

Prognostic value of circulating tumor cells in nonmuscle invasive bladder cancer: a CellSearch analysis

P. Gazzaniga^{1*}, A. Gradilone¹, E. de Berardinis², G. M. Busetto², C. Raimondi¹, O. Gandini¹, C. Nicolazzo¹, A. Petracca¹, B. Vincenzi³, A. Farcomeni⁴, V. Gentile², E. Cortesi⁵ & L. Frati¹

Departments of ¹Molecular Medicine; ²Gynecology, Obstetrics and Urological Sciences, Division of Urology, Sapienza University, Rome; ³Division of Medical Oncology, Campus Biomedico University, Rome; Departments of ⁴Public Health and Infectious Diseases and ⁵Radiological, Oncological and Pathological Sciences, Division of Oncology, Sapienza University, Rome, Italy

Received 24 November 2011; revised 6 December 2011; accepted 14 December 2011

Background: Circulating tumor cells (CTCs) provide prognostic information in patients with metastatic tumors. Recent studies have shown that CTCs are released in circulation in an early phase of cancer disease so that their presence is under investigation in the adjuvant setting. Few studies investigated the prognostic significance of CTCs enumeration in patients with metastatic and advanced bladder cancer. The current study has analyzed the presence of CTC in patients with nonmuscle-invasive bladder cancer (NMIBC).

Patients and methods: Forty-four NMIBC patients were enrolled and included in a 24-month follow-up program. Blood drawings were carried out in all patients at the first diagnosis. CellSearch system (Veridex; LLC, Raritan, NJ) was used for CTCs enumeration.

Results: CTC were detectable in 8/44 patients (18%). Presence of CTC was found significantly associated to shorter time to first recurrence (6.5 versus 21.7 months, $P < 0.001$). Median time to progression was not reached, due to the short follow-up period. CTC presence was found associated to concomitant carcinoma *in situ* and higher T category.

Conclusion: The detection of CTC in this setting of disease may allow to distinguish patients with high risk of recurrence from those with high risk of progression, as well as to early identify patients candidate for adjuvant treatment.

Key words: CellSearch, CTCs, NMIBC, prognosis

introduction

Although most cases of bladder cancer patients present with a disease that is confined to mucosa (Ta) or submucosa (T1), recurrence rate in this cohort of patients is >50%. Furthermore, some nonmuscle-invasive bladder cancer (NMIBC), more frequently T1G3, present with biological features of invasiveness, leading to cancer death after bladder-sparing treatment within 5 years in ~16–23% of cases [1]. Thus, some types of NMIBC represent a clear example of paradoxical discrepancy between clinical and biological features.

Circulating tumor cells (CTCs) play a crucial role for distant failure in different types of solid tumors [2]. Their enumeration through CellSearch system (Veridex) is widely used for prognostic information in patients with metastatic breast, colon and prostate cancer, and provides valuable diagnostic

information for predicting progression-free survival and overall survival earlier than the current standard of care [3].

Recent studies have shown that CTCs are released in circulation in a very early phase of cancer disease where their presence is associated with a worse prognosis of patients. The current hypothesis is that CTC count could reflect the ongoing progression of cancer disease, which is not diagnosed through serum marker or high-resolution imaging technologies. These results have supported the use of CTC analysis in nonmetastatic patients as well, to generate clinically useful prognostic information [4, 5].

To date, few studies have investigated the prognostic significance of CTCs enumeration in patients with bladder cancer, and all have been carried out in metastatic and advanced disease [6–8].

The current study has analyzed the presence and number of CTC in a group of 44 patients with primary diagnosis of NMIBC.

Primary end point of the present study was to investigate the prognostic significance of CTCs in NMIBC patients; to this purpose, the presence of CTCs has been used to predict time to

*Correspondence to: Dr P. Gazzaniga, Department of Experimental Medicine, Sapienza University, Viale Regina Elena 324, Rome 000161, Italy; Tel: +39-6-49973011; Fax: +39-6-4454820; E-mail: paola.gazzaniga@uniroma1.it

first recurrence (TFR) and time to progression (TTP) in a follow-up of 24 months. Secondary end points were the association between CTC presence and known prognostic variables such as T, G and presence of carcinoma *in situ* (CIS).

patients and methods

patient population

In this prospective study, 44 patients were enrolled (38 male, 6 female), mean age 62 years (range 51–78), all with histopathological diagnosis of NMIBC. All tumors were diagnosed as transitional cell carcinomas. According to T, 18/44 (41%) were Ta and 26/44 (59%) were T1. In total, 9/44 (20%) were G1, 9/44 (20%) were G2 and 26/44 (59%) were G3. Concomitant presence of CIS was found in 8/44 (18%) patients. A population of 20 healthy donors were included as negative control when needed. Blood drawings were carried out in all patients at the first diagnosis, 1 h before transurethral resection of the bladder. All patients provided written informed consent for blood drawing and CTCs analysis. Patient characteristics and histopathology are shown in Table 1.

follow-up of patients and clinical outcome

Patients were then included in a follow-up program which consisted of cystoscopy and urinary cytology every 3 months and a urological computed tomograph every 12 months. Cystoscopy was carried out using a D-light system, which provided both white and blue light. The bladder wall was inspected and mapped, first under white light and then by blue light. All tumors and suspicious areas identified were resected or biopsied. The last follow-up was scheduled at 24 months. TFR was defined as time from diagnosis to the date of the first bladder recurrence. TTP to muscle invasive disease was defined as time from diagnosis to the date of first increase to stage T2 or higher disease in the bladder.

CTC enumeration

CellSearch system (Veridex; LLC) was used for CTCs enumeration. Briefly, the method is an immunomagnetic cell enrichment which uses antibodies targeting epithelial cell adhesion molecule (EpCAM) and nucleus labeling with fluorescent dye.

Table 1. Characteristics of patients

	Number (%)
T category	
Ta	18/44 (41%)
T1	26/44 (59%)
Grade	
G1	9/44 (20.5%)
G2	9/44 (20.5%)
G3	26/44 (59%)
Carcinoma <i>in situ</i>	
Yes	8/44 (18%)
No	36/44 (82%)
Recurrence at 24 months	
Yes	20/44 (45%)
No	24/44 (55%)
Progression at 24 months	
Yes	7/44 (16%)
No	37/44 (84%)
Total (patients)	44

Blood samples (7.5 ml) were drawn into CellSave Preservative tubes (Veridex; LLC) containing EDTA and a cell fixative. Samples were maintained at room temperature and processed within 96 h. A semiautomated system (CellPrep) allows the enrichment of the sample for cells expressing EpCAM, and it labels the cell nucleus with the fluorescent nucleic acid dye 4,2-diamidino-2-phenylindole dihydrochloride. Epithelial cells are distinguished from leukocytes using fluorescent-labeled monoclonal antibodies specific for epithelial cells (cytokeratins 8, 18, 19-phycoerythrin) and leukocytes (CD45–allophycocyan).

The identification of CTCs was assessed using the CellSpotter Analyzer, a semiautomated fluorescence-based microscopy system that permits computer-generated reconstruction of cellular images. According to CellSearch instructions, CTCs were defined as nucleated EpCAM-positive cells, lacking CD45 but expressing cytokeratins 8, 18 and 19. CTC identification and enumeration were carried out by trained personnel.

statistical analysis

Statistical analysis was carried out with BMDP statistical software, version 7 (Statistical Solutions, Saugus, MA) and SPSS (Chicago, IL, version 15.00 for Windows). The prognostic significance of CTC was assessed through the log-rank test between CTC presence with both TFR and TTP in 24 months of follow-up. A P value <0.05 was considered as statistically significant. To assess the correlation between the presence of CTC and classical prognostic factors T, G and CIS, the Pearson's Chi-squared test was used. The correlation was considered significant when P value was <0.05 .

results

CTC were detectable in 8/44 patients (18%), (mean number 1.5, range 1–3) (Figure 1) and in 0/20 healthy volunteers. The exact number of CTCs in correlation to clinical and pathological characteristics of tumors is shown in Table 2.

Presence of CTC was found significantly associated to shorter TFR (follow-up: 24 months). In the group of CTC+ patients, 7/8 (87.5%) experienced a local recurrence in the 24-month of follow-up, while in the group of CTC– patients, only 13/36 (36%) local relapses were observed.

Median TFR was found significantly shorter on the group of CTC+ compared with CTC– (6.5 versus 21.7 months, $P < 0.001$) (Figure 2).

Progression to muscle invasive disease occurred in 7/8 CTC+ and in 0/36 CTC– patients. All CTC+ patients with local recurrence of disease experienced a progression to muscle invasive disease as well (Table 2).

Median TTP was not reached, due to the short follow-up period.

CTC were found in 8/26 (31%) patients with T1 tumors, and in 0/18 patients with Ta. This difference was found statistically significant ($P = 0.0275$). CTC presence was also found associated to concomitant presence of CIS; in the group of patients with CIS, CTC were found in 5/8 (62.5%) compared with 3/36 (8.3%) found in the group without CIS ($P = 0.00228$).

Association between CTC presence and tumor grading was found not statistically significant. (G1–2 versus G3, $P = 0.1133$).

discussion

Currently, patients with NMIBC are categorized into risk groups according to clinical and pathological parameters, not always representative for the biological aggressiveness of the

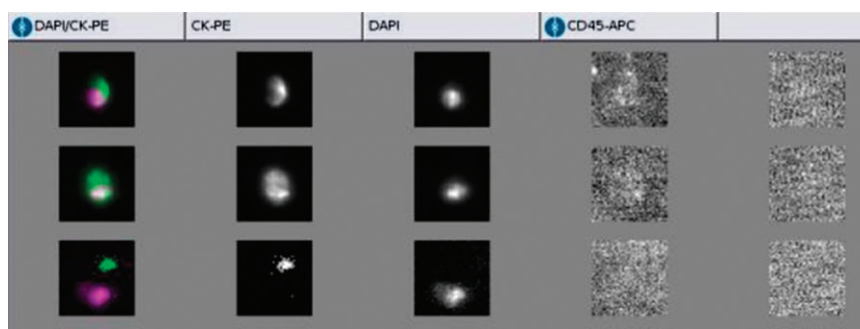


Figure 1. Selected images of a patient with nonmuscle-invasive bladder cancer and presence of two circulating tumor cell (CTCs) in 7.5 ml of peripheral blood. According to CellSearch instructions, a CTC is defined as nucleated (DAPI+), cytokeratin-positive (CK+), CD45-negative (CD45-) object.

Table 2. Correlation between CTC number, tumor characteristics and prognosis

Patient	Circulating tumor cell	T	G	Size	No. of tumors	Time to first recurrence	Time to progression
1	1	T1	3	1-3	3	6	16
2	1	T1	3	>3	1	4	22
3	3	T1	3	<1	1	3	6
4	1	T1	3	>3	>10	3	6
5	1	T1	3	>3	>10	6	18
6	1	T1	3	1-3	5	3	6
7	3	T1	3	<1	4	3	8
8	1	T1	3	1-3	3	-	-

Patient 8 experience neither local recurrence nor progression of disease.

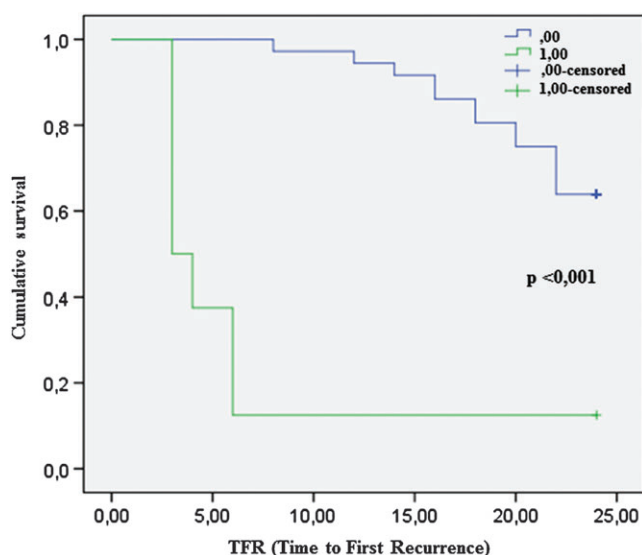


Figure 2. Kaplan-Meier curve of time to first recurrence according to baseline levels of circulating tumor cells.

disease. To define the prognosis of NMIBC is somehow challenging, since the difference between prognostic indicators for local recurrence and progression to muscle invasive disease is still unclear.

In Ta/T1 bladder cancer patients, the probability of recurrence at 1 year from initial diagnosis ranges from 15 to

70%, while the progression rate at 5 years is ~7–40%. It has been reported that the overall rate of at least one recurrence of stage Ta T1 bladder cancer is ~48% in a median follow-up of 3.9 years.

Although several prognostic factors are widely recognized, such as number of tumors, the T category, grade and presence of concomitant CIS, to date no molecular marker with prognostic significance has been proven useful in clinical practice. To date, multiplicity, tumor size, and prior recurrence rates are the most important predictors for recurrence, while tumor grade, stage and CIS are the most important predictors for progression [9].

The prognostic significance of CTCs has been widely demonstrated in metastatic breast, colon and prostate cancer. Furthermore, recent studies have also suggested that CTC enumeration may represent a promising prognostic tool for risk stratification of patients with early staged disease as well.

In breast cancer for instance, preliminary results from SUCCESS trial have demonstrated that the presence of >1 CTC/23 ml of peripheral blood before treatment is a significant prognostic factor in terms of disease-free survival and overall survival [10].

In bladder cancer, Naoe et al. [6] reported a high sensitivity of CellSearch to detect urothelial cancer spiked cells, and found CTCs in 57.1% of metastatic urothelial cancer patients and in 0% of those with nonmetastatic disease. Authors conclude that CellSearch assay is not suitable as a diagnostic tool for early staged urothelial tumors. The main limits of the study were the small number of patients included (14 with metastatic, 12 with nonmetastatic disease), as well as the lack of homogeneity of early staged tumors (stage 0 to III). More recently, Gallagher et al. [7], through CellSearch assay, found CTCs in 44% of patients with metastatic urothelial tumors.

In 2010, the largest study reporting about CTC detection in nonmetastatic bladder cancer patients has been published. Rink et al. [8] detected CTC in 30% of patients with nonmetastatic disease, and showed a significantly worse overall progression-free survival and cancer-specific survival in CTC-positive compared with CTC-negative patients. More recently, the diagnostic value of CTC presence in bladder cancer has been evaluated, highlighting the potential clinical role of CTC detection as an indicator of advanced bladder cancer [11].

To our knowledge, our study for the first time reports about CTC prognostic significance in a homogeneous population of T1 bladder cancer patients using CellSearch. Our group

recently reported about CTC presence in a homogeneous population of patients with T1G3 bladder cancer using RT-PCR characterization [12]. In the present study for the first time, we confirmed the prognostic significance of CTCs using a standardized method which allows to count CTC. Serial assessment of CTC number in these patients may allow a better monitoring of disease status during follow-up. To further integrate the qualitative and quantitative information offered by the two methods may represent an auspicious approach in the clinical management of these patients.

In Ta-staged tumors, we failed to find CTC presence, to further confirm that Ta and T1 represent two distinct diseases from a biological point of view.

Our data show a significantly shorter TFR in CTC+ patients compared with CTC- in a 24-month follow-up. The median TTP was not reached; patients will be followed for further 36 months. At the end of 5 years, a further analysis of TFR and TTP will be carried out. Due to the small sample size, we were not able to perform a multivariate analysis to establish whether CTC detection through CellSearch may represent an independent prognostic factor. A further study with larger patient population is actually ongoing.

To date, molecular markers are not included in the scoring systems and risk tables to predict prognosis of patients affected by superficial bladder cancer, neither in surveillance programs.

Several urine markers are available, but no one has been validated as standard diagnostic procedure in routine urology, since they are not sufficiently sensitive; to date, none of these tests represents an ideal marker which may facilitate reliable bladder cancer detection [13].

Several molecular indicators of aggressive behavior have been also suggested in NMIBC; among them, overexpression of *h-ras*, mutations of *fgf-r* and loss of adhesion molecules, which may account for a reduced cell-cell interactions and increased cell motility. To date, no one of these genetic tests are used in bladder cancer surveillance [14].

Evaluation of CTCs could provide a noninvasive source of representative tumor material.

The detection of CTC in this setting of disease may allow to distinguish patients with high risk of recurrence from those with high risk of progression, as well as to early identify patients candidate for adjuvant treatment.

Moreover, a further molecular characterization of these cells may help to better understand the biological behavior of the disease.

acknowledgements

We thank Dr Serena di Cosimo and Dr Alessio Metere, Department of Surgical Sciences, Sapienza University of Rome.

disclosure

The authors declared no conflict of interest.

references

1. Van den Bosch S, Alfred Witjes J. Long-term cancer-specific survival in patients with high-risk, non-muscle-invasive bladder cancer and tumour progression: a systematic review. *Eur Urol* 2011; 60(3): 493–500.
2. Zhe X, Cher ML, Bonfil RD. Circulating tumor cells: finding the needle in the haystack. *Am J Cancer Res* 2011; 1(6): 740–751.
3. Miller MC, Doyle GV, Terstappen LW. Significance of circulating tumor cells detected by the CellSearch system in patients with metastatic breast colorectal and prostate cancer. *J Oncol* 2010; 2010: 617421.
4. Pantel K, Alix-Panabières C. Circulating tumour cells in cancer patients: challenges and perspectives. *Trends Mol Med* 2010; 16(9): 398–406.
5. Pantel K, Alix-Panabières C, Riethdorf S. Cancer micrometastases. *Nat Rev Clin Oncol* 2009; 6(6): 339–351.
6. Naoe M, Ogawa Y, Morita J et al. Detection of circulating urothelial cancer cells in the blood using the CellSearch System. *Cancer* 2007; 109(7): 1439–1445.
7. Gallagher DJ, Milowsky MI, Ishill N et al. Detection of circulating tumor cells in patients with urothelial cancer. *Ann Oncol* 2009; 20(2): 305–308.
8. Rink M, Chun FK, Minner S et al. Detection of circulating tumour cells in peripheral blood of patients with advanced non-metastatic bladder cancer. *BJU Int* 2011; 107(10): 1668–1675.
9. Sylvester RJ, van der Meijden APM, Oosterlinck W et al. Predicting recurrence and progression in individual patients with stage ta t1 bladder cancer using EORTC risk tables: a combined analysis of 2596 patients from seven EORTC trials. *Eur Urol* 2006; 49: 466–477.
10. Rack BK, Schindlbeck C, Andergassen U et al. Use of circulating tumor cells (CTC) in peripheral blood of breast cancer patients before and after adjuvant chemotherapy to predict risk for relapse: the SUCCESS trial. *J Clin Oncol* 2010; 28: 15s.
11. Msaouel P, Koutsilieris M. Diagnostic value of circulating tumor cell detection in bladder and urothelial cancer: systematic review and meta-analysis. *BMC Cancer* 2011; 11: 336.
12. Gradilone A, Petracca A, Nicolazzo C et al. Prognostic significance of survivin-expressing circulating tumour cells in T1G3 bladder cancer. *BJU Int* 2010; 106(5): 710–715.
13. Tilki D, Burger M, Dalbagni G et al. Urine markers for detection and surveillance of non-muscle-invasive bladder cancer. *Eur Urol* 2011; 60(3): 484–492.
14. Youssef RF, Lotan Y. Predictors of outcome of non-muscle-invasive and muscle-invasive bladder cancer. *Sci World J* 2011; 11: 369–381.