Wash Buffer.

8. Centrifuge at 14-16,000 x g for 3 minutes to dry the column matrix.

4. RNA Elution

1. The dry RB Column was placed in a clean 1.5 ml microcentrifuge tube (RNase-free).

2. About 25-50 µl of RNase-free Water was added into the CENTER of the column matrix.

3. Let stand for at least 3 minutes to ensure the RNase-free Water is completely absorbed by the matrix.

4. Centrifuge at 14-16,000 x g for 1 minute to elute the purified RNA

3.10.7.3. Quantitative real time PCR assay (QRT-PCR) reaction

The amplification of mRNA fragments was performed with the reaction components of cDNA synthesis as in Table (3-9) and the condition of cDNA synthesis as in Table (3-10). Several experiments were done for more appropriate synthesis of cDNA, the qRT–PCR list program in Table (3-11) and the reaction mix of qRT–PCR in Table (3-12) according to (Bioneer lypholyzed kit /Korea).

Analysis is a new, post-PCR analysis method used for identifying genetic variation in nucleic acid sequences. Simple and fast, this method is based on PCR melting (dissociation) curve techniques and is enabled by the recent availability of improved double-stranded DNA (dsDNA)-binding dyes along with next-generation real-time PCR instrumentation and analysis software.

Table (3-10): Reaction volume and components of cDNA synthesis (Kit Bionear lypholyzed/ Korea)

Component	**Volume (**µl)	**Concentration**
RNA	1	100 mg/ ml
Oligo dT 20	1	50 PM
Nuclease free water	18	-----
Total volume	20	-----

Table (3-11): Thermocycling condition of reaction cDNA synthesis

Step	**Temperature (C°)**	**Time (min)**
Primer annealing (oligo dT20)	37	10
cDNA synthesis	60	45
Heat inactivation	95	5

Table (3-12): Quantitative RT–PCR reaction mix

Component	Volume (µl)	Concentration
qPCR master mix	8	2.5 X
$MgCl_2$	0.5	25 mM
Forward primer	0.5	10 PM
Reverse primer	0.5	10 PM
cDNA	2	Variable
Nuclease free water	8.5	-----
Total volume	20	-----

Table (3-13): Thermocycler program for quantitative RT–PCR reaction mix

Steps	Temperature (C°)	Time	Cycles
Hot start activation	95	5 min	1
qPCR Steps			
Denaturation	95	10 S	40
Annealing and elongation: Aquirring on green chanel	60	30 S	40
Milt step 65 – 98 on green chanel	0.5 for each step		1

3.10.7.4. Data Analysis of qRT-PCR

The data of qRT-PCR for target and housekeeping gene were analyzed by the relative quantification gene expression levels (fold change) depending on the method that described by (Livak and Schmittgen, 2001) which consist ΔΔCT using a reference gene. The relative quantities obtained from qRT-PCR experiment must be normalized in such a way that the data become biologically meaningful. In this method, one of the experimental samples is the calibrator such as (control samples) each of the normalized target values (CT values). The ΔCT of control and target gene are calculated as in the following equation:

$$\Delta CT_{\text{target gene}} = Ct_{\text{target gene}} - Ct_{\text{reference gene/target}}$$

$$\Delta CT_{\text{control}} = Ct_{\text{control}} - Ct_{\text{reference gene/control}}$$

ΔΔCT was calculated after the calculation of ΔCT of target gene and control as in the following equation:

$$\Delta\Delta CT = \Delta Ct_{\text{target gene}} - \Delta Ct_{\text{control}}$$

Then $^{2-}\Delta\Delta CT$ was calculated as fold change in expression.

3.11. Statistical Analysis

Statistical analyses were performed using the statistical package for the social science (SPSS) program and Sequence Manipulation Suite online software (http://www.bioinformatics.org/sms2/rest_digest.html). Continuous variables were presented as means and standard deviation. Independent sample T-test was used to compare means between two groups. When *P* values were less than or equal to 0.05 considered as statistical significance, while *P* values were more than 0.05 considered as statistical non-significance (Dowdy *et al.*,2011).

4. Results and Discussion

4.1. Demographic study

4.1.1. Age and gender of the samples

Samples were studies (75) samples they were 29 males and 21 females in the patient group, while in control group they were 16 male and 9 females. The present study results showed that the males' percentage value of myelodysplastic syndrome was more than the females' percentage value (58% and 42%, respectively). Also, the ages of the samples in this study arrange from 20-85 years old in men and women (Table 4-1) and (Fig.4-1). The age and gender of patients and control groups (Fig. 4-2, 4-3 respectively) .

Table (4-1): Age and sex of myelodysplastic syndrome patients and control groups.

Population	Sample size	Age ± SD	Gender	No.	%
Patient	50	57.55 ± 20.35	male	29	58
		65.3 ± 14.7	female	21	42
Control	25	57.44 ± 5.85	male	16	64
		55.56 ± 2.79	female	9	36

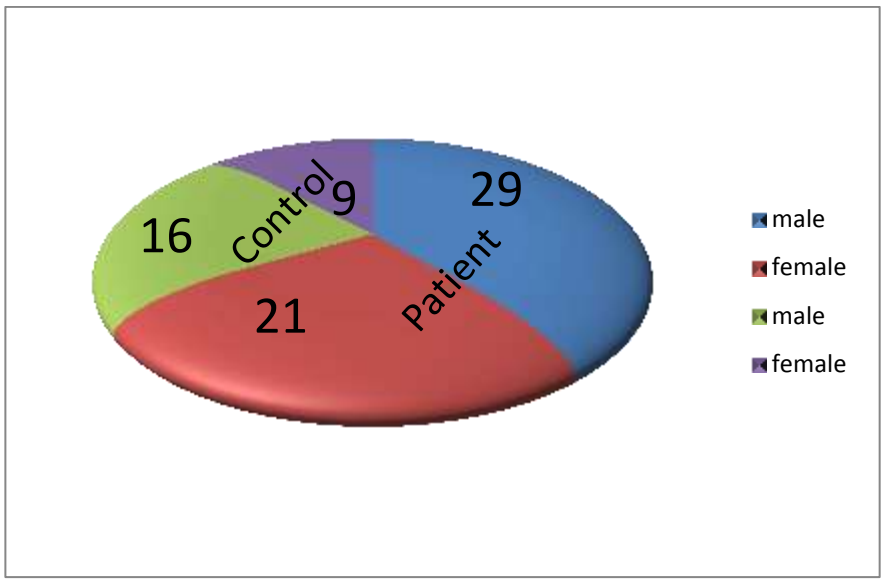


Figure (4-1): Percentage of male and female of myelodysplastic syndrome patients and control groups.

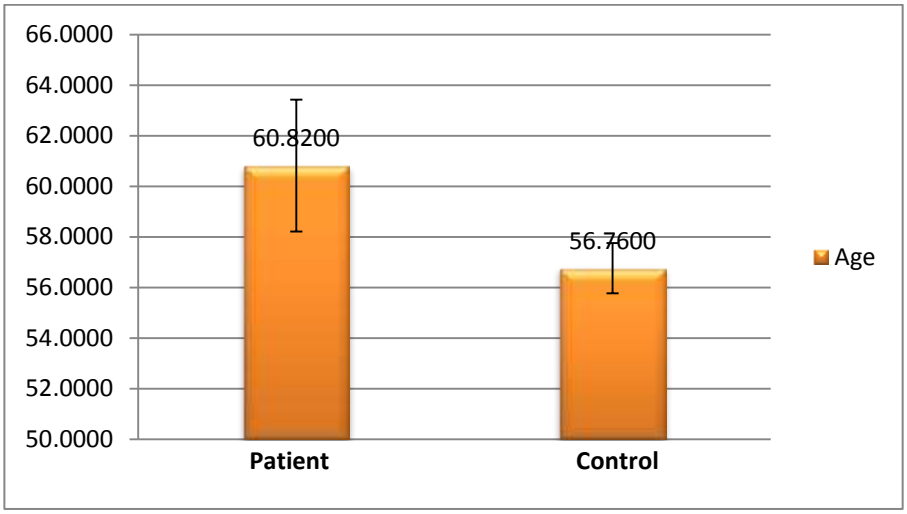


Figure (4-2): Comparison of age between patients of myelodysplastic syndrom and control groups (mean ± SD).

Aging consider the most important risk factors for the development of MDS. Owing to errors in DNA replication and spontaneous mutations from normal metabolic by products (eg, conversion of cytosine to thymidine by oxidative deamination from reactive oxygen species), coding mutations accumulation in hematopoietic stem cells at a mean ± SD of 0.13±0.02 and exonic mutations per year of life (Xie *et al.*, 2014).

Approximately 10% of individual older than 70 years have clonal mutations in genes associated with myeloid neoplasia, such as *DNMT3A, TET2* and *SF3B1* genes and these persons have a 0.5% to 1% chance per year of acquiring additional mutations that lead to progression to MDS or another hematological neoplasm (Jaiswl *et al.,* 2014). Incidence of MDS rises with age, with the majority of cases diagnosed after age 60 years (Ma *et al.,* 2007) (Fig.4-2). The MDS disease more frequently diagnosed in men with the exception of MDS with 5q- syndrome, which is slightly more frequent in women (Climent, 2000).

The present study was inconsisted with the study of Wang *et al.* (2019), showed that the median age of the qualified MDS patients at diagnosis was 76 years (interquartile range, 66-83 years). Female patients were not significantly older than male patients at the time of diagnosis (median 77 years vs. 75 years, respectively). The numbers of male and female patients increased gradually with age. Patients aged ≥80 years had the highest prevalence of MDS compared with those in the <60 years, 60-69 years, and 70-79 years age groups. general annual cases of MDS increased dramatically with advancing ages. Among them, men were shown to have higher annual cases of MDS compared with women (Wang *et al.,* 2019).

Mreno Berggren *et al.*,(2018), found that not only a significant effect of gender on the incident rate of MDS patients but also variation of survival between genders among subgroups by age at diagnosis, race, marital status at diagnosis and MDS subtypes. Male patients had significantly shorter survival compared with that of females, which has been validated by Asian and European MDS cohorts. The higher mortality rates of male patients with MDS might be explained by the increased presence of comorbidities upon diagnosis (Zipperer *et al* .,2014). In addition to the factors described above, other possible explanations of the shorter survival of male MDS patients might include the association with molecular abnormities such as faster methylene aging and shorter telomeres (Hannum *et al.*,2013), both of which often correlate with shorter survival (Barrett and Richardson, 2011). Less benefit

from treatment might be another reason for short survival of male patients. Male MDS patients expressed higher level of cytidine deaminase (CDA) than that of females (DeZern *et al.*,2017), whereas CDA could rapidly inactivate the activities of decitabine (DAC) and azacitidine (AZA), two 5-methylated cytidine analogs commonly used for the treatment of MDS patients (Mahfouz *et al.*,2013). These male-specific responses might affect the outcome of DAC or AZA therapeutics and cause increased mortality. Also found that the increased risk of mortality of male patients was associated with RA, RCMD, MDS 5q-, MDS/MPN and MDS-NOS, but not other subtypes, indicating that survival varied as a function of gender across the MDS histological subtypes (Greenberg *et al.*, 2012).

4.1.2. Relationship of body mass index with myelodysplastic syndrome

The results of body mass index showed a significant decrease ($p<0.05$), in patients with MDS when compared with control group (Figure 4-3).

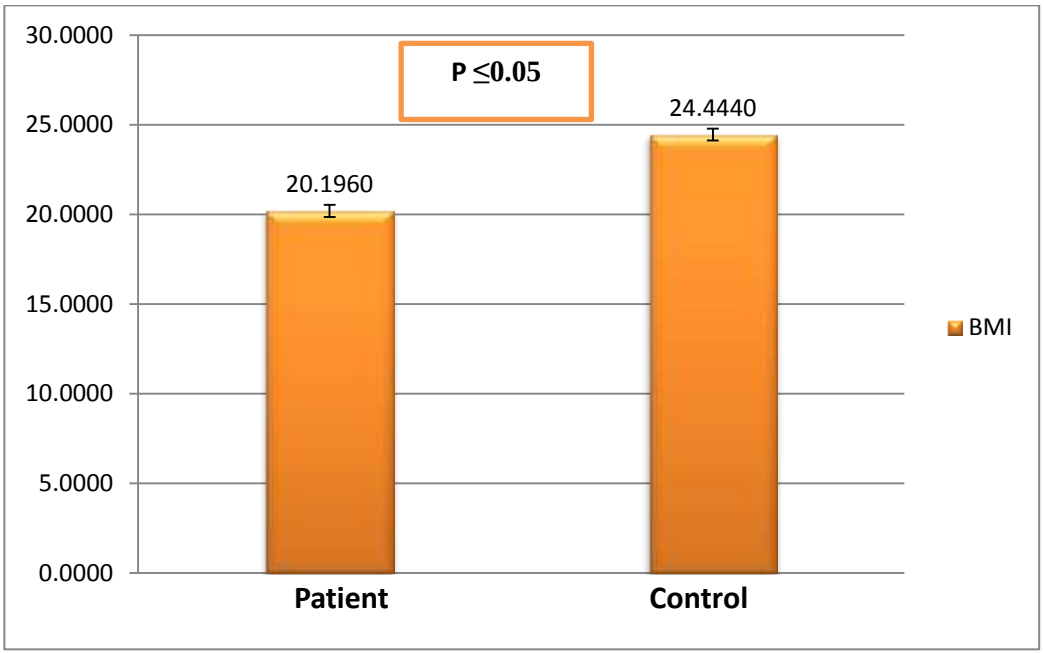


Figure (4-3): Mean ±SD comparison between BMI in MDS patients and control group.

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The body mass index (BMI) is the metric currently in use for defining anthropometric weight/height characteristics in adults. It also is widely used as a risk factor for the development of or the prevalence of several health issues. In addition, it is widely used in determining public health policies. The BMI has been useful in population based studies by virtue of its wide acceptance in defining specific categories of body mass as a health issue. However, it is increasingly clear that BMI is a rather poor indicator of percent of body fat (Nuttall, 2015) .

While the effect of a patient being overweight or obese on cancer prevalence appears to be clear, the impact of excess body fat on patient survival at the time of cancer diagnosis is less well-defined. Kraakman *et al*., (2018). demonstrate that in a mouse model of myelodysplastic syndrome (MDS) obesity improves survival in the absence of treatment, and propose some biological explanations to this survival advantage. A link has recently been established between saturated fatty acids that accumulate in the serum of obese people - the fatty acid binding protein FABP4 to which they bind, which is highly expressed in leukemic cells - and a cellular pathway that leads to DNA hypermethylation and fuels AML cell growth, suggesting innovative therapeutic strategies in this disease (Yan *et al.,* 2018).

As acknowledged by Kraakman *et al.,* (2018), a limitation of their study is the use of Ob/Ob mice in which the Lep gene is disrupted. Leptin levels are elevated in overweight individuals in which this pro-inflammatory adipokine was shown to affect the behavior of tumor cells and their microenvironment. A proliferative and anti-apoptotic effect of leptin has also been depicted on AML blast cells (Konopleva *e al.,*1999). Therefore, the absence of leptin in the tested model may alter the natural history of the disease in an overweight setting, which demands validation in another model of obesity in which leptin secretion is maintained. The demonstration that improved survival in MDS animals is related to the absence of leptin would foster the therapeutic development of leptin antagonists, including leptin analogs and antibodies targeting leptin or its transmembrane receptor (Ray and Cleary, 2017).

The study of Poynter *et al*., (2016) observed significant associations between obesity and MDS only among women.

The biological mechanism linking obesity to hematologic malignancy has not been established to date. Several potential mechanisms have been proposed (Karmali *et al*.,2015), including alterations in the metabolism of endogenous hormones such as sex steroids, insulin, insulinlike growth factors, leptin, adiponectin, and fetuin-A (Damlaga *et al.,* 2013). Insulin-like growth factor-1 (IGF-1) appears to be particularly relevant as it is known to increase in response to obesity-related insulin resistance (Bianchini *et al.*,2002), and exhibits mitogenic activity in both myeloid and lymphoid leukemia cell lines. Leptin levels, which are also increased in obese individuals , have been shown to influence proliferation and differentiation of hematopoietic cells and myeloid leukemia cell lines. It is likely that multiple obesity-related alterations in metabolism are responsible for the association, possibly in the context of changes in the bone marrow microenvironment (Poynter *et al*.,2016). The number of adipocytes in the bone marrow increases with age (Rosen *et al.*,2009), and these cells have been shown to negatively regulate hematopoiesis (Naveiras *et al.*,2009).

Two previous studies and one case control study have reported on overweight and obesity in MDS (Damlaga *et al.*,2008; Ma *et al.*,2009; Murphy *et al.*,2013). The million women study evaluated risk associated with a 10 kg/m2 increase in BMI so the risk estimates are not directly comparable to ours; however, the results do support a significant increase in risk of MDS in obese women (Murphy *et al*.,2013).

4.2. Hematological Study

The results showed significant differences in myolodysplastic syndrome according to hematological parameters. Table (4-4), displays a significant decrease ($p \leq 0.05$), of white blood cells, neutrophils, Eosinophils, lymphocytes, monocytes, red blood cells, hemoglobin, hematocrit, and platelet count when compared with

control group. Additional there were no significant differences between basophils, mean corpuscular volume, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration.

Table (4-2): Mean ± SD of hematological parameters in patient of myelodysplastic syndrome and control groups.

Parameters	Control group (No.25) Mean ±SD	MDS group (No.50) Mean ±SD	P-value
WBCs 10^3/µL	7.6856±2.08543	3.5356±1.66254	*0.000
Neutrophil%	63.32004.06171	42.8406±7.49092	*0.000
Eosinophil%	1.4920±0.79211	0.4832±0.35504	*0.000
Basophil %	0.3772±0.25527	0.3358±0.36455	0.613
Lymphocyte%	33.32006.56836	19.0166±8.26153	*0.000
Monocyte %	4.9680±1.65497	1.3414±0.72036	*0.000
RBCs 10^6/µL	4.6544±0.30293	2.9818±0.72036	*0.000
HCT %	40.5040±3.42789	26.3760±5.15809	*0.000
HGB gm/dl	13.0560±1.56340	8.5458±1.72649	*0.000
MCV Fl	90.3200±3.72424	92.0890±5.83894	0.114
MCH pg	29.7120±1.56053	30.9980±3.52533	*0.032
MCHC g/dl	34.3960±0.94228	34.0140±1.94055	0.255
PLT 10^3/µL	247.2000±63.81092	116.9880±36.71388	*0.000

*** is significant at P≤ 0.05**

SD Standard deviation.

The myelodysplastic syndrome are group of heterogeneous group of clonal bone marrow disorders characterized by ineffective hematopoiesis and peripheral blood cytopenias. Genomic instability of the MDS clone often leads to disease progression, which results in overt leukemia in 0-32% of patients depending on MDS subtype (Germing *et al.*,2006). Eighty percent of patients have a hemoglobin level of less than 10 g/dl at diagnosis, and most of these will become transfusion dependent (Sanz *et al.*,1989).

Anemia (typically macrocytic) is the most common peripheral blood abnormality, occurring approximately 80% to 85% of patients. Thrombocytopenia occurs in 30 to 54% of MDS cases, with approximately 40% of patients found to have neutropenia at diagnosis (Foran and Shammo, 2012). In the study relating to clinical outcomes in MDS, 57% of patients had a hemoglobin level of <10.0 g/dl (27% with severe anemia), 38% of patients had an absolute neutrophil count of <1.500 cells/µl (6% with severe neutropenia), and 37% patients had a platelets count of <100.000/µl (5% with severe thrombocytopenia) (Steesma and Bennett, 2006). These finding is agreed with the results of the present study that mentioned decreased level of hemoglobin, neutrophils and platelets count. The results of the present study agreed with results of Raess *e al.*,(2014) which indicated that patients with MDS have significantly decreased hemoglobin and hematocrit, WBC count, platelets count. MCV, MCH, and MCHC are significantly elevated in patients with MDS. Patients with MDS also showed decreased in the percentage of neutrophils and concordant increased in the percentage of lymphocytes.

Figure (4-5), showed the cells that found in the bone marrow smear from MDS patients of the present study. Bone marrow aspiration/biopsy examinations a few weeks or months, or even years apart in order to firmly establish the diagnosis and to identify cases with rapid disease progression. Dysplastic changes are the most important diagnostic features of myelodysplastic syndrome. A marrow cell lineage is considered picture of MDS if >10% of cells are affected. The cell counting of bone marrow and blood smear should include at least 200 cells in blood smear, 500 cells in bone marrow and 25 megakaryocytes and at least 100 erythroblasts should be evaluated. An optimal staining of blood and marrow slides prepared from freshly drawn aspirates is important for evaluation of dysplasia (Abdul Hamid and Shukry.,2008).

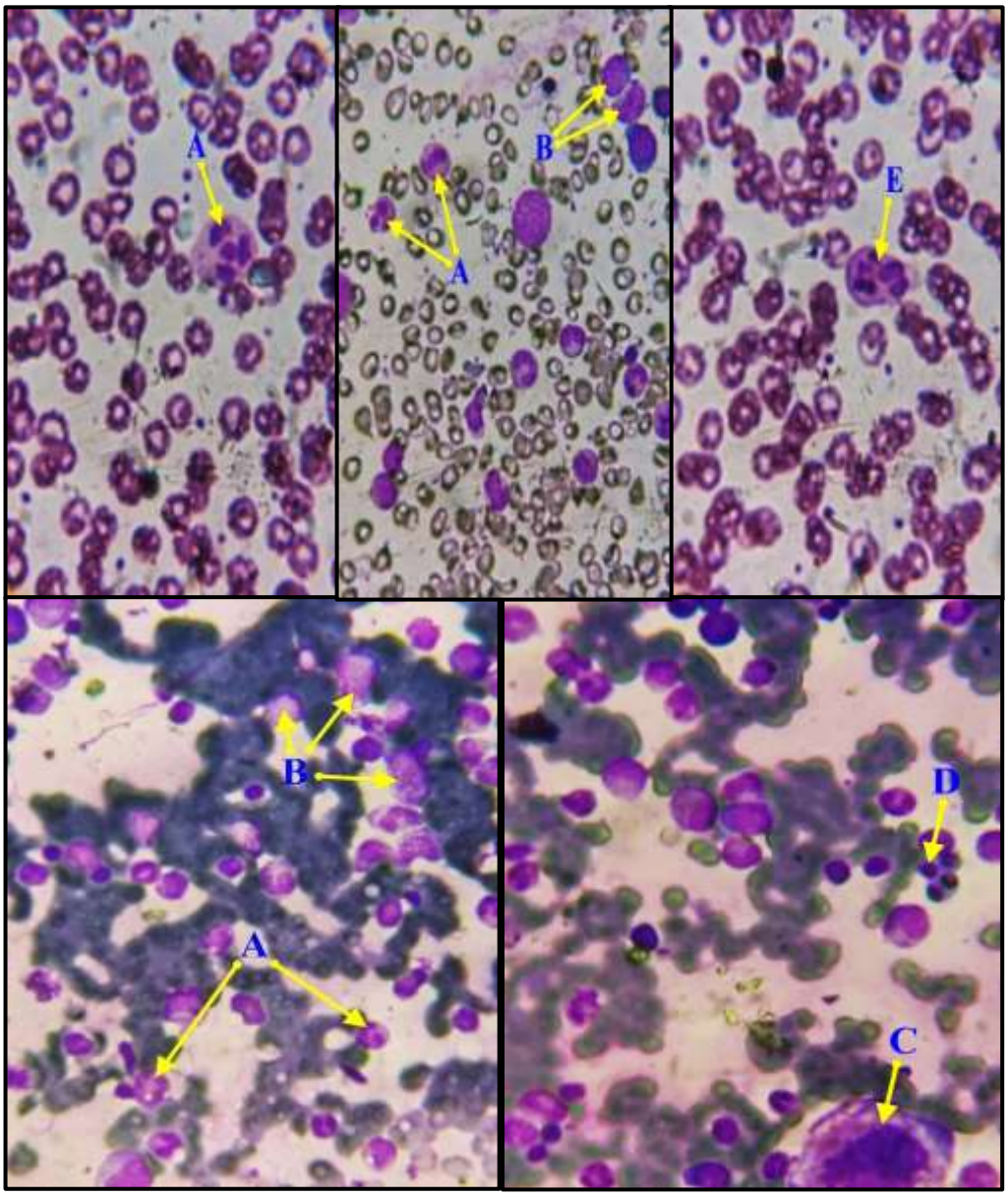

Figure (4-4): Bone marrow smear, A: dysplastic neutrophil, B: mylocyte, C: dysplastic megakaryocyte, D: microerthroblast and E: hypersegmented neutrophil, (Magnification power 400).

4.3.Biochemical Study

4.3.1. Hormone parameters

The results of the present study showed significant differences in some hormones level between the myelodysplastic syndrome patients and control group as shown in table (4-3). Adiponectin and Resistin were a significantly increased ($p \leq 0.05$), in

patients of myelodysplastic syndrome compared with control group. While there was no significant differences in leptin hormone between patient and control groups.

Table (4-3): Mean ± SD of hormones between control and MDS patient groups.

Groups / Hormones (ng/ml)	Control group (No.25) Mean ±SD	MDS group (No.50) Mean ±SD	P-value
Adiponectin	7.1300± 1.29408	31.3014±9.21683	*0.000
Leptin	4.6724 ± 1.73042	3.9872 ± 2.27170	0.152
Resistin	138.1240±40.18569	409.5752± 91.03437	*0.000

*** is significant at P ≤0.05.**

SD Standard deviation.

Adipocytes secrete active biological molecules, mainly including leptin, resistin and adiponectin (Ouchi *et al.*, 2011). The adipose tissue is no longer considered an inert tissue predominantly devoted to energy storage but is emerging as an active endocrine organ secreting several hormones which regulate physiological and pathological processes, such as appetite, insulin sensitivity and resistance, endocrine function, inflammation, hematopoiesis, immunity and angiogenesis (Matarese *et al.*,2005).

The present study agreed with the study of Dalamaga *et al.*,(2007), found higher serum adiponectin level with lower risk of MDS before and after controlling for age, gender, BMI, and serum levels of leptin. These findings are in accordance with a hypothesis generated by previous reports showed that adiponectin is an adipocyte-secreting hormone that induces apoptosis and inhibits proliferation of myeloid cell lineage (Yokota *et al.*, 2000), Also, agreed with similar study responding to adiponectin levels in patients with myeloblastic leukemia (Petridou *et al.*,2006).

Adiponectin which is specifically expressed in human white adipose tissue adipocytes and is inversely released to the degree of adiposity, may suppress proliferation of myelomonocytic progenitors through the induction of apoptosis which is mediated mainly by downregulation of Bcl-2 expression and activation of the caspase group apoptotic enzymes. Exogenously, administration adiponectin suppress the growth of myelomoncytic leukemia cells. Also, adiponectin induce growth inhibition of macrophage precursors and suppression of phagocytosis induced by specific inhibition of TNF-α transcription (Tilija Pun *et al.*,2015). Subsequently, reduced adiponectin levels observed in MDS could be responsible for the TNF-α overproduction in the bone marrow.

Previous studies have demonstrated IL-6 and TNF-α overexpression in progenitor cells in the bone marrow of patients with MDS. Elevated production of TNF-α appears to contributes to an excessive intramedullary progenitor cell apoptosis and to enhanced bone marrow angiogenesis which are considered as main cofactors of inefficient hematopoiesis in MDS and progression to AML (Flores-Figueroa *et al.*,2002 ; Stiffer *et al.*,2005). The pathogensis of early MDS is characterized by intrinsic apoptosis explaining the clinical findings of peripheral cytopenia in spite of hypercellular bone marrow (Bennett *et al.*,2005). In addition, the anti-inflammatory activities of adiponectin extend to inhibition of IL-6 production in part through nuclear factor-$_k$B inhibition (Chan *et al.*,2005 ; Wulster-Radeliffe *et al.*,2004).

The present study found that mean leptin levels were essentially similar in MDS patients and controls in agreement the study of Dalamaga *et al.*, (2007). A another study examining much fewer patients (60) patients and controls with MDS and AML) (Konopleva *et al.*,1999) , with possibly a relatively lower risk observed only in the third quartile in patients with leptin levels in the normal range.

Importantly, low leptin concentrations were observed in low-risk MDS patients with normal or good prognostic karyotype after adjusting for age, gender and BMI. The study have indicated that leptin stimulates leukemic cell proliferation *in vitro*.

Leptin receptor (OB-R, mainly the short isoform), which shows homologies to cytokine and hematopoietic growth factor receptors, is expressed in normal CD34+ progenitor cells in the majority of AML as well as in secondary AML and MDS (Dalamaga *et al.*,2007) . Thus, leptin, produced by bone marrow adipocytes and stromal cells, and its receptor OB-R may function as a growth factor/receptor-ligand system in hematopoietic stem and/or progenitor cells in a paracrine fashion. Furthermore, leptin upregulates phagocytic function and cytokine secretion, such as TNF- (early), IL-6 (late) and IL-12 in monocytes/macrophages (Matarese *et al.*,2005)

Studies have demonstrated that in humans, *in vivo* leptin plays mainly a permissive role in regulating endocrine and immune functions. Thus, in humans, all these activities of leptin are restricted mainly to leptin-sensitive subjects, i.e. those who have leptin levels lower than 10–15 ng/ml, whereas in leptin-sufficient normal subjects, leptin has no effect on their normal neuroendocrine and immune function. Moreover, hyperleptinemic obese subjects are resistant to the biological functions of leptin and in this respect, bone marrow cells of subjects with very high circulating leptin levels due to leptin resistance could respond to circulating leptin in a manner similar to a leptin deficient state (Brennan and Mantzoros, 2006; Papathanassoglou *et al.*,2006).

Fantuzzi and Faggioni, (2000), found reduction in serum leptin level in acute leukemia patients. They suggests that the cause rather seem to be multifactorial and related to the severe illness, altered energy balance, and disease complications. Bruserud *et al*., (2002) found that although increased serum leptin and OB-R expression levels were found in many cancer types with related obesity, there are also reports stating the decrease in serum leptin levels in lymphoid and myeloid malignancies.

The present study disagreed with the study of Aref *et al*., (2013), serum leptin levels were significantly lower in AML as compared to normal controls (P = 0.00).

Wallace *et al.*,(1998), explained the reduction in leptin concentrations in AML patients, although they had normal fat tissue as a defense mechanism from the body in order to keep appetite up to gain weight and to avoid weight loss. This may be due to disregulation in the feedback mechanisms developing in patients with hematologic malignancy. Tabe *et al*.,(2004) suggested that leptin has been shown to stimulate the proliferation of AML cells and to also have an anti-apoptotic effect. It increases the number of progenitor cells and spontaneous AML blast proliferation as well as AML blast release of IL-1beta, IL-6, tumor necrosis factor -alpha, and granulocyte-macrophage colony stimulating factor.

The result of resistin showed significantly increased in MDS patients when compared with control group. Resistin was initially discovered as hormone related to insulin resistance, but study in mice and humans have revealed conflicting data indicating that the physiological role of resistin may mainly be related to inflammation (Lee *et al.*,2003).

The present study disagree with study of Dalamaga *et al.*,(2008), that showed MDS patients have lower level of resistin, probably due to a compensatory response to the upregulation of other inflammatory factors a etiologically linked to myelodysplasia. Moreover, studies in humans have failed to detect higher serum resistin levels in obese or insulin resistant subjects (Lee *et al.*,2003). Resistin is seen mainly as an inflammatory factor which is associated with TNF-a and IL-6, and may upregulate several adhesion molecules and cytokines (Stappan *et al.*,2001).

The present study agreed with the study of Pamuk *et al* .(2006), showed higher level of resistin in patients with lymphoma and this finding suggest that resistin may be associated with disease pathogenesis, immune change and the inflammatory response during the course of disease. Also, resistin increased in multiple myloma patient who used corticosteroids more intensively and more frequently than other point to a role of steroid. Steroid intake affects resistin level (Banerjee and Lazar, 2003).

4.3.2. Level of ferritin

There was a significant increasing ($p \leq 0.05$), of serum ferritin in myelodysplastic syndrome patients and control group (Figure 4-5).

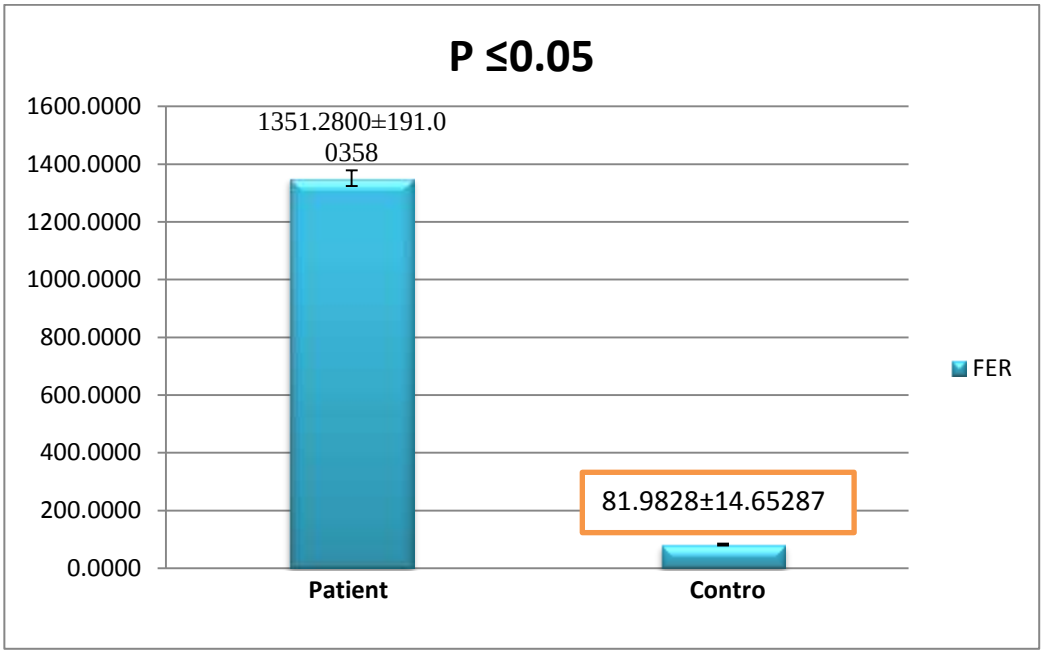

Table (4-5): Mean ±SD comparison of serum ferritin (ng/ml) between control and MDS patient groups.

The majority of patients with MDS (approximately 80%) are anemic, and a large percentage of them will require red blood cell (RBC) transfusional support during their disease course (Shenoy *et al.*,2014 ; Temraz *et al.*,2014). As a result of ineffective erythropoiesis and continued transfusion dependence, MDS patients are prone to excessive iron accumulation and, ultimately, iron overload (Shah *et al.*, 2012; Shenoy *et al.*, 2014). Iron overload (IO) in MDS patients may cause organ deposition of excess iron, resulting in endocrinopathies, liver, and cardiac dysfunction. Accordingly, retrospective and observational studies have suggested patients with MDS and IO have a markedly increased risk of death compared with MDS patients without IO (Shah *et al.*, 2012 ; Adams and Bird, 2013; Mitchell *et al.*, 2013; Steensma and Gattermann, 2013; Wood, 2015). The present study agreed with many studies that indicated iron overload in MDS patients may also be a risk factor

leading to poor outcomes following allogeneic stem cell transplant (Wermke *et al.*, 2012 ; Jacobi and Herich, 2016; Pilleggi *et al.*,2017). Also, there have been conflicting data regarding the role of oxidative stress related to iron over-load in the pathogenesis and progression of MDS,(Goldberg *et al.*,2010), as well as its impact on survival. Recently, a murine model of MDS suggested an increased mutation frequency related to the oxidative stress and mitochondrial iron trapping (Chung *et al.*,2014). Even minimal elevations in serum ferritin are associated with decreased erythroid burst-forming units (Hartmann *et al.*,2013). In addition, apoptotic protease activating factor-1 (APAF1), a key factor in erythroid apoptosis, was found to be elevated in association with iron overload among 35 patients with MDS (Gu *et al.*,2014).

The findings suggest that in MDS patients, serum hepcidin levels are inappropriately low, and the degree of hepcidin response is blunted relative to normal. Specifically, in previous study, the authors hypothesize that tissue hypoxia, resulting from ineffective erythropoiesis, triggers increased erythropoietin production, which then results in low hepcidin; the inappropriately low hepcidin then causes an increase in iron absorption and release from storage, causing saturation of transferrin and resulting in IO (Cui *et al*., 2014). Disruptions in hepcidin levels can have a dramatic impact on iron homeostasis. Ineffective erythropoiesis, a hallmark of MDS, causes a massive expansion of bone marrow erythroblasts due to decreased production of mature RBCs (Sebastiani *et al*., 2016).

4.3.3. Level of reactive oxygen species and antioxidants

The present study showed significant increasing ($p \leq 0.05$) of reactive oxygen species in myelodysplastic syndrome patients and control groups. Also, the antioxidants results showed significant differences in level between the myelodysplastic syndrome patients and the control group, as shown in table (4-6) superoxide dismutase and glutathione peroxidase were significantly increased ($p \leq 0.05$) in myelodysplastic syndrome patients compared with the control group. At

the same time, there was a significant decrease (P≤0.05) in glutathione reductase between patient and control groups.

Table (4-4): Mean ±SD comparison of reactive oxygen species and antioxidants between control and MDS patient groups.

Groups / Antioxidants	Control group (No.25) Mean ±SD	MDS group (No.50) Mean ±SD	P-value
Reactive oxygen species µmol/l	20.8208±2.21840	35.1854±8.42236	*0.000
Superoxide dismutase U/l	3.3208± 1.12327	13.3832 ± 2.01540	*0.000
Glutathione peroxidase U/l	16.3520 ± 2.52816	75.5416± 15.00791	*0.000
Glutathione reductase µg/mg	77.3360 ± 6.77582	48.0846± 6.87792	*0.000

***is significant at P ≤0.05.**

SD Standard deviation.

The present study revealed a significant increase in serum total reactive oxygen species (ROS) in MDS patients compared to the control group. Myelodysplastic syndromes (MDS) are a heterogeneous group of hematopoietic stem cell disorders characterized by the presence of immature myeloid precursors (blasts) and dysplastic hematopoiesis in the bone marrow (BM). High levels of reactive oxygen species (ROS) and consequent oxidative damage in hematopoietic cells report in patients with this disease (Peddie *et al.*,1997, Chung *et al.*,2014, Ivars *et al.*,,2017 and Jiménes-Solas *et al.*,2019), but the consequences are less clear. Regulation of intracellular ROS levels is critical for maintaining the balance between self-renewal, proliferation, and differentiation of progenitor cells, and a loss of this control can lead to diseases characterized by bone marrow failure (Evans, 2005 ; Dalle-Donne, 2009). The potential effects of ROS on hematopoietic cells are particularly relevant because they are acutely vulnerable to oxidative damage associated with the accumulation of free radicals (De Souza *et al.*,2015).

The results of the current study agreed with the results of Ghoti *et al.*(2007); Goncalves *et al.,* (2015); Gonçalves *et al.*, (2016), which showed that BM cells from patients with MDS have increased levels of intracellular peroxide and decreased levels of the antioxidant glutathione (GSH), as compared with normal cells. Importantly, patients with MDS and high ROS or low GSH levels, and a high superoxide/peroxide ratio, have lower overall survival (Goncalves *et al.*,2015). Increased intracellular ROS production effects are well recognized and include direct damage to biomolecules and/or dysregulation of ROS-dependent signaling pathways (Evans *et al.*,2005; Cheresh *et al.,* 2013). In the context of blood cells, an interplay report between oxidative damage of DNA in CD34+ cells and subsequent increased oxidation levels in precursor cells such as blasts or erythroid precursors (Peddie *et al.*,1997).

Also, oxidative stress correlates with DNA hypermethylation in patients with MDS (Gonçalves *et al.,* 2016) and other pathological conditions (Niu *et al.*, .2015). Proteins are essential targets of ROS (Bhatti *et al.*,2017), and oxidation can lead to aggregation, polymerization, unfolding, or conformational changes that cause structural or functional loss. Although several oxidative modifications to proteins are possible, most involve the formation of carbonyl groups, which introduces into proteins (Afiuni-Zadeh *et al.*,2016). Protein carbonylation can occur by several pathways, but the two main contributors are: (i) direct metal-catalyzed oxidation of specific amino acid residues (lysine, arginine, proline, and threonine), and (ii) secondary reactions of nucleophilic amino acid side-chains with ROS-induced lipid peroxidation products such as 4-hydroxynonenal (HNE) (Linares *et al.*,2011). These modifications likely have essential roles in cell signaling (Barrera *et al.*,2018).

Another possible cause of high oxidative stress in MDS patients might be high cytokinesis, mainly TNF-α, known as a producer of oxygen radicals (Kilpatrick *et al.*,2010).

4.3.4. the Correlation among Adiponectin, Leptin, and Resistin with some of the parameters of myelodysplastic syndrome patients.

Correlation analysis among investigated serum parameters revealed a significant negative correlation ($p \leq 0.05$) among adiponectin level and BMI(r=-.534), Hgb(r=-.723), platelets (-.647), while a significant positive correlation with ferritin (r=.800), and reactive oxygen species (r=.757). Also, the results indicated no significant correlation between leptin and BMI, Hgb, platelets, and no correlation with ferritin and reactive oxygen species. Moreover, the resistin showed a significant negative correlation ($p \leq 0.05$) with BMI, Hgb, and platelets, (r=-.501, r=-.667and r=-.697) respectively, while positive correlation with ferritin (r=.772) and reactive oxygen species (r=.588). Table (4-6).

Table (4-5): Correlation among adiponectin, leptin, and resistin hormones level with some parameters studied of myelodysplastic syndrome.

Parameters / Hormones (ng/ml)		BMI (kg/m^2)	Hgb (mg/dl)	Platelets (10^3/µl)	Ferritin (ng/ml)	Reactive oxygen species (µmol/l)
Adiponectin	(r)	-0.534**	-0.723 **	-0.647 **	0.800**	0.757 **
Leptin		0.197237	0.040623	0.11254	--	--
Resistin		-0.501**	-0.667 **	-0.697 **	0.772**	0.588 **

-BMI=Body mass index, Hgb=Hemoglobin.

r: Correlation coefficient

**** is significant at P ≤0.05.**

The study of Pamuk *et al*. (2006), in the control group, leptin level was negatively correlated with hemoglobin level (r= -0.44, p=0.047). In patients with hematological malignancies, leptin level correlated with BMI (r=0.32, p=0.02);

resistin, on other hand correlated with platelets count (r=0.26, p=0.044). In lymphoma patients, leptin level has positive correlated with hemoglobin (r=0.64, p=0.055). The resistin level negatively correlated with BMI for patients with the acute leukemic group (r=- 0.59, p= 0.03). The study of Tungtrongchitr *et al.*(2000) negative correlation between hemoglobin and serum leptin was found in male and female overweight and obese subjects . Adiponectin and leptin had differing relationships with fat mass. The former was negatively correlated with fat mass, while the latter showed a positive correlation (Peng *et al.*,2008). leptin level had a significant correlation with BMI only in hematologic malignancy patients, there was no such correlation in the control group. Leptin and BMI relationship was preserved in hematologic malignancy patients (Pamuk *et al.*,2006).

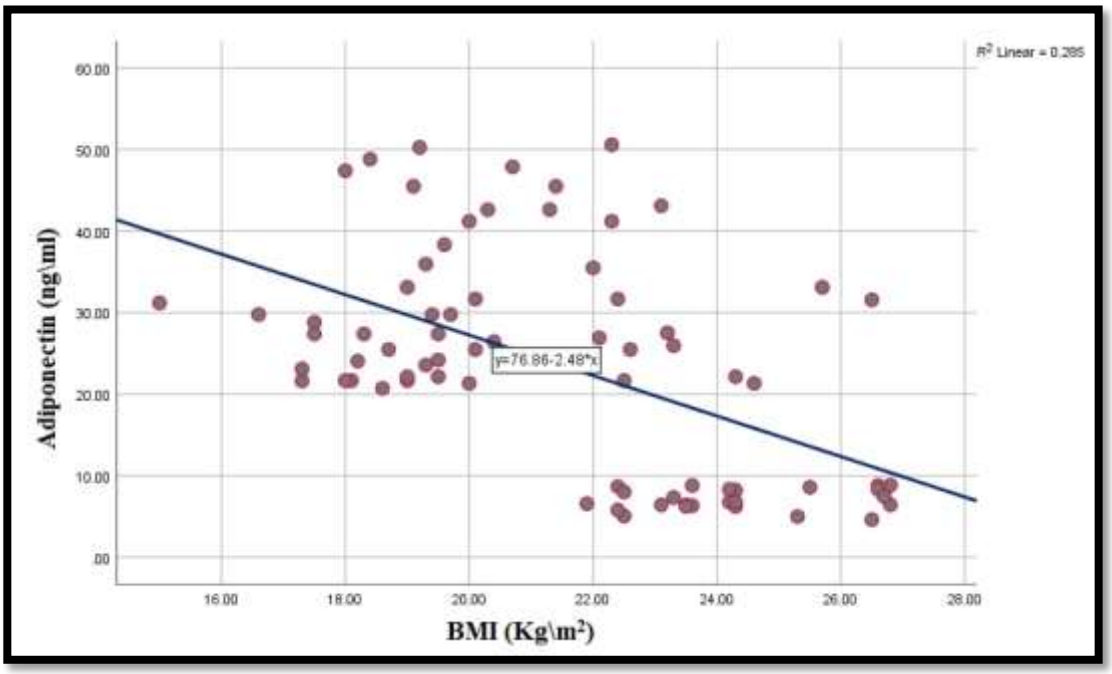

Figure (4-6): The relationship between adiponectin (ng/ml) and BMI (kg/m^2) in myelodysplastic syndrome patients.

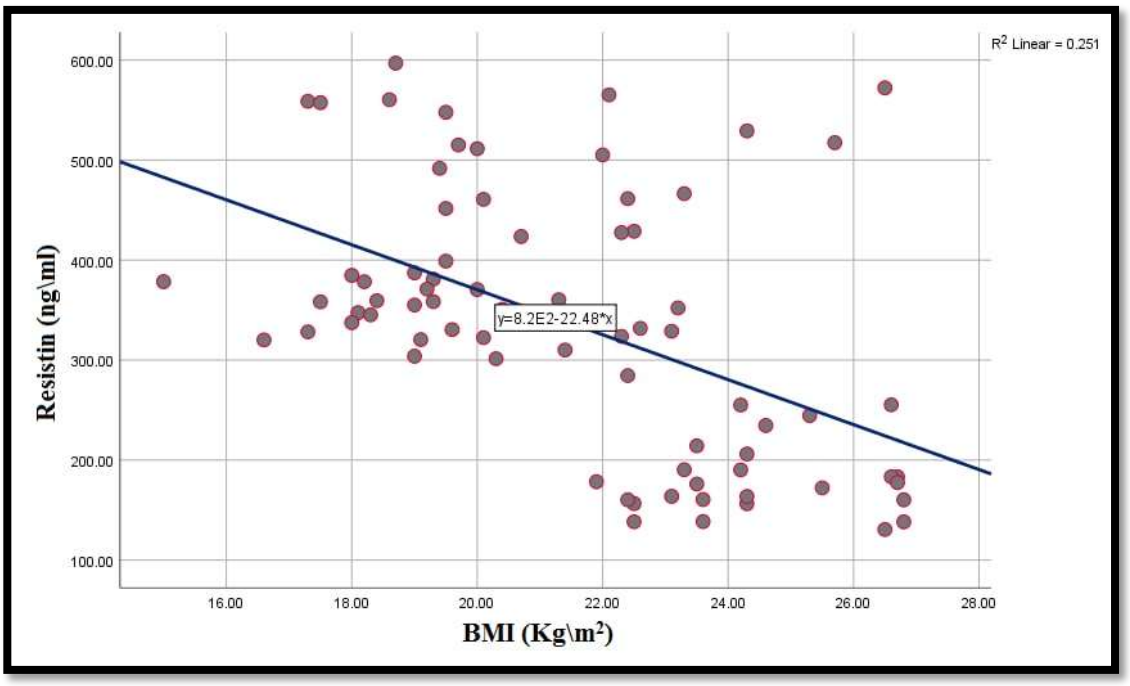

Figure (4-7): The relationship between resistin (ng/ml) and BMI (kg/m^2) in myelodysplastic syndrome patients.

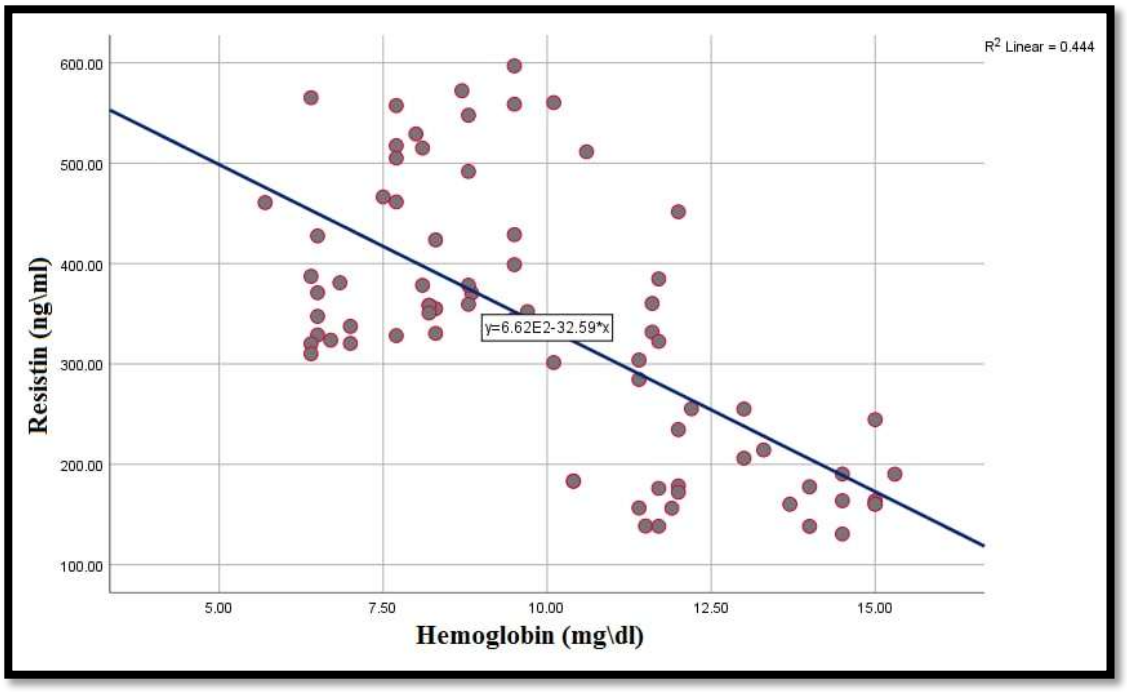

Figure (4-8): The Relationship between resistin (ng/ml) and hemoglobin (mg/dl) in myelodysplastic syndrome patients.

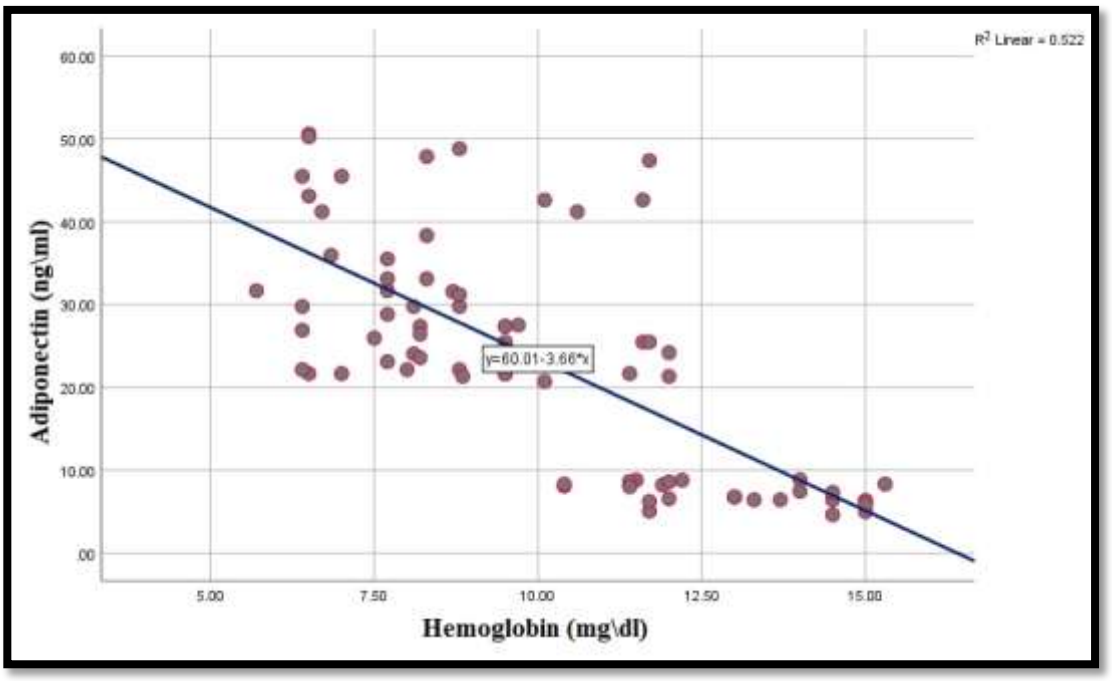

Figure (4-9): The Relationship between adiponectin (ng/ml) and hemoglobin (mg/dl) in myelodysplastic syndrome patients.

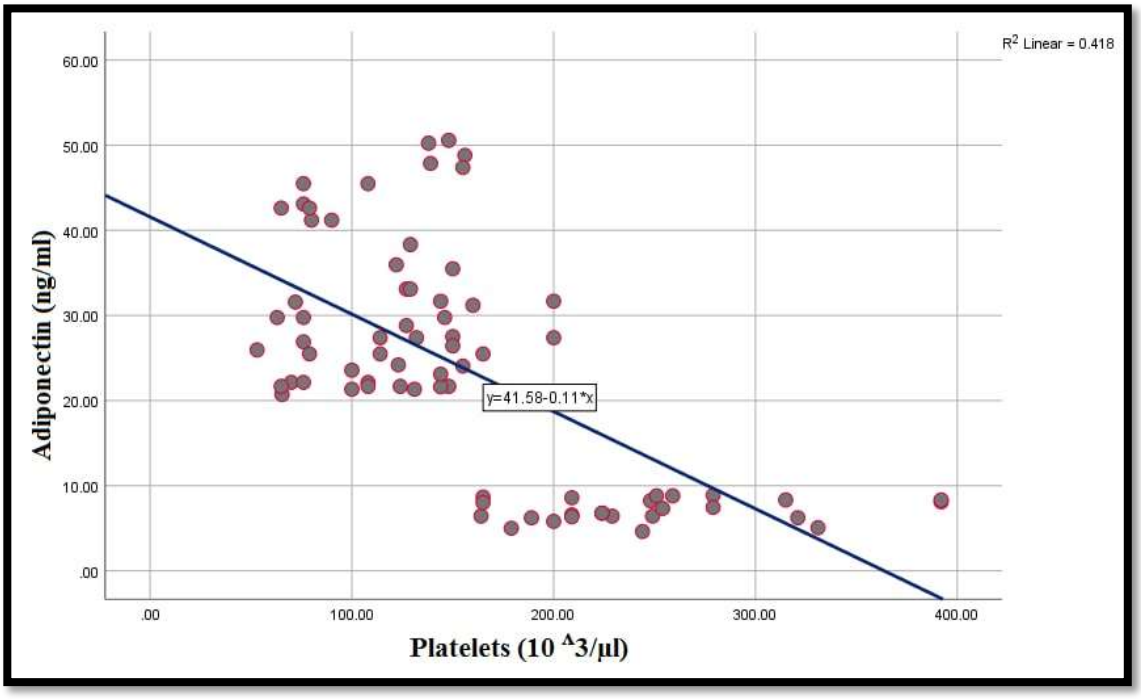

Figure (4-10): The Relationship between adiponectin (ng/ml) and platelets (10^3/µl) in myelodysplastic syndrome.

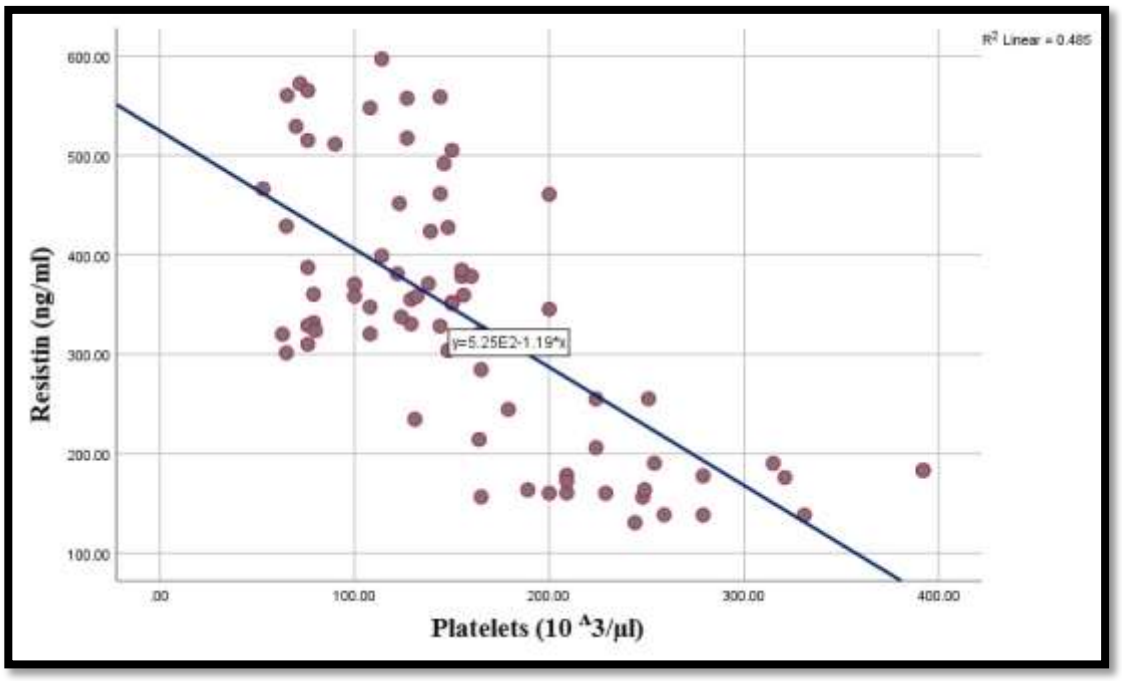

Figure (4-11): The Relationship between resistin (ng/ml) and platelets (10^3/µl) in myelodysplastic syndrome.

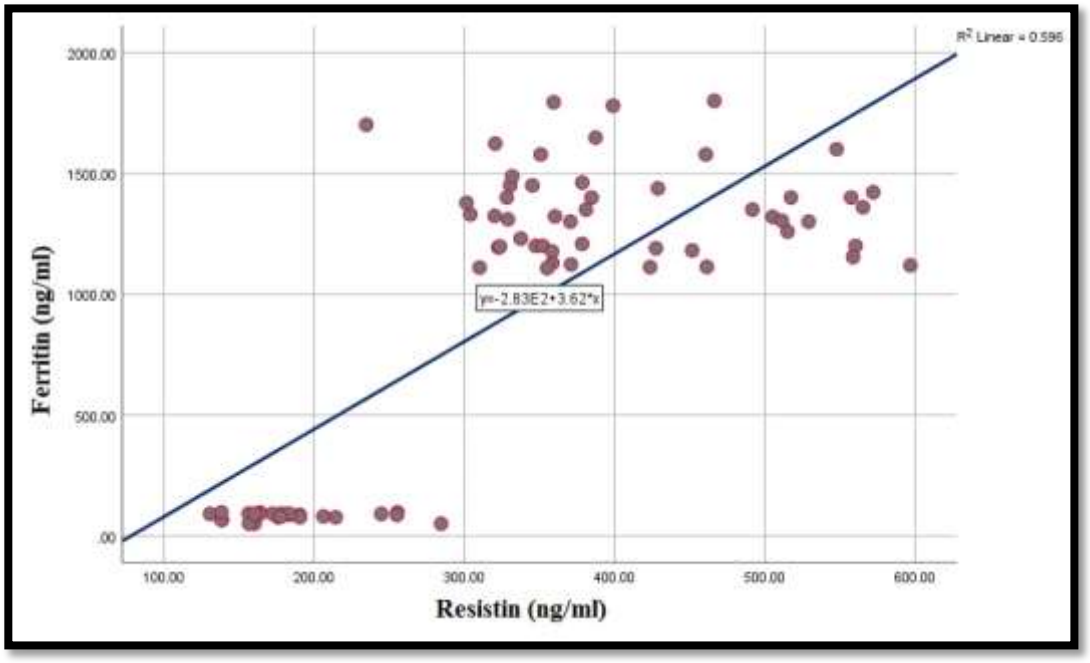

Figure (4-12): The Relationship between ferritin (ng/ml) and resistin (ng/ml) in myelodysplastic syndrome.

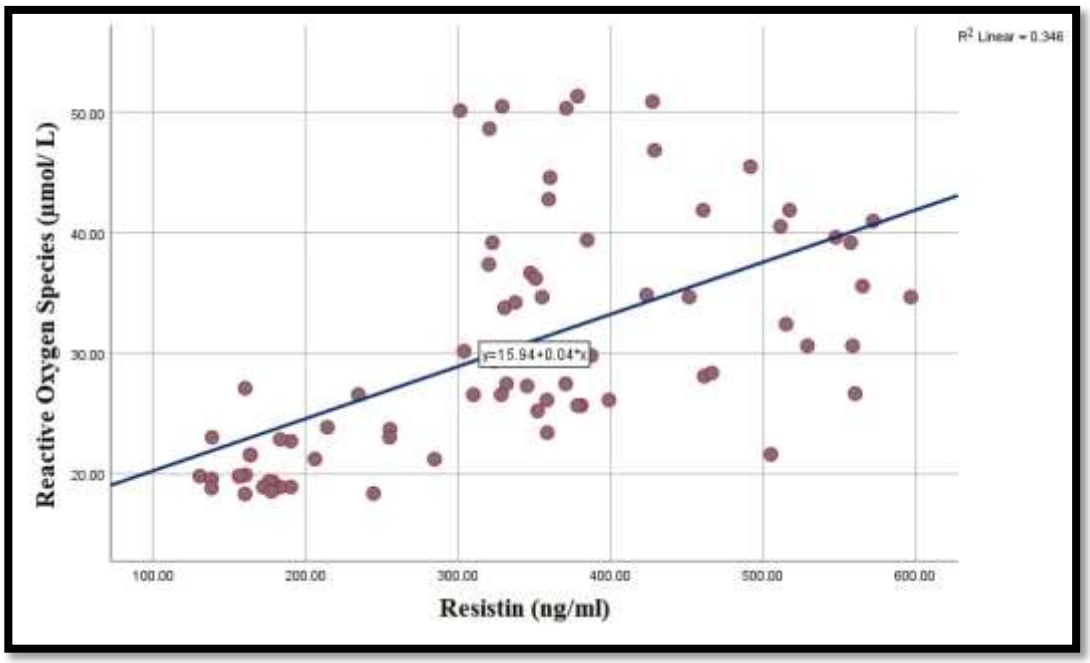

Figure (4-13): The Relationship between reactive oxygen species (µmol/L) and resistin (ng/ml) in myelodysplastic syndrome.

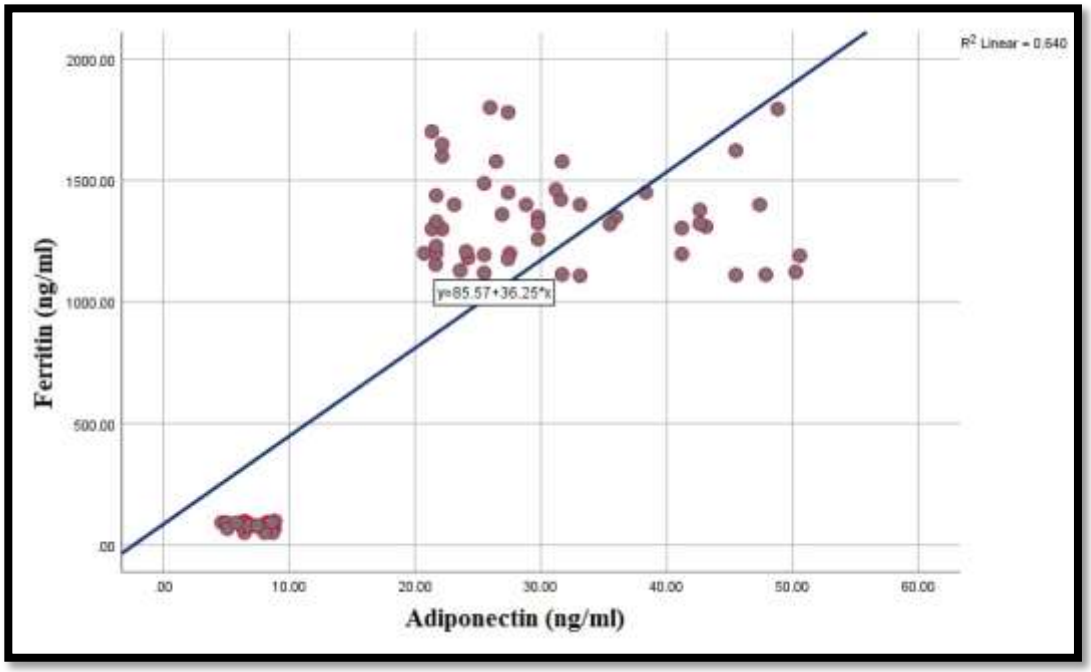

Figure (4-14): The Relationship between ferritin (ng/ml) and adiponectin (ng/ml) in myelodysplastic syndrome patients.

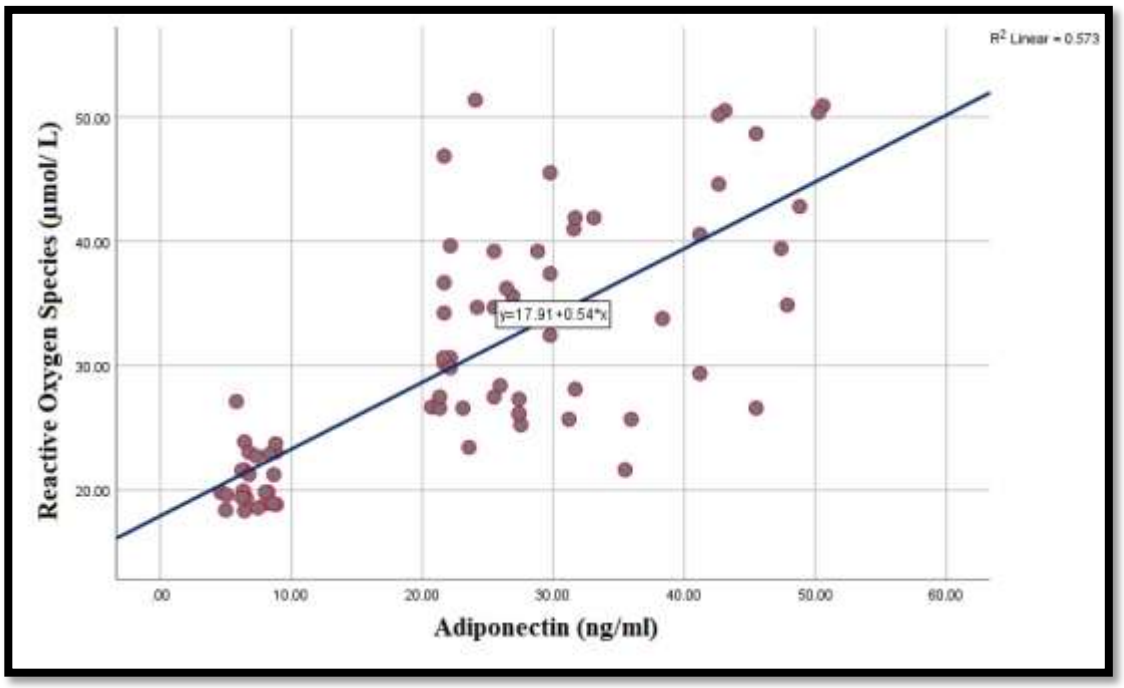

Figure (4-15): The Relationship between reactive oxygen species (µmol/L) and adiponectin (ng/ml) in myelodysplastic syndrome patients.

4.3.6. Correlation between hormones (adiponectin, leptin and resistin)

In the present study the hormones leptin correlated with adiponectin (r=-0.13741), and significantly with resistin (r= -.247). Also, adiponectin with resistin correlated significantly (r= .624). (Table 4-8, Figure 4-17, 4-18, 4-19).

Table (4-6): Correlation of hormones (adiponectin, leptin, and resistin)among MDS patients and control groups.

Hormones (ng/ml)	Mean	Std. Deviation	N	Pearson correlation	P. value
Leptin	4.2156	2.11992	75	-0.13741	0.240
Adiponectin	23.2443	13.72527			
Leptin	4.2156	2.11992	75	-0.247**	0.033
Resistin	334.0915	132.51731			
Adiponectin	23.2443	13.72527	75	0.624**	0.000
Resistin	334.0915	132.51731			

r: Correlation coefficient

**** is significant at P ≤0.05.**

Adipose tissue is a metabolically active organ that secretes multiple adipokines, including classical leptin, adiponectin, and resistin, which exert essential physiological functions. The plasmatic levels of these adipokines, mainly derived from fat depots, are finely regulated under different metabolic conditions such as obesity, fasting, diabetes, etc. Interestingly, these adipokines and their receptors have been found to be also widely expressed in other key endocrine tissues and organs (e.g. hypothalamus, muscle, pancreas or liver), suggesting that circulating and/or locally-produced, adipokines might comprise a relevant regulatory circuit to modulate numerous endocrine functions in multiple cell types (i.e. lipid metabolism, glucose homeostasis, body composition, etc.)(de Oliveira Leal and Mafra, 2013).
Studies on animals suggest that resistin and resistin-like molecules may induce inflammation, angiogenesis, and smooth muscle cell proliferation (Fang *et al.*,2012).

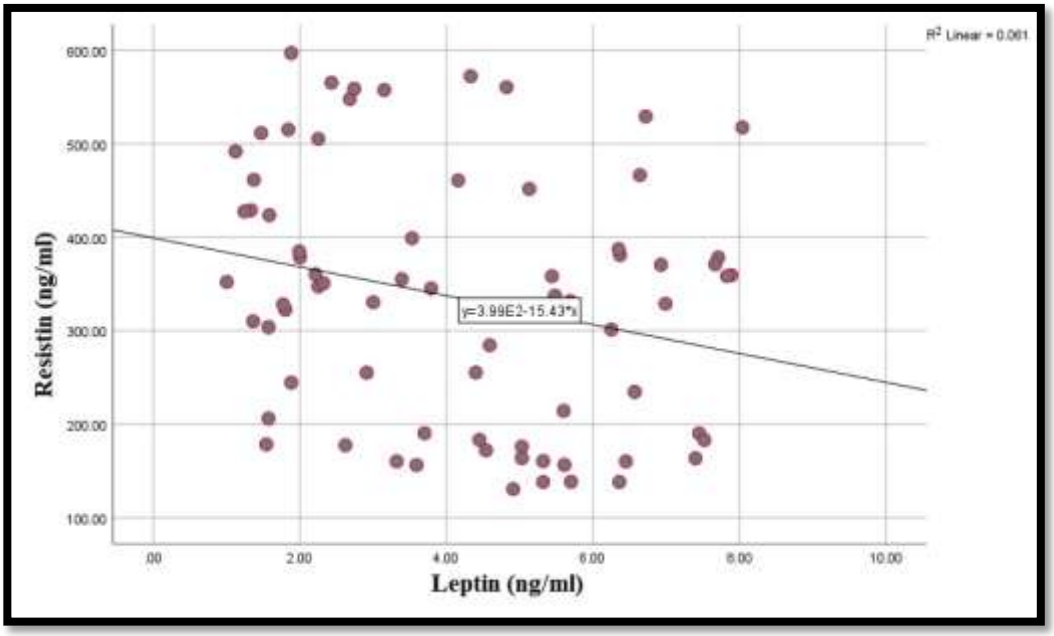

Figure (4-16): The relationship between leptin (ng/ml) and adiponectin (ng/ml) in patients with myelodysplastic syndrome/

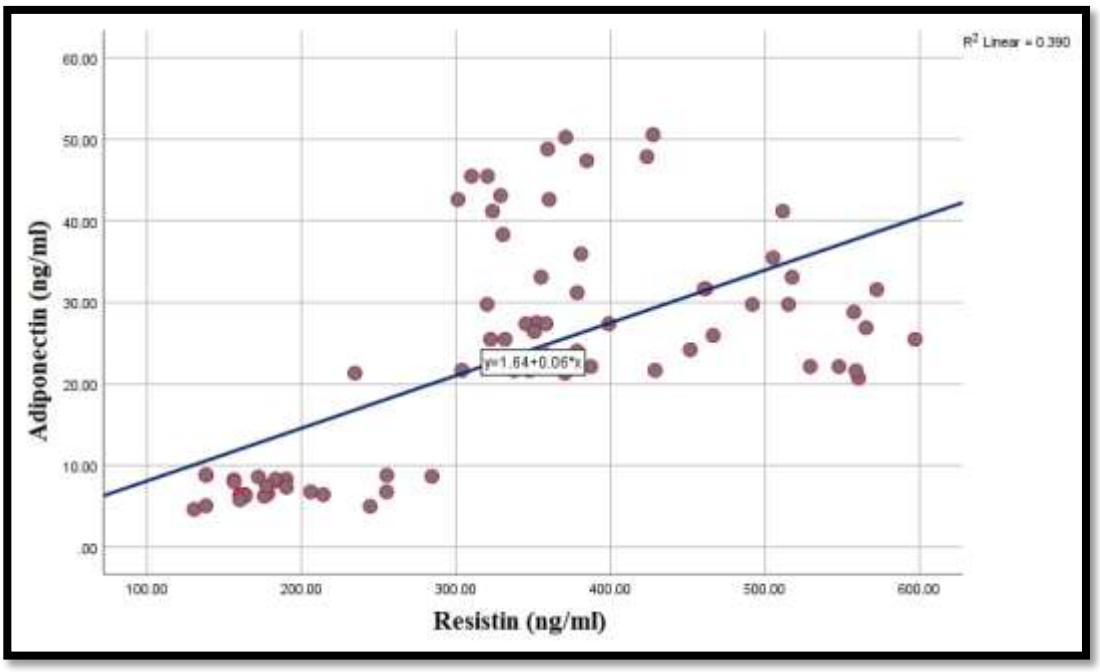

Figure (4-17): The relationship between adiponectin (ng/ml) and resistin (ng/ml) in patients with myelodysplastic syndrome.

4.4.Molecular study

4.4.1. Genomic DNA extraction

The blood samples that have been collected previously, its extracted by use genomic DNA extraction kit (favor gene©). As shown in figure (4-20).

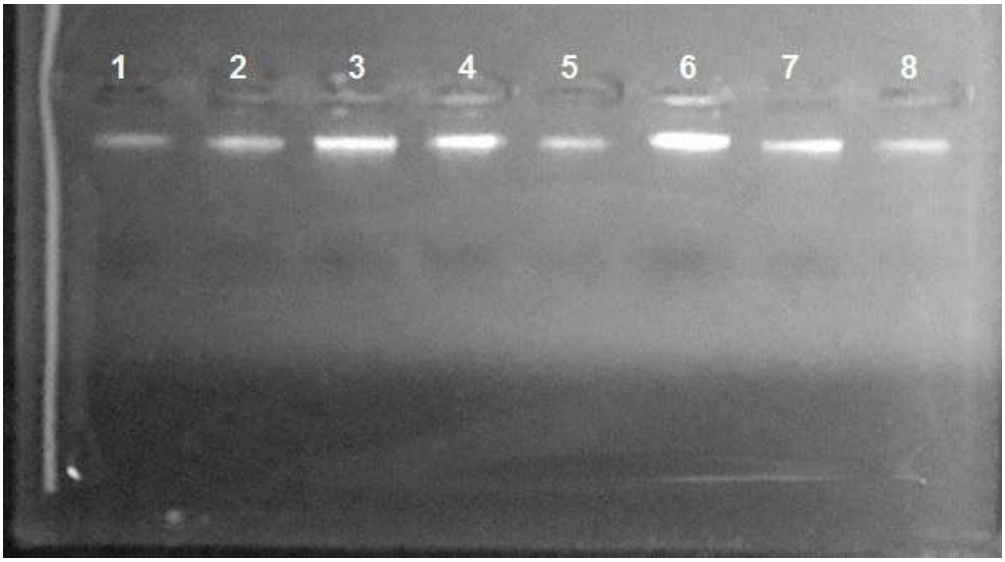

Figure (4-18): DNA extraction of blood samples. The electrophoresis carried on agarose (1.5%), voltage (75)V, current (20) MA for 60 minutes. Samples (1-8) patients.

4.4.2. Detection of polymorphism of adiponectin in Myelodysplastic syndromes

This study detected polymorphism of adiponectin gene by Tetra amplification refractory mutation system polymerase chain reaction (T-ARMS-PCR) (table 4-21).

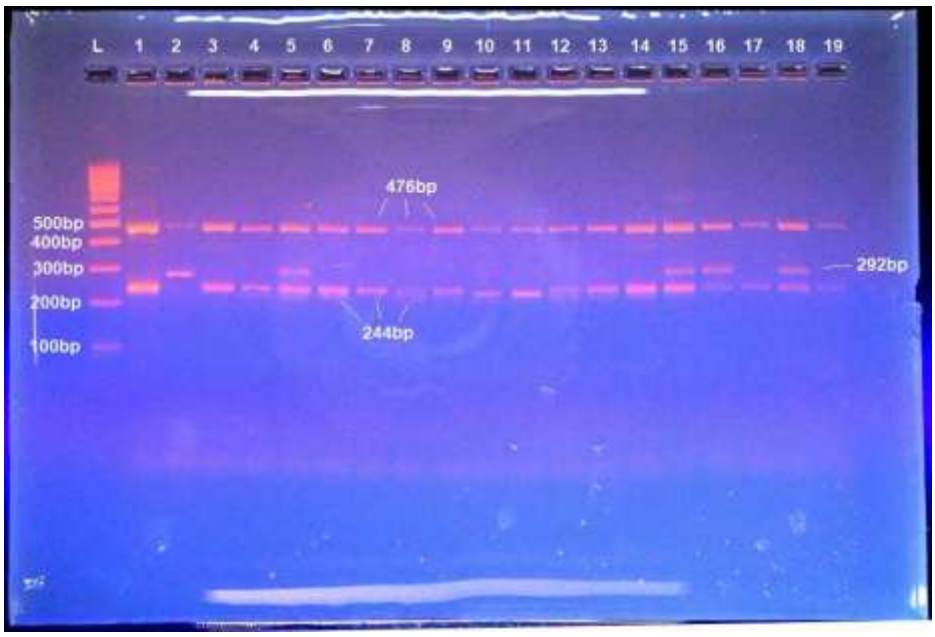

Figure (4-19): Electrophoresis Pattern of PCR Products of T-ARMS-PCR for Detection of Adiponectin rs1501299 G/T Polymorphism. The product sizes were 244 bp for the G allele, 292 bp for the T allele, and 476 bp for the control band. L Lane 100 bp DNA ladder; lane 1, GG; , lane 2 TT genotype; lanes 5, 15, 16, and 18 TG genotype; other lanes GG genotype. (2% Agarose, 75V, 20mA for 1 hour).

4.4.2.1. Genotyping and allele frequency of adiponectin by T-ARMS-PCR in patient

The results of the current study in table (4-7) showed a high percentage of the adiponectin rs1501299 mutated genotype GG and alleles G in MDS patients (54% and 73% respectively) compared with the healthy individuals in control group (36% and 62% respectively) , and this was associated with significant differences ($P \leq 0.05$).

Table (4-7): Genotyping and Allele distribution in MDS patient and control groups.

Model	Genotyping	Control	Patient	OR (95 % CI)	P-value
Codominant	G/G	9 (36 %)	27 (54%)	1.00	0.0399
	G/T	13 (52%)	19 (38%)	0.49 (0.17-1.37)	
	T/T	3 (12%)	4 (6%)	0.44 (0.08-2.38)	
Dominant	G/G	9 (36%)	27 (54%)	1.00	0.0411
	G/T-T/T	16 (64%)	23 (46%)	0.48 (0.18-1.29)	
Recessive	G/G-G/T	22 (88%)	46 (92%)	1.00	0.0755
	T/T	3 (12%)	4 (8%)	0.64 (0.13-3.10)	
Predominant	G/G-T/T	12 (48%)	11 (62%)	1.00	0.0386
	G/T	13 (52%)	19 (38%)	0.57 (0.21-1.49)	
Allele frequency					

Allele	Control		Patient		OR (95 % CI)	P-value
	Count	%	Count	%		
G	31	0.62	73	0.73	1.657 (0.805-3.411	0.0446
T	19	0.38	27	0.27	0.603 (0.293-1.242)	

P≤0.05

95% CI: Confidence interval at 95% level.

The adiponectin synthesis has a strong genetic component, with heritability estimated above 70%. Some single nucleotide polymorphisms (SNPs) have been associated with serum adiponectin levels (Croteau-Chonka *et al.*, 2012). However, there is a controversy between the association of these genetic variants and the adiponectin levels. These discrepancies may relate to the interaction between these genetic variants and nutrients. In particular, heterogeneity of response to fish oil

fatty acids within different patients' groups has been attributed to genetic variation (Kasim-Karakas *et al.,* 2006). One of the most important SNPs at the adiponectin gene (ADIPOQ) locus is a G to T substitution in intron 2 (±276G>T, rs1501299). The G allele has been associated either with increased or decreased concentrations of plasma adiponectin (Kyriakou *et al.,*2008). On the other hand, this genetic variant has been negatively and positively associated with obesity in some populations (Salmenniemi*et al.,*2005).

Adiponectin has been postulated to play an important role in the modulation of glucose and lipid metabolism in insulin-sensitive tissues in both humans and animals. Decreased circulating adiponectin levels have been demonstrated in genetic and diet-induced murine models of obesity (Yamauchi *et al.,*2001), as well as in diet-induced forms of human obesity (Arita *et al.,*1999). Abnormal adiponectin levels have also been strongly implicated in the development of insulin resistance in mouse models of both obesity and lipoatrophy (Yamauchi *et al.,*2001) that may be lead to development MDS as recorded in previous independent studies that found a significant association between increased obesity and the risk of MDS (Murphy *et al.,*2013). In additional, adiponectin receptors are also expressed in the bone marrow environment and result in proliferative effect on the hematopoietic stem cells (DiMascio *et al.,* 2007) and its level correlates inversely with MDS risk, as patients with lower-risk MDS found to have higher adiponectin levels (Dalamaga *et al.,*2007) .

In the current study, increase of adiponectin mutant alleles of SNP rs1501299 appeared in patients , perhaps this slight increase in the mutant alleles plays a role in the pathology of the MDS. It must be mentioned that this gene has been studied in many diseases. Now the current study is the first to determine the role of this gene in MDS . It is worth noting that previous studies dealt with its role in diseases that are associated with the occurrence of the MDS, as stated in the study of Hamoudeh and his colleagues who showed that adiponectin regulates fatty acid oxidation, glucose uptake, and glycogenesis, which is related to the pathogenesis of

diabetes (Hamoudeh *et al.*,2016; Dong *et al.*,2020). Therefore, adiponectin is a candidate gene for the study of metabolic syndrome and T2DM. Rs1501299 is crucial loci in the adiponectin gene. Rs1501299 is located in the second intron of the adiponectin gene, and its polymorphism might affect the function of the neighboring exon. The polymorphism of this site accelerates the occurrence of diabetic mellitus (Al Khaldi *et al.,* 2011). Alternatively, diabetic mellitus when associated with end-organ damage can complicate management of MDS, increase risks of complications, and limit the applicability of intensive therapeutic interventions. Al Khaldi *et al.*, (2011), reported that adiponectin GG genotype of SNP rs1501299 was significantly associated with higher levels of adiponectin and different types of cancer that may be correlated with MDS.

Notably, several studies from multiple populations found that rs1501299 is associated with lower levels of serum adiponectin. The mechanism by which rs1501299 affects serum adiponectin levels remains to be elucidated. Although not part of the promoter, rs1501299 could be located on an enhancer sequence. Enhancer sequences could be located in introns and could thus modulate gene expression (Liu *et al.*,2000). Moreover, rs1501299 could give rise to alternatively spliced mRNA (Wang *et al.,* 2006) or affect mRNA stability (Wang *et al.,* 2005). Song *et al.,* (2018), speculate that the *ADIPOQ* rs1501299 variant alters RNA splicing or stability, leading to allele-specific differential adiponectin expression similar to the effects of intronic SNPs in the *Calpin10* and *collagen type I alpha 1 chain* genes (Song *et al.,* 2018). Another possible explanation is that rs1501299 is in linkage disequilibrium with other *ADIPOQ* SNPs or other genes that have biological effects on adiponectin, tumor or MDS risk (Kawaii *et al.*,2013) in same research of Yang and his colleagues suggesting that SNP rs1501299 may not be a functional SNP but most likely be in linkage disequilibrium with other functional variants (Yang *et al.*,2007).

Moreover, we found that patients with MDS had a markedly increased rate of cardiovascular mortality compared with the age-matched US population, with an

standardized mortality ratios for death attributed to cardiovascular disorders of 2.21. The mechanism behind this relationship is not known, and some have hypothesized that it may be related to the effects of chronic anemia or iron overload which are may be stems from an imbalance in the adiponectin level (Brunner *et al.*,2017).

In the end, it must be mentioned that the genotype GG /rs1501299 is responsible for the MDS in the current study, in contrast to previous studies (Jang *et al.*,2006; Song *et al.*, 2018), that were conducted to determine the role of the gene in diabetes or heart disease, which showed that the genotype TT/ rs1501299 is associated with these diseases, so we hope to conduct other studies that include a larger number from the participants to explain the real role of this gene in the MDS.

4.4.3. Leptin gene (LEP) sequencing analysis

The LEP gene was detected genetically to investigate if there are any genetic defects within this gene. Sequencing of LEP gene for the gene part amplicon with the product (309bp) (Figure **4-20).**

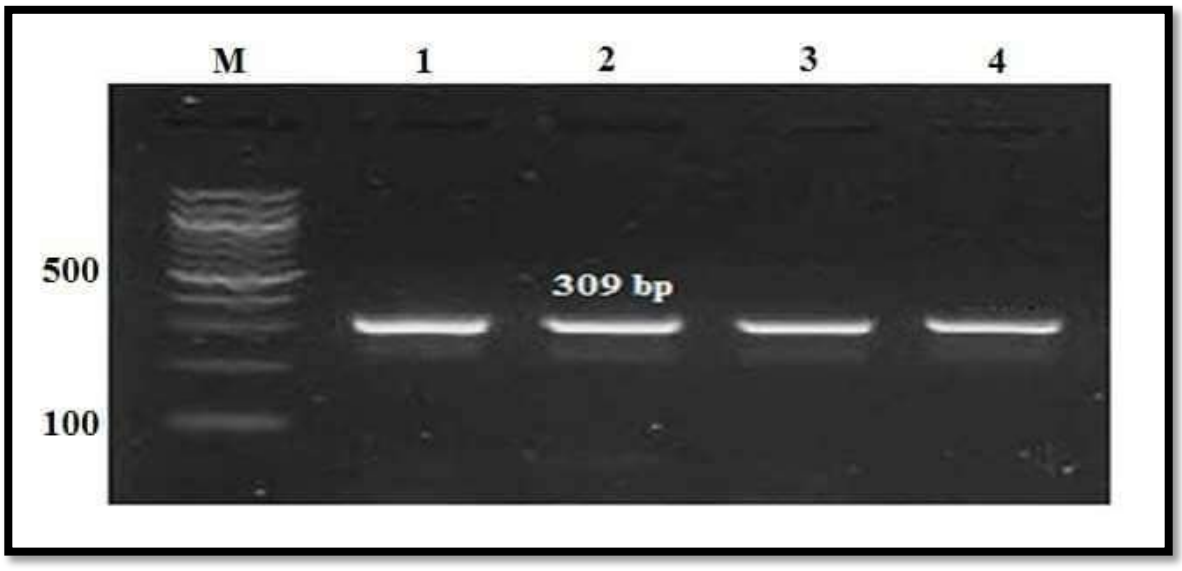

Figure (4-20): PCR product of LEP gene (309bp). L=ladder, lane (1-4) patient, 2% agarose, 75V, 20mA for 1 hour.

4.4.3.2. SNPs of leptin gene

The screening of the SNP (rs10954174) using Sequencing for the LEP gene do for all samples, including control and patient groups. Table (4-8) showed the allele frequency and genotyping for samples, and there is a significant value between patient and control groups.

Table (4-8): Genotyping and allele frequency of *LEP gene* associated with myelodysplastic syndrome patient and control groups.

Model	Genotyping	Control	Patient	OR (95 % CI)	P-value
Codominant	G/G	6 (24%)	26 (59.1%)	1.00	0.016
	A/G	10 (40%)	10 (22.7%)	0.23 (0.07-0.80)	
	A/A	9 (36%)	8 (18.2%)	0.21 (0.06-0.75)	
Dominant	G/G	6 (24%)	26 (59.1%)	1.00	0.0042
	A/G-A/A	19 (76%)	18 (40.9%)	0.22 (0.07-0.65)	
Recessive	G/G-A/G	16 (64%)	36 (81.8%)	1.00	0.0191
	A/A	9 (36%)	8 (18.2%)	0.40 (0.13-1.21)	
Predominant	G/G-A/A	15 (60%)	34 (77.3%)	1.00	0.023
	A/G	10 (40%)	10 (22.7%)	0.44 (0.15-1.28)	

Allele frequency

Allele	Control		Patient		OR (95 % CI)	P-value
	Count	%	Count	%		
G	22	0.44	62	0.7	3.035 (1.474-6.249)	0.012
A	28	0.56	26	0.3	0.329 (0.160-0.678)	

P≤0.05, 95% CI: Confidence interval at 95% level.

The results of the current research identified a clear relationship between the genetic heterogeneity of *LEP* with MDS where the genotype GG and mutant allele G appeared in a high percentage (59.1% and 70% respectively) among MDS patients this means that the *LEP* has become an etiological factor for this syndrome. LEP gene is located in chromosome 7 within q32.1, spanning a transcription element of about 20 kb and containing three exons. This gene encodes for leptin, a multifunctional polypeptide hormone of 16 KDa that is secreted into the blood circulatory system by adipocytes (Londraville *et al.*,2017).

Leptin interacts with many metabolic activities throughout the body, thereby controlling food intake, energy metabolism, body weight, appetite and reproduction (Park and Ahima, 2015). Leptin has been considered as a key regulator for severe insulin resistance and the diabetes phenotype of genetic disorders that impair adipogenesis that causes many disorders as obesity and health complications as renal failure, liver cirrhosis that may for stimulator factors to blood cells abnormalities as MDS (Joffe *et al.*,2001).

Leptin is produced predominantly in the adipose tissue but is also expressed in a variety of other tissues, including placenta, ovaries, mammary epithelium, bone marrow, and lymphoid tissues (Margetic *et al.*,2002), so any genetic variation in leptin gene or their receptors may be lead to serious disorders as MDS. Recently, obesity has been identified as a driver for progressive MDS (Stengel *et al.*,2003). In addition, myeloid precursor cells in the bone marrow have leptin receptors, and leptin plays an important role in the modulation of the innate immune response, inflammation, and hematopoiesis. Leptin is the obesity hormone synthesized mainly by white adipose tissue in humans and its serum level shows strong correlation with body fat mass (Considine *et al.*,1996). Mutations in the leptin gene have been reported to cause severe obesity (Stobel *et al.*,1998) and may also contribute to the complications associated with obesity as MDS. The mechanisms by which obesity can influence the pathogenesis of MDS are unclear, but one possible causal pathway includes elevated insulin and insulin-like growth factor 1 in obese individuals where

the metabolic consequence of obesity is insulin resistance followed by an increase in insulin secretion (Ma *et al.,* 2009). Insulin may promote tumorigenesis of MDS directly through insulin receptors in (pre)neoplastic target cells or indirectly by increasing levels of bioavailable insulin-like growth factor 1 (Calle *et al.,* 2004). Insulin-like growth factor 1 is involved in hematopoiesis and is mitogenic for myeloid cells in bone marrow that maybe represent first pathway for MDS development; almost all normal and neoplastic hematopoietic cells express the insulin-like growth factor receptors (Shimon *et al.*, 1995).

Leptin stimulates the proliferation of cultured glomerular endothelial cells and induces mRNA expression and protein secretion of transforming growth factor-β1 (TGF-β1). Long-term infusion with leptin (3 weeks) has led to increased glomerular expression of type IV collagen (Wolf *et al.*, 1999). Leptin has also been shown to stimulate synthesis of type I collagen in mesangial cells and type IV collagen in glomerular endothelial cells which contributes to extracellular matrix deposition, glomerulosclerosis, and proteinuria (Ballerman, 1999). It is therefore possible that genetic variation/s in the *LEP*, possibly related to variation in serum leptin concentration may be associated with markers of cancers and syndromes such as leukemia, MDS, Scr and eGFR (Ma *et al.,* 2009).

LEP A>G polymorphism known as A19G in 5′UTR variation was detected among Iraqi population in study of Musafer and his co-workers in 2021 (Musafer *et al.,* 2021). Although this *LEP* SNP located in non-coding exon, but it was found to be associated with severe obesity and increase leptin levels due to its critical position in 5′UTR regulatory region (Shimon *et al.,* 1999). In animal and human cell lines, leptin and leptin receptors have also been clearly associated with enhanced in vitro tumor proliferation and/or to in vitro and in vivo promotion of angiogenesis. These effects have been documented in embryonic cells, adipocytes, glia, endothelial cells, hematopoietic cells, and in benign and malignant cells, bone, kidney, colon, liver and pancreas (Snoussi *et al.,*2006). MDS, it was shown that leptin and leptin receptor are both expressed and that they act to favour cancer

proliferation and metastasis. However, the most recent reports indicate that higher leptin serum levels are associated with advanced stage cancer especially leukemia (Caldefie-Chėzet *et al.*,2005). In humans, several polymorphisms have been identified in the *LEP* and *LEP* receptor (*LEPR*) genes: a G to A substitution at nt -2548 upstream of the ATG start site in the LEP gene 5' promoter region, and an A to G substitution at nt 668 from the start codon 223 in exon 6 (Q223R) of the LEPR gene coding for the extracellular region common to all isoforms of LEPR (Boumaiza *et al.*,2012).

This study confirms the presence of an interesting correlation between the polymorphism of the *LEP* gene and MDS, which can serve as a marker for the assessment of several MDS-related parameters in the Iraqi population. Moreover, according to our knowledge, the current study is the first study that dealt with the relationship between genetic variation of *LEP*/SNP rs10954174 and MDS, therefore we did not find sufficient studies in the same field to mention it or compare our results with it. For this reason, our ability to find the pathological effect of this gene in the occurrence of the syndrome was limited.

4.4.4. The genotyping RETN gene polymorphism by PCR-SSCP techniques

The present study includes (75) samples, which group as following: control group (25) blood sample, patients group (50) blood samples.

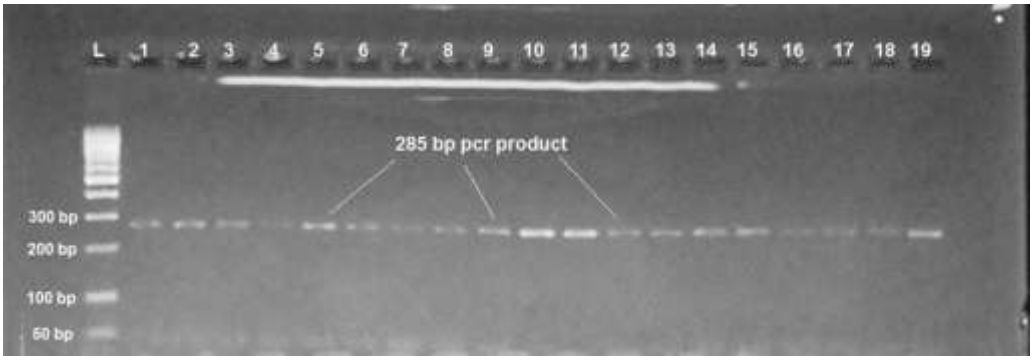

Figure (4-21): PCR product of RETN gene (285 bp), the electrophoresis carried on agarose gel (2%) 75 V, 20 mA for 1 hour. L=ladder (100 bp), lane 1-19 sample PCR product.

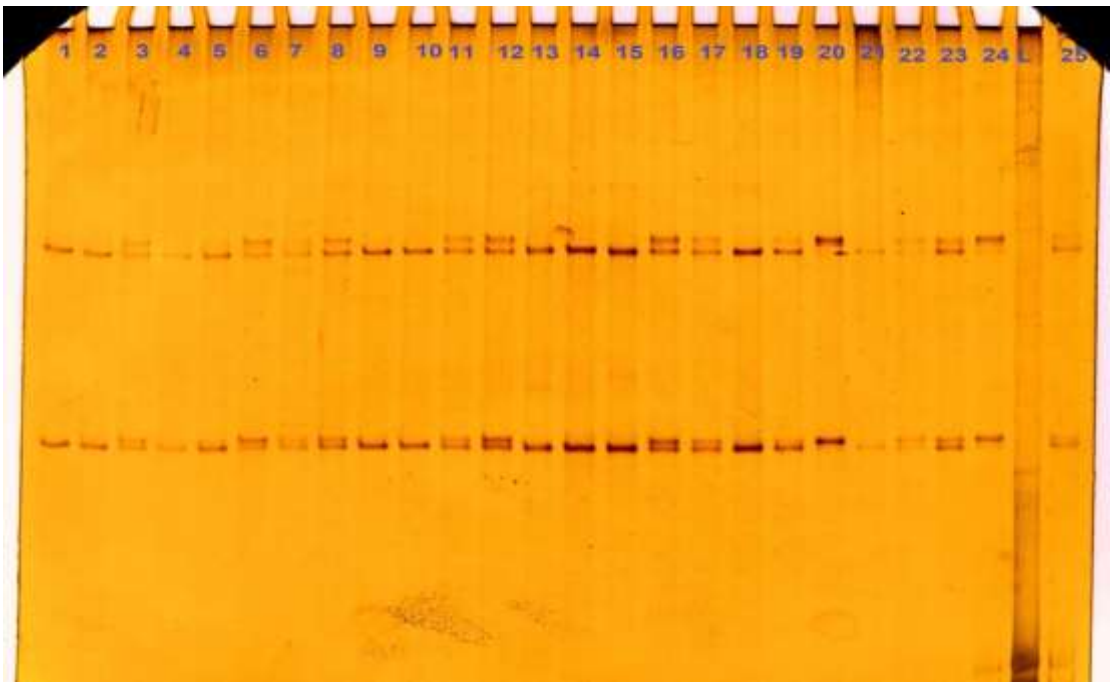

Figure (4-23): SSCP electrophoresis pattern of RETN gene fragment for samples of MDS patients and control, 2% gel 100V, 20Am for 6 h , show the clear three different patterns (lanes 20 and 24 A pattern ; lanes 1,2,4,9,10,13,14,15,18,19,21, B pattern ; other lanes C pattern).

4.4.4.2. SNPs of resistin gene (RETN)

The screening for the SNP (rs3745367) using Sequencing for RETN do for (6) samples, including the control group and patients group samples.

Table (4-9) showed the genotype analysis and allele frequency for the samples, and there is no significant value between the two groups (control and patients).

Table (4-9) Genotype and allele frequency of RENT gene for myelodysplastic syndrome patient and control groups.

Model	Genotyping	Control	Patient	OR (95 % CI)	P-value
Codominant	G/G	9 (36 %)	17 (37.8%)	1.00	0.99
	G/A	12 (48%)	21 (46.7%)	0.93 (0.32-2.71)	
	A/A	4 (16%)	7 (15.6%)	0.93 (0.21-4.03)	
Dominant	G/G	9(36%)	17(37.8%)	1.00	0.88
	G/A-A/A	16 (64%)	28 (62.2%)	0.93 (0.34-2.56)	
Recessive	G/G-G/A	21 (84%)	38 (84.4%)	1.00	0.96
	A/A	4 (16%)	7 (15.6%)	0.97 (0.25-3.69)	
Predominant	G/G-A/A	13 (52%)	24 (53.3%)	1.00	0.91
	G/A	12 (48%)	21 (46.7%)	0.95 (0.36-2.52)	
Allele frequency					

Allele	Control		Patient		OR (95 % CI)	P-value
	Count	%	Count	%		
G	30	0.6	55	0.61	1.048 (0.517-2.124)	0.89737
A	20	0.4	35	0.39	0.955 (0.471-1.936)	

P≤0.05

95% CI: Confidence interval at 95% level.

Several studies suggest that resistin is associated with multiple inflammatory human diseases including diabetes and cardiovascular diseases (Albate *et al.*,2014), but we not found any study link this gene with MDS. Despite the slight increase in the percentage of the mutated genotype or the mutated allele in RETN/SNP rs3745367 in MDS patients, no significant differences appeared in the current study.

Anyway, these simple differences may play a direct or indirect role in the pathogenesis of the MDS. Study of Dalamaga *et al* ., (2008), determined resistinis by radioimmresistiniand found that MDS patients have lower resistin levels and causes for that probably due to a compensatory response to the upregulation of other inflammatory factors etiologically linked to myelodysplasia (Dalamaga *et al.*,2008).

Resistin is a protein hormone produced both by adipocytes and immunocompetent cells, including those residing in adipose tissue. Some evidence suggests that resistin modulates glucose tolerance and insulin action, thereby playing a role in the pathogenesis of obesity and insulin resistance which correlated indirectly with MDS as mentioned in previous sections. RETN, the gene coding for human resistin, is located on chromosome 19p13.3.6 (Boumaiza *et al.,* 2012). The length of the RETN pre-peptide in humans is 108 amino acids (Wang *et al.,* 2009). Up to 70% of the variation in circulating resistin levels can be explained by genetic factors, and several single-nucleotide polymorphisms in the *RETN* gene have been described so far (Engert *et al.,* 2002). One of the most frequently studied polymorphisms, *RETN* rs3745367, was reported to be associated with the regulation of *RETN* gene expression and serum resistin level . Several studies have also associated the *RETN* rs3745367 polymorphism with obesity , insulin sensitivity, type 2 diabetes (Wang *et al.*,2002; Mattevi *et al.,* 2004; Osawa *et al*,.2004), and cerebrovascular disease. As single-nucleotide polymorphisms (SNPs) in the 3′-untranslated region (3'UTR) of genes can affect gene expression and disease susceptibility, the rs3745367 SNP in the 3'UTR of the resistin gene might have an influence on resistin gene expression and thus influences the risk for the development of diabetes and hypertension (Fu *et al.*,2017).

Taking into consideration that diabetes mellitus and obesity are major characteristics of metabolic syndromes (MetS), the resistin gene was considered as a potential candidate gene for MetS in pervious study but other studies in humans have failed to detect higher serum resistin levels in obese or insulin resistant

subjects however, data in humans are controversial (Reseland *et al.*,2009). Additionally, resistin was found to promote the expression of the pro-inflammatory adhesion molecules such as intercellular adhesion molecule 1, vascular cell adhesion molecule 1, and pentraxin 3 in vascular endothelial cells, thereby promoting the adhesion of leukocytes (Ouchi *et al.*,2011). Also resistin increases nuclear factor kappa B-related monocyte expression of pro-inflammatory cytokines such as IL-6 (Lee *et al.*,2014). Thus, the observed increased risk of multiple myeloma in males with lower levels of resistin might reflect a compensatory effect of resistin or a negative feedback loop following the production of IL-6 and/or other cytokines with known effects on proliferation and survival of multiple myeloma cells (Dalamaga *et al.*,2009).

Alternatively, previous studies have shown that TNF-α decreases resistin secretion in murine models. If TNF-α, a well-characterised pro-inflammatory cytokine that stimulates multiple myeloma cell proliferation, also acts as a negative regulator of resistin in humans, this action may explain the observed inverse association between pre-diagnosis resistin levels and multiple myeloma risk. This hypothesis is corroborated by increased resistin levels in multiple myeloma patients after treatment with thalidomide, a TNF-α inhibitor (Santo *et al.*,2017).

Previous studies remember that possible relationship between diseases and resistin according to gender could exist. This could be related to the difference in amount and functionality of adipose tissues in each gender (Cherneva *et al.*,2013). In addition, variations in hormonal signaling pathways and immune response between both genders could account for such relationship (Machura *et al.*,2013). Furthermore, age differences were found in the association between rs3745367 SNP and AD (Robati *et al.*,2014). Results showed only the age group between newborn children and 10 years old is associated with rs3745367 SNP in the *RETN* gene. On other hand, positive association was reported in study from South Korea that indicated lower resistin levels in patients with atopic asthma compared to control

groups (Kim *et al.*,2008). In contrast, a couple of studies on Turkish patients with systemic sclerosis, and Iranian patients with psoriasis, showed higher resistin levels in patients compared to control groups. Thus, alterations in the serum resistin levels might predispose to different sets of diseases (Banihani *et al.*,2018).

4.4.5. Detection polymorphism of splicing factor 3b subunit 1 (SF3B1)

The study performed optimization for the SF3B1 gene, as shown in figure (4-24), followed by specific PCR for exon 13. The optimal PCR condition was following initial denaturation at 94C for 5 min, 35 cycles of 94C for 30 sec, 53C for 30 sec, 72C for 45 sec, and final elongation at 72C for 5 min as figure (4-34)

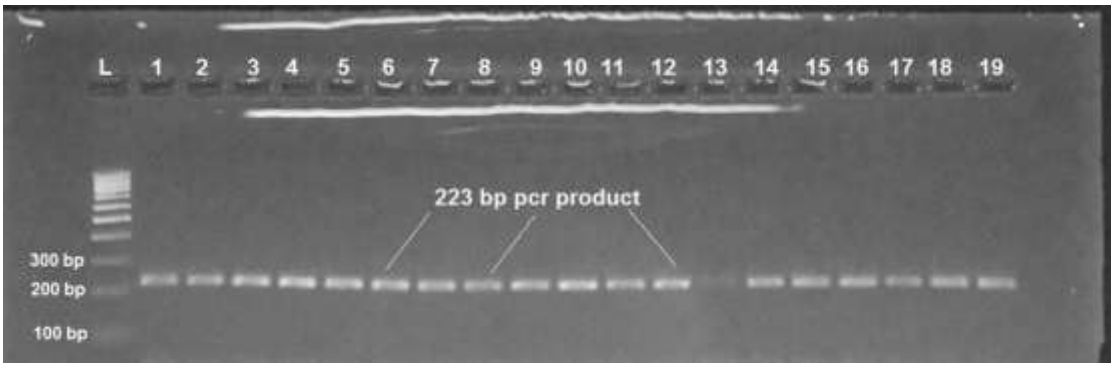

Figure (4-35): PCR product of SF3B1 (exon 13) gene (223 bp), the electrophoresis carried on agarose gel (2%), 75V, 20AM for 1 hour. L=ladder (100 bp), lane 1-19 sample PCR product.

4.4.5.2. Genotyping and Allele frequency of SF3B1 gene

As shown in table (4-17), the prevalence rate of mutant II genotype and I allele were significantly higher among cases with MDS (92.5% and 92% respectively) compared to healthy individuals in control group (60% for each one of them) and this increase occurring of MDS by 8.234 and 8.23 respectively. Moreover mutant II genotype and I allele were responsible for 82% and 81% of incidence rate of new MDS cases while wild DD genotype and I allele mainly appeared in control group at 40% for each variable representing a protective factors at rate 17.7% and 8% respectively.

Table (4-17): Genotyping and allele frequency of *SF3B1* (exon 3) gene in MDS patients and control

***SF3B1* polymorphisms**	**Control N (%)**	**Patient N (%)**	**OR (95 % CI)**	**P value**
Genotypes				
II	15(60%)	37(92.5)	1.00	0.003*
DD	10(40%)	3(7.5)	0.12(0.03-0.50)	
Alleles				
I	30 (60)	74(92)	8.22 (3.006-22.488)	6.577e-06
D	20 (40)	6 (8)	1.113 (0.019-0.25)	

P≤0.05

95% CI: Confidence interval at 95% level.

SF3B1 is the most commonly mutated gene found in MDS (20–28) percent of all patients that is the core component of small nuclear ribonucleoprotein U2 that is part of the branch point sequence recognition (Cazzola *et al.*,2013).

Others studies have shown that splicing factor SF3B1 mutations are found in a high percentage of MDS patients with high serum ferritin conditions(Yoshida and Ogawa, 2014). In our population genetic mutation appeared in *SF3B1* gene of MDS patients and mutant II genotype appeared as etiological factor. Knockdown SF3B1, limits cell development, produces arrest and undermines the differentiation of erythroid. MDS mutations SF3B1 are heterozygous point mutations in particular. The presence of SF3B1 hotspots and the absence of SF3B1 mutations in MDS patients shows that SF3B1 mutations are likely to be (neomorphic) mutations in gain/change of function. A Sf3b1+/ − knockout mouse model has proven to grow sideroblasts in the ring and suggests the development of SF3B1 haploinsufficient (Haferlach *et al.*,2014).

A recent study identified a DNA damage-induced BRCA1 protein complex containing BCLAF1 and SF3B1 (BRCA1–BCLAF1– SF3B1 complex). In response to DNA damage, the complex regulates pre-mRNA splicing of genes involved in

DNA damage signaling and repair and hence affects their transcription and premRNA maturation (Savage *et al.*,2014). It has been demonstrated that abrogation of members of this complex including BRCA1 and BCLAF1 results in genomic instability, a common feature of malignant cells. In MDS patients with mutant SF3B1, the function of this complex may be impaired, with possible downstream effects on the efficiency of DNA damage repair (Doltashade *et al.*,2015).

A recent study showed that defects in the splicing factor gene PRPF8 result in missplicing in myeloid malignancies and are associated with the presence of ring sideroblasts in advanced MDS and AML (Kurtovic-Kozaric *et al.*,2015) Interestingly, we observed differential exon usage of PRPF8 in SF3B1 mutant cases compared with control in our study, indicating a link between SF3B1 and PRPF8 and the ring sideroblast phenotype. Our finding of differential exon usage of multiple RNA processing/splicing genes in the HSPC of cases with SF3B1 mutation points towards an exacerbation of aberrant splicing, with a wider number of downstream target genes affected. Emerging evidence from our study on SF3B1 and the study on U2AF145 thus supports the hypothesis that in MDS patients harboring splicing factor mutations, there is widespread disruption of the splicing machinery as a consequence of downstream effects of the mutant protein. Loss-of-function mutations in zebrafish Prpf8 have been shown to result in missplicing of TP53(Keightley *et al.*,2013). Also, found TP53 to be aberrantly spliced in MDS patients with SF3B1 mutation compared with wild type and control (Doltashade *et al.*,2015).

4.4.5.3. Correlation of ferritin with *SF3B1* SNP

Genetic disorders of MDS effect directly or indirectly on clinical outcomes. Present data (figure 4-39) showed significant effect of MDS *SF3B1* polymorphism on serum level of ferritin when a clear rise in level of ferritin appeared in MDS patients who have genetic mutation in *SF3B* and according to this fact, serum level of ferritin increased in cases with mutant II genotype (1003.41ng/ml) compared

with wild DD genotype (355.04 ng/ml) so that reflected statistical difference (P=0.0019).

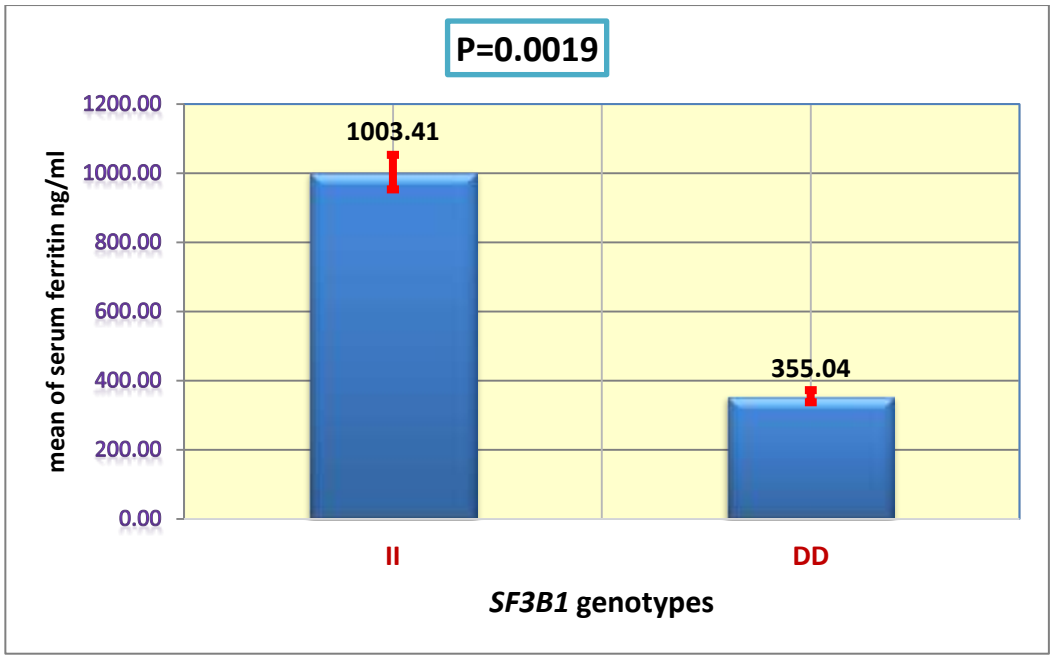

Figure (4-24): Mean serum level of ferritin according to *SF3B1* genotypes.

The current study also found a relationship between high serum ferritin and the mutant II genotype and this result explained by study showed that MDS patients associated with increased levels of oxidative stress, which is further aggravated by iron overload. The oxidative DNA damage that results, which is exacerbated by iron overload, could lead to mutagenesis in the bone marrow (Lu *et al.*,2013). Investigations examining the impact of iron overload on MDS genomic instability back up the theory that iron overload causes genomic instability. The findings also imply that serum ferritin levels above 1000, as well as those between 1000 and the upper limit of normal, have an unfavorable effect on genetic stability (Westhofen *et al.*,2015).

Two genes involved in mitochondria iron metabolism synthesis *PPOX* and *ABCB7* were found be downregulated in *SF3B1* mutated samples. As *PPOX* encoded protoprphyrinogin oxidase, this enzyme located in the mitochondrial membrane and involved in the production of a molecule called heme. Its catalyzed

the 6-electron oxidation of protoprphyrinogen IX to form protoprophyrin IX. It is haplo insufficiency of this gene may induce deficiency heme synthesis and iron accumulation in the mitochondria. ATP binding cassette subfamily B (*ABCB7)* causative gene of congenital sideroblastic anemia, its important mitochondrial erythroid colony growth and decreased mitochondrial ferritin (Clough *et al.*,2021).

In the end, according to our knowledge, studies are few or very limited to determine the relationship between genetic studies of MDS and their effects on clinical outcomes especially ferritin concentration in serum and this is the first study in Iraq to link *SF3B1* polymorphisms of MDS with ferritin rate.

4.4.6. Real-time PCR of SRSF2 gene expression

The results investigated in (figures 4-40, 4-41, and 4-42) showed the gene expression of the SRSF2 gene in control and patients with myelodysplastic syndrome. The gene expression of this gene is significantly high in MDS patients when compared with control groups.

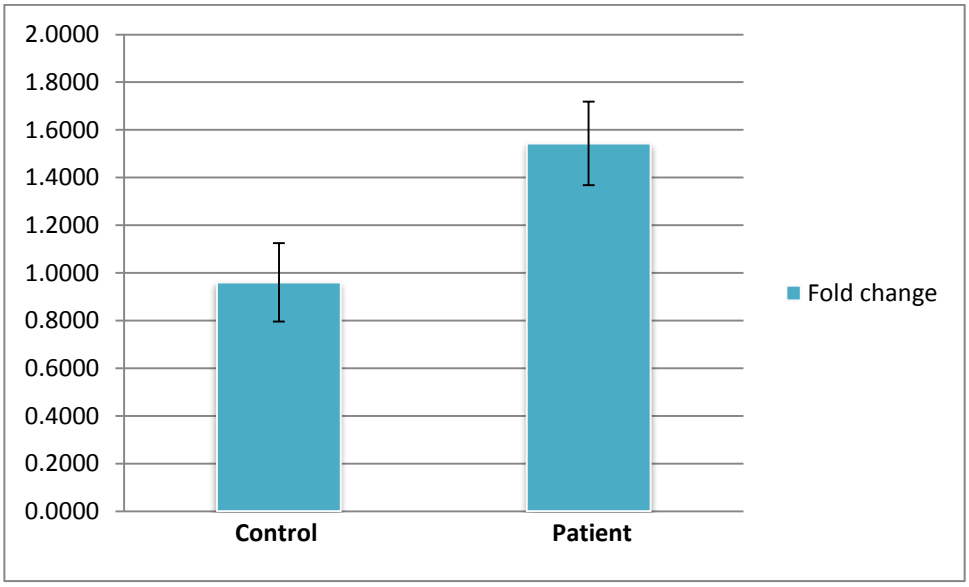

Figure (4-26): Folding change in gene expression of SRSF2 gene for MDS patient and control groups.

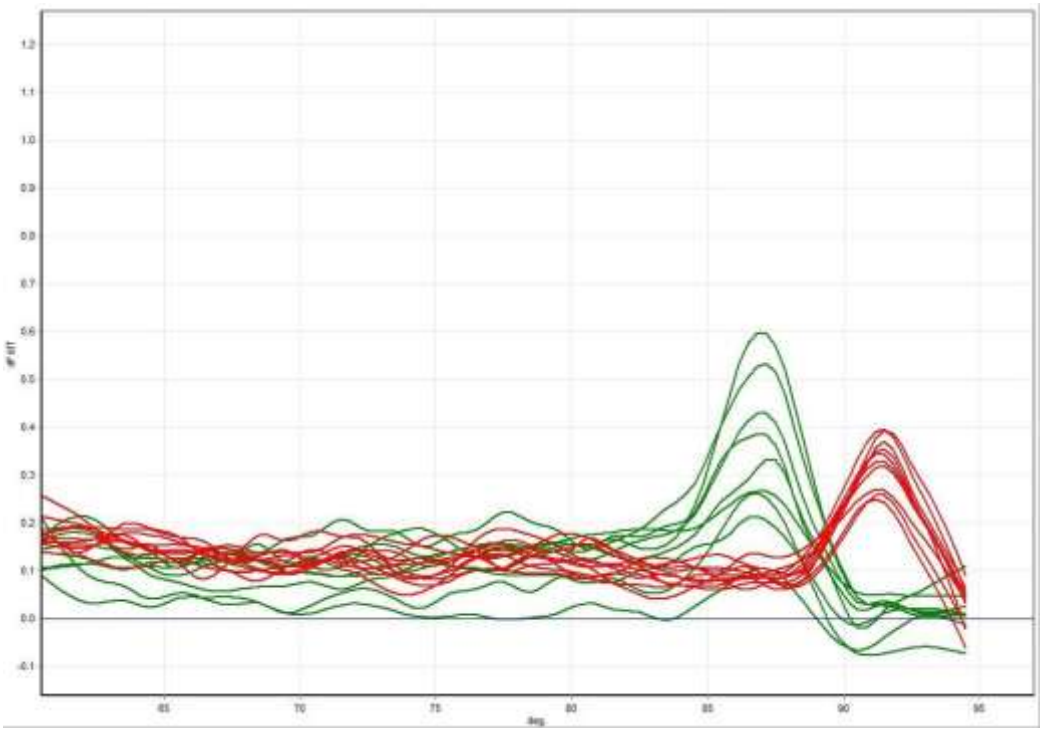

Figure (4-27): Melting curve for sure the specificity of syper green on the target gene (*SRSF2)* and housekeeping gene.

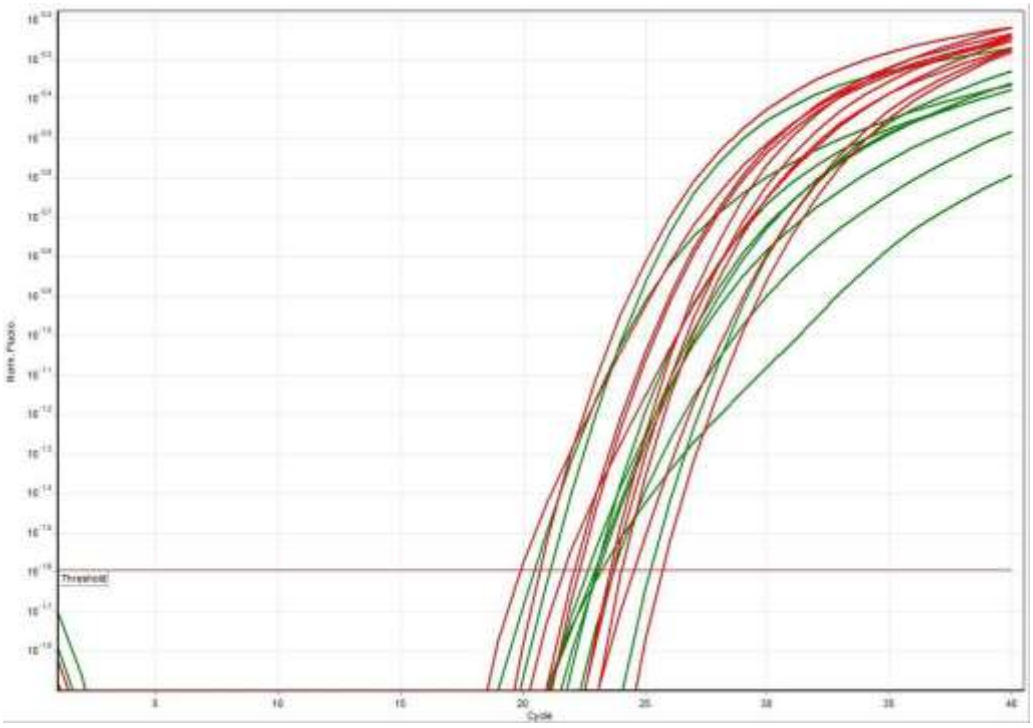

Figure (4-28): Amplification of real-time PCR by the blot of gene *SRSF2.*

Molecular-based diagnostic and prognostic criteria may provide better biomarkers of body disorders as they likely reflect the underlying biology of the disease. Real-time PCR from the best molecular methods for detection folding changes of gene expression (Döhner *et al* .,2015; Hwang *et al*.,2016). Determine of gene expression distinguishes molecular processes from somatic mutations. As such, it may incorporate the effects of diverse somatic and epigenetic lesions into common phenotypes. Gene expression profiling has been used successfully to identify potential biomarkers for the diagnosis and prognosis of different subtypes of disorders of MDS (Papaemmanuil *et al*.,2016; Itzykson *et al*.,2013)

The results of the current study showed that gene expression of *SRSF2* increased among MDS patients compared with healthy controls, and the current result is in agreement with many previous studies (Drexler *et al.*,2009; Matsuoka et al.,2010; Federmann *et al*.,2014; Aujla *et al*.,2018). In present study, Overexpression of *SRSF2* detected derived from folding changes of melting curve and amplification of real-time PCR products.

Furthermore, SRSF2 is a gene encoding critical spliceosomal proteins. SRSF2 mutations appear to play an important role in pathogenesis of myeloproliferative overlap syndromes, particularly in MDS and chronic myelomonocytic leukemia. SRSF2 has a RNA recognition motif and thus enhancing spliceosome assembly at adjacent splice sites to allow appropriate exon inclusion (Zhou and Fu, 2013; Howard and Sanford, 2015).

In addition, SRSF2 was reported to play an active role in transcription elongation and in coupling transcription and splicing processes (Das *et al*.,2007; Lin *et al*.,2008). Over expression of SRSF2 gene in MDS patients may be related to sense mutations in this gene. In 2011, Yoshida *et al.* identified frequently recurring splicing factor mutations in a cohort of adult patients with myeloid neoplasms through performing whole-exome sequencing. SF3B1 (36%) was the most common mutation followed by SRSF2 (25.6%), U2AF35 (16.9%) and ZRSR2 (10.5%) (Yoshida *et al*.,2011). These mutations were more frequent and comparatively more

specific to the diseases with MDS features. Komeno *et al* in 2015 report that SRSF2 is essential for the survival of hematopoietic cells in developing embryos and adults and that its mutant forms switch the RNA splicing profile on a large panel of genes involved in cancer development and apoptosis (Patnaik *et al*.,2013; Komeno *et al*.,2015). Together, these data suggest that SRSF2 mutations identified in MDS are not simply loss-of-function mutations but instead alter SRSF2 function in RNA splicing. Such changes may directly contribute to MDS development and later progression to more aggressive forms of leukemia (Thol *et al*.,2012).

Other researchers found that direct association of SRSF2 in development of myelodysplasia was appeared in SRSF2-P95H mutant mice (Kim *et al*.,2015). P95H is the most common mutation site in the SRSF2 gene and its proximity to RRM site of SRSF2 might play a role in altering RNA binding abilities (Daubner *et al*.,2012; Komeno *et al*.,2015; Zhang *et al*.,2015; Obeng *et al*.,2016). Heterozygous P95H mutant and homozygous SRSF2 deleted bone marrow mononuclear cells led to development of significant leukopenia and anemia in lethally irradiated recipient mice. However, only P95H mutated mice developed macrocytic RBCs and had normal bone marrow cellularity in contrast to bone marrow aplasia seen with homozygous SRSF2 deletion. Peripheral erythroid and myeloid dysplasia was also seen only with P95H mutant mice (Kim *et al*.,2015). These findings correlate with MDS findings in humans .

Other studies suggest that increased expression of SRSF2 mainly affects cell survival but does not disrupt myeloid cell differentiation. More importantly, overexpression of the P95H/ SRSF2and Δ8aa/ SRSF2mutants always showed stronger negative effects on cell survival than overexpression of the WT/ SRSF2 (Wu *et al*.,2012; Komeno *et al*.,2015; Masaki *et al*.,2019). These findings support the possibility that the MDS-associated mutations in SRSF2 promote the development of the disease phenotype with potential to induce a cascade of events that lead to both disease progression and more-aggressive types of blood disorders

therefore, inhibition of splicing may be a new therapeutic approach. E7107, a spliceosome inhibitor, has been shown to differentially inhibit splicing more in SRSF2-mutant cells leading to decreased leukemia burden in mice (Harada and Harada, 2015; Rahman *et al*.,2019).

Conclusion & Recommendation

Conclusion

1. Myelodysplastic syndrome incidence in male was recorded more than in female, and the age risk of disease incidence is begin in 60 years old and increased between 65-75 years old for both sex.

2. Using iron overload as prognosis biomarker in MDS patients.

3. Oxidative stress or reactive oxygen species was recorded higher level and cause DNA damage.

4. rs15001299 and GG genotype of adiponectin gene is responsible for the pathogenesis of MDS patients.

5. Correlation between the polymorphism of *LEP* gene and MDS which can serve as marker for assessment of MDS related parameters in Iraqi population.

6. rs3217350 SNP of splicing factor *SF3B1* showed a strong significant value between the study groups (patients and control) and a significant correlation with ferritin which consider the first study in Iraq to link *SF3B1* polymorphisms of MDS with ferritin rate.

7. *SRSF2* overexpression can affect hematopoietic cells with growth arrest and associated with oncogesis.

Recommendation

1. Studying other hormones that related with disease such as erythropoietin also estimation of hepcedin concentration.

2. Reinforcement the present study with immunological study.

3. Studying SNP in other genes related with hematopoiesis.

4. Studying epigenetic genes like *TET2, DNTM3* that strong relationship in pathopgenesis of myelodysplastic syndrome.

5. Studying target gene (*ABCB7*) of *SF3B1* that related with iron overload in mitochondria.

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Appendix

SEQUENCING REPORT OF THREE *LEP*-GENE BASED PCR AMPLICONS

1. SEQUENCING RESULTS

Sequencing of the 309 bp region within the 3′-UTR of *LEP* gene

In the present study, the *LEP* genetic sequences in chromosome no. 7. This gene is involved in several activities related to body weight regulation (https://ghr.nlm.nih.gov/gene/LEP). The sequencing reactions indicated the exact identity of this genetic fragment after performing NCBI blastn (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Concerning the 309 bp amplicons, the NCBI BLASTn engine shown about 99.5% sequence similarities between the sequenced samples and the intended reference target sequences, which partially cover the 3′-untranslated region (UTR) of this gene. By comparing the observed DNA sequences of these investigated samples with the retrieved DNA sequences (GenBank acc. NC_000007.14), the accurate positions and other details of the retrieved PCR fragments were identified (Fig. 1).

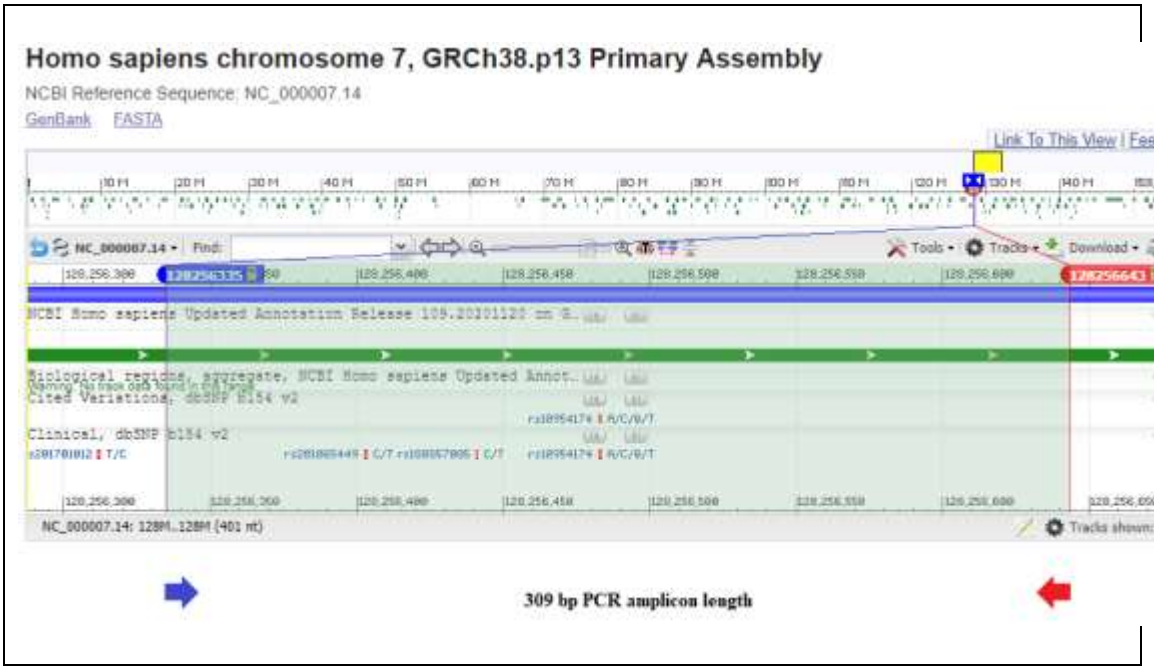


Fig. 1. The exact position of the retrieved 309 bp amplicon that partially covered a portion of the 3′-UTR of the *LEP* gene within chromosome no. 7 (GenBank acc. no. NC_000007.14). The cyan arrow refers to the starting point of this amplicon while the red arrow refers to its endpoint.

Appendix

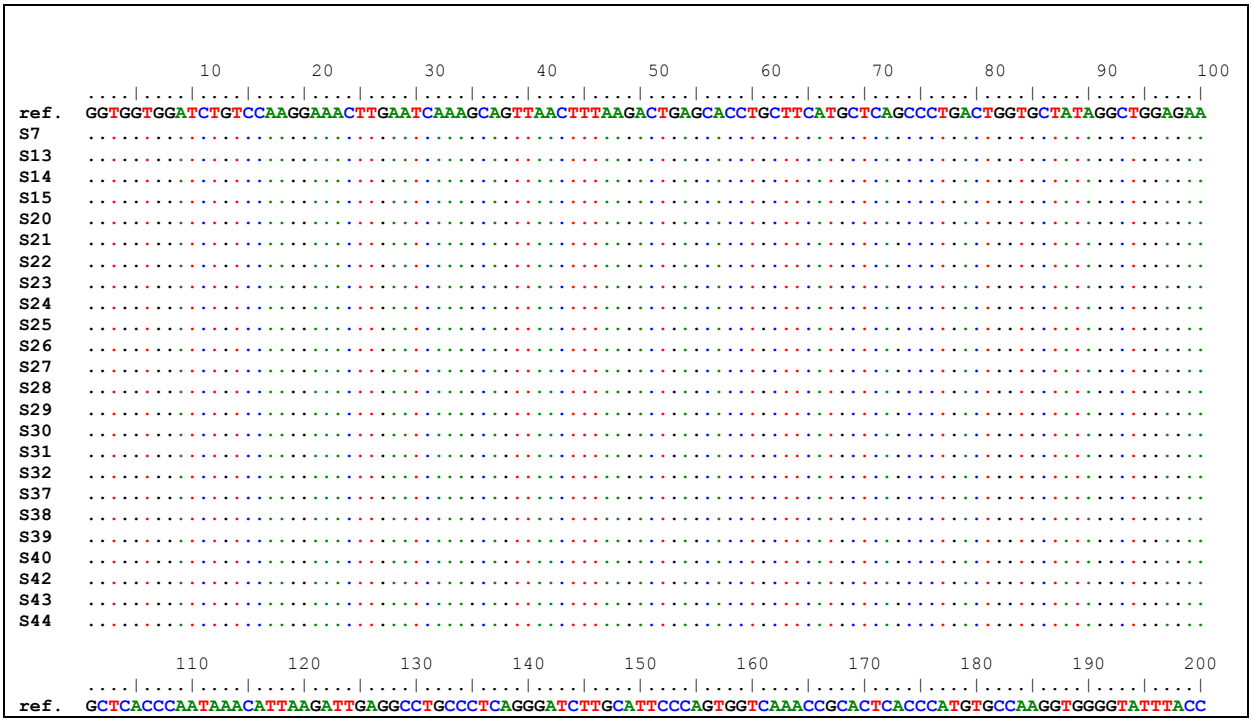
After positioning the 309 bp amplicons' sequences within chromosome no. 7, the details of its sequences were highlighted, in terms of the positioning of both forward and reverse primers of the 309 bp amplified amplicons (Table 1).

Table 1. The position and length of the 309 bp PCR amplicons used to amplify a portion of the 3′-UTR of the *LEP* gene located within chromosome no. 7 (GenBank acc. no. NC_000007.14). The gray-colored sequences referred to the position of the reverse and forward primers, respectively. The red-colored nucleic acid is the position of the targeted high-frequency SNP in the investigated fragment.

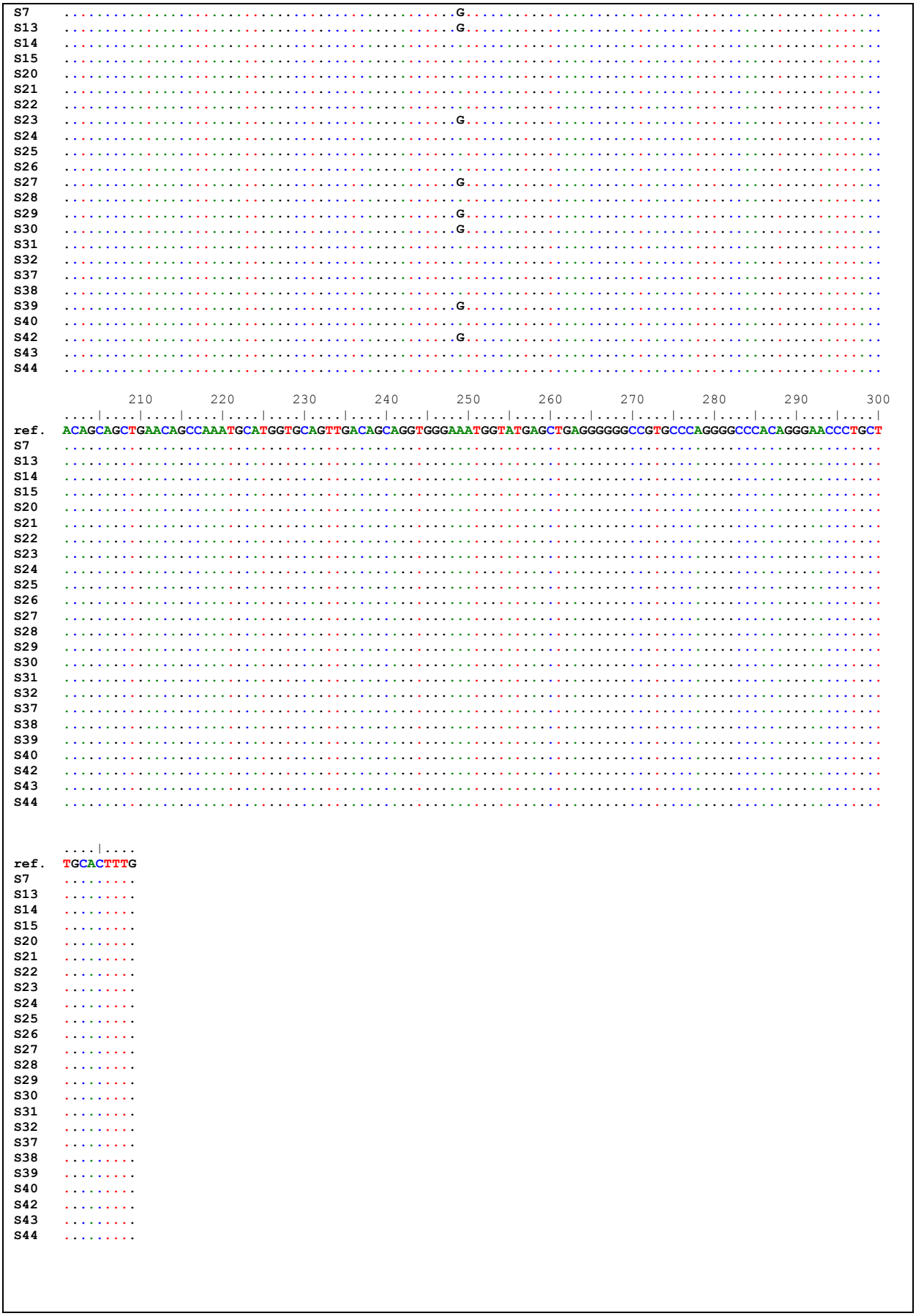
Amplicon	Reference locus sequences (5′ - 3′)	Length
DNA sequences within the 3′-UTR of the *LEP* gene	*GGTGGTGGATCTGTCCAAGGAAACTTGAATCAAAGCAGTTAACTTTAAGAC TGAGCACCTGCTTCATGCTCAGCCCTGACTGGTGCTATAGGCTGGAGAAGCT CACCCAATAAACATTAAGATTGAGGCCTGCCCTCAGGGATCTTGC**A**TTCCCA GTGGTCAAACCGCACTCACCCATGTGCCAAGGTGGGGTATTTACCACAGCAG CTGAACAGCCAAATGCATGGTGCAGTTGACAGCAGGTGGGAAATGGTATGAG CTGAGGGGGGCCGTGCCCAGGGGCCCACAGGGAACCCTGCTTGCACTTTG**	309 bp

* refers to the forward primer sequences (placed in a forward direction)
**refers to the reverse primer (sequences (placed in a reverse complement direction)

The alignment results of the 309 bp samples revealed the presence of only one mutation in some of the analyzed samples in comparison with the referring reference DNA sequences (Fig. 2).



Appendix


S7 G
S13 G
S14
S15
S20
S21
S22
S23 G
S24
S25
S26
S27 G
S28
S29 G
S30 G
S31
S32
S37
S38
S39 G
S40
S42 G
S43
S44
210 220 230 240 250 260 270 280 290 300
ref. ACAGCAGCTGAACAGCCAAATGCATGGTGCAGTTGACAGCAGGTGGGAAATGGTATGAGCTGAGGGGGGCCGTGCCCAGGGGCCCACAGGGAACCCTGCT
ref. TGCACTTTG

Appendix

Fig. 2. DNA sequences alignment of 6 samples with their corresponding reference sequences of the 309 bp amplicons of the 3′-UTR of the *LEP* gene. The symbol "Ref." refers to the NCBI referring sequence, "S" refers to the samples.

A highly interesting nucleic acid polymorphism (SNP) was detected in this study in the investigated S7, S13, S23, S27, S29, S30, S40, and S42 samples, in which Adenine was replaced with Guanine at the position 149, A149G. The sequencing chromatogram of the identified variation region, as well as its detailed annotations, were documented, and the chromatogram of this sequence was shown according to its position in the PCR amplicon. However, this SNP was detected in heterozygous states in S7, S27, S29, S30, S40, and S42, and homozygous in both S13 and S23 (Fig. 3).

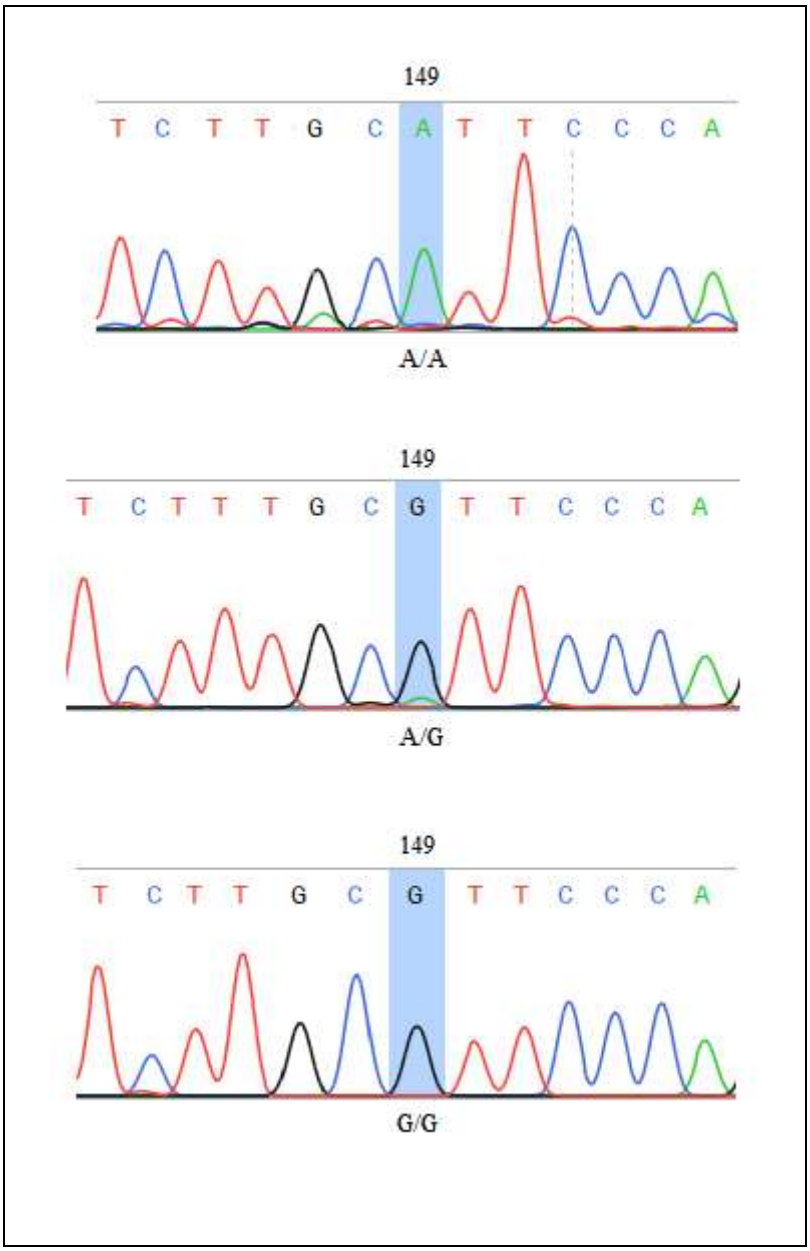


Fig. 3. The pattern of the detected A149G SNP within the DNA chromatogram of the targeted 309 bp amplicons of the 3′-UTR of the *LEP* gene. The identified SNP was highlighted according to its position in the PCR amplicons.

Appendix

To elucidate the position of the targeted SNP concerning their deposited SNP database of the sequenced 309 bp fragment, the corresponding position of the *LEP* gene was retrieved from the dbSNP server (https://www.ncbi.nlm.nih.gov/projects/SNP/). To find out the nature of this SNP, a graphical representation was performed concerning the *LEP* dbSNP database within chromosome no. 7 (GenBank Acc. No. NC_000007.14). By reviewing the dbSNP engine, it was found that this detected SNP was found to be previously known as it was deposited as rs12706832 (Fig. 4). However, this SNP was located in 3′-UTR of the targeted *LEP* gene (https://www.ncbi.nlm.nih.gov/snp/rs10954174). Though this SNP was deposited in very low frequency in different populations, a considerably high frequency was observed in this study for this SNP, which may entail a crucial role for this particular SNP in many potential cellular dysfunctions in the screened population. However, this SNP was reported in many publications that described its effect on many *LEP*-associated metabolic issues (https://www.ncbi.nlm.nih.gov/snp/rs10954174#publications).

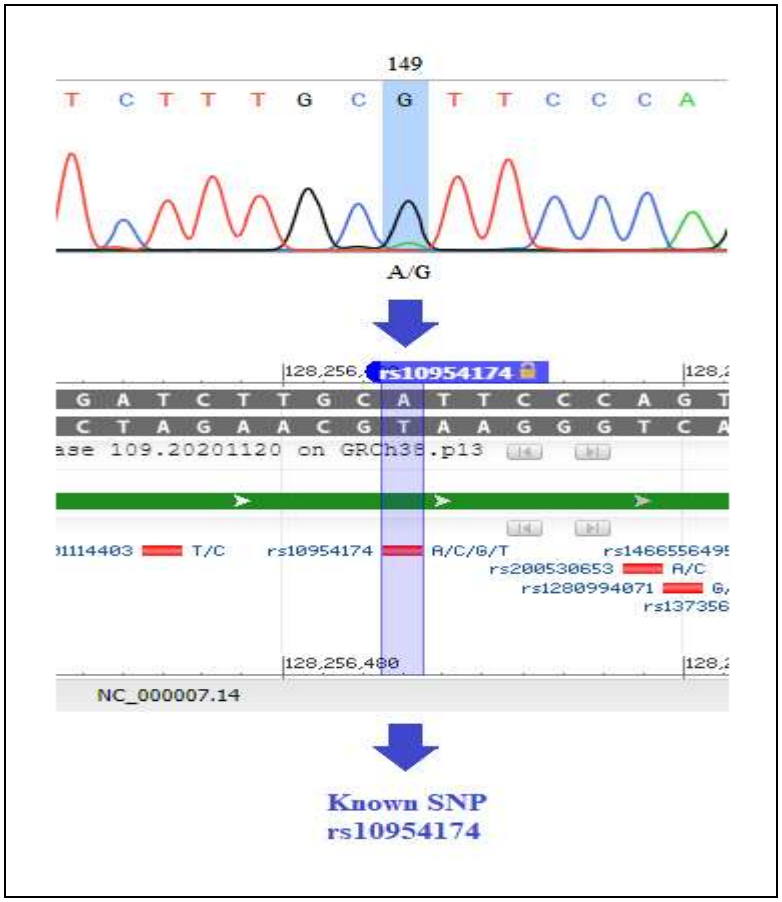


Fig. 4. The SNP's novelty checking of *LEP* genetic single nucleotides polymorphisms using the dbSNP server. The identified SNPs A149G was marked with a blue color. The GenBank acc. no. NC_000012.11 was used in the positioning of the highlighted substitution SNP. The position of the targeted sequences was found in the positive strand.

Appendix

To summarize the results obtained from the sequenced 309 bp fragments, the exact position of the observed variation was described in the NCBI reference sequences (Table 2).

Table 2. The pattern of the observed SNP in the 309 bp amplicons designed to amplify a portion of the 3′-UTR within the *LEP* gene in comparison with the NCBI referring sequences (GenBank acc. no. NC_000007.14). The symbol "S" refers to the sample number.

Sample No.	Zygosity status	Native	Allele	Position in the PCR fragment	Position in the reference genome	SNP type	Variant summary
S13, S23	Homozygous	A	A	149	128256483	UTR variant	rs10954174
S7, S27, S29, S30, S40, and S42	Heterozygous	A	G	149	128256483	UTR variant	rs10954174
Rest of samples	Homozygous	G	G	149	128256483	UTR variant	rs10954174

2. SEQUENCING REPORT OF THE *RENT*-GENE BASED PCR AMPLICONS

SEQUENCING RESULTS

In the present study, the *RENT* genetic sequences in chromosome 19. This gene encodes for resistin, which is a hormone involved in several activities related to the suppression of insulin ability to stimulate glucose uptake into adipose cells (https://www.uniprot.org/uniprot/Q9HD89). The sequencing reactions indicated the exact identity of this genetic fragment after performing NCBI blastn (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Concerning the 285 bp amplicons, the NCBI BLASTn engine shown about 99.5% sequence similarities between the sequenced samples and the intended reference target sequences, which partially cover the downstream portion of exon 2 and the upstream portion of intron 2 of this gene. By comparing the observed DNA sequences of these investigated samples with the retrieved DNA sequences (GenBank acc. NG_023447.1), the accurate positions and other details of the retrieved PCR fragments were identified (Fig. 1).

Appendix

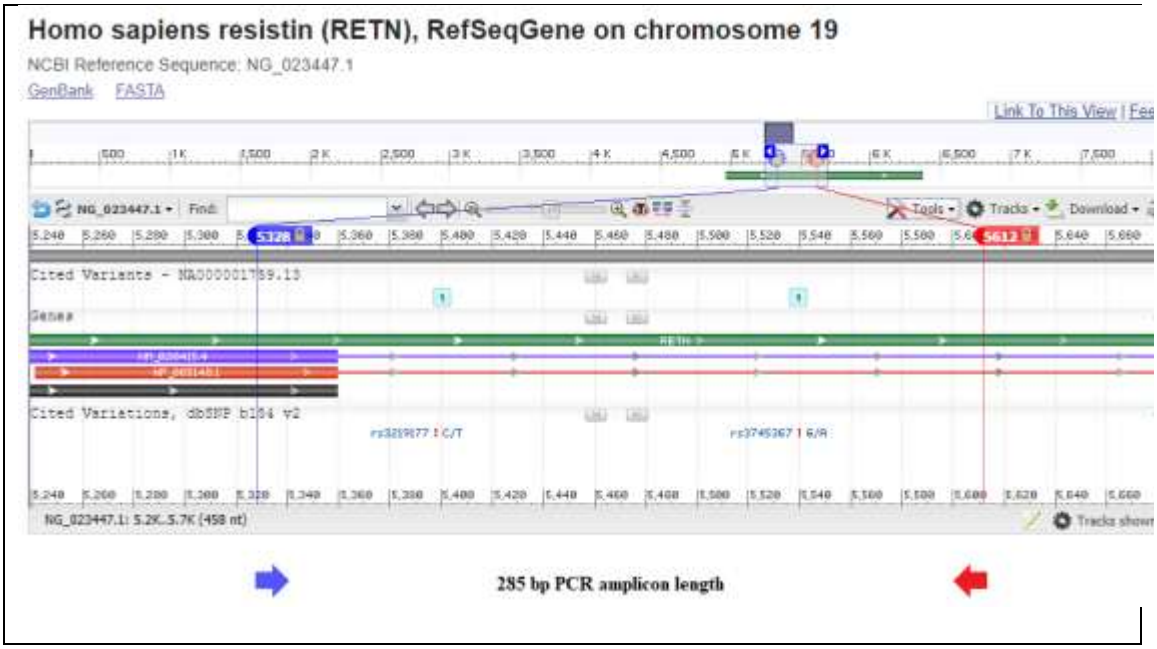


Fig. 1. The exact position of the retrieved 285 bp amplicons that partially covered a portion of the downstream portion of exon 2 and the upstream portion of intron 2 of the *RENT* gene within chromosome 19 (GenBank acc. no. NG_023447.1). The cyan arrow refers to the starting point of this amplicon while the red arrow refers to its endpoint.

After positioning the 285 bp amplicons' sequences within chromosome 19, the details of its sequences were highlighted, in terms of the positioning of both forward and reverse primers of the 285 bp amplified amplicons (Table 1).

Table 1. The position and length of the 285 bp PCR amplicons used to amplify a portion of the downstream portion of exon 2 and the upstream portion of intron 2 of the *RENT* gene located within chromosome 19 (GenBank acc. no. NG_023447.1). The gray-colored sequences referred to the position of the forward and reverse primers, respectively.

Amplicon	Reference locus sequences (5′ - 3′)	length
DNA sequences within the *RETN* gene	*TGAGAGGATCCAGGAGGTCGCCGGCTCCCTAAGTGAGGACCCCCCACTTGG GCAAGCTCCCCAAGGGTCTCAGAGACCTCACTGATCCCTGGCACAGACCTGA CTCCAACCCAGCCCCAGCGCTCACCAAATCTCATCCTCAAATCCAACCAGAT CATAAATTCAACCCCAACTCCACTCCCAACCCCTCCGACTGTCCCCACCTTA TCCACGGCTCCAAACCCAATCCCCGCTCTCACTCCAAACCTTCCCTTACTCC AAAACACCCAACTCAAGACAGGGTCC**	285 bp

* refers to the forward primer sequences (placed in a forward direction)
**refers to the reverse primer sequences (placed in a reverse complement direction)

The alignment results of the 285 bp samples revealed the presence of only one variation in some of the analyzed samples in comparison with the referring reference DNA sequences (Fig. 2). A highly interesting nucleic acid polymorphism (SNP) was

detected in this study in the investigated S3 and S4 samples, in which Guanine was replaced with Adenine at position 213, so that is called G213A.

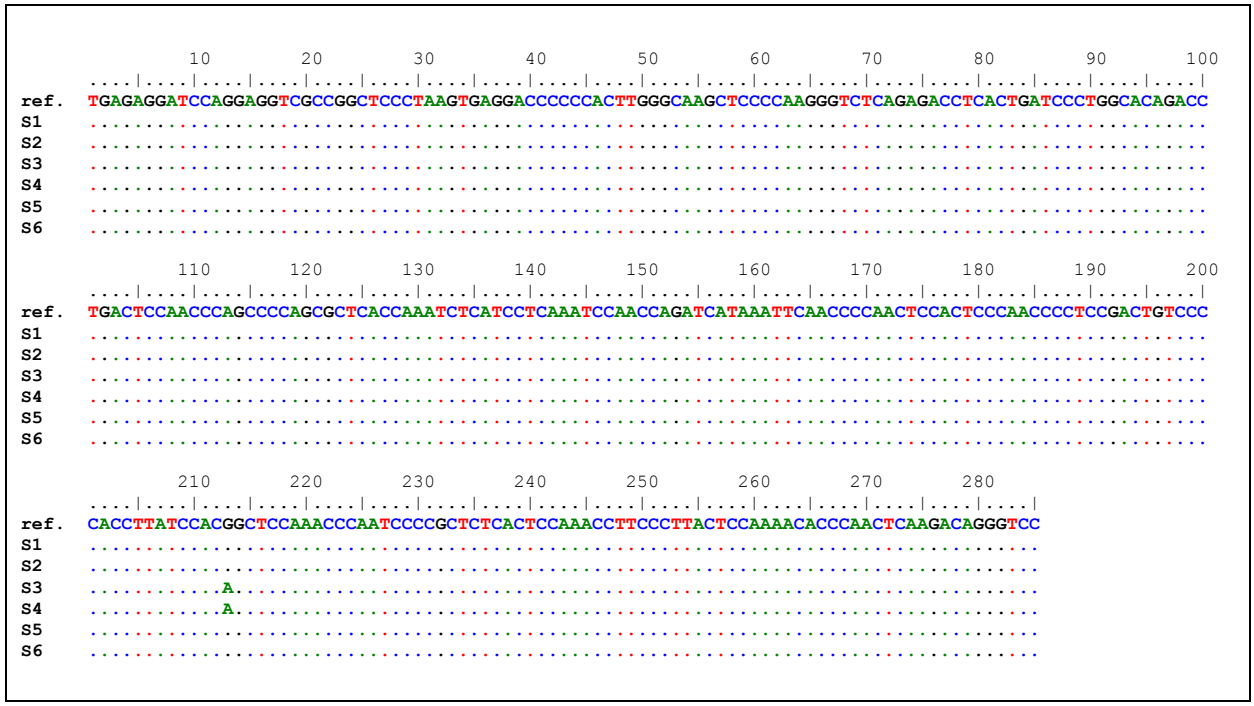


Fig. 2. DNA sequences alignment of 6 samples with their corresponding reference sequences of the 285 bp amplicons of the downstream portion of exon 2 and the upstream portion of intron 2 of the *RENT* gene. The symbol "ref." refers to the NCBI referring sequence, "S1-S6" refer to the samples 1 to 6, respectively.

The sequencing chromatogram of the identified variation, as well as its detailed annotations, were documented, and the chromatogram of this sequence was shown according to its position in the PCR amplicon. However, this SNP was detected in heterozygous G/A status in both S1 and S2, homozygous A/A status in both S3 and S4, and homozygous G/G status in both S5 and S6 (Fig. 3).

Fig. 3. The pattern of the detected G213A SNP within the DNA chromatogram of the targeted 285 bp amplicons of the downstream portion of exon 2 and the upstream portion of intron 2 of the *RENT* gene. The identified SNP was highlighted according to its position in the PCR amplicons. S1 / S2, S3 / S4, and S5 / S6 samples exhibited the G/A, A/A, and G/G states respectively in the highlighted locus.

Appendix

To elucidate the position of the targeted SNP concerning their deposited SNP database of the sequenced 285 bp fragment, the corresponding position of the *RENT* gene was retrieved from the dbSNP server (https://www.ncbi.nlm.nih.gov/projects/SNP/). To find out the nature of this SNP, a graphical representation was performed concerning the *RENT* dbSNP database within chromosome 19 (GenBank Acc. No. NC_000019.10). By reviewing the dbSNP engine, it was found that this detected SNP was found to be previously known as it was deposited as rs3745367 (Fig. 4). However, this SNP was located in intron 2 of the targeted *RENT* gene (https://www.ncbi.nlm.nih.gov/snp/rs3745367). Due to the high frequency of the deposited rs3745367 SNP in the dbSNP database (0.37 to 0.39), it was reported in many publications regarding the potential effect of the *RENT*-associated metabolic issues (https://www.ncbi.nlm.nih.gov/snp/rs3745367#publications).

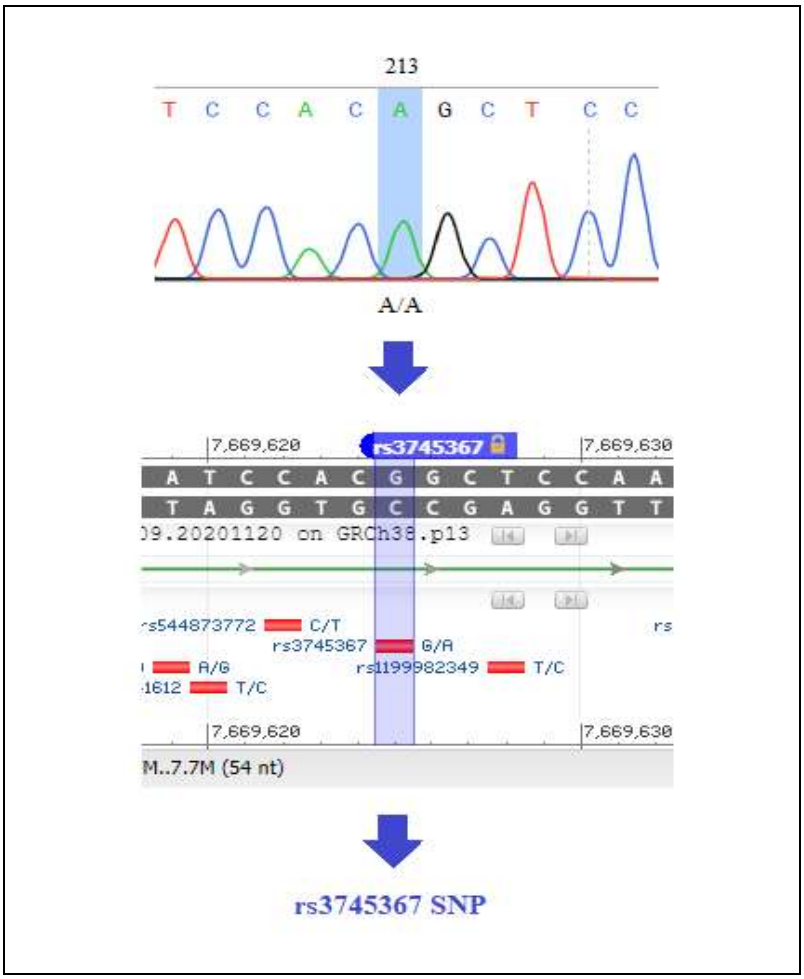


Fig. 4. The SNP's novelty checking of *RENT* genetic single nucleotides polymorphisms using the dbSNP server. The identified G213A SNP was marked with a blue color. The GenBank acc. no. NC_000019.10 was used in the positioning of the highlighted substitution SNP. The position of the targeted sequences was found in the positive strand.

Appendix

To summarize the results obtained from the sequenced 285 bp fragments, the detailed position of the observed variation was described in the NCBI reference sequences (Table 2).

Table 2. The pattern of the observed SNP in the 285 bp amplicons designed to amplify a portion of the downstream portion of exon 2 and the upstream portion of intron 2 within the *RENT* gene in comparison with the NCBI referring sequences (GenBank acc. no. NC_000017.11). The symbol "S" refers to the sample number.

Sample No.	Zygosity status	Position in the PCR fragment	Position in the reference genome	SNP type	Variant summary
S1, S2	Heterozygous (G/A)	213	7669625	Intronic variant	rs3745367
S3, S4	Homozygous (A/A)	213	7669625	Intronic variant	rs3745367
S5, S6	Homozygous (G/G)	213	7669625	Intronic variant	rs3745367

2. SEQUENCING REPORT OF *SF3B1*-GENE BASED PCR AMPLICONS

SEQUENCING RESULTS

In the present study, the Splicing factor 3B subunit 1 (*SF3B1*) gene polymorphism was investigated. This gene encodes for Splicing factor 3B subunit 1 which is involved in pre-mRNA splicing as a component of the splicing factor SF3B complex (https://www.uniprot.org/uniprot/O75533). The sequencing reactions indicated the exact identity of this genetic fragment after performing NCBI blastn (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Concerning the 223 bp amplicons, the NCBI BLASTn engine shown about 99.5% sequence similarities between the sequenced samples and the intended reference target sequences, which completely cover the entire exon 13, and its upstream and downstream intronic portions of the *SF3B1* gene. By comparing the observed DNA sequences of these investigated samples with the retrieved DNA sequences (GenBank acc. NG_032903.2), the accurate positions and other details of the retrieved PCR fragments were determined (Fig. 1).

Appendix

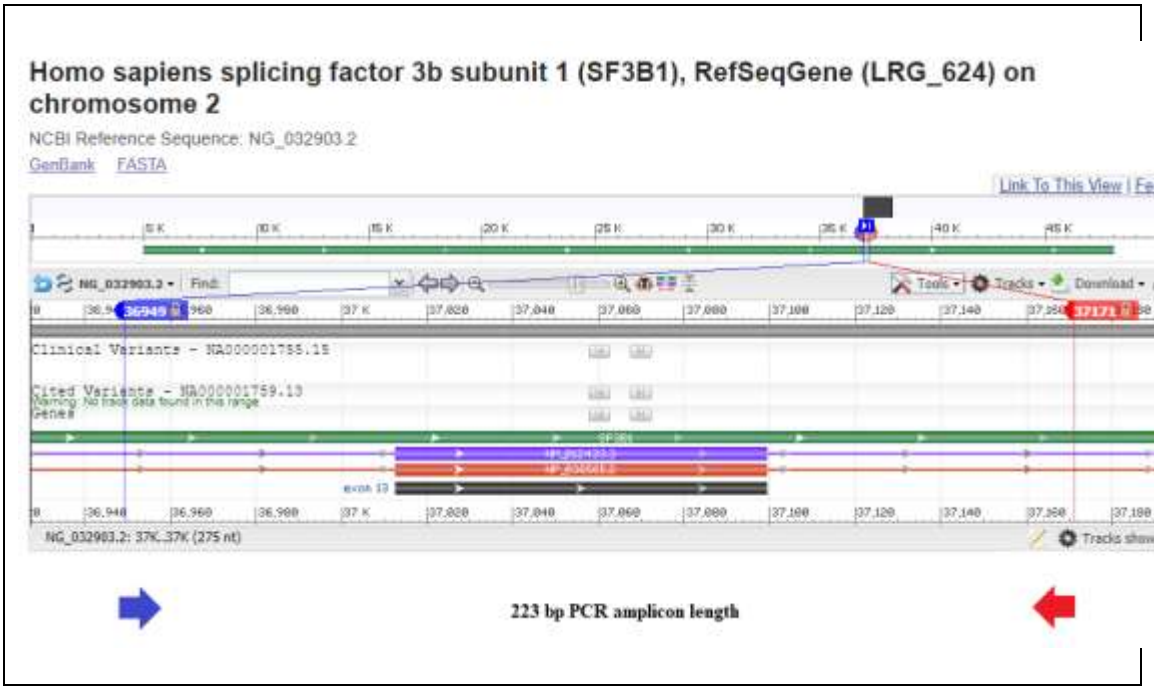


Fig. 1. The exact position of the retrieved 223 bp amplicon that entirely covers the exon 13 of the *SF3B1* gene within chromosome 2 (GenBank acc. no. NG_032903.2). The cyan arrow refers to the starting point of this amplicon while the red arrow refers to its endpoint.

After positioning the 223 bp amplicons' sequences within chromosome 2, the details of its sequences were highlighted, in terms of the positioning of both forward and reverse primers of the 223 bp amplified amplicons (Table 1).

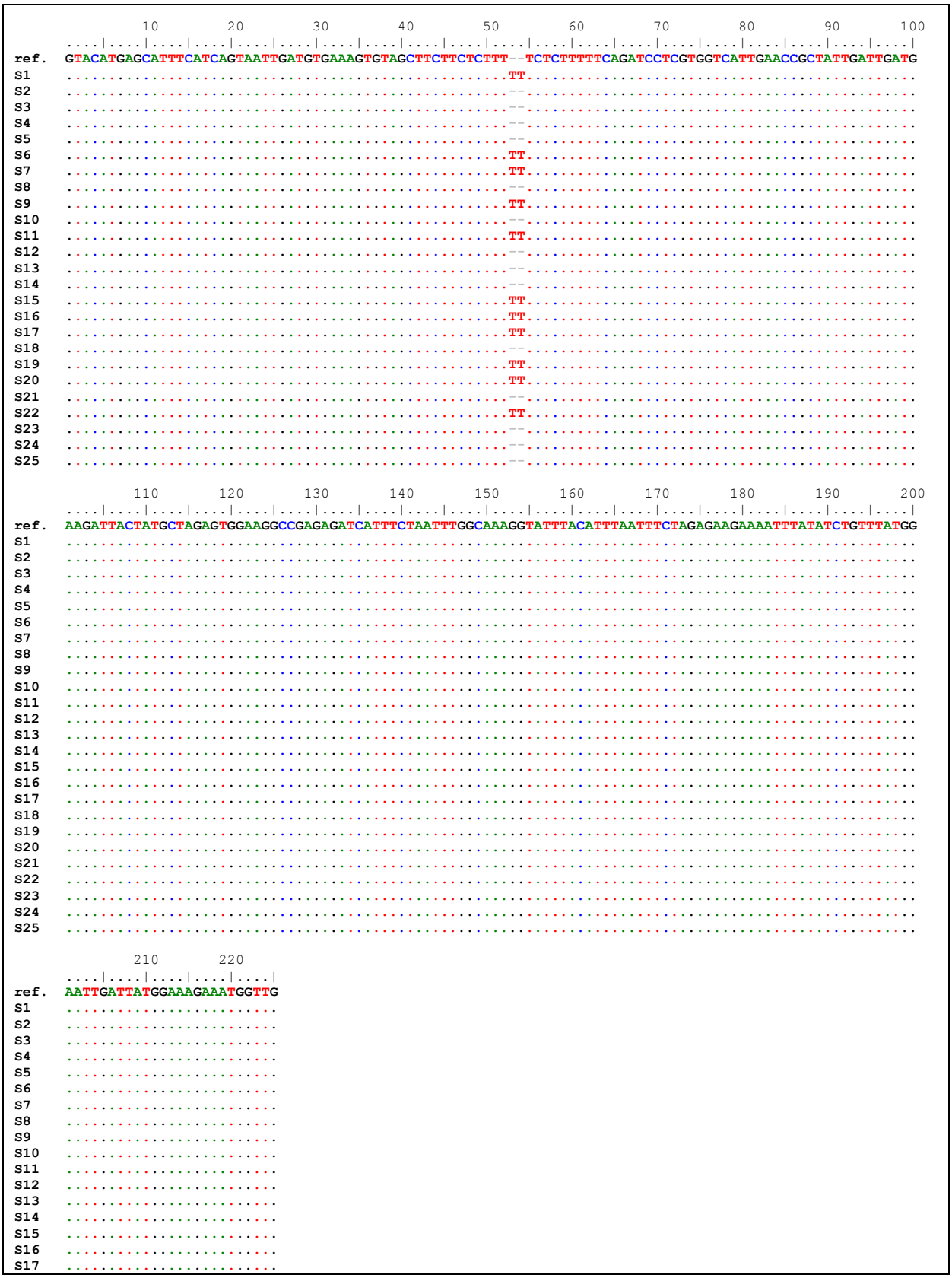
Table 1. The position and length of the 223 bp PCR amplicons that are used to entirely amplify the exon 13, and the upstream and downstream intronic portions of the *SF3B1* gene located within chromosome 2 (GenBank acc. no. NG_032903.2). The gray-colored sequences referred to the position of the forward and reverse primers, respectively.

Amplicon	Reference locus sequences (5′ - 3′)	length
DNA sequences within the exon 13 of the *SF3B1* gene	*GTACATGAGCATTTCATCAGTAATTGATGTGAAAGTGTAGCTTCTTCTCTT TTCTCTTTTTCAGATCCTCGTGGTCATTGAACCGCTATTGATTGATGAAGAT TACTATGCTAGAGTGGAAGGCCGAGAGATCATTTCTAATTTGGCAAAGGTAT TTACATTTAATTTCTAGAGAAGAAAATTTATATCTGTTTATGGAATTGATTA TGGAAAGAAATGGTTG**	223 bp

* Refers to the forward primer sequences (placed in a forward direction)
**Refers to the reverse primer (sequences (placed in a reverse complement direction)

Appendix

The alignment results of the 223 bp samples revealed the presence of only one variant in some of the analyzed samples in comparison with the referring reference DNA sequences (Fig. 2).



Appendix

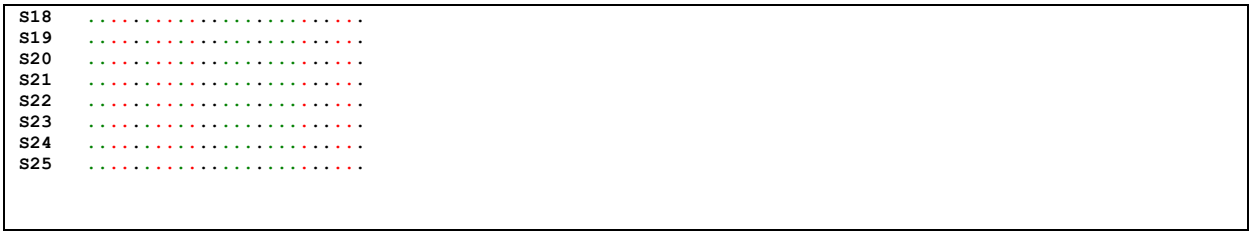


Fig. 2. DNA sequences alignment of 25 samples with their corresponding reference sequences of the 223 bp amplicons of the *SF3B1* gene. The symbol "ref." refers to the NCBI referring sequence, "S1-S25" refer to the samples 1 to 25, respectively.

A highly interesting nucleic acid polymorphism (SNP) was detected in this study, in which a dinucleotide of thymine was inserted between positions 52 and 53, (TT ins 52-53) of the amplified 223 bp *SF3B1* fragments. The sequencing chromatogram of the identified variation region, as well as its detailed annotations, were documented, and the chromatogram of this sequence was shown according to its position in the PCR amplicon. By viewing all chromatograms, it was found eleven samples exhibited this insertion out of 30 investigated samples (Fig. 3).

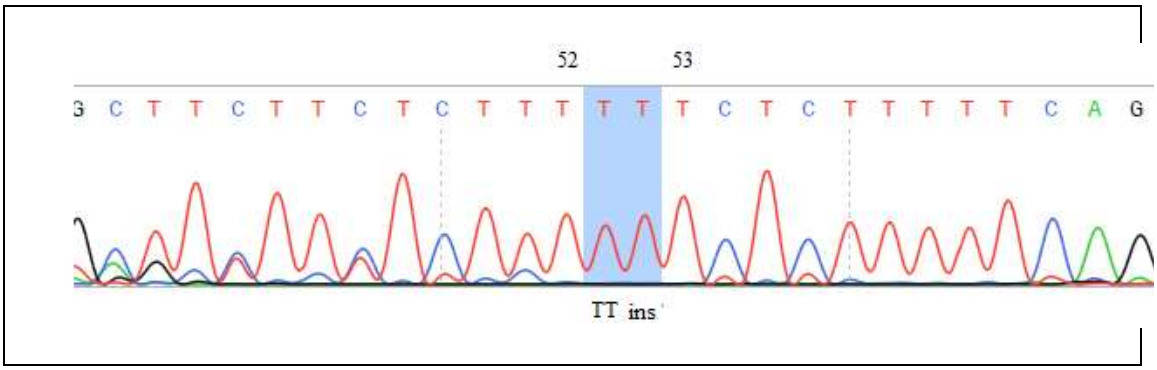


Fig. 3. The pattern of the detected SNPs within the DNA chromatogram of the targeted 223 bp amplicons of the entire exon 13 of the *SF3B1* gene. The identified insertion SNP was highlighted according to its position in the PCR amplicons. The symbol "ins" refers to "insertion mutation"

To elucidate the position of the observed SNP concerning its deposited SNP database of the sequenced 223 bp fragment, the corresponding position of the *SF3B1* gene was retrieved from the dbSNP server (https://www.ncbi.nlm.nih.gov/projects/SNP/). To find out the nature of this SNP, a graphical representation was performed concerning the *SF3B1* dbSNP database within chromosome 2 (GenBank Acc. No. NC_000002.12). By reviewing the dbSNP engine, it was found that this detected SNP was found to be previously known as rs3217350, but our SNP has only two TT insertion (Fig. 4). However, this SNP was located in intron 11 of the targeted *SF3B1* gene (https://www.ncbi.nlm.nih.gov/snp/rs3217350). Though this SNP was not reported in the literature, our study reported an obvious occurrence since it accounted for 37% for all investigated samples.

Appendix

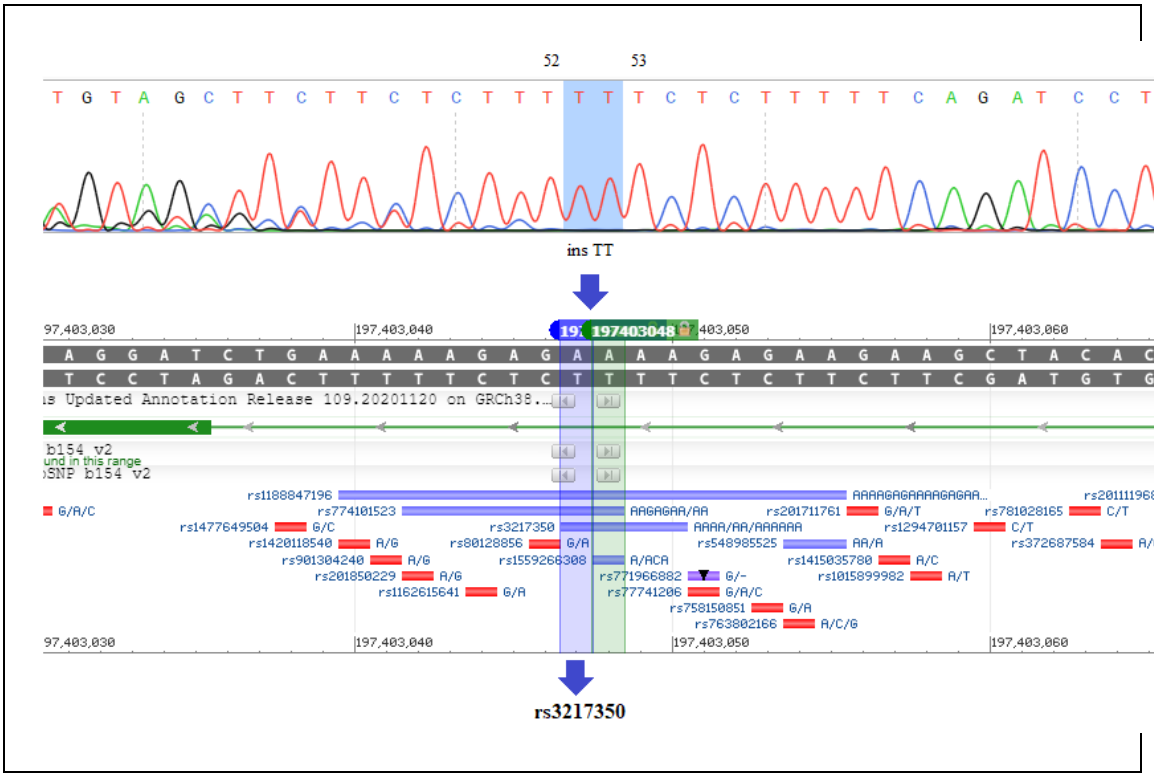


Fig. 4. The SNP's novelty checking of *SF3B1* genetic single nucleotides polymorphisms using the dbSNP server. The identified rs3217350 SNP was marked with blue color. The GenBank acc. no. NC_000002.12 was used in the positioning of the highlighted insertion SNP.

To summarize the results obtained from the sequenced 223 bp fragments, the exact position of the rs3217350-based variation was described in the NCBI reference sequences (Table 2).

Table 2. The pattern of the observed SNP in the 223 bp amplicons designed to amplify the entire exon 13 within the *SF3B1* gene in comparison with the NCBI referring sequences (GenBank acc. no. NC_000002.12). The symbol "S" refers to the sample number.

Sample No.	Position in the PCR fragment	Position in the reference genome	Variant summary
S1, S6, S7, S9, S11, S15, S17, S19, S20, S22	52-53	1974030487-197403048	rs3217350

الخلاصة

متلازمة اعتلال التنسج النقوي (MDS) هي مجموعة متغايرة من الاضطرابات التي تحصل في جينات النسيج المكون للدم و تتميز بمختلف الاعراض السريرية ومخاطر تحول الى سرطان الدم وكذلك قلة فرصة البقاء على قيد الحياة. وهذا يجعل التشخيص والتصنيف الدقيق لمرضى متلازمة اعتلال التنسّج النقوي وثيق الصلة سريريًا

أجريت الدراسة الحالية في مختبر DNA في قسم علوم الحياة كلية العلوم ، جامعة بابل. وتم جمع العينات واكمال الجانب العملي لهذه الدراسة من خلال الفترة الممتدة بين حزيران 2019 إلى شهر تشرين الثاني 2020. العينات التي تم جمعها كانت من مرضى متلازمة اعتلال التنسج النقوي و من مناطق مختلفة من العراق.

تم جمع العينات من الإناث والذكور ضمن اعداد عمرية ممتدة من (20–83)، وكانت عدد العينات (75) عينة دم خلال هذه الدراسة وكانت على النحو التالي: مجموعة السيطرة (25) عينة دم (15 ذكور و 10 إناث) ، مجموعة مرضى تضمنت (50) عينة دم (29 ذكور و 21 إناث).

قسمت الدراسة الحالية إلى أربعة أجزاء الدراسة الاولى (ديموغرافي) اما الثانية تضمنت قياس معايير الدم ، و الثالثة كانت الدراسة الكيموحيوية ، والجزء الاخير تضمن الدراسة الوراثية . يتضمن الجزء الأول العلاقة بين العمر و الجنس ، و مؤشر كتلة الجسم مع المرض .و يتضمن الجزء الثاني تقدير معايير الدم . بينما شمل الجزء الثالث تحديد هرمونات الأديبونكتين ، واللبتين ، والرزستين ، والحديد ، وأنواع الأكسجين التفاعلية من الإجهاد التأكسدي و مضادات الاكسدة .اما الجزء الرابع يدرس التأثيرات الجينية للجينات (*Adiponectin*، *LEP*، *RETN*، *SF3B1*، *SRSF2*) مع المرض.

اوضحت نتائج الدراسة الحالية أن النسبة المئوية للذكور المصابين بمتلازمة اعتلال التنسج النقوي كانت أعلى من قيمة النسبة المئوية للإناث (58٪ و 42٪) على التوالي. كذلك اشارت النتائج الى انه خطر حدوث المرض يبدأ من 60 عامًا ويزداد بين 60–75 عامًا لكلا الجنسين ،اما بالنسبة لمؤشر كتلة الجسم فقد بينت النتائج انخفاضا معنويا لدى مرضى

متلازمة اعتلال التنسّج النقوي بالمقارنة مع مجموعة السيطرة.

اظهرت النتائج قياسات معايير الدم انخفاضا معنويا ($P \leq 0.05$)، في اغلب المعايير الدموية ما عدا متوسط تركيز الهيموجلوبين الذي ارتفع معنويا بالمقارنة مع مجموعة السيطرة و متوسط حجم الكريات و متوسط تركيز الهيموجلوبين للكريات و خلايا القعدة لم تظهر اختلافات معنوية بالمقارنة مع مجموعة السيطرة. بينما أظهرت الدراسة الكيموحيوية ارتفاع معنوي P) ($0.05 \geq$في تركيز الأديبونكتين و الرزستين ، بينما أظهر هرمون اللبتين عدم وجود فروق معنوية بين المرضى و الاصحاء . اما مستوى الحديد لوحظ زيادة معنوية ($P \leq 0.05$) في المرضى الذين يعانون من اعتلال نقي العظم .

أظهرت نتائج الاجهاد التأكسدي ارتفاعا ملحوظ ($P \leq 0.05$) في المرضى الذين يعانون من اعتلال نقي العظم مقارنة مع مجموعة الاصحاء. في حين اظهرت نشاط مضادات الاكسدة (SOD, GPx)، ارتفاع ملحوظ ($P \leq 0.05$) بين المرضى مجموعة الاصحاء ، في حين ظهر نشاط الكلوتاثيون المختزل GSH بشكل منخفض معنويا ($P \leq 0.05$). كذلك لوحظ وجود علاقة سلبية بين الأديبونكتين و الرزستين مع مؤشر كتلة الجسم BMI و الهيموغلوبين Hb و الصفيحات الدموية platelets في حين لوحظ وجود علاقة ايجابية مع الحديد أيضا ، و كان هناك علاقة سلبية ($r = -247$) بين اللبتين و الرزستين اما الأديبونكتين علاقة ايجابية ($r = 624$) مع الرزستين.

الدراسة الوراثية، اظهر جين الاديبونكتين نسبة عالية لتعدد الاشكال الجيني حيث ظهرت نسبة الاليلات GG G) (rs1501299mutant genotype GG and alleles G) 73% , 54%) في مرضى اعتلال نقي العظم بالمقارنة مع مجموعة السيطرة (62%, 36%) و هذا مرتبط بالاختلافات على مستوى ($p \leq 0.05$)، بينما جين ليبتن هناك ى علاقة واضحة ما بين جين اللبتين و مرض اعتلال نقي العظم حيث ظهر الشكل او النمط الجيني GG و الاليل المطفر G بنسبة عالية (70% ,59.1%) ما بين المرضى و يذلك يمكن القول ان جين اللبتين عامل مسبب للمرض او حدوث المتلازمة. كما لوحظ ان جين Resistin لا يوجد فيه اختلاف معنوي بين المرضى الذين يعانون من متلازمة اعتلال التنسج النخاعي بالمقارنة لمجموعة

السيطرة باستخدام تقنيات تفاعل البلملرة المتسلسل (PCR– SSCP) و التي بينت حدوث طفرة في موقع الانترون الثاني.

نتائج تحديد تسلسل (Sequencing) لجين *SF3B1* اظهر وجود طفرة (rs3217350) في الموقع غير المشفر الثاني و بالنسبة لنتائج تعدد الانماط الجينية و تكرار الاليلات حيث اظهر النمط الجيني (II) و الاليل (I) نسبة عالية بين المرضى (92% ,92.5%) بالمقارنة مع مجموعة السيطرة ,(60%), و من جهة اخرى فقد لوحظ زيادة تركيز الحديد في مصل المرضى ذات الشكل الجيني II (1003.41ng/ml) بالمقارنة مع النمط الجيني الاصلي DD (355.04 ng/ml) على مستوى الاختلاف الاحصائي (P=0.0019)

اما نتائج التعبير الجيني لجين *SRSF2* فقد بينت وجود تباين معنوي كبير بين المرضى و الاصحاء من خلال منحني الانصهار و التضخيم عن طريق تفاعل البوليمراز المتسلسل بالزمن الحقيقي Real–Time polymerase chain reaction .

وزارة التعليم العالي و البحث العلمي

جامعة بابل

كلية العلوم

قسم علوم الحياة

دراسة وراثية و كيموحيوية لمرضى متلازمة اعتلال التنسج النقوي في العراق

اطروحة

مقدمة الى مجلس كلية العلوم- جامعة بابل

و هي جزء من متطلبات نيل درجة الدكتوراه فلسفة في العلوم/ علوم الحياة / حيوان

من قبل

ود عبدالخالق عبدزيد صخيل

بكالوريوس علوم الحياة/ جامعة بابل /٢٠١٢

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