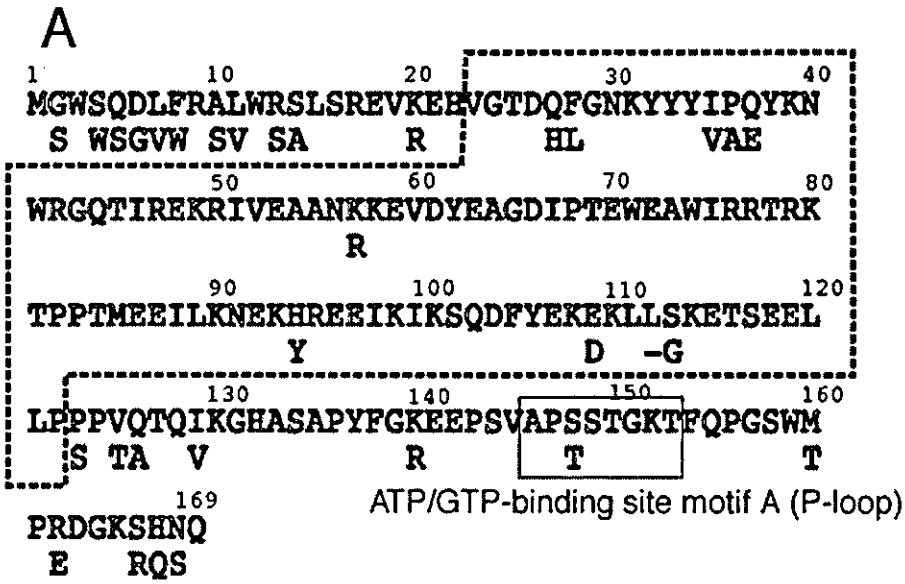
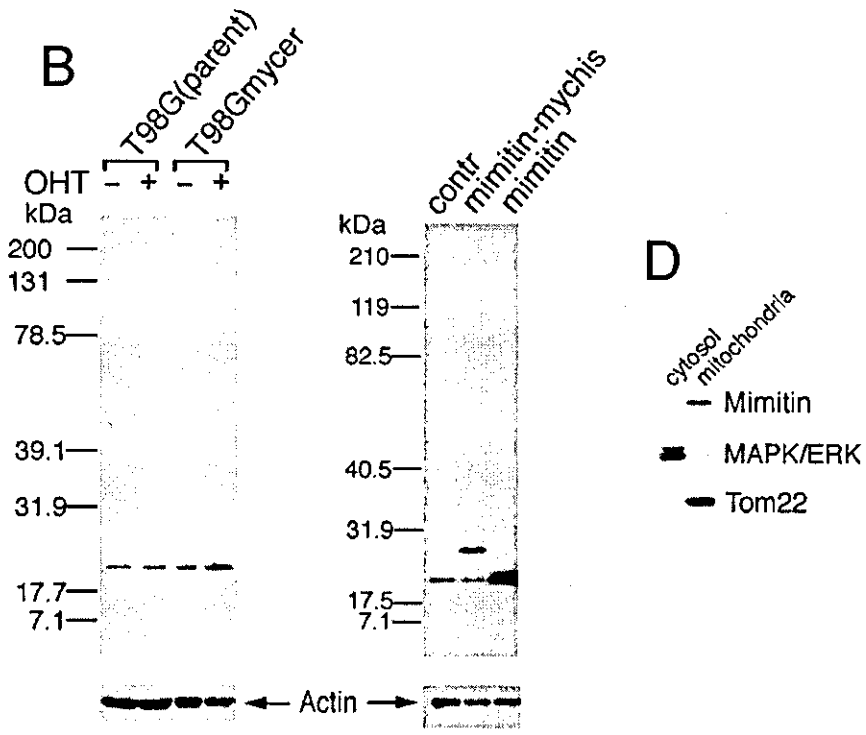
Figure 1


A
1 10 20 30 40
MGWSQDLFRALWRSLSREVKEEVGTDQFGNKYYYIPQYKN
S WSGVW SV SA R HL VAE
50 60 70 80
WRGQTIREKRIVEAANKKEVDYEAGDIPTEWEAWIRRTRK
R
90 100 110 120
TPPTMEEILKNEKHREEIKIKSQDFYEKEKLLSKETSEEL
Y D -G
130 140 150 160
LPPPVQTQIKGHASAPYFGKEEPSVAPSSTGKTFQPGSWM
S TA V R T T
169
PRDGKSHNQ
E RQS
ATP/GTP-binding site motif A (P-loop)


B
T98G(parent)
T98Gmycer
OHT - + - +
kDa
200
131
78.5
39.1
31.9
17.7
7.1
Actin
contr
mimitin-mychis
mimitin
kDa
210
119
82.5
40.5
31.9
17.5
7.1
D
cytosol
mitochondria
Mimitin
MAPK/ERK
Tom22

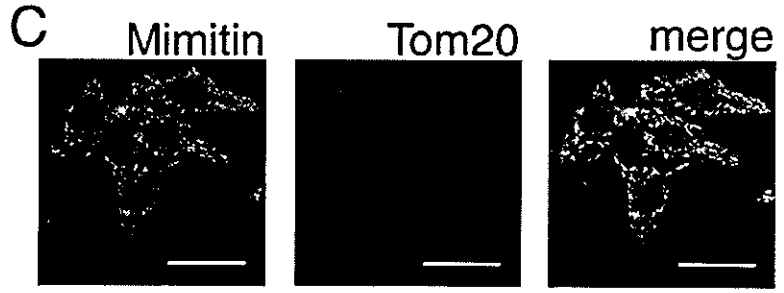

C
Mimitin
Tom20
merge

Figure 2

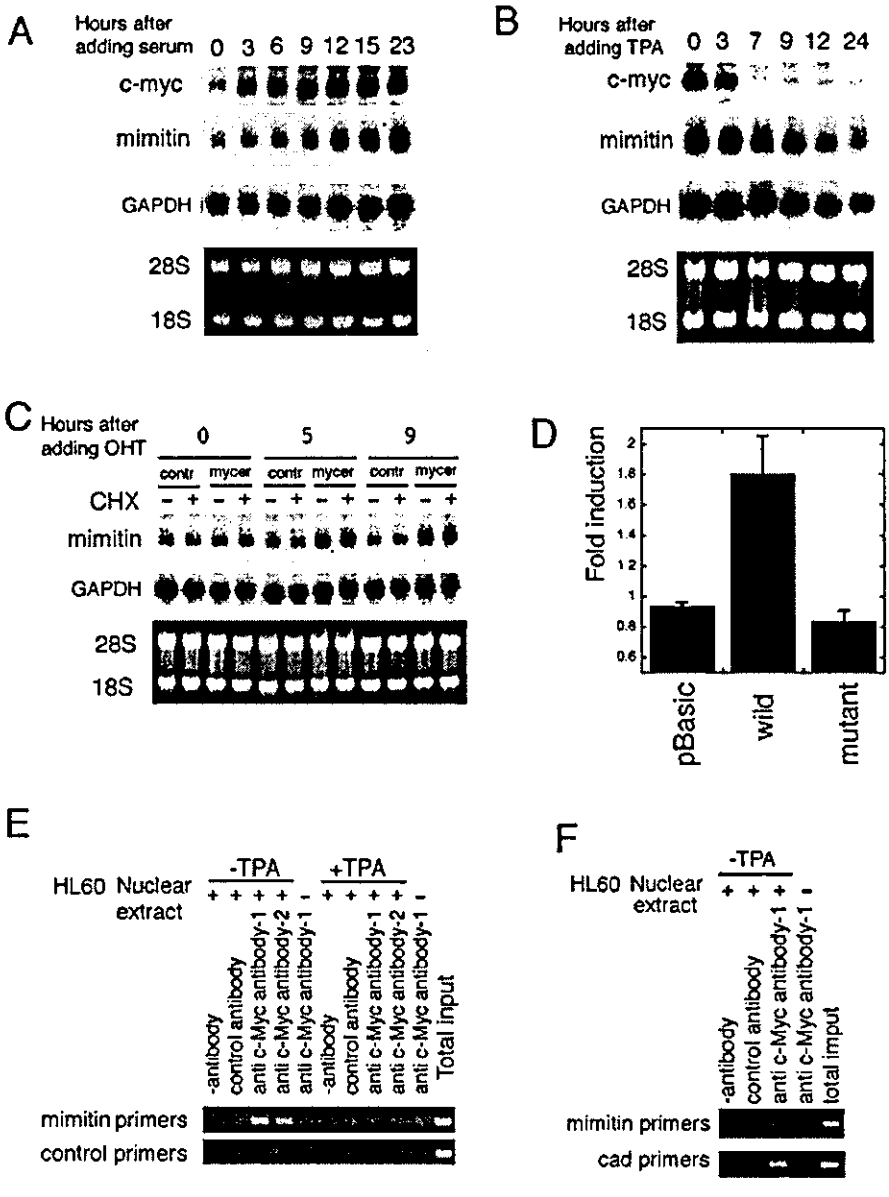

A
Hours after adding serum 0 3 6 9 12 15 23
c-myc
mimitin
GAPDH
28S
18S
B
Hours after adding TPA 0 3 7 9 12 24
c-myc
mimitin
GAPDH
28S
18S
C
Hours after adding OHT 0 5 9
contr mycer contr mycer contr mycer
CHX
mimitin
GAPDH
28S
18S
D
Fold induction
pBasic
wild
mutant
E
HL60 Nuclear extract
-TPA
+TPA
-antibody
control antibody
anti c-Myc antibody-1
anti c-Myc antibody-2
Total input
mimitin primers
control primers
F
HL60 Nuclear extract
-TPA
-antibody
control antibody
anti c-Myc antibody-1
total imput
mimitin primers
cad primers

Figure 3

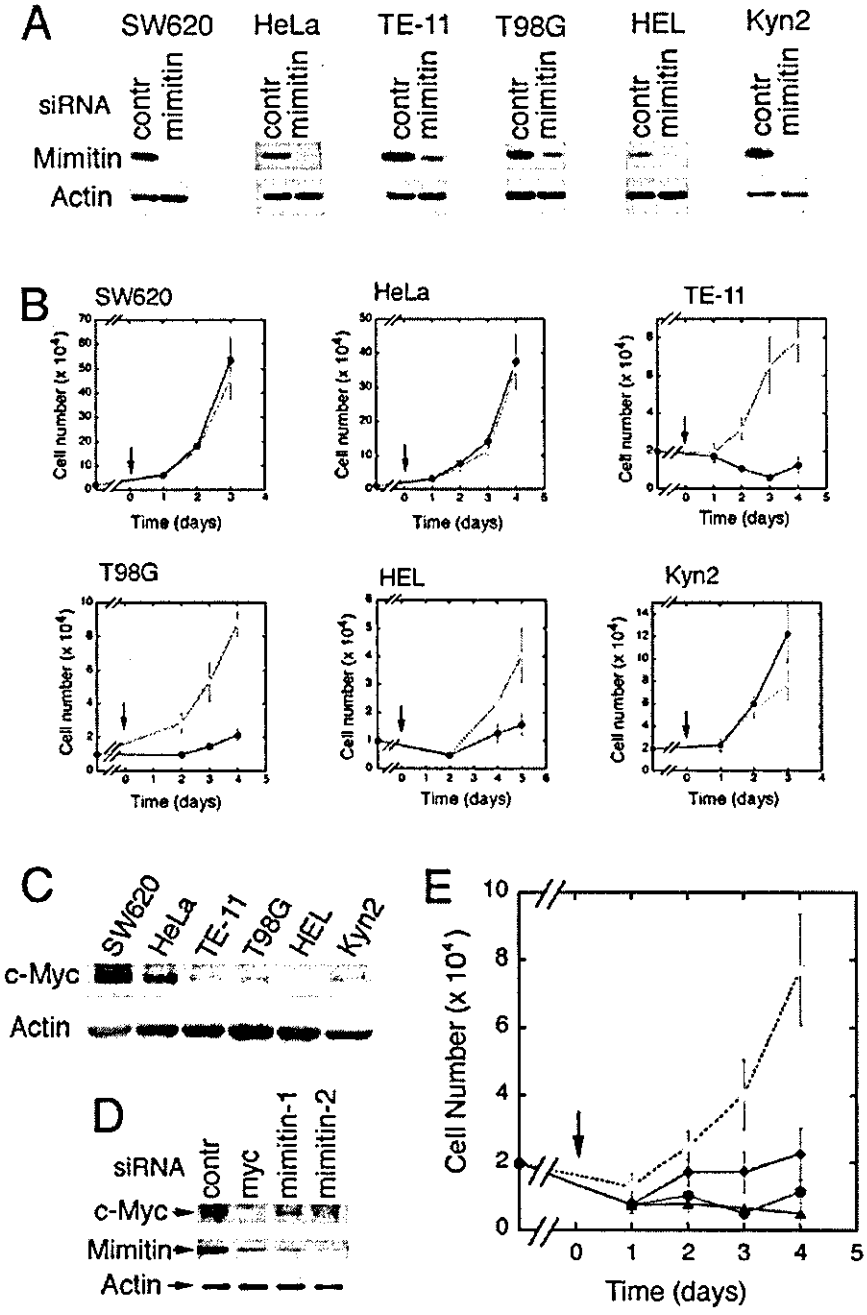
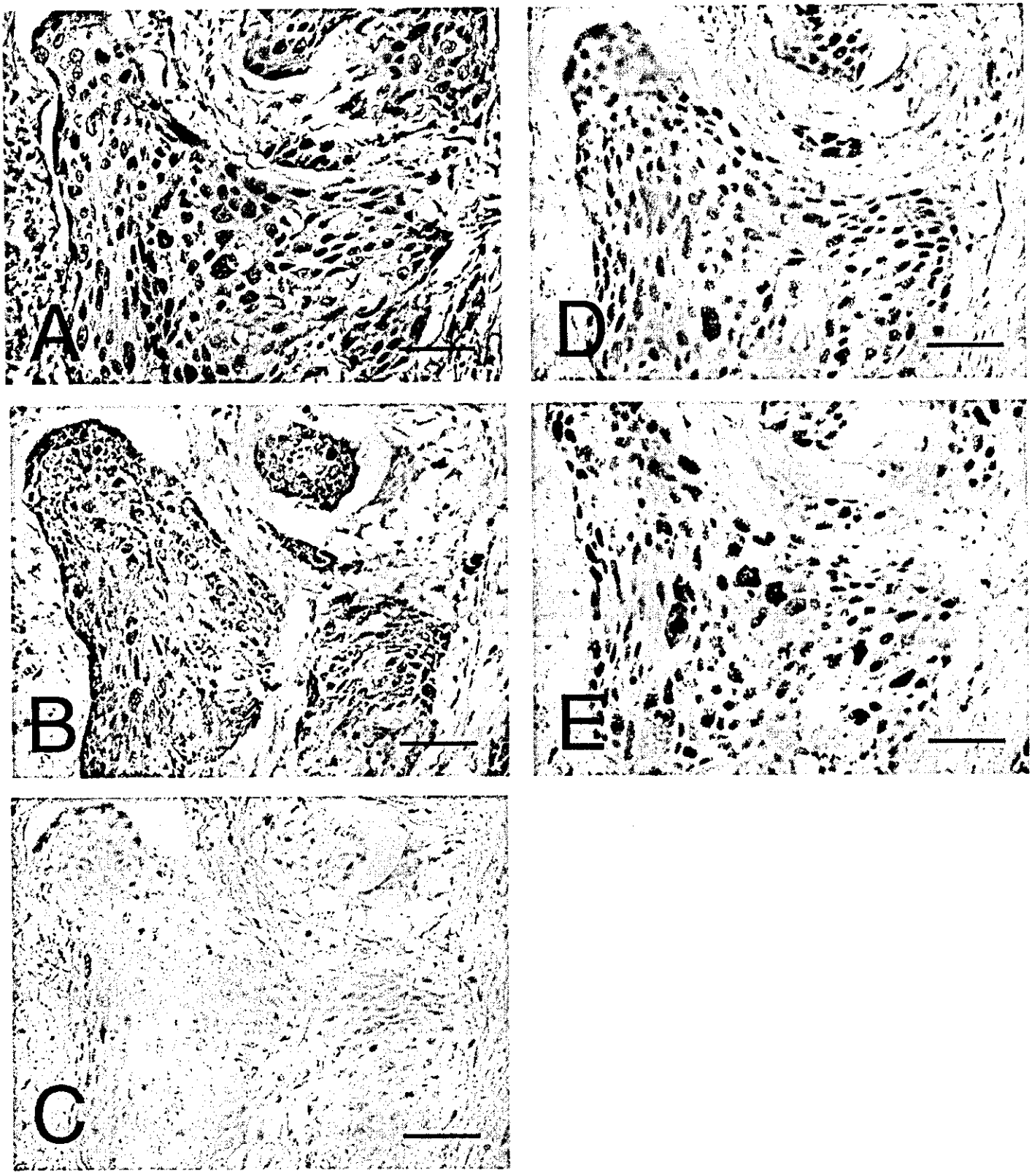

A
SW620
HeLa
TE-11
T98G
HEL
Kyn2
siRNA
contr
mimitin
Mimitin
Actin
B
SW620
HeLa
TE-11
T98G
HEL
Kyn2
Cell number (x 10⁴)
Time (days)
C
SW620
HeLa
TE-11
T98G
HEL
Kyn2
c-Myc
Actin
D
siRNA
contr
myc
mimitin-1
mimitin-2
c-Myc
Mimitin
Actin
E
Cell Number (x 10⁴)
Time (days)

Figure 4



Supplemental information

Table S1. Characterization of ESCC patients and tissues. Tumors from surgical ESCC specimens were immunohistochemically analyzed for Mimitin, Ki-67, and c-Myc. G1, well differentiated; G2, moderately differentiated; G3, poorly differentiated.

Patient No. (Sex, Age)	Stage	Pathological Grade	Mimitin staining			Ki-67 staining			c-Myc staining		
			Intensity	%	Index	Intensity	%	Index	Intensity	%	Index
1 (M, 62)	I	G2	2	86	1.72	3	89	2.67	1	58	0.58
2 (M, 72)	III	G1	1	56	0.56	3	83	2.49	2	82	1.64
3 (M, 77)	I	G3	2	71	1.42	1	12	0.12	2	79	1.58
4 (M, 56)	III	G1	3	93	2.79	3	84	2.52	2	90	1.8
5 (M, 59)	III	G1	2	82	1.64	3	64	1.92	2	85	1.7
6 (M, 60)	IIa	G1	2	91	1.82	3	63	1.89	2	87	1.74
7 (M, 67)	IV	G2	2	91	1.82	3	49	1.47	0.5	19	0.1
8 (M, 67)	III	G2	2	85	1.7	3	39	1.17	0	0	0
9 (M, 69)	III	G2	3	85	2.55	4	71	2.84	3	81	2.43
10 (M, 51)	I	G2	3	34	1.02	0	0	0	0.5	56	0.28
11 (M, 49)	III	G2	3	67	2.01	3	84	2.52	3	94	2.82
12 (M, 47)	I	G3	3	95	2.85	3	65	1.95	3	92	2.76
13 (M, 57)	IV	G1	3	92	2.76	4	60	2.4	0.5	55	0.28
14 (M, 69)	III	G2	3	95	2.85	3	71	2.13	3	90	2.7
15 (F, 71)	IIa	G2	2	95	1.9	1	26	0.26	3	90	2.7
16 (M, 64)	III	G1	2	63	1.26	3	42	1.26	2	97	1.94
17 (M, 59)	III	G2	1	30	0.3	0	0	0	3	98	2.94
18 (M, 46)	III	G2	2	60	1.2	3	51	1.53	3	75	2.25
19 (M, 70)	I	G2	2	82	1.64	4	40	1.6	3	98	2.94
20 (M, 68)	IV	G2	2	44	0.88	0	0	0	2	64	1.28
21 (F, 76)	I	G2	2	95	1.9	4	55	2.2	3	90	2.7
22 (M, 68)	IIa	G1	1	43	0.43	0.5	6	0.03	2	47	0.94
23 (M, 41)	IIa	G3	2	43	0.86	3	30	0.9	1	46	0.46
24 (M, 66)	III	G2	2	32	0.64	2	18	0.36	1	53	0.53
25 (M, 65)	III	G2	3	92	2.76	2	18	0.36	3	57	1.71
26 (M, 66)	IIa	G1	3	88	2.64	4	72	2.88	3	90	2.7
27 (F, 71)	III	G3	3	94	2.82	4	95	3.8	2	79	1.58
28 (M, 50)	IV	G1	2	63	1.26	2	49	0.98	2	91	1.82
29 (M, 61)	IV	G1	4	98	3.92	4	93	3.72	3	93	2.79
30 (M, 67)	IV	G3	2	98	1.96	2	48	0.96	0	0	0
31 (M, 66)	IIb	G3	1	72	0.72	0	0	0	0	0	0
32 (M, 57)	IV	G1	2	78	1.56	4	47	1.88	3	81	2.43
33 (M, 61)	III	G1	0	0	0	0.5	6	0.03	0	0	0
34 (M, 80)	IIa	G1	0	0	0	0	0	0	0	0	0
35 (M, 63)	III	G2	2	95	1.9	3	47	1.41	3	96	2.88

話題

MELASの新しい治療法—L-アルギニン

古賀靖敏[1)]

〔KEYWORDS〕ミトコンドリア病，MELAS，L-アルギニン，脳卒中，電子伝達系酵素欠損，ミトコンドリアDNAの変異，NO，血管内皮機能，ADMA，治療法

1．はじめに

ミトコンドリア病(ミトコンドリア脳筋症)は，細胞のなかのエネルギー産生の中核であるミトコンドリアの異常で脳や筋肉の機能が低下する病気である．この一病型であるMELAS(mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes)は，小児期に脳卒中様の発作を繰り返し，ひいては，精神運動面の退行をきたし，早期に死に至る慢性進行性の難病である．本症における脳卒中発作の成因は，血管説および細胞機能不全説などいまだ不明な点が多い．われわれは，脳卒中発作の成因に血管説が大きく関与しているという仮説のもと，L-アルギニンを投与し，脳卒中に起因する種々の症状が劇的に改善することを発見報告した．MELAS患者では，血管内皮機能が有意に低下しており，本来もっているはずの動脈の拡張機能が傷害されていた．さらに，MELAS患者急性発作時には，血漿中のL-アルギニンや生体内での動脈拡張機能に中心的役割を果たす一酸化窒素(NO)の代謝産物(NOx)が有意に低下しており，かつADMA(asymmetrical dimetyl arginine)が相対的に増加していることがわかった．MELAS患者の脳卒中様発作急性期にL-アルギニンを静注することで，脳虚血からくる神経症状が注射後30分以内に劇的に改善した．また，脳卒中様発作寛解期の患者で，L-アルギニンを内服することで，患者の脳卒中様発作の重症度および頻度を有意に低下することが判明した．MELASに対するL-アルギニン療法は，発作急性期の静注による特効薬的効果のみでなく，発作間歇期の予防的内服薬剤としても期待される．

2．MELASとは？

小児期に発作性の頭痛，嘔吐，半身けいれんで発症し，脳卒中様の発作を特徴とするミトコンドリア病の一病型である．本疾患は，1986年，コロンビア大学の神経内科医Pavlakisにより初めて臨床的に報告された[1)]．2003年の全国調査で，日本においては約233名が罹患し，122名が小児科でフォローアップされている[2)]．80%の患者でミトコンドリアDNAのtRNA$^{Leu(UUR)}$遺伝子のA 3243 G変異が[3)]，また，10%の患者で同じ遺伝子のT 3271 C遺伝子の変異が報告され[4)]，その後多くの点変異が見いだされた．典型的な症状は，20歳前の一過性脳卒中様症状(頭痛，嘔吐，半身けいれん，視野異常，閃輝暗点，視力障害，麻痺など)を特徴とし，同時に筋力低下，感音性難聴，心刺激伝導障害，心筋症を合併するものもある．血液検査では，乳酸ピルビン酸の高値，代謝性アシドーシス，高アラニン血症がみられ，筋生検でミトコンドリアの異常集積像(ragged-red fibers)と中小動脈壁の異常染色性(SSV：strongly SDH hyperreactive blood vessels)が観察される．このSSVは，筋内の中小動脈のみでなく，中枢神経の動脈でも観察され，本症が血管障害を

1) KOGA Yasutoshi　久留米大学医学部医学科 小児科学・助教授

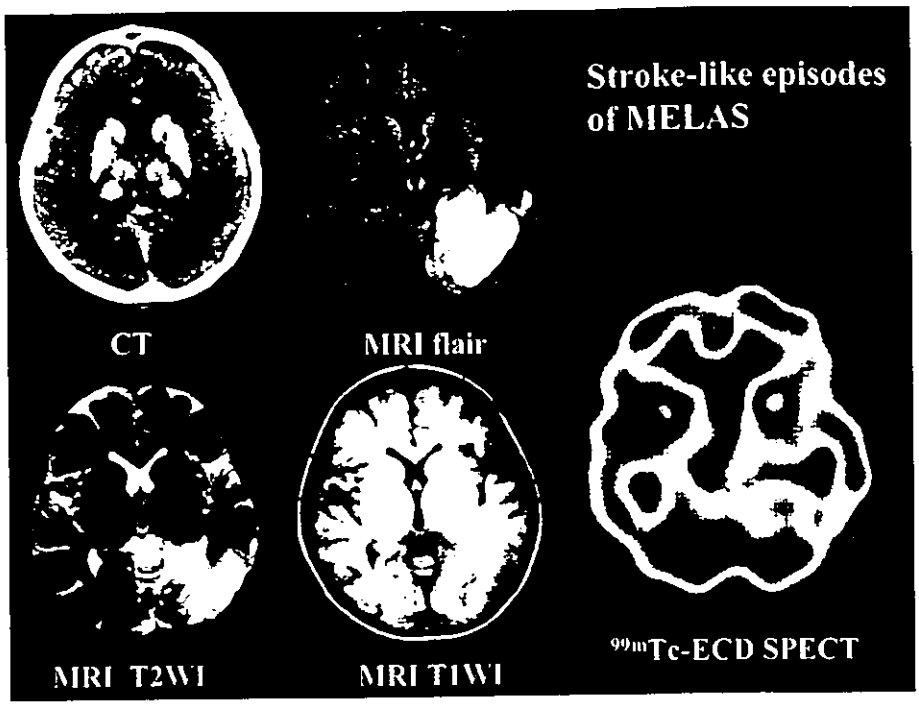


図1　MELAS の頭部画像

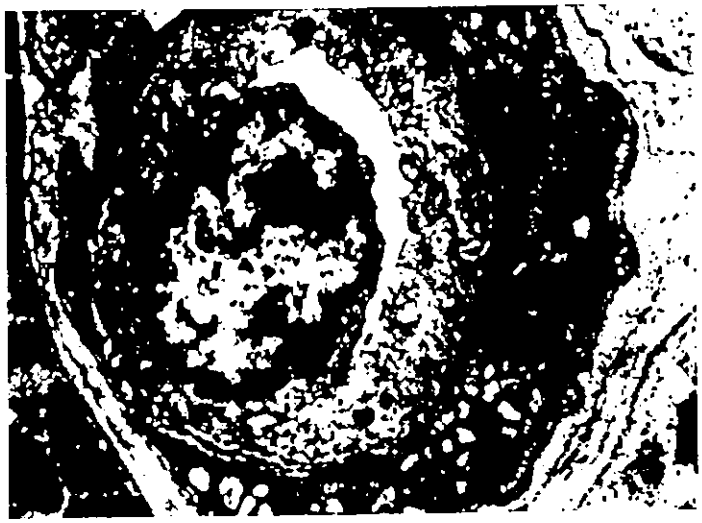


図2　MELAS 患者の筋内動脈の電子顕微鏡所見

有することを示している．

3．頭部画像所見

頭部 CT にて大脳基底核の両側対称性石灰化，脳実質の低吸収域などがみられる．急性期の頭部 MRI では，T 2 強調画像で大脳皮質の高信号域，腫脹などがみられる(図 1)．発作は連続で起こるときもあれば，数か月寛解期をみることもある．発作時みられる症状は一過性の場合もあるが，適切な治療がなされなければ症状は遷延し，血流が障害された末梢脳組織の後遺障害として，半身麻痺，視野障害(同名半盲)，失明をきたす重篤な難治性進行性疾患である．

4．MELAS における脳卒中様発作の成因

MELAS における脳卒中様発作の成因は，血管説および細胞機能不全説などいまだ不明な点が多い．しかし，われわれは，脳卒中様発作の成因に血管説が大きく関与しているという仮説を立てるのに，以下に挙げるようなエビデンスを重要視している．

MELAS 患者では，

（1）ミトコンドリアの機能異常の指標として筋肉での ragged-red fiber (RRF) があり，

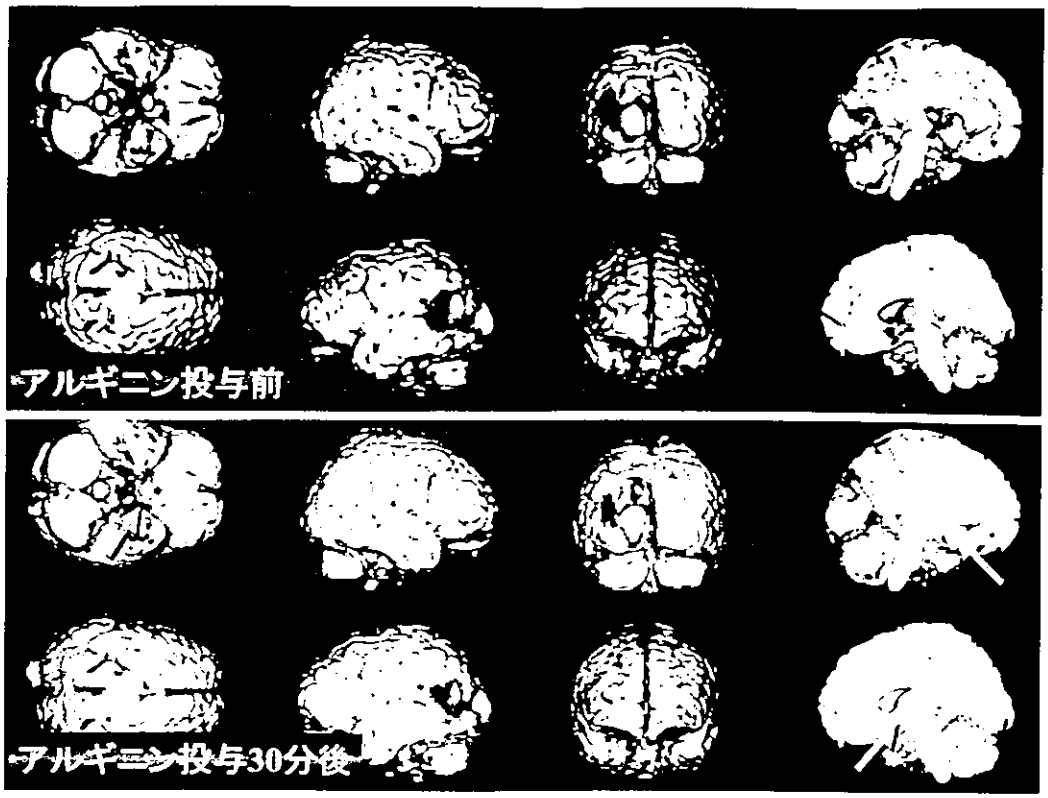


図3　L-アルギニン投与前後のSPM-SPECT画像

その部分の電子顕微鏡的検索ではミトコンドリアの形態異常がみられる．

（2）筋内中小動脈にSSV(strongly SDH positive blood vessels)が観察され，電子顕微鏡的観察で異常ミトコンドリアが中膜平滑筋層および血管内皮細胞に存在し，これがSSVとして観察される．

（3）血管の閉塞性病変が存在する(図2)[5]．

（4）血管内皮の機能異常が存在する．MELAS患者の内皮機能を測定したところ，患者では，同性同年齢のコントロールに比較し，中小動脈の血管内皮依存性血管拡張機能が有意に低下していた．

（5）MELAS患者では，血漿中のL-アルギニンや生体内での動脈拡張機能に中心的役割を果たすNOの代謝産物(NOx)が有意に低下しており，かつADMAが相対的に増加している[6,7]．

（6）L-アルギニンは，動脈の拡張機能に中核的役割を行う．L-アルギニンは，中小動脈における血管の拡張機能において必須となる一酸化窒素産生のもととなるアミノ酸である．このアミノ酸は，MELAS患者の脳卒中様発作急性期では，有意に低下しており，したがって，NO産生の総量を表すNOxは有意に低下していた．ADMAは，虚血性心疾患のリスクファクターとして注目されている物質であり，悪玉L-アルギニンとして，NO合成酵素に抑制的効果を示す．この事実から，MELAS患者では，動脈が拡張しにくい状態にあるといえる[7]．

以上の生化学的基盤をもとに，MELASの脳卒中の成因に，血管障害(特に内皮機能不全)が大きく関与しており，血中のL-アルギニン低値，ADMAの相対的高値がさらなる脳卒中のリスクファクターになっていると考える．

5. MELASの脳卒中様発作急性期におけるL-アルギニン療法の効果

われわれは，脳卒中をきたすミトコンドリア病の急性期にL-アルギニンを投与し，速やかに脳卒中症状が改善したことを報告した[6]．主な内容として，投与量：L-アルギニン10%溶液で5 ml/kg/hr，投与方法：右上腕より点滴静注，有効性：閃輝暗点を除く脳卒中様症状に有効である．安全性：副作用としての頭痛が1例あった

が，肝障害，発疹などの重篤副作用は見られず，頭痛を訴えた症例も投与速度を落としたら頭痛の症状は消失した．血圧に対しては，投与前に比較し最高血圧の10 mmHg以下の血圧低下が投与開始後30分でみられたが，血圧低下による副作用の症状はなかった．

1）L-アルギニン投与による脳卒中様臨床症状の改善

脳卒中様発作症状を発現しているMELAS患者22例にL-アルギニンを静脈内投与し，各急性期症状の改善率を検討した．頭痛は，L-アルギニン投与前に22例全例で高度であったが，投与30分後には14例(64%)が改善し，投与後24時間には全例改善した．臨床的障害(Clinical disability)は，投与前には中等度15例，高度7例であったが，投与終了15分後には3例(14%)が改善し，投与24時間には全例改善した．嘔気は，投与前8例にみられていたが，投与終了30分後には6例(75%)が消失し，24時間後には全例消失した．嘔吐は，投与前10例にみられていたが，投与終了30分後には7例(70%)が消失し，24時間には全例消失した．一過性失明は，投与前7例にみられたが，投与終了15分後にはそのうち3例(43%)が消失し，投与終了30分後に1例再発して改善は2例(29%)となったが，24時間後には全例消失した．半身痙攣は，投与前5例見られたが，投与終了15分後には2例(40%)，投与終了30分後には3例(60%)が消失し，24時間後には全例消失した．意識障害は，投与前1例に見られたが，投与30分後には消失した．閃輝暗点は，投与前9例に見られたが，投与終了30分後には6例(67%)が，24時間後には8例(89%)が消失し，残りの1例は3日目には消失した[7]．

2）L-アルギニン製剤投与後の血中L-アルギニン，L-シトルリン，乳酸，ピルビン酸，NOx，c-GMP，ADMA濃度の推移

MELAS患者におけるL-アルギニン投与前，投与終了後15分，30分，24時間の各血中濃度推移を検討した[7]．

（1）血中L-アルギニンおよびL-シトルリン濃度の推移

投与前の血中L-アルギニン濃度においては，正常値(108.1±27.6 μmol/l)に比べ約1/2(46.99±13.01 μmol/l)に低下していたが，投与15分後には，最大血中濃度(9,542.27±1,169.91 μmol/l)を示し，その後徐々に低下し24時間後には，ほぼ正常値(92.41±15.85 μmol/l)となった．血中L-シトルリンにおいては，正常値(34.6±8.8 μmol/l)とほぼ同じであった投与前値(21.27±9.27 μmol/l)が，L-アルギニンの最大血中濃度より少し遅れて投与30分後に最大血中濃度(41.54±13.04 μmol/l)を示した．

（2）乳酸，ピルビン酸，L/P比の推移

ピルビン酸は，L-アルギニン投与後，徐々に低下し投与24時間後には有意に低下した(0.17±0.03 μmol/l)．乳酸は，L-アルギニン投与後に上昇し投与30分後には最大血中濃度(7.28±0.55 μmol/l)を示したが，投与24時間後には，有意に低下した(3.35±0.49 μmol/l)．L/P値は，乳酸およびピルビン酸の変動に伴い，投与30分後に最大血中濃度(28.02±8.15)を示し，投与24時間後には，ほぼ投与前の値(19.16±4.41)となった．

（3）血中NOx，c-GMP，ADMA濃度の推移

NOxは，投与後30分に有意に上昇し(55.88±20.40 μmol/l)，24時間後も高い血中濃度(29.06±9.68 μmol/l)を示した．c-GMPは，投与後30分に有意に上昇し(0.86±0.16 μmol/l)した．ADMAは，L-アルギニン投与後15分，30分と有意に上昇し，投与30分後に最大血中濃度(0.87±0.11 μmol/l)を示したが，投与24時間後には投与前の値以下まで低下した(図3)．

（4）L-アルギニン投与の頭蓋内血流動態に及ぼす影響

3名のMELAS患者に対して，脳卒中発作時L-アルギニン投与を行い，その前後で頭部^{99m}Tc-ECD-SPECTを実施した．L-アルギニン投与1時間後には，臨床症状の改善とともに，虚血部位の局所的脳血流(rCBF)の改善が認められた．ROIによる虚血部位の血流増加率は，健常側同部位の血流比で算出し，11～13%であった．

（5）副作用

L-アルギニン投与後にL-アルギニン投与によるものと思われる軽度の嘔気が2例に認められたが，経過観察により2～3時間で消失し，特に問

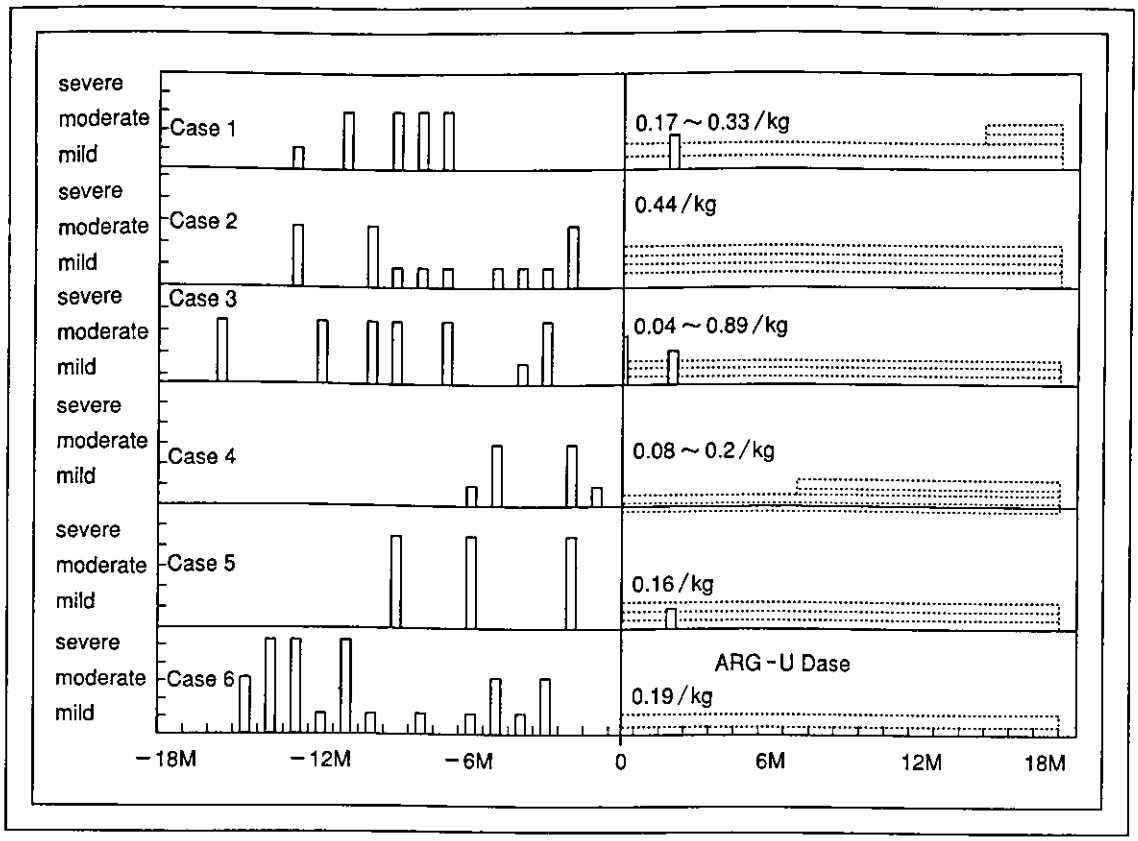


図4　L-アルギニンの脳卒中予防効果

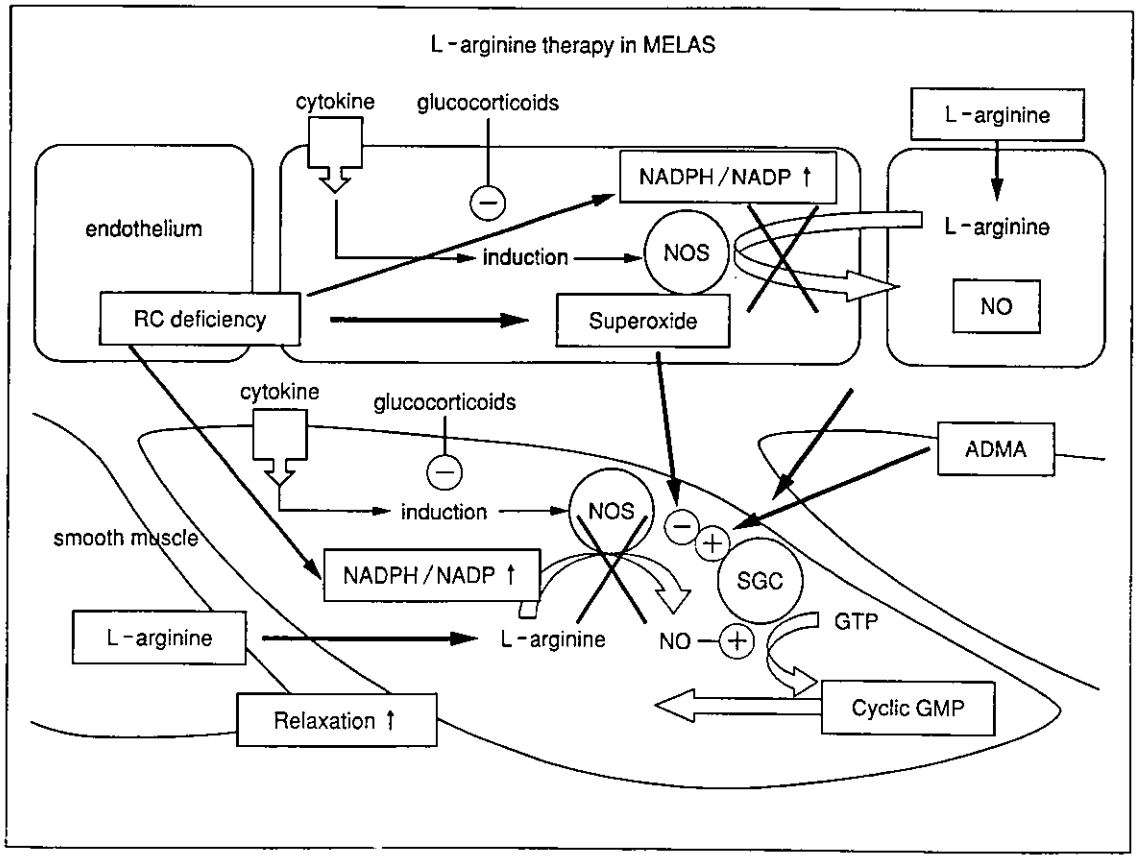


図5　MELAS 患者における血管拡張機能障害
RC deficiency：電子伝達系酵素欠損
ADMA：asymmetrical dimethyl arginine

題となるものではなかった．

6. MELASの発作寛解期における予防効果

過去18か月間に頻回に脳卒中様発作を起こしているMELAS患者6名に対して，L-アルギニンを内服させ，発作予防効果について検討した．その結果，患者では，発作の重症度および頻度共に有意に低下したことが判明した(図4)[7]．

7. おわりに

MELAS患者の脳卒中様発作発現時におけるNOの供与体であるL-アルギニンの投与は，低下している血中L-アルギニン濃度を上昇させ，脳の小中動脈の急性虚血性障害を著明に改善させることが多くの患者で確認され，その効果がより明確となった(図5)．MELAS患者に対するL-アルギニン投与は，MELAS患者の脳卒中様発作急性期のみでなく，発作寛解期の予防にも極めて有効な治療法であると考えられる(図5)．

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AUDIOLOGICAL FEATURES AND MITOCHONDRIAL DNA SEQUENCE IN A LARGE FAMILY CARRYING MITOCHONDRIAL A1555G MUTATION WITHOUT USE OF AMINOGLYCOSIDE

TATSUO MATSUNAGA, MD, PHD
TOKYO, JAPAN

HIROSHI KUMANOMIDO, MD
TOCHIGI, JAPAN

MASAE SHIROMA, PHD
TOCHIGI, JAPAN

YU-ICHI GOTO, MD, PHD
TOKYO, JAPAN

SHIN-ICHI USAMI, MD, PHD
MATSUMOTO, JAPAN

To elucidate the pathophysiological and genetic mechanisms of hearing loss associated with the homoplasmic mitochondrial A1555G mutation in the absence of aminoglycoside exposure, we conducted audiological and genetic analyses on 67 maternally related members of a large Japanese family carrying this mutation. A consistent pattern was evident in the audiograms, with features of sensory presbycusis, cochlear origin at all levels of hearing loss, and a high degree of vulnerability of outer hair cells. That the degree of hearing loss was similar in affected subjects within the same sibling group but differed between sibling groups suggests the involvement of nuclear modifier genes. Total mitochondrial DNA sequences were completely identical among subjects with various levels of hearing loss, and lacked additional pathogenic mutations. For the diagnosis of sensorineural hearing loss, the mitochondrial A1555G mutation should be considered when these features are present even in the absence of aminoglycoside exposure.

KEY WORDS — cochlea, hereditary hearing loss, mitochondria, nonsyndromic hearing loss.

INTRODUCTION

Various mitochondrial DNA mutations have been reported to cause hearing loss, either on their own or in association with other clinical symptoms such as neuromuscular disorders and diabetes.[1] The homoplasmic A1555G mutation in the mitochondrial 12S ribosomal RNA gene has been the first mitochondrial DNA mutation to be associated with nonsyndromic sensorineural hearing loss.[2] The A1555G mutation was initially identified primarily in subjects with hearing loss following aminoglycoside exposure. Indeed, it has been reported that the increased binding affinity of ribosomal RNA to aminoglycosides as a result of the mutation constitutes the pathogenetic mechanism underlying ototoxic susceptibility.[3] Subsequently, this mutation was also found in subjects who developed hearing loss in the absence of aminoglycoside exposure.[4-8] In these cases, the clinical phenotype ranged from profound congenital hearing loss to moderate progressive hearing loss of later onset to only slight hearing loss. Although these phenotypic differences may be the result of additional mutations in the mitochondrial or nuclear DNA, or of unknown environmental factors, the exact mechanism has not been determined. Furthermore, the pathophysiological mechanism of hearing loss due to the A1555G mutation in the absence of aminoglycoside exposure has not been defined, because there are no reports on temporal bone histopathology in patients with this mutation, and the audiological evaluation of patients has been limited to pure tone audiometry (PTA) in most previous studies. Only one study carried out detailed audiological evaluations, but most subjects exhibited profound hearing loss.[9] Thus, such detailed audiological evaluations of subjects with various levels of hearing loss, especially those with mild or moderate hearing loss, remain to be performed to uncover the pathophysiological mechanism underlying the development of hearing loss.

We previously identified a large Japanese family in which the A1555G mutation is prevalent. None of the family members were previously exposed to aminoglycosides, and the prevalence of hearing loss in maternally related members was much higher than that in the general population.[10] To further elucidate the pathophysiological and genetic mechanisms of

From the Department of Otolaryngology, National Tokyo Medical Center (Matsunaga), and the Department of Mental Retardation and Birth Defect Research, National Center of Neurology and Psychiatry (Goto), Tokyo, the Department of Speech-Language Pathology and Audiology, School of Health Science, International University of Health and Welfare, Tochigi (Matsunaga, Kumanomido, Shiroma), and the Department of Otorhinolaryngology, Shinshu University School of Medicine, Matsumoto (Usami), Japan. This work was supported by a Health Science Research Grant from the Ministry of Health, Labor and Welfare of Japan.

CORRESPONDENCE — Tatsuo Matsunaga, MD, PhD, Dept of Otolaryngology, National Tokyo Medical Center, Higashigaoka 2-5-1, Meguro-ku, Tokyo 152-8902, Japan.

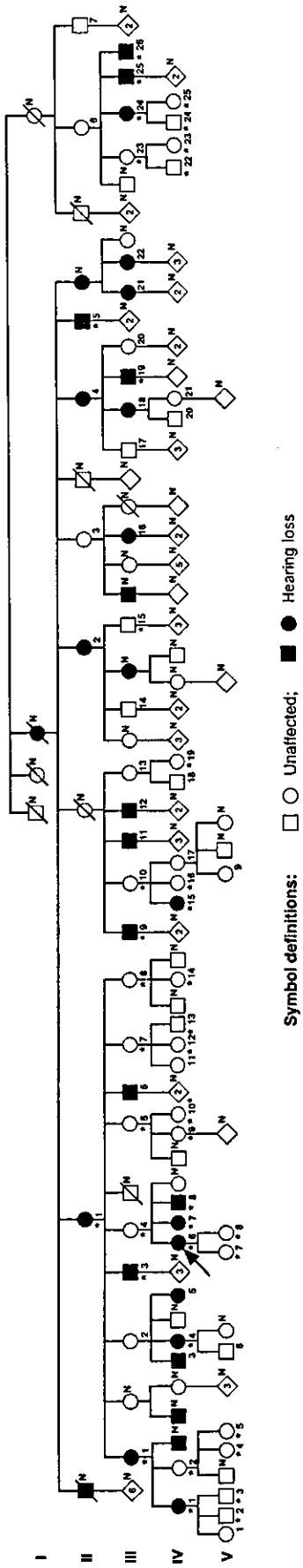


Fig 1. Part of pedigree shows intrafamilial relationship of subjects. Generations are indicated on left in roman numerals, and numbers under symbols represent identification numbers. Family members who were not subjects of this study are indicated by "N" to upper right of symbol. Subjects who reported hearing loss by interview are indicated by solid symbols. Asterisks indicate subjects who were previously tested for A1555G mutation. Arrow indicates proband of family.

the hearing loss due to this mutation, we conducted a battery of audiological tests and sequenced the entire mitochondrial DNA in maternally related members of this family.

MATERIALS AND METHODS

Subjects. The subjects were 67 maternally related members (23 male, 44 female) of a large Japanese family with the homoplasmic mitochondrial A1555G mutation (Fig 1). During interviews prior to PTA testing, 26 of the 67 subjects reported a hearing loss. The original family included 124 maternally related members in 6 generations. The medical histories, clinical phenotypes, and genetic features of these members have been reported previously.[10] In 123 maternally related members whose information about hearing was reliably obtained by interviews, 33 members (penetrance, 26.8%) were considered to have a hearing disability and handicap. The inheritance pattern was maternal and not paternal in this family. Apart from hearing loss, no other significant defects related to mitochondrial mutations were noted in this family. None of the family members had a history of aminoglycoside exposure. All 41 maternally related members who were tested for the A1555G mutation exhibited the mutation in a homoplasmic form. All 41 of these subjects participated in the present study.

Evaluation of Auditory Function. After otoscopic examination, PTA testing was conducted on all subjects. An AA75 audiometer (Rion, Tokyo, Japan) was used in a soundproof room for most subjects. For some subjects, PTA testing was conducted with an AA72B audiometer (Rion) and circumaural earphones in quiet rooms in which background noise was lower than 40 dB sound pressure level (SPL; as measured with an NA29 sound level meter; Rion) with A-weighting. Both air-conducted and bone-conducted thresholds were measured. Subjects who exhibited a pure tone threshold of 30 dB hearing level (HL) or worse at any frequency were given further detailed audiological tests when possible (Table 1). A speech recognition test was conducted with the 67-S monosyllable list (Japan Audiological Society, Tokyo) in 19 subjects. The performance-intensity function was made separately for right and left ears in each subject, and both the maximum speech recognition score and the rollover index were determined.[11] The short increment sensitivity index (SISI) test was performed to examine cochlear dysfunction at 1 or 2 frequencies in 14 subjects. The level of sound stimulation was set at 20 dB above the level of the pure tone threshold at the tested frequencies. Transient evoked otoacoustic emissions (TEOAEs) and distortion product otoacoustic emissions (DPOAEs) were examined to evaluate outer hair cell function with

TABLE 1. SUBJECTS OF DETAILED AUDIOLOGICAL TESTS

Test	*Subjects*
Speech recognition test	II-1, II-5, III-1, III-3, III-9, III-15, III-19, III-21, III-22, III-24, III-25, III-26, IV-1, IV-4, IV-5, IV-6, IV-7, IV-8, IV-15
Short increment sensitivity index test	III-1, III-3, III-9, III-15, III-19, III-22, III-24, III-25, III-26, IV-2, IV-5, IV-7, IV-8, IV-15
Transient evoked otoacoustic emissions and distortion product otoacoustic emissions	II-1, III-3, III-9, III-15, III-19, III-25, III-26, IV-15
Auditory brain stem response	II-1, III-3, III-9, III-26, IV-4, IV-6, IV-8

the ILO292 Otoacoustic Emission Systems (Otodynamics, Hatfield, England) in 8 subjects. For TEOAE analysis, a nonlinear click stimulus train was used at 80 dB SPL, and the number of responses to be averaged was set at 260. The DPOAE measurement was performed at 3 points per octave across the F2 stimulus frequency range of 1,000 Hz to 6,000 Hz with an F2-F1 ratio of 1.221 and at F1 and F2 levels of 70 dB SPL. Each DPOAE result was evaluated with a DP audiogram. The auditory brain stem response (ABR) was evaluated to locate the site of the lesion in the auditory pathway with the Neuropack Σ5504 (Nihon Kohden, Tokyo) in 7 subjects. Alternating click stimulation was presented monaurally at a rate of 10/s through an earphone while the contralateral ear was masked with white noise. The responses were recorded with vertex-earlobe electrodes. A total of 1,000 sweeps were added for each measurement. Thresholds of wave I and wave V were determined, and the latencies of wave I and wave V were measured with the click stimulation presented at 90 dB normal hearing level (nHL).

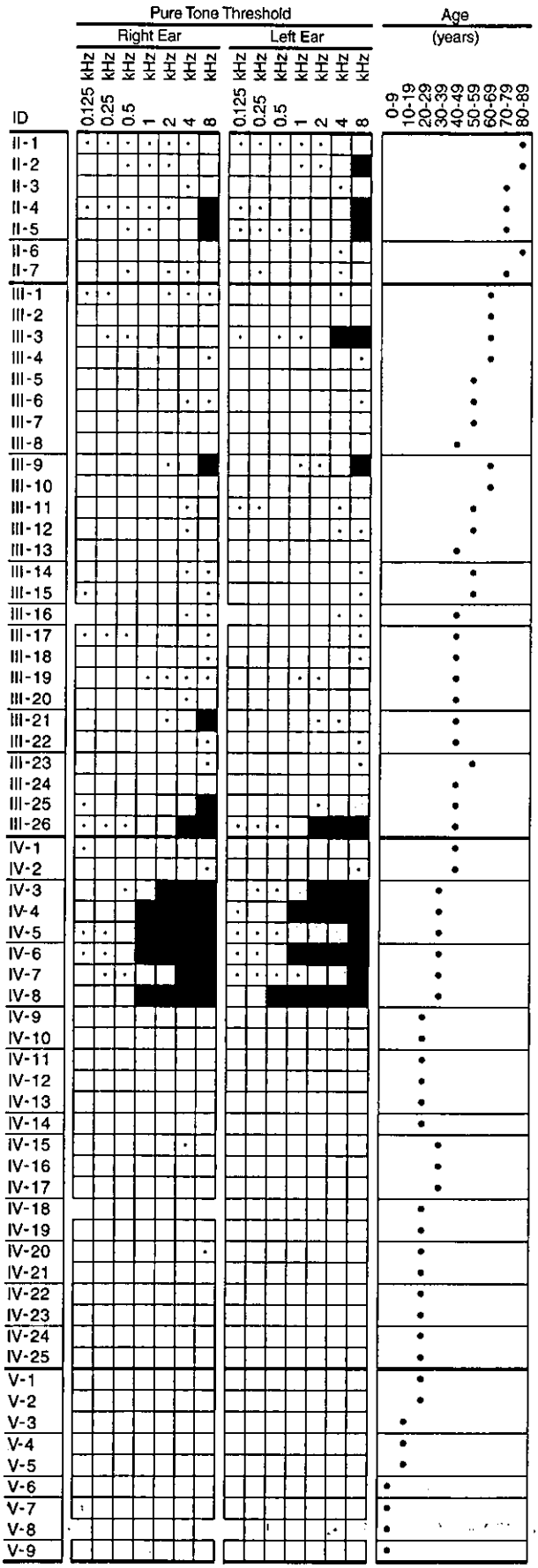
Total Mitochondrial DNA Sequencing. Total mitochondrial DNA was sequenced for 8 subjects with various degrees of hearing loss. The 8 subjects consisted of the proband (IV-6), her daughter (V-7), her mother (III-4), her grandmother (II-1), and 4 siblings (III-23, III-24, III-25, III-26). Genomic DNA was isolated from peripheral leukocytes of the subjects by conventional methods. As in a previous study,[12] to avoid nuclear pseudogene amplification, we applied the long polymerase chain reaction–based sequencing method. With 96 primer sets designed for sequencing, we sequenced the polymerase chain reaction products using the BigDye Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems, Foster City, California). Each reaction product was then analyzed with an ABI 3700 automated sequencer (PE Applied Biosystems) according to the manufacturer's protocol. The sequence data were compared with those in MITOMAP (http://www.mitomap.org),[13] as well as those from 200 unrelated Japanese without hearing loss.

The study protocol was approved by the Ethics Committee of the National Tokyo Medical Center, and the study was conducted according to the principles of the Declaration of Helsinki. Informed consent was obtained from all individuals who participated in the study.

RESULTS

Pure Tone Audiometry. The results of PTA testing in all of the subjects are summarized in Fig 2. Hearing loss was categorized with respect to the mean air-conducted pure tone thresholds at 0.5, 1, and 2 kHz ("PTA 0.5-2 kHz"),[11] by which 59% of the subjects were classified as having normal hearing (≤15 dB HL), 14% had slight hearing loss (16 to 25 dB HL), 9% had mild hearing loss (26 to 40 dB HL), 4% had moderate hearing loss (41 to 55 dB HL), 5% had moderately severe hearing loss (56 to 70 dB HL), 3% had severe hearing loss (71 to 90 dB HL), and 5% had profound hearing loss (>90 dB HL). The PTAs were symmetric in the right and left ears in the majority of the subjects, in that 56 subjects exhibited the same category of hearing loss on both sides. The remaining 11 subjects showed somewhat asymmetric hearing loss, but the categories differed by only 1 level. All subjects with hearing loss exhibited sloping or sharp sloping audiograms except for 1 subject (III-1) who had a history of noise exposure. This subject's audiogram was typical of noise-induced hearing loss (ie, increased bone-conducted thresholds at 4 kHz). The degree of hearing impairment was similar in affected subjects within the same sibling group, but differed between sibling groups.

In 41 subjects who did not report any hearing loss at the time of interview, normal hearing was detected in both ears by PTA over 0.5, 1, and 2 kHz in 32 subjects, slight or mild hearing loss in one or both ears in 8 subjects, and slight hearing loss due to otitis media in 1 subject. The age of the 8 subjects (II-3, II-6, II-7, III-2, III-15, III-17, III-20, III-23) with slight or mild hearing loss ranged from 42 to 80 years. Considering the ages and the degree of hearing loss in these 8 subjects, the lack of reported hearing loss was considered to be reasonable in these subjects. In these 41 subjects, the results of PTA at 8 kHz were analyzed in order to find out whether any subclini-



cal changes existed in their hearing, because the pure tone thresholds at 8 kHz were most prominently affected in the subjects with hearing loss. Thus, subjects whose ages ranged between 18 and 70 years and whose ears were free of otitis media were eligible for this analysis. Sixty-three ears of 32 subjects met this criteria, and 11 of the 63 ears (17.5%) exhibited significantly elevated pure tone thresholds at 8 kHz (>95th percentile) in comparison to the normal range for their respective ages and sexes.[14] A statistical analysis performed with the binomial test on which the threshold probability of the target population is .05 revealed the frequency of the elevated pure tone thresholds at 8 kHz (17.5%) to be significantly higher than the frequency expected in the ears of the otologically normal population ($p < .0001$).

Speech Recognition Test. The relationship between the maximum speech recognition score and PTA 0.5-2 kHz is shown for each ear (Fig 3). The score ranged from 100% in ears with normal hearing to 0% in ears with profound hearing loss. None of the subjects exhibited a disproportionately poor maximum speech recognition score in relation to the magnitude of pure tone thresholds. In 15 of 38 tested ears, the maximum speech recognition score was >50%, and the rollover index of the performance-intensity function could be reliably determined in these 15 ears. Significant amounts of rollover are pathological and are associated with retrocochlear hearing loss. That the rollover index was <40% in all of the 15 ears suggests that retrocochlear dysfunction did not contribute significantly to hearing loss.

SISI Test. The SISI score and the pure tone threshold at the respective frequencies in each ear are shown in Fig 4. We regarded SISI scores of 70% or higher as positive for cochlear dysfunction, while those between 30% and 70% were regarded as semipositive, and those of 30% or lower as negative.[11] The SISI scores were mostly negative at frequencies for which the pure tone threshold was lower than 30 dB HL. In contrast, the SISI scores were predominantly positive at frequencies for which the pure tone threshold was 30 dB HL or higher. A few subjects exhibited

Fig 2. Pattern of pure tone thresholds for all tested frequencies in each subject. Subjects are listed in order of generation and identification number (ID) as designated in Fig 1. Age of each subject is indicated by dot at corresponding division of age scale classified at top. Thin horizontal lines divide different sibling groups, and thick horizontal lines divide generations. Air conduction pure tone thresholds of right and left ears are indicated by following symbols: white square, ≤30 dB HL; dot in white square, 31 to 60 dB HL; gray square, 61 to 90 dB HL; black square, ≥91 dB HL; blank, not tested. Bone conduction pure tone thresholds are shown instead of air conduction thresholds in 2 subjects (IV-18 and V-8) who had otitis media at time of test.

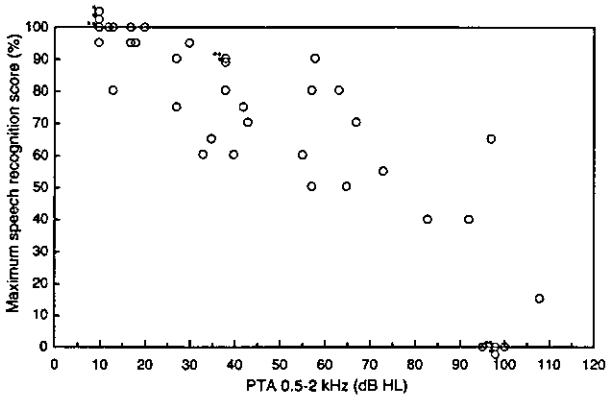


Fig 3. Maximum speech recognition score as function of mean of pure tone thresholds at 0.5, 1, and 2 kHz ("PTA 0.5-2 kHz") for each ear. For ease of visualization, overlapping symbols were moved from original position (indicated by double dots) to neighboring positions (indicated by dot).

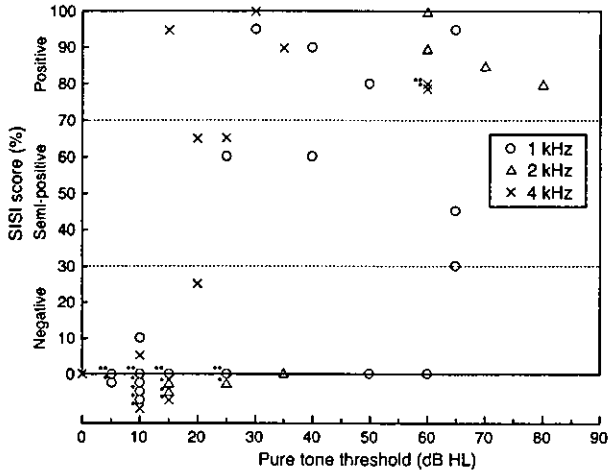


Fig 4. Short increment sensitivity index (SISI) score as function of pure tone threshold at SISI-tested frequency for each ear. Tests were conducted at 1, 2, and 4 kHz. Overlapping symbols were moved as indicated in Fig 3.

semipositive or negative SISI scores despite elevated pure tone thresholds (mostly at 1 kHz). Such occurrences have been noted in previous studies reporting that SISI scores are occasionally semipositive or negative at low frequencies (including 1 kHz) even in ears with cochlear dysfunction.[15]

TEOAE. The TEOAE results were evaluated by the response of the spectral amplitude against noise across a broad frequency range (Fig 5A), as well as by the reproducibility of the time waveform (Fig 5B). The data were plotted against the PTA 0.5-2 kHz in each ear. The response and reproducibility were lower in ears with a PTA 0.5-2 kHz higher than 20 dB HL than in ears with a PTA 0.5-2 kHz of 20 dB HL or lower. No TEOAEs were detected in any of the 6 ears with a PTA 0.5-2 kHz higher than 40 dB HL.

DPOAE. DPOAEs with amplitudes higher than 2 standard deviations above the noise level were considered as positive responses, and DPOAE amplitudes tested at 1, 2, and 4 kHz were compared with the pure tone thresholds measured at the corresponding frequency in each ear (Fig 6). The DPOAE amplitudes were reduced in ears with pure tone thresholds of 20 dB HL or higher at the corresponding DPOAE-tested frequency, and the DPOAE was mostly absent in ears with the pure tone thresholds of 40 dB HL or higher.

ABR. The thresholds of wave I and wave V were determined with the click stimulation, and the latencies of these two waves at 90 dB nHL were measured. The thresholds were then compared with the mean of the air-conducted pure tone thresholds at 2 and 4 kHz ("PTA 2-4 kHz"; Table 2). This frequency range is known to produce the largest ABR components in the cochlea.[16] The relationships of wave I and wave V thresholds and PTA 2-4 kHz were consistent with cochlear dysfunction; ie, the wave V thresholds were almost equal to the PTA 2-4 kHz, and wave I thresholds were higher than wave V thresholds.[17] The wave V latency was within the range predicted by the PTA 2-4 kHz based on the relationship in ears with the corresponding degree of cochlear hearing loss[18] in all but 3 ears (left ear of III-2 and both ears of IV-4) that exhibited relatively long wave V latencies, indicating mild retrocochlear involvement. These 2 subjects were 87 and 62 years old, respectively, and both presented with mild cerebrovascular disease.

Total Mitochondrial DNA Sequence. The mitochondrial DNA sequences were identical in all 8 subjects examined. These subjects exhibited 40 base substitutions relative to the human mitochondrial DNA sequence in MITOMAP, including the A1555G mutation (Table 3). The 39 base substitutions excluding the A1555G mutation were previously reported as polymorphisms in MITOMAP or found in normal Japanese controls — a finding indicating that these substitutions were not related to the observed hearing loss.

DISCUSSION

In our previous study,[10] the proband of the present family exhibited the mitochondrial A1555G mutation in a homoplasmic pattern; ie, all of the mitochondrial genomes in different cells and tissues of the proband harbor the mutation. Because mitochondrial DNA exhibits exclusively maternal inheritance,[19] all of the maternally related members of this family were assumed to carry the A1555G mutation in a homoplasmic form, and this presumption was substantiated by genetic tests that revealed the mutation in a homoplasmic pattern in all 41 maternally related fam-

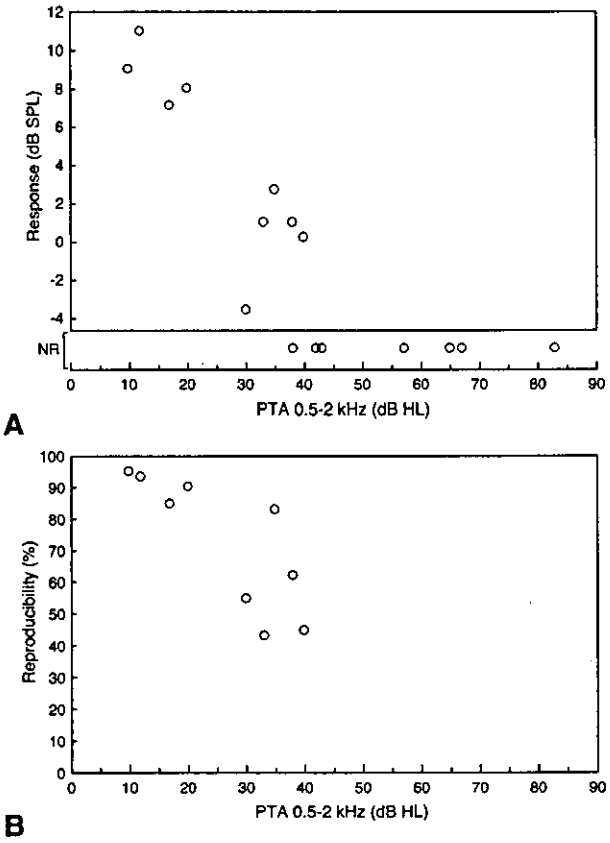


Fig 5. Response (**A**) and reproducibility (**B**) of transient evoked otoacoustic emissions as function of mean of pure tone thresholds at 0.5, 1, and 2 kHz ("PTA 0.5-2 kHz") for each ear.

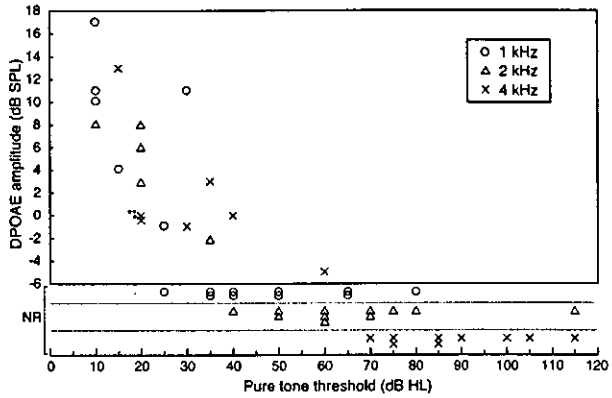


Fig 6. Distortion product otoacoustic emission (DPOAE) amplitude as function of pure tone threshold at DPOAE-tested frequency for each ear. Tests were conducted at 1, 2, and 4 kHz. Symbols between horizontal lines in lower part of Figure (indicated by NR) represent no DPOAE response. Overlapping symbols were moved as indicated in Fig 3.

ily members who were tested.[10] Thus, all of the present subjects who were maternally related members of this family can be considered to carry the A1555G mutation, and all of the present audiological findings can be considered to represent the effects of the A1555G mutation.

A battery of audiological tests conducted in the present study showed a consistent pattern of audiological characteristics, indicating a common pathophysiological mechanism in the development of hearing loss due to the A1555G mutation. Exclusively sloping or sharp sloping audiograms were noted in all subjects with hearing loss except for 1 individual whose hearing loss resulted from long-term noise exposure. In subjects with slight or mild hearing loss according to the PTA 0.5-2 kHz, the pure tone thresholds at 8 kHz were always the most elevated. Even in the subjects who did not report any hearing loss at the time of interview, 11 of the 63 ears (17.5%) exhibited significantly elevated pure tone thresholds at 8 kHz. This frequency was significantly higher than the frequency expected in ears of an otologically normal population. As a result, the relatively frequent occurrence of elevated pure tone thresholds at 8 kHz was considered to be a subclinical audiological feature associated with the mitochondrial A1555G mutation.

These audiogram characteristics have been known in sensory presbycusis, a type of age-related audi-

TABLE 2. CHARACTERISTICS OF AUDITORY BRAIN STEM RESPONSES

	Right Ear					*Left Ear*				
Subject	*PTA 2-4 kHz (dB HL)*	*Threshold* (dB nHL)* *I*	*V*	*Latency† (ms)* *I*	*V*	*PTA 2-4 kHz (dB HL)*	*Threshold* (dB nHL)* *I*	*V*	*Latency† (ms)* *I*	*V*
III-2	55	90	70	1.9	5.9	60		90		7.1
IV-4	80		70		6.4	87.5		70		6.8
IV-11	72.5		80		5.9	67.5	80	70	2.2	6.0
IV-35	92.5	105	100			115		105		
V-7	115					110		105		
V-10	115					115				
V-12	115					115				

PTA 2-4 kHz — average of pure tone thresholds at 2 kHz and 4 kHz.

*Threshold of wave I and wave V.

†Latency of wave I and wave V with click stimulation at 90 dB nHL.

TABLE 3. MITOCHONDRIAL DNA SEQUENCE VARIANTS IN SUBJECTS

Gene Product	*Nucleotide Change*
D-loop	A73G, T152C, A263G, 311insC, T489C
12S rRNA	A750G, A1438G, A1555G
16S rRNA	A2706G, A3145G
NADH dehydrogenase 2	A4715G, A4769G
Cytochrome c oxidase 1	T6632C, A6752G, C7028T, C7196A
Cytochrome c oxidase 2	A8188G
ATP synthase 6	G8584A, A8701G, A8860G, T9090C
Cytochrome c oxidase 3	T9540C
NADH dehydrogenase 3	A10398G, C10400T
NADH dehydrogenase 4	T10873C, G11719A
NADH dehydrogenase 5	C12705T
NADH dehydrogenase 6	C14668T
Cytochrome b	C14766T, T14783C, G15043A, G15301A, A15326G, A15487T, T15784C
D-loop	C16185T, C16186T, C16223T, C16260T, T16298C

tory impairment resulting from the degeneration of sensory hair cells and supporting cells primarily at the basal turn of the cochlea.[20] Several other mitochondrial DNA mutations have been proposed to play roles in age-related dysfunction in organs such as the central nervous system and muscle,[21] and therefore, the A1555G mutation may act analogously to promote auditory dysfunction by a mechanism similar to that of sensory presbycusis.

The speech audiometry results in the present subjects indicated cochlear dysfunction in subjects with slight to severe hearing loss, and these subjects did not exhibit features of retrocochlear dysfunction. The SISI and OAE tests also detected cochlear dysfunction almost simultaneously with or even earlier than the deterioration of pure tone thresholds, indicating that cochlear dysfunction, especially outer hair cell dysfunction, occurred at quite an early stage of hearing loss in the affected subjects. The observed ABR thresholds and latencies also indicated cochlear damage. In agreement with these results, excellent auditory performance with a cochlear implant has been reported in a patient with profound hearing loss due to the A1555G mutation.[22] Given that selective damage to the outer hair cells induces only mild to moderate hearing loss,[23] it would be expected that other cochlear components would thus be damaged in cases of more advanced hearing loss.

The PTA testing confirmed various levels of hearing loss in the present subjects, none of whom had a history of aminoglycoside exposure. To explore possible genetic factors that may have contributed to such phenotypic differences, we sequenced the entire mitochondrial DNA for 8 subjects who presented with various levels of hearing loss. Previously, the coexistence of two mitochondrial mutations, A1555G and G7444A, was identified in Mongolian subjects with hearing loss, and these subjects appeared to present earlier onset and increased severity of hearing loss as compared to patients with the A1555G mutation alone.[24] This finding suggests that an additional new mitochondrial DNA mutation may be responsible for the intrafamilial phenotypic differences in this family. However, our analysis revealed that all 8 subjects had identical mitochondrial DNA sequences, thus indicating that the observed phenotypic differences were not related to any variations in the mitochondrial DNA. In addition, except for the A1555G mutation, no known pathogenic mutations were found in the total mitochondrial DNA sequences; thus, the A1555G mutation is probably the only mitochondrial mutation involved in hearing loss in this family. The degree of hearing loss was similar in the affected subjects within the same sibling group, but varied between the sibling groups. These results suggest that nuclear modifier genes may also be involved in phenotypic differences in the present family, as previously reported in an Arab-Israeli family.[25,26]

In conclusion, our study revealed that various degrees of hearing loss could be caused by an A1555G mutation in the mitochondrial DNA with identical sequences, without any additional pathogenic mutations, even in the absence of aminoglycoside exposure. The affected subjects exhibited audiograms that are characteristic of sensory presbycusis, and also shared common audiological features such as a cochlear origin for all levels of hearing loss and a high degree of vulnerability of outer hair cells. These results further our understanding of the genetic and pathophysiological mechanisms of hearing loss associated with the A1555G mutation, and may aid in the diagnosis and development of new therapies for the treatment of this genetic hearing loss.

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特　　論

ミトコンドリア機能異常と変性性痴呆との関連

Association between mitochondrial dysfunction and degenerative dementia

後藤雄一

Key words：ミトコンドリア，ミトコンドリアDNA，アポトーシス

1. 概念，定義

ミトコンドリア内には，エネルギー代謝に関する多くの酵素が局在している[1]．ミトコンドリア病とは，ミトコンドリア自体およびミトコンドリア内に存在するDNAや蛋白に異常が存在し，ミトコンドリアにおけるエネルギー産生に障害を来した疾患群を総称している．当然のことながらミトコンドリア機能障害が神経細胞に起きると痴呆症状を来す場合があるが，多くは単なる痴呆だけではなく，精神症状・てんかんなどの他の中枢神経症状を同時にもっている．

ミトコンドリア内のエネルギー代謝異常のうち最も頻度の高い電子伝達系酵素の障害は，酵素異常と臨床症状とが必ずしも1対1に対応せず，しかも個々の症例で，極めて多彩な臨床症状が障害度を違えて認められる．また，電子伝達系酵素の一部はミトコンドリアDNA(mtDNA)にコードされており，ミトコンドリア(およびmtDNA)のもつ独自の細胞生物学的特徴を色濃く反映させている[2]．

本稿では，ミトコンドリア機能異常と痴呆との関係を解説する．

2. 痴呆を来すミトコンドリア病の臨床病型

ミトコンドリア病の臨床症状は多彩である．それは，ミトコンドリアが個体の(一部の例外を除き)あらゆる細胞に存在しているために，そのミトコンドリアの障害は種々の異常を引き起こすからである．このような臨床症状の多様性や症例ごとの違いという特徴がある中で，ミトコンドリア病では比較的エネルギー依存度の高い組織や細胞が障害されやすいことは容易に理解できる．実際，エネルギー依存度の高いと考えられる中枢神経，骨格筋，心筋などはミトコンドリア病の主な罹患臓器である．

ミトコンドリア病の代表的な疾患として，慢性進行性外眼筋麻痺症候群(chronic progressive external ophthalmoplegia: CPEO)，赤色ぼろ線維・ミオクローヌスてんかん症候群(myoclonus epilepsy associated with ragged-red fibers: MERRF)，ミトコンドリア脳筋症・乳酸アシドーシス・脳卒中様発作症候群(mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes: MELAS)がある．これら3病型は主症状である中枢神経症状によって分類されているものの，実際には臨床症状を重複してもつ症例や各病型の特徴的症状に乏しい症例などが多数存在している．

3. MELASの病態と痴呆

MELASは，脳卒中様症状を主徴とするミトコンドリア病であり比較的若年で発症する(80%が20歳以前)．臨床症状は，極めて多彩である．卒中様症状を示した急性期や亜急性期に脳

Yu-ichi Goto: National Institute of Neuroscience, NCNP　国立精神・神経センター神経研究所

0047-1852/04/¥50/頁/JCLS