LncRNA and gene expression profiling of human bladder cancer

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Abstract: Bladder cancer (BC) is the most common urologic neoplasms with a high rate of recurrence. The formation of BC is a complicated process, resulting from smoke, drugs, or bladder inflammation. However, the underlying mechanisms of BC tumorigenesis remained unclear. In this study, we performed microarray analysis of three paired paratumor (PT) and tumor tissues from BC patients. In comparison with PT, BC tissues displayed 1136 upregulated and 1199 downregulated lncRNAs, and 1347 upregulated and 953 downregulated mRNAs. In addition, the upregulated mRNAs were focused in SNARE interactions, amino sugar and nucleotide sugar metabolism, and antigen processing pathways. The common downregulated genes were mainly focused in amino acid metabolism and drug metabolism signaling. More importantly, cytokine-related genes were highly expressed in BC, which suggest that the dysregulation of immunoregulatory process and amino acid metabolism played a crucial role in the oncogenesis of BC.

Keywords: amino acid metabolism; bladder cancer; cytokine signaling pathway; lncRNA

1. Introduction

Bladder cancer (BC) is one of the most common cancer worldwide, and 549,000 new cases and 200,000 deaths were estimated worldwide in 2018[1]. The incidence of BC ranks first in China and increases constantly, which becomes a serious threat to public health[2]. Clinically, non-muscle-invasive BC (NMIBC) and MIBC are the major two types of BC. About 70% of BC is NMIBC, characterized by low mortality and high recurrence rate. MIBC accounts for 30% of BC and has a mortality rate more than 50%[3].
Clinically, surgical resection combined with infusion of chemotherapy drugs is the major treatments of BC. For NMIBC, transurethral resection of bladder tumor combined with Bacillus Calmette–Guérin (BCG)-assisted immunotherapy is the standard therapy, with a complete response rate of 70%–80%\(^\text{[4]}\). Chemotherapy combined with surgery, or chemotherapy combined with radiotherapy to reduce local recurrence, is the recommended therapy for advanced, invasive, or metastatic BC. The objective response rate of locally advanced and metastatic BC patients receiving standard chemotherapy regimens containing gemcitabine combined cisplatin chemotherapy or methotrexate, vinblastine, doxorubicin, and cisplatin combination therapy is 50%\(^\text{[5]}\). However, the overall survival (OS) and median progression-free survival were 14 and 8 months, respectively\(^\text{[6]}\). In addition, BCG therapy and chemotherapy may cause severe local or systemic side effects in BC patients. Hence, it is of importance to develop new and effective therapy methods.

The tumorigenesis of BC is a complex process, resulting from smoke, drugs, or bladder inflammation\(^\text{[7]}\). To decipher these mechanisms, researches profiled the DNA mutations and RNA expression of BC extensively. The cancer genome atlas (TCGA) found that the OS of MIBC patients was correlated with DNA mutation load and neoantigen load\(^\text{[8]}\). Patients with low mutation load had shorter OS time than those with high mutation load\(^\text{[9]}\).

By RNA sequencing, TCGA classified MIBC into five types according to molecular phenotype. The luminal-papillary patients had the best prognosis and a 5-year survival rate of 60%. However, neuronal patients only had a 5-year survival rate of 17%\(^\text{[7]}\). In addition, different molecular types of BC have different clinical response, among which luminal type is generally insensitive to cisplatin chemotherapy, basal and squamous type are sensitive to chemotherapy, and luminal-infiltrated type is sensitive to immunotherapy\(^\text{[7]}\). Thus, the high-throughput sequencing combined with the clinical characteristics could provide effective information for carcinogenesis and development of BC, which could be applied to predict the prognosis of patients and explore novel treatment methods. In this study, we profiled RNA expression of three paired paratumor (PT) and tumor tissues from BC patients and found that cytokine-related genes were highly expressed in BC, which suggest that the deregulation of immunoregulatory process played a crucial role in the oncogenesis of BC.

2. Method and Materials

2.1. Patient tissues and mice

Primary human BC and normal bladder tissues were provided by the Second Affiliated Hospital of Kunming Medical University College (Kunming, China) with informed consent, which is approved by the Research Ethics Board at the Second Affiliated Hospital of Kunming Medical University. Detailed clinical information for the BC patients were summarized in Supplementary Table S1.

2.2. Microarray experiment

Arraystar human LncRNA Microarray V3.0 can detect about 30,586 lncRNA and 26,109 encoded transcripts. NanoDrop 2000 was used to assess the quantity and quality of RNA. According to the gene expression analysis protocol based on Agilent monochrome microarray (Agilent technology), the samples were labeled and hybridized by array. Briefly, mRNA was purified from total RNA after removal of tRNA (mRNA-ONLY™ Eukaryotic mRNA Isolation Kit, Epicentre). Then, each sample was amplified and transcribed into fluorescent cRNA along the entire length of the transcripts without 3' bias utilizing a random priming method (Arraystar Flash RNA Labeling Kit, Arraystar). RNeasy Mini Kit (Qiagen) was applied for the purification of labeled cRNAs. NanoDrop 2000 was applied to assess the concentration of the labeled cRNAs. 1 µl of 25 × fragmentation buffer and 5 µl 10 × blocking agent were used for the fragmentation of labeled cRNA. After dilution by 25 µl 2 × GE hybridization buffer, the labeled cRNA was dispensed into the gasket slide. In an Agilent Hybridization Oven, the hybridized arrays were carried out for 17 h at 65°C. Then, the Agilent DNA Microarray Scanner was applied to wash, fix, and scan the slides.

Agilent Feature Extraction software (version 11.0.1.1) was used to analyze acquired array images. Quantile normalization and subsequent data processing were performed with using the GeneSpring GX v12.1 software package (Agilent Technologies). After normalization, mRNAs and lncRNAs that have at least half flags in present or marginal were selected for data analysis. Differentially expressed mRNAs and lncRNAs between tumor and PT were identified by FDR/P-value filtering.

2.3. Statistical analyses

The mean ± standard error of the average was applied to present the data. Statistical methods were described in the figure legends for each data set. The differences between tumor and PT were compared through two-tailed Student’s t-tests. *P < 0.05 was set as statistical significance.

3. Results

3.1. Differentially expressed lncRNA in BC

Differentially expressed lncRNA and genes between human paired para-tumor and tumor tissues from three BC patients were identified by a Arraystar Human LncRNA Microarray analysis (Supplementary Table S1). Agilent Feature Extraction software (version 11.0.1.1) was used to analyze acquired array images. Differentially expressed lncRNAs and mRNAs between the two samples were identified by folding variation filtering. There were 1136 upregulated...
and 1199 downregulated lncRNAs [Figure 1a-d]. The most upregulated lncRNAs including RP11-332K15.1, XX-CR54.1, BC038578, XLOC_002630, XLOC_002630, LINC00348, LOC339535, AC098973.2, RP11-242J7.1, and RP11-414K1.3 and most downregulated lncRNAs including LOC389023, LOC389023, LOC389023, SSTR5-AS1, FAM99A, LOC100506229, RP11-432J5.1, RP3-523K23.2, RP11-372E1.4, and AC104809.2 were also displayed [Figure 1e-f].

3.2. Differentially expressed genes in BC

In comparison with PT, BC tissues displayed 1347 upregulated and 953 downregulated mRNAs [Figure 2a-c]. The most upregulated genes including XAGE5, COX7B2, MAGEB2, MUC15 MUC15, TPTE, DCAF4L2, MUC15, CTAG2, and FOXR2 and most downregulated genes including SLCO1B3, PROM1, BHMT, PROM1, SLC22A1 NR1I3, IFIT1, NR1I3, STMN2, and NR1I3 were also displayed [Figure 2d-e]. Clustered by DAVID tools[9], the 1347 upregulated genes were focused in the SNARE

Figure 1. Differentially expressed lncRNA in bladder cancer. (a) Hierarchical cluster heat map from the microarray results of paratumor (PT) and tumor (T) from three BC patients. (b and c) Scatter plot and volcano plot comparing global lncRNA expression profiles between PT and T. Green lines, 2-fold differences in either direction in lncRNA expression levels. (d) Venn diagram representing overlapping upregulated and downregulated lncRNA in T from three BC patients. (e and f) The most upregulated and downregulated lncRNAs in T compared to PT. Data are presented as mean ± standard deviation, *P < 0.05.
interactions, amino sugar and nucleotide sugar metabolism, and antigen processing pathways [Figure 3a], whereas the 953 downregulated genes were mainly concentrated in the amino acid metabolism and drug metabolism signaling [Figure 3b]. More importantly, cytokine-related genes such as IL1B, IL1R2, IL2RG, IL7, IL15, CCR10, CCL17, CCL18, CCL20, CXCL3, CXCL5, CXCL6, CXCL9, CXCL10, CXCL13, IFNG, IFNAR2, TNFRSF10A, TNFRSF11B, and INHBA were higher in BC than those in PT [Figure 3a and c], which suggest that the dysregulation of immunoregulatory process played a crucial role in the tumorigenesis of BC. Furthermore, amino acid metabolism-related genes such as BHMT, ADH1C, OTC, TAT, CYP2B6, ADH1A, CYP2C19, ADH1B, CYP2C9, ADH4, CYP2C8, UGT1B10, CYP1A2, GPT2, ADH6, SDS, HPD, CYP3A5, CPS1, and CYP3A4, were significantly decreased in BC [Figure 3b and d], which suggest that the abnormality of amino acid metabolism played a pivotal role in the tumorigenesis of BC.

4. Discussion

BC is the most common urologic neoplasms with a high rate of recurrence. However, the therapy regimen of BC has not improved during the past decades [10]. The profiling of DNA mutations and RNA expression of BC could provide novel biomarkers for diagnosis, surveillance, and therapy. Here, we performed RNA sequencing of three paired PT and tumor tissues from BC patients and found that

Figure 2. Differentially expressed genes in bladder cancer. (a and b) Scatter plot and volcano plot comparing global gene expression profiles between paratumor (PT) and tumor (T). Green lines, 2-fold differences in either direction in gene expression levels. (c) Venn diagram representing overlapping upregulated and downregulated genes in T compared to PT. (d and e) The most upregulated and downregulated genes in T compared to PT. Data are presented as mean ± standard deviation, *P < 0.05.
cytokine signaling pathways and amino acid metabolism deregulated in oncogenesis.

LncRNA is over 200 bp but has no protein-coding ability\cite{11}. LncRNA has obvious tissue-specific expression, and spatial and temporal expression pattern\cite{12}. More and more research indicated that lncRNA participated in the regulation of a variety of important physiological processes including X chromosome inactivation, transcriptional activation or inhibition, chromosome modification, protein stability, or activity regulation. LncRNA also contributed to the formation and progression of tumors\cite{13}. For example, HOTAIR was able to reshape the status of chromosomes and ultimately promoted tumor metastasis\cite{11}. LncRNA-p21 could bind to HIF1 and VHL, abrogate the stability of the HIF1-VHL complex, maintain the protein level of HIF1, and promote the Warburg effect and tumor progression\cite{14}. In this study, there were 1136 upregulated and 1199 downregulated lncRNAs in BC. The specific function of those lncRNA in BC tumorigenesis should be validated further.

MIBC was classified into luminal-papillary, luminal-infiltrated, luminal, basal/squamous, and neuronal types by RNA sequencing. Different molecular types of BC have different clinical response\cite{7}. Here, 1347 upregulated and 953 downregulated mRNAs were identified in three BC tissues compared to PT tissues. The upregulated genes focused in the SNARE interactions, amino sugar and nucleotide sugar metabolism, and antigen processing pathways, whereas the downregulated genes were mainly concentrated in the amino acid metabolism and drug metabolism signaling. The mechanisms underlying BC tumorigenesis mediated by these enriched signaling pathways might be the focus of further study.

To uncover novel candidates for the diagnosis and therapy of BC, we found that cytokine-related genes, such as *IL7*, *CCR10*, *IL1R2*, *CCL17*, *IL2RG*, *CCL18*, *CXCL3*, *IFNG*, *CXCL6*, *TNFRSF10A*, *IL4*, *CXCL10*, *INHBA*, *IFNGR2*, *CXCL5*, *TNFRSF11B*, *CXCL13*, *IL15*, and *CXCL10*, were highly expressed in BC. In addition, amino acid metabolism-related genes, such as *BHMT*, *ADH1C*, *OTC*, *TAT*, *CYP2B6*, *ADH1A*, *CYP2C19*, *ADH1B*, *CYP2C9*, *ADH4*, *CYP2C8*, *UGT2B10*, *CYP1A2*, *GPT2*, *ADH6*, *SDS*, *HPD*, *CYP3A5*, *CPS1*, and *CYP3A4*, significantly decreased in BC. These data indicated that dysregulation of immunoregulatory process and amino acid metabolism played a pivotal role in the tumorigenesis of BC. However, the exact effect of those genes in BC oncogenesis should be validated further.

In conclusion, our findings demonstrate the differentially expressed lncRNA and genes of BC compared to PT tissues. The SNARE interactions, nucleotide sugar and amino sugar metabolism, antigen processing, amino acid metabolism, and drug metabolism signaling pathways were significantly enriched. More importantly, cytokine-related genes were

\[ FIGURE 3. \] Pathway enrichment of bladder cancer. (a and b) Gene ontology analysis of 1347 overlapping upregulated and 953 overlapping downregulated genes. (c) Upregulated genes enriched in SNARE interactions, amino sugar and nucleotide sugar metabolism, and antigen processing pathways. (d) Downregulated genes enriched in amino acid metabolism and drug metabolism signaling. Data are presented as mean ± standard deviation, *p < 0.05.
highly expressed in BC, which suggest that the dysregulation of immunoregulatory process also played a crucial role in the oncogenesis. This study provides the underlying targets for the diagnosis and treatment of BC. In future, more tumor tissues and proteomics techniques could be used to obtain comprehensive information on the expression levels of RNA and protein during BC tumorigenesis.

5. Authors’ Contributions

6. Acknowledgments
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Conflicts of Interest
The authors declare no potential conflicts of interest.

References
Supplementary Table

**Supplementary Table 1.** Clinical characteristics of the bladder carcinoma patients.

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