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Fine mapping of *Ahl3* affecting both age-related and noise-induced hearing loss

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Abstract

A region in the vicinity of *D17Mit119* on mouse chromosome 17 harbors a susceptibility gene, designated as *Ahl3*, to age-related hearing loss (AHL). We produced congenic lines of C57BL/6 background that substituted regions around *D17Mit119* with MSM-derived ones, and examined auditory brainstem response (ABR) thresholds for their hearing capacity at 6 and 12 months of age. Three congenic lines carrying the approximately 14-Mb region between *D17Mit274* and *D17Mit183* retained normal hearing at 12 months of age whereas two congenic lines not carrying this region tended to lose hearing at that age. We also investigated noise-induced hearing loss (NIHL) in congenic lines at 1, 7 and 14 days after exposure to the noise of 100 dB for 1 h. Most congenic mice carrying the 14-Mb region did not exhibit permanent threshold shift (PTS) whereas mice not carrying this region exhibited a strong tendency of PTS, indicating the role of *Ahl3* in susceptibility to NIHL. These results indicate that *Ahl3* exists within the 14-Mb region and affects not only AHL but also NIHL.

Keywords: Age-related hearing loss; Noise-induced hearing loss; *Ahl3*; Fine mapping; Auditory brainstem response

Different susceptibility to noise-induced hearing loss (NIHL) has been suggested in human populations. Some susceptible individuals show a greater permanent threshold shift (PTS) in auditory brainstem response (ABR) threshold measurements than others when equal noise exposures are given [1,2]. The range of individual susceptibility is probably affected by genetic variation, but no contributing loci have been identified. One approach for unraveling the genetic basis of NIHL is to use mouse models [3,4], and CBA/CaJ mice exhibit no PTS after acoustic energy exposures whereas C57BL/6(B6) mice show PTS [5,6]. Age-related hearing loss (AHL) is also known to be a complex trait resulting from multiple predisposing genes and environmental factors [3,7]. A locus affecting AHL, *ahl*, was mapped to mouse chromosome 10, and further genetic analysis revealed that *Ahl* is an allele of the *Cdh23* gene, a mutation of which causes a congenital deafness in mice and humans [8]. *Cdh23* encodes a cadherin molecule thought to be a component of the tip links joining adjacent stereocilia at the top of sensory hair cells [9,10]. Genetic basis for susceptibility to NIHL and AHL appears to be correlated, since several studies reported that *Ahl* renders mice more susceptible to NIHL than strains that do not carry this susceptibility allele [5,11–13].

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The B6 mouse strain exhibits AHL beginning at 10 months of age whereas CBA and MSM strains retain normal hearing throughout life. We previously mapped another AHL locus, *Ahl3*, in the vicinity of *D17Mit119* on mouse chromosome 17 [14]. Recent studies have mapped two additional AHL loci [15]. For the *Ahl3* mapping, we used a consomic strain of B6 background substituting the B6 chromosome 17 with the MSM-derived one (B6-Chr17^{MSM}), which showed a prominent resistance to AHL. Since B6-Chr17^{MSM} differs from B6 only at chromosome 17, the MSM chromosome carries the resistant or wild-type *Ahl3* allele. This paper presents fine mapping of the *Ahl3* locus using different sets of congenic mice, which localizes *Ahl3* within a 14-Mb region on mouse chromosome 17. Furthermore, we show resistance to NIHL of the congenic mice carrying the MSM-derived 14-Mb region, indicating the role of *Ahl3* in susceptibility to NIHL as well.

Materials and methods

Mice. A panel of congenic mouse strains were produced by mating B6 with a consomic strain, B6-Chr17^{MSM}. Mice used in this study were maintained under specific pathogen-free conditions in the animal colony of Niigata University.

Noise exposure. Awake mice were housed in separate compartments of a wire cage in a wooden sound box and exposed to noise. The wire cage was divided by a wire mesh partition and one mouse placed in each division. The cage was positioned under the speaker so that the sound pressure within the cage varied by less than 2 dB SPL at 100 dB. Sound pressure levels were measured at the same point within the cage before exposure, using a sound level meter (NA-42, RION Co. Ltd., Japan) to ensure uniformity of stimulus. The noise was a pure tone of 10 kHz frequency, the sound pressure level (SPL) of 100 dB, and the duration was for 1 h. The noise was calibrated before each exposure session.

ABR procedure and assessment of hearing. Auditory brainstem response (ABR) was examined as described previously [14]. The lowest level, at which an ABR pattern could be recognized, was determined in each mouse. Mice at 6 and 12 months of age were examined for AHL and mice at 10 weeks of age were studied before and at 1, 3, 7, and 14 days after noise exposure for NIHL. Prior to measurements, mice were anesthetized with an intramuscular injection of ketamine (75 mg/kg) and xylazine (7.5 mg/kg), and placed in a close acoustic room. A stainless-steel electrode was inserted subcutaneously into the vertex (positive pole), retroauricular region (negative pole), and opposite retroauricular region (background) of each mouse. Acoustic stimuli, consisting of tone burst (0.1 ms rise and fall no plateau) at a frequency of 10 kHz, were presented to each mouse with a sound stimulator (DPS-725, Diamedical System, Tokyo, Japan) and speaker (PT-R9, Pioneer, Tokyo, Japan) in an open field. For each time point, 500 responses for each mouse were recorded and filtered for band widths of 100–3000 Hz. Software Neuropac μ (Nihonkoden, Tokyo, Japan) was used to analyze the response. Individual hearing loss was quantified based upon the threshold shift between pre- and post-exposure ABR thresholds. A PTS was defined as threshold shifts more than 20 dB above that of noise pre-exposure at 14-days post-exposure [11]. The ethics committee for animal experimentation of Niigata University approved all experimental procedures involving the mice.

Genotyping. Isolation of genomic DNA from mouse tail was carried out by following standard protocols. Genotyping of mice were carried out with polymerase chain reaction (PCR) using microsatellite markers as described previously [14]. The position of markers was from the Ensembl database. Separation of PCR products was performed by 8% polyacrylamide gel electrophoresis.

Statistics. χ^2 test was used for statistical analysis with StatView-J 5.0 software on a Macintosh personal computer. Differences were considered to be statistically significant when the P value was less than 0.05.

Histology and scanning electron microscopy. Mice were anesthetized and perfused with 2% paraformaldehyde and 2% glutaraldehyde in 0.15 M cacodylate. Cochlea were dissected from mice at 1 and 14 days after noise exposure at 10 weeks of age, and fixed in 4% paraformaldehyde overnight, and then decalcified in 5% EDTA/PBS. After decalcification for 1 week, the tissues were dehydrated, embedded in paraffin, sectioned (6 μm), and stained with hematoxylin. As for scanning electron microscopy, mice were fixed through the heart with a buffer containing 0.9% saline, 2% glutaraldehyde, and 0.1 M phosphate (pH 7.4). Immediately after perfusion, the cochlea were removed from the body and immersed in the same fixative for more than 4 days at 4 °C. After decalcification for 4 h, they were treated with 1% tannic acid solution for 3 h, washed in distilled water for 1 h, and immersed in 1% OsO_4 solution for 4 h at room temperature. The specimens were dehydrated in a graded ethanol series, transferred to isoamyl-point, and then dried using liquid CO_2. The dried specimens were coated with platinum–palladium in an ion coater and examined on a Hitachi S2380N scanning electron microscopy at an accelerating voltage of 10 kV.

Results

Fine mapping of Ahl3

Fig. 1A displays genetic constitutions of the congenic strains used in this study. Those mice were obtained by mating B6 mice with the consomic mouse strain (B6-Chr17^{MSM}) used in our previous study [14]. Mice of line A carried the MSM-derived chromosomal region from the centromere to *D17Mit141,* line B from *D17Mit274* to the telomere, and line C from *D17Mit74* to *D17Mit41*. Lines A and B, but not line C, contained the *D17Mit119* locus. Only female individuals of these three lines were used for AHL study because AHL was greater in females than in males [16]. Our ABR testing system of hearing showed the greatest elevations above normal values in threshold responses to the 10 kHz stimulus compared to those to the stimuli of 8, 16, and 32 kHz, and therefore, we used this auditory stimulus of 10 kHz by varying the sound pressure level [SPL in decibels (dB)]. Individuals that retained ABR thresholds within normal range (20–25 dB SPL) at 6 months of age were further analyzed at 12 months of age. Fig. 1B summarizes the results. Seven of the eight mice in lines A and B retained normal hearing at 12 months of age, assuming that the normal hearing was defined as elevation of ABR thresholds being less than 15 dB relative to that at 6 months. In contrast, only one of the 11 mice in line C retained normal hearing. This indicated that lines A and B carried the wild-type *Ahl3* but line C did not, suggesting that *Ahl3* exists in a region between *D17Mit274* and *D17Mit141* shared with lines A and B. This is consistent with our previous result [14].

In order to confirm the candidate region of *Ahl3*, we further generated two subcongenic lines, line D and E. Line D carried a region from *D17Mit274* to *D17Mit183* and line E from *D17Mit119* to *D17Mit74* (see Fig. 1A). ABR analysis revealed that nine individuals in line D retained normal hearing at 12 months of age and the remaining two showed

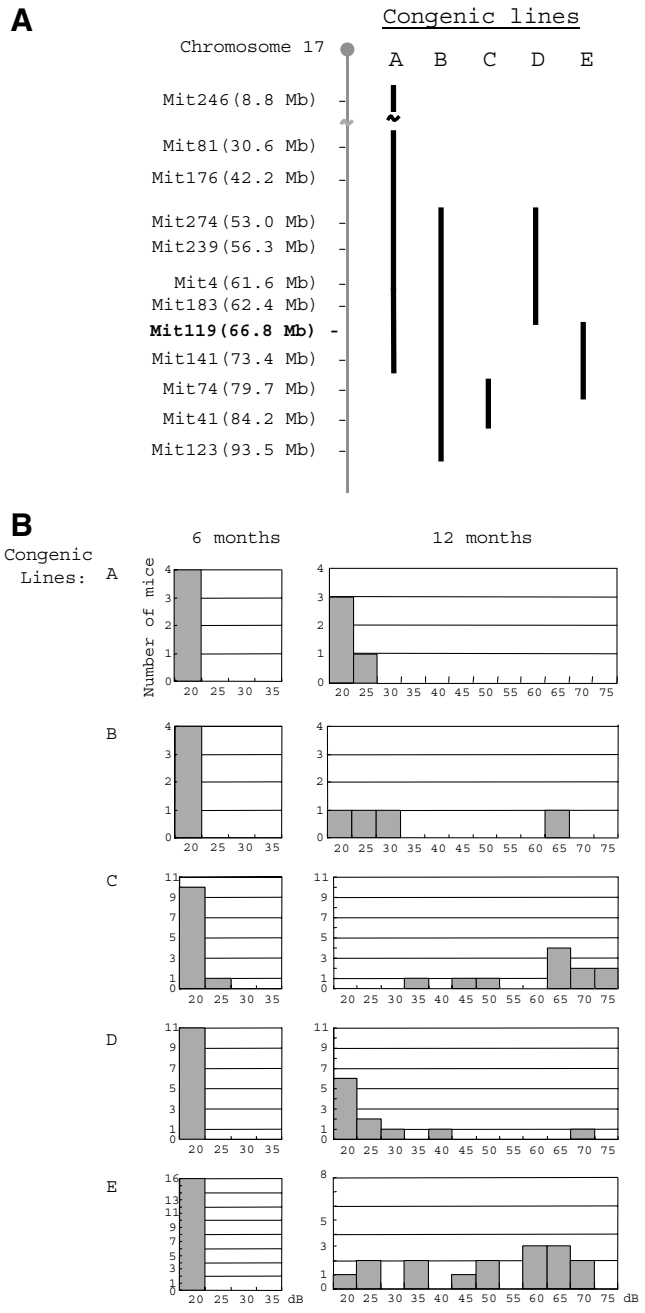

Fig. 1. Congenic mouse lines and AHL. (A) Genetic constitution of congenic mouse lines A–E of B6 background. Mit markers left to the vertical line show genetic positions (in Mb) on the chromosome 17. Vertical bars on the right side represent a chromosomal region carrying an MSM-derived region. The *D17Mit119* position is marked in bold letters, which indicates the peak of *Ahl3* determined by our previous study [14]. (B) Frequency distributions for ABR threshold values among congenic mice. Bars represent number of mice at 6 and 12 months of age that showed various ABR thresholds in increments of 5 dB.

hearing losses (Fig. 1B). In contrast, five of the 16 individuals in line E retained normal hearing at 12 months of age while 11 showed hearing loss. Difference in the number of mice between normal hearing and hearing loss of the two lines was statistically significant ($P = 0.0098$ in χ^2 test in Table 1). The results indicated that the line D mice, but not the line E mice, possessed the wild-type *Ahl3* allele, which localized *Ahl3* in an interval of approximately 14 Mb between *D17Mit274* and *D17Mit183*.

Table 1
Number of affected and unaffected mice in congenic lines of AHL and NIHL

	Congenic line (s)	Unaffected (⩽15 dB)	Affected (⩾20 dB)	*P* value in χ^2 analysis
AHL				
	D	9	2	$P = 0.0098$
	E	5	11	
NIHL				
7 days	A+B+D	16	6	$P = 0.0016$
	E	3	12	
14 days	A+B+D	20	2	$P = 0.025$
	E	9	6	

P values were calculated using χ^2 test.

Ahl3 affects not only AHL but also NIHL

Genetic basis for susceptibility to AHL and NIHL may be correlated, since three AHL mouse strains, C57, WB/ReJ, and BALB/cByJ, that carry the susceptible *Ahl* allele are more vulnerable to the permanent effects of noise overstimulation than CBA/CaJ mice possessing the wild-type or resistant *Ahl* allele [5,6,12]. Thus, *Ahl3* may also render mice more susceptible to NIHL. We tested this possibility by measuring ABR thresholds after noise exposure of 10 kHz frequency at the SPL of 100 dB for 1 h. The two groups of congenic mice were examined, one consisting of 22 mice in lines A, B, and D possessing the wild-type *Ahl3* allele and the other group of 15 mice in line E that did not carry the wild-type *Ahl3* allele. At 1, 3, 7, and 14 days after noise exposure, ABR thresholds were obtained for a specific auditory stimulus for 16 kHz. This combination of the noise exposure and hearing measurement was more efficient than others (data not shown). With this testing system, we were able to detect differences in the influence of noise overstimulation to hearing between CBA/J, BALB/c, and B6 (data not shown).

Fig. 2A shows the threshold shifts at various times after noise exposure. By 1 day after, both groups displayed elevated mean threshold shifts of 47.7 and 48.3 dB, indicating temporary threshold shift (TTS) in all mice, irrespective of genetic difference. Marked differences between the two groups were seen at 7 and 14 days after. Fig. 2B displays distribution of the threshold shifts and separation of individuals into affected and unaffected mouse groups (see Table 1). After 7 days, 16 of the 22 individuals in the lines A, B, and D group possessing the wild-type *Ahl3* allele did not show marked changes in the threshold shift (within less than 15 dB relative to the pre-exposure), suggesting the restoration of normal hearing. In contrast, 12 of the 15 line-E mice exhibited elevations in the threshold shift (more than 20 dB). After 14 days, only two individuals of the lines A, B, and D group showed PTS (9.1%), whereas six individuals

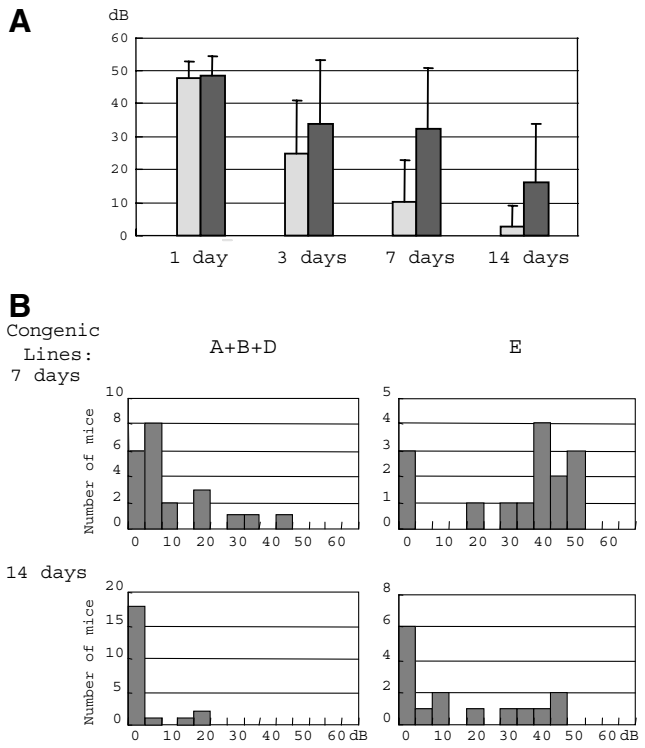

Fig. 2. Frequency distributions for ABR thresholds among congenic mice at various time periods after noise exposure. Individual hearing loss was quantified based upon the shift between pre- and post-exposure ABR thresholds. (A) The *x*-axis displays the time post-exposure and the *y*-axis shows the threshold shift. The light gray bars represent the line A+B+D mice and the dark gray bars represent the line E mice. The error bars indicate standard deviations. (B) The *y*-axis shows the number of mice that showed various ABR thresholds in increments of 5 dB.

of the line E mice exhibited PTS (40%). *P* values in χ^2 test for the differences are summarized in Table 1. The differences in susceptibility to NIHL between the two groups at 7 and 14 days post-exposure were statistically significant. These results indicate that *Ahl3* renders mice more susceptible to NIHL as well as AHL and suggest a mechanistic connection between noise-induced injury and apparent aging.

Intactness of cochlea after noise exposure

Histological examination of hair cells was performed in mice at 1 day after noise exposure. Fig. 3 shows representative micrographs of organ of Corti, spiral ganglia, and electron microscopic pictures of outer hair cells in the middle turn of cochlea. Morphology of organ of Corti and number of spiral ganglia appeared normal (Fig. 3A and B), and soundness of outer hair cells and regular arrays of the stereocilia were seen (Fig. 3C). These findings suggest that the TTS provided by our noise exposure protocol does not require impairment in morphology of the hair cells, consistent with the previous report [17].

Discussion

Genetic factors underlying AHL and NIHL are best treated as quantitative trait loci (QTL) that affect hearing loss susceptibility and not as mutant genes that directly cause hearing loss. An individual mouse with a predisposing genotype may not exhibit AHL and/or NIHL, however mice with the predisposing genotype will on average exhibit a higher incidence of those hearing impairments over the mice without. AHL observed in B6 mice is affected by susceptible (or resistant) alleles in both *Ahl3* and *Ahl* loci and the existence of a resistant allele in either locus markedly reduces the incidence of AHL [14]. We examined the effect of *Ahl3* on AHL and NIHL in mice of the *Ahl*-susceptible background. This study localizes such a QTL of *Ahl3* within the 14-Mb on mouse chromosome 17 through genetic mapping using different sets of congenic mice. In the *Ahl3* region, there are several genes that may be good candidates, including persephin (*Pspn*), neurturin (*Nrtn*), and solute carrier family members (*Slc25a23* and *Slc5a7*). The former two are GDNF family ligands that signal via the activation of the RET receptor tyrosine kinase. Of otological interest is that GDNF is expressed in the inner hair cells of the rat cochlea and induces protective effects on spiral ganglion cells after noise- or drug-induced hearing loss [18–20]. The latter two may be also interesting because mutations of the member of the solute carrier family encoding pendrin, *SLC26A4*, cause sensorineural deafness in Pendred's syndrome [21].

Another objective of the current study was to determine whether or not the *Ahl3* gene affects susceptibility to NIHL. We showed resistance to NIHL of the congenic lines harboring the wild-type *Ahl3* allele, demonstrating genetic evidence for the role of *Ahl3* in susceptibility to NIHL. Studies of knockout mice revealed that some genes

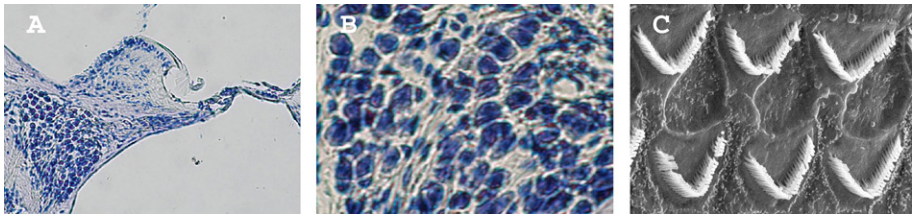

Fig. 3. Representative cochlear morphology and scanning electron micrographs of the line F mice at 10 weeks of age at 1 day after noise exposure. The middle turn of the cochlear duct is shown: (A) cochlear duct, (B) spiral ganglion cells, (C) the apical surface of outer hair cells. Intactness of cochlear morphology and regular arrays of the stereocilia were seen in all mice examined.

for ion regulators and protective mechanisms for stress-triggered factors affect noise susceptibility and AHL [4]. For instance, knockout mice for antioxidant enzyme copper/zinc superoxide dismutase (Cu/Zn SOD) and cellular glutathione peroxidase (GPX) were more vulnerable to noise and showed AHL [22,23]. It may be noteworthy that the candidates of *Ahl3* described above seem to be functionally related with these genes. However, the *Cu/Zn Sod* and *Gpx* genes themselves are not candidates because of the absence within the candidate region on mouse chromosome 17. Our findings will eventually lead to the molecular identification of *Ahl3* that helps to identify a cognate gene in human populations. This will lead to a better understanding of the pathophysiology of age-related and noise-induced hearing impairment and may contribute to the development of improved diagnosis, preventive interventions, and therapies for human presbycusis.

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