Title

Proteomic analysis of urothelium of rats with detrusor overactivity induced by bladder outlet obstruction

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Running Title

Identification of OAB urothelium-specific proteins
Abbreviations

BP, basal pressure; BOO, bladder outlet obstruction; DO, detrusor overactivity; IMP, intermicturition pressure; IVP, intravesical pressure; LUTS, lower urinary tract symptoms; MI, micturition interval; MP, micturition pressure; MV, micturition volume; OAB, overactive bladder; RV, residual volume; SA, spontaneous activity; TP, threshold pressure.
Summary

Overactive bladder (OAB) syndrome is a condition that has four symptoms: urgency, urinary frequency, nocturia, and urge incontinence and negatively affects a patient’s life. Recently, it is considered that the urinary bladder urothelium is closely linked to pathogenesis of OAB. However, the mechanisms of pathogenesis of OAB at the molecular level remain poorly understood, mainly as a result of lack of modern molecular analysis. The goal of this study is to identify a potential target protein that could act as a predictive factor for effective diagnosis and aid in the development of therapeutic strategies for the treatment of OAB syndrome. We produced OAB in a rat model and performed the first proteomic analysis on the mucosal layer (urothelium) of the bladders of sham control and OAB rats. The resulting data revealed the differential expression of 355 proteins in the bladder urothelium of OAB rats compared to sham subjects. Signaling pathway analysis revealed that the differentially expressed proteins were mainly involved in the inflammatory response and apoptosis. Our findings suggest a new target for accurate diagnosis of OAB that can provide essential information for the development of drug treatment strategies as well as establish criteria for screening patients in the clinical environment.

Key Words: overactive bladder, urothelium layer, diagnosis, drug treatment strategy.
Introduction

Overactive bladder (OAB) refers to a complex of symptoms, including urinary urgency, urge incontinence, nocturia, and frequent urination, that has negative impacts on health-related quality of life (1). Data from a study involving 16,776 Europeans revealed that the overall prevalence of OAB was 16.6% (2). In a survey of 5,204 Americans, the prevalence of OAB was 16.0% in men and 16.9% in women (3). Traditionally, OAB was believed to predominantly affect women, but, based on the prevalence of patients presenting with lower urinary tract symptoms (LUTS), OAB is now understood to also affect men (4). Bladder outlet obstruction (BOO), which is predominantly caused by benign prostatic hyperplasia, can induce detrusor muscle overactivity and low bladder capacity, and is the most common cause of OAB in elderly men (5). In addition to the physical aspect of this disorder, OAB is a highly prevalent, often distressing, condition that can have a negative effect on quality of life; furthermore, the disorder may eventually place a severe financial burden on society, not only in medical terms, but also through loss of productivity.

The urinary bladder consists of three distinct tissue layers. The innermost layer of the bladder is the mucosal layer that lines the hollow lumen. Unlike the mucosa of other hollow organs, the urinary bladder is lined with transitional epithelial tissue called the urothelium. The urothelium is able to stretch significantly to accommodate large volumes of urine and protects the underlying tissues from acidic or alkaline urine. The intermediate layer is the submucosa, a layer of connective tissue with blood vessels and nervous tissue that supports and controls the surrounding tissue layers. The outermost layer is the muscularis, which surrounds the submucosa and allows the bladder to expand and contract. The muscularis is commonly called the detrusor muscle and contracts during urination to expel urine from the body (6).

Although the exact pathogenic mechanism that mediates OAB is not completely understood, current understanding indicates that there is contribution from both neurogenic and myogenic sources. It is generally believed that OAB may be related to changes or dysfunction of the muscarinic receptors of the detrusor muscle (7-9). These changes result in a predisposition toward unstable bladder contractions or overactivity of the detrusor muscle. Thus, the major mechanism of
anticholinergic drugs, widely used in treatment of OAB, is their antagonism of the effect of acetylcholine (Ach) on muscarinic receptors in the cholinergically innervated bladder detrusor muscle (10, 11).

The urinary bladder urothelium has been considered to act primarily as a barrier. However, recent studies demonstrated that the urothelium is a responsive structure capable of detecting physiological and chemical stimuli, releasing several signaling molecules and various trophic factors in response to physical or chemical stimulation (12, 13). In addition, the urothelium is considered to be responsible for OAB (13, 14). Since the urothelium can act as a sensor and a transducer, reciprocal communication between the urothelium and other bladder tissue layers is likely. Thus, the functioning of the urothelium is closely related to the functioning of the nervous system, and modulation of the signaling pathways active in the urothelium may be a new therapeutic strategy for the treatment of OAB. However, detailed analysis of the urothelium at molecular level never has been reported.

In this study, we sought to identify urinary bladder urothelial proteins related to OAB syndrome. We established OAB in a rat model and analyzed protein expression in the bladder urothelium. To investigate the pathogenesis of OAB syndrome and discover potential diagnostic markers, an extensive bioinformatics analysis was performed.

**Experimental Procedures**

**Experimental design and statistical rationale**

The aim of this study is to identify urinary bladder urothelial proteins that are related to OAB syndrome by comparing protein expression between sham control and OAB urothelium. To investigate changes of protein expression in OAB urothelium, we produced OAB in a rat model by inducing partial BOO. The urothelium was carefully removed from the smooth muscle layer and its protein expression was analyzed. For proteomic analysis, ten bladder urothelium tissues of each group were divided into two tubes and each tube was analyzed three times by mass spectrometer. The
identified proteins were analyzed to discover canonical pathways, upstream molecules, and potential biomarkers that are associated with OAB by using Ingenuity Pathway Analysis (IPA) tool.

**Animals and experimental procedures**

All animal experiments followed the guideline for care and use of animals and the protocols were approved by the Institutional Animal Care and Use Committee of Chungnam National University, Daejeon, Korea (IRB No. CNU-00706). Female Sprague-Dawley rats (200 ± 30g) were employed in this study. The animals in the study were divided into two groups: the sham-operated group (n=40), the partial BOO group (n=60). Animals were fed standard rat chow and had free access to tap water. They were housed individually in separate cages with wood shaving as bedding at standard laboratory conditions (25±1°C, 55±5% humidity, and 12 hours alternating light-dark cycle).

**Surgical induction of partial BOO**

To induce a partial obstruction of the urethra, we conducted the conventional surgical method (15, 16). The rats were anesthetized by intramuscular injection of xylazine (10 mg/kg) and ketamine (100 mg/kg). The rats were placed on a servo-controlled surgical table to reduce heat loss during the surgery. Through a lower midline incision, the bladder and proximal urethra were carefully exposed. After identifying the urethra, a steel rod with a diameter of 0.9 mm was inserted into the urethra from the meatus. A 4-0 Novafil ligature (monofilament polybutester; Davis & Geck, Wayne, NJ) was placed around the urethra and tied in the presence of steel rod while ligation tension was constantly applied. After suturing, the steel rod was removed, leaving the urethra partially obstructed. The sham operation involved the same technique and exposure urethra rats by loosely tying 4-0 non-absorbable monofilament suture around a dissected urethra.

**Catheter implantation**

The intravesical catheter implantations were performed 3 days before cystometry procedures. Through a lower abdominal incision, a polyethylene catheter (PE-20; A-M Systems, Carlsberg, WA,
USA) with a cuff was inserted into bladder dome and held in place with a circular purse string suture using 6-0 silk. The catheters were tunneled subcutaneously and exteriorized at the upper scapular level anchoring with 4-0 silk ligature. The free end of the catheter was sealed during three days. The incisions were closed by using continuous 5-0 vicryl sutures for peritoneal and muscle incision and 5-0 silk sutures for skin closure. Sham-operated rats were assessed same procedure.

**Bladder cystometry**

Fourteen days after first surgery, cystometric investigations were performed without anesthesia. During cystometries, the end of intravesical catheter was connected via a pressure transducer (PowerLab, AD Instrument, Sydney, Australia) and an infusion pump (Promed-Tech., Bellingham, MA, USA) via a 3-way stopcock, to record intravesical pressure (IVP) and was infused into the bladder at 10ml/h with room temperature saline. The conscious rats were placed in metabolic cages free assessed to the water and feed. These cages also enabled to check the micturition volumes (MVs) using a fluid collector connected to a force displacement transducer (Grass Instruments, Quincy, MA, USA), which was connected via transducer amplifier to the data acquisition software (PowerLab, AD Instrument). After the voiding pattern stabilized, the data on 4–6 representative micturition cycles were collected and the mean values were calculated. IVP and MV were recorded synchronously and continuously using a Chart v 5.5.6 for Windows data acquisition system (PowerLab, AD Instrument) at a sampling rate of 2,000 Hz.

After voiding, we stopped the saline infusion and then we extracted the residual urine (RV) using syringe through the tubes were inserted into the bladder for cystometry (17). The following cystometric parameters were investigated: cystometric pressure and volume parameters, including basal pressure (BP, the lowest bladder pressure between two micturitions), micturition pressure (MP, maximum bladder pressure during micturition), threshold pressure (TP, bladder pressure at onset of micturition), intermicturition pressure (IMP, mean bladder pressure between two micturitions), spontaneous activity (SA, IMP–BP), micturition interval (MI), MV (micturition volume), bladder capacity (BC, MV+ RV) and voiding efficiency (VE, MV/BC micturition volume divided by bladder
capacity) (18). DO was defined as obvious increase in voiding and/or non-voiding contraction, with the latter defined as IVP rises from baseline pressure without a release of fluid from the urethra (19).

Bladder urothelium tissue preparation

After cystometric study, rats were anesthetized, the bladder was removed at the level of the proximal urethra through an abdominal incision. The bladder tissue was weighed and then urothelium was carefully separated from smooth muscle layer under a dissecting microscope by cutting in through the lamina propria, with care taken not to damage the urothelium. Each sample was stored immediately at -70°C for biochemical measurements.

Protein extraction, SDS-PAGE, and in-gel digestion

For proteomic analysis, ten bladder urothelium tissues of each group were divided into two tubes. The bladder urothelium tissue samples were homogenized with micropestle in 700 µL of lysis buffer containing 8 M urea and protease inhibitor cocktails as previously described (20). Tissue debris was removed with low speed centrifugation at 2,000 ×g for 10 min and the supernatants were collected. The protein concentration was determined by bicinchoninic acid (BCA) method and the samples were stored at -70°C for further study. 15 µg of protein samples was separated by 12% SDS-PAGE. The gels were stained with Coomassie Brilliant Blue R-250. In-gel digestion was conducted in accordance with the previously described method (21). Gels were fractionated into 8 parts according to molecular weight. The gel fragments were destained and then digested with trypsin at 37°C for 16 hours after reduction and alkylation of cysteines of the proteins. Digested peptides were extracted by extraction solution (50 mM ammonium bicarbonate, 50% acetonitrile, and 5% trifluoroacetic acid). Digested peptides were resolved in 10 µl of sample solution containing 0.02% formic acid and 0.5% acetic acid.

Protein identification with LC-MS/MS using LTQ-Velos mass spectrometry

Peptides were separated and identified using liquid chromatography integrated with electrospray ionization mass spectrometry (LC-ESI MS). The peptide samples (5 µl) were concentrated on a
trapping column with 75 µm inner diameter, packed with 5 µm C18 particles (Acclaim PepMap100, Thermo Scientific) and analyzed using a 15 cm analytical column packed with 2 µm C18 particles (Acclaim PepMap RSLC, Thermo Scientific). Reversed phase chromatography was performed using an Ultimate 3000 RSLC nano system (Thermo Scientific) with a binary solvent consisting of 0.1% formic acid (solvent A) and 80% ACN in 0.1% formic acid (solvent B). The peptides were separated by a linear gradient of solvent B from 5% up to 95% for 100 min with a flow rate of 300 nl/min. All MS and MS/MS spectra in the LTQ-Velos ESI ion trap mass spectrometer (Thermo Scientific) were acquired in a data-dependent mode. Each full MS (m/z range of 300 to 2,000) scan was followed by three MS/MS scans of the most abundant precursor ions in the MS spectrum with dynamic exclusion enabled. The MS/MS analysis was performed three times for each sample. The raw MS/MS spectra files were converted to mgf files using Proteome Discoverer daemon ver.1.4 (Thermo Fisher Scientific, Waltham, MA, USA). The converted mgf files were used for protein identification by using MASCOT ver.2.4.0 (Matrix science, www.matrixscience.com). The protein quantification was acquired by calculating the exponentially modified protein abundance index Protein quantification (emPAI) (22). The search parameters applied in the database searches were as followed: enzyme specificity: trypsin/P; maximum missed cleavages: 2; carboxymethyl (C) as a static modification; oxidation (M) and N-terminal acetylation as dynamic modifications; a precursor mass tolerance of 0.8 Da; and a MS/MS mass tolerance of 0.8 Da. The MS/MS data was filtered according to false discovery rate (FDR) < 1% criteria that calculating the ratio of false positive matches in Decoy database among the number of matches in database. The UniProt rat proteome database (2015_01., www.uniprot.org) which has a total 29,378 sequences and 15,795,139 residues was used for protein identification. Relative quantitation and significance are provided for all identified proteins (uploaded supplementary file: Supplemental Table S1.xlsx). Raw data are available via ProteomeXchange with identifier PXD007571.

Bioinformatic analysis

Functional analysis of the data set was done using the Ingenuity Pathway Analysis (IPA, Ingenuity
Systems, Redwood City, CA, www.ingenuity.com). The functional analysis identified biological functions and/or diseases that were most significant to the data set. The analysis was done against the Ingenuity Knowledge Base. Fischer’s exact test was used to calculate a $p$-value determining the probability that each biological function and/or disease assigned to that data set is because of chance alone. Canonical pathway analysis identified the pathways from the Ingenuity Pathway Knowledge Base that were most significant to the data set. The significance of the association between the data set and the canonical pathways was measured by the ratio of the number of proteins from the data set that map to the canonical pathway divided by the total number of proteins that map to the canonical pathway and by Fisher’s exact test. The focus genes from the two short lists were overlaid onto a global molecular network developed from information contained in the Ingenuity Pathway Knowledge Base. This generated networks based on the connectivity of the individual proteins. At the same time, protein cluster and the assignment of differentially expressed genes were generated.

**Statistical analysis**

The data were expressed as mean ± standard error of the mean. All statistical analyses were performed using Predictive Analytics Software 18.0 (SPSS Inc., Chicago, IL, USA). Statistical analyses were undertaken with Student’s t-test, Mann–Whitney U-test if data not normally distributed. Differences were considered statistically significant if the null hypothesis could be rejected with > 95% confidence ($p < 0.05$).

**Western blot, immunohistochemistry, and quantitative RT-PCR**

Western blot and immunohistochemistry were performed according to standard protocol as previously described (21). Primary antibodies are as followed: $\beta$-actin (sc-47778), P2RX1 (sc-25692), ATP5B (sc-33618), VAMP2 (sc-13992), EIF4B (sc-82587), PTMA (sc-30037). Total RNA extraction and cDNA synthesis were performed as described previously (21). Real-time PCR was performed with the Exicycler™ 96 (Bioneer, Daejeon, S. Korea) using SYBR Green.
Results

Rat model of OAB

It has been well known that OAB can be induced by BOO and the pathophysiology of OAB can be explained by the effects of BOO on bladder urothelium, detrusor muscle, and neurologic function (23). Since the effects of BOO in humans can be surgically induced in animal models, partial BOO animal models have been used for OAB studies (24-26). To investigate protein expression profiles of OAB urothelium, rat model was used to generate OAB. Partial BOO was induced by surgical method of tying the urethra of rats (15, 16). A significant increase in bladder weight was observed in BOO group compared with controls (Table 1). Representative cystometric tracings from rats in the sham-operated group and BOO group are shown in Figure 1. An unstable basal pressure pattern was observed in the BOO group, and was higher than in controls. Compared with sham animals, micturition interval was shortened in the BOO group. Postvoid residual urine and bladder capacity showed a significant increase in the BOO group compared to controls. Cystometry showed a significant increase in threshold pressure, and spontaneous bladder activity also increased with frequent voiding. Maximal micturition pressure was higher than in controls. Voiding efficiency was significantly decreased in the BOO group (Fig. 1 and Table 1). These results confirmed the surgical induction of detrusor overactivity (DO) in the rat model.

Identification of proteins involved in OAB

To investigate the differences in protein expression between sham control bladder urothelium and OAB urothelium, the urothelium was carefully removed from the smooth muscle layer and proteins were then extracted and analyzed using a LTQ-Velos ESI ion trap mass spectrometer. A total of 507 proteins were detected in sham rat urothelium and 380 proteins in OAB rat urothelium (Supplemental Table S2). Of these, 306 (52.7%) proteins were commonly expressed in sham control and OAB urothelium. By contrast, 201 and 74 proteins were expressed uniquely in sham control and OAB urothelium, respectively. Large numbers of proteins (201 out of 507, 39.6%) expressed in sham
control urothelium were suppressed in OAB urothelium, and 19.5% (74 out of 380) of proteins were expressed in OAB urothelium but not in sham control urothelium (Fig. 2A). In addition, the expression levels of 80 of the 306 commonly expressed proteins (26.1%) were altered by at least 2.0-fold (Fig. 2B). This indicates that protein expression in the urothelium is markedly changed in OAB syndrome and suggests that the normal function of urothelium is disrupted in the OAB rat model.

Cellular component analysis was performed to determine the subcellular localization of the identified proteins. Of the 507 proteins identified in sham rat urothelium, 84 (16.6%) were extracellular, 39 (7.7%) were at the plasma membrane, 259 (51.1%) were present in the cytoplasm, and 66 (13.0%) were found in the nucleus (Supplemental Fig. S1A). The proteins identified in OAB rat urothelium showed a similar pattern of protein localization: 71 (18.7%) were extracellular, 45 (11.8%) were at the plasma membrane, 176 (46.3%) were present in the cytoplasm, and 49 (12.9%) were found in the nucleus (Supplemental Fig. S1B). When comparing the subcellular localization of differentially expressed proteins, however, we noted that plasma membrane proteins were more likely to be newly synthesized or overexpressed in OAB urothelium while proteins from other subcellular regions were generally suppressed (Fig. 2C).

**Functional annotation of differentially expressed proteins**

To perform pathway and network level analysis of the data, the 355 differentially expressed proteins were analyzed using the Ingenuity Pathway Analysis (IPA) tool. The 34 identified canonical pathways, which represent the combined top 20 most significant canonical pathways in sham control and OAB urothelium, are shown in Figure 3. In the OAB urothelium, pathways involved in inflammation, such as the complement system, acute phase response signaling, LXR/RXR activation, and p38 MAPK signaling, were notably up-regulated. By contrast, signaling pathways related to cytoskeletal organization, including ILK signaling, RhoA signaling, and remodeling of epithelial adherens junctions, were commonly down-regulated in OAB urothelium. It is also notable that proteins involved in the unfolded protein response (ER stress) were down-regulated and proteins involved in death receptor signaling were up-regulated.
**Analysis of upstream regulators of OAB**

The results of proteomic analysis demonstrated significant differences in protein expression and related signaling pathways between sham control urothelium and OAB urothelium. To investigate the potential causes of OAB, we performed further bioinformatic analysis using IPA. The results of this analysis identified 17 putative upstream regulators. These regulators are involved primarily in inflammation and cytoskeletal organization (Table 2). Complement component 3b/4b receptor 1-like, huntingtin, and inhibin α act as upstream regulators of Cryab, Aldoa, Tpm2, Myl9, Cnn1, Myh1, and C3, and may cause activation of muscle contraction (Fig. 4A). Six of the upstream regulators, huntingtin, inhibin α, integrin α2, complement component 3b/4b receptor 1-like, HNF1 homeobox B, and platelet derived growth factor family, may also affect positively the cell movement of leukocytes and neutrophils as well as cellular infiltration by leukocytes through the regulation of many other proteins identified in the urothelium (Fig. 4B, C).

**Proteins involved in signaling in bladder urothelium**

The urothelium releases a number of signaling molecules to communicate with closely located bladder nerves, detrusor smooth muscle (DSM), and interstitial cells such as myofibroblasts. The typical signaling molecules released from the urothelium are adenosine triphosphate (ATP), nitric oxide (NO), prostaglandin (PG), protachykinin-1 (TAC1), Ach, and nerve growth factor (NGF) (13, 14, 27). To identify novel regulators of the signaling molecules in the urothelium, we performed protein network analysis of the proteins differentially expressed in OAB rat urothelium. The results identified 52 proteins that may regulate signaling molecules in the urothelium (Fig. 5). Among the identified proteins, 34 may regulate the production or release of ATP, and 18 may regulate the production and release of NO (Fig. 5). Of 34 ATP-related proteins, 7 proteins (ENTPD1, P2RX1, ATP5B, VAMP2, C3, EIF4B, and PTMA) were detected only in the OAB urothelium. Their expression pattern was validated by Western blot and immunohistochemistry experiments (Fig. 6). On the other hands, of 18 NO-related proteins, 7 proteins (RBP4, RPSA, PIN2, CP, SOD2, HSPD1, and
Potential OAB diagnostic markers

Since there are no known molecular diagnostic markers of OAB, clinical diagnosis of OAB is still symptom-based. In this study, we demonstrated that urothelial protein expression is dynamically altered by OAB. These altered proteins in OAB urothelium could be used as potential diagnostic markers for OAB. The urothelium is the outermost barrier tissue that directly contacts urine. Extracellular proteins expressed by urothelium that are released into the urine could also be used as noninvasive OAB diagnostic markers. We identified 37 extracellular proteins that were exclusively expressed in sham control or OAB rat urothelium (Table 3). Of these, 11 proteins were expressed only in the OAB urothelium and are involved mainly in inflammation. The other 24 proteins were expressed only in sham control urothelium and were related primarily to cellular and tissue structure formation (Table 3). Thus, these potential markers are closely related to the pathophysiological changes that occur in OAB. In addition, expression of the up-regulated proteins was verified by real-time PCR experiment (Supplemental Fig. S2). Detecting these proteins or their peptide fragments in urine may be a useful tool for the diagnosis of OAB.

Discussion

Recent studies showed that there is reciprocal communication between the urothelium and adjacent bladder tissue layers. The urothelium can function as a sensor for detecting physiological and chemical stimuli from the other bladder tissues and urine and act as a transducer for transmitting signaling molecules to adjacent bladder tissues (12, 27). The purpose of this study was to identify proteins involved in both the sensor and transducer functions. To discover proteins related to the sensor function of urothelium, we focused on plasma membrane proteins able to sense extracellular stimulation and transmit signals to the intracellular region. The results of our proteomic analysis indicate that, although the total number of expressed proteins in the bladder urothelium is decreased in
the OAB model animal (Fig. 2A), the number of plasma membrane proteins expressed in the urothelium is increased (Fig. 2C). Among the 24 up-regulated plasma membrane proteins, we identified two transporters (Anxa5 and Slc12a7), two ion channels (Cacna2d1 and P2rx1), and two receptors (Lgals3bp and Pgrmc1). This suggests that the OAB urothelium is more capable of detecting and accepting or releasing chemical stimuli. OAB is much more sensitive than sham bladder. Therefore, these up-regulated urothelial plasma membrane proteins may be responsible for OAB pathogenesis and targeting these proteins could be a novel possible therapeutic strategy.

We also identified potential upstream regulators of signaling in the OAB urothelium by extensive bioinformatics analysis. We found three upstream regulators, CR1L, HTT, and INHA, that may regulate muscle contraction (Fig. 4 and Table 2). Historically, although there are many different etiologies for OAB, the hypothesis that has received the most attention is that of increased DSM contractility (overactivity) as the underlying cause (28). This mechanism provides the foundation for the administration of oral anti-muscarinics to block DSM overactivity. We also identified six upstream regulators, HTT, INHA, ITGA2, CR1L, HNF1B, and PDGF, that may regulate the inflammatory response in the bladder. It is known that inflammation of the bladder is directly linked to bladder function (29, 30). In addition, “inflammaging” (inflammation + aging) is highly associated with OAB (31). This suggests that inflammation may be an essential cause of OAB, even in the absence of urinary tract infection. Among these upstream regulators, CR1L, HTT, and INHA are associated with both muscle contraction and inflammation. CR1L (complement component 3b/4b receptor 1-like) is a potential receptor of complement components. Complement component proteins are large glycoproteins that are proteolytically activated and involved primarily in the immune response and host defense (32). In particular, the C3a, C4a, and C5a components, referred to as anaphylatoxins, cause smooth muscle contraction and increase vascular permeability (33). Our proteomic analysis showed that C3 (complement component 3) is exclusively expressed in the OAB urothelium (Supplemental Table S2). INHA (inhibin alpha) is an alpha subunit of inhibin proteins. Inhibin, which plays an opposing role against the effects of activin, is involved in regulating numerous cellular processes, including cell proliferation, apoptosis, immune response, and hormone secretion (34). HTT
(huntingtin) is a disease gene that is linked to Huntington’s disease. Huntington’s disease is a neurodegenerative disorder that is caused by the expansion of a polyglutamine stretch within the HTT protein (35). Although skeletal muscle pathology is a hallmark of Huntington’s disease, the normal function of HTT is unknown. However, association of HTT with inflammation has been demonstrated in previous studies. HTT-induced neurodegeneration is protected against by anti-inflammatory targets (36). Further functional studies of these possible upstream signals and their downstream effectors could provide molecular evidence of the cause of the abnormal physiology of urinary detrusor muscle in OAB.

Next, we investigated proteins related to the transducer function of the urothelium. Further bioinformatic analysis identified 52 proteins that may regulate signaling molecules in the urothelium, including ATP, NO, PG, TAC1, Ach, and NGF. Network analysis showed that there are two notable nodes that have an effect on ATP and NO (Fig. 5). The purine nucleotide ATP is a neurotransmitter responsible for non-adrenergic, non-cholinergic neurotransmission (37). Recently, animal and human studies showed that non-neuronal ATP released from the bladder urothelium is responsible for DO (38, 39). Thus, ATP is considered a possible biomarker and therapeutic target of OAB (40). Among the 34 proteins that may be related to ATP, seven proteins, ENTPD1, P2RX1, ATP5B, VAMP2, C3, EIF4B, and PTMA, are exclusively expressed in the OAB urothelium. ENTPD1 (ectonucleoside triphosphate diphosphohydrolase 1) is a plasma membrane protein that hydrolyzes extracellular ATP and ADP to AMP (41). In the nervous system, ENTPD1 regulates purinergic neurotransmission (42). Purinergic signaling plays a crucial role in normal urinary bladder function (43). P2RX1 (purinergic receptor P2X) is an ATP-gated ion channel that binds to ATP to mediate synaptic transmission between neurons and from neurons to smooth muscle. Expression of P2RX1 is enhanced in bladder biopsy samples of patients with OAB (44). ATP5B (mitochondrial ATP synthase subunit beta) is a subunit of mitochondrial ATP synthase (45). VAMP2 (vesicle-associated membrane protein 2) is a member of the synaptobrevin family of small integral membrane proteins of secretory vesicles. VAMP2 is a key component for neurotransmitter release (46). C3a induces IL-1β production in monocytes by controlling the release of intracellular ATP into the extracellular space during inflammasome
activation (47). Thus, it can be suggested that enhanced expression of these proteins may cause elevated production and release of ATP from the OAB urothelium, leading to overactivity of the bladder. NO also plays an important role in the health of the urinary bladder. Long-term deficiency of NO causes overactivity of the detrusor muscle (48). Network analysis showed that the expression of 12 out of 18 NO-related proteins was suppressed in the OAB urothelium. In particular, the expression of seven proteins, RBP4, RPSA, PIN2, CP, SOD2, HSPD1, and PEF1, was completely blocked in the OAB urothelium. RBP4 (retinol-binding protein 4) is a member of the lipocalin family and functions as a specific carrier for retinol. RBP4 enhances NO production (49), and up-regulation of RBP4 plays a role in myocardial infarction (50).

Previous reports have demonstrated that OAB may be caused by chronic inflammation (51, 52) and proteins related with inflammation can be served as biomarker for OAB syndrome (53). In addition, modulating inflammatory response with anti-inflammation drugs can improve the detrusor contraction disorder (54, 55). In this study, we showed that the urinary bladder urothelium is closely related to inflammation and releases signaling molecules that affect the DO. Therefore, targeting the urothelium is potential treatment strategy for OAB.

Grading urinary urgency and diagnosing OAB are difficult tasks. Generally, multiple questions are used to quantify and grade urgency severity. To overcome this problem, researchers have tried to establish molecular diagnostic markers of OAB. However, the studies have focused on the detrusor muscle or nerve tissue of the urinary bladder. Recently, evidence demonstrating that the urinary bladder urothelium is involved in the pathogenesis of OAB has emerged (12-14, 27). Because the urothelium directly contacts with urine, secreted proteins from the urothelium could be released into urine. Thus, comparing the profile of proteins secreted by OAB urothelium with those secreted by normal bladder urothelium could be a way to identify molecular diagnostic markers for OAB. From our proteomic analysis, we identified 37 extracellular proteins that were differentially expressed in sham control or OAB rat urothelium, 11 of which were expressed only in OAB urothelium and were mainly involved in inflammation (Table 3). Verification of these proteins in the urine of OAB patients may confirm their potential as noninvasive diagnostic markers for OAB.
In conclusion, we identified 355 proteins that were differentially expressed between the sham control bladder urothelium and the OAB urothelium in a rat model. These proteins may be associated with abnormal sensitivity and muscle contraction of the bladder. The proteins identified in this study provide direction for further research toward discovering diagnostic markers and therapeutic targets for the treatment of OAB.
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Author Contributions

E.C.P., J.L., S.K., J.S., and G.H.K. designed all of the experiments and wrote manuscript together. Y.K.T. and S.Y.L. performed bioinformatics analysis. C.W.C., S.Y., J. P., and M.L. performed proteomic analysis. J.S.L., Y.G.N. and J.H.S. performed animal experiments. H.K.C. and K.S.K performed immunohistochemistry experiment. All authors reviewed the manuscript.

Conflict of Interest

The authors declare no competing financial interests.
Figures and Figure Legends

Figure 1

Fig. 1. Representative cystometric tracings in conscious rats. (A) Sham-operated rats. Regular voiding patterns, stable basal pressure (BP) and definite threshold pressure (TP) were observed. (B) BOO rats with detrusor overactivity (DO). Unstable BP, unremarkable threshold pressure (TP), shortening micturition internal (MI), and increased slope were observed. Values are expressed as intravesical pressure (IVP) (cmH₂O) and micturition volume (MV) (ml).
Fig. 2. Identification and quantitative comparison of sham control and OAB rat urothelial proteome. (A) Venn diagram that shows the number of identified proteins from the rat urothelium. (B) Summary of quantitative analysis of the urothelial proteome from sham control and OAB rats. (C) Summary of subcellular localization of 355 differentially expressed proteins between the sham control and OAB rat urothelium.
Fig. 3. Functional annotation of differentially expressed proteins. 355 differentially expressed proteins between the sham control and OAB rat urothelium were analyzed using the IPA tool. The top 20 most significant canonical pathways in sham control and OAB urothelium were shown.
Fig. 4. Representative protein networks of upstream regulators of OAB. Protein network no.1 (A), network no.3 (B), network no.7 (C) of Table 2 were shown.
Fig. 5. Protein network analysis with signaling molecules released from the urothelium. IPA-determined network of identified proteins that are associated with signaling molecules released from the urothelium, such as ATP, NO, PG, TAC1, Ach, NGF. Red, upregulated; green, downregulated.
**Figure 6**

**A**

| Protein   | Sham | OAB |
|-----------|------|-----|
| P2RX1     |      |     |
| ATP5B     |      |     |
| VAMP2     |      |     |
| EIF4B     |      |     |
| PTMA      |      |     |
| β-actin   |      |     |

**B**

![Western blot and immunohistochemistry images](image)

**Fig. 6. Validation of upregulated proteins in OAB.** Western blot (A) and immunohistochemistry (B) of selected proteins in the sham control and OAB bladder urothelium. Uro, urothelium layer; SC, suburothelial connective tissue layer. The scale bar indicates 50 μm (bottom).
### Table 1. Results of bladder weights & cystometric analysis of the experimental animals

| Group                                      | Sham       | OAB        |
|--------------------------------------------|------------|------------|
| Bladder weight (mg)                        | 105.5 ± 9.4| 194.7 ± 12.8*** |
| Micturition interval (MI) (min)            | 8.76 ± 1.27| 4.80 ± 0.40** |
| Micturition volume (MV) (ml)               | 1.43 ± 0.12| 0.78 ± 0.08*** |
| Residual volume (ml)                       | 0.03 ± 0.01| 1.86 ± 0.19*** |
| Bladder capacity (BC, MV+ RV) (ml)         | 1.46 ± 0.12| 2.64 ± 0.17*** |
| Basal pressure (BP) (cmH20)                | 14.3 ± 1.6 | 17.5 ± 2.4* |
| Threshold pressure (TP) (cmH20)            | 25.9 ± 4.1 | 36.2 ± 4.9** |
| Maximal micturition pressure (MP) (cmH20)  | 53.8 ± 6.3 | 68.5 ± 7.5* |
| Intermicturition pressure (IMP) (cmH20)    | 18.4 ± 2.1 | 23.4 ± 3.8 |
| Spontaneous activity (SA=IMP-BP) (cmH20)   | 4.1 ± 0.3  | 5.9 ± 0.4*** |
| Voiding efficiency (VE, MV/BC)             | 0.98 ± 0.01| 0.30 ± 0.04*** |

* p-value < 0.05, ** p-value < 0.01, *** p-value <0.001

Infusion rate = 10 ml/h
Table 2. Protein network analysis of upstream regulators of OAB

| Network | Upstream Regulators | Diseases & Functions | Identified Target Molecules |
|---------|---------------------|----------------------|-----------------------------|
| 1       | CR1L, complement component 3b/4b receptor 1-like, HHT, huntingtin, INHA, inhibin alpha, ITGA2, integrin subunit alpha 2 | Cell movement of leukocytes, Formation of epithelial tissue, Muscle contraction | ALDOA, APOA1, BGN, C3, CD9, CNN1, COL18A1, COL1A1, COL4A1, CRYAB, DCN, FN1, GSN, HSPD1, ITGA5, ITGB1, LUM, MCAM, MYH11, MYL9, PFN1, SERPINA1, SERPINA3, SERPINE1, SERPINF1, Tpm2, YBX1 |
| 2       | CR1L, complement component 3b/4b receptor 1-like, SMAD7, SMAD family member 7 | Advanced stage solid tumor, Metastatic solid tumor | COL18A1, COL4A1, COL6A3, FN1, NID2, SERPINE1 |
| 3       | HNF1B, HNF1 homeobox B, HTT, huntingtin, ITGA2, integrin subunit alpha 2, PDGF (family), platelet derived growth factor | Cellular infiltration by leukocytes | APOA1, BGN, CRYAB, DCN, FGB, FN1, HSPD1, ITGB1, MCAM, SERPINA1, SPARC, YBX1 |
| 4       | APP, amyloid beta precursor protein, NFE2L2, nuclear factor, erythroid 2 like 2 | Cell movement of sarcoma cell lines | CTSB, FN1, SERPINE1, SERPINF1, SOD2, STMN1, VCP |
| 5       | ANGPT2, angiopoietin 2, GH1, growth hormone 1, Phenylbutazone, Prednisolone | Anemia | ANXA1, APOE, C3, C4A/C4B, CFB, CP, CRP, FN1, HBB, LGALS3, PRDX1, PRDX2, PRDX3, RPS6, SOD1, SOD2, TF |
| 6       | miR-122-5p (miRNAs w/seed GGAGUGU) | Carcinoma in lung, Cell viability of tumor cell lines | ALDOA, GPX7, P4HB, PKM, PRDX2, VIM |
| 7       | HNF1B, HNF1 homeobox B | Cell movement of neutrophils, Cellular infiltration by leukocytes | ALB, BGN, FGB, FN1, LUM, SERPINA1, SPARC |
| 8       | ANGPT2, angiopoietin 2, Tanespimycin | Endoplasmic reticulum stress response | CALR, DNAJB4, HSP90AB1, HSPA5, HSPD1, SERPINH1, SOD1 |
| 9       | INSR, insulin receptor, ITGAV, integrin subunit alpha V | Proliferation of muscle cells | CTSB, FHL1, FN1, HSPD1, ITGA5, OGN, SERPINE1, VTN |
| 10      | Phenylbutazone | Anemia, Hemostasis | ANXA1, ANXA2, C4A/C4B, CP, FGB, LGALS3 |
| Symbol | Gene Name |
|--------|-----------|
| **Up-regulated** | |
| C2 | complement component 2 |
| C3 | complement component 3 |
| C4A/C4B | complement component 4B (Chido blood group) |
| CFH | complement factor H |
| CILP | cartilage intermediate layer protein, nucleotide pyrophosphohydrolase |
| COL1A2 | collagen, type I, alpha 2 |
| IGFBP7 | insulin-like growth factor binding protein 7 |
| ITIH1 | inter-alpha-trypsin inhibitor heavy chain 1 |
| MGP | matrixGla protein |
| NID2 | nidogen 2 (osteonidogen) |
| PF4 | platelet factor 4 |
| **Down-regulated** | |
| 1300017J02Rik | RIKEN cDNA 1300017J02 gene |
| A1BG | alpha-1-B glycoprotein |
| CFL2 | cofilin 2 (muscle) |
| COL1A2 | collagen, type I, alpha 2 |
| CP | ceruloplasmin (ferroxidase) |
| CPA3 | carboxypeptidase A3 (mast cell) |
| ECM1 | extracellular matrix protein 1 |
| FBLN5 | fibulin 5 |
| FGB | fibrinogen beta chain |
| FMOD | fibromodulin |
| GPX3 | glutathione peroxidase 3 (plasma) |
| HBA1/HBA2 | hemoglobin, alpha 1 |
| HP | haptoglobin |
| ITIH4 | inter-alpha-trypsin inhibitor heavy chain family, member 4 |
| LAMC1 | laminin, gamma 1 (formerly LAMB2) |
| LTBP4 | latent transforming growth factor beta binding protein 4 |
| PCOLCE | procollagen C-endopeptidase enhancer |
| PRG2 | proteoglycan 2, bone marrow (natural killer cell activator, eosinophil granule major basic protein) |
| PXN | peroxidasin |
| RBP4 | retinol binding protein 4, plasma |
| SERPINA6 | serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 6 |
| SUS2 | sushi domain containing 2 |
| TINAGL1 | tubulointerstitial nephritis antigen-like 1 |
| TNC | tenascin C |