Altered Metabolites in the Plasma of Autism Spectrum Disorder: A Capillary Electrophoresis Time-of-Flight Mass Spectroscopy Study

Hitoshi Kuwabara1, Hidenori Yasumasue2,3*, Shinsuke Koike2,4, Hideyuki Inoue2, Yuki Kawakubo1, Miho Kuroda1,5, Yosuke Takano2, Norichika Iwashiro2, Tatsunobu Natsubori2, Yuta Aoki2, Yukiko Kano1, Kiyoto Kasai2

1 Department of Child Neuropsychiatry, Graduate School of Medicine, The University of Tokyo, Bunkyo-ku, Tokyo, Japan, 2 Department of Neuropsychiatry, Graduate School of Medicine, The University of Tokyo, Bunkyo-ku, Tokyo, Japan, 3 Japan Science and Technology Agency, CREST, Chiyoda-ku, Tokyo, Japan, 4 Office for Mental Health Support, Division for Counseling and Support, The University of Tokyo, Bunkyo-ku, Tokyo, Japan, 5 Department of Psychology, Faculty of Integrated Human and Social Welfare, Shukutoku University, Chiba, Japan

Abstract

Clinical diagnosis and severity of autism spectrum disorders (ASD) are determined by trained clinicians based on clinical evaluations of observed behaviors. As such, this approach is inevitably dependent on the expertise and subjective assessment of those administering the clinical evaluations. There is a need to identify objective biological markers associated with diagnosis or clinical severity of the disorder. To identify novel candidate metabolites as potential biomarkers for ASD, the current study applied capillary electrophoresis time-of-flight mass spectroscopy (CE-TOFMS) for high-throughput profiling of metabolite levels in the plasma of 25 psychotropic-naive adult males with high-functioning ASD and 28 age-matched typically-developed control subjects. Ten ASD participants and ten age-matched controls were assigned in the first exploration set, while 15 ASD participants and 18 controls were included in the second replication set. By CE-TOFMS analysis, a total of 143 metabolites were detected in the plasma of the first set. Of these, 17 metabolites showed significantly different relative areas between the ASD participants and the controls (p<0.05). Of the 17 metabolites, we consistently found that the ASD participants had significantly high plasma levels of arginine (p=0.024) and taurine (p=0.018), and significantly low levels of 5-oxoproline (p<0.001) and lactic acid (p=0.031) compared with the controls in the second sample set. Further confirmatory analysis using quantification of absolute metabolite concentrations supported the robustness of high arginine (p=0.001) and low lactic acid (p=0.003) in the combined sample (n=53). The present study identified deviated plasma metabolite levels associated with oxidative stress and mitochondrial dysfunction in individuals with ASD.

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* E-mail: yamasue-tsky@umin.ac.jp

Introduction

Autism spectrum disorder (ASD) is a neurodevelopmental disorder characterized by social deficits, communication deficits and restricted and repetitive behaviors [1]. Diagnosis or clinical severity of ASD is mostly determined by trained clinicians based on clinical evaluations of observed behaviors [2,3]. As such, this approach is inevitably dependent on the expertise of those administering the diagnostic tests [4]. Thus, more objective methodologies for evaluating diagnosis or clinical severity of the disorder has been sought after [5–9]. These studies have revealed a higher mRNA expression in lymphocytes [5], metabolite alterations in the hippocampal formation [7], atypical brain responses to social stimuli [8], and increased serum levels of anterior pituitary hormones [9]. However, to the best of our knowledge, there are no reliable and practical biological markers for assessing clinical diagnosis or severity of ASD.

Metabolomics is a high-throughput metabolic profiling method to evaluate the concentration of metabolites in a given sample, such as plasma and urine. Because the status of metabolites is considered to be affected by pathophysiological processes, this approach can identify candidate molecules for representing the pathophysiology of developmental and psychiatric disorders [10–12]. To date, previous metabolomics studies using nuclear magnetic resonance (NMR), liquid chromatography mass spectrometry (LC-MS), and gas chromatography MS (GC-MS) have reported possible biomarkers, such as amino acids, antioxidants, gut bacterial metabolites, and sulfur in urine from children with ASD [13,14]. Although urine collection is totally non-invasive and holds promise for the disease biomarkers, a portion of the plasma metabolites are biotransformed in the kidneys before production of
urine [4,15]. Actually, different candidate biological markers for other disorders have been detected from urine and plasma samples [15,16]. Previous literature suggests that changes in potential biomarkers in cerebrospinal fluid might be correlated with those in plasma [6]. However, to our knowledge, there was no previous study reporting metabolic profiles quantified from plasma in individuals with ASD.

Capillary electrophoresis time-of-flight mass spectrometry (CE-TOFMS) is a relatively new MS method that can measure metabolites of higher ionization and lower molecular weight with easy preparation and high throughput [17,18]. While the efficacy of CE-TOFMS has been demonstrated in various human clinical studies [19,20], no previous application of this method for ASD subjects has been reported. To identify novel candidate metabolites as potential biomarkers for ASD, the current study applied CE-TOFMS to plasma samples from subjects with ASD and age-matched typically-developed control subjects. To minimize potential confounding effects of medications and neuropsychiatric comorbidities, including mental retardation and epilepsy, on metabolite levels, the participants with ASD, as well as those with typical development, were confined to psychotropic naive subjects and free from neuropsychiatric comorbidities.

Methods

Ethics Statement

All the participants provided written informed consent after they were given a complete explanation of the study as approved by the ethics committee of The University of Tokyo Hospital (No. 2094-5). Using longitudinal clinical assessments, a trained psychiatrist (H.Y.) confirmed that all of these adult participants had no intellectual disabilities and no need for psychotropic medication, and were capable of providing informed consent. Therefore, no participant needed for someone else to provide consent on their behalf.

Study Participants

A total of 53 Japanese adults (all Asian ethnicity) participated in this study. Considering the relatively low analytical reproducibility of capillary electrophoresis [11], we randomly enrolled two independent sets of samples for exploration and replication. The participants were recruited from April 2010 to July 2012 at the outpatient clinic of The University of Tokyo Hospital. All the study participants were male, since previous studies have indicated the potential contribution of sexual dimorphism to the pathophysiology of ASD [21,22]. Ten ASD participants and ten age-matched controls were assigned in the first set (exploration set), while 15 ASD participants and 18 age-matched control subjects were included in the second set (replication set) (Table 1). All the ASD participants were psychotropic-naive. The diagnostic protocols and clinical assessments were described in detail in our previous papers [23,24]. Briefly, an experienced psychiatrist (H.Y.) carefully diagnosed the participants with ASD on the basis of DSM-IV-TR [1] criteria after more than 2 months of follow-up examinations. Another trained psychiatrist (H.K.) confirmed the clinical diagnoses of the ASD participants using the Japanese version of the Autism Diagnostic Interview–Revised (ADI-R) [3,25]. For the six participants who did not meet the threshold in the ADI-R social domain, the ASD diagnosis was confirmed using the Autism Diagnostic Observation Schedule - Generic [2] by a trained psychologist (M.K.). Moreover the Global Assessment of Functioning (GAF) [1] was evaluated in all the ASD participants. The verbal Intellectual quotients (IQ) of the control group (all the participants in the first set and eight participants in the second set) was estimated using a Japanese version of the National Adult Reading Test [26,27]. Although the National Adult Reading Test can measure IQ accurately in controls, the test is problematic for ASD participants because of the well-known imbalances in their intellectual abilities. Therefore, the IQs of the ASD participants were assessed using the Wechsler Adult Intelligence Scale-revised [28] or Wechsler Adult Intelligent Scale third edition [29].

The exclusion criteria were: neurological illness, traumatic brain injury with any known cognitive consequences or loss of consciousness for more than 5 min, low IQ (i.e. below 80), previous alcohol dependence, previous continuous illegal substance use (e.g., cannabis). Additional exclusion criteria for the control group were any past and present psychiatric disease detected by screening with the modified Mini-International Neuropsychiatric Interview [30] or a family history of axis I disorder within the first-degree relatives.

CE-TOFMS Analysis

Peripheral blood samples were drawn by experienced physicians from a peripheral vein while the participant was fasting (>3 h without any meal and/or nutritious drink). Plasma samples were isolated via centrifugation at 1200 g for 10 min, and then stored at −80°C until use. Plasma samples (100 μL) were immersed into 0.45 mL methanol containing 10 μM each methionine sulfone and 10-camphorsulfonic acid, and mixed well. Then, 200 μL deionized water and 0.5 mL chloroform were added and the solution was centrifuged at 2,300 g for 5 min at 4°C. The upper aqueous layer was centrifugally filtered through a 5-kDa cutoff filter (Human Metabolome Technologies Inc., Tsukuba, Japan) to remove proteins. The filtrate was lyophilized and dissolved in 50 μL of ultrapure water containing reference compounds prior to mass spectrometry analysis. The water was produced by a Milli-Q Academic A10 (EMD Millipore, Billerica, MA, USA).

Samples were applied to a capillary electrophoresis system equipped with an Agilent 6210 time-of-flight mass spectrometer (CE-TOFMS, Agilent Technologies, Santa Clara, CA, USA), as previously described [31]. Raw data files from CE-TOFMS were processed using customized proprietary software written in Java (An extended version of MathDAMP that has been developed in Keio University) [32]. The software performs (1) peak picking, and (2) peak alignment. For (1), all peaks potentially corresponding to metabolites were extracted. After peak picking, the migration time of electrophoresis was normalized using those of the internal standards. For (2), an alignment was applied according to similar mass-to-charge ratios (m/z) and normalized migration times. The tolerance was set to 100 ppm (m/z) and 0.5 min (Normalized migration time). The peak matrix was matched with the annotation table of the metabolomics library (The Basic Scan metabolomics service of Human Metabolome Technologies Inc.) described previously based on their m/z and migration times [33]. All peak areas were divided by the area of the internal standard (i.e. Relative area) to normalize the signal intensities, and to avoid injection-volume bias and mass-spectrometry detector sensitivity bias among multiple measurements.

Statistical Analysis

Comparisons for relative metabolite concentrations between individuals with ASD and controls. All statistical analyses were conducted using the SPSS version 17.0 (IBM Inc., Armonk, NY, USA). As the current sample sizes were relatively small, normal distributions of the present data sets across all metabolites were not warranted in advance. Therefore, non-
parametric statistics were employed for the statistical analyses. The obtained relative area values of each metabolite were regarded as their relative concentration. We tested the differences of these relative concentrations between the ASD participants and the controls using two-tailed Mann-Whitney U test in the first set. For the purpose of exploration, significance levels were set at \( p < 0.05 \) for each metabolite without correction for multiple comparisons. For the purpose of replication, we compared the metabolite levels, which showed significant differences due to diagnosis in the first set, between the participants with ASD and the controls in the second set using one-tailed Mann-Whitney U test. As analyses on the second set were aimed to replicate findings in the first set, the directions of expected effects were known \textit{a priori}. Thus, one-tailed tests (\( p < 0.05 \) were set as significance) were used without correction for multiple comparisons [34].

**Discriminant analyses between ASD and controls based on the identified metabolites.** To preliminarily test the possibility of the detected metabolites as a diagnostic tool, we conducted discriminant analyses using metabolite levels that were significantly and consistently different in the ASD participants in the first and the second sets. Since the discriminant analyses were conducted separately on the first and second sets, the significance levels were set at \( p < 0.025 \) with Bonferroni correction for two data sets.

**Comparisons for absolute metabolites concentrations between ASD individuals and controls.** Analysis by CE-TOFMS in our system enables measurement of the absolute quantities of pre-determined 108 major metabolites, based on the peak area of internal controls of each metabolite. Since the quantity of these metabolites can be reliably compared across different experimental batches, absolute concentrations were quantified for these metabolites for the purpose of further verification in the combined sample of the first and second sets. We compared absolute concentrations of the major metabolites using two-tailed \( U \) test between the 25 ASD individuals and 28 controls. To test the robustness of findings from comparisons using relative metabolite measures, statistical significance was set at \( p < 0.05/N \) (number of metabolites absolutely quantified among the metabolites consistently showed significant differences at relative concentrations in both the first and second sets) employing Bonferroni correction.

**Correlation analyses between metabolites and clinical measures.** To explore clinical significance of the deviated metabolites in the plasma from ASD subjects, we calculated Spearman’s rank correlation coefficients between their clinical characteristics (i.e. ADI-R, GAF, IQ) and absolute value of significant metabolites in the combined sample set. Since the correlation analyses were implicated as exploratory analyses, we adopted absolute values in the combined sample set to increase the statistical power, and then statistical significance was defined as \( p < 0.05 \).

**Results**

By the CE-TOFMS analysis, a total of 143 metabolites were detected in the plasma samples of the first sample set (79 and 64 metabolites for the cation and anion modes, respectively). Of these, 17 metabolites showed significantly different relative areas between the ASD participants and the controls. Of the 17 metabolites which showed significant difference in the first sample set, 14 metabolites were successfully quantified in the second sample set, although a total of 141 metabolites were measurable (76 and 65 metabolites for cation and anion modes, respectively). The lists of detected metabolites in the first and the second sample sets were available in Table S1. For the purpose of validating the findings in the first sample set, we conducted a one-tailed Mann-Whitney U test for the relative areas of the 14 metabolites. Then, we found that the ASD participants had significantly high levels of arginine (\( p = 0.024 \)) and taurine (\( p = 0.018 \)), low levels of 5-oxoproline (\( p < 0.001 \)) and lactic acid (\( p = 0.031 \)) compared with the controls in the second sample set (Table 2 and Figure 1).

Discriminant analysis in the first sample set showed that diagnosis of 80.0% (16 out of 20) of the subjects was correctly classified with the four used metabolites (arginine, taurine, 5-oxoproline, and lactic acid; Wilks’s \( \lambda = 0.447, p < 0.012 \), Figure 2). These four metabolites were differentiated by participants with ASD and controls with an area under the receiver-operating characteristic curve (AUC) value of 0.940 (\( p = 0.001 \)). These discriminant analyses in the second sample set showed that diagnosis of 78.8% (26 out of 33) of the subjects was correctly classified (\( \lambda = 0.437, p < 0.001 \), Figure 2), and AUC value was 0.957 (\( p < 0.001 \)).

Among pre-determined 108 major metabolites, which could measure absolute quantities, 39 metabolites were identified in all the 53 participants (25 ASD participants and 28 controls). Among the four metabolites listed in Table 2, two metabolites (arginine, lactic acid) were included in the 39 major metabolites identified.

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**Table 1.** Demographic characteristics of the study participants.

| First sample set | Second sample set |
|------------------|-------------------|
| **ASD** (n = 10) | **Controls** (n = 10) | **ASD** (n = 15) | **Controls** (n = 18) |
| Age (years) | 32.2±7.0 | 32.9±3.6 | 28.6±5.3 | 28.7±4.0 |
| ADI-R S | 12.3±5.4 | NA | 16.9±6.8 | NA |
| ADI-R C | 9.5±2.0 | NA | 12.8±4.6 | NA |
| ADI-R R | 3.3±2.0 | NA | 4.8±1.8 | NA |
| GAF | 45.5±8.8 | NA | 46.7±6.8 | NA |
| Full Scale IQ | 102.5±11.2 | NA | 109.3±9.5 | NA |
| Verbal IQ | 111.3±11.7 | 115.2±4.7 | 113.0±12.9 | 108.8±9.3 |
| Performance IQ | 90.3±14.1 | NA | 100.9±17.0 | NA |

Values are given in mean ± SD, except for the number of participants. Abbreviations: ASD, autism spectrum disorders; ADI-R, autism diagnostic interview revised; S, social domain; C, communication domain; R, restricted and repetitive behavior domain; GAF, global assessment of functioning; NA, not applicable.

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We thus compared their absolute concentrations using the combined dataset. The significance level was defined at $p<0.025$ with Bonferroni correction for two metabolites. Absolute concentration of arginine was significantly higher ($p=0.001$), while that of lactic acid was significantly lower ($p=0.003$) in the ASD group than in the controls (Table 3).

Correlation analysis using Spearman’s rank correlation coefficients in the combined sample showed significant negative correlation between the concentration of arginine and GAF score ($\rho = -0.413$, $p = 0.040$, Figure 3). There was no significant correlation between metabolites and ADI-R or IQ scores.

**Discussion**

The current metabolomic analysis using CE-TOFMS revealed that plasma levels of several metabolites were significantly different between the participants with ASD and controls. We analyzed two independent sample sets, and consistently identified high levels of arginine and taurine, and low levels of lactic acid and 5-oxoproline in the participants with ASD compared with the controls. Taking advantage of the function of absolute concentration quantification of major metabolites, altered concentrations of arginine and lactic acid in the participants with ASD were further confirmed. To the best of our knowledge, this is the first study that elucidated possible

| Table 2. Results from comparisons for relative metabolite concentrations between individuals with ASD and controls. |
|---------------------------------------------|
| Direction of change | Metabolites | Mode | m/z | MT (min) | First sample set* | Second sample set* |
|--------------------|-------------|------|-----|---------|-------------------|-------------------|
| |                |        |     |        | ASD | Controls | p valueb | ASD | Controls | p valueb |
| Higher Arginine Cation | 175.12 | 7.12 | 0.036 (0.0070) | 0.028 (0.0060) | 0.011 | 0.024 (0.0059) | 0.020 (0.0037) | 0.024 |
| Taurine Cation | 126.02 | 23.31 | 0.0031 (0.00086) | 0.0021 (0.00034) | 0.007 | 0.0026 (0.00081) | 0.0020 (0.00059) | 0.018 |
| Lower 5-Oxoproline Anion | 128.03 | 9.50 | 0.0013 (0.00027) | 0.0015 (0.00026) | 0.035 | 0.00097 (0.00016) | 0.0013 (0.00025) | <0.001 |
| Lactic acid Anion | 89.02 | 10.93 | 0.090 (0.035) | 0.15 (0.058) | 0.009 | 0.10 (0.033) | 0.14 (0.046) | 0.031 |

*Mean relative area value and its SD are given.

Two- and one-tailed Mann-Whitney U tests.

Abbreviations: m/z, mass-to-charge ratio; MT, migration time; ASD, participants with autism spectrum disorder; NA, not applicable; ND not detected.

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biomarkers in peripheral blood plasma to evaluate ASD using metabolomics analysis.

Arginine is an essential precursor for the synthesis of proteins and other molecules of enormous biological importance, including nitric oxide (NO). Excessive arginine is thought to induce oxidative stress via NO production [35]. Though the present CE-TOFMS analyses were not able to measure nitric oxide concentration, NO level was previously reported to be high in ASD children [36].

Figure 2. Results of discriminant analyses. A receiver-operating characteristic (ROC) curve and area under the curve (AUC) according to the results of discrimination analysis in the first (left) and the second sample sets (right) are presented. The results of discriminant analyses between the subjects with autism spectrum disorders and controls are indicated with blue lines.
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Figure 3. Relationship between arginine absolute concentration and GAF score. Scatter plot shows correlation coefficients between arginine absolute concentration and GAF score ($\rho = -0.413$, $p = 0.040$).
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ADI-R scores are calculated mainly based on behavioral for general difficulties due to psychiatric disorders. Besides, since there was no significant correlation with ADI-R scores. Therefore, with GAF score in the current participants with ASD, although the relationship between arginine and oxidative stress in neuro-psychiatric disorders, such as schizophrenia, have reported excess [38] or reduced plasma arginine levels [12]. The precise nature of psychiatric disorders, such as schizophrenia, have reported excess [38] or reduced plasma arginine levels [12]. The precise nature of the relationship between arginine and oxidative stress in neuro-psychiatric disorders is unclear; however, the common susceptibility genes for ASD and schizophrenia, TCF4 [39] and NOS1 [40,41] has been suggested to be involved in the arginine-NO pathway. The arginine concentration was negatively correlated with GAF score in the current participants with ASD, although there was no significant correlation with ADI-R scores. Therefore, the current study could not draw the conclusion that arginine was a specific biomarker for the pathogenesis of ASD rather than that for general difficulties due to psychiatric disorders. Besides, since ADI-R scores are calculated mainly based on behavioral characteristics during childhood, a possibility that arginine concentration reflect the current ASD severity during adulthood rather than that during childhood remains. Future studies should address these issues.

The inhibitory amino acid taurine is an osmoregulator and neuromodulator, also exerting protective actions in neural tissue, including under conditions of oxidative stress [42]. Plasma taurine concentration in children with ASD was reported to be high compared with typically developing children [37,43], and other researches has reported contradictory results that showed low concentrations of taurine in ASD children [44–46]. Similarly, contradictory changes in urine concentrations of taurine in ASD children were reported, with elevated [13] and low concentrations [14]. Although the reason for the contradictory findings in plasma and urine concentrations remains unclear, taurine is hypothesized to be protective for autistic symptoms [47,48]. The present result, which showed elevated plasma taurine level in adults with ASD, might be due to compensatory regulation against pathogenesis of ASD, such as oxidative stress.

The endogenous molecule 5-oxoproline is derived from L-glutamate, being a major intermediate in the γ-glutamyl cycle. This cycle is necessary for the synthesis and breakdown of glutathione, and also for the intracellular transport of free amino acids [49]. Although the pathological role of 5-oxoproline in the human brain is not clarified, it has been presumed from animal studies that 5-oxoproline elicits oxidative stress, which may represent a pathophysiological mechanism in the neuro-pathological disorder in which this metabolite accumulates [50,51]. Direct contribution of 5-oxoproline down-regulation in ASD pathology is not clear, but like taurine and arginine, 5-oxoproline might be a putative biomarker for ASD that has oxidative stress alterations.

Lactic acid is thought to be one of the biochemical markers that may indicate mitochondrial function [52]. Previous studies have suggested that mitochondrial dysfunction and altered energy metabolism may influence the social and cognitive deficits in autism [53–56]. A school-based study of 69 children aged 11 to 14 years with ASD found mitochondrial respiratory chain dysfunction and hyperlactacidemia [57]. The present results showed that the lower lactic acid concentration in ASD participants was contradictory to the results of Oliveira et al. (2005). A possible explanation for this inconsistency is that the present sample sets consisted of adults with ASD. In conjunction with the reported high lactic acid concentration in children with ASD, low lactic acid concentration in adults with ASD in the present results might suggest altered regulation of mitochondrial function during development. There was no significant correlation between lactic acid level and clinical measures, therefore further studies were needed to scrutinize clinical significance of low lactic acid concentration in adults with ASD.

It should be noted that this study has several potential methodological limitations. First, the present study consisted of male subjects only. Though we were able to avoid confounding sex influence on the results, the present findings might not be generalizable to females. Second, longitudinal metabolite measurements with detailed clinical investigations from childhood will be needed to determine if the identified metabolites showed development-dependent changes. Third, the current discriminant analyses successfully classified the participants with ASD and controls using concentrations of the detected four metabolites. However, since the current discriminant analyses used the same sample sets as those for detection of the four metabolites owing to the limited sample size of the present study, the possibility for discrimination remains to be validated in a future study with independent samples. In addition, other psychiatric disorders (e.g. schizophrenia) have to be considered as a clinical control group in future studies, because the pathophysiology of these psychiatric disorders is thought to be shared [58,59]. Comparison among other psychiatric disorders may clarify the pathogenesis more clearly. Fourth, CE-TOFMS is not very effective for the separation of neutral metabolites and large molecules. Concomitant analysis of the same sample by LC-MS, GC-MS, and NMR approaches has the potential to greatly expand the coverage of target metabolites. Finally, the total sample size of the present study (n = 53) was relatively small, although, by conducting power analyses based on the effect sizes of the four altered metabolites detected in the current study (d = 0.72–1.24), the present study provides useful information about required total sample sizes in future studies (n = 58–158) to detect the deviated metabolites with more conservative threshold for statistical significance with power of 80% (e.g. Bonferroni correction).

In conclusion, the present study measured small ionic metabolites in peripheral blood plasma using the CE-TOFMS system, and found elevated and reduced metabolites in plasma samples from ASD participants associated with markers of oxidative stress and mitochondrial dysfunction. These metabolites could become possible biomarkers for differential diagnosis, determination of severity, and prediction of drug response, which could promote better treatment options and result in better prognosis.

### Supporting Information

**Table S1 Detected metabolites list.** Metabolites detected in both the first and second sets, those in the first set only, and those in the second set only were listed respectively.

(DOCX)
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Author Contributions
Conceived and designed the experiments: HK HY KY. Performed the experiments: HY HI Y. Analyzed the data: HK HY. Contributed reagents/materials/analysis tools: SK. Wrote the paper: HK HY SK Y. Kano KK.

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