Expression of miR-146a/b is associated with the Toll-like receptor 4 signal in coronary artery disease: effect of renin–angiotensin system blockade and statins on miRNA-146a/b and Toll-like receptor 4 levels

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ABSTRACT

The TLR4 (Toll-like receptor 4) signal plays an important role in immunity in CAD (coronary artery disease). miR-146a/b (where miR is microRNA) regulates the TLR4 downstream molecules IRAK1 (interleukin-1-receptor-associated kinase 1) and TRAF6 (tumour-necrosis-factor-receptor-associated factor 6). It has also been reported that statins and RAS (renin–angiotensin system) inhibition and have anti-atherosclerotic properties. In the present study, we have investigated whether miR-146a/b was expressed with the TLR4 signal in CAD patients, and whether combined treatment with a statin and RAS inhibition might affect these levels. A total of 66 patients with CAD and 33 subjects without CAD (non-CAD) were enrolled. Patients with CAD were randomized to 12 months of combined treatment with atorvastatin and telmisartan [an ARB (angiotensin II receptor blocker)] or atorvastatin and enalapril [an ACEI (angiotensin-converting enzyme inhibitor)]. PBMCs (peripheral blood mononuclear cells) were obtained from peripheral blood at baseline and after 12 months. Levels of miR-146a/b, IRAK1 mRNA, TRAF6 mRNA and TLR4 mRNA/TLR4 protein were significantly higher in the CAD group than in the non-CAD group (all \( P < 0.01 \)). Levels of miR-146a/b were positively correlated with IRAK1 mRNA and TRAF6 mRNA levels. After 12 months of treatment, these levels were markedly decreased in the ARB and ACEI groups, with the decrease in the ARB group being greater than that in the ACEI group (all \( P < 0.05 \)). In our 12-month follow-up study, high levels of miR-146a and TLR4 mRNA/TLR4 protein at baseline were independent predictors of cardiac events. The present study demonstrates that combined treatment with an ARB and a statin decreases miR-146a/b and the TLR4 signal in CAD patients, possibly contributing to the anti-atherogenic effects of ARBs and statins in this disorder.

Key words: angiotensin-converting enzyme inhibitor (ACEI), angiotensin II receptor blocker (ARB), atherosclerosis, interleukin-1-receptor-associated kinase 1 (IRAK1), microRNA (miRNA), Toll-like receptor 4 (TLR4), tumour-necrosis-factor-receptor-associated factor 6 (TRAF6).

Abbreviations: ACEI, angiotensin-converting enzyme inhibitors; ACS, acute coronary syndrome; AngII, angiotensin II; ARB, AngII receptor blocker; BMI, body mass index; BP, blood pressure; CAD, coronary artery disease; CI, confidence interval; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HbA1c, glycated haemoglobin; HDL, high-density lipoprotein; hsCRP, high-sensitivity C-reactive protein; IL-1, interleukin-1; IRAK1, IL-1 receptor-associated kinase 1; LDL, low-density lipoprotein; MFI, mean fluorescence intensity; miRNA (miR), microRNA; NF-κB, nuclear factor κB; PBMC, peripheral blood mononuclear cell; PCI, percutaneous coronary intervention; RAS, renin–angiotensin system; t-BHP, t-butyl hydroperoxide; TLR, Toll-like receptor; TNF, tumour necrosis factor; TRAF6, TNF-receptor-associated factor.

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INTRODUCTION

TLRs (Toll-like receptors) have been identified as key recognition components of pathogen-associated molecular patterns controlling innate immune responses in mammals [1]. Our previous reports have demonstrated that activation of the TLR4 signal is involved in the downstream release of inflammatory cytokines in circulating monocytes obtained from patients with CAD (coronary artery disease) [2,3]. It has therefore been suggested that a potential pathophysiological link exists between the TLR4 signal and immune response in coronary atherosclerosis.

It has been reported that miRNAs/miRs (microRNAs) are the most abundant family of small non-coding RNAs and regulate mRNA translation of target genes through the RNA interference pathway [4,5]. Taganov et al. [6] have demonstrated a role for one human miRNA family, miR-146, in controlling TLR4 and its downstream cytokine signalling in a human monocytic cell line. In particular, miR-146a/b regulates the NF-κB (nuclear factor κB)-dependent genes, such as IRAK1 [IL-1 (interleukin-1)-receptor-associated kinase 1] and TRAF6 [TNF (tumour necrosis factor)-receptor-associated factor 6], through a negative-feedback regulation loop [6]. Recent reports have indicated elevated miR-146 expression in patients with chronic inflammatory diseases, such as rheumatoid arthritis and psoriasis, which have been shown to have increased levels of inflammatory cytokines [7,8]. It has become evident that coronary atherosclerosis is an inflammatory disease involving an immune response during its initiation and progression [9]. However, it is unclear whether monocytic expression of miR-146a/b is related to the TLR4 signal in patients with CAD.

Clinical studies have reported that RAS (renin–angiotensin system) blockades including ARBs [AngII (angiotensin II) receptor blockers] and ACEIs (angiotensin-converting enzyme inhibitors), have an anti-atherosclerotic effect in patients with cardiovascular risk factors or CAD [10,11]. A recent report found that ARB treatment reduced TLR4 expression in PBMCs (peripheral blood mononuclear cells) obtained from patients with CAD [12]. It has also been reported that statins (3-hydroxy-3-methylglutaryl-CoA reductase inhibitors) have pleiotropic effects including anti-inflammatory properties [13]. Moreover, an experimental study has shown that RAS inhibitors and statins have a combined effect on inflammatory profiles, such as inflammatory cytokines and atherosclerosis [14]. However, it remains uncertain whether combined treatment with RAS inhibitors and statins affects the TLR4 signalling pathway in patients with CAD.

In the present study, our aim was to determine whether miR-146a/b is expressed with its target genes (IRAK1 and TRAF6) and TLR4, and whether it could be modified by combined treatment with RAS inhibition and a statin by comparing the effects of an ARB with those of an ACEI in patients with CAD.

MATERIALS AND METHODS

Study population

A total of 66 patients with stable CAD were admitted to our hospital for PCI (percutaneous coronary intervention) and stent implantation, and none had previous exposure to treatment with RAS inhibitors and statins. All of the enrolled patients underwent initial diagnostic coronary angiography. CAD was diagnosed on the basis of (i) a history of typical chest pain on effort, (ii) documented exercise-induced myocardial ischaemia, (iii) angiographically proven CAD, and (iv) the absence of ACS (acute coronary syndrome) for 3 months before blood sampling. Patients were excluded from the study if they had clinical signs of acute infection, severe renal failure (serum levels of creatine >3 mg/dl), secondary hypertension or rheumatoid disease, or if they were suspected of having a malignant or primary wasting disorder.

The non-CAD group comprised 33 subjects with suspected CAD on the basis of symptoms and/or minor ECG changes. Subsequent coronary angiography and close clinical examination failed to show any evidence of CAD, and these subjects were thus designated as non-CAD. In addition, blood samples were obtained from five healthy volunteers for in vitro study (five males; age, 39.3 ± 9.5 years).

Approval for the study protocol was obtained from the ethics committee of the Iwate Medical University School of Medicine (H17-73). All patients agreed to participate in the study and written informed consent was obtained in each case.

Study design

The present study was designed as a prospective randomized investigator-blinded study. The investigators were blinded to the allocation of each subject’s treatment group and thus did not know whether patients were receiving the study medication. Patient with CAD who met all of the eligibility criteria were randomly assigned using computer-generated random numbers in a 1:1 ratio to receive telmisartan (40 mg/day) and atorvastatin (10 mg/day) (ARB group) or enalapril (5 mg/day) and atorvastatin (10 mg/day) (ACEI group) for a period of 12 months.

Blood sampling

Fasting peripheral blood was collected from patients with CAD in the morning after an overnight fast before PCI and treatment with the RAS inhibitors and the statin for baseline data and again after 12 months of treatment.
with RAS inhibitors and the statin. Fasting peripheral blood was also collected from the non-CAD group in the morning after an overnight fast.

**Clinical follow-up study**

Follow-up coronary angiography was carried out at least 12 months after PCI in all patients with CAD. Cardiac events were defined as target lesion revascularization (the lesion segment with 5 mm margins from each end), target or other vessel revascularization, cardiac death and ACS.

**Cell preparation**

PBMCs were isolated from peripheral blood samples obtained from all subjects by Ficoll–Paque density gradient centrifugation. To exclude platelet contamination, isolated PBMCs were washed and centrifuged twice with PBS containing 5 mM EDTA for 15 min at 400 g at 20 °C [15]. Isolated PBMCs were left to adhere to a plastic dish (120 min at 37 °C) and then detached from the dish by incubation in ice-cold PBS. After washing three times with PBS, the cells were resuspended at a final concentration of 1 × 10⁶ cells/ml in RPMI 1640 (Sigma–Aldrich).

**Real-time PCR for mir-146a/b, IRAK1 mRNA, TRAF6 mRNA and TLR4 mRNA**

Total RNA including the small RNA fraction was extracted from isolated PBMCs using a mirVana™ Paris miRNA isolation kit (Ambion), according to the manufacturer’s recommended protocol (Applied Biosystems). Levels of IRAK1 (assay ID Hs00355570_m1), TRAF6 (assay ID Hs00377558_m1), TLR4 (assay ID Hs01061963_m1) and GAPDH (glyceraldehyde-3-phosphate dehydrogenase; assay ID Hs99999905) mRNA were amplified using TaqMan® Gene Expression assays (Applied Biosystems). The amplification steps consisted of denaturation at 95 °C, followed by 40 cycles of denaturation at 95 °C for 15 s and then annealing at 60 °C for 1 min. All reactions were carried out on the 7500 real-time PCR system (Applied Biosystem) using the TaqMan® Universal PCR master mix and assays on demand (Applied Biosystems). Relative quantification was carried out using the ΔΔ threshold cycle (Ct) method for recurrent compared with primary with U6 or GAPDH as an endogenous control, and fold changes were calculated for each gene [16]. Replicates with a Ct >40 were excluded. The assay was run in triplicate for each case to allow for assessment of technical variability. To account for PCR amplification of contaminating genomic DNA, a control without reverse transcription was included. To improve the accuracy of the real-time PCR for quantification, amplifications were performed in triplicate for each RNA sample.

**Flow cytometric analysis**

The amount of TLR4 and CD14 on the cell surface of PBMCs was measured by FACS. Isolated PBMCs were incubated with FITC-conjugated mouse anti-(human TLR4) antibodies (Santa Cruz Biotechnology) and PerCP-conjugated CD14 antibody (Becton Dickinson). Isotype-matched irrelevant control IgG was used as a control (Becton Dickinson). TLR4 levels in CD14-positive cells were measured by a FACScan flow cytometer (Becton Dickinson) and are shown as the MFI (mean fluorescence intensity).

**Laboratory measurements**

Laboratory data were measured by standard biochemistry methods in our hospital laboratory. All analyses were performed in the same runs from samples that were frozen and stored at −80 °C immediately after centrifugation. hsCRP (high-sensitivity C-reactive protein) was quantified using a latex-enhanced immunonephelometric assay (detection range, 0.035–2.200 mg/dl; CardioPhase hs-CRP; Dade Behring).

**Transfection with mir-146a/b mimic and inhibitor**

Human THP-1 cells, an undifferentiated promonocytic cell line (A.T.C.C., Manassas, VA, U.S.A.) were maintained by twice weekly passage in ATCC-RPMI 1460 medium, 10% (v/v) FBS (fetal bovine serum; Gibco BRL), 100 units/ml penicillin and 100 μg/ml streptomycin at 37 °C with 5% CO₂, and then cultured at a density of 1 × 10⁶ cells/ml. For mir-146a/b mimic and precursor transfections, THP-1 cells were transfected with 20 ng/ml pre-miRTM mir-146a/b precursor molecules (Ambion) and 20 ng/ml anti-miRTM mir-146a/b inhibitors (Ambion) using the NeoFx transfection agent (Ambion). Those cells were used for experiments 24 h after transfection and were exposed at each passage to 0.1 μM t-BHP (t-butyl hydroperoxide; Sigma–Aldrich) for an additional 48 h. Levels of mir-146a/b, IRAK1 mRNA and TRAF6 mRNA were measured using the methods described above.

**Cell culture with oxidant treatment**

Atorvastatin (0.5 μM; Pfizer), telmisartan (30 μM; Sigma–Aldrich) and enalapril (100 mM; Sigma–Aldrich) were dissolved in DMSO (Gibco BRL; DMSO final
concentration, 0.05%). To induce oxidative stress, cultured PBMCs from five healthy volunteers were grown in RPMI 1640 medium (Sigma–Aldrich) with 10% (v/v) heat-inactivated FBS (Gibco BRL), 100 units/ml penicillin, 100 μg/ml streptomycin and each drug or drug vehicle (0.05% DMSO) and were exposed at each passage to 0.1 μM t-BHP (Sigma–Aldrich) or PBS. After 48 h of culture, levels of miR-146a/b, IRAK1 mRNA, TRAF6 mRNA and TLR4 mRNA were measured as described above.

To assess cell viability, we used Trypan Blue (Gibco BRL) exclusion staining of cultured cells; cell viability was over 90% using this method in all experiments.

### Statistical analysis

All values are presented as means ± S.D. Kolmogorov–Smirnov analysis was performed to assess the data distribution. An unpaired Student’s t test was performed for normally distributed data, and the non-parametric Mann–Whitney U test was performed where this was not appropriate. Statistical analysis of categorical variables was also carried out using χ² analysis and Fisher’s exact analysis. After 12 months of combined treatment with RAS inhibitors and statins, comparisons between the two groups (ARB group and ACEI group) were analysed using a two-way repeated measures ANOVA for normally distributed variables and using the Kruskal–Wallis test for non-normally distributed variables. When applicable, significant differences were analysed further with Dunnett post-hoc tests. Pearson’s correlation coefficients were used to examine the relationship between miR-146a/b, TLR4, IRAK1 and TRAF6. Multivariate association between miR-146a/b, TLR4, IRAK1 and TRAF6 levels and clinical outcomes was evaluated with logistic regression models. Relative risks are reported with logistic regression models adjusted for factors that are independently associated with the outcome variable. A value of $P < 0.05$ was considered statistically significant.

### RESULTS

#### Baseline and clinical characteristics

Baseline characteristics of the study populations are shown in Table 1. There were no significant differences in age, percentage of males, BMI (body mass index) or number of subjects with a past history of dyslipidaemia between the CAD and non-CAD groups. There were significant differences in other clinical parameters between the two groups ($P < 0.05$).

#### Clinical characteristics in ARB and ACEI groups

As shown in Table 2, there was no significant difference in baseline characteristics between the ARB and ACEI groups. After 12 months of treatment, BP (blood pressure) and laboratory data [LDL (low-density lipoprotein)-cholesterol and hsCRP] were decreased in both the ARB and ACEI groups. HDL (high-density lipoprotein)-cholesterol levels were increased in both groups.

#### Levels of miR-146a/b, IRAK1 mRNA, TRAF6 mRNA, TLR4 mRNA and TLR4 MFI

There was no difference in average $C_t$ of U6 between the CAD and non-CAD patients ($21.3 ± 2.1$ compared with $21.4 ± 2.0$ arbitrary units; $P$ value was not significant). Levels of miR-146a/b were higher in the CAD group than in the non-CAD group ($P < 0.01$; Figure 1A).

Levels of IRAK1 and TRAF6 mRNA were higher in the CAD group compared with the non-CAD group (both $P < 0.01$; Figures 1B). Levels of TLR4 mRNA and TLR4 MFI were higher in the CAD group than in the non-CAD group (both $P < 0.01$; Figures 1C and 1D). The average $C_t$ of GAPDH did not differ between the two groups ($23.2 ± 2.7$ compared with $23.1 ± 2.5$.)
Table 2 Baseline clinical characteristics and changes in these characteristics after 12 months in the ARB and ACEI groups

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>ARB group (n = 33)</th>
<th>ACEI group (n = 33)</th>
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<tr>
<td>Age (years)</td>
<td>65.5 ± 9.9</td>
<td>66.9 ± 9.4</td>
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<tr>
<td>Male gender (n)</td>
<td>26 (79%)</td>
<td>26 (79%)</td>
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<tr>
<td>BMI (kg/m²)</td>
<td>24.3 ± 3.2</td>
<td>25.3 ± 2.7</td>
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<tr>
<td>Hypertension (n)</td>
<td>20 (61%)</td>
<td>22 (67%)</td>
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<tr>
<td>Diabetes (n)</td>
<td>7 (21%)</td>
<td>8 (24%)</td>
</tr>
<tr>
<td>Dyslipidaemia (n)</td>
<td>12 (36%)</td>
<td>12 (36%)</td>
</tr>
<tr>
<td>Previous CAD (n)</td>
<td>9 (27%)</td>
<td>9 (27%)</td>
</tr>
<tr>
<td>Smoking (n)</td>
<td>9 (27%)</td>
<td>8 (24%)</td>
</tr>
<tr>
<td>Angiographic degree of CAD (n)</td>
<td>33</td>
<td>33</td>
</tr>
<tr>
<td>Single-vessel disease</td>
<td>18 (55%)</td>
<td>20 (60%)</td>
</tr>
<tr>
<td>Multi-vessel disease</td>
<td>35 (105%)</td>
<td>13 (40%)</td>
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<tr>
<td>Systolic BP (mmHg)</td>
<td>141.3 ± 11.8</td>
<td>141.0 ± 14.6</td>
</tr>
<tr>
<td>Follow-up</td>
<td>123.2 ± 11.4*</td>
<td>126.2 ± 15.5*</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>82.6 ± 11.1</td>
<td>80.1 ± 13.2</td>
</tr>
<tr>
<td>Follow-up</td>
<td>70.8 ± 10.1*</td>
<td>70.2 ± 11.7*</td>
</tr>
<tr>
<td>Fasting glucose (mg/dl)</td>
<td>121.6 ± 20.1</td>
<td>125.3 ± 15.3</td>
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<tr>
<td>HbA1c (%)</td>
<td>5.4 ± 0.3</td>
<td>5.5 ± 0.4</td>
</tr>
<tr>
<td>HDL-cholesterol (mg/dl)</td>
<td>48.7 ± 13.7</td>
<td>48.8 ± 13.0</td>
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<tr>
<td>Follow-up</td>
<td>56.6 ± 16.5*</td>
<td>53.5 ± 16.3*</td>
</tr>
<tr>
<td>LDL-cholesterol (mg/dl)</td>
<td>116.7 ± 32.4</td>
<td>112.6 ± 30.0</td>
</tr>
<tr>
<td>Follow-up</td>
<td>81.5 ± 18.0*</td>
<td>79.0 ± 21.4*</td>
</tr>
<tr>
<td>IL-6 (pg/ml)</td>
<td>0.432 ± 0.293</td>
<td>0.411 ± 0.193</td>
</tr>
<tr>
<td>Follow-up</td>
<td>0.093 ± 0.093*</td>
<td>0.114 ± 0.140*</td>
</tr>
<tr>
<td>Medication (n)</td>
<td>33 (100%)</td>
<td>33 (100%)</td>
</tr>
<tr>
<td>Aspirin</td>
<td>13 (40%)</td>
<td>13 (40%)</td>
</tr>
<tr>
<td>Ticlopidine hydrochloride</td>
<td>20 (60%)</td>
<td>20 (60%)</td>
</tr>
<tr>
<td>Clopidogrel sulfate</td>
<td>7 (21%)</td>
<td>8 (24%)</td>
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<tr>
<td>β-Blockers</td>
<td>16 (48%)</td>
<td>14 (42%)</td>
</tr>
<tr>
<td>Calcium antagonists</td>
<td>13 (40%)</td>
<td>15 (45%)</td>
</tr>
<tr>
<td>Nitrates</td>
<td>13 (40%)</td>
<td>15 (45%)</td>
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</tbody>
</table>

Cultured cells transfected with the miR-146a/b mimic and inhibitor

THP-1 cells were transfected with the miR-146a/b mimic and inhibitor followed by challenge with t-BHP for 48 h. Levels of miR-146a/b expression were higher in the miR-146a/b mimic-transfected cells than the mock-transfected cells (Figure 3A). On the other hand, miR-146a/b levels were lower in the miR-146a/b inhibitor-transfected cells than the mock-transfected cells (Figure 3B). To determine the effects of gain-of-function or loss-of-function of miR-146a/b on IRAK1 and TRAF6 expression, samples from transfected cells were analysed for levels of IRAK1 and TRAF6 mRNA. THP-1 cells transfected with the miR-146a/b mimic had a decrease in IRAK1 and TRAF6 mRNA levels compared with mock-transfected cells (Figure 3C). THP-1 cells transfected with the miR-146a/b inhibitor had an increase in IRAK1 and TRAF6 mRNA compared with mock-transfected cells (Figure 3D).

In vitro study with RAS inhibitor and statin treatment

Levels of miR-146a/b, IRAK1 mRNA, TRAF6 mRNA and TLR4 mRNA increased in t-BHP-stimulated PBMCs compared with those treated with PBS (Figure 4). These levels were decreased in t-BHP-stimulated PBMCs treated with the RAS inhibitor (telmisartan or enalapril) and atorvastatin compared with those treated with the vehicle. In addition, these levels were decreased in t-BHP-stimulated PBMCs treated with telmisartan and atorvastatin compared with those treated with enalapril and atorvastatin (Figure 4).

Effect of RAS blockade and statin on miR-146a/b, IRAK1 mRNA, TRAF6 mRNA and TLR4 mRNA levels

Figures 4 and 5 show changes in miR-146a/b, IRAK1 mRNA, TRAF6 mRNA and TLR4 mRNA levels after combined treatment with RAS inhibitors and statin. There was no significant difference in baseline levels of miR-146a/b, IRAK1 mRNA, TRAF6 mRNA and TLR4 mRNA between the ARB and ACEI groups (Figures 5 and 6). For both ARB and ACEI, combined treatment resulted in decreased miR-146a/b, IRAK1 mRNA, TRAF6 mRNA and TLR4 mRNA levels after 12 months (all P < 0.01; Figures 5 and 6). Decreases in miR-146a/b levels, IRAK1 mRNA and TRAF6 mRNA after treatment were greater in the ARB group than in the ACEI group (Figure 5). In addition, decreases in TLR4 mRNA and TLR4 MFI after treatment were greater in the ARB group than in the ACEI group (Figures 6D and 6E).
Figure 1  Comparison of miR-146a/b (A), IRAK1 and TRAF6 mRNA (B), TLR4 mRNA (C) and TLR4 MFI (D) levels between the CAD (n = 66) and non-CAD (n = 33) groups
Levels of miR-146a/b, IRAK1 mRNA, TRAF6 mRNA, TLR4 mRNA and TLR4 MFI were higher in the CAD group than in the non-CAD group. *P < 0.01 compared with the non-CAD group.

Figure 2  Correlation between miR-146a/b and IRAK1 and TRAF6 mRNA levels in CAD and non-CAD groups
There was a positive correlation between miR-146a/b and both IRAK1 and TRAF6 mRNA levels. Correlations are: (A) r = 0.72, P < 0.01; (B) r = 0.79, P < 0.01; (C) r = 0.76, P < 0.01; and (D) r = 0.85, P < 0.01.

Clinical follow-up study
At the time of follow-up, cardiac events were observed in 13 out of 66 patients with CAD at 12 months after PCI. Cardiac events consisted of target lesion revascularization in 11 subjects, and target or other vessel revascularization in three subjects (both target lesion and other vessel revascularizations were shown in one patient). When the CAD group was divided into tertiles according to miR-146a and miR-146b levels, and IRAK1 mRNA, TRAF6 mRNA or TLR4 mRNA levels at baseline, high levels of miR-146a and TLR4 mRNA were independent predictors of cardiac events (miR-146a: relative risk = 6.32 [95% CI = 1.13–35.19], P = 0.04; TLR4 mRNA: relative risk = 9.44 [95% CI = 1.10–88.10], P = 0.04) after adjusting for various clinical parameters [age, gender, culprit lesion (left anterior descending coronary artery), past history of hypertension, diabetes mellitus, previous CAD or smoking, BP, fasting glucose, HbA1c (glycated haemoglobin), LDL-cholesterol and hsCRP].

DISCUSSION
Among the family of TLRs, TLR4 is considered to be an important player in the initiation and progression of atherosclerotic disease [17]. Our previous studies and other reports have shown a relationship between
an activated TLR4 signal and its downstream pathway in circulating monocytes obtained from patients with CAD [2,3,18]. In addition, expression of TLR4 in infiltrating macrophages within the coronary arteries may be an important factor underlying coronary plaque destabilization and rupture in CAD [19]. In agreement with these reports, the present study has shown an increase in TLR4 levels (both mRNA and protein levels) in the CAD group when compared with the non-CAD group. A mouse model has reported that loss of TLR4 expression is associated with reduced atherosclerosis and improved vascular function [19].

Figure 3 Levels of miR-146a/b, IRAK1 mRNA and TRAF6 mRNA in THP-1 cells transfected with a miR-146a/b mimic and inhibitor

(A) Levels of miR-146a/b were higher in the miR-146a/b mimic-transfected cells than in mock-transfected cells. (B) Levels of miR-146a/b were lower in the miR-146a/b inhibitor-transfected cells than in mock-transfected cells. (C) IRAK1 and TRAF6 mRNA levels were lower in the miR-146a/b mimic-transfected cells than in mock-transfected cells. (D) IRAK1 and TRAF6 mRNA levels were higher in miR-146a/b inhibitor-transfected cells than in mock-transfected cells. *P < 0.01 compared with mock transfection.

Figure 4 Effect of RAS blockade (telmisartan or enalapril) and atorvastatin on levels of miR-146a/b (A), IRAK1 and TRAF6 mRNA (B), and TLR4 mRNA and TLR4 MFI (C) in cultured PBMCs stimulated with t-BHP or PBS

All values in t-BHP-stimulated PBMCs treated with vehicle (0.05% DMSO) are defined as 100%. Percentage changes in miR-146a/b, IRAK1 mRNA, TRAF6 mRNA, TLR4 mRNA and TLR4 MFI levels were lower in t-BHP-stimulated PBMCs treated with an RAS inhibitor (telmisartan or enalapril) and atorvastatin than in those treated with drug vehicle. In addition, percentage changes in these levels were lower in t-BHP-stimulated monocytes treated with telmisartan and atorvastatin compared with those treated with enalapril and atorvastatin. *P < 0.05 compared with t-BHP-stimulated PBMCs treated with vehicle. **P < 0.05 compared with t-BHP-stimulated PBMCs treated with enalapril and atorvastatin.
Figure 5  Effect of treatment with an ARB and statin or an ACEI and statin on miR-146a/b, IRAK1 mRNA and TRAF6 mRNA in the CAD group

Levels of miR-146a (A), miR-146b (B), IRAK1 mRNA (C) and TRAF6 mRNA (D) at the time of follow-up (post) were markedly decreased in both the ARB and ACEI groups compared with at baseline (Pre) (P < 0.01). Decreases in miR-146a/b, IRAK1 mRNA and TRAF6 mRNA after treatment were greater in the ARB group than in the ACEI group (P < 0.05).

of TLR4 reduced the severity of atherosclerosis and altered atherosclerotic plaque [20]. It has been suggested that a potential pathophysiological link exists between activation of the TLR4 signal and the progression of coronary atherosclerosis.

It has been shown previously that the miR-146 family (miR-146a/b) regulates downstream TLR4 signalling (IRAK1 and TRAF6) through a negative-feedback regulation loop [6]. The human genome contains miR-146a and miR-146b genes on chromosomes 5 and 10 respectively [6,21,22]. Promoter analysis of miR-146 has demonstrated that both miR-146a and miR-146b function as negative regulators of adaptor molecules of IRAK1 and TRAF6. This suggests that IRAK1 and TRAF6 are targeted for post-transcriptional control by both miR-146a and miR-146b [6]. IRAK1 and TRAF6 activate NF-κB and AP-1 (activating protein-1) transcription factors, and then up-regulate the TLR4-mediated immune response [23]. An in vitro study using human monocyte cell line stimulated with LPS (lipopolysaccharide) has shown that elevation of miR-146 expression occurred in an NF-κB-dependent manner [6]. Therefore miR-146a/b has been shown to be induced by pro-inflammatory stimuli such as TLR4, IL-1 and TNF-α [24]. A novel finding of the present study was that levels of miR-146a/b and its target genes (both IRAK1 and TRAF6) were higher in PBMCs obtained from the CAD group than in the non-CAD group. It has been reported that the key kinases downstream of TLR4, including IRAK1 and TRAF6, are activated in PBMCs from patients with CAD as well as activation of TLR4 [25,26]. A recent study has shown that the expression of miR-146a in PBMCs was significantly increased in patients with ACS [27]. In addition, levels of miR-146a/b were positively correlated with levels of IRAK1, TRAF6 and TLR4 mRNA expression. Our gain-of-function and loss-of-function approaches to miR-146a/b have shown that transfection of miR-146a/b into THP-1 monocytes resulted in a down-regulation of IRAK1 and TRAF6 mRNA expression, but it remains uncertain whether endogenous IRAK1 or TRAF6 mRNA levels were influenced by miR-146a/b expression in humans. This discrepancy between our in vivo and in vitro studies may suggest that the impairment of IRAK1/TRA6 mediation of the prolonged activation of TLR4 and its downstream signalling in patients with CAD. It is therefore speculated that this impairment of IRAK1/TRA6 regulation by miR-146a/b may play a role in the pathogenesis of CAD. Further studies will be
needed to clarify the mechanism by which miR-146a/b dysregulates IRAK1/TRAF6 expression in patients with CAD.

Another important finding of the present study was that combined treatment with RAS inhibitors and a statin reduced monocytc levels of miR-146a/b, IRAK1 mRNA, TRAF6 mRNA and TLR4 mRNA in patients with CAD. RAS inhibitors, including ARBs and ACEIs, exert potent anti-atherosclerotic effects, which are mediated not only by their anti-hypertensive properties, but also by their anti-inflammatory properties [28]. An in vitro study has reported that AngII up-regulated TLR4-dependent signalling and then induced an inflammatory response [28]. The present study has also suggested that RAS inhibitors block the AngII-induced TLR4 signalling pathway [29]. RAS inhibitors may therefore decrease monocytc activation of TLR4 through inhibition of AngII and then decrease miR-146a/b levels due to down-regulation of the TLR4 signal. In particular, decreases in miR-146a/b, IRAK1, TRAF6 and TLR4 levels after treatment were greater in the ARB group than in the ACEI group. It has been reported that treatment with an ARB reduced NF-κB activity [30]. It has been speculated that treatment with an ARB may directly down-regulate NF-κB activity and repress miR-146a/b levels in patients with CAD. It has also been reported that oxidized LDL activated the RAS and increased TLR4 expression in cultured endothelial cells [31]. An in vitro study using PBMCs treated with statins has shown that they decreased several TLR4 transcription factors, such as IRAK1 and TRAF6, and then inhibited the TLR4 signal [32]. Our present in vitro study has shown that telmisartan and atorvastatin had a greater effect than enalapril and atorvastatin on miR-146a/b, IRAK1 and TRAF6. An Apoe (apolipoprotein E-knockout mouse model has reported that combined treatment with an ARB and a statin decreased inflammatory mediators compared with combined treatment with an ACEI and statin or treatment with ARB, ACEI or statin alone [14]. From these observations, combined treatment with telmisartan and a statin may down-regulate IRAK1, TRAF6 and TLR4 expression, and then repress miR-146a/b resulting from the down-regulation of the TLR4 signalling pathway in CAD.

Our 12-month follow-up study showed that high levels of miR-146a and TLR4 mRNA at baseline are
potent independent predictors of cardiac events through regression analysis. Our present study and those of others have reported a marked TLR4 expression in human atherosclerotic plaques [19,33], and indicated that oxidative stress up-regulated TLR4 expression in macrophages [34], suggesting an association between TLR4, inflammatory and immune responses, and coronary atherosclerosis. Therefore activation of the TLR4 signal may induce miR-146a/b expression as a negative regulator, and then induce progression of coronary atherosclerosis in patients with CAD.

A limitation of the present study is the small number of CAD and non-CAD groups. A further limitation is the relatively low dose of enalapril (5 mg/day) compared with other reports from Western countries; this dose was chosen because it is the commonly prescribed dose of enalapril in Japan (the maximum approved dose of enalapril is 10 mg daily) [35]. However, there was no difference between the ARB and ACEI groups with regard to BP after 12 months of therapy, which suggests that enalapril (5 mg/day) and telmisartan (40 mg/day) have the same anti-hypertensive effect. It was an ethical criterion of the study that the statin had to be administered to all patients with CAD because LDL-cholesterol levels in the majority of these patients were over 100 mg/dl. AHA (American Heart Association)/ACC (American College of Cardiology) guidelines for secondary prevention in patients with CAD state that the goal of treatment is an LDL-cholesterol level <100 mg/dl [36]. In addition, the present study could not elucidate the mechanism by which combined treatment with an ARB and statin affects miR-146a/b expression in patients with CAD. Further studies will therefore be needed to determine a causal relationship between these therapeutic agents in CAD.

**Conclusions**

The present study has suggested that dysregulation of miR-146a/b expression may contribute to the prolonged activation of TLR4 and its downstream signalling in PBMCs obtained from patients with CAD. In addition, combined treatment with an ARB and statin decrease miR-146a/b levels, its target genes (IRAK1 and TRAF6) and TLR4 in patients with CAD, possibly contributing to the synergic effects of this therapeutic combination in this disorder.

**AUTHOR CONTRIBUTION**

Yuji Takahashi conceived and designed the study, interpreted the data, and revised the manuscript; Mamoru Satoh conceived and designed the study, and drafted the manuscript; Yoshitaka Minami analysed and interpreted the data; Tsuyoshi Tabuchi conceived and designed the study, and interpreted the data; Tomonori Itoh drafted the manuscript; and Motoyuki Nakamura revised the manuscript.

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**REFERENCES**


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23 Liu, G. and Zhao, Y. (2007) Toll-like receptors and immune regulation: their direct and indirect modulation on regulatory CD4+CD25+ T cells. Immunology 122, 149–156

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