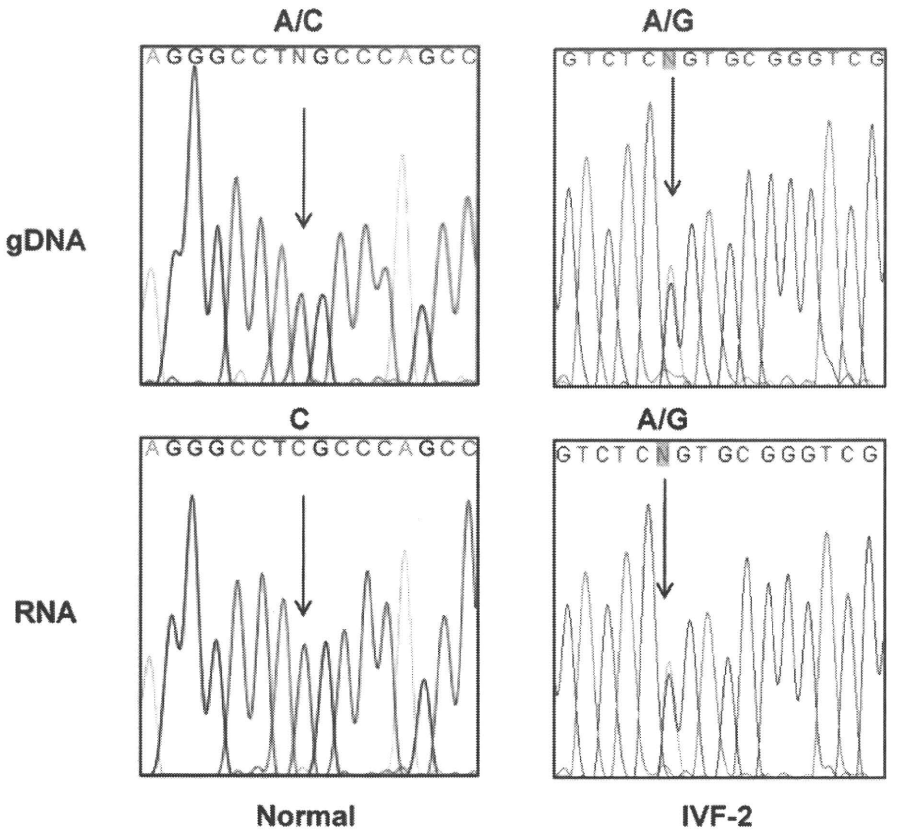

Fig. 5. Allele-specific expression analysis of *Kcnq1ot1* in the lung tissue from normal and IVF-2 cattle. Direct sequencing analysis showed a SNP in the transcribed region of *Kcnq1ot1* in normal and IVF-2 cattle. Direct sequencing analysis of RT-PCR products demonstrated that IVF-2 cattle had biallelic expression of *Kcnq1ot1*.

The use of two restriction enzymes with complementary methylation sensitivities, HpaII and McrBC, is unsurpassed as a simple, rapid method for the analysis of methylation status (Yamada et al., 2004). We therefore employed the HpaII–MspI–McrBC PCR assay to screen the methylation status of the *Cdkn1c* promoter, KvDMR1, and ICR1 in bovines. *Cdkn1c*, an imprinted gene with maternal expression that encodes a cyclin-dependent kinase inhibitor belonging to the CIP/KIP family, has attracted attention as a key gene in BWS and cancer (Higashimoto et al., 2006). The *Cdkn1c* promoter is unmethylated on the both alleles in humans (Diaz-Meyer et al., 2005), whereas it is methylated on the paternal allele in mice (Bhogal et al., 2004). However, methylation status of this region has not been reported in bovines. The bovine *Cdkn1c* promoter was unmethylated according to HpaII–MspI–McrBC PCR assay, and no aberrant CpG methylation was observed in normal, NT, or IVF calves (data not shown).

Kcnq1ot1 is a paternally expressed non-coding RNA associated with a maternally methylated CpG island (KvDMR1) (Horike et al., 2000, 2009). In mice and humans, KvDMR1 is involved in the coordinate control of adjacent imprinted genes including *Cdkn1c* (Fitzpatrick et al., 2002; Horike et al., 2000). In BWS, about 50% of patients demonstrate loss of CpG methylation on KvDMR1, resulting in biallelic expression of *Kcnq1ot1* and subsequent repression of *Cdkn1c* (Higashimoto et al., 2006; Lee et al., 1999). In this study, loss of CpG methylation on KvDMR1 in most organs from two of seven NT calves and one of two IVF calves suffering with LOS, was observed with HpaII–MspI–McrBC PCR assay and bisulfate sequencing analysis. Recently, Couldrey and Lee (2010) have also shown hypomethylation of KvDMR1 in mid-gestation bovine fetuses produced by NT. In their study, Couldrey and Lee used the MassARRAY technology to look at multiple genomic regions and found that the majority of the genome retains normal CpG methylation patterns. However, they found statistically significant differences between NT and artificial insemination in some regions of the genome, particularly KvDMR1 and SNRPN exon 1. On the other hand, we were unable to identify hypomethylation of SNRPN exon 1 (data not shown) although we did observe hypomethylation of KvDMR1 in two of seven full-term NT calves, suggesting that many of the fetuses showing hypomethylation of SNRPN exon 1 may die between mid-gestation and full term.

Differential methylation of ICR1 upstream of *H19* is essential for the reciprocal imprinting of *Igf2* and *H19* in humans and mice (Thorvaldsen et al., 1998). CTCF binds to maternal unmethylated ICR1 and prevents the *Igf2* promoter from interacting with enhancers downstream of

H19, resulting in transcriptional silencing of the maternal *Igf2* allele. In cattle and sheep, the genomic structure and expression profile at the *Igf2-H19* locus is conserved as in humans and mice (Curchoe et al., 2009; Young et al., 2003). In this study, we identified four putative CTCF-binding sites on ICR1 upstream of bovine *H19* (Fig. 1C), and examined their CpG methylation status in NT and IVF animals. We found that this region was differentially methylated in all samples from normal, NT, and IVF animals using HpaII–MspI–McrBC PCR assay, whereas slight hypermethylation was observed in all lung samples from normal (methylation density: 63.1–74.4%), NT (50.8–70.7%), and IVF (69.9–70.0%) calves using bisulfate sequencing analysis. Similar biases in the bisulfate sequencing assay have been reported previously (Curchoe et al., 2009; Kremenskoy et al., 2006). In addition, the expression analysis of *Igf2* using RT-PCR revealed normal expression in all lung samples (data not shown), indicating CpG methylation status of ICR1 was, consistent with HpaII–MspI–McrBC PCR assay results.

RT-PCR revealed that *Kcnq1ot1* was highly expressed in clone-3, -5, and IVF-2, whereas *Cdkn1c* expression was reduced in those calves. *H19* and *Igf2* expression was normal (Fig. 4, Table 3). Moreover, imprinted expression analysis using SNPs showed that *Kcnq1ot1* was biallelically expressed in IVF-2. These findings are consistent with the epigenetic mutation in the *Kcnq1ot1/Cdkn1c* domain of human chromosome 11p15.5 that has been observed in approximately 50% of BWS patients. The biallelic expression of *Kcnq1ot1* and diminished expression of *Cdkn1c* observed in NT- and IVF-derived calves suffering with LOS in this study suggest that aberrant imprinting of the bovine *Kcnq1ot1/Cdkn1c* domain may contribute to LOS calves derived from ART techniques.

To our knowledge, this is the first report to describe aberrant imprinting of the *Kcnq1ot1/Cdkn1c* domain in calves produced by ART techniques. However, no aberrant of DNA methylation in BWS-associated loci were observed in six of nine calves. Although we further investigated three other imprinted genes—*Peg1/Mest*, *Klf14*, and *Gtl2*—no aberrant CpG methylation was found (data not shown). Therefore, LOS in ART animals and the low production rate in NT may not be caused by imprinting defects such as aberrant CpG methylation alone, but other factors might also be involved. However, further epigenetic investigations including not only CpG methylation analysis but also other analyses such as histone modification are needed to increase efficiency of animal production by ART techniques.

In conclusion, imprinting disruption of KvDMR1 and aberrant imprinting of *Kcnq1ot1* and *Cdkn1c* identified in NT and IVF calves may contribute to LOS in animals conceived using ART techniques (Table 3). Our findings and those of Couldrey and Lee suggest that ART techniques might induce an increased risk of epigenetic defects, such as hypomethylation of KvDMR1, because epigenetic changes can be caused by embryo culture itself or the constituents of the culture medium. Therefore, a more thorough understanding of the stability of CpG methylation will be important for the continued safeguarding of ART techniques.

Acknowledgments

We thank the members of the laboratory for valuable suggestions and I. Yamane for statistical analysis and Health Center of Kanazawa city for sample collection. This work was supported by the Program for Improvement of Research Environment for Young Researchers from the Special Coordination Funds for Promoting Science and Technology (SCF), and grants-in-aid for young scientists (B) from the Ministry of Education, Culture, Sports, Science and Technology, Japan (to SH).

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Brain & Development xxx (2009) xxx–xxx

BRAIN & DEVELOPMENT
Official Journal of the Japanese Society of Child Neurology

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Case report

Abnormal glucose metabolism in aromatic L-amino acid decarboxylase deficiency

Shuhei Ide [a,b], Masayuki Sasaki [a,*], Mitsuhiro Kato [c], Takashi Shiihara [c,d], Satoru Kinoshita [a], Jun-ya Takahashi [a], Yu-ichi Goto [b]

[a] Department of Child Neurology, National Center of Neurology and Psychiatry (NCNP), 4-1-1 Ogawahigashi-cho, Kodaira, Tokyo 187-8551, Japan
[b] Department of Mental Deficiency, National Institute of Neuroscience, NCNP, Kodaira, Tokyo 187-8553, Japan
[c] Department of Pediatrics, Yamagata University Hospital, Yamagata, Japan
[d] Department of Neurology, Gunma Children's Medical Center, Shibukawa, Gunma, Japan

Received 7 February 2009; received in revised form 29 April 2009; accepted 10 May 2009

Abstract

We report sibling cases of aromatic L-amino acid decarboxylase (AADC) deficiency, which is a very rare congenital metabolic disorder. These patients were born to healthy and non-consanguineous parents, and presented oculogyric crises, paroxysmal dystonic attacks, and severe psychomotor retardation since early infancy. In cerebrospinal fluid the levels of homovanillic acid and 5-hydroxyindoleacetic acid were very low and the level of L-dopa was very high. The diagnosis was confirmed by the lack of AADC activity in plasma, and a point mutation in the *AADC* gene. MRI revealed a slightly small volume of the prefrontal areas and normal myelination in both patients. Positron emission tomography using 2-deoxy-2[^{18}F] fluoro-D-glucose was performed in one patient, which revealed hypometabolism in the prefrontal cortex and bilateral basal ganglia with a little laterality. These findings suggested that the severe dystonic features were caused by abnormal function of bilateral basal ganglia and severe psychomotor retardation could be due to abnormalities in prefrontal cortical activity.

Keywords: AADC deficiency; MRI; PET; Prefrontal cortex; Caudate nucleus

1. Introduction

Aromatic L-amino acid decarboxylase (AADC or dopa decarboxylase: DDC) deficiency (OMIM #608643) is an extremely rare congenital metabolic disorder and one of the infantile movement disorders, which is very intractable to treat [1–4]. Although less than 100 cases have been reported worldwide [1–8], a relatively high occurrence rate was reported in Taiwan [7]. AADC converts L-dopa to dopamine and 5-hydroxy tryptophan to serotonin, and its deficiency results in the depletion of both dopamine and serotonin in the brain. As a consequence, several characteristic symptoms are caused.

We experienced sibling cases of AADC deficiency, confirmed by enzymatic and genetic analysis. We report magnetic resonance imaging (MRI) findings in both cases, and positron emission tomography (PET) using 2-deoxy-2[^{18}F] fluoro-D-glucose (FDG) between dystonic attacks was performed in patient 1.

2. Case reports

2.1. Patient 1

This 3-year-old boy was born to healthy and unrelated parents with mild asphyxia at full term. He cried

* Corresponding author. Tel.: +81 42 341 2711; fax: +81 42 344 6745.
E-mail address: masasaki@ncnp.go.jp (M. Sasaki).

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doi:10.1016/j.braindev.2009.05.004

Please cite this article in press as: Ide S et al. Abnormal glucose metabolism in aromatic L-amino acid decarboxylase deficiency. Brain Dev (2009), doi:10.1016/j.braindev.2009.05.004

Table 1
The concentration of catecholamine of the CSF.

	L-Dopa	HVA	MHPG	5-HIAA
Patient 1	13.6	5.7	<1.0	<1.0
Patient 2	27.4	12.2	<1.0	<1.0
Normal range	<2.0(ng/ml)	28–200(ng/ml)	6.5–51(ng/ml)	17–116(ng/ml)

HVA, homovanillic acid; MHPG, 3-methoxy-4-hydroxy-phenylglycol; 5HIAA, 5-hydroxyindoleneeric acid.

weakly, was motion-less since birth, and needed tube feeding for 1 week. He first showed oculogyric crisis at 3 months of age, and had similar attacks several times a week. Oculogyric crisis usually lasted about 30 min. He also suffered from generalized dystonic attacks for 30–120 min several times a week. Opisthotonus or bicycle-riding movements were observed during these attacks. He showed visual pursuit at 6 months of age, but had not yet obtained head control or rolling over. He had a severe intellectual and motor developmental delay. He was always nasally congested and his face was frequently running with sweat during wakefulness.

A neurological examination between dystonic attacks revealed general hypotonia, paucity of movement, slightly exaggerated deep tendon reflexes and pathological reflexes. Eye movement was normal. Ordinary blood analyses were normal. An electroencephalogram (EEG) showed no paroxysmal discharges during either dystonic attacks or inter-

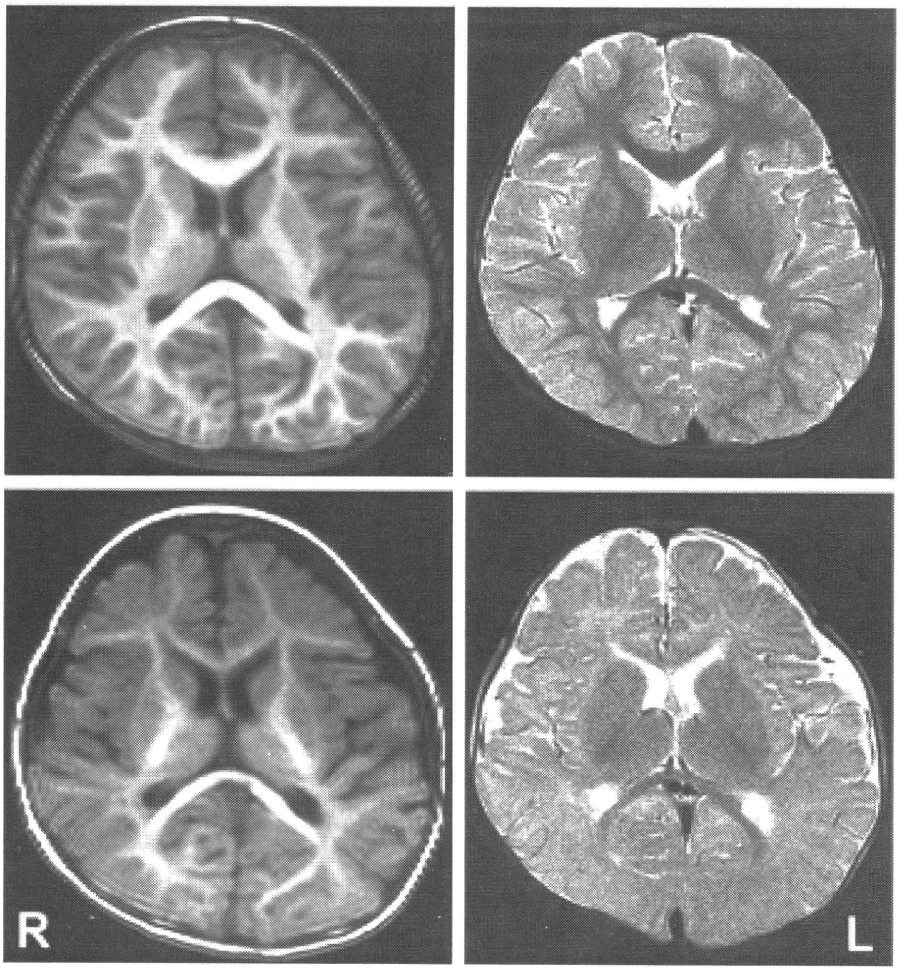

Fig. 1. Axial T1-weighted (left) and T2-weighted (right) MRI at the level of the putamen. Upper row is patient 1 and lower row is patient 2. MRI shows a slightly small volume of the prefrontal areas in both patients. The volumes of basal ganglia and brain cortex are normal, and myelination is also normal. No abnormal intensity areas are seen.

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mittent states. A catecholamine analysis of the cerebrospinal fluid (CSF) revealed a very high concentration of L-dopa and a very low concentration of homovanilic acid (HVA) and 5-hydroxyindolacetic acid (5-HIAA) (Table 1). These results strongly suggested AADC deficiency.

2.2. Patient 2

This 6-month-old girl was the younger sister of patient 1. She was born healthy with no adverse events. She also showed oculogyric crisis since 1 month of age, and paroxysmal general hypertonia lasting for a few hours since 3 months of age but she was alert during the attack. She had not yet obtained head control or rolling over. She also disclosed general hypotonia and paucity of movement between hypertonic attacks. Her CSF revealed a high concentration of L-dopa and a very low concentration of HVA and 5-HIAA (Table 1).

2.3. AADC activity

AADC activity was measured in the serum to confirm the diagnosis using previously reported methods [9]. Serum AADC activity was very low in both patients (AADC activity: 0.5 pmol/min/ml in patient 1, 0.4 pmol/min/ml in patient 2; normal; 50–100).

2.4. Gene analysis

The *AADC* gene mutation was analyzed after obtaining informed consent from the parents of the patients. Genomic DNA from peripheral blood of the patients was extracted according to standard procedures. Each exon of the *AADC* gene was amplified by PCR using primers designed to amplify the coding and flanking non-coding *AADC* regions. Bidirectional cycle sequencing reactions were performed with the ABI Big Dye Terminator Sequencing Kit (Applied Biosystems: Foster city, CA, USA), and the purified products were subject to an automated capillary array sequencer (ABI 3100, Applied Biosystems). Sequencing results revealed a heterozygous point mutation (g.329C > A). The other mutation was not detected. We confirmed that this point mutation was not present in 50 normal Japanese controls.

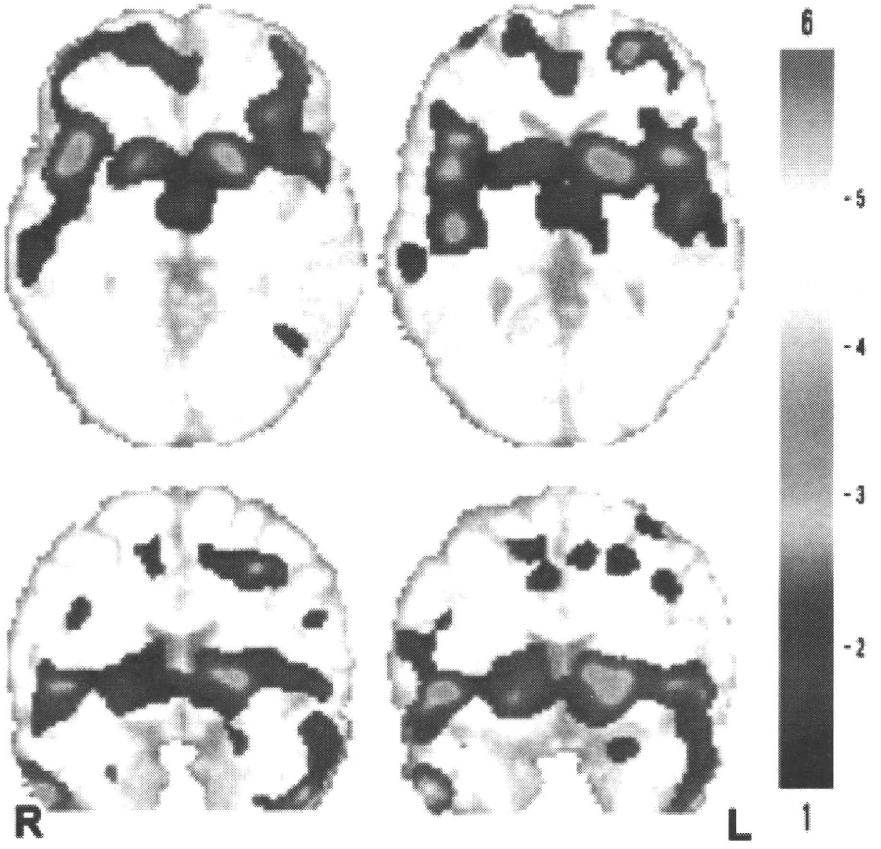

Fig. 2. Easy Z-score imaging (eZIS) analysis of FDG-PET in patient 1. Hypometabolism is observed in bilateral caudate nucleus to putamen (lower in the left side) and insular cortex with some laterality. Upper; axial section, lower; coronal section.

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3. Neuroradiological studies

MRI: Brain MRI in both patients revealed a slightly small volume of the prefrontal areas (Fig. 1) and normal myelination. No abnormal findings in the basal ganglia were observed.

PET: Glucose metabolism was evaluated by FDG-PET in patient 1. We evaluated the results by using an easy Z-score imaging system (eZIS) [10]. eZIS revealed hypometabolism in both caudate nuclei and putamina with some laterality (lower in the left side) (Fig. 2) and prefrontal cortex (Fig. 3). The area in which the level of the area was lower than −2SD compared with the standard is colored with purple or blue and the area lower than −3SD is colored with green.

4. Discussion

Patient 1 was at first assumed to have cerebral palsy (CP) because he was born with mild asphyxia. He had been diagnosed with a dystonic type of CP before patient 2 was born. Patient 2, who was born healthy, showed oculogyric crises and dystonic attacks. Since these symptoms were the same as those in patient 1, it was presumed that they both had a basic disorder. Repeated attacks of dystonia reminded us of childhood movement disorders, especially neurotransmitter diseases, and the catecholamine in the CSF indicated an abnormality in the level of neurotransmitters. The low activity of AADC confirmed the diagnosis of AADC deficiency. The gene analysis of the *AADC* showed heterozygous mutation. Since we examined all exons and intron–exon junctions, there must be other mutation in other area. After the diagnosis was established, both patients were treated with a monoamine oxidase (MAO) inhibitor and a dopamine agonist, but showed no favorable response.

In MRI studies, the volume of the prefrontal area was reduced in both cases by visual inspection, although we did not performed volumetric study. The volume of the basal ganglia was normal.

We performed FDG-PET in patient 1 to investigate the brain glucose metabolism. The eZIS analysis revealed hypometabolism in both basal ganglia and prefrontal cortex. To our knowledge, these findings have not yet been reported in other patients with AADC deficiency [3].

In AADC deficiency, both dopamine and serotonin depletion must have occurred in the brain. Dopamine is mostly involved in substantia nigra and basal ganglia circuits. Hypometabolism in caudate nuclei shown in this FDG-PET study probably could be the cause the symptoms of dystonia and muscle tone abnormality.

The mechanism for the slightly small size and hypometabolism in the prefrontal cortex was not identified. Mesencephalic dopaminergic neurons are known to project to the prefrontal cortex and striatum [11]. The dopamine depletion probably causes dysfunction in dopaminergic innervation, and depleted dopaminergic pathways in the prefrontal cortex probably cause the occurrence of prefrontal cortical dysfunction. Similar dysfunction could occur in the serotonergic pathways. Most patients with AADC deficiency have both severe motor developmental and severe intellectual disability, which might be explained by the prefrontal cortical dysfunction.

Both dopamine and serotonin depletion could produce not only basal ganglia dysfunction but also prefrontal cortical dysfunction, especially in the developing brain.

Acknowledgments

The authors are very grateful to Hiroshi Matsuda M.D. at Saitama Medical Center for providing eZIS analysis for FDG-PET study.

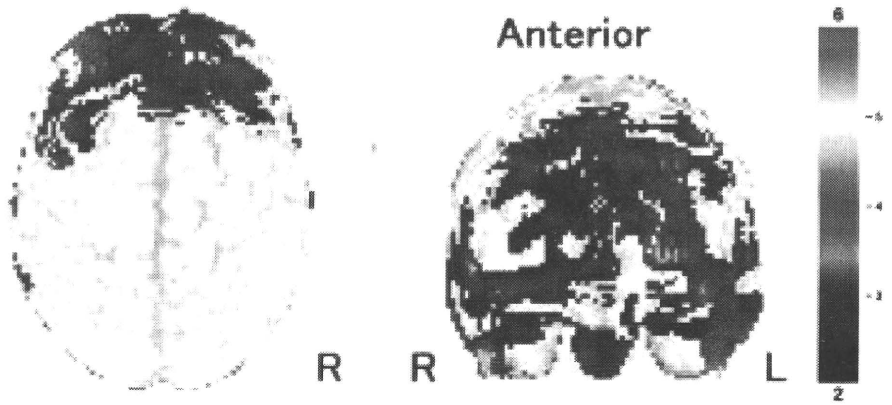

Fig. 3. eZIS analysis of FDG-PET in the projected view show hypometabolism in the prefrontal cortex. Left: from the upper side. Right: from the anterior side.

Please cite this article in press as: Ide S et al. Abnormal glucose metabolism in aromatic L-amino acid decarboxylase deficiency. Brain Dev (2009), doi:10.1016/j.braindev.2009.05.004

This study was supported in part by the Research Grant (20B-14) for Nervous and Mental Disorders from the Ministry of Health, Labor and Welfare.

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Please cite this article in press as: Ide S et al. Abnormal glucose metabolism in aromatic L-amino acid decarboxylase deficiency. Brain Dev (2009), doi:10.1016/j.braindev.2009.05.004