Accepted and presented at the 2ed Medical Research Conference. Faculty of Medicine University of Tripoli, Tripoli, Libya Jan. 2017

A novel single point mutation of the LDL receptor gene in a Libyan hypercholesterolemic family.

Ahmed Zaid*, Ghada Salem.

Department of Biochemistry, Faculty of Medicine, University of Tripoli, Tripoli, Libya.

*Corresponded author A.zaid@uot.edu.ly

ABSTARCT

Autosomal dominant hypercholesterolemia (ADH), a major risk for coronary heart disease, is associated with mutations in the genes encoding the low-density lipoproteins receptor (*LDLR*), its ligand apolipoprotein B (*APOB*) or *PCSK9* (Proprotein Convertase Subtilin Kexin 9). Familial hypercholesterolemia (FH) caused by mutation in the *LDLR* gene is the most frequent form of ADH. Low density lipoprotein receptor plays a key role in regulating plasma low-density lipoprotein cholesterol (LDL-C) levels in human. In the present study we identified a novel polymorphism 24400 G > C in a Libyan HF patient. Genotypes of the *LDLR* 24400 G > C polymorphism were determined via Polymerase Chain Reaction (PCR) and gel electrophoresis, and then confirmed by direct DNA sequencing. This study identified a novel *LDLR* gene polymorphic mutation which is the first to be described here in the Libyan population, increasing the spectrum of ADH-causative mutations.

INTRODUCTION

Atherosclerosis is a disease of the arteries responsible for coronary artery disease (CAD) and myocardial infarction (MI), which underlies most deaths in industrialized countries (1). It is due to multiple genetic factors, environmental factors, and interactions among them. Identification of these genetic and environmental factors will provide valuable information for prevention and control of CAD.

Autosomal dominant hypercholesterolaemia (ADH) (OMIM #143890) is characterized by raised serum LDL-cholesterol levels, and accelerated atherosclerosis that increased the risk of

premature coronary heart disease (CHD) (1). The most prevalent underlying molecular defect of ADH consists of mutations in the LDL receptor gene, but mutations in the *LDL receptor* binding domain of the *apolipoprotein B (apoB)* (familial defective apolipoprotein B) (2), and *PCSK9* have also been associated with familial hypercholesterolaemia (FH) (3). Mutation in the gene encoding the low-density lipoprotein receptor (*LDLR*) is the most common genetic cause of FH (http://www.ucl.ac.uk/fh). The LDL receptor protein is a cell-surface protein which mediates specific uptake and degradation of LDL by its endocytosis, mainly in liver. A wide variety of mutations including insertions, deletions, nonsense and missense mutations has been described in patients with FH. LDLR gene (MIM# 606945) is located on chromosome 19 (19p13.3) and consists of 18 exons which spans about 45 kb with a mature protein of 860 amino acids (6, 7).
Mutation in apolipoprotein B-100 gene (APOB100;MIM 107730) is the second cause of the clinical FH phenotype. This gene is located on chromosome 2p24.1 that codes for the protein component of LDL particles (8, 9). ApoB-100 is an integral component of LDL and functions as the ligand for the LDLR. Therefore mutations in the APOB 100 will drastically alter its functional activity leading to a decrease in its binding to LDLR, thereby delaying the clearance of LDL particles. In contrast to LDLR, only a small number of functional mutations have been identified in APOB gene such as R3500Q (12), R3500 W (13). and R3531C (14). There are no data regarding LDLR and APOB gene mutations in Libyan population. The aim of this study was to characterize the *LDLR* in Libyan FH patient. Other still unknown genes could also be involved.4 The frequency of heterozygous FH in most populations is about 1/500, homozygous FH is rare (1 per million), but in Tunisia, a neighboring country of Libya, higher frequency has been reported (1/165) because of the high levels of consanguinity in this population (6) Eight mutations causing FH in the *LDLR* gene have been reported in the Tunisian population.(7,8) In this study, we report two apparently unrelated families with severe hypercholesterolaemia.

MATERIALS AND METHODS

All subjects provided informed consent, and the institutional review board approved the study.

Blood sampling: Samples were collected from the affected individuals. Blood was obtained from the subjects by venipuncture after obtaining informed written consent from the patient. Blood

was obtained from the subjects by veinopuncture in heparinated tube (Puls blood collection tubes, BD Vacutainer, UK). The collection of the whole blood samples were in the Tripoli Medical Center, Tripoli, Libya.

DNA extraction: Genomic DNA from all patients was isolated from whole blood using QIAamp DNA Mini preps Kit (QIAGEN, USA) in 200 µl or 400 µl of total volume according to Qiagene user protocol. Genomic DNA was administrated in DNA electrophoresis apparatus (TECHNE - multisub, horisontal agarose, UK). The DNA is visualized in the gel by addition of ethidium bromide to check the quality. Concentration of genomic DNA samples was determined by Nanodrop 3300 (Thermo Scientific, USA).

PCR (Polymerase Chain reaction): Primer3 and BLAST software (http://www.ncbi. nlm.nih.gov/ tools/primer-blast/,) used to design primers for the PCR, Each amplification reaction was performed using 100 ng of genomic DNA in 25 µL of reaction mixture consisting of 25 µmol/L of each primer, 200 µmol/L of each deoxynucleotide triphoisphate, 2.5 µL of 10 × PCR buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl, 20 mM MgCl2, 1% Triton), and 2 units of *Taq* polymerase. After initial denaturizing at 94 °C for 5 min, the reaction mixture was subjected to 35 cycles of 30 s denaturation at 94 °C, 30 s annealing at 58 °C and extension 30 s at 72 °C, followed by a final 5 min extension at 72 °C. After electrophoresis on a 1.2% agarose gel with 0.5 µg/mL ethidium bromide (EB), the amplification products were visualized under ultraviolet light for analysis using photographing Apparatus: Chemi Image - Advanced Molecular Vision.

In order to purify PCR product for the 18 *LDLR* exons amplified from the genomic DNA primers, nucleotides, polymerase, and salts we used the QIAquick PCR product Purification Kit (Qiagene, USA). The Purifications were done for PCR product samples and were kept in -20°C in sufficient quantity to perform DNA sequencing.

***LDLR* Gene Primers**

LDLR Gene	*Primers for long-range PCR (5'→ 3'direction)*	L	Start	Stop	Tm	GC%	AF
promoter	F:CACGTGATCGTCCCGCCTA	19			60.0	62.0	263
	R:AAATTCTTGTCAACCTACTTGTGC	24			60.2	37.0	
Exon1	F: TAGGACACAGCAGGTCGTGAT	21	5113	5133	60.89	52.38	160
	R: CCCTCTCAACCTATTCTGGCG	21	5273	5253	60.20	57.14	

Exon2	F: ATTCTGGCGTTGAGAGACCC	20	15800	15819	59.75	55.00	223
	R: GGCGAGACCCTGTCTCTATTAC	22	16023	16002	59.71	54.55	
Exon3	F: TGGGTCTTTCCTTTGAGTGACA	22	18239	18260	59.49	45.45	219
	R: CCACTCCCCAGGACTCAGATA	21	18458	18438	60.06	57.14	
Exon4	F: AGACTTCACACGGTGATGGTG	21	20795	20815	60.27	52.38	476
	R: CCCAGGGACAGGTGATAGGA	20	21271	21252	60.03	60.00	
Exon5	F: CCCTGCTTCTTTTTCTCTGGTTG	23	22120	22142	59.99	47.83	240
	R: AAGCAGCAAGGCACAGAGAAT	21	22360	22340	60.55	47.62	
Exon6	F: TCAGACACACCTGACCTTCCT	21	22967	22987	60.41	52.38	229
	R: CATGTCTCAGTCCCTTTCCTGG	22	23196	23175	60.36	54.55	
Exon7	F: CGAGAGTGACCAGTCTGCATC	21	26235	26255	60.47	57.14	215
	R: TGGTTGCCATGTCAGGAAGC	20	26450	26431	60.90	55.00	
Exon8	F: ATCGCTCCGTCTCTAGCCAT	20	27071	27090	60.54	55.00	238
	R: CTGCCTGCAAGGGGTGAG	18	27309	27292	60.36	66.67	
Exon9	F: CTTGGTTCCATCGACGGGT	19	28841	28859	59.70	57.89	282
	R: CAGGAGCCCTCATCTCACCT	20	29123	29104	60.69	60.00	
Exon10	F: CAGGTGAGATGAGGGCTCC	19	29103	29121	59.17	63.16	291
	R: CTGCTCCCTCCATTCCCTCT	20	29429	29410	60.69	60.00	
Exon11	F: CCTCCAGCCTCACAGCTATTC	21	31677	31697	60.20	57.14	211
	R: GTCTGTCCTCCAGCCTGTG	19	31888	31870	59.71	63.16	
Exon12	F: GGCATCAGCACGTGACCT	18	32437	32454	60.05	61.11	222
	R: ATCCGCCACCTAAGTGCTTG	20	32659	32640	60.39	55.00	
Exon13	F:TCCCAGTGTTTAACGGGATTTGT	23	35666	35688	60.44	43.48	237
	R:TTCCACAAGGAGGTTTCAAGGT	22	35903	35882	59.76	45.45	
Exon14	F:ATAGCTGATGATCTCGTTCCTGC	23	35942	35964	60.31	47.83	243
	R: CAGTTGGAGGACACAGGACG	20	36185	36166	60.32	60.00	
Exon15	F: CGTGGCACTCAGAAGACGTT	20	38759	38778	60.60	55.00	251
	R: ACCCGTCTCTGGGTGAAGAG	20	39010	38991	60.90	60.00	
Exon16	F: CCTTTAGACCTGGGCCTCAC	20	43589	43608	59.75	60.00	169
	R: ACATAGCGGGAGGCTGTGA	19	43758	43740	60.69	57.89	

Exon17	F: AGCTGGGTCTCTGGTCTCG	19	45077	45095	60.68	63.16	283
	R: TTGAGGATCATATGCCTCCAGC	22	45360	45339	59.96	50.00	
Exon18	F:TGTTTCCTGAATGCTGGACTGAT	23	46849	46871	60.25	43.48	179
	R:GCAATGCTTTGGTCTTCTCTGTC	23	47028	47006	60.37	47.83	

Tm: primer melting temperature. L: primer length. AF: amplified fragment

Genetic Analyzer The sequencing of the amplified samples was done by 3130 Genetic analyzer using (Big dye sequencing kit from applied biosystem, USA). GeneScan and GeneMapper 3.0 softwares (Applied Biosystems) were used to determine the genotype of each subject.

Biochemical analysis Blood samples were taken for biochemical analysis following overnight fasting. Serum total cholesterol (TC), triglyceride (TG) and HDL cholesterol (HDL-C) concentrations were determined at accredited clinical laboratories using routine clinical methods. LDLcholesterol (LDL-C) concentrations were calculated using the Friedewald equation [17]. For TG levels _4.6 mmol/L, no LDL-C value was calculated (3 cases).

RESULTS

Clinical and biochemical analyses for blood samples were preformed as shown in Table1 on the family. The father had myocardial infarction (MI) late 2014 and the Mother and daughter were healthy with no CHD. The biochemical analysis shows that the father has a high level of cholesterol and LDL and normal level of trigacylycerol which is an indication for familial hypercholesterolemia (FH) the ratio. The father, mother and daughter are none diabetic with no history of nephrological problems or hyperthyroidism (Tabel 1). Biochemical analysis showed normal values in body mass index (BMI), cholesterol and triacylglycerol for the mother and the daughter indicating that the father can be consider as a potential patient with a familial hypercholesterolemia.

In This study, we succeeded in optimizing the right PCR protocol for amplifying all 18 exon, the software primer 3 for designing PCR prime used for this purpose.

In order to study the cause of (FH) and identify the gene that responsible for the increase of cholesterol, all of the 18 exons and promoter region of *LDLR* gene were amplified by PCR for all

three subjects. PCR product for different exons was applied in electrophoresis analysis to check the quality and size of the amplified fragments of different exons (Figure 1).

All amplified exons were purified and sequenced for identification of mutation in the *LDLR* gene. Our results show no mutation in all exons in the mother and daughter. However, we found a novel mutation at the position 24400 in exon 10 of the father with a change of G to C. This change led to a change in amino acid glycine (Gly) to amino acid arginine (Arg). To our knowledge no other study found this mutation before. In order to confirm the finding, we sequence exon 10 of the father from both direction, the 5' end and 3' ends using exon 10 forward primer and exon 10 reverse primers respectively, (figure 2 A&B). Indeed, the sequencing by using the reverse primer showed a mutation from G to C only. We also can see two peaks G and C at the same place, the peak T represent the wild type allele and the peak C represent the mutated allele, this also indicating that the patient has is a mutation because each peak represents one *LDLR* allele. We have also sequenced the hole gene of *PCSK9* which some mutations in this gene showed a correlation with FH, but no mutation were found in all 12 exons of *PCSK9* gene (Data not shown), suggesting *LDLR* mutation which we reported here responsible about the increase in serum cholesterol in the father

Discussion

The spectrum of *LDLR* mutations causing FH has been studied in many different countries as this information is useful in devising the most efficient laboratory strategy for genetic testing. In genetically heterogeneous countries such as the UK, Italy or the Netherlands, there are many different mutations found [37].

A frequency of FH ranging from 1/411 (0.24%) for North Karelians of Finland [24] to 1/67 (1.5%) for Ashkenazi Jew in South Africa [25]. The frequency of FH is 1/900 (0.11%) for Japanese in Asia [26]. IRAN Very little is known about the frequency of FH in Arabic countries, and our finding is the first report of mutation in one of the gene causing FH in Libya. In addition, the mother and the daughter show no phenotype and were healthy and did not carry any of LDLR gene mutations.

The mature human LDLR proteinof 160 kDa is composed of five domains, exon 1 encodes a short 5' untranslated region and 25 hydrophobic amino acids that are not present in the mature protein. This sequence functions as a signal peptide to direct the receptor synthesising ribosomes to the Endoplasmic Reticulum (ER) membrane. Other functional domains of the peptide correspond to the exons as indicated in Figure (4), exons 2 and 6 encode the Ligand binding domain 292 AA, exons 7 and 14 encode EGF precursor homology approx 400 AA, exon 15 encodes O-linked sugars 58 AA, 41 bp of exon 17 plus exon 16 encode the transmembrane domain (membrane spanning 22AA) and the reminder of exon 17 together with exon 18 encode the cytoplasmic domain (cytoplasmic tail 55AA). (nano)

At present, more than 1,100 variants of *LDLR* gene have been listed in the *LDLR* databases (Al-Allaf FA, 2010) underlying a high genetic heterogeneity of *LDLR* mutations/rearrangements, 65% (n = 689) of which were DNA substitutions, 24% (n = 260) small DNA rearrangements (<100 bp), and 11% (n = 117) large DNA rearrangements (>100 bp). The DNA substitutions and small rearrangements occur along the length of the *LDLR* gene, with 839 in the exons (93 nonsense variants, 499 missense variants and 247 small rearrangements), 86 in intronic sequences, and 24 in the promoter region. The highest proportion of exon variants occurs in the ligand binding domain (exons 2-6) and the EGF (Epidermal Growth Factor) precursor domain (exons 7-14) (Al-Allaf FA, 2010). As shown these mutations are distributed across the 18 exons, introns, and the promoter region of the *LDLR* gene (Romano M, 2011). That's why it is considered the primary causative defects in approximately 85% of FH cases (Dvir H, 2012). The resulting loss of function leads to elevation of plasma LDL and to the development of atherosclerosis at an early age (Dvir H, 2012). Mutations in the ectodomain of LDLR disrupt the binding of LDL to the cell surface, whereas mutations in the cytoplasmic tail of LDLR retain LDL binding but lead to internalization defects by precluding clustering of the receptor in clathrin-coated pits and its subsequent endocytosis (Dvir H, 2012).

According to the nature and location of the mutations within the *LDLR* gene and based on the functional characteristic of the encoded proteins, five different classes of FH-causing mutations have been defined (Go GW, 2012 ; Schaefer JR, 2012). Class I mutations include null alleles

with no detectable LDL receptor protein (receptor synthesis defect). Class II mutations include transport-defective alleles, which disrupt normal folding of the receptor and cause either failure in transport to the cell surface or successful transport of truncated, mutated receptors. (class II a) mutations completely block the transport of the receptor or partially blocked (class II b or leaky LDLRs) in their transport from the endoplasmic reticulum to the Golgi apparatus due to impaired glycosylation (defect in targeting receptor to cell surface). Class III mutations (binding-defective alleles) encode LDL receptors with normal intracellular transport but defective LDL binding. Class IV mutations (internalization-defective alleles) produce LDLR with normal transport and cell surface LDL binding but defective clustering in clathrin-coated pits for internalization. Finally, class V mutations (recycling defective alleles) produce LDLR that internalize normally, but are unable to release bound ligand within the acidic environment of the endosome, and thus do not recycle to the cell surface (Go GW, 2012 ; Schaefer JR, 2012).

In this study the mutation G529R in exon 10 which is one of the exons that coded the EGF precursor domain which is an extracellular domain but not involve in ligand binding. The change from non charged amino acid glycine to the positively charged amino acid arginine would most likely to cause a conformational change in the structure LDL receptor. Further study in *ex-vivo* level is required in order to understand the mechanism of how this new mutation causes FH and which class it belongs. One possible that the amino acid change from glycine to arginine is change from non polar amino acid to a charged one, this can certainly cause a conformational change in LDLR protein and that can disturb the interaction between LDLR and its natural receptor the lipoprotein LDL.

ACKNOWLEGEMENTS

This study was funded by grant for Dr. Ahmed Zaid from the Libyan National Agency for Science and Technology. The Authors thanks the lab assistants at the research unit of the department of biochemistry at the Faculty of Medicine at Tripoli University where the experiments were conducted.

REFERENCES

1- Goldstein JL, Brown MS "History of Discovery:The LDL Receptor"Arterioscler Thromb Vasc Biol. 2009 Apr;29(4):431-8

2- Huang S, Henry L, Ho YK, Pownall HJ, Rudenko G. "Mechanism of LDL binding and release probed by structure-based mutagenesis of the LDL receptor" J Lipid Res.2010 February;51(2):297-308.

3- Hamilton-Craig I, Kostner K, Colquhoun D, Woodhouse S. "Combination therapy of statin and ezetimibe for treatment of familial hypercholesterolemia"Vasc Health Risk Manag.2010 Nov10;6:1023-37

4- Zhao Z, Michaely P." Role of an Intramolecular Contact on Lipoprotein Uptake by the LDL Receptor" Biochim Biophys Acta. 2011 Jun;1811(6):397-408

5- Zhao Z, Michaely P."The Role of Calcium in Lipoprotein Release by the LDL Receptor "Biochemistry. 2009 Aug 4;48(30):7313-24.

6- Mata et al ." Clinical characteristics and evaluation of LDL-cholesterol treatment of the Spanish Familial Hypercholesterolemia Longitudinal Cohort Study (SAFEHEART)Lipids Health Dis.2011;10:94.

7- Go GW, Mani A."Low-Density Lipoprotein Receptor (LDLR) Family Orchestrates Cholesterol Homeostasis"Yale J Biol Med.2012 Mar;85(1):19-28

8- Fahed AC, Nemer GM "Familial Hypercholesterolemia: The lipids or the Genes?".Nutr Metab (lond).2011;8:23.

9- Bambauer R, Bambauer C, Lehmann B, Latza R, Schiel R."LDL -Apheresis:Technical and Clinical Aspects" ScientificWorld Journal. 2012;2012:314283.

10- Brown MS, Goldstein JL."The SREBP Pathway: Regulation of Cholesterol Metabolism by Proteolysis of a Membrane-Bound Transcription Factor".Cell.volume 89,Issue 3, 2 May 1997, 331-340.

11- Kang Q, Chen A."Curcumin suppresses expression of low-density lipoprotein(LDL)receptor, leading to the inhibition of LDL-induced activation of hepatic stellate cells."Br J Pharmacol.2009 Aug;157(8):1345-1367.

12- Schaefer JR, Kurt B, Sattler A, Klaus G, Soufi M."Pharmacogenetic aspects in familial hypercholesterolemia with the special focus on $FH_{Marburg}$." Clin Res Cardiol Suppl.2012 Jun;7(suppl 1):2-6.

13- Lillis AP, Van Duyn LB, Murphy-Ullrich JE, Strickland DK.." The low density lipoprotein receptor-related protein 1: Unique tissue-specific functions revealed by selective gene knockout studies."Physiol Rev.2008 Jul;88(3):887-918.

14- Spuch C, Ortolano S, Navarro C."LRP-1 and LRP-2 receptors function in the membrane neuron. Trafficking mechanisms and proteolytic processing in Alzheimer`s disease."Front Physiol.2012;3:269.

15- Orth M, Bellosta S."Cholesterol: Its Regulation and Role in Central Nervous System Disorders."Cholesterol. 2012;2012:292598.

24. Vuorio AF, Turtola H, Piilahti KM, Repo P, Kanninen T, Kontula K. Familial hypercholesterolemia in the Finnish north Karelia. A molecular, clinical, and genealogical study. ArteriosclerThromb Vasc Biol. 1997;17:3127–38.

25. Seftel HC, Baker SG, Jenkins T, Mendelsohn D. Prevalence of familial hypercholesterolemia in Johannesburg Jews. Am J Med Genet. 1989;34:545–7.

26. Mabuchi H, Haba T, Ueda K, Ueda R, Tatami R, Ito S, et al. Serum lipids and coronary heart disease in heterozygous familial hypercholesterolemia in the Hokuriku district of Japan. Atherosclerosis. 1977;28:417–23.

Table1. Clinical and biochemical characteristics of the studied subjects:

Parameters	Father	Mother	Daughter
Age	55	46	19
Glucose (mg/dl)	91.21 ± 11.12	88.11 ± 10.14	86.23 ± 12.10
BMI	22	24	23
Total cholesterol (mg/dl)	**402.93 ± 34.72**	198.11 ± 22.81	177.44 ± 34.32
Triglycerides (mg/dl)	129.96 ± 112.05	122.73 ± 15.73	130.85 ± 73.71
HDL-cholesterol (mg/dl)	42.52 ± 13.07	43.98 ± 21.74	44.45 ± 34.74
LDL-cholesterol (mg/dl)	**228.47 ± 26.59**	106 ± 23.45	102.33 ± 22.65
Cholesterol/HDL ratio	**9.47 ± 1.98**	4.50 ± 1.11	4.02 ± 1.76

Figure 1 Polyacrylamide of 2 % gel for exon 10 amplified double strand PCR product . Lane 1,Cont. control sample of amplified exon 10. Lane 2, P Patient amplified sample of Exon 10. Lane 3 N negative control and on the right lane 100 λDNA size marker. 291 bp is the size of the amplified fragment which cover exon 10.

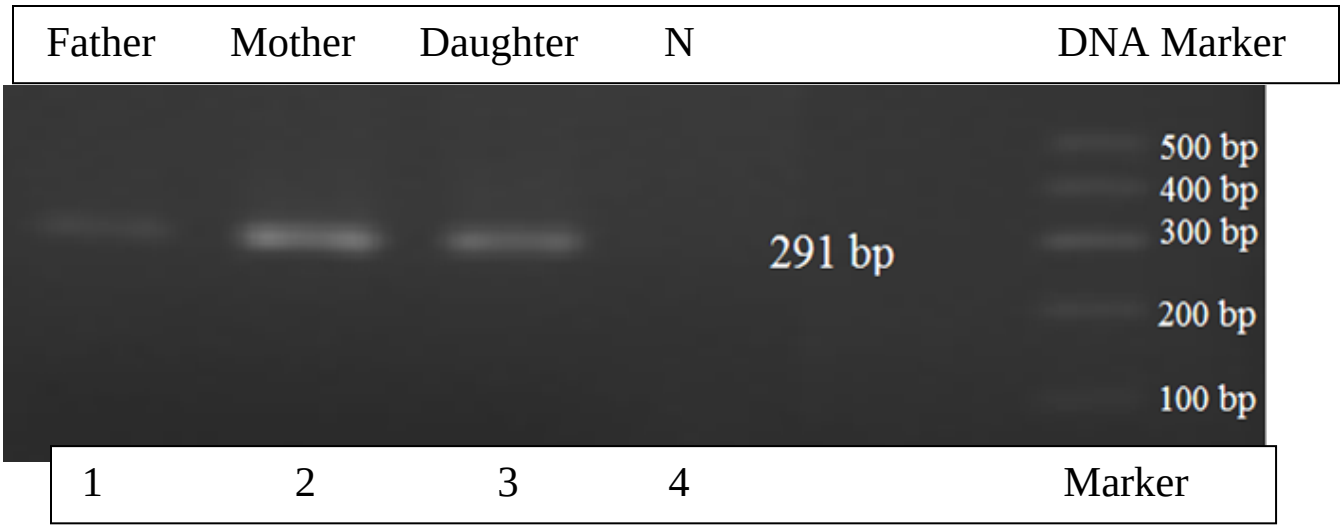

Figure 2. Electropherograms of *LDLR* gene polymorphism in exon 10. a) Forward primer 24400 T > C the arrow indicate the polymorphism. b) Reverse primer 24400 A