



Letter

Rapid and efficient identification of the mouse leptin receptor mutation (C57BL/KsJ-*db/db*) by tetra-primer amplification refractory mutation system-polymerase chain reaction (ARMS-PCR) analysis

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The C57BLKS/J-*Lep^{db}* mouse has a point mutation in the leptin receptor gene and is one of the most useful animal model for non-insulin dependent diabetes mellitus in human. Since the homozygote of C57BLKS/J-*Lep^{db}* mouse is infertile, detection of point mutation in the leptin receptor gene is important for efficient maintaining strains as well as mass production of homozygotes. To develop a rapid and efficient genotyping method for C57BLKS/J-*Lep^{db}* mouse, the tetra-primer amplification-refractory mutation system polymerase chain reaction (ARMS-PCR) was used. The 407 and 199 bp PCR products were amplified from normal (+/+) mice; while the 407 and 268 bp PCR products were amplified from homozygotes (*db/db*) mice; and the 407, 268, and 199 bp PCR products were amplified from heterozygotes (*db/+*) mice. This result showed that the tetra-primer ARMS-PCR analysis by us is suitable to detect point mutation of the leptin receptor gene. Taken together, our method will dramatically reduce animal use for maintenance of strains as well as production of homozygote in the C57BLKS/J-*Lep^{db}* strains.

Keywords: Leptin receptor, genotyping, *db* mouse

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The C57BLKS/J-*Lep^{db}* mouse, which has a mutation in the leptin receptor, is one of the most used animal model for non-insulin dependent diabetes mellitus (NIDDM). The C57BLKS/J-*Lep^{db}* mouse has a G→T point mutation in the leptin receptor gene [1]. Homozygotes of C57BLKS/J-*Lep^{db}* mice are infertile and can be easily distinguished from obese mice. Efficient maintenance of the C57BLKS/J-*Lep^{db}* strain requires an accurate identification method for heterozygotes. However, there is no phenotypic difference between normal and heterozygote C57BLKS/J-*Lep^{db}* mice. Progeny testing is used to identify heterozygotes of C57BLKS/J-*Lep^{db}* mice. For homozygote from a mating pair, both parents are required to be heterozygote, hence researchers spend much time and effort on progeny testing. The Coleman and Hummel incorporated misty gene (*m*) is closely

linked to the leptin receptor and C57BLKS/J-*Lep^{db}* mice [2]. After misty gene incorporation, the heterozygote shows lean black mice and normal shows misty coat color after 3 to 4 weeks of age. The Coleman's method to identify heterozygote of C57BLKS/J-*Lep^{db}* mice has the disadvantage of inability to distinguish between heterozygote and normal offspring until 3 weeks of age. Horvat and Bünger developed a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay to overcome this problem [3]. The Horvet's method is also time consuming and expensive. The tetra-primer amplification-refractory mutation system-polymerase chain reaction (ARMS-PCR) analysis is used to identify known single nucleotide polymorphism in genomic DNA [4,5]. We developed a new method for easy identification of C57BLKS/J-*Lep^{db}* mice genotypes using

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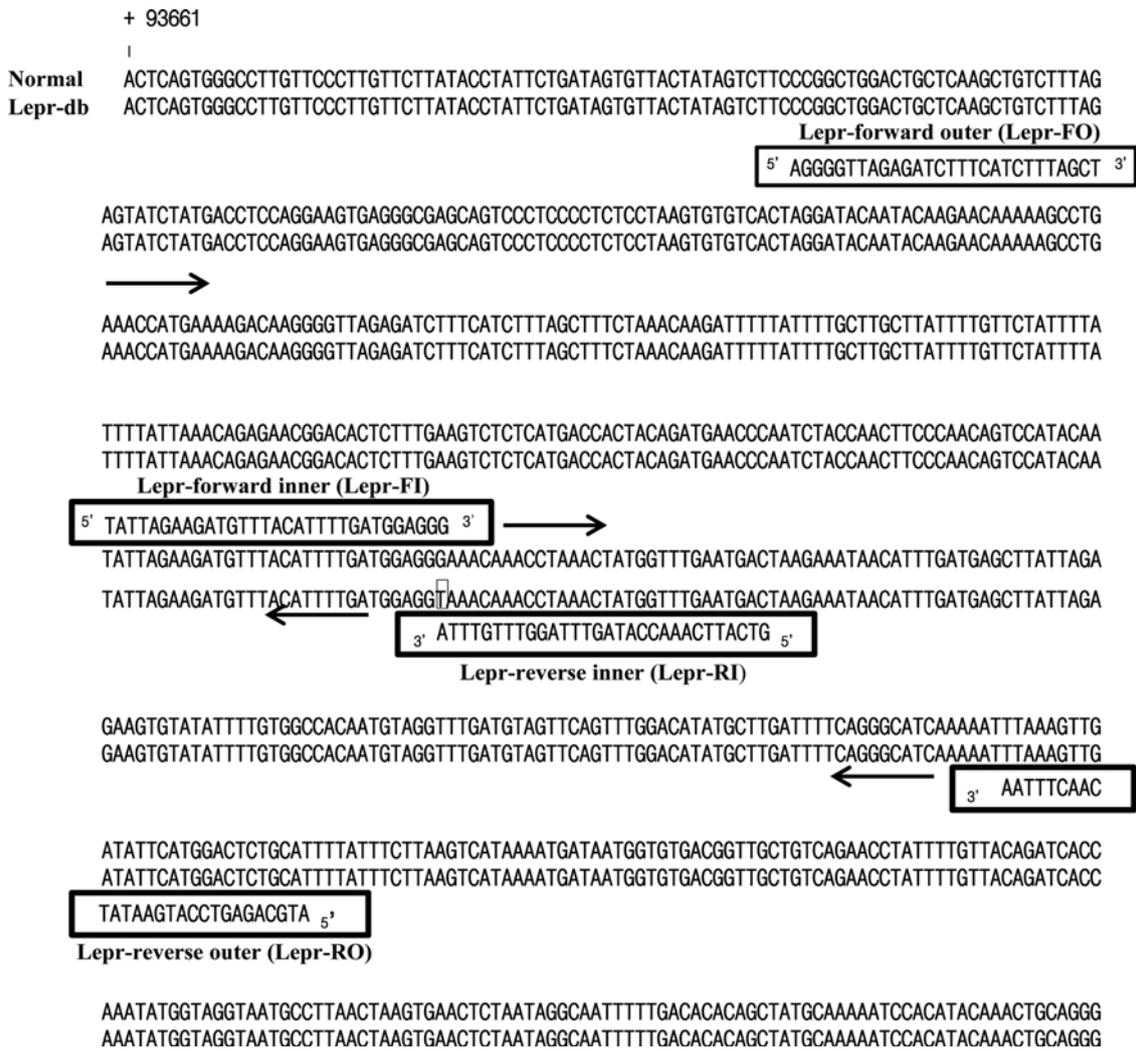


Figure 1. Sequences of the leptin receptor mutation and normal allele (NCBI, Gene ID: 16847). The primers for ARMS-PCR were designed using the primer design web service for tetra-primer ARMS-PCR. Underline indicates transverse point mutation (G→T) in intron 18 of the leptin receptor.

ARMS-PCR.

We used C57BLKS/J-*Lepr^{db}* mice that were supplied from Laboratory Animal Center, Hallym University. Animals were housed in a specific pathogen free room with controlled temperature (23±2°C), humidity (55±10%), and light (08:00 a.m~20:00 p.m.). The animal study was conducted in accordance with Institutional Animal Care and Use Committees of Hallym University (Hallym 2013-133). Genomic DNA was prepared from the mouse tail by standard SDS/proteinase K lysis and the phenol/chloroform extraction method [6]. The design of primers for amplification-refractory mutation system-polymerase chain reaction (ARMS-PCR) used the primer design web service for tetra-primer ARMS-PCR [7]. The PCR samples comprised 50 ng genomic DNA,

6 µL rTaq plus 5× PCR master mix (1 unit/4 µL thermostable DNA polymerases, 50 mM Tris-HCl (PH9.0), 16 mM (NH₄)₂SO₄, 1.75 mM MgCl₂, 2% DMSO, 0.1% Tween 20, 0.1 mg/mL BS, 0.05% bromophenol blue, 12% glycerol), and 2.5 p/mol primers. Target DNA was amplified in a Cyclogene Dri-Block[®] cyclor (Techne Cambridge Ltd., U.K.), with initial denaturation at 94°C for 5 min followed by 30 cycles of 94°C for 30 sec, 49.1°C 45 sec, and 72°C for 45 sec and a final elongation period at 72°C for 10 min. The PCR products were electrophoresed on a 3% agarose gel.

The mouse leptin receptor sequence (C57BL/6J) was retrieved from NCBI (Gene ID: 16847). The C57BLKS/J-*Lepr^{db}* mice have transverse point mutation (G→T) in intron 18 of the leptin receptor [1]. This mutation creates

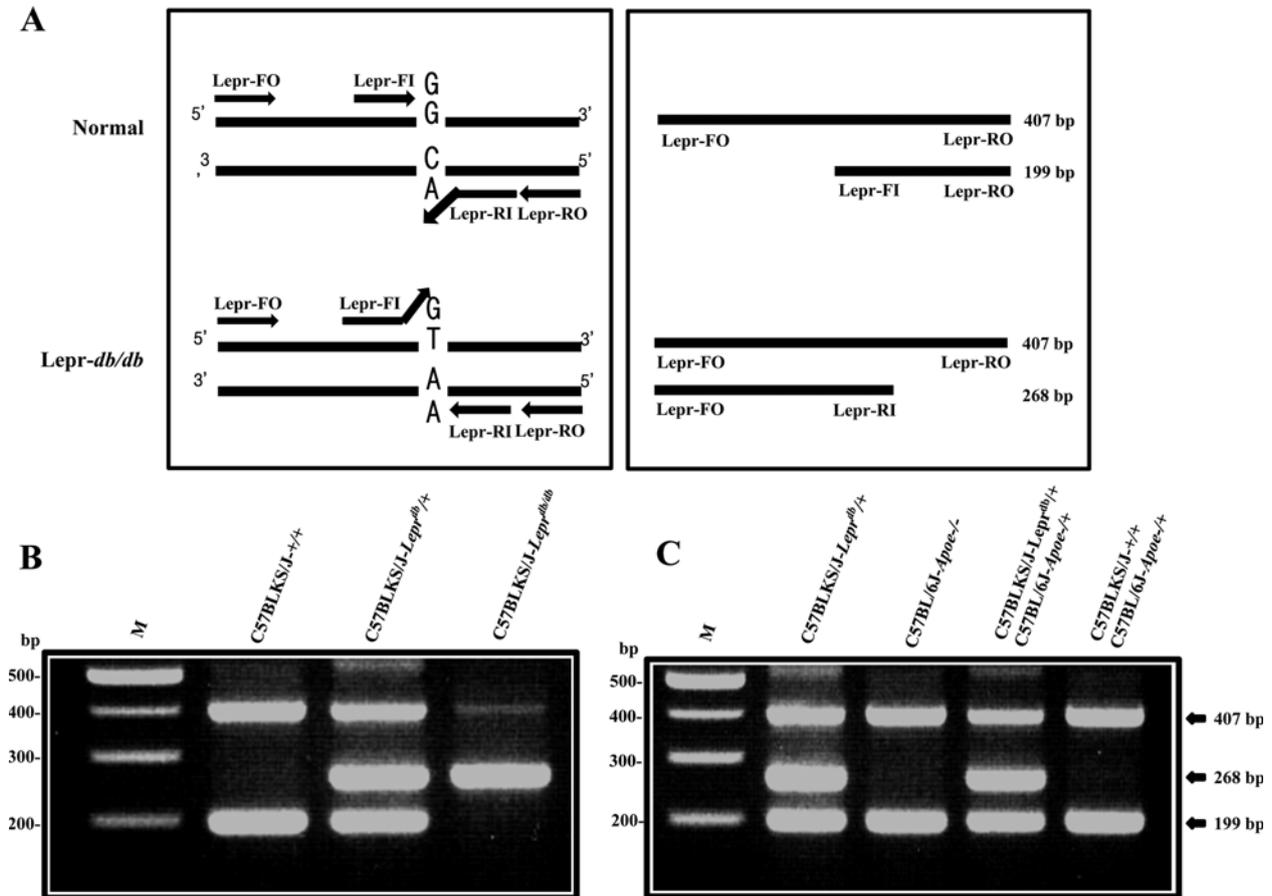


Figure 2. Detection of leptin receptor mutation by the tetra-primer ARMS-PCR analysis. A. Design of PCR products. Leptin receptor mutation (268 bp, Lepr-FO and Lepr-RI), wild type (199 bp, Lepr-FI and Lepr-RO), internal control from both alleles (407 bp, Lepr-FO and Lepr-RO). B. Genotyping of leptin receptor mutant mice by the tetra-primer ARMS-PCR. C. Genotyping of double knock-out mice by the tetra-primer ARMS-PCR. M: 100 bp ladder.

a splicing donor site and produces exclusively short form mutant transcripts. We designed 4 primers for amplification of allele-specific PCR products from leptin receptor mutant and normal alleles using primer design web service for tetra-primer ARMS-PCR (Figure 1) i.e., Lepr-forward outer primer (Lepr-FO); AGGGTTAGAGATCTTTCATCTTTAGCT, Lepr-forward inner primer (Lepr-FI); TTAGAACATGTTACATTTTGATGGAGGG, Lepr-reverse inner primer (Lepr-RI); ATTTGTTTGATTTGATAACCAACTTACTG, Lepr-reverse outer primer (Lepr-RO); AAATTTCAACTATAACTACCTGAGACGTA. The 268 and 199 bp PCR products are amplified from the leptin receptor mutant allele (Lepr-FI and Lepr-RO primers) and normal allele (Lepr-FO and Lepr RI primers), respectively. A 407 bp PCR product is amplified from leptin receptor mutant and normal alleles by Lepr FO and Lepr-RO primers as an internal control (Figure 2A).

We tested our hypothesis by tetra-primer ARMS-PCR on the 3 genotypes i.e., *db/db*, *db/+*, *+/+*. The 407 and 199 bp PCR products were amplified from normal (*+/+*) mice; while the 407 and 268 bp PCR products were amplified from homozygote (*db/db*) mice; and the 407, 268, and 199 bp PCR products were amplified from heterozygote (*db/+*) mice (Figure 2B). Tetra-primer ARMS-PCR identification of heterozygotes was further confirmed by progeny testing. We showed that the tetra-primer ARMS-PCR analysis worked in other C57BL/6J background mice by genotyping progenies from C57BLKS/J-*Lepr*^{*db*}/*+* and C57BL/6J-*ApoE*^{*tm1Unc*} knock-out mating. The 407, 268, and 199 bp PCR products were amplified from double heterozygote (*db/+*, *ApoE*^{*+*}) mice (Figure 2C). This result indicated that the tetra-primer ARMS-PCR analysis was effective in mice with a C57BL/6J genetic background.

Currently, there are 3 methods to identify leptin receptor

mutation. Genotyping by progeny testing is time consuming. The indirect genotyping method by misty gene cannot distinguish between heterozygote and normal mice from birth to 3 weeks of age [2]. Genotyping by PCR-RFLP is expensive and requires several steps such as PCR, restriction enzyme digestion, and electrophoresis [3]. The tetra-primer ARMS-PCR analysis has only 2 steps i.e., PCR and agarose gel electrophoresis. Thus, our tetra-primer ARMS-PCR analysis is the most efficient and rapid genotyping method to identify leptin receptor mutation on a C57BL/6J genetic background. Taken together, the tetra-primer ARMS-PCR analysis is useful for efficient maintenance of C57BLKS/J-*Lepr^{db}* strain and high throughput production of double knock-out mice involving the leptin receptor mutation.

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Conflict of interests The authors declare that there is no financial conflict of interests to publish these results.

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