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Identification of Protein Marker in Vaginal Wall Tissues of Women with Stress Urinary Incontinence by Protein Chip Array

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Abstract

Aim—We sought to investigate protein biomarkers for stress urinary incontinence (SUI) in vaginal tissues using Surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS) and examine if this is a reliable methodology to examine proteins in small tissue specimens.

Methods—We compared protein expression profile of vaginal tissue from women with SUI and continent controls. A 22.6 kDa peak was identified by subsequent weak cation-exchange, reverse-phase fractionation, gel electrophoresis, trypsin digestion, then analyzed by matrix assisted laser desorption/ionization mass spectrometry (MALDI MS) and MALDI MS-MS. Biomarker identity and expression level were confirmed by Western-blotting and immunohistochemistry.

Results—Expression of the 22.6 kDa protein, identified as SM-22 α , was significantly higher in women with SUI versus controls. A 3×3mm tissue sample was sufficient for identification. Western blot/immunohistochemistry confirmed the SELDI-TOS MS findings.

Conclusions—SM-22 α , a marker for myofibroblasts, was identified as a biomarker of SUI. Differential protein profiling by SELDI-TOF MS is a powerful and reliable tool for urogynecological research since it allows us to study an array of proteins simultaneously using small tissue samples.

Keywords

Mass spectrometry; myofibroblast; protein chip array; stress urinary incontinence; SM-22 α

Introduction

Pelvic floor disorders, which include stress urinary incontinence (SUI) and pelvic organ prolapse (POP), are prevalent and disabling conditions for many women. Factors contributing to urinary continence include normal bladder neck mobility or tissue support, urethral sphincter muscle and nerve integrity, urethral smooth muscle, and vascular plexuses. The vaginal wall is the main support tissue of the urethra and bladder neck since it

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prevents urethral and bladder neck descent^{1, 2}. The loss of mechanical stability of the urethra and bladder neck is thought to be an important factor in the development of SUI. This stability is largely provided by intact connective tissues in the pelvis. Women with pelvic floor dysfunction demonstrate abnormal extracellular matrix (ECM) metabolism in their pelvic support tissues³. A major barrier to our understanding of the complex ECM pathways in SUI is our ability to study the important proteins simultaneously.

Studies based on molecular analyses and gene expression profiling of ECM in the pelvic support tissues^{3–9} are now evolving and may provide clues about pathogenic factors leading to SUI. Although genomic and transcriptomic technologies are powerful tools, their disadvantage is that gene expression does not always correlate with protein expression^{10, 11}. Proteins are the most relevant markers of cell functions. Over the years, proteomic studies have used 2-dimensional gel electrophoresis (2-DE) followed by identification of differentially expressed proteins by mass spectrometry. The 2-DE technique has high-resolution capacity but is labor intensive and requires large quantities of intact proteins. More recently, surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS) offers a complementary and efficient platform to detect differentially expressed biomarkers in human diseases, especially smaller peptides and proteins up to 30 kDa)^{12–14}.

In this study, we sought to explore potential biomarkers for SUI in vaginal tissues and investigate whether the SELDI-TOF MS technology can provide useful and reliable data in the analysis of pelvic tissues for research in pelvic floor dysfunction. Because a common limiting factor in pelvic floor dysfunction research is our ability to obtain large tissue specimens, we sought to test this technology with very small tissue sample sizes.

Materials and Methods

Patient /sample information

This study was approved by the Institutional Review Board of Stanford University School of Medicine. All patients had signed a written informed consent before recruitment. We selected women with SUI and continent controls from both the proliferative and secretory phases of the menstrual cycle for this study. Phase of cycle was confirmed by endometrial histology. Women with a history of endometriosis, gynecologic malignancies, pelvic inflammatory conditions, connective tissue disorders, emphysema, prior pelvic surgery and pelvic organ prolapse beyond POP-Q stage 1 were excluded. These conditions could have profound effects on pelvic ECM metabolism.

In women undergoing surgery for SUI, approximately 1 cm² of full-thickness, peri-urethral vaginal mucosa was excised 1 cm lateral to the urethrovesical junction identified by a Foley balloon. Smaller, 0.5 cm² biopsies of vaginal mucosa from a similar area were excised from continent women undergoing benign gynecologic surgeries for fibroids, dysfunctional bleeding, or ovarian cysts. The tissue was frozen immediately in liquid nitrogen and then stored at –80°C for further processing. The epithelial layers were removed with a razor blade before the tissues were processed. A representative cross-section was fixed in 10% buffered formalin for 16 hours, processed with paraffin embedding, and used for immunohistochemistry.

Sample preparation

We recruited 20 participants with SUI (n=10) and controls (n=10) from both the proliferative and secretory phases of the menstrual cycle. All samples were processed simultaneously. The frozen biopsies (small tissue specimens of roughly 3×3 mm in size) were washed in phosphate-buffered saline (Sigma, St. Louis, MO) to rinse off blood and

homogenized in 0.5 ml of lysis buffer (9.0M urea, 2% NP-40, 1% DTT, pH 7.7) using Tissue Tearor (BioSpec Products, Inc, Bartlesville, OK). The samples were rotated at 4°C overnight and then centrifuged at 14,000 rpm for 30 minutes at 4°C to remove the tissue debris. The extracts were aliquoted and stored at -80°C freezer. Before SELDI-TOF MS protein profiling, the protein concentration of the extracts was determined by Bradford protein assay (Bio-Rad, Hercules, CA).

Profiling of tissue extracts on SELDI ProteinChip Arrays

An aliquot of the tissue extracts (2µg) was added to 0.098 ml 0.1 M acetate buffer, pH 4 on CM10 Protein Chip arrays (BioRad, Hercules, CA) in a 96-well bioprocessor in which the wells contain 0.09 ml 0.1 M acetate buffer, pH 4. After binding while shaking on a microplate shaker, the CM10 arrays were washed three times with binding buffer, briefly rinsed twice with purified water, and air-dried for 15 minutes. The energy-absorbing matrix (EAM) sinapinic acid (SPA, 50% saturated solution in 50% acetonitrile, 0.1% trifluoroacetic acid) was applied to the CM10 arrays (two applications of 1 µl) and allowed to dry in air. Mass spectra of the samples were acquired in a Bio-Rad PCS4000 mass spectrometer at low, medium, and high-power laser energies (3000, 4500, and 7500 nJ respectively). The energy setting was optimized for data acquisition in the m/z ranges of 2–5K, 5–20K, and 20–200K, respectively. Following data acquisition, the spectra were de-noised and filtered and the intensities were normalized based on total signal (total ion current) using the vendor's software. Mass spectrum peaks with signal/noise ratios > 4 were located, and clustered across spectra from the different patients when a peak was present in a minimum of 15% of the spectra. Student t-test and Wilcoxon rank sum tests were performed, comparing different groups of patient samples.

Protein Purification and recovery

One classifier of interest (22.6 kDa protein peak cluster) was statistically identified, and this biomarker was biochemically identified. The initial step in the purification of 22.6 kDa peak was to dilute in 0.05M ammonia acetate, pH 4.0 and load on VivaPure mini spin weak cation-exchange columns (VivaScience, Goettingen, Germany) previously equilibrated in the same dilution buffer. Elution of bound proteins was performed by washing columns progressively in 400 µl of 0.05M sodium phosphate buffer, (pH6.0 and pH6.5). The elutions were further purified and concentrated by reversed-phase fractionization and filtration with a YM50 centricon filter. The purification scheme was monitored by enrichment of the peaks in SELDI mass spectra under the same conditions in which the biomarker was first identified. One microliter from each of the fractions was spotted onto a ProteinChip NP-20 surface type to identify a 22.6 kDa peak. The fractions with enriched 22.6 kDa m/z peak were subjected to a 4–12% Bis-Tris NuPage gel (Invitrogen, Carlsbad, CA, USA). The gel was stained with GelCode Blue stain (Pierce, Rockford, IL, USA).

Protein Identification by Tryptic Digest peptide Mapping

The candidate bands on the gel were cut. The proteins were then eluted from the isolated gel slices by extraction into FAPH (50% formic acid, 25% acetonitrile, 15% isopropanol, 10% water) and verifying the biomarker as having the correct m/z. The remaining gel slices of the correct biomarker were then digested with trypsin, and analyzed by MALDI MS to get peptide fingerprint, and MALDI MS/MS to determine the sequence of prominent peptide ions. Coverage map of observed in-gel tryptic peptides was compared with amino acid sequences, as listed in the National Center for Biotechnology information-nonredundant database.

Western-Blot

To verify SM-22 α (22.6 kDa) protein identified by tryptic digestion peptide mapping, the Western-Blot were performed by 10% SDS-PAGE gel electrophoresis. After gel electrophoresis, the proteins were transferred onto the Nitrocellulose paper. The blot was probed with goat anti-SM-22 α polyclonal antibody (0.5 μ g/ml, Abcam, Cambridge, MA) for 1 hour at room temperature. After washing three times with phosphate buffered saline with 0.1% Triton, pH 7.4 (PBS-T), the membrane was then incubated in 1:10,000 dilution of mouse anti-goat IgG conjugated to horse-radish peroxidase (HRP) (GE Healthcare, Pittsburgh, PA, USA) for 1 hour at room temperature, followed by three washes in PBS-T. Blots were developed by chemiluminescence. The blots were re-probed with rabbit anti-Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) polyclonal antibody (1/2500, Abcam, Inc., Cambridge, MA, USA), then 1/10,000 dilution of donkey anti-rabbit IgG conjugated to HRP (GE Healthcare, Sunnyvale, CA, USA). The band density was determined by Bio-Rad Quality One Software (Bio-Rad, Hercules, CA, USA).

Immunohistochemistry

Immunohistochemical staining for SM-22 α , vimentin and desmin was performed on fixed embedded tissue as described previously⁷ to confirm the expression of SM-22 α in vaginal wall specimens. Briefly, paraffin-embedded specimens were cut into 5 μ m sections, de-waxed in Xylene and rehydrated through graded ethanol solutions. After washing with TBS-T, endogenous peroxidases were blocked with 3% H₂O₂ in TBS-T, and non-specific binding was blocked with 1% bovine serum albumin, 5% normal secondary antibody host serum in TBS-T at room temperature for 1 hour. After rinsing with TBS-T, the slides were incubated with goat anti-SM-22 α (1/20) or mouse anti-vimentin or desmin (1/20, Sigma, St. Louis, MO, USA) primary antibody overnight at 4°C. Omission of the primary antibody was used as a negative control. After rinsing with TBS-T, slides were incubated with a secondary antibody, goat anti-rabbit biotin conjugate or horse anti-mouse biotin conjugate (1/50, Vector Laboratory, Forst City, CA, USA). The slides were incubated with Vectastatin ABC Kit (Vector Laboratory) reagent for 30 at room temperature. Slides were counterstained with 25% of hematoxylin (Fisher, Fair Lawn, NJ, USA). They were visualized and photographed with AxioCam (Zeiss, Oberkochen, Germany).

Relative Real-Time Quantitative PCR

Expression of SM-22 α mRNA was analyzed by real-time quantitative PCR (QPCR) in SUI and continent control patients. We recruited total 45 age-matched subjects, including 20 subjects for SELDI Protein Chip Arrays, with SUI (n=11) and controls (n=12) in the proliferative, and with SUI (n=11) and controls (11) in the secretory phases of the menstrual cycle. The extraction of RNA from the tissue sample was carried out with the RNA-STAT-60 reagent (Tel-Test, Inc., Friendswood, TX, USA). The complementary DNA (cDNA) was generated from total RNA, as described previously¹⁵. PCR primers used to amplify cDNA were forward: 5'-GGAGTGGATCATAGTGCAGTGT-3' and reverse: 5'-GCTTGGAGCCATCAGGGTA-3'. Real-time QPCR was carried on the Mx3005P Multiplex Quantitative PCR System with MxPro QPCR software (Stratagene, La Jolla, CA, USA). Brilliant SYBR Green QPCR Master Mix (Stratagene,) was used to perform PCR. The amplifications were carried out following a 10-minute hot start at 95°C in a three-step protocol with 30 seconds denaturation (94°C), 1 minute annealing (60°C), and extension at 72°C for 30 seconds. Forty cycles were performed. Hypoxanthine phosphoribosyl-transferase 1 (HPRT1) was used as an endogenous reference⁷ against which the different template values were normalized. All PCR reactions were performed in duplicate. Cycle of threshold (Ct) methods was used for quantification. Relative quantification of the gene of SM-22 α – corrected for the quantity of the normalizer gene (HPRT1) – was divided by one normalized control sample value (calibrator sample) to generate the relative quantification to

calibrator (Rel. Quant. to Cal.). The PCR products were sequenced to ensure that the correct gene sequence was amplified.

Results

Protein profiling of vaginal wall tissues from women with and without SUI

Mass spectrometric analysis of the proteins that bound to the surface of the Q10 ProteinChip arrays revealed an up-regulated cluster of protein of ~22.6-kDa in the vaginal wall tissue from a woman with SUI (S1) (Figure 1, top A) when compared with a control (C2) (Figure 1, top B) in the secretory phase. A cluster of protein of ~30 kDa (Figure 1A) in the vaginal wall tissue was observed and it matched the size of the subunit of hemoglobin which might come from the tissues.

The ~22.6-kDa intensities of tissue lysates from five women with SUI (S1–S5) were higher than that from five controls (C1–C5) from the secretory phase ($p=0.045$, Figure 2A). The similar pattern was also observed by Western-blot except the subject 1 of the women with SHUI (S1) because of a shortage of sample ($p=0.02$, Figure 2B). This difference in expression of the ~22.6-kDa proteins was not observed in the vaginal wall tissues from the proliferative phase of women with SUI when compared with controls (data not shown).

Identification of ~22.6-kDa protein marker

Because the highest level of difference in the expression of ~22.6-kDa protein was observed on the Q 10 Protein Chip in the tissue lysate from subject 1 with SUI (S1), this lysate was chosen to identify the cluster proteins. We subjected this tissue lysate sequentially to weak cation-exchange columns, reversed-phase fractionization and YM50 centricon concentration. The purification scheme was monitored by enrichment of the peaks on SELDI mass spectra under the same conditions in which the biomarker was first identified. The fractions were spotted onto a ProteinChip NP-20 surface type to identify a 22.6 kDa peak. The fractions with a 22.6 kDa peak were subjected to a 4–12% Bis-Tris NuPage gel (Figure 3). Tryptic in-gel digestion of excised protein bands of ~22.6-kDa yielded peptide that matched well in both peptide fingerprint and MALDI MS/MS analysis to SM-22 α , also known as transgelin.

Confirmation of SM-22 α in the Vaginal Wall Tissues by Western-blot and Immunohistochemistry

To confirm the accuracy of peptide fingerprint and MALDI MS/MS analysis, we perform Western-blot with an anti- SM-22 α antibody in the same lysates from both the proliferative and secretory phases, which were used for Protein Chip arrays. All of them showed the presence of SM-22 α in the vaginal wall tissues except the subject 1 of the woman with SUI (S1) from the secretory phase because of a shortage of sample (Figure 2B). We also confirmed the presence of SM-22 α in the vaginal wall tissues by immunohistochemical staining with an anti-SM-22 α antibody on the fixed embedded tissue (Figure 4). It is co-localized with fibroblast marker (vimentin) and smooth muscle cell marker (desmin) in the vaginal wall tissues.

Gene Expression Levels of SM-22 α in Vaginal Wall Tissue from Continent Controls and Women with SUI

The expression level of SM-22 α in tissues from women with SUI and control groups from the proliferative and secretory phases of menstrual cycle was evaluated using QPCR. The expression level of SM-22 α mRNA in the control groups of both proliferative ($P=0.16$) and secretory phases ($p=0.21$) was not significantly different from that in the SUI group (Figure 5).

Discussion

In this study, SELDI-TOF-MS protein profiling of extracts of the vaginal wall tissues demonstrated that the ~22.6-kDa peak, which was identified as SM-22 α , was differentially expressed in the tissues from women with SUI only (no prolapse) when compared with that from continent controls (no prolapse) during the secretory phase of the menstrual cycle. We found no differential mRNA expression as determined by real-time PCR of secretory phase vaginal wall tissues from women with and without SUI, further supporting the thesis that gene expression does not always correlate with protein expression^{10, 11}.

SM-22 α is the earliest marker of smooth muscle cell (SMC) lineage and it is expressed in myofibroblasts. Fibroblast-to-myofibroblast differentiation in the ECM represents a key event during wound and tissue repair. Myofibroblasts play a central role in tissue repair by closing the wound through contractile forces and increasing synthesis of ECM components. In chronic inflammation, fibrosis occurs when the synthesis of new collagen by myofibroblasts exceeds degradation¹⁶. SM-22 α expression has been observed in injured glomerular epithelial cells¹⁷ and was found to be increased in fibroblasts from keloid scars compared to fibroblasts from normal skin¹⁸. Its co-localization with the fibroblast marker (vimentin) and smooth muscle cell marker (desmin) in the vaginal wall tissues (Figure 4) is consistent with conversion of fibroblasts to myofibroblasts during tissue repair processes.

Our preliminary proteomics data show increased SM-22 α expression in the vaginal tissues from women with SUI during the secretory phase compared to controls. This provides further insight into the mechanisms in pelvic ECM by suggesting that the process of fibroblast-to-myofibroblast transformation is occurring in tissues from women with SUI. This could explain the seemingly contradictory reports of simultaneous collagen deposition and increased degradation within the same pelvic tissue. It is possible that new collagen is being deposited by myofibroblasts while there is ongoing degradation. This progressive ECM remodeling can ultimately destroy and replace normal tissue architecture and mechanical properties. Our previous studies documenting changes in ECM protein expression with cyclic hormonal changes⁷ and observation of increased elastin degradative activity in fibroblasts from the secretory phase support the concept that hormones in the secretory phase increase ECM turnover^{19, 20}. This increase in turnover also alters levels of active and total TGF-beta²¹ which modulates expression of SM-22 α . This is a possible explanation for the difference in SM-22 α expression between the proliferative and secretory phases seen in the present study.

Research in human pelvic floor disorders is difficult due to limited access to sufficient pelvic tissue samples necessary for protein analysis. In this study, we demonstrated that significant data can be obtained with tissue sample sizes of 3×3mm, using the SELDI-TOF MS technology. Our confirmatory assays indicate that the information is reliable. Therefore, this technology could help obviate the need for larger pelvic tissue samples in future studies.

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Figure 1.

A higher expression level of 22.6 kDa protein peak cluster was observed in the vaginal wall tissue from participant #1 with SUI (**S1**) (**A**) compared to that from participant #2 without SUI (**C2**) (**B**) in the secretory phase of the menstrual cycle after SELDI-TOF-MS spectrum profiling retained on CM 10 surface.

Figure 2.

(A), The 22.6 kDa cluster in the vaginal wall tissue lysates from five women with SUI (S1–S5) was higher than that from four of five controls (C1–C5) in the secretory phase of the menstrual cycle determined by SELDI-TOF-MS spectrum ($p=0.045$). (B), Western blotting of SM-22 α in the same vaginal wall tissue lysates as above, except S1 due to a shortage of sample, was performed to identify the 22.6 kDa cluster. The expression pattern of SM-22 α was similar to that observed by SELDI-TOF-MS spectrum ($p=0.02$)

Figure 3.

The purified and concentrated 22.6 kDa peak from S1 subject by weak cation-exchange column, reversed-phase fractionization and YM50 centricon concentration was monitored by enrichment of the peaks on SELDI mass spectra. The fractions with a 22.6 kDa peak were subjected to a 4–12% Bis-Tris NuPage gel, and then stained with GelCode Blue stain. The 22.6 kDa bands were excised from the gel, with one part subjected to elution to verify the biomarker as having the correct m/z. The other part was subjected to in-gel tryptic digestion. Tryptic in-gel digestion of excised protein bands of ~22.6-kDa yielded peptide that matched well in both peptide fingerprint and MALDI MS/MS analysis to SM-22 α .

Figure 4. Immunohistochemical staining of SM-22 α , vimentin and desmin. It shows that SM-22 α co-localizes with vimentin (fibroblast marker) and desmin (smooth muscle) in the vaginal wall tissues. Scale bar=50 μ m.

Figure 5.

Relative real-time QPCR was performed to compare the mRNA expression levels of in the vaginal wall tissue from menstrual-matched women with SUI and controls. Relative quantification of the genes of SM-22 α , normalized to the internal control (HPRT1), was divided by one normalized control sample value (calibrator sample) to generate the relative quantification to calibrator. The numbers represent the mean $\pm$ SEM. mRNA expression level of SM-22 α in the control group (n=12) was not significantly different from that in the SUI group (n=11) during the proliferative phase. The same held true for tissues harvested during the secretory phase of the menstrual cycle.