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Mutational screening in the *PCSK9* gene among Libyan patients presenting familial hypercholesterolemia

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Abstract: Familial hypercholesterolemia (FH) is an autosomal dominant genetic disorder of lipid metabolism, associated with elevated levels of low-density lipoprotein-cholesterol (LDLC), which can lead to premature cardiovascular disease and early death. Early diagnosis and initiation of treatment is important to prevent morbidity and mortality. Autosomal dominant hypercholesterolemia (ADH) is largely due to mutations in the low-density lipoprotein receptor gene (*LDLR*), the apolipoprotein B-100 gene (*APOB*), or the proprotein convertase subtilisin/kexin type 9 (*PCSK9*). In this study, genomic DNA of unrelated Libyan individuals with clinically diagnosed (FH) was analyzed by direct sequencing after dependent specific PCR primers amplification and DNA purification. That led to the identification of *PCSK9* gene mutations for the first time in Libyan population which was compare to other populations. All 12 exons of *PCSK9* gene and boundaries genotyped polymorphisms were sequenced, including leucine repeats coded in exon 1, by fluorescently tagged markers. We identified an allele for the rs67610340 polymorphism: an in-frame deletion, c.61_63delCTG (L8). We also identified another allele rs67610340 polymorphism: an in frame insertion c.61_63InsCTG (L10). The insertion and deletion alleles were both in exon 1 and could be associated with a risk and severity of coronary artery disease (CAD), suggesting a direct effect of PCSK9 on atherogenesis.

Keywords: atherosclerosis, CVS, cholesterol, lipoproteins, proprotein convertases, Libya

Introduction

The discovery in 2003 of the first mutations of *PCSK9* gene causing autosomal dominant hypercholesterolemia (ADH), has revealed the existence of a new important player in cholesterol homeostasis (3). PCSK9 has been shown to enhance the degradation of the LDL receptor (LDLR) at the cell surface (1, 2). Familial hypercholesterolemia (FH) was the first genetic disease of lipid metabolism characterized by increased serum total cholesterol and low density lipoprotein LDL cholesterol (1, 2 3, 5, 6). It is caused by defects in the LDL receptor pathway, which removes LDL particles from the circulation into the liver (1, 3). The majority of FH cases are transmitted in an

autosomal-dominant manner, known as autosomal dominant hypercholesterolemia (ADH). Heterozygotes occur in the general population with a frequency of about one in 500, homozygotes at one in a million (1). Affected individuals have an increased risk of atherosclerosis, premature coronary heart disease and mortality (2). FH can result primarily from mutations in genes that code for proteins involved in hepatic clearance of low-density lipoprotein cholesterol. The genes are the low density lipoprotein receptor gene *LDLR* on chromosome 19, the apolipoprotein B gene *ApoB* on chromosome 2 (6, 7). They were identified as familial hyper-cholesterolemia (FH1 & FH2) respectively. The FH3 locus was identified in 2003 by Abifadel et al. as proprotein convertase subtilisin/kexin type 9 gene (*PCSK9*) on chromosome 1 (1, 40 and 30).Two novel loci for hyper-cholesterolemia have been recognized on chromosome 16q22.1 (FH4) and 8q24.22; however, the cognate genes are yet to be identified (4, 5, and 6). PCSK9 encodes proprotein convertase subtilisin kexin type 9 is a serine protease that belongs to the proteinase K subfamily of the secretory subtilase family. The 12 exons of *PCSK9* encode a 692 amino acid secreted glycoprotein and its high expression levels in liver and small intestine (7-9).

The chromosomal localization of its gene (~22 kb *PCSK9*) on 1p32.3 (1-4). The encoded protein is synthesized as a 72 kDa soluble zymogen and its prodomain is cleaved by its own catalytic activity. The cleaved prodomain forms a protein complex with the rest of the PCSK9 carboxyl terminus within the endoplasmic reticulum and is secreted (28, 29). Secreted protein that binds to the LDL receptor and promotes its degradation (23, 24, 43). Several mutations in the *PCSK9* gene have been identified which are associated with either a hypercholesterolemic or hypocholesterolemic phenotype (25, 26). Gain of function mutations lead to a reduction in the LDLR and carriers of these mutations present with elevated LDLC levels that leads to autosomal dominant hypercholesterolemia. Conversely, “Loss of function” mutations decrease LDLR degradation and patients with these mutations have low LDLC concentrations and appear to be protected from coronary heart disease (8, 9, 11, 12). Thus, PCSK9 has become a new target for cholesterol-lowering drug therapy (34). Earlier Studies have determined that severe dyslipidemia, including both genetic familial hypercholesterolemia (FH) and familial combined hyperlipidemia (FCH) are characterized by elevated plasma LDL (FH) and LDL/triglycerides (FCH) (13-15, 25). *PCSK9* variants might contribute to FCHL phenotype and are to be taken into consideration in the study of this complex and multigenic disease with other genes implicated in dyslipidaemia (1, 20).

The identification of individuals with FH and FCHL have clinically been based on lipid levels. However, phenotypes are overlapping and family history is not always informative. Therefore, DNA diagnostics of a medical condition offers the possibility to start treatment before the disease becomes symptomatic. One of the possible downsides of genetic testing is that a person's test result may affect one's ability to obtain life insurance. A mutation in the *PCSK9* gene has been identified across a number of populations of different ethnicities; however, its existence to the best of our knowledge is not known in the Libyan population. In the present study, we determined human genomic DNA, amplified, purified and sequenced an isolated all 12 exons of the *PCSK9* gene.

This procedure has been performed for FH patients with a clinical diagnosis of familial hypercholesterolemia. Thus, the objectives of this study are to determine the genetic variants in the *PCSK9* gene in Libyan patients. The long term importance of this study is focuses on early detection and control of hyperlipidemia to improve cardiovascular risk prediction, prevention and treatment efficacy.

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 samples was obtained from the subjects by venipuncture after obtaining informed written consent from the patient. The blood sample was collected from the subjects in heparinated tube (Puls blood collection tubes, BD Vacutainer, UK). The collection of the whole blood samples from Libyan patients and controls were done in the Tripoli Medical Center, Tripoli, Libya.

DNA extraction: genomic DNA from all the patients and controls was isolated from the whole blood samples 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 the 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 two units of *Taq* polymerase. After initial denaturizing at 94 °C for 5 min, the reaction mixture was subjected to 35 cycles of 30 sec denaturation at 94 °C, 30 sec annealing at 58 °C and extension 30 sec 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 QIA quick PCR product purification kit (Qiagene, USA). The purifications were done for PCR product samples and were kept in - 20 °C in a sufficient quantity to perform DNA sequencing.

PCSK9 PCR Primers

	Primers for long-range PCR (5'→ 3'direction)	Length	Start	Stop	Tm	GC%	AF
Exon1	F: CACGGCCTCTAGGTCTCCTCGC R: GTCCCGGCTGCGGGTTCG	22 18	5242 5544	5133 5527	60.43 60.41	68.18 77.78	302
Exon2	F: CCTAGGGTTTGCTGGGTTTCTTCCA R: CCCAGCCCTATCAGGAAGTGCC	25 22	9241 9532	9265 9511	58.62 58.69	52.00 63.64	291
Exon3	F:CCAAATGGCTTAAGCAGAGTCCCCC R: AGCTCAGATGGGGTGGGGCA	25 20	11932 12141	11956 12122	59.55 59.53	56.00 65.00	209
Exon4	F: TGCTCATTCCCTCCTCTCCCACAAA R: GCAGAGGCAGCCCTCCCATC	25 20	17692 17901	17716 17882	59.36 59.20	52.00 70.00	209
Exon5	F: GGGGGTCTTTCTCATGTGGTCCTTG R: GTCCAGATGGAGAGAGACCAGCGT	25 24	18065 18286	18089 18263	58.98 59.53	56.00 58.33	221
Exon6	F: TCTCCCCAAGGGGTGACCTTGG R: CCTACCCGCGCCTTGGGG	22 18	21410 21680	21431 21663	59.52 59.41	63.64 77.78	270
Exon7	F: GCTCCTTTCTCTGCCACCCACC R: GTTAGCATCACGGTGGCCGAGG	22 22	22748 23009	22769 22988	59.41 59.86	63.64 63.64	261
Exon8	F: GGCCGGGCCATCACCATCTTTC R: GCCCCAGCCTGGACTTGCC	22 19	23448 23702	23469 23684	60.11 59.96	63.64 73.68	254
Exon9	F: TCCCAGCACCCCCTCCTCATC R: GGCTTGCTGGGGGTCCCTG	21 19	23915 24205	23935 24187	59.35 59.29	66.67 73.68	290
Exon10	F: GAGGGTGCTTGAGTTGATCCTGTCT R: AGACCCCTCCTCACCCCAGG	25 20	24850 25155	24874 25136	58.23 58.57	52.00 70.00	305
Exon11	F: TCCCAGCATTTCACATCTGAGCTGG R:CAGCACCCCACCCACCCG	25 18	26788 27047	26812 27030	59.04 59.56	52.00 77.78	259
Exon12	F: GGGCCACGCTAGACATGTGCTT R:CAGCCCTTGACCCTCCCCAGA	22 21	28779 29081	28800 29061	59.48 59.56	59.09 66.67	302

F - Forward primer; R - reverse primer; L-length of primer; AF-amplified fragment; Tm- annealing temperature.

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. Concentrations of serum total cholesterol (TC), triglyceride (TG) and HDL cholesterol (HDL-C) were determined at accredited clinical laboratories using routine clinical methods. LDL-C concentrations were calculated using the Friedewald equation. For TG levels - 4.6 mmol/L, no LDL-C value was calculated (3 cases). The four patients presented in this study were diagnosed with FH and based on the UK Simon Broome criteria. In order to purify PCR product for five PCSK9 exons amplified from the genomic DNA primers, nucleotides, polymerase and salts we used the QIAquick PCR purification kit. The purification was done for PCR product samples and was kept in - 20 °C in a sufficient quantity and quality for perform sequencing.

Results

In order to read the DNA sequence of the region of *PCSK9* exon 1, GeneScan and GeneMapper 3.0 softwares (Applied Biosystems) were used to determine the genotype of each subject. All PCSK9 gene exons were analyzed for each of the 21 patients and the 10 controls. All of the FH patient analyzed in this work did not have any mutation in *LDLR* or *ApoB* genes (Data not shown Zaid et al). Exon 1 PCR amplified products were purified and applied in electrophoresis to test the fragment size and quality (Figure 1).

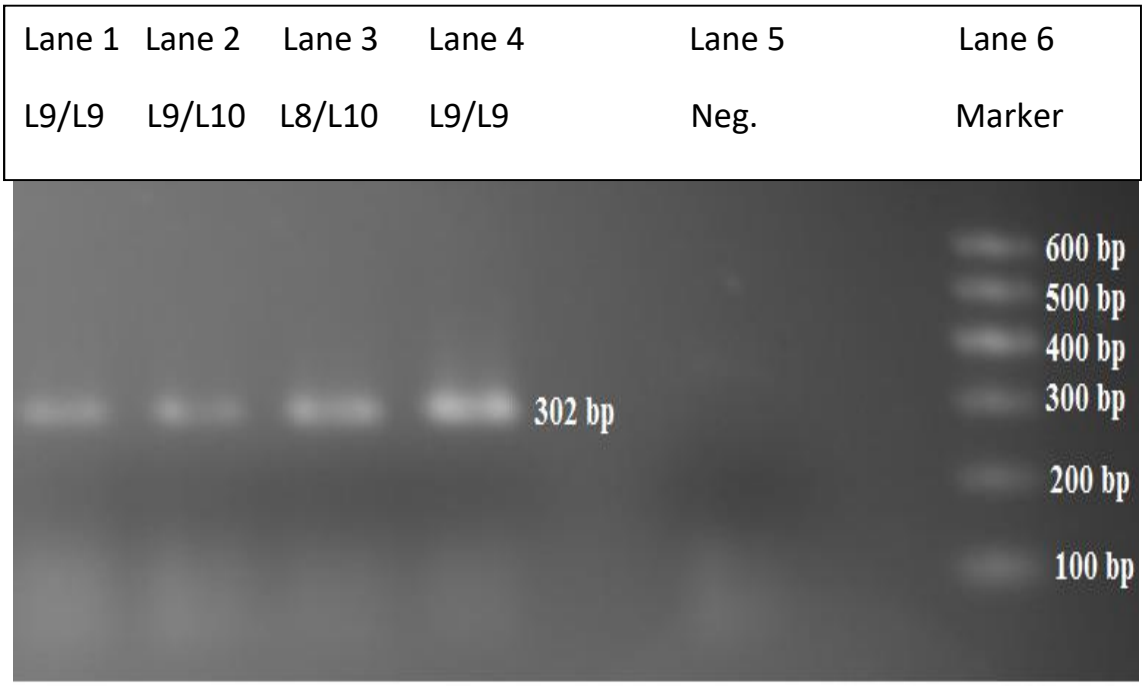

Figure 1: 2 % agaros gel for exon 1 amplified double strand PCR product. Lane 1 amplified exon 1 from L9/L9, positive control sample. Lane 2, Patient amplified sample of exon 1 for FH patient with L9/L10 genotype. Lane 3, Patient amplified sample of exon 1 for patient with L8/L10 genotype Lane 4, Patient amplified sample of exon 1 for FH patient with L9/L9 genotype Lane 5 negative control and on the right lane 100 λ DNA size marker. 302 bp is the size of the amplified fragment which covers exon 1.

We sequenced all exons of *PCSK9* gene and only found mutations in the exon 1 region in all the FH patients. During the sequencing of exon 1 which coded partially for nine leucine stretch, we had to use the GeneMapper 3.0 software to identify nucleotids deletions or insertions (Figure 2).

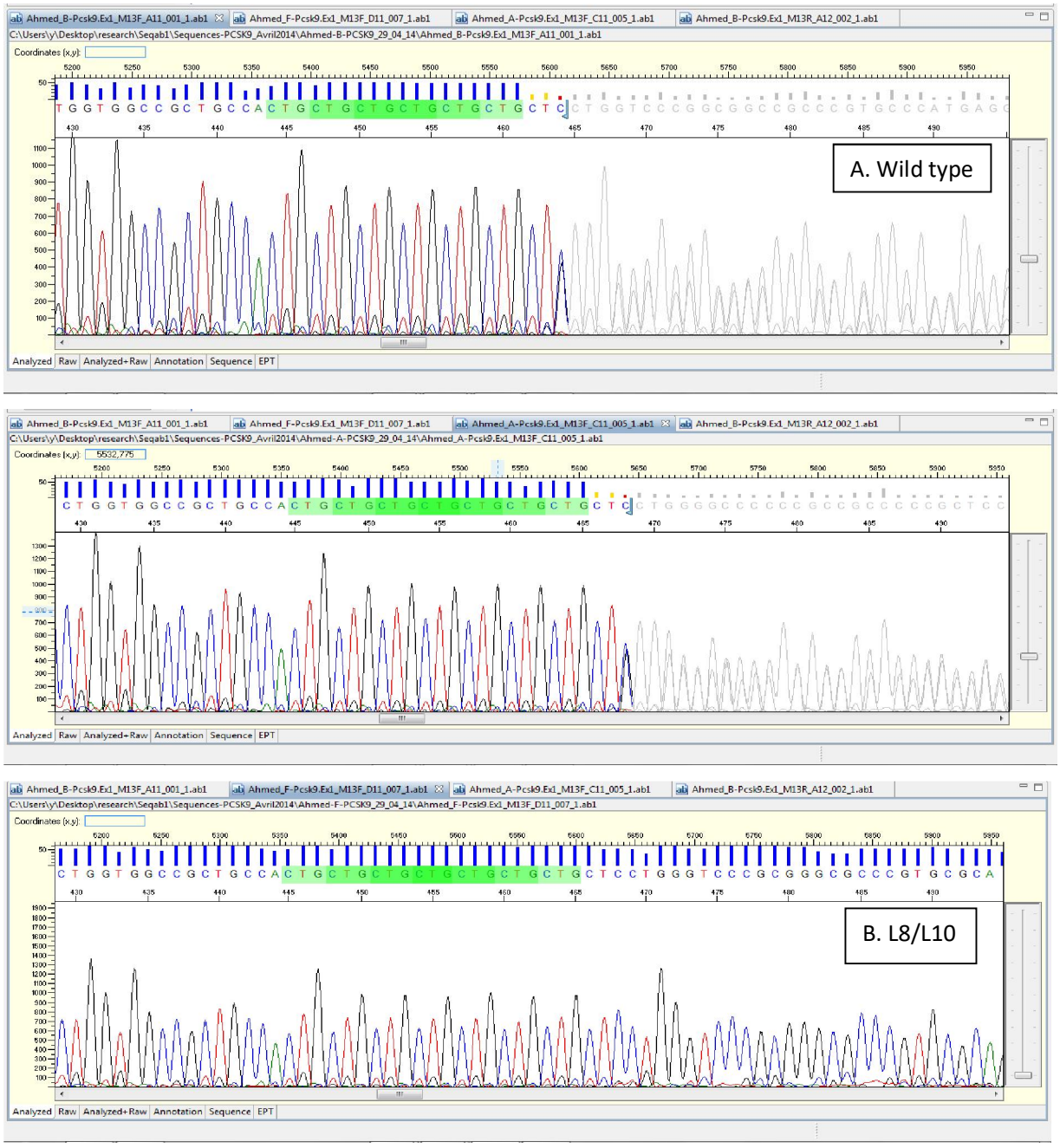

Figure 2: The ABI sequence analysis of different genotypes (L9/L9, L9/L10, L8/L10) for the *PCSK9* exon 1 in A. wildtype (normal or control) which is L9(/L9, in B homozygote mutation L8/L10 and in C heterozygote mutation. The software Sequence scanner ver.3 were used for the analysis.

The GeneMapper shows three different genotypes L9/L9 which is the wild type, L9/L10, which is heterozygous and most interestingly L8/L10 which is a homozygous mutation for the rs72555377 polymorphism in the *PCSK9* gene observed in the studied Libyan population. Labels under each peak provide the polymerase chain reaction (PCR) product size in base pairs (pb) (L9: 158 pb, L10: 162 pb, L8: 154 bp) as shown in Figure 3.

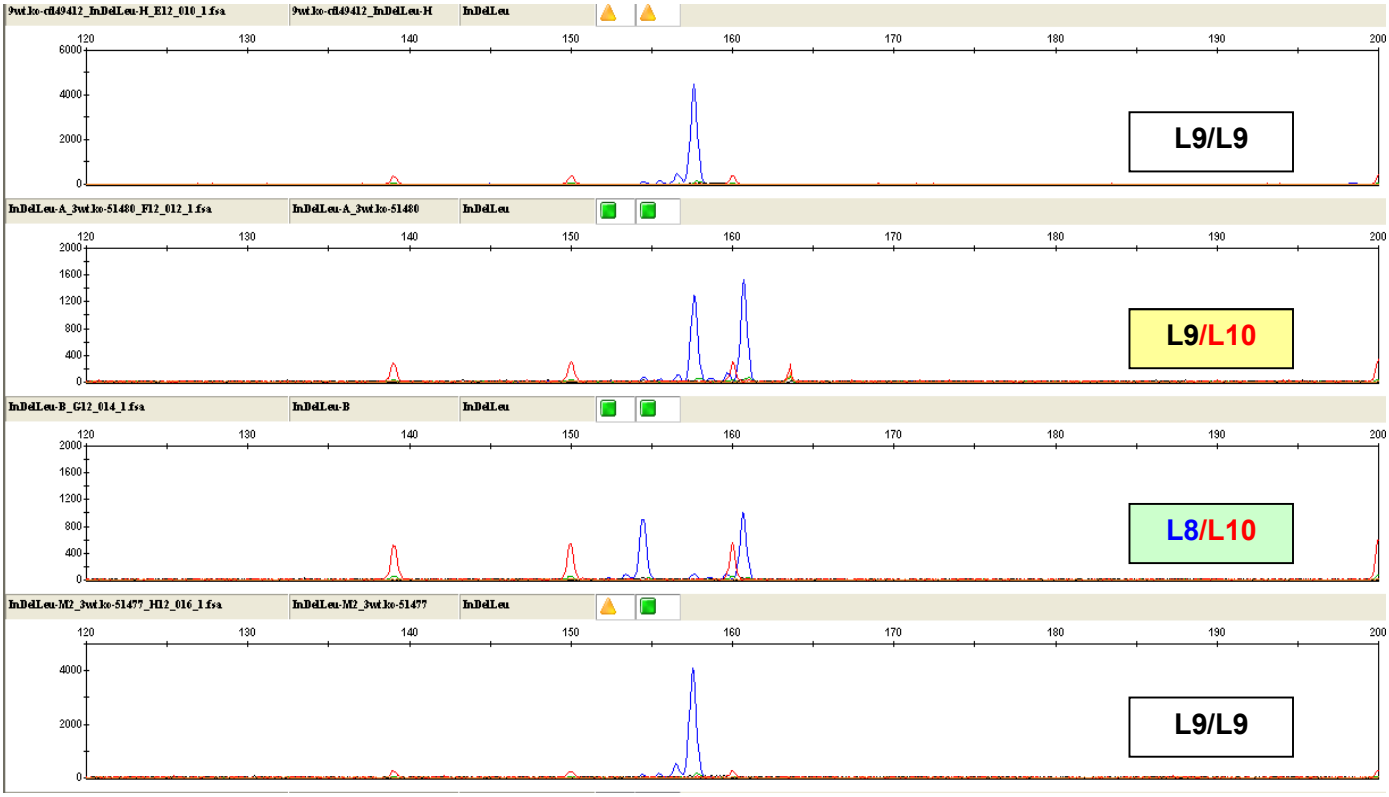

Figure 3: The different genotypes (L9/L9, L9/L10, L8/L10) for the rs72555377 polymorphism in the *PCSK9* gene observed in the studied Libyan population. Labels under each peak provide the polymerase chain reaction (PCR) product size in base pairs (pb) (L9: 158 pb, L10: 162 pb, L8: 154 bp), GeneMapper 3.

One sample for each one of the two different genotypes identified (L9/L10, L8/L10) was confirmed by sequencing. We show here for the first time the existence of a new allele of the rs72555377 polymorphism in Libya population that was called L8 and which was only reported in Tunisian population (46). This allele is an in-frame deletion of one leucine (c.61-63delCTG) within the polyleucine stretch in exon 1 of the PCSK9 gene. Among the 21 patients, 10 were L9/L9, 8 L9/L10, 3 L8/L10. Among the 10 controls, all were L9/L9. We also show here for the first time the existence of homozygote mutation L8/L10 in a Libyan patient. To date, no one reported before the L8/L10 homozygote mutated alleles. We further investigate the association of the new alleles L8 and L10 alleles with risks of CAD coronary artery disease. Table 1 shows the biochemical parameters that been used to investigate the association. Interestingly, the L8/L10 patients showed an increasing level of glucose, TC and TG, which suggest the patient, is FCHC and diabetic. None of other FH patients showed increases in TG and glucose. In addition, patients with L8/L10 allele showed an increase in TC and severe increase in TG levels when compared to wild type L9/L9 (Table 1). However, these results are inconclusive at present considering the small number of L8 carriers. However, our data confirm what the Tunisian group suggested that L8 allele might have

a relation with diabetes (46) since our biochemical analysis to the L8/L10 patients confirm also that he is diabetic.

Table 1: Clinical and biochemical characteristics of the studied subjects

Parameters	L9/L9 Cont.	L9/L10 FH	L8/L10 FH	L9/L9 FH
Number of Patients	10	7	5	10
Average Age	35 ± 8	40 ± 12	41 ± 13	38 ± 12
Glucose (mg/dl)	81.21 ± 10.11	88.11 ± 10.14	**112.23 ± 12.10***	86.23 ± 12.10
BMI	22	24	23	23
Total cholesterol (mg/dl)	177.93 ± 34.72	**284.11 ± 22.81***	**277.44 ± 34.32***	**260.4 ± 24.12***
Triglycerides (mg/dl)	129.26 ± 112.05	122.73 ± 15.73	170.85 ± 73.71	130.85 ± 73.71
HDL-cholesterol (mg/dl)	42.42 ± 12.07	43.88 ± 21.64	40.41 ± 34.74	43.45 ± 14.73
LDL-cholesterol (mg/dl)	108.47 ± 16.59	**166.11 ± 23.45***	**152.33 ± 12.25***	**162.3 ± 24.55***
Cholesterol/HDL ratio	4.21 ± 1.99	**6.60 ± 1.11***	**6.92 ± 1.76***	**6.04± 1.76***

**p*-value is less than 0.05 (shown in bold)

The other FH patients with heterozygote mutated L9/L10 alleles had a normal glucose; high TC levels compared with L9/L9 the wild type. The c.61_63dupCTG (L10) allele of rs72555377 polymorphism in *PCSK9* has been reported to be associated with LDL-C levels and with a decreased risk of CAD. Our patients with L9/L10 alleles had a high CT level, this finding does not fit with what was suggested by other groups before in the literature that L10 allele associated with lower levels of LDL-C.

Discussion

To our knowledge, this study is the first report of trying to identify mutations in the *PCSK9* gene from the Libyan population with and without severely elevated LDL-C levels. However, several groups have reported on the effect of genetic variation in the *PCSK9* gene on the phenotype of FH (30, 31). Abifadel and colleagues showed that individuals who inherited pathogenic mutations in *PCSK9 gene* had higher LDL-C levels. Conversely, Strom and colleagues studied the effect of a loss-of-function *PCSK9* mutation, R46L, in FH (34, 35). The South African Caucasian population presents with unique genetic founder effects either with increased prevalence of unique mutations or mutations found in many other populations (37, 38). A founder effect of the population specific mutations including mutations within the prodomain (S127R) and D129G) for the exon2 and (D129G) for exon3 or catalytic domain (R215H, F216L and R218S) for exon5 are associated with high levels of circulating LDL-C (2, 42, 43). Other mutations, within the catalytic domain (N425S) for exon8, have so far only been identified in families who also carry ***LDLR*** mutations. Based on above information, we chose to start with sequencing all 12 exons of *PCSK9* gene for FH patient with no mutation in *LDLR* (FH1) and *ApoB* (FH2) genes (36, 37). Our first concern was to prepare

and screen for a specific mutation using techniques such as PCR amplification, since the quality of genomic DNA used has an impact on the obtained results. To determine the DNA quality, three important factors need to be determined; the concentration, integrity and purity of the human genomic DNA sample. In this study, we constructed 12 primer pairs for *PCSK9* gene and tested their ability to amplify segments of genomic DNA from suspected individuals. Thus, we have achieved our objective of genetic testing using PCR technology involves the amplification of the regions of DNA containing the *PCSK9* 12 exons which suggesting that might contain the mutations. An early diagnosis of FH would allow a timely monitoring and treatment of this disorder.

Using such method would enable the rapid screening of large populations to determine the prevalence of specific mutations within the Libyan patients. In order to prevent the possibility of false results and detect contamination or non-specific bands, two levels of controls were used which include: negative control (PCR reaction mix without template genomic DNA) and molecular weight size markers. The negative controls were used to detect presence of contamination in PCR reaction mixtures and molecular weight size marker to identify the DNA fragment or band needed for detection of exons. Using these controls in the screening prevent false negative amplifications.

In conclusion, we have identified and analyzed novel and known polymorphisms in *PCSK9* locus, reconstructed haplotypes encompassing the information content of 2 polymorphisms (L8 L10) and shown that they might be is important determinant of plasma LDL-C and TC levels, and is associated with the severity of coronary atherosclerosis in the Libyan population. Identification of molecular mechanism(s) by which *PCSK9* variants affect plasma LDL-C levels could provide new insight into the pathogenesis of atherosclerosis and development of new drug targets. Finally, studying the relationship between *PCSK9* variants might contribute to understand and FH and FCH phenotypes in a complex and multigenic disease with other genes implicated in dyslipidemia.

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