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Original Article

A mir-134-mediated post-transcriptional regulation pathway is involved in the expression of DPYD

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Abstract: Dihydropyrimidine dehydrogenase gene (DPYD) has recently been identified as a novel high risk gene responsible for schizophrenia (SZ) susceptibility. However, the clinical features and potential biological effects of DPYD remain poorly understood. This study aims to better define the role and regulation route of DPYD in SZ. Both mRNA and protein expression of DPYD gene in brain tissues from both SZ patients and healthy persons were firstly examined by real-time polymerase chain reaction PCR (qPCR) and western blot. We found increased DPYD mRNA level yet decreased DPYD protein level in SZ patients, which suggests that DPYD was involved in the post-transcriptional regulation. Through bioinformatics screening, one targeted DPYD MicroRNA (miRNA) miR-134 was selected, and we discovered by luciferase reporter assay that this miRNA bound to the untranslated region (UTR) of DPYD mRNA. Then, the correlation between miR-134 and DPYD was investigated. Our results reveal that our constructed miR-134 expression vectors were able to directly regulate DPYD expression. Furthermore, our results confirmed that the protein expression of the downstream gene including mammalian Sir2 homologue (SIRT-1), cyclic AMP (cAMP), response element-binding protein (CREB) and brain-derived neurotrophic factor (BDNF) were significantly up-regulated after miR-134 had been knocked down. The DPYD expression regulation mediated by miR-134 suggests that there is a complex regulatory mechanism of DPYD biosynthesis in the human brain, and its miRNA regulator may influence the pathophysiology of schizophrenia.

Keywords: DPYD, post transcription, miR-134, development, schizophrenia

Introduction

Schizophrenia is a severe neuropsychiatric disorder characterized by various psychotic symptoms such as delusions and hallucinations, deficits in affective response, social withdrawal, apathy, and cognitive impairment. Genetic factors substantially impact the risk of developing the disease, and its heritability is estimated at more than 80% [1]. Due to the high heritability of schizophrenia, its genetic association has been extensively studied, and numerous candidate genes have been proposed such as those in genome-wide association studies (GWAS). Recently, GWAS have been successful in identifying strongly supported genetic associations for schizophrenia. However, most of those candidate genes have not yet been unequivocally confirmed, and their functional roles in the pathophysiology of the disease remain uncertain.

Dihydropyrimidine dehydrogenase gene (DPYD) is the rate-limiting enzyme in the catabolism of pyrimidine [2], and its activity is particularly important in modulating the production of beta-alanine, a neuromodulator of inhibitory transmission in the brain [3]. DPYD, a newly-identified gene on chromosome 1q21.3 responsible for schizophrenia susceptibility, shows a nearly genome-wide significant association in the most recent schizophrenia GWAS meta-analysis [4, 5]. Furthermore, Xu et al. identified nonsense and missense *de novo* mutations of DPYD in two unrelated probands with schizophrenia by exome sequencing [6]. However, the clinical consequences of DPYD gene in schizophrenia remain unclear so far.

Many reports show that miRNA plays a key role in regulating DPYD protein expression because of the disparity between DPYD mRNA and protein levels [7-10]. MiRNA is a class of endoge-

nous, noncoding RNA molecules about 22 nucleotides long and widely expressed in all tissues and at all stages of development [11]. The miRNAs bind with the 3'untranslated region (3'-UTR), and more than one third of all human genes have been predicted to be miRNA targets [12]. Therefore, miRNAs represent a large and important category of regulatory molecules in post-transcriptional regulation. Recently, it has been shown that miR-134 expression profiles correlate with DPYD [2]. The miR-134 is transcribed from a neuron expressed miR gene cluster at the mouse Dlk1-Gtl2 domain [13] and is specifically expressed in the brain, suggesting that it plays unique regulatory roles in neuronal development and function [13, 14]. Santarelli et al. reported that miR-134 is expressed abnormally in prefrontal cortex Brodmann area 46 among schizophrenia patients, which suggests that miR-134 is involved in the pathogenic mechanism of schizophrenia [15] Moreover, For example, overexpression of miR-134 enhances neuronal differentiation promoted by double cortin (DCX) by negatively regulating Chordin-like 1 (Chrdl-1) and DCX expression [16]. On the basis of these data, we hypothesized that the miR-134 regulation pathway was possibly part of the post-transcriptional regulatory network of DPYD.

Therefore, in the study, we aim to investigate the role of DPYD in schizophrenia by examining both its mRNA and protein expression. We also assessed the correlation between DPYD and miR-134 in order to better define the regulation mechanism of DPYD.

Experimental section

Patients

Four SZ patients fulfilling the criteria as defined by the Diagnostic and Statistical Manual 4th edition (DSM-IV) were prospectively recruited from Qingdao Mental Health Center Army from July 2010 to May 2015. Clinical diagnoses of the patients were made by at least two consultant psychiatrists, and the diagnoses were further confirmed by an additional experienced clinical psychiatrist. In addition, four healthy controls without any family history of major psychiatric disorders (SZ, bipolar disorder and major depression) were recruited. For the use of these clinical materials for research purposes, prior patient's consents and approval from the Institutional Research Ethics Committee were obtained.

Cell culture

Human neuroblastoma cell line (SH-SY5Y) was obtained from ATCC and maintain in DMEM/F12 (HyClone) supplemented with 10% (v/v) fetal bovine serum (HyClone), 1% (v/v) L-glutamine (HyClone) and 1% (v/v) penicillin/streptomycin (HyClone). Human embryonic kidney cell lines (HEK293) were obtained from ATCC and maintained in DMEM supplemented with 10% fetal bovine serum (HyClone) and 1% (v/v) penicillin/streptomycin. The cells were incubated at 37°C in an atmosphere of 5% CO_2.

Quantitative real-time PCR (qPCR)

Expression levels of mRNAs were measured by real-time quantitative (qPCR), using ABI Prism 7900 sequence detection system. PCR cycle parameters were 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s and 59°C or 60°C for 1 min. PCR data were acquired from the Sequence Detector Software (SDS version 2.0). All samples were measured in a single plate for each gene, and their cycles at threshold (C_t) values were in the linear range of the standard curve.

Western blotting analysis

These cells were solubilized in lysis buffer containing protease inhibitors before being subjected to sonication and centrifugation at 4°C for 3 minutes. Loading buffer was added to each of the protein solutions, which were subsequently boiled for 5 minutes and separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis before being transferred to nitrocellulose membranes (Amersham Biosciences, Beijing, China). The membranes were blocked for 1 hour with antibody against human DPYD (kindly provided by Taiho Pharmachemical Co Ltd., Saitama, Japan) and β-actin (SIGMA, St Louis, MO). The immune complexes were additionally reacted with anti-rabbit IgG, peroxidase-linked species-specific whole antibody (Amersham Biosciences, Piscataway, NJ). Membranes were then washed three times in TBS-Tween, and specific bands were visualized using the ECL system (GE Healthcare, Little Chalfont, and Buckinghamshire, UK) according to the manufacturer's instructions. The same

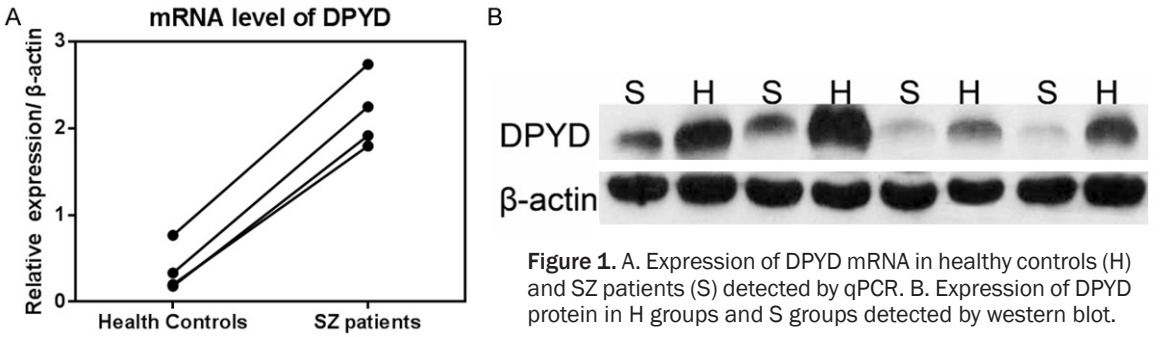

Figure 1. A. Expression of DPYD mRNA in healthy controls (H) and SZ patients (S) detected by qPCR. B. Expression of DPYD protein in H groups and S groups detected by western blot.

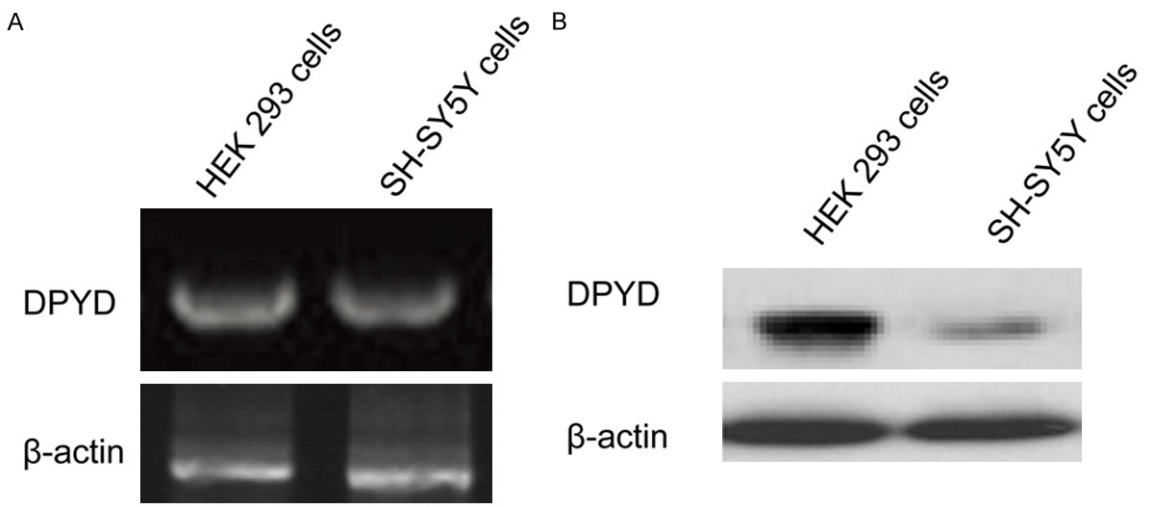

Figure 2. A. Expression of DPYD mRNA in SH-SY5Y cells and HEK293 cells by reverse transcription PCR. B. Expression of DPYD protein in SH-SY5Y cells and HEK293 cells by western blot.

method was used to detect SIRT1, CREB and BDNF protein expression in cells. β-actin detected with an anti β-actin rabbit antibody (1:1,000 dilution; Sigma) was used as the loading control.

Recombinant adeno-associated virus vector construction

Recombinant adeno-associated virus (AAV) vectors were constructed by Biowit Technologies (Biowit, Shenzhen, China). Briefly, the expression sequences encoding premiR-134 or anti-134 were subcloned into AAV-shuffer plasmid which were then co-transfected with two helper packaging plasmid pAAV/Ad and adenoviral plasmid PFG140 to SH-SY5Y cells via the calcium phosphate precipitate method. The recombinant AAV were harvested and concentrated by centrifugation. The titers were determined by dot blot analysis before AAV vectors were used.

Cell transfection

Cells were seeded into dishes without antibiotics 24 h prior to transfection. The infected with AAV-anti-miR-134 vectors or control vector was transiently transfected into cells using the X-treme GENE HP DNA transfection reagent (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's protocol.

Dual-luciferase reporter assay

The fragment from the 3'UTR of DPYP mRNA containing the predicted miR-134-binding sequences was amplified and subcloned into pGL luciferase promoter vector (Promega, Madison, WI, USA). The pGL3 vector containing 3'UTR of DPYP mRNA or mutated forms was co-transfected with AAV-pre-miR-134 or controls in to cells and incubated for 48 h. Then, cells were harvested and lysed, and the luciferase activity was detected using the dual-luciferase reporter

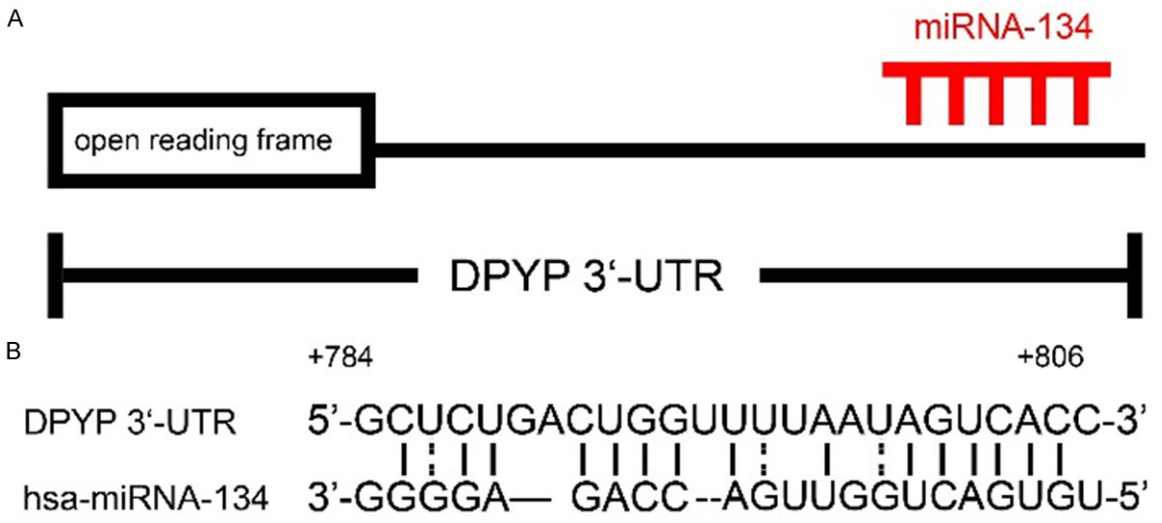

Figure 3. Computational analysis of miR-134 binding sites in the 3'UTR of DPYD gene. A. The locations of miR-134 in the DPYD 3'UTR. B. Homology between the 3'UTR DPYD and the predicted target sequence of miR-134. Numbers indicates distance from the stop codon.

assay kit (Promega, Madison, WI, USA) as per standard protocols. The relative luciferase activities were normalized to that of the control cells.

Statistical analyses

All statistical analysis was carried out using the SPSS 17.0 statistical software package. The quantitative data were expressed as mean ± SD. Statistical analysis was carried out using one-way analysis of variance (ANOVA) followed by Bonferroni test for multiple groups or Student t test between two groups. Differences with a p value less than 0.05 were regarded as statistically significant.

Results

DPYD expression in SZ patients

First of all, to determine DPYD expression in brain tissues, we carried out DPYD expression analysis using qPCR and western blot for 4 SZ patients and 4 healthy controls, respectively. As shown in **Figure 1**, compared to healthy controls, our data shows that the mRNA levels of DPYD in SZ patients significantly increased, but the protein levels of DPYD were down-regulated in schizophrenia patients.

DPYD expression in cell lines

Then, we investigated the DPYD expression in a human neuroblastoma cell line (SH-SY5Y) and human embryonic kidney cell line (HEK293) using reverse transcription PCR and western blot, respectively. **Figure 2A** demonstrates that the levels of DPYD transcription were detected with reverse transcription PCR assays. In agreement with the results of the SZ patients, the DPYD mRNA levels were significantly increased in SH-SY5Y cells, compared with those in the HEK293 cells. Meanwhile, as shown in **Figure 2B**, the DPYD protein level in SH-SY5Y cells were determined using western blot, and a decrease in DPYD protein expression was detected compared with that in the HEK293 cells. The different mRNA and protein expression of DPYD implies that the post-transcriptional regulation played a significant role in DPYD expression.

MiR-134 directly regulates DPYD expression

MiRNA-134 was selected to be a DPYD gene target by the miRecords database. As shown in **Figure 3A**, the miR-134 binding site is located in the 3'UTR region of DPYD mRNA. Furthermore, those binding sites of miRNA-134 targeted at DPYD are demonstrated in **Figure 3B**.

To assess the targeting capability of our constructed miR-134 vectors for the 3'UTR of DPYD mRNA, a dual luciferase reports assay was performed. Cells were transfected with various pGL-miR-134 vectors including those targeted at the DPYD-3'UTR and its mutations. **Figure 4A** demonstrates that the relative lucif-

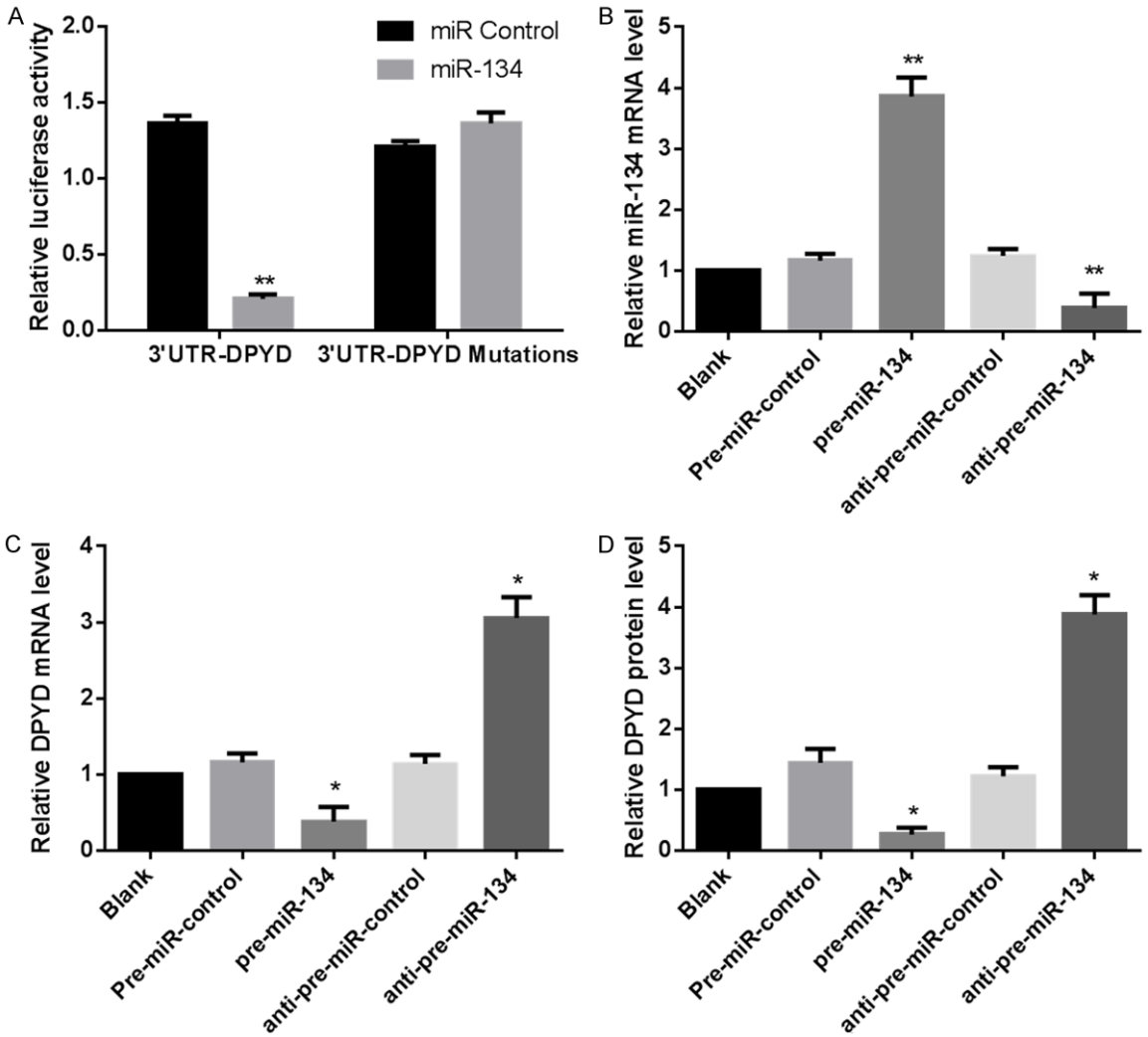

Figure 4. A. Luciferase activity assay was performed to elucidate the interaction between 3'UTR-DPYD and miR-134. B. qPCR analysis of miR-134 expression level after transfection of various miR-134 expression vectors. C. qPCR analysis of effect of miR-134 on DPYD mRNA expression in SH-SY5Y cells. D. Western blot analysis of effect of miR-134 on DPYD protein expression in SH-SY5Y cells. *Indicates $P<0.05$, **indicates $P<0.01$.

erase activity was significantly down-regulated in SH-SY5Y cells after transfection of pre-miR-134 compared with that of the control groups, where there were no obvious changes in the luciferase activity of 3'UTR mutations after transfection of miR-134 controls. These results confirmed that miR-134 was able to bind to the 3'UTR region of DPYD mRNA. Moreover, to assess the effect of our constructed miR-134 vectors on miR-134 expression in cells, miR-134 vectors containing a pre-miR-134 or an anti-miR-134 were transfected respectively into SH-SY5Y cell lines. As shown in **Figure 4B**, miR-134 expression levels were significantly up-regulated or down-regulated in SH-SY5Y cells after transfection of recombinant adeno-associated-virus (AAV)-pre-miR-134 or AAV-anti-miR-134, respectively. These results confirmed that miR-134 recognized the 3'UTR of DPYD mRNA, and then contributed to the repression of DPYD protein expression.

To further explore the correlation between miR-134 and DPYD, we assessed the biological effect of miR-134 on DPYD expression in SH-SY5Y cells using qPCR and western blot. **Figure 4C** shows the mRNA level of DPYD in SH-SY5Y cells significantly increased after transfection of AAV-anti-Pri-miR-134, but the overexpression of miR-134 markedly decreased DPYD mRNA level. Consistently, DPYD protein level was down-regulated when miR-134

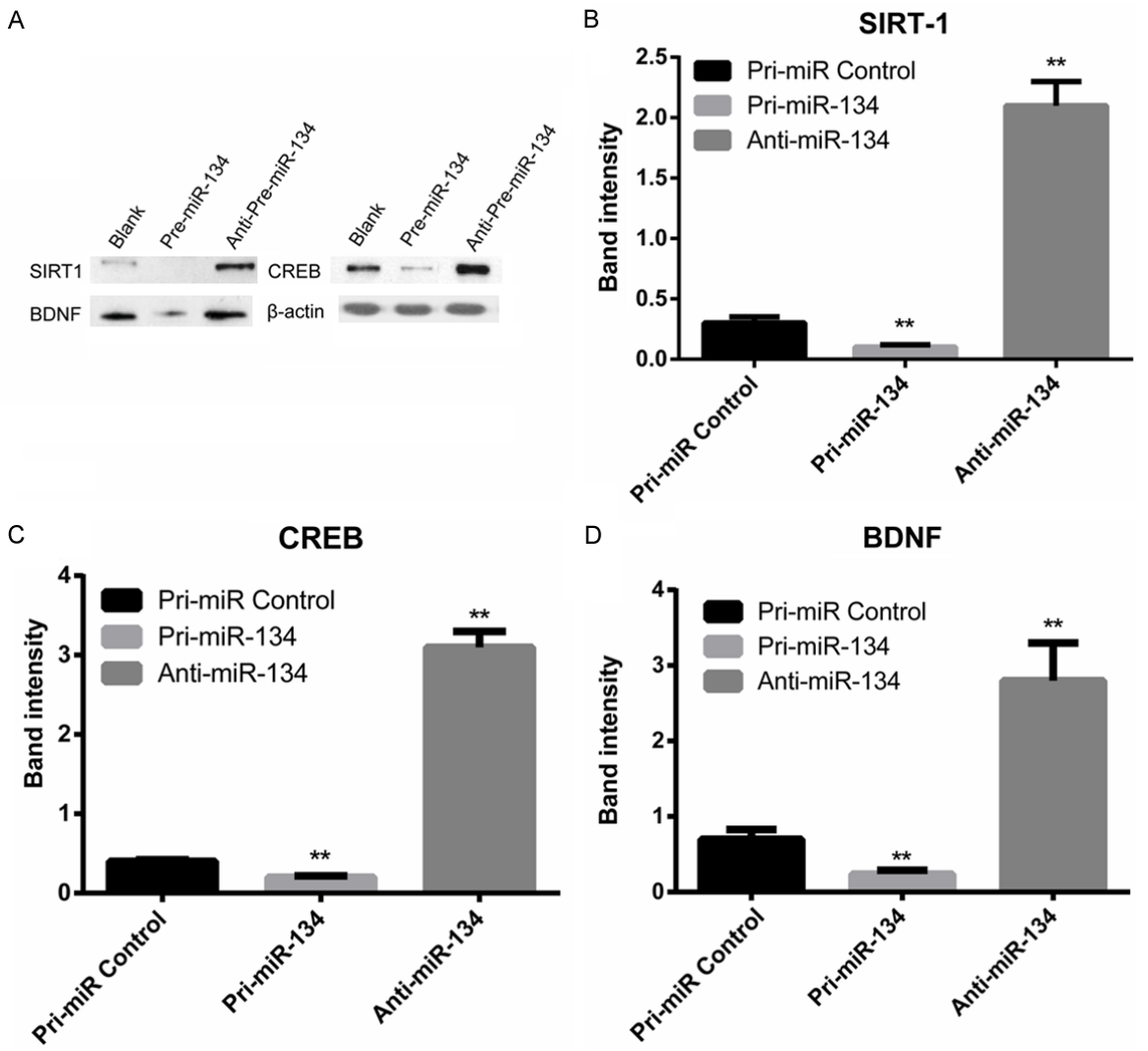

Figure 5. A. western blot analysis of the protein expression levels of SIRT1, CREB and BDNF in different treated groups. B-D. Quantitative analysis of band intensity of relative protein levels using Image-Pro software and normalized to β-actin. N=3, **indicates $P<0.01$.

expression was increased (**Figure 4D**), and up-regulated when miR-134 expression was decreased in SH-SY5Y cells (**Figure 4D**). These results confirmed that miR-134 directly regulated DPYD expression.

MiR-134 is involved in regulating the expression of SIRT1, CREB and BDNF

To further validate the regulatory effect of miR-134 on DPYD, the expression levels of downstream genes including mammalian Sir2 homologue (SIRT1), cAMP response binding protein (CREB), and brain-derived neurotropic factor (BDNF) were examined by western blot analysis. The results show that over-expressed miR-134 significantly decreased the protein expression level of SIRT1, CREB, and BDNF (**Figure 5**) compared to those of the control groups. Moreover, knocking down miR-134 significantly increased the expression levels of SIRT1, CREB, and BDNF in comparison with those of the control groups.

Discussion

Genetic studies suggest that schizophrenia is a complex disorder involving multiple pathways. Recently, a plausible genetic analysis of schizophrenia revealed that DPYD is a significant part of the genome-wide association of schizophrenia [4]. It is noteworthy that a recent paper by

Xu et al. [6] reported two *de novo* mutations in the DPYD gene. However, there have been so far no studies investigating the molecular basis underlying this association. In this study, we investigated mRNA and protein expression of DPYD in brain tissues and explored its possible association with schizophrenia on a molecular level.

First of all, we found no DPYD protein expression in SZ patients, while it was expressed in healthy controls. However, DPYD mRNA levels were significantly higher in SZ patients. Discrepancy between DPYD protein and DPYD mRNA expression has also been reported in cases of gastrointestinal cancer and lung cancer [2, 8]. These results were confirmed by reverse transcription PCR and western blot analysis in SH-SY5Y cells and HEK293 cells. Specifically, DPYD protein level in SH-SY5Y cells was lower than that in HEK293 cells, while both cell lines exhibited a similar level of DPYD mRNA expression. This difference in DPYD expression may be attributable to the lack of strong link between DPYD mRNA levels and protein levels. In fact, it has been reported that some miRNAs could influence the expression of target genes [2]. Thus, post-transcriptional regulation could be of importance to DPYD expression in schizophrenia.

In this study, miR-134, a microRNA screened from the miRecords database, was selected, because miR-134 has been considered to correlate with neuronal proliferation, migration, and differentiation. Recent studies suggest that there is a significant increase of miR-134 expression between E13.5 and E17.5 during mouse embryogenesis *in vivo* [11, 12, 16-18]. Subsequent studies reveal that miR-134 has been identified as a potential regulator of dendritic spine volume and synapse formation in mature rat hippocampal neurons [13]. Furthermore, a number of groups have shown that miR-134 targets and down-regulates the expression of pluripotency markers including *Oct4*, *Sox2* and *Nanog* in mESC and is a powerful inducer of pluripotent stem cell differentiation [16, 19]. Interestingly, miR-134 strongly associated with DPYD expression in this study. After transfecting cells with anti-miR-134, we discovered that the DPYD protein level was also elevated, which suggests that miR-134 plays an important role in regulating DPYD expression in schizophrenia.

In this study, we focused on not only DPYD, but also other miR-134 downstream genes as well, including SIRT1, CREB, and BDNF, which have been shown in previous reports to be closely related to schizophrenia. The SIRT1 is a very important molecule which regulates a variety of complex processes including the regulation of oxidative stress, metabolism control, and circadian rhythms [20, 21]. Recent reports have demonstrated that SIRT1 is involved in neurogenesis and neuroprotection [22, 23]. Moreover, CREB has been reported to play critical roles in various diseases including cerebral ischemic injury, and the activation of CREB could promote neuronal survival by up-regulating BDNF expression [17, 24, 25]. Furthermore, repression of CREB and BDNF was detected in a mouse model lacking SIRT1 catalytic activity [18]. In this study, after down-regulating the miR-134 expression and overexpression of DPYD, we showed that the protein expression of CREB, BDNF and SIRT1 were increased, suggesting that DPYD expression undergoes a miR-134 regulation pathway, which plays an important role in schizophrenia [13].

Conclusions

In summary, compared to that of healthy persons, DPYD protein expression is down-regulated in schizophrenia patients, but DPYD mRNA level in schizophrenia patients is up-regulated. Furthermore, we found that DPYD protein was directly regulated by miR-134, which binds to the 3'UTR of the DPYD mRNA. Our study demonstrated that miR-134 plays a key role in the post-transcriptional regulatory mechanism of DPYD. Although additional studies are necessary to confirm our results, this study supports the DPYD hypofunction model of schizophrenia pathogenesis, and provides new insights into miRNA mediated pathway in the pathophysiology of schizophrenia.

Acknowledgements

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Disclosure of conflict of interest

None.

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