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Genome wide analysis reveals association of a FTO gene variant with epigenetic changes

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ABSTRACT

Variants of the FTO gene show strong association with obesity, but the mechanisms behind this association remain unclear. We determined the genome wide DNA methylation profile in blood from 47 female preadolescents. We identified sites associated with the genes KARS, TERF2IP, DEXI, MSI1, STON1 and BCAS3 that had a significant differential methylation level in the carriers of the FTO risk allele (rs9939609). In addition, we identified 20 differentially methylated sites associated with obesity. Our findings suggest that the effect of the FTO obesity risk allele may be mediated through epigenetic changes. Further, these sites might prove to be valuable biomarkers for the understanding of obesity and its comorbidites.

1. Introduction

Obesity has a strong heritable component that is only in part explained by known single nucleotide polymorphisms (SNPs) [1–4]. The gene that has the strongest association with human obesity is the fat mass and obesity associated (FTO) gene. Although the functional role of FTO remains obscure, it is known that it encodes for an enzyme able to remove methyl groups from DNA and RNA nucleotides *in vitro* with highest affinity for single stranded RNA molecules. This suggests that the association with obesity could be mediated by epigenetic mechanisms [5]. One of the central epigenetic mechanisms is methylation of DNA cytosine residues, which may decrease transcription of genes localized near the methylation site [6]. Interestingly, studies of identical twins have revealed that their DNA methylation profile diverge with increasing age, which supports the notion of a major environmental influence on epigenetic alterations [7]. Increasing amounts of evidence indicate that DNA methylation is sensitive to extrinsic factors, such as certain diets and nutrients [8]. A recent study found that exposure to high-fat diet was not only associated with peripheral insulin resistance, but also influenced DNA methylation of the peroxisome proliferator-activated receptor gamma, coactivator 1-alpha (PPARGC1A) gene in skeletal muscle [9]. Other studies have suggested that the methylation level can be highly associated with specific genotypes, which suggest that the effect of genetic variations can be exerted through epigenetic changes [10]. One such study by Ling et al. identified a polymorphism in the promoter of the NDUFB6 gene that creates a potential new methylation site that was methylated in elderly. Interestingly, this higher methylation reduces NDUFB6 transcription, which was associated with higher peripheral insulin resistance [11]. In a similar way, genetic variations within the MCHR1 gene are affecting DNA methylation levels in an age dependent manner, which contributes to the age dependent obesity association of these genetic variations [12]. Another mechanism for the interaction between genetic and epigenetic interaction is demonstrated by the linkage between polymorphisms in genes involved in the metabolic pathways of DNA methylation that has been associated with changes in these pathways [13{Nordgren, 2011 #113}] and disease [14]. Furthermore, the obesity risk allele of FTO has been associated with higher methylation of sites within intron one of the FTO gene, which was caused by CpG site creating SNPs, implying an interaction between genetic and epigenetic factors for the risk allele [15]. A recent genome-wide investigation study found that the inter-individual variation in methylation level depends on both genetic and environmental factors and that the extent of their influence differs between sites [16]. Since alterations in the DNA methylation profile are closely linked to diseases and pathological conditions including obesity, the determination of such disease-specific methylation patterns can prove to be valuable diagnostic and prognostic biomarkers after clinical validiation [8,17–19]. However, to be useful in the clinical routine the biomarkers should be

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detectable in easily accessible samples, such as peripheral blood. One such methylation marker is the decreased methylation of the IGF2 gene, which can be detected in samples from both colon and peripheral blood and in both cases are associated with higher risk for colorectal cancer [20]. Obesity has been linked to changes in DNA methylation status in blood for several genes through genome-wide methylation profiling studies [21,22] and it has been demonstrated that such markers can predict weight loss outcome [23]. A previous study has linked the obesity risk allele of FTO with methylation changes within the FTO gene itself. However, no previous study has ascertained how the risk allele affects the methylation status of sites related to other genes.

In the present study we determined the methylation profile on a genome-wide scale by sampling DNA from peripheral whole blood of 47 female preadolescents, including an obese and a normal-weight group, both of which contains homozygous carriers of both the FTO normal and risk alleles (rs9939609).

2. Results

We determined the genome wide methylation profile of 47 Greek female preadolescents (Table 1). The influence of the FTO allele and obesity on the methylation profile was identified by fitting a linear model that explains the methylation level for each probe on the array (Fig. 1). The model was corrected for Tanner stage, which is an estimation of physical development, and white blood cell count. Four sites associated with the genes KARS/TERF2IP, DEXI, MSI1 and BCAS3 had a significantly increased methylation level in the carriers of the FTO risk allele and all had a relatively low β-value (Table 2). One site, in proximity to STON1, had a decreased methylation and a β-value close to 50 (Table 2). Notably, two of the five sites, KARS/TERF2IP ($p = 0.0011$) and DEXI ($p = 0.024$) were still significant when applying Bonferroni correction, which is stricter than the method proposed by Benjamini and Hochberg. KARS and TERF2IP share a bidirectional promoter region that lies in conjunction with the identified differentially methylated site. Twenty sites were differentially methylated in the obese individuals compared to the normal-weight controls, 15 sites associated with the genes CERCAM, DPYD, ZNF35, ZNF362, CBX6, FOXF1, PSMD7, PRRC2C, MSI1, NBPF3, USP5, PLOD2, TLE3, RPS24 and POLD3 were hypomethylated and the remaining five sites, associated with the genes DVL3, COL4A1, H1FX, TSC22D2 and IL12A, were hypermethylated (Table 3). All sites that correlated with weight category had a low β-value, indicating that the genes are expressed from most alleles. Notably, the methylation level for the MSI1 site was significantly associated with both weight category and FTO genotype. However, we could not find any significant interaction ($p = 0.22$, unadjusted) between the FTO and weight category for the MSI1 site when adding an interaction term to the linear model.

Table 1
Demographic data stratified for weight category and FTO allele.

FTO[a]		Normal-weight	Obese	P-value[d]
A				
	N	12	11	
	Age(year)	10.55 ± 0.52	11.14 ± 0.88	ns.
	Height (cm)	139.83 ± 5.54	148.09 ± 8.57	0.015
	Weight (kg)	33.07 ± 3.88	61.94 ± 10.62	< 0.0001
	WBC[b] (10^3/mm^3)	7.84 ± 2.35	6.96 ± 1.68	ns.
	Tanner stage[c]	2(1–3)	3(2–5)	0.02
T				
	N	12	12	
	Age(year)	10.53 ± 0.38	10.72 ± 0.47	ns.
	Height (cm)	145.83 ± 5.08	154.00 ± 10.38	0.027
	Weight (kg)	37.70 ± 4.39	61.38 ± 10.39	< 0.0001
	WBC[b] (10^3/mm^3)	6.80 ± 1.62	7.31 ± 1.61	ns.
	Tanner stage[c]	2 (1–3)	2.5 (1–5)	ns.

a — A = risk allele, T = normal allele (homozygous carriers).
b — WBC = White blood cells.
c — estimates physical development, median (range).
d — Indicates p-value for significant or non-significant (ns.) differences between obese and normal-weight individuals.
All values given as Mean ± S.D.

3. Discussion

We have identified five sites, corresponding to six genes that differ in methylation level between homozygous carriers of the normal and the risk allele of the FTO gene and 20 sites that correlate with obesity (Table 2). This is the first evidence showing that the FTO gene, a demethylation enzyme, may influence the methylation level of other genes. The FTO protein is structurally similar to the AlkB family of enzymes that remove methyl groups from DNA residues [24]. The FTO protein has a relatively low affinity for cytosine residues *in vitro,* but a higher affinity for thymine; it has also been shown to prefer single stranded DNA [5]. Thus, it remains unclear whether the observed methylation differences between the two alleles are related to the direct enzymatic function of FTO or is caused by indirect mechanisms. Interestingly, the two strongest associated sites, KARS/TERF2IP and DEXI, are located 21 Mbp and 42 Mbp on each side of the FTO gene on chromosome 16 (Table 2). It is possible that the FTO risk allele affects these genes through changes in the structural or spatial organization of the chromosome even though the genomic distances are large. The KARS and TERF2IP sites are associated with a CpG island near their shared bidirectional promoter [25]. TERF2IP is coding for a multifunctional protein that is found in the nucleus and cytoplasm and has a basic function in regulating telomere length, but is also involved in transcriptional regulation and, when residing in the cytoplasm, functions as an adaptor protein that enhances NF-kB activity and is thereby involved in inflammatory response [26]. FTO is functionally linked to TERF2IP as they both interact or form complexes with the DNA repair protein SLX4/BTBD12 according to the interaction database IntAct [EBI-2371551, EBI-2371041] [27]. SLX4/BTBD12 is an endonuclease that is central for several DNA repair mechanisms and resolves Holliday junctions in the DNA [28]. This interaction is in concert with the role of TERF2IP in telomere maintenance and the DNA demethylase activity of FTO. This functional coupling could suggest that the differences we found in the methylation level of the KARS/TERF2IP site would be a regulatory response to changes in FTO activity or expression to retain homeostasis between the protein complex subunits. The KARS gene codes for a soluble enzyme that catalyzes the attachment of amino acids to tRNA and resides in the cytoplasm as well as the mitochondria and nucleus [29]. However, like TERF2IP, it is a multifunctional protein that is also secreted as a cytokine, which is induced by TNFα stimulation of the cell; adenylates lysine residues of proteins; synthesizes dinucleotides that affect signaling pathways and regulates transcription factors, such as MITF and USF2. The risk allele of FTO has been associated with a higher inflammation status independent of BMI [30], which could potentially be connected to the hypermethylation of the KARS/TERF2IP sites. Also, two SNPs within the TERF2IP locus (rs3784929 and rs8053257) have been associated with a greater risk of ischemic stroke in women, a known co-morbidity of obesity [31]. Hence, it would be of interest to assess the methylation level of the KARS/TERF2IP site as a prognostic marker for ischemic stroke and other cardiovascular diseases. There is no clear connection for the remaining five differentially methylated sites with FTO or obesity. The BCAS3 gene is an estrogen induced transcriptional coactivator that is over expressed in breast cancer [32,33]. Both DEXI and STON1 are poorly characterized genes. The expression of DEXI is induced by glucocorticoids and the gene is proposed to have a role in emphysema [34]. STON1 has no clear function, but its paralog STON2 have a role in synaptic vesicle recycling [35].

Interestingly, the MSI1 site is associated with higher methylation level for the FTO risk allele carriers, but lower in the obese individuals

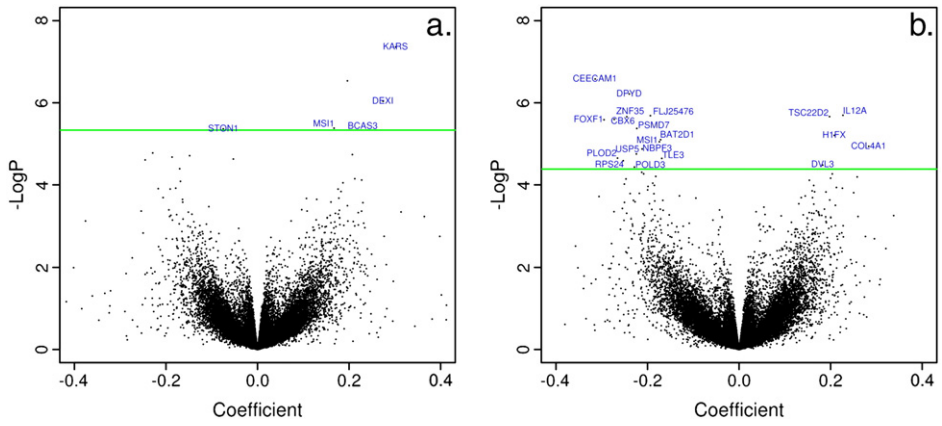

Fig. 1. Volcano plots for linear models of methylation levels comparing FTO alleles (a) and obese and lean individuals (b).Volcano plots that show unadjusted P-values vs coefficients (log-fold change of methylation level) for: a. FTO allele (positive coefficients refer an increased methylation in homozygous carriers of the obesity risk allele (A)), b. Obesity vs normal-weight individuals (positive coefficients refer to an increased methylation in the obese individuals). The green line indicates the adjusted significance threshold.

although with the same effect size of 3.7–3.8% (Tables 1–2). Hence, our results suggest that the loss of methylation that we found in the obese individuals is diminished by the FTO risk allele, which could play a role in the development of obesity. The MSI1 gene binds to mRNA and controls its translation [36]. It is a known stem cell marker involved in cancer progression and is downregulated in the brain of patients with Alzheimer's disease (AD), which reflects a lower number of neuronal progenitor cells [37,38]. Intriguingly, the FTO obesity risk allele has recently been linked to a higher risk of AD, decreased brain volume and cognitive impairment in terms of verbal fluency [39–41]. This could be mediated by the observed epigenetic regulation of MSI1 causing downregulation of the gene and consequently a lower number of neuronal progenitor cells and a decreased cognitive reserve.

In addition to MSI1, we detected 19 sites differentially methylated in the obese individuals of which seven genes are annotated as regulators of transcription (TSC22D2, CBX6, DVL3, FOXF1, TLE3, ZNF35, ZNF362) and consequently can control the expression of a large number of genes. Hence, our results suggest that the epigenetic changes associated with obesity can potentially propagate into large transcriptional alterations. Most of the genes have no known connection to obesity or its co-morbidities. However, the gene COL4A1 is essential for the basement membrane of the extracellular matrix and is important for vascular function [42]. Moreover, it has been associated with myocardial infarction in a Japanese population and has also been linked to other cardiovascular diseases in mice [43,44]. Therefore, we speculate that the methylation level of the COL4A1 gene can be a potential prognostic marker for cardiovascular diseases in obese individuals. Description for the genes not brought up in the discussion can be found in Supplementary 1.

Recently, a study identified four genomic sites where the methylation level covaries with BMI in an elderly Icelandic group [16]. Out of these, three are included in our dataset (MMP9, PRKG1 and RFC5) however the discrepancy for the remaining sites between our studies depends probably on population differences, study design or the relatively small sample size in both studies. Thus, the results of this and previous studies need to be replicated in a larger population, which likely will associate additional methylated sites with both weight and FTO allele.

Herein, we report that the obesity gene FTO is associated with methylation changes in several sites, which suggests that the effect of the risk allele can, at least in part, be mediated through epigenetic alterations. Moreover, we identify 20 sites that are differentially methylated in obese individuals. These are candidate biomarkers which could prove to be of diagnostic and prognostic importance for predicting the progress of obesity, the development of comorbidites and might contribute to a more personalized treatment.

4. Materials and methods

4.1. Ethics statement

All participants and their guardians gave informed written consent and the study was approved by the Greek Ministry of National Education (7055/C7-Athens, 19-01-2007) and the Ethical Committee of Harokopio University (16/ Athens, 19-12-2006).

4.2. Subjects

Twenty three obese and 24 normal-weight preadolescent girls (Table 1) were included in the analysis from the Greek Healthy Growth study, as previously described together with sample collection and preparation procedures [45]. Equal numbers of homozygous carriers of the FTO obesity risk and non-risk allele (rs9939609) were randomly selected from the cohort of the previous study for both the obese and lean group. As the homozygous carriers should presumable

Table 2
Differentially methylated genes between carriers of the FTO TT and AA (risk) allele.

Symbol	Name	Position (NCBI36)	Gene location	Avg meth.[a]	Relative effect[b]	P-value[c]
KARS/ TERF2IP	Lysyl-tRNA synthetase/Telomeric repeat-binding factor 2-interacting protein 1	Chr16:74238612	Intron/uspstream	12.6	8.3%	0.00114
DEXI	Dexamethasone-induced protein	Chr16:10944027	Upstream	4.4	12.8%	0.01198
MSI1	Musashi 1	Chr12:119292001	Upstream	23.8	3.7%	0.02417
STON1	Stonin 1	Chr2:48660278	Intron	57.0	−1.3%	0.02417
BCAS3	Breast carcinoma amplified sequence 3	Chr17:56110037	UTR	11.5	5.6%	0.02417

a — Average methylation (beta) value has range 0–100 (low to high methylation).
b — Percentage methylation change in AA (risk) allele carriers relative to average methylation.
c — Adjusted for multiple comparisons as suggested by Benjamini and Hochberg [18].

Table 3
Differentially methylated genes between obese and normal-weight female preadolescents.

Symbol	Name	Position (NCBI36)	Gene location	Avg meth.[a]	Relative effect[b]	P-value[c]
CERCAM	Glycosyltransferse 25 family member 3	Chr9:130222317	UTR	4.9	−13.7%	0.00687
DPYD	Dihydropyrimidine dehydrogenase	Chr1:98158797	Intron	2.8	−16.4%	0.00796
IL12A	Interleukin 12A precursor	Chr3:161189517	UTR	2.8	15.5%	0.00844
ZNF35	Zinc finger protein 35	Chr3:44665023	Upstream	22.2	−5.5%	0.00844
ZNF362	Zinc finger protein 362	Chr1:33494712	Upstream	5.2	−8.2%	0.00844
TSC22D2	TSC22 domain family 2	Chr3:151609841	Exon	6.4	7.4%	0.00844
CBX6	Chromobox homolog 6	Chr22:37598108	UTR	3.1	−16.7%	0.00844
FOXF1	Forkhead box F1	Chr16:85101840	Exon	4.7	−13.3%	0.00844
PSMD7	Proteasome 26S non-ATPase subunit 7	Chr16:72888397	Intron	7.5	−7.7%	0.01223
H1FX	Histone H1x	Chr3:130517349	UTR	4.1	10.2%	0.0162
PRRC2C	Protein PRRC2C	Chr1:169721292	UTR	4.1	−8.4%	0.0188
MSI1	Musashi 1	Chr12:119292001	Upstream	23.8	−3.8%	0.01937
COL4A1	Collagen alpha-1(IV) chain	Chr13:109758453	Upstream	9.9	8.6%	0.02313
NBPF3	Neuroblastoma breakpoint family member 3	Chr1:21639319	UTR	5.8	−8.4%	0.02483
USP5	Ubiquitin carboxyl-terminal hydrolase 5	Chr12:6831355	Upstream	4.4	−10.4%	0.03069
PLOD2	Procollagen-lysine,2-oxoglutarate 5-dioxygenase 2	Chr3:147362332	Upstream	30.8	−5.4%	0.03476
TLE3	Transducin-like enhancer protein 3	Chr15:68177287	UTR	5.5	−6.9%	0.03476
RPS24	40S ribosomal protein S24	Chr10:79463659	UTR	5.8	−10.0%	0.03736
DVL3	Disheveled 3	Chr3:185356123	UTR	4.4	8.5%	0.04529
POLD3	DNA polymerase, delta subunit 3	Chr11:73981434	Intron	6.1	−8.8%	0.04797

a — Average methylation (beta) value has range 0–100 (low to high methylation).
b — Percentage methylation change in obese female preadolescents relative to average methylation.
c — Adjusted for multiple comparisons as suggested by Benjamini and Hochberg [18].

demonstrate the largest differences in phenotype (i.e. methylation level) heterozygous carriers were not selected. Genomic DNA was isolated from peripheral blood using QiaGen Maxiprep kit (Qiagen, Hilden, Germany) and *FTO* genotyping procedures were performed as previously described [46].

4.3. DNA methylation profiling

The genome-wide Illumina Infinium HumanMethylation27 BeadChip array (Illumina) which allows interrogation of 27,578 CpG dinucleotides covering 14,495 genes was applied to determine the methylation profile of genomic DNA isolated and purified from the peripheral whole blood. This chip has been shown to give a reliable and reproducible estimation of the methylation profile on a genomic scale [47]. First, bisulfite conversion of genomic DNA was performed using the EZ DNA Methylation-Gold™ Kit (Zymo Research) according to the manufacturer's protocol. Briefly, 500 ng of DNA was sodium bisulfite-treated, denatured at 98 °C for 10 min, and bisulfite-converted at 64 °C for 2.5 h. After conversion, samples were desulphonated and purified using column preparation. Approximately 200 ng of bisulfate-converted DNA was processed according to the Illumina Infinium Methylation Assay protocol. This assay is based on the conversion of unmethylated cytosine (C) nucleotides into uracil/thymine (T) nucleotides by the bisulfite treatment. The DNA was whole-genome amplified, enzymatically fragmented, precipitated, resuspended, and hybridized overnight at 48 °C to locus-specific oligonucleotide primers on the BeadArray. After hybridization, the C or T nucleotides were detected by single-base primer extension. The fluorescence signals corresponding to the C or T nucleotides were measured from the BeadArrays using the Illumina iScan scanner. The fluorescence data were then preprocessed using the GenomeStudio 2009.2 (Illumina) software, which assigns a quantitative measure of methylation levels (β-values) for each CpG site, that corresponds to the ratio between the fluorescence signal from the methylated allele (C) and the sum of the fluorescent signals of the methylated (C) and unmethylated (T) alleles, expressed as percentages (0–100%). A total of 26034 probes were included in the analysis, after discarding 535 probes that did not reach the quality threshold (detection P-value<0.01) together with 1009 probes from the sex chromosomes. The HumanMethylation27k BeadChip data can be found in GEO with accession number: GSE27860.

4.4. Data processing and statistical analysis

All downstream data processing and statistical analyses were performed with the statistical software R (www.r-project.org) together with the *methylumi* and *limma* [48] packages of the Bioconductor project [49]. First, data was imported, using the *methylumi* package and normalized between arrays with quantile normalization using the *normalizeBetweenArrays* function in the *limma* package with the method parameter set to "quantile" and otherwise default parameters.

The relationship between methylation level and variables of interests were determined using *limma's* robust regression method (*lmFit* command with setting *method="robust", maxit=1000)* to fit the following linear model for each probe *k*:

$$M_k = a_k + b_{kW}W + b_{kF}F + b_{kB}B + b_{kT}T + \varepsilon_k$$

where M_k is the log2 transformed methylation level (β-value) of probe *k*, *W* is the dichotomized weight category (normal-weight = 0 and obese = 1), *F* is the dichotomized FTO allele (TT = 0 and AA = 1), *B* is the white blood cell count, *T* is the Tanner stage and ε_k represents the unexplained variability. The coefficients b_{kx} summarizes the correlation between the methylation level and the variables of interest. Moderated t-statistics for each contrast and probe was created using an empirical Bayes model as implemented in *limma* (*eBayes* command). P-values were adjusted for multiple comparisons as proposed by Benjamini and Hochberg [50] and an adjusted P-value > 0.05 was considered non-significant (ns).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.ygeno.2011.12.007.

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