NOTE *Laboratory Animal Science*

Anatomic Modifications in the Enteric Nervous System of JF1 Mice with the Classic Piebald Mutation

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ABSTRACT. The Japanese Fancy Mouse 1 (JF1) has a characteristic coat color similar to a very old mutant, *piebald*. The mutation in JF1 and the classic piebald was previously thought to be the same recessive allele in the endothelin B receptor gene (*Ednrb*) according to the haplotype pattern, which is insufficient for this conclusion. In this study, we identified the same insertion of a retroposon-like element in intron 1 of the *Ednrb* gene in JF1 as in the classic *piebald* mutation by PCR. Further, we investigated whether the intestine shows neuronal intestinal malformations such as hypoganglionosis and immaturity of ganglion cells by histochemical staining. Though it has been assumed that the defect of neural crest-derived lineages is restricted to melanocytes in JF1, we found that the enteric innervation and neuronal density were impaired throughout the whole colon in JF1 mice.

KEY WORDS: *Ednrb* gene, hypoganglionosis, JF1 mice, *piebald* mutation.

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Piebald spotting (*s*) is an old classical mouse mutation that affects melanocytes originating from the neural crest during development [8]. Mice homozygous for this recessive mutation, the *s*/*s* animals, exhibit a pigmented and white-spotted coat caused by a lack of the melanin-producing melanocytes in the white areas [10, 12]. The *s* locus on mouse chromosome 14 encodes *Ednrb*, a G protein-coupled, seven-transmembrane domain receptor that recognizes a family of small peptides known as endothelins [7]. Japanese Fancy Mouse 1 (JF1), an inbred strain established at the National Institute of Genetics in Japan was purchased from a market in Denmark in 1987 [9]. JF1 mice show irregular black spots on the white coat, which resembles the classic *piebald* (*s*/*s*) mutation described in 1920 in the U.S.A. [4]. Genetic analysis employing microsatellite markers used for typing mouse subspecies led to the conclusion that JF1 is of the Asian species *Mus musculus molossinus* [9]. Further, the same haplotype patterns in single nucleotide polymorphisms of *Ednrb* were shown in JF1 and *s*/*s* strains, so they were thought to be derived from the same Asian fancy mice. In 2006, a retroposon-like element in intron 1 of the *Ednrb* gene possessing the canonical sequence of a polyadenylation signal and a splice acceptor site was found in *s*/*s* mice, which led to a reduced expression level of the the *Ednrb* mRNA responsible for the partial loss-of-function phenotype [13]. In this study, we designed the allele-specific primers that distinguished normal alleles from retroposon-inserted alleles in *Ednrb* gene, and confirmed the relation between the spotty phenotype of JF1 and the *s*/*s* mutation. The JF1 strain was obtained from the National Institute of Genetics, Mishima, Japan. C57BL/6J (B6) mice were purchased from Japan SLC, Inc. and used as the normal control. All research and experimental protocols adhered to the Regulations for the Care and Use of Laboratory Animals, Hokkaido University, and were approved by the President of Hokkaido University following to a review by the Institutional Animal Care and Use Committee (Approval ID: No. 110226). The primers used were ttgttgccaatgcaattgagttcagggg (F); gccaggcagtgggcactctggat (R-mu) and catgtgattacagacacttacaac (R-wt). PCR was performed for both alleles in the same tube on each sample concomitantly. The primer locations can be seen in Fig. 1A. The F and R-wt primers amplify the 225-base pair (bp) fragment of intron 1 of *Ednrb* in the B6 mouse. The R-mu primer locates in a retroposon-like element of the *s*/*s* mutant and amplifies 318-bp fragments if the JF1 mutation is identical to that of *s*/*s*. As shown in Fig. 1B, each band is amplified in B6, JF1 and F1 hybrid mice, respectively. We next compared the nucleotide sequence of the PCR fragment with the previous reported sequence (accession no. AB242436) and identified an insertion of the same retroposon. Thus, we concluded mutation of JF1 and the classic *piebald* allele are derived from the same origin. To confirm reduction of the transcription level of *Ednrb* mRNA similar to that in the *s*/*s* mutant, semiquantitative RT-PCR was carried out. Primers were designed for *Ednrb* (gaactccacgctgctaagaatcatctac, F; cagcttacacatctcagctccaaatgg, R) and the internal control, *Actb* (tgatggtgggaatgggtcag, F; gaaggctggaaaagagcctc, R). As expected, the expression level of *Ednrb* mRNA in the intestine was significantly reduced in JF1 mice compared with the level in B6 mice (Fig. 1C).

Null mutations of *Ednrb* in mouse and rat strains show severe aganglionic megacolon due to absence of ganglion

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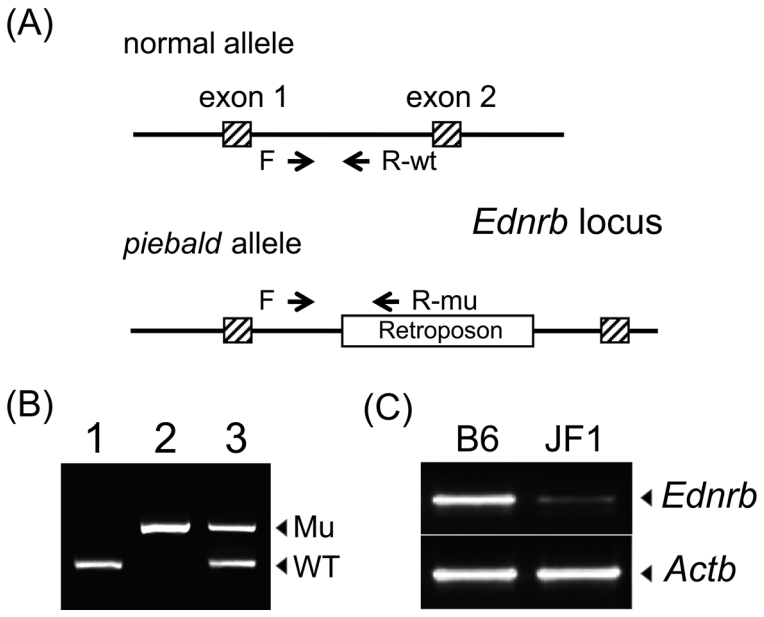

Fig. 1. Structure of the *piebald* (*s/s*) allele and detection of insertion of a retroposon-like element. (A) In the genomic structure of the *Ednrb* gene, exonic sequences that contribute to coding regions are boxed and shaded in stripes, while the 5.5-kp inserted sequence of retroposon are open-boxed. Arrows represent the primers used to identify the mutant and the normal alleles. (B) Genotyping results of the wild-type (WT) and mutant (Mu) alleles. The figure shows results for B6 (lane 1), JF1 (lane 2) and B6/JF1 F1 (lane 3). (C) Semiquantitative RT-PCR of *Ednrb* (175 bp) and *Actb* (668 bp) genes from the intestine of B6 and JF1.

cells in the intestine [2, 6, 7]. However, the *s/s* mice, in which the expression of *Ednrb* mRNA was decreased to about 25–27% of the corresponding wild-type levels, only showed pigmented defects without any megacolon symptoms [13]. It has been assumed that the defect of neural crest-derived lineages is restricted to melanocytes in JF1 mice. In this study, we investigated whether the intestine shows neuronal intestinal malformations such as hypoganglionosis and immaturity of ganglion cells in JF1 mice. To appraise the enteric innervation, we used acetylcholinesterase (AChE) whole-mount staining to visualize enteric neural fibers [5]. The guts from JF1 (n=8) and B6 (n=8) mice at 8 weeks of age, were dissected as a single piece from the proximal esophagus to the distal colon, and then processed for AChE staining. Four segments, each about 2–3 cm long, were taken from various parts of the bowel (proximal and distal intestine and proximal and distal colon) from each animal. Randomly chosen fields within four defined regions of the gut were imaged. The total number of meshes formed by fiber bundles present in each image was counted as a parameter of enteric innervation (Fig. 2A). The results are presented as means ± SD. In control B6 mice, the mean value of the parameter of enteric innervation in the distal colon was 187.75 (Fig. 2B), whereas the mean value was approximately half that in JF1 mice. There was a slight but significant decrease in the amount of enteric innervation in the proximal colon in JF1 mice compared with B6 mice ($P<0.01$; Fig. 2B). We also used histochemical staining for NADPH diaphorase to stain the cell body of the enteric neuron and examine the change in neuronal density according to a previous report [11]. As shown in Fig. 3A and 3B, the NADPH-positive neuron density showed a significant decline from the proximal to distal colon in JF1 mice (JF1, n=8, B6, n=8). These results showed that the *s* mutation also caused the decline in enteric innervation and neuronal density in the region of the colon in JF1 mice, which is consistent with another severe allele, *Ednrb*$^{s/}$ mice. It has been reported that *Ednrb*$^{s/}$ mice lacked the enteric ganglia in the distal portion of the colon because the *Ednrb* mRNA was not expressed [7].

In a previous study, we reported that there are resistant loci to aganglionosis in the genetic background of the F344 strain [3]. Despite the decrease in the expression level of the *Ednrb* gene and hypoganglionosis, JF1 mice do not show any megacolon symptoms (n=150). Interestingly, approximately 3% of the F_2 (JF1 × B6) mice exhibited severe megacolon (data not shown). The results suggested that JF1 mice have also modifier genes to aganglionosis. Several genes have been implicated in the development of human Hirschsprung Disease (HD), including *RET* (OMIM no. 164761), *EDN3* (131242), *GDNF* (600837) and *SOX10* (602229), *ZFHX1B* (605802), *PHOX2B* (603851) and *KIAA1279* (609460). Mutations in these genes explain only a minority of cases, and the vast majority of HD heritability remains

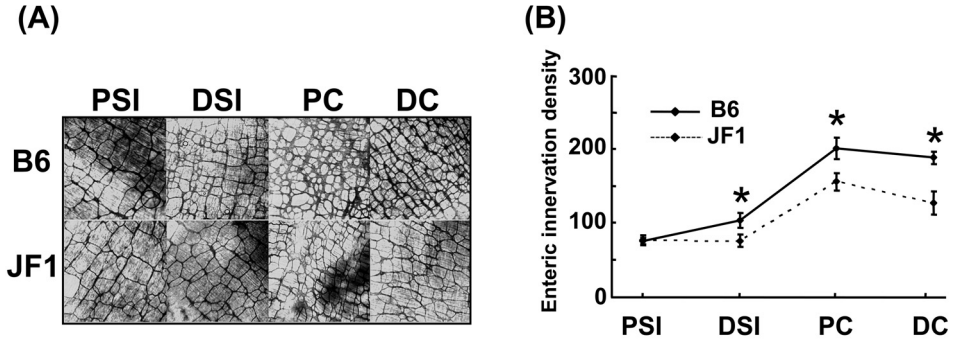

Fig. 2. (A) Whole mount micrograph showing the proximal small intestine to distal colon of JF1 (lower panel) and B6 mice (upper panel) stained for AChE. A sparse network of myenteric ganglia was observed in four parts of the bowel in a JF1 mouse compared with a B6 mouse. PSI, proximal small intestine; DSI, distal small intestine; PC, proximal colon; DC, distal colon. (B) Graphical representation of the relative changes in the density of enteric neural fiber throughout the whole intestine in B6 and JF1 mice. The innervation density in the DSI, PC and DC of B6 mice was significantly higher than that of JF1 mice. A *t*-test was used to compare the mean values for data sets. *, $P<0.01$.

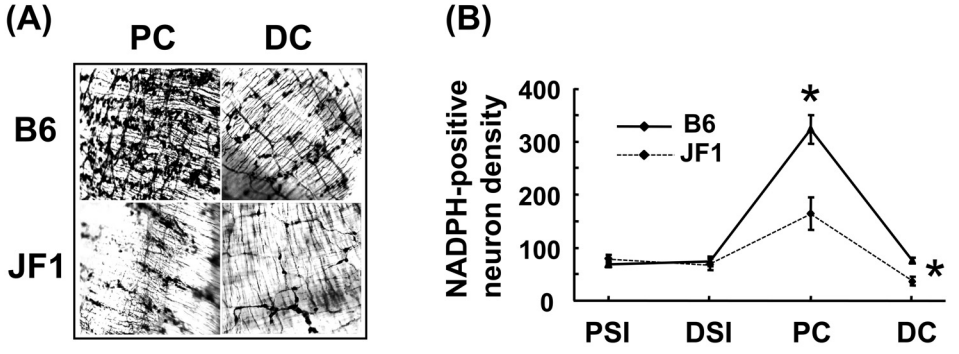

Fig. 3. (A) Whole mount micrograph showing the PSI to DC of JF1 (lower panel) and B6 (upper panel) stained for NADPH. Sparse NADPH-positive neurons were observed in two parts of the bowel (PC and DC) in a JF1 mouse compared with a B6 mouse. (B) Graphical representation of the relative changes in the density of NADPH-positive neurons throughout the whole intestine in B6 and JF1 mice. The neuronal density in the PC and DC of B6 mice was significantly higher than that of JF1 mice. A *t*-test was used to compare the mean values for data sets. *, $P<0.01$.

unknown. Thus, most forms of HD appear sporadically, and the incomplete penetrance and the variable expression observed in familial cases of HD demonstrate that HD is a genetically heterogeneous disease [1]. Therefore, identification of the modifier loci in JF1 mice will provide important new information regarding gene interactions controlling the development of the enteric nervous system.

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