Research paper

Transcripts abundance of TNP 1 and 2 genes in crossbred bull spermatozoa and insight into TNP2 3'-UTR polymorphism of the bta-miR-154 target site

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ABSTRACT

Spermatogenesis involves a sequential replacement of histones with transitional nuclear proteins 1 and 2 (TNP1, TNP2), which are eventually replaced by porotamine 1 (PRM1) and protamine 2 (PRM2). These chromatin-remodeling proteins may decide organism's reproductive success by playing vital role in sperm formation and preserving sperm DNA integrity. The aim was to explore the transcript abundance of TNP1 and TPN2 genes in spermatozoa of normozoospermic (normal) and astheno-normozoospermic (motility impaired) Frieswal bulls semen as a footprint of successful spermatogenesis and gain insight into TNP2 3'-UTR polymorphism (g.1536 C > T) at the bta-miR-154 target site. While sperm from the normozoospermic group showed higher levels of TPN2 mRNAs compared to astheno-normozoospermic, there was no significant difference in transcript abundance (P>0.05) between groups. Almost comparable transcripts of the TPN1 gene were observed in both the groups (P>0.05). Screening of the SNP g.1536 C>T at 3'-UTR of TNP2, which alters the binding to the bta-miR-154 seed sequence, revealed very high frequency of T allele in the populations (HF-0.81, FW-0.91 and Jersey-0.97). The occurrence of frequent TT genotype and T allele in bulls probably indicates that functional seed sequence for bta-miR-154 at 3'UTR of TNP2 gene (due to the presence of T allele) might have selective advantages towards regulation of TNP2 expression at testicular tissue and successful spermatogenesis. At the same time, it seems that the TT genotype does not affect differential TNP2 transcript abundance and sperm motility in mature spermatozoa of crossbred Frieswal bulls.

Keywords: Frieswal, sperm RNA, Percoll, spermatogenesis, Jersey, HF

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INTRODUCTION

In the process of spermiogenesis, both histones and non-histone proteins are initially replaced by transition proteins (TNP-1/2). Later, the transition proteins are being replaced by protamines (PRM-1/2), the major nuclear proteins of elongated spermatids and mature spermatozoa. (Hecht 1990; Oliva and Dixon 1991; Dadoune 1995; Siffroi et al., 1999; Steger 1999). Introduction of protamine results in a highly condensed, transcriptionally innert chromatin (Dadoune 1995), central to germ cell maturation processes and is exclusive to haploid cells (Sassone-Corsi 2002). The transcriptionally silent mature spermatozoa carry a range of mRNA molecules, presumed to be originated from trapped cytoplasmic content remaining after spermiogenesis (Gilbert et al., 2007). These untranslated mRNA may perhaps serve as a “Markers” of spermatogenesis since many of them might directly or indirectly be involved in chromatin packaging, early embryonic development, genomic imprinting and male fertility (Miller et al., 2005).

Transition proteins are necessary for normal processing of protamines and consequently, the completion of chromatin condensation. It has been demonstrated that normal morphology, DNA integrity, as well as progressive motility are reduced in TNP1- and TNP2-null double mutant mice (Miyagawa et al., 2005). Significantly lower levels (P< 0.05) of spermatid-specific linker histone H1-like protein (HILS1), TNP1, and TNP2 transcript levels are observed in spermatozoa isolated from normozoospermic (Grade a + b motility in % 53≠ 21)compared to asthenozoospermic (Grade a + b motility in % 23 ± 12.4) men (Jedrzejczak et al., 2007) indicating their role in sperm motility.

Testicular tissues also express a large number of microRNAs (miRNAs), which are implicated in the regulation of spermatogenesis (Papaioannou and Nef 2010; Huang et al., 2011). The miRNAs are the small (~22 nucleotides) endogenous non-coding RNA molecules found in animals, plants and some viruses. They are mainly involved in RNA silencing and post-transcriptional regulation of gene

expression (Ambros 2004; Barthel 2004; He and Hannon 2004). These miRNAs function via base-pairing with complementary sequence within the 3'-untranslated region (3'UTR) of mRNA molecules and inhibit the target mRNA translation into protein or accelerate mRNA degradation (Barthel 2004; Fabian et al., 2010).

Studies in humans and rhesus monkeys have shown differential expression of miRNA-154 in immature and mature testicular tissues that in turn regulate the expression of a series of genes which are essential for the formation and differentiation of different types of cells especially spermatocytes and spermatids (Yan et al., 2009). In-silico prediction shows the presence of miRNA target sites at 3'UTR of bovine TNP1 (for bta-miR-532 & bta-miR-204) and TNP2 (for bta-miR-154) genes. These functional SNPs at miRNA target sites of TNP1 and TNP2 genes have recently been associated with semen quality traits in Chinese Holstein bulls (Zhang et al., 2015; Gao et al., 2014).

In India, although more than 50 % of young crossbred bulls semen are being rejected straight away due to of poor semen quality, poor libido, poor freezability, non-response to artificial vagina (AV) etc, however, rejection on the basis of poor semen motility was prominent one (Sethi et al., 1989; Varghese et al., 2002; Tyagi et al.,2006). We also observed that a large number (66.26%, 216 of 326) of Frieswal crossbred bulls (5/8 Holstein Friesian- 3/8 Sahiwal) are rejected from semen production stations due to poor semen quality and unacceptable freezability. Among non-freezable quality semen producing bulls, 28.70% were oligo-asthenozoospermic (average sperm concentration below 600 X 10^6 /ml and average initial progressive motility < 40%), 66.67% astheno-normozoospermic (initial progressive motility <40% and average sperm concentration> 600 million/ml) and rest 4.63% normozoospermic (average motility > 40%, average concentration >600 X 10^6 /ml) but rejected due to unsatisfactory semen freezability (i.e. post thaw motility below 40%) (Mandal et al., 2012)

Earlier, we have studied relative transcripts abundance of protamine genes (PRM1, PRM2 and PRM3) in normal and motility impaired semen producing crossbred Frieswal (5/8 HF -3/8 Sahiwal) bulls and observed higher PRM1 transcripts level ($p < 0.05$) in normal bulls compared to poor quality semen producer. However, no significant transcripts difference ($p > 0.05$) was observed between the groups for PRM2 and PRM3 (Ganguly et al., 2013; Kumar et al., 2014). The present research was undertaken to explore relative transcript abundance of TNP1 and TPN2 genes in spermatozoa of normal (normozoospermic) and motility impaired (astheno-normozoospermic) Frieswal bulls semen using real time quantitative PCR. Attempt was also made to have an insight into TNP2 3'-UTR polymorphism of the bta-miR-154 target site with sperm motility in crossbred bulls as well as the status of this polymorphism in Jersey and HF bulls under use in India.

MATERIALS AND METHOD

Sample collection and RNA isolation

Fresh ejaculates from Frieswal bulls were collected and tested for standard seminal parameters such as ejaculate volume (ml), concentration (10^6/ml), initial progressive motility (%) and post thaw motility (%) following standard procedures (Mandal et al., 2012; Kumar et al., 2015). Later, they were categorized into normal (Good/ normozoospermic, % initial progressive motility: 56.54 ± 1.38; n=8) and motility impaired (Poor/ astheno-normozoospermic, % initial progressive motility: 20.12 ± 1.59, n = 9) groups. Semen samples were subjected to discontinuous (40:80) Percoll gradient centrifugation (700 g for 30 min), specifically to eliminate damaged spermatozoa and contaminating somatic cells. The pellets containing the motile spermatozoa were kept at -80^{0}C in RNAlater (Ambion, Austin, TX, USA) until RNA extraction. Buffalo (*Bubalus bubalis*) testis tissues were collected from a local abattoir and transported to the laboratory in RNA later and stored at -80^{0}C before use. Total RNA was extracted from sperm pellet and buffalo testicular tissue samples using hot Tri reagent (Ganguly et al., 2013). From each sample, matching number of spermatozoa (approximately 100 million) were taken for RNA isolation. RNA quantity and purity was measured with Nanodrop ND-1000 spectrophotometer (Nanodrop, Thermo Fisher Scientific, Wilmington, Delaware USA).

Quality assessment of RNA isolated from spermatozoa

Total RNA (~60 ng) extracted from crossbred bull spermatozoa and buffalo (*Bubalus bubalis)* testicular tissues were subjected to reverse transcription using Protoscript First Strand cDNA Synthesis Kit (New England Biolabs, Beverly, MA) as per manufacturer's recommendations. The complementary DNA were then amplified by PCR to check contamination, within isolated Sperm RNA, by g-DNA, testicular germ cells like spermatocytes and epithelial cells using specific primers (Table 1). The reverse transcription PCR (RT-PCR) products were resolved in 1x Tris-acetate EDTA, 2 % agarose gel stained with ethidium bromide. Genomic DNA contamination of the samples was tested by PCR using intron spanning primers specific to bovine deleted azoospermia-like (DAZL) and protamine 1 (PRM1) genes. The positive control was a purified bovine genomic DNA extracted from blood of a Frieswal bull using GenElute™ Blood Genomic DNA Kit (Sigma–Aldrich). Absence of germ cell and epithelial cell in sperm RNA was tested by RT-PCR using primers specific to molecular markers like KIT and CDH1, respectively; where cDNA isolated from buffalo

Table 2: Primer sequences to check the contamination or qPCR amplification of specific transcripts in the RNA isolated from spermatozoa

Contamination	Gene/ marker	Primer name/ sequences**	GenBank ID	Product size size (bp)
For checking of contamination				
Genomic DNA	PRM1	PRM1F- 5'AGATACCGATGCTGCCTCAC3' PRM1R-5'GTGGCATGTTCAAGATGTGG3'	NM_174156	234 (334 with intron)
Genomic DNA	DAZL	DAZL-F 5' CAC CAG CCA AGG CTA TGT TT 3' DAZL-R 5' CAC CAG TTC GAT CCG TGA TT 3'	NM_001081725	158 (573 with intron)
Somatic cell	Somatic cell	CDH1-F 5'CCG TGA GAG TTT TCC CAC AT 3' CDH1-R 5'CAT TGG TGA CTG GGT CTG TG 3'	NM_001002763.1	296
Somatic cell	v-kit oncogene homolog	KIT-F 5'GAC CTG GAG GAC TTG CTG AG3' KIT-R 5'AGG GGC TGC TTC CTA AAG AG3'	XM_612028	316
For qPCR amplification of specific transcripts				
qPCR target	TPN1	TNP1F 5'GACCAGCCGCAAATTAAAGA 3' TNP1R 5' TTTGCTGCCACTTCTTTTGA 3'	NM_174199.2	85
qPCR target	TPN2	TNP2 F- 5'ACAGACACACCATGCACTCC 3' TNP2 R- 5' CTTGATCACCTTTCCCTCCA 3'	NM_174200.1	100
qPCRendogenous control	PPIA	PPIA-F 5' ATG CTG GCC CCA ACA CAA PPIA-R 5' CCC TCT TTC ACC TTG CCA AA 30	XM_001252921.1	100

**Ganguly et al. 2013; Ganguly et al. 2016

testicular tissue was used as positive control. Diluted cDNA (1:10) was used to perform the downsteam PCR amplification and real-time qPCR.

Real time expression analysis

Real-time qPCR (Step One, Applied Biosystems, Foster City, CA, USA) was used to analyze the differential expression of candidate gene TNP1 and TNP2 in mature spermatozoa of normal (good) and motility impaired (poor) crossbred Frieswal bull using SYBR GREEN chemistry. Purified bovine genomic DNA extracted from blood sample of a Frieswal bull was used as a positive control. The PPIA gene was used as an endogenous control. Following the manufacturer's instruction, a final volume of 10 µl reaction mixture contains 2µl of template (50 ng/ µl -cDNA), 5 µl 2X SYBR Green Master Mix (Applied Biosystems® SYBR® Green PCR Master Mix), 0.5 µl of each primer (5 pMol) and 2 µl DNase-/RNase-free sterile water. For PCR cycling conditions, an initial denaturation was carried out at 95°c for 10 min followed by 40 cycles of denaturation at 95°c for 15 s; annealing and extension at 60°c for 1 min. Each sample was run in triplicates and a template negative control was taken for both the genes of interest and endogenous control in each RT-PCR run. Samples were quantified by the ΔΔCt method (Livak and Schmittgen, 2001). The expression values obtained were normalized against PPIA.

Sperm DNA isolation, SNP detection and genotyping

We screened one SNP of TNP2 3'UTR (g.1536 C>T, translation start point is ATG as +1) in 88 bulls (36 HF, 17 Jersey and 35 Frieswal). HF and Jersey bulls were selected randomly with pedigree un-relatedness for 3-4 generations. Frieswal random samples also included bulls from both the groups, categorized for analysis of real-time expression.

All Frieswal bulls were recorded with standard seminal parameters. Genomic DNA was extracted from the sperm samples using standard protocols. The samples were treated with DTT and digested with proteinase K, followed by phenol chloroform extraction and precipitation with isopropanol. DNA content was estimated using Nanodrop ND-1000 spectrophotometer (Nanodrop, Thermo Fisher Scientific, Wilmington, Delaware USA) and the mixture was diluted to 50 ng/µl. All DNA samples were stored at -20^{0}C for subsequent analysis.

We used the proposed PCR-RFLP approach for detecting polymorphism g.1536 C > T at TNP2 3'UTR, the putative site of bta-miR-154 binding (Gao et al., 2014). Polymerase chain reaction (PCR) was carried out from a starting template of around 50 ng of genomic DNA in a final reaction volume of 25µl containing 1× Taq DNA polymerase buffer (Sigma), 1.5 mM $MgCl_2$ (Sigma), 200 µM dNTPs(Sigma), 0.5 µM of each primer and 1 U Taq

polymerase (Sigma). Initial denaturation was kept at 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 54°C for 30 s and 72°C for 30 s and a final extension of 72°C for 5 min. PCR primers and PCR-RFLP tests for genotyping TNP2 gene polymorphism (g.1536 C>T) were shown in table 1. PCR products were visualized in 1.0% agarose gel. The restriction digestion was carried out following manufacturer instructions. Briefly, 10 µl amplicon was digested with 5.0 U of *Hind* III restriction enzyme (HiMedia) in 15 µl final volume overnight at 37°C. Digested products were separated by electrophoresis on a 1.0% agarose gel, stained with ethidium bromide. DNA bands were visualized under ultra-violet light using InGenius 3 gel documentation system (Syngene, UK). Fragment size was estimated by comparing with a co-migratingDNA ladder (2-log DNA ladder-0.1–10.0 kb, NEB, Beverly, MA, USA).

Statistical analysis

Data are presented as mean ± SEM and analyzed by using SPSS statistical program (SPSS 243.0 for Windows; SPSS, Inc., Chicago, IL, USA). Significant differences were determined by one-way ANOVA using the SPSS program. Gene expression patterns of the TNP1 and TNP2 genes between good and poor quality semen producers were compared using Student t-test. Gene (allele) and genotype frequencies were calculated as per standard procedure.

RESULTS AND DISCUSSION

In normozoospermic bulls the ejaculate volume (ml), sperm concentration (10^6 /ml) and initial progressive motility (%) were 5.4±0.22, 970.02±45.32 and 56.54±1.38, respectively; which significantly (P<0.01) differed from astheno-normozoospermic bulls (3.82±0.14, 709.58±47.44 and 20.12±1.59, respectively). In the presence of genomic DNA, the intron-spanning primers produced 573 bp and 334 bp amplicons for targeted genes DAZL and PRM1, respectively (Figure 1, Lane 2 and 5). Corresponding amplicons were of 158 and 234 bp, respectively in pure cDNA. Sperm cDNA samples devoid of genomic DNA contamination were used further for downstream experiments. It is important to mention here that each spermatozoon, haploid spermatid and diploid somatic cell contains ~10–20 fg, ~450 fg and ~10–20 pg of total RNA, respectively (Krawetz, 2005; Gilbert et al., 2007; Goodrich et al., 2007). It shows that each somatic cell holds about 1000 times as much RNA as a mature sperm cell. As a result, somatic RNA contaminants (WBCs, epithelial cells, and immature diploid spermatocytes) may easily alter the sperm transcript profile. Initially, the absence of contamination by somatic and germ cells was confirmed by visual examination of the semen fractions. Later, the absence of contamination of germ cells and epithelial cells in all the RNA extractions

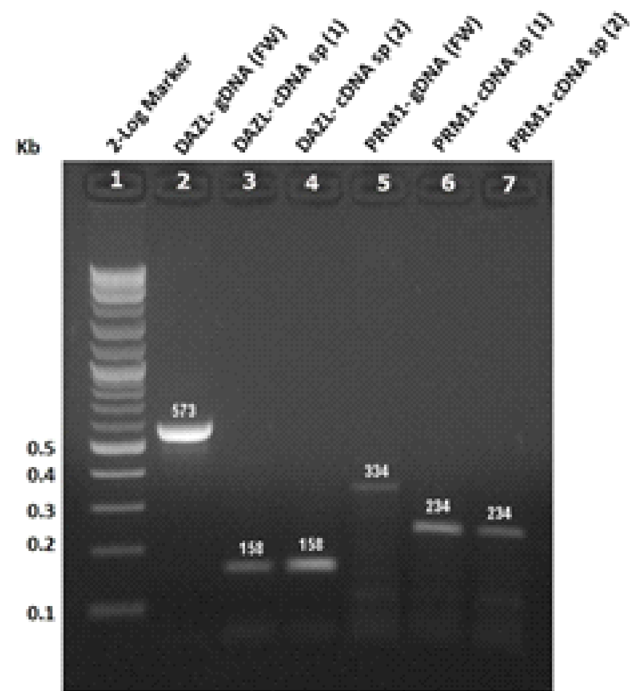

Fig. 1. Checking of genomic DNA contamination from isolated sperm RNA. Spermatozoa cDNA sample giving rise an amplicon of 158 bp corresponding to DazL cDNA without gDNA Contamination. An amplicon of 573 bp is obtained from gDNA with DazL primers; Intron-spanning PRM1primers produce an amplicon of 234 bp from pure spermatozoa cDNA, without gDNA contamination. An amplicon of 334 bp is obtained from gDNA with PRM1primers: M: molecular ladder (2-log DNA ladder-0.1 -10.0 kb, NEB, Beverly, MA, USA)

was tested by RT-PCR by targeting specific markers like KIT and CDH1, respectively (Table 1 and Figure 2). Contamination free RNA sample was chosen for further processing. The presence of clear bands corresponding to transcripts of TNP1 (89 bp) and TNP2 (100 bp) were demonstrated in ejaculated bull spermatozoa (Figure 3).

The mRNA abundance of TNP1 and TNP2 genes were evaluated in mature spermatozoa of good (normal) and poor (motility impaired) crossbred bulls by real-time RT-PCR using SYBR green chemistry. The cDNA synthesized

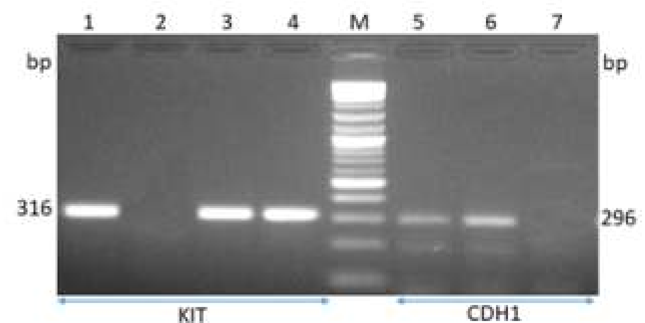

Fig. 2. Contamination of germ cell and epithelial cell in the isolated sperm RNA was tested by PCR targeting specific markers like KIT and CDH1 using an equivalent of 100 ng of sperm cDNA and buffalo testis cDNA samples. The absence of PCR products specific to KIT (lane 2) and CDH1 (lane 7) reveal sample purity. Lane 1,3,4,5 and 6: cDNA from buffalo testicular tissue; Lane 2 and 7: cDNA isolated from spermatozoa of Frieswal bull; M: molecular ladder (2-log DNA ladder-0.1 -10.0 kb, NEB, Beverly, MA, USA)

Table 2. Genotypic and allelic frequencies of TNP2 gene single nucleotide polymorphism at 3'UTR (g.1536 C>T)

Primer Sequences(5'>3')*	Tm	Product size	RE used	Genotype (Number)/ Frequency			Allelic frequency
F:ACTGGACCAATGAACGAA	53.64	535 bp	Hind III	HF-CC(0)/ 0.00	HF-CT (14)/0.39	HF-TT (22)/0.61	HF-T-0.81 HF-C-0.09
				J-CC (0)/0.00	J-CT (1)/0.06	J-TT (16)/0.94	J-T-0.97 J-C-0.03
R: CTCCCTACCCAACCTCTT	54.64			FW-CC (0)/0.00	FW-CT (6)/ 0.00	FW-TT (29)/1.00	FW-T-0.91 FW-C-0.07

*Gao et al., 2014; HF-Holstein Friesian; J-Jersey; FW-Frieswal

from normal buffalo testicular tissue served as positive control. All PCR products displayed a single peak in melting curves and were detected with ethidium bromide stained-agarose gel electrophoresis as single bands of the appropriate size. Samples were quantified by the ΔΔCt method. The expression values obtained were normalized against the housekeeping gene peptidylprolylisomerase A (PPIA) to account for differing amounts of starting material.Good quality semen producing group showed higher level of TPN2 mRNAs expression as compared to the poor quality semen producers, however, transcript abundance was not significantly different between the groups (P>0.05). Almost equivalent expression was observed for TPN1 transcripts in both the groups (P>0.05) (Figure 4). Hence, TNP1 and 2 transcripts abundance in mature spermatozoa may not be a good indicator of progressive sperm motility in Frieswal bulls. Contrary to our findings, spermatozoa from normozoospermic men are found to carry significantly (p<0.05) higher levels of HILS1, TNP1, and TNP2 transcripts compared to asthenozoospermic men (Jedrzejczak et al., 2007).

Hind III digestion of the 535 bp PCR products encompassing TNP2 3'UTR polymorphism (g.1536 C>T) produced only two genotypes: 432- and 103-bp fragments for genotype TT; 535-, 432-, and 103-bp fragments for genotype TC (Figure. 5; Table 2). However, we could not observe genotype CC (535-bp fragment) in any of the bulls under study. Contrary to the finding of Gao et al., 2014, we observed very high frequency of T allele in three populations under study which ranges from 0.81 in HF to 0.97 in Jersey (Table 2). We have recently observed that T allele is fixed in *Bos indicus* breed Sahiwal, Tharparkar and crossbred Karan Fries (HF x Tharparkar) cattle (Ranjan et al., 2018).

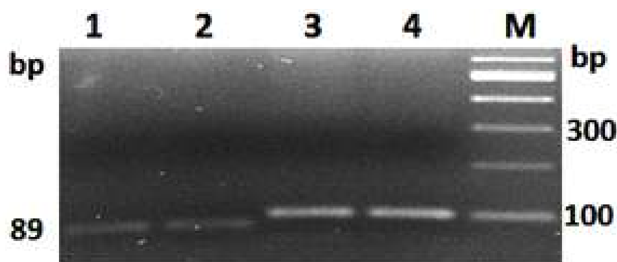

Fig. 3. Agarose gel (1.5%) electrophoresis of 89 bp (lane 1 and 2) and 100 bp (lane 3 and 4) PCR products specific to TNP1 and TNP2 gene, amplified from sperm cDNA. M- Molecular ladder (2-log DNA ladder-0.1–10.0 kb, NEB, Beverly, MA, USA).

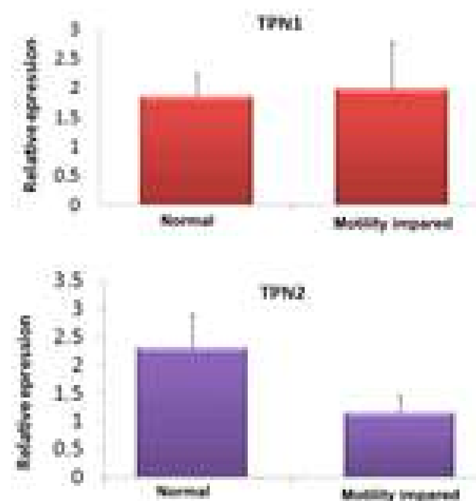

Fig. 4. Relative abundance of (a) TNP1 and (b) TNP2 gene transcripts in bovine spermatozoa obtained from good and poor quality semen producing Frieswal bulls.

No significant differences (P>0.05) were observed between Frieswal bulls with genotype TT and TC for normal semen quality parameters such as ejaculate volume (ml), concentration (10^6/ml), initial progressive motility (%) and post-thaw motility (%) (data not shown). It has earlier been reported that genotype CT displays higher ejaculate volume and post-thaw sperm motility than genotype CC in Chinese Holstein bulls (P< 0.05); however differences between TT-CC and TT-CT genotypes are reported to be non-significant (P> 0.05) for above mentioned traits (Gao et al., 2014). Besides, no differences (P> 0.05) could also be observed between three genotypes (TT, CT and CC) for the initial sperm motility (Gao et al., 2014).

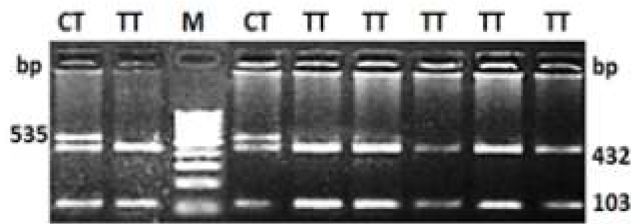

Fig. 5. PCR-RFLP screening of TNP2 3'UTR SNP (g.1536 C>T) in Frieswal, HF and Jersey bulls. TT: 432 and 103 bp; CT: 535, 432 and 103 bp; CC: 535 bp (no bull of this genotype was observed)

Bioinformatics prediction showed that the SNP g.1536 C>T at 3'-UTRs, which alters the binding to the bta-miR-154 seed sequence, may regulate TNP2 gene expression. Only bta-miR-154 has a high likelihood for targeting TNP2 3'-UTR with g.1536 C>T-T. TNP2 binding is found to be absent in g.1536 C>T-C SNP mutation. The same has also been confirmed by luciferase reporter assay in a murine Leydig tumor cell line (Gao et al., 2014). They also observed that the expression of TNP2 mRNA in bull testicles with CT and CC genotypes was significantly higher than that of TT genotype ($P<0.05$), but did not investigate the transcript abundance of TNP2 in mature spermatozoa from bulls of various genotypes (TT, CT and CC). Interestingly, this study revealed that all 17 Frieswal bulls in the normal and motility impaired semen producer category had only one genotype (TT), excluding any plausible function of TT in influencing the differential transcript abundance of the TNP2 gene in mature spermatozoa.

CONCLUSION

Overall, the present study highlights the occurrence of frequent TT genotype and T allele in bulls, which is likely to suggest a selective advantage of bta-miR-154 functional seed sequence at 3'UTR of the TNP2 gene (due to the presence of T allele) in controlling TNP2 expression in testicular tissue and for efficient spermatogenesis. It also shows that the TT genotype in the mature sepmatozoa of Frieswal bulls does not affect the differential TNP2 transcript abundance.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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