Short Communication

Genotype Analysis Using Human Hair Shaft

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Abstract

Analysis of genotypes for genetic predisposition of diseases has profound and widespread clinical application and has become a fundamental tool in the study of molecular epidemiology. In this study, we report a convenient and completely noninvasive approach to perform genotyping using terminal cephalic hair shaft. The average and median DNA amounts purified from the hair shaft are 112 ± 180 (1 SD) pg/mg and 66 pg/mg of hair shaft, respectively. Using hybridization with molecular beacons that can reliably distinguish single nucleotide polymorphisms at different alleles, we are able to analyze the genotypes of *APO-E* and *G-protein β3* (*GNB3*) subunit *C825T* loci in DNA samples obtained from hair shaft in a high throughput format. The amount of DNA obtained from 20 mg of hair shaft is sufficient for multiple independent genotyping assays. These findings demonstrate that terminal hair shaft provides a convenient and reliable genomic DNA source for an analysis of genotypes.

Introduction

Identification of genes causing or predisposing to a host of human diseases will revolutionize the practice of medicine and public health in the postgenomic era. Study of the association between genotype and disease risk will also shed light on the genetic and molecular basis of disease. Although lymphocytes and buccal swab have been routinely used as the sources of genomic DNA for genotype analysis (1, 2), other highly compliant, completely noninvasive and convenient systems for genotyping are of great interest, especially for a population-based genetic screening (2, 3). Human hair has long been known to contain genomic DNA (4) and represents one of the potential DNA sources for noninvasive DNA sampling. Several studies have examined such a possibility using dot-blot hybridization (4, 5), microsatellite markers (6), and PCR-RFLP (7). On the basis of these studies, the analysis of hair genomic DNA is fraught with technical difficulties. The hair DNA, especially when obtained from the terminals, is very limited in quantity, and, accordingly, the genotyping results may not be reproducible, thus limiting its wide clinical application. The enrichment of DNA purified from the root portion of hair including hair follicles is, therefore, mandatory because the root region and hair follicles contain more abundant genomic DNA for multiple assays (4–6). But plucking the hair may not be desirable for many individuals. To overcome these problems, we sought to develop a system to improve genotype analysis using terminal hair samples in a high throughput format. *APO-E* and *GNB3 C825T* loci are selected as the examples in this study because of their well-known association with human diseases. Certain *APO-E* genotypes correlate with the risk to develop cardiovascular disease, late onset of Alzheimer's disease (8, 9), and sleep-disordered breathing in adults (10), and the genotypes of *C825T* polymorphism of *GNB3* correlate with risk for essential hypertension, obesity, and diabetic nephropathy (11–14).

Materials and Methods

Genomic DNA Purification and Quantitation from Hair Shaft. The cephalic hair shaft samples were collected from 48 anonymous volunteers (40 males and 8 females with an age range of 15–66 years) with appropriate consent (Chi-Mei Medical Center, Tainan, Taiwan). The hair shafts were cleaned by alcohol pads, air-dried, and then cut by a new sterile blade from the region 2–10 cm away from the scalp. Hair was also plucked manually and collected as the control. DNA was purified using DNeasy tissue kit with the modified manufacturer's instruction (Quiagen, Valencia, CA). In brief, ~20 mg of hair shafts (equivalent to a total length of 2.5 meter of a single hair) were minced and then incubated with 500 μl of ATL buffer and 50 μl of proteinase K provided by the kit at 55°C overnight. The supernatants were transferred to new tubes containing 550 μl of AL buffer. After vortexing and incubation at 70°C for 10 min, 550 μl of 100% ethanol was subsequently added. The final steps included washing and elution as per the kit instruction. The DNA quantitation was performed using the PicoGreen double-stranded DNA quantitation kit (P-11496; Molecular Probes, Inc.) following the manufacturer's protocol. The fluorescence intensity was measured using a FLUOstar Galaxy fluorescence microplate reader (BMG Labtechnologies Inc., Durham, NC). The results were expressed as the average from six independent measurements.

Real-Time PCR to Assess Degradation of Hair Shaft DNA. Three pairs of oligonucleotide primers that amplified 100 bp (*APO-E* 158 forward and reverse primer in Table 1), 400 bp (forward primer as GCACCACACCTTCTACAATGA and reverse primer as TGTCACGCACGATTTCCC), and 900 bp (forward primer as GACCTTTGAGATTTCAGAGCC and reverse primer as CGCTTTCCGTTTTGGACT) genomic sequences were used for real-time PCR. Equal amounts of DNA (600 ng) from one normal human cell line (IST-1) and five representative hair shaft samples were added to the PCR reaction mixture (10 μl) containing 1 μl of 10× PCR buffer, 0.3 μl of 50 mM $MgCl_2$, 0.2 μl of 10 mM dNTP each, 0.6 μl DMSO, 0.3 μl of SYBRGreen I (Molecular Probes, Inc.), 0.14 μl of Taq

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Table 1 The sequences[a] for primers and molecular beacons

SNPs	*APOE* 112	*APOE* 158	*C825T*
Primer-F[b]	CGGGCACGGCTGTCCAAG	AGGAGCTGCGGGTGCGCC	CTCCCACGAGAGCATCATC
Primer-R	GCATGGCCTGCACCTCGC	GCCCCGGCCTGGTACACT	CCGCCTACTATTCGCTGG
MB-Red	CACGGACGTGTGCGGCCGCCCGTG	CACGCCTGCAGAAGCGCCTGGCCGTG	CACGATCACGTCCGTGGCCTTCCGTG
MB-Green	CACGGACGTGCGCGGCCGCCCGTG	CACGCCTGCAGAAGTGCCTGGCCGTG	CACGATCACGTCTGTGGCCTTCCGTG

[a] SNP is underlined.
[b] F, forward; R, reverse; MB, molecular beacon.

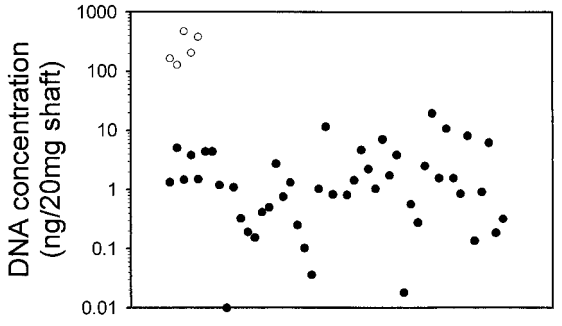

Fig. 1. DNA concentrations of hair from different individuals. The concentration of hair shaft DNA (●) varies among 48 individuals, ranging widely from 0.02 to 20 pg/20 mg of hair. The concentrations of hair DNA isolated from hair follicles (○) are much higher than those from the hair shaft.

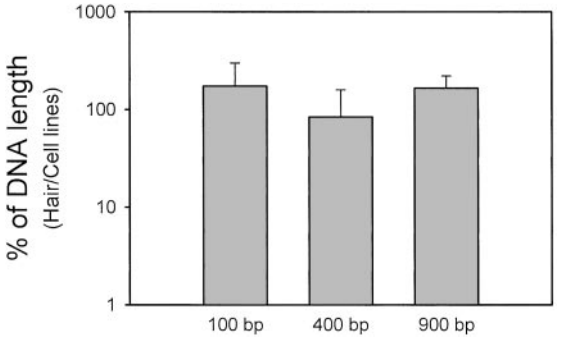

Fig. 2. Determination of DNA length in hair shaft. Real-time PCR was performed to compare the DNA quantity of PCR products in the length of 100 bp, 400 bp, and 900 bp between five representative hair shaft DNA samples and a cell line (IST-1). The abundance of DNA at different lengths of hair shaft is expressed as the percentage of the cell line DNA. *Error bar*, 1 SD.

enzyme, 0.12 μl of 350 μg/ml primers mix (1:1), and 7.34 μl of DNA in water. The relative PCR threshold cycles were recorded using the iCycler iQ multicolor real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA). Assuming the DNA from the cell line was 100% intact, the relative abundance of DNA that could be amplified was calculated from the differences in threshold cycle in the format: 100% $\times$ 2 threshold cycle (cell line DNA hair-shaft DNA).

Analysis of Genotypes by SNP[2] PCR Analysis. The principles and examples of molecular beacons in allelic determination were detailed in previous reports (15–17). The sequences of the primers and molecular beacons for three SNPs including *APO-E* 112, *APO-E* 158, and *GNB3* (*C825T*) were listed in Table 1. Both forward and reverse primers were designed for each SNP, allowing the amplification of ~100 bp of PCR products. Each hair shaft DNA sample (40 pg) was distributed to six wells in a 384-well plate. In addition to all of the essential PCR reagents, the PCR mixture contained a pair of molecular beacons labeled with either fluorescein (green fluorescence) or HEX (red fluorescence) that hybridized with the allele harboring the specific SNP (Gene Link, Thornwood, NY, and Operon Technologies, Inc. CA). An excess of the reverse primer allowed the generation of single-stranded DNA complementary to the molecular beacon. PCR was performed in a single step with the following protocol: 94°C (1 min); 4 cycles of 94°C (15 s), 64°C (15 s), 70°C (15 s); 4 cycles of 94°C (15 s), 61°C (15 s), 70°C (15 s); 4 cycles of 94°C (15 s), 58°C (15 s), 70°C (15 s); 60 cycles of 94°C for (15 s), 55°C (15 s), 70°C (15 s); 94°C (1 min) and 60°C (5 min). The fluorescence intensity in each well was then measured by a Galaxy FLUOstar fluorometer (BMG Lab Technologies, Durham, NC) and the ratio of fluorescein:HEX fluorescence intensity was determined from each well, and the average from six repeats on each sample was determined. The data were converted into genotypes by Microsoft Excel.

PCR-RFLP. The PCR product that contained *C825T* was digested with *Bse*D1 (Fermentas, Hanover, MD) at 55°C overnight (11, 12) and was electrophoresed on a 10% polyacrylamide gel (Novex; Invitrogen Inc.). The different alleles were discriminated by the length of PCR product after *Bse*D1 digestion.

Nucleotide Sequence Analysis. DNA from the wells of the PCR plates was purified using a MiniElute PCR purification kit (Qiagen) and was subject to nucleotide sequencing. The primers used for sequencing were the forward primers listed in Table 1. Sequencing was performed using fluorescently labeled Applied Biosystems Big Dye terminators and an Applied Biosystems 377 automated sequencer (Applied Biosystems, Foster City, CA).

Statistical Analysis. To reassure the new technology in genotyping analysis, χ^2 comparison of proportions was used to analyze the significance of difference in allelic ratio among different populations. *P*s less than 0.05 were considered statistically significant.

Results

The concentration of hair shaft DNA varied among the 48 individuals assessed, and it ranged widely from 0.02 to 20 ng/20 mg of hair. The average and median DNA amounts purified from the hair shaft were 112 $\pm$ 180 (1 SD) pg/mg and 66 pg/mg of hair shaft, respectively (Fig. 1). The hair DNA concentration was similar between male and female hair ($P >$ 0.1). In contrast, the DNA concentrations in plucked hairs containing hair follicles were much higher than those isolated from hair shafts (Fig. 1). On the basis of real-time PCR analysis using different primer pairs, we were able to amplify 100-bp,

[2] The abbreviation used is: SNP, single nucleotide polymorphism.

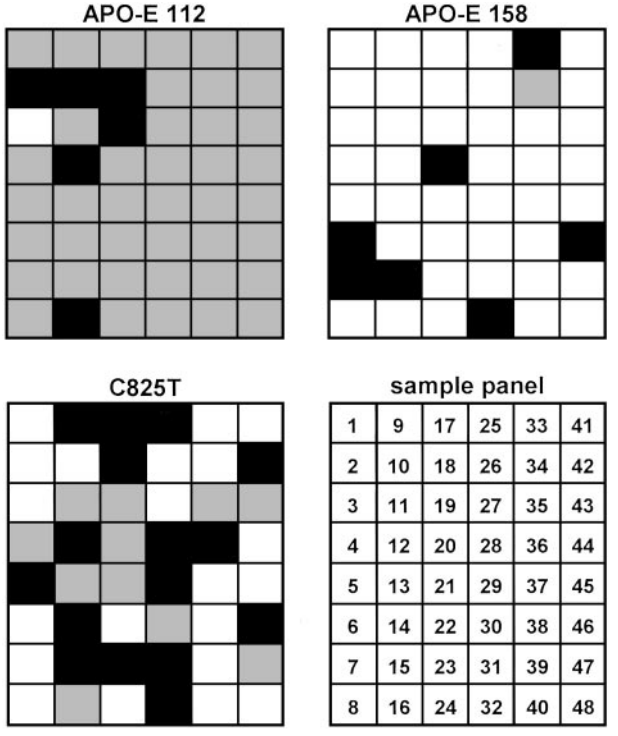

Fig. 3. Allelic distribution for *APO-E* 112, *APO-E* 158, and *C825T*. Hair shaft DNA samples from 48 representative individuals were genotyped using molecular beacon hybridization. Each square represents the genotype of an independent hair DNA sample as shown in a sample panel: *TT* (▨), *TC* (■), or *CC* (□).

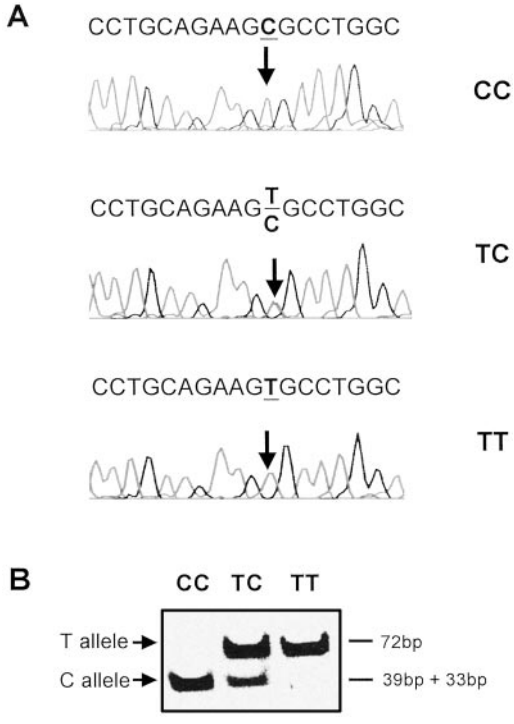

Fig. 4. *A*, nucleotide sequencing of *APO-E* 158 on three representative hair DNA specimens; *underlined*, the SNPs. *B*, PCR-RFLP of *C825T* on three hair shaft DNA samples. PCR products were digested by *Bse*D1 to produce different lengths of DNA fragments. *Bse*D1 cuts at 5′-C ↓ CNNGG-3′ to yield two bands of 33 and 39 bp for the *C* allele and a single (uncut) 72-bp band for the *T* allele. Three bands (33-, 39-, and 72-bp) are present in a heterozygous sample. The 33- and 39-bp bands are very close and appear as a single band.

400-bp, and 900-bp fragments from hair shaft DNA as well as hair containing hair follicles with levels comparable with cell line DNA samples in which the genomic DNA was presumed to be relatively intact (Fig. 2). Five hair samples from other body part were also analyzed. The quality and quantity of DNA isolated from these samples were similar to the cephalic hair (data not shown). The hair DNA was used for *APO-E* and *GNB3* (*C825T*) genotyping in which the loci contained SNPs that were reliably distinguished by a pair of molecular beacons. Using molecular beacon hybridization, we were able to assign individuals with different genotypes based on the ratio of fluorescence intensity of the two molecular beacons (Fig. 3). The genotypes determined by molecular beacons were validated by direct nucleotide sequencing or PCR-RFLP in all of the 48 individuals (Fig. 4). Moreover, based on the genotyping method used in this study, the genotype patterns for *APO-E* and *GNB3* (*C825T*) were identical using both hair and corresponding plasma DNA from the five representative individuals tested. The sensitivity of the method was assessed by analyzing different amounts of hair shaft DNA. Our results demonstrated that as little as 40 pg of DNA gave reproducible genotyping results. Table 2 summarized the results of genotypes of *APO-E* and *GNB3* (*C825T*) in the Taiwanese population. For *APO-E* genotypes, there were two polymorphic sites (codons 112 and 158) in one haplotype, thus resulting in three distinct allelic types (*E2*, *E3*, and *E4*; Table 3; Refs. 18, 19). As compared with the genotypes of Chinese (Mainlander) and Europeans (Icelander, Finnish, and Hungarian; Refs. 13, 20), the percentage of individuals carrying the risk allele (*E4*) in *APO-E* for Alzheimer's disease was similar to that in Chinese but significantly lower than that in Europeans ($P = 0.027$; Table 3).

Table 2 Examples of *APO-E* genotype prediction

Sample No.	Codon 112 158	Genotype Pattern allele 1 / allele 2
34[a]	TT TT	*E2 ($T^{112}T^{158}$) / E2 ($T^{112}T^{158}$)*
1	TT CC	*E3 ($T^{112}C^{158}$) / E3 ($T^{112}C^{158}$)*
3	CC CC	*E4 ($C^{112}C^{158}$) / E4 ($C^{112}C^{158}$)*
6	TT TC	*E2 ($T^{112}T^{158}$) / E3 ($T^{112}C^{158}$)*
12	TC CC	*E3 ($T^{112}C^{158}$) / E4 ($C^{112}C^{158}$)*

[a] The representative samples selected from Fig. 3 are shown.

For *GNB3* (*C825T*), the percentage of the Taiwanese with the risk allele (*TT*) was also similar to the Chinese but was significantly higher than that in Europeans ($P = 0.002$) (Table 4).

Table 3 Genotype and allele frequency of APO-E of Taiwanese, Chinese, and Europeans

Populations	*n*	Genotype (%)						Allele (%)		
		E2/E2	*E2/E3*	*E3/E3*	*E2/E4*	*E3/E4*	*E4/E4*	*ε2*	*ε3*	*ε4*
Taiwanese	48	2.1	14.6	68.8	0.0	12.5	2.1	9.4	82.3	8.3[a]
Chinese (27)	141	1.4	12.1	70.9	0.0	14.9	0.7	7.4	84.4	8.2[a]
Europeans (20)	590	0.8	9.3	57.3	1.9	27.3	3.4	6.4	75.6	18.0[b]

[a] Ref. 27.
[b] Not significant.
[c] $P < 0.03$.

Table 4 Genotype of *C825T* in the populations of Taiwanese, Chinese, and Europeans

Populations	*n*	Genotype (%)		
		TT	*TC*	*CC*
Taiwanese	48	23.0[a]	41.0	36.0
Chinese (13)	960	22.4[a]	50.6	27.0
Europeans (13)	277	10.0[b]	43.7	46.2

[a] Not significant.
[b] $P < 0.001$.

Discussion

We reported a new technique to enrich hair shaft DNA and an improved method using molecular beacons for genotyping. This system has the following useful features in population-based genetic screening. First, it is convenient and economical to collect the hair shaft samples. Because only a small amount of terminal hair shaft is required, it is completely noninvasive and does not require viable tissues obtained by venous puncture, mucosal swabbing, or plucking the hair, all of which can be relatively invasive. For those individuals whose cephalic hair is not available, the hair from other body parts can be the alternative (data not shown). Second, the DNA in the hair shaft is stable at room temperature for a long period of time before DNA extraction (6) because of the dry nature of the hair shaft specimens. For practical consideration, the hair sample, similar to buccal swab/washings, can be self-collected and mailed in a regular envelop directly to a laboratory for analysis (3). This feature is particularly attractive because no personal visits or direct contact with a health care worker are required. Third, with the use of the genotyping technique described here, only a small amount of genomic DNA (~40 pg) is required for this assay, thus minimizing the amount of DNA needed for single analysis and maximizing the information (number of genotypes) gained from each sample. It is estimated that using a 20-mg hair sample that on average yields 2.5 ng of DNA, we will be able to perform at least 50 assays (40 pg of DNA/assay) on different disease-associated SNPs. Finally, our SNP genotyping method is performed in a single step and can be high throughput because a total of 60 different DNA samples could be simultaneously analyzed in one 384-well plate within 4 h. This method may provide an easier and higher throughput assay than the previous methods based on PCR-RFLP analysis.

The nature of wide variation of hair shaft DNA concentrations among individuals is unknown, probably reflecting the region of hair being sampled because the DNA concentration is higher when it is close to the root region (4). On the basis of real-time PCR assays to estimate the length of the hair shaft DNA, the DNA was not substantially degraded, at least within 900 bp, as compared with the DNA isolated from cultured cells or hair follicles; therefore, the hair shaft DNA is not only appropriate for our present method, which amplifies DNA fragments of ~100 bp, but also suitable for other methods that depend on longer PCR fragments for analysis. Although we used two disease loci for demonstration in this study, it should be mentioned that our genotyping method can be applied to other SNPs or deletion polymorphisms associated with diseases because we have successfully genotyped tissue DNA using 30 markers of SNP and deletion polymorphisms (17, 21–23). The technique for isolation of hair shaft DNA can also be combined with a variety of other genotyping methods (24–26). In this study, we demonstrated that other genotyping methods including direct nucleotide sequencing and RFLP might also be appropriate for genotype analysis using hair shaft DNA (Fig. 4). In conclusion, the genotyping system described here is cost-effective, reliable and high throughput and provides an alternative method that can be generally applied in genetic screening and epidemiology studies.

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