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Detection of SNPs of T2DM susceptibility genes by a ligase detection reaction–fluorescent nanosphere technique

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ABSTRACT

Objective: To establish a high throughput, low cost, and simple nanotechnology-based method for the detection of single nucleotide polymorphism (SNP) loci in type 2 diabetes mellitus (T2DM).
Methods: Multiplex ligase detection reaction (LDR) amplification was performed using fluorescently labeled magnetic nanosphere-bound upstream LDR probes and downstream probes labeled with a unique fluorescent group for each SNP locus. The amplified LDR products were separated by magnetic nanospheres and then scanned by fluorescence spectroscopy. Four SNP loci associated with T2DM were detected, including the rs13866634 locus in SLC30A8, rs10811661in CDKN2A/2B, rs1111875 in the HHEX gene, and rs7903146 in the TCF7L2 gene. The SNP genotype was also determined by DNA sequencing as a control.
Results: The SNP genotypes of the four gene loci determined by the nanosphere-based multiplex LDR method were consistent with the DNA sequencing results. The accuracy rate was 100%.
Conclusion: A method based on multiplex PCR and LDR was established for simultaneous detection of four SNP loci of T2DM susceptibility genes.

Introduction

Type 2 diabetes mellitus (T2DM), as a chronic metabolic disease, causes serious mental stress for patients and represents an enormous economic burden for healthcare [1,2]. T2DM is the consequence of multiple genetic factors and various environmental factors [1,2]. Recently, the development of single nucleotide polymorphism (SNP) detection technology, especially its application in genome wide association studies, has greatly promoted efforts to identify T2DM susceptibility genes [3–5].

Many assay methods have been developed for SNP genotyping, including sequencing, restriction fragment length polymorphism, and DNA microarray, etc. [6,7]. Ligation detection reaction (LDR) is a SNP genotyping method that is low cost, simple fast, highly accurate, versatile, and suitable for multiplex assay [8,9]. The principle behind LDR is allele-specific ligation achieved by DNA ligase that is thermostable and of high fidelity [10]. LDR uses two oligonucleotide probes or primers: an allele-specific upstream probe with a 3′ terminal nucleotide as the SNP site and a downstream common probe. The ligation only occurs when the upstream probe perfectly matches the template; thus, the genotype at a site of interest is determined based on the presence or absence of the ligated product [11]. The ligation can occur repeatedly by thermocycling, and thus, the ligation product can be sensitively detected. Because the ligated product is about double the size of the unligated probes, earlier techniques detected the LDR product using high-resolution electrophoresis, which is relatively expensive and complex [11]. The aim of this study was to establish a method for the detection of SNP loci in T2DM that offers advantages of low cost, simple operation, and high throughput, utilizing multiplex LDR in combination with magnetic nanospheres and dye-doped nanospheres.

Materials and methods

Study population

A total of 52 T2DM patients and 48 healthy volunteers were enrolled in this study. Each participant signed an informed consent form. The T2DM group included 30 males and 22 females with an average age

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of 50.04 ± 8.21 years, and the healthy group included 28 males and 20 females with an average age of 48.74 ± 7.60 years. All T2DM patients met the following inclusion criteria: 1. satisfied the 1999 WHO diabetes diagnosis (fasting blood glucose ≥7.0 mmol/L and/or postprandial 2-h blood glucose ≥11.1 mmol/L) and classification criteria; 2. Were without ketosis and other stresses for 6 months; and 3. had no liver or kidney dysfunction. All study participants were from the Han population in Changchun, Jilin Province, China, and none were related.

Materials

The materials and reagents used in this study included: $FeCl_3 \cdot 6\ H_2O$ (purity > 99%, ACROS, Belgium), $FeCl_2 \cdot 4\ H_2O$ (purity > 99%, ACROS, Belgium), ethyl orthosilicate (TEOS, analytical grade, vacuum distilled before use, Beijing Chemical Plant), ammonia ($NH_3{\cdot}H_2O$, 25%, analytical grade, Beijing Chemical Plant), cyclohexane (analytical grade, Beijing Chemical Plant), ethanol (analytical pure, Beijing Chemical Plant), oleic acid (OA, 90%, Shanghai Reagent Co.), Tween-80 (analytical grade, without any purification before use, Shanghai Reagent Co.), cetyltrimethylammonium bromide (CTAB, Sinopharm Group Chemical Reagent Co., Ltd.), sodium dodecyl sulfate (SDS, Sinopharm Group Chemical Reagent Co., Ltd.), acrylic acid (AA, Sinopharm Group Chemical Reagent Co., Ltd.), potassium persulfate (KPS, Sinopharm Group Chemical Reagent Co., Ltd.), sodium bicarbonate (NaHCO3, Sinopharm Group Chemical Reagent Co., Ltd.), isothiocyanide fluorescein (FITC, Aldrich, USA), 3-aminopropyltriethoxysilane (APS, Aldrich, USA), (1,10-trihydrin) ruthenium (Ru (phen) $3Cl_2$, Aldrich, USA), poly (diallyldimethylammoniumchloride) (PDADMAC, Mw = 100000 to 200000 g/mol, 20% water solution, Aldrich, USA), 8-hydroxypyene-1, 3, 6-sulfonic acid trisodium salt (HPTS, Aldrich, USA), 7-hydroxycoumarin-4-acetic acid (HCA, Aldrich, USA).

EDC (Beijing Tripod Biotechnology Co., China), NHS (Beijing Tripod Biotechnology Co.), streptavidin (Beijing Dingguo Biotechnology Company, China), biotin (Beijing Tripod Biotechnology Co.), DNA extraction kit (Tiangen Biotech, Beijing, China), Taq DNA polymerase (Takara, Japan), and dNTP (Takara). The PCR primers and LDR probes were synthesized by Shanghai Shenggong Bioengineering Co. (Shanghai, China).

Extraction of blood genomic DNA

Two milliliters of blood were drawn for each participant from the elbow vein after fasting for more than 8 h, and sodium citrate was added for anticoagulation. The genomic DNA was prepared from the blood samples using a DNA extraction kit, and the extracted DNA sample was stored at -80 ˚C.

Preparation of magnetic nanospheres (MNS) and fluorescent dye-doped nanospheres and their conjugation with streptavidin

Preparation of monodisperse magnetic SiO_2-coated nanospheres

A co-precipitation method was used to prepare Fe_3O_4 magnetic nanospheres. In a 250-ml three-neck flask, $FeCl_2$ (5.6 mM) and $FeCl_3$ (11.2 mM) were added, followed by addition of 150 ml deoxidized ultrapure water and stirring at 500 rpm and 50 ˚C. Then 12.5 ml $NH_3{\cdot}H_2O$ was quickly added into the above mixture, and the reaction were carried out in a 50 ˚C water bath for 30 min. After the reaction, the precipitated Fe_3O_4 nanospheres were attracted to the flask wall with a magnet, and the supernatant was discarded. The nanospheres were washed with deoxygenated water three times.

The precipitated Fe_3O_4 nanospheres were ultrasonically dispersed in 120 ml deoxygenated water and heated to 70 ˚C under bubbling nitrogen gas. Then 1.5 ml OA was added, and the mixture was stirred in a water bath at 70 ˚C for 1.5 h. The magnetic nanospheres were precipitated and washed three times with ethanol to obtain OA-modified Fe_3O_4 nanospheres.

Next 0.11 g OA-modified Fe_3O_4 magnetic nanospheres were dispersed in 1.4 ml cyclohexane to form an oil phase; 0.073 g of sodium dodecyl sulfate was dissolved in 35 ml water to form an aqueous phase. The two were mixed and ultrasonically dispersed for 10 min to form a fine emulsion. The emulsion was heated to 55 ˚C under bubbling nitrogen and stirring for 5 h to obtain Fe_3O_4 magnetic aggregates. Then 0.05 g of magnetic aggregates was added to 5 ml solution containing 3.5 mg Tween-80 and dispersed by ultrasonication for 10 min to obtain Tween-80 modified Fe_3O_4 aggregates for SiO_2 coating. The size and magnetic content of the final nanospheres were controlled by adjusting the size of the aggregates and the thickness of the silica shell.

Preparation of FITC-doped SiO_2 fluorescent nanospheres

The stock SiO_2 nanospheres were prepared using Stöber process as previously described [18,19], by hydrolytic and condensing reaction of TEOS in ethanol, with ammonia as the catalyst. Then 100 ml ethanol, 2 ml water, and 4 ml aqueous ammonia were added to a three-neck flask containing the silica nanospheres and reacted for 12 h at 40 ˚C in a water bath under stirring at 150 rpm; 80 μl APS was then added to continue the reaction for 8 h. The products were separated by centrifugation at 12000 rpm, and 43 nm SiO_2 nanospheres were obtained. The nanospheres were washed three times with ethanol and redispersed in 100 ml ethanol. Then 1.2 mg of FITC (dissolved in 1 ml ethanol) was added to the above nanospheres and reacted for 12 h in a water bath at 40 ˚C under continuous mechanical stirring. The SiO_2-FITC nanospheres were purified by centrifugation, washed three times with ethanol, and dispersed in 100 ml ethanol.

Preparation of Ru (phen) 3-doped SiO_2 fluorescent nanospheres

First 2.4 ml TEOS, 30 ml ethanol, 0.93 M ammonia, and 2.9 M ultrapure water were mixed in a 100-ml three-necked flask and placed in a water bath at 25 ˚C with stirring at 150 rpm. After 3 h of reaction, 3 ml of Ru (phen) 32 + ethanol solution at 0.1 mg/ml was added and the reaction was continued for 12 h. The final product was centrifuged (12000 rpm, 12 min), washed three times with ethanol, and redispersed in 30 ml ethanol.

Preparation of HCA-doped SiO_2 fluorescent nanospheres

As above, 2.4 ml TEOS, 30 ml ethanol, 0.93 M ammonia, and 2.9 M ultrapure water were mixed in a 100-ml three-necked flask and placed in a water bath at 25 ˚C with stirring at 150 rpm. After 3 h of reaction, 3 ml HCA ethanol solution (0.1 mg/ml) was added and the reaction was continued for 12 h. The final product was centrifuged (12000 rpm, 12 min), washed three times with ethanol, and redispersed in 30 ml ethanol.

Preparation of HPTS-doped SiO_2 fluorescent nanospheres

First 10 μl of PDADMAC solution (10 mg/ml) and 300 μl of HPTS aqueous solution (2×10^{-3} M) was added to an ethanol/water (8:1) mixture, for a total volume of 5 ml. The reaction was carried out under magnetic stirring for 20 min. Then 8.4 ml ethanol, 0.1 ml aqueous ammonia, 815 ml water, and 300 μl TEOS were mixed to react for 25 min at 25 ˚C. The above two mixtures were mixed and stirred for