Polymorphism of insulin-like growth factor 1 gene and its relationship with reproductive performances and milk yield in Sarda dairy sheep

Luridiana Sebastianoa, Mura Maria Consueloa, Di Stefano Maria Veronicaa, Pulinas Luisaa, Cosso Giovannia, Nehme Michelleb, Carcangiu Vincenzoa,⁎

aDepartment of Veterinary Medicine, University of Sassari Via Vienna 2, 07100 Sassari, Italy
bDepartment of Veterinary Sciences, Faculty of Agriculture, Deibwaneh, Lebanon

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ABSTRACT

The aim of this research was to detect polymorphisms within the IGF-I gene in dairy sheep and to verify their influence on milk yield and reproductive performances. Four hundred Sarda ewes in their second lactation were selected from 2 farms. Their reproductive traits (fertility rate, interval in days from ram introduction to lambing and litter size) and milk yield were recorded, from the second to the fourth lactation. DNA was extracted from individual blood samples and subjected to amplification and sequencing of the IGF-I gene 5′ UTR and Exon 3 regions. Three polymorphic sites were recorded at positions g184028491C>G and g184028489C>T of the 5′UTR, and g184023223G>A of the Exon 3. The C allele at position g184028491 showed a significant association with higher fertility rate (P < 0.05) and a shorter interval in days from ram introduction to lambing (P < 0.01). In addition, a significant effect of the CC genotype was found with higher milk yield for P < 0.05 in the second and third lactation, and P < 0.01 in the fourth lactation compared to the other genotypes. Even, AA genotype at position g184023223 of the exon 3 showed a positive significant effect on milk yield for P < 0.05, in the second and third lactation, and for P < 0.01 in fourth lactation compared to the other genotypes. In conclusion the found SNPs showed a significant influence on reproductive performances and milk yield in Sarda sheep breed suggesting a possible application in sheep selection plans.

1. Introduction

Insulin-like growth factor-1 is a low-molecular-weight peptide, produced by liver and by many other types of cells, and it plays an important role in regulating postnatal growth, mammary gland development, lactation and reproduction in male and female in several species (Jiang & Lucy, 2001; Lammers, Heinrichs & Kensinger, 1999; Renaville, Hammadi & Portetelle, 2002; Tabecka-Lonczynska, Mytych, Solek, Kowalewski & Koziorowski, 2019). For these important physiological actions, this molecule has received considerable attention, as it can affect the animal's productive career. In fact, a rapid growth in young animal leads quickly to the adult body weight, with a greater chance of entering the production stage in a shorter time (Carcangiu et al., 2013). Furthermore, a more efficient reproduction is essential to reach an adequate production and, in recent years, it has been found that the action of the IGF-I on the ovary is important to improve the fertility of the animals (Gobikrushanth et al., 2018; Monte et al., 2019). In fact, alterations affecting the ovarian IGF-I receptors determine a high degree of apoptosis in large follicles as the FSH (follicle-stimulating hormone) cooperates with IGF-I in the control of growth, survival and maturation of ovarian follicles (Magalhães-Padilha et al., 2012). Recently, with the improvement of analytical tools and molecular biology, the study of the IGF-I gene and its expression has received considerable attention, and same polymorphisms in the IGF-I gene has been shown to be associated with many productive traits in different animal species. Particularly in cattle, a SNPs in the promoter region is linked with greater weight to calf weaning (de la Rosa Reyna et al., 2010; Ge, Davis, Hines, Irvin & Simmen, 2001). In chickens and pigs some polymorphisms in the coding region are associated with growth traits (Bhattacharya et al., 2015; Niu et al., 2013). In cashmere goats, some SNPs are linked to the wool growth (Wu-Jun et al., 2010). Finally, in sheep, variations of the IGF-I gene influence the litter size and the persistence of lactation (He et al., 2012; Scatà et al., 2010). So far, no research has been conducted on the sheep to evaluate association of IGF-1 gene polymorphisms with reproductive performances, with the exception of litter size. The aim of this research was to detect possible polymorphisms within the IGF-I gene and to verify their influence on reproductive
performances and milk yield in Sarda breed sheep.

2. Materials and methods

2.1. Animals management and experimental design

Domestic sheep is the most farmed species in the world, thanks to its adaptability to the difficult climatic and territorial conditions that characterize many less favored areas (poor vegetation, land not suitable for cultivation activity, reduced rainfall). In Italy, sheep have spread more widely in the southern and insular regions because they are able to maximize production even in adverse climatic conditions. Sarda is the most farmed and the best Italian breed of dairy sheep, and Sardinia (with more than 3 million of farmed ewes) has the highest density of sheep in Italy. This breed produces the highest amount of sheep milk in Italy and a considerable quantity of sheep milk at world level. Thus the improvement of their reproductive and productive performances is an important goal for the development of this breed. For the present study, 400 lactating Sarda ewes were selected from two farms located in North Sardinia. Each farm had approximately 800 adult Sarda ewes that were kept under the same management conditions, with grazing on pasture mixture of leguminous and gramineous grasses during the day. Moreover, they received 300 g per head/daily of concentrate commercial food (crude protein 20.4% and 12.5 MJ ME/kg DM) during the milking time. During the night sheep were penned and had free access to water and hay (crude protein 11.1% and 7.2 MJ ME/kg DM). The engaged ewes were all at their second lactation, in a good state of health and nutrition, and had been followed also for the two subsequent lactations. For each animal the reproductive performances and the milk yield of three consecutive lactations were recorded, from the second to fourth. The ewes at their first lambing and lactation were excluded because they do not express the full reproductive and productive potential (Mura et al., 2019). According to the commonly practiced sheep management, males, generally separated from females, were introduced in the flocks, between May 20th and June 1st, to trigger the mating period. Males were removed after 60 days of cohabitation with females. The individual rumen bolus was recorded and each ewe, selected for this study, was individually marked with a numbered collar to avoid identification mistakes. In order to evaluate the reproductive performances over three years, the fertility rate (as percentage of lambed ewes), the interval in days from ram introduction to lambing (IRIL) and the litter size (as number of newborn lambs per ewe) were recorded. Milk yield produced during three years were registered for each animal by ASSONAPA (Associazione Nazionale della Pastorizia).

2.2. Blood sampling and DNA analysis

An individual blood sample (10 ml) was collected, before the males introduction in the first years of the trial, from the jugular vein of the engaged ewes using sterile vacuum tubes with EDTA (ethylenediamine tetraacetic acid) as an anticoagulant (BD Vacutainer Systems, Belliver Industrial Estate, Plymouth, UK). The DNA was extracted from whole blood using a genomic DNA extraction kit (NucleoSpin® Blood, Macherey-Nagel, Germany). An amount of 150 ng genomic DNA was subjected to polymerase chain reaction (PCR) using specific primers (Thermo Fisher Scientific Massachusetts, USA) according to He et al. (2012) and Scatà et al. (2010) for 5’ UTR and exon 3, respectively. According to the Ovis aries IGF-I gene sequence (GenBank accession number X69472.1), the primers were designed to amplify the 5’ UTR sequence: sense primer 5’ – TGA GGG GAG CCA ATT ACA AAG C – 3’; antisense primer: 5’ – CCG GGC ATG AAG ACA ACA ACA A – 3’. Likewise, as regards of Exon 3, the primers were designed using the sequence X69473.1: sense primer: 5’ – CAG ACA CCT TGT TGC ACT CC – 3’; antisense primer: 5’ – AGA GCA TCC ACC AAC TCA GC – 3’. PCR analysis for 5’UTR was carried out in 25 μl total volume containing 2.5 μl of 10X PCR Buffer (200 mM Tris HCl (pH 8.4), 500 mM KCl); 0.75 μl of 50 Mm MgCl2; 4 μl of 1.25 mM dNTPs; 1 μl of 10 μM of both Primers (Forward and Reverse), 0.1 μl of Platinum Taq DNA Polymerase (Thermo Fisher Scientific, Massachusetts, USA). The PCR analysis for exon 3 was carried out in 25 μl total volume containing 2.5 μl of 10X PCR Reaction Buffer B (Mg2+ free) [0.8 M Tris HCl (pH 8.4), 0.2 M (NH4)2 SO4, 0.2% w/v Tween-20]; 2.5 μl of 25 Mm MgCl2, 4 μl of 0.2 mM dNTPs; 1 μl of 0.1 mM of each forward and reverse primers, and 2 U of FIREPol DNA Polymerase (5 U/μl) (Solis BioDyne, Tartu, Estonia). The PCR programs (performed on a Mastercycler ep gradient S, Eppendorf AG, Hamburg, Germany) consist in the following steps: heating lid at 105 °C, Taq activation at 94 °C for 2 min, followed by 30 cycles comprising denaturation at 94 °C for 30 s, annealing at 61 °C for 30 s for 5’ UTR and 30 s at 59 °C for exon 3, extension 72 °C for 30 s, and a final extension at 72 °C for 10 min. PCR products were separated by electrophoresis on a 1.5% (w/v) agarose gel (GellyPhor, Euroclone, Milan, Italy) in parallel with a 100-bp DNA ladder (Thermo Fisher Scientific, Massachusetts, USA). Following electrophoresis (at a constant voltage of 100 V for 30 min) gel was stained with ethidium bromide for 20 min and viewed on a UV Trans-illuminator (UV Tec, Cambridge, UK).

2.3. Sequencing

All the 400 PCR products were sequenced in the forward and reverse directions, using a commercial service. Sequencing was carried out using Applied Biosystems 3730 DNA Analyzer (Perkin-Elmer Applied Biosystems, Foster City, CA, USA), with Dye Terminator 3.1 chemistry. Nucleotide sequence alignments, translations and comparisons were carried out using the Bioedit software (http://www.mbio.ncsu.edu/bioedit/bioedit.html).

2.4. Statistical analysis

Allelic frequencies were determined by direct counting of the observed genotypes. R statistical software (Version 3.6.1 R Core Team 2019 R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/) was used to analyze the associations among genotype and milk yield (individual daily milk production, g/die) or reproductive traits, measured as fertility rate, litter size and interval in days from ram introduction to lambing (IRIL). The following linear model was used:

\[ Y_{jk} = \mu + G_j + e_k \]

Where \( Y_{jk} \) is the trait measured for each animal, \( \mu \) is the overall mean, \( G_j \) is the fixed effect of the genotype and \( e_k \) is the random residual effect of each observation. The farm effect did not show statistical significance and thus, were not included in the model. When \( P < 0.05 \) it was considered statistically significant. In this case, multiple comparisons of the means were performed using Tukey’s method (library Agricolae, R package version 1.3-1. Felipe de Mendiburu (2019): Statistical Procedures for Agricultural Research. https://CRAN.R-project.org/package=agricolae) (de Mendiburu, 2019).

3. Results

The PCR analysis allowed to detect, in all the 400 tested samples, a single band of 294 bp in length for IGF-I gene 5’ UTR, and a single band of 264 bp in length for IGF-I gene Exon 3 amplification. Using the Genomic Context section of NCBI (National Center for Biotechnology Information) we found that the obtained fragments, in reverse complementary, correspond to nucleotides g184025887–g184025894 (5’UTR) and g184023450–g184023187 (Exon 3) of the latest ovine genome version, within the chromosome 3 (Oar_rambouillet_v1.0, GenBank accession number NC_040254.1). The sequencing analysis revealed three polymorphic sites, ordered according to the above said genome version, at positions g184028491C>G and g184028489C>T within...
the 5′UTR, and at position g184023223G > A at the Exon 3 region. The polymorphisms at position g184028491C>G and g184028489C>T were always associated each other. Therefore, 3 genotypes for each SNP were detected in the tested ewes but considering that nucleotide variations at positions g184028491C>G and g184028489C>T were always associated each other. Therefore, 3 genotypes for each SNP were always associated each other and a clear prevalence of 5′ UTR region. In Texel and Dorset breed indeed a similar frequency of different genotypic and allelic frequency for the two polymorphisms at Exon 3 of the IGF-1 gene in Sarda sheep, based on the di
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### Table 2.
Fertility rate (%), litter size (%) and distance in days from ram introduction to lambing (IRIL) in the three considered years regardless of genotypes in Sarda sheep (n = 400).

| Fertility rate | Litter size | IRIL |
|----------------|-------------|------|
| Second lambing | 81.4        | 1.18 | 190.12 ± 20.60 |
| Third lambing  | 83.7        | 1.20 | 193.17 ± 20.53 |
| Fourth lambing | 84.4        | 1.18 | 192.60 ± 20.48 |

### Table 3.
Fertility rate, litter size, and interval in days from ram introduction to lambing (IRIL) according to genotypes at position g184028491 of the IGF-I gene in Sarda sheep (n = 400).

| SNP position | SNP location | Allele frequency (%) | Genotype | Genotype frequency (%) |
|--------------|--------------|----------------------|----------|------------------------|
| g184028491   | 5′ UTR       | C 90                 | CC 85    |                        |
| g184028489   | 5′ UTR       | C 90                 | CC 85    |                        |
| g184023223   | Exon 3       | G 54                 | GG 45    |                        |

Different lowercase letters within the row differ significantly for P < 0.05; Different capital letters within the row differ significantly for P < 0.01.

### Table 4
Means ± standard deviation (SD) of the individual daily milk production (g/die) in Sarda sheep, based on the different genotypes at position g184028491 of the 5′UTR of the IGF-I gene (n = 400).

| Genotype | Second lactation | Third lactation | Fourth lactation |
|----------|------------------|-----------------|------------------|
| CC       | 1580 ± 33        | 1800 ± 40       | 1990 ± 52        |
| CG       | 1370 ± 21        | 1540 ± 34       | 1640 ± 30        |
| GG       | 1160 ± 16        | 1500 ± 19       | 1580 ± 5         |

Different lowercase letters within the row differ significantly for P < 0.05; Different capital letters within the row differ significantly for P < 0.01.

### Table 5
Means ± standard deviation (SD) of the individual daily milk production (g/die) in Sarda sheep, based on the different genotypes at position g184023223 Exon 3 of IGF-I gene.

| Genotype | Second lactation | Third lactation | Fourth lactation |
|----------|------------------|-----------------|------------------|
| GG       | 1340 ± 225       | 1565 ± 300      | 1650 ± 305       |
| GA       | 1330 ± 250       | 1585 ± 285      | 1675 ± 295       |
| AA       | 1430 ± 245       | 1692 ± 150      | 1885 ± 340       |

Different lowercase letters within the row differ significantly for P < 0.05; Different capital letters within the row differ significantly for P < 0.01.

In the present study three single nucleotide variations were found in Sarda sheep breed, two located at position g184028491C>G and g184028489C>T in the 5′ UTR and one located in Exon 3 at position g184023223G > A of the IGF-I gene. The SNPs found at the 5′UTR sequence resulted always associated each other and a clear prevalence of the CC genotype at both positions emerged. The GG genotype is the most represented in the SNP at Exon 3. The allelic and genotypic frequencies for the two SNPs found in the 5′UTR region. In Texel and Dorset breed indeed a similar frequency than in this study was reported, while Chinese breeds have shown a lower G frequency, i.e., in agreement with observation reported by Darwish, El-Shorbagy, Abou-Eisha, El-Din and Farag (2017) and by Bakhtit (2017) in Egyptian and Iranian sheep, respectively. Also, about Exon 3, the allelic and genotypic frequencies found in the present

### Table 6
Means ± standard deviation (SD) of the individual daily milk production (g/die) in Sarda sheep, based on the different genotypes at position g184028491 of the 5′UTR of the IGF-I gene (n = 400).

| Genotype | CC       | CG       | GG       |
|----------|----------|----------|----------|
| Second lactation | 1580 ± 33 | 1370 ± 21 | 1160 ± 16 |
| Third lactation  | 1800 ± 40 | 1540 ± 34 | 1500 ± 19 |
| Fourth lactation | 1990 ± 52 | 1640 ± 30 | 1580 ± 5  |

Different lowercase letters within the row differ significantly for P < 0.05; Different capital letters within the row differ significantly for P < 0.01.

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study agree with what found by Scatà et al. (2010) in Italian breeds. Instead, Bakhtiar et al. (2017) in Iranian sheep found an allelic and genotypic frequency different from Italian sheep. These differences among breeds are certainly linked to the genetic selection operated over time.

In the current study the 5′ UTR SNPs are associated with the Sarda sheep reproductive performance. In fact, animals carrying the C allele, either in homozygous or in heterozygous state, exhibited a greater fertility and a shorter interval in days from male introduction to lambing in all the studied three years. These results agree with what found in Chinese sheep and Chinese and Indian goats, where, precisely, these two polymorphisms improve reproductive performances (He et al., 2012; Thomas, Venkatachalapathy, Aravindakshan & Raghavan, 2016; Wang et al., 2011). Nucleotide variations at the 5′ UTR of the IGF-I gene were also found in cattle and had been correlated with reproductive traits (Mullen et al., 2011; Nicolini, Carrquiry & Meikle, 2013). Also, in other animals’ species a positive effect of some polymorphisms of the IGF-I gene on reproductive activity has been found and many times this effect has been linked to the type of animal management (Hax et al., 2017). The important role of IGF-I hormone has been recognized in several reproductive stages, in fact, the higher levels of this hormone in pregnancy are necessary for fetal development and in parturition favor the recovery of ovarian function (Kawashima et al., 2006; Satue, Marcilla, Medica, Cravana & Fazio, 2018). In addition, in bovine granulose cell, FSH acts in synergy with IGF-I to increase cell number and aromatase expression (Silva, Figueiredo & van den Hurk, 2009). Moreover, the IGF-I enhances the stimulatory effects of FSH on progestosterone production, aromatase/estradiol, luteinizing hormone receptor and inhibin-a (Adashi, Esnick, Svoboda & Van Wyk, 1985). Indeed, studies demonstrated that ovaries lacking IGF-I receptor, had higher levels of apoptosis in follicles from the primary to the large secondary stages (Cox, Navarrete, Carrasco, Dorado & Saravia, 2019). Therefore, it is plausible to think that these polymorphisms can in some way influence the mechanism of action of IGF-I thus determining an improvement in reproductive activity. In this regard it would be crucial to highlight the precise role of the IGF-I gene polymorphisms on the follicular development.

The CC genotypes at the 5′ UTR and the AA genotypes at Exon 3 showed to influence the daily milk yield in the three recorded years. These results agree with what found by Scatà et al. (2010) both in the Sarda and in other Italian breeds. Indeed, Authors found that the SNP at Exon 3 caused a greater persistence of milk production during lactation period. Furthermore, several authors have found positive effects on different productive traits in different animal species thus confirming the important role played by this gene in several physiological mechanisms (Abdolmohammadi & Zamani, 2014; Ge et al., 2001; Gobikrushan et al., 2018; Niu et al., 2013; Sharma et al., 2013).

Regulatory regions are sections in the genome that do not code for proteins but exert a control on the expression of other district, which have coding function. Despite the lack of coding function, the regulatory regions play a crucial role in govern gene activity. Most of them exhibit binding sites for the transcription factors (TFs), proteins able to start and regulate DNA transcription. Therefore, given their crucial role, it is not surprising the great effect that a SNP that occur in the regulatory region can have. The nucleotide substitutions g184028491C>G and g184028489C<T detected in the 5′ UTR in the present research fall within the following sequence: TGAGGGGTTGGAGATCCCGTCGT. This sequence is recognized to be the core consensus binding sequence of the retinoid X receptor heterodimers (RXR) (Scatà et al., 2010). A consensus binding sequence is a short sequence of nucleotides that are shared or similar among multiple distinct DNA sites and is thought to play the same role in its different locations. In the specific case of our research, the consensus binding sequence of the retinoid X receptor heterodimers (RXR), above mentioned, is a member of the superfamily of nuclear factors that includes the vitamin D receptor (VDR). RXR and VDR form a heterodimeric complex and bind to the vitamin D responsive elements (VDREs) to activate or repress a great number of genes, involved in a variety of physiological functions (Lemon, Fondell & Freedman, 1997). This means that, the IGF-I gene could have different functional activity in the different genotypes, as hypothesized also by Scatà et al. (2010). In the present study, a significant effect of the CC genotype was found on milk yield in Sarda sheep.

As stated above, the found allele distribution at positions g184028491 and g184028489 and the almost total lack of animals carrying minor homozygous genotypes could be the results of the intense genetic selection aimed to increase milk production in this breed. Despite further investigations on a higher study population would help to confirm the importance of the IGF-I gene on reproductive efficiency and milk yield in a high production dairy sheep breed. However these results provide new insights into its crucial role in dairy performance. In this research a genotype/phenotype association was found that, carefully detailed and studied, could become an important reference to be used in sheep breeding programs.

5. Conclusions

Considering the IGF-I as a potential candidate gene for reproductive and milk yield, its polymorphisms were investigated in dairy sheep. In Sarda breed, three nucleotide variations in the 5′ UTR region and Exon 3 of the IGF-I gene, were found. The three SNP investigated have shown to be involved in regulating the reproductive activity and milk production in Sarda sheep breed. So, these SNPs can be useful in planning selection programs that can improve the reproductive and productive performances in sheep. However, it will be interesting to conduct further research on a higher number of animals to confirm the effect of the found SNPs and to highlight other SNPs in the sequence of this gene that can also be associated with productive and/or productive parameters in sheep.

Ethical statement

All the animals in this research had veterinary care by the National Health Veterinary Service in accordance with the Animal Welfare Act. Blood samples were collected by veterinarians of the National Health Service for routine health assessments. The farmers involved in the research were informed and provided consent to use the animals in the present study. The approval of the ethics committee was not considered necessary because we did not handle the animals, the biological material was provided by National Health Veterinary Service.

CRediT authorship contribution statement

Luridiana Sebastianino: Data curation, Investigation, Methodology, Software, Validation, Writing - original draft, Writing - review & editing. Mura Maria Consuelo: Formal analysis, Methodology, Supervision, Validation, Writing - original draft, Writing - review & editing. Di Stefano Maria Veronica: Data curation, Formal analysis, Writing - original draft, Writing - review & editing. Pulinas Luisa: Formal analysis, Writing - review & editing. Cosso Giovanni: Formal analysis, Writing - review & editing. Nehme Michelle: Formal analysis, Writing - review & editing. Carcangi Vincenzo: Conceptualization, Funding acquisition, Supervision, Writing - original draft, Writing - review & editing.

Declaration of Competing Interest

None of the authors have any conflicts of interest to declare.

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