Hypomethylation of the Paternally Inherited *LRRTM1* Promoter Linked to Schizophrenia

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Epigenetic effects on psychiatric traits remain relatively understudied, and it remains unclear what the sizes of individual epigenetic effects may be, or how they vary between different clinical populations. The gene *LRRTM1* (chromosome 2p12) has previously been linked and associated with schizophrenia in a parent-of-origin manner in a set of affected siblings (LOD = 4.72), indirectly suggesting a disruption of paternal imprinting at this locus in these families. From the same set of siblings that originally showed strong linkage at this locus, we analyzed 99 individuals using 454-bisulfite sequencing, from whole blood DNA, to measure the level of DNA methylation in the promoter region of *LRRTM1*. We also assessed seven additional loci that would be informative to compare. Paternal identity-by-descent sharing at *LRRTM1*, within sibling pairs, was linked to their similarity of methylation at the gene's promoter. Reduced methylation at the promoter showed a significant association with schizophrenia. Sibling pairs concordant for schizophrenia showed more similar methylation levels at the *LRRTM1* promoter than diagnostically discordant pairs. The alleles of common SNPs spanning the locus did not explain this epigenetic linkage, which can therefore be considered as largely independent of DNA sequence variation and would not be detected in standard genetic association analysis. Our data suggest that hypomethylation at the *LRRTM1* promoter, particularly of the paternally inherited allele, was a risk factor for the development of schizophrenia in this set of siblings affected with familial schizophrenia, and that had previously showed linkage at this locus in an affected-sib-pair context. © 2014 Wiley Periodicals, Inc.

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et al., 2007; Feil and Fraga, 2011]. Mainly located on cytosine bases of DNA [Bernstein et al., 2007], the role of methylation has been particularly studied in the context of gene promoters, where it can interfere with transcription [Jones, 2012; Zhang et al., 2013]. Recent technological developments now permit the precise quantification of methylation on a locus-specific level, or on a genome-wide scale. However, DNA methylation is subject to variation according to factors such as cell and tissue type, genotype, gender, age, stress, alcohol, and drug consumption [Morris et al., 2011; Bleich et al., 2006; Christensen et al., 2009; Cordero et al., 2013; Horvath, 2013; Lim and Song, 2012; Liu et al., 2010a; Liu et al., 2010b; Philibert et al., 2008; Kerkel et al., 2008]. Distinguishing cause from effect is therefore challenging in studies that find links between DNA methylation and human traits, while studies must also be interpreted within the context of the specific tissue being analyzed, and with consideration of potentially confounding variables. However, despite these complexities, whole blood DNA methylation has been shown to be remarkably similar to brain DNA methylation at many loci [Mill and Heijmans, 2013; Klengel

INTRODUCTION

The complex etiology of schizophrenia involves multiple environmental and genetic factors [Insel, 2010]. Epigenetic variation may also be involved, including DNA methylation [Nishioka et al., 2012], which has long been of interest in psychiatric diseases due to a role in gene regulation, and its potential to transduce environmental and genetic effects at the molecular level [Bernstein

et al., 2013; Provencal et al., 2012], leading to the possibility to detect some associations of DNA methylation with psychiatric traits using peripheral tissues [Auta et al., 2013; Davies et al., 2012]. This is because many methylation marks can be stably inherited over generations of cell division, throughout organismal development [Bernstein et al., 2007].

Hypomethylation has previously been suggested to be a global feature of the epigenome in schizophrenia [Nishioka et al., 2012; Bonsch et al., 2012; Bromberg et al., 2008]. An epigenetic study of monozygotic twins discordant for schizophrenia identified a number of loci differentially methylated in peripheral blood [Dempster et al., 2011]. Microarray-based studies, measuring the methylation profiles of thousands of CpG sites across the genome in diverse tissues, have identified various additional candidate genes whose methylation may be associated with schizophrenia [Kinoshita et al., 2013; Wockner et al., 2014]. Other studies using varying methodologies, and targeting a variety of tissues, have found partly consistent evidence for epigenetic alterations associated with schizophrenia at genes including *RELN* [Abdolmaleky et al., 2005; Grayson et al., 2005], *COMT* [Abdolmaleky et al., 2006], *SOX10* [Iwamoto et al., 2005], *HTR2A* [Abdolmaleky et al., 2011], *BDNF*, and *ST6GALNAC1* [Nishioka et al., 2012].

Among the genes previously implicated in schizophrenia by genetic linkage or association, *LRRTM1* (Leucine-rich repeat transmembrane neuronal 1) is of particular interest from an epigenetic perspective [Francks et al., 2007; Francks et al., 2003]. The genomic region spanning *LRRTM1* (chromosome 2p12) was first linked to schizophrenia and schizoaffective disorder in a study of 196 affected-sib-pair families [Francks et al., 2003; DeLisi et al., 2002]. Unusually, the linkage signal was very strong and derived overwhelmingly from paternal identity-by-descent (IBD) sharing (log of the odds, LOD = 4.72, P = 0.0000016), and not maternal IBD sharing (LOD = 0.6, P = 0.048). Haplotype association data within a region directly upstream of *LRRTM1* also pointed to a paternal-specific effect, in analysis of over 1000 families [Francks et al., 2007]. Gene expression data from human brain, mouse-human hybrid cell lines, and human lymphoblastoid cell lines, indicated variable and incomplete imprinting of *LRRTM1*, specifically down-regulation of the maternally inherited copy of the gene [Francks et al., 2007]. Genomic imprinting is often linked to differences in methylation levels between the paternally and maternally inherited alleles [Francks et al., 2007].

LRRTM1 encodes a member of the Leucine-Rich Repeat Trans-Membrane Neuronal (LRRTM) family of proteins, known to interact with neurexins to mediate excitatory presynaptic differentiation [Linhoff et al., 2009; Francks, 2011]. Neurexins have been directly implicated in schizophrenia by genetic deletion mapping [Kirov et al., 2009]. In the mouse, *Lrrtm1* is mainly expressed in neurons of the CA1–CA3 pyramidal layers and the dentate gyrus granular layer of the hippocampus, the cerebral cortex, the striatum, and broadly in the thalamus [Lauren et al., 2003]. This distribution broadly corresponds to that in humans, where *LRRTM1* is mainly expressed in the hippocampus, the striatum, the amygdala and the dorsal thalamus (Allen Brain Atlas: http://human.brain-map.org/microarray/search)[Ball et al., 2012]. *LRRTM1* localises to postsynaptic excitatory synapses and mediates long-term potentiation [Soler-Llavina et al., 2013], probably via an interaction with Post Synaptic Density-95 protein [Bar-Shira and Chechik, 2013], and through modifying the distribution of AMPA receptors and vesicular glutamate transporters [de Wit et al., 2009; Schwenk et al., 2012]. The abundances of different LRRTM-family proteins at synapses may effect fine-tuning of their signaling properties [Francks, 2011].

Since the haplotype association identified by Francks et al. [2007] could only explain a small proportion of the paternal-specific linkage at the *LRRTM1* locus [Francks et al., 2007; Francks et al., 2003], we previously suggested in this journal [Francks, 2011] that alterations of methylation (on the paternally inherited allele) may have been responsible for the unusually strong linkage in the particular set of sibling pairs that was studied. There is no clear *a priori* expectation regarding the sizes of individual epigenetic effects on psychiatric traits and this may vary between different patient populations, but considering the strong genetic linkage signal found previously in this specific set of affected sibling pairs (LOD = 4.72)[Francks et al., 2003], it was reasonable to undertake the present study of methylation using blood DNA samples drawn from the same affected-sib-pair sample [Francks et al., 2007; Francks et al., 2003], with confidence that statistical power was adequate in this context. We performed multiplexed 454-bisulfite sequencing (Roche, Life Technology, Connecticut) to precisely quantify the methylation across a CpG island that is located at the promoter region of *LRRTM1*. CpG islands are genomic regions enriched in CG dinucleotides, the cytosines of which are the primary targets for DNA methylation [Lister et al., 2009]. We also assessed seven additional CpG islands, located at a set of additional genes on chromosome 2p12 and elsewhere in the genome, that would be informative to compare (see below). The resulting data were analyzed i) to determine whether methylation at the *LRRTM1* promoter was linked to paternal IBD sharing across the locus, ii) to test if specific SNP alleles were associated with changes in methylation levels, iii) to test whether affected sibling pairs were more similar in their methylation profiles at the *LRRTM1* promoter than diagnostically discordant siblings, and iv) to test for an overall association between DNA methylation levels within the *LRRTM1* locus and affection with schizophrenia/schizoaffective disorder. Together these lines of evidence would indicate that the level of methylation at the *LRRTM1* promoter, particularly of the paternally inherited allele, is a risk factor for the development of psychosis, independently of common DNA sequence variation at the locus.

MATERIALS AND METHODS
Sampling

We analyzed samples that were drawn from the New York/Oxford set of 196 independent nuclear families that previously showed parent-of-origin linkage and association with psychosis at the *LRRTM1* locus [Francks et al., 2003; DeLisi et al., 2002]. Whole blood DNA was available in sufficient quantity and quality for the present study from 99 subjects, from 41 unrelated sibships. The age range spanned from 12 to 50 years, with a median age of 34 years. We categorized subjects as affected when they were diagnosed with either schizophrenia or schizoaffective disorder. Diagnoses were made according to DSM-III-R criteria, on the basis of structured

interviews, review of medical records, and structured information obtained from at least one reliable family member [DeLisi et al., 2002]. The 41 sibships comprised of: 22 containing at least two affected siblings, 17 with one affected sibling and at least one unaffected sibling, and 2 with no affected subjects according to DSM-III-R. (The latter two sibships contained additional affected sibs, but sufficient DNA was unavailable from the affected members.) All individuals participating in this study gave written informed consent to the analysis of their DNA in studies of schizophrenia.

Selection of Genomic Regions for Methylation Analysis

We targeted the promoter region of *LRRTM1* for methylation analysis, as well as seven other genomic regions that would be informative from different perspectives. As supported above, we necessarily assumed (given the limited tissue availability) that methylation in whole blood DNA would be broadly representative of methylation in different body tissues [Auta et al., 2013; Davies et al., 2012]. In other words we did not have hypotheses regarding functional roles of DNA methylation specifically in whole blood, for any of these eight genomic loci. The precise genomic regions were defined against Human Genome version hg19 [Church et al., 2011] (UCSC: http://genome.ucsc.edu) using previously published findings and publicly available data as summarized here.

Chr2:80531693–80531944 which spans the *LRRTM1* promoter, located directly upstream of *LRRTM1* and including the CpG island there (Supplementary information S1). This was the primary target region for our study. (Unfortunately no common polymorphism is known to be located in this region, which might have facilitated allele-specific methylation studies; see Conclusions).

Chr2:80549616–80550005 which spans a CpG island roughly 20 kilobases (kb) upstream of *LRRTM1*, but is also located within the previously reported region of linkage with schizophrenia [Francks et al., 2007]. Its relative proximity to *LRRTM1*, and its location within the schizophrenia-linked region, made it a particularly useful negative control.

Chr2:79739807–79740072 which spans a CpG island located at the first exon of *CTNNA2*, which is 791 kb from the *LRRTM1* promoter. The *LRRTM1* locus lies within an intron of the larger, surrounding gene *CTNNA2* [Francks et al., 2003], which is also located within the previously reported region of linkage with schizophrenia [Francks et al., 2007]. Again, we included this region as a negative control.

Chr11:2019413–2019741 at the imprinting control center of *H19*, a strongly maternally imprinted gene [Zhang and Tycko, 1992]. We expected to measure roughly 50% methylation of this region, since one inherited copy is known to be strongly methylated while the other copy is non-methylated. We therefore included this as a methodological control.

Chr15:25200038–25200389 which spans a CpG island at the paternally imprinted *SNRPN* locus. Again, we expected to measure roughly 50% methylation of this region, and included it as a methodological control.

ChrX:153363027–153363239 at the *MeCP2* promoter [LaSalle, 2007]. Here we expected to measure different methylation levels in males and females, since *MeCP2* is located on chromosome X and is subject to X-inactivation in females, which is a mechanism linked to methylation [LaSalle, 2007]. This was therefore included as another methodological control.

Chr3:195809003–195809201 at *TFRC* promoter. This 'house-keeping' gene has been proposed as a negative control for analysis of gene expression data in schizophrenia [Silberberg et al., 2009]. We therefore included it as a negative control.

Chr7: 96650540–96650848 which is located at an intronic CpG island of *DLX5*, a transcriptional regulatory factor of *LRRTM1* [Sajan et al., 2011] which may also be imprinted [Okita et al., 2003; Schule et al., 2007], and has been implicated in psychiatric disorders [LaSalle, 2007]. There is no significant linkage of this genomic region to schizophrenia in the New York/Oxford siblings, and therefore we did not investigate the methylation of *DLX5* in relation to IBD sharing (see below). However, we were interested to test for a potential association of methylation at this locus with schizophrenia.

Quantifying Methylation by 454-Bisulfite Sequencing

Details on the 454 sequencing, quality control and methylation quantification are provided in supplementary methods S2. DNA methylation was considered only at CG dinucleotides. Non-CpG methylation was considered to represent the rate of incomplete bisulfite conversion (see supplementary methods S2), which was always less than 2% per sample (although genuine non-CpG methylation cannot be ruled out at a low level). For each subject, and for each of the eight genomic regions analyzed, we derived the average ratio of methylated to non-methylated CpGs. In addition, for higher resolution analysis of the *LRRTM1* promoter, and given highly variable but correlated methylation levels for individual, consecutive CG dinucleotides (supplementary data S3), we averaged the methylation ratios over each set of 10 consecutive CGs, applied as a sliding window, such that the first window spanned CG dinucleotides 1–10, the second window 2–11, and so on. Potentially confounding effects of age, sex, and parental age at birth were investigated and, when appropriate, corrected for (see supplementary methods S2).

Linkage of LRRTM1 IBD Sharing with Schizophrenia

Parent-of-origin affected-sib-pair multipoint linkage analysis with schizophrenia, within the 41 sibships available for this study, was performed using the sib-phase program of the ASPEX package [Hinds and Risch, 1996], which estimates paternal and maternal linkage significance levels empirically by permutation. We used existing genotype data from nine common SNPs (rs6718055, rs6743980, rs6733871, rs1446109, rs1007371, rs920818, rs718467, rs723524, and rs1025947) that span the *LRRTM1* locus and that were previously genotyped in these families (including in the parents of the siblings)[Francks et al., 2007]. As expected due to their close proximity in the genome, no recombination events were identified between these SNPs. The multipoint linkage LOD scores

were therefore identical for all nine SNPs. The basis of this linkage analysis was to test for an increase in IBD sharing in affected sibling pairs relative to the proportion expected by chance (and tested separately for the paternally inherited and maternally inherited chromosomes).

Linkage of LRRTM1 IBD Sharing with DNA Methylation

We randomly selected one pair of siblings from each of the 41 sibships to ensure statistical independence in subsequent pairs-based analysis. For each sibling pair, we estimated maternal and paternal IBD sharing across the *LRRTM1* locus using MERLIN [Abecasis et al., 2002], and using the same set of nine common SNPs as above. IBD sharing could not be unambiguously calculated for 6 of 41 sibling pairs, on the basis of the genotype data available, leaving 35 pairs for the analysis. We regressed the difference in sibling's methylation levels, first for the whole *LRRTM1* promoter and then for each 10-CG sliding window across it, simultaneously but separately on paternal and maternal identity-by-descent (IBD) sharing, using SPSS v.20 (IBM, New York). This was effectively a quantitative sib-pair linkage analysis, with methylation levels as the quantitative trait.

DNA Methylation Levels and Affection

For comparability across different analyses we used the same random selection of one pair of siblings from each of the 41 sibships as above. Six of these pairs were non-affected, fourteen pairs had one affected member, and twenty-one pairs had two affected members. We coded each pair with a binary 'pair-affection' score indicating whether or not both sibs were affected, i.e. we pooled the non-affected and discordant pairs to make two evenly sized groups. We regressed this variable on the average level of methylation of each pair, for each of the eight genomic regions studied, and for each 10-CG sliding window at *LRRTM1*, to test for an overall association of methylation levels with affection. Since our primary hypothesis regarded the *LRRTM1* promoter, we did not correct significance levels at *LRRTM1* for having tested at the other genomic regions. For *LRRTM1*, we also regressed the pair-affection score on the sibling difference in methylation, for each 10-CG sliding window, to pinpoint where in the *LRRTM1* promoter the affected pairs might be most similar in their methylation as compared to discordant and non-affected pairs. Significance testing for this was 1-tailed (SPSS v.20, IBM corporation©).

Allelic Association of LRRTM1 SNPs with Methylation

The nine common SNPs spanning *LRRTM1* were tested for allelic association with methylation levels using the QTDT package [Abecasis et al., 2000], that simultaneously models sib-pair linkage and parent-of-origin allelic association with quantitative traits. 'Total' tests of parent-of-origin association were used, for maximal power [Abecasis et al., 2000]. Again, for comparability across the

different analyses, the same random selection of one pair of siblings from each of the 41 sibships was used as above.

RESULTS

Linkage of LRRTM1 IBD Sharing with Schizophrenia

This analysis was run in order to confirm that the sibships available for this study were representative of the larger dataset from which they were drawn, as regards their linkage at 2p12. A significant increase in IBD sharing in affected sib pairs was detected at *LRRTM1*, LODpaternal = 0.99, $P = 0.016$; LODmaternal = 0.50, $P = 0.064$, that was consistent with the previously reported linkage analyses in the larger dataset [Francks et al., 2003]. (The LODpaternal was 4.72, $P = 0.0000016$, and LODmaternal = 0.6, $P = 0.048$, in the larger dataset, as was reported previously [Francks et al., 2003]).

Methylation Levels

Following quality control 126,099 sequence reads were assigned uniquely to their corresponding genomic regions. Descriptive statistics for the methylation levels for each genomic region analyzed, based on a minimal read depth of 20X, are given in Table I. The imprinted loci *H19* and *SNRPN* showed mean methylation levels close to 50%, consistent with strong imprinting (i.e. heavy methylation of one parentally inherited allele, but little to no methylation of the other allele). This served as a useful validation of our method for quantifying methylation. The six other genes showed profiles of methylation comparable to those recorded in publicly available epigenomic datasets in which whole blood DNA was analyzed (supplementary data S4)[Lister et al., 2009; Heyn et al., 2012; Song et al., 2013; Li et al., 2010; Gertz et al., 2011]. The *LRRTM1* promoter showed generally low levels of methylation while also showing inter-subject variation (Table I). The most heavily methylated 10-CG window at the *LRRTM1* promoter corresponded to CGs 04–13 (minimum methylation = 2%, maximum methylation 27%).

Linkage of Methylation with IBD Sharing

Methylation of the *LRRTM1* promoter was significantly linked to paternal IBD sharing at this locus ($\beta = -0.329$; $P = 0.029$). In other words, siblings who had more similar methylation levels tended to be sharing paternally IBD. This analysis did not require multiple testing correction since it was driven directly by our primary hypothesis, i.e. stemming from the paternal-specific IBD sharing at *LRRTM1* in these sib pairs. The sliding window analysis mapped this effect to the window CG06–CG15 (Fig. 1). Sibling differences in methylation, for this window, were significantly linked paternally after correction for multiple testing across all windows ($\beta = -0.410$; uncorrected $P = 0.0078$; corrected $P = 0.047$ Fig. 1 and supplementary data S5). No significant linkage was obtained with maternal IBD sharing, although there was a weak maternally-derived signal for CG06–CG15 (Fig. 1), and in combination the paternal and maternal sharing produced a stronger linkage (Fig. 1).

TABLE I. Descriptive Statistics of the Methylation Ratios for Each of the Eight CpG Islands

	Chr	Sequenced region	n-CG	n-Ind	Min.	Max.	Mean	S.D.
LRRTM1	2	80531693–80531944	29	99	0.027	0.273	0.050	0.028
20kb CpG Island	2	80549616–80550005	35	75	0.017	0.169	0.066	0.031
CTNNA2	2	79739807–79740072	39	82	0.051	0.232	0.112	0.045
DLX5	7	96650540–96650848	38	86	0.029	0.193	0.067	0.024
H19	11	2019413–2019741	22	73	0.279	0.719	0.502	0.084
MeCP2 (males)	X	153363027–153363239	37	53	0.038	0.606	0.308	0.150
(females)				31	0.300	0.758	0.522	0.128
SNRPN	15	25200038–25200389	26	62	0.214	0.788	0.473	0.108
TFRC	3	195809003–195809201	26	97	0.009	0.266	0.030	0.030

MeCP2 statistics are split by sex because of the gene's location on the X chromosome. N-CG: number of CGs in the sequenced region; n-Ind: number of individuals analyzed for the region.

Methylation and Affection

Reduced methylation of the *LRRTM1* promoter showed a significant association with affection status ($\beta = -0.416$, $P = 0.0038$). In sliding window analysis, the most significant association with affection was obtained for the window CG06–CG15 ($\beta = -0.455$; uncorrected $P = 0.0019$; corrected $P = 0.011$ Figures Fig. 2A and Fig. 3A), although the association also spanned broadly from CG01–CG24 (Fig. 2A). Among the seven additional genomic regions analyzed, only *DLX5* methylation showed a significant association with affection after statistical correction for multiple testing ($\beta = -0.595$, uncorrected $P = 0.00021$; corrected $P = 0.0017$). We discuss *DLX5* further below.

Affected sib pairs were more similar in their methylation at the *LRRTM1* promoter than discordant or unaffected pairs, over two partially overlapping regions from CG01 to CG17 and from CG12 to CG24 (Fig. 2B). The greatest significance was again for the window CG06–CG15 ($\beta = -0.507$; uncorrected $P = 0.00042$; corrected $P = 0.0025$; Figures Fig. 2B and Fig. 3B).

Allelic Association of LRRTM1 SNPs with Methylation

The nine common SNPs spanning *LRRTM1* showed no significant association with methylation levels of the *LRRTM1* promoter (all P-values > 0.05), indicating that the methylation state at this locus is largely or wholly independent of DNA sequence variation.

DISCUSSION

Gene expression studies have previously indicated variable and incomplete imprinting of *LRRTM1*, and specifically down-regulation of the maternally inherited gene [Francks et al., 2007]. The chromosome 2p12 region, where *LRRTM1* is located, was reported to be linked to psychosis in affected sibling pairs, such that IBD sharing of the paternally inherited locus was significantly elevated [Francks et al., 2007; Francks et al., 2003]. SNPs directly upstream of *LRRTM1* were also associated with schizophrenia in a paternal-specific manner, within the same set of sibling pairs [Francks et al., 2007; Francks et al., 2003]. However, the SNP association with schizophrenia was only relevant to a minority of the sibling pairs that showed an increase in paternal IBD sharing at this locus [Francks et al., 2007; Francks et al., 2003].

Here we hypothesized that the epigenetic state of the paternally inherited locus may be involved in disease susceptibility, independently of DNA sequence variation. Epigenetic variability could cause a paternal linkage in affected siblings if, for example, parent-of-origin-specific epigenetic marks are sometimes not correctly erased and re-established during spermatogenesis and early embryonic development [Francks et al., 2007; Francks et al., 2003], possibly due to environmental influences. In a subset of 99
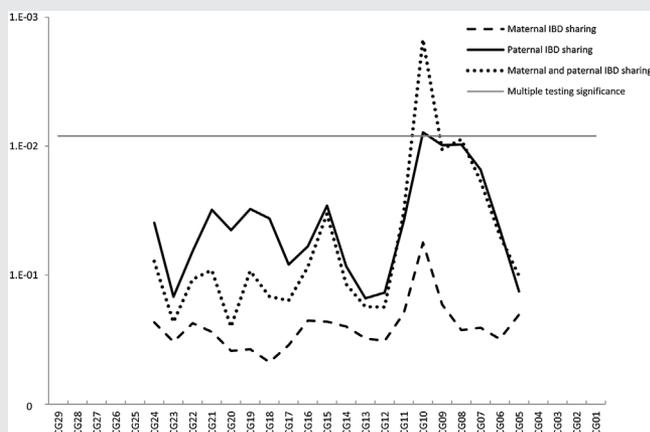

FIG. 1. Linkage of methylation to IBD sharing across the *LRRTM1* promoter. The positions of the CG loci on the x-axis correspond to their order on the Human Genome version hg19. The y-axis shows the negative logarithm (base 10) of the regression *P* value. The value is shown for the mid-point of each 10-CG sliding window. (For example, the value indicated for CG10 represents methylation averaged over CG06–CG15).
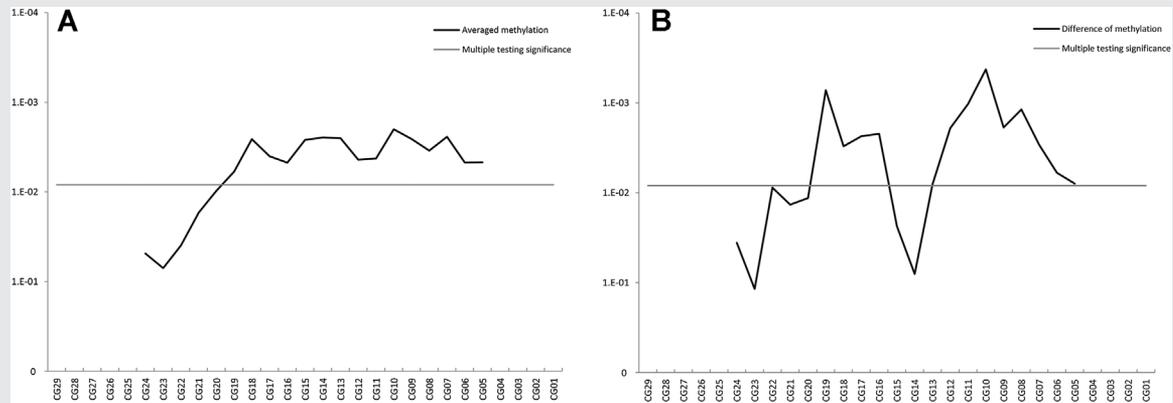
FIG. 2. A. Association across the *LRRTM1* promoter of sib-pair average methylation levels and affection with psychosis. The positions of the CG loci on the x-axis correspond to their order on the Human Genome version hg19. The y-axis shows the negative logarithm (base 10) of the regression *P*-value. The value is shown for the mid-point of each 10-CG sliding window. (For example, the value indicated for CG10 represents methylation averaged over CG06–CG15). B. Association across the *LRRTM1* promoter of sibling differences in methylation with psychosis, across the *LRRTM1* promoter. Affected pairs were more similar in methylation levels than discordant or unaffected pairs, at broadly two locations. Again, the y-axis shows the negative logarithm (base 10) of the regression *P*-value. The x-axis is the same as for A.

individuals from the same New York/Oxford dataset that previously showed linkage and association at this locus, we precisely measured DNA methylation across a CpG island located at the *LRRTM1* promoter, and also at seven additional CpG islands, using 454-bisulfite sequencing. Six of the additional CpG islands were included as negative or methodological controls, while a CpG island at *DLX5* was included for a secondary hypothesis (see below). Although we could not control our data for possibly relevant variables such as medication use or white cell concentration in blood, the profiles of methylation obtained for all of these targets

corresponded well with those reported in publicly available datasets derived from blood DNA (supplementary data S4)[Lister et al., 2009; Heyn et al., 2012; Song et al., 2013; Li et al., 2010; Gertz et al., 2011; Hodges et al., 2011].

We found that methylation at the *LRRTM1* promoter was decreased in sibling pairs affected with psychosis. In addition, the affected sibling pairs showed more similar levels of methylation than diagnostically discordant or unaffected pairs. When analyzed in a linkage context, the methylation was primarily related to paternal IBD sharing, with a trend also observed for maternal
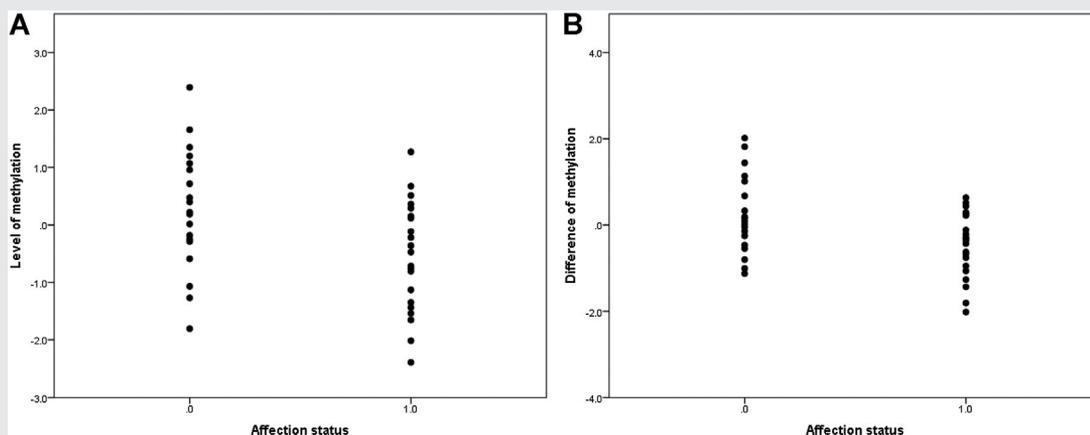

FIG. 3. A. Distribution of sibling pairs according to the averaged level of methylation for the window *LRRTM1* CG06–CG15 and the affection status. 0: discordant or non-affected sibling pair; 1: affected sibling pair. B. Distribution of sibling pairs according to the difference of methylation for the window *LRRTM1* CG06–CG15 and the affection status. 0: discordant or non-affected sibling pair; 1: affected sibling pair. The y-axes values are ranked normalised by Blom's method.

IBD sharing. These complementary ways of analyzing the data were not statistically independent, insofar as they all reflected the hypomethylation in affected pairs. However the linkage analysis was useful in illuminating the paternal bias of the effect, and localizing it primarily between CG06 and CG15.

Strikingly, the effects that we observed were not related to the genotype status of nine common SNPs spanning the *LRRTM1* locus, and which tag the major SNP variation at this locus [Francks et al., 2007]. This is consistent with a model in which the epigenetic state is not merely a secondary phenomenon that arises due to DNA sequence variation, and is consistent with a lack of reported association at this locus in SNP-based genome-wide association studies [Bergen and Petryshen, 2012; Ripke et al., 2013] (which also do not generally target familial forms of psychosis). Our data suggest that disrupted establishment of paternal methylation at *LRRTM1* is involved in the etiology of psychosis in the New York/Oxford siblings, over and above the previously reported, minor effects of SNP variation at this locus. This may possibly arise due to unknown environmental influences, random effects during gametogenesis and early development, or genetic influences elsewhere in the genome. Although this may indicate trans-generational transmission of the epigenetic state of the *LRRTM1* promoter, as several recent studies have established for diverse loci in mammals [Wei et al., 2014; Heijmans et al., 2008], more evidence is needed to establish such a mechanism at this locus.

The overall level of methylation at the *LRRTM1* promoter was lower than that of other known, strongly imprinted loci, including those that were measured here as methodological controls. This was broadly consistent with previous data that indicated variable and incomplete imprinting of *LRRTM1* expression [Francks et al., 2007]. Tissue-specificity of imprinting is also a possibility. The parental imprint of some loci, such as UBE3A, can be detected in only some tissues [Yamasaki et al., 2003]. DNA extracted from whole blood may not necessarily capture *LRRTM1* imprinting strongly, as this is primarily a gene with neuronal functions. However, publicly available data do not indicate a substantial difference between blood and neuronal tissue in methylation at the *LRRTM1* promoter, which supports the use of blood DNA for these analyses (UCSC: http://genome.ucsc.edu, [Gertz et al., 2011; Hodges et al., 2011; Zeng et al., 2012]). Nonetheless, further investigations are warranted using brain tissue samples corresponding to the pattern of *LRRTM1* expression, i.e. particularly the hippocampus, striatum, amygdala, and dorsal thalamus. It is also possible that the *LRRTM1* promoter is not itself an imprinting control center (ICC). The protein-coding regions of *LRRTM1* are also rich in CG dinucleotides, and could be targets for future methylation studies. Further studies would also benefit from using substantially longer sequence reads to simultaneously capture DNA methylation levels at the *LRRTM1* promoter together with a common SNP (the nearest common SNP is located roughly 250 bases from the promoter), which would help directly differentiate the maternal and the paternal alleles.

Our use of sibling pairs derived from the same New York/Oxford set that previously showed linkage and association at *LRRTM1* was a strength of our study, because schizophrenia and schizoaffective disorder are complex and heterogeneous disorders, and different research collections may differ widely in their underlying etiology.

Given the unusual strength of the affect-sib-pair linkage at this locus that was previously reported in these sib pairs (LOD = 4.72, [Francks et al., 2003]), the current study was sufficiently powered to detect an effect mediated by methylation that was hypothesized to drive this linkage, even if such an effect may be relatively unusual and specific to these families. However, our use of the New York/Oxford sibs also meant that we were limited to studying DNA extracted from blood, and also to a reduced number of subjects from whom sufficient DNA was still available from this valuable collection. To understand whether and how our results generalize to a broader patient population, future studies will need to incorporate more diverse approaches to patient recruitment, particularly targeting sporadic patients rather than families with multiple affected individuals, and also using an updated version of the DSM.

LRRTM1 was previously associated in a paternal-specific manner with handedness (relative hand skill) in a set of families affected by dyslexia [Francks et al., 2007]. Unfortunately the handedness data are sparse for the New York/Oxford siblings. Of the 99 subjects included in the present study, questionnaire data on handedness was available for thirty-five, out of which only five were left-handed, preventing reliable statistical analyses.

Of the eight genomic regions analyzed, only *LRRTM1* and *DLX5* showed evidence for an association of methylation levels with schizophrenia after statistical adjustment for multiple testing. The methylation levels at *LRRTM1* and *DLX5* were correlated in our dataset (r = 0.572). This was the strongest correlation of methylation between any pair of loci that we studied. Dlx5 is a transcription factor affecting Lrrtm1 expression [Sajan et al., 2011] and was previously reported to be imprinted [Okita et al., 2003; Schule et al., 2007] and implicated in psychiatric disorders [LaSalle, 2007], which is why we included it here for comparison with *LRRTM1*. Future studies may therefore benefit from a network level analysis of methylation, in which the *LRRTM1-DLX5* interaction can be investigated as a potential factor in the etiology of schizophrenia.

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