

Original Article

Lichen extracts regulate the expression of BMP7 via miR-194-5p targeting to decrease the risk atrial fibrillation

Kailin Zhao^{1*}, Liyun Xiao^{2*}, Meili Zhao², Changjian Ji²

¹Department of Cardiology, Yantai Yeda Hospital, Shandong, China; ²Department of Cardiology, Jinxiang People's Hospital Affiliated to Jining Medical School, Shandong, China. *Equal contributors and co-first authors.

Received December 8, 201; Accepted January 20, 2017; Epub August 1, 2017; Published August 15, 2017

Abstract: Background: Atrial fibrillation (AF), the most common cardiac supraventricular arrhythmia, affects more than 5 million people worldwide. Increasing evidence has demonstrated that genetic factors play an important role in the pathogenesis of AF, and multiple genes responsible for AF have been identified. A better understanding of the genetic mechanism underlying AF would be expected to lead to more accurate risk stratification of AF and optimal clinical treatment strategies. Methods: Immunofluorescence staining was performed to find the components which would have effects on the H9c2 cells development. The changes of BMP7 and miR-194-5p expressions were detected before and after Human Cardiac Myocytes (HCM) were treated with Lichen extraction. In order to confirm whether Lichen could increase the expression of BMP7 through inhibiting the expression of miR-194-5p, the mRNA levels of BMP7 and miR-194-5p were determined in HCM before and after the treatment of Lichen on the conditions that miR-194-5p was over-expressed or not. Results: After 48 hours' treatment with 20 µg/mL Lichen extracts, the Collagen I expression level significantly decreased. The expressions of several genes in H9c2 cells could be changed after the treatment of Lichen extracts and some mRNA of them could also be targeted by miR-194-5p including BMP7. Lichen could depress the expression of miR-194-5p in HCM no matter miR-194-5p was over-expressed or not and correspondingly, the expression of BMP7 could be increased in both conditions. Conclusions: It is indicated that Lichen extracts could regulate the expression of atrial fibrillation-associated genes via miR-194-5a targeting.

Keywords: Atrial fibrillation, lichen, BMP7, miR-194-5p, traditional Chinese herbal preparation

Introduction

Atrial fibrillation (AF) is the most common cardiac supraventricular arrhythmia with the prevalence increasing markedly with age, which ranges from approximately 1% in general population to 10% in those aged over 75 years old [1]. The morbidity of AF is estimated to increase five-fold by 2050 along with the accelerating aging population [2]. The presence of atrial fibrillation can cause serious complications and can independently increase the risk of mortality and morbidity of patients [3]. Atrial fibrillation confers a five-fold increase in the risk of stroke and approximately doubles mortality, resulting in a major financial burden to patients and healthcare systems [4]. Generally, AF is known as a common complication in vari-

ous cardiac and systemic disorders. While in some cases, AF can also exist in the absence of predisposing factors and is defined as lone AF [5].

While the underlying mechanisms of atrial fibrillation are complex and not well understood, multiple potential pathways and risk factors have been investigated. Since the first locus (10q22-q24) was identified in 1997, a number of atrial fibrillation-related genes have been identified and emerging evidences which are strongly implicated with hereditary determinants for AF have been found as well [6]. From then on, researchers have identified and confirmed at least 25 genes which are related with AF. Mutations have also been identified in patient population with AF complicated with

other disorders. Recent data have shown that almost all sodium channel genes and potassium channel genes are involved in the development of AF [7]. Makiyama. *et al* revealed that the *SCN5A* mutation P.M1875T which displays a gain-of-function modulation of cardiac Na⁺ channels is associated with familial AF, it also provided us a novel mechanism that predisposes to increased atrial excitability and familial AF [8, 9]. *KCNA5* encodes a member of the potassium channel producing ultra-rapid delayed rectifier potassium current, on which locus mutations can result in a loss-of-function effect on potassium current and eventually lead to AF [10-12]. However, as we all know that the mechanisms of AF are complex, and the maintenance of sinus rhythm after pharmacological or interventional treatments remains very challenging.

In this case, if we could find some chemical components which can alter expression levels of the AF-related genes resulting in the suppression of AF [13-16], it will be an important step forward to cure AF and relieve the pain that the patients suffer from. In this study, we tried to screen out some chemical components from traditional Chinese herbal preparation which have some effects on regulating the expression of some AF-related genes. *Bmp7*, *Lox*, *Mmp3*, *Mmp8*, *Mmp9*, *Serpine 1*, *Acta2*, *Ctgf*, *Grem1*, *Col1a2*, and *Col3a1* were chosen for their reported functions in AF. *BMP7* encodes bone morphogenetic protein 7, which plays a key role in the transformation of mesenchymal cells into bone and cartilage. It has been reported that it could inhibit the fibrosis of heart and kidney [17].

Lectin-like oxidized low density lipoprotein receptor-1 (LOX-1) has the synergistic effect with TGF- β 1 in myocardial fibrosis and increased expression of LOX has been observed in left atria of patients with AF [18, 19]. MMPs has been reported to correlate with human AF [20]. Patients with AF always have higher *Serpine 1* levels which is implicated in the development of atrial fibrillation. Elevated levels of *Serpine* independently predict development of AF after cardiac surgery as well [21, 22]. *Acta2*, also known as α -SMA, is a major constituent of the contractile apparatus and has a positive correlation with fibrosis [23]. CTGF, also called *CCN2* or connective tissue growth factor, plays an important role in fibrotic diseases, whose over-

expression could promote fibrosis in human [24-26]. *Grem1* is the antagonist of bone morphogenetic proteins (BMPs) which could inhibit *BMP-2*, *BMP-4*, and *BMP-7* in the TGF beta signaling pathway, so that it could accelerate organ fibrosis [27-29]. Collagen alpha-2 (I) chain (*COL1A2*) is one of the chains for type I collagen, the fibrillar collagen which has a positive correlation with human organ fibrosis [30, 31]. *COL3A1* is a fibrillar collagen that is always related to type I collagen [32].

Some of the herbal preparations are usually used to treat systemic disorders or valvar heart disease, as we all know that valvar heart disease, hypertension, ischemic heart disease and hyperthyroidism are the most common causal risk factors of atrial fibrillation [33, 34]. Furthermore, we found that after treating the H9c2 cells with Lichen extracts for 48 hours, the chosen AF-related genes in H9c2 cell lines were significantly changed compared with untreated cells and the cells treated with other extracts or chemical components. It was noted that the expression of *BMP7* was obviously upregulated in both levels of mRNA and protein. It has been speculated that the mRNA of *BMP7* is the target of miR-194-5p which is always upregulated in the patients with heart failure (HF) after acute myocardial infarction (AMI) [35, 36]. While it has been displayed that AF is bidirectional associated with AMI, which means the risk of AF could be bigger when the risk of AMI is bigger [37-40]. According to this, we hypothesized that in patients with AF, *BMP7* was inhibited by overexpressed miR-194-5p to lead to fibrosis, then promoted the progress of AF. In order to prove it, we treated human cardiac myocytes (HCM) with Lichen extracts and performed luciferase assay to reveal the mechanism underlying the inhibition of AF through regulating miR-194-5p or *BMP7*.

Materials and methods

Cell culture and reagents

H9c2 (2-1) rat heart myoblasts [15] were obtained from the American Type Culture Collection. Human cardiac myocytes (HCM) were obtained from Scien Cell Research Laboratories (Catalog Number: 6200, San Diego, CA, USA). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal calf serum, glutamine (2

Lichen extracts decrease the risk of atrial fibrillation

Table 1. Dosing sequence and drug concentration per well

	H9c2 Dosing sequence\drug concentration per well 20 µg/ml							
	1	2	3	4	5	6	7	8
A	Emodin	Lichen	Gegen flavonoids	Juncus roemerianus	Cardamun	Lyciumbarbarum	Salvia miltiorrhiza	Fenugreek
B	Baicalin	Salviae miltiorrhizae	Puerarin	Chrysin	Tetrandrine	Baicalein	Glycyrrhizic acid	Atractylodes
C	Pachyman	Rhodioloside	Clove	Formononetin	Artichoke	Angelica sinensis	Senoside	Salt black bean
D	Tribulus terrestris saponins	Ligustilide	Kudzu	Semen plantaginis	Nutmeg 2	Resveratrol	Spinach	Garlic Polysaccharide
E	Daidzein	Citrus	Radix glycyrrhizae	Soybean isoflavone	Hordenine	Cortex albiziae	Raspberry	Puerarialobata flavone 2
F	Polygonum multiflorum	Hyperforin	Cinnamon	Paecilomyces bepiali chen	Allicin	Hesperidin	TP (tea polyphenol)	Control

Lichen extracts decrease the risk of atrial fibrillation

Table 2. Primers used for Q-PCR

Gene names	Sequence (5'-3')
Col1a2	F GCCAAGAATGCATACAGCCG
	R GACACCCCTTCTGCGTTGTA
Mmp8	F CGGGGTATTGGAGGAGATGC
	R ATGAGCAGCCACGAGAAACA
Mmp9	F GATCCCCAGAGCGTTACTCG
	R GTTGTGGAAACTCACACGCC
Serpine1	F CAACCCACTACGCCTTCACT
	R TCTGTCTATCTGCTGCCCT
Mmp3	F ATGGGCCTGGAATGGTCTTG
	R TGTGGGAGGTCCATAGAGGG
Col3a1	F CAACCCACTACGCCTTCACT
	R GCCACGTTCCACAGTTTAC
LOX	F GACCACAGGGTACTGCTACG
	R CCACTCTCCTCTGTGTGCTG
Bmp7	F CGGAGGTTTCATCTCCGTAGC
	R GCTGTTTTCTGCCACACTGG
Grem1	F GACAGAATGAATCGCACGGC
	R CGCTTCAGGATTTGCGCTC
Ctgf	F ACACAAGGGTCTCTTCTGCG
	R ACTCCTCACAGCATTTCCTCG
Acta2	F ACTCCTCACAGCATTTCCTCG
	R CTGTCAGCAATGCCTGGGTA
GAPDH	F TCACCACCATGGAGAAGGC
	R GCTAAGCAGTTGGTGGTGCA

mM), non-essential amino acids [1% (v/v)] and gentamicin (100 mg/l) under an atmosphere of 5% CO₂ in air saturated with water vapors at 37°C. The medium was replaced every 2 days. Cells were grown to confluence as monolayers and were split by treatment with trypsin. Stock solutions of (-)-isoproterenol, (-)-propranolol and dexamethasone were prepared in water and then diluted directly in DMEM. Untreated cells used as control were grown in the same DMEM to which only water was added.

Immunofluorescence staining

Cells were divided into the following treatment groups (n=10 wells): 1) Rabbit anti-collagen I antibody (1:200 dilution; Catalog Number: Ab6308; Abcam); 2) Rabbit α -actinine antibody (1:400 dilution); 3) 10 mM PBS (control). The cells were cultured for 48 h, fixed in 4% paraformaldehyde, and blocked with goat serum. They were then incubated with the above-mentioned primary antibodies for 1 h and fixed at 4°C in a wet box overnight. Cells incubated with 10 mM

PBS served as the control for the antibody staining. Next, the cells were treated with FITC-labeled goat anti-rabbit IgG (1:100 dilution, Catalog Number: 28176-05-FITC; Seebio), incubated at room temperature in a wet box for 1 hour, and stained in 0.5 μ g/mL DAPI solution for 1 min. After washing the cells with PBS, five visual fields in each slide were randomly selected under a fluorescence microscope (400 \times magnification) and observed. The images were processed using the Image-Pro plus 6.0 (Media Cybernetics, USA) Image Processing Software to obtain the absorbance values at the same time of exposure. All the experiments were performed in the same condition for at least three individual repeats.

Q-PCR analysis

Total RNA from H9c2 cells was extracted by binding to a silica-based membrane using a microspin column technique according to the manufacturer's protocol (Catalog. Number: 15596-026; Invitrogen TRIZOL® Reagent). Remaining traces of DNA were digested in a total volume of 100 μ l with 10 units of DNaseI (FPLCpure, Pharmacia, Freiburg, Germany) in a buffer containing 10 mM Tris/HCl/10 mM MgCl₂/50 mM KCl/10 mM dithiothreitol (pH 9.0) for 20 min at 37°C. With an additional clean-up procedure, DNaseI-treated RNA was recovered by adsorption on silica-based membranes as described in the total RNA extraction procedure.

cDNA synthesis was carried out in a 20 μ l reverse transcription reaction containing 1 μ g of total RNA, and various amounts of the corresponding internal standard RNA. RNA was reverse transcribed in a buffer containing 5 mM MgCl₂, 50 mM KCl, 10 mM Tris/HCl (pH 8.3), 1 mM dNTPs, 2.5 μ M random hexamers, and 20 units of RNase inhibitor and 100 units of RT. The reaction was performed at 42°C for 20 min. Thereafter, the mixture was incubated at 99°C for 5 min to inactivate the enzyme's activity. Quantitative real-time PCR was performed on BioRad Connet Real-Time PCR platform using SYBR Green Master Mix Kit. In brief, each PCR reaction mixture containing 7.5 μ l of 2 \times SYBR premix ex taq, 0.5 μ l of sense and antisense primers (2.5 μ M), 1 μ l of cDNA and 5.5 μ l of ddH₂O, were run for 1 cycle of initial denaturation at 95°C for 30 s, and run for 40 cycles of denaturation at 95°C for 5 s and

Lichen extracts decrease the risk of atrial fibrillation

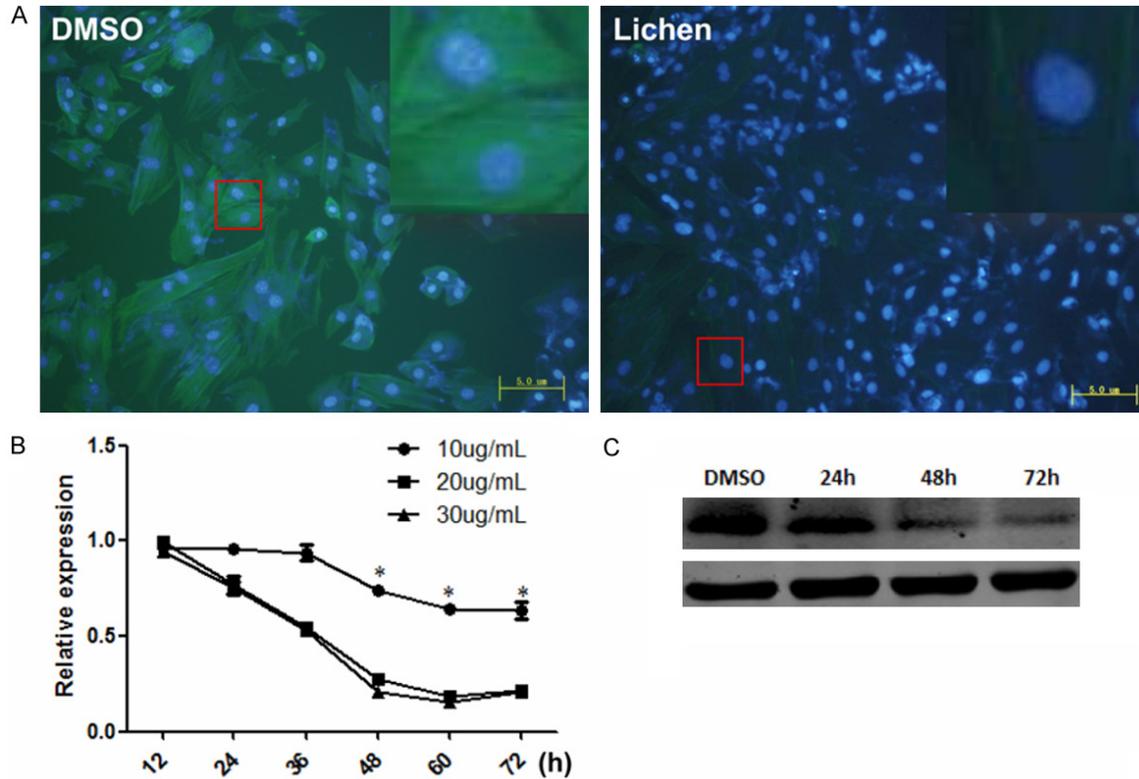


Figure 1. The expression of Collagen I obviously decreased after the treatment of Lichen extracts in H9c2 cells. A. Immunofluorescence staining of Collagen I. A: Lichen. B: DMSO. After 48 h treatment with 20 $\mu\text{g}/\text{ml}$ Lichen extracts, Collagen I expression level significantly down regulated, comparing with the control. B. Collagen I expression level changes with time and concentration of Lichen extracts. (The Collagen I expression level decreased before 48 hrs treatment, after 48 hrs, Collagen I level becomes stable). (Primers used, F: 5'-GGCTTCTTCAAACCACTGCTTT-3', R: 5'-AAAGTCATAGCCACCTCCGCTG-3'). C. Immunoblotting assay (Collagen I decreased with time, comparing with Actin (Control) Collagen I protein level goes down with time. The results are almost the same with the Q-PCR results.

extension at 64°C for 34 s. The expression level of GAPDH was used as an internal control for normalization. All the primers were designed (Sequences of the primers are listed in **Table 2**). Relative gene expression levels were calculated using $2^{-\Delta\Delta\text{CT}}$ analysis. All the experiments were performed in the same condition for at least three individual repeats.

Western blot analysis

H9c2 cells were homogenized in a lysis buffer containing 150 mM NaCl, 50 mM Tris-HCl, 10 mM EDTA, 0.1% Tween-20, 1% Triton, 0.1% β -mercaptoethanol, 0.1 Mm phenylmethylsulfonyl fluoride, 5 $\mu\text{g}/\text{mL}$ leupeptin, and 5 $\mu\text{g}/\text{ml}$ aprotinin (pH 7.4) and allowed to be incubated for 1 h on ice. Homogenates were then centrifuged at 4°C for 10 min at 10,000 g, and supernatants were collected. Protein concentrations were measured in the supernatants using a

protein assay kit (Bio-Rad). Samples with equal amounts of proteins were loaded onto 10% polyacrylamide gel with 0.1% sodium dodecyl sulfate and separated by electrophoresis at 120 V for 1.5 h. Proteins were then transferred onto nitrocellulose membranes. Nonspecific binding sites were blocked for 1 h at room temperature in the Tris-buffered saline solution containing 5% dry milk. The membranes were then probed with primary antibodies (1:5,000 dilution, Abcam). To assure equal loading, band intensities were normalized to actin (1:5,000 dilution, Sigma). After being washed, membranes were incubated with secondary horseradish peroxidase-conjugated antibodies (1:10,000 dilution; Catalog Number: SC-2054; Santa Cruz) for 1 h. Proteins were visualized with enhanced chemiluminescence reagents, and blots were exposed to Hyperfilm. The results were analyzed with the Kodak ID image

Lichen extracts decrease the risk of atrial fibrillation

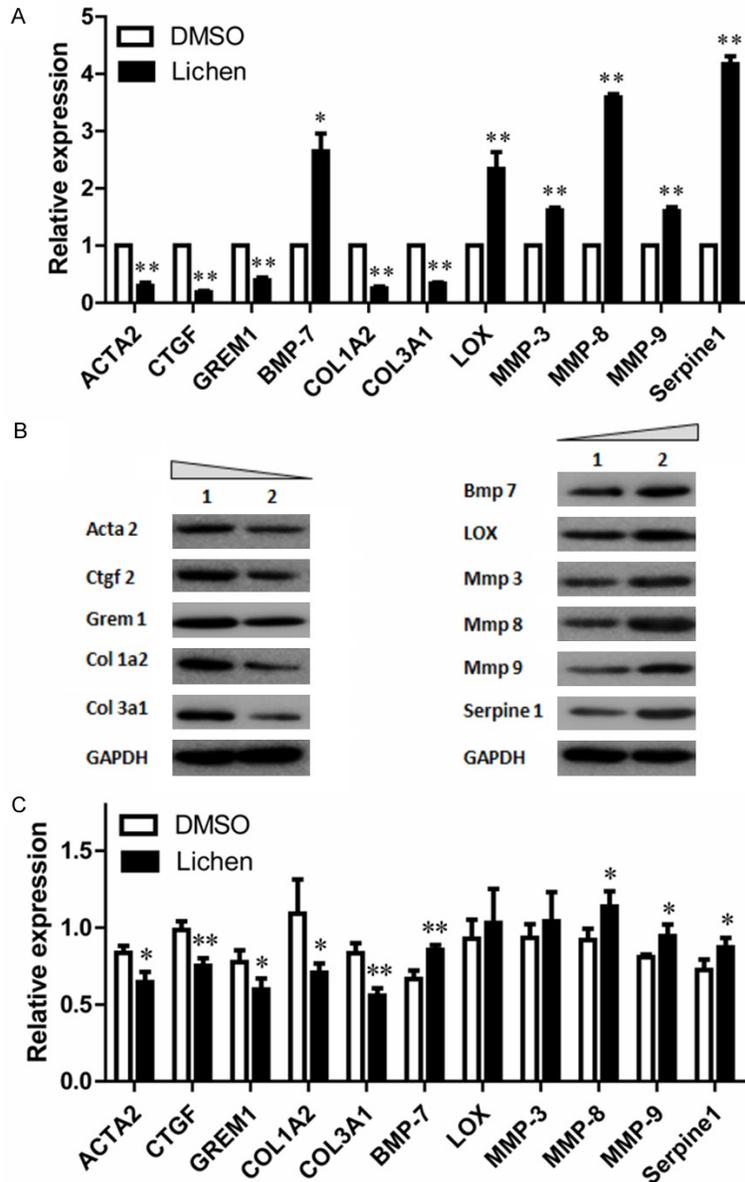


Figure 2. The expression of AF-related genes were changed after 48 h of Lichen extracts treatment in H9c2 cells. A. Real-time quantitative analysis of atrial fibrillation transcription related genes expression (Lichen processing H9C2 cells after 48 hours, using real-time quantitative PCR to detect AF gene transcription level changes ($P < 0.001$ Experimental groups VS. Control groups). B. Western blotting analysis (1: DMSO, 2: Lichen extracts.) Pro-Fibrotic: Acta2, Ctgf, Grem1, Anti-Fibrotic: Bmp7, Extracellular Matrix (ECM) Structural Constituents: Col1a2, Col3a1. Extracellular Matrix (ECM) Remodeling Enzymes: Lox, Mmp3, Mmp8, Mmp9, Serpine1 (mediates inhibition of fibrinolysis by inhibiting the activity of plasminogen activator). C. The column chart results of Western blotting. * $P < 0.05$, ** $P < 0.01$, compared with DMSO treatment.

analysis software. All the experiments were performed in the same condition for at least three individual repeats.

Luciferase activity assay

A fragment of the wild-type (WT) BMP7 3'UTR containing the predicted miR-194-5p binding site was amplified by RT-PCR. The primers used were 5'-CGGAGGTTTCATCTCCGTAGC-3' (forward) and 5'-GCTGTTTTCTGCCACACTGG-3' (reverse). Site-directed mutagenesis of the miR-194-5p target site was carried out using Stratagene Quik-Change site-directed mutagenesis kit (Stratagene, Heidelberg, Germany). The construct was sequenced and named BMP7-UTR-Mut. The pMIR-report luciferase vector was used for the construction of BMP7-UTR or BMP7-UTR-Mut plasmids (Ambion, USA). HCM cells were cultured in 24-well plates. In each well, 10 ng of phRL-TK renilla luciferase vector (Promega, USA) was co-transfected to normalize transfection efficiency. A concentration of 500 ng of BMP7-UTR or BMP7-UTR-Mut plasmids together with 10 nM miR-194-5p mimics or negative control was also co-transfected. Transfection was done using Lipofectamine 2000 and Opti-MEM I-reduced serum medium (Life Technologies, California, USA). Firefly luciferase activity was measured using the Dual luciferase assay kit (Promega). Normalized relative luciferase activity (RLA) was calculated as the following formula: $RLA = \frac{\text{firefly luciferase}}{\text{renilla luciferase}}$ [41].

Statistical analysis

Data were expressed as means \pm SE. Statistical significance ($P < 0.05$) was determined by analysis of variance (ANOVA) followed by Newman-

Lichen extracts decrease the risk of atrial fibrillation

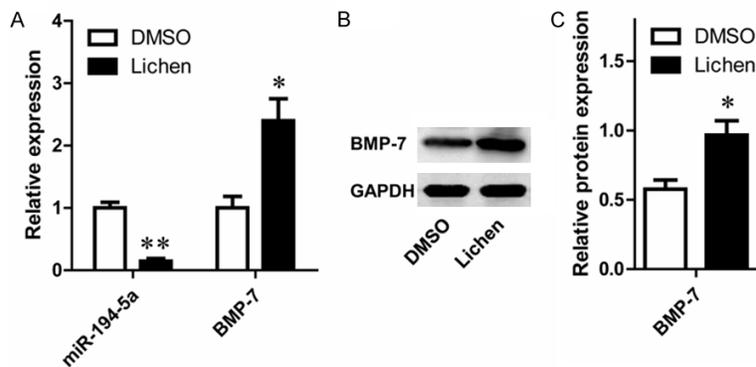


Figure 3. The expression of miR-194-5p and BMP7 were reversely changed after the treatment of Lichen extracts in HCM. A. The mRNA expressions of miR-194-5p and BMP7 were reversely changed after the treatment of Lichen extracts in HCM. B, C. The protein level of BMP7 were significantly increased by Lichen extract in 48 h in HCM. * $P < 0.05$, ** $P < 0.01$, compared with DMSO treatment.

Keuls post hoc testing or Student's *t*-test, where appropriate.

Results

Screening for candidate components

In order to find some candidate components, we selected a series of traditional Chinese herbal preparations (all the details are shown in **Table 1**) and added equal volume with the same concentration per well, i.e. 20 $\mu\text{g}/\text{ml}$ extract or herbal preparation. After the treatment, immunofluorescence staining was performed to find the components which would have effects on the H9c2 cells development. Interestingly, we found that after 48 h of treatment with 20 $\mu\text{g}/\text{mL}$ Lichen extracts, Collagen I expression level was significantly down regulated, comparing with the control (**Figure 1A**).

We also harvested the H9c2 cells treated with different concentrations (10 $\mu\text{g}/\text{mL}$, 20 $\mu\text{g}/\text{mL}$ and 30 $\mu\text{g}/\text{mL}$) of Lichen extracts at different time points and extracted RNA from them to make sure that the immunofluorescence staining results were reliable, and find the optimal conditions for Lichen to inhibit the expression of collagen I in H9c2 cells. As is shown in **Figure 1B**, the Collagen I expression level decreased in the first 48 h treatment and did not go down any more as time went on. The results of western blotting also confirmed the results of q-PCR (**Figure 1C**).

Expression levels of AF associated genes were changed after the treatment of lichen extraction

Although the exact genes responsible for AF in this region remain unclear, the scientific results instigated exploring the genetic characteristics of atrial fibrillation. So, based on the publications, we detected the expression levels of some genes associated with AF, including Acta2, Ctgf, Grem1, Bmp7, Col1a2, Col3a1, Lox, Mmp3, Mmp8, Mmp9, and Serpine1. According to the results of qPCR,

Bmp7, Lox, Mmp3, Mmp8, Mmp9, and Serpine I were upregulated; and Acta2, Ctgf, Grem1, Col1a2, Col3a1 were down regulated compared with control (**Figure 2A**). The results were then confirmed by western blotting analysis (**Figure 2B, 2C**).

Expression levels of BMP7 and miR-194-5p in HCM were changed after the treatment of lichen extraction

In order to investigate the correlation between BMP7 and miR-194-5p during the process myocardial cells are treated with Lichen extraction, we detected the changes of BMP7 and miR-194-5p expressions before and after Human Cardiac Myocytes (HCM) were treated with Lichen extraction. Interestingly, the mRNA level of miR-194-5p significantly decreased after the treatment of Lichen extracts while both the mRNA and protein levels of BMP7 evidently increased simultaneously (**Figure 3**).

Inverse relationship between expression of BMP7 and miR-194-5p

To validate whether miR-194-5p directly targets the 3'UTRs of BMP7 mRNA or not, we cloned a sequence with the predicted target sites of miR-194-5p or a mutated sequence with the predicted target sites to downstream of the pMIR luciferase reporter gene. When the wild-type or mutation-type vector was transfected with miR-194-5p, the luciferase activity of wild-type vector was significantly decreased ($P < 0.01$) compared with mutation-type vector (**Figure 4A**). While the wild-type or mutation-

Lichen extracts decrease the risk of atrial fibrillation

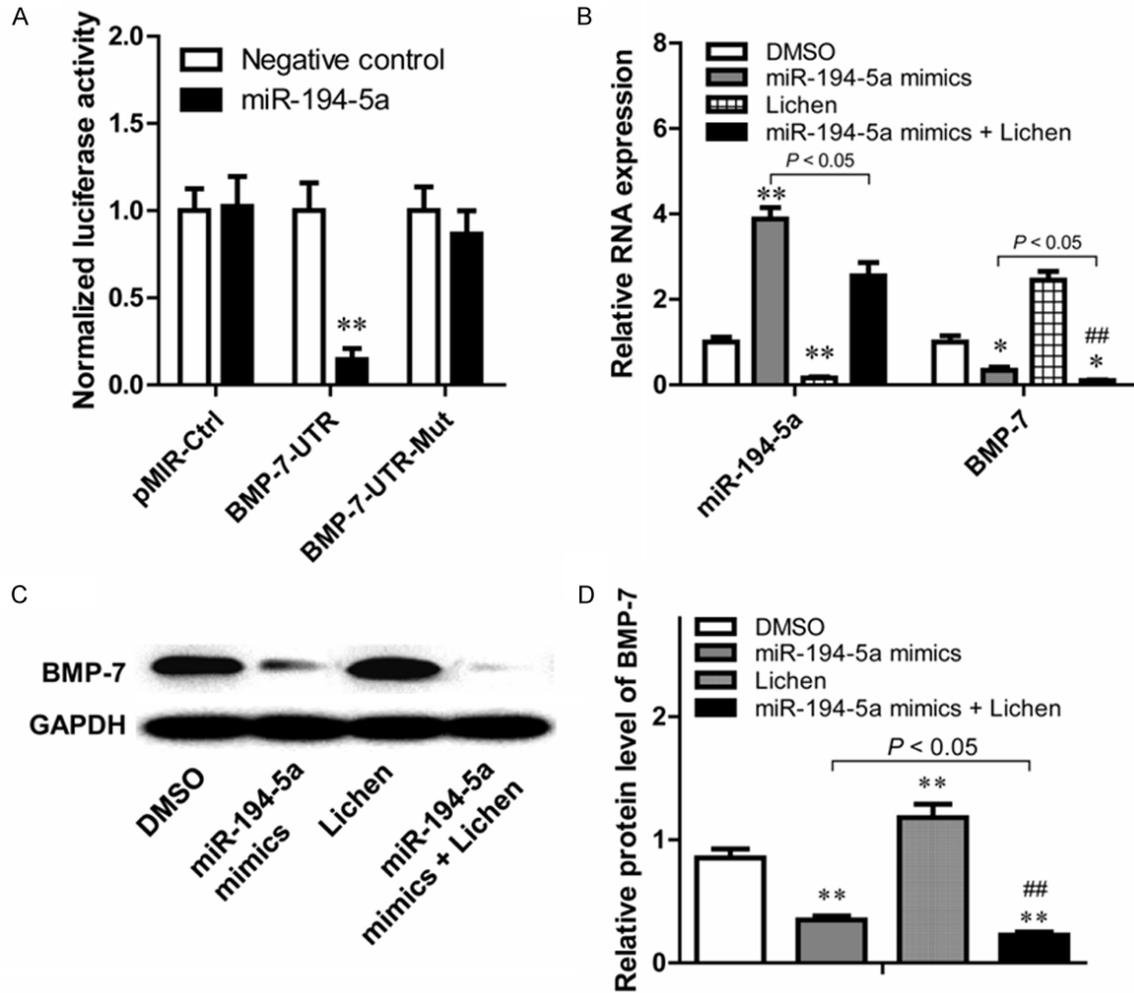


Figure 4. Luciferase activity assay results of miR-194-5p and BMP7, as well as the expression changes of miR-194-5p and BMP7 caused by Lichen extracts in HCM overexpressing miR-194-5p. A. There is a specific targeting reaction between miR-194-5p and BMP7. B. Lichen extracts could reduce the expression of miR-194-5p in both normal HCM and HCM overexpressing miR-194-5p to increase the expression of BMP7. C. The expressions of miR-194-5p and BMP7 at protein level changed in line with the mRNA level. D. The protein level of BMP-7 in the column chart. * $P < 0.05$, ** $P < 0.01$, compared with DMSO treatment; # $P < 0.05$, ## $P < 0.01$, compared with Lichen treatment.

type vector was transfected with negative control miRNA, there was no significant difference between the wild-type or mutation-type vector. These data suggested that miR-194-5p may play a major role in the regulation of BMP7. Taken together, these data indicated that the 3'UTR of BMP7 is a functional target site for miR-194-5p in HCM.

Lichen could always inhibit the expression of miR-194-5p to increase the expression of BMP7 in HCM

As is shown above, it was already proved that BMP7 is the target of miR-194-5p, which

means the increase of BMP7 expression might be the result of miR-194-5p depletion. In order to confirm whether Lichen could increase the expression of BMP7 through inhibiting the expression of miR-194-5p, we determined the mRNA levels of BMP7 and miR-194-5p in HCM before and after the treatment of Lichen on the conditions that miR-194-5p was overexpressed or not. As is shown in **Figure 4B**, Lichen could depress the expression of miR-194-5p in HCM no matter miR-194-5p was overexpressed or not and correspondingly, the expression of BMP7 could be increased in both conditions.

Discussion

AF is a problem of growing proportions that has been termed “epidemic” [42]. Present therapeutic options have limited efficacy and disconcerting adverse-effect risks. Consequently, a variety of novel, mechanism-based approaches are in development. One approach has been developed to target atrial remodeling, with the use of “upstream therapy” interventions that interfere with putative signaling pathways. Interventions, such as omega-3 fatty acids and renin-angiotensin system inhibitors, were reported to work through preventing atrial remodeling in experimental models [43]. While retrospective analysis of clinical data is promising, the few prospective randomized clinical trials which are completed to date have been negative or inconclusive [43]. Even though the notion of remodeling prevention is very attractive, its feasibility remains to be demonstrated. Although the underlying mechanisms of AF are complex and not well understood yet, people have already put forward multiple potential pathways. A better understanding of the genetic mechanism underlying AF will hopefully lead to an accurate risk stratification of AF [44].

Current management strategies for AF have had some developments; However, some challenges still remain to be solved in this field. For example, we still cannot fully understand the early identification of AF in patients who are at risk and little progress has been made in pharmaceuticals. Some groups, for example, Husser *et al* [45] and his group included a total of 195 consecutive patients with drug refractory paroxysmal or persistent AF who underwent AF catheter ablation, and were the first to genotype two common variants, rs2200733 and rs10033464 on chromosome 4q25, which were independently associated with an increased risk of recurrence of AF after catheter ablation. Another group [46] investigated the variations in the human soluble epoxide hydrolase gene responsible for recurrence of AF after catheter ablation and described that the rs751141 polymorphism of the *EPHX2* gene is associated with a significantly increased risk of AF. These results point to a potential role for these common variants and may guide differential therapy in the future. Although, there are many genes related to AF and have been published, there is no prognostic or therapeutic

impact derived from an AF genetic test result. However, it still very valuable if we could find some chemical components which would alter the AF associated genes' expression.

In this study, we have investigated the effects Lichen extracts has on several AF related genes including miR-194-5p and BMP7 in H9c2 cells and HCM, as well as the mechanism underlying the corresponding changes of miR-194-5p and BMP7 in Lichen-treated HCM. According to **Figure 1**, Collagen I expression in H9c2 cells could be significantly decreased by the treatment of Lichen extracts, which could help prevent atrial fibrosis of organs to protect heart from AF. In order to explore more about the effect of Lichen extracts has on preventing AF, expression of more genes concerning AF in H9c2 cells were detected before and after the treatment of Lichen extracts. All the genes expression changed to reduce the risk of AF after the treatment of Lichen extracts as we predicted except MMPs. There is one possibility that the way MMPs influence is complicated and several relevant factors could affect each other through various pathways, so more research needs to be performed to figure out the reason why MMPs were upregulated in H9c2 cells after Lichen extracts treatment.

Because of the predicted targeting action between miR-194-5p and BMP7 [35], we hypothesized that Lichen extracts upregulated the expression of BMP7 through inhibiting the expression of miR-194-5p and performed luciferase activity assay to confirm the targeting reaction between miR-194-5p and BMP7. According to the corresponding changes of expression levels of miR-194-5p and BMP7 in miR-194-5p-overexpressed HCM before and after the treatment of Lichen, it was demonstrated that Lichen could regulate the expression of BMP7 through regulating mRNA level of miR-194-5p in HCM to decrease the risk of AF in human.

Based on the achievements in the past several years, our results, in a way, show the clinical implications of exploring the genetic basis of AF and will have more impacts on screening for pharmaceuticals which would release the pain the patients suffer from.

While, more research needs to be carried out to explore the specific relationship between atrial

fibrosis and AF to solidify our result, and more tests *in vivo* also need to be performed to make our result easier to apply on clinic in the future.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Liyun Xiao, Department of Cardiology, Jinxiang People's Hospital Affiliated to Jining Medical School, No. 117 East Jinfeng Road, Jining, Shandong, China. Tel: +86-15588734110; Fax: +86-54332939; E-mail: xly-sd12@163.com

References

- [1] Hobbs FD, Fitzmaurice DA, Mant J, Murray E, Jowett S, Bryan S, Raftery J, Davies M and Lip G. A randomised controlled trial and cost-effectiveness study of systematic screening (targeted and total population screening) versus routine practice for the detection of atrial fibrillation in people aged 65 and over. *Health Technol Assess* 2005; 9: 1-74.
- [2] Kannel WB and Benjamin EJ. Current perceptions of the epidemiology of atrial fibrillation. *Cardiol Clin* 2009; 27: 13-24.
- [3] Beyerbach DM and Zipes DP. Mortality as an endpoint in atrial fibrillation. *Heart Rhythm* 2004; 1: 8-18.
- [4] European Heart Rhythm A, European Association for Cardio-Thoracic S, Camm AJ. Guidelines for the management of atrial fibrillation: the task force for the management of atrial fibrillation of the European Society of Cardiology (ESC). *Europace* 2010; 12: 1360-1420.
- [5] Munger TM, Wu LQ and Shen WK. Atrial fibrillation. *J Biomed Res* 2014; 28: 1-17.
- [6] Brugada R, Tapscott T, Czernuszewicz GZ, Marian AJ, Iglesias A, Mont L, Brugada J, Girona J, Domingo A, Bachinski LL and Roberts R. Identification of a genetic locus for familial atrial fibrillation. *N Engl J Med* 1997; 336: 905-911.
- [7] Magnani JW, Rienstra M, Lin H, Sinner MF, Lubitz SA, McManus DD, Dupuis J, Ellinor PT and Benjamin EJ. Atrial fibrillation, current knowledge and future directions in epidemiology and genomics. *Circulation* 2011; 124: 1982-1993.
- [8] Chen YH, Xu SJ, Bendahhou S. KCNQ1 gain-of-function mutation in familial atrial fibrillation. *Science* 2003; 299: 251-254.
- [9] Makiyama T, Akao M, Shizuta S, Doi T, Nishiyama K, Oka Y, Ohno S, Nishio Y, Tsuji K, Itoh H, Kimura T, Kita T and Horie M. A novel SCN5A gain-of-function mutation M1875T associated with familial atrial fibrillation. *J Am Coll Cardiol* 2008; 52: 1326-1334.
- [10] Yang Y, Li J, Lin X, Yang Y, Hong K, Wang L, Liu J, Li L, Yan D, Liang D, Xiao J, Jin H, Wu J, Zhang Y and Chen YH. Novel KCNA5 loss-of-function mutations responsible for atrial fibrillation. *J Hum Genet* 2009; 54: 277-283.
- [11] Olson TM, Alekseev AE, Liu XK, Park S, Zingman LV, Bienengraeber M, Sattiraju S, Ballew JD, Jahangir A and Terzic A. Kv1.5 channelopathy due to KCNA5 loss-of-function mutation causes human atrial fibrillation. *Hum Mol Genet* 2006; 15: 2185-2191.
- [12] Yang T, Yang P, Roden DM and Darbar D. Novel KCNA5 mutation implicates tyrosine kinase signaling in human atrial fibrillation. *Heart Rhythm* 2010; 7: 1246-1252.
- [13] Yang YQ, Li L, Wang J, Zhang XL, Li RG, Xu YJ, Tan HW, Wang XH, Jiang JQ, Fang WY and Liu X. GATA6 loss-of-function mutation in atrial fibrillation. *Eur J Med Genet* 2012; 55: 520-526.
- [14] Ellinor PT, Lunetta KL, Albert CM. Meta-analysis identifies six new susceptibility loci for atrial fibrillation. *Nat Genet* 2012; 44: 670-675.
- [15] Nishida K, Michael G, Dobrev D and Nattel S. animal models for atrial fibrillation: clinical insights and scientific opportunities. *Europace* 2010; 12: 160-172.
- [16] Dobrev D, Graf E, Wettwer E, Himmel HM, Hála O, Doerfel C, Christ T, Schüler S and Ravens U. Molecular basis of downregulation of G-protein-coupled inward rectifying K(+) current (I(K,ACh) in chronic human atrial fibrillation: decrease in GIRK4 mRNA correlates with reduced I(K,ACh) and muscarinic receptor-mediated shortening of action potentials. *Circulation* 2001; 104: 2551-2557.
- [17] Weiskirchen R, Meurer SK, Gressner OA, Herrmann J, Borkham-Kamphorst E and Gressner AM. BMP-7 as antagonist of organ fibrosis. *Front Biosci* 2009; 14: 4992-5012.
- [18] Adam O, Theobald K, Lavall D, Grube M, Kroemer HK, Ameling S, Schäfers HJ, Böhm M and Laufs U. Increased lysyl oxidase expression and collagen cross-linking during atrial fibrillation. *J Mol Cell Cardiol* 2011; 50: 678-685.
- [19] Wolke C, Bukowska A, Goette A and Lendeckel U. Redox control of cardiac remodeling in atrial fibrillation. *Biochim Biophys Acta* 2015; 1850: 1555-1565.
- [20] Lewkowicz J, Knapp M, Tankiewicz-Kwedlo A, Sawicki R, Kamińska M, Waszkiewicz E and Musiał WJ. MMP-9 in atrial remodeling in pa-

Lichen extracts decrease the risk of atrial fibrillation

- tients with atrial fibrillation. *Ann Cardiol Angeiol* 2015; 64: 285-291.
- [21] Pretorius M, Donahue BS, Yu C, Greelish JP, Roden DM and Brown NJ. Plasminogen activator inhibitor-1 as a predictor of postoperative atrial fibrillation after cardiopulmonary bypass. *Circulation* 2007; 116: 11-17.
- [22] Maass AH, De Jong AM, Smit MD, Gouweleeuw L, de Boer RA, Van Gilst WH and Van Gelder IC. Cardiac gene expression profiling - the quest for an atrium-specific biomarker. *Neth Heart J* 2010; 18: 610-614.
- [23] Fukui A, Takahashi N, Nakada C, Masaki T, Kume O, Shinohara T, Teshima Y, Hara M and Saikawa T. Role of leptin signaling in the pathogenesis of angiotensin II-mediated atrial fibrosis and fibrillation. *Circ Arrhythm Electrophysiol* 2013; 6: 402-409.
- [24] Jun JI and Lau LF. Taking aim at the extracellular matrix: CCN proteins as emerging therapeutic targets. *Nat Rev Drug Discov* 2011; 10: 945-963.
- [25] Hall-Glenn F and Lyons KM. Roles for CCN2 in normal physiological processes. *Cell Mol Life Sci* 2011; 68: 3209-3217.
- [26] Kubota S and Takigawa M. The role of CCN2 in cartilage and bone development. *J Cell Commun Signal* 2011; 5: 209-21.
- [27] Zuniga A, Haramis AP, McMahon AP and Zeller R. Signal relay by BMP antagonism controls the SHH/FGF4 feedback loop in vertebrate limb buds. *Nature* 1999; 401: 598-602.
- [28] Zuniga A, Michos O, Spitz F. Mouse limb deformity mutations disrupt a global control region within the large regulatory landscape required for Gremlin expression. *Genes Dev* 2004; 18: 1553-1564.
- [29] Bénazet JD, Bischofberger M, Tiecke E, Gonçalves A, Martin JF, Zuniga A, Naef F and Zeller R. A self-regulatory system of interlinked signaling feedback loops controls mouse limb patterning. *Science* 2009; 323: 1050-1053.
- [30] Retief E, Parker MI and Retief AE. Regional chromosome mapping of human collagen genes alpha 2(I) and alpha 1(I) (COLIA2 and COLIA1). *Hum Genet* 1985; 69: 304-308.
- [31] Wenstrup RJ, Cohn DH, Cohen T and Byers PH. Arginine for glycine substitution in the triple-helical domain of the products of one alpha 2(I) collagen allele (COL1A2) produces the osteogenesis imperfecta type IV phenotype. *J Biol Chem* 1988; 263: 7734-7740.
- [32] Hosper NA, van den Berg PP, de Rond S, Popa ER, Wilmer MJ, Masereeuw R and Bank RA. Epithelial-to-mesenchymal transition in fibrosis: collagen type I expression is highly upregulated after EMT, but does not contribute to collagen deposition. *Exp Cell Res* 2013; 319: 3000-3009.
- [33] Anumonwo JM and Kalifa J. Risk factors and genetics of atrial fibrillation. *Cardiol Clin* 2014; 32: 485-494.
- [34] Nguyen TN, Hilmer SN and Cumming RG. Review of epidemiology and management of atrial fibrillation in developing countries. *Int J Cardiol* 2013; 167: 2412-2420.
- [35] Yang JH, Li JH, Shao P, Zhou H, Chen YQ and Qu LH. starBase: a database for exploring microRNA-mRNA interaction maps from argonaute CLIP-Seq and degradome-Seq data. *Nucleic Acids Res* 2011; 39: 202-209.
- [36] Matsumoto S, Sakata Y, Suna S, Nakatani D, Usami M, Hara M, Kitamura T, Hamasaki T, Nanto S, Kawahara Y and Komuro I. Circulating p53-responsive microRNAs are predictive indicators of heart failure after acute myocardial infarction. *Circ Res* 2013; 113: 322-326.
- [37] Stamboul K, Lorin J, Lorgis L, Guenancia C, Beer JC, Touzery C, Rochette L, Vergely C, Cottin Y and Zeller M. Atrial fibrillation is associated with a marker of endothelial function and oxidative stress in patients with acute myocardial infarction. *PLoS One* 2015; 10: e0131439.
- [38] Jabre P, Jouven X, Adnet F, Thabut G, Bielinski SJ, Weston SA and Roger VL. Atrial fibrillation and death after myocardial infarction: a community study. *Circulation* 2011; 123: 2094-2100.
- [39] Schmitt J, Duray G, Gersh BJ and Hohnloser SH. Atrial fibrillation in acute myocardial infarction: a systematic review of the incidence, clinical features and prognostic implications. *Eur Heart J* 2009; 30: 1038-1045.
- [40] Stamboul K, Zeller M, Fauchier L, Gudjoncik A, Buffet P, Garnier F, Guenancia C, Lorgis L, Beer JC, Touzery C and Cottin Y. Incidence and prognostic significance of silent atrial fibrillation in acute myocardial infarction. *Int J Cardiol* 2014; 174: 611-617.
- [41] Shen R, Pan S, Qi S, Lin X and Cheng S. Epigenetic repression of microRNA-129-2 leads to overexpression of SOX4 in gastric cancer. *Biochem Biophys Res Commun* 2010; 394: 1047-1052.
- [42] Cardin S, Libby E, Pelletier P, Le Bouter S, Shiroshita-Takeshita A, Le Meur N, Léger J, Demolombe S, Ponton A, Glass L and Nattel S. Contrasting gene expression profiles in two canine models of atrial fibrillation. *Circ Res* 2007; 100: 425-433.
- [43] Chen LY and Shen WK. Epidemiology of atrial fibrillation: a current perspective. *Heart Rhythm* 2007; 4: 1-6.
- [44] Savelieva I, Kakouros N, Kourliouros A and Camm AJ. Upstream therapies for manage-

Lichen extracts decrease the risk of atrial fibrillation

- ment of atrial fibrillation: review of clinical evidence and implications for European Society of Cardiology guidelines. *Europace* 2011; 13: 308-328.
- [45] Husser D, Adams V, Piorkowski C, Hindricks G and Bollmann A. Chromosome 4q25 variants and atrial fibrillation recurrence after catheter ablation. *J Am Coll Cardiol* 2010; 55: 747-753.
- [46] Wutzler A, Kestler C, Perrot A, Loehr L, Huemer M, Parwani AS, Attanasio P, Özcelik C, Schunck WH, Gollasch M, Haverkamp W and Boldt LH. Variations in the human soluble epoxide hydrolase gene and recurrence of atrial fibrillation after catheter ablation. *Int J Cardiol* 2013; 168: 3647-3651.