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A Point Mutation in a Cadherin Gene, *Cdh23,* Causes Deafness in a Novel Mutant, *Waltzer Mouse Niigata*

T. Wada,*,† Y. Wakabayashi,* S. Takahashi,† T. Ushiki,‡ Y. Kikkawa,§ H. Yonekawa,§ and R. Kominami*,[1]

**First Department of Biochemistry, †Department of Otorhinolaryngology, and ‡Third Department of Anatomy, Niigata University School of Medicine, Asahimachi 1-757, Niigata 951-8510, Japan; and §Department of Laboratory Animal Science, The Tokyo Metropolitan Institute of Medical Science, Honkomagome Bunkyou-ku, Tokyo 113-8613, Japan*

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A novel mouse model for human nonsyndromic hearing loss, *Waltzer niigata* (v^{ngt}), is found and subjected to positional cloning analysis. Genome-wide scan of 1648 backcross mice maps v^{ngt} to the *D10Mit258* locus near *Waltzer (v).* Recombination breakpoints are positioned on a physical map consisting of 13 BACs relative to the flanking markers in the vicinity of v^{ngt}. Allelism test done in parallel shows that v^{ngt} and *v* are allelic. Sequence analysis reveals one-base deletion in the cDNA encoding a cadherin-related protein, Cdh23, mutation of which is recently reported in *v* mutants. The frame-shift change, producing a truncated protein of 51 amino acids, is ascribed to a base-substitution of G to A in the acceptor site of splicing junction which is predicted to cause one-base shift of the splicing position.

Deafness is a relatively common disorder, with approximately 1 in 800 children born with a serious permanent hearing impairment, and very large proportions of the population suffering from progressive age-related hearing loss (1, 2). Over half of the cases of childhood deafness are probably caused by single-gene defects. The majority of such genetic deafness is nonsyndromic, in which hearing loss is not associated with any other abnormalities. Autosomal recessive forms of nonsyndromic deafness (*DFNB*) are mostly due to abnormalities of the sensory neuroepithelia of the inner ear (1, 2). Since the auditory system of mice and humans is well conserved, mutant mice with inner ear disorders represent the most likely candidates for homology with nonsyndromic hearing losses in humans. Therefore, one approach for identifying some of genes involved in deafness is to use mouse mutants as models. Indeed, the genetic analysis of those mutations has proven valuable for the identification of genes causing human *DFNB*, such as MYO7A (3, 4), MYO15 (5–7), and CDH23 (8, 9).

We found a deaf mutant in our mouse colony that could provide a novel model. Homozygotes of the mutant were deaf and showed the typical phenotypes such as circling, headtossing and hyperactivity. The abnormalities were similar to those of *sh-2* and *waltzer* mutants, which were probably due to cochleovestibular dysfunction of the inner ear (10). Thus we designated the mice as *waltzer mouse niigata* (v^{ngt}), and the v^{ngt} mice were subjected to genetic analysis. In this paper we report the establishment of a large intersubspecific backcross segregating for v^{ngt} and the positioning of recombination breakpoints on a physical map consisting of 13 BAC's relative to the flanking markers in the vicinity of v^{ngt}. We also describe that the gene mutated in v^{ngt} mice encodes the Cdh23, a novel type of cadherin which recently has been found by the analysis of *waltzer* mutants (8).

MATERIALS AND METHODS

Mice and genetic cross. The *waltzer niigata* (v^{ngt}) mice were found in our mouse colony at Niigata and their genetic background was of the ICR strain. Homozygotes were mated with MSM and F_1 progeny were backcrossed to v^{ngt} homozygotes. MSM is an inbred strain derived from the Japanese wild mouse, *Mus. musculus molossinus.* A total of 1648 backcross progeny were recovered and homozygotes for v^{ngt} were identified from their heterozygous sibs by observing the phenotypic head shaking, circling, and loss of Preyer reflex characteristics of these mice. The *waltzer*^{2J} (v^{2J}) mice were purchased from the Jackson Laboratory.

Genetic and physical mapping. Genotyping was done with polymerase chain reaction (PCR) as described (6, 11). BAC clones were isolated by PCR screening of a library that were purchased from Research Genetics, Inc. The size of clones was determined by pulsed-field gel electrophoresis using a Sheff mapper apparatus (BioRad, Inc) (11). Each end of BAC inserts was directly sequenced and PCR primers for the ends were synthesized according to sequences obtained. Sequences of primr sets giving polymorphisms are as follows: 189R, 5′-GCAGTTCAGGGAAGTCAGCA-3′ and 5′-ATTCCTGGTC-

[1] To whom correspondence should be addressed. Fax: +81-25-227-0757. E-mail: rykomina@med.niigata-u.ac.jp.

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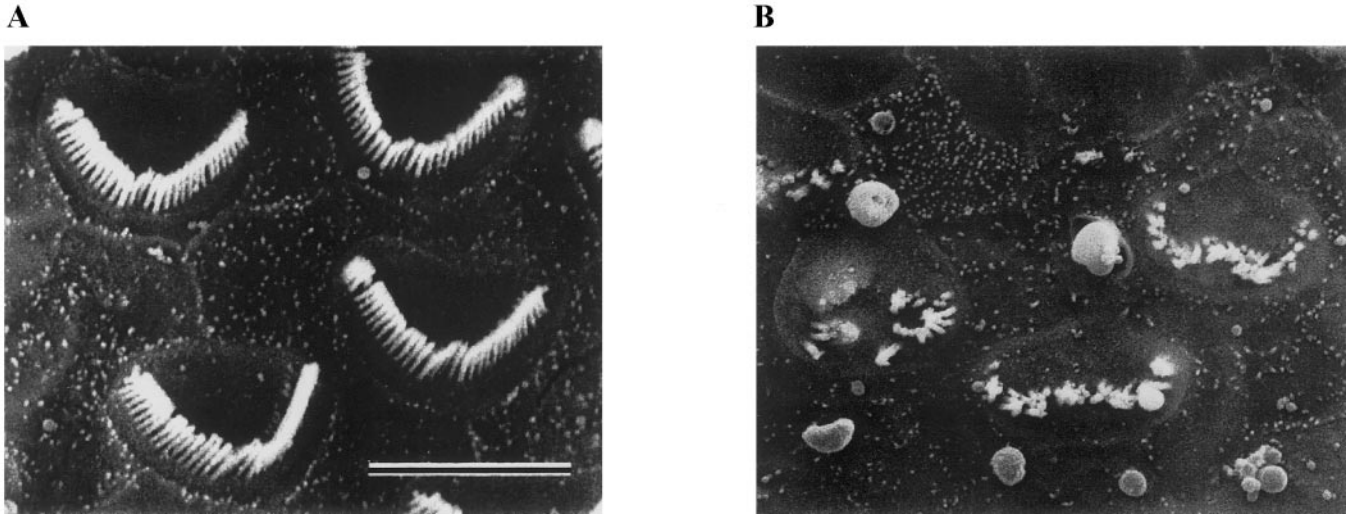

FIG. 1. Scanning electron micrographs of the apical surface of outer hair cells of 7-week-old mice. Phenotypically normal ($+/v^{ngt}$) (A) and v^{ngt}/v^{ngt} (B) littermates were analyzed. v^{ngt}/v^{ngt} homozygotes showed disorganization of stereocilia. Scale bar represents 5 μm.

TCGTGATGGC-3′; 49R, 5′-CCTTCATCAGGCTGTAGCAGG-3′ and 5′-AACCTTCCTAATGCTGCAGGC-3′; 536F, 5′-ACAGACAGATGGACGGATGG-3′ and 5′-GTCTATCTGCCCATCTATCC-3′. The polymorphism of 49R was detected by polyacrylamide gel electrophoresis of PCR products after digestion with *Ssp* I.

cDNA synthesis and sequencing. RNA was purified from heart of mice using ISOGEN (Nippon Gene Co., Japan). cDNA synthesis was primed with random hexamers on 0.01 mg of total RNA according to the manufacturer's protocol. After cDNA synthesis, parts of the *Cdh23*-specific cDNA were amplified with primers synthesized according to the *Cdh23*-sequence (8). Primers used for amplification of the cDNA fragment with mutation are as follows: 5′-GAGGTACTCCCTGGTCACTTG-3′ and 5′-TCCTGCCATGATGTTCTCCAG-3′. DNA products were directly sequenced with Dichloro-Rhodamine Dye Terminators (ABI) and an ABI 310 sequencer. Sequencing of genomic DNA bearing mutation was performed in a similar manner. Primers used are 5′-GAAGTGGAGAGCCTGGTTGC-3′ and 5′-AACACCAAAGGGTCATTGTCC-3′.

Scanning electron microscopy. Mice were fixed through the heart with 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). Dissected inner ears were stored in the same fixative for more than one day at room temperature. They were treated with 1% tannic acid solution for 5 h, washed in distilled water for 1 h, and immersed in 1% OsO_4 solution for 4 h. The specimens were dehydrated in a graded ethanol series, transferred to isoamyl-point dried using liquid CO_2. Specimens were sputter coated with platinum-palladium and examined on a Hitachi S2380N at 10 kV.

RESULTS AND DISCUSSION

Inspection of v^{ngt} homozygotes could distinguish from phenotypically normal littermates by their head shaking, circling, and loss of Preyer reflex. Preliminary genetic analysis indicated that the mutation was autosomal recessive and of complete penetrance. Hence, the v^{ngt} mice were subjected to histological examination of hair cells of the inner ear (Fig. 1). Only irregular arrays of the stereocilia and an incomplete loss of outer hair cells were seen in 7-week old v^{ngt} mice. The observation is essentially consistent with those seen in deafness mutants such as *sh-1* and *sh-2* mice (10), and accordingly the new mutant was shown to be a model for deafness.

Genetic mapping was done by genotyping backcross mice that were obtained by mating F_1 mice between v^{ngt} and MSM to v^{ngt}/v^{ngt} mutants. Analysis of a total of 1648 intersubspecific progeny demonstrated that 850 were found to be heterozygous, whereas 798 were homozygous for v^{ngt}. The segregation ratio was almost 1:1, consistent with that v^{ngt} is an autosomal recessive mutation with complete penetrance. Genome-wide scan of the backcross progeny with microsatellite markers (12) led to the construction of a genetic map that positioned v^{ngt} in a region between *D10Mit59 and D10Mit258/D10Mit172* on mouse chromosome 10. Thirty-six were found to be recombinant at *D10Mit59* while only three were recombinant at *D10Mit258/D10Mit172* (Fig. 2). Thus, *D10Mit258* and *D10Mit172* were used as start points for screening a BAC library. As shown in Fig. 2A, thirteen BAC clones were finally isolated, and their linking was achieved by using sequence-tagged sites (STSs) that were obtained from BAC-end fragments.

To position v^{ngt} on a contig of the BAC clones, we searched polymorphisms in the BAC-end fragments and used them for genotyping the backcross mice. A STS, 49R, from the 49-19L clone did not show recombination to v^{ngt} but the 536F gave one recombinant and the 189R two recombinants. The result delimited the non-recombinant region of v^{ngt} to a region covered by two BAC's (532-13L and 49-19L) (Fig. 2A). Figure 2B shows pulsed-field gel electrophoresis of *Not* I DNA digests of the two BAC's and flanking three clones. This gave an estimate that the nonrecombinant region spanned approximately 200 kb. The BAC of 49-19L was then subjected to random DNA sequencing, which attained to a level of an average of twofold coverage.

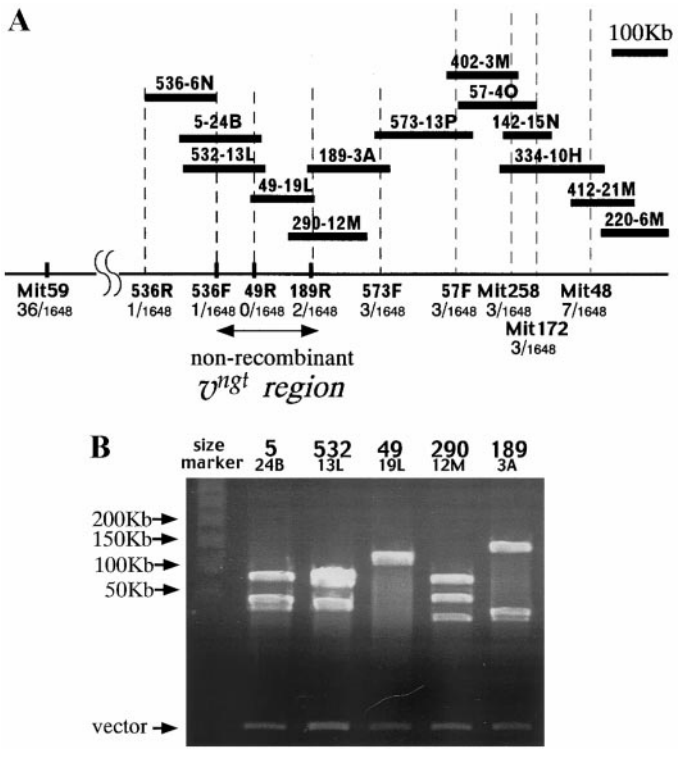

FIG. 2. Physical map of the v^{ngt} locus on mouse chromosome 10. (A) A chromosome walk on BACs across the v^{ngt} locus was completed using the flanking markers, *D10Mit258* and *D10Mit172*, as start points. Bold bars represent BACs and the name of each clone corresponds to the BAC plate number supplied by Research Genetics, Inc. A number below Mit markers indicates the fraction of recombinant mice in the region of v^{ngt} among 1648 meioses scored. The nonrecombinant region is marked by two arrows. (B) Gel staining pattern of *Not* I digests. Five BAC DNAs near the nonrecombinant region marked in the map were digested with *Not* I and subjected to pulsed-field gel electrophoresis. The marker lane contained a 50 kb fragment ladder.

Analysis of obtained sequences showed five groups of ESTs using BLASTn searches of the expressed sequence tag data base (DbEST) and also it revealed many regions showing a high score by the GRAIL computer program, suggestive of coding exons. One of such exon fragments was a part of *Cdh23*, encoding a novel type of cadherin (8).

Mouse chromosome 10 harbors several loci associated with hearing loss such as *waltzer (v)* (13) and age-related hearing loss (*ahl*) (14). The *v* and *ahl* loci were mapped in the vicinity of *D10Mit60* and *D10Mit112*, respectively (15, 16), both of which existed near the *D10Mit258* locus. Therefore, the mapping of v^{ngt} raised the possibility that v^{ngt} may be allelic to *v*. To test this, heterozygotes of v^{ngt} and homozygotes of *v* mated each other. Among 13 progeny obtained, seven showed head shaking, circling, and loss of Preyer reflex, indicating that v^{ngt} and *v* were allelic. Very recently, Di Palma *et al.* (8) have published a paper that describes the cloning of mouse *Cdh23* gene responsible for *v*. Accordingly, we searched mutation for the v^{ngt} allele of *Cdh23* gene. RT-PCR products covering the coding sequence were synthesized using heart mRNA, and their sequences were compared with those of the background ICR strain. We found a one-guanine-base deletion of GGT codon encoding glycine at the 49th in v^{ngt} mice (Fig. 3A). The deletion leads to a frame-shift mutation of *Cdh23* and is predicted to produce a truncated protein of 51 amino acids. The change was confirmed by comparison of genomic DNA sequences. A base-substitution of G to A was detected in the acceptor site of splicing junction which could provide one-base shift of the splicing position (Fig. 3A). The base-substitution was unique to v^{ngt} mice and not found in other laboratory strains (BALB/c, C3H/He, or C57BL/6) and MSM strain (data not shown). These results indicated that the mutation is probably pathologic.

Cdh23 belongs to a member of the cadherin supergene family and the protein is called otocadherin because of the mRNA expressed in the neurosensory epithelium of the organ of Corti and of the loss of function resulting in deafness (8). Cadherins are glycosylated transmembrane proteins consisting of four parts: a signal sequence, an extracellular region, a transmembrane domain, and a cytosolic carboxyl terminus (Fig. 3B) (17–19). The proteins are responsible for Ca^{2+}-dependent cell–cell adhesion, compaction and cellular rearrangement in vertebrate tissues. All members of the cadherin superfamily are characterized by a unique domain in the extracellular region, called cadherin motif or EC domain. The domain contains the negatively charged amino acid sequence motifs, which are involved in Ca^{2+}-binding, and plays a crucial role in the interaction between such adhesion molecules. *Cdh23* proteins contain a large tandem array of EC domains and belongs to a growing subfamily of cadherin-related proteins, designated as Flamingo cadherins (19). The *Cdh23* mRNA is expressed in the neurosensory hair cells of the inner ear that have precisely organized finger-like projections called stereocilia, which are arranged in bundles at their upper surface. Interestingly, mutations of the gene lead to disorganization of the stereocilia bundle (8). Therefore, these findings provide an implication that *Cdh23* is involved in interaction between stereocilia and stereocilia, i.e., the lateral links or the tip links that join adjacent stereocilia.

Three mutations in the *Cdh23* gene are found in *v* mutants; one generates a premature stop codon, another shows deletion of 119 nucleotides, and the rest leads to aberrant splice forms (Fig. 3B). The mutations predict all mutant alleles to cause loss of function. The 4th mutation found in a new mutant allele, v^{ngt} results in a flame-shift which is present in the first EC domain (Fig. 3B). This mutation is also predicted to cause loss of function. The result is

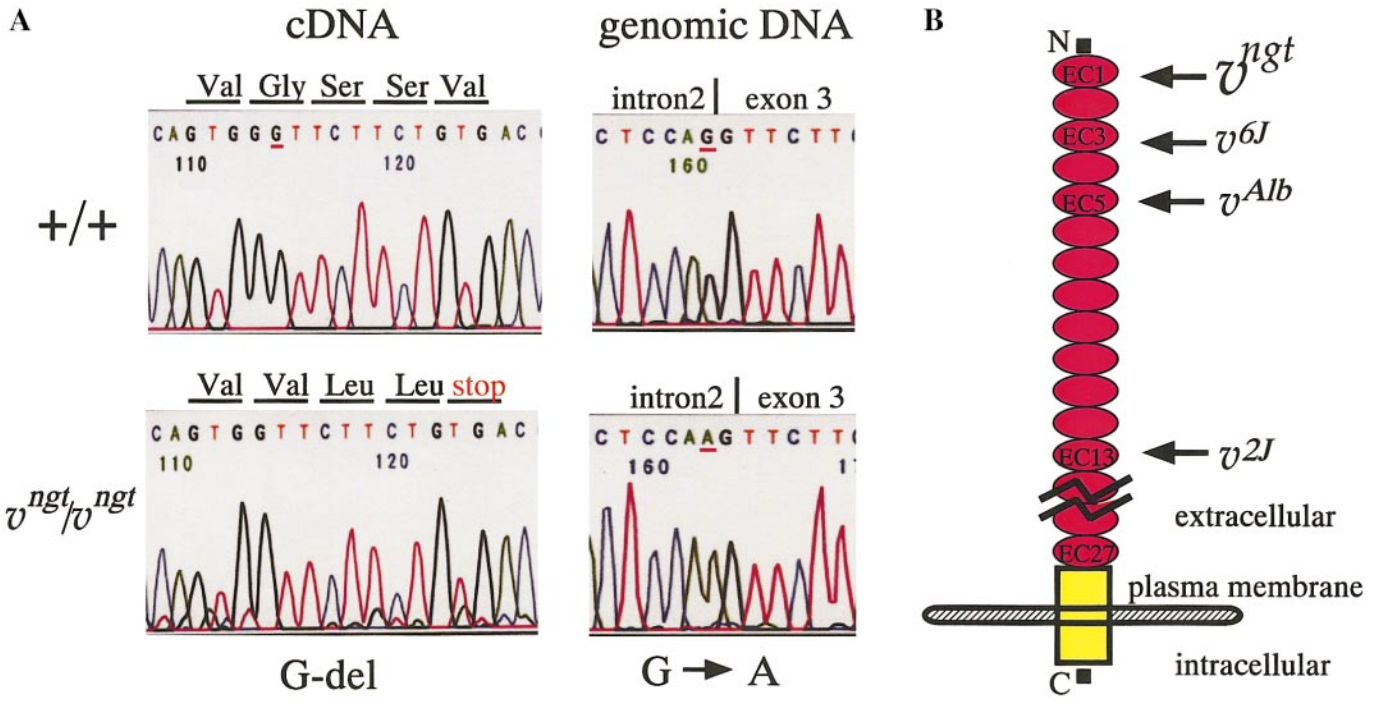

FIG. 3. (A) Sequence analyses of v^{ngt} mutations. cDNA and genomic DNA were examined of ICR(+/+) and ICR(v^{ngt}/v^{ngt}). Deletion of one guanine base is evident in v^{ngt}-derived cDNA, whereas the G to A substitution is seen in genomic DNA. The base substitution occurs in the acceptor site of splicing junction. (B) Schematic representation of the mouse otocadherin protein, Cdh23, with the position of new mutation identified in this study together with those of other allelic mutations.

consistent with similarity in phenotype and morphological changes of stereocila between v^{ngt} and other *v* mutants. Some inbred strains show age-related hearing loss (AHL) which is evaluated by auditory-evoked brain-stem response threshold analysis (14, 16). Genetic linkage analysis mapped a major locus affecting AHL near the *v* locus on mouse chromosome 10. Hence, it is suggested that *Waltzer* may be allelic to *ahl* (16). Since all four DNA changes found in *v* mutants are null mutation, the non-syndromic AHL of inbred strains could be caused by a less severe, allelic form of the *Cdh23* gene.

Importantly, the gene mutation was not only detected in mouse mutants. Mutations in human CDH23 gene were also found in patients with Usher type 1D syndrome (9).

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