Original Article

Detection of circulation tumor cells from peripheral blood in patients with urothelial carcinoma

Komsan Leetanaporn, Watid Karnjanawanichkul, Choosak Pripatnanont, Monthira Tanthanuch, Surasak Sangkhathat

Division of Urology, Department of Surgery, Faculty of Medicine, Prince of Songkla University, Hat Yai, Songkhla, Thailand

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Abstract

Objective: The study was designed to detect CTC in bladder and urothelial cancer patients and find the association of CTC with the staging and grading of urothelial cancer.

Material and Method: Peripheral blood of bladder cancer patients who underwent operations from 2014 to 2015 was collected before the operations. Detection of circulating tumor cells used the quantitative reverse transcription polymerase chain reaction (qRT-PCR) method, using cytokeratin 20 (CK20) (GenBank accession number X73501).

Result: Twenty-seven patients were enrolled in the study; the CK20 gene was detected in every patient's peripheral blood. CTC was higher in >T2 stage compared to the lower stages, but not significantly (0.0056 vs 0.0107, p-value 0.057). Tumor patients who had a high-grade tumor had a higher CTC in their blood significantly compared to patients with low-grade tumors (0.0087 vs 0.0025, p-value 0.006). There was no significant difference in CTC when comparing gender and urine cytology.

Conclusion: CTC is correlated with bladder cancer. CTC of patients with high-grade tumors was found to be significantly higher than in the lower-grade group.

Corresponding author: Komsan Leetanaporn

Address: Division of Urology, Department of Surgery, Faculty of Medicine, Prince of Songkla University, Hat Yai, Songkhla, Thailand

E-mail: kom_lb@hotmail.com

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Introduction

Bladder cancer has become a globally common cancer, bringing with it important causes of morbidity and mortality. The incidence of bladder cancer has been increasing, with an estimated 386,300 new cases in 2008; 430,000 new cases were diagnosed in 2012[1,2]. Transitional cell carcinoma is the most common histological type of bladder cancer. Approximately 95% of primary urothelial cell cancers arise from the bladder, and only a few cases originate from other sites within the urinary tract, such as the renal pelvis and ureter[3-5].

The unique characteristics of bladder cancer are its high recurrence rate, which is up to 80% in high-grade NIBC, and the implantation of the tumor together with the field change effects responsible for explanations[6]. Multifocal lesions are another problem, which make bladder cancer even more challenging to treat. The diagnosis and surveillance follow-up are comprised of the cystoscopy, as per the guidelines[7]. However, the procedure needs experienced hands for the detection of small, viable tumors, and the compliance of patients is problematic because of the busy cystoscopy schedule, even though ancillary equipment was launched for assistance in documenting the presence of tumors during cystoscopy[8]. Nevertheless, the availability of its use is still limited and translation of its findings also requires experienced staff. Urine cytology is one of the less invasive investigations, and is considered for additive parameters, such as the detection of the presence of any significant tumors[9] and circulating tumor cells (CTC), which have been reported as invaluable for bladder tumor detection, and may also be associated with higher stage, non-organ confined diseases[10].

Over the years, many technologies have been utilized in an attempt to identify CTC. The detection of CTC has been well demonstrated in breast, colon, prostate and several other malignancies[11-14], and a variety of methods for detecting CTC have been developed, including nested RT-PCR, which utilizes 2 pairs of PCR primers to amplify a single locus. PCR-based methods are considered highly sensitive. They are also able to demonstrate strong specificity via the design of primers that detect the mRNA expression of tumor-specific genes, such as cytokeratin (CK)-20, uroplakin (UP) II, mucin 7 (MUC7)[15] and epidermal growth factor receptors (EGFR)[16]. There is a study that determined that the expression of CK20 and mucin 7 can be correlated between histological techniques, with an RT-PCR of 95.8%[17,18]. Our purpose was to validate the evidence of CTC in urothelial cancer cases.

Material and Method

A prospective study of bladder cancer patients who underwent surgery was conducted at Songklanagarind Hospital from November 2014 to March 2015. Exclusion criteria included: patients with a history of other cancers, emergent operations, and previous treatment with chemo-radiotherapy. Patient demographic data, family history, and clinical features were collected. The peripheral blood and urine were also collected the day before surgery.

Detection of CTC used the quantitative reverse transcription polymerase chain reaction (qRT-PCR) method, using cytokeratin 20 (CK20) (GenBank accession number X73501) and mucin7 (MUC-7) (GenBank accession number NM_001145006) as marker genes, with cyber Green as the detection system. After collection, each blood sample was stored in a refrigerator in the Central Research Laboratory, Rat-Pratanratnikorn Building, Faculty of Medicine, PSU, and further processed within 24 hours. To separate plasma and cell components, each sample of un-clotted blood was centrifuged at 3,000 rpm for 5 minutes. The plasma supernatant was then aspirated and stored at -80°C. The remaining cell components were subjected to red blood cell lysis, leaving a pellet of white cells, and tumor cells (if any) at the bottom of the tube. The pellet was then re-suspended, with RNA storage media (RNA later) and frozen at -80°C until extraction.

RNA extraction was performed from pellet and plasma fractions, using a RNA extraction kit. Quantity of extracted RNA was estimated using the spectrophotometry method with a Nano-drop spectrophotometer. Quality of the mRNA was evaluated by RT-PCR, of a house keeping gene (GAPDH). An aliquot of RNA equivalent to 1 microgram was used for cDNA construction, using random hexamer and MMLV reverse transcriptase. Each 20 microliters of qRT-PCR reaction consisted of a PCR master mixture, 1 microliter of each specific primer, cyber green and 1 microliter of cDNA sample (to be optimized). The result, in terms of Ct value, was quantitated with RNA from a serial dilution of a urothelial cell carcinoma cell line, as described below.

The qRT-PCR was standardized through both copy number standardization and cancer cell number standardization. In brief, 2 standard curves were constructed. One was plotted between the log copy number and the Ct, while the other was plotted between the log cell number and the Ct value.

Urothelial cancer cell lines used in this study were RT4 and 486P (ATCC, Manassas, Va.), which represent grade 1 and grade 4, respectively. Both cells are propagated in RPMI1640 x standard mammalian cell culture condition (5% CO_2 in 37°C incubator). An amount of 108 cells of each cell line was suspended in 1mL of phosphate-buffered saline, and serially diluted 10 to 108 times. One milliliter of each diluted sample was added to 50 mL of urine, from a healthy volunteer, and subjected to the RT-PCR assay described above.

Patient demographic data and clinical features are reported as mean $\pm$ SD. Categorical variables were analyzed using a Chi-square test, and comparisons of quantitative variables between groups via the use of Student's t test. $p<0.05$ for a 2-tailed test was considered significant. The accuracy of RBG is shown in sensitivity and specificity. Statistical analysis used program R version 2.15.1.

Result

In our population of 26 patients, all had a positive detection of CK20. The surgical procedure included either TUR-BT or radical cystectomy with urinary diversion. Endoscopic surgery with TUR-BT was responsive in almost 80% of cases. Median age was 68.57 years (range from 47-86) and most were male, accounting for 84.6% of the population. Most patients were diagnosed during an early stage of the tumor (T1 or less). Patient characteristics are demonstrated in Table 1.

Positive CTC tended to be related with muscle-invasive bladder cancer (MIBC). However, there was no statistical significance (0.0056 vs 0.0107, p-value 0.057, Figure 1). Interestingly, when compared with the grading of tumors, the results showed that patients with high-grade tumors had significantly higher CTC in their blood than patients with low-grade tumors (0.0087 vs 0.0025, p-value 0.006, Figure 2). There was no significant difference in gender and urine cytology in correlation with the CTC result.

Table 1. Patient characteristics

		Patients	P value
Age (years)	<60	5	
	60-75	14	
	>75	7	
Gender	Male	22	
	Female	4	
Tumor category			0.057
	Ta/Tis/To	5	
	T1	15	
	T2/T3	6	
Tumor Grade			0.006
	Low	10	
	High	13	
Cytology	Positive	8	
	Negative	6	

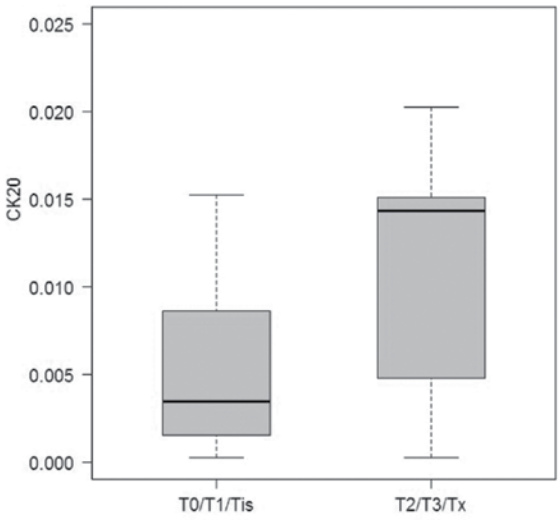

Figure 1. Determination of Circulatory Tumor Cells (CTCs), with tumor stage.

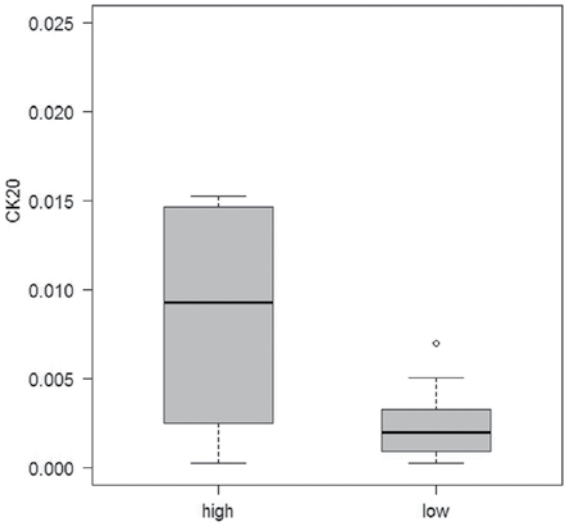

Figure 2. Determination of Circulatory Tumor Cells, (CTCs) with tumor grading.

Conclusion

Bladder cancer presents mostly with non-muscle invasive diseases and has a lower impact with cancer-specific survival. However, the risk of recurrence is high, having been reported as up to 78%. Importantly, risk of disease progression was almost 50% and later leads to cancer morbidity and mortality[19]. Early detection of the tumor will improve disease control, quality of treatment, and lead to lower morbidity. Clinical signs and symptoms always cause a delay in the diagnosis of bladder cancer, and this is always problematic. Therefore, diagnosis during the early period of the disease, coupled with the provision of providing therapeutic treatment, will increase the life expectancy of patients[20]. Urinary cytology has demonstrated a strong correlation with high-grade urothelial cancer, but there is still limited evidence of low-grade tumor detection. Yafi et al.[21] reported a sensitivity of 16% for low-grade bladder tumor, despite detection being as high as 84% for high grades of the disease.

The detection of tumor-like cells in peripheral blood was first reported in 1869 by Ashworth[22]. Additionally, reports on the detection of CTC inothers cancers may serve as prognostic factor for survival, as well as positive lymph node involvement[23-25]. CTC helps in the diagnostics of the presence of tumors, which is demonstrated clearly in breast cancer and is useful for surveillance[26]. A meta-analysis has demonstrated the correlation between the presence of positive CTC and poor prognosis of castrated-resistant prostate cancer (CRPC) patients[27]. In addition, the presence of CTC is valuable, and can be considered as a surrogate marker for micrometastasis[28]. The monitoring for treatment outcomes may therefore be better than a radiographic response[29,30]. The usefulness of this technique is its accurate staging; furthermore, it is appropriate for follow-ups, including monitoring after surgery.

CTC correlation with urothelial cancer was reported in 2007 by Naoe et al.[31], and has since become well known. Our study demonstrated the detection of CTC in all patients, which is different from other studies, which had about one-third detection[32]. Most of the studies included patients with a high grade of the disease in more than half of the populations[32-34]. Moreover, a previous study, from Miller et al.[34], demonstrated the value of CTC in terms of predicting

progression-free and overall survival when compared with the current standard of care. While the recurrence rate depends on multiplicity, tumor size and the prior recurrence rate, this progression was based on tumor grade, stage, and CIS components[32,35]. This corresponds with our findings, in that all CIS components are related to high-grade tumors. The urine cytology was positive in 75% of CIS cases. CTC is also related with the aggressiveness of the disease, which was revealed by the strong positive in high-grade urothelial cancer. The association of CTC with higher tumor stage and higher grades of the disease was demonstrated in our study, in much the same way as previously published studies[36,37].

In the future, CTC may be one of the tools that help in the diagnostic process of urothelial cancer surveillance. The test may also replace current invasive diagnostic procedures while enhancing the compliance of patients, physicians, and economy. The aggressiveness of urothelial cancer can also be predicted by this procedure.

Conflict of interest

The authors declare no conflict of interest.

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