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Polymorphism within growth hormone receptor (GHR) gene in Romanian Black and White and Romanian Grey Steppe cattle breeds

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Abstract. Growth hormone receptor (GHR) is a transmembrane protein encoded by a gene mapped on cattle chromosome 20, which mediates the growth hormone (GH) action in different tissues. In cattle, a T/A substitution located in exon 8, which causes a p.Phe279Tyr in the transmembrane domain of GHR, was associated with higher protein and fat percentages of milk. In an effort to better understand the possible influence of this polymorphism on mammary gland development and function in cattle, we have comparatively studied the frequency this polymorphism on two groups of cattle. The first group was composed of Romanian Black and White (RBW) breed dams, Holstein type, characterised by well developed mammary gland and high milk yield and the second group was composed of Romanian Grey Steppe (RGS) Moldavian strain dams, an ancestral breed characterized by poorly developed mammary gland and very low milk yield, but with higher milk quality as regards protein and fat contents. Surprisingly we found that in RGS breed T allele (Phe) had the highest frequency (T=1), while in RBW breed the calculated frequencies were (T=0.773, A=0.227). Based on the observed frequencies and literature data, according to which A allele (Tyr) was associated with higher milk quality, we concluded that mutations in one gene cannot explain the variability of milk production traits in these two extreme breeds.
Key Words: Cattle, milk, growth hormone receptor, polymorphism, allelic frequency.

Introduction. In the last years understanding of the genetic basis of mammary gland development and function received an increased attention (Carsai & Balteanu 2010). The variations in milk production parameters cannot be attributed just to one gene because the secretor activity of mammary gland is controlled by a cascade of hormones, transcription factors and enzymes affected over the years by mutations, which are the basis these phenotypic variations (Carsai & Balteanu 2010).

The development and function of mammary gland is mainly controlled by growth hormone and prolactin, two protein hormones secreted in anterior pituitary gland. Growth hormone (GH), also known as somatotropin, stimulates milk production in cows (Zhou & Jiang 2006). The action of growth hormone in different tissues is mediated by growth hormone receptor (GHR) a transmembrane protein encoded by a gene mapped on cattle chromosome 20 (Georges et al 1995; Arranz et al 1998). GHR has three domains: extra-cellular that mediates binding of GH, transmembrane and intercellular, thereby interacting with signal transducer and activator of transcription 5 (STAT5) (Zhou & Jiang 2006; Carsai & Balteanu 2010). Binding of GH to GHR causes receptor dimerization that induces activation in cytoplasm of Janus kinase 2 (JAK2). This induces in turn activation trough phosphorylation of STAT5 which translocates to cell nucleus and binds to specific DNA regions activating gene expression (Herrington & Carter-Su 2001; Carsai & Balteanu 2010).

The GHR gene is specifically expressed mainly in liver where GH stimulates the production of growth factors from IGF family, IGF1 being one of the most potent activators of growth and cells proliferation and a potent inhibitor of apoptosis. The GHR gene is also expressed in mammary gland where meditates direct action of GH on mammary gland growth and its secretor activity (Feldman et al 1993).

The existence of QTL affecting milk production segregating on bovine chromosome 20 was suggested in several studies (Georges et al 1995; Arranz et al 1998; Viitala et al 2006). In this respect several polymorphisms were found in GHR gene (Blott et al 2003). In particular a T/A substitution located in exon 8 (referred here as g.T64A), which causes p.Phe279Tyr substitution (formerly called F279Y) in the peptide chain of the transmembrane domain, was found to influence some milk production traits. The presence of Tyr (A allele) was associated with higher protein and fat percentage and to a lesser extent on milk yield in Holstein-Friesian and Jersey cattle breeds (Blott et al 2003). In Finnish Ayrshire the presence of Tyr (A allele) was associated with increased protein and fat percentages (Viitala et al 2006), while in Chinese Holstein was associated with increased protein percentage (Sun et al 2009).

In an effort to better understand the possible influence of several key genes polymorphism on mammary gland development and function in cattle (Carsai & Balteanu 2010), the objective of this research was to comparatively study the frequency of g.T64A (p.Phe279Tyr) polymorphism in Romanian Black and White (RBW), Holstein type, a highly milk productive cattle breed and Romanian Grey Steppe (RGS) Moldavian strain, an ancestral cattle breed with very low milk yield, which seems to be the closest relative to extinct aurochs (Balteanu et al 2010).

Material and Method.

Animals and blood sampling. To infer the frequency of g.T64A polymorphism from GHR exon 8, 120 cattle were selected from two different breeds. The first lot was composed of 60 RBW dams, characterized by a well developed mammary gland and high milk yield (over 10000L/lactation). The second lot was composed of 60 RGS dams, an ancestral breed with poorly developed mammary gland and very low milk yield (about 1200L/lactation), but with higher milk quality as regards protein and fat contents. Peripheral whole blood samples (2 mL) were collected from jugular veins into tubes containing K3-EDTA as an anticoagulant and stored at 4 ^{0}C.

Direct amplification of target DNA from blood. For sequencing of GHR exon 8, a 342 base pairs (bp) fragment containing the entire exon and a part of its flanking regions was amplified from 16 samples (eight from each cattle breed). One set of primers (Viitala et al 2006) was used: a forward primer located in intron 7 (GhRE8-1F: 5`-GTGGCTATCAAGTGAAATCATTGAC-3`) and a reverse primer located in intron 8 (GhRE8-R: 5`-ACTGGGTTGATGAAACACTTCACTC-3`).

The amplification was achieved directly from whole blood without DNA extraction/purification using Phusion Blood Direct PCR Kit (Thermo Fisher Scientific, MA, USA). The PCR amplification reaction and cycling conditions were performed as described in Table 1.

Table 1

PCR reaction setup and cycling conditions

Component	*Volume*	*Cycle (repetitions)*	*Temperature*	*Time*
H2O miliQ	3.9 µL			
2x Phusion Blood PCR Buffer	10 µL	1 (1x)	98^{0}C	5 min
Forward Primer	0.8 µL		98^{0}C	1 sec
Reverse Primer	0.8 µL	2 (35x)	58^{0}C	5 sec
Phusion Blood II DNA Polymerase	0.4 µL		72^{0}C	30 sec
Whole blood	2 µL	3 (1x)	72^{0}C	60 sec
MgCl2	0.6 µL	4 (1x)	4^{0}C	60 sec
EDTA	0.5 µL			
DMSO	1 µL			
Reaction volume	20 µL			

Amplification products were analyzed in 2% agarose gel containing 1X Sybr Safe (Invitrogen, Eugene, OR, USA), in 1X TBE buffer (pH= 8.5) at 65 V constant current for 2 hours. The gel was then analyzed with a Molecular Imager Gel Doc XR System (BioRad Laboratories, Hercules, CA, USA).

Sequencing. The sixteen PCR reactions containing specific 342 bp amplicons were purified using ZR-96 DNA Clean & Concentrator™-5 kit (Zymo Research Corporation, Orange, CA, USA). The sequencing reaction was performed according to the didesoxynucleotide sequencing chain termination method, using BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). Both strands were sequenced with the primers GhRE8-1F and GhRE8-R and analyzed by Capillary Electrophoresis on an Applied Biosystem 3730 (Applied Biosystems, Foster City, CA, USA).

Genotyping for GHR g.T64A polymorphism. For genotyping of all sampled dams a 175 bp fragment was amplified from GHR gene using a new forward primer GhRE8-2F: 5`-AATACTTGGGCTAGCAGTGACAATAT-3` (Sun et al 2009), located in exon 8 and the same reverse primer used for sequencing (GhRE8-R). The amplification protocol was described in the previous paragraph. Because g.T64A mutation is not located in a restriction enzyme site, an artificial restriction site for *SSpI* enzyme was artificially created on the 175 bp fragment amplified by insertion of a single-base mismatch at 23 position of the forward primer (AATAT instead of ATTAT) (Sun et al 2009).

Amplified products (12 μL) were digested with 21μL digest mix containing 1μL *SSpI*, 2μL buffer (Fermentas, Vilnius, Lithuania) and 17μL miliQ water. Samples were incubated at 37 °C for 2 hours and were analysed in 3% agarose gel, as described in the previous paragraph.

Results and Discussion. The sequencing of the 175 bp PCR products amplified from exon 8 of GHR gene revealed the presence of g.T64A substitution characterizing the T and A alleles, respectively (Figure 1).

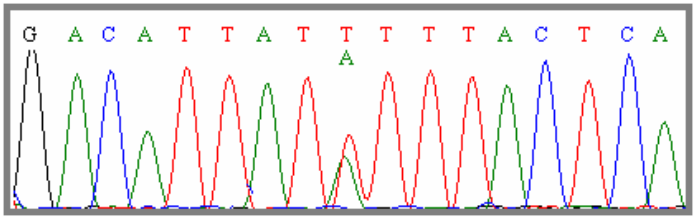

Figure 1. Part of sequencing chromatograms evidencing the g.T64A characterizing T and A allele respectively in cattle (TA genotype).

The genotyping the two cattle populations in GHR locus was done based on g.T64A substitution. The 176 bp amplicon amplified from T allele has one artificially created restriction site for *SSpI* enzyme and therefore two fragments are yielded following digestion: 151 bp and 24 bp, respectively. The g.T64A substitution makes this artificial created restriction site for *SSpI* enzyme inactive and therefore the 176 bp amplicon amplified from A allele remains undigested (Figure 2).

Genotyping of both cattle groups considered in this study was in accordance with sequencing data and revealed two genotypes TT and TA in RBW group: homozygous TT genotypes, characterized by the presence of two fragments 151 bp and 24 bp (invisible due to its small size) and heterozygous TA genotypes, characterized by the presence of an additional uncut fragment (175 bp). Based on these observations alleles and genotypes frequencies were calculated in the two cattle populations (Table 2).

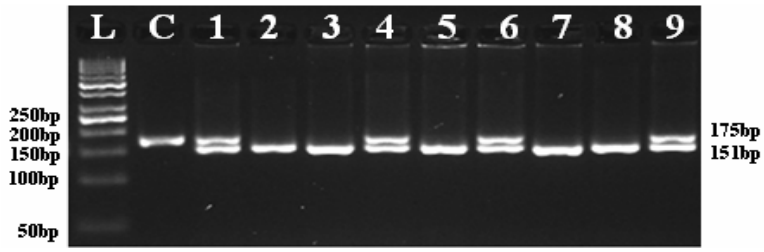

Figure 2. Electrophoresis profile evidencing g.T64A polymorphism located in exon 8 of GHR gene; L: 50bp ADN ladder (Fermentas, Vilnius, Lithuania); C – 175 bp uncut PCR product; Lanes 1, 4, 6 and 9 - TA genotypes; Lanes 2, 3, 5, 7 and 8 – TT genotypes.

Table 2

Genotypic and allelic frequencies in the GHR locus in analysed populations belonging to RBW and RGS cattle breeds

Breed/group	*Number of genotyped individuals*	*Genotypic frequency*			*Allelic frequency*	
		TT	TA	AA	T	A
RBW-high milk yield individuals	60	0.545	0.455	0	0.773	0.227
RGS- low milk yield individuals	60	1	0	0	1	0

RBW - Romanian Black and White, RGS - Romanian Grey Steppe.

The frequency of TT genotype was very close to the frequency of TA genotypes in RBW group. In RGS group only TT genotypes were observed. No AA genotypes were observed in the two cattle groups (Table 2). In RBW group the frequency of T allele is higher as compared with the frequency of A allele (Table 1). In RGS group the very high frequency of T allele (T=1) is surprising since this A allele was associated with higher fat and protein percentages in several cattle breeds as Holstein-Friesian and Jersey (Blott et al 2003), Finnish Ayrshire (Viitala et al 2006) and Chinese Holstein (Sun et al 2009).

Conclusions. We herein report the frequency a T/A substitution located in exon 8 of GHR gene (referred here as g.T64A) which causes p.Phe279Tyr substitution in the peptide chain, that was associated in several studies milk higher milk and fat percentages. This study was comparatively conducted on two groups of cattle expressing extreme phenotypes concerning mammary gland development, milk yield and milk quality. Based on the observed frequencies and literature data we have concluded that a single mutation in one gene cannot explain the variability of milk production traits found in these breeds, which is a polygenic trait with a multitude of factors involved. The polymorphism of GHR in the two Romanian cattle breeds is a part of a more complex study, which is trying to better explain the genetic basis of milk production variability in cattle by studying the polymorphism of several key genes involved in mammary gland development and its lactation capacity.

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