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Research paper

Transcriptomic hallmarks of bone remodelling revealed by RNA-Seq profiling in blood of Arabian horses during racing training regime

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Abbreviations: ACAP2, Arf-GAP with coiled-coil; ACLY, ATP citrate lyase; ADCY9, adenylate cyclase 9; AGPAT2, 1-acyl-*sn*-glycerol-3-phosphate acyltransferase beta; ARFGEF2, ADP ribosylation factor guanine nucleotide exchange factor 2; ARHGEF6, Rho guanine nucleotide exchange factor 6; ATF2, activating transcription factor 2; ATM, serine/threonine kinase; BGLAP, bone gamma-carboxyglutamate (gla) protein, osteocalcin; BUB1, budding uninhibited by benzimidazoles 1; BUB1B, mitotic checkpoint serine/threonine kinase B; C1D, C1D nuclear receptor corepressor; CANX, calnexin; CAPN2, calpain 2; CBL, E3 ubiquitin-protein ligase; CBLB, Cbl proto-oncogene B; CCNA2, cyclin A2; CDC27, division cycle 27; CDC27, cell division cycle protein 27 homolog; cDNA, complementary DNA; CHMP2B, charged multivesicular body protein 2B; CLEC5A, C-type lectin domain family 5; CLTC, clathrin heavy chain; CNOT6L, CCR4-NOT transcription complex subunit 6 like; CNOT7, CCR4-NOT transcription complex subunit 7; CTSK, cathepsin K; DAVID, Database for Annotation Visualization and Integrated Discovery; DDX6, DEAD-box helicase 6; DEGs, differentially expressed genes; DGAT1, diacylglycerol *O*-acyltransferase 1; DGAT2, diacylglycerol *O*-acyltransferase 2; DGKA, diacylglycerol kinase alpha; DHX36, DEAH-box helicase 36; DIAPH2, diaphanous homolog 2; DLAT, dihydrolipoamide *S*-acetyltransferase; DLG1, discs large MAGUK scaffold protein 1; DNAJC10, DnaJ heat shock protein family (Hsp40) member C10; EEA1, early endosome antigen 1; ENOSF1, enolase superfamily member 1; EPS15, epidermal growth factor receptor pathway substrate 15; ETS1, ETS proto-oncogene 1; FBXW8, F-box and WD repeat domain containing 8; FC, fold change; FLNB, filamin B; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GEO, Gene Expression Omnibus; GPD1, glycerol-3-phosphate dehydrogenase 1; HK3, hexokinase 3; HSP90AA1, heat shock protein 90 alpha family class A member 1; HSPD1, heat shock protein family D (Hsp60) member 1; IL6ST, interleukin 6 signal transducer; IQGAP2, IQ motif containing GTPase activating protein 2; ITCH, itchy E3 ubiquitin protein ligase; ITGA4, integrin subunit alpha 4; ITGB1, integrin subunit beta 1; ITPR1, inositol 145-trisphosphate receptor type 1; ITPR2, inositol 145-trisphosphate receptor type 2; KAT2B, lysine acetyltransferase 2B; KEGG, Kyoto Encyclopedia of Genes and Genomes; KHK, ketohexokinase; KIF5B, kinesin family member 5B; LCAT, lecithin-cholesterol acyltransferase; LMAN1, lectin; MAP3K1, mitogen-activated protein kinase kinase kinase 1; MAP3K7, mitogen-activated protein kinase kinase kinase 7; MBOAT7, membrane bound *O*-acyltransferase domain containing 7; MCM4, minichromosome maintenance complex component 4; MGLL, monoglyceride lipase; MSN, moesin; NFATC1, nuclear factor of activated T-cells 1; NFATC1-, nuclear factor of activated T-cells 1; ORC1, origin recognition complex subunit 1; PABPC1, poly(A) binding protein cytoplasmic 1; PAK2, P21 (RAC1) activated kinase 2; PAN3, PAN3 poly(A) specific ribonuclease subunit; PASB, Polish Arabian Stood Book; PDCD4, programmed cell death 4; PDLIM7, PDZ and LIM domain 7 (enigma); PFKFB3, 6-phosphofructo-2-kinase/fructose-26-biphosphatase 3; PGS1, phosphatidylglycerophosphate synthase 1; PIKFYVE, phosphatidylinositol-3-phosphate 5-kinase type III; PIP4K2A, phosphatidylinositol-5-phosphate 4-kinase type 2 alpha; PLA2G4F, phospholipase A2 group IVF; PMM1, phosphomannomutase 1; PNPLA7, patatin like phospholipase domain containing 7; PPP1CB, protein phosphatase 1 catalytic subunit beta; PPP1R12A, protein phosphatase 1 regulatory subunit 12A; PPP2R5E, protein phosphatase 2 regulatory subunit B'epsilon; PPP3CA, protein phosphatase 3 catalytic subunit alpha; PRKDC, protein kinase DNA-activated; PTPN11, tyrosine-protein phosphatase non-receptor type 11; pval, p value; QC, quality control; qPCR, quantitative PCR; RAD21, RAD21 cohesin complex component; RAD23B, RAoD23 homolog B; RASGRP1, RAS guanyl releasing protein 1; RBL2, RB transcriptional corepressor like 2; RDX, radixin; RNA, ribonucleic acid; RNA-Seq, RNA sequencing; RPS6KA3, ribosomal protein S6 kinase A3; RRAS2, Ras-related protein R; SEC23A, 5-Sec23 homolog A coat complex II component; SEC24A, SEC24 homolog A COPII coat complex component; SEL1L, E3 ligase adaptor subunit; SEPT11, Septin 11; SEPT2, Septin 2; SKIV2L2, Ski2 like RNA helicase 2; SLC9B2, solute carrier family 9 subfamily B; SLK, STE20 like kinase; SSH2, slingshot protein phosphatase 2; SSR1, signal sequence receptor subunit 1; STAG2, stromal antigen 2; SUCLG2, succinate-CoA ligase GDP-forming beta subunit; TAZ, tafazzin; TCAP, titin-cap; TGFBR1, transforming growth factor beta receptor 1; THBS1, thrombospondin 1; TIAM1, T-cell lymphoma invasion and metastasis 1; TOB2, transducer of ERBB2; TRIM37, motif containing 37; TTC37, tetratricopeptide repeat domain 37; TWSG1, twisted gastrulation BMP signalling modulator 1; TXLNG, taxilin gamma; TYROBP, TYRO protein tyrosine kinase binding protein; UBA2, ubiquitin like modifier activating enzyme 2; UBA6, ubiquitin like modifier activating enzyme 6; UBE2J1, ubiquitin conjugating enzyme E2 J1; VAV3, vav guanine nucleotide exchange factor 3; VCL, vinculin; VPS36, vacuolar protein sorting 36 homolog; WIPF1, WAS/WASL interacting protein family member 1; XPO1, exportin 1; YWHAG, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein gamma; YWHAZ, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta; ZFYVE16, zinc finger FYVE-type containing 16

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ABSTRACT

The impact of exercises on young developing organisms is still of interest to researchers. Similarly like Thoroughbreds, Arabian horses competing at the race track. The high percent of lameness and loss of days in training are often the result of weakness in the condition of the musculoskeletal system. The objective of the presented study was to identify by RNA-Seq method, the possible skeletal system originating transcriptomic profile in peripheral blood of Arabian horses undergoing race training. Obtained results showed that one of the most significantly deregulated pathway involved in bone homeostasis was those involved in osteoclast differentiation. Among the significantly expressed molecules, we recognized twelve genes potentially involved in the metabolism of the skeletal system: *BGLAP*, *CTSK*, *TYROBP*, *PDLIM7*, *SLC9B2*, *TWSG1*, *NOTCH2*, *IL6ST*, *VAV3*, *NFATc1*, *CLEC5A*, *TXLNG*. The panel of identified genes should be evaluated as candidate biomarkers for bone homeostasis indicators of Arabians performing on race tracks to assess bone remodelling states during training for race track competitions.

1. Introduction

It is well established that Arabians and half-Arabians are mainly endurance horses (Cappelli et al., 2007). However, endurance training requires maturity, which is reached at least by 4 years old. Before that, 2.5-year old horses are introduced to race track training and compete in at least one racing season before they begin further endurance-type workouts. The Arabian racing industry is a well-established high-income business. In general, the 3 year old individuals run a minimum distance of 1400 m whereas the most prestigious Derby Stakes for 4 year old horses is performed at 3000 m. The impact of exercises on young developing organisms is still of interest to researchers. The high percent of lameness and loss of days in training are often the result of weakness in the condition of the musculoskeletal system manifested by the presence of pathological changes that do not show clinical symptoms and may have impacts on future performances in endurance (Turło et al., 2014). Horse blood consists of components that play a key role in supporting the increased metabolic rate during exercise. Blood interacts with each organ and tissue in the body by transporting oxygen, water, electrolytes, nutrients, and hormones. Furthermore, by circulation, products from metabolic processes are removed from cells (Marlin and Nankervis, 2002). According to its functions, blood plays a key role in immunity, inflammation and maintenance of homeostasis and is involved in the pathogenesis of many diseases (Mohr and Liew, 2007). The transcriptomic profile of blood can be a powerful tool to explore disease pathogenesis and physiological homeostasis including rheumatoid arthritis (Oswald et al., 2015), cancer (Dumeaux et al., 2015), and diabetes (Collares et al., 2013). In addition to human research, profiling of the blood transcriptome has been adapted for various animal species due to many functional topics such as reproduction (Kizaki et al., 2013), resistance to infection (Huang et al., 2011), inflammatory response (Burgess et al., 2012) and heat stress (Lewis et al., 2010). Based on the capacity of peripheral blood to reflect changes in the body, we hypothesize that training regime - induced changes may be reflected by the specific transcriptomic profile in blood of trained horses.

Understanding the molecular pathways involved in the ability of the skeletal system to convert environmental signals into biochemical signals (mechanotransduction) should lead to a better understanding of the process of bone remodelling. Furthermore, horse is a valuable animal model in terms of skeletal response to optimized exercise regimens during race training. Thus, the objective of the presented study was to identify the possible skeletal system originating transcriptomic profile in peripheral blood of Arabian race horses after a heavy canter workload before beginning race track performances.

2. Materials and methods

2.1. Animals, samples and study design

The protocol was approved by the Animal Care and Use Committee of the Institute of Pharmacology, Polish Academy of Sciences in Cracow (no. 1173/2015).

All pure breed Arabian horses included in the study belong to lines that have documented origins since at least 1810 and since then, have been maintained without crossing with French lines, and all the horses are registered in the PASB (Polish Arabian Stood Book). To ensure the quality of the samples, all animals were bred by one breeder and recruited with the owner's agreement. They were raised in the same farm and fed in comparable ways including feeding with high quality hay and oats. From a total of 10 horses, 6 (GI) were sampled in September when they were 2.5 years old before the introduction to the race track; thereafter, they were treated as controls. Four (GII) horses were sampled after a 24-week period, included 12 weeks of light work under the saddle with gradual increased intensity, followed by 12 weeks of conditioning exercises prior to the start of race training containing sequences of heavy canters (Ropka-Molik et al., 2017a).

The blood samples (n = 10) were collected by jugular venipuncture into a Tempus™ Blood RNA Tube (Ambion, Life Technologies) and stored at −20 °C until further analysis.

2.2. RNA-Seq performance: RNA isolation and libraries preparation

The RNA was isolated by using the MagMAX™-96 Total RNA Isolation Kit (Ambion, Life Technologies) according to the attached protocol. The quality and quantity of obtained RNA were evaluated on a TapeStation 2200 (Agilent Technologies) by using RNA Screen-Tape and RNA Screen-Tape Ladder and by using a NanoDrop 2000 spectrophotometer (Thermo Scientific). Additionally, RNA was visualized by 2% agarose gel electrophoresis to determine its degradation degree. A 400 ng sample of total RNA was used to construct cDNA libraries with the use of the TruSeq RNA Kit v2 (Illumina) according to the manufacturer's instructions. The quality and quantity of obtained libraries were evaluated by a Qubit 2.0 (Invitrogen, Life Technologies) and a TapeStation 2200 (Agilent D1000 ScreenTape; Agilent). The libraries were sequenced on the HiScanSQ platform (Illumina) using the TruSeq SR Cluster Kit v3-CBOT-HS and TruSeq SBS Kit v 3 - HS chemistry (Illumina), which was performed in 75 single-end cycles.

2.3. Bioinformatics analysis

The bioinformatic analysis was initiated from the quality control (QC) of the obtained NGS sequences using FastQC software. Next, adapter-removal and filtering to retain reads longer than 36 bp and reads with a quality score higher than 20 were performed using Flexbar (Dodt et al., 2012). The quantification of genes and transcripts was