Correlates of Valvular Ossification in Patients with Aortic Valve Stenosis

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Abstract

Valvular calcification may include a regulated process of active ossification; however, the determinants of ossification are unclear. The aim of this study was to identify subject and disease characteristics associated with valvular ossification among patients with calcified aortic valves. Medical records were reviewed for variables associated with aortic stenosis and skeletal bone in a series of 195 patients requiring aortic valve excision. Thirty patients had valvular bone on histopathology. Univariate analyses suggested that warfarin therapy ($p = 0.004$), African American race ($p = 0.006$), height ($p = 0.03$), and male sex ($p = 0.07$) were associated with greater odds of valvular ossification while diabetes was associated with lower odds ($p = 0.07$). Multivariate analysis demonstrated that warfarin use (OR 5.7; 95% CI: 1.5–21.3; $p = 0.009$) and African American race (OR 8.3; 95% CI: 1.8–39.0; $p = 0.007$) were strongly and independently associated with increased odds of valvular ossification. Valvular ossification was not associated with greater ossification of costochondral cartilage on chest radiography. Warfarin therapy and African American race were associated with increased risk of valvular ossification in patients with aortic stenosis. Future studies are needed to confirm this finding and determine if inhibition of matrix GLA protein by warfarin mediates this effect.

Keywords: aortic stenosis, warfarin, ossification, calcification

Introduction

The prevalence of aortic valve stenosis among adults ages 65 and older in the United States is estimated at 2%, and increases with age.[1] Its pathogenesis involves calcification of a normally supple valve into a stiff and stenotic one. Aortic valve calcification has been viewed as a passive process of calcium and phosphate ion crystallization onto lipids already deposited on the valve.[2] However, recent data suggest that valvular calcification, like vascular calcification, includes a regulated process of active bone formation with the identification of skeletal bone matrix proteins—osteopontin, osteocalcin, osteonectin, bone morphogenetic protein (BMP)-2 and BMP-4[3–5]—and identification of cells with osteoblast-like properties[6] from calcified valves. Elucidation of the molecular mechanisms and regulators of valvular calcification are areas of active research.[7]

Mohler et al. reported the prevalence and pathology of heterotopic bone formation and calcification in 347 cardiac valves (256 aortic and 91 mitral) excised from 324 consecutive patients requiring valve replacement surgery.[8] Thirteen percent demonstrated valvular bone formation with active bone remodeling and neoangiogenesis and 83% contained dystrophic calcification. In another large surgical series valvular bone metaplasia was also observed in 10.9% (128 of 1,177).[9] The presence of valvular bone formation suggested the possibility that this process may be subject to factors that affect skeletal bone in general. However, there were no significant associations between patients with and without valvular bone formation.[8] We noted that all of the valves with ossification were calcified and the majority (92%) were derived from aortic valves. The objective of the present study is to re-examine subject and disease characteristics among the subset of patients with calcified, stenotic aortic valves in order to identify factors associated with valvular bone.

Methods

The current study was limited to patients undergoing aortic valve excision due to aortic valve stenosis within the 1994–1998 cohort at the University of Pennsylvania Medical Center and Presbyterian Hospital, as previously described by Mohler et al.[8] Of the 256 aortic valves, 48 were excised due to aortic valve insufficiency, one due to idiopathic hypertrophic subaortic stenosis, and two in subjects with end-stage renal disease. Medical records were not available in an additional 10 subjects. This study describes the remaining 195 patients with aortic valve stenosis, whose inpatient medical charts at the time of valve surgery were reviewed. The study was approved by the Investigational Review Board for Human Studies at the University of Pennsylvania.

Microscopic analysis of valves was performed by three pathologists, as previously reported.[8] Briefly, all specimens were formalin-fixed and demineralized with a 10% formic acid solution to facilitate sectioning and minimize tissue artifact. This brief demineralization does not affect cellular elements such as osteoblasts. Two sections were performed on each valve leaflet. All 195 valves contained calcification. Foci of lamellar bone identified on polarized light microscopy were seen in 30 of the 195 valves. Osteoclasts were identified as multinucleated cells located within resorption spaces (Howship's lacunae). Osteoblasts were identified as polygonal cells actively forming matrix (active osteoblasts). Size of bone foci was not measured.

The medical records were reviewed for factors traditionally associated with skeletal bone mineral density for subject characteristics including sex, race, age, height, body mass index, cigarette smoking, serum creatinine, calcium, and phosphate concentrations at the time of the valve excision. Creatinine clearance was calculated using the method of Cockroft–Gault.[10] Conditions associated with presence of aortic stenosis or related to its progression, such as hypertension, elevated cholesterol (total cholesterol >200 mg/dL), diabetes mellitus, cigarette smoking, sex, and age were noted according to the medical chart. Medications at the time of valve replacement surgery were also recorded including HMG-CoA reductase inhibitor (statin),

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Variable	N	Bone present % (#)	Bone absent % (#)	OR	95% CI	*p*
Warfarin use	195	16.7 (5)	3.6 (6)	5.30	1.59, 17.72	0.004
African American versus Caucasian race	192	13.3 (4)	2.5 (4)	6.08	1.56, 23.7	0.006
Left ventricular hypertrophy	165	84.6 (22)	95.0 (132)	0.29	0.083, 1.01	0.052
Diabetes mellitus	195	16.7 (5)	33.3 (55)	0.40	0.15, 1.07	0.069
Female sex	195	26.7 (8)	44.2 (73)	0.46	0.20, 1.07	0.072
Mitral regurgitation	166	59.3 (16)	74.8 (104)	0.49	0.21, 1.14	0.098
Hypertension	195	40.0 (12)	53.9 (89)	0.57	0.26,1.24	0.160
Current cigarette smoking	194	6.7 (2)	16.5 (27)	0.36	0.00, 1.46	0.167
Statin use	195	3.3 (1)	11.5 (19)	0.26	0.00, 1.62	0.174
Coronary artery disease	195	73.3 (22)	61.2 (101)	1.74	0.74, 4.07	0.206
Carotid artery disease	195	6.7 (2)	15.2 (25)	0.4	0.00, 1.62	0.216
Glucocorticoid use	194	3.3 (1)	7.3 (12)	0.44	0.00, 2.75	0.422
Estrogen replacement therapy	81	0 (0)	6.17 (5)	0	0.00, 7.31	0.445
Bicuspid valve (versus tricuspid)	195	26.7 (8)	20.9 (34)	1.40	0.58, 3.32	0.479
Peripheral artery disease	195	10.0 (3)	7.3 (12)	1.42	0.40, 5.03	0.606
Bisphosphonate use	194	0 (0)	0.61 (1)	0	0	0.669
Aortic valve insufficiency	157	66.7 (16)	66.2 (88)	1.02	0.41, 2.51	0.962

Table 1. Univariate association of dichotomous variables with the presence of valvular ossification.

Variable	*N*	Bone present	Bone absent	OR*	CI	*p*
Height (m)	194	1.71	1.69	2.43	1.09, 5.43	0.030
Creatinine clearance (mL/min)	194	66.43	64.07	1.61	0.74, 3.52	0.234
Body mass index (kg/m^2)	116	27.14	26.46	1.70	0.61, 4.77	0.322
Age (years)	195	72.28	73.19	1.36	0.63, 2.93	0.445
Cardiac output (L/min)	174	4.41	4.40	1.21	0.55, 2.69	0.641
Aortic valve area (cm^2)	186	0.60	0.65	0.85	0.38, 1.87	0.682

*OR generated after dichotomizing continuous variables by the median value, with the exception of BMI. The OR for BMI represents the comparison of BMI >30 with BMI <25.

Table 2. Univariate association of dichotomized variables with the presence of valvular ossification.

warfarin, bisphosphonate, systemic glucocorticoid, and hormone replacement therapy. In addition, data from echocardiography and cardiac catheterization prior to valve replacement surgery were abstracted for variables such as aortic valve area,[6] mitral regurgitation (MR), and left ventricular hypertrophy (LVH).

In order to quantify bone formation on another cartilaginous site, chest radiographs obtained at the time of valve excision were retrieved and evaluated by a radiologist for costochondral calcification using a five point semi-quantitative scale.[11] The radiologist was blinded to valvular bone status. Eighteen films were randomly chosen for replicate scoring, showing good intra-observer reliability with a Spearman coefficient of 0.98. Of note, it has been argued that the term "calcification," as applied to costal cartilage, is a misnomer and that "ossification" denotes the true histopathological state.[11] However, the term "costochondral calcification" is retained to avoid confusion, since it is commonly used in the literature.

Analysis

The data for dichotomous variables were summarized according to the sample proportions in subjects with and without valvular bone formation (*Table 1*). Continuous variables were dichotomized by their median values and also summarized according to sample proportions (*Table 2*). Odds ratios (OR) and their 95% confidence intervals (CI) for the association of each variable with the presence of valvular bone were computed using logistic regression. A type I error rate of 0.05 was used to assess statistical significance in the two-sided univariate hypothesis tests. Chi-squared tests were used to examine confounding between the strongest predictor variables and each of the other possible predictors of valvular bone. A multivariate model was built using all of the variables that initially had *p*-values (p) < 0.10, that were present in at least 95% of the subjects, and that did not show evidence of confounding.[12] Finally, the association between costochondral ossification and each exposure variable was assessed using chi-square tests.

STATA version 6.0 statistical software was used for all analyses and Power version 1 was used for power calculations. Power calculations determined that the sample provided 80% power to detect OR's greater than approximately 3.0 for risk factors that were present in 25–50% of the subjects without valvular bone. The detectable OR increased to 3.9 for risk factors present in only 10% of

control subjects. Similarly, protective factors present in 50–75% of control subjects could be detected for ORs of approximately 0.3.

Results

The mean age at time of surgery was 71 years (range 21–94 years). Most subjects were Caucasian (94%) and 58% were male. Overall, subjects suffered significant comorbidities with a substantial prevalence of hypertension (52%), diabetes mellitus (31%), coronary artery disease (63%), and previous myocardial infarction (19%). Sixty-three percent of subjects were overweight (BMI > 25 kg/m^2) and 23% were obese (BMI > 30 kg/m^2).

Preoperative values for serum calcium concentration were present in only 72% of subjects, phosphorus in 50%, and cholesterol in 28%. Due to missing data, these variables were excluded from subsequent analyses. Of note, among subjects with available data for these variables, there was no evidence of association with valvular bone.

Univariate analyses comparing the 30 subjects with and 165 subjects without valvular bone are shown in *Tables 1* and *2*. Three variables were significantly associated with valvular bone (defined as $p < 0.05$): warfarin use, African American race, and height. Warfarin users were significantly more likely to have valvular bone; 5 of 30 subjects with valvular bone took warfarin as opposed to 6 of 165 without bone ($p < 0.01$). African Americans were more likely than Caucasians to have valvular bone; four of eight African Americans had valvular bone, compared with 26 of 184 Caucasian subjects ($p < 0.01$). Greater height was associated with increased odds of bone ($p < 0.05$); however, height was confounded by sex since men were taller than women ($p < 0.0001$), and men were marginally more likely to have valvular bone ($p = 0.07$), compared with women. After stratification by sex, the association between valvular bone and height was no longer significant, and height was dropped from further consideration. Finally, LVH, diabetes, and MR showed associations with bone within the prespecified level ($p < 0.10$) for potential inclusion into a multivariate model. Subjects with LVH, MR, or diabetes had lower odds of valvular bone. Presence of valvular bone formation did not differ between etiologies of aortic valve stenosis—rheumatic (55%), degenerative (18%), bicuspid (23%), bioprosthetic (0.5%) or other (4%).

Because warfarin and race appeared to be the strongest predictors of valvular bone, bivariate analyses using chi-square tests were constructed between these two variables and with LVH, diabetes, sex, and MR in order to identify possible residual confounding. These analyses showed no significant association between warfarin use with race or between either of these variables with LVH, diabetes, sex, or MR (all $p > 0.3$). Since data on LVH and MR were measured in <95% of subjects, these variables were not included in the multivariate analysis.

The remaining four variables, warfarin use, race, sex, and diabetes, were entered into a multivariate logistic regression model. All four variables achieved or approached statistical significance, so that no modification of the model was attempted (*Table 3*). In the multivariate model, valvular bone remained strongly associated with warfarin use and African American race. Female sex and diabetes remained protective, but the former was marginally significant and the latter approached statistical significance.

Chest radiographs were available for 126 of the 195 (65%) patients, of whom 21 were cases with valvular bone and 105 were controls (*Table 4*). Almost half had no calcification and a quarter had moderate to severe calcification. We pooled the three most severe categories (mild, moderate, and severe) to avoid problems with sparse cells in data analysis. The distributions of costochondral calcification score did not differ among patients with valvular bone compared to those without valvular bone, nor did costochondral calcification scores correlate with warfarin use, race, sex, height, or diabetes using Pearson chi-square tests (all $p > 0.18$). As expected, subjects with costochondral calcification were older versus those without calcification ($p = 0.012$).

Variable	OR	95% CI	p
Warfarin therapy versus no warfarin therapy	5.7	1.5, 21.3	0.009
African American versus Caucasian race	8.3	1.8, 39.0	0.007
Female versus male	0.39	0.15, 0.99	0.047
Diabetes versus no diabetes	0.35	0.12, 1.01	0.052

Table 3. Multivariate analyses of valvular bone.

Degree of costochondral calcification	Bone present % (#)	Bone absent % (#)
None	9 (43)	46 (44)
Minimal	5 (24)	16 (15)
Mild	5 (24)	15 (14)
Moderate	1 (5)	10 (10)
Severe	1 (5)	18 (17)
Total	21 (100)	105 (100)

Table 4. Costochondral calcification and valvular bone.

Discussion

This exploratory study suggests an association between warfarin therapy and African American race with the presence of aortic valve bone formation found at the time of valve excision surgery for treatment of aortic stenosis. Stewart et al.[13] identified clinical risk factors associated with aortic sclerosis or stenosis, many of which are atherosclerotic risk factors such as greater age, male sex, hypertension, cigarette smoking, lipoprotein(a), low density lipoprotein cholesterol, and height. These clinical factors—age, gender, height (after adjusting for gender), history of hypertension, present cigarette smoking, or history of hypercholesterolemia—did not distinguish subjects with valve bone formation from those with calcification only. This is not surprising, as determinants of valvular ossification may not necessarily be those that determine calcification. Our cohort was generally older (mean age 71 years), male (58%), and had a high prevalence of hypertension (52%), cigarette smokers (59% at some point in their lives and 15% currently), coronary artery disease (63%), and previous myocardial infarction (19%), indicating that this cohort had similar atherosclerotic risk factors previously associated with aortic stenosis.[13,14] Clinical predictors of faster rate of progression in aortic stenosis as demonstrated by Palta et. al.[15]—cigarette smoking, hypercholesterolemia, serum creatinine, and serum calcium—did not differ between those with and without valvular bone formation in the present study, suggesting the possibility that presence of valvular ossification did not hasten progression of aortic stenosis.

Aside from warfarin use and African American race, there were 9 potential risk factors for valvular bone formation

whose ORs were >1.0 and *p*-values were >0.1. To have 80% power to detect a risk factor with an OR of ≥3.0 would require prevalence of the factor in the control group <50%. Less than 50% prevalence in controls occurred in all but one variable (aortic valve insufficiency). Since the ORs were <3.0 in all 9 potential risk factors and <2.0 for all except height suggests that the lack of association observed did not reflect a lack of power to detect the association, rather the magnitude of the association in these variables was less than 3.0. Similarly, for potential protective factors against valvular bone formation, aside from diabetes, females, left ventricular hypertrophy, and mitral regurgitation, we observed 8 variables that had ORs <1.0, but whose *p*-values were >0.1. To have adequate power to detect a protective factor with an OR of 0.3 would require a prevalence of the factor in the controls of at least 50%. Prevalence of at least 50% occurred only for aortic valve area. For the remaining variables, prevalence of the potential protective factor was 1–17%. Thus, variables such as statin, glucocorticoid, estrogen, and bisphosphonate use, may indeed have been protective against ossification, but we had poor power to detect the association.

Due to the cross-sectional design of this study, issues of timing between exposure to warfarin therapy and valvular bone formation are unknown, i.e., we cannot ascertain whether warfarin therapy preceded onset of bone formation. We were unable to obtain data on quantitative aspects of warfarin therapy, such as dose, duration, and international normalized ratios, as these data were not generally documented in the medical chart at the time of hospitalization for valve surgery. Such data would be useful in clarifying the association in a cross-sectional study; however, if such data were collected prospectively, determining the timing between exposure and outcome remains unclear, since there is no noninvasive, in vivo method to detect the presence of valve bone formation. Among patients who took warfarin, the indication was usually atrial fibrillation (in 7 patients, and not listed in 4). Atrial fibrillation did not confound the association, as this condition did not differ between subjects with and without valvular bone.

Importantly, we were unable to assess the effect of valvular bone formation on rate of progression of aortic stenosis, a clinically important aspect of the disease. Although pathologic examination of valves was performed without knowledge of potential risks factors, the chart review for potential associations was not performed blinded to the knowledge of valvular bone. The true risk associated with warfarin use may have distorted due to information bias and may be spurious due to confounding factors that were not measured.

Although warfarin users and African Americans in this study were few in number, association of these factors with valvular bone should not be considered invalid solely due to the small numbers of subjects, as small sample sizes do not generally lead to the problem of statistical type 1 error. The possibility of type 1 error due to multiple comparisons is a threat to the validity of our findings, as Bonferonni correction was not employed. However, we decided against adjustment for multiple comparisons, as to do so would have sharply reduced statistical power to detect effects of interest in such an exploratory study. We thoroughly examined the possibility of confounding for each variable that achieved statistical significance and disregarded those variables that appeared significant due to confounding.[16] Nevertheless, this is the first study to examine potential associations of valvular bone formation in a cohort of patients with aortic stenosis, and generates hypotheses for possible determinants in future prospective studies.

Potential mechanisms are considered. Warfarin therapy inhibits vitamin K-dependent gamma-carboxylation of glutamine residues of a number of proteins, including matrix GLA protein (MGP), which is necessary for MGP function. MGP is expressed by smooth muscle cells and macrophages in the artery wall and is an important inhibitor of vascular mineralization.[17] MGP-null mice are born with a cartilaginous matrix that replaces the aortic medial layer and progressively calcifies.[18] The mechanism of MGP action may be through inhibition of BMP-2.[19] In an animal model, warfarin treatment accelerated arterial calcification,[20] and bisphosphonate treatment abrogated this effect.[21] Warfarin therapy in humans was not associated with a decrease in markers of bone formation or bone resorption,[22] bone mineral density or increased fracture rate in humans.[23] In another series of 45 patients undergoing aortic valve replacement surgery for aortic stenosis or insufficiency, those with prior therapy with warfarin had greater cross sectional area of calcified valve versus those without previous warfarin use;[24] the presence of valvular bone was not reported. As previously documented in this cohort of patients, immunohistochemical staining of surgically excised aortic valves demonstrated BMP-2 expression by myofibroblasts and preosteoblasts in foci of ossification.[8] Therefore, it is intriguing to postulate that warfarin therapy promoted valvular bone in patients with aortic stenosis via BMP-2-induced differentiation of mesenchymal cells toward an osteochondrogenic phenotype. Nevertheless, the association between warfarin use and valvular ossification requires confirmation in a prospective study.

The analyses of costochondral calcification were conducted in order to determine if subjects with bone formation at one cartilaginous site, the aortic valve, might also have bone formation at another cartilaginous site, the costal cartilage; and whether correlates of valvular bone formation might also be associated with bone formation at costal cartilage. We were unable to demonstrate these relationships, suggesting that local factors contribute to costochondral calcification. Although chest radiographs were routinely performed on all patients before surgery, only 35% were retrieved, likely because these were discarded after patient expiration, and thus limiting power to find associations.

Many questions remain including whether the appearance of valvular ossification is an early or late occurrence in the natural history of aortic valve stenosis. Does the amount of bone continually grow or vary over time? Does valvular ossification influence the progression of valvular calcification and hemodynamically important valve stenosis and if so what is the direction of the influence?

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