Association of MEG3 Hypermethylation and MiR-21 Upregulation with the Incidence in Iraqi Acute Myeloid Leukaemia Patients

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Abstract

Background: Acute myeloid leukaemia (AML) can be defined as a hematologic malignancy that distinguished by genetic defects and epigenetics alterations. LncRNA MEG3 was shown to play the role of tumour suppressor, and play a pivotal role in leukemogenesis, MEG3 hypermethylation has been reported to be related to different types of haematological malignancies. MIR-21 is regarded as a significant miRNA, it considered to play a vital role in AML progressions.

Results: The levels of methylation in the MEG3 promoter region in AML patients were significantly increased than in healthy controls, as the MEG3 expression levels were significantly lowered ($P \leq 0.05$) in AML patients in contrast with healthy controls. On the other hand, results showed elevated expression levels of miR-21 in AML patients compared with healthy controls.

Conclusion: The present study indicates that the hypermethylation of MEG-3 promoter region could explain MEG-3 expression level loss. Our findings also revealed that the overexpression of miR-21 supports its function as an oncogene.

Key Words: MEG3, MiR-21, DNA Methylation, AML, Epigenetics.

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Introduction

The AML can be distinguished by a rise in the number of the myeloid precursors in the bone marrow and an arrest in their differentiation, which often leads to hematopoietic insufficiency, with or without leukocytosis (Saultz and Garzon, 2016). According to recent reports, AML is characterized by a combination of repetitive genetic and epigenetic changes in hematopoietic stem cells that occur over time (Short *et al*., 2018). The AML is the most common leukaemia type amongst Iraqi population (Iraqi Cancer Board, 2018).

Long non-coding RNAs (lnc RNAs) have been actually reported altered in patients with AML (Gao *et al*., 2020). lncRNA MEG-3 a putative tumour-suppressor gene that is located on the 14q32 chromosome is recognized as a tumour suppressor in haematological malignancies (Ghafouri-Fard and Taheri, 2019; Al-Terehi *et.al* 2020). For example, represses the proliferation of different cancer cells through the regulation of the phosphorylation of retinoblastoma protein and activation of p16INK4a path-way (Lyv *et al*., 2017). Several studies indicated the down-regulation of *MEG-3* expression levels in AML (Lyv *etal*., 2017; He *et al*., 2020).

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Maternally expressed gene 3 (*MEG3*) can be described as an imprinted gene, which is commonly expressed in several normal tissues. None-the-less, it's typically downregulated in non-small cell lung cancer (Lu *etal*., 2013), prostate cancer (Zhou *etal*., 2020), gallbladder cancer (Jin *etal*., 2018), hepatocellular carcinoma (Zhuo *etal*., 2016), cervical cancer (Zhang *etal*., 2016), and nasopharyngeal carcinoma (Chak *etal*., 2017).

MicroRNAs (miRNAs) are about 22 nt small non-coding RNA molecules that are identified to play a critical part in the post-transcriptional regulation of mRNA (Ali Syeda *et al*., 2020). MiR-21 is regarded as a significant miRNA that is often elevated in many cancers, indicating that it plays an important role in cancer progressions (Saini *et al*., 2021), and may result in chemotherapy resistance in leukaemia cells through interfering *PTEN* expression. Elevated expression of miR-21 has been observed in daunorubicin (DNR) resistant cell line K562/DNR by Bai, who also mentioned that the inhibition of miR21 activated cell that have sensitivity to cytotoxicity (Bai *et al*., 2011). This study was conducted to study the correlation between DNA methylation of lncRNA MEG 3 and expression levels in AML patients compared with healthy controls, also, to ensure the levels of expression of miR-21which associated with leukemogenesis in AML patients.

Methods

Subjects

The thirty patients with AML from different ages ranged between 16 and 75 years in addition to thirty normal controls were used in this study. The samples that contain Blood were imparted from newly diagnosed patients with AML, attend the Hematology Center in Baghdad Medical City during the period from 11/2018 to 2/2020. Five-milliliters of blood samples were withdrawn from each case-control subjects and distributed into tubes containing anticoagulant (K_3EDTA) for genomic DNA extraction and other tubes containing TRIizol™ for RNA extraction.

DNA Extraction and MSP-PCR

Genomic DNA has been freshly extracted from the samples of the blood through the use of the ReliaPre Blood gDNA Miniprep kit (Promega, USA). Bisulfite treatment was performed using the Bisulfite Conversion of gDNA kit (Promega, USA). The methylation DNA levels in *MEG-3* promoter region were determined by MSP, by Thermo Cycler (BioRad, USA) with GoTaq Green Master Mix (Promega, USA), MSP-PCR primers have been indicated in table (1). PCR has been performed by the use of the following conditions of the thermo-cycling: initial denaturation one cycle: 95°C (5 m); 35 cycles of 95°C (30 s), 54°C (30 s) and 72°C (30 s); and a finally extended at 72°C (7 m).

RNA Extraction

RNA was isolated from blood samples of AML patients and normal controls according to the protocol of TRIzol™ Reagent (Thermo Scientific, USA) as mentioned by the manufacturer's procedure.

Detection of MEG3 Expression

MEG3 expression was detected in blood samples by using the one-step RT-PCR procedure. Total extracted RNA was converted into cDNA by GoTaq 1-Step RT-qPCR System (Promega, U.S.). Reverse transcription PCR mix components were performed in 10µl final volume containing 1µl of template RNA, 0.5µl of both forward primer and reverse primer shown in table (1), 5 µl of qPCR Master Mix and 0.25µl of RT mixture, 0.25 µl of $MgCl_2$, and 2.5µl of the nuclease-free water. Amplification was achieved by using Quantitative real-time RT–PCR (BioRad/ USA). Triplicate reactions were conducted for each sample, by using conditions as follows: Reverse transcriptase activation at 37 °C (15 min.), initial denaturation at 95 °C (10 m) one cycle, then 40 cycles at 95°C (20sec.), 60°C (20sec.), and 72°C (20sec.). lncRNA MEG3 relative expression was determined by calculating $2^{-\Delta\Delta CT}$.

Detection of microRNA 21 Expression

The miRNA-21expression was achieved by qRT-PCR. By using the GoScript™ kit (Promega, USA) to obtained cDNAs after conversion of extracted microRNAs were converted into according to the instructions of manufacturer. QRT-PCR was done by using of Green Go Taq® qPCR Master Mix (Promega, USA). Thermal cycling conditions as the following: Initial denaturation 1 cycle at 95°C (5 min.), denaturation 40 cycles at 95°C (20sec.), then primer annealing 40 cycles at 60 °C (20 sec.) and extended to 40 cycles at 72 °C (20 sec.). MiRNAs expression was calculated by determining

2-ΔΔCT. Primers sequences indicated in the table (1).

Table 1. Primers Sequences

Gene	Sequence (5'- 3')	Tm (°C)	Ref.
MEG3 - M-F	GCGAATTATTATTTATATAGCCTTC	54	(Li *et al.*, 2018)
MEG3 -M-R	TCACGCGCTACGAAGGGAAACG		
MEG3 -U-F	GTGAGGTGTTATTACCGTATAGTTTGG		
MEG3 -U-R	TTCACACATACAGGTTCCAAACAAT		
MEG3-F	ATCATCCGTCCACCTCCTTGTCTTC	60	
MEG3-R	GTATGAGCATAGCAAAGGTCAGGGC		
miR-21-RT	GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAACACAGCC	60	(Ruiz-Lafuente *et al.*, 2015)
miR-21-F	GTTGCAACACCAGTCGATG		

Analytical Statistical System

The program of analytical Statistical system SAS (2012) was used for the analysis of data analysis [19]. Different gene expression (mean ± standard deviation) was estimated using *p*-value, ($p \leq 0.05$) was accounted as significant and ($p < 0.01$) was increased significantly.

Results

Quantitative Expression of lncRNA-MEG3

The *MEG3* Expression levels of were investigated in AML patients and healthy controls by qRT-PCR. Results illustrated in figure (1) showed that *MEG3* expression levels in patients that with AML have been considerably decreased to (P≤0.05) in comparison to healthy controls.

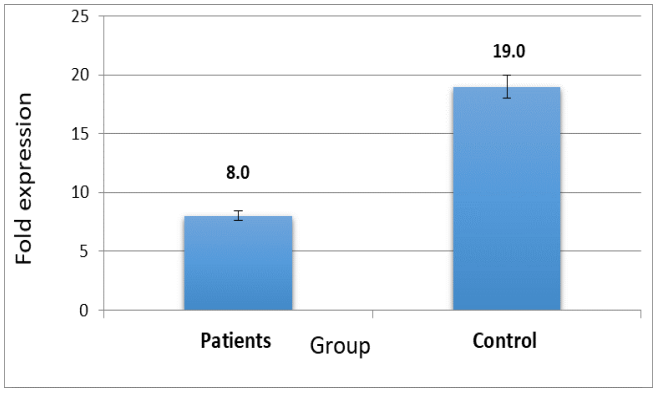

Figure 1. *MEG3* expression levels in AML patients and healthy controls. Error bars denote the standard deviation.

Results indicated in table (2) showed also that the expression fold of *MEG3* was measured to be significant decreasing to ($P \leq 0.05$) from 19.0 in healthy controls to 8.00 in Patients with AML. Several studies also showed downregulation of this lncRNA in various cancers as it controls the expression of many tumour suppressor genes and oncogenes (He *et al.*, 2020; Lu *et al.*, 2013).

Table 2. Expression levels of the *MEG 3* in patients who have AML and healthy controls.

Group	No.	MEG 3 expression*			
		Ct	ΔCt	ΔΔCt	Fold expression
Patients with AML	30	30.79 ±1.38	6.82 ± 1.73	-0.127 ± 1.73	8.00^{b} ± 0.57
Healthy controls	30	30.66 ±1.82	6.93 ± 2.21	-0.014 ± 1.21	19.0^{a} ± 1.13
P-value					0.073 *

*: Expression ± standard deviation; Different letters in fold expression values represents a significant difference (P≤0.05).

DNA Methylation of MEG3

The methylation status of *MEG 3* promoter in patients with AML and healthy controls was investigated by the MSP-PCR. Results revealed that methylated patterns (M) and partially methylated patterns (MU) were detected in 18 (60.0%), and 12 (40.0%) cases of AML patients, respectively. Compared with 10 (33.3%) partially methylated patterns (M & U) and 20 (66.6%) unmethylated patterns (U) observed respectively in healthy controls as shown in figures (2).

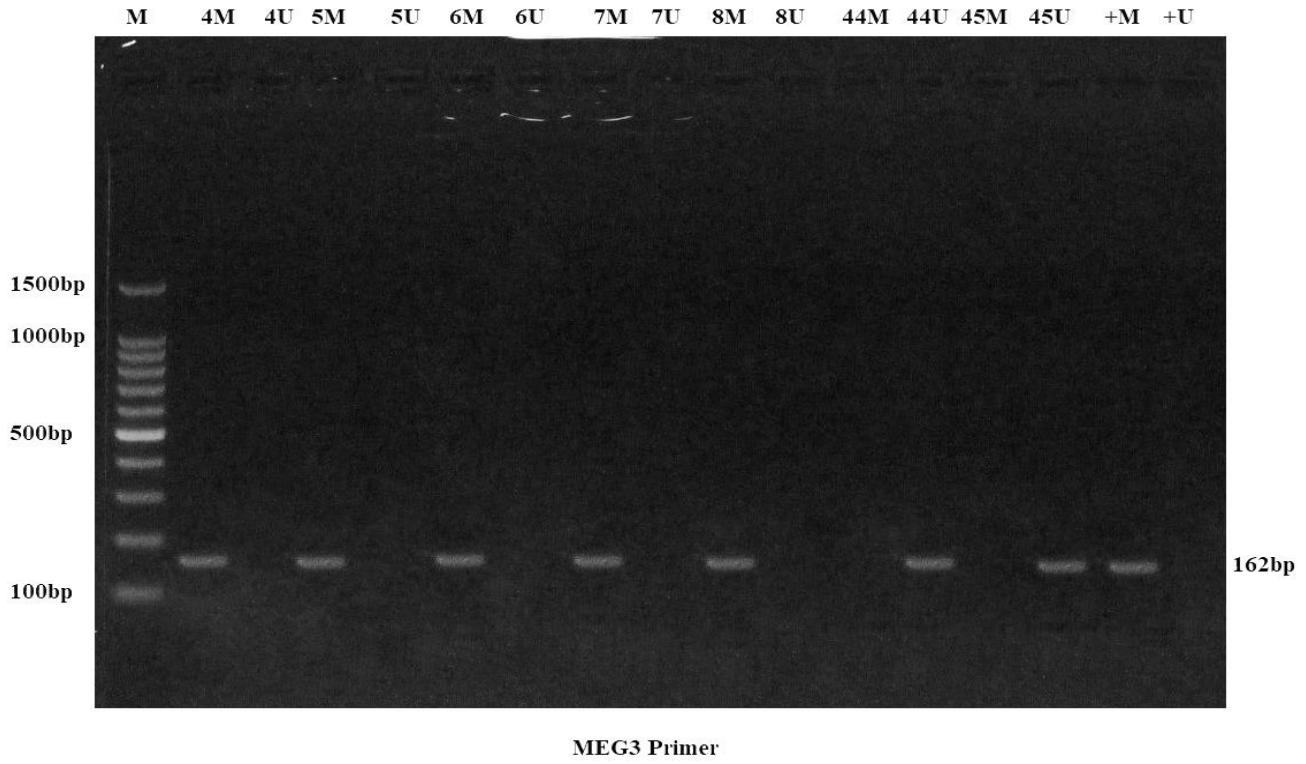

Figure 2. MS-PCR of *MEG3* in AML patients and controls (healthy). Lanes (4 to 8) AML patients, Lanes (44 and 55) normal healthy controls. The methylated and unmethylated patterns designated as M or U, respectively.

The methylation frequency of *MEG3* in blood samples of patients with AML was significantly ($P \leq 0.01$) higher than that in corresponding blood samples of normal healthy controls as shown in (3).

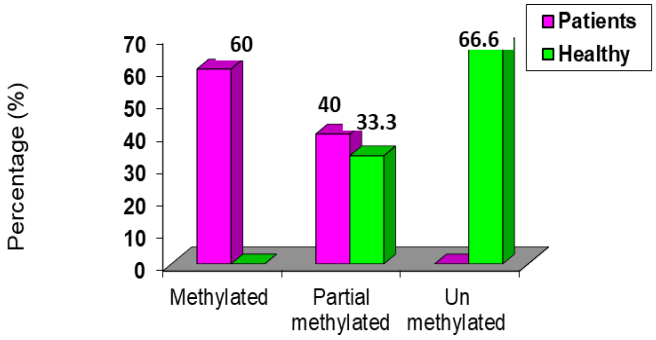

Figure 3. The MEG3Frequency of methylation in region of promoter in patients with AML and normal controls.

The relation between *MEG3* levels of expression and gene methylation promoter was studied. Results illustrated in figure (4) showed that gene expression in patients with AML with highly methylated promoter region (M) was significantly lower ($P \leq 0.01$) than the levels of expression in patients with AML with partially methylated promoter region (MU).

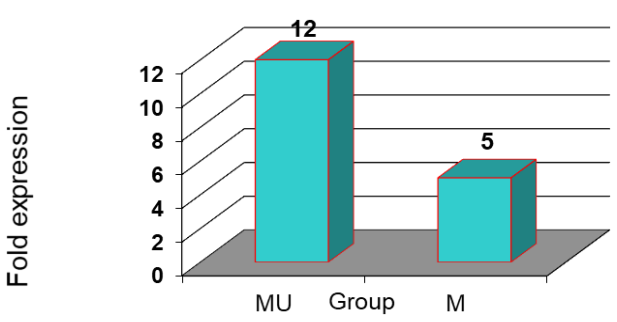

Figure 4. Comparison between partially methylated (MU) and methylated (M) promoter region in fold expression of *MEG 3* in the AML Patients.

These results mentioned at hyper-methylation of *MEG 3* region of promoter correlates with down regulation of gene expression. CpG methylation was noticed in numerous cancer types (Lu *et al*., 2013; Zhou *et al*., 2020).

Quantitative Expression of miR- 21

In this work, the expression of miR-21 in AML patients was detected to determine its role in the development of AML. Results described in figure (5) found that there is a significance increase ($P \leq 0.01$) in miR-21 expressions in AML patients compared with normal healthy controls as the fold expression of miR-21 has been increased from 4.0

in patients with AML to 2.0 in healthy normal controls, which may suggest that miR-21 play a vital part in progression of AML.

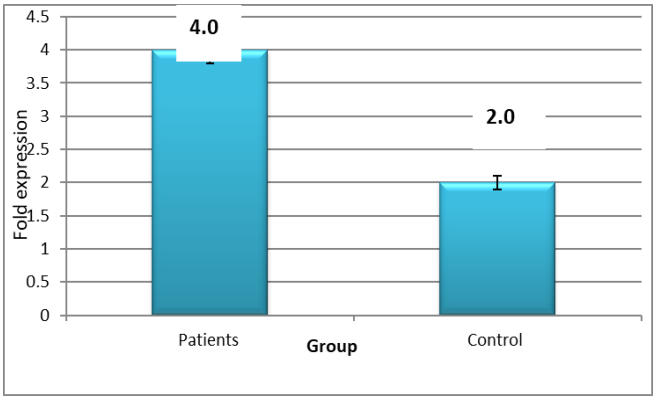

Figure 5. MiR-21 expression levels in AML patients and healthy controls. Error bars represent standard deviation.

Discussions

As it is known that *MEG3* is a potential tumour suppressor gene that represses leukemogenesis and inhibits the proliferation of the leukemic cell, any downregulation in this gene increase the risk for leukemogenesis and incidence of AML (Yu *et al*., 2020).

In this research work, *MEG3* levels of expression in patients with AML were explored. Results illustrated in figure (1) shows a significant decrease (p ≤ 0.0l) in *MEG3* expression levels compared with healthy controls as the fold expression was decreased to 8.0 in patients with AML compared with 19.0 in healthy controls. Downregulation of *MEG3* may promote leukemogenesis due to the reduction of the activity of the *P53* tumour suppressor gene which plays a significant role in regulating cell growth via *P 53* dependent and independent pathways (*RB* pathway) as described by (Lyv *et al*., 2017). Recent studies, also mentioned that there is a reduction of *MEG3* expression levels in patients with AML in comparison to healthy controls (He *et al*., 2020). The dysregulation of the expression of *MEG 3* in the patients who have AML may use as prognostic biomarkers for the incidence of the disease (Lyv *et al*., 2017; Yao *et al*., 2017). Besides, lncRNA MEG 3 could suppress cell growth and invasion then improve the apoptosis via the regulation of the precise signaling pathways. *MEG3* can regulate STAT3, by inhibiting JAK/STAT phosphorylation (Li *et al*., 2018). These studies indicated that the lncRNA MEG3 has anticancer properties in AML. Furthermore, *MEG3* induces cell cycle arrest and apoptosis, thus prevents the proliferation of cervical carcinoma cells (Qin *et al*., 2013).

Downregulation of *MEG 3* may be attributed to hypermethylation of gene promoter as mentioned by Sellers (2019). According to this fact, the relation between *MEG3* downregulation and hypermethylation in *the MEG3* promoter region was detected in Patients with AML by using the MSP-PCR method. Results showed that methylated patterns (M) and partially methylated patterns (MU) in *the MEG3* promoter region were detected in 18/30 (60%) and 12/30 (40%) of Patients with AML respectively, in contrast, 10/30 (33.3%) of only partially methylated patterns were detected in normal controls, while the others 20/30 (66.3%) having the unmethylated patterns shown in figure (3). Aberrant methylation of the promoter region may result in silencing *MEG3* gene expression as a tumour suppressor gene and may promote leukemogenesis. These findings are similar to those obtained by Benetatos who observed that 50% of AML patients with hypermethylation in the *MEG3* promoter region (Bentatos *etal*., 2010; Bentatos *et al*., 2011). Increased methylation in CpG islands within the *MEG 3* promoter region has been reported in AML patients having longer survival and a prognostic marker in AML (Sellers *et al*., 2019). However, the results that have been illustrated in figure (4) show that there has been a significant decrease (P ≤ 0.01) in levels of *MEG3* expression in AML cases with methylated CPG islands within the promoter region compared with AML cases with unmethylated sequences. These results declare that hypermethylation of *MEG3* promoter region is an important biomarker related to decreased expression and gene silencing.

Hypermethylation of *MEG 3* promoter has also been detected in Esophageal cancer (Dong *et al*., 2017), glioma (Li *et al*., 2016), urothelial carcinoma (Greife *et al*., 2014), and retinoblastoma (Gao *etal*., 2017). Lower levels of *MEG3* expression were also found to be related to aberrant hyper-methylation of *MEG3* promoter in those carcinomas.

In the present work, the expression of miR-21 in blood samples of AML patients was detected. Results demonstrated in figure (5) showed that there is a significant increase (p ≤ 0.0l) in miR-21 expression in AML patients compared with healthy controls. Upregulation of miR-21 may promote leukemogenesis due to oncogenic activity. MiR-21 targeting significant tumour suppressor genes and genes involved in carcinogenesis. MiR-21 have a significant impact on the oncogenic process

through suppression of programmed cell death 4 genes (PDCD 4) which involved in the apoptosis process (Bautista-Sánchez *et al*., 2020), which is why, miR-21 could play the role of a possible diagnostic marker and therapeutic target in the AML. These findings are similar to those obtained by Zhang who demonstrated that miR-21 was upregulated in AML patients and promoted the Thp-1 cell proliferation (Zhang *et al*., 2021)

MiR-21 was found to be overexpressed in nucleophosmin 1-mutant AML in a previous study, indicating that miR-21 can play a key role in the progression of the AML (Riccioni *etal*., 2015). Besides, Li revealed that miR-21 promoted proliferation by regulating expressions of transcription factor Kruppel-like factor 5 expressions in AML cells (Li *et al*., 2019). On the other hand, the precise roles of miR-21 in the progression of AML remain unclear. A recent study directed by Moussa Agha reported that miR-21 may play an imported role in AML lymphocytes cells' fragility (Moussa Agha *et al*., 2020).

Conclusions

MEG 3 could play the role of tumour-suppressor gene and abnormal promoter hypermethylation is critical for the silencing of *MEG 3* in AML, which may be a biomarker for AML incidence and a valuable treatment target, while the overexpression of miR-21 is highly related to AML incidence, and may be utilized as one of the prognostic and diagnostic biomarkers and as a potential therapeutic target.

Abbreviations

3′-UTR: 3′ Untranslated region; AML: Acute myeloid leukaemia; cDNA: Complementary deoxyribonucleic acid; CpG: 5'Cytosine-phosphate-guanine3; CT: Cycle threshold; DNR: daunorubicin; EDTA: Ethylene di-amine tetra-acetic acid potassium salt; JAK2: Janus kinase 2; *MEG 3*: Maternally expressed gene 3; MiRNA: MicroRNA; MSP-PCR: Methylation-specific polymerase chain reaction; PTEN: Phosphatase and tensin homolog; qRTPCR: Real-time quantitative polymerase chain reaction; RNA: Total ribonucleic; SMAD7: Mothers against decapentaplegic homolog 7; STAT 3: Signal transducer and activator of transcription 3; STAT 5: Signal transducer and activator of transcription 5; TGFβ Transforming growth factor beta signalling.

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