**KCNJ10 May Not Be a Contributor to Nonsyndromic Enlargement of Vestibular Aqueduct (NSEVA) in Chinese Subjects**

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### Abstract

**Background:** Nonsyndromic enlargement of vestibular aqueduct (NSEVA) is an autosomal recessive hearing loss disorder that is associated with mutations in SLC26A4. However, not all patients with NSEVA carry biallelic mutations in SLC26A4. A recent study proposed that single mutations in both SLC26A4 and KCNJ10 lead to digenic NSEVA. We examined whether KCNJ10 exert a role in the pathogenesis of NSEVA in Chinese patients.

**Methods:** SLC26A4 was sequenced in 1056 Chinese patients with NSEVA. KCNJ10 was screened in 131 patients who lacked mutations in either one or both alleles of SLC26A4. Additionally, KCNJ10 was screened in 840 controls, including 563 patients diagnosed with NSEVA who carried biallelic SLC26A4 mutations, 48 patients with nonsyndromic hearing loss due to inner ear malformations that did not involve enlargement of the vestibular aqueduct (EVA), 96 patients with conductive hearing loss due to various causes, and 133 normal-hearing individuals with no family history of hereditary hearing loss.

**Results:** 925 NSEVA patients were found carrying two-allele pathogenic SLC26A4 mutations. The most frequently detected KCNJ10 mutation was c.812G>A (p.R271H). Compared with the normal-hearing control subjects, the occurrence rate of c.812G>A in NSEVA patients with lacking mutations in one or both alleles of SLC26A4 had no significant difference (1.53% vs. 5.30%, χ² = 2.798, p = 0.172), which suggested that it is probably a nonpathogenic benign variant. KCNJ10 c.1042C>T (p.R348C), the reported EVA-related mutation, was not found in patients with NSEVA who lacked mutations in either one or both alleles of SLC26A4. Furthermore, the normal-hearing parents of patients with NSEVA having two SLC26A4 mutations carried the KCNJ10 c.1042C>T or c.812G>A mutation and a SLC26A4 pathogenic mutation.

**Conclusion:** SLC26A4 is the major genetic cause in Chinese NSEVA patients, accounting for 87.59%. KCNJ10 may not be a contributor to NSEVA in Chinese population. Other genetic or environmental factors are possibly play a role in the etiology of Chinese EVA patients with zero or monoallelic SLC26A4 mutation.

### Citation

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### Introduction

Nonsyndromic sensorineural hearing loss associated with an enlargement of the vestibular aqueduct (EVA) is a common form of inner ear malformation [1]. It occurs either congenitally or following some mild head injury and results in fluctuating and progressive hearing loss. Nonsyndromic enlargement of vestibular aqueduct (NSEVA) is an autosomal recessive hearing loss associated with mutations in the anion transporter SLC26A4, which encodes the anion transporter protein pendrin [2]. Located in the kidney, thyroid, and inner ear, pendrin is responsible for the transport of Cl⁻, HCO₃⁻, OH⁻, I⁻, and formate [2,3]. In the inner ear, pendrin is expressed mainly in the external sulcus, endolymphatic duct and sac, utricle, and saccule, and it maintains the endolymphatic balance by mediating Cl⁻/HCO₃⁻ exchange [4–6]. Malfunction of the SLC26A4 protein can cause NSEVA and Pendred syndrome (PS), a type of syndromic sensorineural hearing loss characterized by EVA, goiter, and in some cases Mondini malformation [7].
The underlying mechanisms for the etiology of NSEVA/PS remain elusive; however, studies that have screened for SLC26A4 mutations in patients with NSEVA/PS and subsequent studies of these mutant proteins have been informative [2]. Some pendrin mutants fail to reach the cell surface and are retained in the endoplasmic reticulum, impairing anion transport and consequently the ionic balance of endolymph [9,9]. This imbalance causes endolymphatic dilatation, enlarges the membranous labyrinth, and disrupts the development of bone structures in the inner ear [9]. In addition, studies using Slc26a4−/− mouse models have demonstrated that the loss of pendrin expression decreases HCO3− secretion into the endolymph [10]. The resulting endolymphatic acidification inhibits Ca2+ reabsorption from endolymph, which further inhibits sensory transduction and promotes the degeneration of the sensory hair cells [10]. In China, NSEVA accounts for 20–25% of hereditary hearing loss cases, and biallelic mutations in SLC26A4 represent nearly 90% of the genetic etiology of NSEVA [11]. However, the detection rate of SLC26A4 biallelic mutations varies among different ethnicities (e.g., 24% in Caucasians and 81% in Koreans) [12,13].

Although most previous studies in patients with NSEVA have focused on SLC26A4, not all patients with NSEVA carry biallelic mutations in SLC26A4. This discrepancy has led to the hypothesis that other genetic modifications or environmental factors may be involved. Yang et al. [14] proposed that single mutations in both SLC26A4 and KCNJ10 lead to digenic NSEVA. KCNJ10 encodes a 42.5-kD member of the inward rectifier potassium channel family and is expressed mainly in the brain, kidney, and inner ear. The KCNJ10 channel family and is expressed mainly in the brain, kidney, and inner ear. The KCNJ10 K+ channel buffers the K+ levels in brain glial cells, and mutations in this gene may lead to the so-called ‘EAST/SeSAME syndrome,’ which is manifested by seizures, sensorineural deafness, ataxia, mental retardation, and electrolyte imbalance [15,16]. In the inner ear, KCNJ10 is expressed mainly in the intermediate cells of the stria vascularis. It maintains the K+ imbalance [15,16]. In the inner ear, KCNJ10 is expressed mainly in the intermediate cells of the stria vascularis. It maintains the K+ imbalance [15,16]. In this study, we aimed to examine whether KCNJ10 may not act for NSEVA.

In this study, we aimed to examine whether KCNJ10 plays a role in the pathogenesis of NSEVA by screening KCNJ10 mutations in Chinese patients with NSEVA who lacked mutations in one or both alleles of SLC26A4. This screening resulted in the identification of common KCNJ10 mutations in the Chinese population. The conclusions from this study contribute important foundations for genetic diagnosis as well as prenatal diagnosis of deafness.

Subjects and Methods

Subjects

The subjects were recruited by the Department of Otolaryngology and Genetic Testing Center for Deafness, PLA General Hospital, Beijing, China. Totally 1056 EVA patients were enrolled in the study. They were all screened the coding region of SLC26A4 firstly. Then a cohort of 131 Chinese patients with NSEVA without mutation or with only one SLC26A4 mutation was recruited for mutation screening for KCNJ10. In addition, we examined 840 controls, comprising the following four groups: 563 patients who were diagnosed with NSEVA and carried biallelic SLC26A4 mutations, 48 patients with nonsyndromic hearing loss and inner ear malformation other than EVA, 96 patients with conductive hearing loss due to various causes (70 cases with chronic otitis media, 11 cases with secretory otitis media, 9 cases with conductive deafness due to unknown causes, three cases with otosclerosis, 2 cases with external auditory canal tumor, and 1 case with a jugular tumor sphere), and 133 normal-hearing individuals with no family history of hereditary hearing loss. Written informed consent was obtained from all subjects or guardians prior to blood sampling and genetic testing. The study protocol, including the consent procedure, was performed with the approval of the Ethics Committee of the Chinese PLA General Hospital.

Mutation screening of KCNJ10

Genomic DNA was isolated from whole blood through a modification of standard procedures [20]. Most DNA samples were collected and stored at the Genetic Testing Center for Deafness. The only coding exon of KCNJ10 was amplified in a 20-μl PCR reaction mixture containing 20 ng of genomic DNA template, 0.0625 units of Taq DNA polymerase (Biomed, Cat: Po01, Lot: 211841XB), and 40 nM primers. The following three sets of PCR primers were used (5′→3′): forward, CTAACCTCATTATGCTG and reverse, AGGATGTGTTG-GACGACCA; forward, TGGCTTCGCTACATCATG and reverse, ACAACTTGGTAAAGGCCTAA; forward, AC-CCTACATTCTATCATG and reverse, GTAGATTCTCT-TACGAGGCG. To increase the specificity and sensitivity of PCR amplification, the technique of touchdown-PCR was employed: one cycle at 95°C for 1 min; 95°C for 30 s, 62°C for 30 s, and 72°C for 45 s, followed by 13 cycles at decreasing annealing temperatures in decrements of 0.5°C; 21 cycles of 95°C for 30 s, 56°C for 30 s, and 72°C for 45 s, with a final extension at 72°C for 7 min. The sizes of the PCR products were confirmed by 1% agarose gel electrophoresis. PCR products were sequenced using forward primers to screen for mutations in KCNJ10 (Doobio Biotechnology Co., China). The detected mutations were confirmed by sequencing using reverse primers.

Statistics

SPSS 17.0 software was used in statistical analysis. Comparisons between groups were tested using the χ2 or Fisher’s exact tests. P value<0.05 was set for statistically significant difference.

Results

SLC26A4 analysis in NSEVA patients

Totally 925 NSEVA patients were found carrying two-allele pathogenic SLC26A4 mutations, SLC26A4 accounts for 87.99%
Table 1. Genotype of NSEVA patients with two-allele SLC26A4 mutations.

| Number | SLC26A4 Genotype | Number of patients |
|--------|------------------|--------------------|
| 1      | IVS7-2A>G        | 237                |
| 2      | IVS7-2A>G        | 103                |
| 3      | IVS7-2A>G        | 40                 |
| 4      | IVS7-2A>G        | 35                 |
| 5      | IVS7-2A>G        | 27                 |
| 6      | IVS7-2A>G        | 23                 |
| 7      | IVS7-2A>G        | 19                 |
| 8      | IVS7-2A>G        | 15                 |
| 9      | IVS7-2A>G        | 11                 |
| 10     | IVS7-2A>G        | 10                 |
| 11     | c.2168A>G(p.H723R)| 9                   |
| 12     | c.2168A>G(p.H723R)| 9                   |
| 13     | c.2168A>G(p.H723R)| 8                   |
| 14     | IVS7-2A>G        | 6                   |
| 15     | IVS7-2A>G        | 6                   |
| 16     | IVS7-2A>G        | 5                   |
| 17     | IVS7-2A>G        | 5                   |
| 18     | c.1174A>T(p.N392Y)| 5                   |
| 19     | IVS7-2A>G        | 5                   |
| 20     | IVS7-2A>G        | 5                   |
| 21     | IVS7-2A>G        | 5                   |
| 22     | IVS7-2A>G        | 4                   |
| 23     | c.1229C>T(p.T410M)| 4                   |
| 24     | c.1975G>C(p.V659L)| 4                   |
| 25     | IVS7-2A>G        | 4                   |
| 26     | IVS7-2A>G        | 4                   |
| 27     | c.2168A>G(p.H723R)| 4                   |
| 28     | c.2168A>G(p.H723R)| 4                   |
| 29     | IVS7-2A>G        | 4                   |
| 30     | c.1540C>T(p.Q514X)| 3                   |
| 31     | c.2027T>A(p.L676Q)| 3                   |
| 32     | IVS7-2A>G        | 3                   |
| 33     | IVS7-2A>G        | 3                   |
| 34     | IVS7-2A>G        | 3                   |
| 35     | IVS7-2A>G        | 3                   |
| 36     | IVS7-2A>G        | 3                   |
| 37     | IVS7-2A>G        | 3                   |
| 38     | IVS7-2A>G        | 3                   |
| 39     | c.1975G>C(p.V659L)| 3                   |
| 40     | IVS7-2A>G        | 3                   |
| 41     | IVS7-2A>G        | 3                   |
| 42     | IVS7-2A>G        | 3                   |
| 43     | IVS7-2A>G        | 3                   |
| 44     | IVS7-2A>G        | 3                   |
| 45     | IVS7-2A>G        | 3                   |
| 46     | c.2168A>G(p.H723R)| 3                   |
| 47     | IVS7-2A>G        | 3                   |
| 48     | c.2168A>G(p.H723R)| 3                   |
| Number | SLC26A4 Genotype | Number of patients |
|--------|------------------|--------------------|
| 49     | IVS7-2A>G        | c.1173C>A(p.S391R) | 3     |
| 50     | c.1229C>T(p.R79X)| c.2168A>G(p.H723R) | 2     |
| 51     | IVS7-2A>G        | c.414delT          | 2     |
| 52     | IVS7-2A>G        | c.1548insC         | 2     |
| 53     | c.1174A>T(p.N392Y) | IVS15+5G>A        | 2     |
| 54     | IVS7-2A>G        | IVS14-2A>G         | 2     |
| 55     | c.235C>T(p.R79X)| c.2168A>G(p.H723R) | 2     |
| 56     | IVS7-2A>G        | c.1699A>T(p.K567X) | 2     |
| 57     | IVS7-2A>G        | c.1371C>A(p.N457K) | 2     |
| 58     | 1240-1243GAGA>AAAG(p.E414K,p.S415G) | IVS14-6T>G | 2     |
| 59     | IVS7-2A>G        | c.1670G>A(p.G557D) | 2     |
| 60     | IVS7-2A>G        | c.665G>T(g.G222V)  | 2     |
| 61     | IVS7-2A>G        | c.1990G>A(p.A664T) | 2     |
| 62     | IVS7-2A>G        | c.1238A>G(p.Q413R) | 2     |
| 63     | c.1079C>T(p.A360V)| c.2168A>G(p.H723R) | 2     |
| 64     | c.1229C>T(p.T410M)| c.1687_1692insA | 2     |
| 65     | IVS7-2A>G        | c.1022delC         | 2     |
| 66     | IVS7-2A>G        | c.907G>C(p.E303Q)  | 2     |
| 67     | IVS7-2A>G        | c.2086C>T(p.Q696X) | 2     |
| 68     | c.2027T>A(p.L676Q)| c.2027T>A(p.L676Q) | 2     |
| 69     | IVS7-2A>G        | c.269C>T(p.S90L)   | 2     |
| 70     | c.1174A>T(p.N392Y) | c.2168A>G(p.H723R) | 2     |
| 71     | IVS7-2A>G        | c.1363A>T(p.I455F) | 2     |
| 72     | c.1174A>T(p.N392Y) | c.2027T>A(p.L676Q) | 2     |
| 73     | c.1673A>T(p.N558I)| IVS14+1G>A         | 2     |
| 74     | c.1318A>T(p.K440X)| c.1327G>C(p.E443Q) | 2     |
| 75     | IVS7-2A>G        | c.1991C>T(p.A664V) | 2     |
| 76     | IVS7-2A>G        | c.1595G>T(p.S532I) | 2     |
| 77     | c.1226G>A(p.R409H)| c.1226G>A(p.R409H) | 2     |
| 78     | c.946G>T(p.G316X) | c.2168A>G(p.H723R) | 2     |
| 79     | IVS7-2A>G        | c.668T>C(p.F223S)  | 2     |
| 80     | IVS7-2A>G        | c.1970G>T(p.S657I) | 2     |
| 81     | c.1522A>G(p.T508A)| c.1522A>G(p.T508A) | 1     |
| 82     | c.1595G>T(p.S532I)| IVS4+7A>G          | 1     |
| 83     | IVS7-2A>G        | c.1720G>A(p.A574T) | 1     |
| 84     | c.1343C>A(p.S448X)| c.2167C>G(p.H723D) | 1     |
| 85     | c.1178delCTCT    | c.439A>G(p.M147V)  | 1     |
| 86     | IVS7-2A>G        | c.400A>G(p.R134G)  | 1     |
| 87     | c.589G>A(p.G197R)| c.1105A>G(p.K369E) | 1     |
| 88     | IVS5+5G>A         | c.1318A>T(p.K440X) | 1     |
| 89     | IVS7-2A>G        | c.1367C>A(p.A456D) | 1     |
| 90     | IVS7-2A>G        | c.249G>A(p.W83X)   | 1     |
| 91     | c.1174A>T(p.N392Y) | c.1975G>C(p.V659L) | 1     |
| 92     | IVS7-2A>G        | c.1964T>A(p.I655N) | 1     |
| 93     | IVS7-2A>G        | c.2081delC         | 1     |
| 94     | c.281C>T(p.T94I) | c.692T>A(p.V231E)  | 1     |
| 95     | IVS7-2A>G        | IVS9+1G>A          | 1     |
| 96     | c.2168A>G(p.H723R)| c.439A>G(p.M147V)  | 1     |
| Number | SLC26A4 Genotype | Number of patients |
|--------|------------------|-------------------|
|        | Allele 1         | Allele 2          |
| 97     | IVS7-2A>G        | IVS10+1G>A        | 1     |
| 98     | c.890delC        | IVS7-2A>G         | 1     |
| 99     | IVS7-2A>G        | c.1829C>A(p.S610X) | 1     |
| 100    | IVS7-2A>G        | c.218delA         | 1     |
| 101    | c.1594A>C(p.S532R) | c.2168A>G(p.H723R) | 1   |
| 102    | c.1229C>T(p.T410M) | c.281C>T(p.T94I) | 1     |
| 103    | IVS7-2A>G        | c.404A>G(p.H135R) | 1     |
| 104    | c.1022insC       | c.1489G>A(p.G497S) | 1     |
| 105    | c.946G>T(p.G316X) | c.1343C>A(p.S448X) | 1   |
| 106    | IVS7-2A>G        | c.230A>T(p.K77I) | 1     |
| 107    | IVS7-2A>G        | c.1327G>C(p.E443Q) | 1   |
| 108    | c.1226G>A(p.R409H) | c.58T>C(p.Y20H) | 1     |
| 109    | c.2027T>A(p.L676Q) | IVS13+9C>T | 1     |
| 110    | c.240G>A(p.W83X) | c.2168A>G(p.H723R) | 1   |
| 111    | c.2037T>C(p.L68P) | IVS5+2T>C | 1     |
| 112    | c.911T>A(p.V304E) | c.1238A>G(p.Q413R) | 1   |
| 113    | c.2027T>A(p.L676Q) | IVS51+5G>A | 1     |
| 114    | c.1343C>A(p.S448X) | c.1339delA | 1     |
| 115    | c.1976T>G(p.V659G) | c.2168A>G(p.H723R) | 1   |
| 116    | c.2027T>A(p.L676Q) | c.1520delT | 1     |
| 117    | IVS10+1G>A       | IVS51+1G>A | 1     |
| 118    | c.1238A>G(p.Q413R) | c.1172G>A(p.S391N) | 1   |
| 119    | IVS7-2A>G        | c.1634T>A(p.V545E) | 1     |
| 120    | IVS7-2A>G        | c.2179C>T(p.L727F) | 1     |
| 121    | c.1343C>A(p.S448X) | c.1178delTCT | 1     |
| 122    | c.2096C>Tqp.E96X) | c.2168A>G(p.H723R) | 1   |
| 123    | c.754T>C(p.S252P) | c.86A>G(p.E29G) | 1     |
| 124    | c.2168A>G(p.H723R) | IVS5+2T>C | 1     |
| 125    | c.1229C>T(p.T410M) | c.1949T>A(p.V650D) | 1   |
| 126    | IVS7-2A>G        | IVS5+2T>A | 1     |
| 127    | c.1229C>T(p.T410M) | c.1693insA | 1     |
| 128    | c.391G>A(p.G131R) | c.2168A>G(p.H723R) | 1   |
| 129    | IVS7-2A>G        | c.2014G>A(p.G672R) | 1     |
| 130    | IVS7-2A>G        | IVS12+1G>A | 1     |
| 131    | IVS7-2A>G        | c.1373T>C(p.L458P) | 1     |
| 132    | c.754T>C(p.S252P) | c.2168A>G(p.H723R) | 1   |
| 133    | c.71G>A(p.R24Q) | c.2168A>G(p.H723R) | 1     |
| 134    | c.1174A>T(p.N392Y) | c.1829C>A(p.S610X) | 1   |
| 135    | c.439A>G(p.M147V) | c.1615A>G(p.S391N) | 1     |
| 136    | IVS7-2A>G        | c.1615A>G(p.S391N) | 1     |
| 137    | c.1174A>T(p.N392Y) | c.1340delA | 1     |
| 138    | c.1975G>C(p.V659L) | c.1238A>G(p.Q413R) | 1   |
| 139    | c.589G>A(p.G197R) | c.589G>A(p.G197R) | 1     |
| 140    | c.1174A>T(p.N392Y) | c.249G>A(p.W83X) | 1     |
| 141    | c.1991C>T(p.A664V) | c.1336C>T(p.Q446X) | 1   |
| 142    | IVS7-2A>G        | c.349delC | 1     |
| 143    | IVS7-2A>G        | c.259G>T(p.D87Y) | 1     |
| 144    | IVS7-2A>G        | c.1687_1692delA | 1     |
| Number | SLC26A4 Genotype | Number of patients |
|--------|------------------|--------------------|
|        | Allele 1          | Allele 2           |
| 145    | c.109G>T(p.E37X)  | c.2168A>G(p.H723R) |
| 146    | c.917insG         | c.2168A>G(p.H723R) |
| 147    | c.2168G>A(p.R409H) | IVS15+5G:A        |
| 148    | IVS7+1G>A         | c.2168A>G(p.H723R) |
| 149    | c.1334T>G(p.L445W) | c.1544T>C(p.F515S) |
| 150    | c.1489G>A(p.G497S) | c.414delT         |
| 151    | IVS7-2A>G         | c.1803G>C(p.K601N) |
| 152    | c.2148A>G(p.H723R) | c.279T>A(p.S93N) |
| 153    | IVS5+5G>A         | c.2145G>T(p.K715N) |
| 154    | c.2192C>T(p.T410M) | c.1343C>A(p.S448X) |
| 155    | IVS7-2A>G         | c.1975G>C(p.K601N) |
| 156    | c.1586T>G(p.I529S) | c.2168A>G(p.H723R) |
| 157    | IVS7-2A>G         | c.279T>A(p.S93N) |
| 158    | c.1223C>T(p.T410M) | c.1343C>A(p.S448X) |
| 159    | IVS7-2A>G         | c.2145G>T(p.K715N) |
| 160    | c.249G>A(p.L676Q) | c.2027T>A(p.L676Q) |
| 161    | IVS7-2A>G         | c.1975G>C(p.K601N) |
| 162    | c.109G>T(p.E37X)  | c.2168A>G(p.H723R) |
| 163    | c.2108T>C(p.F141L) | c.1343C>A(p.S448X) |
| 164    | IVS7-2A>G         | c.227C>T(p.P76L) |
| 165    | IVS7-2A>G         | c.2168A>G(p.H723R) |
| 166    | c.249G>A(p.W83X)  | c.1105A>G(p.K369E) |
| 167    | IVS7-2A>G         | c.754T>G(p.S252P) |
| 168    | c.279T>A(p.S93N)  | c.2183insT         |
| 169    | c.1991C>T(p.A664V) | c.1173C>A(p.S391R) |
| 170    | c.1343C>A(p.S448X) | c.2168A>G(p.H723R) |
| 171    | c.249G>A(p.W83X)  | c.1340delA         |
| 172    | IVS7-2A>G         | c.2168A>G(p.H723R) |
| 173    | c.2174A>T(p.N392Y) | c.1991C>T(p.A664V) |
| 174    | c.1229G>A(p.F223S) | c.1340delA         |
| 175    | c.1229G>A(p.F223S) | c.1340delA         |
| 176    | c.1975G>C(p.K369E) | c.754T>G(p.S252P) |
| 177    | c.1229G>A(p.F223S) | c.1340delA         |
| 178    | c.1229G>A(p.F223S) | c.1340delA         |
| 179    | c.574delC         | c.2168A>G(p.H723R) |
| 180    | IVS7-2A>G         | c.1835delA         |
| 181    | c.1229C>T(p.T410M) | c.1079C>T(p.A360V) |
| 182    | c.1229C>T(p.T410M) | c.1079C>T(p.A360V) |
| 183    | c.1334T>G(p.L445W) | c.2168A>G(p.H723R) |
| 184    | IVS7-2A>G         | c.439A>G(p.M147V) |
| 185    | c.1318A>T(p.K440X) | c.2168A>G(p.H723R) |
| 186    | c.1229C>T(p.T410M) | c.1226G>A(p.R409H) |
| 187    | c.1517T>G(p.L506R) | c.2162C>T(p.T721M) |
| 188    | c.1174A>T(p.N392Y) | IVS13+9C>T        |
| 189    | c.1174A>T(p.N392Y) | IVS13+9C>T        |
| 190    | c.1174A>T(p.N392Y) | IVS13+9C>T        |
| 191    | c.1174A>T(p.N392Y) | IVS13+9C>T        |
| 192    | c.1174A>T(p.N392Y) | IVS13+9C>T        |
| Number | SLC26A4 Genotype | Number of patients |
|--------|------------------|-------------------|
| 193    | c.2027T>A(p.L676Q) | 1                 |
| 194    | IVS5+5G>A       | 1                 |
| 195    | c.387delC       | 1                 |
| 196    | c.946G>T(p.G316X) | 1                 |
| 197    | IVS7-2A>G       | 1                 |
| 198    | IVS7-2A>G       | 1                 |
| 199    | c.1687_1692insA | 1                 |
| 200    | c.1829C>A(p.S610X) | 1             |
| 201    | c.1343C>T(p.S448L) | 1               |
| 202    | c.1174A>T(p.N392Y) | 1             |
| 203    | IVS7-2A>G       | 1                 |
| 204    | c.2168A>G(p.H723R) | 1                 |
| 205    | IVS7-2A>G       | 1                 |
| 206    | c.1540C>T(p.Q514X) | 1             |
| 207    | c.917insG       | 1                 |
| 208    | c.2168A>G(p.H723R) | 1             |
| 209    | IVS7-2A>G       | 1                 |
| 210    | IVS5+5G>A       | 1                 |
| 211    | c.946G>T(p.G316X) | 1                 |
| 212    | c.1927G>T(p.E643X) | 1             |
| 213    | c.2027T>A(p.L676Q) | 1               |
| 214    | c.1336C>T(p.Q446X) | 1             |
| 215    | c.2T>C(p.M1T)   | 1                 |
| 216    | c.1174A>T(p.N392Y) | 1             |
| 217    | c.1174A>T(p.N392Y) | 1               |
| 218    | IVS7-2A>G       | 1                 |
| 219    | c.1226G>A(p.R409H) | 1             |
| 220    | c.917insG       | 1                 |
| 221    | IVS7-2A>G       | 1                 |
| 222    | c.946G>T(p.G316X) | 1                 |
| 223    | c.1173C>A(p.S391R) | 1             |
| 224    | c.1985G>A(p.C662Y) | 1            |
| 225    | c.227C>T(p.P76L) | 1                 |
| 226    | c.768delG       | 1                 |
| 227    | c.1174A>T(p.N392Y) | 1             |
| 228    | c.1225C>T(p.R409C) | 1            |
| 229    | IVS9+1G>A       | 1                 |
| 230    | IVS7-2A>G       | 1                 |
| 231    | c.1174A>T(p.N392Y) | 1             |
| 232    | IVS7-2A>G       | 1                 |
| 233    | c.281C>T(p.T94I) | 1                 |
| 234    | c.1985G>A(p.C662Y) | 1             |
| 235    | c.1548insC      | 1                 |
| 236    | c.235C>T(p.R79X) | 1                 |
| 237    | c.1226G>A(p.R409H) | 1             |
| 238    | c.1229C>T(p.T410M) | 1            |
| 239    | IVS7-2A>G       | 1                 |
| 240    | c.1226G>A(p.R409H) | 1             |
Table 2. KCNJ10 performed further testing for mutation (1.95%, 11/563) and three cases with a heterozygous KCNJ10 mutation were found in our Chinese NSEVA patients. The finding of a heterozygous mutation in one or both alleles of KCNJ10, however, only Patient 195 developed NSEVA symptoms.

Genetic analysis of KCNJ10 among NSEVA patients

Among the 131 NSEVA patients lacking mutations in one or both alleles of SLC26A4, we identified the heterozygous missense mutation c.812G>A in KCNJ10 in two cases (Fig. S1), with a mutation rate of 1.53% (2/131); the KCNJ10 c.1042C>T mutation was not found. In the two cases with the KCNJ10 c.812G>A mutation, Patient 195 carried no mutations in SLC26A4 and Patient 1606 carried a heterozygous mutation in SLC26A4 (c.225C>A). In addition, we screened SLC26A4 and KCNJ10 genes in the parents of Patient 195 (Table 3). Both Patient 195 and his father carried KCNJ10 c.812G>A and wild-type SLC26A4; however, only Patient 195 developed NSEVA symptoms.

Genetic analysis of KCNJ10 among NSEVA patients with two mutations in SLC26A4

As a control, we conducted KCNJ10 mutation screening in 563 patients with NSEVA who carried two mutations in SLC26A4. We found 11 cases with a heterozygous KCNJ10 c.812G>A mutation (1.95%, 11/563) and three cases with a heterozygous KCNJ10 c.1042C>T mutation (0.53%, 3/563) (Fig. S1). We performed further testing for KCNJ10 in one of the pedigrees containing 11 subjects with the KCNJ10 c.812G>A mutation. Our result showed that Patient 2471 shared SLC26A4 c.2027 T>A and KCNJ10 c.812 G>A with his father, and SLC26A4 IVS7-2 A>G with his mother. Patient 2471, with compound heterozygous mutations in SLC26A4, developed NSEVA, whereas his father, with double heterozygosity in both SLC26A4 and KCNJ10, was not affected (Table 3).

We examined three patients with NSEVA in Pedigrees 4769 and 4814 who had biallelic SLC26A4 mutations and the KCNJ10 c.1042C>T mutation (Table 4). Hearing examinations showed that the hearing capabilities of the parents were normal, and high-resolution temporal bone computed tomography (CT) scans showed that EVA was not present in the parents. Because both parents in Pedigree 1134 were deceased, neither hearing examinations nor DNA sequencing could be done. However, the proband from Pedigree 3 reported to us that his parents had no hearing problems. We sequenced the KCNJ10 and SLC26A4 genes from the family members in Pedigrees 4769 and 4814. For Pedigree 4769, the proband’s mother carried the SLC26A4 c.2168A>G (p.H723R) and KCNJ10 c.1042C>T (p.R348C) mutations. For Pedigree 4814, the proband’s father carried the monoallelic SLC26A4 IVS7-2A>G and KCNJ10 c.1042C>T (p.R348C) mutations. IVS7-2A>G and c.2168A>G (p.H723R) are pathogenic mutations supported by both functional and molecular epidemiological studies [21–23]. They are also prevalent SLC26A4 hotspot mutations in Chinese NSEVA. The finding that normal-hearing individuals carried both the KCNJ10 c.1042C>T mutation and a SLC26A4 pathogenic mutation

Table 1. Cont.

| Number | SLC26A4 Genotype | Number of patients |
|--------|------------------|--------------------|
|        | Allele 1         | Allele 2           |
| 241    | c.920C>A         | c.2174insCTAT      | 1 |
| 242    | IVS7-2A>G        | c.1264G>A          | 1 |
| 243    | c.1229C>T        | c.365delT          | 1 |
| 244    | IVS7-2A>G        | IVS7+2T>C          | 1 |
| 245    | c.1229C>T        | c.235C>T          | 1 |
| 246    | c.1174A>T        | c.1174A>T         | 1 |
| 247    | c.754T>C         | c.349insC         | 1 |
| 248    | IVS7-2A>G        | c.387delC         | 1 |
| 249    | c.2014G>A        | c.2168A>G         | 1 |
| 250    | c.249G>A         | c.281C>T          | 1 |
| 251    | c.1262A>C        | c.2054G>T         | 1 |
| 252    | c.1687_1692insA  | c.589G>A          | 1 |
| 253    | IVS7-2A>G        | c.716T>A          | 1 |
| 254    | c.1229C>T        | c.1354C>T         | 1 |
| 255    | IVS7-3C>G        | IVS5+1G>A         | 1 |
| 256    | c.1975G>C        | c.2086C>T         | 1 |
| 257    | c.1225C>T        | c.1225C>T         | 1 |
| 258    | c.2168A>G        | c.404A>G          | 1 |
| 259    | IVS7-2A>G        | IVS3+84G>A        | 1 |
| 260    | c.2006A>T        | c.439A>G          | 1 |
| 261    | IVS7-2A>G        | c.403C>T          | 1 |
| 262    | IVS7-2A>G        | c.1673A>T         | 1 |
| 263    | c.1226G>A        | c.1340delA        | 1 |

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(925/1056) of the genetic etiology in Chinese NSEVA patients. The SLC26A4 mutation data were shown in Table 1 and Table 2.
suggests that KCNJ10 c.1042C>T may not always act together with SLC26A4 mutation in digenic inheritance related to the etiology of NSEVA in Chinese subjects.

Genetic analysis of KCNJ10 among subjects with inner ear malformation
Among 48 patients with NSEVA and non-EVA inner ear malformations, we identified one case (1/48, 2.1%) with the missense mutation c.812G>A in KCNJ10.

Genetic analysis of KCNJ10 among subjects with conductive hearing loss
Among the 96 inpatients with conductive hearing loss, we detected KCNJ10 heterozygous c.812G>A in four cases (4/96, 4.2%), heterozygous c.811C>T in one case (1/96, 1.0%), and heterozygous c.1042C>T in one case (1/96, 1.0%).

Genetic analysis of KCNJ10 among normal-hearing subjects
We completed KCNJ10 mutation screening in 133 normal-hearing control subjects with no family history of hereditary hearing loss. Seven subjects carried a single heterozygous mutation of c.812G>A in KCNJ10 (7/133, 5.3%), and one subject carried a single heterozygous mutation of c.1042C>T in KCNJ10 (1/133, 0.8%). No other mutations in KCNJ10 were detected. No statistical difference in the KCNJ10 c.812G>A and c.1042C>T mutations was observed between the normal-hearing group and the NSEVA cases with zero, one, or two mutations in SLC26A4.

Table 2. Genotype of NSEVA patients with zero or one allele SLC26A4 mutation.

| Number | Genotype | Number of patients |
|--------|----------|--------------------|
| 1      | IVS7-2A>G/wt | 68 |
| 2      | c.2168A>G(p.H723R)/wt | 11 |
| 3      | c.2027T>A(p.L676Q)/wt | 5 |
| 4      | c.1174A>T(p.N392Y)/wt | 5 |
| 5      | IVS15+5G>A/wt | 4 |
| 6      | c.1229C>T(p.T410M)/wt | 4 |
| 7      | c.1226G>A(p.R409H)/wt | 3 |
| 8      | c.147C>G(p.S49R)/wt | 2 |
| 9      | c.235C>T(p.R79K)/wt | 1 |
| 10     | c.487G>C(p.R163L)/wt | 1 |
| 11     | c.471C>T(p.P157P)/wt | 1 |
| 12     | c.1339_1340delA/wt | 1 |
| 13     | c.1286C>A(p.A429E)/wt | 1 |
| 14     | c.1336C>T(p.Q446X)/wt | 1 |
| 15     | IVS18+1G>A/wt | 1 |
| 16     | c.1673A>T(p.N558I)/wt | 1 |
| 17     | c.917insG/wt | 1 |
| 18     | c.946G>T(p.G316X)/wt | 1 |
| 19     | c.1687_1692insA/wt | 1 |
| 20     | wt/wt | 18 |

wt: wild-type.
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Table 3. The KCNJ10 c.812G>A mutation identified in Chinese patients with nonsyndromic enlargement of vestibular aqueduct (NSEVA).

| Pedigree No. | Phenotype | SLC26A4 mutation | KCNJ10 mutation |
|--------------|-----------|------------------|-----------------|
| 195 proband  | NSEVA     | wt/wt            | c.812G>A (p.R271H)/wt |
| P            | Normal    | wt/wt            | c.812G>A (p.R271H)/wt |
| M            | Normal    | unknown          | unknown         |
| 1606 proband | NSEVA     | c.225C>G(p.L75L)/wt unknown | c.812G>A (p.R271H)/wt |
| P            | Normal    | Unknown          | Unknown         |
| M            | Normal    | unknown          | unknown         |
| 2471 proband | NSEVA     | IVS7-2A>G/c.2027T>A(p.L676Q)| c.812G>A (p.R271H)/wt |
| P            | Normal    | c.2027T>A(p.L676Q)/wt | c.812G>A (p.R271H)/wt |
| M            | Normal    | IVS7-2A>G/wt     | wt/wt           |

wt, wild-type; P, father of proband (paternal); M, mother of proband (maternal).
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patients with inner ear malformation, or patients with conductive hearing loss ($\chi^2 = 6.287, P = 0.179$).

Discussion

NSEVA/PS is an autosomal recessive disorder that results in sensorineural hearing loss and is associated with mutations in the SLC26A4 gene. The exact causal relationship between the SLC26A4 genotype and pathological phenotypes remains controversial [24]. Some patients with NSEVA have biallelic mutations in SLC26A4, including homozygous mutations and compound heterozygous mutations, whereas others lack mutations in one or both alleles of SLC26A4. In addition, the ratios of SLC26A4 biallelic and monoallelic mutations vary across different populations. For example, double and single mutation rates are 81% and 11% in South Korea [13], 77% and 13% in France [25], 47% and 13% in Japan [26], and 88% and 10% in China, respectively [27]. Several reasons may explain this seemingly confusing phenomenon. First, the efficiency and effectiveness of mutation screening are limited. Mutations in the promoter and intronic cryptic splicing regions could be missed [28]. Benign SLC26A4 polymorphic variants could be misrecognized as pathogenic alleles [24]. Furthermore, the pathogenesis of NSEVA/PS, as for many complex multigenic disorders, may result from interactions among multiple genes and environmental factors. In patients with NSEVA/PS, mutations in FOXI1, a transcription factor of SLC26A4, have been identified either alone or in combination with mutations in SLC26A4 [28]. Single mutations in both SLC26A4 and KCNJ10 have been suggested as another potential etiological mechanism [14].

Yang et al. [14] proposed that single mutations in both SLC26A4 and KCNJ10, an inward rectifier potassium channel, lead to digenic NSEA. KCNJ10 c.1042C>T was shown to reduce K+ conductance activity and was therefore considered a potential pathological mutation. KCNJ10 c.1042C>T was not found in ESP 6300 and was regarded as a pathogenic SNV by 1000 Genomes. However, in our study, KCNJ10 c.1042C>T was not found in patients with NSEA with zero or one SLC26A4 mutation (n = 131). But the facts that KCNJ10 c.1042C>T was found in a normal-hearing control subject and that normal hearing parents of patients with NSEA with two SLC26A4 mutations carried both KCNJ10 c.1042C>T and SLC26A4 pathogenic mutations suggest that KCNJ10 c.1042C>T might be a benign variant in the Chinese population.

The most frequently identified mutation of KCNJ10 in this study was c.812G>A [p.R271H]. KCNJ10 c.812G>A was not found in ESP 6500 and was regarded as a benign SNV by 1000 Genomes. It was found in patients with NSEA who carried no mutation, a monoallelic mutation, or two mutations in SLC26A4; in patients with inner ear malformation; in patients with conductive hearing loss; and in normal-hearing individuals. The occurrence rate of c.812G>A had no significantly difference among the above five group people, suggest that KCNJ10 c.812G>A is a benign variant in the Chinese population.

Interestingly, the hearing in the two individuals with the SLC26A4 mutations and KCNJ10 c.1042C>T (p.R348C) mutations was normal. It may be due to the likelihood of incomplete penetrance of the p.R348C mutation influenced by additional environmental factors, genetic modifiers or difference in genetic/ethnical background. Our observations also suggested another possibility that KCNJ10 c.1042C>T (p.R348C) is a benign variant in the Chinese population.

Similar to our study, other genetic studies in patients with EVA/PS have failed to find convincing evidence that KCNJ10 mutations contribute to these phenotypes. Jonard et al. [29] screened 25 patients with unilateral deafness and unilateral EVA, but found no mutations in KCNJ10. Mercer et al. [30] screened 51 patients with EVA and found no mutations in KCNJ10. Chen et al. [31] screened SLC26A4 and KCNJ10 in patients with bilateral deafness and inner ear malformations and found no mutations in KCNJ10 in the 15 patients who had one or no SLC26A4 mutations. Landa et al. screened KCNJ10/FOX11 in 68 EVA or Pendred syndrome patients with monoallelic mutations of SLC26A4. They found no evidence for an association between mutations of KCNJ10 or FOX11 with SLC26A4 mutations in the pathogenesis of EVA or Pendred syndrome [32].

To our knowledge, this report is the largest study to screen KCNJ10 in patients with NSEA with two, one, or no SLC26A4 mutations. In addition, we screened KCNJ10 in patients diagnosed with conductive hearing loss having non-EVA inner ear malformations. Interestingly, no statistical differences in KCNJ10 mutation rates were detected among all of the above groups and the normal-hearing control group.

Conclusions

SLC26A4 is the major genetic cause in Chinese NSEA patients, accounting for 87.59%. KCNJ10 may not be a contributor to NSEA. Other genetic or environmental factors are possibly playing a role in the etiology of Chinese NSEA patients with zero or monoallelic SLC26A4 mutation.
Supporting Information

Figure S1 The representative chromatograms of the Sanger sequencing data for wild, c.812G>A and c.1042C>T of KCNJ10. A: wild type of KCNJ10 in the 812 locus. B: sense strand of 812G>A mutation: wild type of KCNJ10 in the 1042 locus. D: sense strand of 1042C>T mutation. E: antisense strand of 1042C>T mutation.

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Author Contributions

Conceived and designed the experiments: PD DH. Performed the experiments: JZ GW DK. Analyzed the data: JZ JC SH BH YY. Contributed reagents/materials/analysis tools: PD. Wrote the paper: JZ YY.