Int J Clin Exp Med 2017;10(1):881-888
www.ijcem.com /ISSN:1940-5901/IJCEM0031122

Original Article

IGF2/IGF2R expression in urothelial bladder cancer and its implications for tumor recurrence and prognosis

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Received April 24, 2016; Accepted May 3, 2016; Epub January 15, 2017; Published January 30, 2017

Abstract: Insulin-like growth factor 2 (IGF2) is a secreted growth factor which has growth-regulating, insulin-like and mitogenic activities. Insulin-like growth factor 2 receptor (IGF2R) is a multifunctional receptor functioning by binding with many ligands such as IGF2, growth factor-β and pro-cathepsin D, etc. To evaluate the expression and clinical significance of IGF2 and IGF2R in urothelial bladder cancer, 126 cases were collected in validation cohort for detection of IGF2 and IGF2R by immunohistochemistry. Moreover, the correlation of IGF2/IGF2R with clinicopathological factors was evaluated with Chi-square test. The association with recurrence and overall survival rate was analyzed with univariate and multivariate analysis. Consequently, the association between IGF2 and tumor recurrence or prognosis was not observed in our study. However, IGF2R overexpression was demonstrated to be significantly correlated with tumor recurrence (P = 0.006) and prognosis (P = 0.019). High expression of IGF2R was identified as an independent indicator of lower recurrence (P = 0.008) and more favorable prognosis (P = 0.010). In conclusion, IGF2R high expression was significantly associated with lower recurrence and better prognosis, suggesting that IGF2R could be considered as a suppressor of bladder cancer by reducing the recurrence rate and improving survival time.

Keywords: Bladder cancer, IGF2, IGF2R, prognosis, recurrence

Introduction

Bladder cancer is the fourth common cancer and the second most common urologic cancer type [1, 2]. Bladder cancer comprised of urothelial bladder cancer, squamous carcinoma and adenocarincoma, etc. Urothelial bladder cancer takes up for more than 90% in all kinds of bladder cancer. Urothelial bladder cancer comprises of two clinical phenotypes, non-muscle-invasive bladder cancer (NMIBC) and muscle-invasive bladder cancer (MIBC), which were considered to have distinct molecular pathways, treatment strategies and clinical outcomes. NMIBC takes up approximately 75%-85% in all bladder cancers and have higher overall survival rate compared with MIBC [3]. Bladder cancer is characterized with its high recurrence rate compared with other cancer types. More than 70% patients with NMIBC would suffer recurrence and up to 15% patients would undergo cancer progression and suffer MIBC eventually [4]. One important way to reduce mortality rate of bladder cancer is to reduce its recurrence rate, thus more effective and predictive biomarker for cancer recurrence and prognosis is essential.

Insulin-like growth factor pathways are gradually considered as potential molecular targets for the treatment of cancers along with the expanding understanding of IGF signaling. Insulin-like growth factor 2 (IGF2) is proved to be potent mitogen and can possess growth-promoting activity with experiments in vitro. Ectopic expression of IGF2 or dysfunction of IGF2 signaling is reported in many kinds of cancers [5-7]. Insulin-like growth factor 2 receptor (IGF2R) is a multifunctional receptor involved in many biological processes, including transferring IGF signaling by binding IGF2 and proteolytic activation of transforming growth factor [8, 9]. Intriguingly, IGF2R was normally considered

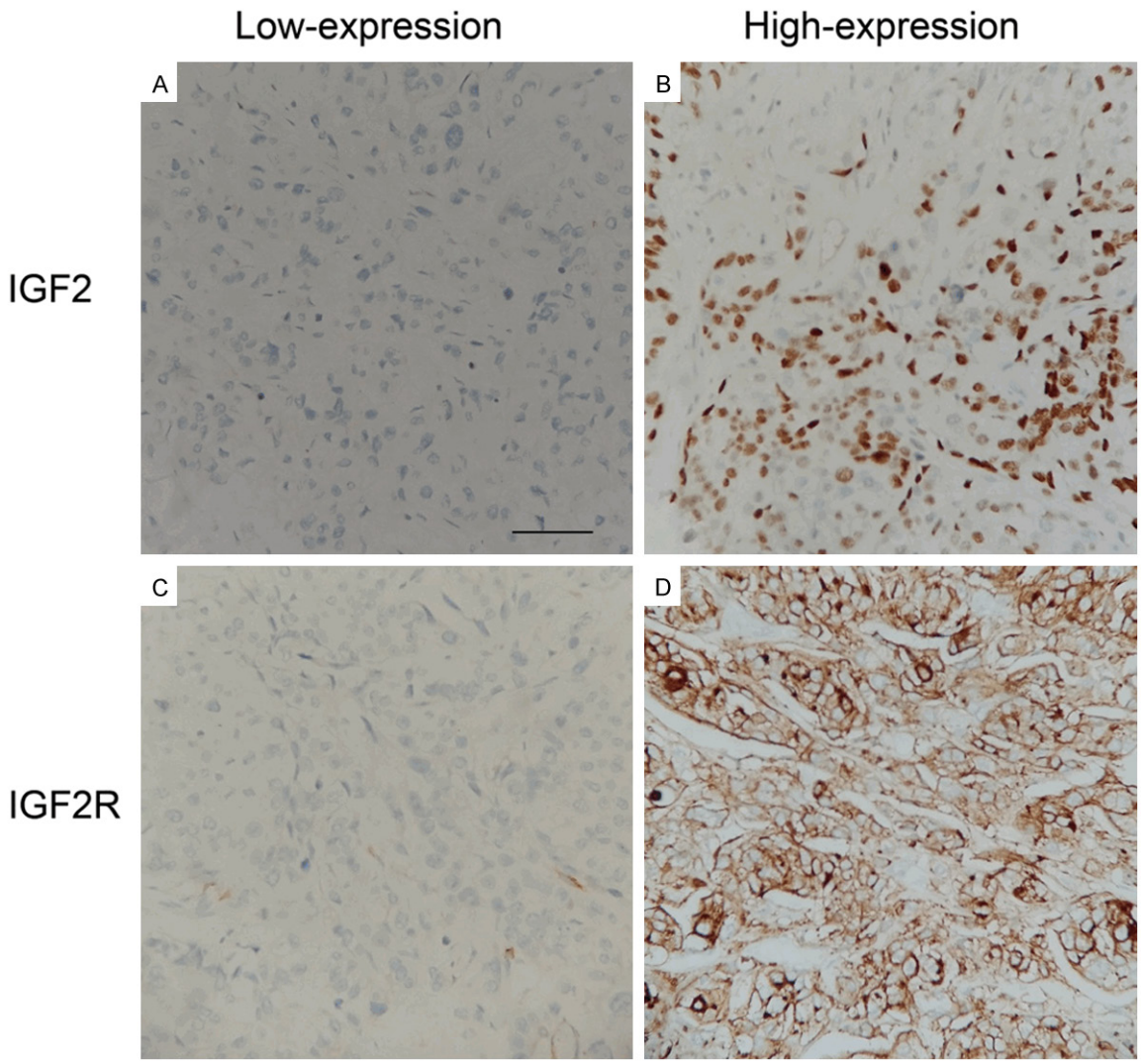

Figure 1. Immunohistochemistry figure of IGF2 and IGF2R. Representative figures of high-expression and low-expression of IGF2 (A and B) and IGF2R (C and D). Scale bar: 50 μm.

as a tumor suppressor in cancer progression, which is different from the IGF2 function.

Previous studies proved that insulin-like growth factor 1 receptor overexpression was a poorer prognostic factor in invasive urothelial cancer [9]. Other independent studies demonstrated that IGF1R could promote motility and invasion of bladder cancer cells with experiments in vitro [10]. Compared to IGF1R, the expression and function of IGF2R in bladder cancer is almost blank, although the underlying value of IGF2R in bladder cancer has been implied in a cDNA microarray study more than ten years ago [11].

In our experiment, we detected the expression of IGF2 and IGF2R in 126 cases of urothelial bladder cancer, consisting of 97 NMIBC and 29 MIBC. With Chi-square test, the correlation between IGF2/IGF2R and clinicopathologic factors of bladder cancer was analyzed. The significance of IGF2/IGF2R in tumor recurrence and prognosis was further evaluated with univariate and multivariate analysis.

Patients and materials

Patients and samples

Our primary cohort consisted of 329 patients who were diagnosed as bladder cancer and underwent surgical resection (transurethral tumor resection or radical total bladder cystectomy) in Yidu Central Hospital of Weifang and

Table 1. Correlations between IGF2/IGF2R and clinicopathologic factors

		IGF2 expression			IGF2R expression		
Factors	Number	Low	High	P*	Low	High	P*
Gender							
Male	99	67	32	0.519	68	31	0.866
Female	27	20	7		19	8	
Age							
< 60	43	30	13	0.900	33	10	0.172
≥ 60	93	57	26		54	29	
Tumor diameter (cm)							
≤ 3	64	41	23	0.218	44	20	0.801
> 3	62	46	16		43	19	
Tumor number							
Single	105	72	33	0.795	74	31	0.445
Multiple	21	15	6		13	8	
pM status							
Negative	116	78	36	0.633	82	33	0.142
Positive	10	9	3		6	6	
Tumor stage#							
Ta-T1	97	67	30	0.991	66	31	0.652
T2-T4	29	20	9		21	8	
Tumour grade#							
Low grade	51	36	15	0.757	33	18	0.386
High grade	75	51	24		54	21	
IGF2							
Low	87	-	-	-	58	29	0.383
High	39	-	-		29	10	
IGF2R							
Low	87	58	29	0.383	-	-	-
High	39	29	10		-	-	

*means calculated by Chi-square test; #means referring to WHO 2004 classification.

Shandong Cancer Hospital and Institute from 2005 to 2012. Total of 126 patients were enrolled into validation cohort according to the criteria: (1) histologic type is urothelial bladder cancer; (2) patients had available follow-ups and paraffin-embedded samples; (3) no history of other tumors and no neoadjuvant chemotherapy. In the validation cohort, 97 patients were confirmed as Ta-T1 in routine pathology, while 29 patients were in T2-T4 and underwent radical total bladder cystectomy. The samples were obtained from the Pathological Department with prior consent of patients and prior approval of the Ethics Committee of the medical centers. Tumor recurrence in our study was defined as new bladder cancer lesion was identified after surgery and the time of recurrence was from the surgery to diagnosis of recurrent tumor.

Immnuohistochemical staining

Immunohistochemistry (IHC) was performed to evaluate the expression of detected proteins in bladder cancer. Briefly, samples were first de-paraffinized and rehydrated first, and then incubated in 0.01 M citric acid (pH 6.0) boiled in microwave for 30 min for antigen retrieval. Endogenous peroxidase activity was blocked with incubation in 0.3% hydrogen peroxide for 10 minutes. Slides were incubated in primary antibody (1:50) of IGF2 (ab9574, Abcam, Cambridge, UK) or IGFR2 (sc-25462, Santa Cruz Biotechnology, CA, USA) at 4°C overnight, and HRP-labeled secondary antibody (Beyotime Institute of Biotechnology, Shanghai, China) at 37°C for 2 hours in sequence. Finally, the proteins were visualized by incubation in Diaminobenzidine, and samples were counterstained with hematoxylin.

Score of IHC

The results of IHC were scored by two senior pathologists who were unaware of the clinical information of the patients, and cases without consensus were re-evaluated by the third pathologist. Staining intensity and positive cell percentage were scored separately and the final IHC score was calculated as the product of staining intensity score multiplied by positive cell percentage score. The staining intensity was defined as weak staining (score 1), medium staining (score 2) or high staining (score 3), and positive stained cell percentage was scored as 25% (score 1), 25%-75% (score 2), 75% (score 3). So the final IHC score ranged from 1 to 9. The cut-off of IHC score was calculated by receiver operating characteristic (ROC) curve. The point with highest sensitivity and specificity was set as the cut-off, dividing cohort into high-expression and low-expression group.

Table 2. Influence of IGF2/IGF2R on recurrence-free survival in NMIBC patients

Characters	Univariate analysis		Multivariate analysis		
	Desease-free rate%	P*	HR	95% CI	P#
Gender					
Male	26.4		-	-	
Female	26.6	0.926	-	-	-
Age					
< 60	40.8		-	-	
≥ 60	20.6	0.313	-	-	-
Tumor diameter (cm)					
≤ 3	28.8		-	-	
> 3	25.5	0.359	-	-	-
Tumor number					
Single	34.9		1		
Multiple	14.6	0.042	1.713	1.01-2.91	0.047
Tumour grade					
Low grade	28.6		-	-	
High grade	13.2	0.080	-	-	-
IGF2					
Low	30.1		-	-	
High	21.0	0.688	-	-	-
IGF2R					
Low	17.0		1		
High	42.5	0.006	0.435	0.24-0.80	0.008

*means calculated by Kaplan-Meier method and log-rank test; #means calculated by Cox-regression model.

Statistical analysis

The software SPSS 17.0 was used to analyze all the data and generated the *P* value. Chi-square test was performed for evaluation of the correlations between IGF2/IGF2R expression and clinicopathological features. Kaplan-Meier method was carried out for recurrence-free survival and overall survival survival curve, and log-rank test was performed to evaluate the statistical difference. In multivariate analysis, Cox-regression proportional hazards model was used to identify the independent factors identified in univariate analysis. P<0.05 was considered as statistically significant.

Results

Expression of IGF2 and IGF2R in bladder cancer

IGF2 and IGF2R expression levels and location were detected by IHC. In all the 126 cases of bladder cancer, IGF2 expression was observed in the cytoplasm, which was also in accordance with its function as a secreted protein (**Figure 1A**). Meanwhile, IGF2R mainly existed in both cell cytoplasm and membrane (**Figure 1B**). As described in Patients and Materials, the validation cohort was divided into high-expression and low-expression group according to the IHC score of detected proteins. In our study, the high expression rate of IGF2 and IGF2R was 30.9% (39/126) and 30.9% (39/126), respectively.

Correlations between IGF2, IGF2R and clinicopathologic factors

With Chi-square test, we further analyzed the associations between IGF and IGF2R expression and clinicopathologic factors including patients' age, gender, tumor grade, tumor number etc. (**Table 1**). In our test, no clinicopathologic factors were proved to be associated with IGF2 or IGF2R expression.

Significance of IGF2, IGF2R in bladder cancer recurrence

Tumor recurrence is the biggest threat to patients with NIMBC, and is also the most characterized feature of bladder cancer. A useful biomarker which can predict tumor recurrence would be very helpful for patients' examination and individual treatment. We analyzed the correlation between IGF2/IGF2R and NIMBC non-tumor survival rate with univariate analysis to evaluate their significance on tumor recurrence (**Table 2**) (**Figure 2A** and **2B**). In our study, tumor number and IGF2R expression were confirmed as high-risk factors in tumor recurrence. High expression of IGF2R was associated with lower recurrence-free survival rate (P = 0.006) and patients with multiple tumors may have high rate of recurrence (P = 0.042). No other clinicopathological factors were observed to be related to recurrence, including IGF2 (P = 0.688). To confirm the independent signifi-

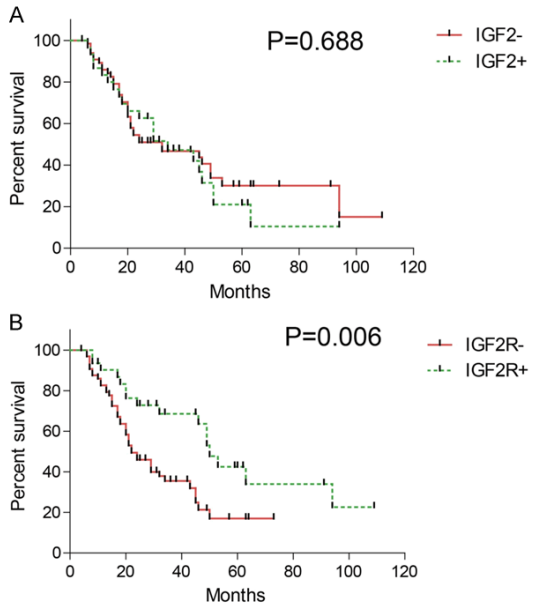

Figure 2. Recurrence-free curve of IGF2 and IGF2R. Recurrence-free curve of IGF2 and IGF2R was plotted by Kaplan-Meier method and analyzed by log-rank test. A. IGF2 is not associated with recurrence of NMIBC (5-year recurrence-free survival rate: low vs. high = 29.0% vs. 19.5%). B. Low expression of IGF2R was significantly related to higher tumor recurrence (5-year recurrence-free survival rate: low vs. high = 17.0% vs. 42.5%).

cance of IGF2R and tumor number in NIMBC recurrence, multivariate analysis was carried out with Cox-regression model. Both IGF2R (P = 0.008, HR = 0.435, 95% CI = 0.24-0.80) and tumor number (P = 0.047, HR = 1.713, 95% CI = 1.01-2.91) were identified as independent predictors of NIMBC recurrence in our experiment.

Prognostic value of IGF2 and IGF2R in bladder cancer

Kaplan-Meier method was first performed to screen out prognostic factor (**Table 3**) (**Figure 3A** and **3B**). IGF2R high expression was demonstrated to be significantly related with more favorable 5-year overall survival rate (P = 0.006, 5-year survival rate: low vs. high = 36.7% vs. 76.2%). Besides IGF2R, tumor number, pM stage, pT stage and tumor grade were also identified as prognostic factors (P = 0.003, P < 0.001, P = 0.001 and P = 0.023, respectively). All the verified prognostic factors were enrolled in Cox-regression model for multivariate analysis to define the independent prognostic parameters. In our cohort, IGF2R expression was identified as an independent prognostic factor and was considered to predict favorable prognosis (P = 0.010, HI = 0.319, 95% CI = 0.13-0.76). Besides IGF2R, only tumor number was also an independent prognostic factor (P = 0.038, HI = 2.25, 95% CI = 1.05-4.84) in bladder cancer.

Discussion

The IGF-axis consists of IGF1 and IGF2, IGF receptors IGF1R and IGF2R, the IGF binding proteinfamily (IGFBP1-6), and the proteins involved in intracellular signaling [12]. It controls a multifunctional signaling network and is involved in biological processes such as maintaining tissue homeostasis, regulating cell proliferation, differentiation and migration [13, 14]. The dysregulation of IGF-axis is observed in many types of cancers. Overexpression of IGF1 or IGF1R was demonstrated to be significantly associated with progression or prognosis in cancer such as hepatocellular carcinoma, lung cancer, pancreatic cancer, breast cancer, etc [15-18]. Unlike IGF1R, IGF2R was generally considered as a tumor suppressor. Depletion or decrease of IGF2R was observed in breast cancer, hepatocellular carcinoma and prostate cancer [19]. Experiments in vitro also support the tumor suppression effect of IGF2R. For example, Nicole et al. demonstrated that IGF2R silencing could enhance tumorigenicity in already transformed breast epithelial cells [20]. However, the study of IGF2/IGF2R function in carcinogenesis and cancer progression is insufficient compared to IGF1/IGF1R, in both clinical and basic medicine. Especially in bladder cancer, the study of expression and clinical significance of IGF2R is almost blank compared with IGF1R.

One feature of IGF2R is its large extracellular domain (2264 amino acids), which confers it the ability to bind multiple ligands, including the mitogenic peptide IGF2, mannose-6-phosphate-bearing proteins such as transforming growth factor-β and pro-cathepsin D and numerous other growth-regulatory molecules, including retinoic acid and the urokinase-type plasminogen activator receptor (uPAR) [20]. The downstream signaling pathway of IGF2R is complicated because of the multiple ligands

Table 3. Prognostic value of clinicopathologic factors

Factors	Univariate analysis		Multivariate analysis		
	5-year survival rate%	P	HR	95% CI	P
Gender					
Male	53.9		-	-	
Female	48.6	0.669	-	-	-
Age					
< 60	57.4		-	-	
≥ 60	41.7	0.804	-	-	-
Tumor diameter (cm)					
≤ 3	54.7		-	-	
> 3	54.4	0.436	-	-	-
Tumor number					
Single	61.4		1		
Multiple	17.2	0.003	2.297	0.94-5.66	0.068
pM status					
Negative	55.8		1		
Positive	0	< 0.001	3.401	0.65-17.9	0.149
pT stage					
Ta-T1	58		1		
T2-T4	23.6	0.001	2.405	0.87-6.67	0.092
Tumour grade					
Low grade	63.6		1		
High grade	43.7	0.023	2.250	1.05-4.84	0.038
IGF2					
Low	55.9		-	-	
High	52.9	0.443	-	-	-
IGF2R					
Low	36.7		1		
High	76.2	0.019	0.319	0.13-0.76	0.010

*means calculated by Kaplan-Meier method and log-rank test; #means calculated by Cox-regression model.

and consequent multiple functions. The tumor-suppress features of IGF2R involve IGF2-dependent and -independent mechanisms. In our study, we did not observe significant correlation between IGF2 and IGF2R, neither the prognostic nor recurrence-predicting value of IGF2. This may be resulted from that IGF2R could suppress bladder cancer recurrence in an IGF2-independent way. As to how IGF2R suppresses bladder cancer recurrence, the underlying mechanism needs further experiment to reveal.

Another feature of IGF2R is the correlation between its polymorphisms and carcinogenesis. More than 1,200 single nucleotide polymorphisms (SNPs) have been identified in IGF2R [21]. Lots of evidence proved that different malignant tumors (breast cancer, ovary cancer, melanoma, lymphoma and renal carcinoma) frequently exhibited loss of heterozygosity at 6q26-27, where IGF2R resides [21, 22]. Previous studies demonstrated that IGF2R polymorphisms contribute to the risks of esophageal-gastric cardia adenocarcinoma, and also to the tumor response to therapy in pancreatic cancer [12, 21]. However, the exact mechanism of how IGF2R polymorphisms lead to ontogenesis is controversy. It was proposed that aberration of CpG methylation due to polymorphism induces expression change. The correlation between IGF2R polymorphisms and bladder cancer progression is still blank. Unfortunately, SNP could not be detected or verified with IHC method. We wish our finding could trigger more interests on IGF2R function in bladder cancer progression and help discover greater breakthroughs.

In conclusion, we detected the expression of IGF2 and IGF2R in 97 patients with NMIBC and 29 patients with MIBC, and subsequently analyzed the correlation between their expression and clinicopathologic factors, recurrence-free survival rate and prognosis for the first time as far as we know. As the result, we demonstrated that IGF2R high expression was significantly associated with lower recurrence and more favorable prognosis. Our results proved that IGF2R is a suppressor of bladder cancer and could reduce the recurrence rate, indicating the potency of IGF2R activator as a targeted drug for bladder cancer therapy.

Acknowledgements

This study was funded by Science and Technology Project of Yantai City (2015WS018),

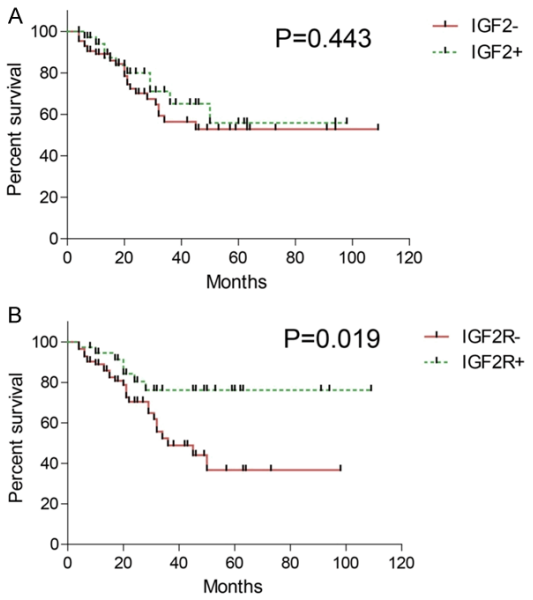

Figure 3. Overall survival curve of IGF2 and IGF2R. 5-year overall survival curve for IGF2 and IGF2R plotted by Kaplan-Meier method. A. IGF2 is not associated with prognosis of bladder cancer (5-year recurrence-free survival rate: low vs. high = 55.9% vs. 52.9%). B. High expression of IGF2R indicates more favorable prognosis of bladder cancer (5-year recurrence-free survival rate: low vs. high = 36.7% vs. 76.2%).

and Youth Research Initiation Fundation of Yuhuangding Hospital (201511).

Disclosure of conflict of interest

None.

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