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QUANTITATIVE DETECTION OF CYTOKERATIN 20 mRNA IN URINE SAMPLES AS DIAGNOSTIC TOOLS FOR BLADDER CANCER BY REAL-TIME PCR

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Aim: **To determine and compare cytokeratin 20 (CK20) mRNA expression in urine of patients with transitional cell carcinomas of bladder (TCCB), urological benign diseases, and healthy volunteers.** ***Methods:*** **Taqman probe was designed according to the sequence of *CK20* cloned gene. The quantitative PCR reaction system was optimized and evaluated. The CK20 mRNA level was screened by real-time polymerase chain reaction (RT-PCR) in 95 urine samples and analyzed according to the following parameters: urinary cytology, nuclear matrix protein 22 (NMP22) expression, tumor stage and grade.** ***Results:*** **For 60 TCCB patients urinary cytology was positive in 28 (46.7%), control group had no false-positive results (specificity 100%). CK20 expression was positive in RT-PCR of 51 cases (85%) of TCCB, but control group was positive in 2 cases (specificity 94.3%) with a cutoff value of crossover point (CT) = 30. Two methods have significant variation in sensitivity ($p < 0.001$), NMP22 expression was positive in 47 cases (78.3%), but control group was positive in 9 cases (specificity 85%). In the simultaneous evaluation of CK20 and NMP22 mRNA expression, there were 54 positive cases (90%). CK20 mRNA values in TCCB group (mean 27712.57 copies/μl) were significantly higher than in non-cancer disease urological group (mean 74.45 copies/μl) and control group (mean 8.47 copies/μl) ($p < 0.001$, $p < 0.001$, respectively). CK20 mRNA values increased gradually with higher tumor grade and stage: G1 differs significantly from G2 ($p = 0.016$); T_{is}/T_a differs significantly from $T_{1–2}$ ($p = 0.022$).** ***Conclusion:*** **Our results indicate that CK20 mRNA expression could be regarded as a potential marker for TCCB. We demonstrated correlation between CK20 expression and the clinicopathologic features of TCCB (tumor stage and grade); simultaneous use of CK20 and NMP22 markers will elevate the sensitivity of the method. CK20 RT-PCR is a sensitive, quantitative, rapid and specific method to detect free cancer cells in the urine, and could be recommended for be wide application in the diagnostics of TCCB and evaluation of therapeutic effect.**

Key Words: **cytokeratin 20, transitional cell carcinoma of bladder, real-time PCR.**

Transitional cell carcinoma of bladder (TCCB) is the most common malignancy of the genitourinary system. Despite the use of the current multimodality approach to treatment of TCCB, the patients with the disease have a very high recurrency rate [1–3]. Therefore, the early detection of new or recurrent TCCB lesions is crucial for successful treatment and favorable prognosis. Currently, diagnosis and monitoring of TCCB is based on cystoscopy, combining the additional information provided by urinary cytology. Despite their great clinical utility, these diagnostic methods still have some limitations mainly related to their sensitivity. Accordingly, alternative methods are urgently required. An objective marker detection that is not based only on morphologic criteria should have been established [4].

An ideal cancer marker should be objective, noninvasive, easy to administer and interpret, and possess high sensitivity and specificity. Bladder tumor antigen (BTA), fluorescence in situ hybridization (FISH), nuclear matrix protein-22 (NMP22) and human telomerase reverse transcriptase (hTERT) have been developed for monitoring patients with TCCB [5-6], and have been approved for clinical use, but their sensitivity depends on tumor differentiation. Regular cystoscopic monitoring thus continues to be the standard in tumor surveillance.

Cytokeratins (CKs) are differentiation intermediate filament proteins, which constitute the cytoskeleton of epithelial cells. More than 20 different CKs are known, and are divided into types I and II based on sequence homology [7]. Moll *et al.* [8] used an immunohistochemistry technique to measure expression of a new cytokeratin 20 (CK20) which is expressed in gastrointestinal epithelium, uroepithelial cells from patients with urothelial cancer and Merkel cells, however, normal urothelial cells do not express the *CK20* gene. These findings suggest that *CK20* expression possibly may serve as a specific marker for detecting urothelial cancer cells [9–10].

Because of its high sensitivity, real-time polymerase chain reaction (RT-PCR) based on the amplification of cell type-specific mRNA have been increasingly used to detect cancer marker [11–12]. In the present study we established a rapid quantitative method for detection of *CK20* mRNA level in urine samples by RT-PCR on the LightCycler, investigated the diagnostic significance of *CK20* mRNA quantification in TCCB patients, and examined whether *CK20* gene expression correlates with the TCCB clinicopathologic features (tumor stage and grade).

MATERIALS AND METHODS

Patients. In the current work, urine samples of 60 patients with histologically proved TCCB and

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Abbreviations used: CK20 – cytokeratin 20; CT – crossover point; CV – coefficient of variation; NMP22 – nuclear matrix protein 22; ROC – the receiver operating characteristics; RT-PCR – real-time polymerase chain reaction; TCCB – transitional cell carcinomas of bladder.

20 patients with non-cancer urological diseases (bladder benign tumor, cystitis glandularis, ureteritis and urinary tract infection) treated at the First Affiliated Hospital of Chongqing Medical University during September 2007 to October 2008 were examined. The study also included 15 healthy volunteers. TCCB cases were staged and graded pathologically according to the International Union Against Cancer 1997 TNM and WHO classifications (Table 1). All patients signed a consent form approved by the Committee on Human Rights in Research of our institution.

Table 1. Characteristics of patients with transitional cell carcinoma, non-cancer urological diseases and control group of healthy volunteers

Group description	Number of cases
TCCB patients (range 35–85 years)	60
Male-to-female ratio	46 : 14
Stage	
pT_{is} / pT_a	18
pT_{1-2}	32
pT_{3-4}	10
Grade	
1	28
2	20
3	12
Urological non-cancer diseases (range 16–78 years)	20
Male-to-female ratio	13 : 7
Healthy volunteers (range 20–40 years)	15
Male-to-female ratio	10 : 5

Sample preparation. First spontaneously voided urine (150–200 ml) was obtained from all patients and controls, enrolled in the study, divided by 50 ml for conventional cytological and nuclear matrix protein 22 (NMP22) examination. The remaining volume immediately was centrifuged at 800 g for 10 min at 4 °C to collect intact exfoliated cells for RT-PCR. The pellets were stored at –80 °C for further use.

Cytological and NMP22 examination. 48 ml of urine sample was centrifuged at 800 g; pellet was stained by hematoxilin/eosin and studied by light microscopy. 2 ml of urine sample was dripped into the sampling bottle, which contained specially stabilized reagent, and then centrifuged at 1000 r/min for 15 min. The supernatant was tested with NMP22 special ELISA kits (Matritech Inc., USA).

Design of the RT-PCR. The cDNA sequences were from Genbank: NM-019010. The regions for primers and probe were chosen based on the conserved areas; a 96-bp fragment of *CK20* encoding mRNA was amplified from the cDNA (Table 2). Plasmid DNA standards for *CK20* were diluted into eight (range 10^2 to 10^9 copies/μl) and analyzed using an ABI 7000 (ABI, USA) sequence detection system and SDS software, A standard curve was constructed by the crossover point (CT).The *CK20* mRNA value in each sample was calculated from this calibration curve.

Table 2. Primers and probe used for CK20 RT-PCR

F/R	Primer sequence
Forward	TTGAAGAGCTGCGAAGTCAGAT
Reverse	TGAAGTCCTCAGCAGCCAGTT
Taqman probe	FAM-TCAACTGCAAAATGCTCGGTGTGTCC-TAMRA

RNA extraction and cDNA synthesis. Total RNA was extracted from urine samples using a commercially available RNA extraction kit (Trizol, Takara, Japan) according to the manufacturer's instructions. RNA concentrations were determined by spectrophotometric analysis at 260 nm wavelength. Then reversely transcribed with a primescript™ RT Reagent kit (Takara, Japan) : 5 × primescript buffer : 4 μl, primescript RT enzyme mix I : 1 μl, oligo dT primer (50 μM) × 1 : 1 μl, random 6 mers (100 μM) × 1 : 1 μl, total RNA : 4 μl (50 ng), RNase free ddH_2O : 9 μl, total volume : 20 μl. RT reactions were performed for 37 °C 15 min, 85 °C 5 s. cDNA was stored at –20 °C for further PCR.

RT-PCR analysis of CK20 mRNA expression level in urine samples. The RT-PCR reactions were performed in 25 μl volumes containing 10 × PCR buffer : 2.5 μl; $MgCl_2$ (25 mmol/l) : 3.5 μl; dNTPs (2.5 mmol/l) : 2 μl, Taq polymerase (5 U/μl): 0.35 μl; forward primer (10 pmol/μl) : 2.2 μl; reverse primer (10 pmol/μl) : 2.2 μl; Taqman probe (10 pmol/μl) : 2.2 μl; cDNA: 2 μl; ddH_2O : 8.05 μl; cycling parameters included 40 cycles of 5 min each at 95 °C, 30 s at 95 °C, and 70 s at 51 °C. Each run consisted of 8 external standards and a negative control. The amplified PCR products were run on 1% agarose gel, stained by ethidium bromide.

Statistical analysis. Statistical analysis was performed using SPSS 15.0 Statistical Package. Comparison of RT-PCR and cytological examination by the chi-square (X^2) test, with $p < 0.05$ considered significant. The Kruskal-Wallis test was performed to analyze the significant variation among carcinoma, benign disease and control groups; CK20 values with reference to tumor staging and grading were analyzed by Kruskal-Wallis, $p < 0.05$ considered significant.

RESULTS

Evaluation of RT-PCR. To estimate the RT-PCR assay, using plasmid DNA control with 10 fold serial dilutions of known quantities from 10^1 to 10^{10} copies/μl, amplifications of standard dilution series were then performed, a standard curve was constructed by plotting the crossover point, at which the signal increased greater than the background level, against the log number of CK20 expression for each run. The RT-PCR assay that we developed showed high sensitivity (10^2 copies/μl). The screening of urine samples of different for CK20 expression by RT-PCR has shown good specificity: CK20 expression has been registered only in urine samples from TCCB patients. The coefficient of variation (CV) value was 1.59% in batch assay, and 2.34% in a day to day assay, and wide range of linearity from $10^2 \sim 10^9$ copies/μl (Fig. 1).

Cutoff value analysis by ROC curve. In present study, different cutoff values were used for discrimination between the presence or absence of CK20 in urine samples of TCCB patients. If after a predefined number of cycles no fluorescent signal was detected on amplification plots, the marker mRNA was assumed absent in the urine samples. The receiver operating characteristics (ROC) curve was used to estimate the cutoff value. The ROC curve for the predicted probabilities of a positive assay (at 0.5 threshold) is shown in Fig. 2, while CT = 30, the area under the ROC curve is 0.896, there was best sensitivity and specificity.

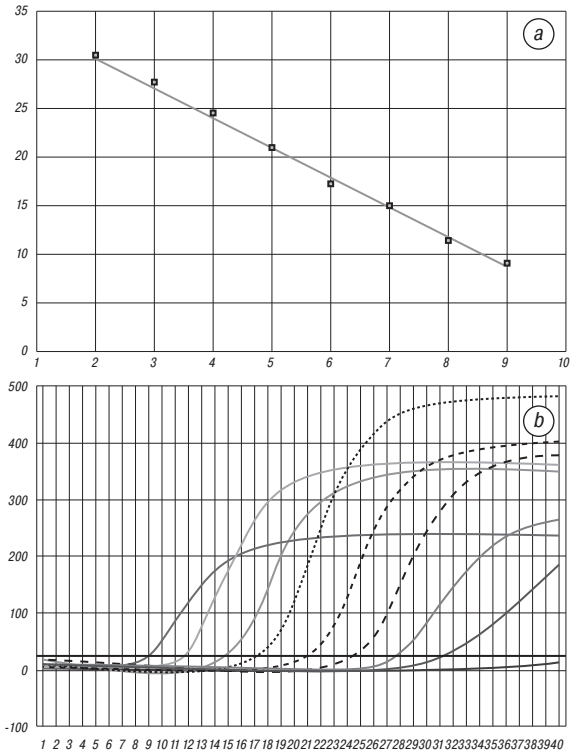

Fig. 1. Standard curve of real time PCR for CK20. *a*, run profile of RT-PCR cycle number, wide range of linearity from eight external standards including $10^2 \sim 10^9$ copies/μl; *b*, calibration curve for CK20 mRNA estimation constructed by plotting cycle number of crossover point against log, including 8 standards as shown in *a*. Relative CK20 mRNA values of unknown samples were calculated with reference to this curve

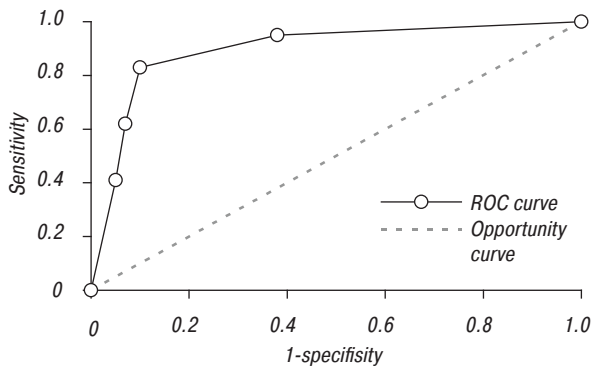

Fig. 2. ROC curve for CK20 cutoff value. ROC curves were constructed by plotting sensitivity and 1-specificity corresponding to each cutoff value for CK20 mRNA

Comparison of sensitivity and specificity of urinary cytology and urinary CK20 mRNA value. Sensitivity and specificity of CK20 expression by RT-PCR in urine sample were 85% and 94.3% respectively, with a cutoff value of 30. Compared with urinary cytology (positive in 28 (46.7%), specificity 100%), RT-PCR had significantly higher sensitivity ($p < 0.001$); from the 32 cytologically negative cases 23 were positive by RT-PCR, and only 9 were negative. All cytologically positive cases were RT-PCR positive (Fig. 3), which indicated improved accuracy of RT-PCR for TCCB diagnostics.

NMP22 and CK20 mRNA expression in urine samples. Detection CK20 expression had shown a higher sensitivity than detection of NMP22 expression (85% *vs* 78.3%, respectively), and higher specificity (94.3% *vs* 85% respectively). As reported in Table 3, sensitivity and specificity according to staging and grading of tumors was higher for CK20 than for NMP22 in the most cases, but in grade 2 tumors there was higher NMP22 expression level than shown for CK20.

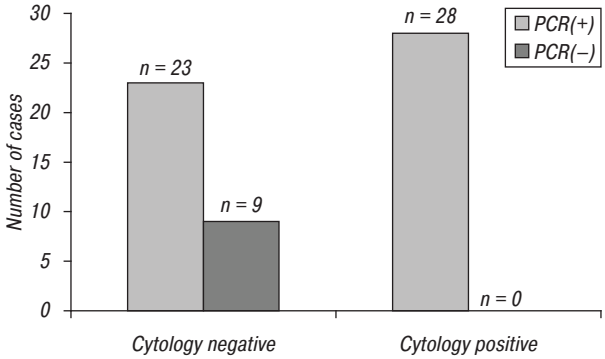

Fig. 3. Comparison of urinary cytology with CK20 results, RT-PCR positive (PCR [+]) or negative ([PCR [–]) was determined based on cutoff value 30. Cytology negative includes patients with classes 1, 2 and 3 cytology, and positive includes classes 4 and 5

Table 3. Expression of CK20 and NMP22 mRNAs and tumor stage/grade

Sample	No.	CK20		NMP22	
		Positive	Negative	Positive	Negative
TCCB patients	60				
Stage					
pT_{is} / pT_a	18	14 (77.8%)	4	11 (61.1%)	7
pT_{1-2}	32	28 (87.5%)	4	28 (87.5%)	4
pT_{3-4}	10	9 (90%)	1	8 (80%)	2
Grade					
1	28	23 (82.1%)	5	20 (71.4%)	8
2	20	17 (85%)	3	18 (90%)	2
3	12	11 (91.7%)	1	9 (75%)	3
Non-cancer diseases	20	2	18 (90%)	7	13 (65%)
Healthy volunteers	15	0	15 (100%)	2	13 (86.7%)

CK20 mRNA value in TCCB cases and control group. Different patients have significant CK20 expression variance. As it is shown on Fig. 4, the variance of CK20 mRNA expression in 9 urine samples from TCCB group analyzed by RT-PCR was 10^4 between the highest and lowest levels. Of the 60 cases of TCCB, mean CK20 mRNA value was 27712.57 copies/μl (range 15.00 to 46523.00), while in non-cancer urological disease group mean value was 74.45 copies/μl (0 to 328.00), and in control group, mean value was 8.47 copies/μl (0 to 35.00), and the variation was significant ($p < 0.001$, $p < 0.001$, respectively). The findings suggested that expression of CK20 is a marker of TCCB.

CK20 mRNA values and tumor staging and grading. Distribution of CK20 mRNA values by tumor grade and stage are shown on Fig. 5: mean CK20 mRNA value for 18 cases of noninvasive transitional cell carcinoma (pTis/pTa) was 20671.78 with individual data (range 15.00 to 32659.00), and for 42 invasive transitional cell carcinoma, including 32 cases pT_{1-2} was 29406.84 (48.00 to 40834.00), and for 10 cases of pT_{3-4} was 35585.30 (86.00 to 46523.00). Mean CK20 mRNA values for histological tumor grades 1, 2 and 3 were: 21901.89 (range 15.00 to 40286.00, n = 28), 30985.45 (66.00 to 40834.00, n = 20), and 35716.00 (48.00 to 46523.00, n = 12), respectively. There was a statistically significant variation in G1 *vs* G2 ($p = 0.016$), while G2 *vs* G3 had no statistically significant variation; there was a statistically significant variation in T_{is}/T_a *vs* T_{1-2} ($p = 0.022$), while T_{1-2} *vs* T_{3-4} had no statistically significant variation. These findings suggested cor-

relation between CK20 and the clinicopathologic features (tumor stage and grade) of TCCB.

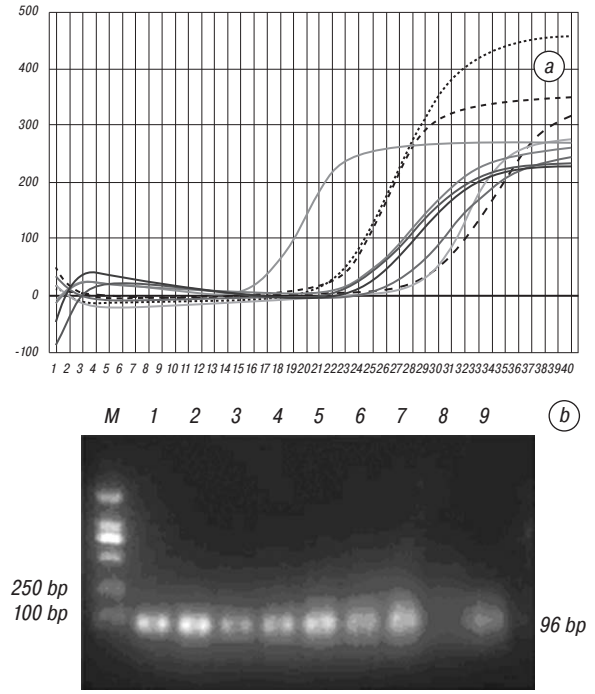

Fig. 4. CK20 mRNA expression in 9 urine samples from TCCB group analyzed by RT-PCR and 1% agarose gel. *a*, amplification curve of urine samples CK20 expression via RT-PCR; *b*, RT-PCR products resolved in 1% agarose gel: M: marker DL2000, 1 ~ 7, 9: CK20 RT-PCR product (+), 8: CK20 RT-PCR product (–). Different samples had different CK20 expression values

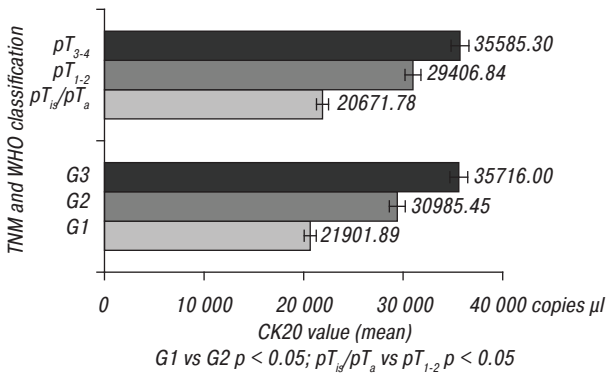

Fig. 5. CK20 expression value and tumor staging and grading. CK20 expression increased gradually, G1 was significantly different from G2 ($p = 0.016$); T_{is}/T_a was significantly different from T_{1-2} ($p = 0.022$)

DISCUSSION

Cystoscopy is still considered the gold standard for the diagnosis and follow-up of TCCB, but it is invasive. Voided urine cytology is the most common noninvasive technique for detecting bladder cancer and has high specificity, however, it has poor sensitivity. Thus, detection of urine markers, which have sufficient sensitivity and specificity, and may be used as diagnostic and surveillance protocols of TCCB [13]. CK20 are intermediate filaments expressed in epithelial cells, early immunological, and Northern blot studies found that CK20 expression was restricted primarily to gastrointestinal tissue, transitional cell carcinoma, and Merkel cells [8]. Due to significantly higher expression in urothelial tumors in comparison with clinical controls including urological benign diseases and healthy volunteers, CK20 mRNA in urine of patients with TCCB is potentially useful urinary marker with diagnostic value [14–16].

Traditional PCR assays had been reported for the detection of CK20 mRNA, Rotem D *et al.* detected CK20 expression of TCCB by traditional PCR, 86.7% of TCCB patients was positive, and only 3.3% of healthy volunteers were positive (specificity 96.7%), but traditional PCR depended on end point data collection, therefore susceptible to large variations that determine the outcome of every single reaction during PCR [17–19]. To overcome these problems, we established a new quantitative detection method for free cancer cells in the urine. Real-time PCR assays to quantify CK20 mRNA was performed with the use of total RNA extracted from the urine samples, and primers and hybridization probe specific for CK20.PCR product was measured during the exponential phase of PCR to eliminate the effect of large variations. To avoid cross-contamination, the analysis was divided into three separate work areas: (1) a plasmid DNA extraction area; (2) a master mix area for diluting and preparing reagent mixes; (3) and a RT-PCR reaction area. The working surfaces and equipment were kept sterile; all reagents were divided into specific storage [12]. With a cutoff value of CT = 30, CK20 mRNA sensitivity and specificity were 85% and 94.3%, respectively. False-positive results were no longer obtained in the healthy volunteer group, but in 2 of 20 patients in the non-cancer urological disease group with cystitis. These findings suggest that real-time PCR provides a rapid quantitative tool allowing appropriate diagnostic for TCCB.

In the present study, we determined cutoff value by the ROC curve that was constructed by plotting the sensitivity and specificity according to each cutoff value for CK20 mRNA. In all studies, additional statistical criteria were explored to dichotomize between positive and negative urine samples. While CT = 30, there were the 95% confidence interval or the 99% percentile of log-normal distribution [14].

In the present study, we aimed to compare results of conventional urinary cytology and quantitative real-time PCR for diagnosis of TCCB. Our results demonstrated that using the best cutoff point determined by the ROC curve, the positivity rate of real-time PCR was significantly higher than that of urinary cytology. Of the 32 cytologically negative cases 23 were positive by real time PCR. These findings suggested that first, CK20 mRNA was positive but urine cytology was negative since some urine samples contain very low number of cancer cells, which may be insufficient for cytological examination but enough for detection of CK20 mRNA by the more sensitive RT-PCR methods; second, RT-PCR false-negative results may be explained by negative or low expression of CK20 in transitional cell carcinoma cells.

The nuclear matrix is a RNA-protein network forming the structural framework of the nucleus. Proteins that bind to RNA in the matrix are called nuclear matrix proteins (NMPs). NMP22 was previously used as

a cancer-specific marker for TCCB. NMP22 specific ELISA kits are available for clinic application [20]. In this study, we compared CK20 and NMP22 expression for diagnose of TCCB. As the result, CK20 detection has shown higher sensitivity and specificity. However, some patients yet could not be diagnosed only dependent on CK20 detection. The simultaneous evaluation of the two tumor markers gave us some benefits. Therefore, to find a proper combination of the available tumor markers for diagnosis of bladder cancer we should also consider the feasibility of each method in clinical practice.

The mean value of urinary CK20 mRNA was significantly elevated in TCCB group compared to non-cancer and control groups ($p < 0.001$). Interestingly, these finding were similar to the research of Inoue *et al.* [21] that CK20 mRNA values in the carcinoma group (mean 35,850) were significantly higher than non-cancer (171) and control groups (4.55, $p < 0.0001$ and < 0.0001, respectively). The study of urine samples of 60 cancer patients showed CK20 value varied significantly between patients: range from 0 ~ 50 000 copies. Also, we found that CK20 value differs significantly between early stage tumors (between T_{is}/T_a and T_{1-2}, G1 and G2), but not in advanced tumors (between T_{1-2} and T_{3-4}, G2 and G3).

In conclusion, our studies demonstrated that urine CK20 mRNA may serve as a marker of TCCB. The obtained data have suggested that quantitative RT-PCR of CK20 is more sensitive method for detection of transitional cell carcinoma cells in the urine than conventional cytology and detection of NMP22 expression, and simultaneous application of both CK20 and NMP22 markers is even more effective. The Light Cycler system allows rapid convenient amplification and on-line data analysis, making it applicable for routine assays in clinical practice.

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