# Detection the correlation between DNA methylation and gene expression in patients with ADPKD in Iraqi Population

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## Abstract

Polycystic Kidney Disease (PKD) is a genetic disease of the kidneys. PKD is one of the most common hereditary kidney diseases affecting millions worldwide. Flix Lejars was the first to use the term PKD in 1888, and PKD was identified as bilateral cysts growing inside the kidneys (ADPKD is caused by mutations in the PKD1 gene in 85% or due to mutations in the PKD2 gene in 15%. Mutations in the PKD1 gene or The PKD2 gene results in indistinguishable disease phenotypes; however, patients with PKD2 gene mutations often present with the disease later in life and have a better prognosis for the disease. Predominant polycystic kidneys in Dohuk city.

The current study included 71 patients between the ages of (20-55) years, who visited the kidney center in Dohuk Governorate, in a period of time ranging from June to September of 2022. The samples are divided into two groups depending on the biochemical results. The first group included 20 samples that were considered as a group. A control and the second group included 71 patients with renal disease. 5 ml of venous blood was taken from the two groups above, the blood sample was separated into three parts, and the first part was placed in tubes with EDTA anticoagulant for DNA extraction, and a part was placed in Eppendorf tubes containing trisol from In order to extract the RNA and the last part, it was placed in tubes devoid of any anticoagulant to separate the blood serum. The genetic variation of the (G/C) PKD1 gene was determined by the Tetra-ARMS-PCR technique, and the nucleotide sequence of the amplified pieces was determined based on the DNA Sequencing technique and the expression level of the PKD1 gene was analyzed based on on technique The RT-PCR and the measurement of DNA methylation in the primer of the PKD-1 gene were measured bilirubin).

The results of the study showed a decrease in the level of gene expression of the PKD1 gene in ADPKD patients compared to the level of gene expression in control samples. Control $\Delta$ $\Delta$CT = 3.2 As for the patient group, the gene expression rate was $\Delta$ $\Delta$CT = 0.35, and the results of the test following the DNA methylation process in the primer of the PKD1 gene showed that methylated DNA was present in all samples of the patients who were tested by 90% compared to the control group In which there was no methylation of the studied gene initiator, while the results showed the presence of bundles belonging to the unmethylated process in all samples, patients with healthy ones, at a rate of 100%. $\leq$0.05. The glucose level in patients was 270 (mg/dl) compared to healthy subjects, when the ratio was 105 (mg/dl). The levels of urea in patients were 107 (mg/dl) compared to healthy subjects, when the ratio was 45 (gm/dl). dl). Creatinine levels in patients were 5.11 (mg/dl) compared to healthy subjects, when the ratio was 0.90 (mg/dl).). Calcium levels in patients were 7.47 (mg/dl) compared to healthy subjects, when the ratio was 10 (mg/dl).)We conclude from this study that the studied genetic variation of the PKD1 gene is one of the main causes of polycystic kidney disease prevalent in the study samples.

**Keywords:** Autosomal Dominant Polycystic Kidney Disease (ADPKD), Epigenetic, Methylation

## Introduction

The most common kind of hereditary kidney disease, autosomal dominant polycystic kidney disease (ADPKD), is a ciliopathy with severe morbidity and mortality that affects one in 400–1000 people. This fatal hereditary. Condition affects around 12.5 million people worldwide (1-3). End-stage renal disease (ESRD) finally develops in the fourth or fifth decade of life as a result of several fluid-filled cysts developing in both kidneys along with renal failure (ESRD)(4) Among the extra-renal symptoms include the development of liver cysts, hypertension, flank discomfort, and a higher risk of cardiovascular conditions and cerebral aneurysms.

Disease development may be predicted using a number of indicators, such as the kind of mutant gene, patient age, gender, renal function, and kidney volume (5-6) Congenital hepatic fibrosis and polycystic liver disease (PLD) can both result from cyst formation in the liver (1) A harmful mutation was discovered in one copy of the PKD1 gene (PKD1) in around 85% of ADPKD families, and in the PKD2 gene in about 15% of cases (PKD2). It has been demonstrated that the protein products of PKD1 and PKD2, respectively, polycystins-1 and -2, bind to one another in the renal tubule epithelium through their C-terminal domains(7-8) In order to control the Ca2+ ion flow through polycystin-2, the extracellular Nterminal region of polycystin-1 protrudes on the primary cilia, which are thought to be triggered in response to renal flow as a mechanical sensor(9) A 52 kb section of chromosome 16p has a big gene called PKD1 that has 46 exons. A 14 kb GCrich transcript is encoded by it(10) Six highly homologous pseudogenes with 97.7% sequence identity to the wild-type gene are produced as a result of the genomic region that contains the majority of PKD1, including exons 1 to 33, being six times duplicated(11) Around 2300 disease-causing mutations in PKD1 have been identified as of this writing(12) The clinical characteristics seen in ADPKD patients are likely explained by the "Two-Hit Model," which has been defined as the underlying mechanism for family malignancies in which two distinct mutations impair appropriate genetic/cellular connections. According to this scenario, even if the sick person acquired the first hit, which is a germline mutation known as PKD1 or PKD2 mutations, cyst development doesn't start until after the second hit(7) There have been several ADPKD instances where neither PKD1 nor PKD2 mutations have been identified(13) Epigenetic modification is one theory for this observation. DNA methylation and histone post-translational modifications are the two primary mechanisms for epigenetic changes, which control gene expression through chromatin remodeling (14) By using the methyltransferase enzyme, DNA methylation is a process that adds methyl groups to the cytosine on a CpG island. While methylation of the promoter frequently suppresses gene production, methylation of the gene body, particularly at the 3′ end, is associated with higher transcription(15) Differentially methylated gene promoters have a function in a number of illnesses(16) Two studies have examined the methylation state of ADPKD tissues using genome-wide profiling, and they discovered that considerable hypermethylation of the PKD1 gene body but not the promoter was associated with lower PKD1 production(17-18).

**The study's objective**, is to detect the Correlation between DNA methylation and gene Expression level of PKD1 in ADPKD patients.

## Case Study

Over the period of June to September 2022, 71 patients of the age range of (20-55) years were reviewed by private pathological analysis laboratories in the city of Mosul. It was based on these clinical instances of the disease to choose Samples. Two groups of the samples were created components determined by biochemical results: The first group, which served as a control group, consisted of 20 people who were among those who did not have any health issues. Based on biochemical findings, the second group includes 71 patients who have renal disease.

## Collection of Blood sample

5 ml of venous blood 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 the PKD1 gene based on the technique the RT-PCR

The process of quantifying the level of gene expression of the PKD1 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, and according to the proven steps:

## Measurement of RNA concentration and purity

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

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

After the completion of the process of extracting the mRNA d, 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 the proven steps used the 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 |
|---|---|
| Pkd1 -RT-F | 5′-CTGCAGGAAGCACTCTACCC-3′ |
| Pkd1 -RT-R | 5′-CTCCCAGCCAACGTCGTAAT-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 | | |

The program shown in the table below was adopted to

perform the RT-PCR reaction:

**Table 3**

| No. | Stage | Temperature | Time | Cycle number |
|---|---|---|---|---|
| 1. | Initial denaturation | 95 | 10 min. | 1 |
| 2. | Denaturation | 95 | 30 sec. | 40 |
| 3. | Annealing | 96 | 30 sec. | |
| 4. | Extension | 58 | 30 sec. | |
| 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 PKD1 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 PKD1 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 PKD1 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 PKD-1 gene

### PKD1methylation by methylation specific polymerase chain reaction (MSP)

The DNA methylation test was carried out according to the method presented by the researcher (Hajirezaei *et al.,* 2020). 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 ( ) based on the PCR technique of the initiator region of the PKD1 gene of sequence bp1241 - bp1340 in the pre-cloning region (TSS) containing four CPG sites (Fatemeh *et al.,* 2020).

**Table 4:** Shows the primers used in the DNA methylation reaction

| Primer | Sequence |
|---|---|
| PKD1 -unmeth-F | 5′-TGGGAGTGTAGTGGTATAATTATGG-3′ |
| PKD1 -unmeth-R | 5′-AAAAACCAACCTAACCAACATAACA-3′ |
| PKD1 -meth-F | 5′-GCGGGAGTGTAGTGGTATAATTAC-3′ |
| PKD1 -meth-R | 5′-ACCAACCTAACCAACATAACGAA-3′ |

**Table 5:** Shows the size of the PCR reaction used in the DNA methylation process

| T | Components | Volume |
|---|---|---|
| 1 | DNA with bisulfate | 5 µl |
| 2 | F_primer | 1 µl |
| 3 | R_primer | 1 µl |
| 4 | 2x super-hot PCR master mix | 10 µl |
| 5 | D.W | 3 µl |
| Total volume 20 µl | | |

**Table 6:** Shows the PCR reaction program for DNA methylation

| No. | Stage | Temperature | Time | Cycle number |
|---|---|---|---|---|
| 1. | Initial denaturation | 95 | ***10*** min. | 1 |
| 2. | Denaturation | 95 | ***30*** sec. | ***40*** |
| 3. | Annealing | ***69*** | ***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 (Urea - Creatinine - Calcium) With the German-made Cobas integra 400 plus device.

### Results of a test measuring the gene expression level of the PKD1 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 |
|---|---|---|---|---|---|
| 28.6 | 28.3 | 19.5 | 17.6 | 10.5 | 9.5 |

The above table shows the values of CT and the level of gene expression for the PKD1 gene and the housekeeping gene for patients with ADPKD 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 that have been studied in addition to the variations The genes that were identified in the DNA Sequence tests, 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 quantity may be too small to meet the needs of the cells.

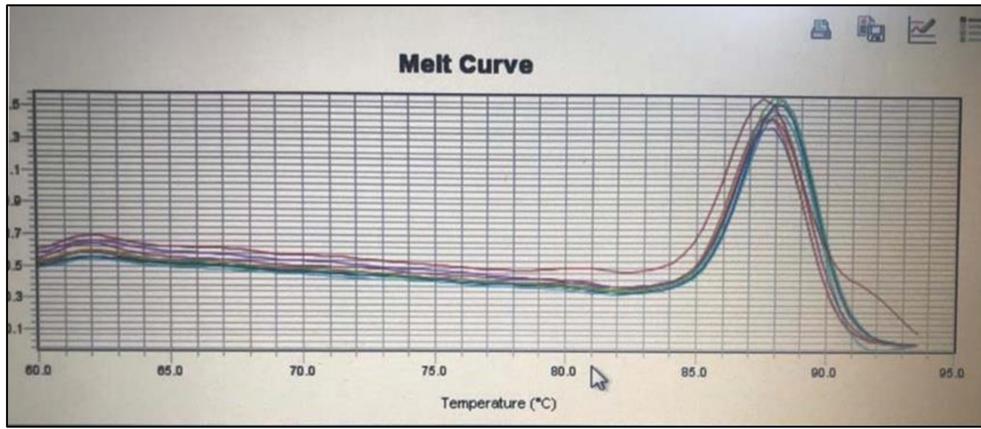


**Fig 1:** Shows the standard curve of the studied genes in the RT-PCR technique

The results of the study showed that there was a decrease in the level of gene expression of the PKD1 gene in ADPKD 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 = 0.38.

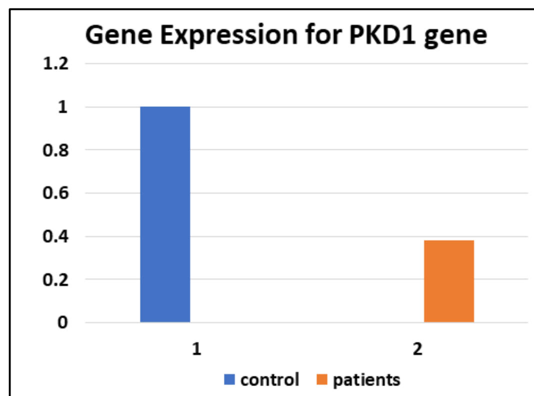


**Fig 2:** Shows the level of PKD1 gene expression in ADPKD patients and control group

From these results, it is clear to us the vital role of the PKD1 gene in the production of polycystin protein in the control group. As for patients with ADPKD, the significant decrease in the rate of gene expression is considered one of the main reasons for the development of the disease and the formation of cysts in the kidney due to the lack of manufactured polycystin protein. By the PKD1 gene, and this decrease in the level of gene expression is either due to an increase in the methylation process in the PKD1 gene promoter. A relationship has been identified between the level of expression of the PKD1 mRNA gene with the state of methylation as a result of this decrease, or because of genetic mutations in the coding region of the studied gene that plays the PC1 complex. PC2 has many roles in regulating intracellular signaling pathways, particularly with regard to mechanosensation. Polycystin also has an important role in interactions with other epithelial cell membrane proteins responsible for maintaining normal differentiated kidney structure and function. And the mutations associated with the genes encoding the polycystin proteins, which reduce the level of genetic expression of the PKD1 gene, have an important effect on the initiation of the formation and growth of the cyst, its expansion, and the secretion of fluids inside the cyst(Berbari *et al*.,2009)[20]

### Results of the DNA methylation test for the primer of the PKD1 gene

The results of the test for the DNA methylation process in the primer of the PKD1 gene showed that methylated DNA was present in all samples of the patients who were subjected to the test by 90% 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 PKD1 gene was determined within the samples included in the study, using special primers for each of the two cases of methylation and its absence, as shown in the figure().

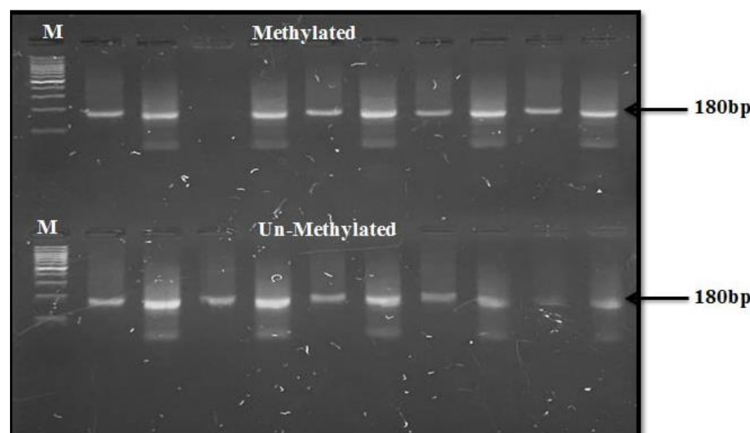


**Fig 3:** Shows the product of the PCR reaction of the DNA-methylated reaction. The size of the reaction product was 180 bp, carried over with a 2% agarose gel.

Likewise, the process of methylation in Promoter gene PKD1 was determined for patients and control samples, and the methylation rate for the gene was 90% in patients, and this percentage is high and negatively affecting the biological function of the gene, as well as for previous studies. It is worth noting that the severity of the pathogenesis of ADPKD patients includes several genetic factors, including epigenetic variations, mainly DNA methylation in gene promoters. Previous studies have indicated that variation in methylation patterns is associated with many tumors and genetic disorders. Also, the increase in the methylation process plays an important role in causing genetic mutations in genes. In turn, it directly affects the function of DNA. Previous studies have indicated that an increase in methylation processes leads to stopping or reducing the process of gene expression, and thus the amount of protein encoded by the gene is insufficient to perform the biological function. Little or ineffective and this case is directly affected by the process of DNA methylation**.** When observing the results of the gene expression of the PKD1 gene, we notice a decrease compared to the level of gene expression of the house keeping genes. The reason for this is that these genes do not contain CPG islands and therefore are not regulated by the methylation process. Hypermethylation in the primer of the PKD1 gene.
Previous studies have indicated an inverse relationship with the gene expression of the PKD1 gene resulting from the epigenitic regulation. The study was conducted on 40 people with ADPKD patients, and the methylation of stimuli in 20 people was high, and this indicates the role of methylation in stopping the gene expression of the PKD1 gene and the formation of cysts in the kidney (Hajirezaei *et al*.,2020)**.**[21]

**Biochemical indicators associated with kidney functions:**
The results showed that there was a significant increase in the level of both creatinine and urea in the serum of ADPKD patients compared to the control group at the level of probability P≤0.05. (gm/dl). Creatinine levels in patients were 5.11 (gm/dl), compared to healthy subjects, when the ratio was 0.90 (gm/dl). The results also showed a decrease in the concentration of calcium in the blood serum of patients, as the ratio was 7.47 (gm / dl). Compared to healthy subjects, the ratio was 10 (gm/dl).

**Table 8:** Results of biochemical variables of kidney function in patients with ADPKD and control group

| Sample | Urea (mg/dl) | Creatinine (mg/dl) | Calcium (gm/dl) |
|---|---|---|---|
| Patient | 107 ± 13.5 | 5.11 ± 0.45 | 7.47 ± 0.78 |
| Control | 43 ± 7.7 | 0.90 0.07 | 10 ± 0.56 |

It is known that serum creatinine and urea are used as diagnostic tests for kidney function, and the high concentration of creatinine and urea in the blood serum of patients with ADPKD may be attributed to the fact that they are two metabolic wastes that are naturally excreted through urine. This leads to less excretion of these residues, so they accumulate and accumulate, and their concentration in the blood serum rises(Vanholder *et al*.,2018)**.**[22] The reason for this may be due to the fact that urea is the main nitrogenous substance from the metabolic waste that was formed in the liver and is excreted outside the body through urine. The increase in urea reflects the failure of the filtration function of the kidney and the speed of urea formation. This increase depends on the severity and progression of ADPKD and the patient's immune status. An increase in the level of creatine and urea and urine retention are among the most important signs of the progression of ADPKD, which eventually leads to renal failure as a result of the loss of the renal functional unit represented by the nephrons
(Pandya *et al*.,2016) [23]

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