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分担研究報告書

精神疾患脆弱性遺伝子と中間表現型に基づく新しい診断法・治療法の開発に関する研究

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研究要旨

統合失調症は多因子疾患のなかでも遺伝因子の関与が比較的強いと考えられ、これまでの研究から複数の発症脆弱遺伝子の候補が提唱されているものの、その分子病態や診断、治療効果判定の客観的指標の開発は不十分である。そこで、神経生物学的で連続的な指標を中間表現型として選択して発症脆弱性遺伝子との関連を調べるために、認知機能検査（WCST: Wisconsin Card Sorting Test、CPT：Continuous Performance Test）、MRI 構造画像を収集した。さらに、BDNF（rs6265）、COMT（rs4680）、YWHAE（rs28365859）、RGS4（rs951436）、DISC1（rs821616）、NRG1（rs6994992）の 6 SNPs の遺伝子型を同定し、認知機能あるいは MRI 構造画像との関連を検討した。

WCST における達成カテゴリー数、保続性の誤りならびに CPT における d' は統合失調症と健常者間では有意な差がみられた。認知機能検査および遺伝子型との関連について解析したところ、有意な関係はみられなかった。

統合失調症で健常者よりも体積が有意に減少していた領域は、前頭葉、側頭葉、後頭葉、海馬、島、基底核などに分布していた。これらの領域と遺伝子型との関係を検討したところ、右海馬および島の GM 体積と COMT(rs4680)のリスクアレル数に有意（$p<0.05$）な相関が見られた。

A. 研究目的

統合失調症は多因子疾患のなかでも遺伝因子の関与が比較的強いと考えられ、これまでの研究から複数の発症脆弱遺伝子の候補が提唱されているものの、その分子病態や診断、治療効果判定の客観的指標の開発は不十分である。そこで、神経生物学的で連続的な指標を中間表現型として選択して発症脆弱性遺伝子との関連を調べるために、認知機能検査（WCST: Wisconsin Card Sorting Test、CPT：Continuous Performance Test）、MRI 構造画像を収集した。さらに、BDNF（rs6265）、COMT（rs4680）、YWHAE（rs28365859）、RGS4（rs951436）、DISC1（rs821616）、NRG1（rs6994992）の 6 SNPs の遺伝子型を同定し、認知機能あるいは MRI 構造画像との関連を検討した。

B. 研究方法

1. 遺伝子型の同定：今回解析した遺伝子と SNP は NRG1(rs6994992)、BDNF(rs6265)、DISC1(rs821616)、COMT(rs4680)、RGS4(rs951436)、YWHAE(rs28365859)である。遺伝子型の同定は PCR-RFLP 法および Taqman 法を用いた。

2. 認知機能との関連

実行機能を測定する Wisconsin Card Sorting Test、注意機能を測定する Continuous Performance Test を実施した。WCST は概念形成、維持、および概念の変換を求める検査で、実行機能の指標と考えられている。本研究では慶応版の WCST（慶應 F-S version）を用い、評価にはカテゴリー達成数(CA)、ネルソン型の保続性の誤り（PEN）、set 把持の障害（DMS）を用いた。注意機能の測定には Continuous Performance Test を用い、視覚刺激として 4 桁の数字を提示した。評価には d' を用いた。

統合失調症群の認知機能検査の結果と Genotype の関係を確認するために、Genotype を独立変数、認知機能検査の結果を従属変数として分散分析を行った。

3. MRI 構造画像との関連

被験者は健常者が 40 名（男：女、21：19、平均年齢、32.3±6.2 才）と、統合失調症患者が 13 名（男：女、10：3、平均年齢、36.1±9.0 才）である。

矢状断 T1 強調画像の撮像は、名大附属病院の 3 テスラ装置（Siemens、Trio）を用いて行った。撮像パラメーターは TR/TE=1420/2.6msec、Flip

Angle=15°、Matrix=256×256、Slices=160、Voxel size=1×1×1mm であった。データ処理は SPM5 と VBM5 を用い、GM 画像を抽出した後に MNI 座標軸に標準化した。さらに modulation 処理と平滑化（FWHM=8mm）を行った。統計処理は SPM5 の two-sample t-test を用い、2 群の GM 体積を全脳で比較した。統計閾値は多重比較である FDR で有意水準が 5%未満、cluster size は 100 voxels とした。

さらに統合失調症群において健常者群よりも有意に GM 体積が減少している領域の中で、特に従来から疾患との関連性が指摘されている部位の GM 体積を抽出した。GM 体積を従属変数とし各遺伝子のリスクアレル数を独立変数とした重回帰分析を行い（強制投入法、$p<0.05$）、GM 体積の変化が各候補遺伝子のリスクアレル数と有意な関係があるかどうかを検討した。

（倫理面への配慮）

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C. 研究結果

1. 認知機能と遺伝子型との関連

・Wisconsin Card Sorting Test（WCST）

統合失調症群 58 名、健常群 157 名の結果を得た。統合失調症群の平均値は、CA は 4.66±1.4、PEN は 3.11±3.8、DMS は 0.89±1.0 であった。健常群の平均値は、CA は 5.31±1.2、PEN は 1.09±2.1、DMS は 0.50±0.8 であった。Wilcoxon 検定を用いて統合失調症群および健常群の平均値を比較したところ、CA（p=7.1E-05）、PEN（p=8.1E-05）、DMS（p=.0048）のすべてにおいて両群の平均値に有意な差が認められた。WCST の各スコアにおいては BDNF, COMT, YWHAE, RGS4, DISC1, NRG1 の各 SNP の genotype に有意な関係はみられなかった。

・ Continuous Performance Test（CPT）

統合失調症群 41 名、健常群 129 名の結果を得た。統合失調症群の平均値は、1 回目 d' は 1.42±0.8、2 回目 d' は 1.69±0.9 であった。健常群の平均値は、1 回目 d' は 2.47±0.8、2 回目 d' は 2.76±0.8 であった。t 検定を用いて統合失調症群および健常群の平均値を比較したところ、1 回目 d'（p=7.5E-11）、2 回目 d'（p=1.3E-09）ともに両群の平均値に有意な差が認められた。CPT の d' と BDNF, COMT, YWHAE, RGS4, DISC1, NRG1 の各 SNP の genotype に関しても有意な関係はみられなかった。

2. MRI 構造画像と遺伝子型との関連

統合失調症で健常者よりも GM 体積が有意に減少していた領域は、前頭葉、側頭葉、後頭葉、海馬、島、基底核などに分布していた。CTL 群で SCH 群より有意に大脳 GM 体積が減少している領域は、今回の解析では認められなかった。次いで海馬、島、前頭極、内側前頭葉などの GM 体積を用いて、リスクアレル数を含めた重回帰分析を行った。その結果では、右海馬および島の GM 体積と COMT(rs4680)のリスクアレル数に有意（$p<0.05$）な相関があった。それらの標準化 β 係数は右海馬で 0.275、島で-0.251 であった（表 1）。

表 1 全 53 例を用いた重回帰分析の結果を示す。数値は標準化 β 値。＊、$p<0.05$、＃、$p<0.1$

領域名	NRG1	BDNF	DISC1	COMT	RGS4
海馬	.029	-.072	.091	.275*	-.002
島	.128	.048	.216#	-.251	.093
前頭極	.009	.094	-.165#	-.233	.009
内側前頭葉	-.049	.138	.018	-.163	-.013

D. 考察

WCST における達成カテゴリー数、保続性の誤りならびに CPT における d' は統合失調症と健常者間では有意な差がみられた。認知機能検査および遺伝子型との関連について解析したところ、有意な関係はみられなかった。

統合失調症患者では脳内の広範な領域で、GM 体積の有意な減少が認められた。中でも認知機能に関連する海馬の体積は患者群で減少しており、さらに

COMT 多型によっても調節されていることが示された。また自律神経系や情動などに関連するとされている島の体積も群間差が有意であり、さらに COMT 多型の影響を受けていた。これらの結果は統合失調症の発達障害仮説を支持する所見ではあるが、COMT 遺伝子が GM 体積に影響を与える機序については不明である。また GM 体積の変化は発症要因として働くだけではなく、発症後の治療経過や予後からも影響を受けると考えられる。

E. 結論

WCST および CPT で計測した認知機能と BDNF (rs6265)、COMT (rs4680)、YWHAE (rs28365859)、RGS4 (rs951436)、DISC1 (rs821616)、NRG1 (rs6994992) の 6 SNPs の遺伝子型との関連は見られなかった。MRI 構造画像と遺伝子型との関連では、COMT 遺伝子多型と海馬体積の関連が計測された。

今後はさらに統合失調症群の被験者数を増やし、検討する予定である。

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G. 知的財産権の出願・登録状況（予定を含む）

1. 特許取得
該当なし。
2. 実用新案登録
該当なし。
3. その他
該当なし。

厚生労働科学研究費補助金（こころの健康科学研究事業）
分担研究報告書

抗精神病薬リスペリドンの治療反応性を規定する遺伝子変異同定に関する研究

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研究要旨

統合失調症治療薬物治療では第二世代抗精神病薬が中心に使用されるようになってきた。しかし個々人の薬物への反応性は、効果・副作用発現ともに大きさ差異があり、それは個体の多様性とくに遺伝的要因に規定されているものと考えられている。昨今薬理遺伝学的手法を用いてこれらの薬物反応性を予測した上で、薬物選択を行う個別化医療の可能性について検討が進められてきている。本研究においては抗精神病薬美投与の初発統合失調症患者１０８名でのリスペリドン単剤治療による反応性に関して特にリスペリドンの作用機序と関連するドパミン受容体・そのシグナル伝達経路・セロトニン受容体に関連する遺伝子との関連を検討した。結果はドパミンＤ２受容体およびＡＫＴ１がリスペリドン治療反応性と関連することを見いだした。これらの治験が集積すればバイオマーカーとしての遺伝子型を用いて最適薬物の選択を可能にするものと期待される。

A. 研究目的

Risperidone (RIS) は代表的な第二世代抗精神病薬の一つであり、従来の定型抗精神病薬に比べ、効果に優れ副作用が少ないとされる。 この有効性はドパミン D2 受容体以外の神経伝達物質受容体への効果が一因と推定されている。本年度の研究では、RIS の親和性プロファイルを考慮し、いくつかの候補遺伝子についてそれらの遺伝子型がRIS治療反応性を予測することが可能か否かを検討した。

候補遺伝子として、ドパミン関連遺伝子（ドパミン受容体遺伝子 (DRD1-DRD5)、AKT1、GSK3 遺伝子）、セロトニン関連遺伝子(HTR1A, HTR1B, HTR1D, HTR2A, HTR2C, HTR6, HTR7) 内の３０の遺伝子多型をタイプした。対象として抗精神病薬投与歴のない 108 例の統合失調症患者を RIS 単剤治療において８週間の治療反応性を PANSS によって評価した。統計解析として他の予測因子（発症年齢・性別・未治療期間・当初の精神症状）を加味した重回帰分析を行うことにより、どの遺伝子型が治療反応性を予測するかを検定した。DRD2 の-241A>G と TaqIA、AKT1 の AKT1-SNP1 と AKT1-SNP5 が有意な予測因子である可能性が示された。他の因子として当初の精神症状の程度が有意差をもっていたがその寄与率よりこれらの遺伝子多型の方が強い寄与率をもっており、臨床上有用な予測因子であると考察する。

B. 研究方法

対象

対象は DSM-VI-TR によって診断された統合失調症患者 177 名。うちこれまでに抗精神病薬の投与歴のない患者 108 名、事前に定型抗精神病薬の投与を受けており、そこからリスペリドンに置換したものが 69 名。治療は抗精神病薬としての単剤投与とし、0 週及び 8 週での症状評価を PANSS で行った。その他の副作用情報も同時に取得した。

サンプルの調整

末梢血から B リンパ球を分離精製し EB ウィルスを感染させることにより不死化させ株化を行った。株化 B リンパ球より通常の方法で DNA を抽出し以後の解析に使用した。

候補遺伝子関連解析

候補遺伝子として、ドパミン関連遺伝子（ドパミン受容体遺伝子 (DRD1-DRD5)、AKT1、GSK3 遺伝子）、セロトニン関連遺伝子 (HTR1A, HTR1B, HTR1D, HTR2A, HTR2C, HTR6, HTR7) 内の３０の遺伝子多型をタイプした。対象として抗精神病薬投与歴のない 120 例の統合失調症患者を RIS 単剤治療において８週間の治療反応性を PANSS によって評価した。統計解析として他の予測因子（発症年齢・性別・未治療期間・当初の精神症状）を加味した重回帰分析を行うことにより、どの遺伝子型が治療反応性を予測するかを検定した。

統計学的解析

抗精神病薬の治療反応性予測因子としてはこれまでの臨床報告から、発症年齢・未治療期間・性別・初診時の症状の程度などが関連すると考えられており、これらの要因も同時に解析する目的で多重回帰解析を用いて行った。

（倫理面への配慮）

本研究課題は、統合失調症患者、健常対照群を対象とした遺伝子解析研究である。したがって、文部科学省、厚生労働省、経済産業省告示第１号の「ヒトゲノム・遺伝子解析研究に関する倫理指針」に即して編成された藤田保健衛生大学倫理委員会において本研究課題に直結するゲノム研究に関する課題、「遺伝子解析によるこころの健康とこころの病気に対するかかりやすさ（発症脆弱性）や薬の効きめや副作用（治療反応性）等の解明に関する研究」の研究計画書を提出し、承認を受け、これまでも研究を遂行してきた。

平成18年度も（平成19年度以降も）、試料提供者へのインフォームド・コンセント、個人情報の厳重な管理（匿名化）などを徹底し、倫理的配慮を持って研究を進める。

また申請者は日本人類遺伝学会の臨床遺伝専門医として藤田保健衛生大学病院遺伝医療相談を担当しており、本研究のみならず様々な遺伝相談に応じる体制を構築し対応する環境を整備している。

C. 研究結果

DRD2 の-241A>G と TaqIA、AKT1 の AKT1-SNP1 と AKT1-SNP5 が有意な予測因子である可能性が示された。

DRD2についての結果を下に示す。この寄与率は6.9%となっており、ベースラインの症状重症度が 3.3%であることを考えると十分有用な予測因子の一つであること考えられた。

AKT/GSK3 cascade の遺伝子群では AKT1-SNP5 の寄与率が 6.0%であり、これもベースラインの症状重症度 5.3%よりも高い予測因子として同定された。

D. 考察

本年度の解析においてこれまで仮説上統合失調症と関連する遺伝子群を検討した。この中でドパミン D2 受容体とそのシグナル伝達経路で特に神経伸張などに関連する AKT1 遺伝子の両遺伝子での遺伝子多型と治療反応性を予測しうる可能性が示された。これはファーマコジェネティックスの方法論は従来の仮説上の統計モデルに当てはめて反応性を予測する手法とは大きく異なり、決定論的にその結果を予測する方法が開発できる可能性を示したと考えることができる。

昨今のゲノム研究での技術革新から簡便・安価に全ゲノム上で遺伝子多型をタイプすることが可能となっている。次年度以降は網羅的解析を行うことにより事前仮説によらない遺伝子多型でのより正確な予測法の確立を目指す。

またこの方法によりこれまでの仮説からは発見できなかった新規の抗精神病薬治療標的分子の同定も期待できる。

E. 結論

抗精神病薬治療反応性を予測する方法をファーマコジェネティックスは高い確度で提供しうる可能性が示された。本年度の検討では DRD2 と AKT1 の遺伝子多型がリスペリドン治療反応性を予測することを明確にした。今後は全ゲノム解析をすすめることにより、より精度の高い治療反応性予測法を確立していきたい。

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G. 知的財産権の出願・登録状況（予定を含む）

1. 特許取得

該当なし。

2. 実用新案登録

該当なし。

3. その他

該当なし。

研究成果の刊行に関する一覧表

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Association study of the *G72* gene with schizophrenia in a Japanese population: A multicenter study

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ABSTRACT

G72 is one of the most widely tested genes for association with schizophrenia. As G72 activates the D-amino acid oxidase (DAO), *G72* is termed D-amino acid oxidase activator (DAOA). The aim of this study is to investigate the association between *G72* and schizophrenia in a Japanese population, using the largest sample size to date (1774 patients with schizophrenia and 2092 healthy controls). We examined eight single nucleotide polymorphisms (SNPs), which had been associated with schizophrenia in previous studies. We found nominal evidence for association of alleles, M22/rs778293, M23/rs3918342 and M24/rs1421292, and the genotype of M22/rs778293 with schizophrenia, although there was no association of allele or genotype in the other five SNPs. We also found nominal haplotypic association, including M15/rs2391191 and M19/rs778294 with schizophrenia. However, these associations were no longer positive after correction for multiple testing. We conclude that *G72* might not play a major role in the risk for schizophrenia in the Japanese population.

1. Introduction

Schizophrenia (MIM181500) is a common neuropsychiatric disorder affecting 0.5–1% of the general population worldwide. Family, twin, and adoption studies of schizophrenia have indicated that there is a strong genetic factor with an estimated heritability of approximately 80% (Cardno and Gottesman, 2000). Several genome-wide linkage scan

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studies of whole-genome linkage scans show suggestive linkages to schizophrenia on chromosomes 1q, 3p, 5q, 6p, 8p, 11q, 13q, 14p, 20q and 22q (Owen et al., 2004). Chumakov et al. (2002) focused on chromosome region 13q22–q34, which have suggested by a number of linkage studies (Blouin et al., 1998; Brzustowicz et al., 1999; Chumakov et al., 2002; Lin et al., 1995). They built a map of 191 single nucleotide polymorphisms (SNPs) in a 5-Mb segment on 13q34 and found robust evidence for genetic association between schizophrenia and several SNPs in the narrowed 65-kb region. Two overlapping genes, *G72* (MIN 607408) and *G30* (MIN 607415), which are transcribed in opposite directions and span approximately 29 and 47 kb of genomic sequences, were annotated in this region (Chumakov et al., 2002). In vitro translation of these genes resulted in a product for *G72* only. Chumakov et al. (2002) demonstrated that the *G72* protein (i.e. LG72), which is only known in higher primates, acts as an activator of the *DAO* protein. The *G72* protein was therefore referred to as *DAO* activator (*DAOA*). Gene expression analysis of *G72* in postmortem dorsolateral prefrontal cortices showed a tendency toward increased expression of *G72* mRNA in schizophrenia than that in control (Korostishevsky et al., 2004), although the reported increase of *G72* expression has yet to be replicated. Furthermore, the activity of DAO was also increased in postmortem cortices from patients with schizophrenia (Madeira et al., 2008). D-serine is an agonist at the glycine modulation site of the N-methyl-D-aspartate (NMDA) -type glutamate receptor and plays a role in neuronal migration and cell death (Scolari and Acosta, 2007). As DAO oxidizes and degrades D-serine, DAO is considered to modulate NMDA function in cortex. Lower serum level of D-serine was revealed in patients with schizophrenia as compared to that in healthy controls. Furthermore, administration of D-serine as add-on medication reduced parts of the symptoms of schizophrenia (Boks et al., 2007). Chumakov et al. (2002) hypothesized that the activation of DAO activity by a *G72* protein product might promote degradation of D-serine and cause a hypofunction of glutamate-signaling through the NMDA receptor in schizophrenia. However, the potential relationship between *G72* and NMDA receptor system still lacks supporting evidence.

Significant associations of *G72* with schizophrenia have been reported in various populations other than Japanese, such as French Canadians, Russians, German, Palestinian Arabs, South African, Ashkenazic Jewish, Chinese, Taiwanese, Scottish, Korean and Irish (Addington et al., 2004; Chumakov et al., 2002; Corvin et al., 2007; Fallin et al., 2005; Hall et al., 2004; Hong et al., 2006; Korostishevsky et al., 2004, 2006; Ma et al., 2006; Schumacher et al., 2004; Shin et al., 2007; Shinkai et al., 2007; Wang et al., 2004; Yue et al., 2006, 2007; Zou et al., 2005). The majority of replication studies of *G72* have indicated significant associations of alleles, genotypes or haplotypes with schizophrenia. However, a minority have reported no association between *G72* and schizophrenia (Bakker et al., 2007; Goldberg et al., 2006; Liu et al., 2006; Mulle et al., 2005; Sanders et al., 2008; Vilella et al., 2008; Williams et al., 2006; Wood et al., 2007). Associations of this gene were also reported with bipolar disorder (Chen et al., 2004; Hattori et al., 2003; Prata et al., 2008; Schumacher et al., 2004; Williams et al., 2006), major depression (Rietschel et al., 2008) and panic disorder (Schumacher et al., 2005).

In this study, we examined possible association between G72 polymorphisms and schizophrenia in a large Japanese population.

2. Materials and methods

2.1. Subjects

The subjects for this study consisted of 1774 patients with schizophrenia [males: 55.5%, mean age of 45.6 years (SD 15.1)] and 2092 healthy controls [males: 49.3%, mean age of 45.0 years (SD 19.7)], which is the largest sample size to date for *G72* association study. There was no significant difference in age between patients and controls groups ($P=0.30$), while the sex ratio differed significantly between groups ($P=0.00014$). All subjects were biologically unrelated Japanese and were recruited at four geographic regions, which were located on the main islands in Japan: Osaka, Aichi, Tokushima and Tokyo. There is little possibility for great ethnic/genetic difference among these regions for feature of homogeneous race in Japan (Yamaguchi-Kabata et al., 2008). Cases were recruited from both outpatients and inpatients at university hospitals and related psychiatric facilities. Controls, including hospital and institutional staffs, were recruited from local advertisements. Each patient with schizophrenia had received a diagnosis and assessment by at least two trained psychiatrists as a part of routine clinical diagnosis and treatment at the university hospitals and the related psychiatric facilities, according to the Diagnostic and Statistical Manual of Mental Disorders, fourth edition (DSM-IV) criteria based on unstructured clinical interviews and other available information including medical records and other research assessments. No patient was diagnosed on the basis of medical records alone. Psychiatrically healthy controls were evaluated using unstructured interviews to exclude individuals who had received psychiatric medication. Written informed consent was obtained for all subjects after the procedures had been fully explained. This study was carried out in accordance with the World Medical Association's Declaration of Helsinki and approved by the Research Ethical Committee of Osaka University, Fujita Health University, Nagoya University, Tokushima University and Juntendo University.

2.2. SNP genotyping and genomic sequencing

Eight SNPs, rs3916965 (M12), rs3916967 (M14), rs2391191 (M15), rs778294 (M19), rs3916970 (M20), rs778293 (M22), rs3918342 (M23) and rs1421292 (M24), were selected from the genomic region of the *G72* gene and its flanking regions. The designations of the SNPs in parentheses are according to Chumakov et al. (2002). To examine the association between schizophrenia and previously associated SNPs in a Japanese cohort, we chose eight SNPs, which had been associated with schizophrenia in previous studies, although our study design using these SNPs does not provide complete *G72* gene coverage. The positions of the eight SNPs analyzed in the present study are indicated in Supplementary Fig. 1. Venous blood was collected from the subjects and genomic DNA was

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Table 1
SNP genotype and allele distribution in patients with schizophrenia and controls

Marker position		M/m[c]	SCZ (%)			CON (%)			MAF		Genotypic	Allelic	OR
SNP number[a]	Kb[b]		M/M	M/m	m/m	M/M	M/m	m/m	SCZ (%)	CON (%)	P-value[d] (df=2)	P-value[d] (df=1)	
M12	0	A/G	57.1	35.5	7.4	55.9	37.6	6.5	25.1	25.3	0.26	0.87	0.99
M14	14	G/A	55.3	36.7	8.0	53.7	39.2	7.1	26.3	26.7	0.21	0.75	0.98
M15	2	A/G	55.1	37.0	7.9	54.3	38.8	6.9	26.4	26.3	0.34	0.91	1.01
M19	23	G/A	72.9	24.4	2.7	71.5	25.9	2.6	14.9	15.5	0.55	0.43	0.95
M20	12	A/G	39.0	46.1	14.9	40.9	45.3	13.8	38.0	36.4	0.4	0.17	1.08
M22	15	A/G	54.7	38.7	6.6	58.9	35.0	6.1	26.0	23.6	**0.034**	**0.019**	1.13
M23	17	T/C	31.7	49.4	18.9	34.7	48.3	17.0	43.6	41.2	0.09	**0.030**	1.11
M24	12	A/T	24.9	50.6	24.5	28.1	49.0	23.0	49.8	47.4	0.07	**0.037**	1.10

SCZ, patients with schizophrenia; CON, healthy controls; m, minor allele; M, major allele; MAF, minor allele frequency; OR, odds ratio.
[a]The db SNP IDs equivalent to the M-SNP IDs designed by Chumakov et al. (2002) are the following: M12 (rs3916965), M14 (rs3916967), M15 (rs2391191), M19 (rs778294), M20 (rs3916970), M22 (rs778293), M23 (rs3918342), and M24 (rs1421292).
[b]Distances inter-SNPs are shown (Kb).
[c]The first shown alleles are major allele. All the alleles are represented according to the forward DNA sequence to make them comparable with the previous published data.
[d]Significant P-values (< 0.05) are in bold face.

extracted from whole blood according to standard procedures. Genotyping of the SNPs was carried out using TaqMan assays (Applied Biosystems, Foster City, California, USA). TaqMan probes and Universal PCR Master Mix were obtained from Applied Biosystems. A 5-μl total reaction volume was used, and allelic-specific fluorescence was measured using an ABI PRISM 7900 Sequence Detector System (Applied Biosystems). In addition, we genotyped eight SNPs in 32 randomly selected subjects (64 chromosomes) by a direct DNA sequencing method to check for typing errors by the TaqMan method. We confirmed that all genotypes determined by the direct sequencing method were in agreement with the genotypes of the TaqMan methods for all eight SNPs. Detailed information on the PCR conditions and the primer pairs are shown in Supplementary Methods and Supplementary Table 1.

2.3. Statistical analysis

Statistical analysis was performed using SNPAlyze V5.1.1 Pro software (DYNACOM, Yokohama, Japan). The presence of Hardy–Weinberg equilibrium (HWE) was examined by using the χ^2 test for goodness of fit. The statistical significance of HWE analysis was defined at $P < 0.01$. The allelic and genotypic distributions of *G72* polymorphisms between patients and controls were analyzed using χ^2 tests for independence. We performed correction for multiple testing in single marker analysis by using the SNPSpD program (Nyholt, 2004). Pairwise linkage disequilibrium (LD) analysis, expressed by D' values, was applied to detect the intermarker relationship in each group using the Haploview software (http://www.broad.mit.edu/mpg/haploview/contact.php). Haplotype frequencies were estimated by the method of maximum likelihood from the genotyping data through the use of the Expectation-Maximization algorithm. Rare haplotypes found in less than 3% of both patients and controls were excluded from the association analysis. We performed 10,000 permutations for the most significant test to determine an empirical significance. We used a 2- to 5-window fashion analysis. Bonferroni corrections were applied for multiple comparisons of the haplotype analysis. All *P*-values reported are two tailed. Statistical significance was defined at $P < 0.05$.

2.4. Power analysis

We performed power calculations using the Power Calculator for Two Stage Association Studies (http://www.sph.umich.edu/csg/abecasis/CaTS/) (Skol et al., 2006). Power estimates were based on allele frequencies of the associated markers ranging from 0.15 (M19) to 0.38 (M20), the odds ratio ranging from 1.33 (M14) to 1.46 (M12) for the markers indicated by Chumakov et al. (2002) and an alpha level of 0.05. Power was calculated under prevalence of 0.01 using an additive or a multiplicative model, assuming various degrees of allele frequencies and the odds ratios of the markers.

3. Results

Our sample size of 1774 cases and 2092 controls had sufficient power (>0.99) to detect an effect of the odds ratio

Table 2
Haplotype analysis of G72 between patients and controls

		Haplotypic global P value			
		Window size			
LD[a]	SNP IDs[b]	2	3	4	5
	M12 (rs3916965)	0.93			
block I	M14 (rs3916967)		0.97		
		0.92		**0.03**	
	M15 (rs2391191)		**0.03**		0.05
		0.03		0.06	
	M19 (rs778294)		0.08		**0.03**
		0.04		0.06	
	M20 (rs3916970)		0.15		0.27
		0.23		0.47	
	M22 (rs778293)		0.28		0.25
		0.09		0.32	
block II	M23 (rs3918342)		0.06		
		0.10			
	M24 (rs1421292)				

LD, linkage disequilibrium.
[a]According to the result of LD analysis, we divided tightly linked SNPs into two LD blocks: block I (M12, M14 and M15), block II (M22, M23 and M24).
[b]The db SNP IDs equivalent to the M-SNP IDs designed by Chumakov et al. (2002) are shown in parentheses.
Haplotypes with frequencies <3% in each group are excluded.
Significant P-values (<0.05) are in bold face.

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