Missense Mutations in the PCSK9 Gene Are Associated With Hypcholesterolemia and Possibly Increased Response to Statin Therapy

Knut Erik Berge, Leiv Ose, Trond P. Leren

Objective—The proprotein convertase subtilisin/kexin type 9 (PCSK9) gene encodes a proprotein convertase that causes degradation of cell surface low-density lipoprotein receptors (LDLRs). Mutations in the PCSK9 gene that disrupt the normal function of PCSK9 could therefore result in increased number of LDLR and hypercholesterolemia. Also, the cholesterol-lowering effect of statins could be increased in subjects carrying mutations in the PCSK9 gene.

Methods and Results—We have screened 38 unrelated hypocholesterolemic subjects as well as 25 unrelated familial hypercholesterolemia (FH) heterozygotes who responded particularly well to statin therapy for mutations in the 12 exons of the PCSK9 gene by DNA sequencing. Six of the 38 (15.8%) hypocholesterolemic subjects were heterozygous for 1 of the 3 mutations R46L, G106R, or R237W in the PCSK9 gene. In the group of 25 FH heterozygotes who responded particularly well to statin therapy, 3 (8.8%) were heterozygous for mutations R46L or N157K in the PCSK9 gene. None of 441 hypercholesterolemic subjects without mutations in the LDLR gene or in the apolipoprotein B-100 gene possessed any of the 4 mutations.

Conclusion—The 4 missense mutations R46L, G106R, N157K, and R237W are associated with hypocholesterolemia and possibly increased response to statin therapy. (Arterioscler Thromb Vasc Biol. 2006;26:0000-0000.)

Key Words: familial hypercholesterolemia ■ hypocholesterolemia ■ mutation ■ PCSK9 ■ statin
Materials and Methods

This study involves 4 groups of subjects (Table I, available online at http://atvb.ahajournals.org). The 38 unrelated hypocholesterolemic subjects had a mean value for total serum cholesterol <4.0 mmol/L. The 441 unrelated hypercholesterolemic subjects had a mean value for total serum cholesterol of 11.15 (±2.58) mmol/L. Nine FH heterozygotes with a particularly good response to statin treatment are referred to as group A responders. A group of 25 FH heterozygotes who had a reduction in total serum cholesterol of >60% on statin monotherapy is referred to as group B responders. Please see http://atvb.ahajournals.org for details on mutations screening of the PCSK9 gene and haplotype analysis.

Results

Hypocholesterolemia

To study whether mutations in the PCSK9 gene may cause hypocholesterolemia, 38 unrelated hypercholesterolemic subjects were screened for mutations in the PCSK9 gene by DNA sequencing. As can be seen from the Table, the mean values for total serum cholesterol and LDL-cholesterol in this group of patients were 3.35 (±0.36) mmol/L and 2.06 (±0.42) mmol/L, respectively. Three different mutations were identified (Table). Four patients were heterozygous for R46L (CGT->CTT) in exon 1, 1 patient was heterozygous for G106R (GGA->AGA) in exon 2, and 1 patient was heterozygous for R237W (CGG->TGG) in exon 5. Thus, 6 of 38 (15.8%) hypercholesterolemic subjects were heterozygous for missense mutations in the PCSK9 gene.

To study whether these mutations were the cause of hypocholesterolemia and not just normal genetic variants, 441 severely hypercholesterolemic subjects with a mean value for total serum cholesterol of 11.15 (±2.58) mmol/L were screened for the 4 mutations by DNA sequencing. None of the 441 subjects who were negative for mutations in the LDLR gene and in the apolipoprotein B-100 gene possessed any of the 3 mutations in the PCSK9 gene. The higher frequency of PCSK9 mutation carriers among hypercholesterolemic pa-

There are not just data to indicate that mutations in the PCSK9 gene result in hypocholesterolemia through a “loss-of-function” mechanism. Several groups have also identified mutations in the PCSK9 gene as the cause of hypercholesterolemia (FH) heterozygotes with extraordinarily good response to statin therapy. The 38 unrelated hypocholesterolemic subjects had a mean value for total serum cholesterol <4.0 mmol/L. The 441 unrelated hypercholesterolemic subjects had a mean value for total serum cholesterol of 11.15 (±2.58) mmol/L. Nine FH heterozygotes with a particularly good response to statin treatment are referred to as group A responders. A group of 25 FH heterozygotes who had a reduction in total serum cholesterol of >60% on statin monotherapy is referred to as group B responders. Please see http://atvb.ahajournals.org for details on mutations screening of the PCSK9 gene and haplotype analysis.

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Functional domains and mutation in the PCSK9 gene. Functional domains of the PCSK9 gene and the mutations in the gene associated with hypercholesterolemia or hypocholesterolemia. The 30 amino acid signal peptide (SP) is followed by a prosegment (Pro) that is cleaved-off in ER. The catalytic domain is followed by a P domain and a cysteine-rich C-terminal domain.

Figure 1. Functional domains and mutation in the PCSK9 gene.

<table>
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Table 1: Functional domains and mutation in the PCSK9 gene.

- **Good Responders to Statin Therapy**

In the group of 9 FH heterozygotes who responded particularly well to statin therapy (group A responders), 1 patient was heterozygous for R46L and 1 patient was heterozygous for N157K (AAC→AAA) in exon 3 of the PCSK9 gene (Table). The N157K mutation was not found in the group of 441 severely hypercholesterolemic subjects. Thus, it is unlikely that the mutation is a normal genetic variant.

The patient heterozygous for R46L (proband in family L2864, Figure 2) was a female who was heterozygous for mutation C210G in the LDLR gene. At age 21, she had a value for total serum cholesterol of 12.5 mmol/L. At age 27, she had a 54% lower value for total serum cholesterol of 5.8 mmol/L on simvastatin 40 mg. The patient heterozygous for N157K (proband in family 0465; Figure 2) was a female who was heterozygous for mutation N804K in the LDLR gene. At age 42, she had a value for total serum cholesterol of 10.0 mmol/L. At age 43, she had a 51% lower value for total serum cholesterol of 4.9 mmol/L on atorvastatin 10 mg. Thus, 2 of the 9 subjects (22.2%) of group A responders were heterozygous for missense mutations in the PCSK9 gene.

In the group of 25 FH heterozygotes who had a reduction in total serum cholesterol >60% on statin monotherapy (group B responders), 1 subject (4.0%) was heterozygous for R46L (proband in family T1987; Figure 2). This subject was a male who was also heterozygous for mutation P664L in the LDLR gene. At age 39, he had a value for total serum cholesterol of 14.9 mmol/L. At age 45, he had a 67% lower value for total serum cholesterol of 4.9 mmol/L on atorvastatin 80 mg. For groups A and B of FH responders together, 8.8% (3/34) had a missense mutation in the PCSK9 gene, which was not found in a group of 441 hypercholesterolemic subjects.

Family members of the 3 unrelated FH heterozygotes of groups A and B responders who were carriers of mutations in the PCSK9 gene were studied with respect to their mutation carrier status, their cholesterol levels, and their response to statins (Figure 2). In family L2684, only the proband was heterozygous for both C210G in the LDLR gene and R46L in the PCSK9 gene. The only other carrier of the R46L mutation in the family was the proband’s mother (subject I:2). At age 53, she had values for total serum cholesterol and LDL cholesterol of 5.0 mmol/L and 2.8 mmol/L, respectively.

In family T1987, only the proband was heterozygous for both P664L in the LDLR gene and R46L in the PCSK9 gene. Among the 5 family members without FH who were tested for R46L, it is apparent that the 3 subjects with the R46L mutation had lower levels of total serum cholesterol and LDL cholesterol than their siblings. This supports the notion that R46L causes hypocholesterolemia.

The only available FH heterozygous family member in family 0465 (subject II:5) did not carry the N157K mutation in PCSK9. At age 22, he had a value for total serum cholesterol of 9.3 mmol/L before lipid-lowering drug therapy was started. At age 26, he had a value for total serum cholesterol of 5.8 mmol/L on simvastatin 80 mg. A 38% reduction in total serum cholesterol was therefore observed on simvastatin 80 mg, which is equipotent to atorvastatin 40 mg. For comparison, the FH heterozygous proband in family 0465 who was heterozygous also for N157K had a reduction in total serum cholesterol of 51% on atorvastatin 10 mg. The levels of total serum cholesterol in the 2 FH heterozygotes before lipid-lowering therapy was started, however, were similar. Thus, even though mutation N157K did not appear to affect the level of total serum cholesterol...
before lipid-lowering drugs were started, it seems to increase the reduction in total serum cholesterol achieved by statin therapy. The proband’s 37-year old sister in family 0465 without FH who carried N157K (subject II:3) had low levels of both total serum cholesterol and LDL cholesterol of 3.9 mmol/L and 2.1 mmol/L, respectively. This finding is consistent with a LDL cholesterol-lowering effect of the N157K mutation in non-FH subjects.

The finding of the N157K mutation carrier among group A responders may suggest that this mutation is a “loss-of-function” mutation. Family studies were therefore also performed in the previously published family 0305, which included a hypercholesterolemic proband who was heterozygous for N157K and D374 years in the PCSK9 gene (Figure 2). It is well-known that the D374 years mutation causes hypercholesterolemia.10–13 Family studies revealed that the 2 mutations were on different alleles. The proband had values for total serum cholesterol of 11.8 mmol/L before lipid-lowering drugs were started. On a combination of atorvastatin 80 mg and ezetimibe 10 mg, the level of total serum cholesterol was reduced by 64% to 4.2 mmol/L. Low levels of total serum cholesterol and LDL cholesterol were observed in the proband’s daughter (subject III:4) and grandchild (subject IV:1), who both carried the N157K mutation but not the D374 years mutation in the PCSK9 gene. The proband’s 55-year-old brother (subject II:2), who carried the N157K mutation only, had low levels of both total serum cholesterol and LDL cholesterol of 4.2 mmol/L and 2.0 mmol/L, respectively. Among his 3 daughters, of whom 1 carried N157K, no clear-cut cholesterol-lowering effect of the N157K mutation could be observed. Based on the findings in families 0465 and 0305, N157K probably causes hypocholesterolemia and an increased response to statins in the same manner as the R46L mutation.

In the families shown in Figures 1 and 2, a total of 46 family members without FH have been studied with respect to the mutation in the PCSK9 gene found in the families. Of these, 28 subjects were heterozygous for one of the mutations R46L, G106R, N157K, and R237W, which might be associated with hypocholesterolemia.10–13 Family studies revealed that the 2 mutations were on different alleles. The proband had values for total serum cholesterol of 11.8 mmol/L before lipid-lowering drugs were started. On a combination of atorvastatin 80 mg and ezetimibe 10 mg, the level of total serum cholesterol was reduced by 64% to 4.2 mmol/L. Low levels of total serum cholesterol and LDL cholesterol were observed in the proband’s daughter (subject III:4) and grandchild (subject IV:1), who both carried the N157K mutation but not the D374 years mutation in the PCSK9 gene. The proband’s 55-year-old brother (subject II:2), who carried the N157K mutation only, had low levels of both total serum cholesterol and LDL cholesterol of 4.2 mmol/L and 2.0 mmol/L, respectively. Among his 3 daughters, of whom 1 carried N157K, no clear-cut cholesterol-lowering effect of the N157K mutation could be observed. Based on the findings in families 0465 and 0305, N157K probably causes hypocholesterolemia and an increased response to statins in the same manner as the R46L mutation.

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Thus, lower values for total serum cholesterol and LDL cholesterol of 12% and 21%, respectively, were found among the PCSK9 heterozygous family members. To determine the prevalence of the missense mutations R46L, G106R, N157K, and R237W in the general population, 100 healthy adult individuals with values for total serum cholesterol between 4.5 mmol/L and 6.5 mmol/L were screened for the 4 mutations. The mean age of these subjects was 37.0 years and levels of total serum cholesterol and LDL cholesterol were 5.06 ($\pm$0.92) mmol/L and 3.2 ($\pm$0.81) mmol/L, respectively. Three subjects heterozygous for the R46L mutation were identified in this group of patients. No one possessed any of the 3 other mutations.

**Haplotypes of the PCSK9 Mutation-Carrying Alleles**

Haplotypes at the PCSK9 locus were constructed by the use of 17 polymorphic markers within the PCSK9 gene to identify the haplotypes of the mutation-bearing alleles (Table I). The R46L mutation was on 3 different haplotypes in the 4 families studied, whereas the N157K mutation was on the same haplotype in the 2 families with the N157K mutation.

In this study, we have found that in a group of 38 healthy unrelated subjects aged 41 to 49 with fasting values for total serum cholesterol $\leq$ 4 mmol/L, 15.8% (6/38) were heterozygous for a missense mutation in the PCSK9 gene. Four of the hypocholesterolemic subjects were heterozygous for R46L, 1 was heterozygous for R237W, and 1 was heterozygous for the novel mutation G106R. We also found that in 2 groups of 9 and 25 FH heterozygotes who responded particularly well to statin therapy, 22.2% (2/9) and 4.0% (1/25), respectively, were heterozygous for either R46L or N157K. None of the mutations R46L, G106R, N157K, and R237W was identified in a group of 441 severely hypercholesterolemic subjects. These data indicate that mutations R46L, G106R, N157K, and R237W may disrupt the normal function of PCSK9 and cause hypocholesterolemia and possibly an increased response to statin therapy.

The failure to detect a clear-cut cosegregation pattern with hypocholesterolemia in most of the families may be because of the fact that levels of serum cholesterol are influenced by environmental factors and genetic factors other than the PCSK9 gene. Moreover, there is an inherent difficulty in

**Discussion**

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The failure to detect a clear-cut cosegregation pattern with hypocholesterolemia in most of the families may be because of the fact that levels of serum cholesterol are influenced by environmental factors and genetic factors other than the PCSK9 gene. Moreover, there is an inherent difficulty in
identifying a trait that is characterized by half-normal levels of LDL cholesterol as compared with a trait that is characterized by a doubling of the levels of LDL cholesterol, as is the case in FH. It should also be noticed that compared with the levels of serum cholesterol in the Norwegian population, the noncarriers in the families studied had relatively low levels of total serum cholesterol and LDL cholesterol. Nevertheless, among family members without FH, significantly lower levels of total serum cholesterol and LDL cholesterol of 12% and 21%, respectively, were found in family members who were carriers of mutations in the PCSK9 gene as compared with family members who were noncarriers. These findings are similar to those of Cohen et al,8 who found a broad range of levels of LDL cholesterol in carriers of 2 nonsense mutations in the PCSK9 gene.

Both the R46L and R237W mutations have previously been reported in normocholesterolemic individuals. Therefore, they have been considered normal genetic variants. However, lipid values were not reported in any of the 2 studies. In the French population, R46L was found in 2.2% of normocholesterolemic subjects10 but was not found in a group of 130 French subjects with autosomal-dominant hypercholesterolemia.21 These findings support the notion that R46L causes hypercholesterolemia. From our study and from previously published reports, R46L, G106R, N157K, and R237W have not been identified in a total of 621 hypercholesterolemic subjects10,13,21,22 but are common in hypercholesterolemic subjects, as shown in our study.

Benjannet et al4 have performed studies on the autcatalytic activity of mutations N157K and R237W. They found that the autodegradative cleavage of PCSK9 was unaffected by both mutations. In contrast, they found that mutations S127R and D374Y in the PCSK9 gene that causes hypercholesterolemia through a “gain-of-function” mechanism had reduced autodegradative activity.7 That the N157K and D374Y mutations have opposite effects on the autodegradative activity was illustrated by the normalization of the autodegradative activity of D374Y mutation when the mutant allele also harbored the N157K mutation.4

Our results also indicate that mutations R46L and N157K in the PCSK9 gene, which are associated with hypercholesterolemia, may increase the response to statin therapy. These data are in agreement with the effect of statin therapy in PCSK9 knockout mice. The FH hypercholesterobod in family 0465, who was heterozygous for the N804K mutation in the LDLR gene and heterozygous for the N157K mutation in the PCSK9 gene, had a 51% reduction in total serum cholesterol on atorvastatin 10 mg. Usually a reduction in total serum cholesterol of 27% to 31% is observed on atorvastatin 10 mg.20,23,24 Her FH heterozygous brother who did not carry the N157K mutation (subject II:5) had a reduction in total serum cholesterol of 38% on a statin dose that is equipotent to atorvastatin 40 mg. Reductions in total serum cholesterol of 54% and 67% in the 2 FH heterozygotes carrying mutation R46L in the PCSK9 gene on simvastatin 40 mg and atorvastatin 80 mg, respectively, are also greater than commonly observed. Usually a reduction in total serum cholesterol of 25% to 30% is observed on simvastatin 40 mg,20,23 and a reduction of 40% to 42% is observed on atorvastatin 80 mg.20,24 Even though the small sample size makes it impossible to draw firm conclusions, it may appear that FH heterozygotes who are PCSK9 mutation carriers may have almost twice the normal reduction in total serum cholesterol on statin therapy. However, further studies are needed to firmly establish that carriers of mutations in the PCSK9 gene are hyper-responders to statin therapy.

If “loss-of-function” mutations R46L and N157K increase the response to statin therapy, “gain-of-function” mutations could have the opposite effect. The finding of Naoumanova et al23 agrees with this hypothesis. They found a particularly severe phenotype in patients heterozygous for the “gain-of-function” mutation D374Y. Moreover, these patients needed both high doses and combinations of lipid-lowering drugs to achieve substantial reductions in levels of total serum cholesterol.25

The finding that the R46L mutation resided on 3 different haplotypes was surprising. Because the mutation does not involve a cytokine of a Cpg dinucleotide that is a hot spot for mutations,26 recombination events most likely have occurred in the 3’ part of the gene. This finding, together with the high frequency of R46L, might indicate that the mutation is very old.

Cohen et al8 reported the presence of nonsense mutations in the PCSK9 gene among blacks with low levels of LDL cholesterol. We could not identify any nonsense mutations in our group of individuals with low levels of total serum cholesterol and LDL cholesterol, and our study is the first to our knowledge to identify missense mutations in the PCSK9 gene associated with hypercholesterolemia. However, studies to determine the mechanisms by which these mutations might disrupt the normal function of PCSK9 are needed to firmly establish that mutations R46L, G106R, N107K, and R237W lead to both hypercholesterolemia and increased response to statin therapy.

References


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Materials and methods

This study involves different groups of subjects and has been approved by The Regional Committee for Ethics in Medicine. Informed consent was obtained from all participants.

Hypocholesterolemic subjects

The 38 hypocholesterolemic subjects were ostensibly healthy unrelated subjects who had participated in a screening program for cardiovascular risk factors among 40-year old subjects in Oslo. The entire population of 40-year olds in Oslo were invited to participate. Of those with a non-fasting screening value for total serum cholesterol < 3.7 mmol/l, 82 subjects consented to participate in a study to identify genetic factors affecting serum cholesterol levels. Of these, DNA was available for study for 38 subjects who also had a fasting value for total serum cholesterol < 4.0 mmol/l at a follow-up visit one to nine years after the screening. The mean age of the 38 subjects was 44.1 (±2.41) years at the follow-up visit. Fasting lipid values obtained at the follow-up visit are given in Table 1. Values for LDL cholesterol were calculated according to the formula of Friedewald et al. (16).

Hypercholesterolemic subjects

The 441 unrelated hypercholesterolemic subjects have been referred to Medical Genetics Laboratory, Rikshospitalet University Hospital for genetic testing for FH. None of the patients possessed a mutation in the LDLR gene that could possibly affect the function of the LDLR, as determined by DNA sequencing of the promoter region and of the translated parts of the 18 exons of the LDLR gene. Moreover, no-one possessed structural alterations in the LDL receptor gene detectable by multiplex ligation-dependent probe amplification of the LDLR gene (17). Nor did anyone possess the mutation R3500Q in the
apolipoprotein B-100 gene as determined by the assay of Hansen et al. (18). Values for total serum cholesterol before lipid-lowering drug therapy was started, are given in Table 1. It should be noted that there are some uncertainties whether these values were obtained in the fasting state or not. Because values for HDL cholesterol and triglycerides were available for only a subset of the patients, presentation of lipid values in Table 1 is restricted to values for total serum cholesterol.

FH heterozygotes who respond well to statin therapy

During the last four years it has been noticed that nine unrelated FH heterozygotes who have been provided with a molecular genetic diagnosis at Medical Genetics Laboratory, Rikshospitalet University Hospital, responded particularly well to statin therapy. They were on different therapeutic regimens, but had a reduction in total serum cholesterol that was larger than commonly observed. This group is referred to as Group A responders.

Of the approximately 1300 unrelated FH heterozygotes who have been provided with a molecular genetic diagnosis at Medical Genetics Laboratory, Rikshospitalet University Hospital, values for total serum cholesterol before and after lipid-lowering drug therapy was started, have been recorded in approximately one third of the patients at the time of genetic testing. Of these, 25 patients had a reduction in total serum cholesterol of more than 60% on statin monotherapy. Many of the patients in this group were on the maximum dose of atorvastatin, 80 mg. This group of 25 subjects is referred to as Group B responders. As for the hypercholesterolemic subjects above, data for total serum cholesterol only are presented for Groups A and B responders in Table 1.
Studies of family members of patients carrying mutations in PCSK9 gene

Family members of patients carrying mutations in the PCSK9 gene were invited for genetic testing with respect to the mutation identified in the family, and for measurement of a non-fasting lipid profile. The concentration of LDL cholesterol was measured directly by the method LDL-C plus 2nd generation (Roche Diagnostics GmbH, Mannheim, Germany). Values for LDL cholesterol are therefore essentially not influenced by the fasting state of the subjects (19).

Mutation screening of the PCSK9 gene

DNA was extracted from EDTA-containing blood by the use of a BioRobot EZ1 (Qiagen GmbH, Hilden, Germany). Mutation screening of the 12 exons of the PCSK9 gene with flanking intron sequences was performed by DNA sequencing as previously described (12).

Haplotype analysis at the PCSK9 locus

Haplotypes at the PCSK9 locus were constructed by the use of 17 polymorphic markers within the PCSK9 gene (12). These were -64, C->T and -8, T->C in the 5’ untranslated region in exon 1, 287_289insCTG and A53V in exon 1, 207+15, G->A in intron 1, 524-11, G->A in intron 3, 657+9, G->A, 658-36, G->A and 658-7, C->T in intron 4, 799+3, G->A and 799+64, A->C in intron 5, V460V and I474V in exon 9, 1680+63, C->T and 1680+64, A->G in intron 10, 1863+94, A->G in intron 11 and E670G in exon 12.

Statistical analysis
Values are given as mean (±SD). Comparisons between groups were performed by Student’s two-sided t-test using Statview software (SAS Institute, Cary, NC). Fischer’s exact test was employed to compare frequencies between groups.

Table

Table I

Haplotypes at the PCSK9 locus of alleles carrying mutations R46L, G106R, N157K or R237W constructed by the use of 17 polymorphic markers within the PCSK9 gene (12).

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