Original Study

Detection and Clinical Significance of Circulating Tumor Cells in Patients Undergoing Radical Cystectomy for Urothelial Bladder Cancer

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Abstract

We studied cytokeratin 7 (CK7) mRNA in circulating cells of patients undergoing radical cystectomy. We found that CK7-positive patients were at higher risk for developing disease recurrence and death, years after surgery. We conclude that CK7 testing is a useful adjunct to define the prognosis of patients undergoing radical cystectomy and to identify candidates for systemic therapy.

Introduction: Estimation of prognosis is patients undergoing radical cystectomy is often unreliable, as occult disease remains undetected by conventional diagnostic tools. The purpose of this study was to evaluate the feasibility and the clinical significance of a polymerase chain reaction assay to detect cytokeratin 7 (CK7) mRNA expression in peripheral blood cells of patients undergoing radical cystectomy for clinically nonmetastatic bladder cancer. **Patients and Methods:** From 2005 to 2009, 59 patients undergoing radical cystectomy and pelvic lymph node dissection were prospectively investigated. Peripheral blood was collected prior to surgery, and a nested polymerase chain reaction assay was developed to identify patients with circulating cells expressing CK7 mRNA. Preoperative, histopathologic data and clinical outcome were compared with CK7 findings. **Results:** CK7 expression was detected in 23 (38.9%) of 59 patients and correlated to T stage and lymph node status. After a median follow-up of 42 months, 29 patients experienced a recurrence, whereas 36 died. The presence of CK7-positive cells was significantly associated with an increased risk for recurrence and decreased survival as compared with patients who were CK7-negative ($P < .001$ and $P < .001$, respectively; hazard ratios of 8.77 and 5.2 for recurrence and overall death, respectively). The detection of CK7-positive cells was an independent predictor of recurrence and death in a multivariable analysis. **Conclusion:** The detection of CK7 mRNA in the circulating cells of patients undergoing radical cystectomy for urothelial cancer identifies those with significantly increased risk of cancer recurrence and death.

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Introduction

Radical cystectomy (RC) with pelvic lymph node dissection (PLND) is considered the treatment of choice for locally invasive bladder cancer (BC). However, more than one-half of the patients with pathologically node-negative BC will face disease progression and die after surgery.[1] This suggests the presence of occult disease that cannot be detected by imaging and conventional pathology. Contemporary cancer diagnostics are gradually relying upon new methods and biological sources for obtaining prognostic information. One promising source is the peripheral blood.

Detection of circulating tumor cells (CTC) in the bloodstream of patients with a solid tumor plays a role in early diagnosis of metastatic disease,[2] as well as to define prognosis and response to systemic treatment in patients with established metastatic disease.[3,4] Several methods have been explored.[5] Most of the evidence results from studies based on immunomagnetic detection technologies. Among those, the CellSearch system (Veridex, Raritan, NJ) is

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based on a positive selection of CTC by an epithelial cell adhesion molecule (EpCAM), and has received US Food and Drug Administration approval for the definition of prognosis in patients with metastatic colon, breast, and prostate cancer.[3,6,7] On the other hand, several studies have successfully employed a variety of reverse transcriptase-polymerase chain reaction (RT-PCR) methods to detect epithelial transcripts as surrogate reporters of CTC.[4,8,9] Studies directly comparing multiple detection methods have shown that RT-PCR results in higher diagnostic sensitivity.[10,11]

Among markers specific for urothelial lineage, cytokeratin 7 (CK7), is highly expressed in several variants of urothelial carcinoma, and commonly used in immunohistochemistry for the identification of metastatic tissue of urothelial origin.[12,13] Therefore, we developed a nested RT-PCR assay for detecting CK7 transcripts from peripheral blood cells of patients undergoing RC. Finally, we explored any correlation between CK7 status and the clinical outcome of our patients.

Patients and Methods

Patients

Starting in 2005, all patients undergoing an RC with PLND for urothelial cancer at our institution were considered potentially eligible for the present study. Eligibility criteria included prior transurethral resection with urothelial carcinoma histology. All subjects were 18 years or older, had Eastern Cooperative Oncology Group performance status ≤ 2, and had to exhibit a recent (within 6 weeks of cystectomy) total body computed tomography (CT) excluding distant metastases and upper urinary tract cancer. Patients excluded from enrollment were those in whom neoadjuvant chemotherapy was either planned or performed, receiving investigational medications, and not suitable for an RC with PLND. At follow-up, all patients were monitored quarterly for the first 2 years, every 6 months until the fifth year, and annually afterwards. For staging purposes, a bone scan at 12 months and a chest and abdominal CT scan to be performed after 6, 12, 18, and 24 months, and yearly thereafter, were scheduled. However, patients with less than a yearly chest and abdominal CT scan were excluded from analysis. Decisions regarding treatment in the adjuvant setting or at the time of recurrence/progression were at discretion of the treating physician. Cells were obtained from the peripheral blood of 8 healthy volunteers without a history of urothelial cancer and were tested as negative controls. In accordance with the precepts of the Helsinki Declaration, written informed consent to the use of biologic material and agreement to participate to the study were obtained from all patients and healthy volunteers prior to sampling and surgery (ClinicalTrials.gov identifier: NCT02345473).

CTC Isolation

Blood Sample Collection and Processing. For each subject, a 4 mL peripheral blood sample was drawn within 24 hours before surgery, and collected into EDTA collection tubes. Ficoll-Hypaque separated nucleated cells were recovered and suspended in 500 μl of TRIzol reagent (Life Technologies, Carlsbad, CA) for total RNA extraction according to the manufacturer's instruction. Before storage at −80°, total RNA amount and integrity (OD_{260}/OD_{280} nm absorption ratio > 1.6) were determined spectrophotometrically (NanoDrop 1000, Thermo Scientific, Waltham, MA).

RT Reaction and Nested PCR. Complementary DNA (cDNA) was obtained from a 20 μL PCR mixture containing 2 μg of total RNA, 25 pmol of random primers, 2.5 mM of each triphosphate deoxynucleotide, 2 μL of 10× RNA PCR Buffer, 1 μL of Multi Scribe Reverse Transcription (all from Life Technologies), and 3.2 μL of Rnase free water. The mixture was incubated at 25°C for 10 minutes, 37°C for 120 minutes, heated to 85°C for 5 seconds, and then chilled on ice. Two sets of CK7 primers (MWG Biotech, Ebersberg, Germany) were designed for the PCR protocol to obtain a final 218 bp PCR product. The primer's sequences were as follows: outer sense, 5′-CGTGCGCTCTGCCTATGG-3′; outer antisense, 5′-GCGGTTAATTTCATCTTCGT-3′; inner sense, 5′-TCCGCAGGTCACCATTAAC-3′; inner antisense, 5′-GCTGCTCTTGGCCGACTTCT-3′. During the first PCR round, 25 cycles of amplification corresponded to 30 seconds of denaturation at 94°, 30 seconds annealing at 60°, and 30 seconds extension at 72°. For the second PCR round, 1 μL aliquot of the first-round PCR product was amplified for 27 cycles under the same conditions, using the outer set of CK7 primers. Adequacy of cDNA synthesis was confirmed by a non-nested RT-PCR of β-actin. A no-template control, omitting any cDNA template, was included in all PCR reactions. Ten μL of second-round PCR products were subjected to 3% agarose gel electrophoresis and stained with ethidium bromide. The present PCR methodology generated a dichotomous result for each sample: CK7-positive or CK7-negative. Each sample was tested twice; a third run was performed when the results were inconclusive.

Cell Line Serial Dilutions and Cell Spikes. A J82 bladder cancer cell line (ATCC, Manassas, VA), highly expressing CK7, was used to set up an in vitro sensitivity test of the nested RT-PCR. The sensitivity of RT-PCR to detect CK7 mRNA was evaluated by adding known numbers of J82 cells (10^6, 10^5, 10^4, 10^3, 10^2, 10, 1, and 0) in the peripheral blood obtained from healthy volunteers, before RNA extraction.

Statistical Analysis

Patients' baseline characteristics according to treatment group were reported as frequency and percentage or median and interquartile range and were compared with the Pearson χ^2 test or the Fisher test or the Mann-Whitney *U* test for categorical and continuous variables, respectively. The Kaplan-Meier method and log-rank test statistic were used to compare probabilities of survival in the 2 treatment groups. Relative risks of death (cancer-specific and overall) and disease recurrence were calculated by univariate and multivariate Cox proportional hazards model, adjusting for the effects of other covariates known to be of prognostic importance. Assumption of proportional hazard was tested using the tests of the nonzero slope. Results are expressed as hazard ratios (HR) with their 95% confidence intervals (95% CIs). All the analyses were performed using Stata Statistical Package Release 14 (StataCorp, College Station, TX). A P value $< .05$ was considered as being statistically significant.

Results

Sensitivity of the Nested RT-PCR for CK7

The sensitivity of RT-PCR to detect CK7 mRNA was evaluated by adding known numbers of J82 cells (10^6, 10^5, 10^4, 10^3, 10^2, 10,