Differential expression of the ghrelin-related mRNAs GHS-R1a, GHS-R1b, and MBOAT4 in Japanese patients with schizophrenia

Shunsuke Nakata1, Yuta Yoshino1, Mitsuo Okita, Kentaro Kawabe, Kiyohiro Yamazaki, Yuki Ozaki, Yoko Mori, Shinichiro Ochi, Jun-ichi Iga*, Shu-ichi Ueno

Department of Neuropsychiatry, Molecules and Function, Ehime University Graduate School of Medicine, Shitsukawa, Toon, Ehime 791-0295, Japan

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ABSTRACT

Objectives: Ghrelin regulates appetite and also plays important roles in cognition and may be involved in vulnerability to SCZ.

Methods: In this study, we measured mRNA expression of the ghrelin-related molecules, growth hormone secretagogue receptor 1a (GHS-R1a) and 1b (GHS-R1b), and the ghrelin activator, membrane bound O-acyltransferase 4 (MBOAT4). Peripheral leukocytes from Japanese patients with SCZ (n = 49; 23 males, 26 females; age = 61.8 ± 13.3 years) and controls (n = 50; 25 males, 25 females; age = 62.0 ± 14.3 years) were recruited according to their clinical information. We also studied the DNA methylation rates of these genes in DNA from leukocytes.

Results: The mRNA expression of GHS-R1a was significantly decreased in SCZ (SCZ vs. control: 0.35 ± 0.081 vs. 1.00 ± 0.059, respectively, p = 0.007), but expression levels of GHS-R1b and MBOAT4 were significantly increased in SCZ (SCZ vs. control: 2.02 ± 0.91 vs. 1.00 ± 0.32, p = 0.023, 1.37 ± 0.21 vs. 1.00 ± 0.11, respectively, p = 0.014). No differences in methylation rates for any genes were found.

Conclusion: We conclude that opposite expression of GHS-R1a and GHS-R1b, and elevated MBOAT4 mRNA expression may reflect the mechanisms of SCZ.

1. Introduction

Schizophrenia is a chronic psychiatric disorder with a heterogeneous genetic and neurobiological background that influences early brain development and combination of psychotic symptoms such as hallucinations, delusions and disorganization and motivational and cognitive dysfunctions (Kahn et al., 2015). The mean lifetime prevalence of the disorder is just below 1%, but large regional differences in prevalence rates are evident owing to disparities in urbanicity and patterns of immigration (Perala et al., 2007). Prospectively designed outcome studies suggested that the course and outcome of schizophrenia is characterized by mainly unexplained heterogeneity rather than uniform poor outcome (van Os & Kapur, 2009). Several hypotheses about the pathogenesis of SCZ, such as the dopamine hypothesis (Seeman & Lee, 1975) and the glutamate hypothesis (Hu et al., 2015), have been well discussed. SCZ is characterized by positive, negative, and cognitive symptoms. Neurocognitive impairment is one of the cognitive symptoms of SCZ (Mesholam-Gately et al., 2009). A wide range of cognitive dysfunctions in the domains of language, memory, and executive functions is seen in SCZ patients (Sumiyoshi et al., 2010). The neurodevelopmental model supports that SCZ genetic and epigenetic risk factors converge on early brain development to perturb neurodevelopmental trajectories. Post-mortem studies of brain gene expression and DNA methylation suggest that risk factors for SCZ, both genetic and epigenetic variations that leave scars in the adult brain, occur principally during early brain development rather than during risk period of onset (late adolescence) (Kahn et al., 2015). Because there is evidence that genetic liability for schizophrenia is mediated in part by differential sensitivity to environments (van Os et al., 2010), endogenous hormones such as ghrelin may be involved in the pathophysiological gene-environmental interplay in schizophrenia.

Ghrelin is a well-known appetite peptide hormone that was originally identified in rodent stomach (Kojima et al., 1999). Ghrelin and its receptor genes are conserved among almost all vertebrates.

Abbreviations: SCZ, schizophrenia; GHS-R1a, growth hormone secretagogue receptor 1a; GHS-R1b, growth hormone secretagogue receptor 1b; MBOAT4, membrane bound O-acyltransferase 4; GH, growth hormone; BPRS, Brief Psychiatric Rating Scale; DIEPSS, Drug-Induced Extrapyramidal Symptoms Scale

* Corresponding author.
E-mail address: iya.junichi.it@ehime-u.ac.jp (J.-i. Iga).
1These authors contributed equally to this work.

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Ghrelin is synthesized and released by gastric neuroendocrine cells and is present in various organs such as the intestine, kidney, hypothalamus, and pituitary (Gnanapavan et al., 2002).

Ghrelin is acylated by membrane bound O-acyltransferase 4 (MBOAT4), which is expressed in several tissues including the hippocampus (Gutierrez et al., 2008; Yang et al., 2008; Murtuza and Isokawa, 2018). Ghrelin was identified as an endogenous ligand of growth hormone (GH) secretagogue receptor 1a (GHS-R1a) (Howard et al., 1996). Acylated ghrelin binds to GHS-R1a, and deacylated ghrelin does not. Most ghrelin-regulated functions are regulated by the acylated or deacylated variants (Kojima & Kangawa, 2010). Additionally, GHS-R1b, which is an alternatively spliced variant of GHS-R, is a strong inhibitor of GHS-R1a. GHS-R1b dimerizes with GHS-R1a and has a dominant negative effect on GHS-R1a-mediated signaling as a direct consequence (Leung et al., 2007). Heterodimers of GHS-R1a and GHS-R1b also block GHS-R1a by inducing a conformational change that does not have a signaling ability (Mary et al., 2013).

GHSR mRNA expression levels are relatively high in the hypothalamus, pituitary, and hippocampus in human brain as seen with in situ hybridization (Guan et al., 1997; Gnanapavan et al., 2002). Ghrelin plays an important role in the regulation of sleep (Kluge et al., 2008), and various parameters was evaluated with the Spearman's rank correlation coefficient. Significant difference is shown in bold (p < 0.05). Values are the mean ± standard deviation. Abbreviations: CP equation: chlorpromazine equivalent, BPRS: Brief Psychiatric Rating Scale, DIEPPS: Drug-Induced Extrapyramidal Symptoms Scale, BMI: body mass index.

### 2. Blood collection, total RNA isolation, and real-time PCR

We collected whole peripheral blood samples in PaxGene Blood RNA Systems tubes (BD, Tokyo, Japan) between 10:00 a.m. to 5:00 p.m. and extracted total RNA. The RNA concentration and purity were determined using absorption spectrophotometry with Nano Drop-1000 (Thermo Fisher Scientific, Yokohama, Japan). RNA (1 μg per sample) was reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) in a total volume of 40 µL. Comparative evaluation of quantitative real-time PCR was performed using TaqMan probes (Assay ID: GHS-R1a, Hs1026313_m1, GHS-R1b, Hs00269780_s1, and MBOAT4, Hs0174954_s1; Applied Biosystems) with the TaqMan gene expression master mix and StepOnePlus real-time PCR system (Applied Biosystems). Each sample was analyzed in duplicate.

### 2.3. DNA isolation and sodium bisulfite conversion of DNA

Genomic DNA was extracted from leukocytes using the QIAcube blood mini kit (Qiagen, Tokyo, Japan) and stored at 4°C. Bisulfite conversion of DNA (1 μg per sample) and subsequent purification were performed using the Epitect Bisulfite Kit (Qiagen) and QIAcube (Qiagen) according to the manufacturer’s instructions.

### 2.4. PCR amplification

JASPAR (http://jaspar.binf.ku.dk/) was used to identify five CpG sites in GHSR and two CpG sites in MBOAT4 that were predicted to bind major transcription factors. Five sites in the GHSR promotor scored the highest number of transcription factors, and two CpG sites in MBOAT4 intron 1 were predicted to bind transcription factors (predictive value > 8). These CpG sites were adjacent to the CpG sites of the hypomethylated region in the GHSR promotor and MBOAT4 intron 1 (http://genome.ucsc.edu/). Primers were designed using Pyromark Assay Design software, version 2.0 (Qiagen). Fig. 1a and b show the CpG sites in the GHSR promotor and MBOAT4 intron 1 and the associated transcription factors. GHSR primer sequences were: 5’-GGTTGG GAGGTTTTAGGTATAT-3’ (forward) and 5’-ACTCAACCCCCAAAATC-3’ (reverse). MBOAT4 primer sequences were: 5’-AGGGGGGAAAGTTATTA TAAGTAGTAT-3’ (forward) and 5’-ACTCAAAAAATCTTCTTACC-3’ (reverse). Bisulfite-converted DNA (100 ng per 2.0 µL) was used as a template for PCR that included 0.2 mM dNTP (Applied Biosystems), 10 × PCR buffer with 15 mM MgCl2 (Applied Biosystems), 0.5 U AmpliTaq gold (Applied Biosystems), and 0.2 µM forward and reverse primers (final volume 25.0 µL).

Each PCR was performed with an initial denaturation for 10 min at 94 °C, followed by 45 cycles of denaturation for 30 s at 94 °C, annealing

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### Table 1

Demographic data of schizophrenia persons.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>GHS-R1a</th>
<th>GHS-R1b</th>
<th>MBOAT4</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>49</td>
<td>49</td>
<td>49</td>
</tr>
<tr>
<td>Sex (male/female)</td>
<td>23:26</td>
<td>23:26</td>
<td>23:26</td>
</tr>
<tr>
<td>Age (years)</td>
<td>61.8 ± 13.3</td>
<td>61.8 ± 13.3</td>
<td>61.8 ± 13.3</td>
</tr>
<tr>
<td>Age of onset (years)</td>
<td>30.3 ± 12.9</td>
<td>30.3 ± 12.9</td>
<td>30.3 ± 12.9</td>
</tr>
<tr>
<td>Duration of illness (years)</td>
<td>31.8 ± 13.5</td>
<td>31.8 ± 13.5</td>
<td>31.8 ± 13.5</td>
</tr>
<tr>
<td>CP equation</td>
<td>563.9 ± 358.0</td>
<td>563.9 ± 358.0</td>
<td>563.9 ± 358.0</td>
</tr>
<tr>
<td>BPRS</td>
<td>31.0 ± 10.5</td>
<td>31.0 ± 10.5</td>
<td>31.0 ± 10.5</td>
</tr>
<tr>
<td>DIEPPS</td>
<td>4.4 ± 3.3</td>
<td>4.4 ± 3.3</td>
<td>4.4 ± 3.3</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>22.2 ± 5.5</td>
<td>22.2 ± 5.5</td>
<td>22.2 ± 5.5</td>
</tr>
</tbody>
</table>

The correlation between mRNA expression of GHS-R1a, GHS-R1b and MBOAT4 and various parameters was evaluated with the Spearman's rank correlation coefficient.
for 30 sec at 56 °C for GHSR or 57 °C for MBOAT4, and elongation for 1 min at 72 °C, followed by a final extension at 72 °C for 10 min.

2.5. Determination of methylation rates

We analyzed each sample in duplicate. The methylation rates of each CpG site were determined by Pyromark Q24 and were accurately analyzed with PyroMark Q24 Advanced software, version 3.0.0 (Qiagen).

2.6. Statistical analysis

Statistical tests were performed using SPSS 22.0 software (IBM Japan, Tokyo, Japan). The Shapiro-Wilk test was used to determine normality. The Mann-Whitney U-test with post-hoc Bonferroni correction was performed to compare the methylation rates of each CpG site between SCZ and control persons. The Fisher’s exact test was performed to evaluate gender differences. Correlations between various clinical parameters and the expression levels of GHS-R1a, GHS-R1b, and MBOAT4 were analyzed using the Spearman’s rank correlation coefficient. $p = 0.05$ was considered statistically significant.
3. Results

3.1. GHSR: GHS-R1a and GHS-R1b, and MBOAT4 mRNA expression

GHS-R1a mRNA expression levels in peripheral leukocytes were significantly lower in SCZ patients compared with those in control persons (0.35 ± 0.081 vs. 1.00 ± 0.059, respectively, \( p = 0.007 \)). However, GHS-R1b and MBOAT4 mRNA expression levels were higher in SCZ than in controls \((2.02 ± 0.91 \text{ vs. } 1.00 ± 0.32, p = 0.023; 1.37 ± 0.21 \text{ vs. } 1.00 ± 0.11, p = 0.014\), respectively) (Fig. 2).

GHS-R1b: \( r = 0.970 \), but we did identify a significant correlation between GHS-R1b and MBOAT4 mRNA expression levels. \( r = 0.115 \); in SCZ. A positive correlation between GHS-R1b and MBOAT4 mRNA expression levels may have contributed to the elevated MBOAT4 mRNA expression via negative feedback to compensate for the reduced ghrelin effects that lead to reduced neuroprotection in SCZ.

3.2. GHSR and MBOAT4 methylation rates

We found no significant differences in GHSR or MBOAT4 methylation rates between SCZ and controls (Tables 2a, 2b).

Table 2a
<table>
<thead>
<tr>
<th>GHSR methylation rates.</th>
<th>SCZ</th>
<th>Ct</th>
<th>( p ) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CpG1</td>
<td>8.9 ± 2.1</td>
<td>8.5 ± 1.4</td>
<td>0.564</td>
</tr>
<tr>
<td>CpG2</td>
<td>12.8 ± 2.9</td>
<td>12.1 ± 1.8</td>
<td>0.415</td>
</tr>
<tr>
<td>CpG3</td>
<td>26.5 ± 5.1</td>
<td>25.8 ± 3.4</td>
<td>0.543</td>
</tr>
<tr>
<td>CpG4</td>
<td>15.9 ± 3.2</td>
<td>16.1 ± 3.4</td>
<td>0.994</td>
</tr>
<tr>
<td>CpG5</td>
<td>8.9 ± 2.2</td>
<td>9.0 ± 2.2</td>
<td>0.774</td>
</tr>
<tr>
<td>Average</td>
<td>14.6 ± 2.8</td>
<td>14.3 ± 2.0</td>
<td>0.698</td>
</tr>
</tbody>
</table>

Values are the mean ± standard deviation. SCZ: schizophrenia persons, Ct: control persons.

Table 2b
<table>
<thead>
<tr>
<th>MBOAT4 methylation rates.</th>
<th>SCZ</th>
<th>Ct</th>
<th>( p ) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CpG1</td>
<td>4.7 ± 1.1</td>
<td>5.1 ± 1.6</td>
<td>0.719</td>
</tr>
<tr>
<td>CpG2</td>
<td>5.9 ± 2.1</td>
<td>5.4 ± 1.3</td>
<td>0.448</td>
</tr>
<tr>
<td>Average</td>
<td>5.3 ± 1.5</td>
<td>5.2 ± 1.3</td>
<td>0.812</td>
</tr>
</tbody>
</table>

Values are the mean ± standard deviation. SCZ: schizophrenia persons, Ct: control persons.

3.3. Correlation between mRNA expression of GHS-R1a, GHS-R1b, and MBOAT4 and their promoter methylation rates

We found no correlations between GHS-R1a, GHS-R1b, and MBOAT4 mRNA expression and their respective promoter methylation rates. Each correlation was as follows: GHS-R1a (CpG1: \( r = 0.171 \), \( p = 0.098 \), CpG2: \( r = 0.13, p = 0.21 \), CpG3: \( r = 0.12, p = 0.247 \), CpG4: \( r = 0.137, p = 0.185 \), CpG5: \( r = 0.065, p = 0.532 \), GHS-R1b (CpG1: \( r = −0.087, p = 0.392 \), CpG2: \( r = 0.0136, p = 0.724 \), CpG3: \( r = 0.176, p = 0.083 \), CpG4: \( r = 0.239, p = 0.018 \), CpG5: \( r = −0.137, p = 0.178 \), and MBOAT4 (CpG1: \( r = −0.054, p = 0.598 \), CpG2: \( r = −0.106, p = 0.299 \), average: \( r = −0.076, p = 0.456 \)).

4. Discussion

This study revealed two major findings. First, mRNA levels of GHS-R1a were significantly lower in SCZ than in controls. However, GHS-R1b and MBOAT4 mRNA expression levels were significantly higher in SCZ than in controls. These findings suggest that lower GHS-R1a and elevated GHS-R1b and MBOAT4 mRNA expression levels may reflect attenuation of the neuroprotective effects of ghrelin in SCZ. According to Birkás Kováts et al. (2005) and Palik et al. (2005), the serum ghrelin levels in SCZ patients are significantly higher than those in healthy persons. On the other hand, Togo et al. (2004) reported the opposite results. Although several studies reported an increase in serum ghrelin levels in response to antipsychotics (Palik et al., 2005; Murashita et al., 2005; Eser-Danaci et al., 2008; Sentissi et al., 2009), others reported no difference (Himmerich et al., 2005; Theisen et al., 2005) or a decrease (Togo et al., 2004; Hosojima et al., 2006; Kim et al., 2008; Tanaka et al., 2008; Wysokinski et al., 2014). In addition, serum ghrelin levels fluctuate with eating (Cummings et al., 2001; Tschop et al., 2001).

Ribeiro et al. (2014) proposed that ghrelin enhances cognition through GHS-R1a, leading to activation of the hippocampus and enhancement of excitatory synaptic transmission and synaptic plasticity by regulating gamma-aminobutyric acid (GABA)ergic neurotransmission. Intracerebroventricular administration of ghrelin in rats increases memory retention, indicating that ghrelin may affect these processes in the hippocampus (Carlini et al., 2002). In this study, GHS-R1a mRNA levels were significantly decreased in SCZ. In addition, the mRNA expression of GHS-R1b, which encodes a strong inhibitor of GHS-R1a and increased GHS-R1b mRNA expression levels may have contributed to the elevated MBOAT4 mRNA expression via negative feedback to compensate for the reduced ghrelin effects that lead to reduced neuroprotection in SCZ. The significant positive correlation between GHS-R1b and MBOAT4 mRNA expression levels indicates that a direct genetic relationship may exist between GHS-R1b and MBOAT4. Further studies are needed to identify the mechanism and determine whether feedback exists between them.

Second, we found a significant negative correlation between MBOAT4 mRNA expression and age in both SCZ and controls (\( r = −0.333, p = 0.021; r = −0.592, p < 0.001 \), respectively.
Table 1. Although Paik et al. (2004) reported a negative association between total plasma ghrelin levels and age, others did not (Villarrasa et al., 2005; Langenberg et al., 2005). However, as mentioned, ghrelin exists as active and inactive forms, and the active form binds GHS-R1a, which has a potent GH-releasing effect (Howard et al., 1996). Broglio et al. (2003) reported that this GH-releasing effect of ghrelin decreases with age. Thus, the negative correlation between MBOAT4 mRNA and age is reasonable.

GHSR and MBOAT4 methylation rates were not different between SCZ and control persons. This may have been because of two reasons. First, the most critical CpG sites of GHSR and MBOAT4 may not have been identified yet. Second, other factors may affect the mRNA expression of GHSR and MBOAT4.

This study has several limitations. First, this study was performed using a relatively small sample size. Hence, our study may contain a beta error. Further studies should address this point. Second, DNA and RNA were obtained from leukocytes, and we did not discriminate expression according to cell type. CpG sites in the target regions were not significantly different among various leukocyte subsets such as neutrophils, B cells, and CD4 + T cells, using a publicly available dataset (UCSC Genome Browser; https://genome.ucsc.edu/). Influences of diurnal variation, socioeconomic status, IQ, food or physical condition (e.g., body mass index, smoking, infection and inflammation) on gene expression were not examined. We did not apply Bonferroni correction to all analysis, which may increase false positive findings. Further studies are necessary to investigate the correlation among the mRNA expression of ghrelin-related genes, methylation, and cognitive function in SCZ.

In summary, GHS-R1a mRNA expression levels were decreased and GHS-R1b and MBOAT4 mRNA expression levels were increased in leukocytes from SCZ patients compared to controls. We found a significant correlation between MBOAT4 mRNA expression and age in both SCZ and controls. We suggest that decreased GHS-R1a and increased GHS-R1b mRNA expression levels in peripheral leukocytes contribute to elevated MBOAT4 mRNA expression to compensate for the reduced ghrelin effects that lead to reduced neuroprotection in SCZ. These findings suggest that disruption in ghrelin systems may be related to one of the pathological conditions of SCZ.

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Conflicts of interest

None to declare.

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Birkat Kováts, D., Faludi, G., Cseh, K., 2005. Possible connection between SCZ and control persons. This may have been because of two reasons. First, the most critical CpG sites of GHSR and MBOAT4 may not have been identified yet. Second, other factors may affect the mRNA expression of GHSR and MBOAT4. This work was partially supported by a Health and Labour Technology, JSPS KAKENHI Grant Numbers 18K07564 and 16K21207.


