A Deletion in the Endothelin-B Receptor Gene is Responsible for the Waardenburg Syndrome-Like Phenotypes of WS4 Mice

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Abstract: The WS4 mouse is an animal model for human Waardenburg syndrome type 4 (WS4), showing pigmentation anomalies, deafness and megacolon, which are caused by defects of neural crest-derived cells. We have previously reported that the gene responsible for the WS4 mouse is an allele of the piebald mutations of the endothelin B receptor gene (Ednrb). In this study, we examined the genomic sequence of the Ednrb gene in WS4 mice and found a 598-bp deletion in the gene. The deleted region contains the entire region of exon 2 and the 5' part of exon 3 and is flanked by inverted repeat sequences which are suggested to trigger the deletion. We concluded that the deletion in the Ednrb gene is the causative mutation for the phenotype of WS4 mice.

Key words: endothelin-B receptor, inverted repeat, Waardenburg syndrome

Waardenburg syndrome (WS) is a human auditory-pigmentary syndrome with hearing impairment and pigmentation anomalies of the skin and iris [10], which are caused by maldevelopment of the neural crest-derived cells. WS has been classified into four types, namely WS1, WS2, WS3, and WS4, with common symptoms being hypopigmentation and sensorineural deafness. In addition to these common symptoms, WS1 is associated with facial deformity, WS3 with upper limb deformity and WS4 with megacolon (Hirschsprung disease), while WS2 is not associated with other symptoms [12]. The WS4 mutant mouse strain, established from hybrids of BALB/c and MSM/Ms strains at the Saitama Cancer Center, shows phenotypes resembling the symptoms of WS4 in humans, including an abnormal coat color (white coat color with black eyes), deafness and megacolon [9]. Therefore, the WS4 mouse is considered to be a good animal model for human WS4. We
have previously reported that the gene responsible for
the WS4 mouse is an allele of the piebald mutations,
which are caused by mutations in the endothelin-B re-
ceptor (Ednrb) gene [9], and the mutant allele of WS4
has been named Ednrb<sup>ws4</sup>. The transcripts of the Ednrb
gene have been shown to lack a 318-bp region corre-
sponding to exons 2 and 3 of the gene in WS4 mice
[9]; however, the causative mutation in the genomic
sequence of the Ednrb gene responsible for the aber-
rant transcripts remains unknown. In this study, we
examined the genomic sequence of the Ednrb gene in
WS4 mice to reveal the structural change of the Ednrb
gene causing the aberrant transcript and to establish an
effective genotyping system for this mutant.

DNA samples were obtained from mice with
Ednrb<sup>ws4</sup>/Ednrb<sup>ws4</sup>, Ednrb<sup>ws4</sup>/+, and +/+ genotypes of
the WS4 strain. The genotypes of these mice were
determined by their phenotypes or progeny tests. Since
the WS4 strain was established from mice obtained from
a cross between BALB/c and MSM/Ms (an inbred strain
of Japanese wild mice), we also obtained DNA samples
of MSM/Ms and BALB/c. The DNA samples were
prepared from the livers of these mice by phenol-chlo-
roform extraction according to the standard protocol.
To examine whether a large-scale change of the ge-
monic sequence, including deletion or insertion, is
responsible for the lack of exons 2 and 3 in the tran-
scripts, these DNA samples were subjected to PCR with
five primer pairs which amplify approximately 500-bp
genomic fragments in the region spanning intron 1 to
intron 3 of the Ednrb gene (Fig. 1). These fragments
correspond to nucleotides 77139525 to 77138809 (F1),
77135550 to 77134932 (F2), 77130635 to 77130527
(F3), 77130098 to 77129937 (F4), and 77129879 to
77128670 (F5) of the mouse genomic contig sequence
containing the Ednrb gene (NCBI accession number:
NT039606). The PCR reaction was performed for 40
cycles consisting of denaturation at 94°C for 30 s, an-
nealing at 58°C for 45 s and extension at 72°C for 45 s,
followed by final extension at 72°C for 10 min. The
reaction mixture contained 0.5 µM of each primer, 1 U
Taq polymerase, 200 µM dNTPs, and 1.5 mM Mg<sup>2+</sup>. Among
the five fragments, F1, F2, and F5 were amplified
from all mice examined with the expected sizes; how-
ever, F3 and F4 were not amplified from Ednrb<sup>ws4</sup>/Ednrb<sup>ws4</sup>
mice but were amplified from Ednrb<sup>ws4</sup>+/+, +/+, BALB/c and MSM/Ms mice with the expected sizes.

These findings suggested that a genomic region includ-
ing F3 and F4 is deleted from the Ednrb<sup>ws4</sup> allele.

To define the expected deletion, a 1,418-bp region
covering the 3’ end of intron 1, exon 2, intron 2, and
intron 3 was amplified with a primer pair P-1 (5’-
TTCAGAGAGTCAGACGAAAAAC-3’) and P-2
(5’-CTTTCCCTTTGTA GTCCGACGTAAT-3’) from
mouse genomic DNA samples. The PCR reaction was
performed for 40 cycles consisting of denaturation at
94°C for 30 s, annealing at 50°C for 45 s and extension
at 72°C for 45 s, followed by final extension at 72°C
for 10 min. As shown in Fig. 1, a fragment of the
expected size was amplified from +/+, BALB/c, and
MSM/Ms mice, but a significantly shorter fragment was
amplified from Ednrb<sup>ws4</sup>/Ednrb<sup>ws4</sup> mice and both shorter
and longer fragments were amplified from Ednrb<sup>ws4</sup>+/+
mice. These results further suggested a deletion in this
region. We therefore cloned PCR fragments of this
region into pGEMT-Easy Vector (Promega) and deter-
mined their nucleotide sequences by the dideoxy chain
terminating method with an automated DNA sequencer
(SQ5500 HITACHI). Comparison of the nucleotide
sequences of the PCR fragments between Ednrb<sup>ws4</sup> and
+/+ alleles confirmed that a 598-bp region consisting of
the 3’ region of intron 1 (304 bp), exon 2 (113 bp),
intron 2 (132 bp) and the 5’ region of exon 3 (49 bp)
was deleted from the Ednrb<sup>ws4</sup> allele (Fig. 2). It is
likely that deletion of the 5’ half of exon 3 including
the splicing acceptor site, as well as the entire region of
exon 2, resulted in aberrant splicing from the splicing
donor site of exon 1 to the acceptor site of exon 4.
Therefore, we concluded that aberrant transcripts of the
Ednrb gene lacking exons 2 and 3 are caused by this
genomic deletion and this deletion is the causative mu-
tation of WS4 mice.

Notably, we found inverted repeat sequences (palin-
dromic sequences) consisting of six complementary
nucleotide pairs at the 5’ end, and five complementary
nucleotide pairs at the 3′ end of the deleted region, and
the breakpoints of the deletion were located in the
middle of these inverted repeat sequences (Fig. 2). The
existence of the inverted repeat sequences at the 5’ and
3’ ends of the deleted region suggests a possible mecha-
nism for the generation of the 598-bp deletion. The
inverted repeat sequences are capable of forming a stem
or a stem-loop structure which would result in the in-
stability of the genomic DNA. For example,
spontaneous deletion mutations often occur at short direct repeats that flank inverted repeat sequences in \textit{Escherichia Coli} [3] and a deletion causing thalassaemia has also been reported to be associated with an inverted repeat sequence in the globin gene cluster [5]. Furthermore, in addition to these inverted repeat sequences in the wild type allele, the nucleotide sequence of the $\textit{Ednrbws4}$ allele resulting from this deletion also possessed an inverted repeat sequence consisting of eight complementary nucleotide pairs at the region flanking the deletion site (Fig. 2). Therefore, it is likely that the inverted repeat sequences might trigger the deletion of the $\textit{Ednrb}$ gene through the formation of stem structures of single strand DNA.

To date, several genes responsible for WS have been identified in humans [10]: PAX 3 mutations have been found in WS1 and WS3 families; MITF mutations in WS2 families; SOX10 mutations in WS4 families; and mutations of the $\textit{EDN3}$ gene and the endothelin 3 gene ($\textit{EDN3}$) in WS4 families [1, 4]. In particular, mutations of $\textit{EDN3}$/\textit{Edn3} genes have been reported in other mammalian species including mice, rats, and horses [2, 6, 8, 11]. Endothelin 3 is a selective ligand for endothelin B receptor [7] and interaction between endothelin 3 and endothelin-B receptor plays an essential role in the migration and proliferation of neural crest cells. Therefore, mutations of these two genes result in white coat color and megacolon caused by defects of neural crest-derived cells, including melanocytes and myenteric ganglion cells [2, 6]. As WS4 mice show phenotypes resembling the typical symptoms of human WS4, including an abnormal coat color and megacolon as well as deafness, the WS4 mouse could be an excellent animal model for human WS4. Our finding of the causative mutation in the genomic sequence of the $\textit{Ednrb}$ gene will further facilitate the use of the WS4 mouse as an animal model. The WS4 strain has been maintained by mating heterozygous male and female mice as homozygous mice of this strain are lethal. This finding of the genomic
deletion in the Ednrb gene enables us to genotype this gene by PCR and facilitate the identification of heterozygous animals. As shown in Fig. 1, Ednrb<sup>ws4</sup>/+ mice showed both 1,418-bp and 820-bp fragments after PCR using primers P1 and P2, while a single 820-bp or 1,418-bp fragment was observed for Ednrb<sup>ws4</sup>/Ednrb<sup>ws4</sup> or +/+ mice, respectively. Currently, this genotyping system is being applied to the breeding of the WS4 strain and the establishment of a congenic strain possessing the Ednrb<sup>ws4</sup> allele with a C57BL/6 genetic background. Since the Ednrb locus has various alleles including Ednrb<sup>ws4</sup>, Ednrb<sup>sl</sup>, and Ednrb<sup>s</sup> which are

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**Fig. 2.** Deletion of a 598-bp region in the Ednrb gene of WS4 mice. A: Genomic structure of the Ednrb gene of the wild type and Ednrb<sup>ws4</sup> alleles. The deleted region is indicated by dotted lines and the expected splicing forms are indicated by thin lines. The bottom part indicates the nucleotide sequences flanking the breakpoints of the deletion. Nucleotides which constitute the inverted repeat sequences are denoted by underlining, dotted underlining, and double underlining. B: Putative stem structures formed by inverted repeat sequences of the wild type and the Ednrb<sup>ws4</sup> alleles. The breakpoints of the deletion are indicated by arrows.
caused by different types of mutations [6, 13], the congeneric strains possessing these alleles will be useful for comparing the precise effects of these alleles on the phenotypes.

References