Circulating level of microRNA-126 may be a potential biomarker for recovery from smoking-related vascular damage in middle-aged habitual smokers

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A B S T R A C T

Background: Cigarette smoking promotes vascular endothelial damage and accelerates progression of atherosclerosis. The purpose of this study was to examine whether the circulating level of vascular endothelium-enriched microRNA-126 (miR-126), which is highlighted as a regulator of gene expression, would serve as a novel biomarker for recovery from smoking-related vascular damage.

Methods: Middle-aged male smokers (n = 30) were enrolled and instructed to stop smoking. Their clinical profiles and laboratory findings including expression of miR-126 were investigated before and after 8 weeks of smoking cessation. Serum levels of cotinine, metabolites of nicotine, were measured to confirm smoking cessation. Endothelial function for peripheral small vessels was assessed and expressed as reactive hyperemia peripheral arterial tonometry (RH-PAT) index. The expression of miR-126 in plasma was analyzed by quantitative real-time PCR.

Results: At baseline, serum cotinine levels were inversely correlated with RH-PAT index (r = −0.48, P < 0.01) and positively correlated with levels of metabolic parameters such as non-HDL cholesterol (r = 0.53, P < 0.01) and HOMA-IR (r = 0.52, P < 0.01). The RH-PAT index was not significantly changed after 8 weeks in all subjects, because only 13 subjects could attain smoking cessation. However, changes in the RH-PAT index showed a significant correlation with those in systolic blood pressure (r = −0.54, P < 0.01). In smokers who completely attained smoking cessation (n = 13), RH-PAT index and plasma levels of miR-126 were significantly increased (P < 0.05, respectively).

Conclusions: Endothelial damage was improved and plasma levels of circulating miR-126 were increased after 8 weeks of smoking cessation. These findings suggested a potential use of miR-126 as a biomarker for recovery from smoking-induced vascular damage.

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1. Introduction

Cigarette smoking promotes vascular endothelial damage and accelerates progression of atherosclerosis [1–4]. Nicotine, the main extract of cigarette smoking, is the major substance underlying its addictiveness, and affects the cardiovascular system itself [5–7]. Cigarette smoking increases cardiovascular risk even at low-doses or with low-tar [8,9]. Thus, cigarette smoking and smoking extracts have a close association with cardiovascular diseases. Moreover, the influence of cigarette smoking may last from a few months to years after smoking cessation. Therefore, numerous efforts have been made to identify reliable and predictive biomarkers to detect the early signs of recovery from smoking-induced endothelial damage. In our previous study, smoking cessation for 8 weeks improved endothelial function although plasma serotonin level was not significantly decreased [10].

On the other hand, circulating microRNAs (miRNAs) are highlighted as new biomarkers for various diseases and pathophysiologicals [11–13]. MiRNAs are a class of short single-stranded non-coding RNAs that regulate cellular functions through degradation and translational repression of miRNAs that contain complementary sequences, and are known to be released from cells into circulation in a stable form, and therefore are explored for their potential as biomarkers in cardiovascular disease [12–14]. Especially, miRNA-126 (miR-126), as an...
endothelium-enriched miRNA, is reported to take an important role in vascular homeostasis and angiogenesis [12,15,16]. Therefore, we hypothesized that the expression of miR-126 would serve as a novel biomarker for recovery from smoking-related endothelial damage in middle-aged habitual smokers.

2. Methods

Apparantly healthy male smokers were enrolled and instructed to stop smoking. Their clinical profiles including endothelial function and laboratory findings including miRNAs were investigated before and after 8 weeks of smoking cessation. The expression of miRNA in platelet poor plasma was analyzed by quantitative real-time PCR. Endothelial function was assessed by reactive hyperemia of peripheral arterial tonometry (PAT) using End-PAT 2000 [17,18].

2.1. Study subjects

Middle-aged male subjects who were habitual cigarette smokers were enrolled. Subjects were recruited from outpatients who came to our hospital with common cold or lifestyle diseases such as low risk hypertension or dyslipidemia. At least 1 month after the first visit, their physical condition was examined and enrolled. Age adjusted apparently healthy subjects were also enrolled as non-smoking controls. Patients with obvious organ damage, diabetes mellitus, complication of malignant neoplasm or active inflammatory disease, medical history of cardiovascular events, or taking medications were excluded.

Informed consent was obtained from each subject. The study was approved by the Ethical Committee of Nagoya City University Graduate School of Medical Sciences and carried out in accordance with the Declaration of Helsinki of the World Medical Association.

After enrollment, endothelial function was assessed by reactive hyperemia, and subsequently peripheral blood was taken for biochemical analysis. Strong precaution was taken to obtain platelet poor plasma samples suitable for serotonin assays as we previously described [19]. Blood pressure was measured in the non-dominant arm using a validated oscillometric technique (HEM-7070; Omron Corporation, Kyoto, Japan) at the seated position on the same day.

**Table 1**

Characteristics of subjects at baseline and after 8 weeks of instruction to stop smoking.

| Variable                        | (n = 30) | P value |
|---------------------------------|----------|---------|
| Age (years old)                 | 42 ± 10  |         |
| BMI (kg/m²)                     | 22.4 ± 3.0 | -       |
| Systolic BP (mm Hg)             | 126 ± 14 | NS      |
| Diastolic BP (mm Hg)            | 80 ± 10  | NS      |
| WBC (/10^-3 μl)                 | 5.7 ± 1.4 | 0.01    |
| Hemoglobin (g/dl)               | 14.8 ± 1.5 | <0.01  |
| Platelet (/10^-3 μl)            | 22.1 ± 5.3 | 0.01    |
| Creatinine (mg/dl)              | 0.76 ± 0.09 |      |
| Total cholesterol (mg/dl)       | 190 ± 30  | 0.01    |
| HDL cholesterol (mg/dl)         | 58 ± 14  | -       |
| non-HDL cholesterol (mg/dl)     | 133 ± 36 | NS      |
| LDL cholesterol (mg/dl)         | 107 ± 29 | NS      |
| Triglyceride (mg/dl)            | 164 ± 165 | 0.01   |
| Fasting plasma glucose (mg/dl)  | 92 ± 9   | NS      |
| HOMA-IR                         | 0.88 ± 0.76 | 0.01  |
| hs-CRP (mg/l)                   | 0.06 ± 0.14 | 0.01  |
| TDS                             | 7.2 ± 1.5 | -       |
| FTND                            | 4.5 ± 2.0 | -       |
| Cotinine (ng/ml)                | 176 ± 200 | 0.01   |
| RH-PAT index                    | 1.80 ± 0.38 | 0.01  |

Data are expressed as mean ± SD. BMI: body mass index, WBC: white blood cell count, BP: blood pressure, HDL: high-density lipoprotein, LDL: low-density lipoprotein, HOMA-IR: homeostasis model assessment of insulin resistance, hs-CRP: high sensitive-CRP, TDS: tobacco dependence screener, FTND: Fagerstrome test for nicotine dependence, RH-PAT: reactive hyperemia of peripheral arterial tonometry.

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**Fig. 1.** Correlation between serum cotinine levels and endothelial function, and metabolic parameters at baseline. a. Correlation between serum cotinine level and reactive hyperemia of peripheral arterial tonometry (RH-PAT) index. b. Correlation between serum cotinine level and high-density lipoprotein cholesterol (HDL-C). c. Correlation between serum cotinine level and non-high-density lipoprotein cholesterol (non-HDL-C). d. Correlation between serum cotinine level and homeostasis model assessment of insulin resistance (HOMA-IR).
Then the subjects were instructed to stop smoking, and endothelial function was assessed after 8 weeks of smoking cessation. Similarly, peripheral blood was taken for biochemical analysis to compare the clinical profiles before and after smoking cessation. Nicotine dependence was assessed by a tobacco dependence screener (TDS) [20]. Serum levels of cotinine, principal metabolites of nicotine, were measured to confirm smoking cessation after 8 weeks [21,22].

2.2. Biochemical analysis

Blood was taken in early morning after overnight fasting. Strict precautions were taken to obtain platelet poor plasma. Serum total cholesterol (total-C), high-density lipoprotein cholesterol (HDL-C) and triglyceride (TG) were determined by standard laboratory assays. High-sensitive-C-reactive protein (hs-CRP) was determined by a latex-enhanced immunonephelometric assay on a BN II analyzer (Dade Behring, Marburg, Germany). Fasting plasma glucose was measured by radioimmunoassay. Serum cotinine levels were measured by ELISA according to the manufacturer’s instructions (Cozart, Abingdon, UK).

2.3. RNA isolation and quantitative real-time PCR

The methods of isolation of total RNA and quantitative real-time PCR of miRNA were performed as previously described [23].

2.4. Determination of vascular endothelial function

Endothelial function in peripheral small vessels was assessed by reactive hyperemia of peripheral arterial tonometry (RH-PAT) in the morning under an overnight fasting condition. RH-PAT was measured using an End-PAT 2000 device (Itamar Medical Ltd., Caesarea, Israel) and expressed as RH-PAT index according to previous reports [17,18].

2.5. Statistic analysis

Data were expressed as mean value ± standard deviation (SD). Comparison of continuous variables was performed by paired or unpaired t-test as appropriate. Correlations between the parameters were assessed by calculating Pearson’s correlation coefficient (r). Parameters not expressing a normal distribution were converted by log-transformation (hs-CRP). Differences of P < 0.05 were considered statistically significant.

3. Results

Characteristics of the subjects enrolled in this study are described in Table 1. At baseline, serum cotinine levels were inversely correlated with endothelial function expressed as RH-PAT index (r = −0.48, P < 0.01) and HDL-C level, and positively correlated with levels of non-HDL-C (r = 0.53, P < 0.01) and HOMA-IR (r = 0.52, P < 0.01) (Fig. 1).

The RH-PAT index was not significantly changed after 8 weeks in all subjects, because only 13 subjects could attain smoking cessation (Table 1). However, % change of systolic blood pressure was significantly correlated with % change of RH-PAT index (Fig. 2a). Furthermore, subjects with decreased systolic blood pressure exhibited a significantly improved RH-PAT index (Fig. 2b).

Next, smokers who completely attained smoking cessation were analyzed separately. Characteristics of the subjects who completely attained smoking cessation are described in Table 2. They indicate

![Fig. 2. Changes of systolic blood pressure (SBP) and endothelial function from baseline to 8 weeks after instruction to stop smoking. a. Percent change of SBP was inversely correlated with percent change of reactive hyperemia of peripheral arterial tonometry (RH-PAT) index. b. RH-PAT index was significantly increased in subjects who decreased SBP.](image-url)
significantly increased RH-PAT ratio ($P < 0.05$) and plasma miR-126 after 8 weeks of smoking cessation ($P < 0.05$, respectively) (Fig. 3). However, RH-PAT index and expression levels of miR-126 did not show a significant difference between subjects who completely attained 8 weeks of smoking cessation and subjects who could not attain smoking cessation.

4. Discussion

The main findings of this study were that (1) serum cotinine levels were correlated inversely with endothelial function and positively with metabolic parameters, (2) % change of blood pressure was significantly correlated with % change of endothelial function, and (3) smokers who completely attained smoking cessation showed improvement of endothelial function and increase in expression of plasma miR-126 after 8 weeks. Recent reports have revealed that upregulation of miR-126 was observed in progression of angiogenesis and maintenance of vascular integrity [24]. Therefore, miR-126 might have a protective role in homoeostasis for vascular endothelium. In fact, atherosclerotic subjects with coronary artery disease and diabetes mellitus were reported to have lower circulating levels of miR-126 in clinical studies [25,26]. Although our previous study did not show a difference in expression of miR-126 between subjects with and without coronary artery disease, the heterogeneity of characteristics of subjects including differences of medications might be involved [23]. Moreover, samples of plasma used in that study might not be platelet poor plasma. In the present study, subjects were non-medicated habitual smokers who might have early atherosclerosis. Strict precautions were also taken to obtain platelet poor plasma. The plasma level of miR-126 increased rapidly after 8 weeks of smoking cessation with concomitant improvement of endothelial function. However, significant correlation was not transversely observed between the level of miR-126 and RH-PAT index. A detailed mechanism was not elucidated but widely interspersed levels of miR-126 expression at baseline might be related. Therefore, the quantitative assessment of miR-126 levels in the platelet poor plasma may have diagnostic potential to detect early recovery of endothelial function particularly in identical subjects who were habitual smokers.

Johnson et al. reported that smoking cessation improved the endothelial function assessed by FMD after 1 year despite body weight gain [27]. We evaluated the endothelial function after 8 weeks of smoking cessation, and confirmed the improvement of endothelial function in subjects who completely attained smoking cessation despite an increase of total-C level. Subjects with decreased systolic blood pressure showed increased RH-PAT index after 8 weeks. The decrease of blood pressure may be due to an improvement of endothelial function and relaxation of vascular tonus. To confirm smoking cessation, we measured serum cotinine levels. At baseline, the cotinine level was positively correlated with atherogenic factors including non-HDL-C and homeostasis model assessment of insulin resistance (HOMA-IR) and inversely correlated with HDL-C and RH-PAT index. This result also supports the favorable effects of smoking cessation and subsequent decrease in cotinine levels.

5. Study limitations

The present study is limited by the small number of subjects studied. Moreover, enrolled subjects were limited to middle-aged smokers and comparatively non-heavy smokers. Further study with a larger population and a longer follow-up period will clarify the relationship of smoking cessation and miR-126, and endothelial function in cigarette smokers.

6. Conclusions

Vascular endothelial damage was improved and plasma levels of endothelium-enriched circulating miR-126 were increased after 8 weeks of smoking cessation. These results suggested a potential use of miR-126 as a sensitive and informative biomarker for recovery from smoking-induced endothelial damage.

Conflict of interest

There are no conflicts of interest to declare.

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Fig. 3. Analysis in subjects who completely attained smoking cessation during 8 weeks and in subjects who could not attain smoking cessation. a. Subjects who completely attained 8 week smoking cessation showed a significant improvement in reactive hyperemia of peripheral arterial tonometry (RH-PAT) index. b. Subjects who completely attained 8 week smoking cessation exhibited a significant increase in circulating levels of microRNA-126 (miR-126).
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