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METHODS

Construction of a Multiplex Allele-Specific PCR-Based Universal Array (ASPUA) and Its Application to Hearing Loss Screening

Cai-Xia Li,[1] Qian Pan,[2] Yong-Gang Guo,[1] Yan Li,[3] Hua-Fang Gao,[3] Di Zhang,[3] Hao Hu,[2] Wan-Li Xing,[1,4,5] Keith Mitchelson,[1,3,5] Kun Xia,[2] Pu Dai,[6]* and Jing Cheng[1,4,5]*

[1]Medical Systems Biology Research Center, School of Medicine, Tsinghua University, Beijing, China; [2]National Laboratory of Medical Genetics of China, Central South University, Hunan, China; [3]CapitalBio Corporation, Beijing, China; [4]State Key Laboratory of Biomembrane and Membrane Biotechnology, Tsinghua University, Beijing, China; [5]National Engineering Research Center for Beijing Biochip Technology, Beijing, China; [6]Department of Otorhinolaryngology Head Neck Surgery, Chinese People's Liberation Army (PLA) General Hospital, Beijing, China

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We demonstrate a new method, using a universal array approach termed multiplex allele-specific PCR-based universal array (ASPUA), and applied it to the mutation detection of hereditary hearing loss. Mutations in many different genes may be the cause of hereditary hearing loss, a sensory defect disorder. Effective methods for genetic diagnosis are clearly needed to provide clinical management. Owing to the broad genetic basis of this condition, clinical assay of such a highly heterogeneous disorder is expensive and time consuming. In ASPUA, the allele discrimination reaction is carried out in solution by multiplex allele-specific PCR and a universal solid phase array with different tag probes is used to display the PCR result. The purpose of developing the ASPUA platform was to utilize the rapidity and simplicity of the amplification refractory mutation system (ARMS) with the detection power of microarray hybridization. This is the first report of the combination of these two technologies, which allow for the completion of allele-specific detection of 11 of the most frequent mutations causing hereditary hearing loss in under 5 hr. The ASPUA platform was validated by accurately analyzing 141 patient samples that had been previously genotyped for GJB2, GJB3, SLC26A4, and MTRNR1. In addition, we also developed a simplified assay by using streptavidin-coated magnetic beads instead of fluorescence for signal display that can be assessed through a conventional light microscope. We demonstrate that the ASPUA platform is rapid, cost-effective, and easily-used, and is especially appropriate for mutation detection in clinical genetic diagnostics. Hum Mutat 29(2), 306–314, 2008. **© 2007 Wiley-Liss, Inc.**

KEY WORDS: hereditary hearing loss; mutation detection; universal array; allele-specific PCR

INTRODUCTION

Congenital hearing loss affects one in 1,000 live births and approximately 50% of these cases are hereditary [American College of Medical Genetics (ACMG), 2002; Morton, 1991]. Sequence variants of many genes can contribute to hereditary hearing loss (Hereditary Hearing loss Homepage; http://webhost.ua.ac.be/hhh). Connexin 26 gene (GJB2; MIM♯ 121011) molecular defects account for up to 50% of recessive nonsyndromic deafness with c.35delG, c.167delT, and c.235delC being the predominant GJB2 mutations in the Caucasian, the Ashkenazi Jewish, and the Japanese populations, respectively [Abe et al., 2000; Estivill et al., 1998; Rabionet et al., 2000] (Connexins and Deafness Homepage, now maintained by the Deafness Research Group [CRG]; http://davinci.crg.es/deafness). Pendred syndrome (PDS) gene mutations in SLC26A4 (MIM♯ 605646) account for as much as 10% of hereditary deafness in diverse populations. The p.Leu236Pro (c.707T>C) and p.His723Arg (c.2168A>G) mutations account for 16% and 53% of all mutant alleles in Caucasoid and Japanese populations, respectively [Campbell et al., 2001; Friedman and Griffith, 2003; Tsukamoto et al., 2003]. In addition, persons with mtDNA MTRNR1 gene (MIM♯ 561000) mutation m.1555A>G are at risk of developing hearing loss if exposed to aminoglycoside antibiotics [Prezant et al., 1993]. Despite the large number of known rare mutations in many genes that may contribute to hearing loss, the patterns of high frequency

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*Correspondence to: Dr. Jing Cheng, Medical Systems Biology Research Center, School of Medicine, Tsinghua University, Beijing 100084, China. E-mail: jcheng@tsinghua.edu.cn and Pu Dai, Department of Otorhinolaryngology Head Neck Surgery, Chinese Peoples Liberation Army (PLA) General Hospital, 28 Fuxing Road, Beijing 100853, China. E-mail: daipu301@yahoo.com

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Cai-Xia Li and Qian Pan contributed equally to this work.

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mutations in populations and their population-wide distributions make it meaningful to initially select hotspot mutations for neonatal screening or genetic testing.

Various techniques are currently used to screen mutations, including restriction fragment length polymorphism (RFLP) [Hurd et al., 2002], amplification refractory mutation system (ARMS) [Newton et al., 1989], denaturing high-performance liquid chromatography (DHPLC) [Lin et al., 2001], sequence analysis [Wang et al., 2002], real-time amplification refractory mutation system quantitative PCR (ARMS-qPCR) assay [Bai and Wong, 2004], and pyrosequencing [Ferraris et al., 2002]. However, most of these methods are low-throughput and time-consuming for multiple gene–based detection. Direct sequencing is the "gold standard" for assay of sequence variations, but is expensive and labor intensive.

In the recent few years microarray technology has had many applications in the areas of disease diagnosis and drug discovery. For example, we have developed protein microarrays for small molecule screening and severe acute respiratory syndrome (SARS) patient sample analysis [Du et al., 2005; Kong et al., 2005], and also DNA microarrays for the early detection of SARS coronavirus as well as for the potential side effect study in developing siRNA-based hepatitis B virus (HBV) inhibiting drugs [Zhang et al., 2005; Guo et al., 2005]. DNA microarrays permit high-throughput mutation detection approaches, such as allele-specific primer extension on microarrays [Pastinen et al., 2000], PCR/ligase detection reaction (LDR) universal array [Gerry et al., 1999], microsphere-based single-base chain extension (SBCE) [Chen et al., 2000], sequence-tagged molecular inversion probes (MIP) [Hardenbol et al., 2003], and combinatorial sequencing-by-hybridization (cSBH) [Cowie et al., 2004]. For the examination of hereditary hearing loss, there are several reports addressing the use of DNA microarray for the high-throughput screening of related DNA mutations [Cremers et al., 2007; Gardner et al., 2006; Siemering et al., 2006]. Although arrayed primer extension array (APEX) has recently been used to detect 198 sequence variations in deafness genes [Gardner et al., 2006] it is relatively expensive. There is great need for still simpler, faster, and more affordable approaches for routine genetic diagnostic laboratories. The purpose of developing ASPUA was to utilize the rapidity and simplicity of ARMS with the detection power of microarray hybridization to provide the allele-specific detection in under 5 hr. Here we used ASPUA to simultaneously screen 11 mutations causing hereditary hearing loss (Supplementary Table S1; available online at http://www.interscience.wiley.com/jpages/1059-7794/suppmat). We also employed streptavidin-coated magnetic beads instead of fluorescence for labeling, to facilitate assay with the naked eye.

MATERIALS AND METHODS

Samples

Various previously genotyped patient DNA samples were provided by the Chinese PLA General Hospital; the National Laboratory of Medical Genetics of China; Thomas Jefferson University; Stanford University School of Medicine, and Charles University, Prague. Genomic DNA was isolated from whole blood and plasmid DNA was extracted from *E. coli* strains with the Wizard® Genomic DNA Purification Kit and Wizard® Plus SV Minipreps DNA Purification System (Promega, Madison, WI), respectively. The extraction of genomic DNA from whole blood takes less than 1 hr. The QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) was used to introduce specific mutations into cloned genes at the 11 loci. Each gene insert was sequenced to verify that selected clones contained the desired mutations. The GenomiPhi V2 DNA Amplification Kit (GE Healthcare, Piscataway, NJ) was used for whole genome amplification of the genomic DNA (10 ng and 1 ng).The whole genome amplification product was purified by the NucleoSpin® Extract II kit (Macherey-Nagel, Düren, Germany).

Oligonucleotides and Universal Array

All the primers and probes were synthesized and purified by Invitrogen (Invitrogen, Shanghai, China). The common primer and the universal primer were fluorescently labeled. Tags were designed and modified according to Chen et al. [2000]. The 5′-amino-tag probes with 15-nt poly T-linker adjacent to the 5′-end were covalently attached to aldehyde-modified glass slides (CapitalBio, Beijing, China) to capture the allele-specific PCR products. Each probe was resuspended at a concentration of 15 μM in 50% dimethyl sulfoxide (DMSO) and printed as five replica spots by SmartArrayer™-48 Microarray Spotter (CapitalBio). The spot diameter was approximately 150 μm at 300 μm center-to-center spacing.

Multiplex Allele-Specific PCR

Multiplex allele-specific PCR was carried out in two tubes to avoid interactions between some primer pairs. Four loci, c.35delG, c.547G>A, c.2168A>G, and c.919–2A>G (IVS7–2A>G) were amplified simultaneously in one tube, and the remaining seven loci were amplified in another tube. Reaction volumes were 15 μl, and contained 0.2 mM dNTPs, 2 mM $MgCl_2$, 0.8 unit of HotStarTaq DNA Polymerase (Qiagen, Hilden, Germany), and 50 ng of genomic DNA or 5 pg of plasmid DNA. Optimal concentrations were determined separately for each primer. Asymmetric PCR was used to obtain sufficient single-strand DNA for hybridization. The amplification was performed in a PTC-225 Thermal Cycler (MJ Research, Watertown, MA). PCR conditions were as follows: 95°C for 15 min; then: 94°C for 30 sec, ramp 68 sec to 55°C, hold for 30 sec; ramp 50 sec to 70°C, hold for 45 sec for 10 cycles; then: 90°C for 30 sec, ramp 60 sec to 55°C, hold for 30 sec, ramp 50 sec to 70°C, hold for 45 sec for 20 cycles; followed by 60°C for 10 min, and 4°C soak. Amplifications were thus completed within 2.4 hr. A negative PCR control (no template) was included for each run. No additional positive PCR control was needed because primers for both alleles are present in each reaction, and even for homozygous templates the product of at least one allele should always appear.

Universal Array Hybridization

The products of both amplification reactions were combined. An aliquot of the mixture (5 μl) was suspended in 10 μl hybridization buffer (6 × SSC, 5 × Denhardt's reagent, 25% (v/v) formamide, 0.1% (w/v) SDS, 5 nM c-PC). After heating for 2 min at 98°C and chilling on ice, the hybridization mixture was added to the subarray. The slide was incubated at 50°C for 1 hr and washed twice (2 min each) at 42°C in 0.3 × SSC/0.1% SDS and in 0.06 × SSC. Finally, the slide was centrifuged to dry.

Data Analysis

The dried slide was imaged with a ScanArray® Express Microarray Scanner (PerkinElmer Life Sciences, Boston, MA). Laser power and photomultiplier tube (PMT) power were 90% and 70%, respectively. The signal intensities of the spots were quantified by GenePix Pro 4.0 (Axon Instruments, Foster City,

CA). The absolute median signal intensity (AMSI) of each spot was calculated by subtracting the local background from the median signal intensity of the spot, and a minimum value of 1,000 was used as the cutoff. To exclude false-positive signals generated by primer dimers, AMSI for one allele was required to be at least 10 times the negative PCR control AMSI for that allele. If these criteria were met, the signal of one allele was considered to be positive.

Magnetic Bead–Labeled ASPUA

The 5′-fluorescent dyes of the relevant oligonucleotides were replaced by biotin. The PCR, hybridization, and washing steps were conducted as described above. Streptavidin-coated MyOne^{TM} Dynalbeads (Dynal Invitrogen, Oslo, Norway) were pretreated according the protocol from the supplier, and then 20% goat blood serum in 1 × binding & washing buffer (5 mM Tris-HCl (pH7.5), 500 nM EDTA, 1 M NaCl) was added to reduce nonspecific background adhesion. The solution was added into the array block. After incubation for 10 min for streptavidin-biotin binding, the array was washed twice (2 min each) in 2 × SSC/0.1% SDS and in 0.2 × SSC. Finally, the image was viewed with Leica DMRE microscope (Leica Microsystems, Wetzlar, Germany) and captured by Canon EOS-1Ds Mark II digital camera EF100 mm f/2.8 Macro USM (Canon, Tokyo, Japan).

RESULTS

An outline of multiplex ASPUA is given in Figure 1. Multiplex allele-specific PCR is carried out using HotStarTaq DNA Polymerase, and then the PCR products are used to hybridize with capture probes on a universal array. Results are determined by the signal intensity and by the position of the tag probe bound on the array. For each locus, two tagged allele-specific primers that differ at their 3′-terminal base define the allele. The HotStarTaq DNA Polymerase used lacks a 3′ to 5′ exonuclease activity, thus if a PCR primer has a mismatch with the template at its 3′-terminal base, the efficiency of amplification will be greatly reduced. The common primer is tailed, and a universal primer that has the same sequence as the tail is also used to increase the amount of the single-strand DNA [Zhang et al., 2005]. In the ASPUA assay, the universal array serves as a decoding tool to display the results of the previous amplification. We first validated the universal array to ensure the specificity of the hybridization results.

Specificity of ASPUA

Figure 2A shows the array format, in which QC and BC were the positive and negative controls of spotting efficiency, and PC and NC were oligonucleotides that served as positive and negative controls of hybridization, respectively. PC was complementary to a TAMRA-labeled oligonucleotide in the hybridization mixture, while NC was noncomplementary to any sequence in the mixture. The rest of the spots on the subarray were tag probes that captured the ASPUA PCR products.

To find out if the allele-specific primers can only amplify their intended target allele and if different alleles of these mutations can be discriminated specifically, a homozygous DNA sample was amplified in the presence of mixtures of all primers. Only the intended target alleles were found to be amplified. For those mutations for which we could not obtain homozygous patient samples, plasmid DNA clones of single alleles were used instead. Genomic DNA samples were used as source templates for the mutations (c.35delG, c.167delT, c.176_191del16, c.235delC, c.299_300delAT, m.1555A>G, and c.919-2A>G) and for the wild-type, and for each type of sample all 11 loci should be detected. The remaining tests were on cloned single-allele templates in which only homozygous signals are detected. The p.Arg180X and p.Glu183Lys mutations occur closely within GJB3 gene (MIM# 603324), and were constructed in a nonoverlapping manner in one plasmid. Other mutations resided on their own plasmid individually. As shown in Figure 2B, only the tags related to the correct primers for the template showed a significant signal. Specific signals appeared for each mutant gene template. Table 1

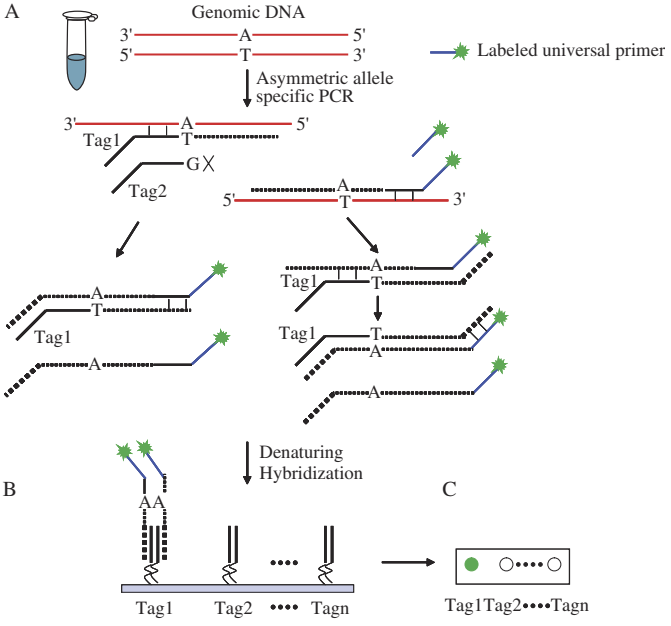

FIGURE 1. **The principles of ASPUA. A: Multiplex AS-PCR was conducted in two tubes (2.5 hr). The amplification was asymmetric and generated sufficient single-stranded labeled DNA for hybridization and detection. B: After denaturing, the PCR products were hybridized with the universal array (1 hr). C: The washed array was read by an array scanner. The image was analyzed to obtain the genotyping results of multiple loci (0.5 hr).**

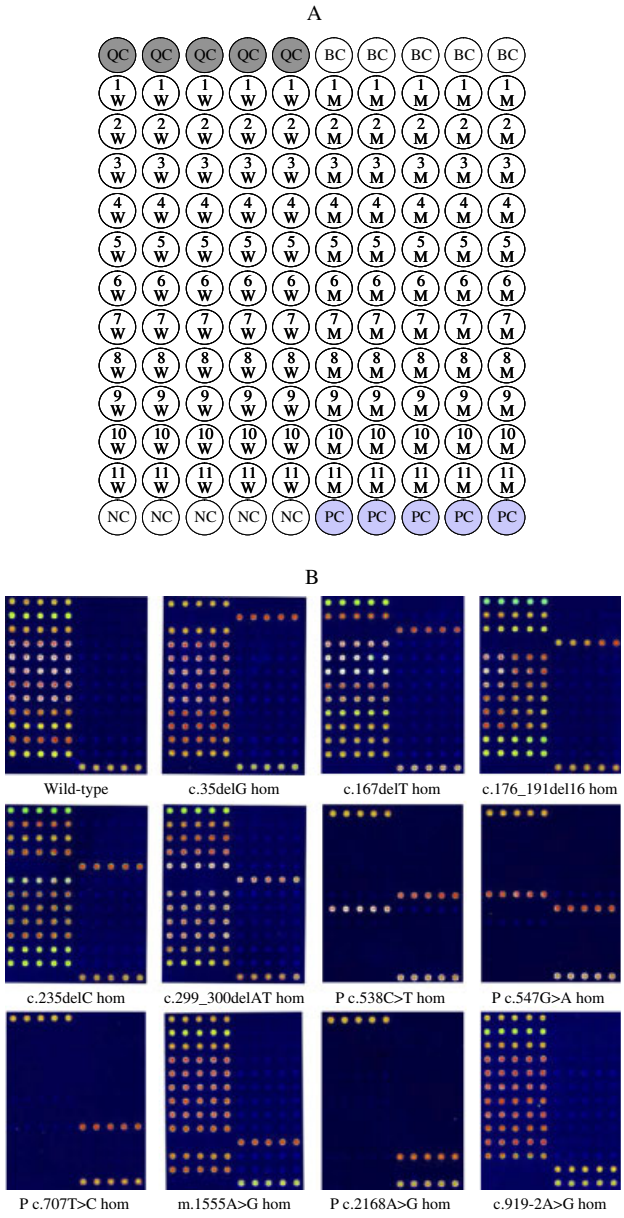

FIGURE 2. **The specificity of ASPUA. A: Each probe was printed as five replicates. QC and BC were positive and negative controls of spotting, and PC and NC were positive and negative controls of hybridization, respectively. All other spots were specific capture probes for each of the different gene alleles. Arabic numerals represent the name of the 11 mutations, which are (in order): c.35delG, c.167delT, c.176_191del16, c.235delC, c.299_300delAT, c.538C>T (p.Arg180X), c.547G>A (p.Glu183Lys), c.707T>C (p.Leu236 Pro), m.1555A>G, c.2168A>G (p.His723Arg), and c.919-2A>G. W and M indicate wild-type and mutant, respectively. B: The image of the hybridization results. P represents a cloned gene template, hom represents homozygous (hom represents homoplasmic for the m.1555A>G mutation).**

shows the AMSI of the specificity study and the standard deviation (SD) data of five repeated experiments. Each of the positive signals in all five repeated experiments meets the two criteria listed in Materials and Methods.

The introduction of artificial mismatches near the 3′-end of the allele-specific primers (AS primers) could enhance the selective hybridization specificity [Lo et al., 1991; Newton et al., 1989]. Reducing the concentration of the AS primer can also increase the discrimination power of this method; however, the efficiency of the two measures varied over a range of PCR conditions. To study these effects we have chosen the wild-type primer of m.1555A>G mutation as the assay target as it has a T residue at the 3′-end and is often poorly discriminated (data not shown). Artificial mismatch was introduced to the AS primer at positions three, five, and nine bases from the 3′-end (called m.1555A>G-W3, -W5, and -W9), respectively. The discrimination ratio (DR) of the natural wild-type primer is low because of the T-G mismatch between the wild-type primer and the mutant target. Decreasing the primer concentration can increase the specificity, but this measure also greatly reduces the amplification efficiency. In contrast, the presence of artificial mismatches near the 3′-end of primers can enhance the discrimination ratio with little deleterious influence on the amplification efficiency. As the artificial mismatch is positioned further from the 3′-end the specificity is seen to decrease. The m.1555A>G-W3 primer maintains high discrimination specificity during signal amplification in the presence of increasing amounts of mutant template, or increasing concentrations of magnesium. In conclusion, the

TABLE 1. The AMSI and the Standard Deviation Data of the Specificity Study*

	Mutation[a]										
Template[b]	1	2	3	4	5	6	7	8	9	10	11
gDNA wild-type											
$AMSI_{WT}$	15905	21724	44585	50224	49514	41497	50181	28473	26537	28466	22313
SD	1323	1291	4513	6428	4926	4825	4056	2473	5471	2596	1463
$AMSI_{MU}$	38	32	70	118	80	108	134	70	43	175	131
SD	11	7	4	16	7	10	18	12	10	9	8
gDNA c.35delG hom											
$AMSI_{WT}$	2	20783	44946	52240	49289	43618	53493	28473	26302	31504	23583
SD	2	1748	4801	7151	6438	6078	7225	2574	5402	3871	3412
$AMSI_{MU}$	34363	27	68	123	87	117	157	73	52	181	136
SD	3165	6	7	15	9	13	20	10	10	10	13
gDNA c.167delT hom											
$AMSI_{WT}$	23830	17	35861	46415	52693	27244	31984	14858	21391	18166	18058
SD	3657	4	7176	9742	7161	4098	3664	4293	5779	3996	4399
$AMSI_{MU}$	66	25029	84	111	84	103	89	47	42	109	135
SD	8	4976	21	33	23	30	12	8	11	27	32
gDNA c.176_191del16 hom											
$AMSI_{WT}$	20787	20922	19	41505	52136	39071	29075	27523	29454	15402	15744
SD	7242	4808	15	8182	7753	7869	4294	5367	7257	5381	4476
$AMSI_{MU}$	50	36	28232	98	90	118	85	83	54	110	122
SD	9	13	6692	37	28	32	23	19	17	14	35
gDNA c.235delC hom											
$AMSI_{WT}$	25066	23478	33339	35	52827	38022	22671	23893	27186	11067	11994
SD	8505	8401	12593	16	11480	7844	3895	4631	8121	3567	2419
$AMSI_{MU}$	64	35	66	36362	92	136	84	80	52	90	103
SD	6	5	14	7835	25	22	27	20	19	24	12
gDNA c.299_300delAT hom											
$AMSI_{WT}$	26315	31857	44561	57688	23	50029	32056	34662	44097	19794	16732
SD	1896	3768	7992	6247	4	7758	3685	4452	5789	2444	3396
$AMSI_{MU}$	75	153	118	204	51331	336	141	136	89	172	203
SD	13	28	14	30	8388	29	6	27	13	46	36
P c.538C>T hom											
$AMSI_{WT}$	10	12	11	24	13	82	45711	39	8	5	4
SD	3	4	4	6	5	45	5812	8	5	2	2
$AMSI_{MU}$	12	14	12	16	18	33294	169	20	8	9	4
SD	3	5	5	5	10	6324	27	4	3	3	2
P c.547G>A hom											
$AMSI_{WT}$	37	46	43	49	41	50127	35	228	20	22	19
SD	71	83	81	91	79	11446	53	223	36	40	34
$AMSI_{MU}$	42	47	44	44	34	173	41516	53	19	18	14
SD	81	89	88	83	66	36	12671	83	31	27	25
P c.707T>C hom											
$AMSI_{WT}$	3	3	4	13	16	111	13	199	3	6	1
SD	4	4	3	11	15	40	11	245	2	10	0
$AMSI_{MU}$	5	4	4	4	3	3	3	40564	3	2	2
SD	5	4	4	4	3	3	3	5462	3	2	2
gDNA m.1555A>G hom											
$AMSI_{WT}$	13090	29393	37165	36649	44896	37375	28068	32263	11	16276	16984
SD	1295	2882	4885	3692	6303	7349	3519	5244	3	7268	9512
$AMSI_{MU}$	97	55	73	106	109	106	88	84	27517	91	162
SD	132	17	16	20	21	16	21	16	5901	54	47
P c.2168A>G hom											
$AMSI_{WT}$	12	8	9	21	11	22	7	12	11	65	5
SD	4	3	3	13	6	15	4	6	4	22	2
$AMSI_{MU}$	9	9	8	8	6	7	5	4	5	29554	5
SD	5	4	3	3	2	2	3	3	2	7923	3
gDNA c.919–2A>G hom											
$AMSI_{WT}$	12203	25979	38365	37275	43530	34834	41694	28927	40962	26381	18
SD	1827	4658	2686	3631	5256	7254	6088	3120	2173	3070	7
$AMSI_{MU}$	38	35	59	106	82	85	118	70	45	129	28946
SD	16	11	7	31	12	12	21	16	9	18	6718

*Standard deviation data (SD) was calculated from five repeated experiments.
[a]Arabic numerals represent the 11 mutations which are, in order: c.35delG, c.167delT, c.176_191del16, c.235delC, and c.299_300delAT (GJB2, NM_004004.4); c.538C>T (p.Arg180X), and c.547G>A (p.Glu183Lys) (GJB3; NM_024009.2); c.707T>C (p.Leu236 Pro) (SLC26A4, NM_000441.1); m.1555A>G (MTRNR1, NC_001807.4); c.2168A>G (p.His723Arg) and c.919–2A>G (SLC26A4, NM_000441.1). Nucleotide +1 in the cDNA reference sequence is the A of the ATG translation initiation codon.
[b]P represents the mutant plasmid template, hom represents homozygous (hom represents homoplasmic for the m.1555A>G mutation).

m.1555A>G-W3 primer appears to perform well over a wide range of PCR conditions.

Patient Study

We tested 141 normal and patient DNA samples that had been previously genotyped. The genotypes detected by ASPUA and the clinical information of these samples are summarized in Supplementary Table S2. Among the 141 individuals examined, 122 samples were from deaf patients and nine were from carriers. One additional patient was found to be homoplasmic for the m.1555A>G mutation but displayed no clinical manifestations, and no mutations were detected in the nine remaining control samples from normal individuals. For each mutation, the AMSI value of the two possible alleles was plotted on each axis. An individual data point falls into one of the three clusters and is assigned the genotype of AA, AB, or BB. All of the expected 1,551 calls were generated for the 141 samples.

To evaluate the minimum amount of sample required for ASPUA, we assayed various amounts of a wild-type gDNA (Supplementary Fig. S1). The AMSI of the sensitivity study and the SD data of five repeated experiments is shown in Table 2. Attenuation of AMSI was observed with decreased amount of target. AMSI values higher than 1,000 were observed at all loci at the 25-ng genomic template, but at 10 ng signals of four of the loci fell below 1,000. We explored using multiple displacement amplification (MDA) to expand the detection sensitivity of ASPUA. Supplementary Figure S1 shows that both 10 ng and 1 ng of starting gDNA could be detected by ASPUA after amplification by MDA, whereas a MDA control reaction with no template was blank and the no target control MDA product showed no significant signals.

Visible Light-Assisted Detection of ASPUA

To achieve visible light-assisted detection, streptavidin-coated MyOne^{TM} Dynalbeads were used to display the hybridization results of the 11 mutations (Fig. 3). The streptavidin-coated beads are 1.05 μm in diameter. They can bind covalently to the biotin-labeled PCR product on the microarray. Those beads that are not specifically bound will be removed by conventional washing. The beads remaining bound on the array can be viewed with an optical microscope or by naked eye. Previously genotyped samples were used for this study. The array format is the same as that shown in Figure 2A. From Figure 3, it is clear that all the samples were detected specifically. Three repeated experiments were conducted to confirm the accuracy of the detection results.

DISCUSSION

ASPCR, also known as ARMS [Newton et al., 1989], is an established method for genotyping mutations. In ARMS, the PCR product is separated by gel electrophoresis. The protocol usually detects one allele per reaction and is laborious when testing many mutations in different samples. To overcome this problem, Roberts et al. [2000] developed multiplex ARMS, in which two separate PCR reactions (ARMS1 and ARMS2) containing a mixture of wild-type or mutation specific primers are performed for each sample. The genotyping was determined by the size of PCR products on the gel image. Gómez-Llorente et al. [2004] combined allele-specific PCR with capillary electrophoresis. This strategy needs different fluorescent dyes and specialized separation equipment, which increases the cost and limits the flexibility of the assay.

Here, we have combined a multiplex allele-specific PCR with a universal array (ASPUA) for rapid and cost effective simultaneous genotyping of 11 mutations causing hereditary hearing loss. The mutations, ranging in size from single-base changes to large 16-bp deletions could be accurately identified. Homozygous DNA samples were used to assess the specificity of the entire assay. The specificity of both the enzymatic reaction and the universal array hybridization conferred the high discrimination power to the entire procedure. Even mutations that are adjacent, such as the

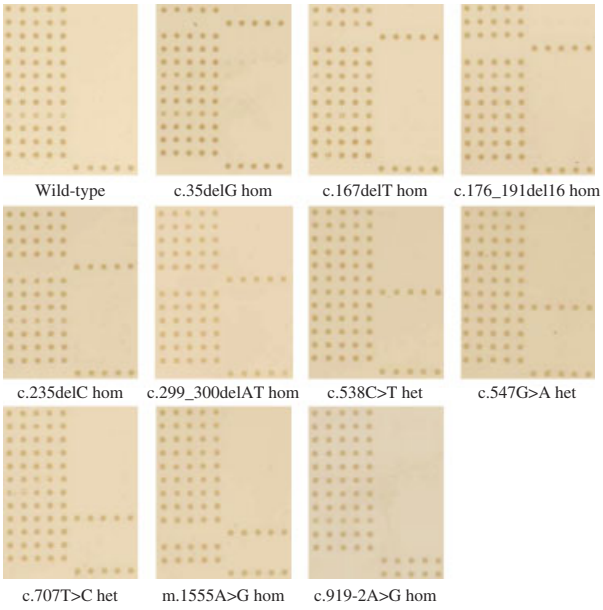

FIGURE 3. **Visible light-assisted detection. The array format is the same as Figure 2A. The magnetic bead-labeled results were captured by Canon EOS-1Ds Mark II digital camera EF100 mm f/2.8 Macro USM. Het stands for heterozygous and hom stands for homozygous (hom represents homoplasmic for the m.1555A>G mutation). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]**

TABLE 2. **The AMSI and the Standard Deviation Data of the Sensitivity Study***

	Mutation[a]										
Template	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
50 ng											
$AMSI_{WT}$	**28626**	**24905**	**38508**	**48381**	**59666**	**40154**	**29625**	**22690**	**34214**	**27437**	**17948**
SD	**5501**	**2306**	**3868**	**7410**	**9138**	**10822**	**5848**	**2837**	**5340**	**4025**	**4231**
25 ng											
$AMSI_{WT}$	**23167**	**16637**	**30988**	**41777**	**50262**	**21552**	**23262**	**8022**	**21352**	**19108**	**3887**
SD	**7802**	**4543**	**4730**	**7514**	**10345**	**7192**	**3678**	**1562**	**2469**	**3028**	**1045**
10 ng											
$AMSI_{WT}$	**16857**	**3485**	**17730**	**30941**	**18393**	**1107**	**4312**	**232**	**5576**	**948**	**157**
SD	**5595**	**602**	**3106**	**4388**	**9217**	**660**	**2698**	**33**	**2731**	**180**	**65**
10 ng-MDA											
$AMSI_{WT}$	**35016**	**10346**	**36858**	**50168**	**59969**	**63678**	**57589**	**23701**	**11417**	**33040**	**22586**
SD	**2299**	**3921**	**6833**	**7614**	**6535**	**6129**	**8835**	**6288**	**6591**	**5430**	**2603**
1 ng-MDA											
$AMSI_{WT}$	**23633**	**8450**	**21934**	**44651**	**56918**	**50928**	**40508**	**17761**	**6860**	**17108**	**6423**
SD	**7713**	**4036**	**8721**	**9627**	**10126**	**15257**	**14510**	**6454**	**1911**	**4943**	**1514**
No target-MDA											
$AMSI_{WT}$	**7**	**6**	**28**	**493**	**5**	**76**	**5**	**5**	**4**	**4**	**3**
SD	**12**	**6**	**20**	**212**	**5**	**12**	**5**	**5**	**5**	**4**	**4**

*Standard deviation data (SD) was calculated from five repeated experiments.
[a]Arabic numerals represents the name of the 11 mutations, which are c.35delG, c.167delT, c.176_191del16, c.235delC, c.299_300delAT, c.538C>T (p.Arg180X), c.547G>A (p.Glu183Lys), c.707T>C (p.Leu236 Pro), m.1555A>G, c.2168A>G (p.His723Arg), and c.919–2A>G.

two loci nine bases apart in the GJB3 gene [Xia et al., 1998], can be accurately detected.

During optimization of the assay we observed that the use of HotStarTaq DNA Polymerase was crucial to performance of multiplex ASPUA, as it can prevent the formation of primer dimers to a large extent. Primer dimers both reduce the efficiency of amplification of some alleles, and cause some false-positive signals to be generated and detected at the PCR negative control. A minimum 25 ng of gDNA is enough for efficient ASPUA detection in the 15-μl reaction volume. If the amount of patient sample is limiting, MDA can be used to increase template abundance before ASPUA allowing as little as 1 ng of starting gDNA to be successfully genotyped. Artificial mismatches can also be introduced close to the 3′-end to enhance the specificity of AS primers by lowering the stability of the primer/template complex, preventing the mismatched primer from extending efficiently. Artificial mismatches can improve the specificity greatly and discriminate efficiently over a wide range of PCR conditions.

In the patient study, we tested 141 samples to validate the accuracy of ASPUA, of which 18 of the samples were blind test samples. The results of 141 samples were fully concordant with the initial genotype information of the supplier laboratories. Signal intensity variation was found among different samples. Because the patient DNA samples were supplied by several laboratories, the DNA quality varied to some degree. But this difference did not influence the assay outcomes and all the positive signals meet the assay criteria. One sample was found to carry the m.1555A>G mutation but the patient showed no clinical manifestations. For such a patient, aminoglycoside antibiotics should be avoided to prevent induced development of deafness. During preparation of this manuscript, we applied ASPUA to a further 515 patient samples and the results were fully concordant with direct sequencing.

We have validated the ASPUA platform with only 11 mutations, many less than reported using APEX methods [Gardner et al., 2006; Cremers et al., 2007]. The low number of abundant mutations detected by the current ASPUA microarray platform was intentional, as it allowed for sufficient numbers of samples of each genotype to be collected for regulatory-requested, statistically-verifiable data. In the future, we need to analyze more samples to ensure the accuracy of the ASPUA platform over a wider diversity of quality of patient samples, and over a wider range of different genotype combinations. Most of the mutations in this work are frequently found in Chinese hearing loss patients. For example, our preliminary study on 1,190 Chinese nonsyndromic hearing impairment (NSHI) patients found that the c.235delC mutation accounts for 88.8% (222/250) of the patients who carry the GJB2 mutation and the c.299_300delAT and c.176_191del16 mutation was found in 24.8% (62/250) and 7.6% (19/250) of these patients, respectively, as some of the patients carry compound mutations. Dai et al. [2006] studied 38 Chinese patients with enlarged vestibular aqueduct (EVA) and found that 91.4% of the patients carry at least one mutation in the SLC26A4 gene, and patients who carry the c.919–2A>G (IVS7–2A>G) and p.His723Arg mutation accounted for 71.9% and 25%, respectively. Liu et al. [2006] reported 3.43% (63/1,836) of the Chinese NSHI patients carry the m.1555A>G mutation. The Connexin 31 gene (GJB3) was first cloned in China, and the p.Arg180X and p.Glu183Lys mutations were associated with hearing loss [Xia et al., 1998]. Three mutations that are prevalent in Caucasoid or Ashkenazi Jewish patients were also chosen to expand the detection spectrum of our methods. For example, the c.35delG mutation accounted for 85% of GJB2 mutations in families with recessive nonsyndromic deafness from Spain and Italy [Estivill et al., 1998] and the c.167delT mutation accounted for 40% of the mutant alleles in the Ashkenazi Jewish population [Sobe et al., 2000]. The p.Leu236Pro mutation accounts for 16% of all SLC26A4-disease causing alleles in the Caucasoid population [Campbell et al., 2001].

We intend to develop a second generation of hereditary deafness microarray by increasing the range of mutations examined by the ASPUA assay. We have shown that separation of amplification reactions into different tubes with compatible combinations of primers and HotStar DNA polymerase permits efficient amplification, even with a range of DNA qualities and quantities. Increasing the number of reaction tubes to provide for additional alleles and gene loci is the simplest approach to increase gene testing, and the addition of a few new gene loci to each tube could also be readily tested, and if efficiency remains high, adopted. We have also demonstrated that alleles with low hybridization efficiency can be markedly improved by inclusion of deliberate mismatches near to the 3′-discriminatory nucleotide—a measure that could be tested with any newly added suboptimal alleles.

The ASPUA platform is simple to use, and although it is currently low-throughput, it can readily be expanded. Other deafness analysis platforms have different design formats. The allele-specific oligonucleotide biochip of Siemering et al. [2006] involves seven steps from PCR to interpretation of results and takes more than 1 day to perform. The APEX method [Gardner et al., 2006] involves six steps and is completed in 6 hr, yet it employs multichannel scanners and four-color fluorescence labeling, which adds considerable expense to the setup and running costs. In contrast, current ASPUA analysis of four samples on a four-subarray chip can be completed in 5 hr with only four steps: DNA extraction (1 hr), PCR (2.5 hr), hybridization (1 hr), scanning and automatic characterization of results (0.5 hr), avoiding the need for amplicon purification and several other steps required by other platforms. The genetic heterogeneity of deafness makes it necessary to undertake multigene detection. Yet, with the current method one could readily increase either the number of genes or the number of mutations to about 50 with almost no increase in the time for analysis. The number of samples analyzed could also be increased with some small time cost. If say 48 samples were analyzed (requiring 12 four-subarray chips) the total analysis time, including scanning and interpretation, would increase by about a further 40 minutes due to sample handling.

Although DNA sequencing may generate fully analyzed genotype data as rapidly as ASPUA, the cost of capital equipment required for sequencing may be several times that of our technology. However, conventional methods such as RFLP or dot blot allele specific oligonucleotide (ASO) both become labor-intensive when the number of mutations or samples is increased. In ASPUA, the PCR products can be used to hybridize directly without the need of post-PCR purification or dye-labeled ddNTP extension, which saves some costs. The flexibility of the universal array also contributes to the cost reduction because the same universal array format can be developed for use for completely different gene mutation assays. It is desirable to avoid the use of expensive instruments and to limit costs significantly for general clinical diagnostic settings and to provide a low cost method for laboratories in less developed regions of China. To this end we have employed magnetic bead–labeling to achieve visible light-assisted detection. The hybridization results can then be visualized through a light microscope or by naked eye. Several groups have also addressed the development of simple and specific methods with the minimal instrumentation requirements. Taton

et al. [2000] developed a sandwich hybridization DNA array using oligonucleotide-modified gold nanoparticle probes coupled with signal amplification by use of silver deposition. The hybridization results could be visualized by a flatbed scanner. However, compared to the sandwich hybridization assay, ASPUA is more flexible and simple.

CONCLUSIONS

In summary, we have combined allele-specific PCR and universal array methodologies for the detection of mutations causing hereditary hearing loss. The specificity and simplicity of the ASPCR reaction provides high genotyping accuracy. ASPUA offers several distinct advantages for routine diagnostic laboratories. Here we have employed it for multiplex detection of 11 mutations causing hereditary hearing loss. The ASPUA universal array makes the multiplex detection highly flexible, and the numbers of gene loci detected can be increased if warranted. The microarray could also be redesigned with a greater number of subarrays so that much larger numbers of patient samples could be tested simultaneously. Finally, the demonstrated feasibility of visible light-assisted detection avoids the need for special detection equipment. All of these features should make it easily acceptable in clinical genetics laboratories.

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