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RESEARCH

Expression of Hedgehog Pathway Components is Associated with Bladder Cancer Progression and Clinical Outcome

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Abstract Hedgehog (Hh) pathway has been implicated in the tumorigenesis of a large number of human tumors. But its effects on the progression and prognosis of bladder cancer remain poorly understood. The aim of this study was to investigate expression patterns of Hh pathway components in bladder cancer and to elucidate their prognostic values in this tumor. The expression of sonic hedgehog (Shh), its receptor Patched (Ptch1), and downstream transcription factor Gli1 in 118 specimens of bladder cancer and 30 specimens of adjacent normal bladder tissue was determined by immunohistochemistry. Statistical analyses were applied to test the relationship between the expression of these three proteins and clinicopathologic features and prognosis. Immunohistochemical staining results showed the localizations of Shh and Ptch1 proteins to be mainly located in the cytoplasm of bladder cancer cells, whereas Gli1 was mainly localized in the nuclear of tumor cells. Additionally, positive expression of Shh, Ptch1 and Gli1 proteins was correlated with pathological stage (P=0.006, 0.006 and 0.008, respectively), venous invasion (P=0.01, 0.01 and 0.012, respectively) and lymph node metastasis (P=0.009, 0.01 and 0.013, respectively), but not with other factors including age, gender, tumor grade and recurrence of superficial cancer. Moreover, patients with positive expression of Shh, Ptch1 and Gli1 proteins respectively showed poorer disease-free (P=0.002, 0.002 and 0.001, respectively) and overall survival (all P<0.001) than those with negative expression of these three proteins. Univariate and multivariate analysis of prognostic factors in bladder cancer patients indicated that the expression patterns of Shh, Ptch1 and Gli1 proteins were independent unfavorable prognostic factors (all P<0.001). This is the first report describing about the correlation between Hh pathway and the prognosis of bladder cancer. Expression of Shh, Ptch1 and Gli1 proteins was greater in bladder cancers than in the adjacent normal tissues. The examination of their expression is potentially valuable in prognostic evaluation of bladder cancer.

Keywords Hedgehog signaling pathway · Sonic hedgehog · Patched · Gli1 · Bladder cancer · Prognosis

Hui-chan He, Jia-hong Chen and Xi-bin Chen offered equal contributions to this study.

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Introduction

In the Western and Asian countries, bladder cancer is one of the most lethal urological malignant tumors. Although the treatment of bladder cancer has improved greatly in recent

years, the incidence of this disease is gradually increasing. More than half of the patients with bladder cancer have advanced stage disease with very poor prognosis. Recently, different biomarker proteins have been investigated to diagnose and prognosticate bladder cancers [1–3]. However, more information about different molecular subtypes and molecular pathways of early stage bladder tumors might ultimately facilitate prediction of disease outcome and treatment response.

Hedgehog (Hh) pathway, a highly conserved system, plays a key role in tissue patterning, cell differentiation and proliferation [4, 5]. Hh protein family, a key regulator of cell growth and differentiation during development, controls epithelial and mesenchymal interactions in many tissues during embryogenesis [6]. It consists of three secreted proteins including Sonic Hedgehog (Shh), Desert Hedgehog, and Indian Hedgehog. Extracellular hedgehog protein binds to patched homologue 1 (PTCH1), a 12-transmembrane receptor, and prevents PTCH1-mediated inhibition of signaling by smoothened homologue (SMO), a 7-transmembrane protein. Signaling by SMO results in the activation of transcription factors encoded by GLI family zinc finger (GLI) and consequent induction of hedgehog target genes, including GLI1 and PTCH1 [7, 8].

Emerging evidence clearly suggesting the activation of Hh pathway has been implicated in the tumorigenesis of a large number of human cancers, including medulloblastomas, basal cell carcinomas, leukemia, lung, gastrointestinal, ovarian, breast and prostate cancers [9–12]. Moreover, because Hh plays a central role in the control of proliferation and differentiation of both embryonic stem cells and adult stem cells, the aberrant activation of Hh signaling could lead to the generation of cancer stem cells and the development of cancer. It has been demonstrated that the Shh-GLI pathway might be one of determinants governing the transition of prostate cancer from an androgen-dependent to an androgen-independent state by compensating, or even superseding androgen signaling [13]. Experimental studies have revealed that the aberrant Hh signalling occurs in pancreatic cancer tumourigenesis and therapeutics that target the transmembrane receptor Smoothened abrogate Hh signalling and may improve the outcomes of patients with pancreatic cancer [14]. Recent study of Chen et al also showed that germ-line genetic variations in the Shh pathway may predict clinical outcomes of non-muscle-invasive bladder cancer patients received transurethral resection and Bacillus Calmette-Guérin [15]. However, there is no published report on the expression of the Hh pathway components in bladder cancer. The aim of this study was to investigate expression patterns of Hh pathway components in bladder cancer and to elucidate their prognostic values in this tumor.

Materials and Methods

Patients and Tissue Samples

This study was approved by the Research Ethics Committee of Guangzhou First Municipal People's Hospital, Guangzhou Medical College, Guangzhou, China. Informed consent was obtained from all of the patients. All specimens were handled and made anonymous according to the ethical and legal standards.

One hundred eighteen paraffin-embedded samples of transitional cell bladder cancer and 30 specimens of adjacent normal bladder tissue were collected between 2002 and 2005 for immunohistochemical assay. All tumors were histologically and clinically diagnosed by Guangzhou First Municipal People's Hospital, Guangzhou Medical College, Guangzhou, China. The disease stage of each patient was classified or reclassified according to the 2002 AJCC staging system [16]. The 118 patients included 105 males and 13 females from 15 to 75 years (mean, 56 years). Of these patients, 22 patients underwent radical cystectomy, 15 patients underwent partial cystectomy, and 81 patients underwent TURBT (transurethral resection of bladder tumor). After partial cystectomy and TURBT, mitomycin C was used in intravesical therapy as weekly intravesical injection beginning within 24 h after surgery. Thirty specimens of adjacent normal bladder tissue distant from the tumor were included for these patients, as well. The clinical and pathologic parameters were obtained from the pathological reports and presented in Table 1.

Immunohistochemistry Analysis

Immunohistochemistry was performed to examine Shh, Ptch1, and Gli1 expression in 118 human bladder cancers and 30 specimens of adjacent normal bladder tissue. The procedures were performed with classical protocols. In brief, the specimens obtained via surgical resection were immediately fixed in 10% formalin and embedded in paraffin blocks. The paraffin sections were serially cut and stained using the streptavidin-biotin immunoperoxidase technique with the LSAB plus kit (Dako Corp, Carpentaria, Calif). The following monoclonal antibodies were applied as primary antibodies; goat polyclonal anti- Shh antibody (1:100; N-19, sc-1194; Santa Cruz Biotechnology, Santa Cruz, Calif), rabbit polyclonal anti-Ptch1 antibody (1:200; H-267, sc-9016; Santa Cruz Biotechnology, Santa Cruz, Calif) and goat polyclonal anti-Gli1 antibody (1:100; N-16, sc-6153; Santa Cruz Biotechnology, Santa Cruz, Calif). The sections were incubated with these primary antibodies overnight at 4°C followed by the secondary antibody. The results were visualized with diaminobenzidine. Specimens of gastric carcinoma in which the Hedgehog pathway is

Table 1 Association of Shh, Ptch1, and Gli1 protein expression with the clinicopathological features

Clinicopathological features	No. of cases (%)	Shh			Ptch1			Gli1		
		Positive (%)	Negative (%)	*P*	Positive (%)	Negative (%)	*P*	Positive (%)	Negative (%)	*P*
Age (years)										
<56	56 (47.5)	36 (64.3)	20 (35.7)	NS	46 (82.1)	10 (17.9)	NS	46 (82.1)	10 (17.9)	NS
≥56	62 (52.5)	42 (67.7)	20 (32.3)		47 (75.8)	15 (24.2)		49 (79.0)	13 (21.0)	
Gender										
Female	13 (11.0)	9 (69.2)	4 (30.8)	NS	10 (76.9)	3 (23.1)	NS	10 (76.9)	3 (23.1)	NS
Male	105 (89.0)	69 (65.7)	36 (34.3)		83 (79.0)	22 (21.0)		85 (81.0)	20 (19.0)	
Grade[a]										
G1/G2	52 (44.1)	37 (61.5)	20 (38.5)	NS	40 (76.9)	12 (23.1)	NS	42 (80.8)	10 (19.2)	NS
G3/G4	66 (55.9)	41 (69.7)	20 (30.3)		53 (80.3)	13 (19.7)		53 (80.3)	13 (19.7)	
Pathological stage[b]										
I/II	46 (39.0)	20 (43.5)	26 (56.5)	0.006	30 (65.2)	16 (34.8)	0.008	32 (69.6)	14 (30.4)	0.008
III	72 (61.0)	58 (80.6)	14 (19.4)		63 (87.5)	9 (12.5)		63 (87.5)	9 (12.5)	
Lymph node metastasis[b]										
No	50 (42.4)	28 (56.0)	22 (44.0)	0.009	33 (66.0)	17 (34.0)	0.01	35 (70.0)	15 (30.0)	0.013
Yes	68 (57.6)	50 (73.5)	18 (26.5)		60 (88.3)	8 (11.7)		60 (88.3)	8 (11.7)	
Venous invasion										
No	52 (44.1)	30 (57.7)	22 (42.3)	0.01	35 (67.3)	17 (32.7)	0.01	37 (71.2)	15 (29.8)	0.012
Yes	66 (55.9)	48 (72.7)	18 (27.3)		58 (32.7)	8 (67.3)		58 (32.7)	8 (67.3)	
Superficial cancer										
Recurrence-free	58 (49.2)	38 (65.5)	20 (34.5)	NS	45 (77.6)	13 (22.4)	NS	45 (77.6)	13 (22.4)	NS
Recurrence	60 (50.8)	40 (66.7)	20 (33.3)		48 (80.0)	12 (20.0)		50 (83.3)	10 (16.7)	

[a] According to the1999 World Health Organization system

[b] According to TNM classification of malignant tumors, 3rd ed. Geneva

known to be activated were used as positive control specimens [17]. Matched negative controls were stained without primary antibody.

Assessment of immunohistochemical staining was evaluated by two independent pathologists. An immunoreactivity score system was applied as described previously [18]. The extensional standard was: (1) the number of positively stained cells <5% scored 0; 6–25% scored 1; 26–50% scored 2; 51–75% scored 3; >75% scored 4; (2) intensity of stain: colorless scored 0; pallide-flavens scored 1; yellow scored 2; brown scored 3. Multiply (1) and (2). The staining score was stratified as negative (0~2 score), positive (3–12 score) according to the proportion and intensity of positively stained cancer cells. Specimens were rescored if difference of scores from two pathologists was >3.

Statistical Analysis

The software of SPSS version12.0 for Windows (SPSS Inc, IL, USA) was used for statistical analysis. Continuous variables were expressed as $\overline{X} \pm s$. The associations between protein expression and different clinical parameters were evaluated using the nonparametric Mann-Whitney *U*-test (when two independent groups were compared) or the Kruskal-Wallis test (when more than two independent groups were compared). Survival curves were plotted by the Kaplan-Meier method, and compared by the log-rank test. We determined that the assumption of proportional hazards was met in all Cox regression models. The significance of various variables for survival was analyzed by the Cox proportional hazards model in the multivariate analysis. Differences were considered statistically significant when p was less than 0.05.

Results

Expression of Shh, Ptch1, and Gli1 Proteins in Paraffin-Embedded Bladder Cancer Samples

To analyze the expression of Shh, Ptch1, and Gli1 proteins in bladder cancer tissues, we performed an immunohistochemical assay in 118 bladder cancer tissues. Representative results are shown in Fig. 1. Shh was mainly localized in the cytoplasm of the urothelial cancer cells (Fig. 1a). Of all the patients, 78 (66.1%) were classified as Shh positive. Turning to the Ptch1, there were 93 specimens (78.8%) with positive Ptch1 expression and

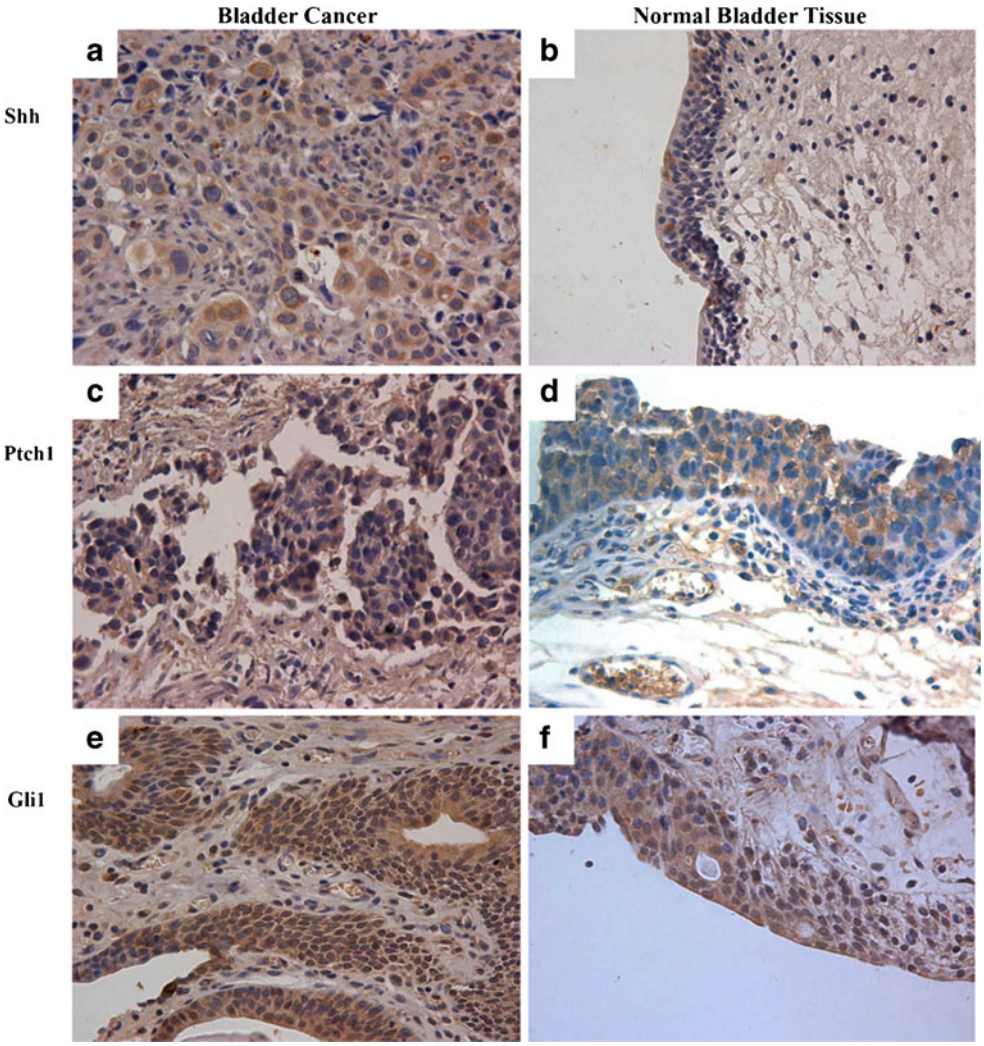

Fig. 1 Representative examples of Shh, Ptch1, and Gli1 proteins immunostaining in bladder cancer (×400). Shh and Ptch1 stainings were both mainly localized in the cytoplasm of urothelial cancer cells, and Gli1 staining was observed in the nuclear of urothelial cancer cells. **a** Positive Shh staining in urothelial cancer tissues; **b** Weak positive Shh staining in adjacent normal bladder tissues; **c** Positive Ptch1 staining in urothelial cancer tissues; **d** Weak positive Ptch1 staining in adjacent normal bladder tissues; **e** Positive Gli1 staining in urothelial cancer tissues; **f** Weak positive Gli1 staining in adjacent normal bladder tissues

strong positivity of Ptch1 staining was also mainly located in the cytoplasm in these specimens (Fig. 1c). Moreover, there were 95 specimens (80.5%) with positive Gli1 expression and the positive lesion was the nuclear (Fig. 1e). Shh (Fig. 1b), Ptch1 (Fig. 1d) and Gli1 (Fig. 1f) were all positively expressed in the adjacent normal bladder tissues with the weak immunostaining. They were all not present in the negative controls with non-immune IgG.

Association of Shh, Ptch1, and Gli1 Expression with the Clinicopathological Features

Table 1 showed the results of statistical analysis of the associations between Shh, Ptch1 and Gli1 protein expression and clinicopathological factors of bladder cancer patients. The expression levels of Shh, Ptch1 and Gli1 protein expression were correlated with pathological stage (P=0.006, 0.008 and 0.008, respectively), venous invasion (P=0.01, 0.01 and 0.012, respectively) and lymph node metastasis (P=0.009, 0.01 and 0.013, respectively), but not with other factors including age, gender, tumor grade and recurrence of superficial cancer.

Survival Analysis

Figure 2 shows the Kaplan-Meier survival curves for bladder cancer patients whose tumors had positive or negative expression of Shh, Ptch1 and Gli1 proteins. The 5-year disease-free survival rates of patients with positive expression of Shh, Ptch1 and Gli1 proteins were significantly lower than those of patients with negative expression of these three proteins (Fig. 2a ~ c; P=0.002, 0.002 and 0.001, respectively). Furthermore, patients with positive expression of Shh, Ptch1 and Gli1 proteins also had significantly lower overall survival rates than those with negative protein expression (Fig. 2d ~ f; all P<0.001).

The results of univariate and multivariate analysis for overall survival of bladder cancer patients are shown in Table 2. Univariate analyses showed that the expression of Shh, Ptch1 and Gli1 proteins (all P<0.001), tumor grade (P= 0.02), lymphnode metastasis (P=0.01) and venous invasion

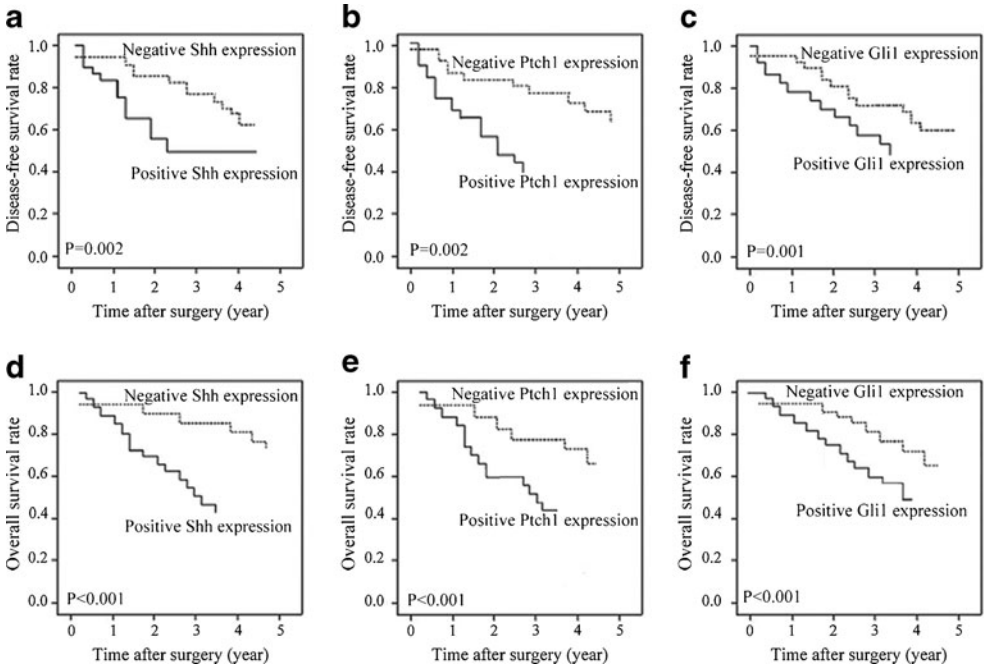

Fig. 2 Kaplan-Meier survival curves of bladder cancer patients according to the expression of Shh, Ptch1 and Gli1 protein. The patients with positive Shh, Ptch1 and Gli1 protein expression tumor showed significantly poorer disease-free (**a**, **b** and **c**, P=0.002, 0.002 and 0.001, respectively; log-rank test) and overall (**d**, **e** and **f**, all P<0.001, respectively; log-rank test) survival rates than those without their expression

(P=0.008) were significant prognostic factors. However, age, gender, and recurrence of superficial cancer had no prognostic significance (all P>0.05). Multivariate analyses of the same set of patients were performed for the expression of Shh, Ptch1 and Gli1 proteins and clinicpathological factors of survival time, which indicated that the expression patterns

Table 2 Univariate and multivariate analysis of prognostic factors for predicting the overall survival of bladder cancer patients in Cox proportional hazards model

Variable	Univariate analysis			Multivariate analysis		
	HR	95% CI	*P*	HR	95% CI	*P*
Age (year)						
>56/<56	0.673	0.456~1.232	NS	1.036	0.609~2.338	NS
Gender						
Female/Male	0.568	0.321~1.189	NS	1.288	0.722~2.798	NS
Grade						
G3~G4/G1~G2	2.418	1.465–4.382	0.02	1.812	1.467–3.688	0.01
Pathological stage						
III/I ~ II	1.789	0.68~3.542	0.008	2.866	1.510~4.558	0.002
Lymph node metastasis						
Yes/No	1.709	1.267~3.658	0.01	2.229	1.436~4.118	0.008
Venous invasion						
Yes/No	2.568	2.121~3.563	0.008	2.871	2.251~4.187	0.006
Superficial cancer						
Recurrence/Recurrence-free	0.897	0.326~1.896	NS	1.233	0.623~2.156	NS
Shh protein expression						
Positive/ negative	3.568	2.689~5.563	<0.001	0.836	0.298–0.979	<0.001
Ptch1 protein expression						
Positive/ negative	3.217	2.386~5.187	<0.001	0.739	0.316–0.921	<0.001
Gli1 protein expression						
Positive/ negative	3.582	2.318~5.679	<0.001	0.867	0.328–0.935	<0.001

HR refers to hazard ratio; 95% CI refers to 95% confidence interval

of Shh (hazard ratio: 0.836; 95%confidence interval: 0.298–0.979; $P<0.001$), Ptch1 (hazard ratio: 0.739; 95% confidence interval: 0.316–0.921; $P<0.001$) and Gli1 (hazard ratio: 0.867; 95%confidence interval: 0.328–0.935; $P<0.001$) proteins were also independent unfavorable prognostic factors.

Discussion

The clinical progression of patients with bladder cancer varies and patients with the same disease stage have different outcomes from the same therapy. Presently, the molecular mechanisms of the initiation and progression of bladder cancers are unclear, although many genetic factors have been found to be associated with bladder cancer. Combination chemotherapy is a strenuous treatment and factors that enable pretreatment evaluation of the probability of a survival benefit are of utmost importance. In this study, we report that three main components of Hh pathway (Shh, Ptch1 and Gli1) are overexpressed in human bladder cancers. The overexpression of these proteins was correlated with pathological stage, venous invasion and lymph node metastasis of tumors. Patients with positive expression of Shh, Ptch1 and Gli1 proteins had poorer disease-free and over-all survival than those without it.

The Hh pathway is a major regulator of cell differentiation, tissue polarity and cell proliferation [19]. It has been demonstrated that this pathway is involved in the initiation and progression of gastrointestinal cancers and it is frequently activated in esophageal [20], gastric [21], and pancreatic cancer [22]. Among the three known Hh genes existing in mammals, Shh is the best studied one of mammalian Hhs with the broadest expression pattern, including in the developing nervous system, limb buds, skin, and gut [23]. Ptch1, a receptor of Hh signaling pathway, can suppress the pathway by inhibiting SMO. Binding of Shh to Ptch1 leads to release of SMO signal transducer and activation of the pathway. Loss of Ptch1 is known to release the SMO signal transducer from Patched-dependent suppression and further activates Gli1. Recently, Chen et al. [15] have genotyped 177 single-nucleotide polymorphisms (SNP) in 11 Shh pathway genes in a study including 803 bladder cancer cases and 803 controls. They also assessed SNP associations with cancer risk and clinical outcomes in 419 cases of non-muscle-invasive bladder cancer and 318 cases of muscle-invasive and metastatic bladder cancer. As their results, it has been demonstrated that germ-line genetic variations in the Shh pathway could predict the clinical outcomes of non-muscle-invasive bladder cancer patients received transurethral resection and Bacillus Calmette-Guérin. However, the expression patterns of the Hh pathway components in bladder cancer and their relationship with the prognosis of patients with this tumor have not been reported.

In the present study, we detected the expression of Shh, Ptch1 and Gli1 proteins in human bladder cancer and adjacent normal bladder tissues by immunohistochemistry. Results showed that the positive expression rates of Shh, Ptch1 and Gli1 proteins were significantly higher in bladder cancer tissues than those in adjacent normal bladder tissues. Thus, Shh, Ptch1 and Gli1 may play important roles during bladder tumorigenesis. Rush et al. [24] reported that Hh pathway activation is common in pediatric pilocytic astrocytomas and may be associated with younger age at diagnosis and tumor growth. Liao et al. [25] showed that abnormal Hh signaling activation plays important roles in the development and progression of ovarian cancers. Gli1 expression is an independent prognostic marker. Inhibition of the Hh pathway molecules might be a valid therapeutic strategy for ovarian cancers. Moreover, Yang et al. [26] found that Hh signaling activation is a very common event in pancreatic cancer and that targeted inhibition of Hh signaling may be effective in treatment of pancreatic cancer. However, the exact mechanisms for activation of Hh pathway in bladder cancer are still unknown and are currently being investigated by our groups. Additionally, we also analyzed the expression patterns of Shh, Ptch1 and Gli1 proteins in relation to clinicopathological factors of bladder cancer patients. To the best of our knowledge, this is the first study to investigate the associations between the expression of Shh, Ptch1 and Gli1 proteins and clinicopathological factors in bladder cancer. Our results suggested that the positive expression rates of Shh, Ptch1 and Gli1 proteins were higher in high-grade cancers (G3/G4) compared to that in low-grade cancers (G1/G2). Moreover, patients with positive Shh, Ptch1 and Gli1 protein expression showed a higher pathological stage than those with negative expression. The high positive expression rates of Shh, Ptch1 and Gli1 proteins were significantly correlated with lymph node metastasis and venous invasion, but not with age, gender and superficial cancer. Furthermore, we analyzed the correlation of Shh, Ptch1 and Gli1 protein expression with survival and prognosis of bladder cancer patients. Patients with positive expression of Shh, Ptch1 and Gli1 proteins showed a lower disease-free or overall survival rate than those with negative expression. These findings were further supported by the univariate and multivariate analysis, which suggested that the expression patterns of Shh, Ptch1 and Gli1 proteins were independent factors for predicting the prognosis of bladder cancer patients.

In conclusion, this is the first report describing about the correlation between Hh pathway and the prognosis of bladder cancer. Expression of Shh, Ptch1 and Gli1 proteins was greater in bladder cancers than in the adjacent normal

tissues. The examination of their expression is potentially valuable in prognostic evaluation of bladder cancer. This study is hypothesis generating, and that further prospective analysis would be worth doing.

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Competing interests There are none competing interests in this study.

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