



A novel splice variant of the bovine *GALNTL5* gene identified in Chinese Holstein bull testis tissue and its mRNA expression

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ABSTRACT. The polypeptide N-acetylgalactosaminyltransferase-like protein 5 (GALNTL5) is a newly identified protein that is specifically expressed in testis tissue and participates in spermatogenesis. In this study, we characterized a novel bovine *GALNTL5* splice variant, designated as GALNTL5-AS, by using real-time polymerase chain reaction (RT-PCR) and clone sequencing methods. The novel GALNTL5 isoform was derived from the complete transcript, GALNTL5-complete, via alternative splicing (AS). The pattern of the splice variant was exon skipping. Bovine GALNTL5 transcripts were expressed in the testis, as demonstrated by RT-PCR. The expression levels of both transcripts were higher in adult testes than in calf testes ($P < 0.05$). In addition, prediction analysis showed that the GALNTL5-AS transcript only encoded 122 amino acids and lost its glycosyltransferase 1 and Gal/GalNAc-T motifs, which may result in a dysfunctional protein compared with the predominant transcript GALNTL5-complete.

This study improves our understanding of the bovine *GALNTL5* gene function during bull sperm formation.

Key words: Alternative splicing; Chinese Holstein bull; Testis; *GALNTL5* gene; Quantitative real-time polymerase chain reaction

INTRODUCTION

The polypeptide N-acetylgalactosaminyltransferase-like protein 5 (*GALNTL5*), also described as pp-GalNAc-T19 (Peng et al., 2010) or GalNAc-T20 (Raman et al., 2012), belongs to the polypeptide N-acetylgalactosaminetransferase (pp-GalNAc-T) gene family. *GALNTL5* possesses the highly conserved catalytic domains of pp-GalNAc-T but uniquely truncates the conserved lectin domain at the C-terminus. The *GALNTL5* mRNA is expressed exclusively in the testes, mainly in the cytoplasm of round and elongated spermatids during spermiogenesis and not in the outermost cells of the seminiferous tubules, which contain spermatogonia and somatic Sertoli cells (Takasaki et al., 2014). *GALNTL5* is indispensable for the formation of mature sperm and may have a unique role in mammalian spermiogenesis. In round spermatids, *GALNTL5* is distributed in the juxtanuclear space and not in the acrosomal vesicles. In elongated spermatids, *GALNTL5* is highly localized in the acroplaxome, which is the region between the developing acrosome and nucleus (Kierszenbaum et al., 2003). During differentiation, the signals are also weakly detected in the transient manchette containing microtubules (Kierszenbaum and Tres, 2004). In epididymal spermatozoa, *GALNTL5* is concentrated to the neck region around the head-tail coupling apparatus and is only weakly detected in the midpiece. Notably, *GALNTL5* also temporarily localizes in the acroplaxome and manchette, which is a transient microtubular/actin structure, during spermatid elongation and finally accumulates in the neck region around the head-tail coupling apparatus of mature spermatozoa. These findings lend strong evidence for *GALNTL5* being involved in spermiogenesis (Takasaki et al., 2014).

Several studies have indicated that the protein components involved in vesicle cargo transport and the ubiquitin-proteasome system alter the distribution of differentiating spermatids through a route similar to that of *GALNTL5* (Kierszenbaum et al., 2011). *GALNTL5* participates in the transport of vesicle cargos and in the ubiquitin-proteasome system in the region of the head-tail coupling apparatus for mature sperm formation. In the spermatozoa of heterozygous mutant mice, some researchers observed impairment of protein loadings into acrosomes and aberrant localization of the ubiquitin-proteasome system. Moreover, co-localization of *GALNTL5* signals with UBC3B, an E2 ubiquitin conjugating enzyme similar to UBC3/CDC34, and ubiquitin signals was observed in the ubiquitin-proteasome system in the neck region of spermatozoa from wild-type mice (Takasaki et al., 2014).

Spermatogenesis is a very intricate and tightly regulated process in which germ cells at each stage, including the proliferative phase (spermatogonia), meiotic phase (spermatocytes), and differentiation or spermiogenic phase (spermatids), are produced in a coordinated manner (Eddy, 2002). Different stages of germ cells possess distinct gene expression levels, which are regulated at transcriptional (Goldberg, 1996), post-transcriptional (Wiszniak et al., 2011), translational (Baker and Fuller, 2007), and post-translational (Lalancette et al., 2006) levels.

Alternative splicing (AS) is an essential function by which post-transcriptional gene regulation occurs in cells (Ochs et al., 2012), with no exception to intrinsic regulation in

germ cells. This phenomenon is universal to numerous protein-coding genes of multicellular organisms. In general, alternatively spliced exons have 5'- and 3'-splice site motifs that differ significantly from the normal motifs. In addition to these splice sites, exons are defined by *cis*-acting regulatory elements, which have been divided into four functional categories: exonic splicing enhancers, exonic splicing silencers, intronic splicing enhancers (also known as intronic activators of splicing), and intronic splicing silencers. These *cis*-acting elements interact directly or indirectly with *trans*-acting activators or repressors of splicing (Keren et al., 2010). In a novel splice variant PLCz1-sv1 in Chinese Holstein bulls, the AS pattern of alternating 5'-splice sites is characterized in the testis tissues (Ju et al., 2014). Thus, AS may be an additional mechanism by which the diverse physiologic functions of the *GALNTL5* gene are regulated. To date, little is known about *GALNTL5* mRNA expression in bull, and splice variants of bovine *GALNTL5* have not yet been characterized. In the present study, we identified *GALNTL5* splice variants in Chinese Holstein bull testis tissue.

MATERIAL AND METHODS

Collection of tissue samples

Tissue samples, including heart, liver, spleen, lung, kidney, and testis, were collected from 13 randomly culled Chinese Holstein cattle, including four calves and nine adult bulls. Of these samples, all collected at slaughterhouses, two calves and five adult bulls came from the Shandong OX Bio-Technology Co., Ltd., whereas all other samples came from the Duoshi Liuyingzi Commercial Slaughter Farm in Jinan, Shandong Province, China. Tissue samples were obtained immediately after slaughter, snap frozen in liquid nitrogen, and transported to the laboratory. The tissue samples were kept frozen in liquid nitrogen until RNA isolation and cDNA synthesis for the relative expression analysis of the *GALNTL5* gene and identification of splice variants.

Identification of the splice variants of the bovine *GALNTL5* gene

To investigate potential splice variants of the bovine *GALNTL5* gene, one pair of specific primers *GALNTL5*-cDNA (Table 1) was designed using Primer Premier v. 5.0 software. These primers amplified the full-length transcript and splice variants, according to the bovine *GALNTL5* reference sequence (GenBank accession No. NM_001076441.1). Total RNA was isolated from the 13 bovine tissue samples using an RNAsimple Total RNA Kit (Tiangen, Beijing, China), following the manufacturer instructions. The RNA concentration was measured using a Biophotometer (Implen, Germany) and the RNA integrity was determined via electrophoresis on 1% agarose gel. Then, a RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Canada) was used to convert ~1 µg RNA from each sample into cDNA according to the manufacturer specifications. A polymerase chain reaction (PCR) was performed with an initial denaturation step at 94°C for 5 min, followed by 35 cycles at 94°C for 30 s, 59°C for 30 s, and 72°C for 1.5 min, with a final extension at 72°C for 10 min. Splice variants were identified based on the method used by Ju et al. (2012). In brief, DNA bands were separated using 1.5% agarose gel electrophoresis and purified using a GenClean kit (Cwbiochem, Beijing, China). The purified products were then cloned into the pEASY-T3 vector (TaKaRa, Dalian, China) and transformed into *Escherichia coli* DH5α cells. The cells

were subsequently propagated in LB medium overnight at 37°C. The positive clones were randomly selected.

Table 1. List of primer sets used.

Primer	Primer sequence (5'-3')	Fragment size (bp)	Target
GALNTL5-cDNA	F: GAGCATAGAGTCCTGCCCAA	1637	GALNTL5 gene full-length cDNA amplification
	R: GGTCCAACTTGTCCTTCTCT		
β -actin gene	F: GCACAATGAAGATCAAGATCATC	173	β -actin gene qRT-PCR
	R: CTAACAGTCCGCCTAGAAGCA		
GALNTL5-complete	F: TCAGTGCACCTACCGAGAGC	188	GALNTL5 gene qRT-PCR
	R: ACGTTTCTGTAGTGGCCCAA		
GALNTL5-AS	F: GTTGTTAAGTTCAGGTGTCA	170	GALNTL5 gene qRT-PCR
	R: ATGTCGTCTACCAAAATAAT		

Quantification of abundance of GALNTL5 transcript variants

Quantitative real-time PCR (qRT-PCR) was performed to determine the relative mRNA expression of the *GALNTL5* splice variants in adult bull and calf testes. We designed the corresponding specific PCR primers to determine the differential expression between GALNTL5-complete and GALNTL5-AS (Table 1). The qRT-PCR analysis was performed in a 20- μ L mixture containing 2 μ L cDNA, 0.4 μ M sense and antisense primers, 6.4 μ L dH₂O, and 10.0 μ L 2X SYBR® Premix Ex Taq™ (TaKaRa). The qRT-PCR was performed under the following conditions: pre-incubation at 95°C for 30 s, 40 amplification cycles of 5 s at 95°C followed by 60°C for 30 s. The conditions for the last stage of the melting curve were as follows: 95°C for 5 s, 60°C for 1 min, and 95°C for 15 s. The PCR was monitored using a Light-Cycler® 480 II Real-time PCR system (Roche, Switzerland). In addition, the bovine housekeeping gene β -actin (GenBank accession No. NM_173979.3) was used as an internal control, to normalize alternatively spliced transcription values. Each sample was run in triplicate.

Data mining

AS detection and sequence analysis were conducted using the BLAST online search resource (<http://blast.ncbi.nlm.nih.gov>) and DNAMAN software package (v. 6.0, Lynnon Biosoft, Quebec, Canada). The ORF Finder in NCBI was used to identify the open reading frame. Cross-species genomic comparisons and functional domain localization within GALNTL5 were conducted using the Ensembl database (www.Ensembl.org). The secondary structure of retained introns was predicted using the RNAfold web server (<http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi>). The 3D structure of the putative GALNTL5 protein was analyzed using SWISS-MODEL (http://swissmodel.expasy.org/workspace/index.php?func=modelling_simple1).

Statistical analyses

The relative quantification of *GALNTL5* mRNA expression was calculated using the standard curve-based method for RT-PCR (Larionov et al., 2005). The analyses were performed using SAS v. 9.3 statistical software (SAS Institute Inc., Cary, NC, USA). Data are presented as means or mean \pm SEM. Statistical significance ($P < 0.05$) for gene expression was tested using a Student's *t*-test.

RESULTS

Identification of the bovine *GALNTL5* splice variants

To investigate the possible splicing variants of the bovine *GALNTL5* gene, RT-PCR was performed on bovine heart, liver, spleen, lung, kidney, and testis tissue samples. We only found two main bands in the testis (Figure 1A), after amplifying the full-length *GALNTL5* transcript with the primers specific for *GALNTL5*-cDNA (Table 1) as templates. The two bands of 1637 and 1516 bp were purified, subcloned, and sequenced. When comparing the sequence obtained from each sample with the bovine *GALNTL5* genomic (GenBank accession No. AC_000161.1) and RNA sequence (GenBank accession No. NM_001076441.1), we found a new splice variant in the testis named *GALNTL5*-AS (Figure 1C) lacking exon 2 (Figure 1B). This sequence was submitted to NCBI (GenBank Accession No. KT763324).

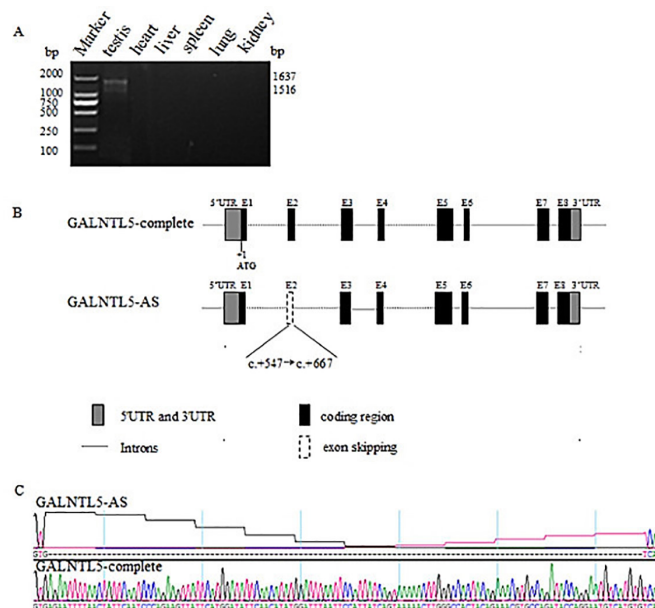


Figure 1. Expression of *GALNTL5* mRNA and identification of bovine *GALNTL5* splice variants. **A.** RT-PCR analysis of *GALNTL5* transcript expression in various adult bull tissues. **B.** Diagrammatic representation of the genomic structure of bovine *GALNTL5* and alternative splicing pattern of *GALNTL5* transcripts. **C.** Sequencing results of *GALNTL5*-complete and *GALNTL5*-AS.

GALNTL5-complete and *GALNTL5*-AS quantification

Selective amplification of *GALNTL5*-complete and *GALNTL5*-AS was achieved by RT-PCR using specific primers (Figure 2A). We then performed relative quantification of the *GALNTL5*-complete and *GALNTL5*-AS transcripts in adult bull and calf testes, using RT-PCR amplification with conditions optimized for each of the isoforms of *GALNTL5*. As demonstrated in Figure 2B, the expression of both transcripts displayed notably higher levels in adult bull testes compared to in calf testes ($P < 0.05$). In addition, in adult bull testes, a

higher expression of GALNTL5-complete than GALNTL5-AS was observed. In contrast, the opposite was observed in calf testes where GALNTL5-AS manifested a higher expression than GALNTL5-complete. These findings were consistent with the RT-PCR results in the current study, suggesting that *GALNTL5* mRNA displayed signs of varying temporal expression.

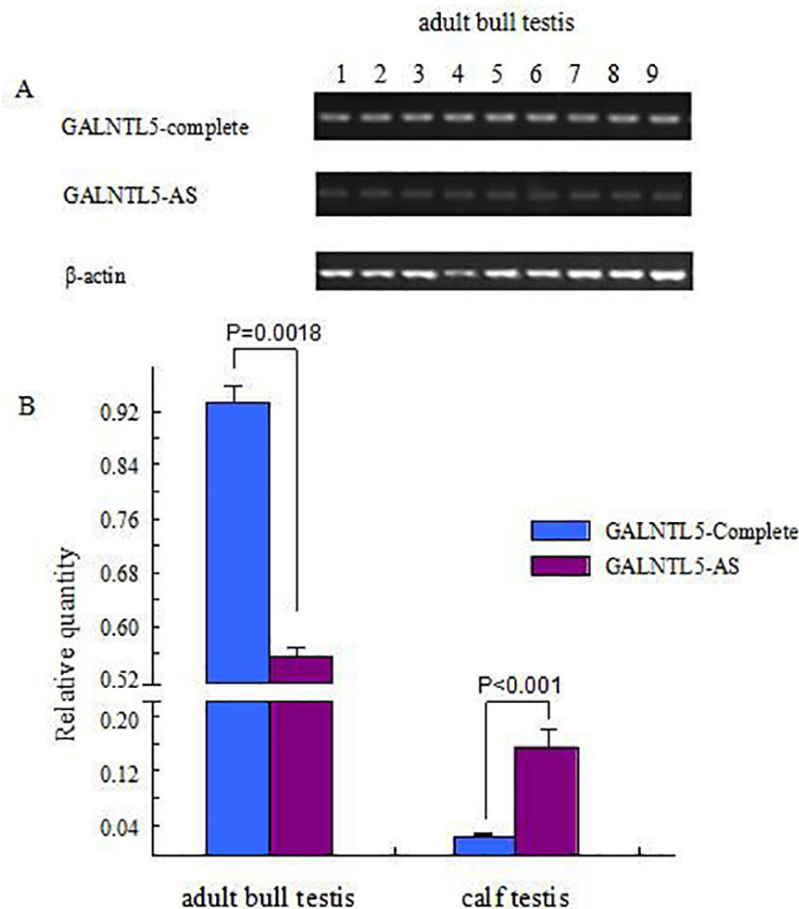


Figure 2. Relative expression of the *GALNTL5* gene. **A.** Expression of the GALNTL5-complete and GALNTL5-AS transcripts in nine adult bull testes using RT-PCR. **B.** Relative expression of GALNTL5-complete and GALNTL5-AS in testis tissue of adult bulls and calves using qRT-PCR. The vertical bars represent standard errors.

In silico analysis of *GALNTL5* mRNA and protein

The open reading frame of GALNTL5-AS with 122 amino acids was predicted by ORF Finder in NCBI. Only the E1 and partial E3 domain were transcribed. The typical domain of the bovine GALNTL5 protein was analyzed utilizing the Ensembl database and protein blast. The structure is presented in Figure 3A. The GALNTL5-AS transcript had a deletion of the glycosyltransferase 1 (GT1) and Gal/GalNAc-T motifs compared with GALNTL5-complete. The 3D structure of the putative GALNTL5 protein also confirmed this finding (Figure 3B).

Cross-species genomic comparisons indicated that the bovine *GALNTL5* protein shared low sequence identity with human (67%) and murine (60%), but *GALNTL5* was conserved in the conserved domains of human, murine, and other bovine species (Figure 4).

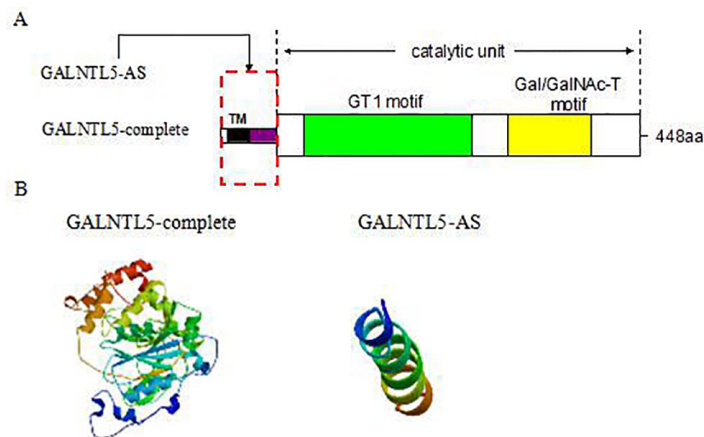


Figure 3. Sketch map of the bovine *GALNTL5* protein. **A.** Linear map of the bovine *GALNTL5* polypeptide shows the domain organization. The red box represents the diagram of *GALNTL5*-AS. **B.** 3D structures of two putative *GALNTL5* proteins produced using the SWISS-MODEL software.

1	MRNVITQCLLCASLTFGINIAVFNIYLYHNVHVKKQEKIQEKNPLIWSLGEKLQSKPDSV	Bovine <i>GALNTL5</i>
1	MKSVITQGLFCGFLAIGLWASMLLLFIH---LEQEDMLENEKEELL---KKRSLGKNA	Mouse
1	MRNAITQGLFYGSLTFGINITALLFIYLYHNVHVSQWKKSQE--PLSAWSPGKAVHQQIIY	Human
61	HLPRAYNVVERASEDEPAEPLKLLSSGENFNYSIFEVIHGYSITYGNSITISNGLHYRN	Bovine <i>GALNTL5</i>
53	H-----QQRHSED-----VTHDEVNFSDPELIQGLRRYGLNAIMSRRLLGIERE	Mouse
59	GSEQIPKPHVIVKRTIDEDKA---KSMGLGTFNHTNFELHKKLLKYGFNVITISRLGIERE	Human
	GT1	
121	VPDIRNVWCQKKMYPAKLPTASIIICFHNEEFNALFRTLSSIMTLIQQYILEEIIILVDDM	Bovine <i>GALNTL5</i>
97	VPDSDKIQQKHYPFNLTASIIICFYNEEFNTILRAVSSVNVLSPOHLLIEEIIILVDDM	Mouse
116	VPDTRSKMCLQKHYPARLPTASIVICFYNEECNALFQTMSSVTNLTPHYFLEEIIILVDDM	Human
181	SDFDLKEKLDYHLEIFRGKIKLIRNKKREGLIRARMTGASHASGDLVFLDSHCEVNRV	Bovine <i>GALNTL5</i>
157	SEFDLKDLDYHLEIFRGKVKLIRNKKREGLIRSKMIGASRASGDLVFLDSHCEVNRV	Mouse
172	SKVDLKEKLDYHLEIFRGKVKIIRNKKREGLIRARLI GASHASGDLVFLDSHCEVNRV	Human
241	WLEPLLNAIAKDPMVVCFLIDVLDYMTLEYQPSPIVRGAFNWRLEFKWHDVLSYEIEGP	Bovine <i>GALNTL5</i>
217	WLEPLLNAIAKDPMVVCFLIDVINELTIDYMAPIVRGAFDWNINLRWNVFAYELDGP	Mouse
236	WLEPLLNAIAKDPMVVCFLIDVIDDRTLEYKPSFLVRGTFDWNLFQKWDNVFSEMDGP	Human
301	EGPTTPIRSPAMAGGIFAINRHYPNEIGQYDKGMNLWGGENLESLRIWMCGGQLFVIIPC	Bovine <i>GALNTL5</i>
277	EGPSTPIRSPAMTGGIFAINRHYPNELGQYDNGMDICGGENVELSLRIWMCGGQLFILPC	Mouse
296	EGSTKPIRSPAMSGGIFAIRRHYPNEIGQYDKMDFWGRENLESLRIWMCGGQLFIIPC	Human
361	SRVGHINRQHVINRFEIMKVVEYNLRIVHTWLDEYKGOFFLRRLKSAAYGNISERVE	Bovine <i>GALNTL5</i>
337	SRVGYNSKALSQHRANQSALSRNLLRVVHTWLDEYKGNFFLQRPSTLYVSCGNISERVE	Mouse
356	SRVGHISKKQTKPSTIISAMTHNYLRVHTWLDEYKEOFFLRKGLKYVTYGNISERVE	Human
	pp-GalNAc-T	
421	LRKRLGCKSFQWYLDITIFFELE	Bovine <i>GALNTL5</i>
397	LRKRLGCKSFQWYLDNIFFELE	Mouse
416	LRKRLGCKSFQWYLDNVFFFELEAS	Human

Figure 4. Comparison of the bovine, murine, and human *GALNTL5* amino acid sequences. The bovine *GALNTL5* amino acid sequence (NCBI: NP_001069909.1) was aligned to the human (NCBI: NP_660335.2) and murine (NCBI: NP_080725.2) sequences using BLAST. Identical amino acid residues within the three species are highlighted in blue.

DISCUSSION

Functionally mature sperm must be motile and have a fully formed acrosome for normal fertilization in mammals. *GALNTL5* is a functional molecule indispensable for mammalian sperm formation. The *GALNTL5* mRNA is mostly expressed in the testes. *GALNTL5* temporarily localizes in the acroplaxome and manchette, finally accumulates in the neck region of mature spermatozoa. Takasaki et al. (2014) found a *GALNTL5* heterozygous mutation in mice. This mutation attenuated the amounts of glycolytic enzymes required for motility, including acrosin, N-ethylmaleimide-sensitive factor, and testicular angiotensin-converting enzyme (Takasaki et al., 2014). The mutation also disrupted protein loadings into acrosomes and caused aberrant localization of the ubiquitin-proteasome system in the neck region of the spermatozoa, which consist of common cytoplasmic constituents shared among the haploid spermatids through the intercellular bridges (Takasaki et al., 2014). In the present study, we found that the expression of *GALNTL5* mRNA was mostly restricted to the bovine testis tissue, as shown by RT-PCR. However, *GALNTL5* in adult bull displayed higher expression than that in calf. Therefore, we speculate that the *GALNTL5* gene is also an vital functional molecule during bull sperm formation.

AS is ubiquitous in bacteria and humans (Keren et al., 2010). It influences the coding sequence, leading to the production of diverse proteins (Graveley, 2001; Swami, 2009). New high-throughput sequencing technology has revealed that 90% of human genes are involved in AS (Wang et al., 2008). Takasaki et al. (2014) also found that the human *GALNTL5* gene had one heterozygous nucleotide deletion at the sixth exon resulting in a stop codon of the sixth exon, which caused a frame shift in the asthenozoospermic sperm of human. This mutation formed a new splice variant that decreased the amounts of *GALNTL5* and contributed to asthenozoospermia. In the present study, a new *GALNTL5* splice variant named *GALNTL5*-AS was identified in Chinese Holstein bulls through cloning of *GALNTL5* mRNA, which showed a skipping of the second exon. In addition, *in silico* analysis showed that the *GALNTL5*-AS transcript only encoded for 122 amino acids. The transcript had lost its GT1 and Gal/GalNAc-T motifs and may ultimately result in disruption of the spermatogenetic function compared with the primary transcript *GALNTL5*-complete. *GALNTL5* is classified as a member of the pp-GalNAc-T family, because it possesses the highly conserved catalytic domains of pp-GalNAc-T but uniquely lacks the conserved lectin domain at the C-terminus. The pp-GalNAc-T family belongs to the type II membrane proteins (Roth et al., 1994; Röttger et al., 1998). Their fundamental structure encompasses a short cytoplasmic tail; a single transmembrane anchoring domain; a stem region with variable length; a catalytic domain with GT1 and Gal/GalNAc-T motifs, which are the putative binding sites for donor and acceptor substrate, respectively; and a C-terminus ricin-type lectin domain (Hazes, 1996). Thus, since *GALNTL5*-AS is lacking the GT1 and Gal/GalNAc-T motifs, it may affect bovine sperm formation.

In conclusion, a novel *GALNTL5* splice variant named *GALNTL5*-AS was identified, and different splicing patterns were detected in bovine testes. We detected expression levels of two transcripts in adult bull and calf testes and found that the *GALNTL5*-AS may be involved in spermatogenesis. This study may ultimately facilitate a better understanding of the *GALNTL5* gene structure and function. However, the biologic significance of the *GALNTL5*-AS transcript requires further exploration, including more replicated studies.

Conflicts of interest

The authors declare no conflict of interest.

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