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Mutations and sequence variants in GDF9, BMP15, and BMPR1B genes in Maremmana cattle breed with single and twin births

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Abstract The families of TGF-β (transforming growth factor-β) proteins are the most important growth factors in the ovary, and three related oocyte-derived members, namely GDF9 (growth differentiation factor 9), BMP15 (bone morphogenetic protein 15), and BMPR1B (bone morphogenetic protein receptor 1B), have been shown to be essential for follicular growth and ovulation. Although the essential role of these genes in determining litter size in sheep and mouse and in controlling folliculogenesis in human has been demonstrated, there is limited information on their action in other species, especially in bovine. Bovine is a monotocous specie, as humans, with one or sometimes two newborns per birth. The twinning is a complex trait determined by both genetic and environmental factors. This study aimed at investigating the nucleotide sequences of different fragments of GDF9, BMP15, and BMPR1B genes in Maremmana cows reared in Castelporziano Presidential Estate (Rome). In this herd, in the period between 1996 and 2008, a twinning rate of 12 % (on average) was observed. We identified nine single-nucleotide polymorphisms (SNPs), five in the coding region, and four in the noncoding region: Two polymorphisms caused non-synonymous mutations, g.6045 G>A (V202I) in the BMP15 gene, and g.231 T>C (L66S) in GDF9 gene. The mutation L66S was found only in cows with double birth. In the literature, there are different evidences that mutations in proregion of GDF9 protein could affect its correct function. A relationship between mutations in this region of protein and granulosa cells proliferation and oocyte development was hypothesized.

Keywords Bovine · Twinning · Single-nucleotide polymorphism · TGF-β

1 Introduction

Bos taurus is a mainly uniparous specie in which twinning occurs at a low frequency. According to Vinet et al. (2012), twinning rate (TR: number of twin birth/100 births) ranged between 0.6 % (in North American Holstein–Friesian cattle breed) and 5 % (in French Maine-Anjou cattle breed). TR is the result of ovulation rate (OR), conception rate, and embryo survival. Gregory et al. (1997) estimated an heritability of 0.35 for OR and a genetic correlation of 0.75 between OR and TR. Ovulation rate is affected by age and parity of the dam, birth season, and feeding (Komisarek and Dorynek 2002). A complex regulatory endocrine network within the ovary and the hypothalamic–pituitary–ovarian axis controls the formation, growth, and selection of follicles due to ovulation (Eppig 2001; Scaramuzzi et al. 2011). Genetic improvement of TR is hampered by the low heritability level of the trait (Komisarek and Dorynek 2002), and few information is available in the literature on genes involved in TR (Silva del Rio et al. 2007).

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In *Homo sapiens*, *Mus musculus*, and *Ovis aries*, three genes: *BMP15* (bone morphogenetic protein 15), *GDF9* (growth differentiation factor 9), and *BMPR1B* (bone morphogenetic protein receptor 1B) were investigated for their effects on reproduction. These genes, related oocyte-derived members of the transforming growth factor-β (*TGF-β*) superfamily, are essential factors for follicular growth and ovulation (Chang et al. 2002; Otsuka et al. 2011; Nilsson et al. 2013; Li et al. 2014).

In humans, a large number of mutations in *GDF9* and *BMPR1B* genes have been identified in mothers of dizygotic twins (Montgomery et al. 2004; Palmer et al. 2006; Hoekstra et al. 2008; Painter et al. 2010; Luong et al. 2011), while Zhao et al. (2008) has demonstrated that variation in *BMP15* gene is not associated with spontaneous human dizygotic twinning. However, the important role of this gene in reproduction has been ascertained by Di Pasquale et al. (2004, 2006) who identified mutations in *BMP15* associated with premature ovarian failure.

In mouse, *GDF9* and *BMP15* genes play a crucial role in ovarian follicular development and they affect granulosa cell proliferation (Yan et al. 2001; Su et al. 2008, 2009).

In sheep, different mutations in *BMP15* (named FecX gene) and in *GDF9* (named FecG gene) genes were associated with increased ovulation rate in heterozygous animals and sterility in homozygous animals (Galloway et al. 2000; Hanrahan et al. 2004; Bodin et al. 2007; Monteagudo et al. 2009; Silva et al. 2011; Shabir and Ganai 2012; Demars et al. 2013; Våge et al. 2013; Mullen and Hanrahan 2014). In several sheep breeds known to be prolific, a single mutation in BMPR1B gene (named FecB gene) was associated with higher litter size (Mulsant et al. 2001; Wilson et al. 2001; Davis et al. 2002, 2006).

To date, genomic research on twinning in cattle consisted in studies on QTL associated with twinning rate and ovulation rate (Bierman et al. 2010; Vinet et al. 2012). Recently, Kim et al. (2009), by using 54 k bovine BeadChip, refined the position of QTL for TR in the North American Holstein population and found a significant association between SNP of *IGF1* (insulin-like growth factor 1) gene and TR. Kirkpatrick and Morris (2012), by using 3 k bovine BeadChip, located on BTA10 a major gene for bovine OR.

Given the scarce knowledge about genetic component in bovine twinning and the association between *GDF9*, *BMP15*, and *BMPR1B* genes with reproductive traits in sheep, human, and murine species, this study aimed to investigate the same genes in one cattle breed to ascertain the association of their SNP, if any exists, on twinning rate. For this study, the autochthonous Italian breed called "Maremmana" was chosen, a breed typically kept at range, well adapted to harsh environments, with a strictly seasonal reproductive cycle.

2 Materials and methods

2.1 Animals

Data from 98 cows of Maremmana breed reared in Castelporziano Presidential Estate (Rome, Italy) with one or more recorded calvings from 1996 to 2008 were analyzed. During those 7 years, 74 cows had only single births, and 24 gave birth to twins at least once. These 24 cows had 142 calvings, 40 of which were twins.

2.2 Blood collection and DNA isolation

Approximately 10 ml of blood was collected from the tail vein of the 98 cows using Vacutainer tubes with sodium heparin as anticoagulant. Samples were stored at −20 °C until the isolation of genomic DNA. Genomic DNA was extracted from frozen blood samples using Wizard DNA Extraction kit (Promega, Madison, WI, USA). DNA was quantified by DTX 800 Multimode detector (Beckman Coulter, CA, USA) using Quant-iT^{TM} PicoGreen dsDNA kit (Invitrogen, UK), and DNA quality was assessed by the spectrophotometer 260/280 ratio.

2.3 PCR amplification

PCR amplification of different fragments of BMP15, GDF9, and BMPR1B genes (Table 1) was performed using 16 DNA samples: eight from cows with single birth and eight from cows with at least one twin birth. The structure of the three genes in bovine was taken in account to select the fragments (http://www.ncbi.nlm.nih.gov/gene/): (a) the I and II exon both for BMP15 and GDF9 and VI exon for BMPR1B were amplified, because in sheep causative mutation in these regions was found (Galloway et al. 2000; Mulsant et al. 2001; Hanrahan et al. 2004); (b) the 3′UTR for GDF9 was amplified, because in bovine a SNP C/T (rs17871989) was identified (http://www.ncbi.nlm.nih.gov/snp?term=rs17871989); (c) the IV, V, VII, and X exon for BMPR1B was amplified, because these regions correspond to parts of the receptor involved in signal transduction (http://www.ncbi.nlm.nih.gov/gene?term=BMPR1B%20bos%20taurus).

For primer design, the following bovine gene sequences extracted by Ensembl database were used: GDF9 (ENSBTAG00000009478), BMP15 (ENSG00000130385), and BMPR1B (ENSBTAG00000002081).

PCR amplification was performed in 30 ul reactions containing 30 ng of genomic DNA, 1× PCR buffer, 0.2 mM each of the four dNTP, 0.8 pmol of each primer, and 0.08 U of GoTaq (Promega). A touch down PCR amplification was performed with an initial denaturation 5 min at 95 °C, followed by 14 cycles of 30 s at 94 °C,

Table 1 Primer designed for PCR amplification of BMP15, GDF9, and BMPR1B genes

Gene	Region of gene	Region of protein	Primer
BMP15	I exon	Proregion	Fw 5′-ATGGTCCTTCTGAGCATCCT-3′
			Rw 5′-TCTGAGAGGCCTTGCTACAC-3′
BMP15	II exon	C-terminal	Fw 5′-GCTCCTGGCACATACAGACC-3′
			Rw 5′-CTGCATGTACAGGACTGGGC-3′
GDF9	I exon	Proregion	Fw 5′-GTGTTCCTTGCTAATTCTTCCA-3′
			Rw 5′-TTCTTCTTCCCTCCACCC-3′
GDF9	II exon	C-terminal	Fw 5′-TGGCATTACTGTTGGATTGTTTT-3′
			Rw 5′-ATAAGCAATTGAGCCATCAG-3′
GDF9	3′UTR	21 aa of C-terminal	Fw 5′-CTGATGGCTCAATTGCTTAT-3′
			Rw 5′-TCATTTAAACATTTGGCCAT-3′
BMPR1B	IV exon	GS domain	Fw 5′-CTACACATAGAGGTTAGCAAGCAGT-3′
			Rw 5′-CTAATTAGGTCAAGGAAAGCTGTG-3′
BMPR1B	V exon	GS domain	Fw 5′-GTGGTTCTGCGTGTTCTGTC-3′
			Rw 5′-AGCGCTTTCCTTTGTCCACT-3′
BMPR1B	VI exon	GS domain	Fw 5′-TCCAGAGGACGATAGCAAAG-3′
		Protein kinase domain	Rw 5′-TTTCATGCCTCATCAACACC-3′
		ATP binding	
BMPR1B	VII exon	Protein kinase domain	Fw 5′-GCTTCATTGCTGCAGATATCA-3′
		H+ acceptor site	Rw 5′-AATAAACTTAACAGCCAAGCCC-3′
BMPR1B	X exon	Protein kinase domain	Fw 5′-AAGCCAGTATCGAGTGCCAG-3′
			Rw 5′-ATGTGCGAGGCTGGTACTGT-3′

30 s at 65 °C (−0.5 °C/cycle), 1 min 30 s at 72 °C; 25 cycles of 30 s at 94 °C, 30 s at 58 °C, 1 min 30 s at 72 °C; a final extension 5 min at 72 °C.

2.4 SNP identification

PCR products were purified using QIAquick PCR Purification kit (Qiagen, Italy) and quantified by DTX 800 Multimode Detector (Beckman Coulter) using Quant-iT^{TM} PicoGreen dsDNA kit (Invitrogen). MWG-Biotech outsourcing service was used to sequence the purified PCR products. The sequences obtained for each fragment were aligned using BioLign software (http://en.bio-soft.net/dna/BioLign.html) to identify putative SNPs.

2.5 SNP genotyping

Genotyping was performed by KBioscience using the proprietary Kaspar methodology. The putative non-confirmed SNPs were discarded. The remaining SNPs were genotyped in all other animals.

2.6 In silico analysis

BLASTP software was used (http://www.ncbi.nlm.nih.gov/BLAST/) to search the homology of the predicted protein sequences with other species. MEGA5 software was used for protein alignment (Tamura et al. 2011, http://www.megasoftware.net/). To determine the potential effect of the amino acid changes on structure and function of the proteins investigated, we used SIFT software (http://blocks.fhcrc.org/sift/SIFT.html). This software uses the protein sequence similarity of different species and the hydrophobic characteristics of amino acids to calculate the probability of a phenotypic effect of a specific amino acid variant (Ng and Henikoff 2003). Wild and mutant sequences were analyzed with the Human Splicing Finder (HSF) (Desmet et al. 2009), which includes several matrices to analyze splice sites, to identify potential SNPs with impact on splicing of the three genes.

2.7 Statistical analysis

For each SNP, the genotype frequencies were calculated and the Hardy–Weinberg equilibrium was verified using the ALLELE procedure in SAS (SAS Institute Inc. 2007).

Birth recording data were used to estimate the allelic substitution effect on number of births (Sherman et al. 2008). The number of copies of each allele of each SNP was regressed against each of the traits separately, using a mixed-effects model with the following equation:

$$Y_i = \mu + b_1 P_i + b_2 \mathrm{SNP}_i + e_i$$

where, Y_i was the trait being modeled per cow: prolificacy (total number of birth/total number of parturition), fertility (total number of birth/total number of mating cows), twinning rate (total number of twin birth/total number of parturition); μ is the overall mean; b_1 is the regression coefficient of the number of births (P_i); b_2 is the regression coefficient of the number of copies of each allele; e_i is a residual random error associated with the individual observation. Models were fitted using the MIXED procedure of SAS software (SAS Institute Inc. 2007). Significance of the allele substitution effect of SNP was tested by Student "*t*" test in the MIXED procedure.

A pedigree analysis was done using Par3 software of PEDIG package (Boichard 2002) to calculate the relationship coefficients within and between single and twin birth groups. Differences were analyzed and tested by one-way ANOVA in the SAS procedure.

3 Results

3.1 PCR fragments

In total, 10 amplicon were amplified. For *BMP15* gene, the following amplicons were obtained: (1) I exon, length 327 bp and (2) II exon, length 853 bp. For *GDF9* gene, the following amplicons were obtained: (1) I exon, length 456 bp, (2) II exon, length 952 bp, and (3) 3′UTR, length 281 bp. For *BMPR1B* gene, the following amplicons were obtained: (1) IV exon, length 305 bp, (2) V exon, length 307 bp, (3) VI exon, length 183 bp, (4) VII exon, length 296 bp, and (5) X exon, length 304 bp.

The sequences of all fragments were submitted to GenBank: *BMP15* accession number EU712722; *GDF9* accession number GQ922451, and *BMPR1B* accession number EU712721.

3.2 SNP discovered

In total, 9 new SNPs were discovered and were submitted to dbSNP. The position (in bp) for each SNP in the gene, the GenBank accession numbers, and amino acid change, when occurred, are listed in Table 2.

The silico analysis highlighted that only two of the nine SNPs were in coding region and were non-synonymous mutations. The polymorphism g.6045 G>A encodes for an amino acid change from Val to Ile (V204I), while the polymorphism g.231 T>C encodes for an amino acid change from Leu to Ser (L66S).

The bovine protein encoded by the BMP15 gene showed a 98 % identity with ovine, 74 % with human, and 69 % with murine proteins. Protein encoded by the *GDF9* gene showed a 95 % identity with ovine, 79 % with human, and 68 % with murine proteins. Protein encoded by the *BMPR1B* gene showed a 99 % identity with ovine, human, and murine proteins. The amino acid substitution V204I was located in the propeptide region, and from the alignment with the protein sequences from other species resulted that the amino acid isoleucine was present. The amino acid substitution L66S was located in the propeptide region, and the alignment with protein sequences from other species showed that the amino acid serine was never displayed.

Three of the nine SNPs (g.2630 C>T, g.23650 C>T, g.49215 C>T) were in noncoding region, and only two of these were in introns. The possible influence of the SNPs localized in the introns on the splicing event was analyzed, but none of the SNPs influenced the canonical donor, acceptor, and branch point sites.

3.3 SNP genotyping

Only 89 animals out of 98 were completely genotyped (the genotype of the remaining 9 animals was lost for technical inconvenients).

Out of the nine studied SNPs, two were monomorphic (*GDF9* r903T>C and *GDF9* r1272T>C) and seven polymorphic but in linkage disequilibrium. Allele substitution effects at polymorphic SNPs of *BMP15*, *GDF9*, and *BMPR1B* genes were estimated for fertility, prolificacy, and twinning rate (Table 3). Only SNP g.231 T>C in *GDF9* gene was significantly associated with prolificacy parameter ($P = 0.007$) and with TR ($P = 0.03$).

The estimated average of relationship within single birth group was 10.41 % and within twin birth group was 12.90 %, while between these groups it was 11.20 %. Between-group variance was 0.0100, and within-group variance was 0.0008, with significant F statistic ($P \geq 0.0006$).

4 Discussion

Reproduction is a fundamental trait for the efficiency of animal production. Nevertheless, little knowledge is available about the genes involved in the expression of reproductive traits, especially in bovine. Twinning has the potential to increase efficiency of beef production, by obtaining more calves from fewer cows (Gregory et al. 1997).

In mammals, the tendency to spontaneously conceive and maintain embryos is a complex trait affected by environmental and genetic factors. The candidate gene approach was very successful to find causal mutations involved in reproductive traits, in sheep and humans. Up to now, *GDF9*, *BMP15*, and *BMPR1B* genes, which play a

Table 2 SNP identified in *BMP15*, GDF9, and BMPR1B genes

Gene	Position of polymorphism[a]	Location in the gene	dbSNP accession number	aa change	Minor allele frequency (%)
BMP15	g.6045 G>A	II exon	ss175327186	V202I	0.090
	g.6113 C>A	II exon	ss175327187		0.090
GDF9	g.231 T>C	I exon	rs110553528	L66S	0.025
	g.2087 T>C	II exon	rs109250106		0
	g.2456 T>C	II exon	rs109422676		0
	g.2630 C>T	3′UTR	rs17871989		0.025
BMPR1B	g.23650 C>T	IV intron	rs109496270		0.006
	g.25125 G>A	V exon	rs109117120		0.026
	g.49215 C>T	IX intron	rs110544775		0.006

[a] The position is referred to following sequences: (a) BMP15 accession number AC_000187 (b) GDF9 accession number AC_000164 (c) BMPR1B accession number AC_000163

Table 3 Estimates of the allele substitution effect of BMP15, GDF9, and BMP15 SNPs

Allele	Trait	Estimate	*P* value
g.6045 G>A (G vs A)	Fertility	−0.0336	ns
	Prolificacy	0.0075	ns
	Twinning	−0.2177	ns
g.6113 C>A (C vs A)	Fertility	0.0397	ns
	Prolificacy	−0.0152	ns
	Twinning	0.3920	ns
g.231 T>C (T vs C)	Fertility	0.094	ns
	Prolificacy	0.207	0.007
	Twinning	1.144	0.031
g.2630 C>T (C vs T)	Fertility	0.024	ns
	Prolificacy	−0.010	ns
	Twinning	−0.190	ns
g.23650 C>T (C vs T)	Fertility	0.019	ns
	Prolificacy	−0.180	ns
	Twinning	−1.971	ns
g.25125 G>A (G vs A)	Fertility	−0.168	ns
	Prolificacy	−0.115	ns
	Twinning	−0.924	ns
g.49215 C>T (C vs T)	Fertility	0.018	ns
	Prolificacy	−0.180	ns
	Twinning	−1.97	ns

key role in regulating fertility in mammals, were mostly investigated for reproduction in this class of vertebrates.

Mulsant et al. (2001) identified a mutation in *BMPR1B* associated with increased ovulation rate in Booroola Merino ewes. Davis et al. (2006) confirmed this mutation in other prolific sheep breeds. Different authors (Galloway et al. 2000; Hanrahan et al. 2004; Bodin et al. 2007; Martinez-Royo et al. 2008; Monteagudo et al. 2009; Demars et al. 2013) discovered six different mutations in *BMP15* gene in eight sheep breeds (Inverdale, Hanna, Belclare, Galway, Lacaune, Rasa Aragonesa, Grivette, and Olkuska). Three different mutations in *GDF9* gene were identified in Belclare, Cambridge, Norwegian White, Thoka, and Embrapa sheep breeds (Hanrahan et al. 2004; Nicol et al. 2009; Silva et al. 2011; Våge et al. 2013).

In *Homo sapiens*, some mutations in *BMP15* gene have been identified in women with premature ovarian failure (POF) (Di Pasquale et al. 2004, 2006; Dixit et al. 2006; Laissue et al. 2006). In addition, rare deletions and missense mutations in the *BMP15* gene have also been identified in mother of dizygotic twins (Montgomery et al. 2004; Palmer et al. 2006). Furthermore, different mutations in *GDF9* gene significantly increased ovulation rate in women (Palmer et al. 2006) or caused the POF phenotype (Dixit et al. 2010; Christin-Maitre and Tachdjian 2010; Persani et al. 2011).

Homozygous *GDF9* knock-out female mice were found infertile, whereas heterozygous *GDF9* knock-out female mice were found fertile (Dong et al. 1996). Yan et al. (2001) demonstrated that homozygous *BMP15* knock-out female mice were found subfertile, with reduced litter size compared with heterozygous and wild-type females.

To date, few studies are available in the literature on these candidate genes in bovine. A research on *BMP15* gene (Zhang et al. 2009) detected a deletion in five Chinese cattle breeds. This deletion alters the reading frame and introduces a stop codon in *BMP15* mRNA, producing a shorter protein supposed non-functional by the authors. Hosoe et al. (2011) compared the expression patterns of *BMP15* and *GDF9* genes in young and adult bovine ovaries, and they showed that mRNA expression was different in calves and cows. Two polymorphisms detected on *GDF9* gene have been associated with superovulation and sperm quality traits in Chinese Holstein cows and bulls (Tang et al. 2013a, b).

Our study ascertained that the Maremmana cattle breed is polymorphic in *GDF9*, *BMP15*, and *BMPR1B* genes.

To the best of our knowledge, mutations in *BMP15* and *GDF9* genes have already been discovered by Zhang et al. (2009) and Tang et al. (2013a); moreover, these ones are different from the newly identified in our investigation.

Pedigree relationship within and between single and twinning birth groups showed in this trial a small difference. Even if significant, this difference should not pose any risk of false associations, since no selective mating for this trait has ever been performed in the past.

Two SNPs (g.6045 G>A in *BMP15* and g.231 T>C in *GDF9*) of the nine identified caused non-synonymous mutations. The SIFT analysis indicated that the amino acid change (V204I) in *BMP15* protein did not affect its structure and function, while the amino acid change Leu to Ser (L66S) in *GDF9* protein caused by g.231 T>C SNP influenced the protein structure and function (SIFT score <0.05). From a multiple alignment with other mammalian, *GDF9* protein resulted that this mutation occurs in a very conserved region (the proregion of protein). Interestingly, Shimasaki et al. (2004) referred that one of the important features in the posttranslational processing of the *TGF-β* superfamily members is that the proregion is necessary for the structure of the mature GDF9 protein. An interesting hypothesis to be investigated is whether the mutation occurring in *GDF9* proregion, as found in two Maremmana cows with twin birth, could influence *GDF9* protein three-dimensional folding and affect its function.

In conclusion, Maremmana cattle breed is polymorphic in *GDF9*, *BMP15*, and *BMPR1B* genes involved in reproductive traits in other species. One of the three polymorphisms of the *GDF9* gene showed an effect on twinning. Due to the size of our sample and the limited number of homozygous detected for the g.231 T>C SNP, these results require to be verified on a wider scale.

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