Novel putative candidate genes associated with umbilical hernia in pigs

L. T. Fernandes[1], R. K. Ono[2], A. M. G. Ibelli[2], E. B. Lagos[3], M. A. Z. Morés[2], M. E. Cantão[2], W. R. Lorenzetti[4], J. de O. Peixoto[2], V. B. Pedrosa[3] & M. C. Ledur[2]

[1] Centro de Diagnóstico de Sanidade Animal - CEDISA, 89715-899, Concordia, SC, Brazil
[2] Embrapa Suínos e Aves, Caixa Postal 321, 89715-899, Concórdia, Brazil
monica.ledur@embrapa.br (Corresponding Author)
[3]Universidade Estadual de Ponta Grossa, Av. General Carlos Cavalcanti 4748, 84030-900, Ponta Grossa, Brazil
[4]Universidade do Estado de Santa Catarina, Rua Beloni Trombeta Zanin 680E, 89815-630, Chapecó, Brazil

Summary

Umbilical hernia is one of the most frequent anatomical defects in pigs in which abdominal contents protrude through the umbilical ring. This condition is considered to have a multifactorial basis in which environmental, infectious and genetic factors play a role. However, a better understanding about the genetic components involved in the umbilical hernia development has not yet been achieved. Although a few studies have mapped QTL for umbilical hernia, just a few candidate genes were reported. Therefore, the aim of this study was to identify genomic regions related to the development of umbilical hernias in pigs and search for potential candidate genes. A GWAS was performed with 92 cases and 233 control crossbred pigs. Five SNPs were associated with umbilical hernia: on SSC4/SSC6/SSC13 and one with unknown position. Candidate genes *TBX15* and *WARS2* were identified close to the SNP on SSC4. Another two candidate genes were located near the SNP associated with umbilical hernia on SSC13 (*LIPI* and *RBM11*). Further validation of these genes should be performed to improve the knowledge about umbilical hernia development in order to improve pig welfare and production by eliminating susceptible animals through genetic selection.

Keywords: candidate genes, congenital defect, GWAS, swine.

Introduction

Umbilical hernia is one of the most frequent anatomical defects in pigs in which abdominal contents protrude through the umbilical ring. This condition occurs due to an acquired weakness of the muscles and connective tissues that comprises the navel and umbilical areas of the animal. It has been reported that the occurrence of umbilical hernias ranges from 0.4 to 1.2% and mainly affects 9 to 14 week-old pigs (Searcy-Bernal *et al*., 1994; Petersen *et al*., 2008). The development of hernias can lead to considerable economic loss and poor animal welfare, and pigs affected by these conditions often show impaired growth, reduced feed conversion rate and reduced carcass quality.

Environmental and infectious factors have been described to contribute to the umbilical hernia occurrence, such as poor hygiene, stretching of the umbilical cord during farrowing, incorrect placing of navel clips, perinatal umbilical infections and navel sucking (Searcy-Bernal *et al*., 1994). However, this condition is considered to have a multifactorial basis, and a better understanding about the genetic components involved in the umbilical

hernia development has not yet been achieved. It has been suggested that this condition may be associated with genetic lines (Rutten-Ramos & Deen, 2006). Also, a few previous studies based on genome-wide scans have identified a number of genomic regions on 11 porcine chromosomes and six copy number variation (CNV) regions in different pig breeds associated with umbilical hernia occurrence (Ding *et al*., 2009; Liao *et al*., 2015; Long *et al*., 2015). Although these studies are informative, just a few candidate genes were reported. Therefore, the aim of this study was to identify genomic regions related to the development of umbilical hernias in pigs and search for potential candidate genes.

Material and methods

Animal population and sample collection

A total of 325 commercial pigs (8-21 weeks-old) were used in this case-control study. Animals were selected from three swine finishing farms with high sanitary status located in the Santa Catarina State in Brazil. The pigs were originated from the same nursery-growing farm and were from the same crossbred commercial line (Agroceres Pic®). Presence of umbilical hernia was clinically diagnosed and confirmed to discard animals with abscesses. A total of 92 pigs was classified as affected with umbilical hernia (cases) and 233 animals were selected as unaffected counterparts (controls). For each case, two controls were kept from the same pen. Ear tissue samples were collected from all animals included in the study and kept at -80°C until usage.

DNA isolation and SNP genotyping

Genomic DNA was extracted from 200 mg of ear tissue samples using Purelink Genomic DNA Mini kit (Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer's instructions. DNA concentration and quality was assessed by spectrophotometry (ND-2000 NanoDrop Technologies, Wilmington, DE, USA). Only DNA samples showing the 260/280 ratio between 1.8 and 2.0 were used for further analyses. Samples were prepared for a final concentration of 500 ng and genotyped at a commercial genotyping service (Neogen, Deoxi operation, Araçatuba, SP, Brazil) with the GGP Porcine BeadChip (Neogen, Lincon, NE, USA) which contains 51,558 SNPs across the swine genome.

Data quality control

The quality control analysis was performed using PLINK software (Purcell *et al*., 2007). Samples were excluded if they had a call rate less than 90% and/or heterozygosity rate more than ± 3 standard deviations from the mean. SNPs were removed for one or more of the following: call rate less than 98%, minor allele frequency (MAF) less than 0.03 and/or deviation from Hardy-Weinberg Equilibrium (HWE) test ($p<1x10^{-6}$). To test for population stratification, a set of independent SNPs were calculated via PLINK software (Purcell *et al*., 2007) using the indep-pairwise option with a window size of 25 SNPs, a step of five SNPs, and a r^2 threshold of 0.2. A total of 7,441 independent SNPs was used to construct a multi-dimensional scalling (MDS) plot. Finally, a total of 46,898 SNPs and 285 samples (82 cases and 203 controls) remained for analysis.

Association analysis

A standard case-control association analysis was performed using Chi-square test implemented in the PLINK software (Purcell *et al*., 2007). The independent SNPs were used to yield a threshold Bonferroni p-value of suggestive significance of $1,343E^{-4}$ (1/7441) and Bonferroni genome-wide 5% significance of $6.720E^{-6}$ (0.05/7441). Furthermore, a moderate association was also considered with a threshold of $5x10^{-5}$ based on the Welcome Trust Case and Control Consortium recommendations (WTCCC, 2007). The Manhattan and the Q-Q plots were constructed using R v3.3 (R Core Team, 2017).

The UCSC (University of California Santa Cruz) genome browser (https://genome.ucsc.edu/) with the genome assembly Sscrofa 10.2, the ensemble and the pig QTLdb (Hu *et al*., 2016) were used to identify candidate genes and QTL regions spanning 300 kb of the associated regions.

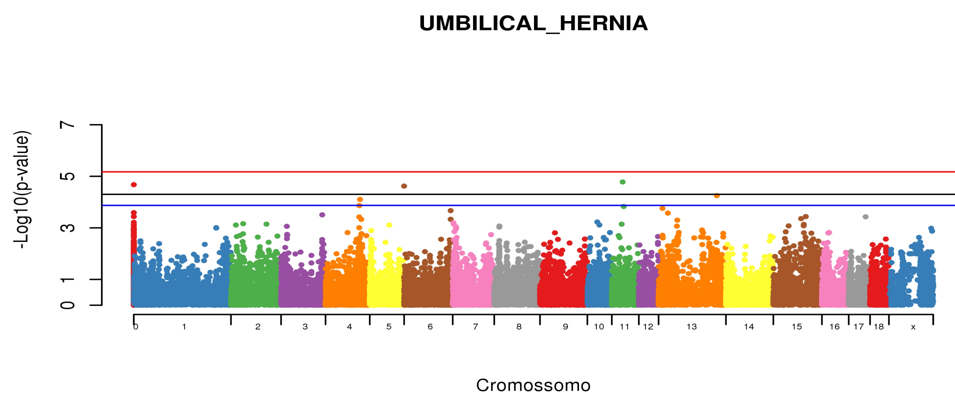
Results and discussion

The Manhattan plot showing the association profile of the SNPs with umbilical hernia and the corresponding Q-Q plot are depicted in Figure 1A and 1B. In the present study, a total of five SNPs was associated with umbilical hernia in commercial pigs. Two SNPs with moderate association were identified in chromosomes SSC6 located at 13,141 bp (rs81337222, p= 2.40 x 10^{-5}) and SSC11 located at 35,056,411 bp (rs80813241, p=1.66 x 10^{-5}). There were no QTLs associated to umbilical hernia neither genes described in these identified regions. However, a QTL for this trait was described in a downstream region (3.8-3.9 Mbp, #120294) of the SSC6 in a CNV analysis (Long *et al*., 2016). This finding suggests that there are regulatory regions in the beginning of the SSC6 that should be further investigated. The other two SNPs had suggestive association with umbilical hernia and were located at 111,916,675 bp in SSC4 (rs334706328, p= 7.89 x 10^{-5}) and at 189,082,499 bp in SSC13 (rs337360700, p= 5.62 x 10^{-5}). Regarding the SNP in the SSC4, one QTL was already described in the region of 122 Mbp (Ding *et al*., 2009, #8749) as being associated to umbilical hernia. For the SNP on SSC13, six genomic regions were also associated to umbilical hernia, and the closest region from this SNP is spanning 206.5-206.9 Mbp (Ding *et al*., 2009). In addition, one SNP with unknown location (*ASGA0069360*) was also moderately associated (p=2.1 x 10^{-5}) with umbilical hernia.

When functional candidate genes were searched spanning a region of 300 kb from the associated SNP on SSC4, two genes were identified: *TBX15* (T-box 15) and *WARS2* (tryptophanyl-tRNA synthetase 2). *TBX15* is a mesodermal transcription factor highly involved in the formation, metabolism and contractile properties of skeletal muscle by the glycolytic fibre type determination. It has been reported that TBX15-deficient mice show marked decrease in number of glycolytic fibres, which leads to a decrease in muscle size and slower myofiber contraction and relaxation (Lee *et al*., 2016). WARS2 is implicated in angiogenesis and participates in the pro-angiogenic signaling by directing cell motility and division to promote endothelial cell migration and proliferation (Wang *et al*., 2015). In this sense, it has been suggested that impaired angiogenesis may play an important role in the pathophysiology of congenital diaphragmatic hernia (Van der Horst *et al*., 2011). Therefore, *TBX15* and *WARS2* genes might also have an important function in the development of umbilical hernia.

Two genes were identified in SSC13, the *LIPI* (lipase I) and *RBM11* (RNA Binding Motif Protein 11). No information about the functions of these genes associated with hernia was previously described. However, *LIPI* is involved in familial hypertriglyceridemia, a condition related with incidence of umbilical hernia in humans (Haghighi *et al*., 2015). This disorder is usually associated with body fat loss, insulin resistance and low leptin levels (Fu *et al*., 2004). The *RMB11* has a biological role related to RNA splicing, cell differentiation and cellular response to oxidative stress (ENSEMBL, 2017). Despite the scarce information available about this gene, it is already known that other RNA-binding motif proteins are involved in myogenesis. One of them, the *RBM24,* is responsible to regulate key steps during myoblast-to-myotube transition, including the myogenin, a major player in the initiation and maintenance of myoblasts (Jin *et al*., 2010). Therefore, it is possible that the *RBM11* gene be involved in the muscle tissue differentiation, and SNPs in this gene might lead to umbilical hernia predisposition.

A)



B)

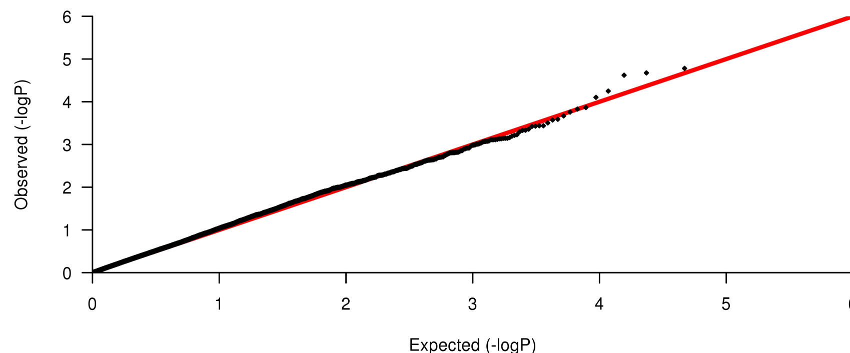


Figure 1. A) Manhattan plot of the genome-wide association for umbilical hernia in crossbred pigs. The red line represents a 5% significant threshold, the black line is a moderate association based on the WTCCC (2007) and the blue line represents suggestive association. B) Q-Q plot showing the observed p-values vs expected for the association of loci with umbilical hernia.

Conclusions

Four novel candidate genes possibly associated to umbilical hernia were identified in

crossbred pigs. Further validation of these findings should be conducted to increase our knowledge about this condition in order to improve pig welfare and production by eliminating susceptible animals through genetic selection. Next steps would be to identify different haplotypes between normal and affected groups and searching for functional SNPs, InDels or CNVs in the candidate genes identified in this study.

Acknowledgements

This study was supported by the CNPq project # 476146/2013-5. RK Ono was funded by a grant from ICASA, SC, Brazil and WR Lorenzetti received a PROMOP/UDESC Scholarship. The authors are grateful to Alexandre L. Tessmann for technical support.

List of References

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.

Fu, M., R. Kazlauskaite, M.F.P. Baracho, M.G.N. Santos, J. Brandão-Neto, S. Villares, F.S. Celi, B.L. Wajchenberg & A.R. Shuldiner. 2004, Mutations in Gng3lg and AGPAT2 in Berardinelli-Seip Congenital Lipodystrophy and Brunzell Syndrome: Phenotype Variability Suggests Important Modifier Effects. The Journal of Clinical Endocrinology & Metabolism. 89:2916–2922.

Haghighi, A., Z. Kavehmanesh, F., Salehzadeh, F. Santos-Simarro, L. Van Maldergem & L. Cimbalistiene, 2015. Congenital generalized lipodystrophy: identification of novel variants and expansion of clinical spectrum. Clin Genet. doi: 10.1111/cge.12623

Jin, D., K. Hidaka, M. Shirai & T. Morisaki. 2010. RNA-binding motif protein 24 regulates myogenin expression and promotes myogenic differentiation. Genes to Cells. 15:1158–1167.

Lee, K.Y., M.K. Singh, S. Ussar, P. Wetzel, M.F. Hirshman, L.J. Goodyear, A. Kispert & C.R. Kahn. 2015. Tbx15 controls skeletal muscle fibre-type determination and muscle metabolism. Nat Commun. 24;6:8054.

Liao, X.J., L. Lia, Z.Y. Zhang, Y. Long, B. Yang, G.R. Ruan, Y. Su, H.S. Ai, W.C. Zhang, W.Y. Deng, S.J. Xiao, J. Ren, N.S. Ding & L.S. Huang. 2015. Susceptibility loci for umbilical hernia in swine detected by genome-wide association. Genetika. 51(10):1163-70.

Long, Y., Y. Su, H. Ai, Z. Zhang, B. Yang, G. Ruan, S. Xiao, X. Liao, J. Ren, L. Huang & N. Ding, 2016. A genome-wide association study of copy number variations with umbilical hernia in swine. Anim Genet, 47: 298–305.

Petersen, H.H., E.O. Nielsen, A.G. Hassing, A.K. Ersboll & J.P. Nielsen. 2008. Prevalence of clinical signs of disease in Danish finisher pigs. Vet. Rec. 162:377–382.

Purcell, S., B. Neale, K. Todd-Brown, L. Thomas, M.A.R. Ferreira, D. Bender, J. Maller, P. Sklar, P.I.W. de Bakker, M.J. Daly & P.C. Sham, 2007. PLINK: A tool set for whole-genome association and population-based linkage analyses. Am. J. Hum. Genet. 81: 559-575.

R Core Team, 2017. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Rutten-Ramos S.C. & J. Deen. 2006. Association between umbilical hernias and genetic line

in a swine multiplication herd and methods to differentiate the role of sire in the incidence of umbilical hernias in offspring. J Swine Health Prod. 14(6):317–322.

Searcy-Bernal, R., I.A. Gardner & D.W. Hird. 1994. Effects of and factors associated with umbilical hernias in a swine herd. J. Am. Vet. Med. Assoc. 204:1660–1664.

van der Horst, I.W., P. Rajatapiti, P. van der Voorn, F.H. van Nederveen, D. Tibboel, R. Rottier, I. Reiss & R.R. de Krijger. 2011. Expression of hypoxia-inducible factors, regulators, and target genes in congenital diaphragmatic hernia patients. Pediatr Dev Pathol. 14(5):384-90.

Wang, M., P. Sips, E. Khin, M. Rotival, X. Sun, R. Ahmed, A.A. Widjaja, S. Schafer, P. Yusoff, P.K. Choksi, N.S. Ko, M.K. Singh, D. Epstein, Y. Guan, J. Houštěk, T. Mracek, H. Nuskova, B. Mikell, J. Tan, F. Pesce, F. Kolar, L. Bottolo, M. Mancini, N. Hubner, M. Pravenec, E. Petretto, C. MacRae & S.A. Cook. 2016. Wars2 is a determinant of angiogenesis. Nat Commun. 8;7:12061.

Welcome Trust Case Control Consortium, 2007. Genome-wide association study of 14,000 cases of seven common diseases and 3000 shared controls. Nature. 447: 661–678.