Am J Transl Res 2020;12(6):2521-2537
www.ajtr.org /ISSN:1943-8141/AJTR0100794

Original Article

Aberrant DNA methylation levels of the *formyl peptide receptor* 1/2/3 genes are associated with obstructive sleep apnea and its clinical phenotypes

Yung-Che Chen[1,2*], Kuo-Tung Huang[1,2*], Mao-Chang Su[1,2,6], Po-Yuan Hsu[1], Chien-Hung Chin[1,2,7], I-Chun Lin[3], Chia-Wei Liou[4], Ting-Ya Wang[1], Yong-Yong Lin[1], Chang-Chun Hsiao[5], Meng-Chih Lin[1,2]

[1]Division of Pulmonary and Critical Care Medicine, [2]Sleep Center, Departments of [3]Pediatrics, [4]Neurology, Kaohsiung Chang Gung Memorial Hospital and Chang Gung University College of Medicine, Kaohsiung 83301, Taiwan; [5]Graduate Institute of Clinical Medical Sciences, College of Medicine, Chang Gung University, Taoyuan 33302, Taiwan; [6]Chang Gung University of Science and Technology, Chia-yi, Taiwan; [7]Chung Shan Medical University School of Medicine, Taichung, Taiwan. [*]Equal contributors.

Received August 10, 2019; Accepted April 15, 2020; Epub June 15, 2020; Published June 30, 2020

Abstract: Background: FPR1 over-expression and insufficiency of FPR2 and FPR3 are associated with disease severity of obstructive sleep apnea (OSA). We hypothesized that epigenetic modification of the *FPR1/2/3* genes may underlie intermittent hypoxia with re-oxygenation (IHR) injury in OSA. Methods: DNA methylation levels over 17 CpG sites of the *FPR1/2/3* genes and their gene expression levels in the peripheral blood mononuclear cells were determined in 40 treatment-naïve OSA patients, 12 severe OSA patients under long-term continuous positive airway pressure treatment, 16 primary snoring (PS) subjects, and 10 healthy non-snorers (HS). Results: Both -524 and -264 CpG sites of the *FPR1* gene were hypomethylated in treatment-naïve OSA versus HS, while -264 CpG site methylation level was negatively correlated with *FPR1/FPR3* gene expression ratio and associated with prevalent diabetes mellitus. Both +8802 and +8845 CpG sites of the *FPR2* gene were hypermethylated in treatment-naive OSA versus HS, while hypermethylated +9132 and +9150 CpG sites were both associated with prevalent hypertension. *FPR3* gene expression and DNA methylation levels over -842/-516 CpG sites of the *FPR3* gene were both decreased in treatment-naive OSA versus HS, while hypermethylated -429 CpG site was associated with elevated serum C-reactive protein level. In vitro IHR stimuli in human monocytic THP-1 cells resulted in gene promoter hypomethylation-mediated *FPR1* over-expression, increased production of reactive oxygen species, and increased cell apoptosis, which could be reversed with re-methylation agent, folic acid, treatment. Conclusions: Aberrant DNA methylation patterns of the *FPR1/2/3* gene promoters contribute to disease severity and diabetes mellitus or cardiovascular disease in OSA patients, probably through regulating *FPR1/2/3* gene expressions.

Keywords: Obstructive sleep apnea, formyl peptide receptor, DNA methylation, intermittent hypoxia with re-oxygenation

Introduction

Obstructive sleep apnea (OSA) syndrome is a breathing disorder characterized by cyclic upper airway collapse and airflow limitation during sleep. The associations of OSA with endothelial dysfunction and cardiovascular morbidities rely on the apnea related multiple cycles of intermittent hypoxia and re-oxygenation (IHR) with increased production of reactive oxygen species (ROS), thereby initiating systemic inflammation and cell apoptosis. Although robust protective effect of short-term continuous positive airway pressure (CPAP) treatment on endothelial function and refractory hypertension has been demonstrated, meta-analysis of the randomized controlled trials showed that long-term CPAP use in OSA patients could not reduce cardiovascular risks or improve glucose control probably due to inadequate use time per night during sleep [1-4]. Thus, developing biological agents that can inhibit chronic systemic inflammation and subsequent cell apoptosis is warranted to improve long-term outcomes in OSA.

Formyl peptide receptors 1/2/3 (FPR1/2/3) belong to a family of G protein-coupled pattern

recognition receptors, which are mainly expressed by mammalian phagocytic leukocytes. FPR1 can trigger phosphatidylinositol-3-kinase (PI3K), protein kinase C (PKC), mitogen-activated protein (MAP) kinases p38, and extracellular signal related kinases 1/2 (ERK 1/2) NADPH-oxidase signaling pathways, resulting in enhanced chemotaxis and ROS production in neutrophils and pro-inflammatory M1 macrophages [5-7]. In contrast, FPR2 homodimerization accelerates resolution of self-limited inflammation, increases IL-10 generation, enhances macrophage efferocytosis, and inhibits ROS production through phosphorylation of p38, MAPK-activated protein kinase (MAPKAPK) and NADPH-oxidase signaling pathways [8-11]. FPR1 and FPR2 have been shown to contribute to endothelial/cardiac/cerebral dysfunction and repair in IHR/ischemia reperfusion injury, respectively [5, 12-14]. On the other hand, constitutive internalization and phosphorylation of FPR3 indicate that it may serve as a decoy receptor that does not transduce signal but regulates the function of one or two other FPRs through undergoing rapid constitutive recycling to bind extracellular ligand and internalize it for degradation [15]. In our recent study, we demonstrated that FPR1 over-expression and FPR2/FPR3 under-expression may play a role in the development of OSA, while underlying mechanisms remains to be clarified [16].

DNA methylation of cytosine residues is an inheritable and reversible epigenetic change, which silences gene transcription by altering accessibility for transcription factors in the gene promoter region, or activates gene transcription by alternative splicing and regulating alternative promoters in the gene body region [17, 18]. Emerging evidence suggests that changes in DNA methylation contribute to pathologies caused by chronic IHR and potentially mediate adaptations to chronic sustained hypoxia by affecting the hypoxia-inducible factor signaling pathway. In our previous whole genome DNA methylation analysis study, we found that *IL1R2* hypomethylation and *AR* hypermethylation may constitute an important determinant of disease severity, whereas *NPR2* hypomethylation and *SP140* hypermethylation may provide a biomarker for vulnerability to excessive daytime sleepiness in OSA [19]. It has been demonstrated that specificity protein 1 (Sp1) binding to the *FPR2* gene promoter region is key for the maximal promoter activity, which is instead suppressed by DNA methylation and histone modifications, while *FPR1* gene promoter activity requires binding of another transcription factor, PU.1. [20-23]. Thus, in this study, we hypothesized that DNA methylation of the *FPR*1/2/3 genes may either contribute to the development of adverse consequences and clinical phenotypes of OSA, or be modified by environmental factors in early life to prime individuals to develop OSA at adulthood.

Methods

Subjects

The study participants were recruited from the sleep center of Kaohsiung Chang Gung Memorial Hospital from August 2014 through July 2017. Ninety eight subjects presenting with loud snoring underwent full-night polysomnography examination and were screened. The exclusion criteria included ongoing infections, autoimmune disease, use of immunosuppressive agent in the past 6 months, narcolepsy, severe obesity (body mass index, BMI, ≥35 kg/m^2), old age (>65 year-old), and those with a BMI<21 kg/m^2. OSA (apnea hypopnea index, AHI, ≥5 events/hour) and primary snoring (PS; AHI<5 events/hour) were diagnosed by full night polysomnography examination at the sleep center of Kaohsiung Chang Gung Memorial Hospital. Nocturnal hypoxemia was evaluated in terms of the percentage of total recording time with an oxyhemoglobin saturation <90% (%time <90% SaO_2), mean SaO_2, minimum SaO_2, and the number of dips >4% of basal SaO_2%//hour (oxygen desaturation index, ODI). Periodic limb movements in sleep (PLMS) were scored and defined as significant limb movement (LM) events occurring in a PLM series according to the AASM scoring rules [24]. PLMS were scored when ≥4 consecutive LMs occurred and were separated by at least 5 sec and at most 90 sec. Periodic limb movement disorder (PLMD) was defined as a PLMS frequency of ≥15/ h. The Epworth Sleepiness Scale (ESS) recorded at the examination was used to measure sleep propensity. Healthy non-snorers (HS) were defined as the absence of loud snoring and OSA symptoms, reported by the subjects and their bed partners.

Isolation of DNA and RNA from blood leukocyte samples

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood of all study subjects using a two-layer Ficoll-Histopaque density gradient centrifugation (Histopaque 1.077 and 1.119; Sigma Diagnostics, St.Louis, MO) method. Samples were stored in RNA*later*® RNA Stabilization Solution (Ambion®) at -80°C until analysis. An RNeasy®Plus Mini Kit (Qiagen, Hilden, Germany) was used for isolation of high quality total RNA, and treated with DNase. DNA was extracted using Puregene Core kit (Qiagen, Maryland, USA).

Measurement of DNA methylation levels of the FPR1/2/3 gene promoter and gene body regions by bisulfite pyro-sequencing

Ten promoter and gene body regions of the FPR1/2/3 genes, including 17 CpG (cytosine guanine dinucleotides with the phosphodiester bond) sites, were amplified based on reference sequence information from NCBI (*FPR1*: NM_001193306.1; *FPR2*: XM_006723120.3; *FPR3*: NM_002030.4). Bisulfite treatment was performed using an EpiTect 96 Bisulfite Kit (Qiagen) and polymerase chain reaction (PCR) amplification was performed using a PyroMark PCR Kit (Qiagen). The PCR conditions were 45 cycles of 95°C for 20 s, 50°C for 20 s, and 72°C for 20 s, followed by 72°C for 5 min. The primer sequences used for PCR amplification and pyro-sequencing for these regions are listed in **Table 1**. The biotin-labeled PCR product was captured by Streptavidin-Sepharose HP (Amersham Pharmacia). Quantitation of cytosine methylation was performed using a PyroMark Q24 system (Qiagen). The amount of C relative to the sum of the amounts of C and T at each CpG site was calculated as a percentage [25]. Representative pyrograms of CpG di-nucleotides assayed of the *FPR*1/2/3 genes in OSA patients are presented in **Figure 1A-G**.

Determination of the FPR1/2/3 gene expressions of isolated PBMCs using quantitative reverse-transcriptase polymerase chain reaction (RT-PCR) method

To determine the expressions of the *FPR1/2/3 genes* of the isolated PBMCs, the gene expressions were analyzed using quantitative RT-PCR in a 96-well format. The house keeping gene *GAPDH* was chosen as an endogenous control to normalize the expression data for each gene. All PCR primers (random hexamers) were designed and purchased from Roche according to the company's protocols (www.roche-applied-science.com), and their sequences are given in **Table 1**. A total of 300 ng RNA was used for synthesis of first strand cDNA with QuantiTectReverse Transcription Kit (QIAGEN, Germany). A total of 5 µl of the reverse transcription reaction was added to 5 µl of master mix (QIAGEN, SYBR Green PCR kit; Roche, Germany). The PCR reactions with 45 cycles of amplification were run in a Roche Light-Cycle 480 machine. Single real time PCR experiment was carried out on each sample for each target gene by the Roch Light CyclerQuantiFast R system. Relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method with the median value for the control group as the calibrator.

In vitro human monocyte cell line culture under IHR stimuli

The human monocytic cell line THP-1 cells obtained from ATCC (1×10^6 cells/ml) were re-suspended in a culture dish containing RPMI 1640 medium, and then exposed to normoxia (NOX) or IHR in a custom-designed, incubation chambers which were attached to an external O_2-CO_2 hand-driven controller as previously described [26]. Air-phase set point for IHR consisted of a 35-min hypoxic period (0% O_2 and 5% CO_2), followed by 25 min of re-oxygenation (21% O_2 and 5% CO_2), 7 hours each day for 2 days. Control cells were maintained in NOX condition for the same durations. To investigate the effect of epigenetic intervention, 500 nM folic acid (FA) (Sigma-Aldrich, St. Louis, MO, USA) was supplemented in the IHR condition. DNA methylation and gene expression levels of the FPR1/2/3 genes were determined using pyrosequencing and quantitative RT-PCR methods, as described above. Cell apoptosis rates were measured by flow cytometry using an Annexin V/Propidium iodide (PI) apoptosis detection kit (BD Biosciences, Franklin Lakes, NJ, USA), and analyzed using the FACScan flow-cytometry system (Becton Dickinson, San Diego, CA, USA). The amount of viable cells was determined via optical density measurement of WST-1 reagent (Roche, Mannheim, Germany)

Table 1. Primer sequences of the FPR1/2/3 genes used in the pyrosequencing and quantitative RT-PCR experiments

Gene name	CpG location relative to transcription start site	Primer	Sequences
FPR1	-431/-433 (region 1)	Forward PCR Primer	GGGTTAATTTTGGTGGTGTGT
		Biotinylated Reverse PCR Primer	ATCCAAATATCCCAACCCCTACTCCC
		Forward Sequencing Primer	ATTTTGGTGGTGTGTA
FPR1	-264 (region 2)	Forward PCR Primer	GATAGTGGGTTGGGTTTGTTTAGA
		Biotinylated Reverse PCR Primer	ATTCTTCTCACCACAATACTTACTACT
		Forward Sequencing Primer	GAGGTTTAGTGTTTTTAATAATAAG
FPR1	+1677 (region 3)	Forward PCR Primer	TGTTATTATGAGGGTTATTGTTTTTATGG
		Biotinylated Reverse PCR Primer	ATAAATATTTTACTCCTACAACCTATCTC
		Forward Sequencing Primer	TTTAAAGTTATTTAATAGTTTAGGT
FPR2	-487/-499 (region 4)	Forward PCR Primer	GGTTGGAGTATAGTGGTGTAAT
		Biotinylated Reverse PCR Primer	AACCAACATATTAAAACCTCACTTCTATT
		Forward Sequencing Primer	AGTTTTTTAAGTAGTTTGAATTAT
FPR2	-348 (region 5)	Forward PCR Primer	GGTTTTTTAAAGTGTTGGGATTATAAGTAT
		Biotinylated Reverse PCR Primer	AAACTACCCAAAACAAAACCACAACTA
		Forward Sequencing Primer	GTGTTGGGATTATAAGTATGA
FPR2	+8802/+8845 (region 6)	Forward PCR Primer	TGGAGAATTGTTTGATATGGAAAAGATTT
		Biotinylated Reverse PCR Primer	ACCCTTCCTTCTTCAAAATTTCATA
		Forward Sequencing Primer	GATGTTAGAAGTATTGTATAAGT
FPR2	+9132/+9134/+9150 (region 7)	Forward PCR Primer	TGTTAGGGGATTGGTTTTAGTTT
		Biotinylated Reverse PCR Primer	AACTCCTTTTCCTATATACTTCTTAACTT
		Forward Sequencing Primer	TTTTTTTTTTATTATTTAGTAGTTT
FPR3	-842 (region 8)	Forward PCR Primer	TTTGTGGGAGTGAGCCCTTTAGCT
		Biotinylated Reverse PCR Primer	GAGTGAGCCCTTTAGCTT
		Forward Sequencing Primer	TTAATTGCAGGTGGCCCAGGTGGT
FPR3	-516/-429/-345 (region 9)	Forward PCR Primer	AGGTGTTTTTTGGGAATGG
		Biotinylated Reverse PCR Primer	CATACCCACTAAAACTAACCTATT
		Forward Sequencing Primer	GGATTGATTTTAGTTTTATTGTT
FPR3	+4147 (region 10)	Forward PCR Primer	TTGTTTTTTTGAGTGTGGTTTTTAGATGT
		Biotinylated Reverse PCR Primer	CCTCAACAACCACCAAAAATCA
		Forward Sequencing Primer	AATATTGTAGTAGTTTTATTGAAGT
FPR1	Quantitative RT-PCR	Forward-qRT-PCR Primer	5'-GTTGGACTAGCCACAATTCAAGT
		Reverse-qRT-PCR Primer	5'-CCAGGAAGAGATAGCCAGCA
FPR2	Quantitative RT-PCR	Forward Primer	5'-CCTCAGGAAAATGCACCAG
		Reverse Primer	5'-GCCAGCAGACTCATAGGACAC
FPR3	Quantitative RT-PCR	Forward Primer	5'-GCTAGTCCACGGAGTCACCT
		Reverse Primer	5'-CAGCCACCCAGATCACAAG

using a microplate reader at 450 nm, with 600 nm as a reference wavelength. Representative pyrograms of -433/-431/-264CpG sites of the *FPR1* genes in NOX, IHR, and IHR plus FA conditions are presented in **Figure 1H**, **1I**.

Statistical analysis

Continuous values were expressed as mean ± standard deviation (SD). The differences between two groups were analyzed using the Mann-Whitney U-test, or X^2-test, as appropriate. Kruskal-Wallis test followed by post hoc analysis was used for comparing distributions of more than two experimental groups. Multivariate linear regression analysis was used to adjust for all potential confounding factors (age, BMI, gender, smoking, alcoholism, co-morbidities, and CPAP use) in case and control comparisons of the biomarkers, and to obtain adjusted *p* values. Spearman's correlation was used to measure the strength and

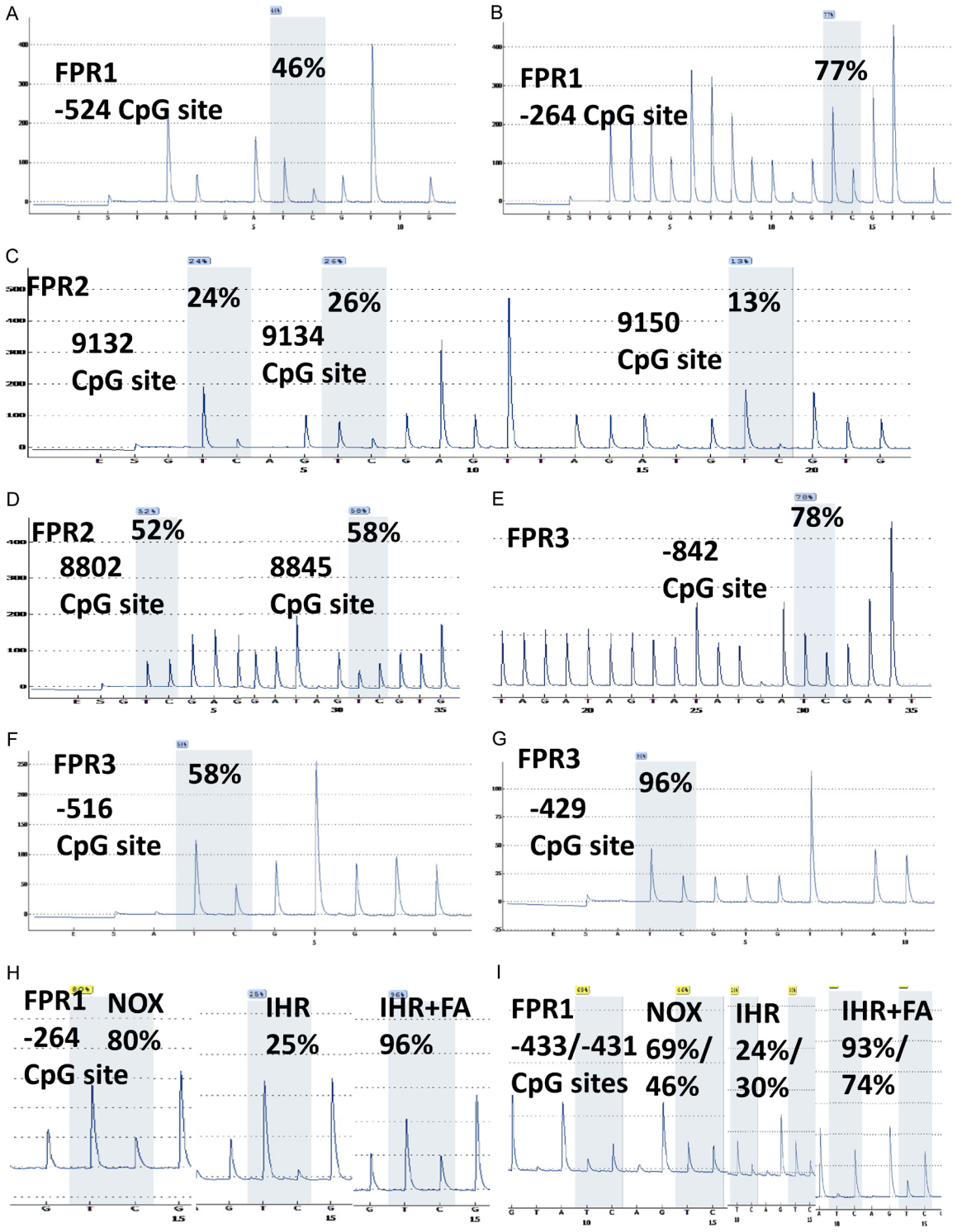

Figure 1. Pyrograms of the representative CpG sites assayed of the *FPR*1/2/3 genes. Representative pyrograms show the percentage of DNA methylation levels over (A) -524 CpG site and (B) -264 CpG sites of the *FPR1* gene in an OSA patient. Representative pyrograms show the percentage of DNA methylation levels over (C) 9132/9134/9150 CpG sites and (D) 8802/8845 CpG sites of the *FPR2* gene. Representative pyrograms show the percentage of DNA methylation levels over (E) -842 CpG site, (F) -516 CpG site and (G) -429 CpG site of the *FPR3* gene. Representative pyrograms show the percentage of DNA methylation levels over (H) -264 CpG site and (I) -433/-431 CpG sites of the *FPR1* gene under in vitro normoxia (NOX), intermittent hypoxia with re-oxygenation (IHR), and IHR plus folic acid (FA) supplement conditions.

Table 2. Demographic, biochemistry, and sleep data of all the 78 study participants

	HS (n=10)	PS (n=16)	Treatment-naïve OSA (n=40)	OSA on CPAP (n=12)	*p* value
Age, years	51.9±9.7	47.1±8.0	47.5±12.9	52.4±10.8	0.366
Male Sex, n (%)	9 (90)	11 (68.8)	34 (85)	10 (83.3)	0.457
BMI, kg/m^2	24.39±2.6	25.9±3.2	26.2±2.6	26.3±3.2	0.476
AHI, events/hour	NA	3.8±3.3	38.3±24.6	60.3±15.6	<0.001
ODI, events/hour	NA	1.2±1.2	27.3±26.8	50.6±20.0	<0.001
Mean SaO_2, %	NA	96.6±1.1	95.1±2.8	93.1±2.3	0.001
Minimum SaO_2, %	NA	91.1±3.1	78.5±12.5	71.9±10.6	<0.001
Snoring index, counts/hour	NA	141.6±177	258±236	239±244	0.223
PLM index, events/hour	NA	13.1±25.9	80.8±151.6	193.6±264	0.615
ESS	NA	8.6±5.2	9.6±4.1	14.8±4.2	0.003
EDS, n (%)	NA	4 (25)	16 (40)	11 (91.7)	0.001
Current smoking, n (%)	0 (0)	4 (25)	4 (10)	1 (11.1)	0.225
Cholesterol, mg/dl	NA	192.1±23.7	182.2±40.9	190±49.4	0.893
Triglycerides, mg/dl	NA	137.6±74.5	132.7±80.7	213.7±159	0.253
Hypertension, n (%)	1 (8.3)	4 (25)	9 (22.5)	7 (58.3)	0.016
Diabetes mellitus, n (%)	1 (8.3)	1 (6.3)	5 (12.5)	2 (16.7)	0.537
Heart disease, n (%)	0 (0)	0 (0)	2 (5)	2 (16.7)	0.196
Stroke, n (%)	0	2 (12.5)	1 (4.5)	0	0.29
CKD, n (%)	0	0	0	0	1

PS = primary snoring; HS = healthy subject (non-snorer); BMI = body mass index; AHI = apnea hypopnea index; ODI = oxygen desaturation index; SaO_2 = arterial oxyhemoglobin saturation; ESS = Epworth Sleepiness Scale; EDS = excessive daytime sleepiness; CKD = chronic kidney disease; PLM = periodic limb movement.

direction of associations between two continuous variables. The null hypothesis was rejected at $P<0.05$. All analyses were performed using SPSS software version 22.0 (SPSS Corp., Chicago).

Results

Demographic data

A total of 68 patients with sleep disordered breathing (SDB) and 10 HS were classified into four groups according to snoring history, AHI, and history of CPAP treatment for more than 1 year (self-reported use of home CPAP>4 hours/night in average). **Table 2** presents subjects' demographic, polysomnography, and blood chemistry data. There were no significant differences among four groups in terms of age, sex, BMI, smoking history, co-morbidity, blood lipid profiles, and fasting blood sugar. There were significant differences in PSG parameters among four groups, and AHI was higher in severe OSA on CPAP group than that in treatment-naive OSA group. ESS was significantly different among three SDB groups. Post hoc comparisons revealed that subjects in severe OSA on CPAP group had higher ESS than subjects in the PS and treatment-naïve OSA groups, but there was no difference in ESS between the PS and treatment-naive OSA groups.

Analysis of the variance revealed significant between-group differences in DNA methylation levels over several CpG sites of the *FPR1/2/3* genes and *FPR3* gene expressions (all *p* values <0.05), but not in *FPR1* or *FPR2* gene expression.

Aberrant DNA methylation of the FPR1 gene promoter and gene body regions in OSA patients and those with diabetes mellitus or PLMD

Post hoc comparisons with corrections for multiple comparisons and adjustment for confounding factors revealed that DNA methylation levels over -524 CpG site of the *FPR1* gene were decreased in treatment-naïve OSA as compared with that in HS (46.8±15.6 versus 63.8±15.8%, adjusted P=0.002, **Figure 2A**). DNA methylation levels over -264 CpG site of

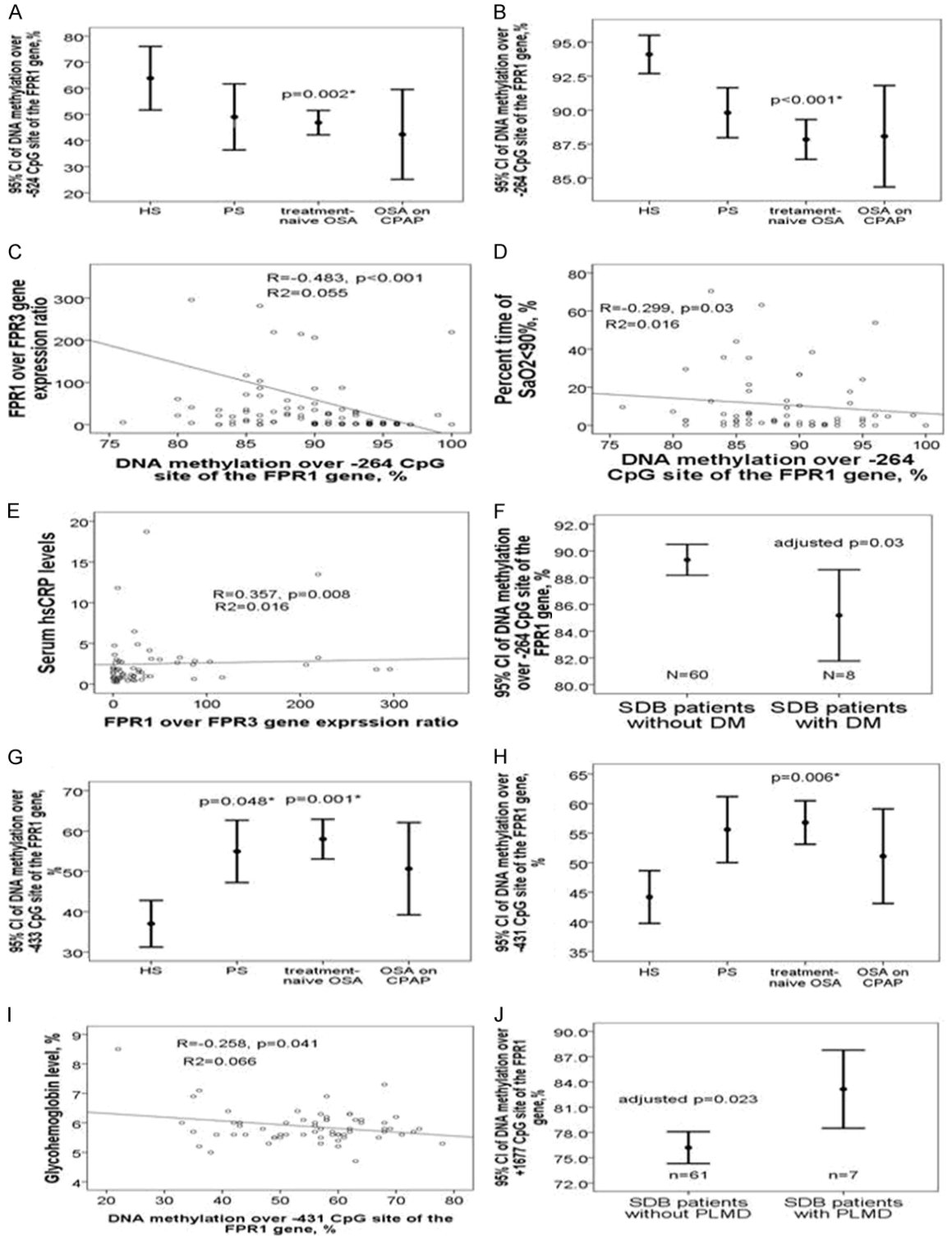

Figure 2. Aberrant DNA methylation of the *FPR1* gene promoter and gene body regions in OSA patients and those with diabetes mellitus or periodic limb movement disorder. DNA methylation levels over (A) -524 and (B) -264 CpG sites of the *FPR1* gene were both decreased in treatment-naïve OSA patients versus healthy subjects (HS), and -264 CpG site methylation was negatively correlated with (C) *FPR1/FPR3* gene expression ratio and (D) percent time of SaO_2<90%. (E) *FPR1/FPR3* gene expression ratio was positively correlated with serum hypersensitive C-reactive protein (hsCRP) levels. (F) DNA methylation levels over -264 CpG site of the *FPR1* gene was further decreased in those with diabetes mellitus. (G) DNA methylation levels over -433 CpG site of the *FPR1* gene were increased in primary snoring (PS) subjects and treatment-naïve OSA versus HS. DNA methylation levels over -431 CpG site

of the *FPR1* gene were (H) increased in treatment-naïve OSA versus HS, and (I) negatively correlated with serum glycohemoglobin levels. (J) DNA methylation levels over +1677 CpG site of the *FPR1* gene were increased in sleep disordered breathing patients with periodic limb movement disorder (PLMD) versus those without PLMD. *P<0.05, compared with HS by Kruskal-Wallis test and adjusted by linear regression analysis model.

the *FPR1* gene was decreased in treatment-naive OSA (87.8±4.5%, adjusted P<0.001) group versus HS (94.1±1.8%, **Figure 2B**), and negatively correlated with both *FPR1/FPR3* gene expression ratios (r=-0.483, P<0.001, **Figure 2C**) and percent of sleep time with SaO_2<90% (r=-0.299, P=0.03, **Figure 2D**). Moreover, *FPR1/FPR3* gene expression ratios were positively correlated with serum hypersensitivity C-reactive protein (hsCRP) levels (r=0.357, P=0.008, **Figure 2E**). Subgroup analysis showed that DNA methylation levels over -264 CpG sites of the *FPR1* gene were further decreased in SDB patients with prevalent Diabetes Mellitus (85.2±5.1%) as compared with that in those without prevalent Diabetes Mellitus (89.3±4.6%, adjusted P=0.03, **Figure 2F**). DNA methylation levels over -433 CpG site of the *FPR1* gene were increased in PS (54.9±13.9%, adjusted P=0.048) and treatment-naive OSA (57.9±14.9%, adjusted P= 0.001) groups versus HS (37±8.1%, **Figure 2G**). DNA methylation levels over -431 CpG site of the *FPR1* gene were increased in treatment-naïve OSA versus HS (56.7±11.1 versus 44.2±6.2, adjusted P=0.006, **Figure 2H**), and negatively correlated with blood hemoglobin levels (r=-0.258, P=0.041, **Figure 2I**). Subgroup analysis showed that DNA methylation levels over +1677 CpG site of the *FPR1* gene were increased in all the SDB subjects with PLMD as compared with that in those without PLMD (**Figure 2J**).

Aberrant DNA methylation of the FPR2 gene promoter region in treatment-naïve OSA patients and those with prevalent hypertension

DNA methylation levels over -499 CpG site of the *FPR2* gene were decreased in PS (71.7±2.7%, adjusted P=0.008) and treatment-naïve OSA (70.2±5.5%, adjusted P=0.039) groups versus HS (75±3%, **Figure 3A**). DNA methylation levels over -348 CpG site of the *FPR2* gene were decreased in treatment-naive OSA (80.4±5%, adjusted P=0.005) group as compared with that in HS (87±3%, **Figure 3B**). DNA methylation levels over +8802 CpG site of the *FPR2* gene were increased in treatment-naïve OSA (42.7±7.3%, adjusted P=0.029) group versus HS (35.1±5.9%, **Figure 3C**). DNA methylation levels over +8845 CpG site of the *FPR2* gene were increased in treatment-naive OSA (50.1±10.5%, adjusted =0.019) versus HS (37.2±9.2%, **Figure 3D**), and negatively correlated with *FPR2* gene expression (r=-0.218, P=0.014, **Figure 3E**), while *FPR2* gene expression was negatively correlated with platelet count (r=-0.231, P=0.035). Subgroup analysis showed that DNA methylation levels over both +9132 (31.7±9.8 versus 24.3±9.7%, adjusted P=0.013, **Figure 3F**) and +9150 CpG (12.8±3.9 versus 10.2±3.9, adjusted P=0.049, **Figure 3G**) sites of the *FPR2* gene were increased in all the SDB patients with hypertension versus that in those without hypertension, while both were positively correlated with percent time of SaO_2<90% (r=0.27, P=0.034; r=0.284, P=0.025) and negatively with mean SaO_2 (r=-0.24, P=0.032, **Figure 3H**; r=-0.236, P=0.035, **Figure 3I**). Additionally, DNA methylation over +9134 CpG site of the *FPR2* gene was positively correlated with both AHI (r=0.224, P=0.026, **Figure 3J**) and percent time of SaO_2<90% (r=0.265, p=0.018), and negatively with mean SaO_2 (r=-0.302, P=0.007).

Aberrant DNA methylation of the FPR3 gene promoter and gene body regions in OSA patients and those with high serum hsCRP levels

FPR3 gene expression levels were decreased in treatment-naïve OSA versus HS (0.2±0.4 versus 2.9±3.8 fold change, adjusted P= 0.001, **Figure 4A**), and negatively correlated with serum hsCRP levels (r=-0.323, P=0.015). DNA methylation levels over both -842 CpG (90.1±5.9 versus 95.4±5.6%, adjusted P= 0.017, **Figure 4B**) and -516 CpG sites (78.8±6 versus 85.2±2.1, adjusted P=0.016, **Figure 4C**) of the *FPR3* gene were decreased in treatment-naive OSA group versus HS, and positively correlated with *FPR3* gene expression (r=0.343, P=0.001, **Figure 4D**; r=0.361, P=0.001, **Figure 4E**), while -842 CpG site methylation was positively correlated with mean SaO_2 (r= 0.246, P=0.033, **Figure 4F**). Subgroup analysis showed that DNA methylation levels over -429

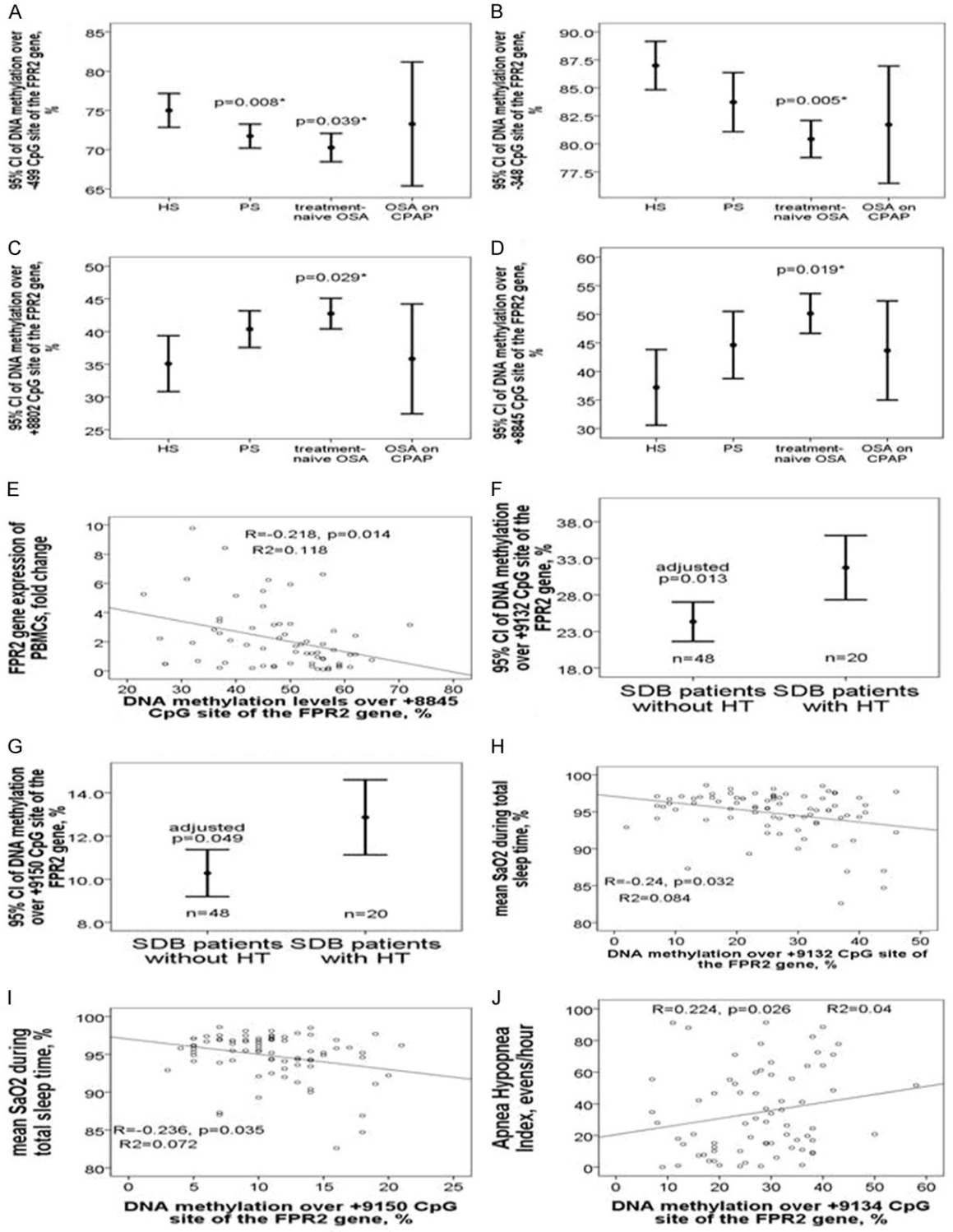

Figure 3. Aberrant DNA methylation levels of the *FPR2* gene promoter region in treatment-naïve OSA patients and those with hypertension. (A) DNA methylation levels over -499 CpG site of the *FPR2* gene were decreased in primary snoring (PS) subjects and treatment-naïve OSA patients versus healthy non-snorers (HS). (B) DNA methylation levels over -348 CpG site of the *FPR2* gene was decreased in treatment-naïve OSA patients versus HS. DNA methylation levels over (C) +8802 and (D) +8845 CpG sites were increased in treatment-naïve OSA patients versus HS, while (E) +8845 CpG site methylation levels were negatively correlated with *FPR2* gene expression levels. DNA methylation levels over (F) +9132 and (G) +9150 CpG sites of the *FPR2* gene were increased in sleep disordered breathing patients with hypertension versus those without hypertension, and (H, I) both were negatively correlated with mean

SaO_2 during total sleep time. (J) DNA methylation levels over +9134 CpG site of the *FPR2* gene were positively correlated with apnea hypopnea index. *P<0.05, compared with HS by Kruskal-Wallis test and adjusted by linear regression analysis model.

CpG site of the *FPR3* gene were increased in SDB patients with high serum hsCRP levels (>3 mg/L, n=18; 78.1±8.1%) versus that in those with low to medium serum hsCRP levels (≤3 mg/L, n=50; 73.4±6.2%, adjusted P=0.023, **Figure 4G**). DNA methylation levels over +4147 CpG site of the *FPR3* gene were decreased in treatment-naïve OSA group (92.5±1.1%) versus PS (93.4±1%, adjusted P=0.016, **Figure 4H**) group.

Effects of in vitro IHR and FA supplement on gene expression and DNA methylation levels of the FPR1/2/3 genes in THP-1 cells

To determine whether IHR per se can affect *FPR1/2* gene expressions and their DNA methylations, human monocytic THP-1 cells were exposed in vitro to either 7 cycles of IHR per day for 4 days or 4 days of continuous NOX condition. IHR treatment in vitro resulted in a significantly increased FPR1 (**Figure 5A**) expression on day 1 through day 3, while decreased FPR2 (**Figure 5B**) expression on day 1 as compared with NOX condition. FPR1/FPR2 expression ratio (**Figure 5C**) was increased on day 1 through day 3. IHR resulted in decreased DNMT 3A (**Figure 5D**) expression on day 2 through day 4, but increased DNMT1 expression on day 3 (**Figure 5E**). IHR resulted in decreased DNA methylation levels over -264 CpG site of the FPR1 gene on day 1 through day 4 (**Figure 5F**), increased DNA methylayion levels over +8802 CpG site (**Figure 5G**) on day 1/day 3 and +8845 CpG site of the FPR2 gene on day 1 through day 3 (**Figure 5H**), and increased DNA methylation levels over both -429 (**Figure 5I**) and +4147 CpG site of the FPR3 gene on day 1 (**Figure 5J**). DNMT3A gene expressions were positively correlated with DNA methylation levels over -264 CpG site of the FPR1 (r=0.553, P=0.005, **Figure 5K**), which in turn were negatively correlated with FPR1 gene expressions (r=-0.497, P= 0.014, **Figure 5L**).

To determine whether FA supplement can affect *FPR1/2/3* gene expressions and their DNA methylations, human monocytic THP-1 cells were exposed in vitro to either 2 days of continuous NOX condition, 7 cycles of IHR per day for 2 days, or 7 cycles of IHR per day plus folic acid supplement for 2 days. IHR stimuli in vitro resulted in increased *FPR1* (**Figure 6A**) and decreased *FPR2* /FPR3 (**Figure 6B** and **6C**) gene expressions as compared with NOX condition, while FA supplement decreased FPR1 gene expression under IHR condition as compared with that in IHR alone condition (all *p* values <0.05). IHR resulted in decreased DNA methylation levels over -264, -431, and -433 CpG sites of the *FPR1* gene versus NOX condition (**Figure 6D-F**), while FA supplement increased methylation levels over these three CpG sites under IHR condition as compared with IHR alone condition (all *p* values <0.05). IHR resulted in increased ROS production (percentage of $H2DCFAD^+$ cells, **Figure 6G**, P<0.05), increased positive percentage of late cell apoptosis marker (percentage of Annexin V^+ and PI^+ double positive cells, **Figure 6H**, P<0.05) and decreased cell viability (mean WST1 absorbance, **Figure 6I**, P<0.05) versus NOX condition, while FA supplement reduced ROS production (**Figure 6G**) and late apoptosis (**Figure 6H**) under IHR condition as compared with IHR alone condition (all *p* values <0.05).

Discussion

Numerous studies have revealed that FPR1 and FPR2 are involved in pro-inflammatory responses and resolution of inflammation, respectively, through regulating the production of ROS [5, 27], while FPR3 serves distinct physiological functions that remain largely unknown [28]. FPR1 up-regulation and FPR2/3 down-regulation of blood immune cells along with defective lipoxin A4/resolvin D1 production have been demonstrated in OSA patients in our recent study [16]. Knowledge of the regulatory mechanisms that drive *FPR1/2/3* gene expressions is a key for the development of innovative anti-inflammatory pharmacology. In mammalian cells, CpG methylation status in gene promoter and body regions is a primary epigenetic mechanism for silencing or activating genes and may thus affect the oxidative stress and low-grade systemic inflammation in response to chronic IHR in OSA patients [17].

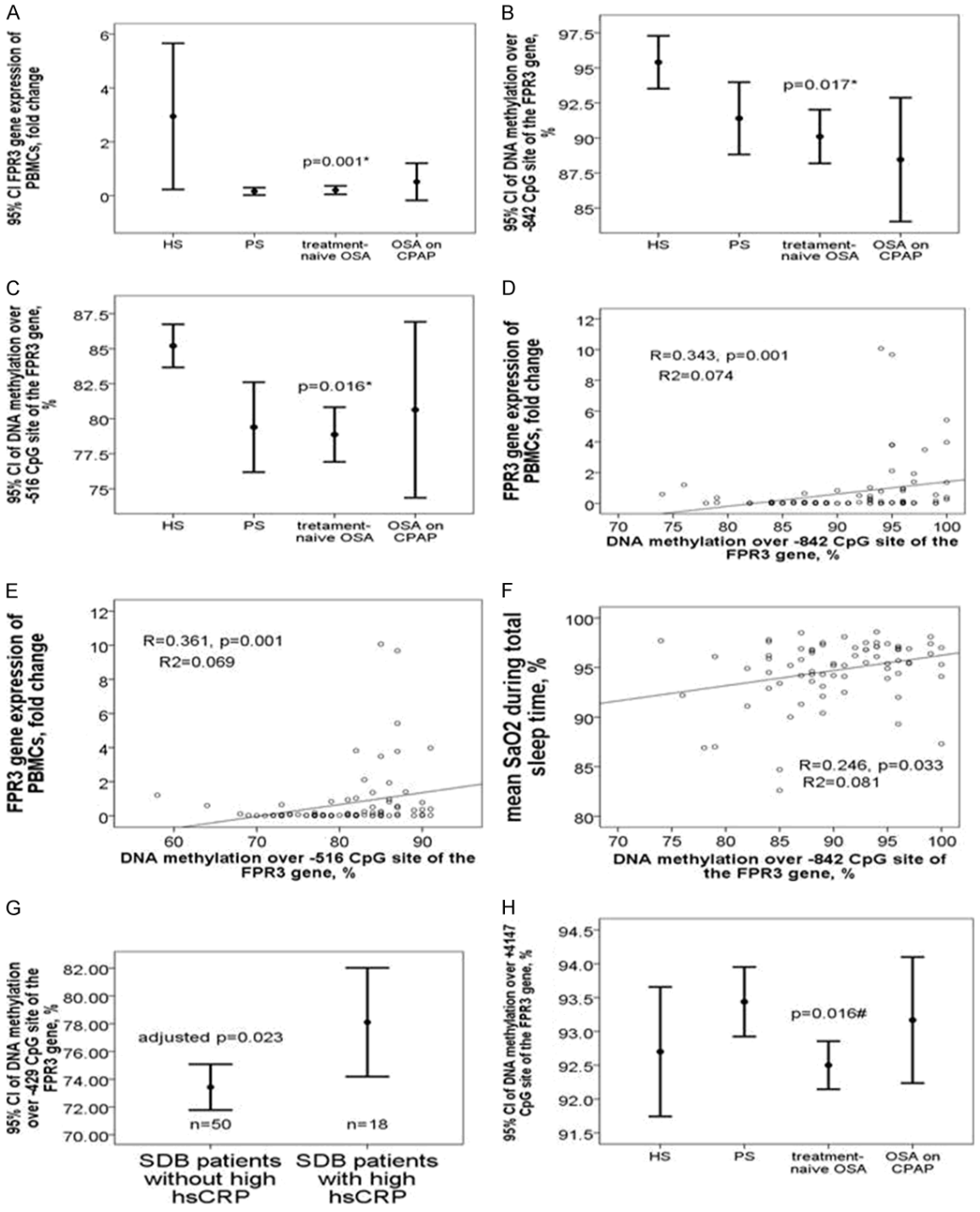

Figure 4. Aberrant DNA methylation levels of the *FPR3* gene promoter and gene body regions in treatment-naïve OSA patients and those with high C-reactive protein levels. (A) *FPR3* gene expression levels were decreased in treatment-naïve OSA patients versus healthy non-snorers (HS). DNA methylation levels over (B) -824 and (C) -516 CpG sites of the *FPR3* gene were decreased in treatment-naïve OSA patients versus HS, and (D, E) both were positively correlated with *FPR3* gene expression levels, while (F) -824 CpG site methylation was positively correlated with mean SaO_2 during total sleep time. (G) DNA methylation levels over -429 CpG site of the *FPR3* gene were increased in sleep disordered breathing patients with high hypersensitive C-reactive protein (hsCRP) levels versus those with low or medium hsCRP levels. (H) DNA methylation levels over +4147 CpG site of the *FPR3* gene were decreased in treatment-naïve OSA patients versus PS subjects. *P<0.05, compared with HS by Kruskal-Wallis test and adjusted by linear regression analysis model, #P<0.05, compared with PS by Kruskal-Wallis test and adjusted by linear regression analysis model.

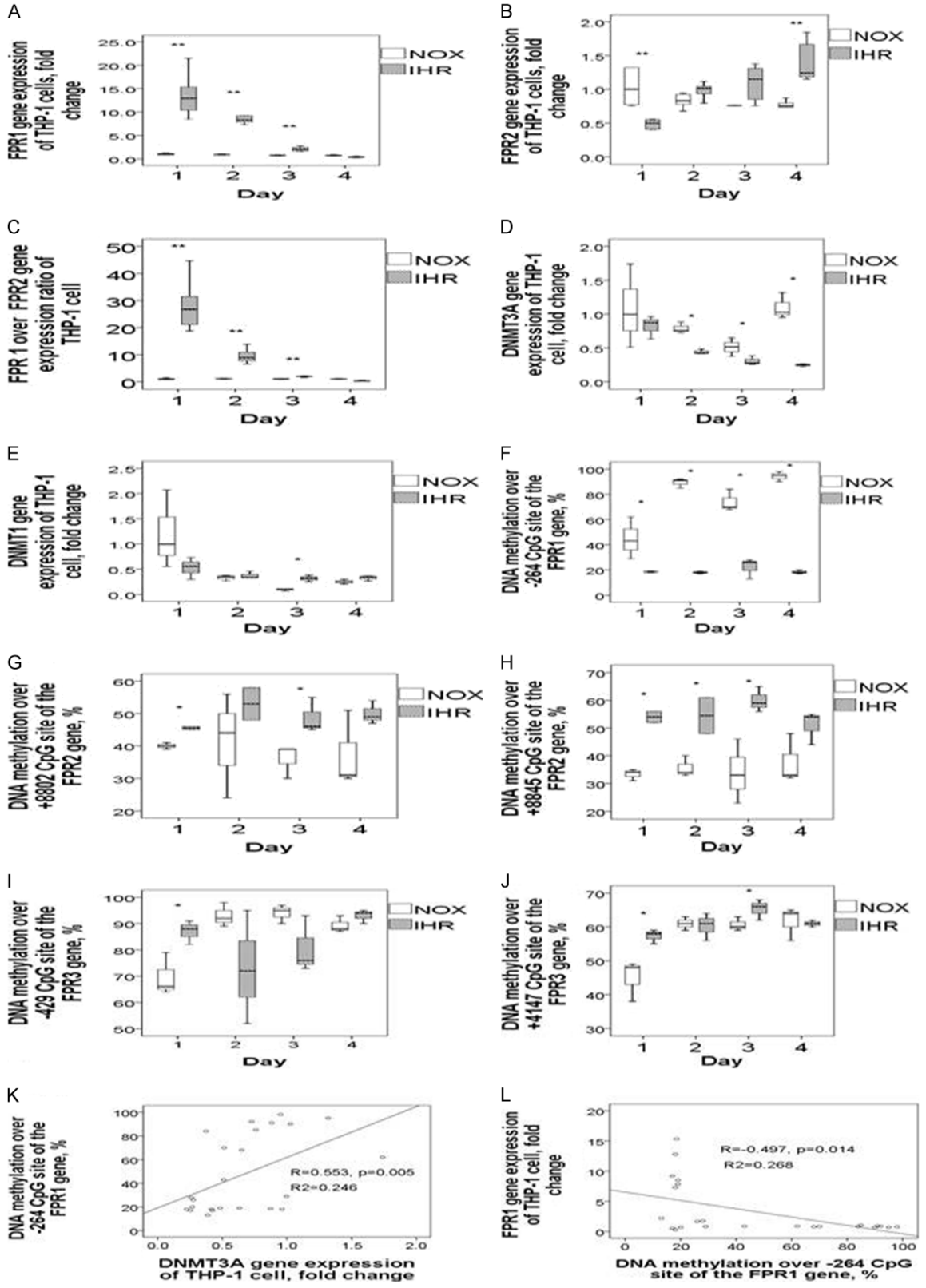

Figure 5. Effects of in vitro intermittent hypoxia with re-oxygenation (IHR) on gene expression and DNA methylation levels of the *FPR1/2/3* genes in THP-1 cells. Compared with normoxic (NOX) condition, IHR exposure (35 minutes of 0% O_2 followed by 25 minutes of 21% O_2/cycle, 7 cycles/day, for 4 days) resulted in (A) increased *FPR1* gene expression on day 1 through day 3, (B) decreased *FPR2* gene expression on day1, (C) increased *FPR1/FPR2* gene expression ratio on day 1 through day 3, (D) decreased *DNMT3A* gene expression on day 2 through day 4, and (E)

increased *DNMT1* gene expression on day 3. IHR exposure resulted in (F) decreased DNA methylation levels over -264 CpG site of the *FPR1* gene on day 1 through day 4, (G) increased +8802 CpG site methylation of the *FPR2* gene on day1/day3, (H) increased +8845 CpG site methylation of the *FPR2* gene on day1 through day 3, (I) increased -429 CpG site methylation of the *FPR3* gene on day 1, and (J) increased +4147 CpG site methylation of the *FPR3* gene on day 1/day 3. DNA methylation levels over -264 CpG site of the *FPR1* gene were (K) positively correlated with *DNMT3A* gene expression levels, and (L) negatively with FPR1 gene expression levels. *P<0.05, compared between NOX and IHR alone conditions.

Our data suggest a potential role of *FPR1/2/3* gene methylation changes in the development of OSA and clinical phenotypes.

This study is the first to find a link between aberrant DNA methylation levels of the *FPR1/2/3* genes and OSA. Previous basic studies have identified and characterized two promoter regions in the *FPR2/ALX* gene, which drive *FPR2/ALX* mRNA expression in monocytes and macrophages through the binding of transcription factors OCT1, IRF and SP1, and are suppressed by DNA hypermethylation [21, 22]. Five CpG sites (+8802, +8845, +9132, +9134, +9150) of the *FPR2* gene assayed in the present study are located within these two promoter regions. Consistent with previous findings from the basic research that *FPR2/ALX* promoter activity is suppressed by DNA methylation, we found that DNA methylation levels over +8845 CpG site of the *FPR2* gene was negatively correlated with *FPR2* gene expression. Moreover, hypermethylated +8802 and +8845 CpG sites were associated with the development of OSA, while hypermethylated +9132 and +9150 CpG sites were associated with the occurrence of hypertension. In vitro experiments showed that short-term IHR stimuli caused *FPR2* under-expression with similar change in the DNA methylation levels over +8802/+8845 CpG sites. We speculate that aberrant epigenotypes of the *FPR2* gene occurring in the early life may contribute to systemic inflammation along with thrombocytosis, and lead to endothelial dysfunction, since FPR2 is able to reduce leukocyte-endothelial interactions, and involved in cardiovascular repair [12, 13]. Further studies are required to clarify underlying mechanisms by which aberrant DNA methylation of the *FPR2* gene may lead to the development of hypertension in OSA patients.

Previous studies have determined the role of FPR1 in chemotaxis and superoxide production in resting and pro-inflammatory M1 macrophages and neutrophils, and identified -395 to +41 sites from transcription start site of the *FPR1* gene as a promoter region through the binding of transcription factors PU.1, IRF1, STAT4, and NF-Y [23, 29]. Up to now, there has been no study exploring the role of DNA methylation in regulating *FPR1* expression. For the first time, we demonstrated that -264 CpG site of the *FPR1* gene was hypomethylated in the OSA patients, especially in those with Diabetes Mellitus, and in response to in vitro IHR stimuli. Furthermore, high concentration folic acid supplement led to increased DNA methylation over the *FPR1* gene promoter region and down-regulation of the *FPR1* gene, in association with the reversion of the late cell apoptosis and excessive ROS production induced by IHR. These findings indicate that hypomethylated -264 CpG site may be originated from chronic IHR stimuli, and lead to *FPR1* over-expression, which in turn may cause increased systemic inflammation and hsCRP levels in the OSA patients. Furthermore, we found that hypermethylated +1677 CpG site of the *FPR1* gene was associated with the occurrence of PLMD in OSA patients. The secondary forms of PLMD may be due to diabetes mellitus, spinal cord tumor, OSA, narcolepsy, uremia, or anemia, but underlying pathogenesis is uncertain. Recent evidence supports neuronal hyperexcitability with involvement of the central pattern generator for gait as the pathophysiology of primary periodic limb movement [30, 31]. Our findings indicate that the aberrant *FPR1* gene methylation pattern may contribute to OSA-related PLMD.

In contrast with previous findings that FPR3 functions as a pathogen sensor and can be up-regulated after stimulation with a bacterial endotoxin [15], we found that OSA patients had defective *FPR3* expression along with aberrant DNA methylation patterns, while *FPR3* under-expression without DNA methylation change was found with IHR stimuli in vitro. We speculate that aberrant epigenotypes of the *PFR3* gene occurring in the early life may cause increased *FPR1/FPR3* gene expression ratios

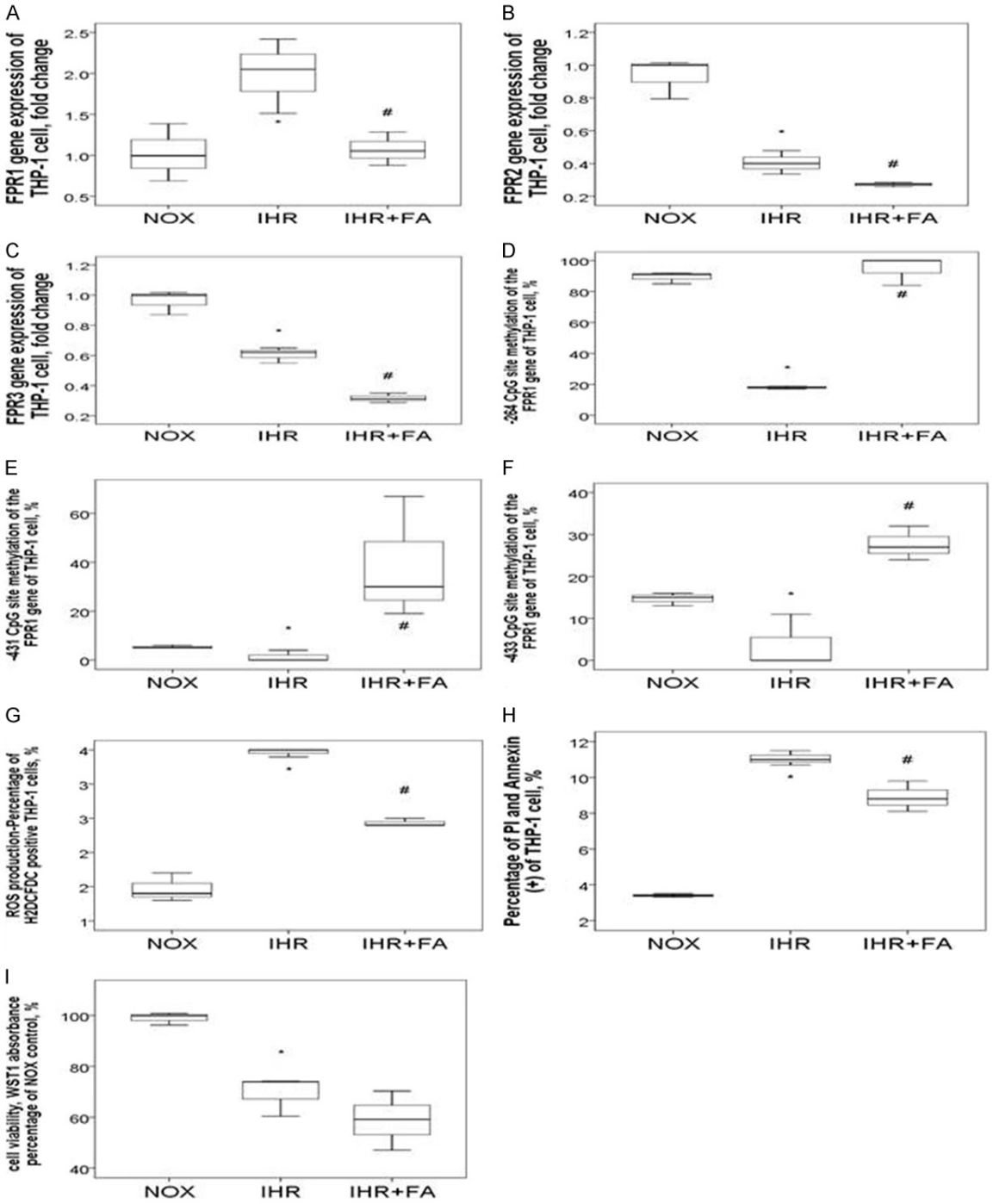

Figure 6. Effects of folic acid (FA) supplement on gene expression/DNA methylation levels of the FPR1/2/3 genes and apoptosis/viability/oxidative stress in THP-1 cells under intermittent hypoxia with re-oxygenation (IHR) condition. Compared with that in normoxic (NOX) condition, two days of IHR stimuli (35 minutes of 0% O_2 followed by 25 minutes of 21% O_2/cycle, 7 cycles/day) in THP-1 cells resulted in (A) up-regulation of the FPR1 gene and down-regulations of the (B) FPR2 and (C) FPR3 genes, while folic acid (FA) supplement reduced FPR1 gene expression under IHR condition as compared with IHR alone condition. IHR stimuli resulted in DNA hypomethylation over (D) -264, (E) -431, and (F) -433 CpG sites of the FPR1 gene, while FA supplement resulted in increased DNA methylation levels over these three CpG sites under IHR condition as compared with IHR alone condition. IHR stimuli resulted in (G) increased ROS production, (H) increased late apoptosis (percentage of Annexin V and PI double positive THP-1 cells), and (I) decreased cell viability (percentage of WST1 positive THP-1 cells), while FA supplement reduced the late apoptosis marker and ROS production under IHR condition as compared with IHR alone condition. *P<0.05, compared between NOX and IHR alone conditions, #P<0.05, compared between IHR alone and IHR plus FA supplement conditions.

and subsequent increased hsCRP levels. Previous studies have identified two promoter regions in the FPR3 gene [32]. Our findings indicate that hypermethylated -429 CpG site and hypomethylated +4147 CpG site of the *FPR3* gene along with chronic IHR exposures may be responsible for the defective *FPR3* expressions in the OSA patients. However, further investigations are required to clarify the cause and effect relationship.

Several limitations of this study should be acknowledged. First, the HS did not undergo full-night polysomnography examinations, because asymptomatic subjects without habitual snoring often hesitate over this bothersome study. However, their bed partners approved the absence of habitual snoring during sleep, and they had no associated symptoms of SDB. Second, the cause and effect relationship between aberrant DNA methylation and OSA is not straightforward, since this is a cross-sectional association study. However, the preliminary in vitro IHR experiments found that DNA methylation levels over certain CpG sites of the *FPR2/3* genes were relatively resistant to external IHR stimuli and belong to the epigenetic variants that do not change after adolescence, while methylation levels of some CpG sites of the *FPR1* gene could change in response to IHR stimuli and lead to corresponding gene expression changes of the *FPR1* gene. Thus, our findings open the possibility of using re-methylation agent to overcome FPR1-triggered cell apoptosis in OSA. Third, several confounding factors could potentially affect DNA methylation and gene expression levels of the *FPR1/2/3* genes in OSA patients. However, we made a linear regression analysis model to reduce these confounding effects to the minimum and get adjusted *p* values.

In conclusions, several CpG sites of the *FPR2* gene promoter regions showed hypermethylation with correspondent *FPR2* under-expression in the OSA patients, and were associated with prevalent hypertension in OSA patients. -264 CpG site of the *FPR1* gene showed hypomethylation with correspondent *FPR1* over-expression in the OSA patients and in response to in vitro IHR stimuli, which was associated with prevalent Diabetes Mellitus. In vitro IHR stimuli resulted in epigenetics-mediated *FPR1* over-expression, increased superoxide production, and increased cell apoptosis, all of which could be reversed with FA supplement. Aberrant DNA methylation over certain CpG sites of the *FPR3* gene may be responsible for decreased *FPR3* expression and subsequent development of OSA along with increased cardiovascular risk.

Acknowledgements

The authors would like to thank for the technical support provided by the Genomic and Proteomic Core Laboratory, and Internal Medicine Core Facility of the Kaohsiung Chang Gung Memorial Hospital. The authors would like to acknowledge Ms. Shu-Jun Kong, Ms. Lian-Rong Liu, and Mr. Wei-Zhe Liu for manual scoring of all PSG. We also thank Chih-Yun Lin and the Biostatistics Center, Kaohsiung Chang Gung Memorial Hospital for statistics work. This work was supported by grants (103-2314-B-182A-094-MY2/105-2314-B-182A-092-MY3/108-2314-B-182A-129-MY3 to M.C. Lin) from the Ministry of Science and Technology, Taiwan, and by grants from the Chang Gung Memorial Hospital (CMRPG8G0281/CMRPG8G0282/CMRPG8J0581/CMRPG8J0231 to M.C. Lin), Taiwan. The funding body has no role in the design of the study, or collection, analysis, and interpretation of data, or in writing the manuscript. All the authors have read the journal's authorship agreement and policy on disclosure of potential conflicts of interest.

This study was approved by the Institutional Review Board of Chang Gung Memorial Hospital, Taiwan (certificate number: 102-5855B). Written informed consent was obtained from each subject participating in the study, who was aged 20 years or older.

Disclosure of conflict of interest

None.

Abbreviations

OSA, obstructive sleep apnea; IHR, intermittent hypoxia with re-oxygenation; ROS, reactive oxygen species; CPAP, continuous positive airway pressure; FPR, formyl peptide receptor; BMI, body mass index; AHI, apnea hyponea index; SaO_2, arterial oxyhemoglobin saturation; ODI, oxygen desaturation index; ESS, Epworth sleepiness scale; PLM, periodic limb move-

ment; PS, primary snoring; HS, healthy subject; PBMC, peripheral blood mononuclear cell; CpG, cytosine guanine dinucleotides with the phosphodiester bond; RT-PCR, reverse transcriptase polymerase chain reaction; NOX, normoxia; FA, folic acid; SDB, sleep disordered breathing; hsCRP, high sensitivity C-reactive protein.

Address correspondence to: Meng-Chih Lin, Division of Pulmonary and Critical Care Medicine, Department of Medicine, Kaohsiung Chang Gung Memorial Hospital and Chang Gung University College of Medicine, 123, Ta-Pei Road, Niao-Sung District, Kaohsiung 83301, Taiwan. Tel: +886-7-7317123 Ext. 8199; Fax: +886-7-7322402; E-mail: linmengchih@hotmail.com; Chang-Chun Hsiao, Graduate Institute of Clinical Medical Sciences, College of Medicine, Chang Gung University, Taoyuan 33302, Taiwan. E-mail: cchsiao@mail.cgu.edu.tw

References

[1] Xu H, Wang Y, Guan J, Yi H and Yin S. Effect of CPAP on endothelial function in subjects with obstructive sleep apnea: a meta-analysis. Respir Care 2015; 60: 749-55.

[2] McEvoy RD, Antic NA, Heeley E, Luo Y, Ou Q, Zhang X, Mediano O, Chen R, Drager LF, Liu Z, Chen G, Du B, McArdle N, Mukherjee S, Tripathi M, Billot L, Li Q, Lorenzi-Filho G, Barbe F, Redline S, Wang J, Arima H, Neal B, White DP, Grunstein RR, Zhong N and Anderson CS; SAVE Investigators and Coordinators. CPAP for prevention of cardiovascular events in obstructive sleep apnea. N Engl J Med 2016; 375: 919-31.

[3] Barbe F, Duran-Cantolla J, Sanchez-de-la-Torre M, Martinez-Alonso M, Carmona C, Barcelo A, Chiner E, Masa JF, Gonzalez M, Marin JM, Garcia-Rio F, Diaz de Atauri J, Teran J, Mayos M, de la Pena M, Monasterio C, del Campo F and Montserrat JM; Spanish Sleep and Breathing Network. Effect of continuous positive airway pressure on the incidence of hypertension and cardiovascular events in nonsleepy patients with obstructive sleep apnea: a randomized controlled trial. JAMA 2012; 307: 2161-8.

[4] Zhu B, Ma C, Chaiard J and Shi C. Effect of continuous positive airway pressure on glucose metabolism in adults with type 2 diabetes: a systematic review and meta-analysis of randomized controlled trials. Sleep Breath 2018; 22: 287-295.

[5] Cattaneo F, Castaldo M, Parisi M, Faraonio R, Esposito G and Ammendola R. Formyl peptide receptor 1 modulates endothelial cell functions by NADPH oxidase-dependent VEGFR2 transactivation. Oxid Med Cell Longev 2018; 2018: 2609847.

[6] Wang W, Li T, Wang X, Yuan W, Cheng Y, Zhang H, Xu E, Zhang Y, Shi S, Ma D and Han W. FAM19A4 is a novel cytokine ligand of formyl peptide receptor 1 (FPR1) and is able to promote the migration and phagocytosis of macrophages. Cell Mol Immunol 2015; 12: 615-24.

[7] Dorward DA, Lucas CD, Chapman GB, Haslett C, Dhaliwal K and Rossi AG. The role of formylated peptides and formyl peptide receptor 1 in governing neutrophil function during acute inflammation. Am J Pathol 2015; 185: 1172-84.

[8] Faour WH, Fayyad-Kazan H and El Zein N. fMLP-dependent activation of Akt and ERK1/2 through ROS/Rho a pathways is mediated through restricted activation of the FPRL1 (FPR2) receptor. Inflamm Res 2018; 67: 711-722.

[9] Skovbakke SL, Heegaard PM, Larsen CJ, Franzyk H, Forsman H and Dahlgren C. The proteolytically stable peptidomimetic Pam-(Lys-betaNSpe)6-NH2 selectively inhibits human neutrophil activation via formyl peptide receptor 2. Biochem Pharmacol 2015; 93: 182-95.

[10] Filep JG. Biasing the lipoxin A4/formyl peptide receptor 2 pushes inflammatory resolution. Proc Natl Acad Sci U S A 2013; 110: 18033-4.

[11] Cooray SN, Gobbetti T, Montero-Melendez T, McArthur S, Thompson D, Clark AJ, Flower RJ and Perretti M. Ligand-specific conformational change of the G-protein-coupled receptor ALX/FPR2 determines proresolving functional responses. Proc Natl Acad Sci U S A 2013; 110: 18232-7.

[12] Heo SC, Kwon YW, Jang IH, Jeong GO, Lee TW, Yoon JW, Shin HJ, Jeong HC, Ahn Y, Ko TH, Lee SC, Han J and Kim JH. Formyl peptide receptor 2 is involved in cardiac repair after myocardial infarction through mobilization of circulating angiogenic cells. Stem Cells 2017; 35: 654-665.

[13] Smith HK, Gil CD, Oliani SM and Gavins FN. Targeting formyl peptide receptor 2 reduces leukocyte-endothelial interactions in a murine model of stroke. FASEB J 2015; 29: 2161-71.

[14] Jang IH, Heo SC, Kwon YW, Choi EJ and Kim JH. Role of formyl peptide receptor 2 in homing of endothelial progenitor cells and therapeutic angiogenesis. Adv Biol Regul 2015; 57: 162-72.

[15] Stempel H, Jung M, Perez-Gomez A, Leinders-Zufall T, Zufall F and Bufe B. Strain-specific loss of formyl peptide receptor 3 in the murine vomeronasal and immune systems. J Biol Chem 2016; 291: 9762-75.

[16] Chen YC, Su MC, Chin CH, Lin IC, Hsu PY, Liou CW, Huang KT, Wang TY, Lin YY, Zheng YX, Hsiao CC and Lin MC. Formyl peptide receptor 1 up-regulation and formyl peptide receptor 2/3 down-regulation of blood immune cells

along with defective lipoxin A4/resolvin D1 production in obstructive sleep apnea patients. PLoS One 2019; 14: e0216607.

[17] Maunakea AK, Nagarajan RP, Bilenky M, Ballinger TJ, D'Souza C, Fouse SD, Johnson BE, Hong C, Nielsen C, Zhao Y, Turecki G, Delaney A, Varhol R, Thiessen N, Shchors K, Heine VM, Rowitch DH, Xing X, Fiore C, Schillebeeckx M, Jones SJ, Haussler D, Marra MA, Hirst M, Wang T and Costello JF. Conserved role of intragenic DNA methylation in regulating alternative promoters. Nature 2010; 466: 253-7.

[18] Lev Maor G, Yearim A and Ast G. The alternative role of DNA methylation in splicing regulation. Trends Genet 2015; 31: 274-80.

[19] Chen YC, Chen TW, Su MC, Chen CJ, Chen KD, Liou CW, Tang P, Wang TY, Chang JC, Wang CC, Lin HC, Chin CH, Huang KT, Lin MC and Hsiao CC. Whole genome DNA methylation analysis of obstructive sleep apnea: IL1R2, NPR2, AR, SP140 methylation and clinical phenotype. Sleep 2016; 39: 743-55.

[20] Simiele F, Recchiuti A, Patruno S, Plebani R, Pierdomenico AM, Codagnone M and Romano M. Epigenetic regulation of the formyl peptide receptor 2 gene. Biochim Biophys Acta 2016; 1859: 1252-8.

[21] Waechter V, Schmid M, Herova M, Weber A, Gunther V, Marti-Jaun J, Wust S, Rosinger M, Gemperle C and Hersberger M. Characterization of the promoter and the transcriptional regulation of the lipoxin A4 receptor (FPR2/ALX) gene in human monocytes and macrophages. J Immunol 2012; 188: 1856-67.

[22] Simiele F, Recchiuti A, Mattoscio D, De Luca A, Cianci E, Franchi S, Gatta V, Parolari A, Werba JP, Camera M, Favaloro B and Romano M. Transcriptional regulation of the human FPR2/ALX gene: evidence of a heritable genetic variant that impairs promoter activity. FASEB J 2012; 26: 1323-33.

[23] Miettinen HM. Regulation of human formyl peptide receptor 1 synthesis: role of single nucleotide polymorphisms, transcription factors, and inflammatory mediators. PLoS One 2011; 6: e28712.

[24] Berry RB, Budhiraja R, Gottlieb DJ, Gozal D, Iber C, Kapur VK, Marcus CL, Mehra R, Parthasarathy S, Quan SF, Redline S, Strohl KP, Davidson Ward SL and Tangredi MM; American Academy of Sleep Medicine. Rules for scoring respiratory events in sleep: update of the 2007 AASM manual for the scoring of sleep and associated events. Deliberations of the sleep apnea definitions task force of the American academy of sleep medicine. J Clin Sleep Med 2012; 8: 597-619.

[25] Chen YC, Hsiao CC, Chen CJ, Chao TY, Leung SY, Liu SF, Wang CC, Wang TY, Chang JC, Wu CC, Lin AS, Wang YH and Lin MC. Aberrant Toll-like receptor 2 promoter methylation in blood cells from patients with pulmonary tuberculosis. J Infect 2014; 69: 546-57.

[26] Chen YC, Su MC, Liou CW, Liu SF, Chen CJ, Lin HC, Hsiao CC, Wang TY, Wang CC, Chin CH, Huang KT, Lin AS and Lin MC. Co-upregulation of Toll-like receptors 2 and 6 on peripheral blood cells in patients with obstructive sleep apnea. Sleep Breath 2015; 19: 873-82.

[27] Zhang L, Wang G, Chen X, Xue X, Guo Q, Liu M and Zhao J. Formyl peptide receptors promotes neural differentiation in mouse neural stem cells by ROS generation and regulation of PI3K-AKT signaling. Sci Rep 2017; 7: 206.

[28] Rabiet MJ, Macari L, Dahlgren C and Boulay F. N-formyl peptide receptor 3 (FPR3) departs from the homologous FPR2/ALX receptor with regard to the major processes governing chemoattractant receptor regulation, expression at the cell surface, and phosphorylation. J Biol Chem 2011; 286: 26718-31.

[29] Gemperle C, Schmid M, Herova M, Marti-Jaun J, Wuest SJ, Loretz C and Hersberger M. Regulation of the formyl peptide receptor 1 (FPR1) gene in primary human macrophages. PLoS One 2012; 7: e50195.

[30] Vetrugno R, D'Angelo R and Montagna P. Periodic limb movements in sleep and periodic limb movement disorder. Neurol Sci 2007; 28 Suppl 1: S9-S14.

[31] Aurora RN, Kristo DA, Bista SR, Rowley JA, Zak RS, Casey KR, Lamm CI, Tracy SL and Rosenberg RS; American Academy of Sleep Medicine. The treatment of restless legs syndrome and periodic limb movement disorder in adults--an update for 2012: practice parameters with an evidence-based systematic review and meta-analyses: an American academy of sleep medicine clinical practice guideline. Sleep 2012; 35: 1039-62.

[32] Ha HS, Huh JW, Gim JA, Han K and Kim HS. Transcriptional variations mediated by an alternative promoter of the FPR3 gene. Mamm Genome 2011; 22: 621-33.