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# Intraleaflet haemorrhage as a mechanism of rapid progression of stenosis in bicuspid aortic valve ☆

Hirokuni Akahori [a], Takeshi Tsujino [b,*], Yoshiro Naito [a], Chikako Yoshida [a], Masaaki Lee-Kawabata [a], Mitsumasa Ohyanagi [c], Masataka Mitsuno [d], Yuji Miyamoto [d], Takashi Daimon [e], Tohru Masuyama [a]

[a] Cardiovascular Division, Department of Internal Medicine, Hyogo College of Medicine, Nishinomiya, Japan
[b] Department of Pharmacy, School of Pharmacy, Hyogo University of Health Sciences, Kobe, Japan
[c] Division of Coronary Heart Disease, Department of Internal Medicine, Hyogo College of Medicine, Nishinomiya, Japan
[d] Department of Cardiovascular Surgery, Hyogo College of Medicine, Nishinomiya, Japan
[e] Division of Biostatistics, Department of Mathematics, Hyogo College of Medicine, Nishinomiya, Japan

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ABSTRACT

*Background:* The mechanisms are unknown why aortic stenosis (AS) progresses faster in patients with bicuspid aortic valve (BAV) than those with tricuspid aortic valve (TAV). The objective of this study is to examine whether neoangiogenesis, haemorrhage in the aortic valve leaflet (intraleaflet haemorrhage) and macrophage infiltration are involved in the mechanisms of rapid progression of AS with BAV.
*Methods:* We retrospectively examined specimens of aortic valve leaflets obtained from patients who had undergone aortic valve replacement for AS (AS with BAV: n = 22, AS with TAV: n = 86). The stenotic valve leaflets were examined by immunohistochemistry to detect vascular endothelial cells, red blood cell remnant and macrophage. We assessed the progression of AS by annualized changes in the aortic valve area (ΔAVA: $cm^2$/year) which was evaluated by serial echocardiography with the continuity equation.
*Results:* Neoangiogenesis, intraleaflet haemorrhage and macrophage infiltration were frequently observed in leaflets obtained from AS patients with BAV (neoangiogenesis: 82%, intraleaflet haemorrhage: 91%, macrophage infiltration 91%). These pathological changes were more severe in AS with BAV than TAV, and they were positively correlated with progression of AS in patients with BAV. Multivariated analysis revealed that bicuspid anatomy was the only factor that predicted neoangiogenesis, intraleaflet haemorrhage and macrophage infiltration when patients with BAV and those with TAV were combined.
*Conclusions:* Neoangiogenesis, intraleaflet haemorrhage and macrophage infiltration are more severe in leaflets from AS with BAV than TAV and associated with rapid progression of AS with BAV. This pathological process may account for rapid progression of AS with BAV.

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## 1. Introduction

Bicuspid aortic valve (BAV) is one of the most common congenital heart defects, and a large proportion of patients with BAV will develop severe AS. Moreover, the progression of aortic valve stenosis with BAV is more rapid than that with tricuspid aortic valves (TAV) [1]. However, our understanding of BAV disease is incomplete and the mechanism of this progression remains unanswered.

In cohort studies, the progression of aortic valve stenosis was associated with many traditional atherosclerotic risk factors [2–7]. In histological studies, stenotic aortic valve and atherosclerotic arterial wall share several common features, including lipid accumulation, calcification, infiltration of inflammatory cells and neoangiogenesis [8–13]. Previous observations have suggested that AS with BAV are also associated with traditional atherosclerotic risk factors [14]. Thus, the mechanisms of progression of AS with BAV may be similar to that of AS with TAV. Recently, we have found that neoangiogenesis, intraleaflet haemorrhage and macrophage infiltration were associated with rapid progression of degenerative tricuspid AS [15]. Hence, we hypothesized that neoangiogenesis, intraleaflet haemorrhage and macrophage infiltration occur in BAV as well as TAV, and these pathological changes contribute to the rapid progression of AS in BAV.

The aims of this study are 1) to demonstrate whether neoangiogenesis, intraleaflet haemorrhage and macrophage infiltration are observed in AS with BAV, 2) to compare BAV and TAV regarding the severity of these pathological findings, and 3) to determine whether these pathological changes are involved in the mechanism of progression of AS with BAV.

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* Corresponding author at: Department of Pharmacy, School of Pharmacy, Hyogo University of Health Sciences, 1-3-6 Minatojima, Chuo-ku, Kobe, 650–8530, Japan. Tel.: +81 78 304 3182; fax: +81 78 304 2812.
*E-mail address:* ttsujino@huhs.ac.jp (T. Tsujino).

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## 2. Methods

### 2.1. Tissue sampling

We examined specimens of aortic valve leaflets obtained from patients who had undergone aortic valve replacement for AS (AS with BAV: n = 22, AS with TAV: n = 86). We excluded patients with rheumatic valvular disease and infectious endocarditis. We collected tissue samples in phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde in PBS within 4 h after surgery. Informed consent was obtained from each patient. The institutional ethics committee reviewed the protocol and approved this study.

### 2.2. Histological analysis

We performed immunohistochemical analyses as previously described [16]. We examined the thickest portion of the leaflet, mostly the mid portion of the leaflet. We detected red blood cell remnant, vascular endothelial cells, and macrophage with antibodies against glycophorin A (an erythrocyte-specific protein that facilitates anion exchange) (DAKO M0819, Tokyo, Japan), von Willebrand factor (vWF) (DAKO A0082, Tokyo, Japan), and anti-CD68 antibody (DAKO M0814, Tokyo, Japan), respectively. We also served non-immune mouse IgG (Mouse/HRP, DAKO, Tokyo, Japan) or non-immune rabbit IgG (Rabbit/HRP, DAKO, Tokyo, Japan) as negative control. We quantified the immunostained tissue area using computer-aided planimetry by two observers and expressed in percentages of the total surface area of the tissue section. We defined neovessels as tubuloluminal vWF positive capillaries recognized in cross sectional and longitudinal profiles, and shown in the number of blood vessels per $mm^2$ of the tissue.

### 2.3. Clinical data assessment

We reviewed clinical data in patients' charts. Diabetes mellitus was defined as fasting plasma glucose ≥126 mg/dl, HbA1C ≥6.5%, or receiving anti-diabetic medications. Hypertension was defined as systolic blood pressure ≥140 mg/dl, diastolic blood pressure ≥90 mg/dl, or receiving anti-hypertensive medications. Dyslipidemia was defined as LDL ≥140 mg/dl, HDL <40 mg/dl, triglyceride ≥150 mg/dl, or receiving anti-dyslipidemic medications. All the patients received coronary angiography before the operation. Coronary artery disease was defined as at least one coronary stenosis of at least 75% detected by coronary angiography before the operation. The types of BAV were classified according to Sievers et al. [16]. The codification for the category is by the following Types: Type 0 for valve with no raphe; Type 1 for valve with one raphe; Type 2 for valve with two raphe.

### 2.4. Transthoracic echo studies

Echo studies were performed by experienced sonographers. Aortic valve areas were calculated with the continuity equation. The progression of AS was assessed from serial echo studies separated by at least 180 days. We also calculated the annualized changes in the aortic valve area ($cm^2$) by dividing the temporal changes in the parameters by the number of the days between the studies and then multiplying 365 (ΔAVA, $cm^2$/year). The degree of calcification of the aortic valve was scored as follows: 1, no calcification; 2, mildly calcified (small isolated spots); 3, moderately calcified (multiple larger spots); 4, heavily calcified (extensive thickening and calcification of all cusp) as previously described [17].

### 2.5. Statistical analysis

We performed all statistical analyses using commercially available statistical software (IBM SPSS Statistics ver. 19, IBM Japan Ltd., Tokyo, Japan). We performed analysis of normality of the continuous variables using Kolmogorov-Smirnov test. We reported continuous variables as mean values (± standard deviations) when normally distributed, or as medians (interquartile ranges) when not normally distributed. We assessed differences in the continuous variables between groups with unpaired Student's *t* test for normal distributions, or Mann–Whitney *U* test for non-normal distributions. We reported categorical variables in numbers and percentages and compared them using the Fisher's exact test. We examined the agreement between the two observers for the score of each histological finding by using intraclass correlation coefficient. We assessed correlations with Spearman's rank correlation coefficient. In addition, to examine the independent relationships between each histological finding and factors influencing the progression of AS, we conducted a multivariate analysis based on linear regression with each histological finding as a dependent variable and other measurements as independent variables. In concrete terms, we included the factors which have been considered as precipitating factor of AS in the linear regression model as the independent variables. All tests were two-sided and the significance was accepted at $P<0.05$.

## 3. Results

### 3.1. Patients characteristics

A total of 108 patients including 57 females (53%) were included in this study (Table 1). The overall mean age was 73 ± 10 years. There were similar patient characteristics in BAV and TAV with a few exceptions when comparing them by valve morphology. As expected, patients with BAV were younger than those with TAV, ΔAVA of the patients with BAV were larger than those with TAV, and there were more male in BAV. These results are not contradictory to the previously report [18]. There were more patients with hemodialysis in the TAV group than in the BAV group. Regarding medical therapy, there were no significant differences between the BAV group and the TAV group (Table 2). The overall mean left ventricular ejection fraction and aortic valve area were 62% and 0.8 $cm^2$, respectively. There were no significant differences in echocardiographic data just before the operation between the BAV group and the TAV group. The types of BAV were as follows: Type 0 (n = 4), Type1 (n = 17), Type2 (n = 1).

### 3.2. Histological findings

Neoangiogenesis, intraleaflet haemorrhage and macrophage infiltration were frequently observed in leaflets obtained from AS patients with BAV: neoangiogenesis 82%, intraleaflet haemorrhage 91%, macrophage infiltration 91% (Fig. 1). There were significant correlations between two of the respective pathological changes (Table 3).

The number of blood vessels was significantly larger in BAV than TAV. The area of glycophorin A staining was significantly larger in BAV than TAV. The area of CD68 staining was significantly larger in BAV than TAV. In our study, intraclass correlation coefficient was 0.81, suggesting a high interobserver agreement in the histological finding. These findings suggested that more neoangiogenesis, intraleaflet haemorrhage and inflammation have occurred in BAV (Fig. 2).

We performed multivariate analysis based on linear regression to assess the independent factors contributing to neoangiogenesis, intraleaflet haemorrhage and macrophage infiltration in the combined population of BAV patients and TAV patients. We included traditional atherosclerotic risk factors and bicuspid anatomy in the linear regression model as independent variables (Table 4). Only bicuspid anatomy significantly contributed to these histological findings, suggesting that bicuspid anatomy induces more neoangiogenesis, intraleaflet haemorrhage and macrophage infiltration than tricuspid anatomy.

**Table 1**
Patient characteristics.

| | Bicuspid (n = 22) | Tricuspid (n = 86) | P value |
|---|---|---|---|
| Age (years) | 66 ± 14 | 74 ± 8 | 0.002 |
| Female | 7 (32%) | 50 (58%) | 0.024 |
| Hypertension | 15 (68%) | 69 (80%) | 0.176 |
| Dyslipidemia | 9 (41%) | 31 (36%) | 0.426 |
| Diabetes mellitus | 3 (14%) | 25 (29%) | 0.112 |
| Hyperuricemia | 3 (14%) | 9 (10%) | 0.459 |
| Hemodialysis | 0 (0%) | 17 (20%) | 0.014 |
| Present smoker | 6 (27%) | 25 (29%) | 0.548 |
| Coronary artery disease | 2 (9%) | 22 (26%) | 0.079 |
| Calcification score 3 or 4 | 21 (95%) | 76 (88%) | 0.297 |
| AVA at the operation | 0.70 (0.58–0.83) | 0.80 (0.60–1.00) | 0.177 |
| LVEF at the operation | 66 (51–70) | 65 (56–70) | 0.924 |
| ΔAVA ($cm^2$/year) | 0.17 (0.05–0.23) | 0.09 (0–0.15) | 0.023 |
| Follow up period (days) | 442 (180–578) | 440 (180–983) | 0.133 |

AVA: aortic valve area, LVEF: left ventricular ejection fraction.
Results shown as numbers and percentages (%) for categorical variables, mean and standard deviation (SD) for age, or median with interquartile range for AVA at the operation ($cm^2$), LVEF at the operation (%), AVA ($cm^2$/year), and Follow up period.
P-value for differences between bicuspid group and tricuspid group.
The differences in the continuous variables between groups were assessed with unpaired Student's t tests for normally distributions (age) or Mann–Whitney *U* test for non-normal distributions (AVA at the operation, LVEF at the operation, AVA, and Follow up period).
Categorical variables were compared using the Fisher's exact test.

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