RESEARCH ARTICLE

Associations of ChREBP and Global DNA Methylation with Genetic and Environmental Factors in Chinese Healthy Adults

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Abstract

Age, gender, diet, gene and lifestyle have been reported to affect metabolic status and disease susceptibility through epigenetic pathway. But it remains indistinct that which factors account for certain epigenetic modifications. Our aim was to identify the influencing factors on inter-individual DNA methylation variations of carbohydrate response element binding protein (ChREBP) and global genome in peripheral blood leucocytes (PBLs). ChREBP DNA methylation was determined by bisulfite sequencing, and genomic 5mdC contents were quantified by capillary hydrophilic-interaction liquid chromatography/ in-source fragmentation/ tandem mass spectrometry system in about 300 healthy individuals. Eleven single nucleotide polymorphisms (SNPs) spanning ChREBP and DNA methyltransferase 1 (DNMT1) were genotyped by high resolution melting or PCR-restriction fragment length polymorphism. DNMT1 mRNA expression was analyzed by quantitative PCR. We found ChREBP DNA methylation levels were statistically associated with age (Beta (B) = 0.028, p = 0.006) and serum total cholesterol concentrations (TC) (B = 0.815, p = 0.010), independent of sex, concentrations of triglyceride, high density lipoprotein cholesterol, low density lipoprotein cholesterol (LDL-C), fasting blood glucose and systolic blood pressure, diastolic blood pressure, PBLs counts and classifications. The DNMT1 haplotypes were related to ChREBP (odds ratio (OR) = 0.668, p = 0.029) and global (OR = 0.450, p = 0.015) DNA methylation as well as LDL-C, but not DNMT1 expression. However, only the relation to LDL-C was robust to correction for multiple testing (OR_{FDR} = 1.593, p_{FDR} = 0.013). These results indicated that the age and TC were independent influential factors of ChREBP methylation and DNMT1 variants could probably influence LDL-C to further modify ChREBP DNA methylation. Certainly, sequential comprehensive analysis of the interactions between genetic variants and blood lipid levels on ChREBP and global DNA methylation was required.
Introduction

DNA methylation is a main epigenetic mechanism that affects gene transcription [1], tissue differentiation [2] and chromatin remodeling [3]. It has been reported that DNA methylation variations are involved in changes of the metabolic status [4–6], while the dietary component could also act as an epigenetic regulation agent against disease [7–11]. However, the underlying mechanisms of how environment or nutrition mediates through epigenetic pathway affecting disease susceptibility are still not clearly understood [12, 13]. These epigenetic modifications are likely to adjust expressions of important genes mediating pathophysiology processes, and are linked with direct benefits of diet and lifestyle, and might offer a rational and simple way to prevent diseases. In fact, investigations have implicated inter-individual DNA methylation variations with age, gender, diet, lifestyle, and genetic variants [14–18] especially single nucleotide polymorphisms (SNPs) in the DNA methyltransferases 1 (DNMT1), which could bind methyl groups to hemi-methylated DNA [19]. These SNPs could affect DNMT1 protein folding, catalytic activity and heterochromatin binding ability, thus leading to the changes of global and loci-specific DNA methylation [20–22]. But substantially less is known about the exact interactions among epigenetic variations, genetic variants and environmental factors.

ChREBP (GenBank accession number: NC_000007.14), is a transcription factor binding with genes of glucose, lipid and redox metabolism, and SNPs in ChREBP gene were reported to be associated with plasma triglyceride levels and coronary artery disease (CAD) in our previous study [23]. Furthermore, we found a distinct inter-individual DNA methylation variation in CpG island of ChREBP in peripheral blood leukocytes (PBLs). Then we speculate either or both of metabolite and heredity would lead to epigenetic modifications in ChREBP. Lipid and glucose levels and blood pressures were chosen as candidate influence factors based on ChREBP’s functions, and SNPs in ChREBP and DNMT1 genes were selected as potential genetic cis-acting elements and trans-acting factors.

In order to reveal the modification factors on methylation variations in ChREBP, we investigated associations among the DNA methylation status of ChREBP gene plus global genome, genetic variations within ChREBP and DNMT1 genes, the metabolite such as blood lipid levels and fasting blood glucose (FBG) etc.

Materials and Methods

Study population

The study population consisted of 309 healthy individuals recruited in Zhongnan hospital (Wuhan, China). General health was established using a general medical checklist. All subjects were free of medication and showed no signs of CAD, hypertension, diabetes mellitus or dyslipidemia based on the physical examination results at the time of enrollment. Informed consent was obtained from all subjects prior to their participation in the study from March/30/2012 to February/25/ 2014. Each subject’s clinical data and blood sample were collected and analyzed anonymously. The authors didn’t have access to identifying information. This study was approved and recorded in Medical Ethics Committee of Zhongnan Hospital of Wuhan University and met the declaration of Helsinki.

Clinical Data

The systolic blood pressure (SBP) and diastolic blood pressure (DBP) were measured using a standard mercury sphygmomanometer. The serum concentrations of fasting blood glucose (FBG), total glyceride (TG), total cholesterol (TC), low density lipoprotein cholesterol (LDL-C), and high density lipoprotein cholesterol (HDL-C) were determined using the AU5400 automatic
biochemical analyzer (Beckman Coulter Co. Ltd). PBLs differential counts were analyzed using the LH750 hematology analyzer (Beckman Coulter Co. Ltd). These analyzers were employed in the Core Laboratory of Zhongnan Hospital using standard techniques.

SNP Selection and Genotyping

The SNPs were selected using Haploview 4.2 program [24] based on Chinese Han Beijing population (CHB) data from the HapMap database (http://www.hapmap.org, phase2). Two tag SNPs spanning ChREBP and five tag SNPs spanning DNMT1 were chosen using pairwise $r^2$ threshold of $\leq 0.8$ and minor allele frequency (MAF) threshold of $\geq 0.05$. The SNPs were rs1051921, rs17145750 within ChREBP, and rs2288349, rs2228611, rs8111085, rs16999593, rs2336691 within DNMT1 (Fig 1A and 1C).

Any of 5 tag SNPs could be chosen as a gene tag in a block with high linkage disequilibrium (LD) pattern, from 1-kb region upstream to 1-kb downstream of ChREBP. And the high LD pattern of the 5 tag SNPs (rs1051921, rs17145750, rs7798357, rs7785479 and rs7800944) plus 1 nonsynonymous SNP (rs3812316) in ChREBP was confirmed in our study population ($D' = 1$, Fig 1B) [23]. On the other side, the picked 5 tag SNPs in DNMT1 captured all 22 SNPs, from 1-kb region upstream to 1-kb downstream of DNMT1 (GenBank accession number: NC_0000019.10). And 4 of the 5 tag SNPs of DNMT1 (rs2288349, rs2228611, rs8111085 and rs16999593) were in linkage disequilibrium in our study population ($D' \geq 0.77$, Fig 1D) [25–27].

Genomic DNA of blood sample was isolated using standard proteinase K digestion and phenol-chloroform extraction. Nine SNPs were genotyped by high-resolution melting (HRM) on LightScanner 32 (Idaho Technology, USA). Two SNPs (rs3812316 & rs7798357) were genotyped by PCR-restriction fragment length polymorphism (PCR-RFLP) method due to G/C transversion. Ten percent of DNA samples were randomly selected for genotype verification using direct PCR sequencing (Qingke Company, Wuhan, China). The detail primer sequences are available in S1 and S2 Tables.

Bisulfite sequencing for ChREBP DNA methylation

After spectrophotometric quantization, 2 ug of genomic DNA was treated with bisulfite as described previously [28]. Genomic DNA of PBLs was treated using CpG M.sSI methyltransferase (New England Biolabs) and was used as the methylated control, whereas the 'C' in non-CpG island ('C' completely transforming to 'T') was considered as the unmethylated control. Bisulfite DNA was amplified by PCR with bisulfite sequencing (BSP) primers designed by Primer 3.0 and listed in S3 Table. PCR products were cloned into the PMD18-T vector (Takara, Dalian, China), and ten positive clones from each sample were randomly selected for sequencing. DNA methylation levels were calculated by the percentage of methylated CpG sites divided by total CpG sites (290 CpG loci) in ten clones.

LC-ESI-MS/MS analysis on genomic 5mdC contents

The capillary hydrophilic interaction chromatography (cHILIC) was performed on a Shimadzu Prominence nano-flow liquid chromatography system (Shimadzu, Tokyo, Japan) with two LC-20AD nano pumps, two vacuum degassers, a LC-20AB high performance liquid chromatography (HPLC) pump, a SIL-20AC HT auto-sampler, and a nano-flow control valve. The electrospray ionization/tandem mass spectrometry (ESI-MS/MS) experiment for detecting the genomic 5-mdC contents was detailly described in the previous study [29]. The results showed linearity within the range of 0.05% - 10% (molar ratio of 5-mdC/dC) with a coefficient value ($R^2$) 0.996.
Fig 1. Linkage disequilibrium (LD)-plot. (A) LD-plot of ChREBP established using Haploview 4.2 program based on HapMap data. Five tag SNPs of ChREBP investigated in this research were highlighted in black boxes; (B) LD-plot of ChREBP in 50 individuals of our study population. The LD-plot was composed by 6 SNPs, including the 5 tag SNPs and 1 nonsynonymous SNP; (C) LD-plot of DNMT1 established using Haploview 4.2 program based on HapMap data. Five tag SNPs in DNMT1 investigated in this research were highlighted in black boxes; (D) LD-plot composed by the 5 tag SNPs of DNMT1 in 287 individuals of our study population.

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Quantitative PCR of DNMT1 expression

The first strand cDNA in PBLs was synthesized using RevertAid™ First Strand cDNA Synthesis Kit (Thermo Scientific Inc.) after mRNA was extracted by RNeapure Blood Kit (CoWin Bioscience Co. Ltd). Quantitative PCR (qPCR) of DNMT1 expression was performed in triplicate using iTaq™ Universal SYBR GREEN mix (BioRad) on a CFX96 Real-Time PCR Detection System (BioRad). The qPCR primer sequences were listed in S4 Table. The mRNA levels were normalized to GAPDH, and the results were expressed as mean ± standard deviation (SD).

Statistical analysis

Continuous variables were expressed as mean ± SD or as median (interquartile range). The comparison of DNA methylation and expression levels among different genotypes was carried out using Mann-Whitney U test or Kruskal-Wallis H test. The correlations between DNA methylation and age, sex, blood pressure, blood index were analyzed by univariate regression and multivariate regression. LD and haplotype construction were analyzed by the Haploview4.2 and the SHEsis software platform (http://analysis.bio-x.cn/myAnalysis.php). The SHEs is a program that uses a partition ligation-combination-subdivision EM algorithm in haplotype reconstruction and frequency estimation. The associations were tested on most likely haplotypes [30, 31]. Data was analyzed with SPSS software (version 16.0) and a p value < 0.05 (two-tailed) was considered statistically significant. False Discovery Rate (FDR) was applied for multiple testing corrections. The pFDR value was calculated by multiplying its p value by the number of tests performed and then divided by the rank order of each p value (where rank order 1 is assigned to the smallest p value). An FDR of 0.05 was used as a critical value to assess whether pFDR value was significant [32].

Results

ChREBP DNA methylation was independently related to age and serum TC concentrations

A description of the study population is reported in Table 1.

We found that ChREBP DNA methylation was correlated with age, TC, TG, LDL-C (all p < 0.05), but was not related to sex, HDL-C, FBG, SBP, DBP, PBLs counts and classifications (all p > 0.05). However, after forward stepwise multivariate linear regression, only age and TC were independent factors associated with ChREBP DNA methylation, explaining 6.9% variation in ChREBP DNA methylation (Table 2).

Associations between ChREBP DNA methylation and DNMT1 haplotype

Because the six SNPs in ChREBP in our study have constructed a high LD pattern (Fig 1B), only 2 SNPs (rs1051921, rs17145750) were chosen to represent the haplotype of ChREBP. However, we didn’t identify any significant association between individual SNP or haplotype and levels of ChREBP DNA methylation (S5 and S6 Tables).

Since DNMT1 plays a major role in the maintenance of methylation patterns, 5 tag SNPs within DNMT1 were genotyped to estimate the trans-effect of genetic variants on ChREBP DNA methylation. Though no significant association was observed between single DNMT1 SNP and ChREBP DNA methylation (S5 Table), significant difference was found in the frequency of the GAAT haplotype of DNMT1 (composed of rs2288349, rs2228611, rs8111085 and rs16999593), between the subgroups with differential levels of ChREBP DNA methylation (p = 0.029, OR = 0.668, 95% CI = 0.465–0.960, Table 3). But after FDR correction, no significant association was observed.
Table 1. Clinical characteristics of the study population.

| Clinical characteristic | N  | Mean ± SD  | Median (interquartile range) |
|-------------------------|----|------------|-----------------------------|
| age, years              | 309| 55.1 ± 10  | 53 (47–62)                  |
| sex, male/female        | 139/170 |          |                             |
| SBP, mmHg               | 309| 116 ± 15   | 115 (106–126)               |
| DBP, mmHg               | 309| 70 ± 9     | 70 (63–76)                  |
| TC, mmol/L              | 309| 4.34 ± 0.54| 4.42 (4.03–4.70)            |
| LDL-C, mmol/L           | 309| 2.55 ± 0.36| 2.61 (2.35–2.83)            |
| HDL-C, mmol/L           | 309| 1.32 ± 0.21| 1.31 (1.18–1.43)            |
| TG, mmol/L              | 309| 0.92 ± 0.34| 0.89 (0.69–1.13)            |
| FBG, mmol/L             | 309| 4.77 ± 0.45| 4.73 (4.47–5.06)            |
| PBLs counts, (×10⁹/L)   | 309| 5.61 ± 1.37| 5.42 (4.71–6.35)            |
| GRAN counts, (×10⁹/L)   | 309| 59.10 ± 8.02| 59.60 (53.80–64.60)        |
| LYM counts, (×10⁹/L)    | 309| 33.91 ± 7.79| 33.3 (28.4–39.05)          |
| MONO counts, (×10⁹/L)   | 309| 7.02 ± 2.59| 7.20 (5.80–8.55)            |
| ChREBP DNA methylation, % | 309| 21.05 ± 13.57| 33.3 (28.4–39.05)       |
| global DNA methylation, % | 159| 4.41 ± 0.84| 4.18 (3.75, 5.02)          |
| DNMT1 expression        | 158| 0.007 ± 0.0078| 0.004 (0.0025, 0.0099) |

SBP, systolic blood pressure; DBP, diastolic blood pressure; TC, total cholesterol concentrations; LDL-C, low density lipoprotein cholesterol concentrations; HDL-C, high density lipoprotein cholesterol concentrations; TG, total triglyceride concentrations; FBG, fasting blood glucose concentrations; PBLs, peripheral blood leukocytes; GRAN, granulocytes; LYM, lymphocytes; MONO, monocytes.

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Table 2. Associations of clinical characteristics with ChREBP DNA methylation.

| Clinical characteristics | ChREBP DNA methylation | Univariate association | Multivariate association |
|-------------------------|------------------------|------------------------|-------------------------|
|                         |                        | B          | p        | B          | p        |
| age                     | 0.030                  | 0.000      | 0.028    | 0.006      |
| sex                     | -0.317                 | 0.060      | -        | -          |
| TC                      | 0.427                  | 0.006      | 0.815    | 0.010      |
| TG                      | 0.517                  | 0.038      | -        | -          |
| HDL-C                   | -0.408                 | 0.304      | -        | -          |
| LDL-C                   | 0.550                  | 0.018      | -        | -          |
| FBG                     | -0.025                 | 0.893      | -        | -          |
| SBP                     | 0.001                  | 0.862      | -        | -          |
| DBP                     | -0.018                 | 0.053      | -        | -          |
| PBLs counts             | 0.024                  | 0.694      | -        | -          |
| GRAN counts             | -0.002                 | 0.872      | -        | -          |
| LYM counts              | 0.002                  | 0.869      | -        | -          |
| MONO counts             | 0.047                  | 0.235      | -        | -          |
| R²                      |                        |            | 0.069    |            |

B, Beta; SBP, systolic blood pressure; DBP, diastolic blood pressure; TC, total cholesterol concentrations; LDL-C, low density lipoprotein cholesterol concentrations; HDL-C, high density lipoprotein cholesterol concentrations; TG, total triglyceride concentrations; FBG, fasting blood glucose concentrations; PBLs, peripheral blood leukocytes; GRAN, granulocytes; LYM, lymphocytes; MONO, monocytes. The levels of ChREBP DNA methylation was sqrt-transformed, \( p < 0.05 \) was considered statistically significant (in bold).

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Associations of \textit{DNMT1} haplotype with global DNA methylation and \textit{DNMT1} expression

To further verify the possible effect of the \textit{DNMT1} haplotype on DNA methylation, the influence of the \textit{DNMT1} haplotype on global DNA methylation was analyzed. We observed a significant difference in haplotype GGGT frequencies between subgroups with the higher and lower levels of global DNA methylation ($p = 0.015$, OR = 0.450, 95% CI = 0.234–0.863, Table 4) before FDR correction.

In order to reveal the mechanism underlying the possible relation of \textit{DNMT1} haplotypes with \textit{ChREBP}, and global DNA methylation, we speculated that the \textit{DNMT1} haplotype may affect global and specific-locus DNA methylation through regulation on the mRNA expression level of \textit{DNMT1}. Sequentially, the mRNA expression level of \textit{DNMT1} was measured and we didn’t reveal any significant association between \textit{DNMT1} haplotypes and expression (Table 4), though we only find 2 SNPs were associated with lipid levels before FDR correction (S8 Table).

Table 3. Comparisons of \textit{DNMT1} haplotype distributions in subgroups with the higher and lower levels of \textit{ChREBP} DNA methylation.

| \textit{DNMT1} haplotype | Haplotype frequencies (N (ratio)) | Group 1 | Group 2 | $p$    | OR     | 95% CI       | $P_{FDR}$ |
|-------------------------|----------------------------------|--------|--------|-------|--------|-------------|---------|
| AGAT                    | 73 (0.255)                       | 68 (0.238) |        | 0.641 | 1.095  | 0.747–1.606 | 0.641   |
| GAAT                    | 75 (0.280)                       | 99 (0.343) |        | 0.029 | 0.668  | 0.465–0.960 | 0.145   |
| GGAT                    | 39 (0.136)                       | 27 (0.092) |        | 0.098 | 1.554  | 0.920–2.625 | 0.163   |
| GGCC                    | 40 (0.140)                       | 49 (0.170) |        | 0.321 | 0.794  | 0.504–1.253 | 0.401   |
| GGGT                    | 48 (0.167)                       | 33 (0.115) |        | 0.070 | 1.553  | 0.962–2.508 | 0.163   |

The population was divided into two subgroups with the lower and higher levels of \textit{ChREBP} DNA methylation by the median level of 18.60%. Group 1 was composed of the individuals with the levels of \textit{ChREBP} DNA methylation less than 18.60%; Group 2 was composed of the individuals with the levels of \textit{ChREBP} DNA methylation more than or equal to 18.60%. Loci for the haplotype analysis: rs2288349, rs2228611, rs8111085, and rs16999593. N = 287; $P_{FDR}$, the adjusted $p$ for multiple testing; Bold letter indicates the $p$ value < 0.05. (All those haplotype frequencies < 0.03 will be ignored in analysis.)

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Associations of \textit{DNMT1} haplotype with global DNA methylation and \textit{DNMT1} expression

To further verify the possible effect of the \textit{DNMT1} haplotype on DNA methylation, the influence of the \textit{DNMT1} haplotype on global DNA methylation was analyzed. We observed a significant difference in haplotype GGGT frequencies between subgroups with the higher and lower levels of global DNA methylation ($p = 0.015$, OR = 0.450, 95% CI = 0.234–0.863, Table 4) before FDR correction.

In order to reveal the mechanism underlying the possible relation of \textit{DNMT1} haplotypes with \textit{ChREBP}, and global DNA methylation, we speculated that the \textit{DNMT1} haplotype may affect global and specific-locus DNA methylation through regulation on the mRNA expression level of \textit{DNMT1}. Sequentially, the mRNA expression level of \textit{DNMT1} was measured and we didn’t reveal any significant association between \textit{DNMT1} haplotypes and expression (S7 Table). However we did find a statistical association between \textit{DNMT1} haplotypes and LDL-C even after FDR correction (Table 5), though we only find 2 SNPs were associated with lipid levels before FDR correction (S8 Table).

Table 4. Comparisons of \textit{DNMT1} haplotype distributions in subgroups with the higher and lower levels of global DNA methylation.

| \textit{DNMT1} haplotype | Haplotype frequencies (N (ratio)) | Group 3 | Group 4 | $p$    | OR     | 95% CI       | $P_{FDR}$ |
|-------------------------|----------------------------------|--------|--------|-------|--------|-------------|---------|
| AGAT                    | 49 (0.310)                       | 35 (0.217) |        | 0.052 | 1.653  | 0.994–2.749 | 0.130   |
| GAAT                    | 43 (0.270)                       | 46 (0.286) |        | 0.798 | 0.937  | 0.572–1.537 | 0.909   |
| GGAT                    | 15 (0.092)                       | 16 (0.097) |        | 0.909 | 0.957  | 0.451–2.030 | 0.909   |
| GGCC                    | 29 (0.185)                       | 27 (0.169) |        | 0.666 | 1.136  | 0.637–2.027 | 0.909   |
| GGGT                    | 16 (0.099)                       | 32 (0.198) |        | 0.015 | 0.450  | 0.234–0.863 | 0.090   |
| AGAT                    | 20 (0.126)                       | 17 (0.106) |        | 0.553 | 1.232  | 0.618–2.455 | 0.909   |

The population was divided into two subgroups with the lower and higher levels global DNA methylation by the median level of 4.18%. Group 3 was composed of the individuals with the level of global DNA methylation less than 4.18%; Group 4 was composed of the individuals with the level of global DNA methylation more than or equal to 4.18%. Loci for the haplotype analysis: rs2288349, rs2228611, rs8111085, and rs16999593. N = 159; $P_{FDR}$, the adjusted $p$ for multiple testing; Bold letter indicates the $p$ value < 0.05. (All those haplotype frequencies < 0.03 will be ignored in analysis.)

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Discussion

In this study, we analyzed the DNA methylation of ChREBP and global genome in PBLs, using BSP and LC-ESI-MS/MS. We found that age and serum TC were independent modification factors of ChREBP DNA methylation, and observed an association related LDL-C to DNMT1 haplotypes which have nominal relationships with the DNA methylation of ChREBP and global genome. As reported, the genetic and epigenetic mechanisms independently involved in the pathophysiological processes and disease developments [12, 13], however they might interact in some processes to determine disease susceptibility together. In our study, we presented a perspective on whether there were interactions between metabolites, genetic variants and epigenetic modifications of DNA methylation.

PBLs are good in vivo target cells for investigating the DNA methylation levels in ChREBP gene and global genome, because the peripheral blood was easy to be collected and assayed. Furthermore, as reported by Davies and Smith et al., the modification of DNA methylation status in PBLs could reflect the modification on DNA methylation in other organs [33, 34]. We found an association between ChREBP DNA methylation of PBLs and serum TC. It might indicate a negative feedback of down-regulation on ChREBP expression mediated by DNA methylation under cell microenvironments with higher serum lipid levels, since ChREBP could activate the transcription of lipid metabolism genes [35]. It also could be a reflection of the DNA methylation modification in liver induced by the elevated cholesterol level.

Bollati et al. also found a complex relationship among the DNA methylation of tumor necrosis factor α (TNFα) in PBLs and blood levels of LDL-C, TC/HDLC and LDL-C/HDLC [36]. And Gillberg et al. found the DNA methylation of peroxisome proliferator activated receptor gamma coactivator 1 alpha (PPARGC1A) in subcutaneous adipose tissue was influenced by high-fat overfeeding in a birth weight dependent manner [37].

Furthermore, we found that the higher level of ChREBP methylation was associated with aging, which was consistent with the previous literatures. Barbara et al. reported that CALCA and MGMT methylation levels increased with age in PBLs[38]. Tra et al. also confirmed that the DNA methylation level of 23 loci elevated with age in T lymphocytes [39], while Fuke et al. found that the genome methylation level decreased during the aging process in PBLs [14]. These results suggested there could be a contrary age-related alteration of DNA methylation between global genome and specific genes in PBLs.

Table 5. Comparisons of DNMT1 haplotype distributions in subgroups with the higher and lower levels of serum LDL-C.

| DNMT1 haplotype | Haplotype frequencies (N (ratio)) | Group 5 | Group 6 | p   | OR  | pFDR | ORFDR |
|----------------|----------------------------------|--------|--------|-----|-----|------|-------|
| AGAT           | 66 (0.228)                       | 74 (0.262) | 0.333 | 0.828 | 0.326 | 0.812 |
| GAAT           | 92 (0.317)                       | 83 (0.2920) | 0.527 | 1.123 | 0.419 | 1.000 |
| GGAT           | 34 (0.117)                       | 31 (0.108)  | 0.691 | 1.111 | 0.644 | 0.994 |
| GGGG           | 34 (0.117)                       | 55 (0.195)  | **0.008** | 0.534 | **0.010** | 0.561 |
| GGGT           | 52 (0.180)                       | 28 (0.100)  | **0.006** | 1.985 | **0.013** | 1.593 |

The population was divided into two subgroups with the lower and higher levels of serum LDL-C by the median level of 2.62 mmol/L. Group 5 was composed of the individuals with serum LDL-C levels less than 2.62 mmol/L; Group 6 was composed of the individuals with serum LDL-C levels more than or equal to 2.62 mmol/L. Loci for the haplotype analysis: rs2288349, rs2228611, rs8111085, and rs16999593. N = 287; pFDR, the adjusted p for multiple testing; ORFDR, the adjusted OR for multiple test. Bold letter indicates the p value < 0.05. (All those haplotype frequencies < 0.03 will be ignored in analysis.)

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Moreover, we observed that serum LDL-C were related to DNMT1 haplotypes, while ChREBP and global DNA methylation were only nominally associated with DNMT1 haplotypes. Actually we reported a risk association of DNMT1 SNP rs2228611 with CAD in Han population, but we didn’t investigate the relation between SNPs and lipid levels in this previous study [40]. The influence of DNMT1 haplotype on LDL-C might be the underlying reason involving DNMT1 SNPs with CAD, and probably ascribed to DNMT1 functions on DNA methylation of specific lipid metabolism genes. The association of LDL-C with the interaction among SNPs as haplotypes, but not a single SNP, was similar to some other studies [41–43]. And several investigations have reported the influence of DNMT1 haplotypes with CAD, and probably ascribed to DNMT1 functions on DNA methylation of specific lipid metabolism genes. The association of LDL-C with the interaction among SNPs as haplotypes, but not a single SNP, was similar to some other studies [41–43].

In addition, serum LDL-C was associated not only with the DNMT1 haplotype (Table 2) but also with ChREBP DNA methylation (Table 5). Whether these indicated that genetic factors indirectly adjusted the ChREBP DNA methylation through influencing the metabolite concentration needed further investigation, and this hypothesis could probably explain associations between the DNMT1 haplotype and ChREBP DNA methylation before FDR correction.

Our study has some limitations. Firstly, because we have investigated only 11 SNPs of ChREBP and DNMT1 genes instead of global genome, we think that comprehensive studies would be more efficient for finding genetic variants affecting DNA methylation variations. Secondly, we didn’t investigate the functional mechanism for the association between ChREBP DNA methylation and serum TC. Thirdly, lipid concentrations could be influenced by other genetic and epigenetic variability, which might be the confusing factors in the association study between DNMT1 haplotypes and lipid levels, and should be included for future researches.

In conclusion, this study explored the complex regulator network among metabolites and epigenetic and genetic variations. The results showed that age and serum TC were the modification factors on inter-individual variation of ChREBP DNA methylation, and genetic variants might indirectly influence ChREBP DNA methylation through adjusting metabolite blood levels. If metabolites could modify an individual’s epigenetic status, it would be a good fundament for diet therapy and a strong support for healthy lifestyle for the benefit of individuals and for the sake of offsprings. And in the future, we might find some way to amend the genetic code in an epigenetic way.

Supporting Information
S1 Dataset. Raw data of all indexes except DNMT1 expression.
(XLSX)

S2 Dataset. Raw data of DNMT1 expression and SNP genotypes.
(XLSX)

S1 Table. Primers used for genotyping and DNA sequencing for ChREBP SNPs.
(DOCX)

S2 Table. Primers used for HRM and DNA sequencing for DNMT1 SNPs.
(DOCX)
S3 Table. Primers used for ChREBP bisulfite sequencing.
(DOCX)

S4 Table. Primers used for DNMT1 mRNA expression analysis.
(DOCX)

S5 Table. Associations of SNPs in ChREBP and DNMT1 with ChREBP and global DNA methylation and DNMT1 expression.
(DOCX)

S6 Table. Comparisons of ChREBP haplotype distributions in subgroups with the higher and lower levels of ChREBP DNA methylation.
(DOCX)

S7 Table. Comparisons of DNMT1 haplotype distributions in subgroups with the higher and lower levels of DNMT1 mRNA expression.
(DOCX)

S8 Table. Associations between DNMT1 SNPs and lipid levels.
(DOCX)

Author Contributions
Conceived and designed the experiments: FZ. Performed the experiments: JJG XPQ. Analyzed the data: JJG FZ. Contributed reagents/materials/analysis tools: XBW CYP. Wrote the paper: JJG FZ. Undertook BSP, genotyping and qPCR: JJG. Undertook LC-ESI-MS/MS: XPQ. Collected samples and extracted DNA and RNA: JJG XPQ. Drafted the manuscript: JJG FZ. Helped with genotyping and contributed to the acquisition of clinical data: XBW CYP. Designed the project: FZ. Read and approved the final manuscript: JJG XPQ XBW CYP FZ.

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