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The Correlation Between miRNA Gene and DNA Methylation On Expression of PER2 Gene in lymphoid leukemia

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Abstract: ALL is a malignant disease, and the most common malignancy in children, for ALL like other cancers are caused by the combination of genetic susceptibility and the interaction of exogenous and endogenous exposure. There are six well-defined epigenetic processes that change chromatin organization: (a) ATP-dependent chromatin remodelling complexes; (b) DNA methylation; (c) covalent histone modification; (d) histone variants; (e) nuclear dynamics; and (f) chromatin remodelling complexes and non-coding RNA (ncRNA), such as long ncRNA, and microRNA (miRNA/miR) (3). Alterations of DNA methylation are the earliest epigenetic events that have been associated with cancer development in cancer cells. The PER (Period) gene family is an essential component of the circadian clock. Highly significant correlations described in various studies between the cancer and PER genes. Abnormal mutations of the PER gene may help initiate and promote malignancies. Method: This study was carried out in Al-Hadbaa adult specialized hospital, which was the private pathological investigation center in Mosul during September and October 2023, included 35 children (3-10) years old. The sample were classified into 2 group the first one include 25 children with leukaemia and the second one include 10 control sample. 2ml from venous blood sample which collected from the Childs and divided into two groups for first group to put it in EDTA tube for laterally used for DNA extraction and methylation and the second contain put in tube with trizol for laterally use for RNA extraction. The research involved three stages. The first level is sensing the gene expression level of the PER2genes. One, quantification of microRNA 44a that regulates gene expression and two, percentage of DNA methylation on the promoter of downstream genes by q-PCR. The value of gene expression for PER2 gene as the result of this study was 0.78 and for microRNA is 3.09.

Key words: clock genes, per 2 gene, DNA methylation, mi RNA, Leukemia

Introduction:

All is a malignant disease, it's a common malignancy in childhood, with the exception of common cancer, all develop when interactions occur between environmental exposure and endogenous susceptibility factors and genetic susceptibility(1). Epigenetic events are crucial for the regulation of gene expression and all biological processes as such, an aberrant epigenetic is associated with the genesis of many diseases including cancer; cancer was the first human disease associated with epigenetic alterations (2). Six established epigenetic processes that modulate chromatin structure comprise: ATP-dependent chromatin remodeling complexes, DNA

methylation, covalent histone modifications, histone variants, nuclear dynamics, and non-coding RNA (ncRNA), encompassing long ncRNA, and microRNA (miRNA/miR) (3). DNA methylation alterations are the first epigenetic mark associated with cancer development in cancer cells (4). The best studied epigenetic mark is DNA methylation. It regulates chromatin architecture and gene expression by a stable gene-silencing mechanism. Covalently adding a methyl group to the C5 cytosine of DNA by DNA methyltransferases (DNMTs) to produce 5-methylcytosine (5mC) (5). DNA methylation fulfills key roles in several processes, including X chromosome inactivation (7), embryonic development (6), transcriptional silencing (9) and genomic imprinting (8). CpG islands commonly methylated in C/G nucleotide-rich genomic sequences in normal tissues (10). CpG islands account for approximately 60% of gene promoters, wherefor these genes can be epigenetically regulated. The genome of cancer cells is known to be hypomethylated compared with normal tissues, but an unexpected increase of CpG methylation of particular sites has been detected within the promoter regions of genes (12). MicroRNAs (miRNAs) are short non-coding RNAs and play a very critical role as a regulator of various biological aspects in animals. miRNAs regulate gene expression by binding to mRNAs to repress translation or induce degradation of the mRNA at the posttranscriptional level(13). miRNAs, approximately 19–25 nucleotides in length, are exquisitely specific for a single mRNA sequence, although they can regulate multiple target genes in theory (14). miRNA genes are transcribed in the nucleus by RNA polymerase II resulting in the primary transcripts called pri-miRNAs (15) which processed by Dicer in the cytoplasm to form re-miRNAs and then complexes with the RNA-induced silencing complex (RISC) (16). These are miRNAs which are the tumor suppressors and they bake the expression of the anti apoptotic factor Bcl-1 (17). There are solid evidence that miRNA and development of cancer (3). miRNA genes both are frequently dysregulated in different tumours, as well as the loss of miRNA genes is detected in other onco-suppressor genes. For instance, miR-15a and miR-16-1 are deleted frequently in B-cell chronic lymphocytic leukemia (18). Mammalian circadian clock is a 24h-period regulation circuit, which drives rhythmic oscillations of many physiological activities. Accumulating evidence links circadian disruption to aberrant clock gene expression that underlies the pathogenesis of several diseases, including cancer (19). clock-controlled genes are the downstream genes that regulated by clock genes and these genes involved in multiple physiological phenomena such as cell cycling, apoptosis, cell proliferating, endocrine signaling, metabolism, and immunity, Dysfunction of clock genes may leads to many diseases, such as malignancy, cardiovascular disease, and endocrine diseases (20). In parallel, the tumor cells show an altered circadian clock as compared to that of normal cells, which contributes to unbalance cell cycle, energy metabolism, repair systems and others. At the molecular level, the biological circadian clock is an array of several genes, the expression of which makes up a time-delayed transcription–translation feedback loop (19). Circadian rhythm is derived from a number of intertwined positive and negative feedback gene translocation and translation loops. Circadian clock genes are Per1, 2, 3 and cry1,2. Per (Per1, Per1 and Per3) is the best known of the core clock genes. Per genes act as core molecules in the regulation of the circadian rhythm,

and possibly have tumor suppressive effects. (21). The Period (PER) gene family is an essential part of the circadian oscillator. Very strong correlations between PER genes and cancers in several studies. The initiation and development of tumors can be promoted by aberrant mutations in PER gene (22). One of them is the 17p13.1 (PER1). 1, an essential circadian clock gene. It was recently reported that PER1 expression was reduced in certain solid carcinomas, and PER1 may modulate downstream cell cycle genes. Moreover, low-expression of mRNA PER1 level may result in asymmetric cell proliferation and apoptosis, which may inevitably contribute to the malignant transformation of the cell (23). The PER2 (period 2) gene on 2q37.3, is a crucial clock gene. PER2 gene is one of the key components of circadian clock, operating as a transcriptional repressor. The PER2 gene is part of the circadian clock, controlling the clock rhythm (24). The expression of Gal-1 during carcinogenesis and evolution of tumor was closely related. PER2 has been found to be decreased in various tumor entities (25). Period 3 (PER3) is a gene of the mammalian circadian system, PER3 (rs57875989) is localised at chromosome 1p36.23 and is polymorphic in the domain which encodes four or five copies of the 54-bp tandem repeat sequence (27). PER3 belongs to the PAS-per ARNT-Sim (PAS-HRNT-SIM) framed protein binding family that can form protein dimers. It dimerizes with other PERIOD (PER) and CRYPTOCHROME (CRY) proteins to form the negative limb of the transcriptional/translational feedback loop and to suppress the expression of core clock genes and clock-controlled genes activated by the heterodimer transcription factor CLOCK/BMAL1 through binding to the promoter E-box motifs (28).

The aim of this study to estimate the correlation between gene expression of per2 gene and miRNA molecules and DNA methylation.

Materials and Methods

Rationale for Study: Participants In Mosul, 35 children aged between 3 and 10 years were involved in the research from September 2023 to October 2023, having been sent to Al-Hadbaa Specialized Hospital by private pathological examination institutes. The samples were categorized into two groups: One group (n=25) included specimens from children with leukemia, whereas another group consisted of 10 control samples.

Collection and preservation of venous blood samples: A 2 ml blood sample was procured from the child's vein by venipuncture and thereafter separated into two segments. The first segment was put in an EDTA tube for DNA extraction and DNA methylation analysis, while the second segment was placed in a Trizol tube for future RNA extraction.

The q-PCR of per2 gene and miRNA 449a involves the gene expression quantification, which is a multi-step technique.

1-mRNA and miRNA 449a: the extract blood from the kids 750 µl then trizol was added to 250 µl of the blood that contain the samples for the patient and the controls then we are used for

extraction RNA kit and kidneys that was prepared by Transgen biotech and miRNA 449a extraction then the purity of the RNA was estimated with Nano drop device.

2-Pelleting of miRNA 449a The miRNA 449a was pelleted as above.

This polymerization of the poly(A) tail at the 3' end of RNA Poly(A) polymerase of E. coli is template-independent and hence may facilitate the sequential addition of ATP to RNA as AMP at the 3' end, effectively adding a poly(A) tail to the 3' end of RNA. Poly(A) polymerase is a very effective tailing enzyme that may append 20 to 200 adenosine bases to the 3' end of RNA. The poly(A) structure enhances the efficiency of mRNA translation. Definition of a unit One unit (1 U) is the amount of enzyme needed to help add 1 nmol of AMP into RNA in 10 minutes at 37°C.

The total reaction volume for miRNA

The total reaction volume for miRNA synthesis was 20 µl. It consisted of 2 µl of master mix poly(A), 1 µl of ATP and GMP, 10 µl of RNA template, 1 µl of poly(A) polymerase, and 6 µl of RNase-free water.

3- Conversion mRNA and miRNA 449a molecules extracted to cDNA molecule: The applied cDNA was performed according to the method of Transgenbiotech kit.

4. Levels of detection the miRNA 449a gene expression: miRNA 449a gene expression level was tested in a quantitative way. Facetion of gene expression of genes for the PER genes by specific had been using the primers housekeeping gene to the gene of the 1 shown in (table 1).

Table 1: The primers of PER2 gene, miRNA 449a gene and housekeeping gene with the specific primer behalf of housekeeping genes.

Primer	Sequence
microRNA-449a-F	AACACGCTGGCAGTGTATTGTTAG
microRNA-449a-R	CAGTGCAGGGTCCGAGGT
U6-F	GTGCTCGCTTCGGCAGCA
U6-R	CAAAATATGGAACGCTTC
PER2-RT-F	CGCAGGGTGCGCTCGTTTGA
PER2-RT-R	GGGCTCTGGAACGAAGCTTTTCG
B-actin-F	TGACCCAGATCATGTTTGAG
B-actin-R	CGTACAGGGATAGCACAG

The reaction volume

The qPCR reaction was prepared in a total volume of 20 µl, consisting of 10 µl of Ultra SYBR Green qPCR Master Mix, 0.5 µl of RT forward primer, 0.5 µl of RT reverse primer, 4 µl of cDNA template, and 5 µl of distilled water (D.W).

The program use in q-PCR reaction

The thermal cycling conditions began with a pre-denaturation stage at 95 °C for 10 minutes, followed by a denaturation step at 95 °C for 15 seconds. This was then followed by the annealing and extension step, carried out at 60 °C for 1 minute. After amplification, melting curve analysis was performed in several steps: 95 °C for 15 seconds, 60 °C for 1 minute, then 95 °C for 15 seconds, and finally 60 °C for 15 seconds.

Calculate the rate of gene expression

Then target gene expression levels were calculated relative to a house-keeping gene with the comparative CT method. For each sample from the Ct values this of the reference (housekeeping gene) was subtracted:

$$\Delta CT (\text{patient sample}) = CT (\text{target gene, patient}) - CT(\text{reference gene, patient})$$

$\Delta CT (\text{treated sample}) = CT (\text{target gene, treated}) - CT (\text{reference gene, treated})$ Data are expressed as $2^{-\Delta Ct}$.

In the present study, CT (target gene) is the cycle number for which the fluorescence exceeds the threshold for the target gene of interest; and CT (reference gene) is the corresponding value for the housekeeping gene in the patients and normal controls.

The fold difference in gene expression between patient and control patients was evaluated as follows.

$\Delta\Delta CT = \Delta CT (\text{patient}) - \Delta CT (\text{control})$ Where, $\Delta CT = CT$ of either target gene – CT of housekeeping gene (GAPDH).

The relative gene expression level was then calculated as fold change:

$$\text{Fold change} = 2^{-\Delta\Delta CT}$$

This approach allows determining the extent of gene expression changes in comparison to a reference group, taking the variation between samples into account normalization by a stable reference gene.

Methylation protocol

DNA extraction DNA sample was treated with Sodium bisulfite for 24 hours according to the transgenbiotech kit procedure based on making all cytosine on the DNA sample to be changed to

uracil expect for the methylated one. The bisulfite modified DNA re-purified and then analyzed by PCR with primers listed in table (2),

Table (2): demonstrates the primers employed for DNA methylation (28).

Primer	Sequence
PER 2	F: GCGGTTTCGTTGCGGTTTAC
	R: GCCGACGCCGTTTCAAACCG

The PCR assay began with the amplification of the gene of interest using five pairs of primers, and the methylation-specific PCR program was applied. Each PCR reaction mixture for DNA methylation had a final volume of 10 μ l, which included 2 μ l of DNA, 0.5 μ l of forward primer, 0.5 μ l of reverse primer, 5 μ l of PCR master mix, and 2 μ l of distilled water. The thermal cycling conditions for methylation-specific PCR started with a pre-denaturation step at 95 °C for 10 minutes, followed by 40 cycles of denaturation at 95 °C for 45 seconds, annealing at 60 °C for 1 minute, and extension at 72 °C for 45 seconds, with a final extension at 72 °C for 4 minutes. After amplification, the PCR products were separated by gel electrophoresis using prepared agarose gel, and the resulting DNA bands were visualized under a UV transilluminator. Additionally, for miRNA analysis, the total reaction volume was 20 μ l, consisting of 2 μ l of poly(A) master mix, 1 μ l of ATP and GMP, 10 μ l of RNA template, 1 μ l of poly(A) polymerase, and 6 μ l of RNase-free water. The qPCR reaction mix for cDNA amplification was also prepared in a 20 μ l final volume, containing 10 μ l of Ultra SYBR Green qPCR Master Mix, 0.5 μ l each of RT forward and reverse primers, 4 μ l of cDNA template, and 5 μ l of distilled water.

Results and discussion:

Based on the q-PCR, the gene expression of miRNA 449a showed 3.09-fold higher in leukemia patients than in the control group 1 as presented in the table below.

Table (1): CT and gene expression level of Micro RNA 449a and Housekeeping gene for patients with leukemia and their control group.

cDNA miRNA 449a Per 2 Gene						
NO.	CT. target	CT. H.K	Δ CT target	Δ CT control	$\Delta\Delta$ CT	Folding
Control	26.43	19.21	7.22	7.22	0	1
Patients	28.9	23.31	5.59	7.22	-1.63	3.09

Also the study result reduced the gene expression of per2 gene for leukemia patients where its average percentage was 0.78 compared to the control group which was on, as indicated in table (2).

Table (2): reveals the CT values and the gene expression level of PER2 gene responsible gene and housekeeping gene in leukemic patients when compared with its control group.

cDNA Per 2 Gene						
NO.	CT. target	CT. H.K	Δ CT target	Δ CT control	$\Delta\Delta$ CT	Folding
1	30.31	22.71	7.6	7.6	0	1
4	30.54	22.59	7.95	7.6	0.35	0.78

MiRNA 449a has a fundamental role in the process of gene expression through the action of distraction of the mRNA of the target gene, such as the direct and valuable role of miRNA 449a in the repression and regulation of the level of gene expression for the per2 gene for children with leukemia.

Hypermethylation in the promoter of the PER2 gene

Hypermethylated promoter of PER2 Due to the DNA methylation of PER2's promoter, the binding of RNA polymerase to the promoter site of genes is not possible. This results in repression of gene expiration, and produces reduced gene expression.

The circadian system is one of the most widely evolved regulatory systems of eukaryotic organisms. Circadian clock genes are crucial regulators of oregnise biological activities. In addition, in addition to being essential for the maintenance of fine-tuned modulation of growth and are involved in intracellular proliferation and apoptotic processes. Accumulating evidence has demonstrated the extensive role of circadian clock genes in tumorigenesis. 7% of the core clock genes act as direct modulators of key cellular processes, including proliferation and apoptotic pathways, as revealed by experimental studies. (29)

A member of the Period gene family, the PER2 gene is a core component of the mammalian circadian clock mechanism, an essential system required to coordinate numerous physiological processes, which also includes stringent hormonal control of the cell cycle. A growing body of data emphasizes its fundamental role in maintaining cellular homeostasis and identifies altered PER2 levels as a major determinant of pathological settings, among which unrestrained cellular growth and apoptotic deregulation.

The PER2 gene is also functionally linked to apoptosis through the evidence showing that its overexpression enhances programmed apoptosis (apoptosis) and its down expression reverses apoptosis signaling. This dysfunction in a PER2-regulated apoptosis is highly associated with pathogenesis, where impaired apoptotic ability leads to uncontrolled cell growth and survival that ultimately contributes to the cancerous conversion. These results put PER2 as being a central player in the etiology of the disease, where its functional integrity can maintain the cellular homeostasis and also be capable of damping the oncogenic processes. The PER2 gene is a transcriptional repressor of cell cycle-associated genes, including cyclin D1. Decrease of PER2 expression lead to

the up-regulation of these regulatory genes and resulted in anomalous cellular proliferation and growth disorder. This unrestrained growth impairs tissue homeostasis, causes pathologies to develop faster and leads to tumours by compromising the control of the cell cycle, and inducing cell transformation prevState. (30). The current study also noticed significant decrease of PER2 gene expression in myeloid leukemia patients compared to the control group. These results suggest PER2 as a promising diagnostic biomarker for leukemia, and its downregulation is highly inversely correlated with the severity of the condition. Moreover, this dysregulation gives an important clue to molecular mechanisms involved in leukemogenesis, implicating for PER2's possible role in disease development and as a target for therapeutic intervention.

The CpG methylation in the promoter region of PER2 gene is the inherited epigenome alteration causing gene silencing with highly reduced expression level. This epigenetic silencing by disruption of the gene regulation, mostly involving the important function in controlling the cell cycle. The results indicated that hypermethylation of the promoter of PER2 is closely related to the reduced level of PER2 expression and then disrupts the cell cycle checkpoint. This dysregulation may lead to uncontrolled growth of neoplastic cells, promoting tumor formation with subsequent progression by reducing genomic stability and ability to escape apoptosis. (31)

Conclusion

According to the study conclusion, miRNA and hypermethylation are essential in regulating gene expression from the PER2 gene in patients with leukemia.

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