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Original Article

Bioinformatic analysis of proteomic data for iron, inflammation, and hypoxic pathways in restless legs syndrome

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ABSTRACT

Objective/Background: We performed bioinformatic analysis of proteomic data to identify the biomarkers of restless legs syndrome (RLS) and provide insights into the putative pathomechanisms, including iron deficiency, inflammation, and hypoxic pathways.

Patients/methods: Patients with drug-naïve idiopathic RLS were recruited at a university hospital from June 2017 to February 2018. Serum samples from patients with RLS (n = 7) and healthy sex- and age-matched controls (n = 6) were evaluated by proteomic analysis. For differentially expressed proteins (DEPs) in patients with RLS, compared to those in controls, the expression profiles and protein–protein interaction (PPI) network were characterized between dysregulated proteins and extracted proteins involved in iron deficiency, hypoxia, and inflammation responses using the String database (http://string-DB.org). The PPI network was visualized by Cytoscape ver. 3. 7. 1. Statistical analyses of the validation Western blot assays were performed using a Student's t-test.

Results: Interactome network analysis revealed a relationship among the eight proteins, their associated genes, and 150, 47, and 11 proteins related to iron deficiency, inflammation, and hypoxic pathways, respectively. All DEPs were well associated with inflammation, and complement 3, complement C4A, alpha-2 HS glycoprotein, and alpha-2 macroglobulin precursor were found to be in hub positions of networks involved in PPIs including iron deficiency, hypoxia pathway, and inflammation. C3 and C4A were verified using western blotting.

Conclusions: We identified key molecules that represent the selected cellular pathways as protein biomarkers by PPI network analysis. Changes in inflammation can mediate or affect the pathomechanism of RLS and can thus act as systemic biomarkers.

1. Introduction

Restless legs syndrome (RLS) is a sensorimotor disorder with established essential and clinical specifiers according to the International Restless Legs Syndrome Study Group (IRLSSG) [1]. However, its assessment is based mainly on self-reporting and no reliable diagnostic biomarkers have been identified. Although the pathomechanism remains unclear, dopaminergic dysfunction with

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brain iron deficiency, inflammation, and central nervous system (CNS) hypoxia are thought to be important contributors to the pathophysiology of idiopathic RLS [2].

For the last decade, mass spectrometry (MS)-based proteomic technologies have been used to measure protein expression, and advanced comparative proteomic analysis of samples from patients and healthy control subjects is commonly applied to identify protein biomarker candidates, which are typically differentially expressed proteins (DEPs) compared to those in healthy controls. This analysis has also been applied to several sleep disorders such as REM sleep behavior disorder, circadian rhythm disorder, and obstructive sleep apnea [3–5]. Recently, numerous studies reported that cellular pathways can serve as better indicators of the disease state than individual molecules, and pathway enrichment analysis provides a list of cellular pathways enriched by the DEPs. A set of cellular pathways related to pathophysiological processes can be selected; network analysis for this subset of DEPs enables effective identification of key molecules that represent the selected cellular pathways as protein biomarker candidates [6]. In this study, we first selected the DEPs of patients with idiopathic RLS compared to expression in healthy controls and then identified key candidate biomarkers through network analysis between DEPs and extracted proteins involved in the pathophysiological process of RLS, including iron deficiency, hypoxia, and inflammation.

2. Methods

2.1. Study participants

Patients with unmedicated idiopathic RLS (iRLS) were recruited at the outpatient clinic of a CHA university hospital from June 2017 to February 2018. The study was approved by the CHA medical center Institutional Review Board. All patients were diagnosed by face-to-face interview with a neurologist in the outpatient neurological clinic. Participants were included if they (1) met the IRLSSG diagnostic criteria [1] and (2) were 30 years of age or older and 45 years of age or younger at the time of diagnosis. Patients were excluded if they had (1) obstructive sleep apnea on polysomnography (PSG) (apnea hypopnea index >10/h), (2) other specific sleep disorders (e g narcolepsy, REM sleep behavior disorder), (3) secondary causes of RLS including iron deficiency, pregnancy, neuropathy, multiple sclerosis, or renal failure, or took medications that caused or exacerbated RLS, (4) disorders with symptoms similar to those of RLS, such as positional discomfort, leg cramps, essential tremor, Parkinson disease, neuroleptic-induced akathisia, vascular claudication, neurogenic claudication, myelopathy, and arthritis, (5) those who had been administered any medication, and other medical disorder. To assess the severity of RLS and evaluate sleep quality, we used the Korean version of the International Restless Legs Syndrome Rating Scale (IRLS) scores [7], insomnia severity index (ISI) [8] and Pittsburg sleep quality index (PSQI) [9]. Age- and gender-matched healthy participants served as controls.

2.2. PSG and blood sampling

All participants were examined by PSG when not under any treatment for RLS. PSG assessments included electroencephalogram (C3/A2, C4/A1, F3/A2, F4/A1, O1/A2, and O2/A1), left and right electro-oculograms, submental electromyogram (EMG), superficial EMG of both the anterior tibialis muscles, and electrocardiogram. Airflow (nasal flow pressure sensor and oronasal thermistor), chest and abdominal breathing efforts, and transcutaneous oximetry were monitored in all patients in a single overnight session. Sleep stage was scored in 30-s epochs according to standard criteria described by the American Academy of Sleep Medicine manual for scoring sleep [10]. Periodic leg movements were scored according to the World Association of Sleep Medicine/IRLSSG criteria [11]. Briefly, limb movements were scored if they were 0.5–10 s long and had an EMG amplitude of 8 μV or more above the resting EMG; periodic limb movements were scored if the limb movements occurred as part of a series of four or more, with 5–90 s between each movement in a series. In the morning after the test, overnight fasting venous blood was collected between 7:00 and 8:00 h to examine hemoglobin, blood urea nitrogen, creatinine, aspartate aminotransferase, alanine aminotransferase, ferritin, iron, total iron binding capacity, C-reactive protein, vitamin D levels. For proteomic analysis, the plasma was separated by centrifugation, aliquoted, and stored frozen at −80 °C.

2.3. Proteomic analysis

2.3.1. Depletion of major abundance proteins with an immunoaffinity column

Depletion of major abundance proteins was carried out essentially as described [12]. The six most abundant proteins (ie albumin, transferrin, IgG, IgA, haptoglobin, and antitrypsin) in the serum were depleted using a MARC (Agilent Technologies, Santa Clara, CA, USA). A 4.6 × 100-mm MARC with binding capacity for 20 μL of human plasma was used. Chromatographic separation of the abundant proteins by MARC was carried out using a mobile phase reagent kit according to a standard liquid chromatography (LC) protocol provided by the manufacturer. Briefly, crude human serum samples were diluted five-fold with Buffer A containing protease inhibitors (COMPLETE™, Roche, Basel, Switzerland) and filtered through 0.22-μm spin filters by centrifugation at 16 000×*g* at room temperature for 1–2 min. The sample was injected, and flow-through fractions were collected and stored at −20 °C until use. To resolve depleted plasma proteins on 2-D gels, the flow-through fractions from MARC were pooled and precipitated with a pre-cooled solution of 10% trichloroacetic acid for 1 h at −20 °C. After washing with ice-cold acetone, the pellets were resolubilized in the sample buffers of two-dimensional electrophoresis (2-DE) and FFE.

2.3.2. Two-dimensional electrophoresis and image analysis

2-DE and image analyses were carried out essentially as described previously [13]. Aliquots in sample buffer (7 M urea, 2 M thiourea, 4.5% CHAPS, 100 mM DTE, 40 mM tris, pH 8.8) were applied to immobilized pH 3–10 nonlinear gradient strips (Amersham Biosciences, Uppsala, Sweden). Isoelectric focusing was performed at 80 000 Vh. The second dimension was analyzed on 9–16% linear gradient polyacrylamide gels (18 cm × 20 cm × 1.5 mm) at constant 40 mA per gel for approximately 5 h. After protein fixation in 40% methanol and 5% phosphoric acid for 1 h, the gels were stained with Coomassie brilliant blue G-250 for 12 h. The gels were stained with H_2O, scanned in a Bio-Rad (Hercules, CA, USA) GS710 densitometer, and converted into electronic files. Spot matching and quantitation were determined by the Image Master Platinum 5.0 image analysis program (Amersham Biosciences). We used a super control, the mixture of plasma from random three samples among six normal samples, and the 2-DE analysis was performed with two different replicates to identify proteins that were differentially expressed.

2.3.3. In-gel tryptic digestion

2-DE spots of interest for analysis were excised from the preparative gel, and the spots were transferred into each 1.5-mL tube. The band was washed with 100 μL of distilled water; then, 100 μL of 50 mM NH_4HCO_3 (pH 7.8) and acetonitrile (6:4) were added to the band and the sample was shaken for 10 min. This process was repeated at least three times until the Coomassie brilliant blue

Table 1
Clinical characteristics of patients with idiopathic restless legs syndrome.

	Patients (n = 7)
Age, year old, mean (range)	39 (34–44)
Onset age of disease, mean (range)	28 (17–40)
Familial history (/total n)	1/7
Laboratory, mean, (range)	
Iron (μg/dL)	79.1 (36–160)
Ferritin (ng/mL)	34.6 (4.2–96.3)
TIBC (μg/dL)	170 (169–328)
CRP (mg/dL)	0.1 (0.03–0.39)
Vitamin D (ng/mL)	21.5 (11.01–32.89)
Questionnaires, mean (range)	
K–ISI	12.1 (6–25)
K-PSQI	10.6 (7–15)
K-IRLS	27 (20–33)
Polysomnography	
PLMSI, mean ± SD (/h),	9.6 ± 10.3

K–ISI, Korean version of insomnia severity index; PSQI, Pittsburg sleep quality index; IRLS, International Restless Legs Syndrome Rating Scale; PLMSI, periodic limb movement during sleep index; SD, standard deviation.

G250 dye disappeared. The supernatant was decanted, and the spot was dried in a speed vacuum concentrator (LaBoGeneAps, Lynge, Denmark) for 10 min and then digested with Pierce trypsin protease, MS grade (Thermo Scientific; enzyme to substrate ratio = 1:50) at 37 °C with shaking for 16 h.

2.3.4. LC-MS/MS for peptide analysis

Nano LC-MS/MS analysis was performed with an Easy n-LC (Thermo Fisher Scientific, Waltham, MA, USA) and LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific) equipped with a nano-electrospray source. Samples were separated on a C18 nanobore column (150 × 0.1 mm, 3 μm pore size; Agilent Technologies). The mobile phase A for LC separation was 0.1% formic acid and 3% acetonitrile in deionized water and mobile phase B was 0.1% formic acid in acetonitrile. The chromatography gradient was designed to linearly increase from 0% B to 60% B in 9 min, 60% B to 90% B in 1 min, and 3% B in 5 min. The flow rate was maintained at 1800 nL/min. Mass spectra were acquired by data-dependent acquisition with a full mass scan (380–1700 *m*/*z*)

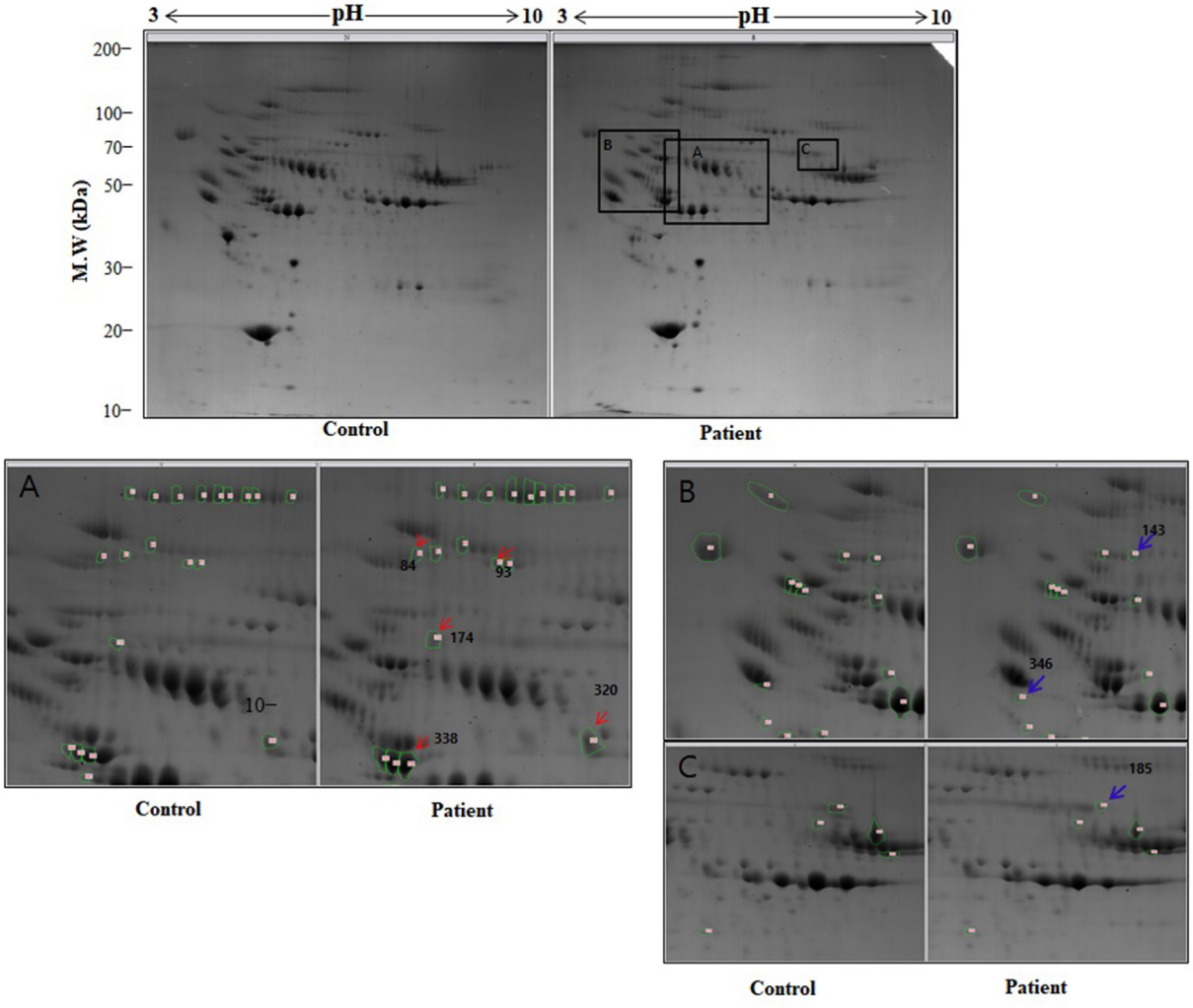

Fig. 1. Representative 2-DE images of serum proteins from a control and patient with idiopathic restless legs syndrome. Concomitantly expressed proteins between the control and patient are encircled in green. Eight protein spots (red and blue arrow) were determined to be overexpressed in 2-DE analysis based on a more than two-fold relative increase and decrease in volume intensity compared to that in the RLS patient and healthy control. Proteins were identified by MALDI-TOF-MS. Upregulated differentially expressed proteins (DEPs) are marked with red arrows (A) and downregulated DEPs are marked with blue arrows (B, C) in the patient. 174, Alpha-2 macrogrobulin; 84, PK-120 precusor; 93, complement C3; 338, chain A, vitamin D binding protein; 320, beta-2-glycoprotein I apolipoprotein H; 143, complement C4A, 346, alpha-2 HS glycoprotein and 185, coagulation factor XII.

followed by 10 MS/MS scans. For MS1 full scans, the orbitrap resolution was 15 000 and the automatic gain control was 2×10^5. For MS/MS in the linear trap quadrupole, the automatic gain control was 1×10^4.

2.3.5. Database searching

The MASCOT algorithm (Matrix Science, Boston, MA, USA) was used to identify peptide sequences present in a protein sequence database. Database search criteria were as follows: taxonomy, *Homo sapiens*; fixed modification, carbamidomethylated at cysteine residues; variable modification, oxidized at methionine residues; maximum allowed missed cleavage, 2; MS tolerance, 10 ppm; MS/MS tolerance, 0.8 Da. The peptides were filtered with a significance threshold of $P < 0.05$.

2.4. Bioinformatics and network analysis

Gene ontology (GO) enrichment analysis was performed using the DAVID bioinformatics resource (https://david.ncifcrf.gov/; PMID: 19131956). Human proteins associated with iron deficiency, hypoxia, and inflammation were extracted from the GO resource (http://geneontology.org/). The total list of proteins directly and indirectly related to DEPs based on protein–protein interactions was obtained from the String version 11.0 database (https://string-db.org/; PMID: 25352553). Construction of the network between DEPs and proteins included in three biological processes was performed using Cytoscape version 3.7.1 software (PMID: 14597658).

2.5. Western blot analysis

Serum samples were separated on 7% SDS-PAGE gels and were transferred to a PVDF membrane (Millipore, Darmstadt, Germany). The membrane was blocked in 5% skim milk (BD, San Jose, CA, USA) in TBS-T buffer (0.05% (v/v) tween-20 in tris-buffered saline) and was incubated with primary antibodies including those against complement C3 (sc-28294), AHSG (sc-133146; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), complement C4A (GTX110413; GeneTex, Irvine, CA, USA), or A2M (ab36995; Abcam, Cambridge, UK). After washing with TBS-T, the membrane was incubated with horseradish peroxidase-conjugated secondary antibodies. We developed Western blot membranes with enhanced chemiluminescence solution and images were captured with a luminescent image analyzer AmershamTM Imager 680 (GE Healthcare, Chicago, IL, USA). The quantification of band intensity on the blot was analyzed with a software program, namely ImageQuant TL (GE Healthcare, Chicago, IL, USA). To ensure equal gel loading, equal amounts (30 μg) of serum samples were separated using 7% SDS-PAGE gels and stained with Coomassie blue stain (Supplementary Fig. 1).

3. Results

3.1. Characteristics of participants

Table 1 shows clinical characteristics of iRLS patients. Eight patients with iRLS were enrolled, and one patient who had severe anemia was excluded. The mean age was 39 years (range, 34–44). All patients were classified as having the early onset form of RLS (age of onset < 45 years), and one patient had a family history of RLS. All patients had severe RLS with poor sleep (PSQI > 5), and one patient had severe insomnia (ISI, 25). In PSG, six of seven patients had periodic limb movements during sleep (mean ± SD, 9.6 ± 10.3/h), and only one patient had pathological PLMS (defined as PLMI > 15/h) that was 30/h.

3.2. Differential protein expression profile

Serum samples were collected from all patients with iRLS and six healthy controls (all female, mean age 39 years, range 34–43) and used for proteomic analysis. A total of 492 proteins with concomitant quantitative data were identified. When protein

Table 2
Identified differentially expressed proteins in patients with iRLS compared to healthy controls by mass analysis.

Acc. number	Protein name	Gene	Score[a]	Matched peptides no.	Sequencing coverage (%)	Fold change[b]	Theoretical MW/pI	Function
Gi|177870	Alph-2 macroglobulin precursor	A2M	220	5	3	+2.8	164600/6.00	Blood coagulation, intrinsic pathway, negative regulation of complement pathway, platelet degranulation
Gi|179674	Complement C4A	C4A	820	19	5	−2.4	194337/6.65	Cellular protein metabolic process, complement activation, inflammatory response, innate immune response
Gi|179665	Complement C3	C3	149	4	1	+2.7	188585/6.02	Amyloid-beta clearance, complement activation, inflammation response, neurophil regulation, regulation of immune response
Gi|28810	Beta-2-glycoprotein I apolipoprotein H	APOH	186	9	5	+3.9	39598/8.34	Blood coagulation, native regulation of endothelial cell migration, proliferation, platelet degranulation
Gi|1402590	PK-120 precusor	ITIH4	763	18	14	+3.9	103521/6.51	Platelet degranulation, response to cytokine, inflammation response
Gi|180357	Coagulation factor XII	F12	40	1	1	−4.3	70055/8.04	Blood coagulation, innate immune response, fibrinolysis, proteolysis
Gi|18655424	Chain A, Vitamin D binding protein	GC	624	57	24	+3.5	54612/5.40	Involved in vitamin D transport and storage, scavenging of extracellular G-actin, enhancement of the chemotactic activity of C5 alpha for neutrophils in inflammation and macrophage activation.
Gi|112910	Alpha-2 HS glycoprotein	AHSG	194	9	11	−3.5	40098/5.43	Cellular protein metabolic process, platelet aggregation, regulation of inflammation response

[a] Score is −10xLog(P), where *P* is the probability that the observed match is a random event; it is based on the NCBInr database using the MASCOT searching program as MS/MS data. b. Average fold changes of proteins: level of protein spot expression change calculated as ratio between the volume intensity in RLS patients and controls: (−) decrease in RLS, (+) increase in RLS.

expression levels in the serum from seven patients with iRLS were compared to those of the six healthy controls, eight proteins that were differentially expressed according to 2-DE analysis based on the relative fold-increase or fold-decrease based on volume intensity ($p \leq 0.05$, Av. Ratio ≥ 2.0) were identified using MALDI-TOF-MS. The expression level was determined by examining the ratio of the relative spot volume of a protein in the gel (Fig. 1). Table 2 shows the identified DEPs.

3.3. Enrichment pathway and network analysis

Fig. 2 shows the protein–protein interaction networks related to the DEPs, along with their associated genes and extracted genes from iron deficiency, inflammation, and hypoxia pathways. Complement 3 (C3), complement 4A (C4A), alpha-2 HS glycoprotein (AHSG), alpha-2 macroglobulin precursor (A2M) were at a hub position in the networks. C3, C4A, AHSG were mainly related to iron deficiency through genes including transferrin, transferrin receptor 2, and ceruloplasmin (Fig. 2A). In the hypoxia pathway, there was no gene directly connecting our DEPs. When we expanded the GO terms extracted from the hypoxia pathway, the DEPs were related to the hypoxia process through a gene for cytochrome B-245 beta chain (*CYBB*), nitric oxide synthase 3 (*NOS3*), and signal transducer and activator of transcription 3 (*STAT3*) (Fig. 2C). C3 and C4A had more interaction with hypoxic pathway than other proteins. All DEPs, particularly C3, C4A, AHSG and A2M, interacted with genes related to the inflammatory process (Fig. 2B).

3.4. Western blot analyses

Western blot validation was performed on four key DEPs including C3, C4A, AHSG, and A2M, from the network analysis. C3 and C4A were validated by Western blot analyses. As shown in Fig. 3, C3 was elevated ($p < 0.05$) and C4A was decreased in RLS patients ($p < 0.005$) compared to levels in controls. A2M and AHSG were detected in both groups and there was no significant alteration in their expression levels (Supplementary Fig. 2).

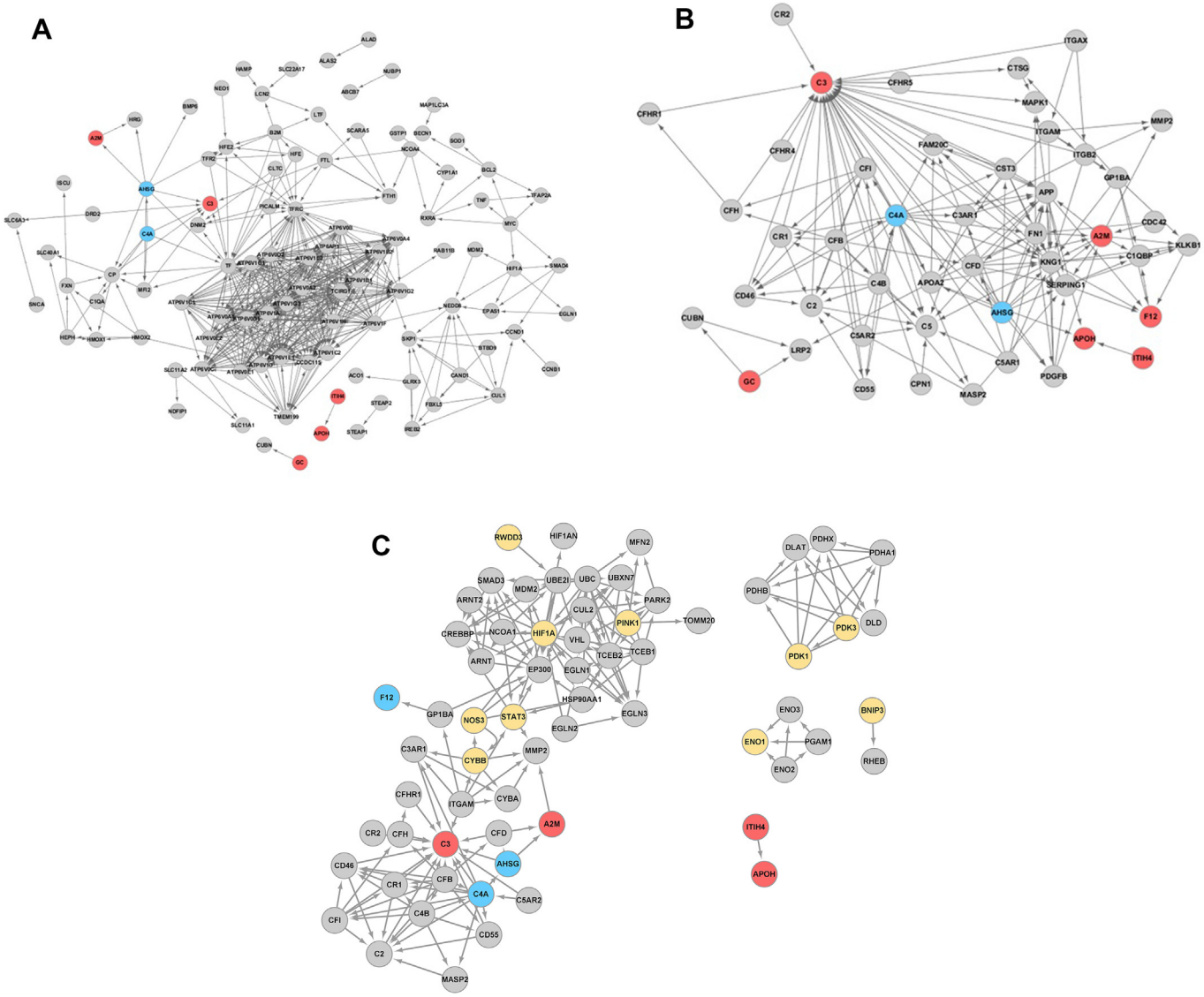

Fig. 2. Protein–protein interaction networks between DEPs, along with extracted genes from iron deficiency (A), inflammation (B), and hypoxia pathways (C). In the hypoxia pathway, there was no gene directly connecting with DEPs, and as we expanded the GO terms extracted from the hypoxia pathway, the DEPs were related to the hypoxia process (C). DEP, differentially expressed protein; red = upregulated DEP; blue = downregulated DEP; yellow = expanded proteins of hypoxic pathway.

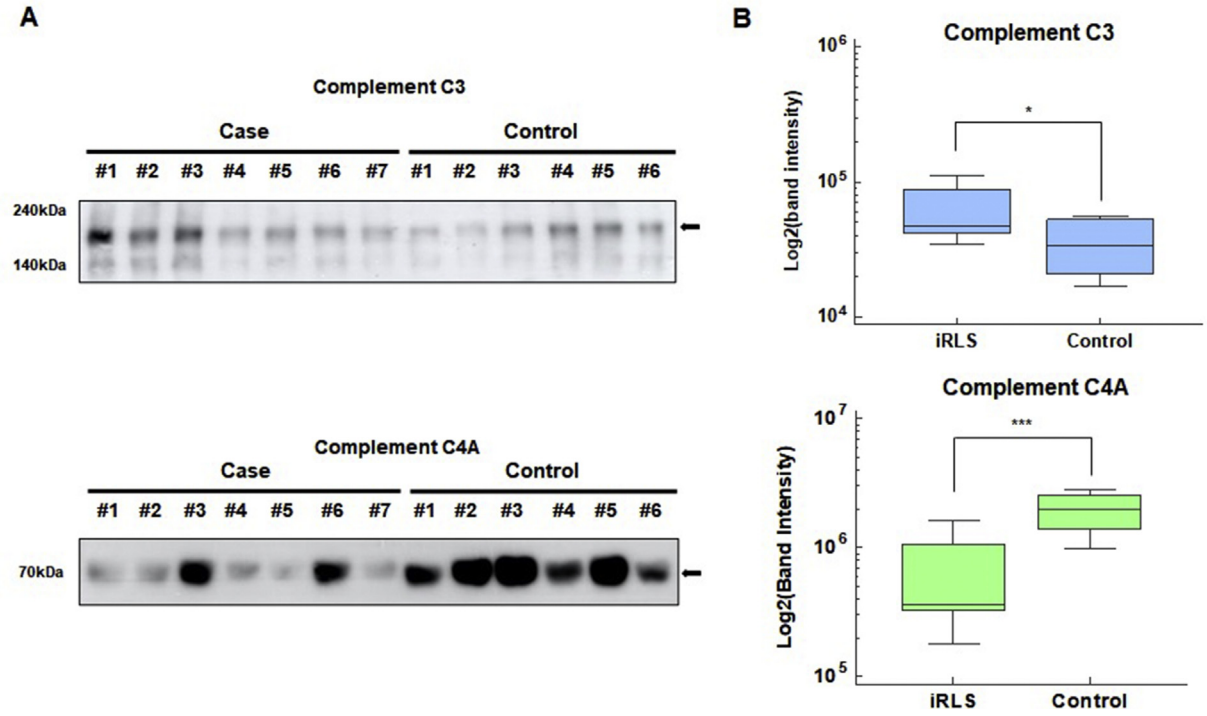

Fig. 3. Western blot analysis of complement C3 (C3) and complement C4 (C4A) for validation. (A) Western blot analysis. (B) Boxplot of the densitometrically determined C3 and C4A expression levels. Statistical analysis was performed using a student t-test (n = 13, p-value < 0.05, *; p-value < 0.005, ***). iRLS, idiopathic restless legs syndrome.

4. Discussion

We identified key protein biomarkers for diagnosis using a network-based approach though protein enrichment pathway analysis, which is a new approach for studying RLS. The eight DEPs mainly function in inflammation and the immune response. To identify hub-like DEPs as biomarker candidates, we analyzed protein–protein interactions between DEPs and disease-specific proteins related to iron deficiency, inflammation, and the hypoxic pathway though functional enrichment analysis. DEPs strongly interacted with the iron and inflammation pathways, and we identified C3, C4A, AHSG, and A2M as candidate biomarkers.

The complement system is part of the innate immune system, plays a key role in regulating inflammation, and is particularly important in the activation of complement component C3 and C4 [14]. In two recent proteomics studies of RLS, inflammation-related proteins were detected as DEPs, and one study showed dysregulation of C3 and C4A [15,16]. Inflammation can contribute to or exacerbate RLS in association with CNS iron deficiency. Weinstock et al. suggested that increased expression of proinflammatory cytokines, such as interleukin (IL)-6 and lipopolysaccharides, increased small intestinal bacterial overgrowth by stimulating the production of hepcidin, the main hormone involved in iron level regulation [17]. Clinically, the incidence and prevalence of RLS in patients with inflammatory bowel disease was found to be approximately 2.5-fold (20–43%) higher than that in the control group [18,19]. Moreover, several studies indicated the contribution of chronic inflammation status related to iron dysregulation of the CNS, and they investigated biomarkers of peripheral inflammation in RLS. In these studies, C-reactive protein (CRP) with IL-6 [20], the neutrophil/lymphocyte ratio [21], and the CRP/albumin ratio [22] were significantly higher in the RLS group than in the non-RLS group.

Conversely, iron deficiency can alter inflammation and immunity. The exact effects or mechanism of action of iron deficiency on the inflammatory and immune systems are not yet known, but some authors have suggested that altered levels of ILs and cytokines might lead to the impairment of both systems in patients with iron deficiency [23–25]. Previously, C3 and C4 were detected at low levels in fetuses and during pregnancy under iron-deficient conditions in the mother [26,27].

Considering that altering peripheral inflammation could cause or affect iron deficiency, C3 and C4A levels could potentially be used as diagnostic or prognostic markers of RLS. We also determined the expression level of C3 and C4A by Western blot analysis and found that C3 was increased and C4A was decreased in RLS patients relative to levels in a healthy person. Further, it is important to investigate the relationship between the degree of the altered level of inflammation and disease severity and augmentation with regards to the duration of RLS.

Brain iron deficiency is associated with important biologic mechanisms in RLS and can alter the dopaminergic state of the CNS to an adenosinergic state. This alteration in the adenosinergic system can cause downregulation of A1R and upregulation of A2R along with disruption of the adenosine-dopamine-glutamate balance uniquely controlled by adenosine and dopamine receptor heteromers in the striatum [28]. However, PPI network analysis did not reveal any relationship among dopamine, adenosine, and DEPs in the iron deficiency pathway. As serum samples were used for proteomic analysis, DEPs might be represented as systemic biological changes of an altered CNS system in RLS. Additionally, MS-based proteome analysis can only be performed for a limited number of proteins and shows reduced accuracy in detecting proteins at lower concentrations. Alterations in protein abundance, associated with inflammation, are commonly observed in several other diseases [6]. Moreover, the sample size in this study was small. Based on these factors, the findings herein should be

interpreted with caution and further confirmation is required based on larger sample studies.

AHSG belongs to the cysteine protease inhibitory factor superfamily and is mainly secreted from the liver. Previously, AHSG has been shown to be inversely associated with atherosclerosis and inflammation [29–31]. More recently, AHSG was shown to be a risk factor of ischemic stroke using proteomic analysis and AHSG polymorphism genotyping [32,33]. A2M, encoded by this gene, is a protease inhibitor and cytokine transporter. It can also bind cytokines including TNF-α, IL-1β, and IL-6. IL-6 is an inflammatory cytokine and plays a crucial role in extracellular matrix deposition and reorganization, which leads to atherosclerosis. In a study using proteomic analysis and enzyme linked immunosorbent assays, A2M was found to be positively correlated with higher vulnerability to carotid plaques [34]. A well-established large prospective cohort study in women and men showed that RLS was significantly associated with a higher risk of cardiovascular (CVD) mortality [35,36]. The mechanism by which RLS caused predisposition to CVD might be the increased activation of inflammatory cytokines such as C-reactive proteins, leading to higher blood pressure and causing sympathetic activation associated with dopamine deficiency [37]. Decreased AHSG with increased A2M would act as valid biomarkers to elucidate the relationship between RLS and CVD and could be used to predict the risk of CVD in RLS.

Proteins related to the hypoxic pathway and DEPs formed an indirect connection through CYBB, STAT3, and NOS3. CYBB is a member of a group of proteins that forms an enzyme complex known as NADPH oxidase, which plays an essential role in the immune and inflammation system [38], and STAT3 is a member of the cytoplasmic protein family that is activated by a large number of extracellular stimuli including IL-6, various cytokines, granulocyte colony stimulating factor (G-CSF), epidermal growth factor, and IL-10 [39]. The relationship between hypoxia and inflammation has been clarified over the last decade, centering on the activity of hypoxia-inducible factors [40]. The endogenous production of nitric oxide (NO), particularly in the cardiovascular system, is mainly dependent on the activity of the enzyme endothelial NO synthase (NOS3) [41]. Several data suggest that oxidative stress and nitric oxide (NO) could play a role in the pathophysiology of RLS [42]. The hypoxia pathway might also be related to RLS based on findings of an exaggerated inflammation response, increased vascular endothelial growth factor, increased tyrosine hydrolase, and alterations in iron regulation [28].

Although proteomic analyses were performed using a small number of samples, pathway enrichment analysis in RLS acts as a better indicator of the disease compared to that with examining individual molecules. Moreover, we verified C3 and C4A among key DEPs using Western blot analysis. With recent advances in high-resolution peptide separation and high-performance MS, functional research such as molecular-based studies is necessary for RLS. This is the first study to perform functional enrichment analysis using proteomics to promote further functional research. Our results might contribute to the development of diagnostic biomarkers and therapeutic strategies.

CRediT authorship contribution statement

Jung-Won Shin: Conceptualization, Investigation, Writing - original draft, Project administration. **Jung-hun Lee:** Software, Formal analysis. **Hyeyoon Kim:** Validation, Resources. **Da-Hye Lee:** Methodology. **Kwang-Hyun Baek:** Methodology, Resources. **Jun-Sang Sunwoo:** Conceptualization. **Jung-Ick Byun:** Data curation, Writing - review & editing. **Tae-Joon Kim:** Writing - review & editing. **Jin-Sun Jun:** Data curation, Writing - review & editing. **Dohyun Han:** Data curation, Supervision. **Ki-Young Jung:** Writing - review & editing, Supervision, Project administration.

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Conflict of interest

None declared.

The ICMJE Uniform Disclosure Form for Potential Conflicts of Interest associated with this article can be viewed by clicking on the following link: https://doi.org/10.1016/j.sleep.2020.09.002

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.sleep.2020.09.002.

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