Published in final edited form as:

Hear Res. 2011 May ; 275(1-2): 150–159. doi:10.1016/j.heares.2010.12.017.

Polygenic inheritance of sensorineural hearing loss (*Snhl2*, -3, and -4) and organ of Corti patterning defect in the ALR/LtJ mouse strain

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Abstract

Progressive sensorineural hearing loss in humans is a common and debilitating impairment. Sensorineural deafness in inbred strains of mice is a similarly common and genetically diverse phenotype providing experimental models to study the underlying genetics and the biological effects of the risk factors. Here, we report that ALR/LtJ mice develop early-onset profound sensorineural hearing loss as evidenced by high-to-low frequency hearing threshold shifts, absent distortion-product otoacoustic emissions, and normal endocochlear potentials. Linkage analyses of a segregating backcross revealed three novel quantitative trait loci named *sensorineural hearing loss* (*Snhl*) -2, -3, and -4. The QTLs achieved very high LOD scores with markers on chromosome 1 (*Snhl2*, LOD: 12), chromosome 6 (*Snhl3*, LOD: 24) and chromosome 10 (*Snhl4*, LOD: 11). Together, they explained 90% of the phenotypic variance. While *Snhl2* and *Snhl3* affected hearing thresholds across a broad range of test frequencies, *Snhl4* caused primarily high-frequency hearing loss. The hearing impairment is accompanied by an organ of Corti patterning defect that is characterized by the ectopic expression of supernumerary outer hair cells organized in rows along the abneural site of the sensory epithelium in the presence of unaltered planar polarity and otherwise normal cochlear duct morphology. Cloning the *Snhl2*, -3, and -4 genes in the ALR/LtJ mice may provide important genetic and mechanistic insights into the pathology of human progressive sensorineural deafness.

Keywords

ALR/LtJ; genetics; sensorineural hearing loss; quantitative trait locus; polygenic inheritance; organ of Corti; patterning defect

1. Introduction

Sensorineural hearing loss (SNHL) is a common sensory impairment in the human population and is a major underlying component of noise-induced hearing loss (NIHL) and age-related hearing impairment (ARHI). In the US, NIHL affects the quality of life of ~26 million citizens and ARHI affects an estimated 40-50% of individuals older than 75 years of age (NIDCD, 2010). SNHL results from the gradual degeneration of the auditory receptors

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in the organ of Corti and the afferent spiral ganglion neurons. The degeneration of the mechano-sensorineural pathway is first seen at the base and high-frequency end of the tonotopic gradient of the cochlea and gradually spreads towards the apex also compromising the perception at lower frequencies.

SNHL is also a common pathology in strains of mice. Phenotypic screens using auditory-brain stem response (ABR) threshold and distortion-product otoacoustic emission (DPOAE) measurements across the phylogenetic tree of inbred strains showed that 35 out of 80 strains exhibited a wide phenotypic variation with respect to the degree of hearing threshold elevation, onset, and temporal progression of hearing loss (Drayton et al., 2006; Jimenez et al., 1999; Martin et al., 2007; Mashimo et al., 2006; Zheng et al., 1999). Genetic linkage analyses of intercrosses, backcrosses and recombinant inbred strains showed that each strain carries one to three common and/or rare alleles that control the phenotype. The *Cdh23*^{753A} allele is common to almost all strains with hearing loss and predisposes to its late onset (Noben-Trauth et al., 2003). Interestingly, additional alleles exist such as *ahl2*, *ahl4*, and *ahl8* that are strain-specific and by epistatically interacting with the *Cdh23*^{753A} allele accelerate the onset of the hearing loss. Other allele pairs (*Phl1*: *Phl2*; *ahl5*: *ahl6*) present in the 101H and Black Swiss strains respectively show co-dominant and cumulative effects (Noben-Trauth et al., 2009). The current data suggest that the phenotypic differences among inbred strains are to a large degree the result of strain-specific *de novo* mutations creating hypomorphic alleles.

The impressive genetic diversity and the wide phenotypic range of hearing loss in common inbred strains provides experimental model systems to systematically decipher the risk factors underlying sensorineural complex hearing loss. The ALR/LtJ and ALS/LtJ inbred strains were developed from ICR mice selected for their resistance or susceptibility to alloxan-induced diabetes, respectively. On the phylogenetic tree, they share a branch with the NOD/LtJ and BUB/BnJ mice (Petkov et al., 2005). Hearing loss in BUB/BnJ mice is due to a mutation in the *Vlgr1* gene on chromosome 13 (Johnson et al., 2005), whereas early-onset hearing loss in NOD/LtJ mice is controlled by an epistatic interaction between the *ahl2* locus on chromosome 5 and the *ahl* locus on chromosome 10 (Johnson et al., 2002). Mice of both the ALR/LtJ and ALS/LtJ strain develop early-onset hearing loss, however the hearing threshold shifts differ from those of the NOD/LtJ and BUB/BnJ mice (Zheng et al., 1999) suggesting that they segregate alleles at different loci. Here, with the aim to identify the hearing loss alleles in the ALR/LtJ strain we refined the hearing pathology and defined the underlying genetics.

Materials and methods

2. Mice and crosses

Strains for this study were obtained from The Jackson Laboratory, Bar Harbor, Maine. F1 and N2 progeny were derived from in-house matings. An institutional review board approved all animal procedures.

2.1. Auditory tests—For auditory-evoked brain stem response (ABR) measurements a computer-aided evoked potential system from Intelligent Hearing System, IHS; Miami, Florida was used. The Smart-EP version 10, modified for high frequency capability and coupled to high frequency transducers generated specific acoustic stimuli to amplify, measure, and display the evoked brainstem responses of anesthetized mice. Subdermal needle electrodes were inserted at the vertex (active), ventrolaterally to the right ear (reference) and the left ear (ground). Specific acoustic stimuli were delivered to the outer ear canal through a plastic tube channeled from the high frequency transducers. Mice were presented with click stimuli and with 8-, 16-, and 32 kHz tone pips at varying intensity, from

high to low (100-10 dB SPL) at a rate of 19.1 times/sec for a total of 350 sweeps. Sound pressure level thresholds were determined for each stimulus frequency by identifying the lowest intensity producing a recognizable ABR pattern of the computer screen (at least two consistent characteristic wave forms). Amplitude and latency measurements were performed using IHS software.

DPOAEs were measured using National Instruments (NI) LabView 8.6 software, operating an NI PCI-4461 Dynamic Signal Analyzer (DSA) sound card, to generate two pure tones, f_1 and f_2 , at the fixed f_2/f_1 ratio of 1.25, which were emitted separately by two Clarion SRU310H high frequency dome tweeters placed in the outer ear canal at the presentation level of $f_2 = f_1$ (Sound Pressure Level). The f_2 component was swept in 1 kHz steps starting from $f_2 = 5$ kHz to 55 kHz. Intensity levels sweeps ranged from 15 dB SPL up to 75 dB SPL, in 10 dB increments. Sound pressure levels were measured using an Etymotic-ER-10B + microphone. The amplitude of the $2f_1 - f_2$ distortion product was plotted in dB SPL against the f_2 frequency where the DP is generated. Clarion speakers and Etymotic ER-10B+ microphone were calibrated using a 1/4 inch microphone 7016 (1/4 inch pre amp 4016 and microphone power supply PS9200, AcoPacific). The AcoPacific 1/4 inch microphone 7016 was calibrated using a QC-10 Sound Calibrator (Quest Technologies).

The endocochlear potential (EP) was measured via the round window. Briefly, the tip of a small glass pipette containing a silver/chloride electrode bathed in 0.1 M KCl was inserted through the round window into the endolymph using a remote controlled motorized micromanipulator (PPM5000, Piezo World Precision Instrument). The electrode was connected to a Warner Dual Channel Differential Electrometer (HiZ-223), which amplified and routed the voltage difference (subdermal 1M KCl reference electrode) to a PC-controlled data acquisition system (Digidata 1440A, Axon Instruments) using AxoScope software, which displayed the measured output. Data were sampled at a rate of 10 kHz for 60 sec. The glass electrode was prepared using a Sutter Instrument (P-97 Flaming/Brown micropipette puller) and measurements were performed in a bench top faraday cage (TMC; Technical Manufacturing Corporation).

2.2. Histology—For gross morphology, the inner ears were dissected in PBS, perfused and fixed with 4% paraformaldehyde, decalcified in 0.1M EDTA pH 8.0 in PBS for three weeks, dehydrated with a graded series of ethanol, and infiltrated with JB-4 polymer (Polysciences, INC.). Serial sections were cut at 4 micron thickness on a RM2265 microtome (Leica), stained with 0.1 % Toluidine Blue O, cleared in xylene, imaged on a DM5000B microscope (Leica) and photographed with a DFC500 digital camera (Leica).

2.3. Phalloidin- and Immunostaining—For immunostaining of organ of Corti whole mounts, cochlea ducts were dissected in Leibovitz medium (Invitrogen), fixed in 4% paraformaldehyde (Electron Microscopy Sciences), permeabilized in 0.5% TritonX-100, and incubated in blocking solution (5% goat serum, 2% BSA in PBS) at 4°C over night. Samples were incubated with anti-acetylated tubulin (Sigma, Clone 6-11B-1, 1:1000 in blocking solution) for 2 hrs at room temperature, washed in PBS and stained with secondary anti-rabbit IgG (1:1000; Alexa Fluor® 594 donkey; Invitrogen) for 1 hr at room temperature. Additional antibodies used include anti-Myo6 (Sigma; 1:100), anti-neurofilament 200 (Sigma, 1: 100), anti-syaptophysin (Sigma, Clone SVP-38, 1:100), anti-CtBP2 (BD Transduction Laboratories, 1:100). For staining the hair bundle, the specimen was dissected as described above and stained with rhodamine phalloidin (Invitrogen) diluted 1:100 in PBS for 30 minutes at room temperature. After washing in PBS, the organ of Corti was removed from the modiolus, mounted in ProLong® Gold antifade reagent (Invitrogen) and imaged using a Zeiss LSM confocal microscope.

2.4. Genome-wide linkage scan—ALR/LtJ mice were crossed to C3HeB/FeJ and F1 hybrids were backcrossed to ALR/LtJ to generate the (ALR/LtJ $\times$ C3HeB/FeJ) $\times$ ALR/LtJ N2 backcross, here abbreviated as ALR.C3H-N2 backcross. Genomic DNA from parental, F1 and N2 mice was extracted from tail biopsies using the DNeasy Tissue kit (Qiagen), and concentration was adjusted to $\sim 100\text{ng}/\mu\text{l}$. Genome-wide genotyping was performed by the Center for Personalized Genetic Medicine, Partners Healthcare® using the Custom Illumina LD panel with 384 SNPs. Linear regression analyses, interval mapping, and interaction tests were performed using QTXb20 software (Manly et al., 2001). Genome-wide significance thresholds were determined by running 1000 permutations. The 95% confidence interval for the QTL location was determined using QTXb20. Centimorgan were converted to base pairs assuming a genetic length of 1528.6 cM and physical length of 2473 Mbp of the mouse genome (www.jax.org).

2.5. Statistical analyses—Unless otherwise indicated groups of data were compared using one-way ANOVA followed by Bonferroni post-tests to correct for multiple testing. GraphPad Prism 4.0b software was used to perform the arithmetic, to compute *p* values and to plot the data.

3. Results

3.1. Hearing phenotype in ALR/LtJ

At five weeks of age ALR/LtJ mice ($n=14$) exhibited significant ABR threshold shifts of 40- and 50 dB SPL for the click and 32 kHz stimuli, respectively (Fig. 1 A). Hearing thresholds at the 8- and 16 kHz stimuli also were significantly increased ($p<0.001$) compared with normal hearing C3HeB/FeJ at four weeks of age mice ($n=8$). The hearing loss was most severe at the higher frequencies and progressed to lower frequencies indicative of a sensorineural hearing impairment (Table 1). Peak-to-peak amplitudes of wave I at a 90 dB SPL and 100 dB SPL click stimulus were $0.1 \pm 0.08 \mu\text{V}$ and $0.35 \pm 0.2 \mu\text{V}$ ($n=11$), respectively (Fig. 1B). Similarly low wave I amplitudes were measured for the 16 kHz (Fig. 1C) and 8 kHz stimulus (data not shown). Amplitudes at all three stimuli and over a range of 80- to 100 dB SPL input levels were significantly lower than in C3HeB/FeJ controls ($n=9$; $p<0.001$). Peak latencies at wave I through V were measured at the click and 16 kHz stimulus at 100 dB SPL input levels. The data were best fitted with a linear regression model following a monotonic curve with slopes near 1 (0.84 and 0.86, respectively) and r^2 values of 0.9 and $r^2=0.94$, respectively ($n=13$). The peak latencies at all five waves in ALR/LtJ mice were not different compared to C3HeB/FeJ control mice ($n=9$; $p>0.05$) (Fig. 1D). To ascertain the functionality of outer hair cells, we measured distortion-product otoacoustic emissions (Fig. 1E). In a cohort of 14 ALR/LtJ mice at an average 5.5 weeks of age (age range: 5-8 weeks), DPOAEs were absent at $f_2=20\text{--}55$ kHz and input levels of $L_2=75$ dB SPL. Only very small distortion-products above noise floor were observed at the lower frequency range at $f_2=10\text{--}18$ kHz, but these were significantly smaller than emissions obtained from control mice. To test for a contribution of the stria vascularis, we measured the voltage potential in the scala media via round window recordings. In seven-week-old ALR/LtJ mice ($n=6$) the endocochlear potential (EP) was 114 ± 3 mV, which was significantly higher than the EP in normal C3HeB/FeJ ears (102 ± 3 mV; $n=6$; $p<0.0001$; *t*-test) (Fig. 1F). The EP in ALR/LtJ is comparable to that in two-months-old C57BL/6J mice (Ohlemiller et al., 2006), suggesting that the slightly higher EP in ALR/LtJ is not adversely affecting the hearing thresholds.

3.2. ABR threshold distribution In ALR.C3H-N2 backcross

To determine the genetic complexity of the hearing loss, we established a small (ALR/LtJ $\times$ C3HeB/FeJ) $\times$ ALR/LtJ backcross. ABR thresholds were measured in 185 N2 mice at an

average 5.8 ± 2.3 weeks of age (age range: 3-8 weeks). Thresholds varied greatly among the N2 progeny. Since thresholds also varied across the stimuli within each progeny, we computed the mean threshold of the four test stimuli for each progeny. The averaged hearing thresholds in the N2 cross followed a normal distribution ($R^2 = 0.82$) with a mean of 64 ± 18 dB SPL ($n=185$). In comparison, the peak distribution of averaged thresholds of the ALR/LtJ parental mice was 87 ± 5 dB SPL ($n=35$) and the mean of the (ALR/LtJ $\times$ C3HeB/FeJ) F1 animals was 39 ± 6 dB SPL ($n=20$) (Fig. 2).

3.3. Genome-wide linkage scan in ALR.C3H-N2 backcross

To map the causative alleles, the 185 N2 progeny were genotyped at 384 SNP marker loci. After adjusting for non-polymorphic markers, 165 markers were mapped on the N2 cross. Linear regression analyses revealed three genomic regions that showed highly significant linkage (Fig. 3A-C; Table 2). The first region was located on the distal half of chromosome 1 and associated with the click and 16 kHz stimulus at a highly significant genome-wide p value ($p < 0.0001$) (Fig. 3A). At the 8 kHz trait, association was significant ($p < 0.01$) and at the 32 kHz stimulus, the association was suggestive of linkage ($p < 0.05$). Interval mapping showed the strongest association with marker *rs13476177* (152,491,007bp, Build NCBI37) at the click stimulus with a LOD score of 12. The 95% confidence interval is an estimated 12 cM (~ 19.2 Mbp) located at position 142 – 161 Mbp on the physical map of chromosome 1. This locus, named *Snhl2*, explained 24% of the phenotypic variation.

The second region with highly significant genome-wide linkage ($p < 0.0001$) mapped to the distal end of chromosome 6 (Fig. 3B). At all four tested stimuli, marker *rs3704289* (131,940,470bp, Build NCBI37) produced very high LOD scores. Linkage was strongest at the 8 kHz with a LOD of 24. This locus, *Snhl3*, explained 43% of the phenotypic variance. The 95% confidence interval for the QTL location is approximately 7cM or ~ 11.2 Mbp and is located at position 125.4 – 136.6 Mbp on chromosome 6.

The third chromosomal region was located on chromosome 10 (Fig. 3C). Interestingly, this region associated with threshold variations at the 32 kHz stimulus only. The other stimuli exhibited no significant or suggestive linkage. Marker *rs3682523* (69,508,558bp, Build NCBI37) achieved a LOD score of 11. This locus, *Snhl4*, explained 23% of the threshold variance. The 95% confidence interval for this QTL is 13 cM (~ 20.8 Mbp) and is located at position 59.1 – 79.9 Mbp on chromosome 10.

A scan for epistatic and synergistic interactions revealed no significant associations. The net combined effect of the three QTLs explained 90% of the ABR threshold variation in the N2 cross.

3.4. Single and combined QTL effects

To reveal single and combined effects of the three QTLs, we plotted the ABR thresholds at the click and 32 kHz against each of the eight possible genotypes. *Snhl2* and *Snhl3* control the phenotype at the click stimulus (Fig. 4A, Table 3). *Snhl2* alone produced thresholds of 48 ± 9 dB SPL ($n=24$) and *Snhl3* alone had thresholds of 54 ± 13 dB SPL ($n=18$; $p > 0.05$). These thresholds were higher than those of mice that were heterozygous at all three loci (37 ± 8 dB SPL; $n=27$; $p < 0.001$). *Snhl2* and *Snhl3* together produced a mean threshold of 78 ± 14 dB SPL ($n=29$). Hence, the thresholds of the combined QTLs were higher than those of the single QTLs ($p < 0.001$) suggesting an additive effect. Presence or absence of *Snhl4* had no effect on the thresholds ($p > 0.05$). Most notably, *Snhl4* alone produced mean thresholds of 35 ± 12 dB SPL ($n=20$), which were not different from heterozygous N2 mice ($n=27$; $p > 0.05$).

At the 32 kHz stimulus, the phenotype was mostly controlled by *Snhl3* and *Snhl4* (Fig. 4B). *Snhl3* alone produced mean thresholds of 79 ± 19 dB SPL ($n=18$) and *Snhl4* had mean thresholds of 79 ± 17 dB SPL ($p>0.05$). These thresholds were higher than those of mice that were heterozygous at all three loci (57 ± 14 dB SPL, $n=27$, $p<0.001$). *Snhl3* and *Snhl4* together produced thresholds of 99 ± 4 dB SPL ($n=21$), which were different from the mean of the individual QTLs ($p<0.001$), indicating a cumulative effect between these two QTLs at the 32 kHz stimulus. *Snhl2* alone produced thresholds of 64 ± 18 dB SPL and had no effect on the threshold elevation when compared with thresholds of mice that were heterozygous at all three loci ($p>0.05$).

The linear regression analyses suggested the segregation of multiple loci with cumulative effects that explained most of the phenotypic variation in the N2 cross. To demonstrate this result, we plotted the averaged thresholds as function of genotype and fitted with to a normal Gaussian distribution (Fig. 4C, Table 3). As shown, the R^2 values ranged between 0.55 and 0.86, suggesting that most thresholds are accounted for by the three-QTL-model.

3.5. Organ of Corti patterning defect in ALR/LtJ

To localize the cellular defect underlying the hearing loss, we examined the gross morphology of the inner ear. In the cochlea of five-week old ALR/LtJ mice, we noticed the presence of additional outer hair cells (OHC) at the abneural side starting at the mid-apical region and reaching up to the apex (Fig. 5A, B). The ectopic outer hair cells (eOHC) were organized in one to three discontinuous rows and appeared supported by Deiter's cells (Fig. 5B-D). Stria vascularis and spiral ganglia showed a normal morphology (Fig. 5E, F).

Fine-dissection of the cochlea revealed a normal morphology and length of the cochlear duct (Fig. 6A). Phalloidin-stained organ of Corti surface preparations in neonatal ALR/LtJ mice (P3-P7) confirmed the findings of the histology, showing a profound disruption of the cellular pattern (Fig. 6B-H). While outer hair cells at the basal and middle part of the organ of Corti were arranged in three rows, there appeared one or more additional discontinuous rows of OHCs at the apical turn. Closer to the apex, the row-pattern of the OHCs became increasingly disorganized. The cellular arrangement of the inner hair cells (IHCs) was largely unaltered along the cochlear coil and was abnormal only at the very apex. We quantified the eOHCs, by counting IHCs and OHCs that emerged in each row. We evaluated specimen from five upper coils that represented an average length of the organ of Corti of $1050 \mu\text{m}$ per coil. We counted on average 41 ± 6 IHCs and 40 ± 19 eOHCs per $300 \mu\text{m}$. Similar numbers were computed when relating the ectopic hair cells to first-, second-, and third-row OHCs (Fig. 6I).

To further address the morphology and functionality of the eOHCs, we stained whole mounts of organs of Corti with various molecular markers. We found that the eOHCs exhibited the hallmarks of typical OHCs. In particular, staining with an antibody against acetylated-tubulin, showed that the kinocilium is present in postnatal ALR/LtJ mice and that the orientation of the stereocilia bundle is unchanged (Fig. 7A). eOHCs were positive for Myosin VI (hair cell marker) (Fig. 7B), and appeared enervated by type II afferent neurons as evidenced by positive staining with antibodies against neurofilament (Nefh), synaptophysin (Syp) and ribeye (Ctbp2, Fig. 7B-E).

4. Discussion

Hearing loss in ALR/LtJ was previously recognized in a large-scale screen ascertaining hearing function in common inbred strains of mice (Zheng et al., 1999). Here, by applying differential hearing tests we refined the hearing loss as early-onset, profound, and sensorineural. In addition, we identified its underlying genetics uncovering three QTLs that

together explain 90% of the phenotypic variation thus, capturing most of the genetic variation. Our data suggest that each of the three QTLs has a strong individual effect and that the combined effects are mainly additive or cumulative. The degree of genetic complexity compares with the oligogenic nature of hearing loss in other inbred and heterogeneous strains, but there are differences that are worthwhile to point out. First, in contrast to *ahl2* (NOD/LtJ), *ahl4* (A/J), and *ahl8* (DBA/2J), the *Snhl2* and *Snhl3* QTLs in ALR/LtJ show no epistatic or synergistic interactions with *Cdh23^{ahl}*. *Snhl4* is too closely linked to *ahl* to reveal or exclude an interaction. Secondly, the *Snhl2-4* QTLs show no mutual genetic interactions either in contrast to the *Phl1:Phl2* and *ahl5:ahl6* pair-wise interactions reported for the 101H and Black Swiss strains, respectively (Drayton et al., 2006; Mashimo et al., 2006). The mono-modal threshold distribution of the combined genotypes of the N2 cross also argues against epistatic or synergistic effects.

The QTL analysis in the ALR/LtJ strain associated increases in hearing thresholds with a region on chromosome 10 that, at its very proximal end of the 95% confidence interval, harbors the *Cdh23* gene. The *Cdh23^{ahl}* variant, which is present in the ALR/LtJ strain (*Cdh23^{753A}*), but not in the C3HeB/FeJ strain (*Cdh23^{753G}*) is a strong contributor to early- and late-onset hearing loss in a number of inbred strains (Johnson et al., 2000; Johnson et al., 2006; Noben-Trauth et al., 2003). It is not possible to rule out *Cdh23^{ahl}* as cause of the *Snhl4* phenotype. However, based upon maximum QTL peak location, *Snhl4* maps more closely to *Phl2* (see *D10Mit15* at map location chr10: 66,475,179-66,475,378bp) and thus outside of the *Cdh23* genomic interval. On the other hand, the strong association with high-frequency hearing loss, which is also a hallmark of the *Cdh23^{ahl}* variant, is indicative of an involvement of *Cdh23^{ahl}* in the *Snhl4* phenotype. The latter interpretation is supported by our observation of high-frequency hearing loss in F1 hybrids. For instance, F1 hybrids that are homozygous at *Cdh23^{ahl}* such as ALR/LtJ×DBA/2J, ALR/LtJ×A/J ALR/LtJ×NOD/LtJ, and ALR/LtJ×ALS/LtJ exhibit high-frequency hearing loss, whereas the F1 hybrids heterozygous at *Cdh23^{ahl}* (ALR/LtJ×C3HeB/FeJ) display normal thresholds (unpublished observations). This result suggests allelism between *Cdh23^{ahl}* and *Snhl4* although other background effects cannot be ruled out.

The 95% confidence intervals of *Snhl2*, -3, and -4 are 19-, 11-, and 21 Mbp, respectively. The distal third of the *Snhl3* interval is found on 12p13.32-p13.2. The autosomal recessive non-syndromic hearing loss locus DFNB62 localizes to a 15Mbp region on human 12p13.2-p11.23 that overlaps with the *Snhl3* interval. Clinically, DFNB62 presents with prelingual, profound (>90 dB HL) hearing impairment (Ali et al., 2006). *Snhl3* is the strongest QTL affecting all test frequencies similar to DFNB62. Hence, *Snhl3* is an excellent candidate locus for DFNB62.

Our histologic analysis of the ALR/LtJ cochlea identified a distinct organ of Corti patterning defect. Similar patterning defects have been demonstrated in a number of genetic models involving genes such as the *Vangl2*, *Foxg1*, *Dll1*, *Jag2*, *Sobp*, *Ptk7*, *pRb*, and others (Chen et al., 2008; Kiernan et al., 2005; Lu et al., 2004; Montcouquiol et al., 2003; Pauley et al., 2006; Sage et al., 2005). However, in most of these models the patterning defect is part of a complex cochlear and organismal phenotype that additionally may include a truncated cochlea coil, ectopic hair cells in Kölliker's organ, planar cell polarity defects, innervation defects, increased number of inner hair cells, or neural tube defects. The patterning defect in ALR/LtJ is most similar to the organ of Corti phenotype in *Spry2^{-/-}* and *Fgfr3^{P244R}* mice, both of which show extra rows of outer hair cells and outer pillar cells (Mansour et al., 2009; Shim et al., 2005). This suggests that the underlying genetic variant(s) in ALR/LtJ may target the *Fgfr3* or *Spry2* signaling pathways. However, it should be noted that our QTL study used hearing thresholds as mapping trait. It is henceforth not clear to what extent, if any, *Snhl2*, -3, and -4 also control the patterning defect. It is possible that the patterning

defect is regulated by other loci or has no effect on hearing thresholds altogether. However, this is less likely given the structural distortion of the organ of Corti of ALR/LtJ mice. Indeed, recent audiologic data on the *Spry2*^{-/-} knock out mice, in which the outer hair cell defect could be separated from the pillar cell defect, suggest, that an additional row of outer pillar cells is more likely to increase hearing thresholds than the extra row of outer hair cells (Shim et al., 2005).

Increased susceptibility to ARHI and NIHL is thought to be the result of the interplay of multiple risk alleles (Konings et al., 2009; Thys et al., 2009; Van Eyken et al., 2007). Typically susceptibility alleles to common diseases show low penetrance, high population frequency and have, when considered individually, only very small, if any, effects (<<1%). Genome-wide association studies on a number of common and genetically complex diseases have been successful in identifying chromosomal regions linked to the phenotype, but have had limited success in uncovering the full plethora of the underlying genetic variation and thus far explain only a minute fraction of the phenotype (McClellan et al., 2010). The success rate in positively identifying the underlying causative nucleotide variant is even lower (Eichler et al., 2010). Given these difficulties, the investigation of animal models with complex inherited progressive hearing loss not only suggests candidate genes but may also point to the mechanism that confers the susceptibility. As recently shown, the DBA/2J-specific *ahl8* allele (*Fscn2*^{326A}), encodes the 109His variant of fascin-2 (Fscn(109His)). Fascin-2 crosslinks actin filaments in developing and mature cochlear hair cell stereocilia (Shin et al., 2010). Whereas the *Fscn2*(109His) variant on a *Cdh23*^{753G} background has no effect on hearing thresholds, it causes early-onset profound hearing loss when expressed on the *Cdh23*^{753A} (*ahl*) background (Johnson et al., 2008). Localization of fascin-2 (tips of stereocilia) and cadherin-23 (tip link) and similar auditory phenotypes of the hypomorphic alleles (*Fscn*^{326A} and *Cdh23*^{753A}, respectively) suggests an interaction on the functional level between tip link and stereocilia treadmilling and/or stiffness (Shin et al., 2010).

In summary, this study demonstrates a progressive sensorineural hearing loss in the ALR/LtJ strain that can be explained by three QTLs. It lays the groundwork for their molecular characterization, in particular of the *Snhl3* QTL, which may represent the mouse model for DFNB62.

Acknowledgments

We thank Glen Martin for help with DPOAEs and Daniel Marcus for advice on EP measurements. We thank Alain Dabdoub and Feng Qian for their comments on the manuscript. The Division of Intramural Research at NIDCD funded this work.

References

- Ali G, Santos RL, John P, Wambangco MA, Lee K, Ahmad W, Leal S. The mapping of DFNB62, a new locus for autosomal recessive non-syndromic hearing impairment, to chromosome 12p13.2-p11.23. *Clin Genet*. 2006; 69:429–433. [PubMed: 16650082]
- Chen Z, Montcouquiol M, Calderon R, Jenkins NA, Copeland NG, Kelley MW, Noben-Trauth K. Jxc1/Sobp, encoding a nuclear zinc finger protein, is critical for cochlear growth, cell fate, and patterning of the organ of corti. *J Neurosci*. 2008; 28:6633–6641. [PubMed: 18579736]
- Drayton M, Noben-Trauth K. Mapping quantitative trait loci for hearing loss in Black Swiss mice. *Hear Res*. 2006; 212:128–139. [PubMed: 16426780]
- Eichler EE, Flint J, Gibson G, Kong A, Leal SM, Moore JH, Nadeau JH. Missing heritability and strategies for finding the underlying causes of complex disease. *Nat Rev Genet*. 2010; 11:446–450. [PubMed: 20479774]
- Jimenez AM, Stagner BB, Martin GK, Lonsbury-Martin BL. Age-related loss of distortion product otoacoustic emissions in four mouse strains. *Hear Res*. 1999; 138:91–105. [PubMed: 10575118]

- Johnson KR, Zheng QY. *Ahl2*, a second locus affecting age-related hearing loss in mice. *Genomics*. 2002; 80:461–464. [PubMed: 12408962]
- Johnson KR, Zheng QY, Erway LC. A major gene affecting age-related hearing loss is common to at least ten inbred strains of mice. *Genomics*. 2000; 70:171–180. [PubMed: 11112345]
- Johnson KR, Zheng QY, Noben-Trauth K. Strain Background Effects and Genetic Modifiers of Hearing in Mice. *Mol Brain Res*. 2006; 1091:79–88.
- Johnson KR, Zheng QY, Weston MD, Ptacek LJ, Noben-Trauth K. The *Mass1^{frings}* mutation underlies early onset hearing impairment in BUB/BnJ mice, a model for the auditory pathology of Usher Syndrome IIC. *Genomics*. 2005; 85:582–590. [PubMed: 15820310]
- Johnson KR, Longo-Guess C, Gagnon LH, Yu H, Zheng QY. A locus on distal chromosome 11 (*ahl8*) and its interaction with *Cdh23* *ahl* underlie the early onset, age-related hearing loss of DBA/2J mice. *Genomics*. 2008; 92:219–225. [PubMed: 18662770]
- Kiernan AE, Cordes R, Kopan R, Gossler A, Gridley T. The Notch ligands DLL1 and JAG2 act synergistically to regulate hair cell development in the mammalian inner ear. *Development*. 2005; 132:4353–4362. [PubMed: 16141228]
- Konings A, Van Laer L, Van Camp G. Genetic Studies on Noise-Induced Hearing Loss: A Review. *Ear Hear*. 2009; 30:151–159. [PubMed: 19194285]
- Lu X, Borchers AG, Jolicoeur C, Rayburn H, Baker JC, Tessier-Lavigne M. PTK7/CCK-4 is a novel regulator of planar cell polarity in vertebrates. *Nature*. 2004; 430:93–98. [PubMed: 15229603]
- Manly KF, Cudmore RH Jr, Meer JM. Map Manager QTX, cross-platform software for genetic mapping. *Mamm Genome*. 2001; 12:930–932. [PubMed: 11707780]
- Mansour SL, Twigg SR, Freeland RM, Wall SA, Li C, Wilkie AO. Hearing loss in a mouse model of Muenke syndrome. *Hum Mol Genet*. 2009; 18:43–50. [PubMed: 18818193]
- Martin GK, Vazquez AE, Jimenez AM, Stagner BB, Howard MA, Lonsbury-Martin BL. Comparison of distortion product otoacoustic emissions in 28 inbred strains of mice. *Hear Res*. 2007; 234:59–72. [PubMed: 17997239]
- Mashimo T, Erven AE, Spiden SL, Guenet JL, Steel KP. Two quantitative trait loci affecting progressive hearing loss in 101/H mice. *Mamm Genome*. 2006; 17:841–850. [PubMed: 16897347]
- McClellan J, King MC. Genetic heterogeneity in human disease. *Cell*. 2010; 141:210–217. [PubMed: 20403315]
- Montcouquiol M, Rachel RA, Lanford PJ, Copeland NG, Jenkins NA, Kelley MW. Identification of *Vangl2* and *Scrb1* as planar polarity genes in mammals. *Nature*. 2003; 423:173–177. [PubMed: 12724779]
- NIDCD. Statistics and Epidemiology. 2010. www.nidcd.nih.gov
- Noben-Trauth K, Johnson KR. Inheritance patterns of progressive hearing loss in laboratory strains of mice. *Brain Res*. 2009; 1277:42–51. [PubMed: 19236853]
- Noben-Trauth K, Zheng QY, Johnson KR. Association of cadherin 23 with polygenic inheritance and genetic modification of sensorineural hearing loss. *Nat Genet*. 2003; 35:21–23. [PubMed: 12910270]
- Ohlemiller KK, Lett JM, Gagnon PM. Cellular correlates of age-related endocochlear potential reduction in a mouse model. *Hear Res*. 2006; 220:10–26. [PubMed: 16901664]
- Pauley S, Lai E, Fritsch B. *Foxg1* is required for morphogenesis and histogenesis of the mammalian inner ear. *Dev Dyn*. 2006; 235:2470–2482. [PubMed: 16691564]
- Petkov PM, Graber JH, Churchill GA, DiPetrillo K, King BL, Paigen K. Evidence of a large-scale functional organization of mammalian chromosomes. *PLoS Genet*. 2005; 1:e33. [PubMed: 16163395]
- Sage C, Huang M, Karimi K, Gutierrez G, Vollrath MA, Zhang DS, Garcia- Anoveros J, Hinds PW, Corwin JT, Corey DP, Chen ZY. Proliferation of functional hair cells in vivo in the absence of the retinoblastoma protein. *Science*. 2005; 307:1114–1118. [PubMed: 15653467]
- Shim K, Minowada G, Coling DE, Martin GR. *Sprouty2*, a mouse deafness gene, regulates cell fate decisions in the auditory sensory epithelium by antagonizing FGF signaling. *Dev Cell*. 2005; 8:553–564. [PubMed: 15809037]

- Shin JB, Longo-Guess CM, Gagnon LH, Saylor KW, Dumont RA, Spinelli KJ, Pagana JM, Wilmarth PA, David LL, Gillespie PG, Johnson KR. The R109H variant of fascin-2, a developmentally regulated actin crosslinker in hair-cell stereocilia, underlies early-onset hearing loss of DBA/2J mice. *J Neurosci.* 2010; 30:9683–9694. [PubMed: 20660251]
- Thys M, Van Camp G. Genetics of otosclerosis. *Otol Neurotol.* 2009; 30:1021–1032. [PubMed: 19546831]
- Van Eyken E, Van Camp G, Van Laer L. The complexity of age-related hearing impairment: contributing environmental and genetic factors. *Audiol Neurotol.* 2007; 12:345–358. [PubMed: 17664866]
- Zheng QY, Johnson KR, Erway LC. Assessment of hearing in 80 inbred strains of mice by ABR threshold analyses. *Hear Res.* 1999; 130:94–107. [PubMed: 10320101]

Abbreviations

SNHL	sensorineural hearing loss
QTL	quantitative trait locus
ABR	auditory brain stem response
DPOAE	distortion product otoacoustic emission
EP	endocochlear potential

Fig. 1.

Auditory phenotype in the ALR/LtJ strain.

A. Shown are ABR thresholds of five-week-old ALR/LtJ (filled symbols) and four-week-old C3HeB/FeJ (open symbols) mice at the stimulus indicated at the X-axis. Each circle represents one measurement of one animal. The line indicates the mean. B, C. Shown are ABR wave I amplitudes (in microVolt) at the click (B) and 16 kHz (C) stimulus at the input level (dB SPL) as indicated on the X-axis for ALR/LtJ (filled symbol; n=11) and C3HeB/FeJ (open symbols; n=9). Values are given as mean $\pm$ SEM. D. Shown are ABR wave latencies (in milliseconds) at click and 16 kHz stimulus at 100 dB SPL levels for ALR/LtJ (filled symbols; n=11) and C3HeB/FeJ (open symbols; n=9). Values are given as mean $\pm$ SEM. Dotted lines = linear regression curve. E. Shown are DPOAE amplitudes at 2f1-f2 frequency at stimulus level L1=75 dB for f2 test frequencies given on the X-axis for ALR/LtJ (filled symbols; n=28 ears) and C3HeB/FeJ (open symbols; n=12 ears); values are given as mean $\pm$ SEM. F. Shown are the mean $\pm$ SD of endocochlear potential measurements (in millivolt) in ALR/LtJ (n=6) and C3HeB/FeJ (n=6); *** $p < 0.001$ (t-test).

Fig. 2.

Averaged ABR threshold distributions in N2 backcross.

Shown are distributions of ABR thresholds (average of four test stimuli) of ALR/LtJ (red square; $n=35$), ALRxC3H-F1 (blue filled circle; $n=20$) and (ALRxC3H) $\times$ ALR-N2 (green open circle; $n=185$) mice. Dotted lines = best-fit-line of normal Gaussian distribution.

Fig. 3.
Chromosomal location of *Snhl2*, -3, and -4.

Chromosome-specific interval mapping blots of *Snhl2* (A), *Snhl3* (B) and *Snhl4* (C) are shown. Each black line represents the LOD profile of the indicated stimulus as function of marker position, which is given on the X-axis. Horizontal lines indicate the genome-wide significance thresholds obtained after 1000 permutations. The grey bars indicate the most likely map position of the QTL obtained after bootstrap tests. The double-arrows indicate the 95% confidence interval. The maximum LOD score is indicated.

Fig. 4.

Single and combined QTL effects.

A, B. Shown are ABR thresholds at the click (A) and 32 kHz (B) stimulus obtained for each of the eight possible genotypes. Bars represent the mean $\pm$ SD. Values are also given in Table 3. Homozygosity at the marker QTL is represented by “A” and the heterozygous genotype is denoted by “H”. The marker used to type the QTL is given on the left and the QTL represented by the marker is given on the right. ** $p < 0.01$; *** $p < 0.001$; n.s. $p > 0.05$ (ANOVA). C. Shown are the distributions of the averaged (all four stimuli) ABR thresholds (X-axis) for each of the eight possible genotypes (indicated in the right legend and coded by color). Each circle represents the number of animals (given on the Y-axis) with the indicated threshold and genotype. The black curve represents the sum of animals exhibiting the indicated threshold, irrespective of the genotype. Distributions were fitted to a normal Gaussian distribution (dotted lines).

Fig. 5.

Gross morphology of the inner ear in ALR/LtJ mice.

A-F. Shown are toluidine-stained plastic sections of organ of Corti (A-D), stria vascularis (E) and spiral ganglion (F) of C3HeB/FeJ at eight weeks of age (A) and ALR/LtJ at five weeks of age (B-F). Images are taken from the mid-apical (A), base (B) mid-apical (C) and apical (D) region of the cochlea. Note supernumerary outer hair cells in C and D as well as distorted organ of Corti in D. ih, inner hair cell; oh, outer hair cell; sc, supporting cell; ipc, inner pillar cell; opc, outer pillar cell; tm, tectorial membrane; bc, basal cell; mc, marginal cell; ic, intermediate cell; sm, scala media; sg, spiral ganglion; scale bar = 20 μ m.

Fig. 6.

Ectopic outer hair cells in the organ of Corti of ALR/LtJ mice.

A. Shown are photographs of C3HeB/FeJ (A) and ALR/LtJ (A') ears at postnatal day P9 (A) and P6 (A') with the cochlear duct (cd) exposed; sac, sacculus; scale bar = 500 μm. B-I. Shown are phalloidin-stained stereocilia bundles of postnatal (P3-P7) organs of Corti of ALR/LtJ (A'-H) and C3HeB/FeJ (I) mice. (B) shows a stained upper cochlear turn indicating the positions of images C-H. Number of rows of OHCs is indicated. IHC, inner hair cell; scale bar = 10 μm. J. Shown are the number of native inner and outer hair cells (grey bars) and ectopic outer hair cells (ect; black bar) per 300 μm length of the organ of Corti. Values are given as mean ± SD.

Fig. 7.

Molecular markers in the sensory epithelium in ALR/LtJ mice.

Shown are confocal microscopy images of postnatal (P3-P7) organ of Corti preparations stained with antibodies against tubulin (A), myosin 6 (B, B'), neurofilament (C, C'), synaptophysin (D) and Ctip2 (E, E') in the red channel and counterstained with phalloidin (green channel) and DAPI (blue channel). Arrows point to positive staining. The number of rows (1-4) of OHCs is indicated. IHC, inner hair cell. Scale bar = 10 μ m.

Table 1
ABR thresholds in parental strains and F1 hybrids

Strain	Cross	wks	click	8 kHz	16 kHz	32 kHz	N
ALR/LAJ	P0	5	78.9 ± 10	75 ± 8	89.3 ± 8	100	14
C3HeB/FeJ	P0	4	26.3 ± 2	30.6 ± 2	18.1 ± 3	42.5 ± 3	8
(ALR.C3H)F1		3	26.3 ± 7	26.9 ± 8	30 ± 5	53.8 ± 9	8
		12	26.3 ± 4	47.5 ± 3	40 ± 4	55.7 ± 7	8
(ALR.C3H)N2			55.6 ± 20	66.7 ± 21	51.6 ± 20	81.7 ± 20	185

wks, weeks of age; kHz, kilohertz; SD, standard deviation; N, number of animals; data are mean ± SD;

Table 2

QTL mapping statistics

	Chr	marker	bp position	LOD	%	p	QTL	95% CI (Mbp)
click	1	rs13476177	152,491,007	12	24	< 0.0001	<i>Snhl2</i>	19
	6	rs3704289	131,940,470	19	36	< 0.0001	<i>Snhl3</i>	
8 kHz	1	rs13476177	152,491,007	6	12	< 0.0001	<i>Snhl2</i>	
	6	rs3704289	131,940,470	24	43	< 0.0001	<i>Snhl3</i>	11
16 kHz	1	rs13476177	152,491,007	6	13	< 0.0001	<i>Snhl2</i>	
	6	rs3704289	131,940,470	17	31	< 0.0001	<i>Snhl3</i>	
32 kHz	1	rs13476177	152,491,007	2	5	< 0.005	<i>Snhl2</i>	
	6	rs3704289	131,940,470	17	31	< 0.0001	<i>Snhl3</i>	
	10	rs3682523	69,508,558	11	23	< 0.0001	<i>Snhl4</i>	20.8

Chr, chromosome; %, percent of the phenotypic variance; bp, base pair; p, ANOVA; QTL, quantitative trait locus; kHz, kilohertz;

Table 3

ABR thresholds as function of *Snhl2*, -3, and -4 genotype

	AAA	AAH	AHA	AHH	HAA	HAH	HHA	HHH
click	77.7 ± 17	77.9 ± 15	52.1 ± 15	48.3 ± 9	55.0 ± 17	53.61 ± 13	35.5 ± 12	37.8 ± 8
8 kHz	86.7 ± 13	85.5 ± 15	65.0 ± 14	53.9 ± 13	74.1 ± 20	72.5 ± 19	43.3 ± 12	48.7 ± 13
16 kHz	73.7 ± 19	66.7 ± 19	50.2 ± 16	41.7 ± 12	57.6 ± 21	50.8 ± 13	31.8 ± 13	36.5 ± 12
32 kHz	97.3 ± 7	89.7 ± 17	92.1 ± 14	63.9 ± 18	99.1 ± 4	78.0 ± 18	78.5 ± 17	57.2 ± 14
averaged	89.1 ± 10	84.6 ± 12	61.4 ± 9	53.0 ± 10	70.8 ± 15	65.3 ± 16	51.4 ± 7	43.1 ± 8
R²	0.61	0.82	0.84	0.86	0.55	0.56	0.61	0.78
N	24	29	22	24	21	18	20	27

kHz, kiloHertz; N, number of N2 progeny; data are mean ± SD; A, homozygous; H heterozygous genotype; R², goodness-of-fit of normal distribution