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Acadl-SNP based genotyping assay for long-chain acyl-CoA dehydrogenase deficient mice

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ABSTRACT

The long-chain acyl-CoA dehydrogenase (LCAD) (*Acadl* = gene; LCAD = protein) deficient mouse model has been important in evaluating the role of mitochondrial fatty acid oxidation of long-chain fatty acids in metabolic disorders. The insertion vector-based gene targeting strategy used to generate this model has made it difficult to distinguish homozygous and heterozygous genotypes containing targeted *Acadl* alleles in LCAD-deficient mice. Herein, we describe the design and validation of *Acadl* SNP genotyping methods capable of distinguishing between heterozygous and homozygous LCAD-deficient mice. The *Acadl* SNP genotyping assays are effective at allelic discrimination of both C57BL/6 and 129 mouse strain-based *Acadl* alleles under conditions including, both low purity and quantity genomic DNA templates. This makes the method practical and provides the necessary tools for genotyping the LCAD-deficient mouse model.

1. Introduction

Long-chain acyl-CoA dehydrogenase (*Acadl* = gene; LCAD = protein) deficiency in mice closely mimics human very long-chain acyl-CoA dehydrogenase deficiency and is a key enzyme involved in mouse fatty acid oxidation of long chain fatty acids [1–5]. LCAD-deficient mice have been distributed around the world for various studies. Unfortunately, due to the insertion vector-based gene targeting strategy used [1], a simple PCR-based genotyping assay is not possible. As previously described, LCAD-deficient ($LCAD^{-/-}$) mice were generated using a targeting strategy that resulted in an insertion duplication of a region containing exons 3 through 4 within the *Acadl* locus [1], which confounds the development of routine genotyping strategies that distinguish targeted and wild-type *Acadl* alleles in $LCAD^{+/-}$ and $LCAD^{-/-}$ mice.

The generation of the LCAD-deficient mouse model led to construction of a chimeric *Acadl* locus comprising sequence regions originating from mouse strains of three distinct genetic backgrounds. An insertion-based targeting vector, p*Acadl*^{tm1Uab}, which contains 129X1 (formerly 129/SvJ) genomic sequence from the *Acadl* locus spanning intron 2 through intron 4 and harbors an 821 base pair deletion in exon 3 was used during the targeted deletion step in TC-1 embryonic stem (ES) cells derived from 129S6 (formerly 129/SvEvTacfBR) mice. Recombinant mice generated from targeted ES cell clones were then backcrossed to C57BL/6 (B6) mice (Taconic Farms) for 10 generations to produce B6 congenic LCAD-deficient ($B6.LCAD^{-/-}$) mice. Ultimately, this targeting approach led to the generation of $B6.LCAD^{-/-}$ mice with an *Acadl* locus consisting of proximal 129X1 and 129S6 sequence and distal B6 sequence (Fig. 1) [1].

In an effort to develop a simple genotyping method for LCAD-deficient mice, we have utilized the chimeric nature of the targeted *Acadl* locus and employed targeted mutant 129-based allele and non-targeted wild-type B6 allele-distinguishing single nucleotide polymorphisms (SNPs). We describe here the validation of a SNP-based genotyping method that enables reliable genotyping and discrimination between B6 wild-type and 129-targeted *Acadl* alleles in $B6.LCAD^{+/-}$ and $B6.LCAD^{-/-}$ mice.

2. Materials and methods

2.1. Mouse genomic DNA

Genomic DNA samples were prepared from $B6.LCAD^{+/+}$ wild-type (Taconic), $B6.LCAD^{+/-}$, $B6.LCAD^{-/-}$, and 129S6 (wild-type) mice. All procedures were approved by the Sanford-Burnham Medical Research Institute (SBMRI) Institutional Animal Care and Use Committee (IACUC). Genomic DNA was isolated from mice by tissue harvest. DNA was isolated from tail or from liver extraction following humane euthanasia methods. Mouse genomic DNA (spleen) from 129X1 (wild-type) mice (formerly 129/SvJ) was acquired from Jackson Laboratory. For genomic DNA extraction, tail tissue was incubated in 200 µl of solution A (0.2 mM EDTA pH 12, 25 mM NaOH) at 95 °C for 45 minutes to 1 hour, followed by the addition of 200 µl of solution B (40 mM Tris pH 5), then vortexed. Liver genomic DNA was extracted using the Genomic DNA Mini Kit (IBI Scientific), followed

Abbreviations: *Acadl*, acyl-coenzyme A dehydrogenase, long-chain; LCAD, long-chain acyl-CoA dehydrogenase.

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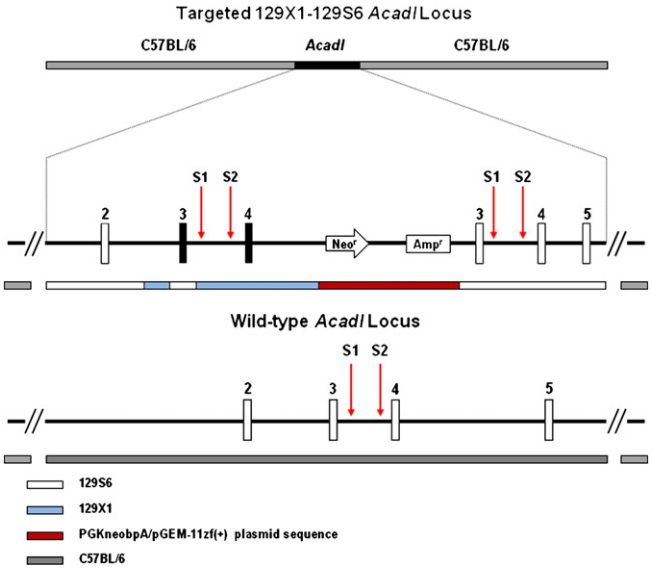

Fig. 1. Chimeric strain-specific sequence of the targeted *Acadl* locus in LCAD-deficient mice. (Top) Targeted 129X1-129S6 *Acadl* locus. *Acadl* locus sequence in LCAD-deficient mice consists of targeted vector comprising 129X1-129S6 chimeric components. Sequence distal to the *Acadl* locus is of C57BL/6 origin. (Bottom) Wild-type *Acadl* locus. Wild-type C57BL/6 *Acadl* locus sequence and distal sequence. (Legend for sequence origin: no color (129S6); blue (129X1); gray (C57BL/6); red (plasmid sequence PGKneobpA/pGEM-11zf(+)). S1 and S2 refer to *Acadl* SNP 1 and *Acadl* SNP 2 locations, respectively, in the targeted *Acadl* (top) locus and wild-type C57BL/6 locus (bottom).

by purification with Wizard® Genomic DNA Purification Kit (Promega), then further purification using standard phenol-chloroform-isoamyl alcohol (Sigma) and ethanol precipitation methods to ensure purity of the genomic DNA with a 260/280 ratio of 1.8–2.0.

2.2. SNP assay development

A region spanning 1.5 kilobases (kb) between exons 3 and 4 within the *Acadl* gene locus was amplified with forward primer 5′-GACTTGCTCTCAACAGCAGTTACTTGGG-3′ (exon 3) and reverse primer 5′-GGCTATGGCACCGATACACT-3′ (exon 4), using either genomic DNA isolated from liver of B6.LCAD$^{-/-}$, B6.LCAD$^{+/+}$, or 129S6 mice, or from 129X1 genomic DNA (Jackson Laboratory). The respective 1.5 kb products were amplified using the Expand Long Template PCR system (Roche). PCR products were separated by gel electrophoresis on a 1% agarose gel, the 1.5 kb fragments then excised and gel purified (Qiagen). Following quantification, the purified products spanning the *Acadl* exon 3 to 4 region were sequenced with primers 5′-GACTTGCTC TCAACAGCAGTTACTTGGG-3′ or 5′GGCTATGGCACCGATACACT-3′ with an ABI 3730 sequencer (Retrogen). Sequence data generated was analyzed and aligned using Vector NTI 11 (Invitrogen Life Technologies). Sequence runs for B6.LCAD$^{-/-}$, B6.LCAD$^{+/+}$, 129S6, and 129X1 *Acadl* exon 3 to 4 regions were aligned together or to known *Acadl* sequence for C57BL/6J (Gene ID: 11363; GenBank ID: NC_000067.5) (NCBI). Single nucleotide polymorphisms (SNPs) identified in B6.LCAD$^{-/-}$, 129S6, and 129X1 sequences aligned with B6.LCAD$^{+/+}$ sequence for the *Acadl* exon 3 to 4 region were selected for SNP genotyping assay development, based on the following criteria: (1) quality of sequence reads, and (2) no other sequence anomalies (i.e. insertions, deletions, or SNPs) within 50 bases of the target SNP.

Primers (5′ GGAGGATTGCCAAGAGCTCAAG 3′ forward; 5′TTTGT-TTTGAAAGCAGCTT CTTGCT 3′ reverse) and probes (5′ TGGCCTA-GAACTGACTAT 3′ FAM; 5′TGGCCTAGAA CTAACTAT 3′ VIC) were selected (*Acadl* SNP 1 assay) to target a 93 base pair amplicon within intron 3 of the *Acadl* locus with the genotyping SNP located specifically at position 66,900,911 (*Acadl* SNP 1) on chromosome 1 (Applied Biosystems). Primers (5′ GTGACT GCGGGTACATTAGAGT 3′ forward; 5′ TCTTACCCTGACACTGCAATTGT 3′ reverse) and probes (5′ AAG-GTGCCCTCCATAAT 3′ FAM; 5′ CAAGGTGCCCTCTATAAT 3′ VIC) were selected (*Acadl* SNP 2 assay) to target a 70 base pair amplicon also within intron 3 of the *Acadl* locus with the genotyping SNP located specifically at position 66,900,172 (*Acadl* SNP 2) on chromosome 1 (Applied Biosystems). *Acadl* SNP 1 and SNP 2 were verified as registered SNPs as rs48198501(dbSNP) and rs30323299 (dbSNP), respectively. Both *Acadl* SNP 1 and 2 assays were then validated by real time PCR using an Applied Biosystems StepOne instrument (ABI). A single reaction consisted of 10 μl of Taqman® Genotyping Master Mix (Applied Biosystems; 4371355), 0.5 μl of either *Acadl* SNP 1 or 2 assay, 7.5 μl dH_2O, and 2 μl of genomic DNA template. Reactions were set up in MicroAmp Fast Optical 96-well plates (Applied Biosystems; 4346906). A SNP genotyping program of (60 °C 30 seconds (1×); 95 °C 10 minutes (1×); 95 °C 15 seconds then 60 °C 1 minute (40×); 60 °C 30 seconds (1×)) was used with genomic DNA samples for known B6.LCAD$^{-/-}$, B6.LCAD$^{+/-}$, B6.LCAD$^{+/+}$, 129S6, and 129X1 mouse strains, as well as no template controls. Genotyping results were represented as allele intensities and calculated as the endpoint fluorescence of reporter dyes normalized to the Rox passive reference dye, minus the starting background reporter fluorescence normalized to the passive reference dye (ΔRn). An allelic discrimination plot of normalized reporter fluorescence (ΔRn) for each allele was then generated using the StepOne algorithm function. The StepOne software then uses an algorithm to plot the normalized reporter fluorescence (ΔRn) as an allelic discrimination plot. Genotype clusters were defined by the ratio of allele 1 (C) ΔRn/allele 2 (T) ΔRn with ratios ~1 being defined as LCAD$^{+/-}$ genotype clusters, ratios >1 defined as LCAD$^{+/+}$ genotype clusters, and ratios <1 defined as LCAD$^{-/-}$ genotype clusters.

2.3. Serial dilution analysis

Serial dilution assays were performed for both *Acadl* SNP 1 and 2 assays. Genomic templates from B6.LCAD$^{-/-}$, B6.LCAD$^{+/-}$, and B6.LCAD$^{+/+}$ mice were diluted serially 2-fold with a maximum of 140 ng/μl of genomic template per reaction and a minimum of 2.18 ng/μl of genomic DNA per reaction. All genomic samples used were purified from liver with a 260/280 ratio of 1.8–2.0. SNP genotyping PCR assays were performed as described above.

2.4. Statistical analysis

Statistical analysis was performed using ANOVA with Tukey post test. Results showing a p-value of $p<0.05$ were considered statistically significant.

3. Results

3.1. Development of an Acadl SNP genotyping assay

In order to develop a SNP-based genotyping method to discriminate between the targeted 129 chimeric *Acadl* allele in B6.LCAD$^{-/-}$ mice from the wild-type *Acadl* allele in B6.LCAD$^{+/+}$ mice, we evaluated sequences for both the targeted 129 and wild-type B6 *Acadl* alleles to identify candidate SNPs. In our sequence analysis of several *Acadl* genomic regions, PCR amplification and sequencing of a 1.5 kb region located between exons 3 and 4 of the targeted 129 chimeric *Acadl* allele and the wild-type *Acadl* allele in B6.LCAD$^{-/-}$ and B6.LCAD$^{+/+}$ mice, respectively, produced sequence runs that could be aligned together or with known sequence for the C57BL/6 *Acadl* locus to search for the presence of candidate SNPs. *Acadl* sequence alignments of the exon 3 to 4 region from B6.LCAD$^{+/+}$ and B6.LCAD$^{-/-}$ genomic