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Expression of the Frizzled Receptors and their Co-receptors in Calcified Human Aortic Valves

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Abstract

The cellular mechanisms that induce calcific aortic stenosis are yet to be unravelled. Wnt signaling is increasingly being considered as a major player in the disease process. However, the presence of Wnt Frizzled receptors (Fzd) and co-receptors LRP5 and 6 in normal and diseased human aortic valves remains to be elucidated. Immunohistochemistry and qPCR were used to determine Fzd receptor expression in normal and calcified human aortic valve tissue, as well as human aortic valve interstitial cells (HAVICs) isolated from calcified and normal human aortic valves. There was significantly higher mRNA expression of 4 out of the 10 Fzd receptors in calcified aortic valve tissues and 8 out of the 10 in HAVICs, and both LRP5/6 co-receptors in calcified aortic valves ($P < 0.05$). These results were confirmed by immunohistochemistry, which revealed abundant increase in immunoreactivity for Fzd3, 7 and 8, mainly in areas of lipid core and calcified nodules of diseased aortic valves. The findings of abundant expression of Frizzled and LRP5/6 receptors in diseased aortic valves suggests a potential role for both canonical and noncanonical Wnt signaling in the pathogenesis of human aortic valve calcification. Future investigations aimed at targeting these molecules may provide potential therapies for aortic valve stenosis.

Keywords: Wnt, LRP, disease, aortic valve stenosis, mRNA, protein, qPCR, immunohistochemistry.

Abbreviations:

HAVICs = Human Aortic Valve Interstitial Cells;

VICs = Valve Interstitial Cells;

VECs = Valve Endothelial Cells,

AVC = Aortic Valve Calcification;

EMT = Endothelial-to-Mesenchymal Transition;

CAV = Calcified Aortic Valve

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Introduction

Aortic stenosis is the most prevalent valvular heart disease in developed countries, affecting 0.2% of adults between the ages of 50 and 59 years, and 2.8% of adults older than 75 years (Otto and Prendergast 2014; Thaden et al. 2014). It is mainly caused by aortic valve calcification (AVC) and is a progressive disease that is currently incurable (Lindman et al. 2013; Maganti et al. 2010). AVC is an active process involving basement membrane disruption, inflammatory and immune cell infiltration, and lipid deposition (Leopold 2012; Li et al. 2013; Merryman and Schoen 2013). Calcific aortic stenosis has been linked to many pathologies, which include the presence of a harsh systolic heart murmur, exercise intolerance, angina, syncope, and heart failure (Das et al. 2000). The aortic valve does not contain any vasculature, and is composed of four cell types: valve endothelial cells (VECs), valve interstitial cells (VICs), smooth muscle cells, and myofibroblasts (Chester and Taylor 2007). VECs function to regulate permeability, thrombogenesis, and cell adhesion, whereas VICs have the ability to contract, secrete cytokines, chemokines, growth factors, matrix components, and remodeling enzymes (Filip et al. 1986). VICs are internally associated with either collagen, glycosaminoglycans, or elastin, and in association form the fibrosa, spongiosa, and ventricularis layers of the aortic valve, respectively (Chen and Simmons 2011; Otto et al. 1994). VICs have been shown to possess the ability to differentiate in both a chondrogenic and osteogenic manner (Chester and Taylor 2007; Mulholland and Gotlieb 1996). Hence, it has been postulated that calcification may arise from the transition of VICs to osteoblast-like bone-forming cells, endothelial-to-mesenchymal transition (EMT) of VECs, and/or matrix vesicle formation resulting in microcalcific nodules (Miller et al. 2010; Piera-Velazquez et al. 2011; Wuthier and Lipscomb 2011).

Mammalian Wnt signaling comprises 19 wingless (Wnt) ligands, 10 frizzled (Fzd) receptors, and 2 low-density lipoprotein receptor-related protein (LRP) co-receptors (Komiya and Habas 2008). Wnt-Fzd binding leads to the activation of intracellular dishevelled (Dsh/Dvl) phosphoprotein, which then modulates various downstream products, triggering one of three major pathways: the canonical (β -catenin-dependent) Wnt pathway, the noncanonical (β -catenin-independent) planar cell polarity (PCP) pathway, or the noncanonical Wnt/ Ca^{2+} pathway (Rao and Kuhl 2010). Wnt signaling has been implicated in heart valve development, specifically regulating EMT and cell proliferation during the initial stages of endocardial cushion and heart valve formation (Flaherty et al. 2012; Tian et al. 2010). Moreover, the canonical Wnt pathway has been shown to play a direct role in AVC (Caira et al. 2006; Gu et al. 2014; Marinou et al. 2012). In active canonical Wnt signaling, Wnt binds Fzd receptors as well as LRP5/6 co-receptors, resulting in the activation of Dsh which inhibits GSK3 activity and disrupts the APC/Axin/GSK3 complex that targets β -catenin for destruction (Gordon and Nusse 2006; Hatsell et al. 2003; Nusse 2012). This results in β -catenin stabilization and accumulation in the cytoplasm and subsequently translocation to the nucleus (Fagotto et al. 1998). In the nucleus, β -catenin associates with members of the TCF/LEF family of transcription factors as well as a number of other factors enabling this complex to act as a transcriptional activator or repressor of Wnt target genes (Brunner 1997; Gordon and Nusse 2006; van de Wetering et al. 1997). The LRP5 Wnt co-receptor regulates bone matrix protein expression in aortic valves and vasculature of mice with hypercholesterolemia and through its interaction with its Wnt ligands, LRP5 activates β -catenin and is thus an important factor in mediating osteogenesis (Caira et al. 2006). More recently, we have shown an important role for noncanonical Wnts in AVC (Albanese et al. 2017). In the present study, we evaluate for the first time the changes in expression of the Fzd receptors in

calcified aortic valves (CAVs) through the examination of human biopsy samples using qPCR and immunohistochemical techniques.

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Materials and Methods

Tissue Collection:

A total of 17 non-calcified aortic valves and 55 calcified aortic valves were collected during cardiac valve replacement surgeries, along with detailed clinical history (Tables 1 and 2). Tissues were fixed in formalin and embedded in paraffin. Five non-calcified aortic valves (mean age 53 ± 19 ; 3 males), and aortic valves with focal calcification ($n=5$) and severely calcified (advanced) aortic valves ($n=16$) were also freshly collected during surgery for valve replacement, snap frozen in liquid nitrogen and stored at -80°C until processed for qPCR. All patients with aortic valve stenosis had severe heart failure (NYHA class III-IV). The study was approved by the ethics committee of the McGill University Health Centre, and informed consent was obtained from all participants involved in this study.

Tissue Expression of Fzd mRNAs:

Total RNAs were extracted from snap frozen aortic valve tissues using TRIzol (Invitrogen)/RNeasy Mini Kit (Qiagen) combining protocol. Total RNAs ($1\mu\text{g}$) were used for first strand cDNAs synthesis using SuperScript III First-Strand Synthesis System (Invitrogen). qPCRs were performed using QuantiFast® SYBR Green PCR kit (Qiagen) on LightCycler 1.5 (Roche). The qPCR data was analyzed using PrimePCR Analysis (Bio-RAD), and the relative mRNA expressions of the genes of interest were normalized using the housekeeping gene GAPDH. Sequences of gene-specific primers are listed in Table 3.

Immunohistochemistry

The paraffin-embedded tissue blocks were cut into $4\mu\text{m}$ sections using a microtome. The sections were incubated for 1 hour in 10% normal goat serum/PBS solution, then incubated overnight with the primary antibodies in 0.1% BSA/PBS solution in humid chambers at 4°C .

Primary antibodies used were: rabbit anti-human Frizzled 3 (Sigma, SAB4503170 at 1:200), Frizzled 7 (Abcam, ab64636 at 1:75), and Frizzled 8 (Sigma, HPA045025 at 1:50). Secondary biotinylated goat anti-Rabbit IgG (Vector Labs, BA1000 at 1:200) were applied followed by Vectastain ABC complex (Vector Laboratories, Burlingame, CA) according to manufacturer protocol. Immunostaining was visualized by 1xDAB/H₂O₂ solution, subsequently counterstained with hematoxylin, and mounted with Permount (Sigma). Immunostaining without primary antibody or with the primary antiserum preabsorbed with its respective antigen was carried out as negative control. Alizarin Red S and Masson's Trichrome staining protocols were used for calcium and collagen detection, respectively.

Isolation and Culture of HAVICs:

Primary HAVIC lines were generated from fresh surgically removed human aortic valve leaflets, and cultured in DMEM high glucose medium containing 10% FBS and 1x streptomycin/penicillin solution (Invitrogen). Briefly, aortic valve leaflets were washed with 1xHBSS buffer (Invitrogen), cut into small pieces, and then incubated in DMEM media with Collagenase Type II (Sigma, 100U/ml) for 3 hours at 37°C in water bath with occasional vortex. The fully digested mixtures were centrifuged at 500g at 4°C for 10 minutes; the supernatant was transferred into new 50ml centrifuge tube and centrifuged at 1000g at 4°C for 10 minutes. Cell pellets were suspended in complete DMEM supplemented with 10% FBS, 1x antibiotic antimycotic (Invitrogen), and seeded in 75 cm² culture flasks. HAVICs at passages 3 to 5 were used for experiments.

Statistical Analysis

Unpaired Student's t-tests were used to assess statistically significant differences in mRNA expression measurements of Fzd receptors between stenotic and non-stenotic HAVICs, normal

and calcified aortic valve tissue, and bicuspid and tricuspid CAV tissues. The same was used to assess the significance of LRP5/6 expression in normal and calcified bicuspid and tricuspid aortic valve tissue. Statistical analysis was performed using SPSS program Version 15. Data are presented as mean $\pm$ SD.

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Results

Expression of Fzd and LRP5/6 mRNAs in Normal and Calcified Aortic Valve Tissue

The mRNA expression of Fzd3, 6, 7, and 8 was significantly increased in calcified human aortic valve tissue, compared to normal human aortic valve tissue (Figure 1; $P < 0.05$). There was no significant difference in mRNA expression of Fzd receptors in bicuspid CAVs compared to tricuspid CAVs (Figure 2). The mRNA expression level of Fzd10 was below the detection limit of the techniques we used, and thus the data has not been included in the study. The mRNA expression of both LRP5 and LRP6 were significantly higher in CAVs (both bicuspid and tricuspid) compared to normal human aortic valves (Figure 3; $P < 0.05$). Between bicuspid and tricuspid CAVs, there was no significant difference of the mRNA expression of LRP5 and LRP6 (Figure 3).

Immunohistochemical Analysis of Fzd3, 7 and 8

There was little-to-no immunoreactivity for Fzd3, 7 and 8 in the normal human aortic valves or normal leaflets of CAVs (Figure 4; A-C). In contrast, there was abundant immunoreactivity for Fzd3 and 7 in the lipid core and calcified nodules of CAVs (Figure 4; D-F). Immunoreactivity for Fzd3 and 7 was also seen in the macrophages and endothelial cells of new microvessels (Figure 4; I-L). There was occasional staining in myofibroblasts as well. There was weak-to-moderate immunoreactivity for Fzd8, mainly in foam cells (lipid core) and in osteoblasts (Figure 4; G-H). Negative control sections for Fzd3, 7 and 8 did not show any immunoreactivity.

Expression of Fzd and LRP mRNAs in Normal and Stenotic HAVICs

There was a significant upregulation of Fzd1, 2, 3, 5, 6, 7, 8 and 9 mRNA expression in stenotic HAVICs compared to normal (Figure 5; $P < 0.05$). Again, the mRNA expression level of Fzd10 was below the detection limit of the techniques we used, and thus has not been included here.

Discussion

It is now clear that, unlike the old belief, AVC is an active cellular process rather than gradual calcium deposition due to aging (Mohler et al. 2001). Hence, studies illuminating the role of bone metabolism pathways in AVC such as the Wnt-Fzd system have gained growing interest. Many studies have been performed in both human and animal models in order to evaluate the effect of canonical Wnt pathways in AVC (Caira et al. 2006; Cheng et al. 2003; Rajamannan 2011b). However, studies focusing on the noncanonical Wnt ligands and their downstream effectors remain limited. We have recently reported significantly increased Wnt5a and b, and Wnt11 mRNA levels, as well as increased Wnt5b and Wnt11 protein and immunohistochemical staining levels in calcified human aortic valves when compared to normal human aortic valves. Moreover, incubation of HAVICs with the above 3 noncanonical Wnt ligands led to a significant increase in HAVIC mineralization and induction of other osteogenic mediators such as BMP-2 (Albanese et al. 2017). Here, we demonstrate an increased expression of the Fzd3, 6, 7, and 8 Wnt receptor mRNAs in calcified human aortic valve tissue, compared to normal aortic valves. Also, we demonstrate an increased expression of the LRP5 and LRP6 co-receptor mRNAs in calcified aortic valve tissue, when compared to normal aortic valves. Furthermore, immunohistochemical evaluation has revealed a transition from minuscule immunoreactivity of Fzd3, 7 and 8 in normal aortic valves, to vastly increased immunoreactivity of the same receptors in CAVs. We also demonstrate for the first time that HAVICs obtained from calcified human aortic valves exhibit a significant increase in the mRNA expression in 8 out of the 10 Fzd receptors. The combined results presented here suggest a possible involvement of the Wnt signaling pathway in the pathogenesis of AVC.

The presence of lipids around calcium deposits along with inflammatory cells (macrophages and T lymphocytes), angiogenic vessels and increased matrix deposition has been documented in CAVs (Olsson et al. 1999). Valves with higher oxidized LDL (oxLDL) have higher densities of inflammatory cells and a higher concentration of inflammatory mediators. The presence of Fzd receptors in areas of lipid core, inflammatory cells, myofibroblasts, osteoblasts, and HAVICs highly suggests the utilization of these receptors in the Wnt signaling cascade, driving the process of lipid deposition and inflammation toward osteoblastic changes, leading to the calcification of the valve. The upregulation of Fzd receptors in aortic valve tissue and especially in HAVICs is therefore highly likely to contribute to the pathogenesis of AVC. In addition, endothelial cells respond to canonical Wnt signaling *in vitro*. Noncanonical Wnt5a has been shown to promote capillary-like endothelial network formation and proliferation (Zerlin et al. 2008). This further demonstrates the vital role of Fzd receptors in the pathological angiogenesis seen in AVC.

Calcific atherosclerosis coexists in over 90% of patients with aortic stenosis, however, calcification is accelerated in the valve compared to regions of aortic and coronary atheroma (Dweck et al. 2013). Like in AVS, the cause of atherosclerosis appears to be related to lipid retention, oxidation, and modification, triggering inflammation at susceptible sites in the walls of major arteries. Both diseases share common risk factors and are initiated by lipid deposition and inflammation, triggered by endothelial damage. The upregulation of Wnt/LRP5 signaling pathway is considered to be a trigger for calcification (Caira et al. 2006; Rajamannan 2011a). LRP5 has been demonstrated to be involved in the innate inflammatory response to lipid infiltration in atherosclerosis, and, on the other hand, LRP5 deficient hypercholesterolemic mice

display attenuated Wnt signaling and aortic lipid infiltration (Borrell-Pages et al. 2015; Borrell-Pages et al. 2011). It has also been determined that LRP5/6 is necessary for AVC in hypercholesterolemic mice. Wnt3a stimulates the production of lipid droplets by endocytosed LDL-derived cholesterol (Scott et al. 2015). Thus, the upregulation of LRP5/6 in calcified aortic valves in our present study is indicative of the involvement of the Wnt signaling pathway and the Frizzled LRP5/6 co-receptors in the pathogenesis of AVC and atherosclerosis.

Approximately 64% of individuals with AVC have bicuspid aortic valves (BAVs), which is the most common congenital heart defect. BAVs are under a significantly higher amount strain compared to tricuspid aortic valves, and increased mechanical stress on VICs contributes to cell injury in AVC (Merryman and Schoen 2013; Szeto et al. 2013). Activation of VICs by shear stress induces inflammatory events and angiogenic activity. Once activated, VICs may then undergo osteoblastic differentiation with bone formation (Lerman et al. 2015). Although insignificant, our results show that expression levels of certain Fzd receptors are higher in calcified BAV tissue. We only employed a limited number of cases in our qPCR analysis therefore, increasing the number of cases studied may lead to significant differences. We previously showed that noncanonical Wnt5b had a significantly higher expression level in calcified BAVs (Albanese et al. 2017). This implies that the Wnt-Fzd pathway may be further upregulated in calcified BAVs.

Unlike the Wnt ligands, which can be categorized as being specific to either the canonical or noncanonical pathway, the Fzd receptors have been shown to be non-specific (Grumolato et al. 2010). This is to say, while the upregulation of Fzd in CAVs is supportive of our previous

finding that the noncanonical pathway is involved in calcific aortic valve stenosis, it does not discredit the possibility that this upregulation could be due to increased signaling through the canonical Wnt pathway as well. The Wnt co-receptors LRP5 and 6 have been shown to associate with Fzd receptors and aid in the binding of Wnt ligands. The noncanonical Wnt pathway does not utilize these co-receptors, however our current results combined with previous results show a simultaneous increase in noncanonical Wnt ligands and LRP5/6 expression (Rao and Kuhl 2010). A potential explanation for this finding is two-fold: a likely possibility is due to increased canonical signaling in calcified valves, and thus an increased expression of LRP bearing Fzd receptors. Alternatively, it is known that there is significant intracellular crosstalk between the two pathways (Weidinger and Moon 2003).

A comprehensive understanding of the mechanism by which the Wnt pathway leads to AVC may allow researchers to exploit key elements as potential drug targets. Secreted frizzled related proteins (SFRP), dickkopfs (DKK), sclerostin (SOST), and Wnt inhibitory factor-1 (WIF-1) have all been shown to be potent modulators of Wnt signaling (Askevold et al. 2012). Hampson et al. demonstrated a significant negative correlation between circulating levels of Wnt inhibitors (DKK and SOST) and the extent of abdominal aortic calcification, even when age, blood pressure, and cholesterol levels were corrected for (Hampson et al. 2013). Repeated demonstration of the involvement of the Wnt pathway in degenerative aortic valve disease onset and progression vindicates the use of canonical and noncanonical Wnt inhibitors as a potential therapy. Further studies are needed to determine which stage of disease and which step in the pathway inhibition would be most effective. In addition, future studies on the effect of increasing

Fzd expression in HAVICs will allow us to further understand the role of the Wnt-Fzd pathway in AVC.

In summary, we demonstrate here increased expression of 8 frizzled family receptors, and co-receptors LRP5/6, in the valves of patients with AVC. Given the lack of research examining the role of the Wnt pathway in AVC, these findings contribute to expanding the current literature, and aid in shedding the light for both clinicians and researchers on an important area of research to target components of the Wnt-Fzd pathway as potential pharmacological intervention targets for AVC.

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Figure Captions:

Figure 1:

Comparison of mRNA expression of frizzled receptors in human aortic valve tissue isolated from normal (n=5) and calcified aortic valves (n=13), *P<0.05.

Figure 2:

Comparison of mRNA expression of frizzled receptors in human aortic valve tissue isolated from calcified bicuspid aortic valves (n=7) and calcified tricuspid aortic valves (n=6).

Figure 3:

Comparison of LRP5/6 mRNA expression in human aortic tissue isolated from normal aortic valves (n=5), calcified bicuspid aortic valves (n=4), calcified tricuspid valves (n=16), *P<0.05.

Figure 4:

Representative images of Fzd immunoreactivity in normal and CAV. Panels A-C show the immunostaining of Fzd3 (A), Fzd7 (B), and Fzd8 (C) in normal human aortic valves. Panels D-F show the abundant immunostaining of Fzd3 (D and E) and Fzd7 (F) in the lipid core (LC) and calcified nodules (CN) of calcified valves. Panel G and H show the moderate immunostaining of Fzd8 in the lipid core and osteoblasts (OS) of calcified valves. Panels I-L show the immunoreactivity of Fzd3 (I and J) and Fzd7 (K and L) in the macrophages (MP) and endothelial cells (EC) of new microvessels (MV), as well as in myofibroblasts (MF). Scale bar: 100 μ m.

Figure 5:

Comparison of mRNA expression of frizzled receptors in cultured human aortic interstitial cells (HAVICs) isolated from non-stenotic (n=5) and stenotic (n=7) aortic valves. Most of the Fzds were upregulated in stenotic HAVICs, *P<0.05.

Tables

Table 1: Clinical data of patients with aortic valve disease used in immunohistochemistry.

Clinical Parameter	Mean $\pm$ SD
Age (years)	67 $\pm$ 13
Female (n)	26 (36.1%)
HbA1c (%)	6.03 $\pm$ 1.34
Total Cholesterol (mmol/L)	3.90 $\pm$ 1.09
LDL (mmol/L)	2.11 $\pm$ 0.92
HDL (mmol/L)	0.98 $\pm$ 0.26
LDL/HDL	2.19 $\pm$ 0.73
Triglycerides (mmol/L)	1.83 $\pm$ 1.97
Creatinine (μ mol/L)	84.65 $\pm$ 21.20
Circulating Calcium (normalized to albumin)	527.94 $\pm$ 88.26
Aortic Valve Area (cm ²)	1.29 $\pm$ 0.84
Parsonnet Score	20.74 $\pm$ 12.94
Ejection Fraction (%)	53 $\pm$ 11
% of Patients with Congestive Heart Failure	63.9%
% of Patients with Diabetes	27.8%
% of Patients with Hypertension	58.3%

Abbreviations: SD: standard deviation; HbA1c: glycated hemoglobin; LDL: low density lipoprotein; HDL: high density lipoprotein

Table 2: Clinical data of patients with aortic valve disease used in qPCR.

Clinical Parameter	Mean $\pm$ SD
Age (years)	68 $\pm$ 10
Female (%)	33.3%
HbA1c (%)	6.07 $\pm$ 1.12
Total Cholesterol (mmol/L)	4.85 $\pm$ 1.66
LDL (mmol/L)	3.32 $\pm$ 1.55
HDL (mmol/L)	1.60 $\pm$ 0.48
Triglycerides (mmol/L)	1.25 $\pm$ 0.24
Creatinine (μ mol/L)	88.70 $\pm$ 20.83
Aortic Valve Area (cm ²)	1.60 $\pm$ 0.86
Ejection Fraction (%)	53 $\pm$ 14

Abbreviations: SD: standard deviation; HbA1c: glycated hemoglobin; LDL: low density lipoprotein; HDL: high density lipoprotein

Table 3: qPCR primer sequences

Gene ID	Forward 5’-	Reverse 5’-
FZD1	GTTACAGGGTGAGTGAGATAACCAA	CAAACCCATACCAGGTTTTAGACAC
FZD2	GCCATCCTATCTCAGCTACAAGTTT	CTCCTCCTGTGAGAAGAACATGGAA
FZD3	TCTGAGGATAGCATTTCTCAAGACC	TCCCAAACCACAGATTCCATCTTAG
FZD4	ATTGTAGCTTCCTCAGAAATCCCTT	GAAAGGAATCTACCAGAAAGGAGGT
FZD5	GAAGCACTTCTTTGTCTCTGTTCTC	TAAGTAGATCCAGTTTCTCTCCCCT
FZD6	AGCAAATTGGCTCCGTTTAAGGATT	CTTTCCTGAAAATGAGTCCTGGGT
FZD7	GCCTGATGTACTTTAAGGAGGAGG	CCAGGTAGGTGAGAACGGTAAAGAG
FZD8	CAAAATTGAGTTTTTGCACCTTCCC	TTGCAGAAGTTCCATTTACATCCAC
FZD9	AAAATTTTTCATGTCACTGGTGGTGG	CCAGGTCTGGAAAGTCTTGGAG
FZD10	AAGTTACCCAAAGGGCTTATTGACT	CAAATATACACACGCAGAAACCACT
GAPDH	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG

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