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FULL REPORT

A Simple and Accurate Genotype Analysis of the *motor neuron degeneration 2* (*mnd2*) Mice: an Easy-to-Follow Guideline and Standard Protocol Applicable to Mutant Mouse Model

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Author contribution; H.A.S. and H.R. managed the project and interpretation of data; H.A.S, H.R. and M.K.N. participated in writing the manuscript; H.A.S. and G.Y.K. performed the experiments; H.G.G. and S.K. provided the sequence data for this manuscript; All authors read and approved the final manuscript.

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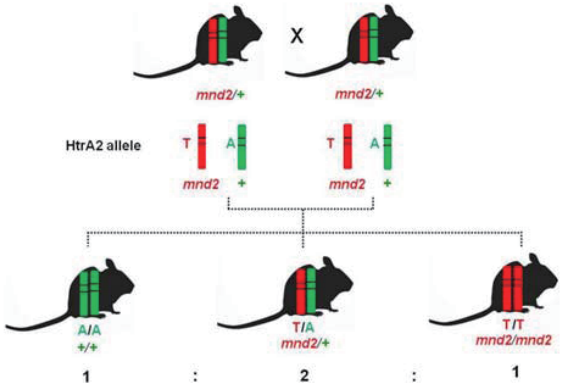
SYNOPSIS

The *motor neuron degeneration 2* (*mnd2*) mice carry a point mutation of A to T nucleotide transversion at the serine 276 residue of high temperature requirement A2 (HtrA2), resulting in losses of an *Alu*I restriction enzyme site (5'AGCT3') and the HtrA2 serine protease activity. Moreover, dysfunctions of HtrA2 are known to be intimately associated with the pathogenesis of neurodegenerative diseases, including Parkinson's disease. Thus, this *mnd2* mouse is an invaluable model for understanding the physiological role of HtrA2 and its pathological role in neurodegenerative diseases. Nevertheless, many molecular and cellular biologists in this field have limited experience in working with mutant mouse models due to the necessity of acquired years of the special techniques and knowledges. Herein, using the *mnd2* mouse model as an example, we describe easy-to-use standard protocols for web-based analyses of target genes, such as HtrA2, and a novel approach for simple and accurate PCR-*Alu*I-RFLP-based genotype analysis of *mnd2* mice. In addition, band resolution of *Alu*I-RFLP fragments was improved in 12% polyacrylamide gel running in 1X Tris-Glycine SDS buffer. Our study indicates that this PCR-*Alu*I-RFLP genotype analysis method can be easily applied by the molecular and cellular biologist to conduct biomedical science studies using the other mutant mouse models.



Key Words: *mnd2* mice; HtrA2; genotyping; web-based analyses; PCR-RFLP; mutant mouse

INTRODUCTION

With the completion of the Genomic Project and rapid growth of biomedical technologies, such as next-generation sequencing, bioinformatics, and gene expression profiling, mutations in specific genes have been identified from various mouse models, in which only disease-like phenotypes have been known, and the gene functions are also in the process of being uncovered[1-4]. Moreover, mouse models have been recognized as a powerful biomedical tool for addressing the biological challenges relevant to various physiological processes, such as cell growth, apoptosis and the pathogenesis of many important human diseases[5-12]. Thus, mouse models, including mice with spontaneous mutations at specific genes, genetically engineered knock-in (KI) and knock-out (KO) mice, have been widely used as valuable research resources for the purpose of better understanding the *in vivo* physiological and pathophysiological functions of specific genes1[3-17].

Recently, the verified mouse models and detailed information on those mice are readily available through various institutions, including the Jackson Laboratory[18,19]. Furthermore, the opportunity to receive such valuable resources has increased for the molecular and cellular biologists to elucidate basic biological processes and address important biological challenges, due to easy access to the worldwide scientific community in the fields of mouse genetics without using genetically engineered processes for creating the mouse models and special expertise in the mouse genetics[20] (http://www.mmrrc.org/). Nevertheless, the scientists, who do not participate in the field of mouse genetics, are still limited to maintain a mouse colony, manipulate the mouse models, and even work in the basic genotyping, which is the process of determining differences in genotypes for individual mice and is a critical step for maintaining and studying with the mouse models.

The *motor neuron degeneration 2* (*mnd2*) has been known to have arisen by a spontaneous, recessively inherited mutation and exhibits abnormal gait, akinesia, bradykinesia, and impaired balance, which are similar phenotypes of neurodegenerative disorders, including PD (Jackson Laboratory, http://jaxmice.jax.org/strain/004608.html)[21,22]. Ten year later, in 2003, the *mnd2* mutation was identified as the missense mutation of serine 276 to cysteine (S276C) in the serine protease domain of the high temperature requirement A2 (HtrA2, known as Omi)[23,24], (refer to Figure 2B). Accumulating evidence indicates that HtrA2 plays pivotal, multifactorial roles in mitochondrial homeostasis and is one of genes causing PD[25-31]. Accordingly, increasing attention has been focused on elucidating the interplay between the HtrA2' role in the mitochondrial biogenesis and dysfunctions in the pathogenesis of important human diseases, such as neurodegenerative diseases and cancer[32-36]. Thus, the utility of the *mnd2* mice will be continuously increased in the near future; however, still little is known on the standard protocol for genotype analysis using *mnd2* mice[24].

Herein, we describe the complete and detailed steps of methodologies used in *mnd2* genotyping analyses, including mouse maintenance, breeding, web-based analyses of genomic

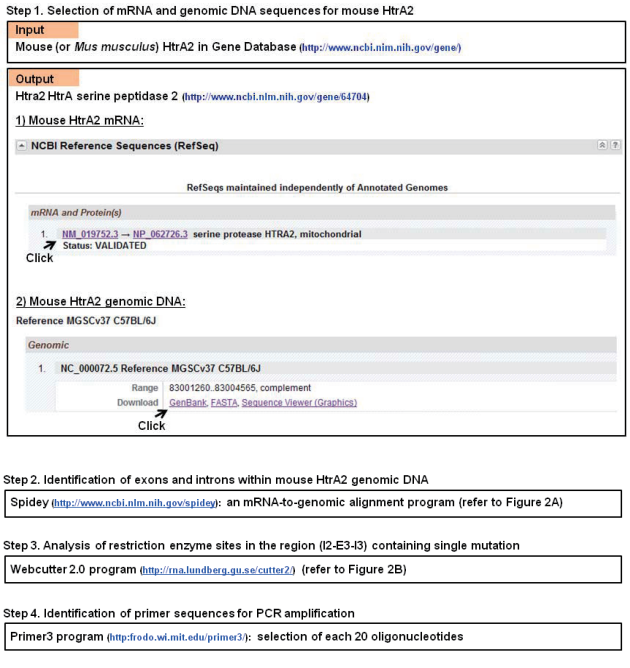


Figure 1. Flowchart of web-based analyses of mouse HtrA2 gene and selection of primers for genotyping of *mnd2* mice.
I2 and E3 in step 3 denote intron2 and exon2, respectively.

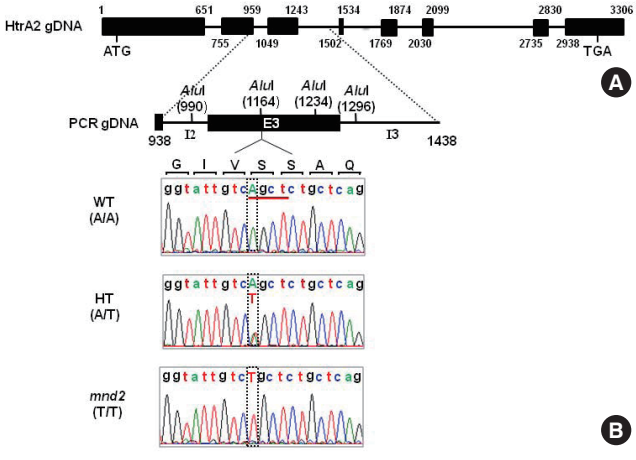


Figure 2. Output of web-based analyses of mouse HtrA2.
(A) Genomic organization of HtrA2 (Steps 1 and 2 in Figure 1). Black boxes represent 8 exons of HtrA2. The numbers indicate the position of nucleotides in the genomic (g) DNA covering the complete coding region of HtrA2. (B) Analysis of *Alu*I restriction enzyme sites (red bar) in the region containing single mutation (Step 3 in Figure 1). The missense mutation (A to T transversion) at residue 276 results in the serine to cysteine (S276C) substitution in the protease domain of HtrA2 in *mnd2*. HT means heterozygous (*mnd2* /+) mouse.

and cDNA of HtrA2, and a flowchart for genotype analysis of *mnd2*. Furthermore, our mouse genotype analysis is an easy-to-use standard protocols, thus could be readily applied by the molecular and cellular biologists to conduct biomedical science studies using the mutant mouse models.

RESULTS AND DISCUSSION

A web-based guideline of genotype analysis using the mnd2 mouse model

First, to obtain information regarding the gene of interest, such as HtrA2, through an online web-based database application, mouse or *mus musculus* HtrA2 was inputted into the Gene Database (http://www.ncbi.nlm.nih.gov/gene) of NCBI (Figure 1, Input in Step 1)[37]. The output screen of the NCBI Database exhibited comprehensive, integrated, non-redundant, well-annotated set of sequences, including genomic (g) DNA, transcripts, and protein data on HtrA2 serine peptidase 2 (Gene ID: 64704) (Step1, Output). Through clicking the GenBank ID: NM_019752.3 found in the *mRNA* and *Protein(s)* panel of the NCBI Reference Sequences (RefSeq) in the lower half of the data screen, information on the *mus musculus* HtrA2 mRNA sequence and protein can be obtained. The HtrA2 gDNA sequence (GenBank ID: NC_000072) was downloaded by clicking on GenBank in the Genomic panel (Step1, Output), exhibited 3,306 bp gDNA sequence in *mus musculus* strain C57BL/6J chromosome 6.

The Spidey program (http://www.ncbi.nlm.nih.gov/spidey), an mRNA-to-genomic alignment program, was run to identify internal exons and introns in the HtrA2 gDNA sequence (Step 2). Aligning the mRNA and gDNA sequences of HtrA2 through the Spidey program allowed determination of the intron-exon structure and 8 exons in the HtrA2 gDNA (Figure 2A)[25,38]. Subsequently, approximately 500-bp region of gDNA containing the specific mutation was analyzed through the Webcutter 2.0 program (http://rna.lundberg.gu.se/cutter2/) to identify the 4-cutter restriction enzyme (RE) sites (Step 3). To conduct PCR amplification of this region, the Primer3 program (http:frodo.wi.mit.edu/primer3/) was used to design PCR primers (Step 4). The proper primer generally has the following characteristics: a GC content of 40-60% to increase the stability of nucleic acid hybridization due to triple hydrogen bond; a melting temperature (Tm) of 55-65°C; and 20-30 nucleotides in length[39]. Accordingly, our forward and reverse primer set was selected as a GC content of 50%, a melting Tm of 60°C and 20 nucleotides in length.

Experiments-based genotype analysis of mnd2 mice

Heterozygous *mnd2* (HT, *mnd2*/+) mice of strain B6(Cg)-*Htra2*^{mnd2}/J (Stock No.: 004608) were purchased from Jackson Laboratory, and HT male were intercrossed with HT female after P42 (Figure 3, Step 1). HT mice were maintained with occasional backcrosses to C57BL/6J (B6) to avoid inbreeding depression, that is, loss of reproductive fitness by inbreeding processes[2,24,40,41]. Because the motor neuron disease-like phenotype did not appear before P15 in the *mnd2* mice, the phenotypic difference between littermates could not be recognized initially. In order to maintain an appropriate number of WT and *mnd2* and manipulate mice at different ages for the purpose of biomedical studies, an accurate genotype analysis is necessary. To do so, P10 littermates before the appearance of phenotype were categorized by genotype analysis as follows: WT (+/+), HT (*mnd2*/+), and homozygous mnd2 (*mnd2*/*mnd2*). Current methods of mouse genotyping include analysis of gDNA by Southern blot hybridization, single-strand conformation polymorphism (SSCP), polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), quantitative PCR, and DNA sequencing[42-49]. Among various analysis methods, the PCR-RFLP method has the advantage of being non-expensive

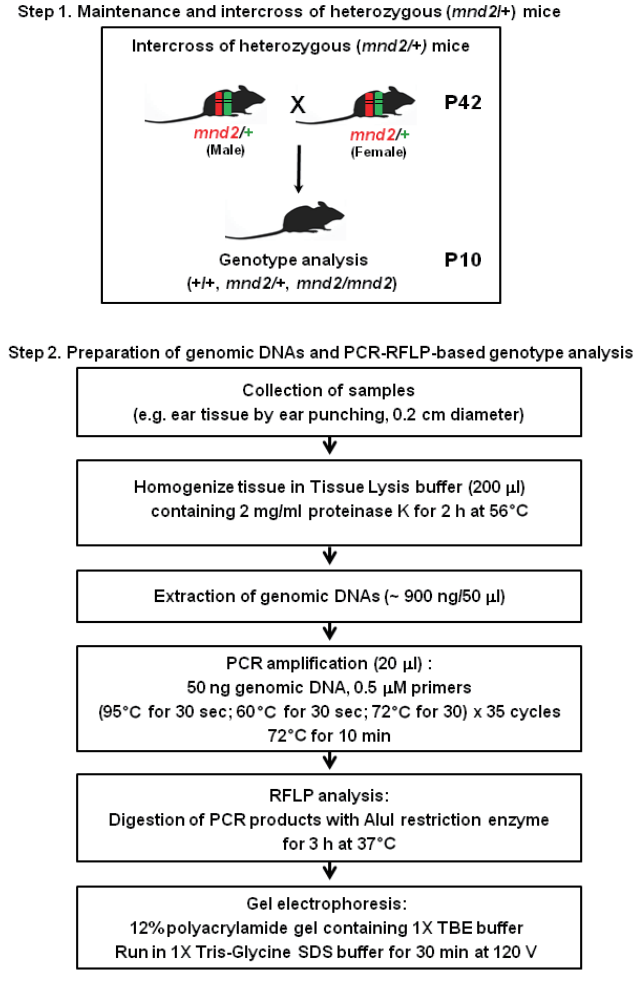


Figure 3. Flowchart summarizing the protocol for rapid genotype analysis. Step 1: HT female and HT male were intercrossed after postnatal day (P) 42 to produce littermate WT, HT, and *mnd2*. Genotype analysis was performed by PCR-RFLP at P10.

and rapid, because a small amount of DNA can be amplified with PCR, and a genetic difference of fragments can be detected by gel electrophoresis after RE digestion of the PCR fragment [50-53]. Thus, the PCR-RFLP assay has been routinely used when the specific RE site destroys or creates due to the mutations in gDNA, for example, the *mnd2* mutation (Figure 2B).

In a previous study, the *mnd2* mutation was identified as an A to T transversion at nucleotide position 1,163 in exon 3 (E3) of HtrA2 (refer to Figure 2B), resulting in mutation of S276C in the serine protease domain of HtrA2[22,23]. The nucleotide sequence differences in this region was confirmed by direct PCR DNA sequencing after isolating the gDNA of littermates from HT intercrossing, and mice were classified into three different genotypes: WT (+/+ or A/A; both A allelic nucleotides), *mnd2* (*mnd2*/*mnd2* or T/T; both T allelic nucleotides), and HT (*mnd2*/+ or A/T, a mixture of two allelic nucleotides) (Figure 2B). The 500 bp region encompassing intron2 (I2)-E3-I3 was analyzed for searching 4-cutter RE sites using the Webcutter 2.0 program, and four *Alu*I RE sites (AGCT) were identified (Figure 1, Step3, Figure 2B). In the case of *mnd2*, the AGCT (nucleotide 1,164, red bar) was changed into TGCT, so that *Alu*I RE could not cleave these sequences. Therefore, the difference was used to perform genotype analysis of littermates produced by HT intercrossing using the PCR-RFLP protocol (Figure 3, Step1).

Nevertheless, there was no detailed information in previous protocols (Jackson laboratory)[23]; in particular, the confusion arose in the genotype determination between HT and mnd2 (Supplementary Figure 1, Supplementary Table 1). In our practical experiments, a more convenient and reliable method of *mnd2* genotyping for *mnd2* users was set up, and this standard PCR-RFLP-based genotyping protocol may be readily applicable to other mutant mouse models (Figure 3, Step 2). First, ear tissue was obtained using a mouse ear punch and homogenized in TL buffer, resulting in preparation of gDNA with an average yield of approximately 900 ng (Figure 3, Step 2). Next, based on the information obtained via the web-based analyses, using the Primer3 program, we selected forward and reverse primers of 20 oligonucleotides that can amplify the 276 bp fragment (nucleotide position 938-1,214) containing two *Alu*I (A to T transversion at 1,164 in *mnd2*) (Figure 4A). This design can give rise to a more convenient and clear discernment of *Alu*I RFLP fragment due to the distinct differences in fragment length between WT and *mnd2* and the presence of *Alu*I RE digestion at nucleotide position 990. For the PCR reaction, 50 ng gDNA was used as a template as described in Step 2, followed by digestion of 75% of the PCR reaction sample with *Alu*I RE for establishing the PCR-*Alu*I-RFLP-based genotype analysis. To optimize the gel electrophoresis conditions in the *mnd2* genotyping, a 12% native polyacrylamide gel system was used because its pore size is smaller than that of the agarose gel, allowing the discernment of 30 bp differences (Supplementary Figure 2). Generally, 1X TBE buffer is used as resolving and running buffer in polyacrylamide gel electrophoresis for DNA identification. Interestingly, in our gel electrophoresis system, the smiling effect disappeared when the running buffer was replaced with 1X Tris-Glycine SDS buffer, resulting in the distinct DNA bands (Supplementary Figure 2). The *Alu*I RE digesting products were resolved with a 12% polyacrylamide gel in 1X Tris-Glycine SDS running buffer to identify the genotype of the mice (Figure 4). In the case of WT, the 174-bp fragment was generated by the *Alu*I RE digestion of the PCR product at the nucleotide position 1,164; whereas, in the case of *mnd2*, this *Alu*I RE site was destroyed due to the specific mutation, and thus generating the undigested 224-bp fragment. Additionally, the 52-bp band was used as a control for the *Alu*I RE digestion. Although HT has two chromosomes that include each allele of A and T (A/T), both 174- and 224-bp fragments were clearly detected by this analysis (Table 1). Also, the fragment difference was sufficiently distinct to resolve even in the 1-1.5% agarose gel. Therefore, the genotype of WT, HT, and *mnd2* mice was distinguishable more easily and accurately through this standard protocol of the PCR-*Alu*I-RFLP-based genotype analysis.

Table 1. Summary of *Alu*I PCR-RFLP using new primer set (refer to Figure 4A)

New primer	WT	HT	*mnd2*
Sequence in alleles	+ allele AG↓CT	*mnd2* allele T[a]GCT	*mnd2* allele TGCT
	+ allele AG↓CT	+ allele AG↓CT	*mnd2* allele TGCT
No. of *Alu*I fragments	3	4	2
Fragment length (bp)	174	174	
	52	52	52
	50	50	
		224	224

[a]Underline indicates the allelic substitution determining the absence or presence of the *Alu*I RE site.

CONCLUSION

The present study provides a simple and rapid *mnd2* genotyp-

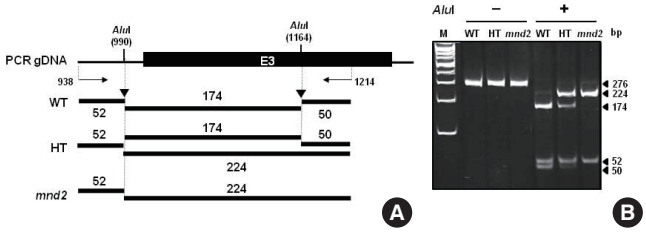


Figure 4. Output of genotype analysis of the *mnd2* mice under optimized conditions.
(A) Schematic diagrams of *Alu*I-RFLP fragments of mice with different alleles. In WT and *mnd2*, single bar was depicted due to the same allele in the two sets of chromosome. Arrowhead indicates the cleavage site of *Alu*I restriction enzyme. (B) An optimal gel electrophoresis pattern of three different genotypes.

ing protocol for scientists, who want to use the *mnd2* mouse model for understanding the *in vivo* roles of HtrA2 as well as the pathogenesis of important human diseases-associated with mitochondria dysfunction, such as neurodegenerative diseases, cancer and diabetes. Furthermore, the two sections on web- and experiment-based genotype analyses are provided as an easy-to-follow guideline and standard protocol to allow application to other studies using different mouse models.

MATERIALS AND METHODS

Animals

All mice used were approved by the institutional Animal Care and Use Committee at the College of Medicine, the Catholic University of Korea (IACUC No.: CUMC-2010-0145-01). Mice were bred and maintained in specific pathogen-free facility according to the 'Guide for the Care and Use of Laboratory Animals (NIH publications No. 85-23)'.

Genomic DNA extraction

A piece of tissue (-0.2 cm diameter) was punched out of the earlap of P10 mouse using the ear puncher and forceps. Ear tissue was homogenized in 200 μL Tissue Lysis (TL) buffer (LaboPass, Korea) containing 2 mg/mL proteinase K (LaboPass, Korea) and incubated for 2 hr at 56°C. Genomic DNA was prepared using a Tissue mini genomic DNA purification kit (LaboPass, Korea).

PCR-RFLP (Restriction fragment length polymorphism)

PCR reactions were set up in a PCR safeseal microcentrifuge tube (Sorenson Bioscience, USA) as follows: each 20 μL reaction contained 50 ng of genomic DNA, 0.2 mM dNTPs, 0.5 μM each of forward and reverse primers, 1X G-Taq buffer, 0.5 unit G-Taq polymerase (LaboPass, Korea). The initial step consisted of heating the reaction sample to 95°C for 3 min. The thermal cycle was followed by 35 cycles of 95°C for 30 sec (denaturation), 60°C for 30 sec (annealing), and 72°C for 30 sec (extension), and a final extension step at 72°C for 10 min using Takara PCR thermal cycler (Takara Bio Inc, Japan). Fifteen microliters of PCR products were digested with 0.2 unit of *Alu*I restriction enzyme for 3 hr at 37°C. The reaction samples were resolved by appropriate gel electrophoresis and run for 30 min at 120V. DNA bands were visualized by staining in 0.4 μg/mL ethidium bromide solution for 10 min under UV light.

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