TECHNICAL NOTE

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Mitochondrial DNA and STR Typing of Matter Adhering to an Earphone

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ABSTRACT: STR typing and mitochondrial DNA (mtDNA) sequencing were performed on the matter adhering to an earphone found at a crime scene. Experimental studies were carried out using the earphones provided by volunteers. By means of immunohistochemistry, keratinocytes and a portion of nucleated epithelial cells were proven to exist in the contents from the earphones. DNA was extracted by means of the phenol/chloroform method, and the low quantity of extracted DNA was found to be highly degraded. Six STR loci, CSF1PO, TPOX, TH01, F13A01, FESFPS and vWA, were PCR amplified and typed by using two triplex systems (CTT and FFv Multiplexes, Promega, WI), and an amelogenin locus was determined as well. Although partial profiles were observed in some experimental samples, all STR loci could be typed when a considerable amount of high molecular weight DNA was obtained (>0.5 ng/μL). Amplification and sequencing of mtDNA hypervariable region I (15997-16401) and hypervariable region II (29-408) were all successful. The mitochondrial DNA sequence of the actual case sample, comprising two hypervariable regions and a total of 785 base pairs, showed eight mutations and two insertions with respect to the standard published reference sequence. The genotype was unique in the three published Japanese databases. These results suggest that it is possible to analyze mtDNA from minute amounts of materials and from degraded materials more effectively and routinely in forensic practice.

KEYWORDS: forensic science, DNA typing, mitochondrial DNA, STR system, crime scene

Typing of short tandem repeat (STR) loci by polymerase chain reaction (PCR) is now widely used in forensic practice, and the validity of this technique has been extensively recognized (1,2). Mitochondrial DNA (mtDNA) is also known to have two highly polymorphic regions, hypervariable region I (HV-I) and hypervariable region II (HV-II), that are located within the control region (D-loop region), and these sites can be detected by means of PCR amplification and direct sequencing techniques (3). Because thousands of copies of mtDNA exist in each cell (4), current studies have indicated that PCR amplification and sequencing of mtDNA is frequently more effective than the STR typing for analyzing highly degraded materials or materials in minute amounts, aged remains or a single hair shaft (5,6).

In the present study, we describe the sequencing of mtDNA and typing of several STR loci from the matter that had adhered to an earphone left at a crime scene. This earphone was found at a robbery scene and was thought to have been used with a cell phone by a suspect. We initially investigated whether or not STR typing and mtDNA sequencing could be performed on the contents adhering to the body of earphones that were used by several volunteers. Then, typing of STR loci and sequencing of mtDNA were performed on the matter adhering to the earphone from the actual case. We mention in this article the results of these experimental DNA analyses and the case study.

Case Report

In June 2000, an attempted robbery of a bank occurred in a local city in Miyazaki Prefecture, Japan. Although the robber (presumed 30–50-years old male), who was masked and threatened to take out the money, fired shots with a pistol, he got away with nothing because a staff of this bank counterattacked the robber. A few days after this event, a motor scooter and a mask that were used in this robbery were found in a coppice along an alley, but the suspect was missing. There was also an earphone inside the mask that belonged to the suspect. Subsequently, we attempted DNA analyses of the matter that had adhered to this earphone (Fig. 1).

Materials and Methods

Earphones that had been used for more than one month and control blood or buccal cells were obtained from five unrelated volunteers (three males and two females). The type of cerumen from two subjects (a male and a female) was wet, and the others were typed dry (Table 1).

The matter that had adhered to the holes of body and surrounding area of each earphone, including that of the actual case sample, was taken out by means of a needle, collected in sterile microcen-

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trifuge tubes, and suspended in 250 μL of extraction buffer (10 mM Tris-HCl, pH 8.0, containing 10 mM EDTA and 0.1 M NaCl).

Fifty microliters of this suspension were centrifuged, and the pellet was fixed with 20% buffered formalin. Fixed cells were immunostained with rabbit anti-human keratin antibody (DAKO A/S, Denmark), followed by peroxidase-labeled anti-rabbit immunoglobulins (ENVISION kit/HRP, DAKO A/S, Denmark). The cells were then counterstained with hematoxylin and mounted in solution for microscopic examination.

Two hundred microliters of the remaining solution were added to 1/9 volume of 10% SDS, 39 mM DTT and 12 μL of proteinase K (20 mg/mL), and incubated at 56°C overnight. The samples were purified by a standard phenol/chloroform extraction method. After ethanol precipitation, the DNA was resuspended in 30 μL of TE buffer (10 mM Tris-HCl, pH 8.0, containing 1 mM EDTA), and 5 μL of an aliquot was applied on a 1% agarose gel and stained with ethidium bromide in order to examine the quality and quantity of extracted DNA. DNA from blood and buccal cells was also extracted using the phenol/chloroform method.

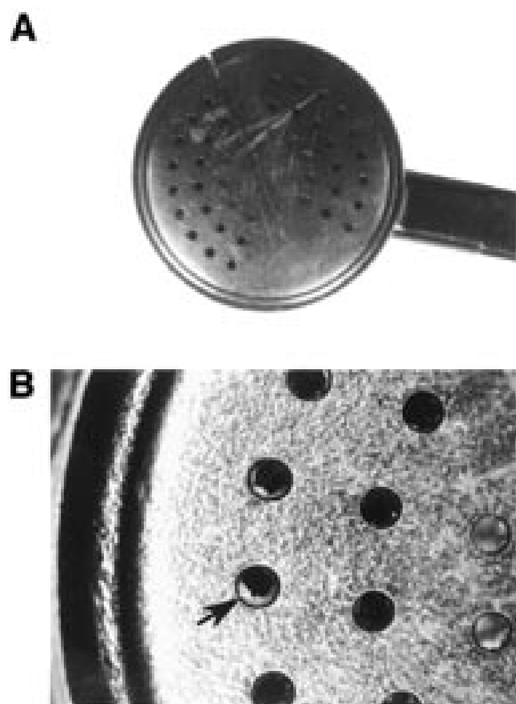

FIG. 1—*The earphone found at crime scene.* A: *It is believed to have been used with a cell phone, and the body is 1.5 cm in diameter.* B: *Arrow shows the contents of a small hole (∅ 0.8 mm) in the body.*

Six STR loci, CSF1PO, TPOX, TH01, F13A01, FESFPS, and vWA were amplified and investigated using GenePrint® STR Systems combined with CTT Multiplex and FFv Multiplex (Promega, WI). Typing of these STR loci was carried out via 4% denatured polyacrylamide gel electrophoresis and silver staining. Amplification and typing were performed according to the manufacturer's recommendations. The X-Y homologous gene, amelogenin, was also amplified and typed as described previously (7).

Two hypervariable segments of mtDNA, HV-I (15997-16401) and HV-II (29-408), were subjected to PCR amplification and purification as described previously (5). Subsequent cycle sequencing was performed using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems, CA). Each mtDNA sequence was confirmed by the forward and reverse sequencing reaction, and the nucleotide positions of point mutation in the extracted DNA from an earphone as well as controls were compared with the reference sequence (8) using Sequence Navigator software (version 1.0.1, PE Applied Biosystems, CA).

Results and Discussion

Histological Examination

A number of cells from all samples were stained with anti-human keratin antibody; therefore, these were demonstrated to be keratinized corneocytes (Fig. 2). The cell particles were assumed to consist of desquamated dead skin of concha auriculae and cerumen. Cerumen also revealed the presence of a large amount of keratin debris (9). A portion of nucleated epithelial cells were identified in these samples (Fig. 2). Herber et al. reported the existence of nucleated epidermal cells in dandruff and suggested that parakeratotic cells were also contained in dandruff as a result of abnormal epidermopoesis with an incomplete terminal differentiation and keratinization (10). In this case, normal epidermal cells were

TABLE 1—*Preparation of samples and results of STR typing.*

Sample	Sex	Origin	Type of Cerumen	Quantity* (ng/μL)	PCR Template (μL)	STRs						Amelogenin (bp)
						CSF1PO	TPOX	TH01	F13A01	FESFPS	vWA	
1	M	Earphone	Dry	0.3	4	ND‡	ND	6.9	4.6	11.12	15.18	106/112
		Blood		5	1	10.11	9.11	6.9	4.6	11.12	15.18	106/112
2	F	Earphone	Wet	<0.2	5	ND	ND	ND	4.4	13.13	14.7	…§
		Buccal cell		5	1	10.13	8.8	7.9	4.4	13.13	14.7	112/112
3	F	Earphone	Dry	<0.2	5	ND	ND	ND	4.4	12.12	16.18	…
		Buccal cell		5	1	12.12	8.11	9.9	4.4	12.12	16.18	112/112
4	M	Earphone	Dry	0.4	4	ND	9.11	7.9	4.4	11.12	16.17	106/112
		Blood		5	1	10.13	9.11	7.9	4.4	11.12	16.17	106/112
5	M	Earphone	Wet	0.6	3	11.12	8.8	7.9	4.4	12.13	18.18	106/112
		Buccal cell		5	1	11.12	8.8	7.9	4.4	12.13	18.18	106/112
Actual Case	M	Earphone	UN†	≥1.0	2	12.13	11.12	7.10	3.2.4	11.13	17.18	…

* Concentration of high molecular weight DNA and each control adjusted 5 ng/μL.
† Unknown.
‡ Not detectable.
§ No determination.

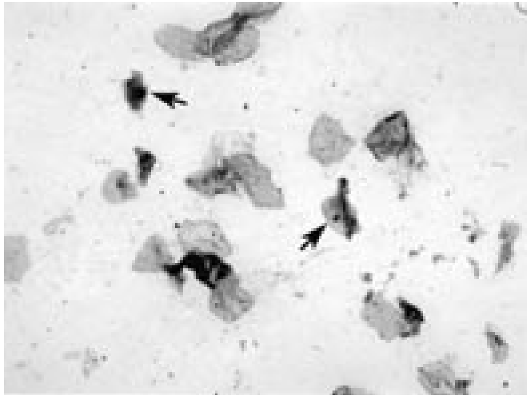

FIG. 2—*Immunohistochemical examination of the contents of an earphone from actual case (×400). Arrows show a portion of nucleated epithelial cells.*

also believed to have been shed and to have adhered to the earphone according to the insertion of the earphone.

DNA Extraction

The quantity and quality of extracted DNA from the earphones were examined on an agarose gel and compared with those of a sample of known concentration. The quantity of extracted DNA from each sample is shown in Table 1. The yield of genomic DNA extracted from the earphones depended on the amount of the matter on each earphone and was not influenced by the type of cerumen. High molecular weight genomic DNA from samples No. 2 and No. 3 was not visible, but more degraded DNA was slightly visible. Sufficiently high molecular weight DNA was confirmed in another two samples (No. 5 and questioned earphone sample); however, highly degraded DNA was also observed.

STR Analysis

Six STR loci were amplified and typed using two triplex STR systems (CTT and FFv Multiplex), and the results of STR typing as well as amelogenin locus are summarized in Table 1. In case No. 2 and No. 3, the amelogenin locus was not established because the remaining volume of the template was insufficient. When CTT (CSF1PO, TPOX, and TH01) loci were amplified and typed, two of the samples (No. 2 and No. 3) yielded no positive results and another two cases (No. 1 and No. 4) showed partial profiles (Table 1). However, typing of FFv (F13A01, FESFPS, and vWA) loci was successful in all cases. Although the reason for the differences in these results was not satisfactorily clarified, it is assumed that the results were affected by the lower amount of template DNA and loss of higher molecular weight genomic DNA. The partial profiles of several multiplex systems have frequently revealed a characteristic loss of high molecular weight loci as the extent of DNA degradation increased, and it has been suggested that PCR amplification is less efficient for significantly degraded genomic DNA (11). The results of CTT loci in this study were thought to support these findsigns. Preferential amplification that yielded relatively stronger signals was frequently observed in amelogenin locus, because amplification of this locus was presumed to require only a relatively short target fragment of DNA to remain intact (12).

The minimum amount of template DNA used for amplification of CTT and HFv (HPRTB, F13A01, and vWA) Multiplex systems were reported to be 0.1 ng for CTT and 0.25 ng for HFv Multiplexes (13). Budowle et al. recommended that a minimum of 0.4–0.45 ng of template DNA be used for PCR with the CTT Multiplex system (14). In fact, all STR loci were clearly detectable when a considerable amount of high molecular weight genomic DNA was obtained (>0.5 ng/μL) (Table 1).

Sequencing of mtDNA

The hypervariable regions of mtDNA subjected to amplification were 15997–16401 (HV-I) and 29–408 (HV-II), respectively, and sequencing of all samples was successful. In cases No. 1 to No. 5, the individual sequences of the matter from each earphone and control (blood or buccal cells) were all identical (data not shown).

The comparison of the mtDNA sequence from the questioned earphone sample with a reference sequence (8) revealed four mutations in HV-I and four mutations in addition to two nucleotide insertions in HV-II (Table 2). Among them, three positions of nucleotide transition at 16290 in HV-I and at 200 and 235 in HV-II are relatively rare in 100 published Japanese sequences (4% for 16290, 2% for 200, and 4% for 235), whereas other positions of mutation are shared 10–100% (15). Although these nucleotide mutations are also observed in other populations, the frequencies of these nucleotide transitions in three European populations were lower or in the same range as the Japanese (16–18). In Koreans, frequencies of nucleotide transitions C to T at 16290 and A to G at 235 were reported to be comparatively higher than in the Japanese population (8.8% for 16290 and 9.5% for 235) (19). However, the genotype of the mtDNA combined with the HV-I and HV-II from the questioned earphone sample is unique, and there is no identical genotype in the published Japanese databases (15,20,21).

In recent years, mtDNA profiling has been used for population genetics (22), archaeology (23), and forensic casework (24). Amplification and sequencing of mtDNA from minute amounts of matter or highly degraded sources (i.e., mummified soft tissue, aged remains, and hair shafts) are well established and validated (25). Because mtDNA is maternally inherited, mtDNA analysis has been used successfully in several forensic identification cases of old skeletal materials from the Romanov family (26), Vietnam war victims (27), and Guatemalan mass graves (28). The successful application of PCR amplification and sequencing of a single hair shaft has also been reported (29), and researchers have been able to analyze a minimum volume of 0.03 mm^3 of axillary hair shafts (6). Our investigations supported the finding that sequencing and analyzing of mtDNA from small amounts of and degraded DNA were more sensitive and effective in comparison with STR typing of single-copy chromosomal DNA. These results suggest that

TABLE 2—*Mitochondrial DNA sequence of the questioned earphone sample compared with reference sequence.*

	Position of Mutation									
	HV-1				HV-II					
Origin of sample	16223	16290	16319	16362	73	152	200	235	309…1	315…1
Reference	C	C	G	T	A	T	A	A	—	—
Questioned earphone	T	T	A	C	G	C	G	G	C	C

mtDNA analysis will become more routinely used in forensic practice and be applicable to various forensic specimens.

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