A new missense mutation in the paired domain of the mouse Pax3 gene

Tamio OHNO1), Tomoki MAEGAWA1), Hiroto KATOH1), Yuki MIYASAKA1), Miyako SUZUKI2), Misato KOBAYASHI3), and Fumihiko HORIO3)

1) Division of Experimental Animals, Graduate School of Medicine, Nagoya University, 65 Tsurumai-cho, Showa-ku, Nagoya, Aichi 466-8550, Japan
2) Department of Applied Molecular Bioscience, Graduate School of Bioagricultural Sciences, Nagoya University, Furo-cho, Chikusa-ku, Nagoya, Aichi 464-8601, Japan

Abstract: Mice with dominant white spotting occurred spontaneously in the C3.NSY-(D11Mit74-D11Mit229) strain. Linkage analysis indicated that the locus for white spotting was located in the vicinity of the Pax3 gene on chromosome 1. Crosses of white-spotted mice showed that homozygosity for the mutation caused tail and limb abnormalities and embryonic lethality as a result of exencephaly; these phenotypes were analogous to those found in other Pax3 mutants. Sequence analysis identified a missense point mutation (c.101G>A) in exon 2 of Pax3 that resulted in a methionine to isoleucine conversion at amino acid 62 of the PAX3 protein. This mutation site was located in the N-terminal HTH (helix-turn-helix) motif of the paired domain of Pax3, which is necessary for binding to DNA and is highly conserved in vertebrate species. Alteration of DNA binding affinity was responsible for embryonic lethality in homozygotes and white spotting in heterozygotes. We named the mutant allele as Pax3Sp-Nag. The C3H/HeN-Pax3Sp-Nag strain may be useful for analyzing the function of Pax3 as a new model of the human disease, Waardenburg Syndrome.

Key words: embryonic lethal, missense mutation, mouse, Pax3 gene, white spotting

Introduction

A number of gene mutations are associated with white spotting in mice, and mutations of orthologous genes have been shown to be responsible for human piebaldism and Waardenburg Syndrome (WS), which causes pigmentation defects and deafness [3, 12]. Many alleles of mouse genes have been identified, and functional analyses of them have provided considerable insights into the regulatory networks of melanocyte development [3, 12]. The present report describes a spontaneous mutation that was first identified in a female mouse that showed small white spotting on its belly; the mouse belonged to the C3.NSY-(D11Mit74-D11Mit229) congenic strain. This new white spotting phenotype showed autosomal dominant inheritance, and we provisionally named the mutation Dws (dominant white spotting). We backcrossed mutant mice to C3H/HeN, the recipient strain of the C3.NSY-(D11Mit74-D11Mit229) congenic strain, and generated the C3H/HeN-Dws strain (Fig. 1). These mice were used to identify and characterize the Dws mutation.

Materials and Methods

Mice

C3.NSY-(D11Mit74-D11Mit229) mice were obtained from the Experimental Animal Division, Riken BioResource Center (Tsukuba, Japan). C3H/HeN (C3H) mice
were purchased from Charles River Laboratories Japan (Yokohama, Japan), and C57BL/6J (B6J) mice were purchased from Japan SLC (Hamamatsu, Japan). All mice were fed a commercial CE-2 diet (CLEA Japan, Tokyo) and had ad libitum access to water. The mice were bred in a pathogen-free facility at the Institute for Laboratory Animal Research, Graduate School of Medicine, Nagoya University, and maintained under a controlled temperature of 23 ± 1°C, humidity of 55 ± 10%, and a light cycle of 12 h light (from 09:00 to 21:00)/12 h dark (from 21:00 to 09:00). Animal care and all experimental procedures were approved by the Animal Experiment Committee, Graduate School of Medicine, Nagoya University, and were conducted according to the Regulations on Animal Experiments of Nagoya University.

Genetic mapping

Approximately half of the (B6J × C3H/HeN-\textit{Dws}) F\textsubscript{1} male and female mice showed white belly spotting, confirming that this character is controlled by autosomal dominant inheritance. White-spotted (B6J × C3H/HeN-\textit{Dws}) F\textsubscript{1} mice were backcrossed to B6J mice. Sixty-four white-spotted and 89 normal (no white spotting) mice were obtained from a total of 153 progeny in the backcross generation. Genomic DNA was prepared from tail tissue by salt/ethanol precipitation. PCR genotyping of simple sequence length polymorphism (SSLP) markers was performed according to standard methods. PCR products were separated by electrophoresis on a 4% NuSieve agarose gel (FMC, Rockland, ME, USA) and visualized by UV light after ethidium bromide staining. Linkage of markers to white spotting was evaluated by \( \chi^2 \)-tests. Genotype distribution was compared with the theoretical expectation based on Mendelian segregation.

Phenotype of homozygous (\textit{Dws}/\textit{Dws}) embryos

Crosses between heterozygous (\textit{Dws}/+) mice failed to produce any overt homozygous offspring in a preliminary study. To confirm the embryonic lethality of the homozygous mutant genotype, we examined 18 pregnant white-spotted (B6J × C3H/HeN-\textit{Dws}) F\textsubscript{1} females that had been crossed with white-spotted (B6J × C3H/HeN-\textit{Dws}) F\textsubscript{1} males. The pregnant females were euthanized by isoflurane inhalation on days 14.5 to 17.5 of pregnancy, and embryos were rapidly extracted for analysis. The genotypes of the embryos were determined as described above.

Sequence analysis

Our mapping analysis identified \textit{Pax3} as a candidate for the mutation. To confirm this, we analyzed the gene structure in the mutant by amplifying the coding region (9 exons) and splice junctions using the primer pairs listed in Table S1. Primer sequences were designed using genome assembly data (GRCh38.p4) as the reference sequence. PCR amplification was performed using a KOD Plus (TOYOBO, Osaka, Japan), and amplification products were purified using a QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany). PCR products were sequenced using the dideoxy chain-termination method with a BigDye Terminator v3.1 Cycle Sequencing Kit.
Results

Genetic mapping

SSLP markers that showed polymorphisms between B6J and C3H were used to scan eight candidate genes (Edn3, Ednrb, Kit, Kitl, Mitf, Pax3, Sox10, and Snai2) for linkage with the white spotting phenotype [3]. The segregation data for the 64 white-spotted mice in the backcross generation are shown for each candidate gene in Table 1. All of the white-spotted mice were heterozygous (B6J/C3H) at the D1Mit215 locus, which is close to Pax3. The other seven markers showed no indication of linkage to white spotting. Our results indicated that the Dws locus was likely located in the vicinity of Pax3. Five SSLP markers on chromosome 1 were used to confirm the location of the mutation and map it more precisely (Table 2). D1Mit215, which was the closest of these markers to Pax3, showed the largest deviation from the expected ratio. These results suggested that Dws was likely a mutation of the Pax3 gene. The presence of 11 heterozygous (B6J/C3H) mice with a normal (no white spotting) phenotype in the backcross generation indicated the incomplete gene penetrance of Dws. The penetrance appeared to be about 85.3% (64/64+11; Table 2).

Embryonic lethality of the homozygous genotype

We analyzed F2 embryos produced by intercrosses of white-spotted (B6J × C3H/Hen−Dws) F1 mice (Table 3). Malformed embryos, characterized by exencephaly and tail and limb abnormalities (Fig. 2), were present from E14.5 to E17.5. The phenotype of the malformed embryos was similar to those described for other Pax3 mutants, such as Pax3Sp, Pax3Sp−1H, Pax3Sp−2H, Pax3Sp−1Xag, Pax3Sp−1Wi, and Pax3Sp−2Osa [2, 5–7, 10, 14]. There was an extremely small number of homozygous (C3H/C3H) (Applied Biosystems, Foster City, CA, USA) and then analyzed on an ABI 3500 (Applied Biosystems) automated DNA sequencer.

Table 1. Segregation ratios of neighboring markers at 8 candidate genes in the 64 white-spotted backcross progeny

| Marker     | Chr. | Position (Mb) | Candidate gene (Position Mb) | Ratio (homo : hetero) | χ²-value |
|------------|------|---------------|-----------------------------|-----------------------|----------|
| D1Mit215   | 1    | 78.21         | Pax3 (78.10–78.20)          | 0 : 64                | 64.00    |
| D2Mit229   | 2    | 168.78        | Edn3 (174.76–174.78)        | 32 : 32               | 0.00     |
| D5Mit355   | 5    | 71.9          | Kit (75.57–75.67)           | 26 : 38               | 2.25     |
| D6Mit149   | 6    | 106.01        | Kitl (100.02–100.10)        | 34 : 30               | 0.25     |
| D10Mit96   | 10   | 99.56         | Sox10 (79.15–79.16)         | 35 : 29               | 0.56     |
| D14Mit92   | 14   | 91.11         | Ednrb (103.81–103.84)       | 29 : 35               | 0.56     |
| D15Mit63   | 15   | 65.25         | Snai2 (14.71–14.71)         | 28 : 36               | 1.00     |

Table 2. Genetic mapping of the Dws locus using SSLP markers around the Pax3 gene

| Marker     | Position (Mb) | White spotting (homo : hetero) | Normal (homo : hetero) | χ²-value |
|------------|---------------|-------------------------------|-----------------------|----------|
| D1Mit484   | 76.39         | 1 : 63                        | 77 : 12               | 107.53   |
| D1Mit132   | 77.15         | 0 : 64                        | 77 : 12               | 114.47   |
| Pax3       | 78.10–78.20   |                               |                       |          |
| D1Mit215   | 78.21         | 0 : 64                        | 78 : 11               | 114.44   |
| D1Mit81    | 85.76         | 0 : 64                        | 74 : 15               | 103.11   |
| D1Mit49    | 87.2          | 1 : 63                        | 74 : 15               | 99.17    |

Table 3. Incidence of malformed embryos of each genotype for D1Mit215 on different days of gestation

| Fetal age | No. of embryos | Average litter size | Number of embryos (B6J/B6J:B6J/C3H:C3H/C3H) | Number of malformed embryos (B6J/B6J:B6J/C3H:C3H/C3H) | Incidence * |
|-----------|----------------|---------------------|---------------------------------------------|-------------------------------------------------------|-------------|
| E14.5     | 33             | 8.3 (33/4)          | 9 : 18 : 6                                  | 0 : 0 : 4                                              | 67% (4/6)   |
| E15.5     | 29             | 7.3 (29/4)          | 12 : 11 : 6                                 | 0 : 0 : 5                                              | 83% (5/6)   |
| E16.5     | 33             | 8.3 (33/4)          | 6 : 21 : 6                                  | 0 : 0 : 5                                              | 83% (5/6)   |
| E17.5     | 39             | 6.5 (39/6)          | 11 : 25 : 3                                 | 0 : 0 : 3                                              | 100% (3/3)  |
embryos, and litter size tended to be reduced, with the occasional occurrence of fetal death (data not shown) at E17.5. Genotyping analysis using D1Mit215 indicated that all the abnormal embryos were homozygous for the C3H-derived allele. The rate of malformed homozygous embryos (C3H/C3H) tended to increase with embryonic age. These facts suggested the embryonic lethality of Dws/Dws mice.

**Sequence of the Pax3 gene in mutants**

A missense point mutation, G to A, was identified at nucleotide 101 in exon 2 of the Pax3 gene (Fig. 3). This alteration resulted in a methionine to isoleucine conversion at amino acid 62 (p.Met62Ile) in highly conserved region among vertebrate species of the Pax3 protein (Fig. 3 and Table 4).

**Discussion**

Pax3 encodes a transcription factor that is important in melanocytes and influences melanocytic proliferation, resistance to apoptosis, migration, lineage specificity, and differentiation [9]. The gene possesses four structural domains: a paired domain, an octapeptide motif, a homeodomain, and a transactivation domain. The paired domain, named after the two helix-turn-helix (HTH) motif-containing sub-domains within it, binds DNA in addition to facilitating interactions with other proteins [9]. The N-terminal HTH motif (amino acid residue 54–94) is necessary and sufficient for binding to DNA, and is highly conserved among vertebrate species [9]. The mutation identified here is located within the N-terminal HTH motif in the paired domain of the Pax3 gene (Fig. 3). Several human PAX3 mutations occur as missense or frameshift mutations within the highly conserved region of exon 2, which gives rise to part of the paired domain [9, 11]. Although exactly the same mutation have not been reported, a mutation on same amino acid (p.Met62Val) within the paired domain has been reported in human PAX3. The patient with this mutation exhibited dystopia canthorum and confluent eyebrows as WS features [8]. It is likely that the amino acid change (p.Met62Ile) on the N-terminal HTH motif identified in this study would alter the DNA binding affinity of the paired domain and would underlie the loss of Pax3 function. We formally named Dws as Pax3<sup>Sp-Nag</sup>.

Modifier genes of Pax3 mutant alleles have been reported [1, 7], and they expand our understanding of the complex network governing melanocyte development. Large size variations in white spotting on the belly were observed among the 64 white-spotted mice of the backcross generation, but such size variation was relatively small in the C3H/HeN-Pax3<sup>Sp-Nag</sup> strain. This suggests that Pax3<sup>Sp-Nag</sup> modifier genes are present in the B6J genetic background. To screen the interaction between modifier and mutant genes, thirteen backcross mice that showed relatively large white spots were selected and genotyped using markers located in the vicinity of seven WS candidate genes (Edn3, Ednrb, Kit, Kitl, Mitf, Sox10, and Snai2). None of the markers showed a distribution
new Missense MuTaTiOn in MOuse

Pax3

249
disequilibrium from the expected ratio (Table S2). This suggests that the size variation in white spotting mediated by Pax3Sp-Nag allele was not a consequence of a direct interaction with other candidate WS genes.

During breeding of C3H/HeN- Pax3Sp-Nag mice, males with white spotting were crossed with wild type females. The incidence of white-spotted offspring was 41.2% (21/51), which is lower than the expected ratio of 50% of the mice with the mutation phenotype. The reduction in the incidence of white-spotted mice from the expected ratio in the C3H/HeN-Pax3Sp-Nag strain is in accordance with incomplete gene penetrance (85.3%). White-spotted mice with tail abnormalities, e.g., looped tails, were rarely observed (<1%) in this strain. The looped tail feature was not passed on to the next generation. Although the cause is not known, a similar phenomenon has been reported for other Pax3 mutant mice such as Pax3Sp-1H, Pax3Sp-1Xzg, and Pax3Rwa [6, 10, 14]. Hetero-

Table 4. Multiple sequence alignments of PAX3 protein from various species

| Species | Number of the amino acid sequence of PAX3 |
|---------|------------------------------------------|
| Mouse (Pax3Sp-Nag/ Pax3Sp-Nag) | H I R H K I V E 1 A H H G I R P C |
| Mouse (Mus musculus) | H I R H K I V E M A H H G I R P C |
| Human (Homo sapiens) | H I R H K I V E M A H H G I R P C |
| Monkey (Macaca fascicularis) | H I R H K I V E M A H H G I R P C |
| Turkey (Meleagris gallopavo) | H I R H K I V E M A H H G I R P C |
| Sea turtle (Chelonia mydas) | H I R H K I V E M A H H G I R P C |
| Frog (Xenopus laevis) | H I R H K I V E M A H H G I R P C |
| Flatfish (Paralichthys olivaceus) | H I R H K I V E M A H H G I R P C |

Fig. 3. (A) Missense mutation in the Pax3 gene of the Dws mutant. A c.101 G>A transition in exon 2 causes the conversion of Met to Ile at amino acid 62. (B) Schematic diagram of the mouse PAX3 protein including homeodomain and paired domain with N-terminal HTH motif. Numbers correspond to the amino acid sequence.
zygous (Pax3<sup>3p-Nag</sup>/+) mice with the p.Met62Ile mutation do not appear to show deafness. Similarly, the patient with the p.Met62Val mutation of Pax3 did not show deafness [8]. The missense mutation at amino acid 62 did not directly affect hearing ability, although deafness is a frequent feature of WS patients and is observed at a rate of about 60% in case of type 1 WS, most of which are caused by PAX3 mutations [11]. The underlying genetic mutation and the phenotype of several Pax3 mutant alleles have been determined and listed in Table 5 [2, 4, 6, 7, 10, 13, 14]. Although a few Pax3 mutants, such as Pax3<sup>3p-d</sup>, Pax3<sup>3p-7H</sup>, and Pax3<sup>3p-1Wli</sup>, have a mutation in paired the domain [4, 13, 14], the Pax3<sup>3p-Nag</sup> allele reported here is the first missense mutant in the N-terminal HTH motif of the paired domain of Pax3 gene in mice.

In conclusion, the C3H/HeN-Pax3<sup>3p-Nag</sup> strain, which carries a new missense mutation in the paired domain of the mouse Pax3 gene, may be useful for analyzing the function of Pax3 with respect to human WS. This strain has been deposited in the Riken BioResource Center (Tsukuba, Japan) under the catalog number RBRC06568.

### Table 5. Pax3 mutations and mutant phenotypes

| Allele     | Mutation                                                                 | Phenotype                        |
|------------|---------------------------------------------------------------------------|----------------------------------|
| Pax3<sup>3p-wa</sup> | 841 bp deletion spanning the promoter region and intron 1              | Embryonic lethal                |
| Pax3<sup>3p-7H</sup> | Missense mutation (V38G) in exon 2 (paired domain)                      | Embryonic lethal                |
| Pax3<sup>3p-d</sup> | Missense mutation (G42R) in exon 2 (paired domain)                      | Perinatal lethal                |
| Pax3<sup>3p-Nag</sup> | Missense mutation (M62I) in exon 2 (paired domain)                      | Embryonic lethal                |
| Pax3<sup>3p-1Wli</sup> | Nonsense mutation (K107X) in exon 2 (paired domain)                      | Embryonic lethal                |
| Pax3<sup>3p-2H</sup> | Point mutation of splice acceptor of intron 3                         | Embryonic lethal                |
| Pax3<sup>3p-3Xg</sup> | 32 bp deletion in exon 5 (homeodomain)                                  | Embryonic lethal                |
| Pax3<sup>3p-7H</sup> | Missense mutation (N269D) in exon 6 (homeodomain)                      | Embryonic lethal                |

### References

1. Asher, J.H. Jr., Harrison, R.W., Morell, R., Carey, M.L., and Friedman, T.B. 1996. Effects of Pax3 modifier genes on craniofacial morphology, pigmentation, and viability: a murine model of Waardenburg syndrome variation. *Genomics* 34: 285–298. [Medline] [CrossRef]
2. Auerbach, R. 1954. Analysis of the developmental effects of a lethal mutation in the house mouse. *J. Exp. Zool.* 127: 305–329. [CrossRef]
3. Baxter, L.L., Hou, L., Loftus, S.K., and Pavan, W.J. 2004. Spotlight on spotted mice: a review of white spotting mouse mutants and associated human pigmentation disorders. *Pigment Cell Res.* 17: 215–224. [Medline] [CrossRef]
4. Bogani, D., Warr, N., Elms, P., Davies, J., Tymowska-Lalanne, Z., Goldsworthy, M., Cox, R.D., Keays, D.A., Flint, J., Wilson, V., Nolan, P., and Arkell, R. 2004. New semidominant mutations that affect mouse development. *Genesis* 40: 109–117. [Medline] [CrossRef]
5. Epstein, D.J., Vekemans, M., and Gros, P. 1991. Splotch (Sp<sup>109</sup>), a mutation affecting development of the mouse neural tube, shows a deletion within the paired homedomain of Pax-3. *Cell* 67: 767–774. [Medline] [CrossRef]
6. Franz, T. 1992. Neural tube defects without neural crest defects in splotch mice. *Teratology* 46: 599–604. [Medline] [CrossRef]
7. Guo, X.L., Ruan, H.B., Li, Y., Gao, X., and Li, W. 2010. Identification of a novel nonsense mutation on the Pax3 gene in ENU-derived white belly spotting mice and its genetic interaction with c-Kit. *Pigment Cell Melanoma Res.* 23: 252–262. [Medline] [CrossRef]
8. Hol, F.A., Geurds, M.P., Cremers, C.W., Hamel, B.C., and Mariman, E.C. 1998. Identification of two PAX3 mutations causing Waardenburg syndrome, one within the paired domain (M62V) and the other downstream of the homeodomain (Q282X). *Hum. Mutat.* 11(Suppl 1): S145–S147. [Medline] [CrossRef]
9. Kubic, J.D., Young, K.P., Plummer, R.S., Ludvik, A.E., and Lang, D. 2008. Pigmentation Pax-ways: the role of Pax3 in melanogenesis, melanocyte stem cell maintenance, and disease. *Pigment Cell Melanoma Res.* 21: 627–645. [Medline] [CrossRef]
10. Ohnishi, T., Miura, I., Ohba, H., Shimamoto, C., Iwayama, Y., Wakana, S., and Yoshikawa, T. 2017. A spontaneous and novel Pax3 mutant mouse that models Waardenburg syndrome and neural tube defects. *Gene* 607: 16–22. [Medline] [CrossRef]
11. 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]
12. Tachibana, M., Kobayashi, Y., and Matsushima, Y. 2003. Mouse models for four types of Waardenburg syndrome. *Pigment Cell Res.* 16: 448–454. [Medline] [CrossRef]
13. Vogan, K.J., Epstein, D.J., Trasler, D.G., and Gros, P. 1993. The splotch-delayed (Sp<sup>)</sup> mouse mutant carries a point mutation within the paired box of the Pax-3 gene. *Genomics* 17: 364–369. [Medline] [CrossRef]
14. Xiao, Y., Zhang, L., He, K., Gao, X., Yang, L., He, L., Ma, G., and Guo, X. 2011. Characterization of a novel missense mutation on murine Pax3 through ENU mutagenesis. *J. Genet. Genomics* 38: 333–339. [Medline] [CrossRef]