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Detection of C677T mutation of MTHFR in subject with coronary heart disease by hairpin probe with enzymatic color on microarray

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ABSTRACT

Molecular beacon (MB) is especially suited for detection of single nucleotide polymorphism (SNP), and the type of MB immobilized on the surface of microarray in particular, may detect multi-sample and multi-locus. However, the majority of MB needs to be labeled with fluorescence and quenching molecules on the two ends of the probe, and observed the reaction of fluorescence or complicated electrochemical signal produced hybridization of MB and target sequence by complex and expensive instruments. The "molecular beacon" and microarray designed appropriately in our study can produce visible light response signal induced by amplification effect of enzymatic color, and are avoided with the marker of fluorescence and quenching molecules and expensive instruments. The "molecular beacon" without fluorescence and quenching molecules is entitled as "hairpin DNA probe" by us for only the "hairpin" structure of traditional molecular beacon is adopted. The merits of two techniques, molecular beacon and amplification effect of enzymatic color, are successfully combined, and the technique is simple, sensitive and specific, to detect and compare the methylenetetrahydrofolate reductase (MTHFR) Gene C677T mutation of subjects between coronary heart disease (CHD) and control group. The results showed that MTHFR Gene C677T polymorphism is an independent risk factor for CHD.

1. Introduction

In this study, a hairpin DNA probe (embryonic structure was entitled as molecular beacon: MB, the expression of "hairpin" is very appropriate due to the changes we made in the structure) was devised according to the principle relationship between nucleic acid base pairing and fluorescence resonance energy transfers (FRET). Space conformation at normal temperature is a stem-loop (hairpin) structure of DNA probe (Weihong et al., 2004; Alevtina et al., 2009). Hairpin probe possess the complementation of ring sequence and target nucleic acid, and the stem formed by target sequence with irrespective sequences. Two ends of hairpin probe are earmarked with fluorescent and quenching molecules. Therefore, fluorescence FRET results in two phenomena. One is too weak in fluorescence signal, due to more effects of quenching molecule on fluorescent molecules, when hairpin probe is an integrity loop-stem structure at normal temperature and are closely combined with fluorescent and quenching molecules. Another phenomenon is strong fluorescence signal, when loop-stem structure of hairpin probe is "on" due to elevated temperature or combined ring sequence and target sequence, which result in the separation between fluorescent and quenching molecules. Hairpin probe is especially applicable to the detection of mutation of single nucleotide polymorphism (SNP), based on high specificity and sensitivity (Chi-Wei et al., 2009; Musa and Lovisa, 2001).

CHD (coronary heart disease) is a horrible threat to human life and life quality. In large number of researches, hyperhomocysteinemia, that is, an amino acid containing sulfenyl and important intermediate product of methionine metabolic cycle, is considered to be the independent risk factor for CHD (Philippa, 2007). N^5, N^{10}-methylenetetrahydrofolate reductase (MTHFR) is a flavoprotein, and can catalyse MTHFR in vivo and prompt the reduction reaction of N^5-methylenetetrahydrofolate (Jiazhong et al., 2005). Hyperhomocysteinemia may result from reduced activity of MTHFR (Rothenbacher et al., 2002). MTHFR Gene C677T mutation may result in high value of homocysteine (Hcy) due to increased thermolability that can obviously reduce the activity of MTHFR and inhibit re-methylation process of Hcy in methionine cycle (Dekou et al., 2001). MTHFR Gene C677T mutation is single base mutation

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and is detected with the methods of high specificity and sensitivity. Of all these developments, MTHFR have become the hotspot of research over the recent years because it is the elected candidate genes for CHD. MTHFR Gene C677T mutation can be detected only by a more sensitive and specific instrument. Besides techniques for PCR-single-strand coaformation polymorphism analysis (PCR-SSCP) (Maria and Elena, 2006), in recent years MB and hairpin probe in particular have proved to be suitable to detect single base mutation for MTHFR Gene C677T (Musa and Lovisa, 2001; Yong-Qiang et al., 2011). The technique "hairpin probe on microarray" in which hairpin probe is correctly immobilized on microarray surface is used for detection, in order to detect many points of MTHFR Gene C677T mutation, and is produced in batches. So far, a report concerning the application of hairpin probe for the detection of MTHFR Gene C677T mutation has not been found in literatures.

In recent years, some reports concerning gene or albumen detected with MB immobilized on the surface of a chip or gold nanoparticles have been found in literatures. However, fluorescence-signal and electrochemical signal accompany the majority of the methods. Combinative ferrocene-labeled MB immobilized on the surface of a chip with T4 DNA ligase has been reported in order to observe the changes in electrochemical signal (Xiaoying et al., 2010). The change of the fluorescent signal produced by MB immobilized on the surface of the gold nano-particle for detecting cystic fibrosis associated mutation was reported (Valerio et al., 2010). The effect of electrochemical signal triggered by hybridization of immobilized MB on nicotinamide adenine dinucleotide (NAD^+) was reported by Xiaoxiao et al. (2011). The method of a target-specific quantum dot-based MB had detected the change of fluorescence in situ hybridization (FISH) in *Escherichia coli* (Sheng-Mei et al., 2010). Modified fluorescent and quenching molecules, and changed fluorescence signal or changed electric signal caused by complex chemical reactions accompanied the majority of all, in order to be detected by Electrochemical Analyzer, fluorescence microscope, spectrophotometer, electrochemical workstation, etc. which are exceedingly complicated and require the use of expensive instruments (Jikui et al., 2009).

The study has been appropriately designed to use hairpin probe with enzymatic color and microarray (HPECM) without the modification of fluorescent and quenching molecules. Hairpin probe, immobilized on the surface of microarray, can produce the signal of visible light response induced by amplification effect of enzymatic color, and directly estimate hybridization reaction for detecting sing-base mismatch (Fig. 1). The absence of observation for fluorescent signal and detection of complicated electrochemical signal showed the advantages of HPECM as a sensitive, specific and simple method without the need for intricately expensive instruments. Moreover, HPECM were detected and compared in MTHFR Gene C677T mutation of subjects between CHD and control group.

2. Materials and methods

2.1. Chemicals and reagents

Hairpin probe and primer used in the study are shown in Table 1. Probe end was decorated with amino, and one end of primers was bedecked with Biotin. Blood cell genomic DNA extraction kit was supplied by Shanghai Baio.Co. The primers and probes were obtained from Shanghai Bio-Engineering Co. Strict NCBI BLAST verification was performed as the probes and primers were synthesized, ensuring the selectivity of amplification and hybridization. PCR Buffer, dNTP and Taq DNA polymerase were purchased from Promega Co. Glue reclaim kit was purchased from Tianwei period Co., China.

Table 1
Alignment of sequences used in experiment.

Name sequence	(5′-3′)
Primer 1	CTCACCTGGATGGGAAAGAT
Primer 2	Biotin-TGACTGTCATCCCTATTGGCAG
Probe 677C	NH_2-TTTTTTTTTCATGAAATCG**G**CTCCCGCACTTAGCATCATGA
Probe 677T	NH_2-TTTTTTTTTCATGAAATCG**A**CTCCCGCACTTAGCATCATGA

The stem sequences of the hairpin probes were shown underlined and in bold text. The design for "*G*" and "*A*" locus of probe target aimed at C → T of MTHFR gene C677. The 5′modifications were shown. "NH_2–", amino group; "Biotin–", biotin group.

Self-made lotion included three types of constituents. Lotion 1 included 100 mL of 20× SSC and 10 mL of 10% Triton X-100 (or 10% SDS) that was sterilized and added to 1000 mL of ddH_2O. Lotion 2 contained 5 mL of 20× SSC and 10 mL of Triton X-100 (or 10% SDS) that was sterilized and added to 1000 mL of ddH_2O. Finally lotion 3 included 0.1 mol of TBS buffer. Antibody solution and color liquid were streptavidin alkaline phosphatase antibody and NBT-BCIP color liquids, respectively.

Self-made hybridization solution buffer included 500 mL of deionized formamide (50% final concentration), 250 mL of 20× SSC (5× final concentration), 100 mL of 50× Denhardt (5× final concentration), 50 mL of 10% SDS (0.5% final concentration), 100 g of dextran sulfate (10% final concentration), and deionized water managed with inactivated enzyme of diethyl pyrocarbonate (DEPC) were added to 1000 mL. The liquid was added with denatured salmon sperm DNA (100 μg/mL final concentration) before application.

Except dextran sulfate, the majority of the component for pre-hybridization buffer solution was similar with self-made hybridization solution buffer. (1) Denhardt's solution (usually, 50× stock solution) included 10 g of saccharosan (Ficoll 400), 10 g for polyvinyl-pyrrolidone (PVP), 10 g of Bovine serum albumin (BSA), and deionized water managed with inactivated enzyme of diethyl pyrocarbonate (DEPC) were added to 1000 mL. (2) Liquor of 20× SSC included 175.3 g (20×) of sodium chloride, 88.2 g (20×) of trisodium citrate and deionized water managed with inactivated enzyme of diethyl pyrocarbonate (DEPC) were added to 1000 mL. In liquor of 20× SSC, 0.2× SSC was diluted to 10 times in turn. Sodium chloride, trisodium citrate, deionized formamide, acetone, glutaraldehyde, sodium borohydride, Triton X-100, and TBS buffer solution were obtained from Shanghai Chemistry Reagent Co. Phosphate buffered saline (PBS), saccharosan, Poly Vingl Pyrrolidone (PVP), Bovine Serum Albumin (BSA), DEPC, streptavidin alkaline phosphatase antibody, NBT-BCIP color liquid, SDS, Phosphate buffered saline (PBS) and 32-aminopropyl trimethoxysilane were purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Instrument and apparatus

Fully-automatic hybridization instrument (e-Hyb) and BE-2.0 biochip reading device were supplied from Shanghai Baio.Co. A GeneAmp PCR System 2400 (ABI, USA). UV spectrophotometer (Smart Spec™ 3000 Spectrophotometer. Bio-Rad, CA, USA). A gel documentation system (Vilber Lourmat, France).

2.3. The extraction of genome DNA and PCR amplification

According to the manufacturer's procedure, genome DNA were extracted from nucleated cells that were extracted from anticoagulation of peripheral blood in patients and the PCR amplification was performed with a Thermal Cycler 2400. The PCR reaction mixture (50 μL) contained genomic DNA extract (2 μL), 0.01 mmol/L of each dNTP, $MgCl_2$ (0.2 mmol/L), Taq DNA polymerase (0.1 U), 0.2 μM of each primer, 10× PCR Buffer (5 μL) and distilled water (35 μL). Amplification was performed with the following cycling