DNA Methylation of the LY86 Gene is Associated With Obesity, Insulin Resistance, and Inflammation

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Background: Previous genome-wide association studies (GWAS) have identified a large number of genetic variants for obesity and its related traits, representing a group of potential key genes in the etiology of obesity. Emerging evidence suggests that epigenetics may play an important role in obesity. It has not been explored whether the GWAS-identified loci contribute to obesity through epigenetics (e.g., DNA (deoxyribonucleic acid) methylation) in addition to genetics.

Method: A multi-stage cross-sectional study was designed. We did a literature search and identified 117 genes discovered by GWAS for obesity and its related traits. Then we analyzed whether the methylation levels of these genes were also associated with obesity in two genome-wide methylation panels. We examined an initial panel of seven adolescent obese cases and seven age-matched lean controls, followed by a second panel of 48 adolescent obese cases and 48 age- and gender-matched lean controls. The validated CpG sites were further replicated in two independent replication panels of youth (46 vs. 46 and 230 cases vs. 413 controls, respectively) and a general population of youth, including 703 healthy subjects.

Results: One CpG site in the lymphocyte antigen 86 (LY86) gene, which showed higher methylation in the obese in both the initial (p = .009) and second genome-wide DNA methylation panel (p = .008), was further validated in both replication panels (meta p = .00016). Moreover, in the general population of youth, the methylation levels of this region were significantly correlated with adiposity indices (p ≤ .02), insulin resistance (p = .001), and inflammatory markers (p < .001). Conclusion: By focusing on recent GWAS findings in genome-wide methylation profiles, we identified a solid association between LY86 gene DNA methylation and obesity.

Keywords: DNA methylation, obesity, GWAS, insulin resistance, inflammation

Obesity is an important risk factor for various diseases, including cardiovascular diseases (CVD) (Poeriot et al., 2006), type 2 diabetes (T2D; Wild et al., 2004), and certain types of cancer (Anderson & Caswell, 2009). Its epidemic has imposed a huge burden on public health worldwide (Danaei et al., 2009; Misra & Khurana, 2008). As a typical, common complex disease, obesity is the result of the interplay between external (environmental) and internal (genetic) factors (Catenacci et al., 2009). Recent fruitful genome-wide association studies (GWAS) have identified a large number of genetic variants contributing to obesity (Loos, 2012). However, a majority of these loci have only a small effect on obesity susceptibility and explain just a fraction of total variance (Loos, 2012). There is growing evidence suggesting that epigenetics, a plastic and heritable (during cell division) mechanism to record cues from external and internal environments (Petronis, 2010) may play an important role in obesity (Drong et al., 2012; Feinberg et al., 2010; Wang et al., 2010). It has not been explored if the GWAS-identified loci contribute to obesity through epigenetics (e.g., DNA (deoxyribonucleic acid) methylation) in addition to genetics.

Taking advantage of our previous genome-wide methylation profilings (Wang et al., 2010; Xu et al., 2013), the current study focused on a group of candidate genes identified by previous GWAS for obesity and its related traits, and examined whether these genes showed differential DNA methylation.
methylations between the obese and lean controls. Using this approach, we successfully identified a repeatable association of the DNA methylation level of one obesity GWAS gene (LY86) with obesity. This is the first study to report the association between LY86 gene methylation and obesity.

Materials and Methods
Study Design
The design of our multistage experiment is shown in Figure 1. We first conducted a literature search for obesity genes previously identified on DNA sequence variants. We then mapped these genes on our initial genome-wide DNA methylation panel (Illumina 27k chip) of seven obese cases versus seven lean controls (i.e., the initial epigenome-wide association studies [EWAS] panel). The CpG sites within these genes showing differential methylation between cases and controls with a raw $p < .01$ in this initial EWAS panel were further checked for validation in our second genome-wide DNA methylation panel (Illumina 450k chip) of 48 obese cases versus 48 lean controls (i.e., the second EWAS panel). The CpG sites that survived the second EWAS panel with a raw $p < .01$ were taken forward to the replication stage. The replicated CpG site was further carried over to a third stage, with the goal of examining the relationships of the CpG sites with more accurate adiposity indices, as well as obesity-related metabolic traits and inflammation in a general population sample of youth.

Literature Search
We focused on three categories: (1) monogenic obesity, typically caused by a single gene mutation with severe early-onset obesity as the main symptom; (2) syndromic obesity, in which patients are clinically obese, yet are additionally distinguished by mental retardation and other developmental abnormalities; and (3) polygenic or common obesity, which is likely caused by the interaction between numerous genes and environmental factors (McCarthy, 2010). The genetic bases for some of the extreme Mendelian human obesity (monogenic and syndromic) have been partially or completely elucidated and well reviewed previously (Ramachandrappa & Farooqi, 2011). For polygenic obesity, we included those genes reported in previous GWAS in terms of obesity and obesity-related traits, including body mass index (BMI), waist–hip ratio (WHR), waist circumference (WC), and weight. The full gene list ($N = 117$) and references are shown in Supplementary Table 1.

Study Subjects
Both the initial EWAS panel and the second EWAS panel have been described previously (Wang et al., 2010; Xu et al., 2013). Briefly, the initial EWAS panel included seven obese and seven age-matched lean controls, all being African-American (AA) males aged 14–18 years, living in the Augusta, Georgia area. The obese cases had a BMI $\geq 99$th percentile and lean controls had a BMI $\leq 10$th percentile for age and gender. The second EWAS panel included 48 obese and 48 age- and gender-matched lean controls, all...
being AAs aged 14–20 years, and with 50% males. Obese cases had a BMI ≥ 95th percentile and lean controls had a BMI < 50th percentile for age and gender. There were two replication panels. The first one included 46 obese (BMI ≥ 30 kg/m² or BMI ≥ 95th percentile for age and gender if age ≤ 18 years) and 46 lean (BMI ≤ 22 kg/m² or BMI ≤ 40th percentile for age and gender if age ≤ 18 years) youths; all were AA males aged 14–30 years recruited from the same area. The second replication panel included 230 obese (BMI ≥ 30 kg/m² or BMI ≥ 95th percentile for age and gender if age ≤ 18 years) and 230 lean (BMI ≤ 22 kg/m² or BMI ≤ 40th percentile for age and gender if age ≤ 18 years) youths; all were AA males aged 14–30 years recruited from the same area, including both AAs (N = 298) and European-Americans (EAs, N = 345), as well as males (N = 289) and females (N = 354). There were no subjects overlapped among the initial EWAS panel, the second EWAS panel, and two replications panels. The third stage involved 703 subjects (aged 13–19 years, 372 EAs, 331 AAs, 351 males, and 352 females) with a BMI ranging from 14.6 to 45.9 kg/m², of which 12 subjects (six obese vs. six lean) were included in the initial EWAS panel (two subjects excluded here because no DNA was available for this panel) and 221 subjects (53 obese vs. 168 lean) were included in the replication stage. More detailed information of the initial EWAS panel, the second EWAS panel, the two replication panels, and the third panel is given in the supplementary material. All subjects from these panels were overtly healthy, free of any acute or chronic illness on the basis of self-report and parental report (if subjects were younger than 18 years), and were not on anti-hypertensive, lipid lowering, anti-diabetic, or anti-inflammatory medications. The general characteristics are shown in Table 1. The Institutional Review Board at the Medical College of Georgia approved the studies. Written informed consent was obtained from all subjects and by parents if subjects were less than 18 years old.

Measurement of DNA Methylation

For all subjects, peripheral blood samples were collected at the visit time. The buffy coat and plasma samples were separated within two hours and stored at -80°C immediately. DNA was extracted from the buffy coat using QIAGEN® Blood Kit. The HumanMethylation27 BeadChip and the HumanMethylation450 BeadChip from Illumina (San Diego, CA, USA) were used for the genome wide DNA methylation profiling in the initial and the second EWAS panels respectively, as described previously (Wang et al., 2010; Xu et al., 2013). The methylation levels of the selected CpG sites for replications were determined by pyrosequencing technology (Wang et al., 2010), which is based on the principle of sequencing by synthesis. For methylation analysis, pyrosequencing not only offers individual methylation high-resolution results for all CpG sites, but also has built-in control for bisulfite treatment to improve accuracy and reproducibility. For each CpG site, a 100–350-bp region was amplified by polymerase chain reaction (PCR) using a pair of primers complementary to the bisulfite-treated DNA sequence. Therefore, in addition to the targeted CpG site, the pyrosequencing assay may involve several surrounding CpG sites.

Additional Measurements

A wide range of metabolic traits were measured in the third panel (N = 703), including fasting glucose and insulin levels, systolic and diastolic blood pressure (SBP and DBP), plasma levels of total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), and triglyceride (TG), as well as fibrinogen and C-reactive protein (CRP). Insulin sensitivity was assessed by quantitative insulin sensitivity check index (QUICKI), calculated as: QUICKI = 1/(Log (fasting insulin, μU/ml) + Log (fasting glucose, mg/dl)). In addition, more accurate indices for adiposity as derived from dual-energy X-ray absorptiometry (DEXA) scans, including visceral adipose tissue (VAT), subcutaneous abdominal adipose tissue (SAAT), and percentage of body fat (%BF), were also available in the same population (Gutin et al., 2007). Detailed information is provided in the supplementary material.

Statistical Analyses

The genome wide methylation analysis has been described previously (Wang et al., 2010; Xu et al., 2013). The Limma package (Smyth, 2004) was used to analyze each CpG site for differential methylation between the obese and lean

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**Table 1**

General Characteristics of the Subjects

<table>
<thead>
<tr>
<th></th>
<th>Initial EWAS panel</th>
<th>Second EWAS panel</th>
<th>Replication panel 1</th>
<th>Replication panel 2</th>
<th>General population panel</th>
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<tbody>
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<td></td>
<td>Cases</td>
<td>Controls</td>
<td>Cases</td>
<td>Controls</td>
<td>Cases</td>
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<td>50</td>
<td>50</td>
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<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Age (years)</td>
<td>15.8 ± 1.0</td>
<td>15.9 ± 1.4</td>
<td>17.5 ± 1.8</td>
<td>17.7 ± 1.8</td>
<td>20.3 ± 5.0</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>39.0 ± 1.7</td>
<td>17.0 ± 0.7</td>
<td>42.0 ± 7.5</td>
<td>18.9 ± 1.3</td>
<td>37.4 ± 6.1</td>
</tr>
<tr>
<td>BMI percentile (%)</td>
<td>99.6 ± 0.1</td>
<td>4.8 ± 2.2</td>
<td>99.2 ± 0.6</td>
<td>20.3 ± 11.1</td>
<td>98.6 ± 1.0</td>
</tr>
</tbody>
</table>

Note: EWAS = Epigenome-wide association studies; AAs = African-Americans; Means ± SD (range); * BMI percentile for age and gender if age ≤ 18.
In this panel, linear regression was used to adjust for the potential effects of age, gender, and race on methylation levels. The gender × group (obese vs. lean) and race × group interaction terms were also built into the analyses to test whether the observed methylation differences between the obese and lean groups were race and gender dependent. The weighted z score-based meta-analysis approach implemented in the package METAL (Willer et al., 2010) was used to combine the results from the two replication panels, as well as the initial and the second EWAS panels. The relationships of the validated CpG sites with more accurate adiposity indices, as well as obesity-related metabolic traits in the third panel, were examined by using partial correlation analyses with adjustment for age, gender, and race. As this panel included samples with pyrosequencing content, we examined by using partial correlation analyses with adjustment for age, gender, and race.

As expected, the meta-analysis with the EWAS panels resulted in stronger associations of the methylation levels with obesity (p = .00016 for LY86_5, the targeted CpG site). In the general population panel, we found significant positive correlations of the methylation levels of the LY86 gene with BMI, and additional indices of adiposity, including %BF, VAT, and SAAT (Table 3). As the methylation levels of these six CpG sites are highly correlated (Pearson r > 0.8, Supplementary Table 2), we also performed a principal component (PC) analysis to combine the six CpG sites into one PC score (LY86_pc), which explained 90% of the variance for all six CpG sites. Similarly, this component was significantly associated with all four indices of adiposity, with the partial correlation coefficients being 0.096 (p = .01), 0.099 (p = .009), 0.138 (p = .004), and 0.109 (p = .02) for BMI, %BF, VAT, and SAAT, respectively, after adjusting for age, gender, race, and batch. These results indicate that the association of LY86 gene methylation is consistent across different measures of adiposity.

### Results

A total of 117 genes were identified from the literature, of which 89 genes were mapped onto the Illumina 27K Beadchip. Four CpG sites showed significant differential methylation between the obese and lean subjects (p < .01) in the initial panel, located at the LEPR, SNRPN, KREMEN1 and LY86 genes (Table 2). In the second EWAS panel, only the CpG site in LY86 gene showed significant differential methylation between cases and controls with p < .01. In both EWAS panels, the obese cases had higher methylation levels of the CpG site in LY86 gene than the lean controls. Pyrosequencing assays were designed to validate the association of this CpG site with obesity in the first replication panel (46 obese vs. 46 lean controls). In addition to the targeted CpG site, the pyrosequencing assays involved five surrounding CpG sites. As shown in Table 3, the methylation level of the targeted CpG site was significantly higher in the obese than that in the lean controls (56.7% vs. 52.2%, p = .009). The methylation levels of the surrounding five CpG sites varied from 20% to 50%, but always showed higher methylation levels in the obese group in comparison with the lean group. All six CpG sites of the LY86 gene were further replicated in the second replication panel (230 obese vs. 413 lean controls). After adjusting for age, gender, and race, all six CpG sites showed significant higher methylation levels in the obese compared to the controls (Table 3). No significant interaction of obesity status with race or gender was found on the methylation levels of these six CpG sites. As expected, the meta-analysis with the EWAS panels resulted in stronger associations of the methylation levels with obesity (p = .00016 for LY86_5, the targeted CpG site).

### Table 2

<table>
<thead>
<tr>
<th>Gene</th>
<th>CpG site</th>
<th>Methylation level (%)</th>
<th>Cases</th>
<th>Controls</th>
<th>Difference</th>
<th>p</th>
</tr>
</thead>
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<td>N = 7</td>
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<td>49.4</td>
<td>-5.1</td>
<td>.009</td>
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<tr>
<td>KREMEN1</td>
<td>cg01791232</td>
<td>23.7</td>
<td>18.1</td>
<td>5.6</td>
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<tr>
<td>LY86</td>
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<td>51.5</td>
<td>43.0</td>
<td>8.5</td>
<td>.009</td>
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<td>Second panel*</td>
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<td>N = 48</td>
<td>N = 48</td>
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</tr>
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<td>52.9</td>
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<td>23.9</td>
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<tr>
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<td>cg02212836</td>
<td>58.4</td>
<td>54.7</td>
<td>3.7</td>
<td>.008</td>
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</tr>
</tbody>
</table>

Note: *Adjusted for age in the initial panel and adjusted for age and gender in the second panel; **Not included in the 450K chip.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Methylation level (%)</th>
<th>Cases</th>
<th>Controls</th>
<th>Difference</th>
<th>p</th>
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<tbody>
<tr>
<td>Initial panel*</td>
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<td>N = 7</td>
<td>N = 7</td>
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<td>12.6</td>
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<td>43.0</td>
<td>8.5</td>
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<tr>
<td>Second panel*</td>
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<td>N = 48</td>
<td>N = 48</td>
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<td>3.7</td>
<td>.008</td>
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</table>

Note: *Adjusted for age in the initial panel and adjusted for age and gender in the second panel; **Not included in the 450K chip.
TABLE 3
DNA Methylation of LY86 and Obesity in the Replication Panels

<table>
<thead>
<tr>
<th>Gene</th>
<th>Cpg site</th>
<th>Cases</th>
<th>Controls</th>
<th>Difference</th>
<th>p</th>
<th>p (adjusted)</th>
<th>p (meta)</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Cases</td>
<td>Controls</td>
<td></td>
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<td></td>
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<td>.02</td>
<td>.004</td>
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</table>

Note: *LY86_5 is the targeted CpG site cg02212836; **Adjusted for age in the replication panel 1 and adjusted for age, gender, and race in the replication panel 2; ***Meta analysis on the replication panels 1 (age-adjusted) and 2 (age-, gender- and race-adjusted).

TABLE 4
Associations of Methylation Levels of the LY86 Gene With Body Mass Index and Additional Indices for Adiposity in the Third Panel (N = 703) Representing the General Population of Youth

<table>
<thead>
<tr>
<th>Cpg sites</th>
<th>BMI</th>
<th>% body fat</th>
<th>VATb</th>
<th>SAATb</th>
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<tr>
<td></td>
<td>Partial correlationc</td>
<td>p</td>
<td>Partial correlationc</td>
<td>p</td>
</tr>
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</table>

Note: BMI = body mass index; VAT = visceral adipose tissue; SAAT = subcutaneous abdominal adipose tissue; **Adjusted for batch; *Log-transformed; **Adjusted for age, gender, and race; ***The first principal component score calculated based on the methylation levels of six CpG sites, adjusted for batch.

We further examined the associations of the methylation levels of the CpG sites with several obesity-related metabolic traits and two inflammatory markers. As shown in Table 5, the PC score of the six CpG sites was significantly associated with fasting glucose (partial r = 0.107, p = .007), fasting insulin (partial r = 0.123, p = .002), and insulin sensitivity (partial r = -0.129, p = .001), as well as fibrinogen (partial r = 0.167, p < .001) and CRP (partial r = 0.147, p < .001), after adjusting for age, gender, race, and batch. These associations attenuated somewhat, but remained significant after further adjustment for BMI, indicating that part of the effects of LY86 gene methylation on insulin resistance and inflammation are independent of body weight. The associations of the metabolic traits with the six individual CpG sites were similar to the results obtained from the PC score (Supplementary Table 3).

To further elucidate the role of methylation in this region on potential function of the LY86 gene, we performed an in silico analysis of the region, including these six CpG sites (122 bases), using TFSEARCH software (Heinemeyer et al., 1998; Figure 2). There were 21 transcription factor-binding sites that passed the threshold (score > 85.0), of which 12 could be found in humans. Interestingly, the third CpG site is located in a region potentially binding with many transcription factors, including USF, c-Myc, and Max. The sixth CpG site is within a binding site for AML-1a. These results indicate that methylation of these CpG sites may affect gene expression by blocking these transcription factor binding sites.

**Discussion**

Taking advantage of our previous genome-wide methylation profiling on obesity (Wang et al., 2010; Xu et al., 2013), in this study we aimed to integrate this data with a hypothesis-based list of obesity genes based on evidence from monogenic, syndromic, and common obesity. Among the 89 GWAS genes mapped on the Illumina 27K methylation platform, the higher methylation level of the LY86 gene in obese cases was successfully confirmed in another genome-wide methylation panel and two replication panels.

In a well-phenotyped general population cohort of youth, we further showed that LY86 gene methylation was not only associated with all adiposity indices but also with insulin...
TABLE 5
Associations of Methylation Levels of the LY86 gene (LY86_pc) with the Obesity-Related Metabolic Traits and Inflammatory Markers in the Third Panel (N = 703) Representing the General Population of Youth

<table>
<thead>
<tr>
<th>Metabolic traits†</th>
<th>Model 1c</th>
<th></th>
<th></th>
<th>Model 2d</th>
<th></th>
<th></th>
<th>Partial</th>
<th>correlation</th>
<th>p</th>
<th>Partial</th>
<th>correlation</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fasting glucose</td>
<td>.094</td>
<td>.02</td>
<td></td>
<td>.082</td>
<td>.04</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fasting insulin</td>
<td>.121</td>
<td>.002</td>
<td></td>
<td>.086</td>
<td>.03</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>QUICKI</td>
<td>-.124</td>
<td>.02</td>
<td></td>
<td>-.091</td>
<td>.02</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SBP</td>
<td>.092</td>
<td>.06</td>
<td></td>
<td>.067</td>
<td>.08</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DBP</td>
<td>.072</td>
<td>.06</td>
<td></td>
<td>.048</td>
<td>.08</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>TC</td>
<td>.030</td>
<td>.44</td>
<td></td>
<td>.024</td>
<td>.55</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>HDL-C</td>
<td>-.058</td>
<td>.14</td>
<td></td>
<td>-.032</td>
<td>.41</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LDL-C</td>
<td>.064</td>
<td>.10</td>
<td></td>
<td>.048</td>
<td>.22</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TG</td>
<td>.050</td>
<td>.21</td>
<td></td>
<td>.025</td>
<td>.52</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Fibrinogen</td>
<td>.170</td>
<td>&lt;.001</td>
<td></td>
<td>.145</td>
<td>&lt;.001</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>CRP</td>
<td>.143</td>
<td>&lt;.001</td>
<td></td>
<td>.114</td>
<td>&lt;.005</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

Note: †All traits are log-transformed except QUICKI and DBP; ‡The first principal component score calculated based on the methylation levels of six CpG sites, adjusted for batch; ‡Adjusted for age, gender and race; ‡Adjusted for age, gender, race and BMI.

FIGURE 2
TFSEARCH search result of the six CpG sites (underlined) in the promoter region of the LY86 gene (122 bases). There were 21 transcription factor binding sites that passed the threshold (score >85.0), of which 12 could be found in human. Interestingly, the third CpG site is located in a region potentially binding with many transcription factors, including USF, c-Myc, and Max. The sixth CpG site is within a binding site for AML-1α.

resistance and inflammation markers, indicating that LY86 gene methylation may also play a role in obesity-related metabolic disorders.

The protein encoded by the LY86 gene is the 'lymphocyte antigen 86', also known as protein MD-1, which is a secreted glycoprotein physically associated with RP105 (a toll-like receptor (TLR) family protein) and playing a crucial role in B cell surface expression of RP105 (Nagai et al., 2002). The RP105/MD-1 complex is expressed on immune cells, including B cells, macrophages, and dendritic cells. Mice deficient in either RP105 or MD-1 exhibit reduced lipopolysaccharide (LPS) responsiveness in B cells (Nagai et al., 2002; Ogata et al., 2000). On the other hand, the expression of RP105/MD-1 in dendritic cells and
macrophages has been shown to serve as a negative regulator
of TLR4/MD-2 in the LPS response (Divanovic et al., 2005). Growing evidence suggests that LY86 may be involved in the
(patho)physiological regulation of the innate immune sys-
tem and inflammation (Sasaki et al., 2012). Most recently,
in a mice model, Watanabe et al. (2012) reported that MD-
1 may contribute to high-fat, diet-induced obesity, adipose
tissue inflammation, and insulin resistance.

Whether the expression of the LY86 gene is regulated by
DNA methylation remains unknown. According to the most
recent release of Genome Browser, the CpG sites showing
higher methylation levels in the obese subjects from the
current study are located in the first exon with a distance of
61–133 bp (first and sixth CpG sites) to the transcription
start site. Further in silico analysis of this region revealed
that the third and sixth CpG sites are located at known
binding sites for several transcription factors. For example,
USF expression has been associated with metabolic traits in
mice, including obesity, plasma lipid, and glucose/insulin
ratio (Wu et al., 2010). In addition, the same study showed
that binding of this factor to its DNA target is controlled
by the methylation level of a single CpG dinucleotide in-
cluded in its targeted sequence. Similarly, c-Myc binds its
target sequence in a methylation-dependent manner, and
over-expression of c-Myc in the liver has been reported to
prevent obesity and insulin resistance in mice (Riu et al.,
2003). The enrichment of transcription binding sites in
this region and the methylation-dependent binding manner
provide a possibility that methylation of these CpG
sites in the LY86 gene may alter gene expression by block-
ing transcription factor binding. Transcriptional profiling
studies in peripheral blood leukocytes may provide some
evidence on the relationship between LY86 gene methyla-
tion and gene expression. We searched the Gene Expression
Omnibus (GEO) database and identified a dataset (Ghosh
et al., 2010; GSE18897) that included genome-wide gene
expression data in peripheral blood samples from 20 obese
and 20 lean adults. In line with our findings that obese cases
had higher methylation levels of the LY86 gene, in this gene
expression dataset, the expression level of LY86 gene was
lower in the obese cases than in the lean controls, although
the difference did not reach significance (309 ± 78 vs.
340 ± 75, p = .18).

The fact that LY86 is one of the GWAS-identified genes
for obesity traits raised a possibility that the observed
methylation difference between obese cases and lean con-
trols may result from the obesity-associated single nu-
cleotide polymorphisms (SNPs). The GWAS-identified SNP
is rs1294421, which is associated with WHR (adjusted for
BMI) and is located 87 kb from the LY86 gene. Data on both
the genotype of this SNP and LY86 gene methylation are
available in 345 of the current study samples. We did not
observe the genotype of this SNP was associated with the
methylation levels of these CpG sites (data not shown),
indicating that the observed methylation difference between
cases and controls is not caused by this SNP. However, we
cannot exclude the possibility that other genetic variants in
or near LY86 gene may influence the methylation levels of
these CpG sites.

There are several strengths to this study. First, by focus-
ing on youth and young adults at a pre-disease stage, we
were optimizing chances to detect disease-specific epige-
netic alterations, as presumably they are not yet masked
by the background of age-related and medication-arising
epigeneic ‘drift’ (Groom et al., 2011). Second, the two
replication panels, with large samples recruited from the
same area, enabled us to replicate the initial findings not
only in AA males, but also in EAs and females. As shown
in the result section, we did not observe significant inter-
actions between obesity status and race or gender on the
methylation levels of LY86 gene. Further stratified analyses
by race and gender revealed similar significant findings in
EAs (p = .001) and females (p = .003). In addition, involv-
ing the third panel enables us to extend our findings to a
general population of youth. Third, the approach of inte-
grating previous genomic and epigenomic data may help
in prioritizing the signals for further validation. Our ap-
proach of replicating candidate genes is hypothesis driven,
thus not requiring the same multiple testing penalty as the
geno-wide approach.

This study also has some limitations. First, epigenetic regu-
lation is tissue specific. In this regard, the target tissue to
identify epigenetic variations responsible for obesity should
preferably be adipose tissue or the hypothalamus. However,
there is increasing evidence that biomarkers derived from
blood may provide an alternative to tissue biopsy for the
diagnosis and prognosis of disease (Dzau & Liew, 2007).
In addition, obesity is multifactorial in origin and several
organs have been implicated in metabolic regulation. In re-
cent years, compelling evidence has emerged pointing to
the involvement of low-grade inflammation in the patho-
physiology of obesity and suggests that the immune system
might be the missing mechanism that links these various
organs with peripheral blood leukocytes as an important
mediator (Pradhan, 2007). Furthermore, methylation pro-
files are typically established during early developmental
stages prior to major cell differentiation, and subsequently
maintained through cell divisions (Petronis, 2010). Thus,
individual-specific, disease-related methylations may ap-
pear not only in the affected tissue(s), but across the human
body, including accessible tissues such as blood. In this case,
to interpret the function of the current finding on LY86 gene
methylation, it will be necessary to conduct gene expression
analysis not only in peripheral blood cells but also in adi-
pose and hypothalamic or other organs implicated in the
metabolic regulation of obesity. Second, we used the DNA
from leukocytes, which represent different cell populations
with distinct epigenetic profiles. A potential concern is that
the results might be biased if the case group and control
group display different cell compositions. Recently, a series
of studies (Houseman et al., 2012; Koestler et al., 2012; Liu et al., 2013; Reinius et al., 2012) were conducted to address this concern by identifying differentially methylated CpG sites among leukocyte subtypes (including CD4+ T cells, CD8+ T cells, CD56+ NK cells, B cells, monocytes and granulocytes) as well as using these CpG sites to predict the cell compositions and adjusting them in the data analysis. The LY86 gene CpG site is within the top 500 CpG sites showing differential methylation among leukocyte subtypes (Supplementary Figure 1; Koestler et al., 2012). We predicted the cell compositions for both the initial and the second EWAS panels using Houseman’s algorithm (Houseman et al., 2012) and observed that the obese group had a higher percentage of granulocytes in the initial panel and a lower percentage of natural killer (NK) cells in both the initial and the second genome-wide panels (Supplementary Tables 4 and 5). We conducted further analysis in the second genome-wide panel adjusting for the predicted cell compositions using linear regression models as suggested by Liu et al. (2013) and observed that this CpG site showed an even stronger association with obesity status ($p = 3.49 \times 10^{-7}$, Supplementary Table 6), indicating that our significant finding on the LY86 gene was not caused by shifts in these leukocyte subpopulations. Due to the limited sample size of the first panel (seven cases vs. seven controls) and the request of five covariates for the adjustment of cell compositions, we did not conduct this analysis in the first panel because we did not have enough sample size to provide reliable correlation estimates. Future studies on DNA methylation profiling of various types of cell populations of leukocytes are warranted to gain a greater understanding of the epigenetic dysregulation in obesity.

In conclusion, by focusing on previous genomic findings on monogenic, syndromic, and common obesity in our genome-wide methylation profiling, for the first time we identified a solid association between our genome-wide methylation profiling, for the first time on monogenic, syndromic, and common obesity in the epigenetic dysregulation in obesity. Further studies are warranted to determine the functional impact of these CpG sites and provide new targets for prevention of obesity related diseases such as CVD and T2D.

**Supplementary Material**

To view supplementary material for this article, please visit [http://dx.doi.org/10.1017/thg.2014.22](http://dx.doi.org/10.1017/thg.2014.22).

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