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A non-invasive genomic diagnostic method for bladder cancer using size-based filtration and microchip electrophoresis

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ABSTRACT

Bladder cancer (BC) cells spontaneously exfoliated in the urine of patients with BC. Detection of exfoliated tumor cells has clinical significance in cancer therapy because it would enable earlier non-invasive screening, diagnosis, or prognosis of BC. In this research, a method for analyzing genetic abnormalities of BC cells collected from urine samples was developed. Target BC cells were isolated by filtration. To find conditions that achieve high cell recovery, we investigated the effects of filter type, concentration of fixative, and flow rate. Cells captured on the filter membrane were completely retrieved within 15 s. Selected genes for genomic analysis, mutated genes (FGFR3, TERT and HRAS) and methylated genes (ALX4, RALL3, MT1A, and RUNX3) were amplified by polymerase chain reaction (PCR), and subsequently, were identified by microchip electrophoresis (MCE). Analysis by MCE reduces the risk of contamination, sample consumption, and analysis time. Our developed approach is economical, effectively isolates cancer cells, and permits flexible molecular characterization, all of which make this approach a promising method for non-invasive BC detection.

1. Introduction

Bladder cancer (BC) is commonly diagnosed in developed countries [1], and is increasingly being diagnosed in developing countries [2]. Upon detection, approximately 75% of bladder cancer tumors are classified as non-muscle invasive BC (NMI-BC). NMI-BC typically recurs after treatment, and in 15–20% of the cases, progresses to muscle-invasive BC (MI-BC) [3,4]. Therefore, patients who have had BC require regular monitoring for recurrence and progression. BC screening and surveillance is traditionally performed with cystoscopy and cytology, each having certain disadvantages. Cystoscopy is an invasive and unpleasant procedure for patients, and although urine cytology has good specificity, it has a poor sensitivity for detection of low-grade BC [5]. Therefore, a sensitive and non-invasive method for BC detection is greatly needed.

Voided urine is an ideal sample for non-invasive BC diagnosis since malignant cells are exfoliated spontaneously when tumors grow. Recently, several non-invasive urine tests for BC have been approved by the Food and Drug Administration (FDA); however, they are not commonly used in clinical practice due to low sensitivity or specificity [6]. Since cancer cells will be scarce in urine, an efficient cell isolation method is needed to improve the sensitivity. Traditional pretreatment methods for BC cells isolation from urine like centrifugation are often associated with significant cell loss [7]. To avoid such cell loss, numerous cell isolation methods have been developed to instead rely on physical or immunochemical properties [8,9]. For example, filtration methods, which are based on variations in both cell size and deformability, provide fast cell isolation from large volume samples and remove the complex background matrix before downstream analysis. One such isolation method using track-etched polycarbonate membrane (PC membrane), originally developed by Vona et al. [10] for epithelial tumor cells (ISET), has been extensively applied to the detection of many kinds of cancers [11–14]. These microfilters of controlled pore distribution, size, and geometry are available in a variety of materials [15–18]. Methods using membranes to isolate cancer cells have shown great results, but have mostly been developed for the isolation of circulating tumor cells (CTCs) in peripheral blood [11–18] as opposed to urine samples [19,20].

Though size-based filtration devices can isolate target cells from urine with high speed, most require that the isolated cells on the membrane be identified by immunostaining [18,20]. The process of fixing, immunostaining, and subsequently imaging is laborious and subjective. An alternative method for identifying the isolated cells is by automatically analyzing tumor-associated

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genomic alterations in DNA [21]. Somatic mutations and hypermethylation of promoter CpG islands in specific genes have been confirmed to be responsible for the transformation of a normal cell into cancer [22], and are promising biomarkers for cancer detection. The most frequently mutated genes include fibroblast growth factor receptor 3 (FGFR3) [23,24] and telomerase reverse transcriptase (TERT) [25,26]. Furthermore, epigenetic perturbation of gene regulation is increasingly being shown to cause early tumorigenesis [27,28]. However, merely applying gene mutation or methylation analysis alone is insufficient to define all types of BC, and different studies have reported highly variable levels of specificity and sensitivity, even for identical markers [29]. Thus, a combination of gene mutation and methylation analysis is recommended to detect BC in clinical samples [30]. Traditional non-invasive BC genomic diagnostic assays consist of genomic sequencing or polyacrylamide gel electrophoresis (PAGE) separation after PCR amplification [16,19,20,31]. Genomic sequencing is time-consuming and costly while the PAGE method is subject to tedious manual operations as well as DNA contamination and low sensitivity, both of which may lead to false results. Most recently, MCE is being explored as a replacement for PAGE for analyzing DNA fragments [32]. Although MCE separations have been used in various biochemical and chemical applications [33–35], few analytical methods have been reported for clinical diagnosis thus far.

The aim of this study was to develop a sensitive and cost-effective cell capture-PCR–MCE system (CCPM) for non-invasive detection of BC. Briefly, BC cells were isolated from urine by a size-based filtration device (Fig. 1A). After cell retrieval and subsequent lysis, target genes were amplified by PCR for gene mutation and methylation analysis (Fig. 1B). A simple and rapid MCE system was utilized to further detect target gene fragments (Fig. 1C). Combining the merits of filtration devices and MCE technology, this system showed good performance in cell recovery and high sensitivity for the detection of genomic alterations, without using expensive instruments and reagents. The developed method is well-qualified in linearity, sensitivity, and repeatability for clinical application for BC detection.

2. Experimental

2.1. Materials and reagents

PC membranes were purchased from Whatman Nucleopore (GE, Kent, UK). The nickel microsieve was manufactured by Shanben Precision Co., Ltd. (Guangzhou, China). Hoechst 33342 and LIVE/DEAD® Cell Viability Kit were purchased from Invitrogen Co., Ltd. (Shanghai, China). Dichloromethane (99.8%, AR) was purchased from Sigma-Aldrich (Shanghai, China). All primers were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). PCR reagents including 10 × PCR Buffer, 25 mM MgCl2, 10 mM dNTPs and HotStart Taq DNA polymerase were purchased from Excell Co., Ltd. (Shanghai, China). QIAamp DNA mini kit was obtained from QIAGEN Inc., (Hilden, Germany). BisuFlashTM DNA Modification kit was bought from Epigentek Inc., (NY, USA). DNA-500 kit, including DNA-500 marker and DNA-500 separation buffer were obtained from Shimadzu Co., Ltd. (Tokyo, Japan). SYBR Gold and 25 bp DNA ladder were purchased from Invitrogen Co., Ltd. (Shanghai, China).

2.2. Apparatus

Scepter™ 2.0 cell counter (Millipore, USA) was used for cell size distribution measurement and cell concentration determination.

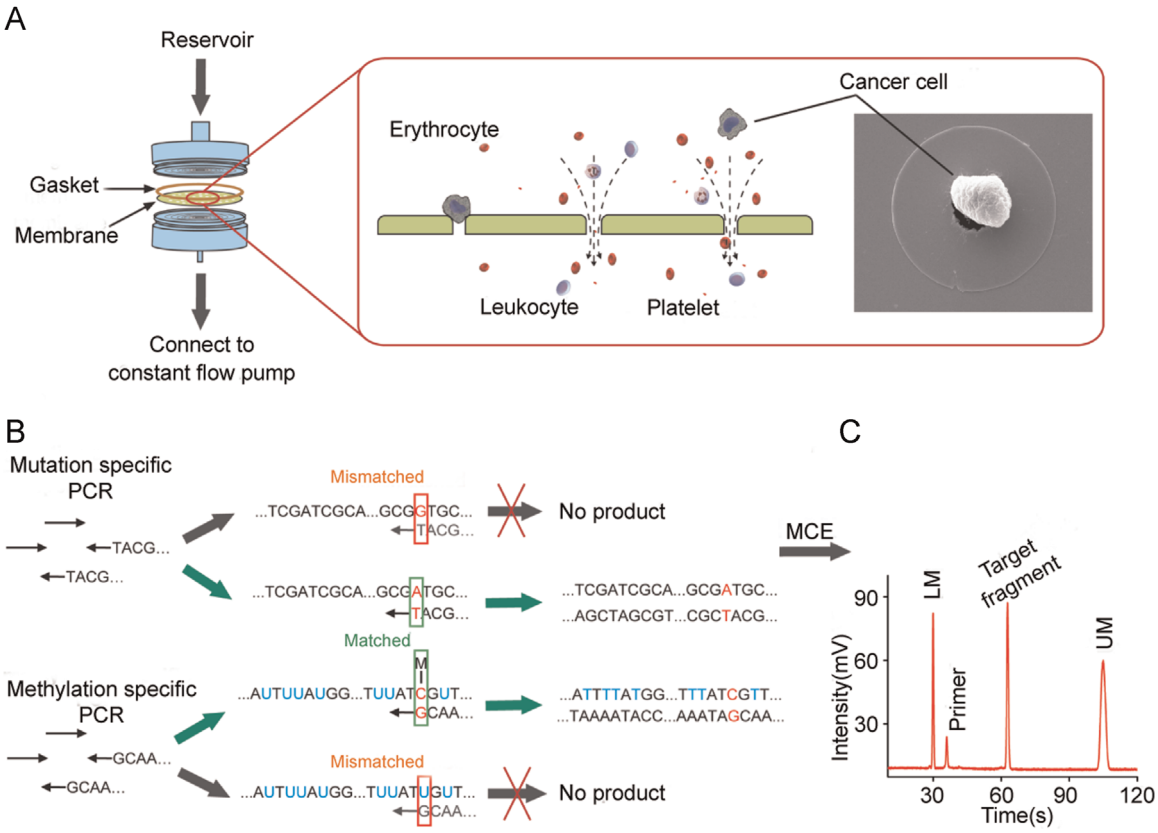

Fig. 1. Schematic diagram of size-based filtration device combined with MCE for genomic abnormality analysis. (A) Tumor cells are isolated by size-exclusion. (B) Cancer-associated mutation and methylation genes are amplified with high specificity. (C) High-throughput MCE separation is carried out for target gene fragment identification. LM: low marker. UM: upper marker.