トピックス編

■2■

ユビキチンとアポトーシス

—IAPを中心に—

高橋 良輔

アポトーシスは発生時期から成熟後を通じて不要，有害な細胞を除去する合目的的な細胞死の一形態である．アポトーシスの実行因子はカスパーゼと呼ばれるシステンプロテアーゼで，何百という基質タンパク質を分解することによってアポトーシスを引き起こす．一方アポトーシスを抑制するタンパク質も生体は用意しているが，その中にアポトーシス阻害タンパク質（IAP）という分子ファミリーが知られている．IAPはカスパーゼに直接結合し，その活性を阻害することによってアポトーシスを阻害する内因性のカスパーゼ阻害因子であるが，最近になってIAPの一部にユビキチンリガーゼ（E3）活性をもつものがあることがわかってきた．ショウジョウバエと哺乳類での最近の知見をもとに，IAPの機能におけるE3活性の意義について述べる．

【キーワード&略語】
IAP：XIAP，DIAP1，Drone，Nヘンドラル，RHC
IAP：inhibitor of apoptosis protein
（アポトーシス阻害タンパク質）
XIAP：X-linked Inhibitor of Apoptosis Protein
（X連鎖アポトーシス阻害タンパク質）
DIAP1：Drosophila IAP1
（ショウジョウバエアポトーシス阻害タンパク質1）
IBM：IAP binding motif

1 アポトーシス阻害タンパク質（IAP）とは

アポトーシスは核の断片化やアポトーシス小体の形成のような特異な形態で定義される細胞死であるが，しばしば「細胞の自殺」に例えられるように，個体の恒常性を維持するために細胞の能動的なシグナル伝達で引き起こされる細胞死でもある．アポトーシスの実行因子はカスパーゼ*と呼ばれるタンパク質分解酵素であり，少なくとも200種類以上の基質タンパク質を分解することによって，アポトーシスを引き起こす．一方，アポトーシスを制御するために，アポトーシスを抑制するタンパク質もいくつか知られている．アポトーシス阻害タンパク質（inhibitor of apoptosis protein：XIAP）は最初，バキュロウイルスでみつかったアポトーシス抑制因子であるが，その後，ショウジョウバエ，哺乳類でも存在が明らかになり，ショウジョウバエでは4種類，ヒトでは8種類のIAPファミリー分子が存在する．IAPは，BIR*ドメインと呼ばれるモチーフをもつことが構造上の特徴である[1)2)]．

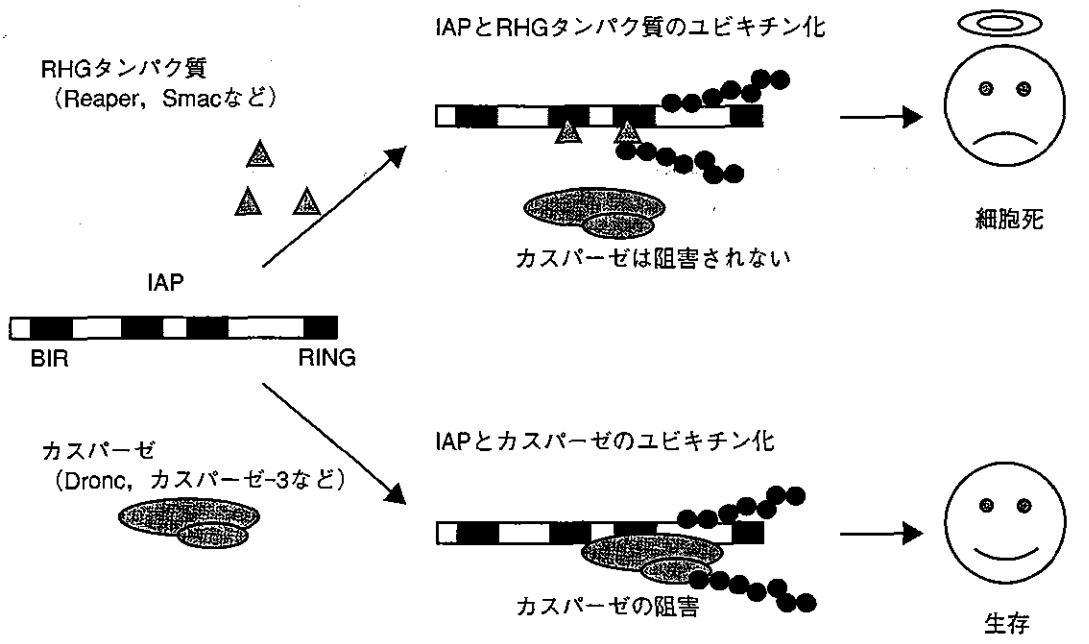
IAPのアポトーシス抑制のメカニズムに関して，ヒトのXIAPがカスパーゼ-3と-7に直接結合して阻害する作用のあることが発見されたことを皮切りに，ほかのほとんどのヒトIAP，さらにはショウジョウバ

＊カスパーゼ

システインプロテアーゼで配列特異的にアスパラギン酸のC末側で限定分解する性質をもつ．ヒトでは14種類が知られ，アポトーシスに関与するものと炎症性サイトカインの産生に関わるものに分類される．

＊BIR

約70アミノ酸から成り，システインとヒスチジンが規則正しく配列され，亜鉛結合能をもつ．BIRを含むタンパク質の中にはアポトーシス抑制以外にサバイビンのように主として細胞質分裂に関与する分子もある．



■概略図■　IAP の E3 活性と細胞死

IAP は内因性のカスパーゼ阻害因子だが，RHG タンパク質が BIR ドメインと結合すると，カスパーゼと結合できなくなり，細胞は死の方向に進む．一方カスパーゼと結合すると，カスパーゼの活性を阻害し，しかも E3 活性でユビキチン化することによって細胞は生存の方向に向かう．両方のケースで IAP の自己ユビキチン化が起こると思われるが，その意義は明らかではない

エの IAP もカスパーゼ阻害作用のあることが示され，IAP が種を超えて内因性のカスパーゼ阻害因子である事実が確立された[1]．興味深いことにヒト，ショウジョウバエでそれぞれ最も強いアポトーシス阻害作用をもち，よく解析されている XIAP と DIAP1 は複数の BIR をアミノ末端側にもつ以外に，カルボキシ末端に多くのユビキチンリガーゼ（E3）を特徴づける RING フィンガーモチーフを有する（図 1）[2]．事実，この 2 種類の IAP は E3 活性を有することが判明し，それがアポトーシス制御のしくみにかなり違いのあるショウジョウバエとヒトの 2 つの種で共通して IAP の生理機能に深く関与していることがわかってきた．

2 ショウジョウバエにおける IAP とその E3 活性

ショウジョウバエでは IAP はアポトーシス制御の中心的役割を担っている[3]．DIAP1 の機能喪失型変異のホモ接合体は原腸形成直後に広汎なアポトーシスを起こして死んでしまう．またショウジョウバエ由来の S2 細胞では DIAP1 の発現を RNAi で抑制するとそれだけでアポトーシスが起こる．これは発生過程の組織でも培養細胞でも常にカスパーゼが活性化しており，それを DIAP1 が抑え込んでいることを反映している．いっぽうショウジョウバエでは細胞死誘導作用をもち，染色体の H99 という領域に局在する Reaper，Hid，Grim という一群の因子が知られていた（Grim Reaper は死神の意，以下これらを RHG タンパク質と省略する）[2][3]．これらによる細胞死を DIAP1 が抑制し，しかもこれらの因子と DIAP1 が直接結合することから，DIAP1 にはカスパーゼ以外に RHG タンパク質による未知の細胞死誘導経路を抑制する作用があるものと最初は考えられていた．ところが，逆に DIAP1 とカスパーゼの結合を RHG タンパク質が阻害するために活性化型カスパーゼの抑制が解かれ，アポトーシスが誘導されることが遺伝学的実験から明白になった．RHG タンパク質は N 末端に IAP 統合モチーフと呼ばれる共通配列をもち（IAP binding motif : IBM*），N 末端で DIAP1 の BIR ドメインに強固に結合するこ

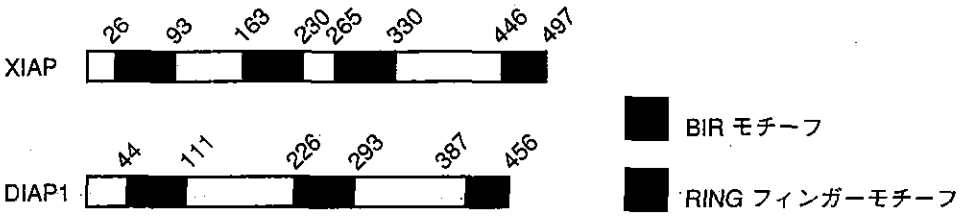


図1 ◆ヒト XIAP とショウジョウバエ DIAP1 の構造比較

とによって，DIAP1 とカスパーゼの結合を妨げる[2]．

アポトーシスに関わるカスパーゼはアポトーシスの引き金を引き下流のカスパーゼを限定分解により活性化する役割をもつ上流のイニシエイターカスパーゼと，さまざまなタンパク質を分解して直接アポトーシス特有の形態変化を引き起こす下流のエフェクターカスパーゼの2種類に分類される．DIAP1 はイニシエイター，エフェクター両方のカスパーゼを阻害する広い特異性をもつが，生理的に特に重要なのはイニシエイターカスパーゼに属する Dronc の阻害作用である．Dronc は生理的条件下で Dapaf-1/DARK というアダプター分子と結合して **apoptosome** *という複合体を形成し，下流のカスパーゼを活性化してアポトーシスを引き起こすが，DIAP1 は Dronc に直接結合して，これを不活性化する．この不活性化が DIAP1 の E3 活性による Dronc のユビキチン化によって行われるのである[4]．DIAP1 は RING 領域を介して UbcD1 というユビキチン結合酵素（E2）および E2 のモチーフはもつが，活性中心が変異している E2 様分子 Morgue と結合する．おそらくこれらの分子，あるいは類似の E2 と協調して Dronc の活性化型およびその前駆体のユビキチン化を促進するものと考えられる[3]．遺伝学的にも DIAP1 の E3 活性による Dronc のユビキチン化が不活性化に必須であることが示された[4]．面白いことにユビキチン化された Dronc はプロテアソームによる分解は受けないらしい．ユビキチン化はプロテアソームによる分解のシグナル以外にも転写制御やエンドサイトーシスなどさまざまな生物学的現象に関与しているが，この場合はカスパーゼの不活性化に働くようである．

また DIAP1 は Dronc 以外に Dcp-1，drICE といったエフェクターカスパーゼに結合しその活性を阻害するが，Dcp-1 または drICE によって N 末端の 20 アミノ酸が切り落とされ，アスパラギン酸が N 末端に露出すると，ユビキチンプロテアソーム系による分解を受けやすくなる[5]．Varshavsky たちは酵母の解析を通じて N 末端のアミノ酸がある種のタンパク質の安定性を決定する要因になることを見出し，N-end rule（N 末端則）と名づけている．カスパーゼによって N 末端を切断された DIAP1 は多細胞生物で初の N-end rule によって分解される生理的基質になることがわかった[5]．意外なことにカスパーゼで切断されなくなった DIAP1 は，RING 変異の DIAP1 同様，アポトーシス抑制効果を失ってしまう．つまり，DIAP1 が分解されることがアポトーシス抑制に必要なのである．おそらく DIAP1 はカスパーゼを道連れにして分解されることによって結果としてアポトーシスを抑制するものと思われる（**図2**）．ショウジョウバエの強力な遺伝学的アプローチから導き出された「IAP の分解が IAP の抗アポトーシス作用に必須」というメッセージは「抗アポトーシス因子である IAP の分解は当然アポトーシス誘導の方向に働くはず」という従来支配的であったドグマを転換することになった．

＊ IAP binding motif（IBM）
アラニンから始まる RHG タンパク質に共通する N 末配列．例えば Reaper は AVAF という配列をもつ．BIR の構造上のポケットの中に入り込み，カスパーゼと IAP の結合を阻害する．

＊ apoptosome
ショウジョウバエでは Dronc と Dapaf-1，哺乳類ではそれぞれに対応するカスパーゼ-9 と Apaf-1，さらにシトクロム-c から成るタンパク質複合体で，哺乳類ではシトクロム-c が形成を促進する．

3 哺乳類における IAP とその E3 活性

哺乳類でもカスパーゼ，IAP，IAP 阻害因子の関係はショウジョウバエと似通っている．DIAP1 に対応する XIAP もイニシエイターカスパーゼ（カスパーゼ -9）とエフェクターカスパーゼ（カスパーゼ -3，-7）をともに阻害するうえ，哺乳類の RHG タンパク質である Smac，Omi/HtrA2 はアポトーシス時にミトコンドリアの膜間スペースから細胞質に放出されて XIAP に結合し，XIAP による阻害からカスパーゼを解き放つ[2]．しかしショウジョウバエとの大きな違いは IAP，IAP 阻害因子のノックアウトマウスが目立った表現型を示さないことである．これは分子のリダンダンシー（redundancy：冗長性）のためと思われるが，IAP の E3 活性の生理的意義の解明を困難にしている．これまでに IAP の E3 活性の意義については，自己ユビキチン化で分解されてアポトーシス進行を促進するという考えと，カスパーゼの分解を早めて，アポトーシスを抑制するという相反する 2 つの考え方が提出されている．前者はアポトーシス誘導時に胸腺細胞で XIAP と，同じく RING を有する c-IAP1 が自己ユビキチン化によって分解されるが，RING を欠いた IAP では自己分解が抑えられ，アポトーシスも抑制されるというデータによって支持されている[6]（ちなみに DIAP1 の自己ユビキチン化は RHG タンパク質である Reaper によって促進されるが[3]，哺乳類 IAP のアポトーシス時の自己ユビキチン化機構は不明である）．しかし自己ユビキチン化しなくなった変異 XIAP のアポトーシス抑制能が野性型と変わらないことがわかり，自己ユビキチン化の意義は再検討を迫られている[7]．一方，後者の考えは XIAP が，活性化型の構造をもつ改変カスパーゼ -3 をポリユビキチン化するうえ，RING に変異のある XIAP は Fas による細胞死抑制効果が野性型より弱くなるというわれわれの結果などに基づいている（図 3）[8]．さらにカスパーゼ -8，-10 の新規 E3，CARPs（caspase-8 and-10 associated RING protein）が報告されており，少なくともカスパーゼのユビキチン化と分解がアポトーシス制御に深く関わっていることは確からしい[9]．最近低分子化合物による IAP の阻害が報告され，癌治療に有効との期待がもたれているが[10]，今後の研究の進展によって E3 機能制御による IAP 阻害が可能になれば，新たな癌治療の手段を提供することになるかもしれない．

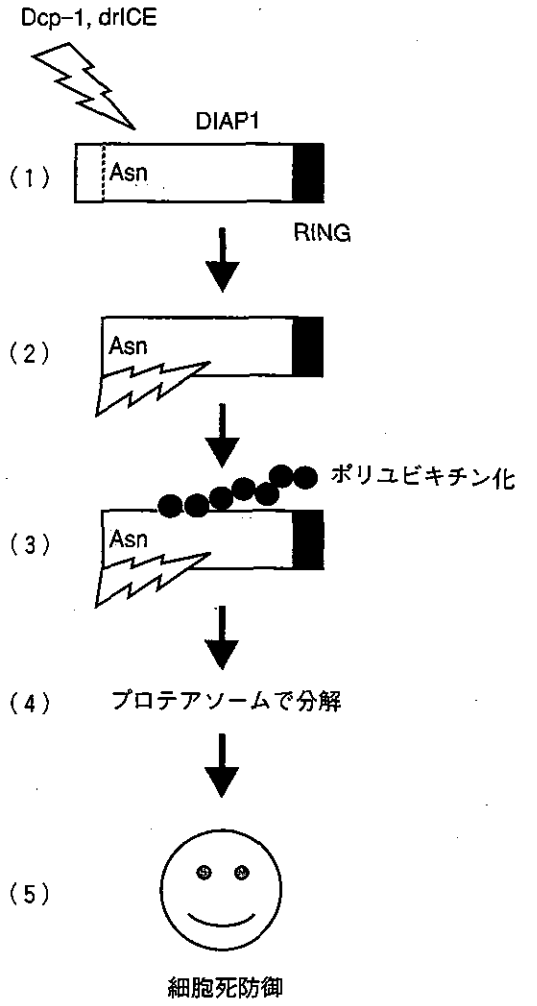


図 2 ◆ N-end rule による DIAP1 の分解
DIAP1 の N 末端の 20 アミノ酸が p35 によって阻害されるカスパーゼ（Dcp-1，drICE など）によって切り落とされると，アスパラギン酸が露出し，N-end rule によってユビキチンプロテアソーム系基質となって分解される．N 末端が切断されなくなる変異型の DIAP1 は細胞死抑制機能を失うことから，N-end rule による DIAP1 の分解はおそらくカスパーゼも同時に分解することによって細胞死防御の方向に働くものと思われる

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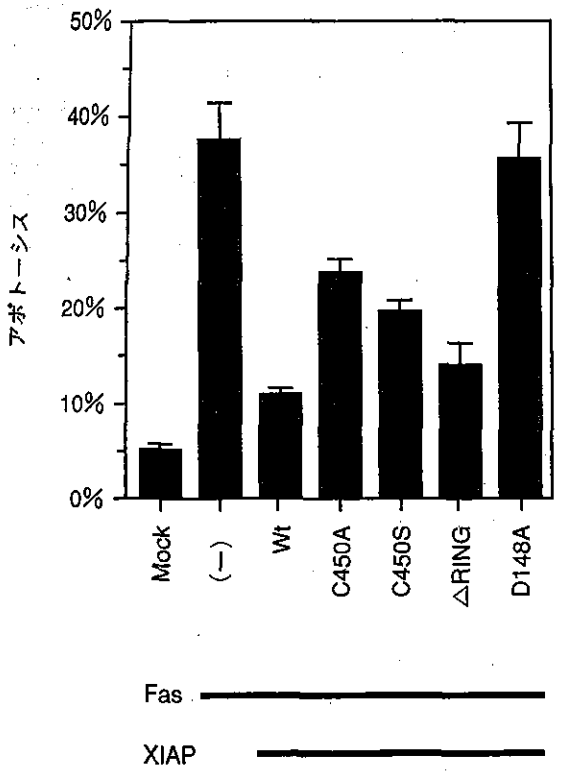

図3◆XIAPのE3活性は細胞死防御の方向に働く
XIAPによるFas誘導性細胞死の抑制効果は野性型（Wt）に比べ，RING点変異（C450A，C450S）またはRING欠損型（Δ RING）で減弱している．D148Aはカスパーゼ-3に結合しないXIAPの点変異（文献8より改変）

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  ≫≫IAPのユビキチンリガーゼ機能についてややくわしく概説．

PARK6-linked autosomal recessive early-onset parkinsonism in Asian populations

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Abstract—The authors performed linkage analysis in 39 families with autosomal recessive early-onset PD (AR-EOPD) *negative for parkin and DJ-1 mutations.* Eight families including three Japanese, two Taiwanese, one Turkish, one Israeli, and one Philippine showed evidence of linkage with PARK6 with multipoint log of the odds (lod) score of 9.88 at D1S2732. The results indicate worldwide distribution of PARK6-linked parkinsonism.

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The *parkin* gene, responsible for autosomal recessive juvenile parkinsonism (ARJP), was identified in 1998.[1] Mutations of this gene have been detected in approximately 50% of cases with autosomal recessive early-onset Parkinson disease (AR-EOPD), indicating that this form is the most frequent among patients with familial PD.[2] Recently, *DJ-1* mutations responsible for PARK7 were reported to cause another type of AR-EOPD.[3] In contrast to PARK2, this gene is unlikely to be of numerical significance in clinical practice.[4] Thus, it is possible that other loci are responsible for the remaining patients with AR-EOPD. Based on this view, it is important to identify the PARK6 gene and to screen AR-EOPD families with no *parkin* or *DJ-1* mutations. The locus for PARK6 was identified on human chromosome 1p35-p36 in several European families.[5,6] The clinical features of PARK6-linked AR-EOPD are similar to those of PARK2- or PARK7-linked AR-EOPD. However, PARK6-linked families frequently lack dystonia at onset.[6,7] Thus, the lack of dystonia at onset might be a distinct sign for differentiating this form from PARK2- or PARK7-linked AR-EOPD. To further narrow the critical region for PARK6-linked AR-EOPD and to define the genotype–phenotype correlations, we performed a linkage study in AR-EOPD families without *parkin* and *DJ-1* mutations.

Patients and methods. *Patients and DNA preparation.* Blood samples and clinical information on patients were obtained from their neurologists in several countries. Diagnosis of AR-EOPD was adopted by the participating neurologists. We investigated 39 AR-EOPD families from seven countries, including 26 Japanese families, 3 Taiwanese, 3 Israeli, 3 Turkish, 2 Moroccan, 1 Philippine, and 1 Brazilian. In the present study, the subjects were from families of consanguineous marriages or at least two affected siblings in the same generation. The study was approved by the ethics review committee of Juntendo University. Blood samples for genetic analysis were collected after obtaining an informed consent from 60 patients and 24 unaffected relatives. DNA was prepared using standard methods. None had mutations in *parkin* or *DJ-1* gene. We analyzed *parkin* mutations by direct sequencing

See also pages 1350 and 1486

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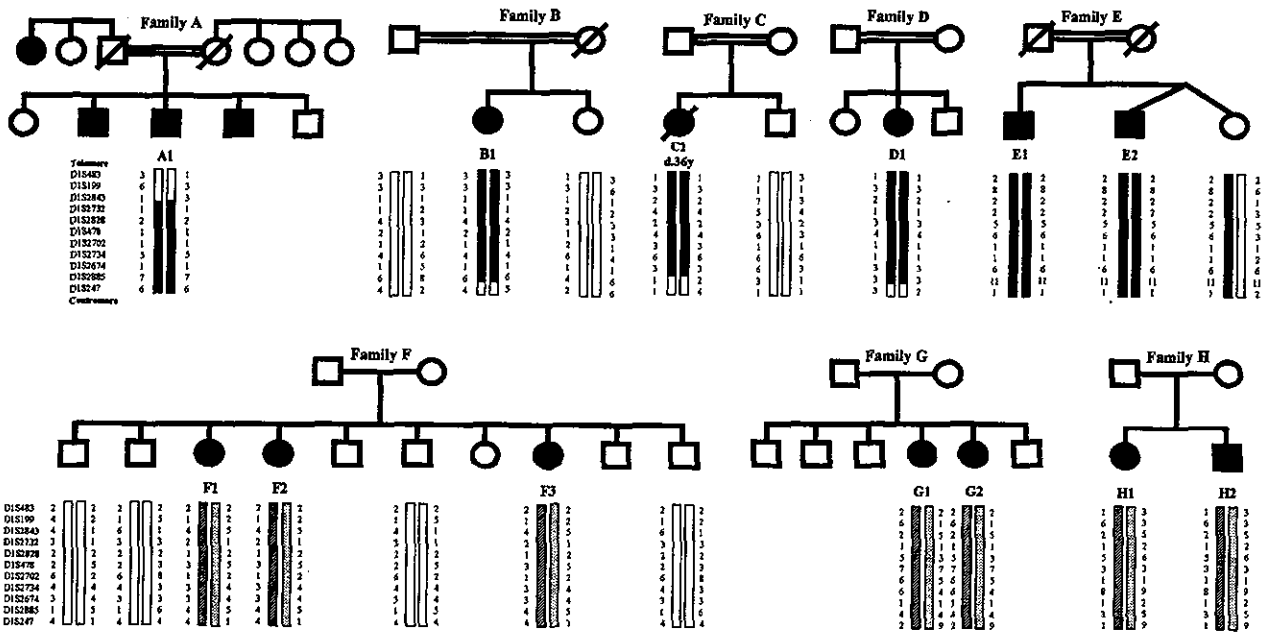

Figure 1. Pedigree structure and haplotype analysis of the families. The affected individuals studied were born to consanguineous parents in five families (Families A, B, C, D, and E) and to non-consanguineous parents in three families (Families F, G, and H). The parents were first cousins in Families A, B, C, D, and E. Haplotypes for 11 polymorphic markers on 1p35–36 are shown. Telomeric to centromeric markers are arranged from top to bottom. Disease-linked homozygous haplotypes are boxed in black. Heterozygous haplotypes shared by affected members of each family are marked in dotted and diagonally striped boxes. The patient, C1, died in a traffic accident.

of the coding regions including exon-intron boundaries using previously described primers.[1] *DJ-1* screening was also performed.[3]

Linkage analysis. We performed linkage analysis in 84 individuals. We used the polymorphic DNA markers D1S483, D1S199, D1S2843, D1S2732, D1S2828, D1S478, D1S2702, D1S2734, D1S2674, D1S2885, and D1S247 selected from the Center for Medical Genetics, Marshfield Medical Research Foundation (http://research.marshfieldclinic.org/genetics/). The primers were labeled by fluorescence and the sequence GTTTCTT was placed on the 5' end of reverse primers for definite genotyping. Pooled PCR products were separated by electrophoresis on 5% polyacrylamide gels on an ABI 377 DNA sequencer (Applied Biosystems). Alleles were sized by GENESCAN and scored with GENOTYPER software. We used the GENEHUNTER program for multipoint parametric log of the odds (lod) scores to find the maximum lod score for each marker. We assumed an autosomal recessive model with complete penetrance in both sexes and a frequency of 0.001 for the disease allele. Since the allele frequencies of the markers were not known, lod scores were calculated by assuming equal allele frequencies.

Results. Haplotypes were constructed using 11 microsatellite markers in the PARK6 region to a 12.5 cM interval. Results are shown in figure 1. Families A, B, C, D, and E showed homozygosity while compound heterozygosity was suggested in Families F, G, and H, who shared the same haplotypes with other affected siblings. Three families were Japanese, two Taiwanese, and one each from Israel, Turkey, and the Philippines. No common haplotype could be detected in the families tested, thus excluding possible single founder effect. Multipoint linkage analysis using the full set of 11 markers, shown in figure 2, indicated a maximum lod score of 9.882 at D1S2732. Multipoint lod scores were >9.7 for five markers spanning ~6.4 cM, with markers D1S2732 and D1S2734 defining telomeric and centromeric boundaries.

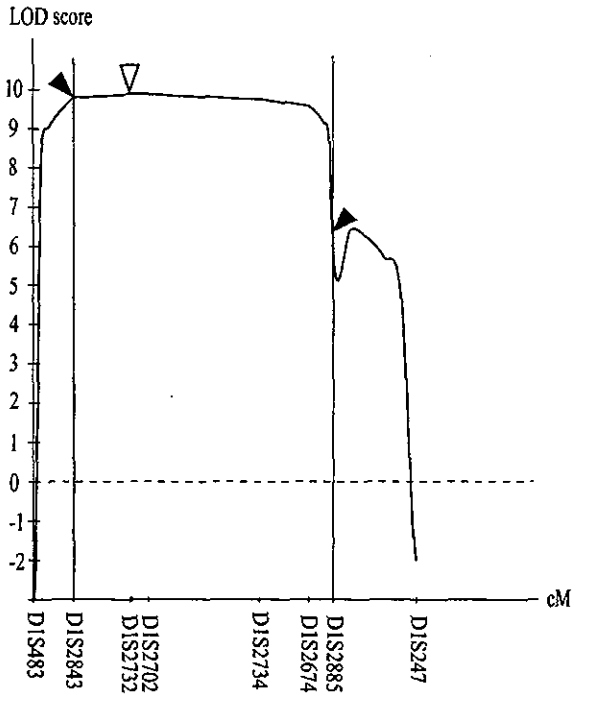

Figure 2. Multipoint linkage analysis of a 12.5 cM region on 1p35–36. The maximum multipoint lod score of 9.882 was obtained at D1S2732 (open arrowhead). We mapped the disease locus to be within an 8.5 cM region extending between markers D1S2843 and D1S2885 (solid arrowheads) by homozygosity mapping.

Table *Clinical features of the patients*

Characteristics	Family A (Japan), A1	Family B (Japan), B1	Family C (Turkey), C1	Family D (Japan), D1	Family E (Israel) E1	Family E (Israel) E2	Family F (the Philippines) F1	Family F (the Philippines) F2	Family F (the Philippines) F3	Family G (Taiwan) G1	Family G (Taiwan) G2	Family H (Taiwan) H1	Family H (Taiwan) H2
Age at onset, y	30	23	28	33	33	25	32	27	27	19	18	56	48
Disease duration, y	17	15	8	8	17	21	19	23	18	24	22	16	19
Sex	M	F	F	F	M	M	F	F	F	F	F	F	M
Clinical presentation													
Resting tremor	−	+	+	PT	−	−	+	+	+	+	+	−	+
Rigidity	+	+	+	+	+	+	+	−	+	+	+	+	+
Bradykinesia	+	+	+	+	+	+	+	+	+	+	+	+	+
Postural instability	+	+	−	+	−	−	+	+	+	−	−	−	+
Frozen gait	−	−	−	−	+	+	−	+	−	−	−	−	−
Wearing off	+	+	−	−	+	+	+	+	+	+	+	+	+
On/off	−	−	−	+	+	+	−	−	−	−	−	−	−
Asymmetry at onset	+	+	+	+	−	−	−	+	+	+	−	−	+
Levodopa-induced dyskinesia	+	−	−	+	+	+	−	+	+	+	+	−	+
Sleep benefit	−	+	+	−	+	+	−	−	−	+	+	−	−
Dystonia at onset	−	−	−	+	−	−	−	−	−	−	+	−	−
Hyperreflexia	+	+	+	+	−	+	−	−	−	−	−	−	−
Psychosis	−	HA	−	−	DE	SC	−	−	−	−	−	−	HA
Dementia	−	−	−	−	+	−	−	−	−	−	−	−	−
Other special comment							FE	DBS					
UPDRS III (on/off)	17/?	20/?	5/34	7/13	29/71	12/16	9/50	25/66	7/59	16/33	8/24	30/44	36/51

All subjects had a good clinical response to levodopa.

PT = postural tremor; HA = hallucination; DE = depression; SC = like schizophrenia; FE = festination; DBS = deep brain stimulation; UPDRS = Unified Parkinson's Disease Rating Scale; ? = information could not be obtained.

The table summarizes the clinical characteristics of the eight families (13 patients, mean ± SD age at onset 30 ± 10.7 years, range 18 to 56 years). Every family presented with at least two cardinal features out of resting tremor, bradykinesia, rigidity, and postural abnormality. In addition to the clinical features of parkinsonism, some of the affected members had various neurologic signs and symptoms such as levodopa-induced dyskinesia, sleep benefit, dystonia at onset, and hyperreflexia. No other clinical signs (gaze palsy, ataxia, rapid progression of disease, and pathologic reflex) were found.

Discussion. Our results indicate that PARK6-linked AR-EOPD is not only distributed in Europe but also in Asia. Five of 39 families tested showed evidence of linkage with PARK6 as homozygous. Families F, G, and H did not show homozygosity, indicating that they may be compound heterozygotes or not linked to this region.

PARK6-linked AR-EOPD was first reported in nine European families.[5-7] The characteristics of PARK6-linked AR-EOPD included wide range age at onset, slow progression, lack of dystonia at onset, and lack of sleep benefit, resembling late-onset PD.[5,6] Families A, F, and H also showed slow progression, lack of dystonia at onset, and sleep benefit, indicating similarity to the European families reported previously.[5-7] With regard to the PARK6-linked AR-EOPD in Japanese families (Families A, B, and D), they showed little similarities to Japanese patients with ARJP. These families were rather similar to the phenotypes of PARK6-linked AR-EOPD in European families described in the original report.[5]

The affected individual in Family C was heterozygous at marker D1S2885, thus defining the centromeric limit of the disease gene interval at this marker. In addition, a recombination event for the telomeric border was observed at D1S2843 in Family A, indicating a reduction of the critical region to 8.5 cM flanked by markers D1S2843 and D1S2885. Moreover, this region is smaller than that described in the original report.[5]

Before identification of *DJ-1* mutations, some families were found to share the haplotypes at two regions of PARK6 and PARK7 due to the proximity of

both loci. Thus, it is difficult to distinguish the families based on each locus. However, after identification of *DJ-1* mutations, it became possible to separate PARK6 from PARK7 using *DJ-1* mutation studies.

In the PARK6 region, several genes have been mapped so far. The genes for USP31 (ubiquitin specific protease 31), HTR6 (serotonin receptor), and ECE1 (neprilysin activity) are located in this locus. Considering the pathogenesis of PD, these genes may be candidate genes for PARK6. USP31 is related to the proteasome system that may be involved in the pathogenesis of PD. HTR6 gene is highly expressed in the striatum and C267T polymorphism in this gene was associated with increased risk for the development of PD.[8] Reduced neprilysin activity may cause Alzheimer disease[9] and the similar mechanism may be related to PD.

These potential candidate genes and numerous other genes located in PARK6 region should be investigated in PARK6-linked families.

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Novel *PINK1* Mutations in Early-Onset Parkinsonism

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***PINK1* was recently found to be associated with PARK6 as the causative gene. We performed mutation analysis in eight inbred families whose haplotypes link to the PARK6 region. We identified six pathogenic mutations (R246X, H271Q, E417G, L347P, and Q239X/R492X) in six unrelated families. All sites of mutations were novel, suggesting that *PINK1* may be the second most common causative gene next to *parkin* in parkinsonism with the recessive mode of inheritance.**

Ann Neurol 2004;56:424–427

The primary cause of Parkinson's disease (PD) is still unknown despite recent progress in research on the molecular mechanism of loss of dopaminergic neurons. Although most patients with PD are sporadic, identification of causative genes of the rare monogenic forms of PD or parkinsonism could provide important insights into the understanding of disease pathogenesis. To date, four genes have been identified as the causative genes for familial parkinsonism: mutations of *α-synuclein* and *UCH-L1* in autosomal dominant forms of parkinsonism and mutations of *parkin* and *DJ-1* in autosomal recessive forms. Among the monogenic forms of parkinsonism, mutations of *parkin* have been detected in approximately 50% of cases with autosomal recessive early-onset parkinsonism (AREP).[1] Although *DJ-1* mutations responsible for PARK7 were reported to cause another type of AREP,[2] it is unlikely to be of numerical significance in clinical practice.[3] Thus, it is possible that other loci are responsible in the remaining patients with AREP.

Recently, mutations of *PINK1* were detected as the causative gene for PARK6.[4] We also performed linkage analysis in 39 families with AREP who were negative for *parkin* and *DJ-1* mutations. Eight of these families showed evidence of linkage with PARK6. *PINK1* is located only 324kb from the D1S2732 at which we obtained multipoint log of the odds score of 9.88.[5] To define the genotype–phenotype relationship, we performed mutation analysis for *PINK1* in these families.

Patients and Methods

Eight families were chosen for *PINK1* mutation screening. Three families were Japanese; two Taiwanese; and one each from Israel, Turkey, and the Philippines. Families A, B, C, D, and E showed homozygosity at the PARK6 region, whereas compound heterozygosity was suggested in Families F, G, and H in our linkage analysis.[5] The clinical characteristics of affected subjects are described in the previous study (mean age at onset ±SD, 30 ± 10.7 years; range, 18–33 years).[5] The study was approved by the ethics review committee of Juntendo University. After obtaining informed consent, we performed mutation analysis of *PINK1* by direct sequencing of the polymerase chain reaction products using the following primers: Ex2 forward 5′-CTGACCTCTCAGATCATTGAGTATTGT-3′, Ex2 reverse 5′- AATCTGTCTTTTCCTACCTACTTCCTG-3′, Ex3 forward 5′-GTTAAGACAGGTCATCTT-ATCTCGAAG-3′, Ex3 reverse 5′-CTACTGTCATA-TCAGACACTGTACCAGG-3′, Ex4 forward 5′-GTACAGTACCTGGCACATAGCAAATCTA-3′, Ex4 reverse 5′- CACTATAGCAAAGTTAGGGGATACA-GAG-3′, Ex5 forward 5′- CTCTTACTTCCTAATT-TGAGGATGGTG-3′, Ex5 reverse 5′- ACTTAGAACA-CAAAACCAGAGAGGAC-3′, Ex6 forward 5′- AAAT-CAAAGTCTCCTGGGGTATAAG-3′, Ex6 reverse 5′- GTTTATGTGACAGGACTTGCATTCT-3′, Ex7 forward 5′- AGAATGCAAGTCCTGTCACATAAAC-3′, Ex7 reverse 5′- GTAACTAGCCTTTACCTTCCTAACACAG-3′, Ex8 forward 5′- ATAGAGGAGACTACTTACCT-GGTTCAAG-3′, and Ex8 reverse 5′- AGACTGAACTCT-CACTCAAGTTCTTCC. Primers for exon 1 were used as reported previously.[4] Dideoxy cycle sequencing was performed with Big Dye Terminator Chemistry (Applied Biosystems, Foster City, CA). This was followed by exon sequencing on ABI377 and 310 automated DNA sequence analyzers (Applied Biosystems). Although the haplotypes of

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the affected members of Family F showed compound heterozygotes, we identified a homozygous point mutation in exon 5. Considering this finding, it is possible that an exonic deletion in the same exon takes place in other alleles of the affected members of this family. Therefore, we performed gene dosage assay in Family F to exclude this possibility using TaqMan real-time quantitative polymerase chain reaction. Primers and probes were designed by Assay-by-Design Service (Applied Biosystems). Sequences of primers and probes and the protocols are available upon request.

Results

We identified four types of homozygous point mutations (R246X, H271Q, E417G, and L347P) involving exons 3, 4, 5, and 6 in *PINK1* of patients from five unrelated families (Fig 1). We also detected two nonsense mutations (Q239X and R492X) as a compound heterozygote in a Taiwanese family (Family G) (Table). All mutations cosegregated with the disease phenotype. In addition, the mutations were not found in 200 normal Japanese chromosomes.

The site of nonsense mutation (c.736 C-to-T transition) was not identical to that reported recently,[4] suggesting a novel mutation site. In addition, although the same mutation was detected in different ethnic groups (one in a Japanese and the other in an Israeli), these families did not share a common haplotype, thus excluding the possibility of a single founder effect. This finding indicates that the point mutation (R246X) may be a hot spot in *PINK1* mutations. Premature termination by this mutation could lead to a truncated protein that lacks 336 amino acids, including a highly conserved protein kinase domain. Two Japanese and one Filipino families carried missense mutations (c.813 C-to-A transversion, c.1040 T-to-C transition, and c.1250 A-to-G transition) in exons 4, 5, and 6 resulting in the substitution of highly conserved amino acids in the putative kinase domain, suggesting that this domain is of functional importance (Fig 2).

Although the affected members of Family F were compound heterozygous, we identified a homozygous missense mutation (c.1040 T-to-C). This finding suggests that the affected members of this family are compound heterozygotes with both a missense and an exonic deletion in the same exon 5. However, we could exclude this possibility because we could not detect the heterozygous exonic deletion in exon 5 using the gene dosage technique. Thus, we conclude that the affected members of this family had a homozygous mutation. For this mutation, we could not exclude the possibility

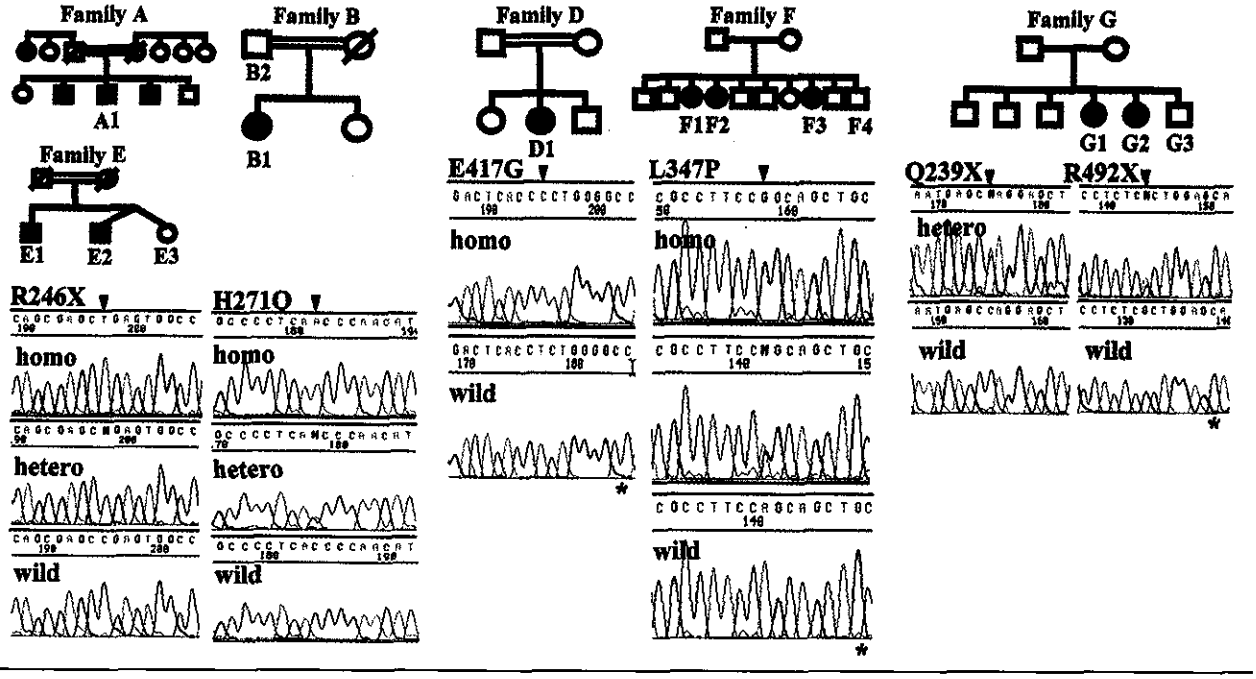

Fig 1. Pedigree and chromatograms illustrating nonsense, missense, and compound heterozygote mutations. Homozygous nonsense mutations (R246X) in exon 3 of affected members (A1, E1, and E2). Homozygous missense mutations (H271Q, E417G, and L347P) in exons 4, 5, and 6 from Families B, D, and F. Compound heterozygote mutation (Q239X/R492X) in exons 3 and 7 of affected members (G1 and G2). Heterozygote states were identified in healthy individuals in Families E (E3), B (B2), and F (F4). One of the unaffected members in Family G (G3) had only a heterozygote mutation (Q239X). (circles) *Women;* (squares) *men;* (solid symbols) *homozygous affected individuals;* (open symbols) *healthy individuals.* (asterisk) *Complementary sequences are presented in exons 5, 6, and 7.*

Table. Mutations in the PINK1 *gene*

	Origin	Nucleotide change	amino acid change	Exon	Zygosity	Mutation type	AAO	DD
Family A	Japan	c.736 C-to-T	R246X	3	*homo*	*nonsense*	30	17
Family B	Japan	c.813 C-to-A	H271Q	4	homo	missense	23	15
Family D	Japan	c.1250 A-to-G	E417G	6	homo	missense	33	8
Family E	Israel	c.736 C-to-T	R246X	3	homo	nonsense	25,33	17,21
Family F	Philippines	c.1040 T-to-C	L347P	5	homo	missense	27,27,32	18,19,23
Family G	Taiwan	c.715 C-to-T/ c.1474 C-to-T	Q239X/R492X	3/7	com/hetero	nonsense/ nonsense	18,19	22,24

homo = homozygous; com/hetero = compound heterozygote; AAO = age at onset (years); DD = disease duration (years)

that this alteration is a rare polymorphism because we could not screen for the mutation among the same races such as normal Filipino controls. However, we consider this mutation to be pathogenic because of the significance linkage to PARK6 of this family,[5] absence of its mutation in 100 normal Japanese controls, and the alteration of highly conserved amino acid among several species.

Several polymorphic variants were identified in normal Japanese controls. In exon 5, a homozygous c.1018G→A substitution (frequency: 10%, n = 100) and a heterozygous c.1018G→A substitution (frequency: 44%, n = 100) were found. Another variant, a C→T homozygous substitution (c.914C→T, P305L), was found in all Japanese controls (frequency: 100%, n = 100) and IVS4-5 G→A was found as homozygous (frequency: 68%, n = 100) and heterozygous (frequency: 29%, n = 100).

All patients with *PINK1* mutations showed early age at onset (mean age at onset ±SD, 26.7±5.9 years; range, 18–33 years), long disease duration (mean, 18.4 ±4.67 years), and good response to L-dopa.[5] There were no distinct clinical signs that could distinguish patients of homozygous mutation from those with compound heterozygous mutation.

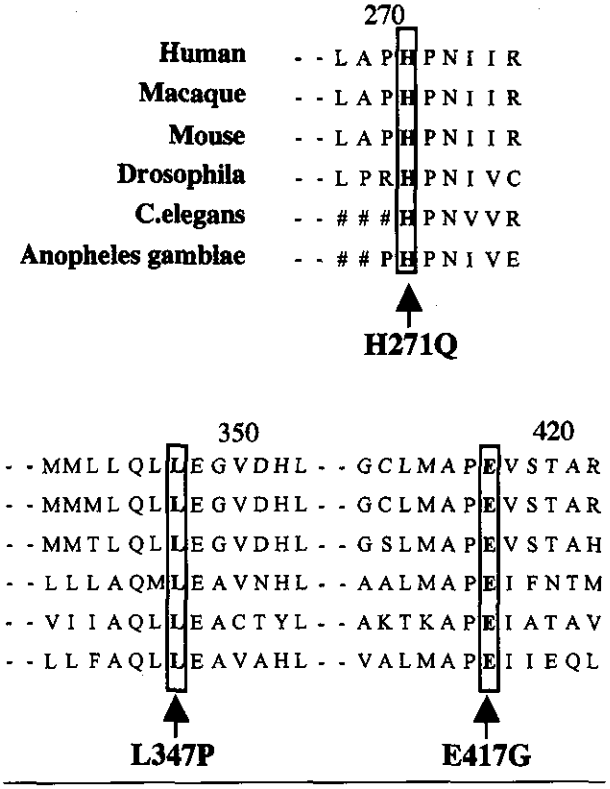

Fig 2. Alignment of PINK1 *homologs showing the conserved amino acid mutated in Families B, D, and F.*

Discussion

Our results indicate that pathogenic mutations in *PINK1*-positive AREP are not limited to Europeans but occur also in Asians, suggesting that *PINK1* mutation is the second most frequent next to *parkin*. Different point mutations seem to be more frequently responsible for the disease phenotype than are deletions.

A homozygous mutation (L347P) was detected in the affected members of the Filipino family of which haplotypes at the PARK6 region showed compound heterozygotes, indicating that the frequency of *PINK1* mutations could be high next to the *parkin* mutations.[5]

In this study, we could not identify the *PINK1* mutation in the protein coding regions including the splicing sites in a Turkish (Family C) and the other Taiwanese families (Family H). Although we cannot exclude the possibility that the patients may have homozygous mutation in the regulatory regions or intron sequences that cause exon skipping, these families may be linked to other loci. Indeed, homozygosity in the *PINK1*-negative families spanned the PARK6 and 9 regions. Thus, these families may have an allelic disorder in the PARK9 gene because the clinical phenotype of PARK9 is a distinct entity from PARK6. We found, based on the comparison between the *PINK1*-positive and -negative families, that the clinical features are very

similar. It is difficult to distinguish *PINK1*-positive AREP from the *PINK1*-negative one. In this regard, the discovery of *PINK1* helps us to provide key clinical information based on the differential diagnosis of AREP. The characteristic clinical features of our *PINK1*-positive families included slow progression and lack of dystonia at onset except for two patients (D1 and G2), indicating similarity to the Italian families described in the original report.[4] Although further studies are needed to determine the frequency of dystonia in *PINK1*-positive AREP, the lack of dystonia might be a distinct clinical sign for differentiating this form from *parkin*- or *DJ-1*–positive AREP. Furthermore, two affected members of Family E showed some psychiatric problems at the onset of the disease. The disease onset is slightly earlier than in patients of the original report, indicating phenotypic variability.

Although PINK1 function is unknown, it originally was reported to be upregulated by the tumor suppressor gene, *PTEN*, in cancer cells.[6] Preliminary results showed that the loss-of-function effect of PINK1 might be associated with mitochondrial dysfunction. There has been considerable progress in our understanding of the molecular mechanisms of nigral degeneration; mitochondrial respiratory failure and oxidative stress appear to play important roles in the progression of the disease.[7] DJ-1 acts as an antioxidant protein, and oxidative stress can damage the 26S proteasome in which parkin acts as an ubiquitin ligase. Thus, all the gene products in AREP may form a common cascade. In summary, the novel mutations identified in this study indicate that *PINK1* is a pathogenic gene in AREP.

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Pathogenetic mechanisms of *parkin* in Parkinson's disease

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Context The cause and pathogenesis of Parkinson's disease remain unknown; mitochondrial dysfunction, oxidative damage, environmental factors, and genetic predisposition might all be involved. Identification of the causative genes for familial Parkinson's diseases allow study of the pathogenesis of the disease at the molecular level.

Starting point Katja Hedrich and colleagues studied 75 Serbian patients with early-onset Parkinson's disease for *DJ-1* mutations (*Neurology* 2004; 62: 389–94). One patient was a compound heterozygote and another had a heterozygous exon deletion. *DJ-1* mutations seem to be rare in this European population. By contrast, *parkin* mutations have been found in about 50% of familial cases and in 10–20% of cases without a positive family history.

Where next The fact that parkin is a ubiquitin ligase gives special meaning to the molecular mechanism of neurodegeneration in general. In Parkinson's disease, Lewy bodies are immunoreactive for ubiquitin. Accumulation of abnormal proteins has also been seen in other neurodegenerative disorders. Disturbance of protein degradation by the ubiquitin-proteasome system might have a critical role in neurodegeneration. Although α-synuclein mutations are infrequent, α-synuclein accumulates in Lewy bodies, and α-synuclein fibrils impair the 26S proteasome function. UCH-L1 is also an abundant deubiquitylating enzyme, and its mutation is linked to *PARK5*. Furthermore, *DJ-1* might interact with SUMO-1 (small ubiquitin-like modifier), which can counteract ubiquitin and stabilise proteins against degradation by the 26S proteasome. Uncovering the mechanisms of protein degradation should add importantly to understanding the neurodegenerative process in these neurodegenerative diseases.

Parkinson's disease is characterised pathologically by selective degeneration of neurons in the substantia nigra and locus coeruleus, and the presence of Lewy bodies in the remaining neurons. Although the cause of the disease remains unknown, the identification of genetic mutations in several forms of familial Parkinson's disease has provided new insights into the mechanism of neuronal loss in the substantia nigra.

In familial disease, *parkin* mutations are the most common cause of autosomal recessive early-onset parkinsonism, including the autosomal recessive juvenile disease. The frequency of the mutation is estimated at 50% in families with autosomal recessive early-onset parkinsonism.[1] The clinical features of the autosomal recessive early-onset disease with *parkin* mutations are highly variable compared with the juvenile disease. Autopsies on patients with parkin-related diseases, except for one case, commonly show lack of Lewy bodies,[2] suggesting that the normal function of parkin is essential for the formation of Lewy bodies. Also, the discovery that parkin is an ubiquitin ligase suggests that the ubiquitin-proteasome system might have an important role in maintaining dopaminergic neurons.[3]

Mutations in *parkin*

Autosomal recessive juvenile parkinsonism is characterised by early-onset parkinsonism (before 40 years of age, average 26·1 years), mild dystonia, diurnal fluctuation, transient improvement of motor disability after sleep or rest, a good response to levodopa, and less frequent resting tremor compared with sporadic Parkinson's disease. With the recognition of the various *parkin* mutations in autosomal recessive early-onset parkinsonism, the clinical features of parkin-related diseases can be similar to those of late-onset sporadic Parkinson's disease, including the age of onset and cardinal features such as postural instability, resting tremor, rigidity, and akinesia.

There are 12 exons spanning 1·4 Mb in *parkin*, encoding a 465-aminoacid protein with moderate homology to ubiquitin at its aminoterminus (ubiquitin-like domain, Ubl) and two RING finger motifs (RINGs) at the carboxy-terminus. Various *parkin* mutations have been identified, including exonic deletion, insertions, and several missense mutations.[4–7] Mutations in *parkin* are not limited to a particular area or race. In fact, early-onset parkinsonism with *parkin* mutations is the most frequent form of familial Parkinson's disease. In addition to homozygous mutations, compound heterozygous states, representing different mutations in each allele, are also not uncommon in young patients with apparently sporadic disease. Compound heterozygotes are difficult to detect by conventional PCR because of the large size of this gene, but can be quantitatively identified by the gene-dosage technique. Point mutations are infrequent in Japanese patients compared with whites, although exonic deletions are common. Katja Hedrich and colleagues[8] studied 75 Serbian patients with early-onset Parkinson's disease for *DJ-1* mutations. One patient was a compound heterozygote and another had a heterozygous exon deletion. Thus *DJ-1* mutations seem to be rare in this European population compared with *parkin* mutations.

The common hot spots for exonic deletions are from exons 2 to 5. By contrast, point mutations have been found from exons 6 to 12, where the two RING finger motifs and the in-between RINGs are located. The clinical phenotypes of parkin-related diseases are expanding. For instance, slowly progressive cerebellar ataxia has been reported.[7] Also, more than a few patients who had psych-

iatric or behavioural symptoms were found to have *parkin* mutations. These atypical clinical symptoms appear before or after the onset of parkinsonism.[7]

Carriers of *parkin* mutations may have a Parkinson's disease phenotype. Indeed positron-emission tomography with fluorodopa revealed reduction of uptake even in carriers.[9] Furthermore, heterozygous *parkin* mutations that seem to be transmitted dominantly have been identified in multiple generations,[10] which suggests that parkin-related diseases might be sometimes dominantly inherited. Single heterozygous mutations in exon 7 might also act as susceptible alleles for the late-onset form of parkin-related diseases.[11] And the association[12] of *parkin* haploinsufficiency in sporadic Parkinson's disease further implicates the role of parkin in the more common form of the disease. The single heterozygous state might exert haploinsufficiency effects rather than dominant negative effects. By contrast, some mis-sense mutations might have a dominant negative effect as mis-sense mutations in functional domains (Ubl or RINGS),[13] resulting in an earlier onset than with mutations in other regions.

Function of parkin and pathogenesis of parkin-related diseases

The ubiquitin-proteasome pathway is important in protein processing and degradation, and contributes to quality control of proteins in cells. Ubiquitin is attached covalently to target proteins. Protein ubiquitination is catalysed by three enzymes, E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme), and E3 ubiquitin ligase. Mutations in *parkin* result in a loss-of-function of E3. Subsequently, substrates for parkin accumulate within dopaminergic neurons, potentially leading to nigral neuronal death, and it is important to identify such substrates.

Nine candidate proteins are degraded by parkin (table).[14–22] Other proteins also interact with parkin, such as E2s, multiprotein ubiquitin-ligase complex (eg, cullin-1),[18] CASK/Lin2, which acts as a scaffolding protein containing postsynaptic PDZ,[23] actin filaments,[24] γ-tubulin,[25] and Rpn 10,[26] which is the binding site for proteasomal proteins. Although further research is needed to elucidate the functional role of the RINGs of parkin, the presence of the proteins listed in the table suggests that parkin might interact with various proteins, including substrates.

Animal *parkin*-knockout models can help to elucidate the mechanism of parkin-related diseases. Mice that are *parkin*-null have motor and cognitive deficits and inhibition of amphetamine-induced dopamine release and glutamate neurotransmission.[27] These mice have high concentrations of dopamine in the limbic areas of the brain, and have a shift of dopamine metabolism towards monoamine oxidase. The finding on monoamine oxidase suggests the presence of oxidative stress in parkin-related diseases (figure). Additionally, *parkin*-null mice show accumulation of parkin substrates, but steady-state concentrations of CDCrel-1, synphilin-1, and α-synuclein, which are substrates for parkin, are not altered.[28] A drosophila model with inactivated orthologue of human *parkin* showed muscular degeneration and mitochondrial pathology,[29] which suggests a physiological role for parkin at a mitochondrial level.[30] However, it remains to be seen why muscular degeneration but not neurodegeneration is observed in this model, although it may represent differences between mice and drosophila.

Most importantly, while it is clear that Lewy-body formation generally does not occur in parkin-related diseases, Lewy bodies were identified in one patient with compound heterozygous mutations,[31] which suggests that some *parkin* mutants with mis-sense mutations might

Substrate	Proposed function
CDCrel-1	Exocytosis (dopamine storage?)
CDCrel-2	
Pael receptor	Stress in endoplasmic reticulum (unfolded protein response)
O-glycosylated α-synuclein	Lewy-body formation
Synphilin-1	Lewy-body formation
Cyclin E	Apoptosis (kainate excitoxication)
α/β tubulin	Microtubules (assembly dysfunction)
p38 subunit	aminoacyl-tRNA synthesis (protein biosynthesis)
Synaptotagmin XI	Fusion or docking, synaptic functions
Parkin-interacting proteins	
UbcH7, UbcH8, Ubc6/7, Ubc4	E2
Actin filament	Morphology
CASK/Lin2	PDZ-containing scaffolding protein
Cullin-1	Multiprotein ligase
γ-tubulin	Centrosome
Rpn 10	Binding of parkin to proteasomal proteins

PDZ=postsynaptic density-95, disc large, zona occludens.

***Table:* Candidate substrates for parkin**

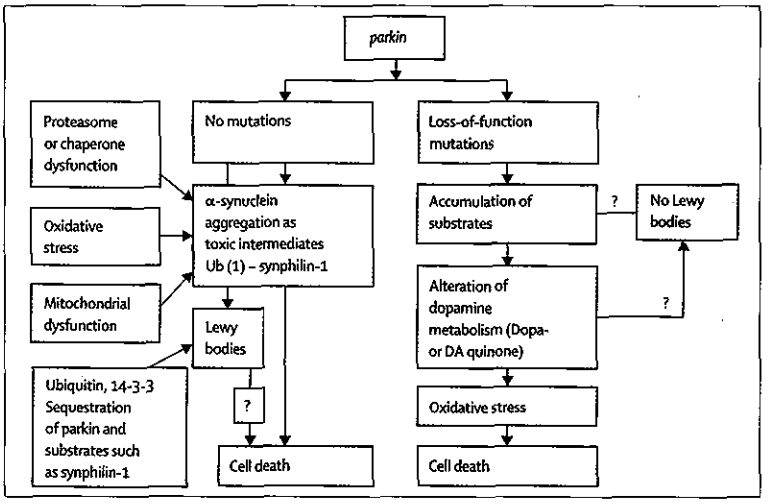

***Figure:* Model for pathogenesis of Park 2 and sporadic Parkinson's disease**
Lewy bodies need parkin for thier formation. By contrast, Park2 lacks Lewy-body formation, suggesting that accumulation of substrates inhibits aggregation of α-synuclein (Lewy bodies). In Parkinson's disease, proteasome dysfunction, oxidative stress, and mitochondrial dysfunction could induce aggregation of α-synuclein, with subsequent formation of intermediate filaments of α-synuclein. Finally, Lewy bodies are formed although whether their formation is cytotoxic is debatable. UB=ubiquitin, Dopa=3,4-dihydroxyphenylalanine, DA=dopamine.

have ubiquitin-ligase activities. Although the pathological findings in the brains of patients with parkin-related diseases include severe neuronal loss and gliosis in the substantia nigra, several atypical findings have been reported, such as accumulation of tau protein as neurofibrillary tangles in the substantia nigra, locus coeruleus, red nucleus, and posterior hypothalamus, and neurofibrillary tangles and thorn-shaped astrocytes in frontal, temporal, and parietal cortices.[2] Accumulation of tau protein as tufted astrocytes, but not neurofibrillary tangles, was reported in a patient with compound heterozygous mutations.[32] In this respect, part of the pathology of parkin-related diseases resembles that of progressive supranuclear palsy, which suggests that tau may be one of the parkin-interacting proteins.

To investigate the toxicity of the substrates for parkin, drosophilia is a suitable model for elucidating the mechanism of dopaminergic neuronal loss. Transgenic flies that express human PAEL receptors (PAEL-R), a parkin substrate, under conditions of altered parkin activity show age-dependent selective degeneration of dopaminergic neurons, despite equal levels of expression of PAEL-R in all neurons.[33] This Pael-R-mediated neurotoxicity in dopaminergic neurons was attenuated by co-expression of human parkin, and exacerbated by blocking the activity of endogenous parkin by RNA interference. Overexpression of parkin can also suppress α-synuclein-induced toxicity.[33] However, drosophila has neither PAEL-R nor α-synuclein proteins. Thus the null background of the ectopic expression of both proteins induces neuropathological changes in the dopaminergic system, which is corrected to some extent by co-expression of parkin. These findings suggest that parkin probably has a central role in maintaining dopaminergic neurons. In other words, parkin is an essential protein for the survival of dopaminergic neurons.

We have no conflict of interest to declare.

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Mitochondrial Genome Variation in Eastern Asia and the Peopling of Japan

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To construct an East Asia mitochondrial DNA (mtDNA) phylogeny, we sequenced the complete mitochondrial genomes of 672 Japanese individuals (http://www.giib.or.jp/mtsnp/index_e.html). This allowed us to perform a phylogenetic analysis with a pool of 942 Asiatic sequences. New clades and subclades emerged from the Japanese data. On the basis of this unequivocal phylogeny, we classified 4713 Asian partial mitochondrial sequences, with <10% ambiguity. Applying population and phylogeographic methods, we used these sequences to shed light on the controversial issue of the peopling of Japan. Population-based comparisons confirmed that present-day Japanese have their closest genetic affinity to northern Asian populations, especially to Koreans, which finding is congruent with the proposed Continental gene flow to Japan after the Yayoi period. This phylogeographic approach unraveled a high degree of differentiation in Paleolithic Japanese. Ancient southern and northern migrations were detected based on the existence of basic M and N lineages in Ryukyuans and Ainu. Direct connections with Tibet, parallel to those found for the Y-chromosome, were also apparent. Furthermore, the highest diversity found in Japan for some derived clades suggests that Japan could be included in an area of migratory expansion to Continental Asia. All the theories that have been proposed up to now to explain the peopling of Japan seem insufficient to accommodate fully this complex picture.

[Supplemental material is available online at www.genome.org.]

Recent analysis of global mitochondrial DNA diversity in humans based on complete mtDNA sequences has provided compelling evidence of a human mtDNA origin in Africa (Ingman et al. 2000). Less than 100,000 years ago, at least two mtDNA human lineages began to rapidly spread from Africa to the Old World (Maca-Meyer et al. 2001). The archaeological records attest that humans reached Japan, at the eastern edge of Asia, around 30,000 years ago (Glover 1980). At that time, Japan was connected to the Continent by both northern and southern land bridges, enabling two migratory routes. As early as 13,000 years ago, pottery appeared in Japan and Siberia for the first time in the world (Shiraishi 2002). Subsequent technical improvements gave rise to the Japanese Neolithic period known as the Jomon period, in which the population growth was considerable. Later, Continental people arrived in Japan from the Korean peninsula, initiating the Yayoi period, with this migration reaching its maximum at the beginning of the first millennium.

With this archaeological framework in mind, it was of an-

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thropological interest to us to know whether the modern Japanese are the result of an admixture between the Paleolithic–Neolithic aborigines and more recent immigrant populations, whether the indigenous population gradually evolved to give rise to the modern Japanese, with subsequent colonizations having strong cultural influences but only minor demographic impact, or even whether the late Neolithic waves entirely replaced the indigenous residents. Morphometric data obtained from the remains of Japanese Paleolithic people are more in accordance with a southern origin for these first immigrants. Subsequent morphological studies on modern indigenous (northern Ainu and southern Ryukyuans) and mainland Japanese favored an admixture model in which the former would be descendants of the Paleolithic Japanese and the latter derived from the Continental immigrants who gave rise to the Yayoi period (Hanihara 1991). Genetic analysis using classical markers assigned a definitive northern origin to the Upper Paleolithic inhabitants of Japan; but whereas some authors favored a homogeneous background for all modern Japanese (Nei 1995), others claimed that although Upper Paleolithic and Yayoi period immigrants had probably a northern Asian origin, they were genetically differentiated (Omoto and Saitou 1997). The application of molecular markers to define maternal and paternal lineages to the peopling of Japan confirmed the dual admixture model but added some interesting novelties. For example, the study of Y-chromosome markers led to the discovery of remarkable Korean and Tibetan influences on the Japanese population (Hammer and Horai 1995); and mtDNA HVS-I sequences also confirmed the Korean input (Horai et al. 1996) and closer affinities of the Japanese to Tibetans than to southern Asians (Qian et al. 2001). In quantitative estimations of maternal admixture, it was found that ~65% of the mainland Japanese gene pool was derived from Continental gene flow after the Yayoi period. However, the indigenous Ainu from the northern island of Hokkaido and the Ryukyuans from southern Okinawa showed <20% Continental specificity, pointing to them as the most probable descendants of the Jomon people. The fact that these indigenous groups were, in turn, genetically well differentiated indicated a notable degree of heterogeneity and/or isolation among the early Japanese immigrants (Horai et al. 1996). However, two handicaps of these studies are the incomplete representation of Asian populations and the relatively small sample size of those analyzed, which weakens the reliance on the relative affinities found by genetic distance methods (Helgason et al. 2001). For mtDNA there are currently enough HVI/HVII data from eastern Asia, including Japan, to test the validity of the above-mentioned results. However, these sequences have been assorted into different clades following different insufficient criteria or even have not been classified at all. Furthermore, the phylogenetic confidence of results based only on sequences from the noncoding region (HVI, HVII) has been recently questioned (Bandelt et al. 2000). This is mainly due to the frequent occurrence of parallel mutations in independent lineages that confuse the correct classification, a source of error that is increased because the basal motif in the noncoding region for the two macrolineages that expanded throughout Asia is the same (16223). In addition, as the noncoding region has not evolved at a constant rate across all human lineages, it is considered inappropriate to use this region for dating evolutionary events (Ingman et al. 2000; Finnilä et al. 2001).

To make reliable use of this important source of available data on the mtDNA noncoding region to contrast the maternal structure and to determine the most probable origin of the modern Japanese, we have undertaken the following approach: First, we used a set of complete mtDNA sequences of 672 Japanese individuals to create a phylogenetic network (Bandelt et al. 1999) that related them to other complete sequences, already published, belonging to the major haplogroups proposed by others (Torroni et al. 1992, 1996; Macaulay et al. 1999; Yao et al. 2002a). Discriminative positions in the noncoding region, defining additional Asian subhaplogroups, were then used to further classify 766 previously published Japanese partial sequences. For this purpose we also included other unambiguously assorted sequence data reported by other research groups (Derbeneva et al. 2002b; Yao et al. 2002a). These HVI sequences thus pooled were then compared with other published Asian sequences. Finally, using all of these classified sequences, we tested the relative affinities of modern Japanese and Continental Asians using global distance methods and phylogeographic approaches framed at different age levels.

RESULTS

Eastern Asia Phylogeny Based on Complete mtDNA Sequences

The phylogenetic network constructed with the complete mtDNA sequences fully coincides with those previously published at worldwide (Maca-Meyer et al. 2001; Herrnstadt et al. 2002) or regional scale (Kong et al. 2003). Moreover, their main branches are well supported by high bootstrap values on a neighbor-joining tree (Supplemental material, condensed by more than 40% bootstrap values).

From the L3 African trunk, two early branches came out of Africa and radiated extensively, originating superhaplogroups M and N, which were defined by the basic mutations depicted in Figures 1A and 2, respectively. Representatives of both superhaplogroups reached Japan. The construction of these phylogenetic trees by using our Japanese complete sequences and other published Asian sequences (Table 1) resulted in a better definition of the known haplogroups and in the identification of new clades at different phylogenetic levels. Characteristic HVI motifs and diagnostic RFLPs in the coding region, and coalescence ages for these haplogroups and subhaplogroups are given in Supplemental Tables A and B. To contribute to the unification of the mitochondrial nomenclature, we revised the previously proposed haplogroups by adding the following new information.

Subdivisions Within Macrohaplogroup M

Haplogroup D

Haplogroup D has been defined by the specific RFLP −5176 AluI (Torroni et al. 1992). Studies on Native American HVI sequences permitted further subdivision of D into subgroups D1 by mutation 16325 and D2 by mutation 16271 (Forster et al. 1996). Additional subdivisions into subhaplogroups D4 and D5 have been proposed for Asian lineages (Yao et al. 2002a). These investigators characterized D4 by position 3010. Two additional mutations, 8414 and 14668, have been proposed to define D4 (Fig. 1B; Kivisild et al. 2002). Whereas these two latter mutations seem to be rare events, 3010 has also been independently detected in haplogroups H and J. A new branch at the same phylogenetic level as D4 and D5 has been detected in Japan (Fig. 1B). It is characterized by mutations 709, 1719, 3714, and 12654 and was named D6. The subdivision of D4 into subgroups D4a and D4b was proposed on the basis of the distinctive mutational motif 152, 3206, 14979, and 16129 for the first and 10181 and 16319 for the second (Kivisild et al. 2002). Both subclades have been detected in our Japanese sample. From our data it can be deduced that mutation 8473 is also basal for D4a. In relation to D4b it seems that its ancestral branch is defined by the 8020 substitution (Fig. 1B). Consequently, the D4b subgroup proposed by Yao et al. (2002a) should be renamed D4b1 harboring 15440 and 15951 as additional basic mutations. A new subgroup character-

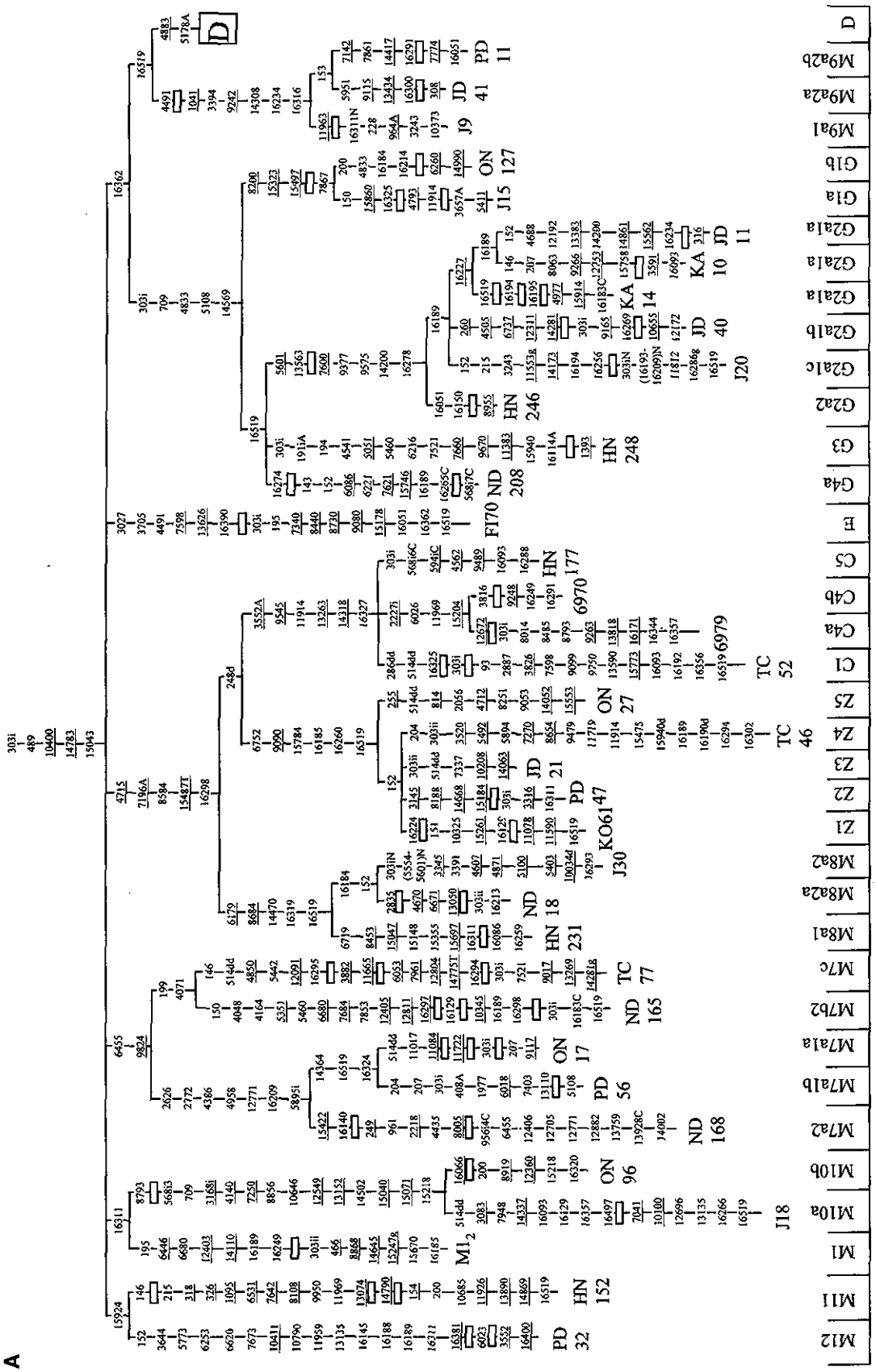

Figure 1 *(Continued on next page)*