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# Gene Expression of the *MTHFR* Gene in a Sample of Diabetic Retinopathy Iraqi Patients

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**Abstract**: Diabetic retinopathy (DR)is a condition in which the walls of the small blood vessels of the retina are damaged by diabetic mellitus (DM), which can lead to blindness. Factors including genetic makeup and nutrient regulation in the body may be the causes of this disease. Methylenetetrahydrofolate (*MTHFR*) plays a key role in cellular processes like regulating homocysteine levels and Oxidative Stress. The present study designed to investigate the association between *MTHFR* gene expression and DR. This study involved One hundred fifty Iraqi subjects in total, including fifty DR patients, fifty DM patients as positive control, and fifty apparently healthy individuals as negative control. Blood samples were collected from all subjects for RNA extraction according to the Trizol-based method using reverse transcription and RT-PCR assay analysis. The results showed that *MTHFR* gene expression according to ΔCt was generally elevated in DR patients with a fold change of 2.45 compared with the apparently control group. The *MTHFR* gene expression according to ΔΔCt confirming these results which showed distinct increase in *MTHFR* gene expression in DR patients in compared with healthy control (2.16), indicating that this gene is a possible cause of severe vascular diseases such as retinal vascular narrowing, an important component of diabetic retinopathy.

**Keywords:** Diabetic retinopathy, *MTHFR* gene, folate metabolism, gene expression, metabolic pathways, RT-PCR.

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## Introduction

Diabetic retinopathy (DR) is a debilitating microvascular complication of diabetes mellitus, affecting millions of individuals worldwide and posing a significant public health burden. It is characterized by progressive damage to the retina's blood vessels, leading to vision impairment and blindness if left untreated.Diabetic retinopathy (DR) is the most common microvascular complication of diabetes mellitus. Vascular endothelial growth factor (VEGF) is a major mediator of vascular permeability and angiogenesis and also an important mediator of retinal ischemia-associated intraocular neovascularization (1) Despite advancements in treatment modalities, the pathogenesis of DR remains incompletely understood, with both genetic and environmental factors playing pivotal roles.(16).The major risk factors for DR development and progression include the duration of

diabetes, Diabetic hyperglycemia influences endothelial cell dysfunction, endothelial cell death by apoptosis, and consequent retinal capillary loss seen in diabetic retinopathy.(3)The MTHFR gene is on the short arm of human chromosome 5 at the p15.2–15.3 position, composed of 15 exons and 14 introns.it encodes the methylenetetrahydrofolate reductase enzyme, which plays a crucial role homocysteine regulation. (11) .The Methylenetetrahydrofolate reductase(MTHFR) gene is one of the most considered candidategenes due to its vital role in folate metabolism(9). MTHFR Gene Expression , is essential for homocysteine metabolism, and its deficiency can lead to elevated homocysteine levels contribute to endothelial dysfunction , which is a risk factor for vascular complications, including diabetic retinopathy, dysregulation of folate metabolism due to reduced MTHFR activity may exacerbate oxidative stress, further damaging retinal cells and contributing to the progression of DR.In the pathogenesis of diabetic retinopathy, oxidative stress is increased in the retina and its vasculature, gene transcription associated with oxidative stress are altered, and apoptosis of capillary cells are accelerated .(10). This study was designed to impact the role of *MTHFR* gene expression in DR development, which can be used as prognostic factors in the future.

## Materials and Methods

### Subjects

In the present study,50 Iraqi patients with DR who were clinically diagnosed at Al-Kadhimiya Medical in Baghdad were included, in addition to 50 apparently healthy individuals as negativecontrol group and 50 diabetic patients without retinopathyas positivecontrol group,who were matched with DR patients in age and sex. All subjects have been informed about the aim of this study and asked to fill in the questionnaire form.

### Blood sampling

From each participant, 1 mL venous blood were drawn into an ethylenediaminetetraacetic acid vacutainer tube and diluted with an equal volume of sterile phosphate-buffered saline. Blood sample (250 µl) was added to 750 µl of trizolfor RNA the estimation of gene expression.The samples were stored at -20 ºC until further processing. The expression levels of *MTHFR* gene were calculated using the ΔCt method where the fold change values were determined for the DR patient group compared with the negative control group.

### Total RNA extraction

RNA was extracted from blood samples according to the protocol of TRIzol™ Reagent using )ELK Biotechnology, Chine, EP013)according to the manufacturer instructions. Quantusfluorometer was used to detect the concentration of extracted RNA in order to detect the quality of samples for downstream applications. For 1 µl of RNA, 199 µl of diluted Quantifluor Dye was mixed. After 5min incubation at room temperature, RNA concentration were detected.

### cDNA synthesis

A total of 2 µgRNA was used for reverse transcription (RT) with the TransCriptor First-Strand cDNA Synthesis Super mix according to the manufacturer's instructions (Synthol /Russia).

### Synthesis of primers

The sequences of genes*MTHFR* were received from NCBI site and the bioinformatic validation of Quantitative Real Time PCR (QRT–PCR) primers were done by the in-silico PCR online-built-in analysis

(https://genome.ucsc.edu/cgi-bin/hgPcr) and delivered to Macrogen Company (Korea). Primers were designed according to Table 1.

**Table (1): Sequences of genesexpression**

| Primer Name | Forward 5'→3' | Reverse 5'→3' | Tm. |
|---|---|---|---|
| ***MTHF*** | GGTGCCACAGGAGATCAAGG | CTCTGTGGTAGCCATCTCGC | **60** |
| **GAPDH** | GAGTCAACGGATTTGGTCGT | TTGATTTTGGAGGGATCTCG | **60** |

**Gene expression:**

Process of real time PCR to study*MTHFR* gene expression was done by The Azure Cielo Real-Time PCR system according to the protocol of Luna® Universal Master Mix (Promega, USA). Based on the protocol, firstly, Master Mix was prepared and added to strip cap microtube and then the cDNA was added to it. The QRT-PCR amplification conditions were: 95°C, 3 min; 95°C, 15sec, 60°C,30 sec for 45 cycles.

**cDNA Concentration (ng/µl)**

cDNA Concentration 4-6ng/µl

**Analysis Gene Expression**

Relative quantification

Folding =2-ΔΔCT

ΔCT= CT gene - CT House Keeping gene

ΔΔCT= ΔCT Treated or Control – Average ΔCT Average Con.

**Results and Discussion**

Most studies on *MTHF* in patients with diabetes focus on single-nucleotide polymorphisms, with very little research on gene expression alteration, particularly in diabetic retinopathy and complications.

The patients exhibited a fold change value equal to 2.45 while the positive control group and negative control group exhibited values 1.30, and 1.00, respectively, showing differing levels of gene expression across the groups. These results were validated with the patient's group 2.16-fold change value using the ΔCt method together ΔCT= CT gene - CT House Keeping gene,with the negative control group. The change value for the positive control group remained at 1.30 while the negative control group stayed at 1.00 value for the negative control group which suggests this gene's putative function in lessening the disease progression and aggravation of the compounding vascular damage (Tables 2, 3, and 4) and Figure 1.

**Table (2): Comparison of *GAPDH* Fold expression between study exposure groups.**

| | Means Ct of GAPDH | $2^{-Ct}$ | Experiment group / control group | Foldchange of gene expression |
|---|---|---|---|---|
| **Patient** | 23.53 | 8.2559E-08 | 8.2559E-08/8.78733E-08 | 0.94 |
| **Control positive** | 23.49 | 8.488E-08 | 8.488E-08/8.78733E-08 | 0.97 |
| **Control negative** | 23.44 | 8.78733E-08 | 8.78733E-08/8.78733E-08 | 1 |

**Table (3): Fold of *MTHF* in expression Depending on ΔCT(normalization Ct values)**

| | Mean Ct of *MTHF* | Mean Ct of GAPDH | ΔCT (Means CT of *MTHF* - Mean CTofGAPDH) | $2^{-\Delta CT}$ | Experiment group / control group | Fold change of gene expression |
|---|---|---|---|---|---|---|
| **Patient** | 27.9 | 23.53 | 4.37 | 0.048 | 0.048/0.020 | 2.45 |
| **Control positive** | 28.77 | 23.49 | 5.28 | 0.026 | 0.026/0.020 | 1.30 |
| **Control negative** | 29.1 | 23.44 | 5.66 | 0.020 | 0.020/0.020 | 1.00 |

**Table (4): Fold of *MTHF*expression in Depending on $2^{-\Delta\Delta CT}$method.**

| | Mean Ct of *MTHF* | Mean Ct of GAPDH | means ΔCT Target (CT of *MTHF* - Ct of GAPDH) | ΔCT Calibrator | ΔΔCT (ΔCT -ΔCT Calibrator) | $2^{-\Delta\Delta CT}$ | Experiment group/control group | Fold change of gene expression |
|---|---|---|---|---|---|---|---|---|
| Patient | 27.9 | 23.53 | 4.37 | 4.46 | -0.09 | 0.94 | 0.94/0.44 | 2.16 |
| Control positive | 28.77 | 23.49 | 5.28 | 4.46 | 0.82 | 0.57 | 0.57/0.44 | 1.30 |
| Control negative | 29.1 | 23.44 | 5.66 | 4.46 | 1.2 | 0.44 | 0.44/0.44 | 1.00 |

(18) Wang and Liu, 2024 confirmed this hypothesis by suggesting that higher levels of homocysteine, which is implicated as a cause of severe vascular diseases such as retinal vascular narrowing, an important component of diabetic retinopathy.

Various changes in cellular methylation metabolism have been shown to directly result in the imbalance of methionine and homocysteine cycles, which has been widely documented in studies related to diabetes complications (2).

On the other hand, other studies contradict these findings, with (6) that some diabetic patient has inhibition of *MTHFR* (due to epigenetic changes, e.g., hypermethylation) that will affect the production of 5-methyltetrahydrofolate, therefore the rising level of homocysteine.

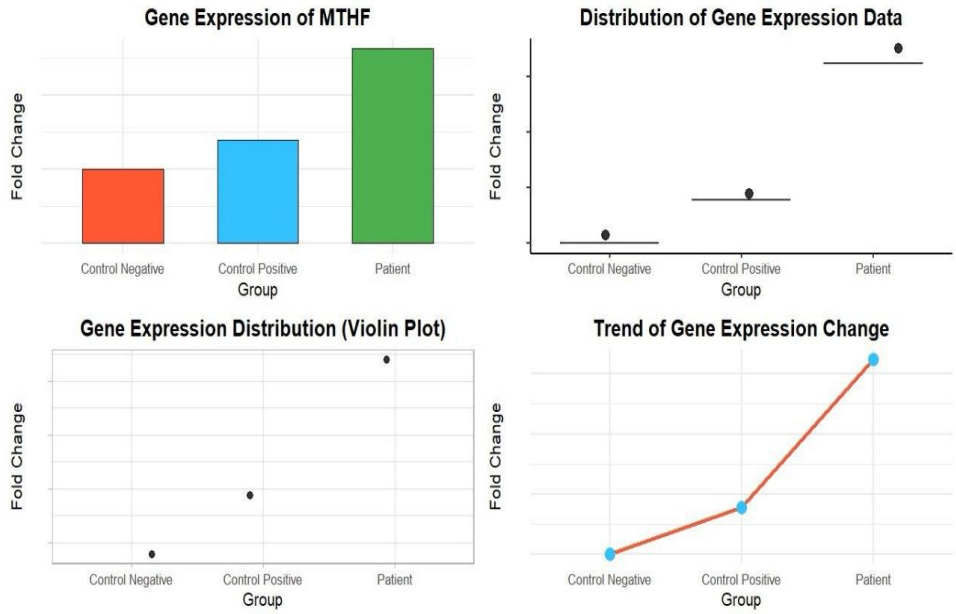


**Figure (1):Graphical Representation of *MTHFR* Gene Expression Among Study Cohorts Using Different Analytical Methods. The above figure illustrates the differences in the expression of the MTHFR gene among the study cohorts by using fold change analysis. The bar chart and trend analysis indicate that the patient group exhibits higher levels of gene expression compared to the two control groups. The distribution plots highlight significant differences in the gene expression values. The noted increase in gene expression lends strength to the implication that MTHFcould play a part in the development of diabetic retinopathy.**

Moreover, *MTHFR* gene expression levels in control positive group suggest that their increased expression (1.30-fold changes) is not a relevant marker for protective effect because it is a physiological response to oxidative

stress, which leads to the disease but will not completely avoid the diabetic course.

Previous studies had confirmed this observation that diabetic individuals with stable degrees of physiological methylation were less likely to suffer complications than those undergoing considerable changes in *MTHF*R activity (5).

The variability of changes in findings indicates other factors impacting the regulation of *MTHF*R, such as genetic and environmental factors (17). There are two factor which the higher levels of *MTHF*R expression in patients with diabetic retinopathy :(i) the retinal hypoxia, causing the induction of hypoxia-inducible factor (HIF-1α), a significant modulator of gene expression in ischemic environments (8) suggested that elevation of HIF-1α activity improves *MTHF*R gene expression as a compensatory response to rely on folate utilization over excessive methylation of cell homeostasis. However, despite the potential adaptive role of elevated *MTHF*R expression, this adaptation may be ineffective in controlling ongoing cellular damage, which may exacerbate diabetic retinopathy. Some studies have suggested that overproduction of 5-methyltetrahydrofolate may lead to an unbalanced increase in DNA methylation, suppressing the expression of other genes essential for vascular protection , and/or (ii) the high glucose level leads to increased oxidative stress, which triggers cytoprotective mechanisms, such as methylation pathways and the folate cycle, as the leading cause of the increased gene expression of *MTHF*R observed in diabetic patients with retinopathy (20 , 12). *MTHF*R is an essential enzyme for folate metabolism, and any modification in the *MTHF*R activity results in derangements of homocysteine and methionine levels, ultimately predisposing to more significant oxidative injury to retinal microvasculature (13, 7, 21).

**Conclusions:**

From the results of the current study , It was concluded that altered *MTHFR* expression can lead to elevated homocysteine, a risk factor for vascular damage. Hyperhomocysteinemia is associated with microvascular dysfunction, contributing to the progression of DR. So, Gene expression of *MTHFR*plays a role in DR and may serve as a prospective biomarker of this blinding disease in its relatively early stages. Finally, Understanding *MTHFR* gene expression in DR may help in developing targeted therapies, such as folate supplementation or homocysteine-lowering strategies, to slow disease progression.

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