Metabolic, Endocrine and Genitourinary Pathobiology

MicroRNAs May Mediate the Down-Regulation of Neurokinin-1 Receptor in Chronic Bladder Pain Syndrome

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Bladder pain syndrome (BPS) is a clinical syndrome of pelvic pain and urinary urgency-frequency in the absence of a specific cause. Investigating the expression levels of genes involved in the regulation of epithelial permeability, bladder contractility, and inflammation, we show that neurokinin (NK)1 and NK2 tachykinin receptors were significantly down-regulated in BPS patients. Tight junction proteins zona occludens-1, junctional adherins molecule -1, and occludin were similarly down-regulated, implicating increased urothelial permeability, whereas bradykinin B₁ receptor, cannabinoid receptor CB1 and muscarinic receptors M3-M5 were up-regulated. Using cell-based models, we show that prolonged exposure of NK1R to substance P caused a decrease of NK1R mRNA levels and a concomitant increase of regulatory micro(mi)RNAs miR-449b and miR-500. In the biopsies of BPS patients, the same miRNAs were significantly increased, suggesting that BPS promotes an attenuation of NK1R synthesis via activation of specific miRNAs. We confirm this hypothesis by identifying 31 differentially expressed miRNAs in BPS patients and demonstrate a direct correlation between miR-449b, miR-500, miR-328, and miR-320 and a down-regulation of NK1R mRNA and/or protein levels. Our findings further the knowledge of the molecular mechanisms of BPS, and have relevance for other clinical conditions involving the NK1 receptor. (Am J Pathol 2010, 176:288–303; DOI: 10.2353/ajpath.2010.090552)

Bladder pain syndrome (BPS) should be diagnosed on the basis of symptoms of pain associated with the urinary bladder, accompanied by at least one other symptom,

such as day-time and/or night-time urinary frequency after exclusion of confounding symptoms, according to the European Society for the Study of Interstitial Cystitis proposal and European Association of Urology guidelines.¹ The term “interstitial cystitis” (IC) was not omitted to avoid problems of reimbursement, disability benefits and so forth. The European Society for the Study of Interstitial Cystitis proposal coined the term BPS, however considered the use of painful bladder syndrome and IC in parallel acceptable. Overall, this disease, which has a significant impact on social and psychological well-being, affects approximately 1 million patients in the United States alone,² with at least 230 confirmed cases per 100,000 females.³ The etiology of BPS is unknown, and its treatment is largely empirical. A multitude of pathogenetic mechanisms have been postulated ranging from neuroinflammatory to autoimmune or possibly infectious or toxic agents, but an inflammatory component is commonly thought to be involved.⁴ Epithelial damage has often been invoked: the mucinous layer of the healthy bladder is often compromised in patients with BPS/IC, as well as in some animal models.^{5,6} An initiating event (toxin) may lead to increased urothelial permeability, which in turn leads to nerve sensitization and possibly up-regulation of neurotransmitter release (tachykinins, glutamate, calcitonin gene-related peptide).⁷ Sensory nerves secrete inflammatory mediators such as substance P (SP), a nociceptive neurotransmitter in the central and peripheral nervous system. It is not clear whether there are differences in urinary SP between IC patients and controls, although several studies demonstrated increased amounts of the released SP in the urine of BPS/IC patients, and there is a suggestion that increased SP-positive nerve fibers may be present in IC patients.^{8–10}

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Activated G protein-coupled receptors are implicated in inflammatory conditions, including BPS/IC and prostatitis.¹¹ Recent results suggest that neurogenic inflammation may be involved in the pathogenesis of BPS/IC in the animal model.¹² However to date this has not been clearly shown in human tissue. Tachykinins (TK), including SP, mediate neurogenic inflammation and smooth muscle contraction in the genitourinary tract through stimulation of neurokinin (NK)1 and NK2 receptors (NK1R and NK2R).¹³ NK1 receptor-expressing sensory neurons are found in the muscle layer of bladder (detrusor), within and just below the urothelium, and around blood vessels, being more abundant in the bladder base than in the dome.¹⁴ In the urinary system, TKs are thought to be responsible for overactivity, hypersensitivity, inflammation, and changes in urothelial permeability.¹⁵ Experiments with NK1R knockout mice demonstrated an obligatory requirement of NK1R in cystitis and participation of these receptors in mast cell degranulation and inflammation.¹² It has previously been shown, using semiquantitative methods, that the NK1R mRNA is increased in bladder biopsies from patients with BPS/IC.¹⁶ In addition, NK2 receptors, which play a pivotal role in the modulation of motor and sensory functions, have been localized in human bladder detrusor muscle.¹⁷ In human urothelium, NK2R expression has not been detected, which is in contrast to animal studies, where NK2 receptors are present in rat urothelium and have a role initiating micturition.^{18,19} The apparent species-specific differences in expression patterns of TK receptors warrant caution in interpreting the data obtained from animal models of BPS/IC, and prompted us to investigate the expression and localization of these receptors in healthy and diseased human bladders.

Changes in bladder function are often concomitant with structural alterations in smooth muscle and urothelial cells, which manifest themselves in changes of gene expression.²⁰ Such changes are indicative of the pathophysiological processes taking place during the progression of a disease; therefore some of the causative factors of BPS/IC might be elucidated by uncovering a link between the expression of proteins, mediating neurogenic inflammation, bladder contractility and epithelial permeability. Although there are several studies examining gene expression changes during experimentally induced cystitis in animals,^{21–25} human data are scarce. In human BPS patients, the molecular markers for bladder permeability and proteoglycan core proteins have been shown to be down-regulated,^{26,27} and there was an increased permeability and decreased tight junction formation of bladder epithelial cell monolayers grown from biopsies in patients with BPS/IC, as compared with cells from normal controls.²⁸

Recently, microRNAs (miRNAs) have been defined as an important class of gene expression regulators, involved in the processes of inflammation and cancer.^{29,30} MiRNAs are noncoding single-stranded RNAs of about 22 nucleotides that regulate the expression of mRNAs, inhibiting the protein production by base-pairing with complementary sequences.³¹ Studies in psoriasis, atopic eczema, and inflammatory bowel disease have shown an

involvement of miRNAs in the pathogenesis of these inflammatory diseases.^{32,33}

We collected biopsy material from 28 BPS patients with a high degree of continuous pain and 8 controls and quantified the disease-related changes in the expression levels of the genes involved in the regulation of epithelial permeability, bladder contractility, and neurogenic inflammation. We identified and validated several miRNA species, significantly up-regulated in BPS patients. Our findings suggest a causative role of miRNAs in the regulation of gene expression in BPS, and possible mechanisms of their induction and targeting.

Materials and Methods

Patients

Permission to conduct this study was obtained from the Ethics Committee of the Canton of Bern (KEK 146/05), and all subjects gave written informed consent. All subjects underwent a complete urological evaluation (including medical history, physical examination, urine culture, flexible urethroscopy). In addition all subjects with the BPS underwent uroflowmetry, post void residual and urodynamic investigations, which were performed according to "Good Urodynamic Practices."³⁴ All methods, definitions, and units conform to the standards recommended by the International Continence Society.³⁵ Based on these findings subjects were divided in two groups:

- 1) Controls—subjects without lower urinary tract symptoms undergoing cystoscopy for other reasons (eg, stent placement for stone disease, microhematuria evaluation).
- 2) BPS—subjects with continuous pain for >3 months at the time of biopsy considered to be located in the bladder and frequency, urgency, or nocturia.

All subjects in group 2 were refractory to treatment with antibiotics (tetracyclines), antiviral agents (Valaciclovir) and at least one antimuscarinic. Cold cut biopsies were obtained in all subjects either in sedation or under spinal anesthesia. Dome bladder biopsies were collected from 8 controls and 28 BPS patients. The biopsies were stored in RNAlater (Qiagen) at -70°C until RNA isolation. Additionally, three control samples after cystectomy were obtained for primary cultures of urothelium and smooth muscle, and studies of receptor expression in bladder layers. Biopsy samples from all subjects underwent a routine microscopic examination by a trained pathologist (H&E staining, staining for mast cell tryptase, staining for S100 proteins, and PGP 9.5 for nerve fibers).

Antibodies and Reagents

Rabbit polyclonal antibodies against human NK1R were from S. Cruz (H-83, sc-15323) and Sigma (S8305), against NK2R (M-48, sc-28951) from S. Cruz. Secondary antibodies goat anti-rabbit Cy3 and goat anti-mouse Cy3 were purchased from Jackson Immuno Research, goat anti-rabbit Alexa 488 from Molecular Probes. Secondary

peroxidase labeled antibodies against rabbit and mouse were obtained from Amersham Biosciences. NK1R and NK2R antagonists L-733'060 and GR 159897 (both used at 10 μ mol/L) were from Tocris Bioscience.

Cell Cultures and miRNA Transfections

Primary cultures of urinary bladder urothelium (UE) were produced following the protocols of CELLnTEC Advanced Cell Systems (Bern, Switzerland). Fresh postoperative bladder material was incubated overnight at 4°C in in CnT-18 defined urothelium medium (CELLnTEC) supplemented with 10 mg/ml Dispase 2 and 2 $\times$ Antibiotics/antimycotics (ABAM) solution. Urothelium sheets there separated from underlying stroma, dispersed by gentle pipetting and seeded at 100 to 200 clumps per 1 cm² on a Petri dish with or without glass coverslips. Cultures were passaged at 60% to 90% confluency by incubating 15 minutes at 37°C in Dispase solution (4 mg/ml in medium).

Primary cultures of bladder smooth muscle cells (SMC) were obtained from isolated smooth muscle bundles, separated from UE and connective tissue. Muscle strips were incubated in 0.2% trypsin in PBS for 30 minutes at 37°C, followed by collagenase I digestion (1 mg/ml in serum-free CnT-5 medium) 30 minutes at 37°C. Cells were dispersed by gentle pipetting, then washed and seeded. Established cultures were passaged by trypsinization.

The UROtsa cell line, an immortalized normal human urothelial cell line kindly provided by Dr. D. A. Sens (University of North Dakota), was grown in serum-containing Dulbecco's Modified Eagle's Medium as previously described.³⁶ The human astrocytoma cell line U373MG was maintained in Dulbecco's Modified Eagle's Medium with 10% fetal bovine serum.

Pre-miR miRNA precursors for miR-192, -199a, -320, -328, -342, -449b, and -500 and validated positive and negative controls for NK1R were from Ambion/Applied Biosystems. The reverse-transfections were done in 12-well plates by mixing the miRNA precursors and controls with 50 μ l of siPORT NeoFX Transfection Agent (Applied biosystems)/Opti-MEM (Invitrogen) per well. The transfected cells were incubated at 37°C for 48 hours before mRNA isolation and analysis.

Total RNA and MicroRNA Isolation

Bladder tissue was homogenized using a TissueRuptor (Qiagen) and total RNA isolated using RNeasy Fibrous Tissue mini kit (Qiagen), including a proteinase K and DNase I digestion. RNA was eluted and stored at -70°C. The cultured cells were harvested in RLT buffer, disrupted using QIAshredder and RNA isolated using RNeasy mini kit (Qiagen). Total RNA concentration was measured using UV spectrophotometry at 260 nm (DU 530, Beckman Coulter), the purity was determined with the 260A/280A ratio and the quality of isolated RNA was confirmed by agarose gel electrophoresis. For microRNA-enriched probes, the RNA was extracted from the bladder biopsies using the mirVana miRNA isolation kit (Ambion) following manufacturer's instructions.

Reverse Transcription and Real-Time PCR Analysis of Receptor mRNA Expression

The reverse transcription reactions were performed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) with random hexamer primers. TaqMan primers (NK1R: Hs00185530_m1; NK2R: Hs00169052_m1; glyceraldehyde-3-phosphate dehydrogenase: Hs99999905_m1; and 18S: Hs99999901_s1) for the real-time PCR were purchased from Applied Biosystems. TaqMan quantitative real-time PCR (QPCR) for each sample was performed in triplicates using 7900HT Fast Real-time PCR System (Applied Biosystems). The Ct values obtained after the real time-QPCR were normalized to the 18S housekeeping gene. The mRNA expression profiling was performed using Custom Microfluidic Card TaqMan arrays for gene expression (Applied Biosystems).

MicroRNA Expression Studies Using Microarrays and Individual miRNA Expression Assays

Reverse transcription reactions were performed with the TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems) with the specific primers Multiplex RT for TaqMan MicroRNA Assays, Human Pool Set (Applied Biosystems), according to the manufacturer's instructions. The eight pools of cDNA were analyzed in TaqMan Low Density Arrays, TaqMan Human MicroRNA Array v1.0 for 384 microRNAs (Applied Biosystems) using 7900HT Fast Real-Time PCR System (Applied Biosystems). The data were normalized to RNU48 endogenous miRNA. The putative target genes for the miRNAs were analyzed using TargetScan, miRBase and miRanda databases. To examine the expression levels of miRNA-449b and miRNA-500, we used individual assays (hsa-miR-449b and hsa-miR-500), and RNU48 as endogenous control.

Immunohistochemistry, SDS-Polyacrylamide Gel Electrophoresis, Western Blotting, and Protein Quantification

Ultracytometry and immunohistochemistry were performed as previously described.³⁷ Briefly, ultrathin cryosections (0.2 μ m) were retrieved on droplets of 2 mol/L sucrose containing 0.75% gelatin, transferred to siliconized glass slides, and then maintained in PBS before immunolabeling. Primary antibodies (rabbit polyclonal anti-NK1R or anti-NK2R) were applied for 1 hour, followed by goat anti-rabbit Cy3 secondary antibodies for 1 hour. Tissue sections were examined at Axiovert 200 M microscope with laser scanning module LSM 510 META (Zeiss). To allow for quantitative assessment of immunofluorescence in different sections, the images were recorded under the same settings (laser power, threshold, magnification, zoom, exposure time). Mean fluorescence intensity was calculated with "Histogram" function of LSM

510 META image analysis software using the total image area and identical threshold settings for all samples.

For SDS-polyacrylamide gel electrophoresis, proteins were extracted using SDS protein extraction buffer (pH 6.8) and concentration determined using the bicinchoninic acid kit (Pierce). Ten microgram probes were analyzed by SDS-polyacrylamide gel electrophoresis followed by Western blotting with specific antibodies. Image analysis was performed using PowerLook 1120 scanner and ImageQuant TL (v2003) software from Amersham Biosciences Europe GmbH.

Intracellular Calcium Measurements

Intracellular calcium imaging recordings were performed as described.³⁸ UE or SM cells were grown on glass coverslips, loaded with 3 μ mol/L Fluo-3 AM, mounted in a recording chamber and observed under an Axiovert 200 M microscope with laser scanning module LSM 510 META (Zeiss) using a $\times 40$ oil immersion lens. Images of the Fluo-3 AM loaded cells were recorded and analyzed using the "Physiology evaluation" software package (Zeiss).

Statistical and Data Analysis

Normality studies were done with Kolmogorov-Smirnov and Shapiro-Wilk tests ($P < 0.05$). Statistically significant differences were determined with a Mann Whitney U -test with an α set to 0.05 for genes not displaying a normal distribution, and with a two-tailed Student's t -test, preceded by a Levene's test, with an α set to 0.05 for genes with a normal distribution. For nonparametric paired data, a Friedman's test ($P < 0.05$) followed by a Wilcoxon signed rank sum test were done. All studies were performed with the SPSS program (version 15.0). Prediction of putative target genes for the significantly deregulated microRNAs was done using TargetScan as the main database, and the results were compared with those obtained from the other databases (miRBase^{39–41} and miRanda⁴²). The heat maps and the hierarchical clustering were performed with GENESIS.⁴³

Results

Selection of Subjects for BPS Study

Overall 36 subjects were recruited in this study: 8 controls and 28 BPS patients. The control group included five males and three females, mean age 47 years (range 24 to 79). All control subjects had normal lower urinary tract function without storage or voiding symptoms. The BPS group included 28 patients, three were male and 25 female, mean age 48 years (range, 23 to 82). Higher incidence of BPS in females led to gender bias in this group of subjects.

All BPS patients had bladder pain for over 3 months, accompanied by frequency, urgency, or nocturia. No patient had an increased postvoid residual. Urodynamic studies showed a median cystometric bladder volume of 200 ml (range 60 to 1000), one patient had a high ca-

pacity bladder with a volume > 1000 ml and two patients had a normal capacity, all of the remaining ones had low capacity bladders. Histopathological evaluation showed chronic inflammation (lymphoplasmocytic infiltration, interstitial edema, and/or hyperemia of the blood vessels with dilated lumina) in all but four patients, and 17 demonstrated increased mast cells in the smooth muscle (≥ 20 mast cells/mm²).

Expression Profiles of Genes Involved the Regulation of Epithelial Permeability, Bladder Contractility, and Inflammation in BPS Patients

The quality of the biopsy samples was assessed, using uroplakin 2 as a marker for the presence of urothelium, and α -smooth muscle actin, smooth muscle myosin heavy-chain, and transgelin/SM22 as markers for smooth muscle. The mRNA for all markers was detected in dome biopsies of controls, and BPS patients (Figure 1A), and there were no statistically significant differences in the expression levels of these genes.

To test whether BPS is accompanied by impaired urothelial permeability, we compared the expression levels of tight junction proteins and observed a significant ($P < 0.05$) down-regulation of mRNA levels of zona occludens-1, junctional adhesion molecule 1, occludin, and claudin 1, and up-regulation of claudin 2 (Figure 1B). These results indicate that there might be significant alterations in the barrier function of urothelium, associated with BPS, leading to increased permeability and resulting in activation of underlying sensory nerves and consequent activation of the receptors involved in motor and sensory functions in the urinary bladder.

In keeping with these findings, we detected a significant up-regulation of the inflammation-induced bradykinin receptor B₁, cannabinoid receptor CB1, and $\beta 2$ adrenergic receptor (Figure 1C). Cannabinoid receptor CB2 was also up-regulated in some subjects, however, this difference was not statistically significant (Figure 1C).

Investigating the expression levels of purinergic and muscarinic receptors, we did not observe significant differences in the expression levels of P2X1 or P2X2 receptors (Figure 1D). Since the expression of P2X3 receptor was consistently below detection level both in control and patient biopsies, this target was excluded from further analysis. We observed a significant ($P < 0.05$) up-regulation of P2Y1 receptor mRNA levels in BPS biopsies (Figure 1D). Similarly, there was a strong increase in the levels of muscarinic receptors M3, M4, and M5, and a slight elevation of M2 (Figure 1E).

Tachykinin Receptor Expression Profiling in Human Bladder

Our data (Figure 1) indicate that BPS in humans is associated with a dramatic alteration of bladder urothelium permeability markers, and an up-regulation of receptors, contributing to pain and increased micturition. To assess potential disease-induced changes in TK receptor expres-

Figure 1. Quantitative evaluation of mRNA levels of selected genes in BPS patients compared with controls; mRNA levels in patients were determined by TaqMan Real-time RT-PCR-based custom gene expression arrays, normalized to 18S RNA and expressed as fold difference relative to healthy controls. The graphs show the average for gene expression in bladder dome biopsies $\pm$ SEM ($n = 8$ for control group; $n = 28$ for BPS group). Statistically significant differences between controls and BPS are indicated (* $P < 0.05$, ** $P < 0.01$). **A:** Marker genes for the presence of urothelium and smooth muscle in collected biopsies. MYH11 smooth muscle myosin 11, heavy chain (Hs00224610_m1), ACTA2 smooth muscle α actin (Hs00426835_g1), TAGLN transgelin/SM22 (Hs00162558_m1), UP2 uroplakin 2 (Hs00171854_m1). **B:** Tight junction proteins. Zona occludens 1 (Hs01551876_m1), ZO-2 zona occludens 2 (Hs00178081_m1), OCLN occludin (Hs00170162_m1), junctional adhesion molecule 1 (Hs00170991_m1), CLD1, CLD2, CLD4: claudins 1, 2, and 4 (Hs00221623_m1, Hs00252666_s1, Hs00533616_s1). **C:** Selected G protein-coupled receptors. BDKRB1 B_1 bradykinin receptor (Hs00664201_s1), ADRB2 β_2 adrenergic receptor (Hs00240532_s1), CB1 and CB2 cannabinoid receptors 1 and 2 (Hs01038522_s1 and Hs00361490_m1). **D:** Purinergic receptors. P2X1 and P2X2 ionotropic purinergic receptors (Hs-00175686_m1, Hs00247255_m1), P2Y1 purinergic G protein-coupled receptor (Hs00704965_s1). **E:** Muscarinic acetylcholine receptors. MuscR2, M2 receptor (Hs00265208_s1), MuscR3 M3 receptor (Hs00265216_s1), MuscR4 M4 receptor (Hs00265219_s1), and MuscR5 M5 receptor (Hs00255278_s1).

sion, we first studied their expression levels and distribution in healthy bladder samples. In the dome biopsies of three control patients, the urothelium was carefully separated from the underlying stroma containing smooth muscle, and samples were analyzed separately. NK1R mRNA was detected in both urothelium and smooth muscle, being relatively higher in urothelium, whereas NK2R mRNA was abundant in bladder smooth muscle, but largely absent from the urothelium (Figure 2A).

These results were confirmed using immunohistochemistry with antibodies against NK1 and NK2 receptors (Figure 2, B and C). The NK1 receptor was detected in smooth muscle, and in urothelium including the umbrella cell layer (Figure 2B). The NK2 receptor was expressed predominantly in bladder smooth muscle, but very weakly in urothelium (Figure 2C).

Figure 2. Expression and localization of tachykinin receptors NK1R and NK2R in normal human bladder. **A:** mRNA levels of NK1R and NK2R in separated urothelium and bladder smooth muscle samples, determined by quantitative real-time RT-PCR. The graph shows the amount of NK1R and NK2R mRNA in bladder layers relative to a whole bladder sample. The data are a mean of three independent experiments $\pm$ SEM. **B:** Immunofluorescence labeling of human bladder sections with an anti-NK1R polyclonal antibody. Nuclei are stained with 4,6-diamidino-2-phenylindole. Scale bar = 5 μ m. **C:** Immunofluorescence labeling of human bladder sections with an anti-NK2R polyclonal antibody. Nuclei are stained with 4,6-diamidino-2-phenylindole. Scale bar = 5 μ m.

Tachykinin Receptor mRNA and Protein Levels are Decreased in Biopsies of BPS Patients

Previous studies failed to elucidate an involvement of NK1 and NK2 receptors in BPS/IC, and allowed for a considerable controversy about their up- or down-regulation in the diseased state.^{16,44,45} In bladder dome biopsies collected in this study, the mRNA levels of NK1R and NK2R receptors showed a significant ($P < 0.05$) down-regulation in BPS patients as compared with controls (Figure 3A).

Up- or down-regulation of mRNA levels is usually accompanied by an increase or decrease in the amount of corresponding protein. We used the cryopreserved material from five controls and six BPS biopsies. Figure 3B shows that NK1R was detected in all samples. However, the small number of samples, and considerable individual variability did not allow determining whether the down-regulation of NK1R protein levels in BPS patients was statistically significant.

Because NK1R and NK2R are unevenly distributed between UE and SMC, as shown in Figure 2, it is possible that the differences in NK1R protein levels in the individual biopsies were due to the predominance of either layer. Therefore, we attempted to use immunohistochemistry to evalu-

Figure 3. Tachykinin receptor mRNA and protein levels are reduced in BPS patients. **A:** The graph shows the levels of NK1R, and NK2R mRNAs in the bladder dome biopsies of 8 healthy controls, and 28 patients with BPS. mRNA levels in patients were determined by QPCR, normalized to 18S RNA and expressed relative to controls. The graph shows the average for NK1R, and NK2R expression in bladder dome biopsies $\pm$ SEM ($n = 8$ for control group; $n = 28$ for BPS group). NK1 and NK2 receptors are significantly ($*P < 0.05$) down-regulated in IC patients compared with controls. **B:** Protein levels of NK1R were determined by Western blotting in biopsies of controls ($n = 5$) and BPS patients ($n = 6$). Ponceau-stained membranes are shown as loading control. **C:** Representative images and the average mean intensities of anti-NK1R-labeled biopsies ($n = 3$ for control group; $n = 3$ for BPS). 4,6-diamidino-2-phenylindole nuclear staining of each section serves as a control of imaging settings. **D:** Representative images and the average mean intensities of anti-NK2R-labeled biopsies ($n = 3$ for control group; $n = 3$ for BPS).

ate the protein levels of NK1R and NK2R. Cryosections of three control biopsies and three biopsies from BPS subjects with low levels of TK receptors' mRNA were stained with anti-NK1R and anti-NK2R antibodies as described in *Materials and Methods*, and immunofluorescence images taken under standardized conditions, ie, using the same laser power, threshold, magnification, zoom, and exposure time. The mean fluorescence signal intensity, corresponding to the protein levels of NK1R and NK2R, was estimated from three individually stained sections of each biopsy as described in *Materials and Methods*. Figure 3, C and D show the results for NK1R and NK2R, respectively. The sections from BPS patients' biopsies revealed thinner UE layer, and significantly ($*P < 0.05$) lower levels of anti-NK1R and anti-NK2R staining, whereas the intensity of 4,6-diamidino-2-phenylindole staining was approximately equal in all of the sections (see graphs in Figure 3, C and D).

Characterization of Primary Cultures of Human Urothelium and Bladder Smooth Muscle Cells

Although biopsy material from healthy and diseased human subjects is invaluable for the evaluation of disease-induced alterations in receptor gene expression, primary cultures are an important tool to gain a mechanistic insight into the underlying changes. Therefore we estab-

lished primary cultures of human urothelium and bladder SMCs and characterized their TK receptor expression levels to evaluate their use for *in vitro* assays. Urothelial and SMC cultures were propagated, and their purity analyzed both morphologically, and by reverse transcriptionPCR with smooth muscle and urothelium-specific markers. Uroplakin 2 was detected only in urothelium, and transgelin/SM22 was over 20 times more abundant in SMCs (Figure 4A).

Early passages of urothelial cultures showed a strong, dose-dependent response to SP (Figure 4B). SP is an agonist of both NK1R and NK2R, with a higher affinity to NK1R. To determine, which of the two TK receptor types was responsible for the SP-mediated intracellular Ca^{2+} release observed in calcium imaging assays, we applied receptor-specific antagonists for NK1R (L 733'060) and NK2R (GR159897) before agonist stimulation (Figure 4C). Application of the NK1R antagonist L733'060 almost completely abolished the response to SP, whereas the NK2R antagonist GR159897 did not have a pronounced effect, suggesting that the predominant TK receptor type expressed in urothelial cells is NK1R.

Primary cultures of both smooth muscle and epithelial origin often fail to retain the features of the original tissue, undergoing a process of de-differentiation on repeated

Figure 4. Primary cultures of normal human urothelium and smooth muscle react to substance P. **A:** mRNA levels of uroplakin 2 (UP2) and SM22 in three independently isolated primary cultures of urothelium and smooth muscle were determined by QPCR, and normalized to 18S RNA. The graphs show values expressed as fold difference relative to either UE cultures (for UP2), or SMC (for SM22). Uroplakin 2 was not detected in smooth muscle cultures, and SM22 was over 20 times higher expressed in the smooth muscle cultures compared with the cultures of urothelium. **B:** Dose-dependent response of urothelial primary cultures to substance P (SP). Primary cultures of healthy human urothelium were grown on coverslips, loaded with 3 μ mol/L Fluo-3 AM and the intracellular Ca^{2+} release on application of increasing concentrations (10 nmol/L to 1 μ mol/L) of SP was monitored by calcium imaging. The graph shows data of a representative experiment. **C:** Effect of selective antagonists of NK1R and NK2R on SP-mediated Ca^{2+} release. Fluo-3 AM-loaded urothelium cells were pretreated with 10 μ mol/L NK1R antagonist L733060, or NK2R antagonist GR159897 for 10 minutes, and exposed to 100 nmol/L SP. Intracellular Ca^{2+} release was monitored by calcium imaging and compared with untreated cells (control). The graph shows data of a representative experiment. **D:** Attenuation of the response to SP in successive passages of urothelial primary cultures. Established primary cultures of healthy urothelium were passaged, seeded on coverslips, and their response to 100 nmol/L SP recorded by calcium imaging. The number of responsive cells, and the amplitude of Ca^{2+} transients decrease on passaging. **E:** Dose-dependent response of bladder smooth muscle primary cultures to SP. Intracellular Ca^{2+} release on application of increasing concentrations (1 nmol/L to 100 nmol/L) of SP was monitored by calcium imaging. The graph shows data of a representative experiment. **F:** Effect of selective antagonists of NK1R and NK2R on SP-mediated Ca^{2+} release. Fluo-3 AM-loaded bladder SMCs were pretreated with 10 μ mol/L NK1R antagonist L733060, or NK2R antagonist GR159897 for 10 minutes, and exposed to 100 nmol/L SP. Intracellular Ca^{2+} release was monitored by calcium imaging and compared with untreated cells (control). Graph shows data of a representative experiment. **G:** Successive passages of bladder SMC cultures retain their TK receptors. Primary cultures of healthy urothelium were passaged, seeded on coverslips, and their response to 100 nmol/L SP was recorded by calcium imaging.

passaging, which renders them unsuitable for *in vitro* studies.^{46,47} In agreement with these data, we observed in consecutive passages of urothelium cells (Figure 4D) that the initially uniform and strong SP-induced Ca^{2+} release in freshly-cultured urothelial cells (passage 0) was progressively attenuated in the consecutive passages, leading to a complete loss of response after the third passage.

Similar to urothelium, SMCs responded to SP application in a dose-dependent manner (Figure 4E). Calcium imaging assays in the presence of the selective antagonist to NK1R showed that blocking this receptor significantly attenuated, but did not completely abolish the response to SP. In contrast to urothelium, the selective antagonist of NK2R also induced a reduction of the SP-mediated Ca^{2+} release (Figure 4F). These data suggest that in bladder smooth muscle both NK1R and NK2R are present and functional. Compared with the urothelial cultures, the bladder SMC primary cultures maintained their

ability to respond to SP longer, though these responses became eventually attenuated, indicating that the bladder primary cultures do not maintain their tissue-specific characteristics indefinitely (Figure 4G). Our data strengthen the importance of using biopsy material rather than primary cultures to investigate the disease-related alterations in their expression levels in BPS/IC patients.

Prolonged Exposure to SP Causes a Down-Regulation of NK1R Expression

Chronic exposure of a receptor to its agonist decreases its expression levels,⁴⁸ and increased levels of substance P have been reported in the urine of patients with BPS/IC.^{8,9} Because primary cultures of bladder urothelium did not sustain NK1R expression (see Figure 4D), and the immortalized normal human bladder UROtsa cell line did not express NK1R when tested (data not shown),

we investigated the effects of chronic exposure to SP on NK1R mRNA levels in the early passages of bladder SMCs. The cells were harvested after 2, 8, and 22 hours of exposure to 100 nmol/L SP, and the amounts of NK1R mRNA studied by real-time QPCR. In two independent experiments, we observed a reduction of NK1R mRNA levels after 22 hours of agonist exposure (Figure 5A). Due to the fact that smooth muscle cultures did not sustain the

Figure 5. Bladder SMC cultures and U373MG cells exposed to SP down-regulate NK1R expression. **A:** Bladder SMC (passage 3) were cultured in normal medium (control), or in medium supplemented with 100 nmol/L SP. After 2, 8, or 22 hours of exposure to the NK1R agonist, cells were harvested and the amounts of NK1R mRNA estimated by TaqMan QPCR. The graph shows the NK1R mRNA levels at the indicated times post application of 100 nmol/L SP, normalized to 18S and relative to the untreated control $\pm$ SDEV. **B:** Prolonged exposure to SP decreases the levels of NK1R mRNA in U373MG astrocytoma cells, without affecting the level of glyceraldehyde-3-phosphate dehydrogenase mRNA estimated by TaqMan QPCR. The graph shows the mRNA levels at the indicated times post application of 100 nmol/L SP, normalized to 18S and relative to the untreated control, $\pm$ SEM ($n = 6$). The statistically significant ($*P < 0.05$) down-regulation of NK1R levels after and 22 hours of SP exposure is indicated. **C:** Calcium imaging assay with U373MG cells exposed to 100 nmol/L SP for 22 hours. The cells were cultured in SP-containing medium, washed and loaded with 3 μ mol/L Fluo-3/AM for 1 hour in the absence of SP. After an additional 30 minutes incubation in Na^+ -Tyrode buffer, calcium imaging experiments were performed by applying 100 nmol/L fresh SP and measuring the intracellular Ca^{2+} release. To account for receptor internalization following the SP treatment, control cells were briefly exposed to 100 nmol/L SP (1 minute), washed and loaded with Fluo-3 AM similar to the chronically treated cells. The graph shows the mean Ca^{2+} transients $\pm$ SEM ($n = 3$).

expression levels of NK1R in successive passages, and could therefore be prone to spontaneous down-regulation of its levels, we sought to confirm these data in a more robust cell-based model of NK1R expression. U373MG human astrocytoma cells endogenously express NK1R and have been extensively characterized in multiple studies of NK1R pharmacology.⁴⁹ Similar to bladder smooth muscle cultures, in U373MG cells there was no significant alteration of NK1R mRNA levels 2 hours after addition of SP, but after 8 hours of exposure the level of NK1R decreased to 40% and remained at 60% of the control after 22 hours of exposure (Figure 5B). In contrast to NK1R, the levels of glyceraldehyde-3-phosphate dehydrogenase mRNA did not change during SP exposure (Figure 5B). The effects of prolonged exposure to SP were further validated in receptor activation assays, which offer an insight into the receptor levels at the plasma membrane. The response to saturating concentrations of SP after 22 hours of previous agonist exposure was decreased compared with untreated control cells

Figure 6. Expression levels of several microRNAs are significantly altered in U373MG cells and bladder SMCs after prolonged exposure to SP. **A:** Heat map of the 12 significantly up-regulated microRNAs in U373MG cells exposed to 100 nmol/L SP for the indicated times. MiRNAs were up-regulated at least twofold during at least one time-point of agonist exposure. The heat map shows the " $-2 \log[\text{expression}]$," ie, the $\Delta\Delta\text{Ct}$ values ($\Delta\text{Ct sample} - \Delta\text{Ct average control}$), representing in green the up-regulations and in red the down-regulations. MiRNAs predicted to have NK1R as target genes are indicated with an arrow. Graphs show relative expression of miR-449b and miR-500 (fold difference compared with the untreated control) in two independent experiments, each performed in duplicates. **B:** Levels of miR-449b and miR-500 were determined in samples of bladder SMCs treated with 100 nmol/L PS for the indicated time periods. MiRNA levels are shown as a heat-map and a graph (fold difference as compared with the untreated control).

(Figure 5C). These results implicate prolonged agonist exposure in the attenuation of NK1R mRNA and protein levels, and correlate well with our findings of a down-regulation of NK1 receptor expression in BPS patients in a chronic phase of the disease.

Expression Levels of Several MicroRNAs Are Increased in U373MG and Bladder SMCs after Prolonged SP Exposure

MicroRNAs are known regulators of gene expression at the level of mRNA stability and translation, therefore we investigated whether the up-regulation of the NK1R-targeting miRNAs could account for the observed down-regulation of NK1 receptor expression in bladder SMCs, and U373MG astrocytoma cells. We screened 365 miRNAs in probes of U373MG cells exposed to SP

for 2, 8, and 22 hours using TaqMan Low Density Arrays, and compared the results to the data obtained using an untreated control. We identified 12 miRNAs that were at least twofold up-regulated, as compared with the untreated control in at least one time point of SP exposure. Figure 6A shows $-2 \log [\text{expression}]$ of these miRNAs as a heat map. Using a miRBase database we identified NK1R as a putative target gene for miR-449b and miR-500. Both miRNAs were at least five times higher in the SP-treated samples (8 hours and 22 hours of exposure) than in the control U373MG cells (Figure 6A, graphs). The other 10 miRNAs, for example MiR-432, were also up-regulated by SP treatment, however, not predicted to regulate NK1R.

We examined the levels of miR-449b and miR-500 in the samples of SP-treated bladder smooth muscle cultures using individual miRNA expression assays (Figure

Figure 7. MiR-449b and miR-500 down-regulate NK1R mRNA and protein levels in U373MG cells, (A–C) U373MG astrocytoma cells, endogenously expressing the NK1 receptor, were transfected with selected miRNAs. NK1R mRNA levels were analyzed 48 hours post-transfection by TaqMan QPCR and compared with scrambled miRNA (negative control) and validated anti-NK1R siRNA (positive control). The graphs show NK1R mRNA levels after transfection with (A) miR-449b $\pm$ SEM ($n = 4$), (B) miR-500 $\pm$ SEM ($n = 4$), (C) miR-432 ($n = 2$). Significant differences to the negative control (* $P < 0.05$, ** $P < 0.01$) are indicated. D–F: U373MG cells, transfected with scrambled miRNA (negative control), validated anti-NK1R siRNA (positive control), or one of the three miRNAs were grown on coverlips, were loaded with Fluo-3 AM, and calcium imaging experiments were performed by applying 0.01 nmol/L SP. The graphs show the average responses recorded in three independent experiments performed in triplicates ($n = 9$). D: Responses in miR-449b-transfected cells compared with the positive and negative control, (E) miR-500-transfected cells, (F) miR-432-transfected cells. G: The graph shows relative responses compared with a negative control. Validated NK1R siRNA, miR-449b, and miR-500 significantly (* $P < 0.05$) down-regulate responses to 0.01 nmol/L SP.

6B). Similar to U373MG cells, elevated levels of miR-500 were detected after 8 and 22 hours of SP treatment. However, the levels of miR449b in cultured bladder SMCs did not significantly change during agonist exposure (Figure 6B, graphs).

MiR-449b and miR-500 Down-Regulate mRNA and Protein Levels of NK1R in the U373MG Cell Line

Having used bioinformatics tools (miRBase) for the prediction of putative miRNA target genes for miR-449b and miR-500, we investigated their role in the regulation of the NK1R expression using a cell-based model. Pre-miR miRNA precursors for miR-449b, miR-500, and miR-432 were reverse-transfected into U373MG cells. Scrambled miRNA was used as negative control, and validated NK1R siRNA as positive control. Only the miRNAs miR-449b and miR-500, predicted to target NK1R, significantly down-regulated of NK1R mRNA levels in all applied concentrations ($P < 0.05$) (Figure 7A and B). Transfection of miR-432 did not reduce the NK1R mRNA levels in the U373MG cell line, consistent with its target prediction, which did not include NK1R (Figure 7C).

To estimate the protein levels of NK1R in U373MG cells, transfected with miRNAs, we used receptor activation assays. In agreement with the down-regulated mRNA levels, there was a significantly reduced the Ca^{2+} release following application of 0.01 nmol/L SP in U373MG cells transfected with NK1R siRNA in all experiments. Both miR-449b- and miR-500-transfected cells also showed reduced receptor activation (Figure 7, D and E), however, miR-432 did not have an effect (Figure 7F). Although both miR-449b and miR-500 reduced the NK1R mRNA levels similar to the validated NK1R siRNA (Figure 7G), they down-regulated the receptor expression on the protein level stronger than the siRNA positive control, in agreement with the dual action of miRNAs on transcription and translation levels of their target genes.⁵⁰

MiR-449b and miR-500 Are Expressed in Human Bladder and Increased during BPS

Having implicated miR-449b and miR-500 in down-regulation of NK1R mRNA and protein levels during chronic exposure to SP in the cell-based models, we investigated whether the same miRNAs might be involved in the down-regulation of NK1R in BPS. We examined the expression levels of these miRNAs, normalized using endogenous RNU48 miRNA, in biopsy samples of 28 BPS patients, and compared them to the levels in 8 control biopsies (Figure 8). Both miRNAs were detected in bladder dome samples of patients and controls, and a statistically significant up-regulation of their expression levels was observed in BPS. These data indicate that the same mechanisms might be involved in regulation of NK1R expression in cells, exposed to SP and *in vivo* in BPS patients with high degree of pain.

Figure 8. MiR-449b and miR-500 are significantly up-regulated in BPS patients, levels of MiR-449b and miR-500 in biopsies of 28 BPS patients compared with 8 controls were determined by TaqMan QPCR after isolation of miRNA-enriched total RNA. miR-449b and miR-500 levels in patients were normalized to endogenous miRNA RNU48 and expressed relative to healthy controls. Both miRNAs are significantly (* $P < 0.05$) elevated in BPS patients.

BPS Is Characterized by a Specific Pattern of miRNA Expression

To gain an insight into the underlying causes of BPS disease, including the mechanisms of gene expression regulation, we screened 365 miRNAs in 8 BPS patients, randomly selected out of 28 subjects of this study, using TaqMan Low Density Arrays, and compared the results to the data obtained from four random controls. Out of 365 miRNAs, three were significantly down-regulated, and 28 up-regulated (Figure 9A). The top three putative targets for the up-regulated miRNAs were identified with Target Scan, miRBase, and miRanda databases (Table 1). The two NK1R-targeting miRNAs miR-449b and miR-500, identified in the earlier screen of 28 patients, were confirmed using the TaqMan Low Density Arrays card. Additionally, four miRNAs expressed in bladder and up-regulated in BPS patients were predicted to have NK1R as a putative target gene: MiR-199a and miR-320 were predicted by TargetScan, miR-192, miR-328, and miR-320 were predicted by miRBase. The expression levels of these miRNAs were two to five times higher in BPS than in control biopsies (Figure 9B).

MiR-328 and miR-320 Down-Regulate the Expression Levels of NK1R in U373MG Cell Line

Having identified four new NK1R-targeting miRNAs from bladder samples (MiR-199a, miR-320, miR-192, miR-328) we investigated their role in NK1R expression at the mRNA and protein levels using a U373MG cell-based model. A statistically significant down-regulation of NK1R mRNA levels was observed only using miR-328 to a final concentration of 10 nmol/L and 100 nmol/L (Figure 10A). Transfection of miR-328 caused a 20% reduction in the NK1R expression in the U373MG cell line. A decreasing trend in NK1R expression was also observed using miR-320 at 100 nmol/L; however, this reduction was not statistically significant (Figure 10B). MiR-129 and miR-199a did not affect the expression levels of NK1R (not shown). Although their effect on NK1R mRNA levels was relatively modest, both miR-328 and miR-320 caused a decrease of NK1R protein levels, as estimated by the NK1 receptor

Figure 9. Expression levels of 31 microRNAs are significantly altered in BPS patients. **A:** Hierarchically clustered (average linkage) heat map of the 31 significantly up- or down-regulated microRNAs in bladder biopsies of 8 random BPS patients compared with 4 random control biopsies. The heat map shows the “ $-2 \log[\text{expression}]$,” ie, the $\Delta\Delta\text{Ct}$ values ($\Delta\text{Ct sample} - \Delta\text{Ct average control}$), representing in green the up-regulations and in red the down-regulations. **B:** Relative expression (fold difference compared with control) of the six miRNAs predicted to have NK1R as a putative target gene. The six miRNAs were up-regulated two to five times in BPS patient biopsies compared with controls. The up-regulation is significant for miR-192, miR-199a, miR-449b, and miR-500 ($*P < 0.05$), and for miR-320 and miR-328 ($**P < 0.01$).

activation assay (Figure 10, C and D). In these assays, miR-320 was similar, and miR-328 more efficient than the validated NK1R siRNA (Figure 10E).

Identification of Further miRNA Targets, Potentially Implicated in BPS Pathogenesis Based on Database Predictions

Most miRNA bind to 3' UTRs of the target mRNAs, and attenuate their translation/destabilize the mRNA leading to its degradation. Figure 11 shows the sequences of the 4 pre-miRNAs, which we validated in this study as negatively influencing the expression levels of NK1R, and their complementary sequences in the 3'UTR of NK1R mRNA, together with the Sanger miRNA Mature Sequence Accession number.

To correlate the BPS-induced miRNAs with their putative targets, we screened the available miRNA databases. Table 2 shows the potential link between the altered genes and some of the BPS-specific miRNAs, identified using the TargetScan 5.0, MiRBase 13.0, and

MiRanda Sep'08 databases. Further research is needed to functionally validate the bioinformatics predictions for these additional miRNAs using the appropriate cell-based models. Our results implicating four miRNAs in regulation of the NK1R expression show that this approach is advantageous for the investigation of novel regulatory mechanisms, activated in disease.

Discussion

In this study aimed to elucidate the molecular mechanisms of BPS, we performed an extensive gene expression profiling using the biopsies from 28 BPS patients and 8 controls. Our results allow us to quantitatively estimate the disease-related changes in mRNA levels for proteins, involved in signal transduction, muscle contraction, and epithelial permeability. To test whether BPS is accompanied by impaired urothelial function, we studied the expression levels of tight junction proteins. We observed a significant down-regulation of mRNA levels of zona occludens-1, junctional adhesion molecule 1, occludin, and claudin 1, indicating a compromised tight junction structure and potentially increased permeability of the urothelium of BPS patients. Claudins are a group of tight junction proteins, regulating paracellular transport. We observed that the samples from BPS patients had unchanged levels of claudin 4, a “tight” claudin normally present in water impermeable epithelia and abundant in normal urothelium,⁵¹ however, there was a significant up-regulation of mRNA levels of claudin 2, associated with permeable, “leaky” epithelia.⁵² Recently, claudin 2 was implicated in formation of cation-selective paracellular pores.⁵³ Thus, the appearance of claudin 2-mediated Na^+ - and K^+ -permeable pores in the urothelium might contribute to the underlying nerve activation and pain sensation during BPS. These results confirm that there are considerable alterations in the barrier function of urothelium, associated with BPS, which might influence the underlying interstitium and cause activation of the receptors involved in motor and sensory functions in the urinary bladder.⁷

The purinergic receptors, expressed in the peripheral nervous system, have been implicated in nociception and pain during BPS/IC in mouse models.⁵⁴ We did not observe any significant differences in the expression levels of P2X1 or P2X2 receptors in the samples of BPS patients compared with the controls. In contrast to previously published data,⁵⁵ we could not reliably detect the expression of P2X3 receptor in either control or patient biopsies, therefore this target was excluded from further analysis. In agreement with the studies showing an increased ATP release in BPS/IC and a concomitant increase in bladder contractility,^{56,57} we observed a significant up-regulation of P2Y1 receptor mRNA levels in BPS biopsies. Similarly, there was a strong increase in the levels of muscarinic receptors M3, M4, and M5, and an elevation of M2. These data correspond very well to the previously published results, showing the presence of all types of muscarinic receptors in human detrusor samples,⁵⁸ and in animal models of BPS/IC, where up to 50

Table 1. List of 31 miRNAs, Significantly Different in the BPS Patient Group Compared with Controls

Predicted target genes of miRNAs, significantly altered in BPS		
miRNA	State	Putative targets
Hsa-let-7g	Up	<i>HMGA2, C14orf28, LIN28B</i>
Hsa-miR-23a	Up	<i>ZNF138, FUT9, SEMA6D</i>
Hsa-miR-23b	Up	<i>ZNF138, FUT9, SEMA6D</i>
Hsa-miR-25	Up	<i>CD69, SLC12A5, FNIP1</i>
Hsa-miR-26a	Up	<i>CHORDC1, POLR3G, ATP11C</i>
Hsa-miR-27a	Up	<i>GLT8D3, PLK2, ACVR1C</i>
Hsa-miR-30e-5p	Up	<i>CELSR3, SNX16, S100PBP</i>
Hsa-miR-95	Up	<i>CUGBP2, SLC12A5, SHOX2, + TACR2</i>
Hsa-miR-130b	Up	<i>MIER1, SLAIN1, C10orf140</i>
Hsa-miR-133b	Up	<i>SYT2, LHFP, CCBL2</i>
Hsa-miR-148a	Down	<i>CCKBR, ATP6AP2, OSBPL11</i>
Hsa-miR-182	Down	<i>RGS17, MTF, ACTR2</i>
Hsa-miR-186	Up	<i>MIB1, GABRA4, USP37, + TACR2</i>
Hsa-miR-192	Up	<i>IKZF2, PABPC4, PABPC4, + TACR1</i>
Hsa-miR-199a(-5p)	Up	<i>ZNF763, ZNF776, ZNF439, + TACR1, TACR2</i>
Hsa-miR-320(a)	Up	<i>ONECUT2, C12orf36, KITLG, + TACR1</i>
Hsa-miR-324-3p	Up	<i>FAM155B, MDGA1, MLL2</i>
Hsa-miR-328	Up	<i>KLHDC5, ARPP-19, CHST7, + TACR1</i>
Hsa-miR-342(-3p)	Up	<i>GLT8D3, CA12, MATN1</i>
Hsa-miR-379	Up	<i>MTMR2, HLCS, LOC285636</i>
Hsa-miR-422a	Up	<i>TOB2, KIAA1522, SDAD1,</i>
Hsa-miR-422b(378)	Up	<i>TOB2, KIAA1522, SDAD1</i>
Hsa-miR-449b	Up	<i>HCN3, FAM76A, UCN2, + TACR1</i>
Hsa-miR-485-5p	Up	<i>TAOK1, EFHC1, PARP11</i>
Hsa-miR-493	Down	<i>MRV1, KRTAP4-1, FAM102B</i>
Hsa-miR-500(*)	Up	<i>RFESD, SERBP1, IPO9, + TACR1, TACR2</i>
Hsa-miR-502(-5p)	Up	<i>C12orf54, LINCR, PRRG4</i>
Hsa-miR-511	Up	<i>ELAVL3, MXI1, C1QTNF7, + TACR2</i>
Hsa-miR-572	Up	<i>FLJ45910, C14orf101, NCAM1</i>
Hsa-miR-594	Up	Not in TargetScan 5.0, miRBase 13.0 or miRanda Sep'08
Hsa-miR-597	Up	<i>GJB7, TLX1, LARP4</i>

miR-148a, miR-182, and miR-493 are down-regulated and the others are up-regulated in the BPS group. The table shows the three top putative target genes for the respective miRNAs, determined using the TargetScan 5.0 database. In bold are the tachykinin receptors as putative target genes for several statistically significant miRNAs (according to TargetScan 5.0 and miRBase 13.0 databases).

times higher levels of M5 receptor were detected in bladders of cyclophosphamide-treated rats.²³

Bradykinin receptor B₁ and cannabinoid receptors CB1 and CB2 are involved in the regulation of pain and inflammatory responses in human bladder.^{59,60} We detected a significant ($P < 0.05$) up-regulation of the inflammation-induced bradykinin receptor B₁ and cannabinoid CB1 receptors. CB2 receptors were up-regulated in some patients; however, this difference was not statistically significant. Here we confirm previous murine data²⁵ in humans, showing that β_2 adrenergic receptor mRNA was significantly higher in BPS patients than in controls.

Substance P and related tachykinins mediate a variety of physiological processes through stimulation of NK1 and NK2 receptors. There is a considerable discrepancy regarding the presence and distribution of tachykinin receptors in the layers of the urinary bladder of different species. We established the presence of NK1R in both urothelium, and underlying stroma and smooth muscle layers, with higher expression rates in the urothelium. In contrast, NK2R was predominantly expressed in smooth muscle, and virtually absent from the urothelium. These data were confirmed by the receptor activation assays performed on the primary cultures of human urothelium and bladder smooth muscle. Our data indicate that two major types of TK receptors have a differential distribution in the layers of healthy human bladder. The presence

of NK1R in both bladder layers suggests its importance for sensory and neuroinflammatory processes.

Investigating the disease-induced changes in the expression levels to the TK receptor genes, we show for the first time that NK1R mRNA levels were significantly ($P < 0.05$) lower in patients with BPS than in controls. Likewise, NK2R mRNA levels were significantly ($P < 0.05$) down-regulated. These observations contradict earlier studies, which relied on semiquantitative methods such as immunofluorescence and *in situ* hybridization.¹⁶ In addition, the limited number of patients recruited in the earlier trials may have influenced the interpretation of the results. We examined biopsy material from a larger group of patients, all of whom were suffering from chronic BPS for extended periods of time. Therefore, our results are not directly comparable with the antigen-induced cystitis in mouse models, still bearing the hallmarks of an acute inflammatory state. It is possible that down-regulation of TK receptor expression levels is an indicator of a chronic state of BPS. Interestingly, in Crohn's disease, the expression levels of NK1R were significantly down-regulated in patients, as compared with controls.⁶¹ Similarly, there was no up-regulation of NK1R gene expression levels in asthmatic lungs, as compared with controls.⁶²

Chronic exposure of a receptor to its agonist has been reported to decrease the expression levels of other G protein-coupled receptors.⁴⁸ Similarly, continuous expo-

Figure 10. miR-328 and miR-320, up-regulated in BPS patients, cause a decrease of NK1R mRNA and/or protein levels in U373MG cells. U373MG astrocytoma cells, endogenously expressing the NK1 receptor, were transfected with selected miRNAs. NK1R mRNA levels were analyzed 48 hours post-transfection by TaqMan QPCR and compared with the scrambled miRNA (negative control) and validated anti-NK1R siRNA (positive control). The graphs show NK1R mRNA levels after transfection with (A) miR-328 ± SEM ($n = 7$). B: miR-320 ± SEM ($n = 6$). Significant differences to the negative control ($P < 0.05$) were seen with anti-NK1R siRNA (positive control, down-regulated to 40%) and when the U373MG cell line was transfected with 10 nmol/L or 100 nmol/L miR-328 (down-regulated to 80% of control levels), but not using miR-320. C–D: U373MG cells, transfected with scrambled miRNA (negative control) and validated anti-NK1R siRNA (positive control) and miRNA were grown on coverlips, loaded with Fluo-3 AM, and calcium imaging experiments were performed by applying 0.01 nmol/L SP and measuring intracellular Ca^{2+} release. The graphs show the average responses recorded in three independent experiments performed in triplicates ($n = 9$). E: Responses in miR-328-transfected cells compared with the positive and negative control. F: Response in miR-320-transfected cells. G: The graph shows relative responses compared with the negative control. Validated NK1R siRNA, and both miR-328 and miR-320 significantly ($*P < 0.05$) down-regulate responses to 0.01 nmol/L SP, by affecting the receptor density at the plasma membrane of transfected cells.

sure to SP causes a reduction of NK1 receptor protein levels, attributed to ubiquitin-dependent degradation,⁶³ which is in line with the increased amounts of SP detected in the urine of patients with BPS/IC.^{8,9} We confirmed these findings using human bladder SMC cultures and U373MG astrocytoma cells, which endogenously express the receptor.⁶⁴ Here we show that in both cell types there was a significant decrease of the receptor mRNA and protein levels after 8 and 22 hours of agonist expo-

sure. These results point out the agonist exposure as an important factor, regulating the receptor density in chronically stimulated cells.

MicroRNAs are known regulators of gene expression at the level of mRNA stability and translation, and have been implicated the processes of inflammation and cancer,³⁰ therefore up-regulation of certain miRNAs could account for the observed down-regulation of NK1 receptor expression in bladder SMC cultures, and U373MG astrocytoma cells. An increase of a miRNA expression level would thus be concomitant with a decrease in its target gene mRNA and its protein product. To gain a mechanistic insight into the underlying causes of NK1R mRNA and protein down-regulation in cell-based models, we screened 365 miRNAs in samples of U373MG cells exposed to SP for 2, 8, and 22 hours using TaqMan Low Density Arrays, and compared the results to the data obtained using an untreated control. We identified 12 miRNAs that were at least twofold up-regulated in at least one time point of SP exposure. Of these miRNAs, only miR-449b and miR-500, which were about five times higher in the SP-treated samples, had NK1R as a predicted target gene. Similar to U373MG cells, in the SP-treated bladder smooth muscle cultures miR-500 was strongly up-regulated after 8 and 22 hours of exposure.

Figure 11. Sequences and binding sites of the four NK1R-specific miRNAs, down-regulating the receptor's mRNA and/or protein levels. Shown are the sequences of the pre-miRNA molecules used in transfection experiments, their miRBase mature accession numbers and predicted binding sites in the NK1R 3' UTR. The nucleotide positions are given counting from the 1 nt in the 3' UTR.

Table 2. Genes with Altered Expression Levels in BPS, and Corresponding Targeting miRNAs, Up-or Down-Regulated in the Patients' Biopsies

Genes	Altered gene expression in BPS and putative regulating miRNAs		
	TargetScan 5.0	miRBase 13.0	miRanda Sep'08
<i>NK1R</i>	—	miR-500*, -320, -328, -449b, -199a-5p, -192	—
<i>NK2R</i>	miR-186, -511	miR-95, -500*, -199a-5p	<i>Not in database</i>
<i>BDKRB1</i>	—	miR-148a	<i>Not in database</i>
<i>ADRB2</i>	Let-7g	Let-7g, miR-27a	miR-27a
<i>CB1</i>	miR-23a, -23b	<i>Not in database</i>	miR-23a, -23b, -328, -148a, -26a, -27a
<i>CB2</i>	miR-328	—	—
<i>ZO-1</i>	miR-23a, -23b	—	miR-186, -27a, -23a, -511, -23b
<i>OCLN</i>	—	—	miR-199a-5p
<i>JAM-1</i>	—	miR-485-5p	miR-493, -149, -182, -485-5p, -328, -199a-5p
<i>CLD1</i>	—	—	493, 186, 320a, 95, 597, 199a-5p, 27a
<i>CLD2</i>	—	miR-379, -328	miR-511, -95, -182, -485-5p, -328
<i>P2Y1</i>	miR-186, -25, -133b, -324-3p	—	miR-25
<i>MuscR3</i>	miR-23a, -23b	—	miR-148a, -511, -328
<i>MuscR4</i>	—	<i>Not in database</i>	<i>Not in database</i>
<i>MuscR5</i>	miR-25	miR-199a-5p	miR-199a-5p, -25, -182

Genes, showing statistically significant up- or down-regulation in BPS patients, and the corresponding regulatory miRNAs, predicted according to TargetScan 5.0, miRBase 13.0, and miRanda Sep'08 databases.

Concomitant up-regulation of miRNAs and down-regulation of their target genes is a strong indication, but not a proof, of their inhibitory function. To obtain conclusive evidence of inhibition, we ectopically expressed miR-449b and miR-500 in U373MG cells and showed that both miRNAs significantly reduced the mRNA and protein levels of the endogenously expressed NK1R. SP treatment and chronic activation of NK1R has far reaching effects on cellular functions, and not all miRNAs, which were up-regulated during sustained agonist exposure, targeted NK1R: miR-432 had no effect on the receptor mRNA and protein levels when examined in the same assay.

Both miR-500 and miR-499b were detected in human bladder biopsies, and their levels were significantly increased in the patients compared with the controls. These results implicate miRNAs in the down-regulation of NK1R in BPS, and prompted us to undertake a broad screening of miRNAs to identify other potential causes of disease-induced changes of gene expression. Out of 365 screened miRNAs, we identified 31 significantly different between the patients and controls, 3 were down-regulated, and 28 up-regulated. Two of the up-regulated miRNAs, miR-23a and -23b, were previously reported to be increased in bladder cancer.⁶⁵ In addition to miR-449b and miR-500, validated in this study, bioinformatic tools predicted NK1R mRNA as a target for miR-192, -199a, -320, and -328 all up-regulated during BPS. To obtain experimental proof of these predictions we studied the effect of the four miRNAs on the NK1R expression levels and show that miR-328 induced a statistically significant decrease in NK1R mRNA and protein levels, whereas miR-320 was only active at the protein level. Consequently, up-regulation of these miRNAs in BPS patients could be contributing to NK1R down-regulation *in vivo*.

Here we show for the first time that the expression and function of a TK receptor may be regulated by a miRNA in a diseased state. An important and still unresolved question is whether miRNAs are a cause or a consequence of BPS. Our data on the agonist-induced down-regulation of NK1R expression show that, at least for this receptor, miRNA elevation is a consequence of prolonged agonist exposure and might therefore represent a secondary adaptive mechanism, allowing the cells to cope with the continuously activated receptor signaling. Our findings further the knowledge of the molecular mechanisms of BPS and have implications for diagnosis and treatment of this enigmatic disorder. They might explain why the TK receptor antagonists proved to be of limited use for treatment of BPS. Our data have also relevance for the pathogenesis of other diseases in which NK1R has been suggested to play a role, such as depression,⁶⁶ cancer,⁶⁷ and pancreatitis.⁶⁸

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