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Investigation of differential *HDAC4* methylation patterns in eating disorders

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Abstract

Objective—To investigate the relationship between methylation patterns of the histone deacetylase 4 gene and eating disorders in a site previously associated with anorexia nervosa.

Methods—Women with anorexia nervosa (N=28) or bulimia nervosa (N=19) were age- and sex-matched to controls (N=45). We obtained saliva-derived DNA and utilized bisulfite pyrosequencing to examine region-specific methylation differences between cases and controls.

Results—The region assayed includes 15 CpGs. We found no significant association between the previously implicated CpG and either anorexia nervosa or bulimia nervosa. We found that three CpGs were nominally associated with anorexia nervosa ($p=0.02$ – 0.03); the largest difference was a 9% hypermethylation in anorexia nervosa. One CpG was nominally associated with bulimia nervosa ($p=0.04$), with 4% hypomethylation. None of these results remained significant after correction for multiple testing.

Conclusion—We did not replicate previous findings, though through expanded coverage, we identified additional CpGs that were nominally associated with eating disorders.

Keywords

Epigenetics; Methylation; Histone deacetylase 4; Anorexia nervosa; Bulimia nervosa

Introduction

Epigenetic modifications like DNA methylation, whereby a methyl group is tagged to a CpG dinucleotide, have been studied in anorexia nervosa at the global genome level (Frieling et al., 2007; Kim et al., 2014) and in candidate gene studies, largely through investigation of promoter regions of genes such as *LEP*, *BDNF*, *SLC6A4*, and *POMC* (Ehrlich et al., 2010;

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Conflicts of Interest

There are no conflicts of interest.

Pjetri E et al., 2013). Yet, to date, methylation differences at the global genome-level or within candidate genes have not been replicated.

Two recent lines of evidence suggest that histone deacetylase 4 (*HDAC4*), which encodes a repressor of transcription factors, might be one gene implicated in the epigenetics of eating disorders. First, through linkage and whole-exome sequencing of two large families with anorexia nervosa (AN), Cui et al. found that an exonic missense mutation A786T in *HDAC4* was associated with AN and predicted to have deleterious effects (Cui et al., 2013). The same team further demonstrated that female mice heterozygous for the same mutation display several feeding and behavioral deficits (Lutter et al., 2017). Second, a study led by Booij et al examined methylation in blood-derived DNA across ~450,000 CpGs in 29 individuals with AN as compared to 15 controls. They found that fourteen CpGs survived a false discovery rate cutoff of $Q < 0.05$, including one within *HDAC4* (genomic location: chr2: 240,171,748, referred to as cg01114124 on the Illumina Infinium HumanMethylation450 BeadChip), which showed 6.25% higher methylation in AN cases than in controls ($p = 4.57 \times 10^{-5}$; false discovery rate = 0.045) (Booij et al., 2015).

HDACs modulate transcriptional regulation in the nucleus, and evidence suggests that the HDAC IIa family, which includes HDAC4, may also regulate substrates independently of deacetylation in the cytoplasm. HDAC4 is involved in energy homeostasis and neuronal plasticity, with widespread distribution in the brain (Darcy et al., 2010; Sando et al., 2012; Clocchiatti et al., 2013).

Given this evidence, our investigation included three goals: 1) to replicate the finding of hypermethylation at the Booij et al *HDAC4* CpG site in a case-control study; 2) to test the association between AN and nearby CpGs in *HDAC4*; and 3) to extend observations to bulimia nervosa (BN).

Materials and methods

Study design

We designed a case-control study using saliva-derived DNA. Case samples were ascertained from the eating disorders inpatient and partial hospital services at the University of Iowa Hospitals and Clinics and University of Texas, Southwestern Medical Center by Dr. Michael Lutter. The Institutional Review Board at both institutions approved all procedures, and participants completing surveys provided written consent. We selected DNA samples from European-American women between the ages of 18–40 years with restrictive type-AN or binge eating-AN. The age group was selected to replicate findings in Booij et al. Clinical information was gathered by research interviews using the Structured Clinical Interview for Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV) Axis I Disorders. The DSM-IV was used to make diagnoses, and BMI at time of onset was recorded. Individuals with binge eating/purging-AN were excluded.

Controls were obtained from the Tissue Procurement Core at University of Iowa, and samples were approved by the University of Iowa's Institutional Review Board. All participants provided written consent. Individuals were volunteers whom we attempted to

match to cases by age, sex, and ethnicity. We matched ages to the nearest age group within five years. In the control group, we excluded those with an eating disorder history and those on psychiatric medications at the time of saliva collection. The Tissue Procurement Core provided BMI at time of saliva collection.

Sodium bisulfite treatment and nested PCR

We collected 400 ng of DNA from each participant's sample for bisulfite pyrosequencing. DNA samples were bisulfite converted using the EZ DNA Methylation-Gold™ Kit (Zymo Research), according to the manufacturer's instructions.

After bisulfite conversion, we performed nested PCR. The outer PCR amplification was done with 1 µl of bisulfite converted DNA. This was done via Taq polymerase using the PyroMark PCR kit. Subsequently, 2 µl of each outer region product was used for inner PCR application. One of the primers in each set was biotinylated at the 5'-end and HPLC-purified. Primers were designed using PyroMark Assay Design software SW 2.0 (Qiagen). For nested PCR primer sequences, refer to table in Supplemental Digital Content 1. The UCSC Genome Browser, GRCh37/hg19, was used to design primers targeting the DNA region 300 bp upstream and downstream of the coordinate chr2:240,171,748 (referred to as cg01114124 on the Illumina Infinium HumanMethylation450 BeadChip), which was previously associated with AN (Booij et al., 2015; Kent et al., 2012).

Bisulfite-pyrosequencing analysis

Ten unique sequencing primers targeting 15 CpGs were created within the 600bp region. Sequencing primers are tabled in Supplemental Digital Content 2. Reactions were conducted using PyroMark Q26 Advanced CpG reagents and a PyroMark Q26 MD instrument (Qiagen). The pyrograms were analyzed using the CpG mode of the PyroMark Q26 Advanced software to determine the methylation percentage of each site for cases and for controls.

To determine how reliable our methylation values were at specific sites, we arbitrarily selected sites sixty-two and fifty-three base-pairs downstream of coordinate chr2:240,171,748 and re-pyrosequenced using DNA from AN cases and controls. There were no statistical differences between the mean percent values when AN- cases and - controls were replicated across methylation assays ($p=0.97$ and 0.99 at site -62; $p=0.72$ and $p=0.92$ at site -53).

Statistical analyses

Age and BMI were compared between cases and controls using a two-tailed Student's t-test. Linear regression was used to evaluate associations of methylation levels with age and BMI. Methylation differences between cases and controls were determined with a two-tailed Student's t-test. We report p-values that are uncorrected. However, we also considered whether our results would stand up to correction for testing two methylated regions, given the correlation structure across the CpGs, using two phenotypes (4 tests).

Results

Clinical characteristics for cases and controls are summarized in Table 1. The average age was 24 years ($\pm$ SD of 6.1 years) and 26 years ($\pm$ 6.3) in AN cases and controls, respectively. Likewise, the average age was 22 ($\pm$ 5) and 28 ($\pm$ 3.8) in BN cases and controls. Average BMI was 16 mg/kg² ($\pm$ 1.9), 20 mg/kg² ($\pm$ 2.1), 25 mg/kg² ($\pm$ 4.5) 28 mg/kg² ($\pm$ 7.8) for AN cases, BN cases, AN-matched controls, and BN-matched controls, respectively.

Table 2 presents the bisulfite pyrosequencing results. Linear regression analyses demonstrated that neither age nor BMI were significantly associated with methylation levels for these CpGs. As a result, we did not control for these in the case-control comparisons of methylation level differences. There was no significant association between AN and the target CpG identified by Booij et al. AN cases showed an average methylation of 93% ($\pm$ SD of 3), and controls averaged 94% ($\pm$ 3, $p > 0.05$). However, three adjacent CpG sites showed average methylation levels that were nominally associated with AN case status ($p < 0.05$). The largest of these differences in methylation between AN cases and controls was 9%. This occurred in a CpG that was hypermethylated in AN with an average methylation level of 25% ($\pm$ 19) in cases vs. 16% ($\pm$ 11) in controls. Two nominally associated sites were hypomethylated in AN as compared to controls: 85% ($\pm$ 6) vs. 88% ($\pm$ 4), respectively and 80% ($\pm$ 9) vs. 85% ($\pm$ 8), respectively.

In our bisulfite pyrosequencing analysis of cases with BN, one unique site was hypomethylated and nominally associated with BN ($p < 0.05$), with average methylation of 71% ($\pm$ 5) in BN cases vs. 75% ($\pm$ 7) in controls (Table 2). We found neither association nor hypermethylation at the Booij et al. CpG site in BN cases.

Discussion

Using bisulfite pyrosequencing, we assessed 15 CpG sites in *HDAC4*, and found that four were nominally associated with EDO (three with AN and one with BN). Notably, none of these associations withstood correction for multiple testing. We neither replicated the significant differential methylation at the CpG dinucleotide at chr2:240,171,748 in AN noted by Booij et al; nor, did we observe the consistent hypermethylation across multiple CpGs in AN observed in that report.

Previous results on DNA methylation variation in EDO have been mixed. Tremolizzo et al. noted hypomethylation in whole blood, while Saffrey et al. demonstrated no individual gene or global DNA methylation differences in buccal tissue (Saffrey et al., 2014; Tremolizzo et al., 2014). These disparate findings may be due to power limitations resulting from the modest number of samples tested in these studies. Additionally, methylation patterns may differ across the tissues assayed.

We note that the region we studied is in an intron, while the interesting functional variant Cui et al. described was in an *HDAC4* exon. Intronic genetic and epigenetic variation may alter gene expression or lead to alternative splicing. The largest genome-wide association study of AN, with 5,551 AN cases and 21,080 controls, identified two intronic variants (rs9839776 in *SOX2OT* and rs17030795 in *PPP3CA*) in their top results, though not at a

genome-wide significant level (Boraska et al., 2014). Analyzing epigenetic differences in intronic regions may aid in the discovery of noncoding regions involved in disease.

We speculate that environmental modifications of *HDAC4* might play a pathogenic role in eating disorders and/or associated phenotypes such as anxiety and increased stress reactivity. Recent evidence demonstrates that transgenic mice carrying the *HDAC4* missense A778T mutation show sex-specific differences in metabolism and behavior; only females manifested reduced response to high-fat, compulsive grooming, increased weight, and/or changes in anxiety-like behavior (Lutter et al., 2017). Another study suggested that overexpression of miRNA-124 leads to behavioral resilience while its inhibition leads to increased susceptibility to stress. The authors identified *HDAC4* as one of miRNA-124's three targets (Higuchi et al., 2016). Finally, a recent study showed evidence for the involvement of *HDAC4* in PTSD in women, and an influence of estrogen on the regulation of *Hdac4* in mice (Maddox et al., 2017).

Several limitations exist in this study. First, our study is cross-sectional; therefore, causation cannot be inferred. Second, while our AN sample had 81% power to detect a methylation difference comparable to that of Booij et al, our BN sample had only 66% power to detect methylation differences greater than ten percent. Third, we used saliva-derived DNA, which may or may not reflect epigenetic profiles in the brain, and which differs from the Booij et al study that used blood-derived DNA. While methylation levels can be tissue specific, many CpGs show methylation levels that are highly correlated across tissues. Although the correlation between saliva and blood at the Booij CpG, cg0111412, is high at 0.824, $p = 1.1 \times 10^{-7}$ (Smith et al., 2014), we acknowledge that the use of blood-derived DNA might have led to different results. Fourth, *HDAC4* comprises 352,780 bp and differential DNA methylation relevant to AN and BN may exist in the gene's 522 CpGs that we did not assay. Fifth, We did not have data on a number of variables that might have influenced methylation levels, including non-psychiatric medications, substance use, and smoking status. Oral contraception use in particular is of concern as changes in estrogen levels have been shown to contribute to variation in *HDAC4* expression (Maddox et al., 2017). Sixth, expression levels using RNA were not testable as we had DNA versus tissue samples.

In summary, our study did not find additional support implicating *HDAC4* DNA methylation variation in eating disorders; though, we cannot rule out the possibility that limitations in study design could have led to false negative results.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Table 1

Clinical characteristics of study samples

Variable	AN (n=28)	Controls-AN (n=27)	p-value ^a	BN (n=19)	Controls-BN (n=18)	p-value ^a
Years old ($\pm$ standard deviation)	24 (6.1)	26 (6.3)	0.21	22 (5.0)	28 (3.8)	2×10^{-4}
Body mass index, BMI mg/kg² ($\pm$ standard deviation)	16 (1.9)	25 (4.5)	2×10^{-12}	20 (2.1) ^b	28 (7.8)	0.04
Age of onset ($\pm$ standard deviation)	17 (3.1)	-	-	18 (6.3)	-	-

^a Calculated by Student's t-test, cases vs. controls^b 6 out of 19 BMIs were recorded and included in analysis

Table 2

Average percent methylation for EDO cases and matched controls

CpG Site ^a	Average % methylation in AN cases ($\pm$ standard deviation)	Average % methylation in controls ($\pm$ standard deviation)	Difference ^b (%)	p-value ^c	Average % methylation in BN cases ($\pm$ standard deviation)	Average % methylation in controls ($\pm$ standard deviation)	Difference ^b (%)	p-value ^c
-119	15 ($\pm$ 14)	13 ($\pm$ 13)	2	0.60	9 ($\pm$ 10)	8 ($\pm$ 2)	1	0.51
-117	25 ($\pm$19)	16 ($\pm$11)	9	0.03	13 ($\pm$ 9)	13 ($\pm$ 5)	0	0.88
-90	30 ($\pm$ 23)	24 ($\pm$ 19)	6	0.34	16 ($\pm$ 6)	17 ($\pm$ 8)	-1	0.68
-88	26 ($\pm$ 18)	24 ($\pm$ 13)	2	0.57	14 ($\pm$ 7)	17 ($\pm$ 6)	-3	0.18
-62	37 ($\pm$ 21)	34 ($\pm$ 24)	3	0.64	29 ($\pm$ 15)	28 ($\pm$ 8)	1	0.87
-53	36 ($\pm$ 18)	31 ($\pm$ 24)	5	0.43	24 ($\pm$ 11)	26 ($\pm$ 10)	-2	0.57
-36	21 ($\pm$ 3)	21 ($\pm$ 4)	0	0.75	30 ($\pm$ 5)	33 ($\pm$ 6)	-3	0.06
-21	86 ($\pm$ 6)	88 ($\pm$ 6)	-2	0.2	83 ($\pm$ 9)	84 ($\pm$ 10)	-1	0.70
0	93 ($\pm$ 3)	94 ($\pm$ 3)	-1	0.22	88 ($\pm$ 6)	89 ($\pm$ 4)	-1	0.57
11	85 ($\pm$6)	88 ($\pm$4)	-3	0.03	85 ($\pm$ 5)	87 ($\pm$ 4)	-2	0.29
23	80 ($\pm$9)	85 ($\pm$8)	-5	0.02	81 ($\pm$ 6)	83 ($\pm$ 6)	-2	0.38
25	74 ($\pm$ 8)	74 ($\pm$ 7)	0	0.99	71 ($\pm$5)	75 ($\pm$7)	-4	0.04
38	76 ($\pm$ 8)	79 ($\pm$ 7)	-3	0.21	75 ($\pm$ 7)	77 ($\pm$ 9)	-2	0.46
58	70 ($\pm$ 7)	70 ($\pm$ 10)	0	0.95	67 ($\pm$ 6)	66 ($\pm$ 8)	1	0.93
154	84 ($\pm$ 3)	83 ($\pm$ 9)	1	0.43	81 ($\pm$ 4)	81 ($\pm$ 4)	0	0.94

^aDistance in bp from the CpG referred to as cg 01114124 on the Illumina Infinium HumanMethylation450 BeadChip, at chr2:240,171,748^bDifference = cases - controls^cNominal p-values calculated using Student's t-test