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SHORT REPORT

Allele-specific regulation of *DISC1* expression by miR-135b-5p

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***Disrupted-in-schizophrenia-1* (*DISC1*) gene has been established as a risk factor for various neuropsychiatric phenotypes. Both coding and regulatory variants in *DISC1* have been identified and associated with these phenotypes in genetic studies. MicroRNAs (miRNAs) are important regulators of protein coding genes. Since the miRNA-mRNA target recognition mechanism is vulnerable to disruption by DNA polymorphisms, we investigated whether polymorphisms in the *DISC1* 3′UTR affect binding of miRNAs and lead to allele-specific regulation of *DISC1*. We identified four predicted polymorphic miRNA target sites in the *DISC1* 3′UTR, and demonstrated that miR-135b-5p regulates the level of *DISC1* mRNA. Moreover, *DISC1* regulation by miR-135b-5p is allele specific: miR-135b-5p only binds to the major allele (A) of rs11122396, not to the minor allele (G). Thus, the G allele may be functionally related to the *DISC1*-associated phenotypes by abolishing regulation by miR-135b-5p, leading to elevated *DISC1* levels.**

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INTRODUCTION

Genetic variants of the *Disrupted-in-schizophrenia-1* (*DISC1*) gene have been associated with various neuropsychiatric phenotypes.[1] The initially identified translocation[2] reduces *DISC1* expression to half normal levels, suggesting haploinsufficiency as the mechanism of disease susceptibility in translocation carriers.[3] In addition, expression quantitative trait loci affecting the expression of DISC1 have been identified[4,5] and shown to associate with age of onset in recurrent major depression.[6] We hypothesized that differential regulation of *DISC1* expression may contribute to the wide range of *DISC1*-associated disorders.

MicroRNAs (miRNAs) are small non-coding RNA molecules that repress gene expression by binding to their target mRNAs. There are no previous reports on miRNA regulation of *DISC1*, although in general miRNAs regulate about half of human genes. We were especially interested in putative polymorphic miRNA target sites in the *DISC1* 3′UTR that could lead to differential regulation of *DISC1*.

MATERIALS AND METHODS

See Supplementary Methods.

RESULTS

We identified four SNPs (rs11122396, rs980989, rs9308481, and rs11803088) within putative binding sites of nine human miRNAs (miR-23a-3p, miR-23b-3p, miR-130a-5p, miR-135a-5p, miR-135b-5p, miR-323-3p, miR-409-3p, miR-548c-3p, and miR-559) (Table 1) through bioinformatic target prediction (see Supplementary Methods). We first tested the effect of each of these miRNAs on endogenous *DISC1* expression *in vitro*. We transfected a commercial miRNA precursor of each miRNA, a negative control miRNA, or a positive control siRNA, into HEK293FT cells and measured *DISC1* mRNA levels by qRT-PCR. We found that two of the nine miRNAs, miR-135b-5p and miR-559, significantly reduced *DISC1* mRNA expression: miR-559 by 23.7% ($P=0.009$) and miR-135b-5p by 16.2% ($P=0.039$), compared with the negative control miRNA (Figure 1). The endogenous expression of miR-135b-5p and miR-559 in HEK293FT cells was found to be low or undetectable, respectively (data not shown).

To determine whether these miRNAs regulate *DISC1* levels by targeting the predicted sites, we used a reporter gene assay, which uses luciferase activity on a protein level as the output measure. We cloned either the full-length *DISC1* Lv isoform 3′UTR (4.4 kb) or the miRNA binding sites with flanking sequences ($\sim$60 nt) into a pmirGLO dual luciferase expression vector. These constructs were co-transfected individually into HEK293FT cells with either the miR-135b-5p or the miR-559 precursor, or with a positive control siRNA or a negative control miRNA. We found that expression from the *DISC1* full-length 3′UTR construct was reduced 32.1% ($P=0.003$) by miR-135b-5p, and by 10.3%, ($P=0.03$) from the construct expressing the 60-nt miRNA binding site and flanking sequences (Figure 2a). The specificity of the repression of *DISC1* expression by miR-135b-5p was further established by demonstrating that co-transfection of miR-135b-5p with anti-miR miRNA inhibitor against both endogenous miR-135b-5p and its miRNA precursor completely abolished the

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Table 1 Predicted polymorphic miRNA target sites of *DISC1*

SNP	*Position*[a]	*MAF CEU*	*Octamer ('seed')*[b]	*Effect*	*SNP allele*	*Targeting miRNA (stem loop)*	*Stem loop location*	*Mature miRNA*
rs11122396	230241891	0.042	caAGCC[A]Taactc	C	A*	miR-135a-1	3p21	hsa-miR-135a-5p
				C		miR-135a-2	12q23.1	
				C		miR-135b	1q32	hsa-miR-135b-5p
					G (ancestral)	*None*		
rs980989	230242818	0.217	tttACA[T]TCAtat	C	T	miR-409	13q32.3	hsa-miR-409-3p
					G* (ancestral)	*None*		
rs9308481	230242929	0.225	taaAAT[G]TGAagt	N	G* (ancestral)	miR-323	14q32	hsa-miR-323-3p
			aaaAT[G]TGAAgt	N		miR-23a	19p13	hsa-miR-23a-3p
				N		miR-23b	9q22.3	hsa-miR-23b-3p
			AAT[G]TGAA	N		miR-130a	11q12	hsa-miR-130a-5p
					A	*None*		
rs11803088	230243392	0.069	cttTTA[C]TTTtaa	N	C* (ancestral)	miR-559	2p21	hsa-miR-559
			cttttA[T]TTTTAa	D	T	miR-548c	12q14.2	hsa-miR-548c-3p

Predictions from Patrocles and PolymiRTS are shown, with miRNAs and SNPs predicted by both programs underlined. The SNP alleles of the wild-type *DISC1 Lv* construct are indicated with an asterisk (*). The effect of the SNP on the target site is denoted as follows: C = derived allele creates a novel miRNA binding site, N = derived allele disrupts a non-conservative miRNA binding site, D = disrupts a conservative miRNA binding site. MAF = minor allele frequency; CEU = HapMap CEPH population.
[a]RefSeq build 36.3.
[b]Genomic sequence 5′ to 3′.

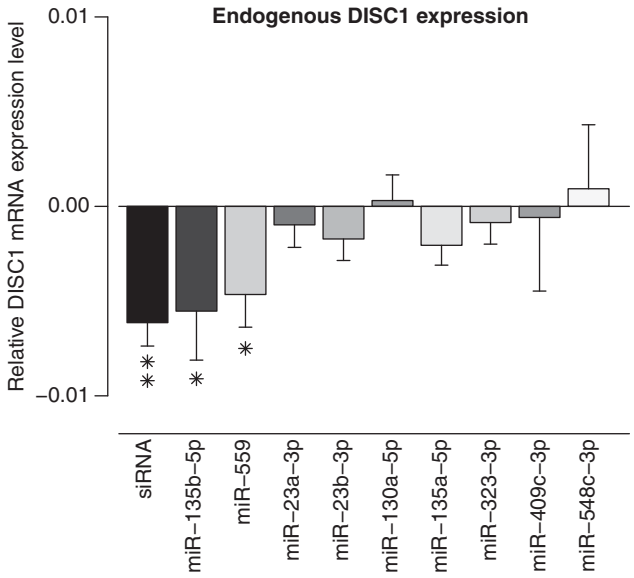

Figure 1 The effect of overexpression of nine miRNAs on endogenous *DISC1* mRNA levels. MiR-135b-5p, miR-559, miR-23a-3p, miR-23b-3p, miR-130a-5p, miR-135a-5p, miR-323-3p, miR-409-3p, and miR-548c-3p were overexpressed in HEK293FT cells and relative levels of endogenous *DISC1* and the housekeeping gene, *GAPDH*, measured using qRT-PCR. We compared the *DISC1* expression levels after miRNA transfection with the *DISC1* expression levels after transfection of a negative control miRNA. We performed three independent experiments and carried out linear regression analysis separately for each batch. We then used the obtained residuals in subsequent linear regression analysis including all the batches to obtain a *P*-value for all three experiments combined, taking the batch effect into account. Out of the nine miRNAs tested, miR-135b-5p and miR-559 reduced *DISC1* mRNA levels significantly. An siRNA against *DISC1* was used as a positive control. Results are expressed as the regression coefficient ± standard error. $^{*}P<0.05$, $^{**}P<0.005$.

repression of *DISC1* expression (Figure 2a). Altogether, these findings establish that miR-135b-5p regulates *DISC1* mRNA expression by targeting the predicted site in the *DISC1* 3′UTR. In contrast, miR-599 overexpression did not affect the expression from the *DISC1* full-length 3′UTR construct or the construct with its 60 nt binding site (Figure 2b). Likewise, none of the other seven miRNAs (miR-23a-3p, miR-23b-3p, miR-130a-5p, miR-135a-5p, miR-323-3p, miR-409-3p, and miR-548c-3p) with predicted binding sites in the *DISC1* 3′UTR were found to have any effect on expression from the full-length *DISC1* 3′UTR reporter construct (Figure 2c).

As polymorphisms may disrupt miRNA binding sites, leading to predisposition to disease phenotypes,[7,8] we next investigated the putative allele specificity of miR-135b-5p binding. The HEK293FT cell line is homozygous for the derived allele A of rs11122396, predicted to create a novel binding site for miR-135b-5p (Table 1). We created constructs with the *DISC1* G allele at rs11122396, to determine whether the repression of *DISC1* expression by miR-135b-5p is allele specific. Strikingly, miR-135b-5p had no effect either on the luciferase activity of the full-length 3′UTR with G at rs11122396 ($P=0.49$), or on the construct with the ~60-nt miRNA binding site with flanking sequences insert ($P=0.18$) (Figure 2a), indicating that miR-135b-5p binding is specific to the derived A allele at rs11122396 of *DISC1*. Thus, the G allele may be functionally related to the *DISC1*-associated phenotypes by abolishing regulation by miR-135b-5p, leading to elevated *DISC1* levels.

DISCUSSION

The rs11122396 G allele of *DISC1*, which we have shown here to disrupt miR-135b-5p binding, has a frequency of 4.4% in the European population,[9] raising the possibility that differential miRNA binding caused by DNA sequence polymorphisms may be a relatively common source of variation of gene expression in humans. The major allele A of rs11122396 is the derived allele that introduces a miRNA-135b-5p binding site, implying that the ancestral G allele may be a risk allele that abolishes miRNA regulation. Unlike in Mendelian diseases where the risk allele is usually the derived allele, in common diseases the ancestral allele sometimes functions as a risk allele under modern environmental conditions (see Di Rienzo and Hudson[10]), for example in the case of the ε4 allele of *APOE* in coronary artery disease[11] and Alzheimer's disease.[12]

Based on the publicly available BrainSpan Atlas mRNA and miRNA sequencing data (www.brainspan.org), miR-135b-5p and *DISC1* are co-expressed in the human brain with the highest levels in the cerebellar cortex and mediodorsal nucleus of thalamus. These brain regions are involved in the regulation of cognitive and affective processes disturbed in neuropsychiatric diseases.[13,14] Thus far, up to

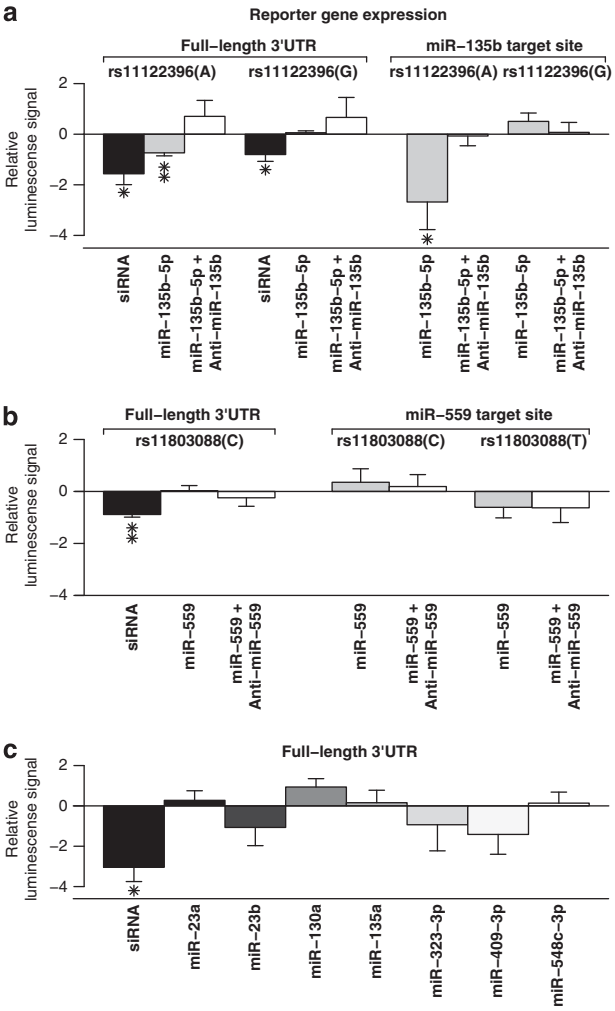

Figure 2 The effect of overexpression of miRNAs on reporter gene activity. (**a**) We measured relative levels of luciferase activity (Firefly/Renilla ratio) after transfecting HEK293FT cells with either the full-length 3′UTR of *DISC1* (full-length 3′UTR) or the predicted miR-135b-5p target site sequence (miR-135b-5p target site) with the two alternative alleles of rs11122396 together with miR-135b-5p precursor. We observed a significant reduction in the reporter gene activity with the A allele constructs, but not the G allele constructs. When anti-miR-135b was co-transfected with miR-135b-5p, reporter gene activity was restored to the control miRNA levels. (**b**) Similar experiment with the two alleles of rs11803088 within the predicted miR-559 target site. MiR-559 did not have a significant effect on the reporter gene expression with either of the two alleles. (**c**) Similar experiment with miR-23a-3p, miR-23b-3p, miR-130a-5p, miR-135a-5p, miR-323-3p, miR-409-3p, or miR-548c-3p and the full-length 3′UTR construct harboring the wild type-alleles at miRNA binding sites. None of these miRNAs reduced the reporter gene activity significantly. For all the analyses, the miRNA effect was compared with the negative control miRNA effect. We performed a minimum of two independent experiments for each miRNA and carried out linear regression analysis separately for each batch. We then used the obtained residuals in subsequent linear regression analysis including all the batches to obtain a *P*-value for all experiments combined taking the batch effect into account. An siRNA against *DISC1* was used as a positive control. Results are expressed as the regression coefficient ±standard error. $^{*}P<0.05$, $^{**}P<0.005$.

44 *DISC1* isoforms have been reported in adult and fetal human brain and in lymphoblastoid cell lines.[15] According to current annotations, four isoforms carry the four SNPs of this study. Previously, rs11122396 was associated with schizophrenia[16] through a rare haplotype. Moreover, the rs11122396 SNP is in complete linkage disequilibrium (LD) in the European population with rs3737597 (G2879A),[9] which has been associated with schizophrenia and recurrent major depression by several independent studies.[6,16,17] In contrast, no association was detected between the rs11122396 SNP and autism spectrum disorders in a family-based study sample.[18] These reports imply that variants in the 3′UTR of *DISC1* may have an important regulatory role in neuropsychiatric phenotypes. We conjecture that variable regulation of *DISC1* by miR-135b-5p in the brain may predispose to neuropsychiatric phenotypes, as has been proposed with, for example, miR-185 in the case of the 22q11.2 microdeletions and schizophrenia.[19]

In conclusion, we show that miR-135b-5p regulates the level of *DISC1* expression *in vitro* in an allele-specific manner, and suggest that the disturbed miRNA-mRNA target recognition mechanism may contribute to the wide range of phenotypic associations of *DISC1*, in accord with comparable natural variations in miRNA target sequences that have been shown to have major phenotypic consequences.[7,8,20]

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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