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Quantification of Circulating Cell-Free DNA in the Serum of Patients with Obstructive Sleep Apnea–Hypopnea Syndrome

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Abstract Serum cell-free DNA concentrations have been reported to increase in many acute diseases as well as in some chronic conditions such as cancer and autoimmune diseases. The aim of this study was to examine whether serum DNA concentrations were elevated in patients with obstructive sleep apnea-hypopnea syndrome (OSAHS). The effects of nasal continuous positive airway pressure (nCPAP) on serum DNA were also investigated. One hundred twenty-seven people diagnosed with OSAHS by polysomnography (PSG) were admitted into the OSAHS group, and 52 subjects without OSAHS were recruited for the control group. The OSAHS group was further divided into mild, moderate, and severe OSAHS subgroups based on their apnea-hypopnea index (AHI) during sleep. Ten patients with moderate and severe OSAHS were treated with nCPAP. Serum DNA, interleukin-6 (IL-6), and malonaldehyde (MDA) concentrations were measured and were found to be significantly higher in patients with moderate and severe OSAHS groups than those in the mild OSAHS and control groups ($p < 0.05$). Univariate analysis showed that serum DNA correlated positively with AHI, oxygen desaturation index (ODI), IL-6, and MDA, and negatively correlated with minimal oxygen saturation ($miniSaO_2$) (all $p < 0.05$). In stepwise multiple regression analysis, only MDA and $miniSaO_2$ were suggested as significant independent predictors for the serum DNA concentrations. After 6 months of nCPAP therapy, serum concentrations of DNA, IL-6, and MDA were significantly decreased ($p < 0.05$). The increasing concentration of serum DNA in patients with OSAHS was positively correlated with disease severity. Serum DNA may become an important parameter for monitoring the severity of OSAHS and effectiveness of therapy.

Keywords Obstructive sleep apnea-hypopnea syndrome · Serum cell-free DNA · Interleukin-6 · Malonaldehyde

Abbreviations

AHI	Apnea-hypopnea index
IL-6	Interleukin-6
MDA	Malonaldehyde
nCPAP	Nasal continuous positive airway pressure
ODI	Oxygen desaturation index
OSAHS	Obstructive sleep apnea-hypopnea syndrome
PSG	Polysomnography

Introduction

Obstructive sleep apnea-hypopnea syndrome (OSAHS), a chronic disorder characterized by sleep-related upper-airway obstruction, is strongly and independently associated with an increased risk for hypertension [1], myocardial ischemia [2–4], and stroke [5, 6]. Patients with OSAHS experience repetitive episodes of hypoxia/reoxygenation during transient cessation of breathing that promote systemic oxidative stress and inflammation, which are fundamental mechanisms underlying tissue and cell damage [7, 8].

Circulating serum cell-free DNA is present in small amounts in the serum of healthy individuals. Recently,

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increased concentrations of cell-free DNA have been found in various clinical conditions, including autoimmune diseases, cancer, stroke, and myocardial infarction [9–13]. Following effective treatment, circulating cell-free DNA concentrations in patients with cancer, acute pancreatitis, and other diseases could decrease, suggesting that quantification of cell-free DNA could be a novel approach to monitoring disease severity and the response to treatment [14–16]. However, the kinetics of circulating cell-free DNA concentrations either during or after therapy remains unknown, and cell death by either apoptosis or necrosis is thought to be the main source of the circulating DNA.

In this study we tested the hypothesis that elevated serum cell-free DNA concentrations are probably due to oxidative stress and inflammation in OSAHS patients, and that treatment with nasal continuous positive airway pressure (nCPAP) could reverse these alterations.

Methods

Study Population

Patients

Patients who came to the Sleep Disorders Center at Ruijin Hospital for evaluation of sleep-disordered breathing from September 2007 to January 2009 were prospectively screened for the study. Patients with newly diagnosed OSAHS, defined as an apnea-hypopnea index (AHI) of five or more obstructive events per hour of sleep, and free of conditions known to affect the serum cell-free DNA concentrations were eligible for the study. Patients with hypertension, coronary artery disease, autoimmune diseases, a history of stroke, diabetes mellitus, chronic obstructive or restrictive pulmonary disease, chronic renal disease, or tobacco use within the past 10 years were ineligible for the study. Data on age, smoking status, alcohol drinking, and medical history of diseases were obtained from questionnaire-based interviews, which were conducted by trained personnel. Diagnosis of hypertension or diabetes mellitus was determined on the basis of medical history and a comprehensive health examination, including measurement of blood pressure and assay of blood glucose.

Control Subjects

Our control subjects were nonsmoking healthy subjects who were not taking any medications or nutritional supplements. Control subjects were matched to patients for gender, age, and BMI. All control subjects underwent polysomnography (PSG) to exclude the presence of sleep-disordered breathing. The Shanghai Jiaotong University Committee on Human Research approved the study. All study participants gave written informed consent.

Study Protocol

All study participants underwent attended nocturnal PSG in the sleep disorders center. Each blood sample of peripheral venous blood was collected after overnight polysomnography from 6 a.m. to 7 a.m. Immediately after blood draw, serum was carefully removed by centrifugation at 3,000 rpm for 10 min and stored at −80°C until analysis.

Polysomnography and nCPAP Therapy

Nocturnal polysomnography was performed as previously described [17]. AHI was defined as the number of obstructive apnea plus hypopnea episodes per hour of sleep. Adherence to nCPAP was defined as nCPAP use for 4 or more hours daily. Adherence was assessed by use of an nCPAP device with compliance software.

Preparation and Measurement of Serum DNA

Serum DNA was extracted from 200 μl serum using a QIAmp Blood Kit (Qiagen, Valencia, CA, USA) according to the protocol recommended by the manufacturer. Serum DNA concentrations were measured using a real-time quantitative polymerase chain reaction (PCR) assay (Applied Biosystems, Foster, CA, USA) for the β-globin gene, which is present in all nucleated cells of the body [18]. The β-globin PCR system included the amplification primers β-globin-354F (5′-GTG CAC CTG ACT CCT GAG GAG A-3′) and β-globin-455R (5′-CCT TGA TAC CAA CCT GCC CAG-3′) [18] and iQ^{TM} SYBR$^{®}$ Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA). All samples were analyzed in duplicate. Thermocycling was performed at 95°C for 30 s, followed by 45 cycles at 95°C for 15 s and 60°C for 10 s to measure the fluorescence signal. For a standard curve, serum DNA was used at concentrations of 10^4–10^8 copies/μl. Of the standard solutions, 2 μl was used in PCR as described above. DNA concentrations in the serum samples were calculated as ng DNA/ml serum.

Measurement of Serum Interleukin-6 (IL-6) and Malonaldehyde (MDA)

IL-6 was measured by enzyme linked immunosorbent assay (ELISA; R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. MDA was measured by the thiobarbituric acid method using the MDA Test Kit (Nanjing Jiancheng, China).

Statistical Analysis

All parameters except serum DNA concentrations were expressed as mean ± standard deviation (SD). DNA concentration was not under normal distribution and was expressed as median (interval of quartile). Analysis of variance was used for the mean of samples among groups. A least significant difference (LSD) test was used for paired comparison between two groups if comparison among multiple groups with analysis of variance showed statistical significance. Univariate analysis was depicted by Pearson's coefficient. A multilinear regression analysis was performed with AHI, oxygen desaturation index (ODI), minimal oxygen saturation ($miniSaO_2$), MDA, and IL-6 as the independent variables and serum DNA as the dependent variable. Comparison of parameters before and after nCPAP treatment was undertaken with a paired t test. $p < 0.05$ was considered statistically significant.

Results

Six hundred fifty-one patients were newly diagnosed with OSAHS during the study period. Five hundred twenty-four patients were not eligible for the study because of the presence of one or more exclusion criteria. The patients in the OSAHS group were further divided into mild, moderate, and severe OSAHS subgroups based on their AHI during sleep. OSAHS and its division by degree were evaluated using international diagnostic criteria [19]. Among all OSAHS patients, ten with moderate and severe OSAHS underwent nCPAP treatment for 6 months. Fifty-two control subjects were studied.

Comparison of Baseline Characteristics Among Groups

The clinical characteristics of the study participants are summarized in Table 1. Patients and control subjects were similar in age, gender, BMI, neck circumference (NC), and systemic blood pressure (all $p > 0.05$). OSAHS patients had significantly lower $miniSaO_2$ and higher ODI during sleep than control subjects. Moreover, ODI and $miniSaO_2$ were all statistically different among the OSAHS subgroups.

Comparison of Serum DNA Among Groups

Serum DNA concentrations in the moderate and severe OSAHS subgroups were significantly higher than those in the mild OSAHS subgroup and the control group (all $p < 0.05$).Similarly, serum DNA concentrations in the severe OSAHS subgroup were significantly higher than those in the moderate OSAHS subgroup. No statistical difference in serum DNA was detected between the mild OSAHS subgroup and the control group ($p > 0.05$) (Table 1).

Comparison of Serum IL-6 and MDA Among Groups

Serum IL-6 and MDA concentrations in moderate and severe OSAHS subgroups were significantly higher than those in the mild OSAHS subgroup and the control group

Table 1 Baseline characteristics of control group and OSAHS subgroups

Variables	Control ($n = 52$)	Mild OSAHS ($n = 43$)	Moderate OSAHS ($n = 39$)	Severe OSAHS ($n = 45$)	F value	p value
Age (years)	45 ± 10	45 ± 12	47 ± 10	44 ± 12	0.41	0.75
Sex (F/M)	15/37	11/32	8/31	6/39	3.70[a]	0.30
BMI (kg/m^2)	26.0 ± 3.2	26.2 ± 3.0	26.6 ± 4.8	26.2 ± 3.1	0.20	0.90
NC (cm)	38.2 ± 2.8	38.0 ± 2.2	38.5 ± 3.2	38.5 ± 2.6	0.69	0.54
SBP (mmHg)	116 ± 10	118 ± 9	119 ± 11	118 ± 8	2.22	0.08
DBP (mmHg)	73 ± 7	75 ± 6	76 ± 7	76 ± 6	1.89	0.13
AHI	2.0 ± 1.4	10.8 ± 4.3*	29.5 ± 5.2*,†	67.1 ± 14.3*,†,§	166.07	<0.01
ODI	3.5 ± 3.5	17.0 ± 11.4*	30.0 ± 11.9*,†	63.6 ± 13.3*,†,§	147.80	<0.01
$MiniSaO_2$	91.4 ± 3.5	85.8 ± 5.5*	81.0 ± 8.5*,†	67.1 ± 11.6*,†,§	117.2	<0.01
IL-6 (pg/ml)	53.6 ± 3.5	64.2 ± 4.2	121.2 ± 9.9*,†	144.5 ± 10.9*,†,§	35.0	<0.01
MDA (nmol/ml)	4.5 ± 1.2	4.6 ± 1.1	6.3 ± 2.1*,†	8.1 ± 2.9*,†,§	25.5	<0.01
Serum DNA (ng/ml)	10.2 (4.3–26.3)	19.8 (4.9–35.3)	31.1*,† (19.0–74.1)	67.4*,†,§ (36.7–137.5)	72.0[a]	<0.01

BMI body mass index, *NC* neck circumference, *SBP* systolic blood pressure, *DBP* diastolic blood pressure, *AHI* apnea-hypopnea index, *ODI* oxygen desaturation index, *miniSaO$_2$* minimal arterial oxygen saturation, *IL-6* interleukin-6, *MDA* malonaldehyde. Values are mean ± SD

[a] represents χ^2 value

* $p < 0.05$ versus control; † $p < 0.05$ versus mild OSAHS; § $p < 0.05$ versus moderate OSAHS

(all $p < 0.05$). Similarly, these concentrations in the severe OSAHS subgroup were significantly higher than those in the moderate OSAHS subgroup. No statistical differences in serum IL-6 and MDA were detected between the mild OSAHS subgroup and the control group ($p > 0.05$) (Table 1).

Relationships Between Serum DNA Concentrations and Polysomnographic Variables, Age, BMI, NC, IL-6, and MDA in all OSAHS Patients

In univariate analysis, serum DNA concentrations were positively correlated with AHI, ODI, IL-6, and MDA ($r = 0.465$, 0.372, 0.553, 0.601, all $p < 0.001$) but were negatively correlated with miniSaO$_2$ ($r = -0.345$, $p < 0.001$). In stepwise multiple regression analysis that included AHI, ODI, miniSaO$_2$, IL-6, and MDA, only MDA and miniSaO$_2$ were significant independent predictors for the serum DNA concentrations (standardized regression coefficients were 0.548 and −0.151, respectively, $p < 0.05$). The normality test of the regressions was passed (Table 2, Figs. 1 and 2).

Comparison Before and on CPAP Treatment

There was a significant decrease in serum DNA, IL-6, and MDA concentrations after CPAP treatment compared with before CPAP treatment (all $p < 0.001$) (Table 3).

Discussion

The β-globin gene measured in our study is the most frequently used gene in previous cell-free DNA studies [18, 20]. Our results showed that cell-free DNA measured in serum of patients with severe and moderate OSAHS was significantly higher than in subjects with mild OSAHS and those without OSAHS. This is the first report that shows that nCPAP treatment can effectively decrease serum DNA concentrations in OSAHS patients.

Table 2 Correlation between serum DNA and baseline characteristics

Variables	Correlation coefficient	p value
Age	−0.033	0.665
BMI	0.050	0.509
NC	0.108	0.152
AHI	0.465	<0.001
ODI	0.372	<0.001
IL-6	0.553	<0.001

BMI body mass index, *NC* neck circumference, *AHI* apnea–hypopnea index, *ODI* oxygen desaturation index, *IL-6* interleukin-6

The mechanisms underlying the presence of circulating DNA are generally unknown. Apoptosis, necrosis, and impaired clearance have been implicated [21–23], but information regarding their role in OSAHS is scarce. Shin et al. [24] first reported that cell-free DNA concentrations

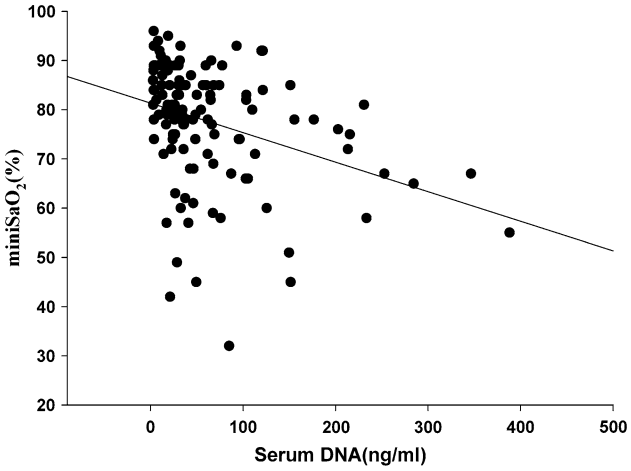

Fig. 1 Negative correlation between serum DNA level and miniSaO$_2$ in all OSAHS patients ($n = 127$, $r = -0.345$, $p < 0.001$). *MiniSaO$_2$* minimal oxygen saturation. Data were analyzed using Pearson's correlation coefficients

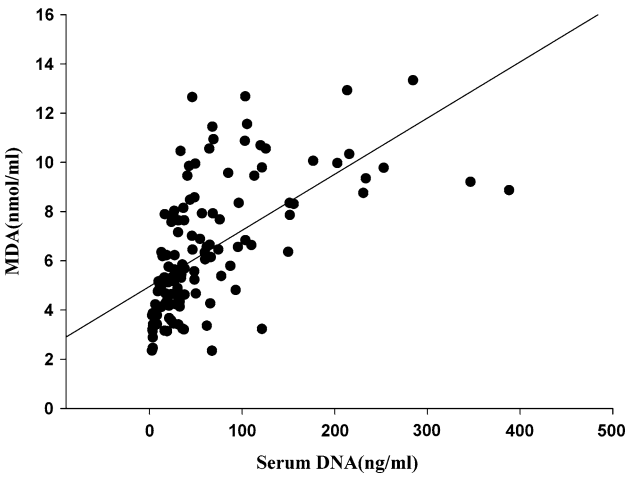

Fig. 2 Positive correlation between serum DNA level and MDA in all OSAHS patients ($n = 127$, $r = 0.601$, $p < 0.001$). *MDA* malonaldehyde. Data were analyzed using Pearson's correlation coefficients

Table 3 Comparison before and after 6 months of CPAP treatment

Variables	Before treatment	After treatment	t value	p value
IL-6 (pg/ml)	131.66 ± 20.39	66.04 ± 10.05	3.86	<0.01
MDA (nmol/ml)	8.11 ± 3.15	5.55 ± 2.10	2.14	0.04
Serum DNA (ng/ml)	117.22 ± 23.12	38.52 ± 8.59	5.14	<0.01

IL-6 interleukin-6, *MDA* malonaldehyde

were increased in patients with severe obstructive sleep apnea (OSA). However, they did not study the association between oxidative stress, inflammation, and circulating cell-free DNA concentrations. Obstructive sleep apnea syndrome with its hypoxia/reoxygenation episodes may be viewed as similar to ischemic/reperfusion injury, causing the generation of reactive oxygen species (ROS) [25]. MDA is regarded as an appropriate biomarker of oxidative stress [26]. Intermittent hypoxia has been associated with systematic inflammation, which is likely to play a crucial role in the activation of proinflammatory factors with consequent production of proinflammatory cytokines such as IL-6 and tumor necrosis factor-α [27, 28].

In this study we found elevated serum DNA concentrations in patients with moderate and severe OSAHS. The serum DNA concentrations were positively correlated with AHI and ODI but negatively correlated with miniSaO_2, suggesting that serum DNA concentrations increased when OSAHS became severe. The severity of OSAHS could thus be reflected by the concentration of serum DNA. In particular, our study demonstrated that the serum IL-6 and MDA concentrations significantly increased in moderate and severe OSAHS patients, and these concentrations were positively correlated with serum DNA concentrations. Then we performed a multiple linear regression analysis for predicting serum DNA concentrations. MDA and miniSaO_2 were significant independent predictors for serum DNA concentrations. It has been suggested that higher cell-free serum DNA concentrations of OSAHS patients may be attributed to increased oxidative stress that occurs under more severe illness conditions. Free serum DNA in OSAHS patients is supposed to originate from the apoptosis or necrosis of tissue [29, 30], and the correlation of serum IL-6, MDA, and serum DNA concentrations also supports this hypothesis. Therefore, we can infer that the serum DNA concentrations of patients with mild disease were not significantly different from those of the controls because they have only limited or recovered oxidative stress. Greater tissue necrosis or apoptosis levels would cause a significant influx of free DNA into circulation, with its concentrations rising during chronic intermittent hypoxia. Although we have excluded patients with other possible sources of free DNA (e.g., infection, trauma, myocardial infarction), we did not provide direct evidence that the free serum DNA originates from tissue necrosis or apoptosis.

Because serum DNA concentrations were positively correlated with AHI and ODI and negatively correlated with miniSaO_2, we evaluated whether reversion of hypoxia using nCPAP treatment could alter serum DNA concentrations. After 6 months of follow-up of ten OSAHS patients on CPAP treatment, there was a remarkable improvement not only in IL-6 and MDA levels but also in serum DNA (all $p < 0.05$). This observation provides further evidence that the elevated serum DNA in OSAHS patients might result from intermittent hypoxemia caused by repeated apnea and hypopnea events, since oxidative stress and inflammatory reactions became weak as abnormally high AHI and low SaO_2 were corrected by CPAP treatment [31]. There were potential limitations in our study. It was not designed as a randomized controlled trial. Of all the OSAHS patients, only ten moderate and severe OSAHS patients underwent nCPAP treatment. Therefore, the present findings need to be confirmed in randomized controlled trials with larger sample sizes.

In conclusion, we have shown that OSAHS patients have significantly increased serum DNA concentrations, which were positively correlated with disease severity. Moreover, the present investigation is the first to report that elevated serum DNA concentrations might be gradually reversed by nCPAP treatment. Consequently, the serum DNA may become an important parameter for monitoring the disease severity of OSAHS and therapeutic effectiveness. The kinetics of serum cell-free DNA concentrations in OSAHS patients need to be further investigated.

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