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Short Communication

Demethylation of the hypoxia induction factor 1 binding site of GPX3 at excess blood ammonia in propionic acidemia

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ABSTRACT

Objectives: Elevated levels of metabolites such as ammonia and propionylcarnitine in propionic acidemia (PA) lead to an increased reactive oxygen species (ROS) production which could activate and stabilize the epigenetic regulated hypoxia-inducible factor-1α (*HIF-1α*). In order to evaluate the DNA methylation status of the *HIF-1α* binding site in PA, we investigated the antioxidant gluthatione peroxidase 3 gene (*GPX3*) promoter region.

Design and methods: Using leukocyte DNA extracted from bloodspots collected 2–4 days after birth from diet free newborns, the cytosine phosphodiester bond guanine (CpG) dinucleotides of a *HIF-1α* binding site (CGTTTTTACG) in the promoter region of *GPX3* was retrospectively analysed. Patients included 7 PA.
and 7 healthy controls (KO) respectively.

Results: A demethylated TGTTTTTTATG allele was detected in 3 PA patients with blood ammonia (NH3) concentrations of 500, 595, and 987 umol/L respectively; a demethylated/partial methylated TGTTTTTTAC/TG allele in 4 PA patients (2 PA with blood NH3 = 213, 271 umol/L respectively); a partial methylated C/TG TTTTTTAC/TG allele in 5 healthy controls respectively; a partial methylated/methylated C/TGTTTTTTACG allele in 2 healthy controls.

Conclusion: Our results suggest that at excess NH3, the DNA methylation status of the *HIF-1α* binding site of *GPX3* in newborns with PA is demethylated (TGTTTTTTATG allele). However, the demethylated allele has to be confirmed as a statistically significant change in more patients.

1. Introduction

PA is caused by an inherited deficiency of propionyl-CoA carboxylase (*PCC*). As a result of the enzyme deficiency, primary biochemical indicators such as propionylcarnitine, 3-hydroxypropionate, and 2-methylcitrate accumulate in PA. These primary biochemical indicators inhibit mitochondrial enzymes resulting in the secondary accumulation of ammonia, lactate, ketone bodies, and metabolic acidosis [1]. The pathophisiology of hyperammonemia in PA shows that propionyl-CoA accumulate and inhibit *N*-acetylglutamate synthase which catalyses carbamoyl-phosphte synthese, an enzyme that catalyses the first rate-limiting step of the urea cycle [2]. Primary biochemical changes and a consequent secondary mitochondrial dysfunction in PA have been shown to result in an increased ROS production [3,4] which could in turn activate and stabilize *HIF1α* [5,6], a transcription factor that regulates oxygen homeostasis, hypoxia, oxidative stress, and the transcription of target genes such as the plasma antioxidant, gluthathione peroxidase (*GPX3*) [7]. Since the biochemical changes in PA are measured in plasma where *GPX3* is expressed as an extracellular antioxidant [1,7], *GPX3* was chosen as an ideal candidate gene for analysis. The promoter region of *GPX3* has a *HIF-1α* binding site with a core ACGT sequence flanked by a CG-rich region [7,8]. Since *HIF-1α* regulated genes are sensitive to cytosine methylation of the *HIF-1α* binding site [8], we investigated the DNA methylation status of the *HIF-1α* binding site in PA and healthy controls respectively. A demethylated *HIF-1α* binding site at excess NH3 in PA was detected.

2. Materials and methods

2.1. Patients

A retrospective analysis of newborn blood spots from the Austrian newborn screening program was carried out. This investigation was approved by the ethics committee of the Medical University of Vienna

Abbreviations: GPX3, gluthatione peroxidase 3; CpG, cytosine phosphodiester bond guanine; PA, propionic acidemia; FC, free carnitine; HIF-1α, hypoxia-inducible factor-1α; ROS, reactive oxygen species; KO, healthy control; *PCC*, propionyl-CoA carboxylase; NBS, newborn screening; NH3, ammonia

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(EK 1085/2015). Samples were collected on days 2 or 3 or 4 after birth. 7 PA term newborns (39–41 gestation week, 2.77–4.12 kg birth weight, C3 ≥ 6.6 μmol/l) with no diet restriction and 7 healthy controls (39–41 gestation week, 2.54–3.86 kg birth weight, C3 < 6.6 μmol/l) were investigated. The cut-off value for low free carnitine (FC) was < 7 μmol/l. A second newborn screening card and urine probe were respectively used to confirm PA.

2.2. Analysis of sample

Samples were analysed using an already described protocol [9]. Amplification primers were:

Fw 5′ CGTTGTGTTTGTTTTTTTTC 3′ and

Rev. 5′ CGACTTACCCTCAACTCCCCA 3′

The 5′ region of *GPX3* (OMIM 138321) (150 bp) which corresponds to a *HIF-1α* binding site (CGCCTCCCACGT) with CpG positions at nucleotides 214 bp and 205 bp respectively were amplified [7].

The propionylcarnitine (C3) concentration (μmol/l) was determined from blood spots by measuring the butylester derivative of C3 at a mass (*m/z*) 274 > 85, using a tandem mass spectrometry (MS/MS) as described [10].

3. Results

4 different *HIF-1α* binding site alleles of *GPX3* were detected in a total of 7 PA and 7 healthy controls respectively at cytosine base positions −214/205 respectively (Table 1). Complete (C/C) methylated alleles were detected in healthy controls only, partial methylated alleles (C/T) in PA and healthy controls respectively, while demethylated alleles (T/T) were detected in PA only (Fig. 1). These included: TGTTT TTTATG allele with a demethylation in PA (6/14 alleles), KO (0/14 alleles); TGTTTTTTAC/TG allele with a demethylation/partial methylation in PA (8/14 alleles), KO (0/14 alleles); C/TGTTTTTTAC/TG allele with a partial methylation in PA (0/14 alleles), KO (10/14 alleles); C/TGTTTTTTACG allele with a partial methylation/methylation in PA (0/14 alleles), KO (4/14 alleles).

4. Discussion

The DNA methylation pattern of the *HIF-1α* binding site of *GPX3* is heterogenous as shown by the 4 different alleles detected in PA and healthy controls respectively. A shifting of the methylation equilibrium to the demethylated status in PA is suggested by the fact that methylated alleles (C/C) were detected only in healthy controls, partial methylated alleles (C/T) in PA and healthy controls respectively, and demethylated alleles (T/T) only in PA. The detection of a demethylated TGTTTTTTATG allele at a low free carnitine/excess ammonia in PA 7 (NH3 = 500 umol/L, FC = 6) and PA 6 (NH3 = 595 umol/L, FC = 5) respectively or high free carnitine/excess ammonia in PA 3 (NH3 = 987 umol/L, FC = 50) suggests that, independent of the free carnitine concentration, excess NH3 could be important for a demethylated state of the *HIF-1α* binding site in PA. Different methylation patterns of the *HIF-1α* binding site were detected in patients with acidosis at excess NH3 in PA 3 (NH3 = 987 umol/L, TGTTTTTTATG allele) and slightly elevated NH3 in PA 5 (NH3 = 271 umol/L, TGTTTTTTAC/TG allele) respectively, further supporting the conclusion that, independent of acidosis, excess NH3 concentration could be important for a demethylated state. The detection of the demethylated TGTTTTTTATG allele before the first hyperammonia presentation in patients with excess NH3 only, but not in patients with an elevated NH3 or healthy patients respectively would suggest that a certain NH3 concentration threshold would be necessary for a demethylated state of the *HIF-1α* binding site. This conclusion is supported by 1). in vitro investigations that show a concentration dependent activation of *HIF-1α* by NH3 [11]. 2). Evidence has shown that Hif-1 activation responds to different ROS levels (as a result of different oxidant levels) which give rise to different cell stress responses, with mild oxidative stress which triggers the binding of Hif-1 and activation of target genes predicted to result in a demethylation of the Hif-1 binding site, while a severe oxidative stress which triggers the blocking of Hif-1 binding and deactivation of target genes predicted to result in a methylation of the Hif-1 binding site [5,8]. Hyperammonemia in PA is an oxidant that results in an increased ROS production [3,4], which in turn has been shown by several studies to induce DNA demethylation [12,13]. Since excess NH3 was determined in 3 newborns on days 3, 7 and 8 after birth respectively, and demethylation was determined on days 2, 3 and 3 after birth respectively in the same patients, our results would suggest that the NH3 concentration threshold was high enough to trigger an increased ROS production and consequently demethylation, before the first hyperammonemia presentation. In vivo susceptibility to ROS depends on the ability to up-regulate the ROS scavenging *GPX3*. Therefore, a demethylation of the HIF-1α binding site which is predicted to enable a binding of HIF-1α and activate *GPX3* could have a beneficial effect [7,8]. Since *GPX3* is an enzymatic antioxidant [7], an implication of these results could be the possibility of using a demethylation of the HIF-1α binding site of *GPX3* as an early marker of an increased ROS production in PA newborns, before the first hyperammonemia presentation. An analysis of the first screening cards of confirmed PA patients for a demethylation could be carried out as indicated in our method. A benefit would be that a demethylation would indicate a PA patient has an increased ROS production at birth and a risk of developing oxidative stress. It has been suggested that oxidative stress could play a role in the development of neurological deficits in inborn errors of metabolism [14].

Table 1
Methylation status of *HIF1* binding site in propionic acidemia (PA) patients and healthy controls (KO) respectively.

Samples	Test results	*HIF1* Binding Site (CGTTTTTTACG)
PA 1	Propionic acidemia NBS (C3 = 9umol/L, day 2 after birth)	TGTTTTTTAC/TG
PA 2	Propionic acidemia NBS (C3 = 17umol/L, day 3 after birth)	TGTTTTTTAC/TG
PA 3	Propionic acidemia NBS (C3 = 22umol/L, day 3 after birth) NH3 = 987umol/L, Acidosis, day 7 after birth (FC = 50)	TGTTTTTTATG
PA 4	Propionic acidemia NBS (C3 = 17umol/L, day 4 after birth) NH3 = 213umol/L, day 3 after birth (FC = 43)	TGTTTTTTAC/TG
PA 5	Propionic acidemia NBS (C3 = 14umol/L, day 3 after birth) NH3 = 271umol/L, Acidosis, day 4 after birth (FC = 246)	TGTTTTTTAC/TG
PA 6	Propionic acidemia NBS (C3 = 18umol/L, day 3 after birth) NH3 = 595umol/L, day 8 after birth (FC = 5)	TGTTTTTTATG
PA 7	Propionic acidemia NBS (C3 = 11,9umol/L, day 2 after birth) NH3 = 500umol/L, day 3 after birth (FC = 6)	TGTTTTTTATG
1ko	Healthy control NBS (C3 = 0.61umol/L, day 3 after birth)	C/GTTTTTTACG
2ko	Healthy control NBS (C3 = 3.3umol/L, day 3 after birth)	C/GTTTTTTACG
3ko	Healthy control NBS (C3 = 1.9umol/L, day 3 after birth)	C/TGTTTTTTAC/TG
4ko	Healthy control NBS (C3 = 2.50umol/L, day 4 after birth)	C/TGTTTTTTAC/TG
5ko	Healthy control NBS (C3 = 2.10umol/L, day 4 after birth)	C/TGTTTTTTAC/TG
6ko	Healthy control NBS (C3 = 0.49umol/L, day 3 after birth)	C/TGTTTTTTAC/TG
7ko	Healthy control NBS (C3 = 0.9umol/L, day 2 after birth)	C/TGTTTTTTAC/TG

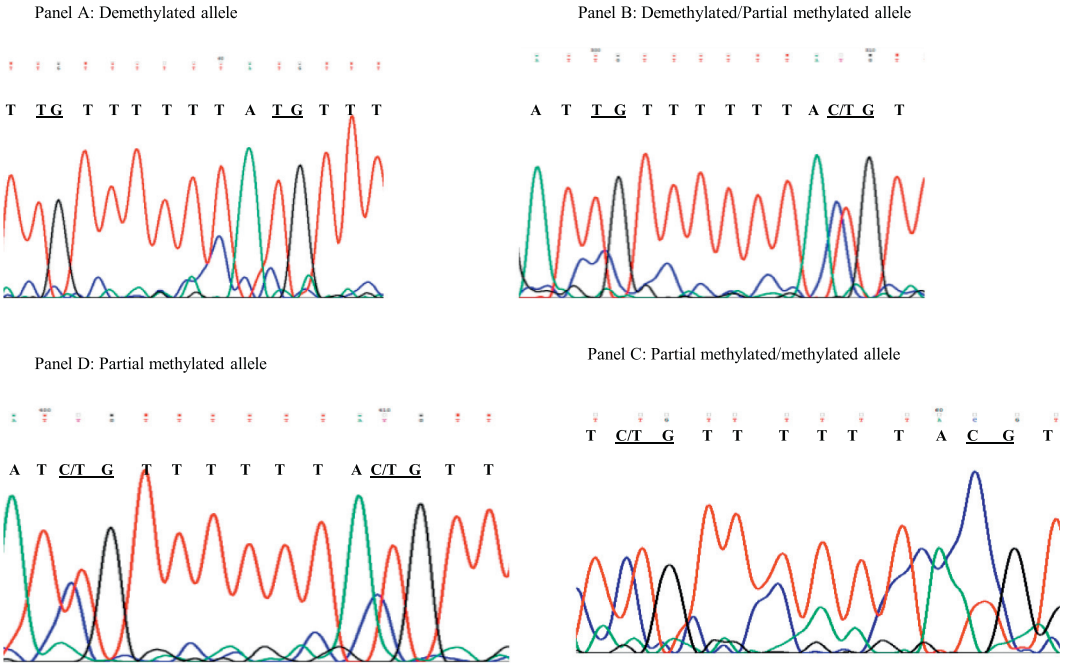

Fig. 1. Panel A: demethylated: TGTTTTTTATG allele. Panel B: demethylated/partial methylated: TGTTTTTTAC/TG allele. Panel C: partial methylated/methylated C/TGTTTTTTACG allele. Panel D: partial methylated C/TGTTTTTTAC/TG allele.
A cytosine base (C/C) at position −214 of the *GPX3* promoter was demethylated (T/T) and partial methylated (C/T) respectively, while cytosine position −205 was methylated (C/C), demethylated (T/T) and partial methylated (C/T) respectively. Colour description: Guanine-G (Black); Thymidine-T (Red); Cytosine-C (Blue). Each curve shows alleles inherited from parents superimposed on each other, when they are similar (C, G, T, A), or differentiated from each other, when they are dissimilar (C/T). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

5. Conclusion

A demethylated allele was specifically detected at hyperammonemia in 3PA patients (PA 3, 6, 7). 4 PA patients (PA 1, 2, 4, 5) with a high propionyl carnitine, but without hyperammonemia, did not show a demethylated allele. We therefore conclude that the observed tendency towards demethylation could be related to hyperammonemia in PA. The demethylated allele has to be confirmed as a statistically significant change at excess NH3 in a large number of patients. Hyperammonemia as an oxidant raises the question of oxidative stress in PA. A better idea of the demethylated status of the HIF-1α binding site as an antioxidant and epigenetic biomarker of an increased ROS production respectively could be obtained by examining the methylation status of PA patients on an antioxidant protein restricted diet [15].

Conflict of interest

The authors do not have any conflict of interests.

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