through RNA transport and translation in the dendrite region (17), a deficit causes MR (18). On the other hand, when CGG repeats are between 55 and 200, this is generally called a premutation (PM), leading to CGG expansion in the next generation causing Fragile X syndrome especially via maternal CGG expansion. FXTAS mostly affects adult men possessing PM after middle age, indicating that PM causes an increase in FMR1 mRNA production, which with aging is toxic to neural and glial cells, and reaches "gain of function" (19, 20).

This is the first case in Japan where FXTAS was diagnosed in a living patient. In Europe and North America, there have been many reports about FXTAS following the report of Hagerman et al in 2001 (1); however, there have been no reports in Asia, or at least in Japan, to date. There are reports of prevalence levels of FXS -- FMR1 full mutations -- within the population of men with MR being from 0.8 (6) to 2.4% (21), which is lower than for the Caucasian man population with MR, with prevalence levels of 2.6-8.7% (22, 23). This suggests a lower prevalence of premutation alleles--one step prior to full mutation--, and thus a lower FXTAS prevalence rate. However, since there is a great difference in the number of reports about FXTAS published in western countries and Japan, it is highly possible that FXTAS may develop but may not be correctly diagnosed in Japan. We need to increase awareness of the existence of FXTAS, and stress the importance of checking for FMR1 premutation in patients above the age of 50 who present with tremor and cerebellar disorder when a T2-weighted MRI shows bilateral MCP lesions.

**Acknowledgement**

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# 脆弱X症候群の分子機構と治療

Molecular mechanism and treatment of fragile X syndrome

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◎脆弱X症候群(FXS)はX染色体上に位置するFMR1遺伝子の異常によって発症し，知的障害，巨大睾丸，細長い顔などを主症状とする．日本人での頻度は欧米よりやや低く，男性で10,000人に1人と考えられる．本疾患では代謝型グルタミン酸受容体(mGluR)のシグナルが異常に亢進し，そのためにシナプスの可塑性が変化し，シナプス樹状突起棘の形態に異常をもたらすことが明らかにされてきている．この異常の機構が詳細に研究され，mGluR理論が確立され，それに基づいた治療法が開発されてきている．動物実験のみならず，ヒトでの臨床治験も行われており，近い将来治療法が確立されることが期待されている．さらに，これらの治療法は他の知的障害や自閉症にも応用できる可能性があり，注目される．日本でも，この治療研究を推進する体制を充実させていくことが重要である．

Key word：脆弱X症候群(FXS)，FMR1遺伝子，CGG繰返し配列延長，グルタミン酸受容体

脆弱X症候群(fragile X syndrome：FXS)は1943年にX連鎖性遺伝形式をもつ知的障害として報告され，Martin-Bell症候群ともよばれた．1969年にX染色体上の脆弱部位が明らかにされ，1991年に原因遺伝子が解明された[1]．遺伝性の知的障害としてはもっとも研究が進んでいる．FXSは巨大睾丸，長い顔などを特徴とし，てんかんや睡眠障害などを合併することも多い．男性患者は重度の知的障害を呈するが，女性では軽度や中等度の場合も多い．まれにfragile X mental retardation(FMR)2遺伝子が原因となるが，そのほとんどはFMR1遺伝子の異常である[2]．FXSの頻度は，男性の4,000人に1人，女性の8,000人に1人と報告されているが，民族によって差がある．日本人では男性の10,000人に1人と推定されている[3]．FXSは知的障害のなかで研究がもっとも進んでおり，病態解明から治療法の開発が行われ，近年では臨床治療研究に到達している．さらに，このFXSで明らかにされてきた脳の病態は，他の原因による知的障害や自閉症とも共通していると考えられ，FXSの研究はひとつの遺伝性疾患の研究にとどまらない．

本稿ではFMR1異常によるFXSの病態と，それに基づく治療法開発の現状を中心に解説する．

## FXSのCGG繰返し配列異常

FXSではX染色体の脆弱部位であるXq27.1に存在するFMR1遺伝子の5′非翻訳領域あるCGG繰返し配列が異常に延長している[1]．このCGG繰返し配列は正常では54以内であるが，患者では200を超える．FXS患者の母親は50～200の繰返し配列(前変異)をもつ保因者である．FXSは，母親の不安定なCGG繰返し配列が患者に遺伝するときに延長する，いわゆるトリプレットリピート病として最初に解明された疾患である[4]．この配列延長がDNAのメチル化をもたらし，その結果，FMR1遺伝子の転写抑制により遺伝子の機能が失われる．一方，FXSの前変異をもつ保因者のなかから50歳以降にParkinson様症状，精神症状などを呈する脆弱X症候群関連振戦/失調症候群(FXTAS)が発症することが知られ，日本でも患者がみつかっている[5]．FXTASの発症機序は

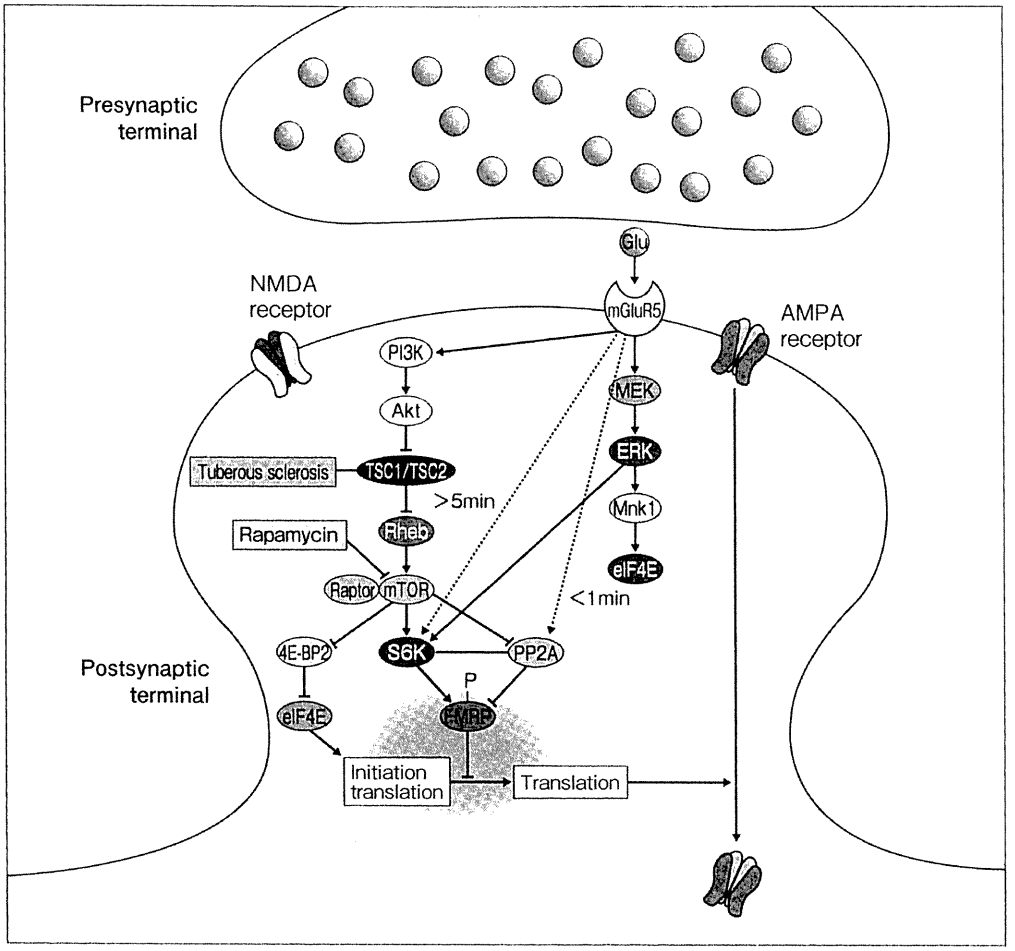


**図 1　mGluR，mTORシグナル伝達系，FMRPの関係[15]**

MEK-ERK-Mnk1 と PI3K-mTOR 経路の 2 つが mGluR5 の下流のシグナル系として存在する．PP2A や PI3K などの FMRP が標的とする mRNA は，ERK によって細胞内の二次メッセンジャーとして働くようになる．リン酸化 ERK は Mnk1 と S6K を介して転写活性をもつ．ERK のリン酸化は PP2A などのホスファターゼにより制御されている．FmR1 KO マウスでは ERK の非活性化が起こるために mGluR5 の刺激に過剰に反応する．このように，ERK 活性の制御異常はシナプスの翻訳調節異常の指標となる．

mTOR の活性化は 4E-BP や S6K のリン酸化を介して転写開始の起始点となる．mGluR5 が刺激されると PI3K が膜の phospholipid PIP2 を PIP3 に転換する．この PIP3 が，Akt を膜に集めてリン酸化させ，PKD1 を活性化させる．Akt で活性化された mTOR は TSC(TSC1 と TSC2 のヘテロダイマー)が抑制する．TSC2 がリン酸化されるとその GAP 活性が減少し，Rheb と mTOR を活性化させる．さらに，この mTOR は Raptor と結合し，4E-BP と S6K に作用する．そして，eIF4F などを介して翻訳が開始する．FMRP は mGluR5 の刺激による S65K や PP2A の活性化を介して制御されている．

MEK：mitogen-activated protein kinase kinase, ERK：extracellular signal regulated kinase, Mnk1：mitogen-activated protein kinase interacting serine/threonine kinase 1, PI3K：phosphoinositide-3 kinase, 4E-BP：4E-binding protein, S6K：S6 kinase, PIP2：phosphatidylinositol 4,5-bisphosphate, PIP3：phosphatidylinositol(3,4,5)-trisphosphate, PDK1：3-phosphoinositide-dependent kinase 1, TSC：tuberous sclerosis complex, GAP：GTPase-activating protein.

FXS とは異なっており，詳細は文献を参照されたい[6]

## 動物モデル

マウスの Fmr1 遺伝子は，ヒトと異なり CGG 繰返し配列をもたない．そのために，CGG 繰返しを延長させることは困難であるが，遺伝子機能を欠

失したモデルマウス〔Fmr1 ノックアウト(KO)マウス〕が開発されている[7]．また，ショウジョウバエなどのモデルも開発されてきた．これらのモデル動物は記憶や行動の異常，巨大睾丸，さらに痙攣を起こしやすいなど，ヒトの症状のかなりの部分が再現されている．

## FMRPの機能とその異常

FMR1 遺伝子がコードする蛋白，FMRP はユビキタスであるが，脳と精巣に比較的強く発現する RNA 結合蛋白である[8]．FMRP は 3 つの RNA 結合部位(2 つの KH ドメインと 1 つの RGG ボックス)をもち，おもに標的 mRNA の 3′ 非翻訳領域に結合する．FMRP は核内 mRNA に結合するが，神経細胞では核内のみならず，シナプス樹上突起や樹状突起棘の局所的 mRNA と結合している．FMRP は標的 mRNA の翻訳を抑制することによりシナプスの機能を維持しており，この機能が失われるとシナプス可塑性に変化をもたらし，知的障害などの症状を呈する．この局所的 mRNA の翻訳調節は，後述する代謝型グルタミン酸受容体(mGluR)からのシグナルが引き金になっている．この mGluR からのシグナル経路の詳細は明らかにされてきている(図 1)．さらに，mGluR5 を刺激すると FMRP が急速に脱リン酸化され，シナプスの局所的な mRNA の急激な増加を引き起こすことが明らかになっている[9]．リン酸化されていない FMRP は，むしろ蛋白翻訳を活性化させ，リン酸化された FMRP のみが蛋白翻訳を抑制できる．FMRP は 499 のセリンが特異的にリン酸化される．この機構には mTOR カスケードが必要で，最終的には S6 キナーゼがリン酸化されることにより FMRP のリン酸化が起こる[10]．

## FXSでのシナプス形態と可塑性の異常

前述の FMRP 異常の機構によりシナプスの異常が引き起こされる．FXS では大きな脳の形態学的変化はないが，シナプス樹状突起棘に異常(数が多い，異常に長く曲がった形)があり，未熟であることが明らかにされている[11]．余談になるかもしれないが，近年，Down 症候群や Rett 症候群などにも同様にシナプス樹状突起の異常がみられることが明らかになっている．また，シナプスの活動状況によってシナプスの伝達効率が変化するシナプス可塑性は記憶や学習に重要な役割があり，シナプス伝達効率が増加する長期増強(LTP)やこの伝達効率が低下する長期抑制(LTD)などの生理的な現象と密接な関係がある．FXS では海馬と小脳の LTD が増強され，大脳や海馬では LTP に変化を起こすことなど，可塑性の異常が報告されている[12]．

## 代謝型グルタミン酸受容体(mGluR)理論

FXS でみられるシナプス形態，可塑性などさまざまの異常を一元的に説明できる画期的な mGluR 理論が，2004 年に Bear らによって報告された[13]．この理論により FXS における，①シナプス棘の数の異常や未熟性，②Fmr1 ノックアウトマウスの神経生理学的異常，③mGluR5 の活性化によるシナプスの樹上突起の蛋白合成の促進，④FXS 患者やマウスモデルの行動異常，などがすべて説明できる．その後も，この理論を支持する研究が次々に報告され，現在の治療法開発へと結びついている．

本理論を理解するためには，mGluR 受容体などの基本的な理解が必要となる．脳のシナプス膜にはイオンチャネル型と代謝型の 2 種類の受容体が存在する．イオンチャネル型受容体は特異的なリガンドと結合し，イオンを通過させ興奮性神経伝達機構を担う．AMPA(γ-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) 型，NMDA(N-methyl-D-aspartic acid)型，さらにカイニン酸などがおもなイオンチャネル型受容体として知られている，脳の可塑性の機構である LTD は，この AMPA 受容体の数の減少によって引き起こされる．

一方，代謝型グルタミン酸受容体(mGluR)はおもには G 蛋白依存で，7 回膜貫通領域(7TMD)をもつ．mGluR は 8 つのサブタイプに分かれ，これらは構造の類似性や薬理学的な作用などから 3 つのグループに分類される(グループⅠ，Ⅱ，Ⅲ)．FXS で重要な mGluR1 と mGluR5 はグループⅠに分類され，Gq 蛋白と結合し，ホスホリパーゼ C を活性化させる．FXS ではグループⅠの mGluR

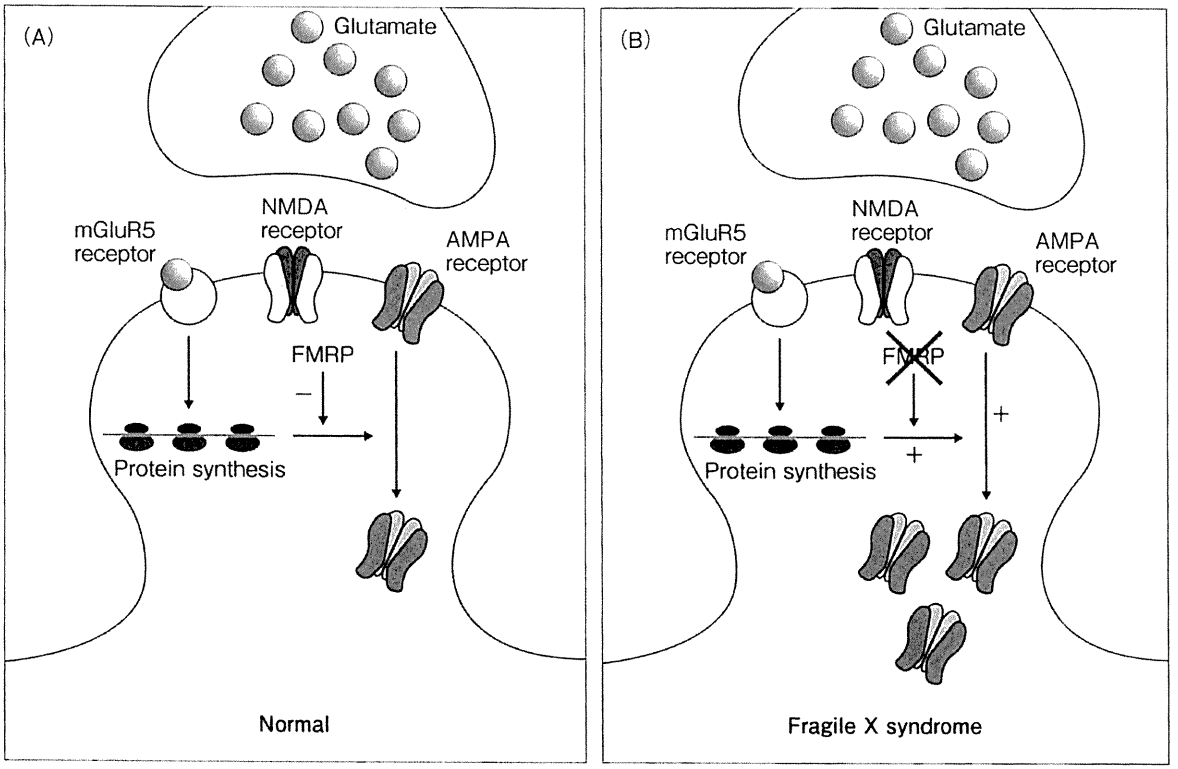


**図 2　mGluR理論[15]**

A：グルタミン酸が mGluR5 を刺激し，シナプスの局所的 mRNA の転写が開始される．この局所的な蛋白合成が，シナプス可塑性に重要な役割を果たしている AMPA 受容体の内在化を促進する．FMRP はこの転写を抑制することにより AMPA 受容体の内在化を阻止している．

B：Fmr1 KO マウスの研究によると，FXS 患者の神経細胞では FMRP が消失することにより AMPA 受容体の内在化が促進され，シナプスの異常が起こる．

の刺激が異常に増強しており，それにより AMPA 受容体の内在化が引き起こされることがこの mGluR 理論の中心である(図2)．グルタミン酸がグループⅠmGluR を刺激すると，FXS では FMRP の転写抑制がないために局所の mRNA の転写が異常に増強する．その結果，局所の蛋白合成が増え，最終的に AMPA 受容体を内在化させてしまう．そしてシナプス可塑性の変化や形態異常を引き起こす．この理論の直接的な実証として，Fmr1 KO マウスにおいて mGluR5 を 50％に減少させると，シナプスの形態，蛋白合成異常，痙攣などの異常が改善された研究が報告されている[14]．さらに，この理論を裏づける多くの研究結果が報告されている．

## 治療法の開発[15]

現段階として，FXS の治療法として最終的に確立したものはないが，mGluR 理論などに基づき，動物のみならず，ヒトにおいて臨床治療研究が進められている．おもな治療薬と作用について図3に示す[15]．さまざまな薬剤の治験の進行についてはホームページで調べることができる(http://www.clinicaltrials.gov)．そのおもなものについて解説する．

MPEP は mGluR5 拮抗薬として動物実験ではさまざまな症状の改善をもたらしたが，薬剤の安定性や毒性などから臨床応用には至らなかった．最初の臨床応用は fenobam が試みられた．この薬剤は，最初は作用機序がよくわからなかったが，その後 mGLuR5 の拮抗薬であることが確認された．Fenobam を用いた臨床試験の第Ⅱ相が最近終了した．12 人の成人の患者に 1 回のみの投与を行い，薬剤の安全性，薬理，一部の認知や行動への効果を検討した．この治験で，不安や音への過剰反応，注意や衝動性なども改善したことが報告されている．これらの結果はすばらしいものであるが，二重盲検ではないため今後の検討が必要である．また，経口投与では fenobam の濃度は変動が大き

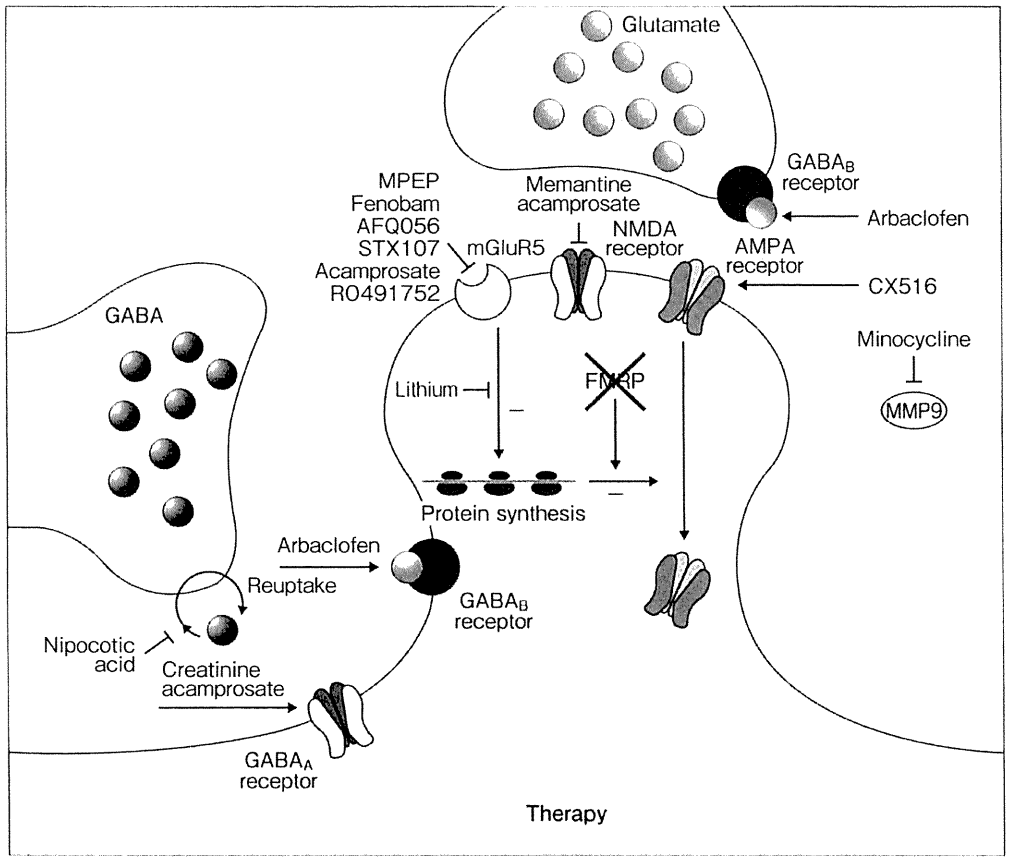


**図 3　FXSの治療戦略**[15]

FMRP の欠失によりグルタミン酸受容体が興奮し，GABA 受容体が抑制されたシナプスを示している．

下記の作用のある薬剤による FXS のシナプス機能の回復が検討されている．シナプス可塑性の異常を改善すると考えられる mGluR5 制御，$GABA_A$作動，$GABA_B$受容体作動，NMDA 受容体拮抗，AMPA 受容体の制御．さらに，リチウム，ミノサイクリン，acamprosate なども検討されている．

く，安定性にも問題がある．小規模治験として，アルコール中毒の治療薬として認められた acamprosate が3人の若年患者に試みられ，言語コミュニケーションや全般的な臨床症状の改善がみられた．さらに，各製薬会社が治療研究に乗り出しており，STX107(Seaside Therapeutics；アメリカで治験が開始される予定)，AFQ056(Novartis；フランス，イタリア，スイスで第Ⅱ相が終了)，RO4917523(Hoffman-LaRoche；アメリカで第Ⅱ相が開始)などの治療研究が進められている．

直接 mGluR を標的にした治療法に加え，mGLuR の上流や下流のシグナル伝達を阻止する方法も検討されている．最初に，気分障害の治療薬として使われているリチウムが用いられた．リチウムはグループ1 mGluR 伝達を含め，さまざまな分子経路に影響することが明らかになっている．

さらに，シナプス前のグルタミン酸の放出を減少させる方法も報告されている．これには$GABA_B$受容体拮抗薬として知られている baclofen が知られており，投与により Fmr1 KO マウスの聴覚過敏性痙攣を減少させることが報告された．この成果をもとにして baclofen の R-異性体である arbaclofen(STX209)が開発され，二重盲検第Ⅱ相の臨床治験が行われている．また，mGluR5 シグナル過剰の影響により matrix metalloproteinase-9 (MMP-9)遺伝子の過剰発現が Fmr1 KO マウスで起こっていることが明らかになった．この結果をもとに，この異常を抑制するミノサイクリン(テトラサイクリンのひとつのアナログ)を用いたマウスの研究では，いくつかの症状に効果があることが示されている．さらに，ヒトの治療研究が進められている．

## おわりに

FXSの治療を考えるときには，正常な脳の発達が変化する時期や可塑性についても考慮する必要がある．マウスの実験では生後かなり経過しても症状が回復する可能性も示唆されているが，早期に治療するほうがよいことは間違いない．そのためには新生児期スクリーニングを検討する必要があり，欧米ではこの研究が開始されている[16]．

FXSではシナプス可塑性の異常が明らかになり，その機構の詳細な検討から治療法の開発に至っている．これは他の知的障害や自閉症などのモデルとしても重要と考えられる．FXSで開発される治療法は自閉症などにも応用が可能と考えられ，すでにその動きもはじまっている．

欧米ではFXSの団体やコンソーシアムを組織して積極的に研究が進められ，治療法の開発を行っている．日本でも，FXS患者への治療に向かって体制を整えていく必要がある．著者らは近い将来，日本人患者への治療も可能になるように体制の整備を進めている．多くの知的障害も治療への時代に入ってきており，日本でも大規模な共同研究体制を充実させ，研究を推進することが重要である．

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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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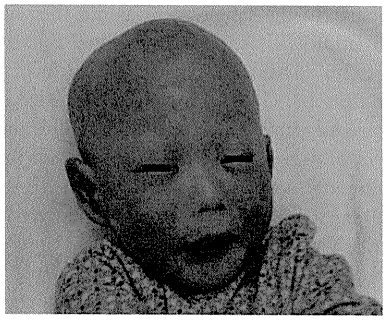

**Figure 1** Photograph of the patient, showing alopecia and absence of eyebrows and eyelashes.

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).

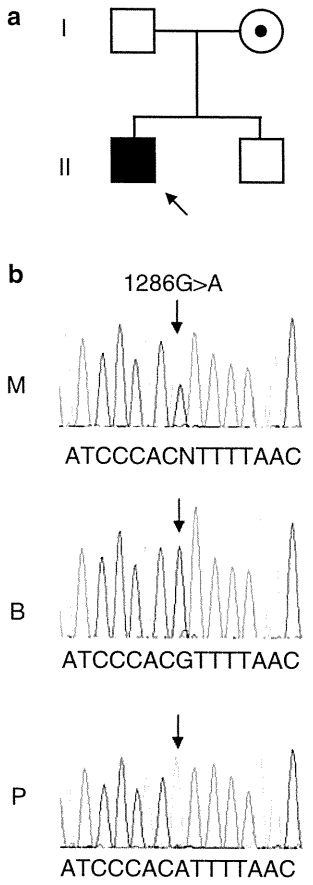


**Figure 2** Family pedigree and mutation analysis. (**a**) Pedigree of the family studied. The circle indicates female individuals, and squares indicate male individuals. The filled symbol denotes the affected individual, and the dot with symbol denotes a carrier individual. An arrow indicates the proband. (**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.

## ACKNOWLEDGEMENTS

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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

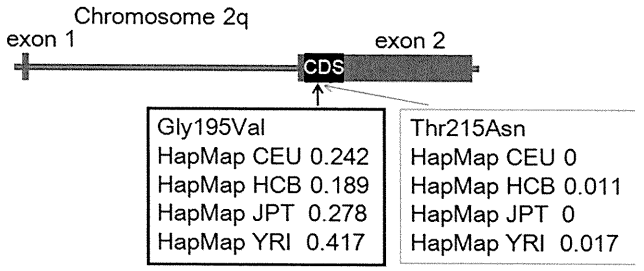


Fig. 1. Genetic structure of *GPR55* gene and its polymorphisms in CDS. The gene has two exons, and a part of second exon codes amino acids (shown in black (CDS)). There are one polymorphism (Gly195Val) and one rare variant (Thr215Asn) in the coding region of the gene. The minor allele frequencies in 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® LR Clonase™ 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 $2 \times 10^5$ 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™ 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

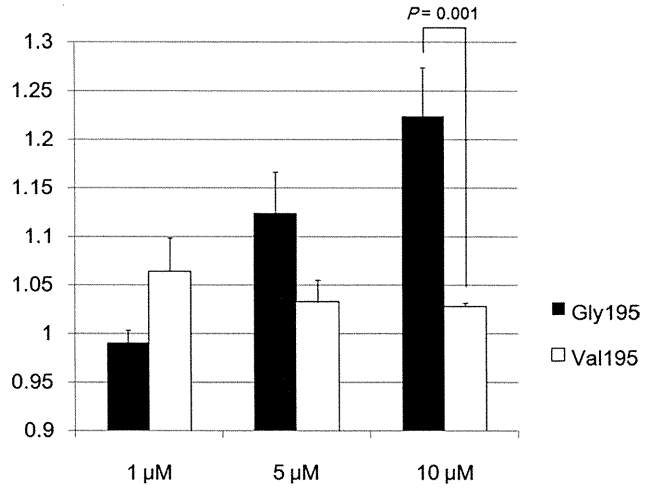


Fig. 2. Functional analysis of the Gly195Val polymorphism of *GPR55* induced by anandamide. Relative phosphorylation levels (mean ± SEM) of ERK1/2 at Thr202/Thr204 in the MAP kinase cascade were compared between two types of transfected polymorphic GPR55 in CHO cells at doses of anandamide (1, 5, 10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

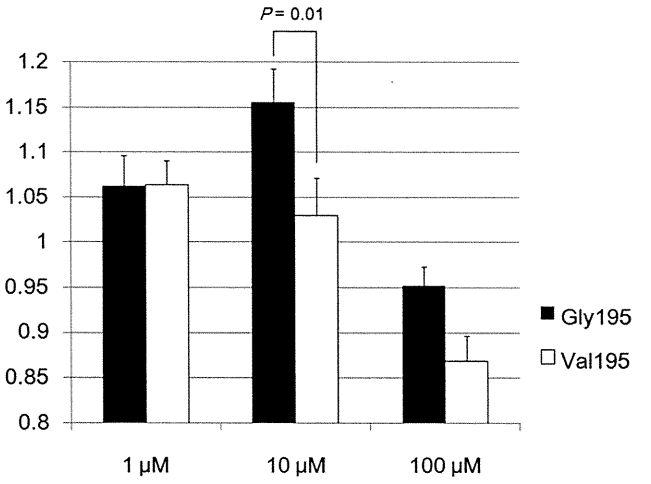


Fig. 3. Functional analysis of the Gly195Val polymorphism of *GPR55* induced by LPI. Relative phosphorylation levels (mean ± SEM) of ERK1/2 at Thr202/Thr204 in the MAP kinase cascade were compared between two types of transfected polymorphic GPR55 in CHO cells at doses of LPI (1, 10, 100 μM) in the cultured 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% Cl = 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% Cl = 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% Cl = 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

further contribute to an understanding of the biology of AN.

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RESEARCH ARTICLE

AMERICAN JOURNAL OF medical genetics PART B
Neuropsychiatric Genetics

# Association of SNPs Linked to Increased Expression of *SLC1A1* With Schizophrenia

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**Glutamate is one of the key molecules involved in signal transduction in the brain, and dysfunction of glutamate signaling could be linked to schizophrenia. The *SLC1A1* gene located at 9p24 encodes the glutamate transporter EAAT3/EAAC1. To investigate the association between the *SLC1A1* gene and schizophrenia in the Japanese population, we genotyped 19 tagging single nucleotide polymorphisms (tagSNPs) in the *SLC1A1* gene in 576 unrelated individuals with schizophrenia and 576 control subjects followed by replication in an independent case–control study of 1,344 individuals with schizophrenia and 1,344 control subjects. In addition, we determined the boundaries of the copy number variation (CNV) region in the first intron (Database of Genomic Variants, chr9:4516796-4520549) and directly genotyped the CNV because of significant deviation from the Hardy–Weinberg equilibrium. The CNV was not associated with schizophrenia. Four SNPs showed a possible association with schizophrenia in the screening subjects and the associations were replicated in the same direction (nominal allelic $P < 0.05$), and, among them, an association with rs7022369 was replicated even after Bonferroni correction (allelic nominal $P = 5 \times 10^{-5}$, allelic corrected $P = 2.5 \times 10^{-4}$, allelic odds ratio, 1.30; 95% CI: 1.14–1.47 in the combined subjects). Expression analysis quantified by the real-time quantitative polymerase chain reaction in the postmortem prefrontal cortex of 43 Japanese individuals with schizophrenia and 11 Japanese control subjects**

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**revealed increased *SLC1A1* expression levels in individuals homozygous for the rs7022369 risk allele ($P = 0.003$). Our findings suggest the involvement of *SLC1A1* in the pathogenesis of schizophrenia.** © 2011 Wiley Periodicals, Inc.

**Key words: transporters; glutamate; postmortem brain; antipsychotics**

## INTRODUCTION

Schizophrenia is one of the most mysterious and costliest mental disorders and it affects 0.30–0.66% of the population. Despite its high heritability estimates, the identification of specific molecular genetic variation has not been easy. Recent findings have suggested that a small proportion of schizophrenia incidence could be explained by rare structural variations [van Os and Kapur, 2009; Vacic et al., 2011].

Glutamate transporters (excitatory amino acid transporters, EAATs) play important roles in maintaining extracellular glutamate concentrations. To date, 5 subtypes of $Na^+$-dependent glutamate transporters—EAAT1 (GLAST, *SLC1A3*), EAAT2 (GLT-1, *SLC1A2*), EAAT3 (*SLC1A1*), EAAT4 (*SLC1A6*), and EAAT5 (*SLC1A7*)—have been identified [Shigeri et al., 2004]. Removal of extracellular glutamate in the forebrain is controlled by three major EAATs, that is, EAAT1, EAAT2, and EAAT3 [Amara et al., 1998; Danbolt, 2001]. EAAT1 and EAAT2 are mainly glial and EAAT3 is mostly neuronal [Rothstein et al., 1994]. EAAT3 is encoded by the glutamate transporter, solute carrier family 1 gene (*SLC1A1*), which is located on chromosome 9p24. EAAT3 (termed EAAC1 in rodents) is predominantly expressed in the cerebral cortex, basal ganglia, and hippocampus.

On the basis of pharmacological evidence, dysfunctions of glutamate neurotransmission have been implicated in the pathophysiology of schizophrenia [Coyle, 2006; Tuominen et al., 2006]. EAAC1 may control activation of some subtypes of *N*-methyl-D-aspartate (NMDA) receptors and vice versa in the hippocampus [Waxman et al., 2007]. Environmental enrichment has been shown to decrease the mRNA expression of EAAC1 in the hippocampus [Andin et al., 2007] and EAAC1-deficient mice have shown reduced neuronal glutathione levels, and, with aging, they developed brain atrophy and behavioral changes including decreased spatial learning abilities and cognitive impairment [Aoyama et al., 2006]. It has also been suggested that EAAC1 deficiency leads to impaired neuronal glutathione metabolism and oxidative stress [Aoyama et al., 2006]. Thus, the glutamate hypothesis [Coyle, 2006], oxidative stress hypothesis [Sarandol et al., 2007], and parallel effects of environmental enrichment and antipsychotic treatment in schizophrenia [Andin et al., 2007] suggest the involvement of EAAT3 in schizophrenia.

Deng et al. [2007] genotyped eight even-spaced single nucleotide polymorphisms (SNPs) that were separated from each other by an average distance of 14 kb in the *SLC1A1* gene in 100 Japanese patients with schizophrenia and 100 Japanese controls. Although a potential association between rs2228622 and schizophrenia was found, the association was not confirmed in an additional sample comprising 300 schizophrenics and 320 controls. Since the average summary odds ratio (OR) of nominally significant effects of 24 genetic variants in 16 different genes was shown to be ~1.23 by systematic meta-analyses [Allen et al., 2008], large sample sizes are required to detect SNPs associated with schizophrenia. The present study aims to investigate associations between SNPs in the *SLC1A1* gene and schizophrenia by a large case–control study of 1,920 Japanese schizophrenic patients and 1,920 Japanese control subjects.

## MATERIALS AND METHODS

### Subjects

The screening groups were comprised 576 unrelated Japanese patients with schizophrenia and 576 mentally healthy unrelated Japanese control subjects. The replication groups were comprised 1,344 unrelated Japanese patients with schizophrenia and 1,344 mentally healthy unrelated Japanese control subjects. Patients with schizophrenia (1,055 men and 865 women; mean age ± standard deviation (SD), 48.2 ± 14.7 years) were diagnosed according to the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV; American Psychiatric Association (APA), 2001) with consensus from at least 2 experienced psychiatrists, and the control subjects (1,051 men and 869 women; mean age ± SD, 47.6 ± 13.4 years) were those whose second-degree relatives were free of psychosis on the basis of self-reporting by the subjects. All the participants provided their written informed consent. The association analysis was approved by the Ethics Committees of the University of Tsukuba, Niigata University, Fujita Health University, Nagoya University, Okayama University, and Seiwa Hospital.

### Human Postmortem Brains

Brain specimens were obtained from Japanese individuals of 43 schizophrenic patients and 11 age- and gender-matched controls. Tissue blocks were cut from gray matter in an area of the prefrontal cortex referred to as Brodmann's area 9 (BA9). The Japanese subjects met the DSM-III-R criteria for schizophrenia. The control subjects had no known history of psychiatric illness. The study was approved by the Ethics Committees of Niigata University, University of Tsukuba, Tokyo Metropolitan Matsuzawa Hospital, and the Tokyo Institute of Psychiatry.

### SNP Selection and Genotyping

The selection of tagSNPs for genotyping in the *SLC1A1* gene was conducted with the use of the International HapMap Project. A total of 19 tagSNPs were selected in this study (Fig. 1, Table I). The SNPs tagged by the selected 19 tagSNPs are shown in the Supplementary Table I.

The SNPs were genotyped by the TaqMan SNP genotyping assay (Applied Biosystems, Foster City, CA). Product information on the TaqMan SNP genotyping assays used in this study is listed in Supplementary Table II. The TaqMan reaction was performed in a final volume of 3 μl consisting of 2.5 ng genomic DNA and Universal Master Mix (Eurogentc, Seraing, Belgium). Genotyping was performed with the ABI PRISM 7900HT Sequence Detection

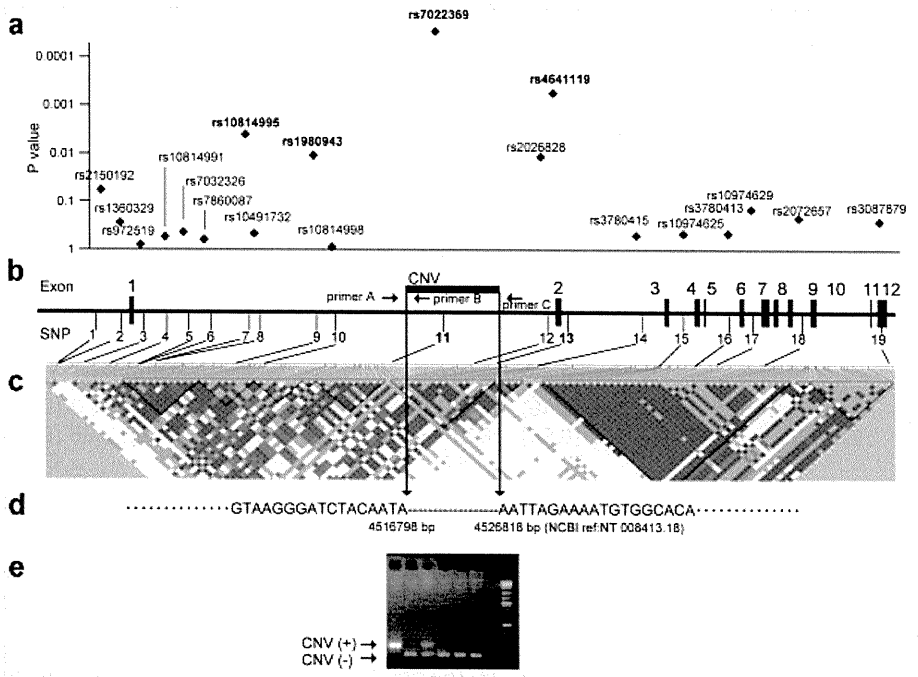


**FIG. 1. The results of SNP association with schizophrenia and the position of the CNV analyzed in the SLC1A1 gene. a: Results of the association study. Squares indicate the allelic *P*-value in the screening population. SNPs in bold letters were also analyzed in the confirmation population and squares of them are the allelic *P*-values in the combined populations. b: Schematic representation of *SLC1A1*. The 12 exons and 11 introns of the *SLC1A1* gene and the approximate location of each polymorphism genotyped in the present study are shown here. The polymorphisms represented in bold showed a positive association in this study. The bold line indicates the copy number variation (CNV) region. c: Linkage disequilibrium and haplotype blocks in the *SLC1A1* gene region. Each box represents the D′ value corresponding to each pair-wise single nucleotide polymorphism combination. D′ is color-coded; the red box indicates D′ = 1.0 between two loci. d: The sequence and position of breakpoints of the CNV. e: An example of genotypes of the CNV amplified by PCR with the primers A, B, and C shown in (d). The ladder marker on the left side lane is 2-Log DNA Ladder (New England BioILabs, MA). [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/ajmgb]**

System (Applied Biosystems). Because the SNPs potentially associated with schizophrenia were in the haplotype blocks that include exon 2, resequencing of SLC1A1 exon 2 was performed by direct sequencing with the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). One-third (1,152) of the samples were genotyped twice for 5 SNPs using TaqMan genotyping (Applied Biosystems), and genotype concordance was 99.5% for rs10814995, 99.4% for rs1980943, 99.8% for rs7022369, 99.7% for rs10758629, 99.9% for rs4641119, respectively. The average missing genotype rate was 1.2% (0.2–1.6%).

## Determination of the Boundaries of the CNV and Genotype

The boundaries of the copy number variation (CNV) region where rs7022369 is located were determined by directly sequencing the genomic DNA around rs7022369. This region was amplified by LA Taq (Takara, Kyoto, Japan) with the primers 5′-AAGATGGAATTGGGGAGGAT and 5′-CGGACGGCTTAAGTGTCAAC, and this produced a product of approximately 14 kb. The CNV was genotyped by the size of the PCR products with the primers 5′-TTAATGCCAGTGTTGCATGAG (common 5′-primer, the primer A in Fig. 1), 5′-GCCCTGGTGTGTGATATTCC (deletion 3′-primer, the primer C in Fig. 1) and 5′-CATTTGCAAAAGTCTCTTTACCTT (wild-type 3′-primer, the primer B in Fig. 1). The 283 and 219 bp PCR product indicated the deletion type and the normal wild-type, respectively.

## Real-Time Quantitative PCR for SLC1A1 Expression in Brains

Total RNA was isolated from human brain tissue (BA9) with an SV Total RNA Isolation System (Promega, Madison, WI). *SLC1A1* expression was quantified by real-time quantitative polymerase chain reaction (PCR) with a TaqMan Gene Expression Assay and an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) as per the manufacturer's instructions. Primers and probes were purchased from Applied Biosystems (Assay ID: Hs00179051_m1). Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as an internal control, and measurement of the threshold cycle ($C_t$) was performed in triplicate. Data were collected and analyzed with Sequence Detector Software (SDS) version 2.1 (Applied Biosystems) and the standard curve method. Relative gene expression was calculated as the ratio of *SLC1A1* to the

**TABLE I. Genotypic and Allelic Distributions of the SLC1A1 Gene Polymorphisms in the Screening Population**

| SNP No. | dbSNP ID | Subjects | n | Genotype count (frequency) | | | $P_{genotypic}$ | Allele count (frequency) | | $P_{allelic}$ | HWE *P* |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | rs2150192 | | | AA | AG | GG | | A | G | | |
| | | Sz | 569 | 259 (0.46) | 238 (0.42) | 72 (0.13) | | 756 (0.66) | 382 (0.34) | | 0.138 |
| | | C | 576 | 267 (0.46) | 253 (0.44) | 56 (0.10) | 0.28 | 787 (0.68) | 365 (0.32) | 0.34 | 0.726 |
| 2 | rs1360329 | | | TT | TG | GG | | G | G | | |
| | | Sz | 568 | 477 (0.84) | 85 (0.15) | 6 (0.01) | | 1039 (0.91) | 97 (0.09) | | 0.318 |
| | | C | 566 | 472 (0.83) | 89 (0.16) | 5 (0.01) | 0.90 | 1033 (0.91) | 99 (0.09) | 0.86 | 0.724 |
| 3 | rs972519 | | | GG | GC | CC | | G | C | | |
| | | Sz | 574 | 503 (0.88) | 66 (0.11) | 5 (0.01) | | 1072 (0.93) | 76 (0.07) | | 0.093 |
| | | C | 558 | 488 (0.87) | 68 (0.12) | 2 (0.00) | 0.52 | 1044 (0.94) | 72 (0.06) | 0.87 | 0.821 |
| 4 | rs10814991 | | | CC | CT | TT | | C | T | | |
| | | Sz | 571 | 119 (0.21) | 275 (0.48) | 177 (0.31) | | 513 (0.45) | 629 (0.55) | | 0.523 |
| | | C | 567 | 123 (0.22) | 282 (0.50) | 162 (0.29) | 0.67 | 528 (0.47) | 606 (0.53) | 0.43 | 0.989 |
| 5 | rs7032326 | | | TT | TC | CC | | T | C | | |
| | | Sz | 572 | 95 (0.17) | 258 (0.45) | 219 (0.38) | | 448 (0.39) | 696 (0.61) | | 0.201 |
| | | C | 565 | 86 (0.15) | 245 (0.43) | 234 (0.41) | 0.54 | 417 (0.37) | 713 (0.63) | 0.27 | 0.102 |
| 6 | rs7860087 | | | GG | GC | CC | | G | C | | |
| | | Sz | 572 | 458 (0.80) | 107 (0.19) | 7 (0.01) | | 1023 (0.89) | 121 (0.11) | | 0.790 |
| | | C | 572 | 473 (0.83) | 92 (0.16) | 7 (0.01) | 0.50 | 1038 (0.91) | 106 (0.09) | 0.29 | 0.299 |
| 7 | rs10814995 | | | AA | AC | CC | | A | C | | |
| | | Sz | 572 | 310 (0.54) | 222 (0.39) | 40 (0.07) | | 842 (0.74) | 302 (0.26) | | 0.976 |
| | | C | 561 | 278 (0.50) | 227 (0.40) | 56 (0.10) | 0.11 | 783 (0.70) | 339 (0.30) | **0.04** | 0.338 |
| 8 | rs10491732 | | | GG | GA | AA | | G | A | | |
| | | Sz | 569 | 417 (0.73) | 137 (0.24) | 15 (0.03) | | 971 (0.85) | 167 (0.15) | | 0.358 |
| | | C | 567 | 402 (0.71) | 148 (0.26) | 17 (0.03) | 0.66 | 952 (0.84) | 182 (0.16) | 0.36 | 0.455 |
| 9 | rs1980943 | | | AA | AG | GG | | A | G | | |
| | | Sz | 572 | 183 (0.32) | 292 (0.51) | 97 (0.17) | | 658 (0.58) | 486 (0.42) | | 0.286 |
| | | C | 571 | 153 (0.27) | 289 (0.51) | 129 (0.23) | 0.03 | 595 (0.52) | 547 (0.48) | **0.01** | 0.737 |
| 10 | rs10814998 | | | AA | AG | GG | | A | G | | |
| | | Sz | 572 | 265 (0.46) | 252 (0.44) | 55 (0.10) | | 782 (0.68) | 362 (0.32) | | 0.660 |
| | | C | 575 | 260 (0.45) | 259 (0.45) | 56 (0.10) | 0.93 | 779 (0.68) | 371 (0.32) | 0.75 | 0.463 |
| 11 | rs7022369 | | | CC | CG | GG | | C | G | | |
| | | Sz | 572 | 432 (0.76) | 115 (0.20) | 25 (0.04) | | 979 (0.86) | 165 (0.14) | | **0.000009** |
| | | C | 566 | 383 (0.68) | 156 (0.28) | 27 (0.05) | **0.01** | 922 (0.81) | 210 (0.19) | **0.01** | 0.04 |
| 12 | rs2026828 | | | AA | AG | GG | | A | G | | |
| | | Sz | 570 | 202 (0.35) | 273 (0.48) | 95 (0.17) | | 677 (0.59) | 463 (0.41) | | 0.865 |
| | | C | 569 | 181 (0.32) | 268 (0.47) | 120 (0.21) | 0.13 | 630 (0.55) | 508 (0.45) | 0.05 | 0.262 |
| 13 | rs4641119 | | | AA | AC | CC | | A | C | | |
| | | Sz | 573 | 431 (0.75) | 128 (0.22) | 14 (0.02) | | 990 (0.86) | 156 (0.14) | | 0.230 |
| | | C | 576 | 384 (0.67) | 170 (0.30) | 22 (0.04) | **0.002** | 938 (0.81) | 214 (0.19) | **0.001**[a] | 0.559 |
| 14 | rs3780415 | | | TT | TC | CC | | T | C | | |
| | | Sz | 574 | 429 (0.75) | 132 (0.23) | 13 (0.02) | | 990 (0.86) | 158 (0.14) | | 0.454 |
| | | C | 568 | 419 (0.74) | 134 (0.24) | 15 (0.03) | 0.89 | 972 (0.86) | 164 (0.14) | 0.64 | 0.283 |
| 15 | rs10974625 | | | GG | GA | AA | | G | A | | |
| | | Sz | 565 | 180 (0.32) | 262 (0.46) | 123 (0.22) | | 622 (0.55) | 508 (0.45) | | 0.134 |
| | | C | 564 | 183 (0.32) | 266 (0.47) | 115 (0.20) | 0.85 | 632 (0.56) | 496 (0.44) | 0.64 | 0.309 |
| 16 | rs3780413 | | | GG | GC | CC | | G | C | | |
| | | Sz | 567 | 289 (0.51) | 223 (0.39) | 55 (0.10) | | 801 (0.71) | 333 (0.29) | | 0.216 |
| | | C | 569 | 299 (0.53) | 218 (0.38) | 52 (0.09) | 0.86 | 816 (0.72) | 322 (0.28) | 0.57 | 0.183 |
| 17 | rs10974629 | | | AA | AG | GG | | A | G | | |
| | | Sz | 571 | 314 (0.55) | 216 (0.38) | 41 (0.07) | | 844 (0.74) | 298 (0.26) | | 0.646 |
| | | C | 569 | 308 (0.54) | 201 (0.35) | 60 (0.11) | 0.12 | 817 (0.72) | 321 (0.28) | 0.26 | 0.002 |
| 18 | rs2072657 | | | TT | TG | GG | | T | G | | |
| | | Sz | 573 | 282 (0.49) | 229 (0.40) | 62 (0.11) | | 793 (0.69) | 353 (0.31) | | 0.135 |
| | | C | 564 | 303 (0.54) | 212 (0.38) | 49 (0.09) | 0.24 | 818 (0.73) | 310 (0.27) | 0.08 | 0.176 |

(*Continued*)