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Expression Profile Analysis and Clone of Lnc RNA $_{NONBT-258}$ from Yak and Cattleyak

Shixin Wu, Chuanping Yi, Chuanfei Xu, Wangsheng Zhao, Mujahid Ali Shah, Xin Cai*

School of Life Science and Engineering, Southwest University of Science and Technology, #59 Qinglong Large Road, Fucheng District, Mianyang, Sichuan 621010, China

***Corresponding author:** Xin Cai
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Abstract | **Original Research Article**

Long non-coding RNA (lncRNA) is a kind of RNA segment longer than 200 nucleotides (nt), with little or no protein-coding capacity. In our previous research on the male sterility of cattleyak, a differentially expressed (DE) lncRNA (id: NONBTAT010258) named lncRNA$_{NONBT-258}$ was identified from the testicular tissues of yak and cattleyak through transcriptome sequencing. Genome alignment revealed that lncRNA$_{NONBT-258}$ was located between *RND2* and *BRCA1* genes on bovine chromosome 19 and encoded by one exon. RT-PCR confirmed the expression of lncRNA$_{NONBT-258}$ and its target gene *BRCA1* in the testis of yak and cattleyak, and RT-qPCR showed that the expression trend of these two molecules was consistent with the sequencing results. Furthermore, molecular cloning and resequencing indicated that the lncRNA$_{NONBT-258}$ presented higher similarity among different cattleyak and yak individuals.

Keywords: lnc RNA, yak, cattleyak, RT-PCR, RT-qPCR.

INTRODUCTION

Non-coding RNAs (ncRNAs) are ubiquitous in mammals, and only 40% of the RNA in animals has the ability to be translated into proteins [1]. LncRNA accounts for 80% of ncRNA, most of them are transcribed by RNA polymerase II, and their base composition ranges from 200 nt to 100 000 nt [2]. Accumulated studies have shown that lncRNA is an important structural molecule in mammalian life activities, and regulates gene expression at epigenetic, transcriptional and post-transcriptional levels [3]. The elucidation of the regulatory mechanism of lncRNA has an important role in the growth and development of animals, metabolism of organisms and disease control. However, lncRNA has complex RNA advanced structure, indefinite subcellular localization and various molecular mechanisms of action [4].

Spermatogenesis is an intricately-regulated biological process in which spermatocytes are subjected to two meiotic divisions and haploid spermatids then differentiate into mature spermatozoa [5]. This process is tightly regulated by specific gene expression under the control of a large quantity of lncRNAs which display more tissue-specific expression patterns and have no apparent protein-coding capability [5, 6]. Meanwhile, accumulated evidences demonstrate that mammalian genomes produce many lncRNAs, which are then involved in numerous important biological phenomena [7]. Up to now, a multitude of lncRNAs were identified from specific developmental stages of testis and spermatogenic cells, and were deemed to play a vital role in testis development and spermatogenesis in mouse [8], rat [9] and human [10]. The catalogues of lncRNA have been established for bovine in early embryos [11], mammary glands [12], skeletal muscle [13], Longissimus thoraci [14], skin [15] and MDBK cell infection [16], and the identification of spermatogenesis-related lncRNAs in the testis of cattleyak and yak has also been done in our previous work, while the regulation research for these lncRNAs need to be studied.

Transcriptome sequencing was used in our previous work to screen the DE lncRNAs between cattleyak and yak testis and to examine their potential roles in spermatogenic arrest of cattleyak. We identified hundreds of DE lncRNAs, and selected a lncRNA candidate (id: NONBTAT010258) named lncRNA$_{NONBT-258}$ and its target gene *BRCA1* to analyze their expression profiles in the testicular tissues of yak and cattelyak.

MATERIALS AND METHODS

Sample collection

The procedure of testis sample collection was similar to our previous studies [17, 18], and the samples obtained from three yaks (Maiwa yak, named Y1, Y2 & Y3) and three cattleyaks (F1 of Simmental×Maiwa yak, named C1, C2 & C3) aged 12 months. These animals were all raised on the same ranch in Hongyuan county, Sichuan province of China. The fresh testicular samples were immersed in liquid nitrogen immediately and stored before further processes.

RNA extraction from testicular tissue of yak and cattleyak

The fresh separated testicular tissues stored in liquid nitrogen tanks were taken out, and then ground into powder by a mortar treated with RNaseZap™ RNase Decontamination Solution and DEPC water (Ambion, USA). The total RNA of bovine tissues was extracted by TRIzol reagent (Invitrogen, CA, USA), and the purity and concentration of the RNA were evaluated with a NanoDrop spectrophotometer (peQLab, Erlangen, Germany). The ratio of D260/280 for all samples was about 2.0, which indicated that RNA has high purity and can be used for subsequent experiments.

RT-PCR and RT-qPCR validation of lncRNA$_{NONBT-258}$ and its target gene *BRCA1*

To confirm the repeatability and reproducibility of lncRNA$_{NONBT-258}$ and its target gene *BRCA1* expression data from RNA-seq, RT-PCR and RT-qPCR were performed on all six testis samples from cattleyaks and yaks. Based on the gene sequence of *Bos taurus*, the transcript-specific primers used for validation were designed using Primer v.5.0.0 and concluded in Table-1. For RT-PCR, 1 μg total RNA was transcribed into cDNA and subsequently amplified using PrimeScript™ One Step RT-PCR Kit Ver.2 (Takara) in each reaction, with *GAPDH* as a control. For RT-qPCR, PrimeScript™ RT reagent Kit with gDNA Eraser (Takara) was used for the reverse transcription of 1 μg total RNA. The amplifications were performed in a CFX96 Touch™ Real-Time PCR Detection System (BIO-RAD, USA) and SYBR Premix Ex Taq (Tli RNaseH Plus) (Takara) was chosen to detect the fluorescence signals. All PCR reactions were performed in triplicate, and the relative-expression levels of lncRNA$_{NONBT-258}$ and its target gene *BRCA1* were quantified based on the $2^{-\Delta\Delta Ct}$ method, with *β-actin* as a reference.

Table-1: Primer sequences of lncRNA$_{NONBT-258}$ and its target gene *BRCA1* used for RT-PCR or RT-qPCR*.

Genes	Primers (5′-3′)	Tm (℃)	PCR product (bp)
lncRNA$_{NONBT-258}$*	lncRNA$_{NONBT-258}$-F: 5′-GTCTGGGCCATAGTTGTTGC-3′ lncRNA$_{NONBT-258}$-R: 5′-AAAGCTCCTTTCCTGGTGGG-3′	59 59	225
BRCA1*	BRCA1-F: 5′-GCGTCAGACATTTGCTCTGT-3′ BRCA1-F: 5′-CGCTACATTTGGCATCGTCA-3′	59 59	245
β-actin*	β-actin-F: 5′-AAGTTCTACAGTGTGGCCGA-3′ β-actin-R: 5′-GACTGGCCCCTTCTCCTTAG-3′	59 59	150
GAPDH	GAPDH-F: 5′-CATGTTTGTGATGGGCGTGA-3′ GAPDH-R: 5′-GCCAGTAGAAGCAGGGATGA-3′	59 59	205

Molecular cloning and analysis of the lncRNA

According to the nucleotide sequence of lncRNA$_{NONBT-258}$ for *Bos taurus*, the primers used for RT-PCR were designed and added the restriction sites of *Kpn* I and *Xho* I enzymes (Table-2). The amplification of lncRNA$_{NONBT-258}$ was performed as described for the RT-PCR validation. PCR product was purified using the MiniBEST Agarose Gel DNA Extraction Kit Ver.4.0 (TaKaRa). We used T-Vector pMD19 (Simple) (TaKaRa) to construct recombinant vectors containing the lncRNA using DNA Ligation Kit <Mighty Mix> (TaKaRa), and recombinant vectors were then transformed to *E. coli* DH5α competent cells. Positive clones were screened and identified using the same primers as those used for RT-PCR amplification of lncRNA$_{NONBT-258}$. Plasmid purification was accomplished by using MiniBEST Plasmid Purification Kit Ver.4.0 (TaKaRa) and the recombinant plasmid was sent to the TsingKe Biological Technology Company for sequencing using M13-RV and M13-M4 primers. All the LncRNA sequences for cattleyak and yak were aligned in ClustalW2 online (https://www.ebi.ac.uk/Tools/msa/clustalo) with parameters set to default using the sequence of lncRNA$_{NONBT-258}$ from *Bos taurus* as the reference.

Table-2: Primer sequences used for molecular cloning of lncRNA$_{NONBT-258}$.

LncRNA	Primers (5′-3′)	Tm (℃)	PCR product (bp)
lncRNA$_{NONBT-258}$	lncRNA$_{NONBT-258}$-Kpn Ⅰ-F: 5′-CGGGGTACCAGGGTGAGCTGGAGGCACAGAGTGG-3′ lncRNA$_{NONBT-258}$-Xho Ⅰ-R: 5′-CCGCTCGAGTGGAAGTGTTTGCTACCAAGTTTAT-3′	72.9 64.5	756

Statistical analysis

All the values were calculated as mean ± SEM (standard error of the mean), and the significance analyses were performed by utilizing Students't-test implemented in GraphPad Prism7.01 software.

RESULTS

Validation of selected lncRNA and its target gene

To confirm the expression pattern of the identified lncRNA and its target gene involved in mitotic cell cycle processes *in vivo*, lncRNA$_{NONBT-258}$ and *BRCA1* were selected for RT-PCR and RT-qPCR validation. As shown in Fig-1, lncRNA$_{NONBT-258}$ and its target gene *BRCA1* could be amplified by RT-PCR from total RNA of the testis samples from each cattleyak and yak. Therefore, the lncRNA and its target gene were truly expressed during spermatogenesis *in vivo*. The results of RT-qPCR showed that the expression levels of lncRNA$_{NONBT-258}$ and *BRCA1* in cattleyak were all lower than those of yak, which validated the expression pattern of lncRNA$_{NONBT-258}$ and its target gene *BRCA1* were consistent with the data from RNA-seq (Fold change(C/Y)=0.26 and 0.37 for lncRNA$_{NONBT-258}$ and *BRCA1*, respectively.) (Fig-2).

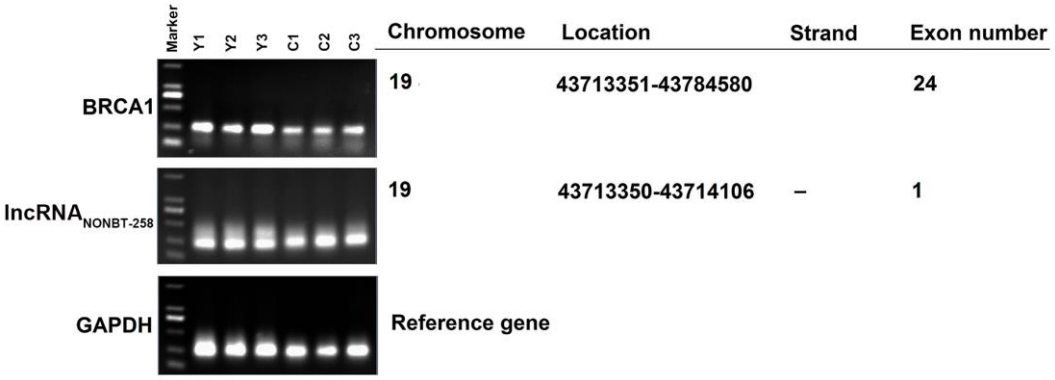

Fig-1: The expression pattern of lncRNA$_{NONBT-258}$ and its target gene *BRCA1* in the testis of yak and cattleyak
The chromosome location, DNA strand and exons numbers for these two molecules were presented. Marker: DL 2000 marker; Y: yak; C: cattleyak.

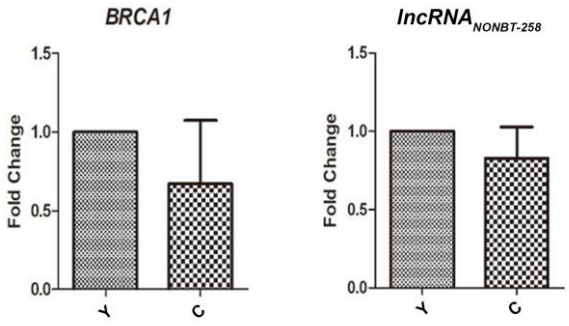

Fig-2: qRT-PCR validation of lncRNA$_{NONBT-258}$ and its target gene *BRCA1* involved in mitotic cell cycle processes:
Y: yak; C: cattleyak.

Molecular cloning and analysis of the lncRNA$_{NONBT-258}$

As the target gene *BRCA1* for lncRNA$_{NONBT-258}$ was identified to be related to the G2/M checkpoint in the regulation of mitosis [19], lncRNA$_{NONBT-258}$ from different cattleyak and yak testis samples was cloned and sequenced. RT-PCR amplification indicated that the length of lncRNA$_{NONBT-258}$ from different individual testis samples were approximately 760 bp (Fig-3). Molecular cloning and resequencing indicated that the length of lncRNA$_{NONBT-258}$ was divergent between cattleyaks and yaks, with a length of 756 bp for the two yaks (Y1 and Y2) and 761 bp for two cattleyaks (C1 and C2) (Fig-4). The sequences of lncRNA$_{NONBT-258}$ presented higher similarity (98.29 %) in different cattleyak and yak individuals using that of *Bos taurus* as the reference (Fig-4).

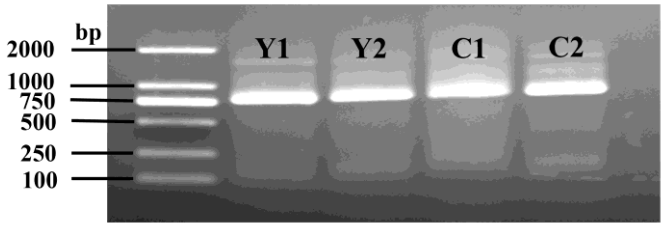

Fig-3: RT-PCR amplification of lncRNA$_{NONBT-258}$ from testis samples of yaks and cattleyaks:
M: DL 2000 marker; Y: yak; C: cattleyak.

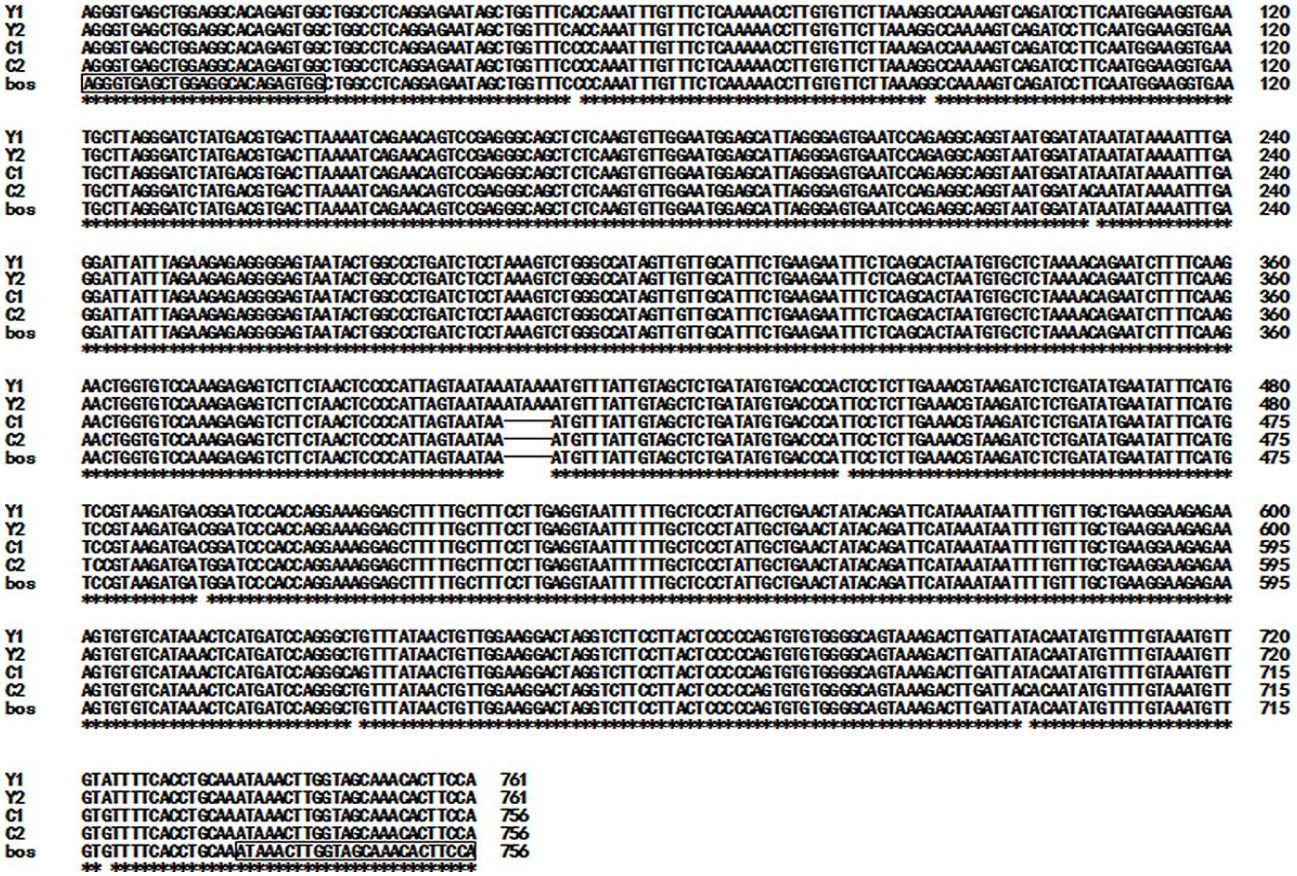

Fig-4: Alignment of the testis lncRNA$_{NONBT-258}$ sequence from two yaks and two cattleyaks using that of *Bos taurus* as the reference
Bos: *Bos taurus*; Y: yak; C: cattleyak.

DISCUSSION

LncRNA is ubiquitous in mammals, and its expression is specific in different growth stages and tissues of individuals [4]. LncRNA not only interacts with genomic DNA or mRNA depending on its primary sequence, but also forms its own advanced structure and then interacts with protein molecules in vivo, thus participating in the regulation of gene expression at all stages [4]. Functionally, compared with other ncRNA such as siRNA and microRNA, LncRNA, which has much more cellular functions, including transcriptional interference, induce chromatin remodeling, modulate alternatively splicing patterns, generate endo-siRNAs, modulate protein activity, alter protein localization, sponge miRNAs, and act as structural component and small RNAs precursor [20]. In addition, the expression abundance of lncRNA in animals is much lower than that of general mRNA, which makes it more difficult to study and reveal the function of specific lncRNA in animals.

In recent years, many studies have shown that lncRNA plays an important role in regulating spermatogenesis in mammals, including spermatogonial proliferation, spermatocyte meiosis and morphological changes of round spermatozoa. Although the regulatory functions of several lncRNAs have been identified at specific stages of spermatogenesis, there are still a large number of lncRNAs related to spermatogenesis and their specific regulatory functions to be studied. In this study, we identified a lncRNA, lncRNA$_{NONBT-258}$, which was DE between cattleyak and yak testis. Furthermore,

bioinformatics analysis revealed that its target gene was *BRCA1*, which was identified to be related to the G2/M checkpoint in the regulation of mitosis [19]. Considering the important role of *BRCA1* in mitosis and the speculation on abnormal mitosis during cattleyak spermatogenesis, we performed a preliminary study on $lncRNA_{NONBT-258}$ and its target gene *BRCA1*.

Bioinformatics analysis revealed that $lncRNA_{NONBT-258}$ and its target gene *BRCA1* were located on bovine chromosome 19, and $lncRNA_{NONBT-258}$ was formed by splicing of a single exon (Fig-1). According to the classification method of lncRNAs [3], $lncRNA_{NONBT-258}$ belongs to bidirectional lncRNA. We designed the primers for RT-PCR and RT-qPCR according to the sequences of these two molecules from *Bos taurus*. RT-PCR showed that $lncRNA_{NONBT-258}$ and its target gene *BRCA1* were truly expressed in the testis of yak and cattleyak (Fig-1), and RT-qPCR validated their expression patterns was consistent with the data from RNA-seq (Fig-2). On the one hand, bioinformatics analysis revealed that $lncRNA_{NONBT-258}$ targeted *BRCA1*, which may play a vital role in the regulation of mitosis [19], and they were all downregulated in the testis of cattleyak with respect to yak. On the other hand, our previous study give a speculation that spermatogenic arrest of cattleyak might occur at the stage of spermatogonial differentiation [17, 18]. Therefore, we speculate that $lncRNA_{NONBT-258}$ may participate in the process of spermatogenesis in yak and cattleyak, and its downregulation may lead to the downregulation of its target gene *BRCA1*, which may lead to the defect of spermatogonia mitosis in cattleyak, thus contributing to the emergence of reproductive problem, namely, spermatogenesis arrest of cattleyak. Furthermore, we cloned $lncRNA_{NONBT-258}$ from two yaks and two cattleyaks. Even though the length of $lncRNA_{NONBT-258}$ was 756 bp for the two yaks (Y1 and Y2) and 761 bp for two cattleyaks (C1 and C2), the sequences of $lncRNA_{NONBT-258}$ presented higher similarity (98.29 %) in these four individuals using that of *Bos taurus* as the reference (Fig-4).

At present, transcriptome sequencing and bioinformatics analysis play a vital role in the functional research of lncRNA for animals. Our study performed the expression profile analysis for $lncRNANONBT_{-258}$ and its target gene *BRCA1* on the premise of these two methods, and laid a foundation for understanding the role of $lncRNA_{NONBT-258}$ as a regulatory factor in cattleyak spermatogenesis through mediating target gene *BRCA1*.

Abbreviations

LncRNA: long non-coding RNA; DE: differentially expressed; RT-PCR: reverse transcription Polymerase Chain Reaction; RT-qPCR: Real-time Quantitative PCR

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Author contributions

CX and WSX conceived the study, WSX, MT, ZWS and MAS analyzed data, WSX, XCF and YCP performed experiments under the supervision of CX. WSX wrote the manuscript with the help of LWJ, YSM and CX. All authors read and agreed the concluding manuscript.

Ethics approval

The experimental animal procedures were according to the Guideline for Care and Use of Laboratory Animals of China and all protocols were agreed by the institution Review Board of Southwest University of Science and Technology.

Competing interests

The authors declare that they have no competing interests.

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