The ubiquitination of serotonin transporter in lymphoblasts derived from fluvoxamine-resistant depression patients

Running Head: Decreased ubiquitination of SERT in depression

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Abstract

There is insufficient serotonergic neuronal function in the pathophysiology of major depressive disorder (MDD). Serotonin transporter (SERT) plays a critical role in terminating the function of serotonergic neurons. SERT is linked to vulnerability to MDD and is an important target for antidepressants. The expression of SERT in lymphocytes and platelets is associated with their expression in central nervous system. Most of the clinical studies that have analyzed the role of SERT in depression have focused on absolute expression of SERT in the brain or peripheral tissue. Our study has shown that the SERT protein is ubiquitinated, which has been implicated through the SERT stability and depressive behaviors in mice. In our study, we have used lymphoblasts derived from the peripheral blood lymphocytes to quantitatively examine SERT protein expression and ubiquitination in fluvoxamine-responsive and fluvoxamine-resistant MDD patients. We found that the protein levels of SERT were higher in the fluvoxamine-resistant MDD patients. Ubiquitinated protein levels of SERT were lower in the fluvoxamine-resistant MDD patients. The proteasome inhibitor failed to increase the protein levels of SERT in both fluvoxamine-responsive and fluvoxamine-resistant MDD patients. In sum, these findings suggest that the downregulation of the ubiquitination of SERT protein induces insufficient degradation of SERT by proteasome, which resulted in the upregulation of SERT protein in fluvoxamine-resistant MDD patients. Although further studies with various populations will be required to generalize results, SERT protein expression, ubiquitination, and the responsiveness of SERT expression to proteasome inhibitor are potential biomarkers for the diagnosis of MDD and antidepressant efficacy.

Keywords:

Major depressive disorder, Serotonin transporter, Ubiquitination, Lymphoblast, Biomarker

Abbreviations:

MDD: Major depressive disorder

SERT: serotonin transporter

MAGE-D1: melanoma antigen gene-D1

EBV: Epstein-Barr virus

HAM-D: Hamilton Depression Scale

UPS: ubiquitin-proteasome system

Introduction

Major depressive disorder (MDD) is a serious illness characterized by a chronically depressed mood, a lack of interest and pleasure, a marked change in body weight, insomnia or hypersomnia, mental decline, psychomotor agitation or retardation, feelings of worthlessness and guilt, a decline of thought and concentration, and suicidal ideation [2]. The prevalence of MDD during the person's lifetime is 16.6 % [13]. Regarding the neurological conceptualizations of MDD, the monoamine hypothesis argues for the decrease in the neural transmission of serotonin, noradrenaline, and dopamine [21]. In particular, the insufficient activity of the central serotonergic nervous system may play a key role in the pathophysiology of MDD [24]. Serotonin transporter (SERT) plays a critical role in terminating the serotonergic transmissions, which is related to vulnerability to MDD [11]. Further, the expression of SERT in the lymphocytes and platelets is associated with its expression in the central nervous system [4, 26]. Higher mRNA levels of SERT in leukocytes, including lymphocytes, are observed in patients with MDD, and these phenomena are reversed by the administration of paroxetine [12]. Moreover, the activity of SERT in the platelet is enhanced during depression in seasonal affective disorders [28]. Conversely, the lower SERT expression in lymphocytes is also observed in patients with MDD [5, 18]. Although SERT is a major target for antidepressants, there is very little evidence of the relationship between SERT expression and its responsibility to antidepressants.

We have previously identified the physiological role of melanoma antigen gene-D1 (MAGE-D1) in the serotonergic nervous system and in emotional behavior using knockout mice [17]. MAGE-D1 binds to SERT and reduces SERT expression and serotonin reuptake activity, as a result of an increase in SERT ubiquitination.

MAGE-D1 knockout mice show depression-like behaviors (a decrease in exploratory behavior in both the home cage and novel apparatus, a decrease in social interaction, increased immobility time during forced swimming and tail suspension, and a decrease in sucrose preference) and serotonergic hypofunction in the prefrontal cortex and hippocampus, accompanied by the hyperexpression of SERT due to a decrease in ubiquitination [17]. Thus, a decrease in SERT ubiquitination has been implicated as a causal mechanism in MDD. Most of the studies on the role of SERT in depression have focused on absolute expression of SERT in the brain or peripheral tissue. There has been no quantitative study of ubiquitinated SERT expression in MDD patients.

Increasing evidence indicates that the gene and protein expression patterns in Epstein-Barr virus (EBV)-transformed lymphoblasts from the blood of patients, can consistently reflect the phenotypes of many psychological and neurological diseases [1, 15, 22, 27]. They are easily established from patients and can be maintained to investigate various cellular and molecular biological functions [22]. Therefore, in our study, we used lymphoblasts derived from the peripheral blood lymphocytes to quantitatively examine SERT protein expression and ubiquitination in the fluvoxamine-responsive and fluvoxamine-resistant MDD patients. Our findings revealed that (1) the expression of SERT protein was higher in the fluvoxamine-resistant MDD patients, (2) the expression of ubiquitinated SERT protein was lower in the fluvoxamine-resistant MDD patients, and (3) the proteasome inhibitor failed to increase the expression of the SERT protein in both fluvoxamine-responsive and fluvoxamine-resistant MDD patients.

Material and Methods

Subject

The sample comprised 12 major depressive disorder (MDD) patients (male = 6, female = 6: age $\pm$ SD = 39.8 ± 18.2) and 6 healthy subjects (male = 3, female = 3: age $\pm$ SD = 33.0 ± 17.6). Detailed information about the clinical phenotype has been described previously [14]. All of the MDD patients were treated with fluvoxamine (for 8 weeks monotherapy) and their depression were evaluated for severity using the Hamilton Depression Scale (HAM-D), at the initial visit (minimal HAM-D score more than 14) and after treatment (8 weeks). A remission was defined as HAM-D scores of less than 7 points after treatment (remitter: male = 3, female = 3, age $\pm$ SD = 42.7 ± 18.6 , non-remitter: male = 3, female = 3, age $\pm$ SD = 38.6 ± 19.0). After explaining the study to the subjects, written informed consent was obtained from each subject. This study was conducted in accordance with the code of ethics of the World Medical Association (Declaration of Helsinki) and approval was given by the Ethics Committee at Fujita Health University School of Medicine.

Cell Culture

After fluvoxamine treatment for 8 weeks, MDD patients were divided into two groups (fluvoxamine-responsive and fluvoxamine-resistant groups) and their blood samples were collected. Each of the blood samples aseptically collected was diluted two folds with a sterile saline. The diluted blood sample was carefully poured in the tube containing 3.5 ml of the Ficoll-Paque liquid (General Electric Company, Uppsala, Sweden), and centrifuged at $600 \times g$ for 30 minutes. Subsequently, the white layer of mononuclear cells formed at the intermediate part of the centrifuged sample, and the

Ficoll-Paque liquid layer immediately below the mononuclear cell layer were collected. The collected sample was suspended in a saline, and centrifuged at 400 x g for 30 minutes. A saline was added to the precipitate obtained by the centrifugation, and was centrifuged at 240 x g for 5 minutes. The liquid medium (RPMI1640) was added to the precipitate obtained by the centrifugation, and was centrifuged again at 240 x g for 5 minutes. Subsequently, the precipitate obtained by the centrifugation was used as the lymphocyte fraction, and cultured in RPMI1640 containing 20% fetus bovine blood serum, 20% of the culture supernatant of Epstein-Barr virus-releasing cell line (B95-8), and 2 µg/ml cyclosporine A. The mixture was cultured for approximately one week, and those that showed proliferation were used as the established lymphocytes, and sub-cultivated in RPMI1640 containing 10% fetus bovine blood serum.

Western blotting analysis

Four hours after the application of MG-132 (20 µM), cells were lysed in ice-cold lysis buffer supplemented with a mixture of proteinase inhibitors (Complete™, Roche Diagnostics, Mannheim, Germany). The protein concentration was determined using a DC Protein Assay Kit (Bio-rad, Richmond, CA). Samples (20 µg of protein) were boiled in Laemmli sample buffer (125 mM Tris-HCl pH 6.8, 10% 2-mercaptoethanol, 4% SDS, 10% sucrose, and 0.004% bromophenol blue). Samples from fluvoxamine-responsive and fluvoxamine-resistant MDD patients and healthy subjects were arranged into pairs matched as close as possible for age and gender. Pairs of subjects were loaded on the same polyacrylamide gel, and then transferred to polyvinylidene difluoride (PVDF) membranes (Millipore Corporation, Billerica, MA). Membranes were blocked with a Detector Block Kit (Kirkegaard and Perry Laboratories,

Gaithersburg, MD) and probed with a primary antibody. Membranes were washed with the washing buffer [50 mM Tris-HCl (pH 7.4), 0.05% Tween 20, and 150 mM NaCl] and subsequently incubated with a horseradish peroxidase-conjugated secondary antibody. The immune complexes were detected by ChemiDoc XRS (Bio-rad) based on chemiluminescence (ECL kit, GE Healthcare, Buckinghamshire, UK). Band intensities were analyzed by densitometry using the ATTO Densitograph Software Library Lane Analyzer (ATTO, Tokyo, Japan). To normalize each sample, membranes were stripped with the stripping buffer [100 mM 2-mercaptoethanol, 2% SDS, and 62.5 mM Tris-HCl (pH 6.7)] at 50 °C for 30 min, and β -actin protein was detected as described above.

The primary antibody was a rabbit anti-SERT (1:1000; Millipore). The secondary antibody that was used at a dilution of 1:2000, was horseradish peroxidase-linked anti-rabbit IgG (Kirkegaard and Perry Laboratories).

Detection of ubiquitinated protein

Detection of ubiquitinated protein was conducted with the agarose-immobilized p62-derived UBA domain (UbiQapture-Q kit[®], Enzo Life Sciences International, Inc., Plymouth Meeting, PA). Equal amounts of protein were incubated with beads that were chemically coupled to the UBA domain. Beads were washed with cold phosphate-buffered saline (PBS) and the supernatants were discarded. Proteins were eluted in Laemmli sample buffer, separated on a polyacrylamide gel, and then transferred to a PVDF membrane. Membranes were blocked and probed with the SERT antibody and detected as described above.

Statistical analysis

All the results were expressed as the mean $\pm$ SEM for each group. The difference between groups was analyzed with a one- or two-way ANOVA, followed by the Bonferroni/Dunn test.

Role of the funding source

Funders had no involvement in data collection, analysis, or interpretation; trial design; patient recruitment; any aspect pertinent to the study; or in the writing of the report or decision to submit for publication. Prof. Toshitaka Nabeshima, the corresponding author, had full access to all the data in the study and final responsibility for the decision to submit for publication.

Results

The expression of SERT protein was higher in lymphoblasts derived from fluvoxamine-resistant MDD patients

We measured SERT protein expression in lymphoblasts derived from MDD patients. The SERT protein was identified by the band (76 kD) detected by Western blotting using the antibody to the SERT. In comparison with the protein levels of SERT in lymphoblasts derived from healthy subjects, the protein levels of SERT from fluvoxamine-responsive MDD patients was 110.4% and had no significant difference, while that from fluvoxamine-resistant MDD patients was 137.0% and was significantly higher (Figure. 1: one-way ANOVA, $F_{(2, 15)} = 7.86$, $p < 0.01$).

The ubiquitination of SERT protein was lower in lymphoblasts derived from fluvoxamine-resistant MDD patients

The stability of the SERT protein is modulated by ubiquitination [16]. We measured ubiquitinated SERT protein expression in lymphoblasts derived from MDD patients. The ubiquitinated SERT protein was identified by the band (76 kD), which was detected by Western blotting using the antibody to the SERT protein by using the ubiquitinated protein bound to the UbiQapture-Q kit. In comparison with the ubiquitinated protein levels of SERT in lymphoblasts derived from healthy subjects, the ubiquitinated protein levels of SERT in fluvoxamine-responsive MDD patients was 88.4% and had no significant difference, while the same in fluvoxamine-resistant MDD patients was 49.6% and significantly lower (Figure. 2: one-way ANOVA, $F_{(2, 15)} = 5.51$, $p < 0.05$).

The proteasome inhibitor failed to increase the expression of SERT protein in lymphoblasts derived from both fluvoxamine-responsive and fluvoxamine-resistant MDD patients

Ubiquitination is often a signal for degradation by the proteasome [10]. We investigated the effect of the proteasome inhibitor on the SERT protein expression in lymphoblasts derived from MDD patients. The MG132 that inhibits the activity of the 26S proteasome was put in the cell culture medium at the final concentration of 20 μ M for 4 hours. In comparison with the protein levels of SERT in lymphoblasts derived from healthy subjects with vehicle, the protein levels of SERT with MG132 was 173.8% and significantly higher (Figure. 3: two-way ANOVA: $F_{\text{MG132}} (1, 30) = 11.53$, $p < 0.01$, $F_{\text{patient}} (2, 30) = 0.64$, $p = 0.54$, $F_{\text{MG132} \times \text{patient}} (2, 30) = 3.12$, $p = 0.06$). However, this phenomenon was not observed in lymphoblasts derived from either fluvoxamine-responsive (129.6%) or fluvoxamine-resistant MDD patients (104.8%) with MG132 (Figure. 3).

Discussion

The expression levels of SERT regulate serotonergic neurotransmission, depression-related behavioral, and histochemical changes [6, 17]. The SERT is a key biological target for antidepressant drugs. The polymorphism of the SERT gene appears to be associated with the therapeutic effect of antidepressants [29, 30]. Firstly, we investigated the protein levels of SERT in lymphoblasts derived from fluvoxamine-responsive and fluvoxamine-resistant MDD patients. We selected fluvoxamine because it has a higher selectivity than fluoxetine and paroxetine for blocking the serotonin reuptake [19]. The protein levels of SERT were lower in lymphoblasts derived from fluvoxamine-resistant depression patients. It has been suggested that the upregulation of SERT expression is related to the severity of depression and/or the efficacy of antidepressants in MDD patients.

The ubiquitin-proteasome system (UPS) controls the stability of most cellular proteins. Polymorphisms of UPS-related genes are associated with depression [7] and the antidepressant response [8]. The expression of the UPS-related gene is impaired in patients with mood disorders including MDD [9, 20]. Further, UPS plays a critical role in behavioral despair [25]. Among substrates of UPS, we have shown that SERT protein is ubiquitinated, and it is implicated in SERT stability and depressive behavior in mice [17]. In the present study, we showed that the ubiquitinated protein levels of SERT were lower in lymphoblasts derived from fluvoxamine-resistant MDD patients. This finding suggests that the downregulation of SERT ubiquitination is related to the severity of depression and/or the efficacy of antidepressants in MDD patients.

The proteasome inhibitor inhibits the degradation of the ubiquitinated SERT protein [17]. In the present study, the expression of SERT protein was increased by

proteasome inhibitor (MG132) in lymphoblasts derived from healthy subjects, but not either fluvoxamine-responsive or fluvoxamine-resistant MDD patients. This datum suggests that the responsiveness of SERT expression to proteasome inhibitor is related to the pathology of MDD.

Conclusion

The present report is first to demonstrate that the ubiquitination of SERT was lower in lymphoblasts derived from fluvoxamine-resistant MDD patients. Present findings suggest that the downregulation of ubiquitination of SERT protein induces insufficient degradation of SERT by proteasome, which resulted in the upregulation of expression of SERT protein in fluvoxamine-resistant MDD patients. Psychiatric disorders including depression are diagnosed by a psychiatrist on the basis of signs, symptoms, and the course of the illness. Searching for biological or physiological markers, rather than relying on behavioral symptoms and signs, might provide a more accurate diagnosis and serve as indicators of normal biological functioning, pathogenic processes, or pharmacological responses to a therapeutic intervention [23]. Moreover, the existence of a number of patients showing treatment-resistant MDD indicates that personalized treatments based on these biomarkers are needed. **There is an interaction between the number of stressful life events and functional polymorphism in the SERT gene promotor**

region (5-HTTLPR) on the risk of MDD in female adolescent subjects [3]. Thus, our study should take into account of combined influence of genotype, sex, age and environmental risk and their interactions on ubiquitination of SERT and MDD.

Although further studies with various populations will be required to generalize the results, SERT protein expression, ubiquitination, and the responsiveness of SERT expression to proteasome inhibitor could be a potential biomarker for the diagnosis of depression and the efficacy of antidepressants.

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Figure legends

Figure 1: The expression of SERT protein was higher in lymphoblasts derived from fluvoxamine-resistant MDD patients

Cell lysates were prepared from lymphoblasts derived from healthy subjects and fluvoxamine-responsive and fluvoxamine-resistant major depressive disorder (MDD) patients. The SERT protein was identified by the band (76 kD) detected by Western blotting (WB) using the antibody to the SERT. Values represent the mean $\pm$ SEM (n = 6). **p < 0.01 vs. lymphoblasts derived from healthy subjects.

Figure 2: The expression of ubiquitinated SERT protein was lower in lymphoblasts derived from fluvoxamine-resistant MDD patients

Cell lysates were prepared from lymphoblasts derived from healthy subjects and fluvoxamine-responsive and fluvoxamine-resistant major depressive disorder (MDD) patients. The ubiquitinated SERT was identified by the band (76 kD), which was detected by Western blotting (WB) using the antibody to the SERT protein by using the ubiquitinated protein bound to the UbiQapture-Q kit. Values represent the mean $\pm$ SEM (n = 6). **p < 0.01 vs. lymphoblasts derived from healthy subjects.

Figure 3: The proteasome inhibitor failed to increase the expression of SERT protein in lymphoblasts derived from both fluvoxamine-responsive and fluvoxamine-resistant MDD patients

Cell lysates were prepared from lymphoblasts derived from healthy subjects and fluvoxamine-responsive and fluvoxamine-resistant major depressive disorder (MDD) patients with or without MG-132. The SERT protein was identified by the band (76

kD) detected by Western blotting (WB) using the antibody to the SERT. Values represent the mean $\pm$ SEM (n = 6). **p < 0.01 vs. each vehicle-treated lymphoblasts.

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