ORIGINAL ARTICLE

Enhanced Carbonyl Stress in a Subpopulation of Schizophrenia

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Context: Various factors are involved in the pathogenesis of schizophrenia. Accumulation of advanced glycation end products, including pentosidine, results from carbonyl stress, a state featuring an increase in reactive carbonyl compounds (RCOs) and their attendant protein modifications. Vitamin B_6 is known to detoxify RCOs, including advanced glycation end products. Glyoxalase I (GLO1) is one of the enzymes required for the cellular detoxification of RCOs.

Objectives: To examine whether plasma levels of pentosidine and serum vitamin B_6 are altered in patients with schizophrenia and to evaluate the functionality of *GLO1* variations linked to concomitant carbonyl stress.

Design: An observational biochemical and genetic analysis study.

Setting: Multiple centers in Japan.

Participants: One hundred six individuals (45 schizophrenic patients and 61 control subjects) were recruited for biochemical measurements. Deep resequencing of *GLO1* derived from peripheral blood or postmortem brain tissue was performed in 1761 patients with schizophrenia and 1921 control subjects.

Main Outcome Measures: Pentosidine and vitamin B_6 concentrations were determined by high-performance liquid chromatographic assay. Protein expression and enzymatic activity were quantified in red blood cells and lymphoblastoid cells using Western blot and spectrophotometric techniques.

Results: We found that a subpopulation of individuals with schizophrenia exhibit high plasma pentosidine and low serum pyridoxal (vitamin B_6) levels. We also detected genetic and functional alterations in *GLO1*. Marked reductions in enzymatic activity were associated with pentosidine accumulation and vitamin B_6 depletion, except in some healthy subjects. Most patients with schizophrenia who carried the genetic defects exhibited high pentosidine and low vitamin B_6 levels in contrast with control subjects with the genetic defects, suggesting the existence of compensatory mechanisms.

Conclusions: Our findings suggest that GLO1 deficits and carbonyl stress are linked to the development of a certain subtype of schizophrenia. Elevated plasma pentosidine and concomitant low vitamin B_6 levels could be the most cogent and easily measurable biomarkers in schizophrenia and should be helpful for classifying heterogeneous types of schizophrenia on the basis of their biological causes.

Arch Gen Psychiatry. 2010;67(6):589-597

SCHIZOPHRENIA IS A DEBILITATING and complex mental disorder with a prevalence of approximately 1% worldwide. Its pathophysiology remains unclear, despite extensive research.[1,2] Biochemical and pharmacological studies using human samples and animal models suggest that oxidative/carbonyl stress contributes to the pathophysiology of schizophrenia.[3-6] Oxidative stress is a central mediator of advanced glycation end product (AGE) formation, and pyridoxamine (vitamin B_6, biosynthesized from pyridoxal in vivo) is known to detoxify reactive carbonyl compounds via carbonyl-amine chemistry. Toxic reactive carbonyl compounds such as α-oxoaldehydes (eg, methylglyoxal, glyoxal, and 3-deoxyglucosone) are formed from sugars, lipids, and amino acids.[7-9] Accumulation of such reactive carbonyl compounds, referred to as carbonyl stress,[10] results in the modification of proteins and the eventual formation of AGEs such as pentosidine. Cellular removal of AGEs hinges largely on the activity of the zinc metalloenzyme glyoxalase I (GLO1).[11] The glyoxalase detoxifi-

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Table 1. Genetic and Biochemical Analyses in Schizophrenic Patients and Control Subjects

Characteristic	No. (%) Schizophrenic Patients (n=1761)	No. (%) Control Subjects (n=1921)	Main Application
Institutions where DNA was collected, No.			
Tokyo Institute of Psychiatry	261	302	Resequence
Tokyo Metropolitan Matsuzawa Hospital (postmortem brain tissue)	70	1	Resequence plasmid construction
RIKEN Brain Science Institute	1156	1502	Resequence
Okayama University	274	116	Resequence
Pentosidine level[a]			
Very high, >130 ng/mL	3 (6.7)[b]	0	HPLC
High, >55.2 ng/mL	18 (40.0)	2 (3.3)	
Normal, <55.2 ng/mL	24 (53.3)	59 (96.7)	
Vitamin B_6, pyridoxal level[a]			
Normal: male, 6-27 ng/mL; female, 4-42 ng/mL	19 (42.2)	54 (88.5)	HPLC
Low: male, <6 ng/mL; female, <4 ng/mL	15 (33.3)	7 (11.5)	
Very low, <3 ng/mL	11 (24.4)[b]	0	

Abbreviation: HPLC, high-performance liquid chromatography.
[a] Forty-five schizophrenic patients; 61 healthy control subjects.
[b] For detailed information, see Table 3.

cation system is ubiquitous in human tissues, including the brain. The GLO1 detoxification system interacts with several metabolizing cascades, and some compounds in these cascades have been reported as candidates for involvement in the etiology of schizophrenia, such as glutathione, homocysteine, and folic acid metabolites (eFigure 1, available at http://www.prit.go.jp/En/PSchizo/TSchizo/archives.html).[12-19]

Recent studies have revealed that dysfunction of GLO1 is involved not only in systemic diseases such as diabetes mellitus[20] and vascular injury,[21] but also in neuropsychiatric disorders such as mood disorder,[22] autism,[23,24] anxiety disorders,[25] alcoholism,[26] and Alzheimer disease.[7] In mice, levels of *Glo1* expression have been associated with anxiety-like behavioral phenotypes.[27-29] *GLO1* has been mapped to chromosome 6p21, a linkage region for schizophrenia.[30-32] A missense polymorphism, Glu111/Ala111, has been reported in 2 multiplex Caucasian pedigrees with schizophrenia spectrum disorders.[33] However, the functional significance of this polymorphism has not been addressed.

The present study examined whether plasma levels of pentosidine and serum vitamin B_6 are altered in patients with schizophrenia. If so, *GLO1* polymorphisms associated with functional deficits could be an underlying substrate of schizophrenia. To the best of our knowledge, this is the first study to suggest enhanced carbonyl stress as an underlying mechanism of schizophrenia.

METHODS

SUBJECTS

Materials for resequencing of the *GLO1* gene were obtained from 1761 schizophrenic patients (mean age, 50.1 years [SD, 13.9 years]) and 1921 healthy control subjects (mean age, 42.5 years [SD, 14.4 years]) (**Table 1**). For genetic study, the affected individuals were randomly recruited from among both inpatients and outpatients. Cases were composed of 961 men (mean age, 49.0 years [SD, 13.4 years]) and 800 women (mean age, 51.4 years [SD, 14.3 years]). Control subjects were composed of 779 men (mean age, 41.2 years [SD, 13.6 years]) and 1142 women (mean age, 43.0 years [SD, 14.8 years]). DNA extracted from 71 postmortem brain tissue specimens was used for resequencing. We did not assess associations between common variants and schizophrenia, as the aim of this study was to focus on rare variations to reveal large biological effects, thus enabling clarification of pathophysiology in rare cases of schizophrenia. These samples were therefore not matched by age or sex. Schizophrenia was diagnosed according to the *DSM-IV* to obtain a best-estimate lifetime diagnosis, with consensus of at least 2 experienced psychiatrists. No structured interviews were performed. Ten percent of patients exhibited discordant subtypes. The available medical records and family informant reports were also taken into consideration. Control subjects were recruited from among hospital staff and company employees documented to be free from mental illness based on brief interviews by experienced psychiatrists. The companies that provided employees as control subjects for our study were biochemical, pharmaceutical, and medical device manufacturers. We personally announced recruitment of volunteers for our research at annual meetings such as those of the Japanese Society of Biological Psychiatry and the Japanese Society of Schizophrenia Research.

Fresh plasma and serum samples were obtained from 45 available schizophrenic patients and 61 healthy controls among the subjects included in the genetic study (Table 1). Diabetes mellitus and renal dysfunction were criteria for exclusion in selecting patients and healthy control subjects, as these diseases may potentially increase pentosidine levels.

All participants provided written informed consent, and the study protocols were approved by the ethics committees of all participating institutions (Tokyo Institute of Psychiatry,[34] Tokai University, RIKEN Brain Science Institute,[35-38] Okayama University,[39] Tokyo Metropolitan Matsuzawa Hospital, Hamamatsu University, Chiba University, and Tohoku University).

RESEQUENCING ANALYSIS OF *GLO1*

All the coding regions and exon-intron boundaries as well as the 5′ upstream region of *GLO1* were examined by direct sequencing of the polymerase chain reaction (PCR) products. Polymerase chain reaction amplification was performed using the sets of

primers listed in eTable 1 and Blend Taq polymerase (Toyobo, Osaka, Japan). Detailed information on the PCR amplification conditions is available from the authors upon request. Sequencing of PCR products was performed using a BigDye Terminator Cycle Sequencing Reaction Kit (Applied Biosystems, Foster City, California) and an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). We read both strands when an inserted or deleted nucleotide yielded dual signals derived from wild-type and mutant-type strands. Moreover, to confirm a single base insertion or deletion, PCR fragments were subcloned into a pTA2 plasmid vector (Toyobo) and sequenced.

GLO1 ENZYMATIC ASSAY

Fresh blood samples were obtained from 45 schizophrenic patients and 61 healthy control subjects (Table 1). Red blood cells (RBC), plasma, and serum were separated by centrifugation and used in subsequent studies. Glyoxalase I enzymatic activity in RBC was determined using the spectrophotometric method described by McLellan and Thornalley.[40] Briefly, washed RBC were lysed with 4 volumes of ice-cold distilled water and kept on ice for more than 30 minutes to complete hemolysis. Debris was removed by centrifugation and the supernatant was assayed for enzymatic activity. Activity of the GLO1 enzyme is given in units/10^6 RBC, where 1 unit is the amount of enzyme required to catalyze the formation of 1 µmol of S-D-lactoylglutathione per minute from hemithioacetal. Hemithioacetal was prepared by preincubation of 2mM methylglyoxal with 2mM glutathione in a 50mM sodium phosphate buffer (pH 6.6) at 37°C for 10 minutes. The increase in absorbance at 240 nm owing to the formation of S-D-lactoylglutathione was measured by spectrophotometry. Prominently low enzymatic activities were confirmed by at least 3 measurements.

MEASUREMENT OF PENTOSIDINE AND VITAMIN B_6

Pentosidine, an AGE, was determined by high-performance liquid chromatography assay as described previously.[41] In brief, the plasma sample was lyophilized, hydrolyzed in 100 µL of 6N of hydrochloric acid for 16 hours at 110°C under nitrogen, neutralized with 100 µL of 5N of sodium hydroxide and 200 µL of a 0.5M sodium phosphate buffer (pH 7.4), filtered through a 0.5-µm filter, and diluted with phosphate-buffered saline (PBS). A sample (corresponding to 25 µg of protein) was injected into a high-performance liquid chromatography system and fractionated on a C18 reverse-phase column. Effluent was monitored at excitation-emission wavelengths of 335/385 nm using a fluorescence detector (RF-10A; Shimadzu, Kyoto, Japan). Synthetic pentosidine was used to obtain a standard curve. We measured pentosidine at least twice, and additional measurements were performed 3 times to confirm 3 outliers. Three forms of vitamin B_6 (pyridoxine, pyridoxal, and pyridoxamine) were measured in serum samples by high-performance liquid chromatography according to a previously described method.[42] Other parameters (glucose, glycohemoglobin A_{1C}, total cholesterol, triglyceride, aspartate aminotransferase, alanine aminotransferase, creatinine, urea nitrogen, total protein, and albumin) were measured in blood samples. Glomerular filtration rate was estimated using the abbreviated Modification of Diet in Renal Diseases study equation.[43]

WESTERN BLOTTING

The GLO1 protein expression in RBC lysate was assessed by Western blotting analysis after sodium dodecyl sulfate–polyacrylamide gel electrophoresis using 5% to 20% polyacrylamide gradient gel. Polyclonal anti-GLO1 sera, designated NT2, were raised in rabbits by immunization with a human GLO1 peptide MAEPQPPSGGLTDEAALSC (corresponding to amino acids 1-19) conjugated to keyhole limpet hemocyanin. Equal volumes of RBC lysates were treated with Laemmli buffer, boiled at 100°C for 5 minutes, applied to the gel, and transferred to polyvinylidene fluoride membranes. Blots were treated with 100% BlockingOne (Nacalai, Kyoto, Japan) to block any nonspecific binding sites at 4°C overnight. The membrane was washed with PBS containing 0.05% Tween 20 (PBS-T) and then incubated with 1-µg/mL rabbit anti-GLO1 antibody (NT2) and mouse anti–glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (1:200; Santa Cruz Biotechnology, Santa Cruz, California) as an internal control in PBS-T containing 5% BlockingOne for 1 hour at room temperature. Anti-GLO1 antibody was affinity-purified using beads coupled with the antigen peptide. The membrane was washed again 3 times with PBS-T and then incubated with peroxidase-conjugated anti-mouse Ig (1:1000) and peroxidase-conjugated anti-rabbit Ig (1:1000) (Vector, Burlingame, California) for 1 hour at room temperature, followed again by a wash and eventual development with 3,3′-diaminobenzidine tetrahydrochloride solution (Sigma, St Louis, Missouri). The GLO1 signals that were normalized to GAPDH were quantified using National Institutes of Health image software (http://rsb.info.nih.gov/nih-image/). Researchers were blind to *GLO1* genotypes during experiments with Western blotting. We performed at least 2 determinations for each sample.

CELL CULTURE

Epstein-Barr virus–transformed lymphoblastoid cell lines derived from patients and normal subjects were established at SRL Inc (Tokyo, Japan). Lymphoblastoid cell lines were grown in RPMI 1640 medium (Wako, Osaka, Japan) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, California) and antibiotic liquid (Nacalai, Kyoto, Japan). Cell lines were cultured at 37°C in a humidified atmosphere incubator under 5% carbon dioxide.

STATISTICAL ANALYSIS

Data were analyzed using PRISM software (GraphPad Software, San Diego, California). Simple comparisons of means and standard errors of data were performed using an unpaired *t* test or the Mann-Whitney test (both 2-tailed). The χ^2 and Pearson correlation tests were used to assess the significance of association between the data. For comparison of more than 2 groups, 1-way analysis of variance was used. If the results of analysis of variance were significant, the Bonferroni procedure was used as a post hoc test. Significance was defined as $P < .05$.

RESULTS

PENTOSIDINE ACCUMULATION AND PYRIDOXAL DEPLETION

We measured plasma pentosidine and serum pyridoxal (vitamin B_6) levels using samples from 45 patients with schizophrenia and 61 mentally healthy subjects (**Figure 1**). Neither schizophrenic patients nor healthy subjects had diabetes mellitus or chronic kidney disease (estimated glomerular filtration rate >60 mL/min), which are 2 major causes of elevated AGEs. An increase in plasma pentosidine (to above the mean plus 2 SDs of control sub-

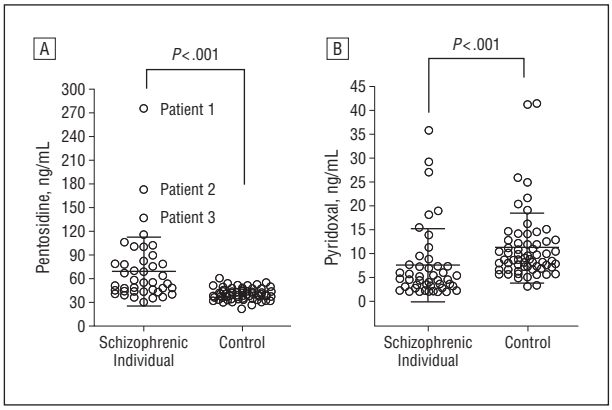

Figure 1. Plasma pentosidine accumulation and serum pyridoxal (vitamin B_6) depletion. Levels of plasma pentosidine (A) and serum pyridoxal (B) were analyzed using high-performance liquid chromatography techniques. Values were compared using the Mann-Whitney *U* test (2-tailed). Error bars indicate standard deviations.

jects, >55.2 ng/mL) was observed in 21 schizophrenic individuals (approximately 47%), as shown in Table 1. Three patients (patients 1, 2, and 3 in Figure 1A) exhibited extremely high pentosidine levels. The mean pentosidine level was 1.73-fold higher in schizophrenic individuals than in control subjects ($P<.001$) (Figure 1A and **Table 2**).

A concomitant marked decrease in pyridoxal levels was found in 11 schizophrenic patients (Table 1), most of whom were hospitalized and had been treated with well-controlled daily nutrition by a registered dietitian approved by the Japanese Ministry of Health, Labour, and Welfare based on the National Dietitian Law. Significant reduction of pyridoxal level was observed in schizophrenic patients compared with healthy control subjects ($P<.001$) (Figure 1B).

Mean values of pentosidine and vitamin B_6 in control samples were 39.6 ng/mL (SD, 7.8 ng/mL) and 11.1 ng/mL (SD, 7.3 ng/mL), respectively. These values do not deviate markedly from the standard levels in adult subjects without diabetes mellitus or renal dysfunction reported in previous studies.[44-46]

GENETIC ANALYSES OF *GLO1*

We next attempted to determine the mechanism underlying the alterations in pentosidine/pyridoxal levels observed in schizophrenia by resequencing analysis (all exons and flanking introns) of *GLO1* using 1761 patients with schizophrenia and 1921 control subjects (Table 1). These subjects included not only those for whom pentosidine/pyridoxal levels were examined, but also many other schizophrenic individuals and controls to ensure thorough genetic scrutiny. This analysis detected 2 heterozygous frameshift mutations. The first was an adenine insertion at nt 79 in exon 1, causing a frameshift starting from codon 27 and introducing a premature termination codon after aberrant translation of 15 amino acid residues (T27NfsX15) in 1 patient with schizophrenia (**Figure 2**A and eTable 2). The second heterozygous frameshift mutation, c.365delC, generated a frameshift from codon 122 in exon 4 and a premature termination after an aberrant 27–amino acid addition (P122LfsX27) (Figure 2B). This mutation was detected in 4 schizophrenic individuals and 10 control subjects (eTable 2). No relatives of subjects exhibiting c.365delC were available for analysis.

Furthermore, we identified 36 nucleotide changes, including 8 common polymorphisms (minor allele frequency >0.03) and 28 rare variants (eTable 2 and eTable 3). We also identified 13 homozygous Ala111 carriers: 9 schizophrenic patients and 4 controls (9 of 1586 schizophrenic patients [0.6%]); 4 of 1685 control subjects [0.2%]) (Figure 2C and eTable 3).

Seven heterozygous frameshift carriers (3 schizophrenic individuals and 4 controls), 10 homozygotes for Ala111 (7 schizophrenic individuals and 3 controls), 22 subjects with Glu111/Ala111 genotype (12 schizophrenic individuals and 10 controls), and 67 subjects with Glu111/Glu111 genotype (23 schizophrenic individuals and 44 controls) were available for biochemical assays (Figure 1 and Table 2).

BIOCHEMICAL ANALYSES OF GLO1

We focused on the heterozygous frameshift mutations and Glu111/Ala111 variation of *GLO1* in an attempt to assess the functional significance of these changes. We first quantified the levels of expression of GLO1 protein in RBC by Western blotting in 45 schizophrenic patients and 61 control subjects. Marked reductions (40%-50%) to full-length GLO1 protein expression were found in 10 subjects carrying heterozygous frameshift mutations ($P<.001$) (Table 2 and eFigure 2A). Significantly reduced (approximately 15%) GLO1 expression was observed in 7 homozygous Ala111 carriers compared with homozygous Glu111 or heterozygous Glu111/Ala111carriers in the schizophrenia group (both $P<.05$) (Table 2). In control subjects, levels of GLO1 protein expression in 3 homozygous Ala111 carriers did not differ significantly from those carrying other genotypes (Table 2).

The GLO1 enzymatic activity in RBC was measured by spectrophotometric assay (Table 2). Marked reductions (40%-50%) in enzymatic activity were found in all individuals carrying heterozygous frameshift mutations ($P<.01$). The 7 homozygous Ala111 carriers also exhibited significantly decreased enzymatic activity (an approximately 20% reduction) compared with homozygous Glu111 carriers in the schizophrenic group ($P<.001$) but not in control subjects.

In addition, we established a cell line from lymphocytes of a heterozygous frameshift carrier and performed functional analysis of these cell lysates (eFigure 2B). They exhibited the same functional abnormalities as identified in RBC, ie, decrease in GLO1 activity and its protein expression.

CONFOUNDING FACTORS AND BIOCHEMICAL DATA

Three patients (patients 1, 2, and 3 in Figure 1A) exhibiting extremely high pentosidine levels had especially severe schizophrenia, though they were free of systemic disease. These 3 schizophrenic individuals had chronic and

Table 2. Samples Used in the Biochemical Analyses

Characteristic	Mean (SD)									
	Schizophrenic Patients					Control Subjects				
	All (n=45)	Glu/Glu (n=23)	Glu/Ala (n=12)	Ala/Ala (n=7)	Frameshift (n=3)	All (n=61)	Glu/Glu (n=44)	Glu/Ala (n=10)	Ala/Ala (n=3)	Frameshift (n=4)
Sex, No., M/F	29/16	13/10	9/3	5/2	2/1	23/38	17/27	3/7	0/3	3/1
Age, y	51.0 (12.2)[a]	47.6 (12.5)[a]	51.5 (12.7)	59.0 (8.6)[a]	57.3 (4.6)[a]	36.0 (9.4)	35.1 (9.4)	41.9 (8.2)	24.3 (1.5)	40.5 (5.7)
Age at onset, y	25.0 (8.7)	24.4 (5.8)	25.8 (11.9)	28.0 (12.7)	20.0 (2.6)					
Relative protein expression	0.95 (0.15)[b]	0.99 (0.11)	1.01 (0.08)	0.86 (0.06)[c]	0.55 (0.09)[d]	0.88 (0.12)	0.91 (0.10)	0.87 (0.09)	0.86 (0.05)	0.60 (0.06)[e]
Enzymatic activity, mU/10^6 RBC	5.43 (1.00)[f]	6.00 (0.75)	5.47 (0.35)	4.70 (0.65)[g]	3.00 (0.20)[h]	5.94 (1.00)	6.18 (0.61)	6.11 (0.69)	5.83 (0.29)	2.90 (0.08)[i]
Pentosidine, ng/mL	68.37 (43.42)[j]	64.73 (32.8)[k]	54.96 (17.83)[l]	97.95 (82.67)[m]	80.91 (53.26)	39.59 (7.82)	39.17 (8.41)	39.27 (6.25)	39.08 (3.24)	45.34 (6.12)
Pyridoxal, ng/mL[n]	7.46 (7.56)[o]	8.20 (8.70)[p]	7.36 (7.66)	6.82 (4.69)	3.60 (2.12)	11.14 (7.31)	11.91 (8.02)	8.45 (2.76)	14.63 (8.95)	6.88 (1.56)

Abbreviation: RBC, red blood cell.
[a] Unpaired *t* test, $P<.05$ (vs controls).
[b] Mann-Whitney test, $P<.01$ (vs controls).
[c] Analysis of variance, $F_{3,41}=21.76$, $P<.001$; Bonferroni multiple comparison test, $P<.05$ in schizophrenic patients (vs Glu/Glu and Glu/Ala).
[d] Analysis of variance, $F_{3,41}=21.76$, $P<001$; Bonferroni multiple comparison test, $P<.001$ in schizophrenic patients (vs Glu/Glu, Glu/Ala, and Ala/Ala).
[e] Analysis of variance, $F_{3,57}=13.71$, $P<.001$; Bonferroni multiple comparison test, $P<.01$ in controls (vs Glu/Glu, Glu/Ala, and Ala/Ala).
[f] Mann-Whitney test, $P<.001$ (vs controls).
[g] Analysis of variance, $F_{3,41}=23.44$, $P<.001$; Bonferroni multiple comparison test, $P<.001$ in schizophrenic patients (vs Glu/Glu).
[h] Analysis of variance, $F_{3,41}=23.44$, $P<.001$; Bonferroni multiple comparison test, $P<.01$ in schizophrenic patients (vs Glu/Glu, Glu/Ala, and Ala/Ala).
[i] Analysis of variance, $F_{3,57}=37.41$, $P<.001$; Bonferroni multiple comparison test, $P<.001$ in controls (vs Glu/Glu, Glu/Ala, and Ala/Ala).
[j] Mann-Whitney test, $P<.001$ (vs controls).
[k] Mann-Whitney test, $P<.001$ (vs controls).
[l] Mann-Whitney test, $P<.01$ (vs controls).
[m] Mann-Whitney test, $P<.05$ (vs controls).
[n] Pyridoxal levels less than 2.0 were calculated as 2.0.
[o] Mann-Whitney test, $P<.001$ (vs controls).
[p] Mann-Whitney test, $P<.001$ (vs controls).

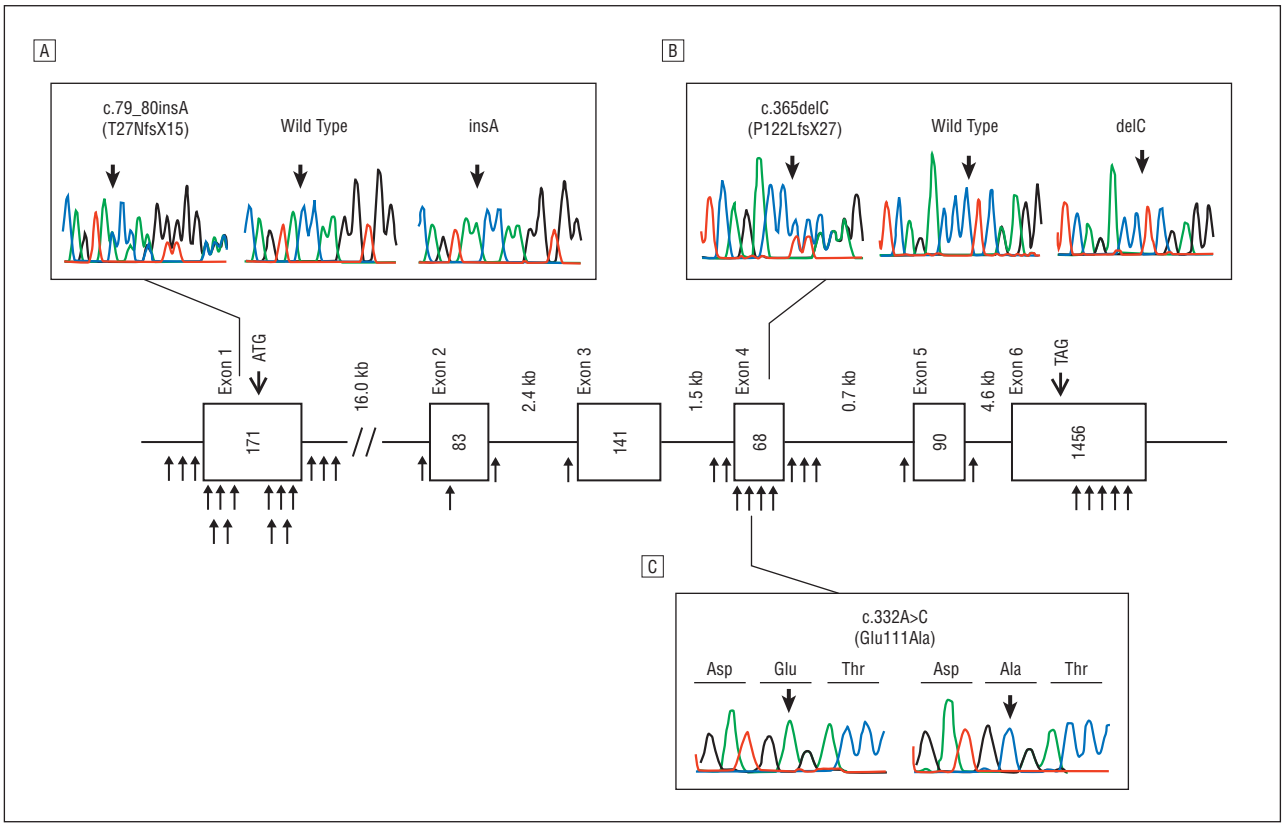

Figure 2. DNA sequence chromatograms showing frameshift and missense variants. Heterozygous sequence traces derived from individuals carrying an adenine insertion within exon 1 (A) and a cytosine deletion within exon 4 (B). TA cloning and subsequent sequencing analyses revealed normal (denoted "wild type") and mutant (denoted insA or delC) sequences. C, Chromatogram showing a Glu111/Ala111 missense variant located within exon 4. Positions of common and rare variants of *GLO1* are indicated by arrows (see also eTable 2 and eTable 3). kb indicates kilobase pairs.

Table 3. Summary of Demographic Data of Patients With High Pentosidine and/or Low Pyridoxal Levels

Characteristic	Patient No.												
	MZ65	TZ5	MZ70	TZ77	NP50	TZ72	MZ192	TZ40	TZ16	TZ41	SF114	SF136	TZ20
Sex	M	F	M	F	F	F	M	M	M	M	M	M	M
Age, y	66	53	60	46	60	59	57	41	60	41	63	60	41
Age at onset, y	17	18	17	16	21	17	18	25	22	19	48	20	19
High pentosidine level	Yes[a]	Yes[b]	Yes[c]										
Very low pyridoxal level, <3.0 ng/mL			Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
GLO1 genotype	Ala/Ala	Glu/Glu	T27NfsX15	Glu/Glu	P122LfsX27	Glu/Glu	Glu/Ala	Glu/Glu	Glu/Ala	Glu/Glu	Ala/Ala	Ala/Ala	Glu/Ala
Enzymatic activity, mU/10^6 RBC	4	5.5	2.8	5.7	3	6.6	5.9	6.6	5.5	6.1	5.5	4.9	5.5
Pentosidine, ng/mL	276.6	172.6	137	106.6	74.7	55.8	49	47.8	46.7	43.3	42.9	40.6	40.6
Pyridoxal, ng/mL	7.3	3.4	2.8	2.4	<2.0	2.3	2.4	<2.0	2.4	2.1	2.8	<2.0	<2.0
Antipsychotics, haloperidol equivalent, mg/d	34.6	54	38	18	7	8	16	20.5	13	9	8	12.3	10.1
Minor tranquilizer, diazepam equivalent, mg/d				10				6.7		6.3			18.8
Benzodiazepine hypnotics, nitrazepam equivalent, mg/d	5	25	20	10	10		10	10	10	10	10	20	7.5
Other medications		CBZ		PB, CBZ, GBP	VPA, CLN	CBZ	VPA	CBZ	Li_2CO_3, CBZ		CLN		
Smoking	No	No	Yes	Yes	No	Yes	Yes	Yes	No	Yes	Past smoker	Yes	Yes
Duration of hospitalization, y	33	10.6	21.4	25.3	14.2	2.7	3.4	0.8	35.8	0	12	35	15
Educational background	HS	College, 2 y	HS dropout	HS	JHS	JHS	U, 8 y	JHS	JHS	JHS	JHS	JHS	College dropout
Case type			Familial	Familial				Familial	Familial	Familial			
Criminal record			Yes			Yes							

Abbreviations: CBZ, carbamazepine; CLN, clonazepam; GBP, gabapentin; HS, high school; JHS, junior high school; Li_2CO_3, lithium carbonate; PB, phenobarbital; RBC, red blood cell; U, university; VPA, sodium valproate.

[a] Patient 1 in Figure 1A.

[b] Patient 2 in Figure 1A.

[c] Patient 3 in Figure 1A.

treatment-resistant schizophrenia (with doses of antipsychotics in haloperidol equivalents of 34.8-54.0 mg/d), with more than a 20-year disease history and more than 10 years of hospitalization each (range, 10.6-33 years) (**Table 3**). Patient 3 (Figure 1A) has an elder brother who committed suicide and 2 maternal uncles, all of whom had schizophrenia; patient 3 killed his mother and exhibited violent behavior against hospital staff.

Most of the patients had been taking multiple medications; we did not control for smoking by subjects. The daily dose of medication in haloperidol equivalents was significantly correlated with plasma pentosidine level (r=0.513, P=.001) but not with serum vitamin B_6 level (r=–0.087, P=.61). The significance of correlation between pentosidine and medication dose disappeared when the data for patients 1, 2, and 3 were excluded (r=0.186, P=.29). The mean value of medication dose in the high-pentosidine group was not significantly different from that in the normal pentosidine group (17.0 mg/day [SD, 12.4 mg/day] vs 12.4 mg/day [SD, 9.1 mg/day], respectively; P=.495). No significant correlation was found between pentosidine and dose of medication (high-pentosidine group, r=0.027, P=.93; normal group, r=–0.067, P=.78). Pentosidine level in smokers was not significantly different from that in nonsmokers (smokers, 65.6 ng/mL [SD, 29.7 ng/mL]; nonsmokers, 80.3 ng/mL [SD, 60.9 ng/mL]; P=.69), nor did vitamin B_6 level differ between these groups (smokers, 5.5 ng/mL [SD, 6.4 ng/mL]; nonsmokers, 7.3 ng/mL [SD, 5.4 ng/mL]; P=.08). Plasma pentosidine and vitamin B_6 levels did not appear to be affected by confounding factors such as duration of hospitalization, since there were no correlations between biochemical data and duration of hospitalization (pentosidine, r=0.295, P=.07; vitamin B_6, r=–0.072, P=.67).

COMMENT

This study revealed that some patients with schizophrenia are predisposed to enhanced carbonyl stress. Pyridoxal is 1 of the 3 forms of vitamin B_6, ie, pyridoxine, pyridoxal, and pyridoxamine. In vivo, pyridoxamine is biosynthesized from both pyridoxal and pyridoxine. Marked decreases in serum pyridoxal levels were found in 11 schizophrenic patients, but not in the control subjects (Table 1 and Table 3). Two schizophrenic patients with heterozygous frameshift mutations displayed markedly lowered pyridoxal levels (Table 3). Depletion of pyridoxal might thus reflect elevated carbonyl stress induced by *GLO1* defects and other unknown factors in these patients. Carbonyl stress and AGEs are known to interfere with cellular functions in various fashions. First, carbonyl compounds are biologically active and initiate a variety of cellular responses.[47] Second, AGEs induce not only structural alterations in proteins, but also influence cellular functions on interaction with receptors for

AGEs.[48] Agents able to inhibit AGE formation or entrap carbonyl compounds may also prove to be of therapeutic value, if carbonyl stress is directly linked to schizophrenic signs and symptoms. Some AGE inhibitory compounds are already clinically available (eg, angiotensin receptor blockers).[49] Others, including pyridoxamine[50] and TM2002,[51] have potent abilities to entrap toxic carbonyl compounds and prevent toxicity. In particular, the markedly lower vitamin B_6 levels in schizophrenic patients with high pentosidine levels suggest that pyridoxamine, a nontoxic, water-soluble vitamin B_6, may prove clinically useful.

To examine the molecular mechanisms underlying the carbonyl stress we observed and determine whether elevated carbonyl stress plays a causative role in schizophrenia, we performed a deep resequencing analysis of one of the target genes, *GLO1*. We focused on *GLO1*, because it is ubiquitous and because a highly active defense against glycation appears to be associated with the risk of development of various disorders,[8] though several enzymes are capable of reduction of α-dicarbonyls, eg, aldose reductase, betaine-aldehyde dehydrogenase, and 2-oxoaldehyde dehydrogenase.[52] We identified rare but drastic genetic variants, 2 different heterozygous frameshift mutations, and a functional Glu111Ala polymorphism. Biochemical analyses revealed that all of these resulted in a 10% to 50% reduction in GLO1 activity in RBC and were linked to attendant biochemical abnormalities, ie, increased plasma pentosidine and decreased serum vitamin B_6. These *GLO1* genetic defects/alterations were also identified in a fraction of control subjects; though in contrast to schizophrenic patients, these controls exhibited normal pentosidine and vitamin B_6 levels, implying the existence of compensatory mechanisms, such as upregulation of other relevant enzymes. Such compensatory mechanisms might not function in schizophrenia owing to additional unknown defects. The mechanisms through which healthy subjects with *GLO1* genetic defects/alterations escape carbonyl stress are of special interest. Elucidation of such mechanisms might clarify not only the sequential events involved in the development of schizophrenia, but also provide clues to novel therapeutic approaches in patients with carbonyl stress. Collectively, our findings suggest a cross-sectional link, albeit incomplete, between *GLO1* defect–elicited carbonyl stress and a subgroup of patients with schizophrenia.

We detected 13 Ala111/Ala111 genotype carriers among 3271 Japanese subjects. The frequency of the Ala111 allele exhibits high population diversity: 0.354 to 0.475 in Europeans, 0.239 to 0.395 in African Americans, 0.267 in sub-Saharan Africans, and 0.033 to 0.125 in Asian populations. The allelic frequency of Ala111 determined in the present study is identical to that described by Thornally.[11] The high prevalence of the Ala111 allele in European and African American populations suggests the existence of a mechanism maintaining normal plasma pentosidine and serum vitamin B_6 levels, despite diminished GLO1 activity, in individuals from these populations.

We estimate that approximately 20% of patients exhibited enhanced carbonyl stress–related schizophrenia based on our biochemical analyses using as criteria both high accumulation of pentosidine (>55.2 ng/mL) and depletion of vitamin B_6 (male, <6 ng/mL; female, <4 ng/mL), as shown in eTable 4. The frequency of such individuals was estimated to be approximately 1% when the criterion was carriage of a heterozygous frameshift mutation or homozygote for Ala111.

There are possible limitations of our study. First, all patients in our study had taken medication. We could not exclude the possibility of an increase of carbonyl stress through antipsychotic medicines. We hope to clarify whether carbonyl stress is involved in psychiatric illnesses using drug-naive patients in the near future. Second, the sample size of biochemical analyses was modest. Further investigations of reciprocal relationships between pentosidine accumulation/vitamin B_6 depletion and genetic defects using large Japanese samples and individuals from different ancestral populations are needed. Third, for biochemical analyses, we arbitrarily selected molecules and cofactors affecting glyoxalase detoxification systems in vivo, as shown in eFigure 1. We thus may have missed important molecules involved in the metabolic cascades maintaining homeostasis by compensating for *GLO1* genetic defects. Fourth, we could not exclude effects of exercise on our biochemical findings, as we were unable to quantify the physical activity of patients in a systematic fashion. In future work, we plan to focus on profiling the metabolomics, genomics, and clinical manifestations of carbonyl stress–related schizophrenia with or without *GLO1* defects. Fifth, the reason why low GLO1 protein expression was observed only in patients with the Ala111/Ala111 genotype in vivo remains unclear.

In summary, our study revealed the pivotal role of carbonyl stress in some patients with schizophrenia, and subsequent intensive resequencing analysis of *GLO1* detected 2 novel frameshift mutations with loss of function and moderate-effect Glu111/Ala111 polymorphism in Japanese cohorts. Additional studies of carbonyl stress in schizophrenia may well pave the way toward novel therapeutic/preventive measures for this devastating disease.

Submitted for Publication: May 11, 2009; final revision received October 9, 2009; accepted October 15, 2009.
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Author Contributions: Drs Arai Makato, Miyata, and Itokawa had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Financial Disclosure: None reported.

Funding/Support: This work was supported in part by grants from the Japan Society for the Promotion of Science (Drs Makoto Arai and Itokawa), the Program for Promotion of Fundamental Studies in Health Sciences of the Pharmaceuticals and Medical Devices Agency (Dr Miyata), and the Mitsubishi Pharma Research Foundation of Japan (Drs Makoto Arai and Itokawa).

Additional Contributions: Naomi Nihonmatsu, MS, Mood Disorders Research Team, Tokyo Institute of Psychiatry, and Yoshitaka Hayashi, Mood Disorders Research Team, Tokyo Institute of Psychiatry, provided technical assistance, and Yoshitaka Tatebayashi, MD, PhD, Mood Disorders Research Team, Tokyo Institute of Psychiatry, Takashi Nonaka, PhD, Molecular Neurobiology Research Team, Tokyo Institute of Psychiatry, Takashi Dan, PhD, Tohoku University Graduate School of Medicine, and Charles van Ypersele de Strihou, MD, PhD, Service de Nephrologie, Universite Catholique de Louvain, participated in helpful discussions. We are also grateful to the staff at Tokyo Metropolitan Matsuzawa Hospital for supporting our study.

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