Rs12976445 Polymorphism Is Associated with Post-Ablation Recurrence of Atrial Fibrillation by Modulating the Expression of MicroRNA-125a and Interleukin-6R

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Background: This study aimed to identify the relationship between miR-125a polymorphism rs12976445 and the post-ablation recurrence of atrial fibrillation (AF), as well as to explore the underlying mechanism of miR-125a in AF recurrence.

Material/Methods: Microarray analysis was performed to search for miRNAs potentially involved in the regulation of AF recurrence, while real-time PCR (polymerase chain reaction) and Western blot analyses were carried out to study the expression of miR-125a (microRNA-125a), IL-6R (interleukin-6 receptor), and IL-16 (interleukin-16) in different experimental groups, so as to understand the regulatory relationships among miR-125a, IL-6R, and IL-16. Subsequently, a logistic regression analysis was utilized to investigate the survival status of recurrent AF in subjects harboring different genotypes of rs12976445.

Results: The subjects in the GG and GC/CC groups of miR-125a polymorphism rs12976445 showed no obvious difference regarding all demographic characteristics that were collected in this study. In addition, 19 miRNAs were identified as potentially involved in the regulation of AF recurrence. Among these miRNAs, 6 were upregulated and 13 were downregulated in the group with early recurrence. According to real-time PCR results, the expression of miR-125a was dramatically upregulated in LRAF (late recurrence of atrial fibrillation) as well as in subjects harboring the GG genotype. On the contrary, the level of IL-6R mRNA was dramatically downregulated in LRAF and subjects harboring the GG genotype. Furthermore, IL-6R was confirmed as a candidate target of miR-125a by a luciferase reporter assay.

Conclusions: MicroRNA-125a polymorphism rs12976445 plays a role in AF recurrence via the regulation of IL-6R.

MeSH Keywords: Atrial Fibrillation • Interleukin-6 • Receptors, Interleukin-6

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Background

Atrial fibrillation (AF) is a persistent arrhythmia with high morbidity and mortality. The overall morbidity of AF can be up to 5.5% and its incidence is 9.9/1000 person-years, with both numbers growing with age. In a Rotterdam study, the morbidity of AF was 0.7% in people ages 55–59 years and its incidence was 1.1/1000 person-years. However, the morbidity of AF increased to 17.8% in people older than 85 years and its incidence increased to 20.7/1000 person-years in people ages 80–84 years [1]. In addition, the overall morbidity of AF and incidence in men (6% and 11.5/1000 person-years) are both higher than that in women of a comparable age (5.1% and 8.9/1000 person-years) [1].

Some pro-inflammatory factors, such as IL-6R, were reported to participate in the recurrence of AF after catheter ablation [2]. IL-6 plays significant roles in a wide range of biological processes. For example, the phosphorylation of the transcription factors STAT1 and STAT3 is induced via their homodimerization with gp130, a co-receptor of IL-6, and subsequent binding of IL-6 to the membrane-bound IL-6R [3]. Additionally, the binding of IL-6 to its free form receptor sIL-6R can activate cells expressing gp130 in the absence of membrane-bound IL-6R [4]. Genetic studies have discovered the role played by the genetic variant of IL6R in the pathogenesis of many human diseases, such as asthma, AF, rheumatoid arthritis (RA), and coronary heart disease (CHD) [5–8].

In addition, as a novel family of endogenous non-protein-coding RNAs that are single-stranded and about 22 nucleotides in length, microRNAs (miRNAs) play important regulatory roles in gene expression at the posttranscriptional level [9]. miRNAs can bind to partially complementary sites in the 3’ untranslated regions (UTRs) of target mRNAs to inhibit subsequent protein synthesis, and/or induce de-adenylation and consequent mRNA degradation [10]. In addition, some reports have demonstrated that a range of different microRNAs are involved in the occurrence of malignancies [11], while genetic variations in the 3’UTRs of miRNA target sites may impact and control the ability of miRNAs to regulate the expression of their target genes [12]. For example, multiple studies have revealed that the single-nucleotide polymorphisms (SNPs) in the miRNA target sites are related to the risk of malignancies, such as gastric, oral, lung and colorectal cancers, as well as leukemia [13–16], while a recent study has demonstrated the regulatory role of miRNAs in the development of AF [17]. In particular, rs12976445 is a SNP (single-nucleotide polymorphism) situated in the precursor of miR-125a that can compromise the production of miR-125a and lead to decreased miR-125a expression [18,19].

In previous computational analyses, we found that IL-6R is a candidate target gene of miR-125a in humans. Therefore, in the present study, we investigated the role of miR-125a and IL-6R in the development of recurrent AF post-catheter ablation and confirmed the association between the genotype of rs12976445 and AF recurrence.

Material and Methods

Sample collection

The Human Research Ethics Committees of our hospital approved this research. In total, 248 atrial, auricle, and peripheral blood samples were obtained from each AF patient in this study that had either early or late AF recurrence after catheter ablation. All study subjects were enrolled from the Department of Cardiology at our hospital. In this study, AF was diagnosed based on the European Society of Cardiology (ESC) Guidelines for the management of AF (Camm et al. 2010) and all patients underwent electrocardiography to determine the recurrence of AF. Demographic information of the subjects, such as family history, medical history, clinical symptoms, history of cigarette smoking, medication, blood pressure, weight, height, and waistline dimension, were collected from all subjects using standardized questionnaires. The patients diagnosed with hypertrophic cardiomyopathy, acute coronary syndrome, left ventricular dysfunction, and thyroid diseases, as well as nephrotic, renal, liver, or major valvular diseases, were excluded from our research. Written informed consent was obtained from all subjects or their first-degree relatives before the study started. The research process was in conformity with the latest vision of the Declaration of Helsinki.

Catheter ablation

In brief, integration of computed tomographic images, fluoroscopic 3-D catheter positioning, and the tagging of ablation sites were performed using a CARTO mapping system (CARTO, Biosense Webster) accompanied by the application of a regular coronary sinus lead. A temperature-controlled, deflectable, and quadripolar catheter (Navistar, Biosense Webster) containing an 8-mm tip was utilized to carried out the ablation.

Follow-up

Follow-up visits were carried out in our outpatient clinic for 12 months following the ablation. During the first 4 weeks after the operation, a 24-h Holter monitor and an event recorder, which was equipped with the ability to transmit signals of symptoms and daily routine indicators, were utilized to assess the status of AF recurrence. At 3, 6, 9, and 12 months following ablation, periodic monitoring was also carried out. An AF episode lasting longer than 30 s was defined as the onset of AF recurrence, while an AF episode occurred during the first 4
weeks following ablation was defined as an event of early recurrence of atrial fibrillation (ERAF). All AF episodes that occurred between 3 and 12 months following ablation were defined as the events of late recurrence of atrial fibrillation (LRAF).

MicroRNA microarray

A Human microRNA microarray V2 (Agilent Technologies, Santa Clara, California, USA) containing 76 human viral microRNAs and 723 human microRNAs (Sanger database V.10.1) was utilized to measure the microRNA expression profiles in the samples. A Mirna Labeling Reagent and a hybridization kit (Agilent Technologies, Santa Clara, California, USA) were utilized to pre-treat the samples. Microarray hybridization was carried out for 20 h at 55°C under a constant rotation of 20 rpm. An Agilent Microarray scanner G2565BA (Agilent Technologies, Santa Clara, CA) was utilized to detect hybridization signals, which were then quantified using Agilent Feature extraction software version 9.5.1 (Agilent Technologies).

TaqMan assay

A commercially available QuantStudio 12K Flex instrument (Thermo Fisher Scientific; formerly Life Technologies, Grand Island, NY) was used to perform TaqMan assays and identify the rs12976445 genotype of all samples.

miRNA isolation and quantitative real-time RT-PCR (qRT-PCR)

Trizol reagent (Invitrogen, CA, US) was used to extract total RNA from HL-1 and 293 cells or tissue samples. RNeasy Qiagen columns (Qiagen Inc., Valencia, CA) were used to purify the extracted RNA, which was then reversely transcribed into cDNA using a PrimeScript II 1st Strand cDNA Synthesis Kit (Takara Bio Inc., Japan) following a standard protocol. The relative expression of miR-125a was measured using qRT-PCR and a mirVanaTM qRT-PCR microRNA detection kit (Ambion, Austin, TX), while the relative expression of IL-6R and IL-16 mRNA was measured using qRT-PCR and a standard SYBR Green RT-PCR Kit (Takara, Otsu, Japan). The expression of miR-125a, IL-6R mRNA, and IL-16 mRNA was quantified using the 2−ΔΔCt method and GraphPad Prism 4.0 software (GraphPad Software, San Diego, CA). Each experiment was run in triplicate.

Cell culture and transfection

HL-1 and 293 cells were cultured at 37°C under 5% CO₂ in minimum essential media (MEM) (Hyclone, China) containing 10% fetal bovine serum (FBS) (Life Technologies, Grand Island, NY, USA), 100 μg/ml streptomycin, 100 units/ml penicillin (Hyclone, China), and 1.2% sodium pyruvate (Hyclone, China). All transfections were performed using Lipofectamine 2000 (Life Technologies, Grand Island, NY, USA) based on the manufacturer’s instruction. Each test was run 3 times.

Luciferase assay

The possible targets of miR-125a were predicted using TargetScan (http://www.targetscan.org/vert50/). The full-length 3’-UTRs of IL-6R and IL-16 genes containing the putative binding sites of miR-125a were inserted into firefly luciferase reporter plasmids. Similarly, the 3’-UTR of IL-6R that contained the mutated binding sequence of miR-125a was inserted into the same firefly luciferase reporter plasmid and served as the mutant construct. These constructs were then transfected into cells using FuGENE HD (Roche, Basel, Switzerland). At 48 h post-transfection, the cells were harvested and their Renilla and firefly luciferase activity was detected using a Dual-Luciferase Reporter Assay System (Promega, Madison, WI, US). All tests were run in triplicate.

Western blot analysis

Tissue samples, as well as HL-1 and 293 cells, were homogenized in an ice-cold protein extraction buffer (Boster Biological Technology, Wuhan, China) and the lysates were centrifuged at 15 000 rpm for 12 min. The supernatants were collected and their protein concentrations were detected using the Bradford method. The target proteins were separated by SDS-PAGE and electro-transferred onto a nitrocellulose membrane (PerkinElmer, Waltham, MA) using an electro-blotting apparatus purchased from Bio-Rad Laboratories (Richmond, CA). Subsequently, the membrane was blocked at room temperature for 90 min in a PBS buffer containing 5% nonfat dry milk before being incubated at 4°C for 12 h in a PBS buffer containing 1% nonfat dry milk, rabbit polyclonal anti-IL-6R, anti-IL-16 (1: 5000 dilution, Santa Cruz, Santacruz, CA) and anti-GAPDH (1: 10 000 dilution, Boster Biological Technology, Wuhan, China) antibodies, followed by an additional incubation at room temperature for 90 min in a PBS buffer containing 1% nonfat dry milk with HRP-labeled rabbit secondary antibodies (1: 12 000 dilution, Boster Biological Technology, Wuhan, China). A NBT/BCIP system was utilized to visualize protein signals, which were then quantified using Gene Snap software (Syngene, Cambridge, UK). Three independent experiments were performed.

Statistical analysis

All experiment results are expressed as mean ± standard deviation (SD). SPSS 15.0 software (SPSS, Chicago, IL) was used to carry out statistical analysis. The difference between 2 groups was analyzed using the t test and a P value of less than 0.05 was considered statistically significant.
miRNAs were differentially expressed in different groups

To identify the miRNAs potentially involved in the regulation of AF recurrence, we used a microRNAs microarray to measure and compare the miRNA expression profiles associated with samples collected from AF patients showing early or late AF recurrence after ablation. As shown in Figure 1, the expression of miR-125a, miR-675, miR-221, miR-29, miR-497, miR-200a, miR-1307, miR-139, miR-486, miR-4775, miR-141, miR-194, miR-215, miR-182, miR-377, miR-191, miR-488, miR-148, and miR-142 expression profiles between ERAF and LRAF.

miR-125a was differentially expressed between ERAF and LRAF groups

In this study, 15 patients with early AF recurrence (the ERAF group) and 15 patients with late AF recurrence (the LRAF group) were enrolled. Using real-time PCR, the expression of miR-125a was compared between the ERAF and LRAF groups, as well as among patients harboring the GG, GC, and CC genotypes of rs12976445. As shown in Figure 2, the LRAF group exhibited a higher level of miR-125a compared with the ERAF group (Figure 2A), while the GG group also displayed a higher level of miR-125a than that in the GC and CC groups (Figure 2B), suggesting that miR-125a is involved in AF recurrence after ablation, and the expression of miR-125a was reduced by the C allele.

IL-6R is a candidate gene of miR-125a

According to the result of our computational analysis, IL-6R and IL-16 were hypothesized as candidate targets of miR-125a because the 3’UTRs of IL-6R and IL-16 contained the complementary sequences for miR-125a (Figure 3A, 3C), which are the “seed sequences” that are highly conserved among different species. Subsequently, the above 4 sequences were mutated using a site-directed mutagenesis kit. As shown in Figure 3B, the first mutation located in the 3’UTR of IL-6R significantly repressed its luciferase activity, whereas the second mutation located in the 3’UTR of IL-6R (Figure 3B) and the 2 mutations located in the 3’UTR of IL-16 (Figure 3D) showed a comparable luciferase activity as that in the scramble control, indicating that miR-125a directly regulated IL-6R expression and this regulation was dependent on the location of the first mutation.
IL-6R was differentially expressed in different groups

As shown in Figure 4A and compared with the ERAF group, the LRAF group showed a lower level of IL-6R expression. In addition, when compared with the GC and CC groups, the GG group exhibited a lower expression of IL-6R mRNA (Figure 4B).

The regulatory relationship among miR-125a, IL-6R, and IL-16

RT-PCR was carried out to further examine the miRNA-mRNA regulatory relationship of miR-125a-IL-6R mRNA and miR-125a-IL-16 mRNA. As shown in Figure 5A, IL-6R mRNA expression was negatively correlated with miR-125a expression and the correlation coefficient was –0.50 (r=–0.50). In addition, miR-125a showed no effect on mRNA expression of IL-16 and the correlation coefficient was 0 (Figure 5B), indicating that miR-125a directly and negatively mediated IL-6R expression but had no effect on IL-16 expression.

**miR-125a regulated the expression of IL-6R**

To investigate the possible in vitro interactions among miR-125a, IL-6R, and IL-16, 2 cell lines (HL-1 and 293) were transfected with miR-125a mimics, miR-125a inhibitors, and IL-6R siRNA. The mRNA and protein expression of IL-6R and IL-16 in these cells was measured by qRT-PCR and Western blot analysis, respectively. As shown in Figure 6, the transfection of
miR-125a mimics and IL-6R siRNA into HL-1 (Figure 6A) and 293 (Figure 6C) cells evidently decreased the mRNA and protein level of IL-6R, but showed no effect on the mRNA and protein expression of IL-16 in these cells (Figure 6B, 6D). In addition, the transfection of miR-125a inhibitors into HL-1 (Figure 7A) and 293 (Figure 7C) cells evidently increased the mRNA and protein level of IL-6R, but showed no effect on the mRNA and protein expression of IL-16 in these cells (Figure 7B, 7D). These results collectively validated the hypothesis that IL-6R is a direct target of miR-125a, which significantly repressed the expression of IL-6R.

Genotypic association between rs12976445 and AF recurrence

A total of 248 AF patients were recruited to have their genotypes of miR-125a rs12976445 SNP determined. The results showed that 129 subjects were genotyped as GG, while 119 subjects were genotyped as GC or CC. The demographic characteristics, such as age, sex, presence of hypertension and diabetes, application of statins and relevant cardiac indicators, of these patients are shown in Table 1. No difference was observed among these patients in terms of age (P=0.63), sex (P=0.29), hypertension (P=0.47), diabetes (P=0.31), statins (P=0.88), ACEI/ARB (P=0.44), BB (P=0.87), paroxysmal (P=0.58), persistent (P=0.45), permanent (P=0.61), lone AF (P=0.06), LAD AF (P=0.66), and LVEF (P=0.41). In addition, a recessive model was used to carry out the survival analysis of AF recurrence in these subjects. As shown in Figure 8, the subjects harboring the GG genotype had a longer disease-free survival time compared with those harboring the GC and CC genotypes.

Discussion

In this study, we collected tissue samples from AF patients and used miRNA microarray analysis to identify differentially expressed miRNAs. We found that the level of miR-125a, miR-675, miR-221, miR-29, miR-497, and miR-200a was upregulated in the ERAF group, while the level of other 13 miRNAs, including miR-1307, miR-139, miR-486, miR-4775, miR-141, miR-194, miR-215, miR-182, miR-377, miR-191, miR-488, miR-148, and miR-142, was downregulated in the ERAF group. Among the above miRNAs, the expression of miR-125a in the ERAF group increased the most and hence the present study focused on the study of miR-125a.

Located on chromosome 19q13.41, miR-125a plays a critical role in both adult tissues and neonatal organ development [20]. Accumulating evidence has shown that miR-125a is associated with the etiology of many human disorders [21]. For instance, the downregulation of miR-125a was observed in some cancers, such as medulloblastoma, lung cancer, leukemia, gastric cancer, and breast cancer, in which the overexpression of miR-125a promoted cancer cell apoptosis and suppressed its proliferation [20–24]. In contrary, Kim et al. observed the upregulation of miR-125a in diffuse large B cell lymphoma, in which miR-125a increased NF-κB activity by inhibiting the expression of TNFAIP3 and hence facilitating the development of anti-apoptotic and malignant B cells [25]. It has also been demonstrated that both miR-125b and miR-125a could induce the irreversible differentiation of human pluripotent stem cells into glial progenitors [26, 27]. Nevertheless, the role of miR-125a in these cells has not been studied, although the expression of miR-125a was also observed in other neural cells, including oligodendrocytes [28]. Furthermore, an earlier study has revealed that the -5p arm of miR-125a acted as a critical modulator of brain endothelial integrity and its expression was elevated in MS brain lesions [29,30]. It has also been demonstrated that relapsing MS patients showed upregulated miR-125a-3p in the CSF when compared with control subjects [31]. In this study, we performed luciferase assay and cell transfection assay to confirm that IL-6R was a direct target of miR-125a.
Figure 6. RT-PCR and Western blot analyses were carried out to further explore the regulatory relationships among miR-125a, IL-6R, and IL-16 in HL-1 and 293 cells. (A) miR-125a and IL-6R siRNA evidently decreased IL-6R level in HL-1 cells. (B) miR-125a and IL-6R siRNA had no effect on IL-16 level in HL-1 cells. (C) miR-125a and IL-6R siRNA evidently decreased IL-6R level in 293 cells. (D) miR-125a and IL-6R siRNA had no effect on IL-16 level in 293 cells.

Figure 7. RT-PCR and Western blot analyses were carried out to further explore the regulatory relationships among miR-125a, IL-6R, and IL-16 in HL-1 and 293 cells. (A) miR-125a inhibitor evidently increased IL-6R level in HL-1 cells. (B) miR-125a inhibitor had no effect on IL-16 level in HL-1 cells. (C) miR-125a inhibitor evidently increased IL-6R level in 293 cells. (D) miR-125a inhibitor had no effect on IL-16 level in 293 cells.
Previous studies have identified miR-125a as a modulator of IL-6R expression. There are 2 hypothetical binding sites for miR-125a (621–627 bp and 91–99 bp) located within the 3'UTR of IL-6R [32]. In addition, IL-6 and its natural receptor, IL-6R, interact and transduce the IL-6 signal through gp130 [33]. The expression of IL-6R is limited to some cell types, such as some lymphocytes, macrophages, monocytes, and hepatocytes [33]. In contrast, nearly all types of cells express gp130. In addition, cells that do not express mIL-6R can still be activated by IL-6 through sIL-6R, which is generated by proteolytic cleavage or alternative splicing of mIL-6R [34]. Furthermore, the binding of sIL-6R to IL-6 renders cells lacking IL-6R responsive to the IL-6 signaling [33]. Additional, IL-6 and its receptor are associated with the development of a variety of inflammatory disorders, including rheumatoid arthritis (RA) and Crohn’s disease, as well as specific cancers, such as hepatocellular carcinoma and multiple myeloma [35]. Nevertheless, the relationship between the IL6R gene and AF has only been discovered recently and the role of inflammation in AF is controversial [36]. Moreover, circulating IL-6 and other pro-inflammatory agents, including fibrinogen and C-reactive protein, have also been implicated in AF [36]. A recent study on the genetic variants of the IL6 gene offered some preliminary data regarding the effect of the IL-6 system in the pathophysiology of AF [37], while the genetic variants in the IL6R locus are closely related to coronary artery disease and diabetes [38,39].

Previous studies have demonstrated that the pre-miR-125a gene harbors a SNP known as rs12976445, which is involved in the processing of pre-miR-125a into mature miRNA, thus elevating the risk of recurrent pregnancy loss and autoimmune thyroid problems [40–42]. Furthermore, rs12976445 is related to unfavorable prognosis in people with esophageal squamous cell carcinoma [41]. A recent report has also shown that the T allele of rs12976445 impairs the expression of mature miR-125a, thus increasing the expression of its target genes, including LIFR and ERBB2 [40,43]. In this study, we performed real-time PCR to determine whether miR-125a and IL-6R were differentially expressed in the ERAF and LRAF groups, as well as in the GG, GC, and CC groups. Our results revealed that miR-125a was significantly downregulated in the ERAF group and subjects genotyped as GG. Similarly, the expression of IL-6R was significantly increased in the ERAF group and subjects genotyped as GG. Furthermore, we recruited 248 AF patients in our study and found that the patients harboring the GG genotype had a longer disease-free survival time.

| Table 1. Demographic and clinicopathological characteristics of the participants of this study. |
|---|---|---|---|
| **Parehreters** | **GG (n=129)** | **GC/CC (n=119)** | **P value** |
| Age (years) | 55.1±8.5 | 56.7±10.4 | 0.57 |
| Sex, female n (%) | 61 (47.3) | 53 (44.5) | 0.66 |
| Hypertension n (%) | 38 (29.5) | 33 (27.7) | 0.76 |
| Diabetes n (%) | 14 (10.9) | 15 (12.6) | 0.67 |
| Statin n (%) | 26 (20.2) | 23 (19.3) | 0.87 |
| ACEI/ARB n (%) | 43 (33.3) | 35 (29.4) | 0.51 |
| BB n (%) | 25 (19.4) | 22 (18.5) | 0.96 |
| **AF category** | | | |
| Paroxysmal n (%) | 49 (40.0) | 43 (36.1) | |
| Persistent n (%) | 71 (55.0) | 59 (49.6) | |
| Permanent n (%) | 9 (5.0) | 17 (14.3) | 0.17 |
| Lone AF n (%) | 75 (58.1) | 73 (61.3) | 0.61 |
| LAD mm | 40.5±5.4 | 41.3±5.8 | 0.49 |
| LVEF% | 61.4±6.1 | 62.4±7.3 | 0.61 |

**Figure 8.** Patients with GG genotype had longer AF recurrence-free survival time compared to that in patients with GC+CC genotypes.

Previous studies have demonstrated that the pre-miR-125a gene harbors a SNP known as rs12976445, which is involved in the processing of pre-miR-125a into mature miRNA, thus elevating the risk of recurrent pregnancy loss and autoimmune thyroid problems [40–42]. Furthermore, rs12976445 is related to unfavorable prognosis in people with esophageal squamous cell carcinoma [41]. A recent report has also shown that the T allele of rs12976445 impairs the expression of mature miR-125a, thus increasing the expression of its target genes, including LIFR and ERBB2 [40,43]. In this study, we performed real-time PCR to determine whether miR-125a and IL-6R were differentially expressed in the ERAF and LRAF groups, as well as in the GG, GC, and CC groups. Our results revealed that miR-125a was significantly downregulated in the ERAF group and subjects genotyped as GG. Similarly, the expression of IL-6R was significantly increased in the ERAF group and subjects genotyped as GG. Furthermore, we recruited 248 AF patients in our study and found that the patients harboring the GG genotype had a longer disease-free survival time.
Conclusions

Our results showed that, by targeting IL-6R, miR-125a plays a key role in the development of AF, while the reduced expression of miR-125a promotes the recurrence of AF after catheter ablation. In addition, the downregulation of miR-125a can, at least partially, attributed to the presence of rs12976445 polymorphism, which compromises the processing of mature miRNA. In summary, our results demonstrate that miR-125a may become a promising and novel diagnostic and therapeutic target for the treatment of AF.

Conflict of interest

None.

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