



# The Effect of *LRP-1* Gene and *Mir-205* on Cardiovascular Disease in Iraqi Patients with Familial Hypercholesterolemia

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## Abstract

Familial hypercholesterolemia (FH) is a genetic disorder characterized by autosomal inheritance in genes related to LDL-C metabolism, with the major clinical features of hyper-LDL-cholesterolemia and premature coronary artery disease. (LRP-1) is a member of the LDLR family. It is a membrane receptor with scavenging and signaling properties. LRP-1 interacts with a wide range of extracellular ligands as well as intracellular scaffolding and signaling proteins, which makes it important in crucial clinical circumstances like cardiovascular disease, cancer, and neurological illnesses. Mir-205 uses these molecules as biomarker for cardiovascular diseases. This study aims to measure gene expression for the LRP-1 gene and its relationship to the development of cardiovascular disease in familial hypercholesterolemia and non-familial hypercholesterolemia. Also, it studies the indication whether mir-205 regulates the action of the LRP-1 gene in terms of increasing or decreasing gene expression. However, the available methods for measuring LRP1 levels are direct and quantitative using Poly Chain Reaction (RT-PCR) in real time, not at its ends. In the present study, blood was isolated from 150 individuals distributed into three groups: Group 1 included: 50 samples from a healthy group; Group 2: 50 samples from non-Familial hypercholesterolemia patients; Group 3: 50 samples Familial hypercholesterolemia patients. The results showed that LRP1 protein expression was significantly reduced in patients with F.H compared with normal control in a small cohort from an Iraqi population. This pilot study suggests that the reduced LRP1 protein expression may be associated with cardiovascular disease progression.

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## 1. Introduction

Familial hypercholesterolemia is a term used to describe a group of inherited and acquired illnesses in which the body's lipid levels are abnormally high [1]. Generally, hypercholesterolemia does not have any obvious symptoms but they are usually discovered during a routine examination or until it reaches the danger stage of a stroke or heart attack [2]. Primary (familial) hypercholesterolemia and secondary (acquired) hypercholesterolemia are the two types of hypercholesterolemia. Primary hypercholesterolemia is caused by a variety of genetic disorders that a patient can inherit at birth. In contrast, secondary hypercholesterolemia is caused by a different underlying cause: poor diet, medications (amiodarone, glucocorticoids), hypothyroidism, uncontrolled diabetes, and/or a poor lifestyle regimen [3, 4]. Cholesterol is an important building block in the formation and repair of cell walls, a function of nerve tissue, and the production of hormones such as testosterone, estrogen, and the stress hormone cortisol [5, 6]. In adults, hypercholesterolemia has been shown to be a major risk factor in developing CVD [7].

Genes are nucleotide sequences in DNA or RNA that code to produce a gene product, such as protein [8]. Low-density lipoprotein receptor-related protein-1 (LRP-1) is a member of the LDLR family. It scavenges and signals actions that are shared by a membrane receptor. LRP-1 interacts with a wide range of extracellular ligands as well as cytoplasmic scaffolding and signaling proteins, making it important not just in essential pathological situations including cardiovascular disease, cancer, and neurological disorders, but also in everyday life [9, 10]. MicroRNA 205 (miR-205) is a well-known miRNA that regulates various biological processes including, cell survival, death, angiogenesis, and metastasis [11].

Gene expression is the process of using a gene's genetic code, or nucleotide sequence, to produce a functioning gene product. It entails a series of stages in which DNA is transformed into RNA then transformed into a protein [12]. Many studies focused on the role of the LRP1 gene and mir-205 in association with cardiovascular disease by using RT PCR [13, 14]. Ischemic heart disease (myocardial infarction, angina) and stroke are both caused by narrowed or blocked (thrombosis) blood arteries, which can lead to ischemic heart disease (IHD) (myocardial infarction, angina) or stroke. This makes it impossible for blood to reach the heart or the brain. The most common cause is a thrombus (clot) that arises when fatty deposits on the inner walls of blood vessels pool and obstruct the arteries. Blood clots or bleeding from a blood artery in the brain can cause strokes [15, 16]. Our study aimed at measuring gene expression for the *LRP-1* gene and its relationship to the development of cardiovascular disease in familial hypercholesterolemia and screening for cardiovascular disease using mir-205 as a biomarker specific and sensitive for the LRP-1 gene. However, it is worthy to mention that, this study is the first in Iraq, which tackles the effect of mir-205 on the lrp-1 gene and affect the genes on patients with familial hypercholesterolemia.

## 2. Methods and Materials

This study was taken place during the period from October 2020 to May 2021. All the study experiments were performed at the University of Technology and Iraqi Hereditary company (IHC) [17].

### 2.1. Study Group

The total number of participants in the study was 150 individuals [18]. The samples were taken randomly according to presence of patients in the place, study groups included the following: -

Group 1: Fifty samples of apparently healthy individuals of both sexes, aged between (30-75 years) were obtained for control.

Group 2: Fifty patients' samples of Iraqi men and women diagnosed with non-familial hypercholesterolemia, aged between (37 and 70 years). The samples were collected at Ibn Al-Nafees Hospital for Cardiovascular Medicine and Surgery in Baghdad/Iraq.

Group 3: Fifty patient's samples of Iraqi men and women diagnosed with familial hypercholesterolemia, aged between (30-70 years) the samples were collected at Ibn Al-Nafees Hospital for Cardiovascular Medicine and Surgery in Baghdad/Iraq.

### 2.2. Blood Sampling

From each participant, 2 ml of whole blood was needed to be collected from the venous blood directly into an EDTA containing tube, this procedure was done under sterile conditions [19].

### 2.3. Total RNA Extraction with Trizol

The RNA extraction from the whole blood of both patient and healthy control with TRIzol subjects using protocol in EasyPure® TransZol Up plus RNA kit (Cat. No.: ER501). TRIzol Lysis Reagent was added after the samples were taken. To separate the aqueous phase, chloroform was added to the lysate. After that, the samples were vortexed, incubated for 3 minutes at room temperature, and centrifuged for 15 minutes at 10,000 rounds per minute. To create adequate binding conditions for all RNA molecules, ethanol was added to the separated aqueous phase. After that, samples were eluted in RNase-free water and kept at -20 °C until further processing [20].

## 2.4. MiRNA Extraction from Whole Blood

The miRNA extraction from whole blood of both patient and healthy control is collected by using the protocol in EasyPure® miRNA kit (Cat. No.: ER601). First, Lysis Buffer10 (LB10) was fully added to the blood mix. Incubate for 5 minutes at room temperature. Then, for the previous components, chloroform was added. Shake the tube briskly by hand for 3 minutes at room temperature, then centrifuge the sample for 15 minutes at 10,000rpm. To create optimal binding conditions for all miRNA molecules, ethanol was added to the separated aqueous phase. After that, miRNA was eluted in RNase-free water and kept at 20 °C until further processing [21].

## 2.5. Primers

The primers designed in this research are obtained using bioinformatics program NCBI (national center biotechnology information). The optimum concentration of genes forward and reverse primers were 10 Picoml. Primers used in this study with their sequences are shown in (Table 1).

**Table (1):** primers used in the study designed by the researchers.

Primer	Sequence (5'→3' direction)
LRP1 Human qPCR expression Primer	
Forward	CAACGGCATCTCAGTGGACTAC
Reverse	TGTTGCTGGACAGAACCACCTC
GAPDH Human qPCR expression Primer	
Forward	TGAGAAGTATGACAACAGCC
Reverse	TCCTTCCACGATACCAAAG
miRNA	
MiR205 F.P.	TCCTTCATTCCACCGGAGTCTG
miRU6 F.P.	AGAGAAGATTAGCATGGCCCCT
MiRNA-universe R.P.	GCGAGCACAGAATTAATACGAC

## 2.6. Real Time PCR (qRT-PCR)

qRT-PCR was performed using the strata gene Real-time PCR System (Analytik Jena Technologies) with qPCRsoft software. Gene polymorphism was quantified by probe color reaction employing the 2xqPCR Master Mix Kits components as shown in (Table 2). A non-template control (NTC), non-amplification control (NAC), and non-primer control (NPC) were included as negative controls for each reaction in duplicate [22]. The expression ratio was calculated without a calibrator sample  $2^{-\Delta Ct}$  according to the following:

$$\Delta CT (\text{test}) = CT \text{ gene of interest (target, test)} - CT \text{ internal control} \quad (1)$$

$\Delta\Delta CT$  was calculated according to the following equation:

$$\Delta\Delta CT = \Delta CT (\text{test}) - \Delta CT (\text{calibrator}) \quad (2)$$

**Table 2.** Reaction Component and volume for RT PCR used in *LRP-1* gene expression experiment.

Component	Volume
Master mix Syper Green	10µl
Forward primer	1µl
Revers primer	1µl
CDNA	2µl
Nuclease free water (N.F.W)	6µl
Total volume	20 µl

### 2.7. The qPCR Reaction Run

The cycling protocol was programmed according to the thermal profile shown in (Table 3).

**Table 3.** Thermal profile of LRP-1 gene expression.

Step	Temperature	Duration	Cycles
Enzyme activation	94°C	30 sec	Hold
Denature	94 °C	5 sec	40
Anneal	64 °C	15 sec	
Extend	72 °C	15 sec	

### 2.8. Housekeeping Gene Amplification

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH). As an internal control, the housekeeping gene was employed to calculate the  $\Delta CT$  value [23]. The Thermal profile presented was used in a qPCR experiment for GAPDH amplification (Table 4).

**Table (4):** Thermal profile of GAPDH gene expression.

Step	Temperature	Duration	Cycles
Enzyme activation	94°C	30 sec	Hold
Denature	94 °C	5 sec	40
Anneal	54 °C	15 sec	
Extend	72 °C	15 sec	

### 2.9. Statistical Analysis

The Statistical Analysis System- SAS (2012) program was used. Least significant difference –LSD test (Analysis of Variation-ANOVA) was used to make significant comparison between means. Chi-square test was used to make a significant comparison between percentage (0.05 and 0.01 probability). Estimation of the correlation coefficient between variables in this study is achieved [24].

### 3. Results and Discussion

The Ct value of *GAPDH*, the housekeeping gene used in this research, is seen in (Table 5). The range of Ct value for *GAPDH* in the healthy group was 16.28-18.43 with a mean  $\pm$  SD ( $17.68 \pm 0.53$ ). It ranged from 17.12-18.43 with a mean  $\pm$  SD ( $17.70 \pm 0.36$ ) for the H group. The F.H group range from 16.08-18.53 with a mean  $\pm$  SD ( $17.71 \pm 0.51$ ). A non-significant difference was found between these groups regarding the mean Ct value of *GAPDH*, ( $p = 0.935$ ;  $p < 0.05$ ) with an LSD value of (0.187).

**Table (5):** Comparison between the different studied groups in the *GAPDH* Ct value of (Mean $\pm$ SD).

Group	No.	Mean $\pm$ SD of Ct value	Range
Group 1 Control	50	$17.68 \pm 0.53$	16.28-18.43
Group 2 Hypercholesterolemia	50	$17.70 \pm 0.36$	17.12-18.43
Group 3 Familial Hypercholesterolemia	50	$17.71 \pm 0.51$	16.08-18.53
LSD	--	0.187 NS	---
P-value	--	0.935	---
NS: Non-Significantly.			

The inherent assumption in using housekeeping genes in molecular studies is that their expression remains constant in the cells or tissue under investigation. One of the most commonly used housekeeping genes in the companion of gene expression data is *GAPDH* [25]. Studying the expression of 1,718 genes using qRT-PCR, the *GAPDH* has been applied as a reference gene in 72 kinds of normal human tissue. It has been found out that using *GAPDH* is quite a reliable strategy for the normalization in qRT-PCR when applied in clinical studies. To improve this, the variance of the total change in *GAPDH* expression was analyzed in different study groups using the  $2^{-Ct}$  value and the ratio of  $2^{-Ct}$  of the different study groups to that of the control group, as shown in (Table 6). The  $2^{-Ct}$  value of the healthy group was  $4.681E-06$ . For the H group, it was  $4.755E-06$  and for the F.H group it was  $4.645E-06$ . The computed ratio for gene fold expression was  $1.00 \pm 0.00$  for the healthy groups,  $1.0159 \pm 0.04$  for the H group and  $0.992 \pm 0.04$  for the F.H group. The results show differences in CT values low for H group and more lower for F.H group. Our findings are also in line with those of this study [26, 27], which looked at the possible association of the LRP-1 gene with genetic damage, hypercholesterolemia, and familial hypercholesterolemia risk.

**Table (6):** Comparison of *GAPDH* Fold Expression between Control, H and F.H groups.

Group	Means Ct of <i>GAPDH</i>	2-Ct	Experimental group/ Control group	Fold of gene expression
Group 1 C.	17.7048	$4.681E-06$	$4.681E-06/4.681E-06$	$1.00 \pm 0.00$
Group 2 H.	17.682	$4.755E-06$	$4.755E-06/4.681E-06$	$1.0159 \pm 0.04$

Group 3 F.H.	17.7158	4.645E-06	4.645E-06/4.681E-06	0.992 ±0.04
LSD value	--	--	--	0.263 NS
NS: Non-Significantly.				

In this study, a quantitative RT-PCR technique was used to examine the LRP-1 mRNA expression and compare it to that of the seemingly healthy control group, the H group, and the F.H group. The fold change in gene expression was calculated using relative quantification. This is dependent on the normalization of Ct values when calculating the  $\Delta Ct$ , which is the difference between the mean Ct values of each case's replica of LRP-1 cDNA amplification and the GAPDH. The result of each group's  $2^{-\Delta Ct}$  was compared to that of the control group to determine the gene expression folds in respect to the housekeeping genes. (Table 7) displays the results. In group H, the gene expression fold was half that of the healthy group. It was much lower (0.1) in the F.H group than it was in the healthy group. The expression of the LRP-1 gene in these populations was found to be significantly lower.

**Table (7):** Fold of *LRP1* expression Depending on  $2^{-\Delta Ct}$  Method.

groups	Means Ct of <i>LRP1</i>	Means Ct of <i>GAPDH</i>	$\Delta Ct$ (Means Ct of <i>LRP1</i> - Means Ct of <i>GAPDH</i> )	$2^{-\Delta Ct}$	Experimental group/ Control group	Fold of gene expression
Group 1 Control.	26.36	17.7	8.66	0.0024722	0.00247/0.00247	1.00 ±0.00 a
Group 2 H.	27.17	17.68	9.49	0.0013907	0.00139/0.00247	0.5625 ±0.06 b
Group 3 F.H.	29.16	17.71	11.45	0.0003574	0.00035/0.00247	0.14459 ±0.08 c
LSD value	--	--	--	--	--	0.278 **
** ( $P \leq 0.01$ ).						

The calculation of the relative expression of the LRP-1 gene in all study groups was achieved by applying the  $2^{-\Delta\Delta Ct}$  results. A calibrator used was one of the samples of the controls with low expression of LRP-1. As shown in (Table 8), the mean of  $2^{-\Delta\Delta Ct}$  values of the Healthy group, H group and F.H group it (0.2191), (0.1232) and (0.0316), respectively. Thus, there was a significant difference between these groups regarding the mean  $2^{-\Delta\Delta Ct}$ , ( $p \leq 0.01$ ). We hypothesize that microRNA, known for gene silencing either through mRNA degradation or translation repression, may contribute in the down-regulation of LRP1 expression, as one microRNA, miR-205, has been reported to regulate LRP1. This miR-205 has been found to suppress LRP1 expression by limiting the translation of LRP1 mRNA into proteins without changing the mRNA level, which is consistent with our findings of lower LRP1 protein expression. As a result, it is thought that miR-205 is overexpressed in the cardiovascular system, suppressing LRP1 translation and lowering LRP1 protein levels [28].

**Table (8):** Fold of LRP1 expression Depending on 2- $\Delta\Delta$ Ct Method.

Groups	Means Ct of LRP1	Means Ct of GAPDH	$\Delta$ Ct (Means Ct of LRP1 - Means Ct of GAPDH)	Mean $\Delta$ Ct Calibrator (ct LRP1 - ct GAPDH)	$\Delta\Delta$ Ct	2- $\Delta\Delta$ Ct	Experimental group/ Control group	Fold of gene expression
Group 1 C.	26.36	17.7	8.66	6.47	2.19	0.2191	0.2191/0.2191	1.00 $\pm$ 0.00 a
Group 2 H.	27.17	17.68	9.49	6.47	3.02	0.1232	0.1232/0.2191	0.5625 $\pm$ 0.06 b
Group 3 F.H.	29.16	17.71	11.45	6.47	4.98	0.0316	0.0316/0.2191	0.14459 $\pm$ 0.08 c
LSD value	--	--	--	--	--	--	--	0.278 **
** (P $\leq$ 0.01).								

#### 4. Conclusions

The expression of the LRP-1 gene was found to be a helpful marker in the prediction of cardiovascular disease. Furthermore, the significant connection between LRP-1 gene and mir-205 suggests that this biomarker may play a role in the pathophysiology of atherosclerotic coronary artery disease (ASCAD) and could be used as a valid diagnostic and therapeutic marker.

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#### Conflict of Interest

The authors declare that they have no conflict of interest.

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