表2 パーキンソン病患者におけるRLSの有病率

報告者(発表年)	調査地	有病率(%)
Ondo et al(2002)[2]	アメリカ	20.8
Krishnan et al(2003)[3]	インド	7.9
Tan et al(2002)[7]	シンガポール	0
Nomura et al(2006)[4]	日本	12
Gomez-Esteban et al(2007)[5]	スペイン	21.9
Lee et al(2009)[6]	韓国	16.3
Calzetti et al(2009)[8]	イタリア	12.7

表3 腎不全患者によるRLSの有病率

報告者(発表年)	調査地	有病率(%)
Winkelman et al(1996)[10]	アメリカ	20
Takaki et al(2003)[11]	日本	20
Mucsi et al(2005)[12]	ハンガリー	14
Kawauchi et al(2006)[13]	日本	23

少ない周期性四肢運動(periodic leg movements in sleep：PLMS)，治療の反応性の悪さを特徴として確認した[9]．

このように，ドパミン機能障害としてはRLSとPDには類似性があるが，特発性RLSとPD合併RLSは異なった側面をもっている．

腎障害とRLS

RLSの有病率について，慢性腎不全，特に血液透析を導入した患者においては，最近14～23%とする報告がある(表3)[10-13]．また人種差はないと思われていたが，アフリカ系アメリカ人の方が白人よりRLSの頻度が少なかったという報告がある[14]．この点に関しては，さらなる大規模な検討が必要である．

慢性腎不全透析患者におけるRLS発症機序については，透析患者がRLSを発症する時期は血液透析の開始直後が最も多く，透析患者の腎移植によりRLS症状が消失し，腎不全の再増悪につれて再び出現したとの報告や，透析時間の延長や血液透析の導入によりRLS症状が改善する症例もあることなどが報告されている．透析の開始自体がRLSの発症に関与するレベルまで慢性腎不全が進行していることを反映しており，尿毒症性の要因がRLSの発症に関連するとの考えが妥当であるが，鉄欠乏や貧血，遺伝，生活様式の要因も関連が考えられる[15]．

さらに，慢性腎不全に至る背景疾患の関与も考慮される．特に腎不全の原因として糖尿病が最も多いことから，糖尿病性ニューロパチーや尿毒症性ニューロパチーの合併もあり得る．したがって，これらのニューロパチーがRLSの発症に関与している可能性も考慮すべきである．

このように腎障害では，種々の原因でRLSが引き起こされている可能性がある．透析患者においてもRLSやPLMSがQOLを悪化させるため[16]，注意が必要である．

リウマチとRLS

RLS患者は下肢の痛みとして症状を訴えることがあり，リウマチ性疾患は鑑別診断として考慮する必要がある一方，リウマチ性疾患でのRLSの報告も稀であるが存在する[17]．

関節リウマチ(rheumaoid arthritis：RA)の患者では，RLSが25%と高率にみられ，背景因子の検討では，変形性関節症患者と比較した結果から，RLS患者では血清フェリチンが低値で，ニューロパチーが高頻度にみられると報告されている[18]．

Gudbjornssonらは，シェーグレン症候群では24%にRLSがみられたのに対し，RAではわずか

表4　妊婦におけるRLSの有病率

報告者(発表年)	調査地	有病率(%)
Goodman et al(1988)[22]	イギリス	19
Suzuki et al(2003)[23]	日本	20
Manconi et al(2004)[24]	イタリア	26

に2%にみられたのみであったと報告している[19]．線維筋痛症でも患者の25%と高率にRLSがみられたと報告され[20]，強皮症の22%がRLSと報告されている[21]．

このような結果には，RLS診断基準作成以前のものも含まれており，診断精度が低い可能性がある．しかしながら，まだまだ他疾患と比べても検討は少なく，RLSの認知を広め，さらなる検討が必要である．

妊娠とRLS

一般的に妊婦では19～26%のRLS有病率の報告がある(表4)[22-24]．日本での検討でもRLSは20%であったと報告されている[23]．また妊娠前0%であったRLSが妊娠後期には23%まで上昇した報告もあり[25]，妊娠とともにRLSが出現する例が多く，出産6カ月後には6%まで軽快した報告もある[24]．このことより，女性では妊娠中にRLSを発症することが多いといえる．初回の妊娠でRLSを発症し出産後にいったん症状が消失しても，妊娠を繰り返すたびに症状が徐々に増悪し，慢性化する症例もあるため，注意が必要である．

妊婦では鉄欠乏を生じやすい状態にあることから，RLSへの鉄欠乏の関与が指摘されているが，妊婦では血清フェリチン値は低下するもののRLS発現との関連が乏しく，むしろ血清葉酸の低下が妊娠中のRLS発症と関連するとの報告もあり[26]，一定の見解は得られていない．

このように，妊娠はRLSのリスク因子として挙げられる．

鉄欠乏とRLS

貧血や鉄欠乏もRLSとの関係が古くから疑われている．鉄欠乏症は高齢患者におけるRLS発症の重要な寄与因子で，鉄サプリメントにより症状が軽減する可能性が示唆されている[27]．RLSの病態における鉄の役割については，中枢神経における鉄の動態が関与していると考えられている．頭部MRIにて線条体や赤核の貯蔵鉄減少の報告や[28]，RLS患者での脳脊髄液中のフェリチン濃度の低下の報告がある[29]．鉄はドパミン合成過程における律速酵素であるチロシン水酸化酵素の補因子として必要であるとともに，鉄がドパミンD_2レセプターの構成要素であることなどより[30]，中枢の鉄欠乏がドパミン系の障害を引き起こしている可能性が考えられる．

これらの病態では，血清フェリチンが50 μg/L以下のときに鉄の投与で軽快する例が多く，RLS患者には精査が必要である．

ミエロパチーとRLS

ミエロパチーによるRLSとしては，外傷による脊髄損傷，脊髄空洞症，炎症性疾患，腫瘍性疾患などによる一過性，あるいは持続性の症状発現の報告がある．RLSの機序として，脊髄に入力しているA11ドパミン神経の関与も考慮されているため(図1)[31]，ミエロパチーでのRLS出現は注意すべきであり，RLSの主な病態機序に関わっている可能性があり得る．

ニューロパチーとRLS

ニューロパチーとRLSの関連についても，一定の見解は得られていない．糖尿病で17.7～27%とRLSの報告があり[32,33]，ポリニューロパチーが増悪因子として挙げられている[34]．

両者の鑑別が困難な場合には，電気生理学的評価が有用であり，ニューロパチーの程度や原因を詳細に把握することが重要である．

SCAとRLS

遺伝性脊髄小脳変性症(SCA)のCAGリピート

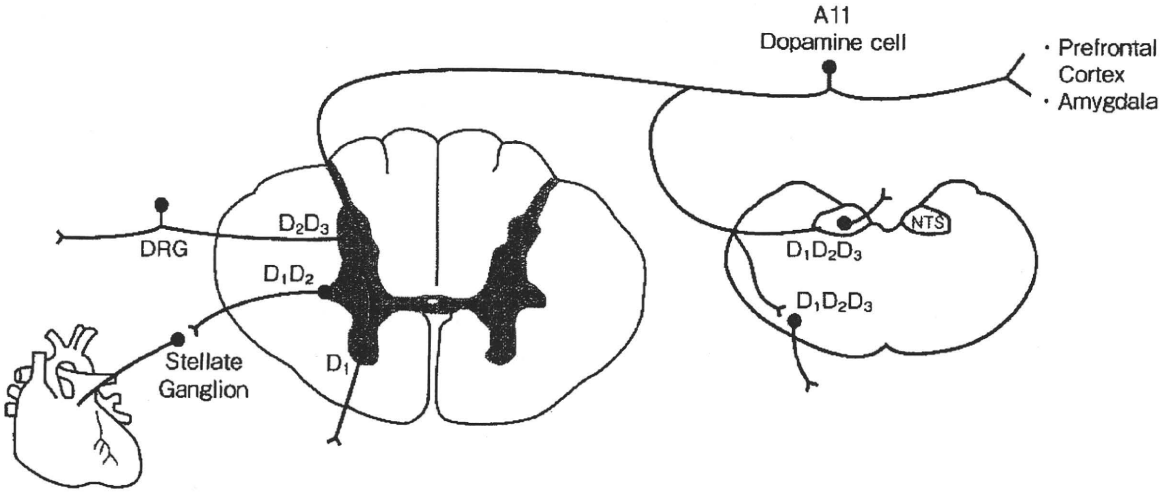

図1　A11ドパミンサーキット[31]

表5　脊髄小脳変性症(SCA)患者におけるRLSの有病率

報告者(発表年)	SCA 1 (%)	SCA 2 (%)	SCA 3 (%)	SCA 6 (%)
Schols et al(1998)[35]	0	18	45	5
Abele et al(2001)[36]	23	27	30	NA
Iranzo et al(2003)[37]	NA	NA	50.5	NA
Boesch et al(2006)[38]	NA	NA	NA	40
Boesch et al(2006)[39]	NA	0	NA	NA
Reimold et al(2006)[40]	25	25	100	NA

SCA：spinocerebellar ataxia，NA：データなし　　Iranzo et al，2007[41]

は脳内の鉄イオンチャンネル機能障害に関連がある．そのためRLSとの関連も示唆されるが，SCAでの調査も小規模なものしかない．その中でSCA 3が最も高頻度であり，30～100%と報告されている(表5)[35-40]．SCAではRLS症状は軽度であり，L-ドーパ治療への反応も良好で，PLMSが高頻度出現することが特徴である．しかし，CAGリピート数や発症年齢，電気生理検査での異常とは関連がなかった．SCAでのRLS出現の病態は明らかでないが，中枢性ドパミン機能障害が疑われる[41]．

その他の神経疾患とRLS

多発性硬化症(multiple sclerosis：MS)では，19%にRLSの合併の報告がある．高齢，長いMSの病歴，原発性進行性タイプ，錐体路徴候などが危険因子として挙げられるが，神経の炎症性損傷が原因と考えられる[42]．またハンチントン病の1家系でのRLSの報告があり[43]，最近RLS症状の4年後にハンチントン病と診断した症例の報告がある[44]．少数の報告であるが，ハンチントン病の早期症状として注意する必要があるのかもしれない．さらに，遺伝性痙性対麻痺の20.5%でRLSの報告もある[45]．このように，神経疾患においてもRLSの合併は多く，今後他疾患での報告の可能性もある．

その他の内科疾患とRLS

神経疾患と同様に，いくつかの内科疾患でのRLS合併の報告がある．クローン病の42.7%[46]，慢性肝疾患の62%[47]，慢性閉塞性肺疾患の29.1%[48]，サルコイドーシスの52%にRLSを認めたとの報告もある[49]．さらに原発性副甲状腺機能亢進症での症例報告もあり[50]，RLSの認知が広がるとともにその他の疾患での報告も増える可能性がある．

おわりに

RLSは頻度の高い病気であることがわかり，病気の認識も広がっている．二次性のRLSには多種の疾患があるが，特発性RLSとの病態が関連している病気も含まれているため，今後も合併疾患との検討が必要であるとともに，RLS症状に注意して診療を行う必要がある．

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多系統萎縮症における睡眠障害—レム睡眠行動障害を含めて*

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Key Words: excessive daytime sleepiness, sleep apnea syndrome, REM sleep behavior disorder

はじめに

多系統萎縮症（multiple system atrophy：MSA）はパーキンソニズム，自律神経障害，小脳症状，錐体路症状等の臨床症状を呈し，レボドパの反応性が乏しく，（黒質線条体，オリーブ橋小脳，自律神経系）等の神経細胞の消失，グリオーシス，嗜銀性細胞封入体の病理変化を特徴とする孤発性の神経変性疾患である．細胞レベルの病因の解明により現在MSAはParkinson病（PD）やLewy小体型認知症と同様にシヌクレイノパチーと分類されている[1]．MSAではしばしば睡眠障害の合併がみられる．PDの51%が睡眠障害を訴えるのに対してMSAでは70%が睡眠障害を訴えている．睡眠分断の訴えが最も多く，53%の患者が睡眠分断を訴えており，早朝覚醒（33%），不眠（20%）と続く[2]．主観的な訴えは客観的な終夜脳波（polysomnography：PSG）で睡眠時間の減少，睡眠効率の低下，レム睡眠や徐波睡眠の減少として確認されている[3~6]．原因としてはLvodopaの低反応による無動症状，疼痛，夜間排尿，周期性四肢運動が睡眠の分断を引き起こし，PSGでの異常として捉えられる[5,7,8]．MSAに特徴的な睡眠障害としては，睡眠関連呼吸疾患とレム睡眠行動障害（REM sleep behavior disorder：RBD）がある．これらは頻度も高く，MSAの一症状と考えられている[1]．

Ⅰ．睡眠関連呼吸疾患

睡眠関連呼吸疾患はMSAに頻度が高く，臨床的にも重大な問題となる[2,5,9~11]．睡眠時呼吸障害は多彩な病像を呈しており，閉塞性無呼吸障害，中枢性無呼吸，脳幹障害による呼吸リズム異常，中枢神経原性の肺胞低換気，低酸素血症における化学受容体の障害[12]などのほか，後輪状披裂筋の神経原性変化による声帯外転麻痺（Gerhardt症候群），および球麻痺や気道感染に基づく呼吸障害であり，これらはしばしば突然死の原因にもなり，生命予後にもかかわる重要な徴候である．夜間の喘鳴や閉塞性無呼吸（objective sleep apnea：OSA）は最も共通の症状であり[4,5]，病態が異なっていてもしばしば同時に起こっている[13]．臨床検査やオーディオモニター付きのPSGで喘鳴は簡単に確認でき，グーグーという低音のいびきのほか，ヒーヒーという高音で“ロバのいななき”と形容される[2,7,11]．喉頭鏡検査で喉頭の狭窄は評価できるが，覚醒時の検査では感度が低く，夜間喘鳴のある患者の声帯運動は正常であったと報告されている[10,11]．そのため，睡眠時を含めた声帯喉頭機能の積極的な評価が必要である．声帯外転麻痺の重症度の評価および治療方針の決定に

* Sleep Disturbances in Multiple System Atrophy Including REM Sleep Behavior Disorder.

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Table 1 Stage and therapy on abductor dysfunction of cord in multiple system atrophy

Stage of abductor paralysis of cord	Movement of cord		Severity of paralysis	Therapy
	Awake	Sleep		
0	normal	No change	normal	observe
1	normal	Paradoxical movement	mild	Nasal CPAP Tracheotomy
2	Limit of abductor	Paradoxical movement	moderate	Tracheotomy
3	Persistent of midline	Persistent of midline	severe	Tracheotomy

は磯崎の分類[14]が有用である．宮本らが示した治療方針をしめす（**Table 1**）[15]．また，この睡眠中の上気道の閉塞の評価には，食道内圧のモニタリングが最も適している[10]．

MSA患者の喘鳴，OSAの正確な頻度は報告によりばらつきがある．多数例の検討では喘鳴はMSAの13～69%[5,18]，OSAは15～37%[4,5]と報告されている．MSA-PよりMSA-Cに喘鳴の頻度が高いとの報告[5]があるが，反対の報告もある[8]．52人に行った検討ではインタビューで19%に夜間の喘鳴があり[2]，PSGで39人に認めていた[4]．MSAの高頻度のOSAは無呼吸による睡眠分断に起因していた[5]．無動症状の重症度を考慮しても重症のMSAが臥位で寝ることが高値のapnea hypopnea index（AHI）に関連していると考えられる．OSA患者の夜間の閉塞イベントは側臥位にすることで改善するとも報告されている[7]．MSAの睡眠呼吸障害の特徴としてMSAでは病気の進行とともにAaDO2開大を伴う低酸素血症が出現し増悪すると共に，声帯のみならずさまざまな部位に気道閉塞が生じていること，無呼吸の指標として頻用されるAHIは罹病期間と相関せず重症度の指標として必ずしも有用でないこと等が示されている[17]．

MSAの睡眠関連呼吸疾患の病因としてもPDと同様に上気道筋の夜間の無動症状である可能性もある[18]．さらに，声帯外転筋の萎縮や麻痺を引き起こす疑核の変性はMSAの吸気性喘鳴を起こすと考えられる[19]．しかし，最近の病理検討でもこれらの所見は確定できていない[20]．また，少数例の筋電図検討（electromyogram：EMG）では，ジストニアと同様に吸気時の声帯内転筋の持続する筋緊張を示し，喉頭の狭窄，吸気流の制限を引き起こしていることが示唆された[21～23]．しかし，大多数のEMGでは麻痺なのか，ジストニアなのかは明らかになっていない．また，喘鳴の存在は死亡率と関連があり，喉頭の閉塞の結果と予想できる突然死のリスクがあげられている[11]．MSAでは，低酸素に対する呼吸応答の障害[12]や睡眠中の呼吸機能を制御するセロトニンやコリン神経の枯渇[24]が呼吸制御の機能障害を起こし，呼吸症状が出現し，喘鳴や無呼吸の増悪をおこしている可能性がある．橋脚被蓋核（pedunclopontine nucleus：PPN）や外背側被蓋核からのコリン神経の変性が視床のコリン神経の欠乏を起こしMSAでの重度なOSAとなっているという報告がある[25]．動物実験の結果の結果もPPNの呼吸パターンの影響が指摘されている[26]．しかし，明確な機序は確立していない．

喘鳴に対する治療としては，気管切開や声門開大術のような侵襲的な治療が現実である．少数例にはボツリヌス毒素が有効であったと報告されているが[23]，日常的な適応には至っていない．現在，喘鳴や無呼吸に対しての非侵襲的な治療としては，continuous positive airway pressure（CPAP）だけが有効な治療である[6,7]．しかし，気管切開術やCPAPでも突然死を完全には防げず，上気道閉塞以外のメカニズムでも突然死が生じることが示されている[27]．さらに，MSAでは睡眠中に中枢性呼吸調節異常が生じ，その結果低酸素血症に伴

う重症不整脈や低酸素脳症，呼吸停止が生じる可能性があることが考えられている[28]．このように，運動症状の重症度が長期間のCPAP治療の制限因子となっているのが現実である[6]．しかし，CPAP療法を行うにあたり治療の効果を十分に発揮しかつ突然死の予防のためにも，CPAPマスクの確実な装着とコンプライアンスの状況に十分な注意が必要である[15]．

II. レム期睡眠行動障害

レム期睡眠行動障害（REM sleep behavior disorder：RBD）はレム睡眠随伴症状であり，MSAで多く認められる睡眠関連症状である．2005年に作成された睡眠障害国際診断分類（international classification of sleep disorders：ICSD）第二版の診断基準[29]において，夢内容の行動化により怪我をしたり，怪我をしてもおかしくないような睡眠中の行動化の病歴があるか，PSG実施中にREM睡眠期に異常な行動化があるかの少なくともどちらかの事象があると共に，PSG上REM sleep without atonia（RWA）の存在が確認されることがRBD診断の必須項目となっている．RBDの約60%は特発性であるが，RBDはシヌクレイノパチーにしばしば関連がある[30]．MSAでは90～100%がPSG確定のRBDである[4,5,31]．特発性RBDの38%までがParkinson関連疾患に発展していた[32]．このように，RBDはパーキンソニズムに数年先行する場合がある[33]．それゆえに，RBDはシヌクレイノパチー等の神経変性疾患の早期症状と考えられている．我々のMSA16人の検討では，11例（68.8%）にRWAを認め，7人が発症前にRBD症状を認めており，発症後にはRWAの出現量は増えるのに対してRBD症状は消失していた．この傾向はTachibanaらにより一症例のMSAで報告されている[34]．この変化は広範な神経変性によるものと考えられる．

現在においてもRBDの病態生理は明らかでない．BoeveらはREM睡眠を促進するREM on（下外側背側核，前青斑核）とREM睡眠を抑制するREM off（中脳水道周辺腹外側灰白質，外側橋被蓋）が相互に干渉してREM睡眠の制御を行い，REM睡眠時には下外側背側核より直接，間接（延髄網様体を介して）的に脊髄前角細胞に抑制を行っているが，下外側背側核の障害により情動系からの出力への抑制が弱くなり，RWAの出現，夢内容の行動化が起こると仮説を立てている（**Fig. 1**）[35]．MSAでは中脳橋のコリンREM on細胞の消失，青斑核ノルアドレナリン細胞の消失，縫線核セロトニン細胞の保持等REM睡眠制御細胞の障害がREM異常を起こしている[36]．このコリンの病態は特発性RBDでのアセチルコリンエステラーゼ阻害薬のdonepezilの効果でも考えられる[37]．また，RBDがドパミン欠乏疾患の一部と考える研究者もいる．機能画像では特発性RBDで黒質線条体のドパミン投射の減少を示している[38,39]．MSA患者でもRBDの重症度と線条体のモノアミン神経の消失とが関連していた[25]．ドパミンの関連は特発性RBDの病理例で黒質，青斑核の神経細胞消失でも見られる[40]．さらに，PDでもRBDはlevodopaの治療後に改善したとの報告もある[41]．黒質線条体投射はPPNへの下方連結によってRBDに関連し，REM制御に重要な役割を示す[42]．MSAでは基底核の機能障害やPPN自体の機能障害，PDと同様の病態である基底核に関連する他の脳幹機能障害があり，RBDの原因となっているのかもしれない[42,43]．動物実験より黒質網様体からPPNへのGABA投射はREM atoniaの制御をしていると考えられている[44]．それゆえに，Parkinson関連疾患で基底核からPPNへのGABAの過剰出力がRBDを引き起こしている可能性はある．しかしながら，MSAでのRBD出現の機序も明確にはなっていない．

RBD治療としては，二重盲検試験は行われていず，RBD関連脱同調を起こすmelatoninの有効性も確立していない[30]．clonazepamはbenzodiazepineがRBDを改善する正確な機序が明らかでなく，OSAを増悪する可能性があるが，治療として選択される[45]．MSAでのアセチルコリンエステラーゼ阻害薬の有効性は不明であるが，検討する価値はあると思われる[30,37]．さらに，ドパミンアゴニストであるpramipexoleで特発性RBDが改善した報告もある[46]が，PDに合併したRBDには効果がなかったとの報告もある[47]．このようにMSAのRBDに対する治療の報告はないが，特発性RBDでの報告より考えると，

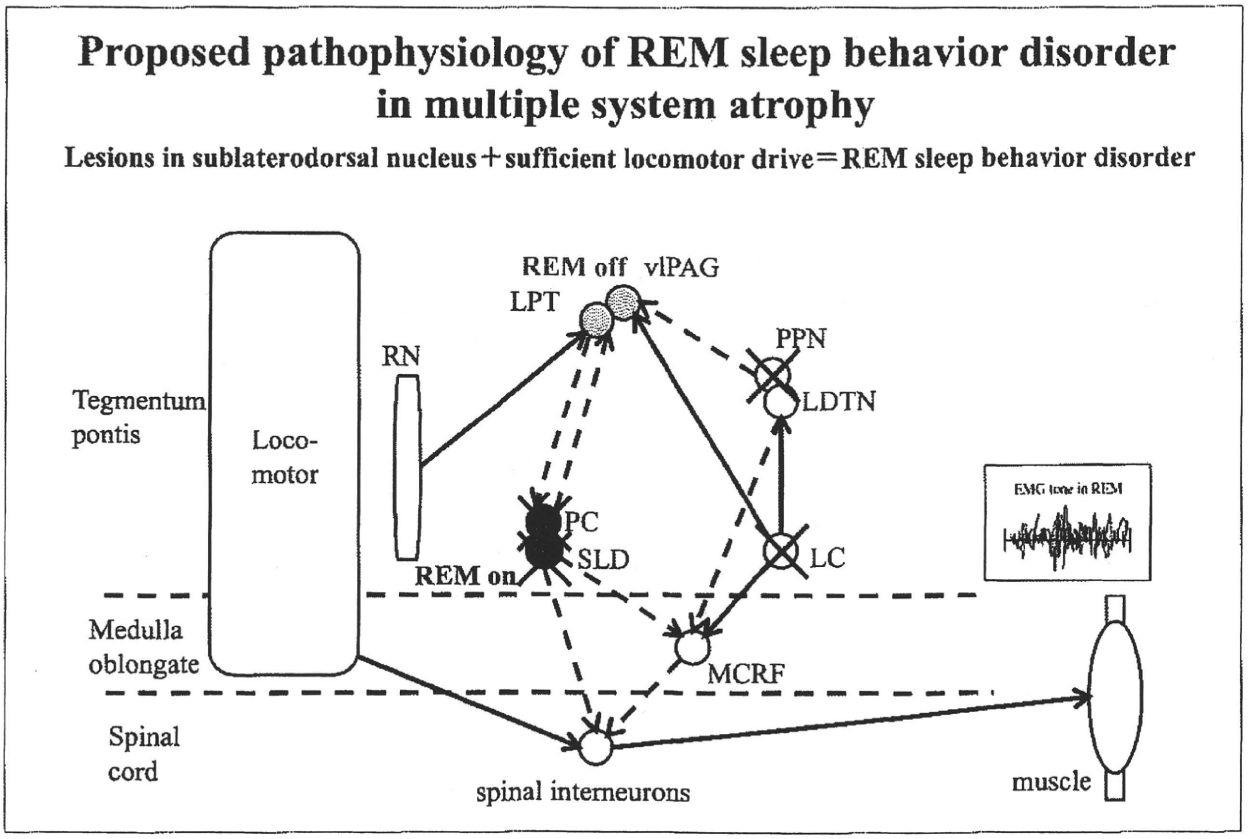

Fig. 1 pathophysiology of REM sleep behaviour disorder in multiple system atrophy.
Excitatory projections represented by black lines, inhibitory projections represented by broken lines. A cross sign reflects ablation of a nucleus in multiple system atrophy. The REM-off region is represented by the vlPAG and LPT in grey, and the REM-on region is represented by the PC and SLD in black. The directly input from SLD to spinal interneurons and the indirect input from SLD through the MCRF to the spinal interneurons, may also contribute to EMG atonia.
EMG = electromyographic, eVLPO = extended part of the ventrolateral preoptic nucleus, LC = locus coeruleus, LDTN = laterodorsal tegmental nucleus, LPT = lateral pontine tegmentum, MCRF = magnocellular reticular formation, PC = pre-coeruleus, PPN = pedunculopontine nucleus, RN = raphe nucleus, SLD = sublaterodorsal nucleus, vlPAG = ventrolateral part of the periaqueductal grey matter.

まずOSAの増悪に注意しながらclonazepamの使用が薦められる．OSAの増悪がある場合にはdonepezil，plamipexoleを考慮してもよいであろう．

III. 日中眠気

MSAでは日中の眠気（Excessive daytime sleepiness：EDS）は一つの有病率の報告しかない．この報告ではPDの30%がEDSを認めるのに対してMSAは半分であった[2]．病気の重症度が主な危険因子であるが，MSAで睡眠疾患の是正がEDSを軽快するかはまだ調査されていない．そのため，MSAのEDSに対する対応は明確にされていない．PDの眠気に関しては，長期の罹病期間，重症例，男性，自律神経障害を有すること，知的障害が存在すること，大量のドパミン作動薬を使用していることが誘発因子と考えられるので，重症のMSA患者においてはドパミン使用を考慮する必要があるかもしれない．

結論

MSAでは，神経変性の経過が生命にかかわる睡眠疾患を高頻度に起こす．臨床，動物実験よりこれらの疾患の病因を解明することがMSAの病

因にも関わり，睡眠制御と基底核の機能障害の複雑な関連を解明するかもしれない．睡眠疾患の認識の向上と管理がこれらのMSA患者のquality of life（QOL）を改善するのに重要である．神経変性疾患の睡眠障害を評価する手法がPSGのスクリーニングとして有用である[48,49]．これらを使用の上高音の鼾に注意することがまず大事である．治療に関しては現状はMSAに特異的な加療というより一般的な睡眠関連呼吸障害，RBDに従って加療することとなる．今後はMSAに特徴的な治療方針を作成する必要がある．

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Sleep Disturbances in Multiple System Atrophy Including REM Sleep Behavior Disorder

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In multiple system atrophy (MSA), sleep disturbances and daytime sleepiness are common complaints. Neurodegeneration of areas that regulate sleep-wake control and respiratory function in MSA may lead to sleep-related respiratory dysfunction and REM sleep behavior disorders (RBD). Sleep-related respiratory dysfunction includes stridor and sleep apnea syndrome. Stridor is an inspiration-associated sound that generates a high-pitched frequency as a result of vocal cord dysfunction. Laryngoscopy can detect and diagnose stridor symptoms. The severity of vocal cord dysfunction is categorized in stages, and therapy is based on the extent of abnormal vocal cord abduction/adduction. Nasal continuous positive airway pressure (CPAP) and tracheotomy are often used as therapy for these symptoms. In comparison, sleep apnea syndrome correlates with morbidity in patients with MSA, and this syndrome is usually cured by nasal CPAP.

RBD is characterized as a dysfunction of REM sleep-associated muscle atonia and was recognized in most MSA patients. Patients with MSA might exhibit a dysfunction of the pedunculopontine nucleus, pre-coeruleus, locus coeruleus, and/or sublaterodorsal nucleus, all of which regulate REM sleep. Although clonazepam, dopamine agonists, and acetylcholine esterase inhibitors are used to treat idiopathic RBD, the efficacy of these agents for RBD therapy in MSA patients is not confirmed.

Neither disorder is commonly associated with MSA pathophysiology. We recommend treating patients with MSA similar to patients with sleep disturbances after diagnosing specific sleep problems in patients with MSA.

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SHORT COMMUNICATION

A Japanese case of ichthyosis follicularis with atrichia and photophobia syndrome with an *MBTPS2* mutation

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Ichthyosis follicularis with atrichia and photophobia (IFAP) syndrome is a rare genetic disorder characterized by the triad of ichthyosis follicularis, alopecia and photophobia. Previous studies have identified five missense mutations in the membrane-bound transcription factor protease, site 2 (*MBTPS2*) gene in European patients with this syndrome. In this study, we detected the 1286G>A (Arg429His) mutation in *MBTPS2* in a Japanese patient with IFAP syndrome. This mutation has previously been detected in a German family with this syndrome. Functional analysis revealed that this mutation was the most severe mutation identified to date for this syndrome. None of the male German patients had been tested for the mutation because they had several visceral and bone anomalies, and had died as neonates or infants. The clinical features of our 5-year-old patient are less severe than those of the German patients. Although he has neurological abnormalities, such as retarded psychomotor development and seizures, he does not have bone or visceral anomalies, except cryptorchidism. This case indicates the existence of other factor(s) that influence the clinical features of this syndrome. Further clinical and genetic studies are required to clarify the relationship between phenotypes and genotypes and to identify such modifying factors.
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Keywords: genotype–phenotype correlation; IFAP syndrome; Japanese; *MBTPS2*; mutation

INTRODUCTION

Ichthyosis follicularis with atrichia and photophobia (IFAP) syndrome (MIM 308205) is a rare congenital disorder characterized by generalized ichthyotic skin changes with follicular hyperkeratosis, congenital hairlessness and photophobia, as well as additional clinical findings.[1] X-linked recessive transmission has been suggested for this syndrome because most patients are male,[2] and the full phenotype is found only in males.[3,4] Oeffner *et al.*[5] performed a linkage analysis using two families of European descent, in which IFAP segregated according to an X-linked pattern of transmission. They identified five missense mutations in the membrane-bound transcription factor protease, site 2 gene (*MBTPS2*; MIM 300294) encoding a membrane-embedded zinc metalloprotease that activates signaling proteins involved in the endoplasmic reticulum stress response and in the sterol control of transcription.[5] In this study, we report the case of a Japanese patient with the IFAP triad, short stature, mental retardation and seizures. The *MBTPS2* Arg429His mutation, which was previously identified by Oeffner *et al.*[5] in male patients most severely affected by this syndrome, was detected in this patient.

CASE REPORT

The patient was a 2-year-old male child who was born to healthy non-consanguineous parents and was referred to our institution for seizures and severe mental and growth retardation. Ultrasonography performed at 21 weeks of gestation revealed fetal intrauterine growth retardation, and delivery occurred at 37 weeks. The birth weight of the patient was 2167 gm and height was 51.5 cm; he lacked scalp hair, eyebrows and eyelashes, and exhibited generalized ichtyosis. At 3 months of age, his serum total immunoglobulin E level was 4945 IU ml^{-1}, and his serum specific immunoglobulin E levels to albumen and milk were elevated. The patient also had bilateral cryptorchidism, which required surgery. Photophobia became apparent during the first year of life. By 1 year of age, he experienced a brief generalized tonic–clonic seizure with high fever. Later myoclonic seizures appeared without fever.

On admission, his weight, height and head circumference were all below the third percentile, and bone age was below chronologic age. Physical examination revealed that there was no visceromegaly, and cardiovascular examination yielded normal results. The patient did not

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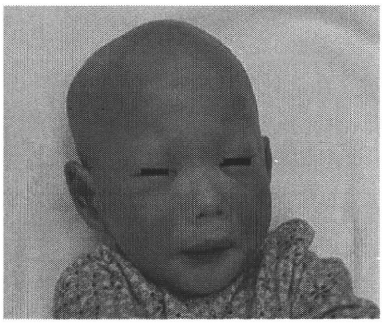

Figure 1 Photograph of the patient, showing alopecia and absence of eyebrows and eyelashes.

have scalp hair, eyebrows and eyelashes (Figure 1). He exhibited generalized skin dryness, which led to severe itching; eczematous changes in the arms and legs; and thickened nails. He had normal teeth. Detailed pathological analysis of the skin biopsy has been reported previously.[6] Ophthalmological evaluation revealed severe photophobia and bilateral corneal vascularization. Neurological examination revealed that the patient was alert but mentally retarded. The patient could smile and visually follow faces but could not talk. He was able to sit unassisted but could not walk. His cranial nerves were unaffected; although a slight decrease in muscle tone was observed, the muscle stretch reflexes were normal. Normal plantar responses and withdrawal to painful stimulation were observed. The results for routine hematological screening tests; liver and thyroid function profiles; plasma amino acid levels; urinalysis; visual- and brainstem auditory-evoked potentials; electrocardiography; and chest, skull and spine radiography were normal or negative. Magnetic resonance imaging revealed enlargement of the cisterna magna and irregular deficiency in the medial occipital lobe seems as schizencephaly, and irregular distortion on the anterior horn of the lateral ventricle, which expanded in the lateral superior direction. Electroencephalography revealed multifocal localization spike on the right central, right parietal and left occipital regions. Spikes were observed on the right parietal region even after antiepileptic therapy. The parents and the younger brother of the patient did not exhibit any of these clinical features.

Currently (age, 5 years 4 months), his weight, height and head circumference are 10.4 kg (<third centile), 88.1 cm (<third centile) and 44.5 cm (<third centile), respectively. He can walk using a walk aid and speak a few words. The seizures were effectively controlled using valproic acid, diazepam and zonisamide. He develops urticaria on exposure to peanuts and tree nuts (that is, hazelnuts and walnuts).

Molecular and cytogenetic studies

Blood samples were collected from the patient and his family after having obtained written and informed consent from unaffected family members according to a protocol reviewed and approved by the ethical committee of the University of Tsukuba. Chromosomal analysis revealed that the patient had a normal karyotype, 46, XY. Sequence analysis of *MBTPS2* was performed according to a previously reported method.[5] We identified a missense mutation, c.1286G>A, that caused a Arg429His substitution in the patient. This mutation was found in the proband and his unaffected mother but not in his unaffected brother (Figure 2).

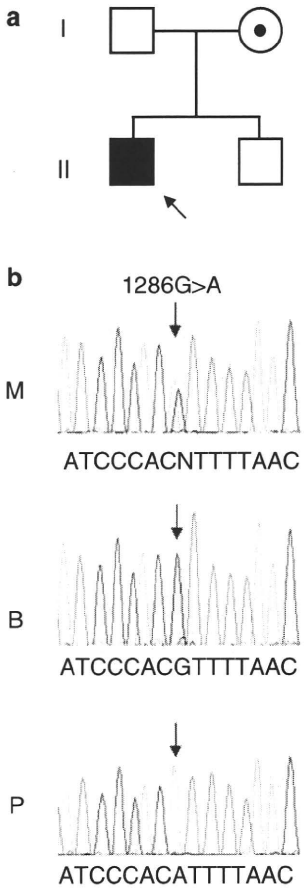

Figure 2 Family pedigree and mutation analysis. (**a**) Pedigree of the family studied. The circle indicates female individuals, and squares indicate male individuals. The filled symbol denotes the affected individual, and the dot with symbol denotes a carrier individual. An arrow indicates the proband. (**b**) Sequence analysis of *MBTPS2*. 1286G>A (Arg429His) mutation was identified in the proband (P) and his mother (M) but not in his brother (B).

DISCUSSION

We detected a Arg429His mutation in *MBTPS2* in a Japanese patient with IFAP syndrome. This is the first case, in which an *MBTPS2* mutation has been identified in a Japanese patient with this syndrome.

The IFAP syndrome was first identified by MacLeod *et al.*[1] in three brothers in 1909. It is a rare X-linked genetic disorder, and <30 cases have been reported. Such patients have a unique appearance because of the alopecia, photophobia and generalized follicular hyperkeratosis. Inconsistent findings include neurological abnormalities, such as retarded psychomotor development, cerebral atrophy, temporal lobe malformation, hypoplasia of the corpus callosum and seizures; failure to thrive; nail dystrophy; atopic manifestations; inguinal hernia; aganglionic megacolon; as well as renal, vertebral and testicular anomalies.[7–12]

Oeffner *et al.*[5] reported that IFAP syndrome is caused by functional deficiency of membrane-bound transcription factor protease, site 2, an intramembrane zinc metalloprotease that is essential for cholesterol homeostasis and the ER stress response.[13–15] They performed a linkage analysis involving two families of European descent, in which IFAP segregated according to an X-linked pattern of transmission, and assigned the *IFAP* locus to the 5.4-Mb region between DXS989 and DXS8019 on Xp22.11–p22.13. They identified five missense mutations

Table 1 Comparison of the main findings of the literature ichthyosis follicularis with atrichia and photophobia cases with Arg429His mutation in MBTPS2 and the present case

	Oeffner et al.[5]		
	3-III:3	*3-III:4*	*Present case*
Ichthyosis	+	+	+
Alopecia	+	+	+
Photophobia	+	+	+
Short statue	−	+	+
Microcephaly	−	+	+
Mental and/or motor retardation	+	+	+
Atopic manifestations	+	−	+
Recurrent respiratory infections	−	+	−
Seizures	−	+	+
Brain abnormalities	−	+	+
Vertebral anomalies	+	+	−
Limb anomalies	+	+	−
Heart malformation	−	+	−
Renal anomalies	+	+	−
Hirschsprung disease	+	+	−
Inguinal hernia	+	+	−
Cryptorchidism	−	−	+
Death in infantile period	+	+	−
Other malformations	Cleft palate; omphalocele	Hydromyelia; choanal stenosis	−

exchanging highly conserved amino acids in *MBTPS2* at Xp22.11 in five unrelated patients of European descent.

Our patient has a missense mutation, 1286G>A, leading to an Arg429His substitution in *MBTPS2*. The same mutation has previously been reported in a German family with IFAP syndrome.[5] The manifestations of two male patients from this family have been compared with those of our patient in Table 1. In this family, three female patients with skin manifestations (that is, dry skin, and atrophoderma with linear lesions) and two unaffected female patients carried this mutation heterozygously. This family included four male patients; however, they had not been tested because they had several visceral and bone anomalies and had died within 2 years after birth. One male patient was a collodion baby who also had motor retardation, a cleft palate, unilateral cleft hand, two butterfly vertebrae, bilateral inguinal hernia, omphalocele, stenosis of the small intestine and Hirschsprung disease; he lacked one kidney. Another male patient had microcephaly, an arachnoid cyst, Arnold–Chiari malformation type I, thoracolumbar hydromyelia, seizures, psychomotor retardation, retrognathia, deficient growth, cleft hands, a butterfly vertebra, a wedge-shaped vertebra, an atrial septal defect, arterial hypertension, recurrent infection of the upper airways, hypospadias, choanal stenosis, inguinal hernia and Hirschsprung disease; he lacked one kidney.

Oeffner *et al.*[5] suggested that missense mutations in *MBTPS2* are responsible for the IFAP phenotype and that the degree of clinical severity correlates with the reduction in activity.They tested the effect of the five *MBTPS2* missense mutations detected in IFAP syndrome patients on the potential to complement S2P deficiency in Chinese hamster CHO-K1-M19 cells and on the stimulation of sterol-responsive elements luciferase reporter. M19 cells in which the orthologous *Mbtps2* is deleted cannot grow in cholesterol and lipid-deficient culture media.[13] Wild-type *MBTPS2* stably transfected into the M19 cells complemented this defect and restored their wild-type growth characteristics. None of the five mutants detected in IFAP patients retained this property to the same extent as did the wild-type gene; however, great variation was observed in residual activity. In mutant Arg429His, almost no survival was detected.[5] A luciferase reporter gene under transcriptional control of sterol-responsive elements was active in cells grown in sterol-deficient media on cotransfection with wild-type *MBTPS2*. However, sterol responsiveness of the cells transfected with the mutants was restored to a lesser extent than that in cells transfected with wild-type *MBTPS2* and also differed considerably among the mutants. The Arg429His mutation had the lowest residual activity.[5] Thus, the Arg429His mutation is considered as the most severe *MBTPS2* mutation till date. Our patient is now 5 years old, and his clinical features are much less severe than those of the German patients reported by Oeffner *et al.* Although he has neurological abnormalities, such as retarded psychomotor development and seizures, he does not have bone or visceral anomalies, except cryptorchidism.

Further clinical and genetic studies are required to clarify the relationship between phenotypes and genotypes and to identify any additional factor(s) that may have a role in the pathogenesis of IFAP spectrum disorders.

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SYNAPSE 65:103–108 (2011)

Functional Polymorphism in the *GPR55* Gene is Associated With Anorexia Nervosa

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KEY WORDS G coupled receptor; ERK phosphorylation; polymorphism; association

ABSTRACT Endocannabinoids, anandamide, and 2-arachidonoyl glycerol are involved in food intake and appetite. Although anandamide is now thought to be a ligand for vanilloid receptor, receptors that are targets of anandamide could play a similar role in eating behaviors and related disorders. This study therefore focused on the receptor, which is called G-protein-coupled receptor 55 (GPR55) that had recently been reported to have binding affinity for endocannabinoids. Functional analysis of the sole missense polymorphism, rs3749073 (Gly195Val) in the *GPR55* gene was performed by detecting the phosphorylation level of extracellular signal-regulated kinase (ERK) in Chinese-Hamster-Ovary (CHO) cells engineered to express human GPR55. Val195 type GPR55 appeared to induce less phosphorylated ERK than Gly195 type GPR55 when CHO cells were treated with anandamide and lysophosphatidylinositol (LPI). An association between the functional Gly195Val polymorphism and anorexia nervosa was tested in a female Japanese population comprising 235 patients and 1244 controls. The Val195 allele and homozygote of the Val195 allele were more abundant in the group of patients diagnosed with anorexia nervosa (P = 0.023, Odds ratio = 1.31 (95% Cl = 1.03–1.37), P = 0.0048, OR = 2.41 (95% Cl = 1.34–4.34), respectively). In conclusion, the low-functioning Val195 allele of *GPR55* appears to be a risk factor for anorexia nervosa. **Synapse 65:103–108, 2011.**

INTRODUCTION

Anorexia nervosa (AN) is characterized by maintenance of a low body weight, and an obsessive fear of weight gain. Familial and twin studies have indicated that genetic factors play a role in the development of eating disorders, including AN (Slof-Op 't Landt et al., 2005). Although the etiology of AN remains unknown, it is associated with other psychiatric disorders, such as depression, anxiety, and substance abuse (Berkman et al., 2007). It has been shown that in female proband relatives of AN sufferers there are significantly higher rates of anxiety disorders (14.6%) and unipolar major depression (8.3%), and in male proband relatives significantly higher rates of "schizo"-spectrum disorders (8.3%) and alcoholism (13.1%), compared with relatives of controls (Grigoroiu-Serbanescu et al., 2003). With regard to the relationship between AN and alcohol misuse disorders, an elevated rate of familial substance use disorders

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occur in patients with restricting AN (Logue et al., 1989; Molgaard et al., 1989). Another study also reported that a history of alcoholism in first-degree relatives is common in patients suffering from AN (Redgrave et al., 2007).

Such high comorbidity between AN and other disorders may be explained by shared genetic factors as well as common environmental factors. The relative risk for AN in proband family members of AN sufferers is 11.3 (Strober et al., 2000). A heritability was estimated of 0.56 (Bulik et al., 2006), and also moderate heritability was found using monozygotic and dizygotic twins, which thus indicate that genetic factors have a larger impact on AN than environmental factors (Mazzeo et al., 2009). Marijuana use in AN patients was found to occur at a rate of 5.9% (Herzog et al., 2006). Similarly, genetic factors appeared to influence the risk for AN and a comorbidity for AN and major depression, in an analysis of 2163 female twins (Wade et al., 2000). Furthermore, depression, anxiety disorders, and alcoholism show high rates of comorbidity with AN and occurrence in first-degree relatives of the disease (Iacovino, 2004; Redgrave et al., 2007).

It is well known that cannabinoids play an important role in appetite and food intake behaviors from newborn age, as endocannabinoids play a vital role in milk suckling behavior as contained in breast milk (Fride et al., 2003). Both exo- (e.g., THC) and endo-cannabinoids (e.g., anandamide), are reported to stimulate feeding behavior (Hao et al., 2000; Rodondi et al., 2006; Williams et al., 1998). Since anandamide activates cannabinoid receptors, which is the reason why anandamide is often called an endocannabinoid, a pharmacological effect of anandamide for food intake/appetite was thought to be mediated by cannabinoid CB1 receptor (Cooper, 2004; Kirkham, 2005). In fact, CB1 receptor antagonist Rimonabant reduces appetite (Wierzbicki, 2006), and an endocannabinoid system may be involved in a common mechanism underlying psychiatric disorders that have high rates of comorbidity with AN. While a regulation of energy homeostasis and feeding behavior in the central nervous system is complex, cannabinoid system may contribute to the regulatory pathways (Harrold and Williams, 2003).

On other hand, anandamide is now frequently referred to as an "endovanilloid," instead of "endocannabinoid," as it binds to vanilloid receptor as a full agonist (Toth et al., 2009). Blood level of anandamide was increased in AN patients but not in bulimia nervosa, while that of 2-AG was not changed (Monteleone et al., 2005). A negative correlation was also found between anandamide levels in blood and plasma leptin levels from female AN patients (Monteleone et al., 2005). Oleoylethanolamide, monounsatured analog of anandamide, decreases food intake and body weight gain through a cannabinoid receptor-independent mechanism (Gaetani et al., 2008). Food deprivation increases intestinal levels of anandamide (which acts in the gut as a "hunger signal"), while it decreases the levels of oleoylethanolamide (which acts in the gut as a "satiety signal") (Capasso and Izzo, 2008).

Interestingly, an unidentified receptor that has binding potential (BP) for a CB1 receptor ligand involved in appetite of pups (Fride et al., 2001) was reported. More recently, it was reported that anandamide also binds to an orphan G-protein-coupled receptor 55 (GPR55) (Baker et al., 2006; Ryberg et al., 2007), a Gq type receptor (Lauckner et al., 2008), although GPR55 appears primarily to be a receptor for endogenous phospholipid L-α-lysophosphatidylinositol (LPI) (Henstridge et al., 2008; Oka et al., 2007). However, a role for the receptor in any particular phenotype remains unknown because the neural circuits in the brain that are regulated by GPR55 remain unknown.

With regard to the genetic association study between endocannabinoid system and AN, no significant association of genetic variations in cannabinoid CB1 receptor (*CNR1*), nor in major endocannabinoid degrading enzymes, fatty acid amide hydrolase (*FAAH*), *N*-acylethanolamine-hydrolyzing acid amidase (*NAAA*) and monoglyceride lipase (*MGLL*) with AN have been found (Muller et al., 2008), while others had reported a weak association with *CNR1* (Aberle et al., 2007, 2008; Monteleone et al., 2009; Siegfried et al., 2004). In addition to those genetic findings, higher levels of *CNR1* mRNA in the blood of patients with AN than in those of controls was found (Frieling et al., 2009).

Although linkage studies have not shown linkage between AN and the locus of *GPR55* on chromosome 2q37 (Klump and Gobrogge, 2005), some vulnerability genes for AN could remain harbored because of the shared small effect on vulnerability to AN. Despite the fact that a genome wide association study with higher density markers could possibly identify such genes, an alternative method was used in this study to directly examine a functional polymorphism of *GPR55* and determine whether it affects susceptibility to AN. In this study, we investigated whether any functional alteration may be caused by this genetic polymorphism of *GPR55*, and an association study was performed between the polymorphism and AN in a population of Japanese women.

MATERIALS AND METHODS

Functional analysis of the Gly196val polymorphism of *GPR55*

We focused on rs3749073, as this is the sole nonsynonymous polymorphism in the *GPR55* gene (NCBI database, http://www.ncbi.nlm.nih.gov/SNP/index.html), and no *cis*- or *trans*-acting single nucleotide polymorphisms

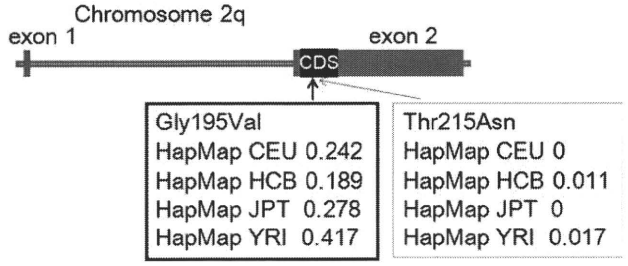

Fig. 1. Genetic structure of *GPR55* gene and its polymorphisms in CDS. The gene has two exons, and a part of second exon codes amino acids (shown in black (CDS)). There are one polymorphism (Gly195Val) and one rare variant (Thr215Asn) in the coding region of the gene. The minor allele frequencies in four populations, including Caucasian (CEU), Chinese (HCB), Japanese (JPT), and African (YRI), respectively. No *cis*- or *trans*-acting SNP was detected in lymphoblast cells (listed in the SNP Browser 1.01 database).

(SNP) resulting in gene expression change was detected in lymphoblast cells (listed in the SNP Browser 1.01 database (Dixon et al., 2007)) (Fig. 1). The rs3749073 polymorphism is located at amino acid position 777 of the mRNA, and alters translation from Gly, to Val in codon195.

GPR55 sequence coding for either Gly195 or Val195 was cloned into the expression vector pDEST26 (Invitrogen, Carlsbad, CA) for transfection into CHO cells. Briefly, full length mRNA of *GPR55* was amplified from lymphocyte cDNA by polymerase chain reaction (PCR), using primers: forward 5′-*GTAGGGATCCA CATGAGTCAGCAAAACACCAGTGGG;* and reverse 5′-*TGTCCTCGAGTTAGCCCCGGGAGATCGTGGTGT.* PCR amplification was carried out in a final volume of 10 μl containing 0.5 U KOD Plus polymerase (Toyobo Co, Tokyo, Japan), 1x KOD buffer, 2.5 mM $MgSO_4$, and 10 mM dNTPs, and 2.5 mM Betaine on a Gene Amp PCR system 9700 (Applied Biosystems, Foster City, CA). PCR conditions consisted of a denaturation at 95°C for 2 min, followed by 35 cycles of 95°C for 30 s, 65°C for 30 s, 72°C for 1 min, followed by a final extension at 72°C for 5 min. The PCR products were purified by QIAEXII Gel Extraction Kit (Qiagen, Valencia, CA), phosphorylated at both edges with T4 polynucleotide kinase (New England Biolabs, Berverly, MA), and inserted at an *Eco*RV site in pBluescript IISK$^+$ (Stratagene, LA Jolla, CA) in order to screen for sequences containing Gly195 or Val195 for cloning. Cloned *GPR55* sequences were then inserted into the pENTR11 vector (Invitrogen, Carlsbad, CA, USA) using the restriction enzymes *Bam*HI and *Xho*I. The *GPR55* sequence was transferred from the pENTR11 vector and inserted into pDEST26 (Invitrogen, Carlsbad, CA) by Gateway^{R} LR Clonase^{TM} II enzyme mix. The correct sequences were confirmed by sequencing on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA).

CHO cells were cultured in 12-well plates (approximately 2 × 10^5 cells/well) with 10% FBS containing Nutrient Mixture F-12 HAM (Sigma Aldrich, Tokyo, Japan) for 24 h at 37°C. One nanogram of vector was transfected to the cell using 4 μl HillyMax transfection reagent (Dojindo Lab., Kumamoto, Japan) as per the manufacturer's instructions at 37°C for 24 h. Two ligands, endocannabinoids anandamide and nonendocannabinoid LPI (Henstridge et al., 2008; Oka et al., 2007), were selected for the experiment, according to the previous findings that both ligands could activate GPR55 to increase intracellular calcium, although another endocannabinoid 2-AG did not (Lauckner et al., 2008). Previous studies had shown doses of these ligands as functional in cultured cells, which are 5 μM for anandamide and 1 μM for LPI (Henstridge et al., 2008; Oka et al., 2007). Therefore, several doses of either anandamide (0, 1, 5, or 10 μM) or LPI (0, 1, 10, or 100 μM) were administered into the medium for 15 min before each analysis. Cellular activation of signaling ELISA: CASE$^{TM\ Kit}$ for ERK1/2 T202/Y204 (Super Array Bioscience Co., Frederick, MD) was used to measure the phosphorylation level of ERK against total ERK in the cultured cells. ELISA analysis was performed using the Wallac 1420 ARVOsx multilabel counter (Perkin Elmer, Yokohama, Japan). The effect of these ligands on the ERK phosphorylation levels was examined in each allele type of the GPR55 receptor expressed CHO cells and compared with the ERK phosphorylation levels in each allele type of the GPR55 receptor expressed CHO cells that were not administered with those ligands respectively, using replicates of each of the four wells.

Subjects

The subjects comprised 235 unrelated Japanese female patients with anorexia nervosa (age 25.2 ± 7.5 years). Diagnosis had been made according to the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV). AN patients comprised 135 patients meeting the criteria for restricting type AN (ANR), and 100 for binge-eating and/or purging type AN (ANBP). An unscreened but gender-matched control group consisted of 1244 unrelated healthy Japanese (age 46.3 ± 12.9 years), who had no known history of psychiatric illness. Written informed consent was obtained from all subjects. The study was approved by the ethics committee of Tsukuba University, the Kurihama Alcoholism Center, and Niigata University.

DNA genotyping

DNA was extracted using the phenol-chloroform method from blood samples. TaqMan SNP genotyping was used. The TaqMan genotyping assay for rs3749073 was synthesized by the Assays-by-Design Service for SNP Genotyping Assays (Applied Biosystems, Foster

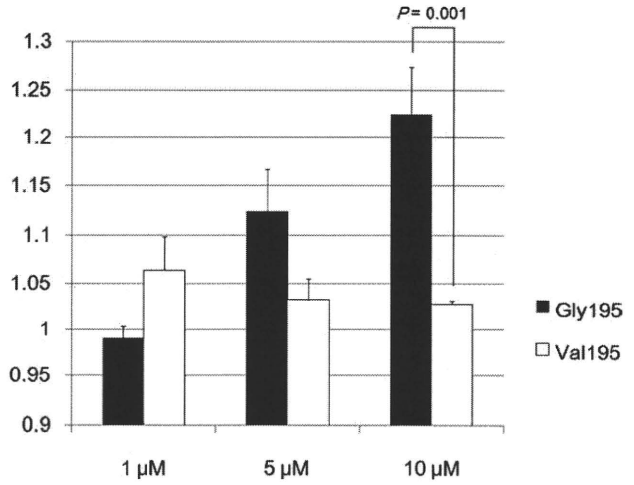

Fig. 2. Functional analysis of the Gly195Val polymorphism of *GPR55* induced by anandamide. Relative phosphorylation levels (mean ± SEM) of ERK1/2 at Thr202/Thr204 in the MAP kinase cascade were compared between two types of transfected polymorphic GPR55 in CHO cells at doses of anandamide (1, 5, 10 μM) in the cultured medium. Nominal *P*-value was shown for significant difference between alleles.

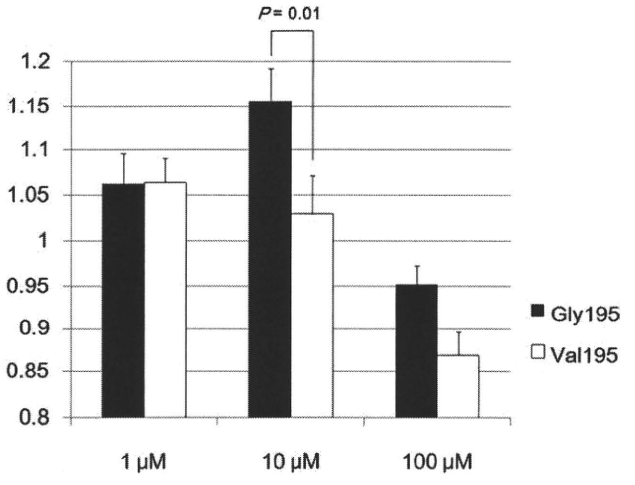

Fig. 3. Functional analysis of the Gly195Val polymorphism of *GPR55* induced by LPI. Relative phosphorylation levels (mean ± SEM) of ERK1/2 at Thr202/Thr204 in the MAP kinase cascade were compared between two types of transfected polymorphic GPR55 in CHO cells at doses of LPI (1, 10, 100 μM) in the cultured medium. Nominal *P*-value was shown for significant difference between alleles.

City, CA), composed of: Forward primer GCCCAGCAGGATGTGGAT; and reverse primer CTG GAGGTGTTTGGCTTCCT; probe labeled with VIC, CTTCCCATGGTCATCAT; and probe labeled with FAM, CCCATGGGCATCAT. The TaqMan reaction was performed in a final volume of 3 μl consisting of 2.5 ng genomic DNA and Universal Master Mix (EUROGENTEC, Seraing, Belgium). Genotying was performed on an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA). Genotyping quality control consisted of 99% successful calls, confirming concordance among repeat genotyping, and documentation of Hardy-Weinberg equilibrium.

Subjects performed personality trait test (TCI)

As higher Harm Avoidance (HA), and lower Self-Direction (SD) and Cooperativeness (CO) scores are consistently reported in AN patients (Karwautz et al., 2003; Klump et al., 2000, 2004), a correlation between those scores and rs3749073 was analyzed. The subjects comprised 177 generally healthy males (mean age: 22.8 years).

Statistical procedures

The phosphorylation levels of ERK detected by ELISA after treatment with the ligands were analyzed between CHO cells transfected with the two *GPR55* allele types by ANOVA to evaluate multiple effects (dose of ligands and genotypes), least mean square method in multiple logistic regression analysis, followed by posthoc analysis using a Student's *t*-test, using JMP ver. 5.1 (SAS Institute Inc, Cary, NC) Correlation between TCI (Cloninger's Temperament and Character Inventory) scores and genotype were calculated using ANOVA one-way testing, using the JMP ver. 5.1 software.

Deviation from predicted Hardy-Weinberg frequency, genotype and allelic associations were calculated using Haploview software version 3.11. The association between homozygote of Val195 and age-onset among patients were calculated by ANOVA test. Logistic regression test was made for age effect on the result of the association between *GPR55* and AN using JMP ver 5.1 software (SAS Institute Inc, Cary, NC). A significant association was defined as when the given *P*-value for allelic or genotypic tests was less than 5%.

RESULTS

Functional analysis demonstrated a difference in phosphorylation level of Extracellular Signal-Regulated Kinase (ERK) between GPR55 with two alleles of the missense polymorphism rs3749073, Gly195Val, in CHO cells. ANOVA revealed significant effects of anandamide administration ($F[1,23] = 4.0$, $P = 0.04$) and of the interaction between anandamide administration and allele ($F[1,23] = 7.6$, $P = 0.004$), while a trend of main effects of allele was observed ($F[1,23] = 3.7$, $P = 0.07$) on ERK phosphorylation levels in cells. Posthoc analysis showed that anandamide administration at a dose of 10 μM induced significant allelic differences of phosphorylation level of ERK in CHO cells ($P = 0.001$) (Fig. 2).

TABLE I. Distribution of Gly195Val polymorphism in GPR55 gene

		Genotype distribution							Allele frequency		
		CC (Gly/Gly)		CT (Gly/Val)		TT (Val/Val)		*P* (TT vs. others)	C	T	
Patients	n = 235	148	63.0%	70	29.8%	17	7.2%	P = 0.0048	366 (77.9%)	104 (22.1%)	P = 0.038
ANR	n = 135	85	63.0%	42	31.1%	8	5.9%	P = 0.0795	212 (78.5%)	58 (21.5%)	P = 0.159
ANBP	n = 100	63	63.0%	28	28.0%	9	9.0%	P = 0.0071	154 (77.0%)	46 (23.0%)	P = 0.087
Controls	n = 1244	837	67.2%	368	29.6%	39	3.1%		2042 (83.3%)	446 (16.7%)	

ANOVA also revealed significant main effects of LPI administration (F[1,23] = 18.3, $P < 0.0001$), of allele (F[1,23] = 6.8, P = 0.018), but not of the interaction between LPI administration and allele (F[1,23] = 2.0, P = 0.16) on ERK phosphorylation in cells. Although ANOVA did not show significant effect of the interaction, 10 μM LPI administration produced different level of the phosphorylation between CHO cells with the two alleles (student t-test P = 0.01) (Fig. 3). Interestingly, 100 μM LPI administration induced significant down regulation of the ERK phosphorylation in CHO cells with GPR55 in comparison to those induced by 10 μM LPI administration or non-administration of LPI. Maximum activation of the receptor induced by LPI administration was observed at different LPI concentrations in each polymorphism (1 μM for Val type and 10 μM for Gly type) (Fig. 3).

The distribution of the Gly195Val polymorphism of the *GPR55* gene is shown in Table I. The Japanese population satisfied Hardy-Weinberg equilibrium (P = 0.25). The T (Val195) allele was significantly more abundant in the AN group than in the control group ($P < 0.04$, OR = 1.30 [95% CI = 1.02–1.66]). When a recessive model for Val195 was tested, homozygotes of Val195 were more strongly associated with AN (P = 0.0048, OR = 2.41 [95% CI = 1.34–4.34]). Although a significant difference in genotype distribution was not observed between the controls and each subgroup of ANR or ANBP (Table I), a significant association was observed between the T (Val195) allele and ANBP (P = 0.007, OR = 3.06 [95% CI = 1.44–6.50]) when the recessive model was accessed. There was no association found between homozygotes of Val195 and age-of-onset. Although there was certain difference of average age between case and control groups, it did not attain statistical significance when included in the regression model as a covariate to control for the effect (Data not shown).

Analysis of association between personality traits and *GPR55*, persons with the TT genotype showed a lower CO score of TCI than others (F = 5.79, P = 0.017, TT: 22.5 vs. others: 27.6). However, there was no difference found for HA and SD scores in this group (P = 0.73, 0.91, respectively) (Table II).

TABLE II. Correlation between GPR55 Val195 homozygote and personality trait

	GPR55 genotype		Correlation	
TCI	Val/Val (n = 8)	Others (n = 174)	F value	P value
HA	18.13 ± 2.39	17.28 ± 0.51	0.12	0.729
SD	25.25 ± 2.50	24.94 ± 0.54	0.01	0.905
CO	22.50 ± 2.10	27.67 ± 0.45	5.79	0.017

DISCUSSION

This study successfully revealed a functional alteration of *GPR55* by its nonsynonymous SNP, Gly195Val. This *cis*-acting functional difference was observed in the phosphorylation level of ERK1/2 that occurs at Thr202/Thr204 in the MAP kinase cascade in cells, which is activated by anandamide and LPI, binding to Gq type G protein coupled receptors. In addition, the *GPR55* polymorphism was associated with vulnerability to AN. The results further suggested a recessive effect of the Gly195 polymorphism of *GPR55* for increased vulnerability to AN.

The effect of the polymorphism for predisposing people to AN seemed to be small, and a previous linkage study failed to find a linkage at the locus. Considering the low effect rate of any susceptibility genes for AN, a larger sample size and dense marker mapping are required for genome wide association study. However, as we could identify the functional polymorphism in the candidate gene, a genetic association between *GPR55* and AN was successfully detected in this study despite the relatively small population of samples.

A weakness of the study could be that if age affects genotype distribution it would not have been seen as controls were not age matched against patients in the association analysis,. Also, this limited sample size may introduce statistical error (Type 1), but we were unable to prepare a second sample set for replication analysis. The association found between the T allele and AN needs to be considered as preliminary, and must be replicated with a larger independent subject group. Finally, a correlation between *GPR55* and CO personality trait score using TCI was found in this study. This is a potentially interesting finding if *GPR55* can explain one of the clinical phenotypes for AN, which was shown in previous studies (Karwautz et al., 2003; Klump et al., 2000, 2004). We analyzed an effect of *GPR55* genotype on personality traits in male and healthy participants who were independent subset from that of patients with AN. Therefore, this study cannot supply direct evidence to explain a possible relationship between personality traits of AN and *GPR55*. Further studies may confirm our findings, and