Original Study

The Prognostic Role of Circulating Tumor Cells (CTC) in High-risk Non–muscle-invasive Bladder Cancer

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Abstract

Circulating tumor cells (CTCs) could represent a promising, noninvasive prognostic and predictive marker in high-risk patients with non–muscle-invasive bladder cancer. We retrospectively evaluated 155 patients with pT1G3 bladder cancer who underwent transurethral resection of bladder tumor after a blood withdrawal for CTC evaluation. In our analysis, the presence of CTCs was significantly associated with time to first recurrence and time to progression.

Introduction: The purpose of this study was to evaluate the impact of circulating tumor cells (CTCs) as a prognostic marker in patients with high-risk non–muscle-invasive bladder cancer (NMIBC) and assess the efficacy and reliability of 2 different CTC isolation methods. **Materials and Methods:** Globally, 155 patients with a pathologically confirmed diagnosis of high-risk NMIBC were included (pT1G3 with or without carcinoma in situ) and underwent transurethral resection of bladder tumor (TURB) after a blood withdrawal for CTC evaluation. A total of 101 patients (Group A) had their samples analyzed with the CellSearch automated system, and 54 (Group B) had their samples analyzed with the CELLection Dynabeads manual system. **Results:** Patients were followed for 28 months, and during this interval, there were a total of 65 (41.9%) recurrences, 27 (17.4%) disease progressions, and 9 (5.8%) lymph node and/or bone metastasis. In our CTC analysis, there were 20 (19.8%) positive patients in Group A and 24 in Group B (44.4%). In our analysis, we found a strong correlation between CTC presence and time to first recurrence; in Group A, we observed an incidence of recurrence in 75% of CTC-positive patients and in Group B of 83% of CTC-positive patients. The time to progression was also strongly correlated with CTCs: 65% and 29%, respectively, of those patients who progressed in those with CTCs in Group A and B. **Conclusion:** The study demonstrates the potential role of CTCs as a prognostic marker for risk stratification in patients with NMIBC, to predict both recurrence and progression.

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Introduction

Bladder cancer is one of the leading causes of death related to cancer, and the majority are transitional cell carcinoma. According to T stage, most of them (75%) are non–muscle-invasive bladder cancer (NMIBC).[1] Although NMIBC is considered a noninvasive tumor, the risk of recurrence is up to 78% and the risk of progression is up to 45%, leading to cancer mortality after conservative, bladder-sparing treatment in 16% to 23% of cases within 5 years.[2] To date, the only methodology for risk assessment is via European Organisation for Research and Treatment of Cancer (EORTC)

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scoring systems and risk tables that take into consideration a variety of tumor characteristics. Further parameters to evaluate prognosis hold promise and include depth and extent of lamina propria invasion (T1 a-b-c), better pathologic evaluation after an en bloc tumor resection, lymphovascular invasion, and identification of subvariants of transitional cell carcinoma (nested, micropapillary, plasmocytoid, sarcomatoid, squamous, and adeno variants).[3,4] Management of high-risk NMIBC can be difficult, as current methods of prediction are inadequate, and a validated tool that accurately predicts risk of progression to guide clinicians to perform more aggressive treatments (such as radical cystectomy) do not currently exist. Recently, several molecular markers have been developed to overcome the limitations of traditional risk-assessment tools.[5] Identifying and describing genetic and molecular cancer alterations could be made by increasing proteomics and genomics knowledge and understanding and releasing new cancer treatments needed to address tumor microenvironment and continuously changing molecular defects in the tumor itself.[6] Currently, 4 main kinds of biomarkers are available: predictive, prognostic, response indicator, and efficacy response, but to date, no markers have been accepted as standard practice. Circulating tumor cells (CTCs), one of the most promising markers, were initially studied for metastatic prostate, breast, and colorectal cancer. CTCs represent a noninvasive prognostic and predictive marker offering information about molecular and phenotypic cancer characteristics. Also, an important aspect of CTCs is that they can be used to assess efficacy of therapy. Many different methods of isolation and count assessment have been developed (immunomagnetic, microfluidics, density gradient centrifugation), but currently, the most widely used antibody to capture CTCs is epithelial cellular adhesion molecule (EpCAM), and the most widely used markers to distinguish CTCs from other nonspecific blood epithelial cells are cytokeratins.[7] We focused on CellSearch (Veridex LLC, Warren, NJ), which is an automated system approved by the United States Food and Drug Administration (FDA), and CELLection Dynabeads (Invitrogen, Carlsbad, CA), a manual system.[8-10]

Therefore, we sought to evaluate CTCs as a prognostic marker in patients with NMIBC at high risk of progression and recurrence and to assess the efficacy and reliability of the 2 different identification methods.

Materials and Methods

A total of 155 patients with primary non–muscle-invasive transitional cell bladder cancer diagnosis underwent transurethral resection of bladder tumor (TURB) after a blood withdrawal for CTC evaluation between April 2006 and October 2013. All procedures were carried out at the Department of Urological Sciences, Sapienza University of Rome, by the surgeons (G.M.B. and E.D.B.), and all CTC identification was carried out at the Department of Molecular Medicine, also at Sapienza University of Rome. The study protocol was approved by our internal ethics committee and the committee for human subjects research. All treatments applied are part of routine standard care, and the study was conducted in accordance with European Urology and Good Clinical Practice guidelines, with ethical principles laid down in the latest version of the Declaration of Helsinki. Every patient signed informed consent to participate in the study.

Only patients that underwent transurethral resection of the primary tumor with a pathologically confirmed high-risk transitional cell tumor were included, pT1G3 with or without carcinoma in situ. All patients underwent a second TURB 6 to 6 weeks later to confirm the pathologic evaluation and to rule out any residual disease. CTCs were evaluated only before the TURB and were not evaluated again with re-TURB and after recurrence or progression. Regarding other molecular parameters, we did not perform any heterogeneity test between the primary tumor and CTCs. Bacillus Calmette-Guerin immunotherapy was started 2 weeks after the second TURB with induction plus maintenance (weekly instillation for the first 6 weeks and then 3-times weekly instillation every 3 to 6 months for the following 3 years). Follow-up was planned with a cystoscopy and urinary cytology every 3 months and a contrast-enhanced computerized tomography scan (CECT) every 12 months.

Blood samples were taken 1 hour before TURB. A total of 101 patients (Group A) had their samples analyzed with the CellSearch automated system, and 54 patients (Group B) had their samples analyzed with the CELLection Dynabeads manual system.

With the CellSearch system, CTCs were isolated from 7.5 mL of blood collected into evacuated blood draw tubes (CellSave, Veridex LLC, Raritan, NJ). The system allows identification of cells expressing EpCAM, labeling the nucleus with fluorescent nucleic acid dye 4,2-diamidino-2-phenylindole dihydrochloride. Monoclonal antibodies specific for CK8, CK18, CK19, and CD45 were used to distinguish epithelial cells from leukocytes. At the end, using a semiautomated fluorescence-based microscopy system, a computer-generated reconstruction of cellular images was constructed.[11,12]

With the other method, CTCs were isolated from 10 mL of peripheral blood by CELLection Dynabeads covered with monoclonal antibody toward the human EpCAM. Enriched cells were then lysed with lysis buffer and 20 μL of Dynabeads Oligo(dT) 25 were added in order to catch poly A + mRNA. Finally, a solid cDNA was realized from the captured mRNA.[9,10] Because CTCs are cells expressing CK8 but lacking CD45, specific primers for them were used (CD45 upstream: 5′CCGTGCAGCTCTACGAGAGG3′; downstream: 5′CAGCGCTTCCAGAAGGGCTC′ - CK8 upstream: 5′ACTGAGATCTCAGAGATCAA3′; downstream: 5′AATACTCATGTTCTGCATCC3′). In addition to CTC identification, a reverse transcription polymerase chain reaction assay was carried out to evaluate survivin expression.[13]

The primary endpoints of the study were time to first recurrence (TFR), defined as the time between CTC identification and first local recurrence and time to progression (TTP), defined as the time between CTC identification and upstage of the disease or appearance of distant metastases. The secondary endpoint of the study was a comparison between the 2 different methods of CTC evaluation.

Statistical Analysis

Descriptive statistics were used to summarize pertinent study information. The association between subgroups was tested by the χ^2 test or Fisher exact test, when appropriate. A multivariate Cox proportional hazard model was also developed using stepwise regression (forward selection) by selecting those predictive variables that were significant upon univariate analysis. Enter limit and remove limit were $P = .10$ and $P = .15$, respectively. Survival was calculated by the Kaplan-Meier product-limit method. The log-rank

Table 1 Patient Characteristics

	Group A N (%)	Group B N (%)	P Value
Total	101	54	
Age, y			.83
≤59	18 (17.8)	9 (16.7)	
60-75	49 (48.5)	28 (51.8)	
≥76	34 (33.6)	17 (31.5)	
Gender			.68
Male	78 (78.7)	44 (81.5)	
Female	23 (23.2)	10 (18.5)	
Hstology			–
T1-G3	101 (100)	54 (100)	
CIS	20 (19.8)	9 (16.7)	.79
Multifocality	77 (76.2)	36 (66.7)	.28
Size			.99
≤1 cm	35 (34.6)	18 (33.3)	
> 1 cm ≤3 cm	55 (54.5)	30 (55.6)	
> 3 cm	11 (10.9)	6 (11.1)	

Abbreviation: CIS = carcinoma in situ.

test was used to assess differences between subgroups. Significance was defined at the $P < .05$ level. Statistical analysis was carried out with the SPSS v. 21.0 software.

Results

In our population of 155 patients, 101 underwent CellSearch CTC evaluation (Group A) and 54 underwent CELLection Dynabeads evaluation (Group B). We found an incidence of carcinoma in situ in 18.7% (19.8% in Group A and 16.7% in Group B, respectively) and an incidence of multifocal tumor in 72.9% (76.2% in Group A and 66.7% in Group B, respectively). The tumor size was evaluated intraoperatively; 34.2% of tumors were less than 1 cm (34.6% in Group A and 33.3% in Group B); 54.8% were greater than 1 cm but less than 3 cm in size (54.5% in Group A and 55.6% in Group B), and 11% were greater than 3 cm (10.9% in Group A and 11.1% in Group B) (Table 1).

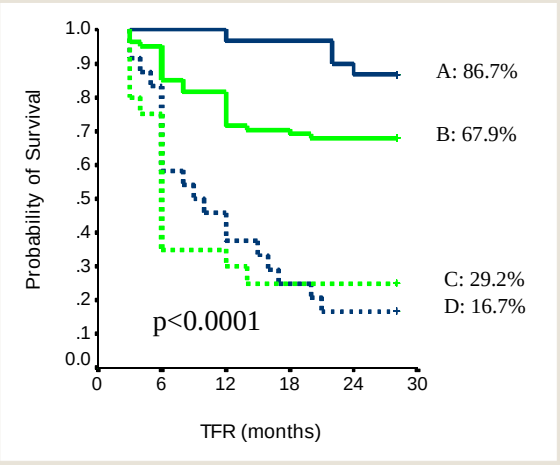
Patients were followed for 28 months, and during this interval, there were a total of 65 (41.9%) recurrences, 41 (40.6%) in Group A and 24 (44.4%) in Group B, respectively. Eighteen (17.8%)

Table 2 Recurrences, Progressions, and CTC-positive Patients

	Group A N (%)	Group B N (%)	P Value
Total	101	54	
Recurrences	41 (40.6)	24 (44.4)	.77
Progressions	18 (17.8)	9 (16.7)	.99
Metastasis	6 (5.9)	3 (5.6)	.99
CTC			.002
Positive	20 (19.8)	24 (44.4)	
Negative	81 (80.2)	30 (55.6)	
CTC count			
1	16 (80)	–	–
> 1	4 (20)	–	
Survivin			
Positive	–	27 (50)	–
Negative	–	27 (50)	

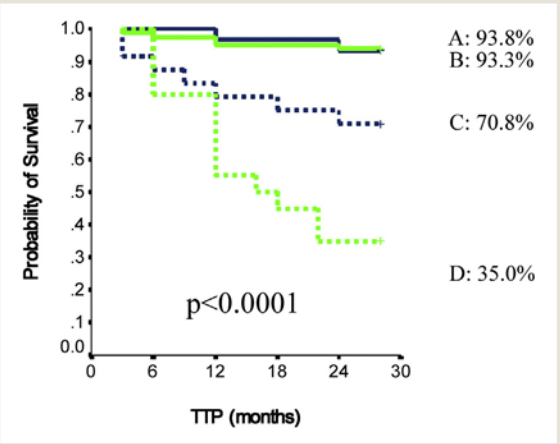
Abbreviation: CTC = circulating tumor cell.

Figure 1 Time to First Recurrence in Accordance With the 2 Different CTC Evaluation Methods. A, CELLection Dynabeds Method, CTC-negative; B, CellSearch Method, CTC-negative; C, CellSearch Method, CTC-positive; D, CELLection Dynabeds Method, CTC-positive



Abbreviation: CTC = circulating tumor cells.

Figure 2 Time to Progression in Accordance With the 2 Different CTC Evaluation Methods. A, CellSearch Method, CTC-negative; B, CELLection Dynabeds Method, CTC-negative; C, CELLection Dynabeds Method, CTC-positive; D, CellSearch Method, CTC-positive



Abbreviation: CTC = circulating tumor cells.

Table 3 Recurrences and Progressions in Accordance With CTC Presence

	CTC-positive (20 Patients Group A; 24 Patients Group B) N (%)	CTC-negative (81 Patients Group A; 30 Patients Group B) N (%)	P Value
Recurrences			
Group A	15 (75)	26 (32)	< .0001
Group B	20 (83)	4 (13)	< .0001
Progressions			
Group A	13 (65)	5 (6)	< .0001
Group B	7 (29)	2 (6)	.02

Abbreviation: CTC = circulating tumor cell.

patients in the CellSearch group and 9 (16.7%) patients in the CELLection Dynabeads group had disease progression, for a total of 27 (17.4%), and 6 (5.9%) in group A and 3 (5.6%) in group B developed lymph node and bone metastasis.

In our CTC analysis, there were 20 (19.8%) CTC-positive patients in Group A and 24 in Group B (44.4%) for a total of 44 (28.4%). In Group A, we found 1 CTC in 16 patients (80%) and more than 1 (maximum, 50) in 4 patients (20%). In Group B, although we were not able to evaluate cell count, positive survivin expression was found in 27 (50%) patients. CTCs were strongly connected with survivin expression because 22 of 24 (92%) CTC-positive patients expressed survivin (Table 2).

In our analysis, we found a strong correlation between CTC presence and TFR; in Group A, it was observed an incidence of recurrence in 75% of CTC-positive patients, and in Group B of 83% of CTC-positive patients (Figure 1).

The TTP was also strongly correlated with CTCs: 65% and 29%, respectively, of those patients who progressed in those with CTCs in Group A and B (Figure 2, Table 3). TFR and TTP were significantly longer, with both evaluation methods, in patients that were found to be negative for the presence of CTCs.

Two multivariate analyses have been conducted, and the first is related to CellSearch patients, whereas the second is related to CELLection Dynabeds; significant parameters are reported in Tables 4 and 5.

Comparing CellSearch and CELLection Dynabeds reliability and efficacy is difficult because, even if the patients are suffering from the same disease at the same pathologic status, there are 2 different populations being compared. Thus, we can only conclude that CellSearch appears to be more reliable and more efficient to correlate with TFR and even more with TTP.

Discussion

The majority of bladder cancers, despite presentation in superficial stages, have a high probability of cancer recurrence, progression, and mortality. More recently, risk stratification of these NMIBCs has been increasingly utilized to predict prognosis. Along the same lines, to date, we know that, owing to inherent heterogeneity, some patients with NMIBCs are at high risk for harboring micrometastatic disease at presentation.[14] The concept of individualized therapy, in accordance with a granular cancer characterization, should become the first step before starting a treatment plan. Considering this, current research is moving to develop new reliable markers or a multimarker panel that could be utilized in clinical practice. At the moment, the PI3K/AKT/mTOR pathway, epigenetic changes in DNA methylation, tissue mitochondrial DNA, exosomes, and CTCs seem to be the most promising developments.[5] Because CTCs can be obtained simply with a blood sample and contain molecular and phenotypic characteristics of the primary tumor, they hold a great deal of promise in prognosticating NMIBCs, as CTCs have been approved by the FDA as prognostic in metastatic colon, breast, and prostate cancer.[15,16]

In the past 10 years, in our consideration for CTCs as a potential biomarker for bladder cancer, we focused on 2 different methods of CTC identification, including the FDA-approved semi-automated CellSearch and the manual CELLection Dynabeads. Our results are encouraging because we found a statistically significant difference with both methods in time to first recurrence between CTC-positive and -negative patients. Also, with regard to tumor progression, we found a statistically significant difference with both methods between CTC-positive and CTC-negative patients.

Comparing the 2 different methodologies was a difficult endeavor. The first major methodologic difference is the capability of the first system to count using a semiautomated system, whereas the second one is based on a more time-consuming polymerase chain reaction-based system (no count). CELLection Dynabeads can be associated with a cell's characterization tool, and the CellSearch cannot characterize cells unless you use the fourth channel to identify tumor receptors.[17] In our experience with CELLection Dynabeads, we found a strong correlation with CTC presence confirmed by 92% of CTC-expressing patients being positive to survivin. In our opinion, CellSearch seems to be more reliable, easier to perform, and allows CTC quantification, but a direct comparison of the reliability, specificity, and sensitivity between the 2 methodologies, considering a different court of patients, is not possible. We could imagine that CELLection Dynabeads is more sensible in identifying circulating cells, but less specific in distinguishing white blood cells in comparison with CellSearch. The previous literature regarding NMIBCs and CTCs are limited, but suggest that CTCs are a negative

Table 4 Multivariate Analysis With Cox Regression Model for CellSearch

	TTP		TFR	
Variable	HR (95% CI)	P	HR (95% CI)	P
Tumor size (continuous variables)	5.41 (2.31-12.68)	< .0001	–	NS
CIS (yes vs. no)	12.36 (4.16-36.70)	< .0001	3.78 (1.99-7.21)	< .0001
Multifocal (yes vs. no)	20.52 (2.75-187.12)	.003	22.81 (3.13-166.21)	.002

Abbreviations: CI = confidence interval; CIS = carcinoma in situ; HR = hazard ratio; NS = not significant; TFR = time to first recurrence; TTP = time to progression.

Table 5 Multivariate Analysis With Cox Regression Model for CELLection Dynabeads

	TTP		TFR	
Variable	HR (95% CI)	P	HR (95% CI)	P
CIS (yes vs. no)	9.97 (2.64-37.66)	.001	5.21 (1.85-14.72)	.002
CTC (yes vs. no)	–	NS	13.79 (4.47-42.56)	< .0001

Abbreviations: CI = confidence interval; CIS = carcinoma in situ; CTC = circulating tumor cell; HR = hazard ratio; NS = not significant; TFR = time to first recurrence; TTP = time to progression.

prognostic factor. The largest trials detailing these findings, by Rink et al, found CTCs in 30% of patients with muscle-invasive non-metastatic bladder cancer.[18] In another experience, they did not find any correlation between CTCs and HER2 expression, although they confirmed that CTC may serve as an indication for therapy and that molecular characterization of CTCs could be, in the future, a "liquid biopsy" guiding individual targeted therapy.[19] In a recent review, Nagata et al recognized the role of CTCs as a prognostic marker for bladder cancer, but available data suggest that a single marker is not adequate for tumor surveillance and perhaps a combination of different markers (epigenetic and genetic) could better predict disease behavior.[20]

The current limitations of CTCs as a prognostic marker must be underscored. First, the small sample size of all previous trials does not allow a correct evaluation of predictive capability and therapy response, and methodologic efficacy has not yet been demonstrated. Large prospective trials are needed to better evaluate how CTCs could reliably predict tumor behavior together with the ability to guide target therapies. Furthermore, present studies never stratified patients in accordance with molecular subtypes, and, for this reason, it is difficult to compare patients that have tumors that are only apparently similar.[14]

Another point of interest is to establish the best evaluation method for CTCs, and this is important, again, to increase clinical use and application. The IsoFlux system showed better sensitivity for CTC detection allowing molecular profiling in prostate and colorectal cancer.[21] It is an immunomagnetic isolation that occurs in a microfluid environment, avoiding carryover with white blood cells. Different antibodies can be used with the magnetic beds and, after every sample of CTC is ready, it is possible to perform a count and a next-generation sequencing. IsoFlux, with a better sensitivity in detection, using next-generation sequencing, can detect genomic alterations present in CTCs.[22]

Last but not least, we have to consider epithelial to mesenchymal transaction, a process that occurs once CTCs have been released in the bloodstream to form micrometastasis, and epithelial to mesenchymal transaction starts as completion of the invasion-metastasis cascade.[23] Unfortunately, at the moment, there is not any instrument able to detect cells that underwent mesenchymal transaction, and research is focusing on markers, like vimentin, expressed by mesenchymal cells.

Liquid biopsies could be a good opportunity to move forward with our understanding of bladder cancer and may help to identify signaling pathways related to cell invasiveness and metastatic process. In a not too distant future, these tests will be likely be important tools used in the diagnosis of cancer, which will revolutionize cancer care, providing urologists and oncologists molecular-level information to optimally guide treatment choices.

Conclusion

The study demonstrates the potential role of CTCs as a prognostic marker for risk stratification in patients with NMIBC, to predict both recurrence and progression. There are 2 specific methodologies that have their respective advantages and disadvantages. Further larger scale prospective trials will be needed to allow for continued assessment of clinical applicability and utility.

Clinical Practice Points

- Patients with NMIBC will experience a non-negligible risk of recurrence and progression over time.
- To date, European Organisation for Research and Treatment of Cancer scoring systems are the only risk assessment tools for treatments strategy and stratification of prognosis.
- CTCs could represent a promising, non-invasive prognostic and predictive marker in high-risk patients with NMIBC.
- Our study demonstrated the potential role of CTCs as a prognostic marker for risk stratification in these patients, predicting both recurrence and progression. Further larger prospective trials are needed to critically assess the role of CTCs in the clinical practice.

Disclosure

The authors have stated that they have no conflicts of interest.

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