Iran. J. Ichthyol. (Special issue 1, 2021): 246-254

P-ISSN: 2383-1561; E-ISSN: 2383-0964
http://www.ijichthyol.org

Research Article

Low-density lipoprotein receptor-related protein-1 and mir-205 expression for cardiovascular disease in familial hypercholesterolemia and non-familial hypercholesterolemia in Iraqi population

Zinah Abdulkareem GHAREEB[1*], Hiba M. Abdel-Hassan AL-KHAFAJI[1], Mohanad K. Aneed AL-SAEDI[2]

[1]*Department of Applied Science, University of Technology, Baghdad, Iraq.*
[2]*Iraqi Hereditary Company, Baghdad, Iraq.*
[*]*Email: Zinah.A.Ghareeb@uotechnology.edu.iq*

Abstract: Familial hypercholesterolemia (FH) is an autosomal dominant lipid metabolic condition that affects people from birth. Low-density lipoprotein cholesterol (LDL-C) levels are extremely high. Patients with familial hypercholesterolemia and hypercholesterolemia have a nearly two-fold increased risk of developing cardiovascular disease (CVD). *LRP-1* serves as a scavenger receptor, a regulatory receptor, and a scaffold receptor. A total of 150 blood samples were taken from people, with 50 of them being patients with familial hypercholesterolemia, non-familial hypercholesterolemia, and healthy control subjects. This study aimed to measure gene expression for *LRP-1* gene and *mir-205* and indicating their relationship to the development of cardiovascular disease in familial hypercholesterolemia and non-familial hypercholesterolemia. Also, screening for familial hypercholesterolemia and its connection cardiovascular disease using *mir-205* as a biomarker specific and sensitive for the *LRP-1* gene was done. The expression of *LRP-1* and *mir-205* in whole blood was estimated using reverse transcriptase quantitative real time polymerase chain reaction. The results showed that the expression of *LRP-1* in the fold of gene expression in F.H patients' group was lower than that of healthy group, while the expression of *mir-205* in the fold of gene expression in F.H patients' group was 14 time higher than that of healthy group. The results also showed low *LRP-1* expression is present in familial hypercholesterolemia and non-familial hypercholesterolemia. The familial hypercholesterolemia group was associated with the lowest expression of *LRP-1* and followed by the non-familial hypercholesterolemia. This reflects an increased susceptibility to cardiovascular disease. overexpression of *mir-205* is found in familial hypercholesterolemia and non-familial hypercholesterolemia. The familial hypercholesterolemia group was associated with the highest expression of *mir-205* and followed by the non-familial hypercholesterolemia. This reflects that *mir-205* is overexpressed in the cardiovascular system, suppressing *LRP1* translation and thereby lowering *LRP1* protein levels.

Keywords: Hypercholesterolemia, *LRP-1* gene, *Mir-205,* Gene expression, RT-PCR.
Citation: Ghareeb, Z.A.; Al-Khafaji, H.M.A. & Al-Saedi, M.K. 2021. Low-density lipoprotein receptor-related protein-1 and *mir-205* expression for cardiovascular disease in familial hypercholesterolemia and non-familial hypercholesterolemia in iraqi population. Iranian Journal of Ichthyology 8(Special issue 1): 246-254.

Introduction

Familial hyperlipidemia is a disorder of lipoprotein metabolism that is passed down through the generations. Despite taking the proper lipid-lowering medication, these patients still have a higher risk of developing cardiovascular disease than the general population. More than 1500 mutations in the LDL receptor gene (LDLR) have been discovered, as well as mutations in other genes that cause the clinical FH phenotype (Wong et al. 2016; Lin et al. 2019). The main clinical manifestation of FH is atherosclerotic lesions in the heart, brain, and peripheral arteries as a

Table 1. Thermal cycler steps.

	Step1	Step2	Step3
Temperature	25°c	42°c	85°c
Time	10min	15min	5seconds
Reaction	Random Primer (N9) binding	Anchored Oligo(dT)18 binding	Inactivate reverse transcriptase enzyme

result of prolonged exposure of the vasculature to high levels of LDL-C (Najam & Ray 2015). LDL-C values, physical signs, family history of high LDL-C and early ASCVD, and more recently, genetic testing are used in the diagnosis of FH (Lee et al. 2019). Patients with FH are up to 16 times more likely than the general population to develop ASCVD (atherosclerotic cardiovascular disease) (Do et al. 2015).

LRP-1 is a membrane receptor serves as a scavenger receptor, a regulatory and scaffold receptor (Potere et al. 2019). *LRP-1* is synthesized as a large glycosylated transmembrane protein of 600 kDa (Lillis et al. 2008). The human *LRP-1* gene comprised 89 exons covering 92 kb of genomic DNA locating on chromosome 12q13-14. In normal tissues, *LRP-1* is expressed by hepatocytes, macrophages, fibroblasts, neurons, and VMSCs (Klar et al. 2015).

MirRNAs are non-coding single RNAs (ncRNAs) that bind to the three primary untranslated regions (3′ UTRs) of target messenger RNAs (mRNAs) and influence gene expression at the post-transcriptional stage (Feng et al. 2020). MicroRNAs are found all over the body and play a key role in the onset and progression of a variety of diseases. MiRNAs are presently being explored extensively as potential biomarkers of numerous diseases due to their high stability and ease of detection (Marrone et al. 2015). MicroRNA-205 (*miR-205*) is a highly conserved miRNA that is found in epithelial tissues of several animals. The pre-miR-205 sequence is found in the final intron/exon junction of the miR-205 Host gene (MIR205HG) gene on chromosome 1q32.2 in the human genome (Lim et al. 2003; Qin et al. 2013). Inflammation and atherosclerosis are induced in vascular endothelial cells by *mir-205*, which targets tissue inhibitor of metalloproteinase 3, which protects vascular endothelial cells by interfering with *miR-205* expression. In endometrial cancer cells, *miR-205* has been shown to directly influence the expression of phosphatase and tensin homolog, as well as block apoptosis (Kim et al. 2014). Furthermore, *mir-205* was shown to be expressed in myocardial apoptosis, which was boosted by *miR-205* upregulation but decreased by *miR-205* downregulation. These findings suggested that downregulation of *miR-205* was one of the major reasons why short-term therapy enhanced cardiac function in chronic heart failure CHF (Xuan et al. 2017).

This study aimed to measuring gene expression for *LRP-1* gene and *mir-205* and indicating their relationship to the development of cardiovascular disease in familial hypercholesterolemia and non-familial hypercholesterolemia and screening for Familial hypercholesterolemia and its connection cardiovascular disease using *mir-205* as a biomarker for the *LRP-1* gene.

Material and Methods

Participants: A total of 100 patients with cardiovascular disease were enrolled in this case-control research (CVD). The patients were divided into two groups based on their clinical condition. The first group consisted of 50 patients with F.H, while the second group consisted of 50 individuals with H. from during October 2020 to May 2021, samples were collected from the Ibn Al-Nafees Hospital for Cardiovascular Medicine and Surgery in Baghdad, Iraq, for diagnosis and treatment, as well as private laboratories in Baghdad, Iraq. Clinical examination

Table 2. Primers used in the study.

Primers	Sequence (5′→3′ direction)
LRP1 Human qPCR expression Primer	
Forward	CAACGGCATCTCAGTGGACTAC
Reverse	TGTTGCTGGACAGAACCACCTC
GAPDH Human qPCR expression Primer	
Forward	TGAGAAGTATGACAACAGCC
Reverse	TCCTTCCACGATACCAAAG
miRNA	
Mir-205 F.P.	TCCTTCATTCCACCGGAGTCTG
miRU6 F.P.	AGAGAAGATTAGCATGGCCCCT
MiRNA-universe R.P.	GCGAGCACAGAATTAATACGAC

Table 3. Comparison between difference groups in Lipid profile.

Group	Mean ± SE (mg/dl)				
	Cholesterol	Triglyceride	HDL	LDL	VLDL
G1: Control	159.74±54.14 c	115.01±5.87 c	54.18±2.53 ab	82.50±4.59b	22.96±1.19c
G2: H.	264.52±5.88 b	388.77±39.59 b	58.86±2.04 a	131.30±8.49a	77.85±7.91b
G3: F.H.	305.68±6.62 a	664.60±18.41 a	49.18±2.01 b	122.80±2.46a	132.92±3.68a
LSD	16.525**	71.096**	6.164**	16.088**	14.223**
P-value	0.0001	0.0001	0.0094	0.0001	0.0001

** ($P \leq 0.01$); Means having with the different letters in same column differed significantly.

was used to make the diagnosis, and a standardized questionnaire was used to acquire the patient's comprehensive medical history. The study included 50 Iraqi control volunteers in addition to the patients.

Blood sampling: Under strict aseptic conditions, sufficient blood samples were taken from each subject. The samples were split into two sections; one was used to test the serum, and for the relative quantification (RQ) of *mir-205* and *LRP-1* expression, another half of the blood was kept in tubes containing EDTA at 20°C.

LRP-1 expression and RQ of Mir-205: Real-time PCR (RTq-PCR) was used to measure the expression of the *LRP-1* gene and the *mir-205* gene. RNA was extracted from the blood of both patients and healthy controls using the EasyPure® TransZol Up plus RNA kit technique, and miRNA was extracted using the EasyPure® miRNA kit procedure. According to the manufacturer's instructions, the expression was reverse transcribed for cDNA synthesis using the EasyScript® One-Step gDNA Removal and cDNA Synthesis SuperMix (Transgen, China) kit. RTq-PCR analysis was performed using the TransStart® Top Green qPCR SuperMix (Transgen, China) according to the manufacturer's procedure to assess the expression. Thermal cycler steps of conditions cDNA Reverse Transcription are show in Table1.

Primers: The primers designed in this research are obtained using bioinformatics program NCBI. Primers used in this study with their sequences are shown in (Table 2).

Gene expression analysis using real-time qRT-PCR: The reference gene was *GAPDH* for *LRP-1* and *miRU6* for *mir-205*, and the quantification gene expression was reported as the ΔCT value (Abed & Al-Khafaji 2021), which was determined by subtracting the CT values of the reference gene from the CT values of the target gene using the equation: CT gene of interest (target, test) – CT internal control = ΔCT (test)

Finally, using the formula, the expression ratio was determined as:

$2^{-\Delta Ct}$ = Ratio of normalized expression

To compare the transcript levels between different samples, the $2^{-\Delta\Delta Ct}$ technique was utilized (Al-Khafaji 2017; Obaid & AL-Saadi 2018). The CT of

Table 4. Fold of LRP1 expression depending on 2-ΔCt method.

groups	Means Ct of LRP1	Means Ct of GAPDH	ΔCt (Means Ct of LRP1 - Means Ct of GAPDH)	2-ΔCt	experimental group/ Control group	Fold of gene expression
Group 1 C.	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 **

** ($P \leq 0.01$); means having with the different letters in same column differed significantly.

Table 5. Fold of LRP1 expression depending on $2^{-\Delta\Delta Ct}$ method.

groups	Means Ct of LRP1	Means Ct of GAPDH	ΔCt (Means Ct of LRP1 - Means Ct of GAPDH)	Mean ΔCt Calibrator (ct LRP1 -ct GAPDH	ΔΔCt	2-ΔΔCt	experimental group/ Control group	Fold of gene expression
Group 1 Control.	26.36	17.7	8.66	6.47	2.19	0.2191	0.2191/0.2191	1.00±0.00a
Group 2 H.	27.17	17.68	9.49	6.47	3.02	0.1232	0.1232/0.2191	0.5625±0.0b
Group 3 F.H.	29.16	17.71	11.45	6.47	4.98	0.0316	0.0316/0.2191	0.14459±0.08c
LSD value	--	--	--	--	--	--	--	0.278 **

** ($P \leq 0.01$); means having with the different letters in same column differed significantly.

gene of interest was adjusted to that of internal control gene. The following formula was used to calculate the difference in cycle threshold (Ct) values between the housekeeping gene (internal control gene) and the *mir-205* and *LRP-1* gene (interest gene):

CT gene of interest (target, test) – CT internal control = ΔCT (test)

CT gene of interest (target, calibrator) – CT internal control = ΔCT (calibrator).

Results

Laboratory finding (Biochemical test): The mean of cholesterol in H, FH and control were 264.52±5.88, 305.68±6.62 and 159.74±54.14, respectively (Table 3), showing the significant difference in the mean cholesterol of patients with F.H compared to H and control (P=0.0001). The mean of triglyceride in H, FH and control were 388.77±39.59, 664.60±18.41 and 115.01±5.87, respectively, revealing significant association between the high triglyceride and F.H. The mean triglyceride was higher in F.H compared to H group and control (P=0.0001). The mean of high density lipoprotein in H, FH and control were 58.86±2.04, 49.18±2.01 and 54.18±2.53, respectively; showing the high significant association between the high HDL and H. The mean of HDL is higher in group with H compared to F.H and control groups (P=0.0094) (Table 3).

The mean of low density lipoprotein in H, FH and control were 131.30±8.49, 122.80±2.46 and 82.50±4.59, respectively, showing the high significant association between the high LDL and H. The mean±SE LDL was higher in group with H compared to F.H group and control (P=0.0001). The mean of very low density lipoprotein in H, FH and control were 77.85±7.91, 132.92±3.68 and 22.96±1.19, respectively, showing the high significant association between the high VLDL and F.H group. The mean VLDL was higher in group with F.H compared to H group and control (P=0.0001) (Table 3).

Fold of LRP1 expression depending on $2^{-\Delta Ct}$ method and $2^{-\Delta\Delta Ct}$ method: The study participants were divided into three groups: healthy controls (n = 50), H patients (n = 50), and F.H patients (n = 50). The relative quantitation approach was used to

Table 6. Comparison between different groups in Ct value of miRU6 (Mean±SD).

Group	No.	Mean±SD of Ct value	Range
Group 1 C.	50	32.10±0.86	30.88-33.69
Group 2 H.	50	30.09±0.93	28.46-31.52
Group 3 F.H.	50	28.24±3.89	21.00-36.50
LSD	--	0.281 NS	---
P-value	--	0.935	---

NS: Non-Significant

Table 7. Comparison of miRU6 Fold expression between study groups.

Group	Means Ct of miRU6	2-Ct	experimental group/ Control group	Fold of gene expression
Group 1 C.	21.9906	2.4E-07	2.4E-07/2.4E-07	1.00±0.00
Group 2 H.	22.0138	2.361E-07	2.361E-07/2.4E-07	0.984±0.08
Group 3 F.H.	22.0088	2.37E-07	2.37E-07/2.4E-07	0.987±0.07
LSD value	--	--	--	0.261 NS

NS: Non-Significant

determine the quantitative expression of the *LRP-1* gene using (RT-qPCR). The gene expression was quantified using the ΔCt value and folding ($2^{-\Delta\Delta Ct}$) method after being normalized to the level of a housekeeping gene (GAPDH). The ΔCt mean of *LRP-1* was significantly lower in H group compared to control (0.5625±0.06 vs. 1.00±0.00) but the difference was significant compared to patients with F.H (0.14459±0.08 vs. 1.00±0.00) (Table 4). However, the relative expression $2^{-\Delta\Delta Ct}$ of *LRP-1* was low expression of *LRP-1* ($P \leq 0.01$) (Table 4).

The F.H group had a much lower gene expression calculation, (0.1) than other groups. The H group had a half-fold lower fold number than the healthy group (Table 5). The low expression in the F.H group was clearly visible when compared to the H group. The H group, on the other hand, has a large number of expressing people. This gene expression study might be repeated every year as a crucial follow-up analysis.

Comparison between different groups in Ct value of miRU6: The Ct value of *miRU6*, the housekeeping gene in the present study is shown in Table 6. The range of Ct value for *miRU6* in the healthy group was 30.88-33.69 (32.10±0.86). For the H group, it ranged from 28.46-31.52 (30.09±0.93). In the F.H group, it ranged from 21.00-36.50 (28.24±3.89). A non-significant difference was found between these groups regarding the mean Ct value of *miRU6* (P=0.935) with an LSD value of 0.281. The inherent assumption in the use of 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 *miRU6.*

To further improve this and although there was a significant difference in the mean Ct value between groups in the present study, the variation of the total change in expression of *miRU6* was studied in different study groups utilizing the 2^{-Ct} value and the ratio of 2^{-Ct} of the different study groups to that of the control group (Table 7). The 2^{-Ct} value of the healthy group was 2.4E-07; for the H group 2.361E-07 and for the F.H group 2.37E-07. The computed ratio for gene fold expression was 1.00 for the healthy groups, 0.984 for the H group and 0.987 for the F.H group. The *miRU6* serves as a helpful control gene due to the minor differences in gene fold expression between the study groups.

Fold of miR-205 expression depending on $2^{-\Delta Ct}$ method and $2^{-\Delta\Delta Ct}$ method: A quantitative RT-PCR technique was used to examine *mir-205* mRNA expression and compare it to that of the seemingly healthy control group, the H group, and the F.H

Table 8. Fold of miR-205 expression depending on $2^{-\Delta Ct}$ method.

Groups	Means Ct of miR205	Means Ct of miRU6	ΔCt (Means Ct of miR205 - Means Ct of miRU6)	2-ΔCt	experimental group/ Control group	Fold of gene expression
Control	32.1	21.99	10.11	0.0009049	0.0009049/0.0009049	1.00±0.00c
Group 2 H.	30.09	22.01	8.08	0.0036955	0.0036955/0.0009049	4.08±0.07b
Group 3 F.H.	28.24	22.008	6.232	0.013304	0.013304/0.0009049	14.70 ±0.15a
LSD value	--	--	--	--	--	1.893 **

Group	Means Ct of miRU6	2-Ct	experimental group/ Control group	Fold of gene expression
Group 1 C.	21.9906	2.4E-07	2.4E-07/2.4E-07	1.00±0.00
Group 2 H.	22.0138	2.361E-07	2.361E-07/2.4E-07	0.984± 0.08
Group 3 F.H.	22.0088	2.37E-07	2.37E-07/2.4E-07	0.987±0.07
LSD value	--	--	--	0.261 NS

NS: Non-Significant.

group. The fold change in gene expression was calculated using relative quantification. This is dependent on the normalizing of Ct values when calculating the ΔCt, which is the difference between the mean Ct values of the *mir-205* cDNA amplification replica in each case and the miRU6 replica.

The result of each group's 2-ΔCt was compared to that of the control group to determine the gene expression folds in respect to the housekeeping genes (Table 8). The fold of gene expression in F.H group was 14 times higher than that of the healthy group. That for the H group was 4 times higher than the healthy group. These results indicate significantly increase expression of *mir-205* gene in these groups. When using the $2^{-\Delta\Delta Ct}$ data to calculate the relative expression of the *mir-205* in all research groups, a calibrator was utilized, which was one of the samples from the controls with high expression *of mir-205*. Gene expression was calculated at a much higher rate in the F.H group as 14 than in the other groups. The H group's fold number was four times higher than the healthy group's (Table 9).

This data revealed that the F.H group has significant gene expression. All study groups were separated into two subgroups: high expression and low expression, with high expression indicating a fold change greater than one and low expression indicating a fold change less than one.

The high expression in the F.H group was obvious when compared to the healthy group. H groups, on the other hand, have a large number of high-expressing individuals ($P \leq 0.01$), indicating that there was a significant difference between the study groups, underlining the significance of careful analysis.

Discussion

The findings of this work were consistent with Haque et al. 2018 found that high serum cholesterol, an increase in LDL-cholesterol levels, and a decrease in HDL-C are all important risk factors for coronary heart disease (CHD). According to Mashali et al. 2018, there was a substantial link between TG and ACS history and family history of heart disease. HDL is recognized to have a direct influence on the atherogenic process and may be linked to atherosclerosis incidence and prevalence (Xu & Fu 2003). According to the findings of Soro et al. 2003, HDL-C levels are generally lower in familial mixed hyperlipidemia. One of the key causes of increasing CVD risks is an inverse association between HDL and the occurrence of CVD (Srivastava 2018). LDL levels have been shown to have a linear association with the risk of cardiovascular disease (Bandyopadhyay et al. 2018). The findings revealed

Table 9. Fold of *mir-205* expression depending on $2^{-\Delta\Delta Ct}$ method.

Groups	Means Ct of mir-205	Means Ct of miRU6	ΔCt (Means Ct of miRNA205 - Means Ct of miRU6)	Mean ΔCt Calibrator (ct IL8-ct miRU6	ΔΔCt	$2^{-\Delta\Delta Ct}$	experimental group/ Control group	Fold of gene expression
Group 1 C.	32.1	21.99	10.11	11.11	-1	2	2/2	1.00 ±0.00 c
Group 2 H.	30.09	22.01	8.08	11.11	-3.03	8.168097006	8.16/2	4.08 ±0.07 b
Group 3 F.H.	28.24	22.008	6.232	11.11	-4.878	29.40521227	29.40/2	14.70 ±0.15 a
LSD value	--	--	--	--	--	--	--	1.893 **

** ($P \leq 0.01$); means having with the different letters in same column differed significantly.

that the severity of dyslipidemia in (FCHL) was determined by the level of VLDL-c (Cruz-Bautista et al. 2015). According to the findings, VLDL from hypercholesterolemic and hyperlipidemic people is more resistant to lipolysis (Wieczorek et al. 2021).

LRP-1 gene expression appears to be induced in part as a result of cardiovascular illness. There was a significant difference between the study groups (P≤0.01) indicating the significance of our findings that the *LRP-1* gene had low expression in all types of hypercholesterolemia studied, which is consistent with the *LRP-1* gene's mode of action. *LRP-1* is involved in the big endocytic and signaling receptors (Gorovoy et al. 2010).

LRP-1, which plays an important role in cellular and organismal cholesterol homeostasis (Van De Sluis et al. 2017), has been found to be abnormally expressed in many cardiovascular diseases. Low expression of *LRP-1* as a marker for arterial wall disruption leading to abdominal aortic aneurysm formation (Chan et al. 2017) is an important finding of this experiment that can be conducted on all cardiovascular patients.

The *mir-205* is shown to be highly expressed in all kinds of hypercholesterolemia studied, which is consistent with the gene's method of action. *Mir-205* is thought to be engaged in a number of cellular biological activities linked to pathological processes like cardiogenesis, oncogenesis, immunological disorders, and hematopoietic differentiation according to Gorovoy et al. 2010. According to Marrone et al. 2015, *mir-205* is overexpressed in a variety of cardiovascular disorders and plays a major role in abdominal aortic aneurysm and inflammation. Based on the abnormal expression of *mir-205* in many cardiovascular disorders, we can consider high expression of *mir-205* gene as a marker for arterial wall disruption leading to abdominal aortic aneurysm formation (Chan et al. 2017). As conclusion, our findings revealed that *miR-205* tightly regulate the expression of *LRP-1* gene in humans mostly through translational inhibition, making it a good marker and more valuable in the prediction of cardiovascular disease.

Acknowledgement

We sincerely thank the faculty and staff members at both Institute of Applied Science Department / Biotechnology division / University of Technology and Iraqi Hereditary Company (IHC).

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