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Fragmentation of Human Heart Mitochondrial DNA Associated with Premature Aging

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Summary: **Point mutations, oxygen damage and deletions in the heart mitochondrial (mt) DNA of a 19-year-old male patient with premature aging, who died of mitochondrial cardiomyopathy, were comprehensively analyzed. With total base-sequencing, one *syn*⁻ mutation in the $tRNA^{Asp}$ gene and one *mit*⁻ mutation in the ND3 gene were demonstrated. Using microHPLC/MS, 0.20% of the total deoxyguanosine (dG) were proved to be converted into its hydroxy-radical adduct, 8-hydroxy-dG, of which amount corresponds to that in normal subjects of 78 years old. The total detection system for mtDNA deletions, using 180 kinds of primer pairs, revealed extensive fragmentation of mtDNA; 235 types of deletions existed with various sizes, 97 of which yielded mtDNA minicircles lacking both of the replication origins of light- and heavy-strands. Deleted mtDNA accounted for 84% of the total mtDNA. In a man died from an accident at age 28 having almost the same mtDNA genotype except *syn*⁻, 50 types of deleted mtDNA, accounting for 15% of the total, were detected in his heart mtDNA. These results will present a clue to an unidentified mechanism of somatic mtDNA replication and the molecular basis of aging heart.**

In 1989, we proposed that the accumulation of mitochondrial genome mutations during life is an important responsible factor to the development of aging and degenerative diseases (1). Since the proposal, accumulating evidence revealed the increase of somatic mutations in mitochondrial (mt)DNA of the post-mitotic cells such as brain (2, 3, 4), heart (5), and muscle cells (6, 7, 8, 9). In human heart mtDNA, we recently quantitatively determined an exponential increase of the 7.4 kilo base pair (kbp) deletion correlative with oxygen damage associated with age (10). Such accumulation of somatic mutations, being also observed among experimental animals, was thought to be the principal cause of aging heart (presbycardia) (11).

As pathogenic mechanisms of idiopathic cardiomyopathy, several point mutations (12, 13, 14) and deletions (15, 16, 17) were demonstrated in the heart mtDNA of some patients, in accordance with characteristic morphological changes of mitochondria and deterioration in mitochondrial function. Therefore, mitochondrial cardiomyopathy, defined as cardiomyopathy caused by mtDNA mutations, has been advocated (18): this disease is a state of premature aging in heart, where the somatic mutations of mtDNA were abnormally accelerated.

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In this paper, whole mutations in the heart mtDNA of a patient with typical mitochondrial cardiomyopathy were comprehensively determined using a total base-sequencing method (19) for germ-line point mutations, a micro high-performance-liquid-chromatography/mass spectrometry (microHPLC/MS) system for somatic oxygen damage (20), and a newly designed total detection system for mtDNA deletions with 180 kinds of primer pairs covering all regions of mtDNA circle. The obtained results demonstrated that a *syn*$^-$ mutation in the tRNA^{Asp} gene initiated substantial accumulation of oxygen damage and deletions of mtDNA. The deletions resulted in over 200 kinds of mtDNA fragments including nearly 100 kinds of mtDNA minicircles lacking both of the replication origins (Or) of light- and heavy- strands (OrL, OrH). Existence of Or$^-$ mtDNA minicircles could present a clue to solve the mechanism of mtDNA replication and the genesis of mitochondrial cardiomyopathy and presbycardia.

Materials and Methods

Total mtDNA base-sequencing: Fluorescence-based direct sequencing was performed according to the method reported previously (19).

Quantitative determination of oxygen damage in mtDNA: Mitochondrial fraction was prepared from 10 grams of heart muscle by the method of Hatefi *et al.* (21), and purified by a discontinuous sucrose density-gradient centrifugation. MtDNA was extracted with phenol and chloroform. A part of mtDNA specimen was subjected to enzymatic hydrolysis with DNase I, spleen exonuclease, snake venom exonuclease, and alkaline phosphatase. Deoxyguanosine (dG), 8-hydroxy-dG (8-OH-dG) and total deoxynucleoside contents in the hydrolysate were quantitatively determined, using a micro high-performance-liquid-chromatography/mass spectrometry (microHPLC/MS) system (20).

Quantitative determination of the wild-type and deleted mtDNAs: As the total amount of deoxynucleosides (ng_t) in the mtDNA hydrolysate derived from both of the wild-type and deleted mtDNAs in the mtDNA specimen was quantified by the microHPLC/MS, the amount of the wild-type mtDNA (ng_w) was quantified by the method with ethidium bromide agarose plates (22). After *Bam*HI digests of mtDNA specimen were electrophoresed, ng_w was obtained from florescence intensity of the 16.5 kbp band. Percentage of the deleted mtDNA was calculated as $100 \times (ng_t - ng_w) / ng_t$.

A total detection system for mtDNA deletions: A total detection system for mtDNA deletions was newly devised. In principle, 30 of L-strand primers distanced from 500 to 600 bp each other were synthesized to cover all around the mtDNA circle. To each L-strand primer, six H-strand primers, distanced 3 kbp each, were synthesized. Thus, overall 180 kinds of primer pairs were used to detect any deletion without exception, and detected deletions were distinguished from the artifact derived from the misanealing of primers by the primer shift PCR method (23), which was automatically built in the system by the combination of these primers. Extracted DNA from the heart muscle was amplified by PCR. The amplified fragments were separated by electrophoresis on 1% agarose gels, stained with ethidium bromide, and detected fluorographically.

Results

The patient was normally delivered after an uneventful pregnancy. From the age of 5, he suffered from neurogenic hearing impairment and growth failure. At age 10, he had a tremor and a generalized convulsion seizure. Elevated levels of lactate and pyruvate in his serum were also recognized. From the age of 12, he had ankylosis, a senile masque, and gradual development of

heart failure upto the 4 degrees according to the New York Heart Association's Standard. He was hospitalized suffering from pneumonia, and died of respiratory and heart failure at age 19. Autopsy revealed that there were severe atherosclerosis in his basilar artery and several infarctions in his putamen. Ragged red fibers and fiber atrophy were recognized in his limb muscle. The myocardial specimen, examined by an electron microscopy, showed that abnormally expanded mitochondria with inclusions (some of them were glycogen granules) proliferated extensively, associated with atrophy and break of muscle fibers, as shown in Fig. 1.

Using the total base-sequencing method (19), subsequently diverged 32 base-substitutions were detected in the patient's mtDNA comparing with the mitochondrial Eve's sequence that is deduced from the total mtDNA sequences of 46 individuals obtained in our laboratory (18). Divergency of the base-substitutions indicated that the patient is a member of the genetic pedigree of mitochondrial cardiomyopathy (18). Among the base-substitutions, seven possible germ-line mutations were demonstrated in his heart mtDNA (Table I): four base-substitutions causing the replacement of non-conserved amino acids, one causing the replacement of a conserved amino acid

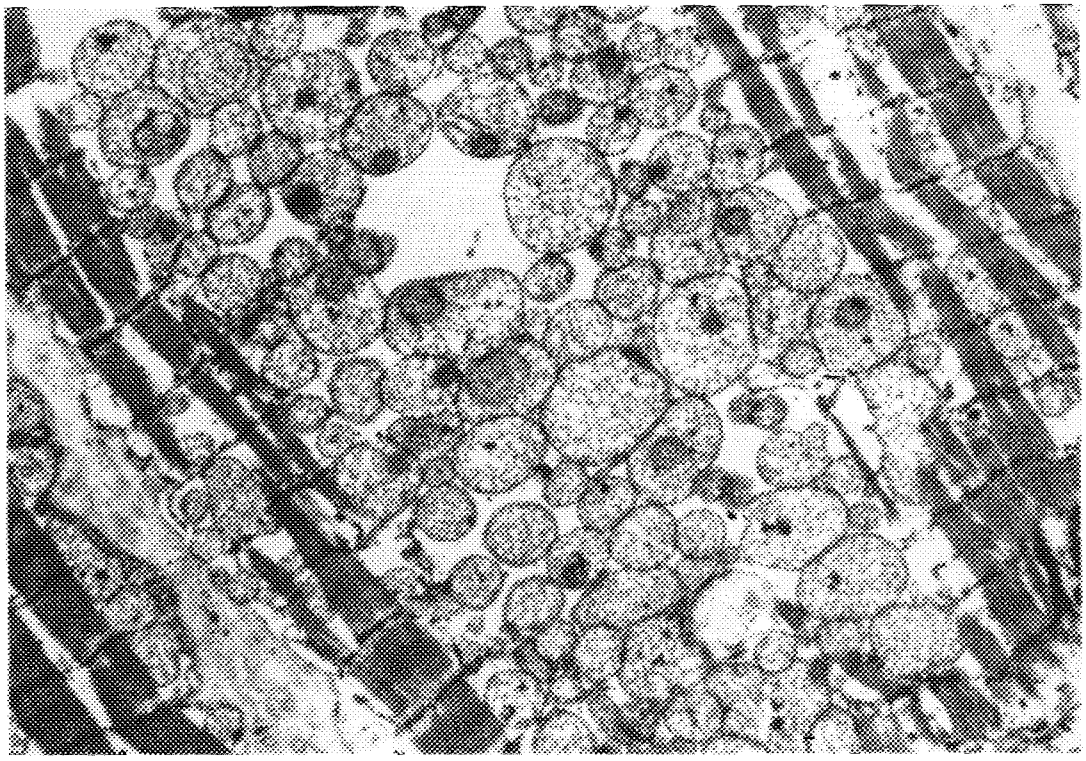

Fig. 1. Abnormal mitochondria in myocardium of the patient.
The patient died of heart failure at age 19. His heart was autopsied one hour after death. A myocardium specimen was examined by electron microscopy. His heart specimen was examined with an electron microscopy. Abnormally expanded mitochondria proliferated and muscle fibers showed severe atrophic and regressive changes.

Table I. Possible point mutations detected in mtDNA of the patient and the *syn*⁻ negative control

	Position	*Gene*	*Std*	*Mut*	*Std*	*Mut*	*N/C*	*Patient*	*Control*
1	5178	ND2	C	A	*Leu*	*Met*	*N*	+	+
2	7579	tRNA-Asp	T	C			*C*	+	-
3	8414	ATP8	C	T	*Leu*	*Phe*	*N*	+	+
4	8701	ATP6	A	G	*Thr*	*Ala*	*N*	+	+
5	10398	ND3	A	C	*Thr*	*Ala*	*C*	+	+
6	10410	tRNA-Arg	T	C			*N*	+	-
7	14979	Cytb	T	C	*Ile*	*Thr*	*N*	+	+

Std, Standard mitochondrial Eve's sequence deduced from the total mtDNA sequence of 46 individuals (18); *Mut*, mutation; *C*, conserved among human, bovine, rat, and mouse; *N*, non conserved; *Leu*, leucine; *Met*, methionine; *Phe*, phenylalanine; *Thr*, threonine; *Ala*, alanine; *Ile*, isoleucine.

in the ND3 gene (thus could be regarded as a *mit*⁻ mutation), one in the $tRNA^{Asp}$ and one in the $tRNA^{Arg}$ gene. The base-substitution in the $tRNA^{Asp}$ gene, 7579 T-to-C transition, resulted in mismatched base-pair with 7524 A. Among all the known biological species, except *S. pombe*, A-T or G-C base pair at this aminoacyl acceptor stem was reported to be conserved (24), suggesting that impaired synthesis of mitochondrial proteins could result from this transition. Thus, this transition could be regarded as a *syn*⁻ mutation. In contrast, 10410 T in the aminoacyl acceptor stem of $tRNA^{Arg}$ gene is not conserved among mammals. The 10410 T-to-C transition changes the T-T base pair into C-T, suggesting that this is a non-significant base-substitution. As a conclusion, the patient's mtDNA possessed two germ-line mutations: one *syn*⁻ mutation in the $tRNA^{Asp}$ gene and one *mit*⁻ mutation in the ND3 gene. A Chinese man, who had an almost similar genotype with that of the patient, died from an accident at age 28. As shown in Table I, his possible germ-line mutations were identical with the patient, except in the two tRNA genes. In addition, 21 out of 27 missense base-substitutions in his mtDNA were also identical with those of the patient. Thus, this subject could be regarded as the *syn*⁻ negative control.

Using a microHPLC/MS system, a substantial amount of oxygen damage in the patient's mtDNA was detected: namely, 0.20 % of dG were converted into 8-OH-dG, a hydroxyl-radical adduct of dG. Even among normal subjects, oxygen damage accumulated exponentially with age [log (8-OH-dG %) = -3.23 + 0.0407 x age, $r = 0.87$, $P < 0.01$] (11). The 8-OH-dG content of the patient, 0.20%, corresponded to that of 78-year-old normal subject, as shown in Fig. 2, and the 8-OH-dG content of the *syn*⁻ negative control was below the detection limit, 0.01%.

A newly designed total detection system for mtDNA deletions revealed that there were 235 types of deletions with various sizes in the patient's heart mtDNA; these were confirmed simultaneously by the primer shift PCR method built in the detection system. As shown in Fig. 3, they included 48 types of deletions where both of the reported OrL and OrH were preserved, 59 types of deletions resulting in mtDNA fragments lacking OrL but not OrH, 31 types resulting in mtDNA fragments lacking OrH but not OrL, and 97 types lacking both OrL and OrH yielding "Or⁻ mtDNA minicircle". As shown in Table II, the precise locations of the numbered deletions (No. 1 to 6) in Fig. 3 were determined by direct mtDNA sequencing across the deletion breakpoints. The

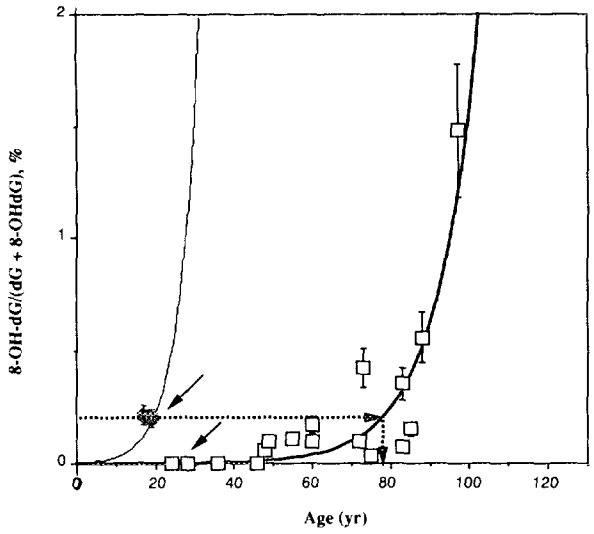

Fig. 2. The 8-OH-dG content in heart mtDNA of the patient.
The 8-OH-dG contents were plotted against the age of subjects. In the case of normal subjects, shown by open squares, the ratio of 8-OH-dG to (dG + 8-OH-dG) increases exponentially with age [log (8-OH-dG %) = -3.23 + 0.0407 x age, $r = 0.87$, $P < 0.01$] (10). The contents of 8-OH-dG of the patient (pointed by an arrow) and another similar patient who died of mitochondrial cardiomyopathy at age 17 were plotted by solid circles. By substituting 8-OH-dG content and age of the patient into the equation obtained with the normal subjects, a curve for the patient was deduced. An arrowed open square stands for the *syn*⁻ negative control's. Arrows with dashed lines indicate that 8-OH-dG content (0.20%) of the patient corresponds to that of a normal subject of age 78.

4,977 bp (No. 1) and the 7,436 bp deletions (No. 2) have been previously shown to occur in an age related manner (2, 5, 25, 11). Ratio of the total amount of deoxynucleosides in the mtDNA hydrolysate and that of the wild-type mtDNA revealed that deleted mtDNA in the patient's mtDNA specimen accounted for 84% of the total, indicating extensive fragmentation of mtDNA. In contrast, the *syn*⁻ negative control possessed 50 types of deletions accounting for 15% of the total mtDNA (Fig. 3).

Discussion

The mitochondrial cardiomyopathy patient comprehensively analyzed in this paper had the remarkable symptoms of progeria, such as arteriosclerosis, a senile masque, and multiple infarctions in the basal ganglia, which were quite different from those in patients with mitochondrial encephalomyopathy. Therefore, this was a quite informative case to elucidate the connection between mtDNA mutations and aging.

Abnormal proliferation of expanded mitochondria and severe atrophic muscle fibers were observed in his myocardium (Fig. 1), corresponding to extensive mtDNA mutations (Figs. 2 and

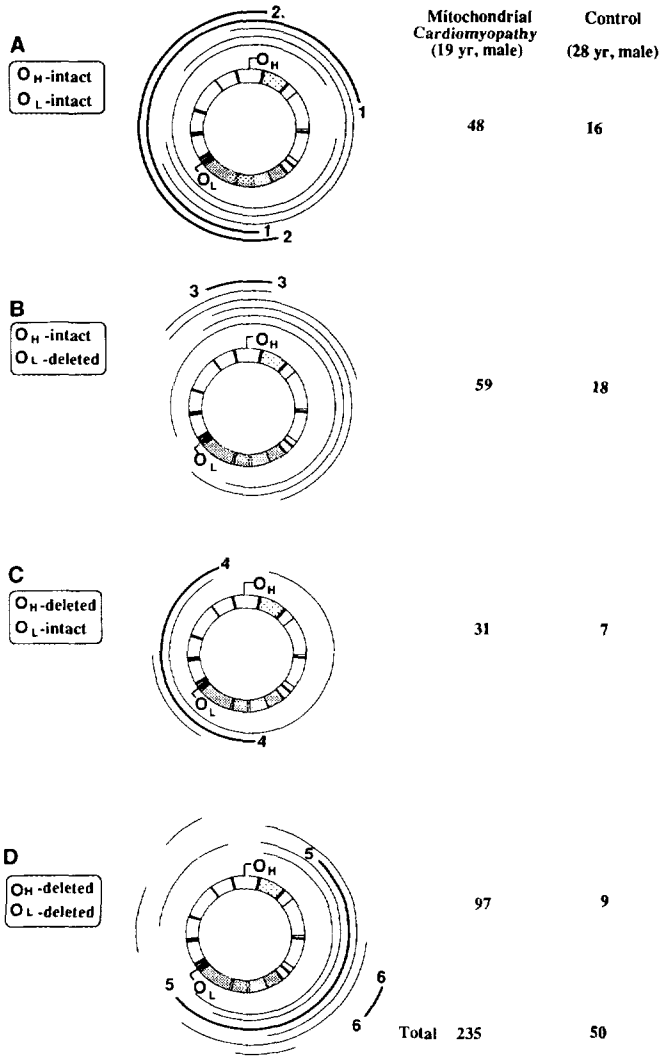

Fig. 3. Various types of deletions detected in heart mtDNA.
A novel total detection system for all kinds of deletions in mtDNA was designed with 180 different primer pairs. In the patient's heart mtDNA, 235 types of deletions are detected, which consist of four groups of deletions; A group of deleted mtDNA having the two preserved replication origins (total number 48), B group having preserved OrH but no OrL (total 59), C group having preserved OrL but no OrH (total 31), and D group having no replication origins (total 97). The deleted mtDNA accounted for 84% of the patient's total mtDNA.
In contrast to the patient, 50 types of deletions were detected in mtDNA of the negative control, which similarly consisted of four groups. The deleted mtDNA accounted for 19% of the total mtDNA.

3), which settled the diagnosis of mitochondrial cardiomyopathy. However, the size of his heart remained normal, not belonging to the conventional category of either hypertrophic or dilated cardiomyopathy.

Table II. Nucleotide sequences of the deletion breakpoint regions

No	bp	Sequence
1	4,977	8470 CACCTACCTCCCTCACCA(*AAGCC*.....*CTTCA*ACCTCCCTCACCA)TTGGC ATPase8 ND5
2	7,436	8637 TATCTCATCAACAACCG(*ACTAA*.....*TCACC*CATCAACAACCG)CTATG ATPase6 D-loop
3	15,217	888 TTTCGTGCCAGCCACC(*GCGGT*.....*ATTAC*TGCCAGCCACC)ATGAA 12S rRNA D-loop
4	8,739	8405 TAATTACCCCCAT(*ACTCC*.....*AAGAC*ACCCCCCACAGTTTAT)GTAGC ATPase8 tRNA-Phe
5	8,047	14411 CCAAGACCTCAACCCCC(*TGACC*.....*ATTTT*ACCTCACCCCC)ACTGA ND6 CO1
6	16,063	11059 CTAATCTCCCTACAA(*ATCTC*.....*ATATC*CTCCCTACTA)TGCCT ND4 ND4L

Direct repeat sequences are underlined and the numbers above each sequence indicate the position of the first nucleotide of repeat. The deleted regions (indicated by italics) are inside the parentheses.

This patient was transmitted 32 germ-line base-substitutions, including one serious point mutation in the $tRNA^{Asp}$ gene. This *syn*$^-$ mutation could be regarded as an important initiator of oxygen damage in his mtDNA (Fig. 2), as another base-substitution in the $tRNA^{Arg}$ gene occurred at a non-conserved position, and one *mit*$^-$ mutation was identical with the *syn*$^-$ negative control (Table I) whose amount of oxygen damage was below the detection limit (Fig. 2). He accumulated 8-OH-dG in his mtDNA even at age 19 equivalent to that of 78-year-old normal subjects. 8-OH-dG has recently been reported to induce random point mutations at the time of replication (25). In yeast mtDNA, it is an experimental observation that *mit*$^-$ mutations are usually associated with a significant rise in the rate of deletions (1). It was our surprise that over 200 different types of deletions including nearly 100 kinds of Or$^-$ mtDNA minicircles existed in mtDNA of one individual. Up to the present, the survey of mtDNA deletions, among several laboratories including ours, has been usually carried out within limited regions of mtDNA eliminating both OrL and OrH regions. This would result from a preconception that both OrL and OrH are essential for mammalian mtDNA replication, despite some evidence that discontinuous synthesis occurs in mammalian mtDNA (26) and in sea urchin oocytes (27). The survey of deletion within the limited regions has provoked curiosity (28) that there is no obvious correlation between the severity of the clinical symptoms or biochemical abnormality and either the location of the deletion or the number of deleted genes.

The presence of 187 mtDNA fragments lacking OrL and/or OrH in the patient's heart, and the presence of 34 mtDNA fragments of the similar type in the *syn*⁻ negative control (Fig. 3) clearly eliminates absolute requirement of both of the replication sites for somatic replication of mtDNA. Actually, until now, two reports have described the existence of large deletions lacking H-strand promoter region (29) or OrL (30) in human mtDNA. Although the replication mechanism of Or⁻ mtDNA minicircles, lacking both of the replication origins, is still obscure, a replication mechanism similar to that observed in stable DNA replication mutants of *Escherichia coli* without *ori C* (31) is likely to operate.

Comparison of the amount of total deoxynucleosides in the mtDNA hydrolysates, with that of wild-type mtDNA revealed that the deleted mtDNA accounted for 84% of the patient's total mtDNA, indicating extensive fragmentation of mtDNA associated with oxygen damage. In the negative control for the *syn*⁻ mutation, 50 types of deletions (Fig. 3) accounting for 19% of the total mtDNA were detected. Thus, it could be concluded that the germ-line *syn*⁻ mutation in the patient's mtDNA initiates oxygen damage that promotes the massive accumulation of mtDNA fragments by vicious cycle as proposed previously (10). There seems to be also a clear correlation between the amount of the oxygen damage and that of deletions, as reported previously (11).

The comprehensive mtDNA mutation analysis presented here will dissolve curious conventional discrepancy (28) between the mtDNA genotype as point mutations or deletions, and its phenotype as mitochondrial diseases or aging. It also presents a clue to elucidate the mechanism of somatic mtDNA replication.

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