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Original Article

Delta-aminolevulinate synthase 2 polymorphism is associated with maximal oxygen uptake after Living-high exercise-high training-low in a male Chinese population

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Abstract: Objective: Each individual shows great variations to hypoxic training, which may be due to individual differences in genotype or gene polymorphism. δ-aminolevulinic acid synthase 2 (*ALAS*$_2$) polymorphism is used as a marker for X-linked sideroblastic anemia. This study assessed the *ALAS*$_2$ polymorphism for an association with response to Living-high exercise-high training-low (HiHiLo) training in Han Chinese males. Methods: A total of 244 healthy young male subjects of Han nationality were recruited from Northern China for detection of the *ALAS*$_2$ polymorphism, 72 of whom were then selected for undergoing a four-week HiHiLo training program (i.e., subject to 10 h of hypoxia training daily at 14.5-14.8% O_2 exposure, three occurrences of hypoxic training every week at 15.4% O_2 level, and normal training at sea level). GeneScan and DNA sequencing were used to analyze *ALAS*$_2$ polymorphism. Before and after training, the maximal oxygen uptake (VO_2 max) in each individual was recorded. Results: A successive cut-point analysis showed that the initial hemoglobin value in individuals with dinucleotide repeats ≤ 166 bp was significantly higher than in individuals with dinucleotide repeats > 166-bp ($P = 0.05$). The ΔVO_2 max and ΔrVO_2 max after HiHiLo training were significantly higher in individuals with dinucleotide repeats ≤ 166 bp than in individuals with dinucleotide repeats > 166 bp ($P < 0.01$). Conclusions: The compound dinucleotide repeat polymorphism in *ALAS*$_2$ intron 7 correlated with response to HiHiLo training. Further study will evaluate this *ALAS*$_2$ SNP as a genetic marker to predict responses to HiHiLo training.

Keywords: δ-aminolevulinic synthase 2, polymorphism, HiHiLo training

Introduction

It is well established that the human body generates more red blood cells or more efficiently utilizes oxygen in response to high-altitude and low-oxygen environments [1]. Living-high training-low (HiLo training) utilizes a similar concept to improve oxygen-carrying capacity, optimize adaptations, and maintain performance for athletes or individuals looking to gain a competitive advantage during endurance events [2]. Living-high exercise-high training-low (HiHiLo) is a modified training program to train individuals in hypoxic conditions. The goal is to increase their body tolerance to hypoxia and strengthen cardiopulmonary functions. To date, such training has been widely used in different sports; however, individual results vary greatly, which may be due to individual difference in genotype or gene polymorphism [3, 4]. However, such information is minimal and our understanding of the correlation between gene polymorphisms, adaptations and outcomes of HiLo training could help us to individualize training programs for each individual.

Mechanistically, the purpose of these training programs is to increase red blood cell levels and optimize adaptations (i.e., cardiopulmonary functions). To this end, heme is a prosthetic group of proteins, most commonly found in hemoglobin, mitochondrial cytochrome, peroxidase, guanylate cyclase, or cytochrome P450 enzyme. As a component of these proteins, heme exhibits important functions in oxygen transport and storage in red blood cells, elec-

tron transport and energy formation, anti-oxidation, cellular signal transduction, and drug metabolism. Heme also participates in globin mRNA transcription and translation as well as in regulation of erythroid differentiation [5-8]. Therefore, heme metabolism is closely related to athletic ability and, in particular, to aerobic capacity. In heme biosynthesis, δ-aminolevulinic acid synthase (ALAS) is the rate-limiting enzyme, which contains two isozymes, $ALAS_1$ and $ALAS_2$. $ALAS_1$ is usually expressed in all tissues in the human body, while $ALAS_2$ is more specifically expressed in erythroid cells and is essential in hemoglobin and myoglobin synthesis. Thus, $ALAS_2$ deficiency can lead to X-linked sideroblastic anemia (XLSA) [9]. Previous studies showed that acute and chronic movement training increases ALAS activity [10, 11]. Under hypoxic conditions, expression of $ALAS_2$ mRNA is upregulated and intracellular heme levels increase [12]. Thus, $ALAS_2$ is closely related to aerobic capacity and adaptation to hypoxia. Previous studies revealed that *ALAS*$_2$ has a compound dinucleotide repeat in intron 7 with the sequence of $(CA)_5T(GC)_2(AC)_4GTA(CA)_{23}(GA)_3CA(GA)_8$, and such a polymorphism has been used as a biomarker for XLSA and for index labeling of a multipoint linkage map of the human X chromosome [13, 14]. Therefore, in this study, we detected this *ALAS*$_2$ polymorphism in a Chinese population as a susceptibility gene to study the association of individual variations with endurance capacity and hypoxic training response.

Subjects and methods

Study subjects

A total of 244 healthy male subjects of Han nationality were recruited from Northern China (including three provinces in Northeast China, five provinces in Northern China, and the Northern part of Anhui province). Their mean age was 20.02 ± 1.76 years (ranged between 18 and 22 years old) with a mean body mass of 65.06 ± 9.59 kg and a mean height of 174.37 ± 6.16 cm. These subjects were healthy after physical examination and acted normally in sports activities, but they had no history of systemic movement exercise or family disease.

After genotyping their *ALAS*$_2$ polymorphisms, 72 of these 244 subjects were randomly selected for HiHiLo training. These 72 subjects had no history of heart or lung diseases, hematopathy disease or history of habitation in hypoxic environments. They were not taking any medication before or during the training program. The mean age was 21.10 ± 1.37 years, mean body mass was 69.80 ± 7.80 kg, and mean height was 177.93 ± 5.26 cm. The Local Institutional Ethics Committee approved this study and each subject provided a written informed consent. This study was carried out in accordance with the ethical standards of the 2004 Declaration of Helsinki.

Gene polymorphism analysis

Genomic DNA was extracted from peripheral blood leucocytes using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA). PCR amplification of the compound dinucleotide repeat was performed using a forward primer fluorescently labeled with FAM (5'-FAM aaa gac aaa gag tca agc ct-3') and a reverse primer (5'-gtg tat gga tcg att gcc tg-3') with a normal PCR product of 174 bp. PCR amplification was performed in a 15 uL reaction volume containing genomic DNA (100 ng), 2 uL of each primer (5 uM), 2 uL of deoxynucleotide triphosphates (10 mM), 0.96 l of Mg^{2+} (25 mM), 2 μL of 10 × buffer, 0.2 L Taq polymerase (5 U/uL), and distilled H_2O to the final volume. The initial denaturation occurred at 95°C for 7 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 57.9°C for 30 s, extension at 72°C for 30 s, and final extension at 72°C for 10 min. A mixture of the fluorescently labeled PCR products and an internal size standard (Genescan-500 Rox) were then separated on a 6% polyacrylamide gel using a 96-well ABI 377 DNA automated sequenator. Gel data were analyzed using GeneScan Analysis 3.1 (Beijing HuaDa Gene Research Center, Beijing, China) and Genotyper 2.0 (PE Applied Biosystems Corporation, Foster City, CA, USA) software packages. We then randomly selected 15 samples from PCR amplifications to directly sequence DNA of the repeat region using an ABI3700 for verification.

HiHiLo training

All 72 subjects underwent 4-week HiHiLo training in the hypoxia-training center (simulated normobaric hypoxia) in Beijing Sport University, China. Specifically, Living-high (Hi) indicated that they were living in normobaric hypoxia

Table 1. Distribution of $ALAS_2$ polymorphism in 244 subjects

Genotype	Number of subjects	Distribution frequency
157 bp	2	0.8%
160 bp	1	0.4%
162 bp	3	1.2%
164 bp	23	9.4%
166 bp	48	19.7%
168 bp	80	32.8%
170 bp	21	8.6%
172 bp	45	18.4%
174 bp	6	2.5%
176 bp	6	2.5%
178 bp	7	2.9%
180 bp	1	0.4%
184 bp	1	0.4%

rooms with simulated altitude of 2800 m to 3000 m and an O_2 concentration of 14.5-14.8% for 10 h every night for four weeks. Hi meant each subject exercised on a bicycle ergometer for 30 minutes with an intensity of 75% VO_2 max in 15.4% O_2 concentration environment (equivalent to 2500 m altitude) three times per week for four weeks. Training-low (Lo) included the remaining time when the subjects endured the same training content and intensity at a lower elevation according to the arrangement of the coach (the average altitude of Beijing city is 43.5 m).

Measurement of hematological index

Fasting venous blood samples were collected before and after HiHiLo training from each individual and levels of red blood cells and hemoglobin and the hematocrit rate were measured by using a Bayer ADVIA120 automatic hematology analyzer (Bayer Corporation, Tarrytown, NY).

Measurement of VO_2 max

For each subject, the VO_2 max was measured before and after HiHiLo training. In brief, each subject ran on a treadmill at 60 cycles/min with increasing loads by starting with 90 W and increased by 30 W every 2 min until exhausted. The standard VO_2 max was designated when the heart rate exceeded 180 beats/min and the respiratory quotient exceeded 1.1 without an increase in oxygen consumption; the subject was unable to do more exercise. The absolute value of VO_2 max was expressed in L/min and rVO_2 max (relative maximal oxygen uptake) in mL/min/kg. The cycle ergometer was a Monark 818 (Monark, Vansbro, Sweden) and the gas metabolism analyzer was a Cortex-Metalyzer II (Leipzig, Germany).

Statistical analysis

The HiHiLo training sensitivity (Δ, in%) for each variable was computed as Δ = (post-training minus baseline/baseline) × 100%.

The compound dinucleotide repeat polymorphism has many short tandem repeats and the genotypes were categorized according to the total length of the repeats, but not the numbers of the repeat. Based on a previous method [15], we used the repeat lengths of 166 bp, 168 bp and 170 bp as cut-off points; two groups were assigned for each cut-off point: long tandem (≤ the chosen cut-off point) and short tandem (> the chosen cut-off point). The difference in hypoxia training response was analyzed between the long tandem and short tandem groups of the same division point.

Before training, the K-S test was used to determine whether the physiological index data met the normal distribution. If these data were normally distributed and their variance was homogeneous, an independent t-test was performed for the initial physiological index value and training sensitivity; otherwise a non-parametric test was used. Data were expressed as mean ± SD, and all statistical analyses were performed using (Statistical Product and Service Solutions) SPSS 11.5 software (SPSS, Chicago, IL, USA). $P < 0.05$ was considered statistically significant.

Results

Distribution of $ALAS_2$ polymorphism

Since *$ALAS_2$* is localized on the X chromosome, male subjects carry only one allele; thus, the allele is also genotype and therefore, there is no need to perform a Hardy-Weinberg equilibrium test.

A total of 13 genotypes of *$ALAS_2$* were detected in this cohort of 244 subjects (**Table 1**). The major *$ALAS_2$* genotypes were repeats of 164 bp, 166 bp, 168 bp, 170 bp, and 172 bp, and their distribution frequencies were 9.4%, 19.7%, 32.8%, 8.6%, and 18.4%, respectively.

Table 2. Association of $ALAS_2$ polymorphism (cut-off point) with the hematological index

Genotype	N = 72 (%)	Pre-HiHiLo			Changed rate after HiHiLo		
		RBC (× 10^9/l)	Hb (g/l)	Hct (%)	ΔRBC	ΔHb	ΔHct
≤ 166 bp	18 (25)	4.95 ± 0.30	155.78 ± 9.05*	44.06 ± 2.21	0.03 ± 0.05	0.04 ± 0.05	0.04 ± 0.05
> 166 bp	54 (75)	4.87 ± 0.25	151.56 ± 8.29	43.36 ± 1.89	0.02 ± 0.04	0.03 ± 0.04	0.03 ± 0.04
≤ 168 bp	43 (60)	4.89 ± 0.25	152.47 ± 7.60	43.51 ± 1.87	0.03 ± 0.05	0.04 ± 0.04	0.04 ± 0.05
> 168 bp	29 (40)	4.89 ± 0.28	152.83 ± 10.07	43.58 ± 2.18	0.02 ± 0.04	0.03 ± 0.04	0.02 ± 0.04
≤ 170 bp	53 (74)	4.88 ± 0.25	151.83 ± 8.24	43.36 ± 1.89	0.03 ± 0.04	0.04 ± 0.04	0.04 ± 0.04
> 170 bp	19 (26)	4.93 ± 0.31	154.79 ± 9.49	44.04 ± 2.19	0.01 ± 0.05	0.02 ± 0.05	0.02 ± 0.04

Notes: *P = 0.05 vs. > 166 bp genotype.

Table 3. VO_2 max changes in 72 subjects before and after HiHiLo training

Index	Pre-HiHiLo	Post-HiHiLo
ΔVO_2 max	3.08 ± 0.47	3.23 ± 0.45*
ΔrVO_2 max	45.02 ± 5.99	47.87 ± 0.59*

Notes: *P < 0.01 vs. Pre-HiHiLo training.

Association of $ALAS_2$ polymorphism with the hematological index in HiHiLo training subjects

In the 72 HiHiLo training subjects, seven *$ALAS_2$* genotypes were detected with distribution frequencies similar to those of the total subjects. The *$ALAS_2$* repeat polymorphisms with 166 bp, 168 bp, 170 bp, and 172 bp genotypes had relatively high frequencies.

The initial value of hemoglobin was significantly higher in the subjects with genotypes ≤ 166 bp than in the subjects with genotypes > 166 bp (P = 0.05). However, there were no other indexes found to have significant associations (**Table 2**).

Association of $ALAS_2$ polymorphism with response to HiHiLo training

After that, these 72 individuals underwent four weeks of HiHiLo training as we measured their aerobic exercise ability and adaptation to hypoxic conditions. The ΔVO_2 max and ΔrVO_2 max both significantly increased after four weeks of HiHiLo training (**Table 3**). After HiHiLo training, the increases in ΔVO_2 max and ΔrVO_2 max were significantly higher in the individuals with genotype repeats of ≤ 166 bp than in the individuals of with genotypes > 166 bp (P < 0.01). There was no significant difference in ΔVO_2 max found when other length segments were used as cut-off points (**Table 4**).

Discussion

$ALAS_2$ is the rate-limiting enzyme in heme synthesis in erythroid cells and is directly related to hemoglobin and myoglobin functions; thus, $ALAS_2$ is closely related to aerobic exercise capacity. *$ALAS_2$* compound dinucleotide repeat polymorphism in intron 7 has been used as a biomarker for XLSA and as an index label for a multipoint linkage map of the human X chromosome [13, 14]. Thus, in this study, we detected this *$ALAS_2$* polymorphism to assess its association with individual responses to HiHiLo training. We detected a total of 13 genotypes with compound dinucleotide repeats lengths between 157 bp and 184 bp in healthy males of Han lineage from Northern China. The 168-bp polymorphism showed the highest frequency (32.8%), followed by the 166-bp (19.7%) and 172-bp (18.4%) polymorphisms; the frequency of the other 10 genotypes was lower than 10%. Moreover, our current study met the requirements for a valid population genetics study in terms of age, region of habitation, race, and sample size; thus, our current data could be used to represent frequency of *$ALAS_2$* polymorphism in young males of Han lineage from Northern China.

We then selected 72 subjects to determine their responses to HiHiLo training, and found that the ΔVO_2 max and ΔrVO_2 max in individuals with genotypes ≤ 166 bp were significantly greater than in individuals with genotypes > 166 bp, suggesting that hypoxia training is most effective in individuals with *$ALAS_2$* genotypes ≤ 166 bp. Our data clearly showed that $ALAS_2$ compound dinucleotide repeat polymorphism was correlated with response to hypoxia training. Further study with a larger sample size could confirm our current data and use this

Table 4. Association of $ALAS_2$ compound dinucleotide repeat polymorphism (cut-off point) with ΔVO_2 max

Genotype	N = 72 (%)	Pre-HiHiLo		Changed rate after HiHiLo training	
		VO_2 max (L/min)	rVO_2 max (mL/kg/min)	ΔVO_2 max	ΔrVO_2 max
≤ 166 bp	18 (25)	2.96 ± 0.39	43.99 ± 4.53	0.13 ± 0.09*	0.14 ± 0.08*
> 166 bp	54 (75)	3.12 ± 0.49	45.36 ± 6.39	0.03 ± 0.11	0.04 ± 0.11
≤ 168 bp	43 (60)	3.09 ± 0.49	44.75 ± 5.33	0.06 ± 0.11	0.07 ± 0.11
> 168 bp	29 (40)	3.06 ± 0.45	45.43 ± 6.92	0.05 ± 0.11	0.06 ± 0.11
≤ 170 bp	53 (74)	3.07 ± 0.48	44.60 ± 5.17	0.06 ± 0.11	0.07 ± 0.11
> 170 bp	19 (26)	3.11 ± 0.47	46.22 ± 7.92	0.05 ± 0.12	0.06 ± 0.12

Notes: *P < 0.01 vs. > 166 bp genotype.

polymorphism as a genetic marker to predict the HiHiLo training response.

Indeed, hypoxia training has been widely used in different sports to improve an athlete's capacity, especially for endurance. The efficacy of hypoxia training obviously improves the level of VO_2 max and performance. Our current study showed that after four weeks of HiHiLo training, VO_2 max and rVO_2 max of each subject were significantly increased. HiHiLo training effectively enhanced the individuals' maximum aerobic capacities, which further confirms the usefulness of hypoxia training. However, previous data demonstrated that hypoxic training efficacy differs greatly between individuals [3] and this difference is associated with gene polymorphism [4]. Our current study thus associated *$ALAS_2$* compound dinucleotide repeat polymorphism with hypoxia training efficacy. We found that, after four weeks of HiHiLo training, the increased ΔVO_2 max and ΔrVO_2 max in individuals with *$ALAS_2$* genotypes ≤ 166 bp were significantly greater than in individuals with *$ALAS_2$* genotypes > 166 bp, suggesting that *$ALAS_2$* genotype affected the response of individuals to hypoxia training. Indeed, $ALAS_2$ is responsible for the heme biosynthesis in red blood cells. Altered $ALAS_2$ expression and function could lead to abnormal levels of heme, and in turn could influence the normal maturation of red blood cells and cell survival [16, 17].

To date, there have been few studies describing $ALAS_2$ polymorphisms, sport capacity and hypoxia. Previous studies showed that, after the endurance exercise, ALAS activity and mRNA levels increase [10, 11, 18]. For example, Town et al. reported that, after 3, 7 and 28 days of the endurance exercise, rat ALAS activity increased by 100%, 150% and 125%, respectively, while cytochrome oxidase increased by 40% after 7 days of exercise, reaching a peak after 28 days. During endurance exercise, the change in activity of aerobic metabolic enzymes lags compared to the change in ALAS activity, indicating that ALAS does play a regulatory role in development of aerobic capacity. Our current data further supported the data from the animal study. Furthermore, *$ALAS_2$* contains a hypoxic response element in the $ALAS_2$ 5'-untranslational region. The hypoxic response elements can mediate transcription of hypoxia inducible factor 1. A previous study showed that when rat erythroleukemia cells were cultured in an environment containing only 1% oxygen, levels of $ALAS_2$ mRNA were increased three-fold. A mutational analysis confirmed that this response depends on a hypoxic response element in the gene promoter region [12]. Therefore, $ALAS_2$ plays a role in aerobic exercise capacity and hypoxia adaptation. Our current data demonstrated for the first time that *$ALAS_2$* compound dinucleotide repeat polymorphism was associated with response to HiHiLo training. The *$ALAS_2$* repeat polymorphisms site is in intron 7 and adjacent to exon 8, a receptor splice site in this junctional zone, affecting *$ALAS_2$* mRNA processing. Previous studies have demonstrated that gene introns can alter gene transcription and expression because the introns may contain gene transcription enhancers and repressors [19, 20]. However, we don't have direct proof that this *$ALAS_2$* genotype with 166 repeats affects heme synthesis. Our current data did show that this polymorphism affects hemoglobin levels in individuals before and after HiHiLo training, and even during pretraining. Our current study does have some limitations including the small sample size and short duration of the HiHiLo training program.

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Disclosure of conflict of interest

None.

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