High-altitude pulmonary hypertension in cattle (brisket disease): Candidate genes and gene expression profiling of peripheral blood mononuclear cells

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ABSTRACT

High-altitude pulmonary hypertension (HAPH) is a consequence of chronic alveolar hypoxia, leading to hypoxic vasoconstriction and remodeling of the pulmonary circulation. Brisket disease in cattle is a naturally occurring animal model of hypoxic pulmonary hypertension. Genetically susceptible cattle develop severe pulmonary hypertension and right heart failure at altitudes >7,000 ft. No information currently exists regarding the identity of the pathways and gene(s) responsible for HAPH or influencing severity. We hypothesized that initial insights into the pathogenesis of the disease could be discovered by a strategy of (1) sequencing of functional candidates revealed by single nucleotide polymorphism (SNP) analysis and (2) gene expression profiling of affected cattle compared with altitude-matched normal controls, with gene set enrichment analysis (GSEA) and Ingenuity pathway analysis (IPA). We isolated blood from a single herd of Black Angus cattle of both genders, aged 12–18 months, by jugular vein puncture. Mean pulmonary arterial pressures were 85.6±13 mmHg STD in the 10 affected and 35.3±1.2 mmHg STD in the 10 resistant cattle, P<0.001. From peripheral blood mononuclear cells, DNA was hybridized to an Affymetrix 10K Gene Chip SNP, and RNA was used to probe an Affymetrix Bovine genome array. SNP loci were remapped using the Btau 4.0 bovine genome assembly. mRNA data was analyzed by the Partek software package to identify sets of genes with an expression that was statistically different between the two groups. GSEA and IPA were conducted on the refined expression data to identify key cellular pathways and to generate networks and conduct functional analyses of the pathways and networks. Ten SNPs were identified by allelic association and four candidate genes were sequenced in the cohort. Neither endothelial nitric oxide synthetase, NADH dehydrogenase, TG-interacting factor-2 nor BMPR2 were different among affected and resistant cattle. A 60-gene mRNA signature was identified that differentiated affected from unaffected cattle. Forty-six genes were overexpressed in the affected and 14 genes were downregulated in the affected cattle by at least 20%. GSEA and Ingenuity analysis identified respiratory diseases, inflammatory diseases and pathways as the top diseases and disorders (P<5.14×10^-14), cell development and cell signaling as the top cellular functions (P<1.20×10^-48), and IL6, TREM, PPAR, NFkB cell signaling (P<8.69×10^-09) as the top canonical pathways associated with this gene signature. This study provides insights into differences in RNA expression in HAPH at a molecular level, and eliminates four functional gene candidates. Further studies are needed to validate and refine these preliminary findings and to determine the role of transcribed genes in the development of HAPH.

Key Words: brisket disease, microarray analysis, hypoxia

INTRODUCTION

High mountain disease (brisket disease) is right heart...
failure due to hypoxic pulmonary hypertension in cattle residing at high altitudes. Hypoxia is the most potent stimulus for pulmonary hypertension, and the hypoxia of high altitude (>7,000 ft) is a well-known cause. Some cattle (Bos taurus) possess a heritable susceptibility to severe high-altitude pulmonary hypertension (HAPH). While most cattle thrive at high altitudes, susceptible cattle develop pulmonary hypertension that is sufficient to cause right heart failure, edema of the brisket and death. Experiments conducted by Grover and Reeves in cattle susceptible and resistant to HAPH suggest an autosomal-dominant mode of inheritance, or inheritance related to a few major genes. No information currently exists on the identity of these genes. HAPH in cattle occurs in about 20% (10–50%) of animals brought to high altitudes (>7,000 ft) to replenish herds suggesting that the gene of interest may be a relatively common polymorphism only expressed with the stimulus of altitude hypoxia. There is no known phenotype of the gene at low altitude.

We hypothesized that the insights into pathogenesis of brisket disease may be gained through two approaches: (1) allelic association analysis of single nucleotide polymorphism (SNP) differences; and (2) a comparative gene expression analysis of hypertensive versus resistant cattle. Therefore, we analyzed DNA and RNA from 20 cattle of the same herd; 10 with severe pulmonary hypertension and 10 with normal pressures. We used an Affymetrix 10K Bovine Gene SNP array and sequenced four genes of interest. Expression data were then analyzed for genes and canonical pathways that were statistically different between the two groups. Gene set enrichment and Ingenuity map analyses were performed to further refine the data. Collectively, the results of this study provide molecular and cellular observations in possible genes of interest in HAPH leading to brisket disease.

MATERIALS AND METHODS

Selection of study animals and blood samples
We obtained blood from a single herd of black Angus cattle of both genders, aged 12–18 months, residing at 8,500 feet, by jugular vein puncture on the same day of right heart catheterization screening for HAPH by one of us (Timothy N. Holt, hereinafter TH). The project was approved by the Vanderbilt Medical Center IACUC. Right heart catheterization is standard practice at altitude to discover hypertensive animals so that they can be shipped down to a low altitude.

DNA and RNA preparation and analysis
DNA was isolated from whole blood using QIAmp DNA mini-kit as directed by the manufacturer’s instructions (Qiagen, Valencia, CA, USA) and subsequently quantified using a spectrophotometer. Total cellular RNA was prepared from blood on silica-gel membranes with on-column DNase treatment (RNeasy Total RNA Isolation Kit; Qiagen). RNA integrity was assessed via agarose gel electrophoresis and samples showing evidence of degradation were discarded.

Affymetrix bovine 10K SNP genotyping and bioinformatic analysis
For SNP comparison, genomic DNA was extracted as described above and hybridized to Affymetrix - GeneChip Bovine Mapping 10KSNP chip according to manufacturer’s instructions (Affymetrix, Santa Clara, Calif., USA). Following bovine genotype collection, we remapped all of the SNP loci represented on the bovine 10K array using the Batch query within dbSNP online (NCBI) in conjunction with the latest bovine genome assembly (Btau 4.0). In addition, SNP loci present on the array more than once (duplications) were identified, and all duplications were removed. Likewise, SNPs with multiple chromosomal assignments were also flagged and subsequently removed. At the conclusion of our bioinformatic analyses, 8,011 unique bovine SNPs with single chromosomal assignments and updated bovine genome annotation were available for genome-wide association (GWA) analysis.

Statistical approach to SNP microarray
Because the precise mode of inheritance related to HAPH in cattle has not been fully elucidated, we used several standard case-control GWA approaches in a preliminary effort to identify new potential candidate genes. All statistical tests were performed using formulae incorporated within the software program HelixTree 6.4.2 (Golden Helix Inc., Bozeman, MT, USA). In the first approach, we performed a simple allelic association test across all unique SNP loci for 10 cattle exhibiting severe HAPH (n=20 alleles) and 10 cattle defined as “resistant” at altitude (n=20 alleles). Thereafter, we also performed standard genotypic association tests in an effort to evaluate whether SNPs implicated by a simple allelic test would systematically assemble into disparate genotypic classes for cattle with severe HAPH, as compared with altitude-matched HAPH-resistant controls. Raw P-values for all tests were corrected by either full-scan permutation and/or the Bonferroni procedure implemented within HelixTree 6.4.2.

Sequencing SNP candidates
TG-interacting factor-2 (TGIF2), endothelial nitric oxide synthetase (eNOS) and NADH coding regions were sequenced using a cDNA-based approach. Briefly, we performed first strand cDNA synthesis using the Superscript First-Strand System (Invitrogen Life Science, Carlsbad, CA, USA). Sequences were obtained using an ABI 3730xl DNA analyzer (Applied Biosystems, Foster City, CA, USA). Sequences were aligned with the bovine genome using the BLAST program.
used to query a global molecular network developed from information contained in the Ingenuity Pathways Knowledge Base. IPA generates models of gene interactions called networks that are presented graphically to show relationships between genes and the pathways they regulate. These networks are ranked according to a score calculated via a right-tailed Fisher’s exact test. In network graph, proteins encoded by genes are represented as nodes and their relationships as edges (links). All edges are supported by references from the literature. The functional analysis of a network identified the biological functions and/or diseases that were most significant to the genes in the network. The network genes associated with biological functions and/or diseases in the Ingenuity Pathways Knowledge Base were considered for further analysis. Fisher’s exact test was used to calculate a P-value, determining the probability that each biological function and/or disease assigned to a network is by chance alone. Canonical pathways analysis identified the pathways from the IPA library of canonical pathways that were most significant to the data set. The significance of the association between the data set and a canonical pathway was measured in two ways. A ratio of the number of genes from the data set that map to the pathway divided by the total number of genes that map to the canonical pathway is displayed. Fischer’s exact test was used to calculate a P-value determining the probability that the association between the genes in the dataset and the canonical pathway is explained by chance alone.

RESULTS

Study animals

Pulmonary arterial (PA) pressures were measured by jugular vein puncture at the time of right heart catheterization. Mean PA pressures were 86.10±5 mmHg STD in the affected and 31.20±0.7 mmHg STD in the resistant cattle, P<0.0001, by two-tailed Upaired t test (Fig. 1). A mean PA pressure of 30–40 mmHg is normal for

Microarray and gene set enrichment analysis analyses

Pulmonary tissue was not available from these animals. It is also possible that the lung tissue expression signature might be overwhelmed by adaptive cellular responses. This would then make the interpretation of the expression data particularly susceptible to false-positive and false-negatives. Our solution to this was to avoid disease effector cells and to use peripheral blood mononuclear cells (PBMCs) in our studies. Peripheral blood mononuclear cells are genetically the same as lung tissue intraindividuals, and are genetically different among hypertensive and resistant cattle and are likely to be free of end-stage disease effects. RNA was extracted from PBMCs. After the RNA was isolated, it was used to probe the Affymetrix GeneChip Bovine Genome Array (900561). Raw data were then analyzed by the Partek software package at the Vanderbilt Functional Genomics Shared Resource to produce expression intensities for each probe set. To study pathway-level differences between groups, GSEA was conducted on the microarray gene expression data. We used the Gene Ontology Biological Process (c5; from the MSigDB database) as our gene set database.

In silico functional analysis HPAH signature using ingenuity pathways analysis

We used IPA in the Ingenuity System to generate networks and conduct functional analyses of the HAPH signature. A data set containing gene identifiers was uploaded into the application. These genes, called focus genes, were
cattle residing at altitude for at least 12 months.[3,8] A mean PA pressure greater than 49 mmHg denotes a high risk for development of brisket disease.[6] Inclusion criteria were animals with a mean pulmonary artery pressure (mPAP) of 72–116 mmHg, thereby considered “affected,” and those with a mPAP of 32–37 mmHg, thereby considered “resistant” at altitude.

**GWA using the a**ffymetrix geneChip bovine mapping 10K array

Among the bovine cohort investigated, 6,344 of the 8,011 unique bovine SNPs with single chromosomal assignments and updated bovine genome annotation were diallelic, thereby making them potentially informative for subsequent analyses. The most robust allelic association was detected on BTA10 (0.0000028 ≤ \( p_{\text{raw}} \) ≤ 0.0000451), with weaker signals detected on additional bovine chromosomes. As expected, full-scan permutation and/or Bonferroni correction for multiple testing revealed a much weaker BTA10 signal for both approximate and exact tests (0.0476 ≤ \( p_{\text{corrected}} \) ≤ 0.2856), respectively. Summary data for the top 10 bovine SNPs implicated by an allelic GWA test are presented in Table 1. Additionally, bovine genes proximal to each SNP are also given, with relevant human–bovine comparative data.

Examination of relevant genotypes determined for the top 10 SNP loci elucidated by an allelic association test revealed six corresponding loci whereby severe bovine HAPH cases and altitude-matched HAPH-resistant controls (Table 1). However, genotypic association tests for all variable loci (n=6344) revealed no statistically significant associations after correction for multiple testing. Relevant bovine genotypic data and corresponding distributions are summarized in (Table 1).

Because of its role in human PAH[20-23] and its place in the TGF-β family of growth and repair receptors, the bovine BMPR2 gene was an obvious initial functional candidate for HAPH. We screened 10 severely affected and 10 unaffected cattle with a set of bovine microsatellites proximal to bovine BMPR2 to evaluate whether local genetic variation exhibited any evidence of segregation with the brisket disease phenotype (HAPH). This microsatellite analysis revealed a total of seven BMPR2 alleles, with no significant differences in the allelic distributions between the HAPH-affected and -resistant cattle, as determined by Fisher’s exact test. No microsatellite allele was found uniformly among severely hypertensive cattle or resistant cattle thus providing no evidence of linkage disequilibrium between any particular allele and the HAPH phenotype. These data suggest, but do not prove, that variation within bovine BMPR2 is not responsible for modulating HAPH in cattle.

Table 1: Top six bovine SNPs implicated by allelic association tests for a small bovine cohort consisting of severe HAPH cases and altitude-matched HAPH-resistant controls

| Bovine SNP^ | Bovine chrom.| Proximal bovine gene(s)^ | Human chrom.| Human Mb range^ | Relevant human gene^ |
|-------------|-------------|--------------------------|-------------|-----------------|---------------------|
| rs29024744  | BTA10       | NRXNIII/DIO2              | 14          | 79,733,622–79,748,2 | Deiodinase          |
| rs29025872  | BTA24       | BRUNOL4                   | 18          | 33,077,828–33,391,941 | RNA binding protein |
| rs29024519  | BTA29       | FOXR2                     | X           | 55,666,558–55,668,483 | Forkhead box R2    |
| rs29016420  | BTA1        | MYH15                     | 3           | 109,730,859          | Myosin heavy chain 15 |
| rs29024944  | BTA24       | NDUFV2/LOC781824          | 18          | 9,092,725–9,124,313  | NADH dehydrogenase  |
| rs29013210  | BTA3        | LOC100138920/FKBP1A       | 20          | 1,297,622–1,321,753  | FK binding protein 1A |

SNP: single nucleotide polymorphism; HAPH: high-altitude pulmonary hypertension; NADH: Nicotinamide adenine dinucleotide phosphate

Two other high-priority functional candidates were screened by cDNA-based sequence analysis, including eNOS and TGIF2.[24,25] Low NO production has been implicated in the pathogenesis of high-altitude pulmonary edema (HAPE)[26,27] while TGIF2 is a homeobox transcriptional repressor and an important downregulator of BMPR2 and TGF-β signaling.[27,28] Sequence comparison of the eNOS and TGIF2 coding regions of HAPH-affected and -resistant cattle did not result in the identification of any statistically significant variants predictive of HAPH in our samples.

**Statistical analysis of microarray data reveals altered expression in HAPH cattle**

Gene expression profiles were determined by oligonucleotide microarray analyses using total RNAs from the PBMCs cells as described in the Methods section. In order to identify candidate genes involved in HAPH or protection from HAPH, we used a hierarchical clustering analysis to analyze the expression signature from the...
PBMCs. A combination of relative level (fold-change) of expression and statistical significance (Student’s t-test) was used to distinguish these genes. This analysis identified 675 genes that were significantly upregulated and 320 genes that were downregulated (the HAPH signature) in affected animals. The top 15 candidates are listed in Table 2. Interestingly, the gene with the highest expression in affected animals is important for antigen presentation while the gene with the lowest expression is important for vessel elasticity as well as regulation of the TGF-beta pathway (Table 2). Genes related to disease process are shown in (Table 3).

**DISCUSSION**

HAPH with right heart failure was first described by Glover and Newsome in 1915 as “Brisket disease: Dropsy of high altitude.”[1] The link of altitude to hypoxic pulmonary hypertension was made after the discovery of hypoxic pulmonary vasoconstriction by von Euler and Liljestrand in 1946.[28] The exact mechanism causing acute hypoxic vasoconstriction remains elusive, although much is known about modifying influences.[29-31] Chronic hypoxia is a powerful stimulus for pulmonary hypertension, which involves not only vasoconstriction but also remodeling[32] of the pulmonary arteries. K-channel function has a central role, as does oxidant stress,[33,34] with Rho–Rho kinases involved in transduction of the hypoxic pressor response.[35-39]

To date, no major genetic variants have been identified that modify the strength of the hypoxic pressor response.[37] Some observations have been made regarding genetic associations with pulmonary responses to altitude in humans.[30-40] For example, eNOS and tyrosine kinase gene variants were overrepresented in a Japanese cohort of individuals susceptible to HAPE as compared with healthy controls at altitude.[27,40] Eddahibi and colleagues detected an association between the LL genotype of the human SLC6A4 (formerly SERT) with pulmonary artery pressure in a cohort of patients with chronic obstructive pulmonary disease as compared with controls.[41] Recent studies implicate HIF2a polymorphism in Tibetans versus Han as a possible difference in successful high-altitude combinations of genetic variations.

| Name             | Fold-change | Molecular function                                           | Functions in cells                                                                 |
|------------------|-------------|-------------------------------------------------------------|------------------------------------------------------------------------------------|
| HLA-DQA2         | +4.95       | MHC Class II receptor                                       | Antigen processing and presentation                                                |
| DNER             | +2.97       | Notch binding and calcium ion binding                        | Differentiation, notch signaling                                                   |
| SLC28A3          | +2.88       | Sodium symporter                                             | Vascular tone                                                                      |
| RYR1             | +2.43       | Voltage-gated calcium channel                                | Response to hypoxia, calcium transport                                            |
| FCAR             | +2.36       | IgA binding                                                  | Immune response, respiratory burst                                                 |
| RAB3IP           | +2.25       | GTPase binding                                               | Golgi to plasma membrane transport                                                |
| ARRD4C           | +2.21       | Unknown binds MLX, MLXIP                                     | Unknown                                                                           |
| UPP1             | +2.17       | Uridine phosphorylase                                        | Growth                                                                            |
| GPD2             | +2.10       | Glycerol-3-phosphate dehydrogenase [NAD+] activity          | NADH metabolic process, oxidation reduction                                        |
| TGM3             | +2.05       | Calcium ion biding, GTPase activity                          | Differentiation, protein modification                                              |
| EMR1             | +2.01       | G protein-coupled receptor                                   | Proliferation, adhesion, calcium response                                          |
| HIST1H1C         | +1.93       | DNA binding                                                  | DNA binding                                                                        |
| PTX3             | +1.93       | Zymosan binding                                              | Inflammatory response, +NOS process                                               |
| NFI3             | +1.78       | Transcription factor                                         | Immune function, particularly T cells                                              |
| DEFB4A           | 1.77        | Chemotaxis, immune response                                  | Immune response                                                                    |
| FBNL1            | −2.03       | Extracellular matrix, regulation of TGF-beta                | Muscle and heart development                                                       |
| KIR3DL1          | −1.6        | Inhibitory MHC Class I receptor                              | Regulation of immune response                                                      |
| BOA              | −1.2        | Class I MHC                                                  | Antigen processing, immune response                                               |
| CRIP3            | −1.15       | Zinc and metal ion binding                                   | T cell function and proliferation                                                  |
| CLGN             | −1.13       | Calcium ion binding, unfolded protein response               | Endoplasmic reticulum chaperone protein                                            |
| IKZF3            | −1.01       | Zinc-binding transcription factor                             | Mesoderm development, lymphocyte development                                       |
| TCRB             | −0.97       | T-cell receptor                                              | Immune response                                                                    |
| RAB30            | −0.89       | GTP binding, GTPase activity                                 | GTP signaling, protein transport                                                   |
| EBF1             | −0.82       | Transcription factor                                         | Apoptosis                                                                          |
| PHACTR1          | −0.78       | Actin binding                                                | Actin function                                                                     |
| CD8A             | −0.75       | MHC Class I                                                 | Immunity, calcium-mediated signaling                                              |
| BCL11A           | −0.74       | Transcription factor                                         | Apoptosis, differentiation                                                         |
| TCRA             | −0.67       | T-cell receptor                                              | Immune response                                                                    |
| MYOSC            | −0.66       | Myosin complex                                               | Muscle function                                                                    |
| VEGFB            | −0.65       | Endothelial growth factor                                    | Angiogenesis, positive regulation of endothelial function                           |
adaptation. Many other signaling pathways possess potential candidate genes.

Relevant to the present study, the risk of acquiring brisket disease in low-altitude cattle brought to very high altitude (10,000 ft) is about 10–50%. Notably, the prevalence of brisket disease among herds chronically residing and bred above 2100 m (c. 7,000 ft) has been reduced to about 1%, presumably because of selective loss of susceptible cattle to heart failure and to removal from herds after HAPH has been identified. Previously, Reeves, Weir and Grover bred a cohort of cattle with HAPH whose mean PA pressure was

| Category | P-value | Genes |
|----------|---------|-------|
| Respiratory disease | 1.61E-11-7.01E-03 | HIST1H1C, SOCS3, TREM1, ACVRL1, ARG2, CD164, HRH1, HMOX1, NFIL3, GPR77, CXCR2, F5, MGD1, RYR1, FOSL2, LTBR, PDHX, FCGR3A, PTX3, IL15, IL1R1, TRL4, CSF2RB, HP, NCF1, S100A9, IL1RN, CEBPD, FBNI, IL1B, CD14, IL2RA, CHI3L1, FCAR, KIR3DL1, SOCS3, TREM1, IGSF6, OLFM4, ETS2, CASP4, BCL6, IL1R2, CD164, HMOX1, HRH1, NFI3, CD47, GPR77, SOD2, HLA-A, CXCR2, F5, S100A8, LTBR, CCL16, FCGR3A, TNSF13B, PTX3, CCR1, MGST1, ATF3, IL15, ALOX5AP, IL1R1, CSF2RB, TRL4, AQ9P, NCF1, HP, S100A9, DUSP1, IL1RN, CEBPD, IL1B, CD14, IL2RA, SIRPA, SOCS3, KIR3DL1, DPYD, ARG2, MAPK13, BCL6, IL1R2, HRH1, GPR77, SOD2, GPD2, FOSL2, DYSF, LTBR, SLC04C1, TNSF13B, ACSV6, MYOF, STX2, ACVRL1, CSF2RB, CLIC2, NCF1, AQ9P, IL1RN, DUSP1, FNBI, CD14, RN1F49, HLA-DQA2, SIRPA, MCTP2, TREM1, RBM47, CASP4, HMOX1, WDFY3, HLA-A, CXCR2, F5, WLS, MXD1, S100A8, C9ORF150, FCGR3A, CCR1, KCNK17, DCK, DNER, IL15, ALOX5AP, IL1R1, TRL4, HRSP12, P2RY13, HP, S100A9, FAM129A, IL1B, IL2RA, C15ORF48, EMRI, CHI3L1, TCF7L2, MAAO SOCS3, DPYD, MAPK13, BCL6, IL1R2, HRH1, GPCPD1, SOD2, GPD2, DYSF, LTBR, SLC04C1, TNSF13B, MYOF, ACVRL1, BCL2B, CSF2RB, NCF1, AQP9, CLIC2, IL1RN, DUSP1, CD14, FNBI, RN1F49, TBXAS1, MTDH, HLA-DQA2, SIRPA, MCTP2, ETS2, GNG7, HMOX1, HLA-A, CXCR2, M2D1, F5, SNX10, RYR1, S100A8, FCGR3A, CCR1, KCNK17, DCK, DNER, IL15, ALOX5AP, IL1R1, TRL4, P2RY13, HP, S100A9, FAM129A, IL1B, IL2RA, RN1F49, CHI3L1, HLA-DQA2, SIRPA, TCF7L2 |
| Connective tissue disorders | 1.07E-07-4.28E-03 | SOCS3, DPYD, SGK1, MAPK13, BCL6, NPL, IL1R2, HRH1, GPCPD1, SOD2, GPD2, DYSF, LTBR, SLC04C1, TNSF13B, MYOF, ACVRL1, BCL2B, CSF2RB, NCF1, AQP9, IL1RN, DUSP1, FNBI, CD14, RN1F49, TBXAS1, MTDH, HLA-DQA2, SIRPA, MCTP2, ETS2, GNG7, HMOX1, HLA-A, CXCR2, M2D1, F5, SNX10, RYR1, S100A8, FCGR3A, CCR1, KCNK17, DCK, DNER, IL15, ALOX5AP, IL1R1, TRL4, P2RY13, HP, S100A9, FAM129A, IL1B, IL2RA, RN1F49, CHI3L1, TCF7L2 |
| Immunological disease | 2.05E-07-5.92E-03 | SOCS3, KIR3DL1, DPYD, MAPK13, FNDC3B, BCL6, IL1R2, HRH1, GPD2, LTBR, DYSF, SLC04C1, TNSF13B, MYOF, ACVRL1, BCL2B, CSF2RB, NCF1, AQ9P, DUSP1, IL1RN, CD14, FNBI, RN1F49, HLA-DQA2, ELL2, SIRPA, TREM1, CASP4, ID1, HMOX1, CD47, HLA-A, WDFY3, CXCR2, S100A8, FCGR3A, CCR1, OA51, KCNK17, DCK, DNER, PSTPI2, IL15, IL1R1, TRL4, HRSP12, P2RY13, HP, S100A9, FAM129A, IL1B, IL2RA, CHI3L1, TCF7L2 |
| Genetic disorder | 1.33E-06-6.72E-03 | GPCPD1, HRH1, SOD2, GPD2, FOSL2, DYSF, ALPK1, PDXX, LTBR, TNSF13B, SLC04C1, MGST1, ATF3, ACS6, MYOF, VLDLR, STX2, SERINC2, KAT2B, CSF2RB, NCF1, IL1RN, DUSP1, FNBI, CD14, RXF2, MTBH, TBXAS1, HLA-DQA2, SIRPA, MCTP2, LITAF, TREM1, RBM47, ETS2, CASP4, VAT1L, E1F4E, GNG7, HMOX1, ID1, CD47, HLA-A, WDFY3, CXCR2, MXD1, F5, WLS, ADAM19, SNX10, ANTX2R, RYR1, S100A8, C9ORF150, MARCKS, AGPAT9, RN144B, RAB8B, FCGR3A, CCR1, MOCS1, KCNK17, DCK, DNER, IL15, DGAT2, ALOX5AP, IL1R1, PPP13B, MARCKSL1, TRL4, HP, S100A9, FAM129A, IL1B, IL2RA, EMRI, C15ORF48, CHI3L1, TCF7L2, ISG20, MAOA |
| Hematological disease | 2.51E-06-6.72E-03 | SOCS3, TREM1, MAPK13, BCL6, HMOX1, HRH1, CD47, SOD2, GPD2, F5, RYR1, FOSL2, LTBR, FCGR3A, ATF3, DCK, IL1R4, TRL5, CSF2RB, S100A9, IL1RN, IL1B, CD14, IL2RA, SIRPA SOCS3, DPYD, KCNU2, KLF6, ACVRL1, ARG2, BCL6, NPL, HRH1, SOD2, GPD2, ALPK1, DYSF, PTX3, MYOF, VLDLR, KL4F, CSF2RB, NCF1, AQ9P, IL1RN, CD14, FNBI, SIRPA, RBM47, ETS2, VAT1L, HMOX1, CD47, F5, ANTX2R, ADAM19, RYR1, S100A8, KCNK17, PSTPI2, IL15, DGAT2, ALOX5AP, IL1R1, TRL4, MARCKSL1, HP, S100A9, IL1B, IL2RA, CHI3L1, ISG20, MAOA |
| Cardiovascular disease | 6.05E-05-6.83E-03 | SOCS3, TREM1, MAPK13, BCL6, HMOX1, HRH1, CD47, SOD2, GPD2, F5, RYR1, FOSL2, LTBR, FCGR3A, ATF3, DCK, IL1R4, TRL5, CSF2RB, S100A9, IL1RN, IL1B, CD14, IL2RA, SIRPA SOCS3, DPYD, KCNU2, KLF6, ACVRL1, ARG2, BCL6, NPL, HRH1, SOD2, GPD2, ALPK1, DYSF, PTX3, MYOF, VLDLR, KL4F, CSF2RB, NCF1, AQ9P, IL1RN, CD14, FNBI, SIRPA, RBM47, ETS2, VAT1L, HMOX1, CD47, F5, ANTX2R, ADAM19, RYR1, S100A8, KCNK17, PSTPI2, IL15, DGAT2, ALOX5AP, IL1R1, TRL4, MARCKSL1, HP, S100A9, IL1B, IL2RA, CHI3L1, ISG20, MAOA |
| Metabolic disease | 1.12E- | ID1, HMOX1, CD47, SOD2, IL1RN, DUSP1, CEBPD, CD14, IL1B, BCL6, KLF4 |
50mmHg (n=8) and a group of resistant animals whose mean PA was 29mmHg (n=11), all residing at 10,000 ft. The 19 cattle were initially taken down to an altitude of 4,916 ft, where the cattle with HAPH recovered, and then the cattle were bred within each phenotypic class. Bulls and cows were equally represented. First-generation offspring were studied at both low and high altitude. Mean PA pressures were 27±4 (STD) at low altitude. After residing at 10,000 ft for 2 months, the offspring of cattle with HAPH (brisket disease) had a mean PA pressure of 87±7 (SE), and those from resistant stock had a mean PA of 44±3 mmHg. Second-generation breeding (breeding experiments not overtly clear) within these two cohorts of calves yielded the same pattern of susceptibility and resistance. Pressures continued to diverge over time at altitude. Proof that the stimulus to pulmonary hypertension was hypoxia and not "altitude" was subsequently demonstrated by exposures in a hypoxic chamber at low altitude, whereby similar pulmonary hypertensive responses were recorded. Arterial blood gas measurements in susceptible and resistant cattle revealed similar severity of hypoxemia, similar alveolar ventilation (defined by the arterial carbon dioxide partial pressure, PCO₂) and only small differences in hematocrit, not considered sufficient to cause viscosity effects. The mode of inheritance of HPAH currently remains unclear. Nevertheless, what does remain clear among several studies is that HAPH in cattle is heritable, which indicates that cattle may be selected for resistance or susceptibility, and that the major gene(s) involved are likely to be discovered using large-scale genomic approaches followed by fine mapping.

Our initial screen using four functional candidate genes (selected because they are involved in human PAH) was negative and suggested that a candidate gene approach may not be efficient. We then employed a 10K bovine SNP array for a GWA aimed at unearthing new potential candidate genes. This analysis revealed six or more genes of interest proximal to bovine SNPs exhibiting the most disparate distributions between severe HAPH and resistant cattle. Of the genes located near the SNPs of interest, NADH dehydrogenase (ubiquinone) flavoprotein 2 (NDUFV), myosin heavy chain 15 (MYH15) and the myocardial signaling protein (FKBP1A) were candidates possibly involved in pulmonary hypertension. NADH dehydrogenase is no different between affected and resistant cattle, and additional studies are needed to further evaluate candidate genes. Furthermore, the recent availability of higher density bovine SNP arrays provides a natural progression to more thorough GWA, which are likely to identify additional candidate genes due to greatly enhanced genomic coverage. The use of these new higher density bovine SNP arrays with larger cohorts of severe HAPH and resistant cattle has the potential to maximize the probability of detecting SNPs that display a nonrandom relationship with either severe HAPH or putative resistance. It is likely that the major gene(s) responsible for bovine HAPH will have identifiable human ortholog(s) that may also modify human pulmonary hypertension related to hypoxia (altitude, emphysema, hypoverilation syndromes), and possibly in other conditions that lead to pulmonary hypertension.

**Biological significance of HAPH signature**

To gain biological insights into resistance or susceptibility to HAPH, we analyzed the expression data using the Gene Ontology and Ingenuity Map database. Our goal was to identify the disease processes, physiological, cellular and molecular functions and the canonical pathways that are enriched in HAPH. These analyses showed that the top disease process reflected in our data is respiratory disease (Table 2); the top three physiological processes are organism survival, hematological system and immune cell trafficking; the top three cellular and molecular pathways are cellular movement, cell signaling and small molecule biochemistry; and the top canonical pathways enriched in HAPH are IL-10 signaling, RXR activation, and, interestingly, immune and endothelial cell function (data not shown but available on request).

We then used the IPA application (IPA version 8.7) to examine the biological context and interactions of our signature. The IPA network analysis identified 10 networks with high P-values (1.0×10⁻²¹) (data not shown but available upon request). The top three networks had many genes (MAP13, STAT5α/b, ERK1/2, Cyclins, VEGF, PDGF, IL1β, ID1, CREB, p38MAPK, IL15, etc.) with a documented role in endothelial and immune cell function through their effects on cellular pathways such as TGF-beta, BMP and MAPK (important in human PAH) and molecular functions such as cell death, survival, inflammatory response and cell morphology. Function annotation of all the networks showed that the top canonical functions associated with these 10 networks are BMP, TGF-beta, PPAR, IL6, endothelial cell function, NOS, TREM1, VEGF, glucocorticoid, ERK/MAPK, NF-kB and HIF1α signaling (data not shown but available upon request). Not surprisingly, many of these pathways are also known to be important in human pulmonary hypertension.

In summary, herein, we have provided the results of the first molecular interrogation of HPAH, including a low-density SNP search, sequencing of four candidate genes and analysis of expression arrays to seek clusters of genes that may be a signature of hereditary bovine HAPH. Our hope is that this initial study of HPAH will spur further discovery and investigation of the molecular mechanisms behind HPAH. Our next challenge will be to attempt whole genome sequencing on the hypothesis that a polymorphism responsible for an autosomal-dominant
phenotype at altitude will emerge from the similar background genomes of the Angus herds.

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Source of Support: Pulmonary Hypertension Association Scientific Award, Newman PI. Elsa S. Hanigan Chair, Dr. Newman. Dr. Hamid is supported by 1R01HL102020-01, Conflict of Interest: None declared.