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Original article

Use of polymerase chain reaction analysis of urinary DNA to detect bladder carcinoma

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Abstract

A cohort of 113 patients underwent prospective evaluation with a panel of seven microsatellites, on chromosomes 9, 13 and 17. Thirty-seven patients had histologically confirmed bladder tumors, 53 patients had a history of previous transitional cell carcinoma of the bladder (TCC) but normal cystoscopies (control Group 1), and 23 patients had no previous history of TCC and normal cystoscopies (control Group 2). Urinary DNA was considered to show a deletion if an allele was reduced by more than 50%, and this was considered diagnostic of bladder carcinoma. The sensitivity of the method was 50%, positive predictive value was 80%, and specificity was 93%. Reducing the threshold for defining allelic loss increases sensitivity, but reduces specificity. The concentration of urinary DNA in the sample did not influence detection rate. The grade and stage of the bladder tumor did not influence the likelihood of detection. This method detects bladder carcinoma with high specificity, and increasing the number of microsatellites used should increase sensitivity.

Keywords: Bladder cancer; Transitional cell carcinoma; Detection; Urine

1. Introduction

At present, the gold standard for the detection of de novo or recurrent carcinoma of the urinary bladder is cystoscopy with direct visualization of the bladder. Tests of the patient's urine may also be used to add to the diagnostic process, but as yet none has been sensitive or specific enough to replace cystoscopy. The ideal test for bladder cancer detection should be noninvasive, 100% sensitive and specific, and produce results instantly. Tests that have been used include the bladder tumor antigen (BTA) test, nuclear matrix protein (NMP22), urinary cytology, fluorescent in situ hybridization (FISH) and telomerase activity [1–6].

Microsatellite analysis can detect chromosomal deletions. Certain chromosomal deletions occur frequently in transitional cell carcinoma of the bladder, such as around the *P16/INK4A* locus on chromosome 9 and in the region of *TP53*, both of which are believed to be integral to tumor development [7]. Tumors may slough cells more readily into urine than normal bladder mucosa as a consequence of their increased cell turnover. Extraction of DNA from these exfoliated cells and detection of allelic losses may be a useful marker of tumor presence or recurrence. Markers used should be based around regions of frequent chromosomal loss in bladder carcinoma, as this will increase the likelihood of tumor detection. The objective of this study was to establish if the allelic ratios produced following polymerase chain reaction amplification of blood and urinary DNA with a panel of seven polymorphic amplimers, could be compared to detect chromosomal deletions. Any deletions identified could diagnose the presence of a bladder tumor. The markers used in this study were clustered around regions of frequent loss in transitional cell carcinoma of the bladder. Two markers were used on each of the long and short arms of chromosome 9, one marker adjacent to the Retinoblastoma gene locus on chromosome 13, and two markers adjacent to the *TP53* locus on chromosome 17.

2. Materials and methods

The Queen's University of Belfast ethics committee provided ethical approval for this study. From each patient,

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3 mL of peripheral blood was sampled into an EDTA tube and 50 mL of voided urine were taken in a sterile 50 mL centrifuge tube. The specimens were processed within 30 min. Patients then underwent cystoscopy, and were classified into three groups. Thirty-seven patients had cystoscopic evidence of bladder tumor, subsequently confirmed on histological analysis of a transurethrally resected specimen (tumor group). Control Group 1 comprised 53 patients who had previously had bladder tumors, but now had no detectable bladder abnormality on cystoscopy. This group was recruited from patients undergoing surveillance cystoscopy to detect recurrences. Control Group 2 consisted of 23 patients with no bladder cancer history, and no bladder abnormality on cystoscopy. This group was undergoing cystoscopy for benign conditions, such as the evaluation of lower urinary tract symptoms.

Extraction of DNA from blood was performed using the Puregene® DNA extraction kit (Gentra systems, Minneapolis, MN), according to the manufacturers protocol. The voided urine specimens were centrifuged at 4000 × *g* for 10 min, to obtain a cell pellet. The urine was then decanted, and the pellet re-suspended in 300 μL of sterile water. The Qiamp® (Qiagen, Sussex, United Kingdom) midi kit filtration protocol was then used for DNA extraction. Primers were selected for polymorphic microsatellite markers on chromosomes that are believed to be lost with high frequency in transitional cell carcinoma of the urinary bladder. The markers used were: D9S164, D9S171, D9S283, D9S285, D13S263, D17S1811 and D17S938.

PCR for the chromosome 9 microsatellites were performed in 5 μL total volume, using template DNA (10 ng) Taq polymerase 0.2 U (Applied Biosystems, Warrington, United Kingdom), 1X Buffer, $MgCl_2$ (2.5 mM), dNTP (200 μM) and primers (0.5 μM). The PCR conditions were: initial denaturation: 95°C for 10 min; followed by 11 cycles of: 94°C for 30 s, 53°C for 30 s, and 72°C for 30 s; followed by 28 cycles of: 89°C for 30 s, 53°C for 30 s, 72°C for 30 s; final extension: 72°C for 10 min.

The chromosome 13 and 17 markers were tested in a multiplex reaction. PCR reactions (5 μL) for this multiplex used template DNA (10 ng), Taq polymerase (0.25 U), D17S938 (0.5 μM), D17S1811 and D13S263 (0.25 μM), dNTP (200 μM), 1μl 10X Buffer and 2μl solution Q. All PCR products were then analyzed using an ABI 3100 sequencer (Applied Biosystems), to establish the relative proportions of each allele in each sample. Urinary DNA was considered to show a deletion if the height of an allele was below 50% of that predicted by the matched genomic DNA sample. Loss of one or more alleles in a urine sample was considered to represent a chromosomal deletion. Detection of one or more chromosomal deletions was considered diagnostic of a bladder tumor.

DNA concentration from each urine specimen was evaluated using the PicoGreen® dsDNA fluorescent detection system (Molecular Probes, Cambridge, United Kingdom), to establish if low urinary DNA concentration was a contributing factor to false positive and false negative results. This was performed with reference DNA concentrations of 0, 50, 200 and 500 ng/mL. For unknown DNA concentrations, 2 μL of DNA was diluted with 80 μL of TE Buffer for each well, and 100 μL of PicoGreen solution diluted by a factor of 200 was added to every sample. The samples were mixed by pipetting up and down. The samples were allowed to incubate under foil (to prevent dye degradation by ambient light) for 3 min at room temperature. The plate was then inserted into the automated plate reader (Cytofluor multiwell plate reader system 4000, PerSeptive biosystems) and a fluorescence reading for each plate well obtained. Using the fluorescence readings from the reference DNA wells to generate a graph of fluorescence against DNA concentration, the DNA concentration of unknown samples could be calculated.

3. Results

The sample tumor population was subdivided by stage into 30 Stage Ta tumors, three Stage T1 tumors and four Stage T2 or greater tumors. When grade was considered, seven were Grade 1, 21 were Grade 2, and nine were Grade 3.

The initial tumor detection rate based on an allele loss in urinary DNA was 49% (18/37 tumors). Positive predictive was 72% (18/25 positive results), and specificity was 89% (68/76 negative results). An example of an allelic deletion consistent with detection of a bladder tumor in a voided urine specimen is shown in Fig. 1, along with an example of a sample that does not exhibit a deletion. Grade and stage of tumor did not statistically significantly influence the likelihood of tumors detection ($P = 0.14$ and 0.09, respectively). No sample from the group who had never had bladder cancer was positive. False positive rate differed significantly between this group and the group with previous bladder cancer, who had eight false positives ($P = 0.04$). The rate of detection of loss of heterozygosity for each marker within each patient group is shown in Fig. 2. Three tumors developed within 24 months of the initial cystoscopy, two of which were predicted by a positive result on microsatellite analysis. If these were included as positive in the detection data, then sensitivity increased slightly to 50% (20/40 tumors), positive predictive value increased to 80% (20/25 positive results), and specificity was 93% (68/73 negative results). The individuals from control Group 1, whose urine demonstrated the presence of a detectable chromosomal deletion had a 25% (2/8) chance of developing a bladder tumor within 24 months. Normal chromosomal analysis conferred only a 2% risk (1/45) of tumor development. The results of each marker are shown for each of the tumor samples in Fig. 3. It is noteworthy that only two of the samples show losses for all the informative markers on chromosome 9 (samples 18 and 20). Most studies of chromosome 9 losses in tumors have shown monosomy 9 rates of 40% to 50%. It is indicative of the limitations of this