Original Article

Exploration of urine metabolic biomarkers for new-onset, untreated pediatric epilepsy: A gas and liquid chromatography mass spectrometry-based metabolomics study

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Abstract

Objective: The discovery of objective indicators for recent epileptic seizures will help confirm the diagnosis of epilepsy and evaluate therapeutic effects. Past studies had shortcomings such as the inclusion of patients under treatment and those with various etiologies that could confound the analysis results significantly. We aimed to minimize such confounding effects and to explore the small molecule biomarkers associated with the recent occurrence of epileptic seizures using urine metabolomics.

Methods: This is a multicenter prospective study. Subjects included pediatric patients aged 2 to 12 years old with new-onset, untreated epilepsy, who had had the last seizure within 1 month before urine collection. Controls included healthy children aged 2 to 12 years old. Those with underlying or chronic diseases, acute illnesses, or recent administration of medications or supplements were excluded. Targeted metabolome analysis of spot urine samples was conducted using gas chromatography (GC)- and liquid chromatography (LC)-tandem mass spectrometry (MS/MS).

Results: We enrolled 17 patients and 21 controls. Among 172 metabolites measured by GC/MS/MS and 41 metabolites measured by LC/MS/MS, only taurine was consistently reduced in the epilepsy group. This finding was subsequently confirmed by the absolute quantification of amino acids. No other metabolites were consistently altered between the two groups.

Conclusions: Urine metabolome analysis, which covers a larger number of metabolites than conventional biochemistry analyses, found no consistently altered small molecule metabolites except for reduced taurine in epilepsy patients compared to healthy controls. Further studies with larger samples, subjects with different ages, expanded target metabolites, and the investigation of plasma samples are required.

Keywords: amino acids; gas chromatography; liquid chromatography; mass spectrometry; new-onset epilepsy

Introduction

The correct diagnosis and appropriate treatment of epilepsy is frequently challenging for physicians, even for epileptologists. The former depends on detailed history-taking on seizures, knowledge of seizure semiology, correct interpretation of electroencephalogram (EEG), and appropriate clinical correlation between EEG findings and clinical history. Mastering these skills requires opportunity and a lengthy intensive subspecialty training. The latter needs not only knowledge of anti-seizure medications and non-pharmacological treatments but also a reliable seizure diary by patients and/or their family members. Information from seizure diaries is frequently unreliable, for example, when seizures occur at night and patients cannot recall them. In such a case, the correct evaluation of therapeutic effects is difficult.

Objective indicators of recent epileptic seizures can aid in the diagnosis and treatment of epilepsy and the ruling out of non-epileptic disorders. They would also be useful to understand the pathophysiology of epilepsy and would aid the discovery of new therapeutic targets. Serum prolactin was reported to increase immediately after a generalized tonic-clonic seizure [1] and its usefulness to differentiate generalized tonic-clonic seizures and focal impaired awareness seizures from non-epileptic seizures was suggested by the American Academy of Neurology [2]. However, clinical samples must be taken with appropriate timing, within 10 to 20 min after an event. In addition, its specificity has recently been questioned because 28.8% of patients with psychogenic non-epileptic seizures have post-event elevation of serum prolactin [3]. The discovery of biomarkers that show a longer-lasting change after an epileptic seizure, like Hb1Ac for diabetes, is necessary.

Metabolomic studies can be helpful for this purpose, because they cover wider ranges of small molecules than focused analyses, such as single compound analysis, amino acid analysis, and organic acid analysis, and are suitable for exploration of the metabolites associated with epilepsy. Past metabolomics studies seeking biomarkers of epilepsy targeted biofluids, including blood (serum or plasma) [4-8], urine [9], and cerebrospinal fluid (CSF) [10-12] samples. Most of these

studies included epileptic patients under treatment and those with various symptomatic etiologies, leaving a question of the potential effects of medications and various etiologies on metabolomic data. CSF may reflect pathological processes in the central nervous system more than blood and urine; however, CSF collection from healthy individuals, especially from children, is not feasible. In addition, the superiority of CSF to urine for estimation of the condition of the central nervous system has not been confirmed and the possibility of skewing the results by the stress response to CSF collection should also be considered [13].

Urine samples can be collected easily and noninvasively, even in young infants. Past studies suggested that urine metabolomics might be utilized for the diagnosis of central nervous system disorders, such as major depressive disorder [14,15], bipolar disorder [16], schizophrenia [17], autism spectrum disorder [18-20], Parkinson disease [21, 22], and brain tumors [23]. In this study, we aimed to explore urine metabolites that reflect the presence of recent epileptic seizures. We conducted gas chromatography-mass spectrometry (GC/MS)-based and liquid chromatography-mass spectrometry (LC/MS)-based metabolome analyses of urine samples obtained from pediatric patients with new-onset, untreated epilepsy and healthy children. We limited the epilepsy patients to those without underlying diseases or treatment to minimize the difference between patient and control groups to see the “pure” effects of epilepsy on the urine metabolome. We hypothesized that changes in the urine metabolome were observable in epilepsy patients, as a subset of small molecules could cross the blood–brain barrier.

Methods

Subjects

This research was conducted as a multicenter prospective study in accordance with the Declaration of Helsinki and was approved by the Ethics Committee of Okayama University Hospital (approval #1604-009). Written informed consent was obtained from the guardians of all patients. We enrolled subjects between June 2018 and March 2020. Patients included children aged 2 to 12 years old with new-onset epilepsy who had not been treated and had had the last

seizure within 1 month before urine sample collection. The diagnosis of epilepsy was made according to the practical clinical definition of epilepsy by the International League Against Epilepsy [24]. Controls included healthy children aged 2 to 12 years old. Exclusion criteria were as follows: (1) presence of underlying or chronic diseases based on clinical history and physical examination, (2) administration of medications, supplements, and/or alcohol within 72 h before sample collection, (3) menstruation within 72 h before sample collection, (4) smoking within 1 week before sample collection, and (5) acute illness within 1 week before sample collection.

Sample collection

Spot urine samples were immediately cooled on ice after collection, and were centrifuged at 2000 to 3000 ×*g* at 4°C for 5 min within 2 h of collection. The supernatant was aliquoted and frozen within 4 h of collection. If the freezing temperature was not below −70°C, the frozen samples were transferred to a deep freezer within 8 h of collection.

Targeted metabolome analysis

All samples were collected at Okayama University Hospital from collaborators and then shipped to the Tohoku Medical Megabank Organization at Tohoku University on dry ice. The creatinine concentration of each urine sample was determined using liquid chromatography-tandem mass spectrometry (LC/MS/MS) according to a past study [25]. Next, urine samples were diluted with ultrapure water to achieve almost the same creatinine concentration in all of the samples. Targeted metabolic profiling was conducted using gas chromatography-tandem mass spectrometry (GC/MS/MS) and LC/MS/MS. GC/MS/MS analysis was performed according to a previously reported method, using a GC-MS TQ8040 system (Shimadzu Corporation, Kyoto, Japan) [26]. Urine samples were pretreated by the same method used for plasma samples in this previous study [26]. The database used in this study includes data on 475 peaks from 333 metabolites. LC/MS/MS analysis was performed using ACQUITY UPLC I-Class and Xevo TQ-S systems (Waters Corporation, Milford, USA). The MxP® Quant 500 kit (biocrates life sciences ag, Innsbruck, Austria) was used as a measurement kit, and targets up to 630 metabolites from 26

biochemical classes.

Metabolomic data analysis

Differences in the urine metabolome between the epilepsy and control groups were examined using MetaboAnalyst 5.0 (https://www.metaboanalyst.ca/). Metabolites with missing values (e.g., below the limit of quantification) were excluded. For LC/MS/MS data, we excluded lipids and analyzed only small molecules (107 compounds) to increase statistical power, as most of the lipids were not quantified in urine samples. The concentration data of each metabolite underwent creatinine correction, logarithmical transformation, and scaling to zero mean and unit variance.

We conducted principal component analysis (PCA) for the metabolome data to detect intrinsic data clustering and outliers. Afterwards we performed orthogonal partial least squares discriminant analysis (OPLS-DA) to determine the metabolites with a high degree of contribution to the group difference. OPLS-DA is a supervised, multivariate classification method that builds a model to predict the categorical factor (epilepsy or control) by multiple, explanatory numerical variables (metabolite concentrations). Model performance was evaluated by leave-one-out cross-validation using calculated R2Y (goodness of fit) and Q2Y (goodness of prediction) values. A Q2Y value ≥ 0.5 was considered a good prediction using a constructed model [27]. The model was also validated by a permutation test to check that group difference did not occur by chance. Subsequently, a metabolite with variable importance for prediction (VIP) score ≥ 1.5 was determined to be a metabolite with a significant change between the two groups. We also conducted a univariate test (Mann-Whitney *U* test) considering multiple comparisons for selected metabolites by OPLS-DA, and calculated *p*-values and corresponding false discovery rates (FDRs). An FDR value <0.05 was considered statistically significant.

Statistical analysis

Statistical analyses for group comparison (Mann-Whitney *U* test, Fisher's exact test) and correlation (Spearman's test) were conducted using R4.3.0 (https://cran.r-project.org/). A *p*-value

<0.05 was considered statistically significant.

Amino acid analysis using a clinical testing system

Based on the result of the metabolomic data analysis described in the Results section, urine samples were analyzed for the absolute quantification of amino acids by LC/MS/MS using ACQUITY UPLC I-Class and Xevo TQ-S micro (Waters) using a Kairos Amino Acid Kit (Waters). Creatinine was measured by an enzymatic assay using a Clinical Chemistry Analyzer JCA-BM 8060 (JEOL Ltd., Tokyo, Japan) and an L type Wako CRE·M kit (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan). These assays used the same systems as clinical testing and were carried out at a registered clinical laboratory (LSI Medience Corporation, Tokyo, Japan).

Results

Clinical profiles of subjects

We enrolled 17 patients with new-onset epilepsy and 21 healthy controls. Subject profiles are summarized in Table 1. The epilepsy group included 8 cases with self-limited epilepsies (self-limited epilepsy with centrotemporal spikes in 6 cases, self-limited epilepsy with autonomic seizures in 1 case, myoclonic epilepsy in infancy in 1 case), 6 cases with idiopathic generalized epilepsy (childhood absence epilepsy), and 3 cases with focal epilepsy of unknown cause. Based on the inclusion and exclusion criteria, these 17 patients had no neurological abnormalities on physical examination, had had the last epileptic seizure within 1 month (0–29 days, median: 5 days) before urine sample collection, and had not been on any medications or supplements.

Differential metabolites between the epilepsy and control groups by metabolome analyses

GC/MS/MS data included 172 metabolites after excluding three metabolites with missing values (Supplementary Table). PCA demonstrated no outliers. There was a minor trend of group separation based on the fifth (4.4%) principal component (Supplementary Fig. 1), which

indicated that most of the metabolome variations were explained by factors other than epilepsy versus control. OPLS-DA (one predictive component and four orthogonal components, R2Y = 0.982 [$p < 5\times10^{-4}$ by permutation test], Q2Y = 0.691 [$p = 0.002$]) exhibited excellent group separation and demonstrated 14 metabolites contributing to group difference (Fig. 1A, 1B, and Table 2). These 14 metabolites had low *p*-values, with 10 metabolites showing significant (<0.05) FDRs.

LC/MS/MS data included 41 metabolites after excluding 65 metabolites with missing values. PCA demonstrated no outliers. There was a minor trend of group separation based on the fourth (6.5%) principal component (Supplementary Fig. 2), which indicated that most of the metabolome variations were explained by factors other than epilepsy verses control. OPLS-DA (one predictive component and four orthogonal components, R2Y = 0.930 [$p = 5\times10^{-4}$ by permutation test], Q2Y = 0.596 [$p < 5\times10^{-4}$]) exhibited group separation and demonstrated four metabolites contributing to group difference (Fig. 1C, 1D, and Table 3). These four metabolites had low *p*-values, with one metabolite (hexoses as a single metabolite group) showing a significant (<0.05) FDR.

Taurine and cysteine were commonly demonstrated in GC/MS/MS and LC/MS/MS datasets, with taurine being reduced in the epilepsy group in both datasets but cysteine showing inconsistent changes. We checked GC/MS/MS data to further investigate the increase in hexoses found in LC/MS/MS data. Galactose and glucose were elevated and tagatose was slightly reduced in the epilepsy group. Galactose showed a low *p*-value (= 0.048) but was not significantly elevated in the epilepsy group based on FDR (= 0.15). Glucose ($p = 0.089$) and tagatose ($p = 0.56$) did not reach statistical significance (Table 2). Ornithine was picked up by LC/MS/MS but it had a low VIP score and insignificant (>0.05) FDR in GC/MS/MS data (Table 2). Among demonstrated metabolites by GC/MS/MS, glutamine and 2-aminoadipic acids were quantified by LC/MS/MS, which showed low VIP scores and insignificant FDRs (Table 3).

Absolute quantification of amino acids

Based on the metabolomic data analysis results suggesting taurine as potentially useful for distinguishing the epilepsy group from the control group, we conducted an absolute quantification of urine amino acids. After excluding those with missing values, 24 amino acids were statistically analyzed. OPLS-DA (one predictive component and one orthogonal component, R2Y = 0.465 [p = 0.047 by permutation test], Q2Y = 0.11 [p = 0.029]) showed poor model fitting, insufficient separation of the two groups, and low performance of prediction (Fig. 1E, 1F), which might have been partially due to the fewer analyzed metabolites than GC/MS/MS and LC/MS/MS. Although four metabolites contributed to the group difference, with two (glycylproline and taurine) having low p-values, none showed significant (<0.05) FDRs (Table 4). Among other amino acids demonstrated by GC/MS/MS and LC/MS/MS, glutamine and 2-aminoadipic acid were quantified and they showed low VIP scores and insignificant FDRs. The results of taurine concentrations determined by GC/MS/MS, LC/MS/MS, and amino acid analysis are demonstrated in Fig. 2.

Discussion

We aimed to explore small-molecule biomarkers that reflect the presence of recent epileptic seizures using GC/MS- and LC/MS-based metabolomics. Confounders, such as variety in etiologies and medication effects, were eliminated as much as possible. This study demonstrated 14 and four differential metabolites based on GC/MS/MS and LC/MS/MS, respectively. Among these metabolites, taurine was consistently reduced in both GC/MS/MS and LC/MS/MS data, with a large contribution (VIP score) to separate epilepsy and control groups. Subsequent absolute quantification of amino acids also suggested the contribution of taurine for group separation. This study did not demonstrate consistently altered metabolites other than taurine between the two groups.

Taurine participates in neurotransmission, osmoregulation, antioxidant activity, trophic factor activity, and modulation of calcium movements. It can serve as an anti-seizure and

neuroprotective agent [28-31]. Lower taurine excretion into urine in epilepsy patients than healthy controls was reported mainly in the 1980s [32-34]. This was ascribed to the increased reabsorption of taurine at the proximal renal tubules. Taurine transporter (TauT) encoded by the *SLC6A6* gene plays the main role in taurine reabsorption [35,36]. The source of taurine in the brain is its transport from the periphery and local *de novo* synthesis, with the former believed to be the main source [37]. TauT is involved in taurine transport at the blood–brain barrier, both influx to and efflux from the brain [38,39]. Therefore, urine taurine concentration may partly reflect taurine status in the brain based on the commonality of the source (blood) and transporter (TauT).

Although the reports in the 1980s discussed the possibility of the genetic influence on taurine transport in the kidney, which might be involved in the polygenic components of idiopathic epilepsies, no genetic link between epilepsy and the *SLC6A6* gene has been reported until now. Considering that the expression of TauT is regulated by several factors, including tumor necrosis factor alpha (TNFα), osmolarity, and extracellular taurine concentration [28,37], it may be reasonable to consider that low taurine excretion is an acquired phenomenon. Chronic epileptic seizures may affect the body directly and/or indirectly via the brain through, for example, peripheral neurotransmission and hormonal effects, causing upregulation of TauT in the kidney. Inflammation should also be considered, as elevated serum TNFα concentration has been reported in epileptic children [40].

Despite the non-invasiveness of sample collection, which is especially relevant for young children, the use of urine metabolites as biomarkers for central nervous system diseases requires great caution. It is possible that the urine metabolome reflects the brain metabolome to some extent, as some metabolites produced in the brain may be transported to the plasma and then into the urine [13]. However, the renal excretion of a metabolite is influenced by its plasma concentration and the expression and function of transporters to control its excretion and reabsorption. The net excretion of a metabolite by the kidney is probably in part under genetic

control, but may be influenced by acute events including epileptic seizures via altered autonomic functions and hormones.

There are several limitations in this study. This study did not demonstrate a causal relationship between epilepsy and reduced taurine. Although we minimized group differences by strict inclusion criteria, the sample size was small. The findings of this study need to be confirmed using other independent sample sets to demonstrate generalizability. We could not investigate plasma samples that might reflect the brain metabolome more directly than urine samples, because it was not ethically feasible to collect blood samples from heathy children. Reasonable setting of the control group (e.g., a different disease group) or a change in target age (e.g., adults and adolescents) would enable investigation of the plasma metabolome difference between epilepsy and control groups. Investigations of other chemical classes such as lipids, peptides, proteins, and inflammatory molecules are also required.

Conclusion

We conducted GC/MS- and LC/MS-based urine metabolomics to discover metabolites as a biomarker of recent epileptic seizures in children aged 2 to 12 years. Among measured small-molecule metabolites, only taurine reduction was associated with epilepsy. We did not find other metabolites consistently altered in epilepsy patients compared with healthy controls.

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Author contributions

Tomoyuki Akiyama designed this research, analyzed the data, and drafted the initial version of this manuscript. Daisuke Saigusa, Eiji Hishinuma, and Naomi Matsukawa conducted metabolome analyses. Takushi Inoue, Chiho Tokorodani, Mari Akiyama, Rie Michiue, Atsushi Mori, Takashi Shibata, and Hiroki Tsuchiya collected clinical samples and information. Katsuhiro

Kobayashi revised this manuscript critically for important intellectual content. All authors contributed to the writing of the final manuscript.

Conflict of interest disclosures

The authors declare no competing interests.

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Table 1. Subject profiles

	Patients (n = 17)	Controls (n = 21)	*p*-value
Age	3y, 0m–10y, 10m (median: 7y, 7m)	2y, 10m–11y, 10m (median: 6y, 1m)	0.319
Sex (male:female)	5:12	12:9	0.111
Body mass index	13.6–17.6 (median: 16.0)	13.2–23.2 (median: 15.8)	0.647
Epilepsy type	Self-limited epilepsy with centrotemporal spikes (6 cases) Childhood absence epilepsy (6 cases) Focal epilepsy of unknown cause (3 cases) Self-limited epilepsy with autonomic seizures (1 case) Myoclonic epilepsy in infancy (1 case)	N/A	
Days from last epileptic seizure	0–29 days (median: 5 days)	N/A	N/A
Hours between last meal and urine sample collection	1–6 h (median: 3.5 h)	0–12 h (median: 4 h)	0.189

N/A, not applicable

Table 2. Differential metabolites in GC/MS/MS data

Metabolites	VIP score	*p*-value	FDR	Fold change	AUC
Increased in epilepsy					
Glutamine	1.88	1.1×10^{-4}	0.0084	1.73	0.857
2-Methyl-3-hydroxybutyric acid	1.77	6.4×10^{-4}	0.028	1.40	0.824
2-Aminoadipic acid	1.74	0.0017	0.040	1.52	0.793
Succinic acid	1.73	0.0023	0.040	1.62	0.784
Hydroxylamine	1.72	0.0019	0.040	1.56	0.790
Benzoic acid	1.64	0.0039	0.056	1.72	0.770
Octanoic acid	1.61	0.0029	0.045	1.47	0.779
Maleic acid	1.59	0.0065	0.070	1.37	0.765
Lauric acid	1.53	0.0086	0.082	1.44	0.748
Cysteine	1.51	0.0048	0.059	1.33	0.770
Decreased in epilepsy					
Taurine	2.59	1.9×10^{-7}	3.2×10^{-5}	0.12	0.947
Quinolinic acid	1.90	1.5×10^{-4}	0.0084	0.50	0.860
N-Acetylneuraminic acid	1.62	0.0019	0.040	0.61	0.790
Catechol	1.55	0.0023	0.040	0.65	0.784
Metabolites demonstrated in LC/MS/MS					
Galactose	1.11	0.048	0.15	1.18	0.709
Glucose	0.88	0.089	0.21	1.61	0.658
Tagatose	0.33	0.56	0.63	0.93	0.588
Ornithine	1.18	0.045	0.14	1.26	0.669

AUC, area under the receiver-operating characteristic curve; FDR, false discovery rate; GC/MS/MS, gas chromatography-tandem mass spectrometry; LC/MS/MS, liquid chromatography-tandem mass spectrometry; VIP, variable importance for prediction

Table 3. Differential metabolites in LC/MS/MS data

Metabolites	VIP score	*p*-value	FDR	Fold change	AUC
Increased in epilepsy					
Hexoses	3.14	4.3×10^{-5}	0.0018	5.64	0.880
Ornithine	1.99	0.012	0.17	1.54	0.737
Decreased in epilepsy					
Cysteine	2.47	0.0039	0.081	0.48	0.770
Taurine	1.64	0.029	0.29	0.69	0.709
Metabolites demonstrated in GC/MS/MS†					
Glutamine	0.79	0.28	0.73	1.13	0.594
2-Aminoadipic acid	0.69	0.32	0.73	1.19	0.602

† Only metabolites that were quantified by LC/MS/MS are demonstrated.

AUC, area under the receiver-operating characteristic curve; FDR, false discovery rate; GC/MS/MS, gas chromatography-tandem mass spectrometry; LC/MS/MS, liquid chromatography-tandem mass spectrometry; VIP, variable importance for prediction

Table 4. Differential metabolites in amino acid quantification

Metabolites	VIP score	*p*-value	FDR	Fold change	AUC
Increased in epilepsy					
Glycine	1.51	0.34	0.82	1.08	0.60
Decreased in epilepsy					
Glycylproline	2.39	0.012	0.28	0.68	0.72
Taurine	1.98	0.049	0.59	0.72	0.68
3-Methylhistidine	0.94	0.075	0.60	0.89	0.64
Amino acids demonstrated by GC/MS/MS and LC/MS/MS					
Glutamine	1.04	0.41	0.87	1.11	0.566
2-Aminoadipic acid	0.54	0.28	0.82	1.20	0.625
Ornithine	Excluded				

AUC, area under the receiver-operating characteristic curve; FDR, false discovery rate; GC/MS/MS, gas chromatography-tandem mass spectrometry; LC/MS/MS, liquid chromatography-tandem mass spectrometry; VIP, variable importance for prediction

Figure 1. Results of statistical analyses.

A and B: GC/MS/MS data analysis. Score plot (A) and permutation test (B) for the OPLS-DA model. C and D: LC/MS/MS data analysis. Score plot (C) and permutation test (D) for the OPLS-DA model. E and F: Amino acid data analysis. Score plot (E) and permutation test (F) for the OPLS-DA model.

GC/MS/MS, gas chromatography-tandem mass spectrometry; LC/MS/MS, liquid chromatography-tandem mass spectrometry; OPLS-DA; orthogonal partial least squares discriminant analysis

Figure 2. Results of taurine concentrations by three different analysis methods

Creatinine-corrected, logarithmically transformed, and normalized taurine concentrations determined by GC/MS/MS, LC/MS/MS, and absolute amino acid quantification are demonstrated.

GC/MS/MS, gas chromatography-tandem mass spectrometry; LC/MS/MS, liquid chromatography-tandem mass spectrometry