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TITLE: Validating Epigenetic and Genetic Biomarkers for Diagnosis of Bladder Pain of Interstitial Cystitis

PRINCIPAL INVESTIGATOR: Jayoung Kim, PhD

CONTRACTING ORGANIZATION: Cedars-Sinai Medical Center
Los Angeles 90048

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14. ABSTRACT The goal of this study is to determine the biosignature consisting of DNA methylation and gene expression status detected in urine from interstitial cystitis/painful bladder syndrome (IC) patients will stratify IC patients from healthy controls. In this funding year, we have focused on (1) identification on baseline of urine collection, (2) expansion of database for better urine biomarker discovery, and (3) technology application to further identify the DNA methylation markers associated with IC. In particular, we were able to optimize the DNA methylation and gene expression assays, which were designed for high-throughput screening (HTS).					
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1. **INTRODUCTION:** *Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.*

The goal of this study is to determine the biosignature consisting of DNA methylation and gene expression status detected in urine from interstitial cystitis/painful bladder syndrome (IC) patients will stratify IC patients from healthy controls.

2. **KEYWORDS:** *Provide a brief list of keywords (limit to 20 words).*

interstitial cystitis, DNA methylation, biosignature, gene expression

3. **ACCOMPLISHMENTS:** *The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.*

What were the major goals of the project?

List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

The **overall goals** of this study are to improve the methodology for diagnosis of IC and progression of disease and to develop new insight into the underlying mechanisms that trigger this condition. In this project, we will determine if urine-based DNA methylation and gene expression signatures associates with pain severity, health-related quality of life, and other comorbid conditions in IC patients.

Approved SOW (by 12months)

Milestone(s) Achieved:

Quantification of epigenetic and genetic biomarker candidates

Subtask 1. Quantification of DNA methylation

- 1.1. Sample preparation and protocol optimization
- 1.2. Targeted DNA methylation analysis

Subtask 2. Quantification of gene expression

- 1.1. Sample preparation and protocol optimization
- 1.2. High throughput gene expression analysis

Subtask 3. Pipeline establishment for computational analysis to test a statistical model

In this funding year, we have focused on (1) identification on baseline of urine collection, (2) expansion of database for better urine biomarker discovery, and (3) technology application to further identify the DNA methylation markers associated with IC.

What was accomplished under these goals?

For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

(1) Major activities

- The subject protocol (version dated 12/03/2019) was approved by the Cedars-Sinai Institutional Review Board (IRB) on 12/3/19.
- The U.S. Army Medical Research and Development Command (USAMRDC), Office of Research Protections (ORP), Human Research Protection Office (HRPO) reviewed the protocol and found that it complies with applicable DOD, U.S. Army, and USAMRDC human subjects protection requirements.
- We got an Approval Memorandum (Proposal Number PR180579, Award Number W81XWH-19-1-0109) in March 4, 2020.
- Trained personnel who will perform biochemical analyses for this project.

(2) Significant results or key outcomes

- We made significant key scientific outcomes since the previous funding period. Several full research papers and review articles were published in this funding period. **Please refer to the APPENDICES.**
- After March 4, 2020 when we got an approval from DoD, we started to establish a laboratory-based protocol and assembled a cohort for this particular study.
- Due to this delay on HPRO approval, we were able to initiate actual lab work a little later than we originally expected.
- Urine samples from IC and healthy controls were pre-processed by the trained lab personnel.
- We optimized the DNA extraction protocols using human urine samples.

- We were able to optimize the DNA methylation and gene expression assays, which were designed for high-throughput screening (HTS). They include CNR2 as planned in the proposed study.
- To perform a pilot study for the MethyLight assay optimization (Dr. Kim).
- Using this optimized assay system, we found that CNR2 DNA methylation is significantly altered in IC patients compared to controls.
- Our pilot study began with evaluating the top five genes (*CNR2*, *PR2Y14*, *GRM6*, *F2R* and *CHRM3*), for which MethyLight assays have already been designed.
- Our ultimate goal is to evaluate the DNA methylation levels of the 20 most hypomethylated genes from preliminary work.
- Our project is conducted through a close collaboration with core facilities in University of Southern California and Genomics Core at CSMC.
- We initiated a contract with NanoString Research team for this particular study. NanoString gene expression analysis is a novel digital technology that is based on direct multiplexed quantification of nucleic acids and offers high levels of precision and sensitivity, in this proposal. NanoString technology employs molecular “barcodes” and single molecule imaging to detect and count target gene expression in a single reaction.
- Unfortunately, due to the current COVID-19 pandemic, we were not able to submit our pro-processed samples to facilities which will perform the HTP analysis.
- Many of my lab personnel were not able to come to work since our lab facility is closed due to the California’s statewide **lockdown** order.
- Facilities, clinical research center, and all other laboratories were closed or minimally maintained since late March, leading to a project hold (May 18, 2020).
- However, recently we establish new collaboration with a big biomarker discovery group at our own institute for further validation of target candidates. While our preliminary are based on small numbers and require validation in a large dataset, they nonetheless demonstrate the feasibility of the current study and provide strong support to the fact that we are likely to identify many clinically and biological important linkages with IC and the various IC phenotypes within this study.

- Luckily, we were able to publish a series of papers relevant to this proposed study. Our recent review paper summarized the underlying mechanisms that induce the chronic pain associated with IC and vulvodynia and explain why these two conditions often coexist. We also developed a statistical model to determine whether the biosignatures to be enhanced by additional systemic changes, such as widespread pain or associated clinical depression.
- We are sorry that this project may be a little bit delayed to complete. It's because that we got the HRPO approval later than we originally expected and the COVID-19 pandemic hold the necessary lab work.
- In the following sections, we will update our progress in detail for your reference.

1. Sexual Pain and IC/BPS in Women

Female sexual dysfunction (FSD) and interstitial cystitis/bladder pain syndrome (IC/BPS) are important factors affecting women's health. A study conducted on the association between sexual and general well-being found that women reported better quality of life (QoL) with higher sexual satisfaction, regardless of age and/or menopausal status.¹ Both FSD and IC/BPS significantly impairs women's abilities to pursue and enjoy sexual relations. Approximately 40-50% of women experience FSD and 0.5-12% experience IC/BPS. Considering these incidence rates in the general population, both these diseases become serious challenges for patients and clinicians.²⁻⁶

Chronic pain deteriorates not only personal health and wellness, but also quality of life. Chronic pain can induce sexual dysfunction, such as arousal disorder and relationship problems.⁷ Furthermore, studies have shown that significantly more women with chronic pelvic pain (CPP) showed FSD compared to those without CPP. Women with CPP and FSD had various types of FSD, such as hypoactive sexual desire disorder, sexual arousal disorder, orgasmic disorder, and sexual pain disorder.⁸

The symptoms of IC/BPS, such as urinary frequency, urgency, and pelvic pain, have a negative impact on sexual activity and life quality.⁹ Women with overactive bladder (OAB) frequently have a risk for sexual dysfunction.¹⁰ In the postmenopausal group, women with scores indicating severe OAB had worse sexual function, mainly in the arousal, lubrication, orgasm, pain, and total domains.¹¹ Despite the known association of sexual dysfunction with bladder diseases, contributing risk factors have yet to be explored. Evaluating the impact of duration, severity, pain localization, sexual trauma history, anxiety, and depression on sexual dysfunction may help elucidate risk factors. Increasing our knowledge about sexual dysfunction as it relates to bladder diseases may aid in clinical diagnoses and play a major role in treatment strategies and overall symptom improvement in this patient population. This review will provide an overview of studies that address FSD in women with IC/BPS.

Both vulvodynia and IC/BPS are common and irritable conditions that disrupt normal life and reduce QoL in women. Vulvodynia also manifests itself along with IC/BPS and there are several reports supporting the association between the two diseases. Unfortunately, knowledge concerning vulvodynia and IC/BPS is inadequate when considering the clinical impact and importance of these two conditions. Therefore, there is an essential need for further studies that delve into discovering the features of vulvodynia and IC/BPS.

2. Pioglitazone Alters the Proteomes of Normal Bladder Endothelial Cells

Thiazolidinediones (TZDs), such as pioglitazone and rosiglitazone, are currently the only oral drugs with a confirmed antihyperglycemic impact in type 2 diabetes mellitus (T2DM) patients. As agonists of peroxisome proliferator-activated receptor γ (PPAR γ), TZDs are known to positively influence insulin sensitivity and β -cell function and potentially alter lipid profiles. Pioglitazone is a synthetic ligand of PPAR γ and is used as a therapeutic treatment for patients with T2DM. PPAR γ , a ligand-activated transcription factor, is expressed in white and brown adipose tissues as well as in the urinary bladder ⁴⁰. Pioglitazone has also been shown to reduce macrophage infiltration through the activation of PPAR γ and induction of apoptotic cell death ⁴¹, which can lead to decreased numbers of macrophages in adipose tissues ⁴². To examine the impact of PPAR γ on bladder cell function, several studies analyzed the expression of PPAR γ in normal bladder and bladder cancer (BC) mucosal samples. They observed different rates of cell migration and invasion in various BC cell lines that express PPAR γ . In addition, prior studies have shown that vascular endothelial growth factor A (VEGF-A), a primary proangiogenic factor, is positively regulated by PPAR γ ⁴³. PPAR γ can also exert its functions via the activation of signaling pathways, interaction with Wnt/ β -catenin, and induction of signaling pathways involved in proliferation and survival, such as IGF-I/PI3K/AKT/mTOR and MAPK ⁴⁴. Furthermore, PPAR γ can regulate the inflammatory response of macrophages through TLR4/NF- κ B signaling ⁴⁵.

There have been contradictory reports on the potential association between diabetes mellitus (DM) and elevated risk of BC ^{46,47}. Studies have demonstrated that pioglitazone can have adverse effects that exaggerate the risk of BC. DM patients, particularly males, on long-term, high doses of pioglitazone, are at a higher risk of developing BC ^{48,49}. The Kaiser Permanente Northern California (KPNC) study determined that inflated dose and length of pioglitazone treatment correlated to increased rates of BC ⁴³. Currently, the U.S. Food and Drug Administration (FDA) allows the use of pioglitazone in patients with special caution to those with a prior history or active BC ⁵⁰. A potential reason for this observation could be that PPAR γ signaling in BC cells may provide a tumor microenvironment that allows for de novo lipogenesis of lipids that can be utilized in increasing tumor mass and energy usage ⁵¹. However, other studies have shown that TZDs posed no risk on survival in BC patients who underwent radical cystectomy ⁵². A recent meta-analysis found no difference in BC incidence among users of pioglitazone and nonusers ⁵³. These inconsistencies between studies have led to questions in the legitimacy of studies and the actual dangers of continued pioglitazone use. Due to the inherent difficulty of accurately assessing the link between TZDs and BC, the association between the two is still in controversy. ⁵⁴

The goal of our study was to determine whether TZDs have any negative or positive effects on the healthy bladder epithelium. Since pathophysiological changes require the actions of a series of key proteins, global proteomic analysis of protein alterations using state-of-the-art mass spectrometry is ideal for acquiring unbiased biological information regarding molecular mechanisms. Using such a method, this study aimed to understand the mechanistic meanings of proteome perturbations in the normal bladder due to TZD treatment. We sought to examine the effects of pioglitazone specifically, being that is the most widely used TZD. Our global quantitative proteomic analyses found that pioglitazone

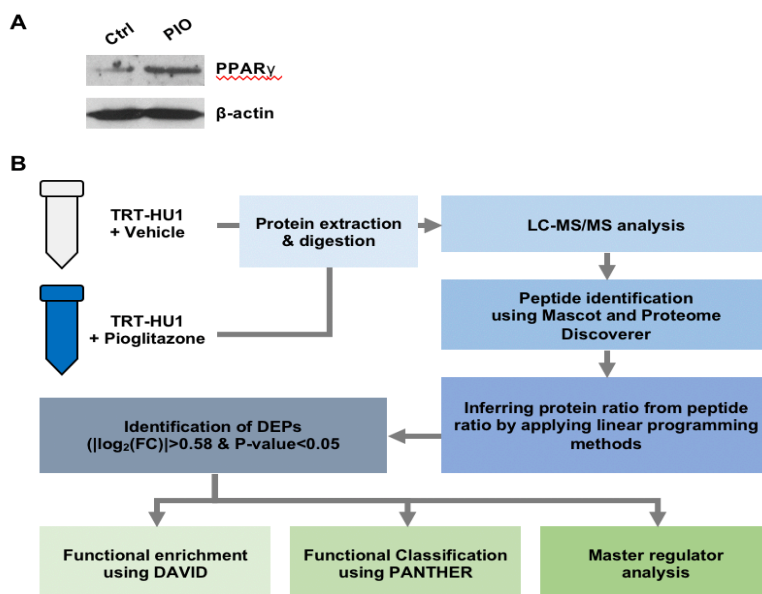


Figure 1. Overview of global proteomics workflow for data generation and analysis. (A) Western blot analysis shows the effects of pioglitazone treatment on PPAR γ expression. **(B)** Proteomics analysis workflow for this study.

3. Classification of the urinary metabolome using machine learning and potential applications to diagnose interstitial cystitis

Running Title: Machine learning for IC diagnosis

INTRODUCTION

Interstitial cystitis (IC), also known as painful bladder syndrome or bladder pain syndrome, is a chronic visceral pain syndrome of unknown etiology that presents itself as a constellation of symptoms, including bladder pain, urinary frequency, urgency, and small voided volumes, in the absence of other identifiable diseases. Urine is in direct contact with the bladder epithelial cells that could be giving rise to IC; as a result, metabolites released from bladder cells may be enriched in urine.

The urinary metabolome was previously investigated by our group for potential IC diagnostic biomarkers. We attempted to identify IC-associated metabolites from urine specimens obtained from IC patients and controls using nuclear magnetic resonance (NMR). Our findings provided preliminary evidence that metabolomics analysis of urine can potentially segregate IC patients from controls. We sought to capture the most differentially detected NMR peaks and discern if there was a significant difference in the peak distribution between IC and control specimens. Based on multivariate statistical analysis, principal component analysis (PCA) suggested that the urinary metabolome of IC patients and controls were clearly different; 140 NMR peaks were significantly altered in IC patients (FDR < 0.05) compared to controls.

Machine learning (ML), originally described as a program that learns to perform a task or make decisions based on data, is a valuable and increasingly necessary tool for modern healthcare [8]. However, this definition is broad and could cover nearly any form of data-driven needs. ML is not a magical approach that can turn data in immediate benefits, even though many news outlets imply that it can. Rather, it is natural extension to traditional statistical approaches. In our present study, we utilized ML and automated performance metrics to evaluate the clinical value of our 140 identified NMR peaks. We used ML algorithms examine the relationship between metabolic expression and disease. We applied logistic regression (LR) and support vector machine (SVM), which are traditionally known to work well even with small sample sizes, to our metabolomics signatures and used this data together with patient clinicopathological features to diagnose IC. We used our dataset of 59 cases to train, test, and validate the model. The results showed that our ML-based algorithms were able to successfully identify IC patients from healthy subjects.

This study aimed to address the question of, “Does utilizing metabolic data in ML play a role in diagnosing IC?”. ML is a form of artificial intelligence (AI) and learns from past data in order to predict the future. Our NMR-based ML algorithm was able to collectively distinguish the IC patient urinary profile from that of controls.

RESULTS

Classification of IC samples with SVM

SVM was applied to the IC dataset with the leave-one-out CV scheme to classify IC samples from controls. The result varied depending on user parameters (*i.e.*, kernel type and kernel parameters). Comparing the numbers, it was found that SVM with polynomial kernel resulted in the best performance when the degree of the polynomial kernel was 3 with 86.4% accuracy, 0.88 AUC of PR curve, and 0.85 AUC of ROC curve. Although the accuracy was the highest when the degree was 5, the AUCs of ROC and PR curves with degrees set to 3 was the highest. Moreover, the degree equal to 3 has less chance of overfitting than a degree of 5.

Here, the usage of linear kernel did not perform well. It may be because the data were not linearly separable or simply the sample size ($N = 59$) was too small compared to the dimension of the data (*i.e.*, 140 features). Performance of RBF kernel was also poor; looking at the accuracy using RBF kernel with SVM (*i.e.*, 72.9%), it was the same as the proportion of IC samples in the dataset (*i.e.*, 43 IC subjects out of 59 subjects) and its recall was 1. This means that the classifier was simply predicting that all the samples belong to IC group and was not able to handle the class distribution imbalance problem.

Classification of IC samples with LR

In addition to SVM experiment, LR was used to classify IC samples with different user parameter settings. LR with l_1 -penalty yielded the best performance when its penalty parameter was set to 10 with 84.7% accuracy, 0.91 for AUC of PR curve and 0.86 for the AUC of ROC curve, which was slight better than the results from SVM.

It was observed that LR worked well despite being a linear model. Notice that the performance of linear SVM was poor; this is because of the l_1 -norm penalty applied to the trained parameter imposing sparsity and behaving as a natural feature selector. When we checked the trained weight of features, most of the weights converged to 0 (a very small number on average of absolute values across the leave-one-out process). When the penalty parameter was 10, the average weights of 133 features was less than or equal to 0.1. This means that we only need a few critical features to predict correct label. In our experiment, feature $ID = 73, 4, 129$, and 35 were the most dominant features with the highest weights regardless of the random initialization. In other words, they were the four most useful NMR features. We have performed further statistical group analysis on these four NMR peaks using two-sample t -test, which resulted in P -values of 0.003, 0.001, 0.057, and 0.036 respectively. It was interesting to see that there were many other NMR peaks with even lower P -values and the peak $ID = 129$ had a P -value greater than 0.05. While these statistical tests are performed independently, our classification results were derived by taking all the peaks at the same time for the analysis and it demonstrates that a linear combination of the features can be more powerful to distinguish IC from controls.

The l_2 -norm constraint did not contribute much in these experiments. This is because the model can robustly operate even without the l_2 -norm regularizer, which typically degrades performance of models in exchange for model robustness. Especially with the l_1 -norm regularizer significantly lowering the dimension of the data (with 133 redundant features), the sample size ($N = 59$) was sufficient to make robust and correct predictions for IC samples.

What opportunities for training and professional development has the project provided?

If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. “Training” activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. “Professional development” activities result in increased knowledge or skill in one’s area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

Nothing to Report

How were the results disseminated to communities of interest?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

Nothing to Report

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

In coming funding period, we will focus on quantification of levels of DNA methylation and gene expression for the IC diagnostic marker panel. We will further analyze patients’ outcomes and construct a statistical model predicting the IC-associated pain severity and to evaluate its association with co-morbid conditions, patient-reported outcomes, and health-related quality of life.

4. IMPACT: Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

What was the impact on the development of the principal discipline(s) of the project?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

- State-of-the-art technologies emerging from recent DNA methylation studies of urine specimens of IC, including the innovative concept that the urine plays an active role modulating bladder microenvironment as a reliable diagnostic biofluid, providing epigenetic resource associated with bladder pain.
- A robust statistical model to obtain comprehensive mechanistic insights into the IC etiology
- Translating DNA methylation and gene expression biomarkers into routine clinical diagnostics for IC risk will be aided by developing automatic quality controls and absolute quantifications for biomarkers in these panels by working with an industry partner who is committed to commercial development and clinical implementation of molecular tests.

What was the impact on other disciplines?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

Nothing to Report

What was the impact on technology transfer?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Nothing to Report

What was the impact on society beyond science and technology?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

Nothing to Report

- 5. CHANGES/PROBLEMS:** *The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, “Nothing to Report,” if applicable:*

Changes in approach and reasons for change

Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

Nothing to Report

Actual or anticipated problems or delays and actions or plans to resolve them

Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

Nothing to Report

Changes that had a significant impact on expenditures

Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

Nothing to Report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

Nothing to Report

Significant changes in use of biohazards and/or select agents

Nothing to Report

6. **PRODUCTS:** *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state “Nothing to Report.”*

- **Publications, conference papers, and presentations**

Report only the major publication(s) resulting from the work under this award.

Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume; year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

1. Kim SJ, **Kim J***, Yoon H*. (2019) Sexual Pain and IC/BPS in Women, *BMC Urology*, 19(1):47, PMID:31170952 ***co-corresponding authors - acknowledgement of federal support (yes).**
2. Kim W-J, **Kim J.** (2019) Innovative technologies for the smart E-Healthcare system *Investig Clin Urol.* 60(1):1-3. PMID:30637354 **acknowledgement of federal support (no).**
3. Myung **NV**, Jung S, **Kim J.** (2019) Application of low-cost, easy-to-use, portable biosensor systems for diagnosing bladder dysfunctions. *International Neurourology Journal.* 23(1):86-87, PMID:30943698 **acknowledgement of federal support (no).**
4. Chung KJ, **Kim J**, Whangbo TK, Kim KH. (2019) The Prospect of a New Smart Healthcare System: A Wearable Device-Based Complex Structure of Position Detecting and Location Recognition System. *International Neurourology Journal.* 23(3):180-184. PMID:31607096, PMCID:PMC6790815 **acknowledgement of federal support (no).**
5. Shahid M, Kim M, Yeon A, You S, **Kim J.** (2020) The Effects of Pioglitazone on Proteome of Normal Bladder Epithelial Cells, *International Neurourology Journal*, 24(1):29-40. PMID:32252184, PMCID:PMC7136443 **acknowledgement of federal support (yes).**
6. **Kim J**, Kim WT, Kim W-J. (2020) Advances in urinary biomarker discovery in urological research, *Investigative and Clinical Urology*, 61(Suppl 1):S8-S22. <https://doi.org/10.4111/icu.2020.61.S1.S8> PMID:32055750 **acknowledgement of federal support (no).**
7. Jung S, **Kim J.** (2000) Biomarker Discovery and Beyond for Diagnosis of Bladder Diseases. *The Bladder*, 7(1):e40, **Selected as a Cover Image acknowledgement of federal support (yes).**
8. **Kim J**, Jin P, Kim WH, Kim W-J. (2000) Utilizing Machine Learning to Discern Hidden Clinic Values from Big Data in Urology, *Investigative and Clinical Urology*, 61:239-24, PMID: 32377598 **acknowledgement of federal support (yes).**
9. Tang F, Shahid M, Peng J, Jung S, Kim WH, **Kim J.** Machine Learning Classifiers of Urine Metabolome and Its Potential Applications to Diagnosis of Interstitial Cystitis, *Bladder*, in press, **acknowledgement of federal support (yes).**

Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

1. Jin F, Shahid M, Kim J. Research Progress of Urine Biomarkers in the Diagnosis, Treatment, and Prognosis of Bladder Cancer, Springer, Nature, in press, **acknowledgement of federal support (yes).**

Other publications, conference papers and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

Nothing to Report

- **Website(s) or other Internet site(s)**

List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

Nothing to Report

- **Technologies or techniques**

Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

Nothing to Report

- **Inventions, patent applications, and/or licenses**

Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

Nothing to Report

- **Other Products**

Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:

- *data or databases;*
- *physical collections;*
- *audio or video products;*
- *software;*
- *models;*
- *educational aids or curricula;*
- *instruments or equipment;*
- *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
- *clinical interventions;*
- *new business creation; and*
- *other.*

Nothing to Report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of

compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate “no change”.

Example:

*Name: Mary Smith
Project Role: Graduate Student
Researcher Identifier (e.g. ORCID ID): 1234567
Nearest person month worked: 5*

*Contribution to Project: Ms. Smith has performed work in the area of combined error-control and constrained coding.
Funding Support: The Ford Foundation (Complete only if the funding support is provided from other than this award.)*

Name:	Jayoung Kim
Project Role:	PI no change
Name:	Jennifer Anger
Project Role:	Co-Investigator no change
Name:	Catherine Breese
Project Role:	Co-Investigator no change
Name:	Muhammad Shahid
Project Role:	Post-doctoral fellow no change

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

Nothing to Report

What other organizations were involved as partners?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

Provide the following information for each partnership:

Organization Name:

Location of Organization: (if foreign location list country)

Partner’s contribution to the project (identify one or more)

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner’s facilities for project activities);*
- *Collaboration (e.g., partner’s staff work with project staff on the project);*
- *Personnel exchanges (e.g., project staff and/or partner’s staff use each other’s facilities, work at each other’s site); and*
- *Other.*

Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: *For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to <https://ers.amedd.army.mil> for each unique award.*

QUAD CHARTS: *If applicable, the Quad Chart (available on <https://www.usamraa.army.mil>) should be updated and submitted with attachments.*

- 9. APPENDICES:** *Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.*

Biomarker discovery and beyond for diagnosis of bladder diseases

Sungyong Jung¹, Jayoung Kim^{2,3*}

¹Department of Electrical Engineering, University of Texas at Arlington, Arlington, TX 76019, USA

²Departments of Surgery and Biomedical Sciences, Cedars-Sinai Medical Center, Los Angeles, CA 90048, USA

³Department of Medicine, University of California Los Angeles, CA 90095, USA

*Corresponding author: Jayoung Kim, Email: Jayoung.Kim@cshs.org

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Abbreviations used: BPS, bladder pain syndrome; CGM, continuous glucose monitors; DID, diabetes interactive diary; IHC, immunohistochemistry; IC, interstitial cystitis; IoT, internet of things; MAPP, multidisciplinary approach to the study of chronic pelvic pain; NIH, National Institutes of Health; ROC, receiver operator characteristic; SMBG, self-monitoring of blood glucose; UI, urinary incontinence; UTI, urinary tract infection

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ABSTRACT

Molecular biosignatures of altered cellular landscapes and functions have been casually linked with pathological conditions, which imply the promise of biomarkers specific to bladder diseases, such as bladder cancer and other dysfunctions. Urinary biomarkers are particularly attractive due to costs, time, and the minimal and noninvasive efforts acquiring urine. The evolution of omics platforms and bioinformatics for analyzing the genome, epigenome, transcriptome, proteome, lipidome, metabolome, etc., have enabled us to develop more sensitive and disease-specific biomarkers. These discoveries broaden our understanding of the complex biology and pathophysiology of bladder diseases, which can ultimately be translated into the clinical setting. In this short review, we will discuss current efforts on identification of promising urinary biomarkers of bladder diseases and their roles in diagnosis and monitoring. With these considerations, we also aim to provide a prospective view of how we can further utilize these bladder biomarkers in developing ideal and smart medical devices that would be applied in the clinic.

Keywords: biomarker; bladder; urine; medical device; biosensor

URINE AND URINARY BIOMARKERS FOR BLADDER DISEASES

Urine is a waste product that is readily produced by all patients and contains a wealth of information. It can be produced in high-volume and procurement of samples is noninvasive. Considering these factors alone, urine is a highly attractive potential resource. However, there are several glaring issues that make urinalysis difficult. Factors such as preanalytical reliability and data analysis can be a major challenge [1,2]. Transport and preservation of urine samples are particularly important. It has been shown that increased time gaps between sampling and analysis, lack of temperature control, and lack of preservatives for samples that cannot be analyzed within two hours after collection can lead to low-quality test results [3]. However, preservatives may also affect the chemical properties and alter the appearance of certain particles [4]. Additionally, urine contains much more complex compounds that

can be affected by a wide range of external factors, including diet and environment [5]. A comparative urinary metabolite profiling study of habitual diet discovered that 417 urinary metabolites were correlated with more than one food, beverage, or supplement [6]. Exposure to different environmental toxins and chemicals have been shown to be reflected in urine. A study of pediatric exposure to pyrethroids, an insecticide, found differing concentrations of the chemical in urine based on each child's level of risk [7]. Fortunately, recent advances in technology and standardization have made urinalysis more of a viable option for a number of clinical issues [8]. Because the uroepithelial-associated sensory web may be related to hypersensitive benign urological disorders [9], it is not always necessary that clinopathological status results in a change in urinary components. As the pathology of genitourinary diseases is being better understood, more diagnostic and prognostic biomarkers are also being identified [10]. A recent study reported that 4 urinary biomarkers were associated with kidney injury [11]. By

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integrating newer technologies with increased knowledge of diseases, novel biomarkers can be discovered.

MULTI-OMICS APPLICATION FOR BLADDER BIOMARKER DEVELOPMENT

Omics involves the high-throughput analysis of different domains of biological information, including the genome, transcriptome, proteome, and metabolome [12,13]. Comprehensive omics analysis of urine can be a potentially valuable source of disease biomarkers. For instance, the proteomic profile of healthy urine can be used as a standard to compare disease-state urine to identify proteins of interest [14]. Recent new types of software are being developed to create workflows that involve distinguishing biomarkers *via* integrated comparative and quantitative analysis [15]. Advanced proteomic analysis has led to high-throughput profiling of bladder cancer-related proteins with both high sensitivity and specificity, which has resulted in a wealth of informative biomarkers [16]. A similar strategy was utilized in a recent study that identified 54 potential protein biomarkers of bladder schistosomiasis by quantitatively comparing urinary samples from humans [17]. Other types of omics applications, such as genomics, epigenomics, transcriptomics and metabolomics, were also applied to determine biomarkers of bladder schistosomiasis. Metabolomic profiling using urine and plasma samples revealed that the perturbed glycerophospholipid and sphingolipid metabolisms are associated with schistosomiasis and its associated-bladder cancer pathologies [18]. Epigenetic regulation on RASSF1A and TIMP3 were found using a quantitative methylation-specific PCR assay in urine sediments of patients with schistosomiasis infection. Hypermethylation of both RASSF1A and TIMP3 shows 77.55% of area under the receiver operator characteristic (ROC) curves ($P = 0.023$) [19]. Another study profiled urinary amino acids to identify potential biomarkers for lower urinary tract symptoms in male patients [20]. As non-invasive disease biomarkers, urinary extracellular vesicles such as exosomes have been discovered to contain a variety of molecular and genetic materials including nucleotides, proteins, metabolites, miRNAs, and they function as a cargo and transfer those materials to nearby neighbor cells [21,22]. Progress in these comprehensive tests continues to increase our understanding of the complexity of biomarkers that underlie diseases and, with technology, it is becoming easier to navigate how to utilize them.

MICROBIOME STUDIES IN UROLOGICAL DISEASES

The microbiome is defined as the collective genome of all microorganisms in an environment [23]. Interest in this field has recently boomed as it has been shown that microbiota and alterations in their communities can contribute to the pathogenesis of chronic urological diseases, such as urothelial carcinoma [24]. A preliminary study found an association between urinary dysbiosis and urothelial carcinoma, suggesting that the ratio for microbiota could be used as a potential diagnostic indicator [25]. Another study observed that bacterial richness increased in the urine of patients with cancer compared to controls [26]. However, despite all the promising exploratory data surrounding microbiome's usage in urological diseases, the field is still relatively new and more comprehensive studies are needed [27]. Studies on the influence of microbiota expand beyond the genitourinary tract as well.

For instance, *Helicobacter pylori* is, well-documented, increasing the risk of duodenal and gastric ulcer disease and gastric cancer [28]. Bacterial pathogenesis is also noted to be potentially associated with colorectal cancer [29]. Based on the extensive role of microbiomes in many diseases, a better understanding of urinary microbes and their roles in urological diseases may prove to be significant.

Aside from potential utilization of the microbiome in diagnostics and prognostics, identifying present microbiota may be important when it comes to various treatments. For instance, gastrointestinal microbes are known to affect the metabolism and toxicity of various agents [30]. *Mycoplasma hyorhinis* has been shown to metabolize and inactivate gemcitabine, a chemotherapeutic drug, which can result in drug resistance [31]. Additionally, reactivation of the inactive metabolites of irinotecan, a topoisomerase I inhibitor, by gastrointestinal bacteria can lead to adverse toxicities, such as severe diarrhea [32]. For urological diseases, there are also some noted interactions between microbiota and treatment. It has been shown that D-mannose, a simple sugar, can hinder bacterial adhesion to the urothelium, thereby reducing risk of urinary tract infections and aiding in acute cystitis management [33].

The urinary microbiome is believed to play an important role in predicting disease status for many different urogenital diseases. Recently, a pilot study looking into the relationship between the urinary microbiome and bladder cancer uncovered that that bacteria belonging to the genus *Fusobacterium* were significantly more abundant in urine specimens from cancer patients [34]. Another exploratory study comparatively surveyed the urine microbiota of female patients with interstitial cystitis (IC)/bladder pain syndrome (BPS) and controls who were enrolled in the National Institutes of Health (NIH) Multidisciplinary Approach to the Study of Chronic Pelvic Pain (MAPP) Research Network. It identified potential negative impacts of the presence of *Lactobacillus gasseri* and protective influence of *Corynebacterium* [35]. It should be noted that a different study on urinary incontinence (UI) found a lack of *Lactobacillus* to be associated with urgency UI and resistance to anticholinergic treatments [36]. However, being that these are two different diseases, the conflicting results are not unexpected. Furthermore, there are many species of *Lactobacillus* and some may contribute to a healthy or disease bladder. On the other hand, another study that collected urinary samples from 21 IC patients and 20 matched controls found no significant differences in urinary microbiota [37]. The conflicting conclusions between these two recent studies highlight controversy surrounding this fairly new field and the need for a more comprehensive longitudinal study.

CHALLENGES AND CONSIDERATION IN URINE BIOMARKER DEVELOPMENT

Despite the promising potential of urinary biomarkers, there are several precautions to consider. One important factor that can affect biomarker outcomes is age. Studies have shown that the maturing kidney can affect biomarker levels and interpretation, suggesting that age-specific biomarker reference ranges may be needed for certain diseases [38]. Furthermore, baseline metabolites have been shown to be different among different age-groups, which may highlight carefully establishing different age groups should be warranted when conducting urinalysis [39]. Gender is another factor to be considered when establishing reference values for urinary biomarkers [14]. Proteomic analysis of female and male urine observed different patterns and variations of proteins

[40]. Given that urine sample can have huge variation in concentration of proteins or metabolites due to the fluid consumption, special care should be taken to data normalization methods to reduce any potential artifacts [41]. Furthermore, external factors that are dependent on individuals can influence the expression of urinary biomarkers. Studies have shown differences in expression of urinary biomarkers in patients who have undergone cisplatin therapy [14]. Certain procedures can also affect urinary levels of metabolites; another study found increased urinary neurotrophin in women with stress urinary incontinence after a midurethral sling procedure [42]. This suggests that in order to effectively utilize urinalysis, there needs to be a comprehensive understanding of the fluctuations in biomarkers that can occur within each individual.

BIOSENSOR FOR THE DETECTION OF URINARY BIOMARKERS

Biosensors are an arising field of great interest when it comes to detecting and monitoring markers in biofluids, such as sweat and urine. Wearable sensors are particularly garnering attention because they can be portable, convenient, non-invasive, and provide real-time evaluation of important biomarkers [43]. In addition to its detection and monitoring benefits, biosensors could also be integrated with therapeutic drugs to monitor for response to treatments [44]. The potential for sensors can extend to many different types of situations. For example, biosensors can be developed into electrochemical sensors or fluid measuring sensors [45]. These biosensors can be constructed to detect various compounds, such as antigens, biomarkers, and bacterial enzymes.

With the advent of smart technologies, there has been exciting developments in utilizing such devices in healthcare as well. In 2015, a team of biomedical engineers at the University of Arizona was able to develop a highly-sensitive and cost-efficient paper-based analytical device (μ Pad) that could monitor urine for urinary tract infection (UTI) and gonorrhea [46]. A recent study developed a similar device that quantified β -glucuronidase, an enzyme released by 95% of *E. coli*, the bacteria that causes UTI [47]. In addition to these urinalysis-based detection devices, several others have been developed to detect other compounds. A study by the Southern Taiwan University of Science and Technology developed an ultraportable microsensor-lined biosensor that can actually quantify the presence of Gal-1, a protein biomarker indicative of multiple oncological conditions, including bladder cancer [48]. These novel devices only scratch the surface of the great potential for biosensors.

The use of technology can also extend beyond detection. Taking advantage of the fact that most people use a smartphone, a study in the United Kingdom crowdsourced members of the public to grade immunohistochemistry stains of bladder cancer tumor microarrays [49]. Surprisingly, this was found to be a potentially accurate way to screen immunohistochemistry (IHC) data and speed biomarker discovery.

DIGITAL APPLICATIONS OF BIOSENSORS

The rise of digital applications of biosensors is also a rising field of great interest. There are incredible possibilities that comes from being able to use everyday technology to monitor health. Not only would this reduce risks to patients and lower healthcare costs, but it could also

lead to an immense wealth of data that can be used to pioneer science even further. The most commonly used interactive app for monitoring has been in diabetes. Currently, there are two major mobile apps that incorporate self-monitoring of blood glucose (SMBG) recording and insulin bolus calculators. These are Diabeo (Voluntis) and Diabetes Interactive Diary (DID) [50]. Studies have shown that monitoring of patients with type 1 diabetes by using Diabeo can lead to substantial improvement in metabolic control in chronic poorly controlled patients without requiring more medical time and at a lower cost than typical standard care [51]. Similar studies with DID show that it can reduce risk of moderate to severe hypoglycemia while also improving quality of life [52]. However, these apps are still a work in progress and have only shown improvements in certain areas of diabetes monitoring. With rapid technological innovation and progress, the focus on making these apps better should be continued.

In addition to real-time monitoring of chronic diseases, digital applications can lead to an enormous wealth of health data that can be used for more comprehensive studies. For instance, adding internet of things (IoT) capabilities to commercially used continuous glucose monitors (CGM) can lead to both the monitoring of patients remotely and crowdsourcing of that data [53]. As personal tech becomes increasingly embedded in the lives of patients, digital phenotypes can be captured to enhance health and wellness [54]. There is one caveat with this integration of technologies with personal health. As information is formed and sourced, careful attention must be paid to decentralizing databases and ensuring that patient health information remains private and protected. With proper cyber security, the promises of digital health monitoring are endless.

CONCLUDING REMARKS

Advances in urine-based molecular profiling technologies, the development of biosensor targeting disease-specific biomarkers and the wirelessly connected medical device would lead to smart diagnosis and monitoring for patients affected by bladder diseases. Thanks to rigorous efforts of scientists and urologists including us to define biomarkers for bladder diseases such as bladder cancer and other types of bladder dysfunction, we have better idea how to manage those bladder diseases. As we discussed in this paper, the current evidence suggests the integration of multi-omics profiling-based characterization of bladder diseases and application of urinary biomarkers into smart medical device could lead future tools for patient care.

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REVIEW

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Sexual pain and IC/BPS in women

Su Jin Kim^{1,5}, Jayoung Kim^{2,3*} and Hana Yoon^{4*}

Abstract

Interstitial cystitis/bladder pain syndrome (IC/BPS) and female sexual dysfunction (FSD) are common conditions that substantially reduce women's health. In particular, women with IC/BPS show vulvodynia, a kind of FDS that originates from consistent pain around the vulvar area. There have been many studies attempting to find the underlying mechanisms that induce the chronic pain associated with IC/BPS and vulvodynia and explain why these two conditions often coexist. Proposed theories suggest that pain hypersensitivity is being mediated by peripheral and central sensitization. However, there are still many unknown factors, such as etiologies, that can evoke pain hypersensitivity and may be linking the casual relationship between IC/BPS and vulvodynia. At present, knowledge regarding IC/BPS and vulvodynia are insufficient when considering their clinical importance. Therefore, efforts are necessary to elucidate the issues surrounding IC/BPS and vulvodynia.

Keywords: Cystitis, interstitial, Sexual dysfunction, Chronic pain, Vulvodynia, Lower urinary tract symptoms, Quality of life

Background

Female sexual dysfunction (FSD) and interstitial cystitis/bladder pain syndrome (IC/BPS) are two conditions affecting women's health. A study on the association between sexual and general well-being found that women reported better quality of life (QoL) with higher sexual satisfaction, regardless of age and/or menopausal status [1]. Both FSD and IC/BPS significantly impairs a woman's abilities to pursue and enjoy sexual relations. Approximately 40–50% of women experience FSD and 0.5–12% experience IC/BPS. Considering these incidence rates, both FSD and IC/BPS present serious challenges for patients and clinicians [2–6].

Chronic pain deteriorates not only personal health and wellness, but also QoL. Chronic pain can induce sexual dysfunction, such as arousal disorder, and relationship problems [7]. Furthermore, studies have shown that significantly more women with chronic pelvic pain (CPP) show FSD compared to those without CPP. Women with CPP and FSD reported various types of sexual dysfunctions, including hypoactive sexual desire disorder, sexual arousal disorder, orgasmic disorder, and sexual pain disorder [8].

The symptoms of IC/BPS, such as urinary frequency, urgency, and pelvic pain, can have a negative impact on sexual activity and QoL [9]. Women with overactive bladder (OAB) frequently have a risk for sexual dysfunction [10]. In the postmenopausal group, women with scores indicating severe OAB reported worse sexual function, particularly in the arousal, lubrication, orgasm, pain, and total domains [11]. Despite the known association between FSD and bladder diseases, contributing risk factors have yet to be explored. Evaluating the impact of duration, severity, pain localization, sexual trauma history, anxiety, and depression associated with sexual dysfunction may help elucidate risk factors. Increasing our knowledge about sexual dysfunction as it relates to bladder diseases may aid in clinical diagnoses, treatment strategies, and overall symptom improvement. This review will provide an overview of studies that address FSD in women with IC/BPS.

Pain in FSD

According to consensus from the 4th International Consultation on Sexual Medicine (ICSM), FSD is classified as hypoactive sexual desire dysfunction, female sexual arousal dysfunction, female orgasmic dysfunction, female-genital-pelvic pain dysfunction, persistent genital arousal disorder, postcoital syndrome, hypohedonic orgasm, or painful orgasm [2, 12]. Guidelines from the 4th ICSM also define pain-associated FSD as female-genital-pelvic pain dysfunction and include all conditions that inhibit sexual

* Correspondence: Jayoung.Kim@csmc.edu; wowhana@ewha.ac.kr

²Departments of Surgery and Biomedical Sciences, Cedars-Sinai Medical Center, Los Angeles, CA 90048, USA

⁴Department of Urology, Medical Research Center, Ewha Womans University School of Medicine, Seoul, Republic of Korea

Full list of author information is available at the end of the article



intercourse or induce negative effects on sexual functions. Female-genital-pelvic pain dysfunction is different from the previous classification characterized as pain associated with FSD, which includes sexual pain disorder including dyspareunia and vaginismus [13]. According to the ICSM, female-genital-pelvic pain dysfunction includes persistent or recurrent difficulties with at least one of the following: (1) vaginal penetration during intercourse, (2) marked vulvovaginal or pelvic pain during genital contact, (3) marked fear or anxiety about vulvovaginal or pelvic pain in anticipation of, during, or as a result of genital contact, or (4) marked hypertonicity or overactivity of pelvic floor muscles with or without genital contact [14]. Painful orgasm also falls under this category and is defined as genital and/or pelvic pain during or shortly after orgasm [2]. These recent new definitions reflect the evolving concept behind pain-associated FSD as it also now includes pain in the vulvovaginal and pelvic area and pelvic floor hypertonicity. The new categorization of female-genital-pelvic pain dysfunction has broadened the view on pain-associated FSD and has differentiated it from its previous classification, which confined symptoms to each organ or single disease. This recent change in classification by the ICSM considers pain-associated FSD as a complex condition influenced by psychological and physical factors and supports the general thought that FSD is multifactorial.

Previously, vulvodynia had been generalized under chronic vulvar pain with no precise discrimination for it. However, recent consensus has redefined chronic vulvar pain as vulvar pain associated with specific diseases, such as inflammation, neoplasm, and/or injury. This was conducted at a conference with the International Society for the Study of Vulvovaginal Disease, the International Society for the Study of Women's Sexual Health, and the International Pelvic Pain Society [15]. Vulvodynia can have diverse pain characteristics; therefore, pain-based classification helps in identification, diagnosis, and treatment. Vulvodynia can be characterized as either general (entire vulva) or localized (parts of the vulva). Additionally, based on the situation of the pain, vulvodynia can be classified as either provoked (triggered by physical contact) or unprovoked (spontaneous occurrence without specific triggers) [16]. Women of all ages can experience vulvodynia, and provoked vulvodynia is the most commonly diagnosed [17, 18]. Provoked vulvodynia is thought to be more widely diagnosed than unprovoked because its symptoms can be better recognized by doctors. Therefore, there is a great need to better identify unprovoked vulvodynia patients.

Sexual pain and IC/bps

IC/BPS is a disorder that induces chronic pain or discomfort in the bladder and surrounding pelvic organs [19, 20]. At present, IC/BPS is not a disease confined to just the

bladder and pelvic area; it is a complex disease that includes the outside of the genitourinary tract. Tripp et al. [21] investigated the pain characteristics of IC/BPS using whole-body diagram pain locators. They found that women with IC/BPS reported significantly more pain all over their body, compared to healthy women without. Only 52 of the 193 IC/BPS diagnosed women (27%) presented pain restricted to the bladder and pelvic area. Moreover, IC/BPS patients had various co-morbidities. Diseases, such as irritable bowel syndrome, fibromyalgia, vulvodynia, chronic pelvic pain, endometriosis, OAB, allergies and chronic fatigue syndrome, were found to coexist in IC/BPS patients [22–28].

In addition, there are several reports that IC/BPS can increase the risk for or worsen other diseases, including FSD. A population-based study found higher prevalence of FSD in women with IC/BPS [29]. FSD incidence also increased depending on the severity of IC/BPS symptoms, suggesting that FSD is a factor may be worsening IC/BPS. In regards to the pain associated with FSD, vulvodynia may be contributing to flare-ups of IC/BPS symptoms and could be the reason why IC/BPS patients avoid sexual activity [9, 30].

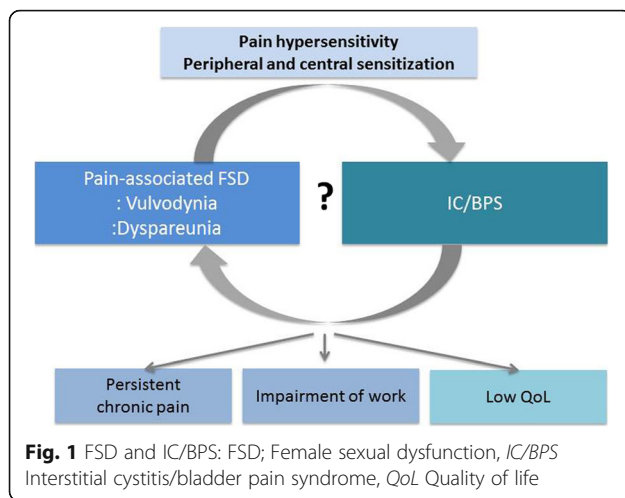
Can FSD and IC/bps be generalized as one disease?

Diseases characterized with lower urinary tract symptoms (LUTS) in females, such as incontinence and OAB, are known to have a negative impact on all domains in female sexual function. Symptoms of IC/BPS can also deteriorate patient's sexual activities and QoL. A significant number of IC/BPS patients avoid sexual activity because of pain. In addition, FSD and IC/BPS share similar clinical characteristics and comorbidities, making it difficult to discriminate between the two [31–33]. As mentioned previously, there are many clinical reports presenting an association between IC/BPS and vulvodynia, mainly due to shared FSD conditions.

Although the mechanisms of the association between IC/BPS and vulvodynia are unclear, visceral nerve cross-talking and the anatomic relationship between genital organs and the bladder offer a simple proximity explanation. Another possible mechanism behind the relationship is abnormal pain hypersensitivity induced by peripheral and central sensitization. The abnormal pain response frequently observed in vulvodynia patients is caused by central or peripheral maladaptive pain processing from local insult, injury, or trauma (Fig. 1).

Role of peripheral sensitization in the pain hypersensitivity of vulvodynia and IC/BPS

Exposure of nociceptors to repetitive pain stimulation reduces the pain threshold and amplifies the responsiveness of nociceptors. Therefore, this can abnormally increase peripheral transduction of sensitivity and lead to the



development of peripheral pain hypersensitivity [34–36]. The potential underlying mechanism of peripheral pain hypersensitivity noted in both vulvodynia and IC/BPS could be due to sensory nerve upregulation. Previous studies have shown that sensory nerve density is significantly increased in the vulva vestibule and bladder. Compared to the normal controls, patients with vulvodynia were found to have increased nociceptors in their vulvar vestibule [37–40]. Consequentially, this increased density of peripheral nociceptors results in increased sensitivity. It was also found that transient receptor potential V1 (TRPV1) exists in these nociceptive nerve endings and enhances pain signaling [37, 41, 42]. Pukall CF et al. [43] and Giesecke J et al. [44] reported increased peripheral tactile, pressure, and pain sensitivity in the patients with vulvodynia and confirmed histologic and molecular changes of peripheral nociceptors is associated with clinical manifestations. Similarly with vulvodynia, it has been reported that IC/BPS patient bladders have upregulated sensory innervation and TRPV1 expression [45–47].

Role of central sensitization in the pain hypersensitivity of vulvodynia and IC/BPS

Central sensitization is an important mechanism underlying various conditions associated with chronic pain and induces pain hypersensitivity through pathologically enhanced pathways that are not normally associated with nociception. For example, the low-threshold Aδ fiber, that is mostly used for temperature and pressure signaling, can be sensitized to pain. Chronic pain induced by central sensitization is persistent even after initiation of the signal and disappearance of the peripheral cause [48]. Studies have shown that the pain characteristics of central sensitization can be found in vulvodynia patients. Foster DC et al. [49] observed that vulvar vestibulitis syndrome patients experienced hyperalgesia and allodynia more often than normal controls after intradermal foot and

forearm capsaicin injections. In addition, pelvic organ crosstalk has an important role in central sensitization because pelvic organs, such as the bladder, colon and vulva, are controlled by the same neural pathway [50]. Thus, afferent signals from other pelvic organs can provoke pain through neural crosstalk even though the initiation and peripheral causes of vulvodynia and IC/BPS have gone. Therefore, central sensitization plays an important role in the chronic pain observed in vulvodynia and IC/BPS. Clinically, similar manifestations of vulvodynia and IC/BPS have also existed. Moreover, recently, there has been an attempt to categorize various pain-associated conditions due to central sensitization as central sensitivity syndrome (CSS). Vulvodynia and IC/BPS are considered subgroups of CSS [51]. Previous studies on clinical findings support the notion that the same mechanisms associated with central sensitization are involved in the pain behind vulvodynia and IC/BPS [52–54].

Recently, evidence supporting central sensitization using functional and structural brain imaging were reported in vulvodynia and IC/BPS. Previously, Pukall et al. [55] showed that increased perception and activation of pain-related brain regions were observed in women with vulvar vestibulitis syndrome, compared to normal women, after tactile stimulation of the vulvar vestibule. Other studies have reported that vulvodynia patients show increased grey matter density in pain-modulating and stress-related regions of the brain as well as alterations in the intrinsic connectivity of regions comprising the sensorimotor, salience, and default mode resting state networks [56, 57]. Similarly, women with IC/BPS showed alterations of oscillation frequency and functional connectivity of brain regions previously reported in other chronic pain conditions [58] and various white matter (right anterior thalamic radiation, left forceps major, and right longitudinal fasciculus, right superior and bilateral inferior longitudinal fasciculi) abnormalities that correlated with severity of pain, urinary symptoms, and impaired QoL [59]. Fig. 2 shows common therapeutic approaches and points of divergence among IC/BPS, IC/BPS + vulvodynia, and vulvodynia patient groups.

Approach and management of vulvodynia and IC/bps patients

Clinically, vulvodynia and IC/BPS often coexists and differentiation between the two is not easy, especially when the patient reports LUTS combined with pain. A majority of patients also seek medical care after relatively longer periods of initially feeling pain, leading to central sensitization that has been firmly established. Furthermore, clinical manifestations of chronic pain found in various other comorbidities can mask vulvodynia and IC/BPS, making it confusing and difficult to officially diagnose.

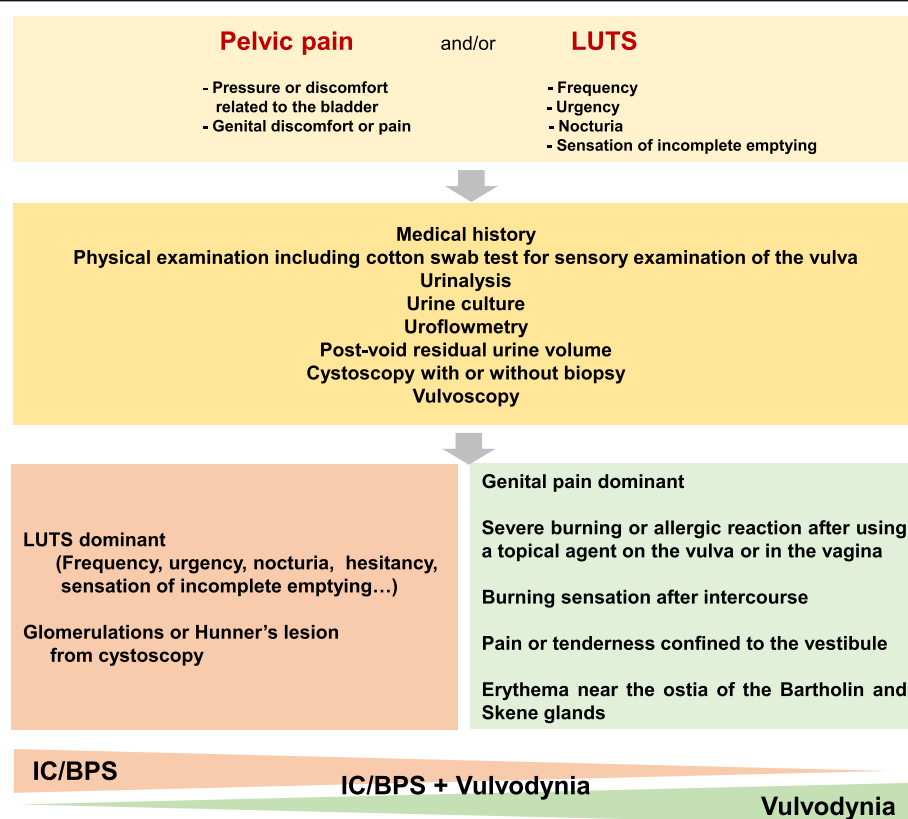


Fig. 2 Approach for differential diagnosis of IC/BPS, vulvodynia, and IC/BPS + vulvodynia (LUTS lower urinary tract symptoms, IC/BPS interstitial cystitis/bladder pain syndrome)

Table 1 Characteristics of FSD observed in women with IC/BPS

	Age	Types of FSD	Prevalence (%)	Other associated conditions
Verit et al. [8] ^a	34.73 ± 8.07 years	NA	67.8 (78 of 112)	NA
Gardella et al. [28]	38.7 ± 12 years	Vulvodynia Dyspareunia	Vulvodynis: 85.1 (40 of 47) Vaginal burning: 65.9 (31 of 47) Dyspareunia: 31.9 (15 of 47)	LUTS (frequency, urgency)
Carrico et al. [24]	52 years (range, 19–90)	Vulvodynia	Vulvar pain: 48.1 (91/189) Pain with intercourse: 66.9 (117/175)	Sexually transmitted infections (genital warts, positive HPV, gonorrhea, chlamydia)
Yoon et al. [9]	51.0 ± 14.7 years	Vulvodynia Dyspareunia	NA	LUTS (frequency, urgency)
Bogart et al. [27]	43.6 ± 16.7 years	NA	Women experienced with sexual dysfunction symptoms: 88 (866/985)	NA
Gardella et al. [23]	38.2 ± 11.3 years	Vulvodynia Dyspareunia	Spontaneous vulvodynia: 23.4 (11 of 47) Provoked vulvodynia: 74.5 (35/47) Localized vulvodynia: 80.9 (38/47) Generalized vulvodynia: 17 (8/47) Dyspareunia: 87.2 (41/47)	NA

^aThis study was done in the women diagnosed of chronic pelvic pain. FSD female sexual dysfunction, IC/BPS interstitial cystitis/bladder pain syndrome, LUTS lower urinary tract symptom, NA Not applicable

Many previous studies have revealed a close correlation between LUTS and impaired sexual function; IC/BPS is not an exception. Besides, it is not uncommon to have trouble differentiating sexual pain and IC/BPS-related genital pain in women. Unfortunately, despite the growing population of affected individuals, there is a lack of comprehensive studies regarding sexual dysfunction and IC/BPS (Table 1). Clinicians should take more concern regarding sexual dysfunction and pain in IC/BPS patients, and more randomized controlled studies should be conducted to better understand correlations and diagnostic differentiations.

Conclusions

Both vulvodynia and IC/BPS are common and irritable conditions that disrupt normal life and reduce QoL in women. Vulvodynia also manifests itself along with IC/BPS and there are several reports supporting an association between the two diseases. Unfortunately, knowledge concerning vulvodynia and IC/BPS is inadequate when considering the clinical impact and importance of these two conditions. Therefore, there is an essential need for further studies that delve into discovering the features of vulvodynia and IC/BPS.

Abbreviations

CPP: Chronic pelvic pain; CSS: Central sensitivity syndrome; FSD: Female sexual dysfunction; IC/BPS: Interstitial cystitis/bladder pain syndrome; ICSM: International consultation on sexual medicine; LUTS: Lower urinary tract symptoms; OAB: Overactive bladder; QoL: Quality of life; TRPV1: Transient receptor potential V1

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Authors' contributions

HY and JK made substantial contributions to conception and design and involved in critically for important intellectual content. SK was involved in acquisition of data, interpretation of data, and drafting the manuscript. All authors gave final approval of the version to be published.

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Availability of data and materials

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Ethics approval and consent to participate

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Consent for publication

Not Applicable.

Competing interests

The author(s) declare(s) that they have no competing interests.

Author details

¹Department of Urology, Seoul St. Mary's Hospital, The Catholic University of Korea College of Medicine, Seoul, Republic of Korea. ²Departments of Surgery and Biomedical Sciences, Cedars-Sinai Medical Center, Los Angeles, CA 90048, USA. ³Department of Medicine, University of California Los Angeles, Los Angeles, CA 90095, USA. ⁴Department of Urology, Medical Research Center, Ewha Womans University School of Medicine, Seoul, Republic of Korea. ⁵Current address: Department of Urology, Yonsei University Wonju College of Medicine, Wonju, Republic of Korea.

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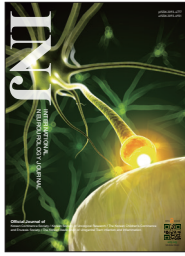
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Pioglitazone Alters the Proteomes of Normal Bladder Epithelial Cells but Shows No Tumorigenic Effects

Muhammad Shahid^{1,*}, Minhyung Kim^{1,*}, Austin Yeon¹, Peng Jin¹, Woong-Ki Kim², Sungyong You^{1,3}, Jayoung Kim^{1,3,4,5}

¹Departments of Surgery and Biomedical Sciences, Cedars-Sinai Medical Center, Los Angeles, CA, USA

²Department of Microbiology and Molecular Cell Biology, Eastern Virginia Medical School, Norfolk, VA, USA

³Samuel Oschin Comprehensive Cancer Institute, Cedars-Sinai Medical Center, Los Angeles, CA, USA

⁴University of California Los Angeles, Los Angeles, CA, USA

⁵Department of Urology, Ga Cheon University College of Medicine, Incheon, Korea

Purpose: Pioglitazone, an antihyperglycemic drug, is widely used in diabetes mellitus patients with insulin resistance. Although pioglitazone is known to have a potential link to bladder cancer (BC), there have been contradictory results. This present study is designed to understand the regulatory mechanisms that drive the effects of pioglitazone on the bladder epithelial cells.

Methods: Labeled liquid chromatography-tandem mass spectrometry-based proteomics profiling characterized the global proteomes of normal human bladder epithelial cells treated with or without pioglitazone.

Results: This approach detected approximately 5,769 proteins in total. Of those 5,769 proteins, 124 were identified as being differentially expressed due to pioglitazone treatment. Further analysis identified 95 upregulated and 29 downregulated proteins (absolute log₂ fold change >0.58 and P-value <0.05). The following functional gene enrichment analysis suggested that pioglitazone may be altering a few select biological processes, such as gene/chromatin silencing, by downregulating BMI1 (B lymphoma Mo-MLV insertion region 1 homolog), a polycomb complex protein. Further cell-based assays showed that cell adhesion molecules, epithelial-mesenchymal transition markers, and major signaling pathways were significantly downregulated by pioglitazone treatment.

Conclusions: These experimental results revealed the proteomic and biological alterations that occur in normal bladder cells in response to pioglitazone. These findings provided a landscape how bladder proteome is influenced by pioglitazone, which suggests the potential adverse effects of diabetes drugs and their links to bladder dysfunctions.

Keywords: Urinary bladder neoplasms; BMI1; Diabetes mellitus; Global proteome; Peroxisome proliferator-activated receptor gamma

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Corresponding author: Jayoung Kim  <https://orcid.org/0000-0002-3683-4627>

Departments of Surgery and Biomedical Sciences, Cedars-Sinai Medical Center, Davis 5071 8700 Beverly Blvd., Los Angeles, CA 90048, USA

E-mail: Jayoung.Kim@cshs.org

*Muhammad Shahid and Minhyung Kim contributed equally to this study as co-first authors.

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INTRODUCTION

Pioglitazone is one of thiazolidinediones (TZDs), which is currently the only oral drugs with a confirmed antihyperglycemic impact in type 2 diabetes mellitus (T2DM) patients. TZDs is well-known agonists of peroxisome proliferator-activated receptor (PPAR) α and γ , TZDs are known to positively influence insulin sensitivity and β -cell function and potentially alter lipid profiles. Pioglitazone is a synthetic ligand of PPAR γ and is used as a therapeutic treatment for patients with T2DM. PPAR γ , a ligand-activated transcription factor, is expressed in white and brown adipose tissues as well as in the urinary bladder [1]. Pioglitazone has also been shown to reduce macrophage infiltration through the activation of PPAR γ and induction of apoptotic cell death [2], which can lead to decreased numbers of macrophages in adipose tissues [3]. To examine the impact of PPAR γ on bladder cell function, several studies compared the PPAR γ levels in normal bladder and bladder cancer (BC) mucosal samples. They found that cell migration and invasion rates are correlated with PPAR γ expression in various BC cell lines. Recently, *in vitro* effects of pioglitazone on normal urothelial epithelium (NUTE) cells and BC (J82) cells were compared [4]. In this paper, pioglitazone inhibited the proliferation and induced apoptosis of NUTE cells, but not J82 cells, suggesting the different effects of pioglitazone on normal bladder cells vs BC cells. In addition, prior studies showed that a primary proangiogenic factor, vascular endothelial growth factor A, is positively regulated by PPAR γ [5]. PPAR γ is involved in proliferation and survival through Wnt/ β -catenin, IGF-I/PI3K/AKT/mTOR, and mitogen activated protein kinase (MAPK) pathways [6]. Furthermore, PPAR γ can regulate the inflammatory response of macrophages through Toll-like receptor 4/nuclear factor- κ B (TLR4/NF- κ B) signaling [7].

There have been contradictory reports on diabetes mellitus (DM) and elevated risk of BC [8]. Studies have demonstrated that pioglitazone can have adverse effects that exaggerate the risk of BC. DM patients, particularly males, on long-term, high doses of pioglitazone, are at a higher risk of developing BC [9]. The KPNC (Kaiser Permanente Northern California) study determined that inflated dose and length of pioglitazone treatment correlated to increased rates of BC [5]. Currently, the U.S. Food and Drug Administration allows the use of pioglitazone in patients with special caution to those with a prior history or active BC [10]. A potential reason for this observation could be that PPAR γ signaling in BC cells may provide a tumor micro-

environment that allows for *de novo* lipogenesis of lipids that can be utilized in increasing tumor mass and energy usage [11]. However, other studies have shown that TZDs posed no risk on survival in BC patients who underwent radical cystectomy [12]. A recent meta-analysis found no difference in BC incidence among users of pioglitazone and nonusers [13]. These inconsistencies between studies have led to questions in the legitimacy of studies and the actual dangers of continued pioglitazone use. Due to the inherent difficulty of accurately assessing the link between TZDs and BC, the association between the two is still in controversy [14].

The goal of our study was to determine whether TZDs have any negative or positive effects on the healthy bladder epithelium. Since pathophysiological changes require the actions of a series of key proteins, global proteomic analysis of protein alterations using state-of-the-art mass spectrometry is ideal for acquiring unbiased biological information regarding molecular mechanisms. Using such a method, this study aimed to understand the mechanistic meanings of proteome perturbations in the normal bladder due to TZD treatment. We sought to examine the effects of pioglitazone specifically, being that is the most widely used TZD. Our global quantitative proteomic analyses found that pioglitazone alters the proteome and biological networks in normal human bladder cells without tumorigenic effects.

MATERIALS AND METHODS

Cell Culture

We used human telomerase reverse transcriptase-immortalized human urothelial cell line (TRT-HU1) for this study [15]. Cell lines that were initially established, frozen, and under a passage number of 10 were used in this study. Culture media was changed after one day of subculturing and cells were passed again when there was 70%–80% confluence. All cells were negative for mycoplasma contamination. For maintaining TRT-HU1, we used Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Invitrogen, Waltham, MA, USA), 1% penicillin/streptomycin, and 1% L-glutamine (Sigma-Aldrich, St. Louis, MO, USA) under a humidified atmosphere of 5% CO₂ at 37°C.

Antibodies and Reagents

Commercial antibodies were obtained from the following sources: actin gamma 2 (ACTG2) (ab189385), myosin heavy chain 3 (MYH3) (ab124205), oxidative phosphorylation (OX-

PHOS) (ab110413) from Abcam; phospho-NF- κ B (3033), phospho-HER2/ErbB2 (2247), phospho-p21 activated kinase 1 (PAK1) (2601), phospho-glycogen synthase kinase-3 β (GSK-3 β) (9336), Snail (3879), N-cadherin (13116), Slug (9585), β -catenin (8480), E-cadherin (14472), Tight Junction Antibody Kit (8683) from Cell Signaling Technology, and β -actin (A1978) from Sigma. Horseradish peroxidase (HRP)-conjugated secondary antibodies (7074, 7076) were purchased from Cell Signaling Technologies. Most of other chemical reagents were procured from Sigma Chemical Corp.

Quantitative Proteomics

For this study, we performed the tandem mass tagging (TMT)-based analysis as we described in previous papers [15]. In brief, cellular proteins from 25 μ M pioglitazone-treated and control TRT-HU1 cells were extracted using a 4% sodium dodecyl sulfate-containing buffer. The protein concentration was measured using the Pierce 660nm Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). The kit was carried out using 60 μ g of protein from each sample. Samples were digested with trypsin via filter-aided sample preparation and labeled with TMT6plex reagents in parallel. Then, the peptides were reconstituted and desalted via C18 spin columns (Thermo Fisher Scientific). High-pH reversed-phase liquid chromatography using an Ultimate 3000 XRS System (Thermo Fisher Scientific) was used to separate samples. Peptides with >30% precursor ion interference were excluded to minimize inaccurate quantifications caused by precursor ion interference and Proteome Discoverer was used.

Identification of Differentially Expressed Proteins

Proteins that have more than 3 nonredundant peptides in each sample were selected for further analysis using a one sample t-test using the log₂ fold changes to compute the significance. False discovery rates (FDRs) of each protein for the 1 sample t-test were then calculated using Storey's correction method. Differentially expressed proteins (DEPs) were identified based on FDR < 0.05 and absolute log₂ fold change $\geq$ 0.58 (1.5-fold). DAVID ver. 6.8 was used for functional enrichment analysis of DEPs. Significantly enriched cellular processes were selected (P < 0.05). Functional classification analysis was performed using PANTHER ver. 11.

Master Regulator Analysis

To identify master regulators, we discerned each DEP and counted their individual partners. The protein-protein interac-

tion information was based on the following 6 interactome databases: the Biological General Repository for Interaction Datasets (BioGRID), the Database of Interacting Proteins (DIP), High confidence protein-protein interactions (HitPredict), the IntAct molecular interaction database (IntAct), the Molecular INTeraction database (MINT), and the STRING database. Fisher exact test was used to calculate the enrichment P-values using. Finally, we selected proteins with P-values lower than 0.01 and prioritized the DEPs based on the number of interaction partners.

Western Blot Analysis

For western blot analysis we used whole-cell lysates. All primary antibodies incubations were done at 4°C overnight. HRP-conjugated secondary antibody incubations were done at room temperature for 1 hour. All western blot experiments were repeated at least 3 times for each antibody and analyzed from different lysates using standard procedures.

Quantification of Reactive Oxygen Species

We measured intracellular reactive oxygen species (ROS) levels by staining with 2', 7'-dichlorofluorescein diacetate (DCF-DA) and sorting with a flow cytometer (FACSCalibur, Becton-Dickinson, Franklin Lakes, NJ, USA). DCF fluorescence intensity was measured with maximum excitation and emission spectra of 495 nm and 529 nm, respectively.

Cell Proliferation Assay

To determine cell growth during exposure to pioglitazone, TRT-HU1 were seeded in 6-well plates (5 $\times$ 10⁴ cells/well). After treatment with varying doses of pioglitazone (0, 25, 50, or 100 μ M) (E6910, Sigma-Aldrich) or vehicle, cell numbers were evaluated after 24, 48, and 72 hours. For crystal violet staining, cells were stained with 0.05% solution of crystal violet. After incubation at room temperature for 15 minutes, the wells were washed thoroughly with phosphate buffered saline and fixed with 4% paraformaldehyde at room temperature for additional 5 minutes. For quantitative analysis, 10% acetic acid solution was used to dissolve the stained cells and absorbance at 570–590 nm were measured. All the data are representative of 3 independent trials.

Statistical Analysis

In order to evaluate differential expression of the proteins between the 2 groups, we used Student t-tests. Variables with nor-

mal distribution were expressed as mean $\pm$ standard deviation. All reported P-values are 2-tailed, with $P < 0.05$ of significance.

RESULTS

Pioglitazone Perturbs the Proteome of Normal Bladder Epithelial Cells

Labeled LC-MS/MS proteomic analysis was conducted to identify the global proteomes of TRT-HU1 bladder cells treated with or without 25 μ M of pioglitazone. We found that pioglitazone treatment increased PPAR γ protein expression (Fig. 1A). Our proteomics analysis workflow is summarized in Fig. 1B.

We detected 5,769 proteins in total, including proteins from the cell part ($n = 2,375$), organelle ($n = 1,663$), macromolecular complex ($n = 1,060$), membrane ($n = 643$), extracellular region ($n = 88$), cell junction ($n = 61$), synapse ($n = 43$), and extracellular

matrix ($n = 29$) (Fig. 2A). To identify DEPs between the control versus pioglitazone groups, we performed a statistical hypothesis test using an empirical null model (see Methods). Among the 5,769 proteins, 124 DEPs (including 95 upregulated and 29 downregulated proteins) were found to have absolute $\log_2$ fold-changes ≥ 0.58 and P-values < 0.05 . Approximately 70% of the DEPs ($n = 95$) showed increased expression due to pioglitazone treatment (Fig. 2B).

Volcano plot displays DEPs between control and pioglitazone groups. The top 5 most significantly up- or downregulated proteins were highlighted with their official symbol (Fig. 2C). HIST1H2BK (histone cluster 1 H2B family member K), ST13P5 (ST13, Hsp70 interacting protein pseudogene 5), and WASH2P (WAS protein family homolog 2 pseudogene) were the most significantly downregulated by pioglitazone treatment. ATPAF1 (ATP synthase mitochondrial F1 complex as-

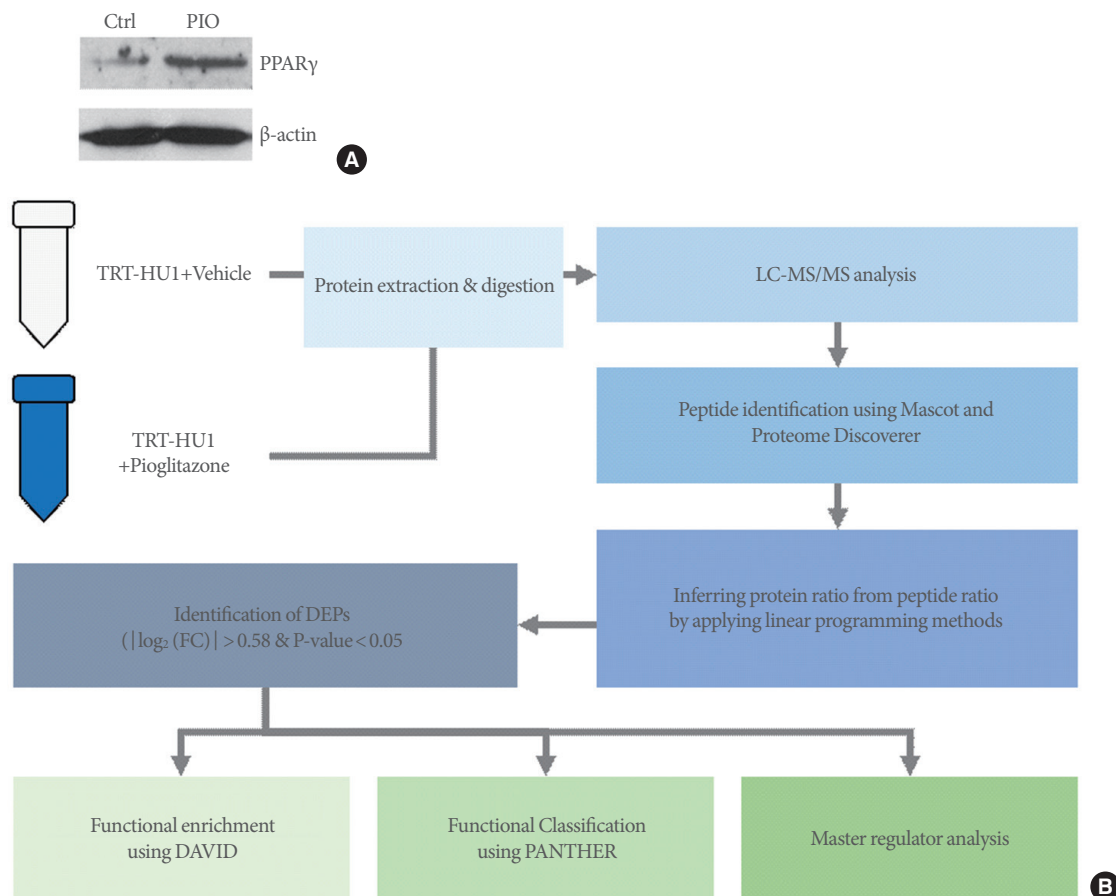


Fig. 1. Overview of global proteomics workflow for data generation and analysis. (A) Western blot analysis shows the effects of pioglitazone treatment on PPAR γ expression. (B) Proteomics analysis workflow for this study. Ctrl, control; PIO, pioglitazone treatment; PPAR γ , peroxisome proliferator-activated receptor gamma; TRT-HU1, human telomerase reverse transcriptase-immortalized human urothelial cells; LC-MS, liquid chromatography-mass spectrometry; DEP, differentially expressed protein; FC, fold change.

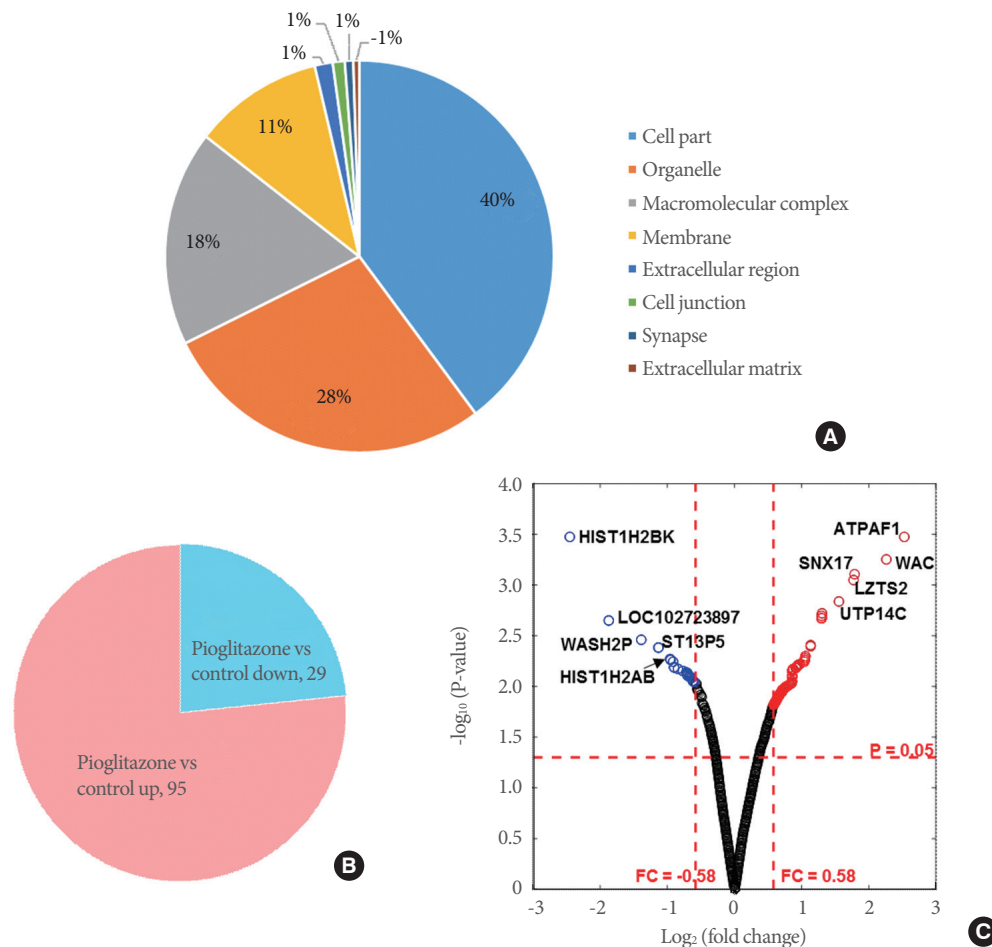


Fig. 2. Total proteins and differentially expressed proteins (DEPs) perturbed in response to pioglitazone treatment. (A) Pie chart depicts the cellular location of the detected DEPs. (B) Pie chart shows proportion of up- and downregulated DEPs in cells treated with pioglitazone. (C) A volcano plot showing the up- or downregulated DEPs by pioglitazone treatment. Red, upregulated DEPs; blue, downregulated DEPs. Protein symbol of the top 5 up- or downregulated proteins are presented in the volcano plot. HIST1H2BK, histone cluster 1 H2B family member K; WASH2P, WAS protein family homolog 2 pseudogene; ST13P5, ST13, Hsp70 interacting protein pseudogene 5; HIST1H2AB, histone H2A type 1-B; ATPAF1, ATP synthase mitochondrial F1 complex assembly factor 1; SNX17, sorting nexin 17; WAC, WW domain containing adaptor with coiled-coil; LZTS2, leucine zipper tumor suppressor 2; UTP14C, small subunit processome component; FC, fold change.

sembly factor 1), SNX17 (sorting nexin 17), LZTS2 (leucine zipper tumor suppressor 2), and UTP14C (UTP14C, small subunit processome component) were the most upregulated in response to pioglitazone. Supplementary Table 1 shows the list of DEPs that were altered in response to pioglitazone along with the transcriptional targets of PPAR γ and test statistics. This data suggest that pioglitazone may induce transcriptional programming through PPAR γ , which contributes to increased protein expression.

Functional Enrichment Analysis Reveals That Pioglitazone Modulates Key Biological Processes

To check the function of proteins perturbed by pioglitazone, we performed functional enrichment and classification using DAVID and PANTHER, respectively. Muscle filament sliding, actin-mediated cell contraction, cellular oxidant detoxification, cell adhesion, purine nucleoside monophosphate metabolic process, ATP metabolic process, and cytoskeleton organization were enriched by upregulated DEPs. However, chromatin silencing, gene silencing, and regulation of gene expression were enriched by downregulated DEPs (Fig. 3A). We listed the DEPs

Table 1. Enriched biological processes and corresponding proteins in upregulated and downregulated proteins

Gene ontology term	Gene	P-value	z-score
Upregulated protein			
Muscle filament sliding	<i>MYH2, MYH3, MYH4, MYH7, MYH8, MYL1, MYL3</i>	3.84E-08	7.41
Actin-mediated cell contraction	<i>MYH2, MYH3, MYH4, MYH7, MYH8, MYL1, MYL3</i>	1.29E-05	4.89
Cellular oxidant detoxification	<i>SOD1, TXN, CCS, PARK7, TXNDC17, SRXN1</i>	7.75E-05	4.11
Cell adhesion	<i>ARHGDIB, DSG1, S100A11, SOD1, PCDHGB4, PCDHGA8, CCS, PARK7, PCDHGA12, PYCARD, PCDHGC5, PCDHGC4, PCDHGB3, PCDHGA9, PCDHGA6, PCDHGA5, PCDHGA4, PCDHGA3, PCDHGA2, PCDHGA1</i>	0.000906	3.04
Cytoskeleton organization	<i>ARHGDIB, CFL1, KRT5, KRT9, KRT16, MYH3, SOD1, PHPT1, PYCARD, SH3BGL3, KRT74, MTPN, WASH3P</i>	0.014298	1.84
Downregulated protein			
Negative regulation of gene expression	<i>BMI1, HIST1H2AD, HIST1H2AJ, HIST1H2AC, HIST1H2AB, HIST2H2AA3, HIST2H2AC, HIST1H4A, HIST1H2AG, H2AFJ, HIST1H2AH, HIST3H2A</i>	5.99E-06	5.22
Regulation of gene expression	<i>ACTG2, BMI1, HIST1H2AD, HIST1H2AJ, HIST1H2AC, HIST1H2AB, HIST2H2AA3, HIST2H2AC, HIST1H4A, HIST1H2AG, H2AFJ, HIST1H2AH, HIST3H2A</i>	0.021575	1.66
Chromatin silencing	<i>HIST1H2AD, HIST1H2AJ, HIST1H2AC, HIST1H2AB, HIST2H2AA3, HIST2H2AC, HIST1H4A, HIST1H2AG, H2AFJ, HIST1H2AH, HIST3H2A</i>	1.13E-15	14.94
Gene silencing	<i>HIST1H2AD, HIST1H2AJ, HIST1H2AC, HIST1H2AB, HIST2H2AA3, HIST2H2AC, HIST1H4A, HIST1H2AG, H2AFJ, HIST1H2AH, HIST3H2A</i>	2.36E-12	11.63

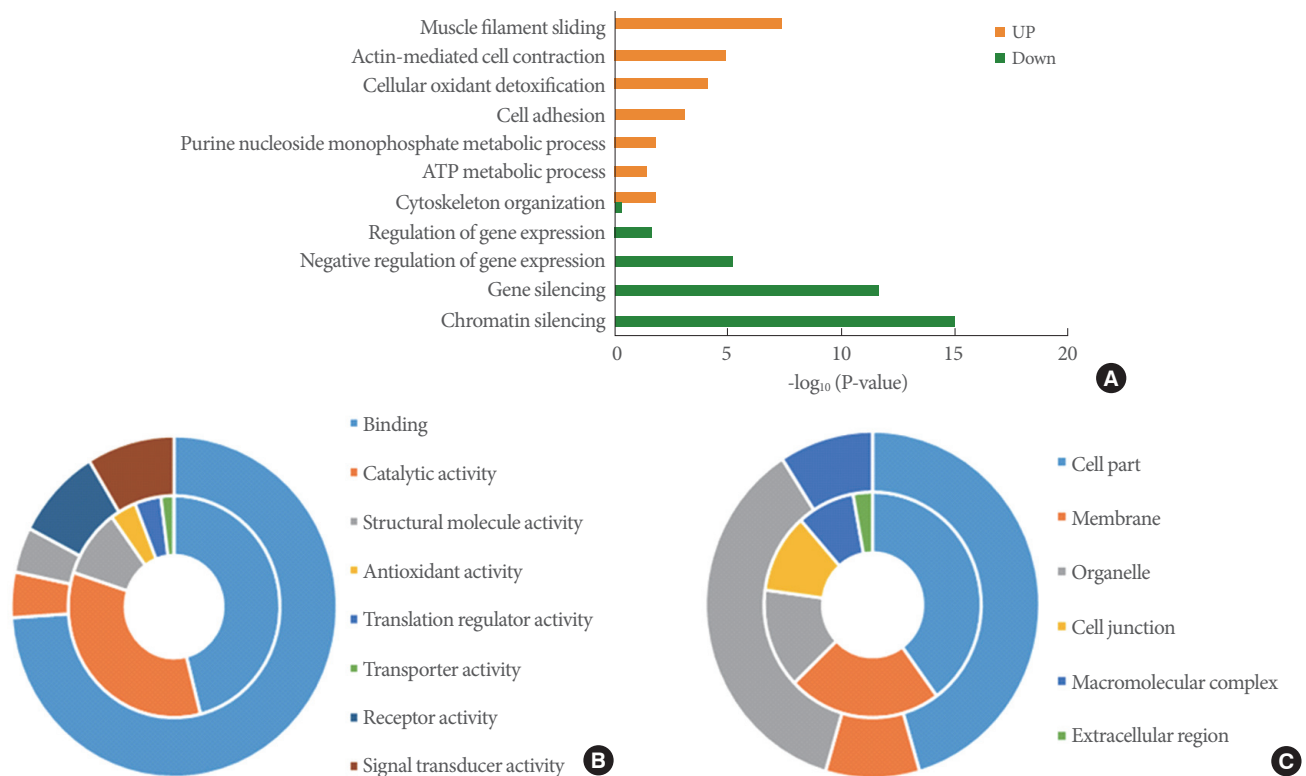


Fig. 3. Functional enrichment and classification of the differentially expressed proteins (DEPs). (A) Bar plot shows biological processes enriched by the up- and downregulated DEPs. (B) Top molecular functions of upregulated (inner circle) and downregulated (outer circle) proteins. (C) Top cellular compartments of upregulated (inner circle) and downregulated (outer circle) proteins. ATP, adenosine triphosphate.

belonging to actin-mediated cell contraction, cellular oxidant detoxification, cell adhesion, purine nucleoside monophosphate metabolic process, and chromatin silencing (Table 1).

Interestingly, the classified molecular functions of the DEPs include binding, catalytic activity, structural molecule activity, antioxidant activity, translation regulator activity, transporter activity, receptor activity, and signal transducer activity (Fig. 3B). Binding function was the largest portion of both the up-regulated and downregulated DEPs. Catalytic, antioxidant, translation regulator, and transporter activity were the major molecular functions in the upregulated DEPs. However, receptor and signal transducer activity were the major functions in downregulated DEPs. The classified cellular compartments of the DEPs were cell part, membrane, organelle, cell junction, macromolecular complex, and extracellular region (Fig. 3C). Membrane, cell junction, and extracellular region were the major cellular compartments in upregulated DEPs. Organelle and macromolecular complex are major cellular compartments in the outer circle.

Identification of Protein Interaction Networks Regulated by Pioglitazone Treatment

Our present study revealed a large set of proteins ($n=5,769$)

with relevant levels of abundance in normal bladder epithelial cells. Their functions are related to various cellular processes, including actin regulation and chromatin remodeling (Fig. 3A). Pioglitazone perturbs and modulates these cellular processes by affecting protein-protein interactions. We therefore performed master regulator analysis using all the proteins detected in our global proteomic analysis to identify key proteins that can be major nodes in the protein-protein interaction networks. To select key proteins, we first identified proteins with a large number of interactions from those detected. We then determined the top 5 proteins with the largest number of interactions and the most significant P-values (Fig. 4A, Supplementary Table 2).

To reconstruct the network model, top 3 proteins were then used. Of note, B lymphoma Mo-MLV insertion region 1 homolog (BMI1), Histone H4A (HIST1H4A), and superoxide dismutase 1 (SOD1) were found to be the top 3 regulators, with P-values <0.01 and large target counts. The network model describes the interactions among the top 3 regulators and their interactors with significant differential expression when treated with pioglitazone. Pioglitazone treatment downregulated expression of BMI1, which was found to interact mainly with histone proteins, such as Histone H2 (HIST2A), Histone H2A type 3-A (H2AA3), Histone H2A type 2-C (HIST2H2AC), Histone H1

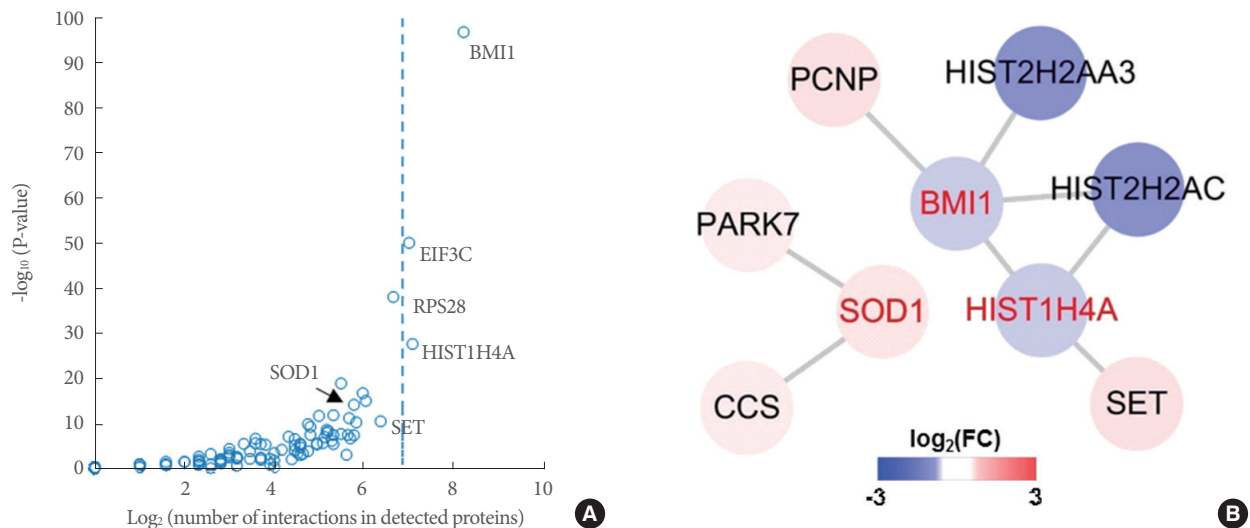


Fig. 4. Potential master regulators suggested by master regulator analysis (MRA). (A) Scatter plot displays distribution of detected proteins by number of interactions and level of significance driven by MRA. (B) Network model describing the interactions of the top 3 master regulators and their interacting differentially expressed protein partners. Red font denotes the top 3 master regulators. BMI1, B lymphoma Mo-MLV insertion region 1 homolog; EIF3C, eukaryotic translation initiation factor 3 subunit C; RPS28, ribosomal protein S28; HIST1H4A, histone cluster 1 H4 family member A; SOD1, superoxide dismutase 1; SET, SET nuclear proto-oncogene; PCNP, PEST proteolytic signal containing nuclear protein; HIST2H2AA3, Histone H2A type 2-A; PARK7, parkinsonism associated deglycase; HIST2H2AC, Histone H2A type 2-C; CCS, copper chaperone for superoxide dismutase; FC, fold change.

type 4-A (HIST1H4A) (Fig. 4B). Expression of SOD1 increased with pioglitazone treatment and it was found to interact with 2 other upregulated proteins, PARK7 (parkinsonism associated deglycase) and CCS (copper chaperone for superoxide dismutase). Interestingly, downregulation of BMI1 and its interacting histone proteins in the network model suggests potential epigenetic regulation by pioglitazone, which is consistent with functional enrichment data indicating “chromatin silencing” (Fig. 3A).

Biological Influences of Pioglitazone on Normal Bladder Epithelial Cells

To further validate the DEPs between the control versus pioglitazone groups, proteins were selected for based on functional enrichment analyses. Western blot analysis showed that expression of MYH3 was upregulated, while expression of ACTG2 was downregulated in cells treated with 25μM of pioglitazone (Fig. 5A). Consistent with morphological changes in pioglitazone-treated cells, we noticed that protein expression of mes-

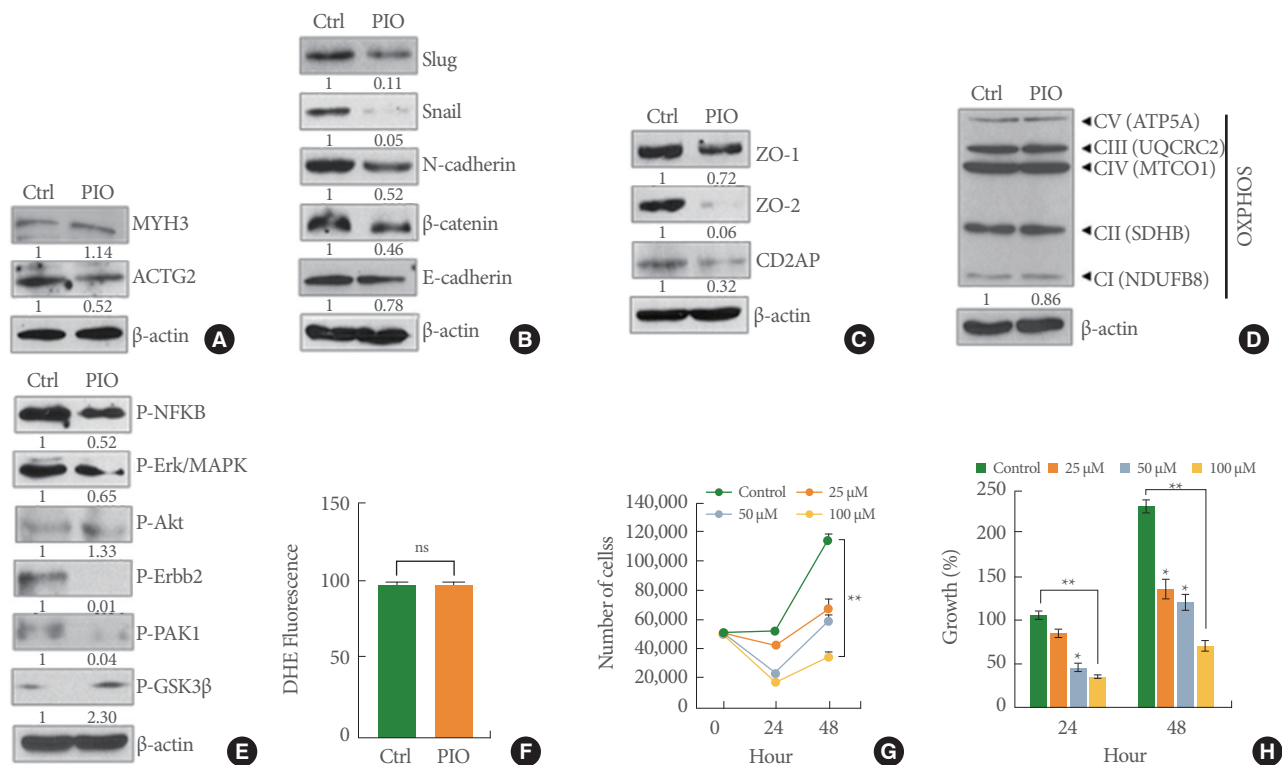


Fig. 5. Effects of pioglitazone treatment on THT-HU1 (human telomerase reverse transcriptase-immortalized human urothelial cells). Cells were incubated in 25μM of pioglitazone-containing Dulbecco's modified Eagle's medium for 72 hours. Whole-cell lysates were immunoblotted with various antibodies as indicated. (A) Immunoblot analyses were performed to validate the expression levels of identified differentially expressed proteins. The protein expression levels of MYH3 and ACTG2 significantly decreased with pioglitazone treatment (PIO), compared to controls (Ctrl). (B) Immunoblot analysis compared several epithelial-mesenchymal transition markers, including Slug, Snail, N-cadherin, β-catenin, and E-cadherin, in pioglitazone treated (PIO) and untreated (Ctrl) cells. (C) Cell junction markers (ZO-1, ZO-2, and CD2-associated protein) were also measured by immunoblot analysis. As the protein loading control, levels of β-actin were shown. (D) Levels of mitochondrial oxidative phosphorylation (OXPHOS) markers were compared between Ctrl and PIO groups. No significant changes were observed in response to pioglitazone treatment. (E) Western blot analysis of phospho-NF-κB (P-NFKB), phospho-Erk/MAPK (P-Erk/MAPK), phospho-HER2/ErbB2 (P-HER2/ErbB2), phospho-p21 activated kinase 1 (P-PAK1), and phospho-glycogen synthase kinase-3β (P-GSK3β) were performed. β-actin was used as an internal control. (F) Reactive oxygen species production was compared between Ctrl and PIO groups. NS, nonsignificant. (G) Cell proliferation was quantified by trypan blue staining. **P < 0.005. (H) Dose- and time-dependent cell growth rates were measured in an independent set of experiments. *P < 0.01. **P < 0.005. NS, nonsignificant. ATP5A, ATP synthase subunit alpha; UQCRC2, ubiquinol-cytochrome C reductase core protein 2; MTCO1, mitochondrially encoded cytochrome C oxidase I; SDHB, succinate dehydrogenase; NDUFB8, NADH:ubiquinone oxidoreductase subunit B8.

enchymal cell markers (N-cadherin, Snail, Slug, and β -catenin) were downregulated in response to pioglitazone treatment (Fig. 5B). In addition, tight junction proteins (zonula occludens [ZO] 1 and 2 and CD2-associated protein) that are involved in cell-cell contact regions were greatly reduced in pioglitazone-treated cells (Fig. 5C).

To determine whether pioglitazone regulates mitochondrial quantity or metabolic respiration-associated proteins, mitochondrial OXPHOS proteins were measured via western blot analysis. The expression levels of OXPHOS subunits (C I subunit [NDUFB8], C II subunit [succinate dehydrogenase, SDHB], C III core protein 2 [ubiquinol- cytochrome C reductase core protein 2, UQCRC2], C IV subunit [mitochondrially encoded cytochrome C oxidase I, MTCO1], and C V alpha subunit [ATP synthase subunit alpha, ATP5A]) were not change by pioglitazone treatment (Fig. 5D). To profile the phosphorylation events in normal bladder cells, human TRT-HU1 cells were treated with or without pioglitazone. A series of western blot analyses showed that several phosphorylation events of specific signaling pathways were regulated by pioglitazone. The phosphorylation levels of NF- κ B, Erk/MAPK, ERBB2, and PAK1 decreased. On the contrary, only the phosphorylation levels of GSK-3 β increased. Additionally, previous studies have shown that the activation of PPAR γ through agonists reduces diabetic myocardial fibrosis by transforming growth factor beta/extracellular signal-regulated kinase pathway and epithelial-mesenchymal transition [16], which were both found to be upregulated in pioglitazone-treated cells compared to controls (Fig. 5E).

To further elucidate on the role of pioglitazone on oxidative stress, ROS levels were measured and analyzed after 72-hour pioglitazone treatment. Levels of ROS were unchanged between the control and pioglitazone groups (Fig. 5F). In addition to our proteomic analysis, we assessed whether pioglitazone affects cell proliferation. The dose-dependent experiments in varying concentrations of pioglitazone (25, 50, or 100 μ M) for 24 and 48 hours, we noticed that the cell proliferation rates of cells treated for 48 hours were significantly suppressed, compared to controls (Fig. 5G, H).

DISCUSSION

There are many discrepancies surrounding the link between TZD usage and side effects on bladder function. Diabetic bladders can have several dysfunctions, ranging from bladder overactivity to impaired contractility, which represent a wide spec-

trum of clinical symptoms. The widespread pathophysiology of bladder dysfunction in DM patients is associated with multiple factors like smooth muscle dysfunction, and urothelial impairment [17]. DM has been shown to be a significant predictor of death from cancer, including BC [18]. Evidence from a meta-analysis study has linked the use of pioglitazone to increased BC risk as well [13]. The tumorigenic effect of pioglitazone is thought to be due to signaling modifications in the PPAR pathway [19]. However, it is still unclear whether the long-term use of pioglitazone increases the chances of developing bladder dysfunctions or disease.

PPAR γ is part of the PPAR subfamily that consists of 2 other subunits, alpha and beta/delta. As the most extensively studied subunit, the functions of PPAR γ have been linked to several crucial biological processes, such as glucose metabolism, lipid biosynthesis, and anti-inflammatory response [20]. The association of PPAR γ to diabetes is based on its role in lipid and glucose metabolism [21]. It has been shown that patients with dominant-negative mutations of PPAR γ have severe hypoglycemia, insulin resistance, and DM [22]. This means that there is a significant genetic link between both PPAR γ and DM. Treatment with TZDs has proven to substantially decrease insulin resistance in diabetes and possibly induces transcription of adipose cell differentiation, which generates smaller insulin-sensitive adipocytes [23]. However, despite their beneficial effects in patients with diabetes, TZDs are known to have adverse effects. This includes weight gain, fluid retention, bone mineral density reduction, and excess myocardial infarction [24]. The fluid retention induced by TZDs has also been proven to significantly increase urine volume and excretion of sodium [25]. Because of this, there is some controversy surrounding the safety of long-term TZD usage to treat diabetes.

In addition to the documented roles of PPAR γ in lipid metabolism and DM, there has been growing research on its functions in both BC and interstitial cystitis (IC). Approximately 20%–25% of human BC is driven by PPAR signaling, PPAR γ gene amplification, or RXRA hot-spot mutations [26]. Furthermore, it was found that the PPAR γ transcription pathway associated with an immune evasion in muscle-invasive BC [27]. Along with this immune escape, it has been proposed that PPAR γ cooperates with the FOXA1 (forkhead box protein A1) and GATA3 (GATA binding protein 3) networks to drive trans-differentiation of basal BC cells into luminal types [28]. PPAR γ has also been identified to be associated with IC. Mahal et al. [29] found that treating their IC rat model with PPAR γ agonists

improved bladder function and urinary health. Based on the complex relationship of PPAR γ with diabetes and urological malignancies, it would be compelling to better understand how patients who routinely take TZDs are affected.

This study revealed that BMI1 is a master node in pioglitazone-regulated networks. Our proteomic profiling of BMI1 exhibited significant downregulation in normal bladder epithelial cells treated with pioglitazone, suggesting a potential inverse relationship between PPAR γ and BMI1. One possible hypothesis for our future study is that PPAR γ signaling induced by pioglitazone may cause loss of BMI1 expression in bladder cells. Expression of telomere maintenance proteins including HIST1H4A, HIST2H2AA3, and HIST2H2AC, which interact with BMI1, are downregulated as well. The presence of an upregulated telomere maintenance mechanism is a hallmark of cancer, and it is interesting to note that HIST2H2AA3 and HIST2H2AC were identified as genes upregulated, differentially expressed in BC [30]. This may signify a stage shift, by pioglitazone treatment, from developing cancer. PPAR γ agonism has also been shown to increase the antitumor effects of histone deacetylase inhibitors in multiple myeloma cells [25].

Most notably, in our study, we found a series of contractile and cytoskeleton proteins, such as myosin light polypeptides, myosin heavy polypeptides, keratins, and ACTG2, as being significantly up- or downregulated in TRT-HU1 bladder epithelial cells treated with pioglitazone. Myosin is a particularly important structural component of muscles that interacts with actin filaments and plays a vital role in actin filament contraction, bundling, motor action, and binding. Furthermore, our network modeling revealed that these actin-binding proteins comprise the central hub. These results suggest the potential pathological mechanisms of pioglitazone's effects.

Our data demonstrated that the activation of PPAR γ signaling pathways suppressed the proliferation of bladder epithelial cells. Interestingly, these findings are consistent with clinical observations showing that the IC bladder has thinner epithelial layers [31] and that IC bladder cells exhibit reduced proliferation due to cell cycle arrest [32]. Our epigenetic remodeling study supports this mechanistic link between IC and defective cell proliferation [15]. Furthermore, previous reports suggest that the antiproliferative role of PPAR γ is through the upregulation of PTEN expression in various cancers, which leads to decreased PI-3K activity [33]. It is currently known that PPAR γ agonists suppress cell cycling by downregulating cyclin D1 in several cancer cells; thereby, inhibiting cyclin D1/CDK-mediated

retinoblastoma (pRb) phosphorylation and maintaining its active form to prevent the G1 to S phase transition [34]. Activation of PPAR γ via agonists in normal human urothelial cells has been shown to link with late/terminal urothelial differentiation by protein induction of uroplakins, cytokeratins, and tight junction constituents [35].

We very much understand certain limitations of the study design arising from performing experiments in normal bladder cells, which cannot truly reflect the diabetic bladder condition [36,37]. Although additional experiments using bladder cells from diabetic condition are actually going on in our laboratory, it is out of scope of this study aiming to understand the pioglitazone effects on normal bladder cells. Another limitation of this study is that we used only one normal bladder cell line. This present study also suffers from limited impact on clinical application mainly due to *in vitro* experiments.

In summary, although pioglitazone is widely used among T2DM patients, its effects on the bladder are still in controversy. While there have been studies examining the effects of pioglitazone on healthy bladder cells, there is no conclusive evidence. Through unbiased quantitative proteomic profiling, our report comprehensively characterized the proteome of normal bladder epithelial cells treated with pioglitazone. We observed suppressed cell proliferation, which may be explained by the decreased expression of tight junction proteins. Taken together, our study provides no experimental evidence to support a tumorigenic effect of pioglitazone on the bladder. This proteomic approach will be useful for examining any potential side effects of the one of the most commonly used drugs.

SUPPLEMENTARY MATERIALS

Supplementary Tables 1 and 2 can be found via <https://doi.org/10.5213/inj.1938186.093>.

AUTHOR CONTRIBUTION

- Conceptualization: JK
- Formal Analysis: MS, MK, AY, PJ
- Investigation: MS, MK
- Methodology: MS, MK, AY, PJ
- Project Administration: JK, W-K K, SY
- Writing – Original Draft: JK, SY
- Writing – Review & Editing: JK

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Utilizing machine learning to discern hidden clinical values from big data in urology

Suppose you are asked to choose the single most important information technology that has given decision-making processes a massive upgrade. Many of us would choose machine learning (ML). The application of ML is defined as “giving computers the ability to learn without being explicitly programmed”. The main purpose of ML is to introduce algorithms that ingest input data, apply computer analysis to predicting output values within an acceptable range of accuracy, identify patterns and trends in data, and to learn from previous experience. ML is often applied to complicated and poorly understood natural phenomena, such as complex biological systems, climate change, astronomy, or particle physics.

The two major pathways in ML are supervised and unsupervised learning. In supervised learning, an algorithm is often provided with data, $X_{N \times P}$ (N samples with P number of features) and a desired target y . The goal is to train a model f (e.g., a decision function), that performs prediction on for X , i.e., $f(X)=y$. Supervised learning primarily deals with classification and regression problems. In unsupervised learning, an algorithm is provided with data, X , without any labels/annotations, to find latent patterns, sometimes producing both answers and questions that may not have been conceived by the investigators. Unsupervised learning typically deals with clustering and dimensionality reduction problems. The patterns identified in unsupervised learning often need to be evaluated for utility by human interrogation or via application within a supervised learning task.

While validation of unsupervised algorithm can only be performed with a hidden ground, the performance of a supervised learning algorithm can be evaluated by various metrics based on the objective of a task. A dataset is typically divided into two independent sets, i.e., training and testing sets; where training of an algorithm is performed using the training set and then the trained model is evaluated using the testing set. In order to remove bias introduced in a single division of training/testing sets, cross validation is often used to evaluate supervised learning algorithms.

HOW IS ML APPLIED TO DEVELOP PRECISION MEDICINE?

Many of us in the healthcare and science field would agree that big data will transform medicine. In recent years, large amounts of data have been accumulated through big omics studies of genome, epigenomes, transcriptomes, proteomes, metabolomes, and other sources. This immense data needs to be analyzed, interpreted, and manipulated to provide biological meaning. Where ML shines is in handling enormous numbers of predictors. ML has become ubiquitous and indispensable for solving complex problems in most sciences. ML will become an indispensable tool for clinicians seeking to truly understand their patients. However, there are several shortcomings when it comes to applying ML to big data [1]. First, algorithms might “overfit” predictions to spurious correlations in the data; multicollinear, correlated predictors could produce unstable estimates. Second, ML algorithms often require a large number of observations to reach acceptable performance levels.

Precision medicine is one of the important developments in modern medicine. It provides clinicians with early intervention by using advanced diagnostic procedures and customizes personalized treatments for patients. Many scientists and physicians are convinced of the importance of information technology and ML in precision medicine. This includes data storage and analysis for disease outcomes, and identification of patient characteristics and optimal treatment. Utilizing ML for pattern recognition, development of statistical models, creation of knowledge bases for existing phenotype categories and diseases, organization of clinical datasets of population size, and development of open software platforms for statistical analysis of high-dimensional healthcare and multi-omics data are crucial for practical realization of precision medicine.

As one can imagine, ML will have a huge impact on disease (especially cancer) diagnostics and prognostics; in par-

ticular, on the development of novel computational tools for stratification, grading, and prognostication of patients with the overall goal of improving care. There are various ML techniques, which have been widely used in disease diagnosis, and prognosis. A series of previous studies have shown how ML can improve diagnostic performance and prediction accuracy in clinically relevant patient cohorts [2]. One study demonstrated how ML can improve on already established standards, such as Gleason scoring; thus, providing more precise prognostication. Another study engineered a ML-based system to predict microsatellite instability in patients with gastrointestinal or endometrial cancers; both accuracies were higher than prediction with molecular markers. Some studies have also shown that ML can lead to higher accuracy in drug response prediction. ML is becoming a popular tool for medical researchers and has demonstrated promising ability to effectively predict future disease outcomes.

So how is ML currently being used in clinical research? For digitalized pathology, various applications incorporating ML are developed to assist in processing pathological diagnoses. Major applications include detection of specific objects, such as cancer cells, cell nuclei, cell division, and blood vessels, classification/grading of tumors, and evaluation of immunostaining. The major obstacle in using ML in pathological imaging is inadequate image annotations. At present, there exist many technologies to address this concern [3]. For example, generative adversarial networks are used for pathological data analysis to automatically prepare image datasets necessary for subsequent deep learning. Pathologists are looking forward to this potential new gold standard technology for processing images.

Applications of ML in radiology are designed to help computers analyze medical imaging data and support diagnoses by associating clinical outcomes. These radiomic techniques can predict diseases with higher accuracy than humans. Using ML to recognize and analyze image data will fundamentally change our understanding of disease risk and treatment. ML can often extract image information that humans cannot recognize, which can lead to the discovery of novel disease patterns and predictive markers.

At present, the use of omics research to drive cancer biomarker discovery is very popular. Because of large datasets, researchers need advanced information technology, such as ML, to analyze and understand data. ML has already been applied to mass spectrometry (MS) data across different biological disciplines, including various cancers. ML can be useful in determining which proteins from MS data can be used as biomarkers to differentiate between classes. ML is also useful for interpreting large genomic datasets and annotating a

wide variety of genomic sequence elements, which has led to the identification of potentially valuable disease biomarkers.

HOW ABOUT APPLYING ML INTO UROLOGICAL RESEARCH?

When it comes to prostate cancer (PC), many technology platforms for diagnosis, prognosis, and treatment have demonstrated potential benefits of ML. The ML methods can be extended into treatment planning and intervention by augmenting surgeries with information, such as tumor localization and other image-guided options. Computer-assisted diagnosis of PC in histopathological slides can be achieved by ML to enhance accuracy. ML can also help genomics research. By identifying specific genes, ML can be used to develop diagnostic and risk stratification tools, determine the best individualized treatments, and generate targeted drug treatment schemes.

ML can be used to read radiological or pathological images of bladder cancer (BC) to provide useful information. Previous studies have used ML models to analyze magnetic resonance imaging data of BC; they were able to identify low and high-grade BC before surgery with 83% accuracy. ML-based methods have been further applied to accurately quantify tumor buds from immunofluorescence-labeled slides of muscle-invasive BC patients. ML algorithms have also been employed to create recurrence and survival predictive models from imaging and operative data. These algorithms can be used to identify genes at initial presentation that are most predictive of recurrence and can be applied as molecular signatures to predict recurrence risk within 5 years after transurethral resection of the bladder tumor [4].

ML technology has been used to analyze the clinical and imaging data of renal cell carcinoma (RCC) to provide disease diagnoses, prognostic information, and assist in treatment plans. Previous studies have shown that the ML model can accurately distinguish high-grade and low-grade RCC by analyzing computed tomography (CT) image feature [4]. In recent years, ML has been developed to identify biomarkers and multiple gene expression-based signatures to predict survival and disease prognosis in clear cell RCC. Moreover, some studies have demonstrated how noninvasive deep learning models constructed from radiomic features have comparable performance to percutaneous renal biopsy in predicting International Society of Urological Pathology grading.

ML has also been applied to various modalities of urinary stone therapy. Computer-assisted detection using image features can support radiologists in identifying stones. With large datasets, artificial neural networks (ANN) can predict

outcomes after various forms of endourologic intervention. ANN has been used to differentiate ureteral stones from phleboliths in thin slice CT volumes due to their similarity in shape and intensity. ANN also can be used for the early detection of kidney stone types and most influential parameters to provide a decision-support system. The model resulted in 97.1% accuracy for predicting kidney stone type. Recently, ML algorithms have been used to predict treatment success after a single-session shock wave lithotripsy in ureteral stone patients.

Additionally, ML can be applied to benign bladder diseases, such as overactive bladder syndrome [5]. A ML model using a random forest-based algorithm was studied to identify patients for whom anticholinergic medications are likely to fail. A validated ML prediction model can predict treatment failure in a 3-month standard anticholinergic treatment experiment with accuracy rate higher than 80%.

HOW WILL ML BE EVOLVED INTO TOMORROW'S UROLOGY?

In today's fast-moving technologically enhanced world, ML is still in its evolution. The steps needed to integrate ML into the clinic are still unknown. How the new algorithms will influence diagnosis and management of patients remains within our realm of decision. Future research should focus on the construction of larger medical databases and further development of artificial intelligence techniques. The predictive precision of ML will continue to provide and enhance personalized medicine with the further inclusion of data and model retraining. There are limitless future applications for artificial intelligence in the field of urology.

CONFLICTS OF INTEREST

The authors have nothing to disclose.

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AUTHORS' CONTRIBUTIONS

Research conception and design: Wun-Jae Kim and Jayoung Kim. Data acquisition: Peng Jin and Won Hwa Kim. Drafting of the manuscript: Peng Jin, Won Hwa Kim, and Jayoung Kim. Critical revision of the manuscript: Wun-Jae Kim and Jayoung Kim. Obtaining funding: Jayoung Kim. Administrative, technical, or material support: Wun-Jae Kim and Jayoung Kim. Supervision: Wun-Jae Kim and Jayoung Kim. Approval of the final manuscript: all authors.

Wun-Jae Kim¹ , Peng Jin² , Won Hwa Kim³ ,
Jayoung Kim^{2,4,5,6} 

Corresponding Author: Jayoung Kim

ORCID: <https://orcid.org/0000-0002-3683-4627>

¹Department of Urology, College of Medicine, Chungbuk National University, Cheongju, Korea, ²Departments of Surgery and Biomedical Sciences, Cedars-Sinai Medical Center, Los Angeles, CA, ³Department of Computer Science and Engineering, University of Texas at Arlington, Arlington, TX, ⁴Samuel Oschin Comprehensive Cancer Institute, Cedars-Sinai Medical Center, Los Angeles, CA, ⁵University of California Los Angeles, CA, USA, ⁶Department of Urology, Gachon University College of Medicine, Incheon, Korea
E-mail: Jayoung.Kim@cshs.org

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Classification of the urinary metabolome using machine learning and potential applications to diagnosing interstitial cystitis

Running Title: Machine learning for IC diagnosis

Feng Tong¹, Muhammad Shahid², Peng Jin^{2,3}, Sungyong Jung⁴, Won Hwa Kim¹, Jayoung Kim^{2,5,6,7*}

¹Department of Computer Science, University of Texas at Arlington, Arlington TX 76019, USA

²Departments of Surgery and Biomedical Sciences, Cedars-Sinai Medical Center, Los Angeles, CA 90048, USA

³Shengjing Hospital of China Medical University, Shenyang 110004, Liaoning, China

⁴Department of Electrical Engineering, University of Texas, Arlington, Arlington, TX 76019, USA

⁵Samuel Oschin Comprehensive Cancer Institute, Cedars-Sinai Medical Center, Los Angeles, CA 90048, USA

⁶University of California Los Angeles, Los Angeles, CA 90095, USA

⁷Department of Urology, Gachon University College of Medicine, Incheon, South Korea

*Correspondence author: Jayoung Kim, Email: jayoung.kim@cshs.org

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Abbreviations used: ADTree, alternative decision tree; AI, artificial intelligence; ANN, artificial neural network; AUC, area under the curve; CV, cross validation; FN, false negative; FP, false positive; IC, interstitial cystitis; LR, logistic regression; ML, machine learning; NFM, neurofuzzy modelling; NMR, nuclear magnetic resonance; PCA, principal component analysis; PR, precision and recall; RBF, radial basis function; ROC, receiver operating characteristic; SVM, support vector machine; TN: true negative; TP: true positive Received March 20, 2020; Revision Received April 30, 2020; Accepted May 12, 2020; Published May XX, 2020

Abstract

With the advent of artificial intelligence (AI) in biostatistical analysis and modeling, machine learning can potentially be applied into developing diagnostic models for interstitial cystitis (IC). In the current clinical setting, urologists are dependent on cystoscopy and questionnaire-based decisions to diagnose IC. This is a result of a lack of objective diagnostic molecular biomarkers. The purpose of this study was to develop a machine learning-based method for diagnosing IC and assess its performance using metabolomics profiles obtained from a prior study. To develop the machine learning algorithm, two classification methods, support vector machine (SVM) and logistic regression (LR), set at various parameters, were applied to 43 IC patients and 16 healthy controls. There were 3 measures used in this study: accuracy, precision (positive predictive value), and recall (sensitivity). Individual precision and recall (PR) curves were drafted. Since the sample size was relatively small, complicated deep learning could not be done. We achieved a 76%–86% accuracy with **leave-one-out** cross validation depending on the method and parameters set. The highest accuracy achieved was 86.4% using SVM with a polynomial kernel degree set to 5, but a larger area under the curve (AUC) from the PR curve was achieved using LR with a l_1 -norm regularizer. The AUC was greater than 0.9 in its ability to discriminate IC patients from controls, suggesting that the algorithm works well in identifying IC, even when there is a class distribution imbalance between the IC and control samples. This finding provides further insight into utilizing

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previously identified urinary metabolic biomarkers in developing machine learning algorithms that can be applied in the clinical setting.

Keywords: interstitial cystitis; biomarker; urine; metabolomics; machine learning; artificial algorithm

INTRODUCTION

Interstitial cystitis (IC), also known as painful bladder syndrome or bladder pain syndrome, is a chronic visceral pain syndrome of unknown etiology that presents itself as a constellation of symptoms, including bladder pain, urinary frequency, urgency, and small voided volumes, in the absence of other identifiable diseases [1-3]. Urine is in direct contact with the bladder epithelial cells that could be giving rise to IC; as a result, metabolites released from bladder cells may be enriched in urine [4].

The urinary metabolome was previously investigated by our group for potential IC diagnostic biomarkers [5-7]. We attempted to identify IC-associated metabolites from urine specimens obtained from IC patients and controls using nuclear magnetic resonance (NMR). Our findings provided preliminary evidence that metabolomics analysis of urine can potentially segregate IC patients from controls. We sought to capture the most differentially detected NMR peaks and discern if there was a significant difference in the peak distribution between IC and control specimens. Based on multivariate statistical analysis, principal component analysis (PCA) suggested that the urinary metabolome of IC patients and controls were clearly different; 140 NMR peaks were significantly altered in IC patients ($FDR < 0.05$) compared to controls [5].

Machine learning (ML), originally described as a program that learns to perform a task or make decisions based on data, is a valuable and increasingly necessary tool for modern healthcare [8]. However, this definition is broad and could cover nearly any form of data-driven needs. ML is not a magical approach that can turn data in immediate benefits, even though many news outlets imply that it can. Rather, it is natural extension to traditional statistical approaches. In our present study, we utilized ML and automated performance metrics to evaluate the clinical value of our 140 identified NMR peaks. We used ML algorithms examine the relationship between metabolic expression and disease. We applied logistic regression (LR) [9] and support vector machine (SVM) [10,11], which are traditionally known to work well even with small sample sizes, to our metabolomics signatures and used this data together with patient clinicopathological features to diagnose IC. We used our dataset of 59 cases to train, test, and validate the model. The results showed that our ML-based algorithms were able to successfully identify IC patients from healthy subjects.

This study aimed to address the question of, “Does utilizing metabolic data in ML play a role in diagnosing IC?”. ML is a form of artificial intelligence (AI) and learns from past data in order to predict the future. Our NMR-based ML algorithm was able to collectively distinguish the IC patient urinary profile from that of controls.

MATERIALS AND METHODS

Ethics statement

For this paper, we used the deposited dataset derived from the published data. This study used the publicly deposited data, which does not need IRB approval.

Dataset

There are 59 samples in total in the IC dataset. In order to acquire IC-associated metabolites, urine samples were collected from 43 IC patient group and 16 healthy control group. Each urine specimen was analyzed using NMR and biomarkers were identified with 140 NMR peaks. The 140 NMR peak feature was utilized to apply the dataset to ML algorithms for classification of IC patients in this paper [5].

Method

Due to limited sample size, we adopted two machine learning algorithms, *i.e.*, support vector machine (SVM) [10,11] and LR [9], that are traditional but work well even with small number of samples. These are supervised learning algorithms, where each data sample is represented by a number of features and comes with a label that tells which group the sample belongs to.

When data is represented as scattered data points in a feature space that consists of two clusters representing individual groups, SVM finds a decision boundary (either linear or non-linear) that separates the different groups. Training an SVM optimizes the decision boundary to maximize the margin between the clusters, and it requires a kernel function train a kernel SVM that learns a non-linear decision boundary, *i.e.*, a non-linear classifier [12]. The model contains a user parameter known as “slack variable” that controls the width of the margin.

LR is also a classifier that learns *via* a linear model. By feeding a set of training samples with a number of features, it learns specific weights associated with features. When a data sample is input into to a LR model, a classification is made by a linear combination between the weights and the data; together with a sigmoid function, the combined value is mapped to a probability between 0 and 1. The predicted label is assigned according to the probability, and by minimizing the classification error (usually formulated using cross-entropy) in the training dataset, the weights are learned. One can add additional regularization terms in the model, such as l_1 or l_2 -norm of the weights, where l_1 -norm controls the sparsity of the weights [13], which will select the most important features, while l_2 -norm controls the smoothness of the weights to make the model more robust [13,14]. Both SVM and LR were implemented using the sklearn package in Python.

Training

Because the sample size was very small, the leave-one-out cross validation (CV) [15] method was utilized to make full use of the data set and to obtain unbiased result from the classifiers. With leave-one-out, we picked one sample as a testing set while using the rest of samples as a training set to train and test the model. The same process was iterated for every sample in the dataset. An illustration of the leave-one-out CV workflow is given in the **Figure 1**.

For SVM, we performed a set of experiments with a linear model, radial basis function (RBF) kernel, polynomial kernel with degree being 3, 5 and 7. The slack variable was set to 1 for all cases. For LR, we tried l_1 and l_2 penalties with different strengths; *i.e.*, the inverse of regularization strength C was set to 1, 5, and 10.

Evaluation

After repeating training and testing the model 59 times with leave-one-out CV, each sample was assigned a predicted label. By comparing these 59 predicted labels with the true labels, we constructed a confusion matrix by counting numbers of true positive (TP), true negative (TN), false positive (FP) and false negative (FN). From these numbers, accuracy, precision

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and recall were calculated to evaluate the performances of the models. Receiver operating characteristic (ROC) curve and precision and recall (PR) curve ~~were~~ plotted, and their area under the curve (AUC) are reported in the result section. Especially when the distribution of labels in the dataset is skewed, the AUC of the PR curve is a suitable measure for evaluating to account for the imbalance.

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RESULTS

Classification of IC samples with SVM

SVM was applied to the IC dataset with the leave-one-out CV scheme to classify IC samples from controls. The result varied depending on user parameters (*i.e.*, kernel type and kernel parameters) as shown in **Figure 2** and **Table 1**. Comparing the numbers, it was found that SVM with polynomial kernel resulted in the best performance when the degree of the polynomial kernel was 3 with 86.4% accuracy, 0.88 AUC of PR curve, and 0.85 AUC of ROC curve. Although the accuracy was the highest when the degree was 5, the AUCs of ROC and PR curves with degrees set to 3 was the highest. Moreover, the degree equal to 3 has less chance of overfitting than a degree of 5.

Here, the usage of linear kernel did not perform well. It may be because the data were not linearly separable or simply the sample size ($N = 59$) was too small compared to the dimension of the data (*i.e.*, 140 features). Performance of RBF kernel was also poor; looking at the accuracy using RBF kernel with SVM shown in **Table 1** (*i.e.*, 72.9%), it was the same as the proportion of IC samples in the dataset (*i.e.*, 43 IC subjects out of 59 subjects) and its recall was 1. This means that the classifier was simply predicting that all the samples belong to IC group and was not able to handle the class distribution imbalance problem.

Classification of IC samples with LR

In addition to SVM experiment, LR was used to classify IC samples and the results are shown in **Figure 3** and **Table 2** with different user parameter settings. LR with l_1 -penalty yielded the best performance when its penalty parameter was set to 10 with 84.7% accuracy, 0.91 for AUC of PR curve and 0.86 for the AUC of ROC curve, which was slight better than the results from SVM. These numbers are the best among several trials because of its randomness with the initial weights being trained, and the results from other trials did not differ much from those reported in **Figure 3** and **Table 2**.

It was observed that LR worked well despite being a linear model. Notice that the performance of linear SVM was poor in **Table 1**; this is because of the l_1 -norm penalty applied to the trained parameter imposing sparsity and behaving as a natural feature selector. When we checked the trained weight of features, most of the weights converged to 0 (a very small number on average of absolute values across the leave-one-out process). When the penalty parameter was 10, the average weights of 133 features was less than or equal to 0.1. This means that we only need a few critical features to predict correct label. In our experiment, feature $ID = 73, 4, 129$, and 35 were the most dominant features with the highest weights regardless of the random initialization. In other words, they were the four most useful NMR features. We have performed further statistical group analysis on these four NMR peaks using two-sample t -test, which resulted in P -values of 0.003, 0.001, 0.057, and 0.036 respectively. It was interesting to see that there were many other NMR peaks with even lower P -values and the peak $ID = 129$ had a P -value greater than 0.05. While these statistical tests

are performed independently, our classification results were derived by taking all the peaks at the same time for the analysis and it demonstrates that a linear combination of the features can be more powerful to distinguish IC from controls.

The l_2 -norm constraint did not contribute much in these experiments. This is because the model can robustly operate even without the l_2 -norm regularizer, which typically degrades performance of models in exchange for model robustness. Especially with the l_1 -norm regularizer significantly lowering the dimension of the data (with 133 redundant features), the sample size ($N = 59$) was sufficient to make robust and correct predictions for IC samples.

DISCUSSION

It comes with no surprise that medicine is awash with claims that ML applications into big healthcare data will create extraordinary revolutions [8,16,17]. Recent examples have demonstrated how big data and ML can create algorithms that can perform on par with human physicians. AI is one ML approach without prerequisites. Various AI techniques already exist, and successful metabolomics analysis has been reported in previous studies [18-20]. Conventional statistical analysis and AI-based methods were used to assess the discrimination capability of quantified metabolites. A multiple logistic regression (MLR) model, alternative decision tree (ADTree), neurofuzzy modelling (NFM), artificial neural network (ANN), and SVM machine learning methods were used [21,22].

Modern advancements in computational and data science, with its most popular implementation in ML, has facilitated novel complex data-driven research approaches. Combined with biostatistics, ML aims at learning from data. It accomplishes this by optimizing the performance of algorithms with immediate previous knowledge. ML can be applied in either a supervised or unsupervised fashion. Supervised learning entails monitoring of the algorithm while it is being trained to learn a correct class assignment from a set of parameters, such as how to make a correct diagnosis from clinical and laboratory information [18].

Current biomarkers for IC diagnosis and prognosis are insufficiently robust for clinical practice using AI. Instead, we used AI to identify IC-related metabolites in an NMR metabolomics dataset from our previous study [5], which was able to collectively distinguish IC patient urinary profiles from that of healthy controls. The development of diagnostic tools using ML may be useful for more accurately identifying IC patients. AI has the potential to manage the imprecision and uncertainty that is common in clinical and biological data. AI or ML-based algorithms can take several different forms. The icons in the presented figures in this paper represent typical ML methods. These include multilayer neuronal networks, decision tree-based algorithms, SVM, and related algorithms that separate classes by placing hyperplanes between them, and prototype-based algorithms, such as k-nearest neighbors that compare feature vectors carried by a case with those carried by other cases and assign classes based on similarities. ML-based algorithms are not being actively applied to IC research. Such applications could lead to a better understanding and deeper knowledge of metabolomics data, which would then provide insights into biomarker discovery.

Although this is out of scope for this study, AI algorithms can be used to predict IC progression or therapeutic responses, too [23,24]. Patient clinicopathological features are commonly used to train AI algorithms to predict patient outcomes in other diseases, such as

cancer [25–27]. For instance, Wong *et al.* developed a prostate cancer patient-specific ML algorithm based on clinicopathological data to predict early biochemical recurrence after prostatectomy [28]. The resulting 3 ML algorithms were trained using 338 patients and achieved an accuracy of 95%–98% and AUC of 0.9–0.94. When compared to traditional Cox regression analysis, the 3 ML algorithms had superior prediction performance. This study demonstrated how AI algorithms, trained with clinicopathological data, imaging radiomic features, and genomic profiling, outperformed the prediction accuracy of D’Amico risk stratification, single clinicopathological features, and multiple discriminant analysis, a type of conventional multivariate statistics [28]. There is also a role for AI in selecting effective drugs for cancer treatment [29]. Using an ML-based algorithm, Saeed *et al.* quantified the phenotypes of castration-resistant prostate cancer cells and tested their response to over 300 emerging and established clinical cancer drugs [30].

We are aware that one of the limitations of this study includes the novelty of using crowdsourcing in medical biomarker development. To our knowledge, there is no previous reference for comparison. Additionally, this study was limited to participants in South Korea and to a 1-time point collection. A major problem associated with medical datasets is a small sample size [5]. Given that sufficiently large datasets are important when creating classification schemes for disease modeling, a relatively larger dataset can result in reasonable validation due to sufficient partitioning of training and testing sets. On the contrary, a smaller training dataset can lead to misclassifications and may result in unstable or biased models. For our study, a major problem was the small sample size. However, the reason for this is that it takes an immense amount of time, effort, and cost to collect a larger amount of medical research data. Furthermore, medical research data is often inconsistent, incomplete, or noisy in nature; thereby, reducing sample sizes even more. Such small sample size for high-dimensional data often leads to “curse of dimensionality”, *i.e.*, failing to properly estimate necessary parameters due to lack of samples, which we also faced with only 59 samples for 140 NMR features. For the SVM used in this study, when casting its objective function as a dual form using Lagrangian multiplier, the optimization problem seeks for a sparse solution that identifies a few “support vectors” and thus greatly reduces the dimension of problem. For the LR, we used two different regularizers on the parameters to estimate, *i.e.*, l_1 and l_2 -norms, to avoid curse of dimensionality and obtain feasible solutions. As demonstrated in the results, as l_1 -norm constraint behaved as a data-driven feature selector reducing the dimension of the problem, the classifier avoided the curse of dimensionality. Although we were able to stay away from the curse of dimensionality in this study, poor analysis may lead to data overfitting and irreproducible results. ML-based algorithms may be manipulated by datasets containing dominant but irrelevant features when the sample number is limited. Also, AI cannot be used as an end-all solution to any question. There are instances where traditional statistics has outperformed AI or where additional AI does not improve results.

In summary, we have found that ML-based algorithms can be applied to developing diagnostic models for IC patients. In the current clinical setting, urologists are generally dependent on cystoscopy and questionnaire-based decisions to diagnose IC due to a lack of objective molecular biomarkers. The purpose of this study was to develop machine learning

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methods for diagnosing IC and assess their performance using metabolomics data. Considering how ML techniques for analyzing omics data can play a role in predicting the diagnosis and prognosis of diseases, future studies should integrate use of a larger multidimensional and heterogenous dataset, application of more accurate validation results, and use of different techniques for classifying and selecting features to pave a promising way toward clinical applications.

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Author contributions

J Kim, M Shahid, P Jin: data collection or management and manuscript drafting; F Tong, S Jung, WH Kim: data analysis and manuscript drafting; S Jung, WH Kim, J Kim: project development, manuscript writing/editing and Data analysis

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Figure 1. IC classification experimental scheme with leave-one-out cross validation.

Figure 2. Classification result evaluation curves using SVM. **A.** Precision-Recall curve. **B.** ROC curve. The values of AUC are calculated for each curve and larger values indicate better performance.

Figure 3. Classification result evaluation curves using LR. **A.** Precision-Recall curve. **B.** ROC curve. The values of AUC are calculated for each curve and larger values indicate better performance.

Table 1. The comparison of results from SVM with different set of parameters.

Parameters	TP	TN	FP	FN	Accuracy	Precision	Recall	AUC of PR	AUC of ROC
Kernel = linear	36	9	7	7	0.763	0.837	0.837	0.82	0.76
Kernel = poly, degree = 3	39	11	5	4	0.847	0.886	0.907	0.88	0.85
Kernel = poly, degree = 5	39	12	4	4	0.864	0.907	0.907	0.88	0.84
Kernel = poly, degree = 7	39	11	5	4	0.847	0.886	0.907	0.87	0.83

Kernel = RBF	43	0	16	0	0.729	0.729	1.000	0.36	0.00
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Table 2. The comparison of results from LR with different set of parameters.

LR	TP	TN	FP	FN	Accuracy	Precision	Recall	AUC of PR	AUC of ROC
Penalty = l_1 , C = 1	39	9	7	4	0.814	0.848	0.907	0.82	0.75
Penalty = l_1 , C = 5	39	10	6	4	0.831	0.867	0.907	0.88	0.84
Penalty = l_1 , C = 10	38	12	4	5	0.847	0.905	0.884	0.91	0.86
Penalty = l_2 , C = 5	38	7	9	5	0.763	0.809	0.884	0.82	0.75
Penalty = l_2 , C = 10	38	7	9	5	0.763	0.809	0.884	0.82	0.75