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Original Article

The role of adipocytokines and their receptors in bladder cancer: expression of adiponectin or leptin is an independent prognosticator

Eiji Kashiwagi[1,2], Tatsuro Abe[1], Fumio Kinoshita[1], Miho Ushijima[1], Hiroyuki Masaoka[1], Masaki Shiota[1], George J Netto[2,3], Masatoshi Eto[1], Hiroshi Miyamoto[2,4]

[1]Department of Urology, Kyushu University Graduate School of Medical Sciences, Fukuoka 8128582, Japan; [2]Department of Pathology and James Buchanan Brady Urological Institute, Johns Hopkins University School of Medicine, Baltimore, MD 21287, USA; [3]Department of Pathology, University of Alabama at Birmingham, Birmingham, AL 35233, USA; [4]Departments of Pathology & Laboratory Medicine and Urology, and James P. Wilmot Cancer Institute, University of Rochester Medical Center, Rochester, NY 14642, USA

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Abstract: Adipocytokines such as leptin and adiponectin have functions in metabolism as well as the development and progression of various types of malignancies. However, little is known about their role in bladder cancer. In this study, we investigated whether leptin, adiponectin, and their receptors have an impact on bladder cancer outgrowth and the mechanisms involved. We performed immunohistochemistry for leptin, leptin receptor (Ob-R), adiponectin, and adiponectin receptors (AdipoR1, AdipoR2) in bladder cancer tissue microarrays. Wound healing assay and western blot were then performed in human bladder cancer lines. The positive rates (0 vs 1+/2+/3+) of Ob-R (P=0.004), adiponectin (P<0.001), AdipoR1 (P=0.016), and AdipoR2 (P<0.001) expression were significantly higher in bladder tumors than in benign urothelial tissues. Strong (3+) leptin expression tended to be present more often in tumors (10.2%; P=0.079) than in benign tissues (3.2%). Multivariate analysis revealed a lower risk of recurrence (hazard ratio [HR]=0.432; 95% confidence interval [CI]=0.198-0.942; P=0.034) in patients with an adiponectin-positive non-muscle-invasive tumor and a higher risk of progression (HR=5.148, 95% CI=1.190-22.273; P=0.028) in patients with a leptin-positive muscle-invasive tumor. Treatment of two bladder cancer cell lines with a synthetic adiponectin inhibited their migration and the expressions of phospho-NF-κB, NF-κB, snail, slug, Y-box-binding protein 1, and COX-2, whereas leptin showed reverse effects. Downregulation of adiponectin expression and upregulation of leptin expression were independent predictors for the recurrence of non-muscle-invasive bladder tumors and progression of muscle-invasive bladder tumors, respectively. In summary, synthetic adiponectin might exhibit antitumor activity against bladder cancer.

Keywords: Bladder cancer, adiponectin, leptin, immunohistochemistry, epithelial-to-mesenchymal transition

Introduction

Bladder cancer (BC) is the ninth most common malignancy worldwide [1]. There are two distinct types of bladder tumor, muscle-invasive (MI) and non-muscle-invasive (NMI). MI tumor represents an aggressive phenotype associated with a poor prognosis even after radical cystectomy and systemic treatment including chemotherapy [2, 3]. NMI tumors are associated with a high risk of recurrence after transurethral surgery. Moreover, long-term follow-up in patients with NMI tumor is required, and the cost of this remains an economic problem [4]. Therefore, the prevention of tumor development and/or recurrence is critical for disease control and cost reduction.

Obesity was reported to be a risk factor for BC development [5] and a poor prognostic factor [6]. Metabolic syndrome was suggested to be associated with an increased incidence of BC [7]. Indeed, two population-based studies in China [8] and the US [9] reported shorter recurrence-free survival rates in obese patients with NMI tumors.

Adipocytokines such as adiponectin and leptin belong to a family of hormones derived from adipose tissue, and are important in metabolism as well as the development and progression of various types of malignancies [10-14]. These obesity-related malignancies include colorectal, breast, endometrial, and renal cancers [15, 16]. Adiponectin interacts with two receptors, AdipoR1 and AdipoR2, to inhibit cell growth and carcinogenesis [10]. In contrast, leptin promotes cancer development by interacting with the leptin receptor (Ob-R) [11]. Epithelial-to-mesenchymal transition (EMT) is a key mediator involved in tumor invasion and metastasis, and its aberrant activation has been implicated in accelerating the progression of various types of cancer [17]. Adiponectin suppressed EMT in prostate cancer [18], but the functional role of adipocytokines in urothelial cancer remains poorly understood. In this study, we investigated whether leptin, adiponectin, and their receptors have an impact on the development and progression of BC and the mechanisms involved.

Materials and methods

Antibodies and chemicals

Anti-COX-2 (160112), anti-leptin (sc-842)/NF-κB (sc-109)/Ob-R (sc-1834)/AdipoR1 (sc-46748)/AdipoR2 (sc-46751), anti-snail (#3879)/slug (#9585)/phospho-NF-κB (#3033), anti-YB-1 (2397-1), and anti-adiponectin (ab22554) antibodies were purchased from Cayman Chemical (Ann Arbor, MI, USA), Santa Cruz Biotechnology (Dallas, TX, USA), Cell Signaling (Danvers, MA, USA), Epitomics Inc. (Burlingame, CA, USA), and Abcam (Cambridge, MA, USA), respectively. AdipoRon (ENZ-CHM101) and leptin (L4146) were purchased from Enzo Life Science (Lausen, Switzerland) and Sigma-Aldrich (St. Louis, MO, USA), respectively.

Cell lines

A human urothelial carcinoma cell line (UMUC3) was obtained from the American Type Culture Collection (Manassas, VA, USA). Another human urothelial carcinoma cell line (647V) was used in our previous studies [19-21]. Both lines, authenticated using the GenePrint 10 System (Promega, Madison, WI, USA) in our institutional core facility, were maintained in Dulbecco's modified Eagle's medium (Mediatech, Herndon, VA, USA), supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere of 5% CO_2.

Immunohistochemistry

A bladder tissue microarray (TMA), comprising 117 cases of urothelial neoplasm and normal-appearing urothelial tissue, was constructed from formalin-fixed paraffin-embedded specimens obtained by transurethral resection performed at the Johns Hopkins Hospital, as described previously [21-23]. Appropriate approval from the institutional review board was obtained before the construction and use of the TMA. These patients included 90 men and 27 women with a mean/median age of 65.7/68 years (range: 30-89). The primary tumors included 7 papillary urothelial neoplasms of low malignant potential (PUNLMPs), 32 non-invasive (pTa) low-grade urothelial carcinomas, 27 NMI (pTa/pT1) high-grade urothelial carcinomas, and 51 MI (≥pT2) high-grade urothelial carcinomas. All 51 patients with MI tumor ultimately underwent radical cystectomy. Cases with radiation or anti-cancer drugs prior to the collection of the tissues were not included in the TMA.

Immunohistochemistry was performed on 5-μm-thick sections from the bladder TMA, using a primary antibody against leptin (dilution 1:100), adiponectin (dilution 1:100), Ob-R (dilution 1:100), AdipoR1 (dilution 1:100), and AdipoR2 (dilution 1:100), and a broad spectrum secondary antibody (Invitrogen), as described previously [24]. All staining of the nucleus and cytoplasm of urothelial cells was manually quantified by a single pathologist (H.M.) blinded to sample identity. German immunoreactive scores were calculated by multiplying the percentage of immunoreactive cells (0%=0; 1%-10%=1; 11%-50%=2; 51%-80%=3; 81%-100%=4) by staining intensity (negative =0; weak =1; moderate =2; strong =3). The scores indicated negative (0; 0-1), weakly positive (1+; 2-4), moderately positive (2+; 6-8), and strongly positive (3+; 9-12) staining.

Western blot

Equal amounts of protein (30 μg) obtained from cell extracts were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride mem-

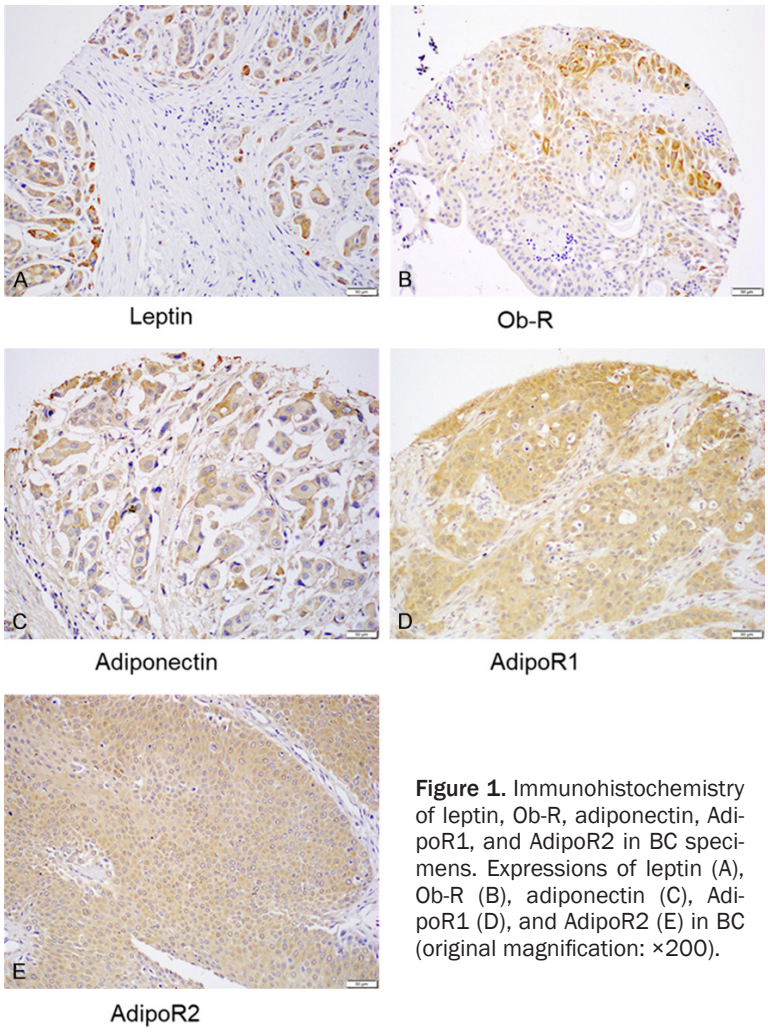

Figure 1. Immunohistochemistry of leptin, Ob-R, adiponectin, AdipoR1, and AdipoR2 in BC specimens. Expressions of leptin (A), Ob-R (B), adiponectin (C), AdipoR1 (D), and AdipoR2 (E) in BC (original magnification: ×200).

brane. Western blot analysis was carried out using each appropriately diluted antibody, and the membrane was developed using a chemiluminescence protocol. Images were obtained using an image analyzer (LAS-3000 mini; Fujifilm, Tokyo, Japan).

Cell migration

A scratch wound healing assay was performed to evaluate the ability of cell migration. Cells at a density of 90%-100% confluence in 12-well plates were scratched manually with a sterile 200 μL plastic pipette tip. After culturing for 12 hours in FBS-free medium, cells were fixed with methanol and stained with 0.1% crystal violet. The width of the wound area was quantitated using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

All statistical analyses were performed using JMP14.2 software (SAS Institute, Cary, NC, USA). Univariate and multivariate analyses were performed using the Cox proportional hazards regression model. Correlations between parameters were examined using χ^2 test or Student's *t*-test. *P* values <0.05 were considered significant.

Results

Expression of adipocytokines and their receptors in BC specimens

We investigated the expression levels of adipocytokines and their receptors, including leptin, Ob-R, adiponectin, AdipoR1, and AdipoR2, by immunohistochemical staining in the bladder TMA, which comprised 117 urothelial tumors and corresponding non-neoplastic bladder tissues. Positive signals were detected predominantly in the cytoplasm of urothelial cells (**Figure 1**).

Table 1 summarizes the expression status of each protein in non-neoplastic urothelium versus urothelial cancer tissues. The positive rates (0 vs 1+/2+/3+) of Ob-R (P=0.004), adiponectin (P<0.001), AdipoR1 (P=0.016), and AdipoR2 (P<0.001) expression were significantly higher in cancers than in benign urothelial tissues. However, leptin positivity was not significantly different (P=0.923), whereas tumors (10.2%; P=0.079) tended to show strong (3+) expression compared with benign tissues (3.2%).

Next, we analyzed the correlations of each expression level with the histopathological profile of the tumors (**Table 2**). The expression level of each protein was not significantly different between tumor grade (PUNLMP + low-grade carcinoma versus high-grade carcinoma) or pathologic stage (NMI versus MI) of the tumors.

Table 1. Expression of leptin, Ob-R, adiponectin, AdipoR1 and AdipoR2 in bladder tissue microarrays.

	n	Expression levels				*P* value		
		Negative	Positive			0 *vs* 1+/2+/3+	0/1+ *vs* 2+/3+	0/1+/2+ *vs* 3+
		0	1+	2+	3+			
Leptin								
Non-neoplastic urothelium	61	7 (11.4%)	31 (50.8%)	21 (34.4%)	2 (3.2%)	0.923	0.921	0.079
Urothelial neoplasm	117	14 (11.9%)	58 (49.5%)	33 (28.2%)	12 (10.2%)			
Ob-R								
Non-neoplastic urothelium	61	43 (70.4%)	17 (27.8%)	0 (0%)	1 (1.6%)	0.004	0.150	0.142
Urothelial neoplasm	117	57 (48.7%)	53 (45.2%)	7 (6.0%)	0 (0%)			
Adiponectin								
Non-neoplastic urothelium	61	32 (52.4%)	21 (34.4%)	8 (13.1%)	0 (0%)	<0.001	0.045	0.358
Urothelial neoplasm	117	24 (20.5%)	63 (53.8%)	29 (24.8%)	1 (0.8%)			
AdipoR1								
Non-neoplastic urothelium	61	14 (22.9%)	30 (49.1%)	17 (27.9%)	0 (0%)	0.016	0.006	0.038
Urothelial neoplasm	117	11 (9.4%)	49 (41.8%)	52 (44.4%)	5 (4.2%)			
AdipoR2								
Non-neoplastic urothelium	61	14 (22.9%)	23 (37.7%)	17 (27.9%)	7 (11.4%)	<0.001	0.276	0.409
Urothelial neoplasm	117	2 (1.7%)	59 (50.4%)	47 (40.2%)	9 (7.6%)			

Moreover, each protein expression was not strongly associated with the status of lymph node metastasis or concomitant carcinoma *in situ* in MI tumors (**Table 3**).

Prognostic significance of the expressions of adipocytokines and their receptors in BC

To determine the prognostic impact of the expressions of adipocytokines and their receptors in patients with NMI or MI tumors, univariate and multivariate analyses were performed (**Tables 4** and **5**). Multivariate analysis using the Cox regression model identified adiponectin expression as an independent predictor for the recurrence of NMI tumors (hazard ratio [HR]=0.432, 95% confidence interval [CI]= 0.198-0.942, P=0.034), but not for progression (**Table 4**). However, leptin/Ob-R/AdipoR1/AdipoR2 expression was not significantly associated with patient outcomes.

In patients with MI tumors, leptin expression was associated with significantly worse progression-free survival (HR=5.148, 95% CI= 1.190-22.273, P=0.028) or marginally worse cancer-specific survival (HR=4.178, 95% CI= 0.955-18.276, P=0.057) (**Table 5**).

Suppression of urothelial cancer cell migration by adiponectin

Immunohistochemical data from the surgical specimens described above showed that adiponectin or leptin expression was associated with a lower risk of NMI tumor recurrence and a higher risk of MI tumor progression, respectively. Therefore, we anticipated that adiponectin possessed an antitumor effect, while leptin promoted tumor progression in urothelial cancer.

We confirmed that two urothelial cancer cell lines, UMUC3 and 647V, expressed Ob-R, AdipoR1, and AdipoR1 (**Figure 2A**). We then examined whether AdipoRon, a synthetic adiponectin [25], had antitumor activity in BC cell lines. An MTT assay showed that treatment with 1-10 μM of AdipoRon for up to 72 hours did not significantly change the viability of UMUC3 and 647V cells (data not shown). However, a wound-healing assay revealed that AdipoRon, even at 0.5 μM, significantly suppressed cell migration (**Figure 2B**, **2C**). Next, we assessed the effect of AdipoRon on the expressions of proteins involving EMT in BC cells [17, 26, 27]. The levels of phospho-NF-κB, NF-κB, snail, slug, COX-2, and YB-1 were reduced dose-dependently by AdipoRon (**Figure 2D**).

Conversely, 5 μg/ml of leptin induced considerable cell migration (**Figure 3A**, **3B**) and the expressions of phospho-NF-κB, NF-κB, snail, slug, and COX-2 (**Figure 3C**). Leptin at 0.1-10 μg/ml did not significantly suppress the cell viability of BC lines (data not shown).

Discussion

Recent epidemiological evidence suggests that obesity is a risk factor for BC [5]. Addi-

Table 2. Correlations between leptin/Ob-R/adiponectin/AdipoR1/AdipoR2 expression and tumor grade/stage of BC

	n	Leptin		*P* value	Ob-R		*P* value	Adiponectin		*P* value	AdipoR1		*P* value	AdipoR2		*P* value
		Negative	Positive	0 *vs* 1+/2+/3+	Negative	Positive	0 *vs* 1+/2+/3+	Negative	Positive	0 *vs* 1+/2+/3+	Negative	Positive	0 *vs* 1+/2+/3+	Negative	Positive	0 *vs* 1+/2+/3+
		0	1+/2+/3+		0	1+/2+/3+		0	1+/2+/3+		0	1+/2+/3+		0	1+/2+/3+	
Tumor grade				0.683[a]			0.694[a]			0.337[a]			0.823[a]			0.624[a]
PUNLMP	7	2 (28.5%)	5 (71.4%)		2 (28.5%)	5 (71.4%)		2 (28.5%)	5 (71.4%)		1 (14.2%)	6 (85.7%)		0 (0%)	7 (100%)	
Low-grade	32	2 (6.2%)	30 (93.7%)		16 (50.0%)	16 (50.0%)		8 (25.0%)	24 (75.0%)		3 (9.3%)	29 (90.6%)		1 (3.1%)	31 (96.8%)	
PUNLMP + LG	39	4 (10.2%)	35 (89.7%)		18 (46.1%)	21 (53.8%)		10 (25.6%)	29 (74.3%)		4 (10.2%)	35 (89.7%)		1 (2.5%)	38 (97.4%)	
High-grade	78	10 (12.8%)	68 (87.1%)		39 (50.0%)	39 (50.0%)		14 (17.9%)	64 (82.0%)		7 (8.9%)	71 (91.0%)		1 (1.2%)	77 (98.7%)	
Pathologic stage				0.096[b]			0.120[b]			0.251[b]			0.443[b]			0.854[b]
pTa	63	5 (7.9%)	58 (92.0%)		27 (42.8%)	36 (57.1%)		14 (22.2%)	49 (77.7%)		5 (7.9%)	58 (92.0%)		1 (1.5%)	62 (98.4%)	
pT1	3	0 (0%)	3 (100%)		1 (33.3%)	2 (66.6%)		2 (66.6%)	1 (33.3%)		0 (0%)	3 (100%)		0 (0%)	3 (100%)	
Non-muscle-invasive	66	5 (7.5%)	61 (92.4%)		28 (42.4%)	38 (57.5%)		16 (24.2%)	50 (75.7%)		5 (7.5%)	61 (92.4%)		1 (1.5%)	65 (98.4%)	
pT2	19	3 (15.7%)	16 (84.2%)		11 (57.8%)	8 (42.1%)		3 (15.7%)	16 (84.2%)		2 (10.5%)	17 (89.4%)		1 (5.2%)	18 (94.7%)	
pT3	24	6 (25.0%)	18 (75.0%)		12 (50.0%)	12 (50.0%)		4 (16.6%)	20 (83.3%)		3 (12.5%)	21 (87.5%)		0 (0%)	24 (100%)	
pT4	8	0 (0%)	8 (100%)		6 (75.0%)	2 (25.0%)		1 (12.5%)	7 (87.5%)		1 (12.5%)	7 (87.5%)		0 (0%)	8 (100%)	
Muscle-invasive	51	9 (17.6%)	42 (82.3%)		29 (56.8%)	22 (43.1%)		8 (15.6%)	43 (84.3%)		6 (11.7%)	45 (88.2%)		1 (1.9%)	50 (98.0%)	

Abbreviations: PUNLMP, papillary urothelial neoplasm of low malignant potential; LG, Low-grade. [a]PUNLMP+LG vs High-grade. [b]Non-muscle-invasive vs Muscle-invasive.

Table 3. Correlations between leptin/Ob-R/adiponectin/AdipoR1/AdipoR2 expression and histopathologic profile of MI tumors

	n	Lymph node involvement			Comcomitant CIS		
		pN0	pN+	*P* value	Absent	Present	*P* value
Leptin				0.337			0.930
Negative (0)	9	4 (44.4%)	5 (55.5%)		4 (44.4%)	5 (55.5%)	
Positive (1+/2+/3+)	42	26 (61.9%)	16 (38.1%)		18 (42.8%)	24 (57.1%)	
Ob-R				0.589			0.151
Negative (0)	29	18 (62.0%)	11 (37.9%)		10 (34.4%)	19 (65.5%)	
Positive (1+/2+/3+)	22	12 (54.5%)	10 (45.4%)		12 (54.5%)	10 (45.4%)	
Adiponectin				0.298			0.670
Negative (0)	8	6 (75.0%)	2 (25.0%)		4 (50.0%)	4 (50.0%)	
Positive (1+/2+/3+)	43	24 (55.8%)	19 (44.1%)		18 (41.8%)	25 (58.1%)	
AdipoR1				0.674			0.216
Negative (0)	6	4 (66.6%)	2 (33.3%)		4 (66.6%)	2 (33.3%)	
Positive (1+/2+/3+)	45	26 (57.7%)	19 (42.2%)		18 (40.0%)	27 (60.0%)	
AdipoR2				0.179			0.284
Negative (0)	1	0 (0%)	1 (100.0%)		0 (0%)	1 (100.0%)	
Positive (1+/2+/3+)	50	30 (60.0%)	20 (40.0%)		22 (44.0%)	28 (56.0%)	

tionally, adipocytokines such as adiponectin and leptin are important for cancer progression [10-14]. However, little attention has been paid to the functional role of adipocytokines in BC. Therefore, we investigated whether adipocytokines are involved in BC initiation and/or progression. Our immunohistochemistry of BC specimens demonstrated associations between adiponectin expression and a lower recurrence rate of NMI tumors as well as between leptin expression and a higher risk of progression of MI tumors. We also found that a synthetic adiponectin inhibited BC cell migration whereas leptin promoted BC cell migration.

Obesity induced local chronic inflammation, resulting in changes in local or systemic adipocytokine overexpression [28] and the subsequent modification of tumorigenesis [29]. Although adiponectin is considered a potential anticancer agent, preclinical studies reported that leptin, as a survival factor, induced tumor growth. However, whether there is a relationship between the level of serum leptin and the risk of malignancies is controversial [11].

We showed that leptin expression correlated with a worse prognosis of MI tumors. In gastric cancer, higher levels of leptin expression were associated with higher tumor stage and worse patient outcome [30]. Elevated leptin was suggested to contribute to carcinogenesis in various types of malignancies [11], including BC as shown in an *in vitro* study [31]. Furthermore, in upper urinary tract urothelial carcinoma samples, leptin receptor expression was associated with worse recurrence-free or cancer-specific survival [32]. Additionally, in The Cancer Genome Atlas pan-cancer dataset, the relative leptin expression was elevated in BC [30]. Our results did not show any significant correlations between leptin/Ob-R expression and tumor grade or stage. Although leptin may be a prognostic factor, its role may differ among cancer types. Further investigation of leptin expression in different grades/stages of BC is required to precisely determine the functions of leptin in urothelial cancer.

Our immunohistochemistry data indicated that higher adiponectin expression, as an independent predictor, was associated with a lower risk for the recurrence of NMI tumors. Bacillus Calmette-Guérin (BCG) immunotherapy is standard for NMI tumors [33]. BCG triggers complex local immune responses by inducing the secretion of cytokines, resulting in the suppression of tumor growth [34]. Interestingly, a higher body mass index is associated with a worse prognosis in BC patients undergoing BCG therapy [35]. However, serum levels of adiponectin involved in local immunoreactions [36] were decreased in obese patients [37]. Adiponectin may thus affect the cancer microenvironment

Table 4. Univariate and multivariate analyses of recurrence/progression in patients with NMI tumors.

parameter	Recurrence-free survival						Progression-free survival					
	Univariate analysis			Multivariate analysis			Univariate analysis			Multivariate analysis		
	HR	95% CI	*P* value	HR[b]	95% CI[b]	*P* value	HR	95% CI	*P* value	HR[b]	95% CI[b]	*P* value
Tumor grade												
LMP+LG	1			1			1			1		
HG	2.148	1.004-4.595	0.048	2.175	0.970-4.880	0.059	6.330	1.342-29.847	0.019	4.950	0.966-25.350	0.054
Pathologic T stage												
pTa	1						1					
pT1	1.833	0.432-7.773	0.410				2.516	0.527-12.003	0.247			
Leptin												
0	1						1					
1+/2+/3+	1.088	0.257-4.604	0.906				2.03E+08	0-Infinity	0.999			
Ob-R												
0	1						1					
1+/2+/3+	0.715	0.334-1.531	0.388				1.142	0.294-4.433	0.847			
Adiponectin												
0	1			1			1			1		
1+/2+/3+	0.468	0.217-1.010	0.053	0.432	0.198-0.942	0.034	0.778	0.200-3.021	0.717	1.103	0.264-4.597	0.892
AdipoR1												
0	1						1					
1+/2+/3+	2.602	0.352-19.230	0.348				2.05E+08	0-Infinity	0.999			
AdipoR2												
0	1						1					
1+/2+/3+	0.542	0.073-4.035	0.550				2.51E+07	0-Infinity	0.999			

Abbreviations: HR, hazard ratio; CI, confidence interval; NMI, non-muscle-invasive. [b]Adjusted for age and sex.

Table 5. Univariate and multivariate analyses of progression/cancer-specific mortality in patients with MI tumors.

parameter	Progression-free survival						Cancer-specific survival					
	Univariate analysis			Multivariate analysis			Univariate analysis			Multivariate analysis		
	HR	95% CI	*P* value	HR[b]	95% CI[b]	*P* value	HR	95% CI	*P* value	HR[b]	95% CI[b]	*P* value
Pathologic T stage												
pT2	1			1			1			1		
≥pT3	3.997	1.629-9.806	0.002	4.035	1.617-10.069	0.002	3.639	1.375-9.633	0.009	3.045	1.116-8.309	0.029
LVI												
negative	1						1					
positive	1.496	0.735-3.046	0.266				1.444	0.672-3.100	0.345			
Concomitant CIS												
negative	1						1					
positive	1.276	0.618-2.632	0.508				1.470	0.671-3.208	0.334			
Leptin												
0	1			1			1			1		
1+/2+/3+	4.242	1.002-17.946	0.049	5.148	1.190-22.273	0.028	3.591	0.843-15.286	0.083	4.178	0.955-18.276	0.057
Ob-R												
0	1						1					
1+/2+/3+	1.188	0.586-2.407	0.631				1.030	0.482-2.201	0.938			
Adiponectin												
0	1						1					
1+/2+/3+	1.522	0.531-4.358	0.433				1.305	0.449-3.789	0.624			
AdipoR1												
0	1						1					
1+/2+/3+	0.928	0.323-2.660	0.890				0.978	0.293-3.258	0.971			
AdipoR2												
0	1						1					
1+/2+/3+	5.20E+08	0-Infinity	0.999				1.90E+08	0-Infinity	0.999			

Abbreviations: HR, hazard ratio; CI, confidence interval; LVI, lymphovascular invasion; MI, muscle-invasive. [b]Adjusted for age and sex.

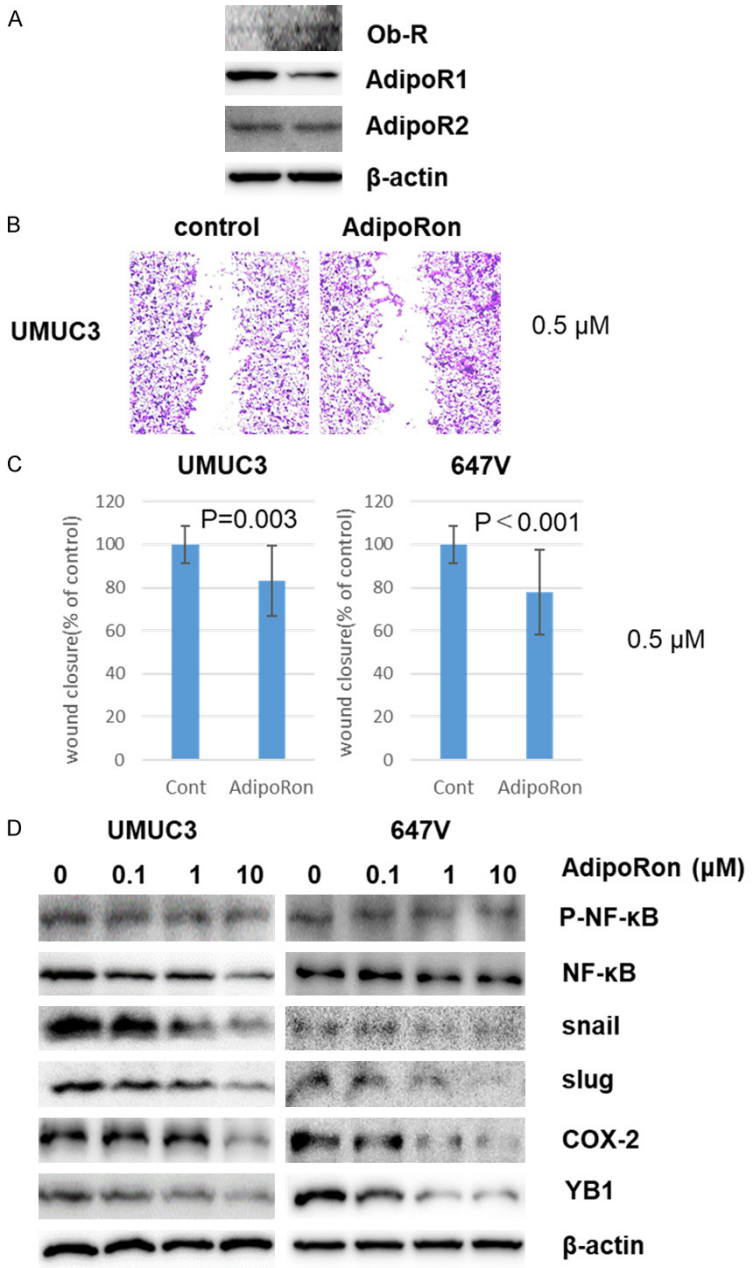

Figure 2. Effects of AdipoRon on BC cell growth. A. Expressions of Ob-R, AdipoR1, and AdipoR2 in UMUC3 and 647V. B. Representative images of wound healing assay in UMUC3. After the cells grew to confluence, confluent monolayers were gently scratched and the wound area was measured after 12-hour culture with ethanol (mock) or AdipoRon (0.5 μM). C. Cell migration determined by the rate of UMUC3/647V cells filling the wound area is presented relative to that of mock treatment in each line (control =100%). Error bars represent standard deviation and each experiment was done in triplicate. D. UMUC3 and 637V were cultured with the indicated concentrations of AdipoRon (μM) for 48 hours. Cell lysates (30 mg) were analyzed for phospho-NF-κB, NF-κB, snail, slug COX-2, and YB-1, using SDS-PAGE and western blotting with specific antibodies. β-actin was used as a loading control.

and contribute to the suppression of tumor recurrence.

AdipoR1 and AdipoR2 expressions in our study did not correlate with the histopathological features of BC, whereas inverse correlations between AdipoR1/AdipoR2 expression and tumor grade or stage were reported in colorectal cancer samples [38]. Additionally, Chou *et al*., reported that AdipoR1 was more likely to be expressed in obesity-related cancers, including BC (n=24), renal cell carcinoma (n=64), hepatocellular carcinoma (n=123), melanoma (n=20), and cholangiocarcinoma (n=20), compared with obesity-unrelated tumors, including ovarian epithelial carcinoma (n=63), cervical carcinoma (n=49), and adrenocortical carcinoma (n=48) [8]. However, in prostate cancer, the expression of AdipoR1 was significantly downregulated and that of AdipoR2 was marginally downregulated compared with non-cancerous tissues [39]. Moreover, serum adiponectin concentrations in men with prostate cancer were lower than in those with benign prostatic hyperplasia [39] and were negatively associated with tumor grade and stage [13]. These observations, in addition to the BC data of the current study, suggest that adiponectin has an anti-tumor effect. However, the functional role of its receptors may differ in cancer types and therefore needs further investigation.

In vitro studies indicated that adiponectin signaling inhibited carcinogenesis and cancer cell growth [10]. miR-222 overexpression, which correlated with a poor prognosis of BC [40], was inversely correlated with AdipoR1 expression in breast cancer cell lines and tissue samples. Knockdown of AdipoR1 in breast cancer cells by a specific siRNA induced their in-

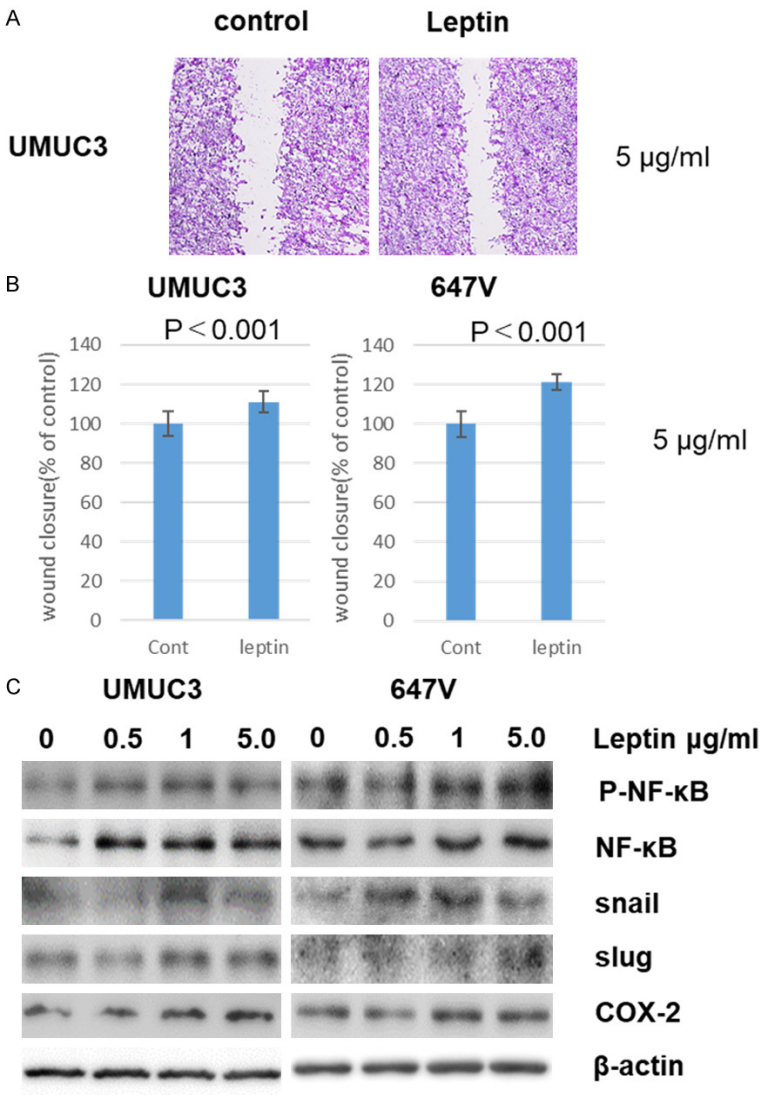

Figure 3. Effects of leptin on BC cell growth. A. Representative images of wound healing assay in UMUC3. After the cells grew to confluence, confluent monolayers were gently scratched and the wound area was measured after a 12-hour culture with ethanol (mock) or leptin (5 μg/ml). B. Migration determined by the rate of UMUC3/647V cells filling the wound area is presented relative to that of mock treatment in each line (control =100). Error bars represent standard deviation and each experiment was done in triplicate. C. UMUC3 and 637V were cultured with the indicated concentrations of leptin (μg/ml) for 48 hours. Cell lysates (30 mg) were analyzed for phospho-NF-κB, NF-κB, snail, slug, and COX-2, using SDS-PAGE and western blotting with specific antibodies. β-actin was used as a loading control.

vasion and EMT, as well NF-κB/vimentin/STAT3 signaling [41]. In our *in vitro* assays, we showed that AdipoRon effectively suppressed phospho-NF-κB, NF-κB and EMT markers, including snail, slug, COX-2, and YB-1, whereas leptin promoted their expressions. Adiponectin was reported to downregulate NF-κB [42], which further activates snail, slug, and COX-2 in BC and non-BC cells [43-46]. In contrast, leptin activated various signals, including STAT3 and NF-κB, which induced cancer development [11], and upregulated snail and slug expression in breast cancer cells [47].

YB-1 is a transcription factor involved in EMT, drug resistance, and cancer progression in BC and other types of cancer cells [27, 48-50]. YB-1 expression was positively correlated with invasiveness and a worse prognosis in bladder cancer samples [51]. Therefore, YB-1 might be a promising therapeutic target, and to the best of our knowledge, this is the first study to report adiponectin suppresses YB-1 expression. However, further research is required to determine the molecular mechanisms involved.

Furthermore, preclinical studies showed that COX-2 overexpression resulted in the promotion of cell invasion and metastasis of BC [52, 53], suggesting that targeting COX-2 might be a therapeutic strategy. These observations are compatible with our data and indicate that AdipoRon may suppress cell migration through the NF-κB pathway.

AdipoRon, a synthetic molecule, [25] is considered a new drug for preventing obesity-related diseases [54]. In our assays, AdipoRon (up to 10 μM) was unable to suppress the cell viability of BC but it might be useful as an adjunctive treatment for BC with intravesical therapy and systemic chemotherapy, because AdipoRon effectively reduced the migration of BC cells and the expression of NF-κB and EMT markers.

In conclusion, this study determined the impact of adipocytokines and their receptors on the development and progression of BC. Our immu-

nohistochemistry data indicated that a loss of adiponectin expression was an independent predictor for the recurrence of NMI tumors. Our findings also suggested that adiponectin activation might be a therapeutic approach for urothelial cancer.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Eiji Kashiwagi, Department of Urology, Kyushu University Graduate School of Medical Sciences, Fukuoka 8128582, Japan. Tel: +81-92-642-5603; Fax: +81-92-642-5618; E-mail: kashiwag@uro.med.kyushu-u.ac.jp

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