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Epigenetic Profiling Identifies Novel Genes for Ascending Aortic Aneurysm Formation with Bicuspid Aortic Valves

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ABSTRACT

Background: Bicuspid aortic valves predispose to ascending aortic aneurysms, but the mechanisms underlying this aortopathy remain incompletely characterized. We sought to identify epigenetic pathways predisposing to aneurysm formation in bicuspid patients.

Methods: Ascending aortic aneurysm tissue samples were collected at the time of aortic replacement in subjects with bicuspid and trileaflet aortic valves. Genome-wide DNA methylation status was determined on DNA from tissue using Illumina 450K methylation chips, and gene expression was profiled on the same samples using Illumina Whole-Genome DASL arrays. Gene methylation and expression were compared between bicuspid and trileaflet individuals using an unadjusted Wilcoxon rank sum test.

Results: Twenty-seven probes in 9 genes showed significant differential methylation and expression ($P < 5.5 \times 10^{-4}$). The top gene was protein tyrosine phosphatase, non-receptor type 22 (*PTPN22*), which was hypermethylated (delta beta range: +15.4 to +16.0%) and underexpressed (log 2 gene expression intensity: bicuspid 5.1 versus trileaflet 7.9, $P = 2 \times 10^{-5}$) in bicuspid patients, as compared to tricuspid patients. Numerous genes involved in cardiovascular development were also differentially methylated, but not differentially expressed, including *ACTA2* (4 probes, delta beta range: -10.0 to -22.9%), which when mutated causes the syndrome of familial thoracic aortic aneurysms and dissections

Conclusion: Using an integrated, unbiased genomic approach, we have identified novel genes associated with ascending aortic aneurysms in patients with bicuspid aortic valves, modulated through epigenetic mechanisms. The top gene was *PTPN22*, which is involved in T-cell receptor signaling and associated with various immune disorders. These differences highlight novel potential mechanisms of aneurysm development in the bicuspid population.

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INTRODUCTION

Bicuspid aortic valve (BAV) is the most common congenital cardiac anomaly, occurring in 1-2% of the population. Patients with BAV have increased risk of proximal aortic dilatation, with aneurysm formation occurring more frequently and at a younger age as compared to patients with normal tricuspid aortic valves (TAV) [Tadros 2009]. Aneurysm formation predisposes to aortic dissection and rupture, both of which can be lethal.

BAV is heritable [Cripe 2004], but the mechanisms underlying the predisposition to aneurysm formation are not fully understood. Several genes and their protein products have been implicated in the pathogenesis of ascending aortic aneurysms in BAV patients, including matrix metalloproteinases [Tadros 2009]. However, epigenetic modifications, which are known to strongly regulate gene expression, have yet to be investigated in this population of patients.

Implicated in the pathogenesis of numerous diseases, epigenetics refers to the study of heritable, reversible DNA or histone modifications that affect gene function without changing the sequence of DNA. DNA methylation, the placement of methyl groups on cytosine, often at cytosine-guanine rich motifs termed CpG islands, is one of the most common and well-understood epigenetic modifications whereby gene expression and function are regulated. These CpG islands frequently occur in regulatory regions of DNA, particularly in transcriptional promoters.

Changes in DNA methylation of these regions have been associated with numerous diseases including lung cancer [Wagner 2013; Brock 2008], colon cancer [Akhtar-Zaidi 2012], and chronic obstructive pulmonary disease [Puig-Vilanova 2014]. Furthermore, DNA methylation is known to regulate cardiomyocyte development and disease [Gilsbach 2014], and mutations in genes involved in controlling DNA methylation have been strongly implicated in congenital heart diseases [Zaidi 2013]. Cardiovascular disease has been identified as a tremendously promising field for which epigenetics could be used to explore the interaction between genotype, environment, and phenotype [Baccarelli 2010].

DNA methylation may be of particular relevance to BAV aneurysm formation given the complex interplay between

environmental factors, hemodynamic changes across BAVs, and the known genetic risk factors for aneurysm formation. A better understanding of these mechanisms could ultimately guide the development of therapies to prevent aneurysm formation and progression. The objective of this study was to explore epigenetic mechanisms predisposing to ascending thoracic aortic aneurysm formation in patients with BAV using a multipronged molecular approach. We hypothesized that variation in DNA methylation contributes to aneurysm formation in BAV patients and that whole-genome epigenetic profiling would identify some of these potential pathways.

MATERIALS AND METHODS

Study Population

This study enrolled patients undergoing surgical ascending thoracic aortic replacement for ascending aortic aneurysms at Duke University Medical Center between April 2006 and December 2008. Patients with bicuspid (n = 16) or tricuspid (n = 16) native aortic valves and ascending aortic aneurysms were identified. All patients met indication for aortic replacement based on American College of Cardiology Foundation/American Heart Association (ACCF/AHA) guidelines [Hiratzka 2010]. Patients undergoing reoperative procedures or patients with connective tissue disorders were excluded. All patients provided consent for inclusion in the study, which was approved by the Duke Institutional Review Board.

DNA Methylation Analysis

Ascending aortic aneurysm tissue samples were collected at the time of surgery, snap frozen in liquid nitrogen, and stored in a -80°C freezer. Tissue samples were weighed and homogenized for DNA extraction in 80 µL PBS per 25 mg tissue using the Omni Bead Ruptor 24 Homogenizer with Hard Tissue Homogenizing bead tubes (OMNI International, Kennesaw, GA, 19-628). DNA extraction was performed on 80 µL of homogenate (25 mg tissue) using the QIAamp DNA Mini kit (Qiagen, Hilden, Germany, 51304) according to manufacturer's protocol. After treatment with sodium bisulfite, DNA was then hybridized to the Ilumina Infinium HumanMethylation450 BeadChip (Ilumina, San Diego, CA) and processed using the Ilumina Infinium Methylation Assay using manufacturer's protocols by the David H. Murdock Research Institute Genomics Core Facility (Kannapolis, NC).

CpG DNA methylation data was interpreted using GenomeStudio to quantify methylated (M) and unmethylated (U) signal intensities for genomic DNA. Overall methylation levels (β) were calculated as the ratio of methylated to total signal (i.e., $\beta = M / (M + U)$) where β ranges from 0 (entirely unmethylated) to 1 (entirely methylated). For quality control, samples with aberrantly low signal intensity (mean < 2,000) and/or fewer CpG loci with detected signal relative to background were removed. Assay controls were inspected to remove samples with poor bisulfite conversion, staining, extension (single nucleotide extension assay), hybridization, or specificity. Additionally, outliers were identified by hierarchical clustering and/or dissimilarity matrices and removed. Methylation probes were excluded if they had missing values or if 80% or more of samples had a detection *P* value > .05. We also equalized β-values obtained from the two different types of probes on the HumanMethylation450 chip by normalizing them separately. Clustering on sex chromosome probes was used to verify the gender of all samples.

Gene Expression Analysis

Frozen tissue samples were processed by Expression Analysis (Durham, NC). Using the frozen aortic aneurysm tissue described above, RNA isolation was performed using the RNeasy Mini Kit (Qiagen, Valencia, CA) using the manufacturer's recommended protocol including DNase treatment. Microarray data were collected at Expression Analysis (Durham, NC). RNA specimens were profiled using the Whole-Genome DASL assay (Illumina, San Diego, CA), which is derived from a previously described cDNA-mediated annealing, selection, extension and ligation (DASL) assay [Fan 2004]. In brief, total RNA was converted to cDNA using biotinylated oligo-dT and random nonamer primers. Two assay-specific oligonucleotides were designed to interrogate a single contiguous 50nt sequence on each cDNA for a total of 24,526 oligonucleotide pairs (probes); which together constituted the whole genome DASL assay pool (DAP), corresponding to 18,626 unique genes, based on well-annotated content derived from the NCBI Reference Sequence Database (Build 36.2, Release 22). The DAP was then annealed to the targeted cDNAs during a 16 h temperature gradient (70-30°C) incubation followed by enzymatic extension and ligation steps. Ligated products were PCR-amplified and labeled with a universal Cy3-coupled primer after which single-stranded labeled products were precipitated and then hybridized to whole genome gene expression BeadChips. BeadChips were then scanned on a BeadArray Reader using BeadScan software, during which fluorescence intensities were read and images extracted. Scanned data were then uploaded into GenomeStudio software (v1.1), via the gene expression module (WG-DASL Assay) for further analysis. Robust multiarray average (RMA) normalization was used as per standard protocol.

Statistics

Gene expression was compared between BAV and TAV patients using unadjusted Wilcoxon rank sum tests at the individual RNA level. Genes were determined to be differentially methylated based on the following criteria: (1) delta beta >10% in the comparison between BAV and TAV groups, (2) genes with 2 or more CpG probes with the same direction of methylation change, and (3) these CpGs separated by ≤ 1 kb. Genes that met these criteria were further filtered by selecting those also showing differential gene expression ($P < 5.5 \times 10^{-4}$ for any RNA probe within that gene). The statistical significance of the differential methylation as measured by delta beta was determined using a Wilcoxon rank-sum test. Methylation profiles of control DNA has correlated well in our previous experiments, generating an average Pearson correlation coefficient (R) of 0.992 both within and between experiments. Differentially methylated probes within *PTPN22* were validated by bisulfite sequencing.

Table 1. Baseline Characteristics of the Study Population*

	TAV (n = 16)	BAV (n = 16)
Age, y	63 ± 7	53 ± 14
Sex	19 (3)	19 (3)
Race, white	75 (12)	94 (15)
BMI, m2	27 ± 6	27 ± 5
History of tobacco use	44 (7)	44 (7)
Hypertension	81 (13)	50 (8)
Diabetes mellitus	0 (0)	0 (0)
Statin use	25 (4)	31 (5)
ACEi or ARB use	44 (7)	25 (4)
Ejection fraction, %	53 ± 4	53 ± 7
Maximum aortic diameter, cm	6.1 ± 1.4	5.4 ± 0.7
Aortic stenosis > moderate	0 (0)	25 (4)
Aortic regurgitation > 2+	31 (5)	31 (5)

*Data are presented as mean ± standard deviation for continuous variables where indicated and as percentage (raw number) for categorical variables. TAV indicates tricuspid aortic valve; BAV, bicuspid aortic valve; BMI, body mass index; ACEi, angiotensin converting enzyme inhibitor; ARB, angiotensin II receptor blocker.

RESULTS

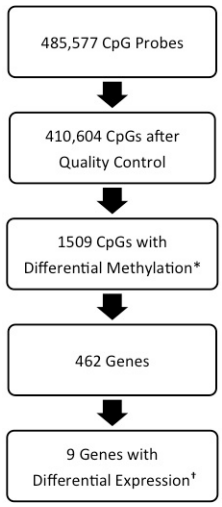
Subjects

Baseline characteristics of the study population are presented in Table 1. As expected, BAV patients were younger than TAV patients (53 ± 14 versus 63 ± 7 y, $P = .03$). Also as expected, BAV patients tended to have smaller aneurysms at the time of operation (5.4 ± 0.7 versus 6.1 ± 1.4 cm, $P = .09$), given the indication for operation at smaller aortic diameters in BAV patients [Hiratzka 2010]. In BAV patients, the most common anatomic configuration was fusion at the commissure between the right and left cusps (Sievers Type I R/L; 88%).

Differentially Methylated and Expressed Genes

We identified 1509 probes in 462 genes showing differentially methylated CpGs in the comparison between BAV and TAV groups (Figure). Nine of these genes also showed significant differential gene expression (Table 2). In all 9 genes showing differential methylation and expression, the direction of change in methylation correlated biologically with the expected change in gene expression. Specifically, in 5 of these genes, we found hypermethylation in the BAV group with a corresponding lower gene expression, and in 4 of these genes we found hypomethylation in the BAV group corresponded to a higher gene expression, as compared with the TAV group.

The strongest finding based on the magnitude of differential methylation and expression was for *PTPN22* (protein tyrosine phosphatase, non-receptor type 22; delta beta range +15.4% to +16.0%) with a resultant significant decrease in gene expression (log 2 gene expression intensity: BAV 5.1 versus TAV 7.9, $P = 2 \times 10^{-5}$). Of the 4 differentially methylated CpGs in *PTPN22*, cg1438573 showed the highest degree of differential methylation (TAV 0.58 versus BAV 0.74, $P = .0009$). Three of the 4 *PTPN22* CpG probes that were differentially methylated in BAV versus TAV patients were located in the 5' regulatory region of the gene, and the other was located adjacent to exon 1.



Filtering process for selecting top differentially methylated genes. *Differential methylation defined as (1) delta beta >10% in the comparison between bicuspid and tricuspid groups, (2) genes with two or more CpG probes with the same direction of methylation change, and (3) these CpGs separated by ≤ 1 kb. †Differential expression defined as $P < 5.5 \times 10\text{-}4$.

Differentially Methylated, but not Differentially Expressed Genes

We also explored genes that showed differential methylation between the 2 groups but that were not differentially expressed. We found CpGs in several genes with relevance to cardiovascular development and function that were differentially methylated, but not differentially expressed (Table 3). For example, *TBX5*, involved in mesoderm differentiation, had 15 CpGs with differential methylation between BAV versus TAV groups (range of delta beta: +10.1% to +13.2%). Other relevant differentially methylated genes included *ACTA2*, which is implicated in a syndrome of familial thoracic aortic aneurysm and dissection, and *GATA4*, a transcription factor involved in myocardial differentiation and function.

DISCUSSION

Using tissue specific whole genome methylation profiling, the current study identified novel genes associated with ascending thoracic aortic aneurysms in patients with BAV, modulated through epigenetic mechanisms. The top candidate gene showing differential methylation and expression, *PTPN22*, is involved in T-cell receptor signaling and is associated with various immune disorders. These differences in epigenetic profiles between BAV and TAV ascending aortic

Table 2. Top Differentially Methylated and Expressed Genes

Gene	Gene Function	CpGs*	Delta Beta‡	TAV Expression†	BAV Expression†	Expression P
PTPN22	T-cell signaling	4	+15.4 to +15.8	7.9	5.1	2.39×10^{-5}
RIPK1	Apoptosis, TNF signaling	3	-10.3 to -11.5	10.7	11.3	2.71×10^{-5}
LIMS2	Protein-protein interactions in extracellular matrix	3	-10.0 to -14.0	11.1	12.0	5.53×10^{-5}
ZBP1	Innate immunity, interferon production	2	+10.5 to +11.4	8.4	4.9	4.24×10^{-4}
MYO18A	Epithelial cell migration	2	-11.0 to -17.7	7.7	9.7	4.44×10^{-4}
GLRX	Antioxidant defense system	3	+14.5 to +17.6	13.0	12.5	4.63×10^{-4}
ERGIC1	Transport between endoplasmic reticulum and Golgi	6	-10.0 to -12.4	7.9	9.0	5.06×10^{-4}
GAPT	B-cell regulation	2	+10.0 to +12.9	9.6	6.6	5.32×10^{-4}
KLHL6	B-cell signaling	2	+10.8 to +12.6	8.8	4.9	5.40×10^{-4}

*Number of CpGs showing differential methylation.
†Log 2 expression.
‡Percent.

aneurysms highlight novel potential mechanisms of aneurysm development in the bicuspid population.

Numerous studies have attempted to identify genes potentially responsible for ascending aneurysm formation in BAV patients, including studies using gene expression, genome-wide association, targeted gene mutation analysis, and proteomic techniques [Prakash 2014]. Although many genes have been identified as potential modulators of ascending aneurysm formation in BAV patients [Tadros 2009; Phillippi 2009] the exact mechanism(s) has not been elucidated. We chose to take an approach of combining epigenetics and gene expression in an exploratory and unbiased approach to discover novel molecular mechanisms of BAV aneurysm formation. DNA methylation is a potent, widespread regulator of gene expression in a tissue specific manner, and had yet to be explored in BAV aneurysm patients. Also, DNA methylation provides a link between the environment and the genome, and BAV aneurysm formation may result from an interaction between the two [Sievers 2011]. Third, BAV is a congenital disease and DNA methylation is known to play a key role in cellular differentiation and embryonic development [Gilsbach 2014].

We identified 9 genes that had differential methylation and expression in BAV versus TAV patients. The top gene was *PTPN22*, located on chromosome 1. *PTPN22* or protein tyrosine phosphatase, non-receptor type 22, encodes the protein lymphoid tyrosine phosphatase (Lyp). This protein is a key regulator of T-cell signaling and is implicated in numerous autoimmune diseases including type 1 diabetes, rheumatoid arthritis, and Graves' disease [Burn 2011]. Variants in this gene result in increased immune cell signaling and lymphocyte and dendritic cell hyperresponsiveness [Zhang 2011]. Relatedly, variants in *PTPN11*, a gene with significant homology to *PTPN22*, are associated with Noonan Syndrome, a disease characterized by pulmonary valve stenosis. Additionally, the protein product of *PTPN11*, SHP-2, is involved in semilunar valvulogenesis.

At this point, one can only speculate about the mechanisms whereby *PTPN22* hypermethylation and decreased *PTPN22* gene expression might be involved in BAV ascending aneurysm formation. Decreased expression of Lyp results in increased T cell signaling. CD3+ T-cells are present in the media and adventitia of ascending aneurysms and are associated with apoptotic markers [He 2008]. Activated T-cells and macrophages can contribute to smooth muscle cell death and degradation of the extracellular matrix [He 2008]. Additionally, it is known that vascular smooth muscle cell apoptosis contributes to BAV aneurysm formation [Tadros 2009]. Thus, it is plausible that increased T-cell activity in BAV patients is associated with increased smooth muscle cell apoptosis and extracellular matrix degradation, resulting in earlier and more frequent ascending aneurysm formation.

In addition to *PTPN22*, probes in 8 other genes demonstrated differential methylation and gene expression, many with biologically plausible roles in aneurysm formation. For example, *RIPK1* is involved in the tumor necrosis factor receptor 1 signaling pathway and plays a role in apoptosis [Duprez 2012]. *LIMS2* encodes a focal adhesion protein involved in protein-protein interaction in the extracellular matrix. *ZBP1* promotes interferon production and is involved in innate immunity. *MYO18A* is a member of the myosin superfamily and plays a role in regulating epithelial cell migration [Hsu 2010]. *GLRX* encodes an enzyme involved in the antioxidant defense system, and it is known that oxidative stress promotes aneurysm formation in BAV patients [Phillippi 2009]. *ERGIC1* encodes a membrane protein involved in endoplasmic reticulum and Golgi apparatus interactions. *GAPT* and *KLHL6* are involved in B-cell regulation and signaling.

DNA methylation is known to be vitally important for proper embryonic development [Guo 2014]. Given this information, as well as the fact that BAV is a congenital disease, we also examined differentially methylated genes that may not have necessarily been differentially expressed at the time of

Table 3. Top Differentially Methylated Genes Related to Cardiovascular Development and Function That Were Not Differentially Expressed

Gene	CpGs	Delta Beta*	Gene Function	Mouse Knockout
TBX5	15	+10.1 to +13.2	Mesoderm differentiation	Heart morphology, function
PRDM16	12	+10.1 to +16.4 (5 probes) -10.1 to -19.3 (7 probes)	Development of brown adipocytes	Ventricular hypoplasia, abnormal morphology
GATA4	9	+10.0 to +16.1	Embryogenesis, myocardial differential and function	Abnormal ventricle morphology
NKX2-6	7	+12.2 to +18.1	Pharyngeal and cardiac embryonic development	Abnormal atrial morphology
TCF21	7	+10.1 to +12.3	Differentiation of epicardial cells	Abnormal vascular morphology
ACTA2	4	-10.0 to -22.9	Cell motility, structural integrity. Familial thoracic aortic aneurysm and dissection	Abnormal aorta morphology
CACNB2	3	-10.4 to -12.8	Voltage dependent calcium channel protein	Abnormal myocardial fiber physiology
MGAT1	3	+10.5 to +11.1	Diet induced obesity	Abnormal vascular development

*Percent.

tissue analysis. The results of this analysis were interesting, as many biologically relevant genes were found to be differentially methylated. For example, *ACTA2*, which when mutated causes the syndrome of familial thoracic aortic aneurysms and dissections [Guo 2007], was hypomethylated in the BAV group. Additionally, *GATA4*, which is involved in myocardial differentiation and function, as well as embryogenesis [Xin 2006], was hypermethylated in the BAV as compared to the TAV group. Thus, it is feasible that methylation changes in genes involved in cardiovascular development may contribute to the pathogenesis of aneurysm formation in BAV patients.

Strengths of this study include its unbiased approach, the integration of genome-wide methylation and expression data, and the validation of certain genes known to be involved in thoracic aortic aneurysm formation. This study also has certain limitations. First, although we have identified methylation differences between BAV and TAV patients, one cannot infer causation. The differences in *PTPN22* methylation and expression that we discovered may be a byproduct of aneurysm formation and not etiologic, as this is a cross-sectional study. Lastly, the 450K chip, although comprehensive, does not analyze all CpGs in the genome, and thus there may be other sites of differential methylation that we have not addressed.

In conclusion, using an integrated, unbiased epigenetic and transcriptomic approach, we have identified novel genetic pathways associated with BAV aortopathy, as compared to acquired degenerative aneurysms and TAV, that appear to be modulated through epigenetic modifications. The strongest finding was for *PTPN22*, a gene involved in T-cell receptor signaling and associated with various autoimmune disorders. These differences highlight novel mechanisms of aneurysm development in the BAV population.

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