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Differential gene expression of epigenetic modifying enzymes between Tibet pig and Yorkshire in high and low altitudes

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ABSTRACT. Epigenetic modifying enzymes play important roles in the adaptation to hypoxia, although no studies have examined their expression levels in Tibet pigs. The lung is an important functional organ in hypoxia adaptation. In this study, we examined the mRNA expression level of 5 enzymes in the lung of Tibet pigs using real-time polymerase chain reaction to determine the epigenetic performance of hypoxia adaptation. We selected four groups of pig as the study object, which were Tibet pig in highland (TH), Yorkshire in highland (YH), Tibet pig in lowland (TL), Yorkshire in lowland (YL). Expression of *Dnmt1* in Tibet pig was higher than that in Yorkshire ($P < 0.01$), although there was no significant difference between different altitudes within each breed. Expression of *Dnmt3a* was higher in Tibet pig than that in Yorkshire ($P < 0.01$), and higher in pigs from highland than that in lowland areas ($P < 0.05$). Expression of *Hdac1* was higher in group TH than in Yorkshire ($P < 0.01$). Expression of *Kdm3a* was higher in group TH than in the rest of the groups ($P < 0.01$). Expression of *Uhrf1* was higher in Tibet pig than in Yorkshire ($P < 0.01$). In conclusion, the expression levels of the 5 epigenetic modifying genes were higher in group TH than in group YH.

Under conditions of oxygen deficiency, breed was the most important factor affecting DNA methylation and gene expression.

Key words: Epigenetic modifying enzyme; Hypoxia adaptation; mRNA expression; Tibet pig

INTRODUCTION

In mammals, DNA methylation and histone modification are primary processes in epigenetic regulation. Stable silencing of 1 gene, which can be inherited, is correlated with DNA methylation in its promoter, along with specific modifications in the N-terminal tails of nucleosomal histones (Quina et al., 2006). Under hypoxia conditions, epigenetic regulation may cooperate with hypoxia-inducible factor-1 (HIF-1) to regulate hypoxia response genes, and the latter is key gene in hypoxia adaptation (Semenza, 2000); it may also play a more important role in maintaining the cell phenotype after HIF-1 initiates the hypoxia response pathway.

Dnmt1 and Dnmt3a are both DNA methyltransferases (DNMTs). Dnmt1 can replicate the DNA methylation pattern during DNA replication, while Dnmt3a is a *de novo* DNA methyltransferase (Bestor, 2000; Goll and Bestor, 2005). Histone deacetylase 1 (Hdac1) is a histone deacetylase and can bind to Dnmt1 to affect DNA methylation (Fuks et al., 2000); its activity is related to 70% HIF-1α response genes (Kasper et al., 2005). Lysine (K)-specific demethylase 3A (Kdm3a) can demethylate H3K9me1/2 specifically to relieve histone suppression. HIF-1 binds to the promoter of Kdm3a in hypoxia, and protein and mRNA expression levels of Kdm3a both increase *in vitro* in a variety of cell lines. Kdm3a is also upregulated in several rat organs *in vivo* (Wellmann et al., 2008). Ubiquitin-like with PHD and ring finger domains 1 (Uhrf1) encodes a member of a subfamily of RING-finger type E3 ubiquitin ligases. The protein binds to specific DNA sequences, and recruits a histone deacetylase to regulate gene expression. In mammals, epigenetic inheritance of DNA methylation requires Uhrf1, which is thought to recruit Dnmt1 to DNA replication forks through unique hemi-methylated CpG-binding activity (Liu et al., 2013). Uhrf1 can target Dnmt1 to maintain DNA methylation by binding to H3K9me2/3 or hemi-methylated CpG.

These epigenetic modifying enzymes play important roles in adaptation to hypoxia. However, only a few studies have been conducted to examine *Hdac1* and *Dnmt1* mRNA expression in pig cells or embryos (Zhao et al., 2011; Ren et al., 2011; Cao et al., 2014), and no studies have examined their expression levels in Tibet pigs. The lung is an important functional organ in hypoxia adaptation. In hypoxia, pulmonary ventilation increases and arterial oxygen pressure rises to relieve the impact of hypoxia on the organism. In this study, we examined the mRNA expression of the 5 enzymes in the lung of Tibet pigs using real-time polymerase chain reaction (PCR) to determine the epigenetic performance of hypoxia adaptation.

MATERIAL AND METHODS

Experimental materials

This experiment included 4 pig groups: Tibet pig from highland (TH) (Linzhi, 3000 m), Tibet pig from lowland (TL) (Beijing, 100 m), Yorkshire from highland (YH) (Linzhi, 3000 m), and Yorkshire from lowland (YL) (Hefei, 100 m). Thirty castrated boars (6-8 each

group) were slaughtered at 6 months of age. Tissue samples were collected from the lung. Samples were immediately frozen in liquid nitrogen and stored at -80°C. All procedures were carried out in strict accordance with the protocol approved by the Animal Welfare Committee of China Agricultural University (Permit No. XK622).

RNA extraction and cDNA synthesis

Total RNA was isolated from lung tissue with Trizol Reagent (Invitrogen, Carlsbad, CA, USA) following manufacturer instructions. RNA solutions were evaluated for concentration and purity using a NanoDrop 2000 Biophotometer (Thermo scientific, Waltham, MA, USA) at 260/280 nm absorbance ratio and on a 1% agarose gel to verify DNA integrity.

Total RNA was reverse-transcribed into cDNA using FastQuant RT Kit (using gDNase, Tiangen, Beijing, China). Next, 1 µg total RNA and 2 µL 5X gDNA Buffer were added into a 200-µL microcentrifuge tube, and RNase-Free ddH_2O was added to 10 µL volume. The tubes were incubated at 42°C for 3 min and then placed on ice. The reaction system contained 2 µL 10X Fast RT Buffer, 1 µL RT Enzyme Mix, and 2 µL FQ-RT Primer Mix. The samples were incubated at 42°C for 15 min, followed by incubation at 95°C for 3 min. The resulting cDNA was stored at 4°C for subsequent use.

Quantitative analysis of mRNA expression of the 5 genes

Expression levels were measured using real-time PCR. Primers were designed using Primer Premier 5.0 (Premier Biosoft, Palo Alto, CA, USA). The house-keeping gene hypoxanthine phosphor-ribosyltransferase was used as an internal standard. Primer information is listed in Table 1.

Table 1. Primer information for 6 genes.

GenBank No.	Gene name	Sequence of primer 5'→3'	Product size (bp)	Tm (°C)
NM_001032376	*HPRT*	F: CAGTCAACGGGCGATATAAAAGT R: CAGTCAACGGGCGATATAAAAGT	95	60
NM_001032355	*Dnmt1*	F: CAGACAATTCAATACCCTCA R: GGTGACAGTTGTGCTGAAAA5	118	5
NM_001097437	*Dnmt3a*	F: GCTACTTCTGGGGAAACCTT R: CCTCACTTTGCTGAACTTGG	119	54
XM_003356305	*Hdac1*	F: ACCTATGTTGATGCTGGGAG R: AAAGTAGTCGTTGTACGGAAG	124	55
XM_003124935	*Kdm3a*	F: AAGCCGTAAAAACGAAACCT R: CGCAGACAGACACACAACAA	152	54
XM_003123079	*Uhrf1*	F: CGCTGGCTCTGAACTGCTTT R: AGGGGCGTACTTGCTGTGCT	128	56

Real-time PCR amplification was conducted using Bio-Rad CFX96 System (Bio-Rad, Hercules, CA, USA). A cDNA pool of all samples was used for calibration. The expression level of each target gene was calculated using the $2^{-\Delta\Delta Ct}$ method ($\Delta\Delta Ct = \Delta Ct_{target\ gene} - \Delta Ct_{housekeeping\ gene}$) (Livak and Schmittgen, 2001).

Statistical analysis of expression level

Two-way analysis of variance (ANOVA) and multiple comparisons were conducted with R 3.0.2. Means ± standard error are reported graphically using SigmaPlot 10.0 (Systat Software, San Jose, CA, USA).

RESULTS

Expression level of *Dnmt1*

Dnmt1 expression in the lung tissue is shown in Figure 1. ANOVA showed that *Dnmt1* expression in the lung was significantly related to breed ($P < 0.01$). *Dnmt1* expression in the Tibet pig was higher than that in Yorkshire, although there was no significant difference between different altitudes within each breed.

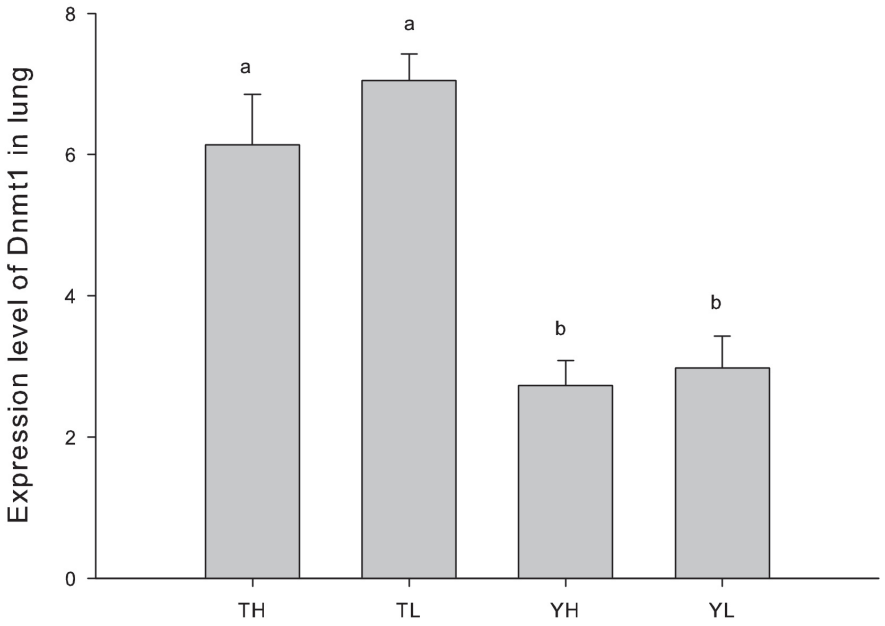

Figure 1. mRNA expression level of *Dnmt1*. Expression level was calculated in lung tissue of 4 groups for this gene. Error bars represent standard error. Letters on bars denote the difference in expression level with significant difference ($P < 0.05$). Group size was 8 for TH, 6 for TL, 8 for YH, and 8 for YL.

Expression level of *Dnmt3a*

Dnmt3a expression in the lung tissue is shown in Figure 2. Two-way ANOVA revealed that the expression level of *Dnmt3a* was significantly related to breed and altitude ($P < 0.01$ for breeds, $P < 0.05$ for altitude). *Dnmt3a* expression was higher in Tibet pig than that in Yorkshire, and higher in highland than in lowland pigs.

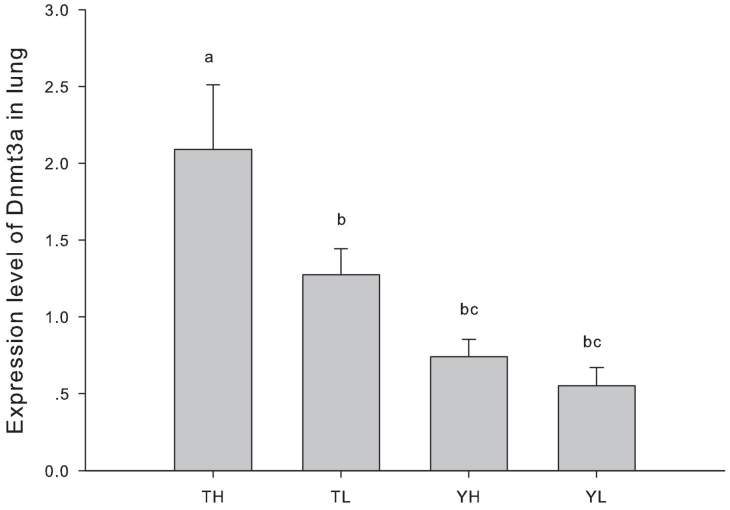

Figure 2. mRNA expression level of *Dnmt3a*. Expression level was calculated in lung tissue of 4 groups for this gene. Error bars represent standard error. Letters on bars denote the difference in expression level with significant difference ($P < 0.05$). Group size was 8 for TH, 6 for TL, 8 for YH, and 8 for YL.

Expression level of *Hdac1*

Figure 3 shows the mean expression of *Hdac1* in the lung tissue. Two-way ANOVA showed that the expression level of *Hdac1* was significantly related to breed ($P < 0.01$), and there was a significant interaction between breed and altitude ($P < 0.05$). Expression of *Hdac1* was higher in group TH than in Yorkshire (both YH and YL), although there was no significant difference between different altitudes within each breed.

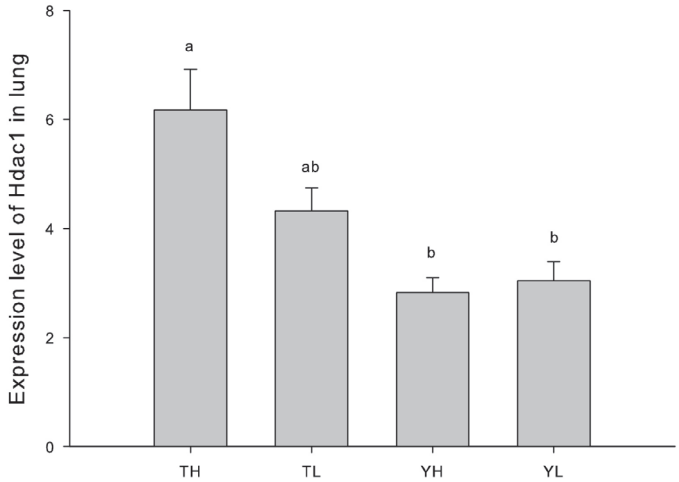

Figure 3. mRNA expression level of *Hdac1*. Expression level was calculated in lung tissue of 4 groups for this gene. Error bars represent standard error. Letters on bars denote the difference in expression level with significant difference ($P < 0.05$). Group size was 8 for TH, 6 for TL, 8 for YH, and 8 for YL.

Expression level of *Kdm3a*

Kdm3a expression in the lung tissue is shown in Figure 4. Two-way ANOVA showed that *Kdm3a* expression was affected both by breed and altitude significantly ($P < 0.01$ for both factors), and there was significant association between breed and altitude ($P < 0.05$). *Kdm3a* expression was higher in group TH than that in the other groups (TL, YH, and YL).

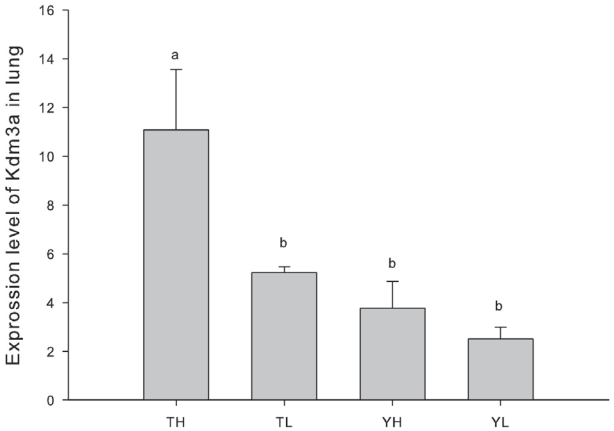

Figure 4. mRNA expression level of *Kdm3a*. Expression level was calculated in lung tissue of 4 groups for this gene. Error bars represent standard error. Letters on bars denote the difference of expression level with significant difference ($P < 0.05$). Group size was 8 for TH, 6 for TL, 8 for YH, and 8 for YL.

Expression level of *Uhrf1*

Figure 5 shows the mean *Uhrf1* expression level in lung tissue. ANOVA results show that *Uhrf1* expression in the lung was significantly related to breed ($P < 0.01$). Expression of *Uhrf1* in Tibet pig was higher than that in Yorkshire, although there was no significant difference between different altitudes within each breed.

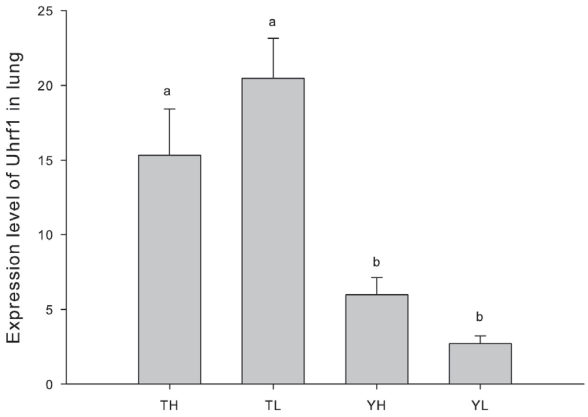

Figure 5. mRNA expression level of *Uhrf1*. Expression level was calculated in lung tissue of 4 groups for this gene. Error bars represent standard error. Letters on bars denote the difference in expression level with significant difference ($P < 0.05$). Group size was 8 for TH, 6 for TL, 8 for YH, and 8 for YL.

DISCUSSION

Analysis of the expression of 5 genes revealed that their expression was significantly higher in the TH group than in the YH group. Dnmt3a is a *de novo* DNA methyltransferase, Dnmt1 maintains the DNA methylation pattern, and Hdac1 and Uhrf1 can cooperate with Dnmt1 to maintain DNA methylation. Thus, the increase of these 4 genes in the TH group suggests that under hypoxia conditions, Tibet pigs increase their DNA methylation level to adapt to hypoxia. In order to adapt to hypoxia, the Tibet pig may downregulate many genes that are irrelevant to the adaptation. Yorkshire was used as a control breed that does not adapt to hypoxia, and many genes are upregulated or activated due to oxygen deficiency.

Expression of *Kdm3a* was higher in TH than in other groups. This was consistent with the results of a previous study (Wellmann et al., 2008). Kdm3a may participate in the response to hypoxia conditions.

In conclusion, the expression of the 5 epigenetic modifying genes were higher in group TH than in group YH, suggesting that to combat hypoxia, the DNA methylation level was higher in Tibet pig than that in the Yorkshire pig. Under conditions of oxygen deficiency, breed was the most important factor affecting DNA methylation and gene expression.

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