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Original Article

Functional analysis of intron 3 in the regulation of gene expression of the human lipoprotein lipase gene

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Abstract

Lipoprotein lipase (LPL) is a key enzyme that hydrolyzes triglycerides (TGs) into free fatty acids. Several genetic variants of LPL are directly or indirectly associated with variations in lipid levels, causing different lipid metabolic disorders. Previous studies on the LPL gene have shown that exons and introns are essential for gene expression and regulation. However, mechanisms through which introns regulate gene expression and function remain unclear. In this study, we successfully designed a protocol to assess the function of LPL intron 3 in LPL regulation. This was accomplished by constructing luciferase reporter vectors, containing full and partial intron 3 fragments from a healthy human DNA sample. These recombinant constructs facilitated the analysis of transcriptional activity using dual-luciferase reporter assays in cell lines. The results showed that the luciferase activity of the chimeric firefly luciferase reporter construct containing the full-length LPL intron 3 was higher than that of other constructs. In this study, a successful protocol was developed to assess the function of LPL intron 3 in regulation of the LPL gene. This protocol provides a novel method for functional analysis of introns and intronic variants that can be applied to other genes.

Keywords: Cloning, Intronic variants, *In vitro* analysis, Lipoprotein lipase, Tissue culture.

1. Introduction

Lipoprotein lipase (LPL) is an enzyme with esterase and phospholipase activities. It is a multifunctional protein that plays a critical role in the metabolism and transport of lipids by catalysing the breakdown of lipids derived from lipoproteins [1-5]. In addition, LPL has a non-catalytic role in the blood, it acts as a monocyte adhesion enhancer, macrophage function activator, nitric oxide producer [6], and an inducer of tumor necrosis factor-alpha (TNF-α) expression [6, 7].

The human *LPL* gene is well documented with numerous genetic variants and other single nucleotide polymorphisms (SNPs) involving *LPL* have been reported. Multiple mutations have been identified in the coding and non-coding regions of the *LPL* gene [8]. However, it has been proposed that variants in the coding regions (exons) explain only a small proportion of the genetic variance in major lipid traits. Hence, attention has shifted to focus on variants that lie in non-coding regions on the basis that approximately 90% of genomic DNA is comprised of non-coding regions [1, 9].

The relationship between numerous genetic variants involving the *LPL* gene locus and variations in lipid levels has been reported in several genetic studies [1, 3, 9-16]. Zhu et al. (2014) reported that the rs326 SNP in intron 8 is associated with variations in serum triglyceride (TG) and high-density lipoprotein cholesterol (HDL-C) levels in the Chinese population. Moreover, Al-Bustan et al. proposed a significant effect of a novel *LPL* variant, 18704C>A, in intron 3 on variations in lipid levels, specifically increased TG and decreased HDL among the Kuwaiti population. In addition, significant effects of genetic variants rs293 and rs295 found in *LPL* intron 6 have been reported to be associated with an increase in body mass index (BMI) in the Kuwaiti population [9].

Furthermore, numerous genetic and genomic studies have reported significant associations involving *LPL* exonic and intronic variants and increased risk of several pathophysiological conditions [17-20]. These conditions include hypercholesterolemia, obesity, coronary artery disease, dyslipidemia, type 2 diabetes mellitus, chylomicronemia, Alzheimer's disease, and atherosclerosis. All of the aforementioned diseases are related to variations in lipid levels as a result of the loss of LPL function [17-19, 21]. Understanding the functional role of significantly associated variants and their role in molecular mechanisms can allow predictions on the efficiency of lipid metabolism and transport, which, in turn, can assess future risks for

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developing metabolic disorders.

It has been demonstrated that certain exonic variants can directly affect protein structure and enzyme activity. Many studies have identified associated variants in *LPL* exon 3, including (264T>A, 272G>A, 286G>C, 287_288 del2, 290_293 del4ins2, 292_295 del4, 292G>A, 300C>A, 306A>C, 337T>C, 337T>G, 373G>A, 382A>G, 384 del1ins6, 394G>A, 397C>T) and in exon 4 (440_443 del4, 488A>G, 496G>A, 506G>A, 541G>A) which are reported to be pathogenic, causing LPL deficiency [22]. It has also been well-established that obesity is influenced by *LPL* intronic variants. These include the promoter variant −93 T<G (rs1800590) and the HindIII (rs320) variant in intron 8 [20, 23]. Also, there are many reported polymorphic intronic variants associated with risk of coronary artery disease (CAD) including the promoter variant T-93G (rs1800590), PvuII (rs285), and HindIII (rs320) [24-26]. Other metabolic disorders are also found to be associated with numerous *LPL* intronic variants of small effect [1, 16, 20, 23-25, 27]. Furthermore, Al-Bustan et al. demonstrated a significant effect of a novel variant, (18704C>A) in *LPL* intron 3, on variations in lipid levels by either increasing TG levels or decreasing HDL levels.

Numerous studies have also reported that specific genetic variants in intron 3 (rs75026342, rs2137497749, rs866034107, rs343, and rs1366457905) are associated with metabolic disorders [1, 15, 27, 28].

LPL intron 3 is 1,360 bp in length and starts from nucleotide position 19,951,949 until 19,953,309 and harbors 386 different genetic variants, as documented in Ensembl Release 107 (July 2022). There are 3 major types of variants found in intron 3. These include 348 single-nucleotide substitutions, 38 indels, and 33 repeat mutations. Four variants in intron 3 have been investigated by different researchers and have been reported to be associated with metabolic disorders [1, 27, 29, 30]. For example, Cho et al. (2008) demonstrated that the rs343 *LPL* SNP (+13836C>A in intron 3), a C-to-A transversion, is significantly associated with type 2 diabetes mellitus (T2DM). They also reported that the rs343 genetic variant is associated with total cholesterol and HDL-C levels as a result of LPL deficiency or dysfunction [27]. However, they were unable to define whether the rs343 variant is a disease marker or a causative SNP, as it is located in a non-coding region. Although this SNP is only 50 bp distant from exon 4 in Ensembl Release 107 (July 2022), it was assumed that the rs343 SNP may be a regulatory variant in binding transcription factors and other proteins [27, 31, 32]. It was also reported that adjacent variants of intron 3, such as rs343 and rs75026342, affect variation in TG and HDL levels [1, 15, 33]. However, functional analyses of these variants have not been conducted.

The main challenge with significant findings on the association of intronic variants is that they are not consistent between different studies. One main reason for these inconsistencies is that intronic variants are never causative; their association can be influenced by other genetic factors or extrinsic factors. Additionally, "rare" variants, which may be specific to certain ethnic groups and may not yield similar results across different populations [1].

Herein, the rationale for focusing on intron 3 is that several *LPL* significantly noncoding variants in intron 3 have been reported to be associated with metabolic disorders [1, 15, 20, 27, 28]. There have been postulations on the functional role of these variants for metabolic disorders. Therefore, we attempted to develop a protocol to investigate the functional role of intronic 3 sites that harbour such variants. In this study, we report the successful designed protocol to assess the function of *LPL* intron 3 in regulation of the *LPL* gene.

2. Materials and Methods

2.1. Cloning of full and partial intron 3 of *LPL* into TOPO vector.

The full (1,360 bp) and partial (515 bp) intron 3 of *LPL* (Supplemental Fig. 1) were amplified by Polymerase Chain Reaction (PCR) using Platinum™ Green Hot Start PCR 2X Master Mix (13001012, Invitrogen™, ThermoFisher Scientific, US) from healthy human genomic DNA using intron 3-specific primers, as shown in Table 1. The health status of the participants was determined based on their medical records and condition at the time of sample collection, as previously reported. [10].

PCR conditions used for amplification of the full and partial intron 3 were set separately as follows: initial denaturation at 94ºC for 2 minutes, followed by 35 cycles of the following steps: denaturation at 94ºCfor 30 seconds, annealing at 63ºC for 30 seconds, extension at 72ºC for 90 seconds/1,500 bp of the full intron and 30 seconds/500 bp of the partial intron; and final extension step at 72ºC for 10 minutes. The PCR products were cloned into linearized pCR 2.1-TOPO TA vector (K450040, Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. Plasmid purification was performed using a GeneJET plasmid miniprep kit (K0502, Thermo Scientific™, ThermoFisher Scientific, US) according to the manufacturer's protocol. The size and orientation of the inserted fragments in each plasmid were confirmed using PCR amplification, restriction endonuclease digestion, and DNA sequencing.

2.1.1. Analysis of recombinant TOPO vector using PCR amplification

Cloning of the desired inserts into TOPO vectors was confirmed by amplifying the purified recombinant vectors using the intronic primers described in Section (2.1). The PCR products were resolved by gel electrophoresis at 120 V for 45 min and analysed qualitatively on 1% agarose gels pre-stained with 10 mg/ml SYBR Safe DNA stain (S33102, Invitrogen).

2.1.2. Analysis of recombinant TOPO vectors using restriction endonuclease digestion

Recombinant pCR 2.1-TOPO TA vectors containing full and partial fragments of *LPL* intron 3 were treated

Table 1. Primers used for cloning targeted regions into TOPO vector.

Targeted Region	Forward Primer sequence (5' – 3')	Reverse Primer sequence (5' – 3')
Partial *LPL* intron 3	TACACCAAACTGGTGGGACA	CTATGCACCTTCCCTTCCAA
Full *LPL* intron 3	TACACCAAACTGGTGGGACA	TGGTCAGACTTCCTGCAATG

with EcoR1-HF restriction enzyme (10,000 units; R3101S, New England Biolabs, US) to confirm cloning of the desired regions into the TOPO vectors because the EcoR1 sites flank the PCR product insertion site. Restriction enzyme digestion reactions were performed separately for each vector. The volume of each recombinant vector was determined based on its concentration. Restriction enzyme reactions were set up (Table 2) to give a final total volume of 50 µL. The reaction mixtures were incubated in a water bath for 1 h at 37°C.

2.1.3. Analysis of recombinant TOPO vectors using DNA sequencing

Definitive confirmation of cloning of the desired products (full intron 3/partial intron3) into TOPO vectors in the correct orientation was performed by Sanger DNA sequencing. The region spanning each insert was amplified using M13 primers, Platinum™ Green Hot Start PCR 2X Master Mix and nuclease-free water reaching a total volume of 50 µL (Table 3). PCR products were purified using the PureLink® Quick Gel Extraction and PCR Purification Combo Kit (K220001, Invitrogen™, US) according to the manufacturer's protocol. BigDye™ Terminator v.3.1 Cycle Sequencing Kit (4337458, Applied Biosystems™, Waltham, MA, USA) was used for DNA sequencing, as shown in Table 3.

Conditions used for sequencing reactions of the full-length and partial fragments of *LPL* intron 3 were set on a thermocycler (4375305, Veriti 96 Well Thermal Cycler, Applied Biosystems, US) with the following conditions: initial denaturation at 96 ºC for 1 min, followed by 25 cycles of the following steps: denaturation at 96ºC for 10 seconds, annealing at 63ºC for 5 seconds, and extension at 60ºC for 4 minutes; and hold at 4ºC.

After DNA sequencing, the BigDye XTerminator™ Purification kit (4376487, Applied Biosystems™, US) was used following the manufacturer's protocol to remove salts, unincorporated dye terminators, dNTPs, and primers.

2.1.4. Data analysis

Purified products from the previous step were analyzed using a Gene Analyzer (Applied Biosystems, Life Technologies Ltd, 3130xL Genetic Analyzer, Thermo Fisher Scientific, UK). The resulting sequences of the forward strand for each purified PCR product were aligned using Clustal Omega against the intron 3 sequence using the https://www.ebi.ac.uk/Tools/msa/clustalo/ website.

2.2. Cloning of full and partial *LPL* intron 3 into Luciferase Reporter Vector pGL4.23 [luc2/minP] (E841A, Promega, Madison, WI, USA).

Full and partial fragments of intron 3 were digested in recombinant TOPO vectors using Sac-HF (R0156S, New England Biolabs) and Xho (R0146S, New England Biolabs) restriction enzymes. The targeted regions were cloned using T4 DNA ligase (M0202S, New England Biolabs, Inc., Beverly, MA, USA) into the digested luciferase reporter vector pGL4.23 [luc2/minP] between the SacI and XhoI sites upstream of the minimal promoter and firefly luciferase gene. Each recombinant pGL4.23 [luc2/minP] vector was expected to contain an insert (full or partial *LPL* intron 3) as shown in Supplemental Fig. 2 (A and B). Plasmid purification was performed using a GeneJET plasmid miniprep kit (K0502, Thermo Scientific™, US) according to the manufacturer's protocol. The size and orientation of the inserted fragments were confirmed by PCR amplification and DNA sequencing.

2.2.1. Analysis of the recombinant pGL4.23 [luc2/minP] vector

Purified recombinant pGL4.23 [luc2/minP] vectors were amplified using intronic primers under the same PCR conditions as described earlier. Then, the PCR products were qualitatively analyzed by 1% agarose gel electrophoresis to identify recombinant vectors carrying the cloned fragments.

Cloning of the desired products (full intron 3/ partial intron 3) into the pGL4.23 [luc2/minP] vector was confir-

Table 2. Reagents and quantities used for restriction enzyme EcoR1 digestion reaction. (NEBcloner® New England BioLabs $_{INC.}$).

Component	Quantity	
	TOPO TA vector + full *LPL* intron 3	**TOPO TA vector + partial fragment of *LPL* intron 3**
Plasmid DNA	6.53 µL of (153 ng/ µL)	44 µL of (22.72 ng/ µL)
10X NE Buffer EcoR1	5 µL (1X)	5 µL (1X)
EcoR1 Enzyme	1 µL	1 µL
Nuclease-Free water	37.47	-
Total	50 µL	50 µL

Table 3. PCR sequencing reaction of full and partial *LPL* intron 3.

Component	Sequencing of full *LPL* intron 3	Sequencing of partial *LPL* intron 3
BigDyeTM Terminator 3.1	Big Dye V3:1 2 µL	Big Dye V3:1 2 µL
Ready Reaction Mix	Seq. buffers 2 µL	Seq. buffers 2 µL
M13 Forward primer: 5'-GAGCTCTCAGACTCGATTCCCCCTCT -3'	1 µl (5 ng/ µl)	1 µl (5 ng/ µl)
Purified PCR product	2 µl (25 ng/ µl)	4 µl (10 ng/ µl)
Nuclease-free water	3 µl	1 µl

med by Sanger DNA sequencing, as described previously. PCR products were purified using the PureLink® Quick Gel Extraction and PCR Purification Combo Kit. The purified products were sequenced using the BigDye™ Terminator v.3.1 Cycle Sequencing Kit and then purified using the BigDye XTerminator™ Purification kit, as described earlier.

Purified products were sequenced using a Gene Analyzer 3130xL. The resulting sequence of the forward strand for the purified PCR product of the partial intron was aligned against the sequence of intron 3 using Clustal Omega and the https://www.ebi.ac.uk/Tools/msa/clustalo/ website. The functional roles of the cloned partial and full intron 3 of *LPL* in regulating gene expression were assessed using luciferase reporter assays in cell cultures.

2.3. Cell culture and transfection

Human Embryonic Kidney (HEK-293) cells were cultured in filtered Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) (10082139, Gibco, Grand Island, NY) and 1% penicillin-streptomycin (15140148, Gibco) at 37 ºC, 5% CO_2. The medium was changed every other day, and the cells were passaged every 3 days. HEK-293 cells were seeded at a density of 1×10^4 cells/well in 96-well plates (92096, TPP, Switzerland) and grown overnight in Opti-MEM reduced serum medium (31985062, Gibco, GrandIsland, NY, USA) under optimal growth conditions. When confluency reached 80%, the cells were co-transfected with 100 ng of the cloned pGL4.23 vector and 20 ng of Renilla vector (E2231, Promega, Madison, WI). Transfections were performed in Opti-MEM reduced serum medium using Lipofectamine™ Transfection reagent (18324012, Invitrogen, Eugene, OR, USA) according to the manufacturer's instructions. 96-well plates containing the transfected cells were incubated for 24 h following transfection at 37°C under 5% CO_2. All transfection experiments were performed in duplicate and repeated twice. The mean values of two independent transfections were calculated.

2.4. LPL luciferase reporter assays

The transfection process and functional analysis of the cloned intronic regions were conducted using the Dual-Luciferase Reporter Assay System (E1910, Promega, Madison, WI, USA) following the manufacturer's protocol. This reporter assay allows for the measurement of firefly luciferase (*Photinus pyralis*) and Renilla luciferase (*R. reniformis* or sea pansy) expressed in HEK-293 cells that were transfected with the following vectors: pGL4.23 cloned with full intron 3, pGL4.23 cloned with partial fragment of intron 3, pGL4.23, and pRL-SV40 control vector. The experiment was carried out as follows: 24 hours post-transfection, the media was gently removed from the transfected and non-transfected cells. 0.02 ml of Dulbecco's phosphate buffered saline (DPBS) was added and removed directly from each well. Then, the cells were lysed by adding 0.02 ml of 1X Passive lysis buffer (PLB) and incubated in a shaker (Thermo-Titer Plate Shaker-4625, serial No. C1882131264777) at 2000 R.P.M. for 15 minutes. After that, the 0.02 ml lysate were then collected into 96 well clear bottom plate (Thermo ScientificTM, catalogue no. 265301) for the measurement of luciferase activity to assess the transcriptional level of each vector using DLR assay system by the Automated Microplate Reader (Clariostar, serial no. 430-1280). 0.1 ml of Luciferase Assay Reagent ll (LAR ll) was added and the first read of firefly luciferase activity was taken. After that, 0.1 ml of Stop & Glo Reagent were added to the same samples and then the second read of Renilla luciferase activity was taken. Non-transfected cells were also processed with Stop & Glo only as background measurements. Relative luciferase activity was determined by calculating the ratio of firefly to *Renilla* activity in each experiment. The experiments were repeated twice under similar conditions to ensure consistency and reproducibility.

2.5. Statistical analysis

The significance of mean value differences between the samples was analysed to assess their impact on luciferase activity. Standard error calculations and P values (set at 0.05) were determined. The samples were compared using two-way ANOVA followed by Tukey's multiple comparisons test. All statistical analyses were conducted using GraphPad Prism 9 (GraphPad Software, San Diego, CA). P values < 0.05 were considered statistically significant.

3. Results

3.1. Confirmation of *LPL* intron 3 fragments cloned into TOPO vectors by PCR.

PCR products from both reactions (primers targeting the full intron and primers targeting the partial intron) confirmed the successful cloning of the desired DNA fragments into the TOPO vector, as visualized on 1% agarose gels (Fig. 1A and Fig. 1B). The amplified PCR products of the cloned full and partial fragments of *LPL* intron 3 were visualized as electrophoretic bands (approximately 1,500 bp and 500 bp) that matched the expected size of each fragment (1,360 bp and 515 bp), respectively. Another band was visualized at approximately 4 kb (Fig. 1 (B)) corresponding to the size of the TOPO vector itself.

3.2. Confirmation of fragments of *LPL* intron 3 cloned into TOPO vector using restriction enzyme digestion.

Undigested and digested recombinant vectors carrying the full and partial fragments of *LPL* intron 3 also confirmed the successful cloning of the desired DNA fragments into the TOPO vector (Fig. 2), as visualized on 1% agarose gels. Fig. 2 shows additional new bands after treating each vector with the restriction enzyme EcoR1 compared to untreated ones. The sizes of each band (1.3 kb and 800 bp) were visualized as being close to the expected size for each cloned fragment (full and partial) of intron 3. Other bands of approximately 4 kb in the undigested and digested forms of each vector were resolved to determine the size of the TOPO vector. The upper faint bands, which were the remaining undigested DNA, were also visualized.

3.3. Confirmation of cloned fragments of *LPL* intron 3 into TOPO vector by DNA sequencing.

DNA sequences of the cloned full and partial fragments of *LPL* intron 3 were successfully generated from the high-quality recombinant TOPO vectors, confirming the cloning of the inserts into the tested vectors (Supplemental Fig. 3) by DNA sequence alignment of the tested *LPL* intron 3 fragments against the reference sequence (sequence of intron 3 from the GenBank database). A complete alignment of the cloned full and partial fragments of intron

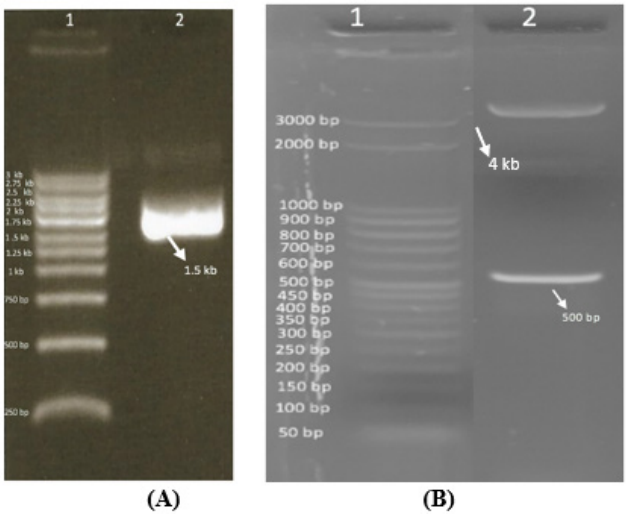

Fig. 1. Agarose gel showing resolution of the desired DNA fragments (full and partial) of *LPL* intron 3 cloned into pCR 2.1-TOPO TA vector. The fragments were resolved on a 1% agarose gel pre-stained with 10 mg/mL SYBR Safe and imaged under UV light showing a specific fragment of *LPL* intron 3. In part (A) lane 1 represents 1 kb (0.25-10 kb) DNA ladder (Thermo Fisher) and lane 2 represents the purified PCR product of the full fragment of *LPL* intron 3 (1,500 bp) from the recombinant TOPO vector. In part (B), lane 1 represents a 3 kb (0.05-3 kb) DNA ladder (Thermo Fisher) and lane 2 represents the purified PCR product of the partial fragment of *LPL* intron 3 (500 bp) from the recombinant TOPO vector.

3 against the reference sequence (Gene ID:4023) is shown in Supplemental Fig. 4 and Fig. 5.

Based on the sequence alignment and comparisons, one variant was identified that could be used to test its functional role. This variant resulted from a T-to-G substitution at nucleotide position 19,951,950 (Supplemental Fig. 5). This variant was previously reported as a splice donor variant (rs1590141773), and was the only variant found in the partial fragment of *LPL* intron 3.

3.4. Confirmation of fragments of *LPL* intron 3 cloned into pGL4.23 [luc2/minP] vector by PCR.

Cloned full and partial fragments of *LPL* intron 3 were successfully amplified from purified recombinant pGL4.23 [luc2/minP] vectors. The amplified PCR products of both the cloned full and partial fragments of *LPL* intron 3 showed clear electrophoretic bands at the expected sizes of 1,500 bp and 500 bp, respectively, confirming the success and overall quality of the cloning (Fig. 3 and Fig. 4).

3.5. Confirmation of fragments of *LPL* intron 3 cloned into pGL4.23 [luc2/minP] vector by DNA sequencing.

The DNA sequence of the partial fragment of *LPL* intron 3 from the purified recombinant pGL4.23 [luc2/minP] vector was successfully obtained. The DNA sequence of the partial fragment of *LPL* intron 3 using the forward strand (Supplemental Fig. 6) showed that the sequence was of high quality, allowing its alignment and comparison with the reference sequence (Gene ID:4023), confirming successful cloning of the full and partial fragments of *LPL* intron 3 into pGL4.23 [luc2/minP]. A complete alignment of the cloned partial fragment of intron 3 against the reference sequence (Gene ID:4023) was performed (Supplemental Fig. 7).

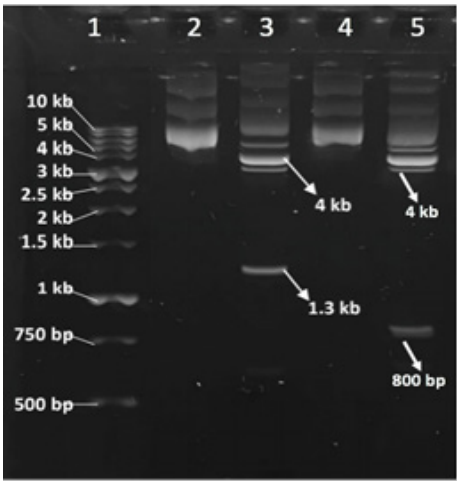

Fig. 2. Agarose gel showing resolution of the restriction digestion products of purified recombinant vectors containing (full and partial) fragments of *LPL* intron 3. The fragments were resolved on 1% agarose gels pre-stained with 10 mg/mL SYBR Safe and imaged under UV light showing the digested recombinant TOPO vector. Lane 1 represents 1 kb (0.25-10 kb) DNA ladder (Thermo Fisher), and lanes 2 and 3 represent the un-digested and digested purified recombinant vectors containing the full intron 3, respectively. Lanes 4-5 represent the un-digested and digested purified recombinant vectors containing the partial fragment of intron 3, respectively.

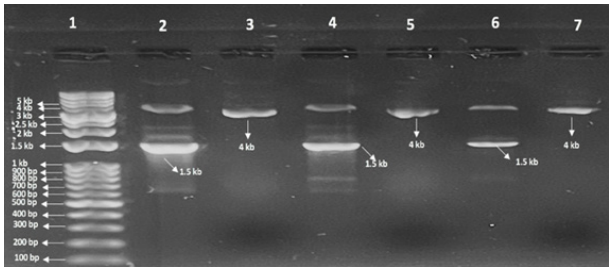

Fig. 3. Agarose gel showing resolution of the amplified PCR products of full-length *LPL* intron 3 from the recombinant pGL4.23 [luc2/minP] vector and imaged under UV light showing full *LPL* intron 3 from the recombinant pGL4.23 [luc2/minP] vector. Lane 1 represents 1 kb (0.1-10 kb) DNA ladder (Thermo Fisher), Lanes 2, 4, and 6 on the same gel represent the amplified PCR product of 1,500 bp using primers of the full fragment of *LPL* intron 3 from purified recombinant pGL4.23 [luc2/minP] vectors. Lanes 3, 5, and 7 represent purified recombinant pGL4.23 [luc2/minP] vector.

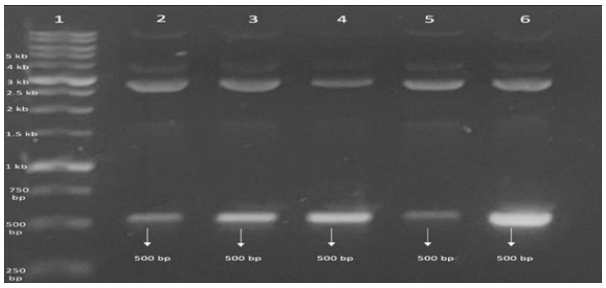

Fig. 4. Agarose gel showing resolution of the amplified PCR products of the partial fragment of *LPL* intron 3 from the recombinant pGL4.23 [luc2/minP] vector and imaged under UV light showing the partial fragment of *LPL* intron 3 from recombinant pGL4.23 [luc2/minP] vector. Lane 1 represents 1 kb (0.25-10 kb) DNA ladder (Thermo Fisher). Lanes 2-6 on the same gel represent 500 bp amplified PCR products using primers for the partial *LPL* intron 3 from purified recombinant pGL4.23 [luc2/minP] vectors.

3.6. *LPL* luciferase reporter assays

The constructed luciferase-based reporter vectors (one containing the full intron 3 of *LPL* and the other containing a partial fragment of intron 3), non-recombinant luciferase-based reporter vector, and *Renilla* vector, as an internal control, were co-transfected into HEK-293 cells. Relative luciferase activity was determined using a dual-luciferase reporter gene. A higher activity value was observed for the pGL4.23 [luc2/minP] vector containing the full intron compared to that containing the partial intron and the empty vector. However, the differences in luciferase ratios between the tested samples (Fig. 5, and Supplemental Tables 1 and 2) were statistically insignificant (P = 0.439).

4. Discussion

The functional roles of numerous intronic variants remain unresolved despite efforts to predict their roles using genetic association studies and/or *in silico* analysis. This study sought to develop a protocol that would provide a means of functional analysis of intronic variants using *LPL* intron 3 as a model. The importance of our findings is that we devised a working protocol that will allow functional analysis of significantly associated intronic sites (harboring a significant SNP) in order to predict their overall risk in the manifestation of metabolic disorders.

Intron 3 was selected based on adjacent exons, which consist of coding sequences that have a direct and active role in the overall catalytic activity of the enzyme. Hence, it could be postulated that intron 3 harbors sequence variants that may play a role in regulating gene expression, based on the numerous variants reported to be significantly associated with metabolic disorders [1, 15, 27, 28]. It has been suggested that intron 3 of *LPL* could be considered to have an important functional role as it lies downstream of exon 2, which includes the protein domain that has been shown to bind to the lipoprotein substrate [2, 34] and is thought to be important for expression of the enzyme at the transcriptional level [35]. Also, exon 2 contains a tryptophan residue at amino acid 55 that encodes an oxyanion hole important for hydrolysis. Moreover, intron 3 is directly located downstream of exon 3 which encodes a ß5' loop. Both exons encode amino acids required for enzymatic activity. Nakamura et al. (1996) suggested the functional role of genetic variants in intron 3 and pointed out that a single mutation in intron 3 that causes an A to T transition 6 bp upstream from the RNA splice acceptor site is associated with hypertriglyceridemia [28]. Therefore, this association is assumed to develop directly because of the point mutation or indirectly because of linkage with other regions of the *LPL* gene or other genes.

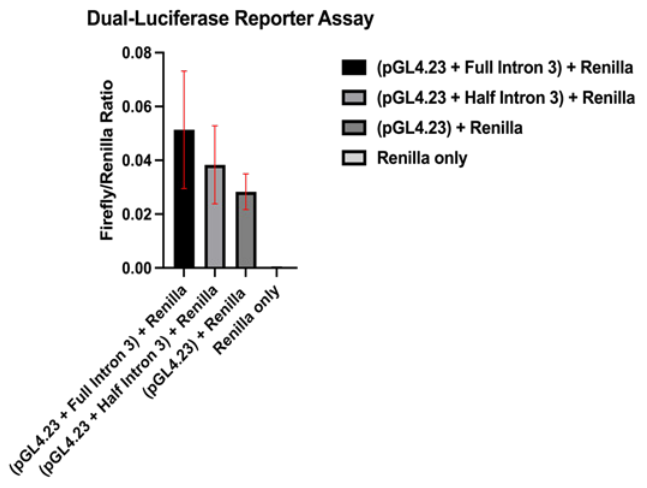

Fig. 5. Comparison of luciferase activity detected between the different tested vectors for the effect of the full and partial fragments of *LPL* on pGL4.23 gene expression in HEK-293 cells. This effect was measured as the mean of the ratio of firefly/*Renilla* luciferase levels. The results presented are from the following constructs pGL4.23 [luc2/minP] vector containing full-length intron 3, another with the partial intron 3, empty pGL4.23 [luc2/minP] vector and *Renilla* control vector. Results are means from two independent duplicate experiments and each bar represents the standard error of the mean of the two experiments.

In a previous publication from 2017 by Al-Bustan et al., a potential mechanism was proposed regarding how elements in the *LPL* intron 3 may influence gene expression levels. The researchers reported that this intronic sequence could contain an important regulatory region of the *LPL* gene, as it resides between the coding sequences of the ApoC2 binding site (exon 4) and the amino acid sequence crucial for *LPL*'s catalytic activity. Additionally, they suggested other mechanisms, such as the interaction of specific sites within the intronic region with various factors that regulate gene expression or splicing. These interactions highlight the complex mechanisms underlying the synchronized expression of *LPL* and its cofactors, *APOCII* and *APOCIII*. However, the researchers were unable to identify specific transcription factors or splice-mediated factors that have distinct binding domains to the intron 3 sequence [35].

In this study, an *in vitro* functional assay was successfully developed by employing two luciferase reporter vectors, pGL4.23 [luc2/minP], which were separately used to clone the full and partial intron 3 of *LPL* and then transfected into HEK-293 cells. This facilitated analysis of the transcriptional activity of the full and partial fragments of intron 3. A dual-luciferase reporter assay was successfully established. Although the luciferase activity of the chimeric firefly luciferase reporter gene construct containing the full fragment of *LPL* intron 3 was higher than that of the other constructs in the tested samples (Fig. 5), the difference was not significant ($P \geq 0.05$). The lack of statistical significance in terms of luciferase activity between the two tested samples may indicate that the reported variant rs1590141773 in intron 3, located in the partial intronic region, does not play any role in gene expression. However, this is merely speculation, and further analyses and studies are needed to validate this. In future studies, we could aim to further develop our protocol by incorporating RNA editing and inducing various mutations in introns 1 and 3.

Most genetic association studies tend to dismiss the functional significance of intronic variants, probably due to the challenges in assessing their role. Few studies have developed methods to evaluate the functional roles of such variants. The protocol developed in this study can be applied to investigate other intronic variants that are significantly associated with genetic traits and diseases.

Although the preliminary results of this study failed to demonstrate any functional role of intron 3 in gene expression, an effective and reproducible experimental protocol was achieved. Furthermore, the modified protocols and methods described in this study have provided an experi-

mental design for the *in vitro* functional analysis of intronic variants that can be used in future studies and for other gene variants, including investigating intronic variants in other genes associated with lipid metabolism and transport, especially within gene families that require synchronized expression. This regulatory mechanism may involve interactions among intronic variants. Key genes of interest in our research include the Apolipoprotein (APO) family, such as *APOCII*, *APOCIII, APOB*, *APOA*1, *APOA5*, and *APOE*. Additionally, it would be advantageous to explore the functional roles of similar introns in the lipase gene family, such as the Endothelial lipase (*LIPG*), Hepatic lipase (*LIPC*) and pancreatic lipase (*PNLIP*) genes. The findings from this study can also serve as a reference for further investigations into the functional role of intronic variants in regulating gene expression and alternative splicing and its regulation.

There are different limitations that have affected our research, for example, using only one DNA sample, the inability to clone the promoter upstream of the intron, and the limited number of natural variants analysed in the tested samples. Our work needs further investigation to properly assess the functional role of intron 3, for example, by analysing the role of intron 3 sequence variants in the regulation of splicing by including a partial sequence of the 3' end of exon 3 and 5' of exon 4. It is recommended that a promoter be included in an analysis of its effect on gene expression. Therefore, we would recommend using different DNA sequences with previously identified variants. We would also recommend performing functional analysis of the reported variants listed in this study by directly inducing mutagenesis to create these specific variants.

Author contributions

NKB was the primary investigator in this project. NKB and SAA contributed to study conception and experimental design. NHS performed experiments and statistical analyses. NHS prepared and wrote a first draft of the manuscript. NKB and SAA contributed to manuscript revision. All authors have read and approved the submitted version.

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Institutional Review Board Statement

The study was conducted in accordance with the Declaration of Helsinki, and approved by the Institutional Review Board of Joint Committee for the Protection of Human Subjects in Research (Health Sciences Center, Kuwait University and Kuwait Institute for Medical Specializations (Reference number: VDR/JC/256 and approved on January 2012) as reported in a previous study conducted by Al-Bustan et al. group [10].

Informed Consent Statement

This study was approved (Reference number: VDR/JC/256) by the Joint Committee for the Protection of Human Subjects in Research (Health Sciences Center, Kuwait University and Kuwait Institute for Medical Specializations) in accordance with established procedures and based on the Helsinki guidelines. The sample and medical data collection protocols and the informed consent practices used were in accordance with the revised version (2000) of the 1975 Helsinki guidelines. Informed consent was obtained from each participant in this study [10].

Data availability statement

The original contributions presented in the study are included in the article or the Supplementary Materials, and further inquiries can be directed to the corresponding author.

Conflicts of Interest

The authors declare that this study was conducted in the absence of any commercial or financial relationships that could be construed as potential conflicts of interest.

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