A Rapid PCR Method of Genotyping LDL Receptor Mutations in WHHL Rabbits

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WHHL rabbits are a valuable model for the study of human familial hypercholesterolemia and atherosclerosis. To use this animal model, it is often necessary to confirm LDL receptor status in WHHL rabbits. Here, we described a simple and rapid PCR method to detect LDL mutations in WHHL rabbits. J Atheroscler Thromb, 2002; 9: 145-148.

Key words: WHHL, LDL receptor, PCR, Hypercholesterolemia, Atherosclerosis, Transgenic rabbit

Introduction

Human familial hypercholesterolemia (FH) is a prevalent autosomal dominant inherited disease of lipid metabolism resulting in premature atherosclerosis with an estimated prevalence of 1 in 500 in most populations in the Western world(1). Classic FH is caused by mutation in the gene encoding the LDL receptor (LDLR) protein, leading to an impaired function of the receptor with respect to cellular binding and uptake of LDL-cholesterol (1). Watanabe heritable hyperlipidemic (WHHL) rabbit is an animal model for human FH, which was reported in 1980 by Dr. Watanabe and his colleagues(2). These rabbits have a defective LDLr due to a spontaneously arising deletion in exon 4 of the LDL gene that encodes a 4-amino acid deletion in the cysteine-rich ligand-binding domain of the protein(3). Homozygous WHHL rabbits are markedly hypercholesterolemic from birth and suffer from tendon xanthoma and atherosclerosis, both of which exhibit a pathological resemblance to human FH(4). In addition to WHHL rabbits, Ishibashi and his colleagues generated LDLr-knockout (KO) mice using a gene targeting technique in 1993(5). Both models have made a great contribution to the study of hypercholesterolemia and atherosclerosis (6, 7). Compared to LDLr-KO mice in which a large number of transgenes were easily introduced, only a few transgenic rabbits have been bred with WHHL rabbits containing human apolipoprotein Al (apoAl), lecithin: cholesterol acyltransferase (LCAT) and apo(a)(8-10). To obtain a sufficient number of WHHL rabbits for experiments, it is necessary to breed WHHL rabbits in one’s own facility. However, homozygous WHHL rabbits have very low fertility (11). Therefore, it is often necessary to breed heterozygous WHHL rabbits (both males and females) into WHHL homozygosity. We usually mate heterozygous WHHL rabbits with transgenic rabbits in order to obtain transgenic rabbits with a homozygous WHHL background. For efficient breeding and/or crossbreeding of WHHL, immediate identification of the LDLr mutation status in WHHL rabbits is required. This is especially important for heterozygous WHHL rabbits since their plasma lipids are just slightly higher than those of wild-type rabbits. LDLr dysfunctions in WHHL can be conventionally analyzed by a defective binding of 125I-labeled LDL to cultured WHHL fibroblasts(12). For routine screening, however, this method is laborious and impractical. For LDLr-KO mice, Gaw et al developed a PCR method to genotype the mouse LDLr allele (13). A PCR method for screening WHHL rabbits was also reported (9) but we found that the method was time-consuming and the product on the gel contained a non-specific band. In
this paper, we described a modified PCR method for the routine determination of LDLr genotype in WHHL rabbits.

Materials and Methods

Genomic DNA was obtained from ear biopsy as described(10). In addition, we attempted to establish a quick method utilizing a drop of blood for PCR analysis. In brief, a drop of blood from the ear of 2-3 week old pups was placed on a piece of Whatman filter paper. Then, a tiny piece of the paper (about 1 mm in size) was cut and loaded in a reaction tube with 50 μl of methanol for 20 min at room temperature. After completely drying, a reaction solution (see below) was loaded and subjected to PCR.

Heterozygosity or homogyzosity for the LDLR defects in WHHL was determined by PCR according to the presence of a 12-nucleotide deletion in exon 4 of the LDLR gene(3). For this purpose, we amplified a 306-bp fragment of exon 4 of the rabbit LDLR gene, including the 12-bp mutant region from 369 to 380 which contains a Bgl I-digestion site with the following primers: 5'-primer (5'-GCC CAA GAC GTG CTC CCA GGA C-3') and 3'-primer (5'-GCC GCT GCC ACA GTG GAA CTC GTG G-3'). Since both primers were G/C-rich (>70%), the reaction conditions including annealing temperature and magnesium concentration were optimized in order to generate specific bands on the gel. The optimized thermal cycling protocol is summarized in Table 1.

To visualize PCR products, we used two methods. For the first method we used a routine approach, and so amplified products were directly applied to a 12% Tris-borate-EDTA (TBE)-polyacrylamide gel and electrophoresed for 1.5 h at 40 mA. The gels were then stained with ethidium bromide for 15 min and visualized with UV light. For the second method, PCR products were directly digested with the restriction enzyme Bgl I (containing 20 μl of PCR product, 4 μl of 10x H buffer, 2 μl of Bgl I and 14 μl of H2O) at 37°C overnight and applied to a TBE-polyacrylamide gel.

In this study, we also cross-bred WHHL rabbits with transgenic rabbits expressing human lipoprotein lipase (14). We analyzed the heterozygous WHHL transgenic rabbits and report preliminary results.

Results and Discussion

The results are shown in Fig. 1. The expected amplified PCR fragment in the wild-type rabbit with normal LDL receptor function (LDLr") is 306 bp in length. In WHHL homozygotes (LDLr""), the PCR fragment was a single and shorter band of 294 bp due to the presence of the 2-allele deletion mutation. In WHHL heterozygotes (LDLr"+), however, there were two fragments (306 bp and 294 bp) representing one normal and one mutant allele. Although these two bands were of the same size by only 12bp, it is not difficult to differentiate LDLr"+ from LDLr" bands on polyacrylamide gel.

To confirm the absence of a Bgl I-digestion site in WHHL rabbits due to the 12-bp deletion in this region, the PCR products were digested with Bgl I. Bgl I-digested products from wild-type rabbits generated two bands, 212 and 94 bp, due to the presence of a digestion site for Bgl I within the 306 bp region. In the case of LDLr"" rabbits, there are three bands; the 212 and 94 bp bands represent the normal allele, while the 294 bp band is undigested and represents the mutant allele. In LDLr"+ rabbits, there is no digestion site for Bgl I, therefore, only 1 band (294 bp) was visible.

We initially performed PCR using the method reported by Brousseau in which there is a need to apply the products to a 10% TBE-polyacrylamide gel and then, purify the desired DNA from the gel. Purified products were further digested with Bgl I and analyzed again with a 20% TBE- polyacrylamide gel (9). We found that the second step was basically not necessary as it was time-consuming.

Table 1. The reaction solution for PCR and thermal cycling protocol.

<table>
<thead>
<tr>
<th>Reaction solution</th>
<th>Genomic DNA 1 μl (1 μg)</th>
<th>10 mM dNTP 1 μl</th>
<th>50 mM MgSO4 1.5 μl</th>
<th>Each primer (concentration: 10 pmol/μl) 1 μl</th>
<th>10× PCR amplification buffer 5 μl</th>
<th>High fidelity Tag DNA polymerase (5U/μl) (Takara, Japan) 0.5 μl</th>
<th>H2O 40 μl</th>
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<tr>
<td>PCR conditions</td>
<td>94°C 1 min 1 cycle</td>
<td>98°C 20 sec (30 cycles)</td>
<td>68°C 1 min (30 cycles)</td>
<td>57°C 10 min</td>
<td>94°C 1 min (30 cycles)</td>
<td>57°C 10 min</td>
<td>94°C 1 min (30 cycles)</td>
</tr>
</tbody>
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Fig. 1. Determination of LDL receptor mutation status by 12% polyacrylamide gel electrophoresis after PCR amplification. Molecular marker is shown in lane 1. Lane 2, wild-type (LDLr""); lane 3, LDLr"+; and lane 4, LDLr"+. PCR products were either directly loaded in the gel (A) or loaded after Bgl I-digestion (B).
and often difficult to purify enough DNA for enzymatic digestion from 20% TBE-polyacrylamide gel. In addition, in the previous report, there was one non-specific band in the wild-type rabbits on the gel. Accordingly, we optimized the PCR conditions and routinely omitted the gel purification of the products, directly applying the PCR products to a 12% TBE-polyacrylamide gel as shown in Fig. 1A. The mutant band (294bp) was 12-bp shorter than the normal allele band (306 bp), therefore, it is easy to differentiate mutant and normal fragments by size. On a chow diet, homozygous WHHL rabbits develop severe hypercholesterolemia and hypertriglyceridemia due to an increase in β-migrating lipoproteins as shown in Fig. 2. It should be mentioned that homozygous LDLr-KO mice have a mild hypercholesterolemia (230 mg/dl; 2-fold higher than in wild-type mice) but their plasma triglyceride levels are basically normal (5), indicating that LDLr plays a vital role in rabbits. Because the crossbreeding of WHHL rabbits requires a lot of effort, we recommend using the current modified method for routine monitoring and screening of WHHL rabbit colonies when crossbreeding WHHL with transgenic rabbits. In serial cross-breeding, we have established WHHL transgenic rabbits expressing human lipoprotein lipase (hLPL) and performed initial analyses on the plasma lipids and lipoprotein profiles with methods described previously (14). At 3-months of age, plasma triglyceride and HDL-C levels were reduced in LDLr+/hLPL-ho as compared to the LDLr-ho control WHHL rabbits (TG: 36 ± 14 mg/dl (LDLr-ho) vs. 13 ± 9 mg/dl (LDLr+/hLPL-ho); HDL-C: 34 ± 11 mg/dl (LDLr-ho) vs 12 ± 5 mg/dl (LDLr+/hLPL-ho); p<0.05) but total cholesterol was decreased although not significantly [48 ± 9 mg/dl (LDLr-ho) vs. 38 ± 13 mg/dl (LDLr+/hLPL-ho), p>0.05]. HPLC analysis showed that overexpression of LPL resulted in a reduction of VLDL and HDL but increase of LDL.

We are now in the process of breeding homozygous WHHL transgenic rabbits expressing hLPL and hopefully, will determine whether increased LPL activity influences LDL metabolism and also atherosclerosis in the setting of LDL receptor deficiency.

**Fig. 2.** Lipoprotein profiles of wild-type and WHHL rabbits. Plasma (2μl) from fasted rabbits was subjected to agarose gel electrophoresis and stained with Fat Red 7B. Homozygous WHHL rabbits show a marked increase in β-migrating lipoproteins and decrease in a-migrating lipoproteins. Five male rabbits were analyzed for total cholesterol (TC), triglycerides (TG) and HDL-cholesterol (HDL-C) in plasma and the data are expressed as the mean ± SD (mg/dl).
Fig. 3. HPLC analysis of plasma lipoprotein profiles of heterozygous WHHL and LPL transgenic rabbits. Plasma levels of triglycerides and cholesterol were determined by HPLC as described previously (14). Note that triglycerides are reduced in all fractions of transgenic rabbits (upper panel), but LDL cholesterol is increased. The HDL peak in transgenic rabbits is shifted to the left (bottom panel), suggesting a reduction in size.

References

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