Proteomic analysis of human serum from patients with temporal lobe epilepsy

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= Abstract =

Purpose: Epilepsy affects more than 0.5% of the world’s population. It has a large genetic component and is caused by electrical hyperexcitability in the central nervous system. Despite its prevalence, the disease lacks definitive diagnostic serological biomarkers. To identify potential biomarkers for epilepsy by a convenient method, we analyzed the expression of serum proteins, reflecting alterations in the patient’s proteomes.

Methods: We compared two-dimensional electrophoretic band patterns of human sera from eight patients with temporal lobe epilepsy (TLE) with those of eight control subjects. The differentially expressed bands were identified using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry and electrospray ionization quadrupole time-of-flight mass spectrometry.

Results: Twelve proteins were differentially expressed in the TLE group, of which 6 were identified. Expression of haptoglobin Hp2, PRO2675, immunoglobulin heavy chain constant region gamma 2, an unnamed protein, and three unidentified proteins were upregulated in serum from the patients with TLE, whereas those of major histocompatibility complex (MHC) class I antigen, plasma retinol-binding protein precursor, and three unidentified proteins were downregulated in these patients. After resection of the epileptogenic zone, the expressions of MHC class I antigen, immunoglobulin heavy chain constant region gamma 2, two of the downregulated unidentified proteins, and one of the upregulated unidentified proteins returned to the normal range.

Conclusion: The 12 serum proteins in this study are potentially useful biomarkers for the diagnosis and monitoring of TLE. (Korean J Pediatr 2009;52:567-575)

Key Words: Proteomics, Proteome, Epilepsy, Temporal Lobe

Introduction

Epilepsy is a neuronal disease characterized by recurrent seizures, caused by a uncontrolled discharge (hyperexcitability) of neurons in either the entire cortex (generalized epilepsy) or localized brain areas (partial epilepsy). In developed countries, the age-adjusted incidence of recurrent, unprovoked seizures ranges from 24 to 53 per 1,000 persons per year, and it is consistent across geographic regions. The age-adjusted prevalence varies from 4 to 8 per 1,000 persons.[1] Epilepsy is a common and heterogeneous neurological disorder originating from biochemical and molecular events that are incompletely understood. The development of molecular markers and genomic resources has facilitated the isolation of genes responsible for rare monogenic epilepsies in human and mouse. Many of the identified genes encode ion channels such as Na+ K+ and Ca2+ channels or other components of neuronal signaling.[2-4] Despite extensive studies on temporal lobe epilepsy, the molecular mechanism underlying the disease pathogenesis is not well understood.

A variety of tests are used to diagnose epilepsy, including semiology, electroencephalography (EEG), and neuroimaging. However, these methods are very complex and can yield inconsistent results. For example, video-EEG monitoring should be performed over several days to obtain accurate EEG recordings and observe symptoms. Magnetic resonance imaging (MRI) does not always detect abnormal lesions in patients with repetitive seizures. These diagno-
stic tools have many other disadvantages. They are time-consuming, expensive, and unable to detect epilepsy in its early phase in order to assess the degree of seizure control or provide prognoses. Thus, the development of serologic marker proteins for epilepsy could simplify diagnosis and assist in screening and post-treatment surveillance, as in the case of alpha-fetoprotein for hepatocellular carcinoma and germ cell tumors. Definitive diagnostic protein biomarkers for epilepsy have not yet been identified. Proteomics has played a central role in clinical diagnosis and monitoring of disease since Hanash et al. first proposed that proteomic study has the potential to identify novel biomarkers from pathologic tissues, biological fluids, and sera. A major resurgence of interest in the human serum proteome has occurred because of evidence that many disease processes are associated with quantitative and functional changes of proteins in body fluids.

Despite research by many groups, serologic biomarker of epilepsy, which could be used as a simple diagnostic tool and in post-treatment surveillance, remains elusive. We analyzed the proteomic profiles in serum of patients with TLE to find an easy and simple diagnostic method and to identify proteins that play a role in the pathogenesis of epilepsy. In the present study, we identified 12 proteins whose expression levels changed in patients with TLE after resection of the epileptic zone or were expressed differentially in these patients compared to normal individuals.

## Materials and methods

### 1. Serum samples

Venous blood was collected from 8 patients with TLE before and 7 days after resection of the epileptic zone, and from 8 control subjects without epilepsy. The serum was separated and frozen at -80°C until its use. The thawed samples were centrifuged at 4,500 rpm for 10 min to remove fibrinogen, and the samples were then filtered (0.45 µm pore size, Millipore, USA). The study was approved by The Ethics Committee of Chonbuk National University Medical School, and written informed consent was obtained from all the subjects.

### 2. Removal of serum albumin by HiTrap Blue column

The preservative was washed out and the HiTrap Blue column (Amersham Biosciences, Uppsala, Sweden) was equilibrated with 10-column volumes of binding buffer (50 mM KH₂PO₄, pH 7.0). The serum samples were loaded onto a 1 ml column at 0.5 ml/min. The column was washed with at least 5-column volumes of a binding buffer, and the serum proteins were eluted with an elution buffer (50 mM KH₂PO₄, 1.5 M KCl, pH 7.0). The eluted samples were then desalted by dialysis tubing (12 kDa molecular cut-off: Sigma, St. Louis, MO, USA) before running 2-dimensional electrophoresis (2-DE).

### 3. Two-dimensional electrophoresis

For 2-DE, 300 µg of serum protein in a rehydration solution (8 M urea, 2% CHAPS, 0.5% immobilized pH gradient (IPG) buffer, 1% DTT and a trace of bromophenol blue) was loaded into an immobiline DryStrip (pH 3–10, 24 cm) (Amersham Biosciences, St. Louis, MO, USA). The first-dimension isoelectric focusing (IEF) was performed using an IPIPhor IEF System (Amersham Biosciences, Uppsala, Sweden) at 20°C. The gels were equilibrated for 30 min in equilibration buffer I [50 mM Tris–Cl (pH 8.8), 6 M urea, 30% glycerol, 2% SDS, 0.1% DTT] and II [50 mM Tris–Cl (pH 8.8), 6 M urea, 30% glycerol, 2% SDS, 0.25% IAA]. The second-dimension IEF was run according to the Ettan DALT II System operating manual (Amersham Biosciences, Uppsala, Sweden). For 2-DE, 12.5% SDS–polyacrylamide slab gels (24 cm) were used. The IPG strips were placed on the surface of the second-dimension gels, and sealed with 0.5% agarose in SDS electrophoresis buffer (25 mM Tris base, 192 mM glycine, 0.1% SDS). The gels were run overnight at 110 V.

### 4. Silver staining

Silver staining of the gels was performed with a modified silver staining protocol and using the Silver Stain PlusOne kit (Amersham Biosciences, Uppsala, Sweden). After electrophoresis, the gels were fixed with 40% methanol/10% acetic acid for 30 min, sensitized by incubation in a sensitizing solution (0.2% sodium thiosulfate, 30% methanol, 68 g/L sodium acetate), and rinsed with 3 changes of distilled water (5 min washes). After rinsing, the gels were incubated in 0.25% silver nitrate for 20 min. Silver nitrate was then discarded and the gels were rinsed twice with distilled water for 1 min and then developed with 0.15% formaldehyde in 2.5% sodium carbonate under intensive shaking. The gel development was terminated with 1.46% EDTA.

### 5. Image analysis

The silver-stained 2-DE gels were scanned with
LabScan software on an Imagescanner (Amersham Biosciences, Uppsala, Sweden). The information was digitized and analyzed using ImageMaster 2D (Amersham Biosciences, Uppsala, Sweden). Band standardization was carried out for all matched bands. The band volume (intensity) was normalized as a percentage of the total band volume using the bands present in all gels.

6. Destaining

The silver stain was removed using chemical reducers as described previously. After the bands of interest were excised from the gels, 30 to 50 µL of a working solution was added to each gel and vortexed occasionally. The stain intensity was monitored until the brownish color disappeared. Gels were then rinsed a few times with water, and 200 mM ammonium bicarbonate was added to the gels for 20 min. Subsequently, the gels were cut into small pieces and dehydrated with repeated changes of acetonitrile until the gel pieces turned opaque white. The gel pieces were finally dried in a vacuum centrifuge for 30 min.

7. Trypsin digestion of proteins in-gel

The proteins were digested enzymatically as described previously. Briefly, the gel pieces were digested with 5 to 10 ng/L of trypsin and 50 mM ammonium bicarbonate, and incubated overnight at 37°C. Following enzymatic digestion, the resultant peptides were extracted 3 times and dried for 30 min using a vacuum centrifuge.

8. Protein identification

The dried samples were analyzed by matrix-assisted laser desorption/ionization time-of flight (MALDI–TOF) mass spectrometry for peptide mass fingerprinting, and by electrospray ionization quadrupole time-of flight (ESI–Q–TOF) mass spectrometry for peptide sequencing.

For MALDI–TOF analysis, the peptides dissolved in 0.5 % trifluoroacetic acid (TFA) solution were desalted using a ZipTipC18 (Millipore, Bedford, MA, USA) tip, and eluted directly onto the MALDI target by using cyan–4–hydroxy–cinnamic acid (CHCA) matrix solution (10 mg/mL CHCA in 0.5% TFA/50% acetonitrile (1:1, v/v)). The MALDI–MS spectra were analyzed using a 4700 Proteomics Analyzer (Applied Biosystems, Framingham, MA, USA) with UV light (355 nm) from a Nd:YAG laser with a repetition rate of 200 Hz.

All MS/MS experiments for peptide sequencing were performed using a Nano–LC/MS system consisting of an Ultimate HPLC system (LC Packings, Netherlands) and a Q–TOF2 mass spectrometer (Micromass, U.K) equipped with a nano–ESI source. Ten microliters of each sample was loaded onto a C18 trap column (ID 300 µm, length 5 mm, particle size 5 µm; LC Packings, Netherlands) for desalting and concentration. The trapped peptides were separated on a C18 nano–column (ID 75 µm, length 150 mm, particle size 5 µm; LC Packings, Netherlands). In the nano–electrospray ionization source, the end of the capillary tubing from the nano–LC column was connected to pico–tip silica tubing (ID 5 µm; New Objectives, USA) and the metal high voltage interface by a three–way tee piece. The applied voltage to produce an electrospray was 1.5 to 2 kV, and the cone voltage was 30 V. Argon was introduced as a collision gas at a pressure of 10 psi.

The database searches were carried out using MASCOT or MS–Fit, accessible at http://kr.expasy.org or http://www.ncbi.nlm.nih.gov.

Results

Protein expression profiles in serum from 8 patients with TLE and control subjects were analyzed by 2–DE, and the proteins were stained with silver. Representative gels (one set) with silver staining are shown in Fig. 1. Routine silver staining distinguished 300 to 600 protein bands of about 300 µg.

By performing 2–DE, we found that the expression of 12 proteins in the serum of the patients with TLE before treatment was significantly and consistently different from that of the control subjects (Fig. 1, 2). These specific protein expression rates seen in the patients with TLE is 5 or 6 times of that of control subjects. Seven of these proteins were upregulated and 5 were downregulated. After surgery, the expression of 4 of these proteins returned to the normal range.

To identify these proteins, the bands were excised from the gels, destained, digested overnight with trypsin, and analyzed by MALDI–TOF MS and ESI Q–TOF MS. The results of this analysis are shown in Table 1. We found that the expression of haptoglobin Hp2, PRO2675, and immunoglobulin (Ig) heavy chain constant region were upregulated in the patients with TLE. However, one band represented an unnamed protein, and three proteins remained unidentified. The downregulated proteins in the patients with TLE were identified as major histocompatibility complex (MHC) class I antigen and,
plasma retinol-binding protein precursor: 3 of these downregulated proteins were unidentifiable. We failed to identify 6 proteins because the expression level was below the limit of detection. These proteins may be novel, and their expression significantly and consistently differed in the 2 groups.

From the 2-DE analysis, upregulation of the specific protein expression seen in the patients with TLE before surgical intervention, expressed as a percentage of that seen in the control subjects, was as follows (Fig. 3): haptoglobin Hp2 (band 113), 232±50 (n=8); PRO2675 (band 115), 715±109 (n=8); Ig heavy chain constant region (band 303), 328±82 (n=8); the unnamed protein (band 375), 235±43 (n=8); unidentified protein (band 135), 278±88 (n=8); unidentified protein (band 188), 293±77 (n=8); unidentified protein (band 252), 472±59 (n=8). The expression levels of Ig heavy chain
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Fig. 3. Seven bands were significantly and constantly upregulated in serum of the patients with temporal lobe epilepsy (TLE) compared with those in serum of the control subjects. In each pair of the two-dimensional electrophoretic gels, one from a control subject and the other from a patient with TLE, the density of the band from the patient with TLE was normalized to the density of the corresponding band obtained from the matched control subject. The averaged volumes of each band are displayed as column bars from the eight pairs in the lower panel. Nor, Pre, and Post denote control, before, and 7 days after resection of the epileptogenic zone, respectively.

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<th>Measured pI/mass</th>
<th>pI/Mw (in database)</th>
<th>Pre op'</th>
<th>Post op'</th>
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Table 1. Protein Expression Significantly Changed in the Serum of Patients with Temporal Lobe Epilepsy

constant region and one of the unidentified proteins (band 188: pI 6.20, molecular weight 48.32 kDa) decreased to levels seen in the control subjects 7 days after surgery. In contrast, expression of haptoglobin Hp2 (band 113) markedly increased after treatment. As shown in Fig. 4, downregulation of specific protein expression seen in the patients with TLE before surgery, expressed as a percentage of that seen in the control subjects, was as follows: MHC class I antigen, 33±3 (n=8); plasma retinol–binding protein precursor, 29±4 (n=8); unidentified proteins (band 61), 21±1 (n=8); unidentified proteins (band 63), 24±3 (n=8); and the third unidentified protein (band 72), 18±12 (n=8). Expression
Fig. 4. Five bands were significantly and constantly downregulated in serum of the patients with temporal lobe epilepsy (TLE) compared with those in serum of the control subjects. In each pair of the two-dimensional electrophoretic gels, one from a control subject and the other from a patient with TLE, the density of the band from the patient with TLE was normalized to the density of the corresponding band obtained from the matched control subject. The averaged volumes of each band are displayed as column bars from the eight pairs in the lower panel. Nor, Pre, and Post denote control, before, and 7 days after resection of the epileptogenic zone, respectively.

Discussion

In the present study, 12 proteins were differentially expressed in serum of 8 patients with TLE compared to serum of 8 normal healthy subjects. Six of these proteins, which were upregulated in the sera from the patients with TLE, were identified as MHC class I antigen, haptoglobin Hp2, PRO2675, plasma-retinol binding protein precursor, Ig heavy chain constant region gamma-2, and an unnamed protein (Accession number: 10433230). The remaining 6 unidentified proteins might be novel. This proteomic information will be useful in the search for candidate biomarkers of TLE, which will simplify diagnosis and monitoring of epilepsy as well as further our understanding of the pathophysiology of this disease.

The MHC class I molecules are members of the Ig superfamily of cell surface proteins that enable the immune system to distinguish normal cells from foreign, mutant, or infected cells. These heterodimeric proteins have a membrane-spanning α-chain bound non-covalently to β2-microglobulin, which does not span the membrane. They are expressed in most tissues and have been studied intensively because of their crucial roles in initiating and regulating immune responses. They are most well known for their ability to bind intracellular-derived peptides and present them for inspection by cytotoxic T cells

The brain has long been considered as an immune privileged organ, and neurons have been thought to express MHC class I molecules only in response to cytokines. However, highly sensitive in situ hybridization and RNase protection techniques have revealed that the MHC class I molecules are expressed throughout the mammalian brains, that different MHC class I molecules have overlapping but clearly distinct neural expression patterns within the brain, and that they are dynamically regulated, during normal development and by neural activity. Recent experiments have demonstrated that proper surface expression of MHC class I proteins is required for normal formation of neuronal connections and normal synaptic plasticity in the mature hippocampus. A study with mutant mice found that deletions of genes encoding β
2-microglobulin and tobacco acid pyrophosphatase 1 dramatically reduced levels of MHC class I proteins on the cell surface. These mutant mice also had abnormal neuronal projections from the retina to the visual area of the thalamus as well as altered hippocampal long-term potentiation and long-term depression. Together, these phenotypes suggest that neuronal MHC molecules are involved in regression and/or elimination of inappropriate synaptic connections.

In addition to synaptic plasticity and development, neuronal MHC class I proteins may play a role in pheromone detection. Two groups have recently shown that several M family MHC molecules are expressed in the mouse vomeronasal organ and are needed for normal mating behavior. These observations suggest that these genes, long studied as mediators of immune function, can also play distinct, non-immune roles in neurons. However, the relationship between MHC class I antigen and epilepsy has never been studied. We found that the expression of this antigen decreased in the serum of patients with TLE but recovered 7 days after surgery to the level seen in the control subjects. This down-regulation of MHC class I antigen expression in epileptic brain cells may be related to disease pathogenesis. If this is true, the protein might also be a biomarker that can be used to diagnosis and monitoring of epilepsy.

Vitamin A (retinal) is an essential micro-nutrient with active derivatives that play key roles in a number of diverse biological processes, such as vision, cell growth and differentiation, and embryonic development and morphogenesis. Retinal is bound to distinct carrier proteins: the plasma retinal-binding protein (PRBP) and cellular retinal-binding proteins (CRBPs), respectively. When complexed with both plasma and cytoplasmic carriers, retinal is bound inside a deep cavity, nearly completely shielded from the external environment, such that it acquires stability drastically higher than that of uncomplexed retinal in the aqueous medium. RBP specifically binds retinol and belongs to a superfAMILY of proteins, known as the lipocalyn superfAMILY, whose members are characterized by a highly conserved basic structural motif, despite the fact that they possess low sequence identity. Retinol is synthesized and complexed with RBP in the liver before it is secreted to various target cells.

Circulating in the plasma, the monomeric RBP molecule (21 kDa) is complexed with tetrameric thyroxine-binding transthyretin (55 kDa). The RBP–transthyretin macromolecular complex is believed to prevent filtration of the relatively small RBP by renal glomeruli. Calamita et al. reported that RBP levels were reduced in patients with liver cirrhosis. To date however, a relationship between epilepsy and RBP or RBP precursor protein has not been established. In the present study, RBP precursor protein expression was decreased in the serum of the patients with epilepsy, and this is the first report to clarify the relationship between epilepsy and the RBP precursor protein. The RBP precursor is thus another potentially valuable biomarker for epilepsy. However, the pathophysiological role of the RBP precursor protein in epilepsy remains unknown.

In humans, the structural gene locus of haptoglobin (Hp) is polymorphic, with two common alleles (Hp1 and Hp2) coding for three phenotypes (Hp 1–1, Hp 2–1, Hp 2–2). Several biological activities have thus far been ascribed to this protein although functional differences have been demonstrated between the three phenotypes. The primary function of Hp is hemoglobin capture to prevent both iron loss and kidney damage during hemolysis. As one of the major acute phase reactants, Hp is involved in inflammation, infection, and malignancy. Hp also possesses immunoregulative properties, inhibits prostaglandin synthesis, and protects against harmful oxidation processes (this effect is less pronounced for Hp 2–2 type). Panter et al. reported that serum total Hp was significantly reduced in patients with familial idiopathic epilepsy or seizures. This hypohaptoglobinemia may be causally associated with a tendency to develop epilepsy. In contrast, the present study found that the serum level of Hp–2 significantly increased in serum taken from patients with epilepsy compared to serum taken from the control subjects. These results suggest that Hp isoforms may have distinct functions. Thus, epilepsy alters Hp expression in an isoform-specific manner, and this could account for differences in reported serum Hp levels. Although the pathophysiological role of Hp–2 in epilepsy is unclear, the protein is a potential diagnostic biomarker for the disease.

The variable and constant regions of Ig or antibody molecules are involved in antigen recognition and effector functions, respectively. The several functional Ig classes are, characterized by different heavy chain constant (CH) regions. CH regions are encoded by genes located in the IgH locus downstream of the variable region (IGHV) genes. The various CH regions interact with different components of the immune system to elicit inflammatory responses and eliminate the antigens. Antibodies characterized by a gamma
heavy chain, or IgG antibodies, exist as monomers and represent the major isotype in blood and extracellular fluids. In humans, IgG antibodies can be subdivided into four subclasses, which are designated IgG1, IgG2, IgG3, and IgG4, and contain a CH region encoded by one of four immunoglobulin heavy gamma (IGHG) genes, designated as IGHG1, IGHG2, IGHG3 and IGHG4 genes, respectively.32, 36)

The constant regions of these Ig molecules, in their secreted forms, consist of three domains (CH1, CH2 and CH3), as well as a hinge region, and differ from each other in sequence, structure, functional properties and levels of expression in response to specific antigenic stimulation.37) The relationship between the constant region of the Ig gamma-2 heavy chain and any disease or pathologic conditions has not been determined. Interestingly, in the present study, the constant region of the Ig gamma-2 heavy chain was up-regulated in serum of patients with TLE. Thus, this protein may be a third useful biomarker of TLE. Further studies are needed to clarify the mechanism underlying this up-regulation. We will try to identify the two functionally uncharacterized proteins (Gi223976 and Gi10433230) and the other six unidentified proteins in future studies using human genomic techniques and human gene libraries. This information will be valuable because the proteins might be novel, and they may have a function in TLE pathogenesis. Data obtained from these studies will establish whether these proteins are economic and simple biomarkers for diagnosing and monitoring TLE.

The information provided by this proteomic analysis will be very useful for our understanding of the pathophysiology of TLE as well as for finding new therapeutic targets or diagnostic biomarker candidates for this disease.
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