Table 1. *KCNQ4* mutations found in this study together with previously reported mutations.

					Functional Prediction								
Nucleotide Change	**Amino Acid Change**	**Exon**	**Position**	**Alleles in Control Chr**	**SIFT**	**P2 D.S.**	**PhyloP**	**LRT**	**Mut Taster**	**GERP++**	**Study location**	**No of Fm**	**Reference**
c.211_223del13	p. Q71fs	1	N-term cyto	?	–	–	–	–	–	–	Belgium	1	Coucke, et al. (1999)
c.211delC	p. Q71fs	1	N-term cyto	0/252	–	–	–	–	–	–	Japan	14	Kamada, et al. (2006), This report
*** c.229_230insGC**	**p.H77fs**	**1**	**N-term cyto**	**0/252**	–	–	–	–	–	–	**Japan**	**1**	**This report**
c.546C>G	p.F182L	4	S3 trans	0/100, 1/252	T (0.00)	B (0.01)	C (0.97)	N (0.999853)	D (0.88)	3.43	Taiwan, Japan	3	Su, et al. (2007), This report
c.664_681del18	p.G215_220del6	4	S4-S5 linker	0/100	–	–	–	–	–	–	Korea	1	Baek, et al. (2010)
*** c.689T>A**	**p.V230E**	**4**	**S4–S5 linker**	**0/252**	**D (1.00)**	**D (0.97)**	**C (0.99)**	**D (0.999999)**	**D (0.99)**	**4.61**	**Japan**	**1**	**This report**
c.725G>A	p.W241X	5	S5 trans	0/100	–	–	–	–	–	–	USA	1	Hildebrand, et al. (2008)
c.778G>A	p.E260K	5	S5 trans	0/100	D (1.00)	D (0.99)	C (0.99)	D (1.00)	D (0.99)	4.73	USA	1	Hildebrand, et al. (2008)
c.785A>T	p.D262V	5	S5 trans	0/100	D (1.00)	D (0.99)	C (0.99)	D (1.00)	D (0.99)	4.73	USA	1	Hildebrand, et al. (2008)
c.821T>A	p.L274H	5	PR (P)	?	D (1.00)	D (0.99)	C (0.99)	D (1.00)	D (1.00)	4.73	Neth	2	Van Hauwe, et al. (2000), De Heer, et al. (2011)
c.827G>C	p.W276S	5	PR (P)	0/252	D (1.00)	D (1.00)	C (0.99)	D (1.00)	D (1.00)	4.73	Neth, Japan	4	Coucke, et al. (1999), Akita et al. (2001), Van Camp, et al. (2002), Topsakal, et al. (2005)
c.842T>C	p.L281S	6	PR (P)	0/96	D (1.00)	Pr (0.84)	C (0.99)	D (1.00)	D (1.00)	5.14	USA	1	Talebizadeh, et al. (1999)
c.853G>T	p.G285C	6	PR (P)	?	D (1.00)	D (1.00)	C (0.99)	D (0.999999)	D (1.00)	5.14	USA	1	Coucke, et al. (1999)
c.853G>A	p.G285S	6	PR (P)	0/150	D (1.00)	D (0.99)	C (0.99)	D (0.999999)	D (1.00)	5.14	France	1	Kubisch, et al. (1999)
c.859G>C	p.G287R	6	PR (P)	0/274	D (1.00)	D (0.99)	C (0.99)	D (1.00)	D (1.00)	5.14	USA	1	Arnett, et al. (2011)
*** c.871C>T**	**p.P291S**	**6**	**PR (P)**	**0/252**	**D (1.00)**	**D (1.00)**	**C (0.99)**	**D (1.00)**	**D (1.00)**	**5.14**	**Japan**	**1**	**This report**
*** c.872C>T**	**p.P291L**	**6**	**PR (P)**	**0/252**	**D (1.00)**	**D (1.00)**	**C (0.99)**	**D (1.00)**	**D (1.00)**	**5.14**	**Japan**	**1**	**This report**
c.886G>A	p.G296S	6	PR	0/100	D (0.99)	D (0.97)	C (0.99)	D (1.00)	D (0.99)	5.14	Spain	1	Mencia, et al. (2008)
*** c.891G>T**	**p.R297S**	**6**	**S6 trans**	**0/252**	**D (1.00)**	**D (0.99)**	**C (0.99)**	**D (1.00)**	**D (0.95)**	**3.89**	**Japan**	**1**	**This report**
c.961G>A	p.G321S	7	S6 trans	?	D (0.99)	Po (0.31)	C (0.99)	D (1.00)	D (0.99)	4.92	Neth	1	Coucke, et al. (1999)

SIFT, Polyphen-2, PhyloP, LRT, Mutation Taster, and GERP++ are functional prediction scores in which increasing values indicate a probable mutation.

Abbreviations: Chr, chromosomes; P2, PolyPhen2; MutTaser, Mutation Taser; Fm, family; cyto, cytoplasmic; trans, transmembrane; PR, Pore region; (P), P-loop; T, tolerated; D, damaging or deleterious; B, benign; Pr, probably damaging; Po, possibly damaging; C, conserved; N, neutral. Neth, Netherlands; *, Novel mutations found in this study.

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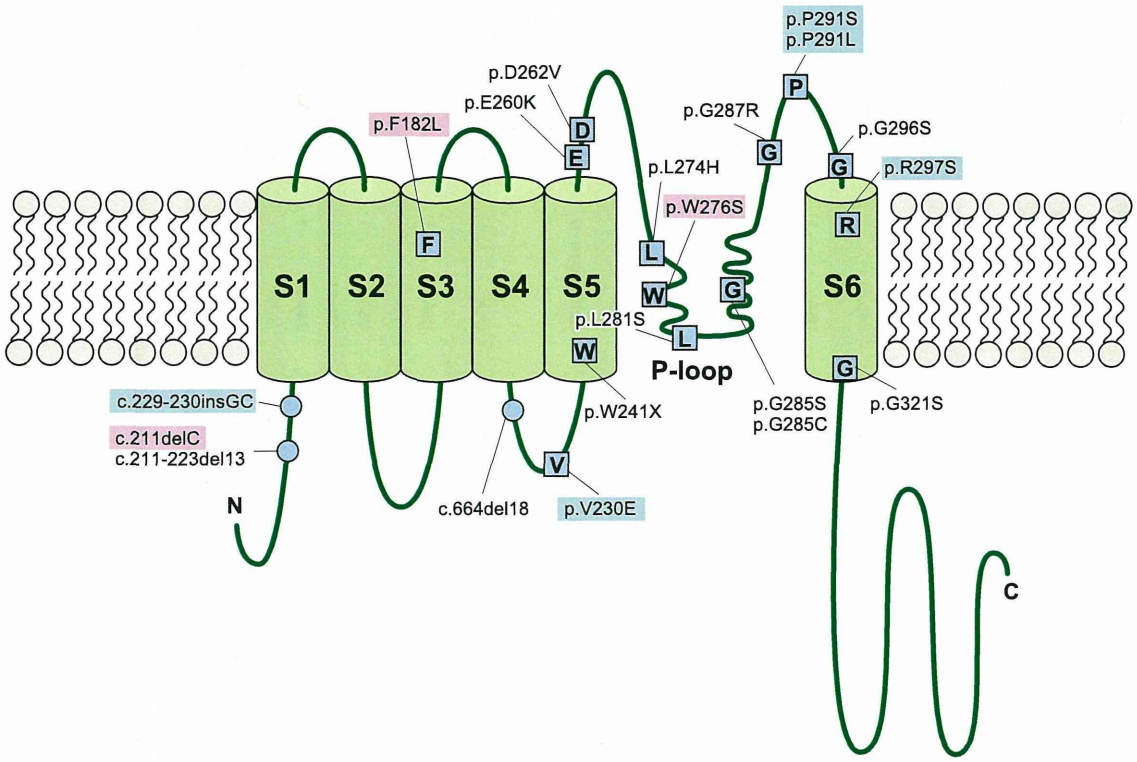

Figure 1. Localization of 20 *KCNQ4* mutations reported in previous studies in the protein. The 6 transmembrane domains (S1–S6) and the P-loop, located between S5 and S6, are shown. 5 mutations are concentrated in a narrow P-loop range. Mutations with pink and blue shadows; possible mutations detected in this study. Blue indicates novel mutations. Original schema is modified from Mencía A (2008) [14].
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from 3 to 40 years old, though the majority became aware when in their teens or younger. Most patients had associated tinnitus, but no vestibular symptoms except in a few cases.

Genotype/phenotype correlations

Concerning type of hearing loss, there were some correlations between genotype and phenotype (audiogram configuration). A variant at the N-terminal site (c. 211delC) showed ski-slope type configuration of audiogram with usually nearly normal hearing at 125–1000 Hz. We found this mutation in 20 patients from 13 families and their overlapped audiogram confirmed a similar configuration (Fig. 3). Onset age was from 10 to 40 years old, with most being in theirs teens and hearing loss was gradually progressive with age (Fig. 3, Table 2). The patients who had a variant in the P-loop region (W276S, P291L, P291S) also had high frequency involved hearing loss, but with some deterioration in the lower frequencies as well (Fig. 3). Most of the patients had earlier onset compared to the former phenotype and a progressive nature (Fig. 3, Table 2). The third audiogram configuration was mid-frequency involved hearing loss found in a family with a variant in the S4–S5 linker region (V230E) (Fig. 3). In most family members, onset was before age ten and gradually progressive (Fig. 3, Table 2). Overlapped audiograms were made for three mutations (W276S, c.211delC, V230E) for which there was a large enough number of patients to be analyzed (Fig. 3).

Therapeutic intervention

Sufficient amplification of hearing aids was obtained in all patients, and no patients received cochlear implantation. An affected subject with W276S (Family-Patient No. 16–2 in Table 2) had used a hearing aid from age 29. Similarly, affected subjects with P291L (Family-Patient No. 18–1) and V230E (Family-Patient No. 15–2) had used hearing aids. None of the affected subjects with c.211delC had a history of hearing aid usage.

Progression analysis

Detailed progression analysis in each frequency showed each affected member's age and their pure tone thresholds for 125, 250, 500, 1000, 2000, 4000, 8000 Hz, respectively (Fig. 4). Linear regression lines calculated by the plots are shown in the graph. Regarding the average progressive rates of hearing loss (db/year) for the patients with c.211delC, 125 (0.15) and 250 Hz (0.078) were shown to be significantly stable compared to the other two mutations (ANCOVA: $p<0.05$). They exhibited milder hearing loss at 500 and 1 KHz (ANCOVA: $p<0.05$). In contrast, at 4 KHz and 8 KHz, the patients with V230E mutations showed milder hearing loss compared to the other two mutations (ANCOVA: $p<0.05$).

Discussion

In this study, we have conducted a comprehensive genetic screening of *KCNQ4* using a large cohort of Japanese ADNSHL patients to establish the mutation spectrum. The *KCNQ4* mutations found in this study together with previously reported mutations (summarized in Table 1) represent an up-dated mutation spectrum for this gene. For missense mutations, we have gone through all reported missense mutations by computer analysis programs, SIFT and PolyPhen2, to predict the effect of

Distance from the c.211delC mutation (bp)	Fm 1		Fm2	Fm 5		Fm 10		Fm 11		Fm 13				Allele frequency				Marker
	Fa (+)	Dau (+)	Dau (+)	Mo (+)	Son (+)	Mo (+)	Son (+)	g.M (+)	Mo (+)	g.M (+)	Mo (+)	Son (+)	Dau (+)					
490912	C	C	T	T	T	C/T	C/T	C	C	T	T	T	T	C	0.80	T	0.20	rs10489431
468938	T	T	T	C/T	C/T	C/T	C/T	T	T	T	T	T	T	C	0.47	T	0.53	rs1846158
441312	A	A	A	A	A	A	A	A	A	A	A	A	A	T	0.31	A	0.69	rs12088482
422378	G	G	G	G	G	G	G	G	G	G	G	G	G	A	0.27	G	0.73	rs3013462
372705	A	A	A/G	A	A	A	A	A	A	A	A	A	A	G	0.68	A	0.32	rs16827291
339980	C	C	C	C/G	C/G	C/G	C/G	C	C	C	C	C	C	G	0.76	C	0.24	rs10489433
333758	C/T	C/T	C/T	C/T	C/T	C/T	C/T	C	C	C	C	C	C	T	0.44	C	0.56	rs209607
333573	G	G	G	G	G	G	G	G	G	G	G	G	G	G	0.89	A	0.11	rs2076493
285371	C/G	C/G	C	C	C	C	C	C	C	C	C	C	C	C	0.48	G	0.52	rs12034162
215165	C/T	C/T	T	T	T	T	T	T	T	T	T	T	T	T	0.44	C	0.56	rs4660167
207908	G	G	G	G	G	A/G	A/G	A/G	A/G	G	G	G	G	G	0.41	A	0.59	rs4660436
201218	C/T	C/T	T	T	T	C/T	C/T	C/T	C/T	T	T	T	T	T	0.36	C	0.64	rs12128397
174767	G	G	G	G	G	G	G	G	G	G	G	G	G	G	0.59	A	0.41	rs500586
173410	A	A	A	A/G	A/G	A	A	A	A	A	A	A	A	G	0.56	A	0.44	rs12217146
168622	G	G	G	G	G	G	G	G	G	G	G	G	G	G	0.60	C	0.40	rs504242
151498	T	T	T	C/T	C/T	T	T	T	T	T	T	T	T	T	0.61	C	0.39	rs542214
140107	C	C	C	C/T	C/T	C	C	C	C	C	C	C	C	C	0.62	T	0.38	rs7520394
9505	T	T	T	T	T	T	T	T	T	T	T	T	T	A	0.42	T	0.58	rs823674
6548	C	C	C	C	C	C	C	C	C	C	C	C	C	T	0.39	C	0.61	rs1327887
3196	A	A	A	A/G	A/G	A	A	A	A	A	A	A	A	G	0.63	A	0.37	rs12405252
2353	T	T	T	T	T	T	T	T	T	T	T	T	T	T	0.70	C	0.30	rs17361386
0	--	--	--	--	--	--	--	--	--	--	--	--	--	-	--	-	--	c.211delC
17282	C	C	C	C	C	C	C	C	C	C/T	C/T	C/T	C/T	C	0.23	T	0.77	rs4660464
20187	A	A	A/T	A/T	A/T	A/T	A/T	A	A	A	A	A	A	T	0.87	A	0.13	rs12408769
25343	G	G	G	C/G	C/G	G	G	G	G	G	G	G	G	G	0.70	C	0.30	rs878043
34533	G	G	G	G	G	G	G	G	G	G	G	G	G	G	0.58	C	0.42	rs2361658
41555	A	A	A/G	A/G	A/G	A	A	A	A	A	A	A	A	A	0.50	G	0.50	rs3767942
43025	A	A	A	A/G	A/G	A/G	A/G	A	A	A	A	A	A	G	0.76	A	0.24	rs6697721
43513	T	T	C/T	C/T	C/T	T	T	T	T	T	T	T	T	T	0.73	C	0.27	rs3767944
43673	T	T	C/T	C/T	C/T	C/T	C/T	T	T	T	T	T	T	C	0.79	T	0.21	rs4660176
58166	C/T	C/T	T	C/T	C/T	T	T	T	T	T	T	T	T	C	0.23	T	0.77	rs1576122
58742	A	A	A/G	A	A	A	A	A	A	A	A	A	A	A	0.64	G	0.36	rs4660472
61431	A/C	A/C	A	A/C	A/C	A/C	A/C	A	A	A	A	A	A	C	0.33	A	0.67	rs4534368
65688	T	T	T	C/T	C/T	T	T	T	T	T	T	T	T	C	0.37	T	0.63	rs11209014
68464	A	A	G	A/G	A/G	A/G	A/G	G	G	G	G	G	G	A	0.46	G	0.55	rs4660473
73906	T	T	T	T	T	T	T	T	T	T	T	T	T	C	0.21	T	0.80	rs913382
75825	G	G	A	A/G	A/G	A	A	A	A	A	A	A	A	G	0.47	A	0.53	rs11209041
101565	A	A	A/T	T	T	A/T	A/T	T	T	A/T	A/T	A/T	A/T	T	0.60	A	0.40	rs6700929
121363	T	T	T	T	T	T	T	T	T	T	T	T	T	T	0.52	C	0.48	rs6684543
122261	T	T	T	T	T	T	T	C	C	T	T	T	T	C	0.68	T	0.32	rs11209145
233975	G	G	C	C/G	C/G	C/G	C/G	G	G	C	C	C	C	G	0.77	C	0.23	rs11209361
237645	A	A	C	A/C	A/C	A/C	A/C	A	A	C	C	C	C	A	0.86	C	0.14	rs6674450
250602	A	A	T	A/T	A/T	A/T	A/T	A	A	T	T	T	T	A	0.84	T	0.16	rs11580656
274693	A	A	A	A	A	A	A	A	A	A	A	A	A	G	0.09	A	0.91	rs4660500
322363	T	T	G/T	G/T	G/T	G/T	G/T	T	T	G	G	G	G	G	0.43	T	0.57	rs548007
334776	G	G	A	A/G	A/G	A	A	G	G	A	A	A	A	G	0.84	A	0.16	rs2284802
369918	G	G	A	A/G	A/G	A/G	A/G	G	G	A	A	A	A	A	0.23	G	0.77	rs213744
487513	C	C	C/T	C/T	C/T	T	T	C	C	T	T	T	T	C	0.48	T	0.52	rs11209779
503189	G	G	G	G	G	G	G	G	G	G	G	G	G	G	0.73	A	0.27	rs12029950

*Fm (n), Family number (n); Mo, Mother; Fa, Father; Dau, Daughter, g. M, grand mother,

Figure 2. The haplotypes around c.211delC mutation of six families constructed using SNPs are shown. Each column shows an affected allele. Each base is defined by pure segregation analysis in the family. Allele frequencies of SNPs are derived from HapMap JPT+CHB samples. Families 2, 5, 10, and 13 shared a large common region of about more than 1 Mb in their haplotypes (blue). Abbreviation: Fm, Family.
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missense variants on *KCNQ4* protein function. A missense mutation (p.F182L) was found in one control patient with normal audiogram and the results showed that it is not likely to be a pathologic mutation.

The present study identified 7 possible disease-causing mutations, including 5 novel mutations, in 19 autosomal dominant families. Based on our unbiased population-based genetic screening, the frequency is 6.62% (19/287) of the overall ADNSHL population. These data indicated that *KCNQ4* is one of the important causative genes among ADNSHL patients, particularly in patients with high frequency-involved hearing loss. This frequency is higher than our recently reported frequency (4/139:

Table 2. Clinical features of affected family members associated with KCNQ4 mutations found in this study.

Amino Acid Change	Family – Patient No.	HL onset age (years)	Age at the first visit (years)	Audiogram frequencies	Progression	Tinnitus	Vertigo
Q71fs	1–1	40	48	Ski slope	N/A	N/A	N/A
	1–2	15	15	Ski slope	+	–	–
	2–1	30	47	Ski slope	+	+	–
	3–1	N/A	31	Ski slope	N/A	–	–
	4–1	12	37	Ski slope	+	+	–
	5–1	32	42	Ski slope	–	+	–
	5–2	10	15	Ski slope	+	+	–
	6–1	14	40	Ski slope	+	+	–
	7–1	11	35	Ski slope	+	+	–
	8–1	18	25	Ski slope	+	+	–
	9–1	18	29	Ski slope	+	+	–
	10–1	17	22	Ski slope	+	+	–
	10–2	20	52	Ski slope	+	+	–
	11–1	40	43	Ski slope	+	–	–
	11–2	N/A	73	Ski slope	N/A	–	–
	12–1	22	38	Ski slope	+	+	–
	13–1	35	55	Ski slope	+	+	–
	13–2	25	33	Ski slope	+	+	+
	13–3	11	14	Ski slope	N/A	+	+
	13–4	–	6	Normal (*)	N/A	N/A	N/A
H77fs	**14**	**22**	**27**	**Ski slope**	**+**	**+**	**–**
V230E	**15–1**	**40**	**78**	**mid freq**	**+**	**+**	**–**
	15–2	**12**	**39**	**mid freq**	**+**	**–**	**–**
	15–3	**5**	**5**	**mid freq**	**+**	**–**	**–**
	15–4	**3**	**3**	**mid freq**	**N/A**	**N/A**	**N/A**
	15–5	**N/A**	**0**	**mid freq**	**N/A**	**N/A**	**N/A**
W276S	16–1	8	65	high freq	+	–	+
	16–2	12	46	high freq	+	–	–
	16–3	7	42	high freq	+	–	–
	16–4	8	8	high freq	+	–	+
	16–5	8	6	high freq	+	–	–
P291S	**17–1**	**20**	**33**	**high freq**	**+**	**N/A**	**N/A**
P291L	**18–1**	**17**	**40**	**high freq**	**N/A**	**N/A**	**N/A**
	18–2	**17**	**15**	**high freq**	**N/A**	**N/A**	**N/A**
R297S	**19–1**	**39**	**39**	**high freq**	**–**	**+**	**–**
	19–2	**5**	**5**	**high freq**	**+**	**–**	**–**

Abbreviations: HL, hearing loss; mid, middle; freq, frequency; N/A, not applicable.
(*) Six-year-old boy's hearing is normal in spite of having the mutation.
doi:10.1371/journal.pone.0063231.t002

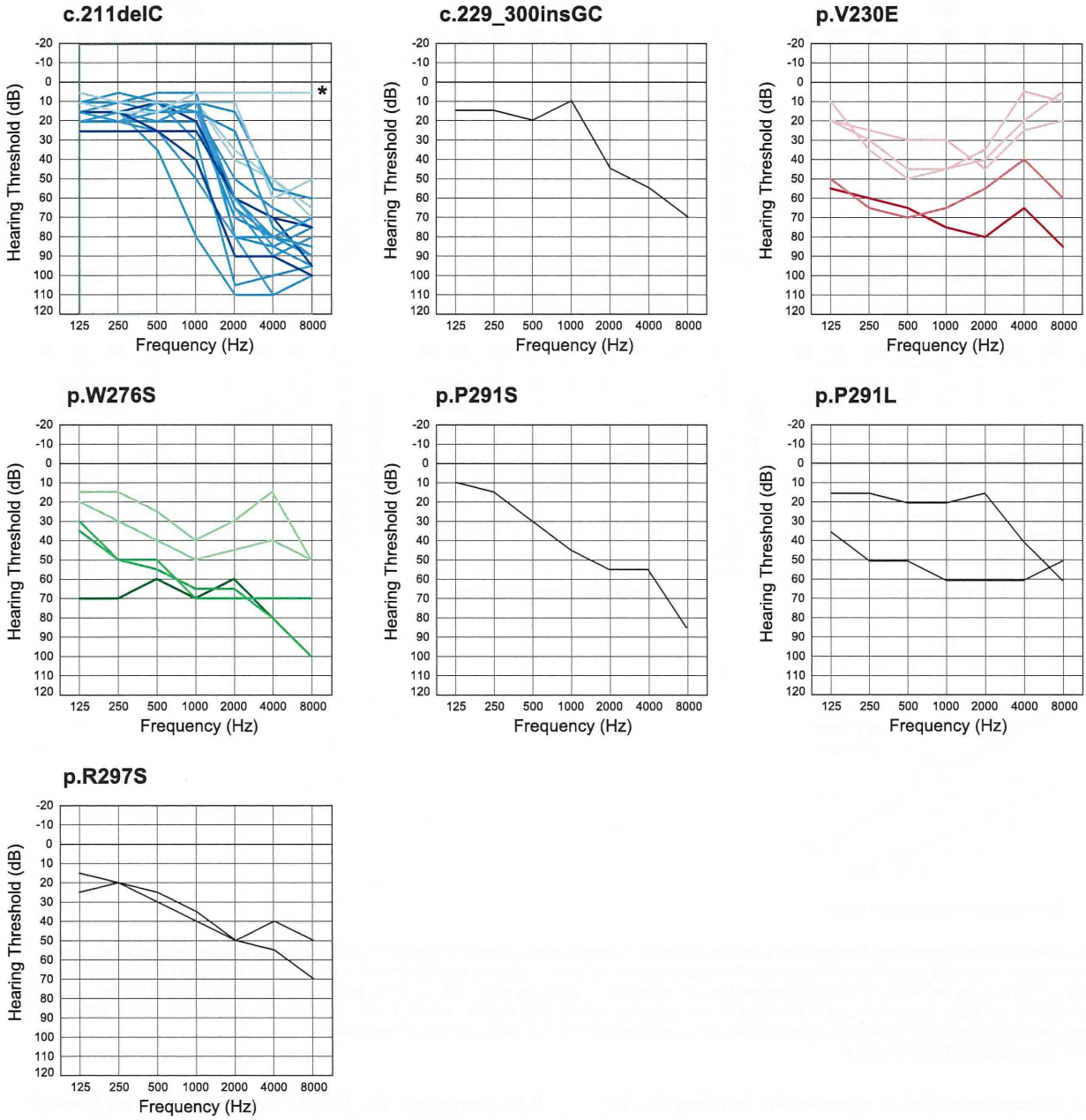

Figure 3. Overlapping audiograms from the better ear for each genotype. In cases of W276S, c.211delC, or V230E, light colored audiograms (green, blue, red) were from individuals aged 19 and under. Dark colored audiograms (green, blue, red) were from the patients aged 20–49 years old, and deep colored audiograms (green, blue, red) are from the patients in their 50 s and over. In family #13 with c.211delC, (*) a six-year-old boy's hearing is normal in spite of having the mutation.
doi:10.1371/journal.pone.0063231.g003

2.9%) of *TECTA* in Japanese ADNSHL families [4], therefore *KCNQ4* is found to be currently the most prevalent gene responsible for Japanese ADNSHL patients, and should be the first in line to be analyzed for ADNSHL patients.

Mutations lie in various domains of the *KCNQ4* protein. While the majority are private mutations, one particular recurrent mutation, c.211delC, was observed in 13 unrelated families. In this gene, we have reported that there is a hot spot mutation, p.W276S, in Belgian, Dutch, and Japanese families [5]. Based on haplotype analysis, in the case for c.211delC, it is not likely a hot spot but rather is suggested to be due to a common ancestor. Such recurrent mutations are common in recessive genes such as 235delC, 35delG, 167delT in *GJB2* [6][7], H723R in *SLC26A4* [8], and P204L in *CDH23* [9]. They are rare in dominant genes, though a mutation in *DFNA5* that causes autosomal dominant sensorineural hearing loss was reported to arise from a common ancestor [10]. Together with specific audiogram configuration, this may facilitate genetic testing for ADNSHL with a particular phenotype.

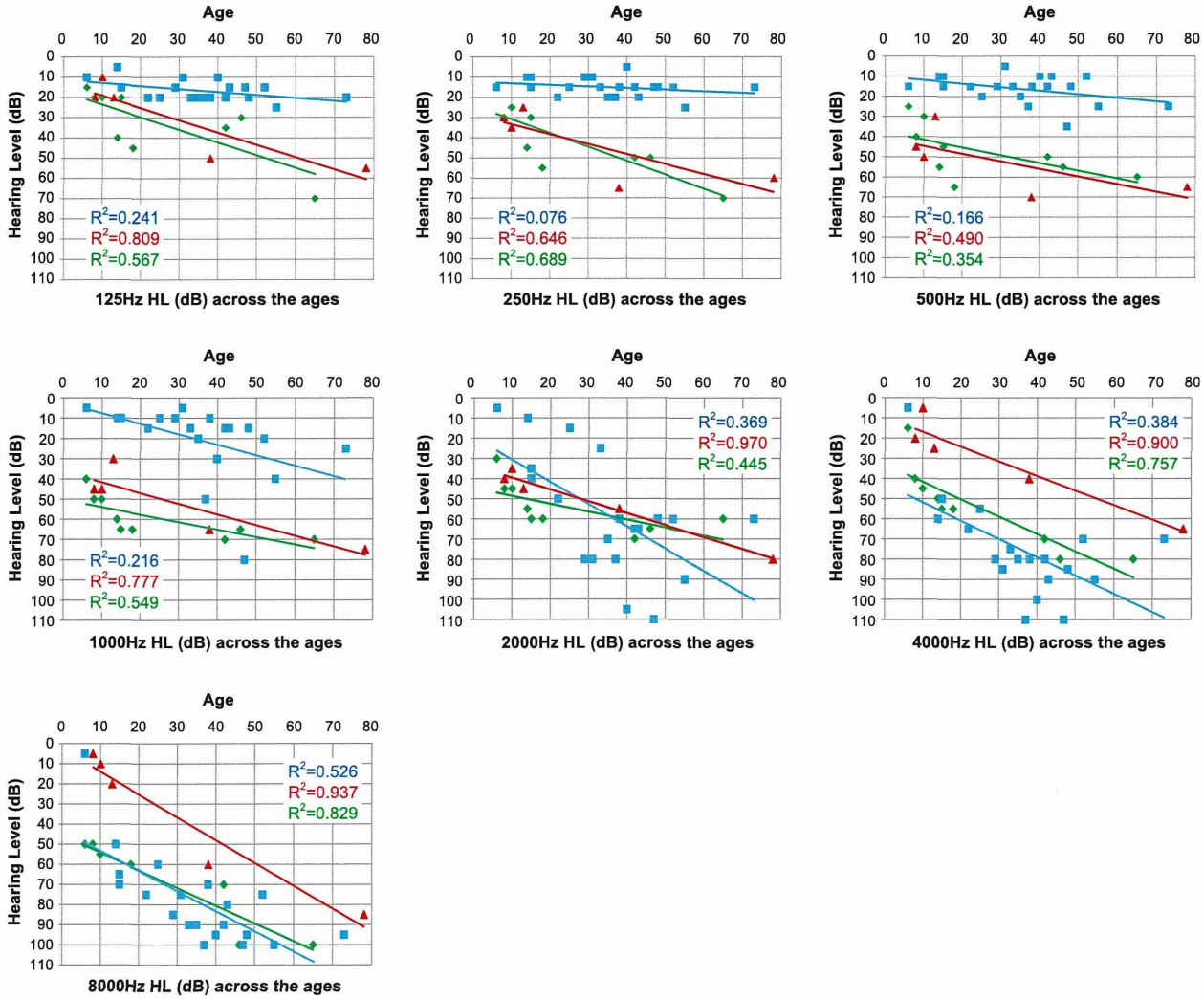

Figure 4. Detailed progression analysis in each frequency. A single audiogram (the better ear) from a single patient was plotted. Gradual progression is characterized regardless of frequency. Average progressive rates of hearing loss (db/year) for the patients with c.211delC, for 125 (0.15) and 250 Hz (0.078) were significantly stable compared to the other two mutations (ANCOVA: $p<0.05$) and they had milder hearing loss at 500 and 1 KHz (ANCOVA: $p<0.05$). In contrast, at 4 KHz and 8 KHz, patients with V230E mutations had milder hearing loss compared to the other two mutations (ANCOVA: $p<0.05$). Each color (green, blue, red) indicates W276S, c.211delC, or V230E, respectively.
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Table 2 summarizes clinical characteristics including hearing threshold, severity, onset age (age of awareness), progressiveness of hearing loss, and vestibular symptoms. Age of onset (awareness of hearing loss) ranged from 3 to 40 years old, though the majority of the patients were in their first decade of life. Many of the mutations were accumulated in the P-loop region as described before [3][11][12], but mutations were also found in the other domains (Table 1, Fig. 1). There were some correlations between genotype and phenotype (Fig. 3). Overlapped audiograms showed characteristic high frequency involved hearing loss in the majority of the patients with *KCNQ4* mutations. Unique audiograms were shown in the patients with c.211delC and p.V230E. The patients associated with c.211delC showed so-called ski slope hearing loss (high frequency involved hearing loss with nearly normal hearing at lower frequencies). Patients with p.V230E showed mid-frequency involved hearing loss.

It has been known that DFNA2 shows high-frequency involved hearing loss [3][13][14]. Based on collected audiograms from the patients with *KCNQ4*, an effective selection algorithm named "Audioprofile" has been proposed and many mutations have actually been successfully identified [13]. The present large cohort study allowed us to confirm and extend the genotype-phenotype correlations. It added a new type of audiogram configuration characterized by mid-frequency predominant hearing loss caused by a *KCNQ4* mutation (Fig. 3). Family #15 had a heterozygous T>A transition at nucleotide 689 in exon 4, which results in a Val to Glu substitution (V230E). This mutation was present in all five affected individuals, and not present in two unaffected family members. None of the 252 normal controls had this mutation. Prediction programs indicated that this mutation is likely to be pathologic. So far mid-frequency predominant hearing loss has been reported with *TECTA* mutations [4]. In this family, we sequenced for *TECTA* to find a mutation, but none were found

(data not shown). A different *KCNQ4* mutation (c.664_681del) within the same domain as this mutation was reported to cause high-frequency involved hearing loss, suggesting that the phenotype is not domain-specific [15]. The V230E mutation is a missense mutation that substitutes a nonpolar and aliphatic valine for a negatively charged glutamate. This single base substitution is located adjacent to the S4 transmembrane domain that has a key role as a voltage sensor. The V230E mutation may therefore change sensitivity of voltage sensor and have an affect on passage of potassium through the cell membrane.

The ski-slope type audiogram configuration found in the patients with c.211delC is also a striking characteristic phenotype (Fig. 3). Single families associated with c.211delC [16] and c.211_223del13 [17] have previously been reported to show ski-slope audiograms. The audiogram collection in this study further generalized this phenotype in the N-terminal site.

Analysis of the different frequencies found evident quickly progressive hearing loss in the middle frequencies, therefore those patients may be at risk for rapid deterioration of speech understanding during the time course. Patients with ski-slope type audiograms sometimes have difficultly in being fitted with hearing aids, but Electric Acoustic Stimulation (EAS) has recently been shown to be effective for those patients with high frequency involved hearing loss [18]. The present data on progression speed showed more stable hearing at low frequencies (125 and 250Hz) (Fig. 4), indicating EAS will be the potential therapeutic intervention for the patients with this particular mutation.

Progressive nature is a common feature of the patients with *KCNQ4* mutations regardless of the particular mutation (Fig. 3). Overlapped audiograms of all subjects with W276S, c.211delC, or V230E mutations showed the progressive nature of hearing loss regardless of the mutation type. However, no patients received cochlear implants in this cohort, suggesting that profound hearing loss may seldom be seen though their hearing loss has a progressive nature.

In conclusion, *KCNQ4* is frequent among ADNSHL patients, and therefore screening for this gene and molecular confirmation of *KCNQ4* mutations have become important in the diagnosis of these conditions.

Supporting Information

Figure S1 Pedigrees of the *KCNQ4* mutation families and detected mutations.
(PDF)

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Author Contributions

Conceived and designed the experiments: SU. Performed the experiments: TN SN YI TY. Analyzed the data: TN SN YI TY SU. Contributed reagents/materials/analysis tools: TN KK SA KI HK AN CO. Wrote the paper: SU TN.

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Massively Parallel DNA Sequencing Successfully Identifies New Causative Mutations in Deafness Genes in Patients with Cochlear Implantation and EAS

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Abstract

Genetic factors, the most common etiology in severe to profound hearing loss, are one of the key determinants of Cochlear Implantation (CI) and Electric Acoustic Stimulation (EAS) outcomes. Satisfactory auditory performance after receiving a CI/EAS in patients with certain deafness gene mutations indicates that genetic testing would be helpful in predicting CI/EAS outcomes and deciding treatment choices. However, because of the extreme genetic heterogeneity of deafness, clinical application of genetic information still entails difficulties. Target exon sequencing using massively parallel DNA sequencing is a new powerful strategy to discover rare causative genes in Mendelian disorders such as deafness. We used massive sequencing of the exons of 58 target candidate genes to analyze 8 (4 early-onset, 4 late-onset) Japanese CI/EAS patients, who did not have mutations in commonly found genes including *GJB2, SLC26A4,* or mitochondrial 1555A>G or 3243A>G mutations. We successfully identified four rare causative mutations in the *MYO15A, TECTA, TMPRSS3,* and *ACTG1* genes in four patients who showed relatively good auditory performance with CI including EAS, suggesting that genetic testing may be able to predict the performance after implantation.

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Introduction

Cochlear Implantation (CI) has been established as a standardized therapy for severe to profound hearing loss [1]. Electric Acoustic Stimulation (EAS) is a hearing implant system combining a cochlear implant and acoustic amplification technology in one device, and has recently become a standard intervention for the patients with partial deafness, defined as a mild to moderate low-frequency sensorineural hearing loss sloping to a profound hearing loss in the higher frequencies [1]. One difficult point is that outcomes of CI/EAS are variable and many factors are thought to be involved in post-implantation performance. Satisfactory auditory performance in the patients with various deafness gene mutations indicates that genetic background would be helpful in predicting performance after CI [2]. When genetic background is involved in intra-cochlear etiology, there is potential for good performance. Therefore, it is important to identify the involved region inside/outside of the cochlea by identifying the responsible gene. Decisions as to whether to undergo EAS surgery and the timing of the surgery, as well as prediction of outcome after EAS is sometimes difficult because of individual differences in progression, which is sometimes of a rather rapid nature but sometimes rather stable. One advantage of genetic testing is that the possible prognosis for hearing, i.e., progressive or not, can be predicted for individual patients.

Etiological studies have shown genetic disorders to be a common cause of deafness, but difficulty lies in the fact that deafness is an extremely heterogenous disorder.

Invader-based multi-gene screening for 13 genes/46 mutations commonly found in Japanese, identified the responsible mutations in approximately 30% of deafness patients [3], accelerating the clinical application of gene screening. However, the etiology of the rest of the patients is still unknown. In addition, the involvement of at least 58 distinct genes sometimes makes the precise diagnosis difficult.

Targeted exon sequencing of selected genes using the Massively Parallel DNA Sequencing (MPS) technology will potentially enable us to systematically tackle previously intractable monogenic disorders and improve molecular diagnosis. We have recently reported that target exon sequencing using MPS is a powerful tool to identify rare gene mutations for deafness patients [4].

In this study, we have chosen 58 deafness-causative genes, and conducted genetic analysis using MPS-based genetic screening to find the rare genes responsible for the patients who received CI or EAS.

Subjects and Methods

Subjects

Eight deafness patients (4 early-onset, 4 late-onset) were randomly selected from among 150 CI or EAS patients (69 male and 81 female, aged 0 to 91), without common *GJB2, SLC26A4*, or mitochondrial 1555A>G or 3243A>G mutations determined by direct sequencing. Four patients with early-onset deafness received CI, and 4 late-onset patients had residual hearing at lower frequencies and received EAS. All subjects or next of kin, caretakers, or guardians on the behalf of the minors/children gave prior written informed consent for participation in the project, and the Ethical Committee of Shinshu University approved the study and the consent procedure.

Auditory behavioral development was assessed by IT-MAIS and LittlEARS, both of which are parent questionnaires regarding a young infant or toddler's auditory behavior [5,6]. IT-MAIS consists of 10 questions, each scored on a 5-point scale: 0 = never, 1 = rarely, 2 = occasionally, 3 = frequently, and 4 = always. LittlEARS has 35 questions, each scored as 1 = yes, and 0 = no.

Amplicon Library Preparation

An Amplicon library of the target exons was prepared with an Ion AmpliSeq™ Custom Panel (Applied Biosystems, Life Technologies., Carlsbad, CA) designed with Ion AmpliSeq™ Designer (https://www.ampliseq.com/browse.action) for 58 genes reported to be causative of non-syndromic hearing loss listed in Table S1 (Hereditary Hearing loss Homepage; http://hereditaryhearingloss.org/) by using Ion AmpliSeq™ Library Kit 2.0 (Applied Biosystems, Life Technologies) and Ion Xpress™ Barcode Adapter 1–16 Kit (Applied Biosystems, Life Technologies) according to the manufacturers' procedures.

In brief, DNA concentration was measured with Quant-iT™ dsDNA HS Assay (Invitrogen, Life Technologies) and Qubit® Fluorometer (Invitrogen, Life Technologies) and DNA quality was confirmed by agarose gel electrophoresis. 10 ng of each genomic DNA sample was amplified, using Ion AmpliSeq™ HiFi Master Mix (Applied Biosystems, Life Technologies) and AmpliSeq™ Custom primer pools, for 2 min at 99°C, followed by 15 two-step cycles of 99°C for 15 sec and 60°C for 4 min, ending with a holding period at 10°C in a PCR thermal cycler (Takara, Shiga, Japan). After the Multiplex PCR amplification, amplified DNA samples were digested with FuPa enzyme at 50°C for 10 min and 55°C for 10 min and the enzyme was successively inactivated for 60°C for 20 min incubation. After digestion, diluted barcode adapter mix including Ion Xpress™ Barcode Adapter and Ion P1 adaptor were ligated to the end of the digested amplicons with ligase in the kit for 30 min at 22°C and the ligase was successively inactivated at 60°C for 20 min incubation. Adaptor ligated amplicon libraries were purified with the Agencourt AMPure XP system (Beckman Coulter Genomics, Danvers, MA). The amplicon libraries were quantified by using Ion Library Quantitation Kit (Applied Biosystems, Life Technologies) and the StepOne plus realtime PCR system (Applied Biosystems, Life Technologies) according to the manufacturers' procedures. After quantification, each amplicon library was diluted to 20pM and the same amount of the 6 libraries for 6 patients were pooled for one sequence reaction.

Emulsion PCR and Sequencing

The emulsion PCR was carried out with the Ion OneTouch™ System and Ion OneTouch 200 Template Kit v2 (Life Technologies) according to the manufacturer's procedure (Publication Part Number 4478371 Rev. B Revision Date 13 June 2012). After the emulsion PCR, template-positive Ion Sphere™ Particles were enriched with the Dynabeads® MyOne™ Streptavidin C1 Beads (Life Technologies) and washed with Ion OneTouch™ Wash Solution in the kit. This process were performed using an Ion OneTouch™ ES system (Life Technologies).

After the Ion Sphere Particle preparation, MPS was performed with an Ion Torrent Personal Genome Machine (PGM) system using the Ion PGM™ 200 Sequencing Kit and Ion 318™ Chip (Life Technologies) according to the established procedures (Publication Part Number 4474596 Rev. B Revision Date 14 July 2012).

Base Call and Data Analysis

The sequence data were processed with standard Ion Torrent Suite™ Software and Torrent Server successively mapped to human genome sequence (build GRCh37/hg19) with Torrent Mapping Alignment Program optimized to Ion Torrent™ data. The average of 412.93 Mb sequences with about 3,200,000 reads was obtained by one Ion 318 chip. The 98.0% sequences were mapped to the human genome and 94.9% of them were on the target region. Average coverage of depth in the target region was 326.5 and 94.2% of them were over 20 coverage.

After the sequence mapping, the DNA variant regions were piled up with Torrent Variant Caller plug-in software. Selected variant candidates were filtered with the average base QV (minimum average base quality 25), variant frequency (40–60% for heterozygous mutations and 80–100% for homozygous mutations) and coverage of depth (minimum coverage of depth 10). After the filtrations, variant effects were analyzed with the wANNOVAR web site [7,8] (http://wannovar.usc.edu) including the functional prediction software for missense variants listed below. PhyloP (http://hgdownload.cse.ucsc.edu/goldenPath/hg18/phyloP44way/), Sorting Intolerant from Tolerant (SIFT; http://sift.jcvi.org/), Polymorphism Phenotyping (PolyPhen2; http://genetics.bwh.harvard.edu/pph2/), LRT (http://www.genetics.wustl.edu/jflab/lrt_query.html), MutationTaster (http://www.mutationtaster.org/), and GERP++ (http://mendel.stanford.edu/SidowLab/downloads/gerp/index.html).

Algorithm

Flow of informatics analysis is shown in Fig. 1. Missense, nonsense, and splicing variants were selected among the identified variants. Variants were further selected as less than 1% of, 1) the 1000 genome database (http://www.1000genomes.org/), 2) the 5400 exome variants (http://evs.gs.washington.edu/EVS/), and 3) the 72 in-house controls. Candidate mutations were confirmed by Sanger sequencing and the responsible mutations were identified by segregation analysis using samples from family members of the patients.

Direct Sequence Analysis

Primers were designed with the Primer 3 plus web server (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). Each genomic DNA sample (40 ng) was amplified using AmpliTaq Gold (Life Technologies) for 5 min at 94°C, followed by 30 three-step cycles of 94°C for 30 sec, 60°C for 30 sec, and 72°C for 30 sec, with a final extension at 72°C for 5 min, ending with a holding period at 4°C in a PCR thermal cycler (Takara, Shiga, Japan). The PCR products were treated with ExoSAP I (GE Healthcare Bio, Buckinghamshire, UK) and by incubation at 37°C for 30 min, and inactivation at 80°C for 15 min. After the products were purified, we performed standard cycle sequencing reaction with ABI Big Dye terminators in an ABI 3130×l sequencer (Life Technologies).

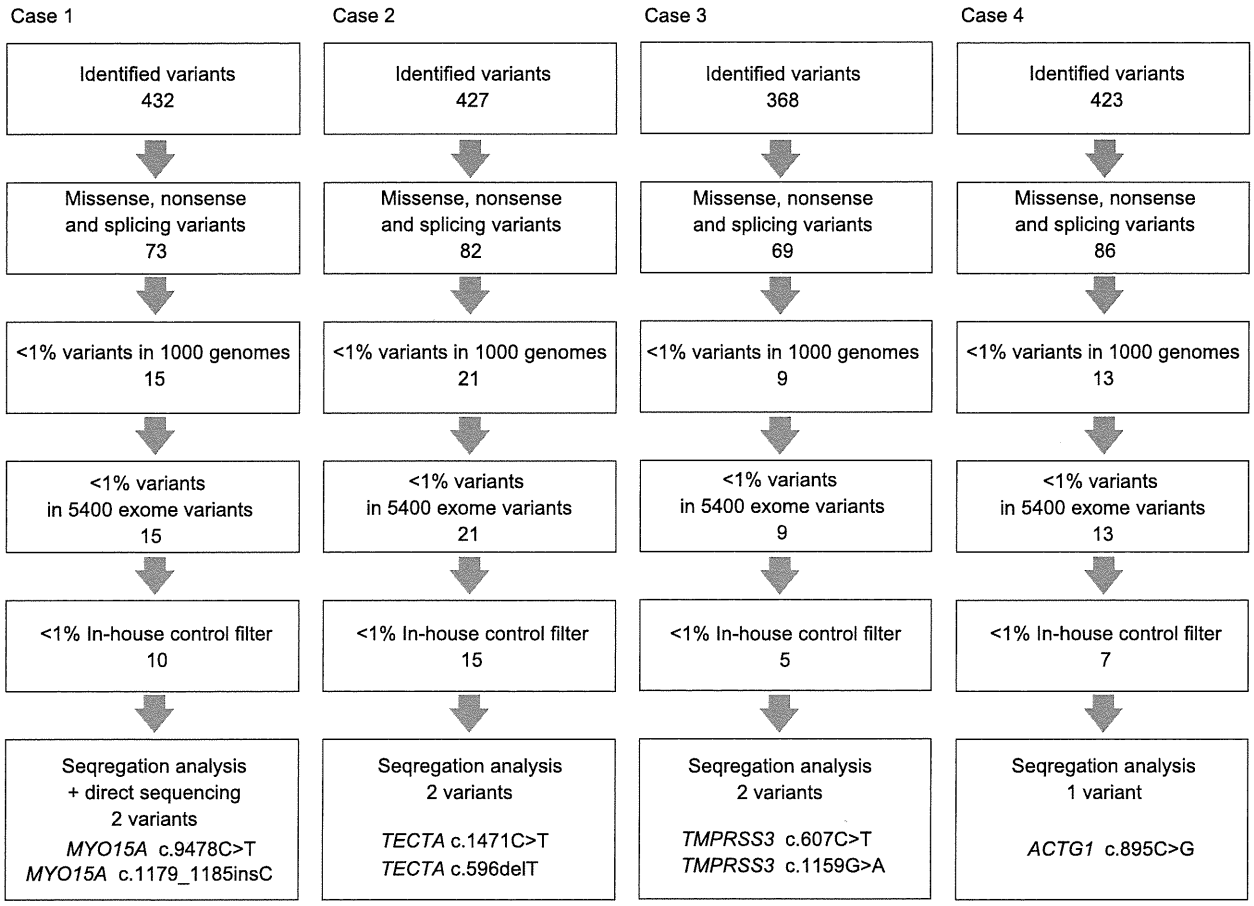

Figure 1. Flow of informatics analysis. Selected missense, nonsense, and splicing variants were filtered with 1) the 1000 genomes, 2) the 5400 exome variants, and 3) the in-house control. Responsible mutations were confirmed by segregation analysis.
doi:10.1371/journal.pone.0075793.g001

Results

After informatics analysis, several candidate variants were identified and segregation analysis confirmed responsible mutations in *MYO15A* (Case #1) and *TECTA* (Case #2) in pre-lingual patients with conventional CI, and mutations in *TMPRSS3* (Case #3) and *ACTG1* (Case #4) were identified in patients with post-lingual deafness with EAS (Fig. 1). All detected mutations were predicted to be pathologic by several software programs (Table 1). In the remaining four cases, there were no conclusive causative mutations found in this study.

Case #1: Severe Hearing Loss caused by *MYO15A* Mutations (Fig. 2)

As in Fig. 1, MPS identified 10 candidate variants in 9 genes. Among the 9 genes, *CDH23* and *MYO15A* are known to be inherited in a recessive manner. Sanger sequencing could not detect the *CDH23* variant. A *MYO15A* mutation (c.9478C>T (p.L3160F)) was confirmed by Sanger sequencing. Consecutive Sanger sequencing analysis identified another mutation, c.1179_1185insC, which was not found by MPS. The inconsistent results between the two methods were due to this mutation being located in the homo-polymer (poly C stretch) region, which is difficult to detect using this system [9] The patient (5y 5 m-old boy) had compound heterozygous *MYO15A* mutations (c.[9478C>T];[1179_1185insC]), and the parents were found to be carriers for these mutations (Fig. 2A). The frameshift mutation c.1179_1185insC, leading to a stop codon, was predicted to be causative, and the missense mutation, c.9478C>T, was predicted to be pathologic by several software programs (Table 1).

His hearing loss was found through newborn hearing screening using OAE. Auditory steady state response (ASSR) and conditioned orientation reflex (COR) evaluated at the ages of 1y 6 m, 2y 3 m, 2y 8 m, and 3y 6 m showed progressive hearing loss. He used hearing aids and some language development was seen, but due to progressive hearing loss, hearing aid amplification was insufficient, and he received a left CI (MEDEL PULSAR CI100/standard electrode) at the age of 4y 9 m. To obtain the final outcome, long-term follow up will be needed, but language was developed after 3 months of CI use (Scores of IT-MAIS: 16/40>25/40, LittlEar: 28>33).

Case #2: Profound Hearing Loss caused by *TECTA* Mutations (Fig. 3)

The patient (a 2-year-old boy) had compound heterozygous *TECTA* mutations (c.[596delT];[1471C>T]), and the parents were found to be carriers for these mutations (Fig. 3A). The frameshift mutation, c.596delT, leading to a stop codon, was predicted to be pathologic. The missense mutation, c.1471C>T

Table 1. Missense mutations found in this study.

Gene	Base Change	AA Change	ESP5400	1000g2012feb	dbSNP135	PhyloP	SIFT	PolyPhen2	LRT	MutationTaster	GERP++
MYO15A	c.9478C>T	p.L3160F	0.007618	0.01	rs140029076	N (0.885983)	D (0.97)	NA (0.754167)	NA (0.981216)	D (0.99518)	0.651
TECTA	c.1471C>T	p.R491C	–	–	–	C (0.998333)	D (0.97)	D (1)	D (1)	D (0.684828)	4.88
TMPRSS3	c.1159G>A	p.A387T	–	–	–	C (0.997807)	D (0.96)	B (0.074)	D (1)	N (0.364687)	4.62
ACTG1	c.895C>G	p.L299V	–	–	–	C (0.978424)	NA (0.750464)	B (0.006)	D (0.99998)	D (0.999635)	1.2

SIFT, Polyphen-2, PhyloP, LRT, Mutation Taster, and GERP++ are functional prediction scores in which increasing values indicate a probable mutation. ESP5400 and 100g2012feb are the allele frequency in each 5400 exome and 1000 genome project.
Abbreviations: C, conserved; N, not-conserved or neutral D, damaging or deleterious; B, benign; NA, not applicable.
doi:10.1371/journal.pone.0075793.t001

(p.R491C), was predicted to be pathologic by several software programs (Table 1).

His hearing loss was found through newborn hearing screening using OAE. ASSR and COR evaluated at the age of 8 m, 1 y 3 m, and 1 y 9 m showed progressive hearing loss. He used hearing aids, but due to insufficient amplification, he received a left CI at the age of 2. Language was developed after 4 months of CI use (Scores of IT-MAIS: 9/40>23/40).

Case#3: Late Onset Hearing Loss with Residual Hearing in Low Frequencies caused by *TMPRSS3* Mutations (Fig. 4)

The patient (a 40-year-old woman) had compound heterozygous *TMPRSS3* mutations c.[607C>T];[1159G>A] (p.[Q203X];[A387T]) (Fig. 4A). The nonsense mutation p.Q203X was predicted to be causative, and the missense mutation (p.A387T) was predicted to be pathologic by several software programs (Table 1). The parents were found to be carriers for these mutations. She had hearing loss detected by mass screening in primary school. It appeared to slowly progress, and by age 25 she suffered inconvenience in hearing and communication. EAS (MEDEL PULSAR FLEXeas) was applied at the ages of 38 and 39. Residual hearing for acoustic amplification could be preserved, and hearing level with bilateral EAS was around 30dB (Fig. 4C–E). Japanese monosyllable test (65dB SPL in quiet) showed dramatic improvement with bilateral EAS from 18% to 90% one year after receiving the second EAS (Fig. 4F).

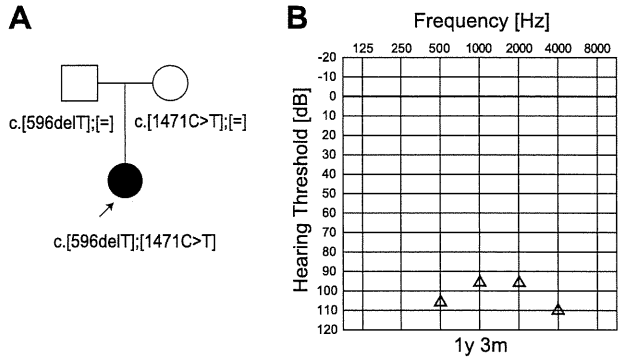

Figure 3. The CI patient with *TECTA* mutations. A: The patient has compound heterozygous *TECTA* mutations (c.[596delT];[1471C>T]), and the parents were found to be carriers for these mutations. B: COR audiogram finding (1y 9 m).
doi:10.1371/journal.pone.0075793.g003

Case #4: Late Onset Hearing Loss with Residual Hearing in Low Frequencies caused by *ACTG1* Mutation (Fig. 5)

The patient (a 41-year-old man) had a heterozygous *ACTG1* mutation, c.895C>G (p.L299V) (Fig. 5A). His pedigree was compatible with autosomal dominant hearing loss. A missense mutation, p.L299V, was predicted to be pathologic by several

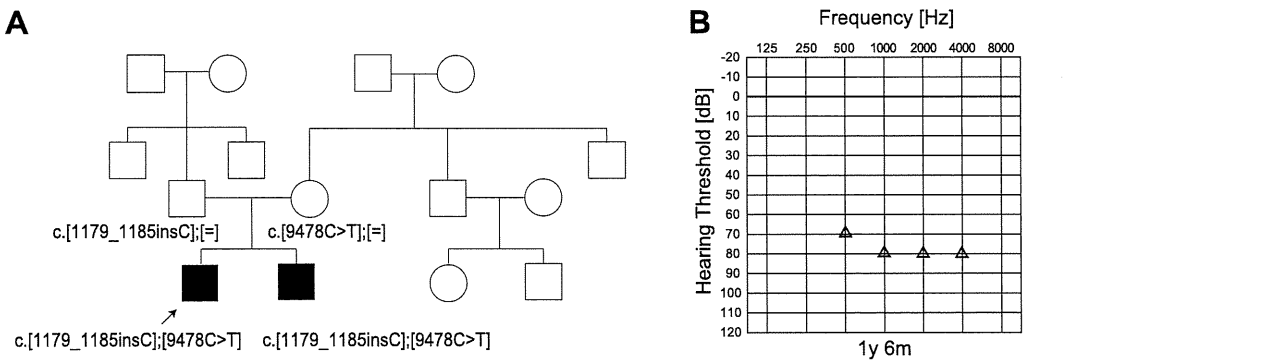

Figure 2. The CI patient with *MYO15A* mutations. A: The patient has compound heterozygous *MYO15A* mutations (c.[9478C>T];[1179_1185insC]), and the parents were found to be carriers for these mutations. B: COR audiogram finding (1y 6 m).
doi:10.1371/journal.pone.0075793.g002

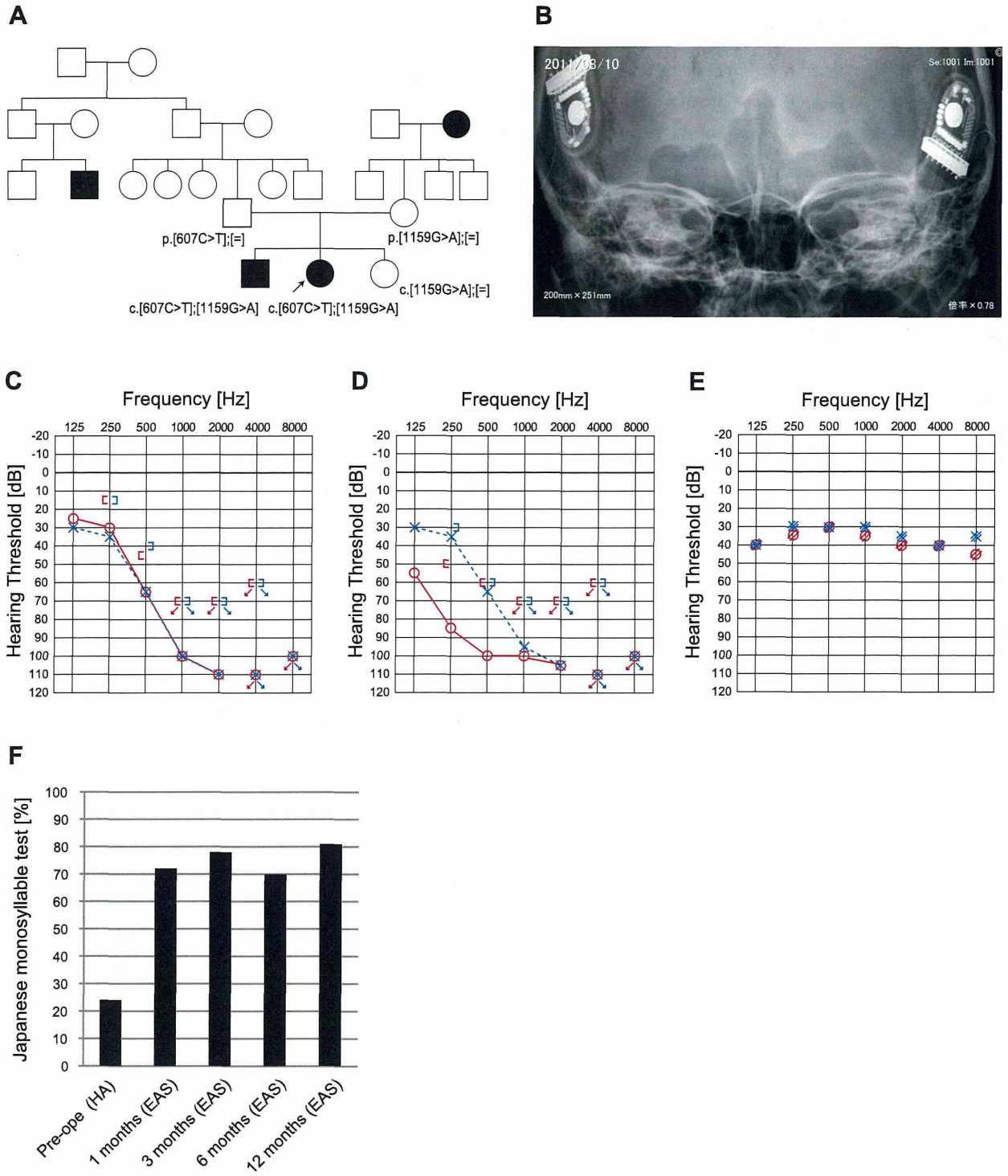

Figure 4. The EAS patient with *TMPRSS3* mutations. A: The patient has compound heterozygous *TMPRSS3* mutations, c.[607C>T];[1159G>A], and the parents were found to be carriers for these mutations. The patient's brother also has the same mutations. B: X-ray imaging after bilateral EAS. C: Pre-operative audiogram. D: Post-operative audiogram (left: 24 months after first EAS, right: 4 months after second EAS). E: Hearing threshold with bilateral EAS. F: Japanese monosyllable test (65dB SPL in quiet) showing dramatic improvement with bilateral EAS.
doi:10.1371/journal.pone.0075793.g004

software programs (Table 1). He noticed his hearing loss at around age 20. He received EAS due to progressive hearing loss. Residual hearing for acoustic amplification could be preserved, and hearing level with bilateral EAS was around 30dB (Fig. 5B, D, E). Japanese

monosyllable test (65dB SPL in quiet) showed dramatic improvement from 20% to 80% one year after receiving EAS (Fig. 5F). His father and brother carried the same mutation. The audiogram of the brother is shown in Fig. 5C. His father also has hearing loss based on anamnestic evaluation. Neither of the patient's sons (aged 10 and 12) have any hearing loss evaluated by pure tone audiogram, although the younger son has the same mutation.

Discussion

The present MPS-based genetic analysis efficiently identified rare causative mutations in four genes, *MYO15A, TECTA, TMPRSS3,* and *ACTG1*. All except *TMPRSS3* were first reported in patients with CI/EAS.

MYO15A has been reported mainly in severe to profound hearing loss [10]. Therefore, it is not surprising the patient with the *MYO15A* mutation was found among the CI patients. However, probably due to being too large to be screened by conventional direct sequencing, the routine screening of this particular gene was hampered in spite of its importance in this particular population. *MYO15A* is known to be responsible for DFNB3 [11]. *Myosin 15a* localizes to the tips of inner ear sensory cell stereocilia and is essential for staircase formation of the hair bundle [12]. Since the etiology is located within the sensory hair cells, comparatively better outcomes can be predicted. This case in fact showed better performance after CI.

TECTA encodes α-tectorin, the major component of non-collagenous glycoprotein of the tectorial membrane. *TECTA* has been reported to be responsible for both autosomal dominant non-syndromic sensorineural hearing loss (ADNSHL) (DFNA8/12) and autosomal recessive non-syndromic sensorineural hearing loss (ARNSHL) (DFNB21). Dominant *TECTA* mutations can cause mid-frequency, high-frequency progressive HL, and *TECTA* is reported to be the commonest causative gene among ADNSHL [13]. Dominant inherited deafness caused by this gene has not been reported to reach the level of profound hearing loss. In contrast, recessive *TECTA* mutations cause more profound hearing loss [14]. The etiology is located within the cochlea, therefore comparatively better outcomes can be predicted. This is the first report of a patient with mutations in this gene showing good outcome as prospected from intra-membranous labyrinth etiology.

In this study, *TMPRSS3* was identified in a patient with post-lingual deafness with EAS (Case #3).

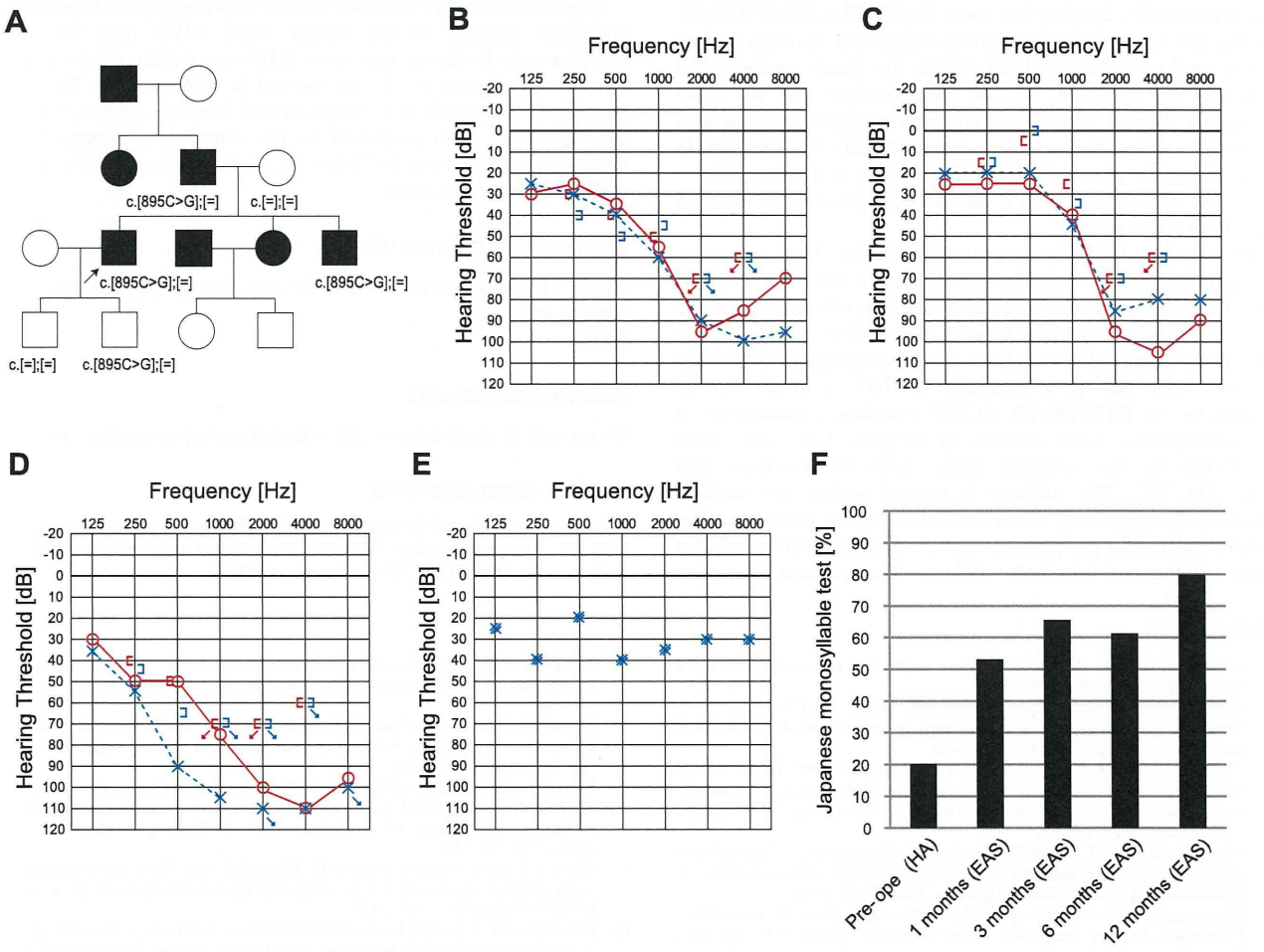

Figure 5. The EAS patient with *ACTG1* mutation. A: The patient has heterozygous *ACTG1* mutation, c.895C>G. Pedigree is compatible with autosomal dominant hearing loss. His father and brother carried the same mutation. B: Pre-operative audiogram. C: Audiogram of brother. D: Post-operative audiogram (6 months after EAS). E: Hearing threshold with EAS. F: Japanese monosyllable test (65dB SPL in quiet) showing dramatic improvement with EAS.
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TMPRSS3 is a member of the Type II Transmembrane Serine Protease family.

TMPRSS3 may be involved in processing proneurotrophins and therefore in the development and survival of the cochlear neurons [15].

TMPRSS3 has been reported to be responsible for DFNB8/10. Typically, the patients show ski-slope type audiograms and progressive HL [16], being compatible with the phenotype of the present patient. Outcome of CI for patients with *TMPRSS3* is controversial [2,16,17]. Two older papers reported good outcome of CI, while a recent report described poorer performance. Eppsteiner et al. [2] reported two cases of 58-year-old patients with a history of progressive hearing loss starting at the age of 5–6 years. Both of their outcomes were poorer compared with other patients, and the authors hypothesized that it was because the encoded protein is also expressed in the spiral ganglion. However, the present 40-year-old patient showed completely different performance after EAS, indicating that CI is not a contraindication and CI and/or EAS can be a recommended therapeutic option. Especially, the previously reported typical phenotype is high frequency involved hearing loss, which is a good indication for EAS. In the literature, there is also a severe phenotype with all frequencies affected [18]. Our 40-year-old patient did not have rapid progressive hearing loss (only 24 dB (125+250+500 Hz/3) during the 7-year follow-up period), supporting that this patient was a good candidate for EAS. Within this family, intra-familial variation was observed, i.e., an elder brother with the same mutations showed early onset (10 y.o.) profound hearing loss. Therefore, other factors may also potentially be involved in determining the phenotype (including severity and progression).

ACTG1 was identified in a patient with post-lingual deafness with EAS (Case #4).

His brother (35 y.o.) also showed similar high frequency involved progressive hearing loss. Together with the previous literature, high frequency involved progressive nature is one of the characteristic features of the patients with *ACTG1* mutations. The present study proved that EAS is a good therapeutic option for the patients with this gene mutation. *ACTG1* is known to be responsible for DFNA20/26. *ACTG1*, encoding gamma-actin, is the predominant actin isoform in auditory hair cells, more specifically in the cuticular plate, adherens junctions and stereocilia [19]. The etiology is located within the cochlea, therefore comparatively better outcomes can be predicted. Our patient's successful performance after EAS is compatible with the intra-membranous labyrinth etiology. The younger son who carried the same mutation will potentially have progressive hearing loss and his hearing is currently checked semiannually.

EAS is a new trend in therapy for the patients with residual hearing in the lower frequencies [20]. Various genes may be involved in the candidates [21], and we have found the mitochondrial 1555 A>G mutation and *CDH23* mutations in the patients receiving EAS [22], suggesting that the patients with those etiologies may have a potential to show good outcomes. Using the new MPS platform based on new generation sequencing enabled us to add two responsible genes, *TMPRSS3*, and *ACTG1*, in the patients with EAS. Identification of those genes may be good predictor when choosing the therapeutic options. Since the speed of progression may depend on the responsible gene, this information may be helpful for timing of EAS surgery and the selection of the electrode.

Overall, the current findings confirmed the importance of genetic information for predicting outcome of the CI/EAS patients, i.e., relatively good performance would be expected if the pathology exists within the cochlea. Such molecular diagnosis is important for the decision making process for selection of appropriate intervention, such as conventional cochlear implantation, EAS, hearing aid, or combination with other communication modes.

In spite of difficulty in discovery of the responsible gene for each individual patient, genetic testing using MPS may be a breakthrough. In the current series, MPS successfully discovered rare causative genes in CI patients and in EAS patients. These genes have not usually been screened and therefore mutations in them have not been diagnosed by the conventional approach. From that point of view, MPS has the potential power to identify such rare genes/mutations.

Supporting Information

Table S1 58 genes reported to be causative of non-syndromic hearing loss.
(PDF)

Acknowledgments

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Author Contributions

Conceived and designed the experiments: SU. Performed the experiments: MM SN. Analyzed the data: MM SN. Contributed reagents/materials/analysis tools: MM TI KF. Wrote the paper: SU.

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Targeted Exon Sequencing Successfully Discovers Rare Causative Genes and Clarifies the Molecular Epidemiology of Japanese Deafness Patients

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Abstract

Target exon resequencing using Massively Parallel DNA Sequencing (MPS) is a new powerful strategy to discover causative genes in rare Mendelian disorders such as deafness. We attempted to identify genomic variations responsible for deafness by massive sequencing of the exons of 112 target candidate genes. By the analysis of 216randomly selected Japanese deafness patients (120 early-onset and 96 late-detected), who had already been evaluated for common genes/mutations by Invader assay and of which 48 had already been diagnosed, we efficiently identified causative mutations and/or mutation candidates in 57 genes. Approximately 86.6% (187/216) of the patients had at least one mutation. Of the 187 patients, in 69 the etiology of the hearing loss was completely explained. To determine which genes have the greatest impact on deafness etiology, the number of mutations was counted, showing that those in *GJB2* were exceptionally higher, followed by mutations in *SLC26A4, USH2A, GPR98, MYO15A, COL4A5* and *CDH23*. The present data suggested that targeted exon sequencing of selected genes using the MPS technology followed by the appropriate filtering algorithm will be able to identify rare responsible genes including new candidate genes for individual patients with deafness, and improve molecular diagnosis. In addition, using a large number of patients, the present study clarified the molecular epidemiology of deafness in Japanese. *GJB2* is the most prevalent causative gene, and the major (commonly found) gene mutations cause 30–40% of deafness while the remainder of hearing loss is the result of various rare genes/mutations that have been difficult to diagnose by the conventional one-by-one approach. In conclusion, target exon resequencing using MPS technology is a suitable method to discover common and rare causative genes for a highly heterogeneous monogenic disease like hearing loss.

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Introduction

Etiological studies have shown that approximately two-thirds of congenital/early-onset sensorineural hearing loss in developed countries is estimated to be due to genetic causes [1]. Deafness is an extremely heterogenous disorder and the involvement of nearly 60 distinct nonsyndromic deafness genes sometimes makes the precise diagnosis difficult. To clarify individual etiology in such heterogenous diseases, one-by-one gene screening based on conventional PCR-based direct sequencing of candidate genes has been developed, and currently *GJB2* has become the first to be screened, followed by several commonly encountered genes. As more comprehensive screening methods, micorarray-based screening [2,3] and Invader assay-based screening [4,5] have also been developed. Recent advances in exome sequencing using Massively Parallel DNA Sequencing (MPS) have revolutionized the elucidation of genetic defects causing monogenic disorders [6–8]. A number of papers regarding gene discovery and successful clinical application for identification of responsible genes for deafness using MPS have recently been published [9–17]. In this study, we have chosen 112 genes (including 54 known deafness causing genes, 22 known syndromic hearing loss causing genes and 36 possible candidate genes which expressed highly in the inner ear) and conducted genetic analysis to 1) confirm the potentiality of MPS -based genetic screening strategies for such a genetically heterogenous disease, and 2) clarify molecular epidemiology by identifying responsible/candidate genes in a large number of patients using MPS technology.

Materials and Methods

Subjects

Two hundred sixteen Japanese patients with bilateral sensorineural hearing loss from 33 ENT departments nationwide participated in the present study. With regard to onset age (the age of awareness), 120 patients had early-onset deafness (below 6 y.o.), and 96 had late-detected deafness. Thirty subjects were from autosomal dominant or mitochondrial inherited families (two or

more generations affected); 98 subjects were from autosomal recessive families (parents with normal hearing and two or more affected siblings) or had sporadic deafness (also compatible with recessive inheritance or non-genetic hearing loss). Hearing loss was evaluated using pure-tone audiometry (PTA) classified by a pure-tone average over 500, 1000, 2000 and 4000 Hz in the better hearing ears. For children who could not undergo PTA, we used an average over 500, 1000, 2000 Hz in either auditory steady-stem response (ASSR) or conditioned oriented reflex audiometry (COR), or the response threshold (dB) from auditory brainstem response (ABR). Computed tomography (CT) scans were performed to check for congenital inner ear anomalies.

The patients had already been evaluated by conventional PCR-based one-by-one gene screening and Invader-based multi-gene screening [5], and 61 out of the 216 (45/120 prelingual, 16/96 postlingual) patients were already found to have *GJB2* (n = 38), *SLC26A4* (n = 15), or mitochondrial 1555 (n = 3) and 3243 (n = 5) mutations. We chose these patients because 1) they were "randomly" selected, and 2) they had already been screened by Invader assay and further fully sequenced by Sanger sequencing for the previously found common and frequent deafness causing genes i.e., *GJB2, SLC26A4, KCNQ4,* and *CDH23*. Therefore, we could simultaneously use these 216 samples for both diagnostic purposes and for verification. As a control for pathogeneity of each genomic variation, 72 Japanese samples were used in this study, because they were 1) ethnically similar, 2) had normal hearing evaluated by pure-tone audiometry, and 3) were collected from throughout the nation, and were able to undergo identical procedures. All subjects or next of kin, caretakers, or guardians on the behalf of the minors/children gave prior written informed consent for participation in the project, and the Shinshu University Ethical Committee as well as the respective Ethical Committees of the other participating institutions of the Deafness Gene Study Consortium (Hokkaido University, Hirosaki University, Iwate Medical University, Tohoku University, Yamagata University, Fukushima Medical University, Jichi Medical University, Gunma University, Nihon University, Nippon Medical School, Nippon Medical School Tama Nagayama Hospital, Jikei University, Toranomon Hospital, Kitasato University, Hamamatsu Medical University, Mie University, Shiga Medical Center for Children, Osaka Medical College, Hyogo College of Medicine, Kobe City Medical Center General Hospital, Wakayama Medical University, Okayama University, Yamaguchi University, Ehime University, Kyushu University, Fukuoka University, Nagasaki University, Kanda ENT Clinic, Miyazaki Medical College, Kagoshima University, Ryukyus University) approved the study.

Targeted Enrichment and DNA Sequencing

One hundred twelve genes listed in **Table S1**, including 54 genes reported to be causative of non-syndromic hearing loss (Hereditary Hearing loss Homepage; http://hereditaryhearingloss.org/) and 22 reported to cause syndromic hearing loss were selected for sequencing. In hopes of finding novel causative genes, we added 36 genes that are highly expressed in the adult human inner ear by microarray analysis [18]. DNA from 12 patients was pooled and 3 μg of each pooled DNA was used as an input material for SureSelect target DNA enrichment (Agilent Technologies, Santa Clara, CA) and Illumina GAIIx sequencing (Illumina, San Diego, CA) according to the manufacturers' procedures. Each genomic DNA pool was fragmented using the Covaris™ S2 System (Covaris, Woburn, MA) to about 200 bp fragment length. After fragmentation, DNA fragments were blunt-ended and phosphorylated at the 5′ end using a Paired End Genomic DNA Sample Prep Kit (Illumina) and successively, adeninylated at the 3′ end and ligated to pre-capture adaptor olligonucleotides containing SureSelect target DNA enrichment kit. After adaptor oligonucleotide ligation, pre-capture amplification was performed with Heraculase II Fusion DNA polymerase (Agilent Technologies). Between each step of sample preparation, DNA pools were purified with the Agencourt AMPure XP system (Beckman Coulter Genomics, Danvers, MA). The Capture library was designed with Agilent's eArray homepage (http://earray.vhem.agilent.com/earray/). The bait cRNA library contained all exons of 112 genes. Exons of selected genes of all variants were selected from RefSeq and Ensembl databases using the University of California Santa Cruz table browser (http://genome.ucsc.edu/). Adaptor ligated and pre-amplicated samples were hybridized to the Capture cRNA library at 65°C for 24 hours with SureSelect Hybridization buffer and successively captured with Dynabeads MyOne Streptavidin T1 beads (Invitrogen) and washed with SureSelect Wash buffer. After target capture, selected product from pooled DNA was post-amplified with Heraculase II Fusion DNA polymerase and Illumina Multiplexing Sample Preparation Oligonucleotide Kit and then submitted to the massive parallel sequencing in a lane on a Illumina GAIIx genome platform (Illumina).

Mapping and Filtering

The sequence data were processed with standard Illumina base calling procedure and successively mapped to human genome sequence (build hg 36) with the Bowtie program and BWA program [19,20]. The two programs were used consecutively, because the Bowtie program cannot detect insertion/deletion efficiently. A total of 55.4 and 8.5 Gb sequences with about 9,000,000 and 1,400,000 reads were obtained by the pair-end method for the patients and the controls, respectively. After alignment, the filtering algorithm shown in **Fig. 1** was applied to collect the responsible genes/mutations. First, because of usage of pooled DNA samples, potential single nucleotide variants (SNVs) were filtered by the frequency of variant reads at each position. For the number of variants in each position, we assumed a binomial distribution with the probability parameter of 1/24, and the size parameter of the number of coverage. The largest integer number that is not larger than the value giving the cumulative distribution function of 0.025 of the binomial distribution was used as the threshold value, and the position was selected when the number of the reads of the variant were not lower than the threshold value indicated in formula (1).

$$P(j \geq k) = 1 - \sum_{i=0}^{k-1} {}_nC_i p^i (1-p)^i \qquad (1)$$

In the formula, n denotes total depth (wild type+mutation allele) of each mapped position, j denotes the observed number of mutational alleles at each mapped position, and p denotes the relative frequency of the mutation allele in the pool. In this study DNA of 12 patients was pooled, and the minimal positive value of the relative frequency of the mutational allele in each pooled DNA sample should be 1/24. Therefore, we employed $p = 1/24$. To reduce false negative cases, we used $P = 95\%$ and after the calculation of this formula, k value indicated the number of minimal mutation allele copies that was used as the threshold for each mapped position. We fixed $p = 1/24$ and $P = 95\%$, and then, k value was dependent only on the total depth n.

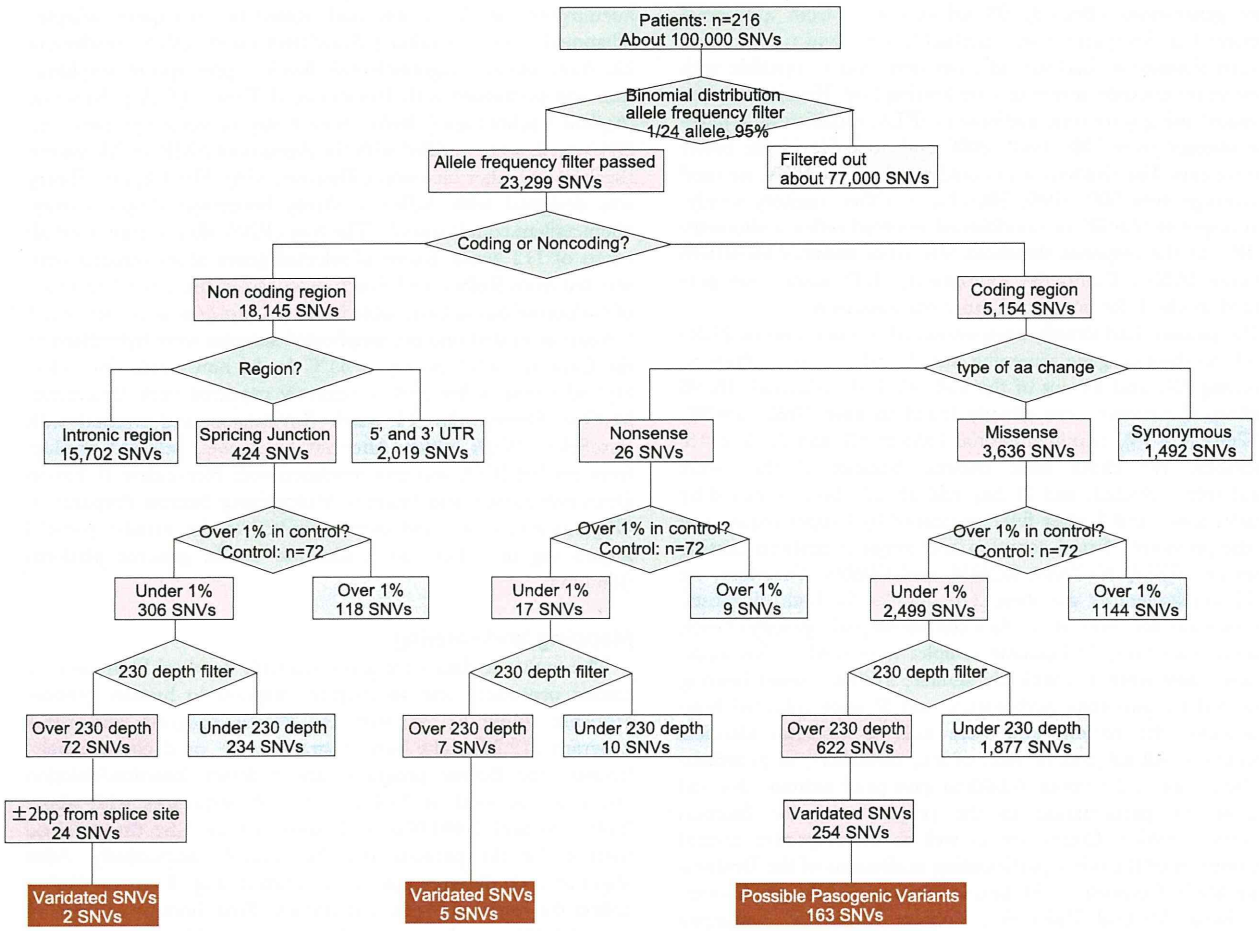

Figure 1. Algorithm applied in this study. Nonsense mutations, splice-site mutations, and missense mutations were chosen according to this algorithm.
doi:10.1371/journal.pone.0071381.g001

When compared with the mutations already identified by Sanger sequencing, this first filtering was effective to detect those mutations (**Fig. S1**).

After the first filtering, the selected SNVs were then classified into the targeted regions (coding region, non-coding region, splicing junction) and types of changes (nonsense mutation, missense mutation, insertion or deletion) (**Fig. 1**). SNVs were then filtered against the sequences observed at over 1% in control subjects because most common *GJB2* deafness causing mutations so far found in Japanese had shown <1% allele frequencies in the control population (**Fig. S2**). Then, the minimum cut off value for the depth was decided to be 230 for each 12-patient pool, based on the data obtained for all exons of the *GJB2, CDH23,* and *KCNQ4* genes by Sanger sequencing and parallel sequencing (**Fig. S2**). For splice-site mutations, 24 possible candidates for causative mutations were selected because SNVs within +/−2base from the exon-intron junction site were considered to be important for splicing [21,22]. After the application of all these filters, the candidate deafness causing mutations were selected, and verified by the subsequent Sanger sequencing. For missense mutations, the Polyphen2 [23] software program was applied to predict the influence on the protein structure by amino acid substitution. Family member genotypes were also used to validate the co-segregations of the deafness trait and the candidate mutations in individual families.

Comparison with Another Algorithm for Pooled DNA Samples

We also analyzed all the data with VIPR, a program established and validated for use with pooled samples [24].

Results

Of 7 selected nonsense mutations, after Sanger sequencing, 2 were not confirmed but 5 actual nonsense mutations in 12 families were identified in *GJB2, EYA1, MIA, TMPRSS3,* and *MYO6* (**Table S2, Fig. 2**).

Of 24 selected splice-site mutations, after Sanger sequencing, 22 were not identified but 2 actual splice-site mutations in 3 families were successfully identified in *KCNQ1* and *SLC26A4* (**Table S2, Fig. 3**). The pathogenic nature was confirmed by 1) segregation within the family and 2) phenotypic configuration (long-QT for *KCNQ1* and enlarged vestibular aqueduct for *SLC26A4*).

Of 27 selected insertion-deletion mutations, after Sanger sequencing, 6 actual mutations in 48 families were successfully identified in *GJB2, MYO15A* and *MYH9* (**Table S2, Fig. 4**).

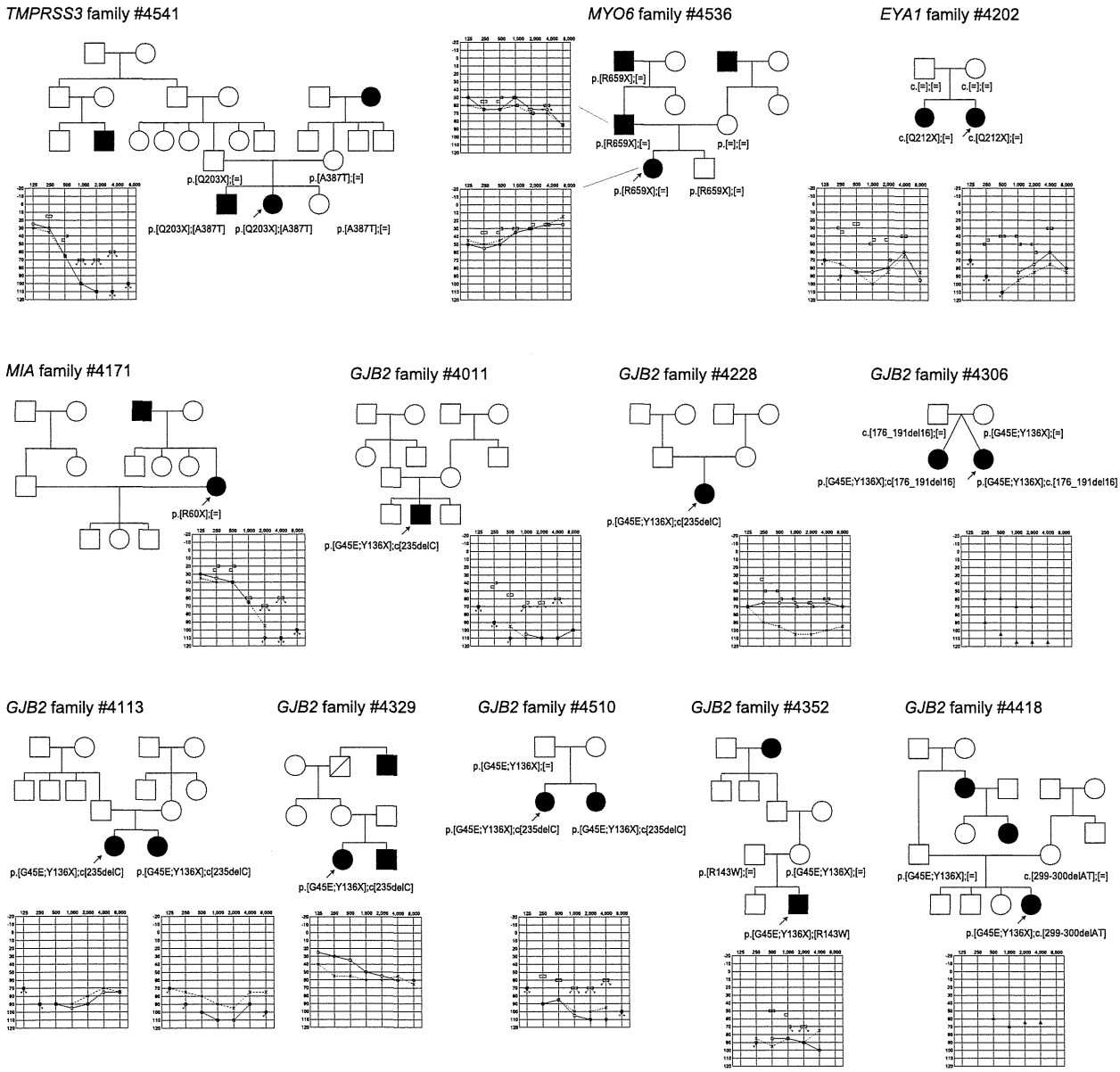

Figure 2. The pedigrees and audiograms of the patients with nonsense mutations after confirmation by Sanger sequencing.
doi:10.1371/journal.pone.0071381.g002

Of 622 missense mutations, 254 mutations were confirmed by Sanger sequencing. By using PolyPhen2 software, 167 were classified as "damaging" or "probably damaging" or "possibly damaging" and 87 were categorized as "benign" (**Table S2**). Of 167 selected missense mutations 163 were <1% allele frequencies in both the 1000 genome project (http://www.1000genomes.org/node/home) and the NHLB grand opportunity exome sequencing project: 6500 exomes (http://esp.gs.washington.edu/drupal/). *TMPRSS3, MYO15A, GJB2, SLC26A4* were found to be responsible for deafness in autosomal recessive or sporadic families. Examples of the families are shown in **Fig. 5, 6**. *TECTA, WFS-1, MYH9, EYA1, COL4A5, COL11A1* were identified as the responsible genes for deafness in autosomal dominant families (**Fig. 5, 6**).

As in **Table S2**, a total of 57 responsible genes were found, and the number of mutations/mutation candidates is shown in **Fig. 1**. *GJB2* was exceptionally higher, followed by *SLC26A4, USH2A, GPR98, MYO15A, COL4A5,* and *CDH23*. In the early-onset group, *GJB2, SLC26A4, GPR98, MYO15A, USH2A, CDH23, and TECTA* were frequently found, in contrast to the late-detected group, where *GJB2, COL4A5, USH2A, MYO15A, CDH23, GPR98, EYA1, and TMPRSS3* were frequently found (**Fig. 7**). The number of possible mutations in the early-onset group vs. late-detected group was 54:22 for *GJB2*, 7:1 for *PCDH15*, 8:3 for *SLC26A4*, 18:2 for *TECTA*, and 3:5 for *TMPRSS3*.

Comparison data between the current algorithm and VIPR, which is widely used for pooled sample analysis due to its higher specificity in mutation detection compared to other programs for pooled samples, is shown in **Table S3.** VIPR is unable to detect

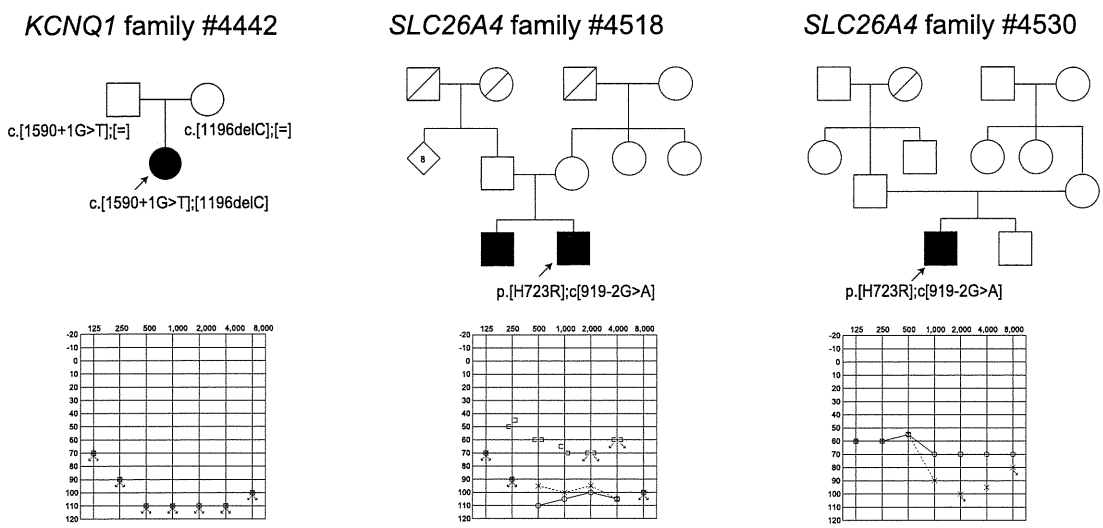

Figure 3. The pedigrees and audiograms of the patients with splice-site mutations after confirmation by Sanger sequencing.
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deletion/insertion mutations, as well as some missense mutations. 93.5% (87/93) and 84.1% (37/44) of the mutations were detected in the *GJB2* and *SLC26A4* genes that had already been fully sequenced by Sanger sequencing, respectively.

Discussion

With regard to sensitivity and specificity, we placed priority on sensitivity because one of the main purposes was clarifying genetic epidemiology. In addition, we used pooled DNA samples because a large number of sample is needed for genetic epidemiology. With the cut off value setting in this study, we could obtain high sensitivity (93.5% sensitivity on the basis of *GJB2, SLC26A4*)(**Fig. S1**). We also analyzed all the data with VIPR, a program established and validated for use with pooled samples [24]. However, sensitivity (84.1%) was not as satisfactory as the current algorithm (**Table S3**). Also, because VIPR is unable to detect deletion/insertion mutations, we used our own algorithm in this study.

On the other hand, it is also true that one problem of the present algorithm is low specificity (high false positive rates: 16% for nonsense, 90% for splice-site, 75% for insertion-deletion mutations and 67% for missense mutations) necessitating time-consuming direct sequencing confirmation afterwards and making it unsuitable for diagnostic purposes. The low specificity was improved by using a more stringent cut off line in the minimum depth of coverage as well as a more stringent p-value in the binomial distribution filtering process. But for diagnosis, more sophisticated methods and algorithms with higher specificity such as bar-code procedures are available for genetic testing for individual patients.

With regard to five nonsense mutations in 12 families (identified in *GJB2, EYA1, MIA, TMPRSS3, MYO6*), two selected splice-site mutations in three families (identified in *KCNQ1* and *SLC26A4*), and six insertion-deletion mutations (identified in *GJB2, MYO15A* and *MYH9*), segregation analysis confirmed they are plausible disease causing mutations (**Fig. 2–4**). For 163 selected missense mutations identified in 33 out of 54 known non-syndromic genes, it is difficult to reach a final conclusion about whether they are really disease causing mutations or not. Although some of the families were too small for segregation study or we failed to collect enough samples from familial members, most cases are consistent with the assumption that these are pathogenic mutations based on the software programs to predict the influence on the protein structure [20]. Actual causative mutations were successfully identified from the selected recessive as well as dominant families in which all the samples of family members were collected (Examples are shown in **Fig. 5, 6**). *TMPRSS3, MYO15A, GJB2, SLC26A4* were found to be responsible for deafness in autosomal recessive or sporadic families, while *TECTA, WFS1, MYH9, EYA1, COL4A5* and *COL11A1* were identified as the responsible genes for deafness in autosomal dominant families.

One interesting result is that a mutation in a novel putative responsible deafness gene, *MIA*, which is highly expressed in the inner ear, was identified in a dominant family (#4171), in the present study. Although the detailed function in the inner ear is currently unknown, genes that are highly expressed in the inner ear, as revealed by cDNA microarray analysis, may have a crucial functional role there [18].

The other interesting result was the mutations in the genes previously reported to be syndromic genes such as *EYA1*. Although re-contact was not possible in all cases, detailed genotype/phenotype correlation study will be an open question. One family was later found to be associated with ear pits (diagnosed as BOR syndrome) (family #4361 in Fig. 5), but the rest of the contacted families did not have any associated branchial disclosure. Interestingly, all families were associated with inner ear anomaly, and therefore these families have slightly different clinical phenotype from typical BOR syndrome. As in this case, the mutation analysis using MPS will potentially expand the phenotypic variations.

Based on the sensitivity, nonsense mutations, splice-site mutations, insertion-deletion mutations or selected missense mutations were found in 57 out of 112 genes (33/56 non-syndromic genes, 12/22 syndromic genes, and 12/36 genes highly expressed in the cochlea). The mutations previously found in Invader assays or direct sequencing were also confirmed effectively in our MPS algorithm. Of 93 previously found *GJB2* and *SLC26A4* mutations, we confirmed 87 (93.5%) of them (Table S3). Approximately 86.6% (187/216) of the patients had at least one mutation.