

Development of a genotyping microarray for Usher syndrome

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ABSTRACT

Introduction: Usher syndrome, a combination of retinitis pigmentosa (RP) and sensorineural hearing loss with or without vestibular dysfunction, displays a high degree of clinical and genetic heterogeneity. Three clinical subtypes can be distinguished, based on the age of onset and severity of the hearing impairment, and the presence or absence of vestibular abnormalities. Thus far, 8 genes have been implicated, which together comprise 347 protein-coding exons. Therefore, sequence analysis and the most routinely used mutation scanning techniques are not cost-effective for molecular diagnostics of Usher syndrome. To improve DNA-diagnostics for patients with Usher syndrome, we developed a genotyping microarray based on the arrayed primer extension (APEX) method.

Methods: Allele-specific oligonucleotides corresponding to 298 Usher syndrome-associated sequence variants known to date, 76 of which are novel, were arrayed. The accuracy of the microarray was analysed using DNAs from 158 patients with known mutations; the efficiency of the microarray was analysed using DNAs from 370 novel European and American patients with Usher syndrome.

Results: Validation of the microarray yielded an accuracy of >98%. Among the novel patients, sequence variants were identified in 64/140 (46%) patients with Usher syndrome type I (USH1), 45/189 (24%) patients with Usher syndrome type II (USH2), 6/21 (29%) patients with Usher syndrome type III (USH3), and 6/20 (30%) patients with atypical Usher syndrome. The chip also identified two novel sequence variants, c.400C>T (p.R134X) in *PCDH15* and c.1606T>C (p.C536S) in *USH2A*.

Discussion:

The Usher genotyping microarray represents a versatile and affordable screening tool for Usher syndrome. Its efficiency will improve with the addition of novel sequence variants with minimal extra costs, making it a very useful first-pass screening tool.

Key words: Usher syndrome, retinitis pigmentosa, sensorineural deafness, mutation analysis, arrayed primer extension

Key points:

- We developed a genotyping microarray for Usher syndrome based on the arrayed primer extension (APEX) method, which includes 298 Usher syndrome-associated sequence variants identified in 8 genes. Seventy-six variants have not been reported previously.
- Validation of half of these variants employing DNAs from the original Usher syndrome patients yielded an accuracy of the microarray of >98%. The efficiency of the microarray was analysed using DNAs from 370 novel unrelated Usher syndrome patients. Sequence variants were identified in 46% of patients with Usher syndrome type I, 24% of patients with Usher type II, 29% of patients with Usher syndrome type III, and in 30% of patients with atypical Usher syndrome.
- We conclude that this genotyping microarray represents a versatile and affordable screening tool for Usher syndrome. Its efficiency will improve with the addition of novel sequence variants with minimal extra costs, making it a very useful first-pass screening tool.

INTRODUCTION

Patients with Usher syndrome (MIM#276900-2; MIM#276905; MIM#605472) suffer from autosomal recessive retinitis pigmentosa (arRP) and sensorineural hearing impairment. In addition, vestibular dysfunction can be seen in a subset of patients. Usher syndrome occurs in ~1/20,000 individuals and represents 50% of all cases with deafblindness [1-3]. Three clinical subtypes can be distinguished [4]. Patients with USH1 show severe to profound congenital hearing loss, RP, and vestibular areflexia. Patients with USH2 suffer from moderate to severe hearing loss, RP, and normal or variable vestibular function (P.L. Huygen et al., unpublished data). USH3 patients present with progressive hearing loss, RP, and variable vestibular function. Eight genes are known to be involved in Usher syndrome which are *CDH23*, *MYO7A*, *PCDH15*, *USH1C*, and *USH1G* for USH1, *USH2A* and *VLGR1* for USH2 and *USH3A* for USH3 [5-15]. The *USH2A* gene is also implicated in arRP [16-18]. Likewise, mutations in *CDH23*, *MYO7A*, *PCDH15*, and *USH1C*, have been reported in patients with non-syndromic hearing impairment [see: Hereditary Hearing Loss Homepage at: <http://webhost.ua.ac.be/hhh/> and refs 7,12,19-22].

Identification of the causal mutations is important for the early diagnosis of Usher syndrome, which is relevant for the decision whether or not to elect for a cochlear implant, for genetic counselling, and for prenatal diagnosis. In addition, gene-specific treatments might become available in the near future, necessitating knowledge of the underlying gene defect. Comprehensive molecular diagnostics for Usher syndrome has been hampered both by the genetic heterogeneity, and the large number of exons for six of the eight known Usher genes. The five USH1 genes collectively contain 179 protein-coding exons, the two USH2 genes comprise 162 protein-coding exons, and the USH3 gene *USH3A* has 6 protein-coding exons part of which are alternatively spliced [UCSC human genome browser at <http://genome.cse.ucsc.edu>]. Thus far, large-scale mutation screening has been carried out using single strand conformation analysis and denaturing gradient gel electrophoresis, with subsequent sequence analysis of fragments with an aberrant migration pattern. For routine analysis, these techniques are both time consuming and expensive.

In this study, we used microarray technology that was previously used for efficient mutation analysis of the *ABCA4* gene, in patients with either autosomal recessive Stargardt disease or autosomal recessive cone-rod dystrophy [23,24], as well as for 7 genes implicated in Leber congenital amaurosis [25,26]. Inherent to the arrayed primer extension (APEX), it detects only known mutations and therefore its efficiency is highly dependent on the extent of earlier mutation analysis efforts. Here, we describe the first comprehensive Usher genotyping microarray which allows the identification of known Usher gene defects in a high-throughput and cost-effective manner.

PATIENTS AND METHODS

Patients

For validation purposes, we obtained genomic DNA from 158 patients with Usher syndrome in which the variants were originally identified. In order to test the efficiency of the Usher microarray (denoted 'evaluation'), 370 patients were analysed. Of these, 170 unrelated Usher syndrome patients were ascertained in 8 European countries: Belgium (20 patients), Denmark (10 patients), England/UK (30 patients), Germany (27 patients), Italy (20 patients), Northern Ireland/UK (4 patients), Spain (5 patients), Switzerland (7 patients), and the Netherlands (47 patients). A further 200 patients from the USA (Boys Town, Omaha) were analysed. In total, we tested DNAs from 140 patients with USH1, 189 patients with USH2, 21 patients with USH3, and 20 patients with atypical Usher syndrome. The patients, except for 104 USH2 patients from the USA, who were previously shown not to carry the *USH2A* c.2299delG (p.E767fs) mutation, were not previously tested for the presence of Usher mutations. Research procedures were in accordance with institutional guidelines and the Declaration of Helsinki. Informed consent was obtained at each Center from all patients after the nature of procedures to be performed was fully explained.

Molecular Methods

Microarrays were designed and manufactured according to the arrayed primer extension (APEX) technology [27,28]. A detailed description of the methodology is found elsewhere [29,30][www.asperbio.com]. In brief, 5'-modified sequence-specific oligonucleotides are arrayed on a glass slide. In general, these oligonucleotides are designed with their 3' end immediately adjacent to the variable site. PCR-amplified and fragmented target nucleic acids are annealed to oligonucleotides on the slide, followed by sequence-specific extension of the 3' ends of primers with dye-labeled nucleotide analogs (ddNTPs) by DNA polymerase. The APEX reaction is, in essence, a sequencing reaction on a solid support.

One hundred and twenty amplicons from eight genes (Table 1) were amplified as described previously [23]. Primer sequences are available on request. In the amplification mixture, 20% of the dTTP was substituted by dUTP [29]. The amplification products were concentrated and purified (GENErALL PCR kit; General Biosystems, Inc., Seoul, Korea). The fragmentation of amplification products was achieved by adding thermolabile uracil *N*-glycosylase (Epicenter Technologies, Madison, WI) and heat treatment [29]. One-sixth of every amplification product was used in the primer extension reaction on the Usher microarray. Each APEX reaction consisted of

fragmented and pooled denatured PCR products, 4 units of ThermoSequenase DNA Polymerase (Amersham Biosciences), 1x reaction buffer and 1.4 μ M final concentration of each fluorescently labeled ddNTP: Texas Red-ddATP, fluorescein-ddGTP (Amersham Biosciences), Cy3-ddCTP, Cy5-ddUTP (NEN). The reaction mixture was applied to a microarray slide for 15 min at 58°C. The reaction was stopped by washing the slide at 95°C in Milli-Q water [31]. The slides were imaged with the Genorama™ Quattrolmager (Asper Biotech, Ltd.) and the sequence variants were identified by Genorama™ Genotyping Software [23,30].

Array-identified variants were confirmed by direct sequencing with the ABI PRISM Big Dye Terminator Cycle Sequencing V2.0 Ready Reaction Kit and the ABI PRISM 3730 DNA analyzer (Applied Biosystems, Foster City, CA, USA).

Table 1. Characteristics of Usher genes

gene	protein coding exons	amplicons for APEX analysis	known pathologic variants
USH1			
<i>CDH23</i>	69	32	52
<i>MYO7A</i>	48	39	118
<i>PCDH15</i>	32	6	7
<i>USH1G</i>	2	2	4
<i>USH1C</i>	28	6	8
USH2			
<i>USH2A</i>	72	26	88
<i>VLGR1</i>	90	6	6
USH3			
<i>USH3A</i>	6	3	9
Total	347	120	292

RESULTS

Construction of the Usher genotyping microarray

We compiled a list of 298 previously published and communicated sequence variants from the coding region and adjacent intronic sequences of the eight Usher genes: *CDH23*, *MYO7A*, *PCDH15*, *USH1C*, *USH1G*, *USH2A*, *USH3A*, and *VLGR1* (Supplementary Table 1). Intronic sequence variants were included when they were predicted to affect splicing, i.e. altering the strictly conserved splice site sequences (nucleotides -2A or -1G of 3' splice sites; nucleotides +1G or +2T of 5' splice sites), transversion of one or a few of the pyrimidines (-5 through -14) of a 3' splice site, changing a -3C nucleotide of a 3' splice site, changing a +3, +4, +5 or +6 nucleotides of a 5' splice site, or exonic nucleotides being part of splice sites, to a less favourable nucleotide. In addition, we included the putatively benign sequence variants c.5156A>G (p.Y1719C) in *MYO7A*, and c.688G>A (p.V230M), c.1434G>C (p.E478D), c.2052A>G (p.Q684Q, possible cryptic splice site), c.2137G>C (p.G713R) and c.2522C>A

(p.S841Y) in *USH2A*. Each sequence variant was queried in duplicate by the software, from both the sense and the antisense strand (Genorama™ Genotyping Software; Asper Biotech, Ltd.).

We also included 4 variants on the chip despite the fact that there are inconsistencies in their notations in the original publications or erroneous genomic sequence data: *CDH23* c.3880>T (p.Q1294X) [12], *MYO7A* c.269G>C (p.R90P) [32], *MYO7A* c.4039_4053del (p.R1347_F1351del) [33], and *USH2A* c.233T>G (p.F78V). The *USH1C_ABCC8* 122,815 bp deletion [7] is not represented on the chip.

Validation of the Usher genotyping microarray

We collected DNA samples from 158 patients with Usher syndrome and at least one known variant. All variants, except two, *CDH23* c.3617C>G and *MYO7A* c.223delG, were reliably identified. For the two unidentified variants (false negatives), primers have been redesigned and will be retested (Supplementary Table 1). For the remaining 140 variants, no DNA samples from the original patients were available as positive controls. However, the wild-type sequences of these variants were robustly identified. Since 102 of these variants represent nucleotide substitutions, the identification of the wild-type sequence can be regarded as a positive control since the same 25-mers are employed for the identification of both the wild-type and mutant nucleotides. However, we cannot exclude the possibility of erroneous sequence variant annotations, which we observed for a number of mutations in the respective original publications. Thirty-eight non-validated variants represent insertions/deletions for which in some instances oligonucleotides linked to the microarray for detection of the wild-type allele differed from that for detection of the mutant allele.

Evaluation of the Usher genotyping microarray

The efficiency of the Usher microarray was evaluated using a total of 370 patients: 140 USH1, 189 USH2, 21 USH3 and 20 patients with atypical Usher syndrome. The results are given both separately and combined for the novel Usher patients from Europe and the USA (Table 2). The European patients originated from 8 Western European countries, i.e. the Netherlands (12.7%), the UK (8.1%), Germany (7.3%), Belgium (5.4%), Italy (5.4%), Denmark (2.7%), Switzerland (1.9%), and Spain (1.4%). The majority of the patients from the USA were of European descent. For USH1, genotyping using the microarray revealed the highest percentage of patients with either one or two mutations, 51% for the European patients, 30% for the patients from the USA and 46% when both groups are combined. When counting mutated alleles, these percentages are 34%, 23% and 31%, respectively. The best results were observed for the USH1 patients of Danish origin in whom a mutation was found in 80% (8/10) of the cases or in 65% (13/20) of the alleles.

Table 2: Identification of Usher mutations in patients from Europe and the USA

Country	USH1 patients				USH2 patients				USH3 patients				Atypical USH patients			
	total	0 all	1 all	2 alls	total	0 all	1 all	2 alls	total	0 all	1 all	2 alls	total	0 all	1 all	2 alls
Belgium, Germany, Netherlands	51	27	12	12	35	25	5	5	3	3	0	0	5	4	1	0
Switzerland					5	5	0	0	2	2	0	0				
Italy, Spain	13	5	8	0	3	2	0	1	1	0	1	0	8	5	2	1
Denmark	10	2	3	5												
UK	29	16	12	1	4	3	0	1	1	0	0	1				
<i>Total Europe</i>	<i>103</i>	<i>50</i>	<i>35</i>	<i>18</i>	<i>47</i>	<i>35</i>	<i>5</i>	<i>7</i>	<i>7</i>	<i>5</i>	<i>1</i>	<i>1</i>	<i>13</i>	<i>9</i>	<i>3</i>	<i>1</i>
<i>Patients with mutation</i>	<i>53/103 = 51%</i>				<i>12/47 = 25%</i>				<i>2/7 = 29%</i>				<i>4/13 = 31%</i>			
<i>Mutant alleles</i>	<i>71/206 = 34%</i>				<i>19/94 = 20%</i>				<i>3/14 = 21%</i>				<i>5/26 = 19%</i>			
USA	37	26	5	6	38	29	7	2	14	9	3	1	7	5	1	1
<i>Patients with mutation</i>	<i>11/37 = 30%</i>				<i>9/38 = 24%</i>				<i>4/14 = 29%</i>				<i>2/7 = 29%</i>			
<i>Mutant alleles</i>	<i>17/74 = 23%</i>				<i>11/76 = 14%</i>				<i>5/28 = 18%</i>				<i>3/14 = 21%</i>			
Total	140	76	40	24	85	64	12	9	21	14	4	2	20	14	4	2
Patients with mutation	64/140 = 46%				21/85 = 25%				6/21 = 29%				6/20 = 30%			
Mutant alleles	88/280 = 31 %				30/170 = 18%				8/42 = 19%				8/40 = 20%			
USA pre-screened					104	80	17	7								
<i>Patients with mutation</i>					<i>24/104 = 23%</i>											
<i>Mutant alleles</i>					<i>31/208 = 15%</i>											

The number of cases with two alleles represent those cases for which two sequence variants were detected in the same gene.

For USH2 the percentages of patients with variants are 25%, 24% and 25%, respectively. The percentages for detected USH2 alleles are 20%, 14% and 18%, respectively. Hundred and four of the patients with USH2 from the USA were pre-screened for the p.E767fs mutation and patients heterozygous or homozygous for this mutation were not included. This pre-screening did not influence the genotyping detection rate for the microarray which was 23% of the patients and 15% of the alleles. The numbers of patients with USH3 or atypical Usher syndrome were relatively low. Consequently, the sensitivity of the microarray for these patient groups, although rather good in the present cohorts of patients, cannot be reliably assessed.

The *USH2A*-variant c.2299delG (p.E767fs) was previously described as a common mutation in Caucasian patients with USH2, comprising between 13 and 39% of *USH2A* alleles [34-38]. The genotyping presented here revealed a second common mutation, c.11864G>A (p.W3955X) in exon 61 of this gene. This nonsense mutation truncates the long isoform of the USH2A protein [39]. Three of 50 European patients with USH2 were heterozygous for this allele, twice in combination with the p.E767fs mutation. In USH2 patients from the USA, 15 alleles with this mutation were detected in 13 of 88 patients. In total, eighteen of 60 mutant alleles (30%) identified in patients with USH2, were p.W3955X. However, since part of the USH2 patients were pre-screened for the common p.E767fs mutation, this percentage is probably higher than would be expected in an unselected patient cohort. For the European patients this percentage was only 17% (3/18). The p.W3955X mutation was also heterozygously present in a patient with atypical Usher syndrome in whom the second mutation was not identified. Haplotype analysis has to be performed to determine whether the p.W3955X mutation is a founder mutation as has been shown for p.E767fs [40].

Of the mutations detected, 191 were re-evaluated by sequence analysis and only two could not be confirmed (false positives) which illustrates the robustness of the method. One mutation, c.496+1G>A in the *USH1C* gene, was not detected by the microarray but detected afterwards by sequence analysis (false negative). Segregation analysis could be performed in relatives of 11 probands in whom at least two variants were detected by chip analysis. In one of these families, the analysis revealed that two *CDH23* variants were present on the same allele. For the remaining 10 probands the results are compatible with both alleles each carrying one mutation.

Identification of novel variants in Usher genes

Since all four nucleotides are available during primer extension, the APEX technology allows the detection of new nucleotide variants for positions that were already known to be variant, and are represented by an assay on the microarray. In this way two novel variants were detected and confirmed by sequence analysis. In *PCDH15*, c.400C>T is predicted to cause the nonsense mutation p.R134X. At this position the substitution of a guanine for a cytosine was already described to cause the missense mutation p.R134G in a family with nonsyndromic hearing loss [21]. In the *USH2A* gene, a c.1606T>A was detected, which is predicted to cause a amino acid substitution p.C536S. The nucleotide substitution c.1606T>C (p.C536R) was reported by Dreyer et al [34] in one of 31 unrelated patients with USH2 who also carried the p.E767fs variant.

Variants in genes known to be associated with a different USH type

In a number of patients, variants were detected that did not correspond with the type of Usher syndrome in the patient (Table 3). In five patients with USH1, one or two mutations were detected in the *USH2A* gene. In USH2 patients, variants were detected in the *MYO7A* gene and also in the *USH3A* gene, and in USH3 patients variants were seen in *MYO7A* and in *USH2A*. Whether the heterozygous mutations in the *MYO7A* gene are indeed causative such that mutations in *MYO7A* can also cause USH2 and USH3, remains to be determined. In many cases, the clinical diagnosis may be ambiguous. This is also true for the heterozygous *USH2A* mutations in patients with USH1 and USH3 and *USH3A* mutations in patients with USH2.

In patients with atypical Usher syndrome, mutations were detected in *MYO7A*, *USH2A* and *CDH23* (Table 3).

Table 3: Variants in genes known to be associated with a different USH type and variants associated with atypical USH (USHA)

patient	phenotype	Allele 1	Allele 2
H2524	USH1	<i>USH2A</i> p.560fs	
JD6381	USH1	<i>USH2A</i> p.E767fs	
03-03704	USH1	<i>USH2A</i> p.L555V	
57	USH1	<i>USH2A</i> p.R4115C	
QUB4/B5	USH1	<i>USH2A</i> p.C536S	<i>USH2A</i> p.H548S
D3900	USH2	<i>USH3A</i> p.N48K	<i>USH3A</i> p.N48K
H2774	USH2	<i>USH3A</i> p.N48K	
0303158	USH2	<i>USH3A</i> p.S50fs	
D9569	USH2	<i>MYO7A</i> p.R1240Q	
0401876	USH2	<i>MYO7A</i> c.736-3C>T	
D8903	USH3	<i>MYO7A</i> p.Q1798X	
D9804	USH3	<i>MYO7A</i> p.E1359fs	
H2156	USH3	<i>USH2A</i> p.V218E	
O2	USH3	<i>USH2A</i> p.E767fs	
H0200	USHA	<i>MYO7A</i> p.R1743W	<i>MYO7A</i> p.R1743W
MM52	USHA	<i>MYO7A</i> p.R1861X	<i>MYO7A</i> p.R1861X
RP683	USHA	<i>CDH23</i> p.T1209A	
21730	USHA	<i>CDH23</i> p.R1060W + p.D1341N	
T56	USHA	<i>USH2A</i> p.L555V	
H1362	USHA	<i>USH2A</i> p.W3955X	

Variants identified in two Usher genes

In nine patients, variants in two different genes were detected, i.e. in four samples used for validation and in five patients that were tested for the evaluation of the chip (Table 4). In three of the nine cases, a variation in a second gene was present in addition to two mutations in one of the other Usher genes. As segregation analysis could not be performed for any of these cases, we cannot conclude that they represent true digenic inheritance, whether the variant in the second gene contributes to the severity of the phenotype, or whether the results are merely a coincidence. However, in one of the

cases with three sequence variants, two in *USH2A* and one in *MYO7A*, the *USH2A* mutations are protein truncating. These truncating mutations are most likely sufficient to cause the disease and thus only a modifying effect for the p.R1343S variant in *MYO7A* seems plausible. A second case had two truncating mutations in *MYO7A* in addition to p.P608R in *USH1C*. Also for this case, the third variant might modify the phenotype but is not likely to be primarily disease causing. The combination of variants in the *MYO7A* and *USH2A* genes were seen in six of the eight cases with variants in two Usher genes, which is not unexpected since these genes are causative in the majority of the patients with USH1 and USH2, respectively, and thus are expected to exhibit the highest carrier frequencies.

Table 4: Usher syndrome patients with sequence variants in two Usher genes

Patient	Phenotype	Allele 1	Allele 2	Allele 3
Val-1	USH1	<i>CDH23</i> p.R2833G	<i>MYO7A</i> p.R302H	
Val-2	USH1	<i>MYO7A</i> p.A1340T	<i>USH1C</i> p.R80fs	
Val-4	USH2	<i>USH2A</i> p.C1002fs	<i>MYO7A</i> p.R302H	
H0151	USH2	<i>USH2A</i> p.C419F	<i>MYO7A</i> p.E1359fs	
H0165	USH2	<i>USH2A</i> p.R317R	<i>MYO7A</i> p.T1566M	
H2264	USH2	<i>USH2A</i> p.E767fs	<i>MYO7A</i> p.R1743W	
Val-3	USH2	<i>USH2A</i> p.K182fs	<i>USH2A</i> p.E767fs	<i>MYO7A</i> p.R1343S
H0900	USH2	<i>USH2A</i> p.V218E	<i>USH2A</i> p.W3955X	<i>MYO7A</i> p.T165M
P99-0345	USH1	<i>MYO7A</i> p.Q121fs	<i>MYO7A</i> p.Q121fs	<i>USH1C</i> p.P608R

USH variants with questionable pathogenicity

Several sequence variants in Usher genes have previously been described for which pathogenicity was uncertain. A number of these were included in the microarray and based on the results of the evaluation experiment, they were regarded as polymorphisms. Nine alleles with the missense variant p.E478D [17,41] in the *USH2A* gene were detected in patients with all three types of Usher syndrome, and in three patients, one of each with USH1, USH2 and USH3, this variant was present in addition to two pathogenic mutations. The frequency in controls was determined for the Jewish population (0/120 alleles) [41]. However, we detected the this variant in three out of 180 control alleles from the Dutch population. The *USH2A* variant p.V230M was detected nine times in patients with all three types of Usher syndrome. In these patients, there was only one putative pathogenic *USH2A* mutation detected in addition to the p.V230M variant. Dreyer et al [34] did not detect this variant in controls, however, Jian Seyedahmadi et al [17] detected similar frequencies in patients and controls. The *USH2A* variant p.G713R was described by Dreyer et al [34] as likely to be pathogenic since it was not detected in controls. However, Seyedahmadi described that the variant did not co-segregate with the disease in some of the families. In the present genotyping, the p.G713R variant was detected as the only variant heterozygously in two patients with USH1, and in one patient with USH3. In an USH2 patient it was detected in addition to a truncating mutation. In an affected sib of one of the USH1 patients this variant was not present. The *USH2A* variant c.2052A>G (p.Q684Q) [42] was described to possibly create a cryptic splice site. This nucleotide substitution was detected once in an USH2

patient as the only variant. The *USH2A* variant p.S841Y was previously described to be present in 2 of 186 control alleles [38]. In the present genotyping this variant was detected in one *USH1* patient with one *MYO7A* mutation and in one *USH1* patient with one mutation in *CDH23*.

In *MYO7A*, the variant p.Y1719C was questioned to be disease-causing by Najera and co-workers [33] since it was detected in 3/168 control alleles. We detected this variant in four patients with all three different types of Usher syndrome, two of which did not have an additional variant in one of the Usher genes. This does not support a causative effect of the mutation.

DISCUSSION

A cost-effectiveness comparison of APEX mutation analysis with other medium and high-throughput mutation detection techniques

The routine mutation analysis of patients suffering from a genetically heterogeneous disease has been severely hampered by the high costs associated with the application of conventional analysis techniques for comprehensive mutation screening. Despite the fact that most of the Usher syndrome patients can be reliably grouped into one of two main clinical groups, a systematic mutation analysis of either the *USH1* or *USH2* genes still requires the analysis of 179 and 162 protein coding exons, respectively. As depicted in Table 5, the APEX-based mutation detection array is an affordable and flexible system as new alleles can be added without the need to design completely new screening platforms. The main disadvantage is its low efficiency in early versions since it relies on the extent of previous mutation analysis studies performed using other techniques. The Affymetrix resequencing technique has two disadvantages. First, heterozygous deletions larger than 1 bp and all insertions and duplications are missed because the complexity of the microarray would increase disproportionately if matching primers are added to the chip. These variants comprise 47/292 (16%) of all pathologic *USH* variants. Second, the screening costs, including development costs, are quite high prohibiting the use of this technique in a routine laboratory setting.

A direct comparison of efficiency with other mutation detection techniques is not straightforward since no comprehensive mutation detection studies have yet been performed for more than one Usher gene. For *USH1*, the most commonly used technique is single strand conformation polymorphism (SSCP) analysis, which yielded between 19 and 48% of mutant alleles in *MYO7A* [32,33,43], which is comparable to our overall efficiency (31% of alleles; Table 2). For *USH2*, SSCP and sequence analysis of exons 1 – 21 of *USH2A* yielded 23% and 38% of mutant alleles, respectively [17,38], which is higher than with the APEX technology (18%). The relatively low yield of *USH2* alleles is due to the fact that the *USH2A* gene was expanded only recently from 21 to 73 exons [39] and that comprehensive mutation analysis of this gene lags behind *MYO7A*.

Table 5: A comparison of mutation detection techniques for Usher syndrome

	Advantages	Disadvantages
APEX	Robust Versatile (addition of new variants with little extra)	Detects only known variants Low efficiency in early stages Less efficient to analyse patients

	costs) Cheap (< EUR 200,-) Medium throughput Not highly dependent on DNA quality	with other ethnic backgrounds Requires specialized expertise Sequence confirmation required
Sequence analysis	Robust Versatile Easy set-up	High costs (> EUR 1000,- for <i>MYO7A</i> or <i>USH2A</i>)
Capillary-based heteroduplex analysis	Robust Medium throughput Relatively cheap	Polymorphic sequences difficult to analyse Sequence analysis necessary for mutation identification
Resequencing (Affymetrix)	Fully automated Detects known and new nucleotide substitutions	High costs (~EUR 600,-) No detection of heterozygous deletions >1 nt and all duplications/insertions, [47/292 (16%) pathologic USH variants] Addition of tested genes requires new chip design

Thus, efficiencies for the Usher chip at the moment can be best compared to the recently developed APEX chip for Leber congenital amaurosis, which is comparable in complexity (contains ~300 variants found in 7 genes) and yields on average 20-28% of LCA alleles [25,26]. The Usher microarray chip is particularly useful for the analysis of patients with atypical Usher syndrome, who have been shown to carry mutations in the *MYO7A*, *CDH23*, and *USH2A* genes.

The overall efficiency of the Usher chip in the coming years is expected to increase proportionally to the addition of new variants. We propose the use of this mutation chip as a first-pass screening tool for all Usher patients. In patients with one or no variant, capillary-based heteroduplex analysis of the larger genes (*CDH23*, *MYO7A*, *PCDH15*, *USH1C*, *USH2A*, *VLGR1*) or sequence analysis of the smaller genes (*USH1G*, *USH3A*) can be performed. Novel variants from these studies can subsequently be added to the chip, resulting in a gradual increase of its efficiency. The Usher syndrome mutation detection chip is available from Asper Ophthalmics [<http://www.asperophthalmics.com/UshersyndromeDNAtest.htm>.]

Deficiencies of the Usher chip and other conventionally used mutation detection techniques

Currently, none of the standard mutation analysis techniques is able to identify heterozygous deletions that span one or both amplicon primers. As a result, we have no knowledge whatsoever on the frequency of deletions of this kind. To fill this caveat, a customised probe set needs to be designed that can identify copy-number changes, e.g. multiplex ligation dependent probe amplification or quantitative PCR [44]. Likewise, deep intronic mutations affecting splicing are missed as RNA analyses are not part of standard procedures due to restricted expression of the Usher genes.

Pathogenicity of variants in Usher genes

When a known mutation is detected in both copies of one of the Usher genes, one can confidently conclude that these mutations are disease-causing. Two alleles were found in 24/140 (17%) USH1 patients, 9/85 (11%) USH2 patients, 2/21 (10%) USH3 patients and in 2/20 (10%) atypical USH patients. However, when one mutated allele is observed, the chance that a false conclusion regarding causality of this particular variant for the phenotype in that patient is made, differs for each of the Usher syndrome genes. For the *MYO7A* gene in USH1 patients, the percentage of patients with a false interpretation based on the presence of one mutated allele for this gene was calculated to be 2.4; for the *USH2A* gene in USH2 patients this percentage was calculated to be 0.8 [45]. For diagnoses based on one allele with a missense mutation this percentage is likely to be higher due to the fact that the pathogenicity of this type of mutations is difficult to prove. Due to the small sizes of the families with Usher syndrome and the fact that samples of other family members are often not available, it is mostly not possible to perform segregation analysis to prove pathogenicity of a specific sequence variant.

We also included on the microarray a number of sequence variants that we assume not to be pathogenic. The results in the present study support this assumption. However, it cannot be excluded that these variants can modulate the disease severity in a way comparable to that of a sequence variant in Bardet-Biedl syndrome. An exonic nucleotide substitution that affects splicing of the *MGC1203* RNA modulates the phenotype of patients with Bardet-Biedl syndrome with mutations in the *BBS1* gene, because the *BBS1* and *MGC1203* proteins interact [46]. Since both the USH1 and USH2 proteins are known to be part of an Usher interactome [47-52] phenotypic modulation might well be possible and was already suggested in a family with homozygous *USH3A* mutations in the affected members and a mutated *MYO7A* gene in one of them [53,54].

Digenic inheritance in Usher syndrome?

Since Usher proteins interact with each other and form dynamic protein complexes, digenic or oligogenic inheritance of Usher syndrome would not be surprising. Indications for digenic inheritance involving mutations in the *PCDH15* and *CDH23* genes have been obtained for two USH1 patients and a patient with atypical Usher syndrome [55] but no elaborate segregation analysis was reported to strengthen this observation. The large number of patient DNAs screened in the present study is specifically suitable for detection of rare cases exhibiting digenic inheritance of the syndrome. From the nine cases with sequence variants in two different genes, one is unlikely to follow digenic inheritance since two truncating mutations in one of the genes were present. *PCDH15* and *CDH23*, previously implicated in digenicity, were not involved in any of the remaining eight cases. Segregation analysis could not be performed for any of the cases and therefore, we cannot confirm or exclude digenic inheritance.

Use of the Usher microarray for patients with non-syndromic arRP and hearing impairment

Mutations in the *USH2A* gene have been described to be a common cause of RP and the p.C759F mutation is almost exclusively detected in patients with non-syndromic RP. Based on their results of screening the exons 1-21 of the gene, Jian Seyedahmadi et al

[17] estimated that *USH2A* mutations are present in 7% of all RP cases in the USA and screening of only one third of the exons 22-73 increased this percentage to 16 for arRP patients [56]. Since all *USH2A* mutations detected in RP patients are represented on the Usher microarray, this is also suitable for this patient population. However, a mutation array with known mutations in a large number of RP genes, including *USH2A*, has been developed [57] and is more suitable for mutation detection in patients with RP.

Mutations in the USH1 genes *CDH23* (DFNB12), *MYO7A* (DFNA11, DFNB2), *PCDH15* (DFNB23), and *USH1C* (DFNB18) can cause non-syndromic hearing loss [51, and references therein]. Among these genes, *CDH23* seems to be the most frequently involved in non-syndromic hearing loss. The hearing loss in DFNB12 patients ranges from moderate to profound but also patients with hearing loss that started as mild in childhood have been described to have mutations in *CDH23* [20]. So far, there are no indications that mutations in the USH1 genes are a common cause of non-syndromic hearing loss. However, more extensive mutation analysis in this patient category is necessary to determine the value of the present microarray for this group of patients.

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Supplementary Table 1: Usher microarray nucleotide variants (page 1 of 3)

#	Exon	Nucleotide change	Amino acid change	Validated	Ref.	#	Exon	Nucleotide change	Amino acid change	Validated	Ref.
CDH23						MYO7A					
1	3	172C>T	Q58X	y	1	1	3	47T>A	L16X	n	6
2	3	193delC	L65fs	y	1	2	3	52C>T	Q18X	y	7
3	4	336G>C	G112G/splice defect	y	2	3	3	73G>A	G25R	n	8
4	4	336+1G>A	splice defect	y	1	4	3	77C>A	A26E	n	9
5	5	371A>G	D124G	y	1	5	3	93C>A	C31X	y	10
6	10	1087delG	V363fs	y	1	6	4	124-2A>G	splice defect	n	11
7	10	1112delT	I371fs	y	1	7	4	199G>A	V67M	n	9
8	13	1355A>G	N452S	y	1	8	4	223delG	D75fs	t/n	10
9	13	1439T>A	L480Q	n	1	9	4	269G>C#	R90P#	n	9
10	14	1450G>C	A484P/splice defect	y	1	10	5	318C>A	N106K	n	7
11	15	1745G>A	R582Q	y	1	11	5	361delC	Q121fs	y	10
12	20	2289+1G>A	splice defect	y	1	12	5	401T>A	I134N	y	9
13	25	2968G>A	D990N	n	3	13	5	448C>T	R150X	y	12
14	25	3105A>C	T1035T/splice defect	y	1	14	5	470+1G>A	splice defect	n	13
15	26	3178C>T	R1060W	y	1	15	6	494C>T	T165M	y	14
16	29	3557G>A	G1186D	n	1	16	7	613A>G	I205V	n	13
17	30	3617C>G	P1206R	t/n	1	17	7	634C>T	R212C	y	10
18	30	3625A>G	T1209A	y	1	18	7	635G>A	R212H	y	10
19	31	3841_3843del	M1281del	n	4	19	7	640G>A	G214R	y	13
20	31	3842_3845dup	V1283fs	y	1	20	7	652_657delGACATC	D218_1219del	n	12
21	31	c.3880>T#	p.Q1294X#	n	3	21	7	700C>T	Q234X	y	12
22	31	4021G>A	D1341N	y	5	22	7	721C>A	R241S	n	6
23	35	4488G>C	Q1496H/splice defect	n	4	23	7	721C>T	R241C	n	9
24	36	4504C>T	R1502X	y	1	24	7	722G>A	R241H	n	7
25	37	4756G>C	A1586P	n	1	25	7	731G>C	R244P	n	11
26	37	4783G>A	E1595K	y	1	26	8	736-3C>T	splice defect	n	7
27	40	5237G>A	R1746Q	y	4	27	8	805_807delAAG	K269del	n	9
28	42	5536G>A	D1846N	n	1	28	9	905G>A	R302H	y	10
29	42	5712G>A	T1904T/splice defect	n	2	29	9	940G>T	E314X	y	10
30	45	6049+1G>A	splice defect	n	3	30	9	999T>G	Y333X	n	10
31	46	6050-9G>A	splice defect	y	1, 2	31	10	1066_1067insATCTCTGC	A356fs	y	7
32	46	6133G>A	D2045N	n	3	32	11	1132C>A	R378S	n	7
33	46	6155delC	T2055fs	y	1	33	11	1157T>C	L386P	n	10
34	47	6307G>T	E2103X	y	1	34	11	1190C>A	A397D	n	13
35	47	6319C>T	R2107X	n	3	35	12	1258A>T	K420X	y	7
36	47	6442G>A	D2148N	n	1,5	36	12	1325A>G	E442G	y	7
37	47	6604G>A	D2202N	n	3	37	13	1348G>C	E450Q	n	10
38	49	6933delT	T2313fs	n	2	38	13	1370C>T	A457V	n	9
39	49	6968delC	P2323fs	y	1	39	13	1373A>T	N458I	y	15
40	51	7362+5G>A	splice defect	n	4	40	13	1403_1404insGCA	R467_H468insQ	y	10
41	52	7393C>T	R2465W	y	1	41	13	1508C>T	P503L	y	10
42	53	7549A>G	S2517G	y	1	42	14	1555-8C>G	splice defect	n	9
43	54	7823G>A	R2608H	y	1	43	14	1555-1G>C	splice defect	n	10
44	54	7872G>A	E2624E/splice defect	n	1	44	14	1556G>A	G519D	n	9
45	57	8230G>A	G2744S	n	1	45	14	1563delC	D521fs	n	8,11
46	58	8497C>G	R2833G	y	1	46	14	1595delA	H532fs	n	10
47	60	8849T>A	I2950N	n	3	47	14	1623_1624insC	K542fs	y	9
48	60	8866C>T	R2956C	n	3	48	15	1797G>A	M599I	n	16
49	62	9175C>A	P3059T	n	3	49	16	1798-14_1798-11 delGTCCinsCCAG	splice defect	n	10
50	66	9510+1G>A	splice defect	n	3						
51	67	9524G>A	R3175H	n	1	50	16	1798delG	G600fs	y	7
52	67	9626_9627insC	I3210fs	y	1	51	16	1884C>A	C628X	n	17

Supplementary Table 1: Usher microarray nucleotide variants (page 2 of 3)

#	Exon	Nucleotide change	Amino acid change	Validated	Ref.	#	Exon	Nucleotide change	Amino acid change	Validated	Ref.
MYO7A						MYO7A					
52	16	1900C>T	R634X	n	14	102	38	5227C>T	R1743W	y	9
53	16	1935G>A	M654I/splice defect	n	16	103	39	5392C>T	Q1798X	y	6
54	16	1935+4A>G	splice defect	n	10	104	39	5411delT	L1804fs	n	7
55	17	1945C>T	R649W	y	7	105	40	5573T>C	L1858P	n	9
56	17	1952T>C	L651P	n	18	106	40	5581C>T	R1861X	y	13
57	17	1969C>T	R657W	n	7	107	40	5618G>A	R1873Q	y	7
58	17	1996C>T	R666X	y	6	108	41	5637-3C>G	splice defect	y	7
59	17	2005C>T	R669X	y	14	109	41	5637-1G>A	splice defect	n	9
60	17	2028C>G	Y676X	n	7	110	41	5648G>A	R1883Q	y	7
61	17	2035_2036insT	E680fs	n	7	111	41	5660C>T	P1887L	n	9
62	18	2171delC	K725fs	n	7	112	43	5886_5888del	F1963del	y	7
63	18	2187+1G>A	splice defect	n	13	113	44	5945-1G>A	splice defect	n	7
64	20	2302A>T	K768X	y	7	114	44	5968C>T	Q1990X	n	7
65	20	2323C>T	Q775X	y	7	115	44	6025delG	A2009fs	y	9
66	21	2461C>T	Q821X	y	17	116	45	6193delC	Q2066fs	n	13
67	21	2476G>A	A826T	n	13	117	47	6410G>A	G2137E	n	7
68	22	2662_2670del	K888_K890del	n	19	118	48	6487G>A	G2163S	n	6
69	23	2766_2679del	K923fs	y	7	119	49	6560G>A	G2187D	n	9
70	23	2863G>A	G955S	n	8	PCDH15					
71	23	2878G>T	E960X	n	6	1	2	7C>T	R3X	n	21,22
72	23	2904G>T	E968D/splice defect	y	9	2	5	400C>G	R134G	n	23
73	25	3109-2A>G	splice defect	n	7	3	8	733C>T	R245X	y	24
74	25	3171C>G	Y1057X	n	7	4	8	785G>A	G262D	n	23
75	25	3238A>T	K1080X	y	20	5	10	1086delT	L362fs	n	22
76	25	3260T>C	L1087P	n	14	6	16	1927C>T	R643X	n	23
77	25	3265delG	A1089fs	y	14	7	28	3686-2A>G	splice defect	n	21
78	28	3508G>A	E1170K	y	20	USH1C					
79	28	3547C>A	P1183T	y	7	1	2	91C>T	R31X	y	25
80	28	3596_3597insT	C1201fs	n	11	2	3	238_239insC	R80fs	y	26,27
81	28	3630+2T>A	splice defect	n	8	3	5	496+1G>A	splice defect	n	25
82	29	3718C>T	R1240W	y	7	4	6	497-2delA	splice defect	y	27
83	29	3719G>A	R1240Q	y	6	5	B	\$32G>T	G431V	n	28
84	29	3750+2T>A	splice defect	n	7	6	D	\$293C>G	P608R	y	28
85	30	3862G>C	A1288P	y	6	7	D	\$329G>T	R620L	n	28
86	31	3979G>A	E1327K	n	17	8	D	\$376C>T	R636C	n	28
87	31	4018G>A	A1340T	y	7	USH1G					
88	31	4029G>C	R1343S	n	6	1	1	143T>C	L48P	n	29
89	31	4036_4038delTTC	F1346del	n	9	2	2	186_187delCA	D62fs	n	29
90	31	4039_4053del#	R1347_F1351del#	n	17	3	2	395_396insG	V132fs	y	29
91	31	4073delC	E1359fs	y	7	4	2	829_848del	S278fs	y	29
92	34	4501_4502delGT	V1501fs	y	7	USH3A					
93	34	4543_4551delGAG ATCATGinsGCA	E1515_M1517delinsA	n	7	1	1	144T>G	N48K	y	30,31
94	35	4697C>T	T1566M	y	17	2	1	149_152delCAGG- insTGTC CAAT	S50fs	y	30
95	35	4770_4771insT	R1591fs	y	7	3	1	165delC	D55fs	n	30
96	35	4805G>A	R1602Q	n	18	4	1	189C>A	Y63X	y	31
97	35	4816delA	K1606fs	n	7	5	1	187_209del	Y63fs	n	31
98	36	4882G>T	A1628S	n	6	6	2	359T>A	M120K	y	32
99	36	4918delG	G1640fs	n	7	7	3	449T>C	L150P	n	30
100	37	5156A>G	Y1719C*	y	20	8	3	459_461delATT	I153_L154delinsM	y	32
101	38	5215C>T	R1739X	y	7	9	3	528T>G	Y176X	y	32

Supplementary Table 1: Usher microarray nucleotide variants (page 3 of 3)

#	Exon	Nucleotide change	Amino acid change	Validated	Ref.	#	Exon	Nucleotide change	Amino acid change	Validated	Ref.
USH2A						USH2A					
1	2	94_130del	S33fs	y	7	53	12	2167+5G>A	splice defect	n	7
2	2	100C>T	R34X	n	33	54	13	2168-9T>G	splice defect	n	7
3	2	187C>T	R63X	y	33	55	13	2242C>T	Q748X	y	35
4	2	233T>G#	F78C#	n	7	56	13	2276G>T	C759F	y	33,40
5	2	238_239insCGTA	Q81fs	y	34	57	13	2282C>G	P761R	n	39
6	2	244C>T	R82W	y	35	58	13	2299delG	E767fs	y	41
7	2	377_378delGT	S126fs	n	7	59	13	2304C>A	C768X	y	7
8	3	486-1G>C	splice defect	n	7	60	13	2431_2432delAA	K811fs	n	7
9	3	488G>A	C163Y	n	33	61	13	2522C>A	S841Y*	y	35
10	3	545_446delAA	K182fs	y	36	62	13	2541C>A	C847X	n	37
11	3	588_589insTC	P197fs	y	7	63	13	2797C>T	Q933X	y	33
12	4	688G>A	V230M*	y	33	64	14	2878_2879delAA	N960fs	n	33
13	4	653T>A	V218E	y	37	65	14	2898delG	T967fs	y	41
14	4	775_776delAG	S259fs	y	36	66	14	2920_2921delGA	D974fs	n	7
15	4	779T>G	L260X	y	37	67	15	3004delT	C1002fs	y	7
16	6	852_853delGA	E284fs	n	36	68	16	3158-2A>C	splice defect	y	7
17	6	905G>T	C302F	n	7	69	17	3368A>G	Y1123C	y	7
18	6	921_922insCAGC	H308fs	y	33,37	70	17	3395G>A	G1132D	y	35
19	6	949C>A	R317R/splice defect	y	35	71	17	3558delT	C1186fs	y	7
20	6	956G>A	C319Y	y	37	72	17	3589delT	S1197fs	y	7
21	6	1000C>T	R334W	n	33,34	73	17	3746C>T	P1249L	y	7
22	6	1019_1023delATCCT	H340fs	n	7	74	18	3840_3841delinsCT	M1280_R1281 delinsIX	y	37
23	6	1036A>C	N346H	y	33	75	18	3883C>T	R1295X	n	33,37
24	6	1042A>C	N348H	n	37	76	18	3896_3897delGC	S1299fs	n	7
25	6	1055C>T	T352I	y	7	77	19	4106C>T	S1369L	y	7
26	6	1110_1111delTA	I371fs	y	7	78	19	4174G>T	G1392X	y	7
27	6	1135C>T	Q379X	y	7	79	19	4251G>T	Q1417H/splice def.	y	7
28	7	1144-2A>T	splice defect	y	7	80	20	4252-1G>A	splice defect	y	7
29	7	1172G>T	S391I	y	7	81	20	4338_4339delCT	C1447fs	y	41
30	7	1215_1216delTA	N405fs	n	7	82	20	4372G>T	G1458X	y	7
31	7	1227G>A	W409X	n	37	83	20	4381C>T	Q1461X	y	7
32	7	1256G>T	C419F	y	37	84	20	4393_4394insAA-AACTTTAGCAG	A1465fs	n	7
33	8	1416_1441del	N472fs	y	7	85	21	4405C>T	Q1469X	y	35
34	8	1434G>C	E478D*	y	36	86	21	4510_4511insA	R1504fs	n	37
35	8	1550G>C	R517T/splice defect	y	36	87	21	4544C>T	T1515M	n	34
36	9	1604G>C	R535T	n	7	88	23	4810G>C	D1604H	y	42
37	9	1606T>C	C536R	y	33	89	44	8723_8724delTG	V2908fs	y	42
38	10	1663C>G	L555V	n	38	90	50	9815C>T	P3272L	y	42
39	10	1679delC	P560fs	y	37	91	61	11864G>A	W3955X	y	42
40	10	1696C>T	Q566X	y	37	92	63	12343C>T	R4115C	y	42
41	10	1829A>C	H610P	y	39	93	63	13274C>T	T4425M	y	42
42	11	1841-2A>G	splice defect	n	39	94	66	14525C>A	S4842X	y	42
43	11	1859G>T	C620F	n	7	VLGR1					
44	11	1876C>T	R626X	y	33,37	1	31	6901C>T	Q2301X	n	43
45	11	1965delT	C655fs	n	33	2	36	8290T>C	S2764P	n	16
46	11	1966G>A	D656N	y	7	3	37	8495C>A	S2832X	n	16
47	12	1985G>T	C662F	n	7	4	38	8713_8717dupAACA	I2906fs	n	43
48	12	2023C>T	Q675X	y	33	5	39	8790delC	M2931fs	n	43
49	12	2052A>G	Q684Q*	y	37	6	89	18732_18750del	Y6244X	n	43
50	12	2100delG	T701fs	y	36						
51	12	2137G>C	G713R*	y	33						
52	12	2154delA	A719fs	y	7						

Legend to Supplementary Table 1

Usher microarray nucleotide variants. The nucleotide and amino acid notations are according to Antonarakis and den Dunnen (<http://www.hgvs.org/mutnomen/>) with the exception of the cDNA ('c.') and protein ('p') annotations. n = not tested; y = validated upon testing; t/n = not validated upon testing. *Presumed polymorphic variants. #Errors in sequence or mutation annotations of the original publications. \$Nucleotide numbering within the exons. These variants thus far were observed in non-syndromic deafness patients. All known mutations in the Usher genes were included, regardless of the phenotype.

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Supplementary Table 1: Usher microarray nucleotide variants (page 1 of 3)

#	Exon	Nucleotide change	Amino acid change	Validated	Ref.	#	Exon	Nucleotide change	Amino acid change	Validated	Ref.
CDH23						MYO7A					
1	3	172C>T	Q58X	y	1	1	3	47T>A	L16X	n	6
2	3	193delC	L65fs	y	1	2	3	52C>T	Q18X	y	7
3	4	336G>C	G112G/splice defect	y	2	3	3	73G>A	G25R	n	8
4	4	336+1G>A	splice defect	y	1	4	3	77C>A	A26E	n	9
5	5	371A>G	D124G	y	1	5	3	93C>A	C31X	y	10
6	10	1087delG	V363fs	y	1	6	4	124-2A>G	splice defect	n	11
7	10	1112delT	I371fs	y	1	7	4	199G>A	V67M	n	9
8	13	1355A>G	N452S	y	1	8	4	223delG	D75fs	t/n	10
9	13	1439T>A	L480Q	n	1	9	4	269G>C#	R90P#	n	9
10	14	1450G>C	A484P/splice defect	y	1	10	5	318C>A	N106K	n	7
11	15	1745G>A	R582Q	y	1	11	5	361delC	Q121fs	y	10
12	20	2289+1G>A	splice defect	y	1	12	5	401T>A	I134N	y	9
13	25	2968G>A	D990N	n	3	13	5	448C>T	R150X	y	12
14	25	3105A>C	T1035T/splice defect	y	1	14	5	470+1G>A	splice defect	n	13
15	26	3178C>T	R1060W	y	1	15	6	494C>T	T165M	y	14
16	29	3557G>A	G1186D	n	1	16	7	613A>G	I205V	n	13
17	30	3617C>G	P1206R	t/n	1	17	7	634C>T	R212C	y	10
18	30	3625A>G	T1209A	y	1	18	7	635G>A	R212H	y	10
19	31	3841_3843del	M1281del	n	4	19	7	640G>A	G214R	y	13
20	31	3842_3845dup	V1283fs	y	1	20	7	652_657delGACATC	D218_I219del	n	12
21	31	c.3880>T#	p.Q1294X#	n	3	21	7	700C>T	Q234X	y	12
22	31	4021G>A	D1341N	y	5	22	7	721C>A	R241S	n	6
23	35	4488G>C	Q1496H/splice defect	n	4	23	7	721C>T	R241C	n	9
24	36	4504C>T	R1502X	y	1	24	7	722G>A	R241H	n	7
25	37	4756G>C	A1586P	n	1	25	7	731G>C	R244P	n	11
26	37	4783G>A	E1595K	y	1	26	8	736-3C>T	splice defect	n	7
27	40	5237G>A	R1746Q	y	4	27	8	805_807delAAG	K269del	n	9
28	42	5536G>A	D1846N	n	1	28	9	905G>A	R302H	y	10
29	42	5712G>A	T1904T/splice defect	n	2	29	9	940G>T	E314X	y	10
30	45	6049+1G>A	splice defect	n	3	30	9	999T>G	Y333X	n	10
31	46	6050-9G>A	splice defect	y	1, 2	31	10	1066_1067insATCTCTGC	A356fs	y	7
32	46	6133G>A	D2045N	n	3	32	11	1132C>A	R378S	n	7
33	46	6155delC	T2055fs	y	1	33	11	1157T>C	L386P	n	10
34	47	6307G>T	E2103X	y	1	34	11	1190C>A	A397D	n	13
35	47	6319C>T	R2107X	n	3	35	12	1258A>T	K420X	y	7
36	47	6442G>A	D2148N	n	1, 5	36	12	1325A>G	E442G	y	7
37	47	6604G>A	D2202N	n	3	37	13	1348G>C	E450Q	n	10
38	49	6933delT	T2313fs	n	2	38	13	1370C>T	A457V	n	9
39	49	6968delC	P2323fs	y	1	39	13	1373A>T	N458I	y	15
40	51	7362+5G>A	splice defect	n	4	40	13	1403_1404insGCA	R467_H468insQ	y	10
41	52	7393C>T	R2465W	y	1	41	13	1508C>T	P503L	y	10
42	53	7549A>G	S2517G	y	1	42	14	1555-8C>G	splice defect	n	9
43	54	7823G>A	R2608H	y	1	43	14	1555-1G>C	splice defect	n	10
44	54	7872G>A	E2624E/splice defect	n	1	44	14	1556G>A	G519D	n	9
45	57	8230G>A	G2744S	n	1	45	14	1563delC	D521fs	n	8,11
46	58	8497C>G	R2833G	y	1	46	14	1595delA	H532fs	n	10
47	60	8849T>A	I2950N	n	3	47	14	1623_1624insC	K542fs	y	9
48	60	8866C>T	R2956C	n	3	48	15	1797G>A	M599I	n	16
49	62	9175C>A	P3059T	n	3	49	16	1798-14_1798-11 delGTCCinsCCAG	splice defect	n	10
50	66	9510+1G>A	splice defect	n	3						
51	67	9524G>A	R3175H	n	1	50	16	1798delG	G600fs	y	7
52	67	9626_9627insC	I3210fs	y	1	51	16	1884C>A	C628X	n	17

Supplementary Table 1: Usher microarray nucleotide variants (page 2 of 3)

#	Exon	Nucleotide change	Amino acid change	Validated	Ref.	#	Exon	Nucleotide change	Amino acid change	Validated	Ref.
MYO7A						MYO7A					
52	16	1900C>T	R634X	n	14	102	38	5227C>T	R1743W	y	9
53	16	1935G>A	M654I/splice defect	n	16	103	39	5392C>T	Q1798X	y	6
54	16	1935+4A>G	splice defect	n	10	104	39	5411delT	L1804fs	n	7
55	17	1945C>T	R649W	y	7	105	40	5573T>C	L1858P	n	9
56	17	1952T>C	L651P	n	18	106	40	5581C>T	R1861X	y	13
57	17	1969C>T	R657W	n	7	107	40	5618G>A	R1873Q	y	7
58	17	1996C>T	R666X	y	6	108	41	5637-3C>G	splice defect	y	7
59	17	2005C>T	R669X	y	14	109	41	5637-1G>A	splice defect	n	9
60	17	2028C>G	Y676X	n	7	110	41	5648G>A	R1883Q	y	7
61	17	2035_2036insT	E680fs	n	7	111	41	5660C>T	P1887L	n	9
62	18	2171delC	K725fs	n	7	112	43	5886_5888del	F1963del	y	7
63	18	2187+1G>A	splice defect	n	13	113	44	5945-1G>A	splice defect	n	7
64	20	2302A>T	K768X	y	7	114	44	5968C>T	Q1990X	n	7
65	20	2323C>T	Q775X	y	7	115	44	6025delG	A2009fs	y	9
66	21	2461C>T	Q821X	y	17	116	45	6193delC	Q2066fs	n	13
67	21	2476G>A	A826T	n	13	117	47	6410G>A	G2137E	n	7
68	22	2662_2670del	K888_K890del	n	19	118	48	6487G>A	G2163S	n	6
69	23	2766_2679del	K923fs	y	7	119	49	6560G>A	G2187D	n	9
70	23	2863G>A	G955S	n	8	PCDH15					
71	23	2878G>T	E960X	n	6	1	2	7C>T	R3X	n	21,22
72	23	2904G>T	E968D/splice defect	y	9	2	5	400C>G	R134G	n	23
73	25	3109-2A>G	splice defect	n	7	3	8	733C>T	R245X	y	24
74	25	3171C>G	Y1057X	n	7	4	8	785G>A	G262D	n	23
75	25	3238A>T	K1080X	y	20	5	10	1086delT	L362fs	n	22
76	25	3260T>C	L1087P	n	14	6	16	1927C>T	R643X	n	23
77	25	3265delG	A1089fs	y	14	7	28	3686-2A>G	splice defect	n	21
78	28	3508G>A	E1170K	y	20	USH1C					
79	28	3547C>A	P1183T	y	7	1	2	91C>T	R31X	y	25
80	28	3596_3597insT	C1201fs	n	11	2	3	238_239insC	R80fs	y	26,27
81	28	3630+2T>A	splice defect	n	8	3	5	496+1G>A	splice defect	n	25
82	29	3718C>T	R1240W	y	7	4	6	497-2delA	splice defect	y	27
83	29	3719G>A	R1240Q	y	6	5	B	\$32G>T	G431V	n	28
84	29	3750+2T>A	splice defect	n	7	6	D	\$293C>G	P608R	y	28
85	30	3862G>C	A1288P	y	6	7	D	\$329G>T	R620L	n	28
86	31	3979G>A	E1327K	n	17	8	D	\$376C>T	R636C	n	28
87	31	4018G>A	A1340T	y	7	USH1G					
88	31	4029G>C	R1343S	n	6	1	1	143T>C	L48P	n	29
89	31	4036_4038delTTC	F1346del	n	9	2	2	186_187delCA	D62fs	n	29
90	31	4039_4053del#	R1347_F1351del#	n	17	3	2	395_396insG	V132fs	y	29
91	31	4073delC	E1359fs	y	7	4	2	829_848del	S278fs	y	29
92	34	4501_4502delGT	V1501fs	y	7	USH3A					
93	34	4543_4551delGAG ATCATGinsGCA	E1515_M1517delinsA	n	7	1	1	144T>G	N48K	y	30,31
						2	1	149_152delCAGG- insTGTCCAAT	S50fs	y	30
94	35	4697C>T	T1566M	y	17	3	1	165delC	D55fs	n	30
95	35	4770_4771insT	R1591fs	y	7	3	1	189C>A	Y63X	y	31
96	35	4805G>A	R1602Q	n	18	4	1	187_209del	Y63fs	n	31
97	35	4816delA	K1606fs	n	7	5	1	359T>A	M120K	y	32
98	36	4882G>T	A1628S	n	6	6	2	449T>C	L150P	n	30
99	36	4918delG	G1640fs	n	7	7	3	459_461delATT	I153_L154delinsM	y	32
100	37	5156A>G	Y1719C*	y	20	8	3	528T>G	Y176X	y	32
101	38	5215C>T	R1739X	y	7	9	3				

Supplementary Table 1: Usher microarray nucleotide variants (page 3 of 3)

#	Exon	Nucleotide change	Amino acid change	Validated	Ref.	#	Exon	Nucleotide change	Amino acid change	Validated	Ref.
USH2A						USH2A					
1	2	94_130del	S33fs	y	7	53	12	2167+5G>A	splice defect	n	7
2	2	100C>T	R34X	n	33	54	13	2168-9T>G	splice defect	n	7
3	2	187C>T	R63X	y	33	55	13	2242C>T	Q748X	y	35
4	2	233T>G#	F78C#	n	7	56	13	2276G>T	C759F	y	33,40
5	2	238_239insCGTA	Q81fs	y	34	57	13	2282C>G	P761R	n	39
6	2	244C>T	R82W	y	35	58	13	2299delG	E767fs	y	41
7	2	377_378delGT	S126fs	n	7	59	13	2304C>A	C768X	y	7
8	3	486-1G>C	splice defect	n	7	60	13	2431_2432delAA	K811fs	n	7
9	3	488G>A	C163Y	n	33	61	13	2522C>A	S841Y*	y	35
10	3	545_446delAA	K182fs	y	36	62	13	2541C>A	C847X	n	37
11	3	588_589insTC	P197fs	y	7	63	13	2797C>T	Q933X	y	33
12	4	688G>A	V230M*	y	33	64	14	2878_2879delAA	N960fs	n	33
13	4	653T>A	V218E	y	37	65	14	2898delG	T967fs	y	41
14	4	775_776delAG	S259fs	y	36	66	14	2920_2921delGA	D974fs	n	7
15	4	779T>G	L260X	y	37	67	15	3004delT	C1002fs	y	7
16	6	852_853delGA	E284fs	n	36	68	16	3158-2A>C	splice defect	y	7
17	6	905G>T	C302F	n	7	69	17	3368A>G	Y1123C	y	7
18	6	921_922insCAGC	H308fs	y	33,37	70	17	3395G>A	G1132D	y	35
19	6	949C>A	R317R/splice defect	y	35	71	17	3558delT	C1186fs	y	7
20	6	956G>A	C319Y	y	37	72	17	3589delT	S1197fs	y	7
21	6	1000C>T	R334W	n	33,34	73	17	3746C>T	P1249L	y	7
22	6	1019_1023delATCCT	H340fs	n	7	74	18	3840_3841delinsCT	M1280_R1281 delinsIX	y	37
23	6	1036A>C	N346H	y	33						
24	6	1042A>C	N348H	n	37	75	18	3883C>T	R1295X	n	33,37
25	6	1055C>T	T352I	y	7	76	18	3896_3897delGC	S1299fs	n	7
26	6	1110_1111delTA	I371fs	y	7	77	19	4106C>T	S1369L	y	7
27	6	1135C>T	Q379X	y	7	78	19	4174G>T	G1392X	y	7
28	7	1144-2A>T	splice defect	y	7	79	19	4251G>T	Q1417H/splice def.	y	7
29	7	1172G>T	S391I	y	7	80	20	4252-1G>A	splice defect	y	7
30	7	1215_1216delTA	N405fs	n	7	81	20	4338_4339delCT	C1447fs	y	41
31	7	1227G>A	W409X	n	37	82	20	4372G>T	G1458X	y	7
32	7	1256G>T	C419F	y	37	83	20	4381C>T	Q1461X	y	7
33	8	1416_1441del	N472fs	y	7	84	20	4393_4394insAA-AACTTTAGCAG	A1465fs	n	7
34	8	1434G>C	E478D*	y	36						
35	8	1550G>C	R517T/splice defect	y	36	85	21	4405C>T	Q1469X	y	35
36	9	1604G>C	R535T	n	7	86	21	4510_4511insA	R1504fs	n	37
37	9	1606T>C	C536R	y	33	87	21	4544C>T	T1515M	n	34
38	10	1663C>G	L555V	n	38	88	23	4810G>C	D1604H	y	42
39	10	1679delC	P560fs	y	37	89	44	8723_8724delTG	V2908fs	y	42
40	10	1696C>T	Q566X	y	37	90	50	9815C>T	P3272L	y	42
41	10	1829A>C	H610P	y	39	91	61	11864G>A	W3955X	y	42
42	11	1841-2A>G	splice defect	n	39	92	63	12343C>T	R4115C	y	42
43	11	1859G>T	C620F	n	7	93	63	13274C>T	T4425M	y	42
44	11	1876C>T	R626X	y	33,37	94	66	14525C>A	S4842X	y	42
45	11	1965delT	C655fs	n	33	VLGR1					
46	11	1966G>A	D656N	y	7	1	31	6901C>T	Q2301X	n	43
47	12	1985G>T	C662F	n	7	2	36	8290T>C	S2764P	n	16
48	12	2023C>T	Q675X	y	33	3	37	8495C>A	S2832X	n	16
49	12	2052A>G	Q684Q*	y	37	4	38	8713_8717dupAACA	I2906fs	n	43
50	12	2100delG	T701fs	y	36	5	39	8790delC	M2931fs	n	43
51	12	2137G>C	G713R*	y	33	6	89	18732_18750del	Y6244X	n	43
52	12	2154delA	A719fs	y	7						

Legend to Supplementary Table 1

Usher microarray nucleotide variants. The nucleotide and amino acid notations are according to Antonarakis and den Dunnen (<http://www.hgvs.org/mutnomen/>) with the exception of the cDNA ('c.') and protein ('p') annotations. n = not tested; y = validated upon testing; t/n = not validated upon testing. *Presumed polymorphic variants. #Errors in sequence or mutation annotations of the original publications. \$Nucleotide numbering within the exons. These variants thus far were observed in non-syndromic deafness patients. All known mutations in the Usher genes were included, regardless of the phenotype.

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