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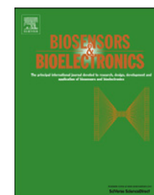

A novel bisulfite-microfluidic temperature gradient capillary electrophoresis platform for highly sensitive detection of gene promoter methylation

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ABSTRACT

The hypermethylated tumor suppressor gene promoters are widely recognized as promising markers for cancer screening and ideal targets for cancer therapy, however, a major obstacle in their clinical study is highly sensitive screening. To address this limitation, we developed a novel bisulfite-microfluidic temperature gradient capillary electrophoresis (bisulfite-μTGCE) platform for gene methylation analysis by combining bisulfite treatment and slantwise radiative heating system-based μTGCE. Bisulfite-treated genomic DNA (gDNA) was amplified with universal primers for both methylated and unmethylated sequences, and introduced into glass microfluidic chip to perform electrophorectic separation under a continuous temperature gradient based on the formation of heteroduplexes. Eight CDKN2A promoter model fragments with different number and location of methylation sites were prepared and successfully analyzed according to their electrophoretic peak patterns, with high stability, picoliter-scale sample consumption, and significantly increased detection speed. The bisulfite-μTGCE could detect methylated gDNA with a detection limit of 7.5 pg, and could distinguish as low as 0.1% methylation level in CDKN2A in an unmethylated background. Detection of seven colorectal cancer (CRC) cell lines with known and unknown methylation statuses of CDKN2A promoter and 20 tumor tissues derived from CRC patients demonstrated the capability of detecting hypermethylation in real-world samples. The wider adaptation of this platform was further supported by the detection of the CDKN2A and MLH1 promoters' methylation statuses in combination. This highly sensitive, fast, and low-consumption platform for methylation detection shows great potential for future clinical applications.

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1. Introduction

The hypermethylation of CpG islands in the promoters of tumor suppressor genes silences their expression, and is thus critically involved in the formation of various types of cancers. During multistage carcinogenesis, DNA methylation is usually observed early and has been recognized as an effective molecular marker for early diagnosis. Moreover, because of drug-inducible reversibility, methylation abnormalities are expected to become ideal targets for tumor treatment (McCabe et al., 2009; Deng et al., 2010). Therefore, promoter methylation testing has a strong potential for cancer screening and therapy, and considerable effort has been devoted to analyzing methylation changes.

Currently, technologies based on the bisulfite treatment of genomic DNA (gDNA) are most commonly used to investigate CpG island methylation. In these methods, bisulfite treatment, in combination with subsequent PCR, can convert differences in methylation status into DNA sequence variation and enable research methods for genetic studies to be employed in detecting the epigenetic changes. The earliest established bisulfite-sequencing was reliable, but its cumbersome steps, high cost, and relatively low sensitivity necessitated the development of a series of prescreening methods (Frommer et al., 1992). As a typical representative of these methods, methylation-specific PCR (MSP) was fast, economical, and capable of detecting 0.1% of methylated templates, but its unsatisfactory specificity and inability to detect methylated CpG sites (CpGs) not covered by primers restricted its applications (Herman et al., 1996; Kristensen and Hansen, 2005).

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Next, denaturing high-performance liquid chromatography (DHPLC) was applied to detect methylation on the basis of the melting temperatures (*T*ms) of the differentially methylated sequences, with high specificity and the capability of assessing all of the CpGs flanked by the primer pairs (Deng et al., 2002; Luo et al., 2006). Besides the expensive and maintenance-intensive instruments, the main limitations of DHPLC were the need to explore optimal temperatures specific to individual known sequences and the relatively low throughput (Omaruddin and Chaudhry, 2010). The newly developed methylation-sensitive high-resolution melting (MS-HRM) is also a *T*m-based commercially available platform, and can perform parallel quantitative DNA methylation detection in closed tube. This method is able to reveal 0.1% methylated template from total 10 ng of input DNA by including a limited number of CpGs into the primer sequences which could reverse the PCR bias and favor the amplification of methylated alleles (Wojdacz and Hansen, 2006; Wojdacz and Dobrovic, 2007; Wojdacz et al., 2008). However, the consequential problem is the methylation status of the CpGs within the primer binding sites can't be determined (Wojdacz et al., 2010). Additionally, to reduce the complexity of the melting profile and thus increase sensitivity, the preferred length of PCR products in MS-HRM assay is around 100-bp with only one melting domain, which inevitably restricted the number of detectable CpGs (Wojdacz and Dobrovic, 2007; Wojdacz et al., 2008). Therefore, although many methods have been established, more capable and robust methylation analysis methods are still sought for use in clinical settings, due to the fairly complicated and low abundance methylated alleles in highly heterogeneous cancer samples and commonly encountered trace quantities of real samples.

Temperature gradient capillary electrophoresis (TGCE), which was initially established as a point mutation detection technology, detectes mutant DNA fragments through a continuous temperature gradient, also based on variations in *T*ms. The main advantage of TGCE is the ability to detect mutations without preexisting knowledge of their exact nature, provided that their *T*ms are sufficiently close to the range of the applied temperature gradient (Gelfi et al., 1994; Murphy and Berg, 2003). Furthermore, TGCE could be utilized to screen mutations in different melting domains of a DNA fragment, which allowed it to detect longer DNA fragments (Murphy and Berg, 2003; Sheffield et al., 1989; Hsia et al., 2005). Considering bisulfite treatment can introduce multiple mutations into the DNA fragment with varying epigenetic modifications, TGCE may be adapted to investigate gene methylation. The utility of TGCE in methylation analysis has not been reported previously, partly because of its sensitivity of only 10% (Li et al., 2002). Over the past decade, microfluidic chips in biological analysis have attracted significant attention with their notably increased speed, throughput, sensitivity, remarkably reduced sample/reagent consumption, and automation (Yeo et al., 2011). Several studies have reported the transplantation of TGCE to the chip platform (Buch et al., 2004, 2005). A more robust microfluidic TGCE (μTGCE) method using a slantwise radiative heating system was developed in our previous work. KRAS mutations in colorectal cancer (CRC) cell lines, paraffin-embedded tissues, cancer tissues, and stool samples of CRC patients were successfully detected, as the system could reveal 0.2% mutant KRAS (Zhang et al., 2007, 2010, 2011).

In this study, μTGCE was combined with bisulfite treatment for the first time to establish a bisulfite-μTGCE platform for methylation analysis, which demonstrated rapid and accurate detection of various model methylated fragments, significantly increased sensitivity, and good capability of detecting the tumor suppressor gene CDKN2A promoter methylation in both CRC cell lines and CRC patients' tissues. In addition, the employment of this platform to analyze CDKN2A and mismatch repair gene MLH1 promoter methylation combinations further supported its wider adaptation.

2. Experimental

2.1. Cell lines and tissue samples

Seven human CRC cell lines (HT29, SW480, LS174T, HCT116, CCL187, Clone A, and CX1) and three human gastric cancer (GC) cell lines (MGC803, BGC823, and MKN45) were cultivated in DMEM supplemented with 10% fetal bovine serum. Twenty surgical primary CRC tissues were collected during the operation. The study was conducted in compliance with China Medical University Institutional guidelines regarding patient information and consent. None of the patients underwent pre-operative radiotherapy and chemotherapy.

2.2. DNA isolation and bisufite treatment

gDNA was extracted from the harvested cells and CRC tissues using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). gDNA samples were linearized with EcoRI (TaKaRa Biotechnology, Dalian, China). The bisulfite treatment of the resulting gDNA was performed with the CpGenome™ DNA Modification Kit (Zymo Research, CA, USA).

2.3. Preparation of model methylated CDKN2A fragments

Eight model methylated CDKN2A fragments were prepared to show the detection capability for methylated templates with different number and location of methylation sites. To obtain completely methylated and unmethylated DNA fragments, DNA samples were isolated from two CRC cell lines in which the methylation statuses of CDKN2A promoters had been determined (Lind et al., 2004). The HT29 cells were completely methylated at all 35 CpGs, while the LS174T cells exhibited no CpG-site methylation. The partially methylated sequences were created with the HhaI and HpaII DNA methylases (New England Biolabs) which modified the internal cytosines (Cs) in the sequences 5′-CGCG-3′ and 5′-CCGG-3′, and the DNA methyltransferase inhibitor 5-aza-2′-deoxy cytine (5-Aza-CdR) (Sigma-Aldrich, MO, USA). The gDNA of LS174T cells was modified by the two methylases. The cultured HT29 cells were treated with 5-Aza-CdR to achieve demethylation. The melting maps of these model fragments were calculated by Melt 94, as the references for choosing the appropriate temperature gradients in the μTGCE assay.

2.4. PCR amplification

A 392-bp fragment of the CDKN2A promoter region containing 35 CpGs and a 294-bp fragment of the MLH1 promoter region containing 15 CpGs were PCR-amplified with universal primers after bisulfite treatment (Deng et al., 2002; Betz et al., 2004). Both forward primers included a 40-bp GC-clamp at the 5′ end (Sheffield et al., 1989). Hot-start PCR was performed in a 25 μl volume containing 80 ng of gDNA treated with bisulfite, 0.2 mmol/L dNTPs, 0.5 μmol/L of each primer, 1 × Taq Plus buffer, and 2.5 U Taq Plus DNA polymerase (Tiangen Biotech, Beijing, China). The initial denaturing step at 95 °C for 5 min was followed by 35 cycles consisting of 60 s at 95 °C, 60 s at 55 °C, and 90 s at 72 °C. The final extension step at 72 °C was for 10 min.

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