Determination of the rdw Rat Genotype by Polymerase Chain Reaction with Allele-Specific Primer (PCR-ASP)

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Abstract: The rat carrying the rdw mutation (rdw rat) is a dwarf mutant with hypothyroidism that is caused by a single G to C transversion in the thyroglobulin gene. Therefore, the development of a simple method for molecular-based genotyping of this mutation has been problematic. We have developed a rapid and simple genotyping method that provides identification of both the rdw and wild-type allele. This polymerase chain reaction with an allele-specific primer (PCR-ASP) method amplifies only the specific allele, wild or mutant type, by using 3'-terminal mismatched primers that pair only with the respective alleles. This assay should be of value for rdw colony control and rapid discrimination of rdw/rdw, rdw/+ and +/+ rats.

Key words: genotyping, rdw rat, thyroglobulin

The rat carrying the rdw mutation (rdw rat) was discovered in a closed colony of Wistar-Imamichi rats as a hereditary dwarf, and was established as an inbred strain [1]. The mutation is autosomal recessive [1] and causes hypothyroidism [2]. The gene responsible for this mutation encodes thyroglobulin, which is a key molecule in the biosynthesis of thyroid hormone, and the mutation is a G to C single nucleotide change (G6958C), resulting in a G2320R missense mutation in the acetylcholinesterase-like domain [3, 4].

For the ordinary breeding of rdw rats, it is necessary to intercross heterozygous rats or cross heterozygous female rats with thyroxine-treated homozygous male rats [5], due to the infertility of rdw rats of both sexes. Therefore, genotyping by a molecular analytical method is indispensable for experiments on the rats before they show their phenotypes, such as characterization at pre- or post-natal stages and pre-implantation genetic diagnosis.

The rdw mutation creates a restriction enzyme MboI-sensitive site, which forms the basis of a previously developed method for molecular analysis [3]. Briefly, the region containing the rdw mutation is amplified by PCR, digested with MboI, and assayed by gel electrophoresis. Cleavage is indicative of the mutant allele, whereas wild-type alleles remain uncleaved. However, this PCR-RFLP (restriction fragment length polymorphism) assay can be problematic because failed or
partial MboI digestion can cause misidentification of a mutant allele as wild type. We therefore developed and report here a polymerase chain reaction with an allele-specific primer (PCR-ASP) method that detects the thyroglobulin mutation and cannot be confounded.

This assay amplifies only the specific allele (wild type or mutant) by using 3’-terminal mismatched primers that pair only with the respective allele.

Tail tips (~0.5 mm) were harvested and digested at 56°C in 10 mM Tris-HCl (pH 8.3), 50 mM NaCl, 2 mM EDTA, 0.4 µg/µl proteinase K, and 0.1% SDS for ~4 h, followed by phenol extraction. Nucleic acid, in the supernatant were precipitated by the addition of 1/10 volume of 5 M NaCl and 2.5 volumes of ethanol. Genomic DNA was spooled out on pipette tips and dissolved in distilled water. The forward primer (WF) was designed as a common primer complementary to the wild and rdw alleles (Fig. 1). Two reverse primers were designed to be allele-specific, one (WR) was specific for the wild-type and the other (rR) was specific for the rdw allele at the 3’ end (Fig. 1). Genomic DNA (50 ng) was subjected to PCR using the two sets of primers (WF × WR and WF × rR) in a total volume of 25 µl of buffer solution containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM dNTP mixture, each primer at 1 µM and 0.65 unit of Taq DNA polymerase (TAkara SHUZOU CO., LTD.). The reaction was performed for 35 cycles under the following conditions: 94°C (denaturation) for 30 s, and 72°C (annealing and extension) for 2.5 min. The PCR products were analyzed by electrophoresis on 1% agarose gels, and stained with ethidium bromide (Fig. 2).

Direct sequencing verified that the 2.4-kb bands that were specifically amplified (Fig. 2) contained a 2.2-2.3-kb intron. Amplification using the wild-type-specific primer (WR) was observed using DNA from wild-type and heterozygous rats (Fig. 2, lanes 1 and 3), while amplification using the rdw-specific primer (rR) was observed for DNA from heterozygous and rdw rats (Fig. 2, lanes 4 and 6). These two allele-specific primers only detect amplification of their complementary allele under the appropriate PCR conditions. To ascertain the accurate identification of several genotypes using this method, we carried out the direct sequencing of genomic DNA. Briefly, the genomic DNAs were used for PCR templates with forward primer 5’-GGCAAGAGAACTACTGTTGTAGTTAGCAGT-3’ and reverse primer 5’-GACCCCCAGTCTGTAGTTAGCAGT-3’ as described in Hishinuma et al. [3]. The amplified 240-bp bands were separated by electrophoresis on low melting point agarose gels followed by phenol extraction and ethanol precipitation. The pellets were suspended in distilled water and subjected to automated
sequencing using a standard protocol and an ABI3700 automated sequencer (Applied Biosystems Japan, Tokyo). The results of the direct genomic DNA sequencing were consistent with the genotypes determined by PCR-ASP (data not shown). These results indicate that the PCR-ASP method reported here can precisely distinguish the point mutation in rdw rats.

PCR-RFLP has been used as a molecular-based genotyping method for the thyroglobulin mutation in rdw rats, but has inherent problems such as the possibility of partial digestion by the restriction enzyme, complicated procedure and high cost. The PCR-ASP method for detection of the rdw mutant reported here does not require a restriction enzyme reaction, and consequently it is cheaper than PCR-RFLP and can save time. Because of its simplicity, this assay should be of value for routine genotyping of rdw rats.

References