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Original Article

Beta-3 adrenergic receptors could be significant factors for overactive bladder-related symptoms

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Abstract: The treatment failure often happens in overactive bladder (OAB) partly owing to its unknown pathogenesis. The purpose of this study is to find significant receptors or biological markers for OAB-related symptoms for establishment of potential order-made therapeutic strategies. The overactive bladder symptom scores (OABSS) and international prostate symptom scores (IPSS)/quality of life (QOL) were questioned in all the 18 patients with OAB diagnosis. Their bladder mucosal tissues were taken from the random biopsy of bladder cancer suspected patients without any finding such as inflammation or carcinoma in situ. They were investigated quantitatively by immunohistochemical (IHC) stainings for inflammatory or immune-system (Interleukin (IL)-6 and cyclooxygenase-2 (Cox-2)), Caspase-3 apoptosis markers, angiogenesis (CD-31), epithelial-mesenchymal transition (E-cadherin) and muscarinic receptor (Muscarine-2 (M)-2), adrenergic receptors (ARs) (alpha 1-d (α1-d) and beta-3 (β-3)). The statistical correlation between the expressions of these 5 markers and 3 receptors and these symptom scores were examined under the comparison between OAB patients and control patients who had urgency score with less than 2 in OABSS. The OABSS and IPSS/QOL was 7.39 ± 2.69 and 21.2 ± 6.59/4.33 ± 1.33, respectively but those of control patients were 2.00 ± 1.41 and 10.1 ± 9.52/2.14 ± 1.46, respectively (P<0.05). Regarding the correlation of those markers' expressions and symptom scores, in OAB patients, OABSS total significantly correlated with β-3 AR expressions (P=0.0457). IPSS post-voiding significantly correlated with β-3 AR expressions (P=0.0308) but no significant relationship in control patients (P>0.05). In conclusion, this study demonstrated that β-3 AR in our tested 8 markers or receptors was correlated strongly with OAB-related symptoms. These data may help elucidate the pathophysiology of OAB and offer possible strategy for its order-made therapies.

Keywords: Overactive bladder, quality of life, symptom scores, beta-3 adrenergic receptor

Introduction

Overactive bladder (OAB) syndrome is defined as lower urinary tract symptom (LUTS) characterized by urgency, with or without urge incontinence, usually with frequency daytime and night time, in the absence of infection or other obvious pathology [1]. Namely, OAB has a symptom-based definition [2]. The prevalence of OAB was estimated as one in six and increased with age [3]. Approximately 16% of adults in the United States are affected by OAB with a somewhat similar prevalence in males and females [3]. OAB negatively affects the patient's quality of life (QOL) [4].

OAB evaluation is still based on subjective symptoms. A new accurate, objective and non-invasive test to diagnose OAB is lacking. Several studies showed potential diagnostic biomarkers were effective [5]. Alpha (α)-adrenergic receptors (ARs) are expressed in the prostate, bladder neck, and internal urethral sphincter, and α1-blockers are often used in clinics to decrease not only intraurethral resistance but contraction in bladder neck and/or internal urethral sphincter [6]. Activation of sympathetic nerves contributes to urine storage by relaxing the detrusor muscle via activation of beta (β)-AR [7]. In humans, however, the detrusor muscle has been recently reported to be predomi-

Table 1. Comparison of symptoms between OAB patients and control patients

			OAB patients	control patients	*p* value
OABSS					
	total		7.39 ± 2.69	2.00 ± 1.41	**<0.0001**
		frequency	1.28 ± 0.56	0.57 ± 0.49	
		nocturia	2.00 ± 0.88	1.00 ± 1.07	
		urgency	3.11 ± 1.45	0.42 ± 0.73	
		incontinence	1.11 ± 1.52	0	
IPSS					
	storage		10.83 ± 3.76	5.14 ± 4.32	**0.0048**
		frequency	4.16 ± 1.22	2.28 ± 1.75	
		urgency	3.16 ± 1.86	1.00 ± 1.60	
		frequency at night	3.78 ± 1.36	1.86 ± 1.46	
	voiding		6.78 ± 3.47	4.43 ± 4.92	0.2112
		intermittency	0.83 ± 1.42	0.28 ± 0.45	
		weak stream	3.28 ± 1.76	1.57 ± 1.92	
		straining	2.67 ± 2.08	1.43 ± 1.84	
	post-voiding (incomplete emptying)		1.44 ± 1.77	0.57 ± 0.90	0.2453
	total scores		21.2 ± 6.59	10.1 ± 9.52	**0.0043**
	QOL		4.33 ± 1.33	2.14 ± 1.46	**0.0022**

bold: statistically significant.

nant site of β-3-AR mRNA expression. The relaxation induced by adrenergic stimulation of human detrusor is mediated mainly through β-3-AR activation [8]. On the other hand, muscarinic receptors are well-expressed in bladder mucosa, especially the bladder trigonal zone [9] which has more vessels comparatively. There are several subtypes of muscarine receptors such as muscarin-1, 2, 3, 4, and 5 (M-1, 2, 3, 4, and -5) [10] and of these, M-2 may have the highest ratio of selectivity for bladder mucosa [11].

Regarding the pathophysiological study of OAB, Juan et al. reported that in their surgical menopause-induced (ovariectomy) OAB using a rat model, anti-apoptotic marker bcl-2 was downregulated and apoptotic marker was upregulated [12]. On the other hand, Liu et al. compared OAB and interstitial cystitis/bladder pain syndrome (IC/BPS) which are considered to have overlapping symptoms and are often hard to discriminate, and showed E-cadherin expression was significantly decreased in IC/BPS but not OAB compared to control patients [13]. Kuo et al. reported in their review that serum proinflammatory cytokines such as Interleukin (IL)-6 and chemokine (IL-8) levels were significantly higher in the serum of patients with IC/BPS than control patients but did not mention those markers in OAB patients [14]. Jang et al. stated that in mice with cyclophosphamide-induced OAB, intravesical treatment by cyclooxygenase-2 (Cox-2) inhibitor suppressed nitric oxide synthase (iNOS) and nerve growth factor (NGF) expressions caused by cyclophosphamide [15]. CD-31 is an angiogenesis marker and may relate to inflammation in urinary symptom-related organs [16].

In this study, we evaluated the relationship between clinical symptom data from OAB patients and the expressions of potentially significant biological markers and receptors, that is, IL-6, Caspase-3 (apoptosis), CD-31, Cox-2, E-cadherin and M-2 receptor, α1-d and β-3 ARs. That is using bladder biopsy samples to determine significant markers or receptors for OAB-related symptoms by quantitative evaluation.

Materials and methods

Patients

We retrospectively examined 18 patients who were diagnosed with OAB and performed bladder biopsy by suspected carcinoma in situ in bladder (but no cancer detection in studied samples) in the Department of Urology at Shinko Hospital and Department of Urology at Hyogo Prefectural Tsukaguchi Hospital (Current name: Hyogo Prefectural Amagasaki General Medical Center). OAB diagnosis was

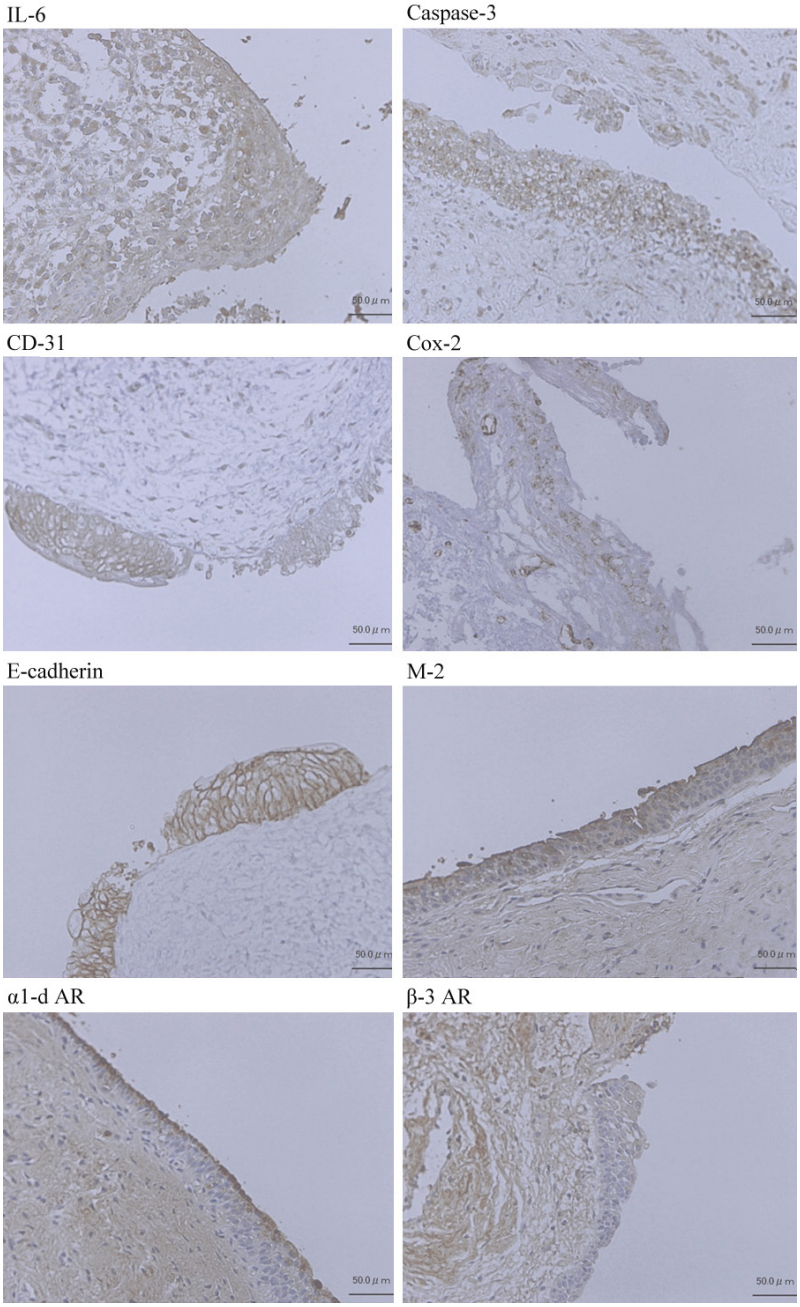

Figure 1. Immunohistochemical stainings showed positive findings in IL-6, Caspase-3, CD-31, Cox-2, E-cadherin and M-2 receptors, α1-d, and β-3 adrenergic receptors with × 100 magnifications.

performed according to OAB criteria [17]. We included the data from 7 control patients without OAB who had urgency score of less than 2 in OABSS. All these patients had no clinically diagnosed BPH and any sign of bladder outlet obstruction, and no infection including control patients. This study was approved by the Institutional Review Board (IRB) members of Shinko Hospital and Hyogo Prefectural Tsukaguchi Hospital. The bladder tissue samples were taken from the ones in random biopsy of bladder cancer suspected patients without any cancer findings.

Clinical factors of OAB patients

The 18 OAB patients and 7 control patients were evaluated for OAB-related symptoms using Overactive Bladder Syndrome Score (OABSS) criteria [18] and International Prostate Symptom Score (IPSS) criteria [19] such as residual sensation, daytime frequency, interruption of urine stream, urgency, voiding disturbance, forced urination or night time frequency, and QOL scores.

Hematoxylin and eosin staining in OAB group or control group

Formaldehyde-fixed, paraffin-embedded tissue sections (cut at 3-5 μm) from bladder biopsy specimens were mounted on glass slides, deparaffinized and stained with hematoxylin and eosin.

Immunohistochemical (IHC) staining of potentially significant markers or receptors for OAB-related symptoms

To detect the significant biological markers or receptors for OAB-related symptoms, we investigated the protein expressions of IL-6, Caspase-3, CD-31, Cox-2, E-cadherin and M-2 receptor, α1-d and β-3 ARs using bladder mucosal biopsy samples referred to the previous work [20]. Briefly, formaldehyde-fixed, paraffin-embedded tissue sections (cut at 3-5 μm) from bladder biopsy specimens were deparaffinized by xylene and rehydrated in decreasing concentra-

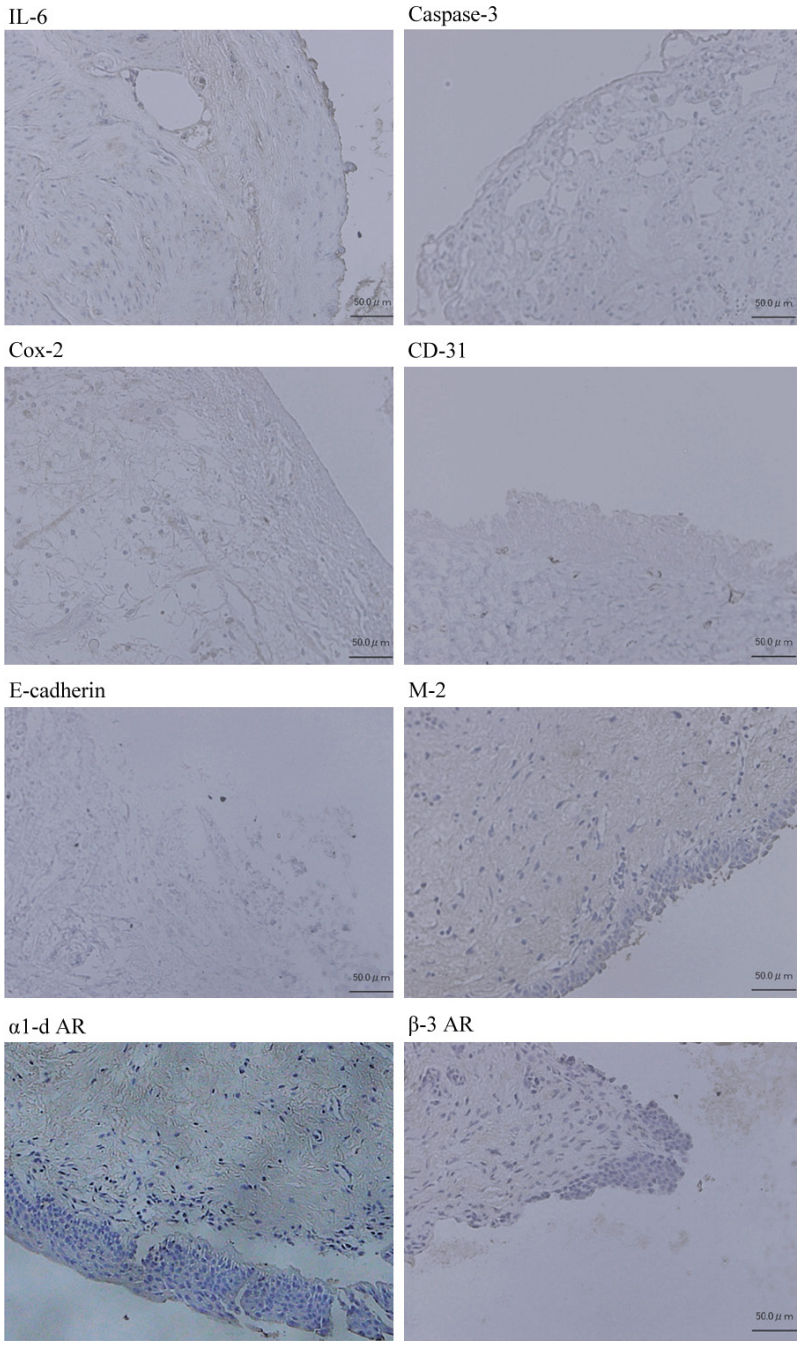

Figure 2. Immunohistochemical staining showed negative findings in IL-6, Caspase-3, Cox-2, CD-31, and E-cadherin and M-2 receptors, α1-d and β-3 adrenergic receptors with × 100 magnifications.

tions of ethanol. The sections were then incubated with anti-human IL-6 rabbit polyclonal antibody (Rockland Immunochemicals, Gilbertsville, PA, USA), anti-human Caspase-3 mouse monoclonal antibody (Novocastra Laboratories Ltd, Newcastle, UK), anti-human CD-31 mouse monoclonal anti-body (Novocastra Laboratories Ltd), anti-human Cox-2 rabbit IgG (IBL Ltd., Fujioka, Gunma, Japan), and E-cadherin mouse monoclonal antibody (Novocastra), and rabbit Anti-Muscarinic Acetylcholine Receptor (M-2) antibody (Sigma-Aldrich, St. Louis, MO, USA) and rabbit Anti-ADRA1D, C-Terminal antibody (Sigma-Aldrich) and rabbit Anti-β-3 AR (Sigma-Aldrich). After incubation in avidin-biotin peroxidase complex for 10 min, the samples were exposed to diaminobenzidine tetrahydrochloride solution and counterstained with methyl green (Vector Laboratories, Burlingame, CA, USA) or hematoxylin. In this study, we used the cases which have been stored as appropriate paraffin blocks to the pathological diagnosis in the past cases. For each protein, we have confirmed the specificity and reactivity of antibodies with known positive control samples.

Evaluation of IHC staining

IHC staining results were scored by observers who were blinded to the clinical data of the OAB patients. As quantitative evaluation for all the biological markers or receptors tested, positive cores of IHC staining were analyzed with Image J, which was produced by National Institute of Mental Health, Bethesda, Maryland, USA. Initial settings of the software were applied to measure area (mm^2). The procedure was referred to the mothod previously described. The software calculated the value of the area in mm^2, ranging from 0 (black) to 255 (total white) from the image [21].

Table 2. Correlation of significant biomarkers for symptoms in OAB patients

	OAB patients IHC score	OABSS Total scores	IPSS storage	IPSS voiding	IPSS post-voiding	IPSS Total scores	QOL
IL-6	105.50 ± 4.95	n.s.*	n.s.	n.s.	n.s.	n.s.	n.s.
Caspase-3	109.35 ± 2.88	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
CD-31	112.64 ± 6.30	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Cox-2	108.48 ± 5.98	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
E-cadherin	80.24 ± 20.51	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
M-2	109.99 ± 4.65	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
α1-d AR**	84.54 ± 13.58	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
β-3 AR	99.05 ± 5.66	**0.0457****	n.s.	n.s.	**0.0308**	n.s.	n.s.

*n.s.: not significant; **AR: adrenergic receptor; ***bold: statistically significant IL-6.

Statistical analyses

Statistical analysis was conducted with the JSTAT, which is a statistics package tool and was produced by Sun Microsystems, Inc., Santa Clara, CA, USA. We used the Spearman rank-order correlation coefficient test to examine the correlation between OABSS and IPSS/QOL scores and the IHC scores in OAB and control patients and the Mann-Whitney U test to compare clinical data of symptom scores of OAB patients and control patients. For these correlations, we adopted the classification under storage symptoms (frequency, urgency and frequency at night), voiding symptom (intermittency, weak stream and straining), and post-voiding (incomplete emptying). We showed mean ± SD in the statistical data. Statistical significance was established at $P<0.05$.

Results

Patients' backgrounds and symptom scores

Those data were shown in **Table 1**. There were 12 males and 6 females with age of 66.3 ± 10.0 (range: 44-82). On the contrary, 7 control patients included six males and one female with age of 56.4 ± 7.23 (range: 48-71). In OAB patients, OABSS total scores were significantly different from the control patients ($P<0.0001$). Likewise, the IPSS storage, total scores and QOL score were also significantly different compared to the control patients ($P=0.0048$, $P=0.0043$, and $P=0.0022$, respectively) (**Table 1**).

Hematoxylin and eosin staining findings in OAB group or control group

The bladder inflammation and abnormality of bladder tissue taken were not shown in both OAB group and control group (data not shown).

IHC staining results in OAB

We tested 8 potential significant biological markers or receptors, IL-6, Caspase-3, CD-31, Cox-2, E-cadherin, and M-2 receptor, and α1-d and β-3 ARs in the 18 OAB cases and 7 control cases (**Figures 1** and **2**). These IHC findings included negatively stained examples. For 18 OAB patients, IHC scores were 105.50 ± 4.95, 109.35 ± 2.88, 112.64 ± 6.30, 108.48 ± 5.98, 80.24 ± 20.51, 109.99 ± 4.65, 84.54 ± 13.58 and 99.05 ± 5.66 in IL-6, Caspase-3, CD-31, Cox-2, E-cadherin, M-2 receptor, α1-d and β-3 ARs, respectively (**Table 2**). For 7 control cases, IHC scores were 128.24 ± 4.08, 153.60 ± 11.56, 145.99 ± 4.33, 156.81 ± 4.95, 142.71 ± 3.19, 150.89 ± 2.51, 159.73 ± 3.03 or 158.88 ± 2.22 in IL-6, Caspase-3, CD-31, Cox-2, E-cadherin, M-2 receptor, α1-d or β-3 ARs respectively (data not shown).

Correlation of OABSS and IPSS/QOL with IHC expressions (IL-6, Caspase-3, CD-31, Cox-2, E-cadherin, and M-2 receptor, α1-d and β-3 ARs)

In OAB patients, OABSS total significantly correlated with β-3 AR expressions ($P=0.0457$). IPSS post-voiding significantly correlated with β-3 AR expressions ($P=0.0308$), suggesting that, taken together, β-3 AR may be a significant makers for OAB related symptoms (**Table 2**). On the other hand, in control patients, there was no significant correlation between OABSS and IPSS/QOL data and IHC data before mentioned in the control patients (data not shown).

Discussion

OAB is a well-established concept diagnosed by the symptoms [1]. As to the treatments, espe-

cially in males, it is often treated by anti-BPH drugs such as-α AR blockers alone or in combination with anti-muscarinic receptor blockers since male LUTS can be caused by BPH-related obstructive symptoms. This study investigated the representative markers or receptors expressed in the bladder mucosa which related to OAB symptoms.

We investigated two adrenergic receptors (ARs) and one muscarinic receptor for this research in OAB patients since the α1-a and α1-d AR subtypes are related to urination, the former mainly located in the prostatic urethra and the latter mainlyin bladder mucosa [8]. Therefore, α1-d AR may affect OAB symptoms such as urgency. β-3 AR also exists in bladder mucosa and induces inhibition of spontaneous contractile activity [8], even though α1-d ARs in particular are found in the bladder neck [8]. The finding in our suggested significant receptors, the expression of β-3 AR significantly correlated with OABSS total scores, even though the study regarding OAB pathophysiology is lacked, was supported by the previous data of more expressions in bladder relaxation as mentioned above [8, 22].

Regarding the research of urinary tract and such markers or receptors, there are several issues and those with β-ARs have been increasing. Limberg et al. suggested β-3 ARs were located in urothelium rather than detrusor [23]. Regarding the subtypes of β-ARs, Yamaguchi et al. showed in their RT-PCR using human bladder tissue, predominant expression of β3-AR with 97% of total β-AR mRNA being represented by the β3-AR subtype but only 1.5% and 1.4% by the β1-AR and β2-AR subtypes, respectively [24].

In other urinary systems, Michel et al. stated that α1-ARs are only poorly expressed and play a limited functional role in the detrusor. α1-a subtype shows a more pronounced expression and promotes contraction of the bladder neck, urethra and prostate as mentioned above. Beta-ARs mediate relaxation of smooth muscle in the bladder, urethra and prostate, and it appears that the β-3- and β-2-subtypes are important in the human bladder and urethra, respectively [25]. These studies may support our data and imply the further importance to take samples from urethra. On the other hand, muscarinic receptors and ARs in bladder neck and membrane are involved in increasing the maximum urethral closing pressure, as in bladder outlet obstruction [8], and stimulate detrusor overactivity (DO) in the diseased bladder, respectively [9].

A detailed evaluation of OAB symptoms is needed for OAB patients, who may suffer from large variety of symptoms including storage symptoms of urgency and/or voiding symptoms like dysuria or straining. In the individual OABSS and IPSS/QOL symptom categories in OAB patients, we showed that β-3 AR was significantly related to OABSS total; IPSS post-voiding but M-2 receptor or α1-d AR was not significantly correlated to OABSS or IPSS/QOL scores. Yamaguchi et al. showed that α1-d AR related to frequency and β-3 AR related to urgency [8]. Ruggieri et al. found that M-2 receptor related to frequency and urgency [26]. Their differences from and similarities to ours may indicate the importance of the establishments of personal order-made therapies. Currently there are several choices for OAB treatments, targeting ARs or muscarinic receptors. However, each patient has his own trend of distribution of these kinds of receptors in the bladder mucosa [8]. Ideally, the first choice for OAB treatments should be individualized accordingly, but biopsy of the bladder mucosa to investigate dominant receptor expression is not a practical alternative. In this situation, our study for which marker or receptor leads to which symptom may be informative for selection of therapeutic drugs based on the patients' particular symptoms.

We would like to emphasize the limitations of this study. First, the number of not only OAB but control cases was not enough to draw definite conclusions. However, as you know, the bladder samples are not easy to take so that even though considering the discussion that these patients may seem far from the common population with OAB, this work has some meanings in OAB pathophysiology. Second, there may be other potentially significant factors for OAB-related symptoms, such as NGFs in addition to our tested markers and receptors. Third, we did not involve molecular expressions in other bladder areas such as the detrusor or urethra. Forth, related to the limitation mentioned above (First limitation), the two populations of OAB patients and control patients were not age-matched. These limitations should be addressed by further studies in the near future.

In conclusion, this study demonstrated that β-3 AR significantly correlated with OAB-related symptoms. These data may help elucidate the pathophysiology of OAB and offer possible strategy for its order-made therapies.

Disclosure of conflict of interest

None.

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