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Genetic basis of Lipomatous Myopathy in Piedmontese beef cattle

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ABSTRACT

In Piedmontese cattle breed, the sporadic detection of Lipomatous Myopathy (LM) has been reported. The phenotypic disease expression consists in degeneration and infiltration of the muscular tissue characterized by replacement of myofibers with adipose tissue. The aim of this study was to investigate the existence of genetic loci associated with lipomatous myopathy in Piedmontese cattle breed through a genome wide association study based on a DNA pooling design. The samples used for the study were collected from a meat cutting plant, pairing cases and controls within farms. Samples of different muscles (diaphragm, superficial and deep pectoral, intercostal, sternocleidomastoid group and *vastus lateralis*) were histopathologically and enzymatically classified as cases and controls. DNA pools of cases and controls were constructed. Equal amounts of DNA were pooled from individuals for the constitution of 4 pools (2 independent biological replicates for cases and 2 for controls). Technical duplicates were also built and all pools genotyped with the Illumina BovineHD BeadChip three time each, for a total of 24 chip array positions. SNPs positions were based on the UMB 3.1 bovine assembly. The B-allele frequencies (BAF) for each array replicate were used in a specific pipeline in R software to perform multiple marker tests after excluding the 5% of SNPs showing the highest BAF variability from the replicate arrays within tail, as well as the monomorphic SNPs. A total of 41 QTLRs were identified on the 29 bovine autosomes, and 4 on the X chromosome. A subset of the identified markers fall inside or nearby genes involved in myogenesis, adipogenesis and cell to cell adhesions. The biological role of these genes in the onset of lipomatous myopathy has been identified looking at the known functions of the encoded proteins on the GeneCards database. Gene networks have been identified using STRING.

1. Introduction

In the Piedmontese cattle breed, the sporadic detection of Lipomatous Myopathy (LM) has been recently histologically well described by Biasibetti et al. (2012) in about 3% of Piedmontese male and female heads. The authors classified the disease as "Lipomatous muscle myopathy", characterized by degeneration and infiltration of the muscular tissue by replacement of myofibers with a different grade of infiltration of adipose tissue, involving multiple or single muscles or rarely the whole carcass.

At present there is no available information on any genetic background of the pathology, and no study has been performed to identify genetic variation connected to the variability disclosed in the Piedmontese carcasses at slaughtering. The availability of dense SNP chips allows a whole genome scan at limited cost and has been recently used to disclose genetic variation related to disease in cattle, e.g. for BRD (Lipkin et al., 2016). The selective DNA pooling experimental design (Darvasi and Soller, 1994) that they used, increases the power of the design and permits the detection of QTLs related to a specific phenotypic variation also in case-control studies, reducing the costs of genotyping.

The aim of this study was to investigate the existence of genetic variation associated with LM in Piedmontese cattle breed through a genome-wide association study using a selective DNA pooling design.

2. Materials and methods

2.1. Sampling of individuals

More than 5500 carcasses of Piedmontese cattle breed (males and females) were inspected in 3 years and collected from a local cutting plant located in the Piedmont region (north of Italy), allowing specific

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visual classification of the individuals (affected and non affected), and tissue sampling. Cases and controls were paired within farms: to select control animals, individuals from the same farms of affected animals were visually inspected at the cutting plant and then submitted to histopathological examinations to validate the negative diagnosis. Different muscles (diaphragm, superficial and deep pectoral, intercostal, sternocleidomastoid group and *vastus lateralis*) were sampled at cutting in order to confirm classification of animals as cases and controls after histological evaluation.

A total of 200 animals were used in this study including affected individuals (i.e. cases) (n = 100) and not affected (i.e. controls) (n = 100).

2.2. Histopathological, histochemical and enzymatic investigations

All muscle tissues, both from cases and control individuals, were processed histopathologically in order to minimize both false positives and false negative classifications. Samples were firstly examined macroscopically, then frozen in isopentane cooled using liquid nitrogen, and stored at −80 °C until subsequent histopathological investigations. Slide Section (10 μm thick) of muscle tissue were obtained using a cryostat and stained for histological and histochemical techniques: haematoxylin and eosin (H & E); modified Gomori trichrome; periodic acid- Schiff (PAS); Red Oil stains, Sudan black, Sudan III. All sections were examined under a transmitted light microscope at 100x and 400x magnification. The adenosine triphosphatase (ATPase), cytochrome C oxidase (COX); succinate dehydrogenase (SDH) and reduced nicotinamide adenine dinucleotide–tetrazolium reductase (NADH-TR) enzymatic activities were also evaluated. Samples were identified as cases when showed a variable infiltration of adipose tissue, not attributed to normal marbling occurrence, involving multiple or single muscles (Fig. 1).

2.3. DNA extraction, pooling constitution and genotyping

All genomic DNA was isolated from muscular tissue in triplicate using the Gentra Puregene Tissue kit® (Qiagen), according to the manufacturer's instructions. All DNA samples were normalized to 50 μl volume using the Concentrator Plus/Vacufuge® plus instrument (Eppendorf).

The quality control was performed on each DNA sample to verify its purity and integrity using the Tecan Infinite 200 NanoQuant (Tecan, Männedorf, Switzerland) and the Invitrogen E-Gel 1% Agarose Gel, respectively. The Qubit® (Life Technologies) instrument with the Qubit® dsDNA HS Assay kit (Life Technologies), were utilized to determine the DNA concentration. Three independent biological replicates were created from each sample. In addition, the DNA concentration for a single replicate was evaluated six times (technical replicates) and each read was verified at 5, 15 and 30 min after samples preparation. As a criteria, samples having DNA concentration diverging from > 6% of the Coefficient of Variation (CV) value, calculated within sample replicates, were not included in the pools. Among the 200 available samples, 39 samples were excluded because of low quality of DNA, leaving 70 controls and 91 cases.

DNA pools were constructed by taking equivalent amounts of DNA from each sample. In order to obtain two independent groups of animals with comparable phenotypic value, the selected samples for each tail (case-control tails) were adjusted randomly into two sub-pools (biological-replicates of pools). Furthermore, for each pool, two DNA duplicate-pools (technical-replicates) were independently constructed from identical samples. Thus, a total of 4 pools per tail were then produced.

The final pools were concentrated to 50 ng/μl, as required for the Illumina array protocol.

Each sub-pool and the relative duplicates were genotyped three times (array-replicates). In total 24 chip positions on different microarrays were used for the pooled genotyping. Genotyping was performed using the Illumina BovineHD BeadChip (777,962 SNPs) according to the Infinium protocol. SNPs position was based on the UMB 3.1 bovine assembly.

2.4. Statistical analysis of pools

One array-replicate, belonging to a control pool, was eliminated from the analysis because its B-allele frequency mean values diverged significantly from the other technical/array-replicates.

Pools were analysed according to the Selective DNA Pooling (SDP) approach using the B-allele frequencies (BAF) for each array replicates, which were obtained from the self-normalization algorithm of Illumina BeadStudio software®, as reported by Strillacci et al. (2014).

A single-marker test for marker-trait association was used, and the P-value for the each marker was calculated as P = 2 x the area of the standard normal curve to the right of:

$$Ztest = Dtest/SD(Dnull),$$

where Dtest is the difference of the B-allele frequencies means among tails; Dnull is the difference of the B-allele frequencies means within tails.

We performed the multiple marker test analysis after excluding monomorphic SNPs, SNPs mapped on BTY, Mitochondrial SNPs, SNP without chromosome position, as well as the 5% of those markers that showed the highest variability of BAF values, as indicated by the size of the SD among measures from the replicate assays within tail. After filtering 662,547 SNPs (27,413 on BTX) were used for the statistical analysis.

According to Benjamini and Hochberg (1995), the nominal P values at different FDR thresholds has been identified and is reported in Table 1, together with the number of significant SNP for autosomes (i.e.

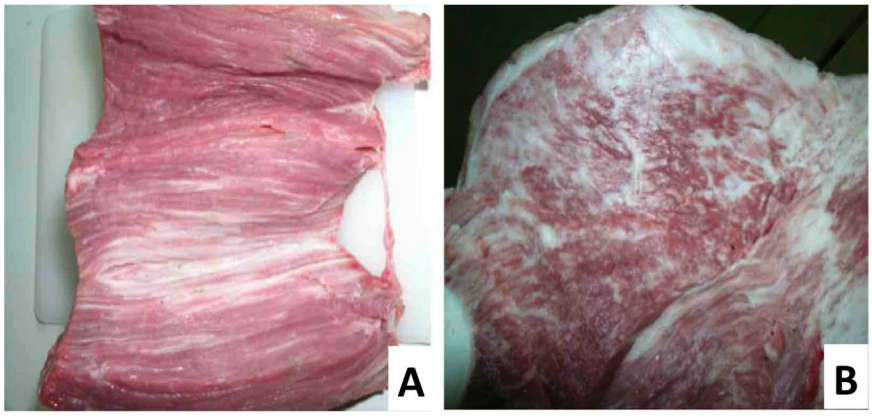

Fig. 1. Muscle with adipose tissue infiltration: sagittal section (A) and transversal section (B).