Integration of a transposon into the Gli3 gene in the Pdn mouse

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ABSTRACT The phenotype of the genetic polydactyly/arhinencephaly mouse (Pdn/Pdn) is similar to Greig cephalopolysyndactyly syndrome (GCPS), whose responsible gene is GLI3. Suppression of Gli3 gene expression has been observed in the Pdn/Pdn and integration of retrotransposon in Gli3 gene in the Pdn mouse has been reported. Thus, the responsible gene for Pdn/Pdn is thought to be Gli3, but the site of mutation within the gene has not been demarcated.

In the present study, we demonstrated that 5442 bp of early retrotransposon was inserted into intron 3 of Gli3 gene in the Pdn mouse (Gli3Pdn). This transposon had almost the same sequence as MMY17106 (EMBL). It had 317-bp long terminal repeat at both ends followed by the identical 6-bp target duplication sequence, GAGACT. Forward and reverse PCR primers were constructed in intron 3 near the insertion point, and a forward primer in the transposon was also constructed. These primers allowed us to discriminate +/+, Pdn+/ and Pdn/Pdn embryos by the PCR products. Morphological determination of the genotypes in the Pdn mouse embryos is impossible before day 12 of gestation. Quick discrimination method of genotypes developed in the present study allows us to investigate the early dysmorphogenetic mechanisms in the brain and limbs in the Pdn/Pdn embryos. Then, the dysmorphogenetic mechanisms in the Pdn/Pdn may be extrapolated to those in GCPS.

Key Words: Gli3, Pdn, transposon, arhinencephaly, polydactyly

INTRODUCTION

Xt/Xt (Extra-toes) (Johnson, 1967) has been clarified as a mouse homologue of the human Greig cephalopolysyndactyly syndrome (GCPS) (Winter and Huson 1988; Vortkamp et al., 1992). Children with GCPS show postaxial polydactyly and broad thumbs of the hands, and preaxial polydactyly of the feet with syndactyly, as well as craniofacial defects that include a broad nasal root, macrocephaly and hydrocephalus (Greig, 1926). The genes responsible for these syndromes are Gli3 for the Xt and Xt mice (Schimmang et al., 1992; Hui and Joyner, 1993; Maynard et al., 2002) and GLI3 for GCPS (Vortkamp et al., 1991) respectively. GLI3 and Gli3 genes have been described as members of a family that are related to the Drosophila segmentation genes of the gap class (Ruppert et al., 1988; Hui et al., 1994). This family has been proposed to play a role in embryonic development and tissue-specific differentiation (Ruppert et al., 1988; Schimmang et al., 1992). Gli3 protein has been shown to be a sequence-specific DNA binding protein (Kinzler and Vogelstein, 1990; Ruppert et al., 1990).

Pdn/Pdn newborns show preaxial polydactyly (Naruse and Kameyama, 1982) and exhibit various brain malformations (Naruse et al., 1990). It has been strongly suggested that Pdn is an allele of Xt from their limb and brain phenotypes and linkage test (Hayasaka et al., 1980; Schimmang et al., 1994; Naruse et al., 2001). Suppression of Gli3 gene expression has been demonstrated in Pdn/Pdn mice (Naruse and Keino, 1995; Naruse et al., 2000). The sequence of the mouse Gli3 cDNA was reported by Thien et al. (1996), but we could not find any alterations of the Gli3 cDNA in the Pdn mouse. Meanwhile, Rüther’s group searched and found an integration of transposon in the Gli3 of the Pdn mouse (Thien and Rüther, 1999).

Recently, the Gli3 genomic DNA sequence database including some unknown nucleotides was published by NCBI Project (NW_000072). In the present study, we determined the insertion point of the transposon into the Gli3 gene in the Pdn mouse using this database, and investigated the structure of the transposon. We designed the primers within the intron 3 and the transposon to develop a quick method to discriminate embryonic genotypes, +/+, Pdn+/and Pdn/Pdn. After the genotyping of the Pdn mouse embryos using this method,
we can analyze the dysmorphogenetic mechanisms of the brain and limbs in the Pdn/Pdn young embryos.

MATERIALS AND METHODS

All experiments were performed in compliance with "Guidelines for Animal Experimentation in Faculty of Medicine, Tottori University" under the "International Guiding Principles for Biomedical Research Involving Animals".

Mice

The Pdn (Polydactylly Nagoya) mouse (Gli3<sup>+/−</sup>) was derived from C3H/ICR (Hayasaka et al., 1980) and has been inbred in Naruse's laboratory, now at 114 generation. Pdn+/− has one extra digit of the distal phalangeal type preaxially in the hindlimb and a deformity of the distal phalanx of the 1st digit in the forelimb, but no other abnormalities (Naruse and Kameyama, 1982). Most importantly, the brain of Pdn+/− is regarded as normal (Naruse et al., 1990). Pdn/Pdn mice were obtained from matings of Pdn+/− x Pdn−/−. Pdn/Pdn exhibits preaxial polydactyly of the duplicated or triplicated metacarpal/metatarsal type both in the fore- and hindlimbs. Pdn/Pdn newborns exhibit various brain malformations including absent olfactory bulbs and hydrocephalus (Naruse et al., 1990; 2002) and they die soon after birth because of sucking dysfunction (Hongo et al., 2000).

Genetic analysis

Oligonucleotide primers were obtained from GENSET KK (Kyoto, Japan). The sequences of the oligonucleotides used in LA-PCR, PCR and RT-PCR are listed in Table 1. Mouse genomic DNA was prepared from whole embryos with proteinase K treatment and phenol/chloroform extraction, according to the standard procedures. LA-PCR amplification of genomic DNA was performed using "enzyme mix" from Expand™ Long Template PCR System (Roche Diagnostics GmbH, Mannheim, Germany) and TaKaRa LA-PCR in vitro Cloning Kit (TaKaRa, Kusatsu, Japan). Some PCR products were subcloned into the pGEM-T vector (Promega Co., Madison, WI, USA). Sequencing of this product was done with the BigDye Terminator v3.0 Cycle Sequencing Ready Reaction Kit according to the manufacturer's instructions (Applied Biosystems, Tokyo, Japan). The sequencing samples were analyzed by an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Tokyo, Japan). The sequence data were analyzed using GENETYX software (Software Development, Tokyo, Japan). These data were referred to nucleotide BLAST of NCBI using the BLASTn algorithm (Altschul et al., 1990).

For the quick determination of genotype of the Pdn mouse embryo, genomic DNA was extracted from the yolk sac membrane of the embryo on day 10 of gestation.

RNA isolation and competitive RT-PCR

Total RNA was isolated from the Pdn mouse embryos on day 10 of gestation using ISOGEN (Nippon Gene, Tokyo, Japan) for the analysis of Gli3 gene expression. The cDNA was synthesized from 3 μg of total RNA that was treated with deoxyribonuclease (Nippon Gene, Tokyo, Japan), M-MLV reverse transcriptase (Gibco BRL, Rockville, USA) and ribonuclease inhibitor (Wako, Tokyo, Japan), using Random Hexamers (Promega Co., Madison, WI, USA). 0.4 μl of the RT reaction was used as a template for PCR. Gli3 gene expression was analyzed with competitive RT-PCR method (Ho et al., 1989). Competitors toward Gli3 and β-actin, which have the same primer binding sites as the target but gave slightly smaller products, were prepared by PCR mutagenesis. These competitors had 10 bp deletion for target PCR products of Gli3 and β-actin genes. The method has been described previously (Ueta et al., 2001). In brief, the PCR reaction solution contained 0.4 μl of cDNA template, 1 μl of DNA competitor, 20 pmol of each primer, 1 μl of 10 × PCR buffer, 5 μl of AmpliTaq Gold (Applied Biosystems, Tokyo, Japan) and 7.4 μl of DDW in a total volume of 10 μl. Relative quantity of the PCR products was analyzed on an ALFRED DNA sequencer (Amersham Pharmacia Biotech, Tokyo, Japan). Gli3 gene expression levels gained with competitive RT-PCR were divided by β-actin gene expression levels also gained with competitive RT-PCR as the internal control.

RESULTS

From the DNA sequence and exon-intron organization of the human Gli3 gene (Wild et al., 1997) and the primer sequences in the Gli3 gene reported by Thien and Rüther (1999), we speculated that a transposon was inserted into intron 3 of the Gli3 gene in the Pdn mouse. Primer sets were designed in exon 3 and 4 for LA-PCR using genomic DNA from wild type of Pdn mouse newborns, and an 11 Kb product was obtained. A 1.8 Kb product in the downstream of exon 3 was obtained by TaKaRa LA-PCR in vitro Cloning Kit. After cloning into pGEM-T vector, 10 and 1.8 Kb of these products were sequenced. These products were the parts of intron 3. These DNA sequence data were analyzed using nucleotide BLAST in the Mouse Genome, and matched to the DNA sequence from the database of NCBI Annotation Project submitted by NCBI, NIH, Bethesda, USA (accession number NW_000072). NW_000072 included the entire coding sequence of exon 3, intron 3 and exon 4 of Gli3 gene.

Exon-intron organization was investigated in the database of NW_000072, and it was determined that the length of intron 3 was 65.8 Kb (Fig. 1A). Intron 3 was separated to 8 regions, A-H, and primer sets were designed to overlap 8 regions. And a LA-PCR method was used in the genomic DNA from +/+ and Pdn/Pdn newborns. Products from the 8 regions were obtained, and the length of the product in F region
was different between +/+ and Pdn+/Pdn (Fig. 1B). Primer set of F1 and R2 was designed again in the F region (F'), and a 4.5 Kb product was obtained from +/+, but 10 Kb product was obtained from Pdn+/Pdn (Fig. 1B), suggesting that about 5.5 Kb transposon was inserted in the F' region. After subcloning the 5.5 and 10 Kb products into pGEM-T vector, the full sequences including transposon were determined. From this sequencing, it was determined that the transposon was inserted into the position 41286/41287 of intron 3 and its length was 5442 bp including 317 bp long terminal repeats (LTR) at both ends followed by the identical 6-bp target duplication sequence, GAGACT (Fig. 1C). This transposon had almost the same sequence as retrotransposon MMY17106 reported by Hofmann et al. (1998).

To develop the genotyping method for the Pdn mouse, primers were designed in intron 3 and the transposon. A 1.8 Kb product was observed with F2 and R3 in +/+ and Pdn+/+, but a 7.4 Kb was observed in Pdn+/Pdn. A 1.8 Kb product was observed with F2 and etn1R in Pdn+/+ and Pdn/Pdn, but not in +/+ . A 5.4 Kb product was observed with etn1F and R3 in Pdn+/ and Pdn/Pdn, but not in +/+ (Fig. 1C).

For quick determination of the genotype, F5 and R6 primers in intron 3, and etn1F in the transposon were designed. As shown in Fig. 2, only the 214 bp product with F5 and R6 was observed in +/+ . The 214 bp product with F5 and R6, and the 180 bp product with etn1F and R6 were observed in Pdn+/+. Only the 180 bp product with etn1F and R6 was observed in Pdn/Pdn. Thus, these primers provide a quick genotyping method for the Pdn mouse embryos.

After determination of the genotype using genomic DNA
from yolk sac membrane, \textit{Gli3} gene expression in the \textit{Pdn} mouse embryo was analyzed by competitive RT-PCR method on day 10 of gestation. The forward primer spanned with the exon 2 and 3, and reverse primer in exon 4 were designed for RT-PCR (Table 1). \textit{Gli3} gene expression in \textit{Pdn/+} was suppressed to about 60\% of +/-, and that in \textit{Pdn/Pdn} was about 20\% of +/- on day 10 of gestation (Fig. 3).

**DISCUSSION**

Integration of transposon sometimes induces mutations in mammals, for example, autoimmune LPR mice (Kobayashi \textit{et al.}, 1993), nude and albino mouse SELH/Bc (Hofmann \textit{et al.}, 1998) and black tremor hamster (Kuramoto \textit{et al.}, 2002). In the present study, the insertion point of a transposon into the \textit{Gli3} gene in the \textit{Pdn} mouse was clarified. The transposon in the \textit{Pdn} mouse was not novel, and was almost the same as the Early Retrotransposon (MMY17106) reported by Hofmann \textit{et al.} (1998). This transposon included a 317 bp long terminal repeat in both ends followed by the identical 6-bp target duplication sequence, GAGACT. This GAGACT was identical to the sequence at position 41281-41286 of intron 3. This identical repeat sequence suggests the invasion system of the transposon into intron 3 of \textit{Gli3} gene (Kuramoto \textit{et al.}, 2002).

\textit{Gli3} gene mutation in the \textit{Pdn} mouse is not null. The phenotype of \textit{Pdn/Pdn} mouse is milder than \textit{Xr'/Xr'} (Naruse \textit{et al.}, 2001) which is null mutation (Hui and Joyner, 1993). It is important to examine how the transposon affects to the \textit{Gli3} transcript. Thien and Rüther (1999) reported that at least 5 species of \textit{Gli3} mRNAs were detectable in the \textit{Pdn/Pdn} mice.

Quick determination of genotypes allows us to investigate the early dysmorphogenetic mechanisms in the brain and limbs in the \textit{Pdn/Pdn} mouse embryos. Then, the dysmorphogenetic mechanisms in the \textit{Pdn/Pdn} may be extrapolated to those in GCPS.

**ACKNOWLEDGMENT**

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**REFERENCES**


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**Table 1** Primers used for the search of transposon in \textit{Gli3}

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**Fig. 3** Analysis of \textit{Gli3} gene expression among +/-, \textit{Pdn}/+ and \textit{Pdn/Pdn} embryos using competitive RT-PCR method after determination of genotypes as shown in Fig. 2 on day 10 of gestation. \textit{Gli3} gene expression levels are indicated as a ratio of \textit{\beta-actin} gene expression. Four embryos in each genotype were examined.

*: Significantly different from +/- by One-way ANOVA (P < 0.01). #: Significantly different from \textit{Pdn}/+ by One-way ANOVA (P < 0.01).

**Table 2** Primers used for the generation of \textit{Gli3} cDNA

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F: forward primer, R: reverse primer, etn: primers in the transposon.


