A g.-1256 A > C in the promoter region of *CAPN1* is associated with semen quality traits in Chinese Holstein bulls

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Abstract

The micromolar calcium-activated neutral protease gene (*CAPN1*) is a physiological candidate gene for sperm motility. However, the molecular mechanisms involved in regulating the expression of the *CAPN1* gene in bulls remain unknown. In this study, we investigated the expression pattern of *CAPN1* in testis, epididymis, and sperm at the RNA and protein levels by qRT-PCR, western blot, immunohistochemistry, and immunofluorescence assay. Results revealed that the expression of *CAPN1* levels was higher in the sperm head compared with that in other tissues. Moreover, we identified a novel single-nucleotide polymorphism (g.-1256 A > C, ss 1917715340) in the noncanonical core promoter of the *CAPN1* gene between base g.-1306 and g.-1012. Additionally, we observed greater sperm motility in bulls with the genotype CC than in those with the genotype AA ($P < 0.01$), indicating that different genotypes were associated with the bovine semen trait. Furthermore, a higher fluorescence intensity of the C allele than that of the A allele at g.-1256 A > C was revealed by transient transfection in MLTC-1 cells and luciferase report assay. Finally, *CAPN1* was highly expressed in the spermatozoa with the CC genotype compared with that with the AA genotype by qRT-PCR. This study is the first report on genetic variant g.-1256 A > C in the promoter region of *CAPN1* gene association with the semen quality of Chinese Holstein bulls by influencing its expression. g.-1256 A > C can be a functional molecular marker in cattle breeding.

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Introduction

The widespread use of bull semen requires high sperm quality, which is economically important in the artificial insemination industry. Molecular breeding technology has become a developmental trend of the world's biological breeding science. The directional breeding of high-quality semen traits is an important developmental direction of dairy cattle breeding. Sperm molecular biomarkers that may better and more stably reflect sperm functions have been developed. In the past, many similar studies were conducted on goats (Wang *et al.* 2011) and boars (Huang *et al.* 2002, Lin *et al.* 2005, Wimmers *et al.* 2005). Recently, several studies focused on candidate marker genes in bulls (Pan *et al.* 2013, Gao *et al.* 2014, Guo *et al.* 2014, Zhang *et al.* 2014, 2015).

During mammalian spermatogenesis, male germ cells undergo a series of differentiation steps that lead to the production of mature haploid spermatozoa. This complex physiological process includes chromatin reorganization, cytoplasm elimination, acrosome formation, and flagellum development in the seminiferous tubules of the testis and epididymis (O'Donnell *et al.* 2001, Bettegowda *et al.*

2010). During storage in the epididymis, spermatozoa are immotile or barely twitching (Yanagimachi 1994). Upon ejaculation, when sperm cells come in contact with secretions from male accessory glands and the female reproductive tract, or when they are suspended in incubation media, spermatozoa become motile, in a process known as "activation." Active motility is important for spermatozoa when passing through several barriers in the female tract. The integrity of the acrosome (the large secretory granule located over the sperm nucleus) is also necessary at several stages in the life of a sperm. Only acrosome-intact sperm can attach to the oviductal wall and penetrate the oocyte vestments (cumulus oophorus and zona pellucida) (Suarez & Pacey 2006), and only acrosome-reacted spermatozoa can bind to the oolemma (Chiu 2014).

Bull fertility traits are quantitative traits of low heritability that are regulated by multiple genes. The micromolar calcium-activated neutral protease gene (*CAPN1*) was reported as a candidate gene related to the semen quality traits of bull based on the early analytical results

of laboratory gene chips, as well as their physiological and biochemical functions (Coureuil *et al.* 2006, Macqueen *et al.* 2010, Hering *et al.* 2014). CAPN1 is a calcium-regulated cysteine protease that has been described in a wide range of cellular processes, including apoptosis, migration, and cell-cycle regulation (Santos *et al.* 2012). Several studies showed that CAPN1 is a motility-related protein, as analyzed by a proteomic approach (Slaughter *et al.* 1989, Rojas *et al.* 1999, Yudin *et al.* 2000), and exhibits genome-wide association with sperm motility or semen biochemistry in Holstein–Friesian bulls (Hering *et al.* 2014). Calcium-dependent proteins, such as calmodulin, are present during mammalian spermatogenesis (Slaughter *et al.* 1989). CAPN1 is expressed in spermatozoa of the cynomolgus macaque, and ultrastructural studies indicated that they are localized between the plasma membrane and the outer acrosomal membrane (Yudin *et al.* 2000). Calpain inhibitors used during *in vitro* fertilization impair the ability of human sperm to fuse and penetrate the oocyte (Rojas *et al.* 1999). These results indicated that CAPN1 may be involved in maintaining sperm motility and function as a sperm-motility marker. However, the detailed expression patterns of CAPN1 in different bull organs and semen have not been fully characterized.

Functional single-nucleotide polymorphisms (SNPs) are the most common forms of genetic variation extensively affecting the mammalian genome, such as protein coding and expression regulation. The 5′-flanking region of the gene, particularly the minimal promoter, is the key transcriptional regulatory region in gene expression (Saeki *et al.* 2011, Amin *et al.* 2012). SNPs in the promoter region may modify the transcription factor binding sites, thereby affecting gene expression (Pan *et al.* 2013, Zhang *et al.* 2015). In some cases, a natural binding site created or abolished by an SNP can account for the differences in gene expression (Schild *et al.* 1994, Wagner *et al.* 1994, Chorley *et al.* 2008).

Based on the abovementioned description, we suggested that bovine *CAPN1* can affect sperm quality traits. To confirm our hypotheses, the following studies were performed. (i) We determined the expression and localization of the *CAPN1* gene in Chinese Holstein bulls using qRT-PCR, western blot analysis, immunohistochemistry (IHC), and immunofluorescence assay (IFA). (ii) We investigated potentially functional genetic variants in the 5′-flanking region of *CAPN1* and their relationship with semen quality traits in Chinese Holstein bulls. (iii) We identified the core promoter region and the effect of the genetic variants on the transcription of the *CAPN1* gene.

Materials and methods

Ethics statement

All experiments were carried out according to the Regulations for the Administration of Affairs Concerning Experimental

Animals published by the Ministry of Science and Technology, China, in 2004. The study involving bull semen and tissue samples was approved by the Animal Care and Use Committee in Shandong Academy of Agricultural Sciences, Shandong, People's Republic of China. Collection of semen and tissue samples was permitted by the animal owners, and the samples were collected by the workers of the companies.

Tissue collection

Semen samples from 206 Chinese Holstein bulls were used in our study, including 128 bulls from the Shandong OX BioTechnology Co., Ltd (Jinan, China) and 78 bulls from the Beijing Dairy Center (Beijing, China). The semen traits, such as ejaculate volume, initial sperm motility, sperm density, post-thaw cryopreserved sperm motility, and sperm deformity rate, were recorded (Liu *et al.* 2011). The mean and standard errors of the sperm traits investigated in the 206 Chinese Holstein bulls are given in Table 1. For each bull, sperm quality traits were measured repeatedly from 2010 to 2014. The ejaculate volume was measured in a semen-collecting vial, and the number of sperm cells was counted by hemocytometer method. The sperm concentration was calculated using a sperm densitometer (Accu-cell; IMV Biotechnology, L'Aigle, France). The motilities of the fresh and post-thaw cryopreserved sperms were viewed on a TV monitor, which was connected to a camera mounted onto a phase-contrast microscope (Olympus-BX40; Optical Co., Ltd., Shinjuku-ku, Tokyo, Japan) at 400× magnification. The percentage of sperm deformities was determined at 400× and 1000× magnification with Giemsa stain (Cassinello *et al.* 1998). After investigating the above traits, the fresh semen was diluted with glycerol–egg yolk–citrate mixture, packaged in 0.25 mL straws and cryopreserved. Two straws were randomly obtained from each sample, ejaculated and thawed at 38°C for 20 s after storage in liquid nitrogen for 5–7 days, and immediately evaluated for the frozen/thawed sperm motility under light microscopy, according to the criteria entitled Frozen Bovine Semen standard (GB/T 4143-2008, China).

Tissue samples, including the testis and epididymis, were collected from three randomly selected adult Chinese Holstein bulls (3 years of age) from the farms of the Dairy Cattle Research Center, Shandong Academy of Agricultural Sciences. The tissue samples were collected and immediately frozen in liquid nitrogen until use.

Immunoblotting, immunohistochemical, and immunofluorescence procedures

Western blot analysis was performed according to the method of our previous report (Guo *et al.* 2014). The tissue samples

Table 1 Mean and standard error (S.E.M.) of sperm quality traits in 206 Chinese Holstein bulls.

Traits	Mean ± S.E.M.
Ejaculate volume (mL)	5.68 ± 0.23
Initial sperm motility (%)	68.83 ± 0.67
Sperm density (× 10 ⁸ /mL)	11.06 ± 0.43
Frozen/thawed sperm motility (%)	43.01 ± 0.53
Deformity rate (%)	16.33 ± 0.49

from the adult bulls were homogenized using radioimmuno-precipitation assay lysis buffer (Beyotime, Shanghai, China). After cooling the lysate on ice for 30 min, it was centrifuged at 12,000g for 10 min at 4°C. After denaturation, the proteins were separated by 12% SDS-PAGE, transferred onto a polyvinylidene fluoride membrane, blocked with blocking buffer (Beyotime), and rotated for 1 h at room temperature. The blots were incubated with monoclonal anti-mouse CAPN1 antibody (1:2000; Abcam, Cat NO. ab3589; <http://www.abcam.cn/calpain-1-antibody-9a4h8d3-ab3589.html>) or polyclonal β -actin (1:500; Beyotime) for 2 h at room temperature. After washing the membranes three times with 0.1% Tween-20 in 1× TBS for 5 min each time, goat anti-mouse secondary antibodies (1:10,000; Beyotime) were incubated with the membranes to detect antigen-antibody complexes.

The testicular and epididymal tissues from two adult bulls were fixed in 4% paraformaldehyde. All tissues were embedded in paraffin and sectioned for IHC (Hou et al. 2012). Deionized water (1 L) and 10 mL citrate buffer solution were used to rehabilitate the antigen. Subsequently, the antigen was washed with 0.01 M PBS (pH 7.4) twice every 3 min. The immunoreaction slides were deparaffinized and hydrated. The slides were blocked with endogenous peroxidase for 10 min, washed with PBS, and incubated with the monoclonal anti-mouse CAPN1 antibody (1:1000; Abcam) for 60 min at room temperature. After washing with PBS, the slides were incubated with goat anti-mouse secondary antibody (1:100; Beyotime) for 15 min at room temperature. The antibodies were visualized with 0.6 mg/mL DAB horseradish peroxidase color development kit (Cwbiochem, Shanghai, China) for brown staining under a microscope (OLYMPUS BX53) according to the manufacturer's instructions. The slides were stained with hematoxylin (Cwbiochem), dried, and photographed with a digital camera.

For IFA, the sperm cells were mounted on slides for 45 min, fixed in 4% paraformaldehyde for 60 min, and washed with PBS three times every 5 min. The slides were sealed with 3% bovine serum albumin for 30 min at room temperature and incubated with monoclonal anti-mouse CAPN1 antibody (1:100; Abcam) overnight at 4°C. After washing with PBS, the slides were incubated with FITC-conjugated AffiniPure Donkey anti-mouse IgG antibodies (1:150; Beyotime, Haimen, China) for 60 min at room temperature and rewashed with PBS. The nucleus was stained with 4',6-diamidino-2-phenylindole. Finally, the cells were photographed using an inverted microscope (Olympus).

Real-time quantitative PCR (qRT-PCR)

To explore the relative expression of CAPN1 in testes, epididymis, and sperm of adult Chinese Holstein bulls, qRT-PCR was conducted. Total RNA was isolated from semen following the same protocol. In brief, the somatic cells existing in semen were first lysed in 1 mL cell lysis solution (0.1% SDS and 0.5% Triton X-100) and then washed in 1 mL rinsing solution (60% Tris-OH and 8.6% sucrose). After the abovementioned steps, the sperm cells were lysed in 1 mL TRIzol (Invitrogen), and the proteins were removed using 200 mL chloroform. Total RNA was precipitated with an equal volume of isopropanol, and the

RNA pellet was washed with 75% ethanol. Finally, the total RNA was dissolved in 20 mL DEPC water. cDNA was synthesized using a transcript first-strand cDNA synthesis kit (Takara Bio). The total RNA was reversed into cDNA according to a previously published protocol (Haas & Beer 1986).

In the qRT-PCR experiment, 20 μ L reaction mixture per well contained 10 μ L SYBR Premix Ex Taq II (2×) (Takara Bio), 0.8 μ M primers, 6.4 μ L H₂O, and 2 μ L cDNA (<200 ng). qRT-PCR was performed in a LightCycler 480 instrument. The qRT-PCR protocol was as follows: 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 30 s. The final stage used for the dissociation curve was as follows: 95°C for 5 s, 60°C for 1 min, and 95°C for 15 s. Each sample was run in triplicate.

Genetic variation screening of the 5'-flanking region in the CAPN1 gene

Sperm DNA was extracted using a high-salt concentration protocol and subsequently stored at -20°C before use. One primer pair (S, Table 2) was designed using the primer PREMIER 5.0 to amplify the 5'-flanking region in the CAPN1 gene based on the GenBank reference sequence of bovine CAPN1 (Accession No. AC_000186.1). The primer pairs were synthesized by Shanghai Sangon Biological Engineering Co., Ltd., Songjiang, Shanghai, China. The corresponding PCR products were sent to a commercial service provider for sequencing. The sequenced results were analyzed with DNAMAN v5.2.2 (Lynnon Biosoft, San Ramon, CA, USA) and DNASTAR LASERGENE 7.1 software to search for SNPs.

Prediction of the core promoter region

The core promoter of bovine CAPN1 was predicted with Genomatix Software (<http://www.genomatix.de/applications/index.html>) and Promoter 2.0 (<http://www.cbs.dtu.dk/services/Promoter/>). The position of the TATA box was predicted using PROSCAN version 1.7 (<http://www.bimas.cit.nih.gov/cgi-bin/molbio/proscan>). The transcription factors were predicted with TFSEARCH (version 1.3) (<http://www.cbrc.jp/research/db/TFSEARCH.html>) and WWW Promoter Scan (<http://www.bimas.cit.nih.gov/molbio/prosan/>). The nucleic acid sequences were analyzed using accepted software formats.

Cloning and construction of CAPN1 promoter-reporter plasmids

To evaluate the promoter activity of the different parts of the 5'-flanking region of the CAPN1 gene, we performed serial truncations of the CAPN1 promoter fragment from -1638 bp

Table 2 Primer information of the bovine CAPN1 gene.

Primer names	Prime sequences (5'-3')	Annealing temperature (°C)
S	F:CAAGATGGGATCCCGCAGTT R:CAACTGAGGACAGGGCCCAA	59 60
P-1F	CGACGCGTCAAGATGGGATCCCGCAGTT(<i>Mlu</i> I)	62
P-2F	CGACGCGTTGCCACAGCCGAGGTAATC(<i>Mlu</i> I)	63
P-3F	CGACGCGTAGCCCTTCCACCCAGATAG(<i>Mlu</i> I)	62
PR	CCCAAGCTTCAACTGAGGACAGGGCCCAA(<i>Hind</i> III)	62

to -769bp. The three pairs of primers (P-1, P-2, P-3 F, and PR) (Table 2), which were progressively located closer to the transcription starting site of *CAPN1*, were employed to construct three plasmids called *pGL3-869* (P1), *pGL3-537* (P2), and *pGL3-273* (P3), respectively. We subsequently analyzed the activity of the reporter constructs. The forward and reverse primers contained restriction sites for *MluI* and *HindIII*, respectively. The amplified promoter fragments were purified, double-digested with the restriction enzymes, and cloned into the *pGL3-Basic* Luciferase Reporter Vector (Promega).

To examine the effect of different genotypes on *CAPN1* promoter activity, a series of reporter plasmids with different genotypes encompassing the P2 fragments was constructed. Each plasmid harbored a core promoter region with a different genotype. The resulting constructs were named *pGL3-A* and *pGL3-C*.

Transient transfection and luciferase reporter assay

The murine Leydig tumor (MLTC-1) cell line was cultured in RPMI-1640 medium (Sigma) supplemented with 10% fetal bovine serum (Invitrogen) containing 10 mg/L penicillin and streptomycin (Invitrogen) at 37°C in a controlled humidified atmosphere with 5% CO₂. For the luciferase reporter assays, MLTC-1 cells were inoculated in 24-well plates and grown to 70–80% confluency. Transfection was performed using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. Cells were cotransfected with 50 ng of the *pRL-TK* vector DNA (Promega) and 400 ng of either the empty *pGL3-Basic* plasmid or one of the promoter constructs with different lengths and genotypes of the *CAPN1* promoter. The plasmid *pGL3-Basic* was adopted as the negative control and *pRL-TK* was used as the internal control to confirm the difference in transfection. After 24 h of incubation, the cells were washed and lysed in 1.5 mL tubes. A Dual-Luciferase Reporter Assay System (Promega) was used according to the manufacturer's instructions to analyze luciferase activity. All transfections were performed in triplicate and repeated at least three times in independent experiments. Promoter activity was analyzed relative to firefly luciferase activity and normalized against Renilla luciferase activity.

Statistical analysis

Data for luciferase activity were analyzed with GRAPHPAD PRISM version 5 (GraphPad Software) using Tukey's multiple comparison test. Values were expressed as the mean ± SE. The genotypic and allelic frequencies, polymorphism information content, heterozygosity, and effective number of alleles were calculated with POPGENE 32 (ver. 1.31). The association between SNP genotypes and sperm quality traits was analyzed using the general least-square model procedure from SAS 9.0 (Statistical Analysis Software, SAS Institute, Cary, NC, USA). The GLM was as follows: $Y_{ijklmn} = \mu + G_i + A_k + P_j + H_l + e_{ijklmn}$ where Y_{ijklmn} was the observed value of each semen quality trait; μ is the overall mean; G_i is the fixed effect of genotype; A_k is the fixed effect of age; P_j is the fixed effect of the origin of bull; H_l is the effect of farm; and e_{ijklmn} is the random residual error. Differences between groups were considered to be significant at $P < 0.05$.

Figure 1 Western blot analysis of CAPN1 in bull spermatozoa of different genotypes using β -actin as the control. Sperm proteins were resolved by SDS-PAGE analysis and transferred onto a PVDF membrane. The blots were incubated with monoclonal antibodies to CAPN1 (1:2000), followed by secondary donkey anti-mouse antibody (1:10,000) and ECL detection. The arrow indicates calpain bands at 80 kDa, as calculated from the migration of known protein standards.

Results

Expression and localization of CAPN1 in bovine spermatozoa

Western blot analysis was carried out in the spermatozoa to validate the expression and localization of CAPN1. The results showed that CAPN1 protein (80 kDa) was expressed in the adult bull spermatozoa (Fig. 1), which was consistent with the reported molecular mass in somatic cells and in mouse spermatozoa (CAPN1 and CAPN2) (Ben-Aharon *et al.* 2005). Ben-Aharon and coworkers showed that CAPN1 is a constituent of the mouse sperm acrosomal membrane and proposed that CAPN1 may be involved in acrosome-related events (Ben-Aharon *et al.* 2005). Using CAPN1 antibody, we examined the immunological localization of CAPN1 polypeptide. This study demonstrated that CAPN1 was localized in the sperm head including acrosome (Fig. 2).

Different expression of CAPN1 in testes, epididymis, and spermatozoa

qRT-PCR results indicated that the bovine *CAPN1* gene expression exhibited tissue variability (Fig. 3). A higher expression of spermatozoa was observed in an adult bull compared with that in the testicular and epididymal tissues (Fig. 3, $P < 0.05$). In addition, the *CAPN1* gene was highly expressed in the epididymis. Using Western blot analysis with anti-mouse CAPN1 antibody, the CAPN1

Figure 2 Arrowheads indicate that CAPN1 protein was mainly localized in the sperm head. (A) CAPN1 protein signals (blue). (B) Sperm phase-contrast counterpart (green). (C) Merging of CAPN1 protein (blue) and sperm phase-contrast counterpart (green). Image was obtained using an inverted microscope (OLYMPUS) at 40×10. Images were obtained on a 5 μ m scale plate.

Figure 3 Relative expression of the *CAPN1* gene in testes, caput epididymis, cauda epididymis, and sperm. The vertical bars represent standard errors. Means with different lowercase superscripts above the error bars are significantly different at $P < 0.01$.

protein was expressed in bull testes and epididymis (Fig. 4). To further understand the CAPN1 protein expression pattern during spermatogenesis, the testicular and epididymal morphologies of the bulls were analyzed by IHC. CAPN1 protein immunoreactivity was detected in the seminiferous epithelium (which included pachytene spermatocytes, primary spermatocytes, and spermatids), as shown in Fig. 5. IHC revealed the CAPN1 protein expression in epithelial cells throughout the entire bull epididymis, including caput epididymis, corpus epididymis, and cauda epididymis.

Identification of genetic variants within the 5'-flanking region of the *CAPN1* gene

We sequenced a 869 bp segment from the 5'-flanking region of the *CAPN1* gene in Chinese Holstein bulls. One SNP (g.-1256 A>C) was discovered using sequence alignment and compared with the *CAPN1* sequence (GenBank accession No. AC_000186.1) (Fig. 6).

The SNP g.-1256 A>C was found in the transcription factor binding site of the promoter core region. This SNP resulted in the presence of the zinc finger protein 263 (*ZNF263*) binding site, which was eliminated by

Figure 4 CAPN1 expressed in different bull tissues. Western blot analysis of CAPN1 in adult bull tissues with β -actin as the control. (1) caput epididymis, (2) corpus epididymidis (3) testes, (4) cauda epididymis, (5) sperm.

Figure 5 Immunolocalization of CAPN1 in bull seminiferous epithelium and epididymis. (A, B and C) Localization of CAPN1 in adult bull caput epididymis (A), cauda epididymis (B), and testis (C), respectively. The brown area indicates the expressed protein, whereas the blue area is the negative control. These cells were photographed using an inverted microscope (OLYMPUS) at 40×10 . Images were obtained on a $50\mu\text{m}$ scale plate.

the presence of the A allele. These results were used to investigate the potential effects of the genetic variations on the regulation of bovine *CAPN1* gene transcription.

Association between single variation and semen quality traits in Chinese Holstein bulls

The SNP g.-1256 A>C in 206 Chinese Holstein bulls was genotyped via PCR-RFLP. The allelic and genotypic frequencies, as well as values of the test of the g.-1256 A>C SNP in the bovine *CAPN1* gene, are presented in Table 3. The results indicated that the g.-1256 A>C position met with the Hardy–Weinberg equilibrium ($P > 0.05$). We analyzed the associations of the g.-1256 A>C SNP with semen quality traits in 206 Chinese Holstein bulls. The initial sperm motility of bulls with genotype CC in position g.-1256 was significantly higher than that of genotype AA ($P < 0.05$) (Table 4), demonstrating that the C allele in g.-1256 may improve initial sperm motility.

Activity analysis of *CAPN1* promoter

We identified a promoter in the 5'-flanking region in the bovine *CAPN1* gene using bioinformatics software. The predicted results demonstrated that the -1638 bp to -769 bp region was the promoter core area. Thus, we carried out serial truncations of the *CAPN1* promoter fragment in this area. As illustrated in Fig. 7A, the promoter activity of the basic *pGL3* vector was significantly lower than that of all the constructs. The relative luciferase activity of the promoter P2 fragment was upregulated by ~ 3.2 -fold compared with that of P3. The P2 fragment was upregulated by ~ 1.6 -fold compared with that of P1. These results indicated that the *CAPN1* noncanonical core promoter was located within the range of g.-1306 to g.-1012. Several inhibitory transcription element binding sites, which inhibit the transcriptional activity of genes, were found with TFSEARCH Online Software in the region from g.-1638 to g.-769. The results indicated that negative regulatory elements were located in the region from g.-1638 to g.-1306 and g.-1012 to g.-769.

The SNP g.-1256 A>C was located in the noncanonical core promoter region (g.-1306 to g.-1012).

Figure 6 Structure of *CAPN1*, location of the identified SNP (g.-1256 A>C), and PCR-RFLP patterns. Patterns for g.-1256 A>C genotypes AA, AC, and CC. M: Marker. Digestion with FokI of the amplified *CAPN1* gene g.-1256 A>C locus produced fragments of the following sizes: 538bp for genotype CC; 538, 489, and 149bp for genotype AC; and 489 and 149bp for genotype AA.

To further investigate the effect of this SNP on *CAPN1* expression, the 5'-flanking region of the *CAPN1* gene, which contained the A or C loci (designated as *pGL3-A* and *pGL3-C*), was subjected to further functional analysis of the promoter activity. The different loci constructs were included in the P2 fragment. A higher fluorescence intensity of the C allele was detected after transient transfection in MLTC-1 cells (Fig. 7B). The SNP g.-1256 A>C could be considered as a functional variant, which played a significant role in the transcriptional activity of *CAPN1* promoter.

CAPN1 expression in spermatozoa of different genotypes

SNP association analysis indicated that the initial sperm motility of bulls with the genotype CC in position g.-1256 was significantly higher than that of the genotype AA ($P<0.01$). Moreover, a higher fluorescence intensity of the C allele was detected after transient transfection in MLTC-1 cells. Therefore, we expected that the *CAPN1* mRNA expression level would be significantly associated with the SNP. To analyze whether the mutation interferes with *CAPN1* mRNA expression, qRT-PCR experiments were performed on spermatozoa of different genotypes. We found that *CAPN1* was highly expressed in the CC genotypes compared with the AA genotypes ($P<0.05$) (Fig. 8). These findings were consistent with our aforementioned predictions. In addition, the western blot results confirmed these findings, indicating that protein varied in spermatozoa of different genotypes (Fig. 1).

Discussion

For the first time, an explorative view of *CAPN1* through the polymorphism in the promoter region altered gene expression and led to biological changes.

In preceding studies performed in mice (Ben-Aharon *et al.* 2005), we speculated that *CAPN1* begins with the early course of spermatogenesis to spermatozoa and leads to fertilization. As expected, we showed that the mRNA for *CAPN1* was expressed in testes, epididymis, and spermatozoa. This result was confirmed by the western blot results, indicating that protein isoform was present in testes, epididymis, and spermatozoa. qPCR analysis on testes, epididymis, and spermatozoa revealed the highest levels of mRNA expression in spermatozoa and low levels of mRNA expression in testes. Mammalian spermatozoa require extensive sperm plasma membrane remodeling during epididymal transit (epididymal maturation) and in the female reproductive tract (capacitation) to acquire their ability to fertilize (Yanagimachi 1994, Abou-Haila & Tulsiani 2000). During storage in the epididymis, spermatozoa are immotile or barely twitch (Yanagimachi 1994). The mRNA expression of *CAPN1* was relatively higher in spermatozoa than that in the epididymis. This period encompasses the capacitation process. IHC revealed positive staining of *CAPN1* compared with the control at all cells and also at the Leydig cells, in which *CAPN1* was formerly detected in testes (Hou *et al.* 2012). IHC results revealed that the *CAPN1* protein was expressed in epithelial cells throughout the entire bull epididymis, including caput epididymis, corpus epididymis, and cauda epididymis.

Table 3 Genotypic frequencies, allelic frequencies, and genetic diversity (PIC, H_e , N_e , and χ^2) of the bull *CAPN1* gene at position g.-1256 A>C.

SNP loci	Genotype	Sample number	Genotypic frequencies (%)	Allelic frequencies (%) (Allele)	χ^2 (P value)
g.-1158 A>C	AA	65	0.32	0.58 (A)	1.14 (0.29)
	AC	108	0.52	0.42 (C)	
	CC	33	0.16		

H_e , heterozygosity; N_e , effectivity of alleles; PIC, polymorphism information content.

Table 4 Least square means and S.E.M. for semen quality traits of different genotypes in the *CAPN1* gene of 206 Chinese Holstein bulls.

Locus	Genotype/sample no.	Ejaculate volume (mL)	Initial sperm motility (%)	Sperm density ($\times 10^8/\text{mL}$)	Deformity rate (%)	Postfrozen/thawed sperm motility (%)
g.-1158 A>C	AA/65	5.33 $\pm$ 0.23	66.75 $\pm$ 0.66 ^a	10.24 $\pm$ 0.42	16.55 $\pm$ 0.50	42.60 $\pm$ 0.98
	AC/108	6.02 $\pm$ 0.16	69.27 $\pm$ 0.46 ^b	11.14 $\pm$ 0.29	16.07 $\pm$ 0.33	42.78 $\pm$ 0.63
	CC/33	5.69 $\pm$ 0.31	70.47 $\pm$ 0.88 ^b	11.81 $\pm$ 0.57	16.36 $\pm$ 0.66	43.38 $\pm$ 0.98

Means in the same column with different lowercase superscripts (a and b) are significantly different at $P < 0.01$.

Previous studies reported that CAPN1 is confined to the head of mammalian spermatozoa at either the acrosomal cap (Rojas *et al.* 1999, Ben-Aharon *et al.* 2005) or the outer acrosomal membrane (Yudin *et al.* 2000). Using confocal immunofluorescence microscopy, we detected CAPN1 at the acrosome of bull spermatozoa. We also found CAPN1 at the spermatozoa head. The acrosome is formed during the early period of spermiogenesis and represents one of the defining features of spermatozoan development. During this process, various proteins are synthesized and incorporated into the acrosome, where they undergo extensive modifications. One of the mechanisms proposed to be involved in these modifications is selective proteolysis, a process that converts an enzymatically inactive precursor form to an enzymatically active mature form (Abou-Haila & Tulsiani 2000). The localization of CAPN1 strongly implies that it possibly participated in the development and function of the acrosome as well. Moreover, we detected CAPN1 at the acrosomal cap of cattle spermatozoa. The integrity of the acrosome (the large secretory granule located

over the sperm nucleus) is also required at several stages in the sperm's life.

Association analysis showed that the SNP g.-1256 A>C was significantly correlated with the sperm quality trait. Adequate sperm motility was reported to be vital for successful fertilization (Olds-Clarke 1996). Our results were also similar to the analytical results in Holstein-Friesian bulls (Chiu *et al.* 2014, Zhang *et al.* 2015). The SNP g.-1256 A>C may regulate the expression of the bovine *CAPN1* gene via transcription factor *ZNF263* and cause significant potential phenotype diversity. For the *CAPN1* SNP g.-1256, the transcription factor *ZNF263* could appear in the presence of the C allele but disappear in the presence of the A allele. As an important transcription factor of eukaryotes, the *ZNF263* factor could bind to their response element in the gene promoter to regulate the expression of different target genes (Dhaouadi *et al.* 2014). A previous study demonstrated that the potential phenotype diversity may be caused by the genetic variation at the transcription factor binding site (Schild *et al.* 1994, Wagner *et al.* 1994, Wang *et al.* 2005). The polymorphisms in the core promoter region can affect gene expression via transcription factors. For example, the promoter region polymorphisms in the human *MBL2* gene control the baseline expression of *MBL2* (Madsen *et al.* 1998). Therefore, we found that the bulls with genotype CC in g.-1256 A>C exhibited relatively higher initial sperm motility than those with

Figure 7 Schema of the 5'-flanking region of the *CAPN1* gene and the identification of the noncanonical core promoter region. (A) Deletion fragment from g.-1638 to g.-769. The 5'-flanking region was divided into four fragments and cloned into *PGL3-basic* vector. The relative luciferase activity of each recombination vector is indicated to the right of the fragment. (B) One variant g.-1256 A>C is located in the P2 region of the *CAPN1* gene. The horizontal bars represent standard errors. Means with different lowercase superscripts above the error bars are significantly different at $P < 0.05$.

Figure 8 Relative expression of the *CAPN1* gene in sperm of different genotypes at g.-1256 A>C locus. The vertical bars represent standard errors. Means with different lowercase superscripts above the error bars are significantly different at $P < 0.01$.

the AA genotype ($P < 0.05$). The core promoter (g.-1306 to g.-1012) of the *CAPN1* gene containing the C loci *pGL3-C* genotype showed 30% higher transcriptional activity than the genotype *pGL3-A*, whereas the empty vector (*pGL3*) provided the lowest level of luciferase activity than the loci constructs. The different genotypes exhibited various promoter activities, thereby implying that genetic variation would likely regulate *CAPN1* expression. To further explore the relationship between *CAPN1* expression and different genotypes, qRT-PCR was conducted. The results showed that *CAPN1* was more highly expressed in the CC and AC genotypes than in the AA genotype. This outcome was confirmed by the western blot results, indicating that protein varied in spermatozoa of different genotypes. Considering that the bulls with the CC genotype exhibited significantly higher initial sperm motility than the bulls with the AA genotype, we suggested that the high expression of the bovine *CAPN1* gene was conducive to the maintenance of high sperm motility.

We first reported the noncanonical core promoter of the bovine *CAPN1* gene. The method of generating truncated constructs was adopted. The P1 and P2 fragments had a significantly higher effect on decreasing promoter activity. Thus, the *CAPN1* noncanonical core promoter was limited to a 295 bp region (g.-1306 bp to g.-1012 bp), which was consistent with the predicted results of bioinformatics analysis. We chose the MLTC-1 cell line for our experiments because of the high transfection efficiency in MLTC-1 and the high amino acid sequence homology (89.53%) between bovine and murine *CAPN1*.

In summary, *CAPN1* expression was related to the capacitation process, and it may be involved in the development and function of the acrosome. Furthermore, functional SNP g.-1256 A>C at the noncanonical core promoter region played a role in regulating *CAPN1* expression in the spermatozoa. Our findings suggested that the bovine *CAPN1* gene may be a sperm motility-related gene.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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References

- Abou-Haila A & Tulsiani DR 2000 Mammalian sperm formation, contents, and function. *Archives of Biochemistry and Biophysics* **379** 173–182. (doi:10.1006/abbi.2000.1880)
- Amin AS, Giudicessi JR, Tijssen AJ, Spanjaart AM, Reckman YJ, Klemens CA, Tanck MW, Kapplinger JD, Hofman N, Sinner MF *et al.* 2012 Variants in the 3' untranslated region of the KCNQ1-encoded Kv7.1 potassium channel modify disease severity in patients with type 1 long QT syndrome in an allele-specific manner. *European Heart Journal* **33** 714–723. (doi:10.1093/eurheartj/ehs473)
- Ben-Aharon I, Brown PR, Etkovitz N, Eddy EM & Shalgi R 2005 The expression of calpain 1 and calpain 2 in spermatogenic cells and spermatozoa of the mouse. *Reproduction* **129** 435–442. (doi:10.1530/rep.1.00255)
- Bettgowda & Wilkinson MF 2010 Transcription and post-transcriptional regulation of spermatogenesis. *Philosophical Transactions of the Royal Society B, Biological Sciences* **365** 1637–1651. (doi:10.1098/rstb.2009.0196)
- Cassinello J, Abaigar T, Gomendio M & Roldan ER 1998 Characteristics of the semen of three endangered species of gazelles (*Gazella dama mhorr*, *G. dorcas neglecta* and *G. cuvieri*). *Journal of Reproduction and Fertility* **113** 35–45. (doi:10.1530/jrf.0.1130035)
- Chiu PC, Lam KK, Wong RC & Yeung WS 2014 The identity of zona pellucida receptor on an unresolved issue in developmental biology. *Seminars in Cell & Developmental Biology* **30** 86–95. (doi:10.1016/j.semcdb.2014.04.016)
- Chorley BN, Wang X, Campbell MR, Pittman GS, Noureddine MA & Bell DA 2008 Discovery and verification of functional single nucleotide polymorphisms in regulatory genomic current and developing technologies. *Mutation Research* **659** 147–157. (doi:10.1016/j.mrrev.2008.05.001)
- Coureuril M, Fouchet P, Prat M, Letallic B, Barroca V, Dos Santos C, Racine C & Allemand I 2006 Caspase-independent death of meiotic and postmeiotic cells overexpressing p53: calpain involvement. *Cell Death and Differentiation* **13** 1927–1937. (doi:10.1038/sj.cdd.4401887)
- Dhaouadi N, Li JY, Feugier P, Gustin MP, Dab H, Kacem K, Bricca G & Cerutti C 2014 Computational identification of potential transcriptional regulators of TGF- β 1 in human atherosclerotic arteries. *Genomics* **103** 357–370. (doi:10.1016/j.ygeno.2014.05.001)
- Gao Q, Ju Z, Zhang Y, Huang J, Zhang X, Qi C, Li J, Zhong J, Li G & Wang C 2014 Association of TNP2 gene polymorphisms of the bta-miR-154 target site with the semen quality traits of Chinese Holstein bulls. *PLoS ONE* **9** e84355. (doi:10.1371/journal.pone.0084355)
- Guo F, Yang B, Ju ZH, Wang XG, Qi C, Zhang Y, Wang CF, Liu HD, Feng MY, Chen Y *et al.* 2014 Alternative splicing, promoter methylation, and functional SNPs of sperm flagella 2 gene in testis and mature spermatozoa of Holstein bulls. *Reproduction* **147** 241–252. (doi:10.1530/REP-13-0343)
- Haas GG Jr & Beer AE 1986 Immunologic influences on reproductive sperm gametogenesis and maturation in the male and female genital tracts. *Fertility and Sterility* **46** 753–766. (doi:10.1016/S0015-0282(16)49808-0)
- Hering DM, Olenski K & Kaminski S 2014 Genome-wide association study for poor sperm motility in Holstein-Friesian bulls. *Animal Reproduction Science* **146** 89–97. (doi:10.1016/j.anireprosci.2014.01.012)
- Hou Q, Huang J, Ju Z, Li Q, Li L, Wang C, Sun T, Wang L, Hou M, Hang S *et al.* 2012 Identification of splice variants, targeted microRNAs and functional single nucleotide polymorphisms of the BOLA-DQA2 gene in dairy cattle. *DNA and Cell Biology* **31** 739–744. (doi:10.1089/dna.2011.1402)
- Huang SY, Chen MY, Lin EC, Tsou HL, Kuo YH, Ju CC & Lee WC 2002 Effects of single nucleotide polymorphisms in the 5'-flanking region of heat shock protein 70.2 gene on semen quality in boars. *Animal Reproduction Science* **70** 99–109. (doi:10.1016/S0378-4320(01)00202-0)

- Lin CL, Ponsuksili S, Tholen E, Jennen DG, Schellander K & Wimmers K 2006 Candidate gene markers for sperm quality and fertility of boar. *Animal Reproduction Science* **92** 349–363. (doi:10.1016/j.anireprosci.2005.05.023)
- Liu X, Ju Z, Wang L, Zhang Y, Huang J, Li Q, Li J, Zhong J, An L & Wang C 2011 Six novel single-nucleotide polymorphisms in SPAG11 gene and their association with sperm quality traits in Chinese Holstein bulls. *Animal Reproduction Science* **129** 14–21. (doi:10.1016/j.anireprosci.2011.10.003)
- Macqueen DJ, Delbridge ML, Manthri S & Johnston IA 2010 A newly classified vertebrate calpain protease, directly ancestral to CAPN1 and 2, episodically evolved a restricted physiological function in placental mammals. *Molecular Biology and Evolution* **27** 1886–1902. (doi:10.1093/molbev/msq071)
- Madsen HO, Satz ML, Hogh B, Svejgaard A & Garred P 1998 Different molecular events result in low protein levels of mannan-binding lectin in populations from southeast Africa and South America. *Journal of Immunology* **161** 3169–3175.
- O'donnell L, Robertson KM, Jones ME & Simpson ER 2001 Estrogen and Spermatogenesis. *Endocrine Reviews* **22** 289–318. (doi:10.1210/edrv.22.3.0431)
- Olds-Clarke P 1996 How does poor motility alter sperm fertilizing ability? *Journal of Andrology* **17** 183–186. (doi:10.1002/j.1939-4640.1996.tb01773.x)
- Pan Q, Ju Z, Huang J, Zhang Y, Qi C, Gao Q, Zhou L, Li Q, Wang L, Zhong J *et al.* 2013 PLCz functional haplotypes modulating promoter transcriptional activity are associated with semen quality traits in Chinese Holstein bulls. *PLoS ONE* **8** e58795. (doi:10.1371/journal.pone.0058795)
- Rojas FJ, Brush M & Moretti-Rojas I 1999 Calpain-calpastatin: a novel, complete calcium-dependent protease system in human spermatozoa. *Molecular Human Reproduction* **5** 520–526. (doi:10.1093/molehr/5.6.520)
- Saeki M, Kurose K, Hasegawa R & Tohkin M 2011 Functional analysis of genetic variations in the 5'-flanking region of the human MDR1 gene. *Molecular Genetics and Metabolism* **102** 91–98. (doi:10.1016/j.ymgme.2010.08.019)
- Santos DM, Xavier JM, Morgado AL, Solá S & Rodrigues CM 2012 Distinct regulatory functions of calpain 1 and 2 during neural stem cell self-renewal and differentiation. *PLoS ONE* **7** e33468. (doi:10.1371/journal.pone.0033468)
- Schild TA, Wagner V & Geldermann H 1994 Variants within the 5'-flanking regions of bovine milk protein I. κ -casein-encoding gene. *Theoretical and Applied Genetics* **89** 116–120. (doi:10.1007/BF00226992)
- Slaughter GR, Meistrich ML & Means AR 1989 Expression of RNAs for calmodulin, actins, and tubulins in rat testis cells. *Biology of Reproduction* **40** 395–405. (doi:10.1095/biolreprod40.2.395)
- Suarez SS & Pacey AA 2006 Sperm transport in the female reproductive tract. *Human Reproduction Update* **12** 23–37. (doi:10.1093/humupd/dmi047)
- Wagner VA, Schild TA & Geldermann H 1994 DNA variants within the 5'-flanking region of milk-protein-encoding genes II. The β -lactoglobulin-encoding gene. *Theoretical and Applied Genetics* **89** 121–126. (doi:10.1007/BF00226993)
- Wang PQ, Deng LM, Zhang BY, Chu MX & Hou JZ 2011 Polymorphisms of the cocaine-amphetamine regulated transcript (CART) gene and their association with reproductive traits in Chinese goats. *Genetics and Molecular Research* **10** 731–738. (doi:10.4238/vol10-2gmr1091)
- Wang X, Tomso DJ, Liu X & Bell DA 2005 Single nucleotide polymorphism in transcriptional regulatory regions and expression of environmentally responsive genes. *Toxicology and Applied Pharmacology* **207** 84–90. (doi:10.1016/j.taap.2004.09.024)
- Wimmers K, Lin CL, Tholen E, Jennen DG, Schellander K & Ponsuksili S 2005 Polymorphisms in candidate genes as markers for sperm quality and boar fertility. *Animal Genetics* **36** 152–155. (doi:10.1111/j.1365-2052.2005.01267.x)
- Yanagimachi R 1994 Fertility of mammalian its development and relativity. *Zygote* **2** 371–372. (doi:10.1017/S0967199400002240)
- Yudin AI, Goldberg E, Robertson KR & Overstreet JW 2000 Calpain and calpastatin are located between the plasma membrane and outer acrosomal membrane of cynomolgus macaque spermatozoa. *Journal of Andrology* **21** 721–729. (doi:10.1002/j.1939-4640.2000.tb02140.x)
- Zhang X, Wang C, Zhang Y, Ju Z, Qi C, Wang X, Huang J, Zhang S, Li J & Zhong J 2014 Association between an alternative promoter polymorphism and sperm deformity rate is due to modulation of the expression of KATNAL1 transcripts in Chinese Holstein bulls. *Animal Genetics* **45** 641–651. (doi:10.1111/age.12182)
- Zhang S, Zhang Y, Yang C, Ju Z, Wang X, Jiang Q, Sun Y, Huang J, Zhong J & Wang C 2015 The g.-165 T>C rather than methylation is associated with semen motility in Chinese Holstein bulls by regulating the transcriptional activity of the HIBADH gene. *PLoS ONE* **10** e0127670. (doi:10.1371/journal.pone.0127670)

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