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Quantitative trait loci on chromosome 5 for susceptibility to frequency-specific effects on hearing in DBA/2J mice

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Abstract: The DBA/2J strain is a model for early-onset, progressive hearing loss in humans, as confirmed in the present study. DBA/2J mice showed progression of hearing loss to low-frequency sounds from ultrasonic-frequency sounds and profound hearing loss at all frequencies before 7 months of age. It is known that the early-onset hearing loss of DBA/2J mice is caused by affects in the *ahl* (*Cdh23^{ahl}*) and *ahl8* (*Fscn2^{ahl8}*) alleles of the cadherin 23 and fascin 2 genes, respectively. Although the strong contributions of the *Fscn2^{ahl8}* allele were detected in hearing loss at 8- and 16-kHz stimuli with LOD scores of 5.02 at 8 kHz and 8.84 at 16 kHz, hearing loss effects were also demonstrated for three new quantitative trait loci (QTLs) for the intervals of 50.3–54.5, 64.6–119.9, and 119.9–137.0 Mb, respectively, on chromosome 5, with significant LOD scores of 2.80–3.91 for specific high-frequency hearing loss at 16 kHz by quantitative trait loci linkage mapping using a (DBA/2J × C57BL/6J) F₁ × DBA/2J backcross mice. Moreover, we showed that the contribution of *Fscn2^{ahl8}* to early-onset hearing loss with 32-kHz stimuli is extremely low and raised the possibility of effects from the *Cdh23^{ahl}* allele and another dominant quantitative trait locus (loci) for hearing loss at this ultrasonic frequency. Therefore, our results suggested that frequency-specific QTLs control early-onset hearing loss in DBA/2J mice.

Key words: *ahl* locus, chromosome 5, DBA/2J, early-onset hearing loss, quantitative trait loci (QTL)

Introduction

Hearing loss is the most common sensory disease in the human population and severely affects the quality of

life. Although the causes of hearing loss are diverse, involving both genetic and environmental factors, half of hearing loss cases are considered to have a genetic origin [23]. Indeed, many genetic loci responsible for

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hearing loss have been mapped in humans by linkage analyses [Hereditary Hearing Loss Homepage: <http://hereditaryhearingloss.org/>]. Moreover, many mutations responsible for hearing loss have been recently identified in humans. Most of these mutations are primarily associated with congenital hearing loss caused by a single gene; however, little information exists concerning acquired hearing loss such as progressive hearing loss (PHL), age-related hearing loss (AHL), and presbycusis.

Acquired hearing loss is a complex disease and occurs through the effects of several quantitative trait loci (QTLs) and environmental risk factors, such as exposure to noise, aging, ototoxic drugs, and viral and bacterial infections, which are major causes of hearing loss [6, 21]. The effects of environmental risk factors pose a particular challenge to genetic analysis because understanding all of the negative effects of hearing caused by environmental factors throughout a patient's life is impossible. Therefore, the association of QTLs with acquired hearing loss is only defined at a chromosomal locus [7], although there are several challenges associated with genome-wide association studies (GWASs) [e.g., 5, 8, 34, 37].

To identify QTLs associated with acquired hearing loss, inbred strains of mice offer important advantages as bioresources because of the controlled handling environment, ability to maintain genetic stability [3], well-characterized genetic polymorphisms [16, 33], and hearing abilities among the strains [19, 38]. Within the inbred strains, several strains have been characterized as models of acquired hearing loss through measurements of hearing [13, 19, 38]. Moreover, previous studies have reported that the inbred strains exhibit variable hearing ability and onset times of hearing loss [13, 38]. For example, the C57BL/6J (B6J) strain is a well-known mouse model of late-onset AHL and develops severe hearing loss with respect to stimuli at mid frequencies (1–10 kHz) at 9–15 months of age, stimuli at high frequencies (10–20 kHz) at 4–10 months of age, and stimuli at ultrasonic frequencies (<20 kHz) at 2–6 months of age [10, 22]. By contrast, the DBA/2J (D2J) strain shows early-onset AHL (PHL). The hearing loss of D2J mice rapidly progresses to the low frequencies from the ultrasonic frequencies and to profound levels at approximately 8 months of age [15].

Previous studies have confirmed that the *ahl* mutation (*Cdh23^{ahl}*, c.753G>A) of cadherin 23 (*Cdh23*) is responsible for AHL in multiple inbred mouse strains [26].

Although both the B6J and D2J strains have a homozygous allele of *Cdh23^{ahl/ahl}*, AHL occurs significantly much earlier in D2J than B6J. This difference in AHL onset time is suggested to contribute to other genetic factors in addition to the *Cdh23^{ahl}* allele in D2J mice. Johnson *et al.* [15] found that the early-onset AHL of D2J mice was significantly associated with a locus on chromosome 11, designated *ahl8*. Moreover, Shin *et al.* [31] confirmed that the responsible mutation of *ahl8* is the c.326G>A missense mutation (p.Arg109His) in the fascin 2 gene (*Fscn2*), which encodes an actin cross-linking protein. In addition, Nagtegaal *et al.* [25] reported that the *ahl9* locus on chromosome 18 is tightly linked to early-onset and low-frequency-specific AHL in D2J mice. Thus, these studies suggest that the onset of AHL is accelerated by the effects of several susceptibility QTLs.

In this study, we reported that the hearing loss in D2J mice is more severe with high-frequency (16 kHz) and ultrasonic-frequency (32 kHz) stimuli than with mid-frequency (8 kHz) stimuli. Moreover, we also report that the early-onset AHL in D2J mice is associated with QTLs on chromosome 5 that only show high-frequency-specific effects on hearing loss.

Materials and Methods

Mice and ethics statement

The DBA/2Jcl (D2J) and C57BL/6Jcl (B6J) strains were purchased from Clea Japan (Tokyo, Japan) and were maintained at the animal facility at the Tokyo Metropolitan Institute of Medical Science and the Tokyo University of Agriculture. All of the procedures involving animals met the guidelines described in the Proper Conduct of Animal Experiments, as defined by the Science Council of Japan, and were approved by the Animal Care and Use Committee on Ethics of the Tokyo Metropolitan Institute of Medical Science and the Tokyo University of Agriculture.

Measurements of the auditory brain stem response

An auditory brain stem response (ABR) workstation (TDT System III, TDT, Alachua, FL, USA) was used to test mice for ABR thresholds as previously described [22]. The ABR thresholds from both ears in all of the mice were measured using a tone pip stimulus at 8, 16, and 32 kHz.

Linkage quantitative trait locus analyses

Genetic mapping of the susceptibility loci in D2J mice linked to early-onset AHL was performed using (D2J × B6J) F₁ (BDF₁) × D2J backcross progeny (N₂). DNA samples from 90 N₂ mice were genotyped using 103 polymorphic microsatellite markers located throughout the mouse genome (Supplementary Table 1), which were selected from the Microsatellite Data Base of Japan (<http://www.shigen.nig.ac.jp/mouse/mmdbj/top.jsp>) based on size variation between PCR products from D2J and B6J mice. PCR amplification was carried out using a KAPA2G Fast PCR Kit (Kapa Biosystems, Woburn, MA, USA), and the cycle conditions were as follows: 1 cycle at 95°C for 2 min and 40 cycles at 95°C for 15 s, 58°C for 20 s, and 72°C for 5 s. The PCR products were then subjected to 4% agarose gel electrophoresis. ABR thresholds at 8, 16, and 32 kHz at 3 months of age (± 5 days) were evaluated as quantitative traits for genetic mapping in the N₂ mice. The linkage mapping was performed by QTL interval mapping using the R/qtl program of the R Statistical package to perform single-QTL genome scans by Haley–Knott regression using a step size of 1.0 cM [2]. The logarithm of odds (LOD) scores for significance were calculated by 1,000 permutations testing using R/qtl. Additional statistical comparisons were evaluated using the Student's *t*-test with Welch's correction for the differences between two groups and one-way ANOVA with Tukey's post hoc multiple comparison test for differences among multiple groups. GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA) was used to calculate column statistics and compute *P* values. All of the results are presented as the mean ± standard deviation (SD).

Mutation screening

Mutation analysis was performed on cDNA synthesized as previously described [22] from cochleae at 3 weeks of age and brain stems at 12 weeks of age in D2J and B6J mice using primers (Supplementary Table 2) designed to amplify and sequence the coding exons and partial UTRs of the urocortin (*Ucn*), glutaredoxin, cysteine rich 1 (*Grxcr1*), purinergic receptor P2X, ligand-gated ion channel, 2 (*P2rx2*), *Sad1* and UNC84 domain containing 1 (*Sun1*), Fascin 1 (*Fscn1*), and actin, beta (*Actb*), genes. For partial sequencing of *P2rx2* and *Sun1*, we used genomic DNA to screen the mutations because we failed to sequence the 3' region of their cDNAs for alternative splicing. Amplification was carried

out at 94°C for 2 min followed by 45 cycles of 98°C for 10 s and 68°C for 3 min using KOD-FX Neo (Toyobo, Osaka, Japan). The products were purified using a QIAquick Gel Extraction Kit (Qiagen, Valencia, CA, USA), sequenced using a BigDye Terminator Kit (Life Technologies, Grand Island, NY, USA), and analyzed using an Applied Biosystems 3130xl Genetic Analyzer.

Quantitative RT-PCR

The cDNAs from the cochleae in D2J and B6J mice at 3 weeks were used for quantitative RT-PCR (qRT-PCR). *Grxcr1*, *P2rx2*, *Sun1*, *Fscn1*, and *Actb* were amplified and quantified using a QuantiTect SYBR Green PCR Kit (Qiagen, Valencia, CA, USA) and LightCycler 480 Instrument (Roche Diagnostics, Tokyo, Japan), respectively, according to the manufacturer's protocol. The primers used for the detection of these transcripts are shown in Supplementary Table 3. The signal values were normalized to the hypoxanthine-guanine phosphoribosyl transferase (*Hprt*) median signals, and the geometric means of target signals were calculated in triplicate. The gene expression levels were compared between the D2J and B6J mice using the Student's *t*-test with Welch's correction.

Results

Hearing assessment of D2J mice

Although data for ABR thresholds in DBA/2J mice have been reported by several research groups, there are moderate differences [13, 15, 25, 31, 38]. To evaluate hearing abilities, we measured the ABR to tone-pip stimuli at 8, 16, and 32 kHz in mice from a D2J strain. Figure 1 shows the representative ABR waveforms with 8-, 16-, and 32-kHz stimuli recorded from B6J and D2J mice at 2 months of age. In the waveforms from B6J mice, the latency peak responses for peaks I–V were detected at all frequencies, but the peak response was decreased at 32 kHz. By contrast, the waveforms from D2J mice lack peak V and obviously showed decreased peak responses at all frequencies compared with those of B6J mice (Fig. 1). We next determined the ABR thresholds for tone-pip stimuli at 8, 16, and 32 kHz in D2J mice at 1–12 months of age at 1-month intervals compared with our previous data for B6J mice [22]. At 1 month of age, the ABR thresholds of the D2J mice were significantly higher than those of B6J mice for all frequency stimuli, and the mice exhibited levels of se-

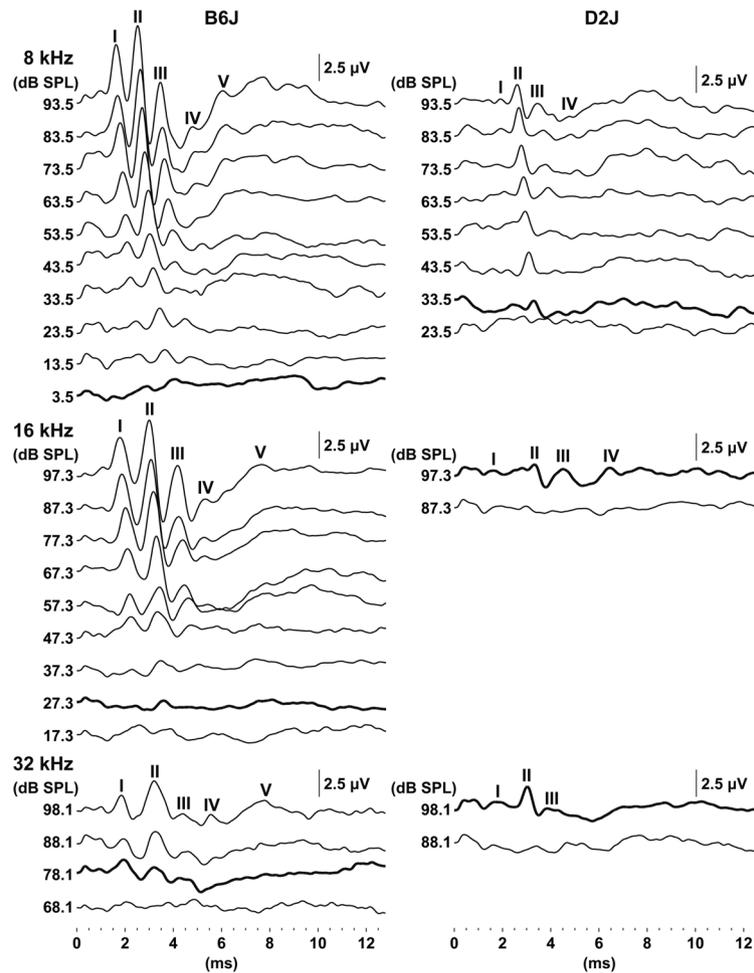


Fig. 1. Representative ABR waveforms with 8-, 16-, and 32-kHz stimuli recorded from B6J and D2J mice at 2 months of age. The waveforms represent the ABR in response to the intensities of tone-pip stimuli decreasing from 93.5 to 3.5 dB SPL at 8 kHz, from 97.3 to 17.3 dB SPL at 16 kHz, and from 98.1 to 68.1 dB SPL at 32 kHz. Bold lines represent the detected thresholds. The locations of peaks I–V are indicated.

were (71–90 dB SPL) and profound (<91 dB SPL) hearing loss at 16 and 32 kHz, respectively (Fig. 2 and Supplementary Table 4). The hearing loss of the D2J mice rapidly progressed to the low frequencies from the high frequencies and to profound levels at the mean thresholds for 8-kHz stimuli at 7 months of age and 16-kHz stimuli at 3 months of age (Fig. 2 and Supplementary Table 4). Although the ABR thresholds in D2J mice were slightly high compared with those in previous reports, the patterns of hearing degeneration were similar to those in most reports [13, 15, 31, 38].

Genetic analyses for early-onset AHL in D2J mice

Previous studies have reported that high-frequency and low-frequency early-onset AHL are linked to the *ahl8* locus [15] on chromosome 11 and to the *ahl9* locus [25] on chromosome 18. To confirm the genetic effects in early-onset AHL in D2J mice, we produced F₁ and backcross N₂ progeny by crossing with B6J mice and measured the ABR thresholds for 8-, 16-, and 32-kHz stimuli at 3 months of age. The mean ABR thresholds at 8 and 16 kHz in BDF₁ mice were 16.8 ± 10.1 dB SPL and 21.4 ± 6.3 dB SPL, respectively, and were not significantly different compared with the thresholds (22.9 ± 4.7 dB SPL at 8 kHz and 27.4 ± 6.9 dB SPL at 16 kHz)

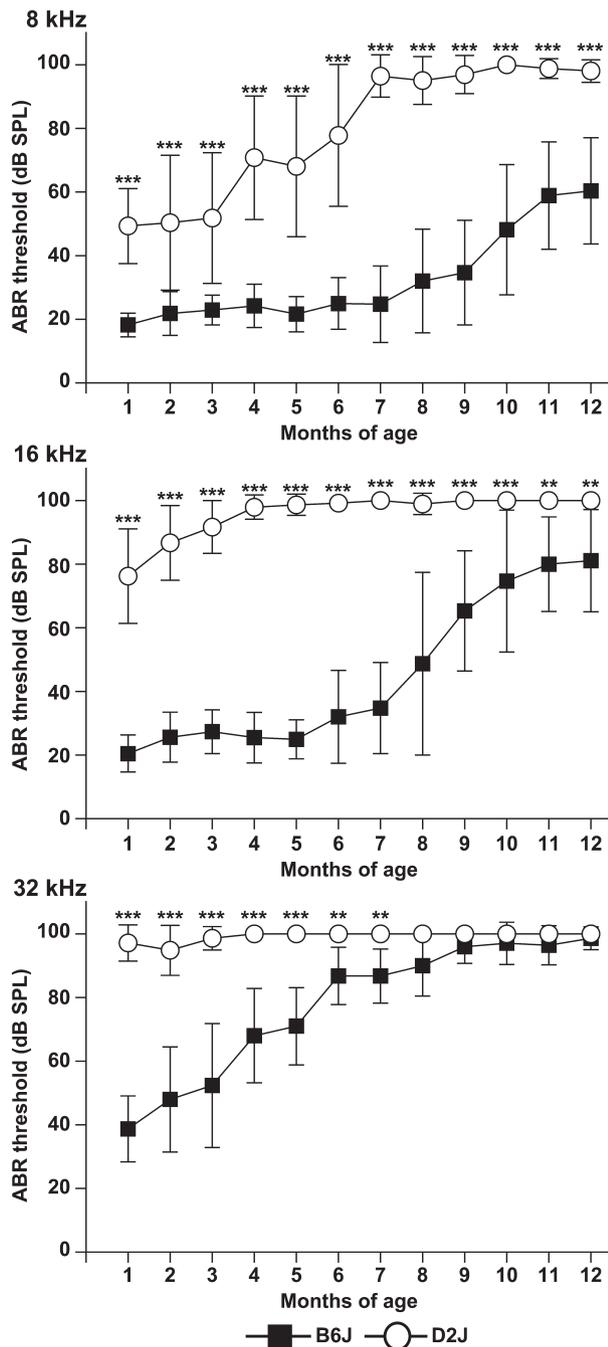


Fig. 2. Early-onset progressive hearing loss in D2J mice. The means (black squares in B6J and white circles in D2J) and SDs (error bars) of ABR threshold measurements for 8-, 16-, and 32-kHz stimuli are shown for each mouse strain at 1 to 12 months of age. The number of ears tested is listed in Supplementary Table 4. ** $P < 0.01$; *** $P < 0.001$.

[22] of B6J mice (Fig. 3A and Supplementary Table 4). The ABR thresholds at 8 and 16 kHz in (BDF₁ × D2J) N₂ mice were 31.9 ± 20.5 and 62.1 ± 25.7 dB SPL, re-

spectively, which were slightly higher than those of B6J mice (Supplementary Table 4). Although the distribution of ABR thresholds for 8-kHz stimuli in the (BDF₁ × D2J) N₂ mice barely followed a normal distribution ($R^2 = 0.7525$), the thresholds of the N₂ population were widely distributed from high to low levels (Fig. 3B). By contrast, the mean ABR thresholds at 32 kHz in the BDF₁ and (BDF₁ × D2J) N₂ mice were 91.4 ± 7.4 and 94.0 ± 7.8 dB SPL, respectively, and were similar to the threshold (98.6 ± 3.7) detected in D2J mice (Fig. 3A and Supplementary Table 4). These data for the ABR thresholds from the F₁ and N₂ populations suggested that the QTLs responsible for hearing loss in D2J mice differed according to the sound frequency.

To define the susceptibility locus underlying hearing loss in D2J mice, we performed genome-wide QTL linkage mapping based on the ABR thresholds with 8- and 16-kHz stimuli, which showed a normal distribution. QTL linkage analysis of the (BDF₁ × D2J) N₂ progeny revealed a strong QTL on chromosome 11 with significant LOD scores of 5.02 at 8 kHz and 8.84 at 16 kHz (Fig. 4A and Table 1). The locus (marker *D11Mit103*) was in the same position as *ahl8*, indicating a strong effect of the homozygous *Fscn2^{ahl8}* allele on hearing loss in D2J mice. Although we could not detect the other QTL with significant LOD scores at 8 kHz, including the *ahl9* locus on chromosome 18, QTL peaks were revealed on chromosome 5 with significant LOD scores of 2.80–3.91 at 12 markers located approximately within the 52.7–126.1 Mb region by analysis using ABR thresholds at 16 kHz (Fig. 4B and Table 1). The peak LOD score of 3.91 on chromosome 5 was detected at marker *D5Mit233* (52.7 Mb; Fig. 4B and Table 1). Subsequently, the second and third peak LOD scores of 3.89 and 3.78 were detected at markers *D5Mit214* (126.1 Mb) and *D5Mit7* (93.3 Mb), respectively, suggesting that there are multiple QTLs on chromosome 5 that contribute to hearing loss at 16 kHz in D2J mice (Fig. 4B and Table 1).

Next, we analyzed the associations between phenotypes and genotypes in (BDF₁ × D2J) N₂ mice to confirm the effects of the *Fscn2^{ahl8}* and QTLs on chromosome 5 because it is possible that the QTLs on chromosome 5 are false positives due to the limited sample size and distribution across a wide range on chromosome 5 (Fig. 4 and Table 1). First, we reconfirmed this analysis using *D11Mit103* to mark the *Fscn2^{ahl8}* locus and evaluated its effect on hearing loss at 16 kHz in N₂ mice. Most N₂ mice exhibited elevated ABR thresholds from the DD

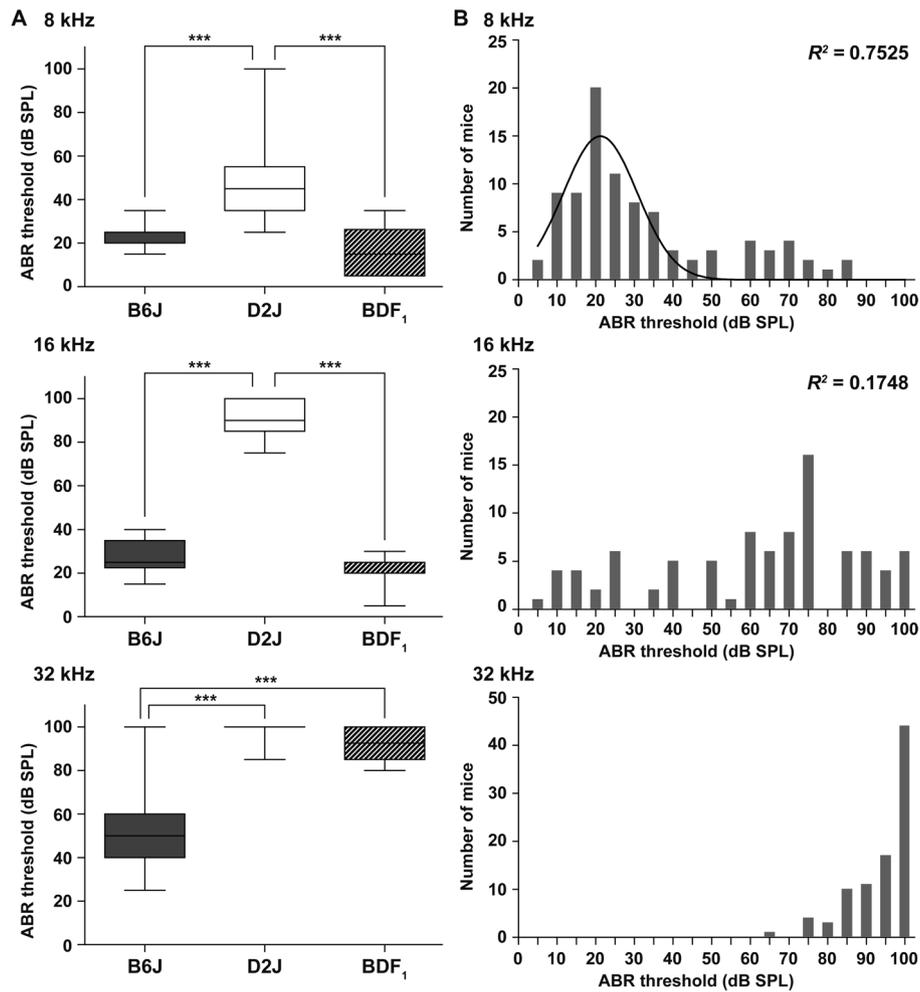


Fig. 3. Distributions of ABR thresholds in B6J, D2J, BDF₁, and (BDF₁ × D2J) N₂ mice. (A) Distributions of ABR threshold measurements for 8-, 16-, and 32-kHz stimuli in B6J, D2J, and BDF₁ mice at 3 months of age. The lines within boxes and error bars indicate the median and max/min thresholds, respectively. *** $P < 0.001$. (B) Distributions of ABR thresholds for 8-, 16-, and 32-kHz stimuli among the (BDF₁ × D2J) N₂ mice. The best-fit curves for a Gaussian distribution are shown in black. The regression coefficient for the curve is given as R^2 calculated by the D'Agostino and Pearson tests.

homozygous genotype for the D2J allele at this locus (Fig. 5). Although some N₂ mice with the DB heterozygous genotype at the *Fscn2^{ahl8}* locus developed hearing loss, the ABR thresholds from the DD and DB genotypes were significantly different (mean difference: 31.5 dB SPL, $P < 0.001$, Fig. 5). Second, we analyzed the association between hearing loss and genotypes of *D5Mit233*, which showed the highest peak LOD on chromosome 5 in the QTL linkage analysis (Fig. 4 and Table 1). The mean difference between the DD and DB genotypes at this locus was 21.9 dB SPL. The difference was smaller than that of the *Fscn2^{ahl8}* locus, but it showed a significant difference (Fig. 5). Third, we investigated the indi-

vidual effects of the genotypes of QTL and the interaction between the D2J alleles at both *D11Mit103* and *D5Mit233*. The mean thresholds of individuals with hearing loss were decreased with the DB heterozygous genotype at both *D11Mit103* and *D5Mit233* compared with the DB heterozygous genotype at *D11Mit103* only (Fig. 5). Moreover, the threshold in N₂ mice with the DD genotype at *D5Mit233* was significantly higher than that of the N₂ mice with the double DB heterozygous genotype at both *D11Mit103* and *D5Mit233*, even if the genotype was DB heterozygous at *D11Mit103* (Fig. 5). Thus, these results suggest that the DD homozygous allele at *D5Mit233* contributed to the hearing loss at

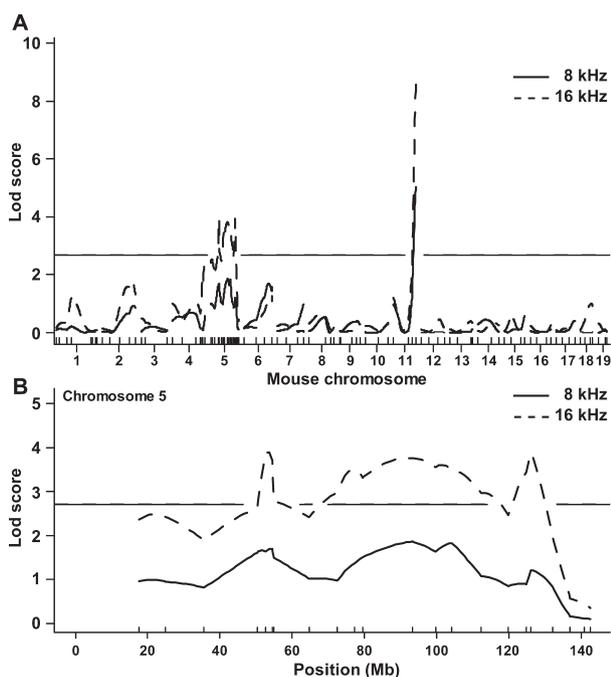


Fig. 4. Genome linkage analysis for early-onset hearing loss in D2J mice. (A) Genome-wide interval mapping for susceptibility genes associated with early-onset hearing loss for 8- (solid curve) and 16-kHz (dotted curve) stimuli of the (BDF₁ × D2J) N₂ mice. Chromosome numbers and marker positions (vertical bars) are given below the linkage map. The horizontal lines indicate significant LOD thresholds at 8 (solid, LOD = 2.69) and 16 (dotted, LOD = 2.71) kHz, respectively. (B) Magnified view of the significant QTLs at chromosome 5.

16 kHz in N₂ mice but that the effect of the DD homozygous allele at the *Fscn2^{ahl8}* locus was significant and was not affected by the genotypes at *D5Mit233* (Fig. 5). Additionally, a similar effect was exerted by DD homozygous alleles in the other QTLs on chromosome 5 (Supplementary Fig. 1).

Most N₂ mice with the DD homozygous genotypes at both *D11Mit103* and *D5Mit233* exhibited elevated ABR thresholds, and the mean threshold was identical to that of the DD homozygous genotype at *D11Mit103* only (Fig. 5).

Analyses of candidate genes on chromosome 5

There are many genes related to hearing loss that function on chromosome 5 in mice [The Jackson Laboratory, Hereditary Hearing Impairment in Mice: <http://hearingimpairment.jax.org/>]. Because D2J mice showed degeneration of stereocilia in inner ear hair cells and because more severe degradation of stereocilia in the

Table 1. Detected susceptibility QTLs for early-onset AHL in (BDF₁ × D2J) N₂ mice

Frequency (kHz)	Chr*	Marker name	Ensembl position (bp)	LOD score
8	11	<i>D11Mit203</i>	116363361	4.95
	11	<i>D11Mit103</i>	117167319	5.02
16	5	<i>D5Mit233</i>	52697226	3.91
	5	<i>D5Mit300</i>	54486435	3.71
	5	<i>D5Mit255</i>	54954339	2.80
	5	<i>D5Mit336</i>	72488531	3.00
	5	<i>D5Mit113</i>	77255215	3.52
	5	<i>D5Mit309</i>	79502721	3.34
	5	<i>D5Mit7</i>	93297205	3.78
	5	<i>D5Mit155</i>	99690609	3.58
	5	<i>D5Mit10</i>	104239005	3.50
	5	<i>D5Mit24</i>	112317683	2.98
	5	<i>D5Mit95</i>	124829235	3.46
5	<i>D5Mit214</i>	126090176	3.89	
11	<i>D11Mit203</i>	116363361	8.35	
11	<i>D11Mit103</i>	117167319	8.84	

*Chromosome.

basal turn is consistent with the initial onset of high-frequency hearing loss [29, 31], we screened genes on chromosome 5 with known relationships with the development and maintenance of stereocilia as candidates that could be responsible for hearing loss in D2J mice. We selected six genes—*Ucn*, *Grxcr1*, *P2rx2*, *Sun1*, *Fscn1*, and *Actb*—as candidates, and their expressions were observed in stereocilia [1, 11, 27, 28, 35, 36]. Although the LOD scores were extremely low in the immediate vicinity of markers with *Sun1*, *Fscn1*, and *Actb* (Fig. 6A), we performed resequencing analysis of the coding region corresponding to the published sequences of their genes on cDNA and/or genomic DNA from D2J and B6J mice. However, resequencing of the coding regions of their genes did not show any sequence variation in either strain. Next, we carried out qRT-PCR analysis to validate the differences in gene expression in their genes using RNA isolated from the cochleae of D2J and B6J mice at 3 weeks of age because it is possible that mutations in the *cis*-acting elements of their genes affect gene expression. We performed qRT-PCR analyses in five genes because the bands of *Ucn* were not detected via RT-PCR analysis using RNAs isolated from the cochleae of both mice. Four of these transcripts—*Grxcr1*, *P2rx2*, *Sun1*, and *Actb*—did not exhibit significant expression changes between the D2J and B6J mice, but *P2rx2* was up-regulated by ~1.5-fold at the mean expression level in D2J mice (Fig. 6B). Although the significant downregulation of ~2.0-fold in D2J mice was only detected in

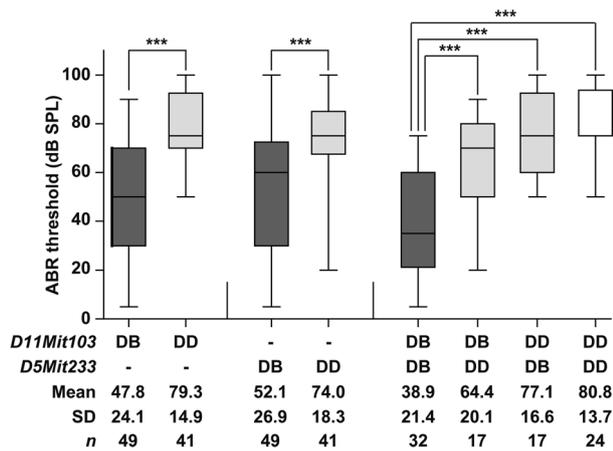


Fig. 5. Effects of the *Fscn2^{ahl8}* locus and a susceptibility locus on chromosome 5 on ABR thresholds at 16 kHz in (BDF₁ × D2J) N₂ mice. The lines within boxes and error bars indicate the median and max/min thresholds, respectively. The *Fscn2^{ahl8}* locus and a susceptibility locus on chromosome 5 were genotyped with *D11Mit103* and *D5Mit233*, respectively. ****P* < 0.001.

Fscn1, a member of the Fascin family, in addition to *Fscn2* [9] (Fig. 6B), we found that the LOD scores of the mapped position were extremely low (Fig. 6A).

Discussion

The D2J strain is a well-known mouse model of early-onset AHL. In this study, we demonstrated that hearing loss increased to mid frequencies from ultrasonic frequencies according to the in ABR thresholds (Figs. 1 and 2; Supplementary Table 4). Previous studies have reported that early-onset AHL in D2J mice is controlled by the QTLs *Cdh23^{ahl}* and *Fscn2^{ahl8}* [15]. We also confirmed the significant effect of *Fscn2^{ahl8}* on chromosome 11 for hearing loss with 8- and 16-kHz stimuli by QTL linkage analysis using (BDF₁ × D2J) N₂ mice (Fig. 4 and Table 1). Using QTL analysis, we also found significant QTLs that contributed to hearing loss with only 16-kHz stimuli on chromosome 5. These QTLs were primarily detected as two comparatively sharp curves at peak LOD scores of the markers *D5Mit233* (52.7 Mb) and *D5Mit214* (126.1 Mb) and a gently sloping curve at peak LOD scores of the marker *D5Mit7* (93.3 Mb). Among the QTLs, a QTL in the distal region (119.9–137.0 Mb) on chromosome 5 was also detected as suggested by previous studies on early-onset hearing loss with 16-kHz stimuli in BXD recombinant inbred strains [15, 25]. Moreover, the individual effect of each QTL on chromo-

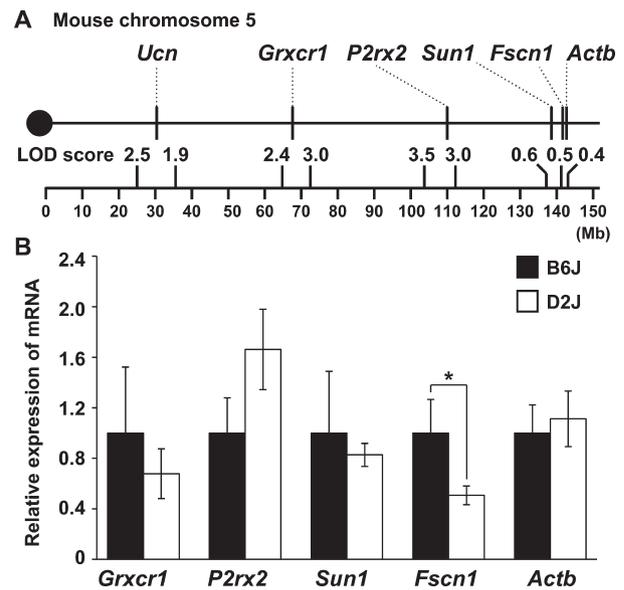


Fig. 6. Expression analysis of five selected candidate genes associated with the degeneration of stereocilia of the inner ear hair cells on chromosome 5. (A) The chromosomal locations of five candidate genes, *Grxcr1*, *P2rx2*, *Sun1*, *Fscn1*, and *Actb*, for the susceptibility to early-onset PHL in D2J mice. The LOD scores (16 kHz) of markers in the vicinity of their candidate genes are indicated. (B) The relative levels of mRNA in the cochlea of B6J (black bars) and D2J (white bars) mice at 3 weeks of age. mRNA expression was detected using real-time quantitative RT-PCR analysis. **P* < 0.05.

some 5 was observed on early-onset hearing loss with 16-kHz stimuli (Fig. 5 and Supplementary Fig. 1), although we could not distinguish whether the QTLs effected an additive or nonadditive influence on early-onset hearing loss in D2J mice because the recombinants in the QTL regions on chromosome 5 of the N₂ mice were insufficient to analyze the interactions among the QTLs.

The auditory systems of the mammals that perceive sounds in air are organized based on the separation of complex sounds into component frequencies [20]. This process begins at the level of the auditory sensory epithelium, where specific frequencies are distributed along the tonotopic axis of the mammalian cochlea such as the apex, middle, and base areas of the cochlea that correspond to the detection of sounds at low, mid, high, and ultrasonic frequencies, respectively [20, 24]. QTLs contributing to frequency-specific hearing loss have been reported in mice [17, 18]. D2J mice represent a typical model for frequency-specific hearing loss according to the contributions of different QTLs. Nagtegaal *et al.* [25]

suggested that *Cdh23^{ahl}* and *Fscn2^{ahl8}* alleles make a large contribution to early-onset high-frequency hearing loss in D2J mice. The study also suggested that the *ahl9* locus on chromosome 18 affects early-onset hearing loss with only low-frequency (4 kHz) stimuli in D2J mice. In this study, we indicated that the QTLs on chromosome 5 affect early-onset hearing loss with only high-frequency (16 kHz) stimuli (Fig. 4), although their effect can be demonstrated by phenotypic analyses for hearing in congenic mice in QTL regions on chromosome 5. In addition, we demonstrated that the contribution of *Fscn2^{ahl8}* towards early-onset hearing loss with an ultrasonic frequency stimulus of 32 kHz is extremely low. This was because most *Fscn2^{ahl8/+}* heterozygotes from BDF₁ and (BDF₁ × D2J) N₂ mice showed profound hearing loss at 3 months of age (Fig. 3). *Cdh23^{ahl}* is believed to play a major role in this process, as all mice belonging to the BDF₁ and (BDF₁ × D2J) N₂ groups displayed the homozygous *Cdh23^{ahl}* allele. However, the ABR thresholds of BDF₁ and (BDF₁ × D2J) N₂ mice were similar to that observed in D2J mice and extremely high compared with that of B6J mice (Fig. 3). Therefore, we speculated that another dominant modifier gene(s) may have resulted in early-onset hearing loss at ultrasonic frequency. Altogether, these data indicated that the hearing loss of D2J mice is controlled by frequency-specific functional QTLs.

Many genes and several mutations in different genes are contained in the QTLs on chromosome 5 [Wellcome Trust Sanger Institute, Mouse Genomes Project: <http://www.sanger.ac.uk/resources/mouse/genomes/>]. We performed mutation screening and expression analysis for six of these genes, which are expressed in the stereocilia of cochlear hair cells [1, 11, 27, 28, 35, 36]. Of these genes, *Fscn1* is a functional strong candidate gene because it is a paralogous gene of *Fscn2* and binds to crosslinks of actin filaments like *Fscn2* [9]. *Actb* is also a functional candidate. *Actb*-flox Foxg1-cre mice show age-related degeneration of stereocilia similar to the short stereocilia phenotypes in D2J mice [28]. Although sequence variations were not detectable in the coding sequences of either gene, we found that expression of the *Fscn1* transcript was significantly downregulated in the cochlea in D2J mice (Fig. 6B). However, the LOD scores (0.4–0.5) of the immediate vicinity markers with *Fscn1* were extremely low (Fig. 6A). *Fscn1* is abundant in stereocilia bundles [32]; therefore, we speculated that the reduction in *Fscn1* may be caused by structural de-

fects in the stereocilia of D2J mice. Additionally, there were no sequence variations and significant expression changes in the other functional candidate genes of the QTL regions on chromosome 5.

In mice, the *ahl2* locus is mapped to chromosome 5, with a peak LOD score of 5.5 for *D5Mit309* (79.5 Mb), and is associated with early-onset AHL in NOD/LtJ mice [14]. The LOD score of *D5Mit309* is 3.34 for hearing loss with 16-kHz stimuli by QTL linkage analysis using (BDF₁ × D2J) N₂ mice (Table 1), and the *ahl2* position is included in the interval (64.6–119.9 Mb) of a QTL (Fig. 4B). Therefore, the QTL may be allelic to the recessive deafness gene at the *ahl2* locus.

In humans, several loci associated with hearing loss are mapped to the syntenic region with mouse chromosome 5 [Hereditary Hearing Loss Homepage: <http://hereditaryhearingloss.org/>]. Within their loci, possible mutations in a human gene orthologous to the QTLs include two loci, DFNB40 and DFNB55, for recessive non-syndromic early-onset AHL. The DFNB40 and DFNB55 loci are included in the interval of a QTL (64.6–119.9 Mb) for early-onset AHL in D2J mice [4, 12]. Moreover, the QTL overlaps with the DFNA27 locus for dominant early-onset AHL [30]. Although the causative mutations of the loci remain unknown, D2J mice are likely to provide useful information regarding cloned mutations from their loci if causative mutations for the QTLs associated with early-onset AHL in D2J mice can be identified in future genetic analyses.

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