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Methods paper

A potentially functional polymorphism in the regulatory region of let-7a-2 is associated with an increased risk for diabetic nephropathy

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ABSTRACT

Diabetic nephropathy (DN) is a major diabetic complication. However, the initiating molecular events triggering DN are unknown. MicroRNAs (miRNAs) have recently been identified as regulators that modulate the target gene expression and are involved in DN. However, the evidence of the mechanism is still insufficient in human samples. In this study, microRNA microarray assay was used to study gene differential expression profiles in DN and diabetes mellitus (DM) patients. One of the specific differentially expressed microRNAs, let-7a, was down-expressed in DN. Additionally, the expression of let-7a was also decreased in DN by real-time RT PCR in the patients' samples. Moreover, single nucleotide polymorphism (SNP) analysis was used to evaluate the relationship between three SNPs in the regulatory region of let-7a-2 gene and the risk of DN in the Chinese Han population by means of PCR-restriction fragment length polymorphism (RFLP-PCR). Also, the genotype and allele frequencies of let-7a-2 polymorphism were tested in 274 individuals, including 108 DN, 104 DM patients and 62 health control individuals (CON). It was found that a variant rs1143770 and the distributions of CT/TT genotypes were significantly different in three groups, and the CT + TT genotypes frequencies were significantly higher in DN and DM groups than that in CON group. In conclusion, let-7a-2 might participate in the regulation of the occurrence of DN, and a potential variant rs1143770 was significantly associated with the increased risk for DN.

1. Introduction

Diabetes mellitus (DM) is a multifactor disorder that results from the interaction of genetic and environmental factors. Diabetic nephropathy (DN), as a most common chronic microvascular implication of diabetes, has become the main factor results of the chronic renal failure. However, the mechanism of DN is not fully understood. MicroRNA(miRNA) is a class of small non-coding RNAs which negatively regulate gene expression by inhibiting translation of target mRNAs. Recent studies have revealed that miRNAs play important roles in progression of DN diseases (Alvarez and DiStefano, 2012).Our previous study showed that miR-21 and miR-451 play a potential role in early DN in mouse. miR-21 and miR-451 expression were down-regulated in response to early DN in vitro and in vivo (Zhang et al., 2009; Zhang et al., 2012). However, the research of miRNAs in human DN is less by far. It was reported that miR-377 (Wang et al., 2008), miR-21 (Dey et al., 2011),miR-29a (Du et al., 2010),miR-192 (Krupa et al., 2010),miR-200b/c and miR-205 (Gregory et al., 2008) have a close relation with human DN disease.

Lawrie et al. (2008), firstly presented his conception of circulating miRNAs, which are present in plasma and serum with tissue specificity and high stability that is protected from endogenous RNase activity (Mitchell et al., 2008). Since then the circulating miRNA as a novel biomarker has been studied in a variety of diseases, including acute myocardial infarction, hypertension, acute coronary syndrome, heart failure and type 2 diabetes (Zampetaki et al., 2012). However, it has no reports about circulating miRNAs in DN up to now. In this study, we identified 22 DN-related circulating miRNAs in type 2 DM with or without DN patients by miRNA array. Also, let-7a is the one.

The let-7 family of miRNAs is encoded by 12 genes in human genome; all let-7 family members are believed to exert similar functions because they share a common seed region (nucleotides 2–8). They have been widely studied as a tumor suppressor by targeting RAS, HMGA2 and k-ras/c-Myc (He et al., 2010; Johnson et al., 2005; Watanabe et al.,

Abbreviations: DM, diabetes mellitus; DN, diabetes mellitus with diabetic nephropathy; SNP, single nucleotide polymorphism; PCR-RFLP, PCR-restriction fragment length polymorphism; LD, linkage disequilibrium; MAF, minor allele frequency; UARE, urinary albumin excretion rate; HbA1c, hemoglobin a1c; CREB, cyclic adenosine monophosphate response element-binding protein.

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2009). However, in recent study it was supported that let-7 family was a potential target for type 2 DM to regulate glucose metabolism and insulin synthesis secretion (Kumar et al., 2012). Also, the whole body and pancreas-specific over-expression of let-7 in mice resulted in impaired glucose tolerance and reduced glucose-induced insulin secretion (Frost and Olson, 2011). Furthermore, over-expressing let-7 in let-7a/d/f/g transgenic mice generated growth retardation and impaired glucose tolerance by lin28-mediated pathway (Zhu et al., 2011). Therefore, let-7a was established the potential candidate microRNA involving in the susceptibility to DN.

Additionally, let-7a-2 gene regulatory region was evaluated by single nucleotide polymorphism (SNP) analysis in this study. Three tag SNPs (rs1143770, rs547008 and rs562052) with a change of transcription factors were selected as candidate SNPs by bioinformatics prediction. Moreover, we found that the genotype frequency of rs1143770 polymorphism was remarkably different in type 2 DM, DN, and health control cases. All data indicated that let-7a may participate in DN, and a potential variant rs1143770 in let-7a-2 regulatory region may significantly associate with occurrence of DN.

2. Subjects and methods

2.1. Experimental subjects

A total of 212 patients were enrolled in this study with type 2 diabetes mellitus who were hospitalized in the Department of Endocrinology and Department of Nephrology at the first affiliated hospital of Chongqing Medical University from 2010 to 2012, including 104 type 2 DM patients without nephropathy (urinary albumin excretion rate (UAER) <20 μg/min) (DM group), and 108 type 2 DM patients with DN (UARE: 20–200 μg/min) (DN group). All patients with DM and DN had more than five-year history of diabetes, and the UARE in urine test was measured twice during 6 months. 62 control adult individuals who were healthy and had no evidence of any chronic illness, excluding renal, hepatic, thyroid or any other metabolic diseases (CON group). The patients and control individuals were all from the Han ethnic group aged 35–65 years. The early stages of DN were determined according to the stage of diabetic nephropathy diagnostic reference as noted by Mogensen et al. (1983), the emergence of urinary albumin is a sign of this period (20 μg/min ≤ UARE ≤ 200 μg/min), and routine urine test protein remains negative. The study had been approved by the hospital ethics review committee; all subjects were informed and agreed to provide blood samples. Each eligible participant was interviewed to obtain data regarding age, sex, ethnicity, duration of diabetes, glycosylated hemoglobin and UARE status.

2.2. Total RNA isolation

The 7 ml venous blood on empty stomach in the early morning was collected and immediately transferred into PreAnalytiX PAXgene™ blood RNA tubes (Qiagen, Valencia, CA, USA), and total RNA was extracted by Trizol reagent (Invitrogen) according to the manufacturer's instructions.

2.3. MicroRNA microarray assay

Microarray assay was performed by LC sciences (Houston, TX, USA). μParaflo™ micro-fluidic chip contains the probe sequence of Version 10.0 microRNA database. Small RNAs (<300 nt) were isolated when 2 to 5 μg total RNA sample was size-fractionated using a YM-100 Microcon centrifugal filter (from Millipore), and the ends of the small RNAs were extended with a poly(A) tail for later fluorescent dye staining. Hybridization was performed overnight on a μParaflo™ microfluidic chip using a micro-circulation pump (Gao et al., 2004). The hybridization melting temperatures were balanced by chemical modifications of the detection probes. Hybridization used 100 μl 6×SSPE buffer (0.90 M NaCl, 60 mM Na2HPO4, 6 mM EDTA, pH 6.8). After hybridization, the tag-specific Cy3 and Cy5 dyes as fluorescence labeling were used for detecting the miRNAs. Data were analyzed by first subtracting the background and then normalizing the signals using a LOWESS filter (locally-weighted regression) (Bolstad et al., 2003). *P*-values of the *t*-test were performed to identify the different miRNA expression. $P < 0.05$ was considered as having significant difference between case and control.

2.4. Real time reverse transcription (RT)-PCR

TaqMan miRNA assays (Applied Biosystems, California, USA) were used for semiquantitative determination of the expression of miRNAs according to the manufacturer's instructions. Briefly, 10 ng of total RNA was reverse transcribed using miRNA-specific stem-loop RT primers, MultiScribe reverse transcriptase, RT buffer, dNTPs, and RNase inhibitor (Applied Biosystems) in the GeneAmp 9700 PCR system (Applied Biosystems) under the following conditions: 16 °C for 30 min, 42 °C for 30 min, 85 °C for 5 min. Real-time PCR was performed on the resulting complementary DNA (cDNA) using miRNA-specific TaqMan primers and TaqMan Universal PCR Master Mix in a 7500 real-time PCR system (Applied Biosystems) as the following conditions: 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The relative expression was normalized to the expression of snoRNA202 (Applied Biosystems). Relative fold changes of gene expression were calculated by the ΔΔCT method and the values are expressed as $2^{-\Delta\Delta Ct}$. Real-time RT-PCR was performed on the matched 8 cases of DN, 7 cases of DM and 9 normal controls.

2.5. Candidate SNPs selection

The upstream of the 5′ fragment 2.8 kb of the let-7a-2 gene including the transcriptional start site and the regulatory activity had been validated in other research (Guan et al., 2011). In our study, let-7a-2 gene regulatory region has been extracted based on the information from UCSC Genome browser (Fujita et al., 2011)(http://genome.ucsc.edu/index.html). All related information from miRNA SNP (http://www.bioguo.org/miRNASNP/) and NCBI online database should be for reference. The tag SNPs of let-7a gene regulatory region (<2.8 kb) were screened by Hapmap phase II database and Haploview4.1 software (Gibbs et al., 2003). TFSEARCHver1.3 software was used to predict transcription factor binding sites. The candidate SNPs were selected by the values, including in Han population, minor allele frequency (MAF) >0.05 and transcription factors.

2.6. Genetic analysis

DNA was extracted and purified from whole blood (EDTA) by using the TIANamp Blood DNA Kit (TIANGEN, Beijing, China), and stored at −20 °C for later use. The genotypes of rs1143770, rs547008 and rs562052 were analyzed by RFLP-PCR. PCR primers were as follows, (1) rs1143770: F 5′-AAAACCTTGGCTTGAGATTACC-3′ and R 5′-TTCAGTTTTACCAGAGG AACATTTA-3′; (2) rs547008: F 5′-GC GGTCCCAAAAGGGTCAGTAGTCAACTCTGGAAAAAGGTGC-3′ and R 5′-GCGGTCCCAAAAGGGTCAGTAGATTAAGAAATACCGCAAAAGC-3′; (3) rs562052: F 5′-ACCTCATCAAAGCATAAGGAAAT-3′ and R 5′-TT CCTGATACTTCT ACTGGACTGAT-3′. PCR reactions were run in a 15 μl final volume containing 10× buffer solution 1 μl,dNTP mix (2.5 mmol/l) 0.3 μl, $Mgcl_2$ 1.2 μl, Taq polymerase(5 U/u) 0.1 μl, PCR primers (50pmol/l) 0.2 μl and genomic DNA(20 ng/μl)0.5 μl. PCR program was initiated by 5 min at 95 °C, followed by 20 cycles of 30 s at 95 °C, 45 s of annealing at 68 °C and 30 s at 72 °C, the other 20 cycles of 30 s at 95 °C, 30 s at 58 °C, and 40 s at 72 °C, and a final elongation step of 6 min at 72 °C. PCR products of rs1143770, rs547008 and rs562052 polymorphisms digested the enzymes Hpy8I, Alw44I and TspRI (MBI Fermentas) respectively.