Decreased gene expression activity as a result of a mutation in the calreticulin gene promoter in a family case of schizoaffective disorder

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Abstract Accumulating evidence of population association studies support the hypothesis that the high heritability of major psychiatric disorders is a combination of relatively common alleles of modest effect, and rare alleles some with relatively larger effects. We have previously reported low frequency mutations in the proximal promoter of the human calreticulin (CALR) gene that co-occur with the spectrum of major psychiatric disorders. One of those mutations at –205C>T (rs556992558) was detected in an isolate case of schizoaffective disorder. In the current study, the functional implication of mutation –205T is studied in the human neuronal cell lines LAN-5, BE(2)-C and HEK-293. In contrast with other mutations in the promoter region which increase gene expression activity, the –205T mutation significantly decreased gene expression in those cell lines in

comparison with the wild-type –205C nucleotide ($p < 0.000001$, $p < 0.0005$, and $p < 0.017$, respectively). Treatment of the cell lines with the mood-stabilizing drug, valproic acid (VPA) resulted in differential gene expression activity in the mutant –205T versus the wild-type –205C construct. VPA increased gene expression activity in both constructs, while a significantly higher expression activity was observed in the mutant construct ($p < 0.01$), indicative of the creation of a positive effector binding site for VPA as a result of the –205T mutation. We conclude that deviation from normalcy in the level of CALR in either direction is associated with major psychiatric disorders.

Keywords Calreticulin · Mutation · Promoter · Valproic acid · Expression · Psychiatric disorder

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Abbreviations

CALR	Calreticulin
DSM-IV-TR	Diagnostic and Statistical Manual-IV-Test Revised
ER	Endoplasmic reticulum
ERSE	Endoplasmic reticulum stress response elements
GABA	Gamma aminobutyric acid
MDD	Major depressive disorder
PCR	Polymerase chain reaction
SNP	Single nucleotide polymorphism
VPA	Valproic acid

Introduction

Aberrant expression of the genes related to the chaperone/immune system has been reported to be linked with psychosis (Arion et al. 2007; Harrison and Weinberger 2005;

Iwamoto and Kato 2006; Kakiuchi et al. 2003). Calreticulin (CALR) is an endoplasmic reticulum (ER) Ca(2+)-binding chaperone that interacts with 4-aminobutyrate type A (GABAA) receptor-associated protein, which also interacts with integrins, suggesting the involvement of CALR in synaptogenesis (Mohrluder et al. 2007). CALR is specifically and maximally expressed in the brain gray matter in late-adolescence and early adulthood in the animal primate model *Macaca fuscata* (Higashino and Kageyama 2008). The timing of this tissue-specific expression coincides with the spectrum of psychosis. CALR has a pivotal role in the differential trafficking of certain glutamate receptor subunit splice variants (Jaskolski et al. 2005; Nakamura et al. 2004), and also in the GABAergic interneurons migration and early neocortical network activity (De Lima et al. 2009).

Convergence of the glutamate and GABA-ergic pathways has been proposed as a mechanism for the pathophysiology of schizophrenia (Harrison and Weinberger 2005). Pharmacological evidence of the therapeutic effect of CALR (Bodmer and Bonilla 2008; Bown et al. 2002; Chen et al. 2000; Kim et al. 2005; Shao et al. 2006; Yuan et al. 2001) in psychosis, further strengthen the role of CALR as a promising candidate in the causation of major psychiatric disorders. We have previously reported cases of psychosis-associated promoter mutations with estimated frequencies of less than 0.0007 in the general population (Aghajani et al. 2006; Farokhashtiani et al. 2011; Olad Nabi et al. 2010). Those mutations occurred at positions –220 (rs138452745), –205 (rs556992558), and –48 (CR061331). The function of mutations –48C and –220A is to increase gene expression (Esmailzadeh-Gharehdaghi et al. 2011; Nunes et al. 2008). Mutation –205T was observed in an isolate case of schizoaffective disorder (Olad Nabi et al. 2010). In the current study, we examined a possible role of this mutation in the expression of the CALR gene in the human neuroblastoma cell lines BE(2)-C and LAN-5, and HEK-293, and show that in contrast with mutations –48C and –220A, this mutation results in constitutive decrease in gene expression activity. We also analyze the effect of valproic acid (VPA), one of the most widely-used mood stabilizing drug, on this mutation in the LAN-5 neuroblastoma cell line.

Materials and methods

Experimental procedures

Subjects

The patient included in this study carried a heterozygous mutation at position –205C>T (rs556992558) from the transcription start site. This patient is a 32-year-old female with a 16-year history of schizoaffective disorder (bipolar

type) (DSM-IV-TR), and is currently on treatment with valproic acid (VPA), and has responded to this medication. In a clinical assessment of the available family members (Fig. 1), the proband's mother, also heterozygous for the –205T mutation (Fig. 2), was diagnosed with major depressive disorder (MDD), and is currently under Sertraline anti-depressant treatment. Written informed consent was taken from the family members for their participation in this study. The University of Social Welfare and Rehabilitation Sciences Ethics Board approval was also obtained for this study.

Promoter cloning

The promoter region of CALR was amplified from peripheral blood genomic DNA, by PCR using the following primers: Forward: 5' AAA CTCGAG TGG GTT CAG GTC TGG TCA CAT G 3', Reverse: 5' AAA AAGCTT AGC GGC TCT GCA GTA CGG AC 3'. The fragment generated spanned the proximal promoter (Fig. 3), containing the TATA box and the three known endoplasmic reticulum stress response elements (ERSE), and has been shown to be transcriptionally active (Yamazaki et al. 2004). Briefly, PCR reactions were carried out in 25 µl volume, containing 100 ng genomic DNA, 10 pmol of each primer, 75 mM Tris–HCl pH 8.8 at 25 °C, 1.5 mM MgCl₂, 200 µM each of dNTP and 1 U of Taq DNA polymerase. PCR parameters were 30 s at 94 °C, 1 min at 58 °C, 45 s at 72 °C, for 30 cycles, followed by 5 min final extension at 72 °C. The resulting PCR products were cloned into pGL3 basic vector (Promega, UK) using the restriction sites *XhoI* and *HindIII* (Invitrogen, UK).

Cell culture, transfection, and luciferase analysis

Three cell lines BE(2)-C, LAN-5, and HEK-293 were purchased from Pasteur Institute in Tehran. Cells were cultured in DMEM, 10 % FBS, and 1X penicillin–

Fig. 1 Family of the proband. The mother, also carrying the –205T mutation, is affected with MDD

Fig. 2 The –205T mutation descended from the mother

Fig. 3 The constructs used for luciferase analysis. The promoter region of CALR was amplified from peripheral blood genomic DNA of the proband by PCR as described in experimental procedures

streptomycin (Biosera, UK) in an incubator with 5 % CO₂ and 95 % humidity. Each cell line in 12-well plates was transfected with 1 µg of pGL3-basic vector containing the wild-type –205C (pGL3wt) or –205T mutant (pGL3 mut) CALR promoter constructs, and pGL3-basic vector without insert as a control (Fig. 3), using FuGENE HD reagent according to the manufacturer's instructions (Roche Biochemicals, Germany). For normalization, all cells were co-transfected with 100 ng Tk-Renilla vector. To control for transfection efficiency, the beta-galactosidase (beta-Gal) reporter assay was carried out using the pSVβ-Gal Control Vector (Promega, USA), and the beta-Gal Staining Set Kit (Roche Applied Sciences, Germany). Transfection efficiencies were ~15, ~10, and ~50 % in BE(2)-C, LAN-5, and HEK-293, respectively, as measured by parallel transfection with beta-Gal. Concomitantly, cells were treated with 0.6 mM VPA. Twenty four hours post-transfection, cells were lysed in lysis buffer (Promega, UK),

firefly and Renilla luciferase activities were sequentially measured using Dual-Glo reagents (Promega, UK) in a Sirius Berthold luminometer.

Statistical analysis

Test of ANOVA was used to analyze results using OpenEpi software (<http://www.openepi.com/OE2.3/Menu/OpenEpiMenu.htm>).

Results

Transfection and dual luciferase analysis

We have recently reported a mutation in the human CALR gene promoter at –205T in a female case of schizoaffective disorder (Olad Nabi et al. 2010). In the current study, we compared the expression of the CALR –205T mutation with the wild-type –205C in the LAN-5, BE(2)-C, and HEK-293 cell lines. LAN-5 revealed the most dramatic decrease in gene expression of over fivefold ($p < 0.000001$; Fig. 4a). Over threefold decrease in gene expression was observed as a result of the –205T allele in the BE(2)-C cell line ($p < 0.0005$; Fig. 4b). 4.5-fold decrease in gene expression as a result of this allele was also detected in HEK 293 ($p < 0.017$; Fig. 4c).

Effect of VPA treatment of the cell lines harboring mutant –205T or wild-type –205C constructs

Following transfection of the human neuroblastoma cell line LAN-5 with the wild-type or mutant –205 constructs, cells were treated with VPA at 0.6 mM concentration. Differential effect to increase gene expression activity was observed in the cells with the mutant versus wild-type construct. VPA increased gene expression activity in both constructs, whereas a significantly higher expression activity of threefold was observed in the mutant construct ($p < 0.01$; Fig. 5).

Discussion

With low frequency mutations that co-occur with major psychiatric disorders and do not exist in the control pool, CALR offers a prime model for the pathogenesis of those disorders.

Two of the mutations identified in the promoter sequence of the CALR gene increase gene expression activity in neuronal cell lines. Neuroblastoma cell line models have been widely used for the analysis of gene expression patterns in major psychiatric disorders (Altar

Fig. 4 Effect of the $-205T$ mutation on transcriptional activity of the CALR promoter. **a** LAN-5, **b** BE(2)-C, and **c** HEK-293 cell lines were transfected with firefly luciferase-based reporter DNA together with wild-type or $-205C$ mutant CALR promoter constructs or pGL3 empty vector as control (see Fig. 2). To control transfection efficiency phTK-Renilla luciferase was also included. 24 h post-transfection, firefly and Renilla luciferase activities were sequentially measured in a Sirius luminometer (Berthold, Germany). Results are shown as the fold change in luciferase activity from the wild-type or mutant promoters in respect to luciferase activity from empty pGL3 vector. The $-205T$ mutation significantly decreased gene expression in those cell lines in comparison with the wild-type C-nucleotide ($p < 0.0005$, $p < 0.000001$, and $p < 0.017$, respectively). Data were analyzed by ANOVA test (OpenEpi). Results are mean $\pm$ SD, from three independent experiments carried out in triplicate

Fig. 5 Effect of VPA treatment of the cell line harboring mutant and wild-type constructs. Transfected LAN-5 cell line with the wild-type or mutant construct were treated with VPA in two independent experiments and each time in triplicate. The $-205T$ construct had a differential effect to increase gene expression threefold more than the wild-type construct ($p < 0.01$)

et al. 2008; Deslauriers et al. 2011; Lee et al. 2010). In the current study, we used LAN-5, BE(2)-C, and HEK-293 cell lines to examine the effect of the CALR $-205T$ mutation on gene expression. In contrast to the previously identified CALR promoter mutations, $-48C$ and $-220A$, which increase gene expression (Esmailzadeh-Gharehdaghi et al. 2011; Nunes et al. 2008), the $-205T$ mutation significantly decreases gene expression activity. Remarkably, the proband is a positive responder to VPA. These findings are in line with the pharmacological effect of VPA and lithium, which increase the expression of CALR (Bodmer and Bonilla 2008; Chen et al. 2000; Kim et al. 2005; Shao et al. 2006). Increase in gene expression activity was significantly higher in the cell lines with the mutant $-205T$ construct ($p < 0.01$). This indicates that $-205T$ recruit positive effectors of VPA, and may be considered a pharmacogenomic target for the augmentation of CALR.

We have recently identified an inhibitory effect of mutation $-220A$ on the action of VPA, which uniquely represses the function of VPA (Ohadi et al. 2012). Mutation $-48C$, on the other hand, has no effect on the action of VPA (Nunes et al. 2008). The mother, also carrying the $-205T$ mutation, is diagnosed with MDD. The clinically different diagnosis from the proband may be explained by the genetic overlap within the spectrum of psychiatric disorders (Gratacos et al. 2009; Green et al. 2009; Ohadi et al. 2007).

Previous research suggests that bipolar disorder is associated with significant reduction in brain volume and cell number in cerebral cortical and limbic regions. The

presence of cell loss and brain atrophy raises the possibility that neuroprotection may be involved in the mood stabilizing effect of pharmacological treatments of this disease. The mood stabilizer VPA activates mitogen-activated protein kinases and promotes neurite growth (Yuan et al. 2001).

For rare variants and deleterious mutations, it will nearly always be the case that the functional effect is due to the mutation itself (Bodmer and Bonilla 2008). This is because of the choice of candidate gene, the assessment of the effect of the mutation on the function of the gene product, and the extremely low probability of finding two rare variants with comparable functional effects in closely linked genes. In addition to the promoter mutations, we have recently detected a mutation in the first intron of the CALR gene in a case of substance-induced psychosis (Ohadi et al. 2012). Mutations identified by our group in the CALR gene are all non-existent in the control population pool. Their frequencies fall below the 0.1 % threshold, which positions them as deleterious mutations (Arion et al. 2007). Those findings in addition to the functionality of the mutations strengthen the possibility that the spectrum of major psychiatric disorders are, at least in part, a collection of very low frequency mutations (below the 0.1 % threshold for rare variants) that are absent from the control population. Re-sequencing of the candidate gene regulatory regions is warranted to test this model.

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