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PCSK9 post-transcriptional regulation: Role of a 3'UTR microRNA-binding site variant in linkage disequilibrium with c.1420G

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Abstract

Background and aims Proprotein convertase subtilisin/kexin type 9 (PCSK9) plays a crucial role in cholesterol homeostasis. A common variant, the G allele in position c.1420 (c.1420G), has been associated with a decrease of both plasma PCSK9 and LDL-cholesterol concentrations. However, the functional effect of this variant is currently not well understood. We hypothesized that it could be explained by functional variants in linkage disequilibrium (LD), more specifically, by variants located in the *PCSK9* 3' UTR as targets for miR regulation of *PCSK9* expression.

Methods Variations in LD with c.1420G were studied in 1029 patients followed for dyslipidaemia. *In silico* studies identified potential miRNA binding sites induced by *PCSK9* 3'UTR variants in LD with c.1420G. Their functionality was studied by a luciferase reporter assay in *HuH-7* cells and confirmed by cotransfection of antimiRNAs.

Results The c.*571C and c.*234T variants located in *PCSK9* 3'UTR were found in tight LD with c.1420G (D'=0.962; LOD= 163.06). The haplotype carrying c.*571C showed a 6.7% decrease in luciferase activity (p=0.003). Inhibition of hsa-miR-1228-3p and hsa-miR-143-5p counteracted their effect on the haplotype carrying c.*571C allele, suggesting that *PCSK9* expression was decreased by the endogenous binding of hsa-miR-1228-3p and hsa-miR-143-5p on its 3'UTR.

Conclusions This post-transcriptional regulation might contribute towards the association between plasma PCSK9 levels and c.1420G. Such regulation of *PCSK9* expression may open new perspectives for the treatment of hypercholesterolemia and atherosclerosis cardiovascular diseases.

Introduction

Familial hypercholesterolemia (FH) is the most common autosomal dominant inheritable disease caused by a defective catabolism of LDL particles. Among causative genes, different variations in *PCSK9* gene can lead to either FH or familial hypobetalipoproteinemia (FHBL), depending on whether they are gain (GoF) or loss (LoF) of function variations[1–3]. Indeed, PCSK9 shifts the LDL receptor (LDLR) trafficking from plasma membrane recycling to lysosomal degradation depending on its affinity for LDLR, therefore modulating the level of LDL-cholesterol (LDL-c). *PCSK9* GoF variations decrease LDLR expression in the hepatocytes, resulting in high levels of LDL-c in the plasma[4], whereas LoF variations improve LDLR recycling, increasing LDL clearance, leading to lower LDL-c levels and protection from cardiovascular diseases[5,6].

However, the effect of common p.(Ile474Val) (c.1420A>G; rs562556) allele varies according to the populations. This variation has been associated with a decrease in plasma PCSK9 (-9.6%, p=0.033) and LDL-c levels (pooled standardized mean difference -0.34mmol/L, p=0.002). However, this effect depends on ethnicity [7–10]. Currently, the mechanism of the hypocholesterolemic effect of the c.1420G variation is not well understood: despite a reduction of the steric hindrances, the variation does not drastically affect the PCSK9 tertiary structure *in silico*[11]. This substitution is located in a β-hairpin motif of the C-terminal domain. In this region, 2 out of 8 missense variations, p.(Ser465Leu) and p.(Pro467Ala), were functionally characterized, and as GoF[12,13]. Additionally, the effect of p.(Ile474Val) was not reported *in vitro*.

Considering the little evidence regarding a potential LoF effect of this *PCSK9* variation *in silico*, we considered if other functional SNPs in LD with c.1420G could explain the apparent LoF inferred from the decrease in LDL-c and the variability between populations. Accordingly, previous studies have already shown that key genes involved in the regulation of lipids metabolism could be post-transcriptionally regulated by 3'UTR variants creating or suppressing binding sites for miRs[14–17]. Therefore, we aimed to assess whether *PCSK9* variants in LD with c.1420G and located in the 3'UTR could be linked and involved in the LoF effect of this variant.

Materials and methods

Population

A total of 1029 probands and 50 of their relatives were studied (Supplemental Materials). Written informed consent was obtained in accordance with the principles of the Declaration of Helsinki, the French bioethics law and approved protocols by the Hospices Civils de Lyon.

PCSK9 polymorphisms analysis

After genomic DNA extraction, the *PCSK9* gene was sequenced and analysed as detailed in Supplemental Materials.

The cis-conformation was determined using two different technologies: a sub-cloning of PCR products thanks to a TopoTA reaction (n=2 probands) and a long read sequencing (n=1 propositus) (Supplemental Materials).

Minor allele frequencies (MAF) of probands were compared to MAF in European and mondial population (Table 1 and Supplemental Table 1).

Linkage disequilibrium

Linkage disequilibrium analyses were performed using Haploview 4.2 software[18]. A D'>0.8 with LOD>2 were considered to be strong LD.

Bioinformatic studies for 2d structure and 3d partial structure of *PCSK9* 3'UTR

We compared the *PCSK9* 3'UTR secondary of each 3'UTR haplotype with RNAfold WebServers software[19] and the partial structures (c.*124_c.*324 and c.*471_c.*671) of the different alleles (c.*234C; c.*234T; c.*571T and c.*571C) with SimRNAweb software[20] as detailed in Supplemental Materials.

Bioinformatic studies of miR binding-site predictions

The miR binding-site predictions were compared with seven different softwares as detailed in Supplemental Materials.

Expression of hsa_miRs was checked in the Human protein Atlas[21].

An in-house predicted score was calculated as detailed in Supplemental Materials and Supplemental Table 2.

Human *PCSK9* 3'UTR luciferase constructs

The miR expression regulation of the three *PCSK9* 3'UTR haplotypes was studied using a firefly luciferase reporter assay as described in Supplemental Materials.

Targeted miRs expression determination

Hsa-miR sequences were obtained from miRbase available online (www.mirbase.org). Total RNA was extracted from *HuH-7* cell lines and quantitative PCR was performed as described in Supplemental Materials.

Luciferase assays

HuH-7 human hepatic cell lines were transfected as detailed in Supplemental Materials with the *PCSK9* 3'UTR wild type [c.*234C; c.*571T] or *PCSK9* 3'UTR variant: haplotype [c.*234C; c.*571C] or haplotype [c.*234T; c.*571C].

Then, cells were co-transfected with each of the *PCSK9* 3'UTR and with anti-hsa-miR to test the specific effect of endogenous predicted miRs in comparison with a negative control (see Supplemental Materials).

Statistical analyses

T test or ANOVA plus Bonferroni test were performed when appropriate for variable with Gaussian distribution, Kruskall-Wallis, Mann-Whitney and Wilcoxon tests were performed as non-parametric tests when appropriate for variables with non-Gaussian distribution. The categorical variables were analysed with the Chi-squared test (Supplemental Materials). A p value < 0.05 (two sided) was considered as significant.

Results

Haplotype analysis

In this population, a total of 138 single nucleotide polymorphisms (SNPs) were identified in *PCSK9* (Supplemental Figure 1A). 11 SNPs were in high LD with c.1420G (Supplemental Figure 1B and Supplemental Table 1) leading to 10 different haplotypes (frequency, f>0.1%) (Table 1). The haplotype with the higher frequency was considered as the wild type (WT, haplotype A, f=0.682). The c.1420G was found in LD with c.1380A (p.(Val460=)) (haplotypes B, E, F), c.*234T (haplotype E), c.*571C (haplotypes B, E, F), and c.*1360_*1361insTGATG (haplotypes B and E) in haplotypes with a frequency above or greater than 1% (Table 1).

Sanger sequencing confirmed a coinheritance of c.1420G with c.*571C in 31 relatives and coinheritance of c.1420G with c.*234T and c.*571C in two relatives. Cis conformation of c.1380A, c.1420G, and c.*571 variants was further confirmed.

In silico, the c.1380A (p.(Val460=)) is predicted to have no effect neither at protein level nor on natural donor site of intron 8, and the g.30401_30402insTGTGA is located after the polyadenylation signal. Consequently, we focused on the c.*234T and c.*571C 3'UTR variations and hypothesized that they might create illegitimate binding sites for miRs, thus resulting in *PCSK9* post-transcriptional inhibition.

Allele-specific *PCSK9* 3'UTR structures

The c.*571C allele changes the predicted secondary structure conformation and decreases significantly the RNA entropy from -492.81kcal/mol to -494.50kcal/mol. Addition of the c.*234T modifies the predicted RNA conformation and restores entropy (-494.50kcal/mol to -492.72kcal/mol) (Figure 1). The tertiary partial structure modelling predicts strong differences in conformation (Figure 2).

Luciferase activity downregulation with the *PCSK9* c.*571C allele in *HuH-7* cells

Because *PCSK9* is mostly expressed in the liver, liver-expressed miRs were considered. Consequently, we sought to determine whether luciferase activity was regulated by the two most frequent *PCSK9* 3'UTR haplotypes in LD with c.1420G, i.e. [c.*234C; c.*571C] and with [c.*234T; c.*571C] in human hepatic cells *HuH-7*.

Compared to wild type [c.*234C; c.571T], the *PCSK9* 3'UTR containing the [c.*234C; c.*571C] haplotype showed a mild but consistent decrease in luciferase activity (-6.7±1.4%; p=0.003) (Figure 3). Meanwhile, the luciferase expression of the [c.*234T; c.*571C] haplotype was similar to the wild type haplotype (p=0.803).

Endogenous miRs selection

Endogenous miRs to be tested were identified by bioinformatic predictions followed by miRs expression, according to the decision tree shown in Supplemental Figure 2.

Among 1921 different miRs predicted to bind the *PCSK9* 3'UTR, 171 miRs were identified by three or more software and only 26 were predicted to bind on the c.*551_c.*591 position (Supplemental Table 3).

According to the prediction score, the c.*571C allele creates or suppresses a binding site for only two miRs, hsa-miR-3178 and hsa-miR-1228-3p, respectively. We also tested three additional miRs with the highest predicted scores (hsa-miR-143-5p, hsa-miR-139-5p, hsa-miR-25-3p) (Supplemental Table 3). Sequences alignments with *PCSK9* 3'UTR WT or with c.*571C allele, total binding base and hydrogen bonds are shown on Supplemental Figure 3. Hsa-miR-1228-3p, hsa-miR-3178 and hsa-miR-25-3p showed a better affinity for *PCSK9* 3'UTR c.*571C and the global affinity of hsa-miR-143-5p was unchanged.

Finally, hsa-miR-25-3p, hsa-miR-1228-3p and hsa143-5p were found to be expressed in total RNA extract of HuH-7 cells, whereas hsa-miR-3178 and has-miR-139-5p were not detected (Supplemental Figure 4).

Endogenous downregulation of luciferase activity by hsa-miR-1228-3p and hsa-miR-143-5p with the *PCSK9* c.*571C allele in HuH-7 cells

We first tested, as a positive control in our cellular model, the functionality of hsa-miR-30c on the *MTTP* 3'UTR regulation. As previously demonstrated in HuH-7 cells by Soh et al. [22] a 21±1.2% decrease in *MTTP* expression by hsa-miR-30c was observed, counteracted by the anti-miR30 (regain of 19±1.6%, p<0.001) (Supplemental Figure 5).

Then, the specific effect of endogenous miRs on the luciferase activity of the *PCSK9* 3'UTR was studied by using anti-miRs in comparison with a negative control (miRCTRL) (Supplemental Figure 6). Following transfection of the anti-hsa-miR-25-3p, the luciferase activity in [c.*234C; c.*571C] *PCSK9* 3'UTR construct *versus* [c.*234C; c.571T] wild type was similar to that observed with miRCTRL (4.1±0.8% decrease, p<0.001) (Figure 4): in

these experiments, the anti-hsa-miR-25-3p transfection did not reverse the luciferase activity of *PCSK9* 3'UTR c.*571C allele. However, the anti-hsa-miR-143-5p and anti-hsa-miR-1228-3p fully reversed the decrease in luciferase activity of *PCSK9* 3'UTR c.*571C allele (p=0.647 and p=0.798) exposed to endogenous miRs. These results suggested that in HuH-7 cells, *PCSK9* expression was regulated by endogenous hsa-miR-1228-3p and by hsa-miR-143-5p binding to the 3'UTR.

Discussion

Since *PCSK9* discovery, more than 220 variations were reported in the Clinvar database and several of these have been well characterized. We focused on the c.1420G variant, which had little evidence of a LoF effect *in silico*, whereas it was associated with a LDL-c decrease in many but not all populations.

The LDL-c decrease associated with c.1420G varies among different studies

The c.1420G variation was associated with a decrease in plasma LDL-c levels in Caucasians (p=0.05), ancestry European ($p=6 \times 10^{-21}$) and Japanese populations (p=0.0007) [8–10]. Nevertheless, no effect was described in Caucasians from Northern Europe, nor in American, Thai, and Brazilian populations[23–29]. Similarly, an in-house French meta-analysis including the MONA LISA[30] and ELISABET cohorts [31] (n=3357) did not show any significant association between c.1420G and LDL-c levels, neither after comparing all haplotypes in the global population (Supplemental Table 4) nor between homozygous c.1420A *versus* homozygous c.1420G carriers (Supplemental Table 5). However, an association of c.1420G with a mild but significant decrease of LDL-c levels was demonstrated in a recent meta-analysis[7] and in a large study including 172,970 individuals[32], prompting us to study if other variants, especially in regulatory and untranslated regions, in LD would be part of this effect.

In vitro miR regulation by c.*571C allele

Previous studies have proven that SNPs in 3'UTRs could modify mRNA regulation and protein expression[14]. We focus on the two most frequent variations in LD with c.1420G, namely c.*234 (allele C or T) and c.*571 (allele C or T). We found a low but significant decrease in luciferase activity induced by c.*571C in the HuH-7 cell line.

In vitro, the *PCSK9* c.*571C minor allele creates an illegitimate and functional binding site for hsa-miR-143-5p and hsa-miR-1228-3p leading to a specific inhibition of *PCSK9* expression counteracted by anti-miR treatment. Paradoxically, *in silico,* the binding site for hsa-miR-1228-3p was predicted to be removed in the presence of the c.*571C allele. This contradictory result, based on complementarity of the primary sequence between miR and 3'UTR, highlights the limitations of *in silico* studies and supports the need for *in vitro* studies[33].

Biological and clinical relevance

Since hsa-miR-143-5p and hsa-miR-1228-3p were expressed in HuH-7 and in human liver tissue[34], they might downregulate *PCSK9* at the posttranscriptional level, mediating the association between PCSK9 plasma concentrations and c.1420G variation. Moreover, the 6.7% decrease in luciferase activity observed in this study is in line with the 10% decrease in PCSK9 plasma concentration associated with the c.1420G allele[9,10]. It should lead to a decrease in LDL-c concentration of 0.07 mmol/L according to a correlation found in control subjects[35], and is consistent with the 0.066 mmol/L decrease observed by Hopewell et al. in the c.1420G allele carriers[8]. In recent years, polygenic risk scores (PRS), based on multiple common genetic variants with small to moderate effects, have enabled the diagnosis of polygenic dyslipidemia and have contributed to explain the variable expressivity of monogenic dyslipidemia[36,37]. It would be of interest to evaluate the effect of the c.*571C itself on LDL-c and if the addition of this variant to PRS improves their performance.

To date, miR-1228-3p and miR-143-5p were not reported to affect cholesterol homeostasis. It is possible that the involvement of these particular miRs in the regulation of LDL-c metabolism was elusive since *PCSK9* c.*571 regulation would appear only upon the creation of a functional illegitimate target site. However, miR-143-3p, the miR excised from the 3' stem-loop of pre-miR143 during miR maturation, has been shown to be up-regulated in dyslipidemic rabbits with coronary heart disease and may has been associated with an increased LDL-c plasma concentration in obese subjects[38]. Conversely, the miR-143-3p inhibitor reduced triglycerides and LDL-c plasma concentration in CHD rabbits, demonstrating a specific effect of this miR on lipids [39]. In literature, other miRs with strand-specific biological effects, opposite in some case, have been previously described[40,41]. Although the two mature 5p and 3p miRs are transcribed from the same pre-miR, they may have different stability, target genes and biological functions[42].

Other miR binding sites on *PCSK9* 3'UTR

In this study, the regulation of *PCSK9* expression based on variations in LD with c.1420G allele is significant but mild. Recently, another variation, rs17111557 (c.*614C>T), was described as non-significantly associated with an increase of LDL-c (p=0.059) by Zambrano's team in the Brazilian population[43]. In our study, this variation was also found in LD with c.1420G at a low frequency, in a specific haplotype including a total of six variations in *PCSK9* 3'UTR (haplotype G, Table 1). Unfortunately, the haplotype found in this Brazilian population was not described. It would be of great interest to test *in vitro* the effect of this particular haplotype to determine if it would regulate *PCSK9* expression. Indeed, in our study, the effect of c.*571C alone is lost when both c.*234T and c.*571C polymorphisms are present, probably due to an alteration of miR binding on c.*571C in the modified structure due to the presence of both variants (Figure 2)[44].

Interestingly, in our study, more than 1900 miRs were predicted to bind the 3'UTR of the *PCSK9* mRNA. Among them, miR-191, miR-222 and miR-224 have been shown to bind to target sites other than c.*571[45,46]. A significant 20-30% down-regulation of luciferase activity was demonstrated *in vitro.* However, the overexpression of these miRs was non-physiological [45,46] and the association between these miRs levels and LDL-c was not established.

Limitations of the study

We cannot completely exclude a regulation by a miRNA not detected by our bioinformatics analysis even if we used a combination of several softwares, not included in *in vitro* studies, or not expressed in the cellular model. Moreover, effect of the c.*571C itself on LDL-c has to be evaluated in large genome-wide association studies to distinguish it from the effect of variants in LD.

Conclusion

miRs provide a sophisticated mechanism of gene regulation which coordinates a broad spectrum of steps in the regulation of lipid metabolism. We provide evidence that the miR binding site created by the c.*571C rare allele in *PCSK9* should modulate *PCSK9* expression in HuH-7 cells. Such *3'*UTR *PCSK9* post-transcriptional regulation may open new therapeutic

perspectives for the management of hypercholesterolemia and atherosclerosis cardiovascular disease

Conflict of interest

The authors declared they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

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Author contributions:

Clinical assessments: SC BC PM.
Experiments: conception and design: CD AJ NC OM LD AM CB MDF; performed the experiments: CD MMo SD ED.
Analyzed the data: CD MMo MMa AJ OM SC PM MDF.
Wrote the paper, approve the version to be published, agree to be accountable for all aspects of the work: all authors.

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Titles and legends to Figures and Table

Figure 1: *PCSK9* 3'UTR mRNA secondary structure representation.
(A) *PCSK9* 3'UTR mRNA [c.*234C; c.*571U]; (B) *PCSK9* 3'UTR mRNA [c.*234C; c.*571C] (C) *PCSK9* 3'UTR mRNA [c.*234U; c.*571C]. The secondary structure was represented with entropy value (red=0 to purple=3,5). Total entropy for the construct [c.*234C; c.*571U] was -492,81kcal/mol; -494,50kcal/mol for the construct [c.*234C; c.*571C] and -492,72kcal/mol for the construct [c.*234U; c.*571C]. This representation was obtained with RNAfold webserver with Andronescu model and RNA G-quadruplex prediction.

Figure 2: *PCSK9* 3'UTR mRNA tertiary structure representation.
Partial 3D structures (A) and (B): [c.*134_c.*334] and (C) and (D): [c.*471_c.*671] of *PCSK9* 3'UTR mRNA were visualized with PyMol software *[47]*. First nucleotide of each partial structure was labeled in blue and last in red. Nucleotides c.*234 or c.*571 are represented with spheres (green= carbon; blue=nitrogen and red =oxygen).

Figure 3: Endogenous hsa-miRs down regulate luciferase activity of the *PCSK9* 3'UTR c.*571C allele in HuH-7 cells.
Luciferase activity is expressed as relative to that of the most frequent haplotype [c.*234C; c.571T]. The *PCSK9* 3'UTR with the c.*571C allele showed a decrease of luciferase activity whereas it was not different for the construct with [c.*234T; c.*571C]. Each experiment has been performed at least five-time and n represented the total number of replicates. Subgroups were compared using a Student t -test.

Figure 4: *PCSK9* 3'UTR luciferase activity after the co-transfection with specifics anti-hsa-miR.
n represented the number of replicates. The construct c.*234C/c.*571T transfected was consider as the reference for data normalisation for each hsa-miR or anti-hsa-miR. Each experiment has been conducted at least four-time and n represented the total number of replicates. Subgroups were compared using Mann-Whitney tests.

Figure 1

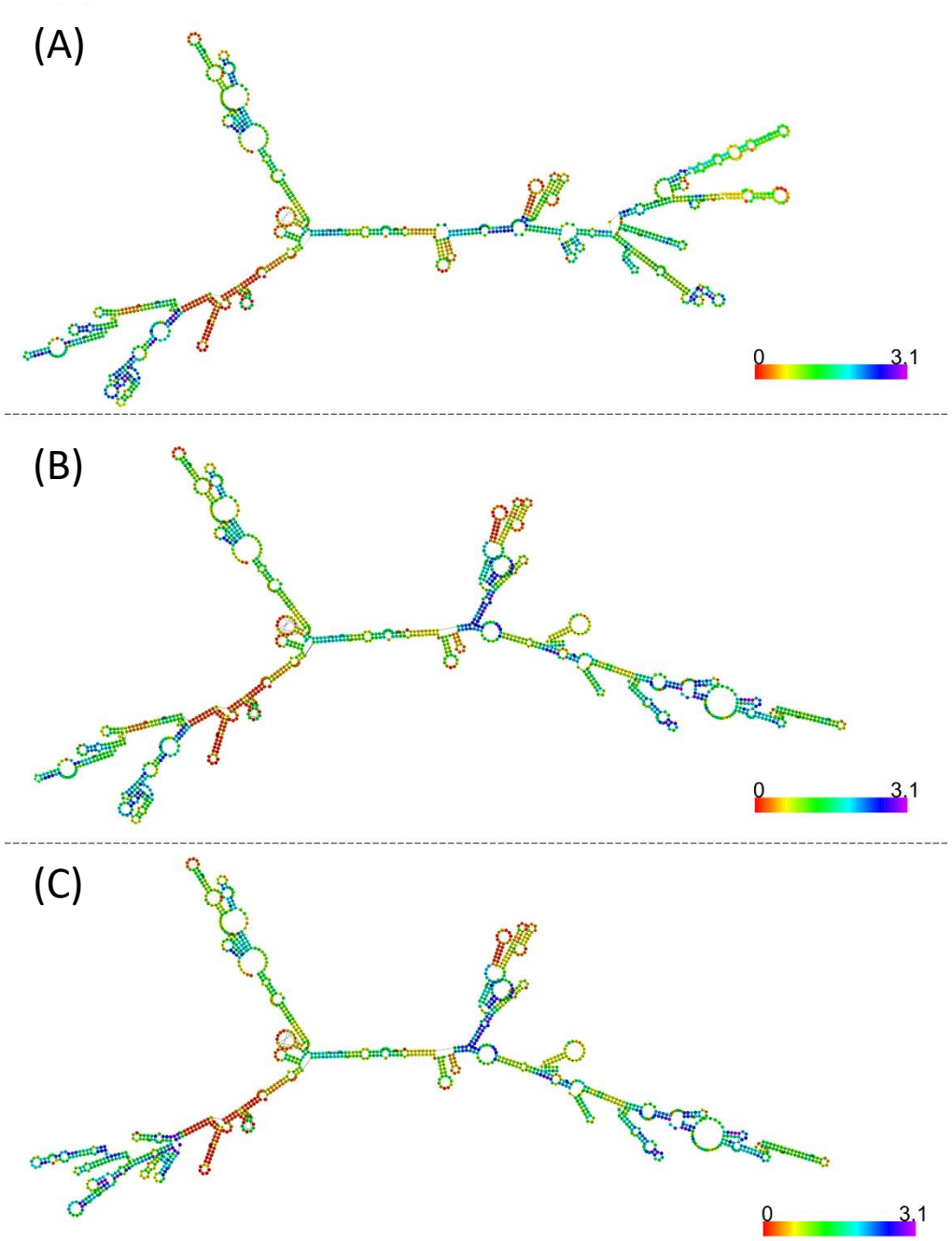


Figure 2

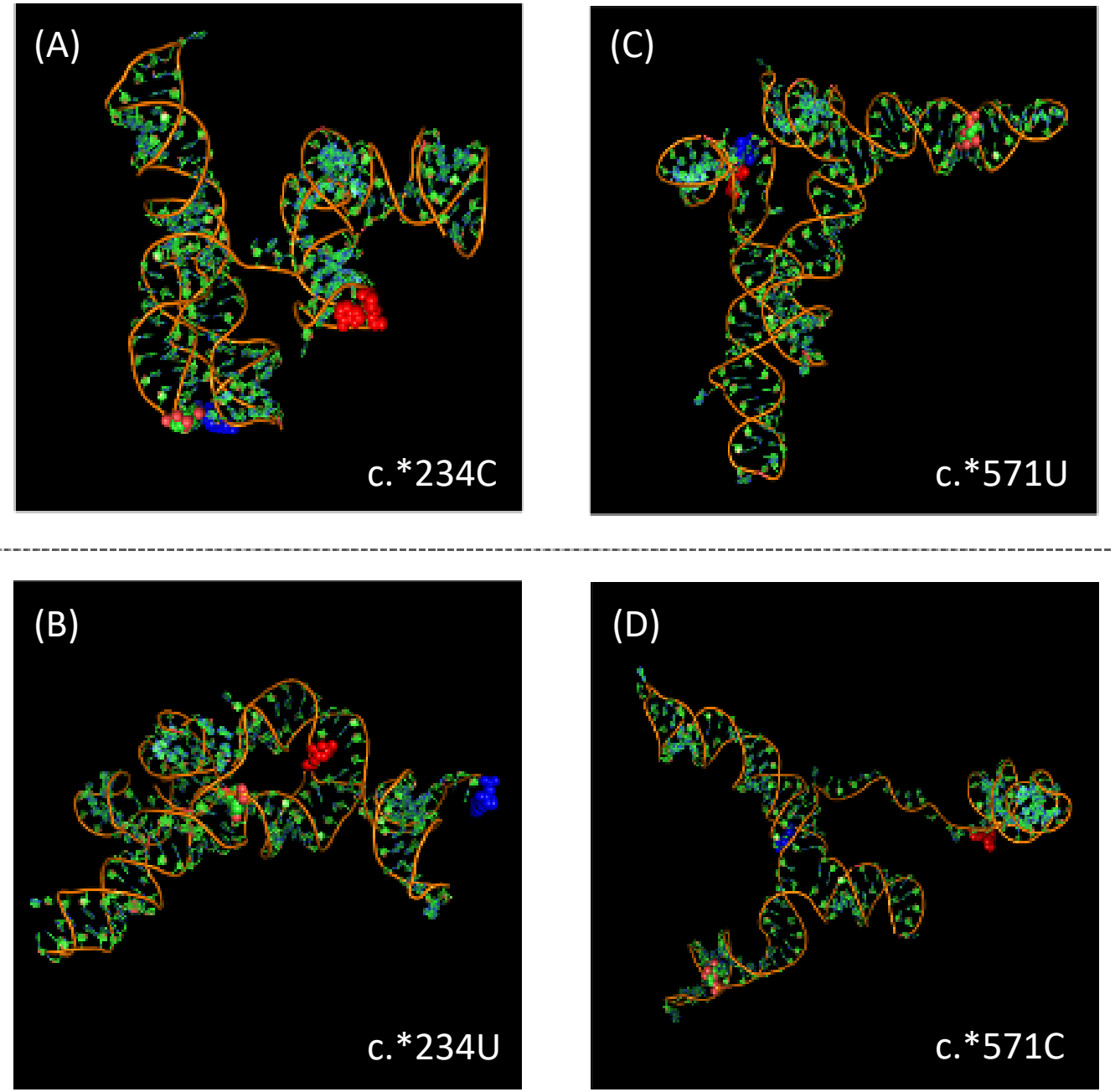
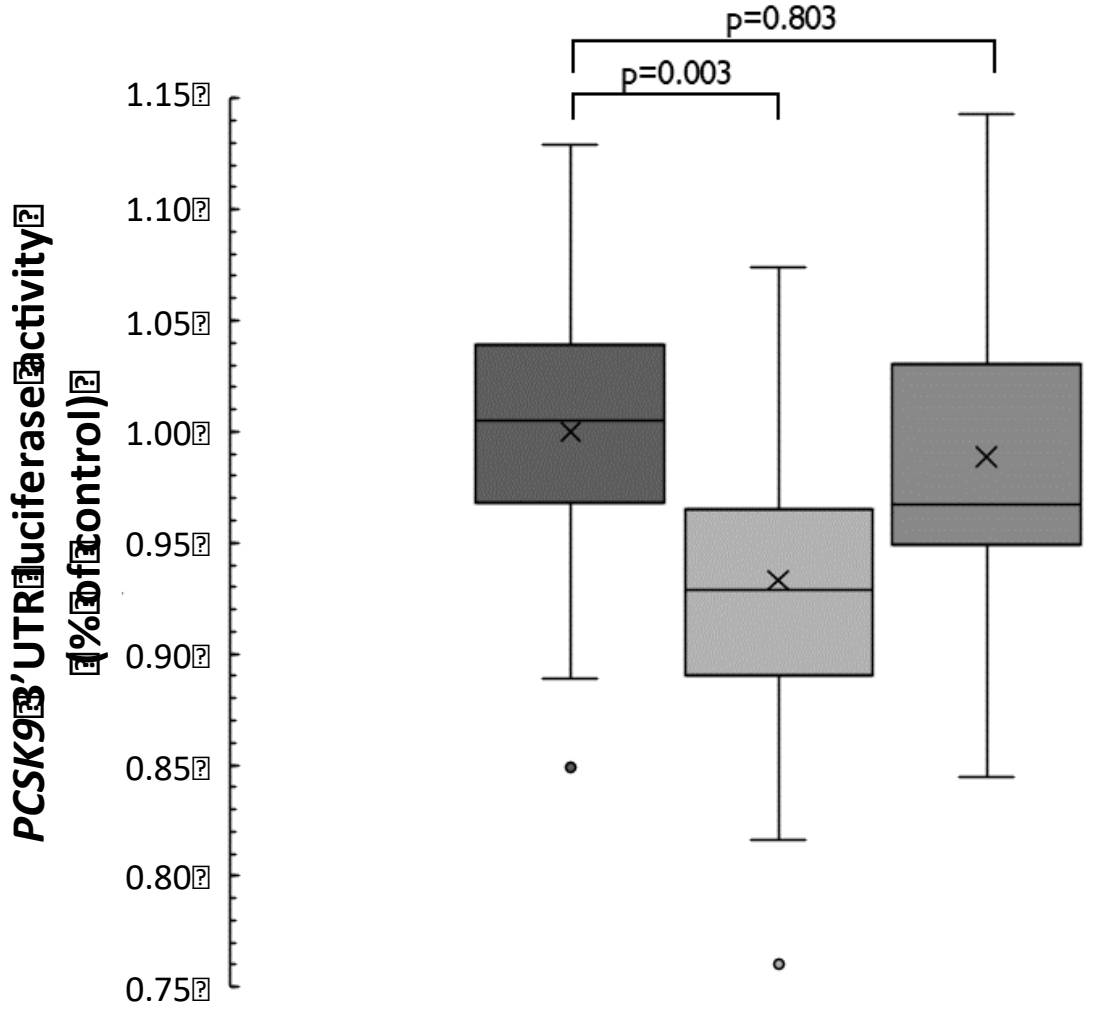
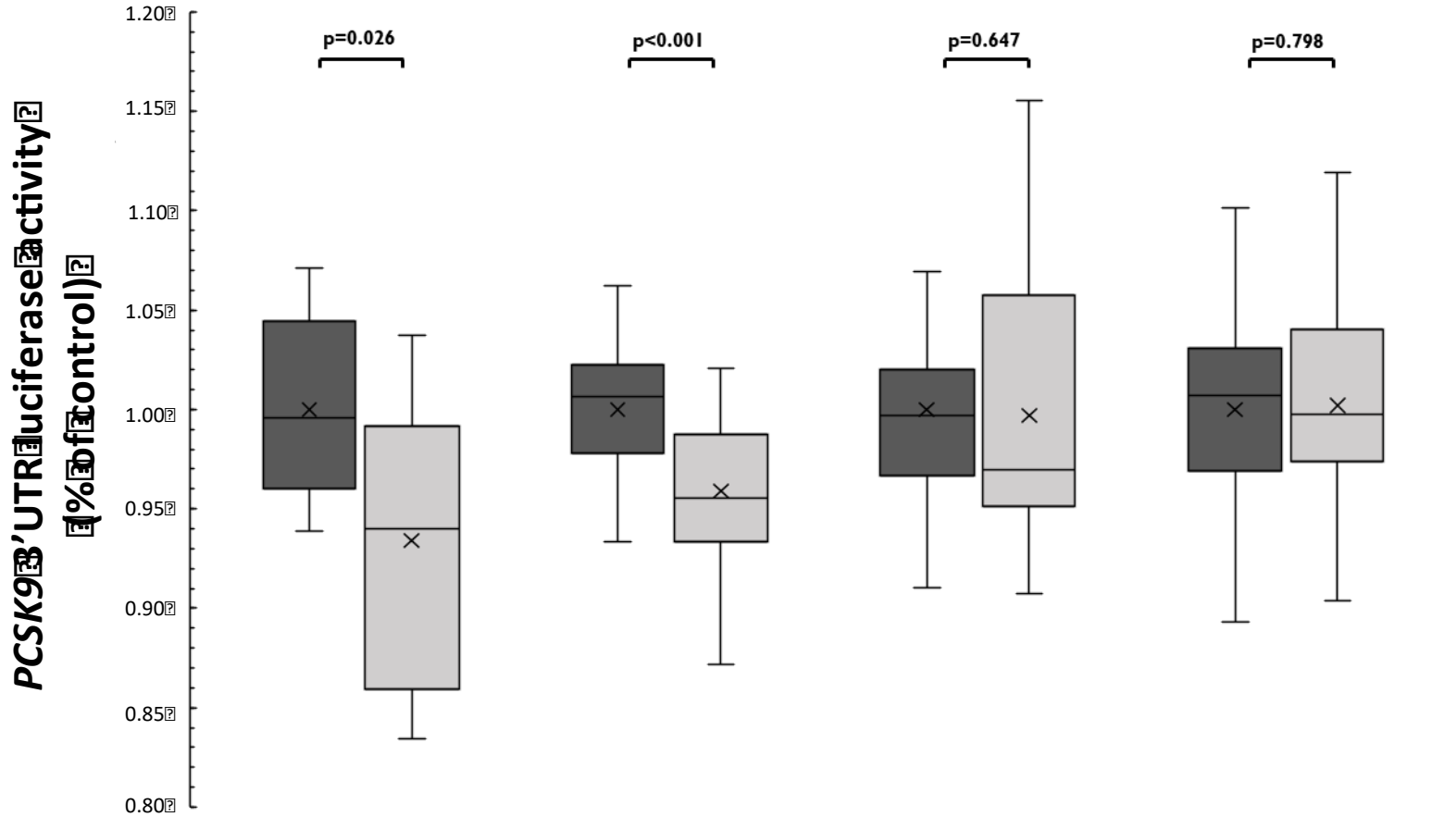


Figure 3



Construct	pGlo-3'UTR-*PCSK9* [c.*234C c.*571T]	pGlo-3'UTR-*PCSK9* [c.*234C c.*571C]	pGlo-3'UTR-*PCSK9* [c.*234T c.*571C]
n	40	28	27

Figure 4



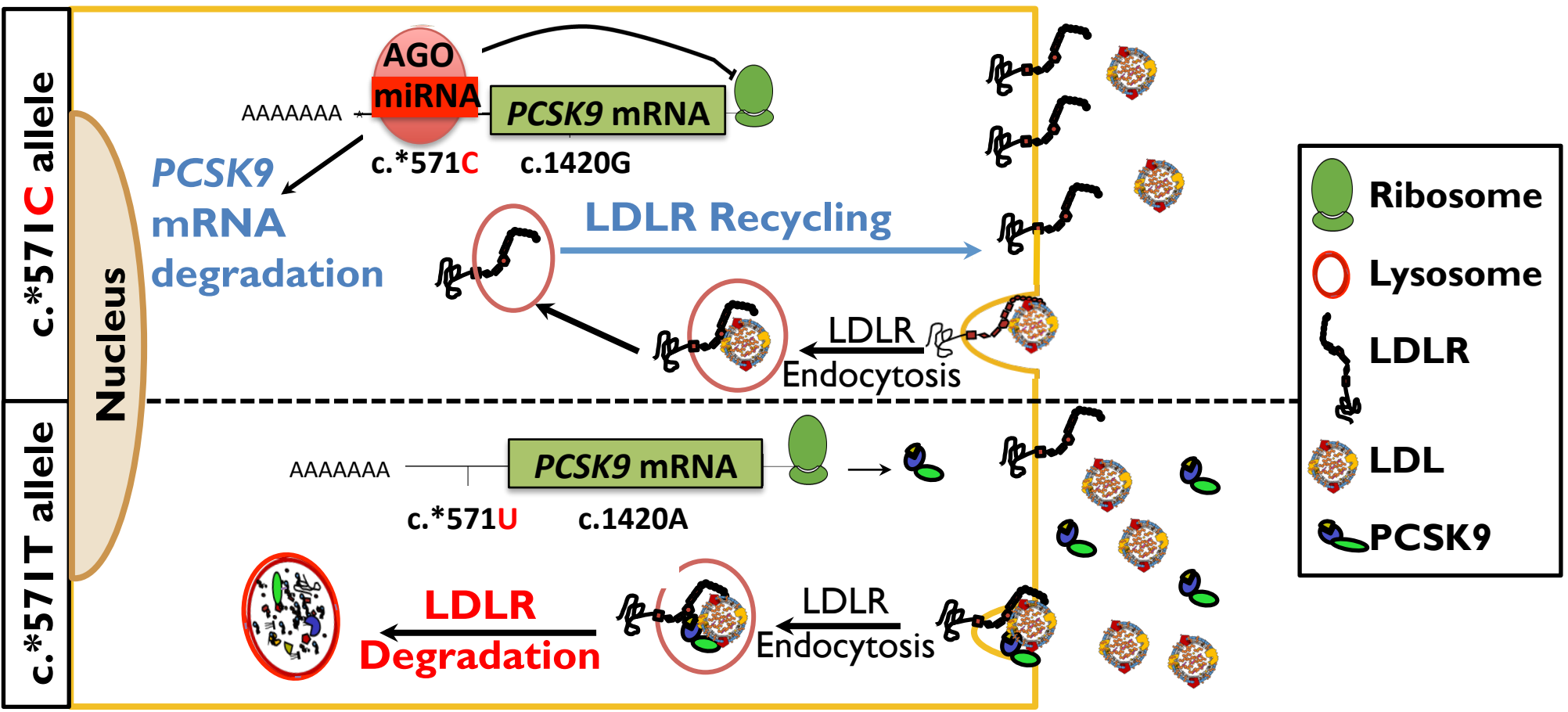
Transfect	hsa-miR-CNTR		Anti-hsa-miR-25-3p		Anti-hsa-miR-143-5p		Anti-hsa-miR-1228-3p	
Construct	pGlo-3'UTR-*PCSK9* [c.*234C c.*571T]	pGlo-3'UTR-*PCSK9* [c.*234C c.*571C]	pGlo-3'UTR-*PCSK9* [c.*234C c.*571T]	pGlo-3'UTR-*PCSK9* [c.*234C c.*571C]	pGlo-3'UTR-*PCSK9* [c.*234C c.*571T]	pGlo-3'UTR-*PCSK9* [c.*234C c.*571C]	pGlo-3'UTR-*PCSK9* [c.*234C c.*571T]	pGlo-3'UTR-*PCSK9* [c.*234C c.*571C]
n	13	10	25	20	20	20	25	20

Table 1 Haplotypes with a frequency >0.1% in all patients and comparison of the frequencies between patients with FH or FHBL phenotype.

	c.996+44delA	c.1380A>G	c.1420G>A	c.*75C>T	c.*234C>T	c.*345C>T	c.*414C>T	c.*537delT	c.*571C>T	c.*614C>T	g.30401_30402insTGTGA	g.30445G>A	Haplotype frequency	Patient with suspected FH	Patient with suspected FHBL	Chi-square	*p*-value
Haplotype A	A	G	A	C	C	C	C	T	T	C	-	G	68.2%	69.1%	65.5%	2.006	0.1567
Haplotype B	A	A	G	C	C	C	C	T	C	C	TGATG	G	13.9%	13.7%	14.6%	0.217	0.6412
Haplotype C	A	G	A	C	C	C	C	T	C	C	-	G	8.4%	8.5%	8.0%	0.126	0.7229
Haplotype D	-	G	A	C	C	C	C	T	T	C	-	G	6.3%	5.9%	7.7%	1.954	0.1621
Haplotype E	A	A	G	C	T	C	C	T	C	C	TGATG	G	0.9%	0.8%	1.6%	2.167	0.141
Haplotype F	A	A	G	C	C	C	C	T	C	C	-	G	0.9%	0.9%	1.1%	0.173	0.6771
Haplotype G	A	A	G	T	C	T	T	G	C	T	-	A	0.7%	0.6%	1.2%	1.882	0.1701
Haplotype H	A	A	G	C	C	C	C	T	T	C	-	G	0.4%	0.4%	0.2%	0.27	0.6036
Haplotype I	A	G	A	C	C	C	C	T	C	C	TGATG	G	0.2%	0.1%	0.2%	0.184	0.6683
Haplotype J	A	A	G	C	C	C	C	T	T	C	TGATG	G	0.1%	0.1%	0.0%	0.494	0.4821

Nucleotide numbers are derived from *PCSK9* cDNA sequence [Genbank *PCSK9*: NM_174936.3] and NG_009061.1.

Subgroups were compared using a Chi-squared test


c.*571C allele
c.*571T allele
Nucleus
AGO
miRNA
AAAAAAA
PCSK9 mRNA
c.*571C
c.1420G
PCSK9
mRNA
degradation
LDLR Recycling
LDLR
Endocytosis
AAAAAAA
PCSK9 mRNA
c.*571U
c.1420A
LDLR
Degradation
LDLR
Endocytosis
Ribosome
Lysosome
LDLR
LDL
PCSK9