(Hahnemann University, Philadelphia, PA) for providing the ribosomal DNA clone.

References

1. Chinnery PF, Turnbull DM. Mitochondrial DNA and disease. Lancet 1999;354:SI17–SI21.
2. Servidei S, Zeviani M, Manfredi G, et al. Dominantly inherited mitochondrial myopathy with multiple deletions of mitochondrial DNA: clinical, morphologic, and biochemical studies. Neurology 1991;41:1053–1059.
3. Suomalainen A, Majander A, Wallin M, et al. Autosomal dominant progressive external ophthalmoplegia with multiple deletions of mtDNA: clinical, biochemical, and molecular genetic features of the 10q-linked disease. Neurology 1997;48: 1244–1253.
4. Kaukonen J, Zeviani M, Comi GP, et al. A third locus predisposing to multiple deletions of mtDNA in autosomal dominant progressive external ophthalmoplegia. Am J Hum Genet 1999; 65:256–261.
5. Kaukonen J, Juselius JK, Tiranti V, et al. Role of adenine nucleotide translocator 1 in mtDNA maintenance. Science 2000; 289:782–785.
6. Suomalainen A, Kaukonen J, Amati P, et al. An autosomal locus predisposing to deletions of mitochondrial DNA. Nat Genet 1995;9:146–151.
7. Spelbrink JN, Li FY, Tiranti V, et al. Human mitochondrial DNA deletions associated with mutations in the gene encoding Twinkle, a phage T7 gene 4-like protein localized in mitochondria. Nat Genet 2001;28:223–231.
8. Van Goethem G, Dermaut B, Lofgren A, et al. Mutation of POLG is associated with progressive external ophthalmoplegia characterized by mtDNA deletions. Nat Genet 2001;28: 211–212.
9. Stepien G, Torroni A, Chung AB, et al. Differential expression of adenine nucleotide translocator isoforms in mammalian tissues and during muscle cell differentiation. J Biol Chem 1992; 267:14592–14597.
10. Li K, Warner CK, Hodge JA, et al. A human muscle adenine nucleotide translocator gene has four exons, is located on chromosome 4, and is differentially expressed. J Biol Chem 1989; 264:13998–14004.
11. Gross A, McDonnell JM, Korsmeyer SJ. BCL-2 family members and the mitochondria in apoptosis. Genes Dev 1999;13: 1899–1911.
12. Nishino I, Kobayashi O, Goto Y, et al. A new congenital muscular dystrophy with mitochondrial structural abnormalities. Muscle Nerve 1998;21:40–47.
13. Moraes CT, Shanske S, Tritschler HJ, et al. mtDNA depletion with variable tissue expression: a novel genetic abnormality in mitochondrial diseases. Am J Hum Genet 1991;48:492–501.
14. Akanuma J, Muraki K, Komaki H, et al. Two pathogenic point mutations exist in the authentic mitochondrial genome, not in the nuclear pseudogene. J Hum Genet 2000;45: 337–341.
15. Moraes CT, Schon EA. Detection and analysis of mitochondrial DNA and RNA in muscle by in situ hybridization and single-fiber PCR. Methods Enzymol 1996;264:522–540.
16. Moslemi AR, Melberg A, Holme E, Oldfors A. Clonal expansion of mitochondrial DNA with multiple deletions in autosomal dominant progressive external ophthalmoplegia. Ann Neurol 1996;40:707–713.
17. Oldfors A, Moslemi AR, Fyhr IM, et al. Mitochondrial DNA deletions in muscle fibers in inclusion body myositis. J Neuropathol Exp Neurol 1995;54:581–558.
18. Nishino I, Spinazzola A, Hirano M. Thymidine phosphorylase gene mutations in MNGIE, a human mitochondrial disorder. Science 1999;283:689–692.

Electrophysiological Findings in X-Linked Myopathy with Excessive Autophagy

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We report electrophysiological features and magnetic resonance imaging muscle findings in 4 patients and 1 female carrier of X-linked myopathy with excessive autophagy. Motor units were polyphasic with high mean amplitude and normal duration. The thigh muscles were most severely involved, but myotonic discharges were abundant in both clinically affected and unaffected muscles. Along with the clinicopathological features, these electrophysiological findings distinguish X-linked myopathy with excessive autophagy from other limb-girdle myopathies.

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X-linked myopathy with excessive autophagy (XMEA) is a hereditary myopathy originally described in a Finnish kindred in 1988.[1–3] The onset is in childhood, manifesting as mild muscle weakness. Proximal lower limb muscles are most severely affected. The progression is slow, and life expectancy is normal. The main pathological findings are abundant sarcoplasmic vacu-

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Atypical muscle pathology and a survey of *cis*-mutations in deaf patients harboring a 1555 A-to-G point mutation in the mitochondrial ribosomal RNA gene

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Abstract

We investigated three families with maternally inherited deafness associated with a 1555 A-to-G substitution in the 12S ribosomal RNA gene. Probands in these families developed deafness following streptomycin treatment, whereas several family members who did not receive aminoglycoside showed onset of deafness in middle age. One proband had a non-synonymous A14062G mutation in the ND5 gene and the other had a non-synonymous G15221A mutation in the cytochrome *b* gene and a T1391C mutation in the 12S ribosomal RNA gene, whose importance in disease expression remains to be clarified. Two muscle biopsies from the patients with and without streptomycin treatment, showed similar findings; a moth-eaten appearance with decreased cytochrome *c* oxidase activity and abnormal mitochondrial morphology. These findings suggest that even without exposure to aminoglycoside the A1555G mutation may impair mitochondrial function and that the mitochondrial abnormalities associated with the A1555G mutation may be expressed in tissues other than those of the auditory system.

Keywords: Mitochondrial DNA; Aminoglycoside; Deafness; Myopathy; Streptomycin

1. Introduction

Human mitochondrial DNA (mtDNA) is a small 16.6-kilobase (kb), double-stranded molecule that encodes 13 structural proteins, two ribosomal RNAs (rRNAs), and 22 transfer RNAs (tRNAs). Point mutations of tRNA genes and large-scale deletions of mtDNA generally are associated with multisystem mitochondrial diseases characterized by ragged-red fibers (RRFs) as a morphologic hallmark. These include myoclonic epilepsy with RRFs (MERRF); mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes (MELAS); and chronic progressive external ophthalmoplegia (CPEO), including Kearns–Sayre syndrome. In contrast, point mutations of protein-coding genes are responsible for encephalopathies with rare pathologic and biochemical muscle abnormalities, such as Leber's hereditary optic neuroatrophy; maternally inherited Leigh disease; and neurogenic muscle weakness, ataxia, and retinitis pigmentosa [1,2].

The mutation in the mitochondrial rRNA gene, an A-to-G transition at nucleotide (nt) 1555 of the 12S rRNA gene, is associated with maternally inherited non-syndromic deafness in a large Arab–Israeli pedigree [3] and aminoglycoside-induced deafness in other families [4,5]. Deafness associated with the A1555G mutation is considered to be caused by cochlear involvement [5]. Several questions remain to be answered about the relationship between genotype and phenotype in this disease. These can be classified as two major clinical and biological issues; penetrance and tissue specificity [6].

Elsewhere, we used a ρ^0 cell system and showed that cells carrying the homoplasmic A1555G mutation had very high susceptibility to streptomycin [7]. This suggests that the A1555G mutation mainly is responsible for the phenotype and that nuclear DNA mutation(s), if present, have a less

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important role. Therefore, to determine the possibility of significant *cis*-mutation(s), which may influence the disease expression, we sequenced the whole mtDNAs of three Japanese patients with the A1555G mutation. Evaluation of the functional relevance of new mutation is difficult in the absence of a convincing in vitro or in vivo assay of its effect on mitochondrial function. It has then to rely on the analysis of the mutation frequency in a sufficiently large control population.

Does the A1555G mutation affect tissues other than the cochlea? Although most patients reported as having the A1555G mutation had deafness as the sole clinical manifestation, recent studies suggest the possibility of phenotypic heterogeneity. Santorelli et al. [8] reported a family with maternally inherited cardiomyopathy ascribed to a heteroplasmic A1555G mutation, in which the skeletal muscle biopsy of the proband showed central or paracentral minicores and reductions in NADH-dehydrogenase and cytochrome *c* oxidase (COX) activities. Shoffner et al. [9] described a family harboring the A1555G mutation in association with both deafness and Parkinson's disease, complex I activity being decreased in muscle samples from several of the family members. To determine whether the effects of the A1555G mutation are expressed in tissues other than the cochlea even in patients with deafness as the sole clinical symptom, we obtained muscle biopsy specimens from two patients; one with streptomycin-induced, the other with streptomycin-unrelated deafness. These specimens showed such morphological abnormalities as a moth-eaten appearance with atypical mitochondrial morphology and decreased COX activity, and a few typical RRFs. Taken together with the results obtained with ρ^0 cells [7] and lymphoblastoid cell lines, [10] this finding supports the speculation that the effects of the A1555G mutation may be expressed in tissues other than the cochlea. Abnormal mitochondrial morphology was present even in a patient with no history of aminoglycoside treatment, indicating that the mutation itself can cause mitochondrial dysfunction or with aging accelerate the deterioration of mitochondrial function, manifesting as deafness of middle age onset.

2. Patients

In family 1, the proband (III-5) was a 44-year-old man who became deaf in both ears after several administrations of streptomycin for treatment of tetanus at age 17. His maternal aunt (II-2) also had streptomycin-induced deafness. His mother (II-3), grandmother (I-2), brother (III-7), and maternal cousin (III-3), as well as his maternal cousin's daughter (IV-5) had bilateral deafness, but they had no history of aminoglycoside treatment (Fig. 1).

In family 2, the proband (III-16) was a 27-year-old woman who at age 5 lost hearing after receiving several streptomycin injections for treatment of an upper respiratory tract infection. Her hearing acuity continued to deteriorate thereafter. Her brother (III-15), mother (II-11), maternal uncles (II-5, II-7), and grandmother (I-2) all had sensorineural hearing loss of unknown cause.

In family 3, the proband (III-2), a 19-year-old woman experienced tinnitus after being administered 22 g of streptomycin for treatment of tuberculosis. At that time, a puretone audiogram showed a slight increase in her hearing threshold at 8 kHz only. Although the treatment was immediately stopped, her hearing continued to deteriorate over the next 6 months, becoming profoundly impaired at all frequencies. Her maternal aunt (II-5) also had streptomycin-induced deafness, whereas her grandmother (I-2) and another aunt (II-11) had deafness of unknown cause.

None of these probands had been aware of dizziness, vertigo, or oscillopia. None of the family members in these pedigrees had clinical symptoms characteristic of mitochondrial encephalomyopathy.

3. Methods

3.1. Mitochondrial DNA analysis

Total DNA was isolated from the peripheral leukocytes of

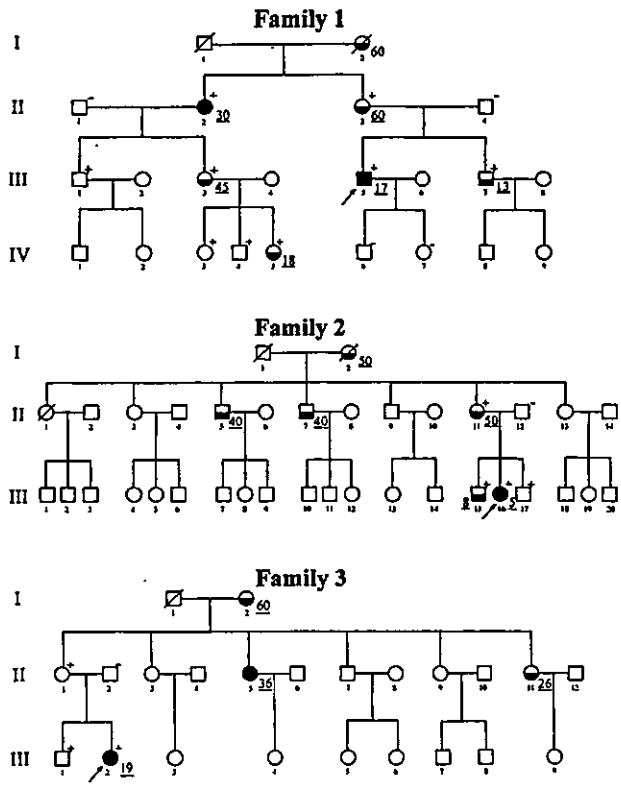

Fig. 1. Pedigrees of the families examined. Arrows indicate probands; crossed symbols, deceased subjects. In these families, shading of upper half = a history of streptomycin treatment; shading of lower half = hearing loss. The numbers underlined are onset ages of the probands and their family members. Plus and minus signs indicate the subjects receiving the sequence analysis or dot blot examination of A1555G mutation with positive and negative results, respectively.

the three probands and several of their family members. To determine whether the A1555G mutation was present, the mtDNA fragment surrounding the 12S rRNA mutation site was amplified by the polymerase chain reaction (PCR) which used the sense primer 5′-AAACTCAAAG-GACCTGGCGG (nt 1160–1179) and antisense primer 5′-CGTCCAAGTCGACTTTCCAG (nt 1598–1579). The fragments were subcloned into a plasmid vector by means of a TA cloning kit (Invitrogen, San Diego, CA, USA) then were directly sequenced with an ABI DNA sequencer. To detect the mutation in the remaining relatives, 2 μl of the PCR products were processed by the dot-blotting method. Hybridization was done with a probe of 19-mer oligonucleotides 5′-TACGACTTGCCTCCTCTAT (nt 1564–1545) labeled with γ-^{32}P-ATP, after which autoradiography was performed.

Large-scale deletions were examined by Southern blot and long PCR analyses [11,12]. We sequenced the whole mtDNA of each of three probands as described previously, [13] and compared each sequence with the standard mtDNA sequence and polymorphism data in MITOMAP. Data used to prepare the table were derived from MITOMAP: the Mitochondrial Human Genome Database at Emory University in Atlanta (http://www.gen.emory.edu/mitomap.html) on March 7, 1999 at 10 AM EST. If the base changes were not described in MITOMAP, we made mismatch primers in order to detect the mutation in control subjects by PCR-restriction fragment length polymorphism (RFLP) analyses.

3.2. Audiological and neurotological examinations

The probands and several of their family members were examined by the following types of audiological and neurotological testing. Audiological examinations consisted of pure-tone and Bekesy's audiometry, speech recognition, acoustic threshold test (ART), and an assessment of auditory brainstem response (ABR) and evoked and distortion-product otoacoustic emissions (EOAE and DPOAE). The stimuli for ABR consisted of alternating clicks, generated by square wave electrical pulses lasting 0.1 ms. They were presented monoaurally at 9.5/s and 90 dB HL. Two averages of 1000 trials were obtained for the stimulation of each ear. EOAE and DPOAE were recorded and analyzed, respectively, with ILO 88 and ILO 92 (Otodynamics, London). The stimulus for obtaining EOAE was a non-filtered click 80 ms long at 80 dB SPL. The click rate was 50/s, and the analysis period 20 ms. Two hundred and sixty responses were averaged. A passband of 500–6000 Hz was used. 2f1–f2 DPOAE was measured at 1/4-octave intervals across the stimulus frequency range of 700–6000 Hz with a f2/f1 ratio of 1.21:1.23 and f1 and f2 sound pressure levels of 70 dB SPL. Stimulus presentation, data recording, averaging, and spectrum analysis were done with otodynamic ILO 88 and ILO 92 software and hardware. Neurotological tests were performed to assess the righting reflex, pathologic nystagmus (change of fixation, gaze, and head position), the vestibulo-ocular reflex function (caloric testing), and visual-ocular control (saccade and smooth pursuit). Informed consent for the testing was obtained from all the patients.

3.3. Muscle biopsy

Biopsy specimens were obtained from the biceps brachii of two deaf patients (III-5 and II-3) in family 1. The former, a 44-year-old man had received streptomycin, whereas the latter, a 69-year-old woman had not. The specimens were quickly frozen in isopentane chilled in liquid nitrogen, then subscribed to histochemical and electron microscopic observations. Frozen serial sections were stained with hematoxylin and eosin (H&E), modified Gomori trichrome (mGT), and a battery of histochemical agents that included COX, succinate dehydrogenase (SDH), myofibrillar adenosine triphosphatase with preincubation at pH 10.8, 4.6 and 4.2, and NADH dehydrogenase tetrazolium reductase [14]. For the electron microscopic studies, muscle samples were fixed in phosphate buffer containing 2% glutaraldehyde. After being washed in phosphate buffer solution, the samples were postfixed in 1% osmium tetroxide, embedded in epoxy resin and sectioned. The ultrathin sections were double-stained with uranyl acetate and lead citrate and examined by electron microscope (H-7000, Hitachi).

4. Results

4.1. Mitochondrial DNA analysis (Table 1)

No large-scale deletions were detected by the long PCR and Southern blot analyses. All the point mutations detected by the whole sequence survey are listed in Table 1. Three non-synonymous mutations were found in family 1, two of which were also present in 1% of the normal controls, indicative of polymorphism. The other A14062G mutation in the ND5 gene was not present in any of the 100 controls. Two non-synonymous mutations were found in family 2, both of which were present in two of the 100 controls. This family also had a homoplasmic T2626C mutation in the 16S rRNA gene, which was also present in 12 of 58 controls. A non-synonymous G15221A mutation in the cytochrome *b* gene and the T1391C mutation in the 12S rRNA gene were found in family 3. Neither mutation was present in the approximately 200 controls.

4.2. Audiological and neurotological findings

The family pedigrees show that hearing loss related or unrelated to aminoglycoside treatment was maternally inherited over three or four generations (Fig. 1). Members with bilateral deafness of unknown cause had experienced hearing loss since their second or third decade, the loss being slowly progressive. The proband had bilateral deafness, the higher frequencies being more severely affected.

Table 1
Sequence analysis of whole mitochondrial DNA

Family 1						Family 2						Family 3					
663	A→G	12S rRNA	MITOMAP			709	G→A	12S rRNA	MITOMAP			750	A→G	12S rRNA	MITOMAP		
750	A→G	12S rRNA	MITOMAP			750	A→G	12S rRNA	MITOMAP			827	A→G	12S rRNA	MITOMAP		
1438	A→G	12S rRNA	MITOMAP			1438	A→G	12S rRNA	MITOMAP			1391	T→C	12S rRNA			(0/196)
1555	A→G	12S rRNA				1555	A→G	12S rRNA				1438	A→G	12S rRNA	MITOMAP		
1736	A→G	16S rRNA	MITOMAP			1598	G→A	12S rRNA	MITOMAP			1555	A→G	12S rRNA			
2706	A→G	16S rRNA	MITOMAP			2626	T→C	16S rRNA			(12/58)	2706	A→G	16S rRNA	MITOMAP		
3106	C-del	16S rRNA	MITOMAP			2706	A→G	16S rRNA	MITOMAP			3423	G→T	ND1	MITOMAP		
3408	C→T	ND1	CGC→CGU	Arg		3106	C-del	16S rRNA	MITOMAP			3981	A→G	ND1	AUA→AUG	Met	
3423	G→T	ND1	MITOMAP			3423	G→T	ND1	MITOMAP			4769	A→G	ND2	MITOMAP		
4248	T→C	ND1	MITOMAP			4769	A→G	ND2	MITOMAP			4820	G→A	ND2	GAG→GAA	Glu	
4769	A→G	ND2	MITOMAP			4985	G→A	ND2	MITOMAP			4985	G→A	ND2	MITOMAP		
4824	A→G	ND2	MITOMAP			6962	G→A	COI	MITOMAP			5585	G→A	non-coding			
4985	G→A	ND2	MITOMAP			7028	C→T	COI	MITOMAP			7028	C→T	COI	MITOMAP		
5177	G→A	ND2	AAG→AAA	Lys		8281-9	9bp deletion non-coding					8281-9	9bp	del	non-coding		
7028	C→T	COI	MITOMAP			8584	G→A	ATPase 6	MITOMAP			8860	A→G	ATPase 6	MITOMAP		
8348	A→G	tRNA-Lys			(2/135)	8829	C→T	ATPase 6	AAC→AAU	Asn		9559	G→C	COIII	MITOMAP		
8409	C→T	ATPase 8	CCC→CUC	Pro→Leu	(1/99	8856	G→A	ATPase 6	MITOMAP			11335	T→C	ND4	MITOMAP		
8459	A→G	ATPase 8	AAC→GAC	Asn→Asp	(1/100)	8860	A→G	ATPase 6	MITOMAP			11518	G→A	ND4	CUG→CUA	Leu	
8794	C→T	ATPase 6	MITOMAP			9559	G→C	COIII	MITOMAP			11719	G→A	ND4	MITOMAP		
8860	A→G	ATPase 6	MITOMAP			9950	T→C	COIII	MITOMAP			13590	G→A	ND5	MITOMAP		
9559	G→C	COIII	MITOMAP			10103	A→G	ND3	UUA→UUG	Leu		13702	G→C	ND5	MITOMAP		
11335	T→C	ND4	MITOMAP			10398	A→G	ND3	MITOMAP			13818	T→C	ND5	ACU→ACC	Thr	
11719	G→A	ND4	MITOMAP			11335	T→C	ND4	MITOMAP			13934	C→T	ND5	MITOMAP		
12705	C→T	ND5	MITOMAP			11719	G→A	ND4	MITOMAP			14199	G→T	ND6	MITOMAP		
13702	G→C	ND5	MITOMAP			12361	A→G	ND5	MITOMAP			14272	G→C	ND6	MITOMAP		
14062	A→G	ND5	AUC→GUC	Ile→Val	(0/100)	13702	G→C	ND5	MITOMAP			14365	G→C	ND6	MITOMAP		
14067	C→T	ND5	ACC→ACU	Thr		14199	G→T	ND6	MITOMAP			14368	G→C	ND6	MITOMAP		
14199	G→T	ND6	MITOMAP			14272	G→C	ND6	MITOMAP			14569	G→A	ND6	MITOMAP		
14272	G→C	ND6	MITOMAP			14365	G→C	ND6	MITOMAP			14971	T→C	Cyt b	UAU→UAC	Tyr	
14365	G→C	ND6	MITOMAP			14368	G→C	ND6	MITOMAP			15221	G→A	Cyt b	GAC→AAC	Asp→Asn	(0/192)
14368	G→C	ND6	MITOMAP			15223	C→T	Cyt b	GAC→GAU	Asp		15326	A→G	Cyt b	MITOMAP		
15317	G→A	Cyt b	MITOMAP			15326	A→G	Cyt b	MITOMAP			15535	C→T	Cyt b	MITOMAP		
15326	A→G	Cyt b	MITOMAP			15508	C→T	Cyt b	GAC→GAU	Asp		16136	T→C	D-loop		MITOMAP	
16223	C→T	D-loop		MITOMAP		15662	A→G	Cyt b	AUC→GUC	Ile→Val	(2/100)	16182	A-del	D-loop		MITOMAP	
16234	C→T	D-loop		MITOMAP		15758	A→G	Cyt b	AUC→GUC	Ile→Val	(1/100)	16183	A→C	D-loop		MITOMAP	
16319	G→A	D-loop		MITOMAP		15927	G→A	tRNA-Thr	MITOMAP			16183	CC-ins	D-loop	MITOMAP		
16362	T→C	D-loop		MITOMAP		16140	T→C	D-loop		MITOMAP		16189	T→C	D-loop		MITOMAP	
73	A→G	D-loop		MITOMAP		16183	A-del	D-loop		MITOMAP		16217	T→C	D-loop		MITOMAP	
152	T→C	D-loop		MITOMAP		16183	CC-ins	D-loop	MITOMAP			16218	C→T	D-loop		MITOMAP	
207	G→A	D-loop		MITOMAP		16189	T→C	D-loop		MITOMAP		16298	T→C	D-loop		MITOMAP	
235	A→G	D-loop		MITOMAP		16243	T→C	D-loop		MITOMAP		16362	T→C	D-loop		MITOMAP	
514	C-del	D-loop		MITOMAP		16519	T→C	D-loop		MITOMAP		16519	T→C	D-loop		MITOMAP	
515	A-del	D-loop		MITOMAP		73	A→G	D-loop		MITOMAP		73	A→G	D-loop		MITOMAP	
						103	G→A	D-loop		MITOMAP		152	T→C	D-loop		MITOMAP	
						189	A→G	D-loop		MITOMAP		263	A→G	D-loop		MITOMAP	
						203	G→A	D-loop		MITOMAP		303	CC-ins	D-loop		MITOMAP	
						263	A→G	D-loop		MITOMAP		311	C-ins	D-loop		MITOMAP	
						310	C-ins	D-loop		MITOMAP		499	G→A	D-loop		MITOMAP	
						311	C-ins	D-loop		MITOMAP							
						514	C-del	D-loop		MITOMAP							
						515	A-del	D-loop		MITOMAP							

Speech recognition correlated well with the PTA. Bekesy's audiometry and ART showed the presence of recruitment phenomena in all the probands' ears. An ABR examination did not show retro-cochlear abnormalities such as interpeak latency elongation, and neither EOAE nor DPOAE was observed in any of the probands' ears. These results indicate that hearing loss involved the cochlea. None of the probands showed any abnormalities in the vestibular examinations including caloric testing. Audio-vestibular findings for the other family members who had deafness of unknown cause were similar to those of the probands. Hearing loss was more pronounced at high frequencies and accompanied by recruitment phenomenon, indicative of cochlear involvement, whereas no vestibular abnormalities were found.

4.3. Muscle pathology

Muscle biopsies done on two patients in family 1; one (Patient III-5) with streptomycin-induced deafness, the other (Patient II-3) with deafness unrelated to aminoglycoside, gave very similar findings. There was moderate variation in size in the type 1 fibers. Modified mGT and SDH stains showed RRFs in a few type 1 fibers, but they did not exceed 0.5% of the total muscle fibers. Type 1 and 2 fibers were clearly distinguishable on NADH stain. The intermyofibrillar networks were disorganized with a moth-eaten appearance in approximately 20% of the type 1 fibers (Fig. 2). In Patient II-3, RRFs had negative COX activity. In Patient III-5, RRFs had positive COX activity. In both biopsy specimens, most of the moth-eaten fibers detectable on the SDH or NADH stain showed slightly decreased COX activity (Fig. 2). No strongly SDH-reactive blood vessels were found in either biopsy. The electron microscope showed atypical morphological changes in mitochondria in the moth-eaten fibers; enlarged mitochondria containing amorphous materials and sometimes inclusions in both muscle biopsies (Fig. 3). No such morphological abnormalities were found in the fibroblasts from Patient II-3 (data not shown).

5. Discussion

Several *cis*-mutations were found in our families but all except three mutations are considered to be polymorphism because of their presence in controls. An A14062G mutation found in Patient III-2 in family 1 converts an isoleucine to a valine of the 576th amino acid residue in the ND5 gene. Because the wild-type isoleucine and mutant valine are both amino acids with very similar non-polar side chains and this type of conversion is frequently in a panel of reported polymorphism, we speculate that the mutation has little effect on disease expression. A non-synonymous G15221A and a T1391C mutation were found in the propositus in family 3. The G15221A mutation converts an aspartic acid to an asparagine of the 259th amino acid residue in the cytochrome *b* gene. The T1391C mutation was located in the same 12S rRNA gene as the A1555G mutation. In this study, we stress that the A1555G mutation without significant *cis*-mutation can induce deafness as seen in family 2.

Moreover, the mtDNA haplogroups and polymorphisms were reported to be associated with some multifactorial diseases such as male infertility [15] and type 2 diabetes mellitus [16]. Based on the D-loop sequence and the presence of 9-bp deletion in the COII/tRNA-Lys intergenic

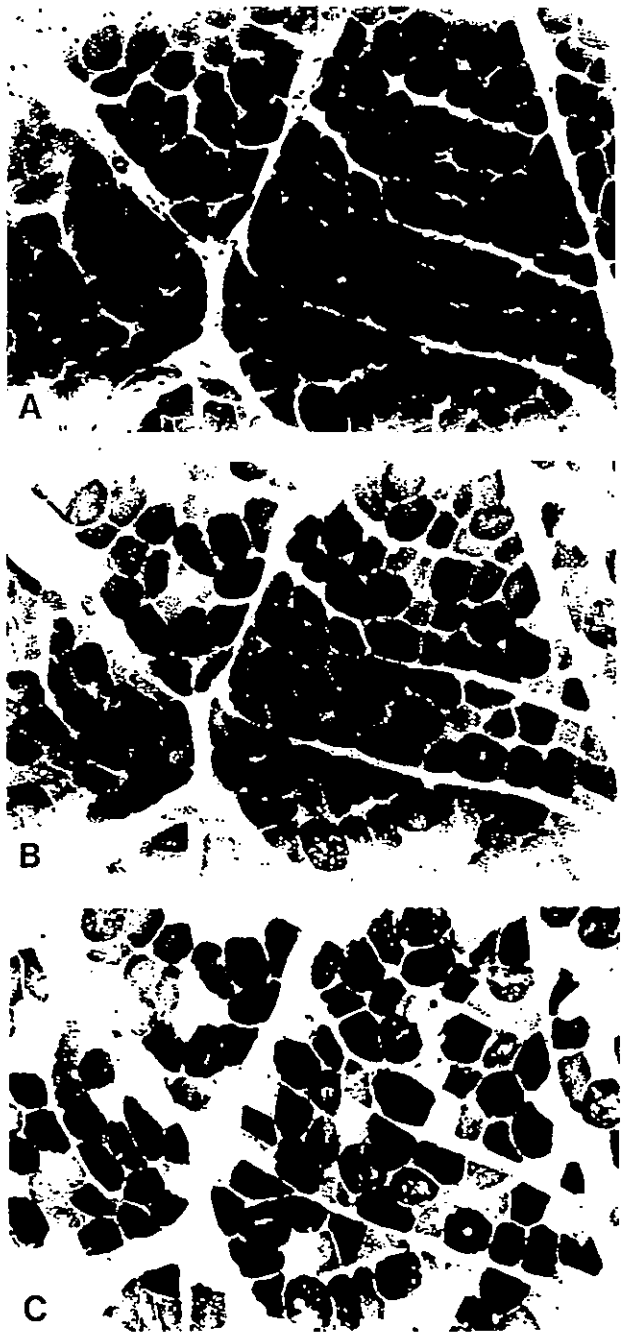

Fig. 2. Muscle pathology of Patient III-5 in family 1 at the age of 44. Stains: mGT (A), SDH (B), and COX (C). Type 1 fibers with a moth-eaten appearance show uneven distribution of oxidative enzymes, and COX activity is decreased as compared to that in normal type 1 fibers (×100).

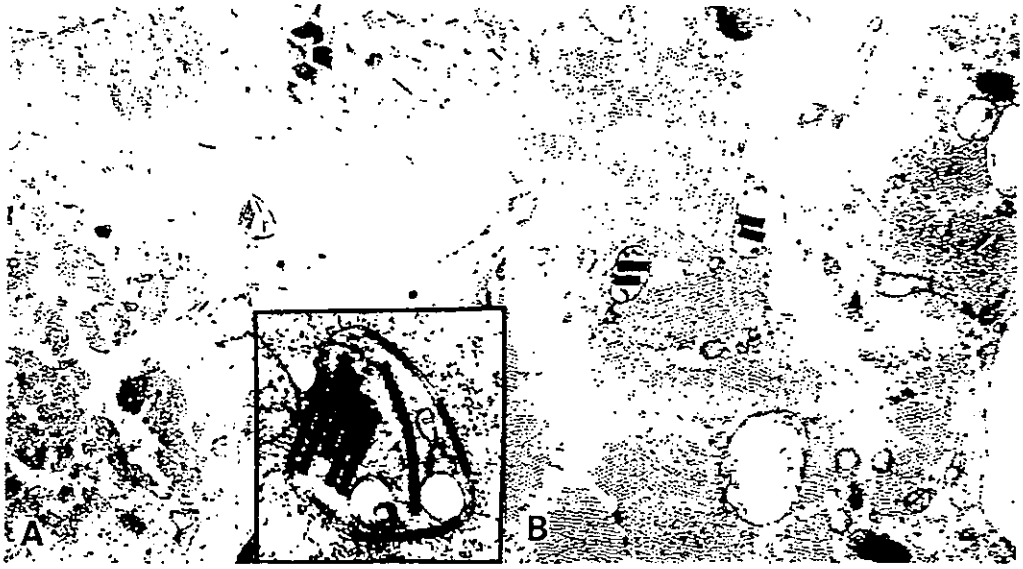

Fig. 3. Electron micrographs of enlarged mitochondria containing amorphous materials and inclusions (inset) in the moth-eaten fibers in Patient II-3 of family 1. (A: ×8000, inset: ×40,000, B: ×23,000).

region, family 1 is included in C10 cluster, and families 2 and 3 in C2 cluster, which were designated in the previous report [17]. The biological evaluation of the mtDNA haplogroups and polymorphisms as well as the three mutations found in this study, however, remains to be clarified.

Morphological mitochondrial alterations such as a moth-eaten appearance with atypical mitochondrial morphology and decreased COX activity, and a few RRFs in two patients showed the presence of muscle mitochondrial dysfunction in patients with isolated deafness. A moth-eaten appearance is seen where the intermyofibrillar networks on oxidative enzyme stains are disturbed and is more common in myopathy than in neuropathy [18]. Moth-eaten fibers have been found in approximately one-third of patients with CPEO [11]. In the present study, the moth-eaten fibers showed decreased COX activity and contained enlarged mitochondria with amorphous materials and inclusions, indicating that this change occurs because of mitochondrial dysfunction associated with the A1555G mutation. On COX stain, one patient with streptomycin-induced deafness showed positive enzymatic activity in the RRFs, whereas another deaf patient with no history of aminoglycodside treatment lacked COX activity. The difference in COX activity in the RRFs of our patients may reflect age differences, the presence/absence of a history of aminoglycoside treatment, or both.

Pathophysiology of the deafness due to mitochondrial DNA alterations should be discussed. Deafness is frequent in patients with mitochondrial encephalomyopathy characterized by the presence of RRFs ascribed to point mutations of mitochondrial tRNA genes or large-scale deletions of mtDNA [1,2]. Deafness is the main symptom in maternally inherited diabetes and deafness associated with a 10.4 kb mtDNA deletion [19] or a point mutation in the $tRNA^{Leu(UUR)}$ gene of mtDNA [20]. In contrast, deafness typically is absent in pure encephalopathies with no gross morphological muscle abnormalities ascribed to point mutations of the protein encoding mtDNA [1,2]. Because oxidative phosphorylation presumably is impaired in both types of disorder, decreased mitochondrial protein synthesis appears somehow to act in the manifestation of deafness, as it does as a signal for mitochondrial proliferation or RRF formation [21]. This speculation is supported by recent reports [8,9] and our present findings, which provide evidence that the A1555G mutation may also cause mitochondrial morphological abnormalities in muscle.

Aminoglycoside administration seems critical for the patients with the A1555G mutation because they can readily develop profound and progressive hearing loss after being given a low dose of aminoglycoside administration. However, patients with streptomycin-induced deafness and those with deafness of middle age onset unrelated to aminoglycoside were found in our families, as reported in other families [3,4,5,22]. Estivill et al. [22] reported that in families with A1555G mutations all patients who received aminoglycoside became deaf but that approximately 80% of patients with deafness onset in middle age had not been treated with aminoglycoside. This suggests that the A1555G mutation has age-dependent penetrance for deafness and that aminoglycoside merely enhances the progression of deafness. Inhibition of mitochondrial protein synthesis with chroramphenicol potentiates hair cell loss after exposure to two different ototoxicants, gentamicin, and acoustic trauma, [23] which suggests that mitochondrial function regulates the probability of survival after metabolic cochlear damage and that cochlea with decreased mitochondrial protein synthesis is more vulnerable to ototoxicants than is normal cochlea. This also may explain why patients with the A1555G mutation tend to develop deafness.

In this study, we demonstrated the mitochondrial abnormality in the muscle of the patients with the A1555G mutation irrespective of aminoglycoside administration. The presence of the A1555G mutation itself can induce the mitochondrial dysfunction in tissues other than cochlea and is indicative of a determinant for the disease. The genetic and environmental modifying factors such as *cis*-mutations found in this study and aging process should be studied to understand the pathophysiology of the disease.

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References

[1] Wallace DC. Diseases of mitochondrial DNA. Annu Rev Biochem 1992;61:1175–1212.
[2] Zeviani M, Antozzi C. Defects of mitochondrial DNA. Brain Pathol 1992;2:121–132.
[3] Prezant TR, Agapian JV, Bohlman MC, et al. Mitochondrial ribosomal RNA mutation associated with both antibiotic-induced and non-syndromic deafness. Nat Genet 1993;4:289–294.
[4] Hutchin T, Haworth I, Higashi K, et al. A molecular basis for human hypersensitivity to aminoglycoside antibiotics. Nucleic Acids Res 1993;21:4174–4179.
[5] Usami S, Abe K, Kasai M, et al. Genetic and clinical features of sensorineural hearing loss associated with the 1555 mitochondrial mutation. Laryngoscope 1997;107:483–490.
[6] Fischel-Ghodsian N. Mitochondrial RNA processing and translation: link between mitochondrial mutations and hearing loss? Mol Genet Metab 1998;65:97–104.
[7] Inoue K, Takai D, Soejima A, et al. Mutant mtDNA at 1555 A to G in 12S rRNA gene and hypersusceptibility of mitochondrial translation to streptomycin can be co-transferred to ρ0 HeLa cells. Biochem Biophys Res Commun 1996;223:496–501.
[8] Santorelli FM, Tanji K, Manta P, et al. Maternally inherited cardiomyopathy: an atypical presentation of the mtDNA 12S rRNA gene A1555G mutation. Am J Hum Genet 1999;64:295–300.
[9] Shoffner JM, Brown MD, Huoponen K, et al. A mitochondrial DNA mutation associated with maternally inherited deafness and Parkinson's disease. Neurology 1996;46(Suppl):A331.
[10] Guan MX, Fischel-Ghodsian N, Attardi G. Biochemical evidence for nuclear gene involvement in phenotype of non-syndromic deafness associated with mitochondrial 12S rRNA mutation. Hum Mol Genet 1996;5:963–971.
[11] Goto Y, Koga Y, Horai S, Nonaka I. Chronic progressive external ophthalmoplegia: a correlative study of mitochondrial DNA deletions and their phenotypic expression in muscle biopsy. J Neurol Sci 1990;100:63–69.
[12] Goto Y, Nishino I, Horai S, Nonaka I. Detection of DNA fragments encompassing the deletion junction of mitochondrial genome. Biochem Biophys Res Commun 1994;202:1624–1630.
[13] Nishino I, Seki A, Maegaki Y, et al. A novel mutation in the mitochondrial tRNA(Thr) gene associated with a mitochondrial encephalomyopathy. Biochem Biophys Res Commun 1996;225:180–185.
[14] Novikoff AB, Goldfischer S. Visualization of peroxisomes (microbodies) and mitochondria with diaminobenzidine. J Histochem Cytochem 1969;17:675–680.
[15] Ruiz-Pesini E, Lapeña A-C, Díez-Sánchez, et al. Human mtDNA haplogroups associated with high or low reduced spermatozoa motility. Am J Hum Genet 2000;67:682–696.
[16] Poulton J. Does a common mitochondrial DNA polymorphism underlie susceptibility to diabetes and the thrifty genotype? Trends Genet 1998;14:387–389.
[17] Horai S, Murayama K, Hayasaka K, et al. mtDNA polymorphism in east Asian populations, special reference to the peopling of Japan. Am J Hum Genet 1996;59:579–590.
[18] Dubowitz V, Brooke MH. Muscle biopsy: a modern approach. London: W.B. Saunders, 1973.
[19] Ballinger SW, Shoffner JM, Hedaya EV, et al. Maternally transmitted diabetes and deafness associated with a 10.4 kb mitochondrial DNA deletion. Nat Genet 1992;1:11–15.
[20] Yamasoba T, Oka Y, Tsukuda K, Nakamura M, Kaga K. Auditory findings in patients with maternally inherited diabetes and deafness harboring a point mutation in the mitochondrial transfer RNA$^{Leu(UUR)}$ gene. Laryngoscope 1996;106:49–53.
[21] Moraes CT, Ricci E, Petruzzella V, et al. Molecular analysis of the muscle pathology associated with mitochondrial DNA deletions. Nat Genet 1992;1:359–367.
[22] Estivill X, Govea N, Barcelo E, et al. Familial progressive sensorineural deafness is mainly due to the mtDNA A1555G mutation and is enhanced by treatment of aminoglycosides. Am J Hum Genet 1998;62:27–35.
[23] Hyde GE, Rubel EW. Mitochondrial role in hair cell survival after injury. Otolaryngol Head Neck Surg 1995;113:530–540.

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Leber's hereditary optic neuropathy with intracranial arteriovenous malformation : a case report

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Abstract

We reported a patient with Leber's hereditary optic neuropathy (LHON) with an intracranial arteriovenous malformation (AVM). Genetic analysis of this patient revealed a point mutation in mitochondrial DNA (mtDNA) at nucleotide position 11778 in the ND4 subunit of complex I. Although the relationship between intracranial AVM and mtDNA mutations remains uncertain, some patients with intracranial AVM may be associated with mitochondrial abnormality. Further study is necessary to confirm whether the above conditions are coincidental or closely interrelated.

Key words : Leber's hereditary optic neuropathy (LHON) ; arteriovenous malformation (AVM) ; mitochondrial DNA.

Introduction

Leber's hereditary optic neuropathy (LHON) is a maternally inherited disorder characterized clinically by an acute or subacute onset of central visual loss prevalently in young males. Several point mutations in mitochondrial DNA (mtDNA) have been identified in patients with LHON and the most frequent mutation is a substitution at nucleotide position (np) 11778 (11778 mutation) in the ND4 subunit of complex I (Wallace *et al.*, 1988 ; Newman, 1993).

Intracranial arteriovenous, malformation (AVM) has been considered to originate from intrauterine maldevelopment of cerebral vessels. Familial cases have been rarely reported (Laing *et al.*, 1974 ; Snead OC III *et al.*, 1979 ; Aberfeld *et al.*, 1981 ; Boyd *et al.*, 1985 ; Yokoyama *et al.*, 1991 ; Amin-Hanjani *et al.*, 1998), though a genetic defect has not yet been established.

We report here, a patient with LHON associated with intracranial AVM. Genetic analysis of this patient revealed the 11778 mutation in mtDNA common to LHON. Although we could not ascertain how the mtDNA mutation is related to AVM, this disease combination was of interest to explain vascular abnormalities in both diseases.

Case report

A previously healthy 9-year-old Japanese boy suddenly developed severe headache and vomiting, and was admitted to our hospital. His parents were healthy with no consanguinity. The patient had one brother and one sister who were healthy with no visual disturbance. On admission, he was lethargic and showed inward deviation of both eyes, left hemiparesis and hemiataxia. Brain computed tomography revealed right thalamic and intraventricular hemorrhage. Angiography revealed AVM located from the right posterior thalamus to the midbrain with feeders from the posterior thalamoperforate artery (Fig. 1). Ventricular drainage was performed and all symptoms were improved except for upward gaze palsy of both eyes. Since a conventional direct operation was difficult in this area, he underwent gamma radiation in the U.S.A. Six months after this therapy, the AVM disappeared on angiography, though he developed left hemiparesis, and postural and intention tremor of the left upper limb, which were thought to have been induced by radiation injury. He underwent a right thalamotomy (nucleus ventralis intermedius) 8 years after the radiation, then the tremor disappeared.

At the age of 18 years, he first noticed a visual disturbance on the left, and the right 8 months after the onset. He visited the ophthalmologic clinic in our hospital and was referred to the neurology section for further evaluations.

He was 163 cm in height and 62 kg in weight. His mental status was normal with normal speech. He had mild left hemiparesis and hemiataxia, but no tremor. Deep tendon reflex was mildly hyperactive on the left side, but no pathologic reflexes were seen. He showed mild hypesthesia in the left upper limb.

On ophthalmological examination, his visual acuity was 0.03 OD and 30 cm/nd OS. He had bilateral central scotoma, which was more severe in the left than the right eye. Optic disc examination showed mild optic atrophy in the temporal side of

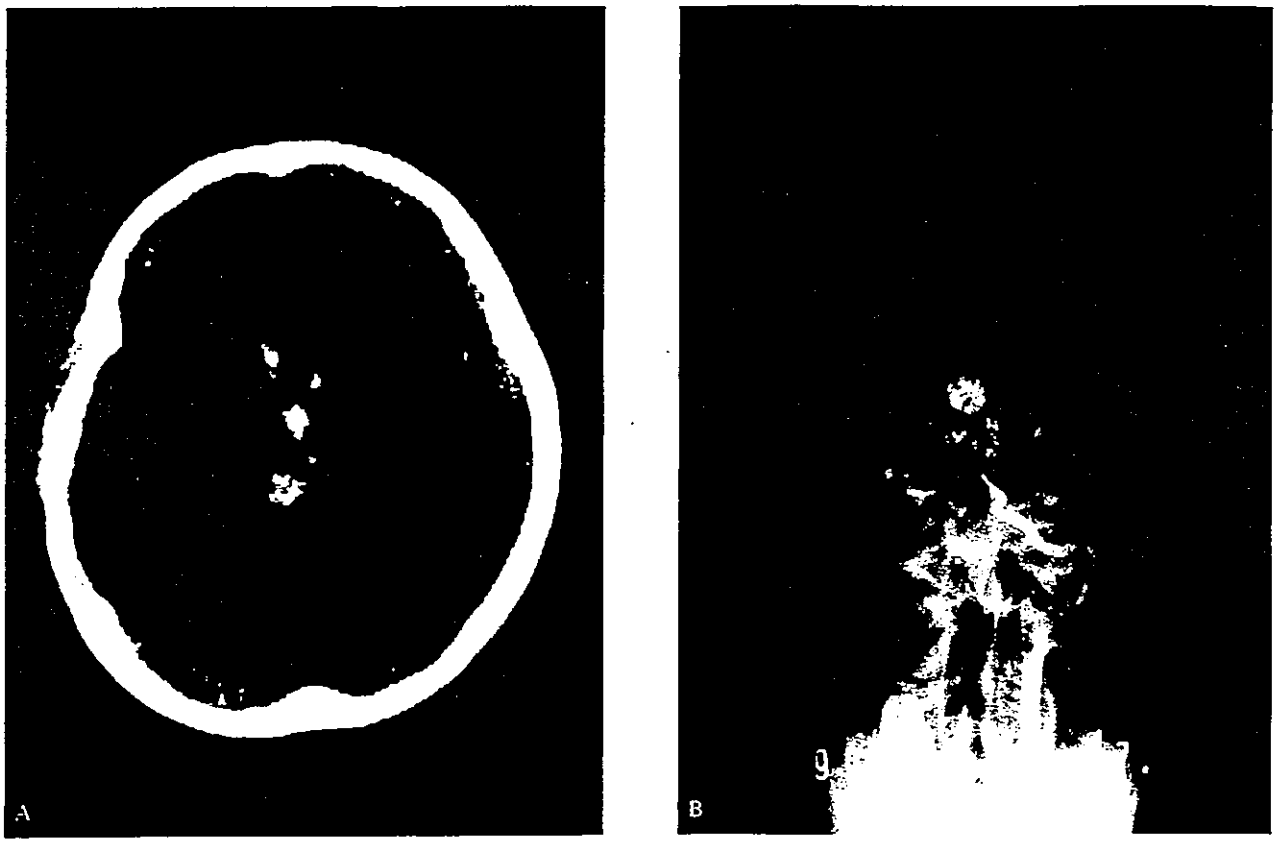

FIG. 1. — (A) Computed tomography of the brain shows a right thalamic hemorrhage and intraventricular rupture.
(B) Left vertebral angiography reveals arteriovenous malformation (AVM) at the right posterior thalamus to the midbrain with feeders from the posterior thalamoperforate artery.

the left eye, but normal in the right. Retinal microangiopathy was not recognizable during the course. Critical fusion frequency (CFF) was 25 cycles/sec OD and 13 cycles/sec OS. Fluorescein fundus angiography showed neither dye leakage nor dilated capillaries. Pattern-reversal visual evoked potentials failed to exhibit P-100 waves in both eyes.

His routine blood analysis (complete blood count, liver enzyme activities, electrolytes, glucose, urea nitrogen, etc) yielded normal results. Lactate and pyruvate levels in the blood were 8.4 mg/dl (normal 3.7-16.3) and 0.86 mg/dl (0.1-0.9), respectively. The cerebrospinal fluid was normal including a cell count of 2/mm^3, protein level of 24 mg/dl, lactate of 15.1 mg/dl (11.1-16.6), and pyruvate of 1.10 mg/dl (0.75-1.29). Electrocardiography was normal. Electroencephalography showed a slow background of 7-8 Az and θ waves.

A small area in the right thalamus and midbrain had a low signal on the T1-weighted image and a high signal on the T2-weighted image of magnetic resonance imaging (MRI), thought to be caused by the ruptured AVM and gamma radiation.

He was placed on corticosteroid pulse therapy (methylprednisolone 1000 mg div/day for 3 days per week, three times) with the tentative diagnosis of optic neuritis. After LHON was diagnosed, coenzyme Q_{10} supplement therapy was started. About two weeks after the steroid pulse therapy, his visual acuity improved to 0.08 OD and 0.04 OS. His visual acuity became 0.04 OD and 0.01 OS some months later, but it further improved to 0.20 OD and 0.03 OS after the next 2 years.

MtDNA analysis

To detect the LHON mutation, DNA was extracted from peripheral lymphocytes. Two large mtDNA fragments were amplified using long PCR by a method described previously that avoid the amplification of nuclear mtDNA-like sequences (Akanuma *et al.*, 2000). The PCR products were directly purified using Microspin S-400 HR columns (Amersham Pharmacia Biotech). With 96 primer sets designed for sequencing, fragments were subject to the BigDye Terminator Cycle Sequencing reaction (PE Applied Biosystems) and sequence determination on an ABI 3700 automated sequencer according to the manifacturer's protocol. We identified the 11778 G $\rightarrow$ A mutation but did not find any other LHON mutation or new polymorphism (Table 1). To determine whether the mutation was homoplasmic or heteroplasmic, we applied PCR-RFLP method using SfaNI restriction enzyme. The all PCR products were not cut by the enzyme indicative homoplasmy.

TABLE I

Sequence analysis of whole mtDNA

region	mutation	amino acid replacement	frequency in normal controls
D-loop	a73g		98%
	c150t		14%
	t199c		10%
	a263g		83%
	ins-303c		44%
	ins-311c		85%
	t489c		62%
12SRMA	a750g		99%
	a1438g		92%
16SRMA	a2706g		99%
	ins-3168c		0%
ND1	g4048a.	D → N	3%
	c4071t	syn	5%
	a4164g	syn	4%
ND2	a4769g	syn	99%
	a5351g	syn	4%
	g5460a	A → T	5%
CO I	c6455t	syn	13%
	t6680c	syn	4%
	c7028t	syn	99%
CO II	t7684c	syn	3%
	g7853a	V → A	4%
ATP 6	a8701g	T → A	45%
	a8860g	T → A	100%
CO III	t9540c	syn	52%
	t9824c	syn	14%
ND3	t10345c	I → T	4%
	a10398g	T → A	70%
	c10400t	syn	63%
ND4	t10873c.	syn	63%
	g11719a	syn	100%
	g11778a	R→ H	0%
ND5	c12405t	syn	4%
	c12705t	syn	73%
	t12811c	Y → H	4%
cyt b	c14766t	I → T	100%
	t14783c	syn	65%
	g15043a	syn	63%
	g15301a	syn	49%
	a15326g	T → A	99%
D-loop	g16129a		21%
	c16223t		75%
	t16297c		4%
	t16298c		10%

syn : synonymous.

Discussion

Our patient presented the typical symptoms of LHON, including subacute onset of bilateral visual loss at the age of 18 years. He had no family history, however, and mtDNA analysis disclosed the 11778 mutation. The most interesting feature of this patient was an association with intracranial AVM.

It is well known that some pedigrees with LHON have exhibited some additional neurological findings and have been called Leber's 'plus'. The symptoms include dystonia, tremor, parkinsonism, spasticity and polyneuropathy (Meire *et al.*, 1995 ; Nikoskelainen *et al.*, 1995 ; Schoffner *et al.*, 1995). Multiple sclerosis-like symptoms and MR findings have also been reported (Harding *et al.*, 1992). However, AVM has not been complicated with LHON, with myoclonus epilepsy associated with ragged-red fibers (MERRF) nor mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes (MELAS). Although it is difficult to conclude whether the combination with AVM and LHON was coincidental or interrelated, further cumulative study of the combination may resolve this issue. It would be interesting to screen the many reported families with LHON for the occurrence of intracranial AVM by CT or MRI study.

Our patient's visual acuity improved from 0.03 OD and 30 cm/nd OS to 0.20 OD and 0.03 OS

after about 2 years suggesting a beneficial effect of coenzyme Q_{10} therapy. The treatment of LHON is not yet established. Newman (1993) described in the review of LHON that attempts to treat or prevent the acute phase of visual loss with systemic steroids, hydroxycobalamin, or cyanide antagonists have, in general, proved not efficacious. He also stated that use of coenzyme Q and succinate in patients with LHON and visual loss had been limited, but his preliminary results were not particularly encouraging. Nakamura *et al.* (1994) reported a patient with LHON with a mtDNA mutation at np 3460, whose visual acuity improved after coenzyme Q_{10} supplemention, steroid pulse therapy and hydroxycobalamine injection. Mashima *et al.* (1992) reported a patient who had a remission of LHON with idebenone, which is a quinor that stimulates net ATP formation in cerebral metabolism and inhibits lipid peroxidation in the mitochondrial membrane. Nishikawa *et al.* (1989) reported a case of mitochondrial encephalomyopathy with cytochrome c oxidation deficiency, treated with high doses of coenzyme Q_{10}. Abnormal elevation of serum lactate over pyruvate ratio and the increased concentration of serum lactate plus pyruvate induced by exercise decreased with coenzyme Q_{10} treatment. They suggested that coenzyme Q_{10} had clinical value in the long-term management of patients with mitochondrial encephalomyopathies, even though there were clinical limitations to the effects of this therapy.

Familial occurrence of AVM is rarely reported. Yokoyama *et al.* (1991) reported 3 families in which a father and his son, cousin, and a mother and her son were respectively affected. Amin-Hanjani *et al.* (1998) reviewed familial AVM patients reported in the literature, consisting of parent-child combinations from 7 families (41%), including 2 mother-daughter, one mother-son, 4 father-son, as well as sibling combinations in 7 families (41%), and cousin combinations in 3 families (18%). Analysis of familial patients has revealed that some appear to have autosomal dominant inheritance and others mtDNA (maternal) inheritance or autosomal recessive inheritance.

Genetic mechanisms that contribute to pathogenesis and phenotype of intracranial AVM are still unknown. A lot of research is currently going on exploring the molecular aspects of vascular proliferation/malformation. Recently, a gene and mutations causing cerebral cavernous malformations (CCM) in a subset of families with the disease were identified. CCMl mapping to 7q encodes KRIT1, Krev-1/rap1a binding protein (Laberge-le Couteulx *et al.*, 1999 ; Sahoo *et al.*, 1999). Hereditary hemorrhagic telangiectasia (HHT) is an autosomal dominant disorder characterized by cutaneous vascular dysplasia and a high propensity to develop systemic and intracranial vascular lesions or AVMs. Endoglin, a transforming growth factor beta binding protein of endothelial cells, has been proved to be the gene for HHT type 1 mapping to 9q3 (McAllister *et al.*, 1994).

Blood vessel involvement has been observed in some mitochondrial diseases, especially MELAS in which abnormal mitochondria are accumulated in the small arteries showing "strongly succinate dehydrogenase-reactive blood vessels" (SSV) (Hasegawa *et al.*, 1991 ; Goto *et al.*, 1992). Sakuta *et al.* (1989) examined biopsy specimens of muscles from patients with mitochondrial myopathy and revealed that some pericytes around a few arterial capillaries contained aggregates of slightly enlarged mitochondria. Kishi *et al.* (1988) reported an autopsy case of MELAS, describing that the cerebral cortex was in spongy state and capillary proliferation and fibrillary gliosis were noted. Bertrand *et al.* (1996) also reported a case of mitochondrial encephalomyopathy of mixed MELAS type, showing numerous focal and so called pseudolaminar cortical necrosis in the brain with characteristic proliferation of capillary vessels in a microscopic study. Microangiopathy is the first abnormal finding in the optic disc in LHON. In conjunction with these findings, some patients with intracranial AVM may have an associated mitochondrial abnormality. A link could be found between molecular mechanisms of AVM and the mitochondrial defect. Further genetic research is necessary to understand the relationship between AVM and LHON.

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REFERENCES

1. Aberfeld D. C., Rao K. R. Familial arteriovenous malformation of the brain. *Neurology*, 1981, **31** : 184-186.
2. Akanuma J., Muraki K., Komaki H., Nonaka I., Goto Y. Two pathogenic point mutations exist in the authentic mitochondrial genome, not in the nuclear pseudogene. *J. Hum. Genet.*, 2000, **45** : 337-341.
3. Amin-Hanjani S., Robertson R., Arginteanu M. S., Scott R. M. Familial intracranial arteriovenous malformations. Case report and rewiew of the literature. *Pediatr. Neurosurg.*, 1998, **29** : 208-213.
4. Bertrand E., Fidzianska A., Schmidt-Sidor B., Mendel T. Mitochondrial encephalomyopathy of mixed MELAS type. *Folia Neuropathol.*, 1996, 34 : 193-198.
5. Boyd M.C., Steinbok P., PatY D.W. Familial arteriovenous malformations. Report of four cases in one family. *J. Neurosurg.*, 1985, **62** : 597-599.
6. Goto Y., Horai S., Matsuoka T., Koga Y., Nihei K. *et al.* Mitochondrial myopathy, encephalopat by, lactic acidosis, and stroke-like episodes (MELAS) : A correlative study of the clinical féatures and

mitochondrial DNA mutation. *Neurology*, 1992, **42** : 545-550.
7. HARDING A. E., SWEENEY M. G., MILLER D. H., MUMFORD C. J., KELLAR-WOOD H. *et al.* Occurrence of a multiple sclerosis-like illness in women who have a Leber's hereditary optic neuropathy mitochondrial DNA mutation. *Brain*, 1992, **115** : 979-989.
8. HASEGAWA H., MATSUOKA T., GOTO Y., NONAKA I. Strongly succinate dehydrogenase-reactive blood vessels in muscles from patients with mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes. *Ann. Neurol.*, 1991, **29** : 601-605.
9. KISHI M., YAMAMURA Y., KURIHARA T., FUKUHARA N., TSURUTA K. *et al.* An autopsy case of mitochondrial encephalomyopathy : biochemical and electron microscopic studies of the brain. *J. Neurol. Sci.*, 1988, **86** : 31-40.
10. LABERGE-LE COUTEULX S., JUNG H. H., LABAUGE P., HOUTTEVILLE J.-P., LESCOAT C. *et al.* Truncating mutations in CCM1, encoding KRIT1, cause hereditary cavernous angiomas. *Nat. Genet.*, 1999, **23** : 189-193.
11. LAING J. W., SMITH R. R. Intracranial arteriovenous malformations in sisters : a case report. *J. Miss. State Med. Assoc.*, 1974, **15** : 203-206.
12. MASHIMA Y., HIDA Y., OGUCHI Y. Remission of Lever's hereditary optic neuropathy with idebenone. *Lancet*, 1992, **340** : 368-369.
13. MCALLISTER K. A., GROGG K. M., JOHNSON D. W., GALLIONE C. J., BALDWIN M. A. *et al.* Endoglin, a TGF-β binding protein of endothelial cells, is the gene for hereditary haemorrhagic telangiectasia type 1. *Nat. Genet.*, 1994, **8** : 345-351.
14. MEIRE F. M., VAN COSTER R., COCHAUX P., OBERMAIER-KUSSER B., CANDAELE C. *et al.* Neurological disorders in members of families with Leber's hereditary optic neuropathy (LHON) caused by different mitochondrial mutations. *Ophtalmic Genetics*, 1995, **16** : 119-126.
15. NAKAMURA M., TANIGAWA M., YAMAMOTO M. A case of Leber's hereditary optic neuropathy with a mitochondrial DNA mutation at nucleotide position 3460. *Jpn. J. Ophtalmol.*, 1994, **38** : 267-271.
16. NEWMAN N. J. Leber's hereditary optic neuropathy. New genetic considerations. *Arch. Neurol.*, 1993, **50** : 540-548.
17. NIKOSKELAINEN E. K., MARTTILA R. J., HUOPONEN K., JUVONEN V., LAMMINEN T. *et al.* Leber's "plus" : neurological abnormalities in patients with Leber's hereditary optic neuropathy. *J. Neurol. Neurosurg. Psychiatry*, 1995, **59** : 160-164.
18. NISHIKAWA Y., TAKAHASHI M., YORIFUJI S., NAKAMURA Y., UENO S. *et al.* Long-term coenzyme Q_{10} therapy for a mitochondrial encephalomyopathy with cytochrome c oxidase deficiency : a ^{31}P NMR study. *Neurology*, 1989, **39** : 399-403.
19. SAHOO T., JOHNSON E. W., THOMAS J. W., KUEHL P. M., JONES T. L. *et al.* Mutations in the gene encoding KRIT1, a Krev-1/rap1a binding protein, cause cerebral cavernous malformations (CCM1). *Hum. Mol. Genet.*, 1999, **8** : 2325-2333.
20. SAKUTA R., NONAKA I. Vascular involvement in mitochondrial myopathy. *Ann. Neurol.*, 1989, **25** : 594-601.
21. SCHOFFNER J. M., BROWN M. D., STUGARD C., JUN A. S., POLLOCK S. *et al.* Leber's hereditary optic neuropathy plus dystonia is caused by a mitochondrial DNA point mutation. *Ann. Neurol.*, 1995, **38** : 163-169.
22. SNEAD O. C. III, ACKER J. D., MORAWETZ R. Familial arteriovenous malformation. *Ann. Neurol.*, 1979, **5** : 585- 587.
23. YOKOYAMA K., ASANO Y., MURAKAWA T., TAKADA M., ANDO T. *et al.* Familial occurrence of arteriovenous malformation of the brain. *J. Neurosurg.*, 1991, **74** : 585-589.
24. WALLACE D. C., SINGH G., LOTT M. T., HODGE J. A., SCHURR T. G. *et al.* Mitochondrial DNA mutation associated with Leber's hereditary optic neuropathy. *Science*, 1988, **242** : 1427-1430.

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Atypical MELAS Associated With Mitochondrial tRNA^{Lys} Gene A8296G Mutation

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We report on a unique patient with mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS) presenting optic atrophy, cardiomyopathy, and bilateral striatal necrosis before stoke-like episodes became apparent. Skeletal muscle total mitochondrial DNA analysis identified a heteroplasmic A to G point mutation in the tRNA^{Lys} gene at position 8296. Skeletal muscle pathology revealed typical MELAS findings, including ragged-red fibers cytochrome *c* oxidase positive strongly succinate dehydrogenase-reactive blood vessels. Recent reports describe the 8296 mutation identified in patients with diabetes mellitus or myoclonus epilepsy with ragged-red fibers, not MELAS. We conclude that the 8296 mutation is likely to be pathogenic and that it may be not only a mutation responsible for diabetes mellitus or myoclonus epilepsy with ragged-red fibers but also for MELAS.

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Introduction

Mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS) are characterized by recurrent neurologic deficits including seizures, headache, dementia, and psychomotor retardation that initiate in childhood. Although symptomatic heterogeneity among MELAS patients has been known, the combination of chronic progressive external ophthalmoplegia, hearing loss, and diabetes mellitus is shared by most of them [1]. It is rare to have optic atrophy of early childhood onset with cardiomyopathy and bilateral striatal necrosis before stoke-like episodes become apparent.

We performed direct sequencing of all mitochondrial DNA (mtDNA) and identified the A8296G substitution in the tRNA^{Lys} gene. In recent reports the A8296G mutation has been identified in patients with diabetes mellitus and myoclonus epilepsy with ragged-red fibers (MERRF). Herein we report on the first patient with atypical MELAS associated with the A8296G mutation and its causal link to the clinical features.

Case Report

A 14-year-old Japanese male was born by normal spontaneous delivery at 38 weeks of gestation weighing 2,675 gm. His mother, 39 years old, was healthy, but his 65-year-old maternal grandmother had hearing loss and noninsulin-dependent diabetes mellitus. In his prenatal and perinatal periods and through early infancy, no abnormal characteristics were observed. At 4 years of age, he temporarily lost central vision in both eyes without noticeable pain. However, the loss of vision was transient, lasting for a few days; his visual acuity was limited to perceiving hand movements in both eyes. The optic disc pallor developed after a few months, and he was thus diagnosed as having optic atrophy. His visual acuity was gradually reduced. Hypertrophic cardiomyopathy was found at a school check-up when he was 6 years of age. However, he did not develop heart failure. Then he developed persistent fatigue and weakness of skeletal muscles between 8 and 9 years of age. At that time, he was noted to have short stature, sensorineural hearing loss, a variable speech disorder, poor short-term memory, and clumsiness. A computed tomography brain scan revealed a low-density area in bilateral striatal lesions. At 14 years of age, he had an episode of recurrent headache with nausea accompanied by focal motor and generalized epileptic seizures and lactic acidosis. He was referred to our hospital for further examination, including muscle biopsy, biochemical analysis, and DNA analysis. On admission, physical examination revealed short stature and body weight both below the second percentile. His pubertal development was normal. General examination findings were otherwise unremarkable. Optic atrophy was observed, but there was no retinal pigmentation. He had myoclonus with hypertonicity. There was no objective sensory deficit. Brain magnetic resonance imaging demonstrated diffuse cortical

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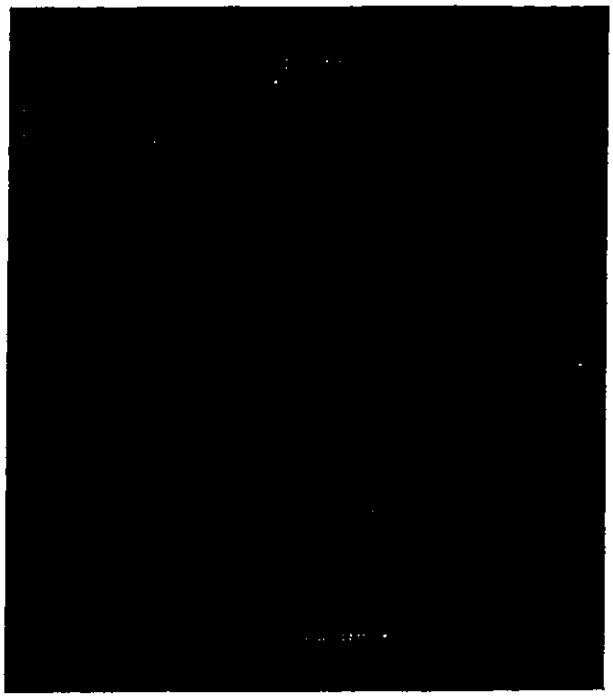

Figure 1. Axial T_1-weighted brain magnetic resonance image (TR = 400 ms, TE = 15 ms) is presented. Note the diffuse cortical atrophy and bilaterally symmetric lesions (low-intensity area) of the putamen and caudate nucleus.

atrophy and bilaterally symmetric lesions at the putamen and caudate nucleus (Fig 1). Echocardiography revealed hypertrophy of the anterolateral and posterior walls of the left ventricle (Fig 2). We treated the patient with cytochrome *c* and dichloroacetate for mitochondrial dysfunction, without significant effects.

Informed consent from the patient and patient's mother was obtained for all investigations.

Muscle Biopsy

We obtained biopsied muscle from the left biceps brachii muscle. Serial frozen sections were stained with hematoxylin and eosin, modified Gomori trichrome, succinate dehydrogenase (SDH), cytochrome *c* oxidase (COX), and a battery of histochemical methods.

Figure 2. Echocardiography reveals hypertrophy of the anterolateral and posterior walls of the left ventricle at 14 years of age (parasternal long-axis view).

DNA Analyses

Total DNA was extracted from a part of the muscle biopsy specimen. DNA sample was analyzed for the mutations previously identified in MELAS or MERRF, as described [2-6]. For sequencing analysis, 12 sets of primers were used to amplify the DNA fragments covering all of the mitochondrial tRNA regions. Polymerase chain reaction products were purified and their nucleotide sequences determined by an ABI 377 Automatic Sequencer (Perkin Elmer Biosystem, Foster City, CA) using the DNA Sequencing Kit and the Dye-Terminator Protocol (Perkin Elmer Biosystem, Foster City, CA).

Based on the sequencing result a simple screening method was devised to detect the A8296G mutation. This method utilizes an *AciI* restriction site that is only present in the mutated allele. The mtDNA fragment surrounding the $tRNA^{Lys}$ was amplified by polymerase chain reaction using a sense primer, 5';-AACCAAACCACTTTCACCGCT-3'; (8123–8143), and an antisense primer, 5';-CATACGGTAGTATTTAGTTGG-3'; (8391–8371). The polymerase chain reaction was performed in a total volume of 5 μL containing 5 ng of genomic DNA, 50 nM of two primers, 62.5 μM of dNTP, 50 mM KCl, 10 mM Tris-HCl, 2.0 mM $MgCl_2$, and 0.25 U of the Taq DNA polymerase. The polymerase chain reaction was performed for 30 cycles consisting of 15 seconds denaturation at 94°C, 15 seconds annealing at 50°C, and 30 seconds primer extension at 72°C in a Thermal Cycler (Perkin-Elmer Biosystem, Foster City, CA).

Results

Biochemical and Histologic Characteristics

Routine hematologic and biologic investigations were indistinguishable from normal. Glucose tolerance test was normal. However, lactate levels of fasting blood and cerebrospinal fluid were elevated to 40 mg/dL and 80 mg/dL (normal range = 3.3–14.9 mg/dL), respectively.

The muscle fibers varied in size from 20 to 80 μm in diameter. Some ragged-red fibers (RRFs) and strongly SDH-reactive blood vessels (SSVs) were recognized. All the muscle fibers were COX positive. RRFs and SSVs demonstrated increased levels of COX activity (Fig 3). Enzymes involved in mitochondrial respiratory chain were not analyzed.

DNA Analyses

The DNA sample was screened for the mutations in mitochondrial tRNA genes that were previously found to be associated with MELAS or MERRF. They included the 3243, 3271, 3291 mutation of the $tRNA^{Leu(UUR)}$ gene, and the 8344 and 8363 mutation of the $tRNA^{Lys}$ gene [2-6]. However, none of these were detected. Sequence analysis identified a number of nucleotide substitutions, A1438G, T2626C, A2706G, C2772 torr, G3423 torr, A4769G, A4958G, G4985A, C7028 torr, A8296G, A8701G, A8860G, T9540 torr, T11335C, C12705 torr, and G14365C, in comparison to the Cambridge sequence. With the exception of the A8296G mutation, these substitutions have been viewed as polymorphisms without negative effects on function. Cleavage of the 268-bp polymerase chain reaction product containing the mutated region by *AciI* revealed a heteroplasmic proportion (Fig 4). With this simple and specific screening method, the

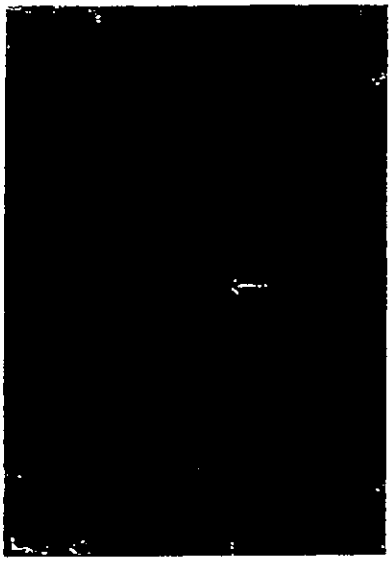

Figure 3. Frozen cross-sections are illustrated with cytochrome c oxidase (COX) stain. There was a mild variation in fiber size. Some ragged-red fibers (RRFs)(*) and strongly SDH-reactive blood vessels (SSVs)(←) were recognized. All the muscle fibers were COX positive. RRFs and SSVs demonstrated increased levels of COX activity (bar = 50 μm).

A8296G mutation was not detected in 100 normal control DNA samples.

Discussion

The diagnosis of MELAS was determined for this patient based on the following: (1) stroke-like episodes with recurrent headache and vomiting, (2) high lactate level presenting both in blood and cerebrospinal fluid, (3) muscle biopsy findings including RRFs and COX-positive SSV. The presence of COX-positive SSV is a characteristic vascular finding for MELAS. In MERRF, SSVs also appear in muscle specimens, but COX activity in SSVs is almost negative [1].

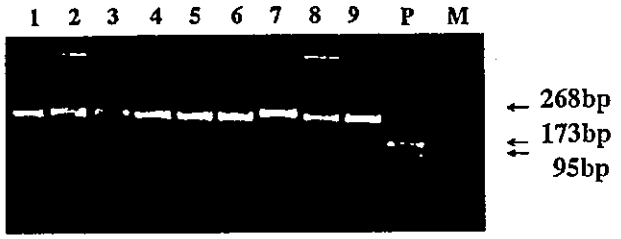

Figure 4. The A8296G mutation and normal alleles of the $tRNA^{Lys}$ gene detected by polymerase chain reaction followed by AciI digest are depicted. The DNA fragments were stained with ethidium bromide and visualized using an ultraviolet light transilluminator. The polymerase chain reaction products from the mutant mtDNA of the patient (lane P) and wild type of mtDNA in control subjects (lane 1-9) were fractionated by electrophoresis through a 3% Nusieve agarose gel. The larger DNA fragment of 268-bp in size is derived from the nonmutated allele. The mutated allele contains an AciI site and thus generates two smaller fragments (173 and 95 bp) by an AciI cleavage. The proportion of the two alleles in the patient DNA sample indicates a characteristic of a heteroplasmic mutation. M lane is DNA size marker.

The current patient is unusual in that the symptoms are accompanied by optic atrophy, cardiomyopathy and bilateral striatal necrosis [7], which appeared in early childhood before the onset of stoke-like episodes. Ophthalmologic impairments usually observed in MELAS are pigmentary retinopathy, ophthalmoplegia, and ptosis [8]. However, optic atrophy observed in this patient has been rarely reported in association with MELAS [9] and thus makes this patient unique. Pulkes et al. reported on a patient having combined syndromes of Leber's hereditary optic neuropathy (LHON) and MELAS [10]. A recent report described a family in which the affected members had late-onset optic atrophy, ataxia, and myopathy associated with a mutation of a complex II gene. These patients did not have stroke-like episodes [11]. LHON cause of permanent blindness in otherwise healthy young adults is more common in men. LHON is mostly associated with mtDNA point mutation in the mitochondrial complex I subunit genes ND1, ND4, and ND6 [12]. Our patient developed blindness in his early childhood; however the loss was only temporary. Because of the disparity of symptoms, our patient was not diagnosed as LHON.

None of the mutations previously associated with MELAS were detected in our patient, and sequencing of all mtDNA was necessary to identify any potentially causal mtDNA mutation. Extensive sequence analysis of mtDNA identified only one nucleotide substitution, A8296G, in the $tRNA^{Lys}$ gene of a MELAS patient who presented with an unusual combination of symptoms. More than 80% of MELAS patients are found to have a single nucleotide substitution at position 3243 from A to G (A3243G mutation) in the $tRNA^{Leu(UUR)}$ gene of the mitochondrial genome [1]. Mutations at position 3251, 3256, 3271, and 3291 of the $tRNA^{Leu(UUR)}$ gene may also be responsible for various forms of MELAS [3,4]. Thus the $tRNA^{Leu(UUR)}$ gene may be a genetic hot spot with a specific link to MELAS.

In contrast, many of the mitochondrial DNA mutations in $tRNA^{Lys}$ are associated with MERRF [5,6]. Its unique link to and the atypical nature of clinical features imply that the mutation is pathogenic. This notion is supported by the following three additional factors: (1) the mutation is heteroplasmic, (2) the mutated site is located in the aminoacyl acceptor stem of $tRNA^{Lys}$, in which the wild type of nucleotide adenine is highly conserved between a diverse range of species, and (3) the mutation is not present in 100 healthy control subjects.

The A8296G mutation may be a part of the complex cause of diabetes, and 1% of diabetic patients have this particular mutation [13]. Moreover, a recent report documented a double mutation, A8296G and G8363A, being associated with myoclonus epilepsy with MERRF [14]. In this context, because the 8296 mutation was nearly homoplasmic, the authors concluded that G8363A mutation is pathogenic; the co-occurrence of the 8296 mutation is of unclear significance and is likely to be a rare polymorphism. However, this 8296 mutation alters the double

helix of the stem and secondary structure of tRNA. This effect is the impairment of the activities of aminoacyl-tRNA synthase and peptidil-tRNA hydrolase, which play a central role in the mitochondrial translation process. Thus this study reports the first patient of MELAS associated with the A8296G mutation.

We conclude that the A8296G mutation is likely to be pathogenic and may have a causal link not only to diabetes mellitus and MERRF but also to MELAS.

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References

[1] **Goto Y.** Clinical features of MELAS and mitochondrial DNA mutations. Muscle Nerve 1995;3:S107-S12.

[2] **Goto Y, Nonaka I, Horai S.** A mutation in the tRNA(leu)(UUR) gene associated with the MELAS subgroup of mitochondrial encephalomyopathies. Nature 1990;348:651-3.

[3] **Goto Y, Nonaka I, Horai S.** A new mtDNA mutation associated with mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes (MELAS). Biochim Biophys Acta 1991;1097:238-40.

[4] **Goto Y, Tsugane K, Tanabe Y, Nonaka I, Horai S.** A new point mutation at nucleotide pair 3291 of the mitochondrial $tRNA^{leu(UUR)}$ gene in a patient with mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS). Biochem Biophys Res Commun 1994;202:1624-30.

[5] **Shoffner JM, Lott MT, Lezza AM, Seibel P, Ballinger SW, Wallace DC.** Myoclonic epilepsy and ragged-red fiber disease (MERRF) is associated with a mitochondrial DNA tRNA(Lys) mutation. Cell 1990;61:931-7.

[6] **Ozawa M, Nishino I, Horai S, Nonaka I, Goto YI.** Myoclonus epilepsy associated with ragged-red fibers: a G-to-A mutation at nucleotide pair 8363 in mitochondrial tRNA(Lys) in two families. Muscle Nerve 1997;20:271-8.

[7] **Campos Y, Martin MA, Rubio JC, Gutierrez del Olmo MC, Cabello A, Arenas J.** Bilateral striatal necrosis and MELAS associated with a new T3308C mutation in the mitochondrial ND1 gene. Biochem Biophys Res Commun 1997;238:323-5.

[8] **Sue CM, Mitchell P, Crimmins DS, Moshegov C, Byrne E, Morris JG.** Pigmentary retinopathy associated with the mitochondrial DNA 3243 point mutation. Neurology 1997;49:1013-7.

[9] **Hwang JM, Park HW, Kim SJ.** Optic neuropathy associated with mitochondrial tRNA[Leu(UUR)] A3243G mutation. Ophthalmic Genet 1997;18:101-5.

[10] **Pulkes T, Eunson L, Patterson V, et al.** The mitochondrial DNA G13513A transition in ND5 is associated with a LHON/MELAS overlap syndrome and may be a frequent cause of MELAS. Ann Neurol 1999;46:916-9.

[11] **Birch-Machin MA, Taylor RW, Cochran B, Ackrell BAC, Turnbull DM.** Late-onset optic atrophy, ataxia, and myopathy associated with a mutation of a complex II gene. Ann Neurol 2000;48:330-5.

[12] **Harding AE, Sweeney MG.** Leber's hereditary optic neuropathy. Mitochondrial disorders in neurology, 1st ed. Oxford, England: Butterworth-Heinmemann, 1994:181-98.

[13] **Kameoka K, Isotani H, Tanaka K, et al.** Novel mitochondrial DNA mutation in tRNALys (8296A→G) associated with diabetes. Biochem Biophys Res Commun 1998;245:523-7.

[14] **Arenas J, Campos Y, Bornstein B, et al.** A double mutation (A8296G and G8363A) in the mitochondrial DNA tRNALys gene associated with myoclonus epilepsy with ragged-red fibers. Neurology 1999;52:377-82.

ORIGINAL ARTICLE

Age related expression of Werner's syndrome protein in selected tissues and coexpression of transcription factors

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Aims: Werner's syndrome (WS) is an uncommon autosomal recessive disease resulting from mutational inactivation of human WRN helicase, Werner's syndrome protein (WRNp). Patients with WS progressively develop a variety of aging characteristics after puberty. The aim of this study was to determine the distribution of WRNp and the expression of the transcription factors regulating WRN gene expression in a variety of human organs in an attempt to understand the WS phenotype.
Methods: Tissue specimens were obtained from 16 controls aged from 27 gestational weeks to 70 years of age and a 56 year old female patient with WS. The distribution of WRNp and the expression of the transcription factors regulating WRN gene expression—SP1, AP2, and retinoblastoma protein (Rb)— were studied in the various human organs by immunohistochemical and immunoblot analyses.
Results: In the healthy controls after puberty, high expression of WRNp was detected in seminiferous epithelial cells and Leydig cells in the testis, glandular acini in the pancreas, and the zona fasciculata and zona reticularis in the adrenal cortex. In addition, the SP1 and AP2 transcription factors, which regulate WRNp gene expression, appeared in an age dependent manner in those regions where WRNp was expressed. In controls after puberty, SP1 was expressed in the testis and adrenal gland, whereas AP2 was expressed in the pancreas.
Conclusions: These findings suggest that the age specific onset of WS may be related to age dependent expression of WRNp in specific organs.

Werner's syndrome (WS) is an uncommon autosomal recessive disease resulting from mutational inactivation of the human WRN helicase gene, which encodes both helicase and exonuclease activities, and is found at 8p11–12.[1 2] WRN helicase is one of five human RecQ helicases, and is one of the three such genes that have been related to a heritable human disease; namely: Bloom syndrome (BLM)[3] and Rothmund-Thomson syndrome (RTS).[4]

The characteristic phenotype of WS is premature aging. After puberty, affected individuals rapidly develop greying and loss of hair, scleroderma-like skin changes, osteoporosis, atherosclerosis, non-insulin dependent diabetes mellitus, and hypogonadism; these patients also have an increased risk of cancer.[5] At the cellular level, cultured cells from patients with WS have a shortened life span and prolonged S phase of the cell cycle.[6] In addition, increased mutation frequency, chromosomal instability, and rapid senescence of fibroblast cultures are seen in cells from patients with WS.[7–9]

> "The characteristic phenotype of WS is premature aging"

The 5′ upstream region of the WRN gene contains three cis regulating elements: an SP1 element, an AP2 element, and a retinoblastoma (Rb) control element (RCE). WRN expression is regulated mainly by the SP1 transcriptional control system.[10] Despite this molecular information we do not understand why the WS phenotype is only expressed after puberty. The hypotheses are that: (1) WRN is expressed after puberty in specific tissues, and/or (2) the transcription factors regulating WRN gene expression are manifested after puberty in those tissues that express WRN. Thus, in our present study, using immunohistochemical and immunoblot analyses of various postmortem human tissues we investigated the expression of WS protein (WRNp) and transcription factors, SP1, AP2, and Rb in an attempt to clarify the pathogenetic importance of WRN.

MATERIALS AND METHODS

Postmortem tissues

Sets of postmortem tissue specimens (brain, liver, pancreas, testis, ovary, kidney, adrenal gland, heart, spleen, and intestine) were obtained from 16 controls and a female patient with WS. The ages of the controls ranged from 27 gestational weeks to 70 years and the patient with WS was 56 years old.[11] In these cases, informed consent was obtained in writing. The necropsies were performed within 24 hours of death, and usually within 12 hours.

Immunohistochemistry

The sections for immunohistochemistry were dewaxed and pretreated with 0.3% hydrogen peroxide in methanol for 15 minutes to abolish endogenous peroxidase activity, and then incubated for 30 minutes in the presence of 10% normal rabbit or goat serum to block non-specific binding. The sections were then incubated with a mouse monoclonal antibody, 8H3, which is specific to the N-terminus of human WRNp (diluted 1/500),[12] a goat polyclonal antibody against human SP1 (diluted 1/10) (PEP2-G; Santa Cruz Biotechnology, Santa Cruz, California, USA), or a rabbit polyclonal antibody against human AP2 (diluted 1/500) (C-18, Santa Crutz Biotechnology) overnight at 4°C. This was followed by incubation with biotinylated rabbit antimouse IgG/A/M, goat antirabbit IgG, or

Abbreviations: BLM, Bloom syndrome; DHEA-S, dehydroepiandrosterone sulphate; Rb, retinoblastoma protein; RCE, retinoblastoma control element; RTS, Rothmund-Thomson syndrome; WS, Werner's syndrome; WRNp, Werner's syndrome protein

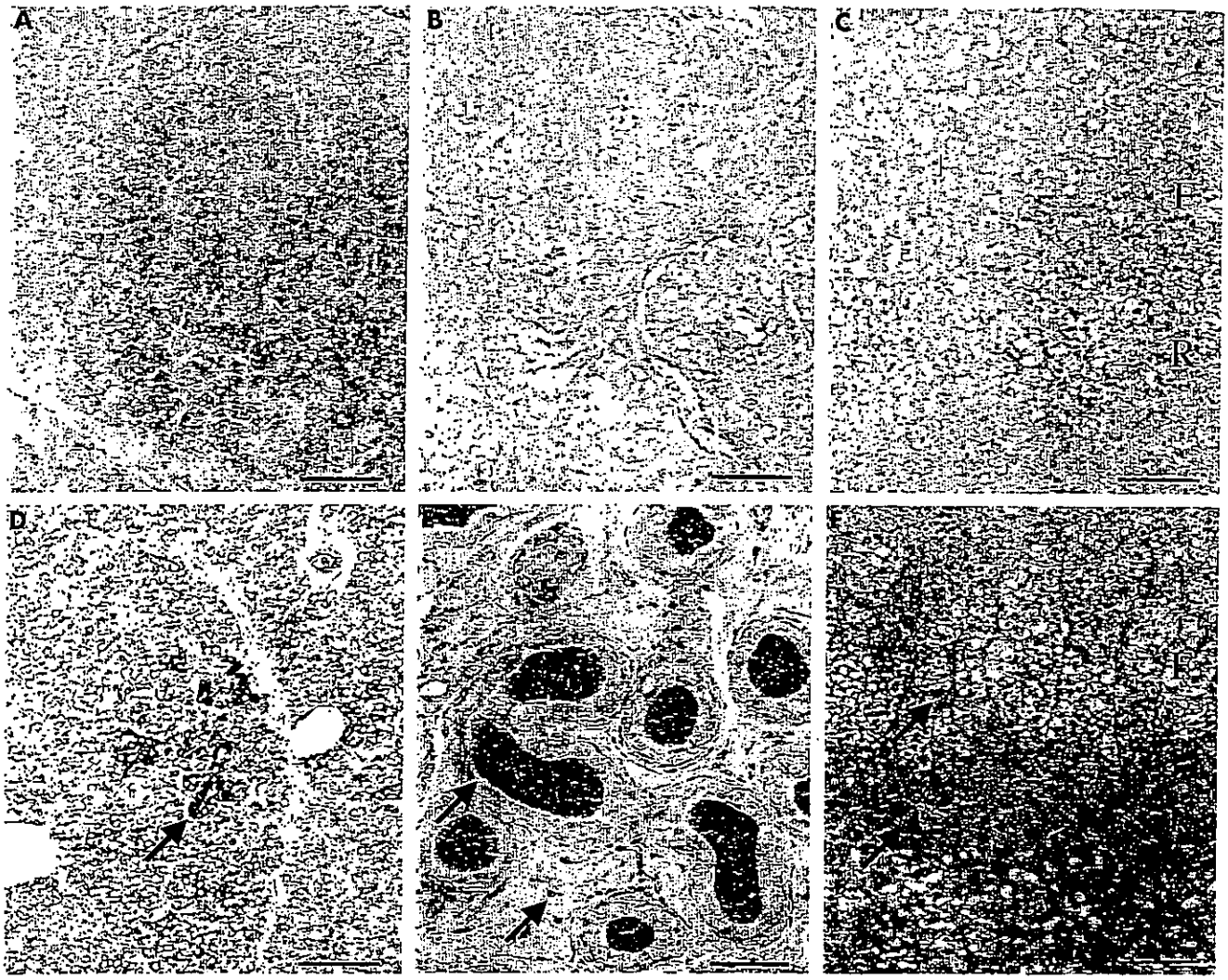

Figure 1 Immunoperoxidase staining of the organs, showing age dependent changes in Werner's protein (WRNp) immunoreactivity. Pancreas at (A) 11 years and (D) 32 years; seminiferous tubules in the testis at (B) 11 years and (E) 32 years; cortex of the adrenal gland at (C) 11 years and (F) 32 years. Arrows indicate the WRNp positive cells. F, zona fasciculata; R, zona reticularis. Scale bar, 1 μm.

rabbit antigoat IgG (Nichirei, Tokyo, Japan), respectively, for one hour at room temperature and then incubation with peroxidase conjugated streptavidin (Nichirei) for 30 minutes at room temperature. After using the TSA Plus kit (NEN Life Products, Boston, Massachusetts, USA), the immunoproducts were visualised by the addition of 0.02M diaminobenzidine tetrahydrochloride in 0.05M Tris buffer, pH 7.6, containing 0.006% hydrogen peroxide. Nuclei were counterstained with haematoxylin. The detailed procedures for preparing the monoclonal antibody 8H3, and its biochemical and immunocytochemical characterisation, have been published elsewhere.[12 13] In addition, all specimens were stained with haematoxylin and eosin as a routine examination.

Immunoblot analysis

Frozen sections of frontal lobes, pancreas, and kidney from the controls aged 27 gestational weeks, 11 years, and 64 years were used for nuclear and cytoplasmic protein extraction. The samples were thawed and homogenised in the buffer from the nuclear and cytoplasmic extraction reagents kit (Pierce, Rockford, Illinois, USA), according to the manufacturer's instructions. After the protein concentration was measured, the samples were incubated for two minutes at 95°C. The protein samples were separated by 7.5% polyacrylamide gel electrophoresis and then transferred electrophoretically to a polyvinylidene difluoride membrane (Immobilon; Millipore, Bedford, Massachusetts, USA). The membrane was incubated overnight at 4°C with primary antibody against human WRNp diluted 1/1000, followed by antimouse IgG and horseradish peroxidase labelled whole antibody diluted 1/5000 (Amersham Pharmacia Biotech, Amersham, Buckinghamshire, UK). The colour was developed with the aid of an ECL kit (Amersham Pharmacia Biotech).

RESULTS

Immunohistochemistry to detect WRNp and relevant transcription factors in human tissues

We examined the expression of WRNp in brain, liver, pancreas, testis, ovary, kidney, adrenal gland, heart, spleen, and intestine obtained from controls of various ages by immunohistochemical analyses. Immunoreactivity for WRNp was not detected in the organs from the controls during the neonatal period and childhood (fig 1A, B, C). Appreciable amounts of WRNp first appeared in selective tissues—such as glandular acini of the pancreas, seminiferous epithelial cells and Leydig cells in the testis, and the zona fasciculata and zona reticularis in the adrenal cortex—from the age of 17 in controls, and was detected persistently after adolescence (fig 1D, E, F). However, immunoreactivity for WRNp was not detected in the other organs at all ages. WRNp was not detected in these organs from the 56 year old female patient with WS (data not shown).

Next, we studied the expression of the transcription factors regulating WRN gene expression in the pancreas, adrenal gland, and testis by means of immunostaining. SP1 and AP2 could not be detected in the tissues from young controls (fig

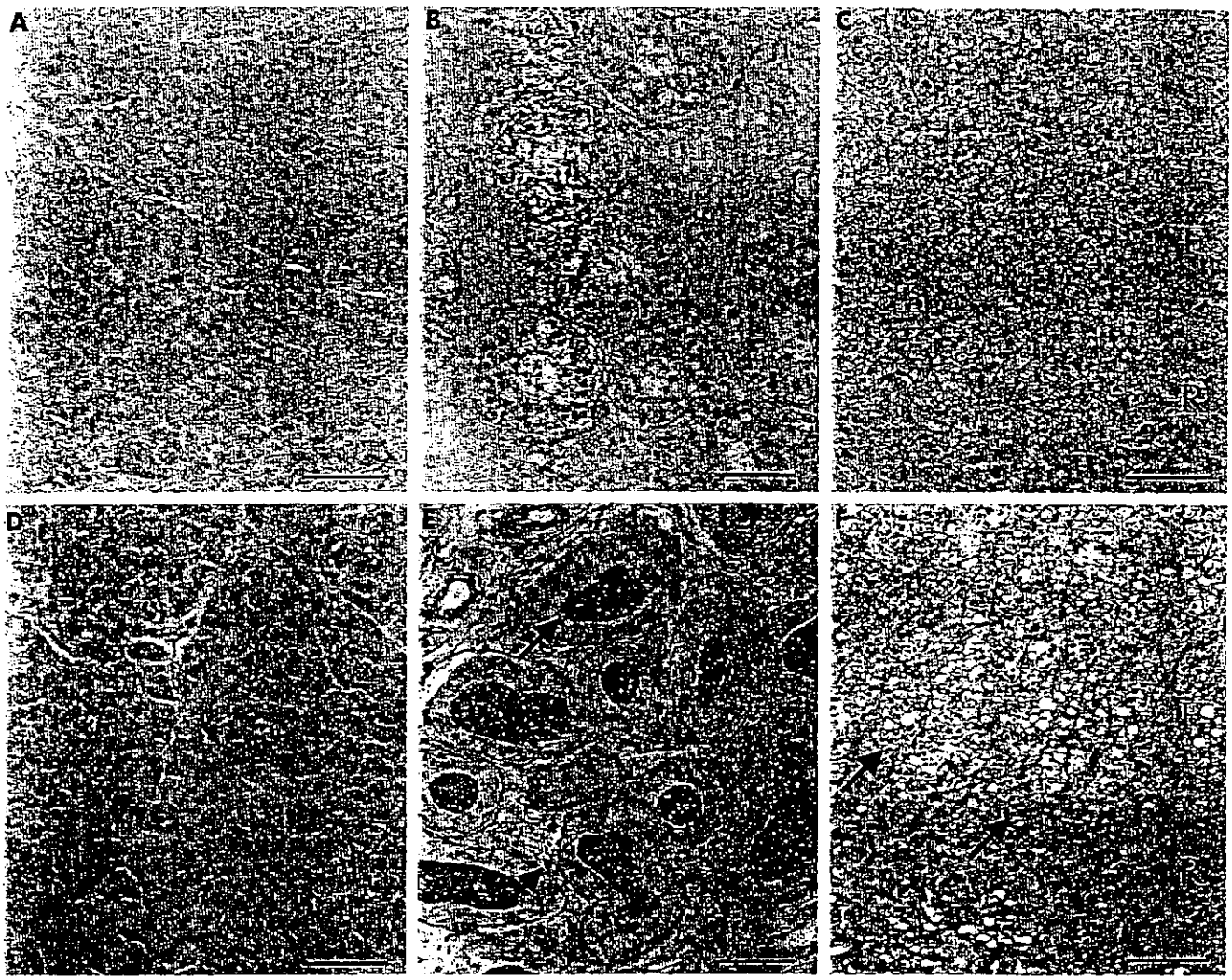

Figure 2 Immunoperoxidase staining of the organs, showing age dependent changes in SP1 immunoreactivity. Pancreas at (A) 11 years and (D) 32 years; seminiferous tubules in the testis at (B) 11 years and (E) 32 years; cortex of the adrenal gland at (C) 11 years and (F) 32 years. Arrows indicate SP1 positive cells. F, zona fasciculata; R, zona reticularis. Scale bar, 1 μm.

2A, B, C and 3A, B, C), whereas SP1 was seen in seminiferous epithelial cells and Leydig cells in the testis (fig 2E) and the zona fasciculata and zona reticularis in the adrenal cortex (fig 2F), but not in the glandular acini of the pancreas (fig 2D) from controls after puberty. Immunoreactivity for AP2 was detected in the glandular acini of the pancreas (fig 3D), but not in the seminiferous epithelial cells and Leydig cells in the testis (fig 3E), or in the zona fasciculata and zona reticularis in the adrenal gland (fig 3F) from controls after puberty. In contrast, immunoreactivity for the Rb protein was not seen in the pancreas, adrenal cortex, or testis at all stages (data not shown).

Immunoblot analysis for WRNp in tissues from young and old healthy subjects

Immunoblot analysis showed that WRNp was abundant in the cytosol fraction of the pancreas from a 64 year old control (fig 4; lane 8). In contrast, bands were not detected in the cytosol fraction from young controls (fig 4; lanes 1–6) and in the frontal lobe and the kidney from the 64 year old control (fig 4; lanes 7, 9). Surprisingly, the WRNp specific bands were not detected in the nuclear fraction from all controls when 50 μg of nuclear protein was used (data not shown).

DISCUSSION

In our study, we first demonstrated the age related expression of WRN in selected tissues and the simultaneous expression of some transcription factors involved in the upregulation of the WRN gene. High expression of WRNp after puberty was seen in the seminiferous epithelial cells and Leydig cells in the testis, glandular acini in the pancreas, and the zona fasciculata and zona reticularis of the adrenal cortex in controls. Immunoblot analyses also showed that WRNp appeared in these tissues after puberty. We found that WRNp was abundant in the cytosol obtained from the frozen specimens; this expression pattern implies that WRNp has a dual nuclear and cytoplasmic localisation in the cell. It has been reported that p53 is stored in the cytoplasm, closely associated with the cytoskeletal actin filaments, and that some of this p53 moves into the nucleus to initiate gene activation in resting fibroblasts.[14] On the basis of this evidence, we speculate that WRNp is stored in the cytoplasm and some of the WRNp moves into the nucleus for DNA repair. The expression profiles of the five human RecQ helicases differ. As measured by northern blot analysis, the WRN gene is expressed highly in the pancreas and the testis, whereas the BLM gene is expressed predominantly in the thymus and the testis, like the RTS helicase gene. The RecQl gene appears to be expressed ubiquitously, like RecQ5.[15] The present immunological results with WRNp are in good agreement with those previously published by Kitao *et al* using northern blot analysis.[15]

"We speculate that Werner's syndrome protein is stored in the cytoplasm and some of this protein moves into the nucleus for DNA repair"