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The point mutation of mitochondrial DNA characteristic for MERRF disease is found also in healthy people of different ages

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The A-to-G transition mutation in the $tRNA^{Lys}$ gene of mitochondrial DNA (mtDNA), characteristic for the maternally inherited MERRF syndrome (myoclonic epilepsy with ragged red fibers), has been identified by point mutation-specific polymerase chain reaction in extraocular muscle from 11 of 16 healthy people of different ages. No mutation was found in navel-string samples from 5 newborns, in HeLa cells, and in 2 individuals younger than 20 years. On the other hand, the mutation is present in all 5 tested 74–89-year-old individuals and in 6 of 9 20–70-year-old individuals. The amount of mutated from total mtDNA was estimated by 'mispairing PCR' in extraocular muscle of 2 individuals of 74 and 89 years to 2.0 and 2.4%, respectively. In most tissue samples the MERRF mutation occurs together with the 'common deletion' of mtDNA, which was previously shown to accumulate in healthy individuals with increasing age. It is proposed that during aging, deletions and point mutations of mtDNA accumulate, which could impair mitochondrial energetics.

MERRF syndrome; Mitochondrial DNA; Polymerase chain reaction; Point mutation-specific PCR; Aging; Common deletion

1. INTRODUCTION

The MERRF syndrome (myoclonic epilepsy with ragged red fibers) is a maternally inherited encephalomyopathy characterized by decreased activities of respiratory enzymes in skeletal muscle mitochondria [1,2]. Its molecular basis could be related to an A-to-G transition mutation at nucleotide pair (np) 8,344 in the gene for $tRNA^{Lys}$ of mitochondrial DNA (mtDNA) [3]. Results on the incorporation of [^{35}S]methionine into mitochondrial proteins of cultured fibroblasts from MERRF patients have shown an impaired synthesis of enzyme complexes involved in respiration and oxidative phosphorylation [1,4], indicating an impaired function of the mutated $tRNA^{Lys}$.

It has been proposed that somatic mutations of mtDNA during aging will decrease the capacity of energy generation in tissues [5]. In addition it has been proposed that the final death of each individual is related to the accumulation of statistic somatic mutations of mtDNA, in particular of the heart, due to the constant turnover of mtDNA also in non-dividing cells [6]. This proposal was based on the demonstration of focal cytochrome *c* oxidase-defective cells in the heart [7] and in skeletal muscle fibres [8] with increasing age. By using the polymerase chain reaction (PCR), several recent publications have shown the occurrence of deletions of mtDNA in tissues of elderly people [9–15]. These deletions have so far been described only in mtDNA from patients with mitochondrial myopathies without proven maternal inheritance, such as Kearns Sayre syndrome or CPEO (chronic progressive external ophthalmoplegia) [16–20]. In other patients with different forms of mitochondrial diseases lacking deleted mtDNA, point mutations of various mitochondrial tRNA genes have been described [21].

We have applied a point mutation-specific PCR (PS-PCR) to identify low contents of the A-to-G transition mutation at np 8,344 in mtDNA, characteristic for the maternally inherited MERRF syndrome, in healthy people of increasing age. This method uses a primer containing a mismatched 3′ residue, and corresponds to the 'allele-specific PCR' (ASPCR) [22–23] or the 'amplification refractory mutation system' (ARMS) [24].

2. EXPERIMENTAL

2.1. *Preparation of DNA from human tissues*

Post mortem specimen of extraocular muscle and of skeletal muscle were obtained from human individuals of different ages, with no indications for a mitochondrial disease, between 3 and 19 h after death. Navel strings were obtained immediately after birth from the women's hospital. Total DNA was extracted and purified according to Wallace et al. [1], followed by digestion of RNA with RNase A, and the amount was quantified spectrophotometrically.

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Abbreviations: MERRF, myoclonic epilepsy with ragged red fibers; np, nucleotide pair; mtDNA, mitochondrial DNA; PCR, polymerase chain reaction.

2.2. *PCR amplifications*

PCR amplifications were carried out in 100 μl volumes with 100 ng total DNA of the indicated tissue, 200 μM of each dNTP (dATP, dCTP, dGTP, dTTP), 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 0.01% (w/v) gelatine, and 30 pmol each of the forward primer, P1 (np 8,259–8,280 of the mtDNA sequence [25]: TACCCTATAGCACCCCCTCTA), and the MERRF point mutation-specific reverse primer, P2 (np 8,369–8,344: CATTTCACTGTAAAGAGGTGTTGGC). Primer P2 included at its 3′ end the mutated nucleotide and will amplify under selected conditions only the mutated mtDNA. After 5 min at 94°C (hot start) 1 U *Taq* polymerase (Amersham) was added. The reactions were carried out in a Perkin Elmer Cetus DNA thermal cycler by 'touch down PCR' [26], including 1 min each of denaturation at 94°C, elongation at 72°C, and annealing from 72 to 70°C during the first 3 cycles, and at 69°C for a further 30 cycles.

Amplifications by 'mispairing PCR' [27] were performed with 10 ng total DNA with the P1 primer and the mispairing P3 primer (np 8,372–8,345: GGGGCATTTCACTGTAAAGAGGTGCCGG), in the presence of [α-^{32}P]dATP. The mispairing primer is homologeous to the 5′ region adjacent to the mutated nucleotide except for 2 nucleotides which create a restriction site for *Nae*I in the amplified fragment including the mutated nucleotide. The amplified fragments were precipitated with ethanol, treated with *Nae*I and separated on 8% polyacrylamide gels. The gel was silver stained for subsequent isolating and counting of the bands. The 'common deletion' was amplified with the forward primer, P1, and the reverse primer, P4 (np 13,795–13,774: TTAGGTAGAGGGGGATTGTTG). This primer pair, under the applied conditions, will only amplify the region around the common deletion (np 8,482–13,459), resulting in a DNA fragment of 560 np [28]. The PCR was performed for 35 cycles after hot start at 94°C denaturation, 58°C annealing and 72°C elongation temperature for 1 min each. From each reaction 5 μl were loaded on the 1% agarose gel.

3. RESULTS AND DISCUSSION

We have systematically investigated the optimal conditions for specific amplification of very small amounts of the mutated mtDNA at np 8,344 in the presence of excess wild-type mtDNA, and obtained specific amplification under the following conditions: hot start (i.e. incubation without *Taq* polymerase for 5 min at 94°C), touch down PCR [26], and annealing temperature of 69°C. Under these conditions no amplification of the fragment of 111 np was obtained with 5 different navelstring DNA samples and with DNA from HeLa cells, as shown in Fig. 1. In contrast, with DNA from extraocular muscle of 3 young and 8 elderly, and from skeletal muscle of 2 elderly people, the expected fragment was amplified under otherwise identical conditions. No amplification was obtained with DNA of extraocular muscle from 3 young and 2 elderly and from skeletal muscle of 1 elderly individual. This result indicates the accumulation with increasing age of the MERRF mutation in mtDNA of muscle tissue. The lack of mutated mtDNA in some tissue samples from elderly people indicates either the absence, or too small amounts, of this mutation to be detected by our method. It should be pointed out that the mutated mtDNA was found in males as well as in females.

To verify that the PCR-amplified bands represent the correct DNA fragment, the bands were electroeluted, digested with *Dde*I and separated on 8% acrylamide gels. The expected double bands of 52 and 59 np size were obtained (not shown).

In order to estimate the amount of mutated mtDNA, we amplified total mtDNA by mispairing PCR, which introduces a restriction site into the amplified DNA, and digested the amplified fragment with the restriction enzyme, *Nae*I, which cleaves only mutated mtDNA [27].

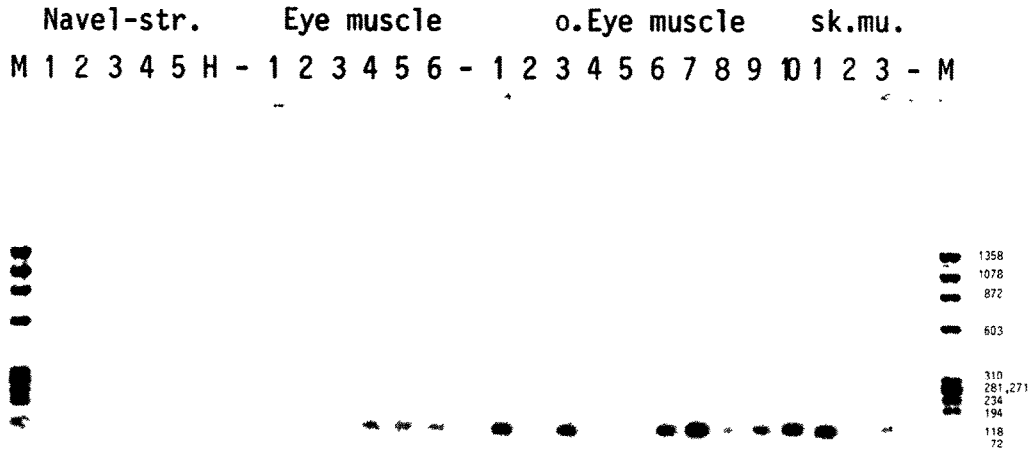

Fig. 1. Point mutation-specific PCR of mtDNA from different tissues of humans of variable age. The negative image of the ethidium bromide-stained 1% agarose gel is shown. M, marker (*Hae*III-digested PhiX 174 DNA) with the fragment size on the right side in np; Navel-str. (lanes 1–5), navel-string DNA from 5 different newborns; H, HeLa cell DNA; Eye muscle (lanes 1–6), DNA of extraocular muscle of younger people (sex in brackets) aged 17, 18, 20, 20 (m), 24 (m), and 30 (f), respectively; o.Eye muscle (lanes 1–10), DNA of extraocular muscle from old people aged 67 (f), 69 (m), 70 (f), 70 (m), 70 (f), 75 (f), 74 (m), 76 (m), 79 (m), and 89 (m), respectively; sk.mu. (lanes 1–3), skeletal muscle DNA of individuals aged 67–72 years.

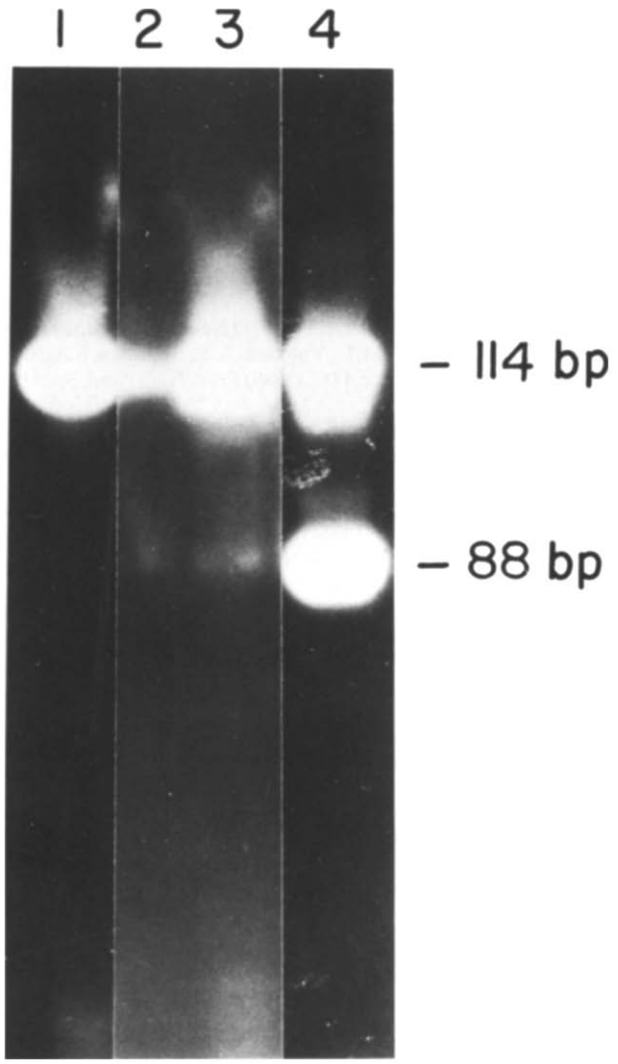

Fig. 2. Autoradiography of DNA fragments amplified by mispairing PCR and digested with the restriction endonuclease, *Nae*I. The reverse image of the autoradiography is shown. Lane 1, navel-string DNA; lane 2, DNA of extraocular muscle (age 89, male); lane 3, DNA of extraocular muscle (age 74, male); lane 4, DNA of skeletal muscle from a MERRF patient (patient IV-2 of [4]).

As shown in Fig. 2, no digestion of the amplified 114 np fragment from navel-string DNA, but a small amount of digested DNA (88 np) from amplified extraocular muscle DNA of 2 individuals, can be seen. In contrast, the amplified fragment from muscle DNA of a MERRF patient was largely digested by *Nae*I (Fig. 2, lane 4). The amount of mutated mtDNA was quantified by counting the radioactivity of the cleaved and uncleaved fragments, which had been labelled by [α-^{32}P]dATP during PCR. Table I shows that 2.4 and 2.0% of total mtDNA from extraocular muscle of 2 people, aged 89 and 74 years, respectively, were mutated at np 8,344. No radioactivity was found at the corresponding position of 88 np in lane 1 of Fig. 2 to which navel-string DNA was applied.

Table I

Content of mtDNA mutated at np 8,344 in individuals of different age and of a MERRF patient, as determined by mispairing PCR [27]

Lane of Fig. 2	cpm		% mutated mtDNA
	Upper band	Lower band	
1	3,474	0	0
2	817	16	2.4
3	3,911	61	2.0
4	2,603	5,657	73.6

The silver-stained bands of 114 and 88 np size of Fig. 2 were cut out and counted in a scintillation counter. The % of mutated mtDNA was calculated based on the adenosine content of the fragments.

We have also investigated the common deletion of mtDNA, which is characteristic for some cases of Kearns Sayre Syndrome and CPEO [16–20], and which was shown to occur also in healthy humans with increasing age [9–15]. In Fig. 3 is shown the amplified fragment of 560 np in most cases of extraocular muscles from elderly people but not from 17–30-year-old people, and not in skeletal muscle, HeLa cells and navel-string tissue.

These results demonstrate that aging is also associated, in addition to deletions of mtDNA, with point mutations in the tRNA^{Lys} gene of mtDNA, supporting the proposed accumulation of statistic somatic mutations of mtDNA with aging [5,6]. These mutations of mtDNA may contribute, in addition to other as yet unknown point mutations, to the defective respiratory

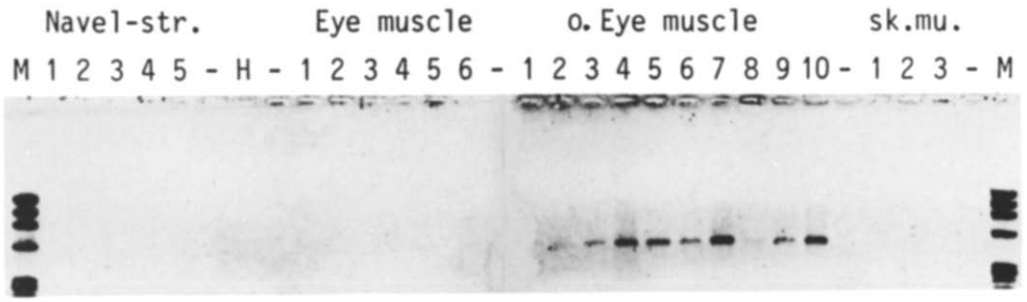

Fig. 3. PCR amplification of the region adjacent to the common deletion of mtDNA of various tissue samples. The negative image of the ethidium bromide-stained 1% agarose gel is shown. The same samples were applied to the indicated lanes as described in the legend to Fig. 1.

function of individual cells in aging tissue [7,8], and thus to the decrease of energetic capabilities of old people.

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REFERENCES

[1] Wallace, D.C., Zheng, X., Lott, M.T., Shoffner, J.M., Hodge, J.A., Kelley, R.I., Epstein, C.M. and Hopkins, L.C. (1988) Cell 55, 601–610.
[2] Lombes, A., Mendell, J.R., Nakase, H., Barohn, R.J., Bonilla, E., Zeviani, M., Yates, A.J., Omerza, J., Gales, T.L., Nakahara, K., Rizzuto, R., Engel, W.K. and DiMauro, S. (1989) Ann. Neurol. 26, 20–33.
[3] Shoffner, J.M., Lott, M.T., Lezza, A.M.S., Seibel, P., Ballinger, S.W. and Wallace, D.C. (1990) Cell 61, 931–937.
[4] Seibel, P., Degoul, F., Bonne, G., Romero, N., Francois, D., Paturneau-Jouas, M., Ziegler, F., Eymard, B., Fardeau, M., Marsac, C. and Kadenbach, B. (1991) J. Neurol. Sci. 105, 217–224.
[5] Linnane, A.W., Marzuki, S., Ozawa, T. and Tanaka, M. (1989) Lancet i, 642–645.
[6] Kadenbach, B. and Müller-Höcker, J. (1990) Naturwissch. 77, 221–225.
[7] Müller-Höcker, J. (1989) Am. J. Pathol. 134, 1167–1173.
[8] Müller-Höcker, J. (1990) J. Neurol. Sci. 100, 14–21.
[9] Cortopassi, G.A. and Arnheim, N. (1990) Nucleic Acids Res. 18, 6927–6933.
[10] Ikebe, S., Tanaka, M., Ohno, K., Sato, W., Hattori, K., Kondo, T., Mizuno, Y. and Ozawa, T. (1990) Biochem. Biophys. Res. Commun. 170, 1044–1048.
[11] Linnane, A.W., Baumer, A., Maxwell, R.J., Preston, H., Zhang, C. and Marzuki, S. (1990) Biochem. Int. 22, 1067–1076.
[12] Hattori, K., Tanaka, M., Sugiyama, S., Obayashi, T., Ito, T., Satake, T., Hanaki, Y., Asai, Y., Nagano, M. and Ozawa, T. (1991) Am. Heart J. 121, 1735–1742.
[13] Katayama, M., Tanaka, M. and Yamamoto, H. (1991) Biochem. Int. 25, 47–56.
[14] Sugiyama, S., Hattori, K., Hayakawa, M. and Ozawa, T. (1991) Biochem. Biophys. Res. Commun. 180, 894–899.
[15] Yen, T.C., Su, J.H., King, K.L. and Wei, Y.H. (1991) Biochem. Biophys. Res. Commun. 178, 124–131.
[16] Holt, I.J., Harding, A.E. and Morgan-Hughes, J.A. (1988) Nature 331, 717–719.
[17] Ozawa, T., Yoneda, M., Tanaka, M., Ohno, K., Sato, W., Suzuki, H., Nishikimi, M. and Horai, S. (1988) Biochem. Biophys. Res. Commun. 154, 1240–1247.
[18] Zeviani, M., Moraes, C.T., DiMauro, S., Nakase, H., Bonilla, E., Schon, E.A. and Rowland, L.P. (1988) Neurology 38, 1339–1346.
[19] Shoffner, J.M., Lott, M.T., Voljavec, A.S., Soueidan, S.A., Costigan, D.A. and Wallace, D.C. (1989) Proc. Natl. Acad. Sci. USA 86, 7952–7956.
[20] Obermaier-Kusser, B., Müller-Höcker, J., Nelson, I., Lestienne, P., Enter, C., Riedele, T. and Gerbitz, K.-D. (1990) Biochem. Biophys. Res. Commun. 169, 1007–1015.
[21] Lauber, J., Marsac, C., Kadenbach, B. and Seibel, P. (1991) Nucleic Acids Res. 19, 1393–1397.
[22] Wu, D.Y., Ugozzoli, L., Pal, B.K. and Wallace, R.B. (1989) Proc. Natl. Acad. Sci. USA 86, 2757–2760.
[23] Ruano, G. and Kidd, K.K. (1989) Nucleic Acids Res. 17, 8392–8392.
[24] Newton, C.R., Graham, A., Heptinstall, L.E., Powell, S.J., Summers, C., Kalsheker, N., Smith, J.C. and Markham, A.I. (1989) Nucleic Acids Res. 17, 2503–2516.
[25] Anderson, S., Bankier, A.T., Barrell, B.G., de Bruijn, M.H.L., Coulson, A.R., Drouin, J., Eperon, L.C., Nierlich, D.P., Rose, B.A., Sanger, F., Schreier, P.H., Smith, A.J.H., Staden, R. and Young, J.G. (1981) Nature 290, 457–465.
[26] Don, P.H., Cox, P.T., Wainwright, B.J., Baker, K. and Mattick, J.S. (1991) Nucleic Acids Res. 19, 4008–4008.
[27] Seibel, P., Degoul, F., Romero, N., Marsac, C. and Kadenbach, B. (1990) Biochem. Biophys. Res. Commun. 173, 561–565.
[28] Seibel, P., Mell, O., Hannemann, A., Müller-Höcker, J. and Kadenbach, B (1991) Methods Mol. Cell Biol. 2, 147–153.