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I. 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。

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分担研究報告書

SSRIによるパニック障害の治療における薬理遺伝学的研究

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研究要旨

本研究計画はパニック障害の病態生理への関連が想定され、また、セロトニン再取り込み阻害薬(SSRI)の作用部位と考えられているセロトニントランスポーター(5HTT)遺伝子およびセロトニン(5HT)受容体遺伝子における遺伝子変異、薬物代謝酵素遺伝子変異、薬物代謝の個体差などの変数とSSRIであるパロキセチンを投与された各個体の臨床効果や副作用出現との関連を検討することにより、治療反応性や副作用出現の投与前予測、SSRIによるパニック障害のオーダーメイド（個別化）治療の確立を目指したものである。モデル細胞(5HTTを発現させた培養細胞(HEK293))を用いたパロキセチンの5HTT阻害効果の研究ではCYP2D6の代謝欠損を生じさせる遺伝子多型であるCYP2D6*5の有無により、パロキセチン血漿中濃度が同程度であっても、5HTT阻害率は大きく異なっており、治療初期段階の臨床効果発現に影響を与える可能性が示唆された。パロキセチンの治療反応性に影響を与える因子の探索をパロキセチンにて治療中のパニック障害の患者で行なった結果、パロキセチン血中濃度が高値、5HTT L/S遺伝子型を保有していること、身体合併症を有していることが、パロキセチンによる治療反応性が不良であることと関連していることが判明した。このことからパロキセチン血中濃度の調整を5HTT遺伝子型を参考にしながら行なうことで、パニック障害患者の初期治療反応性を向上させることが示唆された。

A.　研究目的

目的

代表的な不安性障害であるパニック障害では選択的セロトニン再取り込み阻害薬が薬物治療として推奨されている。しかしながら、その臨床効果には個体差があり、吐き気などの消化器症状のために、その投与をあきらめざるを得ない場合も少なくない。このようなSSRIの臨床効果や副作用の個体差を事前に予測し、それによって合理的な薬物治療計画を立案することが求められている。本研究計画ではパニック障害の病態生理への関連が想定され、また、SSRIの作用部位と考えられている5HTT遺伝子および5HT受容体遺伝子における遺伝子変異、薬物代謝酵素遺伝子変異、薬物代謝の個体差などの変数とパロキセチンを投与された各個体の臨床効果や副作用出現との関連を検討することにより、治療反応性や副作用出現の投与前予測をめざし、SSRIによるパニック障害のオーダーメイド（個別化）治療の確立を目的とする。

B.　研究方法

1．モデル細胞を用いたパロキセチンの5HTT阻害効果の研究

パロキセチン10mg/日内服中のパニック障害患者42例より得られた末梢血からDNAを抽出し、

PCR法によりパロキセチンの薬物代謝に関わるCYP2D6の遺伝子多型の解析を行い、高速液体クロマトグラフィー（HPLC）にてパロキセチン血漿中濃度を測定した。また、5HTTを発現させた培養細胞(HEK293細胞)を用い、血漿成分の5HTT阻害効果を[^{3}H]5-HTの阻害率を測定することにより評価した。臨床効果は、パニック障害・広場恐怖評価尺度(Panic and agoraphobia scale observer-rated: PAS)の得点の推移によって評価し、CYP2D6遺伝子型とパロキセチン血漿中濃度、5HTT阻害率および臨床効果との関連を解析する。

2. パロキセチンの治療反応性に影響を与える因子の検討

獨協医科大学精神科通院中のパニック障害患者でパロキセチン(10mg/day)を服用しており、本研究の趣旨を説明し同意が得られた 27 名（男性 6 名、女性 21 名）で、年齢は 34.7 ± 11.1 歳、体重は 54.9 ±8.7kg であった。パニック障害の臨床評価尺度として PAS を使用し、パロキセチン血中濃度は HPLC にて測定した。5-HTT の L、S 多型については、末梢血から genomic DNA を抽出し、Heilis ら(1996)の方法により同定した。

（倫理面での配慮）

「パロキセチンの治療反応性に影響を与える因子の検討」においては参加者には研究内容を説明し、文書にて同意を得ている。本研究内容は獨協医科大学病院倫理委員会の承認を得て、また文部科学省・厚生労働省・経済産業省「ヒトゲノム・遺伝子解析研究に関する倫理指針」を遵守して行った。

C. 研究結果

1．モデル細胞を用いたパロキセチンの 5HTT 阻害効果の研究

CYP2D6の代謝欠損を生じさせる遺伝子多型であるCYP2D6*5の有無により、パロキセチン血漿中濃度、5HTT阻害率、臨床効果を比較した。CYP2D6*5保有群では非保有群と比較して、パロキセチン血漿中濃度に有意差は認められなかったが、5HTT阻害率は有意に高かった。また、臨床効果に関しては、CYP2D6*5保有群では非保有群と比較して、2週間後のPAS改善率が有意に低い結果であった。

2. パロキセチンの治療反応性に影響を与える因子の検討

初診時 PAS 得点=21.6 ± 6.9 点あり、2 週間後の PAS 改善率 (初診時 PAS－2 週間後の PAS / 初診時 PAS) は、15.1 ± 6.9 %あった。5-HTTLPR 遺伝子型は、L/S 型が 9 例、S/S 型が 18 例で、PAS 改善率を従属変数、性別、年齢、体重、大うつ病の合併、空間恐怖の合併、喫煙の有無、飲酒、初診時 PAS 得点、初診時での 1 週あたりのパニック発作回数、ベンゾジアゼピン投与の有無、副作用の有無、パロキセチン血漿中濃度、5HTT 遺伝子型、身体合併症の有無多変量解析(ステップワイズ法)を行ったところ、PAS 改善率とパロキセチン血中濃度、5HTT 遺伝子型、身体合併症の有無が有意な独立変数としてピックアップされた。具体的にはパロキセチン血中濃度が高値、5HTT L/S 遺伝子型を保有していること、身体合併症を有していることが、パロキセチンによる治療反応性が不良であることと関連していることが判明した。

D. 考察

1．モデル細胞を用いたパロキセチンの 5HTT 阻害効果の研究

パロキセチンの5HTT阻害率は、同程度のパロキセチン血漿中濃度であってもCYP2D6遺伝子型により大きく異なっていることが明らかになった。これは、CYP2D6遺伝子型の違いによるパロキセチンのmetabolic patternの違いが関連していると考えられた。

2. パロキセチンの治療反応性に影響を与える因子の検討

パロキセチンが臨床効果を発揮するには5HT1A受容体のdown regulationが必須と考えられているが、5HT1A受容体のdown regulationが起こる前にパロキセチン血中濃度が高値になると一時的に5HT神経系の活動を低下させる結果となり、臨床効果が低下すると考えられる。また5HTT L/S遺伝子型を保有している個体では、S/S遺伝子型を保有している個体に比してシナプス間隙の5HT量がもともと低く、5HT1A受容体密度が高い（つまり、S/S遺伝子型を保有している個体ではもともとdown regulation様の現象が起こっている）ことが治療反応性の差異と関連している可能性があると考えられた。

E. 結論

1．モデル細胞を用いたパロキセチンの5HTT阻害効果の研究

CYP2D6の代謝欠損を生じさせる遺伝子多型であるCYP2D6*5の有無により、パロキセチン血漿中濃度が同程度であっても、5HTT阻害率は大きく異なっており、治療初期段階の臨床効果発現に影響を与える可能性が示唆された。

2. パロキセチンの治療反応性に影響を与える因子の検討

パロキセチン血中濃度が高値、5HTT L/S 遺伝子型を保有していること、身体合併症を有していることが、パロキセチンによる治療反応性が不良であることと関連していることが判明した。このことからパロキセチン血中濃度の調整を5HTT遺伝子型を参考にしながら行なうことで、パニック障害患者の初期治療反応性を向上させることが示唆された。

F. 健康危険情報

特に無し

G. 研究発表

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第18回日本臨床精神神経薬理学会・第38回日本神経精神薬理学会合同年会、東京2008年10月1日－3日

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大栗有美子、佐伯吉規、石川里子、下田和孝
セルトラリン投与によりStevens-Johnson症候群が惹起された症例
日本臨床薬理学会、２００８年１２月４日－６日、東京

H.知的財産の出願・登録状況（予定を含む）
1. 特許取得
なし

2. 実用新案登録
なし
3. その他
なし

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分担研究報告書

向精神病薬によるQTc延長に関する研究

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疾病研究第三部・室長（H20年9月まで）

研究要旨

抗うつ薬、特に三環系抗うつ薬は時に心電図上のQT間隔の延長を引き起こし、突然死の原因となりうることが知られている。しかし、実際の臨床場面でどの程度の気分障害患者にQT延長が見られ、その異常が抗うつ薬とどの程度関連しているかについての報告はほとんどないため、これらに関して検討を行った。426人の気分障害患者（大うつ病：294人、双極I型障害：65名、双極II型障害：23名、その他の気分障害：44名）を対象に、内服している向精神薬と心電図上のQT間隔（Bazettの補正式[QTc: $QTc=QT/RR^{1/2}$]で補正）を比較した。その結果、女性は男性に比較してQTcが長いこと（$p<0.01$）が見出され、クロルプロマジン換算した抗精神病薬の量が多いほどQTc間隔が大きくなる傾向が見られた（$p<0.06$）。抗精神病薬を服用していない群の中では、イミプラミン換算した抗うつ薬の服用量とQTc間隔が関連する傾向がみられた（$P<0.09$）。個々の抗うつ薬とQTc間隔との関連をステップワイズ法による重回帰分析で解析すると、トラゾドンがQTc間隔の延長と関連していた（$p<0.01$）。以上の結果から、気分障害患者に薬物治療を行う際、QTc間隔延長予防という観点からは、抗精神病薬の併用時に特に注意をすること、トラゾドンはQTc延長の因子となりやすいこと、女性では特に注意が必要であることがわかった。

A. 研究目的

気分障害（うつ病や躁うつ病など）の治療において向精神薬、とりわけうつ薬の投与は欠かせないものである。しかし、300mg以上の三環系抗うつ薬の投与は突然死の危険性を2.5倍にすることが知られているなど、抗うつ薬が突然死のリスクになることが知られている。突然死の原因は主に心室性不整脈であると考えられており、心室性不整脈から突然死に至る危険性の指標として、心電図上のQT間隔の延長が知られている。突然死を起こす向精神薬はQT間隔の延長を引き起こすことが知られているが、実際の臨床場面での個々の薬剤のQT間隔

延長に対する危険性の報告は少なく、特に新規の抗うつ薬に関してはほとんど報告されていない。さらに本邦に限れば、実際にどの程度の患者で QT 間隔の延長が見られるか、どのような因子が特に危険であるかなどに関しても報告されていない。そこで本研究では、気分障害患者を対象に心室性不整脈の予測因子である心電図上の QT 間隔の延長が実際どの程度の患者で見られるかの実態調査を行うと同時に、QT 延長と関連する内服薬のデータや生化学データを収集することで QT 延長の危険因子を明らかにし、予防方法を検討することを目的とする。

B. 研究方法 (倫理面への配慮)

2003 年から 2007 年の間に国立精神・神経センター病院にて入院もしくはうつ病専門外来で治療を受けた気分障害患者 426 人(男性 209 人。女性 217 人)、DSM-IV-TR 診断ごとの分類は、大うつ病性障害 294 人、双極 I 型障害 65 人、双極 II 型障害 23 人、その他の気分障害 44 人であった。これらを対象に年齢、性別、治療期間、内服内容を調べ、心電図上の QT 時間の延長との関連を、統計学的に検討した。QT 時間は心拍数に影響を受けるため、Bazett の補正式($QTc=QT/RR^{1/2}$)で補正した値を用いた。内服薬に関しては、抗うつ薬はイミプラミン、ベンゾジアゼピン系薬物はジアゼパム換算、抗精神病薬はクロルプロマジン換算、抗パーキンソン薬はビペリデン換算し、比較した。気分安定薬は換算せずに検討した。なお本研究は国立精神神経センター倫理委員会の承認を得て行われた。

C. 研究結果

QTcが470msec$^{1/2}$ 以上の値を示した患者は4名 (0.9%、男性1名、女性3名) 、440msec$^{1/2}$ 以上の値を示した患者は27名 (6.3%、男性9名、女性18名) であった (470msec$^{1/2}$は家族性QT延長症候群の診断基準でカットオフポイントとされる値、440msec$^{1/2}$は、一般に異常値とされる値) 。年齢、性別、抗うつ薬 (イミプラミン換算) 、抗精神病薬 (クロルプロマジン換算) 、抗パーキンソン薬 (ビペリデン換算) 、ベンゾジアゼピン系薬物 (ジアゼパム換算)、リチウム、バルプロ酸、カルバマゼピンを独立変数として、QTc間隔延長との関連を重回帰分析で解析したところ、女性であることとQTc間隔の異常が有意に関連しており ($p<0.01$)、抗精神病薬の服用と関連する傾向が見られた ($p=0.06$)。抗精神病薬を服用していない患者のみを対象に再び重回帰分析を行ったところ、やはり女性であることとQTc間隔延長との間に有意な相関がみられ ($p<0.01$)、抗うつ薬との間に有意な傾向が見られた ($p=0.09$)。さらに各抗うつ薬、気分安定薬とQTc時間の延長を重回帰分析 (ステップワイズ法) すると、女性であることと、トラゾドンを服用していることの二点が、QTc 間隔延長と関連していた (各々 $p<0.01$)。

D. 考察

今回の研究より、気分障害患者に対する薬物療法を行う際の QTc 間隔延長は、性別が最も重要な因子であった。この結果はこれまでも繰り返し報告されているものであり、本研究でも確認された。次に注意するべきは抗精神病薬の併用である。また、抗

精神病薬を併用していない場合には、抗うつ薬の総量がQTc間隔延長と関連していた。この順番で、QTc間隔延長の危険性を考慮するべきであることが明らかになった。今回トラゾドンがQTc間隔延長と関連しているとの結果を得た。トラゾドンの過量服薬がQTc延長をきたすとの症例報告や、HERGチャネルの阻害作用があるなどと報告されているが、QTc間隔に関するまとまった報告はない。我々の結果を確認する意味でも、検体数の増加による検討および、複数の機関よりの研究報告が待たれる。

E. 健康危険情報

なし

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H.知的財産権の出願・登録状況

1．特許取得　なし

2．実用新案登録　なし

3．その他　なし

厚生労働科学研究費補助金
（創薬基盤推進研究事業：ヒトゲノムテーラーメード研究）

分担研究報告書

抗精神病薬の治療効果に関する薬力学的研究

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研究要旨　ギャバ神経伝達系の遺伝子の中でGABRG3遺伝子と遅発性ジスキネジアの関連について詳しく関連解析を行った。GABRG3 遺伝子の 110 のタグ SNPs を解析し，そのなかで rs2286946 と rs741124 の２つの SNP で関連を見出した。これは以前同定していた関連 SNP rs2061051 とは違う連鎖不平衡ブロックに存在していた。死後脳前頭前野ではリスク遺伝子型では統合失調症では有意に発現レベルが低かった。ハロペリドール長期投与後のマウス脳では Gabrg3 遺伝子の発現は有意に亢進していた。これらの結果は，抗精神病薬投与による GABRG3 の発現が亢進しない遺伝子型をもっている人が遅発性ジスキネジアのリスクになっていることを示唆している。

A.　研究目的

遅発性ジスキネジアに関連する遺伝子を同定するため，ゲノムワイド SNP 解析を行い，ギャバ神経伝達系に関わるタンパク質をコードしている遺伝子に関連 P 値の低い SNP が有意に集積していることを発見していたが，各遺伝子で解析した SNP 数は少なかった。本研究はギャバ受容体シグナル伝達系に関わる分子の中で特に GABRG3 に的を絞って解析を行い，GABRG3 の遅発性ジスキネジアに果たす役割についてさらに解析をすすめルことを目的とした。

B．研究方法

統合失調症患者で遅発性ジスキネジア群 86 人と非遅発性ジスキネジア群 188 人を対象とした。死後脳はオーストラリアブレインバンクよりの 17 例(コントロール 9，統合失調症 8)と新潟大より提供の 17 人(コントロール 11，統合失調症 6)を対象とした。マウスは C57/BJ6 雄で 40 週ハロペリドール投与と生食投与で比較した。遺伝子型判定は，TaqMan 法，遺伝子発現は real-time PCR を使用した。統計処理は遺伝子関連は Haploview，遺伝発現は Wilcoxon テストを使用した。

なお，本研究は，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」を遵守した研究計画書を作成し，研究施設での倫理委員会において承認を受けた上で研究を行った。

C．研究結果

GABRG3 遺伝子の 110 の１塩基多型(SNPs)を用いてスクリーニングした結果，rs2286946 で $P=0.002$ (permutation $P = 0.08$), rs741124 で $P = 0.002$ (permutation test で $P = 0.07$)の関連を同定した。ゲノムワイド関連解析ですでに同定していた rs2061051 は $P = 0.0006$ (permutation test で $P = 0.03$)であった。ヒト死後脳前頭前野では遺伝子型別の GABRG3 遺伝子発現レベルに有意差はなかったが，rs741124 の遺伝子型と診断グループに交絡作用があり，防御的遺伝子型では統合失調症群は有意に遺伝子発現レベルが上昇していたのに対して，リスク遺伝子型群ではそのような傾向はなかった。マウスに 40 週間ハロペリドールを投与した結果，Gabrg3 遺伝子の発現は上昇していた。

D．考察

本研究において，GABRG3 遺伝子と遅発性ジスキネジアの関連が確認された。マウスのハロペリドール長期投与後の結果から，抗精神病薬により GABRG3 遺伝子の発現上昇は「正常な」反応であり，この反応が乏しいことが遅発性ジスキネジアの脆弱性に関わっていると推測された。

E．結論

GABRG3 遺伝子は遅発性ジスキネジアの関連遺伝子であることが示唆された。今後さらに病態生理との関連で検討を加える予定である。

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G．知的財産権の出願・登録状況

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。

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Genetic Variations of Human Neuropsin Gene and Psychiatric Disorders: Polymorphism Screening and Possible Association with Bipolar Disorder and Cognitive Functions

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Human neuropsin (NP) (hNP) has been implicated in the progressive change of cognitive abilities during primate evolution. The hNP gene maps to chromosome 19q13, a region reportedly linked to schizophrenia and bipolar disorder. Therefore, hNP is a functional and positional candidate gene for association with schizophrenia, mood disorders, and cognitive ability. Polymorphism screening was performed for the entire hNP gene. The core promoter region was determined and whether or not transcriptional activity alters in an allele-dependent manner was examined by using the dual-luciferase system. Allelic and genotypic distributions of five single-nucleotide polymorphisms (SNPs) were compared between patients with schizophrenia ($n = 439$), major depression ($n = 409$), bipolar disorder ($n = 207$), and controls ($n = 727$). A possible association of the hNP genotype with memory index (assessed with Wechsler Memory Scale, revised, WMS-R) and intelligence quotient (IQ assessed with Wechsler Adult Intelligence Scale, revised; WAIS-R) was examined in healthy controls ($n = 166$). A total of 28 SNPs, including nine novel SNPs, were identified. No significant effects on transcriptional activity were observed for SNPs in the promoter region. A significant allelic association was found between several SNPs and bipolar disorder (for SNP23 at the 3′ regulatory region; odds ratio 1.48, 95% confidential interval 1.16–1.88, $P = 0.0015$). However, such an association was not detected for schizophrenia or depression. Significant differences were observed between SNP23 and attention/concentration sub-scale score of WMS-R ($P = 0.016$) and verbal IQ ($P < 0.001$). Genetic variation of the hNP gene may contribute to molecular mechanisms of bipolar disorder and some aspects of memory and intelligence.

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Keywords: neuropsin; polymorphism screening; association study; bipolar disorder; memory; intelligence

INTRODUCTION

Neuropsin (NP, MIM: 605644), also called as kallikrein 8 (KLK8), is one of the secreted-type serine proteases, which was first cloned by our group in mice (Chen *et al*, 1995). NP mRNA is expressed specifically in the limbic system of mouse brain and is localized at the highest concentration in pyramidal neurons of the hippocampal CA1-3 sub-fields. Direct hippocampal stimulation and kindling induced by amygdaloid stimulation caused a significant bilateral change in NP mRNA level in the hippocampal pyramidal neurons. The activity-dependent changes and the specific localization indicate that NP is involved in hippocampal plasticity (Chen *et al*, 1995). Indeed, NP has a regulatory effect on Schaffer-collateral at the early phase of long-term potentiation (LTP) (Komai *et al*, 2000). Mice lacking NP were significantly impaired in the Morris water maze and Y maze, suggesting that NP has an important role in learning and memory (Tamura *et al*, 2006). The human NP (hNP) gene was cloned by Yoshida *et al* (1998), and then localized to chromosome 19q13.3–q13.4 (Gan *et al*, 2000; Harvey *et al*, 2000). It consists of six exons and the first exon is non-translational. Four alternative splicing variants have been identified (Mitsui *et al*, 1999; Magklara *et al*, 2001). The regular form is called type1, and type 2 contains a 135-bp

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insertion of 5′ upstream region of exon 3 (Mitsui *et al*, 1999). Interestingly, type 2 is a hominoid-specific splicing form (Li *et al*, 2004) and is expressed as abundantly as the type 1 in human brain (Mitsui *et al*, 1999). These findings points to the possibility that type 2 hNP may contribute to progressive change of cognitive abilities during primate evolution. Moreover, dysfunctions in hNP may be involved in psychiatric diseases of cognitive abilities, including schizophrenia and mood disorders.

Family, twin, and adoption studies clearly suggest that genetic components play an important role in the pathogenesis of schizophrenia and mood disorders (reviewed by Shih *et al*, 2004). These psychiatric diseases demonstrate substantial cognitive deficits such as learning and memory (reviewed by Sharma and Antonova, 2003; Robinson *et al*, 2006; Green, 2006). A genome screen of linkage with bipolar disorder pedigrees provided evidence for susceptibility locus on chromosome 19q13 (Badenhop *et al*, 2002). Another genome scan in schizophrenia and bipolar pedigrees obtained an LOD ratio score of 1.5 at 19q13 in schizophrenic families (Macgregor *et al*, 2004). Therefore, the hNP gene is a good candidate gene for association with schizophrenia and mood disorders. Here we performed, for the first time, a polymorphism screening and association analysis of the hNP gene with schizophrenia, major depression, and bipolar disorder in a Japanese sample. A possible association of hNP with memory and intelligence in healthy subjects was also examined. In addition, we determined a core promoter region of the hNP gene and examined whether transcriptional activity varies in an allele-dependent manner.

MATERIALS AND METHODS

Subjects

Subjects for the association study were 439 patients with schizophrenia (240 males, mean age of 44.6 years (SD 14.0)), 409 patients with major depression (136 males, 53.3 years (15.9)), 207 patients with bipolar disorder (80 males, 50.2 years (14.7)), and 727 healthy controls (324 males, 43.5 years (16.4)). Among these, 104 patients with bipolar disorder and 108 controls were recruited around Shiga prefecture, approximately 350 km to the west of Tokyo, while the remaining 1570 subjects were recruited around Tokyo. Consensus diagnosis by at least two psychiatrists, one of whom was in charge of the patients, was made for each patient according to the Diagnostic and Statistical Manual of Mental Disorders, 4th edition (DSM-IV) criteria (American Psychiatric Association, 1994), on the basis of unstructured interviews and information from medical records. Control subjects were healthy volunteers who had no current or past contact to psychiatric services. Among them 213 controls were screened by the Japanese version of the Mini-International Neuropsychiatric Interview (Sheehan *et al*, 1998; Otsubo *et al*, 2005) by a research psychiatrist, whereas the remaining controls were not screened by such a structured interview. Participants were excluded if they had prior medical histories of central nervous system disease or severe head injury, or if they met the criteria for substance abuse or dependence, or mental retardation. All subjects were biologically unrelated Japanese. After description of the study, written informed consent was obtained from every subject. The study protocol was approved by institutional ethics committees.

Neuropsychological Test Measures

Among controls, 166 (53 males, 37.6 years (12.4)) were subject to memory and intelligence tests to detect possible association with the hNP genotype. These individuals were all screened by the Mini-International Neuropsychiatric Interview with respect to their psychiatric history and confirmed that they had no current or past history of psychiatric illness. To assess memory and intelligence, Japanese full versions of the Wechsler Memory Scale-Revised (WMS-R) (Sugishita, 2001; Wechsler, 1987) and the Wechsler Adult Intelligence Scale-Revised (WAIS-R) (WAIS-R, Shinagawa *et al*, 1990; Wechsler, 1981), respectively, were administered. Testing and scoring were performed by psychologists who were blind to genotypic data.

Polymorphism Screening and Genotyping

Venous blood was drawn from the subjects and genomic DNA was extracted from whole blood according to standard procedures. The genomic structure of hNP was determined from the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/) and the University of California at Santa Cruz (UCSC) database (http://genome.ucsc.edu/cgi-bin/hgBlat). To screen for polymorphisms, we used direct sequencing with the Genome Lab-DTCS (Dye Terminator Cycle Sequencing) kit and CEQ8000 Genetic Analysis System (Beckman Coulter, Fullerton, CA). The entire 7428-bp genomic region containing all the exons, introns, the 1078-bp 5′ flanking region upstream to exon 1, and the 655-bp 3′ flanking region downstream to exon 6 were amplified from the genomic DNA of 24 randomly selected schizophrenic subjects. Sequences of 24 sets of primers for the polymorphism screening are listed in Supplementary Table S1.

The examined 7428-bp region seemed to constitute of single haplotype blocks (Supplementary Figure S1). We genotyped five single-nucleotide polymorphisms (SNPs) using TaqMan 5′-exonuclease allelic discrimination assay. They were A-807>G (ss73688625, SNP3), G-658>C (rs1722550, SNP4), IVS2-101T>A (rs1701947, SNP6), IVS3-10A>G (rs1701946, SNP7), and A5229>G (rs1612902, SNP23) (Figure 1). SNPs 3 and 4 were chosen from the 5′ regulatory region since they may have some effects on transcriptional activity, and SNPs 1, 2, 4, and 5 were in absolute linkage disequilibrium (LD) (ie, genotypes were completely the same) with each other. SNPs 6 and 7 were chosen because they were SNPs located close to the splicing sites of exon 3a (ie, an exon specific to type 2 hNP) and exon 4, respectively, and may have some effects on splicing. SNP23 was chosen from the 3′ region, since SNPs15, 16, 17, 19, 21, 23, 24, 25, 27, and 28 were in absolute LD with each other. TaqMan probes and Universal PCR master mix were obtained from Applied Biosystems (Foster City, CA). Thermal cycling conditions for polymerase chain reaction (PCR) were 1 cycle at 95°C for 10 min followed by 50 cycles of 92°C for 15 s and 60°C for 1 min. After

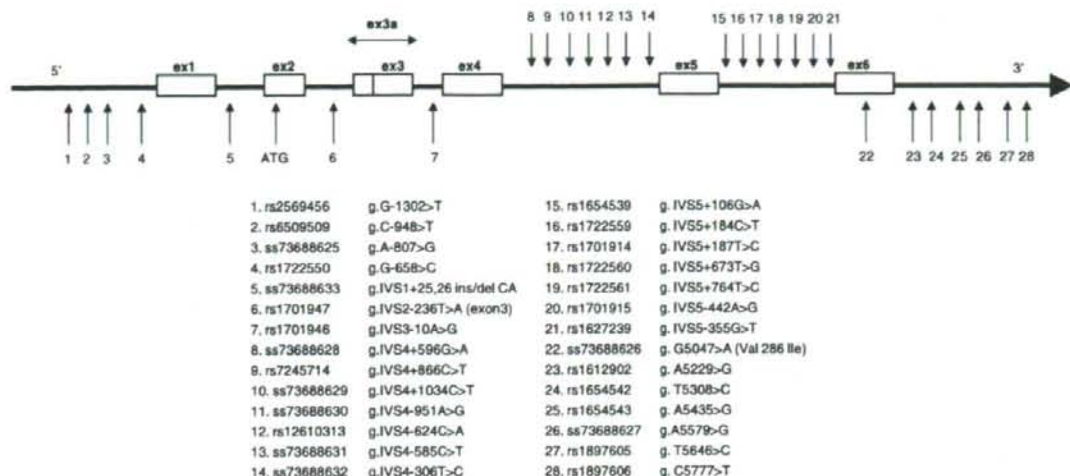

Figure 1 Genomic structure and identified polymorphisms in the human NP gene. A total of 28 SNPs, including one insertion/deletion (ins/del) polymorphism, were identified. The A of the translational start ATG is designated + 1. Nine SNPs were novel and have been registered in the dbSNP (ss-tagged numbers). ex, exon; ex3a, exon3 in hNP type2.

amplification, the allele-specific fluorescence was measured with ABI PRISM 7900 Sequence Detection Systems (Applied Biosystems). Genotype data were read blind to the case–control status. Ambiguous genotype data were not included in the analysis.

Promoter Assay in Primary Cultured Neurons

Primary dissociated cultures were prepared from the brain cortex of postnatal 2-day-old rats (SLC, Shizuoka, Japan) as described previously (Numakawa *et al*, 2002). To generate plasmids for luciferase gene reporter assay, two differentially sized (964 and 128 bp) fragments of the 5′ flanking region of hNP were amplified by PCR with primers 5′-CGACGCGTGCGTGTGCTGGGTTTGAA-3′ (forward) and 5′-GAAGATCTCTAGAGCCTGGGGAGCTTCT-3′ (reverse) for the 964-bp fragment, and 5′-CGACGCGTCCTCCTCTCCCTAGCCTCAG-3′ (forward) and 5′-GAAGATCTCTAGAGCCTGGGGAGCTTCT 3′ (reverse) for the 128-bp fragment. These primers were designed to incorporate *Mlu*I (forward) and *Bgl*II (reverse) restriction sites, and the PCR product was inserted into the multiple cloning site upstream of the luciferase coding region in the pGL3-Basic vector (Promega, Madison, WI). The inserted sequence was confirmed with the auto sequencer CEQ8000 in both directions using primers 5′-TCTCCATCAAAACAAAACGAA-3′and 5′-TTCCATCTTCCAGCGGATA-3′.

Among the four SNPs (SNPs 1–4; see Figure 1) in the 5′ upstream region (ie, putative promoter region) of the hNP gene, the genotypes of SNPs 1, 2, and 4 were completely the same for all the 24 schizophrenic subjects, and we found a significant association of bipolar disorder with SNP4 but not SNP3 (see results). In addition, haplotypes containing the A allele (the major allele), but not the G-allele, of SNP3 showed some evidence for association with bipolar disorder in haplotype analysis (see Table 2). We therefore made two allele-specific promoter fragments (haplotypes consisting of SNPs 1-2-3-4 were G-C-A-G and T-T-A-C) of 964- and 128 bp upstream from the transcription initiation site, which were subject to the luciferase reporter gene assay. The plasmid constructs were transfected into cultured neurons at 5 days *in vitro*. Cells on 24-well plates were co-transfected with 3200 ng of pGL3-Basic firefly luciferase reporter vectors, which included allele-specific promoter fragments of 964 and 128 bp, and 100 ng of phRL-TK *Renilla* luciferase vector (Promega, Tokyo, Japan) as an internal control using Lipofectamine 2000 regent (Invitrogen, Tokyo, Japan). As negative control, an empty pGL3-Basic vector was simultaneously transfected in all the experiments. At 24 h after transfection, luciferase activity was measured using a Dual-Luciferase Reporter Assay System (Promega) and a Lumat LB 9507 luminometer (Berthold, Bad Wildbad, Germany), as described previously (Tadokoro *et al*, 2004; Okada *et al*, 2006). Firefly and *Renilla* luciferase activities were quantified sequentially as relative light unit (RLU) by addition of their respective substrates according to the protocol of the supplier. The ratio of firefly RLU to *Renilla* RLU of each sample was automatically computed. The activity of each construct was expressed at the relative value compared with that of pGL3-Promoter (as a positive control), and these relative values were computed by *t*-test. Primary cultured cells were prepared six times and transfection was performed quadruplicate for each cell culture.

Statistical Analysis

Deviations of genotype distributions from Hardy–Weinberg equilibrium were assessed with χ^2-test for goodness of fit. Genotype and allele distributions of each SNP were compared between patients and controls using χ^2-test for independence. The association of the hNP genotype with

memory and intelligence was examined by multiple analysis of variance (MANOVA) controlling for possible confounders (age, sex, and education years). These tests were performed with the SPSS software version 11 (SPSS Japan, Tokyo, Japan). The LD (D') between polymorphisms was examined using the Haploview program (http://www.broad.mit.edu/mpg/haploview/) (Barrett *et al*, 2005) and haplotype-based association analyses were performed with COCAPHASE software version 2.4 (http://www.mrc-bsu.cam.ac.uk/personal/frank/software/unphased/; Dudbridge *et al*, 2000). The expectation-maximization (EM) and 'droprare' options were used. Haplotypes with frequencies less than 3% were considered to be rare. We examined associations by permutation procedure (10 000 replications) to determine the empirical significance. All *P*-values reported are two-tailed. Statistical significance was considered when $P<0.05$.

RESULTS

Polymorphism Identification and Genotyping

The 7428-bp genomic region containing all the exons, introns, 5′ flanking, and 3′ flanking regions of hNP were screened for polymorphisms in 24 schizophrenic patients. A total of 28 SNPs, including one insertion/deletion (ins/del) polymorphism, were identified (Figure 1). Among them 19 SNPs had already been listed in the NCBI dbSNP database, whereas nine SNPs were novel. Four SNPs were located in 5′ upstream, one SNP in exon 6, six SNPs in 3′ downstream, and the remaining 17 polymorphisms in introns. There was only one SNP that resulted in an amino-acid change, SNP22 (G5047 > A; Val286Ile: the number of the amino acid is according to NP_653088), which gave rise to a restriction site for *Acy*I and was located at an evolutionarily conserved (rodents through humans) residue. This non-synonymous polymorphism was found in only one schizophrenic patient. Additional genotyping was performed for 178 individuals with schizophrenia; however, there was no individual carrying the 286Ile allele, indicating that this amino-acid change is a rare mutation. The LD between SNPs is shown in Supplementary Figure S1, indicating that the entire genomic region consists of single haplotype block. Genotypes for SNPs 1, 2, 4, and 5, those for SNPs 8 and 9, those for SNPs 10 and 13, and those for SNPs15, 16, 17, 19, 21, 23, 24, 25, 27, and 28, respectively, were completely the same as each other for the 24 individuals.

Promoter Assay

We identified four SNPs in the 1078-bp 5′ upstream region of the hNP gene, and SNPs 1, 2, and 4 were found to be associated with bipolar disorder, memory, and intelligence quotient (IQ) (see below). Furthermore, to our knowledge, there is no information in the literature on the location of core promoter of the hNP gene. We therefore performed a promoter assay using the dual-luciferase system (Promega) in rat cultured cortical neurons and examined whether transcriptional activity alters in an allele-dependent manner. As shown in Figure 2a, pGL3-Basic vectors containing 128- and 964-bp fragments, which consisted of major alleles for the four SNPs, demonstrated substantially higher RLEs

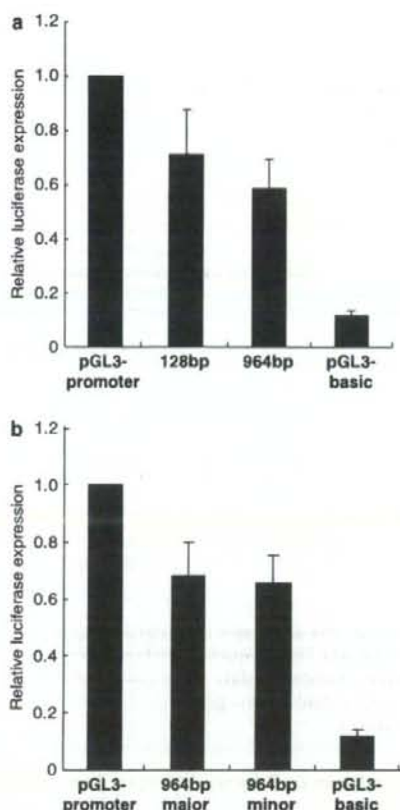

Figure 2 Promoter assay. (a) RLE for pGL3-Basic vector with insertion of 128 and 964 bp of the hNP 5′ flanking regions in comparison with pGL3-Basic vector, which dose not contain a promoter sequence. The RLE for pGL3-promoter vector containing SV40 promoter (positive control vector) was assigned a value of 1. Both 128- and 964-bp fragments showed substantially higher RLE compared with pGL3-basic vector without promoter sequence. (b) Comparison of RLE between the major (G-C-A-G for SNPs 1-2-3-4) and minor (T-T-A-C) alleles. No significant difference was found between the two alleles.

(relative luciferase expression) than that of pGL3-basic empty vector, suggesting that the core promoter region is located within the 128-bp fragment. We then cloned the 964-bp allele-specific promoter fragments (SNP1-2-3-4; major allele: G-C-A-G, minor allele: T-T-A-C) and compared RLEs between the two alleles (Figure 2b); however, we found no significant difference in the RLE between the two haplotype fragments. These results suggest that SNPs 1, 2, and 4 might not influence the transcriptional activity of the hNP gene.

Association with Psychiatric Diseases

We genotyped five SNPs (SNPs 3, 4, 6, 7, and 23) to examine possible association with schizophrenia, major depression, and bipolar disorder. Genotype and allele distributions in the diagnostic groups are shown in Table 1. Genotype

Table 1 Genotype and Allele Distributions of the five SNPs of the hNP Gene in Patients with Schizophrenia, those with Major Depression, those with Bipolar Disorder, and the Controls

SNP	Diagnosis	N	Genotype frequency (GF)			Allele frequency (AF)		Odds ratio (95% CI)	χ^2-Test vs controls		
									GF vs HW	GF (df=2)	AF (df=1)
SNP3			A/A	A/G	G/G	A	G				
	Controls	696	462 (0.66)	208 (0.30)	26 (0.04)	1132 (0.81)	260 (0.19)		0.67		
	SZ	421	277 (0.66)	126 (0.30)	18 (0.04)	680 (0.81)	162 (0.19)	1.06 (0.85–1.33)	0.45	$\chi^2=0.21$ $P=0.90$	$\chi^2=0.11$ $P=0.74$
	MD	382	276 (0.72)	90 (0.24)	16 (0.04)	642 (0.84)	122 (0.16)	1.18 (0.93–1.51)	0.02	$\chi^2=4.94$ $P=0.08$	$\chi^2=2.48$ $P=0.12$
	BD	202	139 (0.69)	56 (0.28)	7 (0.03)	334 (0.83)	70 (0.17)	1.09 (0.82–1.46)	0.65	$\chi^2=0.4$ $P=0.81$	$\chi^2=0.36$ $P=0.54$
SNP4			G/G	G/C	C/C	G	C				
	Controls	683	388 (0.57)	243 (0.36)	52 (0.08)	1019 (0.75)	347 (0.25)		0.11		
	SZ	406	234 (0.58)	150 (0.37)	22 (0.05)	618 (0.76)	194 (0.24)	1.1 (0.90–1.36)	0.75	$\chi^2=1.97$ $P=0.37$	$\chi^2=0.62$ $P=0.43$
	MD	371	219 (0.59)	126 (0.34)	26 (0.07)	564 (0.76)	178 (0.24)	1.1 (0.89–1.36)	0.19	$\chi^2=0.5$ $P=0.78$	$\chi^2=0.58$ $P=0.47$
	BD	198	91 (0.46)	90 (0.45)	17 (0.09)	272 (0.69)	124 (0.31)	1.33 (1.04–1.7)	0.43	[illegible]	[illegible]
SNP6			T/T	T/A	A/A	T	A				
	Controls	711	316 (0.44)	306 (0.43)	89 (0.13)	938 (0.66)	484 (0.34)		0.27		
	SZ	422	195 (0.46)	192 (0.45)	35 (0.08)	582 (0.69)	262 (0.31)	1.17 (0.97–1.41)	0.20	$\chi^2=4.86$ $P=0.09$	$\chi^2=2.15$ $P=0.14$
	MD	378	171 (0.45)	164 (0.43)	43 (0.11)	506 (0.67)	250 (0.33)	1.06 (0.88–1.29)	0.70	$\chi^2=0.31$ $P=0.86$	$\chi^2=0.21$ $P=0.65$
	BD	197	70 (0.36)	99 (0.50)	28 (0.14)	239 (0.61)	155 (0.39)	1.25 (0.99–1.58)	0.46	$\chi^2=5.03$ $P=0.08$	$\chi^2=3.8$ $P=0.051$
SNP7			A/A	A/G	G/G	A	G				
	Controls	718	325 (0.45)	314 (0.44)	79 (0.11)	964 (0.67)	472 (0.33)		0.81		
	SZ	433	209 (0.48)	190 (0.44)	34 (0.08)	608 (0.70)	258 (0.30)	1.16 (0.96–1.40)	0.31	$\chi^2=3.26$ $P=0.20$	$\chi^2=2.36$ $P=0.12$
	MD	387	182 (0.47)	163 (0.42)	42 (0.11)	527 (0.68)	247 (0.32)	1.05 (0.87–1.28)	0.55	$\chi^2=0.33$ $P=0.85$	$\chi^2=0.21$ $P=0.65$
	BD	203	72 (0.35)	103 (0.51)	28 (0.14)	247 (0.61)	159 (0.39)	1.31 (1.04–1.65)	0.36	[illegible]	[illegible]
SNP23			A/A	A/G	G/G	A	G				
	Controls	714	428 (0.60)	241 (0.34)	45 (0.06)	1097 (0.77)	331 (0.23)		0.16		
	SZ	421	267 (0.63)	135 (0.32)	19 (0.05)	669 (0.79)	173 (0.21)	1.17 (0.94–1.44)	0.71	$\chi^2=2.25$ $P=0.32$	$\chi^2=2.13$ $P=0.14$
	MD	388	240 (0.62)	127 (0.33)	21 (0.05)	607 (0.78)	169 (0.22)	1.08 (0.87–1.34)	0.44	$\chi^2=0.56$ $P=0.75$	$\chi^2=0.56$ $P=0.45$
	BD	204	98 (0.48)	86 (0.42)	20 (0.10)	282 (0.69)	126 (0.31)	1.48 (1.16–1.88)	0.86	[illegible]	[illegible]

Abbreviations: 95% CI, 95% confidence interval; BD, bipolar disorder; df, degrees of freedom; hNP, human neuropsin; HW, Hardy–Weinberg; MD, major depression; SNP, single-nucleotide polymorphism; SZ, schizophrenia.Significant p-values are gray colored.

distributions of these SNPs did not deviate significantly from Hardy–Weinberg equilibrium, except for SNP3 in patients with major depression ($P=0.02$). There was no significant difference in genotype or allele distribution for any SNP between patients and controls for schizophrenia or major depression. However, there was a significant difference in genotype distributions between patients with bipolar disorder and controls for three SNPs, that is, SNPs 4, 7, and 23. Allele frequencies for these SNPs also differed significantly between the two groups. P-values, odds ratios, and their 95% confidence interval (CI) are shown in Table 1. Then we performed haplotype-based analysis with a two-marker sliding window method. We obtained no evidence of a significant association for schizophrenia or major depression (data not shown). With respect to bipolar disorder, we obtained significant individual P-values for all combinations of two markers; however, significant global P-value (0.0068) was obtained only when haplotype consisted of SNPs 7 and 23 (Table 2). Furthermore, overall global P-value ($P=0.083$), considering all multiple testing for all the combinations of two-marker haplotypes, just failed to reach statistical significance. Thus, we did not obtain any stronger evidence for association in the haplotype-based analysis than in the single-marker analysis of SNP23 ($P=0.0015$).

Association with Memory and IQ

Among the 166 controls whose memory scale and IQ were measured, SNP23 (A/G) was successfully genotyped in 163 individuals. Mean (SD) index scores of verbal memory, visual memory, general memory, attention and concentration, and delayed recall in the 163 controls were 110.9 (13.7), 109.9 (9.0), 112.2 (12.1), 103.9 (13.5), and 112.1

Table 2 Two-Marker Haplotype Analysis in Patients with Bipolar Disorder and Controls.

Markers					Haplotype frequency		P-value		
SNP3	SNP4	SNP6	SNP7	SNP23	BD	Controls	Individual	Global[a]	Overall global[a]
A	C				0.31	0.26	0.028	0.073	
	C	A			0.30	0.24	0.030	0.10	
		A	G		0.39	0.33	0.028	0.11	
			G	G	0.30	0.23	0.0068	0.014	0.083

Abbreviations: BD, bipolar disorder; SNP, single-nucleotide polymorphism.
[a]Global P-value for each combination of two markers and overall global significance for all combinations of two markers were calculated by permutation of 10 000 simulations.

(12.0), respectively. Mean (SD) full-scale IQ, verbal IQ, and performance IQ were 109.3 (11.6), 107.3 (12.9), and 110.3 (11.7), respectively. Since SNP23 showed the strongest association with bipolar disorder (G-allele was the risk allele) among the 5 SNPs examined, memory and IQ were compared between those who carried the G-allele (carrier, G/G or A/G, $N=64$) and those who did not (non-carrier, A/A, $N=99$) (Figure 3). Since the number of individuals with G/G genotype was very small ($N=8$), they were combined with those with the A/G genotype. With respect to sub-scales of WMS-R, the mean score of attention/concentration was significantly lower in carriers than in non-carriers ($P=0.016$); however, there were no significant differences between the two groups for the remaining sub-scales (verbal memory, visual memory, general memory, and delayed recall). With respect to WAIS-R, there was a significant difference in full-scale IQ ($P=0.018$) between the two groups. When verbal and performance IQ were examined separately, there was a highly significant difference in verbal IQ ($P<0.001$), but not in performance IQ, between the two groups. The mean verbal IQ (SD) for carriers and non-carriers was 103.4 (12.9) and 109.8 (12.3), respectively. As for the other SNPs, similar results are obtained (data not shown) because of the tight LD across the SNPs.

DISCUSSION

In the present study, we performed polymorphism screening and identified 28 SNPs, including nine novel SNPs, in the 7428-bp region of the whole hNP gene, including the 5′ and 3′ flanking regions. Then we performed promoter assay and determined a core promoter region of the hNP gene, although failing to find significant effects of SNPs on transcriptional activity. Association analysis using five SNPs as markers revealed significant difference in genotype and allele distributions for some of the SNPs between patients and controls for bipolar disorder, but not for schizophrenia or major depression. When a possible association of the SNPs with memory and IQ was examined in healthy control subjects, we found significant differences in attention/concentration sub-scale score of the WMS-R and verbal IQ between genotypes.

Among the 28 SNPs identified, there was only one SNP in the exons; SNP22 was a Val286Ile missense mutation in exon 6, which was detected in a patient with schizophrenia. Additional genotyping for 178 schizophrenic subjects did not find anyone carrying this variant, indicating that this is a rare mutation. Thus, whether this mutation is pathogenic or not is unclear. Since we examined only 24 individuals for polymorphism screening, we may have missed some rare mutations as the SNP22.

Our promoter assay in rat primary cultured neurons suggested that the core promoter is present in the 128-bp 5′ upstream region of the hNP gene. Since the RLE of pGL3-vector containing the 964-bp fragment was somewhat lower than that of pGL3-vector containing 128-bp fragment, a silencer-like region may be present between 128- and 964-bp positions upstream of the hNP gene. Then we examined whether transcriptional activity differs in an allele-dependent manner; however, we found no significant difference between alleles. These results suggest that SNPs 1, 2, and 4 might not influence the transcriptional activity of the hNP gene. According to the TFSEARCH database (http://mbs.cbrc.jp/research/db/TFSEARCHJ.html), these SNPs are not located on any of the binding sites of transcriptional factors, which is in line with our finding of no significant difference between the alleles.

In our association study with psychiatric diseases, we found, for the first time, significant differences in genotype and allele distributions between patients with bipolar disorder and controls. The best *P*-value was obtained for SNP23 in allele distribution ($P=0.0015$, odds ratio 1.48, 95% CI 1.16–1.88). This *P*-value remained significant even after correcting the critical *P*-value for Bonferroni's multiple testing (15 comparisons: 5 SNPs $\times$ 3 diseases). Haplotype-based analysis also yielded nominally significant results particularly when SNP23 was included in markers of analysis. These results suggest that SNP23 or other unknown SNPs in LD with SNP23 confers susceptibility to bipolar disorder. Since hNP is a part of a gene cluster (kallikreins), there remains a possibility that variations of some other kallikrein gene might be truly responsible to giving susceptibility to bipolar disorder. The results are in line with a previous study reporting a susceptibility locus for bipolar disorder on chromosome 19q13 (Badenhop *et al*, 2002). A possible limitation is that a portion of patients with bipolar disorder and controls were recruited in a geographically different area (ie, Shiga prefecture but not in Tokyo), which may have resulted in a population stratification; however, the minor allele frequency of SNP23 was very similar in controls from Shiga and those from Tokyo (0.233 in Shiga and 0.232 in Tokyo), suggesting that the effect of stratification is unlikely. Another limitation might be that