Expression signatures of exosomal long non-coding RNAs in urine serve as novel non-invasive biomarkers for diagnosis and recurrence prediction of bladder cancer

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Abstract

Recently, expression signatures of exosomal long non-coding RNAs (lncRNAs) have been proposed as potential non-invasive biomarkers for cancer detection. In this study, we aimed to develop a urinary exosome (UE)-derived lncRNA panel for diagnosis and recurrence prediction of bladder cancer (BC). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed to screen and evaluate the expressions of eight candidate lncRNAs in a training set (208 urine samples) and a validation set (160 urine samples). A panel consisting of three differently expressed lncRNAs (MALAT1, PCAT-1 and SPRY4-IT1) was established for BC diagnosis in the training set, showing an area under the receiver-operating characteristic (ROC) curve (AUC) of 0.854. Subsequently, the performance of the panel was further verified with an AUC of 0.813 in the validation set, which was significantly higher than that of urine cytology (0.619). In addition, Kaplan-Meier analysis suggested that the up-regulation of PCAT-1 and MALAT1 was associated with poor recurrence-free survival (RFS) of non-muscle-invasive BC (NMIBC) (p < 0.001 and p = 0.002, respectively), and multivariate Cox proportional hazards regression analysis revealed that exosomal PCAT-1 overexpression was an independent prognostic factor for the RFS of NMIBC (p = 0.018). Collectively, our findings indicated that UE-derived lncRNAs possessed considerable clinical value in the diagnosis and prognosis of BC.

Keywords: Bladder cancer, Urine exosomes, LncRNA, Non-invasive biomarkers, Diagnosis, Recurrence prediction

Bladder cancer (BC) is the most common malignancy of the urinary tract worldwide [1]. About 75% of patients are classified as non-muscle-invasive BC (NMIBC), which features a high recurrence rate. Moreover, roughly half of the muscle invasive BC (MIBC) patients will progress to metastasis and die within 3 years [2]. The poor prognosis of BC is partially due to lack of an effective means for early diagnosis. At present, the diagnosis of BC mainly relies on cystoscopy. However, the invasiveness of such procedure limit its use in mass cancer screening. Urine cytology has been proved to be ineffective as a tool to detect low-grade malignancy. Therefore, discovery of effective biomarkers for detection and recurrence prediction of BC can play pivotal roles in improving the prognosis of BC patients.

Exosomes are small vesicles with a diameter of approximately 30–150 nm. They are distributed in nearly all body fluids, including blood and urine. Exosomes can act as vehicles in cell-to-cell communication by transferring oncogetic molecules and play significant roles in tumorigenesis, progression and metastasis [3]. These functional contents are not stochastically packed into exosomes, which may rely on signature motifs, leading to the variation of exosomal contents under different pathological conditions or in different original cell types [4]. Studies have shown that BC cells can secrete exosomes into the urine and lncRNAs have been found to be stably present in exosomes [5]. Therefore, analyzing the expression profiles of urinary exosome (UE)-derived lncRNAs would provide valuable clues
for diagnosis of BC. In this study, we systematically analyzed the expression profiles of UE-derived IncRNAs in BC patients and established a three-IncRNA panel for BC detection. Finally, we explored the prognostic value of the selected exosomal IncRNAs.

Results and discussion

Characterization of UEs

Transmission electron microscopy (TEM) showed UEs have a diameter of 60–150 nm with a cup-shaped membrane (Fig. 1a). Western blotting of UEs demonstrated the presence of CD9 and TSG101, which are exosome markers (Fig. 1b). Nanoparticle tracking analysis (NTA) found that particles ranging from 20 nm to 200 nm in diameter accounted for 98.1% (Fig. 1c). The flow cytometry showed that the positive rate of CD63 and CD81 specific antibodies on the exosome surface was 90.9% and 93.6%, respectively (Fig. 1d). Collectively, these data indicated that exosomes existed in urine, which laid a foundation for further study of exosomal biomarkers. The methods are explained in Additional file 1.

Selection and evaluation of candidate UE-derived IncRNAs in BC patients

Eight IncRNAs (MALAT1, PCAT-1, SPRY4-IT1, UCA1, MEG3, H19, UBC1 and TUG1), which have been reported to play functional roles in tumorigenesis, were selected as candidate molecules [6–8]. Then, in the training set, the expressions of eight IncRNAs in 104 BC patients and 104 healthy controls were assessed by qRT-PCR. MALAT1, PCAT-1 and SPRY4-IT1 were significantly up-regulated in BC patients compared with the healthy controls (p < 0.001) (Additional file 3: Table S2). To evaluate the performance of the identified IncRNAs for BC detection, we performed ROC curves in training set. The diagnostic accuracy of MALAT1, PCAT-1 and SPRY4-IT1, measured by AUC, was 0.844 (95% CI = 0.787 to 0.890, sensitivity = 72.1% and specificity = 84.6%), 0.832 (95% CI = 0.774 to 0.880, sensitivity = 72.1% and specificity = 81.7%) and 0.760 (95% CI = 0.696 to 0.817, sensitivity = 66.3% and specificity = 76.9%), respectively (Additional file 4: Figure S1 a-c).

Analysis of the stability of identified IncRNAs in UEs

Next, two experiments were performed to verify the stability of UE-derived IncRNAs (MALAT1, PCAT-1 and SPRY4-IT1), considering that this is an essential prerequisite for biomarkers. Firstly, urine samples and exosome isolated nucleic acids were incubated with RNase A for 0, 30, 60 and 90 min. Strikingly, RNase A had no effect.
on the level of exosomal lncRNAs in the urine group (Additional file 6: Figure S3 a-c). However, the exosome isolated nucleic acids group was completely degraded by the treatment of RNase A within 30 min (Additional file 6: Figure S3 d-f). Secondly, urine samples were stored at −80 °C for 1, 2 and 3 months. Results indicated that this treatment had no effect on the expression levels of MALAT1, PCAT-1 and SPRY4IT1 in UEs (Additional file 6: Figure S3 g-i). Collectively, our data indicated that the exosomal membrane can protect lncRNAs from being degraded, and their excellent stability makes exosomal lncRNAs ideal biomarkers for tumor diagnosis.

Establishment of the UE-derived lncRNA panel for BC diagnosis
Considering that combinations of tumor markers can improve the diagnostic accuracy, multivariate logistic regression model was performed in the training set to establish the selected exosomal lncRNA panel. The predictive probability of being diagnosed with BC was calculated using the equation as follows: Logit \((P) = 0.6577 - 0.0695 \times MALAT1 - 0.0686 \times PCAT-1 - 0.0015 \times SPRY4-IT1\). The AUC of the panel was 0.854 (95% CI = 0.799–0.899, sensitivity = 70.2% and specificity = 85.6%, Fig. 2a).

Validation of the diagnostic performance of the lncRNA panel
To further verify the diagnostic performance of the panel, ROC analysis was performed in the validation set. Results showed that the AUC of the panel was 0.813 (95% CI = 0.744–0.870, sensitivity = 62.5% and specificity = 85.0%) (Fig. 2b). Currently, urine cytology is widely used in clinical practice, but it has relatively poor sensitivity. Therefore, we compared the diagnostic performance

![Graphs showing the diagnostic performance of the lncRNA panel and urine cytology for BC diagnosis.](image)

**Fig. 2** Evaluation the diagnostic performance of 3-lncRNA panel and urine cytology for BC diagnosis. ROC analysis was used to evaluate the performance of 3-lncRNA panel for the detection of BC in the training set (a) and in the validation set (b); ROC analysis revealed the diagnostic performance of urine cytology for BC diagnosis in the validation set (c).
between the panel and urine cytology. As expected, the AUC of urine cytology for BC detection was 0.619 (95% CI = 0.539–0.694, sensitivity = 25% and specificity = 98.7%) (Fig. 2c), which was significantly lower than that of the panel.

Correlation between the three UE-derived IncRNAs and clinicopathological characteristics

Next, we analyzed the correlation between the three UE-derived IncRNAs and clinicopathological characteristics of the BC patients. Results demonstrated overexpression of UE-derived PCAT-1 and SPRY4-IT1 were correlated with advanced TNM stage (all at p < 0.05). However, we did not find any significant association between the three IncRNAs and age, sex, tumor grade or positive lymph node metastasis (all at p > 0.05) (Additional file 7: Table S3).

Correlation between the three UE-derived IncRNAs and recurrence-free survival (RFS)

To explore prognostic value of the three IncRNAs, BC patients were followed-up in the validation set. In the NMIBC group, results showed that patients with up-regulated MALAT1 and PCAT-1 had a significantly lower RFS (p = 0.002 and p < 0.001, respectively, Fig. 3a-b) compared with their corresponding counterparts. Afterwards, univariate Cox proportional hazards regression showed that there was a significant correlation between RFS of NMIBC and PCAT-1 (p = 0.001), MALAT1 (p = 0.005) or tumor stage (p = 0.001). Multivariate analysis revealed that PCAT-1 (p = 0.018) and tumor stage (p = 0.036) were independent prognostic factors for the RFS of NMIBC (Additional file 8: Table S4). However, none of the three IncRNAs were correlated with the recurrence of MIBC patients (all at p > 0.05, Additional file 9: Table S5).

Researchers have revealed UE-derived HOTAIR could serve as a biomarker for BC. However, their study are only conducted in a very small amount of urine samples, which are not verified in a larger population [9]. Moreover, considering that the progression of BC is a complex pathophysiological process, thus no single lncRNA can stand alone as a biomarker, but instead panel consisting of several lncRNAs will be necessary for BC diagnosis.

Conclusions

We established a three-IncRNA panel for BC diagnosis through analyzing UE-derived IncRNAs, and identified that PCAT-1 could act as an independent risk factor for RFS of NMIBC. Further multi-center studies are necessary to verify the diagnostic efficiency of such panel before it could be adopted into clinical practice.
Availability of data and materials
The datasets used and/or analyzed during the current study are available within the manuscript and its supplementary information files.

Authors’ contributions
YZ and LTD: performed experiments, analyzed data and drafted the manuscript; LTD, YSW and CXW: initiated, organized and supervised the study; LTD, LSW and XMJ: critically revised the manuscript; YZ, WLD and SJZ: collected urine samples; KQY: provide clinical information; LLW, JL, YHZ and WLD: provided technical support. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate
This study was reviewed and approved by the Ethics Committee of Qilu Hospital of Shandong University, and all of the participants signed an informed consent form.

Consent for publication
All the patients involved in our study obtained written consent for publication.

Competing interests
The authors declare that they have no competing interests.

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References
1. Siegel RL, Miller KD, Jemal A. Cancer statistics 2018. CA Cancer J Clin. 2018; 68:7–30.
2. Babjuk M, Bohle A, Burger M, Capoun O, Cohen D, Comperat EM, et al. EAU guidelines on non-muscle-invasive urothelial carcinoma of the bladder: update 2016. Eur Urol. 2017;71:447–61.
3. Zhang X, Yuan X, Shi H, Wu L, Qian H, Xu W. Exosomes in cancer: small particle, big player. J Hematol Oncol. 2015;8:83.
4. Santangelo L, Guarato G, Cicchini C, Montaldo C, Mancone C, Tarallo R, et al. The RNA-binding protein SYNCRP is a component of the hepatocyte exosomal machinery controlling microRNA sorting. Cell Rep. 2016;17:799–808.
5. Beckham CJ, Olsen J, Yin PN, Wu CH, Ting HJ, Hagen FK, et al. Bladder cancer exosomes contain EDIL-3/Del1 and facilitate cancer progression. J Urol. 2014;192:583–92.
6. Liu L, Liu Y, Zhuang C, Xu W, Fu X, Lv Z, et al. Inducing cell growth arrest and apoptosis by silencing long non-coding RNA PCAT-1 in human bladder cancer. Tumour Biol. 2015;36:7685–9.
7. Fan Y, Shen B, Tan M, Mu X, Qin Y, Zhang F, et al. TGF-B-induced upregulation of malat1 promotes bladder cancer metastasis by associating with suz12. Clin Cancer Res. 2014;20:1531–41.
8. Liu D, Li Y, Luo G, Xiao T, Yao D, Wu X, et al. LncRNA SPRY4-IT1 sponges miR-101-3p to promote proliferation and metastasis of bladder cancer cells through up-regulating EZH2. Cancer Lett. 2017;388:281–91.
9. Berrondo C, Flax J, Kucherov V, Siebert A, Otinski T, Rosenberg A, et al. Expression of the long non-coding RNA HOTAIR correlates with disease progression in bladder cancer and is contained in bladder cancer patient urinary exosomes. PLoS One. 2016;11:e0147236.