Piebald mutation on a C57BL/6J background

Sanae FUKUSHIMA1), Kimie NIIMI1) and Eiki TAKAHASHI1)*

1) Research Resources Center, RIKEN Brain Science Institute, Saitama 351-0198, Japan

(Received 7 August 2014/Accepted 2 October 2014/Published online in J-STAGE 20 October 2014)

ABSTRACT: The classic piebald mutation in the endothelin receptor type B (Ednrb) gene was found on rolling Nagoya genetic background (PROD-s/s) mice with white coat spotting. To examine whether genetic background influenced the phenotype in the piebald mutant mice, we generated a congenic strain (B6.PROD-s/s), produced by repeated backcrosses to the C57BL/6J (B6) strain. Although B6.PROD-s/s mice showed white coat spotting, 7% of B6.PROD-s/s mice died between 2 and 5 weeks after birth due to megacolon. The PROD-s/s, s/s and Japanese fancy mouse 1 (JF1) strains, which also have piebald mutations on different genetic backgrounds with B6, showed only pigmentation defects without megacolon. In expression analyses, rectums of B6.PROD-s/s with megacolon mice showed ~5% of the level of Ednrb gene expression versus B6 mice. In histological analyses, aganglionosis was detected in the rectum of megacolon animals. The aganglionic rectum was thought to lead to severe constipation and intestinal blockage, resulting in megacolon. We also observed an abnormal intestinal flora, including a marked increase in Bacteroidaceae and Erysipelotrichaceae and a marked decrease in Lactobacillus and Clostridiales, likely inducing endotoxin production and a failure of the mucosal barrier system, leading ultimately to death. These results indicate that the genetic background plays a key role in the development of enteric ganglion neurons, controlled by the Ednrb gene, and that B6 has modifier gene(s) regarding aganglionosis.

KEY WORDS: aganglionic rectum, Ednrb gene, genetic background, piebald mutation

doi: 10.1292/jvms.14-0408; J. Vet. Med. Sci. 77(2): 161–166, 2015

The autosomal recessive ataxic rolling Nagoya strain (PROD-rol/rol) mice, which exhibit white spots on an agouti coat, were found among descendants of a cross between the SII and C57BL/6Nga strains and have been maintained by intercross mating [16]. In our previous study, PROD-rol/rol was shown to be a double-mutant strain with an amino acid change at R1262G in the Cacna1a gene on chromosome 8, causing neuronal Ca\(_{2+}\)-dependent channel dysfunction, and the piebald mutation in the endothelin receptor type B (Ednrb) gene on chromosome 14, causing the coat pigment defect [23]. The PROD-rol/rol has a mutation in the voltage-sensing S4 segment of the third repeat in the Ca\(_{2+}\)-Ca\(_{2+}\) current amplitude exhibits a 40% reduction in PROD-rol/rol compared to wild-type rolling Nagoya (PROD-s/s) [14]. The PROD-s/s mice have no apparent abnormal behaviors [20, 21] and two silent nucleotide substitutions in the coding region and insertion of a retroposon-like element in intron 1 of the Ednrb gene [23]. The same mutation in the Ednrb gene was detected in the Japanese fancy mouse 1 (JF1) mice [4, 12, 13], derived from a Japanese wild strain, and in the s/s mice [7, 22], derived from laboratory strain with SSLE/Le background. They have a spotting defect without megacolon [4, 22].

In addition to the coat spotting, null mutations of the Ednrb gene in rats (Ednrb\(^{null}\) rats) [19] and mice (s/s mice) [9] cause megacolon owing to aganglionosis. Interestingly, genetic backgrounds strongly affect the penetrance and severity of aganglionosis in Ednrb\(^{null}\) rats. Thus, 90% of Ednrb\(^{null}\) rats with the LEH/Hk genetic background (LEH-Ednrb\(^{null}\) rats) showed aganglionosis [6]. However, about 40% of Ednrb\(^{null}\) rats with the F344 genetic background (F344-Ednrb\(^{null}\) rats) showed aganglionosis [6].

The Ednrb\(^{null}\) rats and s/s mice are known as an animal model for human Hirschsprung’s (HSCR) disease [6, 9]. HSCR disease shows aganglionosis and causes severe constipation and intestinal blockage, resulting in megacolon [1, 2, 8, 10]. Incomplete penetrance and inter-familial variation are commonly observed in HSCR disease [1, 17], suggesting that genetic background is an important factor in the development of HSCR disease.

However, it remains unknown whether megacolon phenotype is influenced by the genetic background of the piebald mutant mice. Thus, in this study, we examined whether genetic background change can lead to the occurrence of megacolon in the piebald mutant mice.

MATERIALS AND METHODS

Animals: The research was approved by the Animal Experiments Committee of RIKEN (Approved ID: No. H24-2-206). To generate a congenic strain having classic piebald mutation with C57BL/6J (B6) mice (Charles River Japan, Yokohama, Japan), PROD-s/s strain [16, 23], backcrossed to B6 for 12 generations, produced B6.PROD-s/s. The mice were allowed ad libitum access to water and food pellets (CRF-1; Oriental Yeast, Tokyo, Japan) and kept at room temperature (23 ± 1°C) and 55 ± 5% humidity under a 12/12-hr light/dark cycle (light from 8:00 am to 8:00 pm).
Microsatellite genotyping: To confirm the establishment of the congenic strain with a B6 background, microsatellite markers located on chromosome 14 (D14Mit30, D14Mit225, D14Mit93, D14Mit94, D14Mit170, D14Mit42 and D14Mit267) were typed using genomic DNA obtained from the tails of B6.PROD-s/s (male, female: n=8, 6), PROD-s/s (male, female: n=8, 6) and B6 (male, female: n=8, 7) mice. The polymerase chain reaction (PCR) protocols and primers of microsatellite markers were reported in the Mouse Microsatellite Data Base of Japan (MMDBJ, http://www.shigen.nih.ac.jp/mouse/mmdbj/).

Genomic and gene structure analysis: To distinguish normal alleles from those with the insertion of a retroposon-like element in intron 1 of the Ednrb gene (GenBank ID: AB242436.1), the PCR products were amplified with genomic DNA from the tails of B6.PROD-s/s (male, female: n=8, 6), PROD-s/s (male, female: n=8, 6) and B6 (male, female: n=8, 7) mice. The polymerase chain reaction (PCR) protocols and primers of microsatellite markers were reported in the Mouse Microsatellite Data Base of Japan (MMDBJ, http://www.shigen.nih.ac.jp/mouse/mmdbj/).

Total RNAs from the brains of B6.PROD-s/s (n=8), PROD-s/s (n=8) and B6 (n=8) male mice were isolated using the TRizol reagent (Invitrogen, Burlington, Canada). According to a previous study [23], the amplified fragments of Ednrb cDNA were sequenced.

Real-time quantitative RT-PCR (real-time qRT-PCR): The levels of Ednrb mRNA in the colon and rectum of B6.PROD-s/s with megacolon (n=8), B6.PROD-s/s (n=8) and B6 (n=8) male mice were measured using Applied Biosystems TaqMan Gene Expression Assays (Ednrb, Assay ID: Mm01224433_m1) and normalized relative to the 18S ribosomal RNA (Assay ID: Hs99999901_s1) as reported previously [23].

Histochemistry: The large intestine of B6.PROD-s/s with megacolon (n=5), B6.PROD-s/s (n=6) and B6 (n=5) male mice was dissected after perfusion with saline followed by 4% paraformaldehyde, fixed with tissue fixative (Gonostaff, Co., Ltd., Tokyo, Japan), embedded in paraffin wax and cut into sections of 6 µm for hematoxylin and eosin (H&E) staining and in situ hybridization (ISH). The protocol, including the probe sequence for the Cacna1a gene, was reported previously [18, 23].

Bacteriological analysis: The dilated region of the intestines from anesthetized B6.PROD-s/s with megacolon
Fig. 2. Phenotypes of B6.PROD-s/s with megacolon and B6.PROD-s/s mice. (A) Representative photographs showing white spots on agouti coat of B6.PROD-s/s with megacolon (upper, left) and B6.PROD-s/s (upper, center) mice are presented. Representative photographs of the open abdomen of mice are shown (middle). Representative photographs of the symptoms of aganglionosis of PROD-s/s with megacolon mice (lower, left). (B) The expression levels of Ednrb mRNA in the colon determined by real-time qRT-PCR. (C) The expression levels of Ednrb mRNA in the rectum determined by real-time qRT-PCR. The Ednrb mRNA expression level for each strain was calculated relative to that in B6 mice. **P < 0.01, *P < 0.05, vs. the appropriate control (Dunnett’s test).

Fig. 3. H&E staining and in situ hybridization. Representative photographs of H&E staining (upper) and in situ hybridization (lower) in the rectum are shown. Arrows indicate enteric nerve plexus (upper) and localization of Ca_{2,1}α_{1} mRNA (lower). The scale bar is 50 µm.
(n=7) and B6.PROD-s/s (n=14) male mice was removed aseptically, and the contents of the intestines were isolated. We used terminal restriction fragment length polymorphism (T-RFLP) analysis as reported previously [15] to examine the intestinal microflora.

**Statistical analysis:** The data are presented as the means ± standard error of the mean (SEM). Statistical analyses were conducted using Excel Statistics 2006 (SSRI, Tokyo, Japan). The data were analyzed using Dunnett’s test between groups where appropriate. In all analyses, P<0.05 was taken to indicate statistical significance.

**RESULTS**

*Generation of congenic strain:* A congenic strain (B6.PROD-s/s) was produced by repeated backcrosses to B6 for 12 generations with selection for white spotting as a marker from the PROD-s/s. Because the Ednrb gene is located on chromosome 14 in the mice, we typed microsatellite loci on chromosome 14 to confirm the changed genetic background. The location order and distances among the loci are presented in Fig. 1A. As shown in Fig. 1B, fragments were amplified from B6.PROD-s/s, PROD-s/s and B6 mice. According to MMDBJ, MGI and our previous report [23], different sizes between PROD and B6 strains are produced in D14Mit30 (PROD, B6: size=154 bp, 154 bp), D14Mit225 (PROD, B6: size=101 bp, 118 bp), D14Mit93 (PROD, B6: size=189 bp, 147 bp), D14Mit94 (PROD, B6: size=108 bp, 104 bp), D14Mit170 (PROD, B6: size=163 bp, 146 bp), D14Mit42 (PROD, B6: size=142 bp, 152 bp) and D14Mit267 (PROD, B6: size=114 bp, 114 bp) loci. The amplified fragment sizes were similar between B6.PROD-s/s and B6 mice in the D14Mit30, D14Mit225, D14Mit170, D14Mit42 and D14Mit267 (Fig. 1B) loci.

PCR analyses showed the insertion of a retropon-on-like element in intron 1 of the Ednrb gene of B6.PROD-s/s and PROD-s/s mice (wild type, mutant type: size=225 bp, 318 bp; Fig. 1C) and two silent nucleotide substitutions in the coding region (data not shown).

**Influence of genetic background on intestinal malformation:** The PROD-s/s and B6 showed no megacolon symptoms until 12 months after birth. However, ~7% (male: n=12, female: n=10) of B6.PROD-s/s (male: n=153, female: n=131) showed megacolon symptoms (B6.PROD-s/s with megacolon; Table 1) and died between 2 and 5 weeks after birth. The symptoms in B6.PROD-s/s mice (wild type, mutant type: size=225 bp, 318 bp) gene of B6.PROD-s/s and B6 mice. According to MMDBJ, MGI and our previous report [23], different sizes between PROD and B6 strains are produced in D14Mit30 (PROD, B6: size=154 bp, 154 bp), D14Mit225 (PROD, B6: size=101 bp, 118 bp), D14Mit93 (PROD, B6: size=189 bp, 147 bp), D14Mit94 (PROD, B6: size=108 bp, 104 bp), D14Mit170 (PROD, B6: size=163 bp, 146 bp), D14Mit42 (PROD, B6: size=142 bp, 152 bp) and D14Mit267 (PROD, B6: size=114 bp, 114 bp) loci. The amplified fragment sizes were similar between B6.PROD-s/s and B6 mice in the D14Mit30, D14Mit225, D14Mit170, D14Mit42 and D14Mit267 (Fig. 1B) loci.

**PCR analyses** showed the insertion of a retropon-on-like element in intron 1 of the Ednrb gene of B6.PROD-s/s and PROD-s/s mice (wild type, mutant type: size=225 bp, 318 bp; Fig. 1C) and two silent nucleotide substitutions in the coding region (data not shown).

**Influence of genetic background on intestinal malformation:** The PROD-s/s and B6 showed no megacolon symptoms until 12 months after birth. However, ~7% (male: n=12, female: n=10) of B6.PROD-s/s (male: n=153, female: n=131) showed megacolon symptoms (B6.PROD-s/s with megacolon; Table 1) and died between 2 and 5 weeks after birth. The symptoms in B6.PROD-s/s mice (wild type, mutant type: size=225 bp, 318 bp) gene of B6.PROD-s/s and B6 mice. According to MMDBJ, MGI and our previous report [23], different sizes between PROD and B6 strains are produced in D14Mit30 (PROD, B6: size=154 bp, 154 bp), D14Mit225 (PROD, B6: size=101 bp, 118 bp), D14Mit93 (PROD, B6: size=189 bp, 147 bp), D14Mit94 (PROD, B6: size=108 bp, 104 bp), D14Mit170 (PROD, B6: size=163 bp, 146 bp), D14Mit42 (PROD, B6: size=142 bp, 152 bp) and D14Mit267 (PROD, B6: size=114 bp, 114 bp) loci. The amplified fragment sizes were similar between B6.PROD-s/s and B6 mice in the D14Mit30, D14Mit225, D14Mit170, D14Mit42 and D14Mit267 (Fig. 1B) loci.

PCR analyses showed the insertion of a retropon-on-like element in intron 1 of the Ednrb gene of B6.PROD-s/s and PROD-s/s mice (wild type, mutant type: size=225 bp, 318 bp; Fig. 1C) and two silent nucleotide substitutions in the coding region (data not shown).

**Ednrb gene expression levels in the colon and rectum:** Real-time qRT-PCR analysis was performed to determine the levels of Ednrb mRNA in enteric neurons of B6.PROD-s/s with megacolon, B6.PROD-s/s and B6 mice. In the colon, B6.PROD-s/s with megacolon and B6.PROD-s/s showed about 75% of the level of Ednrb expression compared to control B6 mice (Fig. 2B). In the rectum, although the B6.PROD-s/s showed ~75% of the level of Ednrb expression versus the control B6 mice, B6.PROD-s/s with megacolon mice showed ~5% of the level of Ednrb gene expression versus the B6 mice (Fig. 2C).

**DISCUSSION**

Hirschspring’s (HSCR) disease is a congenital intestinal disease, characterized by the loss of ganglion cells in the intestinal tract [1, 2, 8, 10]. This disorder occurs in about 1/5,000 live births. Due to the lack of ganglia, the stool cannot be passed through the colon, resulting in megacolon. Several susceptibility genes have been identified for HSCR disease: the RET proto-oncogene, endothelin-3 (EDN3) gene, endothelin receptor B (EDNRB) gene, glial cell line derived neurotrophic factor (GDNF) gene and SRY-related HMG-box 10 (SOX10) gene [17]. These genes encode ligands, receptors and transcription factors and play important roles in the formation of the enteric nervous system [1, 2, 8, 10]. There are both incomplete penetrance and inter-familial variation in HSCR disease [1, 17], suggesting that genetic background or multiple molecular interactions are important in the development of HSCR disease.

The EDNRB is a G-protein-coupled seven-transmembrane receptor that interacts with a family of ligands known as the endothelins [11]. The piebald mutation has an insertion of a...
Mutations in the gene encoding the EDNRB cause three symptoms: aganglionosis, pigmented disorder and hearing loss [1, 2, 17]. In addition, the Ednrb null mice and rats show abnormal splenic microarchitecture with lymphopenia [3, 5]. In this study, we have not examined the phenotypes of hearing ability and lymphopenia in the B6.PROD-s/s mice. We will study them in the next study.

In considering the cause of death in the B6.PROD-s/s with megacolon, it is interesting to examine the condition of the intestinal microflora after severe intestinal blockage, because a defense system in the intestine is believed to affect the balance of intestinal bacteria. In B6.PROD-s/s with megacolon, we observed an abnormal composition of the intestinal bacteria, suggesting that they might induce excess harmful products (e.g., endotoxins) and/or cause failure of the mucosal barrier system, ultimately leading to death.

In conclusion, the present study has shown that genetic background contributes importantly to the phenotypes of piebald Ednrb gene mutation and that use of different strains may facilitate understanding of a complex disease, such as HSCR disease.

ACKNOWLEDGMENT. We thank Takuro Yoshimoto for technical assistance and for helpful comments.

REFERENCES

1. Amiel, J., Sproat-Emison, E., Garcia-Barcelo, M., Lantieri, F., Burzynski, G., Borrego, S., Pelet, A., Arnold, S., Miao, X., Griseri, P., Brooks, A. S., Antinolo, G., de Pontual, L., Clement-Ziza, M., Munnic, A., Kashuk, C., West, K., Wong, K. K., Lyonnet, S., Chakravarti, A., Tam, P. K., Ceccherini, I., Hofstra, R. M., Fernandez R., Hirschsprung Disease Consortium 2008. Hirschsprung disease, associated syndromes and genetics: a review. J. Med. Genet. 45: 1–14. [Medline] [CrossRef]

2. Borrego, S., Ruiz-Ferrer, M., Fernandez, R. M. and Antiholo, G. 2013. Hirschsprung’s disease as a model of complex genetic etiology. Histol. Histopathol. 28: 1117–1136. [Medline]

3. Cheng, Z., Wang, X., Dhall, D., Zhao, L., Bresce, C., Doherty, T. M. and Frykman, P. K. 2011. Splenic lymphopenia in the endothelin receptor B-null mouse: implications for Hirschsprung associated enterocolitis. Pediatr. Surg. Int. 27: 145–150. [Medline] [CrossRef]

4. Dang, R., Sasaki, N., Torigoe, D. and Agui, T. 2012. Anatomic modifications in the enteric nervous system of F1 mice with the classic piebald mutation. J. Vet. Med. Sci. 74: 391–394. [Medline] [CrossRef]

5. Dang, R., Sasaki, N., Nishino, T., Nakanishi, M., Torigoe, D. and...
Agui, T. 2012. Lymphopenia in Ednrb-deficient rat was strongly modified by genetic background. Biomed. Res. 33: 249–253. [Medline] [CrossRef]

Dang, R., Torigoe, D., Suzuki, S., Kikkawa, Y., Moritoh, K., Sasaki, N. and Agui, T. 2011. Genetic background strongly modifies the severity of symptoms of Hirschsprung disease, but not hearing loss in rats carrying Ednrb(sl) mutations. PLoS ONE 6: e24086. [Medline] [CrossRef]

Dunn, L. C. 1920. Independent Genes in Mice. Genetics 5: 344–361. [Medline]

Furness, J. B. 2012. The enteric nervous system and neurogastroenterology. Nat. Rev. Gastroenterol. Hepatol. 9: 286–294. [Medline]

Hosoda, K., Hammer, R. E., Richardson, J. A., Baynash, A. G., Cheung, J. C., Giaid, A. and Yanagisawa, M. 1994. Targeted and natural (piebald-lethal) mutations of endothelin-B receptor gene produce megacolon associated with spotted coat color in mice. Cell 79: 1267–1276. [Medline] [CrossRef]

Heanue, T. A. and Pachnis, V. 2007. Enteric nervous system development and Hirschsprung’s disease: advances in genetic and stem cell studies. Nat. Rev. Neurosci. 8: 466–479. [Medline] [CrossRef]

Horinouchi, T., Terada, K., Higashi, T. and Miwa, S. 2013. Endothelin receptor signaling: new insight into its regulatory mechanisms. J. Pharmacol. Sci. 123: 85–101. [Medline] [CrossRef]

Koide, T., Moriwaki, K., Uchida, K., Mita, A., Sagai, T., Yokemaka, H., Katoh, H., Miyashita, N., Tsuchiya, K., Nielsen, J. T. and Shiroishi, T. 1998. A new inbred strain JF1 established from Japanese fancy mouse carrying the classic piebald allele. Mamm. Genome 9: 15–19. [Medline] [CrossRef]

Kumagai, T., Wada, A., Tsuzuki, M., Nishimura, M. and Kunieda, T. 1998. Nucleotide sequence of endothelin-B receptor gene reveals origin of piebald mutation in laboratory mouse. Exp. Anim. 47: 265–269. [Medline] [CrossRef]

Mori, Y., Wakamori, M., Oda, S., Fletcher, C. F., Sekiguchi, N., Mori, E., Copeland, N. G., Jenkins, N. A., Matsushita, K., Matsuyama, Z. and Imoto, K. 2000. Reduced voltage sensitivity of activation of P/Q-type Ca2+ channels is associated with the ataxic mouse mutation rolling Nagoya (tg(rol)). J. Neurosci. 20: 5654–5662. [Medline]

Nagashima, K., Hisada, T., Sato, M. and Mochizuki, J. 2003. Application of new primer-enzyme combinations to terminal restriction fragment length polymorphism profiling of bacterial populations in human feces. Appl. Environ. Microbiol. 69: 1251–1262. [Medline] [CrossRef]

Oda, S. 1973. [The observation of rolling mouse Nagoya (rol), a new neurological mutant, and its maintenance (author’s transl)]. Jikken Dobutsu 22: 281–288. [Medline]

Pingault, V., Ente, D., Dastot-Le Moal, F., Goossens, M., Marlin, S. and Bondurand, N. 2010. Review and update of mutations causing Waardenburg syndrome. Hum. Mutat. 31: 391–406. [Medline] [CrossRef]

Sakuraoka, Y., Sawada, T., Shiraki, T., Park, K., Sakurai, Y., Tomosugi, N. and Kubota, K. 2012. Analysis of hepcidin expression: in situ hybridization and quantitative polymerase chain reaction from paraffin sections. World J. Gastroenterol. 18: 3727–3731. [Medline] [CrossRef]

Suzuki, T., Won, K. J., Horiuchi, K., Kinoshita, K., Hori, M., Torihashi, S., Momotani, E., Itoh, K., Hiromiya, K., Ward, S. M., Sanders, K. M. and Ozaki, H. 2004. Muscularis inflammation and the loss of interstitial cells of Cajal in the endothelin ETB receptor null rat. Am. J. Physiol. Gastrointest. Liver Physiol. 287: G638–G646. [Medline] [CrossRef]

Takahashi, E., Niimi, K. and Itakura, C. 2011. Role of Ca(V)2.1-mediated NMDA receptor signaling in the nucleus accumbens in spatial short-term memory. Behav. Brain Res. 218: 353–356. [Medline] [CrossRef]

Takahashi, E., Niimi, K. and Itakura, C. 2011. Emotional behavior in heterozygous rolling mouse Nagoya Ca v 2.1 channel mutant mice. Neurobiol. Aging 32: 486–496. [Medline] [CrossRef]

Yamada, T., Ohtani, S., Sakurai, T., Tsuji, T., Kunieda, T. and Yanagisawa, M. 2006. Reduced expression of the endothelin receptor type B gene in piebald mice caused by insertion of a retroposon-like element in intron 1. J. Biol. Chem. 281: 10799–10807. [Medline] [CrossRef]

Yoshimoto, T., Aoyama, Y., Kim, T. Y., Niimi, K., Takahashi, E. and Itakura, C. 2014. Rolling Nagoya mouse strain (PRODrol/rol) with classic piebald mutation. J. Vet. Med. Sci. 76: 1093–1098. [Medline] [CrossRef]