Testicular gene expression of Anti-Müllerian hormone, Androgen receptor and Inhibin alpha subunit in porcine cryptorchidism

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Abstract

Cryptorchidism, one of the common congenital abnormalities in boars, results in a boar taint in the carcass and economic loss. However, knowledge of gene expression of hormones controlling the testicular descent process, such as anti-Müllerian hormone (*AMH*), androgen receptor (*AR*) and inhibin alpha subunit (*INHA*), in porcine testicular cryptorchidism is still limited. The present study aimed to investigate the gene expression profiles of *AMH*, *AR* and *INHA* in undescended testes (UDT) and descended testes (DT) in unilateral cryptorchid pigs of suckling (1–2 weeks of age), nursery (6 weeks of age) and growing-finishing pigs (15–20 weeks of age) and in normal testes (NT) at 1–2 weeks of age, using quantitative real-time reverse transcription PCR (qRT-PCR). A significant decrease in expression of *AMH* and *AR* genes in the UDT group was observed compared with the DT group at 15–20 weeks. Within each group, the highest expression of *AMH* was observed at 6 weeks of age, whereas the lowest expression was shown at 15–20 weeks compared with other ages. For the expression of *AR*, the lowest expression was revealed only in the UDT group at 15–20 weeks compared with other ages. Prominent expression of *AR* and *INHA* was observed in the DT group at 20 weeks. In conclusion, this study unveiled the decreased expression of *AMH* and *AR* genes in UDT compared with DT in growing-finishing pigs. Markedly increased expression of *AR* and *INHA* was observed in DT when pigs reached puberty.

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Introduction

Cryptorchidism is the failure of one or both testes to descend from its intra-abdominal location into the scrotum. This abnormality is one of the most common congenital abnormalities observed in boars, with the prevalence of porcine litters of different breeds with unilateral and bilateral cryptorchidism being 2.2 and 0.2%, respectively (Dolf *et al.*, 2008). The major concerns of cryptorchidism are the abnormalities of spermatogenesis caused by the aberrant high temperature of the undescended testis in the abdomen and boar taint, which is an objectionable odor on the meat owing to the retained testis. This odor can be the cause of economic loss because consumers avoid buying meat with boar taint and flavor (Meier-Dinkel *et al.*, 2016).

Testicular descent is composed of two main stages: transabdominal and inguinoscrotal. In pigs, these stages begin at 55 and 85–90 days of gestation respectively and the testis has already been in the scrotum since birth. Multiple proteins control testicular descent, such as androgen receptor (AR), anti-Müllerian hormone (AMH) and inhibin alpha subunit (INHA) (De Kretser *et al.*, 2004; Hutson and Lopez-Marambio, 2017; Yimpring *et al.*, 2019). AR has been reported to be important in both stages of testicular descent (Oprins *et al.*, 1988). It regulates cranial suspensory ligament regression during the transabdominal stage and gubernacular eversion at onset of the inguinoscrotal stage (Kaftanovskaya *et al.*, 2012). AMH [also known as Müllerian inhibitory substance (MIS)] is a homodimeric disulfide-linked glycoprotein of the transforming growth factor-β superfamily (Murase *et al.*, 2015). It functions to determine male sex. AMH protein expression has been shown to be a marker of Sertoli cell maturation not only in pigs but also in horses and humans (Ball *et al.*, 2008: Boukari *et al.*, 2009; Ford and Wise, 2009). For INHA, it is a potent activin inhibitor. This hormone plays an important role in negative feedback to the hypothalamo-pituitary-gonadal (HPG) axis, hence it can cause undescended testis, as inhibin is accountable for down-regulation of the follicle-stimulating hormone (FSH) in boars (Ohnuma *et al.*, 2007). The study of gene expression involved in cryptorchidism is beneficial in helping to understand the defect mechanism of testicular descent, which affects breeding programs, animal welfare and economics as mentioned above.

The objective of this study was to investigate the gene expression profile of *AMH*, *AR* and *INHA* in undescended testes (UDT) and descended testes (DT) in unilateral cryptorchid pigs at the ages of 1–2, 6 and 15–20 weeks and in normal testes (NT) at the age of 1–2 weeks by quantitative real-time reverse transcription PCR (qRT-PCR). The gene expression was normalized against 3 reference genes including beta-actin (*ACTB*), beta-2-microglobulin (*B2M*) and 18S ribosomal RNA (*Rn18s*), which have been widely used in qRT-PCR for porcine samples (Li *et al.*, 2011; Xiang-Hong *et al.*, 2011).

Materials and Methods

Sample collection: Twenty-seven UDT (twelve at 1–2 weeks, five at 6 weeks, five at 15 weeks and five at 20 weeks of age) and 29 DT (twelve at 1–2 weeks, six at 6 weeks, five at 15 weeks and six at 20 weeks of age) were recruited from cryptorchid pigs on private farms in Thailand. Eight NT were collected from healthy pigs at 1–2 weeks. Samples were collected when piglets were routinely castrated on the farms, kept in RNALater solution (Thermo Fisher Scientific, Waltham, MA, USA) overnight at 4°C and stored at –20°C until processed. The procedures used and the care of animals were approved by the Chulalongkorn University Animal Care and Use Committee (CU-ACUC), Thailand.

RNA extraction: One hundred micrograms of testicular tissues in RNALater solution were cryogenically ground under liquid nitrogen. Total RNA was extracted from testes by homogenization with TRIzol reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. Briefly, 1 ml of TRIzol reagent was added to 50–100 mg of homogenized tissue. After centrifugation at 12,000 rpm for 10 mins, chloroform was added to the supernatant for nucleic acid separation. Isopropanol was used to collect gel-like pellets of the RNA samples. Finally, the RNA samples were washed using 75% ethanol and dissolved in 60 µL of RNase-free water. Genomic DNA traces were removed from the RNA with TURBO DNase (Thermo Fisher Scientific) to purify the total RNA, as reported previously (Pisamai *et al.*, 2017). Briefly, total RNA samples were treated twice with TURBO DNase for 30 mins at 37°C. Supernatants were collected and stored at –20°C. A Nanodrop ND-1000 Spectrophotometer V3.7 (Thermo Fisher Scientific) was used to analyze the DNase-treated RNA quality and concentration. The RNA integrity of each sample was analyzed using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

Primer design and testing: Primer sequences were developed using Primer 3 software (Rozen and Skaletzky, 2000) and checked for specificity against the porcine genome (SGSC Sscrofa10.2/susScr3, August 2011) using the In-Silico PCR program (http://genome.ucsc.edu/) and the Basic Local Alignment Search Tool (http://www.ncbi.nlm.nih.gov/blast). The primer sequences, accession numbers and amplicons are shown in Table 1. PCR products were purified using a QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) and sequenced with an automated DNA sequencer and the sequenced data was submitted to the Basic Local Alignment Tool from the National Center for Biotechnology Information to validate the gene results.

Quantitative reverse transcription PCR (qRT-PCR): cDNA was synthesized by qRT-PCR using the SuperScript III First-Strand Synthesis System (Thermo Fisher Scientific) according to the manufacturer's protocol, using 1 µg of total RNA as a template. The qRT-PCR was performed using a KAPA SYBR Fast qPCR Master Mix Universal kit (Hoffmann-La Roche,

Basel, *Switzerland*) and a Rotor Gene 3000 Thermal Cycler (Qiagen) according to the manufacturer's protocol, with the PCR reactions performed as described previously (Theerawatanasirikul *et al.*, 2012). Briefly, cDNA at 25 ng and primers at 200 nM each were mixed with the 2× qPCR Master Mix Universal. Reactions were started at 95°C for 2 mins followed by 40 cycles at 95°C for 3 s and 60°C for 20 s. Each reaction was performed in duplicate in three independent runs. A melting curve analysis was used to determine the purity of the amplified products. Relative expression levels were calculated using REST 2009 (Relative Expression Software Tool) software with a detection threshold of 0.1 (Pfaffl *et al.*, 2002; Pisamai *et al.*, 2017).

Table 1   Primers used for the two-stage qRT-PCR in the present study.

Gene	Accession number	Primer (5′–3′)	Amplicon (bp)	Tm (°C)
ACTB	AY550069.1	Fwd 5′-gatgagattggcatggctttt-3′	122	60.04
		Rev 5′-caccttcaccgttccagttt-3′		60.01
AMH	NM_214310.2	Fwd 5′-aggatgcagggtccttctct-3′	162	60.22
		Rev 5′-gttccagtcccagtggaaga-3′		60.09
AR	NM_214314.2	Fwd 5′-ggcaaaagcaacgaagagac-3′	174	60.00
		Rev 5′-tgctacatcgtccagtgctc-3′		60.02
INHA	NM_214189.1	Fwd 5′-acctgctggcactatcatcc-3′	122	60.10
		Rev 5′-gactgggtgggtcaacaaag-3′		60.40
B2M	(Almeida *et al.*, 2013)	Fwd 5′-ttcacaccgctccagtag-3′	166	55.70
		Rev 5′-ccagatacatagcagttcagg-3′		55.00
Rn18s	NR_046261.1	Fwd 5′-aattccgataacgaacgagac-3′	141	59.99
		Rev 5′-ggacatctaagggcatcacag-3′		60.20

Statistical and expression stability analyses: Statistical analysis of real-time PCR results was performed with REST 2009 software with a pairwise fixed reallocation randomization test to test for significant differences between groups as target gene expression against the reference genes *ACTB*, *B2M* and *Rn18s* in UDT, DT and NT. Results were considered significant at $p < 0.05$.

Results

Gross morphology of UDT and DT at 20 weeks of age was demonstrated (Fig. 1). Differential gene expression of *AMH*, *AR* and *INHA* in UDT, DT and NT was shown. At 15–20 weeks, markedly decreased expression of *AMH* and *AR* was exhibited in UDT compared with DT (Table 2). Within each group of UDT and DT, pigs at 15–20 weeks showed remarkable down-regulation of *AMH* expression, whereas pigs at 6 weeks showed the highest expression levels (Table 3). For the expression of *AR*, the lowest expression was revealed only in the UDT group at 15–20 weeks compared with other ages, whereas in DT prominent expression was noticed at 20 weeks (Tables 3 and 4). Aberrant expression of *INHA* was also observed in the DT group at 20 weeks (Table 4).

Table 2   Fold change in gene expression of anti-Müllerian hormone (*AMH*), androgen receptor (*AR*) and inhibin alpha subunit (*INHA*) genes in pigs with undescended (UDT), descended (DT) and normal testes (NT) at 1–20 weeks of age. P-values are in parentheses.

Age of pigs (weeks)	*AMH*	*AR*	*INHA*
UDT vs. DT			
1–2	1.013 (0.971)	1.027 (0.923)	0.397 (0.285)
6	1.156 (0.262)	1.434 (0.184)	0.651 (0.534)
15–20	0.270* (0.026)	0.320* (0.023)	0.601 (0.089)
UDT vs. NT			
1–2	0.920 (0.842)	0.985 (0.962)	0.396 (0.276)
DT vs. NT			
1–2	0.908 (0.796)	0.959 (0.887)	1.000 (1.000)

* denotes data with $p < 0.05$

Table 3   Gene expression differences of anti-Müllerian hormone (*AMH*), androgen receptor (*AR*) and inhibin alpha subunit (*INHA*) genes in pigs with undescended testes (UDT) or descended testes (DT) at 1–2, 6 and 15–20 weeks of age. P-values are in parentheses.

Age of pigs (weeks)	AMH	AR	INHA
Undescended testes (UDT)			
1–2 vs. 6	2.671* (0.032)	1.968 (0.077)	0.578 (0.588)
1–2 vs. 15–20	20.118* (< 0.001)	2.446* (0.002)	0.810 (0.757)
6 vs. 15–20	53.726* (0.001)	4.815* (< 0.001)	0.468 (0.057)
Descended testes (DT)			
1–2 vs. 6	2.341* (0.019)	0.547 (0.274)	0.352 (0.272)
1–2 vs. 15–20	5.355* (< 0.001)	0.762 (0.520)	1.227 (0.753)
6 vs. 15–20	12.538* (< 0.001)	1.074 (0.912)	0.432 (0.095)

* denotes data with $p < 0.05$

Table 4   Gene expression differences of anti-Müllerian hormone (*AMH*), androgen receptor (*AR*) and inhibin alpha subunit (*INHA*) genes in pigs with undescended testes (UDT) or descended testes (DT) at 1–2 and 6 weeks of age compared with those at 15 and 20 weeks of age. P-values are in parentheses.

Age of pigs (weeks)	AMH	AR	INHA
Undescended testes (UDT)			
1–2 vs. 15	0.188* (0.014)	0.457* (0.027)	1.799 (0.567)
1–2 vs. 20	0.013* (< 0.001)	0.366* (0.014)	0.848 (0.870)
6 vs. 15	0.070* (0.005)	0.232* (0.002)	3.112* (0.009)
6 vs. 20	0.005* (0.006)	0.186* (0.001)	1.467 (0.357)
15 vs. 20	0.070* (0.023)	0.800 (0.617)	0.471 (0.115)
Descended testes (DT)			
1–2 vs. 15	0.189* (< 0.001)	0.158* (0.011)	0.455 (0.424)
1–2 vs. 20	0.185* (< 0.001)	1.350 (0.599)	1.325 (0.777)
6 vs. 15	0.081* (0.001)	0.289* (0.006)	1.293 (0.671)
6 vs. 20	0.079* (< 0.001)	2.467* (0.002)	3.763* (0.010)
15 vs. 20	0.980 (0.952)	8.536* (<0.001)	2.911* (0.001)

* denotes data with $p < 0.05$

Figure 1   Gross morphology of undescended (left) and descended testes (right) from unilateral cryptorchid pigs at 20 weeks of ages. Scale bar = 1 cm.

Discussion

The present retrospective study shows that cats In the present study, *AMH* expression was shown to be increased in UDT and DT at birth and the nursery stages compared with that in the late growing-finishing stage. The results were similar to that in cryptorchid horses, where strong AMH immunohistochemistry labeling presented up to 3 years of age, whereas faint labeling was exhibited at 4 years of age (Ball *et al.*, 2008). In humans, decreased AMH levels were observed in prepubertal children with primary testicular disorders, reflecting Sertoli cell disorders (Condorelli *et al.*, 2018). AMH malfunction has been reported to be an indicator of cryptorchidism and persistent Müllerian duct syndrome (PMDS) (Hutson and Lopez-Marambio, 2017). Mutations of *AMH* and *AMH-RII* have been shown to be associated with PMDS and cryptorchidism (Lindhardt Johansen *et al.*, 2013). In our study, decreased expression of *AMH* in both UDT and DT at 15–20 weeks was possibly due to defects of Sertoli cells in adult pigs and the effect did not depend on testis temperature. Since no significant difference was observed in the expression of *AMH* in UDT, DT and NT of suckling piglets, it might not serve as a suitable biomarker of cryptorchidism in young pigs.

In our study, the pattern of *AR* expression in UDT was similar to that of *AMH*. The *AR* gene and protein expression in UDT of stallions has been reported to be decreased compared with that in DT (Almeida *et al.*, 2013). Similar protein expression of AR has been demonstrated in UDT and DT of pigs at 2 months of age (Manee-in *et al.*, 2011). However, in the present study, *AR* levels were remarkably higher in DT at 20 weeks of age than in UDT. Since the temperature of an abdominal testis was 4–5°C higher than a scrotal testis, the higher temperature in UDT possibly affected testicular function, such as decreased *AR* transcription at 20 weeks of age (Harrison and Weiner, 1949). The high body temperature of UDT might be the etiology of defective spermatogenesis (Cobellis *et al.*, 2014). Cryptorchidism has been developed in mice with conditional inactivation of the *AR* gene in the gubernacular ligament (Kaftanovskaya *et al.*, 2012). *AR* gene and AR protein expression has been reported to be associated with spermatogenesis in lambs and humans (Monet-Kuntz *et al.*, 1987; Regadera *et al.*, 2001). The expression of testicular AR has been shown to be increased in pigs at 2 months of age with decreased testicular estrogen synthesis (Ramesh *et al.*, 2007). Low protein expression of AR has been revealed in UDT and DT of suckling piglets compared with NT (Yimpring *et al.*, 2019). The paradoxically increased *AR* gene expression of suckling piglets in the present study

might be due to post-transcriptional regulation that possibly inhibited the protein expression of AR. In addition, gene mutation and isoforms for the gene may be able to diminish protein expression, regardless of effects on the transcription level (Chen *et al.*, 2002). AR truncated isoforms resulting from post-translational processing have been revealed in humans, with an association with prostate cancer (Mudryj and Tepper, 2013). Furthermore, the CAG-insertion/deletion polymorphism of *AR* gene in pigs has been shown to be associated with fatness traits, uterus and ovary sizes (Wimmers *et al.*, 2005). However, the isoforms of porcine AR and gene mutation, if any, and their association with cryptorchidism are needed for further investigation. In our study, expression of *INHA* was shown to be prominent in DT at 20 weeks of age compared with UDT, probably owing to the effect of temperature similar to that of *AR* expression. To the best of our knowledge, this is the first study to describe the association of INHA in porcine cryptorchidism. In rats at 15 and 45 days of age, decreased *INHA* gene expression was noted in cryptorchid testis (Rivier *et al.*, 1989). Plasma INHA has been found to be decreased in dogs with cryptorchidism compared with normal dogs (Kawakami *et al.*, 2007).

In conclusion, this study unveiled the differential expression of *AMH*, *AR* and *INHA* genes in pigs with UDT and DT at 1–20 weeks of age. The expression of *AMH* and *AR* was significantly decreased in UDT compared with DT in growing-finishing pigs. Markedly increased expression of *AR* and *INHA* was observed in DT when pigs reached puberty, possibly owing to different temperatures of abdominal and scrotal testes. For future work, the mechanisms of the gene expression should be studied and the protein expression in the blood of affected pigs should be further investigated.

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