

Jingjie Zhao, Xu Guo, Yi Du, Yu Han, Yongzhi Wang, Li Li^{a,*}, Jialin Qian, Mingzhen Li, Huijuan Wu, Teresa Golden and Ning Wu^{a,*}

Correlative study of peripheral *ATP1A1* gene expression level to anxiety severity score on major depressive disorder patients

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Abstract

Background: Major depressive disorder (MDD) frequently co-occurs with other psychiatric problems. Our previous study showed that *ATP1A1* gene expression level was significantly decreased in MDD patients. This research explores the potential correlations between the *ATP1A1* expression level reduction and MDD patients' clinical manifestation.

Methods: All participant patients were diagnosed by Diagnostic and Statistical Manual of Mental Disorders – 4th edition (DSM-IV). Hamilton rating scale for depression (HAM-D) and anxiety (HAM-A) were applied to group patients into different categories. *ATP1A1* expression level was measured by reverse transcript real-time polymerase chain reaction.

Results: *ATP1A1* expression levels of all MDD subgroups showed significant reduction compared to the control group ($p < 0.01$). Further, the trend of *ATP1A1* expression level reduction is significantly related to MDD patients' HAM-A scores ($p < 0.01$). However, there was no significance between *ATP1A1* level and HAM-D scores ($p > 0.05$).

Conclusions: *ATP1A1* expression level reduction is related to MDD anxiety score, which may be an explanation for the clinical manifestations and the underlining physiological mechanisms.

Keywords: anxiety; *ATP1A1*; gene expression; major depressive disorder (MDD).

Introduction

Major depressive disorder (MDD) is a highly prevalent psychiatric disorder which is becoming an increasingly serious public health concern [1, 2]. Previous studies showed that MDD affects the central nervous system and thereafter affects multiple systems to cause both psychic and somatic symptoms [3–5]. Accordingly, gene expression levels in peripheral blood leukocytes will be affected and changed to reflect the changes in the central nervous system [4]. Currently, about 150 genes in peripheral blood leukocytes have been identified to demonstrate expression level differences in MDD patients [6]. Our previous study confirmed that the expression level of *ATP1A1* gene, coding for Na^+/K^+ ATPase $\alpha 1$ subunit – the most abundant Na^+/K^+ ATPase subunit in cerebrum and cerebellum [7], in MDD patient peripheral blood showed a significant reduction compared to normal controls [8]. Na^+/K^+ ATPase is a membrane protein which plays a key role in the maintenance of ion homeostasis for regular neuronal excitability, secondary transport, and neurotransmitter uptake [9]. The $\alpha 1$ Na^+/K^+ ATPase possesses both pumping and signaling functions [10]. Its primary physiological role is the maintenance of cellular transmembrane sodium and potassium ion gradients necessary for the control of resting membrane potential [7]. In addition, the $\alpha 1$ Na^+/K^+ ATPase nucleotide binding domain is actively involved in cellular signal transduction and cell growth [10]. The inhibition of Na^+/K^+ ATPase activity decreased the uptake of norepinephrine [11], serotonin, and dopamine [12], and, therefore, altered neuronal firing [13]. On the other hand,

^aLi Li and Ning Wu contributed equally to this work.

***Corresponding authors:** Dr. Li Li, Department of Traditional Chinese Medicine, Beijing Friendship Hospital, Capital Medical University, 95 Yong-An Road, Beijing 100050, P.R. China, Phone: +86 10 6313 8328, E-mail: liliqin_1999@yahoo.com; and Dr. Ning Wu, Department of Biological Sciences, Southeastern Oklahoma State University, Durant, OK 74701, USA, Phone: +1 580 745 2564, E-mail: nww@se.edu

Jingjie Zhao, Xu Guo, Yi Du, Yu Han and Yongzhi Wang: Department of Traditional Chinese Medicine, Beijing Friendship Hospital, Capital Medical University, Beijing, P.R. China

Jialin Qian, Mingzhen Li and Huijuan Wu: Beijing Center for Physical and Chemical Analysis, Beijing, P.R. China

Teresa Golden: Department of Biological Sciences, Southeastern Oklahoma State University, Durant, OK, USA

Na^+/K^+ ATPase α subunit functional deficiency will interfere with the interaction and regulation of the cellular Src pathway, and therefore, inhibit ouabain-activated signal transduction and cause the depression-like symptoms [14, 15].

Major depressive disorder frequently co-occurs with other psychiatric problems. Among the MDD patient population, nearly 50% of lifetime MDD patients were reported to have, at least, one type of lifetime anxiety disorder [16]. Besides the general MDD diagnosis, additional clinical evaluation was performed to assess the comorbidity of MDD by using hamilton rating scale for depression (HAM-D) [17] and hamilton anxiety rating scale (HAM-A) [18]. Previous studies showed that there were three types of Na^+/K^+ ATPase α subunit isoforms expressed in the central nervous system encoded by the genes of *ATP1A1*, *ATP1A2*, and *ATP1A3*, respectively [7, 19]. The decreased expression level of *ATP1A3* gene that codes for the $\alpha 3$ subunit isoform plays an important role in the MDD's depression-like endophenotypes [15], while the *ATP1A2* gene that codes for the $\alpha 2$ subunit isoform has a significant association with bipolar disorder and anxiety-related behavior [20, 21]. The expression level reduction of *ATP1A1* gene that codes for $\alpha 1$ subunit isoform has also been proven to be involved in MDD [8]. However, the role of *ATP1A1* in MDD endophenotypes (anxiety-like or depression-like) remains unclear. This study combines MDD patients' clinical HAM-D and HAM-A evaluations with peripheral blood *ATP1A1* expression levels to investigate the correlations between *ATP1A1* gene expression level and MDD endophenotypes.

Materials and methods

Subject selection

All participants were from the Chinese Han ethnic group. A total of 40 drug-free MDD patients (25 females, 15 males, age 29–60 years) were included in the experimental group, while an additional 40 healthy subjects (25 females, 15 males, age 30–60 years) participated as the normal control. All subjects were recruited from the clinic of Beijing Friendship Hospital, Capital Medical University (Beijing, China) and were clinically screened to eliminate possible diseases of other systems. The MDD patients were diagnosed and enrolled into study group according to the criteria of Diagnostic and Statistical Manual of Mental Disorders – 4th edition (DSM-IV) (American Psychiatric Association) through “The Mini International Neuropsychiatric Interview” (MINI) structured clinical interview [22]. All research procedures were reviewed and approved by the Institutional Research Ethics Committee of Capital Medical University and a written informed consent statement was obtained from each participant.

The inclusion criteria for MDD patients were: (1) diagnosed by two well-trained senior psychiatrists in accordance with the DSM-IV

criteria; (2) absence of evidence and history of any other systemic diseases; (3) medication-free for at least 2 weeks; (4) HAM-D 24 and/or HAM-A 17 scores (HAM-D ≥ 20 and/or HAM-A ≥ 14). For normal control subjects, the scores of HAM-D 24 and HAM-A 17 were < 8 and 7, respectively.

Peripheral venous blood collection and total RNA extraction

Following 12 h fasting, 5 mL of peripheral venous blood was collected from both MDD patients and normal controls. The collected blood was stored in EDTA and was processed for total RNA extraction within 2 h of collection. Whole blood total RNAs were isolated by using PureLink® RNA Mini Kit (Life Technologies, Carlsbad, CA, USA) according to manufacturer's instructions. The isolated total RNAs were subjected to the BioSpec-nano Spectrophotometer (Shimadzu, Kyoto, Japan) for quantization of optical density (OD) at OD_{260} and quality assessment at $\text{OD}_{260}/\text{OD}_{280}$ and $\text{OD}_{260}/\text{OD}_{230}$, respectively. In addition, 1% agarose gel electrophoresis was also used for total RNA quality control.

Reverse transcription and real-time PCR

Total RNAs were reverse transcribed into cDNAs by using PrimerScript™ RT Reagent Kit (Takara Bio Inc., Shiga, Japan) according to the manufacturer's instructions. Briefly, the 10 μL reaction system was composed of 500 ng total RNA, 25 pmol Oligo dT primer, 50 pmol Random 6 mers, 2 μL of 5 $\times$ PrimerScript buffer, 0.5 μL PrimerScript™ RT Enzyme Mix, and RNase free dH_2O . The reaction was performed on S1000™ Thermal Cycler (BIO-RAD, Hercules, CA, USA) by the program of 37 °C for 15 min and 85 °C for 5 s.

Beacon Designer™ software (PREMIER Biosoft, Palo Alto, CA, USA) was applied to design the polymerase chain reaction (PCR) primers for *ATP1A1* (target) and β -actin (control) genes according to GenBank nucleotide sequence records. The forward and reverse primers for *ATP1A1* gene (Accession number: NM_000701) were GGTCCTCAACGCGCTCACTC and ACCACACCCAGGTACAGATTATCG, respectively. The forward and reverse primers for β -actin gene (Accession number: NM_001101) were GCGGGCACCACCATGTACCCT and AGGGGCCGGACTCGTCATACT, respectively. The primer sequences of *ATP1A1* gene were subjected to nucleotide BLAST investigation against GenBank “Human genomic” and “transcript” databases to ensure the specificity (i.e. no sequence overlapping with other Na^+/K^+ ATPase α subunit isoforms). The real-time PCR was performed by using Applied Biosystems 7500 Fast Real-Time PCR System (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instruction. Briefly, the 25 μL reaction system was composed of 2 μL of diluted cDNA template, 12.5 μL SYBR® Green Real-Time PCR Master Mix (Life Technologies, Carlsbad, CA, USA), and 0.2 mmol/L of each primer. The reaction conditions were 95 °C for 20 s, followed by 40 cycles of 95 °C for 3 s, 60 °C for 30 s, and then 95 °C for 15 s, 60 °C for 1 min, 95 °C for 15 s, and 60 °C for 15 s. The amplified fragments were checked by running 1% agarose gel electrophoresis of the PCR products. A 10-fold dilution series of cDNA (10^{-3} – 10^{-10} times) for each target gene was applied as a standard to construct a relative standard curve and to determine the PCR efficiency that would be used in converting quantification cycle into relative quantity.

Data analysis

The real-time PCR data was processed and analyzed by using 7500 Fast Real-Time PCR System Sequence Detection Software (version 1.4) (Life Technologies, Carlsbad, CA, USA). The standard curve of each target gene and their amplification (R_n vs. Cycle) and dissociation curves were derived. The equations of each target gene amplification were then calculated for optimal experimental condition evaluation. The final absolute copy number of each target gene was calculated by using the formulation of target gene absolute copy numbers = sample concentration/sample molecular weight $\times 6 \times 10^{14}$ and then was converted to target gene relative copy numbers by using the formulation of target gene relative copy numbers = target gene absolute copy numbers/reference control gene absolute copy numbers. The data of both control and MDD groups were statistically assessed by using Microsoft Excel T-test program (Microsoft, Seattle, WA, USA). The comparative studies of *ATP1A1* gene expression levels to MDD patients' HAM-A and HAM-D scores were performed, respectively. Further, the correlative statistical analysis between *ATP1A1* expression level and HAM-A score in all 40 patients' samples was also performed by using Microsoft Excel CORREL program (Microsoft, Seattle, WA, USA).

Results

Demographics

The general information about MDD and normal control groups is listed in Table 1. There were no significant differences between the control and each patient groups regarding age ($p > 0.05$). However, there were significant differences in both HAM-A and HAM-D scores between the normal control and the MDD patients ($p < 0.01$) (Table 2). According to the clinical survey scores of HAM-A and

HAM-D, the MDD patients were reorganized into HAM-A1 (HAM-A score ≥ 15) and HAM-A2 (HAM-A score ≤ 14) groups, and HAM-D1 (HAM-D score ≥ 24) and HAM-D2 (HAM-D score ≤ 23) groups in further (Table 2). Additional analysis of MDD group regarding HAM-A and HAM-D scores showed that there was a significant difference in HAM-A scores between HAM-A1 and HAM-A2 groups ($p < 0.01$), but no significant difference in HAM-D scores between those two groups ($p > 0.05$) was observed. The comparative of HAM-D1 and HAM-D2 groups showed a significant difference in HAM-D scores ($p < 0.01$) and no significant difference in HAM-A scores ($p > 0.05$) (Table 2).

Real-time PCR reactions of target and control genes

Both *ATP1A1* and β -actin genes demonstrated satisfactory amplification and unique PCR product in each individual gradient reaction with a single sharp amplification peak and a smooth amplification curve. Both genes' standard equations showed a linear relationship with R^2 -values larger than 0.99 and the slopes less than -3 , which indicated the success of reactions for both genes' amplifications.

Comparative study of *ATP1A1* expression among control and MDD patient groups

The absolute and relative copy numbers of *ATP1A1* and β -actin genes of each individual subject among the control and MDD groups were calculated. The distribution of *ATP1A1* gene relative expression levels among control and MDD groups were shown in Figure 1. There was a significant difference on *ATP1A1* expression level between control and whole MDD patient groups ($p < 0.01$). Additional studies showed that the *ATP1A1* gene expression levels of all MDD subgroups showed significant reduction compared to that of control group

Table 1: General information of MDD and control groups.

Groups	Sex		Age, years (range)
	Male	Female	
Control (n=40)	15	25	46.95 $\pm$ 9.99 (30–60)
MDD (n=40)	15	25	46.95 $\pm$ 9.86 (29–60)

Table 2: HAM-A and HAM-D scores of MDD and control groups.

Groups	HAM-A score (range)	HAM-D score (range)
Control (n=40)	2.05 $\pm$ 1.62 (0–6)	2.56 $\pm$ 1.92 (0–7)
MDD (n=40)	20.05 $\pm$ 11.34 (6–43)	25.43 $\pm$ 4.20 (20–34)
HAM-A1 (score ≥ 15) (n=20)	29.80 $\pm$ 7.63 (15–43)	24.30 $\pm$ 3.87 (20–34)
HAM-A2 (score ≤ 14) (n=20)	10.30 $\pm$ 2.95 (6–14)	26.55 $\pm$ 4.21 (20–34)
HAM-D1 (score ≥ 24) (n=23)	19.78 $\pm$ 12.59 (6–43)	28.22 $\pm$ 3.37 (24–34)
HAM-D2 (score ≤ 23) (n=17)	20.41 $\pm$ 9.36 (6–39)	21.65 $\pm$ 1.13 (20–23)

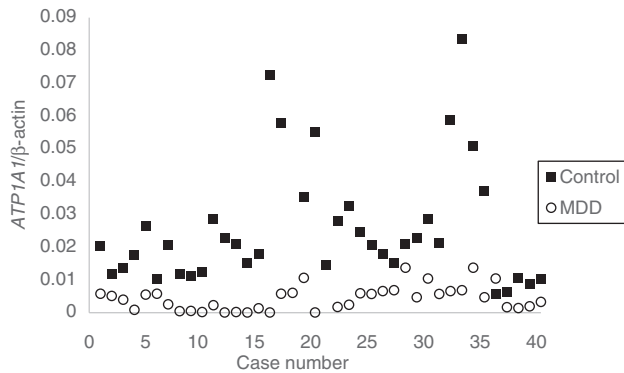


Figure 1: The distribution of *ATP1A1* gene relative expression level among control and MDD groups.

($p < 0.01$). Further, there was a significant difference in *ATP1A1* gene expression level reduction between HAM-A1 and HAM-A2 groups ($p < 0.01$). However, there was no significant difference in *ATP1A1* gene expression level changes between HAM-D1 and HAM-D2 groups ($p > 0.05$). In addition, the correlation statistical analysis between *ATP1A1* and HAM-A scores of all 40 patients showed a weak negative correlation between two groups (-0.3234) (Figure 2A).

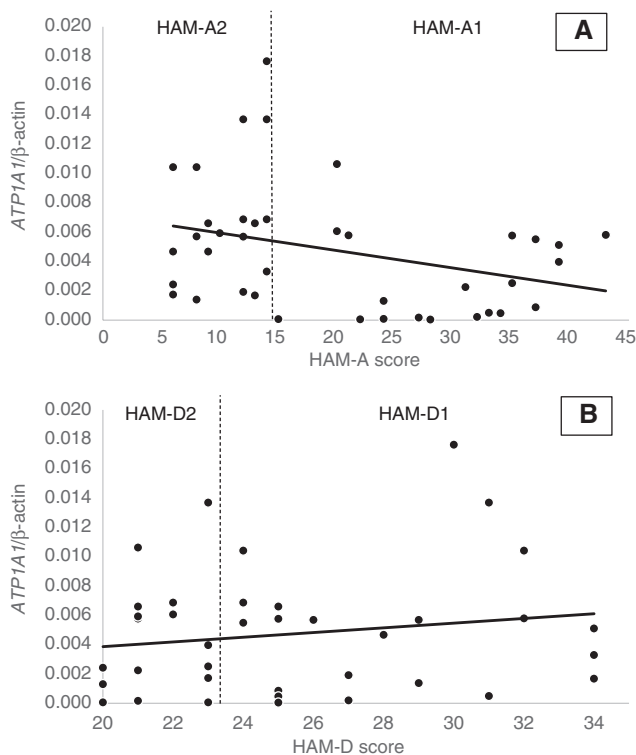


Figure 2: The relations and trends (solid line) of *ATP1A1* gene expression level changes among HAM-A (A) and HAM-D (B) groups.

Discussion

ATP1A1 is one of the three members of sodium/potassium pump ($\text{Na}^+/\text{K}^+\text{-ATPase}$) family genes expressed in brain, which maintains the sodium and potassium concentrations across the cellular membrane [23]. Past studies confirmed that α subunit isoforms of brain $\text{Na}^+/\text{K}^+\text{-ATPase}$ are involved in different behavioral aspects, especially $\alpha 2$ and $\alpha 3$ subunits that were involved in human mental and behavioral disorders [15, 20, 24, 25]. However, there are not many studies that deal with the relationship between $\alpha 1$ subunit and MDD. Our previous study confirmed that *ATP1A1* gene expression level demonstrated a significant reduction in clinically diagnosed MDD patients' peripheral blood [8], which indicated that $\alpha 1$ subunit was also involved in a human mental disorder.

The results of this study provide an additional evidence that *ATP1A1* expression level is closely related to MDD (Figure 1). Additional analysis between the clinical diagnostic criteria – HAM-A and HAM-D rating scales and *ATP1A1* expression levels were performed in this study. The results showed that *ATP1A1* expression level reduction is positively correlated to MDD patients' clinical anxiety score level with a significant difference between the two-scaled HAM-A groups ($p < 0.01$) (Figure 2A). However, there was no significant difference observed in HAM-D groups ($p > 0.05$) (Figure 2B). Past studies also confirmed that *ATP1A1* regulated sodium and potassium homeostasis in pyramidal cells and, therefore, contributed to the generation of resting membrane potential locally [23]. In addition, $\text{Na}^+/\text{K}^+\text{-ATPase}$ regulates the formation of a cohesive neuroepithelium and the restriction of neuroepithelial permeability [26]. Reduction of *ATP1A1* expression level in brain neuronal cells may cause an increase of intracellular Na^+ concentration by reducing the pumping capability of sodium from inside of cell to the outside, and increasing the intracellular potassium concentration [27]. The overall effects may elevate the resting potential level of neuronal cell inner membrane, and, therefore, facilitate the generation of neuronal action potentials with a relatively reduced threshold level and lesser stimulation strength than in a normal neuron. An MDD patient with anxiety is more easily irritated by external environment alternation. Even a slight or mild change of the environment may cause the anxious reactions and symptoms. In other words, such patients are sensitive to external environmental stimulation and generate the neuronal innervated symptoms accordingly. In conclusion, the results of this study, that the *ATP1A1* expression level reduction is related to MDD anxiety score, may explain these clinical manifestations and the underlining physiological mechanisms.

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