FEBS Letters 354 (1994) 79–83

FEBS 14676

Ageing-associated tandem duplications in the D-loop of mitochondrial DNA of human muscle

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Received 11 July 1994; revised version received 8 September 1994

Abstract Using PCR techniques, we detected a ~260 bp (type I) and a ~200 bp (type II) tandem duplications in the mtDNA of muscle biopsies from aged individuals. Only one 70-year-old subject was found to harbour the type I and 28 out of 58 subjects had type II duplication. About 90% of the subjects harbouring the duplicated mtDNAs also had the 4,977 bp deletion. Moreover, the incidence and quantity of the type II duplication were found to increase with age. The proportion of the type II duplicated mtDNA in the muscle of a 71-year-old subject was 3.1% while that of a 55-year-old individual was only 0.78%. We suggest that the tandem duplications occur alone or with mtDNA deletions in human tissues in an age-dependent manner, and thereby cause synergistic deleterious effects on mitochondrial respiratory functions in human ageing.

Key words: Mitochondrial DNA; D-loop; Duplication; Deletion; Human ageing; Muscle

1. Introduction

Human mitochondrial DNA (mtDNA) is a circular double-stranded DNA of 16,569 bp in size [1]. The genome contains genes coding for 13 polypeptides involved in respiration and oxidative phosphorylation, 2 rRNAs and a set of 22 tRNAs that are essential for protein synthesis of the mitochondria [1]. In sharp contrast to the nuclear DNA, mtDNA is a naked compact DNA molecule without introns, and is replicated with much higher rate without proof-reading and efficient DNA repair mechanisms [2–6]. These characteristics have rendered mtDNA more vulnerable to attack by reactive oxygen species and free radicals that are generated by the electron leak of the respiratory chain of mitochondria [7].

In the past few years, a number of point mutations, deletions and insertions have been found in mtDNA from patients with specific neuromuscular diseases [8,9]. Recently, two point mutations at nucleotide position (np) 3,243 and np 8,344 of mtDNA, which are respectively associated with mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes syndrome (MELAS) and myoclonic epilepsy and ragged-red fibers disease (MERRF), were detected in the muscle mtDNA of old humans [10,11]. Moreover, several mtDNA deletions have also been identified in various tissues of old humans [12–20]. The most common one is the 4,977 bp deletion with a 13 bp direct repeat flanking the 5′- and 3′-end breakpoints at np 8470/8482 and np 13447/13459. This deletion was originally observed in the muscle of patients with mitochondrial myopathies, including the Kearns–Sayre syndrome (KSS) and chronic progressive external ophthalmoplegia (CPEO) [21,22]. However, the proportions of the age-related mtDNA deletions in various tissues of old humans are not so high as those of the patients with mitochondrial myopathies that they could not be detected by Southern hybridization and are usually detected by the polymerase chain reaction (PCR) technique.

Recently, Brockington et al. [23] reported a heteroplasmic tandem duplication with an approximate size of ~260 bp in the D-loop of human mtDNA and proposed that it is associated with large-scale deletions in mtDNAs of patients with mitochondrial myopathies. They further suggested that the duplicated mtDNA might be responsible for mild mitochondrial dysfunction in the muscle of the mothers of some patients, and is a predisposition to mtDNA deletions.

Accumulation of mutations in mtDNA has been recently proposed to be the major cause of respiratory function decline during human ageing process [24]. On the basis of the observations of the existence of disease-associated mutations in the mtDNA of old humans, we have investigated if the tandem duplicated mtDNA also exists in aged individuals. Interestingly, we found two types of tandem duplicated mtDNAs: type I is identical to the ~260 bp duplication, while type II is a novel ~200 bp partially duplicated mtDNA. The relationship between the duplications and the common 4,977 bp deletion in mtDNA during human ageing process is discussed.

2. Materials and methods

2.1. Preparation of samples

We obtained 58 muscle biopsies from Chinese subjects of different ages in the Veterans General Hospital, Taipei. All the samples were obtained with consent of the patients during exploratory laparotomy or surgical operations. All the patients were screened by clinical, biochemical and genetic criteria to exclude the possibility of being associated with any of the known mitochondrial diseases. In the genetic approach, we had examined all the samples by reported methods [25,26] to exclude the np 3,243 A-to-G and np 8,344 A-to-G point mutations that are associated with MELAS and MERRF syndromes, respectively. Large-scale deletions other than ageing-associated 4,977 bp and 7,436 bp deletions were also examined by PCR using primers encompassing the region between replication origin for heavy strand (O_H) and that for light strand (O_L). Total DNA was prepared from the specimens by the proteinase K/SDS protocol similar to that described by Sambrook et al. [27].

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Abbreviations: PCR, polymerase chain reaction; mtDNA, mitochondrial DNA; np, nucleotide position; MELAS, mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes syndrome; MERRF, myoclonic epilepsy and ragged-red fibers disease; KSS, Kearns–Sayre syndrome; CPEO, chronic progressive external ophthalmoplegia.

SSDI 0014-5793(94)01063-3

2.2. Polymerase chain reaction (PCR)

Each desired mtDNA fragment was amplified in a 50 μl reaction medium containing 200 μM of each dNTPs, 1 unit of Taq DNA polymerase (Perkin-Elmer/Cetus), 0.4 μM of each primer, 50 mM KCl, 1.5 mM $MgCl_2$ and 10 mM Tris-HCl, pH 8.3. The sequences of primers were identical to those described by Brockington and coworkers [23]: L336, 5′-AACACATCTCTGCCAAACCC-3′ (np 336–355 of mtDNA); L467, 5′-CCCATACTACTAATCTCATC-3′ (np 467–486); H335, 5′-TAAGTGCTGTGGCCAGAAGC-3′ (np 335–316); and H466, 5′-AGTGGGAGGGGAAAATAATG-3′ (np 466–447). The DNA amplification reactions were carried out for 30 cycles in a Perkin-Elmer/Cetus DNA thermal cycler. The first cycle was done by 3 min denaturation at 94°C, 3 min annealing at 55°C and 1 min extension at 72°C. The thermal profile of the following PCR cycles was as follows: denaturation at 94°C for 40 s, annealing at 55°C for 40 s and extension at 72°C for 50 s.

2.3. DNA sequencing

The PCR product was treated with the Klenow fragment and then cloned by blunt-end ligation to an *Eco*RV digested pGEM-5zf(−) plasmid (Promega Co., Madison, WI). DNA sequencing was performed using the SequiTherm cycle sequencing kit (Epicentre Technologies Co., Madison, WI) according to the instructions of the manufacturer.

2.4. Semi-quantitative PCR

Total DNA from each muscle biopsy was serially diluted by two fold with distilled water. The range of dilution was usually between 2^0 to 2^{22}. The primers L3108 and H3324 were used to amplify a 236 bp DNA fragment from total mtDNA, and the primers L336 and H335 were used for the amplification of a ~200 bp PCR product from the type II duplicated mtDNA. The sequences of the primers L3108 and H3324 were 5′-TTCAAATTCCTCCCTGTACG-3′ (np 3108 to 3127 of mtDNA) and 5′-TGGGTACAATGAGGAGTAGG-3′ (np 3343 to 3324), respectively. Amplified DNA fragments were separated by electrophoresis on 1.5% agarose gels at 100 V for 40 min and were detected fluorographically under UV light transillumination after staining with ethidium bromide. The proportion of the type II duplicated mtDNA was determined by the ratio of the highest dilution fold that allowed the ~200 bp PCR product amplified from the type II duplicated mtDNA to be visible on the gel to that which allowed the 236 bp PCR product to be visibly amplified from the total mtDNA under identical conditions [19].

3. Results

By the PCR method using primer pairs L336–H335 and L467–H466, we first screened for the ~260 bp tandem duplication in mtDNAs of the muscle biopsies from 58 normal Chinese subjects. The ages of the subjects ranged from 1 to 79 years, which represent a broad age spectrum. Only one 70-year-old subject was found to harbour the ~260 bp duplicated mtDNA in his muscle. Further, we detected another novel ~200 bp tandem duplication in the D-loop of mtDNA from 28 out of the 58 subjects. Although the exact size of the novel duplication was not known, the length of the PCR fragment amplified from the novel duplicated mtDNA was about 200 bp (Fig. 1). The PCR products obtained from the two primer pairs were identical in size. In a separate experiment by using the primers L467-H335, we obtained a less intense ~70 bp PCR product from the mtDNAs harbouring the ~200 bp tandem duplication (data not shown). These results indicate that the duplication is authentic and that the size is very close to 200 bp.

By cloning and sequencing, we demonstrated that the sequence of the ~260 bp fragment was similar to that reported by Brockington et al. [23]. The sequence from np 302 to 447 contained two additional cytosines (C) near the 5′ end of this region, which was inserted at np 567 (Fig. 2). Moreover, we found that the sequence of the novel ~200 bp fragment was

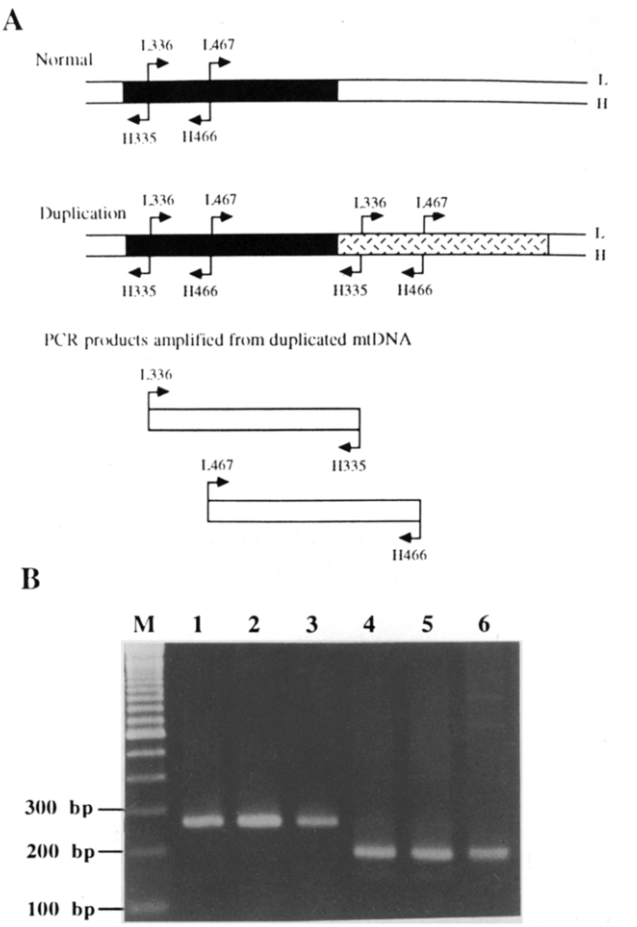

Fig. 1. PCR amplification of the ~260 bp and ~200 bp tandem duplications in the D-loop of mtDNA from muscle biopsies of old humans. (A) Two pairs of primers L336–H335 and L467–H466 were used to amplify the D-loop sequence of human mtDNA. Since the primers were such designed (back to back) that no PCR product could be generated from normal mtDNA and only those mtDNA molecules containing the putative tandem duplications were amplified to give rise the PCR product with a distinct size. (B) The ~260 bp PCR products amplified with primers L336–H335 (lanes 1 and 2) and L467–H466 (lane 3) for 30 cycles from mtDNAs of muscle biopsies from a 36-year-old subject with mitochondrial myopathy (lane 1) and that from a 70-year-old normal subject (lanes 2 and 3). Lanes 4 to 6 represent the ~200 bp PCR products amplified with primers L336–H335 (lanes 4 and 5) and L467–H466 (lane 6) from mtDNAs of the muscle biopsies from two normal subjects of 71 (lane 4) and 75 (lanes 5 and 6) years old, respectively. M indicates the 100 bp ladder DNA size marker.

different from that of the ~260 bp fragment. There were also two additional C nucleotides near the 5′ end of the segment from np 302 to 447, which was inserted at np 493 of mtDNA (Fig. 2).

In order to test the hypothesis that tandem duplication is a predisposition to the large-scale deletion of mtDNA, we also screened for the 4,977 bp deletion in mtDNAs of the muscle biopsies from the 58 subjects. The results showed that about one-third of the subjects who had the 4,977 bp deletion did not harbour the ~200 bp tandem duplication and that near 10% of the subjects who had the tandem duplication did not carry the 4,977 bp deletion in their muscles (Table 1). On the other hand, we found that the ~200 bp tandem duplication occurred more frequently and abundantly in subjects of older age groups than

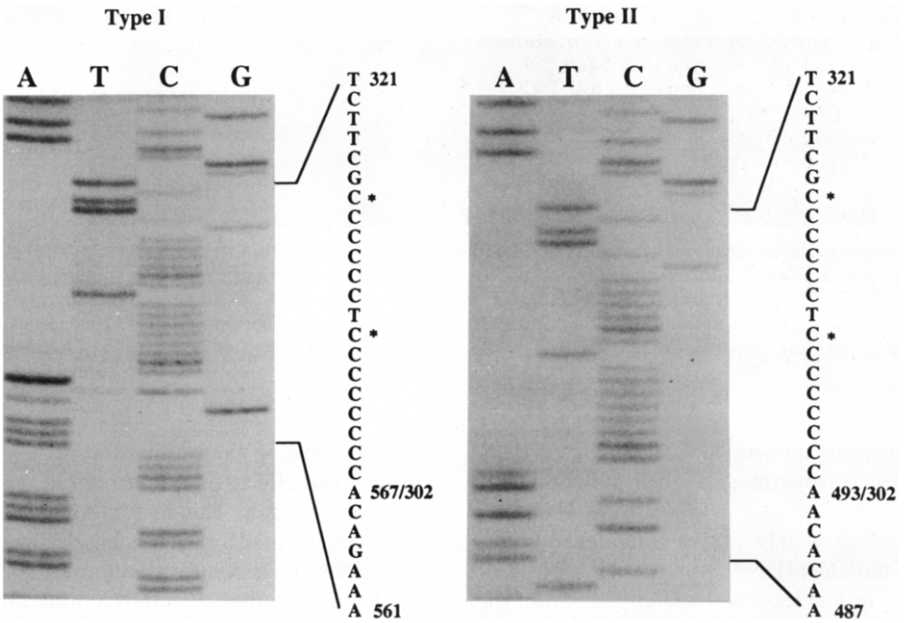

Fig. 2. Light strand nucleotide sequence of mtDNA showing the 5′-end junction of the type I and type II tandem duplications. Type I (left panel) duplication of ~260 bp was inserted with a junction sequence of ACCCCCCC to the np 567 near the heavy strand promoter region. Type II (right panel) duplication, with ~200 bp in size, was inserted by an identical AC_7 sequence to np 493 between the $tRNA^{Phe}$ and heavy strand promoter. The additional C residues are marked with asterisks at the end of the poly C run in the inserted duplicated sequence.

did in younger subjects (Fig. 3). The frequency of occurrence of the type II duplication in mtDNAs of the muscle biopsies from 58 subjects was 0% (0/4), 16.7% (1/6), 50% (3/6), 27.3% (3/11), 100% (3/3), 56.3% (9/16) and 75% (9/12) for the subjects in the age groups of 0–9, 10–29, 30–39, 40–49, 50–59, 60–69 and 70–79, respectively. The youngest subject that was found to harbour the type II duplication was 18. The type II duplication was not detectable by the two primer pairs in 3 of the 12 individuals above 70 years of age. However, one of the three subjects was found to harbour the ~260 bp duplicated mtDNA in his muscle biopsies (Table 1).

Using a semi-quantitative PCR method [19], we determined the proportion of the type II duplicated mtDNA from some samples that harboured the ~200 bp duplication. We found that about 3.1% of the type II duplicated mtDNA was present in the muscle of a 71-year-old subject, while only about 0.78% of the duplicated mtDNA was detected in the muscle of an individual of 55 years old.

4. Discussion

The D-loop of human mtDNA contains the replication origin O_H and two transcriptional promoters for each strand of mtDNA [5]. The replication origin O_L is nested within a cluster of five tRNA genes well away from the D-loop. Initiation of L-strand synthesis occurs after O_L is exposed as a single-stranded template by displacement accompanying the synthesis of a nascent H-strand during mtDNA replication. Thus, O_H is the dominant element for initiation of mtDNA replication. On the other hand, the promoters in the D-loop region are the only established promoters regulating the transcription of human mtDNA [28,29]. Therefore, the D-loop is the most important control region in human mtDNA. Any types of mutation in the regulatory elements in the D-loop of mtDNA can cause alterations of mitochondrial DNA replication and/or transcription. Brockington et al. [23] detected a ~260 bp tandem duplicated mtDNA in the muscle from some of the patients with mitochondrial myopathies. The duplication results in additional copies of conserved sequence blocks (CSB) II and III, and light strand and heavy strand promoters. They proposed that the duplication may account for a paucity of H strand transcripts but normal or slightly increased amounts of L strand transcripts of mtDNA as detected by in situ hybridization. Moreover, they suggested that large-scale mtDNA deletions are asso-

Table 1
Coexistence of 4,977 bp deletion and tandem duplications in the D-loop of human muscle mtDNA from Chinese subjects of different ages

Age	1	1	6	8	10	10	17	18	18	25	35	35	36	36	38	39	40	41	42	42	42	45	46	46	47	47	49	55	55
4,977 bp deletion	−	−	−	−	−	−	+	−	+	+	+	+	+	+	−	−	−	−	−	−	+	−	+	+	+	+	+	+	+
Type I duplication	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Type II duplication	−	−	−	−	−	−	−	−	+	+	−	−	−	+	+	+	−	−	−	+	−	−	−	−	−	+	+	+	+
Age	55	60	60	60	60	61	62	64	64	65	65	65	65	66	67	67	68	70	70	70	71	71	71	71	72	74	75	76	79
4,977 bp deletion	+	+	+	+	+	+	+	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Type I duplication	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−
Type II duplication	+	−	−	+	+	+	+	−	−	+	−	+	+	−	−	+	+	−	+	+	−	+	+	+	+	+	+	−	+

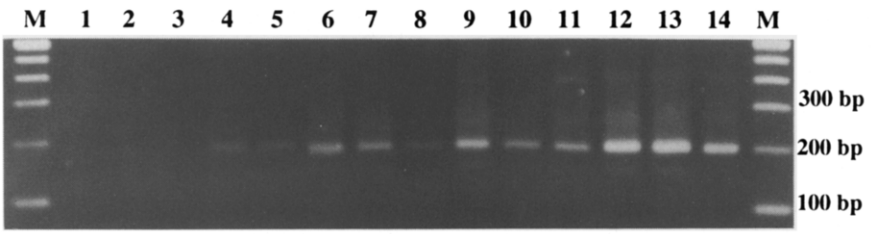

Fig. 3. Age-dependent increase in the incidence and abundance of the ~200 bp duplicated mtDNA in human muscle. The ~200 bp PCR products were amplified with the primer pair L336–H335 for 30 cycles from mtDNAs of the muscle biopsies from subjects (lanes 1 to 14) with ages of 1, 8, 35, 36, 38, 39, 49, 62, 65, 68, 71, 71, 75, and 79, respectively. M indicates the 100 bp ladder DNA size marker. Equal amounts of total DNA from each of the muscle biopsies were used for PCR amplification under identical conditions as described in section 2. It is noteworthy that the intensity of the ~200 bp band increased with the age of the subjects.

ciated with the heteroplasmic tandem duplications in some patients with mitochondrial myopathies and their maternal relatives.

In this study, we demonstrated two types of tandem duplications in the D-loop of muscle mtDNA from normal subjects of different ages (Fig. 1). The incidence and abundance of the type II duplication in mtDNAs of the muscle biopsies were found to increase with age (Fig. 3). Moreover, quantitative PCR analysis indicated that the proportion of the type II duplicated mtDNA of older subjects was higher than that of younger ones. By contrast, the type I duplication was only detected in the muscle of a 70-year-old subject.

We have reported an age-related increase in the incidence and the proportion of the 4,977 bp-deleted mtDNA of the same set of muscle biopsies examined in this study [19]. The 4,977 bp deletion and the tandem duplications were found to coexist in muscle mtDNAs of 60% of the subjects (Table 1). The incidence of the 4,977 bp deletion and that of the tandem duplication in mtDNA both increased with age. However, it is worth mentioning that 15 subjects that harboured the 4,977 bp-deleted mtDNA did not carry either tandem duplications and that 4 subjects who had the type II duplicated mtDNA did not exhibit the 4,977 bp deletion. No clear relationship was found between the 4,977 bp deletion and the tandem duplications.

It is important to note that the two types of tandem duplications are inserted at the site with perfect direct repeats of $A(C)_n$ and that the sequence near the 5′ junction regions of the duplications contained an extra C in the bridging repeat and a further C in the polyC run directly downstream from it. The number of the extra C flanking this bridging region was noted to vary among different old individuals (data not shown). In cultured human KB cells, the length variation of the np 302–308 region has been shown to be due to a homopolymer sequence which can range in length from 6 to 12 residues [30]. The significance and mechanism causing the additional C nucleotides in the tandem duplications in the D-loop of mtDNA are not clear.

Accumulation of mtDNA mutations has been proposed to be an important contributor to the ageing process of the human [24]. A number of point mutations and deletions in mtDNA have been established to increase in an age-dependent manner in old humans [8,10,11]. In this study, the type II tandem duplication in the D-loop of mtDNA was detected in the muscle tissues of a number of clinically normal old individuals. There may exist other types of duplications in addition to these well-characterized mtDNA mutations in human ageing. These mutations, existing alone or in combinations, will cause synergistic deleterious effects on mitochondrial respiration and oxidative phosphorylation. This may account, at least in part, for the well-documented age-dependent decline of human bioenergetic functions. In conclusion, our results support the hypothesis that accumulation of mtDNA mutations in tissue cells is a contributory factor in the ageing process of the human.

Acknowledgements: This work was supported by research grants from the National Science Council (no. NSC83-0412-B010-031-M02) and the Department of Health (no. DOH83-HR-219), Executive Yuan, Republic of China. Y.H. Wei wishes to express his appreciation of the National Science Council and the Ching-Ling Medical Foundation for research awards received during this study.

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