A role of pre-mir-10a coding region variant in host susceptibility to coxsackie virus-induced myocarditis

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Abstract. – OBJECTIVE: Acute viral myocarditis (VM) is an important cause of sudden cardiac death and heart failure in healthy young person. Direct virus-mediated injury and secondary immune reactions, including inflammatory and autoimmune responses, have been reported both in animal models and in humans. Recently, genetic variation has been confirmed related to myocarditis process and susceptibility to VM. In this study, we scanned 339bp of pri-miR-10a coding region in CVB3 VM patients, want to found genetic relations between miR-10a and VM susceptibility.

PATIENTS AND METHODS: The secondary structure of two genotype 220 bp pri-miR-10a sequences was predicted using RNAfold web server. In vitro biological functional study concluded dual luciferase assay and Western blotting.

RESULTS: We found the rare allele T of rs3809783 was accumulated in VM patients and related to VM significantly. Subsequently, we confirmed that ITCH, a NK-kB signaling suppressor, is a direct target of miR-10a. In vitro biological functional study indicated that this site variation reduced mature miR-10a expression and induced a down-regulated cytokine secretion in the cell culture supernatant.

CONCLUSIONS: The results suggest that the rare allele T in pri-miR-10a coding region should be involved in the CVB3 caused VM pathogenesis through weakening host anti-virus immune response. This site may be used for clinical genetic evaluation for VM susceptibility.

Key Words: Viral myocarditis, microRNA, Coxsackievirus, ITCH, NF-kB.

Introduction

Myocarditis is considered as a non-familial form of heart muscle disease1. It is defined as an inflammation of the heart muscle, identified by clinical or histopathologic criteria2-4. Virus infection is a major cause of myocarditis and Coxsackievirus B3 (CVB3), a kind of enterovirus, is believed to be the most common causative agent in human viral myocarditis. Viral myocarditis (VM) affects 5-20% of the human population who can be fatal in infants as well as children5,6. The pathogenesis of VM is based on an adverse immune response evoked by infection of the cardiac muscle by cardiotropic viruses, which leads to viral elimination as well as cardiac myocyte destruction, reparative fibrosis, and heart failure. The lack of effective therapies to treat myocarditis mandates a better understanding of the basic molecular mechanisms that govern the adequate and autodestructive inflammatory signaling pathways within the immune system7.

MicroRNAs (miRNAs) are short, noncoding RNA sequences that regulate gene expression at the posttranscriptional level by targeting the 3’-untranslated region of mRNA sequences. Bioinformatical study7 indicated that more than 60% of human genes may be regulated by miRNAs. Loss-of-function studies in mice firmly established that miRNAs control a variety of cellular processes essential to the heart8. miRNAs play very important roles in maintaining normal human body physiology conditions, and abnormal miRNA expressions have been found related to many human diseases spanning from psychiatric disorders to malignant cancers9-12.

Recently, genetic variation has been considered related to myocarditis process and susceptibility to VM. Wiltshire et al13,14 analyzed genes expression using microarray and found genetic variants of Tnni3k, Fpgt, or H28 may control susceptibility to viral myocarditis. Gorbea et al14 screened TLR3 coding region in VM patients and
found two nucleotides variation are associated with host susceptibility to entero viral myocarditis and dilated cardiomypathy. MiR-10a gene is located in chromosome 17, and its function was first described\(^1\) as a tumor suppressor gene in myeloproliferative neoplasm. Recently\(^2\), miR-10a\(^-\) has been found can up-regulates coxsackievirus B3 biosynthesis and might be involved in the CVB3 cardiac pathogenesis. In this study, we scanned 220bp of pri-miR-10a coding region and found polymorphism site rs3809783 is recruited in 77 VM patients want to understand whether there are some relations between miR-10a and VM susceptibility.

**Patients and Methods**

**Patient Enrollment**

77 patients with CVB3 infection caused myocarditis were enrolled at the Affiliated Longyan First Hospital of Fujian Medical University. Blood samples were obtained from all patients after informed consent. The process of material collection was in accordance with the Declaration of Helsinki and the Ethical Committee of The Affiliated Longyan First Hospital of Fujian Medical University. Controls comprised 110 patients without inflammatory cardiac disease and a population control consists of 248 Chinese-Han people.

**DNA Collection and Genotyping**

DNA from blood samples were extracted by using TIANamp Blood DNA Kit (TIANGEN, Beijing, China). DNA specimens were amplified by using standard PCR protocols. The PCR products were sequenced in forward direction with the ABI 3730xl sequencing platform. The sequencing results were analyzed by using DNA-MAN and Chromas Lite software.

**Secondary Structure Prediction**

The secondary structure of two genotype 220 bp pri-miR-10a sequences was predicted using RNAfold web server (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi).

**MiR-10a Expression Vectors**

To construct miR-10a expression vectors, fragments (339nt) corresponding to pre-miR-10a and its flanking regions (previously determined to have the two genotypes) were amplified from cDNA and cloned into the pcDNA3.1 vector (Invitrogen, Carlsbad, CA, USA). The sequences of these two genotypes vectors were confirmed by direct sequencing.

**Cell Culture**

HEK293T and Hela cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS, Gibco, Gran Island, NY, USA), 100 IU/ml penicillin and 10 mg/mL streptomycin. All cells were maintained at 37°C under an atmosphere of 5% CO₂.

**Real-time Reverse Transcriptase Quantitative PCR**

Quantitative RT-PCR analysis was used to determine the relative expression of miR-10a. Total RNA was extracted from clinical or cell samples, using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. The expression levels of miR-10a-5p and miR-10a-3p were detected using TaqMan miRNA RT-Real Time PCR. Single-stranded cDNA was synthesized by using TaqMan MicroRNA Reverse Transcriptation Kit (Applied Biosystems, Foster City, CA, USA) and then amplified by using TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA) together with miRNA-specific TaqMan MGB probes (Applied Biosystems, Foster City, CA, USA). The miR-16 was used for serum miRNA normalization and U6 snRNA was used for cell samples. Each sample in each group was measured in triplicate and the experiment was repeated at least three times.

**Dual Luciferase Assay**

Full length of ITCH 3’UTR were cloned into downstream of firefly luciferase coding region in pmirGLO vector (Promega, Madison, WI, USA) to generate luciferase reporter vector. For luciferase reporter assays, HEK293T cells were seeded in 48-well plates. miRNA mimics or miRNA antagonists or their corresponding controls and luciferase reporter vector were co-transfected by using lipofectamine 2000 (Invitrogen, Carlsbad, CA USA). Two days after transfection, cells were harvested and assayed with the Dual-Luciferase Assay (Promega, Madison, WI USA). Each treatment was performed in triplicate in three independent experiments. The results were expressed as relative luciferase activity (Firefly luciferase activity/Renilla luciferase activity).

**Western Blotting**

Protein extracts were boiled in SDS/β-mercaptoethanol sample buffer, and 20 µg samples were
loaded into each lane of 10% polyacrylamide gels. The proteins were separated by electrophoresis, and the proteins in the gels were blotted onto PVDF membranes (Amersham Pharmacia Biotech, St. Albans, Herts, UK) by electrophoretic transfer. The membrane was incubated with rabbit anti-ITCH polyclonal antibody (Abcam, Cambridge, MA, USA) and mouse anti-β-actin monoclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) for 1 h at 37°C. The specific protein antibody complex was detected by using horseradish peroxidase conjugated anti-rabbit or anti-mouse antibody. Detection by the chemiluminescence reaction was carried using the ECL kit (Pierce, Appleton, WI, USA). The β-actin signal was used as a loading control.

Preparation of Viruses
CVB3, strain Nancy, was propagated in mycoplasma free HeLa cell cultures. Seven to 8 hours after CVB3 infection with 1 multiplicity of infection (MOI), the culture medium was removed and the cells were disrupted by three freezing (-20°C) and thawing cycles. After centrifugation at 1500g, the cell debris was removed and virus particles were purified by chloroform extraction. Aliquots of purified virus were stored at -70°C.

Determination of Infectious Virus Particles
Infectious CVB3 particles were determined in the supernatants of infected cell cultures by the TCID50 assay or by a standard plaque assay as cytopathic effect on confluent cultures of mycoplasma-free HeLa cells, using a 1% agarose overlay.

Infection and Culture Conditions
HeLa cells were exposed to CVB3 at 5 MOI in serum-free medium at 37°C. The virus-containing medium was removed after a 1 h absorption period. The monolayers were carefully washed three times, 1 ml of fresh serum free medium was added, and the incubation was continued for time periods as indicated in the results section. Thereafter, the cell culture supernatants were removed, rendered cell free by centrifugation, and aliquots were stored at -70°C until assayed for cytokines.

Cytokine Assay
Levels IL-6 of cell culture supernatants were determined by enzyme-linked immunosorbent assay (ELISA) (Abcam, Cambridge, MA, USA) following the manufacturers’ instructions.

Statistical Analysis
Data were analyzed by using SPSS Statistical Package version 16 (SPSS Inc., Chicago, IL, USA). Independent two group’s analyses are used t-test. p < 0.05 was considered statistically significant.

Results

Genotypes and Risk of VM
To understand whether there are some relations between miR-10a and VM susceptibility, we scanned 339bp of the pri-miR-10a coding region. Only one single-nucleotide polymorphism (SNP) was found in this population in this region and the genotype frequencies of rs3809783 in controls conformed to the Hardy-Weinberg equilibrium (p = 0.44 for 110 patients without inflammatory cardiac disease and p = 0.22 for 248 healthy controls). After statistical analysis, we found the rare allele A of rs3809783 was associated with VM occurrence significantly (p < 0.01) (Tables I and II).

Table I. Genotype frequencies of the rs3809783 in pri-miR-10a coding region in VM patients and controls (110 patients without inflammatory cardiac disease).

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Patients (77), n%</th>
<th>Controls (110), n%</th>
<th>OR (95% CI)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs3809783</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>127 (0.82)</td>
<td>205 (0.93)</td>
<td>OR=0.34 [0.18, 0.67]</td>
<td>0.0012</td>
</tr>
<tr>
<td>T</td>
<td>27 (0.18)</td>
<td>15 (0.07)</td>
<td>OR=2.91 [1.49, 5.67]</td>
<td>0.0026</td>
</tr>
<tr>
<td>AA</td>
<td>50 (0.65)</td>
<td>95 (0.86)</td>
<td>OR=0.29 [0.14, 0.60]</td>
<td>0.0026</td>
</tr>
<tr>
<td>A T</td>
<td>27 (0.35)</td>
<td>15 (0.14)</td>
<td>OR=3.42 [1.67, 7.01]</td>
<td>0.0026</td>
</tr>
<tr>
<td>T T</td>
<td>0 (0.00)</td>
<td>0 (0.00)</td>
<td></td>
<td>0</td>
</tr>
</tbody>
</table>

P: Level of significance (<0.05).
Allele of rs3809783 and host susceptibility to viral myocarditis

The Mutation site Enhanced the Predict pri-miR-10a Stability and Reduced miR-10a Expression

To further explore the function of the mutation site, we first compared the predicted secondary structures of two genotypes pri-miR-10a molecules. As shown in Figure 1B, rare allele T causes an apparent change in stem-loop structure and a lowering of the predicted ΔG from -58.40 kcal/mol to -60.20 kcal/mol.

By using qRT-PCR, we detected the expression of mature miR-10a-5p and -3p generated by differ-
ent genotypes miR-10a expression vectors in HEK293T cells. As shown in Figure 1C, the rare allele T made a 34.9% reduction in mature miR-10a-5p expression and a 14.9% reduction in mature miR-10a-3p expression (Figure 2C), the result of which was in coincidence with the data from miR-10a-5p expression in human serum (Figure 3).

**ITCH, a NF-κB Signaling Suppressor, is a Direct Target of miR-10a**

To investigate the relation between disturbed miR-10a expression and VM, we first predicted the potential miR-10a target gene by using miRanda (http://www.microrna.org/micro/home.do). To our surprise, miR-10a targets ITCH 3'UTR directly according to the online prediction. To validate whether ITCH is indeed the target gene of miR-10a, full length human ITCH 3'-UTR containing was cloned into the downstream of the firefly luciferase reporter gene in the pmirGLO for the dual luciferase assay. HEK293T cells were co-transfected with pmirGLO-ITCH and miR-10a mimics or inhibitor. As shown in Figure 3B (left), compared with the miRNA control, the luciferase activity was significantly suppressed by the miR-10a mimic, about 51.8% ($p < 0.01$). Furthermore, the luciferase activity was significantly up-regulated by the miR-10a inhibitor compared with the anti-miR control, about 27.2% ($p < 0.05$). These results indicate that miR-10a targets the 3'-UTR of ITCH, leading to the change of firefly luciferase translation.

Seed sequence mutation clone was also used to further confirm the binding site for miR-10a (Figure 3A). The vector containing putative miR-10a binding region in the 3'-UTR of ITCH with 3 mutant nucleotides (designated as pmirGLO-ITCH-Mu) was constructed. The histogram in Figure 3B (right) showed that the enzyme activity was reduced not changed significantly in cells co-transfected with pmirGLO-ITCH-Mu and miR-10a mimics compared with miRNA control.

Table II. Genotype frequencies of the rs3809783 in pri-miR-10a coding region in VM patients and controls (248 healthy controls).

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Patients (77), n%</th>
<th>Healthy Controls (248), n%</th>
<th>OR (95% CI)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs3809783</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>127 (0.82)</td>
<td>460 (0.93)</td>
<td>OR=0.37 [0.22, 0.63]</td>
<td>0.0017</td>
</tr>
<tr>
<td>T</td>
<td>27 (0.18)</td>
<td>36 (0.07)</td>
<td>OR=2.72 [1.59, 4.64]</td>
<td>0.0026</td>
</tr>
<tr>
<td>AA</td>
<td>50 (0.65)</td>
<td>212 (0.85)</td>
<td>OR=0.31 [0.17, 0.57]</td>
<td></td>
</tr>
<tr>
<td>AT</td>
<td>27 (0.35)</td>
<td>36 (0.15)</td>
<td>OR=3.18 [1.77, 5.72]</td>
<td></td>
</tr>
<tr>
<td>TT</td>
<td>0 (0.00)</td>
<td>0 (0.00)</td>
<td></td>
<td></td>
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</tbody>
</table>

Figure 2. miR-10a expression in clinical serum samples was detected using qRT-PCR. Serum samples miR-10a from 20 age and sex compared individuals was detected using stem-loop qRT-PCR in VM patients (A), 110 patients without inflammatory cardiac disease (B) or 248 healthy controls (C).
could regulate endogenous ITCH expression. Hela cells were transfected with miR-10a mimics or inhibitor to see whether the dysregulation of miR-10a expression affected endogenous ITCH expression. As shown in Figure 3C, compared with corresponding control, the level of ITCH protein was significantly suppressed by miR-10a mimics and up-regulated by miR-10a inhibitor.

The rare allele of rs3809783 Reduced NF-κB signaling by Down Regulated miR-10a Expression in CVB3 Infected Hela cells

To understand whether the effect of nucleotide variation of rs3809783 can be reflected on changed ITCH expression, Hela cells were co-transfected with ITCH 3’UTR reporter vector and one of the two genotypes pri-miR-10a expression plasmids. As shown in Figure 4A, although the relative luciferase activity was significantly reduced compared with empty vector, the luciferase activity of cells transfected pri-miR-10a-TT vector was raised up to 1.28 fold when compared with pri-miR-10a-AA.

The NF-κB family of transcription factors is a key in inflammatory responses and playing crucial role in virus resistance and autoimmune effect. Itch is an essential component of the ubiquitin-editing complex that is required for termination of proinflammatory activation of the kinase JNK and the transcription factor NF-κB. To further investigate the biological function of overexpressed miR-10a, we detected IL-6 levels of Hela cells culture supernatants. As shown in Figure 4B, CVB3 infection up-regulated IL-6 levels, meanwhile, when transfected with pri-miR-10a-AA or pri-miR-10a-TT vector, the concentration of IL-6 was raised significantly (p < 0.05). But when compared with pri-miR-10a-AA group, the concentration of IL-6 reduced to 80.4% (p < 0.05).

Discussion

Virus infection of the heart can cause severe damage to cardiomyocytes through multiple mechanisms. Direct virus-mediated injury and secondary immune reactions, including inflammatory and autoimmune responses, have been reported both in animal models and in humans.17,18 Since viral infection of the heart is almost exclusively accompanied by histological inflammation of heart muscles, ‘viral myocarditis’ has become standard terminology to express virus-mediated...
pressed cytokines also contribute to the cardiomyopathy.

In this study, we scanned 339bp of pri-miR-10a coding region in CVB3 VM patients, want to found genetic relations between miR-10a and VM susceptibility. We found the rare allele T of rs3809783 was accumulated in VM patients and related to VM significantly. Subsequent biological functional study indicated that this site variation reduced mature miR-10a expression and induced a down-regulated cytokine secretion in the cell culture supernatant. The results suggest that the rare allele T in pri-miR-10a coding region should be involved in the CVB3 caused VM pathogenesis through weakening host anti-virus immune response.

There is also a report that miR-10a-3p is positive for CVB3 biosynthesis by targeting the 3D-coding sequence. Although the rare allele T of rs3809783 can also reduce miR-10a-3p expression, the expression of miR-10a-3p is pretty rare in the serum samples and according the deep sequencing data in miRbase (http://www.mirbase.org/index.shtml) the expression of miR-10a-3p is very low. So we guess the reduced miR-10a-3p may not affect the CVB3 biosynthesis.

**Conclusions**

We first report the rare allele T of rs3809783 is related to CVB3 caused VM. We also confirmed ITCH is a direct target of miR-10a. This site variation reduced miR-10a expression in vitro and in vivo, and related to weakened anti-virus response. So this site may be used for clinical genetic evaluation for VM susceptibility.

**Conflict of Interest**

The Authors declare that they have no conflict of interests.

**References**


2) Heart Failure Society of America, Lindenfeld J, Albert NM, Boehmer JP, Collins SP, Ezekowitz JA,
Allele of rs3809783 and host susceptibility to viral myocarditis


