

MUTATION IN BRIEF

## Identification of Three Mutations in the Low-Density Lipoprotein Receptor Gene Causing Familial Hypercholesterolemia Among French Canadians

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### INTRODUCTION

Familial hypercholesterolemia (FH) is an autosomal codominant disorder caused by mutations in the low-density lipoprotein (LDL) receptor gene (Goldstein and Brown, 1989). The heterozygous FH form is characterized by a two- to three-fold elevation in plasma LDL concentration, tendinous xanthomatosis, and premature atherosclerotic coronary artery disease, usually occurring at 35–55 years of age, whereas homozygous or compound heterozygous patients show a six- to eight-fold increase in plasma LDL concentration and typically present manifestations of coronary artery disease before the age of 20 years.

FH is also one of the most common inherited metabolic traits, with a worldwide frequency of 1 in 500 for heterozygotes and one per million for homozygotes. In the province of Quebec, the prevalence of homozygous FH is approximately sixfold higher, and the minimal estimated frequency of heterozygotes ranges from 1:81 to 1:154 in northeastern Quebec (Moorjani et al., 1989). While six mutations in the LDL receptor gene are responsible for 85% of French Canadian (FC) heterozygous FH patients, defined on the basis of clinical and biochemical criteria (Simard et al., 1994), 15% of suspected cases are still uncharacterized at the molecular level. Two of those mutations, a > 15-kb deletion at the 5' end of the gene and a missense mutation (W66G) in exon 3, are present in approximately 60% and 18% of FH patients who attend our lipid clinic in Quebec city, respectively.

The present study was designed to characterize further the spectrum of LDL receptor gene mutations in the FC population. We have examined the 18 ex-

ons of the LDL receptor gene, the exon–intron splicing boundaries and 290 bp of the 5' noncoding region, including the putative promoter by single-strand conformation polymorphism (SSCP) analysis in 15 unrelated FC adult patients with FH and in 15 normocholesterolemic FC subjects who were negative for the six known mutations.

### METHODS

#### Subjects

In order to probe for novel mutations, 15 unrelated adult FH patients, who did not have any of the six known mutations were selected as index patients. The relative frequency of the three mutations described in the present study was then calculated in a cohort of 48 unrelated FC children (2–18 years old) with suspected heterozygous FH based on the following common criteria: plasma LDL-cholesterol concentrations above the 95th percentile for age and sex, premature coronary artery disease (below age 60 years), and presence of tendinous xanthomas in a first- or second-degree relative. All these children were negative for the six previously known FC mutations in the LDL receptor gene (Simard et al., 1994) and were then screened for the three mutations. The ApoB 3500 mutation (Soria et al., 1989) was not found in any of the 15 adult FH patients or in any of

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the 48 children, as determined by the assay that was previously described (Hansen et al., 1991). Additional 100 normocholesterolemic FH men were also screened for the C152W and C347R mutations in order to exclude that these mutations are not linked polymorphisms.

#### PCR-SSCP and DNA Sequence Analyses

Selective polymerase chain reaction (PCR) amplification of each individual 18 exons, the exon-intron splicing boundaries, and 290 bp of the 5' non-coding region of the LDL receptor gene were performed using primers previously described (Simard et al., 1994). For SSCP analysis, [<sup>35</sup>S]dATP was used for labeling PCR fragments. Each PCR reaction was subjected to 35 cycles with a temperature cycle consisting of 1 min at 95°C, 1 min at 62°C and of 1 min at 72°C. The PCR product was added to formamide loading buffer, heated at 95°C for 5 min, and electrophoresed (8 W for 16 hr at room temperature) in a Hydrolink-MDE polymer gel (AT Biochem, Malvern, PA). Gels were dried and exposed to X-ray film. DNA sequencing of the exons displaying aberrant electrophoretic patterns by SSCP analysis were performed as previously described (Simard et al., 1994).

#### Mutation Detection Using Restriction Fragment Analysis

Symmetric PCR is performed under the same conditions described above for all three mutations, using the primer pair 4u-mismatch 5'-TGTGGGCCTGCGACAACGACCCCGACTA-3', 4d 5'-AGTTTTCTCTCGTCAGATTTGTCCTTGCA-3' or the primer pairs 7u, 7d; 8u, 8d previously described (Hobbs et al., 1992). The PCR products were digested with the appropriate enzyme, as recommended by the supplier (New England Biolabs, Beverly, MA) and the resulting fragments were size-separated by electrophoresis on a 1.5% agarose gel.

### RESULTS AND DISCUSSION

When compared with results of control subjects, SSCP analysis of the promoter and the 18 exons of the LDL receptor gene revealed aberrant electrophoretic patterns in exons 4, 7, and 8 in one, one, and three FH patients, respectively.

Direct sequencing of the PCR fragments from exon 4 of the LDL receptor gene in the index case showed that the aberrant electrophoretic pattern in exon 4 (Fig. 1A) was due to the presence of the novel missense mutation C152W. This mutation is caused by a C-to-G transversion at nucleotide 519 (Yamamoto et al., 1984), thus converting codon 152 (TGC) encoding cysteine into TGG-encoding tryptophan (Fig.

1B). Since the C152W mutation does not alter any restriction site we have developed a rapid detection method using a mismatch primer approach with primers 4d and 4u-mismatch, which contains an A instead of a G at position 518 creating a *Bfa*I site that is abolished in control individuals due to the presence of a C at position 519. The resulting PCR digested fragments were 175 bp and 26 bp for the mutant allele and 201 bp for the normal allele (Fig. 1D). The C152W mutation changed a highly conserved cysteine at the C-terminal end in the fourth of the seven tandem cysteine-rich repeats that form the binding site for LDL (Bieri et al., 1995; Mehta et al., 1991). Most of the missense mutations located in this region produce class 2 (transport defective) phenotypes (Hobbs et al., 1992; Yamamoto et al., 1986). Another missense mutation involving the same codon (C152R) has already been reported in a Greek patient with compound heterozygous FH (Hobbs et al., 1992), thus also supporting our hypothesis that this mutation yields to a pathological allele. Moreover, the C152W mutation was not detected in 100 normocholesterolemic men, suggesting that it is not a rare sequence variant in normal FC population.

Determination of the nucleotide sequence of the PCR amplicon from exon 7 in the index case showed a C-to-T transition at nucleotide 1048 (Yamamoto et al., 1984) in one allele (Fig. 2B). This mutation converts codon 329 (CGA) encoding arginine into a TGA-stop codon. Because this mutation generates a *Bsr*I site, digestion of PCR products amplified using primers 7u and 7d with this enzyme yielded two fragments, of 129 bp and 41 bp, for the mutant R329X allele and of a single fragment of 170 bp for the uncut normal allele (Fig. 2D). The R329X mutation has recently been reported in a Norwegian patient with FH (Solberg et al., 1994) and occurs at a CpG dinucleotide that is a well-established hotspot for point mutation in the human genome (Cooper and Youssoufian, 1988).

Figure 3B shows the partial nucleotide sequence of PCR fragments from exon 8 exhibiting a T-to-C transition at nucleotide 1102 (Yamamoto et al., 1984) in one allele converting the codon 347 (TGC)-encoding cysteine into CGC-encoding arginine. This mutation generates an *Acc*I site, thus digestion of PCR products obtained with primers 8u and 8d lead to fragments of 100, 66, 7, and 3 bp for the mutant C347R allele and 166, 7 bp and 3 bp for the normal allele (Fig. 3D). The C347R mutation changed a cysteine in the second cysteine-rich repeat of the epidermal growth factor (EGF) precursor homology domain. It has been reported that missense muta-

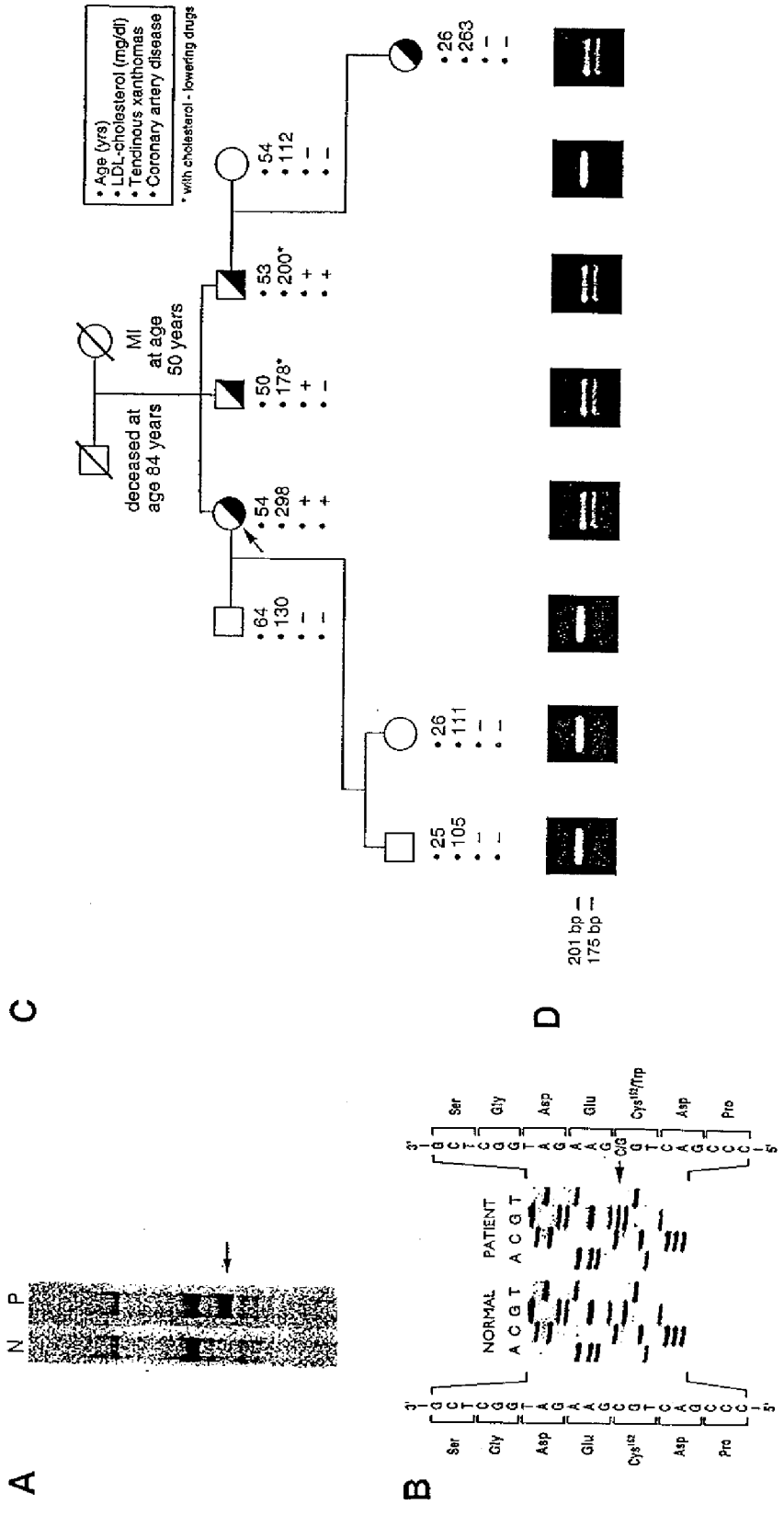


FIGURE 1. A: SSCP analysis of PCR fragment encompassing exon 4 of the LDL receptor gene in control subjects (N) and patients (P). Arrow, abnormal fragments, corresponding to the C152W mutation. B: Partial nucleotide sequence of PCR fragments from exon 4. C: Partial pedigree and individual clinical data. D: Detection of the missense mutation C152W in the LDL receptor gene. Numbers at left indicate the size of the restriction fragments. MI, myocardial infarction.

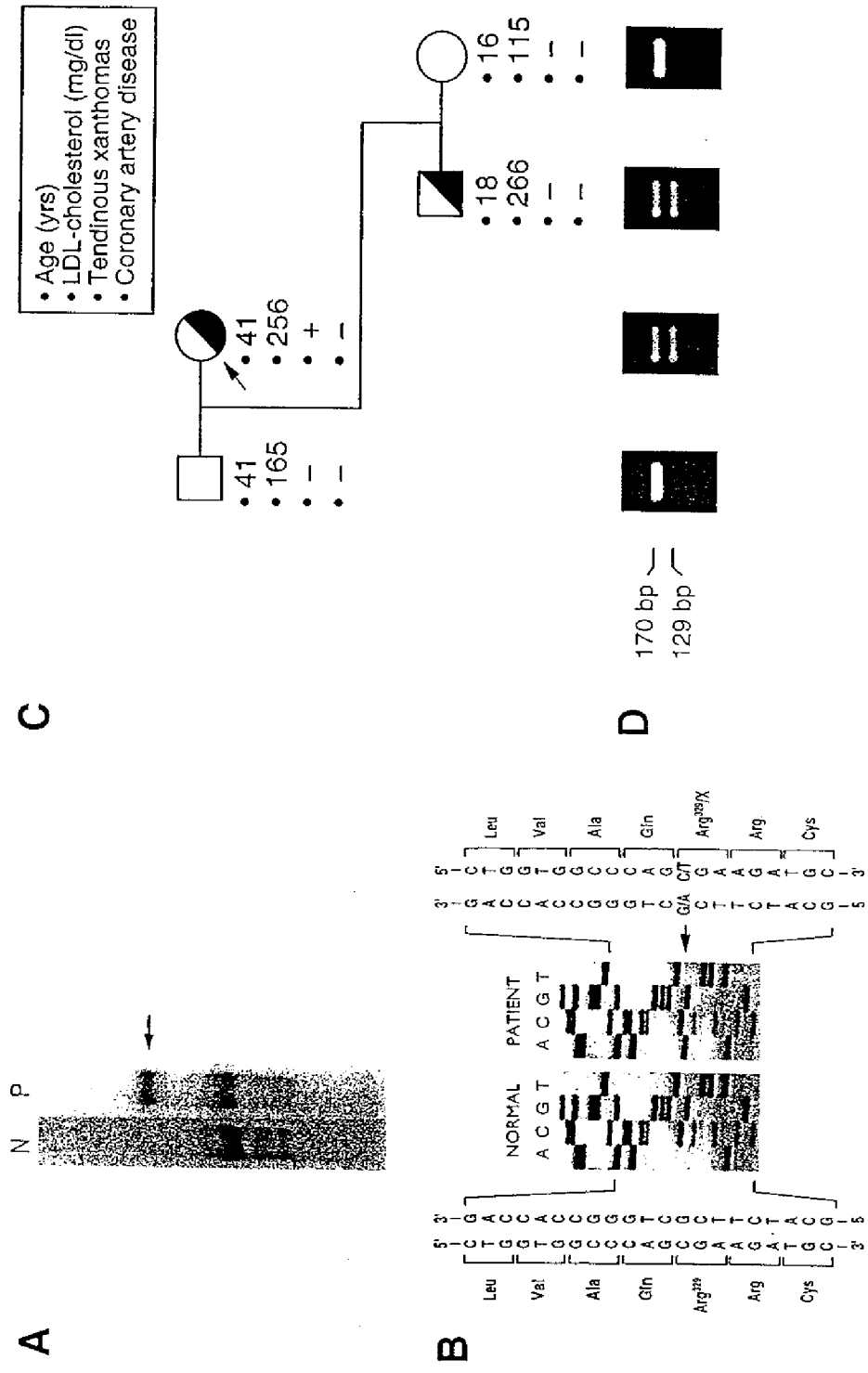


FIGURE 2. **A:** SSCP analysis of PCR fragment encompassing exon 7 of the LDL receptor gene in control subjects (N) and patients (P). **B:** Partial nucleotide sequence of PCR fragments from exon 7. **C:** Partial pedigree and individual clinical data. **D:** Detection of the nonsense mutation R329X in the LDL receptor gene. Numbers at left indicate the size of the restriction fragments.

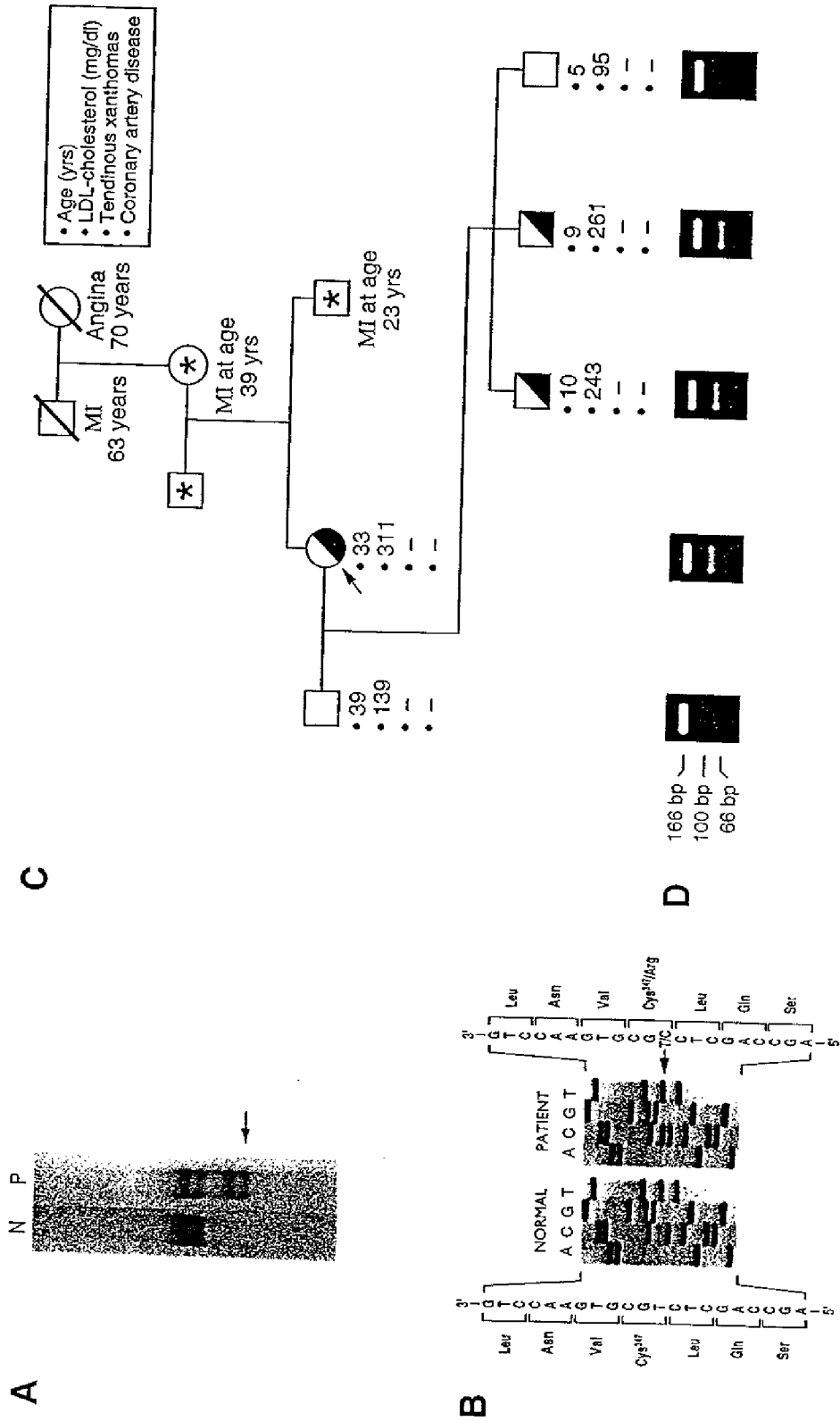


FIGURE 3. **A:** SSCP analysis of PCR fragment encompassing exon 8 of the LDL receptor gene in control subjects (N) and patients (P). **B:** The partial nucleotide sequence of PCR fragments from exon 8. **C:** Partial pedigree and individual clinical data. **D:** Detection of the missense mutation C347R in the LDL receptor gene. Numbers at left indicate the size of the restriction fragments. MI, myocardial infarction; asterisk (\*), subjects not sampled.

tions located in the EGF precursor homology domain usually encode mutant proteins that yield to class 2 (transport defective) or class 5 (recycling defective) phenotypes (Hobbs et al., 1992). The C347R mutation was not detected in 100 normocholesterolemic men, suggesting that this mutation is not a rare polymorphism in normal FC population.

We have finally compared the prevalence of the C152W, R329X, and C347R mutations in our cohort of 48 unrelated children of FC origin with suspected heterozygous FH. The C152W, R329X, and C347R mutations were found in two, two, and three children, respectively.

Our attempt to detect the mutation in the LDL receptor gene in 15 FC patients with a clear clinical diagnosis of FH was successful in only five of these subjects, leaving 10 individuals with no detectable mutation. This low success rate can be explained by the lack of sensitivity of SSCP analysis. In order to identify new mutations in these 10 remaining FH patients, it will be important to demonstrate the cosegregation of a particular haplotype at the LDL receptor gene locus with the clinical phenotype of FH in the families studied. If a linkage does exist, it will be essential to completely sequence the coding region of the gene and the proximal part of the promoter containing the sequence of regulatory significance. However, in at least some of these cases it is probable that the defects occurs elsewhere in the LDL receptor gene possibly in noncoding regions that are important for mRNA expression, splicing, and stability.

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