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Argininosuccinate synthetase gene is silenced by CpG methylation in children with phenylketonuria

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ABSTRACT

Objectives: The concentration of tyrosine and the ratio of branch-amino acid to the aromatic amino acid in phenylketonuria (PKU) patients are much lower than that of normal people, which reveal that PKU patients have amino acid metabolism disorder. The aim of the present study was to investigate the arginine level in blood, the expression of argininosuccinate synthetase (ASS), the rate-limiting enzyme in arginine synthesis pathway, and the methylation of ASS in patients with PKU.

Design and Methods: Twenty-five children with PKU and 65 healthy controls were investigated in this study. Blood concentration of arginine was analyzed by automatic amino acid analyzer. The methylation of ASS gene promoter was evaluated by using methylation-specific polymerase chain reaction (MSP) and bisulfite sequencing PCR (BSP) methods, and the mRNA level of *ASS* was evaluated by semi-quantitative RT-PCR.

Results: Blood concentration of arginine in PKU patients without dietary control was 0.017 ± 0.009 mmol/L while in normal persons was 0.129 ± 0.007 mmol/L, which is statistically significant ($P < 0.001$). The promoter of *ASS* was methylated in PKU (15/15, 100%) but not in normal persons (0/15). The mRNA level of *ASS* in PKU patients was lower than that of normal people, which was well correlated with its methylation status.

Conclusions: The silencing of *ASS* due to aberrant promoter CpG methylation may be an important mechanism for arginine biosynthesis disorders in PKU. High levels of phenylalanine and low levels of arginine are common characteristics in PKU patients. These findings would extend the current understanding of arginine, *ASS* in the development of PKU disease.

Introduction

Phenylketonuria (PKU) is an autosomal recessive metabolic hereditary disease characterized by a mutation in the gene located at the long arm of chromosome 12 for the hepatic enzyme phenylalanine hydroxylase (PAH), which leads to the loss of activity of liver phenylalanine hydroxylase and phenylalanine (Phe) metabolism disorders [1]. The concentration of phenylalanine in phenylketonuria patients is 15 times higher than normal level [2]. Agitation, overactivity, depression and abnormal mental behavior always occur in children with disease, and finally lead to intermediate or severe oligophrenia (low intelligence). Thus, the early and accurate diagnosis and therapy of PKU are of importance. Since its discovery, there have been many advances in its treatment. Early cases of PKU were treated with a low-phenylalanine diet. More recent research has now shown that diet alone may not be enough to prevent the negative effects of elevated phenylalanine levels.

Amino acids are the building blocks of life. Amino acid composition change in various body fluids can directly reflect health condition and nutritional status, and besides relate to a lot of pathological processes. Arginine plays important roles in the metabolism of an organism [3–5]. It is the precursor for the synthesis of proteins and other molecules of great biological importance, including nitric oxide, ornithine, polyamines, agmatine, proline, glutamate, creatine, dimethylarginine, and urea. Arginine is an indispensable amino acid to children, but one which is semi-essential to adults. Argininosuccinate synthetase (ASS) is a rate-limiting enzyme in the synthesis of arginine [6]. ASS converts citrulline to argininosuccinate which is then converted to arginine by argininosuccinate lyase [7,8]. This metabolic pathway allows cells to synthesize arginine from citrulline, making this amino acid non-essential for the growth of humans and most mammalian cells. The ASS gene that involved in argininosuccinate synthase expression locates on chromosome 9 [22]. ASS cDNA has been cloned in 1981. The kinetic property of ASS enzyme has been extensively studied and the crystal structure in bacteria has been identified. The mechanisms of transcriptional and translational control of ASS gene are not well understood and appear to be tissue specific [9].

Here, we report that the concentration of arginine in the serum of PKU patients without dietary control was decreased, the promoter of *ASS* was methylated resulting in down regulation of its expression at

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mRNA level in PKU patients compared with that of healthy control, suggesting that high levels of phenylalanine and low levels of arginine are common characteristics, the silencing of *ASS* due to aberrant promoter CpG methylation maybe an important mechanism for arginine biosynthesis disorder in PKU patients.

Materials and methods

Patients

From May 2009 to May 2011, 25 patients with PKU and 65 normal children with the age from 8 months to 2 years at the NingXia Maternal and Child Care Service Center were investigated in this study. All PKU patients had been diagnosed by "neonatal screening", which screened for high Phe concentrations in blood taken by heel puncture [4], the phenylalanine level of which more than 120 μmol/L. There was no dietary control for patients and healthy controls. The study was approved by the Institution Ethics Committee of Peking University, and written consent was obtained from the parents.

Quantitative analysis of Phe and Arg

Fasting venous blood (1 mL) was drawn from each of participants, centrifuged at 3000 rpm for 15 min, and 0.5 mL supernatant was collected and mixed with 5% sulfosalicylic acid (1:1), centrifuged at 10000 rpm for 10 min. The supernatant was used for Phe and Arg examination by using an 835-50 automatic amino acid analyzer (Hitachi Ltd., Tokyo, Japan). Results were calculated with amino acid standard (Sigma) as internal calibrator.

DNA extraction

DNA extraction was performed as described previously [27]. Briefly, fasting venous blood (1 mL) was drawn from each of the participants and placed on ice. The genomic DNA was extracted by using phenol–chloroform extraction method. The genomic DNA was precipitated and dissolved in 20 μL sterilized water and stored at −20 °C.

DNA bisulfite treatment and methylation analysis

DNA bisulfite treatment was performed according to the manufacturer's instruction by using EZ DNA Methylation Kit™ (Zymed Research, Orange, CA). Methylation-specific PCR (MSP), and bisulfite sequencing PCR (BSP) were carried out as described previously [10]. The methylation of the ASS promoter was determined by MSP and BSP using the specific primers designed according to the online primers program "MethPrimer" (http://www.uro-gene.org/methprimer/). The PCR analysis was performed by using FailSafe™ PCR Kit (Epicentre Biotechnologies, Madison, WI). The PCR for MSP was carried out in a 20 μL reaction mixture containing 2.0 μL 10× Buffer, 0.5 μL dNTP, 0.5 μL of each primer (10 μmol/L), 0.25 μL Taq DNA polymerase, 14.25 μL ddH_2O and 2 μL DNA template. The PCR conditions were 94 °C predenaturation for 3 min, 32 cycles of each consisting of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 60 s, followed by an extension at 72 °C for 10 min. The PCR products were 116 bp. The following were the primers used for MSP: ASS M1: 5′-TAGGAGGTAAGTTTTTCGAAGGAC-3′ and ASS M2: 5′-GATTCCTAACCCTAACCCGC-3′. MSP primers were tested previously for not amplifying any unbisulfited DNA and the specificity of MSP was further confirmed by direct sequencing of some PCR products, which is known as BSP. In order to enrich template to facilitate sequencing, two pairs of nested PCR primer were designed. P1/P2 was used to amplify the long sequences of methylated DNA region while P3/P4 was used to amplify the short sequences of methylated DNA region, in which the amplification products of P1/P2 used as template. The first round of nested PCR was carried out in a 20 μL reaction mixture containing 2.0 μL 10× Buffer, 0.5 μL dNTP, 0.5 μL of each primer (10 μmol/L), 0.25 μL Taq DNA polymerase, 14.25 μL ddH_2O and 2 μL DNA template. The PCR conditions were 94 °C for 3 min, 32 cycles of each consisting of 94 °C for 30 s, 62 °C for 30 s and 72 °C for 60 s, followed by an extension at 72 °C for 10 min. The PCR products were used as the templates for the second round of PCR. The second round of nested PCR was carried out in a 20 μL reaction mixture containing 2.0 μL 10× Buffer, 0.5 μL dNTP, 0.5 μL of each primer (10 μmol/L), 0.25 μL Taq DNA polymerase, 15.25 μL ddH_2O and 1 μL DNA template. The PCR conditions were 94 °C for 3 min, 32 cycles of each consisting of 94 °C for 30 s, 62 °C for 30 s and 72 °C for 60 s, followed by an extension at 72 °C for 10 min. The following were the primers used for BSP: P1: 5′-TATTGAGGTTATGGTTGGGGAG-3′; P2: 5′-CCCAAATCTCCATATAAAAACTTCA-3′; P3: 5′-GGTTTTGGGGGTTGTAGAAGGTT-3′; and P4: 5′-AAAACCCCTTCCCTCCTACCTC-3′. The PCR products of "P3/P4" primer were cloned into Trans GenPpEASY2TVector, with 6 to 15 colonies randomly chosen and sequenced.

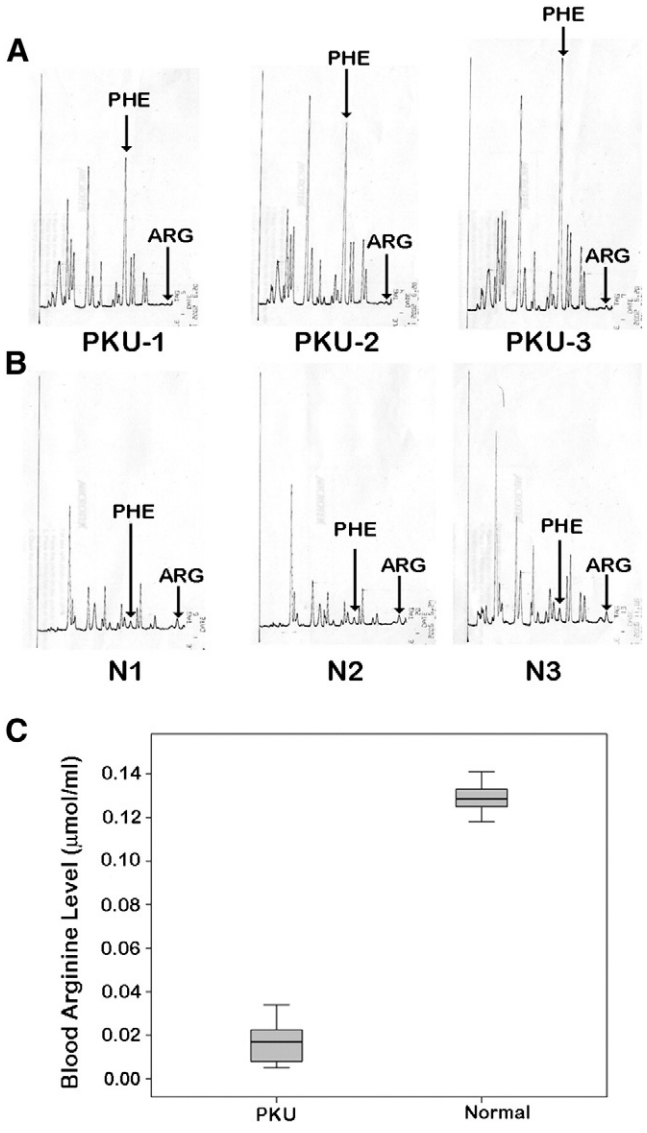

Fig. 1. Blood arginine level is significantly decreased in PKU patients. (A, B) Representative chromatograms detected by automatic amino acid analyzer. (A) PKU patients. (B) Normal people. The arrows indicated that the peak of phenylalanine (Phe) in A was much higher than that of B, while the peak of arginine (Arg) in A was significantly lower than that of B. (C) Quantification of the arginine concentration. Chi-square criterion and t test were conducted with SPSS 18.0 software. The mean blood arginine level in PKU patients and normal people were 0.017 ± 0.009 and 0.129 ± 0.007 (μmol/mL) (mean ± s.d.) respectively.

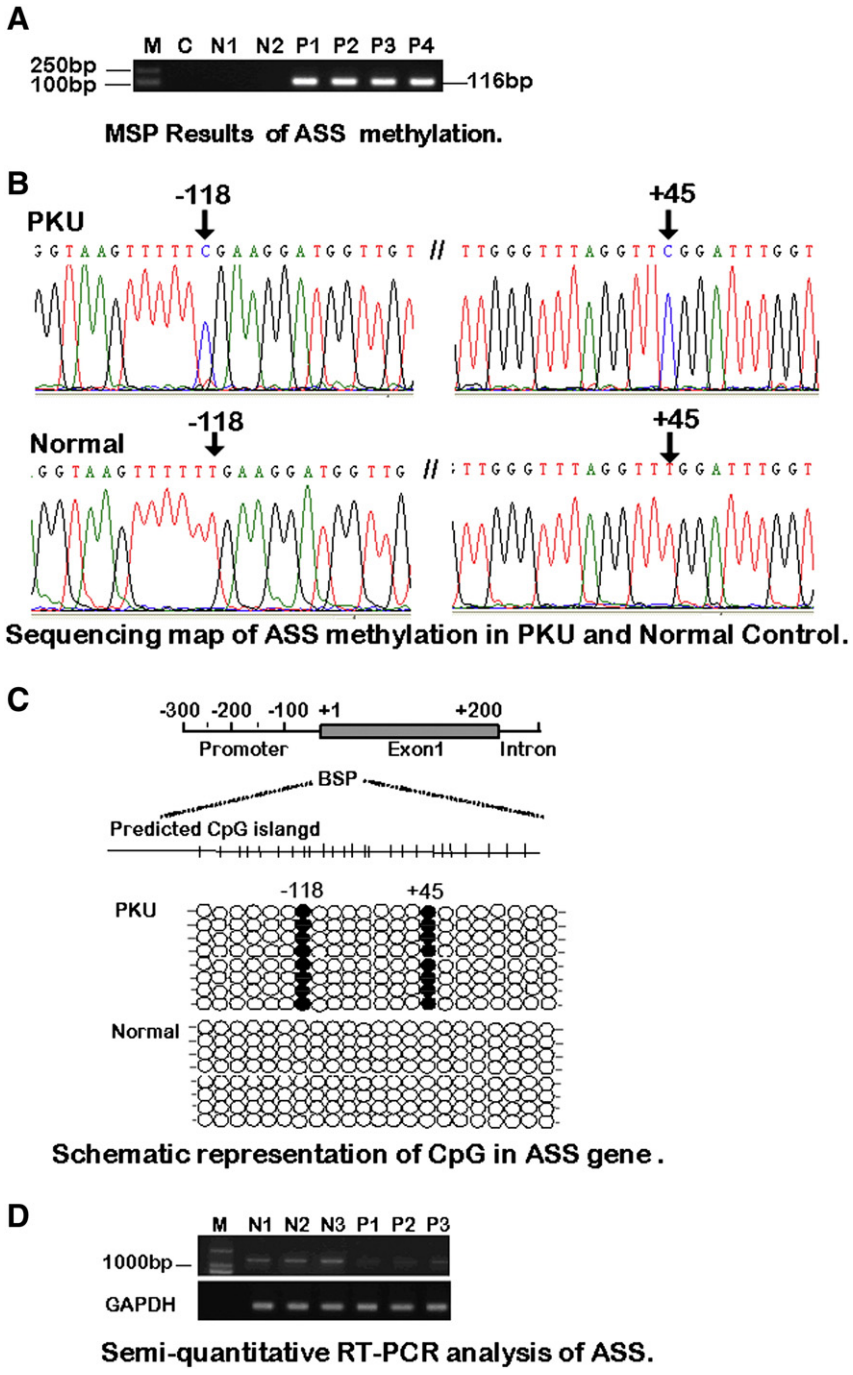

Fig. 2. Methylation of *ASS* in PKU patients. (A) MSP results of ASS methylation. One 116 bp-stretch sequence was amplified. M, marker; C, ddH_2O; N1–N2, normal people; P1–P3, PKU patients. (B) Sequencing maps of ASS methylation determined by BSP. (C) Schematic representation of ASS gene CpG from PKU patients and normal people. The white circles were non-methylated CpG sites; the black circles were methylated CpG sites. The methylated CpG sites of all the 15 PKU patients located at nt +45 and nt −118. (D) Semi-quantitative RT-PCR analysis of ASS gene in PKU patients and normal people. M, marker; N1–N2, normal people; P1–P3, PKU patients. The mRNA level of ASS gene in the same 15 PKU patients was lower than that of normal people.

Semi-quantitative reverse transcription-PCR

The expression level of ASS gene was determined by semi-quantitative RT-PCR as described previously [28]. Total RNA was extracted according to the manufacturer's instruction using NucleoSpin® RNA II (MACHEREY-NAGEL). First-strand cDNA was synthesized from 1 μg of total RNA using a Superscript III Reverse Transcriptase Kit (Invitrogen, Carlsbad, CA). cDNA was stored at −20 °C. PCR mixtures contained 5.0 μL 10× Buffer, 3 μL $MgCl_2$ (2.5 mM), 1 μL dNTP, 0.25 μmol/L of forward and reverse primers, 0.5 μL Taq DNA polymerase, 36.5 μL ddH_2O and 2 μL cDNA template, for a total volume of 50 μL. PCR conditions were as follows: hold for 3 min at 94 °C, followed by 35 cycles of each consisting of 30 s at 94 °C, 30 s at 58 °C and 60 s at 72 °C, then followed by an extension at 72 °C for 10 min. The amplified products were 250 bp, and subjected to electrophoresis in a 1% agarose gel. Amplification of GAPDH was used as an internal control. Primers used in the PCR were as follows: ASS forward: 5′-TTCACTGCACTGTGAAAACAGA-3′; ASS reverse: 5′-CTCATTGTACA CGGGTCTATTT-3′; GAPDH forward: 5′-GAAGGTGAAGGTCGGAGTC-3′; and GAPDH reverse: 5′-GAAGATGGTGATGGGATTTC-3′.

Statistical analysis

The blood samples from 25 PKU patients and 65 normal people were analyzed to determine the arginine concentration. Chi-square criterion and t test were conducted with SPSS 18.0 software (SPSS Inc., Chicago, IL). P values < 0.05 were defined as statistically significant.

Results

The blood arginine level is significantly decreased in PKU patients

The concentration of arginine in the serum was analyzed by automatic amino acid analyzer. 25 PKU patients and 65 normal persons without dietary control were tested. As shown in Fig. 1A, B, the peak of phenylalanine (Phe) was much higher, while the peak of arginine (Arg) was significantly lower in PKU (A) than that of in normal people (B). The quantification of the arginine concentration was shown in Fig. 1C. The blood arginine level in normal people was 0.129 ± 0.007 (μmol/mL) (mean ± s.d.), while decreased to 0.017 ± 0.009 (μmol/mL) in PKU patients. The data were normally distributed within each group ($P_1 = 0.182$, $P_2 = 0.69$) and the two population variances were equal at the significance level 0.05 ($F = 3.21$, $P = 0.081$). The t test of two independent-samples resulted in $t = 45.022$, $P < 0.001$, the difference was statistically significant ($P < 0.05$). It should be noted that, although circulating levels of most AA undergo marked changes during neonatal period, under catabolic conditions and in disease [11], the concentration of arginine in blood was not significantly different within the age from 8 months to 2 years in PKU patients without dietary control.

The promoter of ASS was methylated in PKU patients

As ASS is the rate-limiting enzyme in the synthesis of arginine, and the promoter of ASS contains a typical CpG island (Fig. 2C), we analyzed its methylation by MSP in 15 PKU patients and 15 normal children. Results showed that patients with PKU had methylated promoter whereas no methylation was detected in normal people (Fig. 2A). We also examined the detailed methylation status of individual CpG sites in the promoter of ASS by BSP and results were consistent with those of MSP (Fig. 2B, C). The exact methylated sites in the promoter of ASS gene were located at nt +45 of the first exon (the first nucleotide sequence in the first exon designated as +1) and nt −118 of the promoter. Aberrant promoter CpG methylation is related to gene silencing. The nt +45 point in the first exon was located at the inner of ASS gene. So we hypothesize that methylation of this site would prevent RNA polymerase to recognize the base correctly, and hence prevent its extension. Besides, the nt −118 point was not in the nt −10 and −35 regions of the promoter which had critical influence on gene expression. Therefore, methylation of this site did not shutdown the ASS gene. The methylation of nt +45 point and nt −118 point may inhibit the expression of ASS gene through synergistic effect. In short, the methylation of these two sites did not completely shutdown, but partly suppress the transcription of ASS gene, which was confirmed by the blood arginine concentration from PKU patients in vivo. As shown in Fig. 1, the expression of arginine was reduced to 10% in PKU patients.

The mRNA level of ASS was decreased in PKU patients

Next, the expression of ASS gene was confirmed in the same 15 PKU patients by semi-quantitative RT-PCR. Results showed that the mRNA level of ASS gene in all the 15 PKU patients was lower than that of normal people (Fig. 2D). ASS gene is the key gene involved in the metabolism of arginine, without it arginine cannot be synthesized [10]. The decrease of ASS mRNA led to substantial reduction of ASS protein synthesis and hence restricted arginine synthesis. Results from semi-quantitative RT-PCR confirmed the relatively weak expression of ASS in PKU patients, supporting the notion that the methylation of ASS gene was well correlated with its transcriptional down regulation, which consequently disturbs the synthesis of arginine.

Discussion

Arginine is an ampholytic amino acid, whose side chain closest to the main chain is longer, organic and hydrophobic, and the other end of the side chain is a guanidine [12]. In addition to providing nutrition, arginine has many other physiological functions in the human body. For example, it is the precursors of nitric oxide, urea, and ornithine and is the key element of muscle pigment synthesis. Besides, arginine can assist vasodilatation, suppress viral replication, improve sperm quality, and increase sperm motion energy [13–17]. In this study, our results showed that the concentration of arginine in PKU patients without dietary control is significantly decreased, which is consistent with the previously published report from Schulpis K.H. [17] and Kanzelmeyer N. et al. [18]. These results indicated that patients with PKU have an obstacle of arginine synthesis. High levels of phenylalanine and low levels of arginine are common characteristics in PKU patients.

ASS is a rate-limiting enzyme which converts citrulline to arginine [20,21]. As a key enzyme of arginine synthesis pathway, ASS plays an important role in urea synthesis, nitric oxide synthesis, polyamine synthesis, creatine synthesis and other metabolic pathways [7,22–25]. The expression changes of this enzyme will affect vascular contraction and expansion, the formation of sperm and other physiological and metabolic functions. The expression of ASS gene usually reduced in tumors which include hepatocellular carcinoma, melanoma, some mesotheliomas and some renal cell cancer types [26]. To the best of our knowledge, this is the first study concerning ASS expression in PKU patients. Our results found that the promoter of *ASS* was methylated and the exact methylated sites were located at nt +45 of the first exon and nt −118 of the promoter in all the 15 PKU patients tested. Aberrant promoter CpG methylation of *ASS* results in its reduced expression at mRNA level in PKU patients compared with that of healthy control, suggesting that the down regulating of *ASS* due to aberrant methylation may be an important mechanism for arginine biosynthesis disorder in PKU patients. We noticed that the methylation of *ASS* has already been detectable before obvious symptoms of phenylketonuria appear in patients with PKU. But the exact factors that result in the methylation of *ASS* still remain unclear.

Taken together, our findings revealed that *ASS* is silenced by CpG methylation in children with PKU, resulting in the arginine synthesis deficiency contributing to develop some arginine deficiency symptoms in PKU patients. Therefore, when phenylketonuria patients were treated, not only low-phenylalanine diet is needed, but also a good arginine supplementation is required. Thus our study extends the current understanding of arginine, ASS in the development of PKU disease.

Conflict of interest

The authors declare no conflict of interest.

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