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A candidate gene association study of cryptorchidism and scrotal hernia using canine and porcine models

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A candidate gene association study of cryptorchidism and scrotal hernia using canine and porcine models

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ABSTRACT

Cryptorchidism and scrotal hernia, both being sex-limited complex defects, are the most common congenital defects observed in humans, dogs and pigs. It is believed that these two defects are controlled by multiple genes as well as affected by environmental factors.

In this thesis, 22 or 14 functional and positional candidate genes, respectively, have been evaluated to identify the possible associations for crytorchidism in Siberian Huskies and scrotal hernia in pigs. In the canine cryptorchidism study, 76 single nucleotide polymorphisms (SNPs) were found and 51 out of 76 were genotyped by the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method or directly sequencing in 156 Siberian Huskies. A Sibling-Transmission Disequilibrium Test (sib-TDT) was first applied to the discordant sib sets (n=38), and then to all 156 dogs including 47 families and 15 individuals. Sib-set analyses showed six SNPs distributed in the collagen type II α 1 (*COL2A1*) gene were significantly associated with cryptorchidism ($P < 0.05$). Using a sib-TDT analysis on all 156 dogs, SNP rs23389020 in *COL2A1* was suggestively significant ($P < 0.06$), but not significant after permutation tests (n=1000). Neither of the two haplotypes formed by the five SNPs in *COL2A1* was significantly associated with cryptorchidism.

In the porcine scrotal hernia study, a total of 1,534 pigs were used including a data set of 692 individuals from 298 pig nuclear families and another data set of 340 unaffected and 502 affected male pigs. The SNPs of all candidate genes were analyzed by using PCR and genotyped by using Sequenom MassArray™ technology. Statistical analyses were performed on the family-trio and the case-control data, respectively. Two genes involved in collagen

metabolism including homeobox A10 (*HOXA10*) and matrix metalloproteinases 2 (*MMP2*), and one gene as zinc finger protein, multitype 2 (*ZFPM2*), which is regarded as a candidate gene with the development of diaphragmatic hernias, were significantly associated with scrotal hernia incidence ($P < 0.01$, $P < 0.05$ and $P < 0.05$, respectively). Animals with these genotypes have relatively high odds ratio (OR) of developing scrotal hernias, 2.21 (1.58-3.09) and 2.43 (1.73-3.42) for the two *HOXA10* SNPs, and 4.3 (2.78-6.64) and 4.45 (2.88-6.88) for the two *ZFPM2* SNPs, respectively. Another gene, collagen type II α 1 (*COL2A1*) was potentially involved in hernia development ($0.05 < P < 0.1$).

Based on the statistical results, we suspect *COL2A1* may be a potentially associated gene with cryptorchidism in Siberian Huskies. *HOXA10*, *ZFPM2*, and *MMP2*, as well as *COL2A1* could possibly have important roles in pig scrotal hernia development. The potential associations found in the *COL2A1* gene with both cryptorchidism and scrotal hernia can be explained as both defects might be partially regulated by the same group of genetic loci. Moreover, the significant genetic variants found in pig scrotal hernia are possibly useful for the marker assisted selection to remove scrotal henia from the pig industry. Due to the physiological and genome similarities between dogs and pigs with humans, genes associated or suggestively associated with cryptorchidism and scrotal hernias uncovered in this study may be valuable in understanding the molecular mechanisms underlying these defects in humans. Further association studies for both defects are needed to validate our results.

CHAPTER 1. GENERAL INTRODUCTION

GENERAL INTRODUCTION

Two common congenital defects, cryptorchidism and scrotal hernia, could be the results of abnormal anatomical structures formed during the testicular descent process. Cryptorchidism is defined as the failure of the testis to descend from its intra-abdominal location into the scrotum. It affects about 3% of full-term human male infants and 30% of preterm infants (Klonisch et al., 2004). Scrotal hernia is a condition in which part of the small intestine passes through the internal inguinal ring and the inguinal canal, then goes into the scrotum, and may remain positioned next to the testicle. Inguinal hernia refers to the hernia contents presenting in the inguinal canal. Both an inguinal hernia and a scrotal hernia are likely to be treated as an inguinal/scrotal hernia. Without treatment, cryptorchidism can disturb spermatogenesis, lead to infertility and even testicular cancers (Hutson et al., 1997); and scrotal hernia can cause the strangulation of the hernia contents. For livestock breeders and the animal industries, both of them require more time and energy in the management and treatment of affected individuals, increasing substantially the possible economic loss.

Cryptorchidism can be caused by the abnormalities in the following processes including the swelling and outgrowth of the gubernaculum, the regression of the gubernaculums and the cranial suspensory ligament, and the elevation of the intro-abdominal pressure as well as the extension of the processus vaginalis (Hutson and Hasthorpe, 2005). Inguinal/scrotal hernias, on the other hand, could be the result of the failed closure of the internal inguinal ring and the incomplete obliteration of the processes vaginalis after the testicular descent (Hutson et al., 1997; Tomiyama et al., 2005), the weak triangle of the

inguinal region (Friedman et al., 1993), an abnormality in the smooth muscle differentiation in the wall of the hernial sac (Tanyel et al., 1999; Tanyel et al., 2002), or increased intra-abdominal pressure.

Genetic studies have revealed that these two defects are complex ones controlled by multiple genes, as well as affected by environmental factors (Czeizel et al., 1981; Rothschild et al., 1988; Cox et al., 1978; Nielen et al., 2001). For certain purebred dogs, a high incidence of cryptorchidism exists. It is essential to remove the affected dogs and carriers from a dog breeding program to decrease its incidence which will benefit the dog breeders. Culling pigs with inguinal/scrotal hernia from a nucleus breeding population will decrease the economic loss caused by this defect in the pig industry which is a significant problem in many herds each year. With the dog genome being completely sequenced (Lindblad-Toh et al., 2005), and pig genome soon as well, the search for underlying genetic factors for these two defects will be greatly advanced. Due to the high anatomic and physiological similarities to humans (Khanna et al., 2006; Ibrahim et al., 2006), as well as the high structural similarity between the human and pig or the dog genome (Rettenberger et al., 1995; Meyers et al., 2005; Murphy et al., 2005), dogs and pigs can serve as animal models for human cryptorchidism and scrotal hernia studies.

The complex involvement of the genetic factors and their interplay with environmental factors increase the difficulty to identify true causative genetic variants for these defects, even though genome-wide linkage scan studies have found several quantitative trait loci (QTL) regions associated with inguinal/scrotal hernia in pigs (Plastow et al., 2003; Du et al., 2004; Grindflek et al., 2006; Knorr et al., 2006). As a pilot study, we chose a candidate gene approach to uncover genes contributing to susceptibility for crytorchidism

and inguinal/scrotal hernias. We integrated information from biological pathway and comparative genomics to select functional and positional candidates, which can be complemented by genome-wide association studies in the future.

RESEARCH OBJECTIVES

There are both crytorchidism and scrotal hernia in humans, dogs and pigs. These two defects can contribute to reduced fertility or testicular malignancy or even death. Identification of the genetic factors involved in these two defects is extremely important. The objective of Chapter 2 is to present an association study of candidate genes with cryptorchidism using Siberian Huskies. The objective of Chapter 3 is to present an association study of genetic markers in candidate genes involved in the development of scrotal hernia in the pig.

THESIS ORGANIZATION

The following section of this chapter provides a literature review that coincides with the research conducted herein. The subsequent chapters of this thesis are individual papers that have either been accepted for publication or submitted to a peer-reviewed journal. Chapter 2 is a manuscript that was submitted to the Journal of Heredity. This working title is "Association of *COL2A1* with Cryptorchidism in Siberian Husky Dogs" and it provides insight on the associations of genetic markers with cryptorchidim in pure Siberian Huskies. The research was conducted and manuscript written by Xia Zhao under the supervision of Dr. Max Rothschild. The manuscript comprising Chapter 3 "Biological Candidate Gene Analyses Identify that *HOXA10, ZFPM2* and *MMP2* are Associated with Scrotal Hernias in

Pigs" has been accepted for publication in American Journal of Veterinary Research. The research was conducted and the manuscript was written by Xia Zhao under the supervision of Dr. Max Rothschild. Postdoctoral associate Zhi-Qiang Du contributed many components of this research from help with genotyping and data analyses as well as manuscript revisions.

LITERATURE REVIEW

1. Testicular descent

The testicles are developed from the urogenital ridge of male mammal embryos under the regulation of the testis determining gene, the sex-determining region Y chromosome gene (SRY) (Sinclair et al., 1990). Stimulated by developmental signals specific to different mammals, the general testicular descent process is that the testis migrates down from the kidney position, passes through internal ring, inguinal canal, external ring, and eventually stays at an extra-abdominal location in the scrotum. This position maintains a lower temperature environment, keeping the testes several degrees cooler than the core body temperature which is necessary for normal spermatogenesis and future fertility (Jung et al., 2005).

1.1 The mechanism of testicular descent

Following the process of testicular descent, two main theories have been proposed. The main difference is the number of phases and the regulation of hormones. The first theory has been widely accepted by many researchers and revolves around two basic steps (Baumans et al., 1983; Hutson et al., 1997). The first step is a trans-abdominal phase, constituted by the enlargement of the caudal gubernaculum and the regression of the cranial suspensory ligament. The enlargement of the gubernaculum is under the control of insulin-like hormone 3 (INSL3) which is produced by the Leydig cells (Nef and Parada, 1999; Emmen et al., 2000) and the regression of the cranial suspension ligament is controlled by testosterone, which was also secreted by leydig cells (van der Schoot and Elger, 1992; Yuan et al., 2006). At this step, the testis is clinging to the future internal inguinal ring. The

inguinoscrotal phase is the second step at which the gubernaculum grows out from the inguinal abdominal wall and migrates into the scrotum (Heyns, 1987). The caudal tip of the processus vaginalis also actively elongates (Ramasamy et al., 2001). With the growth of the gubernaculum and the elongation of the processus vaginalis, the testis is directed into the bottom of the scrotum. The calcitonin gene-related peptide (CGRP), a neurotransmitter released by the genitofemoral nerve (Beasley and Hutson, 1987), and stimulated by androgen (Shenker et al., 2006) controls the direction of the migration of gubernaculum and testis.

The second theory proposed by Amann and his colleague (Amann and Veeramachaneni, 2007) divided the testicular descent process into three phases, the abdominal testis translocation, the transinguinal testis migration and the inguinoscrotal testis migration. The combination of the last two phases in this theory corresponds to the second phase in the first theory. During the phase of abdominal testis translocation in this theory, the thin and short gubernaculm gradually enlarges, while the cranial suspensory ligament regresses. Therefore, the testis is anchored by the cranial suspensory ligament and the gubernaculum and located near the internal inguinal ring. The process of INSL3 stimulating the growth of the gubernaculum is also believed to be involved in this phase, but testosterone is not required, which is different from the first theory. In the second phase or transinguinal testis migration, with the help of gubernacular bulb enlargement and regression of the cranial suspensory ligament, the testis passes the inguinal canal and is located just external to the inguinal canal or plane of the abdominal wall. Neither INSL3 nor testosterone is considered required for this phase. In the last phase, the testes migrate from the external inguinal ring to the final scrotal location which requires the extension of the gubernucular bulb and enclosed processus vaginalis to the bottom of the scrotum. The CGRP acts as a neurotransmitter and

plays an important role in this process by directing the gubernacular growth, the elongation of the processus vaginalis, the growth of the cremaster muscle, and the regression of the gubernaculum (Amann and Veeramachaneni, 2007).

1.2 Anatomical structures involved in the testicular descent

The descent of the testis through the inguinal canal and going down into the scrotum requires the coordination among many factors including abdominal pressure, the processus vaginalis, the cranial suspensory ligament and androgen-induced gubernacular regression. In mice, the cremasteric muscle, as a part of the gubernaculums, is also involved in this process (Hrabovszky et al., 2002). Therefore, five anatomical structures are described in detail here to elucidate the mechanisms of the testicular descent and to help compare the etiology of cryptorchidism and inguinal/scrotal hernia, two abnormal defects associated with the testicular descent. In Figure 1 is shown several anatomical structures involved in testicular descent in a typical mammal.

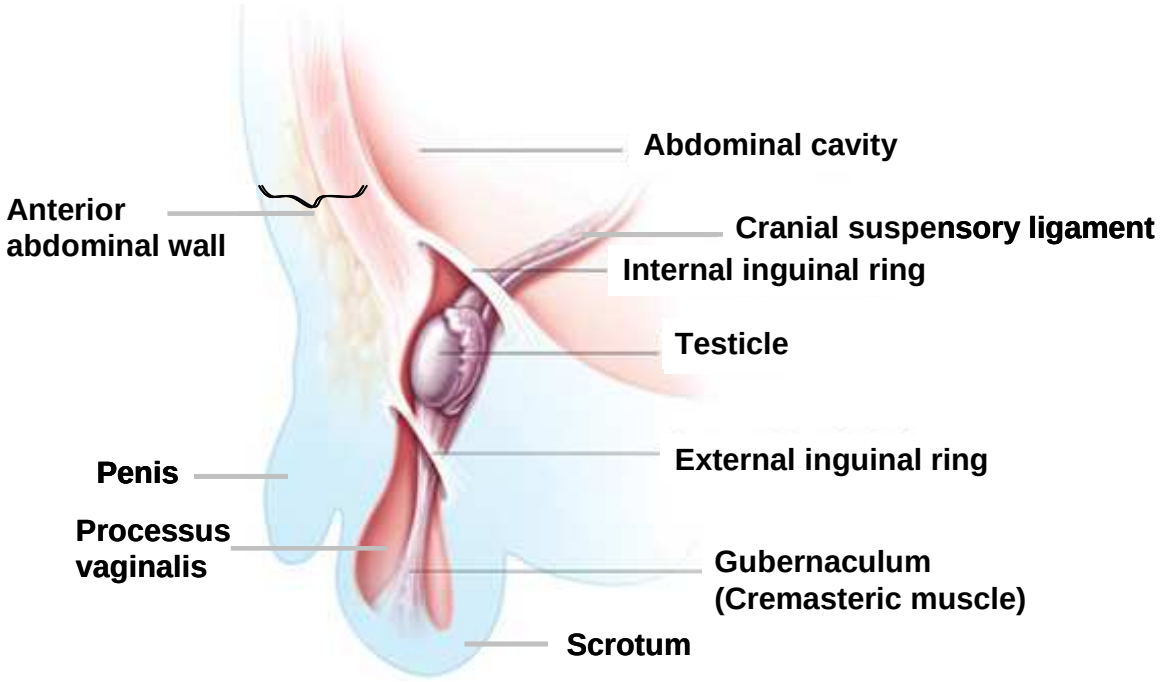

Figure 1. Structures involved in testis descent in humans.

This is a sagittal section of a male reproductive region. The gubernaculum and the cranial suspensory ligament attach to each side of the testis. The testis is now descending into the inguinal canal through the internal inguinal ring and will continue going all the way through the external inguinal ring down into the final position-scrotum. The peritoneal lining inverts as a vaginal process within the outer limits of the portion of the gubernaculum within the abdominal wall (Weiss, 2007).

The gubernaculum

The gubernaculum was first described by John Hunter as the folded peritoneum which attaches to the caudal end of the gonad and presents during the development of the urinary and reproductive organs in mammals (Hunter, 1786). Two regions on the gubernaculum called the cranial gubernacular cord (the proximal attachment part) and the caudal gubernacular bulb (the distal attachment part) exist. The cranial gubernacular cord

contactes the testicle and the epididymis. On the other end, the caudal gubernacular bulb expands to the retro-abdominal wall (Vigueras et al., 2004). Histologically, the gubernaculum is composed of mesenchymal cells and rich in collagen, abundant with glycosaminoglycans and hyaluronic acid (Heyns and de Klerk, 1985; Wensing, 1986). In males, the gubernaculum plays an important role in the testicular descent. During the transabdominal descent of the testis, the caudal gubernaculum enlarges and is known as the "gubernacular outgrowth" to provide an anchor for the testis. Along with the regression of the craninal suspensory ligament and fetal growth, the testes are retained in the inguinal region. Then during the transinguinal phase or inguinoscrotal phase, the gubernaculum bulb enlarges progressively to dilate the inguinal canal and facilitate the passage of the testes (Heyns, 1987). A significant migration of the paired gubernaculum to the scrotum controlled by the genitofemoral nerve and following regression are required for the testis go down to the bottom of the scrotum (Huynh et al., 2007). The enlargement of gubernaculum bulb is caused by cell division and an increase in glycosaminoglycans and hyaluronic acid. The relative wet mass and water content of the gubernaculum increases during the testicular descent, which makes the end of the gubernaculum bulky and gelatinous. After descent of the testis, the wet mass and water content of the gubernaculum decrease, leaving a fibrous remnant that attaches the testis and caudal epididymis to the scrotum (Heyns et al., 1990).

Cranial suspensory ligament

The cranial suspensory ligament (CSL), also called the craninal gonadal ligament, plays an important role for the normal testicular descent with the help of the gubernaculums. The CSL and gubernaculum are attached on opposite ends of the testis in males. Regression of the CSL development is a part of the mechanism of testicular descent. It has been learned

from feminized male mice studies, that mice have an intra-abdominal testis retention with a clear CSL remaining which demonstrates the necessity of the regression of the CSL in the normal testicular descent process (Hutson, 1986). However, some authors believe the CSL has a limited role in some species (Barthold et al., 1994; Husmann and Levy, 1995).

Cremaster muscle

The cremaster muscle(s) is formed by myoblasts, migrating from the abdominal wall muscle and differentiating from mesenchymal cells of the gubernaculums. It invades the vaginal portion of the gubernuculum and generates propulsive force to help in the testis descent through the processus vaginalis (Tanyel, 2004a). The anatomic structure of the cremaster muscle differs among species. The strip-like cremaster muscle exists in humans and companion or food-producing animals such as bulls, horses, pigs and dogs. In rodents or rabbits, the cremaster muscle is like a cup encompassing the proper portion of the gubernacular bulb (Hrabovszky et al., 2002; Amann and Veeramachaneni, 2007). Due to the different structures of the cremaster muscle and the close relationship with the gubernaculums, Amann and Veeramachaneni have suggested using the term “gubernacular-cremaster complex” in future studies. It has been found that CGRP released from the genitofemoral nerve with estrogen has an effect on the cremaster muscle in the gubernaculum (Yamanaka et al., 1993; Donaldson et al., 1996). Several genes related to muscle development, including myogenin (Myog), troponin T2 (Tnnt2), follistatin (Fst), insulin-like growth factor 1(Igf1), insulin-like growth factor binding protein 5 (Igfbp5), inhibitor of DNA binding 2 (Id2), and homeobox, msh-like 1(Msx1) have been found to be differently expressed between rats with cryptorchidism and those rats with normal testicular descent

(Barthold et al., 2008). This past functional study suggests an important role of cremaster muscle in the normal testicular descent.

Abdominal pressure

Abdominal pressure has an additional role in helping the testis exit from the abdomen during the inguinoscrotal descent (Frey and Rajfer, 1984; Kaplan et al., 1986). Abdominal wall defects such as gastroschisis, omphalocele and umbilical hernia can increase the incidence of crytorchidism in humans (Kaplan et al., 1986). However, a similar result could not be replicated in an animal model using the rat by excising the anterior abdominal wall to simulate the abdominal wall defects (Quinlan et al., 1988). The likely reason was that the proximal attachment of the gubernaculum to the testis is critical and both the gubernacular attachment and the intra-abdominal pressure act synergistically to aid testicular descent (Attah and Huston, 1993; Clarnette et al., 1996).

Processus vaginalis

The processus vaginalis is an embryonic developmental outpouching of the peritoneum underlying the gubernaculum with the abdominal wall (Amann and Veeramachaneni, 2007). In males of all species it provides a channel for the testis going through from the abdominal cavity and descent down to the scrotum (Shrock, 1971). The timing of the genitofemoral nerve division in the rat has been found to be crucial for the development of the processus vaginalis. The developmental morphology of the processus vaginalis is also different in certain species. In the human fetus, the processus vaginalis is surrounded by a strip-like cremaster muscle, and in rodents there is a bilaminar cremasteric sac within the processus vaginalis (Clarnette et al., 1996). Inguinoscrotal testicular descent requires the extension of the processus vaginalis. The force for the testis movement from the

intra-abdominal pressure is also via the lumen of the processus vaginalis (Amann and Veeramachaneni, 2007). The closure of the processus vaginalis occurs sometime from a few weeks before birth, to a few weeks after birth in different species. Usually, the remaining portion around the testes becomes the tunica vaginalis. Failure of closure of the processus vaginalis leads to the possible development of a number of abnormalities, including hydrocele, and inguinal/ scrotal hernias (Tanyel, 2004b).

2. Cryptorchidism

Cryptorchidism refers to testes retained in the abdominal cavity after the normal time for testicular descent for the species of interest. It can happen as a unilateral (one-side testicle retained) cryptorchid or a bilateral cryptorchid (both side testes retained). This defect is one of the most common congenital defects in humans, dogs, cats, pigs and many other eutherian mammals (Fig. 2). The estimated incidence in these species is in a range of 0.1-12%. For one particular species, such as the Sintka Black-Tailed Deer (SBTD) placed on Kodiak Island, Alaska, the incidence is even as high as 75%. Additionally, for almost all species, unilateral cryptorchidism is more common than bilateral cryptorchidism (Amann and Veeramachaneni, 2007). Major health concerns of cryptorchidism are reduced fertility or infertility and significantly increased risk of testicular malignancies (Pendergrass and Hayes, 1975). Therefore, hormonal therapy or surgery to help the retained testis move down into the scrotum is necessary for humans (Zivkovic et al., 2006; Kave and Palmer, 2008). To avoid the development of testicular cancer happen, the removal of the retained testicle is required in affected dogs or cats (Yates et al., 2003).

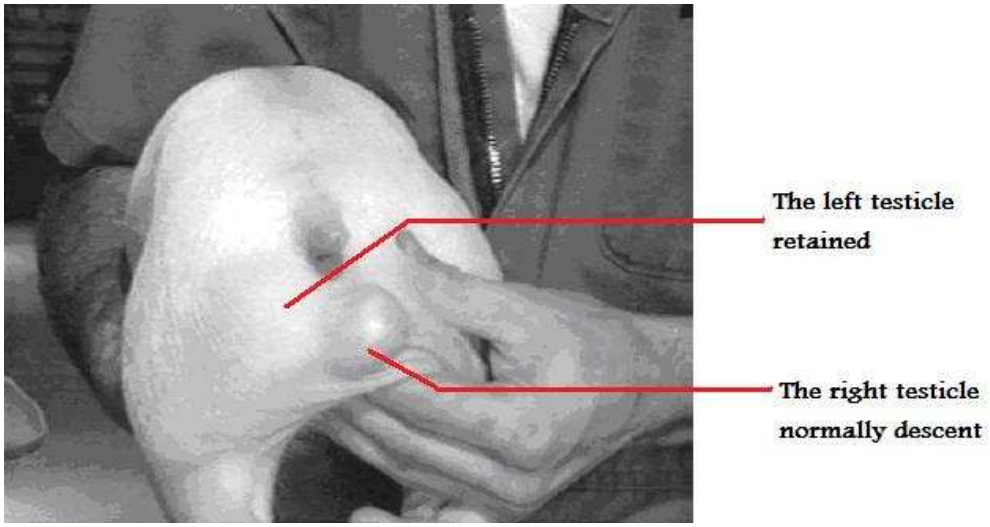

Figure 2. A unilateral cryptorchidism in a male pig.

The left testicle is retained and the right testicle has normal descent into the scrotum. This photo was modified based on the published paper: See et al., 2001. PIH-97.

Cryptorchidism is a sex-limited trait. Evidence found in humans (Czeizel et al., 1981), pigs (Rothschild et al., 1988) and dogs (Cox et al., 1978, Nielen et al., 2001) suggests that cryptorchidism is a defect controlled by multiple genes. Genetic pathways affecting the swelling and outgrowth of the gubernaculum, the regression of the cranial suspensory ligament and the gubernaculums, and the increase of the intro-abdominal pressure caused by the contraction of the abdominal muscles, can create developmental problems of the male reproductive organs which include cryptorchidism.

Various genes in these genetic pathways potentially vital to the development of cryptorchidism have been investigated. The gene named Müllerian inhibiting substance (*MIS*), or anti-Mullerian hormone (*AMH*), encoding a 140-kDa glycoprotein produced by Sertoli cells is critical for the mullerian duct during male sexual differentiation (Bashir and

Wells, 1995). It is believed to be associated with the swelling reaction of gubernaculum occurring during the first phase of testicular descent (Kubota et al., 2002). Deletion of this gene with the testicular feminization (Tfm) mutation has been suggested as a cause of undescended testis in mice (Behringer et al., 1994). Insulin-like factor 3 gene (*INSL3*) is a member of the insulin-like hormone superfamily. It is expressed in the pre-and postnatal Leydig cells of the testis and postnatal theca cells of the ovary (Zimmermann et al., 1997). In the initial phase of transabdominal testicular descent, the *INSL3* gene plays an important role for the normal descent of the testes. Targeted disruption of this gene prevents gubernaculum growth and causes bilateral cryptorchidism in mice (Nef and Parada 1999; Zimmermann et al., 1999). The leucine-rich repeat-containing G protein-coupled receptor 8 gene (*LGR8*) encodes the only receptor for the INSL3 peptide (Bogatcheva et al., 2003). This gene is highly expressed in the gubernaculum and is responsible for mediating hormonal signals that affect testicular descent (Gorlov et al., 2002). Mice lacking this gene exhibit bilateral intra-abdominal cryptorchidism (Tomiyama et al., 2003; Bogatcheva et al., 2007). The homeobox A10 (*HOXA10*) gene has been found highly expressed in the male gubernaculum and probably is essential for the gubernacular migration during testicular descent (Nightingale et al., 2008). Disruption of the gene (Overbeek et al., 2001) has been implicated in development of cryptorchidism in mice. The mutated gonadotropin-releasing hormone receptor (*GNRHR*) gene has initiated undescended testes in male mice as well (Pask et al., 2005). Estrogen receptor 1 (*ESR1*) and estrogen receptor 2 (*ESR2*), two ligand-activated transcription factors recognized as “candidate” genes of reproductive traits, have also been found to be important in the process of testicular descent (Donaldson et al. 1996; Przewratil et al., 2004). *ESR1* knockout male mice had retracted gonads which suggested that estrogens played a direct role

in the scrotal positioning of the testis (Donaldson et al., 1996). Moreover, the180A>G variant in another HOX gene, *HOXD13*, has been identified to be significantly different in healthy control individuals and those exhibiting cryptorchidism in humans (Wang et al., 2007). Androgen receptor (*AR*) was found to be strongly expressed in the gubernaculums (George and Peterson, 1988). The presence of androgens and AR together with INSL3 are believed to be important in normal transabdominal testicular descent (Emmen et al., 2000). The GGN length variant in the *AR* gene was associated with the risk of cryptorchidism in an Iranian population (Radpour et al., 2007). Moreover, the calcitonin gene-related peptide includes CGRP-alpha (CALCA) and CGRP-beta (CALCB). Exogenous CGRP injection into the scrotum can change the direction of gubernacular migration in the mutant trans-scrotal (TS) rat (Griffiths et al 1993; Clarnette and Hutson 1999). However, one mutation association analysis in humans suggested that *CGRP* gene was not a major factor for cryptorchidism (Zuccarello et al., 2004). Furthermore, several genes in the collagen metabolism process, e.g. collagen type I α 1 (*COL1A1*), collagen type II α 1 (*COL2A1*), collagen type IX α 1 (*COL9A1*) and metalloproteinase inhibitor 2 (*TIMP2*) are important candidate genes in the inguinal/scrotal hernia studies (Rosch et al., 2002; Grindflek et al., 2006; Abci et al., 2005). The adjustment of collagen contents has been found to be involved in the gubernaculum change during testicular descent or testis development (Heyns et al., 1989).

Cryptorchidism, as a complex defect, is influenced by genetic effects as well as epigenetic and environmental components (Spencer et al. 1991; Klonisch et al., 2004; Thonneau et al., 2003; Hutson and Hasthorpe 2005). It has been proposed that environmental factors having estrogenic or anti-androgenic effects have a role in the happening of male reproductive health including cryptorchidism (Sharpe and Skakkebaek, 1993; Toppari et al.,

1996). Both the animals and humans studies have supported this fact (Norgil et al., 2002; Fisher, 2004, Gill et al., 1979). However, further studies to elucidate the final role of environmental factors in the etiology of cryptorchidism remains to be done.

3. Inguinal/scrotal hernia

The inguinal/scrotal hernia, described as the bulging of abdominal contents in the inguinal canal or in the scrotum is one of the most common congenital and developmental undesirable defects in humans and animals (Fig. 3). Inguinal/scrotal hernias are further divided into the more common indirect hernia, in which the inguinal canal is entered via the internal inguinal ring, and the direct hernia, where the hernia contents invade through a weak spot in the back wall of the inguinal canal. The formation of an inguinal/scrotal hernia is believed to be caused by the failure of the embryonic closure of the internal inguinal ring, the incomplete obliteration of the processes vaginalis (Hutson et al., 1997; Tomiyama et al., 2005), the weak triangle of the inguinal region caused by the alternated collagen subtype proportions (Friedman et al., 1993; Kral and Levine, 1995), an abnormality in the smooth muscle differentiation in the wall of the hernial sac (Tanyel et al., 1999; Tanyel et al., 2002) or from increased intra-abdominal pressure (Stoppa et al., 1987). The mode of genetic inheritance is still unclear, but hernias are likely sex-limited autosomal traits with partial penetrance (Sawaguchi et al., 1975).

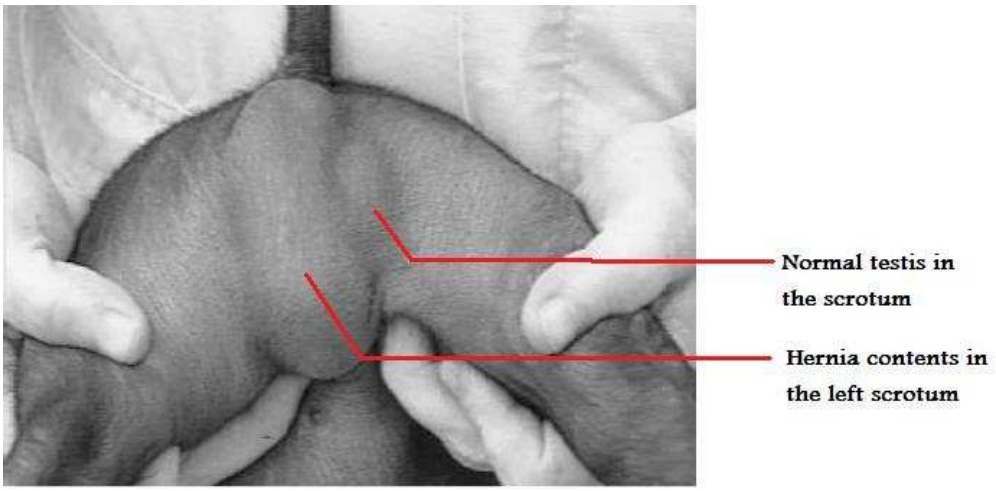

Figure 3. Scrotal hernia in a male pig.

The left side of the scrotum is much larger than the right one because hernia contents are in the left side of the scrotum and the right side of the scrotum is normal with a testis inside. The photo was modified based on the published paper: See et al., 2001. PIH-97.

In order to discover the true genetic model and some susceptibility genes causing an inguinal/scrotal hernia, several groups have devoted considerable efforts in studying this defect by using the pig model. Geneticists at the Pig Improve Company (PIC) have used a classical BLUP EBV system to select against scrotal hernia in purebred lines. Two markers in two different genomic regions were discovered by using candidate gene approach (over 40 markers were investigated). A genome scan approach also showed strong associations with scrotal hernia in one of the pure lines (Plastow et al., 2003). The research group from the Monsanto Company (2004) performed a whole-genome scan (microsatellite markers) using independent scrotal hernia-affected paternal families from three commercial pig lines. QTL on SSC2 and SSC12 were identified and were confirmed to be associated with scrotal hernia in pigs (Du et al., 2004). Moreover, Grindflek et al. (2006) detected 9 significant QTL ($P <$

0.01) on 8 porcine chromosomes in a population of 194 individuals including 103 affected sib pairs (ASP) through a genome-wide linkage analysis. The most promising QTL is on the pig chromosome SSC1, SSC2, SSC5, SSC6, SSC15, SSC17 and SSCX. It is worthy to note that several promising candidate genes, including *INSL3, MIS,* and *CGRP*, are located within the highly significant QTL regions. Knorr et al.(2006) also performed a genome-wide scan based on affected porcine half-sib families. Significant QTL on chromosomes SSC3, SSC6, SSC7, SSC12 and SSC15 were mapped by non-parametric linkage analysis (Knorr et al., 2006).

A number of candidate genes were selected in the previous inguinal/scrotal hernia studies including mullerian inhibitory substance (*MIS*) (U.S. patent 20040126795), insulin-like 3 (*INSL3*) (Knorr et al., 2004), beta-glucuronidase gene (*GUSB*) (Beck et al., 2006), *HOXA10* and *CALCA* (Knorr et al., 2001), and the SRY-box 9 (*SOX9*) gene (Ding 2006). Over expressed INSL3 made female mice display bilateral inguinal hernia as well as the descent of the ovary into the scrotal position based on the disruption of the proper development of the muscular and connective tissue structures of the abdomen (Zimmermann 1999; Adham, et al., 2002; Koskimies, 2003). Several genes might be good candidate genes such as estrogen receptor α (*ESR1*), zinc finger protein, multitype 2 (*ZFPM2*), G-protein coupled receptor affecting testicular descent (*GREAT*) and gonadotropin-releasing hormone receptor (*GNRHR*), as they are potentially involved in the incomplete obliteration of the processes vaginalis by aberrant sex hormone signaling pathways which usually control testicular development and the descent process or diaphragmatic hernia (Tanyel, 2004b; Clugston et al., 2008). The *ESR1* gene is located on SSC1 (60cM) where one QTL region exists for inguinal hernias in Grindflek's study. Estrogen treatment has profound effects on

the development of the internal genitalia of a male marsupial, preventing inguinal closure and interfering with testicular descent (Coveney et al., 2002).

Another important characteristic of inguinal/scrotal hernias found in human studies is the weak triangle of the inguinal region caused by the alterted collagen subtype proportions, which can profoundly change the tissue elasticity and resistance to tensile stress (Bendavid, 2004; Junge et al., 2004; Taniguchi et al., 2006). The decrease of type I collagen and/or the increase in type III collagen have been found in recurrent human hernia patients, which suggests that the relative composition of collagen types determines the elastic properties of tissue (Junge et al., 2004). Furthermore, the *MMP2*, *HOXA10* and elastin (*ELN*) genes have been found to be related to the collagen metabolism process in humans (Bellón et al., 2001; Kolon et al., 1999; Bertini et al., 2004; Rodrigues et al., 2006) and *COL2A1* is in the QTL region on SSC5 (90cM) associated with inguinal hernias in pigs (Grindflek et al., 2006). Therefore, genes including collagen type I α 1 (*COL1A1*), collagen type I α 2 (*COL1A2*), *COL2A1*, *MMP2*, *HOXA10* and *ELN* could also be good candidate genes for the inguinal/scrotal hernia study.

4. Common factors for the development of cryptorchidism and inguinal/scrotal hernia

Cryptorchidism is an abnormal condition associated with testicular descent. It is the case of one or two testes not successfully going down to the scrotum. To complete the normal testicular descent process, several factors are involved including the swelling and outgrowth of the gubernaculum (genitoinguinal ligament), the regression of the gubernaculums and the cranial suspensory ligament, and the increase of intro-abdominal

pressure caused by the contraction of the abdominal muscles as well as the extension of the processus vaginalis. Moreover, hormonal regulations affected by INSL3, androgen, or CGRP also play a critical role in this process. The inguinal canal is the correct way for the testis passing through from the abdominal position to the final outside body position in the scrotum. In the case of an inguinal/scrotal hernia, part of the small intestine may pass through the internal inguinal ring and the inguinal canal and then it goes into the scrotum and may remain positioned next to the testicles (scrotal hernia) or it presents in the inguinal canal (inguinal hernia). The formation of an inguinal/scrotal hernia is believed to be caused by the failure of the embryonic closure of the internal inguinal ring, the incomplete obliteration of the processes vaginalis, the weak triangle of the inguinal region caused by the alternated collagen subtype proportions, an abnormality in the smooth muscle differentiation in the wall of the hernia sac or from increased intra-abdominal pressure. Therefore, by comparing the etiology of the two defects, cryptorchidism and inguinal/scrotal hernia, it is apparent that they share some of the same anatomic structures and risk factors. In table 1 are shown the common factors involved in the development of these two defects.

Table 1. Comparison of the common factors involved in the development of cryptorchidism and inguinal/ scrotal hernia

Factors	Genes involved	Cryptorchidism	Inguinal/Scrotal hernia
Gubernaculum	*INSL3* (Emmen et al., 2000) *CGRP*(Clarnette and Huston, 1999)	a. Failure of growth of the gubernaculums (Emmen et al., 2000) b. Change of the direction of the gubernaculums' migration (Clarnette and Huston, 1999)	-
Processus vaginalis	*Androgen* (Tanyel, 2004b) *CGRP* and *HGF* (Ting et al., 2005)	Premature decrease of androgen diminishing the amount of smooth muscle to cause early obliteration of processus vaginalis (Tanyel, 2004b)	Absence or inadequacy decrease of androgen-Incomplete obliteration (Tanyel, 2004b)
Intra-abdominal pressure	Myog, Tnnt2, Fst, Igf1, Igfbp5, Id2, and Msx1(Barthold et al., 2008)	Abdominal wall defects causing decreased intra-abdominal pressure (Kaplan et al., 1986)	High intro-abdominal pressure (Zhou et al., 2007)
Abdominal wall	Type I/III collagen (Friedman et al., 1993; Junge et al., 2004)	-	Increased type III collagen synthesis (Friedman et al., 1993) Decreased type I collagen synthesis (Junge et al., 2004)

5. Animal models and comparative genomics

5.1 The dog model

The domestic dog has been extensively used in research as a model organism because of its similarities to humans in many anatomic and physiological aspects, particularly in the cardiovascular, urogenital, nervous and musculoskeletal systems (Khanna et al., 2006). The similar manifestation of some diseases, similar genome content and genetic predisposition, similar treatment and clinical trials between the dog and human and an advantageous population structure in dogs can help to map human complex diseases in dog models. The well known complex diseases include cancers (Khanna et al., 2006), diabetes (Srinivasan and Ramarao, 2007), heart disease (Kohler et al., 1984), kidney disease (Aresu et al., 2008) or other diseases which occur in both humans and dogs. The dog genome is less divergent from the human compared to the mouse genome and is relatively small (2.4 Gb) but with almost the same number of genes as humans (Lindblad-Toh et al., 2005). Because of the long term domestication history, a total of only 15,000 SNPs has proven sufficient for genome-wide association in breeds. By knowing the patterns of limited LD across whole dog populations and extensive LD within breeds, a two-stage mapping steps including identification of the disease locus in a breed and then finding the narrowed genomic region using multiple breeds is a reasonable strategy (Karlsson and Lindblad-Toh, 2008).

Testicular descent is usually completed in 10 days after birth in normal dogs. However, cryptorchidism is one of the most common congenital developmental defects in purebred dogs. Its estimated incidence in dogs is in a range of 1-11% (Amann & Veeramachaneni 2007). The incidence is even higher in some families within more inbred

breeds. According to the Siberian Husky Health Foundation's (SHHF) 2005/2006 health survey, nearly 14% of Siberian Husky males were affected (http://siberianhuskyhealthfoundation.org/researchresults.asp). Major health concerns of cryptorchidism are reduced fertility or infertility and significantly increased risk of testicular malignancies which could be caused by the common etiologic factors shared with the development of cryptorchidism. (Pendergrass and Hayes 1975; Weissbach and Ibach 1975; Romagnoli 1991; Husmann, 2005). The AKC (American Kennel Club) has a rule that "a male which does not have two normal testicles normally located in the scrotum, may not compete at any show". Therefore, removing the affected dogs and carriers from the dog breeding program to decrease the incidence of the defect and avoid the development of testicular cancer will benefit the dog breeders. Moreover, considering the similarities of the development of the genital tract, testis descent, and tunic relationships between dogs and humans (Pendergrass and Hayes 1975), the outcome of a dog study may be valuable for further investigation of the causes of human cryptorchidism.

5.2 The pig model

Pigs, also called hogs or swine, are under the genus *Sus* within the family *Suidae*. Phylogeographic analysis reveals that pigs were domesticated a long time ago, as far as 9,000 years ago (Larson et al., 2005). In modern society, the pig industries play a critical role in maintaining and improving quality of human life by not only providing meat but also being a primary source of more than 20 drugs and pharmaceuticals including pig insulin (Clark et al., 2007).

Most importantly, organs of the pig such as kidney, heart and cardiovascular system, liver, lungs and respiratory system, and even pancreatic islets are very similar to the organs

on humans (Ibrahim et al., 2006). Therefore, there are considerable researches ongoing to genetically engineer pigs so they can be used in the xenoimmunobiology of pig-to-nonhuman primate transplantation (Ekser et al., 2008) and then used for human transplantation eventually.

There is a high level of structural and sequence similarity between the pig and human genomes (Rettenberger et al., 1995; Meyers et al., 2005; Murphy et al., 2005). The matching frequency of BAC (bacterial artificial chromosome) end sequences between the pig and human genomes is approximately three-fold greater than that observed between the mouse and human (Gregory et al., 2002; Humphray et al., 2007).

Pigs with inguinal and scrotal hernias (also named *hernia inguinalis* and *hernia scrotalis*) (Fig. 3) may require additional health considerations including labor and veterinary treatments, which will lead to reduced animal welfare and large economic losses for pig producers. Most pig producers combine *hernia inguinalis* and *hernia scrotalis* as scrotal hernia. Reports suggest that this combined defect exists in certain pig breeds with varying incidence (1.7%-6.7%), and the heritability estimates vary widely (0.12-0.86) (Vogt and Ellersieck, 1990; Thaller et al., 1996; Mikami and Fredeen, 1979). With the fast development and application of molecular genetic technology, animal breeders have been applying molecular genetic methodologies to assist in the elimination of genetic defects and improve performance in commercial herds by using marker assisted selection (MAS) in pig industry (Plastow et al., 2003). Because pigs and humans share considerable physiological and genomic similarities, the domestic pig can be a good system for modeling human disease, such as inguinal/scrotal hernia or crytorchidism.

5.3 Comparative genomics

Comparative genomics is the analysis and comparison of genomes from different species. It gives important information about how closely different species are related in evolutionary terms (Hardison, 2003). We can learn sequence similarity, gene location, the number of exons within genes, the amount of noncoding DNA and highly conserved regions by using the "BLAST" procedure at the National Center for Biotechnology Information web site on sequence in other model organisms with the species of interest. With the completion or near completion of genome projects for more than 30 species including humans, animals, bacteria, yeasts and plants, it is very convenient to study gene functions by using simple model organisms which offer a cost-effective way to do the research. For example, a series of gene functional studies conducted in the mouse can successfully supply valuable information for the researching of human diseases by taking advantage of the genetic similarity between human and mice (Yan et al., 2004; Tybulewicz and Fisher, 2006; Girard et al., 2009). Domestic animals such as pigs, dogs, chicken and cattle are appropriate models for studying human complex diseases. There are several reasons. First, less genetic heterogeneity within breeds of domestic animals exists compared with large outbred human populations. Second, large family sizes in domestic animals often make it possible to deduce the QTL genotype of the parents with confidence by using progeny testing (Andersson and Georges, 2004). The completion of draft genome sequences for key domestic animals such as dogs, chickens and cattle and the nearly finished pig genome sequence, will offer us further understanding of complex traits in humans.

6. Candidate gene method and genome wide association approach

To discover the susceptibility gene(s) for a disease or trait, the candidate gene method has been extensively used. This method is performed to determine whether genetic variants

in the candidate genes are associated with a disease or trait. For some diseases, it is abviously that the biochemical structures or pathways that are likely sites of causative mutations. In such cases, a direct search for mutations at the DNA sequence level in suspected candidate genes can be an effective strategy. The candidate gene method is good at detecting genes underlying common and more complex diseases where each candidate gene contributes a relatively small effect (Kwon and Goate, 2000). The major difficulty with this approach is to choose suitable potential candidate genes. There are several ways to select candidate genes. The genes implicated in the biological pathway or containing variants capable of influencing the regulation of genes in the development of a disease or trait are the first choices. Another important way is to select candidate genes (positional candidate genes) from regions where quantitative trait loci (QTL) that have been detected from genome-linkage scans. Previous association data can also provide some good candidate genes (Hattersley and McCarcy, 2005). By using the candidate gene method, many genes have been identified in the previous studies, such as *APOE* for the Alzheimer's disease (Corder et al., 1993), *PPARG, KCNJ11* for the type 2 diabetes (Altshuler et al., 2000; Gloyn et al., 2003), *MC1R* for red/black color (Kijas et al, 2001) and *ESR1* for litter size (Short et al., 1997).

However, this method sometimes has limited power to detect all the true susceptibility genes or exclude the involvement of a gene. It also often happens that there is a failure to replicate findings in various studies conducted on different data sets. Small sample sets, limited genotyping quality, different analytical methods, and populations with distinctive environments may explain the limitations of some candidate gene association studies. To successfully uncover causasive genetic components involved in a given disease or trait, a genome-wide association approach can be a complementary method to detect more

susceptibility genes. This approach uses a large number of genetic variants, such as single nucleotide polymorphisms (SNPs) which are spread throughout the genome to uncover the genetic loci by using the case and control data sets. The completion of the genome sequence projects for many species and the commercial SNP-chip products facilitate the application of this approach. By using a genome-wide association (GWA) study, many susceptibility genes found in previous researches have been validated, including *KCNJ11* (Tabara et al., 2008), and *MC1R* (Han et al., 2008).

The candidate gene method for association studies is an efficient approach to detect genetic loci for the given disease or trait. At the same time, combining the application of the GWA method will improve the power to detect the true susceptibility gene locus (loci).

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CHAPTER 2. Association of *COL2A1* with Cryptorchidism in Siberian Husky Dogs

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ABSTRACT

Cryptorchidism (retained testicles) is one of the most common developmental defects in purebred dogs. It creates health concerns of reduced fertility and increases risk of testicular malignancies. The incidence is estimated to be as high as 14% in Siberian Husky. A total of 51 single nucleotide polymorphisms (SNPs) discovered from 22 candidate genes was investigated to analyze their associations with cryptorchidism. The Sibling-Transmission

Disequilibrium Test (sib-TDT) on 38 discordant full sibs revealed six SNPs in the collagen type II α 1 (*COL2A1*) gene were significantly associated with cryptorchidism ($P<0.05$), and another one was suggestively associated ($P<0.10$). Further analyses on 156 Siberian Huskies showed that only the SNP rs23389020 remained suggestively significant ($P\leq0.06$), but not significant after permutation tests (n=1000) to account for multiple testing. Neither of the two haplotypes on *COL2A1* gene was significantly associated with cryptorchidism. Based on the statistical results and the involvement of *COL2A1* in the testicle development and descent, we can only propose that *COL2A1* may be a potential gene for cryptorchidism in Siberian Huskies. Further association studies with more dog samples or other dog breeds for this gene are necessitated to validate this result.

Keywords: **Cryptorchidism, candidate gene, *COL2A1*, sib-TDT**

INTRODUCTION

Cryptorchidism (CO) refers to the failure of one or both testes to descend into the scrotum. It is one of the most common congenital defects in purebred dogs. The estimated incidence in dogs ranges from 1-11% (Amann and Veeramachaneni 2007) and is even higher within certain highly inbred breeds. According to the Siberian Husky Health Foundation's (SHHF) 2005/2006 health survey, nearly 14% of Siberian Husky males were affected (http://siberianhuskyhealthfoundation.org/researchresults.asp). Major health concerns of CO are reduced fertility or infertility, and significantly increased risk of testicular malignancies (Pendergrass and Hayes 1975; Weissbach and Ibach 1975; Romagnoli 1991). To avoid the risk of developing testicular cancer, the removal of retained testicles in affected dogs is often required.

Cryptorchidism is a sex-limited trait, and its exact pattern of inheritance has been debated. In the early years, some researchers have proposed that CO was caused by recessive genes at one locus in pigs (Huston et al. 1978; Johnston et al. 1958). Later more evidence found in humans (Czeizel et al. 1981), pigs (Rothschild et al. 1988) and dogs (Cox et al. 1978, Nielen et al. 2001) suggested that cryptorchidism was controlled by multiple genes. Most recently, researchers believed that it was a complex trait caused not only by genetic component but also by epigenetic and environmental factors (Klonisch et al. 2004; Thonneau et al. 2003; Hutson and Hasthorpe 2005). Aberrant genetic pathways affecting the swelling and outgrowth of the gubernaculum, the regression of the cranial suspensory ligament, and the elevation of intro-abdominal pressure caused by contractions from the abdominal muscles, will create developmental problems of the male reproductive organs which include CO.

Various genes potentially vital to the development of CO in these genetic pathways have been investigated. A series of evidence from the animal models showed genes including the müllerian inhibiting substance (*MIS*), insulin-like factor 3 (*INSL3*), the leucine-rich repeat-containing G protein-coupled receptor 8 (*LGR8*), homeobox A10 (*HOXA10*), gonadotropin-releasing hormone receptor (*GNRHR*) and the estrogen receptor (*ESR*) (Cederroth et al. 2007) could be potential candidates. *MIS* is critical for the formation of the mullerian duct during male sexual differentiation. Deletion of this gene combined with the testicular feminization (Tfm) mutation has been suggested to cause undescended testis in mice (Behringer et al. 1994). Targeted disruption of genes encoding *INSL3* (Nef and Parada 1999), *LGR8* (Tomiyama et al. 2003) and *HOXA10* (Satokata et al. 1995) have been implicated in the development of cryptorchidism in mice. A mutation in *GNRHR* initiated undescended testes in male mice as well (Pask et al. 2005). Moreover, an *ESR1* disrupted gene mutation in mice has been found to affect the development of the gubernaculum which is one of the key structures in the process of normal testicular descent (Donaldson et al. 1996). Besides the animal models, quite a few previous clinical studies also revealed several genes might be potential candidate genes, and excluded some other genes for the risk of CO. One genetic variant (180A>G) in another HOX gene, *HOXD13*, has been found to be significantly different in healthy control individuals and those exhibiting cryptorchidism in humans (Wang et al. 2007). The GGN length variant in the androgen receptor (*AR*) gene has been found to be associated with the risk of cryptorchidism in Iranian population (Radpour et al. 2007). In addition, the Gly146Ala polymorphism in steroidogenic factor-1(*SF-1*), also named nuclear receptor subfamily 5, group A, member 1(*NR5A1*) has been found significantly different between human affected patients and controls (Wada et al. 2006). Nevertheless, the

calcitonin gene-related peptide (*CGRP*) gene has been suggested as only a minor factor for the sporadic cryptorchidism based on a mutation association analysis in humans even though it acts as a neurotransmitter to control the direction of gubernacular migration (Zuccarello et al. 2004; Clarnette and Hutson 1999).

Several genes involved in the collagen metabolism process, e.g. collagen type I α 1 (*COL1A1*), collagen type II α 1 (*COL2A1*), collagen type IX α 1 (*COL9A1*) and metalloproteinase inhibitor 2 (*TIMP2*) were discovered as important candidate genes in the previous inguinal/scrotal hernia studies (Rosch et al. 2002; Grindflek et al. 2006; Abci et al. 2005). The adjustment of collagen contents have been found to be involved in the gubernaculum changes during testicular descent or testis development (Heyns et al. 1989), but no genetic studies have been conducted for determining the role of these collagen genes with cryptorchidism.

Since the genetic components of cryptorchidism in dogs still remain unclear, we decided to perform the current study. Our goal was to investigate the relationships between genetic variants in potential candidate genes and cryptorchidism in Siberian Huskies. In total, 22 candidate genes were selected including 13 biological functional candidates and 9 positional candidates chosen by using a comparative genomics approach from our cryptorchidism study in pigs (unpublished data) (Table 1). We hypothesized that the associated genetic variants identified in this study can help to pinpoint the real causative loci, which will be used to design a diagnostic test and then facilitate the removal of affected individuals and carriers from the dog breeding program and decrease the incidence of the defect. Moreover, considering the similarities of the development of the genital tract,

testicular descent, and tunic relationships between dogs and humans (Pendergrass and Hayes 1975), the outcome from our study may be valuable for human CO investigations.

MATERIALS AND METHODS

Animals

All dogs were sampled by their owners under conditions approved by the Iowa State University Animal Care Committee. In total, 156 Siberian husky dogs were included in this study. Males (n = 118) were diagnosed as CO affected (n = 56) (including unilateral and bilateral) or not by palpation performed by veterinarians or owners at 6 months of age. There are 15 unrelated dogs, while the other 141 dogs came from 47 nuclear families. Among the dog nuclear families, 16 out of 47 had discordant CO affected full sibs (n=38), 8 had concordant ones and 23 had only one offspring. Twenty-five families had both parents and the remaining had only one parent included.

Buccal samples were collected by owners with sterile cytology brushes (Fisher Scientific, Hampton, NH, USA). DNA was extracted using the QIAamp DNA Mini Kit (QIAGEN, Valencia, CA, USA) from two cytology brushes for each of these 156 dogs and the DNA concentration was adjusted to 12.5 ng/μl.

SNP Discovery

The candidate gene symbols, full names and their chromosome locations as well as the SNP information are listed in Table 1 and Supplemental Table 1. Primer sets were designed using Primer 3 (Rozen and Skaletsky 2000) for each candidate gene to preferentially amplify SNPs in exons, promoters and splicing sites. The whole genomic sequences, including 10 kbp upstream and downstream of *COL2A1* from six species (human,

chimpanzee, rat, mouse, cow, and cat), were retrieved and aligned using UCSC genome browser (Kent et al. 2002) to detect the most conserved sequences across multi-species (Fig 1. upper panel). More primers were designed to amplify some of these conserved sequences and try to find more genetic variants in *COL2A1* (Supplemental Table 2).

A panel of DNA pool of four individuals with or without cryptorchidism was used for SNP discovery. A 10 μl PCR system was employed which included 1x GoTaq reaction buffer containing 1.5 mM Mg^{++} (Promega, Madison, WI, USA), 0.125 mM of each dNTPs, 2.5 pM of each primer, 12.5 ng of genomic DNA, and 0.25 U of GoTaq DNA polymerase (Promega, Madison, WI, USA). The PCR program was 35 cycles of 30 s at 94°, 30 s at the primer-specific annealing temperature and 30 s at 72°C with additional 3 min denaturation in the first cycle and 5 min extension in the last cycle. PCR products were verified by 1.5% agarose gel electrophoresis and treated with ExoSAP-IT® (USB, Cleveland, OH, USA), and sequenced commercially by using a 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA, USA). The sequences were aligned and compared using Sequencher software version 3.0 (Gene Codes Ann Arbor, MI USA). Potential SNPs were confirmed by restriction fragment length polymorphism (PCR-RFLP) tests using suitable restriction endonucleases (New England Biolabs, Beverly, MA, USA) based on the presence or absence of a restriction site at the polymorphic sites in the PCR-amplified DNA fragments (Supplemental Table 1 and Supplemental Table 2).

Genotyping Assay

SNPs distributed in 22 candidate genes were first genotyped on the 38 discordant full sibs by using PCR-RFLP test or direct sequencing and were analyzed to get preliminary

results. Then additional genotyping was conducted in the remaining dogs for potential SNPs associated with cryptorchidism found in the preliminary results.

Statistical Analysis

A DFAM procedure in the PLINK v1.05 program (Purcell et al. 2007) was undertaken. This method is a family-based association analysis for disease traits which implements the sib-TDT (Sibling-Transmission Disequilibrium Test) and also allows for unrelated individuals to be included. A preliminary statistical analysis was first performed on the genotyping results from the 38 discordant full sib dogs for all SNPs discovered. A "nonfounders"option in the DFAM procedure was used because of a sibling-only sample applied in this analysis. No multiple testing corrections were conducted in the preliminary results since we did not want to miss any potential candidate genes at this level. More efforts were made for the *COL2A1* gene after the outcome of the preliminary analyses. Furthermore, the DFAM procedure was then carried out for the 8 SNPs in *COL2A1* with the genotyping results from all Siberian Huskies (n=156). To correct the multiple testing problem, empirical p-value 1 (EMP1, pointwise) produced from the max (T) permutation procedure in PLINK program was applied.

The pairwise linkage disequilibrium (LD) status for the 8 SNPs within *COL2A1* was estimated. Haplotype analysis using TDT was performed in Haploview 4.0 (Barrett et al. 2005) to identify whether specific haplotypes in *COL2A1* were associated with CO based on the genotyping results of all 156 dogs.

RESULTS

SNP discovery and genotyping

By using DNA pools to do PCR and sequencing, 76 SNPs in total were discovered from 22 candidate genes, while 31 out of 76 SNPs were identified in the second round within the *COL2A1* gene (Supplemental table 1 and Supplemental table 2). About one third of the SNPs have previously been published in the NCBI SNPs database. Fifty one SNPs distributed in the 22 candidate genes including 8 SNPs covering the most parts of the whole gene sequence of *COL2A1* were genotyped on 38 discordant full sibling Siberian Huskies. Additional genotyping was done on the remaining 118 dogs for the 8 SNPs within *COL2A1* (Table 3).

Association analyses

The result of the preliminary DFAM test on 38 full sibling dogs shows that 7 out of 8 SNPs in *COL2A1* were significantly ($P<0.05$) or suggestively significantly ($0.05<P<0.10$) associated with CO (Table 2). The synonymous SNP C2A1-I2 (196C>T) in exon 2 yielded a P-value of 0.06. Another synonymous SNP C2A1-I20-2 (rs23335235 in dbSNP, 368A>G) located in exon 20 and four intronic SNPs including C2A1-I4-6-1 (rs23389020, 219A>G) in intron 5, C2A1-I12 (rs23358342, 157A>G) in intron 12, C2A1-E39-3 (rs23382149, 425C>T) in intron 39 and C2A1-E52-1 (rs23379923, 142A>G) in intron 51 were significantly associated with cryptorchidism ($P<0.05$). Additionally, the SNP C2A1-3U in the 3'UTR region of COL2A1 was also significantly associated with CO ($P<0.05$). Besides the *COL2A1* gene, one intronic SNP C1A1-I23 (241A>C) in *COL1A1* gene also showed significance in this data set ($P\leq 0.05$) (Table 2).

Based on the preliminary results of full-sib analyses, the *COL2A1* gene was likely associated with CO. All eight SNPs within *COL2A1* were then analyzed on all dog samples (n=156) using the DFAM procedure (Table 3). Only SNP C2A1-I4-6-1 (rs23389020,

219A>G) was suggestively significantly associated with crytorchidism (0.05<P<0.10). However, after the permutation test (n=1000), the corrected empirical P-values for this SNP turned to be 0.27 (Table 3).

Pairwise LDs were estimated for the 8 SNPs within *COL2A1*. Two LD blocks constructed by 5 SNPs were observed (Figure 1. Middle and lower panels; Table 4). However, neither of the haplotypes were significantly associated with cryptorchidism (P>0.05, Table 4).

DISCUSSION

This is the first time that an association study for canine cyptorchidism has been conducted in the Siberian Husky breed. Our statistical analyses showed that *COL2A1* might be a potential gene associated with CO. Previous functional studies for *COL2A1* indicated that it was involved in the normal testicle development and descent. *COL2A1* encodes two isoforms, the long and short forms. Collagen type IIA as the long form is expressed in noncartilage tissue; collagen type IIB is the short form and is found in mature cartilage (Ryan and Sandell 1990; Sandell et al. 1994). Nonchondrocytic type II collagen have been found to play specific roles for development of testis cords via interaction with members of the transforming growth factor beta superfamily of signaling molecules in mice (McClive and Sinclair 2003). *COL1A1* encodes type I collagen and the ratio of type I and type III collagens is an important parameter for hernia occurrence (Junge et al. 2004). In our results, one SNP within *COL1A1* was also found to be significantly associated with CO in the preliminary statistical analyses. However, it is far from addressing a conclusion for this gene to be associated or not. More SNPs and dog samples are needed for further analysis. Collagens are

important components in several anatomic structures involved in the testicular descent, such as the internal inguinal ring and gubernaculums. The outgrowth and subsequent regression of the gubernaculums is fundamental to the process of testicular descent (Baumans et al. 1981). Collagen could accumulate in the gubernaculums during and after the period of testicular descent even though an increase in the collagen content was not verified to be a cause of testicular descent (Heyns et al. 1989). Therefore, the aberrant ratio of subtypes or quantity of collagen in particular tissues related to the testicular descent could be a critical reason for the development of CO.

The DFAM as a family based analysis method was applied for the single maker association in our study. A large scale recruitment of canine cohorts is very difficult since the Siberian Huskies used in current study came from individual dog owners which constrained us from collecting the whole dog families in a limited time. Some dogs had samples from both parents, but some individual samples were collected without any parent's information. The DFAM procedure was considered the best choice for analyzing our current data set because it implements a sib-TDT test and can be used to combine discordant sibship data, parent-offspring trio data and unrelated case/control data into a single analysis. No information was lost from our data set. The TDT test proposed by Spielman et al. (1993) has been extensively used which measures the over-transmission of an allele from the parents to affected offspring, to test for the presence of an association between a genetic marker and a trait. The use of within family controls in the TDT offers a solution to the population stratification problem which often arises in the case-control analysis. The sib-TDT compares the marker genotypes in affected and unaffected offspring instead of using marker data from affected offspring and their parents (Spielman and Ewens 1998). Therefore, the sib-TDT in

the DFAM procedure should be a suitable and accurate analysis method when only incomplete data is available.

In this study, the genotypes of discordant full sibs with similar genetic and environmental background were first used to do the preliminary association analyses, and further statistical analyses were then performed for these associated SNPs by using more dogs. This strategy helps to check many SNPs distributed in the candidate genes at limited cost. However, since the number of full sib pairs is relatively small, it does not have a strong power to detect all the associated SNPs in our candidate genes in the current study. Findings from laboratory animal models and clinic studies suggested that *MIS, INSL3, LGR8, HOXA10, GNRHR, ESR1, HOXD13* and *NR5A1* are all potential candidate genes for the risk for CO. However, the variants found in these genes were not associated with CO in our preliminary results, and no variant was found in *HOXD13*. Besides the reasons explained above, the inconsistency between the previous studies and ours may be due to the severity in phenotype. More serious symptoms occur in the mice deficient in these genes when compared to our affected dogs, such as twisted ductus deferens, small testes and scrotal sacs or abnormal inguinal canals (Wang et al. 2007). Adding more discordant full sib pairs in the preliminary test will increase the power for gene marker detection. Moreover, the reason that the candidate genes chosen did not show highly significant genetic contributions to cryptorchidism susceptibility may also be due to the modulation by environmental risk factors.

In the future, additional studies are needed to verify our results by increasing the number of Siberian Huskies. Dogs from other breeds are also needed to examine the association analyses of these interesting SNP markers. Environmental factors, their complex

interaction and their effects on testicular development and descent should also be studied. To comprehensively understand the genetic variant effects, a systemic genomic study using a canine SNP chip covering the whole dog genome may help reach this goal.

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Table 1. Summary of gene symbols, located chromosomes and names for candidate genes

Gene symbol	(CFA)[a] Dog chromosome	Gene name
ESR1	CFA1	Estrogen receptor 1
MMP2	CFA2	Matrix metallopeptidase 2
ESR2	CFA8	Estrogen receptor 2
TIMP2	CFA9	Metallopeptidase inhibitor 2
GPRC5C	CFA9	G protein-coupled receptor, family C, group 5, member C
SYNGR2	CFA9	Synaptogyrin 2
COL1A1	CFA9	COL1A1 collagen, type I, alpha 1
NR5A1	CFA9	Nuclear receptor subfamily 5, group A, member 1
COL9A1	CFA12	COL9A1 collagen, type IX, alpha 1
GNRHR	CFA13	Gonadotropin-releasing hormone receptor
HOXA10	CFA14	Homeobox A10
HSD17B12	CFA18	Hydroxysteroid (17-beta) dehydrogenase 12
PDHX	CFA18	Pyruvate dehydrogenase complex, component X
APIP	CFA18	APAF1 interacting protein
ELF5	CFA18	E74-like factor 5 (ets domain transcription factor)
ACTN3	CFA18	Actinin, alpha 3
INSL3	CFA20	Insulin-like factor 3
SERPINH1	CFA21	Serpin peptidase inhibitor, clade H (heat shock protein 47), member 1
CALCA	CFA21	Calcitonin/calcitonin-related polypeptide, alpha
COL2A1	CFA27	Collagen, type II, alpha 1
INHA	CFA37	Inhibin, alpha
TIMP1	CFAX	Metalloproteinase inhibitor 1

[a] CFA, *Canis familiaris* chromosome.

Table 2. Results of association analyses for 51 SNPs with 38 Siberian Huskies

Gene symbol	SNP	DFAM * P-value
COL2A1	C2A1-I4-6-1	0.01
	C2A1-I12	0.02
	C2A1-E39-3	0.02
	C2A1-I20-2	0.02
	C2A1-E52-1	0.02
	C2A1-3U	0.03
	C2A1-I2	0.06
	C2A1-E29-31-1	0.36
COL1A1	C1A1-I23	0.05
ESR2	ESR2-I2	0.15
HOXA10	HOXA10-I3-4-2	0.12
	HOXA10-I3-4-4	0.15
	HOXA10-I3-4-3	0.19
	HOXA10-I3-4-1	0.24
	HOXA10-E3	0.69
ESR1	ESR1-I7-1	0.36
	ESR1-I7-2	0.64
	ESR1-I2-1	0.39
INSL3	INSL3-5U-3	0.21
	INSL3-E1b-4	0.31
	INSL3-E1b-5	0.31
	INSL3-E1b-1	0.40
	INSL3-E1b-2	0.40
	INSL3-E1b-3	0.40
	INSL3-E1b-6	0.64
	INSL3-E2-1	0.81
	INSL3-E2-2	1.00
	INSL3-3U	0.89
ELF5	ELF5-E4-1	0.36
	ELF5-E4-3	0.36
	ELF5-E4-2	1.00
GNRHR	GNRHR-I1	0.36
SYNGR2	SY-I4	0.44
NR5A1	NR-I4	0.44
ACTN3	ACTN-I9-2	0.48
TIMP2	TIMP2-5U	0.49
HSD17B12	HSD17-I1	0.49
	HSD17-I4	0.82
GPRC5C	GPRC5C-I4-1	0.64
COL9A1	COL9A1-5U-2	0.66
	COL9A1-I38	1.00
PDHX	PDHX-E1-1	0.77
MMP2	MMP2-5U-1	1.00
	MMP2-5U-2	0.79
SERPINH1	SER-I4-2	0.81
CALCA	CALCA-5U-1	0.81
APIP	APIP-I5-1	0.82
	APIP-I1-3	1.00
	APIP-I4	1.00
INHA	INHA-E2-1	1.00
TIMP1	TIMP1-I1	1.00

* DFAM: a family-based association for disease traits in the Plink program which implements the sib-TDT test and also allows for unrelated individuals to be included. There were 16 Siberian husky full sib groups including 38 dogs (some of the full sibs are triples or quadruples).

Table 3. Results of the family based association analyses (DFAM) and permutation tests for 8 SNPs within COL2A1

SNP	156 Siberian Huskies	
	DFAM P-value	Max(T) permutation(1000) EMP1 *
C2A1-I4-6-1	0.06	0.27
C2A1-I12	0.14	0.33
C2A1-E39	0.37	0.59
C2A1-I20-2	0.60	0.72
C2A1-E52	0.62	0.69
C2A1-3U	0.76	0.86
C2A1-I2	0.82	1.00
C2A1-E29-31	0.84	1.00

* EMP1, corrected empirical P-value (pointwise) applying Max (T) permutation test procedure in the PLINK program.

Table 4. Results of haplotype analyses for the genetic associations between *COL2A1* and cryptorchidism

Haplotype Block	Haplotype	Frequency	T:U*	Chi Square (TDT)	P-value
C2A1-I12+ C2A1-I20-2	AA	0.38	12.0 : 15.0	0.33	0.56
	GG	0.33	8.0 : 8.0	0.00	1.00
	GA	0.29	11.0 : 8.0	0.47	0.49
C2A1-E39-3 + C2A1-E52-1+ C2A1-3U-4	TGG	0.39	14.0 : 13.0	0.04	0.85
	CAA	0.34	5.0 : 10.0	1.67	0.20
	CGG	0.27	10.0 : 6.0	1.00	0.32

* **T:U,** the ratio of the Transmitted and Untransmitted haplotypes in affected.

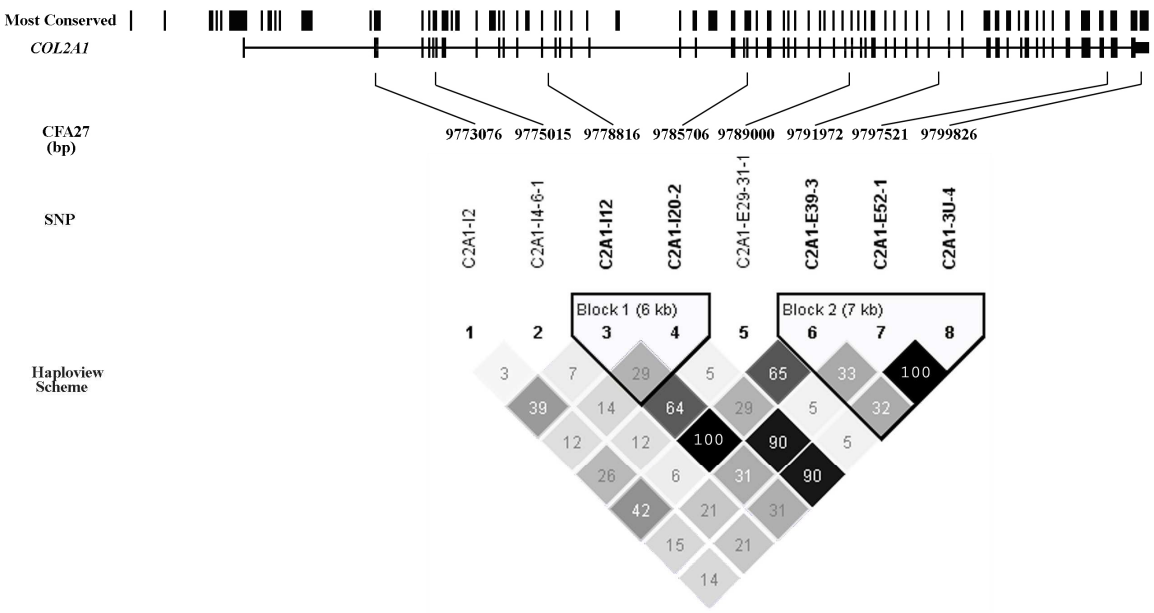

Figure 1. Distribution of genotyped SNPs and two haplotypes in *COL2A1*.

The most conserved regions of canine *COL2A1* with its partial genomic sequences upstream and downstream were found in the UCSC genome browser (upper panel). Eight single nucleotide polymorphisms (SNPs) within this gene were genotyped and analyzed. The chromosomal positions for each SNP are listed (middle panel). The linkage disequilibrium (LD) structure for these SNPs and two constructed haplotype blocks are shown (lower panel). The intensity of the shading of each numbered box indicates the LD relationship. The denser the shading color with larger number (100 x r^2), the higher the LD values are.

Supplemental Table 1. Primer sequence, annealing temperature, PCR amplicon size and 45 uncovered SNPs in 22 candidate genes.

Primer name	Primer sequence (5'-3')	T_A* (ºC)	Size (bp)	Contig accession	SNP name	SNP database ID	SNP†	SNP position	Endo-nuclease
ESR1-I2	TCCCCCAAGCCTCTTATTCT CCCTTCCTGTTGAGTTGCTC	60	549	NW_876269.1	ESR1-I2-1	rs21960513	271C>T	Exon 2	no
ESR1-I7	CACAAACGGAGGACTCACCT	58	386	NW_876269.1	ESR1-I7-1	no	105G>T	Intron 7	BtsCI
	TCACCACAGGTTGCCAATAA				ESR1-I7-2	no	141A>G	Intron 7	no
MMP2-5U	CCTCTACCATTTGCGAGCTG	56	321	NW_876292.1	MMP2-5U-1	rs22807868	161A>C	5'UTR	no
	TGCCCCTAGAGCAACTCTGT				MMP2-5U-2	no	291A>G	5'UTR	no
ESR2-I2	GGCATAGACTCCCGTAACCA TGAAACAATCCACTCCACCA	61	579	NW_876327.1	ESR2-I2	no	461C>T	Intron 2	no
TIMP2-5U	GACCTCTTCCCGTCTCCTTC TAACTGGGCCACAATCATCA	62	598	NC_006591	TIMP2-5U	no	321A>C	5'UTR	Fnu4HI
GPRC5C-I4	CAACCTGTGTGACCGTTCTG GGAAGTTGAGGGCAAAGACA	58	311	NC_006591.2	GPRC5C-I4-1	no	137A>G	Exon 4	HgaI
SY-I4	CCTTTCCACTTACCCGAACA CCACACCCAGTTCCTACCTG	58	375	NC_006591.2	SY-I4	rs22657440	119C>T	Intron 4	AciI
C1A1-I23	CCTTTGGGGCATCTAACCTT GCAAAGATGGAGAAGCAGGA	60	483	NW_876332.1	C1A1-I23	rs24560587	241A>C	Intron 23	no
NR5-I4	CTCCCCTCTCCTGCTCTCTT TGAGAGCAACCATCAAGCAC	62	464	NC_006591.2	NR5-I4	no	415C>T	Intron 4	no
C9A1-5U	GGCTCACTCCCTTTGCTTTA CTCACGTTTCCAGCTCCTTC	62	551	NW_876254.1	C9A1-5U-2	no	262C>T	5'UTR	BtsI
C9A1-I38	GTCTCCATCCATCCTTTCCA TTCGGTGGTGTTTCTCCTTC	60	398	NW_876254.1	C9A1-I38	no	85A>C	Intron 37	HpyCH4III
GNRHR-I1	GGCAGAAAGAGAGGCAGAGA CTCAAGTCAAGCTCAGGGAAA	58	410	NW_876327.1	GNRHR-I1	rs22272057	133C>T	Intron 1	BtsCI
HOXA10-I3-4	AAACGCTCTTGAGCTCTTGG AATGTGAAAGACGGCTGGAC	58	504	NW_876258.1	HOXA10-I3-4-1	no	75C>T	Intron 3	no
					HOXA10-I3-4-2	rs22319567	255A>G	Intron 3	AvaI
					HOXA10-I3-4-3	rs22340569	262A>G	Intron 3	BgIII
					HOXA10-I3-4-4	no	287C>T	Intron 3	no
HOXA10-E3	GGAGCCCTCTTTTCTGCTCT AGCGGGTTGGCTGAGTAGTA	65	494	NC_006596.2	HOXA10-E3-1	no	165A>G	Intron 2	StuI
HSD17-I1	CCATTCTGTGGAGTGGGTTT GCCTTCAACCCCTGATGTAA	58	345	NC_006600.2	HSD17-I1	rs22662289	59C>T	Intron 1	BsgI
HSD17-I4	GGAGGCCCAAGGTCAAGG CTGGGTTCAGGTATTGCAGAC	58	429	NC_006600.2	HSD17-I4	rs22643127	141C>G	Intron 4	ApaI
PDHX-E1	GAGGGCCTTGACGACTGG GAAGACGTCCCCATGGAA	48	618	NC_006600.2	PDHX-E1-1	no	158A>T	Exon 1	MsII
APIP-I5	AGGATTGTCATTGCTTCAAGG ATGTGACTTGTCGTCCCAAG	58	510	NW_876266.1	APIP-I5-1	no	412C>T	Exon 6	HhaI
APIP-I1	TTTGCCTCTTACAGCACCAA TGTCCTTGGGTAATGGTGGA	56	431	NW_876266.1	APIP-I1-3	rs9142978	215C>T	Intron 2	SspI
APIP-I4	AGGAGCAGGTGCAGTGATTC	56	506	NW_876266.1	APIP-I4	rs22658615	438A>G	Intron 5	BsmBI

Continued

Primer name	Primer sequence (5'-3')	T_A* (ºC)	Size (bp)	Contig accession	SNP name	SNP database ID	SNP†	SNP position	Endo-nuclease
	TCATGGATACCAAGTGAAGGTG								
ELF5-E4	GGGATGTGGATGCTGATAGAA	58	426	NW_876266.1	ELF5-E4-1	no	23C>G	Intron 4	no
	GTGGGTTGGTTGGTTTGTTG				ELF5-E4-2	rs22633264	41C>T	Intron 4	SfoI
					ELF5-E4-3	no	59A>G	Intron 4	no
ACTN3-I9	GAGCAGCCAGTCCTCATAGC	64	326	NW_876266.1	ACTN3-I9-2	no	138A>G	Intron 9	BtsCI
	GGAGGACTTTCGGGACTACC								
INSL3-5U	TAAGTGCCAGAAGGGGAAAA	58	419	NC_006602	INSL3-5U-3	no	273C>G	5'UTR	BanII
	GATGGGGATGGAACATTCTG								
INSL3-E1b	CAGAATGTTCCATCCCCATC	58	579	NC_006602	INSL3-E1b-1	no	68A>G	5'UTR	no
	AGCAATGCAAAAATTCACGA				INSL3-E1b-2	no	94A>G	Exon1	no
					INSL3-E1b-3	no	156A>G	Intron 1	no
					INSL3-E1b-4	no	333C>G	Intron 1	no
					INSL3-E1b-5	no	408A>G	Intron 1	no
					INSL3-E1b-6	no	549C>T	Intron 1	no
INSL3-E2	GCCCAAGATGAGTGTTTGGT	58	419	NC_006602	INSL3-E2-1	no	35A>G	Intron 1	BmgBI
	CTCCCAGATAGGGCTTGATG				INSL3-E2-2	no	394C>T	Exon 2	no
INSL3-3U	TGGAAGATCCTTGGTTTTGC	58	568	NC_006602	INSL3-3U	no	513C>T	3'UTR	no
	CCAGGTGGGTCTTGACAGAT								
SER-I4	TTTCCTGCAAGCCTCAGTTT	58	449	NC_006603.2	SER-I4-2	no	392C>T	Exon 5	EcoRV
	GGGGTGGTCAGCATAGAAGA								
CALCA-5U	CTAACTGCGCTCCAGCATC	62	426	NW_876273.1	CALCA-5U-1	no	84C>T	5'UTR	SnaBI
	CCCAAGCCTCCATTCATCT								
C2A1-I2	CCTCCTTCCCTTTGCTCTCT	60	424	NW_876284.1	C2A1-I2	no	197C>T	Exon 2	BsoBI
	CAATTTCAACCTGCAAGCAA								
C2A1-I4-6	AAGGAGAACCCGGAGACATC	60	535	NW_876284.1	C2A1-I4-6-1	rs23389020	219A>G	Intron 5	Cac8I
	GTCCCAGGCTCTCCATCTCT								
INHA-E2	GTCCTGGTGCTGCTGCTG	64	495	NC_006619.2	INHA-E2-1	no	258A>G	Exon2	TspRI
	GTTGGGCACCGTCTCATACT								
TIMP1-1	CTTAACCCCCTTCCAAGCTG	62	525	NC_006621	TIMP1-1	no	360C>T	Intron 2	TaqαI
	GGGGGTCAGGAATACTAGGG								

* Annealing temperature. † SNP position in the current amplified fragment.

Supplemental Table 2. New primer sequence, annealing temperature, PCR amplicon size and 31 SNPs information for the *COL2A1* gene

Primer name*	Primer sequence (5'-3')	T_A (°C)	Size (bp)	SNP name†	SNP position	SNP database ID	SNP	Endo-nuclease
C2A1-5U	GCGGGGTCTCAGGTTACAG GGCGACTTACGGACATCCT	60	434	no	-	-	-	-
C2A1-5U-3	TGCCCGTCTCCAAGATTTAG GGGGCTGTAACCTGAGACC	62.5	468	no	-	-	-	-
C2A1-I1	CCTGGTTTCTCACCTTTCCA CAAAGTCACCCCCAAACCTA	58	402	no	-	-	-	-
C2A1-I2-2	TGTGTGGAGGGAAGTGTTGA TAGGACACAACCCAGGAAGG	62.5	462	C2A1-I2-2-1	Intron 2	no	180A>G	Bsu36I
C2A1-E3-4	CCTTCCTGGGTTGTGTCCTA CAACCCCTGTTGCGAGTAAT	58	408	no	-	-	-	-
C2A1-I7	TCGTGGTGACAAAGGTGAAA GCCCTTAGCACCACAGTCTC	58	490	C2A1-I7	Intron 6	no	92C>T	BtgZI
C2A1-E8	TTGGCAAGTCTGTTCAATGC TCACTCCAGCGCATCATTAG	58	500	C2A1-E8	Intron 8	no	267->T	-
C2A1-E11	CCACTCAGACCCCAAGACTG CCGGACACTCGTAAGCTAGG	58	595	no	-	-	-	-
C2A1-I12	GGCCCTCAGGTAAAGTCCTC CTTCCAAAACCCTTGTCTGC	58	507	C2A1-I12	Intron 12	rs23358342	157A>G	NIaIII
C2A1-E14-15	GGGTTCATCCCAGAAGTGTG GCATTTATAGCGGAGCCATC	58	543	no	-	-	-	-
C2A1-I15	TTGAAATAGCCCTGTGAGCA CCAGGATGACAACACCCTCT	61	504	C2A1-I15	Intron 15	rs23354654	137A>G	BsaXI
C2A1-E17	ACAAGGAGAACATGCCCACT CATCCTCCTCCCCATCCT	58	415	C2A1-E17-1 C2A1-E17-2	Intron 16 Intron 16	no rs23372864	22C>G 101C>T	BsrI BfuAI
C2A1-I18	AGAAGTCCCACCCAGAAACA CCCCATTCATCCTCGCTAAC	58	515	no	-	-	-	-
C2A1-E19	CTGGCCTGTGAGAGGTCTGT TACCCTGGAAAAGGGAGAGG	58	467	C2A1-E19	Intron 18	rs23335233	35A>G	BbsI
C2A1-I20	CTCTCTACAGCACCCCCATC GGACTTGGGGTTCACTCTGA	61	427	C2A1-I20-1 C2A1-I20-2	Intron 19 Exon 20	no rs23335235	181C>T 368A>G	BccI HpyCH4V
C2A1-I21	TCAGAGTGAACCCCAAGTCC	58	390	no	-	-	-	-

Continued

Primer name*	Primer sequence (5'-3')	TA (°C)	Size (bp)	SNP name†	SNP position	SNP database ID	SNP	Endo-nuclease
	ACGTGGCTCAAACCAAACAT							
C2A1-E23-25	CGGTTTCTCTTTCCTCCTGA	58	708	C2A1-E23-25-1	Intron 23	no	C365>T	no
	ATTAGCTCCCTTGGGACCAG			C2A1-E23-25-2	Intron 23	no	426C>G	no
				C2A1-E23-25-3	Intron 23	no	444A>G	no
				C2A1-E23-25-4	Intron 24	no	581G>T	no
C2A1-I23-2	CCTCCCCTGATACCCATCTT	58	617	C2A1-I23-2	Intron 23	rs23381390	403G>T	no
	ACTTACGTTGGCACCTTTGG							
C2A1-I23-3	ATCTGATTCTTGGGCCTCCT	58	387	no	-	-	-	-
	GCCAGAAGTCTGAGGGTTCA							
C2A1-E29-31	AGGGTCTTCCAGGCAAAGAT	58	693	C2A1-E29-31-1	Intron 30	no	501A>G	BpmI
	TCACCTGGTTTTCCACCTTC			C2A1-E29-31-2	Intron 30	no	503C>G	no
C2A1-E32-34	TCACCTGTCCCTCTTTGTCC	58	601	C2A1-E32-34	Intron 32	no	188(GCTT) Indel	-
	GGGGAGCTCAACCTGGTACT							
C2A1-E36	CTGGGGACTCTCCTGACAAG	58	503	C2A1-E36-1	Intron 36	rs23384580	217C>T	no
	GACGAGAGTCACGGAGAAGG			C2A1-E36-2	Intron 36	no	356G>T	BtsI
C2A1-E39	CACACCCACACTGACCTCAC	60	525	C2A1-E39-1	Intron 38	rs23359656	94C>T	no
	CCCTACCCCAAGACAATGAA			C2A1-E39-2	Intron 39	rs23382148	399A>G	no
				C2A1-E39-3	Intron 39	rs23382149	425C>T	AciI
				C2A1-E39-4	Intron 39	no	A478>G	no
C2A1-E42-43	GGGGCAGGGTAAGATGAGTT	60	540	no	-	-	-	-
	CACCAGAGGAAGGGGTATCA							
C2A1-E46-48	CAACTGGAAAGCAGGGAGAC	58	658	no	-	-	-	-
	TAGCAGCATGGGACACTCAG							
C2A1-E51	CAGGGGACTGGAAGACGATA	58	396	C2A1-E51	Exon 51	no	132C>T	no
	CATTCAGGGTGGCAGAGTTT							
C2A1-E52	ACCTGCAAGAGGGTCTCCTT	58	575	C2A1-E52-1	Intron 51	rs23379923	142A>G	BsmI
	TGTTAGGGCAGACAGCAGTG			C2A1-E52-2	Intron 52	rs23379922	466C>T	no
C2A1-3U	CATGGAACCCAGGAGTGAAC	58	509	C2A1-3U-1	3'UTR	no	116C>G	no
	TCACACCCACCATACCCTCT			C2A1-3U-2	3'UTR	no	117A>G	no
				C2A1-3U-3	3'UTR	no	C214>T	no
				C2A1-3U-4	3'UTR	no	394A>G	AciI

*The primer sets of C2A1-I2, C2A1-I4-6 for *COL2A1* were not included. † "no" means no SNP has been found in this fragment in our population.

CHAPTER 3. Association of *HOXA10, ZFPM2* and *MMP2* genes with scrotal hernias via biological candidate gene analyses in pigs

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ABSTRACT

Objective — To evaluate the associations of 14 biological candidate genes with scrotal hernias in pigs.

Sample population — A total of 1,534 Pietrain-based pigs, including 692 individuals from 298 pig families and 842 male pigs without family information.

Procedures — Pigs were classified as affected or unaffected for scrotal hernias. Single nucleotide polymorphisms (SNPs) of candidate genes were analyzed via PCR assays and genotyped. Statistical analyses were performed on the family-trio and the case-control data.

Results — Two genes involved in collagen metabolism (homeobox A10 [*HOXA10*] and matrix metalloproteinases 2 [*MMP2*]), and zinc finger protein, multitype 2 (*ZFPM2*), important in the development of diaphragmatic hernia, were significantly associated with hernias. Pigs with these genotypes had high odds ratios of developing scrotal hernias (2.21 [95% confidence interval, 1.58 to 3.09] and 2.43 [1.73 to 3.42] for 2 *HOXA10* variants, and 4.3 [2.78 to 6.64] and 4.45 [2.88 to 6.88] for 2 *ZFPM2* variants, respectively). Another gene, collagen type II α 1 (*COL2A1*), was potentially involved in hernia development (the *P* value approached significance).

Conclusions and Clinical Relevance — *HOXA10, ZFPM2*, *MMP2,* and *COL2A1* could have important roles in pig hernia development and potentially be useful for marker-assisted selection in the pig industry.

Impact for Human Medicine — Pigs are used as a model system for the study of many human diseases because of their physiological similarities. Genes associated with scrotal hernias in this study may be directly used in understanding the molecular mechanisms underlying this defect in humans.

INTRODUCTION

Pigs with inguinal and scrotal hernias may require health care including extra labor and veterinary treatments, which, if not provided, may result in reduced animal welfare, or, if provided, may result in economic losses for pig producers. Inguinal hernia is characterized by bulging of abdominal contents into the inguinal canal. If abdominal contents enter the scrotum, a scrotal hernia occurs. Most pig producers combine these conditions and call the problem scrotal hernias. Such hernias are likely affected by multiple genes and environmental factors. Reports suggest that these are common

complex defects in certain pig breeds with low incidence (1.7% to 6.7%), and the heritability estimates vary widely (0.12 to 0.86).[1-2] The mode of genetic inheritance is still unclear, but hernias are likely sex-limited autosomal traits with partial penetrance.[3]

The formation of scrotal hernias is believed to be caused by failure of embryonic closure of the internal inguinal ring, incomplete obliteration of the processes vaginalis,[4] weakness of the triangle of the inguinal region caused by altered collagen subtype proportions,[5] an abnormality in smooth muscle differentiation in the wall of the hernial sac,[6] or increased intra-abdominal pressure.[7]

Initially, 8 genes were selected for study, including mullerian inhibitory substance (gene *MIS*), insulin-like 3 (gene *INSL3*) and its sole receptor, G-protein coupled receptor affecting testicular descent (gene *GREAT*), estrogen receptors α and β (genes *ESR1* and *ESR2*), gonadotropin-releasing hormone receptor (gene *GNRHR*), calcitonin gene related peptide (gene *CGRP*), and gene *ZFPM2* (important for development of diaphragmatic hernia).[8] These genes could be potentially involved in the incomplete obliteration of the processes vaginalis via aberrant sex hormone signaling pathways, which usually control the testicular development and descent process. The *ESR1* gene is located on SSC1 (60cM) and *MIS*, *INSL3*, and *CALCA* are located on SSC2 (60 to 80 cM) regions for inguinal hernias detected in pigs.[9]

Another important characteristic of scrotal hernias found in human studies is the weakness of the triangle of the inguinal region caused by the altered collagen subtype proportions, which can profoundly change tissue elasticity and resistance to tensile stress. A decrease of type I collagen, increase in type III collagen, or both, have been found in humans with recurrent hernias, which suggests that the relative composition of collagen

types determines the elastic properties of tissue.[10] Furthermore, *MMP2*, *HOXA10* and elastin (*ELN*) genes have been found to be related to the collagen metabolism process in humans,[11-13] and *COL2A1* is located in one identified pig inguinal hernia associated QTL region on SSC5 (90 cM).[9] Therefore, 6 genes including collagen type I α 1 (*COL1A1),* collagen type I α 2 (*COL1A2), COL2A1, MMP2, HOXA10* and *ELN* were also chosen for this study.

With the rapid development and application of molecular genetic technology, animal breeders have been applying molecular genetic methodologies to assist in elimination of genetic defects and improve performance in commercial herds. The *Pvu*II polymorphisms in *ESR*[14] and variants in *MC4R*[15] have been successfully used in MAS for increasing reproductive ability and meat quantity in the pig industry.[16] The main objective of the study reported here was to elucidate the possible associations of 14 selected biological candidate genes with scrotal hernias in commercial pig lines. In addition, because pigs and humans share considerable physiological similarities,[17] genes associated with scrotal hernia in pigs are strong candidates for the study of this defect in humans.

MATERIALS AND METHODS

Pigs — Samples for DNA extraction were collected from a total of 1,534 pigs born between 1991 and 2002. Pigs were classified as affected or unaffected for scrotal hernias. A total of 692 individuals from 298 pig nuclear families (including sire, dam and male offspring) were from a Pietrain-based line. The remaining pigs, 340 unaffected and 502 affected males, were derived from Pietrain-based lines or from crossbred matings of Pietrain-based boars to commercial females collected from 7 different farms. Full

pedigree information was available for purebred animals from the nuclear families. DNA of all animals was extracted and adjusted to12.5 ng/μl.

SNP Discovery and Genotyping — Candidate genes were listed with their comparative position information in *Sus scrofa* and *Homo sapiens* (Table 1). To find SNPs in these candidate genes, pig homologous sequences (Table 2) were identified by evaluating correspondence of the human sequence as listed in the Ensembl Genome Browser site [28] and blast pig genome resources in the National Center for Biotechnology Information site.[29] Primers were designed preferentially to amplify exons, promoters, and splicing sites of these genes by use of a PCR primer designing program.[30] Several primer sets for *ESR1* and *MIS* were directly obtained from the published literature.[31,32] The gradient PCR approach was used to optimize the primers in a 10-μL system, which included 1X PCR reaction buffer,[a] 0.125 mM of each deoxynucleotide triphosphate,[a] 2.5 pM of each primer,[b] 12.5 ng of genomic DNA, and 0.25 units of DNA polymerase.[a] The gradient PCR profile was 94°C for 3 minutes, 94°C for 30 seconds, 52 to 66°C for 45 seconds, and 72°C for 50 seconds; then steps 2 through 4 were repeated 35 times, followed by 72°C for 5 minutes, and 4°C for 1 minute.

A panel of 5 individuals from each of the primary pig lines included in this study was used for SNP identification. The PCR protocol was 35 cycles of 30 seconds at 94°, 30 seconds at the primer-specific annealing temperature, and 30 seconds at 72°C with additional 3 minutes of denaturation in the first cycle and a 5-minute extension in the last cycle. The PCR products were verified via 1.5% agarose gel electrophoresis, and treated with a reagent for PCR product clean up (ExoSAP-IT®)[c] before pooling PCR products and sequencing commercially by use of a high throughput genetic analysis system

(3730x1 DNA Analyzer).[d] The sequences were aligned and compared by use of a DNA sequence analysis software (Sequencher v3.0).[e] Potential SNPs were confirmed by use of PCR-RFLP tests that used suitable restriction endonucleases.[f]

The SNP genotyping was performed commercially by use of a leading technology of SNP genotyping system (MassARRAY® iPLEX Gold).[g] Two positive controls and 2 negative controls were put on the same plate for quality control. Primers were designed by use of the assay designing software within this genotyping system. After PCR amplification, samples were spotted onto a 384-well microarray chip (SpectroChip®) [g] by a robot dispenser, which enabled an automated readout by laser excitation in a compact matrix-assisted laser desorption/ionisation-time of flight mass spectrometer. Spectral data were then analyzed by the software named Typer 3.4 [g] in this system.

Statistical Analysis — Transmission disequilibrium tests were performed on the family trios data (298 nuclear families), by use of two software packages named Haploview[33] and PLINK[34] that have been widely used in human disease studies. Transmission disequilibrium tests measure the over-transmission of an allele from the parents to affected offspring, to test for the presence of association between a genetic marker and a trait.[35] The parental transmission disequilibrium test called parenTDT, based on the between-within-sibship association model, was used in this study because the incorporation of parental phenotype can increase power, compared with the standard transmission disequilibrium test.[36]

For the male pigs without pedigree information, allele-wise case-control analyses were completed by using the same two software packages. Quality control was performed on each SNP to estimate Hardy-Weinberg equilibrium and MAF in the case and the

control pigs, respectively. Those deviating from Hardy-Weinberg equilibrium or with MAF < 0.05 in the control group were excluded. Associations of the genetic polymorphisms in the candidate genes with scrotal hernias in pigs were determined by evaluating significant difference of allele frequencies between affected and unaffected groups. The FDR control was conducted as well to correct *P* values for each SNP, by using of the linear step-up procedure of Benjamini and Hochberg[37] in the PLINK software. In addition, *P* values and ORs were calculated for each SNP with farm distribution treated as a covariate by use of the conditional logistic regression analysis. For all analyses, $P < 0.05$ was considered significant.

RESULTS

SNP Discovery and Genotyping — Twenty-nine SNPs were discovered in the 14 genes (Table 3). The genotyping success rates were > 85% for 25 SNPs. Three SNPs, HOXA10-5U, COL1A1-2-5 and MIS-2-1, were excluded because of the low genotyping success rate (< 85%). Four other SNPs, ELN-5U-3, GREAT-1, COL1A1-4-2 and MMP2-E13-1 were eliminated because of low MAF (MAF < 0.05), and the SNP HOXA10-E1a was excluded because it significantly deviated from Hardy-Weinberg equilibrium in the control group of case-control data.

Association analysis — In the family-based association analyses, 2 SNPs in the same gene *HOXA10* (HOXA10-I2-2 C/T and HOXA10-I2-3 C/T) were significantly ($P < 0.01$) associated with scrotal hernias, both with the allele C over-transmitted to the affected individuals (Table 4). Two SNPs (ZFPM2-5U-1 G/C and ZFPM2-5U-2 A/G) in *ZFPM2* were also significantly ($P < 0.05$) associated with hernia, with over transmitted alleles C and G, respectively. Furthermore, *MMP2* was significantly associated with hernias, with

the allele G from the SNP MMP2-P2-2 A/G over-transmitted to affected pigs. The SNP C2A1I28-5 A/G in *COL2A1* had a *P* value that approached significance regarding the risk to develop hernias (Table 4).

In case-control analyses on another set of 842 pigs, the genetic variants in *HOXA10, ZFPM2* and *MMP2* were significantly different between affected pigs and controls (Table 5). Associations were observed for the HOX10-I2-2 and HOX10-I2-3 SNPs with the associated T alleles ($P < 0.001$). The SNPs ZFPM2-5U-1 and ZFPM2-5U-2 were associated with C and G alleles ($P < 0.001$). In addition, SNP MMP2-P2-2 was observed with a significantly increased frequency of the G allele in affected pigs with hernia, compared with controls. Additional SNPs (ESR2-1, ESR1-E4, ESR1-1, COL1A1-2-1, MIS-1-4, GNR5U-1, GNR5U-2, C2A1I28-1, C2A1I28-2 and C2A1I28-6) in other genes were also significantly ($P < 0.001$) associated with hernia. After the FDR evaluation, 15 of the 16 SNPs were still significantly associated with hernia, and analysis of MMP2-P2-2 yielded $P = 0.06$. The SNP ZFPM2-5U-1 with the C allele and ZFPM2-5U-2 with the G allele were significant risk factors for hernia occurrence (OR, 4.3 [95% confidence interval, 2.78 to 6.64; and OR, 4.45 [95% confidence interval, 2.88 to 6.8], respectively). Other SNPs such as HOXA10-I2-2, HOXA10-I2-3, ESR2-1 and GNR-E1-2 had significant ($P < 0.001$) associations and ESR1-E4, ESR1-1, COL1A1-2-1 and MIS-1-4 had significant ($P < 0.05$) associations with hernia. (Table 5)

DISCUSSION

By use of candidate gene methods, 3 genes, *HOXA10, ZFPM2* and *MMP2*, were found to be associated with hernia susceptibility in commercial pig lines, and analysis of another gene, *COL2A1,* yielded a *P* value that approached significance. Family-based

association analyses were first used to discover the association signal, and the results were replicated in a different set of pigs via case-control analyses. This method is widely used in human disease studies to lower the FDR.[38] The overtransmitted alleles of the significantly associated SNPs from *ZFPM2* and *MMP2* in the family-trio analysis were the same as those in the case-control analysis. However, for the two SNPs in *HOXA10* (HOXA10-I2-2 and HOXA10-I2-3), the over-transmitted alleles were different; C in the family-trio analysis and T in the case-control analysis. The reason could be that neither of these two SNPs is causative but they are in linkage disequilibrium with the true causative genetic variants and the phase is different in these two samples. Additionally, several genetic variants in *ESR1, ESR2, COL1A1, GNRHR,* and *MIS* were not significantly associated with hernia by use of the family-based analyses, but were significantly associated by use of the case-control analyses. This could be because of the hidden population substructure not accounted for in the case-control samples, which combined data from several crossbred matings of Pietrain-based boars to commercial females (female information was not available). However, SNPs significantly associated in both datasets suggested that these genes could have important effects on hernia development. Additionally, those genes without significant associations can not be ruled out at present because it is possible that some SNPs may not have been discovered or, in other populations, these genes could be causative. Further comprehensive genetic analysis would help clarify these points.

The gene *COL2A1*, an important cartilage collagen gene, is expressed mostly in cartilage, but also in the Sertoli cells in the developing testicular cord in mice.[39] The aberrant expression of this gene may have a relationship with the formation of inguinal

and scrotal hernias. *MMP2* is a type IV collagenase gene encoding a 72-kD protein which is a member of zinc-dependent endopeptidases. The protein encoding by *MMP2* specifically cleaves type IV collagen, the major structural component of basement membranes.[40] Increased amounts of MMP2 protein in the fibroblasts of the fascia of the transversalis muscle were found in human patients with direct hernias in which hernia contents does not pass through the internal inguinal ring but directly enter the inguinal canal through a weak point in the fascia of the abdominal wall.[11] Furthermore, the changed proportion of elastic fibers and collagen in the transversalis fascia is responsible for development of two types of inguinal hernias in humans.[40] One mutation of the *ELN* gene is significantly associated with inguinal hernia in humans.[13] The *ZFPM2* gene had a significant association with hernias via family-based analyses as well as case control analysis in the present study. Two reports indicate that *ZFPM2* is required in the normal development of lung and may be important in the progression of diaphragmatic hernias in mice and humans.[41]

Hernia defects may not be caused by a single mutation or combinations of mutations in collagen genes, but by a quantitative aberration of collagen protein synthesis resulting from a gene mutation within transcription factors (eg, the homeobox [*HOX*]) genes).[42] The *HOX* genes consist of 4 clusters, A, B, C, and D, and are spatiotemporally expressed during embryonic development. The *HOXA10* gene is one member of this large transcription factor family and it is a critical regulator involved in normal hematopoiesis by effects on hematopoietic stem cells and erythroid and megakaryocyte development.[43] Overexpression of *HOXA10* initiates profound perturbations in human hematopoiesis, followed by homeobox gene-associated leukemogenesis. The expression

of collagen type III α (*COL3A1*) gene could be up regulated when *HOXA10* is overexpressed in human umbilical cord blood cells.[44] Because the ratio of type I and type III collagens is an important factor in hernias, expression of *HOXA10* may be implicated in the formation of scrotal hernias by regulating different types of collagen. Furthermore, *HOXA10* is potentially associated with another defect, cryptorchidism, [12] which shares some anatomic structures with hernia development.

Several genes selected to study the incomplete obliteration of the processus vaginalis are not significantly associated with scrotal hernia susceptibility. These genes are thought to affect the development and function of gubernaculum (*INSL3*, *GREAT*, *CGRP*).[45- 47] The genes *ESR1*, which are involved with scrotal positioning of the testes through down regulation of *INSL3* expression in Leydig cells[48] and *MIS*,[49] which also affects the gubernaculum, were significantly associated with hernia in case control analysis. Results of analysis of *ESR1* and *MIS* were consistent with the previous results.[9] It should be mentioned that the SNP in the promoter region of *INSL3* was not associated with scrotal hernia in our pig population, which was consistent with findings from another study.[50]

Although the disruption caused by intronic variants on enhancer or repressor which is to increase or decrease the rate of the gene transcription (*HOXA10*), and the interference of SNPs in 5'-UTR on transcription (*ZFPM2*) could partially explain the molecular mechanism, further evaluation needs to be carried out in the candidate genes significantly associated with scrotal hernias in pigs with the objective to confirm or identify the causal mutations. Specifically, high-throughput sequencing and genotyping of additional pig populations, as well as functional studies such as determining the

differential expression pattern of associated genes in affected and unaffected pigs are warranted. The associated SNPs can potentially be applied by the breeding industry for marker-assisted selection aimed at reduction of scrotal hernia incidence. Furthermore, the causal mutations found in our candidate genes may provide clues for understanding the development of scrotal hernias in humans.

ABBREVIATIONS

ABBREVIATIONS	
SNP	Single nucleotide polymorphism
PCR	Polymerase chain reaction
cM	Centimorgan
QTL	Quantitative trait locus
MAS	Marker assisted selection
PCR-RFLP	Polymerase chain reaction- restriction fragment length polymorphism
MAF	Minor allele frequency
OR	Odds ratio
FDR	False discovery rate

FOOTNOTES

a. Promega, Madison, WI
b. Integrated DNA Technologies, Coralville, IA
c. USB, Cleveland, OH
d. Applied Biosystems, Foster City, CA
e. Gene Codes Ann Arbor, MI
f. New England Biolabs, Beverly, MA
g. Sequenom, San Diego, CA

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closely correlated with deregulated HOX gene expression in human acute myeloid leukemia. *Blood* 2008;111:309-319.

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Table 1. Symbols, names, and corresponding chromosomes of genes evaluated in a study of biological candidate genes for scrotal hernias in pigs

Gene symbol	Gene name	Chromosome	
		Pig (SSC [#])	Human (HAS)
*ESR1***	Estrogen receptor alpha	SSC 1[18]	HSA 6
ESR2	Estrogen receptor beta	SSC 1[19]	HSA 14
*MIS***	Muellerian inhibiting factor precursor	SSC 2[20]	HSA 19
*INSL3***	Insulin like 3	SSC 2[21]	HSA 19
*CALCA***	Calcitonin gene-related peptide , alpha	SSC 2[22]	HSA 11
ELN	Elastin	SSC 3[††]	HSA 7
ZFPM2	Zinc finger protein, multitype 2	SSC 4[††]	HSA 8
*COL2A1***	Collagen, type II, alpha 1	SSC 5[23]	HSA 12
MMP2	Matrix metallopeptidase 2	SSC 6[†]	HSA 16
GNRHR	Gonadotropin-releasing hormone receptor	SSC 8[24]	HSA 4
COL1A2	Collagen, type I, alpha 2	SSC 9[25]	HSA 7
GREAT(RXFP2)	G protein coupled receptor affecting testicular descent	SSC 11[†]	HSA 13
COL1A1	Collagen, type I, alpha 1	SSC 12[26]	HSA 17
HOXA10	Homeo box A10	SSC 18[27]	HSA 7

[#]The pig chromosome symbol; reference number associated with the pig candidate gene mapping information. *The chromosome symbol for human..**Implied genes in QTL regions in published literature. [†]Personal communication for gene mapping information. [††] Chromosome location expected by the comparative map between pigs and humans.

Table 2. Primers associated with sequencing and genotyping candidate genes for scrotal hernias in pigs.

Primer name*	Primer sequence	T_A (ºC)	Size (bp)	Primer location	Accession No.
ESR1-F [31]	5'-CCTGTTTTTACAGTGACTTTTACAGAG-3'	61	121	intron9	gnl|ti|1008624340
ESR1-R [31]	5'-CACTTCGAGGGTCAGTCCAATTAG-3'				
ESR1-E4F	5'- AATACTAATTCTGTCCTCTTGCTTTTG-3'	54	310	exon4 - intron 4	gnl|ti|1008624339
ESR1-E4R	5'-AAAAATAAACACCCTCTGCTATAAAAA-3'				
ESR2-F	5'-AAAATACTGATACCCACCCCACAT-3'	61	218	exon 5	Ti|765498129
ESR2-R	5'-CGCCACATCAGCCCCACCAT-3'				
MIS-1F [32]	5'-GGACTCCACCTCTGCCTTCCTC-3'	60	1450	5'UTR-intron 1	Patent :
MIS-1R [32]	5'-GGAACTTCAGCAAGGGTGTTGG-3'				20040126795 †
MIS-2F [32]	5'-CCAGCAACAGACAAATACACG-3'	60	194	intron 1	Patent :
MIS-2R [32]	5'-GCTCCAGGTGCCAAACCTGC-3'				20040126795†
INSL3-5UF	5'- GGGGATTTAAAGGGCAAGAA -3'	60	953	5'UTR	X73636
INSL3-5UR	5'- GGGGATTTAAAGGGCAAGAA-3'				
CALCA-3F	5'- TTCCTGGCTTTCAGCATCTT-3'	60	384	exon 2-intron 2	BF443390
CALCA-3R	5'- AGGCGACCTTTCCTTCTCACT-3'				
ELN-5UF	5'-GCCCCCACCCTAGCACTC-3'	60	501	5'UTR	gnl|nisc|RP44-
ELN-5UR	5'-GGGAACGAGCCTGACCTT-3'				363K13
ZFPM2-5UF	5'-TGGGAAAACAGCAGATGACA-3'	56	407	5'UTR	CU462936
ZFPM2-5UR	5'-AAAGGAAAGGGAGGGCTGTA-3'				
ZFPM2-E9F	5'-CCACTTTGGCTTCCAGACTC-3'	56	418	exon 9	CU151861
ZFPM2-E9R	5'-GATGTCTTGGGGAAAAGCAA-3'				
COL2A1-I28-29F	5'- GCAAAGATGGCGAGACAGGT-3'	67	440	intron28	AF201724
COL2A1-I28-29R	5'- CAGATGGCCCAGGAGCAC-3'				
MMP2-P2F	5'- AAAATGCTCTTCAGGCAGGA-3'	60	509	5'UTR	emb|CU442722.2
MMP2-P2R	5'- AGGAGATGGGACTGGGAGTT-3'				
MMP2-E13F	5'- GCTGCCACACTTTAGGCTCT-3'	60	591	exon 13	emb|CU442722.2
MMP2-E13R	5'-TGCAGCGAGCAGAGATTTAG-3'				

Continued

GNRHR-5UF	5’-CATCAAGCACTCACCATGAAA-3’	61	700	5’UTR	AF227685S1
GNRHR-5UR	5’-ACACATTCTGAGGGCCAAAC-3’				
GNRHR-E1F	5’- TCCCACCCTGACCTTATCTG-3’	61	599	exon 1	AF227685S1
GNRHR-E1R	5’- TGCCCAAGAGTAGAGCCAGT-3’				
COL1A2-E17-19F	5’- GGTGAACTTGGACCTGTTGG-3’	61	450	exon17-exon19	AC104481
COL1A2-E17-19R	5’- ACCAGTAGCACCAGCAGCA-3’				
GREAT-1F	5’-CTGGTAGGACCGCAACTGTC-3’	61	500	intron 15	gnl|ti|1420979027
GREAT-1R	5’-GAGTGGGTCTCAGGTCATGG-3’				
COL1A1-2F	5’- AGGGCGACAGAGGCATAAAG-3’	62	903	exon 45-exon47	AK236626
COL1A1-2R	5’-ACAGGACCAGCATCACCAGT-3’				
COL1A1-4F	5’-TCCCACTCTTTAGCCCACAG-3’	58	657	5’UTR	gnl|ti|767748265
COL1A1-4R	5’-CCATCTCCCTTCATCCTTGA-3’				
HOXA10-5UF	5’-TCAAACAGACCCCAAAGGAG-3’	58	506	5’UTR	AC129962
HOXA10-5UR	5’-GCCGTGGGCGTAGTAACC-3’				
HOXA10-I2F	5’-GTGTAGATTGGAGGCCCACT-3’	61	411	intron1-exon2	AC129962
HOXA10-I2R	5’- AGTTTCATCCTGCGGTTCTG-3’				
HOXA10-E1aF	5’- CTCATCAGCTCCGGCAGA-3’	56	445	exon 1	AC129962
HOXA10-E1aR	5’- CTTTGGGGCATTTGTCAGTC-3’				

F = Forward; R = Reverse. *Refers to the reference number associated with the primer.
T_A =Anealing temperature. † United States patent application 20040126795.[32]

Table 3. Single nucleotide polymorphisms detected in biological candidate genes evaluated for associations with scrotal hernias in pigs.

PCR fragment	Name of SNP	SNP	SNP location	Relative position*
ESR1-1	ESR1-1	A/G	intron 9	711bp
ESR1-E4	ESR1-E4	C/T	intron 4	506 bp
ESR2-1	ESR2-1	A/G	exon 5	532 bp
MIS-1	MIS-1-4	A/G	intron 1	1031 bp
MIS-2	MIS-2-1	C/T	intron 1	1101 bp
INSL3-5U	INSL3-1	A/C	5'UTR	795 bp
CALCA-3	CALCA-3	C/T	intron 2	165 bp after exon2
ELN-5U	ELN-5U-3	C/T	5'UTR	864 bp
ZFPM2-5U	ZFPM2-5U-1	G/C	5'UTR	78681 bp
	ZFPM2-5U-2	A/G	5'UTR	78440 bp
ZFPM2-E9	ZFPM2-E9-1	C/T	exon 9	32887 bp
COL2A1-I28	COL2A1-I28-1	C/T	intron 28	38 bp after exon28
	COL2A1-I28-2	A/G	intron 28	114 bp after exon28
	COL2A1-I28-5	A/G	intron 28	206 bp after exon28
	COL2A1-I28-6	A/G	intron 28	232 bp after exon28
MMP2-P2	MMP2-P2-2	A/G	5'UTR	122851 bp
MMP2-E13	MMP2-E13-1	C/T	exon 13	95361 bp
GNRHR-5U	GNR5U-1	G/T	5'UTR	310 bp
	GNR5U-2	C/T	5'UTR	593 bp
GNRHR-E1	GNR-E1-2	A/G	exon 1	1756 bp
COL1A2-E17-19	COL1A2-E18	C/T	exon 18	64315 bp
GREAT-1	GREAT-1	A/G	intron 15	673 bp
COL1A1-2	COL1A1-2-1	A/G	intron 45	31 bp after exon45
	COL1A1-2-5	C/G	intron 46	137 bp after exon 46
COL1A1-4	COL1A1-4-2	A/G	5'UTR	523 bp
HOXA10-5U	HOXA10-5U	C/G	5'UTR	23592 bp
HOXA10-I2	HOXA10-I2-2	C/T	intron 1	21292 bp
	HOXA10-I2-3	C/T	intron 1	21263 bp
HOXA10-E1a	HOXA10-E1a	C/T	exon 1	23043 bp

*Positions are given relative to the corresponding sequences with GenBank accession numbers**.**

Table 4. Results of association analyses based on family trio data in a study of biological candidate genes for scrotal hernia

Gene	SNP	Family-based analysis*		
		Overtransmitted allele	*P* - value *	*P* - value **
HOXA10	HOX10-I2-2	C	0.003	0.002
	HOX10-I2-3	C	0.003	0.003
ZFPM2	ZFPM2-5U-1	C	0.024	0.034
	ZFPM2-5U-2	G	0.011	0.023
	ZFPM2-E9-1	C	0.191	0.233
MMP2	MMP2-P2-2	G	0.019	0.024
COL2A1	C2A1I28-5	A	0.080	0.058
	C2A1I28-6	G	0.302	0.297
	C2A1I28-1	C	0.326	0.297
	C2A1I28-2	G	0.372	0.265
CALCA	CALCA-3	T	0.167	0.084
COL1A1	COL1A1-2-1	G	0.191	0.170
GNRHR	GNR5U-1	G	0.121	0.352
	GNR5U-2	C	0.712	0.572
	GNR-E1-2	A	0.803	0.317
MIS	MIS-1-4	G	0.269	0.270
ESR1	ESR1-1	G	0.452	0.845
	ESR1-E4	C	0.627	0.615
INSL3	INSL3-1	A	0.502	0.917
ESR2	ESR2-1	A	0.734	0.689
COL1A2	COL1A2-E18	T	0.753	0.706

†Family-based analyses are the association analyses that use the family trio data, which included 692 Pietrain pigs from 298 nuclear families. * *P* value produced by using Haploview statistical analysis software. ** *P* value produced by using PLINK statistical analysis software

Table 5. Results of association analyses, FDR and logistics analyses with the covariate based on case-control data

SNP	Associated allele	Case (n=502)		Control (n=340)					Logistic analysis		
		HW * *P* value	MAF	HW *P* value	MAF	*P* – value **	*P* - value ***	FDR_BH †	OR†	95% CI †††	*P* - value ****
HOX10-I2-2	T	0.001	0.249	1.00	0.128	<0.001	<0.001	<0.001	2.21	1.58-3.09	<0.001
HOX10-I2-3	T	0.001	0.248	0.887	0.122	<0.001	<0.001	<0.001	2.43	1.73-3.42	<0.001
ZFPM2-5U-1	C	<0.001	0.216	0.910	0.048	<0.001	<0.001	<0.001	4.30	2.78-6.64	<0.001
ZFPM2-5U-2	G	<0.001	0.218	0.913	0.048	<0.001	<0.001	<0.001	4.45	2.88-6.88	<0.001
ZFPM2-E9-1	C	0.304	0.269	0.579	0.295	0.249	0.204	0.227	0.87	0.67-1.14	0.324
ESR2-1	A	<0.001	0.335	0.842	0.175	<0.001	<0.001	<0.001	2.03	1.49-2.77	<0.001
ESR1-E4	C	0.002	0.173	0.634	0.094	<0.001	<0.001	<0.001	1.60	1.11-2.31	0.012
ESR1-1	G	0.003	0.171	0.685	0.095	<0.001	<0.001	<0.001	1.51	1.04-2.17	0.029
COL1A1-2-1	G	1.00	0.476	0.555	0.413	<0.001	<0.001	<0.001	1.33	1.04-1.68	0.021
MIS-1-4	G	0.203	0.093	0.878	0.167	<0.001	<0.001	<0.001	0.62	0.44-0.88	0.007
GNR-E1-2	A	<0.001	0.321	0.081	0.092	<0.001	<0.001	<0.001	3.93	2.73-5.66	<0.001
GNR5U-1	G	<0.001	0.372	0.835	0.463	<0.001	0.001	0.001	0.87	0.67-1.12	0.284
GNR5U-2	C	<0.001	0.377	0.744	0.465	0.001	0.001	0.001	0.87	0.67-1.11	0.258
MMP2-P2-2	G	1.00	0.156	1.00	0.197	0.032	0.048	0.052	0.94	0.69-1.27	0.680
C2A1I28-1	T	0.645	0.339	0.998	0.420	0.001	0.001	0.002	0.87	0.68-1.11	0.258
C2A1I28-2	A	0.564	0.336	1.00	0.420	0.001	0.001	0.002	0.89	0.69-1.13	0.336
C2A1I28-6	A	0.643	0.340	0.958	0.424	0.001	0.001	0.002	0.89	0.70-1.14	0.348
C2A1I28-5	G	1.00	0.094	0.329	0.067	0.048	0.034	0.045	1.16	0.75-1.79	0.508
INSL3-1	C	0.837	0.206	0.164	0.222	0.438	0.310	0.400	1.03	0.78-1.36	0.841
CALCA-3	C	<0.001	0.407	0.707	0.389	0.484	0.453	0.471	1.00	0.77-1.29	0.983
COL1A2-E18	T	0.198	0.285	0.987	0.284	0.960	0.974	0.982	0.88	0.68-1.15	0.353

*HW *P* value is the Hardy-Weinberg equilibrium *P* value, which is the probability that the SNP genotyping data deviation from Hardy-Weinberg equilibrium could be explained by chance. ** *P* value of case –control association analyses by using Haploview statistical analysis software. *** *P* value of case –control association analyses produced by using PLINK statistical analysis software. **** *P* value from logistic analyses. † FDR_BH is the Benjamini & Hochberg-step-up FDR control. †† OR= Odds ratio. †††95% CI = 95% Confidence interval.

CHAPTER 4. GENERAL CONCLUSIONS

The recognized high level of similarities between humans and dogs or pigs in anatomic, physiological and genetic aspects is of assistance for using dogs or pigs as animal models for human complex diseases. The main objective of this study was to uncover genetic markers associated with two complex defects, cryptorchidism and inguinal/ scrotal hernia which are two of the most common congenital developmental defects for not only humans but also dogs or pigs. The method applied here is a candidate gene approach by using single nucleotide polymorphism markers to do the association analyses.

In Chapter 2, the candidate gene discovery was performed in a population of Siberian Husky dogs as genetic models for cryptorchidism. To investigate associations of candidate genes with this defect, a total of 51 SNPs from 22 functional and positional candidate genes were investigated. The functional candidates are related to the normal testicular descent and potentially vital to the development of cryptorchidism including Müllerian inhibiting substance (*MIS*), insulin-like factor 3 (*INSL3*), the leucine-rich repeat-containing G protein-coupled receptor 8 (*LGR8*), nuclear receptor subfamily 5, group A, member 1(*NR5A1*), homeobox A10 (*HOXA10*), homeobox D13 (*HOXD13*), gonadotropin-releasing hormone receptor (*GNRHR*), estrogen receptor 1 (*ESR1*), estrogen receptor 2 (*ESR2*), androgen receptor (*AR*), CGRP-alpha (*CALCA*), CGRP-beta (*CALCB*) and several genes in the collagen metabolism process, e.g. collagen type I α 1 (*COL1A1*), collagen type II α 1 (*COL2A1*), collagen type IX α 1 (*COL9A1*) and metalloproteinase inhibitor 2 (*TIMP2*). In addition, eight positional candidates were selected using a comparative genomics approach from our study on cryptorchidism in

pigs (unpublished data). The Sibling-Transmission Disequilibrium Test (sib-TDT) on 38 discordant full sibs revealed 6 SNPs in the collagen type II α 1 (*COL2A1*) gene were significantly associated with cryptorchidism ($P < 0.05$), and another one suggestively associated ($P < 0.10$). Further analysis on all 156 Siberian Husbies with only the SNP rs23389020 had suggestive significance ($P < 0.06$), but was not significant after permutation tests (n=1000). Neither of the two haplotypes formed by the 5 SNPs in *COL2A1* was significantly associated with cryptorchidism. Based on the statistical results and the possible involvement of *COL2A1* in the development of cryptorchidism, *COL2A1* may be a potential gene for cryptorchidism in Siberian Huskies.

Candidate gene discovery was also conducted in 1,534 commercial pigs which were used as genetic models for the inguinal/scrotal hernia study in Chapter 3. There were eight functional genes being selected including *MIS, INSL3, GREAT, ESR1, ESR2*, *GNRHR*, *CGRP*, and *ZFPM2*. Among which, some were chosen also due to their overlap with QTL regions for inguinal hernias detected in pigs, such as *ESR1* located on SSC1 (60cM); *MIS, INSL3*, and *CALCA* located on SSC2 (60-80 cM). Another six functional candidate genes involving in the collagen machnisim were also chosen. These genes includes matrix metalloproteinases 2 (*MMP2*), *HOXA10*, elastin (*ELN*), *COL1A1*, collagen type I α 2 (*COL1A2*) and *COL2A1*. The last one is in the QTL region on SSC5 (90 cM) related with inguinal hernias in pigs. In total, 29 SNPs were discovered distributed in the above 14 functional candidate genes. The results showed in this study that two SNPs (HOXA10-I2-2, 56C>T and HOXA10-I2-3, 86C>T) in *HOXA10* were highly significantly associated with hernias and two SNPs (ZFPM2-5U-1 106G>C and ZFPM2-5U-2 337A>G) in ZFPM2 were significantly associated with scrotal hernias in

the family-based association analyses as well as in the case-control analyses. Moreover, *MMP2* was found to be associated with hernias ($P < 0.05$), with an increased allele "G" from the SNP MMP2-p2-2 (242A>G) being over-transmitted to affected animals. The SNP C2A1I28-5 (251A>G) in COL2A1 was suggestively associated with the increased risk to develop hernias (average p value: $0.05<p<0.1$). Besides the above associated variants, additional genetic variants in *COL2A1, ESR1, COL1A1, MIS* and *GNRHR* were also discovered to be significantly associated with hernias in the 842 animals by using case-control analyses even after the FDR evaluations. In summary, SNPs found in *HOXA10, ZFPM2* and *MMP2* were significantly associated with hernia incidence. The gene COL2A1 was identified to be potentially involved in hernia development. Molecular mechanisms underling the associations for these SNPs may be explained by the disruption of intronic variants on enhancers or repressors (*HOXA10*), and the interference of SNPs in the 5'-UTR on transcription (*ZFPM2*) for gene regulation. These variants might also closely link with the true causal mutations nearby.

Since the two defects, cryptorchidism and inguinal/scrotal hernia share some anatomic structures involved in the process of the testicular descent including the inguinal ring, the inguinal canal, processes vaginalis and the scrotum as well as several risk factors such as androgen and its receptor (AG), INSL3, CGRP or the ratio of different types of collagen, some common candidate genes were selected for both of these two defects. The results coming out from Chapter 2 and Chapter 3 show that *COL2A1* gene are associated or suggestively associated with both defects. The long form of *COL2A1* is collagen type IIA and is expressed in noncartilage tissue and has specific roles for testicle development via interaction with TGFβ family. Our results based on the

understanding of the function of the *COL2A1* gene help us raise a hypothesis that cryptorchidism and inguinal/scrotal hernia can be partially regulated with the same group of genetic loci. One allele for the causal mutation (such as a SNP caused mutation) can initiate the development of cryptorchidism and another allele in the same causal locus might be associated with or trigger an inguinal/scrotal hernia. This hypothesis needs to be confirmed by using the same animal model on these two defects.

The DFAM as a family based analysis method was applied for the single maker association in the canine cryptorchidism study. This procedure located in the PLINK program procedure could be the best choice for analyzing our current data set because it implements a sib-TDT test and can be used to combine discordant sibship data, parent-offspring trio data and unrelated case/control data in a single analysis. Usually, recruitment of a large canine cohort is very difficult. This statistical analysis method is more accurate than others when no complete or a large number of animal samples is available.

FURTHER RESEARCH

There are several popular candidate genes such as *INSL3, GREAT, CGRP* and *AG* suspected of being related in the development of scrotal hernia and cryptorchidism. However, no significant associations were uncovered within these genes in our studies. This may be due to the inadequate SNP discovery for the genes, small data sets, non-identical phenotypes or modulation by environmental risk factors. Further studies are needed with increasing the sample size, seeking novel genetic variants across the whole genome or using other populations.

For the genetic markers found with associations, more studies with other animal populations or breeds are needed to verify our results. Environmental factors, their complex interaction and their effects on testicular development and descent should also be studied. To comprehensively understand the genetic variant effects, a systemic genomic study using canine or porcine SNP chips covering the whole genomes may help us understand the real factors associated with the molecular mechanisms behind these defects.

CONCLUSIONS

In conclusion, several genetic markers including *HOXA10*, *ZFPM2*, and *MMP2* were identified to be significantly ($P < 0.05$) associated with scrotal hernia in pigs. The collagen gene *COL2A1* showed a suggestive association with both scrotal hernia in pigs and cryptorchidism in dogs. Genetic markers found in the pig study could potentially being used in a selection system for commercial swine production to discrease the incidence of swine scrotal hernia.

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