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CpG methylation analysis at the putative differential methylated region of *Xist* gene in goats (*Capra hircus*) somatic cells

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ABSTRACT

X chromosome inactivation is a complex epigenetic process that ensures equal expression of most X-linked genes in both sexes. *Xist* (X-chromosome inactive specific transcript) is a crucial development related gene that initiates X chromosome inactivation in mammalian females during embryogenesis. DNA methylation is a major chromatin modification involved in transcriptional repression of genes, regulating X chromosome inactivation, genomic imprinting, and cell or tissue specific gene expression and in many other processes during embryo development. Appropriate DNA methylation of cytosine residues at CpG dinucleotide within the differential methylated region of many imprinted genes is required for normal post implantation development of a mammalian embryo. An aberrant pattern of DNA methylation because of incomplete or anomalous nuclear reprogramming of somatic cells is a reason for poor success rate of somatic cell nuclear transfer in farm animals. In the present study, a putative differential methylated region was identified for first time in goats (*Capra hircus*) followed by study of the DNA methylation status of this region, using bisulfite sequencing analysis taking fibroblast and cumulus cells from goats. The putative differential methylated region contains 10 CpG sites with a mean methylation level of 92% and 94% for fibroblast and cumulus cells respectively. Phylogenetic analysis of this region revealed goats and sheep positioned in a single cluster distinct from others studied. This study will be a definite help in future research on molecular events responsible for complete nuclear reprogramming of goat somatic cells in SCNT.

Key words: *Xist* gene, DNA methylation, epigenetic reprogramming, bisulfite sequencing, somatic cell nuclear transfer, goats

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Introduction

In many sexually dimorphic species, a mechanism is required to ensure equivalent levels of gene expression from the sex chromosomes. In mammals, dosage compensation of X-linked genes, between male (XY) and female (XX) is achieved by the transcriptional silencing of one of two X- chromosome in the females (XX) during early development, a process known as X-chromosome Inactivation (XCI) or Lyonisation (LYON, 1961) . This XCI is initiated by an X-linked imprinted gene, *Xist* (X-inactive specific transcript) located at the X-Inactivation Centre (XIC) on both X-chromosomes and expressed only from inactive X chromosome in females (BROWN et al., 1991). The *Xist* gene from the future inactive X chromosome encodes a large, polyadenylated noncoding nuclear RNA, which progressively coats the same chromosome, spreading out from the XIC, resulting in exclusion of transcription machinery. Subsequent DNA methylation of the cytosine within CpG dinucleotides and hypoacetylation of histone H4 act synergistically in maintaining X chromosome inactivation (CSANKOVSZKI et al., 2001).

Methylation of DNA occurs on a cytosine base, especially on 5'CpG3'dinucleotides enriched in small regions of DNA known as CpG island. They are usually located in the promoter region or the exon-1 of most of the genes and are associated with gene inactivation (STRATHDEE et al., 2004). DNA methylation is the best understood epigenetic mark regulating expression of imprinted genes (LUCIFERO et al., 2006). Incomplete or inappropriate reprogramming of this epigenetic mark in donor nuclei of somatic cells is one of the reasons for poor survival of somatic cell nuclear transfer (SCNT) derived clones (DANIELS et al., 2000). Nuclear reprogramming in SCNT is a process by which a differentiated somatic cell re-acquires developmental potential and the consequent recapitulation of development, ultimately giving rise to a cloned newborn (SINGH and ZACOUTO, 2010).

Xist is an important gene during embryo development in mammals, and it is one of the first imprinted genes to be expressed in the early embryo with expression begins at zygotic genome activation (WELLS, 2012; ZUCCOTTI et al., 2002). Among many genes expressed aberrantly in clone embryos, *Xist* is one (FUKUDA et al., 2010). Atypical patterns of X chromosome inactivation may affect the SCNT efficiency and development patterns of clones (JIE et al., 2008; RIDEOUT et al., 2001). There is a great deal of literature mentioning abnormal methylation patterns at differential methylated regions (DMR) of the *Xist* gene in aborted SCNT-derived cloned bovine calves (XUE et al., 2002; LIU et al., 2008). Although the *Xist* gene and its methylation profile at DMR have been studied in humans, mice, cattle (DINDOT et al., 2004; JIE et al., 2008), sheep (ZHAO et al., 2011) and many other domestic animals it is yet to be explored in goats. In most of the studies, fibroblast cells and cumulus cells were used as donor cells for SCNT in bovines (KUBOTA et al., 2000; TIAN et al., 2000), pigs (BOQUEST et al., 2002) and goats (PARK et al., 2007). Among

the somatic cell types tested, the consensus from numerous laboratories is that cumulus cells give the highest cloning efficiency and result in the least number of abnormalities in the cloned animals (TIAN et al., 2003). Again, DNA methylation in many genes is tissue specific (LI, 2002). In this context, the current study was designed to identify the putative DMR region of the *Xist* gene and its methylation profile in goat somatic cells *viz.*fibroblast and cumulus cells, and to find the differences in methylation patterns in putative DMR of the *Xist* gene in both somatic cells, if any.

Materials and methods

Development of skin fibroblast and cumulus cell lines of goat. A fibroblast primary cell line was developed from ear tissue pieces from female goats of the Jamunapari breed (a famous dairy breed in India), collected aseptically from a nearby animal slaughter house. The tissue pieces were processed and primary explants were prepared. A complete medium, Dulbecco's modified eagle medium (DMEM) with 15% FBS, was used and subsequently subcultured after attaining 70-80% confluency. The cumulus cell line was procured directly from the Embryo Biotechnology Laboratory of the Biotechnology Centre, JNKVV, Jabalpur, India.

DNA extraction and bisulfite treatment. Genomic DNA was extracted from cultured cells using a DNeasy Blood and Tissue kit (Qiagen,USA) as per the manufacturer's instruction. The bisulfite treatment was carried out using the EpiTect Bisulfite kit (Qiagen, USA) as per the given protocol, with some modifications. Considering the high degree of DNA fragmentation and subsequent loss of DNA during purification due to the high salt concentration, low pH and high temperature during bisulfite treatment, the quantity of input genomic DNA was optimized to 2µg. Bisulfite treated DNA was stored at -20 °C for later use.

Primer designing and amplification. The nucleotide sequence of the *Bos taurus Xist* gene (accession no NR_001464.2) was used for CpG island prediction, as well as designing the required primers for the study. The CpG island was predicted using MethPrimer software (http:/www.urogene.org/methprimer) as per the criteria defined by LI and DAHIYA (2002). The CpG island should be a DNA stretch at least 200bp long with GC content more than 50% and an [observed CpG/expected CpG] ratio $\geq$0.6. A CpG island was obtained, spanning from 1296bp to 1502 bp. The normal primers and bisulfite sequencing primers (BSP) were designed for amplification of this region, before and after bisulfite treatment of genomic DNA, respectively. The softwares Primer Express 3.0 and MethPrimer were used for designing normal primers (X1) and BSP primers (X2) respectively (Table 1). Methylation analysis of the CpG island within *Xist* gene was performed by bisulfite sequencing. PCR amplifications were carried out in a reaction volume of 25 µL containing a 1X reaction buffer, including 25 mM $MgCl_2$, 100 ng of

DNA, 5 μmol/L each of forward and reverse primers, each deoxyneucleotide of 200 μM and 1.25 units of DNA Taq polymerase (Fermentas, Germany). The PCR was performed under the following thermal conditions: an initial denaturation at 95 °C for 5 minutes, followed by 30 cycles of PCR consisting of 30 seconds at 95 °C, 30 seconds at 57 °C/ 55 °C (X1/ X2 respectively) and 30 seconds at 72 °C, and one cycle of final extension at 72 °C for 10 minutes.

Table 1. Primer sequences used for amplification of putative DMR of Xist gene in goat

Primer set	Primer name	Primer sequence (5′-3′)	Annealing temperature (Tm)	Amplicon size (bp)
X1	XIST-Fn1	TTTGCTGCAGGGACAATATGGCTGAC	57 °C	221
	XIST-Rn1	CCACCCTTTCTGATTGGGTGGGACAC		
X2	XIST-Fm2	TTTGTTGTAGGGATAATATGGTTGAT	55 °C	221
	XIST-Rm2	CCACCCTTTCTAATTAAATAAAACAC		

Gel elution, cloning and DNA sequencing of PCR products. The PCR products underwent gel electrophoresis on a 1.4% agarose stained with ethidium bromide. The gel elution of PCR products was performed using a MinElute Gel Extraction kit (Qiagen, USA) as per the manufacturer's instructions. Further PCR products were cloned into the InsT/A cloning $p^{TZ57R/T}$ vector (MBI Fermentas, Germany). All clones containing the appropriate inserts were sequenced using an automated DNA sequencer (ABI 310 Genetic Analyser, USA).

Statistical analysis. Methylation analysis of the amplified CpG island of *Xist* gene for both cell types was performed by independent t-test using SPSS software at 95% level of confidence, i.e. differences were considered to be statistically significant at $P<0.05$ and insignificant at $P>0.05$.

Phylogenetic analysis. The obtained nucleotide sequence was submitted to GenBank.

The available *Xist* gene nucleotide sequences of other species such as sheep (*Ovis aries*: Accession No: GU372693), cattle (*Bos taurus*: Accession No: AF104906) and pigs (*Sus scrofa*: Accession No: KC753464) were retrieved from GenBank and further aligned with that of goats, using Mega 5.1 software (TAMURA et al., 2011).

Results

Initially, the size of the amplified products from genomic DNA both before and after bisulfite treatment was approximated by distinct bands above 200 bp in agarose gel electrophoresis (Fig. 1). After sequencing, the amplified products of 221 bp were confirmed.

The sequences of the amplified fragment from untreated and bisulfite treated genomic DNA were compared and it was found that all the cytosine residues, except those at CpG motifs, had been replaced with thymine residues, indicating complete and efficient bisulfite treatment (Fig. 2). Although the somatic cells contain both active and inactive allele of the X chromosome, the bisulfite sequencing results showed that only active allele (methylated at DMR of *Xist* gene) was amplified in all the samples. The 221 bp fragment was found to contain 10 CpG sites. In the case of fibroblast cells, out of ten samples subjected to bisulfite sequencing, three samples showed 100% methylation at the CpG island, nine samples showed 90% and one sample showed 80% methylation (Fig. 3A). In the case of cumulus cells, four samples showed 100% and the remaining six samples showed 90% methylation at the CpG island (Fig. 3B).

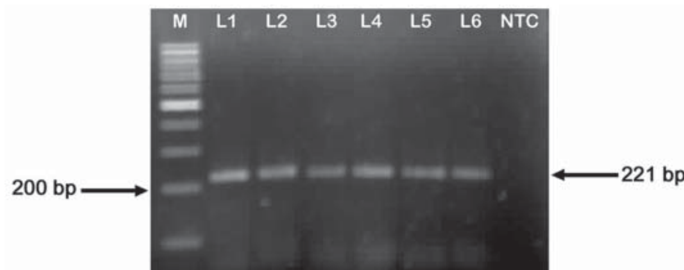

Fig. 1. PCR amplified products from bisulfite treated genomic DNA. Lane M - 100 bp ladder, L1 to L3 and L4 to L6 are amplified fragment using genomic DNA extracted from goat fibroblast cells and cumulus cells respectively and Non-template control (NTC).

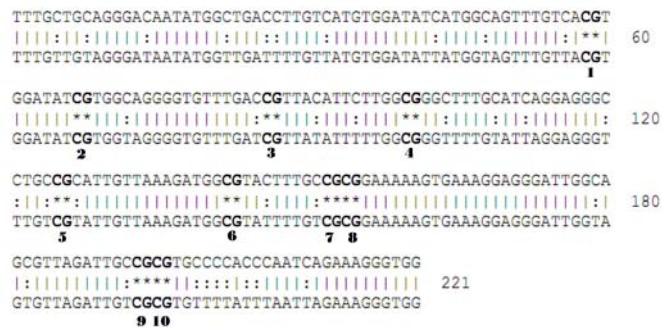

Fig. 2. Sequence of the amplified region using untreated genomic DNA (upper strand) and bisulfite treated genomic DNA (lower strand). Amplified 221 bp fragment contains 10 CpG sites shown in bold. Cytosine residues in the upper strand replaced with thymine residues in the lower strand except at CpG dinucleotides.

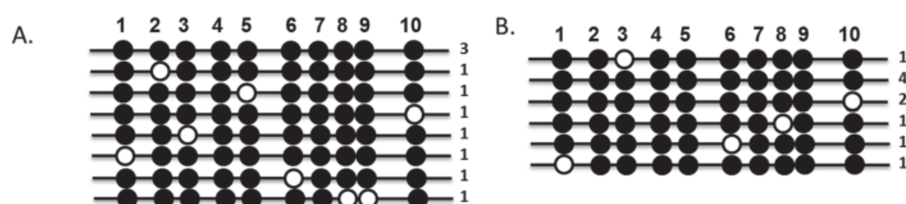

Fig. 3. Lollipop diagram showing methylation status of CpG sites at *Xist* gene putative DMR for goat fibroblast (A) and cumulus cells (B). Numbers at the end of lines indicate the number of identical samples sequenced. Open circles and closed circles represent unmethylated and methylated CpG sites respectively.

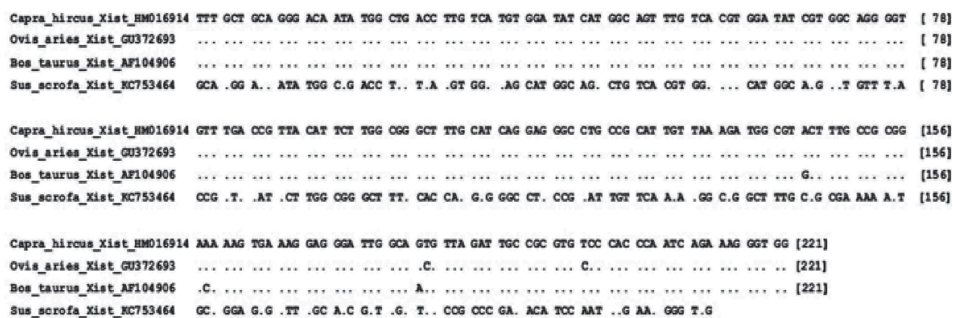

Fig. 4. Nucleotide alignment of partial sequence of goat (*Capra hircus*) *Xist* gene with sheep (*Ovis aries*), cattle (*Bos taurus*) and pigs (*Sus scrofa*). Identity to the goat sequence is indicated by a dot, and differences by the corresponding nucleotide.

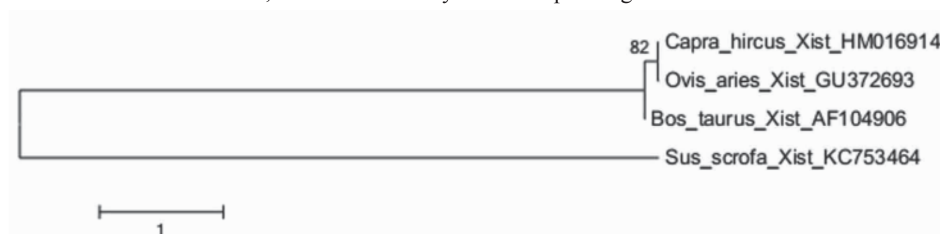

Fig. 5. Phylogram illustrating the evolutionary relationship of partial sequence of goat (*Capra hircus*) *Xist* gene with sheep (*Ovis aries*), cattle (*Bos taurus*) and pig (Sus scrofa). The phylogenetic tree was constructed using Neighbour-Joining analysis. Numbers represent bootstrap values (given as percentages) for a particular node. 1000 replicates were used in bootstrap analysis for good statistical support. The branch lengths are scaled to represent the relative number of substitutions occurring along each branch. The scale bar indicates the evolutionary distance of 1 nucleotide substitution per site for a unit branch length.

The submitted sequence was later assigned with the accession number HM016914. The nucleotide alignment report of goat sequences with other studied sequences is given below (Fig. 4). The alignment of this partial sequence of *Capra hircus Xist* gene nucleotides with other species revealed maximum similarity with *Ovis aries* followed by *Bos Taurus* and *Sus scrofa* and the values of similarity as a percentage for validation were 99.1, 98.6 and 91.5 respectively. Phylogram was shown based on nucleotide sequences (Fig. 5), where it was evident that sheep, goats and cattle belong to one clad and pigs are in a different clad.

Discussion

Dairy goats are ideal for transgenic production of therapeutic recombinant proteins (LAN et al., 2007) and the lower incidence of scrapie in goats, relative to sheep, adds to the attractiveness of latter for human recombinant protein production (BAGUISI et al., 1999). A high level of human antithrombin III was obtained from the milk of transgenic cloned goats (BAGUISI et al., 1999). The application of SCNT in the propagation of transgenic goats could be a reliable method. However, in ruminants SCNT is frequently associated with pathological changes in the placenta and the phenotype of foetus and also has significant consequences for development, both before and after birth (LIN et al., 2008). In the SCNT technique, the most critical factor is the correct epigenetic reprogramming of the transferred somatic cell nucleus from its differentiated status into the totipotent state of the early embryo, in order to support early development (REIK et al., 2001). DNA methylation is the major epigenetic modification required to be reprogrammed in somatic nuclei for proper development of the embryo, and it also determines post natal health (LI et al., 2004). It has been reported that in cloned embryos, the frequency of the normal or abnormal methylation pattern closely reflects the developmental ability of reconstructed embryos (SANTOS et al., 2003; DEAN et al., 2001) and in the process of implantation and placenta formation (MALASSINE et al., 2003).

Many studies have correlated aberrant methylation patterns at DMR regions of the *Xist* gene resulting in aberrant X chromosome inactivation with abnormalities associated with SCNT derived bovine calves. Previous reports have shown the existence of different degrees of methylation at the DMR region *i.e.* hypomethylation (<50% of methylated CpG sites) (LIU et al., 2008; XUE et al., 2002) and hypermethylation (>50% of methylated CpG sites) (JIE et al., 2008; DINDOT et al., 2004) in aborted bovine cloned calves. All these studies indicate the importance of the methylation pattern, which should be studied in the case of the goat *Xist* gene, to make SCNT successful in goats.

Comparative sequence analysis of XCI in humans, mice and bovines was performed by CHUREAU et al. (2002) and they reported the presence of a highly conserved region at the 5' ends of the *Xist* gene in these animals. In cattle, one CpG island is present within the

exon-1 of the *Xist* gene, representing its DMR (DINDOT et al., 2004). *Xist* DMR of cattle shows 94.8% similarity with that of sheep (ZHAO et al., 2011). The current phylogenetic study based on the nucleotide sequence, suggested the presence of sheep and goat in a single monophyletic group, and the studied sequence was highly conserved among these two species as compared to cattle and pigs. Sheep and goat clad was more related to cattle clad than pigs. Pigs in whole total belong to a more distant clad, indicating their recent divergence from other mammalian species studied, with respect to the DMR of the *Xist* gene.

The amplified CpG island in the present study may represent *Xist* DMR in goats, and which notion is bolstered by some earlier reports given above and the present phylogenetic analysis.

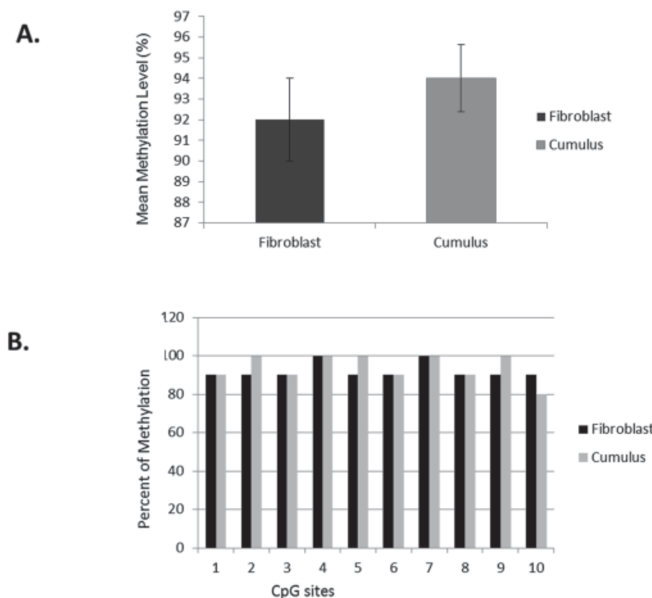

Fig. 6. Methylation analysis of *Xist* putative DMR in fibroblast and cumulus cells. (A) Showing mean methylation level of CpG island. (B) Histogram showing percentage of methylation of each CpG sites in CpG island.

Methylation analysis of amplified putative DMR of the Xist gene in fibroblast and cumulus cells. When the mean methylation level at the putative DMR of the *Xist* gene was considered, the fibroblast and cumulus cells showed 92% ± 1.63 and 94% ± 2.0 of methylation, respectively (Fig. 6A). This suggested no significant difference (P>0.05) in mean methylation at the putative DMR in both somatic cell types. The methylation

pattern was analyzed of individual CpG sites within the CpG island in both cell types. Out of 10 CpG sites in the putative DMR, the CpG motifs at positions 1,3,4,6,7 and 8 showed similar methylation patterns in both fibroblast and cumulus cells, with 90-100% of methylation, but CpG motifs at the remaining positions showed different methylation pattern (Fig. 6B). However this minor difference in the methylation pattern was found to be insignificant (P>0.05)

Increased methylation of the CpG island within gene regulatory regions is associated with transcriptional repression of the gene (STRATHDEE et al., 2004). Methylation at CpG sites within the DMR region of the *Xist* gene prevents the gene being expressed. In the absence of *Xist* expression, the contained X chromosome is active. Our sequencing results showed that most of the time amplification had occurred from the active X allele DNA strand. JIE et al. (2008) in their study on lung somatic cells of both post-fertilization and SCNT derived aborted bovine clones, reported that *Xist* DMR in bovine contains 10 CpG sites and nearly all the CpG sites were methylated in both cases. In another study, ZHAO et al. (2011) identified the *Xist* DMR in exon1 and studied the methylation profile of the gene in five different organs, spermatozoa and mature oocytes of a two-day old female lamb. The identified *Xist* DMR in sheep contained 11 CpG sites, and bisulfite sequencing revealed that both active and inactive X alleles were amplified and methylation was either complete or nearly complete in the organs, whereas in the sperm it was completely unmethylated.

This pioneer study on the *Xist* gene of goat will be helpful in elucidating the detailed methylation profile of *Xist* DMR at different stages of the development of SCNT derived goat clones, and give a way to manipulate them to improve SCNT in goats.

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SAŽETAK

Inaktivacija X kromosoma složeni je epigenski postupak kojim se osigurava jednaka ekspresija većine X-vezanih gena kod oba spola. *Xist* (X kromosom specifični inaktivirajući transkript) je ključni gen povezan s razvojem koji potiče inaktivaciju X kromosoma kod ženki sisavaca tijekom embriogeneze. Metilacija DNA glavna je modifikacija kromatina uključena u transkripcijsku regresiju gena, regulaciju X kromosomske inaktivacije, genomskog utiska, specifičnu staničnu ili tkivnu gensku ekspresiju odnosno u mnoge druge procese tijekom embrionalnog razvoja. Prikladna DNA metilacija citozin-rezidua na CpG dinukleotidu, unutar metilacijske regije mnogih utišanih gena, prijeko je potrebna za normalni postimplantacijski razvoj embrija sisavaca. Nepravilan oblik DNA metilacije zbog nekompletnog ili poremećenog reprogramiranja u jezgi somatskih stanica razlog je manje uspješnosti njezinog prijenosa kod farmskih životinja. U ovom radu istražena je moguća druga metilacijska regija koja je utvrđena prvi put u koze (*Capra hircus*). Navedeno je omogućeno nakon istraživanja statusa DNA metilacije u toj regiji, primjenom analize bisulfit sekvenciranja, uz upotrebu fibroblasta i stanice kumulusa koze. Moguća nova metilirajuća regija sadrži 10 CpG mjesta s prosječnom razinom metilacije od 92% i 94% za fibroblaste odnosno stanica kumulusa. Filogenetske analize regije pokazale su da su koze i ovce smještene u jedan odvojeni “grozd”. Istraživanje će pomoći u budućem radu vezanom za molekularnu razinu koja je odgovorna za potpuno reprogramiranje jezgre somatskih stanica koza u SCNT.

Ključne riječi: *Xist* gen, DNA metilacija, epigenetsko reprogramiranje, bisulfit-sekvenciranje, prijenos jezgre somatskih stanica, koze