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An Allele-Specific Genotyping Method for Rat *Lyst* (Lysosomal Trafficking Regulator) Gene

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Abstract: Two spontaneous mutant beige rats, with phenotypes resembling human Chediak-Higashi syndrome (CHS), were found independently in two inbred strains. Both beige mutations were identified to be recessive alleles in the *Lyst* locus on rat chromosome 17 and the alleles were denoted *Lyst*^{tg} and *Lyst*^{tg-Kyo}. As it is almost impossible to discriminate these mutations phenotypically, we developed an allele-specific genotyping method for the *Lyst* gene. The nested PCR amplification was followed by restriction fragment length polymorphism (RFLP) analysis. By this method, we could discriminate the mutant *Lyst*^{tg}, *Lyst*^{tg-Kyo} alleles, and the normal *Lyst* allele, easily and accurately.

Key words: *Lyst* alleles, nested PCR-RFLP analysis

Human Chediak-Higashi syndrome (CHS) is a rare autosomal recessive genetic disorder characterized by partial oculocutaneous albinism, mild predisposition to pyogenic infections, and giant granules in various cells [2, 3, 14]. Increased susceptibility of human CHS patients to infection may be explained by defects observed in T-cell cytotoxicity and natural killer (NK) cell activity as well as in chemotaxis and bacteriocidal capacity of granulocytes and monocytes [1, 5, 7, 15].

Two spontaneous beige rat mutations were found occurring independently. One was found in 1989 in an inbred colony of DA/Ham strain rats maintained at

Hamamatsu University School of Medicine [13]. Another was found in 1999 in an ACI/N rat colony maintained at the Institute of Laboratory Animals, Graduate School of Medicine, Kyoto University [10]. The genetic and phenotypic characteristics of these beige mutant rats were investigated and both mutants were regarded as useful animal models for human CHS. Both beige mutations resulted from a genomic deletion of about 20 kb containing exons 28, 29, and 30, which constitute the 578-bp sequence missing in the *Lyst* cDNA on rat chromosome (Chr) 17 [8, 9, 12]. Each deletion junction was formed by a recombination be-

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tween the different sites of the same two Long Interspersed Nucleolar Element 1s (L1) within the *Lyst* gene [10]. Accordingly, these *Lyst* mutations were demonstrated to be alleles of the *Lyst* locus and were denoted *Lyst*^{bg} and *Lyst*^{bg-Kyo}. However it is almost impossible to discriminate between the *Lyst*^{bg} allele and the *Lyst*^{bg-Kyo} allele phenotypically, except that each beige mutant comes from a different genetic background. We developed a method for discriminating the rat *Lyst* alleles and a simple genotyping method for the rat *Lyst* locus.

Firstly, female DA/Slc rats were mated with male DA/Ham-*Lyst*^{bg}/*Lyst*^{bg} homozygotes at 8 weeks of age and thirteen progenies were obtained in all. Similarly, female ACI/N rats were mated with male ACI/N-*Lyst*^{bg-Kyo}/*Lyst*^{bg-Kyo} homozygotes. Using standard techniques with phenol-CHCl₃, the genomic DNA was isolated from the tissue of a tailpiece taken out from each progeny and their parents.

Three pairs of primers Bg41C//Bg50, Bg39A4//Bg52A4, and Bg39A4nes//Bg52A4nes were synthesized for the genotyping of the *Lyst* locus (BEX CO., LTD). The Bg41C//Bg50 primer pair was designed based on the nucleotide sequence of the mouse, and rat *Lyst* cDNA was used for detecting the normal *Lyst* allele. Each primer sequence was as follows: Bg41C, 5'-CGGC TGCAGAGGCTCACCATCTTAGCTGTGAACA-3' (nucleotide positions 7885 to 7918 of GenBank AB020019 deposit), and Bg50, 5'-GAGGTCTT GCTTTGGGATGT-3' (nucleotide positions 7978 to 7997 of GenBank AB020019 deposit) [9, 11]. The PCR analysis by the Bg41C//Bg50 primer pair was carried out with initial incubation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 68°C for 4 min, with a final extension at 72°C for 7 min. The Bg39A4//Bg52A4 and the Bg39A4nes//Bg52A4nes primer pairs were used for detecting the mutant *Lyst*^{bg} and *Lyst*^{bg-Kyo} alleles. The Bg39A4//Bg52A4 primer pair was designed based on the cDNA sequence of the rat *Lyst* gene and each primer sequence was as follows: Bg39A4, 5'-CTACCACCATTCAGTGCCTCAGAA GAGGAGAAGCATTGC-3' (nucleotide positions 7701 to 7739 of GenBank AB020019 deposit), and Bg52A4, 5'-ATCAGCTCTGACAAATACAAAACCAACTT GGCTCC-3' (nucleotide positions 8323 to 8357 of GenBank AB020019 deposit) [16]. The Bg39A4nes//Bg52A4nes, which is the inner primer pair for the nested

Fig. 1. Partial nucleotide sequence of genomic DNA amplified with the Bg39A4//Bg52A4 primer pair and the positions of the primer pairs used for the genotyping of the *Lyst* gene. White boxes represent exons. The numbers on the upsides of the boxes indicate the nucleotide positions on the rat *Lyst* cDNA. The recombination sites of the *Lyst*^{bg} and the *Lyst*^{bg-Kyo} alleles are indicated by *b* and *K*, respectively.

PCR analysis, was designed as follows. Using genomic DNA isolated from the DA/Ham-*Lyst*^{bg}/*Lyst*^{bg} homozygote, PCR was carried out with the Bg39A4//Bg52A4 primer pair. As a result of the amplification, a DNA fragment of about 13 kb was obtained. We sequenced about 1 kb of both ends of the genomic DNA fragment by the Dye terminator method from BEX CO., LTD (Fig. 1). The Bg39A4nes inner primer was designed within one of the sequenced regions and the Bg52A4nes inner primer was within the other sequenced region and each primer sequence was as follows: Bg39A4nes, 5'-CCATGTAGGAGCTGTGGCAGCAATCAAGGTAGAC-3' and Bg52A4nes, 5'-AAATCCCGTGGAAAGGGAGA GCTAGACCTTCAGAG-3'. For detecting both mutant alleles, the first round of PCR amplification by the Bg39A4//Bg52A4 primer pair was carried out with initial incubation at 94°C for 1 min, followed by 25 cycles of denaturation at 94°C for 30 s, annealing and extension at 68°C for 18 min with a final extension at 72°C for 10 min according to the manufacturer's instruction

of LA *Taq* (TAKARA SHUZO CO., LTD). Each first PCR product was diluted one-tenth with distilled water and was used as a template in the subsequent round of the nested PCR. The second PCR amplification by the Bg39A4nes//Bg52A4nes inner primer pair was carried out with the same conditions of the first PCR analysis. The PCR products were electrophoresed on 1% agarose gels (SeaPlaque GTG Agarose, Bio Whittaker Molecular Application, Inc.) in 1 × TAE buffer and were stained with ethidium bromide.

Using PCR analysis by the Bg41C//Bg50 primer pair, a DNA fragment of about 3.5 kb was amplified from ACI/N-*Lyst*^{bg-Kyo/+} heterozygotes, DA-*Lyst*^{bg/+} heterozygotes and their wild-type inbred strains (Fig. 2A), but this targeted PCR product was not obtained from ACI/N-*Lyst*^{bg-Kyo/Lyst}^{bg-Kyo} homozygotes and DA/Ham-*Lyst*^{bg/Lyst}^{bg} homozygotes. On the other hand, the nested PCR analysis by the Bg39A4//Bg52A4 and the Bg39A4nes//Bg52A4nes primer pairs amplified about 11 kb of the DNA fragment in ACI/N-*Lyst*^{bg-Kyo/+} heterozygotes, DA-*Lyst*^{bg/+} heterozygotes and their mutant homozygotes (Fig. 2B), while this targeted PCR product was not obtained from their normal wild-type inbred strains. On electrophoresis using agarose gel, however, the 11 kb DNA fragment amplified from the *Lyst*^{bg} allele was indistinguishable from that amplified from the *Lyst*^{bg-Kyo} allele. The nested PCR product of each mutant allele was therefore digested with restriction endonucleases according to the respective manufacturer's instructions and each individual digest was electrophoresed on 1% agarose gel. The restriction endonucleases used for the RFLP analysis were as follows: *Bam*HI, *Bgl*II, *Bse*DI, *Bss*HII, *Dde*I, *Eco*RI, *Eco*RV, *Mbo*I, *Msp*I, *Sal*I, *Stu*I, *Taq*I, *Tru*9I, *Tsp*45I, and *Xba*I. Only the electrophoretic pattern of the *Stu*I digests could discriminate between the *Lyst*^{bg} allele and the *Lyst*^{bg-Kyo} allele. In addition to two DNA fragment of about 5.5 kb and 0.3 kb, which were equally observed in both mutant alleles, a DNA fragment of about 5 kb observed in the *Lyst*^{bg-Kyo} allele was divided into two DNA fragments of about 4 kb, and 0.7 kb in the *Lyst*^{bg} allele (Fig. 2B).

In this study, we designed several primers for the genetic analysis of the *Lyst* locus [4]. None of the primers designed on the L1 sequences amplified the DNA fragments specific to the mutant alleles because L1 is highly repeated and dispersed in the mammalian

Fig. 2. Electrophoretic patterns obtained by nested PCR-RFLP analysis for discriminating rat *Lyst* alleles. (A) PCR products obtained by the Bg41C//Bg50 primer pair. Lane 1: ACI/N. Lane 2: ACI/N-*Lyst*^{bg-Kyo/+} heterozygote. Lane 3: ACI/N-*Lyst*^{bg-Kyo/Lyst}^{bg-Kyo} homozygote. Lane 4: DA/Slc. Lane 5: DA-*Lyst*^{bg/+} heterozygote. Lane 6: DA-*Lyst*^{bg/Lyst}^{bg} homozygote. (B) Nested PCR products of the Bg39A4//Bg52A4 and Bg39A4nes//Bg52A4nes primer pairs (Lane 1-6) and the nested PCR products digested with *Stu*I (Lane 7-10). Lane 1: ACI/N. Lane 2, 7: ACI/N-*Lyst*^{bg-Kyo/+} heterozygote. Lane 3, 8: ACI/N-*Lyst*^{bg-Kyo/Lyst}^{bg-Kyo} homozygote. Lane 4: DA/Slc. Lane 5, 9: DA-*Lyst*^{bg/+} heterozygote. Lane 6, 10: DA-*Lyst*^{bg/Lyst}^{bg} homozygote. Lane M1: Lambda *Hind* III DNA size marker. Lane M2: 1kb DNA Ladder size marker.

genome [6]. A Bg41C//Bg50 primer pair amplified the specific PCR product only in the normal *Lyst* allele, since the Bg41C primer sequence designed on exon 28 and the Bg50 primer sequence on exon 29 are in the deleted regions of both the mutant alleles. However, the primer pairs for the mutant alleles did not amplify

the targeted PCR products in the normal *Lyst* allele owing to the long size of the fragment sequence. The position of the Bg39A4 primer is over 30 kb distant from the position of the Bg52A4 primer in the genomic sequence of the non-deleted normal *Lyst* allele which is the same as that of the Bg39A4nes//Bg52A4nes inner primer pair.

The PCR analysis using the Bg39A4//Bg52A4 primer pair did not show sufficient sensitivity and reproducibility for the detection of the mutant alleles in the *Lyst*^{bg-Kyo/+} and the *Lyst*^{bg/+} heterozygotes. However, nested PCR analysis made it possible to detect both the beige mutant alleles in the *Lyst* locus with high sensitivity, reproducibility and specificity. Moreover, combining RFLP with *StuI* digestion made it possible to accurately discriminate among the three alleles on the *Lyst* locus. The nested PCR-RFLP analysis used in this study will be useful for developing and maintaining congenic strains with the *Lyst*^{bg} gene and/or the *Lyst*^{bg-Kyo} gene.

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