SCIENTIFIC REPORTS

OPEN

QTL analysis of modifiers for pigmentary disorder in rats carrying *Ednrb^sl* mutations

Jieping Huang[1,2,*], Ruihua Dang[2,*], Daisuke Torigoe[3,5], Anqi Li[2], Chuzhao Lei[2], Nobuya Sasaki[4], Jinxi Wang[3] & Takashi Agui[3]

Pigmentary variation in animals has been studied because of its application in genetics, evolution, and developmental biology. The large number of known color loci provides rich resource to elucidate the functional pigmentary system. Nonetheless, more color loci remain to be identified. In our previous study, we revealed that two different strains, namely, AGH rats and LEH rats, but which had the same null mutation of the *Ednrb* gene (*Ednrb^sl*) showed markedly different pigmented coat ratio. This result strongly suggested that the severity of pigment abnormality was modified by genetic factor(s) in each strain. To elucidate the modifier locus of pigment disorder, we carried out whole-genome scanning for quantitative trait loci (QTLs) on 149 F$_2$ (AGH-*Ednrb^sl* × LEH-*Ednrb^sl*) rats. A highly significant QTL, constituting 26% of the total pigmentation phenotype variance, was identified in a region around *D7Got23* on chromosome (Chr) 7. In addition, investigation on epistatic interaction revealed significant interactions between *D7Got23* and *D3Rat78* and between *D7Got23* and *D14Mit4*. Results suggested that a modified locus on Chr 7 was mainly responsible for the variance of pigmentary disorder between AGH-*Ednrb^sl* rats and LEH-*Ednrb^sl* rats, and two modifier loci showing epistatic interaction may, in part, influence pigment phenotype.

Genetic studies on coat color mutations in mammals have a long history in biomedical research because of their viable and visible phenotypes. Today, a wealth of information about the pathways and genes involved in the pigmentation has been revealed. Nearly 130 genes with approximately 1000 different alleles have been detected to affect coat color[1]. Early in the 19th century, coat color mutation was used to prove Mendel's laws[2]. Then, coat color mutations were used to generate different inbred lines of visible markers[3,4]. Melanin-based pigmentation is highly conserved across vertebrates[5]; thus, color mutations in mammals can provide models for some human diseases. A large number of diseases in humans are associated with pigmentary abnormalities, such as Waardenburg syndrome[6], Hirschsprung's disease[7], oculocutaneous albinism I[8], and piebaldism[9]. Moreover, the pigmentation system is a classical tool in ecological studies. Selective forces such as aposematism, crypsis, thermoregulation, and sexual signaling drive variation in the pigmentation pattern[10].

The endothelin3 (Edn3)/endothelin receptor B (Ednrb) ligand–receptor pair is involved in pigmentation[11,12]. *Ednrb*-deficient mice exhibit an almost completely white coat, and they develop megacolon[11]. *Ednrb^sl* is a spontaneous null mutation characterized by deletion of 301 bp in the *Ednrb* gene in rats, resulting in Hirschsprung's disease and pigmentary disorder[13]. In our previous study, we established two strains with different genetic background but carrying the same *Ednrb^sl* mutation, namely, AGH-*Ednrb^sl* and LEH-*Ednrb^sl*[14]; these two strains showed different pigment phenotype. AGH-*Ednrb^{sl/sl}* rats showed almost no pigmentation all over the body, whereas a large pigmented spot appeared on the head of LEH-*Ednrb^{sl/sl}* rats. Therefore, we hypothesized that modifier loci in the genetic background of LEH modulated the severity of the pigmentary disorder.

[1]College of Life Sciences, Northwest A&F University, Yangling, Shaanxi, China. [2]College of Animal Science and Technology, Northwest A&F University, Yangling, Shaanxi, China. [3]Laboratory of Laboratory Animal Science and Medicine, Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, Hokkaido, Japan. [4]Laboratory of Laboratory Animal Science and Medicine, School of Veterinary Medicine, Kitasato University, Aomori, Japan. [5]Division of Microbiology and Genetics, Center for Animal Resources and Development, Kumamoto, Japan. *These authors contributed equally to this work. Correspondence and requests for materials should be addressed to R.D. (email: dangruihua@nwsuaf.edu.cn) or C.L. (email: leichuzhao1118@126.com)
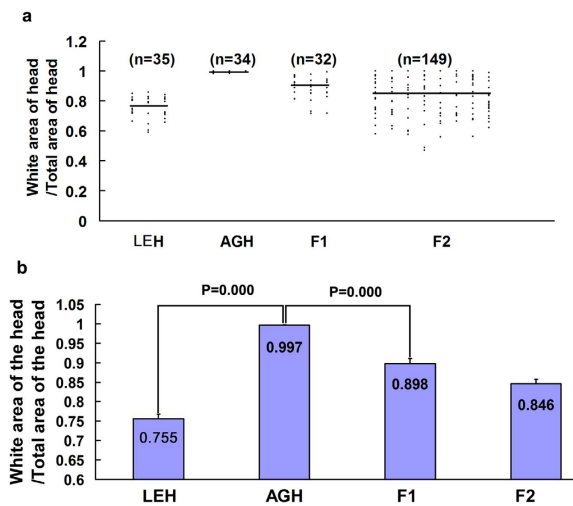
AGH-*Ednrb*$^{sl/sl}$ **LEH-*Ednrb*$^{sl/sl}$**
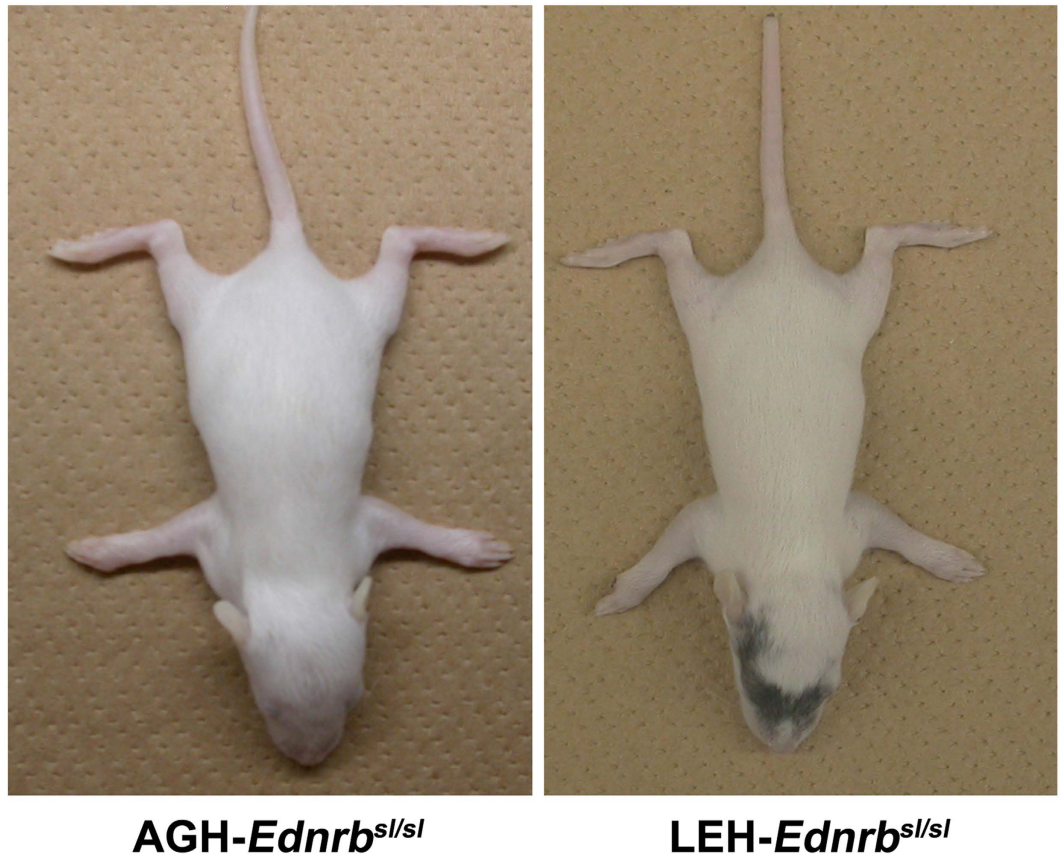
Figure 1. Comparison of the expressivity of the pigment disorder. In 14-day-old rats, no pigmentation on the head was observed in AGH-*Ednrb*$^{sl/sl}$ rats, and pigmentation was observed in LEH-*Ednrb*$^{sl/sl}$ rats.

In this study, we analyzed the difference in pigmentation between the *Ednrb*^{sl}-mutated rats; we performed quantitative trait locus (QTL) analysis using the intercross descendants with varying severity of pigmentation disorder, and we tried to search the modifier gene(s) affecting the phenotype.

Results

Evaluation of pigmentation in F$_2$ *Ednrb*$^{sl/sl}$ pups.   Homozygous *Ednrb*$^{sl/sl}$ rats showed a pigmentary disorder. We reported previously that variations of the pigmentary disorder was observed in *Ednrb*$^{sl/sl}$ rats with different genetic backgrounds[14]. AGH-*Ednrb*$^{sl/sl}$ rats almost had no pigmentation on their heads, whereas a large pigmented spot on the head was observed in LEH-*Ednrb*$^{sl/sl}$ (Fig. 1). We concluded that a modifier locus in the LEH background rescued the pigmentary disturbance to some extent[14]. We calculated the unpigmented coat ratio of AGH, LEH, F$_1$, and F$_2$ *Ednrb*$^{sl/sl}$ rats by using a camera and Photoshop and revealed the degree of variation of the pigmentary disorder. The severity of pigmentary disorder was calculated as a ratio of the unpigmented area in the head (pigment area/total area), which could be used as quantitative trait of the individual. The range of the severity of pigmentary disorder for each homozygous *Ednrb*$^{sl/sl}$ rat was presented in a scatter plot. Figure 2a shows that spots for F$_2$-*Ednrb*$^{sl/sl}$ rats were fairly scattered in the range of two extreme values compared with that of both AGH-*Ednrb*$^{sl/sl}$ and LGH-*Ednrb*$^{sl/sl}$ rats. Moreover, we calculated the mean value of the pigment disorder ratio for each of the AGH, LEH, F$_1$, and F$_2$ *Ednrb*$^{sl/sl}$ rats, which were 0.997, 0.755, 0.898, and 0.846, respectively (Fig. 2b). The values obtained in AGH-*Ednrb*$^{sl/sl}$ rats were highly different from that of LEH-*Ednrb*$^{sl/sl}$ rats with significance of P = 0.000, suggesting that the role of modifier(s) in the variance of pigment disorder observed in the LEH strain.

QTL analysis of the modifiers of pigment disorder in the F$_2$ *Ednrb*$^{sl/sl}$ rats.   MapManager QTXb20 software was used for QTL scan of the genome to determine the severity of pigmentary disorder in 149 F$_2$-*Ednrb*$^{sl/sl}$ rats with 91 microsatellite markers (Table 1), which showed polymorphism between AGH and LEH rats. As many as 5,000 random permutations in 1-centiMorgan (cM) steps were performed for each chromosome to calculate the likelihood ratio statistic (LRS). This LRS can be interpreted as a $\chi 2$ statistic or as a LOD score. In addition, the LRS can be converted to the conventional base-10 LOD score by dividing it by 4.61 (twice the natural logarithm of 10)[15]. Results of interval mapping were suggestive, significant, and highly significant linkages, that is, the LRS values were 9.6, 16.5, and 25.6, respectively. A highly significant QTL was detected in the region around *D7Got23* on chromosome (Chr) 7 (Figs 3 and 4), which explained 26% of the total phenotypic variance (Table 2).

Figure 2. Range of pigment disorder. (**a**) The range of pigment disorder in 14-day-old pups in AGH-*Ednrb*$^{sl/sl}$, LEH-*Ednrb*$^{sl/sl}$, F$_1$, and F$_2$. Horizontal lines indicate mean values. (**b**) ANOVA results of the pigment disorder ratio in AGH-*Ednrb*$^{sl/sl}$, LEH-*Ednrb*$^{sl/sl}$, F$_1$, and F$_2$. Each bar indicates the mean ± S.E.M.

| Microsatellite Markers	Position (Mbp)	Microsatellite Markers	Position (Mbp)	Microsatellite Markers	Position (Mbp)	Microsatellite Markers	Position (Mbp)
D1Rat392	22	D4Rat183	187	D9Rat153	107	D14Rat94	89
D1Rat343	99	D4Rat204	243	D10Mgh27	12	D15Rat5	25
D1Rat269	133	D5Rat125	22	D10Rat217	17	D15Rat6	37
D1Rat159	218	D5Rat196	107	D10Rat177	29	D15Rat48	66
D1Got225	255	D5Rat44	162	D10Got60	40	D16Rat78	21
D2Rat252	42	D6Got15	30	D10Rat163	50	D16Rat3	44
D2Rat116	52	D6Got74	71	D10Mit2	65	D16Got63	69
D2Mgh14	62	D6Rat165	103	D10Rat154	75	D16Rat55	78
D2Rat201	69	D6Rat11	124	D10Rat7	104	D17Rat2	68
D2Mit33	73	D7Rat31	32	D11Got45	67	D17Rat12	29
D2Rat385	79	D7Got23	36	D11Rat63	72	D17Rat24	50
D2Mit5	86	D7Got36	47	D11Rat43	90	D17Rat175	87
D2Rat21	95	D7Rat73	61	D12Rat58	1	D18Rat132	26
D2Rat123	132	D7Rat143	105	D12Rat76	34	D18Rat34	48
D2Got114	191	D7Rat131	115	D12Rat14	34	D18Got63	68
D2Rat241	243	D8Rat68	21	D12Rat86	46	D18Rat86	66
D3Rat57	8	D8Rat33	79	D13Rat150	21	D19Rat15	27
D3Mgh7	45	D8Rat18	99	D13Rat149	50	D19Rat27	30
D3Rat34	89	D8Rat8	121	D13Rat180	67	D19Got53	62
D3Rat287	111	D9Got6	4	D13Rat131	88	D20Mit4	34
D3Rat78	159	D9Rat41	14	D14Got35	29	D20Rat55	46
D4Mgh16	61	D9Got27	20	D14Mit4	44	D20Got47	52
D4Rat26	135	D9Mit3	63	D14Rat45	70		

Table 1. Microsatellite markers used for genotyping F$_2$ intercrossed progenies.

Epistatic interaction analysis was performed using the interaction function of MapManager QTXb20. The LRS values of suggestive, significant, and highly significant interactions were 29.2, 38.2, and 49.9, respectively. Three two-locus interactions were identified for pigmentary disorder, namely *D7Got23* (Chr 7) and *D14Mit4* (Chr 14), *D7Got36* (Chr 7) and *D3Rat78* (Chr 3), *D7Rat143* (Chr 7) and *D3Rat78* (Chr 3) (Table 3). We performed ANOVA to confirm the significant epistatic interactions in these microsatellite loci and the vicinal loci. ANOVA results revealed that the *D7Got23* locus showed highly significant interaction with *D3Rat78* locus. Thus, we chose the *D7Got23* locus located at the peak position of the QTL on Chr 7 to represent the loci showing significant epistatic interactions.

Allelic effects of *Ednrb*$^{sl/sl}$ modifier loci.   Modifier loci influence the phenotype but cannot revert the effects of a predisposed mutation such as *Ednrb*$^{sl/sl}$ [16]. To estimate the effects of a modifier locus on the pigmentary
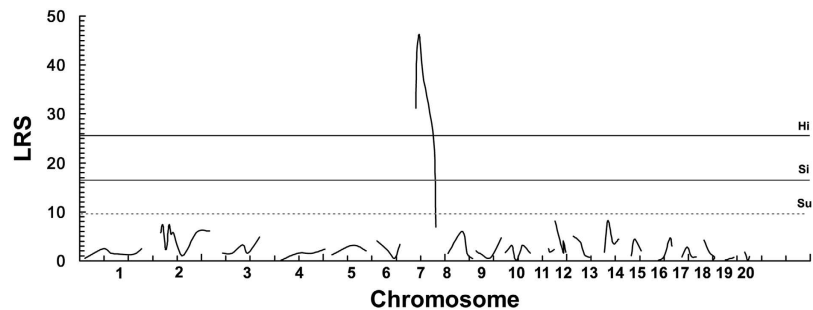
Figure 3. Result of interval mapping by MapManager QTXb20 in F$_2$ rats. Analyses of the linkage of the unpigmented area in F$_2$ populations to chromosomal loci were performed using the MapManager QTXb20 software. Recombination frequencies (%) were converted into genetic distance (in cM) by using the Kosambi map function; linkage data were provided as LRS scores. Genome-wide significance thresholds were calculated in terms of LRS by carrying out permutation tests for 5,000 permutations. The thresholds for suggestive (Su, LRS = 9.6), significant (Si, LRS = 16.5), and highly significant (Hi, LRS = 25.6) linkages were indicated using dotted, thin, and thick lines, respectively.
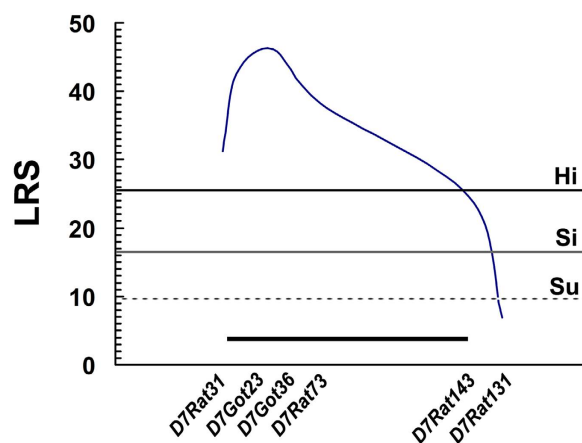

Figure 4. Details of QTL for the severity of pigment disorder on chromosome 7. The QTL on chromosome 7 showed highly significant linkage to the pigment disorder ratio. The dotted, thin, and thick lines represent suggestive (Su, LRS = 9.6), significant (Si, LRS = 16.5), and highly significant (Hi, LRS = 25.6) thresholds, respectively, calculated by 5,000 times permutation tests. The microsatellite markers used for determining genotypes of F$_2$ rats are presented along the X-axis. The black bars on the graph indicate approximately 95% confidence intervals.

Chr	Locus	Position (Mbp)	LRS	%	P	CI	Add
Chr 2	D2Rat116	52	7.3	5	0.02576	74	0.04
Chr 2	D2Mit33	73	6.1	4	0.04794	89	0.02
Chr 2	D2Rat385	79	7.4	5	0.02431	73	0.02
Chr 7	D7Rat31	32	30.0	18	0.00000	19	−0.08
Chr 7	D7Got23	36	45.0	26	0.00000	14	−0.10
Chr 7	D7Got36	47	44.0	26	0.00000	14	−0.10
Chr 7	D7Rat73	61	34.6	21	0.00000	17	−0.08
Chr 7	D7Rat143	105	24.6	15	0.00000	23	−0.07
Chr 12	D12Rat58	1	8.1	5	0.01759	67	0.03
Chr 14	D14Mit4	11	8.2	5	0.01681	67	0.03

Table 2. Characteristics of QTLs detected for variance of pigment disorder in F$_2$ intercrossed progenies. %: Percentage of total variance attributable to locus. CI: 95% confidence interval of QTL location as calculated by QTX software. Add: Addictive effect of the allele from LEH strain compared with that from AGH strain.

Chr 1	Locus 1	Position 1 (Mbp)	Chr 2	Locus 2	Position 2 (Mbp)	LRS	P	IX	Main 1	% 1	Main 2	% 2
Chr 7	D7Got23	36	Chr 14	D14Mit4	11	68.6	0.00000	13.6	45.0	26	8.2	5
Chr 7	D7Got36	47	Chr 3	D3Rat78	159	65.0	0.00000	17.7	44.0	26	4.9	3
Chr 7	D7Rat143	105	Chr 3	D3Rat78	159	45.8	0.00000	14.2	24.6	15	4.9	3

Table 3. Interaction results. LRS: Total LRS for association; IX: Interaction LRS; Main 1: LRS for locus 1 main effect; Main 2: LRS for locus 2 main effect; % 1: Percentage of total variance attributable to locus 1; % 2: Percentage of total variance attributable to locus 2.
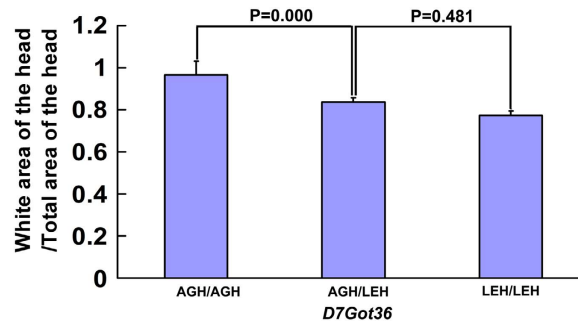

Figure 5. Allelelic effects of *Ednrb*^{sl/sl} modifier loci on the severity of pigment disorder. Homozygous *Ednrb*^{slsl} genotypes of the 149 experimental rats obtained from the marker closest to the modifier were used to assess the effects of individual loci on the severity of phenotype in F_2 population. The mean of the pigmentary disorder ratio (white area of the head/total area of the head) is plotted for each genotype class to show the relationship of the number of AGH or LEH alleles to the ratio of pigment disorder for this locus. Markers used to generate genotype information are listed beneath the plot. Genotype groups are defined as AGH/AGH, AGH/LEH, and LEH/LEH. Each bar indicates the mean ± S.E.M.

disorder in *Ednrb*^{sl/sl} individuals, a complete evaluation of the genotypic information in all F_2-*Ednrb*^{sl/sl} progenies was performed. Figure 5 shows the extent of pigment disorder that was evidently modified by the LEH alleles at the modifier locus on Chr 7. The ratio of pigmentary disorder was higher in homozygous AGH alleles compared with that in heterozygotes or homozygotes for LEH alleles. Significant difference was found between the ratios of homozygous AGH alleles and heterozygotes (P = 0.000), whereas no significant difference (P = 0.481) was found between heterozyotes and homozygous LEH alleles. Thus, we considered that the effect of LEH allele was approximately dominant.

Discussion

Coat color generally depends on the amount of melanin produced by melanocytes derived from neural crest cells[17,18]. Endothelin receptor B (EDNRB) is a G-protein-coupled receptor with seven transmembrane domains that is necessary during the development of neural crest and melanocytes[19]. Mouse with null *Ednrb* gene appeared *albino*[11]. However, *Ednrb*^{sl/sl} rats with different genetic background showed variation in their extent of pigmentation[14]. More severe *albino* phenotype was observed in AGH-*Ednrb*^{sl/sl} strain, whereas the pigmentation disturbance was reverted to some extent in LEH-*Ednrb*^{sl/sl} rats (Fig. 1). This observation suggested that the modifier loci in the LEH allele affected the phenotype.

We performed a genome-wide scan in F_2 progenies (AGH-*Ednrb*^{sl/sl} × LEH-*Ednrb*^{sl/sl}) to examine the effect of the modifier loci in pigment disorder in rats using QTL analysis. Results revealed a highly significant QTL around *D7Got23* on Chr 7 (Fig. 3); it has an extremely high LRS value and has 26% contribution to the total phenotypic variance (Table 2). Therefore, we assumed that one or more modifier genes responsible for pigment disorder may be located in the same region in Chr 7. The LEH allele in the modifier locus on Chr 7 increased the extent of the pigmented area (Fig. 5). Highly significant difference was observed between the ratios of homozygous AGH alleles and heterozygotes indicating that LEH allele was dominant (Fig. 5). While significant difference was detected between the ratios of homozygous LEH alleles and heterozygotes revealing other loci could influence pigment phenotype as well. Furthermore, a synteny analysis in other mammals revealed that the QTL around *D7Got23* locus on Chr 7 in rats corresponded comparatively to a region on Chr 10 in mouse, which housed an *Ednrb*^s modifier locus (*k10*) determining the expressivity of a white forelock and dorsal hypopigmentation[20]. This result suggested that *D7Got23* locus is a region of conserved synteny between rat and mouse genomes. In addition, *D7Got23* locus showed epistatic interactions with *D14Mit4* locus and *D3Rat78* locus. When *D7Got23* locus had LEH-homozygous genotype, the LEH-homozygous genotype of *D3Rat78* locus showed the highest extent of pigmented coat in F_2 progenies, followed by AGH/LEH-heterozygous and then the AGH-homozygous genotypes (Fig. 6). The LEH allele resisted the pigmentary disorder in both *D7Got23* and *D3Rat78* loci. We noted that the physical position of *D14Mit4* (43.8 Mb, RGSC 5.0) is close to that of *D14Got40* (35.3 Mb, RGSC 5.0), which was detected as the hooded locus[21]. The hooded phenotype is one of coat color phenotypes in rat with many alleles causing different extents of pigmented coat area[22–24]. Therefore, the modifier locus we detected for
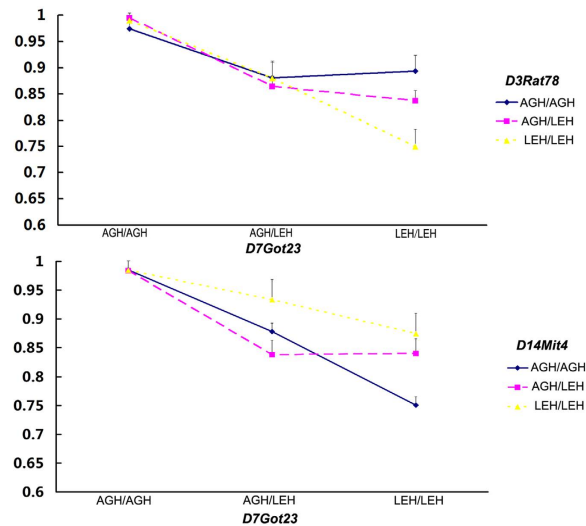
Figure 6. Highly significant epistatic interactions influence the extent of pigment disorder in F₂ rats.
Two highly significant epistatic interactions were detected between *D3Rat78* locus and *D7Got23* locus (**a**) and between *D14Mit4* locus and *D7Got23* locus (**b**). Each bar indicates the mean ±S.E.M.

the pigmentary disorder on Chr 7 might show epistatic interactions with the hooded locus. Interestingly, the effects of *D7Got23* locus and *D14Mit4* locus seemed to be opposite. LEH allele of *D7Got23* locus resisted the pigmentary disorder, while that of *D14Mit4* locus increased the extent of the disorder (Fig. 6). The *D7Got23* locus was the main one responsible for the variation of pigmentary disorder. The *D14Mit4* locus showed significant effect on the pigmentary phenotype when the *D7Got23* locus was homozygous for LEH allele. The F₂ rats owning the LEH-homozygous genotype at the *D7Got23* locus and the AGH-homozygous genotype at the *D14Mit4* locus showed the highest extent of pigmented coat.

Possible modifier genes responsible for the pigmentary disorder within the identified chromosomal region were identified using some bioinformatics methods, such as genome annotation combined with literature searches to check the confidence interval of the QTL on Chr 7 for potential genes that might be involved in the development of melanocytes[25]. More than 70 genes were identified (Supplementary Table S1). Among these candidates, *Lgr5* and *Wif1* attracted our attention. Both of them are associated with *WNT* signal pathway, which is responsible for the development of melanocytes[26,27]. Mass spectrometry demonstrated that Lgr4 and Lgr5 associate with the Frizzled/Lrp Wnt receptor complex[28]. However, the relationship between *Lgr5* gene and pigmentation was unknown. The *Wif1* gene, a Wnt inhibitory factor 1, was expressed not only in the melanocytes of normal human skin but also in cultured melanocytes and promoted melanogenesis in normal melanocytes[29]. In addition, we sequenced the coding region of these genes; however, we failed to find any difference between AGH and LEH strains. The causal difference might exist within the regulatory region of *Lgr5 or Wif1* gene. But it was possible that other causative genes had not been sequenced in our study. The main reason that we failed to identify the modifier gene(s) was the broad confidence interval of the QTL on Chr 7 (between *D7Rat31* and *D7Rat143*, from 28.5 Mb to 79.0 Mb, RGSC 3.4). We tried hard to find more markers showing polymorphism between AGH and LEH strains in this interval to improve the confidence interval, but we found none. The origin of this interval in AGH and LEH strains might be the same. In addition, the low number of the F₂ rats limited the precision of the result. To elucidate the pigment disorder, other biological methods must be employed to identify the modifier gene(s) in this region.

In conclusion, we have identified a highly significant QTL on Chr 7 by using two pigmentary disorder strains. Other two loci, namely, *D14Mit4* locus on Chr 14 and *D3Rat78* locus on Chr 3, show interaction with the main QTL on Chr 7 and may synergistically affect pigmentation.

Methods

Animals. The F₁ family was produced by crossing 2 AGH/Hkv (aganglionosis Hokkaido)-*Ednrb^{sl/+}*[14] males and 8 LEH/Hkv (Long-Evans Hokkaido)-*Ednrb^{sl/+}*[14] females. Five heterozygous males and 20 heterozygous females of F₁ rats were bred to generate the F₂ animals ($n = 592$), from which 149 *Ednrb^{sl/sl}* pups were selected by genotyping through PCR amplification using a pair of specific primers (F-CCTCCTGGACTAGAGGTTCC and R-ACGACTTAGAAAGCTACACT) and then flanking the site of deletion with 301 bp in *Ednrb* gene. PCR products were electrophoresed in 1.5% agarose gels to distinguish the wild (511 bp) type and the mutant (210 bp) type. AGH ($n = 35$), LEH ($n = 34$), and F₁ rats ($n = 32$) were raised for the determination of the severity of pigment disorder in each strain. Animals were maintained in specific pathogen-free conditions, fed, and supplied with water *ad libitum*. All research and experimental protocols were in accordance with the Regulation for the Care and Use of Laboratory Animals, Hokkaido University and approved by the President of Hokkaido University following to the review of the Institutional Animal Care and Use Committee (Approval ID: No. 110226).

Measurement of unpigmented coat ratio.   Photographs from the dorsal side of the rats were taken with a COOLPIX 4500 digital camera (Nikon, Tokyo, Japan). The total area of the head and of the pigmented area in the head were traced with lasso tools and calculated as pixels using the histogram function of Photoshop Elements 4.0 (Adobe Systems, California, USA). The ratio of unpigmented area of the head (1-pigmented area/total area) was then calculated as the phenotypic value of pigment disorder.

Microsatellite genotyping.   A total of 149 intercross progenies were selected for the genome-wide scan. Extraction of genomic DNA from the tail clips was performed using the standard methods. A total of 91 polymorphic microsatellite markers covering all 20 autosomes were selected from the National Center for Biotechnology Information <NCBI; http://www.ncbi.nlm.nih.gov/> (RGSC 5.0) for the genome-wide scan at 10–30 Mbp resolution (Table 1). The X chromosome was not scanned in our study because gender bias of pigment phenotype was not found in F_2 rats. The PCR procedure is described as follows: each $10\,\mu L$ reaction contains $0.5\,\mu L$ (10 ng) DNA, $0.5\,\mu L$ (5 pmol) of each primer, $5.5\,\mu L\,2\times$ Taq MasterMix (CWBIO, Beijing, China), and $3.0\,\mu L$ ddH$_2$O. Touchdown-PCR was performed as follows: 2 min at 95 °C, followed by 10 cycles for 30 s at 95 °C, 30 s at 63–54 °C, 30 s at 72 °C, then 25 cycles for 30 s at 95 °C, 30 s at 54 °C, 30 s at 72 °C, and a final extension at 72 °C for 10 min. PCR products were electrophoresed using 10% acrylamide gels at 160 V for 1.5–3 h, stained with ethidium bromide, and photographed under an ultraviolet lamp (Supplementary Fig. S1).

Linkage analysis.   Genotyping results and phenotypic values were analyzed using MapManager QTXb20 to identify the pigment disorder modifier loci[30]. A maximum likelihood algorithm with interval mapping was performed to examine linkage probability. Permutation tests were done in 1-cM steps for 5,000 permutations to determine the suggestive, significant, or highly significant levels of statistics. Interactions between all pairs of markers were also screened using MapManager QTXb20 program. We used 5,000 permutations in 1-cM steps to detect possible interactions using regression.

Two-locus interaction analysis.   Interaction effects or epistasis of all pairs of marker loci were tested with the interaction function of Map Manager QTXb20[15]. According to the manual, pairs of loci had to pass two tests to claim significant interaction effects. First, the total effect of the two loci had a p-value $< 10^{-5}$. Second, the interaction effects itself had a p-value < 0.01.

Statistical analyses.   Statistical analyses was performed using the SPSS 19 software and R. Significance test for pigment disorder ratio in AGH-*Ednrb^{sl/sl}*, LEH-*Ednrb^{sl/sl}*, F_1, and F_2, as well as in three types of alleles of *D7Got23* located on the peak were analyzed using ANOVA in SPSS 19. Significance test for the corresponding phenotypic mean values of nine types of alleles of two pairs loci showing epistatic interactions were analyzed by ANOVA in R.

References

1. Steingrímsson, E., Copeland, N. G. & Jenkins, N. A. Mouse coat color mutations: from fancy mice to functional genomics. *Dev. Dyn.* **235,** 2401–2411 (2006).
2. Silver, L. M. In *Mouse Genetics*: *Concepts and Applications*. (Oxford University Press, New York, 1995).
3. Zheng, B. *et al.* Engineering a mouse balancer chromosome. *Nat. Genet.* **22,** 375–378 (1999).
4. Hentges, K. E. & Justice, M. J. Checks and balancers: balancer chromosomes to facilitate genome annotation. *Trends Genet.* **20,** 252–259 (2004).
5. Bennett, D. C. & Lamoreux, M. L. The color loci of mice – a genetic century. *Pigment Cell Res.* **16,** 333–344 (2003).
6. Wang, C. *et al.* A PAX3 polymorphism (T315K) in a family exhibiting Waardenburg Syndrome type 2. *Mol. Cell. Probes.* **12,** 55–57 (1998).
7. Shanske, A., Ferreira, J. C., Leonard, J. C., Fuller, P. & Marion, R. W. Hirschsprung disease in an infant with a contiguous gene syndrome of chromosome 13. *Am. J. Med. Genet.* **102,** 231–236 (2001).
8. Tomita, Y., Miyamura, Y., Kono, M., Nakamura, R. & Matsunaga, J. Molecular bases of congenital hypopigmentary disorders in humans and oculocutaneous albinism 1 in Japan. *Pigment Cell Res.* **13,** 130–134 (2000).
9. Richards, K. A., Fukai, K., Oiso, N. & Paller, A. S. A novel KIT mutation results in piebaldism with progressive depigmentation. *J. Am. Acad. Dermatol.* **44,** 288–292 (2001).
10. Cott, H. B. In *Adaptive Coloration In Animals*. (Methuen, London, 1940).
11. Hosoda, K. *et al.* Targeted and natural (piebald-lethal) mutations of endothelin-B receptor gene produce megacolon associated with spotted coat color in mice. *Cell* **79,** 1267–1276 (1994).
12. Baynash, A. G. *et al.* Interaction of endothelin-3 with endothelin-B receptor is essential for development of epidermal melanocytes and enteric neurons. *Cell* **79,** 1277–1285 (1994).
13. Gariepy, C. E., Cass, D. T. & Yanagisawa, M. Null mutation of endothelin receptor type B gene in spotting lethal rats causes aganglionic megacolon and white coat color. *PNAS.* **93,** 867–872 (1996).
14. Dang, R. *et al.* 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 (2011).
15. Chmielewicz, K. M. & Manly, K. F. In *User manual for QTX*. (Roswell Park Cancer Institute, New York, 2002).
16. Nadeau, J. H. Modifier genes and protective alleles in humans and mice. *Curr. Opin. Genet. Dev.* **13,** 290–295 (2003).
17. Donoghue, P. C. J., Graham, A. & Kelsh, R. N. The origin and evolution of the neural crest. *BioEssays.* **30,** 530–541 (2008).
18. Steingrímsson, E., Copeland, N. G. & Jenkins, N. A. Melanocyte stem cell maintenance and hair graying. *Cell* **121,** 9–12 (2005).
19. Shin, M. K., Levorse, J. M., Ingram, R. S. & Tilghman, S. M. The temporal requirement for endothelin receptor-B signalling during neural crest development. *Nature* **402,** 496–501 (1999).
20. Rhim, H. *et al.* Spatially restricted hypopigmentation associated with an Ednrbs-modifying locus on mouse chromosome 10. *Genome Res.* **10,** 17–29 (2000).
21. Torigoe, D. *et al.* Genetic analysis of modifiers for the hooded phenotype in the rat. *Jpn. J. Vet. Res.* **57,** 175–184 (2010).
22. Gumbreck, L. G., Stanley, A. J., Macy, R. M. & Peeples, E. E. Pleiotropic expression of the restricted coat-color gene in the Norway rat. *J. Hered.* **62,** 356–358 (1971).
23. Palmer, M. L., Allison, J. E., Peeples, E. E. & Whaley, G. D. Coat-color restriction gene in rats: its effect in the homozygous condition. *J. Hered.* **65,** 291–296 (1974).

24. Robinson, R. An extreme allele of hooded spotting in the Norway rat. *Genetica* **79,** 139–141 (1989).
25. Cozma, D. *et al.* A bioinformatics-based strategy identifies c-Myc and Cdc25A as candidates for the Apmt mammary tumor latency modifiers. *Genome Res.* **12,** 969–975 (2002).
26. Roelink, H. & Nusse, R. Expression of two members of the Wnt family during mouse development--restricted temporal and spatial patterns in the developing neural tube. *Genes Dev.* **5,** 381–388 (1991).
27. Dorsky, R. I., Moon, R. T. & Raible, D. W. Control of neural crest cell fate by the Wnt signalling pathway. *Nature* **396,** 370–373 (1998).
28. De Lau, W. *et al.* Lgr5 homologues associate with Wnt receptors and mediate R-spondin signalling. *Nature* **476,** 293–297 (2011).
29. Park, T. J. *et al.* Wnt inhibitory factor (WIF)-1 promotes melanogenesis in normal human melanocytes. *Pigment Cell Melanoma Res.* **27,** 72–81 (2014).
30. Manly, K. F., Cudmore, R. H. & Meer, J. M. Map Manager QTX, cross-platform software for genetic mapping. *Mamm. Genome.* **12,** 930–932 (2001).

Acknowledgements

Author Contributions

Conceptual and experiment designs by R.D. and T.A. Experiments were conducted by J.H., R.D., D.T., A.L. and J.W. Data analysis performed by J.H., R.D. and C.L. Reagents/materials/analysis tools were contributed by R.D., N.S. and T.A. and the report was written by J.H. and R.D.

Additional Information

Supplementary information accompanies this paper at http://www.nature.com/srep

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Huang, J. *et al.* QTL analysis of modifiers for pigmentary disorder in rats carrying *Ednrb^sl* mutations. *Sci. Rep.* **6**, 19697; doi: 10.1038/srep19697 (2016).