The involvement of muscle-related genes in the occurrence of scrotal hernia in pigs

G. S. Romano[1], W. R. Lorenzetti[2], A. M. G. Ibelli[3], J. O. Peixoto[3], M. A. Z. Morés[3], I.R. Savoldi[4], K.B. Carmo[4], J. S. Lopes[5], V. B. Pedrosa[6], M. E. Cantão[3], L. L. Coutinho[7]& M. C. Ledur[3]

[1]Universidade Federal da Bahia, Av. Adhemar de Barros 500, 40170-110, Salvador, Brazil
[2]Universidade do Estado de Santa Catarina, Rua Beloni Trombeta Zanin, 680-E. Bairro Santo Antônio, Chapecó, SC, Brazil.
[3]Embrapa Suínos e Aves, Rodovia BR 153 s/n, 89715-899, Concórdia, SC, Brazil
monica.ledur@embrapa.br (Corresponding Author)
[4]Universidade do Contestado, Rua Victor Sopelsa, 3000, 89700-970, Concórdia, SC, Brazil
[5]BRF S/A, Rodovia BR 277, 3001 – Mossunguê, 82305-100, Curitiba, PR, Brazil
[6]Universidade Estadual de Ponta Grossa, Av. General Carlos Cavalcanti 4748, 84030-900, Ponta Grossa, Brazil
[7]Escola Superior de Agricultura "Luiz de Queiroz", Universidade de São Paulo, Av. Pádua Dias, 11, 13418-900, Piracicaba, São Paulo, Brazil

Summary

The incidence of scrotal hernia is still a problem in the swine production, leading to economic losses and reducing the animal welfare. Although some QTLs and candidate genes have already been associated to scrotal hernia, the genetic mechanisms and genes controlling this pathology remain to be clarified. Therefore, this study aimed to identify genes involved in the scrotal hernia occurrence in pigs. Thus, two experiments were performed: 1) RNA-Seq analysis of the inguinal ring tissue from eight 60-days-old Landrace pigs (4 healthy and 4 affected with scrotal hernia) that were sequenced in the Illumina HiSeq 2500 and 2) quantitative PCR (qPCR) of 8 candidate genes chosen from the inguinal ring transcriptome in 18 MS115 30-days-old pigs (9 normal and 9 affected with scrotal hernia). Based on the differentially expressed (DE) genes in this transcriptome, some candidate genes related to muscle development biological processes (BP), including the muscle structure (*ACTA1*), intracellular transport (*RYR1, MYBPC1*), cell adhesion (*COL13A1*) and apoptosis (*MAP1LC3*) were found. The expression profile of the DE genes was consistent with the anatomical alterations related to the scrotal hernia development, such as the weakness of the inguinal canal and non-obliteration of the *processus vaginalis*. In the qPCR analysis, the *MYH1* was the only DE gene identified. The downregulation of the *MYH1*gene might be one of the possible causes of scrotal hernia in swine since its expression had already been reduced in 30-days-old affected pigs. Therefore, the DE genes identified in our study related to muscle development bioprocesses are possibly involved in the occurrence of scrotal hernia in pigs.

Keywords: congenital disorder, qPCR, RNA-Seq, transcriptome

Introduction

Congenital disorders in livestock production, such as scrotal hernia in pigs, lead to economic losses and reduced animal welfare (Sevillano et al., 2015). Although some QTLs and

candidate genes have already been associated to scrotal hernia (Sevillano et al., 2015; Grindflek et al., 2006), the genetic mechanisms and genes controlling this pathology remain to be clarified. Despite hernias have been considered as a congenital disorder controlled by a reduced number of genes, recent genomic studies have indicated a complex genetic architecture for this defect. Sevillano et al. (2015) identified several significant SNPs explaining a small proportion of variance for hernia, reinforcing the use of genomic estimated breeding values to reduce hernia incidence in pigs. Although scrotal hernia etiopathogenesis is not completely understood (Ding et al., 2009), physiological changes such as the inguinal canal loosening, posterior inguinal wall weakness or non-obliteration of the *processus vaginalis* have been associated to the inguinal/scrotal hernias (Beck et al., 2006, Sevillano et al., 2015). Therefore, functional genomic studies can contribute to identify transcriptome alterations in the inguinal canal tissue related to hernias. Hence, this study aimed to identify differentially expressed genes involved with occurrence of scrotal hernia in pigs.

Material and methods

Animals, Sample Collection and RNA extraction

Experiment 1. A total of eight pedigreed Landrace pigs with 60 days of age from the same nucleus farm, located in Santa Catarina State, Brazil, was used in this study. Water and feed were supplied *ad libitum* and the management conditions followed the recommendations for this line. The pigs were transported to Embrapa Swine and Poultry National Research Center, Concórdia, SC, Brazil for necropsy and sample collection. Tissues from the inguinal ring were collected from 4 healthy (control group) and 4 animals with scrotal hernia (affected group), stored in liquid nitrogen and frozen at -80ºC for further analysis. The control animals were selected from families without history of hernia in the last 3 generations.

Experiment 2. A total of 18 pigs with 30 days of age from a synthetic male line (MS115) developed by Embrapa Swine and Poultry was used in this study. The animals were reared at Embrapa following the recommendations for this line. The tissues from the inguinal ring were collected from 9 healthy (control group) and 9 animals with scrotal hernia (affected group) and then frozen at -80ºC.

For both experiments, euthanasia was performed by electrocution followed by exsanguination, according to the ethical guidelines of the Embrapa Swine and Poultry Ethics Committee on Animal Utilization, under the protocol # 011/2014. After sample collection, the total RNA extraction from 100 mg was performed using the Trizol reagent (Invitrogen). The quality of total RNA was evaluated in a Bioanalyzer Agilent 2100 equipment, considering only samples that presented RIN values (RNA integrity number) above 8 for further analysis.

Library preparation, RNA Sequencing, global gene expression and functional analysis

The cDNA libraries were prepared using the TruSeq™ Stranded RNA Sample Prep Kit (Illumina, USA). Sequencing were performed in the Illumina HiSeq 2500 platform (lllumina, USA) with a 2x100bp paired-end protocol, at the Functional Genomics Center, ESALQ-USP, Piracicaba, São Paulo, Brazil. After the sequencing, the data quality control was performed using the SeqyClean software (https://github.com/ibest/seqyclean). All reads were mapped against the swine reference genome (SusScrofa 10.2, Ensembl 84) using the BWA-MEM software (Li & Durbin, 2010). Reads counting were performed in the HTseq-count script

(Anders et al., 2015) and differential expressed (DE) genes (FDR ≤0.05) were obtained using the edgeR package (Robinson et al., 2010). Negative and positive fold-changes indicate downregulated and upregulated genes, respectively, comparing the affected and non-affected groups. DAVID Bioinformatics Resources 6.8 (http://david.abcc.ncifcrf.gov) was used for functional annotation.

Quantitative PCR (qPCR)

For the experiment 2, eight DE muscle-related genes in the transcriptome were chosen to be evaluated by qPCR: myosin heavy polypeptide 1 (*MYH1*), desmin (*DES),* actin alpha 1 *(ACTA1),* actin gamma 2 *(ACTG2*), myosin binding protein C *(MYBPC1),* fibroblast growth factor 1 (*FGF1),* calponin 1 *(CNN1),* and microtubule associated protein 1 light chain 3 gamma *(MAP1LC3C)*. Primers in exon-exon junctions were designed using the Primer-Blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) and the qPCR reactions were carried out in 15 µL final volume containing 1X of Maxima SYBR Green/ROX qPCR Master Mix (2X) (Thermo Fisher Scientific), 0.05 to 0.13 µM of each primer and ~20 ng of cDNA. The analysis was performed in the Quantstudio 6 (Thermo Fisher Scientific) using SYBR Green as fluorescent dye. The cycle threshold (Ct) means for each sample and gene was obtained and normalized to a reference gene (*RPL19*), and the relative quantification was performed using the Relative Expression Software Tool (REST©) (Pfaffl et al., 2002).

Results and discussion

Regarding the RNA sequencing (experiment 1), 13,035 genes were expressed in the analyzed tissues, being 644 DE between normal and herniated pigs (FDR ≤ 0.05). Based on the DE genes, 409 biological processes (BP) were found. Out of those, it was possible to highlight some candidate genes related to muscle development BP, including the development of muscle structure (*ACTA1*), cell differentiation (*FGF1*), protein signaling (*DESMIN, MYH1*), intracellular transport (*RYR1, MYBPC1*), cell adhesion (*COL13A1*), apoptosis (*MAP1LC3*) and regulation of ATP (*TPM2*) activity. The expression profile found in the analyzed tissue is consistent with the anatomical alterations related to the scrotal hernia development reported in the literature, such as the weakness of the inguinal canal or non-obliteration of the *processus vaginalis* (Beck et al., 2006, Sevillano et al., 2015).

To the correct obliteration of the *processus vaginalis,* the smooth muscle apoptosis is required (Tanyel et al., 2003), avoiding hernia development. In the transcriptome analysis, the *MAP1LC3* was the most upregulated gene in the affected group when compared to the normal group (Fold change = 7). This gene is involved in cell autophagy (Shpilka et al., 2011), a process that can inhibit the apoptosis (Thorburn, 2008). Therefore, the high expression level of *MAP1LC3* in the affected group could reduce the smooth muscle apoptosis, leading these animals to be more susceptible to scrotal hernia. The posterior inguinal canal weakness, due to the fibers and muscles degeneration, has already been related to the predisposition of hernia in humans (Bendavid, 2004). However, the *MAP1LC3* gene was not DE in the qPCR analysis, with 30d-old pigs. Another DE gene clustered in the muscle development BP is the *ACTA1*, which encodes the main actin isoform present in the thin filaments of the sarcomere in the skeletal muscle. Several congenital myopathies have been associated with the *ACTA1* gene, resulting in hypotrophy and muscle weakness, and cell catabolism (Feng & Marston, 2009; Laing et al., 2009). This gene was upregulated in pigs affected with scrotal hernia at 60 days of age. However, in an early age (30d) this expression pattern was not observed.

In the qPCR analyses, the myosin heavy polypeptide 1 (*MYH1*) gene was the only DE gene in the 30 days-old pigs ($p<0.05$), being downregulated in the affected group and also, following the same pattern observed in the 60-days-old pigs transcriptome (FDR< 0.05). *MYH1* was -11.94 and -10 times less expressed in the affected than in the normal group at 60 and 30 days of age, respectively. Individuals with scrotal hernia have a decrease in type I collagen, which makes the inguinal affected tissue loosen (Beck et al., 2006). The *MYH1* function is associated with the tight junction pathway and its downregulation may alter the transcription of other genes, such as the *RHOA* (Ras Homolog Gene Family, Member) gene (Zhu et al., 2013). The unbalanced expression of these two genes might affect extracellular matrix components, including type I collagen (Zhu et al., 2013). Furthermore, the myosin and troponin proteins interact with the actin filaments generating ATP release conferring force and muscle movement (Lie & Hutson, 2011), which might help in the tissue strengthen. According to our results, the muscle related DE genes are candidates to be involved in the scrotal hernia occurrence. The downregulation of the *MYH1* gene might be one of the possible causes of the incidence of this hernia in swine since its expression had already been reduced in 30-days-old affected pigs.

Conclusion

Differentially expressed genes related to muscle development bioprocesses are possibly involved in the occurrence of scrotal hernia, being the *MYH1* a strong candidate to trigger this anomaly in pigs.

Acknowledgements

This work was supported by project # 476146/2013 from CNPq, Brazil. LL Coutinho is a CNPq fellow. GS Romano received a Scholarship from FAPESB, Ba, Brazil. IR Savoldi and KB Carmo are recipients of a PIBIC/CNPq scholarship.

List of References

Anders, S. & W. Huber, 2010. Differential expression analysis for sequence count data. Genome Biology, 11:R106–R106.

Beck, J., K. Bornemann-Kolatzki, C. Knorr, H. Taeubert, & B. Brenig, 2006. Molecular characterization and exclusion of porcine GUSB as a candidate gene for congenital hernia inguinalis/scrotalis. BMC Vet. Res. 2(1):2-14.

Beuermann, C., J. Beck, U. Schmelz, H. Dunkelberg, E. Schütz, B. Brenig & C. Knorr, 2009. Tissue calcium content in piglets with inguinal or scrotal hernias or cryptorchidism. J. Comp. Pat. 140(2): 182-186.

Bendavid, R., 2004. The unified theory of hernia formation. Hernia 8(3): 171-176.

Ding, N. S., H. R. Mao, Y.M. Guo, J. Ren, S. J. Xiao, G.Z. Wu, H.Q. Shen, L.H. Wu, G.F. Ruan, B. Brenig, & L.S. Huang, 2009. A genome-wide scan reveals candidate susceptibility loci for pig hernias in an intercross between White Duroc and Erhualian. J. Anim. Sci. 87(8): 2469-2474.

Feng, J. J. & S. Marston, 2009. Genotype-phenotype correlations in ACTA1 mutations that cause congenital myopathies. Neuromuscul. Disord. 19(1): 6-16.

Laing, N. G., D.E. Dye, C. Wallgren Pettersson, G. Richard, N. Monnier, S. Lillis, T.L. Winder, H. Lochmuller, C. Graziano, S. Mitrani-Rosenbaum, D. Twomey, J.C. Sparrow,

A.H. Beggs & D. Twomey, 2009. Mutations and polymorphisms of the skeletal muscle alpha-actin gene (ACTA1). Hum. Mutat. 30(9): 1267-1277.

Lie, G. & J.M. Hutson, 2011. The role of cremaster muscle in testicular descent in humans and animal models. Ped. Surg. Inter., 27(12): 1255-1265.

Li, H. & R. Dubin, 2009. Fast and accurate short read alignment with Burrows-Wheeler Transform. Bioinformatics. 25(14):1754–1760.

Robinson, M.D., D.J. McCarthy & G.K. Smyth, 2010. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics. 26: 139-140.

Pfaffl, M.W., G.W. Horgan & L. Dempfle. 2002. Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Research.30(9):e36.

Rozenblum, G. T. & M. Gimona, 2008. Calponins: adaptable modular regulators of the actin cytoskeleton. Int. J. Biochem. Cell. Biol. 40(10):1990-1995.

Sevillano, C. A., M. S. Lopes, B. Harlizius, E.H. Hanenberg, E. F. Knol & J.W. Bastiaansen, 2015. Genome-wide association study using deregressed breeding values for cryptorchidism and scrotal/inguinal hernia in two pig lines. Genet. Sel. Evol. 47(1): 1-8.

Shpilka, T., H. Weidberg, S. Pietrokovski & Z. Elazar, 2011. Atg8: an autophagy-related ubiquitin-like protein family. Genome Biol. 12(7): 226.

Sparrow, J. C., K. J. Nowak, H. J. Durling, A. H. Beggs, C. Wallgren-Pettersson, N. Romero, I. Nonaka & N.G. Laing, 2003. Muscle disease caused by mutations in the skeletal muscle alpha-actin gene (ACTA1). Neuromuscul. Disord. 13(7): 519-531.

Zhu, J., D. Nguyen, H. Ouyang, X. H., Zhang, X. M. Chen, & K. Zhang, 2013. Inhibition of RhoA/Rho-kinase pathway suppresses the expression of extracellular matrix induced by CTGF or TGF-β in ARPE-19. Int. J. Ophthalmol. 6(1): 8-14.

Tanyel, F. C., S. Erdem, N. Büyükpamukçu & E. Tan, 2002. Smooth Muscle within Incomplete Obliterations of Processus vaginalis Lacks Apoptotic Nuclei. Urol. Int. 69(1): 42-45.

Thorburn, A., 2008. Apoptosis and autophagy: regulatory connections between two supposedly different processes. Apoptosis, 13(1), 1-9.