厚生労働科学研究費補助金（創薬基盤推進研究事業）
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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 20-24 年度　総合研究報告書

研究代表者　　後　藤　雄　一

国立精神・神経医療研究センター

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Ⅰ．総合研究報告書

厚生労働科学研究費補助金（創薬基盤推進研究事業）
総合研究報告書

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

研究代表者
後藤　雄一（国立精神・神経センター医療研究センター神経研究所　疾病研究第二部長）

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24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25年3月31日までに、710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、さらにセカンドコホートとして統合失調症9例、健常者10例のデータを追加検討し、バイオマーカーの候補となりうるタンパク質を23個見いだした。そこで、別の統合失調症患者40例、健常者40例でELISA法を用いたバリデーションを行い9個で何らかの統計的有意なものを見つけており、疾患もしくは創薬標的バイオマーカーとなる可能性が高い。今後、疾患特異性の検討、血液での検討などを行い、臨床応用を目指す。（D）を目標としてきたが、結果としてそこまで研究が進展できなかった。また、他の精神疾患やパーキンソン病のバイオマーカーの探索研究は、端緒の段階に止まった。

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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、「タンパク質レベル」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年度ー平成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タンパク質の同定が可能で、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タンパク質を見いだし、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、さらにはそのタンパク質及び関連するタンパク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 法を用いたプロテオーム解析を行う（林）。

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）

ELISA を用いた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。その結果を踏まえて、40 例程度のバリデーション研究を行う。

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タンパク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（1st コホート）。その際、cICAT 法のスタンダード（L 鎖標識）として、購入髄液を用いた。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。

さらに、別の統合失調症群 9 名と健常者 10 名のプロテオーム解析を行い（2nd コホート）、1st コホートで有意差のみられたタンパク質を絞り込んだ。その際、cICAT 法のスタンダード（L 鎖標識）として、正常圧水頭症患者のプール髄液を用いた。

その上で、40 例の別の患者群と 40 例の健常者について、多検体を用いて ELISA 法で検討した。

（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、健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。

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、3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（図1）。

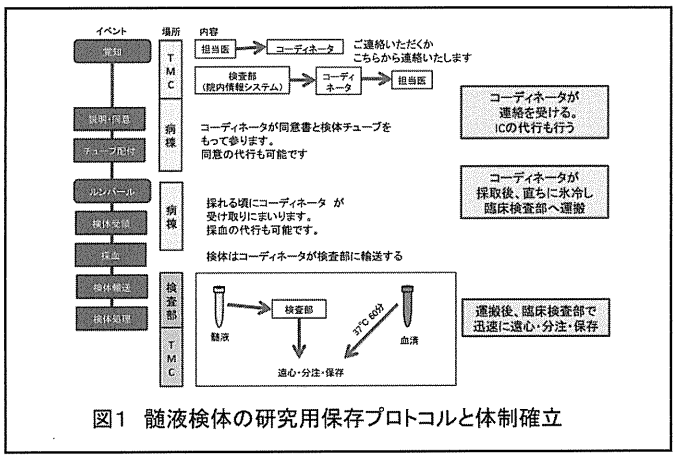

図1 髄液検体の研究用保存プロトコルと体制確立

また、臨床情報及び検体情報を登録するデータベースシステムを構築した（図2A、図2B）。

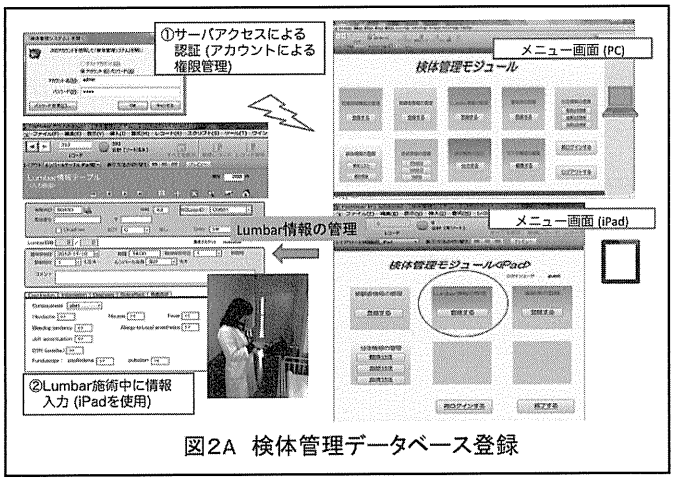

図2A 検体管理データベース登録

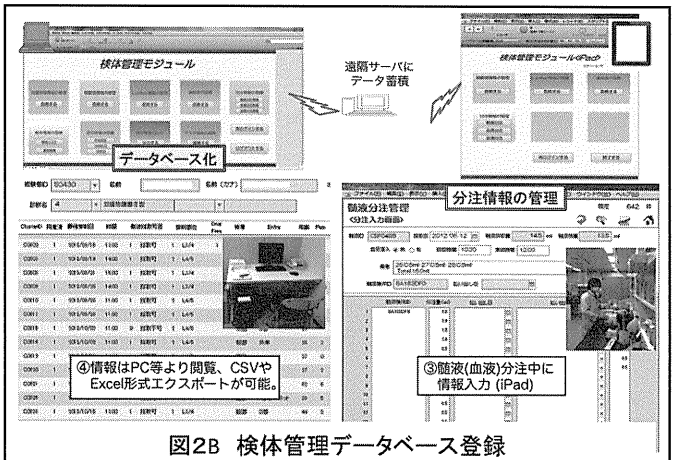

図2B 検体管理データベース登録

NCNP内サーバーに検体管理モジュールを作成し、アクセスはアカウント制限の認証を用いてセキュリティー管理を行った。また、デスクトップPCとiPadからの入力管理を行えるシステムを構築した。

具体的には、コーディネーターチームが病棟に赴いて、説明と同意を行う際や実際に腰椎穿刺に立ち会う際に病棟でiPadを用いて情報を入力できるようにした（図3）。

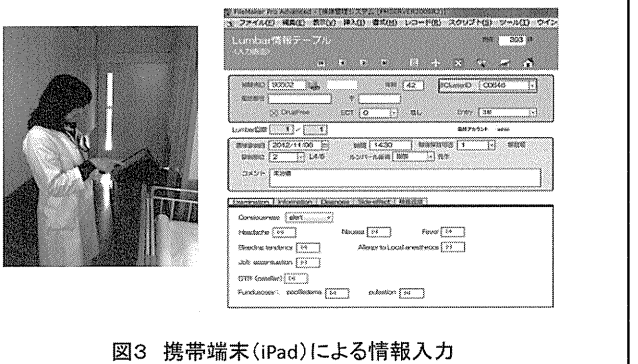

図3 携帯端末(iPad)による情報入力

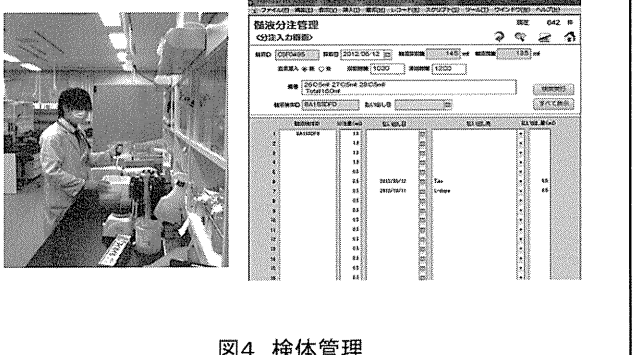

図4 検体管理

また、髄液検体をラボに搬入し、処理を行った上で、分注してフリーザーに保存する際に検体情報を iPad を用いて入力できるようにした（図４）。

入力された情報は PC 等で閲覧が可能で、CSV ファイルやエクセルファイルで出力できる（図５）。

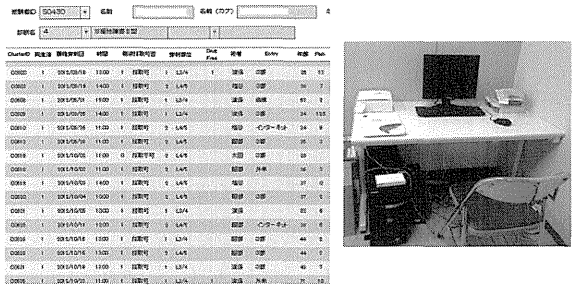

図5 データベース化

(2) 患者試料の登録

平成 25 年 3 月末までに 576 例(710 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、2012 年 3 月で 270 検体、2013 年 3 月で 710 検体の登録を行う事ができた。特に統合失調症の検体は、3 年間で 150 検体以上に達した。その内訳を次表に示す。

分類	内訳	検体数	症例数	測定数
精神疾患（159症例）	統合失調症	159	83	30
	大うつ病	93	72	17
	双極性障害	46	36	5
	健常対照	92	72	25
神経内科疾患（69症例）	パーキンソン病	76	76	5
	脊髄小脳変性症	7	7	2
	正常圧水頭症	31	24	5
	認知症	54	54	2
	その他	132	132	5
小児神経疾患（20症例）	てんかん	11	11	0
	精神遅滞	5	5	0
	その他	4	4	0
Total		710	576	96

表1 髄液検体の内訳 （2013年3月末現在）

2）プロテオーム解析

(1) 髄液 2mL からのプロテオーム解析

平成 23 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。この作業は研究補助者 4 人を雇い、マニュアルで行うため相当の時間がかかった。

(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（1st コホート）。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。

さらに、男女を含む、別な統合失調症患者 9 名、健常者10名の結果を比較検討した(2nd コホート)。

1st コホート

	N	Sex (M/F)	Age
Schizo	10	10/0	43.0±12.2
Control	10	10/0	41.4±13.3

未治療1例を含む

2nd コホート

	N	Sex (M/F)	Age
Schizo	9	5/4	40.8±10.6
Control	10	6/4	39.7±12.2

未治療1例、Drug free 1例を含む

表2 検体の内訳

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、1st コホートでは、結果としてのべ 682 個のタンパク質が同定できた。

そこで、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。

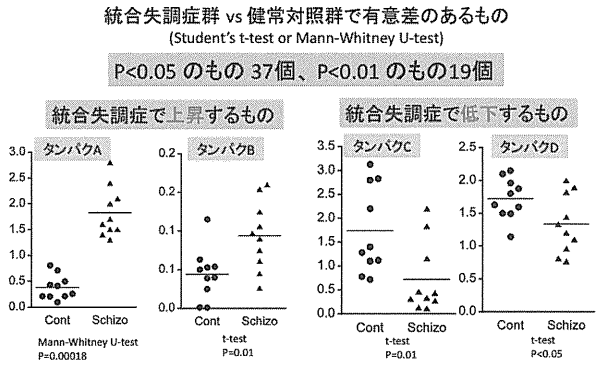

図6 統合失調症に共通のバイオマーカーの探索

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、それらの解析を分散比とF-testで行い、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た。

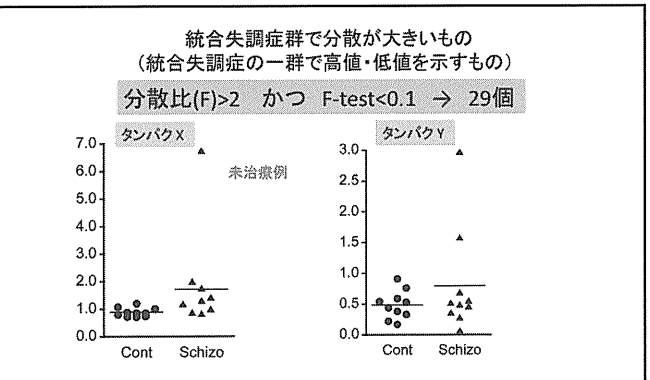

図7 統合失調症のサブタイプの探索

次いで、2nd コホートの解析結果を合わせ、以下の基準で候補となるタンパク質を選び出した。

1) ランクA：1st コホートと2nd コホートが明らかに同じ傾向を示すもの → 7種
ランクA-：症状評価から残したもの
→ 6種
2) ランクB：1st コホートと2nd コホートがある程度同じ傾向を示すもの → 4種
3) ランクC：上記以外のもの。ランクAやランクBで同定されたタンパク質から、病態に関わる可能性のあるパスウェイが見出された場合に関連のある分子など。 → 6種

これら24種のバイオマーカー候補に関して、さらに、患者群 40 例、健常者群 40 例を用いて、ELISA 法を用いた多検体での検証を行った。

その際、タンパク量の結果を用いた、T-test, F-test に加え、PANSS の陰性症状・陽性症状及び総合症状の評価、使用薬剤の等価計算、BACS の評価項目を用いての解析も追加した。

その結果、表4のような結果を得た。すなわち、T-テストで有意な結果を得たものが5種、BACS の症状との関連性を示したものが 4 種（1種は両方で相関あり）であった。

タンパク番号	評価	1st	2nd	多検体解析
1	A	T-test	F-test	T-test
2	A	T-test	T-test	有意差なし
3	A	T-test	T-test	BACS
4	A	T-test	T-test	T-test
5	A	F-test	T-test	T-test
6	A	T-test	T-test	抗体なし
7	A	T-test	T-test	有意差なし
8	A	T-test	T-test	有意差なし
9	A-	総合	総合	抗体なし
10	A-	陰性	陰性	有意差なし
11	A-	陰性	陰性	BACS
12	A-	総合	総合	T-test, BACS
13	A-	陽性	陽性	有意差なし
14	A-	陰性	陰性	抗体なし
15	B	陽性	陽性	抗体なし
16	B	F-test	F-test	抗体なし
17	B	T, F-tests	F-test	有意差なし
18	B	T-test	F-test	抗体なし
19	C	病態	病態	T-test
20	C	病態	病態	抗体なし
21	C	病態	病態	有意差なし
22	C	病態	病態	BACS
23	C	病態	病態	抗体なし

表4 多検体解析の結果

これら 9 種のバイオマーカー候補に対して、1）疾患特異性の評価、2）薬剤との関連性から、drug-free 例での解析、3）血液や尿などのアクセスしやすい検体での評価、など臨床応用に向けてのさらなる検討が必要であり、現在進行中である。

E．結論

平成24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が、(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が、バイオマーカー候補が 9 種に絞られたところまでの研究に終わり、D) の臨床応用にまで到達できなかった。また、当初の全体計画では多種類の疾患を対象としてあげていたが、大幅に進捗が遅れたことは否めない。

一方で、精神疾患を中心に、多数の高品質の髄液を収集できたことは、今後の研究に積極的に利用することで、新たなバイオマーカーを同定できると確認している。NCNP に登録させた検体を広く共同研究を行って利用促進を図る予定である。

F．健康危険情報

特になし

G．研究発表

1．論文発表

1) Sasayama D, Wakabayashi C, Hori H, Teraishi T, Hattori K, Ota M, Ishikawa M, Arima K, Higuchi T, Amano N, Kunugi H. Association of plasma IL-6 and soluble IL-6 receptor levels with the Asp358Ala polymorphism of the IL-6 receptor gene in schizophrenic patients. Journal of Psychiatric Research, 45:1439-1444, 2011

2) Hattori K, Tanaka H, Wakabayashi C, Yamamoto N, Uchiyama H, Teraishi T, Hori H, Arima K, Kunugi H. Expression of Ca^{2+}-dependent activator protein for secretion 2 is increased in the brains of schizophrenic patients. Prog Neuropsychopharmacol Biol Psychiatry. 35:1738-1743, 2011

3) Sasayama D, Hori H, Teraishi T, Hattori K, Ota M, Iijima Y, Tatsumi M, Higuchi T, Amano N, Kunugi H. Possible association between Interleukin-1beta gene and schizophrenia in a Japanese population Behav Brain Funct. 7: 35, 2011

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7) Fujii T, Ota M, Hori H, Sasayama D, Hattori K, Teraishi T, Yamamoto N, Hashikura M, Tatsumi M, Higuchi T, Kunugi H. Association between the functional polymorphism (C3435T) of the gene encoding P-glycoprotein (ABCB1) and major depressive disorder in the Japanese population. Journal of Psychiatric Research 46: 555-559, 2012

8) Sasayama D, Hori H, Teraishi T, Hattori K, Ota M, Matsuo J, Kawamoto Y, Kinoshita Y, Hashikura M, Amano N, Higuchi T, Kunugi H. More severe impairment of manual dexterity in bipolar disorder compared to unipolar major depression. Journal of Affective Disorders 136:1047-1052, 2012.

2．学会発表　なし

H．知的財産権の出願・登録状況

「統合失調症の判定方法」(出願準備中)

Ⅱ．研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

雑 誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Sasayama D, Wakabayashi C, Hori H, Teraishi T, Hattori K, Ota M, Ishikawa M, Arima K, Higuchi T, Amano N, Kunugi H.	Association of plasma IL-6 and soluble IL-6 receptor levels with the Asp358Ala polymorphism of the IL-6 receptor gene in schizophrenic patients.	Journal of Psychiatric Research	45	1439-1444	2011
Hattori K, Tanaka H, Wakabayashi C, Yamamoto N, Uchiyama H, Teraishi T, Hori H, Arima K, Kunugi H	Expression of Ca2+-dependent activator protein for secretion 2 is increased in the brains of schizophrenia.	Progress in Neuro-Psychopharmacology & Biological Psychiatry	35	1738-1743	2011
Sasayama D, Hori H, Teraishi T, Hattori K, Ota M, Iijima Y, Tatsumi M, Higuchi T, Amano N, Kunugi H	Possible association between Interleukin-1beta gene and schizophrenia in a Japanese population	Behavioral and Brain Functions	7	35	2011
Hori H, Teraishi T, Sasayama D, Matsuo J, Kawamoto Y, Kinoshita Y, Kunugi H	Relationships between season of birth, chizotypy, temperament, character and neuocognition in a non-clinical population.	Psychiatry Research	195	69-75	2012
Sasayama D, Hattori K, Teraishi T, Hori H, Ota M, Yoshida S, Arima K, Higuchi T, Amano N, Kunugi H	Negative correlation between cerebrospinal fluid oxytocin levels and negative symptoms of male patients with schizophrenia.	Schizophrenia Research	139	201-206	2012

Hattori K, Tanaka H, Yamamoto N, Teraishi T, Hori H, Kinoshita Y, Matsuo J, Kawamoto Y, Kunugi H	Blood CADPS2 Delta Exon3 expression is associated with intelligence and memory in healthy adults.	Biological Psychology	89	117-122	2012
Fujii T, Ota M, Hori H, Sasayama D, Hattori K, Teraishi T, Yamamoto N, Hashikura M, Tatsumi M, Higuchi T, Kunugi H	Association between the functional polymorphism (C3435T) of the gene encoding P-glycoprotein (ABCB1) and major depressive disorder in the Japanese population	Journal of Psychiatric Research	46	555-559	2012
Sasayama D, Hori H, Teraishi T, Hattori K, Ota M, Matsuo J, Kawamoto Y, Kinoshita Y, Hashikura M, Amano N, Higuchi T, Kunugi H	More severe impairment of manual dexterity in bipolar disorder compared to unipolar major depression	Journal of Affective Disorders	136	1047-1052	2012

Ⅲ. 主な刊行物・別刷

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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).

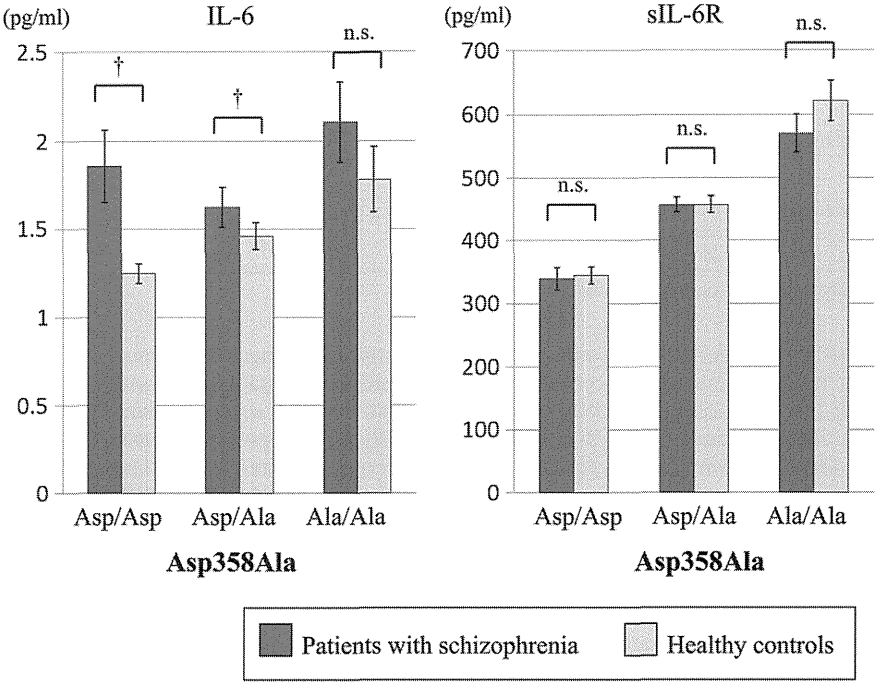

Fig. 1. The mean plasma levels of IL-6 and sIL-6R in patients with schizophrenia and controls are shown for each genotype of the IL-6R Asp358Ala. Error bars indicate the standard error of the means. †Significant difference between schizophrenia and controls (Mann–Whitney's test, $p < 0.05$); n.s.: no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