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MicroRNA-499 Rs3746444 polymorphism and biliary atresia

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ABSTRACT

Background: Single nucleotide polymorphisms within microRNAs are known to affect the risk in development and prognosis of many diseases. This study was designed to investigate whether polymorphism of microRNA-499 (miR-499, rs3746444 A>G) is associated with risk to biliary atresia (BA).
Methods: A hospital-based cases–control study was performed on a total of 507 Han Chinese (207 BA cases and 300 ethnically-matched healthy controls without any evidence of liver diseases) so as to analyze the association between miR-499 rs3746444 polymorphism and BA risk as well as liver function remission (LFR) after liver transplantation.
Results: A significant higher frequency of the rs3746444 G alleles was found in the BA cases than the control group (odd ratio, 1.55, 95% confidence intervals [CIs], 1.15–2.10). This polymorphism was also observed to correlate with some clinic-pathological features of BA cases such as liver inflammatory. Further research found both higher levels of IL-6 ($P < 0.05$) and TNF-α ($P < 0.05$) in removed liver as well as in serum. What is more, the miR-499 rs3746444 polymorphism significantly affected the status of LFR (hazard ratio, 1.37; 95% CI, 1.08–1.83).
Conclusions: MiR-499 (rs3746444) gene polymorphisms may be genetic determinants for increased risk of BA and prolonged recovery of BA patients after liver transplantation in Han Chinese.

1. Introduction

Biliary atresia (BA) is a rare neonatal cholestatic disorder caused by obstruction of extra- and intra-hepatic bile ducts. If untreated, progressive liver cirrhosis will lead to death within 2 years. As contemporary strategy towards BA (including Kasai surgery and liver transplant) is relatively successful, the exact etiology of BA remains obscure, since none of the existing hypothesis, including intrauterine viral infection, maternal immune cell infiltration, genetic mutation, etc. can perfectly explain the pathogenesis.

In the past decades, the microRNAs (miRNAs), a type of small noncoding RNAs that regulate gene expression at post-transcriptional levels by facilitating degradation and/or inhibiting translation of target mRNAs, have been reported to play an important role in the development of biliary system and liver function [1–5]. Previous studies have suggested that rs3746444 polymorphism might affect susceptibility to hepatocellular carcinoma [6]. It was also believed to play an important role in host immune reaction in the development of rheumatoid arthritis and osteotitis [7]. Zhi et al. concluded that coronary artery disease was associated significantly with miR-499 polymorphisms in Chinese [8]. The targets of miR-499 include several immunal cytokines which has been proved to associate with BA [9–12], thus making this miRNA polymorphism a suspect in our research. In this study, we investigated whether a genetic polymorphism in the microRNA-499 (miR-499), rs3746444 A>G, correlated with BA risk and liver function remission (LFR) after liver transplantation.

2. Materials and methods

2.1. Study subjects

The protocol was approved by the Research Ethics Committee of the Renji Hospital, School of Medicine, Shanghai Jiaotong University. This hospital-based case–control study recruited 207 BA

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cases referred to Ren Ji Hospital, Shanghai Jiao Tong University School of Medicine during the period from January 2006 through July 2014. All cases [101 males, 106 females; age range, 0–6 yrs] received routine laboratory test including blood type testifying, hematology analysis, blood coagulation test, liver and kidney function test and CMV or EBV antibody screening on their admission day. Pediatric End-Stage Liver Disease (PELD) score was calculated based on bilirubin level, international ratio index, albumin level, age and condition of nutrition and development. Among these 207 recruits, 203 received final liver transplant treatment (LT), including living donors (82.0%) and dead cardiac donors (18.0%). At the same time, 300 gender and age (±5 months) matched children [177 males, 123 females; age range, 0–6 yrs] without any evidence of liver diseases were set for controls. Their diagnosis varied among appenditis, hernia, sleep apnea hypopnea syndrome, tethered cord syndrome, fracture and trauma, skeleton deformity, cleft palate, hydrocele testis, hypospadia, enorchia, and hydronephrosis. In this study, the response rate for the cases has been about 100%. After informed consent was obtained, 1 mL of peripheral blood samples were collected from all subjects for genotyping analysis, and surgically removed liver samples of these BA cases pathological scoring analysis.

2.2. Genotyping assay

Laboratory personnel were blinded to case and control status. Genomic DNA was detracted from the leukocytes of the peripheral blood samples using the DNA extraction kit (C.W. Biotech, Beijing, China) according to manufacturer's instructions. The miR-499 rs3746444 genotypes were analyzed using the TaqMan-PCR on iCycler iQ real-time PCR detection system (CFX96, Bio-Rad Laboratories Inc., Hercules, CA). Primer and probe sets were obtained from the Applied Biosystems (ABI), Carlsbad, CA. Each PCR was carried out in a total volume of 5 μL containing 1 × TaqMAN Universal Master Mix II (cat#4440041, ABI), 1 × TaqMan probe and primer Mix, and 50–100 ng of genomic DNA. The PCR program had an initial denaturation step of 2 min at 95 °C followed by 50 cycles of 10 s at 95 °C and 1 min at 60 °C. For quality control, controls were included in each run, and repeated genotyping and sequencing of a random 10% subset yielded 100% identical genotypes.

2.3. Pathology scoring analysis

Among those 203 transplantation-received patients, 199 had histology slices of removed livers sent for Masson staining. The inflammatory and fibrotic grade was evaluated by two independent pathologists according to ISHAK histological scoring system [13].

2.4. The IL-6 and TNF-α expression assay

The level of IL-6 and TNF-α expression in serum was evaluated by corresponding enzyme-linked immunosorbent assay (ELISA) kits purchased from Neobioscience Biotechnology Company (Beijing, PR China) following the instructions of the manufacturer. The absorbance was read using ELISA reader (SpectraMax) at 450 nm. The detection ranges for TNF-α were 15.62–1000 pg/mL and IL-6 was 7.8–500 pg/mL. All the samples were thawed only once and assayed in duplicate.

The expression and localization in liver tissue was determined by immunohistochemical (IHC) staining. Slides of liver samples were paraffin-embedded and cut at a thickness of 4 μM. After deparaffined, the slides underwent antigen retrieval process in 10 mM sodium citrate buffer. Then, the staining was performed with IHC kits provided by Maixin Biotech, Fuzhou, China following manufacturer's instructions. Primary rabbit-origin antibodies were purchased from Proteintech Group, Inc., Chicago, USA and diluted according to recommended concentration. Positive IHC staining was observed as brown stains under a light microscope. Then each slide was given a semiquantitative score with regard to the intensity of the dye color and the number of positive cells. The intensity of the dye color was graded as 0 (no color), 1 (light yellow), 2 (light brown), or 3 (brown), and the number of positive cells was graded as 0 (<5%), 1 (5–25%), 2 (25–50%), 3 (51–75%),or 4 (>75%). The final score was the multiplication of the two grades.

2.5. The LFR evaluation

For survival analysis, these BA cases receiving LT treatment ($n = 203$) were followed and underwent serial monitoring of liver function including the serum levels of ALT, AST, and TB, and the dose and serum level of FK506, an immunologic suppressing agent tacrolimus, every 24 h for the first week and every one week thereafter for detection of the LFR. In the present study, the liver function remission point (LFRP) was defined as following status: ALT ≤ 40 IU/L, AST ≤ 70 IU/L, and TB ≤ 35 mol/L for more than 3 days; Whereas the liver function remission time (LFRT) was defined as the date of LT treatment to the date of LFRP.

2.6. Statistical analysis

To assess differences between groups, demographic characteristics, clinic-pathological data, and miR-499 rs3746444 genotypes were compared using Student t test, the Mann–Whitney U test, the Kruskal–Wallis test, or the χ^2 test. Adjusted odds ratios (ORs) with 95% confidence intervals (95% CIs) were calculated by non-conditional logistic regression analysis. Kaplan–Meier survival analysis (with the Log-Rank test) was used to evaluate the association between miR-499 rs3746444 polymorphism and the LFR status of BA cases. In this analysis model, LFRP was treated as events, and median LFRT was used to evaluate the effects of this polymorphism on the LFR. All statistical tests were two-sided, and a probability level of $P < 0.05$ was considered to be statistically significant. Data analysis was done using SPSS 16.0 software (SPSS, USA).

3. Results

3.1. The miR-499 rs3746444 polymorphism increased BA risk

Table 1 summarizes the genotypic and allele distribution of miR-499 rs3746444 polymorphism among BA cases and controls. The genotypic and allele distribution of this polymorphism in the controls was in Hardy–Weinberg equilibrium ($P > 0.05$). The frequencies of the rs61992671 G allele were higher in cases (25.8%) than controls (18.2%). For allele analysis, those carrying G alleles of miR-499 (rs61992671-AG) featured an increasing BA risk (OR = 1.55, 95% CI, 1.15–2.10), compared with those not. We set dominant model of GG + AG vs. AA, recessive model of GG vs. AA + AG, codominant model of AG vs. AA + GG and additive model of AA vs. GG. Significant results were found in recessive model ($P = 0.002$, OR = 2.78, CI: 1.46–5.28) and additive model ($P = 0.002$, OR = 2.82, CI: 1.47–5.41), with the risk of BA increased to almost 3 times. It seemed that a loss of A allele gave risk to BA susceptibility.

3.2. The miR-412 rs61992671 polymorphism correlated with the clinic-pathological features of BA

We next analyzed the association between the miR-499 rs3746444 polymorphism and the clinic-pathological features of BA cases, and found this polymorphism was related with the increasing number of inflammatory scores (Fig. 1, upper graph), Represent graphs exhibited this difference (Fig. 1, below pictures). However, significant differences in the distributions of the