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The Simultaneous Use of Telomerase, Cytokeratin 20 and CD4 for Bladder Cancer Detection in Urine

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Abstract

Objective: Because of the low sensitivity of urinary cytological diagnosis of urinary bladder carcinoma, new molecular diagnostic methods have been proposed. We decided to verify the expression of telomerase mRNA coding for the catalytic component (hTRT), cytokeratin 20 (CK20) and CD4 antigen mRNAs in urine as possible diagnostic tool.

Methods: Evaluation of hTRT, CK20, CD4 mRNAs was performed in 50 ml of naturally voided urine of 205 patients of which 153 with bladder cancer (Tis, $n = 11$; TaGx, $n = 4$; TaG1, $n = 25$; TaG2, $n = 26$; TaG3, $n = 8$; T1G1, $n = 16$; T1G2, $n = 17$; T1G3, $n = 20$; T2G2, $n = 6$; T2G3, $n = 13$; T3G3, $n = 7$) and 52 controls. A quantitative expression of hTRT at mRNA level versus TRAP (telomeric repeat amplification protocol) assay was performed in 20 patients and 14 controls. The expression of RT-PCR for hTRT, CK20, CD4 versus urinary cytology was analysed in 44 patients with bladder cancer. Evaluating the three molecular markers together, the result was considered correct when at least two of the markers were positive, suspected when only one marker was positive and negative for diagnosis of tumour when all markers were negative. The performance of the diagnostic model resulted from the logistic analysis evaluated with receiver operating characteristics (ROC) curve analysis.

Results: The sensitivity detected for each tumour marker was as follows: for hTRT 90.8%, for CK20 84.3% and for CD4 was 64.7%, while the specificity was 94.2% for CD4 and 78.8% for both hTRT and CK20. When a simultaneous evaluation of the three tumour markers was considered, 88.2% of the diagnoses were correct, 11.8% were suspected for tumour and none were mistaken. When compared with cytology, the simultaneous use of the three markers allowed reaching a correct diagnosis in 88% of the cases in comparison to 25% by urinary cytology. The sensitivity in the detection of bladder cancer was higher for hTRT at mRNA level in comparison with the enzymatic activity detection with TRAP (90% vs. 35%) while the specificity for both markers resulted very high (100%).

Conclusions: These data show that in the future the diagnostic improvement of urine based molecular markers for the detection of bladder cancer in the urine could improve the sensitivity of urinary cytology reducing the need of a cystoscopy.

Keywords: Urinary bladder; Transitional cell carcinoma; Telomerase; Cytokeratin 20; CD4

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1. Introduction

Bladder cancer is the second most common urologic cancer [1]. Recurrence rate of superficial bladder cancer varies from 50% to 70% and of these 10%–15% progress to muscle invasion [2]. In the past 50 years urologists used voided urine cytology as the major diagnostic method for detection of bladder cancer despite the very low sensitivity for low-grade tumours [3]. Several molecular markers have been recently proposed, to improve diagnostic sensitivity in urine. Among these an increasing attention is focused on the role of telomerase in bladder cancer detection [4]. Telomerase is a ribonucleoprotein whose main activity is to maintain the telomere length. The RNA component of telomerase (hTR) is widespread, and only the expression of the mRNA encoding the catalytic protein subunit (hTRT) is correlated with telomerase activity. Telomerase enzymatic activity can be detected also by telomeric repeat amplification protocol (TRAP) in exfoliated cells collected in normally voided urine or in bladder washing. The overall sensitivity of this method is reported to vary between 0% and 100% and the specificity between 24% and 96% respectively [5,6]. We decided to study the telomerase (hTRT) expression at mRNA level together with other molecular markers such as cytokeratin 20 and CD4.

Cytokeratins are intermediate filaments expressed in epithelial cells [7]. One of these, cytokeratin 20, is higher in urothelial tumours in comparison with normal transitional epithelium so it can be considered as marker of urothelial differentiation [7].

CD4 antigen is specific for tumour infiltrating lymphocytes. The CD4 specific T cells play an important role in tumour related immunity [8–10].

Here, the authors supposed that the combined analysis of these three markers with different functional molecular targets could improve the general sensitivity and specificity of transitional bladder cancer diagnosis in urine.

2. Materials and methods

2.1. Patient characteristics

We evaluated hTRT, CK20 and CD4 at mRNA level in 50 ml of naturally voided morning urine, of which 153 bladder cancer affected patients with primary or recurrent transitional cell carcinoma with specified staging and grading (Tis, $n = 11$; TaGx, $n = 4$; TaG1, $n = 25$; TaG2, $n = 26$; TaG3, $n = 8$; T1G1, $n = 16$; T1G2, $n = 17$; T1G3, $n = 20$; T2G2, $n = 6$; T2G3, $n = 13$; T3G3, $n = 7$), and 52 in patients suffering from hematuria due to non-neoplastic causes as clinical controls, (cases of cystitis, urethral calculosis and acute prostatitis). All urine specimens of subjects with bladder cancer were obtained prior to trans-urethral resection of bladder tumour (TURB). The quantitative expression of the catalytic component of telomerase (hTRT) at mRNA level versus telomeric repeat amplification protocol (TRAP) assay was compared in 20 patients with bladder carcinoma and in 14 clinical controls. These analyses were performed using the same voided urine samples. The quantitative expression of hTRT, CK20 and CD4 performed by RT-PCR were also compared with urinary cytology in 44 patients affected by bladder cancer (TaG1, $n = 9$; TaG2, $n = 9$; TaG3, $n = 5$; T1G1, $n = 3$; T1G2, $n = 4$; T1G3, $n = 7$; T2G3, $n = 7$). The cases of transitional cell carcinoma (TTC) after trans-urethral resection were staged and graded pathologically according to 1997 TNM and WHO classifications [11].

2.2. Urine sampling and RNA extraction

We analysed 50 ml of first morning urine. The samples were analysed as soon as possible or conserved for a maximum of three hours in the refrigerator at +4 °C.

Urine samples were filtrated through a vacuum filter system. Exfoliated cells were collected on a filter membrane and put directly into the lysis solution and stored at −20 °C for a maximum period of 1 month.

The total RNA was extracted using a new experimental kit (R&D Lab. of Macrochip s.r.l., Area Science Park, Trieste, Italy). In order to completely eliminate genomic DNA from the RNA solution a step of digestion with DNase I was performed. RNAs were resuspended in 1× DNase I Buffer, 80 units of RNase inhibitor (Ambion, Austin, TX, USA), 10 units of DNase I RNase free (Amersham Biosciences, Uppsala, Sweden). Reaction was left to proceed at 37 °C for 20 minutes. To purify RNA from DNase I an extraction with phenol-H_2O/chloroform was carried out. The final concentration of RNA in the solution was made by precipitation with iso-propanol using 5 μl of glycogen 1 mg/ml as precipitation carrier. Purified RNA was stored at −80°.

2.3. Reverse Transcriptase Polymerase Chain Reaction assay (RT-PCR)

The specific mRNA sequences were transcribed in cDNA using an AMV Reverse Transcriptase with specific primer and then short fragments were amplified in log linearity conditions to perform a relative quantification of the expression [12]. Quantitative RT-PCR analysis was performed separately for each molecular marker. Standardisation on a housekeeping gene is needed for each sample in order to evaluate the RNA degradation level. In this study we used GAPDH mRNA sequence as housekeeping gene. The RT-PCR was performed for hTRT, CK20 and CD4 as previously reported [13]. The primers for RT-PCR were: CK20 sense-GGC GTC ATC ATG AATGAA; CK20 antisense-CAG AAC TGC AGT CTG TCTCT; CD4 sense-CGG CAA GGC CAC AAT GAA; CD4 antisense-TTT CCC TGA GTG GCT GCT; hTRT sense-CAA GGC TGG GAG GAACAT; hTRT antisense: AGG CTG TTC ACC TGC AAA T; GAPDH sense-CCAAGGTCATCCATGACA; GAPDH antisense-ATC CAC AGT CTT CTG GGT (designed by the R&D Lab. of Macrochip s.r.l., Trieste, Italy).

The amplified fragments were tested by dot blot hybridisation using as probe oligonucleotides related to sequences inside the amplification fragments, labelled with ^{32}P-ATP (Amersham Biosciences Europe, Freiburg, Germany). The hybridised membranes were then analysed using a Cyclone apparatus (Camberra Packard, Meriden, CT, USA).

A cut-off (positive result vs. negative result) equivalent has been established for CK20 and CD4. The values corresponded to 23000 cpm for CK20 and 20000 cpm for CD4. These quantitative cut-offs were compared with the same quantity of T24 cells (that express CK20) and with a specific amount of lymphocytes for the