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Increased Methylation of Interleukin 6 Gene Is Associated with Obesity in Korean Women

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Obesity is the fifth leading risk for death globally, and a significant challenge to global health. It is a common, complex, non-malignant disease and develops due to interactions between the genes and the environment. DNA methylation can act as a downstream effector of environmental signals; analysis of this process therefore holds substantial promise for identifying mechanisms through which genetic and environmental factors jointly contribute to disease risk. To assess the effects of excessive weight and obesity on gene-specific methylation levels of promoter regions, we determined the methylation status of four genes involved in inflammation and oxidative stress [interleukin 6 (*IL6*), tumor necrosis factor α (*TNFα*), mitochondrial transcription factor A (*TFAM*), and glucose transport 4 (*GLUT4*)] in blood cell-derived DNA from healthy women volunteers with a range of body mass indices (BMIs) by methylation-specific PCR. Interestingly, the samples from obese individuals (BMI ≥ 30 kg/m^2) showed significantly increased hypermethylation for *IL6* gene compared to normal weight (BMI < 23 kg/m^2) and overweight samples (23 kg/m^2 ≤ BMI < 30 kg/m^2) (*P* = 0.034 and *P* = 0.026). However, there was no statistically significant difference in promoter methylation of the other 3 genes between each group. These findings suggest that aberrant DNA methylation of *IL6* gene promoter may play an important role in the etiology and pathogenesis of obesity and *IL6* methylation could be used as molecular biomarker for obesity risk assessment. Further studies are required to elucidate the potential mechanisms underlying this relationship.

INTRODUCTION

DNA methylation is an epigenetic modification that effectively regulates gene expression via gene silencing, and may significantly contribute to the risks of many complex diseases, including cancer, cardiovascular, and metabolic diseases (Esteller et al., 2001; Ozanne and Constancia, 2007; Rodenhister and Mann, 2006). In cancer and certain other diseases, DNA methylation alterations have mainly been observed at the tissue level (Esteller et al., 2001; Ozanne and Constancia, 2007; Rodenhister and Mann, 2006). Furthermore, an increasing number of studies have reported that cancer-related genes are hypermethylated or hypomethylated in the peripheral blood cells (PBCs) of cancer patients (Li et al., 2012; Terry et al., 2011). Data related to whether DNA methylation changes in PBCs can serve as useful, informative biomarkers for different health outcomes is rapidly emerging (Heyn and Esteller, 2012), but information in complex non-malignant disease is much more limited.

The prevalence of obesity (body mass index, BMI ≥ 30 kg/m^2) has risen to epidemic proportions and continues to be a major health problem worldwide (Danaei et al., 2009; Mirsa and Khurana, 2008). Obesity as a common, complex, non-malignant disease is closely linked to the increased incidence of various diseases, including type 2 diabetes, hypertension, cardiovascular disease, and certain types of cancer (Anderson and Caswell, 2009; Kopelman, 2007). Obesity is the result of the interplay between external (environmental) and internal (genetic) factors (Catenacci et al., 2009). Recent genome-wide association studies (GWAS) have identified a large number of genetic variants contributing to obesity-related traits (Loos, 2012). However, a majority of these loci have only a small effect on obesity susceptibility and their accuracy in predicting obesity is poor (Loos, 2012). Importantly, DNA methylation is dynamic and plastic in response to cellular stress and environmental cues, controlling insulin sensitivity and metabolism in obesity (Foley et al., 2009). Emerging evidence suggests that gene-specific DNA methylation in blood DNA may play an important role in obesity etiology (Carless et al., 2013; Dick et al., 2014; Milagro et al., 2012; Relton et al., 2012; Su et al., 2014; Wang et al., 2010). Recently, epigenetic epidemiology is an area of great research interest and we have demonstrated a differential influence of BMI on global DNA methylation in healthy women (Na et al., 2014). Therein, to investigate the influence of obesity on methylation status of genes involved in inflammation and oxidative stress, we have determined the methylation levels of glucose transport 4 (*GLUT4*), interleukin 6 (*IL6*), tumor necrosis factor (*TNF*) α, and mitochondrial transcription factor A (*TFAM*) in blood DNA from 284 healthy women volunteers with a range of BMIs using methylation-specific PCR (MSP).

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MATERIALS AND METHODS

Study population

The study subjects included 284 apparently healthy volunteers aged between 16 and 60 years (mean 31.9 ± 7.8 years). This study was approved by the Institutional Review Board of Kyungpook National University Hospital. Additionally, informed written consent was obtained from all subjects before they participated in the study. Demographic information and lifestyle factors were determined for all participants by trained interviewers using a standardized questionnaire via face-to-face interviews. Height and bodyweight were measured using standard methods with participants wearing light clothes. BMI is calculated by weight divided by height squared [kg/m^2] and is a convenient surrogate measure of total fat mass for defining overweight and obesity. BMI has also been shown to be directly related to health risks and mortality in many populations. Based on the current international standard (WHO 1998) and slight modifications for Asian populations (Low et al., 2009), we divided the participants into 3 categories based on BMI as previously described: normal weight (BMI $<$ 23 kg/m^2), overweight (23 kg/m^2 $\leq$ BMI $<$ 30 kg/m^2), and obese (BMI $\geq$ 30 kg/m^2) (Na et al., 2014). Blood samples were obtained via venipuncture after overnight fasting, and serum samples were separated by centrifugation and transferred to sterile bottles with Teflon-coated caps. All samples were kept frozen at -70°C until analyses were conducted. Clinical laboratory values were determined by standard biochemical automatic or semi-automatic methods.

Blood DNA extraction, bisulfite treatment and methylation analysis

Genomic DNA was extracted from whole-blood samples using the QIAamp DNA Blood Kit (Qiagen, USA). One microgram of DNA was bisulfite-modified using the EZ DNA Methylation-Gold Kit (Zymo Research, USA) according to manufacturer's instructions. Final elution was performed with 30 μl of M-Elution Buffer (Zymo Research) and DNA was stored at -70°C until analyzed. The methylation status of the each gene was determined by nested methylation-specific PCR (MSP). External PCR was performed with the flanking primers of target gene promoter, diluted at 1:200 and then subjected to the internal PCR that incorporated unmethylated or methylated primers. The respective primer sequences are listed in Table 1. All PCR amplifications were carried out using reagents supplied in the GeneAmp DNA Amplification Kit with AmpliTaq Gold as the polymerase on a PTC-100 thermal cycler (MJ Research, USA). CpGenome™ Universal methylated and unmethylated DNA (Chemicon, USA) was used as a positive control for the methylated and unmethylated genes, respectively. Negative control samples without DNA were included for each set of PCR. PCR products were analyzed on 2% agarose gel, stained with ethidium bromide, and visualized under UV light. Each MSP was repeated at least once to confirm the results.

Pyrosequencing

Methylation status of *IL6* gene promoter was quantitatively confirmed by pyrosequencing (PS) method. Briefly, bisulfite-modified DNA was amplified using forward primer (5′-AGGGATAATTT-AGTTTAGAGTTTATTTGT-3′) and reverse primer (biotin-5′-CTCCCTCTCCCTATAAATCTTAATT-3′) through PCR, enabling the conversion of the PCR product to a single-stranded DNA template suitable for PS. All samples were heated to 95°C for 5 min and then amplified for 45 cycles of 95°C 45 s, 54°C 45 s, and 72°C 45 s, followed by a final extension at 72°C for 5 min. The quality and lack of contamination of the PCR products were checked on 2% agarose gels with ethidium bromide staining. After purification of PCR product using Sepharose beads on PyroMark Vacuum Prep Workstation (Qiagen), PS was performed according to the manufacturer's specifications with sequencing primer (5′-ATAAGAAATTTTTGGGTGT-3′) using the PyroMark Q96MD System (Qiagen). A mean methylation index (MI) was calculated from the mean of the methylation percentage for all observed CpG sites. To set the controls for PS, we used

Table1. Primer sequences for nested MSP

Primer	Forward primer	Reverse primer	Size (bp)
External PCR			
GLUT4	GTTTTTGGTTTGTGGTTGTG	CCTATCTATTAAAAACCCAAC	188
IL6	GGTTTTTGAATTAGTTTGATT	CCCTATAAATCTTGATTTAAAAT	132
TFAM	GTTTTAGTTTTGGTTTGAATT	CCAAAAAAAATAATAAAAAAACC	181
TNFα	GGGTTTTATATATAAATTAGTTAG	TAATAAACCCTACACCTTCTA	187
Internal PCR			
GLUT4			
U-MSP	GGTTTGTTTTTGTATGTTATTTT	CTAAACACACAAAAACAACA	117
M-MSP	GGTTCGTTTTCGTACGTTATTTC	CTAAACGCGCAAAAACGACG	
IL6			
U-MSP	GAAATTTTTGGGTGTTGATGT	AAAACTACAAACACAAACACA	67
M-MSP	GAAATTTTTGGGTGTCGACGC	AAAACTACGAACGCAAACACG	
TFAM			
U-MSP	TTGAGATGTTTTGTTGGGTGT	AAAAAAACCACAACAACAACC	149
M-MSP	TTGAGACGTTTCGTTGGGCGC	AAAAAAACCGCGACGACGACC	
TNFα			
U-MSP	GTTTAGAAGATTTTTTTTGGAATT	TCAATTTCTTCTCCATCACA	138
M-MSP	GTTTAGAAGATTTTTTTCGGAATC	TCGATTTCTTCTCCATCGCG	

M-MSP, MSP for the methylated allele; U-MSP, MSP for the unmethylated allele

Table 2. Correlation between promoter methylation and BMI

	Normal weight (n = 97)	Overweight (n = 85)	Obese (n = 102)	F/X^2	*P*
Age, years (SD)	31.23 (8.75)	31.65 (5.50)	32.86 (8.58)	1.16	0.314 [a]
IL6 methylation, n (%)	73 (75.3)	63 (74.1)	90 (88.2)	7.38	0.025 [b]
TNF methylation, n (%)	88 (90.7)	77 (90.5)	94 (92.2)	0.18	0.912 [b]
TFAM methylation, n (%)	1 (1.0)	0 (0.0)	2 (2.0)		0.777 [c]
GLUT4 methylation, n (%)	14 (14.4)	17 (20.0)	24 (23.5)	2.67	0.264 [b]

[a]ANOVA(Analysis of variance); [b]Chi-square test; [c]Fisher's exact test

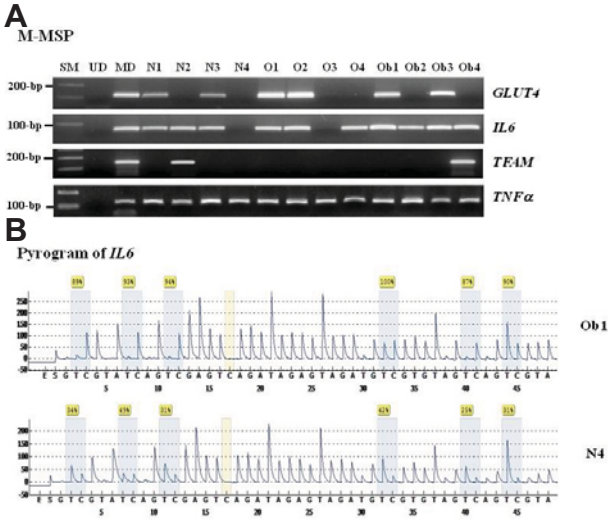

Fig. 1. Methylation analysis of *GLUT4, IL6, TFAM,* and *TNF*α genes in peripheral blood of healthy volunteers with normal weight (N), overweight (O), and obese (Ob) participants. (A) Representative data of the MSP analysis of 4 genes. Their methylation status was analyzed in whole blood DNA by using nested MSP. PCR amplification was carried out with methylation-specific (M) primers (M-MSP). CpGenome™ Universal methylated and unmethylated DNA (Chemicon) was used as positive controls for the M and U forms, respectively. (B) Representative pyrogram of *IL6* gene. The letters on the axis represent the dispensation order; E, enzyme mix; S, substrate; A, G, C, and T, nucleotide. Shaded bars encompassing T/C pairs, indicate six interrogated CpG sites. The methylation of each CpG site was calculated as a percentage of C incorporation.

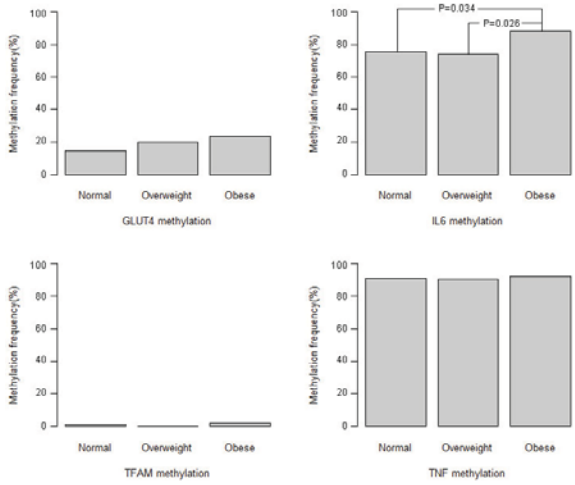

Fig. 2. Effect of body mass index on *IL6* methylation. Methylation frequency of *GLUT4, IL6, TFAM,* and *TNF*α genes was expressed in normal weight, overweight, and obese subjects. Pairwise comparison identified statistical difference following Bonferroni correction.

universal methylated and unmethylated DNA that were consistently positive or negative with stable levels of methylation.

Statistical analysis

Statistical analysis and plotting was performed using R version 3.1.0 (http://www.**r**-project.org). Mean age difference between BMI groups was compared by ANOVA. Comparisons of proportion of each BMI groups were conducted using Chi-square or Fisher's exact test and pairwise comparison identified statistical difference following Bonferroni correction. $P < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Growing evidence indicates that pro-inflammatory and oxidative stress molecules produced by adipose tissue have been implicated in obesity and its comorbidities (Fruhbeck, 2008). We thus analyzed the methylation profile of *GLUT4, IL6, TNF*α, and *TRAM* in the peripheral blood DNA of 284 healthy volunteers with a range of BMIs using MSP. Although the *IL6, TFAM,* and *TNF*α promoters do not contain classical CpG islands, recent reports have demonstrated the occurrence of CpG methylation in the control of their expression (Gemma et al., 2010; Gower et al., 2011; Niel et al., 2008). In addition, Yokomori et al. (1999) have shown that changes in DNA methylation of the *GLUT4* promoter can account for the differences in gene expression. We thus designed MSP primers covering these CpG sites. Methylated alleles of representative samples are shown in Fig. 1A. Unmethylated bands were detected in most samples (data not shown), thus confirming the integrity of the DNA in those samples. Moreover, methylation status of *IL6* gene was confirmed by pyrosequencing of representative PCR products showed that all cytosines at non-CpG sites were converted to thymine (Fig. 1B), ruling out the possibility of incomplete bisulfite conversion. Interestingly, the frequency of *IL6* methylation was significantly higher in obese participants (88.2%) than in normal weight (75.3%) and overweight (74.1%) ($P = 0.034$ and $P = 0.026$, respectively) (Table 2 and Fig. 2), inconsistent with recent observation that levels of *IL6* promoter methylation is not

significantly correlated with BMI (Zhang et al. 2012). These divergent results may be due to different sub-classification of the experimental population. The Zhang et al. study (2010) divided subjects into 2 groups according to a BMI = 25. Alternatively, the specific racial/ethnic characteristics of the study population or the small sample size may account for these differences. Moreover, there was no statistically significant difference in promoter methylation of the other genes depending on BMI levels (Table 2 and Fig. 2). Furthermore, gene-specific methylation was not correlated with smoking status and alcohol consumption (data not shown). Therefore, these results suggest that *IL6* promoter hypermethylation may be a BMI-associated event in obesity etiology.

Although the exact mechanism underlying elevated *IL6* hypermethylation observed in the blood cells of obese people is not known, several possible explanations have been suggested. First, it is noteworthy that circulating levels of IL-6, mainly secreted by adipose tissue, are significantly correlated with BMI (Browning et al., 2008). Recently, the peripheral blood mononuclear cells (PBMCs) from obese subjects showed a decreased response of IL6 after stimulation with Toll-like receptor ligand compared with PBMCs from non-obese participants (Teran-Cabanillas et al., 2013). Thus, it is tempting to speculate that the higher basal levels of IL6 can induce *IL6* hypermethylation via a negative feedback loop to contribute this poor response. Second, obesity is associated with a chronic inflammatory response, characterized by the abnormal production of adipokines and the activation of certain pro-inflammatory signaling pathways (Fruhbeck, 2008). Interestingly, persistent inflammation may cause global DNA hypermethylation in peripheral blood cells via IL6 signaling (Stenvinkel et al., 2007). Simultaneously, we recently demonstrated in the same cohort that a significantly higher level of *Alu* methylation is found in obese participants compared with normal weight or overweight participants (Na et al., 2014). Thus, it is likely that elevated *IL6* methylation in people with high BMI is a consequence of a low but continuous inflammatory drive in obesity. Third, a plausible hypothesis is that sleeping disturbance might contribute to increases in *IL6* methylation in obese people. Importantly, the levels of systemic inflammatory markers are higher in obstructive sleep apnea (OSA) patients compared to control subjects (Nadeem et al., 2013). Moreover, significant hypermethylation of inflammatory genes in blood DNA is linearly associated with the severity of OSA (Kim et al., 2012). Indeed, we found that scored sleep management was higher in normal and overweight subjects than in obese ones (data not shown), indicating that obese individuals may have poorer sleep quality than non-obese people. Studies with a larger sample size and quantitative analysis with pyrosequencing are therefore required to confirm these findings and make a clearer interpretation. Further studies to understand the biological mechanisms underlying observed relationship are also required.

Our study has a few limitations. First, because of practical difficulties in obtaining tissues from living individuals, methylation levels were tested in PBCs, but not directly from the primary affected adipose tissues. Therefore, our results may not provide a direct index of DNA methylation in the system of adipose metabolism. In this respect, it is noteworthy that Dick and colleagues have addressed an association between BMI and *HIF3A* methylations in both blood and adipose tissue DNA (Dick et al., 2014). Second, DNA methylation usually influences disease risk through silencing gene expression. Unfortunately, *IL6* expression was unable to be evaluated in this study because of the lack of fresh PBCs. Lastly, because our study was cross-sectional, we are unable to determine whether DNA methylation is likely to be a result of increased BMI or the part of causal pathways leading to an increased prevalence of obesity.

Nonetheless, this is the first study to demonstrate the association of aberrant DNA methylation in the promoter region of *IL6* gene with obesity in Korean women, suggesting that the perturbation of IL6 pathways could have an important role in the etiology and pathogenesis of obesity. In addition, *IL6* hypermethylation could become useful measure for overweight and obesity and for initial screening of other obesity-related diseases. Therefore, the present data are an excellent starting point for a deeper analysis of molecular diagnostics. Moreover, considering reversibility of DNA methylation, our result would suggest the potential for lifestyle or therapeutic interventions for obesity.

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