



Lack of association to a NRG1 missense polymorphism in schizophrenia or bipolar disorder in a Costa Rican population

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ABSTRACT

A missense polymorphism in the NRG1 gene, Val>Leu in exon 11, was reported to increase the risk of schizophrenia in selected families from the Central Valley region of Costa Rica (CVCR). The present study investigated the relationship between three NRG1 genetic variants, rs6994992, rs3924999, and Val>Leu missense polymorphism in exon 11, in cases and selected controls from an isolated population from the CVCR. Isolated populations can have less genetic heterogeneity and increase power to detect risk variants in candidate genes. Subjects with bipolar disorder (BD, n = 358), schizophrenia (SZ, n = 273), or unrelated controls (CO, n = 479) were genotyped for three NRG1 variants. The NRG1 promoter polymorphism (rs6994992) was related to altered expression of NRG1 Type IV in other studies. The expression of NRG1 type IV in the dorsolateral prefrontal cortex (DLPFC) and the effect of the rs6994992 genotype on expression were explored in a postmortem cohort of BD, SZ, major depressive disorder (MDD) cases, and controls. The missense polymorphism Val>Leu in exon 11 was not significantly associated with schizophrenia as previously reported in a family sample from this population, the minor allele frequency is 4%, thus our sample size is not large enough to detect an association. We observed however an association of rs6994992 with NRG1 type IV expression in DLPFC and a significantly decreased expression in MDD compared to controls. The present results while negative do not rule out a genetic association of these SNPs with BD and SZ in CVCR, perhaps due to small risk effects that we were unable to detect and potential intergenic epistasis. The previous genetic relationship between expression of a putative brain-specific isoform of NRG1 type IV and SNP variation was replicated in postmortem samples in our preliminary study.

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1. Introduction

Schizophrenia (SZ) is a debilitating brain disease characterized by delusions, hallucinations, decreased emotional affect, paranoia, and motor deficiencies (Liddle et al., 1994). Though the exact neurobiology of schizophrenia is not wholly understood, family, twin, and adoption studies have demonstrated that schizophrenia is a complex disease with a significant genetic component. First-degree biological relatives of patients with schizophrenia have an estimated 10% risk of developing

the disease, compared to 1% for the general population (Gottesman and Erlenmeyer-Kimling, 2001). In twin studies, concordance rates of 41%–65% have been seen in monozygotic twins compared to 0%–28% in dizygotic schizophrenic twins, suggesting heritability as high as 85% (Tsuang et al., 2001). In light of this evidence, much effort has been directed toward the discovery of genes that increase the risk of schizophrenia.

Schizophrenia linkage to chromosome 8p has been identified in multiple studies (Pulver et al., 1995; Levinson et al., 1996; Kaufmann et al., 1998; Shaw et al., 1998; DeLisi et al., 2002). Neuregulin 1 (NRG1) is located at 8p12 and is involved in neurodevelopment, regulation of glutamate, and synaptic plasticity (Tosato et al., 2005). Stefansson et al (Stefansson et al., 2002) first reported an association

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between NRG1 and schizophrenia in an Icelandic population via a haplotype (HAP_{ICE}) consisting of five SNPs (SNP8NRG221132, SNP8NRG221533, SNP8NRG241930, SNP8NRG243177, and SNP8NRG433E1006) and two microsatellite markers (478B14-848 and 420M91395) located at the 5' end of the gene that doubled the risk for the disorder. Since this initial report, confirmatory studies of NRG1 association have been reported in different populations in Scotland (Stefansson et al., 2003), China (Li et al., 2004), Hungary (Keri et al., 2009), Japan (Fukui et al., 2006), Sweden (Alaerts et al., 2009), and a second Scottish cohort (Thomson et al., 2007), though the associated haplotype varies between studies. Negative studies of association have also been reported in Japan (Iwata et al., 2004; Ikeda et al., 2008), Ireland (Thiselton et al., 2004), Denmark (Ingason et al., 2006), Spain (Rosa et al., 2007), and the United States (Crowley et al., 2008).

Variation in NRG1 has been found to be associated with various biological indices that are known to underlie schizophrenia. For example, brain imaging measurements of the anterior cingulate (Wang et al., 2009), prefrontal functioning (Mechelli et al., 2008; Mechelli et al., 2009), overall white matter integrity (Zuliani et al., 2011), excitatory synapse development (Ting et al., 2011), GABA interneuron dysfunction (Ting et al., 2011), and immune system dysregulation (Marballi et al., 2010; Shibuya et al., 2010) have been linked to genetic variation in NRG1. These observations contribute to the validity of the association of NRG1 with schizophrenia and its likelihood to contribute to the underlining basis for the development of this illness.

A novel missense mutation present in NRG1 (Val>Leu in exon 11) that increased the risk of schizophrenia in individuals from Costa Rica was reported in a family based association analysis (Walss-Bass et al., 2006a). Our research group has been independently studying the genetics of schizophrenia in families and unrelated individuals from the same region of the Central Valley of Costa Rica (CVCR) (DeLisi et al., 2002; Cooper-Casey et al., 2005; Bertisch et al., 2009); therefore we attempted to replicate the missense association with the Val>Leu polymorphism in exon 11 in this study. We do not believe that we have any overlap in samples with those reported in (Walss-Bass et al., 2006a), although we have not made a formal comparison due to potential IRB ramifications. We also selected rs3924999 for analysis as it has been linked to lower prepulse inhibition, an endophenotype of schizophrenia (Hong et al., 2008), and for its association with schizophrenia in a Chinese Han cohort (Zhang et al., 2009). In addition rs6994992 was selected based on its inclusion in the HAP_{ICE} risk haplotype (Stefansson et al., 2002), and based on evidence that the T/T genotype is associated with decreased activation of frontal and temporal lobe regions and increased risk of psychosis (Hall et al., 2006). This SNP was also selected for investigation for its function in promoting expression of the NRG1 type IV transcript in postmortem tissue (Law et al., 2006); (Shamir and Buonanno, 2010). Therefore, we studied the expression of NRG1 type IV in postmortem brain sample and association with genotypes of rs6994992. Though there is NRG1 intragenic epistasis between 5' and 3' markers (Nicodemus et al., 2010) in functional imaging studies, our group selected the present markers to attempt replication of implicated SNPs that were also functionally relevant to the CVCR collection. The SNPs chosen for this study were selected primarily based on conclusions published in the literature when designing the study. As associations between schizophrenia and the HAP_{ICE} haplotype have been both supported and refuted across varying populations, we were interested in adding the CVCR results to the pool of association data. rs6994992 was also selected based on reports of its function in promoting expression of the NRG1 type IV transcript in postmortem tissue. Finally, cost of the materials needed to test a wider range of markers was also a factor in determining which SNPs to investigate. As this was a preliminary association study to test our samples with these three implicated SNPs, the present data can be added to meta-analysis using SNPs in NRG1.

In addition to schizophrenia and control subjects, we have included bipolar subjects in the genotyping and brain gene expression as well. Bipolar disorder (BD) illness affects approximately 0.8–1.6% of the population (Kessler et al., 1997) and is characterized by cyclical episodes of mania and depression, with a return to normal state between episodes (Berns and Nemeroff, 2003). There is a significant genetic component to BD based upon twin studies; BD has an estimated heritability as high as 93% (Kieseppa et al., 2004). Though schizophrenia and bipolar disorder have been historically categorized as divergent psychopathologies, there is a growing body of evidence suggesting that causative commonalities exist between the two disorders (Badner and Gershon, 2002; Berrettini, 2003). To date, multiple studies have shown schizophrenia implicated loci and genes having a positive association to BD (Harrison and Weinberger, 2005; Craddock et al., 2006), including 8p12 (Park et al., 2004) and NRG1 (Green et al., 2005; Prata et al., 2009). NRG1 in particular has also been shown to possibly play a role in bipolar psychopathology, pointing to a common involvement of this cell–cell interaction and growth involved protein in both bipolar disorder and schizophrenia (Thomson et al., 2007; Georgieva et al., 2008).

A population sample from the central valley of Costa Rica was chosen for this study due to its geographical isolation and genetic homogeneity. The Costa Rican genome is comprised of largely European and Amerindian ancestry (Morera et al., 2003), as a result of six waves of Spanish colonization and admixture with the indigenous Amerindians (DeLisi et al., 2001). It is estimated that fewer than 1000 families gave rise to the three million residents of the CVCR (Mathews et al., 2004), and due to mountainous boundaries and dense lowland jungle, the CVCR population has remained isolated from immigration and emigration for 500 years. Consequently, the CVCR region presents a homogenous population perfect for studying genes underlying complex genetic disorders. Chromosomal areas of interest have already been identified in this cohort for both schizophrenia (DeLisi et al., 2002; Cooper-Casey et al., 2005; Walss-Bass et al., 2006b) and bipolar disorder (Freimer et al., 1996).

While large association studies have shown the relative value of identifying common variants that contribute statistically significant associations, there are usually small relative risks for disease attributable to an individual SNP (e.g. Purcell et al., 2009; Ruderfer et al., 2011). However, family based studies in relatively isolated populations can offer knowledge about regions of interest that might contain rare variants in pathways that have etiologic relevance to schizophrenia. Thus, the CVCR collection offers a resource for exploring the effects of genes that have been largely implicated in multiple studies, and perhaps can increase the association signal by increased genetic homogeneity that is lacking in larger association studies. Thus, as sample sizes for schizophrenia and bipolar disorder are projected to be larger in the future, the relative risks attributable to a single variant will most likely be decreasing due to genetic heterogeneity. With a modest sample size pursued in the present study, there is adequate power in pursuing biologically based genes on an a priori basis. The purpose of this study is to examine NRG1 SNPs previously associated with SZ in a geographically isolated, relatively homogenous population from the CVCR.

2. Materials and methods

2.1. Sample collection

Subjects were recruited with the approval of the Ministry of Health of Costa Rica and the ethics committee for the Hospital Nacional Psiquiatrico and by the Institutional Review Board at the University of California at Irvine. Unrelated individuals with diagnoses of schizophrenia (n = 273) and bipolar disorder (n = 358) whose four grandparents were of Spanish descent were obtained by screening patients admitted to the National Psychiatric Hospital of Costa Rica as previously described (DeLisi et al., 2001; DeLisi et al., 2002). Control subjects (n = 479) with the same ancestral criteria were recruited from large companies via questionnaires

administered by the companies' health services department. Controls were selected if no family history of schizophrenia, bipolar disorder, suicide or hospitalization for psychiatric reasons were present, and if self-reports of psychosis, diagnosis of schizophrenia, bipolar disorder, use of medications for depression or psychiatric conditions, and suicide attempts were negative. Interviews of affected subjects were conducted with the Diagnostic Interview for Genetic Studies (Nurnberger et al., 1994) using a translated Spanish DIGS 2 (DeLisi et al., 2001) and diagnoses were based upon DSM-IV criteria (Association, 1994) by a consensus of two independent local psychiatrists. A third independent psychiatrist made a final diagnosis, based on family history, medical records, and a summary of personal interviews. All participants gave written informed consent for participation.

2.2. DNA extraction

Blood samples were collected from participants in Costa Rica and sent to the laboratory at UC Irvine. DNA was isolated from these samples via 10% SDS and Proteinase K digestion, phenol–chloroform extraction, followed by a sodium acetate precipitation (Bell et al., 1981). Purity and concentration were assessed by 260 nm and 280 nm absorbances on the SpectraMax Plus spectrophotometer (Molecular Devices, Sunnyvale, CA) and aliquots were diluted to a working concentration of 2.5 ng/μl.

2.3. Genotyping

DNA samples were genotyped for NRG1 SNPs rs3994992 and rs6994992 using pre-validated TaqMan 5'-allele discrimination assays (Applied Biosystems, Foster City, CA). The third polymorphism G/T in exon 11 (Val>Leu) was genotyped with a custom TaqMan assay using the following reference sequence in the Walss-Bass study (Walss-Bass et al., 2006a): GAACATGGACAATGTATGCAGCATGCCACTGTTGGTTG-TAGTCAGTCTGGCAAGTGGAAAGTGACCTGTGATGACATCTGCTCT-CATCCCTTCCAGAGCGGAGGAGCTGTACCAGAAGAAGTCTGACCA-TAACCGGCATCTGCATCGCCCTCCTTGTGGTCCGCATCATGTGT[G/T] TGGTGGCTACTGCAAAACCAAGTAAACCTTCTTTCTC-CATGCCTTCTCTCCTTCATGCAGAGACAGCTTAGATGCCAGGCTTTG-CAGAATCTGAGCTCCACAGCCTAGTCTTGGGG. The Walss-Bass assay was performed on an ABI Prism 7000 Sequence Detection system, in a total reaction of 25 μl (4 μl DNA, 12.5 μl TaqMan Universal PCR Master Mix, No AmpErase UNG, 1.25 μl 40× TaqMan assay, 7.25 μl H₂O) using the following amplification conditions: denaturation at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and at 55 °C for 1 min. Individual PCR reactions for rs3924999 and rs6994992 were carried out on an ABI Prism 7900 Sequence Detection system in a total reaction of 12 μl (4 μl DNA, 5 μl TaqMan Universal PCR Master Mix, No AmpErase UNG, 0.5 μl 40× TaqMan assay, 2.5 μl H₂O) using the following amplification protocol: denaturation at 95 °C for 10 min, followed by 50 cycles at 92 °C for 15 s and at 58 °C for 1.5 min. The genotype of each sample was determined by measuring allelic-specific fluorescence using SDS 2.3 software for allelic discrimination (Applied Biosystems).

Genotype and allelic statistical analyses were performed using Yates corrected χ^2 for continuity and Fisher's Exact Test for analyses that contained low cell numbers. Deviation from Hardy–Weinberg equilibrium (HWE) was tested using on line calculator (<http://ihg.gsf.de/cgi-bin/hw/hwa1.pl>).

2.4. Brain gene expression

Brain samples were obtained at the UC Irvine/UC Davis Brain Repository through a uniform process approved by the Institutional Review Board. Postmortem diagnoses were made through an extensive review of multiple sources of information including the medical examiner's conclusions, coroner's investigation, medical and psychiatric records, toxicology results, interviews of the decedents' next-of-kin and a neuropathological examination as previously

Table 1

The number of subjects for expression of NRG1 type IV isoform by genotype shown in Fig. 1. There were no subjects with SZ that are homozygous for the T allele, so those subjects were not included in the analysis.

Diagnosis * rs6994992 genotype	C/C	C/T	T/T	Total
BP	2	5	3	10
Control	8	11	3	22
MD	4	6	3	13
Total	14	22	9	45

described (Tomita et al., 2004; Vawter et al., 2006). The human brain dissection and freezing protocol were performed as previously described (Jones et al., 1992; Vawter et al., 2006) and brains were stored in –80 °C freezers until further dissected. RNA was extracted from 100 mg samples of dissected brain using a standard Trizol (Life Technologies, Carlsbad, California) procedure. Integrity of total RNA was evaluated via 18S and 28S ratios and RNA integrity numbers (RIN) using the 2100 Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA). Only samples with pH values higher than 6.5 were included in the analysis. cDNA was synthesized with oligo dT primers using the Superscript procedure (Invitrogen, Carlsbad CA). Expression levels of NRG1 IV were interrogated by TaqMan expression assay (ABI) using the same primer and probe sequence provided in the Law et al. study in which differences in gene expression for this subtype were observed (Law et al., 2006): forward 5'GCTCCGGCAGCAGCAT3'; reverse 5'GAACCTGCAGCCGATTCT3'; internal Probe 5' FAM ACCA-CAGCCTTGCCCT-MGB-3'. Since the NRG1 E187 exon has high homology with other genes (Steinthorsdottir et al., 2004), a TaqMan probe provided specificity for amplification of the NRG1 gene as the probe spanned the junction of the E187–Igl1 exons (Law et al., 2006).

After amplification, the PCR product was run on a 2% agarose gel, the amplicon's specificity was confirmed by sequencing using the same primers used in the TaqMan assay. We compared the NRG1 type IV TaqMan expression assay results for diagnoses groups (Table 1) to controls by an analysis of covariance taking into account age, sex, and RNA quality. The TaqMan expression was normalized with GAPDH. Brain gene expression levels were compared in bipolar disorder (n = 10), control (n = 22), MDD (n = 13) for a total of 45 postmortem subjects that had genotypes for NRG1rs6994992.

3. Results

3.1. Association testing of polymorphisms

The genotype counts for each SNP are shown in Table 2. The recessive and dominant association tests for three NRG1 SNPs

Table 2

Genotype frequencies for three SNPs in association study of CVCR subjects with bipolar disorder (BD) and schizophrenia (SZ).

NRG1 Exon 11 (Val>Leu)	BD	SZ	Control	Total
G/G	327	256	446	1029
G/T	28	17	33	78
T/T	3	0	0	3
Total	358	273	479	1110
rs6994992				
C/C	149	116	201	466
C/T	159	119	218	496
T/T	50	38	60	148
Total	358	273	479	1110
rs3924999				
A/A	40	27	62	129
A/G	159	132	209	500
G/G	159	114	208	481
Total	358	273	479	1110

genotyped were not significant with either BD or SZ (Table 3). There was a trend for SNP (Val>Leu exon 11) in BD cases only to not be in Hardy–Weinberg equilibrium (nominal $p=0.043$).

3.2. Expression of NRG1 Type IV in brain

Two brain regions (hippocampus and DLPFC) were analyzed for expression differences of NRG1 Type IV. For the hippocampus results, there were no statistically significant differences in expression for SZ, BD, or MDD cases compared to controls; however, in the DLPFC, the MDD cases ($n=12$) showed decreased expression ($p=0.004$) compared to controls ($n=22$) when pH, age, RIN, and gender are included in the ANCOVA model. The RIN factor was significant ($p=0.05$). The hippocampus showed very low levels of amplification indicating low expression of intact poly-adenylated mRNA.

We next investigated the rs6994992 genotype effect on NRG1 type IV expression in hippocampus and DLPFC. The genotype effect was significant ($p=0.040$) only in DLPFC; however there were no homozygous T carriers in the SZ group. We did confirm that NRG1 type IV expression was increased in the TT compared to the CC group in the DLPFC and that the effect was significant in the direction previously reported as the TT genotype showed a significantly increased expression ($p=0.024$) of 15.3 fold compared to CC genotype (Fig. 1). The CT group also showed a significant difference compared to the TT group ($p=0.023$); again, the direction supported the dominant effect of the T allele increasing expression of the NRG1 type IV expression. The CT group did not show a significant difference from the CC group ($p=0.83$). Interestingly, the MDD subjects showed a decreased expression compared to controls for both the CC and CT genotype group comparisons ($p=0.009$, $p=0.018$), the TT genotypes were not different comparing expression of NRG1 type IV between MDD and controls ($p=0.21$).

4. Discussion

Within the isolated CVCR population, this study failed to find an association with schizophrenia or bipolar disorder testing three NRG1 SNPs. Thus while our study had 80%–86% power for SZ and BD, respectively to find association for two of the more common SNPs (Skol

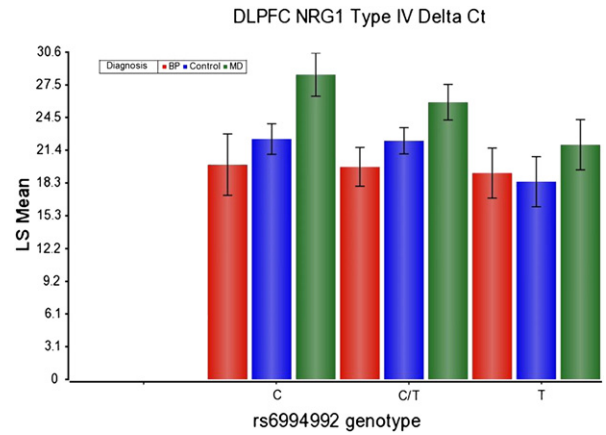


Fig. 1. The delta Ct values for NRG1 type IV expression in DLPFC by genotype and diagnosis were analyzed with an ANCOVA. The Diagnosis main effect was significant ($p=0.0015$), Diagnosis \times Genotype was not significant (p -value = 0.68). The Diagnosis main effect was largely due to a decrease in MDD DLPFC NRG1Type IV expression reduction of 33.5 fold compared to controls ($p=0.002$) while BD was not changed significantly in DLPFC ($p=0.51$). The Genotype effect was significant ($p=0.040$). The TT genotype showed a significantly increased expression ($p=0.024$) of 15.3 fold compared to CC genotype. Since schizophrenia subjects did not have any TT genotypes, they were omitted from this analysis. The LS Mean (y-axis) is the delta Ct values adjusted for age, RIN, and sex. The bar graph is an inverse to the relative amount of the NRG1 type IV expression, higher bar indicates lower expression since the figure uses Ct values. The levels have been normalized to the endogenous reference gene GAPDH.

et al., 2007) with risk ratio of ≥ 1.37 , it was not adequately powered for the rarer exon 11 missense SNP. This minor allele frequency requires a larger sample to definitively test for association. Though there are positive reports for NRG1 as both a schizophrenia and bipolar susceptibility gene for one or more of these SNPs, this hypothesis has not been consistently proven in the literature as shown in the introduction. Although we tested the same missense mutation previously associated in a family sample in CVCR (Walss-Bass et al., 2006a), we did not replicate these findings in a larger case – control analysis from the same CVCR population, perhaps due to this rare SNP frequency of 4%.

Additionally, we confirmed a prior report of an association between higher expression of NRG1 type IV and the rs6994992 T/T genotype in the DLPFC as previously reported (Law et al., 2006) (Shamir and Buonanno, 2010). We also report the preliminary finding of decreased NRG1 type IV expression in MDD in the DLPFC. Although previous studies have not found an association between NRG1 SNPs and MDD in a large sample of European ancestry (Schosser et al., 2010), there was decreased NRG1-alpha protein in MDD and SZ in the prefrontal cortex (Bertram et al., 2007). Since NRG1 has strong pleiotropic effects related to growth factor signaling in the brain, the findings of decreased NRG1 expression in prefrontal cortex could lend support to the overall growth factor hypothesis of depression (Evans et al., 2004).

Two caveats to the postmortem findings are that we cannot rule out the effect of antidepressant medications without animal studies as a potential cause of this decrease in MDD. The decreased expression in DLPFC of NRG1 type IV in MDD requires additional postmortem studies in part based upon small sample size, for validation, but is consistent with a reported decrease of NRG1 protein in MDD frontal cortex. In conclusion, while the present findings do not support association of the NRG1 variants in the CVCR population with schizophrenia or bipolar disorder, the tested SNPs might have some intergenic epistasis or be of a smaller effect size that we do not have power to detect. The present findings continue to show that there is a robust effect of genetic variation on NRG1 type IV expression which is thought to be brain-specific and with multiple impacts on brain function.

Table 3
The NRG1 association results for BD and SZ were not significant for the study of CVCR subjects.

Model	SNP	Disorder			
	NRG1 Exon 11	BD		SZ	
Recessive	rs6994992	Yates Chi-Square/p	Pearson Chi-Square/p	Yates Chi-Square/p	Pearson Chi-Square/p
		0.68/0.40	0.91/0.34	0.04/0.84	0.12/0.72
Dominant	rs6994992	Fisher exact p		Fisher exact p	
		0.077		1	
Recessive	rs3924999	Yates Chi-Square/p	Pearson Chi-Square/p	Yates Chi-Square/p	Pearson Chi-Square/p
		0/1	0.01/0.92	0/1	0.02/0.88
Dominant	rs3924999	Yates Chi-Square/p	Pearson Chi-Square/p	Yates Chi-Square/p	Pearson Chi-Square/p
		.26/.61	.37/.54	.19/.66	.3/.58
Recessive	rs3924999	Yates Chi-Square/p	Pearson Chi-Square/p	Yates Chi-Square/p	Pearson Chi-Square/p
		.45/.50	.6/.43	1.28/0.25	1.55/0.21
Dominant	rs3924999	Yates Chi-Square/p	Pearson Chi-Square/p	Yates Chi-Square/p	Pearson Chi-Square/p
		0.05/.82	.08/.77	.13/.71	.2/.65

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Contributors

Authors EM, BR, WEB, LED, WB, and MPV conceived and designed the study. EM, BR, LED, WB, and MPV carried out the computational analyses and candidate gene selection. EM, BR, AM, LED conducted subject screening. EM, BR, carried out the genotyping. EM and MPV performed the statistical analysis of the genotyping and expression data. RMM, HA, SJW, JB, EG, AS, WEB provided the guidance and additional support on this project. EM, BR, WEB, LED, WB, and MPV wrote the first draft of the paper, all authors revised the current paper. LED, AM, and WB recruited, diagnosed, and gathered patients and controls. BR, EM, LED, AM, WB, MPV contributed to the collection and preparation of control DNA samples.

Conflict of interest

All authors have no conflicts of interest to declare.

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