Original Article
In vitro culturing of viable circulating tumor cells of urinary bladder cancer

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Abstract: Objective: Approximately one third of patients diagnosed with muscle-invasive urinary bladder cancer (UBC) have undetected metastases at the time of treatment of the primary tumor. Currently there are no reliable specific serum markers for monitoring and evaluating risk profiles of urothelial cancers. Several studies suggest that detection of circulating tumor cells (CTCs) may correlate with the disease status and prognosis at baseline and early in the treatment of cancers. In this study a new way of isolation and in vitro cultivation of CTCs of urinary bladder cancer was introduced. Materials and methods: Peripheral blood (PB) samples from 53 patients who had undergone urological procedure were evaluated using the MetaCell device (MetaCell s.r.o., Ostrava, Czech Republic). The patients enrolled in the study were both oncological patients with UBC and non-oncological patients with inflammation (14 patients). The sensitivity and quantification of CTCs were evaluated. The separated CTCs were cultured in vitro. Results: 39 patients with confirmed UBC were enrolled in the study. CTCs were detected in 25 (64%) patients, and most of these patients had between 6 and 10 cells. The separated CTCs were successfully cultured in vitro. Conclusion: CTCs were detected in a higher percentage of patients than in other studies. This paper describes the first successful culturing of human UBC cells. The MetaCell approach used in this study enabled the capture of viable intact virgin CTCs (virgin CTC) suitable for next in vitro culturing, single cell analysis or drug testing.

Keywords: Urinary bladder cancer, circulating tumor cells, CTC, MetaCell, urothelial tumors

Introduction

Urinary bladder cancer (UBC) is one of the most common malignancies among men in Western countries. A characteristic feature of the UBC is its ability to metastasize to distant organs or tissues [1]. Approximately one third of patients diagnosed with muscle-invasive UBC have undetected metastases at the time of treatment of the primary tumor [2], and 25% of patients treated by radical cystectomy present with lymph node involvement at the time of surgery [3]. In addition, approximately 50% of patients with muscle-invasive urothelial cancer develop metastases within 2 years of operation and subsequently die of the disease [4]. Therefore, early diagnosis and initiation of appropriate therapy are essential for increasing the life expectancy of such patients. So, there is a definite need for new diagnostic tools that would more accurately reflect the patient's actual disease status.

Currently, there are no reliable specific serum markers for monitoring and evaluating risk profiles of urothelial cancers. Several studies suggest that detection of circulating tumor cells (CTCs) may correlate with the disease status and prognosis at baseline and early in the treatment of cancers [5]. CTCs have been demon-

Table 1. Patient characteristics and CTC examination results

Patient characteristics	Total patients (N)	With detected CTC (N)	%
	39	25	64
pT stage			
pTa	17	9	35
pT1	14	10	71
pT2	2	1	50
pT3	3	2	67
pT4	3	2	67
Grading			
1	11	5	45
2	15	9	60
3	13	11	85
Disease stage			
0a	17	10	59
I.	14	8	57
II.	1	1	100
III.	4	3	75
IV.	3	3	100
Number of separated cells			
1-5 cells	5		
6-10 cells	10		
10-50 cells	0		
More 50 cells	9		

strated in various malignancies, including breast, prostate, and colon cancer [6]. The presence of CTCs in whole blood before and during radical cystectomy could provide further information on the disease status, and could be used as an indicator to determine the need for adjuvant or even perioperative chemotherapy.

Recently, detection of CTCs has also been reported in patients with metastatic UBC urothelial carcinoma [7]. Currently, no literature exists regarding the ability to cultivate and facilitate proliferation of isolated CTCs *in vitro*. The purpose of our study was to introduce a new method of isolation and cultivation of UBC CTCs.

Materials and methods

Patients

To this date, 39 patients who underwent the urological surgery (TURB, uretrotomy) or cystoscopies have been enrolled in the study. The final diagnoses were made based on histopa-

thology samples. Clinical data were collected from all patients based on their informed consent. The patient characteristics are provided in **Table 1**. Peripheral blood (PB) was collected prior to the surgery. For each patient, approximately 8 mL of venous PB was drawn from the antecubital veins and placed into S-Monovette tubes (Sarstedt AG & Co., Numbrecht, Germany) containing 1.6 mg EDTA/mL blood as an anticoagulant. The samples were processed at room temperature using an isolation procedure completed within 24 hours after the PB draw.

The ethics committees of all universities and hospitals participating in the study approved the study protocol in line with the requirements of the Declaration of Helsinki. All patients provided their written consent.

CTCs enrichment and culture

Recently, a new size-based separation method for viable CTC enrichment from unclothed PB has been introduced (MetaCell®, MetaCell s.r.o., Ostrava, Czech Republic). The size-based enrichment process is based on the filtration of PB through a porous polycarbonate membrane (pores of 8 μm in diameter). The minimum and maximum volume of the filtered PB may be adjusted up to 50 mL of fluid. Standardly, 8 mL PB from patients with UBC was transferred into the filtration tube. The successive blood transfer in several steps is recommended to prevent blood clotting on the membrane filter. The PB flow is supported by capillary action of the absorbent touching the membrane filter. Afterwards, the membrane filter kept in a plastic ring is transferred into the 6-well cultivation plate, 4 mL RPMI media is added to the filter top and CTCs are cultured on the membrane *in vitro* under the conditions of standard cancer cell cultures (37°C, 5% atmosphere of CO_2,) and observed by inverted microscope (**Figure 1**). The CTCs grow in the FBS enriched RPMI medium (10%) on the membrane for a minimum of 14 days. The grown cells are analyzed by histochemistry (May-Grünwald staining) and immunohistochemistry using specific antibodies to prove the cell origin (anticytokeratin 18 - FITC conjugated antibody (Sigma) and unspecific, DAPI staining (see **Figures 1** and **2**). Alternatively, the enriched CTCs fraction can be transferred from the membrane and cultured directly on any plastic surface or a microscopic slide. Microscopic slide culture is preferred if the immunohistochemistry/immunofluorescence analy-
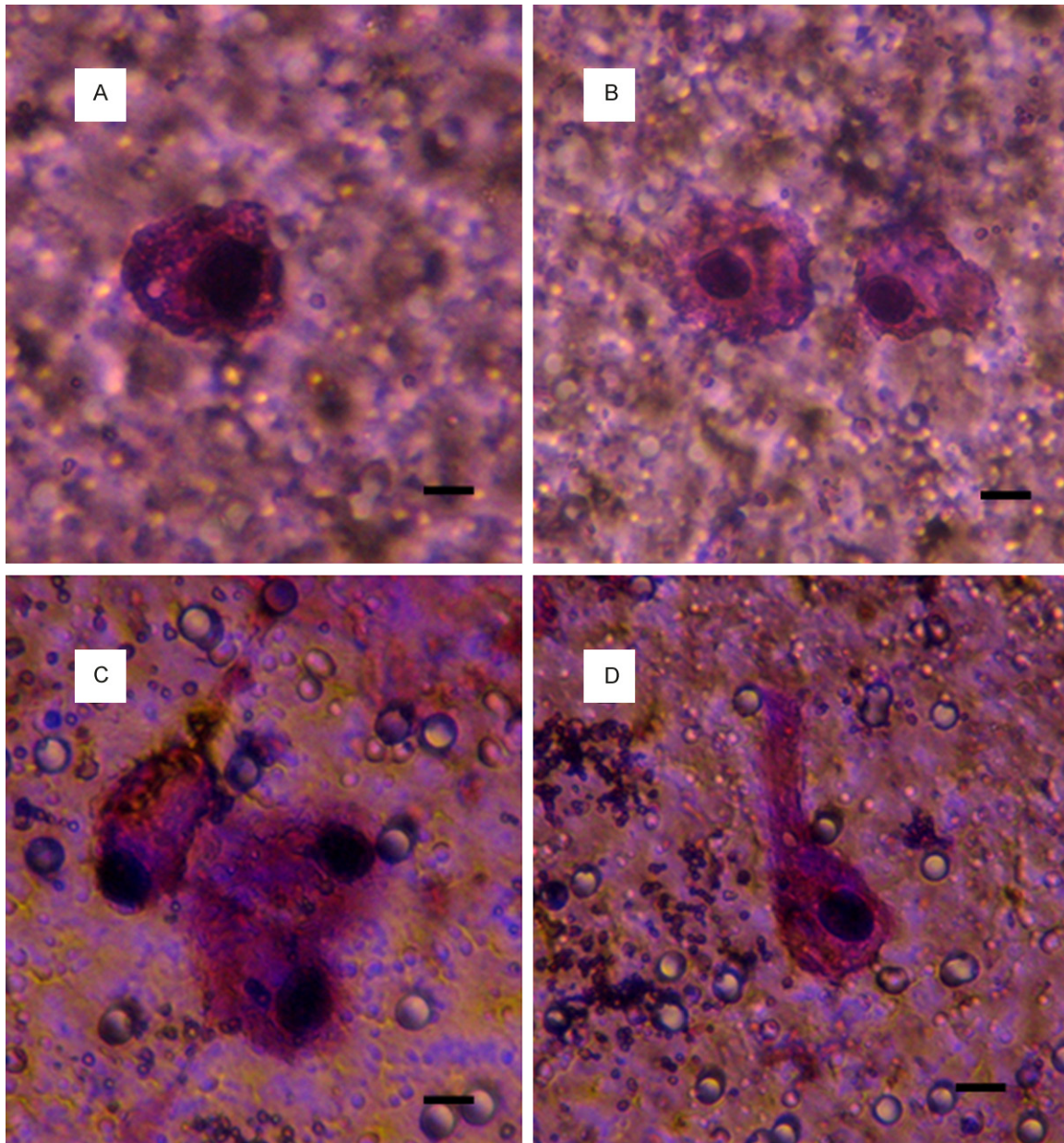
Figure 1. CTCs enriched from peripheral blood of patient with urinary bladder cancer were grown on the separation membrane in vitro for 14 days. The cells visualized on the membrane by May-Grünwald stain are reaching size of more than 17 μm in average, the nuclei are bigger than 10 μm. Bar scale is 10 μm.

sis is planned. If an intermediate CTCs-analysis is awaited, the CTCs-fraction is transferred in PBS (1.5 mL) to the cytospin slide. The slide is then dried for 24 hours and analyzed immunohistochemically.

Cytological analysis

Each immunostaining experiment included a positive control (a positive cell line stained with a specific antibody). The fixed and stained cells on the membranes were examined using light microscopy in two steps: (i) screening at x20 magnification to locate the cells, (ii) observation at x40 magnification for detailed cytomorphological analysis. Isolated cells and/or clusters of cells of interest (immunostained or not) were selected, digitized, and examined by an experienced researcher and/or pathologist. CTCs were defined as cells presenting the following characteristics: (i) nuclear size equal or larger than 10 μm); (ii) irregularity of the nuclear contour; (iii) presence of a visible cytoplasm;
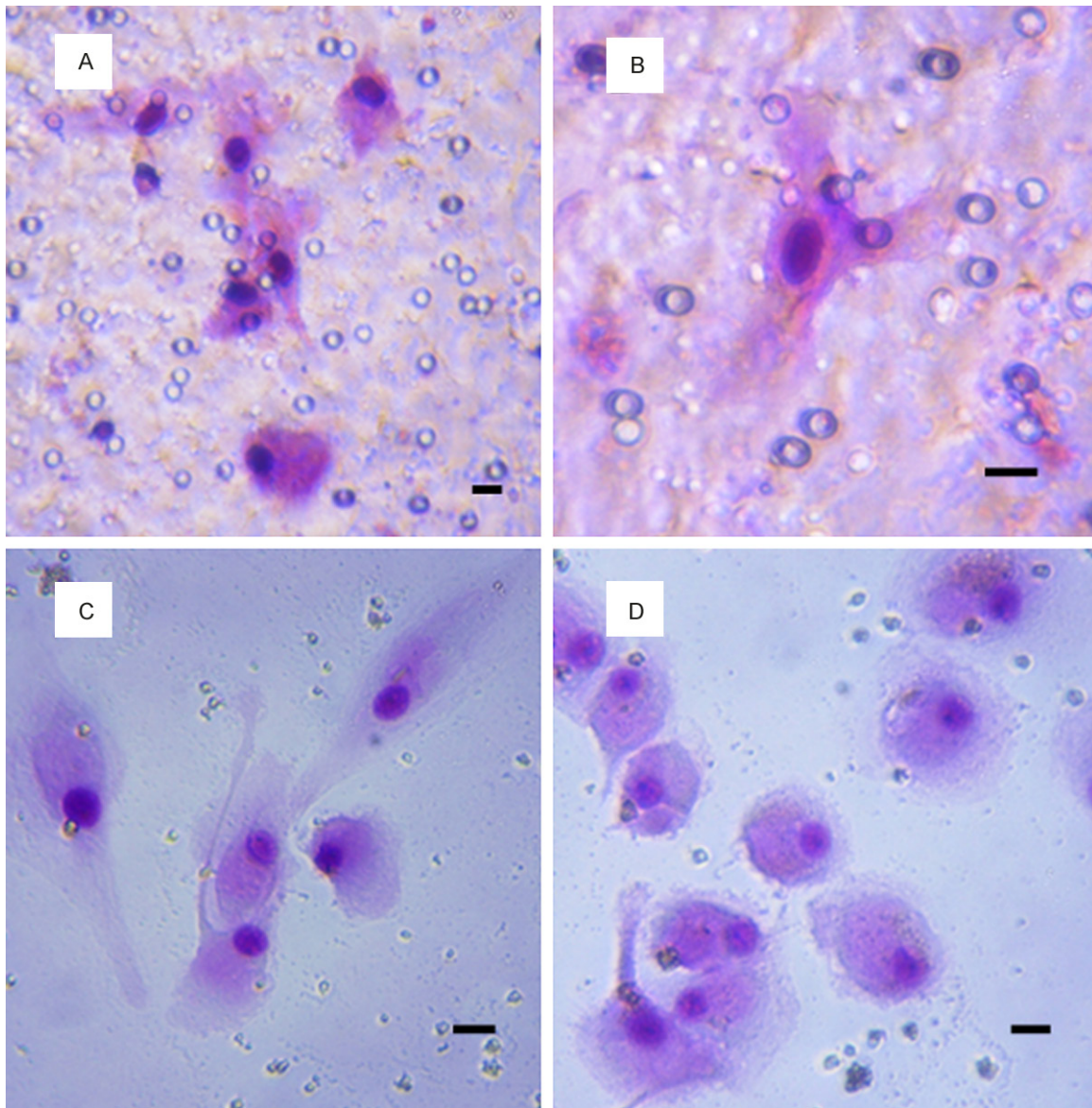
Figure 2. CTCs enriched from peripheral blood of patient with urinary bladder cancer were grown in vitro for 14 days. The CTCs were organized in 2 subcultures. The first one of them grows on the separating membrane (A, B), the second one is created by cells which are growing through the membrane pores to the bottom of the cultivation flask (C, D). Bar scale 10 μm.

(iv) prominent nucleoli. (v) high nuclear-cyto-plasmatic ratio.

The patients with detected CTCs were divided into 4 groups according to the number of sepa-rated cells: under 5 cells, 6-10 cells, 11-50 cells and more than 50 cells.

Results

Thirty-nine patients who underwent urological procedure (TURB, TUC, Uretherottomy) were enrolled in the study. The median age was 71.7 years (between 54 and 89 years), with 30 male and 9 female patients. All patients had patho-logically confirmed papillary carcinoma (See **Table 1** for more information about the stage of the disease). The preprocedure characteristics of the study cohort are detailed in **Table 1**. The mean age of the study population was 71.6 years (54-89). The pathology and CTC results for the study cohort are detailed in **Table 1**. The majority, i.e., eighty per cent (80%) of patients had pTa (44%) or pT1 (36%) stage.
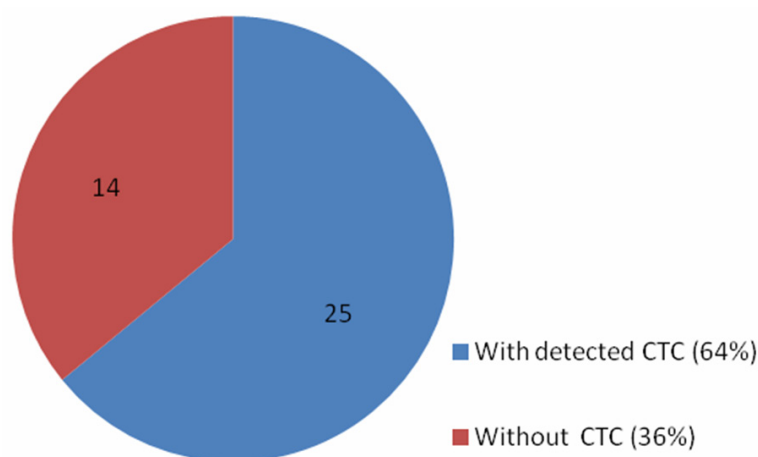
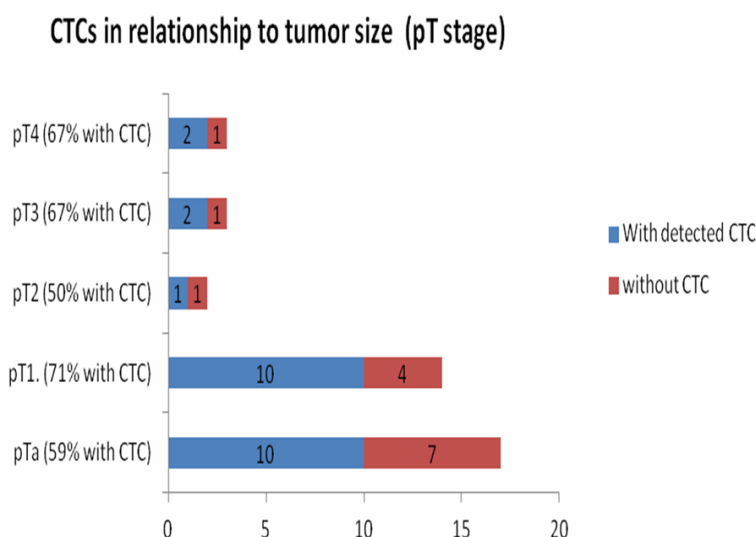Figure 3. Patients with detected CTCs.

Figure 4. CTCs in UBC patients at different patient pT stage.

Of the 39 patients, 25 (64%) had CTCs detected in their serum sample, mostly between 6 and 10 cells. None of the patients had between 10 and 50 cells, as shown in **Table 1**. There was no difference in CTC status (positive vs. negative) based on age, gender, prior BCG history, or neoadjuvant chemotherapy history.

The frequency of the CTCs positivity is summarized for different patient subgroups in **Figure 3**. Due to the low number of patients in the different disease stage groups, the CTC-positivity does not reflect the tumor size (**Figure 4**) or disease stage (**Figure 5**). CTC relationship to the G grade is noted in **Figure 6**.

We report a successful isolation of CTCs with proliferation potential in patients with UBC. The cells captured by size-based filtration approach were enriched viable, which enabled setting up cell cultures from viable CTCs which were unaffected by antibodies or lysing solutions. The CTCs were next cultured *in vitro* for further downstream applications.

The size of the cells guided us in the process of cancer cell identification even without any additional staining (e.g. May-Grünwald stain-MG). However, this standard staining method (MG) enabled us to identify and analyze the nuclei with nucleoli. Generally the nucleus is bigger than 10 µm itself and the cells do not present much of cytoplasm. The nuclear-cytoplasmatic ratio is relatively high in cancer cells, but not in the *in vitro* cultured CTCs. The CTCs got big and long in the culture, which changed the nuclear-cytoplasmatic ratio. The cytoplasm of CTCs is rather pale than rigid. Due to the cell size (> 15 µm), nucleus size (> 10 µm) and shape and expressive nucleoli visualized by simple DAPI-stain in the formerly fixed cells, it was possible to detect cancer cells not only on the separating membrane (**Figure 1A**), but also on the plastic bottom of the 6-well plate (**Figure 1B-D**). The results indicate that the captured cancer cells display plasticity enabling them to overgrow the separating membrane. The shape of the cancer cells in the "bottom fraction" suggests that these cells present a more invasive CTC-fraction. Immunohistochemical analysis has shown the abundance of cytokeratin-18 in the "membrane" fraction as well as in the "invasive" fraction proving the carcinoma origin. Gene expression analysis has enormous potential, and could reveal the epithelial-mesenchymal char-
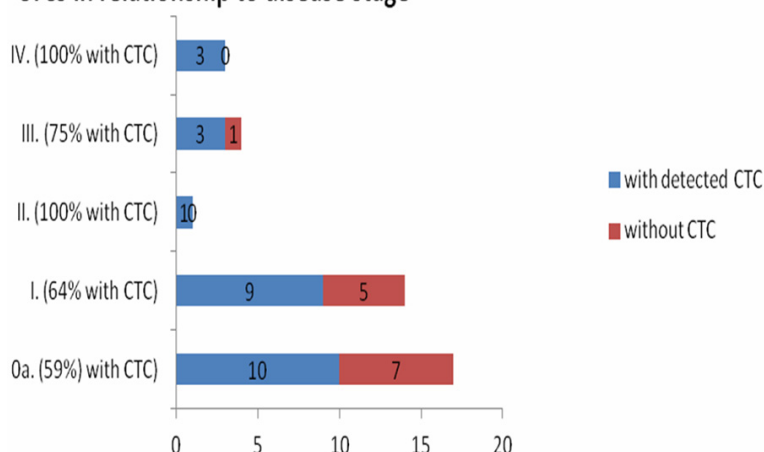
CTCs in relationship to disease stage

Figure 5. CTCs in UBC patients at different stages of the disease.
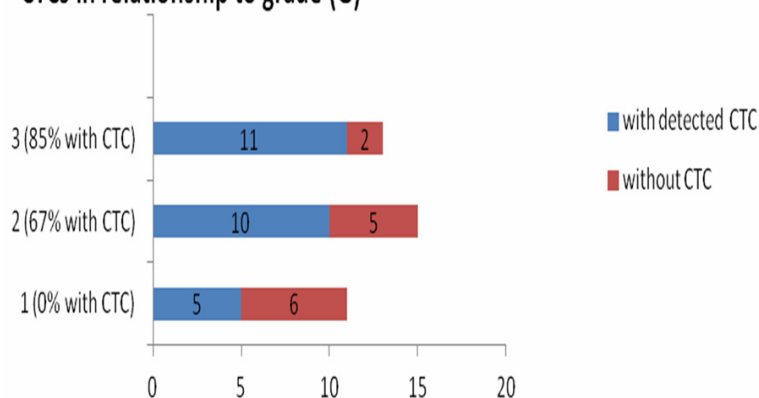
CTCs in relationship to grade (G)

Figure 6. Patients with detected CTCs divided according to the G grading.

acter of the detected cancer cells based on the RNA.

Discussion

The classification most widely used for UBC staging is the American Joint Committee on Cancer (AJCC) tumor, lymph node, and hematogenous metastasis (TNM) system [8]. According to this classification, extravesical disease is categorized as either stage III or IV tumors invading adjacent tissues and/or metastasizing to lymph nodes or to distant sites, whereas stage ≤ II cancers are localized (organ-confined). However, initial clinical staging can be imprecise and a considerable proportion of patients thought to have localized disease will be upstaged to extravesical cancer

following surgical treatment [9]. Inaccurate clinical staging may lead to suboptimal treatment, particularly since extravesical disease at the time of surgical therapy is a known predictor of poor prognosis [10] and patients who are thought to have localized disease may not receive potentially beneficial neoadjuvant therapy. Increased accuracy of initial clinical staging would thus facilitate risk stratification and preoperative decision making.

UBC is being increasingly recognized as a disease that cannot be treated based on pathological staging alone; management strategies will need to focus on molecular alterations associated with individual tumors. The various molecular events that lead to urothelial tumorigenesis and progression are now increasingly understood, and several of these alterations manifest as deregulations in cell-cycle progression.

Tumorigenesis is clearly a multistep process, and while determination of individual molecular alterations offers some insight into its biology, combined assessment of pathway aberrations is essential to determine the aggressiveness of the disease process. Multimarker investigations in deregulations associated with cell-cycle progression have indicated that the burden of molecular alterations in the tumors of individual patients may be more prognostic than the knowledge of the specific aberrations. Future UBC management will be based on the employment of consensus marker panels that will be able to provide accurate predictions of outcome and also define the appropriate therapeutic regimens and targets in individual patients.

To date, molecular markers have not been included in the scoring systems and risk tables to predict prognosis of patients affected by

superficial UBC or in surveillance programs. Several urine markers are available, but none of them has been validated as standard diagnostic procedure in routine urology, since they are not sufficiently sensitive; none of these tests represents an ideal marker which could facilitate reliable UBC detection [11].

The present study has demonstrated that CTCs are frequently detected in patients with UBC and metastatic disease. Survival analysis for the patients with metastatic disease suggests a prognostic role of CTCs in this setting and is hypothesis-generating for the design of future UBC studies [12]. The largest study reporting CTCs detection in nonmetastatic UBC patients was published in 2010. Rink et al. detected CTCs 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 [13]. More recently, the diagnostic value of CTCs presence in UBC was evaluated, and the potential clinical role of CTCs detection as an indicator of advanced UBC was highlighted. Guzzo et al. assessed the PB of 43 patients with clinically localized disease before a planned cystectomy. CTCs were detected in low numbers in a small percentage (21%) of patients [6].

The role of the CTCs as a preoperative marker has been assessed in a limited manner in UBC. One report from Germany described the detection of CTCs in 1 of 5 preoperative patients with UBC [14]. Flag et al. demonstrated CTCs in 5 (18%) of 28 preoperative patients with UBC [12].

In all these studies CTCs were detected in less than 30% of patients. In our study CTCs were detected in 64% of patients, which is a significantly higher number. This result is the effect of using a novel separation method. Unlike other studies which used immunomagnetic separation methods dependent on the antigens expressed by CTCs (mainly EpCAM), our study utilized a size-based filtration method.

There are several ways to detect CTCs in the PB, with RT-PCR being the most common method employed over the last several decades. They allow detection of expression of the so-called epithelium-specific genes, which are expressed in cancer cells but not in nucleated cells of the blood. While the detection of mRNA markers specific for CTCs by RT-PCR is sensi-

tive, its specificity may be significantly different depending on the marker. Several tumor-specific genes have been targeted for such detections. Although the sensitivity of this technique has been well documented, its specificity for diagnostic purposes remains a problem because of the high incidence of false-positive and false-negative results [15]. The other limitations of using PCR to detect CTCs include amplification of nonspecific products and lack of consistent protocol and primer design between investigators necessary for interlaboratory comparisons.

More recently, immunomagnetic methods of capturing CTCs have shown potential to overcome the shortcomings of RT-PCR. Epithelial cell in the blood specimen gains magnetic properties through incubation with iron particles coated with anti-EpCAM. Then, the use of anti-CK, anti-CD45 antibodies and the nuclear dye DAPI results in microscopic visualization and identification of epithelial carcinoma cells and white blood cells. Contamination with blood cells or normal exfoliated cells by urinary infection may result in false-positive results.

It should also be noted that the widely used cell search assay is based on EpCAM and cytokeratin expression; it is therefore possible that a certain fraction of metastatic urothelial CTCs have de-differentiated to the point where they do not express these surface antigens and cannot be detected by this method. Other disadvantage of immunomagnetic method is that captured CTCs are killed in the process of separation and identification. In contrast, our paper describes the first successful culturing of humane UBC cells. The method used in this study (MetaCell approach) enabled the capture of viable intact virgin CTCs (virgin CTC) suitable for next *in vitro* culturing, single cell analysis or drug testing. These procedures are very important for the studies into heterogeneous CTCs and new treatment possibilities.

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Disclosure of conflict of interest

VB and KK declared consultancy for MetaCell s.r.o.

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