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Mass spectrometry identification of potential biomarker proteins in the 150-kD electrophoretic band in patients with schizophrenia

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Abstract

Background: Diagnosing schizophrenia is primarily based on the presentation of defined signs and symptoms, none of which is pathognomonic for this group of syndromes. However, few significant genome-wide associations between schizophrenia and individual have detected. Protein profiling of candidate serum biomarkers in schizophrenia is therefore an area of great interest.

Methods: In the present study, we used a combination of 7% polyethylene glycol (PEG) enrichment of immune complexes and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to separate abnormal band, then analyse the band with liquid chromatography mass spectrometry (LC-MS).

Results: There is a special 150-kD electrophoretic band in patients with schizophrenia, bipolar disorder, or depression relative to healthy controls (each 30 samples). Analysis of the band using LC-MS resulted in the identification of 11 serum proteins whose abundance was altered between patients and controls. Among them, 8 proteins (CFH, CFB, cDNA FLJ75416, zinc finger protein 729, isoform 2 of nidogen-1, diaphanous-1, cDNA FLJ77762, and cDNA FLJ58411) were up regulated, while one protein (isoform 1 of collagen alpha-1 (II) was down regulated in patients with schizophrenia, but only zinc finger protein 729 has statistics significance ($P < .05$). No differences were noted with regard to thrombospondin-1 or collagen alpha-2 (I) among the 3 groups. These proteins take part in several biological functions such as focal adhesion, complement cascades, ECM-receptor interaction, and Staphylococcus aureus infection.

Conclusions: The 150-kD electrophoretic band or zinc finger protein 729 may become biomarkers in patients with schizophrenia. In the future increasing sample size and function research of zinc finger protein 729 should be executed continuously.

Abbreviations: CFB = complement factors B, CFH = complement factors H, IPA = ingenuity pathway analysis, LC-MS = liquid chromatography mass spectrometry, PEG = polyethylene glycol, SDS-PAGE = sodium dodecyl sulfate-polyacrylamide gel electrophoresis, THBS-1 = thrombospondin-1, ZNF729 = zinc finger protein 729.

Keywords: biomarkers, protein, schizophrenia

1. Introduction

Schizophrenia is a severe, complex neuropsychiatric condition that affects approximately 0.7% of the population worldwide.[1] The current standards for diagnosis are based on criteria outlined in the Diagnostic and Statistical Manual of Mental Disorders IV or the International Statistical Classification of Diseases and Related Health Problems,10th revision (ICD-10).[2,3] Such rigid definitions leave clinicians with the pervasive problem of diagnosing schizophrenia primarily based on the presentation

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There is no competing interest to declare.

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of defined signs and symptoms, none of which is pathognomonic for this group of syndromes.[4]

Given these issues, the availability of tests for molecular biomarkers based on the underlying pathology would greatly facilitate disease diagnosis and stratification. Several studies have been conducted in the search for genetic mutations linked to schizophrenia. However, few studies have detected significant genome-wide associations between schizophrenia and individual single nucleotide polymorphisms (SNPs).[5,6] Protein profiling of candidate serum biomarkers in schizophrenia is therefore an area of great interest and potential with regard to diagnosis and individualized patient treatment. Recent studies have indicated that some patients with schizophrenia exhibit proteomic or metabolomic alterations. Such studies have reported altered levels of phosphorylated proteins, selected apolipoproteins, and plasma and serum proteins as well as abnormal immune system function in patients with schizophrenia.[7–10] However, attempts to utilize these biomarkers to secure a diagnosis in clinical settings have been largely unsuccessful.

In the present study, we used a combination of 7% PEG enrichment of immune complexes and SDS-PAGE in order to identify potential biomarker proteins in the 150-kD band of samples obtained from patients with schizophrenia.

The results were expected to find an objective index for diagnosis, therapy, and effect of schizophrenia. They are also explored to explain the pathogenesis of schizophrenia.

2. Materials and methods

The Ethical Committee of the Longgang Central Hospital reviewed and approved the protocol of the present study as well as the procedures for sample collection and analysis. All study participants provided written informed consent.

2.1. Clinical samples

Thirty patients with paranoid schizophrenia (20 men/10 women, mean age: 37.27 ± 12.4 years) diagnosed according to ICD-10 criteria and thirty patients with bipolar disorder (n = 17) or depression (n = 13) (20 men/10 women, mean age: 38.14 ± 13.7 years) were recruited from Kangning Hospital in Shenzhen, China. In addition, 30 healthy, age- and sex-matched control participants (20 men/10 women, mean age: 37.04 ± 10.7 years) with no family history of schizophrenia or other detectable psychiatric, neurological, or medical history were recruited from among hospital staff. No participants had been diagnosed with diabetes mellitus, hyperlipidemia, or other severe physical diseases (see Table 1).

Venous blood was drawn from each participant in the morning prior to breakfast at least 8 hours, collected in 7.5 mL serum S-Monovette tubes (BD, UK), and allowed to clot for 2 hours at 21°C. Centrifugation (4000 × g for 5 minutes at 21°C) was performed to remove any remaining clotted or cellular material. The supernatants were transferred to 1.5 mL microcentrifuge tubes (Eppendorf, UK) and stored at −70°C until use.

2.2. Preparation and separation of immune complex

A total 100 μL of serum was mixed with an equal volume of 7% PEG6000 (Sigma-Aldrich) in PBS (Phosphate Buffered Saline, Gibco) and vortex mixed for 1 minute. The sample was then incubated overnight at 4°C. Centrifugation was then performed at 1500 rpm for 20 minutes at 4°C in order to produce a clear supernatant with precipitate at the bottom. The supernatant was discarded, and the sample was then resolubilized in 50 μL deionized water and vortex mixed for 1 minute.[11]

Electrophoresis was conducted at 40 mA per gel for 1 hour in buffer containing 24 mM Tris (Sigma), 0.2 M glycine (Sigma), 0.1% w/v SDS (Sigma) (pH 8.6). The concentration of the separation gel was 10%. Following electrophoresis, the gel was placed directly into the stain solution (0.2% CBB R-250, 83% methanol/17% acetic acid).[12] The gel was stained for 4 hours under gentle agitation, and then destained with 50% methanol/10% acetic acid for 4–8 hours under gentle agitation and rinsed with deionized water for at least 15 minutes prior to image acquisition.

Following imaging, we analyzed differences between the patient and control groups with regard to the electrophoretic bands observed. In cases of significant difference, the molecular weight of the protein was determined and compared to known protein markers. The appropriate area was then extracted, including the 150 kD band and related negative area, placed in a sterilized Eppendorf tube, and stored at −70°C for mass spectrometry analysis.

2.3. Component analysis of the 150 kD electrophoretic band by using LC-MS

LC-MS was performed by BGI-Tech (Shenzhen, China). Proteins were digested using trypsin (Pierce, US) at a ratio of 1:50 (w/w, trypsin/protein) for 20 hours at 37°C. Nano-LC-MS/MS experiments were performed on an HPLC system comprised of 2 LC-20AD nano-flow LC pumps, an aSIL-20 AC auto-sampler, and LC-20AB micro-flow LC pump (all Shimadzu, Tokyo, Japan) connected to an LTQ-Orbitrap mass spectrometer (Thermo-Fisher, San Jose, CA). The mass spectrometry data were matched

Table 1

Basic characteristics of subjects in schizophrenia, bipolar disorder, or depression and healthy controls.

Variables	Schizophrenia	Bipolar disorder or depression	Healthy controls
Age, years			
Mean ± SD	37.27 ± 12.4	38.14 ± 13.7	37.04 ± 10.7
Range	22–65	17–62	23–60
Gender			
Male	20	20	20
Female	10	10	10
Clinical types			
Manic	16		
Depression	14	13	
Bipolar disorder		17	

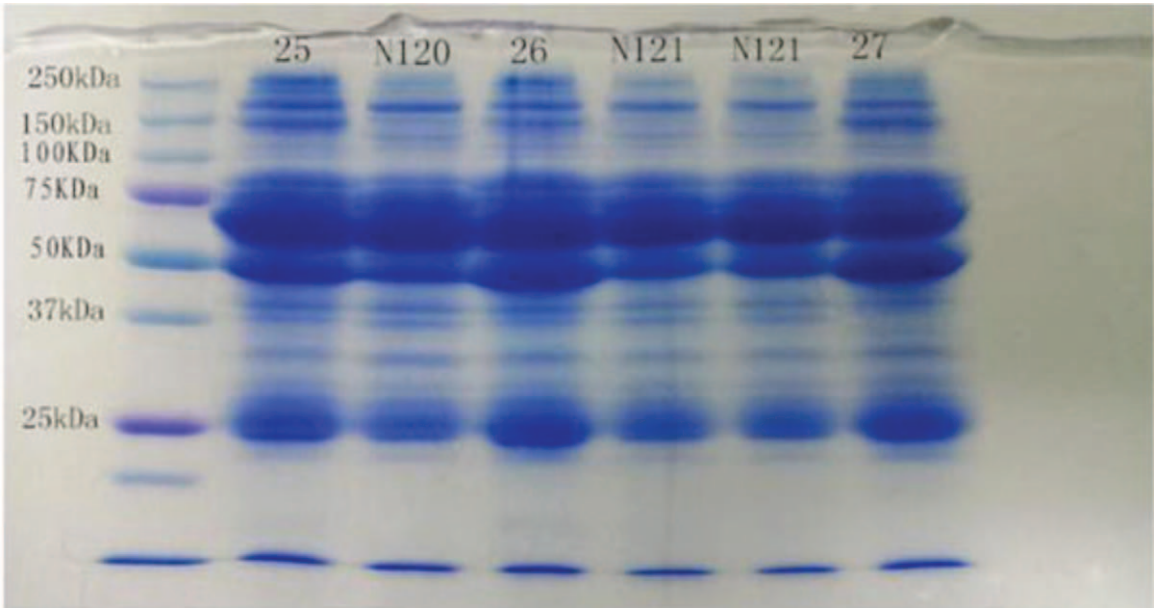

Figure 1. SDS-PAGE in patients with schizophrenia and healthy controls. M indicates a molecular protein marker with a scope of 10-250 kD. Samples 25, 26, 27, 41, and 42 obtained from patients with schizophrenia. Samples N120, N121 were obtained from healthy controls. SDS-PAGE = sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

with data simulated by the Swiss-Prot/UniProt database with Mascot 2.3.02 in order to obtain protein result. Gene ontology (GO), clusters of orthologous groups of proteins (COG), and Kyoto Encyclopedia of Genes and Genomes (KEGG) data were also retrieved and analyzed.

2.4. Molecular networks

In order to explore the biological function of proteins for which significant differences were identified, we utilized the Ingenuity Pathway Analysis (IPA) Knowledge Base (Ingenuity Systems, Redwood City, CA, www.ingenuity.com).

2.5. Statistical analysis

All statistical analyses were performed using SPSS Version 13 software (SPSS Inc., Chicago, IL). The non-parametric Kruskal–Wallis test was used to evaluate differences in the abundance of each protein among the 3 groups. The level of statistical significance was set at $P < .05$.

3. Results

3.1. The 150 kD electrophoretic protein band

See Figure 1.

3.2. Mass spectrometry of abnormal protein band

All 150 kD electrometric protein bands, including positive and negative regions, were analyzed using LC-MS. Differences in the density of each protein were noted (Table 2).

3.3. Molecular networks

In order to explore the biological function of the proteins for which significant alterations were observed, we utilized the IPA Knowledge Base. This platform enables researchers to visualize potential interactions between the molecules of interest and others that may not have been detected in a particular analysis. Using the IPA, we were able to determine that 8 of the 11 proteins

Table 2

Density of the 11 identified proteins in patients with schizophrenia, bipolar disorder, or depression and healthy controls.

		Total number of peptides detected			*T*-test (*P*-value)		
Protein accession number	Name	① Schizophrenia	② Mania or depression	③ Healthy control	①②	②③	①③
sp|08603	Complement factor H	206.25	201.43	124.75		*P* = .235	
sp|A6NN14	Zinc finger protein 729	1.0	0.43	0.25	*P* = .157	*P* = 1.000	*P* = .050
P14543–2	Nidogen-1 (isoform 2)	0.8	1.14	0.25		*P* = .256	
tr|A0RZB8	Diaphanous-1	0.5	0.14	0		*P* = .051	
tr|A8K8U1	cDNA FLJ77762	1.25	0.14	0		*P* = .123	
tr|A8K5T0	cDNA FLJ75416	157.37	48.57	59.12		*P* = .137	
tr|B4E1Z4	Complement factor B	9.5	4.71	5.62		*P* = .678	
tr|B4DZ36	cDNA FLJ58441	0.63	0.57	0.13		*P* = .357	
sp|P02458	Collagen alpha-1 (II) (isoform 1)	0.63	0.57	1.75		*P* = .763	
tr|Q59E99	Thrombospondin	42.38	40.86	39.5		*P* = .933	
sp|P08123	Collagen alpha-2 (I)	5	0.29	4.38		*P* = .568	

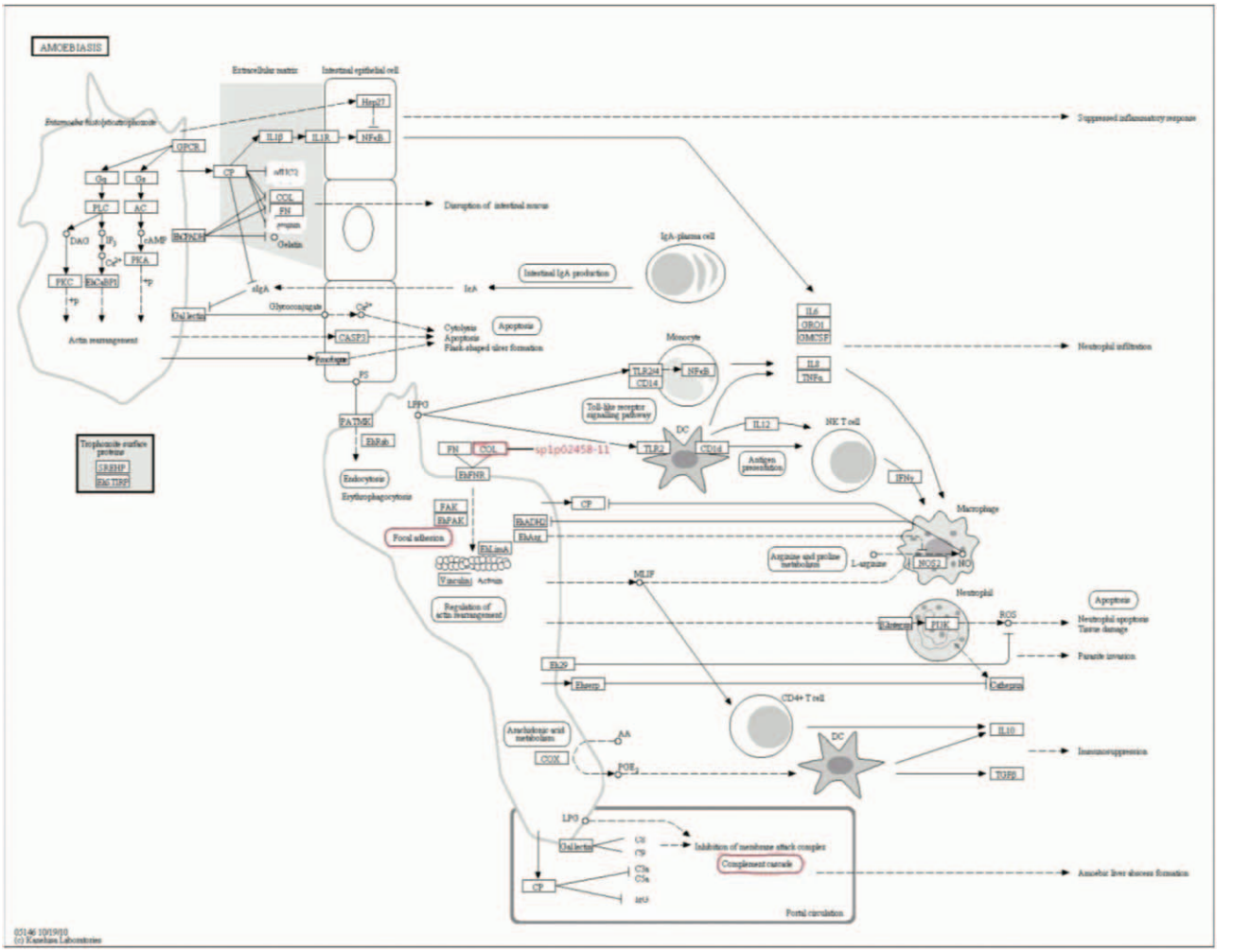

Figure 2. Amoebiosis. The amoebiosis included focal adhesion pathway, complement cascade pathway and isoform 1 of collagen alpha-1 (II) (sp|P02458).

identified in the schizophrenia cohort can be mapped onto a specific molecular network (see Figs. 2–4).

The significantly altered proteins as well as those proteins linked to them are not part of a single biological pathway, but rather take part in several biological functions such as focal adhesion, complement cascades, ECM-receptor interaction, and *Staphylococcus aureus* infection.

Our analysis revealed that BF, HF, THBS-1, and diaphanous-1 are key nodes in the network. Apart from ZNF729, isoform 2 of nidogen-1, and isoform 1 of collagen alpha-1 (II), a direct link was observed between each of the identified molecules, which may indicate an interaction between them.

4. Discussion

In the present study, a PEG precipitation method was utilized for large scale enrichment of serum immune complexes. Label-free relative quantitative proteomics analyses were used to assess the serum proteomic profiles of patients with schizophrenia, bipolar disorder, depression, and healthy controls.

The present study is the first to report the identification of a special 150-kD electrophoretic band in patients with schizophrenia, bipolar disorder, or depression relative to healthy controls. Analysis of the band using LC-MS resulted in identification of 11 serum proteins whose abundance was altered between patients and controls. Among them, eight proteins (CFH, CFB, cDNA FLJ75416, zinc finger protein 729, isoform 2 of nidogen-1, diaphanous-1, cDNA FLJ77762, and cDNA FLJ58411) were up-regulated, while one protein isoform 1 of collagen alpha-1 (II) was down-regulated in patients with schizophrenia, but only zinc finger protein 729 has statistics significance ($P < .05$). No differences were noted with regard to thrombospondin-1 or collagen alpha-2 (I) among the 3 groups. Our findings were further supported by ELISA validation in crude serum.

4.1. Zinc finger protein 729

Chromosome 19 contains members of several different ZNF gene subfamilies, though most of the clustered genes belong to the KRAB-ZNF subtype.[13] The function of the zinc fingers primarily involves the recognition of DNA and activation of transcriptional processes. In addition to DNA binding, domains containing C2H2 zinc fingers have been implicated in nuclear localization, protein-protein interaction, and DNA bending.[14]

Currently, more than 10 families of ZNFs have been identified. Though no data with regard to ZNF729 have been reported for schizophrenia, ZNF804A has been identified as a candidate gene for Schizophrenia (SCZ), ASD (autism spectrum disorders), and

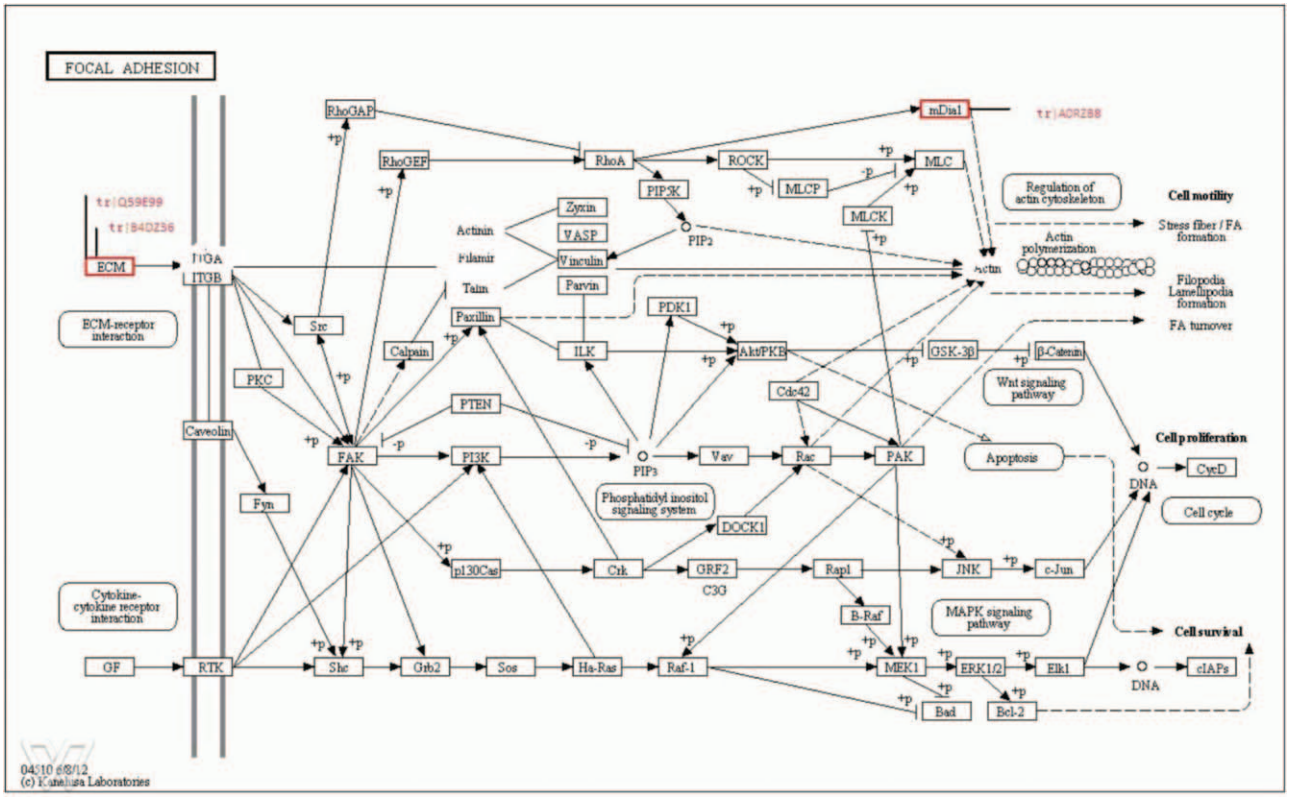

Figure 3. Focal adhesion pathway. The focal adhesion pathway outline above includes thrombospondin (tr|Q59E99), cDNA FLJ61587 (tr|B4DZ36), and diaphanous-1 (tr|A0RZB8).

BD (bipolar disorder) in replicated GWAS (genome wide association studies) and via CNV (copy number variation) analysis, although its function has not been well characterized.[15] Furthermore, Jaros et al[7] reported decreased phosphorylation of ZNF148 in patients with schizophrenia, though no changes in overall levels of ZNF148 were observed. We found that ZNF 729 obviously increased in schizophrenia than other groups. Further study will focus on ZNF729 to explore its function in schizophrenia etiology.

4.2. Complement factors B and H

Three of the 11 proteins for which significant alterations in abundance were observed (CFB, CFH, cDNA FLJ75416) belong to the complement super family. CFH, a regulator of complement activation, is involved in protection from thrombotic microangiopathies and advanced macular degeneration.[16] CFH competes with B or Bb for binding to C3b and accelerates the displacement of Bb from C3b, thereby inhibiting the formation of C3 convertase (C3bBb) in the alternative pathway.[17] Constitutive high expression of CFH has been detected in the eye, and a *CFH* polymorphism is strongly associated with age-related macular degeneration.[18] Individuals with a congenital deficiency of factor H are susceptible to microangiopathies,[19,20] and those with senile deficiency are susceptible to early development of advanced macular degeneration.[21,22] CFB is an enhancer of an alternative pathway of complement activation and is increased and augmented in many inflammatory diseases.

Nevertheless, the findings regarding the role of CFH and CFB in schizophrenia remain inconsistent. Singer et al[23] reported a significant increase in the frequency of the FB*F allele of CFB among patients with paranoid schizophrenia, while Rudduck et al[24] provided contrary data supporting a decrease in the frequency of this allele in familial schizophrenia. Furthermore, Yang et al[25] and Karine[26] reported increased plasma levels of CFB in schizophrenia, while Jaros et al[7] reported unchanged levels of CFH and increased levels of CFB. In the present study, we observed increases in CFH, CFB, and cDNA FLJ75416 in patients with schizophrenia. Such conflicting findings lead us to speculate that the methods utilized for serum preparation in some studies may have resulted in the depletion of some high-abundance proteins, selectively enriching results for phosphorylated proteins.

4.3. Thrombospondin-1 (THBS-1) and Nidogen-1

THBS-1 is a protein encoded by the *THBS1* gene in humans. Research has indicated that THBS-1 is involved in cell adhesion, signaling, proliferation, and angiogenesis.[27,28] Further reports suggest that THBS-1 is involved in tumorigenesis[29] and promotes the induction of immune regulatory cells.[30–32] In humans, 2 nidogen proteins, nidogen-1 (150 kD), and nidogen-2 (200 kD), have been identified. The 2 proteins share a 46% primary sequence identity and a similar three-dimensional structure, consisting of 3 globular domains (G1, G2, G3) connected by a flexible link and a rod.[33] The nidogens bind and form a ternary complex with laminin-1 and collagen type IV, connecting the 2 networks and stabilizing and maintaining the structure of the basement membrane.[34] Physiologically, nidogens have been shown to interact with cell receptor molecules and also control cell polarization, migration, and invasion.[35]

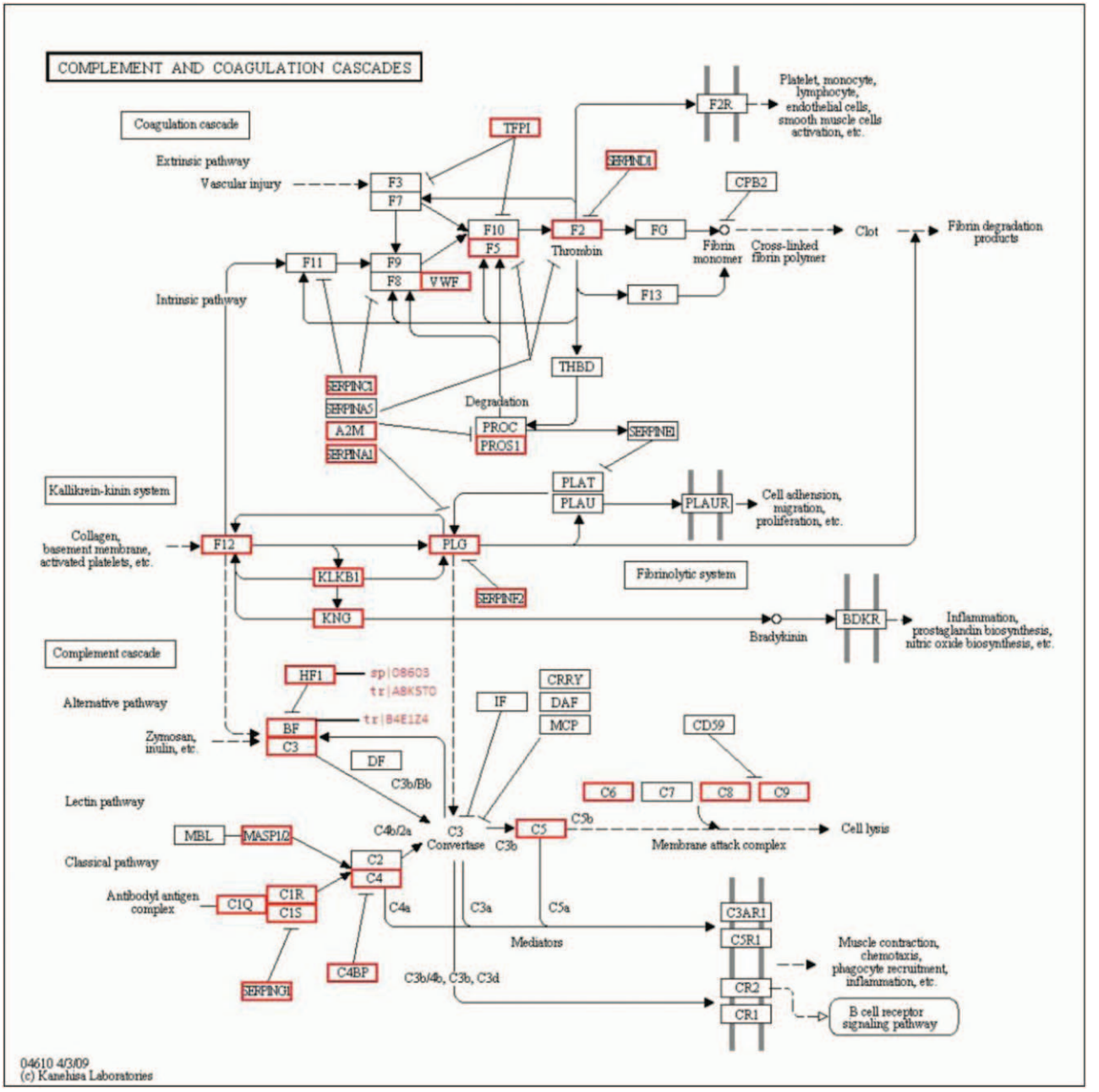

Figure 4. Complement cascade pathway. Complement factor H (sp|08603), cDNA FLJ75416 (tr|A8K5T0), and complement factor B (tr|B4E1Z4) are included in the pathway outlined above.

Schwarz et al[36] reported that THBS-1 was significantly increased at the time of relapse between the short- and long-term relapse groups in a study involving patients with schizophrenia. Jaros et al,[7,37] however, reported no change in levels of THBS-1 in patients with schizophrenia. In contrast, Katsel et al[38] reported that THBS-1 was significantly reduced in the anterior cingulate gyrus in patients with schizophrenia.

Though no data is available regarding serum nidogens in schizophrenia, Ning et al[39] observed that both THBS-1 and nidogen levels appear to increase over time following oxidative stress. Though we observed increases in levels of nidogens, our results indicated no changes in serum levels of THBS-1 in patients with schizophrenia, bipolar disorder, or depression when compared to healthy controls. Such differences may be due to the various tissues affected by and complex pathology of schizophrenia.

4.4. Collagen alpha-1 (II) and collagen alpha-2 (I)

Type I collagen is a heterotrimer consisting of 2 a1 (I) chains and one a2 (I) chain, encoded, respectively, by the COL1A1 and COL1A2 genes. The type I alpha 2 collagen gene, located on chromosome 7q22.1, is 38 kb in length and has 52 exons.[40,41] Collagen is the most abundant extracellular matrix protein in humans and is the major structural protein of skin, bone, tendon, ligaments, and the cornea.[42] Various studies have revealed that collagen is associated with intracranial aneurysm and its redisposition. Type II collagen forms fibrils with the same

periodicity in cartilage and the vitreous humor, and is a homotrimer composed of 3 α1 (II) chains, which show a high sequence homology to α1 (I).[43]

In the present study, we observed decreases in collagen alpha-1 (II) and collagen alpha-2 (I) chains in patients with schizophrenia, bipolar disorder, and depression when compared to controls. Though no previous data have been published with regard to collagen alpha-1 (II) and collagen alpha-2 (I) chains in schizophrenia, Doknic et al[44] reported that a potential long-term consequence of asymptomatic hypogonadism due to risperidone-induced hyperprolactinemia may cause a slight rise in the C-terminal telopeptide of type I collagen (CTX), particularly in patients with schizophrenia who have been treated with long-acting injectable risperidone (LAIR) for 12 to 24 months. Liang et al[45] also reported significantly increased levels of the amisulpride group in patients with schizophrenia after 4 weeks of antipsychotic treatment.

4.5. Diaphanous-1

Mammalian diaphanous-related formin (mDia1, diaphanous-1) is a regulator of linear actin polymerization and mediator of Ras homolog family members in mammals. Loss of this protein results in aberrant myeloid differentiation and hyperactivity of the immune system.[46] In the present study, we observed significant increases in diaphanous-1 in patients with schizophrenia, though further research is required in order to elucidate the potential role of this decrease in the pathology of the disorder.

4.6. Changes in cDNA FLJ77762 and cDNA FLJ58441

Research has confirmed that cDNA FLJ77762 is highly similar to cullin-associated and neddylation-dissociated 1 (CAND1) mRNA in humans. CAND1, a negative regulator of SKP1-Cullin1-F-box (SCF) ubiquitin ligases,[47] interacts with cullin in a mutually exclusive fashion via substrate adaptor binding and neddylation.[48]

On the other hand, cDNA FLJ58441 is highly similar to attractin. Attractin has a collagen-binding domain and, similar to CD26, is co-stimulatory for T cells to recall antigens.[49] Increasing experimental evidence indicates that attractin plays a role in the regulation of immune responses.[50] Furthermore, higher levels of these proteins have been observed in patients with schizophrenia, bipolar disorder, and depression patients than in controls.[51] cDNA FLJ58441 in our research also had a higher level as above, which indicated that such patients may exhibit abnormal immune system function.

In general, a special 150-kD electrophoretic band with high positive rate in patients with schizophrenia, bipolar disorder, or depression relative to healthy controls was found, and 11serum proteins whose abundance was altered between patients and controls were analyzed with LC-MS for it.

In conclusion, a special 150-kD electrophoretic band in patients with schizophrenia, bipolar disorder, or depression relative to healthy controls was found. In this band, the abundance of 11 serum proteins were altered between patients and controls. Even though only zinc finger protein 729 has statistics significance, other proteins maybe also have, because the case is seldom and the result information was not made best of due to the statistics method. Also these proteins take part in amoebiosis pathway, which includes focal adhesion, complement cascades, ECM-receptor interaction, and *Staphylococcus aureus* infection. In the upcoming work, we will increase sample size and identify these protein function in animal experiments to explore the pathogenesis of schizophrenia.

5. Conclusion

The present study suggests that there is a special 150-kD electrophoretic band in patients with schizophrenia, bipolar disorder, or depression relative to healthy controls (each 30 samples) with a combination of 7% PEG enrichment of immune complexes and SDS-PAGE. Analysis of the band using LC-MS resulted in identification of 11 serum proteins whose abundance was altered between patients and controls. Among them, 8 proteins (CFH, CFB, cDNA FLJ75416, zinc finger protein 729, isoform 2 of nidogen-1, diaphanous-1, cDNA FLJ77762, and cDNA FLJ58411) were upregulated, while one protein (isoform 1 of collagen alpha-1 (II) was downregulated in patients with schizophrenia, but only zinc finger protein 729 has statistics significance ($P < .05$). No differences were noted with regard to thrombospondin-1 or collagen alpha-2 (I) among the 3 groups. These proteins take part in several biological functions such as focal adhesion, complement cascades, ECM-receptor interaction, and *Staphylococcus aureus* infection. The 150-kD electrophoretic band on zinc finger protein 729 may become biomarkers in patients with schizophrenia. In the future, increasing sample size and function research of zinc finger protein 729 should be executed continuously.

Author contributions

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Writing – review & editing: Ruihuan Xu.

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