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Prostate cancer-associated urinary proteomes differ before and after prostatectomy

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Abstract

Background: A wide range of disorders can be detected in the urine. Tumor-modifying proteins in the urine may serve as a diagnostic tool for cancer patients and the alterations in their profiles may indicate efficacies of chemotherapy, radiotherapy, and surgery.

Methods: We focused on urinary proteomes of patients with prostate cancer and identified tumor-modifying proteins in the samples before and after prostatectomy. Protein array analysis was conducted to evaluate a differential profile of tumor-promoting cytokines, while mass spectrometry-based global proteomics was conducted to identify tumor-suppressing proteins.

Results: The result revealed striking differences by prostatectomy. Notably, the urine from the post-prostatectomy significantly decreased the tumorigenic behaviors of prostate tumor cells as well as breast cancer cells. We observed that angiogenin, a stimulator of blood vessel formation, was reduced in the post-prostatectomy urine. By contrast, the levels of three cell-membrane proteins such as prostasin (PRSS8), nectin 2 (PVRL2), and nidogen 1 (NID1) were elevated and they acted as extracellular tumor-suppressing proteins. These three proteins, given extracellularly, downregulated tumorigenic genes such as Runx2, Snail, and transforming growth factor beta and induced apoptosis of tumor cells. However, the role of NID1 differed depending on the location, and intracellular NID1 was tumorigenic and reduced the percent survival.

Conclusions: This study demonstrated that prostatectomy remarkably altered the profile of urinary proteomes, and the post-prostatectomy urine provided tumor-suppressive proteomes. The result sheds novel light on the dynamic nature of the urinary proteomes and a unique strategy for predicting tumor suppressors.

Keywords: angiogenin, nectin 2, nidogen 1, prostasin, prostate cancer, prostatectomy, urine

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Introduction

Prostate cancer is a major men's health problem[1] and the third most common cause of cancer-related mortality among men in the United States.[2,3] Advanced prostate cancer nearly always metastasizes to the bone, and bone metastasis leads to a significantly decreased survival rate.[4] Bone is fertile soil for migratory prostate cancer cells, and their invasion into the bone initiates a vicious cycle in which bone-resorptive osteoclasts destruct the bone matrix and release tumor-promoting factors such as transforming growth factor beta (TGFβ).[5,6] The current treatment includes the administration of anti-androgenic compounds as well as anti-resorptive agents such as bisphosphonates, and radiotherapy to relieve pain.[7–9] However, there is an unmet need to develop an effective therapy to prevent tumor-invaded bone destruction.

Bio-fluids are frequently used to detect disease-identifying biomarkers. For instance, the prostate-specific antigen (PSA) test is a blood examination for detecting prostate cancer. In the

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diagnosis of phosphate disorders such as chronic kidney diseases, the level of FGF23 can be monitored both in the serum and urine.[10] While it is generally considered that urine in any form is not therapeutic for cancer patients, an intriguing question is whether urine from patients with cancer may contain oncogenic factors as well as anti-oncogenic factors. If it contains both, the next question is whether their levels might be affected by cancer progression or environmental stimuli. We have previously reported that the urinary level of dopamine, which acts as a tumor suppressor, is elevated by physical exercise.[11] We have also shown that physical exercise can reduce the level of cholesterol, a stimulator of cancer progression, in urinary volatile organic compounds (VOCs). Physical exercise can thus enhance the antitumor capability of the urine. These results allowed us to revisit a commonly perceived claim that urine does not contain any form of antitumor factors.[12]

In bladder cancer, few urinary biomarkers are available for diagnostic purposes,[13] except for nuclear matrix protein 22 and bladder tumor antigen, which are used as supplementary diagnostic tools.[14] Regarding prostate cancer, PSA in the blood and its derivatives, including PSA kinetics, PSA density, and percentage of free PSA, are commonly employed. However, the low specificity of PSA results in a considerable number of unnecessary prostate biopsies, and a urine-based test tool such as SelectMDx, which detects the mRNA levels of HOXC6 and DLX1, is shown to improve the accuracy of diagnosis.[15] Furthermore, detecting prostate cancer antigen 3 and a gene fusion between TMPRSS2 and ERG in the urine is becoming popular.[16–18] The marker candidates also include annexin A3, matrix metalloproteinase 9, and vascular endothelial growth factor,[19] in which the role of annexin A3 is reported context dependent, acting as a tumor promoter as well as a tumor suppressor.[20] Obesity is a risk factor for prostate cancer, and thus the accumulation of metabolic intermediates and an increased expression of genes in the tricarboxylic acid cycle, and the induction of *de novo* lipogenesis are proposed to provide a novel set of biomarkers.[21] Our previous studies have also shown that tumor-modifying proteins such as Hsp90ab1, moesin, and enolase 1 are location dependent, and they can serve as a tumor promoter intracellularly and as a tumor suppressor extracellularly.[22–24] However, the urinary proteomes associated with prostate cancer have not been fully elucidated.

Here, we conducted mass spectrometry-based global proteomics using two sets of urine samples from patients with prostate cancer. One set was collected before prostatectomy and the other set after prostatectomy. The serum level of PSA was undetectable in post-prostatectomy patients. As a non-tumor control, we employed urine samples from healthy individuals. These control samples were collected before and after 30-min step aerobics since our previous study showed that physical exercise converted urine into an antitumor agent.[25] Our previous studies using a mouse model of mammary tumor and bone metastasis have shown that the administration of Pitavastatin, a cholesterol-lowering agent, altered the profile of urinary VOCs.[26] We thus hypothesized that physiological changes associated with the progression of prostate cancer by prostatectomy may alter the urinary levels of tumor-modifying proteins. We also hypothesized that the action of some of those proteins may be context dependent, and they may act as tumor promoters intracellularly and tumor suppressors extracellularly.

Protein array analysis was conducted to evaluate a differential profile of tumor-promoting cytokines. The result with the protein array indicated that the urine from post-prostatectomy patients and the post-exercise controls commonly downregulated angiogenin that would stimulate the formation of vessels, an important process for the cancer progression including prostate cancer. Besides protein array analysis, mass spectrometry-based global proteomics predicted 10 putative tumor suppressors in post-prostatectomy samples, including prostasin (PRSS8), nectin 2 (PVRL2), and nidogen 1 (NID1), as well as CD14. Prostasin is a membrane-anchored serine protease and has been known as a tumor suppressor,[27,28] and nectin 2 is a single-pass membrane glycoprotein for building adherens junctions.[29,30] NID1 is a basement membrane protein that binds to extracellular matrix (ECM) proteins,[31,32] and CD14 is a lipopolysaccharide-binding protein at the cell surface that activates monocytes in the immune system.[33,34] The prediction of extracellular nectin 2 and NID1 as tumor suppressors was unexpected since these proteins are considered the potential targets to be inhibited for many cancers.[29,35–37] The result herein indicated that their actions were context dependent, and they acted as tumor-promoting and suppressing proteins in the intracellular and extracellular domains, respectively.

Materials and methods

Urine collection

The use of human urine was approved by the Indiana University Institutional Review Board. This study was conducted in accordance with the Declaration of Helsinki of the World Medical Association. The urine samples were collected from 10 treated patients with prostate cancer (average age, 53 years in the range of 49–80), who received prostatectomy and the PSA level was below 0.04 ng/mL. The urine samples were also collected from 10 untreated patients with prostate cancer (average age, 50 years in the range of 52–80), who had a PSA level above 2.57 ng/mL and were yet to receive prostatectomy. Besides patients with prostate cancer, urine samples were collected from seven healthy male participants with no history of cancers (average age, 28 years in the range of 18–41) before and 1 h after 30-min step aerobics as described previously.[11] The urinary levels of proteins, urobilinogen (an indicator of liver problems), ketone (an indicator of diabetes and ketosis), nitrite (an indicator of urinary tract infection), and bilirubin (an indicator of liver or gallbladder problems) were detected using urinalysis test strips (Nebunox; Diagnox Health, Plano, TX, USA).

Cell culturing

TRAMP-C2ras prostate tumor cells,[38] EO771 mouse mammary tumor cells (CH3 BioSystems, Amherst, NY, USA),[39] and 4T1.2 mouse mammary tumor cells (obtained from Dr. R. Anderson at Peter MacCallum Cancer Institute, Melbourne, Australia)[40] were cultured in Dulbecco's modified eagle medium. MDA-MB-231 breast cancer cells (ATCC)[41] were grown in α-MEM, and PC-3 human prostate cancer cells (ATCC)[42] were cultured in RPMI-1640 (Gibco, Carlsbad, CA, USA). MLO-A5 osteocytes (obtained from Dr. L. Bonewald at Indiana University, IN, USA) and RAW264.7 pre-osteoclast cells (ATCC) were grown in α-MEM. The culture media was supplemented with 10% fetal bovine serum and antibiotics (50 units/mL penicillin, and 50 μg/mL streptomycin), and cells were maintained at 37°C and 5% CO_2.

MTT, EdU, scratch, and transwell invasion assays

Cell viability was examined using an MTT assay (Invitrogen, Carlsbad, CA, USA).[43] The optical density (OD) values were read at 490 nm by a microplate reader (Molecular Devices, LLC, Sunnyvale, CA, USA). Percent viability was defined as the relative absorbance of intervened cells versus control cells. Cellular proliferation was examined using a fluorescence-based EdU proliferation kit (Thermo Fisher Scientific, Waltham, MA, USA). After fluorescent labeling, the number of fluorescently labeled cells was counted, and the ratio of the number of fluorescently labeled cells to the total number of cells was determined.[11] A transwell invasion assay was conducted to detect invasive ability, and a wound-healing scratch assay was utilized to evaluate two-dimensional motility.[44]

Western blot, protein array analyses, plasmid transfection, and enzyme-linked immunosorbent assay

Western blot analysis was conducted as previously described.[44] We used antibodies against cleaved caspase 3 (9661S), Snail (3879S), TGFβ (3711S), albumin (4929S) (Cell Signaling, Danvers, MA, USA), MMP9 (sc-393859), angiogenin (sc-74528) (Santa Cruz, Dallas, TX, USA), Trail (NB500-220) (Novus, Centennial, CO, USA), p53 (MA5-12557) (Invitrogen, Carlsbad, CA, USA), PRSS8 (H00005652-M11A) (Abnova, Taibei, Taiwan, CHN), and β-actin (Sigma, Saint Louis, MO, USA). We employed a human XL cytokine array (R&D Systems, Minneapolis, MN, USA) and determined the levels of 105 cytokines and chemokines in PC-3 human prostate cancer cells. We also employed a mouse XL cytokine array (R&D Systems) and determined the levels of 111 cytokines and chemokines in TRAMP tumor cells. The overexpression of NID1 was achieved by transfecting NID1 plasmids (#12016, Addgene, Cambridge, MA, USA). The urinary levels of prostasin, nectin 2, and NID1 were determined using enzyme-linked immunosorbent assay (ELISA) kits (MBS2706071, 2889707, 2021468, respectively; MyBioSource, San Diego, CA, USA), and their concentrations were normalized per unit urinary volume.

Global proteomics analysis

In all, 10 urine samples (five post- and five pre-prostatectomy samples) were analyzed in the Dionex UltiMate 3000 RSLC nano-system combined with the Q-exactive high-field hybrid quadrupole orbitrap mass spectrometer (Thermo Fisher Scientific). Proteins were digested on beads using trypsin/LysC as described previously[45] except digestion was performed in 50 mM ammonium bicarbonate buffer instead of urea. Digested peptides were desalted and separated using a trap and 50-cm analytical

columns.[45] Raw data were processed using MaxQuant (v1.6.3.3)[46] against the Uniprot mouse protein database at a 1% false discovery rate allowing up to two missed cleavages. MS/MS counts were used for relative protein quantitation. Proteins identified with at least one unique peptide and two MS/MS counts were considered for the final analysis. To evaluate the predicted tumor suppressors, we employed recombinant proteins. There were PVR (A42503) (Thermo Fisher Scientific), TFF1 (MBS143343), TFF2 (MBS143344), KNG1 (MBS2034317), CD146 (MBS9139698), ALDOB (MBS847085), QPCT (MBS141058), GDF15 (MBS2010943), HBB (MBS2012150), PVRL2 (MBS2029543), PIK3IP1 (MBS2553092), PTGDS (MBS2029824), NID1 (MBS2030194), FBN1 (MBS2086777, Mybiosource), PGLYRP1 (2590-PGB-050), PRSS8 (4599-SE-010), AXL (154-AL-100), ROBO4 (2366-RB-050) (Novus), and CD14 (593004) (Biolegend, San Diego, California, USA). The mass spectrometry result is shown as the total number of identified peptide spectra matched for the protein.

Evaluation of the percent survival in TCGA dataset: A patient-driven genomic database was analyzed using the GEPIA (Gene Expression Profiling Interactive Analysis) server.[47] For the newly identified tumor-suppressing proteins, such as prostasin (PRSS8), nectin 2 (PVRL2), and NID1, their potential role in the overall survival rate of all cancer patients was evaluated. The statistical significance was examined between the two groups of patients with low transcript levels (bottom 25%) and high transcript levels (top 25%) for each of the three proteins.

Statistical analysis

For cell-based experiments, three or four independent experiments were conducted, and data were expressed as mean ± SD. Statistical significance was evaluated using a one-way analysis of variance. Post hoc statistical comparisons with control groups were performed using Bonferroni correction with statistical significance at $p < 0.05$. The single and double asterisks in the figures indicate $p < 0.05$ and $p < 0.01$, respectively.

Results

Tumor-suppressing capability by post-prostatectomy urine samples

We collected a pair of 10 urine samples from patients with prostate cancer before and after prostatectomy and named them the un-treated/uncured (pre) and treated/cured (post) samples, respectively (Supplemental Figure 1(a)). Urea is a major component of urine with an estimated concentration of 9–30 mg/mL.[48,49] We observed that the effect of urea in our MTT assay for TRAMP and P3 prostate cancer cells was negligible with its amount in 2% urine (180–600 μg/mL) (data not shown). No significant differences in pH (~6.7) and protein concentrations, as well as the levels of urobilinogen, ketone, nitrite, and bilirubin, were observed in the two sets of samples (Supplemental Figure 1(b) and (c)). The pre-prostatectomy samples presented positive PSA levels, while PSA was undetectable in the post-prostatectomy samples. In the MTT-based viability assay as well as the EdU-based proliferation assay, the inclusion of 2% post-prostatectomy urine in culture media significantly reduced the viability and proliferation of TRAMP prostate tumor cells (Figure 1(a) and (b), Supplemental Figure 2(a)). In the scratch-based migration assay, the culture media with 2% post-prostatectomy urine suppressed the motility of TRAMP prostate cancer cells (Figure 1(c), Supplemental Figure 2(b)). No reduction was observed with the pre-prostatectomy urine or the control urine. Furthermore, the post-prostatectomy urine downregulated the selected protumorigenic genes such as TGFβ and upregulated an apoptosis marker, cleaved caspase 3, in TRAMP cell lines (Figure 1(d)). The response of viability and proliferation to the three sets of urine samples was the same with PC-3 prostate cancer cells (Figure 1(e) and (f)).

Interestingly, the tumor-suppressing effects were also observed with MDA-MB-231 breast cancer cells, and EO771 and 4T1.2 mammary tumor cells (Supplemental Figure 2(c)–(f)). In the scratch-based migration assay, the culture media with 2% post-prostatectomy urine suppressed the motility of MDA-MB-231 and 4T1.2 cancer cells (Supplemental Figure 3(a)–(d)). Furthermore, the post-prostatectomy urine downregulated the selected protumorigenic genes such as TGFβ and upregulated an apoptosis marker, cleaved caspase 3, in MDA-MB-231, 4T1.2, and EO771 cell lines (Supplemental Figure 3(e)). Taken together, the results indicated that the post-prostatectomy urine showed the tumor-suppressing capability in all 10 samples, indicating the alteration in their components after prostatectomy.

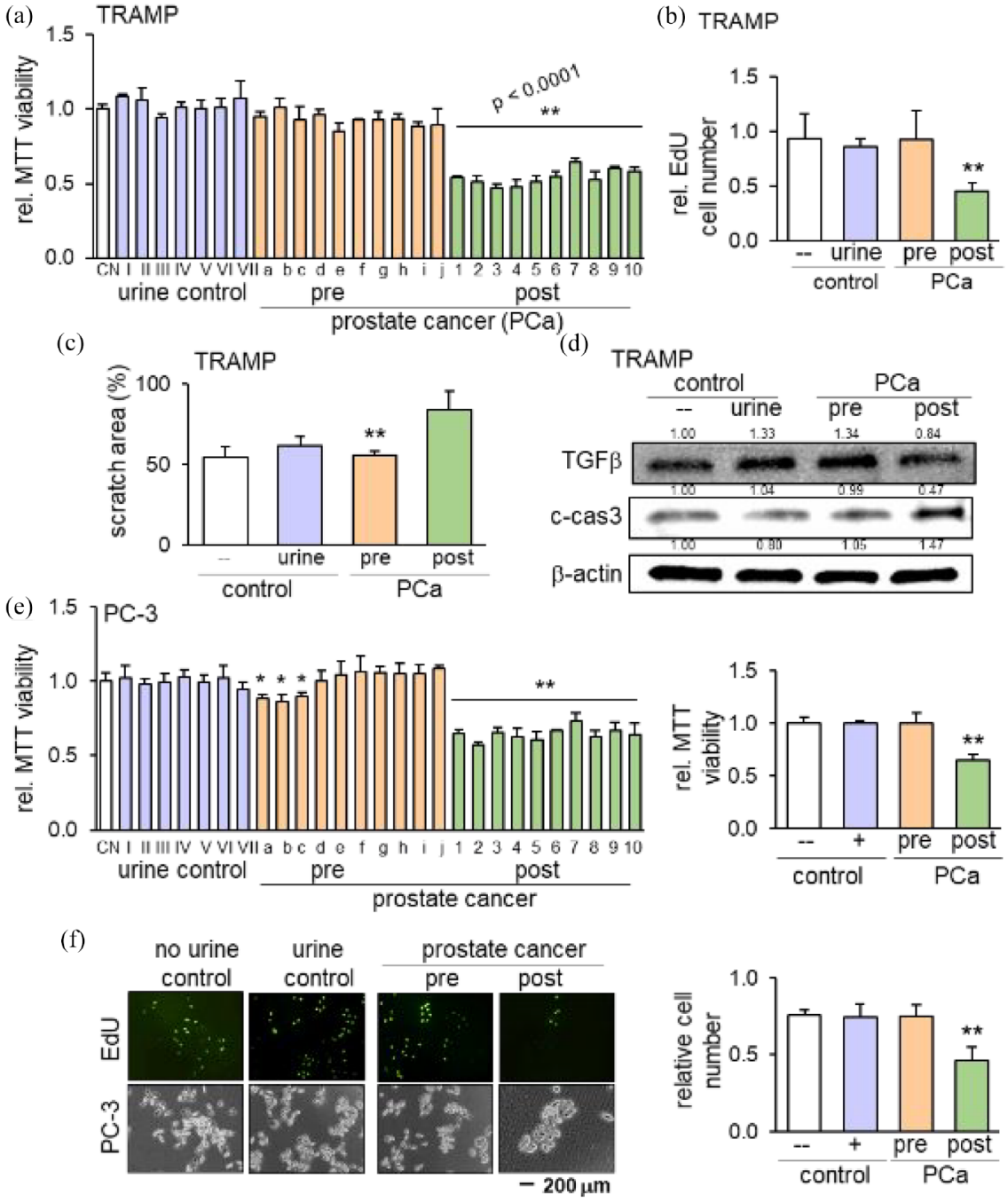

Figure 1. Prostate tumor-suppressing capability of the urine samples from the post-prostatectomy patients. The single and double asterisks indicate $p < 0.05$ and 0.01, respectively. (a and b) Reduction in the MTT-based cell viability and EdU-based proliferation of TRAMP prostate tumor cells by the addition of 2% urine from the post-prostatectomy patients in the culture medium. (c) Significant decrease in scratch-based motility of TRAMP cells by the addition of 2% urine from the post-prostatectomy patients in culture medium. (d) Downregulation of TGFβ, and upregulation of c-cas3 in TRAMP cells by the addition of 2% urine from the post-prostatectomy patients. (e and f) Decrease in the MTT-based cell viability and EdU-based proliferation of PC-3 prostate tumor cells by the addition of 2% urine from the post-prostatectomy patients.
c-cas3, cleaved caspase 3; PCa, prostate cancer; post, post-prostatectomy; pre, pre-prostatectomy; TGFβ, transforming growth factor beta.

Protein array analysis and angiogenin regulation

To examine the role of urine in the regulation of cytokines, we conducted the protein array analysis using two sets of urine samples, a pair of pre- and post-prostatectomy urine samples, and a pair of before and after the step aerobics. We have previously shown that the human urine, collected after 30-min step aerobics, possesses the tumor-suppressing capability. Of note, the murine urine, collected after 5-min mechanical loading, also presented the antitumor action by reducing the MTT-based cell viability and scratch-based motility (Supplemental Figure 4(a)–(d)). The post-loading urine samples showed reduced levels of MMP9, TGFβ, and Snail (Supplemental Figure 4(e)). The level of angiogenin (Ang) was also reduced, while the levels of p53 and Trail were elevated (Supplemental Figure 4(f)). In the array analysis, the post-prostatectomy urine and the urine after step aerobics downregulated several cytokines and chemokines (Figure 2(a) and (b)). Among them, angiogenin, a stimulator of angiogenesis, was commonly downregulated by prostatectomy and step aerobics (Figure 2(c)). In our previous study, osteocytes have been shown to possess innate antitumor capabilities. Interestingly, the conditioned medium, derived from MLO-A5 murine osteocytes, downregulated angiogenin in PC3, TRAMP, EO771, and MDA-MB-231 cancer cells (Supplemental Figure 5(a) and (b)).

The treatment with recombinant angiogenin proteins promoted transwell invasion and scratch-based motility of TRAMP cells and elevated the levels of TGFβ (Figure 2(d)–(f)). Recombinant angiogenin proteins also downregulated c-caspase 3 and p53 (Figure 2(f)). The tumor-stimulating actions were observed in response to angiogenin in EO771 and 4T1.2 mammary tumor cells (Supplemental Figure 5(c)–(f)), The angiogenin elevated the level of TGFβ and downregulated the levels of c-caspase 3 and p53 (Supplemental Figure 5(g) and (h)). Collectively, the level of tumor-promoting angiogenin is one of the major differences between the pre- and post-prostatectomy urine samples.

Enrichment of tumor suppressors in the post-prostatectomy urine

While the reduction in tumor-promoting factors is preferable in the post-prostatectomy urine, the tumor-suppressing capability should be associated with the upregulation of tumor suppressors. We thus conducted a global proteomics analysis and determined proteins that were enriched in the post-prostatectomy urine. Mass spectrometry-based protein identification was conducted using five pre- and five post-prostatectomy urine samples and we identified 41 proteins that were significantly elevated in the post-prostatectomy urine (Figure 3(a)). Of note, the detailed proteomics data are provided with the p value (Supplemental Table 1). Based on the availability of recombinant proteins, we conducted an MTT-based viability assay in TRAMP cells using 19 recombinant proteins. The result showed that 10 recombinant proteins indicated their tumor-suppressing capabilities. Among them, prostasin (PRSS8), nectin 2 (PVRL2), and NID1 presented the most potent antitumor ability. These three proteins also reduced the MTT-based cell viability of 4T1.2 and EO771 tumor cells (Figure 3(b)). We conducted an ELISA assay and determined the concentrations of prostasin, nectin 2, and NID1 in urine samples. As expected, their levels in the post-prostatectomy samples were significantly higher than those in the pre-prostatectomy samples (Figure 3(c)–(e)). Hereafter, we mostly focused on the role of these proteins.

Tumor-suppressing capabilities of prostasin, nectin 2, and NID1

The enrichment of prostasin, nectin 2 (PVRL2), and NID1 in the post-prostatectomy urine samples was confirmed by Western blotting (Figure 4(a)). As expected, the addition of these recombinant proteins in the medium of TRAMP cells reduced the levels of TGFβ and elevated the levels of c-caspase 3 and p53 (Figure 4(b) and (c), Figure 5(a)). The EdU-based proliferation and scratch-based motility of TRAMP cells were also reduced by these three recombinant proteins (Figure 4(d)–(g), Figure 5(b) and (c)). By contrast, the EdU-based proliferation and scratch-based motility of TRAMP cells were stimulated by the overexpression of NID1 (Figure 5(d) and (e)). Furthermore, the overexpression of NID1 in TRAMP cells elevated TGFβ and reduced c-caspase 3 (Figure 5(f)). The result indicated that extracellular NID1 acted as a tumor suppressor, while intracellular NID1 as a tumor promoter.

Unlike the dichotomous role of intracellular and extracellular NID1 as a tumor promoter and suppressor, respectively, prostasin and nectin 2 did not present the opposing actions in the intracellular and extracellular domains. The overexpression of prostasin in TRAMP tumor cells downregulated

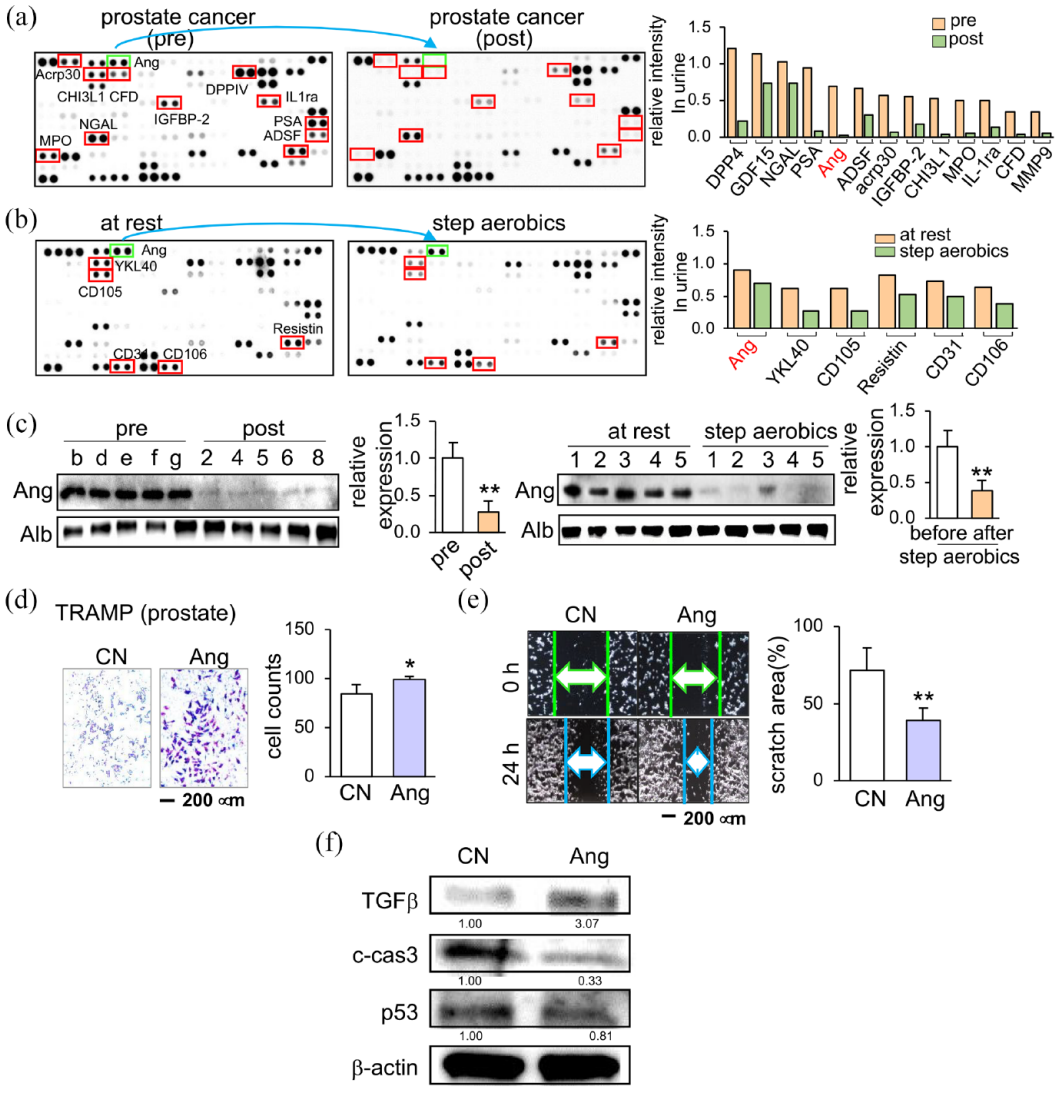

Figure 2. Downregulation of tumorigenic angiogenin by the urine samples from the post-prostatectomy patients and the samples from healthy individuals after step aerobics. The single and double asterisks indicate $p < 0.05$ and 0.01, respectively. (a) Cytokine and chemokine array staining for the urine samples from the pre- and post-prostatectomy patients. (b) Cytokine and chemokine array staining for the urine samples from a healthy individual before and after step aerobics. (c) Reduction of angiogenin (Ang) by the urine samples from the post-prostatectomy patients and the samples from healthy individuals after step aerobics. (d and e) Elevation of transwell invasion and scratch-based motility of TRAMP cells by 100 ng/mL angiogenin. (f) Upregulation of TGFβ, and downregulation of caspase 3 and p53 in TRAMP cells by 100 ng/mL angiogenin.
Alb, albumin; Ang, angiogenin; CN, control; TGFβ, transforming growth factor beta.

TGFβ and Snail, and upregulated c-caspase 3, whereas the overexpression of nectin 2 did not alter the levels of TGFβ and Snail, but elevated the level of c-caspase 3 (Figure 5(g) and (h)). Taken together, the common feature of prostasin, nectin 2, and NID1 is that they all act as tumor-suppressing proteins in the extracellular domain. Besides prostasin, nectin 2, and NID1, 10 potential tumor-suppressing proteins were identified (Figure 2(b)). Among them, the treatment of TRAMP cells with CD14 recombinant proteins downregulated TGFβ and elevated c-caspase 3 and p53 (Supplemental Figure 6). It also reduced the scratch-based motility of TRAMP cells.

Tumor selectivity

To evaluate the selectivity of inhibitory actions, we defined the tumor selectivity using MTT-based metabolic activity as a ratio of 'reduction in

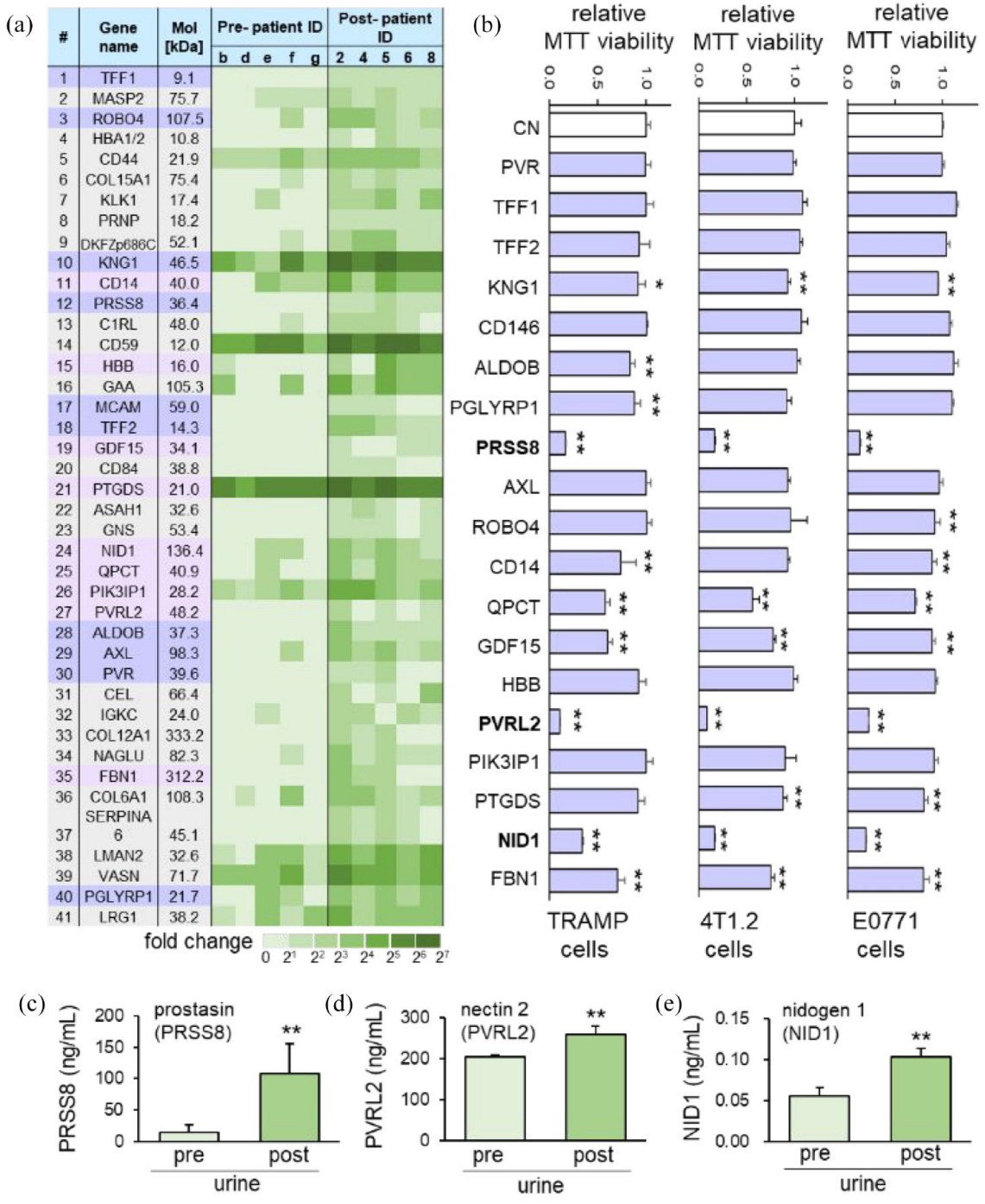

Figure 3. Prediction of the potential tumor suppressors in the urine samples from the post-prostatectomy patients. The single and double asterisks indicate $p < 0.05$ and 0.01, respectively. (a) List of 41 potential tumor suppressors that were significantly enriched in the urine samples from the post-prostatectomy patients. (b) MTT-based cell viability of TRAMP prostate tumor cells, and 4T1.2 and E0771 mammary tumor cells in response to 19 recombinant proteins at 5 μg/mL. Three proteins (PRSS8, PVRL2, and NID1) were selected for further analysis. (c–e) Concentrations of prostasin (PRSS8), nectin 2 (PVRL2), and NID1 in urine from the pre and post-prostatectomy patients.
NID1, nidogen 1.

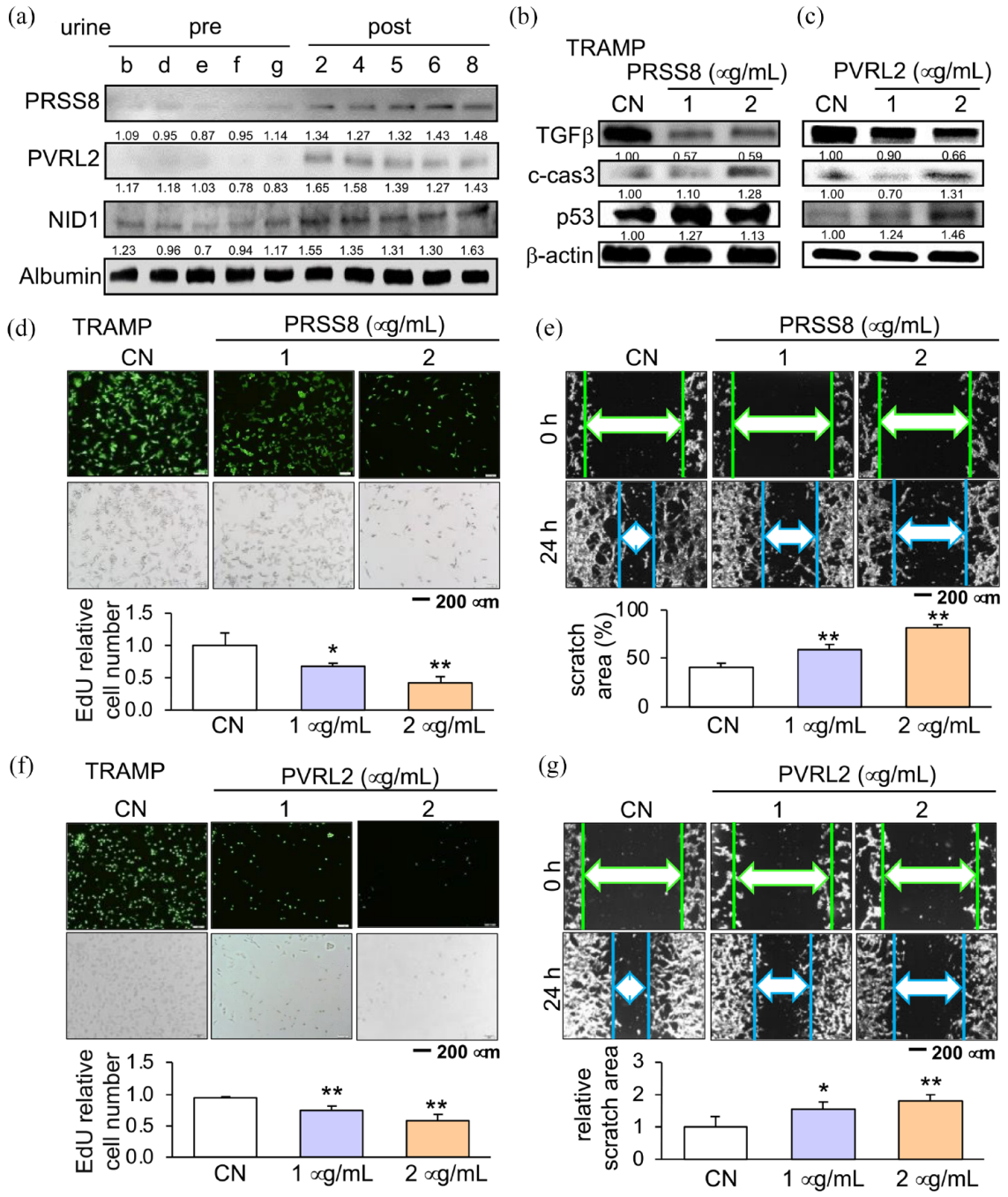

Figure 4. Antitumor effects of PRSS8, PVRL2, and NID1 on TRAMP prostate tumor cells. The single and double asterisks indicate $p < 0.05$ and 0.01, respectively. (a) Elevated levels of PRSS8, PVRL2, and NID1 in the urine samples from the post-prostatectomy patients. (b and c) Downregulation of TGFβ, and upregulation of c-cas3 and p53 by the incubation of TRAMP prostate tumor cells with 1, 2 μg/mL of PRSS8 and PVRL2. (d and e) Reduction in the EdU-based proliferation and scratch-based migration of TRAMP cells by PRSS8 recombinant proteins. (f and g) Reduction in the EdU-based proliferation and scratch-based migration of TRAMP cells by PVRL2 recombinant proteins.

c-cas3, cleaved caspase 3; CN, control; NID1, nidogen 1; PRSS8, prostasin; PVRL2, poliovirus receptor-related 2 or nectin 2; TGFβ, transforming growth factor beta.

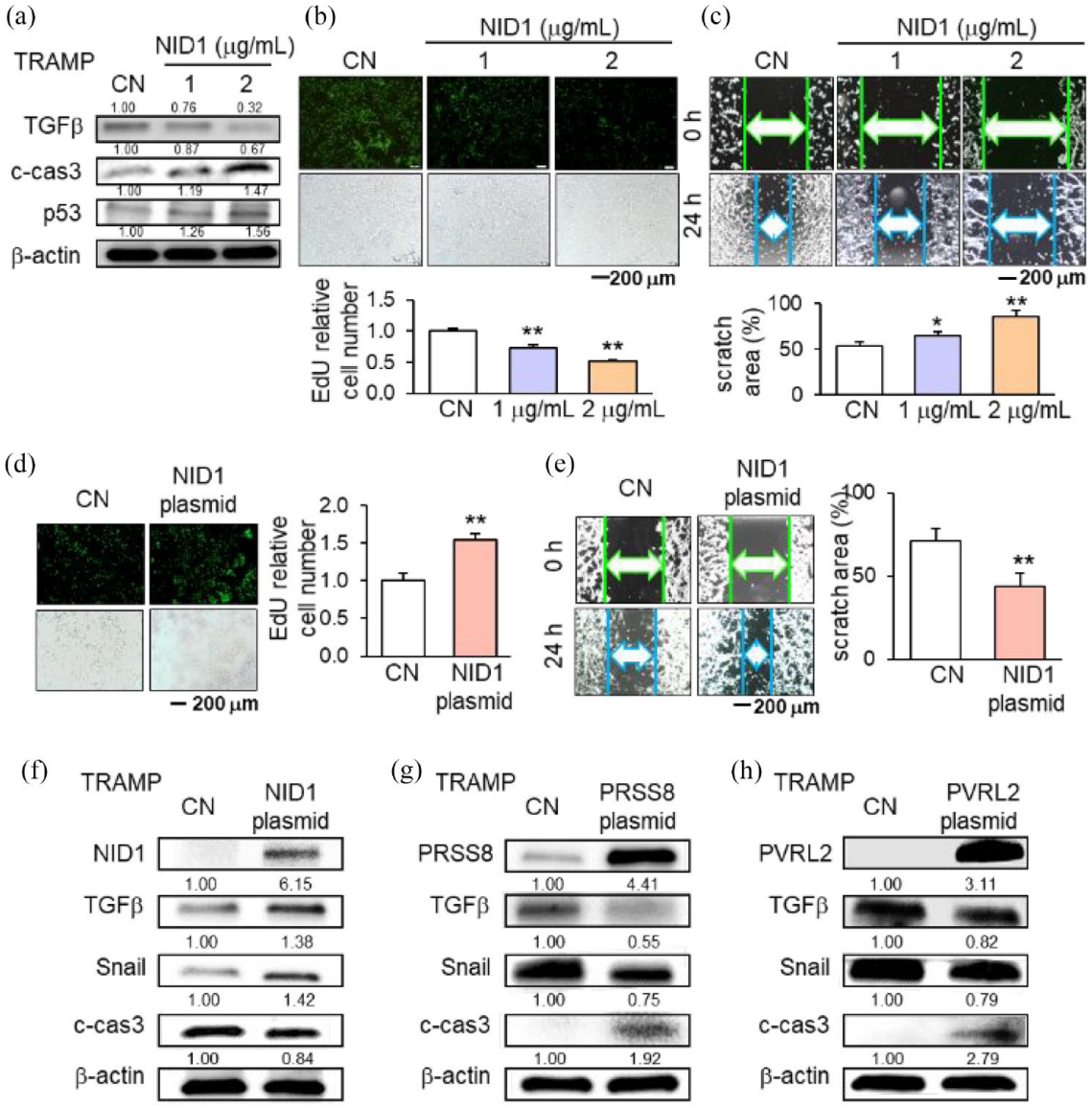

Figure 5. Differential role of NID1 in the extracellular and intracellular domains for TRAMP prostate tumor cells. The single and double asterisks indicate $p < 0.05$ and 0.01, respectively. (a) Extracellular NID1 as a tumor suppressor by downregulating TGFβ and upregulating c-cas3 for apoptosis induction and p53 for tumor suppression. (b and c) Reduction in EdU-based proliferation and scratch-based motility of TRAMP cells by extracellular NID1. (d and e) Elevation in the EdU-based proliferation and scratch-based migration of TRAMP cells by the overexpression of NID1. (f) Upregulation of TGFβ and Snail, and downregulation of c-cas3 in TRAMP cells by the overexpression of NID1. (g and h) Downregulation of TGFβ and Snail, and upregulation of c-cas3 in TRAMP cells by the overexpression of PRSS8 and PVRL2.
CN, control; c-cas3, cleaved caspase 3; NID1, nidogen 1; TGFβ, transforming growth factor beta.

MTT-based activity of tumor cells' to 'reduction in MTT-based activity of non-tumor cells'. A value larger than 1 indicates that the inhibition is stronger in tumor cells than in non-tumor cells. Using two tumor cells (TRAMP and PC-3) and three non-tumor cells (MLO-A5 osteocytes, adipose-derived MSCs, and MC3T3 osteoblasts), the result showed a clear tumor selectivity of prostasin, nectin 2, and NID1 (Figure 6(a), Supplemental Figure 7).

(a)

tumor selectivity (tumor cells vs. non-tumor cells)	prostasin (PRSS8)		nectin 2 (PVRL2)		nidogen 1 (NID1)	
	TRAMP cells	PC-3 cells	TRAMP cells	PC-3 cells	TRAMP cells	PC-3 cells
A5 osteocytes	> 10	> 10	4.84	3.78	N.D.	N.D.
adipose MSCs	> 10	> 10	> 10	> 10	6.41	5.01
MC3T3 osteoblasts	> 10	> 10	9.00	7.03	6.49	5.07

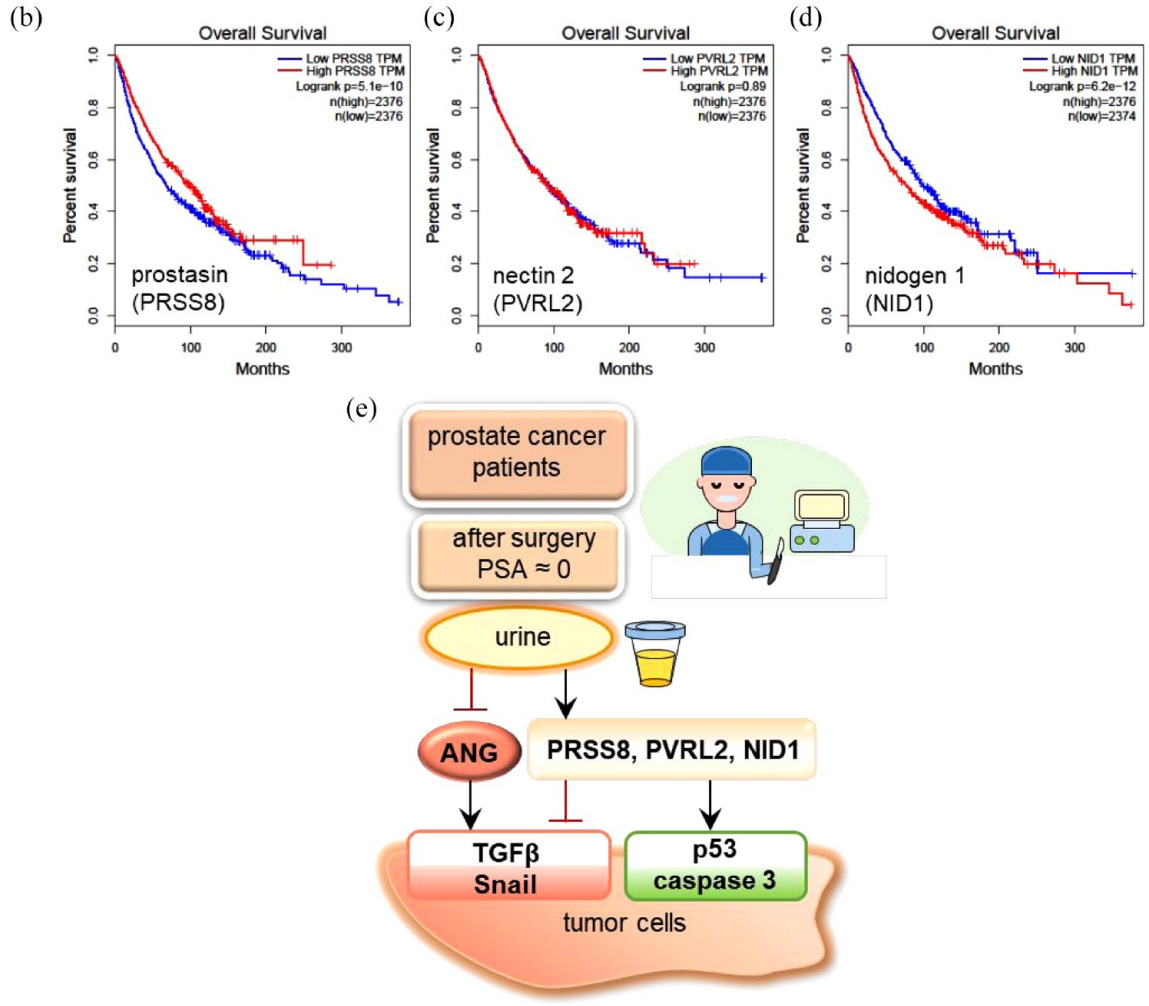

Figure 6. Tumor selectivity, effects on percent survival, and the putative regulatory mechanism. (a) Tumor selectivity. The tumor selectivity was defined as the ratio of 'reduction in MTT-based viability of tumor cells' to 'reduction in MTT-based viability of non-tumor cells'. The double asterisk indicates $p < 0.01$. Two tumor cell lines (TRAMP and PC-3 cells) and three non-tumor cell lines (A5, adipose, and MC3T3 cells) were employed. Of note, N.D. means that the viability of non-tumor cells was stimulated. (b–d) Percent survival of all cancer patients with the low and high mRNA levels of PRSS8, PVRL2, and NID1. (e) Putative tumor-suppressing mechanism by urine from the post-prostatectomy patients.
NID1, nidogen 1; PSA, prostate-specific antigen.

Effects on percent survival by prostasin, nectin 2, and NID1

We have so far shown that prostasin, nectin 2, and NID1 act as tumor-suppressing proteins in the extracellular space. Lastly, we examined their role in the percent survival of cancer patients focusing on the transcript levels in cancer tissues since NID1 presented the location-dependent

pro and antitumor actions (Figure 5(b)–(e)). Notably, the effects of these three proteins on the survival rate differed (Figure 6(b)–(d)). The high level (top 25%) of prostratin transcripts elevated the percent survival ($p = 5 \times 10^{-10}$). However, the high level of nectin 2 did not significantly alter the percent survival ($p = 0.89$) and that of NID1 reduced the percent survival ($p = 6.2 \times 10^{-12}$).

Discussion

We presented for the first time that the human urine from post-prostatectomy patients was able to suppress the growth and migration of prostate and breast cancers, When 2% of the post-prostatectomy urine samples were given to culture media of multiple prostate and breast cancer cell lines, their tumorigenic behaviors, including MTT-based metabolic activities, EdU-based cellular proliferation, and scratch-based motility, were significantly inhibited. In contrast, with the urine collected from pre-prostatectomy patients, no tumor-suppressing capability was observed. Of note, the tumor-suppressing activity was also observed in the urine collected after 30-min step aerobics from healthy individuals.[11] Global proteomics analysis revealed that the post-prostatectomy urine samples were enriched with prostasin, nectin 2, and NID1, and they acted as extracellular tumor suppressors. Their recombinant proteins reduced the levels of protumorigenic proteins such as TGFβ, and Snail, and promoted the expression of p53 and c-caspase 3 for inducing apoptosis. Collectively, we demonstrated the striking difference in the antitumor capability of urinary proteins, which was caused by prostatectomy.

The striking tumor-suppressive capability of the post-prostatectomy urine samples was associated with the downregulation of angiogenin, as well as the upregulation of prostasin, nectin 2, and NID1. In this study, we observed antitumor actions of the three independent urine samples, such as the post-prostatectomy urine from the patients with prostate cancer, the human urine after step aerobics, and the urine after mechanical loading to the tibia in the mouse model. These three sets of urine samples uniformly downregulated angiogenin, a member of the ribonuclease superfamily as well as a potent stimulator of new blood vessels.[50] Angiogenin is reported upregulated in a variety of cancers, including prostate cancer.[51–53] The result herein indicates that the urinary antitumor proteomes can be built by surgical treatment, physical exercise, and mechanical loading, and one of the common responses is the suppression of angiogenin-driven angiogenesis, which is essential for the progression of tumors.

Among three tumor-suppressing proteins in this study, the tumor-suppressing action of prostasin was consistent with the previous report.[54] Prostasin is highly expressed in prostate epithelia and is known to suppress tumor growth and metastases in hepatocellular carcinoma and inhibit breast cancer invasiveness.[55] However, the antitumor actions of nectin 1 and NID1 were unexpected. Nectin 2 is involved in the immune checkpoints and the activation of natural killer cells and T-cells, and its high level is reported to correlate with poor outcomes in acute myeloid leukemia.[56] As a basement membrane protein, NID1 is reported to regulate EMT for metastasis and chemoresistance of ovarian cancer cells and stimulate metastasis of lung cancer.[35] The result herein indicates that intracellular NID1 can act as a tumor promoter by interacting with ECM, while extracellular NID1 serves as a tumor suppressor by blocking interactions of tumor cells with ECM.

The putative regulatory mechanism is depicted for the antitumor capability of post-prostatectomy urine samples (Figure 6(e)). We have previously shown that a tumor promoter inside the cell may act as a tumor suppressor outside the cell.[57] For instance, the activation of Wnt signaling promotes tumor progression, while the proteome derived from Wnt-activated osteocytes presents antitumor capabilities.[58] Extracellular enolase 1 and ubiquitin C proteins are reported to inhibit the proliferation and migration of breast cancer cells, whereas their overexpression in breast cancer cells promotes their tumorigenic behaviors.[22] Also, the activation of PI3K signaling in mesenchymal stem cells generated tumor-suppressive proteomes, while PI3K signaling is oncogenic and a target of chemotherapy.[23] These moonlighting proteins may function as a tumor suppressor extracellularly and a tumor promoter intracellularly.[59,60] In this study, we have shown that the overexpression of NID1 in TRAMP cells promoted their proliferation and migration, while extracellular NID1 serves as a tumor suppressor. We also showed that extracellular CD14 acts as a tumor suppressor, whereas it is reported to serve as a stimulator of EMT intracellularly.[61,62] Collectively, the observations in this study indicate that inhibiting specific target proteins such as NID1 may block their

extracellular tumor-suppressing action as well as their intracellular tumor-promoting action. Consistently, the percent survival of cancer patients was not correlated with the transcript levels of the described tumor-suppressing proteins, indicating their context-dependent role.

Using a mouse model of breast cancer and bone metastasis, we have previously shown that the treatment with Pitavastatin, a cholesterol-lowering drug, inhibits the growth of mammary tumors and alters the profile of the urinary VOCs.[63] We have also reported that the VOC profiles alter depending on the progression of mammary tumors.[64] Here, we demonstrated that prostatectomy significantly altered the profile of the urinary proteomes. Although the results in this study present a novel urine feature, the study has limitations. The sample size of 10 for each group of patients provided statistically significant results, but further analysis is needed because of variations in hormonal status, gene mutations, etc. Urine contains non-protein factors including metabolites and they also contribute to tumor progression. Urinary components are altered not only by the state of prostate cancer, but also by physical activities, diet, and daily cycle. The distribution of tumor suppressors may depend on the stage of prostate cancer. Patients may receive radiotherapy and chemotherapy, which should also alter the urinary secretomes.

It is reported that a prostatic systemic inflammatory marker score, which is calculated from the ratios of neutrophils, platelets, monocytes, and eosinophils to lymphocytes, is elevated for advanced prostate cancer.[65,66] It is thus possible that pro-inflammatory and anti-inflammatory cytokines are linked to the post-prostatectomy urinary tumor-suppressive proteomes. In summary, this study demonstrated that the urine from post-prostatectomy patients presented the tumor-suppressing capability to prostate and breast cancer cells. The post-prostatectomy urine suppressed cellular viability, proliferation, and migration of cancer cells. The observed antitumor action was associated with the elevation of prostasin, nectin 2, and NID1, as well as the reduction in angiogenin. However, the percent survival of cancer patients was not always correlated with their transcript levels in cancer tissues. The observed inconsistency between the action of extracellular tumor-suppressing proteins and percent survival is likely based on the context-dependent roles of these proteins. Extracellular NID1 acted as a tumor suppressor, whereas its intracellular counterpart served as a tumor promoter. The results support the possible urine-based monitoring of the post-surgery conditions and the extracting of antitumor proteins and identifying a novel cell-membrane target for the treatment of prostate cancer.

Declarations

Ethics approval and consent to participate

The Indiana University Institutional Review Board approved study #1910596457.

Consent for publication

No patient identifiable information was used in this study.

Author contribution(s)

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Hiroki Yokota: Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Project administration; Supervision; Validation; Visualization; Writing – original draft.

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Competing interests
The authors declare that there is no conflict of interest.

Availability of data and materials
The datasets generated or analyzed during the current study are available on request from the corresponding author.

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Supplemental material for this article is available online.

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