# Original Article Girdin protein is a novel prognosis predictor for patients with non-muscle invasive bladder cancer

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**Abstract:** This study aimed to determine the expression status of actin-binding protein Girdin in non-muscle invasive bladder cancer (NMIBC) tissues, and to explore the relationships between Girdin expression and the clinicopathological characteristics of bladder carcinoma. The correlations between Girdin expression and the clinicopathological parameters were examined by Chi-square test. Recurrence-free survival (RFS) and progression-free survival (PFS) were analyzed using Kaplan-Meier method. The prognostic significance of Girdin expression was assessed using univariate and multivariate Cox regression analysis models. Results showed that 69 (43.1%) of the 160 NMIBC tissue samples displayed positive expression of Girdin; Girdin positivity was more frequently seen in high-grade tumors than in low-grade tumors (P = 0.019), and in undifferentiated tumors than in differentiated tumors (P = 0.028). In Kaplan-Meier analysis, expression of Girdin was significantly associated with lower RFS (P = 0.007) and PFS (P = 0.024) rates. The univariate analysis revealed that Girdin expression was a risk factor for both recurrence and progression of NMIBC. Further multivariate analysis identified Girdin as an independent predictor of tumor recurrence (hazard ratio [HR]: 2.056, 95% CI: 1.213-3.483, P = 0.007). The present study suggests that Girdin is differentially expressed in bladder tumors and may represent an independent predictor of prognosis for patients with NMIBC.

Keywords: Girdin, NMIBC, recurrence, progression

#### Introduction

Bladder cancer is the most common malignancy of the urinary tract worldwide [1]. Approximately 75% of patients with bladder cancer are diagnosed as non-muscle-invasive bladder cancer (NMIBC) [2], and routinely treated with transurethral resection of bladder tumor (TURBT) with or without intravesical therapy. As NMIBC is characterized by high probability of recurrence and progression, with a 5-year recurrence rate ranging from 15% to 90% and a 5-year progression rate from 7% to 50% [3-5], stratification of patients into distinct prognostic groups will greatly contribute to personalized treatment and follow-up plan. Although previous studies identified the clinicopathological factors including gender, tumor stage, lymph node involvement, lymphovascular invasion, and surgical margin status as

prognostic factors of NMIBC, inconsistencies exist between the studies regarding the performance of these factors on prognosis prediction [6-8]. Hence, novel predictors of prognosis are eagerly anticipated for NMIBC. In addition to the conventional clinicopathological factors, the newly emerging molecular markers have shown their clinic significance in evaluation of prognosis [9-11].

Intracellular signaling pathways are involved in almost all aspects of cellular functions, such as proliferation, differentiation, apoptosis, and etc. Therefore, dysregulation of key components in signaling pathways may lead to initiation and progression of cancers. For instance, the Girdin protein, which serves as a scaffold protein and locates at the crossroad of G protein signaling and tyrosine kinase receptor signaling [12], is abnormally expressed in malignant tumors derived from various types of tissues including the uterine cervix, breast, lung, and thyroid gland. Moreover, it has been shown that expression of Girdin predicts patient survival in colon cancer and that Girdin may be a useful biomarker for tumor stage in colorectal carcinoma [13]. Although our knowledge about the roles of Girdin in cancer is accumulating, the relationships between expression of Girdin and the clinicopathological factors of bladder tumors remain unknown.

In the present study, we aimed to determine the expression status of Girdin protein in tumor tissues from patients with NMIBC, as well as the clinical implications of Girdin protein in NMIBC.

# Materials and methods

# Patients and tissue specimens

This study included 160 patients with NMIBC who underwent surgery from January 2006 to January 2011 at Second Hospital of Tianjin Medical University. All patients were pathologically confirmed to have urothelial bladder carcinoma. 153 out of the 160 tissue specimens were acquired via TURBT, and the other 7 specimens via cystoscopy biopsy. None of the patients had received chemotherapy or radiotherapy before surgery. Two pathologists who were blind to patient information independently reviewed all sections for tumor staging and grading. The tumors were graded in accordance with the World Health Organization (WHO)/ International Society of Urological Pathology (ISUP). Pathological stages were determined according to the Union for International Cancer Control (UICC) TNM classification. Nodal and metastatic profiles were not included in this study due to the incomplete clinical history of patients.

# Follow-up and clinical outcomes

Postoperative follow-up was conducted with rigid cystoscopy every 3 months for the first two years, every 6 months thereafter, and annually after the fifth year according to the US and European guidelines. The end points for patients in this study were tumor recurrence and progression. Outcomes of interest were recurrence-free survival (RFS) and progressionfree survival (PFS). The period for RFS was estimated from the date of surgery to the date of initial recurrence confirmed (any grade, stage category or CIS). The PFS duration was calculated from the date of surgery to the date of disease confirmed with development into higher histological or pathological stage and/or to metastasis. For patients without recurrence and progression, the end point was the date of the last available follow-up cystoscopy. No patients died until the last available follow-up in the study. The present study was approved by the Ethics Committee of Second Hospital of Tianjin Medical University.

# Immunohistochemistry

Polyclonal rabbit anti-human Girdin antibody (T-13: sc-133371; dilution 1:100) was from Santa Cruz Biotechnology (Santa Cruz, Biotechnology, CA, USA). Thin slices of tumor tissue of all cases, which were fixed in 4% formal-dehyde solution (pH 7.0), were from the histopathology unit at the hospital. The fixation for all slices did not exceed 24 h. The tissues were processed routinely for paraffin embedding, and 4  $\mu$ m-thick sections were cut and placed on glass slides coated with 3-aminopropyl triethoxysilane for immunohistochemistry. Tissue samples were stained with hematoxylin and eosin to determine the histological type and grade of tumors.

The number of immunoreactive cells was quantified as 'proportion score', including 0; no immunoreactive cells, 1; the numbers of immunoreactive cells were less than 1%, 2; less than 10%, 3; less than one-thirds, 4; less than twothirds, 5; more than two-thirds. The highest number of immunoreactive tumor cells was graded as 'intensity score', including 0; no immunoreactivity, 1; weak staining intensity, 2; intermediate, 3; strong. The IHC results were grouped based on the IHC score (proportion score plus intensity score): 0-2, negative (-); 3-8, positive (+) [14].

# Statistical analysis

Statistical analysis was performed using SPSS software (IBM Company, version20.0). Relationships between Girdin expression and the clinic parameters were studied using the Chisquare test and Fisher's extract test or independent t test. RFS and PFS rates were analyzed using the Kaplan-Meier method. The logrank test was used to analyze the differences



**Figure 1.** Immunohistochemical staining of Girdin protein in human bladder carcinoma tissue samples. Protein expression of Girdin was mainly evident in the cytoplasm or membrane with yellow or brown-yellow staining. A showed negative (IHC score 1) stain of Girdin in NMIBC sample. B showed positive (IHC score 3) stain of Girdin in NMIBC sample. C showed positive (IHC score 6) stain of Girdin in NMIBC sample. D showed positive (IHC score 8) stain of Girdin in NMIBC sample. A showed positive (IHC score 8) stain of Girdin in NMIBC sample. C showed positive (IHC score 6) stain of Girdin in NMIBC sample. D showed positive (IHC score 8) stain of Girdin in NMIBC sample. C showed positive (IHC score 8) stain of Girdin in NMIBC sample. C showed positive (IHC score 8) stain of Girdin in NMIBC sample. C showed positive (IHC score 8) stain of Girdin in NMIBC sample. C showed positive (IHC score 8) stain of Girdin in NMIBC sample. C showed positive (IHC score 8) stain of Girdin in NMIBC sample. C showed positive (IHC score 8) stain of Girdin in NMIBC sample. C showed positive (IHC score 8) stain of Girdin in NMIBC sample. C showed positive (IHC score 8) stain of Girdin in NMIBC sample. C showed positive (IHC score 8) stain of Girdin in NMIBC sample. C showed positive (IHC score 8) stain of Girdin in NMIBC sample. C showed positive (IHC score 8) stain of Girdin in NMIBC sample. C showed positive (IHC score 8) stain of Girdin in NMIBC sample. C showed positive (IHC score 8) stain of Girdin in NMIBC sample. C showed positive (IHC score 8) stain of Girdin in NMIBC sample. C showed positive (IHC score 8) stain of C score 8) stain 0 score 8) stain

in survival between groups. The prognostic significance of Girdin expression was assessed using univariate and multivariate Cox regression analysis models. *P* value < 0.05 was considered statistically significant.

# Results

# The expression of Girdin in tumors from patients with NMIBC

We detected the Girdin expression in tumors from an independent primary NMIBC cohort

comprising 160 patients by using immunohistochemical staining with polyclonal antibodies against human Girdin protein. As depicted in **Figure 1**, Protein expression of Girdin was mainly evident in the cytoplasm or membrane with yellow or brown-yellow staining. Additionally, the demographic characteristics of the cohort are summarized in **Table 1**. Girdin expression was dichotomized (negative versus positive) based on the staining area and intensity (**Figure 1**), with 69 (43.1%) and 91 (56.9%) tissue samples showing positive and negative expression of Girdin, respectively.

	Girdin ex		
Variables	Negative	Positive	P
Total no. (%)	91 (56.9)	69 (43.1)	
Age, years (no. [%])			0.121
≤ 62	43 (47.3)	42 (60.9)	
> 62	48 (52.7)	27 (39.1)	
Gender (no. [%])			0.753
Male	55 (60.4)	40 (58.0)	
Female	36 (39.6)	29 (42.0)	
Smoking (no. [%])			0.343
No	54 (59.3)	46 (66.7)	
Yes	37 (40.7)	23 (33.3)	
Tumor size (no. [%])			0.078
< 3 cm	69 (75.8)	60 (87.0)	
≥ 3 cm	22 (24.2)	9 (13.0)	
Multifocality (no. [%])			0.084
Single	56 (61.5)	33 (47.8)	
Multiple	35 (38.5)	36 (52.2)	
Histological differentiation (no. [%])			0.028
Differentiated	39 (42.9)	18 (26.1)	
Undifferentiated	52 (57.1)	51 (73.9)	
Intravesical therapy (no. [%])			0.720
No	37 (40.7)	30 (43.5)	
Yes	54 (59.3)	39 (56.5)	
T stage (no. [%])			0.617
Та	6 (6.6)	6 (8.7)	
T1	85 (93.4)	63 (91.3)	
Grade (no. [%])			0.019
Low	43 (47.3)	20 (29.0)	
High	48 (52.7)	49 (71.0)	
Recurrence (no. [%])			0.008
Absent	66 (72.5)	36 (52.2)	
Present	25 (27.5)	33 (47.8)	
Progression to MIC			0.022
Absent	82 (90.1)	53 (76.8)	
Present	9 (9.9)	16 (23.2)	

**Table 1.** Association between girdin expression and clinicopathological characteristics

\*The immunohistochemical score was based on both staining area and intensity. Its expression was dichotomized (negative vs. positive) because such grouping showed the most significant survival difference in the Kaplan-Meier analysis.

# The association of Girdin expression with clinicopathological factors

The relationships between the expression of Girdin and clinicopathological characteristics are summarized in **Table 1**. Girdin expression was significantly associated with histological differentiation (P = 0.028), tumor grade (P =

0.019), recurrence (P = 0.008), and progression to MIBC (P = 0.022), but not related to age, gender, tumor size, multiplicity, smoking history, tumor stage, or intravesical therapy.

# Prognostic significance of Girdin expression in NMIBC

The overall median follow-up duration was 65.0 months (interquartile range (IQR) 29.0-97.8). With respect to patients with Girdin-positive and Girdin-negative tumor, the median follow-up time was 49.0 months (IQR 20.5-90.0) and 74.0 months (IQR 49.0-98.0), respectively. The overall RFS and PFS rates of the cohort were 63.8% and 84.4%, respectively.

The Kaplan-Meier analysis showed that the Girdin-positive patients had significantly lower RFS (P = 0.007) and PFS (P = 0.024) rates than did the Girdin-negative patients (Figure 2A and 2B). The univariate Cox regression analyses showed that Girdin expression was a significant risk factor for tumor recurrence and progression (Tables 2 and 3). Compared with Girdin-negative tumor, Girdin-positive tumor was associated with a 2.024-fold (95% confidence interval, 1.202-3.408) and 2.481fold (95% confidence interval, 1.096-5.616) increased risk for recurrence (P = 0.008) and progression (P =0.029), respectively.

In multivariate analysis with adjustment for Girdin expression and multiple clinicopathological factors, Girdin expression remained to be a significant predictor of tumor recurrence (**Table 2**). Patients with Girdinpositive tumor were associated with a 2.056-fold (95% confidence inter-

val, 1.213-3.483) increased risk for tumor recurrence, compared with those with Girdinnegative tumor (P = 0.007). Although the analysis also implied the correlation of Girdin-positive tumor with a 2.272-fold (95% confidence interval, 0.999-5.171) increased risk for tumor progression, the association was marginally significant (P = 0.050).



**Figure 2.** Kaplan-Meier survival curves for recurrence-free survival (A) and progression-free survival (B) according to Girdin expression. Expression was dichotomized appropriately according to the IHC score which was based on staining area and intensity.

#### Discussion

Girdin is a novel protein identified in 2005, of which the encoding gene locates at chromosome 2p16.1 [12, 15]. Previous studies have demonstrated Girdin interactions with the key components in intracellular signaling pathways that regulate a number of biological processes, such as Akt, Gαi/s, dynamin, and guanosine triphosphate hydrolase enzyme (GTPase) [16, 17]. Accumulating evidence support that Girdin plays an important role in tumor development and progression, which implies its potential as a biomarker for diagnosis and prognosis in clinic oncology. In breast cancer, Girdin has been reported to be a potential predictor for distant metastasis [18]. Wang et al. [19] determined the Girdin expression in 105 gastric cancer tissues and 72 para-cancer tissues. Based on the results, Girdin might be considered as a novel biomarker for metastasis and prognosis in gastric cancer.

To determine the expression of Girdin in tumor tissues from patients with NMIBC, we performed immunohistochemistry analysis. We observed the positive expression of Girdin in 43.1% of tumor tissues in the cohort. In a previous study, Jiang et al. [20] detected high expression of Girdin in breast, colorectal, lung, cervical and thyroid cancer tissues. The positive ratio of Girdin expression varied between 10 and 50% among different types of cancer. Basically, our observation was in accordance with Jiang et al.'s.

To the best of our knowledge, this is the first study that explored the relationships between expression of Girdin and the clinicopathological parameters of NMIBC. Because patients with NMIBC are of high probability suffering from tumor recurrence and progression to muscle invasive cancers [21], we focused on the prognostic implication of Girdin in NMIBC. The uni-

	Univariate analysis		Multivariate analysis		
RISK factor	HR (95% CI)	Р	HR (95% CI)	Р	
Age, years	0.783 (0.467, 1.311)	0.352			
Gender	1.273 (0.759, 2.136)	0.361			
Smoking	1.197 (0.709, 2.020)	0.501			
Tumor size	1.086 (0.575, 2.052)	0.799			
Multifocality	1.884 (1.120, 3.169)	0.017	1.735 (1.028, 2.927)	0.039	
Histological differentiation	1.570 (0.883, 2.793)	0.125			
Intravesical therapy	0.530 (0.303, 0.927)	0.026	0.495 (0.282, 0.870)	0.014	
T stage	0.850 (0.340, 2.126)	0.728			
Grade	0.721 (0.430, 1.209)	0.215			
Girdin expression	2.024 (1.202, 3.408)	0.008	2.056 (1.213, 3.483)	0.007	

 Table 2. Univariate and Multivariate analysis of the factors associated with recurrence

Table 3.	Univariate a	nd Multivariate	analysis of the	factors associated	with progression to M
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Dial factor	Univariate analysis		Multivariate analysis	
RISK TACLOI	HR (95% CI)	Р	HR (95% CI)	Р
Age, years	0.869 (0.384, 1.916)	0.728		
Gender	0.959 (0.431, 2.136)	0.919		
Smoking	1.655 (0.754, 3.633)	0.209		
Tumor size	1.270 (0.507, 3.181)	0.610		
Multifocality	2.304 (1.018, 5.216)	0.045	2.090 (2.918, 4.757)	0.079
Histological differentiation	0.952 (0.421, 2.155)	0.906		
Intravesical therapy	1.179 (0.528, 2.632)	0.688		
T stage	0.548 (0.164, 1.832)	0.328		
Grade	0.946 (0.425, 2.107)	0.892		
Girdin expression	2.481 (1.096, 5.616)	0.029	2.272 (0.999, 5.171)	0.050

variate analysis shows that Girdin expression is a risk factor for both tumor recurrence and progression in the cohort. Further, multivariate analysis indicates that Girdin is an independent predictor of tumor recurrence for patients with NMIBC. However, the prognostic significance of Girdin in the present study should be interpreted with cautions because of the single-institution retrospective study design and the relatively small sample size of the cohort. Future prospective studies in collaboration with multiple centers should be performed to validate our results.

Although the association between Girdin expression and tumor recurrence of NMIBC has been shown in this study, the detailed mechanism by which Girdin acts in NMIBC remains largely unknown. To be noticed, the study by Zhang et al. [22] showed that knockdown of Girdin enhanced chemosensitivity of colorectal cancers cells to oxaliplatin. This finding projects a potential correlation between Girdin protein and the chemoresistance of cancer cells. We reason that high expression of Girdin may confer the survival advantages of bladder tumor cells under the intravesical chemotherapy, thereby resulting in a higher rate of recurrence in patients with Girdin-positive tumors. Although Girdin-G protein-AKT axis has been shown to be associated with anti-apoptotic effects and chemoresistance in cancers [23] the impact of this axis on prognosis of NMIBC remains to be evaluated.

In conclusion, the present study demonstrates that overexpression of Girdin correlates with the tumor recurrence and malignant progression in patients with NMIBC. Moreover, Girdin may represent a novel independent predictor of tumor recurrence for patients with NMIBC.

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# Disclosure of conflict of interest

# None.

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