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An improved fluorescence polarization assay in 5'-nuclease reaction for gene promoter methylation detection

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ABSTRACT

The detection of gene promoter methylation plays increasing roles in personalized medicine. In this study, an improved gene promoter methylation assay based on fluorescence polarization in 5'-nuclease reaction was developed. The novel assay offered a homogeneous annealing and cleavage reaction fully integrated with PCR which used a probe labeled with fluorescence without quencher to obtain the decreased fluorescence polarization values. In this platform, gene promoter methylated and unmethylated alleles were detected simultaneously in a tube. O^6-methylguanine-DNA methyltransferase gene promoter methylation in 103 glioma tissue samples and epidermal growth factor receptor gene promoter methylation in 116 primary non-small-cell lung carcinoma tissue samples were detected by the novel assay and sequencing, absolute quantitative analysis of methylated allele in parallel. The accuracy of the results measured by the improved fluorescence polarization assay was evaluated using the paired-samples *t* test. No significant difference was found ($P > 0.05$). Therefore, the improved fluorescence polarization assay in 5'-nuclease reaction demonstrated a homogeneous, reliable and cost-effective method for gene promoter methylation analysis in clinic. That would provide a scientific basis for applying a reasonable therapeutic regimen in future treatment.

1. Introduction

DNA methylation only takes place at position 5' of the cytosine ring in cytosine-guanine dinucleotides (CpG) and 15% of the total CpG is located in CpG islands. In 60% of human genes, the CpG islands reside in promoters, first exon and 5′untranslated region. CpG islands are usually maintained free of methylation. Gene promoter methylation is a critical signal defining heritable epigenetic states of transcription and is associated with transcriptional repression (Taby and Issa, 2010). Moreover, gene promoter methylation is reversible. Gene promoter methylation plays an important role in normal development as well as disease processes. Transcriptional inactivation by cytosine-5 methylation at promoter CpG islands of tumor suppressor genes is thought to be an important mechanism in human carcinogenesis. Aberrant cytosine-5 methylation at gene promoter CpG islands results in cancer progression and chemotherapy resistance (How Kit et al., 2012; Normanno et al., 2013; Ren et al.,2011). The detection of gene promoter methylation plays increasing roles in personalized medicine (Mikeska et al., 2012). O^6-methylguanine-DNA methyltransferase gene (MGMT) and epidermal growth factor receptor gene (EGFR) promoter methylation are prognosis and prediction molecular biomarkers in the monitoring of carcinogenesis and the personalized cancer treatment. The DNA repair protein MGMT removes alkyl adducts from the O^6 position of guanine and MGMT promoter methylation has been associated with loss of MGMT protein. The *MGMT* promoter methylation has relation not only with glioma progression-free survival but also with outcome treated with alkylating agents (Cankovic et al., 2013; Hegi et al., 2008; Jacinto and Esteller, 2007). *EGFR* promoter methylation results in transcriptional silencing of *EGFR* in non-small-cell lung carcinoma (NSCLC). It is of clinical interest that

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NSCLC cells with *EGFR* promoter hypermethylation are resistant to EGFR inhibitor (Anglim et al., 2008; Jakopovic et al., 2013; Li et al., 2013; Montero et al., 2006).

The detection methods for DNA methylation, such as methylation-specific polymerase chain reaction (MS-PCR), methylation-specific multiplex ligation-dependent probe amplification (MLPA), combined bisulfite restriction assay (COBRA), sequencing, pyrosequencing, MethyLight and absolute quantitative analysis of methylated allele (AQAMA), have been developed (Eads et al., 2000; Herman et al., 1996; Hernández et al., 2013; Nygren et al., 2005; Wu et al., 2011; Shaw et al., 2006; Xiong and Laird, 1997; Zeschnigk et al., 2004). A detection method for gene promoter methylation in clinical diagnosis should be reliable, cost-effective, and a one step format is preferred to prevent crossover contamination. Enhancement and innovation for DNA methylation detection technologies are currently in progress. The fluorescence polarization (FP) technique can be used to detect any significant change in molecular weight of a fluorescent molecule (Ohiso et al., 2000; Bao et al., 2010). FP is a good detection method for 5'-nuclease reaction, where a fluorescent probe is cleaved by 5'-nuclease activity of Taq DNA polymerase during PCR only when it is annealed to a perfectly complementary template. The FP assay in 5'-nuclease reaction has been used as a homogeneous and reliable method for genotype in one step (Sherif et al., 2001). The detection format of FP assay in 5'-nuclease reaction avoids crossover contamination resulting from sample separation and is adaptable to automated analysis. FP plate reader can detect more than one kind of fluorophore in one tube. In our study, the inclusion of a pair of methylated and unmethylated allele-specific probes labeled respectively with reporter fluorophores 6-carboxyfluorescein (FAM) and tetramethyl-6-carboxyrhodamine (TAMRA) at the 5′-end without quenchers allowed the simultaneous, homogeneous detection of methylated and unmethylated alleles in one step.

In this study, the successful adaptation of FP assay in 5'-nuclease reaction to a reliable, cost-effective gene promotor methylation detection were reported. To evaluate the feasibility of the improved FP assay, MGMT promoter methylation in 103 glioma tissue samples and EGFR promoter methylation in 116 primary NSCLC tissue samples were detected by the novel assay and sequencing, AQAMA in parallel. The research confirmed the improved FP assay in 5'-nuclease reaction would be applicable in clinical diagnosis.

2. Material and methods

2.1. Primers and probes

Two pairs of universal primers (*MGMT* -forward and *MGMT* -reverse, *EGFR* -forward and *EGFR* -reverse) and the methylated and unmethylated allele-specific probes were designed based on GenBank accession nos. AL355531 and X17054. The universal primers amplified the bisulfite-modified DNA target but did not discriminate between methylated and unmethylated alleles, as they did not cover any potential CpG dinucleotide sites. It was possible to amplify both methylated and unmethylated alleles simultaneously in one tube. Most current researches for the detection of methylation are based on the conversion of unmethylated cytosine into uracil after sodium bisulphite treatment, and the uracil is converted to thymidine during subsequent PCR. In this study, FP technique and PCR-annealing-cleavage were combined to develop gene promoter methylation assay. The methylated and unmethylated allele specific probes recognized their target sequences in the same promoter as the binding sites of the probes covered the four target CpG and TpG dinucleotide sites (Table 1). DNA methylation was discriminated when the two differently-labeled methylated and unmethylated allele specific probes annealed to their target sequences and were cleaved in the same tube (Fig. 1). FAM-unm *MGMT*-probe and TAMRA-m*MGMT*-probe were respectively specific for the unmethylated and methylated alleles of the *MGMT* promoter. FAM-unm*EGFR*-probe and TAMRA-m*EGFR*-probe were respectively specific for the unmethylated and methylated alleles of the *EGFR* promoter.

Table 1
Sequences of primers and probes.

Name Sequence
MGMT-forward primer 5'-ttggatatgttgggatagtt-3'
MGMT-reverse primer 5'-cccaaacactcaccaaatc-3'
EGFR-forward primer 5'-ggttttttgatttygtttagta-3'
EGFR-reverse primer 5'-ccttacctttctttttcctcc-3'
FAM-unmMGMT-probe FAM-5'-taggttttt ***gtg***gtg ***tg***tat***tg***-3'
TAMRA-mMGMT-probe TAMRA-5'-aggtttt ***cgcg***gtg ***cg***tat ***cg***- 3'
FAM-unmEGFR-probe FAM-5'-atg ***tg***attttt ***tg***gga ***tg***gt ***tg***-3'
TAMRA-mEGFR-probe TAMRA-5'-tg ***cg***atttttt ***cg***gga ***cg***gt ***cg***-3'
MGB-unmMGMT-probe FAM-5'- ***tgtg***gtg ***tg***tat ***tg***-BHQ-3'
MGB-mMGMT-probe VIC-5'- ***cgcg***gtg ***cg***tat ***cg***-BHQ-3'
MGB-unmEGFR-probe FAM-5'- ***tg***attttt ***tg***gga ***tg***gt ***tg***-BHQ-3'
MGB-mEGFR-probe VIC-5'- ***cg***atttttt ***cg***gga ***cg***gt ***cg***-BHQ-3'

The CpG dinucleotide sites were highlighted in bold and underlining.

In AQAMA, the methylated and unmethylated allele discrimination occurred using the specific minor-groove-binding (MGB) molecule containing probes covered the same CpG dinucleotide sites of the methylated and unmethylated alleles (Table 1) (Zeschnigk et al., 2004). The AQAMA was based on real-time PCR using fluorescence quenching detection. The MGB probe was fitted with a minor groove binder to keep the probe length to a minimum and thus maximize the effect of the one-base mismatch (Afonina et al., 1997). DNA methylation was discriminated when two differently-labeled MGB probes hybridized with their target sequences in the same tube (de Kok et al., 2002).

All primers and probes were synthesized and labeled by Invitrogen (Shanghai, China).

2.2. Standard plasmid construction

In order to develop the improved FP assay, standard controls with methylated and unmethylated target DNA sequences should be detected first, and standard plasmids should be constructed. Bisulfite-modified DNA from peripheral blood leukocytes of healthy volunteers was amplified using the two pairs of primers to create standards for the unmethylated allele; to create standards for the methylated allele, bisulfite-modified DNA from peripheral blood leukocytes of healthy volunteers was treated in vitro with SssI methyltransferase (New England Biolabs, M0226S, Boston, USA) before amplification. The PCR products of methylated and unmethylated alleles (*MGMT* promoter, 100 bp; *EGFR* promoter, 156 bp) were introduced into pGEM-T-easy Vector (Promega, A1360, USA) to construct recombinant plasmids, according to the manufacturer's instructions. The recombinant plasmids were identified by sequencing. The plasmids p-methylated- *MGMT* and p-methylated- *p16INK4a* contained the PCR products of methylated alleles. The plasmids p-unmethylated- *MGMT*, p-unmethylated-*EGFR* contained the PCR products of unmethylated alleles. The plasmids were purified using a Quick Plasmid Miniprep kit (Tiangen, DP105, Beijing, China), the concentration of DNA was determined by measuring the optical density at 260 nm.

2.3. Template preparation

One hundred and three glioma tissue samples were obtained from patients undergoing surgery in the Department of Neurology, Xijing Hospital and the Tangdu Hospital of The Fourth Military