Identification of a Novel Mitochondrial Mutation in Dupuytren's Disease Using Multiplex DHPLC

Ardeshir Bayat, Ph.D., M.R.C.S.(Eng.), A.F.R.C.S.(Ed.), Joanne Walter, B.Sc.(Hons.), Helen Lambe, B.Sc.(Hons.), James S. Watson, M.R.C.P., F.R.C.S.(Eng.), John K. Stanley, M.Ch.(Orth.), F.R.C.S.(Eng.Ed.), Michael Marino, Ph.D., Mark W. J. Ferguson, Ph.D., F.F.D., F.Med.Sci., C.B.E., and William E. R. Ollier, Ph.D., F.I.B.M.S., F.R.C.Path.

Manchester, Cheshire, and Lancashire, United Kingdom

Dupuytren's disease is a familial fibroproliferative disorder of late onset affecting the hands. It is extremely common in individuals of Northern European extraction. Genetic studies have yet to identify the genes involved in the formation of the disease. Mitochondria play a critical role in cell metabolism and apoptosis. It is known that defective mitochondria generate abnormally high levels of reactive oxygen species by means of electron leak and that antioxidant enzyme activities decrease with age in skin fibroblasts. Respiratory function of mitochondria is also impaired in aging human tissues. Oxidative stress and production of free radicals may be important factors in the pathogenesis of Dupuytren's disease. Mitochondrial genes are also included in the regulation of apoptosis. Diseased tissue contains large numbers of myofibroblasts, which disappear by apoptosis during normal wound healing. High numbers of mitochondria have been observed in fibroblasts derived from diseased tissue. In the light of this evidence, the mitochondrial genome represents a potential location for candidate susceptibility genes for this late-onset disorder. In this study, the authors investigated the presence of mutations within the mitochondrial genome in 40 subjects; 20 Caucasian Dupuytren's disease patients with a maternally transmitted inheritance pattern and 20 control subjects were matched for age, sex, and race using a multiplex denaturing high-performance liquid chromatography approach. A hitherto unknown heteroplasmic mutation located within the mitochondrial 16s rRNA region was evident in 90 percent of patients and absent from all control subjects ($p < 0.001$; $\chi^2 = 16.1$). This mutation may be important in the pathogenesis of Dupuytren's disease. (*Plast. Reconstr. Surg.* 115: 134, 2005.)

Dupuytren's disease is a nodular palmar fibromatosis that causes permanent contracture of the digits. It is a progressive and irreversible disorder with a high rate of recurrence after surgical excisional treatment.[1] It is a familial disorder that is highly prevalent in individuals of North European extraction.[2] It is considered to be one of the most common heritable disorders of connective tissue in Caucasians.[3]

Cases are sporadic, and a variety of inheritance patterns with variable penetrance have been proposed,[2] although no specific gene has been identified. It is, however, unclear whether Dupuytren's disease is a complex oligogenic

From the Center for Integrated Genomic Medical Research, University of Manchester; Transgenomic Ltd.; the Department of Plastic, Reconstructive, and Hand Surgery, Wythenshawe Hospital; the Upper Limb Unit, Wrightington Hospital for Joint Diseases; and the School of Biological Sciences, University of Manchester. Received for publication August 25, 2003; revised March 19, 2004.

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condition or a simple monogenic Mendelian disorder. The identification of susceptible gene loci would provide an approach to unraveling the hereditary component of this common disease. We have observed that some patients demonstrate apparent maternally inherited disease. As mitochondrial disorders are maternally inherited,[4] it is possible that the inheritance pattern of at least some forms of Dupuytren's disease is explained by transmission of mutant mitochondrial DNA through the maternal lineage.

The mitochondrial genome may contain candidate susceptibility genes for this late-onset disorder. The ability to scan human populations with high mutation detection efficiency and maximum sensitivity is important. The technology of choice has been shown to be denaturing high-performance liquid chromatography (DHPLC).[5–7] In this study, we investigated the presence of mutations within the mitochondrial genome using a multiplex DHPLC approach.

PATIENTS AND METHODS

Patients

Dupuytren's disease patients with apparent maternal inheritance (n = 20, 10 men and 10 women) were enrolled in the study. The mothers of all affected patients had demonstrated evidence of advanced Dupuytren's disease either by direct examination or from clinical records. Patients ranged in age from 38 to 78 years, with a mean age of 59.05 years (SD, 10.01 years). Patients were all unrelated Caucasians from the Northwest region of England; patients in the United Kingdom were mostly identified through operative record clinical codes from the South Manchester University Hospital Trust and Wrightington Hospitals in the North West region. All patients were seen by the author who took a full medical history using a pro forma and examined both hands and feet of each individual patient. All patients had a confirmed diagnosis of Dupuytren's disease preoperatively, with the presence of characteristic Dupuytren's nodules in the palm of the hand or digits with contracture of either the metacarpophalangeal joint or the proximal interphalangeal joint.

Control Subjects

Control subjects (n = 20, 10 men and 10 women) were all age (within 3 years)-, sex-, and ethnicity-matched healthy Caucasian men and women selected from the same region of the northwest of England. The age range of control subjects was 34 to 78 years, with a mean age of 57.8 years (SD, 10.04 years). All control subjects were seen by the author, who took a full medical history using a pro forma and examined for scars and both hands and feet of each individual subject to rule out the presence of any skin fibrotic disorders. In particular, all control subjects were questioned about the presence of Dupuytren's disease in either parents or siblings. None of the subjects had any evidence of Dupuytren's disease and none reported any family history of Dupuytren's disease. The local and hospital ethical committees gave approval for the study. Written consent was obtained from all individuals.

DNA Extraction

Blood samples were collected from subjects using standard venesection technique; 15 ml of venous blood was collected from every subject. DNA was extracted from peripheral blood cells using a commercially available DNA extraction kit (Qiagen, United Kingdom). DNA concentrations were measured and diluted in buffer to 100 ng/μl using sterile Qiagen buffer (Qiagen, Crawley, United Kingdom).

Fragment Construction and Design

The mitochondrial genome sequence was obtained from http://infinity.gen.emory.edu/mitomap.html. The fragments were designed to create amplified products that yielded, on digestion with a combination of only five restriction enzymes, fragments between 100 and 600 bp that could be analyzed by DHPLC at a minimum number of temperatures. Further design features were the creation of distinct fragments to cover the major mitochondrial genes and which could be easily amplified. Primers were designed using the Primer 3 software available at http://www.genome.wi.mit.edu/cgi-bin/rimer/primer3www.cgi and obtained from Invitrogen (Carlsbad, Calif.). The complete list of the fragments generated and primers used are summarized in Table I.

Polymerase Chain Reactions for DHPLC Analysis

Polymerase chain reactions were performed in a 100-μl volume containing approximately 100 ng of genomic DNA, 200 μM each dNTP (Cruachem Ltd., Glasgow, United Kingdom), 30 pmol of each forward and reverse primer,

TABLE I

Fragment Numbers, Position, and Gene in the Mitochondrial DNA and Fragment Length and Relevant Sequences*

Fragment	Position	Length (bp)	Segment of Gene Covered	Sequence	
				Forward Primer	Reverse Primer
1	15974–16409	436	Hypervariable region	ctc cac cat tag cac cca aag c	gag gat ggt ggt caa ggg acc
2	16341–102	331	Hypervariable region	tac agt caa atc cct tct cgt cc	tcc agc gtc tcg caa tgc tat c
3	29–480	452	Hypervariable region	ctc acg gga gct ctc cat gca t	att agt agt atg gga gtg gga gg
4	368–1713	1346	12s rRNA	acc cta aca cca gcc taa cca g	ttg tct ggt agt aag gtg gag tg
5	1650–2841	1192	16sRNA	aac tta act tga ccg ctc tga gc	agg ttg ggt tct gct ccg agg
6	2415–3811	1397	16srRNA, ND1	ctc act gtc aac cca aca cag g	tgt gtt gtg ata agg gtg gag ag
7	3429–4428	1000	ND1	ccc tac ggg cta cta caa ccc	ccc gat agc tta ttt agc tga cc
8	4180–5488	1309	ND2	act tcc tac cac tca ccc tag c	gga gat agg tag gag tag cgt g
9	5347–6382	1036	COI	cct acg cct aat cta ctc cac c	ccc taa gat aga gga gac acc tg
10	6318–7707	1390	COI, COII	ctg gag cct ccg tag acc taa c	ggc ata cag gac tag gaa gca g
11	7644–8784	1141	ATPase 8, ATPase 6	tat cac ctt tca tga tca cgc cc	gtc cga gga ggt tag ttg tgg c
12	8643–9458	816	ATPase 6, COIII	aac cga cta atc acc acc caa ca	gga tta tcc cgt atc gaa ggc c
13	9397–11397	2001	COIII, ND3, ND4L, ND4	aag cac ata cca agg cca cca c	gtg gag tcc gta aag agg tat c
14	11322–12852	1531	ND4	ctc ctg agc caa caa ctt aat atg	gga ttg ctt gaa tgg ctg ctg tg
15	12753–13264	512		ctg ttc atc ggc tga gag ggc	agt tga ctt gaa gtg gag aag gc
16	13172–14610	1439	ND6	ctt agg cgc tat cac cac tct g	taa gcc ttc tcc tat tta tgg gg
17	14427–15590	1164	Cytb	cca tgc ctc agg ata ctc ctc a	cgg aga att gtg tag gcg aat ag
18	15424–16451	1028	Cytb	aaa gac gcc ctc ggc tta ctt c	agc gag gag agt agc act ctt g

* Primer sequences were used to amplify the mitochondrial genome yielding fragment from 300–2000 bp. The correlation between primer set and mitochondrial gene analysis is shown.

and 2.5 units of Optimase polymerase (Transgenomic Ltd., Omaha, Neb.). Amplification took place using a MJ Research Thermal Cycler (MJ Research, Watertown, Mass.). Polymerase chain reaction conditions were as follows: an initial denaturation cycle of 95°C for 3 minutes followed by 14 cycles of 95°C for 30 seconds, touchdown annealing commencing at 63°C and decreasing by 0.5°C per cycle for 1 minute, and 72°C extension at 1 minute per 500 bp. A further 20 cycles were performed at 95°C for 30 seconds, 56°C for 1 minute, and 72°C at 1 minute per 500 bp. A final extension of 72°C for 5 minutes completed the amplification. Verification of amplification took place by analyzing 5 μl of sample using 2% agarose 0.5 M TBE gel electrophoresis stained with ethidium bromide and viewed under ultraviolet light.

Restriction Enzyme Digestion

The five restriction enzymes indicated in Table II were purchased from New England BioLabs (Beverly, Mass.); 88.5 μl of sample were mixed with 10 μl of the relevant restriction

TABLE II

Enzymes Used for Production of Multiplex Fragments for DHPLC and Their Analysis Temperatures

Fragment	Restriction Enzyme	Restriction Buffer Used	Enzyme Recognition Site	Fragments from Digestion	DHPLC Analysis Temperatures (°C)
1	None	None	–	436	58
2	None	None	–	331	60
3	None	None	–	452	57; 59
4	MboI	NEB-3	↓GATC	211+276+372+487	57; 58
5	HaeIII	NEB-2	GG↓CC	273+394+525	56; 59
6	DdeI	NEB-3	C↓TNAG	125+210+278+342+442	57; 58; 59
7	HaeIII	NEB-2	GG↓CC	109+179+242+470	57; 58; 60
8	MspI	NEB-2	C↓CGG	135+247+396+531	55; 56; 57
9	HaeIII	NEB-2	GG↓CC	122+190+233+491	58; 59
10	MspI	NEB-2	C↓CGG	117+162+253+354+504	55; 56; 57; 58
11	HaeIII	NEB-2	GG↓CC	141+181+212+243+364	56; 59
12	DdeI	NEB-3	C↓TNAG	187+239+390	57; 59
13	AluI	NEB-2	AG↓CT	248+312+366+487+588	54; 57;
14	HaeIII + MspI	NEB-2	GG↓CC + C↓CGG	178+366+435+552	54, 56, 58
15	None	None	–	512	59
16	AluI + DdeI	NEB-2	AG↓CT + C↓TNAG	129+177+289+382+462	55, 57, 59
17	MboI	NEB-3	↓GATC	191+235+297+441	57; 59
18	AluI	NEB-2	AG↓CT	218+353+457	55; 57

DHPLC, denaturing high-performance liquid chromatography.

buffer and 1.5 μl of enzyme (Table II) and incubated at 37°C for 2 hours. Verification of restriction digestion completion and the presence of restriction site mutants took place by analyzing 5 μl of sample using 2% agarose, 0.5 M TBE gel electrophoresis stained with ethidium bromide and viewed under ultraviolet light.

DHPLC Analysis

Before DHPLC analysis all samples underwent a heteroduplex formation step consisting of heating to 95°C for 5 minutes and then cooling at a rate of 1.5°C per minute until a temperature of 25°C was reached using the MJ Tetrad thermal cycling instrument. Sample analysis temperatures were predicted using the Wavemaker software (Transgenomic). The gradient mobile phase consisted of buffer A (0.1 M triethyl ammonium acetate, pH 7.0; Transgenomic), and buffer B (0.1 M triethyl ammonium acetate, pH 7.0; Transgenomic), and 25% acetonitrile (BDH/Merck HiPerSolv grade). These buffers were mixed to produce a linear gradient varying buffer B between 45% and 67% over a 12-minute period. After each analysis the cartridge was cleaned for 30 seconds with 75% acetonitrile and equilibrated at buffers A:B, 61:39 for 2 minutes before the next sample analysis.

Sequence Analysis

Samples were amplified for sequencing as indicated above using the relevant primer set in a 25-μl reaction volume. This was then treated with ExoSAPIT (US Biochemicals, Cleveland, Ohio) to remove excess primers and nucleotides; 10 μl of ExoSapIT was added to the 25-μl polymerase chain reaction, incubated at 37°C for 15 minutes, and inactivated at 80°C for 15 minutes. Ethanol precipitation took place using 0.1 volumes of 3 M sodium acetate pH 5.2 and 3 volumes of 100% ethanol. Cycle sequencing, according to manufacturer's protocols, was performed at the Biopolymer Lab (University of Maryland at Baltimore, Baltimore, Md.) using Applied Biosystems 3100 Sequencer (Foster City, Calif.). Sequencing primers were ordered from Invitrogen. Primer sequences used were the amplification primers indicated in Table I.

Statistical Analysis

The association of this mutation with Dupuytren's disease was investigated by comparing the frequency of mutations in the affected Dupuytren's disease patients and control subjects using the McNemar chi-square test.

RESULTS

Noncoding Region

The primer sets (MT1 through MT4) amplified the noncoding region also known as the D-loop or hypervariable region. Multiple DHPLC pattern variations suggestive of a large number of random mutations were detected throughout the affected and control samples in primer sets 1 through 4. In particular, a high degree of DHPLC pattern variability was located in primer set 3. As there was no single dominant pattern present in the Dupuytren's disease compared with control samples, this indicated no obvious disease-causing mutations in this region.

Coding Region

The primer sets (MT4 through MT18) amplified the rest of the mitochondrial genome referred to as the coding region. Primer design had necessitated a degree of overlap to ensure full coverage of the mitochondrial region, and thus the beginning of primer 4 had also covered 300 bp of the terminal portion of D-loop region. Several DHPLC pattern changes suggestive of a multiple number of random mutations were detected again throughout the affected and control samples in primer sets 4 through 18. The majority of these changes were of no disease interest, as there was no single dominant pattern present in the Dupuytren's disease compared with control samples, suggesting an absence of disease-causing mutations in this region.

DHPLC analysis showed a major change between the control and affected population in fragment 4 from primer set 6 as illustrated in Figure 1. In the affected individuals there was a much more pronounced two-peak pattern in this fragment compared with the control samples that showed a major peak and shoulder. The mutant DHPLC pattern was present in 90 percent of our Dupuytren's disease patients in comparison with the wild-type pattern in all the control subjects. None of the control subjects have the mutant pattern. Four samples (two affected and two control subjects) were submitted as representative for sequencing. This interesting finding was confirmed by sequencing and found to be an unknown heteroplasmic

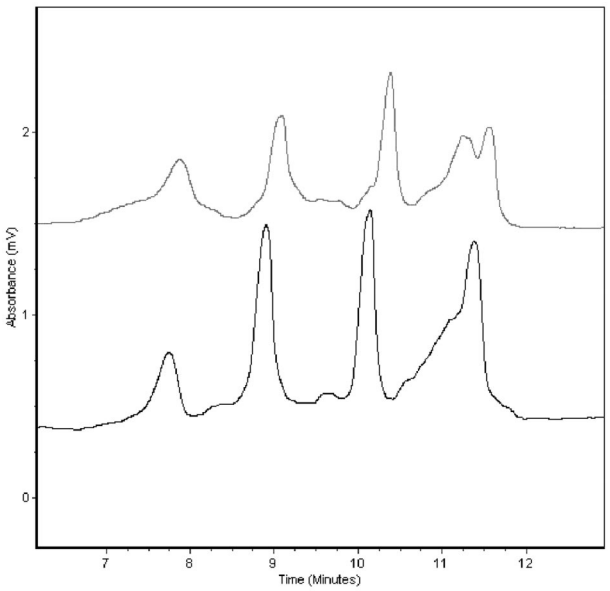

FIG. 1. A denaturing high-performance liquid chromatography chromatogram showing a mutation in primer set 6. This chromatogram shows an affected (11; *top line*) versus a control sample (3; *bottom line*) with the mutation being evident as an enhanced double-peak pattern in fragment 4 of the primer set 6 (corresponding to the 16s rRNA gene).

mutation at position C2839A base pair in the 16s rRNA region of the mitochondrial genome.

There was a statistically highly significant difference in the frequencies ($p < 0.001$; $\chi^2 = 16.1$) between patients and control subjects for the mutation in the mitochondrial 16s rRNA gene. Presence of the mutation may be associated with an increased risk of developing Dupuytren's disease.

Known restriction site mutations were found using primer sets 9, 11, 13, 16, and 17. There were no restriction site mutants in the samples analyzed using other primer sets. Both disease and control samples had the restriction site mutations, which appeared to not be disease related. All the sequencing data from our identified mutations were checked against the mitochondrial polymorphism reference database (http://infinity.gen.emory.edu/mito map.html).

DISCUSSION

The etiopathogenesis of Dupuytren's disease remains unexplained. Factors such as smoking, alcohol, and medical conditions including epilepsy and diabetes have been associated with Dupuytren's disease formation.[8–10] Interestingly, both epilepsy[11] and diabetes[12] have been associated with mutations in mitochondrial DNA. The relevance of some of these etiologic factors in the pathology of Dupuytren's disease has been questioned; none has been proven to contribute to the disease's etiopathogenesis.[13] There are, however, two elements in the etiology of Dupuytren's disease that clearly stand out. One is the common occurrence of this condition in Caucasians and the other is the familial nature of the disease.[2] Genetic studies have yet to identify the genes involved in the formation of the disease.

There is accumulating evidence indicating that mitochondrial mutations may underlie a large percentage of the common, late-onset, heterogeneous disorders.[14,15] The possibility of mitochondrial DNA mutations inducing the pathogenesis of Dupuytren's disease is suggested by the following observations. First, Dupuytren's disease is preferentially co-expressed in individuals with diabetes and epilepsy,[13] which are both shown to be associated with pathogenic mitochondrial DNA mutations.[16] The myofibroblast in the palmar aponeurosis of patients in different stages of Dupuytren's disease was found by electron microscopy to contain a large number of mitochondria.[17,18]

Mitochondria play a critical role in cell metabolism and apoptosis. Reactive oxygen species are generated by mitochondria as byproducts of respiration.[19] The respiratory function of mitochondria is impaired in aging human tissues.[20] It is known that defective mitochondria generate more reactive oxygen species by means of electron leak and that antioxidant enzyme activities decrease with age in skin fibroblasts.[21] Mitochondrial DNA mutations have been shown to accumulate with age in postmitotic tissues in association with age-related decline. Interestingly partial depletion of mitochondrial DNA under genetic stress induces stress signaling to the nucleus leading to phenotypic changes and tumor progression. Transforming growth factor (TGF) β_1 is overexpressed in cells subjected to mitochondrial and metabolic stress.[22] TGFβ_1 has been shown to be overexpressed in Dupuytren's disease tissue.[23] Increased free radical generation has been implicated by finding a sixfold increase in hypoxanthine concentrations in Dupuytren's disease compared with control palmar fasci.[24] Reactive oxygen species can activate latent TGFβ_1 and are important in the pathogenesis of radiation wound fibrosis. Therefore, mitochondrial mutations could simultaneously increase the level of free radicals and increase the expression of TGFβ_1 because of the activation of the secreted latent TGFβ_1 protein lead-

ing to normal levels of active TGFβ_1, which in turn could promote myofibroblast differentiation, survival, and collagen synthesis, in turn producing the characteristic fibrotic nodule of Dupuytren's disease tissue.

An unknown heteroplasmic mutation in the primer set 6 at position 2839 bp, which was a C to C/A base pair change in the 16s rRNA region of the mitochondrion, was identified. The DHPLC pattern change was evident in 90 percent of the patients and absent from all control subjects. The high prevalence of the DHPLC pattern change and its marked differentiation between patients and control subjects indicated a mutation of potential disease relevance. There was a statistically significant difference in the frequencies ($p < 0.001$; $\chi^2 = 16.1$) between patients and control subjects for the mutation in the mitochondrial 16s rRNA gene. Presence of the mutation is associated with an increased risk of developing Dupuytren's disease. Despite the small numbers involved in this study, we have estimated that a sample size of 27 subjects would be able to give 80 percent power to detect an odds ratio of 8.0 as being significant at the 5 percent level. Therefore it is unlikely for this finding to be a result of chance alone. It must be noted, however, that all such studies would require confirmation by replication of the study in another sample of subjects. We would like to emphasize that, although suggestive, the above finding cannot be definitely linked to the pathogenesis of Dupuytren's disease until this finding is demonstrated by further confirmatory and functional studies.

Other known disease mutations have been described in the 16s rRNA region, including diabetes (3203G;3204T),[25] Rett syndrome (2835T),[26,27] mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (or MELAS) (3093G),[28] and Alzheimer's disease/Parkinson's disease (3196A).[29,30] Ribosomal RNAs have multiple functions including RNA polymerase activity,[31] ribonuclease activity,[32] and directing the synthesis of peptide bonds.[33] Single point mutations in the 16s rRNA region may interfere with protein refolding and recovery of enzymatic activity of denatured proteins.[34] It is already known that mutations accumulate in the mitochondrial genes, including the 16s rRNA region, with aging.[34]

The inheritance patterns in mitochondrial disorders can range from maternal to Mendelian or be a combination of the two. A wide variety of mitochondrial conditions have been linked to mutations in both nuclear and mitochondrial genes.[35] We have solely investigated the mitochondrial DNA in maternally inherited pedigrees for the presence of mutations in relation to our patients. The identification of a novel heteroplasmic polymorphism in the 16s rRNA region that is present in 90 percent of the patients and absent from control subjects is a potentially important finding. It is possible that this mutation is pathogenic and may cause an oxidative phosphorylation defect by interfering with mitochondrial protein synthesis. This novel polymorphism is not present in existing mitochondria databases, and the functional relevance of this polymorphism is unclear. This heteroplasmic polymorphism is absent from our control population. The degree of heteroplasmy within our family pedigrees has not been determined; however, clinical examination of affected members of some of our pedigrees demonstrates variation in phenotypic expression in Dupuytren's disease. This corresponds well with the possibility of mitochondrial DNA heteroplasmy and the threshold effect in this condition.

Mitochondrial mutations leading to functional impairment of the respiratory chain are pathogenic.[35] Nonetheless, the genotype and phenotype relationships in mitochondrial DNA and affected disorders are difficult to understand. It is unclear why defects in the metabolic pathway that result in energy crisis can present with such a range of symptoms and in a variety of tissues. The same mitochondrial DNA mutation can produce very different phenotypes and different mutations can produce similar phenotypes. This may be a result of the varying ratios of wild-type to mutant mitochondrial DNA, the modulating effect of other mitochondrial and nuclear genes, and the different thresholds of biochemical expression for the tissue and the particular mutation involved.

Dupuytren's disease can affect both the palms of the hands and soles of the feet in the same individual. This combination is particularly common in individuals with a positive family history. Interestingly a known familial condition that mainly affects the palm and the sole (palmoplantar keratoderma) is attributable to a mitochondrial mutation (A7445G).[36] Patients with Dupuytren's disease show a variety of phenotypes, as some only develop the nodule whereas others develop the full contracture. This clinical expression may be explained by the threshold effect that is seen in

mitochondrial disorders. Dupuytren's disease is currently best treated by surgical excision of the nodules; however, recurrence rate after treatment is very high. This is particularly so in patients with a positive family history.[2]

It has been proposed that mitochondrial DNAs with substantial mutations replicate more rapidly and therefore have a selective advantage over normal mitochondrial DNAs.[29] This may account for the observation that high proportions of mutated mitochondrial DNA were found in specific affected tissues, such as muscle, as compared with other unaffected tissues, such as the liver, which shows a lower proportion of mutations. The percentage of mutated mitochondrial DNA is presumed to increase with age in muscle.[37] If the total number of muscle fibers does not change appreciably after birth, mitochondrial DNA replication is dissociated from cell division and there is positive selection for defective cells with mutated mitochondrial DNA. The variation in age of onset is thought to be attributable to the percentage of mitochondrial DNA present at birth, and thereafter during birth it is the rate of accumulation of mutated mitochondrial DNA compared with normal mitochondrial DNAs.

SUMMARY

Maternal transmission in a proportion of Dupuytren's disease patients suggests that a mutation in the mitochondrial genome may be predisposing to formation of the disease. We have demonstrated for the first time a novel mitochondrial heteroplasmic mutation that may be associated with the pathogenesis of Dupuytren's disease. Understanding the genes involved in the pathology of Dupuytren's disease is of importance in helping to develop future diagnostic, therapeutic, and prognostic therapies.

Ardeshir Bayat, Ph.D., M.R.C.S. (Eng. Ed.)
Center for Integrated Genomic Medical Research
University of Manchester
Stopford Building
Oxford Road
Manchester M13 9PT, United Kingdom
ardeshirbayat@doctors.org.uk

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