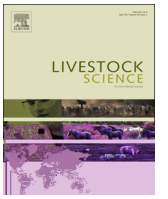

Short communication

High level of hepcidin mRNA expression is associated with high production of immune factors in Tibetan pigs

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ABSTRACT

Hepcidin, an antimicrobial peptide, is considered to be a key homeostatic regulator of iron metabolism. Tibetan pigs are a Chinese indigenous plateau breed with strong disease resistance. It is therefore of interest to look for porcine hepcidin (pHepc) gene expression in Tibetan pigs in order to clarify the involvement of pHepc in interaction of host defenses and iron homeostasis. In this study, the mRNA expressions of pHepc and the immune response-associated factors were determined by real-time PCR in 30-day-old Tibetan pigs and Landrace pigs were chosen as control. The results showed that the expression of pHepc mRNA in most tissues were higher in Tibetan pigs than those of Landrace pigs, which corresponded to the higher serum iron concentration in Tibetan pigs. In addition, compared to Landrace pigs, higher levels of pro-inflammatory cytokines (TNF-α, IL-1α, IL-1β and IFN-γ), pattern recognition receptors (TLR-2, TLR-4, NOD-1 and NOD-2) and chemokines (MCP-1 and IL-8) were detected in Tibetan pigs, which suggested that Tibetan pigs have a strong immunity. The high expression of pHepc may affect the production of immune factors by regulating iron homeostasis, which may partly explain the strong disease resistance of Tibetan pigs.

1. Introduction

Hepcidin, a liver derived 20–25 amino acid peptide hormone, possesses antifungal and antibacterial activities and closely resembles the cysteine-rich antimicrobial peptides involved in host defense (Park et al., 2001; Ganz, 2003). It has also been identified as the key regulator of iron homeostasis (Lee and Beutler, 2009). Hepcidin negatively regulates the main points of entry into the plasma compartment by decreasing the absorption of iron in the duodenum and limiting the release of recycled iron from macrophages (Ganz and Nemeth, 2011). The existence of hepcidin is not limited to human. Indeed, hepcidin has beenh31.6(um)-2.5()-52d ohomitenumemts-6(uuen)2(r)(com)74.9(mom)74.9(l)2ica

Tibetan pig is a local pig breed, which is mainly distributed over the drainage basin around the middle of the Bramaputra River and the high-mountain and deep valley areas of East Tibet in China (with an average altitude of more than 3000 m) (Li and Luo, 1993). This animal is characterized by disease resistance, which is able to tolerate and survive under the adverse local conditions (Cheng, 1984; Qi et al., 2009). However, the mechanism is unclear until now. We made a hypothesis that these characteristics may be related to the iron homeostasis adapted to high-altitude. Therefore, we determined the mRNA gene expressions of pHepc in various tissues for 30-day-old Tibetan pigs and Landrace pigs, and the immune response-associated factors mRNA expression were compared between the two breeds.

2. Material and methods

2.1. Animals and tissue sample collection

All animals' protocols were approved by the animal care committee of Zhejiang University in accordance to guidelines stated in the Guide for the Care and Use of Agricultural animals in Research and Teaching. Five 30-day-old healthy Landrace pigs and five 30-day-old healthy Tibetan pigs were obtained from Breeding Pig Farm of Chengdu, China. The pigs were housed in floored indoor pens and given ad libitum access to feed and water. They were fasted 12 h and killed by exsanguinations. Tissues were collected and snap-frozen in liquid nitrogen for RNA isolation including liver, thymus, bone marrow, spleen, lung, mesenteric lymph nodes (mln), duodenum, jejunum and ileum. To obtain serum, the blood from precaval vein was stored in coagulating tubes and centrifuged 1000 rpm for 30 min. The samples were then stored in −80 °C until used.

2.2. Measurement of iron concentration in serum

Total iron in serum was determined by microwave digestion and flame atomic absorption spectrophotometer (AA6501, Shimadzu Ltd., Kyoto, Japan; Rodgerson and Helfer, 1966).

2.3. Total RNA extraction and reverse transcription

Total RNA was extracted from each tissue by using Total RNA isolation kits (Bioer Technology, Hangzhou, China) according to the manufacture's instruction. The purity and concentration were measured by NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA) at 260 and 280 nm. The integrity of RNA was detected by using 1.5% agarose gel electrophoresis. Then total RNA was reverse-transcribed using Superscript system (PrimeScriptTM RT reagent Kit, TAKARA) according to the manufacturer's directions. The RT products (cDNA) were stored at −20 °C for relative quantitative real-time PCR.

2.4. Quantitative real-time PCR

Quantitative real-time PCR analysis was used to measure relative levels of transcript abundance for hepcidin in different tissues and cytokines, pattern recognition receptors (PRRs) and chemokines in Landrace and Tibetan pigs. SYBR Green PCR assays were performed by using a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Inc., Foster City, CA, USA) with following profile: 30 s at 95 °C, followed by 40 cycles of 5 s at 95 °C, and 34 s at 60 °C. Specific primers were designed based on the published gene sequences of hepcidin, 18S ribosomal RNA (18S), tumor necrosis factor (TNF-α), interleukin-1α (IL-1α), interleukin-1β (IL-1β), Interferon gamma (IFN-γ), toll-like receptors (TLR-2, TLR-4 and TLR-5), nucleotide-binding oligomerization domain-containing proteins (NOD-1 and NOD-2), monocyte chemotactic protein 1 (MCP-1) and interleukin-8 (IL-8) in pigs using Primer 5.0 and GenBank (Table 1). The $2^{-\Delta\Delta T}$ method (Schmittgen and Livak, 2008) was used to determine the data. All the data were normalized to the housekeeper gene 18s rRNA from the same individual sample.

2.5. Statistical analysis

Statistical analyses were performed using SPSS 16.0 (SPSS Inc., Chicago, IL). T-test was used to compare the differences between two breeds of pigs. Data were presented as mean ± SEM. The results were considered significantly different at $p \leq 0.05$.

3. Results

3.1. Tissue distribution of porcine hepcidin mRNA

In order to determine the tissue specificity of pHepc gene expression, pHepc mRNA was examined in most tissues by real-time quantitative PCR analysis in Landrace and Tibetan pigs (Fig. 1). In Landrace pigs, pHepc was expressed at high levels in liver, thymus and bone marrow, and at low levels in small intestine (duodenum, jejunum, and ileum), mln and lung. In liver, thymus and bone marrow expression of pHepc mRNA were ~13,284-fold, 14-fold and 15-fold higher ($p < 0.05$), respectively, than those in the duodenum (control tissue) of Landrace pigs. In Tibetan pigs, pHepc was also expressed at high levels in liver, thymus and bone marrow, and at low levels in lung, duodenum and ileum. In addition, pHepc was also expressed at high levels in 54 eh.3159TD-.0241Tc(

Table 1
Oligonucleotide primers used for real-time RT-PCR analysis.

mRNA	Primer sequence (5′–3′)	Amplicon size (bp)	Accession number
Hepcidin	F: GAGCCACCGCTGGTTTGAC R: ACATCCCACAGATTGCTTTGC	108	AF516143.1
TNF-α	F: CCAATGGCAGAGTGGGTATG R: TGAAGAGGACCTGGGAGTAG	116	NM_214022.1
IL-1α	F: CCCGTCAGGTCAATACCTC R: GCAACACGGGTTCGTCTTC	170	NM_214029.1
IL-1β	F: ACAAAAGCCCGTCTTCCTG R: ATGTGGACCTCTGGGTATGG	105	NM_214055.1
IFN-γ	F:CAAAGCCATCAGTGAACTCATCA R: TCTCTGGCCTTGGAACATAGTCT	100	NM_213948.1
TLR-2	F:TCACTTGTCTAACTTATCATCCTCTTG R: TCAGCGAAGGTGTCATTATTGC	162	NM_213761.1
TLR-4	F: GCCATCGCTGCTAACATCATC R: CTCATACTCAAAGATACACCATCGG	108	NM_001113039.1
TLR-5	F: CAGCGACCAAAACAGATTGA R: TGCTCACCAGACAGACAACC	122	NM_001123202.1
NOD-1	F: ACCGATCCAGTGAGCAGATA R: AAGTCCACCAGCTCCATGAT	140	NM_001114277.1
NOD-2	F: CCTTTTGAAGATGCTGCCTG R: GATTCTCTGCCCCATCGTAG	100	NM_001105295.1
MCP-1	F: ACCAGCAGCAAGTGTCCTAAAG R: GTCAGGCTTCAAGGCTTCGG	146	NM_214214
IL-8	F: TTCGATGCCAGTGCATAAATA R: CTGTACAACCTTCTGCACCCA	176	NM_213867.1
18S	F: CCCACGGAATCGAGAAAGAG R: TTGACGGAAGGGCACCA	122	AY265350.1

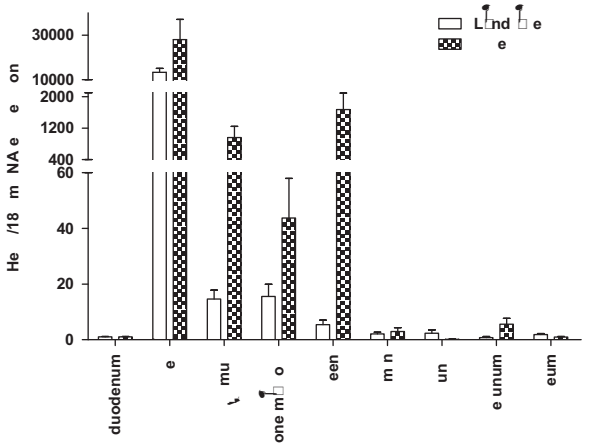

Fig. 1. Tissue distribution of pHepc mRNA in Landrace and Tibetan pigs. The pHepc PCR products were normalized to 18s rRNA as the reference genes, and data shown are pHepc mRNA expression as a ratio of duodenum tissue. The values are depicted as relative pHepc/18s levels plus SEM ($n=5$).

3.3. Comparison of immune response-associated factors mRNA expression between Landrace and Tibetan pigs

To determine whether the up-regulation of pHepc mRNA expression correlated with immune response, the transcripts of cytokines, chemokines and PRRs were measured (Fig. 3). This analysis showed that cytokines were highly abundant in Tibetan pigs including TNF-α, IL-1α, IL-1β and IFN-γ (Fig. 3A). The expression of IL-1β and IFN-γ mRNA in Tibetan pigs were ~5.9-fold and 4.5-fold higher ($p<0.05$), respectively, than those in Landrace pigs. Furthermore, chemokines MCP-1 and IL-8 were also at high levels in Tibetan pigs (Fig. 3A). In addition, the mRNA expressions of PRRs TLR-2, TLR-4 and NOD-2 were mostly higher in Tibetan pigs than those of Landrace pigs ($p<0.05$) (Fig. 3B).

4. Discussion

Antimicrobial peptides are an important component of innate immunity in many species, including plants, insects, fish, amphibians and mammals (Hancock and Scott, 2000). Although especially the latter have a highly efficient system of adaptive immunity, there is growing evidence that in animals antimicrobial peptides such as defensins, protegrins and cathelicidins represent a considerable part of the immune system in the defense of cellular pathogens (Kokryakov et al., 1993; Kin et al., 2011). As an antimicrobial peptide, hepcidin has broad spectrum activity against Gram-positive and Gram-negative bacteria (Drakesmith and Prentice, 2012). But little is known about the tissue distribution and its relationship with immune responses in pigs. In the present study, higher mRNA expression of pHepc was observed in liver, thymus, spleen and bone marrow, while the lower expression was tested in all other tested tissues. It was most abundant in liver, which was consistent with the expression described before in pigs, humans and other animals (Park et al., 2001; Sang et al., 2006). Furthermore, the transcript levels of pHepc were high at thymus, bone marrow and spleen, which are importantly central immune organs. This was in agreement with other antimicrobial peptides, β-defensins and cathelicidins in pigs (Chen et al., 2010; Ma et al., 2014).

The comparison of pHepc between Landrace and Tibetan pigs showed that the expression of pHepc of Tibetan

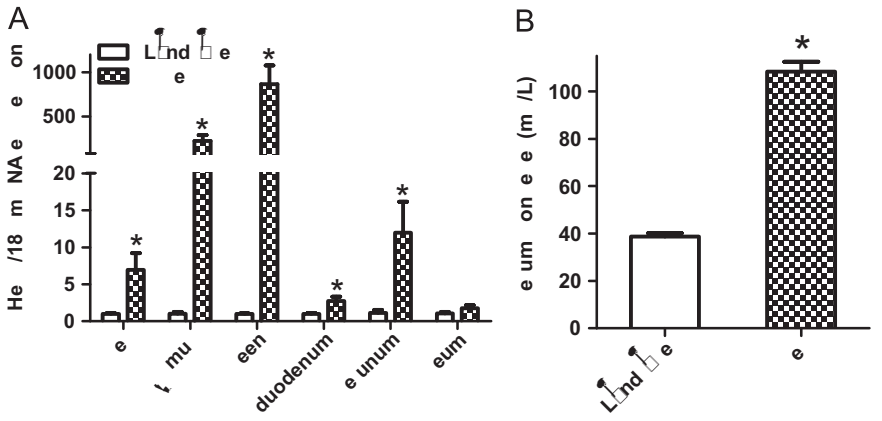

Fig. 2. Comparison of pHepc mRNA expression in tissues (A) and serum iron levels (B) of Landrace and Tibetan pigs. The pHepc PCR products from the liver, thymus, spleen, duodenum, jejunum and ileum were normalized to 18s rRNA as the reference genes. pHepc mRNA expression of the Landrace was used as the control in each tissue. Data plotted represent the mean ratio value ± SEM ($n=5$). Asterisks indicate significant differences ($p \leq 0.05$).

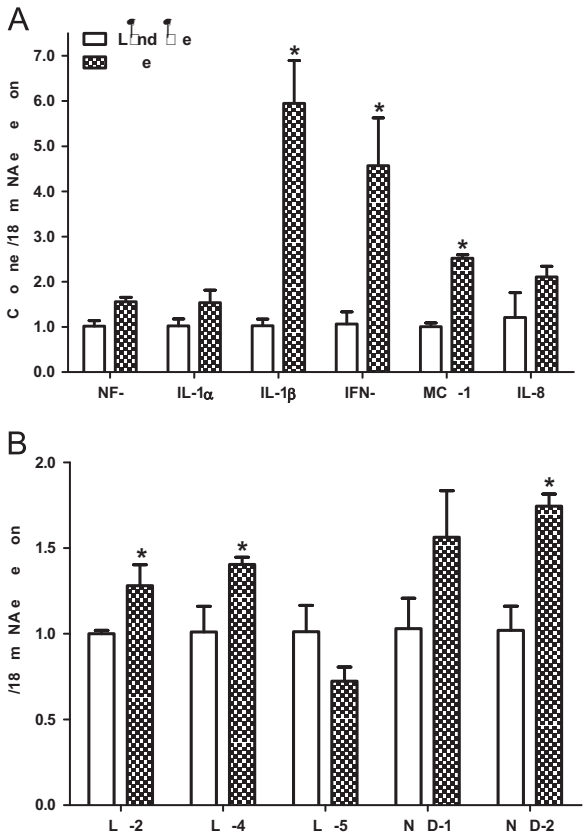

Fig. 3. Analysis of immune response-associated factors mRNA expression in the ileum of Landrace and Tibetan pigs. (A) Comparison of the expression of pro- and anti- inflammatory cytokines and chemokines. (B) Comparison of the expression of pattern recognition receptors. The PCR products were normalized to 18s rRNA as the reference genes, and data shown are cytokines mRNA expression as a ratio of Landrace. Data plotted represent mean ± SEM ($n=5$). Asterisks indicate significant differences ($p \leq 0.05$).

pigs in most tissues including liver, thymus, spleen, jejunum and duodenum were higher ($p < 0.05$) than those of Landrace pigs. These findings have extended our current knowledge and understanding of the diversity of hepcidin expressions between Landrace pigs and Tibetan pigs. Hepcidin production is regulated by several stimuli, including iron (as a negative feedback loop), the inflammatory cytokine interleukin-6 (IL-6), endoplasmatic reticulum stress, active erythropoiesis, anemia, and hypoxia (Ganz and Nemeth, 2011). In order to identify the regulation of iron by pHepc, serum iron levels were determined in both breeds. As expected, serum iron level of Tibetan pigs was significantly higher ($p < 0.05$) than that of Landrace pigs. Tibetan pig is the only Plateau type and grazing type pig according to the classification of pig breeds in China (Zhang et al., 1986). At high altitude a significant decrease in the oxygen saturation and the partial oxygen pressure was observed. As an adaptation to a decreased oxygen pressure, erythropoiesis increases the iron demand in the erythropoietic compartment and induces adaptive changes in the human body such as increased intestinal iron uptake, augmentation of serum iron-binding capacity, and enhanced mobilization of iron from cellular stores (Haase, 2010). Enhanced erythrocyte production under hypoxic conditions requires an increased hemoglobin pool and, therefore, improved heme synthesis (Beall et al., 1998). Therefore, Tibetan pigs might increase hemoglobin synthesis with hypoxia in high altitude adaptation. pHepc gene expression seemed to be regulated mainly by active erythropoiesis and high iron concentration in Tibetan pigs.

The link between innate immunity and iron homeostasis was gradually established with the discovery that increased hepcidin expression was observed during infection and inflammation (Ong et al., 2006). In this study, the wide expressions in most tissues of healthy pigs suggest that pHepc may contribute to systemic host defenses. Therefore, the mRNA expressions of cytokines, PRRs and chemokines were measured in both breeds. Cytokines play crucial roles in the modulation of the inflammatory response in animal gastrointestinal tracts. Numerous proinflammatory cytokines (e.g., TNF-α and IFN-γ) are essential in the mediation of the inflammatory response caused by pathogen infection (Lippolis, 2008). Our results showed higher levels of pro-inflammatory cytokines including TNF-α, IL-1α, IL-1β and IFN-γ were detected in Tibetan pigs when compared to Landrace pigs, which suggested that more cytokines was

reserved and released by ileum and the intestinal mucosal immune system might be more active in Tibetan pigs. A well-known component of innate immunity uses PRRs to recognize pathogen-associated molecular patterns (PAMPs) (Medzhitov and Janeway, 1997) in the detection and eradication of pathogens. In the present study, most tested PRRs including TLR-2, TLR-4, NOD-1 and NOD-2 were all expressed higher in Tibetan pigs. The PRRs serve as frontline surveillance molecules and may trigger downstream processes to accelerate pathogen clearance. As chemical messengers to guide the migration of cells, chemokines are produced in acute and chronic inflammation to activate leukocyte growth, differentiation, and activity (Laing and Secombes, 2004). Similar to other cytokines, chemokines MCP-1 and IL-8 were also at high levels in Tibetan pigs, which might affect biological activities such as the growth of immune cells and the immune responses.

In summary, gene expression for pHepc in Tibetan pigs and Landrace pigs were determined and showed a characteristic expression pattern. Contrary to Landrace pigs, the higher expression levels of Tibetan pigs were also found in spleen. pHepc expression of Tibetan pigs in most tissues were higher than those of Landrace pigs. Furthermore, elevated cytokines, PRRs and chemokines were detected in ileum of Tibetan pigs. The high expression of pHepc may affect the production of immune factors by regulating iron homeostasis, which might be the part of reason of Tibetan pigs have higher immunity.

Conflict of interest statement

None.

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