



## Heparanase Gene Hypomethylation as a Potential Biomarker for Precision Screening of Bladder Cancer

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### Abstract

**Background:** Epigenetics has been playing an increasingly important role in the study of the origin and development of bladder cancer (BC). This study aimed to investigate the correlation between promoter hypomethylation of the heparanase (HPSE) gene and clinicopathologic characteristics of bladder cancer (BC).

**Materials and Methods:** The promoter hypomethylation profile was evaluated by methylation-specific polymerase chain reaction (PCR) using 27 BC tissue specimens and 15 normal control specimens. The aim was to help decipher the underlying relationship between the clinicopathologic characteristics and the hypomethylation status.

**Results:** Experimental results showed that 16 (59.26%) BC specimens demonstrated the promoter hypomethylation of HPSE, including 2 cases with complete demethylation. For normal control groups, only 3 specimens (20%) indicated hypomethylation ( $P < 0.05$ ). In addition, the occurrence of hypomethylation increased with the metastasis of positive lymph nodes ( $P < 0.05$ ). Importantly, no significant correlation was found between the hypomethylation of HPSE and the profile of patients including gender, age, tumor size, cancer stage, or histologic grade ( $P > 0.05$ ).

**Conclusion:** The promoter hypomethylation of HPSE gene is a common epigenetic event occurring in BC and is positively correlated with a poor prognosis. This study suggested that the promoter hypomethylation could be used as a potential biological marker for the early screening of BC.

**Keywords:** Bladder cancer, Cancer diagnosis, Hypomethylation, Methylation-specific polymerase chain reaction, Heparanase gene

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### Introduction

During the last few years, epigenetics played an increasingly important role in the study of the origin and development of bladder cancer (BC). The current studies on the mechanism of epigenetics mostly focus on two aspects: gene deactivation via DNA hypermethylation and the overexpression of the target gene. During the early-stage tumorigenesis, promoter hypomethylation of an oncogene occurs primarily at the CpG5' cytosine of the promoter CpG island. Disordered DNA methylation has been reported throughout the origin and development of urocyt cancer, suggesting that DNA methylation impacts tumor development via the chromatin structure and the anti-oncogene expression (1). Heparanase (HPSE) has been reported to be the only matrix-degrading enzyme capable of degrading the side chain of heparin sulfate proteoglycan within the extracellular matrix. Previous studies demonstrated that heparinase can significantly determine pathophysiological processes including tumor metastasis, angiogenesis, and many other processes (2,

3). Moreover, these studies also validated that HPSE only over-expressed tumor-associated cells not normal somatic cells. However, there still seems to be a lack of research on hypomethylation of the HPSE gene promoter in BC. A study aiming to unveil the potential correlation between HPSE hypomethylation and tumor progression, differentiation, lymph node metastasis, and other clinicopathologic characteristics would shed further light on this promising field. In this study, the clinical significance of promoter HPSE hypomethylation in BC was evaluated by the methylation-specific polymerase chain reaction (MSP). The obtained results generated sound correlations between the hypomethylation of HPSE and the clinicopathologic profile of BC using 27 BC clinical samples.

### Materials and Methods

#### Study Subjects

Study samples including 27 BC tissues and 15 normal tissues were all collected through the Fifth Affiliated

Hospital of Sun Yat-Sen University. All samples were confirmed by the standard pathologic examinations and stored in liquid-nitrogen sample containers at  $-80^{\circ}\text{C}$ . Patients were informed and their consent was obtained. For the BC samples, 21 and 6 samples were collected from males and females, respectively, with an age spectrum ranging from 30 to 83 (median age was 63-year-old).

Noting that these BC samples were staged according to the UICC classification: 10 cases were Ta-T1, and 17 cases were T2-T4. The tumor size was measured and calculated by computed tomography (CT). Further, these samples were graded according to the WHO classification: 7 cases were I-II, and 20 cases were III-IV. Samples were collected from patients who have not undergone chemo- or radiation therapy. For the control group, the participants have an age spectrum range from 32 to 77 years old, with a median age of 53.3, suggesting that no significant difference occurred in age between these two groups ( $P > 0.05$ ).

### Reagents

The DNA methylation modification kit was purchased from Zymo Research (EZ DNA Methylation-Gold™ Kit, USA). Further, the tissue genomic DNA extraction kit was obtained from KangWei Corporation (Tissue Gen DNA Kit, China). The Hot start Tap DNA polymerases were purchased from Sangon Biotech (Tap DNA Polymerase SK 2082, Shanghai, China), and primers were synthesized by Sangon Biotech (Shanghai, China) and used as received.

### Tissue Genomic DNA Extraction and Bisulfite Modification

Tissue genomic DNA of cancerous and the normal bladder samples were extracted using the Tissue Gen DNA Kit (KangWei Corporation, Beijing, China). After extraction, we prepared DNA samples at a concentration of  $2\ \mu\text{g}/\mu\text{L}$  and OD260/OD280 between 1.8 and 2.0. All samples were stored at  $-20^{\circ}\text{C}$ . To perform analysis, genomic DNA was modified by the EZ DNA Methylation-Gold™ Kit (ZYMO Research, USA), and  $10\ \mu\text{L}$  of modified DNA was collected from each modification reaction. The modified DNA samples were stored at  $-20^{\circ}\text{C}$ .

### Polymerase Chain Reaction Mixture and Primers for Bisulfite-Modified DNA Samples

In total,  $1\ \mu\text{L}$  of modified genomic DNA sample was added to  $50\ \mu\text{L}$  polymerase chain reaction (PCR) mixture containing  $22\ \mu\text{L}$  ddH<sub>2</sub>O,  $2\ \mu\text{L}$  primers ( $10\ \mu\text{mol}/\text{L}$ ), and  $25\ \mu\text{L}$   $2\times$  HiFi-PCR Master ( $3\ \text{mmol}/\text{L}$  MgCl<sub>2</sub>,  $0.2\ \text{mmol}/\text{L}$  dNTP,  $0.1\ \text{U}/\mu\text{L}$  Taq DNA Polymerase, and  $2\times$  PCR buffer). The primers were designed using the Meth Primer program (<http://www.urogene.org/methprimer/indexl.html>). The primers included the left M primer ( $5^{\prime}\text{-TATTTCGAGGGTTAGAGGGATATTC-3}^{\prime}$ ), right M

primer ( $5^{\prime}\text{-GAAAATAACCGAAATCCAAACG-3}^{\prime}$ ), left U primer ( $5^{\prime}\text{-TTTATTTGAGGGTTAGAGGGATATTT-3}^{\prime}$ ), and right U primer ( $5^{\prime}\text{-ACAAAAATAACCAAAATCAAACAC-3}^{\prime}$ ). The product sizes of the methylated primers and unmethylated primers were 193 bp and 197 bp, respectively.

### Methylation-Specific Polymerase Chain Reaction

The promoter methylation status of the HPSE gene was detected by the MSP. Primer pairs (LMP-RMP and LUP-RUP) were used for the detection of methylated and unmethylated target sequences, respectively. Modified DNA was used as the template, and ddH<sub>2</sub>O was used as the blank control. The PCR cycles were  $95^{\circ}\text{C}$  for 5 minutes; 35 cycles of  $94^{\circ}\text{C}$  for 40 seconds,  $56^{\circ}\text{C}$  for 30 seconds,  $72^{\circ}\text{C}$  for 1 minute, and  $72^{\circ}\text{C}$  for 10 minutes. The PCR amplification product was analyzed by 2% agarose gel electrophoresis and a multi-function gel imaging system.

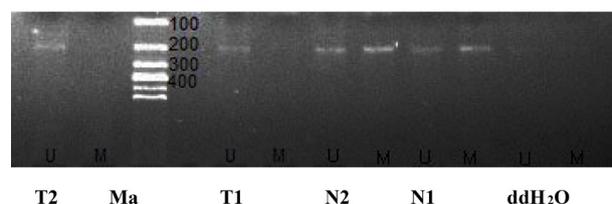
### Statistical Analysis

Statistical analysis was performed using SPSS 13.0 software. The  $\chi^2$  test was used for qualitative data analysis, the  $t$  test was used for quantitative data analysis, and Fisher's exact test was used when the data were not in accordance with  $\chi^2$  test conditions. Further, a  $P$  value of less than 0.05 was regarded to be statistically significant.

### Results

#### Methylation Profile of the HPSE Gene in BC and Normal Bladder Samples

To understand the characteristic methylation profile of the HPSE gene in BC, we compared the methylated degree of the HPSE gene both in BC and normal bladder samples. According to Figure 1, the obtained results showed that 16 BC samples (59.26%) were tested positive for demethylation, specifically including 2 cases with complete demethylation, while in normal bladder tissues, only 2 cases (20.00%) were tested positive for hypomethylation. This study, therefore, validated that the methylation profile of the HPSE gene in BC could be significantly differentiated from that in the normal bladder samples ( $P < 0.05$ ). Promisingly, these data implicated that the methylation profile of the HPSE gene could be exploited as a potentially viable genetic



**Figure 1.** HPSE Gene Promoter Hypomethylation in BC and Normal Control Checked by MSP. Note. HPSE: Heparanase; BC: Bladder cancer; MSP: Methylation-specific polymerase chain reaction. T1-T2: BC samples; N1-N2: Normal bladder samples; Ma: Marker (100 bp DNA ladder); M: Methylated; U: Unmethylated.

biomarker for the investigation of the pathologic profile of BC pathology.

**Correlation Between the Promoter Hypomethylation Profile of the HPSE Gene and the Clinicopathological Features of BC**

To further evaluate whether the methylation profile of the HPSE gene is related to the occurrence of BC, we investigated the correlations between the promoter hypomethylation of the HPSE gene and the pathologic characteristics of BC. To begin with, we compared the median age of BC patients who tested positive for demethylation with control group participants who tested negative for demethylation. It was found that the median age of the demethylation-positive patients with BC was slightly higher than that of the subjects in the normal control group (Table 1). Then, we discussed how the promoter hypomethylation of the HPSE gene is affected by relevant subjects' variables including gender, clinical stage, pathologic grade, tumor size, and tissue histology. Importantly, it was found that these factors posed no significant impact on the positive rate of promoter hypomethylation of the HPSE gene ( $P > 0.05$ ) (see Table 1). Furthermore, we evaluated how lymph node metastasis affects the promoter hypomethylation of the HPSE gene. As illustrated in Table 1, of 17 cases with lymph node metastasis, 16 cases (94.12%) were tested positive in hypomethylation, while among 10 cases without lymph node metastasis, only 3 cases (30.00%) were demethylated, indicating that lymph node metastasis can dramatically increase the occurrence of promoter hypomethylation of the HPSE gene. In sum, the findings of this study suggested that promoter hypomethylation of the HPSE gene is not statistically dependent on factors

such as tumor size, histopathological type, clinical stage, or pathologic grade but is impacted by the lymph node metastasis.

**Discussion**

For BC, epigenetic alterations play an important role in the tumor formation process and tumor development process that is regulated by gene transcription [4, 5]. In certain types of tumors, particular genes demonstrated abnormal methylation profiles: the genes are found to be hypomethylated in malignant tumors such as BC [6]. Promisingly, the investigation of DNA hypomethylation could be used as an effective biomarker for the identification of malignant tumors (7). Previous reports have proved that CpG methylation could be the major evidence for the presence of cancer cells, and DNA hypomethylation abnormalities could serve as one of the emerging biomarkers for the study of carcinoma progression (8). For instance, Sharrard et al (9) found ubiquitous DNA hypomethylation in some premalignant lesions, including those that occurred in the tissues of the benign colon, gland, and metastatic carcinoma. A previous study also found a similar phenomenon in rats, that is, rats fed with diets lacking methyl donors were tested positive for DNA demethylation, which in turn led to the occurrence of liver carcinoma [10]. Therefore, DNA hypomethylation could be a feasible biomarker for the investigation of the occurrence and development of tumors.

As a sign of the occurrence of cancer, the gene methylation profile showing abnormality is usually accompanied by overall gene hypomethylation and segmental gene hypermethylation (11). Hypermethylation and hypomethylation, two independent processes during

**Table 1.** The Relationship Between Clinicopathologic Features in BC and HPSE Gene

Item	Variance	Cases	HPSE Gene Hypomethylation		P Value
			Positive	Negative	
Gender					
Male	—	21	14	7	0.187
Female	—	6	2	4	
Age (y)	30~83	27	63.38	63.55	0.767
Tumor size (mm <sup>3</sup> )	14~120	27	65.31 ± 31.71	69.36 ± 24.35	0.537
Stage (N)	T <sub>a</sub> ~T <sub>4</sub>				
T <sub>a</sub> ~T <sub>1</sub>	—	10	5	5	0.453
T <sub>2</sub> ~T <sub>4</sub>	—	17	11	6	
Grade (N)	I~IV				
I~II		7	4	3	1
III~IV		20	12	8	
Lymph node metastasis (N)					
+		17	15	2	0.004
-		10	3	7	

Note. BC: Bladder cancer; HPSE: Heparanase.

tumor onset and tumor progression, work on different targets during different periods (12). Hypermethylation and gene silencing can regulate the process of cell proliferation, which in turn impacts the early formative period of the tumor, while hypomethylation and certain gene activation events could significantly influence the occurrence and development of tumors (13). Up to now, the role of hypermethylation and deactivation of suppressor genes in malignant cancers has been well-studied. However, there have been few studies exploring the impacts of DNA hypomethylation in highly malignant and metastatic cancers.

In this study, we firstly compared the promoter demethylation profile of the HPSE gene between BC tissues and normal control tissues, then investigated the possible correlations between genetic demethylation and clinicopathologic characteristics. As mentioned previously, MSP was analyzed to evaluate the promoter demethylation status of the HPSE gene. The results indicated that the rate of hypomethylation was positively correlated with the occurrence of BC: 59.26% (16.27) of BC samples were tested positive for hypomethylation, while only 20.00% (3.15) of the control group were tested positive. Importantly, no statistically significant difference was detected between the two study groups ( $P > 0.05$ ). According to different UICC stages, T2-T4 (17.27) showed a slightly higher rate of hypomethylation compared to Ta-T1. Although hypomethylation may contribute to the development of tumor, no significant difference was found in this regard. Further, a similar result was achieved when the impacts of WHO grouped grades and tumor sizes on the degree of promoter demethylation were both investigated ( $P > 0.05$ ).

It is worthwhile to note that lymph node metastasis could be reflected by the methylation status of the HPSE gene. Results of this study found that metastatic BC samples were characterized by a higher degree of methylation, implying that there existed a positive relationship between promoter hypomethylation of the HPSE gene and the progression of BC, subsequently leading to a poor prognosis.

The described HPSE biomarker represents progress towards the precision screening of BC. However, this BC diagnostic approach is still at a very early stage of development, and more work is required for successful translation into viable precision clinical screening tools, including further validation of HPSE as a feasible biomarker, clinical verification of this proposed method, and acceleration of validation process. The time frame for comprehensive clinical validation and translation of novel BC currently takes 5-10 years, which needs to be effectively accelerated to provide benefits for patients.

## Conclusion

In summary, the obtained results indicated that the

promoter hypomethylation status of the HPSE gene could increase the HPSE expression level in BC, contributing to the occurrence and development of BC. Additionally, it was found that the methylation level was closely correlated with the lymph node metastasis. However, the underlying mechanism of methylation and mRNA expression of the HPSE gene remain unknown, and further investigations are needed. Furthermore, promoter hypomethylation of the HPSE gene was found to be a viable biomarker for the analysis of BC samples. This study explored the use of HPSE gene hypomethylation as a feasible strategy for the guided clinical diagnosis and prognosis as well as the precision therapy and medical treatment of BC.

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## Authors' Contributions

Study concept and design: BsX, YyJ, CzL, and MbY. Data acquisition: BsX and YyJ. Data analysis: BsX, YyJ, and CzL. Drafting of the manuscript: BsX and CzL. Critical revision of the manuscript: BsX and CzL.

## Conflict of Interest Disclosures

All authors disclose no conflict of interests.

## Consent for Publication

Informed consent was obtained from all subjects before the study.

## Disclaimer

The views expressed here are those of the authors and do not necessarily reflect the views of the first people in Hospital of Xiushui, Creative Biosciences (Guangzhou Co., Ltd.) and the fifth Affiliated Hospital of Sun Yat-Sen University.

## Ethical Statement

All experimental protocols were assessed and approved by the Medical Ethics Committee of the fifth Affiliated Hospital of Sun Yat-sen University (ID: FAHSYU20180888), and written informed consent was obtained from all subjects. All experiments and analyses were carried out in accordance with the principles embodied in the Declaration of Helsinki and in accordance with local statutory requirements.

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## References

1. Zhu J, Yao X. Use of DNA methylation for cancer detection: promises and challenges. *Int J Biochem Cell Biol.* 2009;41(1):147-54. doi: 10.1016/j.biocel.2008.09.003.
2. Yingying X, Yong Z, Zhenning W, Xue Z, Li J, Yang L, et al. Role of heparanase-1 in gastric carcinoma invasion. *Asian Pac J Cancer Prev.* 2009;10(1):151-4.
3. Ilan N, Elkin M, Vlodavsky I. Regulation, function and clinical significance of heparanase in cancer metastasis and angiogenesis. *Int J Biochem Cell Biol.* 2006;38(12):2018-39. doi: 10.1016/j.biocel.2006.06.004.
4. Wolff EM, Liang G, Jones PA. Mechanisms of disease: genetic and epigenetic alterations that drive bladder cancer. *Nat Clin Pract Urol.* 2005;2(10):502-10. doi: 10.1038/ncpuro0318.

5. Gibney ER, Nolan CM. Epigenetics and gene expression. *Heredity* (Edinb). 2010;105(1):4-13. doi: 10.1038/hdy.2010.54.
6. Ogishima T, Shiina H, Breault JE, Terashima M, Honda S, Enokida H, et al. Promoter CpG hypomethylation and transcription factor EGR1 hyperactivate heparanase expression in bladder cancer. *Oncogene*. 2005;24(45):6765-72. doi: 10.1038/sj.onc.1208811.
7. De Smet C, Lorient A. DNA hypomethylation in cancer: epigenetic scars of a neoplastic journey. *Epigenetics*. 2010;5(3):206-13. doi: 10.4161/epi.5.3.11447.
8. Ahuja N, Li Q, Mohan AL, Baylin SB, Issa JP. Aging and DNA methylation in colorectal mucosa and cancer. *Cancer Res*. 1998;58(23):5489-94.
9. Sharrard RM, Royds JA, Rogers S, Shorthouse AJ. Patterns of methylation of the c-myc gene in human colorectal cancer progression. *Br J Cancer*. 1992;65(5):667-72. doi: 10.1038/bjc.1992.142.
10. Szyf M. The DNA methylation machinery as a target for anticancer therapy. *Pharmacol Ther*. 1996;70(1):1-37. doi: 10.1016/0163-7258(96)00002-2.
11. Das PM, Singal R. DNA methylation and cancer. *J Clin Oncol*. 2004;22(22):4632-42. doi: 10.1200/jco.2004.07.151.
12. Szyf M, Pakneshan P, Rabbani SA. DNA demethylation and cancer: therapeutic implications. *Cancer Lett*. 2004;211(2):133-43. doi: 10.1016/j.canlet.2004.04.009.
13. Esteller M. DNA methylation and cancer therapy: new developments and expectations. *Curr Opin Oncol*. 2005;17(1):55-60. doi: 10.1097/01.cco.0000147383.04709.10.