f. CMT4F　Periaxin（*PRX*）

Dejerine-Sottas typeまたはCMT4Fの原因としてミエリンと軸索の接着に関与する*PRX*（periaxin）が同定された[12]．上肢のMCVはそれぞれ2.1〜20m/secで，非常に遅延し，病理学的には異常な髄鞘と部分的な低形成，さらにオニオンバルブ形成を認めている．電子顕微鏡では脱髄やミエリンの菲薄化，また，tomaculaやfocally folded myelinが認められ，paranodeでミエリンと軸索をつないでいるseptate-like junctionの欠損がある．Periaxinはミエリンループと軸索をつなぎ止める働きをしていると考えられる[13]．本邦から3家系の報告が行われたが，いずれもR1070Xのホモ接合体で，発症は幼児期で早いものの，その後の進行はゆるやかである例などを報告している．

g. CMT4H　Frabin（*FGD4*）

CMT4Hの原因としてguanine nucleotide exchange factor（GEF）である*FGD4*が同定された．アクチン細胞骨格や微小管のネットワークはRho familyのsmall GTPaseによりコントロールされている．本症は，Rho GTPaseシグナリングの異常をひき起こすことにより，Schwann細胞のミエリン形成，維持に異常を与えることが考えられている．同様にRho GTPase GEFであるARHGEF10の異常症も神経伝導速度を遅くすることが知られている．

症例の発症年齢は乳幼時期で，四肢遠位部の筋力低下と側彎を認める．上肢のMCVはそれぞれ15m/sec以下で，非常に遅延し，ミエリン低形成や軸索障害を認める[14)15]．

h. CMT4J（*FIG4*）

FIG4は，リン酸化イノシトールの代謝にかかわるFIG4がCMT4Jの原因として同定された．本遺伝子のミスセンス変異のヘテロ接合体がALSのリスクになることも近年報告されている．リン酸化イノシトールの代謝という点ではCMT4Bとの機序的な共通点もある．細胞内での顆粒の輸送の障害が考えられている．発症年齢はさまざまであるが，発症後の筋力低下の進行は早い．左右の非対称性もある．上肢のMCVも19〜50m/secと幅が広く，病理学的に有髄神経の減少が認められる[16]．

3. 常染色体優性遺伝形式の軸索障害型CMT（CMT2）（図6）

CMT2の原因遺伝子・蛋白は，軸索変性をひき起こすこともあり，hereditary motor neuropathy（HMN）のようなmotor neuron diseaseと原因がオーバーラップしているものも多い．原因は，一般的には優性遺伝によるdominant-negativeやtoxicな変異もあり，構造蛋白や転写因子を除けば必ずしも蛋白の機能と疾患が関連しないことも多い．CMT2の原因蛋白は軸索の主構造物であるニューロフィラメント関連の蛋白もあるが，機能のはっきりしない蛋白も多く存在する．

a. CMT2A1　*KIF1B*（Kinesin family member 1B）
b. CMT2A2　*MFN2*（mitofusin 2）

CMT2Aの原因として，軸索での輸送にかかわる*KIF1B*が報告された．しかし，*KIF1B*の異常がモデルマウスに疾患をひき起こすことは証明された．CMT2A1は日本の1家系の報告のみである．

一方，この遺伝子が存在する1p36領域に連鎖を示した家系のほとんどはミトコンドリアの融合・分裂に関与する*MFN2*が原因として同定された．MFN2はCMT2の原因としてはもっとも頻度が高い．MFN2はミトコンドリアの膜上に分布し，先に述べたCMT4Aの原因のGDAP1とともにミトコンドリアの形態，大きさ，数などの調節にかかわっていると考えられる．発症年齢は5〜26歳で，四肢遠位部の筋力低下を示す．HMSN Vという CMT with pyramidal featureの臨床型やHMSN VIのoptic atrophyを示した症例もMFN2のミスセンス変異である．また，認知機能障害など中枢神経障害を呈する例の報告もある．末梢神経障害がなく，中枢神経の障害のみの例も報告され，ミトコンドリア病でみられるさまざまな病態をひき起こす可能性がある．

c. CMT2B　*RAB7*（RAS-associated protein RAB7）

CMT2Bの原因としてvesicular transportとmembrane traffickingの調節機能をもつ*RAB7*が同定された[17]．エンドゾームとライソゾーム間の輸送に関与すると考えられている．20〜30歳代で発症し，足の潰瘍や感染症を起こし，足の変形も強い．

d. CMT2D　*GARS*（glycyl tRNA synthetase）

CMT2Dの原因としてグリシンのtRNAを合成する*GARS*が報告された[18]．GARSは，現在さまざまな疾患でとり上げられている，アミノアシルtRNA合成酵素の一つであり，グリシンをコードするtRNAを合成する．この遺伝子の変異はhereditary distal motor neuropathy（dSMA-V）の原因としても報告された．発症は16～30歳で，手に強い筋萎縮と筋力低下を呈する．

e. CMT2E/CMT1F　*NEFL*（neurofilament light chain）

先ほどCMT1Fの原因としても報告したNEFLは，はじめはCMT2Eの原因として報告された[19]．CMT2Eも比較的報告例が多く，発症も1歳から50歳代までにわたり，臨床像は多様である．CMTの基本的な症候はもち，MCVも20m/secから正常まで幅が広い．

f. CMT2F　*HSPB1*（HSP27）

CMT2Fの原因としてニューロフィラメントの形成に関与する*HSPB1*（HSP27）が報告された[20]．本遺伝子はdistal HMNの原因としても報告されている．HSPB1は，細胞骨格の調節や維持を行っている．発症年齢は6～64歳と幅が広く，軽症例が多い．進行は緩徐である．

g. CMT2L　*HSPB8*（HSP22），

CMT2Lの原因として*HSPB8*（HSP22）が報告されている[21)22)]．dHMN IIの原因としても報告されている．15～33歳で下肢の筋力低下での発症例の報告がある．

4. 常染色体劣性遺伝形式の軸索障害型CMT（AR-CMT2）

常染色体性劣性遺伝形式のCMT2の原因として7つの原因遺伝子が同定されているが，純粋なニューロパチーだけを示すものはない．AR-CMT2A＋hoarseness（CMT2K）の原因は*GDAP1*であるが，これは先に述べたCMT4Aの原因でもある．失調などの中枢神経症状や毛髪の異常を合併するgiant axonal neuropathyの原因として，microtubuleのnetworkと関連する*GAN1*（gigaxonin）が報告されている．Polyneuropathyとagenesis of corpus callosum, mental retardation, dysmorphic featuresをもつAndermann症候群の原因としてpotassium-chloride cotransporterである*KCC3*が

報告されている．小脳失調を伴う遺伝性ニューロパチーは特殊であるが，EAOH/AOA1および小脳失調症を伴う軸索型ニューロパチー（SCAN1）は，それぞれ*APTX*（aprataxin），*TDP1*（tyrosyl-DNA phosphodiesterase）が原因で，single strand DNA修復障害が原因である[1]．これらの疾患は，活性酸素やトポイソメラーゼによるDNA切断点の修復の遅延によりひき起こされると考えられている．また，2疾患と類似の臨床像をもつAOA2は，肝腫瘍マーカーであるAFPの上昇が特徴で本法での報告も数例に及ぶ．原因は，RNA/DNA helicaseの活性をもつ*SETX*（senataxin）でALS4の原因ともなる．これらの疾患の病態は，転写障害による蛋白合成の低下が神経症候をひき起こしている可能性が高い．

5. X染色体優性・劣性遺伝形式のCMT（CMTX）

X染色体性のものでは，CMTX1の原因として*GJB1*（gap junction protein, beta-1；connexin32；Cx32）が，CMTX5の原因として*PRPS1*（phosphoribosyl pyrophosphate synthetase 1）が報告されている．

a. CMTX1（*GJB1*）

*GJB1*遺伝子異常によるCMTX1はCMT1Aについで2番目に頻度が高い．X染色体優性で女性も軽症であるが発症する．Gx32はSchwann細胞でgap junctionを形成し，ミエリンと軸索の間の栄養物質の交換にも関与する．*GJB1*の異常によるCMTは，CMT1とCMT2の中間的な伝導検査の所見をとり，病理学的にはthin myelinationが多い[4]．臨床的には上肢の障害がやや強い傾向にある．末梢病変に加え中枢神経症状を合併した症例報告も多い[23]．

b. CMTX5（*PRPS1*）

PRPS1（phosphoribosyl pyrophosphate synthetase 1）の異常は，CMTX5の原因として報告された．*PRPS1*はプリンおよび核酸代謝で働く重要な酵素である．本症は10歳前後で発症し，難聴や視神経萎縮を伴うことがある[24]．

c. Intermediate type のCMT

Intermediate CMTと呼ばれ，現在までに6つの病型が報告されている．10q24.1に連鎖するものはDI-CMT type A（CMT DIA；dominant intermediate type A），19q12に連鎖するものはCMT

DIB, 1p34-p35に連鎖するものはCMT DICと名づけられ，CMT DIBの原因として*DNM2*が報告された[25]．そのほかにも*MPZ, GJB1, NEFL, GDAP1*の変異による例も報告されている．

d．DI-CMT type B

Dynamin 2(DNM2)はGTPaseの一つで，細胞融合分裂の機能をもつと考えられている[25]．また，DNM2はactinと結合することが示され，actin-mediated membrane dynamicsと関連していることが推定されている．発症は10歳前後で，遠位部優位の筋力および感覚の低下がある．MCVは24～54m/secである．また，先天性白内障などの眼症状を伴うCMT2の原因としても同定された．

e．DI-CMT type C　Tyrosyl-tRNA synthetase（YARS）

この遺伝子もまたtRNA合成酵素であり，CMT2Dの原因であるGARSの異常症と機序的に共通している．多くは1～20歳で発症し，下肢優位の筋力低下を認めた．進行は非常にゆっくりで，車いすを用いることはほとんどない．

6．その他の病型

Mini fascicular neuropathyは小さなfasciculusを特徴とするが，46, XY partial gonad digenesis accompanied by minifascicular neuropathyが報告され，原因として*DHH*(desert hedgehog)の変異が同定された．

以上，遺伝子異常のはっきりしているものを概説した．いくつかの遺伝子異常はCMT1およびCMT2の両方の臨床型を呈し，重症度も多彩である．遺伝性疾患は遺伝子変異の強さや修飾因子により，より重症例や軽症例が報告されることも多いため注意が必要である．さらにintermediate formのCMTも確立され，臨床症状による軸索型，脱髄型という分類と遺伝子異常による分類は必ずしも一致せず，互いにオーバーラップしている．

CMTの正確な遺伝子変異の検出はCMTの診断を確定し，遺伝子カウンセリングなどに使えるだけでなく，今後の治療をみすえた対策を立てるための基礎データとして重要と考えられる．

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劣性遺伝性脊髄小脳変性症の
治療と具体的事例

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脊髄小脳変性症は大きく遺伝性のものと非遺伝性のものに分けられます。非遺伝性のものの半分以上は多系統萎縮症であり、小脳症状に加えてパーキンソン症状（体の固さなど）や自律神経症状（立ちくらみなど）を伴う特徴があります。また、脊髄小脳変性症のうち、遺伝性のものは約3分の1を占め、その中のほとんどは常染色体優性遺伝性の病気であり、その多くはSCA1、SCA2などのSCA(脊髄小脳失調症)という記号に番号をつけた名前で表されています。常染色体性劣性遺伝形式の脊髄小脳変性症は、頻度的には1.8％とまれであるにもかかわらず、数多くの病型があります。近年、それらの原因遺伝子の同定や病態の解明も進んできており、疾患の理解が深まってきています。
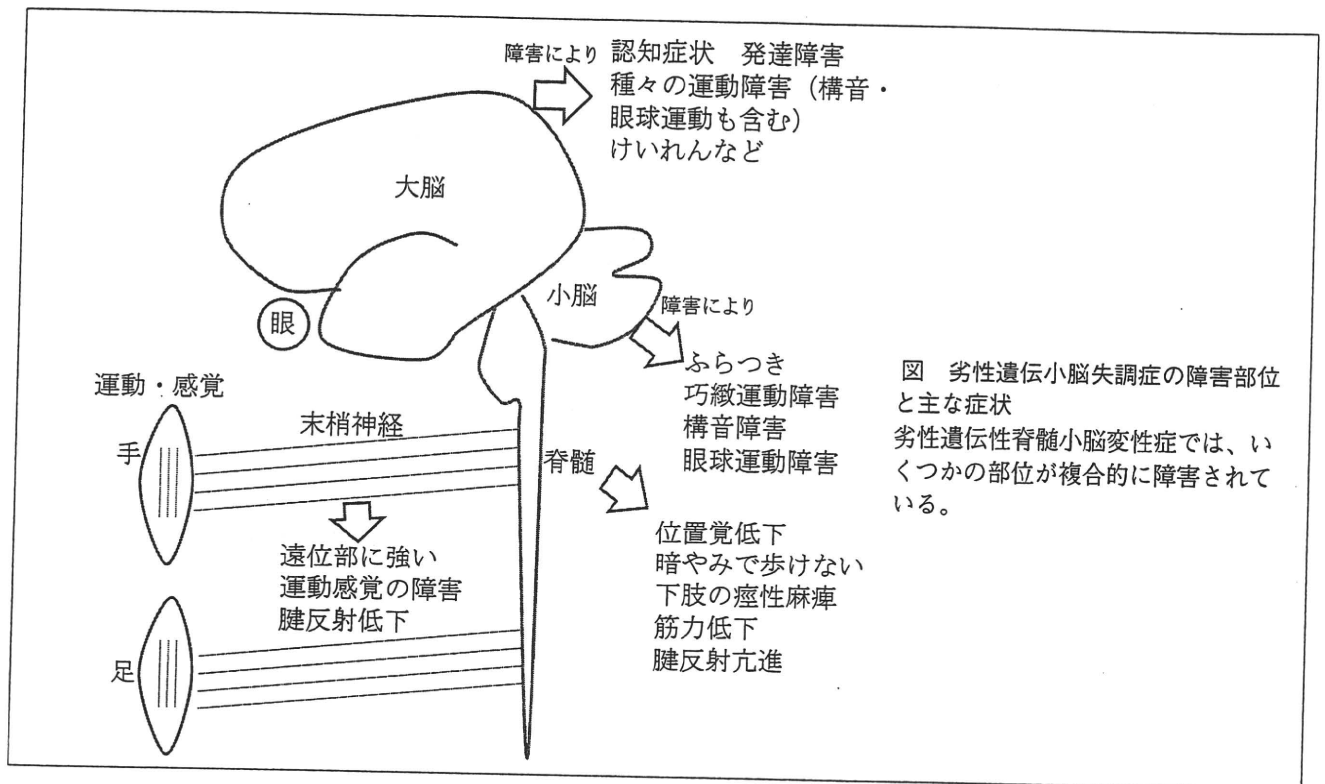
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疾患の基本的特徴

常染色体優性遺伝形式が通常2本ずつある遺伝子の1本に異常があって病気が起こるのに対し、常染色体劣性遺伝形式というのは、2本の遺伝子とも異常になって起こるものであります。それゆえ、同じ遺伝子上に異常が重なるためには、両親とも異常を持っている必要があり、一般には起こりにくく、近親婚でよりおこりやすくなっています。近親婚をしない限りは、通常は次の世代には受け継がれません。

発症機序を大きな視点から述べますと、優性遺伝性の小脳変性症の多くが、遺伝子のトリプレットリピートの延長（正常3塩基の繰り返し配列の増加）が原因であり、その延長により作り出された、ポリグルタミンを持った異常蛋白の蓄積が神経細胞毒となり病気が起こっています。

それに対し、劣性遺伝性の小脳変性症は、2本の遺伝子とも悪いため、それらの異常遺伝子から正常

機能を持った蛋白質が作れず、機能が欠落することにより、神経細胞が悪くなっているといえます。このことは、治療法を考える上では全く別のことを考える必要があり、優性遺伝性の小脳失調症は、ポリグルタミンの毒性をどのように取り除くのかが研究の中心であるのに対し、劣性遺伝病では、どのように正常遺伝子を入れて、正常の働きをとりもどすのかということが治療研究の中心となっています。

代表的疾患

常染色体劣性遺伝性の小脳失調症は、小脳失調だけではなく、他の症状を合併している病型がほとんどであります。その中には、末梢神経障害（ニューロパチー）、知的機能障害、てんかん、筋障害、視力障害、網膜異常、眼球運動障害、不随意運動、皮膚異常など様々な症候があり、小脳失調以上に身体的影響が大きい症候もあります。このなかでも先天性や乳幼児期の発症の疾患の多くは、知能障害を伴うことが多く、重度の障害を持つことが多いようです。

小脳失調主体に日常生活に支障がでている疾患として、アプラタキシン欠損症（EAOH/AOA1），セナタキシン異常症(SCAR1/AOA2)、ビタミンE単独欠乏性運動失調症(AVED)、シャルルボア-ザクネ型痙性失調症(ARSACS) 軸索型末梢神経障害を伴う小脳失調症(SCAN1)、フリードライヒ失調症(FRDA)などがあります。今回は、これらの病気の中でも本

図　劣性遺伝小脳失調症の障害部位と主な症状
劣性遺伝性脊髄小脳変性症では、いくつかの部位が複合的に障害されている。

邦でみられる4疾患について概説いたします。

1）ビタミンE単独欠乏性運動失調症

　脊髄小脳変性症の治療法は、対照的なものに限られているのが実情です。しかし、劣性遺伝性の疾患の中に、ビタミンE単独欠乏性運動失調症（Ataxia with vitamin E deficiency (AVED)）という病気があり、本症は、日本でもみられる治療可能な小脳変性症として重要であります。本症は、ビタミンE転送蛋白（α-tocopherol transfer protein)という遺伝子の異常で引き起こされます。この蛋白の異常により、腸で吸収されたビタミンEを、肝臓を介して血中に運び出しているのですが、その運び出しがうまくいかなくなるため、血中および体の組織のビタミンEが足りなくなり、病気になることがわかってきています。

　それにより、歩行障害、構音障害などの小脳失調に加えて、高率に脊髄の後索症状、具体的には手足の位置覚の障害のため暗いところが歩きにくいことや、反射の低下、足の変形がみられます。頭の震えや視力障害（網膜色素変性）を伴うこともあります。発症年齢は、遺伝子異常の強さにより様々ですが、小児期から60歳くらいまでにわたっています。診断は、血中のビタミンEの測定で著明な低下をみるため、採血により行うことができます。そのほか体性感覚誘発電位などの電気生理学的検査でも異常をと

らえることができます。

　治療は、ビタミンE大量摂取により、症状の改善や進行の防止が期待されます。1日約800mgのビタミンEをとれば、多くの例で血中のビタミンEが上昇し、改善が期待できると報告されています。

事例紹介

(1) 56歳　男性

　主訴は歩行障害。近親婚あり。48歳のとき暗やみで歩きにくくなる。また、洗顔時によろめくようになってきた。52歳の時しゃべりにくくなってきた。53歳の頃から歩きにくくなり、55歳には、足を広げて歩行するようになった。病院での診察では、ゆっくりとしたしゃべりで、手足の腱反射が消失。足先に触覚、痛覚、振動覚および位置覚の低下がみられた。ロンベルグ徴候陽性（足をそろえて起立し、目をつぶると強くふらつく症状）。
臨床症状および血中ビタミン 0.8μg/ml（正常値5～15)からビタミンE単独欠乏性運動失調症と診断されビタミンE　1日あたり800mgを内服開始した。その6ヶ月後、血中ビタミンE値 11.5μg/mlと上昇し、小脳失調、感覚障害もすこしずつ改善している。また、体性感覚誘発電位も改善。1年後には、ロンベルグテスト陰性化し、歩行も早く歩けるようになってきた。ビタミンEは、300mgを続けている。

2）アプラタキシン異常症（EAOH/AOA1）

海外では、劣性遺伝性運動失調症で足の変形や感覚障害を伴うものの多くはフリードライヒ失調症で、しかし、本邦では多くの遺伝子学的検討が行われましたが、フリードライヒ失調症は確認されておりません。それゆえ、本邦においてその症状を見かけたときには、アプラタキシン異常症（EAOH/AOA1）やセナタキシン異常症(SCAR/AOA2)が疑われます。

眼球運動失行と低アルブミン血症を伴う早発型脊髄小脳失調症は、小児期発症の特徴的な眼球運動失行と下肢のむくみを特徴とする疾患で、我が国の劣性遺伝性の脊髄小脳変性症でもっとも頻度の高いものであります。原因は、一本鎖DNA修復酵素の1つであるアプラタキシン(ataxin)の遺伝子異常により発症することが、新潟大学により明らかにされました。本症の典型的な場合には、乳児期から小児期にかけて眼球運動障害で発症するが、特徴ある眼球運動を行うため、本症を疑うことは比較的容易であります。

この眼球運動は、ものを見るのに先立ってまばたきを行ったり、見たい方向を見るときに頭を回旋させたりするものであります。次に小脳失調と末梢神経障害とが徐々に明らかとなり、20歳を超えると低アルブミン血症が進行します。アルブミンは血管内に体の水分を保つ働きがあるため、低アルブミンになると血管から水分が漏れ出るため、体（特に下肢）にひどいむくみが発生することとなります。一方、遺伝子変異が強くない場合には、成人での発症となり、この場合には特徴的な眼球運動障害が無い場合があり、診断は容易ではありません。アプラタキシンの機能はわかってきていますが、現在根治的な治療法はありません。対照的にはセレジストの効果は個人差がありますので試してみるのも良いとおもいます。低アルブミン血症に対するアルブミンの点滴治療も考えられますが、効果は一過性であり、適応は慎重に選ばれます。

(2) 症例　60歳女性

主訴は、歩行障害。家族歴に兄にも同様の症状がある。高校生の頃から走るのが遅くなり、運動が苦手になってきた。18歳のころ歩行時のふらつきを指摘される。症状は少しずつ進行し、25歳の頃、下肢の変形、筋萎縮、構音障害などの末梢神経障害と小脳失調の症状からフリードライヒ失調症と診断された。35歳頃には伝い歩き、40歳には四つ這い移動になった。50歳代に手の震えも出現し、日常的に介助が必要となり、入院となった。

神経内科では、知能は正常、眼球運動時に眼振をみとめ、両手先の筋萎縮、筋力低下、下肢の筋力低下と著明な浮腫を指摘された。感覚も上肢でやや弱く、下肢で強く障害され、深部腱反射は消失していた。MRIでは著明な小脳の萎縮像、神経伝導検査では高度の軸索障害型の末梢神経障害、血清アルブミン値は3.0g/dlと低下、高コレステロール血症(270mg/dl)が認められている。

小脳失調症の遺伝子検査の結果、本邦の軽症例に多い、アプラタキシン遺伝子のアミノ酸コドン206番のプロリンがロイシンへの変異（P206L）のホモ接合体の異常が検出され、EAOH/AOA1と診断された。治療は、車いすを日常的に使用しているため、リハビリテーションを中心に、療養を行っている。投薬では、セレジストは以前使用していたが、効果が明らかでなかったため、現在は使用していない。高脂血症は、HMGCoA還元酵素阻害剤により改善している。足のむくみは、時間おきの下肢の挙上で対応している。

3）セナタキシン異常症（眼球運動失行を伴う失調症; SCAR1/AOA2）

小児期にアプラタキシン異常症と類似の眼球運動失行をとる疾患としてセナタキシン異常症（眼球運動失行を伴う失調症ataxia oculomotor apraxia；AOA2）と呼ばれる疾患があり、本症も日本において確認されています。本症もまた特異な眼球運動障害に末梢神経障害と小脳失調を呈する疾患で、アプラタキシン異常症と似ていますが、本症では眼球運動失行が見られないことが多いようです。それ故、劣性遺伝性の末梢神経障害とAOA2という呼び方よりも脊髄小脳失調症（劣性遺伝）という意味でSCAR1とも呼ばれています。

セナタキシン欠損症も遺伝子異常の強さが弱い場合には、発症も遅く眼球運動失行が見られないことが多いので、末梢神経障害による遠位筋の筋萎縮と筋力低下、感覚障害と小脳失調が重要な症状です。血液検査では、アプラタキシン異常症とは異なり、

低アルブミン血症はないものの、代わりに肝臓の腫瘍マーカーであるAFPという検査値の上昇を見るのが特徴であります。もちろん肝臓に悪性腫瘍があるわけではありません。MRIでは、著明な小脳の萎縮を認めます。確定診断は遺伝子診断でありますが、セナタキシンの遺伝子が非常に大きいため、診断は容易ではありません。根治的な治療法は、確立しておらず対照的な治療が主体となっています。

このSCAR1/AOA2およびEAOH/AOA1といわれる病気と類似の疾患として、軸索型ニューロパチーを伴う脊髄小脳失調症（SCAN1）という疾患が報告されています。この病気は15歳前後に発症する末梢神経障害を伴う脊髄小脳変性症であり、眼球運動失行はないものの、AOA2やEAOHの軽症例に似た症状を示します。血液検査では低アルブミン血症や高コレステロール血症をともなう点がEAOHと共通であります。

本症は、一本鎖DNA修復酵素のTDP1の異常により起こりますが、これまでに世界でサウジアラビアの1家系の報告しか無く、493番目のヒスチジンがアルギニンにかわるH493R異常しか報告がありません。近年、このH493Rの遺伝子異常の特殊性により発症することがわかってきました。それ故、本症が日本および世界でそれほど多くはないと考えられていますが、TDP1は、アプラタキシンとともに一本鎖DNA修復に関わっていることがわかっており、互いの疾患メカニズムを比較する形で研究が進んでいます。

現在のところ、セナタキシンの機能もはっきりとはしていませんが、構造からはDNA修復に関与すると考えられており、DNA修復と小脳変性の関連性が急速に解き明かされつつあるところです。劣性遺伝性の脊髄小脳変性症の大きな機序として、DNA修復異常やそれに伴う転写障害が中心となっていることは間違いなさそうであります。この研究の発展が、他の小脳失調症の病態解明に影響を与えると考えられています。

4）シャルルボア-ザクネ型痙性失調症（ARSACS）

シャルルボアーザクネ型痙性失調症(Autosomal recessive spastic ataxia of Charlevoix-Saguenay；ARSACS)劣性遺伝性運動失調症は、カナダのケベック州に多く報告されていた病気で、原因は

SACS遺伝子の異常により起こることがわかっています。劣性遺伝性の小脳失調症は、今まで述べてきたように、末梢神経障害があり、深部腱反射が低下する型が多いのですが、本症は小脳失調に加えて、痙性麻痺があるため、下肢の突っ張りの症状もある場合に特に鑑別にあがります。特に、眼底検査で網膜有髄線維の増生があれば、本症の可能性がかなり高くなります。確定診断は、遺伝子診断になりますが、本症の原因であるSACS遺伝子が大きいため、遺伝子診断は容易ではありません。

一般的に遺伝子異常症ではよくあることですが、遺伝子変異の強さや個人の体質、環境要因などにより、重症度や症状が変わります。実際、ARSACSにおいても、網膜有髄線維の増生が無い例は以前より報告されていましたが、近年下肢の痙性までない場合もあることがわかり、臨床症状から遺伝子異常を推定するには限界があるといえると思います。現在、病態は少しずつわかってきていますが、根治的な治療法はまだありません。

おわりに

劣性遺伝性脊髄小脳失調症は、長年神経内科では未知の領域でありましたが、ここ数年の研究で、病気の原因、診断、病態がかなりわかってきました。これらの病気の多くは、小脳失調だけでなく、下肢の萎縮、感覚障害など、二重三重の障害を引き起こすため、より重篤ともいえますが、一方、他の小脳失調症と比べて、小脳失調症の進行はそれほど早くはありません。

今までの小脳失調症で蓄積されてきた、リハビリテーション、在宅ケアに加えて、末梢神経障害に行われる下肢の変形のケア（手術も含めて）、下肢脱力への適切な装具使用（短下肢装具など）などが必要と思われます。

これらの病気の解明への取り組みは日々行われており、今後の発展が期待されています。

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ORIGINAL ARTICLE

Clinical and genetic characterization of 16q-linked autosomal dominant spinocerebellar ataxia in South Kyushu, Japan

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16q-ADCA (OMIM no. 117210) is an autosomal dominant spinocerebellar ataxia (AD-SCA) characterized by late-onset pure cerebellar ataxia and −16C>T substitution of the *puratrophin-1* gene. Recently, a series of single-nucleotide polymorphisms (haplotype block) were found to be specific to 16q-ADCA. We screened patients with ataxia and found 62 patients, including four homozygotes who carry the C–T substitution of the *puratrophin-1* gene. By further analysis of the patients with the haplotype block, we observed a single-founder effect for 16q-ADCA, even in patients who are supposed to be sporadic late cortical cerebellar atrophy (LCCA). We also observed slippage mutations of microsatellite markers, GATA01 and 17msm, in the pedigrees. We compared the clinical course of 16q-ADCA in heterozygotes and homozygotes with the haplotype block and observed no apparent gene dosage effect. 16q-ADCA accounts for 27% of AD-SCAs and is the most frequent AD-SCA in South Kyushu, Japan.
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Keywords: 16q-ADCA; haplotype block; homozygote; microsatellite slippage; *puratrophin-1* mutation; SCA

INTRODUCTION

Autosomal dominant cerebellar ataxias (ADCAs) are clinically classified into three subtypes, and ADCA type III was defined as pure cerebellar ataxia with late onset.[1] Until date, 16 genes and 10 additional loci are reported to cause ADCAs.[2,3] SCA4 belongs to ADCA type I, characterized by cerebellar ataxia with sensory axonal neuropathy.[4] Its locus was originally mapped to 16q22.1 in a family from Utah and was confirmed in SCA4 families from Germany.[4,5] The 16q-ADCA from six Japanese families was mapped to the same region as SCA4;[6] however, it was categorized as ADCA type III on the basis of clinical features.[6–8] A single-nucleotide substitution (−16C>T) in the 5′-untranslated region of the *puratrophin-1* gene is strongly associated with 16q-ADCA.[9] However, three patients from families having 16q-ADCA (−16C>T) did not have the substitution.[10–12] Haplotype analysis suggested that the mutation was in a region centromeric to the −16C>T substitution.[10–12]

We had reported that this disease locus was telomeric to the *puratrophin-1* gene.[8] To clarify the disease locus in these patients, we reexamined the disease haplotype using microsatellite markers and a series of single-nucleotide polymorphisms (SNPs) specific to 16q-ADCA. Moreover, we reported the clinical features of 49 patients, including four homozygous patients, and described the frequency of 16q-ADCA in South Kyushu, Japan.

MATERIALS AND METHODS

We studied 147 patients with a family history of ataxia and 331 patients with sporadic ataxias, referred to our department (Department of Neurology and Geriatrics, Kagoshima University Graduate School of Medical and Dental Sciences) from South Kyushu (Kagoshima, Miyazaki and Oita) and Okinawa Island during the past decade until January 2008. All patients were referred by their primary physicians or neurologists and signed informed consents were obtained from the patients. The study was approved by the Institutional Review Board of Kagoshima University. Genomic DNA was extracted from peripheral blood leukocytes. We screened for the expansion of triplet repeats associated with SCAs (SCA1, -2, -3, -6, -7, -8, SCA12, -17 and DRPLA) by standard PCR methods and GeneScan analysis software using an ABI Prism 377 DNA sequencer (Foster City, CA, USA) as described earlier.[8] We used PCR-direct sequencing or PCR-restriction fragment length polymorphism to examine the C–T substitution of *puratrophin-1* and the Gerstmann–Sträussler–Scheinker syndrome (GSS) mutation.[9,13] For patients who had a C–T substitution of the *puratrophin-1* gene, PCR product sizes of the microsatellite markers, GATA01 and 17msm, were determined using the ABI Prism 377 Genetic Analyzer, (Foster City, CA, USA).[8] Using PCR-direct sequencing, we typed SNP02, -04, -05 and -06, which are highly specific to the diseased chromosome.[12] The SCA5, SCA10, SCA14 and FGF mutations were not analyzed in this study. Expert neurologists provided information on the clinical features of the 16q-ADCA patients. The Kaplan–Meier method was used to draw the curve of clinical severity for *puratrophin-1* alteration of 43 heterozygotes and four homozygotes, with the end point being wheelchair bound. The log-rank test was used for

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comparison between the two groups because of the small number of homozygotes. Patient data were statistically processed using StatView version 5.0.

RESULTS

Of the 478 patients, 62 carried the *puratrophin-1* C–T substitution. Of the 62 affected individuals, 43 patients were from 17 families with an autosomal dominant inheritance. Eleven patients were from seven families who had no proof of autosomal dominant inheritance, but their siblings had similar neurological symptoms. Eight patients had either sporadic ataxias or unclear family histories. We examined all the patients with *puratrophin-1* alteration to determine whether they had a haplotype block (SNP05 and SNP06, highly specific to the 16q-ADCA allele), but neither of the SNPs was identified in 200 control chromosomes.[12] All patients had changes in SNP05 (G–A) and SNP06 (A–G), whereas none of the 96 controls had the haplotype block (Table 1).

The average age of onset of ataxia for the 49 patients with the C–T change was 59 years (range, 43–80 years), and the oldest asymptomatic carrier (K01 pedigree) was 80 years old (Table 2).

The initial symptoms of ADCA are gait ataxia or dysarthria. On neurological examination, all individuals were affected with truncal and limb ataxia. Although deep-tendon reflexes were generally increased, spasticities or pathological reflexes were rare. Hearing impairment and dementia were poorly represented in our cohort. Brain magnetic resonance imaging revealed that 97% of the affected individuals had cerebellar atrophy, especially in the vermis.

We compared the clinical features between the four homozygous and 45 heterozygous patients. One homozygous patient had a clear family history, and both his parents, who were maternal/paternal cousins of each other, had medical histories of ataxia. Three siblings homozygous for the substitution were found by our genetic analysis. The mother of these patients reported a history of ataxia, but their father did not have ataxic symptoms and died of old age at 82 years. Similar to the heterozygous patients, the mean age of the four homozygous patients at the time of onset was 59.3 years (range, 50–63 years). However, within each family, the disease onset of the homozygotes tended to be earlier than that of the heterozygotes. The duration of the period until being wheelchair bound was determined for four homozygotes and 43 heterozygotes by the Kaplan–Meier method. There was no clinical difference between the two groups ($P=0.9689$) (Figure 1).

The results of the family-based study in 116 families diagnosed by gene tests for all patients revealed that 16q-ADCA (27%) was the most prevalent ADCA in South Kyushu and Okinawa, followed by SCA6

Table 1 Haplotype analysis with SNPs and microsatellite markers

Haplotype analysis with SNPs and microsatellite markers in families with 16q-ADCA

SNP/ marker	Common haplotype on 16q-ADCA	Frequency in 192 control chromosomes	Family no.											
			Large families											
			M1	M2	M3	M4	M5	K01	K02	K03	K04	M6	M7	M8
SNP02	C	21.4%	C	C/A	A	C/A	C/A	C	A	C/A	C/A	C/A	A	C/A
SNP04	T	28.7%	T	T	C	T/C	T/C	T	C	T	T/C	T/C	C	T/C
SNP05	A	0.5%	A	A	A	A	A	A	A	A	A	A/G	A/G	A/G
SNP06	G	0.0%	G	G	G	G	G	G	G	G	G	G/A	G/A	G/A
GATA01	158	34.9%	158	158	158	158	158	162	154/	158/	154/	158/	158/	158/
	162	16.7%							162	154	162	146	162	158
Puratro-phin-1	T	0.0%	T	T	T	T	T	T	T	T	T	T/C	T/C	T/C
17msm	189	2.1%	191	191	191	191	191	189	189/	191/	191/	191/	191/	191/
	191	8.9%							197	195	195	195	193	195

Family no.																			
Small families or sporadic cases																			
M9	M10	M11	M12	M13	K05	K06	K07	K08	K09	K10	K11	K12	K13	K14	O1	O2	O3	O4	O5
C/A	A	C/A	C/A	C/A	C/A	C	C	C/A	C/A	C/A	C	C	C/A	C	C/A	A	C/A	C/A	C/A
C	C	T/C	T/C	T/C	T/C	T	T	T/C	T/C	T/C	T	T	T	T	T/C	C	T/C	T/C	T/C
A/G	A/G	A/G	A/G	A/G	A/G	A/G	A/G	A/G	A/G	A/G	A/G	A/G	A/G	A/G	A/G	A/G	A/G	A/G	A/G
G/A	G/A	G/A	G/A	G/A	G/A	G/A	G/A	G/A	G/A	G/A	G/A	G/A	G/A	G/A	G/A	G/A	G/A	G/A	G/A
158/	158/	154/	158/	154/	162/	158/	158/	154/	158/	158/	158/	162/	154/	158/	158/	162/	154/	154/	158/
158	162	158	158	162	158	158	154	162	154	158	158	162	158	162	162	154	158	158	154
T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C
191/	191/	191/	191/	193/	189/	191/	191/	191/	191/	191/	191/	189/	191/	191/	191/	191/	191/	191/	191/
191	193	195	195	197	198	203	195	195	191	191	193	207	195	205	193	195	195	193	195

Abbreviation: SNP, single-nucleotide polymorphism.
The number of microsatellite markers represents the size of the PCR products. All families carried the haplotype block, A-G-T (SNP05-SNP06-*puratrophin-1*[C/T]), which is highly specific to 16q-ADCA. Note that compared with most pedigrees, K01, K05 and -O2 have a differently sized molecular GATA01 marker that is between SNP06 and the puratrophin C–T change. In addition, pedigrees K01 and K05 have a differently sized 17msm marker that is at the telomeric side of the puratrophin C–T change. A single allele of SNPs or sizes of microsatellite markers represents disease-associated allele. Two alleles represent that the alleles cannot be determined to be associated with the disease because of the limited number of the family members.

Table 2 Comparison between homozygous and heterozygous patients

(a) Clinical features of patients with 16q-ADCA

Patients examined (*N*)	45	4
Puratrophin-1 C/T change	C/T	T/T
Age at examination (years)	71 ± 8.7 (range, 54–91)	72.3 ± 6.7 (range, 67–82)
Age at onset (years)	58.9 ± 7.7 (range, 43–80)	59.3 ± 6.2 (range, 50–63)
Disease duration (years)	11.9 ± 7.6 (range, 1–41)	12.5 ± 6.5 (range, 6–19)
Initial symptoms (%)		
Unsteadiness of gait	84	50
Dysarthria	16	50
Clinical features (%)		
Ataxic gait	100	100
Dysarthria	96	100
Spasticity	9	0
Brisk DTRs	67	75
Decreased vibration sense	82	100
Hearing impairment	12	0
(b) Brain MRI findings of patients with 16q-ADCA		
Patients examined (*N*)	32	4
Brain MRI findings (%)		
Cerebellar atrophy	97	100
Leukoaraiosis	6	0
Cerebral atrophy	9	0

Abbreviation: DTR, deep tendon reflexes; MRI, magnetic resonance imaging.

(21%), MJD/SCA3 (15%), DRPLA (9%) and GSS (9%). The results of the patient-based study also revealed that 16q-ADCA was the most prevalent ADCA. The 16q-ADCA accounted for 65% of the ADCAs in Miyazaki Prefecture and was the most frequent ADCA in Kagoshima Prefecture (Figure 2). Characteristically, GSS accounted for 17.5% of ADCAs in Kagoshima Prefecture. In Oita Prefecture, SCA6 was the most frequent ADCA, followed by 16q-ADCA. DRPLA and SCA2 consisted mostly of ADCAs, and 16q-ADCA was not found in Okinawa Prefecture.

DISCUSSION

The 16q-ADCA has been mapped to 16q22.1.[6–8] It has been reported that the −16C>T substitution of the *puratrophin-1* gene makes it possible to genetically diagnose 16q-ADCA.[9] 16q-ADCA families have been reported from various parts of Japan after the discovery of *puratrophin-1* alteration, and it is one of the most common ataxic diseases in Japan.[10,11,14–17] Although the pathogenicity of the −16C>T substitution is controversial because of the discovery of two patients from families having 16q-ADCA, who did not have the −16C>T substitution,[10,11] it remains useful for the screening of 16q-ADCA in Japanese patients, as the −16C>T substitution has never been seen in Japanese controls thus far.[12]

We found 32 pedigrees within 62 affected individuals who carried the −16C>T substitution of *puratrophin-1*. Nineteen patients were believed to be recessive for SCA or sporadic LCCA, but we revealed that all 19 had the haplotype block (SNP05-SNP06-−16C>T substitution of *puratrophin-1*), which is highly specific to 16q-ADCA. This indicates that all of our 16q-ADCA patients originated from a single founder. Therefore, these sporadic patients are not *de novo* in origin, but their diagnoses might be because of limited ancestral information. 16q-ADCA patients diagnosed by the *puratrophin-1* alteration among sporadic LCCA have also been reported in earlier studies.[14–16] We agree with their proposal that screening for the
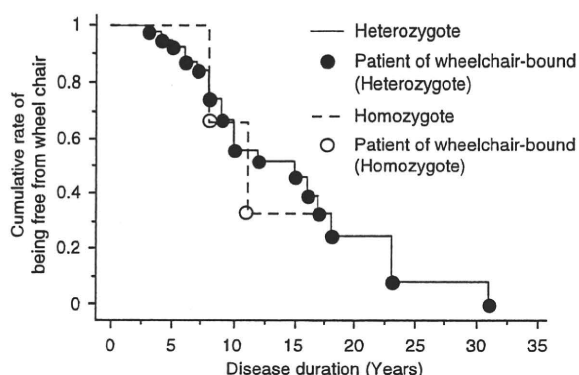

Figure 1 Comparison of the disease duration until wheelchair bound between homozygotes and heterozygotes by the Kaplan–Meier method. The log-rank test revealed no significant difference between the two groups (*P*=0.9689).

−16C>T substitution of *puratrophin-1* should be considered for SCA diagnosis, even in cases believed to be sporadic LCCA.[14,16]

Earlier, we excluded a region centromeric to 17msm as critical for 16q-ADCA, because, compared with other pedigrees, patients with the K01 pedigree have a differently sized marker at GATA01 and 17msm on both sides of the −16C>T substitution.[8] Nevertheless, the K01 pedigree has the haplotype block specific to 16q-ADCA. The disease haplotype of pedigree K01 is A-G-162-T-189 at SNP05-SNP06-GATA01-*puratrophin-1*-17msm. Conversely, most pedigrees have the haplotype, A-G-158-T-191. If the human genome sequence is correct in this critical region, the difference in microsatellite marker size is interpretable as being either multiple recombinations among SNP06, GATA01, *puratrophin-1* and 17msm, or a slippage mutation of the microsatellite markers; however, multiple recombinations are less likely to be based on the limited distance (approximately 0.75 Mb) from SNP06 to 17msm. As there is only a single-repeat unit difference between GATA01 and 17msm, a slippage mutation might have occurred. Microsatellite slippage mutations in GATA01, D16S397 and GGAA10 were also reported in 16q-ADCA families.[12] When the point mutation rate was 10^{-8} per nucleotide per generation, the slippage mutation rates ranged from 10^{-7} to 10^{-2}.[18] It might be better to use SNPs for 16q-ADCA mapping rather than microsatellite markers, as there are three disease-specific SNPs, which suggests a greater occurrence of slippage mutations around the SNPs. On the basis of haplotype analysis with these SNPs, we reassign SNP04 to its centromeric border and the *puratrophin-1* alteration to its telomeric border. The chromosomal position should be from 64 982 677 to 65 871 433 on chromosome 16 of the human genome sequence (NCBI Build 36.1) (Figure 3).

In agreement with other studies, the clinical features of our 16q-ADCA patients were late-onset pure cerebellar ataxia and brisk deep-tendon reflex.[11,14–17] We found that hearing impairment, dementia and leukoaraiosis varied on the basis of patient location, suggesting that environmental factors or genetic backgrounds other than *puratrophin-1* mutations are responsible for 16q-ADCA symptoms.

In an earlier study, four patients from one pedigree showed homozygosity for the *puratrophin-1* C–T substitution, and their mean age at onset was earlier than that of heterozygotes within the same pedigree.[14] In this study, we found four homozygotes from two pedigrees and compared their phenotypic severity with that of heterozygotes over several years. We did not find differences between homozygotes and heterozygotes with regard to age at onset or phenotypic severity. The four homozygotes had the A-G-T haplotype at SNP05-SNP06-*puratrophin-1*, indicating
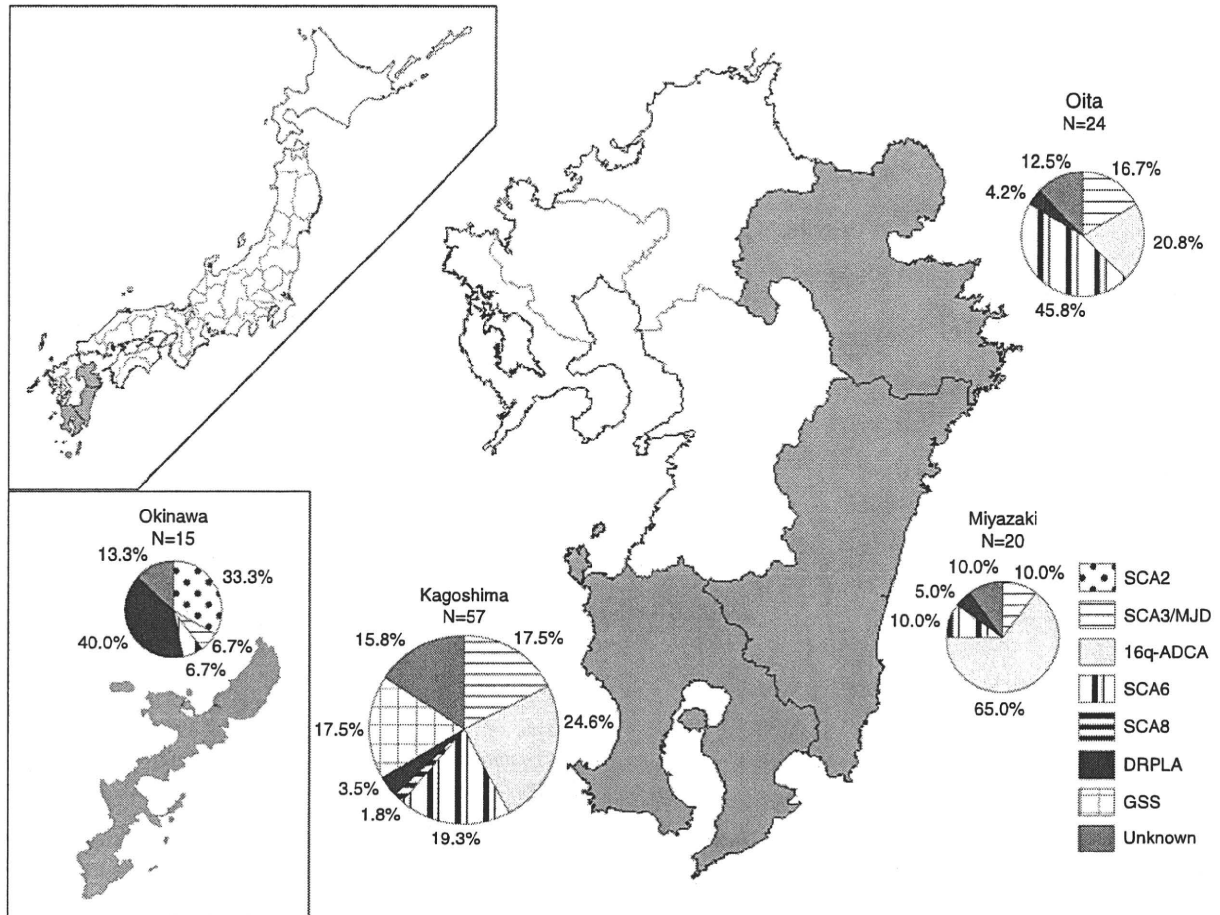
Figure 2 Relative frequency of ADCAs in the prefectures of South Kyushu, Japan. For Kagoshima, patients were referred from the whole area of Kagoshima, hence the cohort represents Kagoshima Prefecture. On the other hand, the data for Miyazaki, Oita and Okinawa prefectures were collected from a limited number of hospitals; therefore, these data may not represent the exact frequency of the disease in each prefecture.

homozygosity for the 16q-ADCA mutation. The gene dosage effect for AD-SCAs varies by gene. If the mechanism of 16q-ADCA is haploinsufficiency caused by the total or partial loss of function of a single allele, then a homozygote should show a severe phenotype. As we found no homozygotic patients with severe phenotypes, this suggests an alternative mechanism. Homozygotes with triplet-repeat expansions of SCA2, SCA3 or SCA6 showed modified or severe phenotypes.[19–24] Conversely, homozygotes of SCA3, SCA6, SCA8 and SCA12 did not have severe clinical phenotypes compared with heterozygotes.[19,25–28] On the basis of our results, there does not seem to be a strong gene dosage effect in 16q-ADCA. As some homozygotes with SCAs caused by gain-of-function mutations did not always show as severe a phenotype as the heterozygote, it is reasonable to suggest that 16q-ADCA operates through a gain-of-function mechanism.

The 16q-ADCA prevalence in Miyazaki and Kagoshima districts was higher than the average prevalence in Japan. The GSS syndrome (Prion protein (*PRNP*) Pro102Leu mutation) accounted for 18% of the AD-SCAs in Kagoshima Prefecture. Although the inclusion of GSS as an AD-SCA might be controversial, we included it on the basis of prominent cerebellar ataxia that has led physicians to recognize it as an ADCA. This epidemiological study should help neurologists gain an understanding of the frequency of ADCAs in the district.

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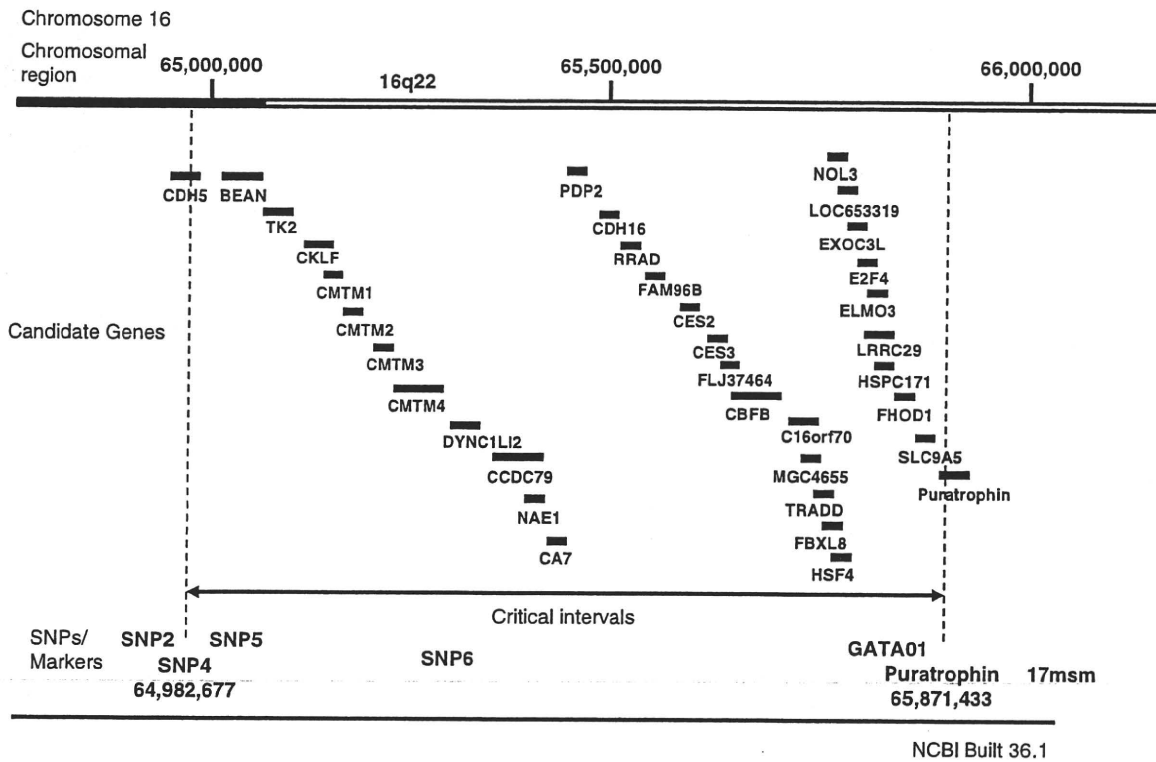
Figure 3 Physical map of the critical region of 16q-ADCA. This map shows the critical interval of 16q-ADCA in our study and is based on the human genome sequence Built 36.1 (NCBI). There are 35 candidate genes in the interval.

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Diagnostic value of serum peptidome analyses for protease activated pathological conditions beyond cancer diagnosis

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SUMMARY

Human serum contains thousands of proteolytically derived low-molecular-weight peptide fragments (serum peptidome). The concept of utilizing the serum peptidome for cancer diagnosis has been developed. A pathological serum peptidome appears when the homeostatic balance between proteases and protease inhibitors is disrupted. We hypothesize if analyses of the serum peptidome are of diagnostic value as information on which molecules are disrupted, and the pathological course it will take in unknown pathological conditions and disseminated intravascular coagulation (DIC). We analyzed the serum peptidome in 3 stages (early stage, pre-DIC and DIC stages) in one patient with POEMS (polyneuropathy, organomegaly, endocrinopathy, M protein and skin changes) syndrome, an intractable disease with unknown pathology, using a 1-dimensional gel electrophoresis/matrix-assisted laser desorption/ionization-mass spectrometry (1-DE/MS)-based rapid quantitative approach. A very large number of peptide fragments appeared in the DIC stage, compared to pre-DIC. In addition, we identified fragments of transthyretin (ALGISPFHEHAEVVFTANDSGPR, m/z 2451.18) and α1-antitrypsin (EDPQGDAAQKTDT-SHHDQDHPTFN, m/z 2691.02) that significantly increased in the DIC stage, compared to those in the pre-DIC stage. Rapid analyses of the serum peptidome may lead to a diagnostic method that can predict on-going protease activated pathological conditions and help to decide on multilateral strategies including nutritional support and drug therapy.

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Introduction

It has been well established that human serum contains thousands of proteolytically derived peptides (serum peptidome) [1–4], yet it remains unclear to date whether this complex peptidome shows a robust correlation with some biological events occurring in the entire organism [4]. An attempt to utilize peptide fragments that specifically appear in the serum of cancer patients as pathological information for the diagnosis has been reported by Petricoin et al. [5], who utilized it for the diagnosis of early-stage ovarian cancer. Villanueva et al. [4] also reported the possibility of the early diagnosis of breast, prostate, and urinary bladder cancers based on the same concept. Broadening the concept above, we formed a hypothesis that, by analyzing the peptidome, information on which molecules are disrupted and the pathological course it will take would be available. To investigate the hypothesis, we ana-

lyzed peptide fragments that appeared in serum in the early, pre-DIC (disseminated intravascular coagulation), and DIC stages in a patient with an intractable disease and a poor prognosis, POEMS (polyneuropathy, organomegaly, endocrinopathy, M protein and skin changes) syndrome, using a 1-DE/MS-based rapid quantitative approach [6]. A very large number of peptide fragments appeared in the circulation with diverse pathological conditions, showing a characteristic pattern of pathology, which may be utilized for early multilateral decisions regarding nutritional support and the therapeutic policy, in addition to the diagnosis.

POEMS syndrome (hereafter, POEMS) is a rare multisystemic disorder usually associated with plasma cell dyscrasia and characterized by the combination of polyneuropathy, organomegaly, endocrinopathy, M protein and skin changes. POEMS is an intractable disease with a mean life expectancy of 33 months (6 months–7 years) [7–9]. We previously reported that the serum VEGF-A level was markedly elevated from the early pathological stage in this syndrome, being involved in the pathology [10–13]. However, the essential pathogenesis of POEMS is still unclear.

High-abundance serum carrier proteins such as albumin may act to sequester low-molecular-weight peptide fragments in the

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Table 1
Clinical data of platelet number, blood coagulation and fibrinolysis of the patient with POEMS syndrome.

Parameters	Platelets (×10⁴/μl)	PT (%)	APTT (s)	Fig (mg/dl)	TAT (μg/l)	D-Dimer (μg/ml)
Normal range	<32.0	80–125	<50	170–410	<3.0	<1.0
Early stage	49.0	65	32.1	299	<2.0	2.5
Pre-DIC stage	27.7	40	39.7	440	4.8	3.3
DIC stage	18.2	51	>180	275	10.9	13.9

PT, Prothrombin time; APTT, activated partial thromboplastin time; Fig. fibrinogen; TAT, thrombin–antithrombin complex.
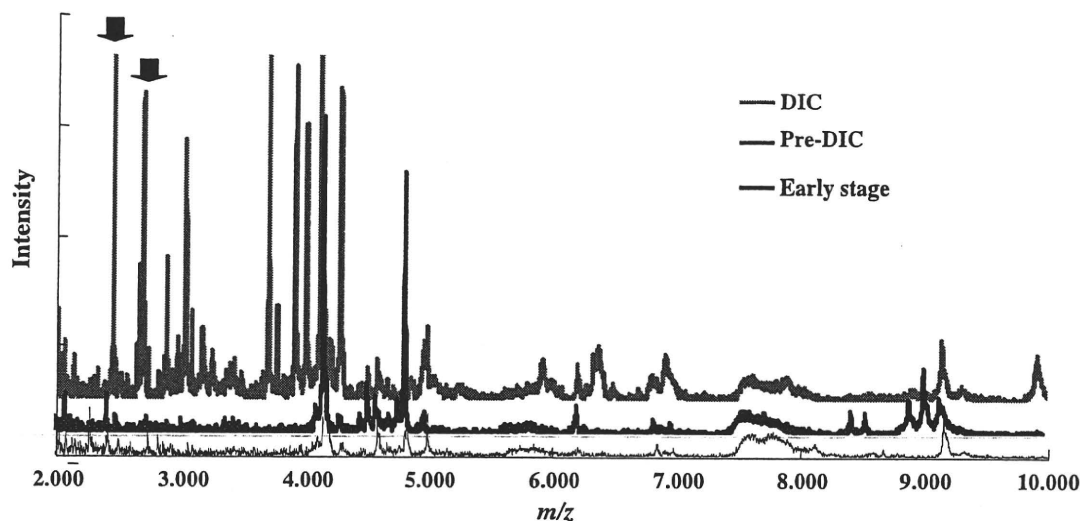
Fig. 1. Differential profiling of the patient with POEMS syndrome at the stages of early, pre-DIC and DIC. Each spectrum is the average of four independent experiments. Two identified ions; transthyretin fragment (m/z 2451.18) and α1-antitrypsin fragment (m/z 2691.02), are indicated by arrows. Black line: early stage, Blue line: pre-DIC stage, Red line: DIC stage.
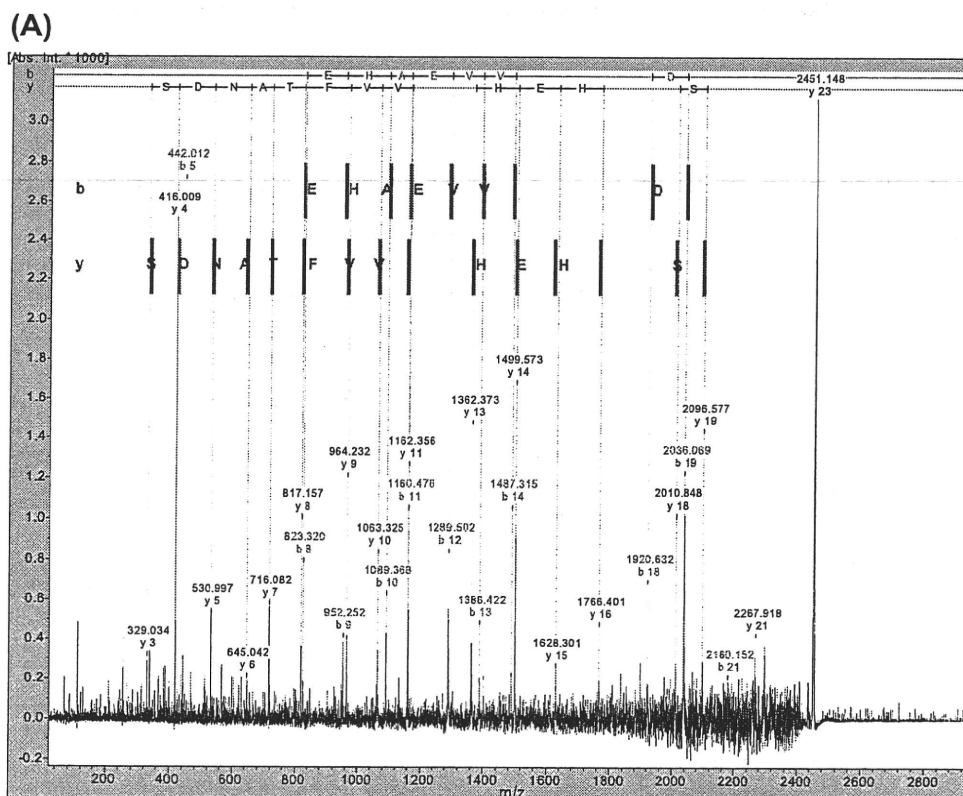
Fig. 2. (A) Identification of the peptide of m/z 2451.18. A representative MS/MS spectrum assigned to transthyretin fragment (ALGISPFHEHAEVVFTANDSGPR; calc. Mr 2450.20) (MOWSE score 82). (B) Identification of the peptide of m/z 2691.02. A representative MS/MS spectrum assigned to α1-antitrypsin fragment (EDPQGDAAQKTDTSHHDQDHPTFN; calc. Mr 2690.12) (MOWSE score 63).
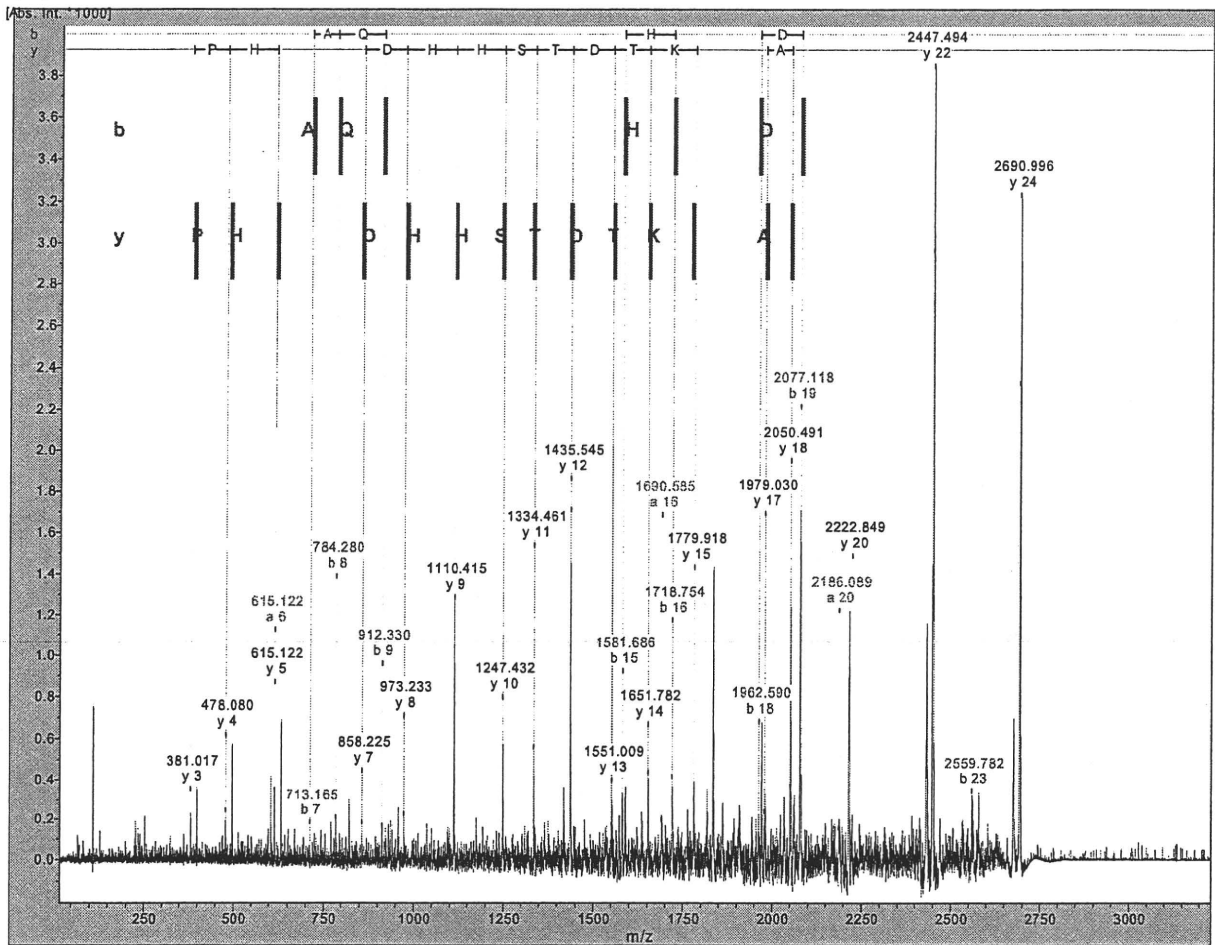
Fig. 2 (continued)

blood. Such sequestered peptides may provide a potentially rich source of candidate disease-associated or diagnostic biomarkers for subsequent clinical validation, and provide a new opportunity to expand the knowledge base regarding the molecular composition of the circulation [14]. The peptidome analytical method employed in this study does not require the removal of albumin from serum or serum digestion by trypsin [6].

The hypothesis

We hypothesized herein that serum peptidome analyses may be useful not only for cancer diagnosis but also providing various pathological information, including that on tissue damage, DIC, and organ failure, leading to an early optimum therapeutic strategies.

Empirical data

Serum peptidome analyses

The research protocol and consent form were approved by the Institutional Review Board of Kagoshima University. Serum peptidome was analyzed by employing a 1-dimensional gel electrophoresis/matrix-assisted laser desorption/ionization-mass spectrometry (1-DE/MS)-based rapid quantitative approach, as previously reported [6] in 3 stages (early stage, six days before DIC (pre-DIC)

and DIC stages) in one patient with POEMS who showed a subacute course, progressed to the DIC stage, and died 178 days after serum sampling in the early stage [13]. Clinical data on blood coagulation, fibrinolysis, and the platelet number of the patient in each stage are summarized in Table 1.

Serum peptidome in early stage, pre-DIC and DIC stages

The peptide fragments showed dynamic patterns characteristic of the three pathological stages: early stage, pre-DIC and DIC stages. Much larger numbers of peptide fragments were observed in the DIC compared to the pre-DIC stage. Differential profiling between the pre-DIC and DIC stages is shown in Fig. 1, in which transthyretin (ALGISPFHEHAEVVFTANDSGPR, m/z 2451.18) and α1-antitrypsin (EDPQGDAAQKTDTSHHDQDHPTFN, m/z 2691.02) fragments that appeared in the DIC stage were identified (Fig. 2A and B).

Evaluation of the hypothesis

On the application of peptidome analysis in this study, many peptide fragments that appear in the circulation with diverse pathological conditions including pre-DIC and DIC showed a characteristic pathological pattern, reflecting a multitude of enzymatic and proteinase activities, and its analysis may contribute to the multilateral diagnosis of not only cancers but also various pathological conditions that will develop. Although some dismissed the pepti-

dome as "noise", "biological trash" or too small and unstable to be biologically relevant, others have proposed that peptidome might contain a rich, untapped source of disease-specific diagnostic information [4,15,16]. A transthyretin fragment, ALGISPFHEHAEVVF-TANDSGPR (m/z 2451.18), and a α1-antitrypsin fragment, EDPQGDAAQKTDTSHHDQDHPTFN (m/z 2691.02), were identified in the DIC stage. Transthyretin is important, being involved in homeostasis via thyroid hormone, and so is α1-antitrypsin, involved in the control of lung inflammation. The degradation of these molecules was thought to be associated with the breakdown of homeostatic regulation in the body. The serum level of transthyretin fragment (m/z 2451.18) was previously reported to be significantly elevated in early breast cancer [4], suggesting a similar mechanism in protease activation in the tumor microenvironment of breast cancer to the DIC pathology. The tumor microenvironment consists of tumor, immune, stromal, and inflammatory cells which produce cytokines, growth factors, and adhesion molecules that promote tumor progression and metastasis [17]. DIC is diagnosed based on the elevation of blood levels of fibrinogen/fibrin degradation products induced by the activation of coagulation factors, and multiple organ failure (MOF) is diagnosed as a result of organ dysfunction. Both DIC and MOF are states showing a marked activation of proteases, and their onsets may be predicted very early by applying peptidome analyses to the DIC/MOF pathological conditions.

In summary, peptidome analyses shows characteristic patterns in not only the early diagnosis of cancer but also various pathological conditions, suggesting that it may develop into a diagnostic method sensitive to homeostatic abnormalities.

Conflict of interest statement

The authors state that they have no conflict of interest.

Acknowledgements

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Original Article: Complications

Serum VEGF increases in diabetic polyneuropathy, particularly in the neurologically active symptomatic stage

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Abstract

Aim To identify the relationship between vascular endothelial growth factor (VEGF) and diabetic polyneuropathy (DPN).

Methods Two hundred and twenty diabetic patients participated, 113 with DPN and 107 without DPN. All patients were also classified according to the four stages of DPN (no neuropathy: stage 0; asymptomatic neuropathy: stage 1; symptomatic neuropathy: stage 2; disabling neuropathy: stage 3). Serum VEGF concentration was measured using an enzyme-linked immunosorbent assay (ELISA) and levels between the patients with and without DPN and also between the different stages of DPN, were compared.

Results The mean serum VEGF level in all patients was 264.6 ± 218.8 pg/ml. The mean serum VEGF level was higher in patients with DPN (310.1 ± 224.3 pg/ml) than in the patients without DPN (216.5 ± 204.0 pg/ml, $P = 0.0014$). Serum VEGF was higher in the 'symptomatic' stage (stage 2, 364.8 ± 225.9 pg/ml) in comparison with the 'asymptomatic' (stage 1, 256.7 ± 224.4 pg/ml, $P = 0.015$) and 'disabling' (stage 3, 180.3 ± 109.4 pg/ml, $P = 0.042$) stages. The mean serum VEGF level in patients with diabetic retinopathy (261.1 ± 210.6 pg/ml) and in patients with diabetic nephropathy (241.5 ± 185.7 pg/ml) was not increased.

Conclusions The serum VEGF level is increased in patients with DPN, particularly in patients in the neurologically active 'symptomatic' stage.

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Keywords diabetes, neuropathy, vascular endothelial growth factor

Abbreviations DPN, diabetic polyneuropathy; HbA_{1c}, glycated haemoglobin; VEGF, vascular endothelial growth factor

Introduction

Vascular endothelial growth factor (VEGF)-A is one of the potent angiogenic and vascular permeability factors induced in ischaemic organs [1]. As VEGF production is stimulated by hypoxia [2], hyperglycaemia, advanced glycation end products and oxidative stress [3–6], VEGF has been recognized as a key cytokine related to the development of complications of diabetes

mellitus such as retinopathy [7,8] and nephropathy [9–11]. In diabetic retinopathy, VEGF is increased in the vitreous and aqueous fluids [7] and a recent report has shown that VEGF in the aqueous fluid is increased in both pre-proliferative and proliferative diabetic retinopathy [12]. In nephropathy, urinary VEGF excretion rates are increased and recent reports have focused on the relationship between VEGF and the stage of diabetic nephropathy [9,13,14]. As other reports have identified a significant correlation between VEGF and diabetic microangiopathy [14,15], one might predict a close relationship between VEGF and diabetic polyneuropathy (DPN). There is, however, little clinical evidence available regarding the potential role of VEGF in DPN [16,17].

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Moreover, there are no reports regarding the relationship between VEGF and the stages of DPN. This study, therefore, examined serum VEGF in diabetic polyneuropathy and investigated how it relates to the status of neuropathy.

Patients and methods

Two hundred and twenty diabetic patients (105 men, 115 women, mean age 63.5 ± 12.6 years, 11 with Type 1 and 209 with Type 2 diabetes) were enrolled. All of the patients attended the diabetes outpatient clinic of Kagoshima University hospital and at the Southern-Region hospital from 2001 to 2005. The mean duration of diabetes was 10.4 ± 9.2 years and mean glycated haemoglobin (HbA$_{1c}$) 8.0 ± 2.1%. One hundred and twenty-one patients had diabetic retinopathy and 99 patients had overt proteinuria. All patients gave their informed consent to participate in this study.

Diagnosis and staging of DPN

The criteria for the diagnosis and staging of DPN were previously proposed by Dyck [18]. Recently in Japan, a new approach to establish abbreviated diagnostic criteria and staging for diabetic polyneuropathy has been developed for daily practice [19]. Thus we modified Dyck's criteria by using the Japanese abbreviated criteria and all patients were classified into four stages; stage 0 (no neuropathy), stage 1 (asymptomatic neuropathy), stage 2 (symptomatic neuropathy) or stage 3 (disabling neuropathy). For the staging of DPN, each patient completed a detailed questionnaire and neurological evaluation. These included inquiries about subjective symptoms and determination of presence or absence of muscle weakness, ankle jerk, vibratory sensation, sensory disturbance, autonomic neuropathy and disorders affecting quality of life. Other causes of neuropathy were excluded by measuring vitamin B12, thyroid-stimulating hormone, anti-nuclear antibody and serum protein electrophoresis. Individuals with a family history of neuropathy or a disease known to cause neuropathy were excluded by taking a detailed history. Laboratory investigations, spinal X-rays and diagnostic imaging systems such as magnetic resonance imaging were performed where appropriate. In addition, patients with diseases known to affect serum VEGF, such as cancer, inflammatory diseases and peripheral vascular disease, were also excluded. The effect of age was taken into consideration when examining elderly patients and symptoms and deficits from entrapment neuropathies or mononeuropathies were not staged. All examinations and questionnaires were performed by two neurologists certified by the Japanese Society of Neurology.

Measurement of serum VEGF

Early morning fasting serum was obtained from each patient. Serum was rapidly cryopreserved. VEGF concentrations were measured in duplicate using a commercial enzyme-linked immunoabsorbent assay (ELISA) kit (R&D Systems, Minnea-

polis, MN, USA) that recognizes the soluble isoforms VEGF$_{121}$ and VEGF$_{165}$ as previously described [20,21]. The assay system used is sensitive to 15.6 pg/ml VEGF and does not cross-react with platelet-derived growth factor (PDGF) or other homologous cytokines. The optical density at 450 nm was measured on the ImmunoMini NJ-2300 (Nippon Inter Med, Tokyo, Japan) and the VEGF concentration was determined by linear regression from a standard curve using the VEGF supplied with the kit as a standard.

Statistical analysis

All data are presented as mean ± SD unless otherwise indicated. Differences between data sets were assessed by analysis of variance (ANOVA), with the Scheffe test *post hoc*. Paired data were compared by paired Student's *t*-test. All statistical analyses were carried out using the STATVIEW J-5.0 software program (SAS Institute, Cary, NC, USA) for Windows; *P*-value < 0.05 was considered to be statistically significant.

Results

Characteristics of subjects

By the diagnostic criteria and staging of DPN [18,19], 113 of the 220 patients had DPN (males 51, females 62, mean age 65.4 ± 11.3 years, mean duration of diabetes 11.6 ± 7.7 years, mean HbA$_{1c}$ 8.2 ± 2.0%) while 107 did not (males 54, females 53, mean age 61.5 ± 13.6 years, mean duration of diabetes 9.1 ± 10.4 years, mean HbA$_{1c}$ 7.7 ± 2.2%). Patients without DPN were included in stage 0. One hundred and thirteen DPN patients were included in stages 1–3. Thirty-five patients were classified as stage 1, 65 patients were stage 2 and 13 patients were stage 3 and the characteristics of patients in each stage of DPN are shown in Table 1. There was a significant correlation between the stages of DPN and the duration of diabetes, however, no significant correlation was found between the severity of DPN and age or HbA$_{1c}$.

The number and characteristics of patients with or without other diabetic complications are shown in Table 2. One hundred and twenty of the 211 patients had diabetic retinopathy and 99 of the 211 patients had overt proteinuria. The incidence of diabetic microvascular complications, paticularly retinopathy and nephropathy, significantly increased with age and duration of diabetes; however, there was no relationship with HbA$_{1c}$.

Comparison of serum VEGF level

There was a significantly higher level of serum VEGF in patients with DPN (310.1 ± 224.3 pg/ml) in comparison with those without DPN (216.5 ± 204.0 pg/ml) (*P* = 0.0014). In contrast, no significant difference in serum VEGF was found between patients with diabetic retinopathy (261.1 ± 210.6 pg/ml) and those without diabetic retinopathy (268.1 ± 237.3 pg/ml) (*P* = 0.82). In addition, in the patients with diabetic

Table 1 The characteristics of diabetic patients in each stage of diabetic polyneuropathy

	DPN (−)	DPN (+)		
	Stage 0	Stage 1	Stage 2	Stage 3
n	107	35	65	13
Age (years)	61.5 ± 13.6	66.4 ± 12.1	65.5 ± 11.3	62.3 ± 8.2
Sex (male)	54 (50.7)	18 (51.4)	24 (36.9)	7 (53.8)
Duration of diabetes (years)	9.1 ± 10.4*	8.4 ± 5.2†	11.9 ± 7.8	14.9 ± 12.9*,†
HbA$_{1c}$ (%)	7.7 ± 2.2	7.8 ± 2.1	8.3 ± 2.0	8.5 ± 1.5
Neuropathy (%)‡	0	100	100	100
Subjective symptoms of DPN (%)§	17.8	0	100	100
ATR (−)/(±) (%)	15.0	100	64.6	100
Vibration ↓ (%)	14.8	100	61.7	84.6
Autonomic¶	7.8	5.7	38.8	92.1
Motor**	0.2	0.2	7.7	100
Retinopathy (%)	34.6	51.4	66.2	84.6
Nephropathy (%)	18.7	34.3	52.3	84.6

Data are the means ± SD or number or per cent, unless otherwise indicated. There was a significant dierence in duration of diabetes between stage 0 and stage 3* ($P = 0.008$) and between stage 1 and stage 3† ($P = 0.013$). There was no significant dierence in HbA$_{1c}$ by stage of DPN (Schee's *post hoc* test with ANOVA).
‡Meets the abbreviated diagnostic criteria for DPN.
§Bilateral numbness, spontaneous pain, paraesthesia, decreased sensation, etc.
¶Orthostatic hypotension, abnormal sweating, severe diarrhoea and constipation, etc.
**Weakness and/or atrophy.
ANOVA, analysis of variance; ATR, Achilles tendon reflex; DPN, diabetic polyneuropathy; HbA$_{1c}$, glycated haemoglobin; SD, standard deviation.

Table 2 The number and the characteristics of patients with diabetic retinopathy and nephropathy

	Retinopathy			Nephropathy		
	(−)	(+)	P	(−)	(+)	P
n	91	120		112	99	
Age (years)	61.6 ± 14.6	65.0 ± 10.6	0.048	61.0 ± 13.5	66.3 ± 10.9	0.0023
Duration of diabetes (years)	6.9 ± 5.9	13.0 ± 10.3	< 0.0001	8.5 ± 6.9	12.7 ± 11.1	0.0013
HbA$_{1c}$ (%)	7.8 ± 2.5	8.1 ± 1.8	0.29	7.8 ± 2.3	8.2 ± 1.9	0.25

Data are the means ± SD.
The incidence of diabetic retinopathy and diabetic nephropathy increased with age and the duration of diabetes.
There was no significant correlation between diabetic microangiopathies and HbA$_{1c}$.
HbA$_{1c}$, glycated haemoglobin; SD, standard deviation.

nephropathy, there was no significant difference of serum VEGF level between patients with overt proteinuria (241.5 ± 185.7 pg/ml) and those without overt proteinuria (284.1 ± 248.8 pg/ml) ($P = 0.16$; Fig. 1).

Serum VEGF level was 216.5 ± 204.0 pg/ml in the 'no neuropathy' stage 0, 256.7 ± 224.4 pg/ml in the 'asymptomatic' stage 1, 364.8 ± 225.9 pg/ml in the 'symptomatic' stage 2 and 180.3 ± 109.4 pg/ml in the 'disabling' stage 3. The serum VEGF level in stage 2 was higher than in stage 0 ($P = 0.0002$) and stage 3 ($P = 0.04$; Fig. 2).

Discussion

In the present study, serum VEGF levels in the patients with DPN were significantly higher than in the patients without DPN.
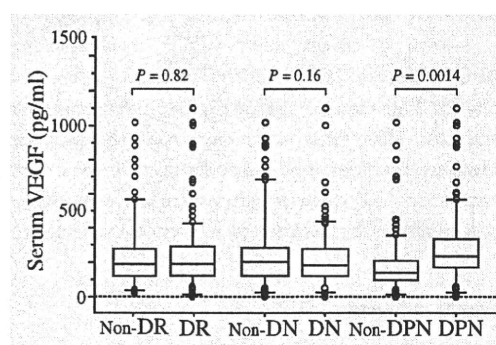
FIGURE 1 Serum vascular endothelial growth factor (VEGF) levels in patients with and without diabetic retinopathy (DR), diabetic nephropathy (DN) and overt proteinuria (DPN).