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Association of osteopontin gene with milk production traits in Gir native and HF crossbred cows

SHINGNE D F[1], SAWANE M P[1], CHOPADE M M[2], SORTE S D[3] and PAWAR V D[1]✉

Mumbai Veterinary College (MAFSU), Mumbai, Maharashtra 400 012 India

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ABSTRACT

In the present investigation, relationship between bovine osteopontin (*OPN*) gene variants and milk production traits of Gir native and Holstein Friesian (HF) crossbred cattle was studied. A total number of 30 each from Gir native and HF crossbred cows were genotyped by using PCR-RFLP method. The *Bsr*I/ PCR-RFLP analysis of 290 bp *OPN* Intron 4 amplicons revealed two genotypes (TT and CT) in Gir native cow while, three genotypes (TT, CT and CC) in HF cows. The observed genotypic and allelic frequencies were TT (0.63), CT (0.37) and T (0.82), C (0.18) for Gir native cows while, TT (0.20); CT (0.57); CC (0.23) and T (0.48); C (0.52) for HF crossbred cows, respectively. There was no significant association between *OPN* genotypes with the lactation milk yield and fat per cent of the cows studied. Molecular screening of the larger Gir and HF crossbred population might be helpful in discovering the association between *OPN* variants and milk traits in studied breeds.

Keywords: Gir, HF crossbred, Milk fat, Milk yield, Osteopontin gene polymorphism

India is the global leader in milk production with 198.4 million metric tonnes of milk/year. The livestock industry contributes 4.19% to the national GDP and 28.63% to the total agricultural GDP (Anonymous 2020). The dairy sector alone is a significant contributor with 5.13% to India's total agricultural GDP. Even though the country is having the largest cattle population and milk production, the overall milk productivity of individual cattle is significantly low as compared to the other leading milk-producing nations. Genetic improvement of the dairy cattle is the key to enhance the productivity and meet up the increasing demands of milk and milk products. The conventional selection methods limit the annual genetic gain. However, incorporation of molecular markers such as SNPs in the selection process (marker assisted selection/genomic selection) have drastically improved the economics of dairy traits of the cattle (Weigel 2017).

Bovine Osteopontin (*OPN*) is one amongst the numerous identified candidate genes associated with milk production and its components. It is also known as Secreted phosphoprotein-1 (SPP1) or T-lymphocyte activation protein 1 (ETA-1) gene located on chromosome 6 and is made of seven exons (Chakraborty *et al.* 2010) and encodes a 278-AA protein (Kerr *et al.* 1991). *OPN* encodes for multifunctional osteopontin protein found in body fluids and most tissues. Ostepontin protein also influences mammary gland proliferation, differentiation and ultimately milk production traits (Nagatomo *et al.* 2004).

The *OPN* variants are reported to have significant association with milk traits (Leonard *et al.* 2005, Raschia *et al.* 2018), somatic cell counts in milk (Kowalewska-Łuczak and Kulig 2013) and lactation persistency (Bissonnette 2018). In purebred HF cattle, *OPN* gene association with milk yield has been widely studied (Kulaj *et al*. 2019), but very inadequate information is available in case of Gir indigenous cattle and crossbred cows in India. Therefore, the present investigation was proposed to study the polymorphism of *OPN* gene variants in Gir and HF crossbred cows and its association with milk traits.

MATERIALS AND METHODS

The blood samples and milk production records were collected from the herd maintained at Centre of Excellence for Dairy, KVK, Baramati, Pune.

Collection, isolation of genomic DNA and DNA quantification: The blood samples were collected from 30 Gir native and 30 HF crossbred cows with minimum one lactation record and maintained in cold chain. Genomic DNA was isolated from the blood using Phenol: Chloroform: Isoamyl alcohol (P: C: I) protocol described by Sambrook and Russell (2006). The quantity and quality of DNA was examined by using spectrophotometer (NanoDrop ND-2000, Thermo, USA) and by agarose gel

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Present address: [1]Department of Animal Genetics and Breeding, Mumbai Veterinary College, Mumbai, Maharashtra. [2]College of Veterinary and Animal Sciences, Parbhani, Maharashtra. [3]Department of Animal Husbandry, Animal Husbandry College, Baramati, Maharashtra. ✉Corresponding author email: vdpawar1@gmail.com

electrophoresis. DNA sample with OD 260:280 ratio of 1.8 to 2.0 were diluted with nuclease free water to gain approximate 50-100 ng/µL final DNA concentration.

PCR amplification of OPN gene: The fourth intron region of *OPN* gene was amplified using primer sequences (F: 5′- GCAAATCAGAAGTGTGATAGAC -3′; R: 5′- CCAAGCCAAACGTATGAGTT -3′) designed by Leonard *et al.* (2005). The final 25 µL reaction mix consisted of 12.5 µL PCR master mix (SMOBIO, ExcelTaq™ 2X PCR Master Mix), 10.5 µL ultrapure nuclease free water, 0.5 µL each of forward and reverse primer and 1 µL of genomic DNA (~100 ng/µL), was added in each PCR tube to make the final volume 25 µL. The optimized touchdown PCR conditions were: initial denaturation at 95ºC for 5 min, followed by 32 cycles of each denaturation at 94ºC for 45 s; touchdown annealing at 63°C-50°C (−2°C/cycle) for 45 s; extension at 72ºC for 45 s and final extension at 72ºC for 7 min. The amplified PCR products were electrophoresed on 1.7% agarose gel and documented by gel documentation system (UVP, UK).

BsrI/PCR-RFLP *analysis of OPN (intron 4) gene fragment:* The 290 bp *OPN* (intron 4) DNA fragment were digested with *Bsr*I (*BseN*I) restriction enzyme at 65ºC for 4 h and 30 min. Digested PCR products were electrophoresed on 2.5% agarose gel and restriction patterns were analyzed by gel documentation system.

Association studies between OPN genotypes and milk production traits: The association of *OPN* genotypes with milk traits in HF crossbred was studied by using one way ANOVA. The linear model used to study association between genotypes and milk traits was:

$$Yij = \mu + \tau i + \epsilon ij$$

where, Yij, milk trait of j-th animal with i-th genotype; μ, overall mean, τi, i-th genotype effect and ϵij, the random error.

While, two mean student's 't' test by William Gosset were used for comparing means of genetic groups of Gir cows.

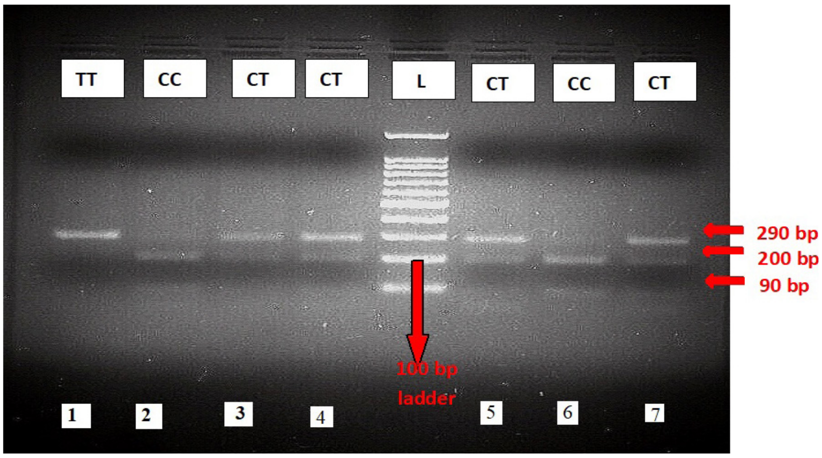

Fig. 1. Agarose gel electrophoresis of *Bsr*I/PCR-RFLP intron 4 amplified fragment of *OPN* gene in HF crossbred cow [Lane L- 100 bp DNA ladder, Lane 1- (TT genotype), Lane 2 and 6- (CC genotype), Lane 3, 4, 5 and 7- (CT genotype)].

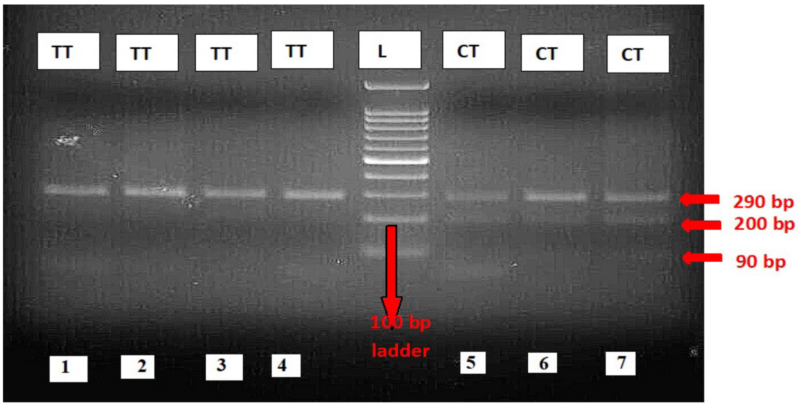

Fig. 2. Agarose gel electrophoresis of *Bsr*I/PCR-RFLP intron 4 of amplified fragment of *OPN* gene in Gir cow [Lane L- 100 bp DNA ladder; Lane 1, 2, 3 and 4- (TT genotype); Lane 5, 6 and 7- (CT genotype)].

Table 1. Gene and genotypic frequencies of *OPN* (intron 4) gene in HF crossbred and Gir cows

Breed	Animal number	Genotype	Genotype frequency	Gene frequency		Genotype frequency HWE χ^2 test ($p \leq 0.05$)
				T	C	
HF crossbred (n = 30)	6	‘TT’	0.20	0.48	0.52	0.765^{NS}
	17	‘CT’	0.57			
	7	‘CC’	0.23			
Gir (n = 30)	19	‘TT’	0.63	0.82	0.18	0.474^{NS}
	11	‘CT’	0.37			

RESULTS AND DISCUSSION

The present study was undertaken to ascertain association of Osteopontin gene with milk production traits in Gir native and HF crossbred cattle. A total of 30 Gir cows and 30 HF crossbred cows were genotyped using *Bsr*1/ PCR RFLP technique and statistical analysis was performed. The 290 bp *OPN* intron 4 DNA fragment was successfully amplified using touchdown PCR annealing temperature between 63ºC-50ºC (-2ºC/cycle).

BsrI/ PCR-RFLP assay for intron-4 of OPN region: The *Bsr*I/PCR-RFLP analysis revealed polymorphism at *OPN* intron 4 (c.8514 C>T locus) locus in HF crossbred and Gir cows. Three genotypes TT, CT and CC were observed in the HF crossbred population (Fig. 1), while only TT, CT genotypes were observed in Gir cows (Fig. 2). The ‘TT’ genotype was characterized with 290 bp single fragment; CC with 200 bp and 90 bp fragments, while 290 bp, 200 bp and 90 bp fragments indicated ‘CT’ genotype. The observed allelic and genotypic frequencies were T (0.48); C (0.52) and TT (0.20); CT (0.57); CC (0.23) for HF crossbred cows, while T (0.82), C (0.18) and TT (0.63), CT (0.37) for Gir cows, respectively. *OPN* allelic and genotypic frequencies and chi-square (χ^2) value are given in Table 1. These findings are in close agreement with Leonard *et al.* (2005) and Khatib *et al.* (2007). In HF bulls and cows, Leonard *et al.* (2005) reported gene frequencies as T (0.51) and C (0.49) while, genotypic frequencies as TT (0.27), CT (0.48) and CC (0.25). Similarly, Khatib *et al.* (2007) also observed frequencies of T and C alleles as 0.515 and 0.485 while, genotypic frequencies as 0.26, 0.51 and 0.23 for CC, CT and TT genotypes, respectively. The *OPN* intron 4 polymorphism with TT, CT and CC genotypes were also reported by Pareek *et al.* (2008) in Hereford and Polish Holstein cattle; Pasandideh *et al.* (2015), Kopaaee *et al.* (2016), Lali *et al.* (2020) and Yang *et al.* (2023) in Holstein cattle. All these researchers reported intermediate frequencies of ‘C’ and ‘T’ alleles along with higher frequency of ‘CT’ genotype in the said animals.

In case of Gir cows only, ‘TT’ and ‘CT’ genotypes were noted with predominance of ‘T’ allele. Similar findings were observed by by Oztabak *et al.* (2008) in East Anatolian Red (EAR) cattle; Kate *et al.* (2023) in Gaolao cattle and Karwa (2023) in Dangi cattle. The observed frequencies of ‘TT’ and ‘CT’ genotypes by them were 0.675, 0.325 in EAR cattle, 0.99, 0.01 in Gaolao cattle and 0.97, 0.03 in Dangi cattle. The predominance of ‘T’ allele with frequency ranging between 0.84 to 0.97 was observed by them. However, monomorphic nature of *OPN* gene with presence of only ‘T’ allele was recorded by Rahmatallah *et al.* (2015) in Sudanese dairy cattle breeds and Sharma *et al.* (2019) in Sahiwal and Hariana cattle breeds.

Association studies between OPN genotypes and milk production traits: The statistical analysis revealed non-significant association of *OPN* genotypes and lactation milk yield and fat per cent of studied HF crossbred and Gir population (Table 2). Like present findings, non-significant effect of *OPN* genotypes on milk and its components were noted by Kowalewska-Łuczak and Kulig (2013) in Jersey cattle. De Mello *et al.* (2012) showed that milk production was independent of *OPN* genotypes in Girolando cows, though they observed relatively higher milk production in cows carrying at least one copy of ‘T’ allele. Previous studies also recorded no association between *OPN* genotypes and breeding values of milk production, fat or protein per cent traits in Brown Swiss cattle (Zakizadeh *et al.* 2015); milk production of Iranian Holstein cattle (Koopaee *et al.* 2016); milk parameters and effect on frequent cattle diseases in milk samples of Holstein-Friesian cattle (Kulaj *et al.* 2019); milk yield and milk composition traits of Holstein Friesian crossbred cattle of Kerala (Lali *et al.* 2020); milk traits of Dangi cows (Karwa 2023).

In contrast to the present findings, several association studies reported significant influence of *OPN* genotypes on milk yield and milk traits of bovines. Leonard *et al.* (2005) first time reported SNP in intron 4 (C/T) and noted ‘C’ as a favourable allele for higher milk fat and protein percentage as compared to ‘T’ allele. Correspondingly, Khatib *et al.* (2007) confirmed the significant additive effects of *OPN* variants on milk fat and protein percentage as well as

Table 2. Association of *OPN* genotype with average milk yield and Fat % of HF crossbred and Gir cows

Genotype	Milk yield (kg) (Mean±SE)	Fat % (Mean±SE)
HF crossbred cows		
TT (n = 6)	5028 ± 268.72	3.650 ± 0.043
CT (n = 17)	5032.53 ± 221.07	3.659 ± 0.040
CC (n = 7)	5287.43 ± 157.17	3.671 ± 0.048
**p*-value (≤ 0.05)	0.752	0.964
Gir cows		
TT (n = 19)	2502.43 ± 78.12	4.13 ± 0.11
CT (n = 11)	2417.19 ± 138.14	3.87 ± 0.13
**p*-value (≤ 0.05)	0.582	1.574

fat yield. Pasandideh *et al.* (2015) showed significant association of *OPN* genotypes with adjusted per cent milk fat content for two milkings and per cent per day milk protein content adjusted for 305 days of Holstein cattle. Additive effect of *OPN* 'C' allele on expected breeding value of protein percentage in Czech Fleckvieh cattle was reported by Boleckova *et al.* (2012), however, no significant differences for breeding values of milk, protein yield, fat percentage and fat yield were reported. Besides above, *OPN* SNPS at different loci were also found to be associated with milk yield and milk components. Significant association *OPN* exon 4 genotypes (g.38329758, T > C) with milk protein content of Niliravi buffalo (Manzoor *et al.* 2018), *OPN* variants in 3'UTR (SNP rs132812135) with 305-day milk production in Argentinean Holstein and Holstein × Jersey cows (Raschia *et al.* 2018); lactational persistency in dairy cows with *OPN* (SPP1c.-1301G) variants (Bissonnette 2018) and *OPN* SNP (g.2916G>A and g.58899C>A) and haplotype Hap6/4 with increase in butter-fat percentage in Chinese Holstein cows (Raza *et al.* 2021).

Previous reports suggested *OPN* as a candidate gene for milk yield and milk traits in Holstein Friesian and other exotic cattle, however there are no published reports in Gir cows. Present investigation revealed polymorphic nature of *OPN* gene in HF crossbred and Gir cows. The chi-square test (×2) revealed no deviation from Hardy-Weinberg equilibrium in the studied population. Even though *OPN* genotypes did not exhibit any association with the milk traits, still these findings encourage further study to establish the relationship between the *OPN* gene and production traits of indigenous cows.

In conclusion, present investigation revealed *OPN* (c.8514 C>T) polymorphism in studied HF crossbred and Gir cows. The study showed no significant association between the Osteopontin genotypes and milk traits, which might be attributed to smaller proportion of screened animals. The studied population was under Hardy-Weinberg equilibrium. To confirm association findings and recommend c.8514 C>T SNP as molecular marker, detailed screening of extended population is essential, hence continuation of the similar studies in larger population is suggested.

REFERENCES

Anonymous. 2020. National Accounts Statistics, Press Information Bureau, Government of India, Ministry of Statistics and Programme Implementation, Pushpa Bhawan, New Delhi. (https://pib.gov.in/PressReleasePage.aspx?PRID=1693205).

Bissonnette N. 2018. Genetic association of variations in the osteopontin gene (SPP1) with lactation persistency in dairy cattle. *Journal of Dairy Science* **101**: 456–61.

Boleckova J, Matejickova J, Stipkova M, Kyselova J and Barton L. 2012. The association of five polymorphisms with milk production traits in Czech Fleckvieh cattle. *Czech Journal of Animal Science* **57**: 45–53.

Chakraborty D, Sharma A, Tantia M S, Singh A and Yathish H M. 2010. Role of secreted phosphoprotein 1 (*spp1*) gene in bovines – A review. *Agricultural Review* **31**: 184–93.

De Mello F, Guimarães M F M, Cobuci J A and Gualberto M V. 2012. Analysis of genetic diversity of osteopontin gene in Girolando cattle. *Revista Brasileira de Zootecnia* **40**(11): 2374–7.

Karwa S. 2023. 'Osteopontin gene polymorphism and its association with milk traits in Dangi cattle.' M.V.Sc. thesis submitted to MAFSU, Nagpur, Maharashtra.

Kate R, Kale D, Singh J, Patil D and Bahiram K. 2023. Polymorphisms within intron-4 and exon-4 regions of *SPP1* gene and their association with milk traits in Gaolao cattle. *The Indian Journal of Animal Sciences* **93**(1): 51–55.

Kerr J M, Fisher L W, Termine J D and Young M F. 1991. The cDNA cloning and RNA distribution of bovine osteopontin. *Gene* **108**: 237–43.

Khatib H, Zaitoun I, Wiebelhaus-Finger J, Chang Y M and Rosa G J M. 2007. The association of bovine PPARGC1A and OPN genes with milk composition in two independent Holstein cattle populations. *Journal of Dairy Science* **90**: 2966–70.

Koopaee K H, Pasandideh M, Dadpasand M, Koshkoiyeh E A and Abadi M M R. 2016. Joint analysis of the DGAT1, OPN and PPARGC1A genes effects on variation of milk production and composition in Holstein cattle population. *Iranian Journal of Applied Animal Science* **6**: 797–803.

Kowalewska-Łuczak I and Kulig H. 2013. Genetic polymorphisms of *FAM13A1*, *OPN*, *LAP3*, and *HCAP-G* genes in Jersey cattle. *Turkish Journal of Veterinary and Animal Sciences* **37**: 631–35.

Kułaj D, Pokorska J, Ochrem A, Dusza M and Makulska J. 2019. Effects of the c.8514C>T polymorphism in the osteopontin gene (OPN) on milk production, milk composition and disease susceptibility in Holstein-Friesian cattle. *Italian Journal of Animal Science* **18**(1): 546–53.

Lali A, Kulangara A and Aravindakshan T V. 2020. Effect of Osteopontin gene variants on milk production traits in Holstein Friesian crossbred cattle of Kerala. *Turkish Journal of Veterinary and Animal Sciences* **44**: 695701.

Leonard S, Khatib H, Schutzkus V, Chang Y M and Maltecca C. 2005. Effects of the osteopontin gene variants on milk production traits in dairy cattle. *Journal of Dairy Science* **88**: 4083–86.

Manzoor S, Nadeem A, Maryam J, Hashmi A S, Imran M and Babar M E. 2018. Osteopontin gene polymorphism association with milk traits and its expression analysis in milk of riverine buffalo. *Tropical Animal Health Production* **50**(2): 275–81.

Nagatomo T, Ohga S, Takada H, Nomura A, Hikino S, Imura M, Ohshima K and Hara T. 2004. Microarray analysis of human milk cells: Persistent high expression of osteopontin during the lactation period. *Clinical and Experimental Immunology* **138**: 47–53.

Oztabak K, Un. Cemal, Dawit T, Iraz A and Ahmet M. 2008. Genetic polymorphisms of osteopontin (*OPN*), prolactin (*PRL*) and pituitary-specific transcript factor-1 (*PIT-1*) in South Anatolian and East Anatolian Red cattle. *Animal Science Papers and Reports* **58**: 109–12.

Pareek C S, Czarnik U, Pierzchala M and Zwierzchowski L. 2008. An association between the C>T single nucleotide polymorphism within intron IV of Osteopontin encoding gene (*SPP1*) and body weight of growing Polish Holstein Friesian cattle. *Animal Science Paper and Report* **26**: 251–57.

Pasandideh M, Mohammadabadi M R, Esmailizadeh A K and Tarang A. 2015. Association of bovine *PPARGC1A* and *OPN* genes with milk production and composition in Holstein cattle. *Czech Journal of Animal Science* **60**: 97–104.

Rahmatallah S A, Rei Bmann M, Mueller U and Brockmann G A. 2015. Identification of genetic variants influencing milk production traits in Sudanese dairy cattle. *Research Journal Animal Science* **9**: 12–22.

Raschia M A, Nani J P and Maizon D O. 2018. Single nucleotide polymorphisms in candidate genes associated with milk yield in Argentinean Holstein and Holstein × Jersey cows. *Journal of Animal Science Technology* **60**: 31.

Raza S H A, Khan R, Pant S D, Shah M A, Quan G, Feng L, Cheng G, Gui L S and Zan L 2021. Genetic variation in the OPN gene affects milk composition in Chinese Holstein Cows. *Animal Biotechnology* **15**: 1–7.

Sambrook J and Russell D W. 2006. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press. Cold Spring Harbor, N.Y.

Sharma M, Singh S P, Tiwari M, Sharma D, Kumar A, Goel R and Yadav B. 2019. Fixation of T allele in C>T polymorphism in intron IV region of secreted phosphoprotein 1 (*SPP1*) gene in Indian cattle breeds. *International Journal of Livestock Research* **9**(9):116–21.

Weigel K. 2017. Genomic selection of dairy cattle: A review of methods, strategies, and impact. *Journal of Animal Breeding and Genomics* **1**(1): 1–15.

Yang Z, Sun Y, Wu X, Ma Y, Liu D, Lu X and Zhao T. 2023. Molecular marker-assisted selection of *ABCG2, CD44, SPP1* genes contribute to milk production traits of Chinese Holstein. *Animal (Basel)* **13**(1): 89.

Zakizadeh S, Gholami H, Vakili R and Ghods rohani M. 2015. The effect of osteopontin gene polymorphism on milk production traits in Brown Swiss cattle. *Journal of Animal Science* **105**: 283–90.