VARIANTS OF *PCSK9* ARE ASSOCIATED WITH TRIGLYCERIDE LEVELS IN THE OLD ORDER AMISH

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Elevated levels of plasma low-density lipoprotein (LDL) cholesterol are a major risk factor for coronary artery disease and stroke. Recently, sequence variants in the proprotein convertase suntilisin/kexin type 9-serine protease gene (*PCSK9*) have been identified and found to be associated with plasma levels of LDL cholesterol. *PCSK9* is a secreted enzyme of the serine protease family and is expressed most abundantly in the liver, kidney, and small intestine. Overexpression of *PCSK9* or the mouse orthologue in the livers of mice results in a marked reduction in LDL receptors, which is the main pathway for the removal of LDL from the plasma, and a corresponding increase in circulating LDL cholesterol levels. For this study, we hypothesized that variants of *PCSK9* are associated with LDL levels in the Old Order Amish population.

In order to test the hypothesis, we chose specific variants to genotype in the Old Order Amish population. Using hapmap, a scientific website that lists specific single nucleotide polymorphisms (SNPs), we were able to capture the variation in the entire gene. We refer to these common SNPs as tag SNPs. We selected eight tag SNPs from this database based on linkage disequilibrium, as well as minor allele frequencies >.05. TaqMan was the method used to genotype these eight tag SNPs in the *PCSK9* gene. This method uses different probes that detect specific alleles within the sequence. For each of the eight tag SNPs genotyped, the results were displayed as three clusters: one representing homozygotes for the common allele, one for the heterozygotes, and the third for the homozygotes for the rare allele. The eight tag SNPs of *PCSK9* gene were not found to be associated with fasting low-density lipoprotein levels in the Amish. There were, however, two SNPs, rs10888897 and rs557435, which were associated with triglyceride levels following the consumption of a high fat diet. The results indicated that common SNPs within *PCSK9* may play a modest role in lipid metabolism.

BACKGROUND

The *PCSK9* gene is a secreted enzyme of the serine protease family and plays a role in regulating the low-density lipoprotein receptor (LDLR) concentration in the liver. The LDLR regulates the uptake of cholesterol into hepatocytes. Thus, when hepatic cholesterol levels are decreased, a group of regulatory proteins known as sterol regulatory element-binding proteins (SREBPs) are activated and subsequently induces expression of low-density lipoprotein receptors and increased cellular uptake of cholesterol from the plasma. Consequently, variations within the *PCSK9* gene may lead to decreased amounts of LDLR and higher levels of LDL. Research has shown that an elevated plasma level of LDL cholesterol is a primary causal factor in the pathogenesis of coronary heart disease.

METHODS

Researchers in the endocrinology, diabetes and nutrition division at the University of Maryland, Baltimore, study complex diseases such as coronary heart disease. Some of their research is conducted on a large group of the Old Order Amish population from Lancaster, Pennsylvania. They chose to work with the Amish because it is a large, homogenous group in which their genetic make-up is very similar. The Amish can be traced back 14 generations to a few individuals. This study included over 600 Amish individuals whose lipoproteins were measured after an overnight fast. The subjects' triglyceride levels were measured at one, two, three, four, and six hours after consuming a high-fat meal.

We chose the single nucleotide polymorphisms (SNPs) by referencing the hapmap database. The boundaries with which we narrowed our search for SNPs included those within the *PCSK9* gene among the Caucasian population. The hapmap database compiled a list of the SNPs in the gene we wanted to study. We chose the eight tagging SNPs with the highest allele frequencies in order to interrogate most or all of the common variation in the genes.

We used a procedure known as TaqMan to genotype the eight SNPs in the *PCSK9* gene in the Amish DNA samples. TaqMan is an advanced genotyping method using two different probes. One probe attaches to the common allele and the other attaches to the rare allele. Before analyzing SNPs using TaqMan, the DNA region containing the gene of interest must be amplified so that the variants within the gene can be detected with this method. The gene amplification method utilized is called the polymerase chain reaction (PCR). PCR allows for amplification of a very specific region of DNA, eg, the region coding for the *PCSK9* gene. This specificity is obtained by using both the forward and reverse primers flanking this gene. In so doing, the double stranded piece of DNA is first separated into two single strands, known as templates. The next part of the reaction involves annealing of the aforementioned primers to these template strands. Finally, an elongation period allows for the remainder of the new DNA strand to form, starting with the primer and continuing through the remainder of the strand, base pairing

Table 1. Associations between *PCSK9* Variants and LDL levels

SNPs	Minor Allele	Frequency	Fasting Low Density Lipoprotein Mean±SD (mg/dL)			P-value
			11	12	22	
rs631220	A	0.12	153.78±12.6	158.45±13.12	153.66±12.67	0.51
rs505151	G	0.05	141.73±40.22	154.77±6.56	153.24±3.65	0.89
rs10888897	C	0.49	151.46±4.89	155.68±4.68	150.27±5.7	0.83
rs535471	T	0.32	154.23±6.73	157.24±4.18	151.82±4.18	0.39
rs630431	G	0.27	150.96±7.64	156.03±4.08	151.54±4.08	0.56
rs557435	A	0.12	143.02±13.47	151.17±13.63	151.87±13.48	0.21
rs10888896	G	0.23	145.89±7.81	153.18±4.33	154.85±3.96	0.32
rs11583680	T	0.29	157.97±8.13	154.99±4.13	150.9±4.18	0.25

with the template strand along the way. This cycle is repeated many times in order to provide satisfactory amplification of the gene. After amplification was complete, we genotyped the SNPs using TaqMan. This system integrates the PCR-based assay with laser scanning technology to excite the fluorescent dyes that are attached to the allele-specific probes. During the extension step in PCR, a reporter dye attaches to the primers. Cleavage separates the reporter dye and quencher dye. The quencher dye is then separated from the PCR product. This results in increased fluorescence of the reporter dye. When there is an accumulation of PCR product, it is detected by the increase fluorescence of the reporter dye.

RESULTS

We were able to successfully genotype all eight SNPs in the Amish samples. TaqMan results are displayed as three separate clusters. One cluster represents those individuals who were homozygous for the common variant at that locus, the second represents those who were heterozygous and having one common allele and one rare allele, and the third cluster represents those who were homozygous for the rare allele. When we analyzed the mean and standard deviation for the fasting low-density lipoprotein, we found that there was no difference between the minor and major alleles. Statistically, this meant that a P-value $>.05$ was obtained for each SNP. Our results showed that there was no association between variants in the *PCSK9* gene and LDL levels in the Amish (Table 1). However, two SNPs did show positive results, $P<.05$. Interestingly, these associations were with triglyceride levels following the high-fat meal. We generated a linkage disequilibrium (LD) structure plot for the *PCSK9* gene. This structure illustrated that the two SNPs, rs10888897 and rs557435, were not in high LD with each other. As a result, these SNPs were not associated with each other; and they are both associated with triglyceride levels.

CONCLUSION

In conclusion, two SNPs in *PCSK9*, rs10888897 and rs557435, are associated with triglyceride levels following the intake of a high-fat meal. Furthermore, none of the *PCSK9* SNPs we analyzed were associated with LDL levels.

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For more information:.

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