ORIGINAL ARTICLE

Sonic hedgehog expression in *Gli3* depressed mouse embryo, *Pdn/Pdn*

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ABSTRACT **The phenotype of the genetic polydactyly/arhinencephaly mouse (*Pdn/Pdn*) is similar to Greig cephalopolysyndactyly syndrome (GCPS), which is induced by mutation of *GLI3*. Suppression of *Gli3* gene expression has been observed in *Pdn/Pdn*. Thus, the gene responsible for *Pdn/Pdn* has been considered to be *Gli3*. Recently, the mutation point was demarcated, that is, a transposon was inserted into intron 3 of the *Gli3* gene in the *Pdn* mouse. Forward and reverse primers were constructed in intron 3 near the insertion point. A forward primer in the long terminal repeat region of the transposon was also constructed. Now we can discriminate +/+, *Pdn*/+, *Pdn/Pdn* embryos from the PCR products. After genotyping of the *Pdn* embryos, *Gli3* and other correlated gene expressions, such as sonic hedgehog (*Shh*), *Bmp-2*, *Bmp-4*, *ptc-1*, were analyzed by real-time PCR method. *Gli3* gene expression in *Pdn/Pdn* was suppressed to 20–30% of +/+, and that in *Pdn*/+ was about 60% of +/+ through all the embryonic and neonatal periods examined. As *Shh* has been considered to be an antagonist of *Gli3*, *Shh* expression was analyzed, and a difference among genotypes was observed only on day 9 of gestation. We could not detect any alterations among genotypes in other gene expressions examined. *Gli3* and *Shh* gene expression were also analyzed on day 9 by whole-mount *in situ* hybridization in the +/+ and *Pdn/Pdn* embryos. Neuroectoderm was positive by *Gli3* probe in +/+ but not in *Pdn/Pdn*. Notochord, floor plate and prechordal mesoderm were positive by *Shh* probe both in +/+ and *Pdn/Pdn* embryos, but ectopic and/or overexpression of *Shh* were not observed in *Pdn/Pdn* embryos.**

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INTRODUCTION

The genetic polydactyly/arhinencephaly mouse (*Pdn/Pdn*) exhibits polysyndactyly, absence of olfactory bulbs, hydrocephalus, telecanthus and frontal bossing. These phenotypes are very similar to the human genetic disease, Greig cephalopolysyndactyly syndrome (GCPS) (Greig 1926; Naruse & Kameyama 1982; Naruse *et al.* 1990; Naruse & Keino 1995). *Gli3* is the gene responsible for *Pdn* mouse (Thien & Rüther 1999; Naruse *et al.* 2000, 2001; Ueta *et al.* 2002) and *GLI3* is responsible for GCPS (Vortkamp *et al.* 1991, 1992; Wild *et al.* 1997). From these facts, we have considered that *Pdn/Pdn* is the mouse homolog of GCPS (Naruse & Keino 1995; Naruse *et al.* 2000, 2001).

Meanwhile, it has been reported that sonic hedgehog (*Shh*) gene expression depresses *Gli3* gene expression (Wang *et al.* 2000), *Gli3* and *Shh* depress the expression each other (Litingtung & Chiang 2000; Litingtung *et al.* 2002), and *Gli3* and *Shh* play a critical role in the dorsoventral patterning of the intermediate neural tube (Persson *et al.* 2002; Rallu *et al.* 2002; Meyer & Roelink 2003). The gene responsible for a type of holoprocencephaly and cyclopia is *Shh* (Belloni *et al.* 1996; Roessler *et al.* 1996; Edison & Muenke 2003). From these reports, we speculated that over-expression of *Shh* by depressed *Gli3* may induce brain malformations in *Pdn/Pdn*.

Recently, we developed a quick genotyping method using PCR for the *Pdn* mouse (Ueta *et al.* 2002). This allowed us to analyze the alteration of gene expression of *Gli3* and related genes such as *Shh* in early *Pdn* mouse embryos.

MATERIALS AND METHODS

All experiments were performed in compliance with the *Guidelines for Animal Experimentation in Faculty of Medi-*

cine, Tottori University under the *International Guiding Principles for Biomedical Research Involving Animals.*

Mice

The *Pdn* (Polydactyly Nagoya) mouse (*Gli3*^{Pdn}) was derived from Jcl : ICR (Hayasaka *et al.* 1980) and has been inbred in Naruse's laboratory, now at the 119th generation. *Pdn/+* has one extra digit of the distal phalangeal type preaxially in the hindlimb and a deformity of the distal phalanx of the 1st digit in the forelimb, but no other abnormalities (Naruse & Kameyama 1982). The brain of *Pdn/+* is regarded as normal (Naruse *et al.* 1990). *Pdn/Pdn* mice were obtained from matings of *Pdn/+* with *Pdn/+*. *Pdn/Pdn* exhibits preaxial polydactyly of the duplicated or triplicated metacarpal/metatarsal type both in the forelimbs and hindlimbs. *Pdn/Pdn* newborns exhibit various brain malformations including absence olfactory bulbs and hydrocephalus (Naruse *et al.* 1990; Naruse & Ueta 2002) and they die soon after birth because of a suckling dysfunction (Hongo *et al.* 2000). Gene expressions were examined in four embryos of each genotype from day 8 to 18 and in four newborns of each genotype.

Genotyping method

As shown in Fig. 1, for genotyping, the forward primer (F5) in 167 bp upstream of the insertion point of the transposon and the reverse primer (R6) in 46 bp downstream of the insertion point, and the forward primer (etn11F) in the long terminal repeat region of the transposon were constructed as described in the previous report (Ueta *et al.* 2002). Only a 214-bp PCR product by F5 and R6 was observed in +/+ embryos. A 214-bp product by F5 and R6, and a 180-bp product by etn11F and R6 were observed in *Pdn/+* embryos. And, only a 180-bp product by etn11F and R6 was observed in *Pdn/Pdn* embryos. From the PCR products, we determined the genotypes of the young *Pdn* mouse embryos using genomic DNA extracted from the yolk sac membrane (Fig. 2). After day 13, *Pdn* embryos were genotyped by their limb morphology (Naruse & Kameyama 1982).

Analyses of gene expressions

As *Gli3* related genes, we inspected *Shh*, *Bmp-2*, *Bmp-4* and *ptc-1* gene expressions (Ming *et al.* 1998; Theil *et al.* 1999; Garrett *et al.* 2003; Motoyama *et al.* 2003) in the present study. After genotyping, total RNA was isolated from the *Pdn* mouse embryo proper on days 8, 9, 10 and 11 (Fig. 2) using ISOGEN (Nippon Gene, Tokyo, Japan) for the analyses of gene expressions. The cDNA was synthesized from 3 mg of total RNA that was treated with deoxyribonuclease (Nippon Gene, Tokyo, Japan), M-MLV reverse transcriptase (Gibco BRL, Rockville, USA) and ribonuclease inhibitor (Wako, Tokyo, Japan), using Random Hexamers (Promega, Madison, WI, USA). The synthesized cDNA products

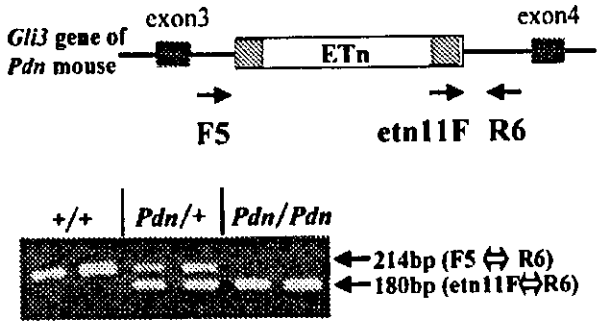

Fig. 1 Quick discrimination of the genotypes in the *Pdn* mouse. Primers F5 and R6 were constructed in intron 3 just upstream and downstream of the insertion point of the transposon. The etn11F primer was also constructed in the long terminal repeat of the transposon. Using these primers, we obtained the 214 bp product with F5 and R6 in +/+ embryos, 214 bp product with F5 and R6, and 180 bp product with etn11F and R6 in *Pdn/+* embryos, and only the 180 bp product was obtained with etn11F and R6 in *Pdn/Pdn*.

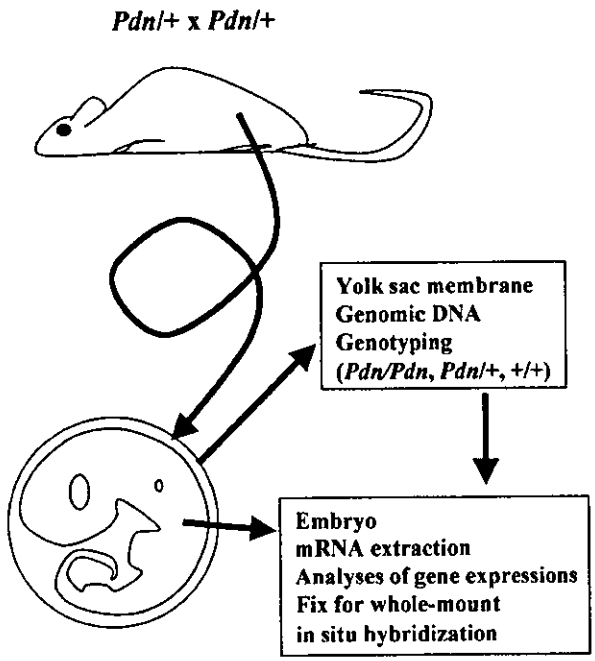

Fig. 2 Experimental design in the present study. Mouse embryos and newborns were recovered from *Pdn/+* × *Pdn/+* on various days. Genomic DNA was extracted from yolk sac membrane for genotyping, *Pdn/Pdn*, *Pdn/+* and +/+. mRNA was extracted from the embryo proper for the analyses of gene expression by real-time PCR and embryos were also fixed for whole-mount *in situ* hybridization.

(0.1 μL) was subjected to real-time PCR in a reaction mixture (10 μL) containing FastStart DNA Master SYBR Green I mix (Roche Molecular Biochemicals, Mannheim, Germany), 3 mM $MgCl_2$ and 10 μM of each primer. *Gli3*, *Shh*,

Bmp-2, *Bmp-4* and *Ptc-1* primers were designed from the published nucleotide sequences (GenBank Database). All real-time amplification reactions were performed on a Light-Cycler Instrument (Roche Molecular Biochemicals, Mannheim, Germany). The settings for the thermal profile were an initial denaturation (10 min at 95°C) followed by 40 amplification cycles: denaturation for 15 s. at 95°C; annealing for 5 s. at 55°C; and elongation for 10 s. at 72°C. Final melting curve analysis followed PCR was performed with a temperature profile slope of 0.1°C/s. from 65 to 95°C. A negative control without a cDNA template was run with each assay to assess overall specificity. Gene-specific standard curves were generated using 10-fold serial dilutions. The amount of target mRNA was expressed as the ratio of β-actin mRNA.

As shown in Fig. 2, after genotyping, embryos were also fixed in 4% paraformaldehyde. *In situ* hybridization on whole-mount mouse embryos was performed as described by Xu & Wilkinson (1998) using *Gli3* and *Shh* riboprobes. Those specimens were inspected for the ectopic and/or varied expression of *Gli3* and *Shh*.

RESULTS

Depression of *Gli3* gene expression in the *Pdn/Pdn* was observed throughout all of the developmental stages examined. *Gli3* expression in *Pdn/+* was about 60% of +/+, and that in *Pdn/Pdn* was about 20–30% of +/+ from day 8 to 11 (Fig. 3). The same tendency was observed in the *Pdn* embryos from day 12 to 18 and in newborns (data is not shown). *Gli3* gene expression level was not dependent on the developmental stage. Double over-expression of *Shh* gene in the *Pdn/Pdn* compared with +/+ was observed only on day 9 (Fig. 4). *Shh* gene expression level was not different among the genotypes on the other days and did not depend on the developmental stage. In other genes, *Bmp-2*, *Bmp-4* and *ptc-1*, we could not detect any alterations among genotypes and developmental stages (data is not shown).

We searched the ectopic and/or varied expression of *Gli3* and *Shh* using whole-mount *in situ* hybridization method in the +/+ and *Pdn/Pdn* embryos on day 9. *Gli3* expression was observed in the neuroectoderm in +/+ but not in *Pdn/Pdn* on day 9 (Figs 5A.B). *Shh* expression was observed in the notochord, floor plate and prechordal mesoderm in both +/+ and *Pdn/Pdn* embryos on day 9. No ectopic and/or over-expression of *Shh* was detected in *Pdn/Pdn* embryos on day 9 (Figs 5C.D).

DISCUSSION

The *GLI3* gene, which is responsible for GCPS, was mapped on Chromosome 7p13 (Vortkamp *et al.* 1991, 1992; Wild *et al.* 1997). Deletions were detected in the *Gli3* gene in the *Xt* and Xt^{J} mouse (Schimmang *et al.* 1992; Hui & Joyner 1993; Maynard *et al.* 2002), and the *Gli3* gene was mapped on chromosome 13A2 which is the synteny region of human 7p. From this knowledge, *Xt/Xt* and Xt^{J}/Xt^{J} were considered

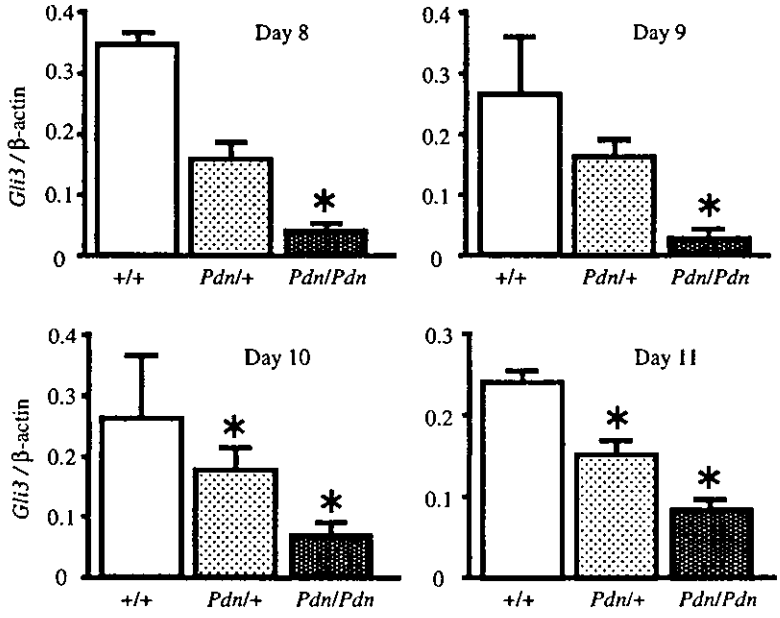

Fig. 3 Expression of the *Gli3* gene in *Pdn* mouse embryos. *Gli3* gene expression was analyzed using the real-time PCR method from day 8 to 11 of gestation. The expression of *Gli3* in the *Pdn/Pdn* and *Pdn/+* were repressed in the early embryos. Increase or decrease of *Gli3* gene expression was not observed on different embryonic days, regardless of genotype. (*) Significantly different from +/+ by one-way ANOVA ($p < 0.01$).

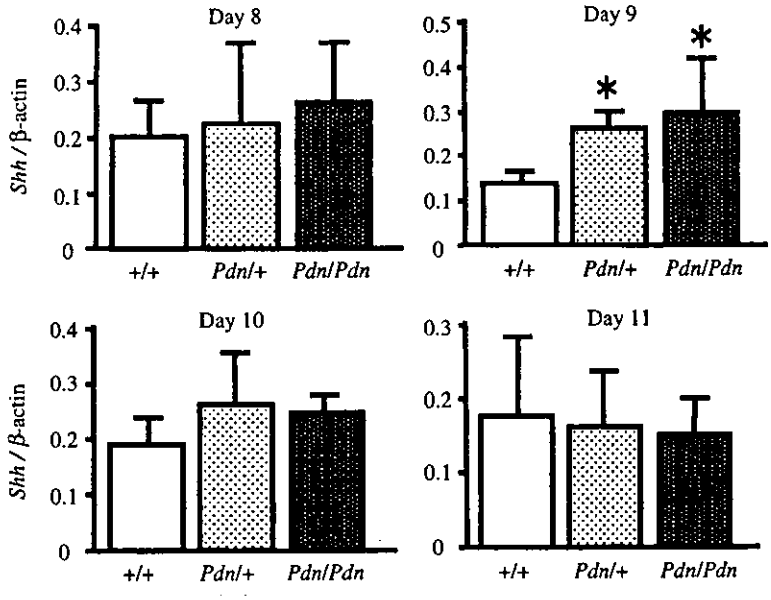

Fig. 4 Expression of the *Shh* gene in the *Pdn* mouse embryos. *Shh* gene expression was analyzed with real-time PCR method from day 8 to 11 of gestation. Increase of *Shh* expression was observed in *Pdn/Pdn* and *Pdn/+* compared with +/+ only on day 9. Increase or decrease of *Shh* expression was not observed on different embryonic days, regardless of genotype. (*) Significantly different from +/+ by one-way ANOVA ($p < 0.05$).

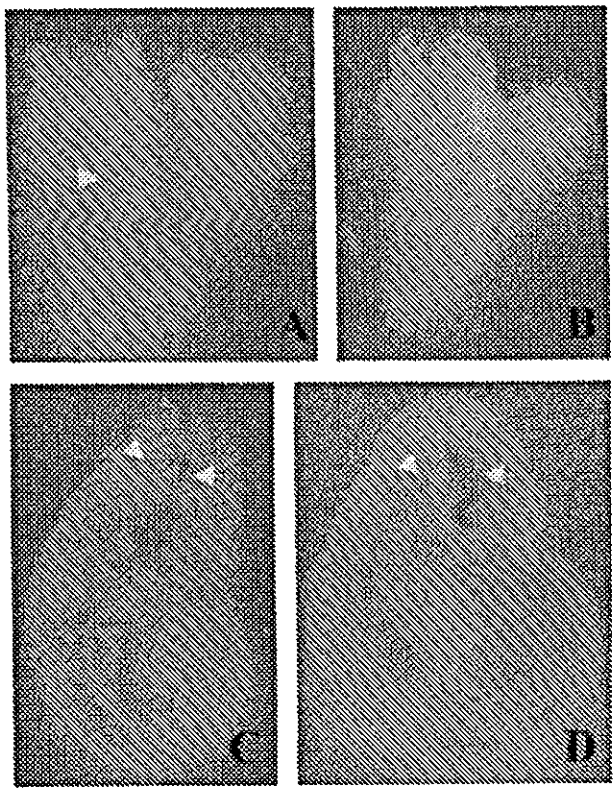

Fig. 5 Whole-mount *in situ* hybridization specimens. Neuroectoderm (arrowhead) was positive in +/+ (**A**) but not in *Pdn/Pdn* (**B**) embryos on day 9 with *Gli3* probe. Notochord and floor plate (arrowheads), and prechordal mesoderm (arrows) were positive in both +/+ (**C**) and *Pdn/Pdn* (**D**) embryos on day 9 with *Shh* probe. We could not detect ectopic and/or over-expression of *Shh* in *Pdn/Pdn*.

to be the mouse homolog of GCPS (Winter & Huson 1988). As the *Pdn* mouse has been considered to be an allele of *Xt*, alteration of the *Gli3* gene has been analyzed. Severe depression of *Gli3* gene expression was observed in *Pdn/Pdn* embryos suggesting the gene responsible for *Pdn* was *Gli3* (Naruse & Keino 1995; Naruse *et al.* 2000, 2001), and *Pdn/Pdn* has been considered to be the mouse homolog of GCPS (Naruse & Keino 1995). Insertion of a transposon into intron 3 of the *Gli3* gene in *Pdn* mouse has been reported (Thien & Rüther 1999; Naruse *et al.* 2000, 2001), and we developed the quick genotyping method using PCR primers in intron 3 and the transposon (Ueta *et al.* 2002).

In the present study, double over-expression of *Shh* was observed only on day 9 in the *Pdn/Pdn* compared with +/+. Day 9 is a critical period for brain and limb morphogenesis. But, it is not clear that, though the depression of *Gli3* expression was observed in *Pdn/Pdn* throughout all of the developmental stages, *Shh* expression was not altered in any period except on day 9 in the *Pdn/Pdn*. These data do not support the concept that *Gli3* and *Shh* depress the expression each other (Büscher *et al.* 1997; Litingtung & Chiang 2000; Litingtung *et al.* 2002). As *Shh* is upstream of *Gli3* in the signaling pathway (Ming *et al.* 1998), depression of *Gli3* expression may be affected indirectly or through another pathway to *Shh* (Aoto *et al.* 2002; Lamm *et al.* 2002; te-Welscher *et al.* 2002; Altaba *et al.* 2003; Kushels *et al.* 2003). In the present study, even on day 9, over-expression of *Shh* in *Pdn/Pdn* was just double that of +/+, although expression of *Gli3* in *Pdn/Pdn* was 1/4 of +/+.

In the whole-mount *in situ* hybridization specimens, we could not detect ectopic and/or over-expression of *Shh* in

Pdn/Pdn embryos on day 9. Double over-expression level may not be detectable by whole-mount *in situ* hybridization.

The responsible gene for GCPS is *GLI3*, but we do not know how the phenotypes of GCPS appear. It is our strategy that the mechanism of the manifestation in the homologous mouse, *Pdn/Pdn*, may be extrapolated to that of GCPS. Investigation of gene expression in the young embryos before the malformations appear is of particular importance.

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特集

児童・小児精神障害

自閉症の遺伝に関する研究：最近の知見を中心に

難波栄二*

KEY WORDS

・自閉症
・遺伝的要因
・ゲノム刷り込み
・セロトニン

SUMMARY

自閉症の遺伝的研究について最近の研究を中心に概説した．自閉症は精神神経疾患のなかでも遺伝的要因の強い疾患で，近年遺伝に関する研究が非常に増えてきている．染色体異常，連鎖解析，関連遺伝子解析などのオーソドックスな方法に加えて，ゲノム刷り込みが重要な鍵を握ると考えられる．多くの染色体領域が関連するが，7q，15qなどゲノム刷り込みの領域，またセロトニン関連遺伝子，脳の発生に関係する遺伝子などを中心にさまざまな遺伝子が解析されてきている．しかし，自閉症に重要な遺伝子は現段階では明らかではない．今後は脆弱X症候群などの研究成果も参考に，DNAチップなど新しい方法も導入され，飛躍的に研究が進むと考えられる．

はじめに

近年，ほかの多くの疾患と同様に，精神神経疾患に関しても遺伝的研究が注目を集めるようになってきた．従来は，比較的まれな一つの遺伝子の異常で発症する疾患（単一遺伝子病）の研究が中心であったが，近年は統合失調症（精神分裂病）や躁うつ病に関する遺伝的研究も盛んにおこなわれるようになってきた．自閉症は精神神経疾患のなかでは，最も遺伝要因の強い疾患と考えられている．自閉症に関連する遺伝子が明らかにされることは，患者さんにとって大きな朗報であると同時に，神経科学の大きなブレークスルーと考えられる．近年，自閉症の遺伝に関する研究報告が飛躍的に多くなってきている．本稿を執筆している最中にも，新しい報告が次々となされており，すべてをフォローすることさえ容易でない状況である．これらの報告のなかには，いわゆる「ネガティブデータ」も多い．しかし，これらの膨大なデータの蓄積から新しい方向が見出され，近い将来自閉症の本質に迫る発見がなされると予想される．

本稿では，できるだけ最新の報告を大まかに分類し紹介する．本稿により，今までの研究の流れを把握していただき，今後の膨大な研究報告を理解する一助になれば幸いである．

1．遺伝的特徴

自閉症の発症頻度は1/500から1/2,500と報告されており，男性に多い（男女比：4対1）[1)2)]．同胞での再発危険率は約2%から4%であるが，一卵性双生児の発症一致率は約60%から80%と著しく高いことが大きな特徴である[3)4)]．さらに，自閉症の罹患同胞には双生児が

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多いという特徴も明らかになっている[5]．これらの事実が，自閉症の原因には遺伝的背景が強いという根拠になっている．しかし，この特徴はメンデルの法則では説明できず，複雑な遺伝メカニズムと推測される．

2．遺伝研究の流れ

まず，自閉症では染色体異常が検討された．つぎに家系例のDNAを用いて多くのマーカー遺伝子を解析することにより，原因遺伝子の染色体上の位置をさぐる研究（連鎖解析）が進められている．さらに，染色体の位置や生化学的特徴などから候補遺伝子を推測し，関連を直接調べる研究も多い（関連遺伝子解析）．しかし，これらの比較的オーソドックスな遺伝研究の手法では確実な結果は得られておらず，新たな手法や考え方の導入も必要と思われる．そのひとつとして，ゲノム刷り込み（後述）があり，新たなアプローチとして注目される．また，自閉症の症状と関連する単一遺伝子病（結節性硬化症，脆弱X症候群，レット症候群）の研究も自閉症の解明のヒントになると考えられる．以下，それぞれの研究方向ごとに概説する．

3．染色体の異常

染色体異常は自閉症患者の約3%にみられ，14番と20番の染色体を除くすべての染色体の異常が報告されているが，15番染色体と性染色体の異常の報告が多い[6)7)]．これらの例は，そのほとんどが個発例であり，遺伝的解析まで至る例は少ない．

1）15番染色体

同腕二動原体染色体や15q11-13の部分的な重複の報告が多く，この部位の微細な欠失が自閉症で多いとの報告もある[8)9)]．この領域は，ゲノム刷り込みを受ける遺伝子が多く存在している（後述）．

2）X染色体

自閉症は男性に多く，X染色体との関連も研究されてきた．1997年，Skuseら[10)]はターナー症候群患者のX染色体の由来と認知能力に注目して研究を進め，ゲノム刷り込み機構をうける認知に関連した遺伝子（CGF-1）の存在を示した．連鎖解析では自閉症との連鎖は示されないものの，CGF-1遺伝子は何らかの関係があるかも知れない．

3）そのほかの染色体異常

そのほかの染色体の異常では2q染色体の染色体末端のテロメアに近い部分の欠失の報告がある[11)]．また最近，マーカー遺伝子の解析から7番，8番の染色体の一部の欠失の頻度が高いという報告もなされている[12)]．

4．連鎖解析

1980年代半ばから，原因不明の遺伝性疾患では，染色体上の位置を明らかにし，遺伝子を単離するポジショナールクローニングとよばれる方法によってつぎつぎと原因が明らかにされてきている．この方法では，多くの家系サンプルを集め，ゲノムDNA上のマーカー遺伝子解析をおこなう連鎖解析が必要になる．自閉症では家系内の患者は多くないために，両親と同胞のサンプルで解析可能な罹患同胞対解析という連鎖解析のひとつを用いる．近年，全ゲノム領域を対象にした研究が次々に発表されてきている[13)~17)]．ヨーロッパでの共同研究では，6つの染色体上（4番，7番，10番，16番，19番，22番）で，ある程度の関連が示唆され，7qが比較的強い関連領域であった[13)]．そのほかの報告でも，共通してある程度の連鎖（LODスコア-3以下）が示唆される領域として7qが挙げられる．さらに，父由来と母由来の遺伝子により連鎖解析の結果が異なり，父由来の遺伝子のみ関連しているとする報告もあり，ゲノム刷り込みを示唆する[18)]．最近，さらに2番染色体との関連を示す報告もある[19)]．これらの研究を総合すると，自閉症には染色体の多くの領域が関連する可能性が高い．さらに，これらの関連する領域の差は民族ごとに原因遺伝子が異なる可能性もあり，いまだ報告のない日本人での連鎖解析を早急に進める必要がある．

5．自閉症の候補遺伝子

1）15番染色体の遺伝子

15q11-q13領域の遺伝子との関連について多くの研究がなされている[20)~27)]．Cookら[20)]は脳の興奮を制御す

るGABA（γ-aminobutyric acid）受容体β3サブユニット多型との関連を示したが，その後の報告ではこの遺伝子の関連を示す結果は得られなかった[20)~22)]．しかし最近の報告では，Angelman症候群の原因遺伝子UBE3A遺伝子やATP10C遺伝子，GABRB（γ-aminobutyric acid type-A receptor beta 3 subunit）3遺伝子などの解析がなされ，その関連を示唆する報告も増えている[23)~26)]．さらに，この部位に新しい遺伝子も同定されてきている[27)]．

2）7q染色体の遺伝子

7qは，連鎖解析から最も注目される領域である．7qの腕内逆位の家系から，7q31領域において言葉に関連するSPCH1領域が明らかにされ，さらにこの領域からFOXP2遺伝子が単離された[28)29)]．この遺伝子は，ハンチントン舞踏病などの遺伝子にみられるグルタミンのくり返し配列をもつ興味深い遺伝子であるが，自閉症との関連はない[30)]．さらに，7番染色体にはセロトニン受容体5-HT2A遺伝子，脳の形成に関連するWNT2遺伝子，Reelin遺伝子なども存在している．WNT2やReelin遺伝子は，自閉症と関連する報告がある[31)32)]．WNT2やReelin遺伝子は脳の発生に関連する遺伝子としても注目される．

3）セロトニン関連遺伝子

自閉症では血小板や尿中のセロトニン（5-HT）値が高いことや，脳でのセロトニン代謝異常の報告などから，セロトニン関連遺伝子に注目した研究も多い．Cookら[33)]はセロトニントランスポーター（5-HTT）遺伝子のプロモーター領域多型が自閉症と関連する可能性を最初に報告した．しかしその後多くの研究では，その結果は必ずしも一致しない[34)~38)]．最近，この領域の連鎖や遺伝子の一塩基多型（SNP）を利用した研究によると自閉症との関連を示唆している[39)40)]．そのほかのセロトニン関連遺伝子としては，Tryptophan Hydroxylase遺伝子，5-HT2A，5-HT2B，5-HT7などは関連がないと報告されている[41)~43)]．X染色体で上の5-HT2Cは関連する可能性も報告されている[44)]．

4）脳の発生に関連する遺伝子

Ingramら[45)]は，サリドマイドによる脳奇形の発症時期が自閉症と関連すると考え，HOX1A，HOX1B遺伝子に注目した．彼らはHOX1A遺伝子が関連するとの報告をおこなったが，その後それを否定する報告がなされている[46)]．われわれは，日本人自閉症患者にHOX1A遺伝子の特徴的な異常があることを最近見出している．

前述の，7番染色体上に存在するWNT2遺伝子，Reelin遺伝子も脳の発生に重要な遺伝子であり，これらとの関連を示す報告がなされている[31)32)]．

5）そのほかの遺伝子

X染色体上のGRPR（gastrin-releasing peptide receptor）遺伝子，神経線維腫症のNF1遺伝子，甲状腺ホルモン受容体複合体遺伝子HOPA遺伝子，VIPR2（vasoactive intestinal peptide receptor type 2）遺伝子，免疫不全症の原因adenosine deaminase遺伝子，glutamate receptor 6遺伝子核内など，多くの遺伝子との関連が研究されている[47)~51)]．

6．ゲノム刷り込み

ゲノム刷り込みは父親と母親由来の対立遺伝子が識別され，異なる発現を示す現象であり，15q領域の異常によって発症するPrader-Willi症候群とAngelman症候群をはじめとして，多くの疾患の病態に関連すると考えられている．7q32の領域でもゲノム刷り込み遺伝子が発見されており，多くのゲノム刷り込み遺伝子が自閉症候補遺伝子として研究の対象になっている（前章参照）．「ゲノム刷り込み」は，今後の自閉症研究のキーワードの一つである．

7．単一遺伝子病

結節性硬化症は常染色体性優性遺伝病で，16番染色体上のTSC1遺伝子か9番染色体上のTSC2遺伝子の異常によって発症する．結節性硬化症は自閉症の症状を示すことも多い（17-68%）[52)]．脆弱X症候群はX染色体に存在するFMR-1遺伝子の異常によって発症する[53)]．この疾患は精神遅滞が中心であるが，自閉的な傾

向をもつために自閉症との関連も研究されてきた．自閉症の患者での遺伝子異常の報告は多くないが，われわれは自閉症患者に遺伝子異常を見出した例を経験している[54]．X染色体に存在するMECP2遺伝子異常によりレット症候群が発症する．レット症候群は女性に発症する疾患ではあるが，男性の精神遅滞患者のなかにもMECP2遺伝子の異常を示す例が発見されている．自閉症患者でもMECP2の異常が検討されたが，異常は発見されていない[55]．

以上のように，これらの疾患は自閉症患者と直接関係は少ないが，その病態には共通したものがあると考えられている．これらの疾患では，遺伝子異常がなぜ脳機能異常を引き起こすかの分子メカニズムの研究が進んでいる．自閉症に共通した分子メカニズムの解明が，自閉症の研究にとっても重要な課題である．

8．研究の問題点と今後の展開

今までの連鎖解析や関連遺伝子解析の報告では，報告ごとに結果が一致しない場合も多い．これは，一つには自閉症の診断基準の問題があげられる．しかし，より厳密な診断基準を適応するだけでは解決しない問題もある．たとえば，自閉症の家系内には，しばしばADHDなどほかの診断名のついた患者が存在する．これらの患者は，遺伝的異常には共通のものが存在すると推測され，これらを除外して遺伝的研究を進めても決して正しい結果を得ることができない．また，民族的な遺伝的背景の違いを考えて研究することも重要である．われわれは5-HTT遺伝子多型の分布が日本人では欧米とは大きく異なっていることを見出したが，近年，多くのDNA多型には民族的な差があり，この差を考えながら研究することが重要とされてきている[56]．さらに，ゲノム刷り込みに注目して研究を進めることは重要だが，ゲノム刷り込み遺伝子の多くが明らかになっていない．ゲノム刷り込みに関連する遺伝子を効率的に解析し，自閉症の候補遺伝子として研究していくことが求められる．

現在ゲノム研究の手法は飛躍的に進歩している．全ゲノムに存在する多数の一塩基多型（SNP）をいっきに解析し，正確な関連領域を検討する方法が開発されてきている．また，DNAチップを用いた研究も盛んになってきている．自閉症の脳を用いたDNAチップによる，発現遺伝子解析がなされており，グルタミン酸などの神経伝達物質の異常を示唆する報告もなされている[57]．脆弱X症候群など自閉症と深く関連する疾患の病態に関連する遺伝子もDNAチップで解析されており，これらの遺伝子のなかに，自閉症に関連する遺伝子が存在する可能性も高い[58]．さらに本稿では述べることができなかったが，動物モデルを開発して研究を進めることも重要になる．

日本での研究は，残念ながら諸外国より遅れていると言わざるを得ない．しかし，自閉症の関連遺伝子は民族ごとに異なっている可能性があり，日本人自閉症での研究を積極的に推進する必要がある．

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Association Between the Neurofibromatosis-1 (NF1) Locus and Autism in the Japanese Population

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Autistic patients have a 100 to 190-fold increased risk of neurofibromatosis compared to the general population. This suggests that the two diseases may share a common etiological background. Recently, a new allele (or the six-repeat allele) of the $(AAAT)_n$ repeat polymorphism in an Alu sequence in the neurofibromatosis-1 (*NF1*) gene was observed exclusively in severe autistic patients, not in controls, in Caucasians of French ancestry. This suggests a role of the *NF1* gene in the development of autism. We investigated three microsatellite polymorphisms within the intron-27b and intron-38 of the NF1 region, including the $(AAAT)_n$ and two (CA)n repeat polymorphisms, in Japanese subjects with autism (n = 74) and controls (n = 122). The six-repeat allele of the $(AAAT)_n$ polymorphism was not found either in patients or controls, possibly indicating an ethnic difference in the polymorphism. However, significant differences were observed in the allele distributions of the $(AAAT)_n$ and a $(CA)_n$, which were located at intron-27b, between patients and controls, although an association was not significant between autism and another polymorphism at intron-38. This may suggest an involvement of the NF1 locus in susceptibility to autism, although further investigations are recommended. © 2004 Wiley-Liss, Inc.

KEY WORDS: autism; genetic association study; neurofibromatosis-1; Alu; tetranucleotide repeat

INTRODUCTION

Autistic disorder, also known as infantile autism, is a severe developmental disorder of early childhood. The disorder is characterized by sustained impairments in reciprocal social interaction, communication deviance, and stereotyped behavioral patterns. Abnormal functioning in the areas appears in the first 3 years of life and persists into adulthood. Mental retardation (MR) is observed in two third of the patients. The disorder usually starts before childhood, with a three times more frequency in boys than in girls. Estimates of the population prevalence of autism are in the order of 5–10 per 10,000 [Bryson et al., 1988; Fombonne, 1999].

Etiological factors remain to be clarified in autism, however family and twin studies strongly suggest a genetic effect. Prevalence of autism in siblings of patients with the disorder is 50–100 times greater than in the general population [Rutter et al., 1999]. The concordance rates of the disorder in monozygotic and dizygotic twins were reported to be 36 and 0% [Folstein and Rutter, 1977], and more recently, 91 and 0% [Steffenburg et al., 1989], and 60 and 0% [Bailey et al., 1995], respectively.

Autistic patients have a 100 to 190-fold increased risk of neurofibromatosis compared to the general population [Gillberg and Forsell, 1984; Lenoir, 1989], which suggests that the two diseases may share an etiological factor. *NF1* gene, a susceptibility gene for neurofibromatosis-1, is located at 17q11.2. A chromosomal region 17q11.1-2 close to the *NF1* gene has been suggested for linkage with autism in a study [IMGSAC, 2001]. Its product, neurofibromin, regulates Ras proteins, the

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product of protooncogene H-Ras markers. Previous studies observed a significant association of the H-Ras markers with autism [Herault et al., 1995; Comings et al., 1996]. These observations indicate the NF1 region as a candidate locus for autism.

The NF1 region contains a GXAlu polymorphism of $(AAAT)_n$ repeats in intron-27b [Xu et al., 1991]. Four alleles corresponding with seven to ten (AAAT) repeats were found in the polymorphism [Xu et al., 1991]. A study in Caucasians of French ancestry observed a new allele of the polymorphism (corresponding to six (AAAT)s) exclusively in severe cases of autism [Mbarek et al., 1999]. This suggests that the NF1 locus may be associated with susceptibility to autism, although a study in Caucasian and Africans from North-American populations did not find the allele in autistic patients or control subjects [Plank et al., 2001].

In the present study, we investigated the $(AAAT)_n$ polymorphism in Japanese subjects with autism and healthy controls. The allele distributions, including the frequencies of the six-repeat allele, were compared between patients and controls. In addition to the $(AAAT)_n$ polymorphism, two other microsatellite (or $(CA)_n$ repeat) polymorphisms at the NF-1 locus were studied. The two polymorphisms, IVS27AC28.4 at intron-27b [Lazaro et al., 1993] and IVS38GT53.0 at intron-38 [Lazaro et al., 1994], had not previously been investigated in autistic patients. These two microsatellite markers have been observed to associate with NF1 [LOD = 2.76–3.64, Fang et al., 2002].

SUBJECTS AND METHODS

Subjects comprised 74 unrelated Japanese patients with autism (62 males and 12 females, age range: 9–43 years (mean = 25.0 years)). The patients were recruited from the Outpatient Clinic for Autism and Mental Retardation, Department of Psychiatry, University of Tokyo and from several rehabilitation facilities for people with developmental disorders, including autism, in Tokyo. All patients met the DSM-IV criteria for autistic disorder. All of the patients had been diagnosed with autism previously by child-psychiatrists at our clinic or elsewhere. However, the diagnosis was reconfirmed for the present investigation according to the following procedure. Patients and at least one of the parents (mostly mothers) were interviewed by two of authors (O.H and M.I), who were child-psychiatrists and had more than 10 years of experience in diagnosis and treatment of pervasive development disorders (PDD) including autism. Clinical records were also reviewed. The diagnosis of autism was reached when the subject's condition met the DSM-IV criteria for autistic disorder. The Child Behavior Questionnaire Revised (CBQ-R) [Isutsu et al., 2001] was used as a supplementary scale for the diagnosis. We used the CBQ-R to find out characteristic behavior of autism in the patients. Parents were asked to rate their child on each item. The CBQ-R is a supplementary scale for the diagnosis of PDD in child and adult patients. Reliability and validity of the questionnaire as a supplement tool of diagnosis have been confirmed in 269 PDD subjects (age: 2–26 years) and 76 MR subjects (age: 3–26 years) [Isutsu et al., 2001].

IQ levels, assessed by using the Tanaka–Binet test (Japanese-specific modification of the Binet test, validated and the most widely used in public-health-service settings in Japan), were below 50 in 48 patients and below the borderline level (or below 70) in the rest. Most patients were at mental age above 18 months, except for two adult patients. Apparent dysmorphic features were not observed in any of the subjects.

Control subjects consisted of 122 unrelated healthy subjects (51 males and 71 females, age range: 21–65 years (mean = 48.3 years)). They were recruited from hospital staff of general and mental hospitals in Tokyo and adjacent prefectures. The hospitals included the Tokyo University Hospital, Tokyo, Saitama-Saiseikai Konosu Hospital, Saitama, and Haryugaoka Mental Hospital, Fukushima. All the patients and control subjects were ethnically Japanese. The study was approved by the Ethical Committee of the Faculty of Medicine, University of Tokyo. Objective of the study was explained and written informed consent was obtained from all the subjects. No symptoms of neurofibromatosis were observed in the present patients and the controls.

The whole region of the $(AAAT)_n$ repeat was amplified using PCR (forward-primer: CAAGAAAAGCTAATATCGGC; reverse-primer: GGAACCTTAAGTTCACTTAG, annealing temperature 52°C, product size: around 400 bp). PCR samples contained 200 ng genomic DNA, 200 μmol/L each dNTP, 20 pmol of each primer, ExTaq buffer, and 2 U Ex-Taq buffer and 2 U Ex-Taq polymerase (Takara, Kyoto, Japan) in a final volume of 20 μl. After the initial denaturation the reaction mixtures were subjected to 35 cycle of PCR consisting of 30 sec at 94°C, 60 sec at 52°C, 90 sec at 72°C. To identify the alleles, the region containing the polymorphic site of the initial PCR product was amplified using Cy-5 labeled forward-primer: AGAGCAAGACTCTGTATGAAATAA and the reverse-primer: GGAACCTTAAGTTCACTTAG (annealing temperature 52°C, product size: around 120 bp). This was to make the PCR product shorter and to enable reliable identification of the alleles. The size of the final PCR product was determined using the Alfred automatic sequencer with the Allele Link software (Amersham Biosciences, Piscataway, NJ, USA). To confirm the sequence and the number of the repeats, the PCR products in several subjects were directly sequenced using the ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). Regions containing the CA repeats of the IVS27AC28.4 and the IVS38GT53.0 were amplified by PCR. For the IVS27AC28.4, the primer seqences were: GTTCTCAACTTAAATGTAAGT (forward) and GAACATTAACAACAAGTACC (reverse), and the annealing temp was 55°C [Lazaro et al., 1994]. For the IVS38GT53.0, the primer sequences were: CAGAGCAAGACCCTGTCT (forward) and GAACATTAACAACAAGTACC (reverse), and the annealing temperature was at 52°C [Lazaro et al., 1993]. The forward-primers were labeled with Well Red dyes D4-PA (Proligo, La Jolla, CA, USA). The sizes of these PCR products were determined using SEQ 8000 sequencer (Beckman-Coulter, Fullerton, CA, USA).

Allele distributions of the polymorphisms were compared between the patients and control subjects, using chi-square test.

RESULTS

Table I summarizes allele frequencies of the GXAlu polymorphism at the intron-27b. Four alleles with seven to ten (AAAT) repeats were observed in patients and controls. The six-repeat allele, which was found exclusively in severe autistic patients in a previous study [Mbarek et al., 1999], was not observed in the present patients or controls. However, a significant difference in the allele frequencies was found between the patients and controls ($\chi^2 = 11.57$, df = 3, $P = 0.009$). An increase of the nine-repeat allele with a decrease of the eight-repeat allele was observed in the patients (61.5 and 36.5%, respectively) compared with the controls (44.2 and 54.2%, respectively; $\chi^2 = 11.45$, df = 1, $P = 0.0007$), when the frequencies of the nine-repeat allele and the eight-repeat allele were compared).

As summarized in Tables II and III, eight and nine alleles were observed in the IVS27AC28.4 and IVS38GT53 polymorphisms in the Japanese subjects. A significant difference in allele distribution was also observed in the IVS27AC28.4 polymorphism at the intron-27b between patients and controls (Table II, $\chi^2 = 21.1$, df = 9, $P = 0.012$). There appeared to be an increase of the 209 bp-allele, with a decrease of the 205 bp-allele, in the patients (10.14 and 12.84%, respectively) compared with the controls (3.69 and 20.84%, respectively; $\chi^2 = 9.68$, df = 1, $P = 0.002$, when the frequencies of the 209 and the 205 bp-alleles were compared). In contrast to the two polymorphisms in intron-27b, no significant difference was observed in the allele distributions of the IVS38GT53 polymorphism at intron-38 between the patients and controls ($\chi^2 = 9.80$, df = 9, $P = 0.367$, Table III). When rare alleles in each table were combined not to make sparse cells, essential changes were not found in the statistical results. Genotype distributions of any three markers in patients or controls were not significantly deviated from the Hardy–Weinberg equilibrium ($P > 0.05$). Allele frequencies were not different between males and females in patients or controls in any of the three markers.

TABLE I. Allele Frequencies of the GXAlu Polymorphism in Intron-27b of the *NF1* Gene in Chromosomes From Japanese Patients With Autism and Control Subjects

	Allele frequencies	
Allele	Patients (n = 148)	Controls (n = 240)
1 (407 bp, 10 times repeats)	2 (1.35%)	3 (0.13%)
2 (403 bp, 9 times repeats)	91 (61.49%)	106 (44.17%)
3 (399 bp, 8 times repeats)	54 (36.49%)	130 (54.17%)
4 (395 bp, 7 times repeats)	1 (0.67%)	1 (0.42%)

$\chi^2 = 11.57$, df = 3, $P = 0.00901$; $\chi^2 = 11.45$, df = 1, $P = 0.000714$, when the frequencies of the 399 and the 403 bp-allele are compared; $\chi^2 = 11.70$, df = 1, $P = 0.00062$, when the 395 and 403 bp-allele are combined with the 399 and 407 bp-allele, respectively.

TABLE II. Allele Frequencies of the IVS27AC28.4 Polymorphism in Intron-27b of the *NF1* Gene in Chromosomes From Japanese Patients With Autism and Control Subjects

	Allele frequencies	
Allele (bp)	Patients (n = 148)	Controls (n = 244)
219	3 (2.03%)	2 (0.82%)
217	3 (2.03%)	0 (0%)
215	2 (1.35%)	1 (0.41%)
213	1 (0.68%)	6 (2.46%)
211	1 (0.68%)	3 (1.23%)
209	15 (10.14%)	9 (3.69%)
207	102 (68.92%)	164 (67.21%)
205	19 (12.84%)	51 (20.90%)
203	0 (0%)	4 (1.64%)
201	2 (1.35%)	4 (1.64%)

$\chi^2 = 21.11$, df = 9, $P = 0.012$; $\chi^2 = 9.68$, df = 1, $P = 0.002$, when the frequencies of the 209 and the 205 bp-allele are compared; $\chi^2 = 18.51$, df = 5, $P = 0.002$, when the 201, 211, and 215 bp-alleles are combined with the 203, 213, and 217–219 bp-alleles.

DISCUSSON

The present study investigated three microsatellite polymorphisms within the *NF1* gene in Japanese subjects with autism and the controls. Significant differences were observed in allele distributions of the two polymorphisms (GXAlu and IVS27AC28.4), between the patients and controls. These polymorphisms are both located at the intron-27b of the NF1 region. Regarding the GXAlu polymorphism, four alleles were observed in the Japanese patients and controls. Among the four alleles, the nine-repeat allele was increased and the eight-repeat allele was decreased significantly in the patients compared to the controls. Those alleles were the most frequent alleles in Japanese as well as Caucasian and African populations [Plank et al., 2001]. Regarding the IVS27AC28.4 polymorphism, a trend of an increase of the 209 bp-allele with a decrease of the 205 bp-allele was observed in the patients, compared to the controls. These observations might suggest an involvement of the NF-1 locus in autism, although, no significant association was observed between another

TABLE III. Allele Frequencies of the IVS38GT53.0 Polymorphism in Intron-38 of the *NF1* Gene in Chromosomes From Japanese Patients With Autism and Control Subjects

	Allele frequencies	
Allele (bp)	Patients (n = 146)	Controls (n = 238)
191	1 (0.68%)	0 (0%)
189	2 (1.37%)	6 (2.52%)
187	45 (30.82%)	59 (24.79%)
185	17 (11.64%)	20 (8.40%)
183	9 (6.16%)	13 (5.46%)
181	49 (33.56%)	104 (43.70%)
179	2 (1.37%)	7 (2.94%)
177	15 (10.27%)	32 (13.45%)
175	0 (0%)	1 (0.42%)
173	6 (4.11%)	6 (2.52%)

$\chi^2 = 9.80$, df = 9, $P = 0.367$; $\chi^2 = 6.37$, df = 6, $P = 0.383$, when the 173, 177, and 187 bp-allele are combined with the 175, 179, 189–191 bp-alleles.

polymorphism (the IVS38GT53 at intron-38) and the disease. In the present subjects, no apparent symptoms of the skin or other dysmorphic features, which suggest neurofibromatosis, were observed. Therefore, the significant associations might be related with the development of autism, not only with co-morbidity of neurofibromatosis in autism.

It is now known that high linkage disequilibrium (LD) could be observed among markers of, sometimes, hundreds kb distance. The observed significant association between autism and one of the added markers (IVS27AC28.4), in addition to the association of the initial (AAAT)n marker, could be due to LD between the two markers within the same intron of the *NF1* gene, whereas a previous study did not observe high LD between the two markers in Caucasians [Valero et al., 1996]. The results should be carefully interpreted.

The six-repeat allele of the GXAlu polymorphism, which was specifically found in severe Caucasian patients of the French ancestry [Mbarek et al., 1999], was not observed in the present patients or controls. A substantial portion of the present patients consisted of severe cases, the discrepancy might not be due a difference in severity of the disease between the French and present Japanese patients. Also, this may not necessarily be considered contradictory to the observation in the French–Caucasian [Mbarek et al., 1999]. Ethnic differences in allele distributions of genes are frequent in general, nonetheless it should be acknowledged that the six-repeat allele was not observed in Caucasians (133 patients and 134 controls), Africans (65 patients and 66 controls), and a small number of subjects with other ethnic origins in a North-American study [Plank et al., 2001].

Several limitations of the present study may be acknowledged. First, the ADOS or ADI, which may be currently major tools of the diagnosis of autism in the North-America and West-Europe, was not available in the present study. However, the diagnosis, according to the DSM-IV criteria, was cautiously confirmed by well-experienced child-psychiatrists; patients and the parents were interviewed using a widely used questionnaire in our country (the CBQ-R) and clinical records were carefully reviewed. Nonetheless, the method of the present diagnosis may be cautiously acknowledged.

Secondly, the results may be vulnerable to limitations of the case-control design. All patients and control subjects were ethnically Japanese and recruited from a relatively confined area, around the Kanto District of Japan, in the present study. The Japanese population has experienced no major migrations for more than a thousand years, due to its geographical and cultural isolation, and may have been well mixed. Nonetheless, it is possible that the observed association between the NF-1 locus and autism was due to biases in the case-control design including the population stratification. Weakness of the present study may be that the controls were not well-matched to the patients for the aspects of age and sex. These, especially the latter, may be cautiously borne in mind, although the *NF1* gene is not located on the sex chromosomes and no gender difference was observed in the allele distributions of any of the polymorphisms in the present subjects. Additionally, the control subjects were recruited from hospital staff. This could to some extent affect the result, although the effect might not be very significant.

Another issue to be acknowledged may be that the present patients consisted mostly of those with moderate to severe MR, as indicated in the IQ levels and their verbal dysfunction. Thus, the NF1 locus could be related with the lower level of intelligence, not directly with the development of autism. Furthermore, the sample size is relatively small (74 patients and 122 controls), which could increase a risk of statistical errors. Finally, the chromosomal region of 17q, where the *NF1* gene is located, has been suggested for comparatively weak linkage with autism only in the IMGSAC study [2001], not in the other studies. Therefore, the role of the NF-1 locus in autism, when it really does exist, may be minor. Larger samples might be desirable to obtain adequately reliable statistical power.

The present study may have several methodological limitations. Nonetheless, the result might add a support for a role of the NF-1 locus in the development of autism. The result is of interest and further investigations may be recommended, when the devastating effect of autism on patients' life and evidence for an etiological role of genetic factors in the disease are considered.

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Original article

Gastrin-releasing peptide receptor (GRPR) locus in Japanese subjects with autism

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Abstract

Gastrin-releasing peptide receptor (GRPR) gene is considered a candidate locus for infantile autism for several reasons. The present study investigated two polymorphic sites (C/450/T and C/661/T) in the second exon of the GRPR gene in Japanese patients with autism (DSM-IV) and healthy subjects. The two polymorphic sites were at high linkage disequilirium, consistent with a previous study in a North American population. The C450–C661 allele, which was observed in one-third of the chromosomes from the North American subjects, was less frequent (6–7%) in the Japanese subjects, suggesting a large ethnic difference in the frequency of the polymorphism. The allele frequencies and genotype distributions were not significantly different between the patients and controls. However, further studies are required to exclude the GRPR locus as a candidate locus for autism, considering the low frequency of the polymorphism in the Japanese subjects.

Keywords: Autism; Gastrin-releasing peptide receptor; X-chromosome; Ethnic difference

1. Introduction

Autism (or autistic disorder) is a severe developmental disorder of early childhood, characterized by sustained impairments in reciprocal social interaction, communication deviance and stereotyped behavioral patterns. Etiological factors remain to be clarified in autism. However, family and twin studies strongly suggest a genetic effect. Prevalence of autism in siblings of patients with the disorder is 50–100 times greater than in the general population [1]. The concordance rates of the disorder in monozygotic and dizygotic twins were reported to be 91 and 0% [2] and 60 and 0% [3], respectively.

In light of the consistent observation of a three times more prevalence in males than in females, a role of the X chromosome is of interest in autism. A case study found a family of autism linked with an X:8 balanced translocation, which suggested the breakpoint regions of the translocation as candidate loci for autism [4]. The breakpoint on the X chromosome was in the first intron of the gastrin-releasing peptide receptor (GRPR) gene. Another case report observed a patient with autism associated with a duplication of Xp22.1–p22.3, where the GRPR gene is located [5]. These reports suggest a role of the GRPR locus in autism.

Gastrin-releasing peptide (GRP) is a bombesin-like peptide, which is widely distributed in the central nervous system in addition to gastrointestinal systems. GRPR-mRNA is expressed at early embryonic stages in nervous and other systems [6]. In most of the locations, GRPR-mRNA levels increase steadily throughout embryonic development, but suddenly disappear at birth. Thus, appropriate development of central nervous systems may require GRPR-mediated signaling. The expression of GRPR-mRNA in the brain appears most intensive in the limbic system, which plays a major role in emotion, affection and attention [7]. GRPR-deficient mice showed a significant increase of locomotor activity and response to a

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newcomer [8]. These observations also indicate the GRPR locus as one of candidate loci of autism.

Recently, two high-frequent biallelic polymorphic sites were found in the exon-2 of the GRPR gene [9]. We investigated the GRPR gene in autistic patients, focusing on these polymorphic sites.

2. Subjects and method

Subjects comprised 71 unrelated Japanese patients with autism (59 males and 12 females, age range: 9–40 years (mean = 24.3 years)). All the patients met the DSM-IV criteria for autism. The patients were recruited from the Outpatient Clinic for Autism and Mental Retardation, Department of Psychiatry, University of Tokyo and from several rehabilitation facilities for people with developmental disorders, around Tokyo. All subjects had been diagnosed with autism during their childhood. To reconfirm the diagnosis, the patients and one or two of their parents (mostly mothers) were again interviewed by one of the authors. A questionnaire in the Japanese language was filled up by the parents for the evaluation of the present status of language function, social interaction and response to environments (including repetitive-stereotyped behaviors and insistence on sameness). IQ levels, assessed by using the Tanaka–Binet test (Japanese-specific modification of the Binet test), were below 50 in 48 patients and below the borderline level (or below 70) in the rest. Twenty-four patients generated no meaningful verbal expressions. Apparent dysmorphic features were not observed in any of the subjects. All chromosomes of the patients were studied using the method of G-banding. No chromosomal aberration was found.

Control subjects consisted of 79 unrelated healthy subjects (30 males and 49 females, age range: 20–66 years (mean = 48 years)). They were recruited from hospital staff of several general and mental hospitals around the Kanto District of Japan, where Tokyo is located.

All the patients and control subjects were ethnically Japanese. Objective of the study was explained and written informed consent was obtained from all the subjects. Genomic DNA was extracted from whole blood using a standard protocol.

The region containing the polymorphisms was amplified by PCR (forward-primer: ATACAAAGCCATTGTCCGGCCA, reverse-primer: CCCTTCCACGGGAAGATTGTAAGC, annealing temperature = 52°C), using a normal PCR machine (PC808, ASTEC, Tokyo). Then, the direct sequence of the PCR product was conducted in all subjects.

3. Results

The two polymorphic sites were at high linkage disequilibrium. C450 was exclusively associated with C661, and T450 with T661, respectively, in male subjects. In female subjects, the (C-C), (T-T) and (C-T) genotypes at the position 450 were exclusively associated with the same pattern ((C-C), (T-T) and (C-T)) at the position 661, respectively.

Allele and genotype distributions of the polymorphims are summarized in Table 1. No difference in the allele frequencies was observed between the patients and controls. The C450–C661 allele was less frequent in the Japanese subjects (6% in the controls and 7% in the patients) than the previous report in the North American (Californian) subjects (33–36%) [9]. The C450–C661 allele appeared less frequent in male patients and more frequent in the female patients than male controls and female controls, respectively, which were not statistically significant.

4. Discussion

We investigated two polymorphisms, which were previously found in patients with Rett syndrome and control subjects from the Californian population [9]. The polymorphisms result in the transversion at the wobble positions of a serine codon (Ser151) and an isoleucine codon (Ile221). Two polymorphic sites were at high linkage disequilibrium in the present subjects; C450 and T450 were exclusively associated with C661 and T661, respectively. This was consistent with the previous report [9]. The frequency of the C450–C661 allele reached approximately one-third (33–

Table 1
Genotype and allele frequencies of the C/450/T–C/661/T polymorphism in the second exon of the GRPR gene in Japanese subjects with autism and controls

	Genotype distribution					Combined allele frequency	
	Male		Female				
	C	T	C-C	C-T	T-T	C	T
Patients (n = 71)	2 (3%)	57 (97%)	1 (8%)	2 (17%)	9 (75%)	6 (7%)	77 (93%)
Controls (n = 79)	4 (13%)	26 (87%)	1 (2%)	2 (4%)	46 (94%)	8 (6%)	120 (94%)

C, C450-C661 allele; T, T450-T661 allele.

36%) in the Californian subjects [9], the frequency was rather low in the Japanese subjects (6–7%). A large ethnic difference in the frequencies of the polymorphisms may exist, although the ethnicity of the subjects was not specified in the previous study [9].

In this study, no evidence was provided for an association between the GRPR locus and autism. However, we should acknowledge the limited statistical power due to the unexpectedly low information of the polymorphism in the Japanese subjects. Another limitation of the present study is that the control subjects were not fully matched to the patients for aspects including age and sex. Although the GRPR gene is not located on the sex chromosomes, this could make a major weakness of the study. Further studies in other populations or of other polymorphic sites of the gene may be recommended for exclusion of the GRPR gene as a candidate locus for autism.

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