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Original Article

Interaction network analysis of differentially expressed genes and screening of cancer marker in the urine of patients with invasive bladder cancer

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Abstract: Objective: To detect the expression profile of bladder cancer and to delineate the interaction network of these genes in invasive bladder cancer. Methods: A total of 126 differentially expressed genes were identified, and input into STRING online database to delineate interaction network. The network data were screened with central nodes. The expression of genes with the most evident change in the exfoliated cells of urine was detected. RNA markers with over-expression in stage Ta tumor and/or T_1 to T_4 tumors but low expression in blood or inflammatory cells were characterized. Results: On the basis of assay of 21,639 whole-genome oligonucleotide microarray, a total of 126 differentially expressed genes were identified, of which 69 had up-regulated expression and 57 had down-regulated expression. STRING screening showed there were interactions among 103 genes in the bladder cancer which formed a complex network. A total of 23 central nodes were screened with Cytoscape and are involved in multiple signaling pathways related to tumorigenesis. The test specificity was 80% for the 30 control patients with urinary tract infections. The combination of BLCA-4 and HOXA13 could distinguish between low and high grade tumors, with specificity and sensitivity of 80%. Conclusion: The interaction network of differentially expressed genes, especially the central nodes of this network, can provide evidence for the early diagnosis and molecular targeted therapy of invasive bladder cancer, and combined detection of IGF-1, hTERT, BLCA-4 and HOXA13 genes is helpful to evaluate BTCC at different stages.

Keywords: Bladder transitional cell carcinoma, interaction network, central nodes, urine marker, quantitative real-time PCR

Introduction

Bladder transitional cell carcinoma (BTCC) is one of the leading causes of cancer-related morbidity and mortality and the sixth most common cancer worldwide [1]. In China, the incidence of BC continues to rise [2]. About 30% of BTCC have invaded the lamina propria or myometrium at diagnosis (T_2-T_4 stage), the 5-year disease free survival rate reduced from 42.5% for BTCC at T_a-T_1 stage (or carcinoma in situ) to 11% for BTCC at T_2-T_4 stage, and about 6% of patients with BTCC at T_2-T_4 stage have distant metastasis [3]. The incidence of recurrence is very high after therapy for BTCC patients and is about 50-70%. Once recurrence is present, the biological behaviors of BTCC change and it may progress to advanced pathological grade and clinical stage [3].

Biopsy of suspected lesions and subsequent cystoscopy has been regarded as the gold standard in the diagnosis of bladder cancer and for the follow-up of these patients. A variety of studies have focused on developing new non-invasive methods for the diagnosis of bladder cancer and monitoring its progression. New biomarkers that would increase the detection rate of transitional cell carcinoma before the invasion of bladder muscle layer would reduce BTCC-related morbidity and mortality [4]. Although the detection of telomerase and hyaluronic acid hyaluronidase in the urine is sensitive and specific, their roles in the diagnosis of

bladder cancer still require to be validated in multicentered clinical studies. Therefore, it is important to find the specific biomarkers for bladder cancer, which may improve the early diagnosis and effective postoperative follow-up.

High-throughput microarray assay has shown that gene expression profile is helpful for the identification of bladder tumor cells from normal urothelial cells and the staging of bladder cancer. The gene expression data provided by the high-throughput microarray assay are benefit for the diagnosis of bladder tumors and present some characteristics of diseases and information for diagnosis [5, 6]. To investigate the molecular mechanism underlying the pathogenesis of BTCC, 70-mer oligonucleotide microarray assay was performed to detect the gene expression profiles of invasive BTCC cells and normal bladder transitional epithelial cells. A total of 126 differentially expressed genes were identified. To further elucidate the interaction among the products of these genes and their role in the pathogenesis of BTCC, bioinformatics database was employed to delineate the interaction network of these differentially expressed genes. The central nodes of this network were screened and then their expressions were validated in exfoliated cells in the urine by real-time quantitative RT-PCR.

Patients and methods

Clinical samples

All samples were collected from patients treated at the Department of Urology, People's Hospital of Gansu Province from 2009 to 2012. The staging of cancer was performed according to the TNM classification [7] and grading to the WHO criteria [8] independently by two pathologists blind to this study. Voided urine samples were obtained from consecutive patients with BTCCs in whom 32 samples were obtained from patients receiving during transurethral resection by cold-cup biopsy, and 40 from patients under the monitoring for bladder cancer recurrence. Eighty-three voided urine samples were also obtained as controls from patients with non-malignant diseases in whom bladder tumors were excluded by flexible cystoscopy. These control patients were diagnosed as having urinary tract infections (n=30), and other non-malignant urological diseases including benign prostate hyperplasia, urolithiasis and neurogenic bladder (n=53). The mean age of BTCC patients and controls was 72 and 69 years, respectively. Eighty-six percent of BTCC patients and 76% of controls were male. Written informed consent was obtained from each patient before surgery and the whole protocol was approved by the Ethics Committee of our hospital.

Extraction of high-quality RNA from samples

RNA was extracted from samples according to manufacturer's instructions (Agilent), and the A260/A280 of extracted RNA was assured to be >1.80. The integrity of purified mRNA was determined with the Agilent2100 Bioanalytical System and the RNA integrity number (RIN) was >6.5 for all the samples. In the present study, the mRNA from 6 invasive BTCC tissues met the requirements for further detection, and used in the subsequent mRNA expression profiling.

Scanning of microarray and data extraction after hybridization

After hybridization, microarray was scanned with the Agilent Microarray Scanner and Agilent Scan Control at a resolution of 5 mm. Then, the images were transformed into data with Agilent Feature Extraction 10.5.1. The Grid Template Browser was "019118-D-F-20130608", FE Protocol Browse was "GE1-v5-91-0806", the extracted were stored, and a quality-control report was generated. The quality control report of each microarray showed the hybridization signals were favorable.

Delineation of interaction network of differentially expressed genes in BTCC

A total of 126 differentially expressed genes were identified between invasive BTCC and normal bladder transitional epithelial cells, of which 69 had up-regulated expression and 57 had down-regulated expression. These genes were input into Bioinformatics Database STRING (http://string-db.org), and the interactions of proteins encoded by these genes were analyzed. The nodes without interactions with other proteins were deleted, and the location of central nodes was adjusted to completely expose the interaction network. Then, the weighted gene co-expression network analysis

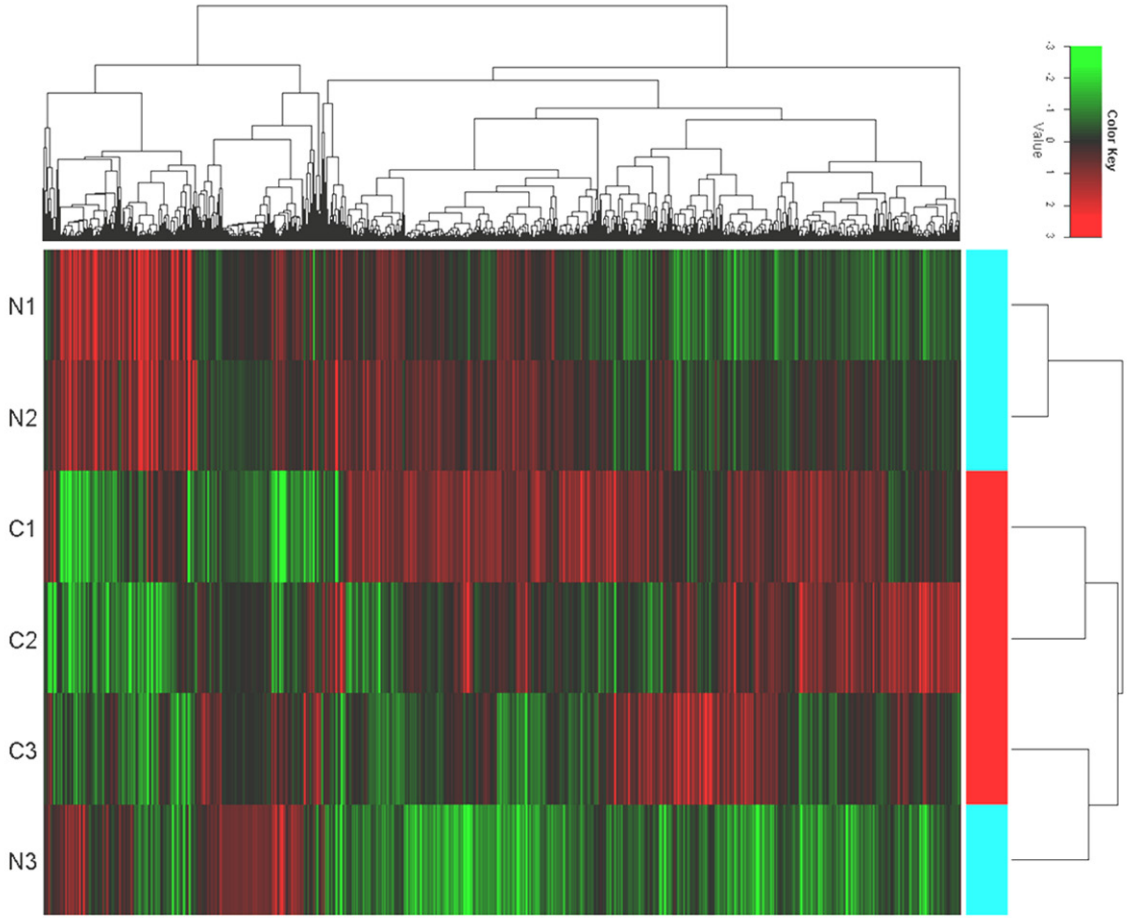

Figure 1. Treeview after cluster analysis of differentially expressed genes in several samples (Red: up-regulation; green: down-regulation).

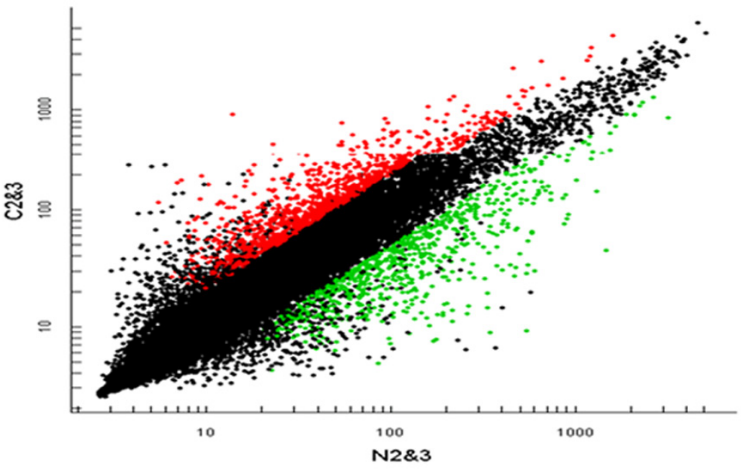

Figure 2. Normalized data of BTCC 22K 70-mer Oligo microarray assay.

(WGCNA) was performed to delineate the interaction network.

Selection of central nodes and investigations of their functions

The network data were output and stored in the form of txt and then input into Cytoscape 2.6.2. Hierarchic Layout was employed for analysis, and the central nodes were exposed and marked. After applying the organic mode, it is attempted to delete the nodes at the center on the basis of connections from simple to complex, and the influence of these nodes on the network was observed. When the network structure became slack or even disrupted after deleting a node, it was confirmed as a central node and then marked.

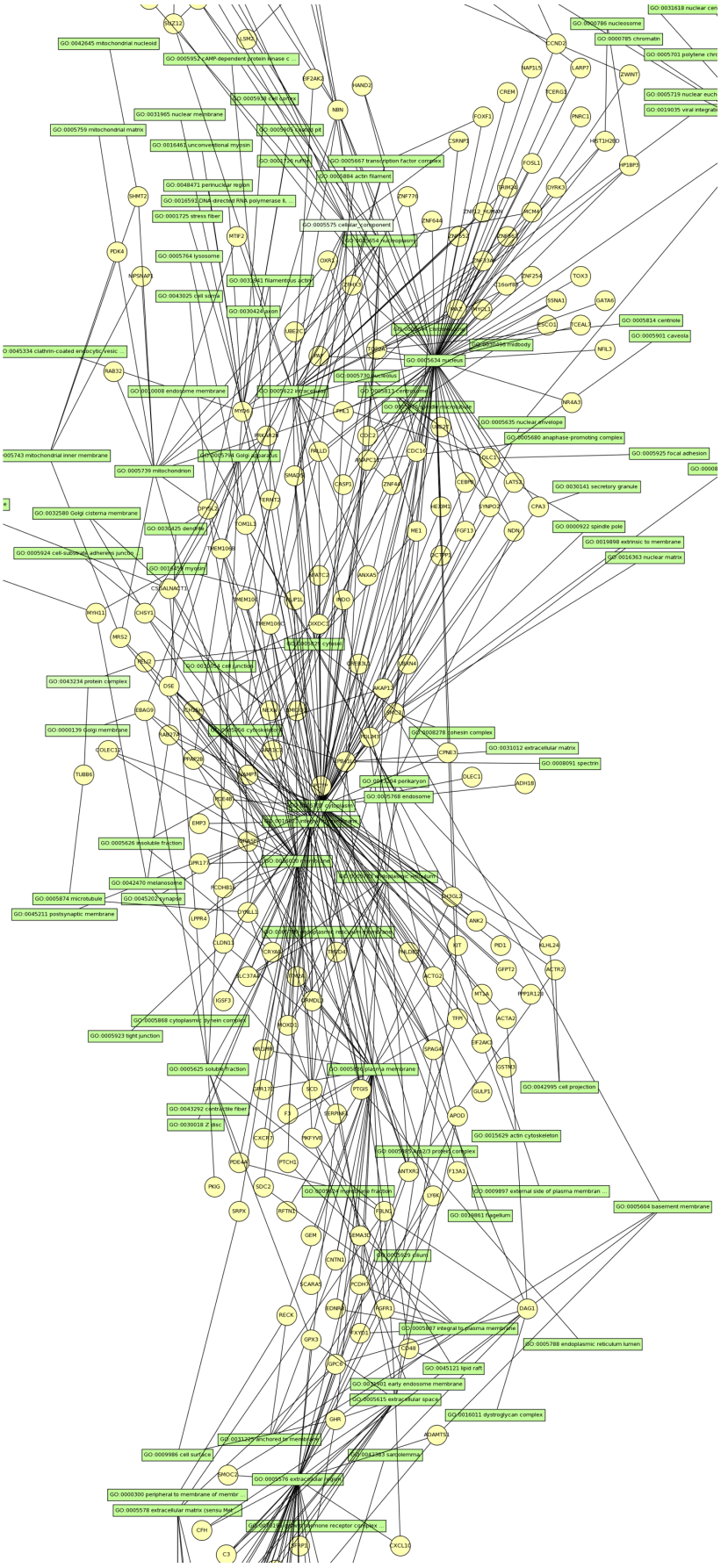

Figure 3. Interaction network of differentially expressed genes in BTCC.

Validation of central nodes in the network

Real time RT-PCR was employed to confirm the expression of central nodes with definite connection with the tumor. RNA markers with high overexpression in stage T_a tumors and/or T_1 to T_4 tumors but low expression in blood or inflammatory cells were characterized by quantitative reverse transcription-PCR using 2 mL of voided urine from 72 BTCCs patients and 83 controls with other urological diseases. Primers for PCR were synthesized in Invitrogen company. Qiagen Rotor-Gene SYBR Green RT-PCR kit was used for PCR. The mixture (25 ul) used for PCR included 2×Rotor-Gene SYBR Green RT-PCR Master Mix (12.5 ul), 1 umol/L forward primer, 1 umol/L reverse primer, Rotor-Gene RT Mix (0.25 ul), RNA (1 ul; 50 ng), and nuclease-free water. Rotor Gene 6000 thermal cycler was used for PCR at reverse transcription at 55°C for 10 min. Then, the mixture was heated at 95°C for 5 min to activate Hot Star Taq Plus DNA polymerase. Two-step method was employed for PCR (denaturation at 95 °C for 5 s, annealing/extension at 60°C for 10 s.

Statistical analysis

PCR results were quantitatively determined throu-

Table 1. Central nodes of interaction network and their fold changes in BTCC by microarray assay

Change	Differentially expressed genes	Fold
Up-regulation	BUB1B, IGF-1, NEK2, ST14	≥10 fold
	PRKR (EIF2AK2), EZH2, hTERT, VEGF	≥5 fold <10 fold
	JUP, CCNB2, PPP1CA	≥2 fold <5 fold
Down-regulation	ACTA2, MYLK, FN1, TGFBR2, HOXA13, TAGLN	≥10 fold
	CAV1, VCL, COL4A2, SPARC, BLCA-4	≥5 fold <10 fold
	ROCK2, ITGAV, ABL1	≥2 fold <5 fold

Table 2. Specificity of individual markers and uRNA-D

	Test Positives/total true Positives* (%)					Cytology positives (%)
	IGF-1	hTERT	BLCA-4	HOXA13	uRNA-D	
Control group	14/83 (16.9)	19/83 (22.9)	14/83 (16.9)	1/83 (1.2)	16/83 (19.3)	0/83 (0)
BTCC group	61/72 (84.7)	65/72 (90.3)	65/72 (90.3)	64/72 (88.9)	65/72 (90.3)	61/72 (84.7)
Overall	85%	85%	85%	85%	85%	80%

Footnotes: *True Positives were defined as samples from patients who were positive for BTCC by flexible cystoscopy and biopsy.

gh Fisher linear discriminant analysis (LDA). This generated a formula (the linear discriminant, LD1) that is a linear combination of ΔCT values and can be used for discrimination of non-BTCC from BTCC. The sensitivity and specificity, as well as 95% confidence interval (CI), were determined using the LD1 values (for each marker or a combination of markers) and the distribution of these values across training runs. 95% CI for the sensitivity at a given specificity was obtained by calculating the 95% confidence limit for the cutoff value (corresponding to that specificity) followed by calculation of 95% CI for the sensitivity at a extreme cutoff value. LD1 values of each sample were assumed to be normally distributed across different training runs. All analyses were done with the R package for statistical analysis (http://www.r-project.org/). LDA and logistic regression (generalized linear model) analysis were performed with MASS package, and Receiver Operating Characteeristic (ROC) ROC package was used to analyze the sensitivity and specificity.

Results

Microarray assay and Treeview of differentially expressed genes

The hybridization was detected and results showed the effective hybridization rate was higher than 99%. Of 6 samples, the number of identified genes (genes with signal >200 in more than 50% of samples) was 8613. Systemic cluster analysis showed Treeview of differentially expressed genes (**Figure 1**) and normalized data of microarray assay (**Figure 2**).

Interaction network of differentially expressed genes in BTCC

The microarray includes a total of 21,639 probes, and finally 126 differentially expressed genes were identified in BTCC of which 69 had up-regulated expression and 57 had down-regulated expression. Of these genes, the products of 103 genes showed interactions as shown after STRING screening, and they formed complex, multicentered interaction network (**Figure 3**). These genes located at the center of this network, and had extensive interactions with peripheral nodes and other central nodes.

Selection of potential diagnostic markers

After screening with Cytoscape, a total of 26 central nodes were selected from the network (**Table 1**). Deleting these nodes caused slack structure. KEGG pathway analysis showed these genes involved a variety of biological processes, and played important roles in the cell cycle regulation, cell signal transduction, tumorigenesis, cell proliferation and differentiation, cytoskeleton formation, cell adhesion, focal adhesion formation, metabolism and

Table 3. Sensitivity of individual markers and combined assay uRNA-D in the differentiation of BTCC at different stages and grades at 85% specificity

BTCC presentation	n	Mean percentage (95% confidence interval)					Cytology positives (%)
		IGF-1	hTERT	BLCA-4	HOXA13	uRNA-D	
Stage T_a	31	52 (50-54)	66 (64-69)	91 (89-94)	34 (32-35)	68 (67-69)	4/31 (13)
Stage T_1	15	65 (64-67)	90 (90-90)	90 (90-90)	36 (32-37)	90 (90-94)	6/15 (40)
Stage T_2~T_4	26	80 (80-80)	100 (100-100)	80 (80-80)	100 (10-100)	100 (100-100)	17/21 (81)
Grade 1	32	64 (63-67)	81 (79-83)	96 (94-97)	46 (43-47)	68 (67-69)	5/32 (16)
Grade 2	24	72 (70-75)	90 (89-93)	91 (88-93)	44 (44-44)	61 (60-62)	6/24 (25)
Grade 3	16	78 (75-80)	96 (95-98)	80 (78-82)	38 (36-39)	97 (95-98)	7/16 (44)

Footnotes: The mean sensitivities and 95% confidence intervals determined by LDA and cross-validation are shown. Fifty-three non-BTCC samples were included in this analysis.

modification of nucleic acids and proteins, and ubiquitin-mediated proteolysis. Centroid clustering was performed for an unsupervised hierarchical cluster analysis of selected genes with fold change >3. Cluster analysis revealed that the refined genes formed clusters on the basis of their sensitivity to early diagnosis of BTCC and also successfully identified the bladder carcinoma at different stages or grades.

To test the abundance of these markers in the urine, QPCR was carried out using total RNA extracted from the urine samples of patients with confirmed BTCC and control patients. The human runt-related transcription factor (RUNX3) and bladder cancer specific nuclear matrix protein 4 (BLCA-4) had the highest up-regulation in both stage >T_1 tumors and stage T_a-T_1 tumors. Topoisomerase II A (TOP2A) and cell division cycle 2 (CDC2) showed the highest up-regulation in both stage >T_1 tumors and stage T_a-T_1 tumors, and also showed the least overlap between normal tissues and tumor tissues, and their expressions in 93% of >T_1 tumors and 88% of T_a-T_1 tumors were greater than the 95th percentile of the expressions in normal urothelium. Ras association domain family I Agene (RASSF1A) showed a high up-regulation in normal samples but had a poor ability to differentiate BTCC from normal tissues, and only 45% of T_2-T_4 tumors had RASSF1A expression higher than the 95th percentile of expression in normal samples. Four candidate markers, insulin-like growth factor 1 (IGF-1), human telomerase reverse transcriptase (hTERT), BLCA-4 and homeobox A13 (HOXA13), showed greater up-regulation in stage T_a-T_1 tumors than in stage >T_1 tumors, with the greatest differential expression of BLCA-4 and HOXA13. Based on the reproducibility of real time PCR, those genes that should be included in further difference analysis of marker accumulation between BTCC patients and controls were determined. IGF-1, hTERT, BLCA-4 and HOXA13 were finally confirmed in BTCC tissues, and used to stratify patients into low- or high-risk groups.

Quantitative real-time PCR analysis

QPCR was performed to detect the candidate markers (IGF-1, hTERT, BLCA-4 and HOXA13). In addition to assessing the performance of individual marker, LDA was used to develop a multivariate analysis of all four markers (designated uRNA-D). Sensitivities and specificities for each marker and uRNA-D were determined, and the characteristics of each analysis were further evaluated using ROC curves. Because of the potential effects on the characteristics of samples from patients with urinary tract infections, sensitivities and specificities were analyzed in the absence or presence of 30 samples from patients with infections. Results showed the ROC curves for the detection of BTCC at stage T_a-T_1, >T_1, grade 1, grade 2, and grade 3 in the absence of these samples (72 BTCC and 53 normal controls). The sensitivity of each marker and their combination in this cohort of 72 BTCC and 53 normal controls was shown in **Table 2** with a specificity of 85%. At this specificity, hTERT and IGF-1 were the unique markers with the highest sensitivity for high-stage/high-grade BTCC, and 87% of grade 3 tumors, 90% of stage T_1 tumors and 100% of stage >T_1 tumors could be identified with hTERT.

At the same specificity used in **Table 3**, the inclusion of patients with infection into the non-BTCC control group had minor effects on the

Table 4. Sensitivity of individual markers and combined assay uRNA-D in the differentiation of BTCC different stages and grades at 85% specificity in the presence of samples from patients with urinary tract infections

BTCC presentation	n	Mean percentage (95% confidence interval)				
		IGF-1	hTERT	BLCA-4	HOXA13	uRNA-D
Stage T_a	31	48 (45-49)	65 (62-66)	92 (90-94)	40 (38-40)	67 (65-68)
Stage T_1	15	65 (64-67)	90 (90-90)	90 (90-90)	40 (36-40)	89 (86-89)
Stage T_2~T_4	26	80 (80-80)	100 (100-100)	80 (80-80)	100 (100-100)	100 (100-100)
Grade 1	32	62 (60-64)	80 (79-83)	97 (95-97)	49(47-50)	67 (66-69)
Grade 2	24	68 (65-69)	88(85-88)	93 (89-93)	44 (44-44)	61 (61-61)
Grade 3	16	77 (75-80)	93 (90-93)	80 (78-82)	46 (44-46)	94 (90-94)

Footnotes: The mean sensitivities and 95% confidence intervals determined by LDA and cross-validation are shown. All 83 non-BTCC samples, including 30 infection samples, were included in the analysis.

sensitivity of BLCA-4, HOXA13 and IGF-1. BLCA-4 and HOXA13 showed small improvements in sensitivity, and hTERT showed no more than a 3% loss in the performance for cancers at any stage or grade. There was, however, a significant change in IGF-1 (an average 10% decrease) in the sensitivity across tumors at different stages and grades. The effect of uRNA-D was intermediate for the single marker analysis, showing 2% and 5% decrease in the sensitivity in the detection of BTCC of grade 1-2 and grade 3, respectively (**Table 4**). Analysis of ROC curves, generated from 72 BTCC samples and either 53 non-infection controls or 30 infection samples alone, confirmed that infection samples had no negative effects on the overall sensitivity of BLCA-4 and HOXA13 in the 80%-100% specificity range and only a minor effects on the IGF-1 and uRNA-D (**Figure 4**).

The specificity of each marker and uRNA-D for different non-BTCC control groups was shown in **Table 2**. When the specificity for all control samples was set at 85%, the specificity for infection samples alone ranged from 75% for BLCA-4 to 100% for HOXA13; uRNA-D exhibited an intermediate specificity for 80% of infection samples. No significant difference was found in the marker specificities between patients with non-BTCC cancers and benign urinary tract diseases. In summary, these results indicated that the combination of these markers (IGF-1, hTERT, BLCA-4 and HOXA13) leads to a better sensitivity than one marker alone does. In each case, the greatest accuracy in discriminating difference classes was provided through the introduction of HOXA13 and BLCA-4 to the analysis. These findings suggest that IGF-1, hTERT, BLCA-4, HOXA13 and their combination may provide greater sensitivity than single marker and also provide a means to differentiate low-grade T_a tumors from those of high grade or having an invasive phenotype.

Real time quantitative RT-PCR showed the detection rates of some known genes (IGF-1, hTERT, BLCA-4 and HOXA13) for BTCC at T_a, T_1 and $>T_1$ stages were 48%, 90% and 100%, respectively, with a specificity of 85%. The test specificity was 80% for 30 control patients with urinary tract infections. The combination of BLCA-4 and HOXA13 could distinguish between low- and high-grade tumors, with specificity and sensitivity of 80%.

Discussion

The identification of bladder cancer by detecting urine samples has two distinct problems. First, the cancer ranges from small highly differentiated polyps with low malignant potential to high grade *in situ* carcinomas and highly invasive, poorly differentiated tumors. These different types of tumors are usually characterized by differences in gene and protein expression patterns. Second, the cellular content of a urine sample is affected by the size and histologic characteristics of the tumor, the presence of blood and inflammatory cells, and the number of exfoliated non-malignant urothelial cells in the urine [9]. The challenge is to develop a test that not only accounts for the tumor heterogeneity but exhibits a high specificity in a clinical setting where frank and occult urinary tract infections and hematuria are common.

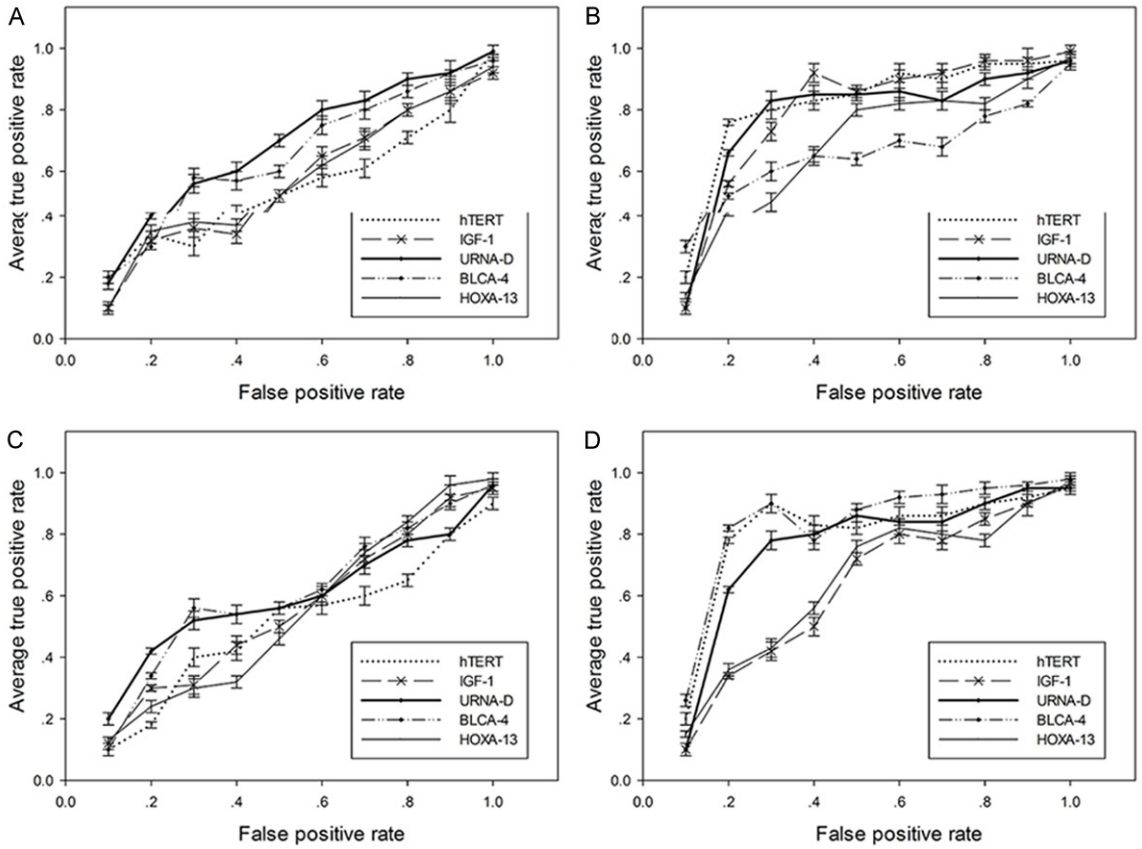

Figure 4. ROC for the detection of stage T_a-T_1 tumors (A), stage T_2-T_4 tumors (B), grade 1-2 tumors (C), and grade 3 tumors (D). Seventy-two TCC and 53 non-TCC samples were used in each analysis. Dotted vertical lines in A and C showed results at 90% specificity.

We reasoned that a combination of over-expressed markers with low expression in the blood and inflammatory cells would provide the basis for a urine test with these markers.

In the present study, a genome-wide gene expression database was employed for the marker selection to avoid any selection bias. A total of 126 differentially expressed genes were input STRING database and screening showed about 2/3 of differentially expressed genes had interactions. KEGG pathway analysis revealed 26 central nodes of the interaction network of these genes were involved in a variety of biological processes related to tumorigenesis and play important roles in a lot of signaling pathways. The four genes with the largest alteration in their expression between BTCC and normal bladder mucosa were IGF-1, hTERT, BLCA-4 and HOXA13, all of which are crucial for the tumorigenesis. This suggests that the tumorigenesis of BTCC is dominated by some genes as in other cancers and has involvement of changes in gene expression and regulation of multiple signaling pathways.

Highly sensitive qPCR has shown its ability to determine the stage and grade of tumors and to distinguish malignant tumors from benign tumors. Among the highly over-expressed genes selected for qPCR analysis, four were further verified, particularly in tumors at stage T_1-T_4. Results showed the results of quantitative PCR were consistent with those from microarray assay. Genes (such as IGF-1, a gene involved in cell growth and proliferation and a potent inhibitor of programmed cell death) showed high expression in a majority of tumors examined [10-13]. Whereas the hTERT expression was variable, and its up-regulation was only observed in a fraction of tumors [14]. HOXA13, a transcriptional factor, is involved in the morphogenesis and differentiation of genitourinary tracts [15]. BLCA-4 is a bladder cancer specific

nuclear matrix protein, which is a cancellated component and affects diverse processes including development, differentiation and survival of cancer cells [16, 17]. Our findings indicated the expressions of HOXA13 and BLCA-4 were elevated in stage T_a tumors. The protein expressions of IGF-1, hTERT, BLCA-4 and HOXA13 were also examined by Western blot assay. The protein and mRNA expressions of target genes were also detected in the exfoliated urothelial cells of BTCC patients and controls, indicating a strong correlation in the results between microarray assay and protein detection.

ROC showed that, in the 90%-100% specificity, HOXA13 and BLCA-4 showed a better performance in the detection of T_a/low-grade tumors than either IGF-1 or hTERT. In contrast, IGF-1 and hTERT were closely related to the highly invasive and high-grade tumors. To develop a test with good performance for both early and late stage cancers, LDA was used to develop algorithms that incorporated all four markers. The combined test, uRNA-D, showed more sensitive or specific than any individual gene. The sensitivity of uRNA-D was higher than urine cytology across all stages and grades of BTCC as uRNA-D detected 90% of stage T_1 cancers (85% specificity) compared to 40% by cytology (**Table 3**). With this caution in mind, comparison of data presented here with those published suggested that uRNA-D might have better performance than the UroVysion fluorescence *in situ* hybridization (FISH) assay in the detection of primary TCC at equivalent specificities: uRNA-D detected 60% and 92% of primary T_a and T_1 tumors, respectively, compared to 59% and 73% by FISH assay [18-20]. Compared to the UroVysion FISH assay, uRNA-D had comparable performance across grades. Because the characteristics of BTCC patients and controls were different in a variety of studies, the reported results would be only used as references in the screening of BTCC patients with these markers.

The contamination of blood or inflammatory cells is one of limitations in the clinical application of many screening tests for bladder cancer, including urine cytology, BTA-Stat, and NMP22 [21, 22]. Given the known inaccuracy in discriminating BTCC at stage T_a from those at stage T_1 with clinical utility, the prediction of stage or grade at the time of initial screening may enable the patients with suspected BTCC to receive urgent cystoscopy. Though the results presented here are required to be further validated in future studies, particularly in patients with hematuria and other urological conditions, our findings may provide evidence for the early diagnosis of BTCC in clinical practice.

Taken together, this study delineates the interaction network of differentially expressed genes in BTCC, and central nodes are identified from this network and further validated in the urine samples. Our findings provide evidence for not only the elucidation of pathogenesis of invasive BTCC, but for the early diagnosis and molecular targeted therapy of bladder cancer.

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Disclosure of conflict of interest

None.

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References

[1] Jemal A, Siegel R, Xu J and Ward E. Cancer statistics, 2010. CA Cancer J Clin 2010; 60: 277-300.

[2] Liu E, Xiang Y, Jin F, Zhou S, Sun L, Fang R, Yuan Z, Gao L and Gao Y. Cancer incidence trends in urban Shanghai, 1972-1999. Tumor 2004; 24: 11-15.

[3] Parekh DJ, Bochner BH and Dalbagni G. Superficial and muscle-invasive bladder cancer: principles of management for outcomes assessments. J Clin Oncol 2006; 24: 5519-5527.

[4] Villicana P, Whiting B, Goodison S and Rosser CJ. Urine-based assays for the detection of

bladder cancer. Biomark Med 2009; 3: 265-274.
[5] Sanchez-Carbayo M, Socci ND, Lozano J, Saint F and Cordon-Cardo C. Defining molecular profiles of poor outcome in patients with invasive bladder cancer using oligonucleotide microarrays. J Clin Oncol 2006; 24: 778-789.
[6] Brinkman AB, Simmer F, Ma K, Kaan A, Zhu J and Stunnenberg HG. Whole-genome DNA methylation profiling using MethylCap-seq. Methods 2010; 52: 232-236.
[7] Sauter G, Algaba F and Amin M. WHO Classification of Tumour of urinary system and male genital organs. Lyon: IARCC Press; 2004.
[8] Sobin LH, Gospodarowicz MK and Wittekind C. TNM classification of malignant tumours. John Wiley & Sons; 2011.
[9] Cowell JK and Hawthorn L. The application of microarray technology to the analysis of the cancer genome. Curr Mol Med 2007; 7: 103-120.
[10] Maggio M, De Vita F, Lauretani F, Buttò V, Bondi G, Cattabiani C, Nouvenne A, Meschi T, Dall'Aglio E and Ceda GP. IGF-1, the cross road of the nutritional, inflammatory and hormonal pathways to frailty. Nutrients 2013; 5: 4184-4205.
[11] Cappola AR, Xue QL, Ferrucci L, Guralnik JM, Volpato S and Fried LP. Insulin-like growth factor I and interleukin-6 contribute synergistically to disability and mortality in older women. J Clin Endocrinol Metab 2003; 88: 2019-2025.
[12] Chia DJ, Varco-Merth B and Rotwein P. Dispersed chromosomal Stat5b-binding elements mediate growth hormone-activated insulin-like growth factor-I gene transcription. J Biol Chem 2010; 285: 17636-17647.
[13] Serel TA, Turan T, Soyupek S, Aybek Z and Perk H. Urine and serum free IGF-1 levels in patients with bladder cancer: a brief report. Urol Res 2003; 31: 297-299.
[14] Xie XY, Yang X, Zhang JH and Liu ZJ. Analysis of hTERT expression in exfoliated cells from patients with bladder transitional cell carcinomas using SYBR green real-time fluorescence quantitative PCR. Ann Clin Biochem 2007; 44: 523-528.
[15] Scott V, Morgan EA and Stadler HS. Genitourinary functions of Hoxa13 and Hoxd13. J Biochem 2005; 137: 671-676.
[16] Van Le TS, Myers J, Konety BR, Barder T and Getzenberg RH. Functional characterization of the bladder cancer marker, BLCA-4. Clin Cancer Res 2004; 10: 1384-1391.
[17] Santoni M, Catanzariti F, Minardi D, Burattini L, Nabissi M, Muzzonigro G, Cascinu S and Santoni G. Pathogenic and Diagnostic Potential of BLCA-1 and BLCA-4 Nuclear Proteins in Urothelial Cell Carcinoma of Human Bladder. Adv Urol 2012; 2012: 397412-397415.
[18] Bayani J and Squire JA. Application and interpretation of FISH in biomarker studies. Cancer Lett 2007; 249: 97-109.
[19] Kipp BR, Tanasescu M, Else TA, Bryant SC, Karnes RJ, Sebo TJ and Halling KC. Quantitative fluorescence in situ hybridization and its ability to predict bladder cancer recurrence and progression to muscle-invasive bladder cancer. J Mol Diagn 2009; 11: 148-154.
[20] Bubendorf L, Grilli B, Sauter G, Mihatsch MJ, Gasser TC and Dalquen P. Multiprobe FISH for enhanced detection of bladder cancer in voided urine specimens and bladder washings. Am J Clin Pathol 2001; 116: 79-86.
[21] Guo A, Wang X, Shi J, Sun C and Wan Z. Bladder tumour antigen (BTA stat) test compared to the urine cytology in the diagnosis of bladder cancer: A meta-analysis. Can Urol Assoc J 2014; 8: e347-352.
[22] Miyake M, Goodison S, Giacoia EG, Rizwani W, Ross S and Rosser CJ. Influencing factors on the NMP-22 urine assay: an experimental model. BMC Urol 2012; 12: 23.