厚生労働科学研究費補助金（創薬基盤推進研究事業）
創薬バイオマーカー探索研究事業

精神・神経疾患関連バイオマーカー探索による創薬基盤研究

（H20-バイオ-一般-010）

平成23年度　総括研究報告書

研究代表者　　後　藤　雄　一

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平成 24（2012）年 5 月

総括研究報告

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後藤雄一

厚生労働科学研究費補助金（創薬基盤推進研究事業）
総括研究報告書

精神・神経疾患関連バイオマーカー探索による創薬基盤研究

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研究要旨　本研究計画は（A）髄液等の患者試料と情報の収集、（B）プロテオーム解析、（C）疾患特異的バイオマーカー同定に向けた研究、（D）臨床応用と創薬研究、という研究内容の区分がある。平成23年度は、（A）に関しては、病院検査部及び各診療科との連携で、髄液試料確保のシステムを整備し、正常対照者、統合失調症等の精神疾患患者を中心に髄液採取が順調に登録された（平成24年3月31日までに、420検体）。（B）（C）に関しては、統合失調症10例、健常対象者10例のプロテオーム測定を終了し、バイオマーカーの候補となるタンパク質を見いだしている。他の統合失調症患者でELISA法を用いた確認実験が継続中である。また、気分障害についても解析が進んでおり、正常対照者に対して増加しているタンパク質、低下しているタンパク質を見いだしている。

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A．目的

精神・神経疾患はその病因、病態の複雑さのために、治療薬開発が最も遅れている分野である。ヒトゲノムプロジェクトの成果を受けて、網羅的なゲノム解析手法で疾患関連遺伝子及びその産物が同定されてきているが、それらが病態にどう関わるかについて理解し、さらに創薬に結びつけるには、「たんぱく質レベル」の動態の把握が必要なことが周知の事実となっている。平成15年度～平成19年度に行った精神・神経疾患プロテオーム研究において、血液を用いた解析に比べ、髄液を用いた解析では、数多くの神経特異的たんぱく質の同定が可能で、中枢神経の状態を直接的に反映していることが実証された。その手法を最大限活用し、各種の精神・神経疾患患者から採取した髄液のプロテオーム解析を出発点として疾患特異的に変動するたんぱく質を見いだし、診断、病勢、薬効を判定する際に有効なバイオマーカーを同定し、さらにはそのたんぱく質及び関連するたんぱく質の機能解析を行うことで創薬に結びつけることが本研究の目的である。

B．研究方法

全体計画

1） 髄液等の患者試料と情報の収集

国立精神・神経医療研究センター病院（もしくは共同研究病院）で、IC 取得後に試料と情報を収集、登録する。精神疾患担当（有馬、吉田、功刀）、神経疾患担当（村田、山村）、小児精神・神経疾患担当（中川）で行う。

2）プロテオーム解析

当センターにおいて cICAT 法を中心としたプロテオーム解析を行い（林）、また試料の一部をプロテオーム・リサーチ・センター（PRC）に送付し、測定結果を返してもらう。

3） 疾患特異的バイオマーカー同定

ア．バイオインフォマティクス（後藤、金子）

当センターでのデータ及び PRC より返されたデータを解析し、候補バイオマーカーを選択する。

イ．バリデーション研究（村田、有馬、沼知、中川、和田、功刀、山村、後藤）

簡易測定系を開発し、バイオマーカーとして有用かどうかを判定するために、血液や尿などの採取しやすい試料での検討を行う。その結果を踏まえて、100 例程度の血液等を用いたバリデーション研究を行う。

ウ．候補バイオマーカー関連物質の探求（後藤）

候補バイオマーカーに関連する物質の探求のために、それら物質のゲノム解析及びトランスクリプトーム解析等を行う。これにより、バイオマーカーとしての信憑性が高められることが期待できる。

エ．候補バイオマーカーの機能解析（和田、功刀、山村、有馬）

有力な候補バイオマーカーに関して、生物学的な機能を検討する。実験動物、ヒト由来の培養細胞、剖検脳などを使用して研究する。

4）臨床応用と創薬研究

ア．バイオマーカーの臨床応用（村田、吉田、有馬、山村、中川）

多数例を用いて臨床的な有用性の確認を行う。

イ．創薬研究（疾患担当者、小紫、茶木）

有力なバイオマーカーに関連するたんぱく質の探求やそれらの生物学的機能の理解を踏まえて、新薬開発に関する研究を行う。

本年度の研究方法

1）髄液等の患者試料と情報の収集

(1) 髄液等の検体採取と受け入れシステム化

髄液採取コーディネーターチームを作り、研究への参加意思の確認から、髄液採取の実施もしくは援助、得られた検体の運搬・処置、匿名化、臨床情報の取得などを行う。

(2) 患者試料の登録

収集した試料をプロテオーム解析まで小分けしてディープフリーザー（-80℃）に凍結保存する。

2）プロテオーム解析

(1) 髄液 2mL からのプロテオーム解析

確定した解析手法で前処理を行い、QSTAR による質量分析を行う。

(2) 統合失調症症例のプロテオーム解析

統合失調症の男性患者 10 名のプロテオーム解析を行い、健常者10名での結果を比較検討した。その際、統合失調症群と健常対照群の年齢を合わせるとともに、すべて男性症例で検討した。通常の統計的比較（Student’s t-test もしくは Mann-Whitney U-test）に加えて、分散が大きいものを（統合失調症の一群で高値・低値を示すもの）捉える分散比および F-test を実施した。

(3) 気分障害症例 9 例のプロテオーム解析

うつ病患者の症例9名のプロテオーム解析を行い、健常者 13 例と比較検討した。

（倫理面への配慮）

研究者の所属する施設の倫理委員会に本研究に関する倫理申請を行い、承認を得て行った。診療上、髄液を採取する必要のある疾患患者に研究参加を依頼することを基本に研究計画を作成した。すでに、認知症及び神経疾患全般に関しては、余剰髄液を用いて行う研究が動いていたので、それに加えるプロ

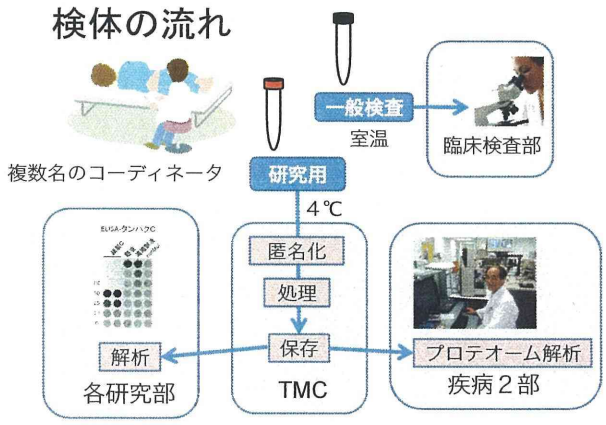
トコールで研究計画を構築した。さらに平成21年度後半には、ボランティアにて髄液採取に協力していただける疾患患者、正常者からの髄液を研究利用するプロトコールも倫理委員会の承認を得た。購入した米国人髄液（VITAL PRODUCT社）は、検査後余剰プール髄液（ICあり）であり、米国FDAも購買リストに名が連なっている。

C．研究結果と考案

1）髄液等の患者試料と情報の収集

(1) 髄液等の検体採取及び受け入れのシステム化

患者への説明、同意取得、検体採取等の一連の流れと必要書類、必要物品をパッケージとして病棟に配布し、それらを用いた「検体採取のプロトコール」を作成し使用している。今年度は、2名の臨床検査技師、2名の臨床心理士を含む髄液コーディネーターチームを組織し、検体採取の流れを促進させた。



(2) 患者試料の登録

平成24年3月末までに370例(420検体)の髄液採取ができた。精神疾患患者からの検体数が飛躍的に伸び、小児神経科、神経内科、脳神経外科からの登録も得た。2011年2月末では総数が150検体であった、13ヶ月で270検体の登録を行う事ができた。特に統合失調症の検体は、1年間で80検体も増加した。その内訳を次表に示す。

髄液検体の内訳（2012年3月末現在）

分類	内訳	検体数	症例数
精神疾患（203症例）	統合失調症	117 (37)	80
	気分障害	92 (28)	92
	健常対照	66 (30)	60
	その他	5	5
神経内科疾患（113症例）	パーキンソン病	9	9
	脊髄小脳変性症	6	6
	正常圧水頭症	36	29
	多発性硬化症関連	21	21
	その他（認知症等）	48	48
小児神経疾患（20症例）	てんかん	11	11
	精神遅滞	5	5
	その他	4	4
Total		420 (150)	370

括弧内は2011年2月末の検体数

2）プロテオーム解析

(1) 髄液2mLからのプロテオーム解析

昨年度までの研究で確定した初期量2mLからのプロテオーム解析のプロトコールを実施した。

その後、QSTAR-XLを用いて質量分析を行い、データをプロテオームファクトリーで開発した質量分析データ処理ソフト（Mascotベース）を用いて解析した。しかし、自動的に2つのラベル化ペプチド（HとL）の量比（H/L）が出ない場合が多く、その場合はペプチド量の生データから値を引き出してくる必要があった。この作業はマニュアルで行うため相当の時間がかかることになった。

(2) 統合失調症症例のプロテオーム解析

統合失調症の男性患者10名のプロテオーム解析を行い、健常者での結果を比較検討した。健常対照者の場合と同様に、一回の質量分析で、270～300前後のタンパク質を同定できた。

統合失調症10例と健常対照者10例の測定結果

統合失調症			タンパク数	健常者			タンパク数
初発	26	M	321	C01	25	M	337
S10	27	M	348	C21	26	M	277
S06	35	M	307	C09	35	M	306
S08	38	M	337	C16	35	M	295
S11	40	M	286	C18	36	M	353
S07	43	M	364	C08	38	M	283
S01	50	M	356	C10	43	M	278
S03	53	M	389	C14	54	M	279
S12	55	M	299	C04	57	M	333
S13	63	M	283	C05	65	M	290
平均	43.0			平均	41.4		

全10例で測定結果が自動で実測できているのではなく、生データからマニュアルで当該タンパク質データを抽出する作業を継続中。

680個の同定タンパク質のうち、データ解析が可能な280余で解析

個々の質量分析で同定できているタンパク質の中で、タンパク解析ソフトで自動的に同定されるタンパク質が複数例で同定できているタンパク質は少なく、結局のべ680個のタンパク質が同定された。

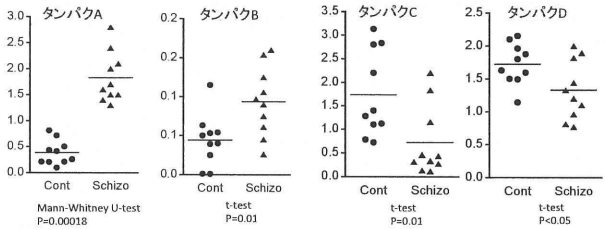
統合失調症群で上昇している、もしくは低下しているタンパク質は、P<0.05のものが37個、P<0.01のものが19個存在した。その中から代表的なタンパク質の例を以下に示す。

解析1　統合失調症に共通のバイオマーカーの探索

統合失調症群 vs 健常対照群で有意差のあるもの
(Student's t-test or Mann-Whitney U-test)

P<0.05 のもの 37個、P<0.01 のもの19個

統合失調症で上昇するもの　　統合失調症で低下するもの

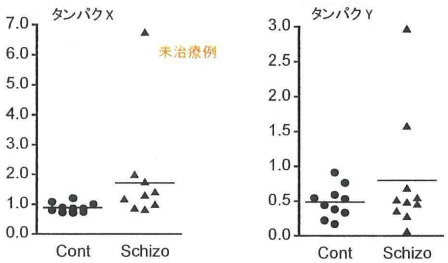


また、統合失調症にはいくつかのサブグループが存在すると想定されており、それらの解析を行ういとで、他の検体と飛び抜けて値の違うタンパク質を探索することで、サブグループ特異的なマーカーを探し出す試みをした。その中で以下の図に示すようなタンパク質が同定でき、今後その検証を行ってゆく予定である。

解析2　　統合失調症のサブタイプの探索

統合失調症群で分散が大きいもの
（統合失調症の一群で高値・低値を示すもの）

分散比(F)>2　かつ　F-test<0.1　→　29個



(3)　気分障害症例9例のプロテオーム解析

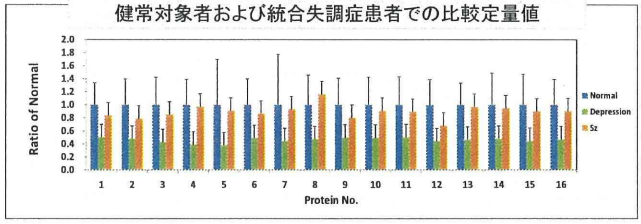
気分障害症例9例と健常対照者13例の測定結果をみると、同定できた550個のタンパク質において、変動が2倍以上のタンパク質が44個（そのうち低下したものは34種類）、変動が1.67倍以上のタンパク質が93種類（そのうち低下したものは82種類）であった。これは、うつ病で変動するタンパク質の種類が多いことを示しており、特に低下するものが圧倒的であるというが判明した。

うつ病9例と健常対照者13例の測定結果

同定できている550個程度のタンパク質で比較　有意差 (p<0.05)

変動が2倍以上 (Ratio <0.5 or >2.0):　44種類（低下:34種類）
変動が1.67倍以上 (Ratio <0.6 or >1.67):　93種類（低下:82種類）

うつ病で2倍以上低下したタンパク質の
健常対象者および統合失調症患者での比較定量値



1. うつ病では、変動するタンパク質が多い
2. うつ病で低下したタンパク質は統合失調症では無変化

さらに、これら低下していたタンパク質は、統合失調症では変動していないことが判明した。

今後はこれらのデータに電気けいれん療法で効果のある症例の治療前後のプロテオームプロファイルの違いなどを手がかりに、新たなバイオマーカーの同定を行う予定である。

E．結論

平成23年度においては、(A) 髄液等の患者試料と情報の収集、(B) プロテオーム解析、(C) 疾患特異的バイオマーカー同定に向けた研究、(D) 臨床応用と創薬研究、という研究内容の区分のうち、(A) 髄液等の患者試料と情報の収集が格段に進んだ上、(B) プロテオーム解析の結果から、(C) 疾患特異的バイオマーカー同定の研究に踏み込むことができた。しかし、当初の全体計画では多数の疾患を対象としてあげていたが、その意味では大幅に進捗が遅れていることは否めない。しかし平成24年度は最終年度として、特に統合失調症の疾患特的マーカーの同定、さらに創薬に関連するマーカーの同定を行うべく最大限の努力を行うこと、加えて気分障害においても疾患特異的タンパク質変化を多数例で検証する研究を推進させる予定である。

F．健康危険情報

特になし

G．研究発表

1．論文発表

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2．学会発表　なし

H．知的財産権の出願・登録状況

なし

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Association of plasma IL-6 and soluble IL-6 receptor levels with the Asp358Ala polymorphism of the IL-6 receptor gene in schizophrenic patients

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ABSTRACT

Recent studies indicate a role of excessive interleukin-6 (IL-6) signaling in the pathogenesis of schizophrenia. A previous study reported a significant association of schizophrenia with the IL-6 receptor (IL-6R) gene Asp358Ala polymorphism, which is known to regulate circulating IL-6 and soluble IL-6R (sIL-6R) levels in healthy subjects. To further examine the influence of the polymorphism in schizophrenic patients, we compared the plasma levels of IL-6 and sIL-6R between schizophrenic patients and healthy controls for each genotype of the Asp358Ala polymorphism. Asp358Ala genotyping and plasma IL-6 level measurements were performed in 104 patients with schizophrenia and 112 healthy controls. Of these participants, 53 schizophrenic patients and 49 controls were selected for the measurement of plasma sIL-6R levels. A two-way factorial analysis of covariance was performed with the transformed plasma levels as the dependent variable, diagnosis and genotype as independent variables, and sex and age as covariates. No significant diagnosis × genotype interaction was observed for IL-6 and sIL-6R levels. The Ala allele of Asp358Ala was significantly associated with higher levels of both IL-6 and sIL-6R. IL-6 levels were significantly elevated in schizophrenic patients compared to those in controls, whereas no significant difference in sIL-6R levels was observed between schizophrenic patients and controls. Our findings suggest that the presence of schizophrenia is associated with elevated IL-6 levels, whereas sIL-6R levels are mainly predetermined by the Asp358Ala genotype and are not associated with the disease status. Increased IL-6 levels without alterations in sIL-6R levels may result in excessive IL-6 signaling in schizophrenia.

1. Introduction

Inflammatory immune processes have been strongly implicated in the etiology of schizophrenia (Watanabe et al., 2010). Elevated serum or plasma levels of interleukin-6 (IL-6) is observed in patients with schizophrenia (Potvin et al., 2008), suggesting a role of excessive IL-6 signaling in the pathogenesis of this disorder. IL-6 binds to the soluble IL-6 receptor (sIL-6R) to form an IL-6/sIL-6R complex that is capable of binding to gp130 in the cellular membrane to mediate intracellular signaling. As membrane-bound IL-6R is expressed selectively on monocytes, neutrophils, T and B lymphocytes, and hepatocytes, other cells require the IL-6/sIL-6R complex for IL-6 signaling. Therefore, it could be inferred that sIL-6R plays an important part in the pathogenesis of schizophrenia.

An increased IL-6 level is one of the most robust findings in the study of inflammatory markers in schizophrenia, as evidenced by a meta-analysis of 19 studies comprising 1219 subjects (Potvin et al., 2008). Furthermore, one study showed a positive correlation between the severity of symptoms and plasma IL-6 levels in antipsychotic-free schizophrenic patients (Pae et al., 2006). However, findings regarding changes in the circulating levels of sIL-6R in patients with schizophrenia have been equivocal. Some studies reported increased sIL-6R levels in patients with schizophrenia (Lin et al., 1998; Maes et al., 1997), whereas one study reported lower sIL-6R levels (Maes et al., 1994). Others reported no significant differences in sIL-6R levels between patients and controls (Maes et al., 1995; Muller et al., 1997; O'Brien et al., 2008). Non-significant effect size estimates were obtained for sIL-6R in a meta-analysis of 7 studies (Potvin et al., 2008).

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Table 1
Subject characteristics.

	Patients with schizophrenia				Healthy controls			
	Asp/Asp	Asp/Ala	Ala/Ala	Statistical test results	Asp/Asp	Asp/Ala	Ala/Ala	Statistical test results
All subjects								
Gender (males/females)	15/17	26/23	14/9	$\chi2 = 1.1$, $p = 0.59$	14/24	31/29	11/3	$\chi2 = 7.3$, $p = 0.03$
Age (years)	38.8 ± 13.9	40.8 ± 12.3	35.3 ± 8.5	$F = 1.64$, $p = 0.20$	41.0 ± 13.0	38.4 ± 13.1	39.2 ± 11.2	$F = 0.48$, $p = 0.62$
Duration of illness (years)	16.7 ± 11.0	17.7 ± 10.7	15.4 ± 8.8	$F = 0.39$, $p = 0.68$				
Duration of treatment (years)	13.0 ± 8.6	15.1 ± 10.1	12.3 ± 7.8	$F = 0.91$, $p = 0.41$				
CP equivalent dose	852 ± 685	993 ± 883	1030 ± 667	$F = 0.44$, $p = 0.65$				
Subjects selected for the measurement of sIL-6R levels								
Gender (males/females)	9/9	9/9	8/9	$\chi2 = 0.04$, $p = 0.98$	6/12	7/10	11/3	$\chi2 = 7.1$, $p = 0.03$
Age (years)	41.2 ± 10.2	40.6 ± 12.1	36.1 ± 7.6	$F = 1.28$, $p = 0.29$	43.5 ± 10.1	37.8 ± 13.3	39.2 ± 11.2	$F = 1.15$, $p = 0.32$
Duration of illness (years)	17.0 ± 7.3	18.1 ± 11.2	16.3 ± 7.7	$F = 0.17$, $p = 0.84$				
Duration of treatment (years)	14.8 ± 8.1	16.4 ± 10.8	12.2 ± 6.2	$F = 1.02$, $p = 0.37$				
CP equivalent dose	922 ± 688	1020 ± 788	1094 ± 746	$F = 0.24$, $p = 0.79$				

Continuous values are shown as mean ± standard deviation, CP: chlorpromazine.

sIL-6R is generated by shedding of the membrane-bound IL-6R. This process is influenced by the single nucleotide polymorphism (SNP) Asp358Ala of the IL-6R gene (rs8192284), which results in an amino acid substitution in the proteolytic cleavage site. The Ala allele of this polymorphism in healthy subjects is known to be strongly associated with higher levels of circulating sIL-6R (Galicia et al., 2004; Rafiq et al., 2007; Reich et al., 2007) and IL-6 (Jiang et al., 2010; Rafiq et al., 2007; Reich et al., 2007). Therefore, possession of the Ala allele may result in constitutively elevated IL-6 signaling.

A previous genetic association study reported a significant association of the Ala allele of the IL-6R Asp358Ala polymorphism with schizophrenia (Sun et al., 2008). It can be hypothesized that the excessive IL-6 signaling associated with Ala alleles may increase the susceptibility to schizophrenia. However, the increased IL-6 levels without significant change in sIL-6R levels in schizophrenic patients (Potvin et al., 2008) could not be explained solely by the increased Ala allele frequency in schizophrenia.

To our knowledge, the possible associations of the IL-6R Asp358Ala polymorphism with circulating sIL-6R and IL-6 levels in patients with schizophrenia have not yet been examined. Further investigation of the influence of this polymorphism in schizophrenia is necessary to elucidate the roles of IL-6 and sIL-6R in this disorder. Thus, we compared the plasma levels of IL-6 and sIL-6R between patients with schizophrenia and healthy controls for each genotype of the Asp358Ala polymorphism.

2. Materials and methods

2.1. Subjects

Asp358Ala genotyping and plasma IL-6 level measurements were performed in 104 patients with schizophrenia (55 men and 49 women; mean age ± standard deviation: 39.0 ± 12.2 years), and 112 healthy controls (56 men and 56 women; age: 39.4 ± 12.8 years), frequency-matched for sex and age. Of these participants, 53 schizophrenic patients (26 men and 27 women; age: 39.4 ± 10.2 years) and 49 controls (24 men and 25 women; age: 40.3 ± 11.6 years), matched for the number of cases and controls for each genotype, were selected for the measurement of plasma sIL-6R levels. All subjects were biologically unrelated Japanese individuals and were recruited from the outpatient clinic of the National Center of Neurology and Psychiatry Hospital, Tokyo, Japan or through advertisements in free local information magazines and by our website announcement. Consensus diagnosis by at least 2 psychiatrists was made for each patient according to the Diagnostic and Statistical Manual of Mental Disorders, 4th edition criteria (American Psychiatric Association, 1994), on the basis of unstructured interviews and information from medical records. All patients were under treatment with antipsychotic medication, of which 25 were receiving inpatient treatment. The average chlorpromazine equivalent dose converted from daily doses of antipsychotics using published guidelines (American Psychiatric Association, 1997; Inagaki et al., 1999) was 957.0 ± 776.7 mg/day (typical antipsychotics, 528.8 ± 747.7 mg/day; atypical antipsychotics, 428.2 ± 458.0 mg/day). The mean age at onset was 21.9 ± 7.1 years, and the mean durations of illness and antipsychotic treatment were 16.9 ± 10.3 and 13.8 ± 9.2 years, respectively. The controls were healthy volunteers with no current or past histories of psychiatric treatment and were screened using the Japanese version of the Mini International Neuropsychiatric Interview (Otsubo et al., 2005; Sheehan et al., 1998) by a research psychiatrist to eliminate the possibility of any axis I psychiatric disorders. Participants were excluded if they had prior medical histories of central nervous system diseases or severe head injury or if they met the criteria for substance abuse or dependence or mental retardation. The self-reports indicated that none of the participants suffered from any inflammatory or infectious diseases at the time of assessment. The study protocol was approved by the ethics committee at the National Center of Neurology and Psychiatry, Japan. After describing the study, written informed consent was obtained from every subject.

2.2. Genotyping

Genomic DNA was prepared from venous blood according to standard procedures. The Asp358Ala polymorphism was genotyped using the TaqMan 5′-exonuclease allelic discrimination assay.

Table 2
Distribution of the Asp358Ala variants.

	Genotype count (frequency)						Allele count (frequency)					HWE	
	N	Asp/Asp	Asp/Ala	Ala/Ala	$\chi2$ (df = 2)	*P* value	N	Asp	Ala	$\chi2$ (df = 1)	*P* value	$\chi2$ (df = 1)	*P* value
Patients	104	32 (0.31)	49 (0.47)	23 (0.22)	3.52	0.17	208	113 (0.54)	95 (0.46)	1.80	0.18	1.69	0.19
Controls	112	38 (0.34)	60 (0.54)	14 (0.13)			224	136 (0.61)	88 (0.39)			0.27	0.61

HWE: Hardy–Weinberg equilibrium.

Table 3
IL-6 and sIL-6R levels in patients with schizophrenia and healthy controls.

		Patients with schizophrenia			Healthy controls			Mann–Whitney's test
		N	Mean	S.D.	N	Mean	S.D.	
IL-6 (pg/ml)	Both genders	104	1.80	0.99	112	1.43	0.56	$U = 4296$, $P = 0.00087$
	Males	55	1.87	1.00	56	1.53	0.64	$U = 1261$, $P = 0.10$
	Females	49	1.73	0.99	56	1.33	0.44	$U = 879$, $P = 0.0015$
sIL-6R (pg/ml)	Both genders	53	453	127	49	463	136	$U = 1273$, $P = 0.86$
	Males	26	461	124	24	511	137	$U = 244$, $P = 0.18$
	Females	27	446	132	25	416	120	$U = 279$, $P = 0.28$

IL-6: interleukin-6; sIL-6R: soluble interleukin-6 receptor, S.D.: standard deviation.

The thermal cycling conditions for polymerase chain reaction were as follows: 1 cycle at 95 °C for 10 min followed by 50 cycles of 92 °C for 15 s and 60 °C for 1 min. The allele-specific fluorescence was measured with ABI PRISM 7900 Sequence Detection Systems (Applied Biosystems, Foster City, CA). Ambiguous genotype data were not included in the analysis.

2.3. Laboratory methods

Plasma samples were collected between 1100 and 1200 h in tubes containing ethylenediaminetetraacetic acid. The samples were stored at −80 °C until they were assayed. Plasma levels of IL-6 were determined by the BD™ Cytometric Bead Array system using the BD FACSCanto II system (BD Biosciences, San Jose, CA), according to the manufacturer's instructions. Data analysis was performed using the FCAP Array software (BD Biosciences). Plasma levels of sIL-6R were measured using a commercially available immunoassay kit (Quantikine; R&D Systems, Inc., Minneapolis, MN), according to the manufacturer's instructions.

2.4. Statistical analysis

Deviations of genotype distributions from Hardy–Weinberg equilibrium (HWE) were assessed using the χ^2 test for goodness of fit. Genotype and allele distributions were compared between patients and controls by using the χ^2 test for independence. Comparison of continuous variables was analyzed using one-way analysis of variance or Mann–Whitney's test, according to the data distribution. Non-continuous variables were analyzed using χ^2 tests. To determine the possible interaction effects between diagnosis and Asp358Ala genotype, two-way factorial analysis of covariance (ANCOVA) was performed with the transformed plasma levels as the dependent variable, diagnosis and genotype as independent variables, and sex and age as covariates. Because the plasma IL-6 and sIL-6R levels were not normally distributed, the aligned rank transformation method was used to transform the data prior to conducting ANCOVA (Wobbrock et al., 2011). Post-hoc comparisons between genotypes were Bonferroni-corrected for multiple comparisons. Correlations between continuous values were assessed using Spearman's correlation coefficient. Statistical analyses were performed using the Statistical Package for the Social Sciences version 11.0 (SPSS Japan, Tokyo, Japan). All statistical tests were two-tailed, and $P < 0.05$ indicated statistical significance.

3. Results

The subject characteristics for each genotype of the IL-6R Asp358Ala polymorphism are shown in Table 1. Table 2 shows the genotype and allele frequencies of the Asp358Ala polymorphism in the patients and controls. The genotype distribution did not significantly deviate from HWE in the patient or control group. No significant differences were found in the Asp358Ala genotype or allele distribution between the patients and controls. However, analysis under the recessive genetic model suggested a trend of higher frequencies of the Ala/Ala genotype in schizophrenic patients ($\chi^2 = 3.51$, $P = 0.061$). Table 3 shows the overall mean plasma IL-6 and sIL-6R levels. IL-6 levels were significantly higher in patients with schizophrenia compared to those in the controls. In contrast, no significant difference in sIL-6R levels was observed between patients and controls. The associations of IL-6 and sIL-6R levels with clinical characteristics are shown in Table 4. The IL-6 and sIL-6R levels were significantly higher in healthy men than in healthy women. Age exhibited significant correlations with IL-6 levels in the patients and controls. The duration of illness and treatment also exhibited significant correlations with IL-6 levels in the patients; however, after controlling for age, these correlations with IL-6 levels were not significant any more (duration of illness: $\rho = 0.10$, $P = 0.30$; duration of treatment: $\rho = 0.16$, $P = 0.11$). The chlorpromazine equivalent dose was not significantly correlated with IL-6 or sIL-6R levels.

The plasma IL-6 and sIL-6R levels of patients with schizophrenia and healthy controls in each genotype of the Asp358Ala polymorphism are shown in Fig. 1. Table 5 presents the results of the two-way ANCOVA performed with the transformed plasma levels

Table 4
Associations of IL-6 and sIL-6R levels with clinical characteristics.

	Statistical test results			
	Patients with schizophrenia		Healthy controls	
	IL-6 ($N = 104$)	sIL-6R ($N = 53$)	IL-6 ($N = 112$)	sIL-6R ($N = 49$)
Gender (males/females)	$U = 1264$, $p = 0.59$	$U = 328$, $p = 0.68$	$U = 1170$, $p = 0.020$[a]	$U = 169$, $p = 0.009$[a]
Age (years)	$\rho = 0.32$, $p < 0.0009$[b]	$\rho = -0.19$, $p = 0.18$	$\rho = 0.32$, $p = 0.0006$[b]	$\rho = -0.15$, $p = 0.32$
Duration of illness (years)	$\rho = 0.33$, $p = 0.0008$[b]	$\rho = 0.029$, $p = 0.84$		
Duration of treatment (years)	$\rho = 0.33$, $p = 0.0007$[b]	$\rho = 0.012$, $p = 0.94$		
CP equivalent dose	$\rho = 0.042$, $p = 0.67$	$\rho = 0.039$, $p = 0.78$		

IL-6: interleukin-6; sIL-6R: soluble interleukin-6 receptor.
Age, duration of illness, and duration of treatment were significantly correlated with IL-6 levels in patients with schizophrenia (Spearman's rank correlation test).
[a] The IL-6 and sIL-6R levels were significantly higher in healthy males compared to healthy females (Mann–Whitney's test).
[b] Age was significantly correlated with IL-6 levels in healthy controls (Spearman's rank correlation test).

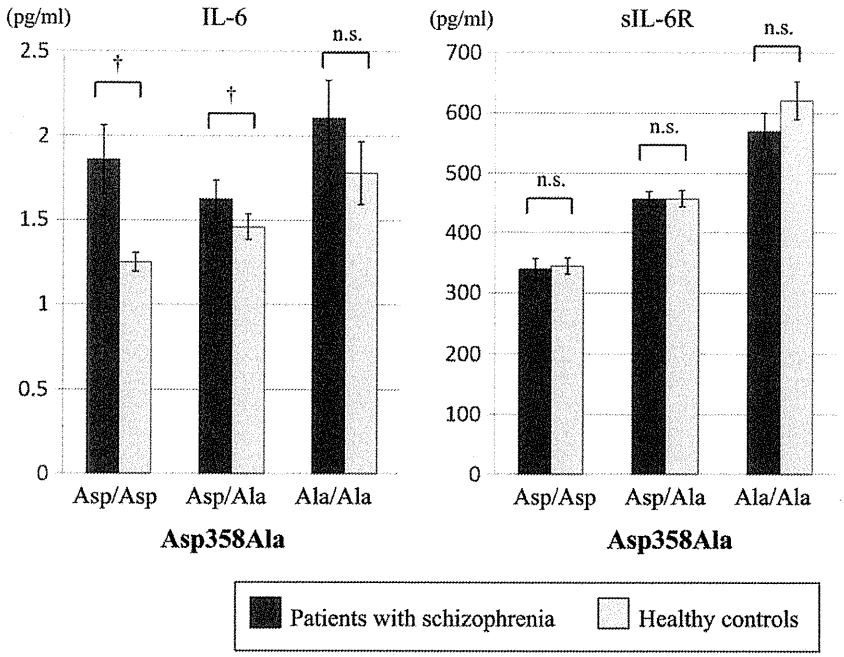

Fig. 1. The mean plasma levels of IL-6 and sIL-6R in patients with schizophrenia and controls are shown for each genotype of the IL-6R Asp358Ala. Error bars indicate the standard error of the means. †Significant difference between schizophrenia and controls (Mann–Whitney's test, $p < 0.05$); n.s.: no significant difference; IL-6: interleukin-6; sIL-6R: soluble interleukin-6 receptor.

as the dependent variable, diagnosis and the genotype as independent variables, and sex and age as covariates. Because the recessive genetic model suggested a trend of higher frequencies of the Ala/Ala genotype in schizophrenic patients, we used the recessive model as well as the co-dominant model in the ANCOVA analysis. Regarding IL-6 levels, two-way ANCOVA revealed significant effect of diagnosis and of Asp358Ala genotype with no significant diagnosis × genotype interaction. In the co-dominant model, Bonferroni's post hoc tests revealed a significant difference between Asp/Ala and Ala/Ala genotypes and a trend toward significance between Asp/Asp and Ala/Ala genotypes. Regarding sIL-6R levels, two-way ANCOVA demonstrated a significant effect of the genotype but not of the diagnosis and no significant diagnosis × genotype interaction. Post hoc tests revealed significant differences across genotypes.

4. Discussion

The results show that the Ala allele of the IL-6R Asp358Ala polymorphism is associated with higher plasma levels of IL-6 and sIL-6R in both patients with schizophrenia and healthy controls. The overall IL-6 levels were elevated in schizophrenic patients with no significant change in sIL-6R levels. Consistent with our findings, previous studies of healthy subjects also showed that higher levels of sIL-6R and IL-6 levels were associated with the Ala allele of Asp358Ala (Galicia et al., 2004; Rafiq et al., 2007; Reich et al., 2007). A two-way factorial ANCOVA revealed no significant diagnosis × genotype interaction for IL-6 and sIL-6R levels. Taken together, our findings suggest that the IL-6 levels of schizophrenic patients are elevated compared to those of their healthy counterparts irrespective of the Asp358Ala genotype and that the increase in IL-6 levels without alterations in sIL-6R levels results in excessive IL-6 signaling in schizophrenic patients.

Consistent with the previous finding that the Ala allele was associated with susceptibility to schizophrenia (Sun et al., 2008), our results indicated a trend of higher frequencies of the Ala/Ala genotype in schizophrenic patients. Taken together with the finding that the Ala allele was associated with higher IL-6 and sIL-6R levels, our results lend support to the evidence that excessive IL-6 signaling could cause neurodevelopmental abnormalities

Table 5
The results of the two-way ANCOVA.

	Diagnosis		Genotypes of Asp358Ala		Interaction		Post hoc tests between genotypes					
							Asp/Asp vs Asp/Ala		Asp/Ala vs Ala/Ala		Ala/Ala vs Asp/Asp	
	F value	*P* value	*F* value	*P* value	*F* value	*P* value	*F* value	*P* value[a]	*F* value	*P* value[a]	*F* value	*P* value[a]
Co-dominant model (Asp/Asp vs Asp/Ala vs Ala/Ala)												
Plasma IL-6 levels	7.03	0.01	3.96	0.02	1.07	0.35	0.74	1.0	6.59	0.03	5.14	0.08
Plasma sIL-6R levels	0.36	0.55	105	<0.0001	0.25	0.78	65.4	<0.0001	40.4	<0.0001	237	<0.0001
Recessive model (Asp/Asp + Asp/Ala vs Ala/Ala)												
Plasma IL-6 levels	6.74	0.010	8.11	0.0048	1.00	0.32						
Plasma sIL-6R levels	0.03	0.86	82.5	<0.0001	0.63	0.43						

ANCOVA was performed with the transformed plasma levels as the dependent variable, the diagnosis and the genotype as independent variables, and sex and age as covariates.
ANCOVA: analysis of covariance; IL-6: interleukin-6; sIL-6R: soluble interleukin-6 receptor.
[a] Bonferroni-corrected *P* values.

associated with schizophrenia (Gilmore et al., 2004; Marx et al., 2001). To conclude the possible genetic association between the Asp358Ala polymorphism and schizophrenia, further studies in a larger sample size are required.

Recent studies have shown that IL-6 signaling functions as a risk factor for the development of schizophrenia. Behrens et al. (2008) reported that IL-6 production by neurons induces NAPDH oxidase and subsequently leads to the degeneration of parvalbumin, the dysfunction of which is considered one of the key features in the brain pathology of schizophrenia (Lewis et al., 2005). In an animal study, injection of IL-6 alone into mothers was sufficient to cause schizophrenia-like behavioral abnormalities in the offspring, whereas anti-IL-6 antibody blocked the development of such abnormalities (Smith et al., 2007).

The two-way ANCOVA revealed that IL-6 levels in schizophrenic patients were elevated compared to those in controls with no significant diagnosis × genotype interaction, suggesting that some schizophrenia-related factors other than the Asp358Ala polymorphism are associated with elevated IL-6 levels. Conversely, similar sIL-6R levels between schizophrenic patients and the controls suggest that sIL-6R levels are mainly predetermined by the IL-6R Asp358Ala polymorphism and are unrelated to the disease status.

Because IL-6 levels are affected by a number of environmental and genetic factors, various conditions may be attributed to increased IL-6 levels in schizophrenic patients. For example, acute mental stress could induce a significant increase in plasma IL-6 levels (von Kanel et al., 2006). Therefore, stressful life events triggering the exacerbation of psychotic symptoms, as well as psychological stress caused by the onset of the disease, may have contributed in the elevation of IL-6 levels. Stimulation of the peripheral immune system can result in activation of microglia in the central nervous system. According to the recent microglia hypothesis of schizophrenia (Monji et al., 2009), activated microglia release pro-inflammatory cytokines and free radicals, thereby causing neuronal degeneration, white matter abnormalities, and decreased neurogenesis associated with the pathophysiology of schizophrenia. Genetic variations other than the one examined in this study may also play a role in the regulation of IL-6 levels in schizophrenic patients. For example, a well-known functional polymorphism, −174G/C of the IL-6 gene, known to affect the circulating levels of IL-6 (Bonafe et al., 2001; Fishman et al., 1998; Olivieri et al., 2002), was found to be associated with schizophrenia in a Caucasian population (Paul-Samojedny et al., 2010). Although this SNP is reported as monomorphic in the HapMap Japanese population, there may still be other unknown genetic polymorphisms attributable to higher IL-6 levels in patients with schizophrenia.

Previous studies have suggested the influence of antipsychotic treatment on the IL-6 and sIL-6R levels in schizophrenic patients. Xu et al. (1994) reported higher plasma IL-6 levels in schizophrenic patients taking antipsychotic medication than in neuroleptic-free patients. Loffler et al. (2010) reported that treatment with clozapine increased plasma IL-6 levels. In line with this, van Kammen et al. (1999) reported that exacerbation after haloperidol withdrawal resulted in decreased plasma IL-6 levels. In contrast, a larger study by Zhang et al. (2004) demonstrated no significant influence of risperidone or haloperidol on serum IL-6 levels in their schizophrenic patients. Regarding sIL-6R, one study reported a significant decrease of the serum levels after neuroleptic treatment (Muller et al., 1997). The present study obtained no evidence of association between the antipsychotic dose and the levels of IL-6 and sIL-6R, which is consistent with Zhang et al. (2004). Although the duration of antipsychotic treatment and the duration of illness correlated significantly with IL-6 levels, which is consistent with some previous studies (Ganguli et al., 1994; Kim et al., 2000), these significant correlations appeared to reflect the influence of age, as the correlations disappeared after controlling for age. Further studies are required to draw conclusions as to the possible influence of antipsychotic medication on IL-6 and sIL-6R levels.

Some limitations must be considered when interpreting the results of this study. First, the cross-sectional design did not allow for any definitive conclusions regarding whether the increased IL-6 levels in schizophrenic patients were premorbid or the result of illness onset. Secondly, only the IL-6R Asp358Ala polymorphism was examined in the present study. Future studies should examine gene-wide tagging polymorphisms of IL-6 and IL-6R genes and their associations with the circulating levels of IL-6 and sIL-6R in schizophrenic patients. Thirdly, we did not assess inflammation markers such as C-reactive protein. As the presence or absence of inflammatory diseases was based only on self-reports, the results may have been affected by unrecognized inflammatory processes in some participants.

In conclusion, the Ala allele of the IL-6R Asp358Ala polymorphism was found to be associated with higher plasma levels of both IL-6 and sIL-6R in schizophrenic patients and controls. The overall IL-6 levels were elevated in schizophrenic patients with no significant change in sIL-6R levels, supporting the role of excessive IL-6 signaling in schizophrenia. The finding that the IL-6 levels in schizophrenic patients were elevated with no significant diagnosis × genotype interaction suggests that some schizophrenia-related factors, other than the effects of the polymorphism, are associated with increased IL-6 levels in schizophrenic patients. In contrast, the sIL-6R levels are mainly predetermined by the polymorphism and are not influenced by the disease status.

Contributors

D.S., C.W., and H.K. designed the study and D.S. wrote the draft of the manuscript. D.S., H.H., T.T., K.H., and M.O. screened the study participants using the Mini International Neuropsychiatric Interview (M.I.N.I.). C.W. measured the IL-6 levels and D.S. measured the sIL-6R levels. D.S. performed the genotyping. D.S. and H.H. undertook the statistical analysis. H.K. supervised the data analysis and writing of the paper. M.I., K.A., T.H., and N.A. also supervised the writing of the paper and gave critical comments on the manuscript. All authors contributed to and have approved the final manuscript.

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Conflict of interest

The authors report no conflicts of interest.

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Expression of Ca^{2+}-dependent activator protein for secretion 2 is increased in the brains of schizophrenic patients

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ABSTRACT

Ca^{2+}-dependent activator protein for secretion 2 (CADPS2), a secretory granule associate protein, mediates monoamine transmission and the release of neurotrophins including brain-derived neurotrophic factor (BDNF) which have been implicated in psychiatric disorders. Furthermore, the expression of CADPS2deltaExon3, a defective splice variant of CADPS2, has been reported to be associated with autism. Based on these observations, we examined whether expression levels of CADPS2 and CADPS2deltaExon3 are altered in psychiatric disorders. Quantitative polymerase chain reaction analysis was performed for postmortem frontal cortex tissues (BA6) from 15 individuals with schizophrenia, 15 with bipolar disorder, 15 with major depression, and 15 controls (Stanley neuropathology consortium). The mean CADPS2 expression levels normalized to human glyceraldehyde-3phosphate dehydrogenase (GAPDH) or TATA-box binding protein levels was found to be significantly increased in the brains of the schizophrenia group, compared to the control group. On the other hand, the ratio of CADPS2deltaExon3 to total CADPS2 was similar in the 4 diagnostic groups. We then analyzed CADPS2 expression in blood samples from 121 patients with schizophrenia and 318 healthy controls; however, there was no significant difference between the two groups. Chronic risperidone treatment did not alter the expression of CADPS2 in frontal cortex of mice. The observed increase in the expression of CADPS2 may be related to the impaired synaptic function in schizophrenia.

1. Introduction

Ca^{2+}-dependent activator protein for secretion (CADPS) family, which consists of two members, CADPS1 and CADPS2, is a secretory granule-associated proteins involved in Ca^{2+}-dependent exocytosis of large dense-core vesicles containing diverse array of modulators including neurotrophines, monoamines and neuropeptides (Liu et al., 2008; Sadakata et al., 2004). CADPS2 mediates the release of neurotrophins such as brain-derived neurotrophic factor (BDNF) and neurotrophin-3. Mouse CADPS2 protein is associated with BDNF-containing secretory vesicles and promotes activity-dependent release of BDNF (Sadakata et al., 2004). BDNF release is significantly reduced in cultured neurons prepared from the brain of CADPS2 deficient mice (Sadakata et al., 2007a,b).

A number of findings suggest that BDNF action is impaired in psychiatric disorders including schizophrenia, bipolar disorder and depression. Several studies have shown decreased levels of BDNF or its receptor, TrkB, in the postmortem brains of patients with schizophrenia (Hashimoto et al., 2005; Iritani et al., 2003; Weickert et al., 2003), although there are contradictive reports (Chen et al., 2001; Dunham et al., 2009; Durany et al., 2001; Takahashi et al., 2000). The contribution of BDNF in depression has been suggested from animal studies that demonstrated stressful environments decrease, and antidepressive treatments increase BDNF levels in the brain (Duman and Monteggia, 2006; Martinowich et al., 2007). Also, centrally administered BDNF has an antidepressant-like effect in rat models (Siuciak et al., 1997). Thus, the molecules that contribute to the trafficking and release of BDNF may be a culprit of these disorders.

CADPS family also mediate monoamine transmission. Both CADPS1 and CADPS2 mediate the refilling of catecholamine to the releasable vesicles, and catecholamine secretion is significantly suppressed in the CADPS1/2 double deficient cells. (Liu et al., 2008). Another study supports that CADPS family are involved in monoamine storage as antibodies against CADPS1 or 2 inhibit monoamine

Abbreviations: ANCOVA, Analysis of covariance; BDNF, Brain-derived neurotrophic factor; CADPS2, Ca^{2+}-dependent activator protein for secretion 2; CCK, Cholecystokinin; DSM–IV, Diagnostic and Statistical Manual of Mental Disorders, 4th edition; FST, Freezer storage time; M.I.N.I., Mini-International Neuropsychiatric Interview; NT, Neurotensin; PCR, Polymerase chain reaction; PMI, Postmortem interval; SD, Standard deviation; TBP, TATA-box binding protein.

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sequestration by synaptic vesicles from mice brain (Brunk et al., 2009).

Dysregulation of monoamine neurotransmission has been hypothesized to play a central role in the etiology of psychiatric disorders including schizophrenia and mood disorders. In schizophrenia, not only classical evidence that dopamine agonists induce and dopamine D2 receptor antagonists ameliorate psychoses but also brain imaging studies on drug naïve patients have suggested that dopamine transmission is affected in this disorder (Lyon et al., 2011). In major depression, reduced monoamine transmission hypothesis was derived from the finding that most anti-depressants increase monoamine levels in the synaptic cleft and that reserpine, a monoamine-depleting drug, worsen depressive symptoms in a subset of patients with mood disorder (Krishnan and Nestler, 2008), although imaging, postmortem, or cerebrospinal fluid studies have yet to find the definitive evidence for altered monoamine neurotransmission in this disorder (Belmaker and Agam, 2008; Nikolaus et al., 2009).

While, to our knowledge, CADPS2 expression in schizophrenia or mood disorders have not yet been examined, aberrant splicing of CADPS2 mRNA was reported in autism (Sadakata et al., 2007b). In this study, an exon-3 skipped isoform, CADPS2ΔExon3, was detected in the bloods of several autistic patients but not in those of healthy controls. They also showed that CADPS2ΔExon3 was deficient in proper axonal transport, which results in the loss of local synaptic BDNF release. Though the CADPS2ΔExon3 expression in the brains of patients with autism is unclear, the aberrant splicing of CADPS2 could contribute to susceptibility to autism by affecting neurotrophin release.

Based on above findings, the present study was aimed to examine whether the expression of CADPS2 transcripts is altered in the frontal cortex of patients with psychiatric disorders including schizophrenia, major depression and bipolar disorder. The CADPS2 expression levels in the blood of schizophrenia were also examined.

2. Materials and methods

2.1. Brain samples

Frozen postmortem samples of frontal cortex (BA6) were obtained from the Stanley Foundation Neuropathology Consortium (Torrey et al., 2000). The collection consists of 60 subjects: 15 with schizophrenia, 15 bipolar disorder, 15 major depression and 15 unaffected controls. All groups were matched for age, sex, race, pH and hemispheric side (Table 1), although postmortem interval (PMI) and freezer storage time differed across the groups. The brain tissues obtained were coded. Once our blind study was complete, we sent the data to the Stanley Foundation who then returned the codes, demographic and clinical data. In a cold-room, each frozen brain tissue was broken into powder in the plastic bag using dry-ice block and dry-ice-cold hammer. The powder was then transferred and kept in dry-ice-cold tubes. Temperature of the tubes and instruments that directly contacted to the samples was frequently measured by infrared–thermometer (AD-5613A, A&D Company, Japan) and kept under −20 °C. Then, 30 to 40 mg of brain powder was used for cDNA synthesis. RNA was extracted using RNAqueous (Applied biosystems, Foster City, CA) according to manufacturer's instructions with a slight modification, i.e., after homogenization, samples were washed twice with 500 μl of chloroform, and then applied to the spin-column. Extracted RNA was quantified by optical density reading at 260 nm using NanoDrop ND-1000 (Thermo Scientific, Rockford, IL). Then, the obtained RNA (14 μl) was used for cDNA synthesis using SuperScript VILO cDNA Synthesis Kit (Invitrogen, Carlsbad, CA).

Table 1
Demographic information on brain specimens of Stanley Neuropathology Consortium.

	Control	Schizophrenia	Bipolar disorder	Major depression
Age (years)	48.1 (29–68)	44.2 (25–62)	42.3 (25–61)	46.4 (30–65)
Gender (M/F)	9/6	9/6	9/6	9/6
Race	14 C, 1 AA	13 C, 2 A	14 C, 1 AA	15 C
PMI (hours)	23.7 (8–42)	33.7 (12–61)	32.5 (13–62)	27.5 (7–47)
pH	6.3 (5.8–6.6)	6.1 (5.8–6.6)	6.2 (5.8–6.5)	6.2 (5.6–6.5)
Side of brain frozen (R/L)	7/8	6/9	8/7	6/9
Freezer storage time (months)	11.3 (1–26)	20.7 (2–31)	20.7 (7–28)	14.5 (3–31)

AA, African American; A, Asian; C, Caucasian; F, female; M, male; and PMI, postmortem interval.

2.2. Blood samples

Subjects were 121 patients with schizophrenia (84 males and 37 females; age 44.1 ± 13.7 (mean ± SD) years) and 318 controls (90 males and 228 females; age 43.1 ± 15.3 years). All subjects were biologically unrelated Japanese and recruited from the same geographical area (Western part of Tokyo Metropolitan). Consensus diagnosis by at least two psychiatrists 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. The controls were healthy volunteers recruited from the community, through advertisements in free local magazines and our website announcement. Control individuals were interviewed by the Japanese version of the Mini-International Neuropsychiatric Interview (M.I.N.I.) (Otsubo et al., 2005; Sheehan et al., 1998) and those who had a current or past history of psychiatric treatment were not enrolled in the study. After the nature of the study procedures had been fully explained, written informed consent was obtained from all subjects. The study was conducted in accordance with the Declaration of Helsinki and approved by the ethics committee of the National Center of Neurology and Psychiatry, Japan.

Blood collection and RNA isolation were performed using the PAXgene blood RNA system (Qiagen, Valencia, CA). Blood samples were collected around 11 A.M. Extracted RNA was quantified as described above. Samples that contained more than 40 ng/μl of total RNA were used for analysis; 8 μl from each sample was reverse transcribed using SuperScript VILO cDNA Synthesis Kit (Invitrogen, Carlsbad, CA).

2.3. Chronic risperidone treatment to mice

C57BL/6J male mice aged 10 weeks were purchased from Crea Japan. Chronic oral risperidone treatment was performed according to Belforte et al., (Belforte et al., 2010). In brief, 2.5 mg/kg/day of risperidone (Rispadal liquid, Janssen Pharmaceutical, Tokyo, Japan) in drinking water freshly made every 72 h had been administered continuously for 3 weeks. Control mice received solvent (1.4 mM tartaric acid neutralized to pH 6–7). All experimental procedures were in accordance with the guidelines of the United State's National Institutes of Health (1996) and were approved by the Animal Care Committee of the National Institute of Neuroscience, NCNP.

2.4. Quantitative real-time polymerase chain reaction

Polymerase chain reaction (PCR) amplifications were performed in triplicate (5 μl volume) on 384-well plates using ABI prism 7900HT (Applied Biosystems, Foster City, CA). Each reaction contained 0.28 μl of cDNA sample, qPCR QuickGoldStar Mastermix Plus (Eurogentec, Seraing, Belgium) and a primer of the target, i.e. human CADPS2 (Hs01095968_m1 at Exon 4–5 on NM_017954.9), mouse CADPS2 (Mm00462577_m1), human CADPS2ΔExon3 (Forward primer: GTAGCTGACGAAGCATTTTGCA,

Reverse Primer: TGATCTGGGCTGCTTGTTCAT, Reporter: CTGCGTTATC-CAGCTCAT) and a primer of the housekeeping gene human glyceraldehyde-3phosphate dehydrogenase (GAPDH, 4326317E), mouse GAPDH (4352339E) and human TATA-box binding protein (TBP, Hs99999910_ml) all purchased from Applied Biosystems (Foster City, CA). Negative control reactions were carried out with "no RNA" samples. The real time PCR reactions ran at 50 °C for 2 min, at 95 °C for 10 min and in 40 or 45 cycles changing between 95 °C for 15 s and 60 °C for 1 min. A standard amplification curve was made by serial dilution of a "standard" pooled cDNA sample in each plate. The mean value of triplicate of each sample was normalized to the standard curve. Then, the values of CADPS2 and CADPS2ΔExon3 from each sample were normalized to those of GAPDH.

2.5. Statistical analyses

Data analyses were performed with SPSS software (Version 11, SPSS Japan, Tokyo, Japan). Effect of age, brain pH, postmortem interval (PMI), and freezer storage time on each brain analysis was assessed by Pearson's correlations (Table 2). Variables showing significant correlations were included as covariates in the main analysis. Levene's test was used to assess the equality of variances across diagnostic group. Analysis of covariance (ANCOVA) was used to identify overall effects of diagnosis and significant main effects of diagnosis were investigated by planned post hoc contrasts. In the blood sample analyses, CADPS2 expression levels were converted to 10-log scale before statistical analysis in order to obtain a normal distribution (Castensson et al., 2005). The effect of diagnosis on blood CADPS2 expression was assessed by ANCOVA with sex and age as covariates after Levene's test. The effect of diagnosis on blood CADPS2ΔExon3 expression was assessed by logistic regression, controlling for sex and age as covariates. The effect of risperidone on CADPS2 expression in mice brain was assessed by student's *t*-test after F-test.

3. Results

3.1. CADPS2 expression levels in the postmortem brain (BA6)

We first analyzed the effects of age, brain pH, postmortem interval (PMI), and freezer storage time (FST) on each expression analysis (Table 2). Brain pH was significantly correlated with GAPDH expression levels or raw CADPS2 expression levels. PMI also tended to be correlated with GAPDH expression levels or raw CADPS2 expression levels. If the effects were analyzed separately within each diagnostic group, no significant correlation was detected.

CADPS2 expression levels normalized to GAPDH expression levels (CADPS2/GAPDH) in each sample are shown in Fig. 1A. ANCOVA with brain pH as covariates detected a significant effect of diagnosis on CADPS2/GAPDH levels ($F=3.4$, $df=3$, $p=0.025$) and post hoc test detected a significant difference between schizophrenia and control groups ($p=0.03$). Even if PMI was added as another covariate, the difference was significant ($p=0.002$). There was no significant difference between bipolar disorder and controls or between depression and controls. There was no significant correlation between CADPS2/GAPDH levels and lifetime dose of antipsychotic drugs (data not shown). There was a significant effect of diagnosis on GAPDH expression levels ($F=3.4$, $df=3$, $p=0.023$, Fig. 1B). GAPDH levels in the control group was significantly higher than that of schizophrenia ($p=0.012$), bipolar disorder ($p=0.009$) or major depression group ($p=0.013$). Raw CADPS2 levels did not differ among the diagnostic groups ($F=1.0$, $df=3$, $p=0.38$, Fig. 1C). There was a significant correlation between GAPDH expression levels and raw CADPS2 expression levels (Pearson's correlation 0.69, $p<0.001$, Fig. 1D).

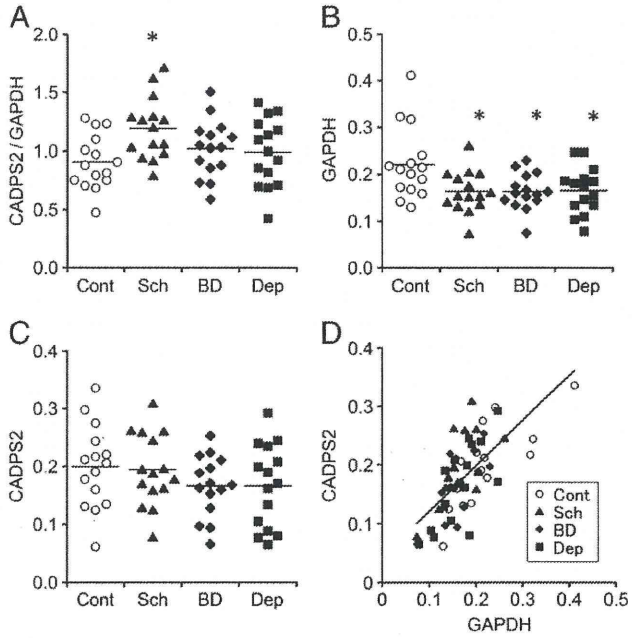

Fig. 1. CADPS2 expression levels in the postmortem brains of psychiatric disorder. (A) CADPS2 expression levels normalized by GAPDH levels. Scatter plots display the variability and differences in the CADPS2 mRNA expression levels normalized by each GAPDH expression levels. A crossbar on each scatter plot represents mean expression levels for each group. (B) GAPDH expression levels (C) Raw CADPS2 expression levels. (D) Correlation between GAPDH levels and raw CADPS2 levels. Cont, control; Sch, schizophrenia; BD, Bipolar Disorder; and Dep, Depression. *, statistically significant difference ($p<0.05$).

We compared relative CADPS2 expression levels among diagnostic groups using another endogenous control, TATA-box binding protein (TBP), and obtained similar result (Fig. S1, this experiment was done after uncode the sample). ANCOVA with brain pH as covariates detected a significant effect of diagnosis on CADPS2/TBPlevels ($F=3.3$, $df=3$, $p=0.027$) and post hoc test detected a significant

Table 2
The effect of age, pH, postmortem interval, and freezer storage time on each brain expression analysis.

		GAPDH	CADPS2	ΔExon3	CADPS2/GAPDH	ΔExon3/G APDH	ΔExon3/C ADPS2
Age	Pearson's	0.013	−0.13	0.19	−0.18	0.088	0.27
	P	0.92	0.34	0.37	0.16	0.51	0.041
pH	Pearson's	0.36	0.26	0.25	0.031	0.12	0.090
	p	0.005	0.048	0.058	0.81	0.38	0.50
Post mortem Pearson's interval (hours)	−0.23	−0.22	−0.13	−0.040	0.039	0.15	
	P	0.076	0.098	0.30	0.76	0.77	0.25
Freezer storage time (months)	Pearson's	−0.22	−0.034	−0.041	0.21	0.12	0.052
	P	0.092	0.80	0.75	0.11	0.36	0.69

ΔExon3, CADPS2ΔExon3; and Pearson's, Pearson's correlation.

difference between schizophrenia and control groups ($p=0.019$). Even if PMI was added as another covariate, the difference was significant ($p=0.012$).

With respect to CADPS2ΔExon3/GAPDH level (Fig. 2A), the effect of age was detected in the control group (Pearson's correlation 0.58, $p=0.023$) and the effect of pH was detected in the bipolar disorder group (Pearson's correlation 0.60, $p=0.018$). ANCOVA with age and brain pH as covariates detected the marginal effect of diagnosis ($F=2.8$, $df=3$, $p=0.050$) and the mean expression level was significantly increased in the schizophrenia group, compared to the control group ($p=0.030$). When the ratio of CADPS2ΔExon3 to raw (total) CADPS2 expression levels was compared, the ratio was similar in the 4 diagnostic groups ($F=1.1$, $df=3$, $p=0.36$, Fig. 2B). Neither the effect of diagnosis on raw CADPS2ΔExon3 levels was observed ($F=1.9$, $df=3$, $p=0.15$, Fig. 2C). There was a significant correlation between GAPDH expression levels and raw CADPS2ΔExon3 expression levels (Pearson's correlation 0.66, $p<0.001$, Fig. 2D).

3.2. Cortical CADPS2 expression after chronic antipsychotic treatment in mice

To see whether antipsychotics alter the mRNA expression of CADPS2, we measured the CADPS2 levels in the frontal cortex of mice, following chronic treatment with an antipsychotic risperidone. Oral administration of risperidone (2.5 mg/kg, $n=15$ for the controls and 16 for the risperidone group) for 3 weeks did not alter CADPS2 expression ($F=1.5$, $df=29$, $p=0.61$).

3.3. CADPS2 expression in blood sample

Since we observed increased expression of CADPS2 in postmortem brains of schizophrenia patients, we then examined whether such an alteration exists in peripheral blood samples. The CADPS2/GAPDH expression levels were converted to 10-logarism before statistical analyses to obtain normal distribution. The mean (Standard deviation) CADPS2 expression level was 0.17 (1.29) in the control group and 0.32 (1.46) in the schizophrenia group. ANCOVA controlling for age and sex did not detect the significant effect of diagnosis on CADPS2/GAPDH level ($F=1.67$, $df=1$, $p=0.20$). We also measured CADPS2ΔExon3 levels in the blood samples. Compared to brain samples, the expression levels were quite low and could not detect in the majority of samples. Thus, we defined "expressed" when at least 2 tubes in triplet analyses of each sample were detected until 45 cycles. CADPS2ΔExon3 expression was detected in 36 of 318 control samples (ratio = 0.11), and 21 of 121 schizophrenia samples (ratio = 0.17). There was no significant effect of diagnosis on CADPS2ΔExon3 expression by the logistic regression analysis controlling for age and sex (odds ratio 1.51, [95% CI 0.80–2.86], $p=0.21$). Even when men and women were examined separately, there was no significant difference between the patients and controls for each sex (data not shown).

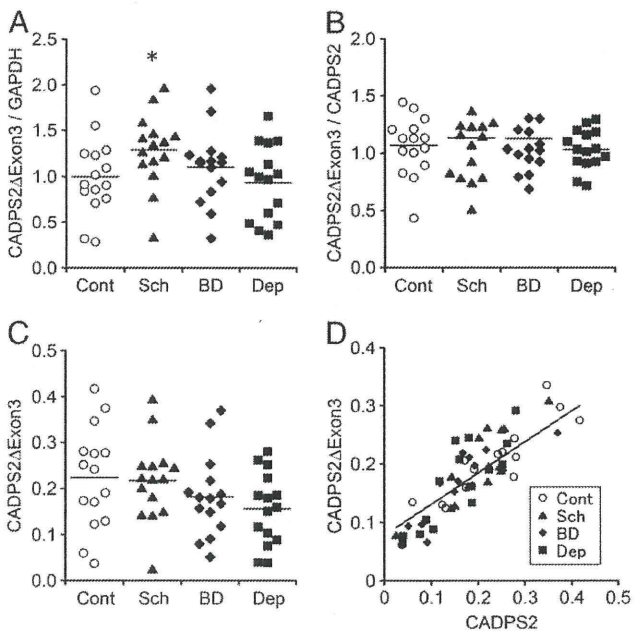

Fig. 2. CADPS2ΔExon3 expression levels in the postmortem brains of psychiatric disorder. (A) CADPS2ΔExon3 expression levels normalized by GAPDH levels. Scatter plots display the variability and differences in the CADPS2ΔExon3 mRNA expression levels normalized by each GAPDH expression levels. A crossbar on each scatter plot represents mean expression levels for each group. (B) CADPS2ΔExon3 levels normalized to each total CADPS2 expression levels. (C) Raw CADPS2ΔExon3 expression levels. (D) Correlation between GAPDH expression levels and raw CADPS2ΔExon3 expression levels. Cont, control; Sch, schizophrenia; BD, Bipolar Disorder; and Dep, Depression. *, statistically significant difference ($p<0.05$).

4. Discussion

4.1. Main findings

In the present study, we analyzed the expression of CADPS2 mRNA in the postmortem brains (BA6) of psychiatric patients (schizophrenia, major depression and bipolar disorder) and controls. A significant increase in the CADPS2 expression was detected in the brains of the schizophrenia group, compared to the control group. No change was detected in other disease groups. While a CADPS2 splice variant, CADPS2ΔExon3 showed a non-significant increase in the schizophrenia group, its ratio to the total CADPS2 levels was not different from the control group. Chronic risperidone treatment did not alter the CADPS2 levels in mice brain. We also analyzed CADPS2 or CADPS2-ΔExon3 expression levels in the blood samples of schizophrenia and control subjects; however, the levels were not significantly different between the two groups.

4.2. Brain analysis

4.2.1. Drug effect

A large number of gene expressions in the brain are affected by antipsychotic treatments (Girgenti et al.,; Mehler-Wex et al., 2006; Thomas, 2006). Therefore, the observed increase in CADPS2 mRNA in the schizophrenia group could be the result of antipsychotic treatment. However, our results did not support this assumption because the CADPS2 levels did not correlate to life-time antipsychotic dose and chronic risperidone treatment in mice did not alter CADPS2 expression on their cortices, although caution is required for the interpretation of those results because we don't have data for the latest dose before death and other drugs such as chlorpromazine, haloperidol and clozapine might be used in the patients.

4.2.2. Possible relevance to BDNF secretion, dopamine transmission, and neuropeptide release

Considering that defective BDNF signaling has been suggested in schizophrenia and mood disorders (Angelucci et al., 2005) and that CADPS2 mediates BDNF release in neurons (Sadakata et al., 2004), we initially expected that CADPS2 levels would be decreased in frontal cortex in patients with these psychiatric disorders. However, in our results, CADPS2 levels were not altered in mood disorders but increased in schizophrenia. In addition, the relative levels of defective CADPS2 isoform, CADPS2ΔExon3 were not altered in those disorders. Thus, it is unlikely that altered CADPS2 expression might be a cause of BDNF deficits in schizophrenia. It may be rather a compensatory consequence of reduced BDNF signaling.

CADPS2 also promotes monoamine storage in neurons (Brunk et al., 2009; Liu et al., 2008). CADPS2 is highly expressed in the dopamine-rich brain areas such as ventral tegmental area and substantia nigra of mice brain (Sadakata et al., 2006) and it is reported to interact with dopamine D2 receptor (Binda et al., 2005). Growing evidence has demonstrated increased presynaptic dopamine levels in the striatum of schizophrenia patients (Lyon et al., 2009). If the observed increase in the expression of CADPS2 occurs in the subcortical regions including striatum and midbrain as well as frontal cortex, it might be the cause of hyper-dopamine transmission that reflects psychotic state (Howes et al., 2009).

Furthermore, large dense-core vesicles contain not only neurotrophins and monoamines but also neuropeptides (Salio et al., 2006). Neuropeptides such as endorphins, cholecystokinin (CCK), neurotensin (NT), somatostatin, Neuropeptide Y and neuregulin 1 have been implicated in schizophrenia (Caceda et al., 2007). Especially reduced levels of CCK and NT have been repeatedly reported in the disorder (Caceda et al., 2007), which may have caused compensatory increase in the CADPS2 expression in schizophrenia.

4.3. CADPS2 expression in the blood

4.3.1. CADPS2 expression and diagnosis

Following the report that 4 of 16 patients with autism expressed CADPS2ΔExon3 in peripheral bloods but none in 24 normal subjects (Sadakata et al., 2007b), another group reported that they detected CADPS2ΔExon3 in some control subjects (Eran et al., 2009). Thus we assumed that the ratio of CADPS2ΔExon3 to total CADPS2 rather than whether CADPS2ΔExon3 exists or not is important and therefore we applied quantitative real-time PCR to measure their expression. The pilot experiment in the present study indicated that our quantification method using SuperScript VILO and random-hexamer, was 4 to 8 fold more sensitive than one step real-time PCR using gene specific primers and could detect l0 to 100 clones of CADPS2 or CADPS2-ΔExon3 sequence-containing vector. Compared with the brains, CADPS2 expression was 32 to 128 fold lower in the blood. Unlike in the brain, CADPS2ΔExon3 could not be detected in most blood samples. So we performed qualitative analysis for each subject. As a result, we didn't detect any significant difference in the expression of CADPS2ΔExon3 in the blood between patients with schizophrenia and controls. The CADPS2ΔExon3 was abundantly expressed in the brain and the levels were unchanged across the diagnostic groups. Thus, it is unlikely that the expression or the splicing balance should relate to diseases we analyzed.

5. Conclusion

In conclusion, we found increased mRNA expression of CADPS2 in the postmortem frontal cortex of schizophrenia patients which might have some relevance to dysregulation in the release of dopamine, neurotrophins, and/or neuropeptides in the disorder. This increase was unlikely to be attributable to antipsychotic medication. We also analyzed the CADPS2ΔExon3 in human brains and found that it is abundantly present in the frontal cortex in any diagnostic group. We obtained no evidence for the specific role of the splice variant in schizophrenia or mood disorders. Future research should include the evaluation of CADPS2 expression in other brain areas, and basic studies on the cause and consequence of increased CADPS2 expression.

Supplementary materials related to this article can be found online at doi:10.1016/j.pnpbp.2011.05.004.

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Possible association between *Interleukin-1beta* gene and schizophrenia in a Japanese population

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Abstract

Background: Several lines of evidence have implicated the pro-inflammatory cytokine interleukin-1beta (IL-1β) in the etiology of schizophrenia. Although a number of genetic association studies have been reported, very few have systematically examined gene-wide tagging polymorphisms.

Methods: A total of 533 patients with schizophrenia (302 males: mean age ± standard deviation 43.4 ± 13.0 years; 233 females; mean age 44.8 ± 15.3 years) and 1136 healthy controls (388 males: mean age 44.6 ± 17.3 years; 748 females; 46.3 ± 15.6 years) were recruited for this study. All subjects were biologically unrelated Japanese individuals. Five tagging polymorphisms of *IL-1β* gene (rs2853550, rs1143634, rs1143633, rs1143630, rs16944) were examined for association with schizophrenia.

Results: Significant difference in allele distribution was found between patients with schizophrenia and controls for rs1143633 ($P = 0.0089$). When the analysis was performed separately in each gender, significant difference between patients and controls in allele distribution of rs1143633 was observed in females ($P = 0.0073$). A trend towards association was also found between rs16944 and female patients with schizophrenia ($P = 0.032$).

Conclusions: The present study shows the first evidence that the *IL-1β* gene polymorphism rs1143633 is associated with schizophrenia susceptibility in a Japanese population. The results suggest the possibility that the influence of *IL-1β* gene variations on susceptibility to schizophrenia may be greater in females than in males. Findings of the present study provide further support for the role of IL-1β in the etiology of schizophrenia.

Background

Several lines of evidence suggest that pro-inflammatory cytokine interleukin-1beta (IL-1β) is implicated in the etiology and pathophysiology of schizophrenia. Although studies investigating peripheral levels of IL-1β in schizophrenic patients have reported inconsistent results [1-6], a study examining the cerebrospinal fluid has shown a marked elevation of IL-1β in patients with first-episode schizophrenia compared to healthy controls [7]. Kowalski et al [8] reported that the release of IL-1β by peripheral monocytes was increased before treatment and then normalized by antipsychotic medication in patients with schizophrenia. Recently, Liu et al. [9] showed that IL-1β in the peripheral blood mononuclear cells was overexpressed not only in schizophrenia patients but also in their siblings, suggesting the involvement of the hereditary factors. Furthermore, previous findings suggested that IL-1β may be involved in the possible link between prenatal exposure to infection and schizophrenia [10,11].

The *IL-1β* gene is located in a region on 2q14. This region has consistently shown positive linkage findings in schizophrenia. Many studies have reported this region among their largest results [12,13]. Furthermore, Lewis et al [14] have shown in their meta-analysis of 20 genome scans that 2p12-q22.1 was associated with a genomewide significant *P* value. Linkage of this region with schizophrenia in an Asian population has also been reported [15].

A number of genetic association studies have suggested that genetic variation of the *IL-1β* gene might confer susceptibility to schizophrenia. Three studies in Caucasian populations reported a significant association of schizophrenia with an *IL-1β* gene polymorphism

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