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Original Paper

Circulating MiR-146a May be a Potential Biomarker of Coronary Heart Disease in Patients with Subclinical Hypothyroidism

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Key Words

Coronary heart disease • Subclinical hypothyroidism • Circulating miRNAs • microRNA-146a

Abstract

Background/Aims: Subclinical hypothyroidism (SCH) plays a crucial role in the development and progression of coronary heart disease (CHD). However, any associated changes in the circulating microRNAs (miRNAs) levels and slightly elevated thyroid stimulating hormone (TSH) levels in CHD patients are unknown. miR-146a is a well known miRNA associated with inflammatory autoimmune diseases. Here, we evaluated miR-146a expression in patients, with the goal of re-evaluating the effect of SCH on CHD. ***Methods:*** A total of 192 study subjects who underwent coronary angiography for either suspected or confirmed CHD were enrolled in 3 groups: CHD with SCH, CHD alone, and healthy controls. The circulating levels of miR-146a were quantified using qRT-PCR. ***Results:*** Levels of miR-146a were positively correlated with CHD severity, as indicated by the Gensini score (r=0.354). The relative expression of miR-146a in the CHD+SCH, CHD and healthy control groups was 2.223±0.827, 1.588±0.726 and 0.632±0.309, respectively. Plasma TSH levels were positively correlated with miR-146a levels (r=0.321). According to multivariate logistic regression analyses, miR-146a levels were associated with the incidence of CHD in patients with SCH. For diagnosing CHD, the area under the ROC curve (AUC) of miR-146a and TSH was 0.779 and 0.752, respectively. When the TSH and miR-146a levels were combined to form a composite panel, the AUC of the panel was 0.858. ***Conclusion:*** Plasma miR-146a levels correlated with the severity of coronary atherosclerosis and increased with TSH slightly elevated in patients with CHD. Thus, miR-146a may have good predictive value for CHD among individuals with elevated TSH levels.

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Introduction

Subclinical hypothyroidism (SCH) is defined as elevated thyroid stimulating hormone levels (TSH) above the upper limit of reference concomitant with normal free thyroid hormones [i.e., free T3 (FT3) and free T4 (FT4) [1]. The prevalence of SCH is relatively high and ranges from 4% to 20% among the adult population [1, 2]. SCH has various causes, the most common of which is chronic autoimmune thyroiditis (60% to 80%) [3].

The association between SCH and cardiovascular disease has received increasing attention in recent years [4, 5]. A Korean study demonstrated that SCH individuals with an intermediate-to-high risk of coronary heart disease (CHD) had higher coronary calcium scores than euthyroid individuals [6]. Higher TSH levels are associated with an increased risk of myocardial infarction in patients with established cardiovascular disease [7]. Moreover, SCH can cause abnormal lipid metabolism [8], oxidative stress [1], and endothelial dysfunction [9].

There is growing evidence showing that cardiovascular disease patients with SCH have increased cardiac-related and all-cause mortality [10, 11].

MicroRNAs (miRNAs) are endogenous, small (18-22 nucleotides) non-coding RNA molecules capable of regulating gene expression at the post-transcriptional level by binding to target mRNA, resulting in either mRNA degradation or translational repression [12]. MiRNAs are remarkably stable in circulating blood and may be useful as biomarkers for detecting disease [13]. miR-146a is a relatively well known miRNA in inflammatory autoimmune diseases [14, 15]. Studies have indicated that miR-146a plays an important role in the pathogenesis of several autoimmune disorders, such as rheumatoid arthritis, systemic lupus erythematosus, and multiple sclerosis [16-18]. miR-146a was also proven to be associated with the development of autoimmune thyroid diseases [19, 20]. miR-146a targets multiple genes in the Toll-like receptor 4 (TLR4)/nuclear factor-κB (NF-κB) pathway, an important immune and inflammatory response pathway, that regulates the inflammatory response [21]. Furthermore, miR-146a is also expressed in vascular endothelial cells, smooth muscle cells and monocytes/macrophages, and regulates the development of atherosclerosis by acting on different target genes [22-24].

Endothelial dysfunction, which is an early step of the atherosclerotic process, has been reported in patients with SCH [25]. In this study, we assessed miR-146a expression in patients with CHD and slightly elevated TSH levels as a novel perspective for re-evaluating the effects of SCH on CHD.

Materials and Methods

Study design

We enrolled 192 consecutive patients who underwent coronary angiography for either suspected or confirmed CHD at The First Hospital of Xi'an between October 2015 and September 2016. In this study, all subjects were patients with newly diagnosed SCH who did not receive any intervention. SCH is defined as elevated TSH levels above the normal upper limit concomitant with normal levels of free T4 [26]. The exclusion criteria were: (1) myocardial bridge, cardiomyopathy, valvular heart disease, acute myocardial infarction; (2) clinical hyperthyroidism and hypothyroidism, hypothalamic or pituitary disease and other endocrine diseases; (3) the presence of cancer, acute cerebrovascular disease, severe infection, liver or kidney dysfunction, and hereditary hyperlipidemia; and (4) using medications that affect thyroid function (such as an amine iodine ketones and other iodine-containing drugs, lithium agents, hormone preparations, interferon, phenytoin, dopamine and hormones) within the previous three months prior to enrollment.

The research protocol was approved by the Ethics Committee at Xi'an First People's Hospital. All the recruited patients provided their informed consent prior to their inclusion in the study.

Clinical parameters

The demographic and clinical parameters assessed were age, gender, and coronary risk factors, which consisted of hypertension, diabetes mellitus (DM), dyslipidemia, current smoking, and body mass index (BMI). Hypertension was defined as blood pressure ≥ 140/90 mmHg or the use of antihypertensive medications. Diabetes was defined as a fasting blood glucose level ≥7.0 mmol/L or a diagnosis of diabetes requiring diet or anti-diabetic therapy. Hyperlipidemia was defined as a total cholesterol (TC) level of ≥5.72 mmol/L and/or a triglyceride (TG) level of ≥1.70 mmol/L, or treatment with a lipid-lowering medication [27]. Individuals who formerly or currently smoked ≥10 cigarettes per day for at least 2 years were defined as smokers. We used standard laboratory protocols to determination the fasting glucose (FPG), TC, TG, low-density lipoprotein cholesterol (LDL-c), high-density lipoprotein cholesterol (HDL-c), high-sensitivity C-reactive protein (hs-CRP) and serum creatinine (Scr) levels. FT3, FT4 and TSH levels were measured using an electrochemical luminescence method with E170 (Elecsys module) immunoassay analyzers (Roche Diagnostics, Mannheim, Germany). The reference intervals for FT3, FT4 and TSH were 3.1-6.8 pmol/L, 12-22 pmol/L, and 0.27-4.2 IU/mL, respectively.

Coronary angiography and Gensini score

Coronary angiography was performed via radial artery access using the Judkins technique and the angiograms were visually evaluated by two experienced cardiologists who were blinded to the participants and to the other's decisions. Coronary artery disease was defined as >50% stenosis in the luminal diameter of at least one major epicardial coronary artery [28].

The Gensini scoring system was utilized to quantify the severity of CHD [29]. When the severity of coronary artery stenosis was 25%, 50%, 75%, 90%, 99% or complete, the assigned score was 1, 2, 4, 8, 16 and 32 points, respectively. The coefficient was determined based on the locations of coronary artery lesions: the left main coronary artery ×5; the proximal segment of left anterior descending coronary artery (LAD) ×2.5; the proximal segment of the circumflex artery ×2.5; the mid-segment of the LAD ×1.5; the right coronary artery, the distal segment of the LAD, the posterolateral artery, and the obtuse marginal artery ×1; and all others ×0.5.

RNA isolation

Fasting blood samples (5 mL) were collected in EDTA coated tubes in the early morning. Samples were separated by centrifugation at 3000 g for 15 min. A further centrifugation (1200 g for 15 min at 4°C) was performed to remove cell debris and then stored at −80°C until use.

Total RNA containing miRNAs was isolated from plasma using the miRNeasy serum/plasma kit (TIANGEN: catalog number DP503, China). Briefly, 200 μL of plasma were mixed with denaturing buffer in the volumes according to the manufacturer's instructions. The homogenate was incubated for 5 min at room temperature, 25 fmol of synthetic cel-miR-39 (TIANGEN; catalog number: CD200-01, China) was spiked in. Subsequently, the RNA was extracted according to the manufacturer's protocols. Total RNA was eluted in 30 μL of RNase-free water. The level of hemolysis in plasma samples was assessed by spectrophotometry (NanoPhotometer P300, Implen).

Reverse transcription (RT) and quantitative PCR (qPCR)

RNA was reverse transcribed to cDNA with reverse transcriptase kit (TIANGEN; catalog number: KR211, China). The reaction system contained total RNA 2 μg, miRNA RT reaction buffer 10 μL, Enzyme Mix 2 μL, RNase-free water up to 20 μL. The mixture was incubated at 42°C for 60 min, 95°C for 3 min, and then held at 4°C. A no-RT negative control was included in each experiment to ensure that PCR products were not due to contamination by genomic DNA.

Plasma miR-146a quantification was measured by SYBR Green-based real-time PCR using a miScript SYBR Green PCR kit (TIANGEN; catalog number FP411，China). The reaction contained 2×miRcute Plus miRNA Premix 10 μL, 0.2 μL PCR Forward Primer, 0.4 μL PCR Reverse Primer, 3.0 μL cDNA, RNase-free water up to 20 μL. The reactions were incubated at 95°C for 15 min, 94°C for 20 sec, 60°C for 30 sec, 72°C for 34 sec. All reactions were run in duplicate. The average of the Ct value was calculated after the PCRs were run in duplicate for each sample. The cel-miR-39 value from the duplicate was used as the internal control [13, 30]. The relative expression of each miR-146a after normalization to cel-miR-39 is displayed as $2^{-[Ct\ (miRNA)-\ Ct\ (cel\text{-}miR\text{-}39)]}$.

Statistical analysis

Statistical analyses were performed using SPSS version 18.0 software. Continuous variables were expressed as the mean ± SD, and categorical variables were expressed as numbers and percentages. Student's t-test was used to compare continuous variables, and the Chi-square test was used to compare categorical variables. For data that were normally distributed, one-way ANOVA and the least significant differences (LSD) post hoc multiple comparisons test were applied, whereas the Kruskal-Wallis test was performed to compare data that were not normally distributed. The Spearman correlation test was performed to explore the relationships between TSH, miR-146a and Gensini score. Univariate analysis and multivariate logistic regression analysis were performed to determine the variables that independently contributed to the presence of CHD. The receiver operating characteristic (ROC) curve analysis was performed with plasma miR-146a and TSH to distinguish between CHD patients and healthy controls. The AUC was estimated to assess the diagnostic performance of miR-146a and TSH. All tests were two-sided, and P<0.05 was considered statistically significant.

Results

Clinical characteristics of the patients

A total of 192 individuals participated in this study. The clinical characteristics of the CHD+SCH patients (n=60), CHD patients (n=73) and healthy controls (n=59) are listed in Table 1. No significant differences were observed regarding gender, BMI, SBP, DBP, medications, HDL-c, FPG, Scr and FT4 among the 3 groups (P>0.05). Patients in CHD+SCH group (69.83±9.21) were significantly older than those in the CHD group (65.67±11.59) and healthy control groups (59.67±9.86). The concentrations of TC, TG, LDL-c and hs-CRP were highest in the SCH+CHD compared to the CHD and control groups (P<0.05). The FT3 levels in CHD+SCH patients (3.82±0.68) were lower than those in CHD patients (4.16±1.72) and healthy controls (4.59±0.74). The TSH and Gensini score in CHD+SCH patients were higher than those in CHD patients and healthy controls (P<0.05).

Table 1. Clinical characteristics of each group. ACEI, angiotensin converting enzyme inhibitors; ARB, angiotensin II receptor blockers. The values are expressed as either the mean±SD or numbers and percentages. P<0.05 was considered statistically significant

	Controls	CHD	CHD+SCH	P Value
Gender (M/F)	59 (32/25)	73 (42/31)	60 (35/25)	0.863
Age (years)	59.67±9.86	65.67±11.59	69.83±9.21	<0.001
SBP (mmHg)	135.09±28.01	134.03±19.95	138.13±19.88	0.433
DBP (mmHg)	81.03±15.22	78.95±12.53	76.98±9.5	0.698
BMI (kg/m^2)	23.55±3.82	23.55±3.59	24.13±3.48	0.566
Risk factors				
Smoking (%)	13 (22.03)	16 (21.92)	13 (21.67)	0.899
Hypertension (%)	24 (40.68)	26 (35.62)	22 (36.67)	0.828
Diabetes (%)	10 (16.95)	13 (17.81)	10 (16.67)	0.984
Hyperlipidemia (%)	19 (32.20)	16 (21.92)	21 (35.00)	0.655
Medications				
Aspirin (%)	48 (81.36)	65 (89.04)	55 (91.67)	0.100
Beta-blocker (%)	13 (22.03)	21 (28.77)	19 (31.67)	0.486
ACEI/ARB (%)	18 (30.51)	26 (35.62)	23 (38.33)	0.664
Statins (%)	42 (71.19)	58 (79.45)	50 (83.33)	0.182
Laboratory parameters				
TC (mmol/L)	3.83±0.84	3.91±0.94	4.52±1.02	0.003
TG (mmol/L)	1.41±0.82	1.46±0.89	1.72±0.69	0.034
HDL-c (mmol/L)	1.14±0.29	1.11±0.31	1.08±0.28	0.591
LDL-c (mmol/L)	2.19±0.83	2.46±0.87	2.86±0.94	0.005
FPG (mmol/L)	6.31±3.12	5.72±2.41	5.88±1.69	0.496
Scr (μmol/L)	78.48±18.65	78.69±19.55	82.63±19.67	0.445
hs-CRP (mg/L)	1.28±0.43	1.89±0.62	2.39±0.97	<0.001
FT3 (mmol/L)	4.59±0.74	4.16±1.72	3.82±0.68	0.011
FT4 (mmol/L)	16.03±2.29	15.79±3.66	15.33±2.54	0.113
TSH (mIU/mL)	2.11±0.93	2.31±1.01	7.01±2.39	<0.001
Gensini score	1.45±1.36	22.74±7.76	31.59±8.62	<0.001

The correlation of plasma miR-146a levels with the severity of coronary atherosclerosis

The Spearman correlation analysis demonstrated positive correlations of miR-146a levels with the severity of CHD, as quantified by the Gensini score (r=0.354, P=0.002; Fig. 1A).

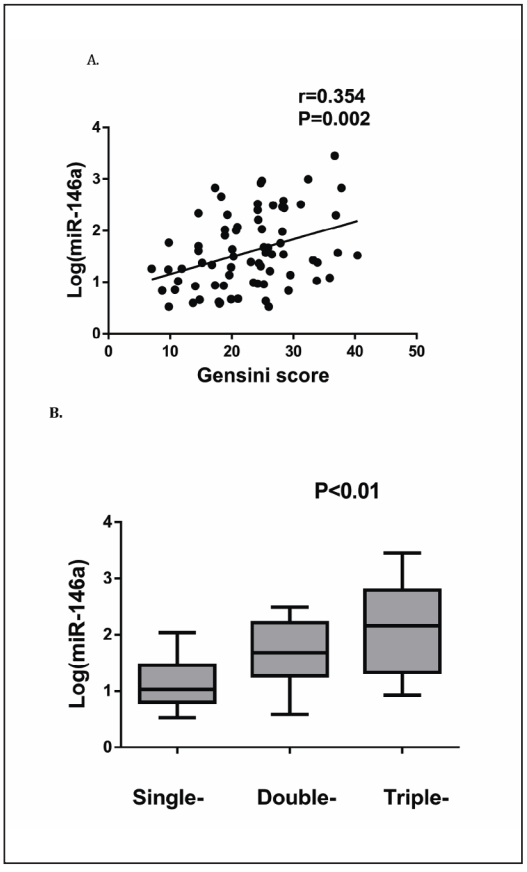

Fig. 1. Correlation between miRNA-146a levels and CHD severity. The correlation between miRNA-146a levels and CHD severity: (A) The Spearman's correlation analysis showed that the plasma miR-146a levels were positively correlated with the severity of coronary stenosis (r=0.354, P=0.002); (B) The plasma miR-146a levels increased as the number of affected vessels increased (P<0.01).

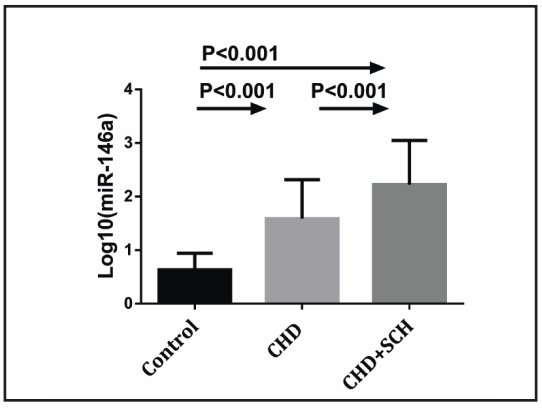

Fig. 2. Plasma levels of miR-146a among the 3 groups. The circulating levels of miR-146a (shown in a log10 scale) in the healthy controls, CHD and CHD+SCH groups.

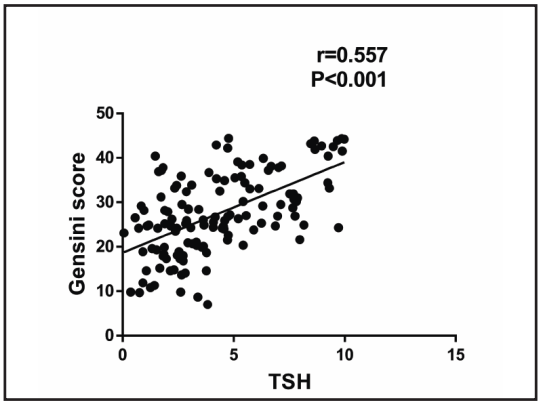

Fig. 3. Correlation between TSH levels and Gensini score. The correlation between the TSH levels and Gensini score in CHD and CHD+SCH patients. The plasma levels of TSH were positively correlated with the Gensini score (r=0.557, P<0.001).

CHD patients were subdivided into 3 subgroups (single-, double- and triple-vessel disease) based on the number of affected coronary arteries. As the number of affected vessels increased, the plasma miR-146a levels significantly increased (means ± SD: 1.124±0.422 vs. 1.654±0.562 vs. 2.077±0.756, respectively; P<0.01; Fig. 1B).

Expression levels of miR-146a among the 3 groups

Compared with those of healthy controls, CHD and CHD+SCH patients exhibited significantly increased levels of circulating miR-146a, with the levels in the CHD+SCH patients showing the largest increase (P<0.001; Fig. 2). The means ± SD of the relative miR-146a expression were 2.223±0.827 for CHD+SCH patients, 1.588±0.726 for CHD patients and 0.632±0.309 for healthy controls.

Correlation between TSH and Gensini score

Serum TSH levels were significantly correlated with the Gensini score in CHD and CHD+SCH patients (n=133, r=0.557, P<0.001) (Fig. 3).

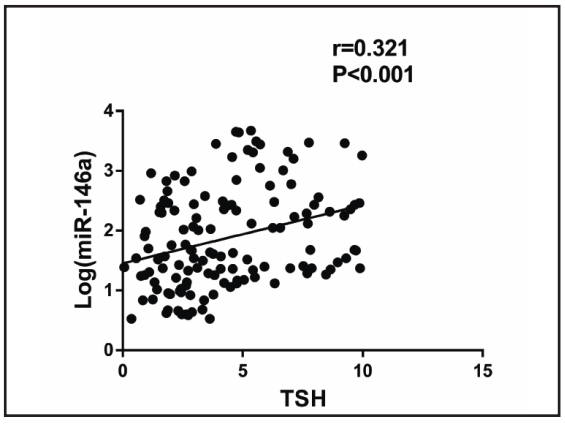

Fig. 4. Correlation between TSH and miR-146a levels. Correlation between TSH and miR-146a levels in the CHD and CHD+SCH groups. The plasma levels of TSH were positively correlated with miR-146a expression (r=0.321, P<0.001).

Table 2. Univariate analysis and multiple logistic regression analysis for the risk of CHD in patients with SCH. [a]Model 1: Adjusted for age, gender and BMI. [b]Model 2: Adjusted for model 1, smoking, hypertension, diabetes and hyperlipidemia. [c]Model 3: Adjusted for model 2, TC, TG, LDL-c, hs-CRP and TSH

Circulating miR-146a	OR (95% CI)	P-value
Univariate analysis	2.277 (1.385-3.742)	0.009
Adjusted, model 1[a]	2.067 (1.141-3.744)	0.017
Adjusted, model 2[b]	2.001 (1.073-3.730)	0.029
Adjusted, model 3[c]	2.530 (1.231-4.202)	0.012

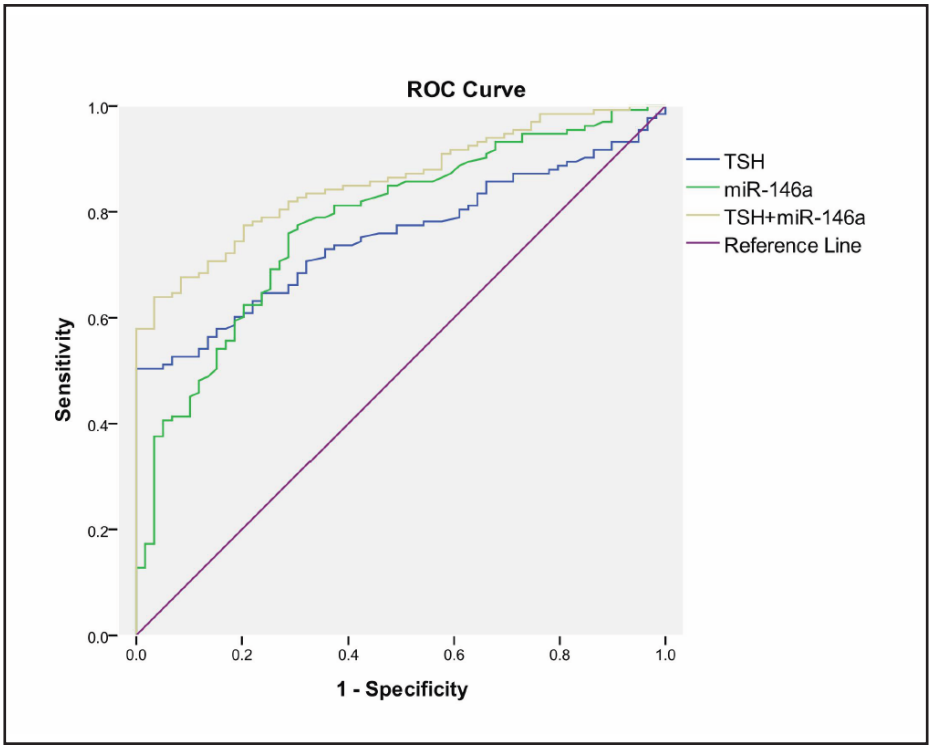

Fig. 5. ROC curves for the predictive value of miR-146a and TSH regarding CHD. The ROC curves for the predictive values of miR-146a, TSH and TSH+miR-146a to identify CHD patients from healthy controls.

Correlation between TSH and miR-146a levels

The results showed that the levels of circulating miR-146a were significantly correlated with TSH in the CHD and CHD+SCH groups (r=0.321, P<0.001; Fig. 4).

Increased plasma levels of miR-146a are associated with the presence of CHD in patients with SCH

Following further analysis using multivariate logistic regression with adjustment for age, gender and BMI, additionally for smoking, hypertension, diabetes and hyperlipidemia, and further for TC, TG, LDL-c, hs-CRP and TSH, miR-146a levels were independently associated with CHD (Table 2).

ROC curve for CHD

We performed a ROC analysis to determine the predictive values of miR-146a and TSH for CHD. The AUCs of miR-146a and TSH were 0.779 (95% CI 0.711-0.848; P<0.001) and 0.752 (95% CI 0.686-0.818; P<0.001), respectively. Importantly, when the TSH and miR-146a values were combined to form a composite panel, the resulting AUC was 0.858 (95% CI 0.808-0.908; P<0.001). This indicated that the diagnostic performance of the TSH and miR-146a panel was superior to either TSH or miR-146a alone (Fig. 5).

Discussion

Thyroid hormone plays a major role in cardiovascular system function and helps maintain cardiovascular homeostasis [10]. The results of our analysis showed that the FT3 levels in CHD+SCH patients were lower than those in CHD patients and healthy controls. However, both simple and multivariate logistic regression analyses indicated that FT3 was not associated with CHD. These data are inconsistent with the results of other studies that have assessed these associations [31-33]. We thought that discrepancy might originate from the study design, i.e., the study subjects and inclusion criteria. We also observed that patients in the CHD+SCH group were older than patients in the CHD group and healthy controls. Colorado reported that TSH increased with age for men and women [34], with Hollowell et al [35]. confirming these results. Thus, in patients older than 60 years of age, a diagnosis of SCH poses a challenge whether this change is a normal adaptive response associated with senescence or reflects an actual increase in abnormal function requires further study. Hypercholesterolemia and LDL-c levels are two important factors that contribute to the association between hypothyroidism and CHD [3, 36-38], and our results are in accordance with previous findings.

SCH is a well-known risk factor for atherosclerosis. The present study demonstrated that high serum TSH levels are associated with the Gensini score and that the serum TSH levels could predict CHD. The notion that SCH serves as a precursor to CHD may be related to the following factors. Most studies have suggested that patients with SCH have abnormal blood lipid levels, which may accelerate atherosclerosis [39-41]. It has also been proposed that the coagulation and fibrinolytic systems are disturbed in patients with SCH [42, 43]. Moreover, patients with SCH tended to exhibit increased platelet activation and platelet activity which enhances the risk of atherothrombosis in CHD [44]. Furthermore, SCH could contribute to endothelial dysfunction in atherosclerosis due to decreases in the levels of nitric oxide, a vascular endothelium-derived relaxant factor involved in the pathophysiology of the cardiovascular system [45].

An accumulating number of miRNAs, including miR-181a, miR-30d, miR-126-5p, miR-221, miR-130a, miR-155, have been showed potential as biomarkers for cardiovascular diseases [46-49]. miR-146a was the first miRNA found to exert a regulatory role in inflammatory autoimmune diseases. In this study, we used the Gensini score to assess CHD severity and found that plasma miR-146a levels were positively correlated with the Gensini score, indicating that plasma levels of miR-146a increased as the severity of CAD increased. This suggests that miR-146a may be released into the coronary circulation from vulnerable coronary plaques and therefore may be useful as a biomarker of CHD.

These findings are compatible with those of a previous study showing that the expression of miR-146a is highly up-regulated in atherosclerotic plaques [50]. Oerlemans et al. showed,

using real-time quantitative PCR analysis, that miR-146a expression levels in acute coronary syndrome patients were significantly higher than those in patients with unstable angina with the highest expression levels in patients with non-ST-segment elevation myocardial infarction [51]. Patients with CHD were randomly assigned to 12 months of combined treatment with atorvastatin and telmisartan or enalapril. Peripheral blood mononuclear cells were obtained from peripheral blood at baseline and after 12 months. At baseline, the levels of miR-146a/b were significantly higher in the CHD group than those in the non-CHD group. After 12 months of combined treatment, these levels were markedly decreased in both the statin/ARB and statin/ACEI groups. During the 12-month follow-up period, the levels of miR-146a/b decreased in CHD patients [52]. Dong et al. reported that miR-146a expression was increased in proliferative vascular smooth muscle cells [23], whereas if miR-146a was knocked down *in vitro*, vascular smooth muscle cell proliferation and migration were inhibited. Using gene chip and real-time quantitative PCR analysis, Vasa-Nicotera et al. observed that aging human umbilical vein endothelial cells have significantly reduced miR-146a expression [53], which results in increased expression of the target gene NOX4, elevated levels of reactive oxygen species, and the accelerated atherosclerosis progression.

In the current study, we found that the plasma levels of miR-146a were positively correlated with TSH and that the increased miR-146a and TSH levels were associated with the presence of CHD independent of other established cardiovascular risk factors. Zhang et al. reported that patients with SCH present atherosclerosis-specific circulating miRNA expression profiles [54]. Tian et al. found that elevated TSH may cause endothelial cells dysfunction [55, 56], stimulate vascular smooth muscle cell proliferation, therefore, we speculated that elevated TSH levels increase the expression of miR-146a in cells, which induces an inflammatory response via NF-κB signaling [21] and promotes the occurrence and development of atherosclerosis. These phenomena require further study *in vitro.*

This study had several limitations. First, this study did not report serum thyroid antibody levels, and TSH levels were only measured once. Second, this was a cross-sectional study that suggested hypotheses but failed to describe the relationship between the putative cause and effect. Third, this was single-center study involving a small number of patients, and large-scale multicenter studies are necessary. Finally, the mechanisms of miR-146a that regulate CHD in humans were not fully elucidated. The potential role of miR-146a and TSH in CHD needs to be further determined via basic research.

Conclusion

In conclusion, the present study may provide new insight into the significance of circulating miRNA expression profiles in predicting CHD among patients with SCH. The results of this study showed that plasma miR-146a levels correlated with the severity of coronary atherosclerosis. Furthermore, plasma miR-146a levels increased with slight elevations in TSH in CHD patients. Therefore, miR-146a may have good predictive value for CHD among individuals with elevated TSH levels.

Acknowledgements

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Disclosure Statement

No potential conflicts of interest were disclosed.

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