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Association of cigarette smoking with serum microRNA expression among middle-aged Japanese adults

Koji Suzuki, PhD[1], Hiroya Yamada, PhD[2], Ayuri Nagura, MMSc[3], Koji Ohashi, PhD[3], Hiroaki Ishikawa, PhD[3], Mirai Yamazaki, BHSc[3], Yoshitaka Ando, MMSc[4], Naohiro Ichino, PhD[5], Keisuke Osakabe, PhD[5], Keiko Sugimoto, PhD[5], Nobuyuki Hamajima, MD, PhD[6], Takashi Inoue, PhD[1]

[1]Department of Public Health, Fujita Health University School of Health Sciences, Toyoake, Aichi, Japan, [2]Department of Hygiene, Fujita Health University School of Medicine, Toyoake, Aichi, Japan, [3]Department of Clinical Biochemistry, Fujita Health University School of Health Sciences, Toyoake, Aichi, Japan, [4]Department of Joint Research Laboratory of Clinical Medicine, Fujita Health University Hospital, Toyoake, Aichi, Japan, [5]Department of Clinical Physiology, Fujita Health University School of Health Sciences, Toyoake, Aichi, Japan [6]Department of Healthcare Administration, Nagoya University Graduate School of Medicine, Nagoya, Aichi, Japan

Abstract

Objectives: Circulating micro (mi)RNA profiles are influenced by various medical conditions, and miRNAs have been examined as potential biomarkers for cancer, cardiovascular disease, and diabetes. However, few studies have investigated whether circulating miRNAs and cigarette smoking are correlated. Our aim was to determine the association between smoking status and expression of various miRNAs in a Japanese population sample.

Methods: We conducted a cross-sectional analysis of 526 subjects (219 men and 307 women) aged 39 years and older who had undergone a health examination at a clinic in Yakumo, Hokkaido in August 2012. We used quantitative real-time polymerase chain reaction to determine serum miRNA expression. We then calculated the odds ratios for elevated serum miRNA levels according to smoking status using never-smokers as the reference group.

Results: Expression of lethal (let)-7d, miRNA(miR)-150, miR-192, miR-197 and miR320 was significantly higher in current smokers than in never-smokers. Multivariate logistic regression analyses showed that while current smokers were significantly more likely to have elevated serum levels of miRNA let-7d, miR-21, miR-122, miR-146, miR-150, miR-192, miR-197, and miR320 than never-smokers, former smokers had significantly higher odds of having elevated miR-1, miR-146, miR-150, miR-195, and miR-320 levels in their sera.

Conclusions: We found that cigarette smoking is associated with elevated expression of various serum miRNAs. Our results suggest that it is necessary to consider the confounding effect caused by smoking when evaluating expression of serum miRNAs for diagnosing pathological conditions.

Keywords: circulating microRNA, smoking status, cross-sectional study

Introduction

MicroRNAs (miRNAs) are small, noncoding, single-stranded RNAs that are typically 22 nucleotides long. They regulate gene expression post-transcriptionally through suppression of messenger RNA (mRNA) translation or by degradation of mRNA. They have been shown to be involved in the regulation of various biological processes, including cell proliferation, apoptosis, stress responses, angiogenesis and oncogenesis.[1-4] More than 1000 miRNAs have been identified in the genome to date.[5,6] Various conditions, including cancer, are known to influence their transcription and thereby alter miRNA expression profiles.[7] Recent studies have reported that miRNAs can be detected in bodily fluids including serum, plasma, urine, breast milk, and tears, and may be useful as biomarkers for disease.[8-13] Circulating miRNAs are widely considered as potential biomarkers for detecting cancers,[14,15] cardiovascular diseases (CVD)[16,17] and diabetes.[18]

Health behaviors such as cigarette smoking, alcohol consumption, and poor diet are strongly associated with the onset and progression of various diseases. Although a large body of work has shown the association between smoking and the risk of certain cancers, such as lung cancer and CVD,[19-21] the exact mechanisms through which this occurs remain to be fully elucidated. One hypothesis may be that smoking-induced changes in miRNA expression may influence oncogenesis.

Although a recent study on a small sample of healthy subjects (11 smokers and 7 non-smokers) showed that chronic cigarette smoking induces changes in circulating miRNA profiles,[22] few large-scale studies have investigated the association between circulating miRNAs and smoking status. We therefore sought to determine the association between smoking status and serum levels of various miRNAs in a Japanese population sample.

Methods

Study subjects

Our study sample consisted of 556 residents who attended health examinations in Yakumo, a town in Hokkaido, Japan, in August 2012. After excluding 30 subjects who declined to participate in the study, data from the remaining 526 subjects (219 men and 307 women aged 39 years and older) were included our analysis. All participants provided written informed consent. The Ethics Review Committee of Fujita Health University

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Corresponding author : Koji Suzuki, PhD
Department of Public Health, Fujita Health University School of Health Sciences, 1-98 Dengakugakubo, Kutsukake-cho, Toyoake, Aichi, 470-1192 Japan
E-mail: ksuzuki@fujita-hu.ac.jp

approved the study protocol (approval number 11-101).

Data collection

Trained nurses administered a questionnaire assessing health behaviors including smoking status, alcohol consumption (with participants categorized as regular drinkers, ex-drinkers, or never drinkers) and history of major illnesses. The questions regarding smoking habits, which related to cigarette smoking only, included items on current smoking status (current smoker, former smoker, and never smoker), age at which smoking started, and number of cigarettes smoked per day. The total number of cigarette-years was calculated for current smokers by multiplying the average number of cigarettes smoked per day by the duration of smoking in years. Participants were categorized according to number of cigarettes smoked daily (1–19 and ≥ 20), years of smoking (<20 and ≥ 20) and cigarette-years of cumulative exposure (<400 and ≥400) for the purposes of analysis. Height, weight, and blood pressure were also measured during the health examination. Body mass index (BMI) was calculated as body mass (kg) divided by height (m) squared.

Blood biochemistry

Fasting blood samples were taken during the health examination and sera were separated from blood samples by centrifugation within 1 hour of collection. Serum samples were stored in a deep freezer at − 80° C until analysis.

We used quantitative real-time polymerase chain reaction (qPCR) to detect levels of expression for 16 miRNAs in the sera as previously described.[23] These miRNA have been associated with lifestyle-related diseases including cancer and CVD.[18,24-32] The relative expression of each miRNA was calculated using the comparative cycle threshold method ($2^{-\Delta\Delta CT}$)[24,33] normalized to spiked-in cyn-39 levels. The levels of miRNA expression were then calibrated relative to those among the never-smokers. Biochemical analyses of sera were performed using an auto-analyzer (JCM-BM9130, Nihon Denshi Co., Ltd., Tokyo, Japan) at Yakumo General Hospital.

Statistical analysis

All statistical analyses were conducted using JMP version 10.0 (SAS Institute, Cary, NC, USA). Because serum levels of miRNAs and triglyceride were distributed logarithmically, these were log-transformed for the purposes of analysis. We estimated relative geometric least squares means and 95% confidence intervals for each hypothesized confounding variable, including age, sex, BMI, systolic blood pressure (SBP), hemoglobin A1c (HbA1c), serum total cholesterol levels, estimated glomerular filtration rate (eGFR = 194 × serum creatinine$^{1.094}$ × age$^{0.287}$ (× 0.739 in women)) and drinking status, according to smoking status using analysis of covariance (ANCOVA). Serum triglyceride levels were presented as geometric means with their interquartile range. Normally distributed variables were presented as mean ± standard deviation (SD). Analysis of variance (ANOVA) and the Tukey–Kramer HSD test were used to compare continuous parameters according to smoking status while the chi-square test was used to compare categorical variables. We then performed multivariate logistic regression analyses to estimate adjusted odds ratios (ORs) with 95% confidence intervals (CIs) for each confounding factor. We calculated the ORs for elevated serum miRNA levels (greater than the 75th percentile) by cigarette smoking status using the never smokers as the reference group. Two-tailed p-values were given for each covariate, and p-values <0.05 were considered statistically significant.

Results

Our study sample included 280 never smokers (53.2%), 76 current smokers (13.5%) and 170 former smokers (32.3%). The characteristics of our subjects are shown in Table 1 according to their smoking status. A significantly greater proportion of the current smokers were male compared with the never smokers. Current smokers were also younger than never smokers. While serum triglyceride levels and eGFR were significantly higher in current smokers, diastolic blood pressure (DBP) was significantly higher in former smokers than in never smokers.

Table 2 shows participants' serum miRNA levels according to smoking status after adjustment for confounding variables. Current smokers had significantly higher serum levels of lethal (let)-7d, miRNA(miR)-150, miR-192, miR-197, and miR-320 compared with never-smokers. Serum miR-150 levels were significantly higher in former smokers.

Table 3 shows adjusted ORs with 95% CIs for elevated serum miRNAs according to smoking status. Current smokers had significantly higher odds of elevated let-7d, miR-21, miR-122, miR-146, miR-150, miR-192, miR-197, and miR320 than never smokers. Former smokers, meanwhile, had a significantly higher odds of elevated serum miR-1, miR-146, miR-150, miR-195, and miR-320.

Serum miRNA levels among current smokers were compared according to number of cigarettes smoked per day, years of smoking, and cigarette-years of exposure (Table 4). Heavy

Table 1. Sample characteristics

	Current smoking status			
	Never smoker	Former smoker	Current smoker	p
N	280	170	76	
Sex, % male	16.4	73.5	63.2	<0.001
Current drinker, %	19.7	60.6	55.3	<0.001
Age, years (SD)	65.6 (9.6)	63.4 (9.1)	58.6 (10.4)[a]	<0.001
BMI, kg/m^2 (SD)	23.4 (3.3)	23.9 (3.0)	23.6 (3.7)	0.302
SBP, mmHg (SD)	133.4 (19.4)	136.6 (18.6)	132.9 (18.7)	0.169
DBP, mmHg (SD)	74.0 (11.5)	79.1 (12.4)[b]	77.3 (12.6)	<0.001
Hemoglobin A1c, % (SD)	5.4 (0.4)	5.6 (0.7)	5.4 (0.9)	0.147
Total cholesterol, mg/dl (SD)	212.9 (31.9)	209.4 (31.2)	209.2 (32.4)	0.443
Triglyceride, mg/dl (IQR)	87.0 (65.0–115.0)	95.5 (64.8–130.8)	102.0 (67.3–152.5)c	0.026
HDL-cholesterol, mg/dl (SD)	61.5 (13.5)	58.1 (13.4)[c]	56.8 (15.2)[c]	0.005
eGFR, ml/min/1.73m^2 (SD)	66.7 (13.0)	69.4 (14.3)	74.3 (12.8)[a, d]	<0.001

Values are represented as means with standard deviations (SD) where applicable, except triglyceride levels, which are presented as geometric means interquartile ranges (IQR).

BMI: body mass index, SBP: systolic blood pressure, DBP: diastolic blood pressure, HDL: high-density lipoprotein, eGFR: estimated glomerular filtration rate.

[a] p<0.001(vs. never and former smokers).

[b] p<0.001 (vs. never smokers).

[c] p<0.05 (vs. never smokers).

[d] p<0.01 (vs. former smokers).

Table 2. Adjusted relative serum miRNA levels by smoking status

	Current smoking status					
miRNA	Never		Former		Current	
let-7d	1.00	(0.76 – 1.31)	0.91	(0.65 – 1.28)	2.14	(1.33 – 3.43) [a, d]
miR-1	1.00	(0.75 – 1.33)	1.21	(0.86 – 1.69)	1.43	(0.88 – 2.31)
miR-17	1.00	(0.82 – 1.23)	1.20	(0.93 – 1.56)	1.27	(0.88 – 1.83)
miR-20a	1.00	(0.80 – 1.25)	1.06	(0.80 – 1.39)	1.04	(0.70 – 1.54)
miR-21	1.00	(0.80 – 1.24)	1.33	(1.01 – 1.73)	1.68	(1.14 – 2.46)
miR-27a	1.00	(0.80 – 1.25)	0.97	(0.73 – 1.27)	1.07	(0.73 – 1.58)
miR-92	1.00	(0.77 – 1.30)	1.10	(0.80 – 1.52)	1.58	(1.00 – 2.51)
miR-122	1.00	(0.78 – 1.28)	1.23	(0.90 – 1.67)	1.86	(1.20 – 2.87)
miR-126	1.00	(0.83 – 1.21)	1.04	(0.82 – 1.31)	1.10	(0.79 – 1.53)
miR-130a	1.00	(0.77 – 1.30)	0.99	(0.72 – 1.36)	1.23	(0.78 – 1.93)
miR-146	1.00	(0.78 – 1.29)	1.70	(1.24 – 2.32)	1.80	(1.15 – 2.82)
miR-150	1.00	(0.84 – 1.19)	1.78	(1.43 – 2.22) [b]	2.00	(1.47 – 2.73) [b]
miR-192	1.00	(0.81 – 1.24)	1.15	(0.88 – 1.50)	1.99	(1.36 – 2.92) [a, c]
miR-195	1.00	(0.74 – 1.35)	1.03	(0.71 – 1.49)	1.08	(0.63 – 1.83)
miR-197	1.00	(0.85 – 1.18)	1.24	(1.01 – 1.52)	1.66	(1.24 – 2.23) [a]
miR-320	1.00	(0.88 – 1.13)	1.28	(1.09 – 1.49)	1.56	(1.26 – 1.94) [e]

Data are shown as relative values (with 95% confidence intervals) adjusted for age, sex, BMI, SBP, HbA1c, serum total cholesterol levels, eGFR and current drinking status.

[a] $p<0.05$ (vs. non smokers)

[b] $p<0.001$ (vs. non smokers)

[c] $p<0.05$ (vs. former smokers)

[d] $p<0.01$ (vs. former smokers)

[e] $p<0.01$ (vs. non smokers)

Table 3. Multivariate-adjusted odds ratios and 95% confidence intervals for elevated serum miRNA according to smoking status

	Current smoking status			
miRNA	Never	Former		Current
let-7d	1.00	1.29	(0.70 – 2.39)	3.22 (1.66 – 6.31)
miR-1	1.00	2.79	(1.21 – 6.66)	2.14 (0.83 – 5.47)
miR-17	1.00	1.18	(0.67 – 2.08)	1.52 (0.77 – 2.94)
miR-20a	1.00	1.18	(0.67 – 2.09)	1.54 (0.78 – 2.98)
miR-21	1.00	1.34	(0.74 – 2.40)	2.23 (1.14 – 4.33)
miR-27a	1.00	1.18	(0.67 – 2.10)	1.12 (0.55 – 2.21)
miR-92	1.00	0.88	(0.49 – 1.58)	1.85 (0.97 – 3.52)
miR-122	1.00	1.18	(0.65 – 2.12)	2.14 (1.09 – 4.19)
miR-126	1.00	1.50	(0.85 – 2.65)	1.27 (0.63 – 2.49)
miR-130a	1.00	1.06	(0.60 – 1.88)	1.21 (0.61 – 2.37)
miR-146	1.00	1.95	(1.12 – 3.41)	2.31 (1.20 – 4.46)
miR-150	1.00	2.29	(1.31 – 4.03)	2.80 (1.46 – 5.38)
miR-192	1.00	1.12	(0.62 – 2.01)	2.39 (1.24 – 4.61)
miR-195	1.00	1.78	(1.03 – 3.08)	1.52 (0.78 – 2.92)
miR-197	1.00	1.70	(0.97 – 2.99)	2.69 (1.40 – 5.16)
miR-320	1.00	1.99	(1.15 – 3.45)	2.15 (1.13 – 4.11)

Odds ratios and 95% confidence intervals were adjusted for age, sex, BMI, SBP, HbA1c, serum total cholesterol levels, eGFR and current drinking status.

smokers (≥20 cigarettes per day) had significantly lower levels of serum miR-21 and miR-150 than light smokers (<20 cigarettes per day). Smokers with <400 cigarette-years of exposure had significantly lower levels of serum miR-17 compared with those who had ≥400 cigarette-years. There was no significant dose–response relationship between serum miRNA levels and years of smoking.

Discussion

Although numerous clinical studies have investigated the association between serum miRNA and various pathological conditions,[18,24,28,30-32] there is currently a paucity of studies on the effects of smoking on serum miRNA levels in the general population In the present study, we observed significant and independent correlations between smoking and serum levels of some miRNAs such as let-7d, miR-1, miR-21, miR-122, miR-146, miR-150, miR-192, miR-195, miR-197, and miR-320 in a sample of middle-aged adults drawn from the general Japanese population. However, we found no significant differences in serum levels of miRNAs among current smokers according to number of cigarettes smoked per days, years of smoking, and cigarette-years of exposure. This suggests that smoking status has a greater influence on serum miRNA levels than cumulative exposure to tobacco smoke over time.

Several reports have shown an association between cigarette smoking and intracellular and extracellular miRNA expression in human subjects. Schembri et al. found that expression of 28 miRNAs, including miR-128, miR-130a, and miR-146a, in human bronchial epithelial cells differed between smokers and non-smokers.[34] Maternal cigarette smoking during pregnancy has also been associated with the downregulation of miR-16, miR-21, and miR-146a in the placenta.[35]

Recent small-scale studies[22,36] have reported associations between cigarette smoking and expression of circulating miRNA. Takahashi et al.[22] also reported that cigarette smoking affects the expression of 43 miRNAs in plasma. Their finding that plasma levels of miR-21 and let-7d were significantly higher in smokers than in non-smokers is in agreement with our own. Furthermore, another study has shown that expression of let-7i-3p and miR-154-5p are significantly downregulated in the sera of smokers.[36]

Table 4. Adjusted[a] relative serum miRNA levels according to number of cigarettes smoked per day, years of smoking, and cigarette-years among current smokers

	Number of cigarettes smoked per day			Years of smoking			Cigarette-years		
miRNA	<20 (N = 98)	≥ 20 (N = 143)	p	<20 (N = 58)	≥ 20 (N = 184)	p	<400 (N = 91)	≥ 400 (N = 149)	p
let-7d	3.26 (1.87 – 5.69)	1.61 (0.84 – 3.10)	0.121	0.67 (0.06 – 7.84)	2.51 (1.66 – 3.80)	0.296	3.86 (1.85 – 8.06)	1.90 (1.14 – 3.17)	0.133
miR-1	1.44 (0.82 – 2.54)	0.84 (0.34 – 2.05)	0.346	0.44 (0.07 – 2.72)	1.29 (0.83 – 2.00)	0.250	1.46 (0.63 – 3.38)	1.06 (0.55 – 2.07)	0.603
miR-17	1.68 (0.98 – 2.87)	0.92 (0.51 – 1.68)	0.161	0.38 (0.04 – 3.84)	1.33 (0.90 – 1.96)	0.288	2.40 (1.18 – 4.86)	0.96 (0.60 – 1.53)	0.041
miR-20a	1.35 (0.78 – 2.34)	0.77 (0.40 – 1.46)	0.207	0.29 (0.03 – 3.32)	1.10 (0.73 – 1.65)	0.286	1.74 (0.85 – 3.57)	0.82 (0.49 – 1.35)	0.103
miR-21	2.87 (1.69 – 4.88)	1.12 (0.62 – 2.04)	0.030	0.77 (0.07 – 8.02)	1.93 (1.31 – 2.86)	0.443	2.92 (1.40 – 6.10)	1.53 (0.95 – 2.48)	0.168
miR-27a	1.21 (0.68 – 2.14)	0.88 (0.47 – 1.67)	0.489	0.15 (0.01 – 1.70)	1.11 (0.74 – 1.66)	0.109	1.50 (0.71 – 3.18)	0.88 (0.53 – 1.46)	0.260
miR-92	1.70 (0.78 – 3.68)	1.52 (0.63 – 3.64)	0.858	1.53 (0.05 – 45.7)	1.62 (0.93 – 2.83)	0.974	3.21 (1.19 – 8.67)	1.14 (0.58 – 2.26)	0.105
miR-122	2.64 (1.47 – 4.74)	1.16 (0.60 – 2.25)	0.081	0.64 (0.05 – 8.47)	1.89 (1.23 – 2.90)	0.413	3.44 (1.59 – 7.45)	1.34 (0.80 – 2.27)	0.058
miR-126	1.44 (0.93 – 2.25)	0.88 (0.53 – 1.44)	0.157	0.50 (0.07 – 3.45)	1.18 (0.86 – 1.63)	0.387	1.64 (0.91 – 2.97)	0.98 (0.66 – 1.45)	0.166
miR-130a	1.32 (0.68 – 2.57)	0.99 (0.48 – 2.07)	0.586	0.41 (0.03 – 6.66)	1.20 (0.75 – 1.92)	0.453	2.35 (0.99 – 5.58)	0.83 (0.47 – 1.47)	0.062
miR-146	2.11 (1.11 – 3.99)	1.19 (0.57 – 2.49)	0.271	1.47 (0.09 – 24.5)	1.65 (1.04 – 2.63)	0.935	3.21 (1.42 – 7.26)	1.17 (0.66 – 2.06)	0.055
miR-150	2.97 (1.83 – 4.83)	1.36 (0.79 – 2.34)	0.046	1.14 (0.13 – 9.98)	2.13 (1.49 – 3.04)	0.575	3.16 (1.64 – 6.09)	1.71 (1.11 – 2.65)	0.141
miR-192	2.03 (1.16 – 3.54)	1.73 (0.93 – 3.23)	0.722	0.65 (0.06 – 7.10)	1.95 (1.31 – 2.90)	0.369	3.01 (1.45 – 6.25)	1.51 (0.93 – 2.46)	0.141
miR-195	1.39 (0.73 – 2.67)	0.65 (0.31 – 1.40)	0.155	0.44 (0.02 – 8.07)	1.03 (0.63 – 1.68)	0.564	1.28 (0.54 – 3.06)	0.89 (0.48 – 1.63)	0.504
miR-197	1.64 (1.06 – 2.56)	1.63 (0.98 – 2.71)	0.987	1.28 (0.18 – 8.96)	1.65 (1.20 – 2.28)	0.798	2.25 (1.27 – 3.99)	1.39 (0.94 – 2.07)	0.191
miR-320	1.57 (1.17 – 2.11)	1.62 (1.16 – 2.26)	0.899	1.39 (0.38 – 5.09)	1.60 (1.29 – 1.98)	0.833	2.16 (1.48 – 3.14)	1.37 (1.05 – 1.77)	0.060

[a] Adjusted for age, sex, BMI, SBP, HbA1c, serum total cholesterol levels, eGFR and current drinking status.

Smoking is implicated in the onset of lifestyle-related diseases such as cancer and CVD.[19-21] The harmful health effects are considered to result from exposure to the complex mixture of organic and inorganic chemicals in cigarette smoke and their carcinogenic, pro-inflammatory, and proatherogenic properties.[37] Both oxidative stress and the direct effects of nicotine have been reported to cause dysregulation in expression of intracellular miRNAs such as miR-21 and miR-133.[38,39] However, although the effects of individual components of cigarette smoke have been investigated in epigenetic studies, the biological mechanisms for these effects have yet to be fully characterized.

Aberrant intracellular expression of certain miRNAs, which can act as tumor suppressors or oncogenes[40], has been implicated in oncogenesis.[7] Different cancers have also been shown to have different miRNA profiles. For example, among the miRNAs investigated in the present study, miR-21, miR-130a, and miR-146 have been found to act as onco-miRNAs while let-7d and miR-122 have been identified as tumor suppressor-miRNAs for various cancers.[25] Moreover, some studies have shown that dysregulation of miRNA may be implicated in cardiac hypertrophy and myocardial infarction (MI). In particular, previous work has shown that elevated expression of miR-21[26] and miR-195[27] in cardiac tissue is associated with cardiac hypertrophy and heart failure. Moreover, downregulation of miR-1 and miR-133a has also been observed in cardiac tissue from subjects who experienced MI when compared with healthy controls.[41]

Given that various cells in the body release miRNA into blood, investigators have been able to detect not only cellular miRNAs but also circulating miRNAs. Although their function has yet to be clearly understood, circulating miRNAs, which are contained within vesicles such as exosomes or in high-density lipoprotein particles, have been investigated as potential biomarkers for various diseases, such as cancer,[14,15] CVD,[16,17] and diabetes.[18] They are also considered to play a role in cell-to-cell communication.[42]

Circulating miRNAs, whose levels are not necessarily correlated with those of intracellular miRNAs, have been implicated in the development of several smoking-related diseases. While elevated levels of miR-1 and miR-195 are associated with acute MI,[28,29] circulating miR-21 levels have been positively associated with lung cancer and peripheral arterial diseases.[30] Previous work has also shown that increased circulating levels of miR-146 and decreased levels of miR-21, miR-150, miR-197 are correlated with type 2 diabetes mellitus.[18,31] Furthermore, circulating miR-192 levels may be inversely correlated with impaired glucose tolerance.[32]

The present study has several limitations. First, although our cross-sectional analysis has identified 10 miRNAs that were associated with cigarette smoking, further longitudinal studies are required to prove a causal relationship between expression of these miRNAs and smoking-related pathology. Second, although we were able to appropriately adjust for a range of confounding factors in our analyses, residual confounding cannot be completely ruled out.

In summary, we found that cigarette smoking is associated with elevated expression of various serum miRNAs. However, duration of smoking and number of cigarettes smoked per day showed little association with serum miRNA levels. Cigarette smoking may therefore be a factor influencing serum miRNA levels. Our findings suggest that it is necessary to consider the effect of confounding caused by smoking when evaluating expression of serum miRNAs to diagnose pathological conditions.

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Conflict of interests

The authors declare no conflicts of interest.

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