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Sequence characterization and expression analysis of mammary gland derived osteopontin gene of river buffalo

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ABSTRACT

Sequencing of RT-PCR amplified osteopontin (*OPN)* gene of buffalo mammary gland revealed its ORF to be of 843 nucleotides, coding for 280 amino acids long protein. Sequence comparison with cattle, sheep and pig showed 4 buffalo specific nucleotide changes. Two additional triplet nucleotides 273ACG275 and 681CAA683 in buffalo *OPN* as compared to cattle resulted in insertion of 2 amino acids N95 and N227. Phylogenetic analysis based on both nucleotides and amino acids sequence showed buffalo grouping more close to cattle and pig being placed most distantly from other 3 livestock species. Most of functional domains were conserved in buffalo *OPN* except that one of the 4 chymosin cleavage sites reported in cattle was missing in buffalo. Expression analysis of osteopontin gene by real-time PCR revealed approximately 5 fold increase in lactating buffalo mammary gland as compared to non-lactating, indicating its importance in milk production.

Key words: Buffalo, Expression analysis, ORF, Real-time PCR, Sequencing

Osteopontin (*OPN*) is a highly phosphorylated acidic glycoprotein, associated with multiple biological activities, viz. tissue mineralization, cellular transformation and cancer, immune response, inflammation etc. (Denhardt and Guo 1993, Sodek *et al.* 2000). *OPN* encoded by single copy gene, is modified extensively through a number of post-translational events. In mouse mammary gland, *OPN* expression is linked to mammary involution and a transgenic mouse model has shown osteopontin to be necessary for mammary gland development and normal milk production (Rittling and Novick 1997, Nemir *et al.* 2000).

A large number of hormones, cytokines and growth factors regulate the expression of *OPN* by influencing transcription as well as translation of the protein (Mazzali *et al.* 2002). OPN is found to be present in a number of tissues and body fluids such as milk, plasma, urine, bile (Sodek *et al.* 2000). Important biological properties of *OPN* secreted in cow milk were reported and bovine *OPN* gene was found present within a major milk production QTL, recently mapped to bovine chromosome 6 (BTA6) using microsatellite markers and direct sequencing of 12.3 kb region harboring osteopontin gene (Bayless *et al.* 1997, Schnabel *et al.* 2005). A significant association of a single nucleotide polymorphism in intron 4 (C/T) of *OPN* with milk protein percentage and milk fat percentage in cattle has also been observed (Leonard *et al.* 2005). White *et al.* (2007) showed that *OPN* promoter region polymorphism to be associated also with post-weaning growth in cattle. Previously buffalo OPN gene were characterized but unlike cattle, no much information is available about water buffalo osteopontin gene even though it is one of the major dairy animals of South-east Asia (Tantia *et al.* 2008). In this study an attempt was therefore made to generate more information buffalo osteopontin gene and its role in different stages of lactation.

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MATERIALS AND METHODS

Total RNA was extracted from 100mg of buffalo mammary gland tissue of 3 stages (lactating, non-lactating and heifer) using the 'TRIzol' method, following the procedure described by the manufacturer. Quality and quantity of RNA was checked spectro-photometrically by measuring its OD at 260 and 280 nm. Approximately 1µg of total RNA extracted from different tissues was reverse transcribed using strata script RT-PCR system in a reaction volume of 20 µl at 42°C for 30 min. First strand cDNA template was amplified using *OPN* gene specific primers (Forward 5'-AGGGGACTGGACTCTTCTCG-3', nt position 15–34; Reverse 5'-CTCCCACCCTGCTTTAA TGTATCC-3', nt position 969–946) to amplify 955 bp

sequence comprising complete ORF. PCR amplification was carried out using cycling conditions: initial denaturation for 2 min at 95°C, followed by 30 cycles of 30 sec at 95°C, 30 sec at 55°C 2 min at 72°C and final extension of 10 min at 72°C. The PCR product was analyzed in 1.5% Ethidium Bromide stained agarose gel.

Full length RT-PCR generated product was purified using PCR purification kit and both strand sequencing was performed using both forward and reverse primers. Buffalo *OPN* gene sequence was analysed by comparison with cattle (Acc. NM_174187), goat (Acc. EU295699), sheep (Acc. AF152416), pig (Acc. NM_214023) and horse (Acc. XM_001496186). Sequences were aligned using the 'MegAlign' programme of 'Lasergene' software. The buffalo *OPN* cDNA sequence generated was submitted to GenBank (Acc. No. DQ415660). Buffalo secondary structure was analysed by online psipred program.

A semi quantitated RT-PCR analysis of buffalo *OPN* gene was carried out using equal quantity of RNA extracted from different mammary gland tissues, as described above using a primer set to amplify 333 bp fragment within ORF. Primers used were: F 5'- GACGCTGAAACCACTGATGACC -3' nt position 376–397 and R 5'- TTTGGAAAGCTCGCTACTGTTGG -3' nt position 708–686, designed from the cattle sequence (Acc. No. M66236). House keeping gene GAPDH was included as an internal control co-amplifying 119 bp fragment. Two microlitres of each stage cDNA was amplified using same reactants as above with the 27 cycles of 95°C-30 sec. 58°C-30 sec and 72°C-30 sec without final extension at 72°C. The samples were analysed by running in 2.5% ethidium bromide stained agarose gel. cDNA synthesized from equal amounts of RNA (1µg) extracted from different buffalo mammary gland tissues were also subjected to real-time PCR analysis for expression profiling by using Brilliant SYBR Green QPCR Master Mix on Stratagene *Mx3000* machine. GAPDH and RPS15 were included as control for normalization of data and to check PCR efficiency standard curves were drawn using 10 fold serially diluted cDNA of OPN, GAPDH and RPS15 internal control. Relative gene expression analysis was carried out by $2^{-DDC}{}_{T}$ method (Livak and Schmittgen 2001).

RESULTS AND DISCUSSION

Sequence analysis of buffalo mammary gland derived osteopontin mRNA revealed 843 nucleotides long ORF. Two triplet insertions were present in buffalo at 273ACG275 and 681CAA683 in comparison to cattle. Percent similarity of buffalo *OPN* at nucleotide levels was 97.8% with cattle, 91.2% with goat, 94.2% with sheep, 80.0% with pig and 69.9% with sheep. Buffalo secondary structure was analysed by online psipred program revealed distribution of alpha helices and beta sheets (Fig. 1)

Translated amino acid sequence analysis revealed buffalo osteopontin to be of 280 aa with 16 aa signal peptide and 264 aa long mature peptide. Two specific amino acid changes, D at 94 and N at 227 in buffalo *OPN* were present

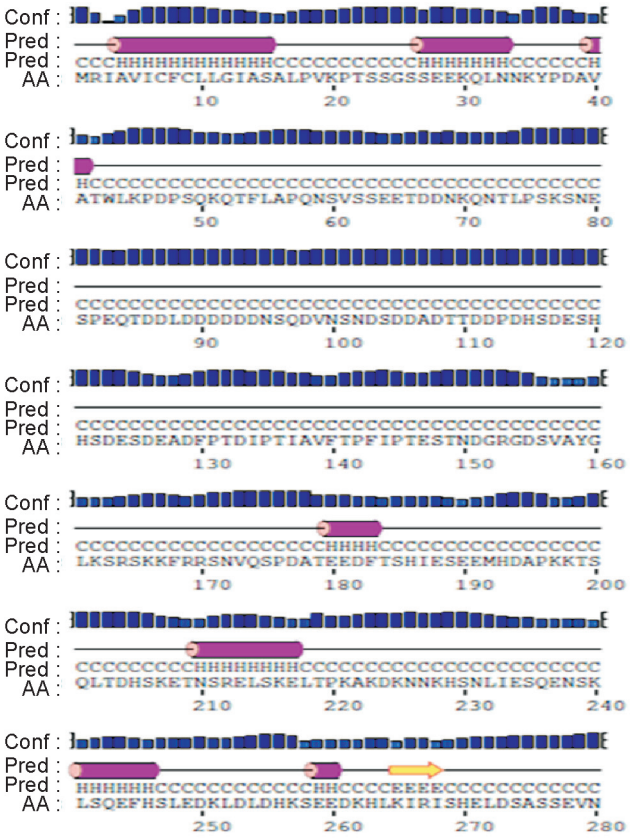

Fig. 1. A curtailed output of secondary structure elements in osteopontin: Alpha helix, beta sheet and random coil location in the curtailed output.

as compared to other ruminant livestock species. Overall, cattle and buffalo *OPN* had seven amino acid differences. Triplet nucleotide insertions at two places in buffalo as compared to cattle resulted in insertion of two amino acids N at 95 and N at 227 positions. Amino acid similarity of buffalo *OPN* with cattle (97.1%) was maximum, followed by sheep (91.8%) goat (91.4%), pig (72.1%) and lowest with horse (64.7%). Nucleotide sequence based phylogenetic analysis grouped cattle and buffalo close to each other away from sheep, goat while pig and horse placed in a separate lineage (Fig. 2).

Cell binding domain 'RGDS' was found to be conserved across all livestock species, which has been postulated to be associated with important function of osteopontin in cellular differentiation and proliferation in luminal epithelial

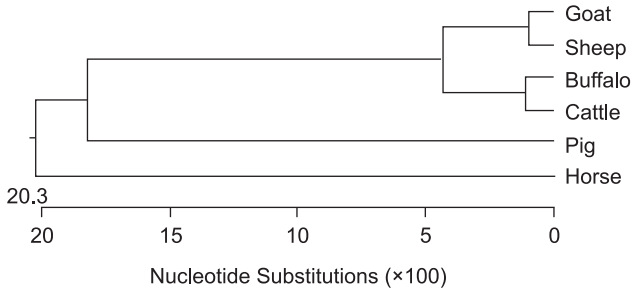

Fig. 2. Phylogenetic tree of osteopontin gene based on nucleotide sequence analysis of four species using 'MegAlign' programme of 'Lasergene' software.

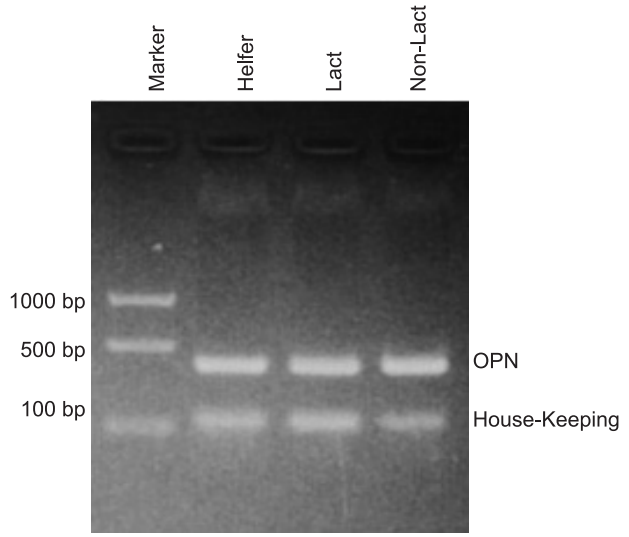

Fig. 3. Agarose gel electrophoresis of buffalo osteopontin gene RT-PCR amplified from mammary gland of three different lactation stages co-amplified along with GAPDH house-keeping gene.

Table 1. Fold changes in expression of *OPN* gene in lactating, non-lactating vs heifer stages of buffalo mammary gland

Tissue	Av. Cp value		ΔCp	ΔΔCp OPN		Fold
	OPN	RPS15	OPN			change
Lac	17.3	22.63	2.31	Lac vs Nlac	2.31	4.96
Nlac	15.1	22.73	7.63	Lac vs Heifer	1.35	2.55
Heifer	16.03	22.71	6.68	Nlac vs Heifer	–0.96	1.95

of several organs (Brown *et al.* 1992). As in other species, 2 transglutaminase reactive Gln residues G34 and G36 reported to be conserved in mature peptide were also conserved in buffalo reconfirming functional importance of this motif (Sorensen *et al*. 1994). Twenty-eight phosphorylation sites composed of 27 serine and 1 threonine residues, reported in cattle mammary gland osteopontin, were found to be maintained in buffalo *OPN*. Besides 3 *O*-glycosylated threonine residues (T115, T124 and T129) in mature peptide were also conserved which is considered to be essential for function of the protein (Sorensen *et al.* 1995). Earlier 4 chymosin cleavage sites, I26-W27, L72-D73, R152-R153 and L223-S224 were reported in the *OPN* secreted in bovine milk, having importance in adsorption of colostrum secreted *OPN* through the abomasums of calf (Kumura *et al.* 2004). Out of these 4 sites, 3 were conserved with I26-W27 being replaced by T26-W27 in buffalo protein.

Semi quantitative expression analysis of *OPN* mRNA across physiological stages, viz. lactating and non-lactating revealed almost equal expression during different stages (Fig. 3). However, real-time PCR analysis, being more sensitive, in 3 stages of buffalo mammary gland tissues, confirmed approximately 5 fold higher *OPN* expression during lactation in buffalo, reflecting its possible role in milk production (Table 1). Persistently high expression of osteopontin through out the lactation in human beings has been observed earlier, also suggesting its potential role in immunity in infants (Nagatomo *et al.* 2004). When non-lactating and heifer were compared 1.95 times higher expression was found in non-lactating explaining its potential role in normal functioning and development of mammary gland other than milk production, as reported by other workers (Rittling and Novick 1997, Nemir *et al.* 2000). Also the expression of OPN gene was observed to be in declining pattern from colostrums period to dry period with an apparent increase in OPN expression in the late lactation period as compared to peak lactation period in Yak (Bai *et al.* 2012). Thus the role of *OPN* in milk production and mammary gland development implies its important function in buffalo milk production.

This report is an attempt to characterize *OPN* and its expression pattern in different physiological stages of buffalo mammary gland. Further characterization of its functional role will lead to subsequent exploitation of osteopontin gene polymorphism for milk production traits in water buffalo.

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