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ORIGINAL PAPER

Phylogenetic analysis of mitochondrial DNA in Japanese pedigrees of sensorineural hearing loss associated with the A1555G mutation

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Thirteen Japanese families (ten of which were from the northern part of Japan), with sensorineural hearing loss associated with the 1555 A to G (A1555G) mitochondrial mutation, a known cause of non-syndromic hearing loss, were phylogenetically analysed using data obtained by restriction fragment length polymorphism (RFLP) and D-loop sequencing of mitochondrial DNA (mtDNA). Various types of mtDNA polymorphism were detected by restriction enzymes and D-loop sequence. No common polymorphic pattern throughout the 13 families was found, though three families exhibited the same restriction patterns and the same sequence substitution in the D-loop. To find where each of the 13 families are situated in the phylogenetic tree, the 482-bp of D-loop sequence were compared with those of 62 normal Japanese subjects. Despite the three families mentioned above appearing to be clustered, the remaining 10 families were scattered along the phylogenetic tree. This indicates that there was no common ancestor for the 13 Japanese families bearing the A1555G mutation except three families, and that the A1555G mutation occurred sporadically and multiplied through evolution of the mtDNA in Japan. The present results showed that the common pathogenicity (hearing loss associated with the A1555G mutation) can occur sporadically in families which have different genetic backgrounds, even in the Japanese population.

Keywords: phylogenetic analysis; polymorphism; mitochondrial DNA; D-loop; sensorineural hearing loss; aminoglycoside

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Introduction

Mitochondrial mutations are known to be a factor in non-syndromic sensorineural hearing loss (SNHL). Among them, the A1555G mutation has been reported to cause susceptibility to aminoglycoside induced hearing loss.[1–3] According to our clinical studies[4] this mutation causes SNHL even without aminoglycoside exposure.

This mutation was first reported within populations in restricted areas, including Arab-Israeli, Chinese, and Japanese.[1–3] The regional predominance in Asian populations has lead us to hypothesize that there was a common ancestor of such families with the A1555G mutation. However, recent reports demonstrate that this mutation has also been found in Greek, English/Irish, Italian, Mexican, Puerto Rican, and Vietnamese,[5] Zaïrean,[6] Spanish,[7] and Mongolian populations.[8] This suggests that the A1555G may exist all over the world. This widespread existence of the A1555G mutation gave rise to an interesting question as to the origin of the A1555G mutation. With regard to occurrence of this mutation, phylogenetic analysis of 10 independent families with the A1555G mutation from Africa and Asia indicated it had multiple origins, occurring on each racial-specific mtDNA haplotype.[9] However, there still remains a question as to whether this mutation occurs sporadically even within individual racial populations or occurs on some specific haplogroups within individual populations (eg Japanese). Within the Asian populations the A1555G mutation has been reported to occur predominantly on a specific haplogroup, which is rare in the Caucasian population.[10] Hutchin and Cortopassi postulated that the type II haplogroup[10] may have the potential risk for deafness. We therefore examined whether the A1555G families belong to some specific haplogroups.

Polymorphism found in Japanese pedigrees was studied on the basis of

1) restriction enzyme analysis (restriction fragment length polymorphism – RFLP),
2) the presence of the 9-bp deletion, which is known to be a valuable marker in examining populations with Asian affinities,[11,12] and
3) the sequence of the D-loop region, which is more rapidly evolved than other parts and therefore more variable and a useful evolutional marker.[13]

The polymorphism of the 13 Japanese families revealed by the present study provided informative results as to the origin of the A1555G mutation within the Japanese population.

Materials and Methods

Samples

Blood samples obtained with informed consent from 13 subjects from 13 Japanese families (one subject per family) with the A1555G mutation were used in the present study. Ten families originated from Aomori Prefecture, in the northern area of the main Japanese island, one family from Tokyo, in the central part of Japan, and two families from Kumamoto Prefecture, in the southern area. The control samples were from 62 subjects from the main island of Japan.[14] Total genomic DNA was prepared by means of a GENOMIX Kit (Talent, Trieste, Italy).

The A1555G Mutation

The A1555G mutation was identified as previously described.[4] In brief, PCR products (the nucleotide of 1252 to 1726) were digested with the restriction enzyme (Alw 26I), electrophoresed and visualised on an agarose gel. The 1555 mutant mtDNA was visualised as the loss of a restriction enzyme site. The PCR-amplified products were also sequenced with an ABI sequencer 373A (Perkin Elmer Co Ltd, Foster City, CA, USA) to confirm the conversion of the nucleotide of 1555 from A to G.

Restriction Enzyme Analysis

Amplification of mtDNA by PCR was carried out using 7 sets of primers according to Torroni *et al.*[15] 5'->3' primer coordinates for RFLP analysis were as follows (upper, lower): (533–553, 722–701), (5140–5161, 5469–5451), (10280–10298, 10548–10529), (13200–13219, 13537–13518), (16286–16306, 16547–16529), (8196–8215, 8316–8297), (7888–7908, and 8288–8310). The Cambridge Reference Sequence[16] was used for primer design.

The PCR was performed in a Perkin Elmer 9600 thermocycler using 200 ng genomic DNA, 10–1000 pmol of each primer, 1–2 mM $MgCl_2$, 2 mM dNTP, 10 × buffer, 5 U Taq Gold (Perkin Elmer, Foster City, CA, USA) and enough H_2O to create a 100 μl PCR reaction mixture. Ten minutes preheating at 95°C was followed by 35 three-step cycles (95°C for 30 s, 55°C for 1 min, 72°C for 1 min) ending with 10 min at 72°C in a Perkin Elmer/Cetus thermal cycler. Amplified fragments were digested with eight restriction enzymes (AluI, DdeI, HaeIII, HincII, RsaI, AvaII, Sau3AI, and TaqI),[15,17–19] electrophoresed on 2–3% agarose gels stained with ethidium bromide, and observed under UV light.

The 9-bp Deletion

Mitochondrial DNA 9-bp deletion of one of the two tandem repeats (CCCCCTCTA) between nt 8272 and 8289 within the COII/tRNA$^{(Lys)}$ region[11,12,20] was examined. Fragments including the 9-bp deletion region were amplified using primers nt 8196–8215 and nt 8316–8297.[12] The PCR products were electrophoresed using 6% polyacrylamide gels (40% acrylamide stock fluids, 1 × TBE, TEMED, 10% APS, 18 hours). After staining with ethidium bromide, the length of these fragments were visualised by UV light.

DNA Sequencing

The D-loop region is located at 16024–16569 and 1–648. To amplify the D-loop region, primer pairs (nt15945–15969, 748–767) were used for PCR. The nucleotide sequence was determined by using a dye termination method. For sequencing the large fragments, a series of 8 primers was used. Primers for D-loop region sequencing were as follows (5'- > 3'): 15945–15965, 16238–16257, 16560–10, 316–335, 767–748, 452–432, 130–111, and 16389–16329.

Phylogenetic Analysis

The nucleotide sequences of a 482-bp fragment of the D-loop region (positions 16129–16569 followed by positions 1–41 in the reference sequence of Anderson *et al*[16]) from the present family subjects together with 62 normal subjects[14] were aligned and compared. The number of nucleotide substitutions per site between individual sequences was estimated by the Kimura two-parameter model of nucleotide substitutions.[21] On the basis of the estimated number of nucleotide substitutions, a phylogenetic tree was constructed using the unweighted pair-group method with arithmetic mean (UPGMA).[22]

Results

Table 1 summarises polymorphism of mtDNA in all 13 pedigrees revealed by

1) restriction enzyme analysis,
2) the presence of 9-bp deletion, and
3) sequence substitution between nt 1252 and 1726 by means of direct sequencing.

Various patterns revealed by the RFLP analysis were evident. Among the 13 families, 5 families (Nos 1, 2, 6, 11 and 12) showed identical patterns with regard to the RFLP analysis. The 9-bp deletion was found in 6 families (1, 2, 6, 11, 12 and 13). Direct sequencing confirmed the A1555G mutation for all the families. In families 7 and 9, the A1382C mutation was also found, but no other mutations between nt 1252 and 1726 were revealed in our samples. Whilst each family showed two to six mutations, no mutated site except for the A1555G mutation was common among all 13 families (Table 1).

Table 2 shows mutations found in the D-loop region by direct sequence analysis. A total of 45 mutations were found and each family possessed an average of 11.4. The A73G and A263G mutations were common among the families studied. Three families (1, 2 and 6) out of the five mentioned above (families 1, 2, 6, 11 and 12) showed the same set of mutations.

Mean pairwise divergences of total D-loop between all families were calculated to be from 0 to 1.59% (mean 1.11%). In the most variable part in the D-loop (16024–16393), the sequence divergence was from 0 to 3.51% (mean 2.13%).

Figure 1 shows the phylogenetic analysis of the 13 families by comparison of 482-bp of D-loop sequences in them with those of 62 normal Japanese subjects.[14] The 13 families were not clustered but were scattered along the phylogenetic tree.

Discussion

Mitochondrial DNA polymorphism of Japanese has been demonstrated using various restriction enzymes as

Table 1 Polymorphism in 13 families

	–AluI 5176	*+AluI 10397*	*+AluI 13262*	*+DdeI 10397*	*+HaeIII 663*	*+HaeIII 16517*	*–HineII 13259*	*–HineII 16329*	*9bp deletion*	*+AvaII 8249*	*+Sau3AI 8019*	*+TaqI 8022*	*1382 A→C*	*1555 A→G*
Family 1	–	–	–	+	–	+	–	–	D	–	–	–	–	+
Family 2	–	–	–	+	–	+	–	–	D	–	–	–	–	+
Family 3	–	–	–	–	+	–	–	–	N	–	–	–	–	+
Family 4	–	–	–	–	+	+	–	–	N	–	–	–	–	+
Family 5	–	–	–	–	–	+	–	–	N	–	–	–	–	+
Family 6	–	–	–	+	–	+	–	–	D	–	–	–	–	+
Family 7	+	+	–	+	–	+	–	–	N	–	–	–	+	+
Family 8	–	–	–	+	–	–	–	–	N	–	–	–	–	+
Family 9	–	–	–	+	–	+	–	–	N	–	–	–	+	+
Family 10	–	–	–	+	–	–	–	–	N	–	–	–	–	+
Family 11	–	–	–	+	–	+	–	–	D	–	–	–	–	+
Family 12	–	–	–	+	–	+	–	–	D	–	–	–	–	+
Family 13	–	+	–	+	–	–	–	–	D	–	–	–	–	+

Polymorphism found in 13 families associated with the A1555G mutation, defined by 8 restriction enzymes, the presence of 9-bp deletion, and the sequence substitution between nt 1252 and 1726. Thirteen families showing various polymorphic patterns, with an identical pattern in five (families 1, 2, 6, 1 and 12).
+: mutated, –: not mutated, D: the presence of 9-bp deletion, N: the absence of 9-bp deletion.

Table 2 Sequence substitution in the D-loop region

Family:	1	2	3	4	5	6	7	8	9	10	11	12	13
(nt)515–524:AC deletion	+	+	+	+	+	+	+	–	+	+	+	+	–
489							T→C	T→C	T→C	T→C			T→C
381								C→A					
263	A→G	A→G	A→G	A→G	A→G	A→G	A→G	A→G	A→G	A→G	A→G	A→G	A→G
235			A→G	A→G									
210												A→G	
207	G→A	G→A				G→A							
204	T→C	T→C				T→C					T→C	T→C	
199													T→C
194							C→T		C→T				
186			C→T										
152			T→C	T→C			T→C						
150													C→T
146								T→C					
131	T→C	T→C				T→C					T→C		
93												A→G	
73	A→G	A→G	A→G	A→G	A→G	A→G	A→G	A→G	A→G	A→G	A→G	A→G	A→G
16519	T→C	T→C		T→C	T→C	T→C	T→C		T→C		T→C	T→C	
16463	A→G	A→G				A→G					A→G		
16362							T→C	T→C	T→C	T→C			
16356				T→G									
16344					C→T								
16319			G→A	G→A						G→A			
16311					T→C								
16304					T→C				T→C				
16298													T→C
16297													T→C
16296					C→T								
16291									C→T		C→T		
16290			C→T	C→T									
16266													C→A
16249					T→C								
16243	T→C	T→C				T→C					T→C		
16234	C→T	C→T				C→T					C→T		
16223				C→T	C→T			C→T	C→T	C→T		C→T	
16215									A→G				
16189	T→C	T→C			T→C	T→C					T→C	T→C	T→C
16187			C→T									C→T	
16183	A→C	A→C			A→C	A→C					A→C		
16182					A→C						A→C		
16140	T→C	T→C				T→C					T→C	T→C	
16129					G→A							G→A	G→A
16124			T→C										
16111	C→T	C→T				C→T					C→T		
16093							T→C						
Total 45 sites	14	14	10	10	14	14	9	7	11	7	15	13	9

The sequence substitution found in the D-loop region (compared with the sequence reported by Anderson *et al*[16]). Each family shows 7–15 polymorphic sites. No common substitution pattern is evident except for families 1, 2 and 6.

well as D-loop sequence.[17,20,23,24] A series of observations revealed the characteristic features of Japanese ethnic background. Horai *et al*[17] classified 62 different combinations of restriction types by using 9 restriction enzymes, and showed a clear separation into two major clusters (groups I and II). This grouping was also compatible with the phylogenetic analysis using D-loop sequence.[14] The thirteen families were examined to see which haplogroup they belonged to by various restriction sites as well as D-loop sequences. Although RFLP indicated that there were completely identical patterns in five families (Table 1, families 1, 2, 6, 11 and 12), there were no common polymorphic patterns within the 13 families. According to the above classification (group I/II), families 1, 2, 6, 11 and 12 belong to group I. Since the other eight families belong to group II, the

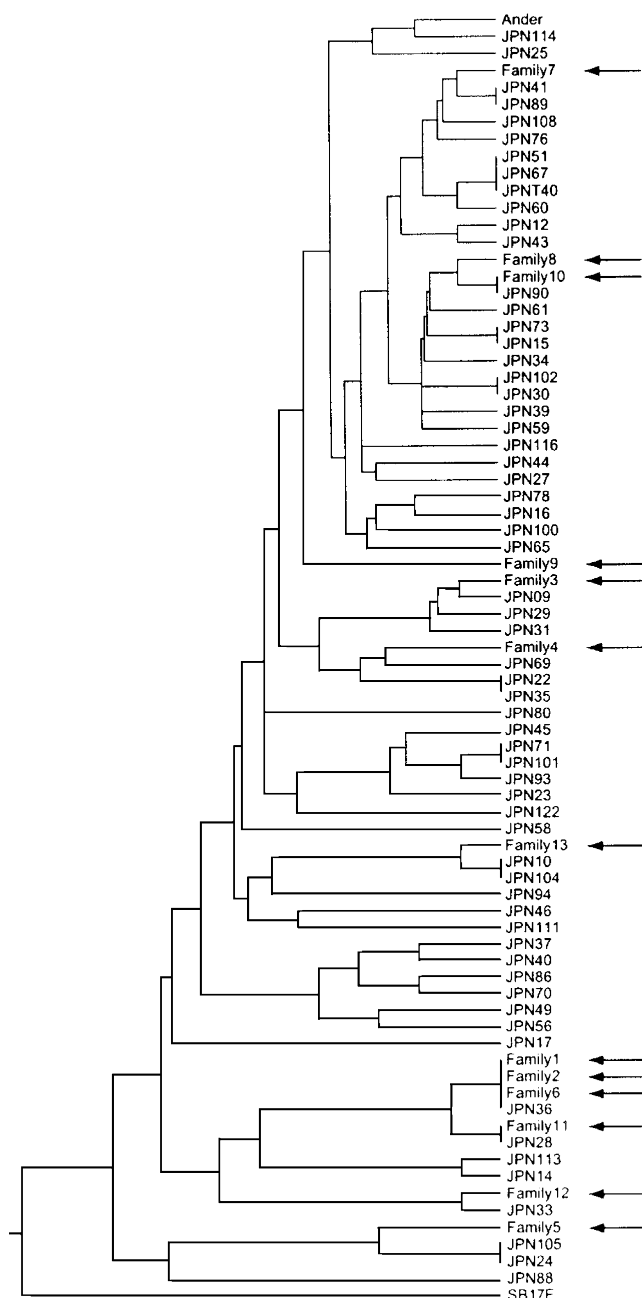

Figure 1 *UPGMA-tree based on 482-bp sequences. Phylogenetic tree showing the 13 families (arrows) compared with 62 normal Japanese subjects, 1 European (Ander), and 1 African (SB17). Three families (1, 2 and 6) belong to the same haplotype, though the remaining ten families are found throughout the phylogenetic tree.*

A1555G mutation may not be related to those haplogroups. In contrast to the data by Hutchin *et al*[9,10] who indicated that there is a high frequency of A1555G carriers in persons with group II mtDNA, our data did not show any preferential occurrence of the A1555G mutation in group II.

The intergenic COII/tRNA$^{(Lys)}$ 9-bp deletion has been reported in various racial populations with varying frequency, and to be an especially valuable anthropological marker for people of Asian origin.[11,12,20] In the present study, although six out of the 13 families (46%) showed 9-bp deletion, if families 1, 2 and 6 were to have a common ancestor as discussed later, the corrected frequency might be 36% (4/11), which is comparable with the reported frequency of 28% in northern Japan.[25] Therefore there is unlikely to be a relationship between the haplogroup with 9-bp deletion and the A1555G mutation.

A recent study indicated that the D-loop region, which is more rapidly evolved than other parts and therefore more variable, has been used as an evolutional marker.[13] We therefore analysed the D-loop region of mtDNA by the direct sequencing method, in addition to restriction enzyme analysis of mtDNA and the 9-bp deletion. In the present study, the sequences of the mitochondrial D-loop were variable and no common patterns were seen in the 13 families, except in three (families 1, 2 and 6) from the same region (the northern part of Japan).

Within D-loop sequences of the 13 Japanese families studied, an A to G conversion at both sites 73 and 263 was commonly found. One or both of these two mutations may possibly be associated with the A1555G mutation responsible for hearing impairment. However, since these two mutations have been reported to be relatively common in the Japanese,[26] they are unlikely to be relevant to hearing impairment; in fact, non-affected family members also have those two base conversions. Further study will be necessary to determine if these two mutations are markers of a more mutagenic mitochondrion.

Mean pairwise divergence of D-loop in this study (mean 1.11%: range 0–1.51%) is compatible with that previously reported in Mongoloid subjects.[14] Mean pairwise divergence of the hyper variable part of the D-loop (mean 2.13%: range 0–3.51%) is not lower than that (mean 2.2%: range 0.51–4.3%) reported in African and Asian subjects bearing the A1555G mutation by Hutchin *et al.*[9] If the 13 families in this study had a common ancestor, the mean pairwise divergence would be smaller, but the variable tendency in pairwise divergence suggested the multiple mutational occurrence.

Evolutionary relationships between families represented by the UPGMA-tree based on 482-bp sequences show that families with the A1555G mutation are

distributed widely across all the postulated branches of the Japanese evolutionary tree (Figure 1). Although the three families (1, 2 and 6 from the northern part of Japan) which have the same polymorphic patterns belong to the same group, the other families were found sporadically. In Figure 1, families next to each of the 13 families did not possess the A1555G mutation, suggesting this mutation may be a recent and sporadic mutational event during the mtDNA divergence.

With regard to the polymorphism found in the subjects associated with the A1555G mutation, recent phylogenetic analysis of the mtDNA of African and Asian patients bearing the A1555G mutation indicated that the A1555G mutation occurred in each racially specific mtDNA haplotype.[9] Results from our phylogenetic analysis of 13 Japanese families were also compatible with the multiple occurrence of this mutation, but did not support A1555G mutation occurrence on some Asian-specific haplotypes.[9,10] Our data indicated that this mutation occurred sporadically during the mtDNA evolution even within the Japanese population. In addition, it is likely that some minor families may have had a common ancestor.

As to association of disease-related mitochondrial mutations with particular haplogroups, differing results have been reported. In Leber's hereditary optic neuropathy (LHON)[27,28] several pathogenetic mitochondrial mutations have been reported. Among these, some were found to be associated with some particular haplogroups and some were found to not be so.[29] The A4336G mutation, which is known to be related to Alzheimers and Parkinson's disease, was found to be associated with some particular haplogroups.[9] However, the A1555G mutation was found in this study to be randomly distributed throughout the phylogenetic tree.

A question remains as to why the A1555G mutation has been reported mainly in Asian ancestry. The higher incidence of the A1555G mutation in the Japanese population may be interpreted in two ways. First, aminoglycoside antibiotics were commonly used in Japan in 1950–1970. Since aminoglycoside exposure has been more common in Asiatic populations, it is perhaps reasonable to expect a higher frequency of deletion of A1555G-associated aminoglycoside deafness. However, recent wide screening in our population resulted in our finding several hearing-impaired patients bearing the A1555G mutation who had no history of aminoglycoside injection.[4] An alternative explanation is that there is some genetic background in the Japanese population which exacerbates the effect of the A1555G mutation. It is possible, as hypothesised by Jabor *et al*,[30] that there is an additional recessive gene. The involvement of a secondary mutation is another possibility that has not yet been proven.

However, the present results from three different approaches (restriction enzyme analysis, the presence of the 9-bp deletion, and the sequence of the D-loop region) did not provide evidence of any possible haplogroups which may be related to the A1555G mutation in the Japanese population.

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