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Original

Hypervariable region structure and polymorphism of mtDNA from dental pulp and a family analysis

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Abstract: Nucleotide sequences of the hypervariable region in the D-loop of mitochondrial DNA (mtDNA) were analyzed using DNA extracted from 140 old dental pulp samples. These sequences were compared with the sequence reported by Anderson et al. Nucleotide substitution in the HV1 region was identified at 77 positions. A C-to-T transition at position 16223 (C16223T) was most frequently detected (77.9%). Fourteen types of C-stretch sequence patterns were detected and the same sequence as Anderson had the highest frequency (57.9%). In the HV2 region, base transitions were identified at 56 positions. A263G was identified in all samples. Seven types of C-stretch were detected, but none had the same sequence as Anderson. In the HV3 region, base transitions were identified at 21 positions. T489C was most frequently identified (64.3%). Five types of C-stretch were detected, and the same sequence as Anderson accounted for 92.9%. The 140 samples were classified into 128 kinds by the sequence patterns of the HV region. Next, using the blood and oral mucosa epithelium from 23 subjects comprising four generations in a family line, the hereditary relationship of mtDNA was examined. All mtDNA types of the first-generation mother were infallibly inherited by the fourth generation. (J. Oral Sci. 48, 145-152, 2006)

Keywords: forensic odontology; dental pulp; mtDNA; hypervariable region; C-stretch; family line.

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Introduction

The nucleotide sequences of human mitochondrial DNA (mtDNA) were reported by Anderson et al. in 1981 (1). Of these, hypervariable region 1 (HV1), hypervariable region 2 (HV2) and hypervariable region 3 (HV3) which are rich in polymorphisms in the control region including the displacement loop region (D-loop region) were discovered (2-6).

There are several thousand copies of mtDNA in any cell, which is beneficial since analysis may be undertaken from minute specimens or from old specimens that are contaminated or degraded (7,8). Therefore, the utility of mtDNA in the field of forensic medicine is extremely high. Moreover, mtDNA is transferred via cytoplasmic inheritance or in other words, maternal inheritance. For this reason, it is potentially applicable in the identification of maternal relationship, but since the mtDNA of the father (sperm) is eradicated upon fertilization, mtDNA cannot be used for identification of a paternal relationship (9-11).

The specimens used in forensic investigation are often bloodstains and hair, however, in the case of corpses that have decayed significantly or skeletons, other specimens must be used. Dental pulp that is covered with hard tissue such as dentin or enamel is highly capable of protecting the DNA and is thus extremely useful. Although dental pulp has been used as a sample previously, there have not been any reports which examined polymorphisms of HV1, HV2 and HV3 including base sequences of C-stretch using this sample.

In this study, using DNA extracted from old dental pulp, the nucleotide sequence polymorphisms of HV1, HV2 and HV3 in the control region of mtDNA were determined in a Japanese population, and its application for personal identification was analyzed. In addition, the genetic

relationship of the hypervariable region in mtDNA was examined using blood or oral mucosa obtained from 23 people comprising four generations in a family line.

Materials and Methods

Sample preparation

Dental pulp samples from 140 unrelated Japanese subjects were used to analyze the nucleotide sequence polymorphism of the hypervariable region in mtDNA. Dental pulp samples were obtained from permanent teeth that had been stored at room temperature for 1 to 23 years. Blood or oral mucosa samples from a total of 23 people comprising four generations in a family line which had been stored in the frozen state were also obtained. DNA was extracted using the phenol-chloroform method (12). Prior to DNA analysis, the Ethics Review Committee of Nihon University School of Dentistry had granted ethical approval for the study.

PCR method

Nucleotides in the mtDNA sequence are numbered according to the sequence reported by Anderson et al. (1), for example nt16000.

According to the report of Tsutsumi et al. (13), nt15997 to 16401 of HV1 were amplified by synthesizing forward primer: 5'-CACCATTAGCACCCAAAGCT-3' and reverse primer: 5'-TGATTTCACGGAGGATGGTG-3', and nt29 to 408 of HV2 were amplified by synthesizing forward primer: 5'-GGTCTATCACCCTATTAACCAC-3' and reverse primer: 5'-CTGTTAAAAGTGCATACCGCCA-3'. Moreover, nt162 to 639 of HV3 were amplified by synthesizing forward primer: 5'-CGCACCTACGTTC AATATTAC-3' and reverse primer: 5'-GGTGATGTGAG CCCGTCTAA-3'. The PCR reaction mixture used was PCR mixture 3.0 μl, 2.0 mM dNTP mix 3.0 μl, 20 pmol of each primer, Gold Taq DNA polymerase 2U and 15 ng template DNA to which sterilized water was added to make a total volume of 30 μl. The initial step of PCR was set at 95˚C for 9 min, and then thirty cycles (60 sec at 95˚C for denaturation, 60 sec at 55˚C for annealing, and 120 sec at 72˚C for extension) were performed. After the last cycle, the samples were incubated for an additional 7 min at 72˚C.

Analysis of nucleotide sequence

The DNA templates for sequence analysis were refined from the PCR products. A total of 3 regions in HV1 (nt16081-16390), HV2 (nt73-354) and HV3 (nt438-594) were sequenced using ABI PRISMTM 310 Genetic Analyzer (Applied Biosystems).

Results

The nucleotide sequence of each hypervariable region in the 140 samples of dental pulp DNA was compared with the sequence reported by Anderson et al. (1).

Hypervariable region 1

In nt16081-16390, nucleotide variations (transition, transversion, insertion) occurred at 77 positions (24.8%). Table 1 - HV1 shows the parts of high frequency. A C-to-T transition at nt16223 (C16223T) that was identified in 109 of the 140 cases (77.9%) was most frequently found. This was followed by T16362C with 74 cases (52.9%), T16189C with 51 cases (36.4%), A16183C with 35 cases (25.0%) and G16129A with 29 cases (20.7%) in this order.

There were 14 types (10.0%) of C insertion between nt16193 and nt16194 (*16193.1C: the asterisk indicates nucleotide absence and 1C means the insertion of one nucleotide C).

Moreover, as shown in Table 2 - HV1, 14 cases of sequence patterns of homopolymeric cytosine stretches (C-stretch) were detected between nt16184 and nt16193. Eighty one cases (57.9%) with the same structure (CCCCCTCCCC: 5C1T4C) as that reported by Anderson et al. (1) appeared to be the most frequently observed pattern. Following this was 10C with 28 cases (20.0%) and 11C with 13 cases (9.3%) in this order.

The 140 samples were classified into 86 kinds based on the sequence pattern of the HV1 region.

Hypervariable region 2

In nt73-354, nucleotide variations (transition, transversion, insertion, deletion) occurred at 56 positions (19.9%). Of these, the parts with a high frequency are shown in Table 1 - HV2. A263G was the mostly frequently observed transition, which was detected in all specimens. This was followed by T310A with 92 cases (65.7%), C311A with 63 cases (45.0%), C150T with 31 cases (22.1%) and C312A with 24 cases (17.1%) in this order. Examples of insertion were *315.1C in 135 cases (96.4%), *315.2C (2C means the insertion of two nucleotides C) in 87 cases (62.1%) and *315.3C in 20 cases (14.3%). Moreover, cases of nucleotide deletion were found in 0.7% to 5.0% of nt248, nt249, nt316 and nt317 (A248del, A249del, G316del, C317del: del means nucleotide deletion).

On the other hand, the C-stretch sequence patterns for nt303-315 were composed of 7 types as shown in Table 2 - HV2. The pattern with the highest frequency was 8C1T6C with 63 cases (45.0%). This was followed by 7C1T6C with 48 cases (34.3%) and 9C1T6C with 20 cases (14.3%). There were no cases that indicated the

Table 1 Characteristic nucleotide variations and frequencies in HV1, HV2 and HV3 regions

HV1

Nucleotide position	Replacement	n	%
16129	G → A	29	20.7
16172	T → C	14	10.0
16182	A → C	11	7.9
16183	A → C	35	25.0
16189	T → C	51	36.4
16193.1	* → C	14	10.0
16209	T → C	15	10.7
16217	T → C	13	9.3
	→ A	1	0.7
16223	C → T	109	77.9
16249	T → C	10	7.1
16278	C → T	8	5.7
16290	C → T	8	5.7
16291	C → T	7	5.0
16304	T → C	12	8.6
16311	T → C	10	7.1
16324	T → C	15	10.7
16325	T → C	11	7.9
16362	T → C	74	52.9
16390	G → A	7	5.0

HV2

Nucleotide position	Replacement	n	%
76	A → C	7	5.0
	→ G	2	1.4
150	C → T	31	22.1
152	T → C	23	16.4
194	C → T	12	8.6
199	T → C	20	14.3
207	G → A	8	5.7
248	A → del	1	0.7
249	A → del	7	5.0
263	A → G	140	100.0
310	T → A	92	65.7
311	C → A	63	45.0
312	C → A	24	17.1
315.1	* → C	135	96.4
315.2	* → C	87	62.1
315.3	* → C	20	14.3
316	G → del	1	0.7
317	C → del	6	4.3

HV3

Nucleotide position	Replacement	n	%
489	T → C	90	64.3
522	C → del	46	32.9
523	A → del	46	32.9
568.1	* → C	7	5.0

n: No. observed, *: Nucleotide absence, del: Nucleotide deletion, N = 140

Table 2 C- stretch sequences and frequencies of HV1, HV2 and HV3 regions

HV1

Nucleotide position	16184	16185	16186	16187	16188	16189	16190	16191	16192	16193	16193.1	n	%
Anderson's sequence	C	C	C	C	C	T	C	C	C	C			
This study's sequence	T	C	C	C	T	C	C	C	C	del		1	0.7
	T	C	C	C	C	T	C	C	C	C		1	0.7
	C	T	C	C	C	T	C	C	C	C		2	1.4
	C	C	C	T	C	T	C	C	C	C		4	2.9
	C	C	C	T	C	C	C	C	C	del		1	0.7
	C	C	C	T	C	C	C	C	C	C		1	0.7
	C	C	C	C	T	C	C	C	C	C		1	0.7
	C	C	C	C	T	C	C	C	C	C	C	1	0.7
	C	C	C	C	C	T	C	C	T	C		1	0.7
	C	C	C	C	C	T	C	C	C	C		81	57.9
	C	C	C	C	C	C	C	C	T	C		2	1.4
	C	C	C	C	C	C	C	C	C	del		3	2.1
	C	C	C	C	C	C	C	C	C	C		28	20.0
	C	C	C	C	C	C	C	C	C	C	C	13	9.3

HV2

Nucleotide position	303	304	305	306	307	308	309	310	311	312	313	314	315	315.1	315.2	315.3	n	%
Anderson's sequence	C	C	C	C	C	C	C	T	C	C	C	C	C					
This study's sequence	C	C	C	C	C	C	T	C	C	C	C	C	C				1	0.7
	C	C	C	C	C	C	C	T	C	C	C	C	C	C			48	34.3
	C	C	C	C	C	C	C	C	T	C	C	C	C	C	C		63	45.0
	C	C	C	C	C	C	C	C	C	T	C	C	C	C	C		4	2.9
	C	C	C	C	C	C	C	C	C	T	C	C	C	C	C	C	20	14.3
	C	C	C	C	C	C	C	C	C	C	C	del	del				1	0.7
	C	C	C	C	C	C	C	C	C	C	C	C	C				3	2.1

HV3

Nucleotide position	568	569	570	571	572	573	573.1	573.2	573.3	573.4	573.5	573.6	573.7	n	%
Anderson's sequence	C	C	C	C	C	C									
This study's sequence	C	C	C	C	C	C								130	92.9
	C	C	C	C	C	C	C							1	0.7
	C	C	C	C	C	C	C	C	C					4	2.9
	C	C	C	C	C	C	C	C	C	C	C	C		2	1.4
	C	C	C	C	C	C	C	C	C	C	C	C	C	3	2.1

n: No. observed, del: Nucleotide deletion, N = 140

same structure as that reported by Anderson et al. (1).

The 140 samples were classified into 75 kinds based on the sequence pattern of the HV2 region.

Hypervariable region 3

In nt438-594, nucleotide variations (transition, deletion, insertion) occurred at 21 positions (13.4%). T489C was the most frequently detected nucleotide transition with 90 cases (64.3%) (Table 1 - HV3). This was followed by nt522 and nt523 that showed nucleotide deletion with 46 cases each (32.9% for both) (C522del, A523del). In the case of nt568.1, nucleotide insertion was identified (*568.1C).

In nt568-573, there were 5 types of C-stretch sequence patterns as shown in Table 2-HV3. Of these, 130 cases (92.9%) showed the same structure (6C) as that reported by Anderson et al. (1), and the others indicated nucleotide insertion of between 1 and 7 nucleotides. Moreover, in HV3, there were 46 cases that indicated both C522del and A523del and of these, 33 cases were 8C1T6C with the highest frequency (45.0%) of C-stretch nucleotide sequence in nt303-315 of the HV2 region as shown in Table 2 - HV2.

The 140 samples were classified into 20 kinds based on the sequence pattern of the HV3 region.

As stated above, the 140 samples were classified into 128 kinds based on the sequence patterns of the three HV regions.

Polymorphism in a family line

PCR amplification was performed on the three HV regions in the mtDNA from 23 people comprising four generations in a single family line (first generation: father and mother; second generation: 5 children and 4 spouses of the children; third generation: 9 grandchildren and 1 spouse of a grandchild; fourth generation: 2 great-grandchildren).

The nucleotide sequence of the mother in the first generation was compared with the findings reported by Anderson et al. (1). With respect to the HV1 region, nucleotide transition was identified as C16223T and T16362C (Fig. 1 - HV1). The variations identified in the HV1 region were limited to this part and other sequences coincided with those reported by Anderson et al. (1). The frequency was 14 cases (10%) among 140 cases of dental pulp DNA. Moreover, C-stretch of 5C1T4C was identified in nt16184-16193. The frequency was 81 (57.9%) among the 140 cases.

With respect to the HV2 region, variations were identified at 6 positions that had the sequence A73G-C194T-A263G-T310C-C311T-*315.2C (Fig. 1 - HV2) and of the other sequences coincided with the report of Anderson et al. (1) in 5 (3.6%) of the 140 cases. Moreover, C-stretch was identified for 8C1T6C at nt303-315 in 63 (45.0%) of the 140 cases.

Furthermore, in the HV3 region, variations were identified at three positions with the sequence of T489C-C522del-A523del (Fig. 1 - HV3) and the other sequences coincided with the sequence reported by Anderson et al. (1) in 10 (7.1%) of the 140 cases. Moreover, C-stretch was identified for 6C at nt570-575 in 130 (92.9%) of the 140 cases.

From the above findings, 2 samples of the 140 cases of dental pulp DNA (1.43%) showed the same pattern as the HV1, HV2 and HV3 regions in the mother of the first generation.

The genealogical tree of the 23 people is shown in Fig. 2. The sequence comparison between this study and Anderson's results indicated that the sequence of the mother in the first generation was inherited by the children [five: 3 males (No.3, 5, 7), 2 females (No.10, 11)] in the second generation, grandchildren [2 females (No.19, 20)] in the third generation and the great-grandchildren [two: 1 male (No.23), 1 female (No.22)] in the fourth generation (Fig. 2: bold line).

Discussion

In the area of forensic investigation, the specimens used are usually highly damaged and difficulties are often experienced in DNA identification. However, even if examination using the genetic polymorphism of nuclear DNA with few copies as reference is not feasible, there are a number of cases in which examination using the genetic polymorphism of mtDNA with several thousand copies as reference is in fact possible. This is because by viewing the variations such as nucleotide transition in the hypervariable region found in 3 positions of the control region as haplotypes, precision of personal identification may improve (14).

As dental pulp DNA is enclosed in hard tissue, the state of storage of DNA is extremely good, and thus is an ideal specimen for identification. Successful identification was reported using dental pulp from a corpse that had been left underwater for 7 months (15). While the reliability of the results of identification is high if the specimen is blood, when the dental pulp of a decomposed or skeletonized body is the specimen, the impact of heteroplasmy due to various bacteria cannot be disregarded (16). For this reason, mtDNA polymorphism using old dental pulp was analyzed in the present study for personal identification of decomposed or skeletonized bodies.

First, with a total of 140 cases of old dental pulp, analysis of polymorphism in the HV1 region was performed.

Sequence analysis was possible for all specimens. As shown in Fig. 1 - HV2, there were cases in which heteroplasmy at nt316 of C-stretch was identified, but this did not affect the analysis. The proportion of manifestation of heteroplasmy is said to differ not only among relatives on the maternal side (17) but also within individuals (16,18-20), and hence, caution is advised during analysis. In actual identification, multiple analyses are necessary so that the possibility of variation by an error of Taq polymerase is considered (21).

Compared to the nucleotide sequence identified by Anderson et al. (1) using blood, as shown in Table 1,

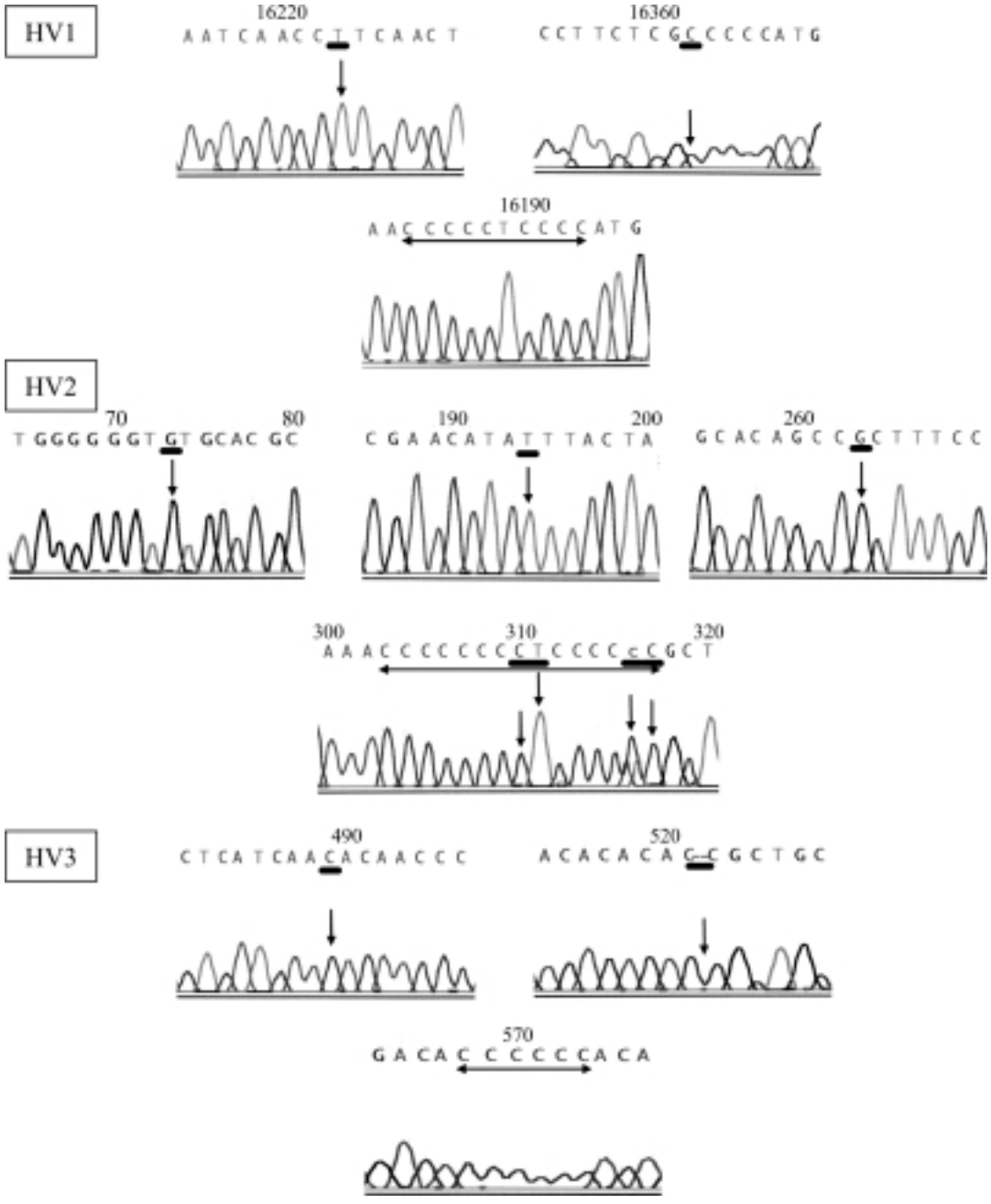

Fig. 1 Sequence variations of HV1, HV2 and HV3 regions in the mother of the first generation. In HV1 region, nucleotide transitions were identified from C toT (↓) at nt16223 and from T to C at t16362 (↓). C-stretch of nt16184 to nt16193 (⟷) showed the same structure (CCCCCTCCCC: 5C1T4C) as that reported by Anderson et al. In HV2 region, nucleotide transition was identified from A to G at nt73, C to T at nt194, A to G at nt263, T to C at nt310 and C to T at nt311. Moreover, two bases C insert at nt315.1 and nt315.2. Heteroplasmy was detected at nt315.1. C-stretch of nt303 to nt317 was 8C1T6C. In HV3 region, nucleotide transition was identified from T to C at nt489. Cytosine and adenine delete at nt522 and at nt523, respectively. C-stretch of nt568 to nt573 showed the same structure (6C) as that reported by Anderson et al. (1).

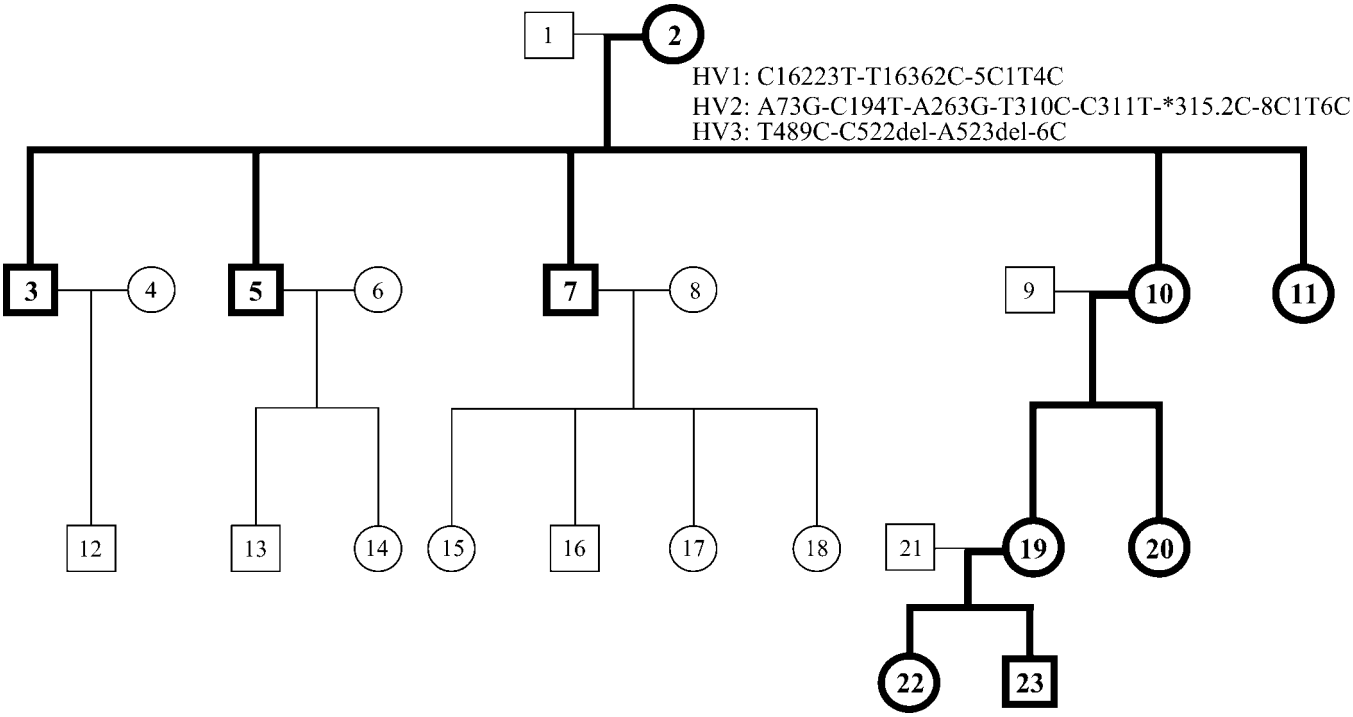

Fig. 2 Genealogical tree and the heredity relations of hypervariable region (HV1, HV2 and HV3) in mtDNA. Bold numbers (heavy line) indicate heredity of the sequence of the mother in the first generation.

C16223T that is reported to have high frequency (13) accounts for 77.9%. This is followed by T16362C with 52.9%, T16189C with 36.4% and A16183C with 25.0% in this order. Nishimaki et al. (22) who studied 150 blood samples of Japanese people reported that the frequency of C16223T was 82.6%, T16362C was 43%, G16129A was 31.1% and that of A16183C was 14.7% in this order. There was no significant difference between the two results. Moreover, they also studied 120 blood samples of Chinese people. While the frequencies of T16362C, G16129A and A16173C were about the same, they reported that the frequency of C16223T was 62.5%. This indicates that the high frequency of a C-to-T transition at nt16223 is characteristic of Japanese people. On the other hand, a T-to-A transversion at nt16217 is infrequent, but is extremely effective for personal identification, if detected.

Fourteen types of sequence patterns of C-stretch were identified for nt16184-16193 (Table 2 - HV1). Eighty-one cases or 57.9% showed the same structure (5C1T4C) as that reported by Anderson et al. This was followed by 41 cases (29.3%) of combined 10C and 11C being manifested and these three types accounted for about 90% of the total. Therefore, it is understood that if a DNA type other than these three types is detected, this would be useful for personal identification.

Next, having analyzed polymorphism in the HV2 region, A263G was observed in all 140 cases. For this reason, it may be considered that nt263 is guanine in the case of the Japanese people. In the event of a different nucleotide being found in this region, it may thus be considered that the identity of the body is other than ethnic Japanese. It seems that a difference in the position of nucleotide substitution is helpful in analysis in human genetics to characterize races. Moreover, 135 cases (96.4%) of *315.1C were identified. This finding is also believed to be characteristic of the Japanese. On the other hand, in nt76, samples of A-to-C transversion and of A-to-G transition were identified. Moreover, the frequencies of A248 and of G316del were low and thus, these deletions may be effective for personal identification.

With respect to the sequence pattern of C-stretch in nt303-315, there were no cases that showed the same structure (7C1T5C) as that reported by Anderson et al., and this finding is thought to be characteristic of the Japanese. The 8C1T6C, 7C1T6C and 9C1T6C patterns amount to approximately 95% showing a slant, but these were detected more or less equivalently. In addition, the samples that showed 6C1T6C and 11C2del accounted for only 0.7% (1 case). Detection of such a finding will enhance the reliability of identification.

Moreover, as a result of performing analysis on the polymorphism in the HV3 region, T489C was detected in 90 cases (64.3%). C522del and A523del were detected in 46 samples each, and these findings were characteristic.

With respect to the sequence pattern of C-stretch in nt568-573, 130 cases (92.9%) had the same structure (6C) as that reported by Anderson et al. and the fact that almost all exhibited this pattern may also be considered a characteristic specific to the Japanese.

Of the 46 cases in which nucleotide deletion was recognized at nt522 and nt523, 33 cases showed C-stretch of 8C1T6C in the HV2 region. This finding can be considered important in personal identification.

The 140 samples were classified into 128 kinds based on the sequence patterns of the three HV regions. As stated above, it was recognized that personal identification by polymorphism analysis of the hypervariable region in mtDNA extracted from old dental pulp is extremely useful. The use of DNA analysis is not limited to the area of forensic medicine and it is often used as a method in personal identification and confirmation of blood relationship in the areas of anthropology and archeology (23-25). In particular, analysis of mtDNA polymorphism is an effective method in tracking relationships with a deceased person. This is because mtDNA types are inherited from the mother. In 1994, it was reported that the analysis of autosomal STR and mtDNA was performed to confirm the identity of the corpses of Nicholas II, the last Czar of Imperial Russia and his family (11). There have apparently been no other reports investigating a single family tree using the hypervariable region in mtDNA. Hence, the samples gathered from 23 people comprising 4 generations of a single family line were considered extremely valuable in investigating a family line.

MtDNA types of the mother in the first generation were determined as C16223T-T16362C-5C1T4C in the HV1 region, A73G-C194T-A263G-T310C-C311T- *315.2C-8C1T6C in the HV2 region and T489C-C522del-A523del-6C in the HV3 region. These haplotypes of the mother in the first generation were inherited without mutation by all five brothers and sisters in the second generation (3 male children and 2 female children). One girl got married and delivered two girls who comprise the third generation. Furthermore, girl No.19 delivered one male and one female who make the fourth generation. It was reported that the mutation rate of mtDNA was 5 to 10 times higher than that of nucleus DNA (26), but, in this study, all mtDNA types of the first-generation mother were inherited by the fourth generation without fail (Fig. 2: bold line). In the future, we plan to analyze a wider range of nucleotide sequences and examine the presence of variations.

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