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ORIGINAL ARTICLE – CANCER RESEARCH

The long-term prognostic value of survivin expressing circulating tumor cells in patients with high-risk non-muscle invasive bladder cancer (NMIBC)

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Abstract
Objectives Long-term follow-up study to evaluate the impact on disease-free survival and cancer-specific survival of survivin expression in tissue and CTCs from T1G3 bladder cancer patients.
Patients and methods The study was conducted using tumor tissue and blood samples from 54 patients with a primary diagnosis of T1G3 NMIBC. Survivin was evaluated by reverse transcription-polymerase chain reaction in tumor tissues. CTCs were isolated from blood by CELLection™ Dynabeads (Invitrogen, Carlsbad, CA, USA). Cells were lysed and cDNA was synthesized and analysed for the expression of CD45, CK8 and survivin. The endpoints of this long-termanalysis were disease-free survival, DFS and cancer-specific survival, CSS.
Results Here, we report that, at 9 years of median follow-up, disease-free survival and cancer-specific survival are both significantly influenced by the expression of survivin in tumor tissue ($p = 0.006$), by the presence of CTCs ($p < 0.0001$) and by the expression of survivin in CTCs ($p < 0.0001$).
Conclusion The statistically significant impact of survivin expressing CTCs on cancer-specific survival that we observed might be interpreted as the result of the persistence of a subpopulation of highlander cells in the blood of T1G3 bladder patients over time.

Keywords Non-muscle invasive bladder cancer · T1G3 bladder cancer · Circulating tumor cells · Survivin · Prognosis

Abbreviations

CSS	Cancer-specific survival
CTCs	Circulating tumor cells
DFS	Disease-free survival
IAP	Inhibitor of apoptosis
NMIBC	Non-muscle invasive bladder cancer
PPV	Positive predictive value
TFR	Time to first recurrence
TTP	Time to progression

Introduction

Bladder cancer is a complex malignancy with a variable natural history and clinical behavior. When initially diagnosed, according with T stage, most of bladder cancers are non-muscle invasive (NMIBC) (Burger et al. 2013). Although NMIBC is considered a non-invasive 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 16–23% of cases within 5 years (Sylvester et al. 2006). Thus, NMIBC remains a challenge in oncology, representing an ideal candidate for research on biomarkers that could identify patients at increased risk of recurrence, progression, and death (De Berardinis et al. 2011). Prediction of non-muscle invasive bladder cancer outcomes is currently based on well-known

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clinico-pathological parameters such as tumor grade and stage, number and size of tumors, multifocality and the presence of carcinoma in situ. However, these models have poor discrimination for prognostic outcomes and provide a low positive predictive value (PPV) for progression, especially in patients with high-grade disease (EORTC 21%; CUETO 24%) (Kluth et al. 2015). Management of high-risk NMIBC can be difficult as current methods of prediction are inadequate and an accurate estimation of the outcome risk in the individual patient would help identifying the most appropriate therapy to avoid tumor progression. Recently, several molecular markers have been identified to overcome the limitations of traditional risk assessment tools (Sanguedolce et al. 2015). Recent sequencing studies have brought new insights into bladder cancer molecular background, revealing its unique genetic complexity (Knowles and Hurst 2015). Bladder cancer has been shown to elude programmed cell death with altered expression of both pro-apoptotic and anti-apoptotic proteins. Survivin is the smallest member of the inhibitor of apoptosis (IAP) family of proteins, it is prominently expressed during embryonal development, absent in most normal, terminally differentiated tissues but upregulated in a variety of human cancers. Although mainly involved in inhibition of apoptosis and regulation of cell cycle, survivin is emerging as a multifunctional molecule, able to support angiogenesis, metastasis and chemo-resistance of tumor cells and to promote survival of cancer stem cells (Garg et al. 2016). Survivin has been reported to correlate with the presence of higher grade and advanced stage bladder cancer and is associated with tumor recurrence and decreased cancer-specific survival. In NMIBC, survivin is overexpressed in almost 50% of cases; it shows great potential for patients stratification into distinct risk groups for disease recurrence and progression to muscle invasive disease (Kim et al. 2015). The expression of survivin in circulating tumor cells (CTCs) has been previously reported and might interpreted as a mechanism by which a subset of cancer cells that escape from primary tumor protect themselves from the hostile environmental conditions in the blood, via resistance to apoptosis, and from the attack from the host immune system, via resistance to cytotoxic T-cells mediated lysis (Garg et al. 2016; Gradilone et al. 2010; Noman et al. 2014). There is reliable evidence that the detection of CTCs is a powerful predictor for unfavorable outcomes in bladder cancer patients. Particularly, evidence has been provided that the presence of CTCs has an independent prognostic value in high-risk non-muscle invasive bladder cancer and possibly identify patients with micrometastatic disease. Molecular characterization of CTCs holds further potential to give insights into the intricate biology of bladder cancer, allowing a real-time surveillance of tumor dynamic changes (Soave et al. 2015). We have previously reported that the presence of CTCs is an independent prognostic factor for disease-free survival in T1G3 bladder cancer patients and that a major proportion of CTCs, in this subgroup of patients, do express survivin at the mRNA level. We here report that the presence of survivin-positive CTCs, in the same cohort of patients, accurately predicts long-term disease-free and cancer-specific survival, thus reinforcing the notion that liquid biopsy might reveal systemic dissemination of disease in a proportion of patients diagnosed with non-muscle invasive bladder cancer (Gradilone et al. 2010).

Patients and methods

We previously published the prognostic significance of survivin expression in tumor tissues and CTCs in patients with T1G3 disease after a follow-up of 24 months. The study was conducted using tumor tissue and blood samples from 54 patients with a primary diagnosis of T1G3 NMIBC. Details of the study design and methods have been reported previously (Gradilone et al. 2010). In brief, we recruited patients aged 18 years or older who had T1G3 non-muscle-invasive bladder cancer with tumor size <3 cm, absence of carcinoma in situ and multifocality. All patients provided written informed consent. Survivin was evaluated by reverse transcription-polymerase chain reaction in tumor tissues. CTCs were isolated from blood by CELLection™ Dynabeads (Invitrogen, Carlsbad, CA, USA) coated with the monoclonal antibody towards the human epithelial cell adhesion molecule. Cells were lysed and Dynabeads Oligo (dT) was used to capture poly A+ mRNA. cDNA was synthesized and analysed for the expression of CD45, CK8 and survivin. The endpoints of this long-term, median follow-up 108 months (range 9–108), analysis were disease-free survival, DFS (defined as the interval from the date of diagnosis to the date of high-grade disease tumor recurrence or tumor progression to muscle invasive bladder cancer) and cancer-specific survival, CSS (defined as the interval from the date of diagnosis to the date of death from bladder cancer or related causes or to the last follow-up date).

Statistical analysis

Survival analysis was calculated by the Kaplan–Meier product-limit method. Log-rank test was used to assess differences between subgroups. All reported p values are based two-sided tests, and a p value of less than 0.05 was considered to indicate statistical significance. SPSS software was used for all statistical evaluations (SPSS version 21.0, SPSS Inc., Chicago, Illinois, USA).

Results

The study cohort was composed by 54 T1G3 bladder cancer patients. Baseline demographic and disease characteristics have been previously reported (Gradilone et al. 2010). At the time of first analysis, expression of survivin was found in half of the tumors (27/54 patients) and patients with survivin-negative tumors had a longer DFS than those with survivin-positive tumors ($p = 0.029$). CTCs were found in 24/54 patients (44%) and 92% of CTC expressed survivin. The difference in DFS between CTC negative and CTC positive patients was statistically significant ($p < 0.001$). The presence of CTC was an independent prognostic factor for DFS ($p < 0.001$). Here, we report long-term results of the analysis, which confirm that disease-free survival is significantly influenced by the expression of survivin in tumor tissue ($p = 0.006$), by the presence of CTCs ($p < 0.0001$) and by the expression of survivin in CTCs ($p < 0.0001$). At 9 years of median follow-up, all the three parameters have meaningful impact on cancer-specific survival ($p = 0.04$, $p = 0.02$ and $p = 0.002$, respectively). Results are summarized in Table 1 and Kaplan–Meier curves for DFS and CSS are depicted in Figs. 1 and 2, respectively.

Table 1 Correlation between outcome of patients and survivin expression in tumor tissue, CTC status and survivin expression in CTCs

Outcome	Survivin tissue			CTCs			Survivin CTCs		
	Neg	Pos	*p* value	Neg	Pos	*p* value	Neg	Pos	*p* value
DFS (months) Median (CI 95%)	79 (64–95)	41 (24–57)	0.006	89 (78–100)	23 (9–37)	<0.0001	90 (80–101)	16 (6–26)	<0.0001
CSS (months) Median (CI 95%)	ne	98 (89–108)	0.04	ne	97 (86–108)	0.02	ne	96 (84–108)	0.002

ne not evaluable

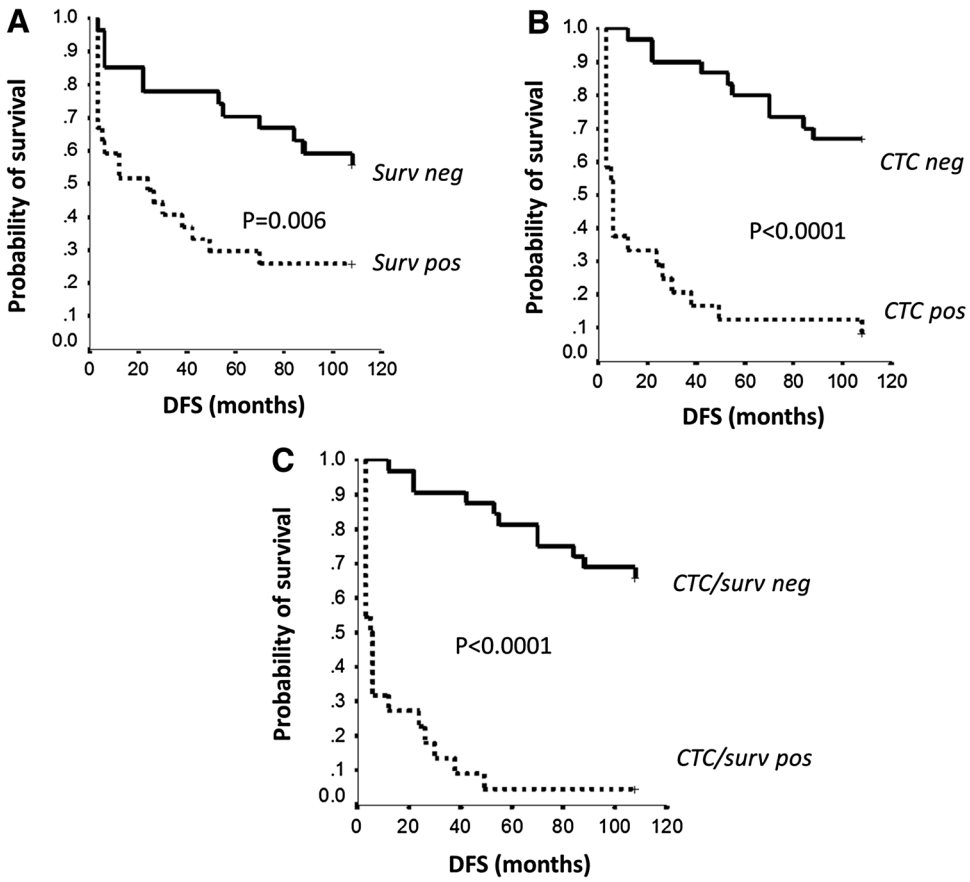

Fig. 1 Kaplan–Meier curves of disease-free survival (DFS) of 54 patients with T1G3 bladder cancer, according to the expression of survivin in tumor tissue (**a**), $p = 0.006$ at log-rank test, the presence or absence of CTCs (**b**), $p < 0.0001$ at log-rank test, and the expression of survivin in CTCs (**c**), $p < 0.0001$ at log-rank test

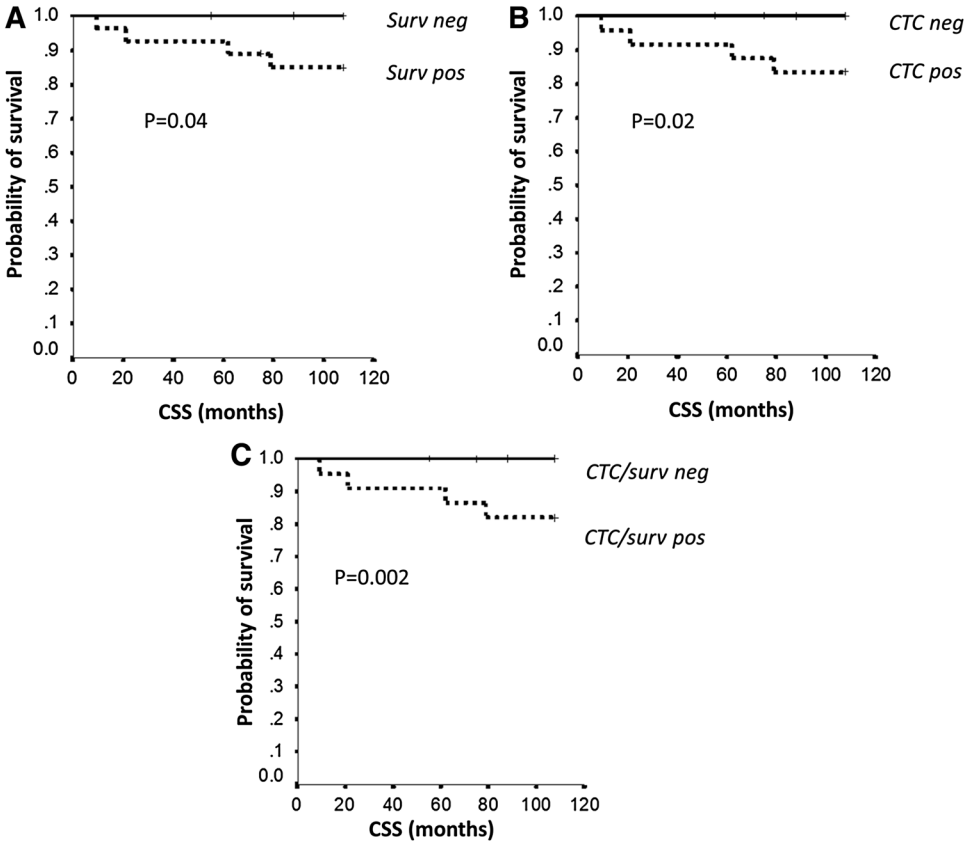

Fig. 2 Kaplan–Meier curves of cancer-specific survival (CSS) of 54 patients with T1G3 bladder cancer, according to the expression of survivin in tumor tissue (**a**), $p = 0.04$ at log-rank test, the presence or absence of CTCs (**b**), $p < 0.02$ at log-rank test, and the expression of survivin in CTCs (**c**), $p < 0.002$ at log-rank test

Discussion

Although associated with an overall favorable survival rate, rates of recurrence and progression are highly heterogeneous among patients affected by non-muscle invasive bladder cancer. Although accurate risk stratification is essential for determining evaluation, treatment and surveillance of NMIBC patients, the tools at our disposal for measuring this risk are yet imperfect. As our understanding of the molecular biology of bladder cancer advances the paradigm of prediction based on clinical and pathological parameters will be complemented by the molecular-guided approach (Bolenz and Lotan 2010). The use of molecular biomarkers for non-invasive disease surveillance has demonstrated potential clinical applicability in different tumor types (Alix-Panabières and Pantel 2016). Patients with NMIBC may already harbor micrometastatic disease, which is notoriously suboptimal to be diagnosed. It is anticipated that liquid biopsy will be routinely used in various clinical settings, including identification of risk for recurrence and progression in NMIBC patients, as well as monitoring treatment response in patients with advanced or metastatic bladder cancer (Kang and Ku 2016). Development of assays for surveillance using genomic variants in cell-free tumor DNA from plasma and urine has been recently reported. This retrospective pilot study demonstrated that tumor-specific genomic variations are detectable in plasma from patients with bladder cancer, even in non-muscle invasive disease, and that the level of tumor DNA in plasma may be a useful tool for disease surveillance (Birkenkamp-Demtröder et al. 2016). Similarly, Rink et al. demonstrated that CTCs are detectable in approximately 20% of patients undergoing radical cystectomy for bladder cancer and predict recurrence, cancer-specific survival and overall mortality (Soave et al. 2017). We previously reported that CTCs are detectable in a similar proportion of patients with primary diagnosis of high-risk NMIBC (T1G3) and predict both decreased time to first recurrence (TFR) and time to progression (TTP) (Gazzaniga et al. 2014). We here report the results of a long-term analysis [108 months (range 9–108) median follow-up] conducted to evaluate the impact on disease-free survival and cancer-specific survival of survivin expression in tissue and CTCs from T1G3 bladder cancer patients. Our results show that expression of survivin in tumor tissue, CTCs detection and the presence of survivin expressing CTCs all significantly decrease DFS and CSS. At the time of the first analysis, little was known about circulating tumor cells and their biology had been only partially explored. The past few years have witnessed a remarkable progress in CTCs isolation technologies and

an improved understanding of the molecular portrait of CTC. With a median follow-up of 9 years, our data confirm that the expression of survivin in CTCs, not necessarily concordant with the expression of the same marker in tumor tissue samples, is a common trait among cancer cells floating in the blood. This seems not surprising in view of the multifaceted role which is currently being attributed to survivin. While its role in cell division and apoptosis has been substantially investigated, it is only recently that the contribution of survivin to angiogenesis and metastasis, cancer stem cells survival and immune evasion by tumor cells is being appreciated (Knowles and Hurst 2015). Once in the bloodstream, CTCs face several obstacles that hamper the metastatic process and, to protect themselves during transit, they acquire traits that increase their ability to survive. Among those, survivin expression might equip CTCs with a protection against programmed cell death and immune system attack. The statistically significant impact of survivin expressing CTCs on cancer-specific survival that we observed might thus be interpreted as the result of the persistence of a subpopulation of highlander cells in the blood of T1G3 bladder patients over time, which in fact were already present at the time of the initial diagnosis. Apparent limits of this study are represented by the non-standardized method used at that time to isolate CTCs, which indeed might have overestimated the presence of CTCs suffering from non-adequate specificity, and the lack of any re-evaluation of the CTCs status over the course of time. We might envisage that different methods, such as the customized use for survivin of the free channel of the CellSearch® platform or the immunofluorescent staining of survivin expression in CTCs isolated by non-antigen dependent assays, as well as an ad-hoc design of a larger study will enable to draw definitive conclusions. Due to the key role of survivin in mediating diverse roles in cancer cells, the therapeutic blockade of this molecule in tumor cells holds promise to yield benefits (Mobahat et al. 2014). The emergence of immune checkpoint inhibitors as effective cancer immunotherapy in bladder cancer, in particular, provides exciting opportunities for the treatment of bladder cancer when applied to combination approaches.

Compliance with ethical standards

Conflict of interest The authors declared that they have no competing interest.

Funding Nothing to declare.

Ethical approval All procedures performed were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

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