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Metylation of Promoter and Gene Expression of CDKN2B Gene in Iraqi KIDS suffered with Leukemia

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Abstract:

Leukemia is a malignant hematologic disorder characterized by abnormal white blood cell differentiation leading to increased rates of cancer cells in the bone marrow and peripheral blood. It is a common malignancy that occurs in children and adults when changes occur in the regulatory processes of normal cells and cause uncontrolled proliferation of hematopoietic stem cells. in the bone marrow.The CDKN2B gene, which is located in chromosome 9P21, is a tumor suppressor gene that plays an important role in the cell cycle through its inhibition of cyclin complexes and cyclin dependent kinase 4/6, and the inactivation of this gene is due to mutations or by a process of methylation for promoter of gene leads to the occurrence of the disease.This study aims to determine the relationship between the genetic variation of the CDKN2B gene and the incidence of leukemia among children in the city of Mosul.This study included (71) children between the ages of (1-15) years old who were referred to Ibn Al-Atheer Hospital in the city of Mosul for a period ranging from September to October of 2022, and the samples were divided into two groups. The first included this category 20 children and counted As a control group, the second group included 71 children from among the children with leukemia. (5.0) ml of venous blood was drawn from the children and divided into three parts. The first part was placed in tubes containing EDTA anticoagulant, which is used for DNA extraction, and the second part was placed in Eppendorf tubes containing On trisol for the purpose of extracting the RNA, and the last part was placed in two gel tubes free of any anticoagulant substance to obtain blood serum. Determining the genetic variation of the CDKN2B gene using by analyzing the gene expression level of the CDKN2B gene based on the RT-PCR technique. also measured DNA methylation in the promoter of gene CDKN2B. In addition, the level of the cell cycle-regulating protein CDKN2B was measured based on ELISA technology. The results of the test for the DNA methylation process in the promoter of the CDKN2B gene showed that methylated DNA was present in all samples of the patients on whom the test was conducted at a rate of 100% compared to the control group in which there was no methylation of the studied gene primer, while the results showed the presence of bundles belonging to the unmethylated process in all samples Patients with healthy people by 100%, and if it is noticed that there is a decrease in the level of gene expression for patients compared to the control group, , as it was found A significant decrease in the level of CDKN2B protein at the level of probability $p \leq 0.05$, the ratio in the control group was 40.1 ± 2.34 compared to the patients group 28.5 ± 1.24.We conclude from this study that genetic variations and mutations and an increase in methylation led to a decrease in the level of gene expression of CDKN2B gene with A decrease in the level of the encoded protein, which is one of the main causes of the development of leukemia.

Keywords: CDKN2B gene, Mutation, T-ARMS-PCR, Gene expression, DNA methylation

Introduction:

Leukemia is a common malignant illness of the hematological system that is brought on by unbalanced hematopoietic cell growth and death [1]. Leukemia progresses and develops in a complicated way. Leukemia may be categorized into the four most frequent forms based on the rate of disease progression and the types of white blood cells affected: acute myeloid leukemia (AML), chronic myeloid leukemia (CML), acute lymphocytic leukemia (ALL), and chronic lymphocytic leukemia (CLL) [2]. Although great attempts have been made to pinpoint the causes of leukemia [3], the pathophysiology of the disease is still not entirely understood [4]. Leukemia has been linked to environmental variables such radiation exposure, electrical work, and excessive benzene exposure [5]. Meanwhile, it is recognized that leukemia is linked to an accumulation of errors in a variety of cancer genes.Leukemia development is connected to this Meanwhile, it is recognized that leukemia is linked to an accumulation of errors in a variety of cancer genes[6].

The tumor suppressor gene CDKN2B (cyclin-dependent kinase inhibitor 2B) is located on chromosome 9p21 (inhibitor of CDK4) and codes for the CDKN2B (INK4B) protein, which is a member of the INK family. The cell cycle is controlled by the cyclin-dependent kinase CDK4 enzyme[7]. CDKN2B is largely activated in response to TGF-, and its suppression is presumably specifically advantageous for the development of tumor cells[8]. Due to the fact that TGF- primarily activates CDKN2B, it is likely that its inhibition is particularly helpful for the growth of tumor cell [9].In the absence of CDKN2B, pRb is phosphorylated by CDK4 and CDK6 in a manner that inhibits its interaction with E2F and encourages cell proliferation. When CDKN2B is overexpressed, the cell cycle is arrested in the G1 phase and CDK4 is redistributed from cyclin D-CDK4 complexes to CDK4-CDKN2B complexes, which causes unbound cyclin D to be degraded via the ubiquitin-dependent proteasome degradation pathway[10].

The complicated phenomena of epigenetics' molecular base includes the activation or suppression of certain genes without changing the DNA's basic genetic sequence[11]. Differentiated cells can have their own unique shape and function thanks to the machinery involved in these alterations[12].While most epigenetic modifications only happen during mitosis, a mechanism known as transgenerational epigenetic inheritance [13]. allows for some alterations to be passed on to the organism's progeny, Specific epigenetic mechanisms include chromosome X inactivation, Para mutation, imprinting, and bookmarking. [14,15].

Numerous genetic and epigenetic changes were shown to have a significant impact in the development of leukemia. Previous research has linked abnormal DNA methylation to leukemogenesis [16]. an ordinary epigenetic alteration , Aberrant tmethylation, a common epigenetic alteration, was seen in lymphoid/hematopoietic malignancies, includingAML CML, ALL ,and CLL[17] .Advances in science suggest that DNA methylation is an epigenetic covalent modification that only occurs within CpG dinucleotides (CpG islands) in the eukaryotic genome, particularly in humans. It involves the addition of a methyl group (CH3) at position 5 of the cytosine base by the activity of DNA methyltransferase (DNMT) enzymes[18,19]. Recently, specialists have connected aberrant DNA methylation to a variety of negative effects, including cancer[20]. DNA methylation is a major epigenetic alteration that affects biological processes such as cell development and gene expression without changing DNA sequencing[21]. Epigenetic factors and enzymes control how lymphoid and myeloid lineages differentiate as well as how many distinct cell types are produced[22]. Hematological cancers are the result of mutation or dysregulation in the end Researchers claim that the DNA methylation profile signature associated with particular promoter genes varies from other cancer types associated with the same gene[23].+

The aim of this study to detect the correlation between DNA methylation and Gene expression of CDKN2B in child with leukemia in Mosul city.

Case Study:

71 kids between the ages of 1 and 15 were examined between August and October 2022 by private pathological investigation facilities in Mosul. These clinical cases of the condition served as the basis for selecting the Samples. The samples were developed in two groups. components selected based on biochemical findings: Twenty persons who were among those without any health difficulties made up the first group, which functioned as the control group. The second group, which consists of 71 kids with leukemia was determined by biochemical results.

Collection of Blood sample

5 ml of venous blood was drawn from these patients and divided into two groups; the first component was put into tubes containing EDTA anticoagulant to extract DNA, and the second group

was put into tubes devoid of any anticoagulant mixed with trizol for RNA extraction. The blood was allowed to coagulate in the tubes for an hour before the blood was centrifuged for ten minutes at a speed of three thousand cycles per minute to extract the blood serum for the biochemical testing.

Analysis of the gene expression level of CDKN2B gene based on the technique the RT-PCR:

The process of quantifying the level of gene expression of the CDKN2B gene includes several stages:

RNA extraction:

Immediately after the process of drawing blood from the samples included in the study, 250 µl of the blood sample was mixed with 750 µl of Trizol After that, the mRNA was extracted, depending on the analysis kit prepared by Trans.

Measurement of RNA concentration and purity:

The purity of the extracted RNA is evaluated using Biodrop device,

The process of converting the extracted mRNA molecule into cDNA molecule:

After the completion of the process of extracting the mRNA, it was converted into a molecule of cDNA, depending on the effectiveness of the reverse transcriptase enzyme, using the analysis kit prepared by Trans Company, according to protocol of kit :

RT-PCR reaction:

To conduct a quantitative test for the level of gene expression, the special primers of the gene were used with the primers of the housekeeping genes, as proven in the table below.

Table (1) shows the primers used for the genes under study in the RT-PCR technique

Primer	Sequence
CDKN2B -RT-F	5′-AACGGAGTCAACCGTTTCGG-3′
CDKN2B -RT-R	5′-TGTGCGCAGGTACCCTGCAA-3′
housekeeping-S9-F	5′-GATGAGAAGGACCCACGGCGTCTGTTCG-3′
housekeeping-S9-R	5′-GAGACAATCCAGCAGCCCAGGAGGGACA-3′

The final reaction volume was 20 µl (Housekeeping gene:ribosomal protein S9(RPS9)

Table (2) shows the final reaction volume components in the RT-PCR reaction:

T	Components	Volume
1	cDNA	5 µl
2	S9-F_primar	1 µl
3	S9-R_primar	1 µl
4	Green q PCR super Mix	10 µl
5	D.W	3 µl
Total volum 20 µl		

Table (3): The program shown in the table below was adopted to perform the RT-PCR reaction:

No.	Stage	Temperature	Time	Cycle number

1.	Initial denaturation	95	5 min.	1
2.	Denaturation	95	1 min.	35
3.	Annealing	55	1 min.	
4.	Extension	72	1 min.	
5.	Final extension	72	5 min.	1
6.	Stop reaction	4	5 min	1

How to calculate gene expression rate:

The gene expression rate of the CDKN2B gene is calculated based on the CT value of the target gene with the housekeeping gene for both patient and control samples using the following laws:

ΔCT (test)= CT (target, test) – CT (ref, test).

ΔCT (control)=CT (target, control) -CT (ref, control).

CT (target, test) indicates the mRNA transcripts of the CDKN2B gene from patient samples

CT (ref, test) indicates the mRNA transcripts of the housekeeping gene from patient samples

CT (target, control) indicates the mRNA transcripts of the CDKN2B gene in control samples

CT (ref, control) indicates the mRNA transcripts of the housekeeping gene of control samples

Equate the ΔCT of the treated sample with respect to the ΔCT of the control sample using the following law:

ΔΔCT =ΔCT(test) -ΔCT(control).

Measurement of DNA methylation in the primer of the CDKN2B gene:

CDKN2B methylation by methylation specific polymerase chain reaction (MSP)

The DNA methylation test was carried out according to the method presented by the researcher As described in the following steps:

DNA extraction:

Treating the DNA with Bisulfite Modification Bisulfite. Sodium bisulfite was used to treat DNA samples for 24 hours using a QIAGEN assay kit following the manufacturer's protocol whose primary function is to convert all unmethylated cytosine into uracil, While all methylated cytosine was retained. The bisulfite-treated DNA was re-extracted before PCR assay,

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Perform a methylation test using the technique **PCR Methylation-specific PCR (MSP):**

Methylation assay analysis was carried out using two sets of primers as shown in Table (3-1) based on the PCR technique of the initiator region of the CDKN2B gene of sequence bp1241 - bp1340 in the pre-cloning region (TSS) containing four CPG sites (مصدر)

Table (4) shows: shows the primers used in the DNA methylation reaction

Primer	Sequence
CDKN2B -unmeth-F	5′-TGTGATGTGTTTGTATTTTGTGGTT-3′
CDKN2B -unmeth-R	5′-CCATACAATAACCAAACAACCAA-3′
CDKN2B -meth-F	5′-CGTTCGTATTTTGCGGTT-3′
CDKN2B -meth-R	5′-CGTACAATAACCGAACGACCGA-3′

Table (5): shows the size of the PCR reaction used in the DNA methylation process

T	Components	C
1	DNA with bisulfat	5 µl
2	F_primer	1 µl
3	R_primar	1 µl
4	2x super-hot PCR master mix	10 µl
5	D.W	3 µl
Total volum 20 µl		

Table (6): shows the PCR reaction program for DNA methylation

No.	Stage	Temperature	Time	Cycle number
1.	Initial denaturation	95	5 min	1
2.	Denaturation	95	30 sec.	35
3.	Annealing	61	30 sec.	
4.	Extension	72	30 sec.	
5.	Final extension	72	5 min	1
6.	Stop reaction	4	5 min	1

After that, the bundles of methylation are separated using an agarose gel at a concentration of 3%, and then photographed with the Gel documentation.

Estimation of biochemical indicators:

The biochemical indicators were measured the level of the cell cycle-regulating protein CDKN2B was measured based on ELISA technology.

Result and discussion

Measuring the gene expression level of the CDKN2B gene:

Table (7): Results of a test to determine the level of gene expression based on the RT-PCR technique

CT-target CONTROL	CT-target PATIENTS	CT-housekeeping CONTROL	CT-housekeeping PATIENTS	Δ CT-target	Δ CT-control	Gene expression

34.7	35	20.5	19.5	15.4	14.5	0.66

The above table shows the values of CT and the level of gene expression for theCDKN2B gene and the housekeeping gene for patients with leukemia with the control group. If there is a decrease in the level of gene expression for patients compared with the control group, this is due to several reasons, including the genetic mutations in addition to the variations in CDKN2B genes, in addition to the different methylation patterns in the Promoter gene, and thus lead to the manufacture of a weak protein that is unable to perform the biological function, or its The quantity might not be adequate to satisfy the cells' demands.

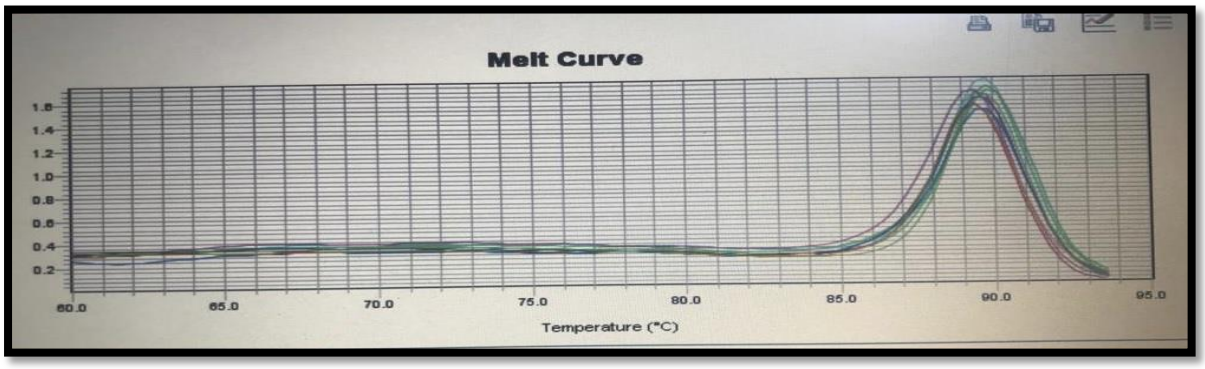

Figure (1): shows the standard curve of the studied genes in the RT-PCR technique.

The results of the study showed that a decrease in the level of gene expression of the CDKN2B gene in leukemia patients compared to the level of gene expression in control samples. As the rate of gene expression for the control group was Δ ΔCT = 1.0, while for the patient group, the rate of gene expression was low with an amount of Δ ΔCT = 0.66

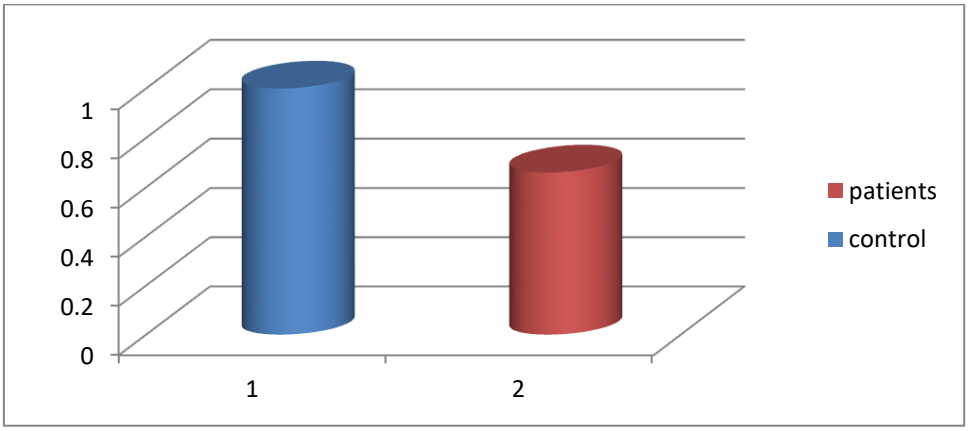

Figure (2): shows the level of CDKN2B gene expression in leukemia patients and control group

From these results, it is clear to us the vital role of the CDKN2B gene in the manufacture of the CDKN2B protein in the control group. As for leukemia patients, the marked decrease in the rate of gene expression is considered one of the main reasons for the development of the pathological condition, due to the lack of CDKN2B protein manufactured by the CDKN2B gene, and this decrease in the level of gene expression Either it is due to the methylation process in the CDKN2B gene promoter, and a relationship has been identified between the level of CDKN2B mRNA gene

expression with the methylation status as a result of this decrease, or due to genetic mutations in the region indicating the studied gene.

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Since the CDKN2B gene is one of the CDK inhibitors that loses its expression in a very high percentage of patients with acute leukemia and that this gene in its normal state encodes the CDKN2B protein, which belongs to the INK4 protein family, it is from the family of proteins that play a vital role, In cell cycle regulation, it is known that cell cycle regulator molecules (24)Therefore, these variations that have been determined in the study samples directly contribute to the loss of the biological function of the CDKN2B gene. Therefore, the continuous self-renewal of these cells results from the abnormal cell cycle, and this is due to the large deviations of the cell cycle regulators in their expression patterns in different types of cancers, including malignant hematomas. In the normal situation, cell cycle regulation is highly dependent on the presence of adequate amounts of CDKIs as well as their function in the nucleus. Various studies have shown that any variation in CDKI plays an essential role in cancer and is an early event during tumorigenesis., the CDKN2B gene has been repeatedly altered, and this in turn leads to disruption of the RB / p16 pathway by genetic changes in the CDKN2B gene, and this illustrates the important role of this gene in the pathways of leukemia cells , And that the inhibition of the CDKN2B gene is achieved by 5% hypermethylation of the CpG islands in exone 1 of the gene in many tumors.(25)

Result of DNA methylation test for the primer of the CDKN2B gene:

The results of the test for the DNA methylation process in the primer of the CDKN2B gene showed that methylated DNA was present in all samples of the patients on whom the test was conducted at a rate of 100% compared to the control group in which there was no methylation of the studied gene primer, while the results showed the presence of bundles belonging to the unmethylated process in all samples Patients with healthy and by 100%, the difference in the methylation pattern of the CDKN2B gene was determined within the samples included in the study, using special primers for both cases of methylation and its absence, as shown in the figure (3-1) :

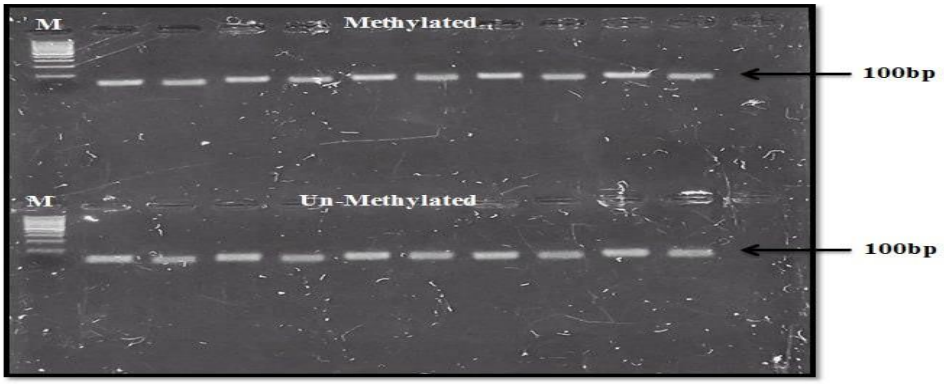

Figure (3): shows the product of the PCR reaction of the DNA-methylated reaction. The size of the reaction product was 100 bp, carried over with a 2% agarose gel.

The promoter of CDKN2B gene was found to be methylated in patient samples; in patients, the methylation rate for the gene was 100%, which is high and detrimental to the gene's biological activity as well as for earlier research. It is important to note that various genetic variables, including epigenetic alterations, primarily DNA methylation in gene promoters, contribute to the severity of the pathogenesis in leukemia patients. Variation in methylation patterns has been linked to a variety of cancers and genetic problems, according to earlier research. Additionally, the increased methylation process is a significant factor in the development of genetic mutations in genes. It therefore has a direct impact on how DNA functions. Prior research has shown that
Deletion of the CDKN2B allele and hypermethylation of the gene's promoter have been found to be common in a variety of hematologic malignancies, including leukemias. That's the CDKN2B gene Disruption of transcription by methylation of CpG islands of regulatory sequences is an epigenetic mechanism for the inactivation of several tumor suppressor genes, including the CDKN2B gene.(26)

Biochemical results :

The table (8): shows the results of biochemical tests among samples of children with leukemia compared to healthy children:

groups	CDKN2B µg \ ml
control	40.1 ± 2.34
patients	28.5 ± 1.24

The results of the study showed, as shown in Table (8), that there was a significant decrease in the level of CDKN2B protein at the level of probability p≤0.05. The ratio in the control group was 40.1 ± 2.34 compared to the patients group 28.5 ± 1.24, as shown in the table.

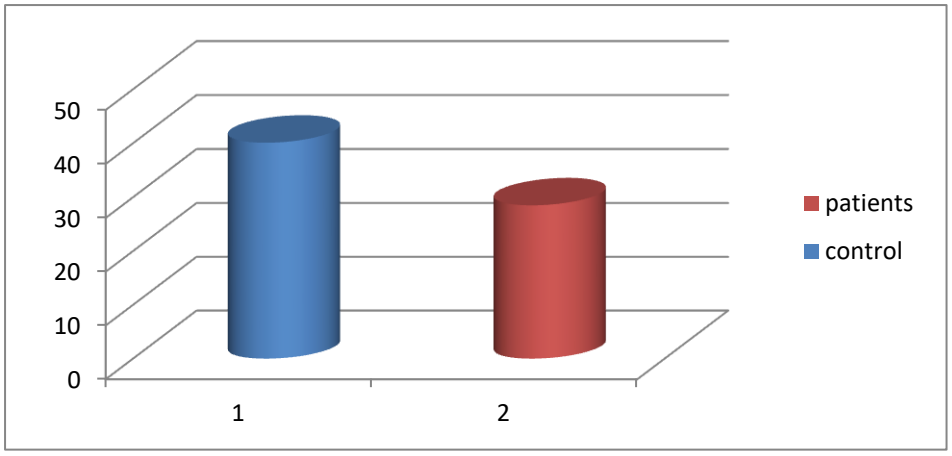

Figure (4): It shows the level of CDKN2B protein in patients with leukemia and the control group.
As we mentioned earlier, the decrease in the level of gene expression of the CDKN2B gene resulting from an increase in methylation process for CDKN2B gene promoter in patients compared to the control group, and thus a decrease in the rate of CDKN2B protein synthesis, and this in turn corresponds with our results obtained in measuring the protein level based on the ELISA technique, as described previously.

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