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Original Article

Diagnostic value of long non-coding RNA H19, UCA1, and HOTAIR as promising biomarkers in human bladder cancer

Wei Wang[1], Zhihua Yin[2]

[1]*Department of Drug Control, Criminal Investigation Police University of China, Shenyang, P. R. China;* [2]*Department of Public Health, China Medical University, Shenyang, P. R. China*

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Abstract: Aim: Currently, there are no satisfactory markers for bladder cancer available in clinics. In this study, we selected 3 long noncoding RNAs, H19, UCA1, and HOTAIR, to assess their diagnostic value in bladder cancer screening. Method: In this research, a total of 96 cancer tissues and paired non-cancer tissues from patients with bladder cancer (BC) were collected. Total RNA was isolated from tissues using TRIzol reagent and performance of three selected lncRNAs as cancer markers were analyzed by reverse transcription-PCR. A new lncRNA-based score was conducted by Logistic regression model. Receiver operating characteristic (ROC) curves and area under the ROC curve (AUC) was generated to assess the diagnostic values of the lncRNAs. Result: Expression levels of lncRNAs H19, UCA1 and HOTAIR were remarkably increased in bladder cancer tissues compared with those in normal tissues (all P<0.001). The AUC (95% CI) of H19, UCA1, and HOTAIR were 0.717 (0.647-0.779), 0.787 (0.722-0.843), 0.713 (0.643-0.776), respectively. We conducted a new score named lncRNA-score, based on three selected LncRNAs: Lnc-Score =0.48*H19+0.49*UCA1+1.2*HOTAIR, which had the best diagnosability performance with AUC of 0.870 (0.814-0.914), sensitivity of 70.8%, specificity of 88.5%, +LR of 3.67 and -LR of 0.22. Conclusion: The 3 selected lncRNAs were remarkably increased in bladder cancer tissues and have great potential to be new sensitive, reliable biomarkers for diagnosis of bladder cancer.

Keywords: lncRNA, bladder cancer, diagnose, ROC analysis

Introduction

As one of the most common types of genitourinary tumors, human bladder cancer accounts for one half of all tumors of the urinary system [1, 2]. Despite increased early detection of bladder cancer and more frequent surgery, the incidence and mortality of bladder cancer has not changed significantly in the past several decades [3]. Therefore, an urgent need of searching new sensitive, reliable biomarkers is emphasized for bladder cancer.

Deep sequencing recently facilitated the discovery of thousands of novel transcripts, now classified as long noncoding RNAs (lncRNAs), in many vertebrate and invertebrate species [4]. Like microRNAs, lncRNA are also proving to be the key mediators of cellular differentiation, cell lineage choice, organogenesis and tissue homeostasis [4, 5]. Dysregulation of lncRNA expression has been shown to be important in carcinogenesis and cancer metastasis [6-8]. A study that considered samples from 5,037 human tumor specimens from the cancer genome atlas project, demonstrated that, lncRNAs are highly cancer type specific compared with protein-coding genes, which provides a resource for investigating lncRNA in cancer and lays the groundwork for the development of new sensitive, reliable biomarkers for diagnosis and treatment.

Recently, most studies focus on revealing the molecular mechanism of lncRNA in cancer mechanism. In the present study, we determined the expression of selected 3 candidate lncRNAs (H19, UCA1 and HOTAIR) in human bladder tissues and assess their diagnostic values in BC screening by comparing their expres-

Table 1. Characteristics of bladder cancer patients

Characteristics	BC patients (n=96)
Age	61.3±6.2
Male	69 (71.8%)
Alcohol use	
Yes	32 (33.3%)
No	64 (66.6%)
Tobacco use	
Yes	28 (29.2%)
No	68 (70.8%)
Family history of BC	
Yes	10 (10.4%)
No	86 (89.6%)
TNM	
I+II	39 (40.6%)
III+IV	57 (59.4%)

sion level in cancer tissues and pair-matched adjacent normal tissues.

Materials and methods

Subjects

Total of 96 cancer tissues and paired non-cancer tissues (defined as >2 cm distance from tumor edge) from patients with urothelial carcinoma were collected. Then tissues were aliquoted into microcentrifuge tubes, marked and stored at -80°C within 2 hours of collection. Clinical data were collected, including gender, age, smoking, drinking and TNM stage. This research was conducted in strict accordance with the protocol approved by the Ethics Committee of Peking Union Medical College Hospital, and a written informed consent was obtained from each subject before their participation in the study.

Diagnosis of primary BC was clinically confirmed by histopathology or biopsy and patients who meet the following criteria were excluded 1) who has severe infection, active clinical comorbidities, or a history of any other malignancy; 2) who has received any chemotherapy, radiotherapy, or operation; 3) older than 75 years or younger than 40 years.

RNA isolation

Total RNA was isolated from tissues using TRIzol reagent according to the manufacturer's protocol (Invitrogen). Briefly, 1 ml TRIzol was added to 100 mg of sample tissues, and then homogenated completely by a power homogenizer. 0.2 mL isopropanol was added to the mixture and incubate at room temperature for 10 minutes. Then the sample was centrifuged at 12,000×g for 10 minutes at 4°C. Wash the pellet, with 1 mL of 75% ethanol and centrifuge the tube at 7500×g for 5 minutes at 4°C. Finally, the RNA pellet was resuspended with RNase-free water. The concentration and purity of the RNA solution was measured by detecting its absorbance at 260/280 and 260/230 nm with NanoDrop 1000A spectrophotometer. (NanoDrop Technologies, Wilmington, DE). All the purified RNA samples were stored at -80°C for further processing.

Reverse transcription and quantitative real-time PCR (q-RTPCR)

To synthesize single-stranded cDNA from total RNA, we used the High Capacity cDNA Reverse Transcription Kits (Thermo). Prepare the 2×RT master mix using the kit components before preparing the reaction plate. 2 μg of total RNA was added to 20 μL reaction. The reverse transcription was performed on a MJ Research PTC-200 Peltier Thermal Cycler (Global Medical Instrumentation) at 25°C for 10 min, 37°C for 120 min, and 85°C for 5 min.

Then qRT-PCR was performed to quantify the expression level of Long non-coding RNA H19, UCA1, and HOTAIR with SYBR Green PCR Master Mix (Thermo) following the manufacturer's instructions. The amplifications reaction contained: master mix, forward primer, reverse primer, and nuclease-free water. GAPDH was used as an intrinsic control. H19 forward, 5'-ATCGGTGCCTCAGCGTTCGG-3' and reverse, 5'-CTGTCCTCGCCGTCACACCG-3'; UCA1 forward, 5'-ACGCTAA CTGGCACCTTGTT-3' and reverse, 5'-TGGGGATTACTGGGGTAGGG-3'; HOTAIR forward, 5'-GCTGCTCCGGAATTTGAGAG-3' and reverse, 5'-TGCTGC CAGTTAGAAAAGCG-3'; GAPDH forward, 5'-AGCCA CATCGCTCAGACAC-3' and reverse, 5'-GCCCAATACGACCAAATCC-3'. The RT-PCR reaction was performed at 95°C for 2 min and in 40 cycles at 95°C for 15 s and 60°C for 1 min on an ABI 7500 thermocycler (Applied Biosystems). Relative gene expression level of each LncRNA was analyzed using the $2^{-\Delta\Delta Ct}$ method.

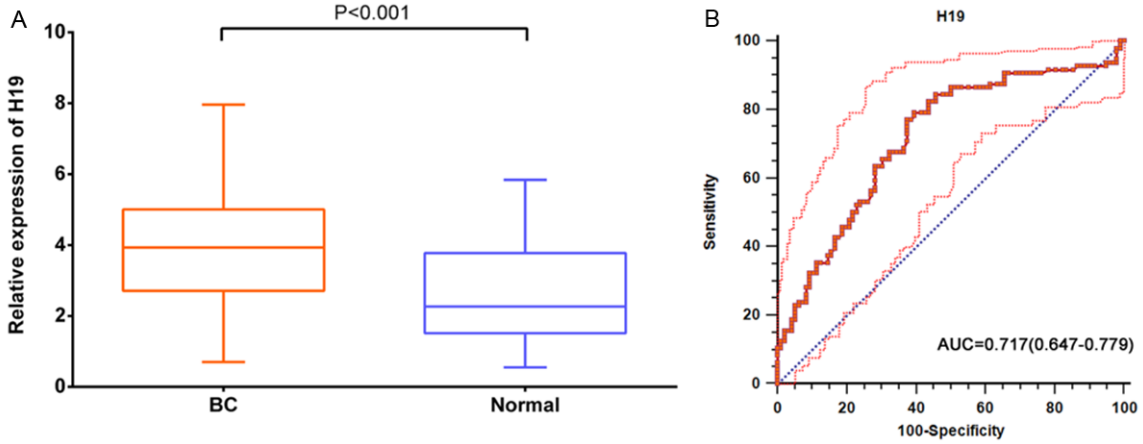

Figure 1. Diagnostic performance of lncRNA H19 in human bladder cancer (BC) tissue. A: Relative expression levels of lncRNA H19 in BC tissue and normal tissues. B: ROC curve analysis of lncRNA H19 in BC tissue and normal tissues.

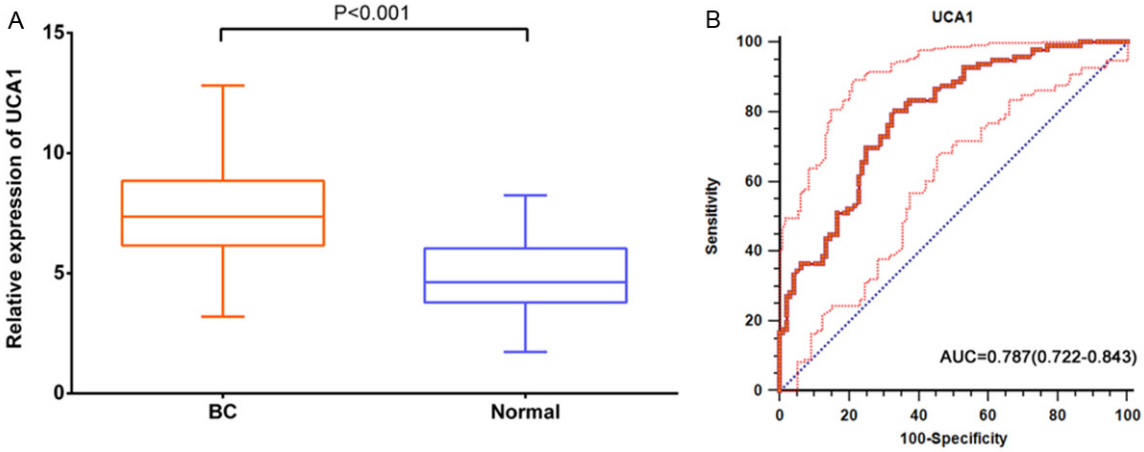

Figure 2. Diagnostic performance of lncRNA UCA1 in human bladder cancer tissue. A: Relative expression levels of lncRNA UCA1 in BC tissue and normal tissues. B: ROC curve analysis of lncRNA UCA1 in BC tissue and normal tissues.

Statistical analysis

Continuous variables are expressed as mean ± standard deviation and categorical variables are expressed as absolute relative frequencies. We compared two groups using the t test for continuous variables and χ^2 test for categorical variables. Receiver operating characteristic (ROC) curves were constructed and area under the ROC curve (AUC) was generated to assess the diagnostic values of the candidate RNAs. We used the Logistic regression model to do the multivariable analysis, and calculate a new LncRNA-based score using the three selected LncRNAs. *P* value <0.05 was considered as statistically significant. All statistical analysis were performed by R software version 3.0.1 (MathSoft Inc., USA), and the graphs were obtained from GraphPad Prism 5.0 (GraphPad Software Inc., CA).

Results

Clinical characteristics of study population

Table 1 shows detailed clinical characteristics of the 96 patients. There were 69 males and 27 females, aged from 42 from 73 (average 61.3). Among these BC patients, alcohol use accounted for 32 (33.3%), tobacco use accounted for 28 (29.2%). 10 patients had a family history of BC. Additionally, 39 patients were diagnosed as TNM I+II and 57 diagnosed as TNM III+IV.

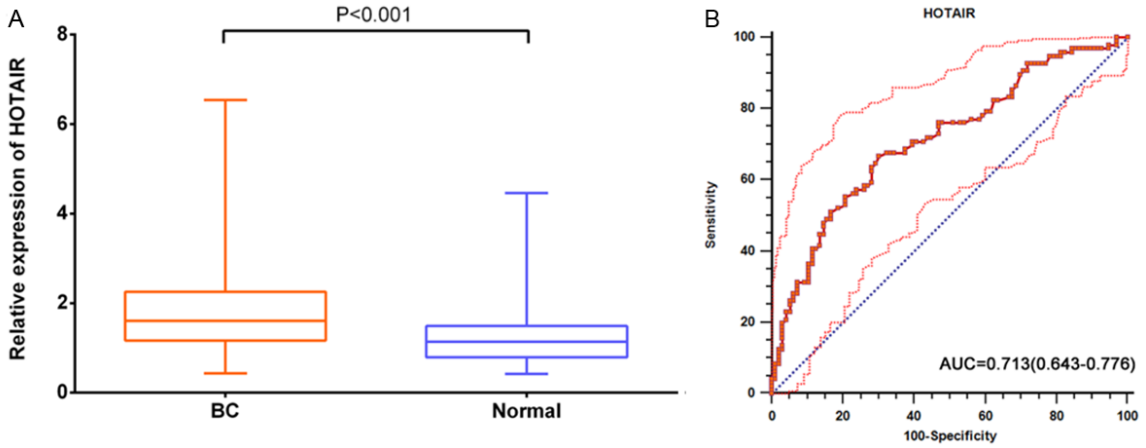

Figure 3. Diagnostic performance of lncRNA HOTAIR in human bladder cancer tissue. A: Relative expression levels of lncRNA HOTAIR in BC tissue and normal tissues. B: ROC curve analysis of lncRNA HOTAIR in BC tissue and normal tissues.

LncRNAs level in bladder cancer tissues

The expression level of three selected lncRNAs was detected by quantitative real-time PCR in BC tissues and pair-matched adjacent normal tissues form 96 BC patients. As **Figures 1A**, **2A**, **3A** shows, the relative expression of H19, UCA1, and HOTAIR in BC tissues were 3.93 (2.75, 5.00), 6.83 (5.25, 8.94), 1.61 (1.17, 2.22), respectively. While the relative expression in the normal tissues were lower, with the media (quartile) of 2.26 (1.53, 3.75), 3.99 (2.46, 5.66), 1.14 (0.80, 1.49), respectively. Statistically significant difference can be observed between BC tissues and pair-matched adjacent normal tissues (all P<0.001). Then we performed the Logistic regression model to calculate a new LncRNA-based score using the three selected LncRNAs: Lnc-Score =0.48*H19+0.49*UCA1+1.2*HOTAIR.

Diagnostic performance of LncRNAs in bladder cancer detection

Receiver operating characteristic (ROC) curves were constructed and area under the ROC curve (AUC) was generated to assess the diagnostic values of the three selected LncRNAs and the Lnc-Score. As **Figures 1B**, **2B**, **3B** shows, the AUC of H19, UCA1, and HOTAIR were 0.717 (0.647-0.779), 0.787 (0.722-0.843), 0.713 (0.643-0.776), respectively. **Table 2** shows the detail information about the ability of the three LncRNAs and Lnc-Score to diagnose the BC in patients. Among 3 selected LncRNAs, UCA1 had the highest sensitivity of 80.2% while HOTAIR had the highest specificity of 69.9%. We also can observe that UCA1 had the highest +LR of 2.41 and the lowest -LR of 0.30. **Figure 4A** shows the distribution of risk for BC, which suggested that BC tissues had higher Lnc-Score than that of adjacent normal tissues. Compared with these 3 separate LncRNAs, Lnc-Score showed the highest AUC, with value of 0.870 (0.814-0.914). Also, the best diagnosability parameters were observed in Lnc-Score, with relative higher sensitivity of 70.8%, the highest specificity of 88.5%, highest +LR of 3.67 and lowest -LR of 0.22.

Discussion

Early diagnosis and surveillance for metastasis of bladder cancer are critical issues. Deregulation of long non-coding RNAs (lncRNAs) has been implicated in urologic malignancies and represents potential markers. In this study, we detected the relative expression levels of lncRNAs H19, UCA1 and HOTAIR in tissues from patients with bladder cancer. Higher expression of these three lncRNAs were observed in the BC tissues than those in normal tissues. Then we performed receiver operating characteristic (ROC) curves and area under the ROC curve (AUC) to assess the diagnostic values of the three lncRNAs. We can observe ideal diagnosability of the three selected lncRNAs, with AUCs of 0.717, 0.787 and 0.713, respectively. Furthermore, we constructed a new score based on these lncRNAs, named lncRNA-score, which show greater diagnosability than 3

Table 2. The receiver operating characteristic (ROC) analysis of lncRNA in human bladder cancer

miRNAs	AUC	95% CI	*P* value	Youden	Cut-off	Sensitivity	Specificity	+LR	-LR
H19	0.717	0.647-0.779	<0.001	0.395	2.65	77.8%	62.5%	2.06	0.37
UCA1	0.787	0.722-0.843	<0.001	0.469	4.94	80.2%	66.7%	2.41	0.30
HOTAIR	0.713	0.643-0.776	<0.001	0.365	1.34	66.7%	69.9%	2.21	0.48
Lnc-Score	0.870	0.814-0.914	<0.001	0.594	6.53	70.8%	88.5%	3.67	0.22

Note. AUC, area under the receiver operating characteristic curve; CI, confidence interval; +LR, positive likelihood ratio; -LR, negative likelihood ratio; *P*-value, compared with AUC of 0.5. LncRNA-Score =0.48*H19+0.49*UCA1+1.2*HOTAIR.

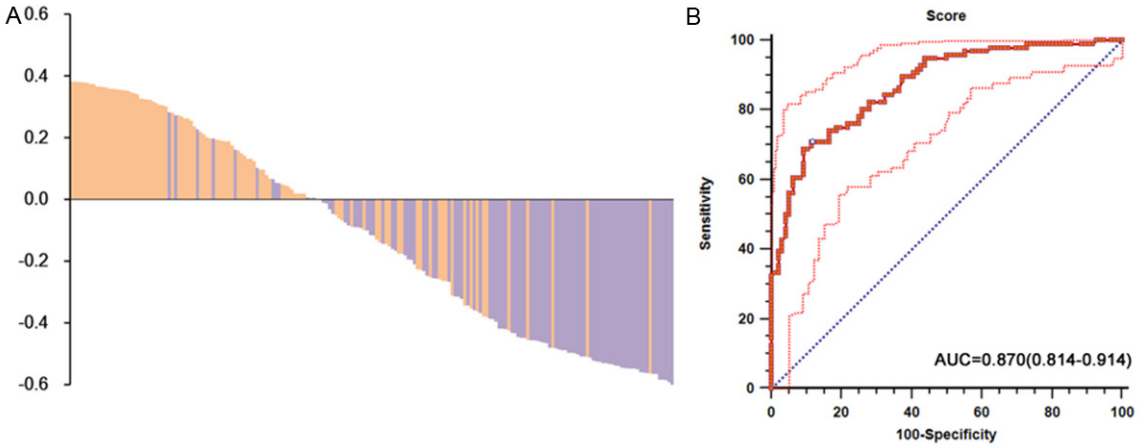

Figure 4. Diagnostic performance of LncRNA-Score in human bladder cancer tissue. A: Relative risk of LncRNA-Score in BC tissue and normal tissues. B: ROC curve analysis of LncRNA-Score in BC tissue and normal tissues.

selected miRNAs alone. Collectively, our study provides evidences that serum level of lncRNAs H19, UCA1 and HOTAIR have great clinical value as promising biomarkers in BC.

Overexpression of non-coding RNAs are implicated in metastasis of human tumors, but most are shorter non-coding like microRNAs (miRNAs) [9]. Recently, an accumulating body of evidence has have linked specific lncRNA gene mutations with cancer, either by overlap of sequencing libraries with previously annotated GENCODE lncRNAs, or by de novo assembly of all available public datasets, raising the possibility of lncRNA-based cancer diagnostics and therapy [6, 10-13]. A study including more than 7,000 RNA-Seq libraries from 25 independent studies found that among 91,013 expressed genes, over 68% (58,648) of genes were classified as lncRNAs, of which 79% (48,952) were previously unannotated [12]. Sequencely, summary of lncRNAs with experimental data supporting the expression and functions of promoting tumor invasion and metastasis was made in some studies [6, 14].

Recently, a number of lncRNAs proved to be able to modulate Urologic neoplasms. The lncRNA RCCRT1 is upregulated remarkably in renal cell carcinoma (RCC) compared with adjacent noncancerous tissues, particularly in high-grade RCC tissues. Furthermore, siRNA-induced depletion of RCCRT1 expression suppressed migration and invasion in RCC cell lines [15]. Another study, which identified a novel lncRNA named SChLAP1, demonstrated that SChLAP1 expression increases with prostate cancer progression, and high SChLAP1 expression is associated with poor outcome after radical prostatectomy by both univariate and multivariate analysis [16].

In present study, we investigated three selected lncRNAs H19, UCA1 and HOTAIR in the bladder cancer tissues and pair-matched adjacent normal tissues from 96 patients. H19, the oldest known lncRNA, was widely studied in cancer biology even before lncRNAs had gained the attention of cancer researchers [6]. The oncogenic properties of H19 were strongly associated with antagonism of the tumor suppressor miRNA let-7 and forced EMT mediated

by the non-histone chromosomal transcriptional regulator HMGA2 [17, 18]. Also, H19 expression is directly induced by the v-myc avian myelocytomatosis viral oncogene homolog (c-MYC) and loss of the p53 tumor suppressor further supporting the importance of H19 as a potent oncogene [19, 20]. Urothelial carcinoma-associated 1 (UCA1), another upregulated lncRNA in bladder cancer, is significantly correlated with greater tumor depth and apoptosis escape [21]. Overexpression of UCA1 enhances ERK1/2 MAPK and PI3-K/AKT kinase activity, causing increased expression of the coactivator p300 and its coactivator cAMP response element-binding protein (CREB), which promotes cell cycle progression, carcinogenesis, and cancer invasion [22, 23]. HOTAIR is a noncoding 2.2-kb RNA gene located downstream, acts as a scaffold for histone modification complexes [6]. In cancer cells, HOTAIR partners with Polycomb Repressive Complex 2 (PRC2, a histone methyltransferase) to induce genome-wide gene silencing and increased cancer invasiveness and metastasis in a manner dependent on PRC2 [24, 25]. Additionally, HOTAIR has been shown to regulate several genes involved in epithelial-to-mesenchyme transition (EMT) including Snail family zinc finger 1 (SNAI1), Poly r(C)-Binding Protein 1 (PCBP 1), Junctional adhesion molecule 2 (JAM2) and ABL proto-oncogene 2 (ABL2) [25-27]. These findings indicate that lincRNAs have active roles in modulating the bladder cancer and may be important targets for cancer diagnosis and therapy.

Conclusion

In conclusion, above all, our results extend the findings of previous studies about lncRNAs H19, UCA1 and HOTAIR in bladder cancer patients. Our data provide the 3 selected lncRNAs were significantly higher in bladder cancer tissues compared to normal tissues, and demonstrated the ideal diagnostic value to distinguish cancer tissues from normal tissues. However, whether this correlation is exactly proportional requires carefully scrutiny and study on a larger sample is needed to confirm this results.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Wei Wang, Department of Drug Control, Criminal Investigation Police University of China, No. 83, The Tawan Street, Huanggu District, Shenyang 110035, P. R. China. Fax: +86-024-86982468; E-mail: zhigangji352@sina.com

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