Expression of survivin mRNA in urine exfoliated cells of patients with bladder transitional cell carcinoma detected by real-time PCR

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[Abstract] Background and Objective: Bladder cancer is the most malignant cancer of the urinary system. However, a noninvasive and sensitive method of the early diagnosis for bladder cancer has not been developed. This study was to explore the expression of survivin mRNA in urine exfoliated cells of preoperative and postoperative patients with bladder transitional cell carcinoma (BTCC), and to analyze its value in early diagnosis and postoperative monitoring. Methods: Urine of 30 patients with initially diagnosed BTCC was collected before operation and one week, one month, six months and 15 months after operation. Urine of 10 healthy volunteers and 15 patients with cystitis was used as control. Expression of survivin mRNA in urine exfoliated cells was detected by real-time fluorescent quantitative polymerase chain reaction (real-time PCR). Results: The relative copy number of survivin mRNA in the patients was (96.01± 42.33) before operation, which was significantly higher than that of healthy volunteers and cystitis patients (P <0.05). The level of survivin mRNA was apparently declined one week after operation (25.30± 1.51) compared with its preoperative level (P <0.05); and the level became as low as the control group after one month (13.20± 1.49) and six months (13.90± 1.36) (P >0.05). Patients were followed up for 15 months, and three patients recurred, whose survivin mRNA level (97.83± 27.47) was significantly higher than that at six months after operation (P <0.05). Conclusion: Detecting survivin mRNA in urine exfoliated cells is sensitive for the diagnosis of BTCC. Detection survivin mRNA after operation can monitor recurrence.

Key words: bladder transitional cell carcinoma, survivin mRNA, real-time fluorescent quantitative polymerase chain reaction

In China, bladder cancer is the most common malignant tumor in the urinary system. The incidence of bladder cancer significantly increases in recent years, and it is prone to recur even after surgery and postoperative intravesical perfusion treatment. Therefore, early diagnosis, early treatment and dynamic follow-up after treatment are very important. Recently, searching for a highly sensitive, specific and non-invasive method for the early diagnosis of bladder cancer is an important direction in Urological research. In this study, real-time fluorescent quantitative PCR method was applied to detect the expression level of survivin mRNA in urine exfoliated cells in patients with bladder transitional cell carcinoma (BTCC) before and after surgery, in order to assess its clinical significance in early diagnosis and postoperative monitoring of tumor recurrence.
Data and Methods

Clinical information. This study included a total of 30 patients with BTCC who were hospitalized for surgery in the First Affiliated Hospital of Soochow University and Suzhou Municipal Headquarters Hospital from April 2006 to December 2006. There were 21 cases of male and nine cases of female, aged 52–85 years old, with a median age of 66.5 years old. At before operation, one week, one month and six months after operation, 150 mL clean midstream morning urine samples were collected. Regular follow-up was performed up to 15 months after surgery, and another 150 mL urine samples were collected from postoperative recurrent patients before their second surgery. There were a total of 25 subjects in the two control groups, of which 10 cases were healthy volunteers, seven cases of males and three cases of females, aged 55 to 76 years old, with a median age of 65.5 years old. Another 15 cases were cystitis patients, eight cases of males and seven cases of females, aged 50 to 79 years old, with a median age of 64 years old.

Major reagents. Major reagents included TRIZOL RNA extraction reagent, M–MLV reverse transcriptase, RNAsin, dNTP and Taq enzyme. Primer sequences for survivin gene were as follows: upstream 5′-TGCCTGCGACCCCTTTC-3′, downstream 5′-GCG CAGCCCTCAAGAA-3′; and probe 5′-FAM-CAAGGACACGGCATCTCATTCAAAG-TAMRA-3′. Primer sequences for Ab1 gene were as follows: upstream 5′-TCTCCAGCTGTATCTGGAAG-3′; downstream 5′-ACTACAGCCTGAGGCTAAAG-3′; and probe 5′-FAM–AAGCCCTTCAGCGGCCAAG TAG CAT-TAMRA-3′. Main instruments used were a low-temperature high-speed centrifuge, a PE 9600 PCR amplification instrument, a MJ DNA Engine OPTICON2 system and a UV–spectrophotometer.

Methods. Sample preparation; Urine of subjects (150 mL) was centrifuged at 3000 r/min for 5 min. After discarding the supernatant and a rinse with PBS solution once, exfoliated cell pellets were collected.

RNA extraction; Total cell RNA in cell pellets were extracted according to the protocol provided in the TRIZOL kit, and mRNA was quantified by measuring the absorbance at 280 nm on a UV spectrophotometer. RNA concentration was adjusted to 0.5 μg/μL.

Synthesis of cDNA; random primers (2 μL), 4 μL RNA and 9 μL DEPC-H2O were added into the tube ①, incubated at 70°C for 5 min, 8 μL 5 × Buffer, 1 μL MMLV, 0.5 μL RNAsin, 1.25 μL dNTP and 14.25 μL ddH2O were added into tube ②. Then tubes ① and ② were mixed, incubated at 37°C for 1 h, then at 95°C for 5 min, and finally stored at 4°C.

Real-time PCR reaction; The PCR system included 5 μL 5 × Buffer, 0.3 μL Mg2+, 0.75 μL dNTP, 0.3 μL Taq Man probe, 0.5 μL Primer1, 0.5 μL Primer2, 0.25 μL Ex–hotstart Taq enzyme, 15.4 μL DEPC-H2O and 2 μL sample. Reaction conditions were: pre–denaturation at 95°C for 5 min, then 95°C 15 s, 60°C 1 min followed by plate reading, for a total of 50 cycles, and the fluorescent signal was collected at 80°C. The relative copy number was calculated using the following formula: relative copy number = (copy number of survivin gene / copy number of abl) × 10^4.

Statistical methods. Quantitative data were presented as mean±SD, and statistical analysis was performed using SPSS 12.0 statistical software. Comparisons between two groups were performed using t test and among multiple groups using single-factor analysis of variance. P < 0.05 was set as the criteria for statistically significant difference.

Results

Among 10 subjects in the normal control group, expression levels of survivin mRNA in urine exfoliated cells were all low, with the relative copy number of (13.12 ± 2.25). Among 15 subjects in the cystitis control group, the expression level of survivin in one case was relatively high, up to 46.25, while all other patients had low expression levels, with the relative copy number of (15.73 ± 8.55). There was no significant difference between the two groups (P > 0.05).

The 30 cases of BTCC patients in the experimental group had high expression levels of survivin mRNA before operation, with the relative copy number of (96.01 ± 42.33), which was significantly higher than that of the normal control group and cystitis group (P < 0.05). At postoperative week one, the relative copy number of survivin mRNA in 30 cases of BTCC patients was (25.30 ± 1.51), which was significantly decreased compared to its preoperative level (P < 0.05). At one month and six months postoperatively, the relative copy numbers of survivin mRNA were (13.20 ± 1.49) and (13.90 ±
1.36) respectively. There were no statistical differences compared to the healthy control group (13.12 ± 2.25) (P > 0.05). Follow-up lasted for 15 months after surgery, and three patients were confirmed as recurrence by cystoscopy and imaging examination at 7.5, 12.5 and 15 months after surgery, respectively. Before the second surgery, the relative copy number of survivin mRNA was significantly increased to (97.83 ± 27.47), which was significantly different to the level at six months after the first surgery (P < 0.05).

In the patient group, one case of a 71-year old male patient was diagnosed as stage T1 bladder cancer, who was subjected to transurethral electrical resection of the bladder tumor. Pathological diagnosis confirmed this case as grade II BTCC. At six months postoperatively, the relative copy number of survivin mRNA in this patient was 42.55, significantly higher than that of other patients at the same time point. Previous cystoscopy failed to detect abnormal findings, but cystoscopy and imaging examination at 7.5 months postoperatively confirmed tumor recurrence. The postoperative expression level of survivin mRNA in urine exfoliated cells changed from a high to a low level, but rose again before clinical diagnosis of tumor recurrence (Fig. 1).

Discussion

Cystoscopy and bladder biopsy are still the gold standard for bladder cancer diagnosis and follow-up. However, since cystoscopy is an invasive examination, it not only causes pain in patients, but may also lead to many complications, such as urethral injury, bleeding and urinary tract infection and so on, therefore its clinical application is limited. Meanwhile, the sensitivity of urinary cytology is low, thus an ideal method for early diagnosis of bladder cancer in clinic is in need. Recently, many studies on urinary biomarkers to detect bladder cancer were underway in China and overseas, which identified some tumor markers, such as bladder tumor antigen (BTA), urinary bladder cancer marker (UBC) and nuclear matrix proteins (NMPs), and so on. However, these markers show relatively low sensitivities and specificities. The high false-positive rate could be caused by cystitis, urinary tract infections, urethral stone and other benign diseases.

Survivin is a member of the inhibitor of apoptosis protein family (IAP), which is the strongest apoptotic inhibitor discovered so far. It is not expressed in normal terminally differentiated tissues and cells, but highly expressed in most malignant tumor tissues, including bladder cancer. Previous studies by our group confirmed that survivin expression in urine exfoliated cells of bladder cancer patients had a sensitivity of 100% and specificity of 77.8%. Shariat et al. tested the expressions of survivin and NMP22 in urine and compared the results with urinary cytology. They believed that urinary survivin is the most sensitive independent indicator for bladder cancer. Smith et al. divided newly diagnosed and recurrent patients into five groups and detected their urinary survivin expression statuses using the one-step antibody reaction method, confirmed western blot and RT-PCR. They found that the sensitivity of this antibody reaction method to diagnose new episode and recurrent bladder cancer is 100% and the specificity is 95%. This suggests that detection of survivin mRNA in urine exfoliated cells provides a new noninvasive method to diagnose bladder cancer.

This study found that survivin mRNA detected by real-time fluorescent quantitative PCR was highly expressed in newly diagnosed BTCC patients before operation, with a sensitivity up to 100%, consistent with our earlier studies and study by Smith et al. In BTCC patients, urinary expression of survivin mRNA changed from a high level preoperatively to a low level postoperatively, which was elevated again in recurrent patient after operation, indicating that it is also valuable for monitoring postoperative tumor recurrence.

During the dynamic follow-up, one patient was found to have increased expression of urinary survivin mRNA at six months postoperatively. Although no abnormal findings were detected by cystoscopy at
that time, tumor recurrence was confirmed at 7.5 months after surgery by cystoscopy and imaging examinations. Urinary expression of survivin mRNA in this patient was increased before cystoscopically diagnosis of recurrence, suggesting that urinary survivin mRNA can be used not only for the diagnosis of postoperative recurrence of BTCC, but also as an early diagnostic marker to predict tumor recurrence prior to cystoscopy and imaging studies. However, this claim needs to be confirmed by studies including a larger sample size, using extended follow-up and more frequent time points at peak tumor recurrent period, in order to gain more data in for statistical analysis.

Using real–time fluorescence quantitative PCR to detect the expression of survivin mRNA in urine is highly sensitive, convenient and noninvasive, so it is of good application value in the early diagnosis and postoperative monitoring of BTCC patients.

References


