にヘテロも加えたグループと比較すると，Ser のホモは左側海馬の灰白質が小さかった．統合失調症を対象に^{1}H-MRSI 法を用いて海馬体の N-アセチルアスパラギン酸（NAA）を測定した結果，Ser のホモの患者は Cys をもつ患者と比較して，左側海馬体で NAA が有意に低下していた．健常者を対象に fMRI を用いて N-back 作業記憶テストを行うと，Ser のホモの人は Cys をもつ人と比較して両側海馬体の活性が非特異的に上昇していた．宣言的記憶テストでは，Ser のホモの人は Cys をもつ人と比較して両側海馬体の活性が減少していた．Ser のホモの統合失調症は，Wechsler 記憶尺度の得点が低く，Wisconsin Card Sorting Test（WCST）のカテゴリー尺度が低かった．

Thomson ら[15]は，1921 年に行われたスコットランドの大規模試験（Lothian Birth Cohort, 1921）を利用して，425 例の対照健常者を対象に Moray House Test（MHT）を 11 歳と 79 歳の時点で 2 回行い，認知機能と Ser 704 Cys との関連を解析した．Callicott ら[11]の報告では Ser がリスクアレルだったが，Thomson ら[15]の解析では 11 歳の成績で補正した 79 歳の値が，Cys のホモの人で有意に女性のほうが男性よりスコアが低かった．

Burdick ら[16]は，白人と黒人の 250 例の統合失調症者を用いて認知機能と hCV 1650649 の関連を解析した．リスクアレルをホモでもつ患者は，リスクアレルをもたない患者と比較して，有意に Trial Making Test-A（TMT-A）と Digits backward の成績が低かった．

Hennah ら[7]は，統合失調症，統合失調感情障害，双極性感情障害の 215 家系を用いて，HEP 2，3 と認知機能の関連を解析した．統合失調症で HEP 3 が有意に低いスコアの visual attention と visual working memory と関連した．性差を検討すると，visual working memory の低いスコアは男性において有意に関連した．

3．DISC 1 結合蛋白との関連（表 1）

Lyons-Warren ら[17]は，双極性感情障害，統合失調感情障害，大うつ病の 307 家系の 1,012 名を用いて，*Citron* のイントロン 5～46 にわたる領域から 7 つの SNP を選び，TDT により関連を検討した．その結果，イントロン 9 の rs 435136 とイントロン 13 の rs 203368 が有意に関連していた．また，7 つから 2～4 SNP を選んでハプロタイプ解析を行うと，6 つの SNP で関連が認められた．rs 435136 と rs 203368 は，それぞれ DISC 1 結合部位のエクソン近傍にあり，また *Citron* は双極性感情障害と連鎖が複数報告されている染色体 12 q 24 にコードされている．

われわれは，360 例の統合失調症，119 例の双極性感情障害，年齢・性比を一致させた健常対照者（360 例 vs 統合失調症，140 例 vs 双極性感情障害）を用いて，*FEZ1* 遺伝子全長から 8 個の SNP を選んで症例・対照研究を行った[18]．その結果，イントロン 2 の rs 559688 とエクソン 3 の rs 597570 において，統合失調症と有意な関連が認められた．双極性感情障害とは，いずれの SNP も関連を示さなかった．

おわりに

HEP 1～4 など，関連が追認されているものもあるが，報告間で関連疾患が異なったりハプロタイプのリスクが異なったりと，一致しない点も多い．性差が複数のグループで報告されているが，報告間で関連する性別が不一致なケースもみられる．これまでの有意な関連の報告は，欧米人を対象としたものがほとんどである．日本人を対象としたものでは，Zhang ら[19]が 338 例の統合失調症を用いて *TRAX* の 3 SNP，*DISC1* の 12 SNP について症例・対照研究を行い，関連を認めなかったことを報告している．われわれも，*DISC1* の 5′上流領域を解析し，新規の 2 つを含む 4 つの SNP と 2 つのくり返し多型を同定した[20]（**表 1**）．198 例の統合失調症と 198 例の健常対照者を用いて症例・対照研究を行った結果，rs 3738398 と rs 3054533 で有意な関連を認めた．性差を検討したところ，男性で有意な関連がみられた．関連を確実にするために別の 532 例の統合失調症者と 519 例の健常対照者で検討したところ，同多型に有意差は認められなかった．また，同多型について，148 例の双極性感情障害者，77 例の単極性感情障害者と 359 例の健常対照者を用いて検討したが，有意な関連は認められなかった[21]．

これまでの報告を概観すると，大きく 3 つのリスク領域があるようにみえる（図 1）[7]．一番目は，*DISC1* の

エクソン2からプロモータ領域を含んで*TRAX*のイントロン4に至る比較的広い領域で，Thomsonら[12]のregion 1，Hennahら[8]のHEP 2〜3に相当し，両報告間で一致するSNPも含まれている．2番目は，エクソン9および*DISC2*を中心とした領域で，Hennahら[8]のHEP 1，Hodgkinsonら[9]のHap 2〜3，Ekelundら[10]のハプロタイプ，Thomsonら[12]のregion 3が含まれ，この領域でも複数のSNPが追認されている．3番目は，エクソン13を中心とした領域で，Hennahら[8]のHEP 4，Thomsonら[12]のregion 2が含まれ，ここでも複数のSNPが追認されている．DISC 1は数多くの結合蛋白が同定されており，それぞれの結合ドメインはDISC 1の全長にわたって分散している[12]．ハプロタイプのほとんどがイントロンのSNPで機能的意義は不明だが，3つのどの領域のリスクでも何らかの結合蛋白との相互作用に影響を与える可能性は考えられる．特にC末側の機能的重要性は*in vitro*で明らかにされており[3]，*in vivo*ではMillarら[2]の報告した転座家系とSachsら[13]の報告した4 bp欠失家系で確認されている．

今後，追認されている多型の生物学的意義とDISC 1自身の機能が解明されていくにつれ，統合失調症と双極性感情障害の病態が明らかにされていくであろう．

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REVIEW ARTICLE

Genetic or epigenetic difference causing discordance between monozygotic twins as a clue to molecular basis of mental disorders

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Classical twin research focused on differentiating genetic factors from environmental factors by comparing the concordance rate between monozygotic (MZ) and dizygotic twins. On the other hand, recent twin research tries to identify genetic or epigenetic differences between MZ twins discordant for mental disorders. There are a number of reports of MZ twins discordant for genetic disorders caused by genetic or epigenetic differences of known pathogenic genes. In the case of mental disorder research, for which the causative gene has not been established yet, we are trying to identify the 'pathogenic gene' by comprehensive analysis of genetic or epigenetic difference between discordant MZ twins. To date, no compelling evidence suggesting such difference between MZ twins has been reported. However, if the genetic or epigenetic difference responsible for the discordant phenotype is found, it will have impact on the biology of mental disorder, in which few conclusive molecular genetic evidences have been obtained.
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Introduction

Evidence proving the etiological roles of genetic factors in schizophrenia and bipolar disorder was derived mainly from twin studies. While the monozygotic (MZ) twins have identical genotypes, dizygotic (DZ) twins share only 50% of their genotypes on average. Thus, higher concordance rate in MZ twins compared with DZ twins is a hallmark of the role of genetic factors in a disease. Using this classical approach, the concordance rate in MZ twins in schizophrenia and bipolar disorder was found to be significantly higher than that in DZ twins. However, in spite that many candidate loci and candidate genes were proposed and analyzed, these findings are not yet conclusive. *DISC1* (disrupted in schizophrenia 1), cloned from a break point of balanced chromosomal translocation linked with mental disorders in a large pedigree, may be only one exception. In this situation, an alternative or complementary approach to study the molecular basis of mental disorders has been pursued.

Since the MZ twins provide a valuable opportunity of studying the role of genetic factors, methodology used for the twin study has been continuously evolving. If we refer the classical twin research noted above as 'first-generation twin research', second-generation twin research may be the study to identify environmental risk factors causing discordance, or to identify endophenotypes associated with the disease. The former strategy was used, for example, to identify the role of birth complications in the etiology of schizophrenia.[1,2] Using the latter approach, decreased hippocampal volume in schizophrenia[3] was established as an intermediate phenotype.[4] These studies were based on an assumption that there is no difference of genomes between MZ twins.

The first study in which the presence of genetic or epigenetic difference was pre-assumed in MZ twins was reported by Polymeropoulos *et al.*[5] Since then, several groups also have tried to identify genetic or epigenetic difference between MZ twins.[6–10] These studies seem to have induced a paradigm shift to the third-generation twin research, from focusing on the higher concordance rate in MZ twins, to looking for the genetic or epigenetic difference between MZ discordant twins.[11]

In this review, the theoretical concept of such study to search for genetic or epigenetic difference between MZ discordant twins is explained and its application to schizophrenia and bipolar disorder is summarized.

What is epigenetics?

Epigenetics is defined as the study of mitotically or meiotically heritable variations in gene function that

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cannot be explained by changes in DNA sequence.[12] For such mechanisms, methylation of the cytosine residue in the DNA molecule, and acetylation, methylation, and other modifications of histones have been well described. These modifications stably affect gene expression through alteration of DNA–protein interaction. These events refer to the inheritance from a parental cell to daughter cells. With regard to the inheritance from parents to the offspring, DNA methylation status is once totally reprogrammed at the fertilization. However, the DNA methylation can be conserved throughout the process of fertilization in some special cases.[13,14]

As evidenced by the study of clone animals, a different phenotype can be produced from the genomes having completely the same DNA sequences. The phenotypic difference between cloned animals and donor should have arisen from epigenetic differences. Similarly, we can postulate that epigenetic difference is responsible for the discordance of phenotypes between MZ twins.

Tsujita *et al*[6] showed that electrophoresis patterns of the genomic DNA digested with a methylation-sensitive restriction enzyme were different between MZ twins discordant for schizophrenia. This finding raised a possibility that phenotypic discordance between MZ twins may be caused by some epigenetic difference rather than 'genetic' difference, that is difference of DNA sequence.[15] This does not always preclude a possible effect of environmental factors in discordant phenotypes of MZ twins, since environmental factors may affect the DNA methylation status.[16–18]

Cause of discordance between MZ twins

A number of case reports have revealed that phenotypic discordance between MZ twins can be arisen from several kinds of genetic or epigenetic differences, which are inter-correlated each other.[19] For example, expansion of triplet repeat can alter DNA methylation status, and reduced DNA methylation of transposon can cause transposition and finally causes disruption of a gene. Thus, dichotomy of 'genetic' and 'epigenetic' is difficult, and only a tentative classification is given below.

Genetic

Point mutation To our knowledge, there are only two cases of MZ twins, in which a point mutation causative for the discordance of a disease was identified. One case is the MZ twin pair discordant for Darier's disease. Darier's disease is an autosomal dominantly inherited dermatological disease caused by mutations in the *ATP2A2*, encoding endoplasmic reticulum Ca^{2+}-ATPase.[20] In this pair of MZ twins discordant for Darier's disease, a point mutation of *ATP2A2* was identified. This mutation, G23E, was not found in the healthy co-twin and their parents, suggesting that it was a *de novo* mutation. In the other report, a mutation, Glu92X, in interferon regulatory factor 6 (*IRF6*) was identified in a MZ twin having Van der Woude syndrome characterized by the cleft lip and palate with lip pits, whose healthy co-twin did not have this disease.[21] This mutation was not found in healthy co-twins and their parents. They reported this finding as one of the evidences to prove the causative role of *IRF6* in Van der Woude syndrome.

This kind of genetic difference may happen at or after the twinning. In these reports, however, it cannot be ruled out that both twins had the mosaic mutation whose percentage is different.

Chromosomal Abnormality Chromosomal abnormality is sometimes seen as mosaicism. Since the percentage and the tissue distribution may differ between the MZ twins, mosaicism of chromosomal abnormality can cause discordance between MZ twins. Machin[19] extensively reviewed the MZ twin cases with discordant phenotype caused by chromosomal abnormalities. They reviewed 16 pairs of MZ twins discordant for Turner syndrome. In addition, other discordant MZ twins, such as trisomy 21 and trisomy 13, were also reported. After that, a number of cases of MZ twins having discordant phenotypes arisen from *de novo* mosaic chromosomal abnormalities were reported. These include Turner syndrome,[22] skin pigmentation,[23] minor anomalies,[24] and sex phenotypes.[25,26]

Since there are a number of case reports of MZ discordant twins caused by mosaicism of chromosomal abnormality, it may be a frequent cause of discordance in MZ twins. However, it might be biased by methodology. While discordance of DNA sequence is difficult to identify, the methods to detect chromosomal abnormality are well established and can be tested in clinical settings. This might be the reason of higher number of case reports on discordance caused by chromosomal abnormality.

Phenotypic discordance is known in the MZ twins with chromosome 22q11 deletion. This could be explained by epigenetic mechanism,[27] rather than difference in chromosomal abnormality.

Mitochondrial DNA (mtDNA) heteroplasmy

In the mitochondrial encephalomyopathies, mutated mtDNA usually coexists with wild-type mtDNA. This phenomenon is referred to as heteroplasmy. Clinical phenotype alters with the ratio and tissue distribution of the mutation. *De novo* heteroplasmic 11778 mutation of mtDNA reportedly caused the discordance of Leber's disease in MZ twins.[28] An MZ twin pair discordant for chronic progressive external ophthalmoplegia was also reported. In this pair, both twins had a small amount of 4115 base pair deletion in muscles, but the affected twin had much higher amount of deletion.[29] Discordant phenotypes due to uneven amount of heteroplasmic mutations were also found in DZ twins with myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS)[30] and myoclonic epilepsy with ragged-red fibers (MERRF).[31] However, effects of nuclear genes could not be ruled out in these DZ twin cases.

Triplet repeat Triplet repeat expansion is known to cause inherited disorders, such as Huntington's disease.[32] Triplet repeat expands during meiosis due to slippage of the DNA polymerase. This causes expansion of the repeat from generation to generation, which is known as the molecular basis of anticipation. On the other hand, triplet repeat also expands during somatic cell mitosis, which causes mosaicism of the length of the repeat. Thus, length of the triplet repeat may be different between MZ twins.

Phenotypic discordance of fragile X syndrome caused by the difference of length of CGG repeat in the 5′-UTR of the *FMR-1* gene was reported in a male MZ twin pair.[33] Repeat expansion of *FMR-1* causes methylation of the CpG island, and results in the inactivation of this gene.

Epigenetic

X-chromosome inactivation While males have only one X chromosome, females have two X chromosomes. To avoid the unbalance of expression levels of the genes on the X chromosome between sexes, one of two X chromosomes in females is inactivated in humans. In females, the X chromosome of paternal origin is once inactivated during embryogenesis. After being once reactivated, one of the X chromosomes is randomly inactivated. Since this phenomenon randomly occurs during the development, it causes heterogeneity of X chromosome inactivation among tissues. Due to the mosaicism of X chromosome inactivation, X-linked genetic diseases are sometimes discordant between female MZ twins.[19] Discordant phenotypes were reported in X-linked mental retardation, Duchenne type muscle dystrophy,[34,35] red–green color blindness, Hunter disease,[19] and Fabry's disease.[36] For example, in female MZ twins discordant for fragile X syndrome, the length of CGG repeat did not differ, but its methylation status was different. The normal allele of FMR-1 was methylated and inactivated in the affected twin, while mutant allele was methylated and inactivated in healthy twin.[37]

Loss of imprinting Genomic imprinting is a phenomenon in which one of two alleles, from maternal or paternal origin, is inactivated by DNA methylation. Methylated and inactivated allele is referred to as 'imprinted'. Many of the imprinted genes are related to the cell growth, and loss of imprinting is known to be one of the causes of cancer.

Beckwith–Wiedemann syndrome (BWS) is a congenital disorder characterized by hyperplasia of organs and tumor susceptibility, caused by several different genetic or epigenetic mechanisms such as chromosomal abnormalities, point mutations, and loss of imprinting of the genes on 11p15, the most studied imprinted chromosomal region. Since the phenotype is not so severe, it does not always cause clinical problems.

Among female MZ twins, prevalence of BWS is higher than expected, and they are mostly discordant. *KCNQ1* (voltage-gated potassium channel 1) in the imprinted region on 11p15 is regulated by an antisense RNA gene, *KCNQ1OT1* (*KCNQ1*-overlapping transcript 1), which is also regulated by imprinting. Among five pairs of MZ twins discordant for BWS, lack of DNA methylation of *KCNQ1OT1* in fibroblasts was observed only in the affected twins.[38] Thus, loss of imprinting is the cause of BWS in these cases. A similar finding, discordant DNA methylation, was also seen in lymphocytes. However, it is of note that loss of imprinting was observed in both twins in some of the MZ twin pairs. This could be due to the blood transfusion *in utero*. Since lymphocytes with loss of imprinting have enhanced growth, these cells may be selectively amplified in the healthy co-twin.

It has been postulated that loss of imprinting itself caused twinning. This can explain the higher rate of twins in BWS.

Recently, it was reported that children born by *in vitro* fertilization (IVF) is more frequently seen in BWS (4%, 6/149) compared with general population (less than 1.2%).[39] All were sporadic cases with loss of imprinting of differentially methylated region (KvDMR1) within the *KCNQ1*.[40] In the other study, four of 37 cases with BWS was born by IVF, while IVF was identified as the method of conception in only one of 148 matched controls.[41] Angelman syndrome and retinoblastoma are also reportedly associated with IVF.[42] These findings suggest that IVF is a risk factor of abnormality in DNA methylation.

Mobile elements Approximately 40% of the human genome is comprised of retroelements such as retrotransposon, transposon, and endogenous retrovirus. While retrotransposon transposes after transcribed into RNA, transposon transposes as DNA. In the case of endogenous retroviruses, expressed RNA is reverse transcribed and assembled into the genome by reverse transcriptase. Since transposition of transposon was first discovered in corn, transposition of transposon or retrotransposon is known to be active in plants.

Kazazian *et al*[43] first described a disease caused by the transposition of mobile element in humans. They found the insertion of the mobile element, LINE1 (L1), into the factor VIII gene, in two of 240 unrelated patients with hemophilia A. *De novo* insertion of Alu into an intron of *NF1* causing neurofibromatosis was also reported.[44] Such mechanism could explain the discordance between MZ twins in some cases. Since transposition of transposon is regulated by DNA methylation, abnormal DNA methylation of transposon may be associated with activity of transposition. Thus, transposon is related to both epigenetic and genetic mechanisms.

A possible role of human endogenous retrovirus (HERV) is also suggested in schizophrenia. Karlsson et al[45] examined the expression levels of endogenous retroviruses in cerebral spinal fluid (CSF) in patients with schizophrenia and found that their expression is higher in the CSF of schizophrenic patients. HERV-W-related RNA was detected in plasma of patients with schizophrenia.[46]

Retroviruses Retrovirus infection, that can be integrated into genome, could also explain the discordance between twins.[47,48]

Relevance of epigenetics in mental disorders
The field of epigenetics is too broad to be covered by this short review on discordant twins. Epigenetic regulation of gene expression is used in a wide variety of biological functions, such as tissue-specific gene expression, differentiation of cells, epigenetic memory, suppression of retroelements, and genomic imprinting. If epigenetics is related to mental disorders, all of them may be relevant. Although tissue-specific gene regulation and epigenetic memory need to be studied in the brain, it is difficult to obtain brain samples from discordant twins. Practically, we can obtain only blood cells or other non-neuronal cells from discordant twins. Abnormalities of imprinting might be detected in non-neuronal cells, according to the case of BWS.[38] Thus, we mainly focused on imprinting.

Bipolar disorder Petronis proposed that epigenetic mechanisms might be relevant to the pathophysiology of bipolar disorder based on several lines of evidence such as the relatively high degree of discordance in MZ twins, characteristic age at onset, parent-of-origin effects (POE), and fluctuation of the disease course.[49] Especially, POE in the transmission of bipolar disorder suggests the role of genomic imprinting. In bipolar disorder, several reports suggested the involvement of POE.[50,51] POE refers to the phenomenon that the sex of the parent transmitting the disease affects the severity or age at onset of the offspring. These include higher number of affected mothers compared with affected fathers, higher prevalence rate of the disorder among maternal relatives compared with paternal relatives, and lower age at onset in the proband with affected father compared with those with affected mother, and higher number of maternally inherited pedigrees compared with paternally inherited pedigrees.[50,51] However, some of these findings were not replicated.[52] On the other hand, linkage of bipolar disorder with chromosome 18 (18q22 and 18p11) was observed only in the paternal transmission,[53] which was replicated in several studies.[54] POE was also reported in other chromosomes such as 6q,[55] 13q12, and 1q41.[56] POE is seen in the diseases caused by the imprinted genes. Thus, imprinted genes on these chromosomes are suggested to have a role in the etiology of bipolar disorder.

It is also suggested that epigenetics may be relevant to bipolar disorder, since some drugs can affect the DNA methylation. Among the mood stabilizers, valproate is known to be a histone deacetylase (HDAC) inhibitor. Histone acetylation is coupled with DNA methylation and plays a role in the epigenetic regulation of gene expression. If inhibition of HDAC by valproate is related to its efficacy, it may suggest that such epigenetic gene regulation may be relevant to bipolar disorder.[57] On the other hand, S-adenosyl methionine (SAM) is known to be effective for bipolar depression.[58] SAM supplies methyl residue in DNA methylation reaction, and was reported to enhance DNA methylation *in vitro*.[59] The effect of SAM on bipolar disorder may also be mediated by alteration of DNA methylation status. However, such evidence is too circumstantial to prove the role of epigenetic factors in this disorder.

Schizophrenia Gottesman and Bertelsen[60] examined the risk of schizophrenia in the offspring of MZ twins discordant for schizophrenia. The risks in the offspring of twins did not differ, suggesting that the cause of discordance is not heritable, but rather environmental or epigenetic. The possible role of epigenetics has been postulated in pathophysiology of schizophrenia from several clinical features such as inheritance pattern, discordance of MZ twins, and fluctuating course.[61]

One of the findings suggestive of POE, higher number of affected mothers compared with affected fathers, is also seen in schizophrenia. In paternally transmitted cases, anticipation, lower age at onset in offspring compared with the parent, was more prominent than maternally transmitted cases.[62,63] However, this finding is not replicated in other studies and might be due to selection bias.[64] Twin is one of the risk factors for schizophrenia.[65] This has been regarded as reflecting birth complications such as anoxia. However, this could also be explained by epigenetic abnormality as discussed above.

Costa and colleagues[66] have been studying the epigenetic animal model of schizophrenia. They applied methionine to the mice and found that reelin is hypermethylated in these mice and these mice showed altered behavior resembling schizophrenia.

Search for epigenetic discordance between MZ twins
To date, a number of studies have been performed to reveal genetic or epigenetic difference between MZ twins discordant for mental disorders.

Schizophrenia
DNA sequence: To date, the difference of DNA sequence between the MZ twins discordant for schizophrenia has been searched for. No difference of genotypes was found between twins, by genotyping 94 microsatellite markers in five pairs of MZ twins discordant for schizophrenia.[5] No difference of DNA sequence between discordant MZ twins was found, by random amplification of polymorphic DNA

(RAPD) method using 10 retroviral related primers as well as eight random primers,[67] or genomic representational difference analysis (RDA) using six different enzyme digest representations.[10] No difference of repeat length was found between discordant MZ twins in the CAG repeat in atrophin-1, the causative gene for dentatorubral-pallidoluysian atrophy (DRPLA).[68] The CAG/GAA repeat was not expanded in discordant MZ twins using the repeat expansion detection (RED) method.[7]

More recently, Nguyen et al[8] separated the DNA fragment obtained from the sequences surrounding the CAG repeat using the high-performance liquid chromatography-based method, targeted genomic differential display (TGDD), and reported that the difference of peaks was larger between the discordant twins compared with the concordant twins. However, the locus causative for this difference has not been identified yet.

DNA methylation: Deb-Rinker et al[47,48] analyzed the lymphocytes obtained from MZ twins discordant for schizophrenia using RDA and found a new retrovirus, which was expressed only in the affected twin. They named this as schizophrenia-related retrovirus-1 (SZRV-1) and SZRV-2. Loss of DNA methylation of SZRV-2 was detected in this patient with schizophrenia. It has not been established whether or not SZRV-2 is a cause of schizophrenia in this case, or in general.

Tsujita et al[6] used the restriction landmark genome scanning (RLGS) method[69] to screen the difference of genomes between MZ twins discordant for schizophrenia.[6] In this method, genomic DNA was digested with a methylation-sensitive restriction enzyme, *Not*I, the fragments were analyzed by two-dimensional electrophoresis, and the pattern seen in the gel was compared between twins. They identified two spots showing different intensities between twins, suggesting genetic or epigenetic difference. The results obtained by RLGS might reflect the difference of DNA sequence, but more likely reflected difference of DNA methylation.

Petronis et al[12] have been studying the possible role of epigenetic factors in mental disorders. They analyzed the DNA samples obtained from two pairs of twins discordant or concordant for schizophrenia and examined the DNA methylation status of the upstream region of the dopamine D2 receptor.[9] They examined the difference of the DNA methylation status between twins, which was named 'epigenetic distance'. They reported that epigenetic difference was larger between discordant twins compared with concordant twins.

McDonald et al[10] used genomic RDA to identify discordance of DNA sequence or DNA methylation between twins discordant for schizophrenia. For two of six enzyme digest representations, methylation-sensitive enzyme, *Hpa*II, was used. They identified an apparent difference of one gene when two enzymes, *Hpa*II and *Mbo*I, were used for digestion. However, this DNA sequence was derived from bacterial genomic fragment of *Pseudomonas aeruginosa*, suggesting possible contamination. They concluded that there is no genetic or epigenetic difference between MZ twins discordant for schizophrenia.

Gene expression: Using the DD method, Friedhoff et al[70] cloned a new gene of unknown function from lymphocytes of MZ twins discordant for schizophrenia. The expression level of this gene, *oksc12b*, was lower in affected twin compared with healthy cotwin. However, the expression level of this gene in the brains of patients with schizophrenia did not differ from controls, suggesting no pathophysiological significance.[71]

Summary of the findings: In summary, difference of DNA sequence has been searched for in MZ twins discordant for schizophrenia, but most of the studies did not support the genetic difference. The results in the difference in DNA methylation seem a little more promising. However, no conclusive evidence has been obtained yet. Gene expression difference was also searched for, but any effort to reveal genetic or epigenetic difference has not been taken yet.

Bipolar disorder Compared with schizophrenia, few molecular genetic studies of discordant twins have been published in bipolar disorder, possibly because MZ twins completely discordant for bipolar disorder are quite rare. Although the summary of published studies reported that the concordance rate of bipolar disorder in MZ twins is approximately 70%, this largely depends on the definition of concordance. For example, in the study by Bertelsen et al,[72] 46 of 69 MZ twins were completely concordant. However, 14 of other 23 were partly concordant, that is, the others had some mental disorder or had committed suicide. Thus, MZ twins completely discordant for bipolar disorder are rarely seen.

Gene expression: In an attempt to identify the genetic or epigenetic difference between twins, the authors examined two pairs of MZ twins discordant for bipolar disorder and a pair of healthy twins.[73] Intracellular calcium response was different between the lymphoblastoid cells obtained from discordant twins. By DNA microarray analysis, two genes, *XBP1* and *GRP78* (*HSPA5*), both of which have pivotal roles in endoplasmic reticulum (ER) stress signaling, were commonly downregulated in affected twins.[73] *XBP1* is located at 22q12, the common linkage locus for bipolar disorder and schizophrenia. *GRP78* is regulated by *XBP1* and induced by valproate.

Based on this finding in twins, we further examined the role of this pathway in pathophysiology of bipolar disorder by case–control studies. Response of *XBP1* and *GRP78* to ER stress was attenuated in bipolar disorder. This difference was partly explained by the functional polymorphism in the promoter of *XBP1*, named −116C/G. The functional disturbance caused by −116G was improved not by lithium but by valproate. The genotype was associated with treatment response to lithium in Japanese bipolar patients.[74] This polymorphism was associated with bipolar disorder in Japanese case–control samples. Although it was also associated in Caucasian trios obtained from NIMH in the first report, it was not replicated in an extended NIMH trio samples, triads from Bulgaria and the UK, as well as case–control samples from various European populations.[75] A case–control study in Chinese also did not support the association.[76]

On the other hand, it was recently reported that this polymorphism was associated with schizophrenia in Japanese[77] and Chinese.[76] The other gene in this cascade, *GRP78*, was also associated with bipolar disorder, suggesting that there are more than one functional polymorphisms affecting ER stress response.[78]

DNA sequence and DNA methylation: In these MZ discordant twins, the genotype was the same between the twins; one pair had C/G and the other pair had G/G. Thus, the cause of discordance was not due to this polymorphism. No other difference of genomic DNA sequence could be identified in the *XBP1* gene. We also quantified the copy number of *XBP1* using quantitative genomic PCR, but there was no difference. We further analyzed the DNA methylation status of the CpG island of *XBP1*. However, the CpG island was not methylated in both affected twins and their healthy co-twins. Thus, the discordance of endophenotype, reduction of *XBP1* expression, was not caused by genetic or epigenetic difference of *XBP1* itself.

Although we selected the genes commonly altered in both of these twins, the primary discordance may not be caused by these genes. Especially, it is of note that one of the discordant twins is also discordant for several somatic diseases, including ossification of the posterior longitudinal ligament (OPLL).[79] Thus, there might be discordance in some gene upstream to the molecular cascade of bipolar disorder and that of OPLL. We are now searching for the epigenetic discordance causing altered gene expression in these discordant twins.

Methodological considerations and future strategies

Subjects To search for the genetic or epigenetic difference between discordant twins, identification of a suitable twin pair would be the most important point. Firstly, if one of the twins had onset of the disease just several years before, they may not be truly discordant, since the other twin may have disease onset soon. Thus, the ages of the subjects should be 10 or 20 years after the age at onset of the affected twin. Secondly, in the case with marked environmental insult such as infection, perinatal complication, or head trauma, such cases may not be suitable for the search of genetic or epigenetic difference. Thirdly, phenotypic discordance should be complete. In the case of mental disorders, phenotype definition is not a dichotomy of 'disease' and 'healthy'. If the index case had schizophrenia and the other twin had schizoid personality disorder, they are incompletely discordant, and the possibility of genetic or epigenetic difference may be smaller than completely discordant cases. Fourthly, discordance of comorbid genetically determined somatic disease or intermediate phenotype might also be a hallmark of genetic or epigenetic difference.

Tissue The ideal source for epigenetic analysis would be the brain tissue, but it is practically impossible in the study of discordant twins. Peripheral blood cells are usually used for this kind of study.

In the case of peripheral blood cell, effects of medication are difficult to control, since the affected twin is usually medicated with various psychotropic drugs, some of which can affect histone acetylation and DNA methylation. In addition, the subpopulation of white blood cells, such as granulocytes, B lymphocyte, or T lymphocyte, can be altered by mental status, hormones, or medication. In lymphocytes, difference of genome rearrangement status between cells can obscure other more important genetic difference.

Using lymphoblastoid cell lines, some of the above-noted problems, such as effects of drugs and cellular heterogeneity can be minimized. However, Epstein–Barr virus that is used for transformation may alter the DNA methylation status of some genes.[80] Transposon may become active during cell culture, especially when the DNA methylation inhibitor, 5-aza-deoxycitidine (5-aza-dC), is applied.[81] In both cases, the possible effects of blood transfusion *in utero* might obscure the difference between twins.[38]

Most of the above-noted problems can be overcome by using fibroblasts, which can also be cultured and stored. Although it is somewhat more invasive to perform skin biopsy than drawing blood, it does not cause pain and scar at all, when adequately performed.[82]

Methodology To identify the epigenetic discordance between twins, several methodologies could be used. Among these methods, RLGS is the most established method, although it is laborious. Although it had been difficult to identify the gene with DNA methylation difference responsible for the spot detected, the recent development of *in silico* RLGS has made it easier.[83] Microarray-based comprehensive analysis of CpG island would be an ideal method to screen the DNA methylation difference between discordant twins.[84]

We are currently searching for the DNA methylation difference using two different strategies. One is methylation-sensitive representative differential analysis (MS-RDA).[85] In this method, the genomic region with different methylation status between two genomes can be selectively amplified using methylation-sensitive restriction enzymes. We also applied 5-aza-dC to lymphoblastoid cells obtained from discordant twins to unmethylate all DNAs in these cells. Before and after the 5-aza-dC treatment, gene expression patterns were examined by DNA microarray. Although this method has an apparent disadvantage of the difficulty of excluding false positives caused by secondary effect of drug treatment, this method has been successfully applied to the identification of hypermethylated CpG islands in cancer cells.[86] We checked the DNA methylation status of the candidate genes that upregulated after the 5-aza-dC treatment only in one of the twins, and found the differences in methylation status between MZ twins, although their pathophysiological significances remain elusive (Iwamoto *et al*, in preparation).

Conclusion

Search for genetic or epigenetic difference between MZ twins discordant for mental disorders might be a promising strategy to identify the genes responsible for mental disorders. Once the responsible mutation or epimutation was found in the affected twin, this information would become a clue to study the pathophysiology of mental disorders. Further studies are warranted to identify genetic or epigenetic difference between MZ twins responsible for discordant phenotypes.

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Munchausen's Syndrome With 20-Year Follow-Up

To the Editor: We report a 20-year follow-up for a patient with Munchausen's syndrome simulating torsion dystonia that was first presented in 1985 (1). Long-term follow-up for patients with Munchausen's syndrome is rare because of the secretive nature of the disease and the limitations in sharing confidential patient information.

Ms. A is now a 56-year-old woman who had worked as a teacher until she reportedly suffered a traumatic brain injury when she was pushed by a student. Neuroimaging after the incident was unremarkable, and a neurological examination revealed only bilateral ankle inversion consistent with her history of torsion dystonia. Two weeks later, she was found severely dehydrated and unable to walk or dial for help because of an apparent exacerbation of her torsion dystonia. Her symptoms progressed upon hospitalization. She developed severe language deficits, including the inability to read or write. When transferred to a rehabilitation facility, she reported auditory hallucinations and paranoid delusions and fled the facility several times. During psychiatric consultation, she described a recent suicide attempt by drug overdose but reported no history of psychiatric conditions or hospitalizations.

Upon admission to a psychiatry unit, Ms. A's psychotic symptoms and suicidal ideation immediately resolved. A search of medical records revealed that she had been described in a case report 20 years earlier. At the age of 19, she feigned suicide to gain admission for psychiatric care. In 1979 she submitted to bilateral ventrolateral thalamotomy for an intractable dystonia that lacked typical electromyographic findings. She had a devastating subsequent course of aphonia lasting 18 months, intractable seizures leading to tracheostomy, trismus requiring a gastrostomy tube, and severe weight loss that was thought secondary to surreptitiously discarding her gastrostomy tube feedings. With psychotherapy she eventually admitted to fabricating all of her symptoms.

Ms. A later moved to our city and taught for nearly 20 years. In 1990 she had two hospital admissions for Behçet's disease, a diffuse vasculitis often associated with uveitis and aphthous ulcers. There were no objective findings, and she was discharged with probable factitious disorder. She has otherwise led a productive life, free of hospital admissions, for the last 15 years.

Patients with factitious disorder can suffer considerable iatrogenic morbidity and place immense strain on the health care system. Health care providers may develop a sense of therapeutic nihilism. Factitious disorders are often refractory to psychotherapy, and confronting patients about their symptoms is rarely beneficial (2). With psychotherapy this once severely disabled woman achieved relative independence for many years. Whether she sustained a traumatic brain injury before her most recent admission is questionable, but the acute stress of an assault at work may have caused an exacerbation of this patient's factitious disorder.

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Neuroleptic Malignant Syndrome Related to a Switch to Perospirone and Anticholinergic Withdrawal

To the Editor: We report the first case, to our knowledge, of neuroleptic malignant syndrome related to perospirone. The patient's medication had been switched from olanzapine and an anticholinergic drug approximately 2 weeks previously. When treated with perospirone without anticholinergics, the patient had a fulminant neuroleptic malignant syndrome that was complicated with rhabdomyolysis and acute renal failure.

Mr. A, a 43-year-old man, had suffered from paranoid schizophrenia for 27 years. He had been admitted to a psychiatric hospital 11 times between the ages of 21 and 33 because of insomnia, psychomotor agitation, and persecutory delusions. His illness had been maintained with a dose of 9–15 mg/day of bromperidol for more than 20 years. Bromperidol is a butyrophenone neuroleptic that shows a similar pharmacodynamic response as haloperidol. Six weeks before his admission, Mr. A's medication was switched to risperidone and biperiden, and bromperidol was discontinued because of the drug-induced extrapyramidal symptom of a hand tremor.

Three weeks later, risperidone was switched to olanzapine because Mr. A felt that risperidone did not effectively treat his symptoms. After Mr. A had been taking the medication for 3 weeks, olanzapine and biperiden were discontinued, and perospirone, 16 mg/day, was introduced because of psychiatric symptoms, such as auditory hallucinations and diarrhea. On the 12th day of perospirone treatment, Mr. A suddenly became confused and obtunded with severe muscle rigidity. When he was admitted to a psychiatry department, he had a high fever, tachycardia, and severe extrapyramidal symptoms. Laboratory tests showed an elevation of creatine phosphokinase (33,878 IU/liter) and leukocyte levels (30.9×10^9/liter). A urine analysis showed myoglobinuria. A brain computerized tomography scan showed no remarkable change, and the possibility of infectious disease was excluded.

Mr. A had a fulminant neuroleptic malignant syndrome complicated with rhabdomyolysis and acute renal failure. On the third day of admission, he was transferred to an intensive care unit. He received dantrolene treatment and hemodialysis. On the 19th day, the fever disappeared, and his serum creatine phosphokinase level was normal (112 IU/liter). On the 44th day, his muscle rigidity was resolved, and no psychiatric symptoms were observed. He was discharged on the 46th day.

This case report shows that neuroleptic malignant syndrome can occur after a switch to perospirone treatment from other antipsychotics and anticholinergic withdrawal. Perospirone is a novel serotonin-dopamine antagonist and also a partial serotonin (5-HT_{1A}) agonist (1). The receptor binding profile and pharmacological property of perospirone resemble those of risperidone, and side effects, such as extrapyramidal symptoms, tend to occur less often with perospirone (2). This patient did not develop neuroleptic malignant syndrome during previous risperidone and olanzapine treatment with anticholinergics. It has been reported that abrupt withdrawal of anticholinergic agents is associated with neuroleptic malignant syndrome (3). Special caution with regard to concomitant drugs is therefore necessary when switching between antipsychotic drugs.

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Trimipramine for Refractory Panic Attacks

TO THE EDITOR: Trimipramine, a tricyclic tertiary amine pharmacologically related to imipramine, which was the first pharmacological agent noted to treat panic disorders (1), provides significant rapid antipanic effects with minimal side effects at a low dose. Tricyclic drugs are less widely used than selective serotonin reuptake inhibitors (SSRIs) because tricyclic drugs generally have more severe adverse effects at the higher doses required for effective treatment of panic disorders (2). This is a case report about a man with a 20-year history of panic disorders who was unresponsive to all agents tried, with the exception of imipramine, which was discontinued because of side effects. Tripramine at 50 mg/day induced remission of the panic attacks without adverse effects.

Mr. A, a 41-year-old white man, had suffered from panic attacks since age 21. According to Mr. A, the medications tried to no avail (either were ineffective or were discontinued because of side effects) included amitriptyline, bupropion, amoxapine, fluoxetine, sertraline, citalopram, paroxetine, fluvoxamine, duloxetine, escitalopram, carbamazepine, valproic acid, lithium, olanzapine, aripiprazole, olanzapine plus fluoxetine, aripiprazole plus fluoxetine, clonazepam, lorazepam, alprazolam, and combinations of alprazolam with multiple SSRIs, atypical neuroleptics, tricyclics, tetracyclics, and anticonvulsants.

Mr. A was initially seen while taking fluoxetine, 20 mg/day, in addition to 8 mg/day of alprazolam with olanzapine, 10 mg/day, and he continued to experience 1–2 panic attacks every 10 days. He had been taking alprazolam for 14 years. Fluoxetine and olanzapine were discontinued, and mirtazapine, 15 mg at night, was begun with the longstanding alprazolam, 2 mg/day. Mirtazapine caused stimulation and was discontinued. Quetiapine, 25 mg/day, was tried in conjunction with 2 mg/day of alprazolam. Quetiapine had to be discontinued because of gastrointestinal side effects. Desipramine was started at 10 mg at bedtime. The dose was increased to 20 mg at bedtime within a 2-week span, but it had to be discontinued because of overstimulation and worsening of the panic attacks. Trimipramine was begun at 25 mg/day, together with alprazolam, 2 mg/day. In view of no side effects, the dose of trimipramine was increased to 50 mg in the morning together with 2 mg/day of alprazolam. Within 5 weeks, Mr. A indicated he was "much calmer" and had not experienced any panic attacks. He remained free of panic attacks for 6 months. Ongoing tapering of alprazolam was being pursued.

On the basis of this case report, further use of trimipramine may be warranted in the treatment of panic disorders unresponsive to the more commonly used treatment modalities.

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Letter to the Editors

A missense polymorphism (H204R) of a Rho GTPase-activating protein, the chimerin 2 gene, is associated with schizophrenia in men

Dear Editors:

Schizophrenia is a complex genetic disorder characterized by profound disturbances of cognition, emotion and social functioning. This disease is believed to involve genetic abnormalities in developmental/plasticity related processes (DeLisi, 2000). The pathophysiology of schizophrenia is still unclear; however, the high incidence developing schizophrenia was observed in mental retardation, suggesting common pathophysiological basis of these two diseases including genetic basis. As several X-linked mental retardation genes are involved in Rho signaling pathways (Ramakers, 2002), Rho GTPase related genes could be strong candidate genes for schizophrenia. The Rho family of GTP binding proteins act as a key regulator for developing neuronal network, e.g. neurite and growth cone formation (Negishi and Katoh, 2002). Chimerin 2 gene, CHN2, is one of the GTPase-activating proteins expressed in a variety of human tissues with the highest expression levels in brain (Yuan et al., 1995). Therefore, genetic variability of the chimerin 2 gene is of considerable interest in the evaluation of risk of schizophrenia. To our knowledge, however, there is no study examining the possible association between the CHN2 gene and schizophrenia.

The CHN2 maps to chromosome 7p15.3 and consisted of 13 exons and 12 introns, spanning 318 Kb (Yuan et al., 1995). We searched for polymorphisms in the CHN2 gene in silico and detected a common single nucleotide substitution (A611G; NCBI SNP ID: rs3750103) (Haga et al., 2002) in exon7 giving rise to an amino acid change of histidine to arginine at codon 204 (H204R) (amino acid numbering is according to NCBI protein data base accession NP_004058). In our search there was no other missense polymorphism reported in the CHN2 gene. Since this polymorphism may alter functions of the CHN2, we performed an association analysis between this polymorphism and schizophrenia in a Japanese sample of 293 patients (162 males and 131 females with mean age of 43.7 years [S.D. 14.2]) with schizophrenia and 450 healthy controls (222 males and 228 females with mean age of 36.5 years [S.D. 12.6]). Consensus diagnosis was made for each patient by at least two psychiatrists according to the Diagnostic and Statistical Manual of Mental Disorders, 4th edition(DSM-IV) criteria. After description of the study, written informed consent was obtained from every subject. The study protocol was approved by institutional ethical committees.

The H204R polymorphism genotypes were determined using the TaqMan 5′-exonuclease allelic discrimination assay, described previously (Hashimoto et al., 2004). Briefly, probes and primers for detection of the SNP are: forward primer, 5′-CAGATCTCCTCCCTGGTTCGA-3′, reverse primer, 5′-TGCTTACCTTAAAGTTGTGTGTCTTCT-3′, probe 1, 5′-VIC-CCCTCACACACAACGA-MGB-3′, and probe 2, 5′-FAM-CCTCACACGCAACGA-MGB-3′.

Genotype distributions and allele frequencies of the H204R missense polymorphism of the chimerin 2 gene among the patients and controls are shown in Table 1. The genotype distributions for the two groups and those of male and female patients as well as controls were in Hardy–Weinberg equilibrium (data not shown). There was a trend towards an increased frequency of the R204 allele in the patients than in the controls ($\chi^2=3.74$, $df=1$, $p=0.053$, odds ratio = 1.29, 95%CI 1.00–1.66). The individuals homozygous for the R204 allele were significantly

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Table 1
Genotype and allele distributions for the H204R polymorphism of the chimerin 2 gene between the patients with schizophrenia and controls

Group	N	Genotype distribution				Mantel Haenszel	Allele frequency			odds ratio (95%CI)
		His/His	His/Arg	Arg/Arg	P value	P value	His	Arg	P value	
Male										
Patients	162	95 (58.6%)	57 (35.2%)	10 (6.2%)	0.047	0.018	247 (76.2%)	77 (23.8%)	0.018	1.53 (1.07–2.19)
Controls	222	152 (68.4%)	65 (29.3%)	5 (2.3%)			369 (83.1%)	75 (16.9%)		
Female										
Patients	131	80 (61.2%)	44 (33.6%)	7 (5.3%)	0.703	0.678	204 (77.9%)	58 (22.1%)	0.681	1.08 (0.75–1.56)
Controls	228	141 (61.9%)	79 (34.6%)	8 (3.5%)			361 (23.8%)	95 (20.8%)		
Total										
Patients	293	175 (59.7%)	101 (34.5%)	17 (5.8%)	0.087	0.052	451 (77.0%)	135 (23.0%)	0.053	1.29 (1.00–1.66)
Controls	450	293 (65.1%)	144 (32.0%)	13 (2.9%)			730 (81.1%)	170 (18.9%)		

more common in the cases than in the controls ($\chi^2=3.94$, $df=1$, $p=0.049$, odds ratio $=2.07$, 95%CI 0.99–4.33). The observed frequency for the minor allele (R204) in our control group (19%) was quite similar to that reported by Haga et al. (2002) (18%) estimated from 48 Japanese chromosomes. Thus, the observed significant difference in the allele frequency between the cases and controls cannot be ascribed to an unusually lower frequency of the R204 allele in our control subjects.

As gender differences occur in various aspects of the disease, including earlier age of onset, poorer course and medication response in men, we examined males and females separately. The R204 allele was excess in our cases when compared to controls among males ($\chi^2=5.57$, $df=1$, $p=0.018$, odds ratio $=1.53$, 95%CI 1.07–2.19). Genotype distributions also revealed significant difference between male controls and male patients with schizophrenia ($\chi^2=6.12$, $df=2$, $p=0.047$; $\chi^2=5.56$, $df=1$, $p=0.018$ by Mantel Haenszel test). However, there was significant difference in neither allele frequency nor genotype distribution between the schizophrenics and controls in females.

CHN2 protein acts as a receptor of diacylglycerol/phorbol esters and regulates the activity of the Rac GTPase, one of the Rho GTPase family proteins (Caloca et al., 2003). The CHN2 inhibits Rac-GTP activation by the stimulation of epidermal growth factor (EGF). EGF protein levels were decreased in the prefrontal cortex of schizophrenic patients, and conversely, EGF receptor expression was elevated in the prefrontal cortex (Futamura et al., 2002). Serum EGF levels were markedly reduced in schizophrenic patients, even in young, drug-free patients (Futamura et al., 2002). Neonatal perturbation of EGF in rats resulted in abnormal sensorimotor gating and social interaction in adults (Futamura et al., 2003). In addition, neuregulin-1, one of the EGF family proteins, was reported as a schizophrenia susceptibility gene (Harrison and Owen, 2003) and the abnormal expression of neuregulin-1 has been observed in schizophrenic brain (Hashimoto et al., 2003). Therefore, the CHN2 H204R polymorphism might lead to the abnormality of neuregulin signaling pathways. As the location of H204R is close to diacylglycerol/phorbol ester binding domain (214–264 amino acid), this polymorphism could alter the protein structure of the region, which may change the second messenger signaling. H204R polymorphism, next to a casein kinase II phosphorylation site, might also play a potential role in the CHN2 phosphorylation state, although the physiological phosphorylation status is unclear.

We demonstrated, for the first time, the possible association between a missense polymorphism (H204R) of the CHN2 gene and schizophrenia in a Japanese population. A false-positive association due to population stratification could not be excluded in our case control designed study, despite the precaution of ethnic matching of this study. Therefore, it is necessary to carry out further investigations to confirm our findings in other samples. If our results are replicated, functional analysis of the CHN2 H204R polymorphism might contribute to understanding the molecular mechanisms of schizophrenia.

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No association of haplotype-tagging SNPs in TRAR4 with schizophrenia in Japanese patients

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Abstract

Recent study of linkage disequilibrium mapping showed one of the trace amine receptor (TRAR) genes, TRAR4, was associated with schizophrenia. We conducted a replication study of TRAR4 with schizophrenia in Japanese patients. We used two large independent sets of samples in a first-set analysis (cases = 405, controls = 401) and second-set analysis (cases = 503, controls = 440). In the first-set analysis, one Marker (Marker5) showed a significant association, but this significance was not seen in the second-set analysis. Our results indicate that TRAR4 may not play a major role in Japanese schizophrenia patients, and that it is important to examine the possibility of false positives in genetic association analysis.

Keywords: Schizophrenia; Trace amine receptor; Linkage disequilibrium; Haplotype-tagging SNP

1. Introduction

Trace amines (TAs) are endogenous amine compounds that are chemically similar to classic biogenic amines. TAs were thought to be 'false transmitters' which displace classic biogenic amines from their storage and act on transporters in a fashion similar to the amphetamines, but the identification of brain receptors specific to TAs indicates that they also have effects of their own effects, and TA receptors bind several psychostimulants such as amphetamine and D-lysergic acid diethylamide (LSD) (Parker and Cubeddu, 1986).

A recent study of linkage disequilibrium (LD) mapping showed significant association between SNPs in one trace amine receptor (TRAR) gene TRAR4 and schizophrenia (SCZ) (Duan et al., 2004). By genotyping 192 pedigrees with SCZ of European or African American ancestry, from samples that previously showed linkage evidence to 6q13–

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q26, an association of schizophrenia with 1 SNP (rs4307545: Marker16) within the TRAR4 gene remained significant after correction for multiple testing. The authors also showed that TRAR4 is preferentially expressed in several brain regions including the hippocampus that have been implicated in the pathophysiology of SCZ.

In this study, we conducted a replication study of TRAR4 with SCZ in Japanese patients. However, because SNP variations and LD patterns differ among populations, we included a systematic mutation search around TRAR4 and an LD evaluation. After the selection of haplotype-tagging (ht) SNPs, we performed a two-stage association analysis using two independent sets of samples in a first-set analysis (cases=405, controls=401) and second-set analysis (cases=503, controls=440).

2. Materials and methods

2.1. Subjects

In the association analyses, two independent sets of samples were examined: for the first-set screening scan, 405 patients with SCZ (206 male and 199 female; mean age ± standard deviation (S.D.) 42.6 ± 14.9 years) and 401 controls (213 male and 188 female; 34.1 ± 13.1 years), and for the second-set confirmation analysis, 503 patients with SCZ (284 male and 219 female; 52.8 ± 13.8 years) and 440 controls (223 male and 217 female; 40.7 ± 14.2 years).

The subjects for mutation search and LD evaluation were 37 patients with SCZ and 64 controls, respectively. These subjects were also included in the first-set screening scan. Characterization details and psychiatric assessment of these subjects were identical to those published elsewhere (Ikeda et al., 2005). All subjects were unrelated to each other and ethnically Japanese.

After the study had been described, written informed consent was obtained from each subject. This study was approved by the Ethics Committee at Fujita Health University and Nagoya University School of Medicine.

2.2. Mutation search

Primer pairs were designed using information from the GenBank sequence (accession number: NT-025741.13) and 5 amplified regions, which covered the coding exon and 5′ flanking region upstream 500 bp. A more detailed description can be seen in a previous paper (Suzuki et al., 2003). Sequences of primer pairs are available on request.

2.3. SNP selection and LD evaluation

For the evaluation of LD, we included the positive SNPs shown in the original paper (Duan et al., 2004) (Fig. 1) in addition to the SNPs we detected. First we determined 'LD blocks' with criteria based on 95% confidential bounds on D' using HAPLOVIEW ver 3.0 software (Barrett et al., 2005). Next, htSNPs were selected within each LD block for 90% haplotype coverage using SNPtagger software (Ke and Cardon, 2003).

2.4. SNP genotyping

We used TaqMan assays (Marker5, 12, 16, 19 and 21), primer extension using denaturing high performance liquid chromatography (Marker3, 4, 6, and 14) and direct sequencing (MarkerA and B). Sequences of primer pairs are available on request.

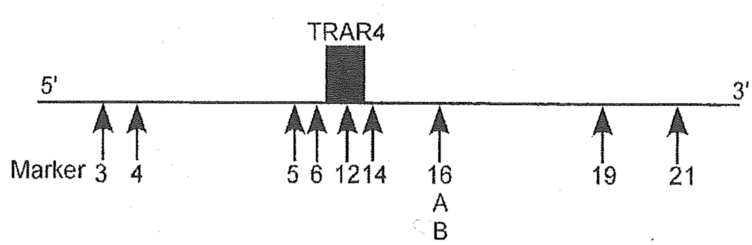

Fig. 1. Genomic structure of TRAR4. Numbers under arrows represent marker IDs. Vertical bar represents exon.

2.5. Statistical analysis

Genotype deviation from the Hardy–Weinberg equilibrium (HWE) was evaluated by χ^2 test (SAS/Genetics, release 8.2, SAS Institute Japan Inc, Tokyo, Japan).

Marker-trait association was evaluated allele/genotype-wise with χ^2 test (SPSS 10.0J, SPSS Japan Inc). In the first-set screening scan, we used a recently developed software program, SNPSpD, which could reflect the correlation of markers (LD) on corrected P-values, in order to control inflation of the type I error rate (Nyholt, 2004).

Power calculation was performed using a statistical program prepared by Ohashi et al. (2001). We estimated the power for our sample size under a multiplicative model of inheritance.

The significance level for all statistical tests was 0.05.

3. Results

Searched variants in the coding exon and 5′ flanking region among this ethnic group, two SNPs were identified in the 5′ upstream and 3′ downstream of TRAR4 (Marker6 and 14). None of the other SNPs were discovered.

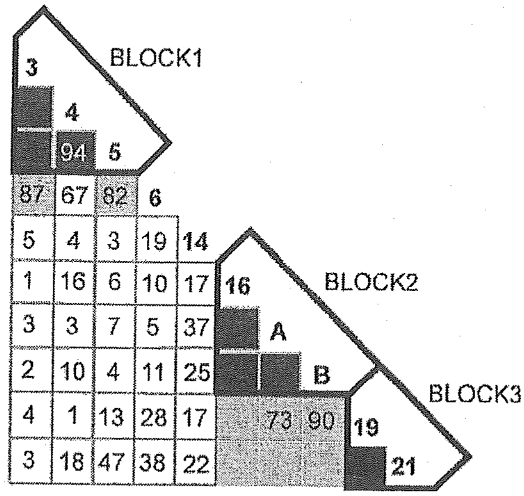

Fig. 2. Linkage disequilibrium evaluation. Numbers in box represents D' values after decimal point. D' values of 1.0 are not shown. Other information is provided at Haploview's website.

Nine SNPs of the Duan's report and two SNPs we detected were genotyped for evaluation of LD. All genotype frequencies of these SNPs were in HWE. Marker12 was excluded from the LD evaluation due to its low minor allele frequency (0.8%). Three LD blocks were defined and six SNPs (five htSNPs and Marker12) were selected (Table 1 and Fig. 2).

In the association analysis, we first genotyped these htSNPs and Marker12 for first-set samples (cases=405,

Table 1
htSNPs and first-set screening scan

Marker	SNP ID[a]	Blocks[b]	MAF[c] (%)	Genotype distribution[d]									
				M/M		M/m		m/m		P–value[e]		Corrected P–value[f]	
				SCZ	CON	SCZ	CON	SCZ	CON	Genotype	Allele	Genotype	Allele
3	rs4473885 (G > A)		18.0										
4	rs4085406 (A > G)	BLOCK I	19.5										
5	rs6907909 (A > G)		24.2	233	266	150	119	22	16	**0.0354**	**0.0122**	0.204	0.0702
6	rs9373026 (C > G)		48.4	143	132	179	181	83	88	0.749	0.437		
12	rs8192625 (A > G)		0.80	394	389	11	12	0	0	0.814	0.815		
14	rs7772821 (T > G)		28.1	243	231	143	145	19	25	0.572	0.350		
16	rs4305745 (G > A)		21.9	151	162	181	186	73	53	0.165	0.102		
A	rs17078770 (–/ A)	BLOCK II	20.3										
B	rs7452939 (C > T)		21.1										
19	rs6903874 (T > C)		14.1										
21	rs6937506 (G > A)	BLOCK III	13.3	298	292	96	96	11	13	0.901	0.716		

[a]Gray box represents 'haplotype-tagging (ht) SNPs' for association analysis.
[b]Blocks were defined by HAPLOVIEW.
[c]MAF=minor allele frequency of 64 controls.
[d]M=major allele, m=minor allele, SCZ=schizoprenia, CON=control.
[e]Bold numbers represent significant P-values.
[f]Corrected P-values were calculated by SNPSpd.