Int J Clin Exp Med 2014;7(5):1244-1254
www.ijcem.com /ISSN:1940-5901/IJCEM0000255

Original Article

Gene microarray analysis of the lncRNA expression profile in human urothelial carcinoma of the bladder

Huarong Luo*, Xin Zhao*, Xiaodong Wan, Shengsong Huang, Denglong Wu

*Department of Urology, Tongji Hospital of Tongji University, Shanghai 200065, China. *Equal contributors.*

Received March 17, 2014; Accepted May 11, 2014; Epub May 15, 2014; Published May 30, 2014

Abstract: Objective: To analyze the expression profile variation of lncRNAs in normal urinary bladder tissue and urothelial carcinoma of the urinary bladder through microarray technology. The differentially expressed lncRNAs were identified and classified, and their biological information was analyzed. The data obtained in the study will prove helpful for the diagnosis, treatment, and prevention of urothelial carcinoma of the bladder. Materials and methods: Three specimens of urothelial carcinoma of the bladder and three specimens of normal bladder tissue were identified by histology. The total RNA was isolated from the bladder urothelial carcinomas and normal tissue and purified. The targets were mixed and hybridized with the genes on the microarray, which contained thirty thousand lncRNAs. The bladder urothelial carcinomas were then compared with the normal bladder tissue. The lncRNAs that were differentially expressed between the two groups were identified based on the signal-to-noise ratios using the Agilent Feature Extraction software and were analyzed with the Agilent Genespring GX software (Agilent). The outcome was obtained, and the biological information of these genes was deposited in GenBank. Results: The expression profile of lncRNAs was significantly different between normal bladder tissue and urothelial carcinoma of the bladder. Compared with normal bladder tissue, 1,122 lncRNAs exhibited at least a twofold, significant difference ($P < 0.05$) and are thus regarded as differentially expressed lncRNAs. Of these, 734 and 388 lncRNAs were upregulated and down regulated. The differentially expressed lncRNAs in the urothelial carcinoma of the bladder are distributed on every chromosome, and most of these lncRNAs are distributed on chromosomes 1, 2, 3, 4, 6, and X. Conclusions: Urothelial carcinoma of the bladder is a complicated disease that involves the regulation of multiple genes and the participation of multiple chromosomes. Some of the differentially expressed lncRNAs that were upregulated, such as AK124776, lincRNA-RAB12-1, KRT8P25, RP11-474J18.4, AC000110.1, KRT8P13, KRT8P10, BC072678, and downregulated, such as nc-HOXB9-206, RP11-160A10.2, nc-HOXA11-86, nc-HOXD10-7, nc-HOXB9-205, CES4, nc-HOXD12-3, systematic research on these lncRNAs will help clarify the mechanisms of urothelial carcinoma of the bladder and guide the early diagnosis and treatment of this cancer in the future.

Keywords: lncRNA, gene microarray, urinary bladder, urothelial carcinoma, expression profile

Introduction

Carcinoma of the bladder is the ninth most common malignancy of the genitourinary tract [1] and has several pathological types, including urothelial cell carcinoma (UCC), adenocarcinoma, and squamous cell carcinoma. UCC is the most common pathological type of bladder carcinoma. Early diagnosis and early treatment is important for the cure of UCC. The objective of our research is to identify a suitable tumor marker with high sensibility and high specificity to allow the early detection of UCC. It is well known that the pathogenesis of UCC is complicated and is the result of the interaction of multiple factors, including heredity, environment, and metabolism factors. Among these, smoking remains a major risk factor [2, 3]. The germination of carcinoma is related to multiple cytokines and proteins that are regulated by many types of genes. In recent years, we have started to determine the cellular- and molecular-level changes induced by UCC and have attempted to investigate the cellular and molecular mechanism to ultimately find a UCC-specific marker. To date, we have obtained a large number of valuable experimental evidence. However, it is difficult to explain the germination of UCC through a single molecule or gene. The procedure involves multiple processes and multiple

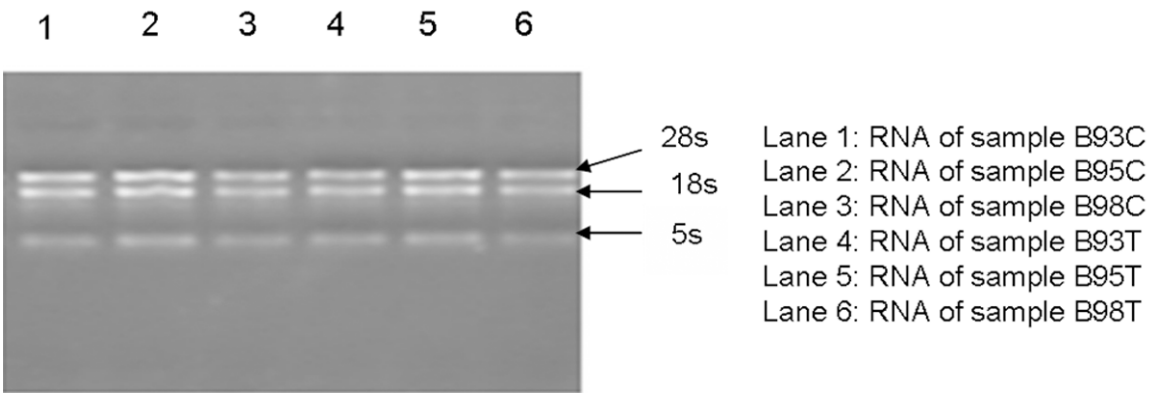

Figure 1. Analysis of RNA integrity and genomic DNA contamination through denaturing agarose gel. *The 28s and 18s rRNA bands are clear and intact. However, the 28s band is twice as intense as the 18s band.

Table 1. Quantification and quality assurance of RNA using the NanoDrop ND-1000

Sample ID	OD260/280 Ratio*	OD260/230 Ratio*	Conc. (ng/uL)	Volume (uL)	Quantity (ng)	QC result (Pass/Fail)
B93C	1.88	2.04	352.40	10	3524.00	Pass
B95C	1.88	2.03	254.01	10	2540.10	Pass
B98C	1.90	2.08	620.42	10	6204.20	Pass
B93T	2.02	2.03	1257.93	10	75475.80	Pass
B95T	1.84	1.97	127.72	10	1277.20	Pass
B98T	1.93	1.98	521.22	10	20848.80	Pass

*The OD260/280 ratio measured with a spectrophotometer should be closer to 2.0 for pure RNA (ratios between 1.8 and 2.1 are acceptable). The OD260/230 ratio should be higher than 1.8.

genes. With the development of the combination of genomic and proteomic studies, recent studies are limited principally to DNA, coding RNA, and protein. As a result, an increasing number of markers that are likely related to UCC have been found and reported [4-18]: (1) DNA methylation markers (e.g., NMP22), (2) mRNA markers (e.g., survivin, PSCA, p63, and c-FLIP), and (3) cytokine or protein markers (e.g., BLCA-1, BLCA-4, DJ-1, HSP90a, CCL 18, OCT4, COX-2, IL-8, and HER-2). Nevertheless, we have not found a specific marker for UCC. With the development of sensitive microarrays, numerous non-coding RNAs (ncRNA) have been found, and this number far exceeds the number of coding RNAs in the mammalian transcriptome. Increasing studies have revealed that long non-coding RNAs (lncRNA) have a variety of important functions, including roles in transcription, splicing, translation, nuclear factor trafficking, imprinting, genome rearrangement, and chromatin modification [19-21]. Thus, we hypothesize that lncRNAs, in combination with mRNAs, are also involved in the germination and development of tumor. The aim of this study is to analyze the expression profile variation of lncRNAs in normal urinary bladder tissue and urothelial carcinoma of the bladder through microarray technology to explore the function of lncRNAs in UCC and to attempt to identify a specific gene that will prove helpful to the diagnosis, treatment, and prevention of UCC.

Materials and methods

Samples

The RNA quantity and quality were measured using a NanoDrop ND-1000, and the RNA integrity was assessed by standard denaturing agarose gel electrophoresis.

Microarray

The Arraystar Human LncRNA Array v2.0 was designed for researchers who are interested in profiling both LncRNAs and protein-coding RNAs in the human genome. A total of 33,045 LncRNAs were collected from authoritative data sources, including RefSeq, UCSC Known Genes, Ensembl, and the related literature.

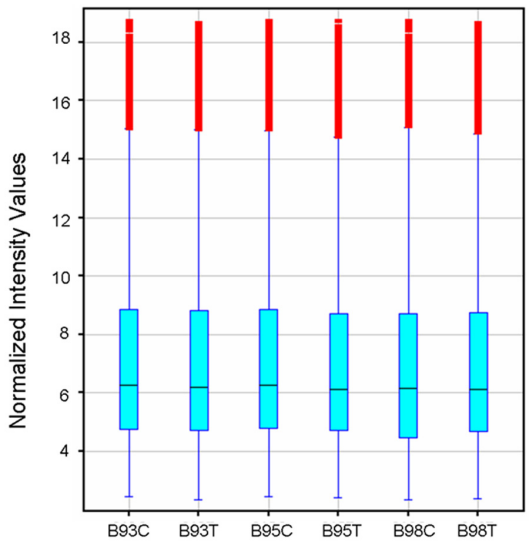

Figure 2. Box plot of all of the tested samples in the dataset after normalization. All six samples in the dataset were normalized, and the medians are all close to six. It is clear that the distributions of the log2-ratios are nearly the same.

RNA labeling and array hybridization

The sample labeling and array hybridization were performed according to the Agilent One-Color Microarray-Based Gene Expression Analysis protocol (Agilent Technology) with minor modifications. Briefly, the mRNA was purified from the total RNA after the removal of rRNA (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. The labeled cRNAs were purified with a RNeasy Mini Kit (Qiagen). The concentration and specific activity of the labeled cRNAs (pmol Cy3/μg cRNA) were measured using a NanoDrop ND-1000. A mass of 1 μg of each labeled cRNA was fragmented through the addition of 11 μl of 10X blocking agent and 2.2 μl of 25X fragmentation buffer. The mixture was then heated at 60°C for 30 min. Then, 55 μl of 2X GE hybridization buffer was added to dilute the labeled cRNA, and 100 μl of the hybridization solution was dispensed into the gasket slide and assembled on the lncRNA expression microarray slide. The slides were incubated for 17 hours at 65°C in an Agilent hybridization oven. The hybridized arrays were washed, fixed, and scanned using an Agilent DNA microarray scanner (part number G2505B).

Data analysis

The Agilent Feature Extraction software (version 10.7.3.1) was used to analyze the acquired array images. The quantile normalization and subsequent data processing were performed using the GeneSpring GX v11.5.1 software package (Agilent Technologies). After the quantile normalization of the raw data, the lncRNAs and mRNAs with flags in Present or Marginal ("All Targets Value") in at least 2 of the 6 samples were chosen for further data analysis. The differentially expressed lncRNAs and mRNAs that exhibited statistically significant differences were identified through Volcano Plot filtering, and the differentially expressed lncRNAs and mRNAs were identified through fold-change filtering. Hierarchical clustering was performed using the Agilent GeneSpring GX software (version 11.5.1).

Results

Integrity of the sample RNA

The integrity of RNA can be assessed by electrophoresis on a denaturing agarose gel. Intact total RNA run on a denaturing gel will exhibit sharp 28s and 18s rRNA bands (eukaryotic samples). The 28s rRNA band should be approximately twice as intense as the 18s rRNA band. This 2:1 intensity ratio indicates that the RNA is intact (**Figure 1**).

Purity and concentration of the sample RNA

The NanoDrop ND-1000 was used to accurately measure the concentrations (OD260), protein contamination (ratio of OD260 to OD280), and organic compounds contamination (ratio of OD260 to OD230). The OD260/OD280 ratios of total RNA should be higher than 1.8 (**Table 1**).

Quality assessment of LncRNA data after filtering

Box plot: Box plots are a convenient way to quickly visualize the distributions of a dataset. These are commonly used to compare the distributions of the intensities from all samples. After normalization, the distributions of the log2-ratios among all of the tested samples were nearly the same (**Figure 2**).

Scatter plot: The scatter plot is a visualization method used to assess the LncRNA expression variation (or reproducibility) between two sam-

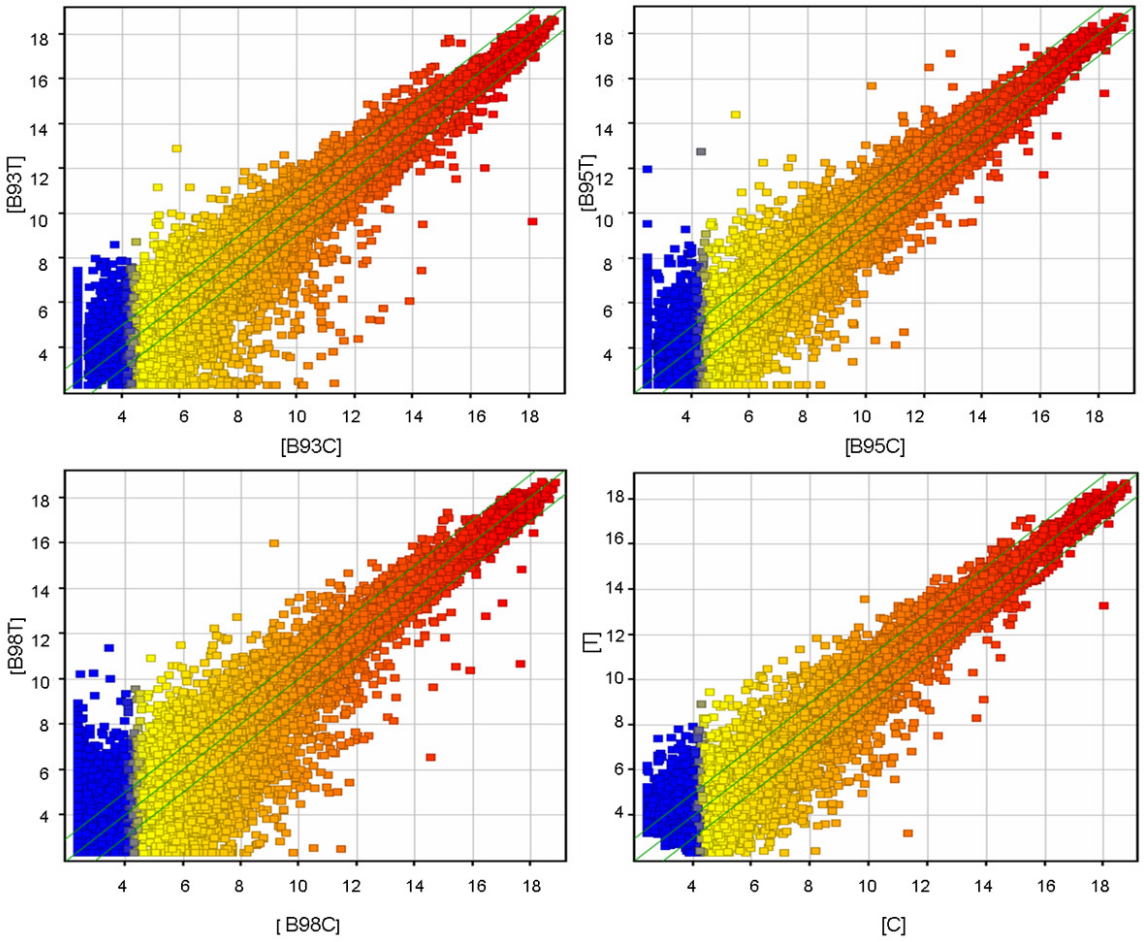

Figure 3. Scatter plots for B93T vs. B93C, B95T vs. B95C, B98T vs. B98C, and T vs. C. The green lines are the fold-change lines (the default fold-change value is 2.0). The LncRNAs above the top green line and below the bottom green line are those that exhibit at least a 2.0-fold difference between the two compared samples.

ples or two groups of samples. The values of the X and Y axes in the scatter plot are the normalized (log2-scaled) signal values of the samples or the average normalized (log2-scaled) signal values of a group of samples. The green lines are the fold-change lines (the default fold-change value is 2.0). The LncRNAs above the top green line and below the bottom green line are those that exhibit at least a 2.0-fold difference between the two compared samples or the two compared groups of samples. Four scatter plots are provided in the data report (**Figure 3**).

Heat map and hierarchical clustering

Hierarchical clustering is one of the most widely used clustering methods to analyze LncRNA expression data. Cluster analyses arrange samples into groups based on their expression levels, which allows us to hypothesize the relationships among the samples. A dendrogram shows the relationships between the LncRNA expression patterns of different samples. Four clusters are provided in the data report (**Figure 4**).

Screening of differentially expressed LncRNAs

To identify the lncRNAs that are significantly differentially expressed (Fold Change ≥ 2.0, *P*-value ≤ 0.05), we performed a volcano plot filtering between the two groups. To identify the differentially expressed LncRNAs, we performed a fold-change filtering between two samples (Fold Change ≥ 2.0). The expression profiles of lncRNAs were significantly different between normal bladder tissue and urothelial carcinoma of the bladder. Compared with normal bladder tissue, 1,122 lncRNAs exhibited at least a 2-fold significant difference ($P < 0.05$)

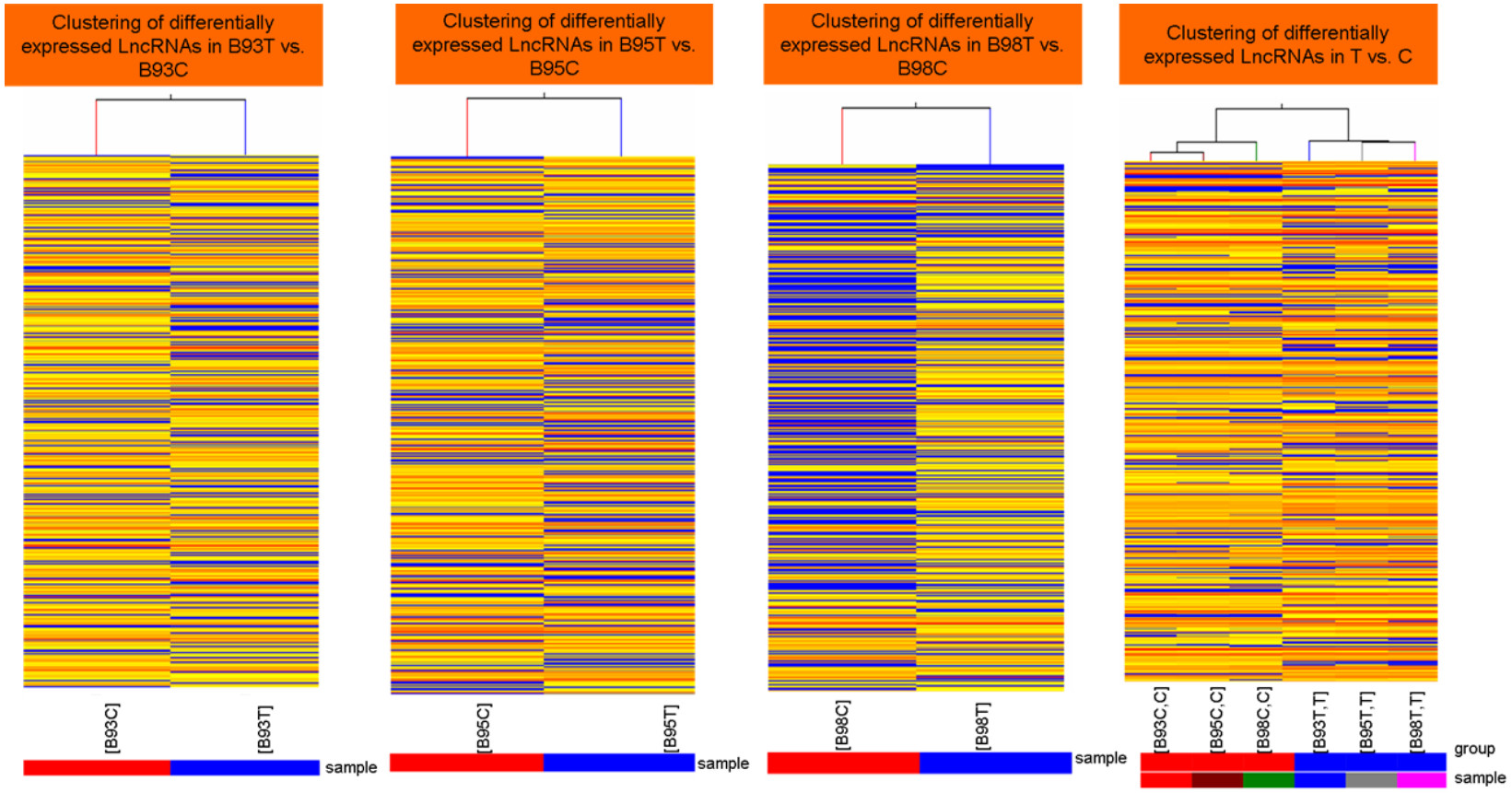

Figure 4. Hierarchical clustering of the differentially expressed LncRNAs in B93T vs. B93C, B95T vs. B95C, B98T vs. B98C, and T vs. C. The red color indicates a high relative expression, and the blue color indicates a low relative expression.

Table 2. LncRNAs that were upregulated by at least 10-fold in urothelial carcinoma of the bladder

Sequence Name	Gene Name	Chrom.	FC Absolute	Probe Name
uc003 cmy.1	AK124776	chr3	26.29291	ASHG19A3A022520
C20652	lincRNA-RAB12-1	chr18	18.844774	ASHG19A3 L0003342
AK000839	/	chr17	16.210579	ASHG19A3A008508
ENST00000473150	KRT8P25	chr3	15.875776	ASHG19A3A022860
ENST00000512863	RP11-474J18.4	chr5	15.131286	ASHG19A3A027163
ENST00000431957	AC000110.1	chr7	15.049078	ASHG19A3A035014
ENST00000463294	KRT8P13	chr3	14.981011	ASHG19A3A023526
ENST00000450021	KRT8P10	chr2	14.825374	ASHG19A3A014732
uc001rjo.1	BC072678	chr12	14.747757	ASHG19A3A048481
ENST00000448540	RP11-111E2.1	chr9	14.659423	ASHG19A3A038816
ENST00000504509	RP11-789C1.1	chr4	14.507504	ASHG19A3A026300
ENST00000414328	RP11-110J1.3	chr1	14.4582815	ASHG19A3A032876
ENST00000399149	SFRS9P1	chr21	14.17717	ASHG19A3A018851
ENST00000449217	RP3-452 M16.1	chrX	14.110057	ASHG19A3A040138
ENST00000413425	KRT8P18	chr3	13.99092	ASHG19A3A020870
ENST00000440275	KRT8P7	chr11	13.342168	ASHG19A3A045784
ENST00000451609	RP11-543F8.1	chr10	13.288165	ASHG19A3A042424
EC495588	lincRNA-RCN2	chr15	12.822171	ASHG19A3 L0002752
ENST00000367074	GS1-309P15.4	chrX	12.674266	ASHG19A3A040786
ENST00000424882	GS1-309P15.3	chrX	12.63172	ASHG19A3A039699
ENST00000485873	RP4-621B10.3	chr1	12.627898	ASHG19A3A048157
ENST00000398745	KRT8P26	chr11	12.41178	ASHG19A3A046607
ENST00000511504	RP11-790I12.1	chr4	12.265042	ASHG19A3A025622
nc-HOXB4-176+	nc-HOXB4-176	chr17	12.118851	CUST_54_PI426075208
ENST00000454391	AL358913.1	chr14	11.904219	ASHG19A3A050730
ENST00000497538	RP11-111F10.2	chr3	11.498124	ASHG19A3A023430
ENST00000392131	AC079305.6	chr2	11.317965	ASHG19A3A016645
ENST00000510090	RP11-618I10.2	chr4	11.099051	ASHG19A3A025620
ENST00000434750	AC108171.5	chrX	10.894489	ASHG19A3A041704
ENST00000453086	RP5-1091N2.3	chrX	10.1777935	ASHG19A3A040026

and were thus regarded as differentially expressed lncRNAs. Of these lncRNAs, 734 increased by more than twofold, and 388 decreased by more than twofold. We also determined the lncRNAs that were upregulated or downregulated more than 10 fold in urothelial carcinoma of the bladder (**Tables 2** and **3**) and determined the chromosomal distribution of the differentially expressed lncRNAs (**Figure 5**).

Discussion

The large amount of clinical observations and experimental studies on lncRNAs demonstrates that lncRNAs play an important role in the tumorigenesis and progression of urologic oncology, including bladder cancer. It has been reported that there is a significant difference in the expression of lncRNAs between normal tissue and tumor tissue [22] and between different stages of the same tumor [23]. Although its function has not been deeply studied, the mechanism through which lncRNAs participate in or influence the tumorigenesis and progression remains unclear, but its potential value in future studies is self-evident. We have found that the majority of lncRNAs are highly conserved and have specific gene sequences. These are widely expressed in both normal cells and tumor cells of human being, have many copies, and are distributed in specific chromosomal loci and tumor-associated regions. Certain lncRNAs are closely associated with the tumorigenesis and progression of

Table 3. LncRNAs that were downregulated by at 10-fold in urothelial carcinoma of the bladder.

Sequence Name	Gene Name	Chrom.	FCAbsolute	Probe Name
nc-HOXB9-206-	nc-HOXB9-206	chr17	272.16144	ASHG19A3H0000077
ENST00000426074	RP11-160A10.2	chr6	52.299904	ASHG19A3A032028
nc-HOXA11-86+	nc-HOXA11-86	chr7	37.83364	CUST_4_PI426075208
AL109700	/	chr15	36.111774	ASHG19A3A052515
AK123324	/	chr8	28.818373	ASHG19A3A035994
nc-HOXD10-7-	nc-HOXD10-7	chr2	28.742731	ASHG19A3H0000169
BC042023	/	chr1	27.417946	ASHG19A3A047528
AK026384	/	chr2	26.26481	ASHG19A3A016486
nc-HOXB9-205-	nc-HOXB9-205	chr17	25.701212	ASHG19A3H0000076
ENST00000421606	CES4	chr16	24.578844	ASHG19A3A054608
nc-HOXD12-3-	nc-HOXD12-3	chr2	21.637152	ASHG19A3H0000174
uc002tjx.3	AY343891	chr2	20.35203	ASHG19A3A014229
ENST00000416700	RP11-58A12.3	chr9	17.737352	ASHG19A3A038722
AL109696	/	chr15	15.587008	ASHG19A3A052517
uc010 mam.2	AF131817	chr8	15.372687	ASHG19A3A035947
uc003tcg.2	AK124304	chr7	15.336274	ASHG19A3A034154
ENST00000442797	AC007064.21	chr22	15.284027	ASHG19A3A020031
AL390167	/	chr13	14.648443	ASHG19A3A049319
ENST00000514910	RP11-451F20.1	chr4	14.038315	ASHG19A3A026355
uc003xjb.2	BC034319	chr8	13.934998	ASHG19A3A036526
CF129145	lincRNA-LOC100506581-1	chr16	13.665986	ASHG19A3 L0000894
uc001awc.1	AK055853	chr1	13.640614	ASHG19A3A012055
AK125472	/	chr13	13.590306	ASHG19A3A049853
uc001 gla.1	CR621436	chr1	12.739795	ASHG19A3A035511
uc004ags.1	AK130904	chr9	12.102253	ASHG19A3A038744
AF087976	/	chr3	11.665831	ASHG19A3A022767
G65639		chr5	10.440295	ASHG19A3A026776
AK307235	lincRNA-SRD5A2	chr2	10.403127	ASHG19A3 L0001181
ENST00000439715	RP11-282E4.1	chr9	10.397835	ASHG19A3A038699
ENST00000434499	RP11-145A3.4	chr1	10.215157	ASHG19A3A040793

bladder tumors and thus may contribute to the prevention, diagnosis, and treatment of bladder tumors in the future.

Research on chromosomal distribution of lncRNAs in bladder cancer

It is well recognized that cancer is a hierarchical disease that is caused by a synergy of oncogenes, tumor-suppressor genes, changes in repair genes, and other factors. Recent studies have found that tumorigenesis is not only related to the activation of oncogenes and the inactivation of tumor-suppressor genes but also connected with gene methylation, microsatellite genetic abnormalities, and telomere abnormal expression, which can occur on any chromosome. According to the abovementioned data, we hypothesize that lncRNAs may also participate in these changes. Our studies have shown that the differentially expressed lncRNAs in bladder urothelial carcinoma are distributed on each of the chromosomes, although the majority of lncRNA are found on chromosomes 1, 2, 3, 4, 6, and X. In the tumorigenesis and progression of bladder cancer, the chromosomes show chromosomal abnormalities, which are not confined within a particular chromosome. All of the chromosomes, including the X and Y chromosomes, can experience different quantities and degrees of changes in bladder cancer, and our experimental results are consistent with this assertion. The chromosomal abnormalities include gene amplifications,

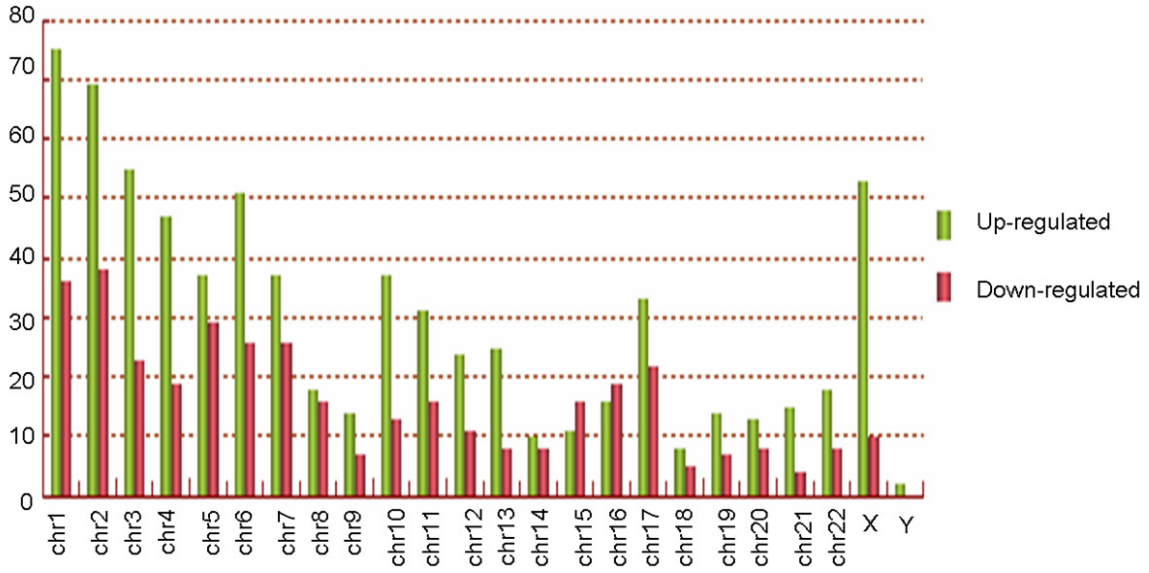

Figure 5. Chromosomal distribution of differentially expressed lncRNAs. Most of the differentially expressed lncRNAs are distributed on chromosomes 1, 2, 3, 4, 6, and X.

gene rearrangements, gene deletions, and numerical and structural anomalies. It has been reported in the literature that chromosomal abnormalities are mainly distributed in chromosomes 3, 7, 8, 9, 11, 13, 16, 17, and Y in bladder cancer [24-26], and we hypothesize that the degrees and quantities of these abnormalities are associated with the grade and stage of the tumor, the depth of invasion, and the recurrence of tumors. We also took the experimental methods into account because the main experimental method the is currently used to detect chromosomal abnormalities is FISH technology, which emerged in the late 1980s and is a non-radioactive *in situ* hybridization method that was developed based on radioactive *in situ* hybridization. The basic principle of FISH is the use of a fluorescent dye-labeled specific nucleic acid as a probe, which, based on base complementary, will specifically bind a nucleic acid in the sample to form a hybrid double-stranded nucleic acid. Fluorescence microscopy is then used to identify and count the fluorescent signals, which allows the qualitative positioning and quantitative analysis of the chromosomes in the sample. In addition to the differences in the experimental methods used, we took into consideration the possible relationship with the sample size. The objective of our experiments was to screen the differentially expressed lncRNAs in bladder cancer to identify genes that are specific to bladder cancer. We aim to screen large samples though microarray technology, but were limited by the high cost of microarray technology. Hence, the methodology, the sample size, and the intrinsic factors of the tumors themselves, including the different stages and grades, are likely to cause differences in the distribution of chromosomal aberrations. However, it is clear that the tumorigenesis and the progression of bladder cancer is not the result of a single chromosomal aberration but of multiple chromosomal abnormalities.

Research on differentially expressed lncRNAs in bladder cancer

In the current literature on bladder cancer, lncRNAs are rarely mentioned. The only relevant lncRNA that has been reported is urothelial cancer-related genes (UCA1), and this lncRNA was first reported in 2006 by Wang [27]. This lncRNA was initially considered an urothelial cancer-specific gene, but it was later also found in placental, gallbladder, and epithelial tissue [28-30]. UCA1 is positioned within the cytoplasm and is located in human chromosome 19p13.12, which has three exons and two introns. There are three different splice bodies with lengths of 1.4 kb, 2.2 kb, and 2.7 kb [31], and the first splice body was found mainly in bladder urothelial carcinoma. Wang [32] first used suppression subtractive hybridization to

analyze BLS-211 and BLZ-211 cells and obtained a 1,442-bp UCA1 gene. The function of this lncRNA may be associated with the regulation of the downstream molecular processes of protein synthesis. The UCA1 gene can increase the malignant phenotype of the bladder urothelial carcinoma cell line BLS-211 and increases its proliferation *in vitro*. In addition, this gene significantly increased the invasiveness and drug resistance markedly this cell line, causes significant tumorigenesis in nude mice, upregulates the gene expression of WNT6, CYP1A1, and AURPK, and downregulates the expression of MBD3 and SRPK1 [33]. In the detection of urinary sediments of different pathological grades in urothelial carcinoma patients, it was found that this gene expression pattern is closely related to G2-G3 superficial bladder cancer: 40% similar to G1, 90.9% similar to superficial G2, 64.3% similar to invasive G2, 91.7% similar to superficial G3, and 100% similar to invasive G3 [27]. In our experiment, UCA1 was expressed, but the data analyses demonstrated that the two groups did not exhibit differential expression of UCA1, likely due to the sample size, pathological differences, methodology used, and other factors. Microarray technology is highly sensitive; however, whether a highly expressed lncRNA or a related RNA is our bladder cancer-specific target remains controversial. Further validation, including RT-PCR analyses, is required, and a variety of tissues (not just bladder cancer tissue) need to be verified to ensure that the target is specific to bladder cancer.

At present, research on lncRNAs is just beginning, and many of the features of lncRNAs, including their mechanism of action and their relationship with tumor development, are still unclear. Our experiment obtained differentially expressed lncRNAs that have not been previously reported in the literature. These include some lncRNAs that were upregulated, such as AK124776, lincRNA-RAB12-1, KRT8P25, RP11-474J18.4, AC000110.1, KRT8P13, KRT8P10, and BC072678, and some lncRNAs that were downregulated, such as nc-HOXB9-206, RP11-160A10.2, nc-HOXA11-86, nc-HOXD10-7, nc-HOXB9-205, CES4, and nc-HOXD12-3. In future research, we will continue to search for bladder cancer-specific or -related lncRNAs. This study sets the basis for future research on lncRNAs in bladder carcinoma.

Acknowledgements

This work was supported by the Shanghai Education Commission Research and Innovation projects (No. 12ZZ034).

Disclosure of conflict of interest

None.

Abbreviations

UCC, urothelial cell carcinoma; LncRNA, long noncoding RNA.

Address correspondence to: Dr. Denglong Wu, Department of Urology, Tongji Hospital of Tongji University, 389 Xincun Road, Shanghai 200065, China. Tel: 86-021-66111532; Fax: 86-021-66111533; E-mail: wudenglongtj@163.com

References

[1] Karim Kader. Bladder Cancer. ScientificWorldJournal 2011; 11: 2565-2566.
[2] Braisch U, Meyer M, Radespiel-Tröger M. Risk of tobacco-related multiple primary cancers in Bavaria. Germany BMC Cancer 2012; 12: 250.
[3] Burger M, Catto JW, Dalbagni G, Grossman HB, Herr H, Karakiewicz P, Kassouf W, Kiemeney LA, La Vecchia C, Shariat S, Lotan Y. Epidemiology and Risk Factors of Urothelial Bladder Cancer. Eur Urol 2013; 63: 234-241.
[4] Reinert T. Methylation Markers for Urine-Based Detection of Bladder Cancer: The Next Generation of Urinary Markers for Diagnosis and Surveillance of Bladder Cancer. Adv Urol 2012; 6: 503271.
[5] Santoni M, Catanzariti F, Minardi D, Burattini L, Nabissi M, Muzzonigro G, Cascinu S, Santoni G. Pathogenic and Diagnostic Potential of BLCA1 and BLCA4 Nuclear Proteins in Urothelial Cell Carcinoma of Human Bladder. Adv Urol 2012; 7: 397412.
[6] Lee H, Choi SK, Ro JY. Overexpression of DJ-1 and HSP90α, and loss of PTEN associated with invasive urothelial carcinoma of urinary bladder: Possible prognostic markers. Oncol Lett 2012; 3: 507-512.
[7] Urquidi V, Kim J, Chang M, Dai Y, Rosser CJ, Goodison S. CCL18 in a multiplex urine-based assay for the detection of bladder cancer. PLoS One 2012; 7: 37797.
[8] Zhao P, Liu C, Xu K, Zheng S, Li H, Xu Y, Xu A, Li B, Huang P. Expression of OCT4 protein in bladder cancer and its clinicopathological implications. Nan Fang Yi Ke Da Xue Xue Bao 2012; 32: 643-646.

[9] Thanan R, Murata M, Ma N, Hammam O, Wishahi M, El Leithy T, Hiraku Y, Oikawa S, Kawanishi S. Nuclear localization of COX-2 in relation to the expression of stemness markers in urinary bladder cancer. Mediators Inflamm 2012; 4: 165879.

[10] Urquidi V, Chang M, Dai Y, Kim J, Wolfson ED, Goodison S, Rosser CJ. IL 8 as a urinary biomarker for the detection of bladder cancer. BMC Urol 2012; 4: 12.

[11] Johnen G, Gawrych K, Bontrup H, Pesch B, Taeger D, Banek S, Kluckert M, Wellhäußer H, Eberle F, Nasterlack M, Leng G, Stenzl A, Brüning T; UroScreen Study Group. Performance of survivin mRNA as a biomarker for bladder cancer in the prospective study UroScreen. PLoS One 2012; 7: 35363.

[12] Fu YP, Kohaar I, Rothman N, Earl J, Figueroa JD, Ye Y, Malats N, Tang W, Liu L, Garcia-Closas M, Muchmore B, Chatterjee N, Tarway M, Kogevinas M, Porter-Gill P, Baris D, Mumy A, Albanes D, Purdue MP, Hutchinson A, Carrato A, Tardón A, Serra C, García-Closas R, Lloreta J, Johnson A, Schwenn M, Karagas MR, Schned A, Diver WR, Gapstur SM, Thun MJ, Virtamo J, Chanock SJ, Fraumeni JF Jr, Silverman DT, Wu X, Real FX, Prokunina-Olsson L. Common genetic variants in the PSCA gene influence gene expression and bladder cancer risk. Proc Natl Acad Sci U S A 2012; 109: 4974-4979.

[13] El Gehani K, Al-Kikhia L, Emaetig F, Syrjänen K, Al-Fituri O, Elzagheid A. Over-expression of HER-2 is associated with the stage in carcinomas of the urinary bladder. Libyan J Med 2012; 7: 14694.

[14] Hosseini J, Golshan AR, Mazloomfard MM, Mehrsai AR, Zargar MA, Ayati M, Shakeri S, Jasemi M, Kabiri M. Detection of recurrent bladder cancer: NMP22 test or urine cytology. Urol J 2012; 9: 367-372.

[15] Feng CC, Wang PH, Guan M, Jiang HW, Wen H, Ding Q, Wu Z. Urinary BLCA-4 is highly specific for detection of bladder cancer in Chinese Han population and is related to tumour invasiveness. Folia Biol (Praha) 2011; 57: 242-247.

[16] Choi W, Shah JB, Tran M, Svatek R, Marquis L, Lee IL, Yu D, Adam L, Wen S, Shen Y, Dinney C, McConkey DJ, Siefker-Radtke A. p63 expression defines a lethal subset of muscle-invasive bladder cancers. PLoS One 2012; 7: 30206.

[17] Kim TS, Rhew HY, Hwang HY. Pilot study of the clinical significance of serum and urinary her-2/neu protein in bladder cancer patients. Korean J Urol 2011; 52: 815-818.

[18] Ewald F, Ueffing N, Brockmann L, Hader C, Telieps T, Schuster M, Schulz WA, Schmitz I. The role of c-FLIP splice variants in urothelial tumours. Cell Death Dis 2011; 22: 245.

[19] Gibb EA, Brown CJ, Lam WL. The functional role of long non-coding RNA in human carcinomas. Mol Cancer 2011; 13: 38.

[20] Orom UA, Derrien T, Beringer M, Gumireddy K, Gardini A, Bussotti G, Lai F, Zytnicki M, Notredame C, Huang Q, Guigo R, Shiekhattar R. Long noncoding RNAs with enhancer-like function in human cells. Cell 2010; 143: 46-58.

[21] Bian S, Sun T. Functions of noncoding RNAs in neural development and neurological diseases. Mol Neurobiol 2011; 44: 359-373.

[22] Gibb EA, Vucic EA, Enfield KS, Stewart GL, Lonergan KM, Kennett JY, Becker-Santos DD, MacAulay CE, Lam S, Brown CJ, Lam WL. Human cancer long non-coding RNA transcriptomes. PLoS One 2011; 6: 5915-5917.

[23] Zhu Y, Yu M, Li Z, Kong C, Bi J, Li J, Gao Z, Li Z. ncRAN, a newly identified long noncoding RNA, enhances human bladder tumor growth, invasion, and survival. Urology 2011; 77: 510-515.

[24] Panzeri E, Conconi D, Antolini L, Redaelli S, Valsecchi MG, Bovo G, Pallotti F, Viganò P, Strada G, Dalprà L, Bentivegna A. Chromosomal aberrations in bladder cancer: fresh versus formalin fixed paraffin embedded tissue and targeted FISH versus wide microarray-based CGH analysis. PLoS One 2011; 6: 24237.

[25] Minner S, Kilgué A, Stahl P, Weikert S, Rink M, Dahlem R, Fisch M, Höppner W, Wagner W, Bokemeyer C, Terracciano L, Simon R, Sauter G, Wilczak W. Y chromosome loss is a frequent early event in urothelial bladder cancer. Pathology 2010; 42: 356-359.

[26] Zieger K, Wiuf C, Jensen KM, Ørntoft TF, Dyrskjøt L. Chromosomal imbalance in the progression of high-risk non-muscle invasive bladder cancer. BMC Cancer 2009; 16: 149.

[27] Wang XS, Zhang Z, Wang HC, Cai JL, Xu QW, Li MQ, Chen YC, Qian XP, Lu TJ, Yu LZ, Zhang Y, Xin DQ, Na YQ, Chen WF. Rapid identification of UCA1 as a very sensitive and specific unique marker for human bladder carcinoma. Clin Cancer Res 2006; 12: 4851-4858.

[28] Xie XJ, Li X, Wang F, Chen W. Cellular localization and tissue expression pattern of UCA1, a non-coding RNA. Nan Fang Yi Ke Da Xue Xue Bao 2010; 30: 57-60.

[29] Kaneko K, Ito Y, Ono Y, Tainaka T, Tsuchiya H, Shimoyama Y, Ando H. Gene expression profiling reveals upregulated UCA1 and BMF in gallbladder epithelia of children with pancreaticobiliarymaljunction. J Pediatr Gastroenterol Nutr 2011; 52: 744-750.

[30] Qiao HP, Gao WS, Huo JX, Yang ZS. Long Noncoding RNA GAS5 Functions as a Tumor Suppressor in Renal Cell Carcinoma. Asian Pac J Cancer Prev 2013; 14: 1077-82.

[31] Tsang WP, Wong TW, Cheung AH, Co CN, Kwok TT. Induction of drug resistance and transfor-

mation in human cancer cells by the noncoding RNA CUDR. RNA 2007; 13: 890-898.

[32] Wang F, Li X, Xie X, Zhao L, Chen W. UCA1, a non-protein-coding RNA up-regulated in bladder carcinoma and embryo, influencing cell growth and promoting invasion. FEBS Lett 2008; 582: 1919-1927.

[33] Rafiee P, Shi Y, Su J, Pritchard KA Jr, Tweddell JS, Baker JE. Erythropoietin protects the infant heartagainst ischemia-reperfusion injury by triggering multiple signalingpathways. Basic Res Cardiol 2005; 100: 187-197.