NOTCH1 Genetic Variants in Patients with Tricuspid Calcific Aortic Valve Stenosis

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Background and aim of the study: **Calcific aortic valve stenosis (AS) affects 2-5% of the population aged >65 years. Functional DNA variants at the NOTCH1 locus result in bicuspid aortic valve (BAV) and severe valve calcification. The contribution of these variants to AS in the population with tricuspid aortic valve (TAV) remains to be determined.**

Methods: **Fourteen genetic variants surrounding the NOTCH1 gene were genotyped, including rare mutations previously reported, and common polymorphisms. The study involved 457 French Canadian patients with severe tricuspid AS. Genotyping was carried out using the Illumina® BeadXpress platform. Allele frequencies of common single nucleotide polymorphisms (SNPs) for patients with AS were compared to a shared control group of European ancestry (n = 3,294). In total, 88 ancestry-informative markers were used to correct for population stratification.**

Results: **The mutation R1107X, previously associated with AS and BAV, was identified in a relatively young patient (aged 58 years). The mutations R1279H and V2285I were detected in 18 and 14 heterozygotes, respectively. A common polymorphism (rs13290979) located in intron 2 was significantly associated with AS (p = 0.003), which remained significant after correction for multiple testing. However, this association was no longer significant after accounting for population stratification (p = 0.088).**

Conclusion: **In this study, rare functional variants were found in the NOTCH1 gene in a French Canadian population of patients with severe tricuspid AS. This also suggests, for the first time, the presence of a common polymorphism in this gene conferring susceptibility to AS.**

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Calcific aortic valve stenosis (AS) is the most frequent valvular heart disease, and affects 2% of the population aged over 65 years (1). The condition shares many clinical risk factors with atherosclerosis, including age, hypertension, and dyslipidemia (2). Nowadays, AS is known as a disease implicating many cellular processes such as inflammation, lipid deposition, and calcification (3,4). Currently, there is no treatment available other than aortic valve replacement (AVR) surgery or transcatheter valve implantation (5).

A number of genetic studies were performed to identify genes associated with AS (6). For most genes investigated so far, replication in larger populations is needed and functional causality remains to be established. The most compelling evidence of a genetic predisposition to AS was observed with the NOTCH1 gene. Two mutations that have functional consequences on the Notch1 protein have been identified. A mutation labeled R1107X was identified in a five-generation family affected with aortic valve disease that resulted in a premature stop codon (7). The mutation (H1505del) identified in a smaller Hispanic family and characterized by bicuspid aortic valve (BAV), causes a frameshift that alters 74 amino acids of the protein before ending its translation prematurely. The absence of these functional mutations in unaffected family members and healthy controls strongly suggested NOTCH1 as the causal gene. These specific mutations have not been reported

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Table I: Clinical characteristics of case subjects (n = 457).

Parameter	Males	Females
Gender (%)	57 (259)	43 (198)
Age (years)	70.7 ± 9.0	73.8 ± 7.9
Body weight (kg)*	78.6 ± 13.5	68.6 ± 4.9
Waist circumference (cm)*,[a]	102.5 ± 12.0	98.5 ± 14.8
Body surface area (m^2)*	1.9 ± 0.2	1.7± 0.2
Body mass index (kg/m^2)*	27.7 ± 4.3	28.7 ± 6.1
Mean transvalvular gradient (mmHg)*,[b]	40.8 ± 16.5	42.5 ± 16.1
Aortic valve area (cm^2)*	0.82 ± 0.24	0.68 ± 0.20
Coronary artery disease (%)	62.4 (161)	41.1 (81)
Hypolipemic drugs (%)	81.4 (210)	76.1 (150)
Hypertension (%)	66.3 (171)	75.1 (148)
Diabetes (%)	26.3 (68)	31.5 (62)

*Values are mean ± SD.
Values in parentheses are patient numbers.
[a], n = 393 patients.
[b], n = 362 patients.

in other populations. Another group has reported two different likely pathogenic missense mutations (p.T596M and p.P1797H) in two sporadic cases of BAV (8). Whether these mutations are private or are restricted to a few families in isolated geographic regions remained to be confirmed. In the present study, it was hypothesized that known mutations in the NOTCH1 gene are found among patients undergoing AVR surgery for tricuspid calcific AS. It was also hypothesized that more subtle, but common (minor allele frequency [MAF] > 0.05), polymorphisms in the NOTCH1 gene influence susceptibility to AS.

Clinical material and methods

Patients

A total of 457 patients with severe AS undergoing AVR surgery were recruited at the Institut universitaire de cardiologie et de pneumologie de Québec (Quebec City, Canada). All patients provided their written, informed consent, and the study was approved by the local ethics committee.

The patients were French Canadians, which are known as a homogeneous population of European ancestry (9). Only subjects with tricuspid non-rheumatic AS were considered. All patients had severe AS and an aortic regurgitation grade ≤ mild. Patients with a history of rheumatic disease, endocarditis or an inflammatory disease were excluded. Clinical data included diagnoses of hypertension (patients receiving antihypertensive medications or having known, but untreated, elevated blood pressure (≥140/90 mmHg)), and diabetes (patients with established diagnoses currently receiving oral hypoglycemic medication or insulin). Every patient underwent a coronary angiogram. A significant coronary artery disease (CAD) was considered to be present when at least one coronary artery had a lesion ≥50%. All patients underwent a comprehensive Doppler echocardiographic examination preoperatively. Doppler echocardiographic measurements included transvalvular gradients calculated using the modified Bernoulli equation (10) and the aortic valve area (AVA) calculated with the continuity equation. The clinical characteristics of the patients are listed in Table I.

Control population

Genotype data for 3,294 white control subjects typed on the HumanHap550 BeadChip were obtained from the Illumina Genotyping Control Database (www.illumina.com/science/icontroldb.ilmn). Cardiovascular phenotypes were not available for this shared control group, which consisted of 2,034 women (61.7%) and 1,260 men (38.3%) with a mean age of 32.2 ± 21.5 and 20.9 ± 20.8 years, respectively. In addition, DNAs for this control population were not available, so no additional genotyping could be carried out.

DNA extraction in patients with AS

Genomic DNA was isolated from the Buffy coat using QIAmp® DNA Blood Midi Kit (Qiagen). DNA quality was assessed by the UV A260/A280 ratio. DNA quantification was assessed by PicoGreen® assay (Invitrogen™; Carlsbad, CA, USA) using a Synergy™ HT fluorometer (Biotek®; Winooski, VT, USA). Every sample was diluted to a final concentration of 50 ng/µl.

Table II: SNPs selected for genotyping at the NOTCH1 locus.

rs number	Function	Location*	Reason for selection	Genotyped+
rs4379550	3′ region	chr9:138,496,247	Present on Hap550 BeadChip	X
rs61751489	Exon 33	chr9:138,511,159	Srivastava et al. (23), Missense, V2286I	
rs41309766	Exon 25	chr9:138,519,657	Garg et al. (7), frameshift, H1505del	
rs61751543	Exon 23	chr9:138,521,054	Srivastava et al. (23), Missense, R1280H	
rs41309764	Exon 20	chr9:138,522,511	Garg et al.(7), nonsense R1108X	
rs3124599	Intron 18	chr9:138,523,591	Tag	X
rs35962301	Exon 18	chr9:138,524,162	Srivastava et al. (23), Missense, R939Q	
rs11145766	Intron 17	chr9:138,524,716	Tag	X
rs35136134	Exon 16	chr9:138,525,470	Srivastava et al. (23), Missense, E848K	
rs61755997	Exon 11	chr9:138,529,872	Mohamed et al. (8) , missense, T596M	
rs13290979	Intron 2	chr9:138,545,455	Tag	X
rs11145770	Intron 2	chr9:138,546,887	Tag	X
rs7034799	5′ region	chr9:138,576,033	Present on Hap550 BeadChip	X
rs7849014	5′ region	chr9:138,577,327	Present on Hap550 BeadChip	X

*NCBI36/hg18
+On Illumina HumanHap550 BeadChip

Single nucleotide polymorphism (SNP) selection

To facilitate genetic association testing with Illumina controls, 12 SNPs genotyped on the HumanHap550 BeadChip located within 20 kilobases upstream and downstream of NOTCH1. Nine of these SNPs were identified as tagging SNPs and three as singletons, using the European-derived (CEU) genotype data from the HapMap project (11) and a pairwise tagging algorithm implemented in Haploview 4.2 (12). The MAF and r^2 thresholds were set at 0.05 and 0.8, respectively. Seven additional SNPs were selected from the literature and known to alter Notch1 protein (nonsense, missense or frameshift), even if they were not present on the HumanHap550 array. Using Illumina's Assay Design tool, a first panel was designed that contained 19 SNPs in the NOTCH1 locus. Four tagging SNPs failed Hardy-Weinberg (HW) equilibrium in the controls and were excluded. One tagging SNP with an assay design final score of 0, reflecting a low designability of the assay for this locus, was excluded. Accordingly, a total of 14 SNPs was selected and a multiplex assay was manufactured by Illumina to interrogate these. A list of SNPs genotyped in this study, and the reason for their selection, is provided in Table II.

Genotyping

Genotyping of the 457 patients was performed using the VeraCode GoldenGate genotyping assay. Microbeads were read on the Illumina BeadXpress Platform (Illumina, San Diego, CA, USA). The results were analyzed and genotype reports produced with the Illumina GenomeStudioV2010.1 Genotyping Module 1.6.3. DNAs from one Coriell trio (Camden, NJ, USA) were included as positive controls. In order to monitor repeatability, one AS sample and a single Coriell subject were included in all five 96-well genotyping plates.

Quality control of genotyping data

Clustering of genotyping data was performed using the Illumina GenomeStudio software. One singleton SNP (rs7849014) failed the genotyping assay. A rare variant rs35136134 failed HW equilibrium (p-value = 5.44E-18) and was excluded from further analysis. Seven samples with a call rate ≤0.90 were excluded. No replicate or parent-child errors were found from the Coriell trio and replicates tested. The software STRUCTURE version 2.3.2.1 (13) was used to identify population outliers using 88 ancestry-informative markers (AIMs) (14). Cases, controls and four reference populations from HapMap (11) (CEU, CHB, JPT, and YRI) were used. As shown in Figure 1, the majority of cases, controls and CEU individuals were contained in a major cluster. Three cases and 82 controls located outside the major cluster were removed. One sample for which AIMs genotyping data were not available was excluded.

Sequencing

The single heterozygote patient carrying the R1107X mutation was confirmed by sequencing. A 357 bp fragment of the NOTCH1 gene was amplified with the sense primer 5′-GTCCACCAGGTCCTCACAGT-3′ and the antisense primer 5′-TGTGCACTGGTGTGACTCCT-3′. The polymerase chain reaction (PCR) was performed in a final volume of 20 µl containing 30 ng of genomic

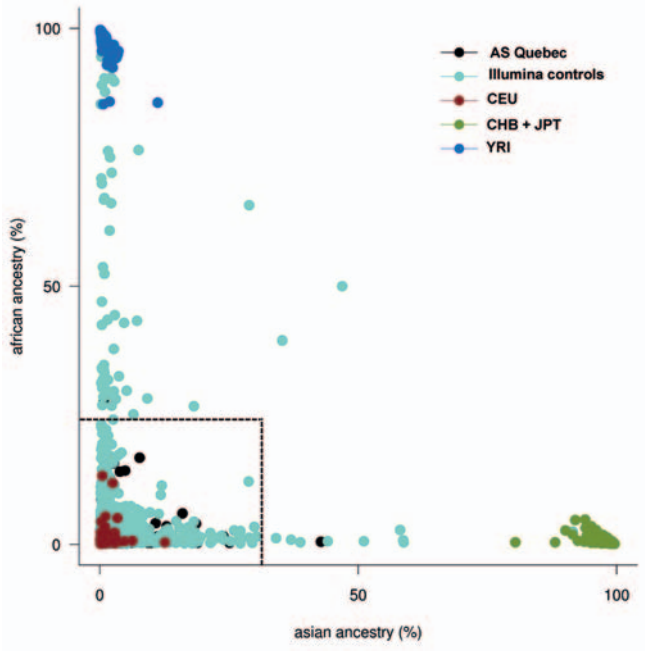

Figure 1: Triangular plot showing the genetic background of cases and controls. The plot was generated using STRUCTURE version 2.3.2.1 with HapMap subjects as internal controls. Black, AS (calcific aortic valve stenosis) cases; Cyan, Illumina controls; Red, CEU population (Utah residents with ancestry from northern and western Europe); Green, CHB + JPT population (Han Chinese in Beijing, China + Japanese in Tokyo, Japan); Violet, YRI population (Yoruba in Ibadan, Nigeria). The dotted lines delimited the boundaries for European ancestry.

DNA, 2 U of HotStar Taq DNA polymerase (Qiagen), PCR buffer 1X, 200 µM of each dNTP, and 0.2 µM of each primer. The PCR reaction was carried out on the GeneAmp® PCR system 9700 (Applied Biosystems) with the following cycling conditions: 15 min at 95°C, 35 PCR amplification cycles (15 s at 94°C, 30 s at 56°C, and 30 s at 72°C), and 7 min at 72°C. The sequencing reaction was then performed using standard procedures, and the product was run on the ABI 3730xl DNA Analyzer (Applied Biosystems). Sequencing files were assembled and analyzed using the Phred/Phrap/Consed System.

Statistical analyses

Allele frequencies of common SNPs between 446 cases and 3,212 controls that passed quality controls were compared using chi-square tests in PLINK version 1.06 (15). The method of Nyholt was used to correct for multiple testing (16), leading to a p-value threshold of 0.0085 or lower to consider polymorphisms as statistically significant. The AIMs were further used to calculate the genomic inflation factor in order to correct for population stratification (17). Population stratification can exist when the

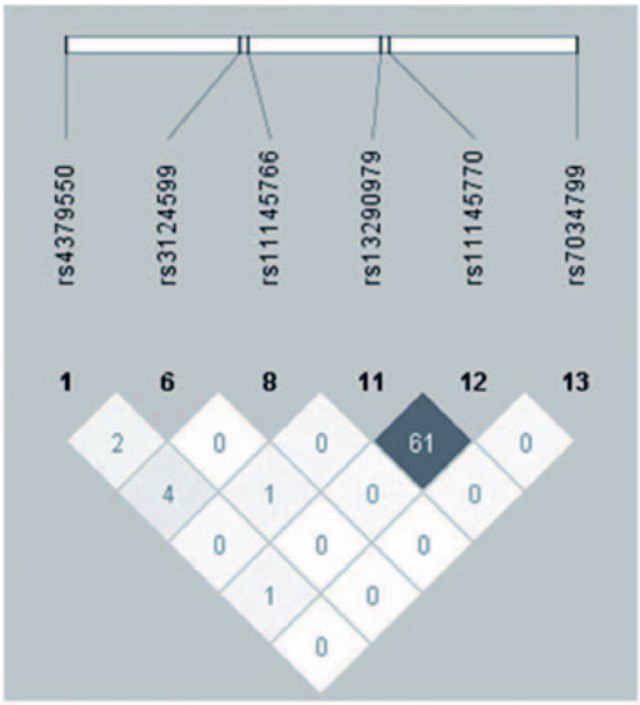

Figure 2: LD-plot showing r^2 values for the common NOTCH1 SNPs in the case/control population.

populations under study consist of subpopulations with different marker allele frequencies and disease prevalence, caused by different ancestry, which may increase the rate of false positives in case-control studies (18). Linkage disequilibrium (LD) was calculated using Haploview. The influence of SNPs on disease severity, as assessed with AVA and mean transvalvular gradient, was tested using linear regression models adjusted for age and body surface area (BSA). Age at surgery was additionally analyzed using linear models adjusted for BSA. Disease severity was also analyzed between carriers and non-carriers of non-synonymous sequence variants. Disease severity analyses were performed with R software version 2.8.1. The population-attributable risk was calculated as PAR% = 100% × P × (OR - 1)/[P × (OR - 1) + 1], where P is the frequency of the risk allele associated with AS in the control group, and OR is the odds ratio calculated in the case-control cohort.

Results

The assay conversion rate was 93% (13/14 SNPs). Call rates for the remaining SNPs were above 95%, with a mean of 99.8 ± 0.3. The six common SNPs tested for association in the case-control cohort are listed in Table III; the LD plot for the common SNPs in the case-control cohort is shown in Figure 2.

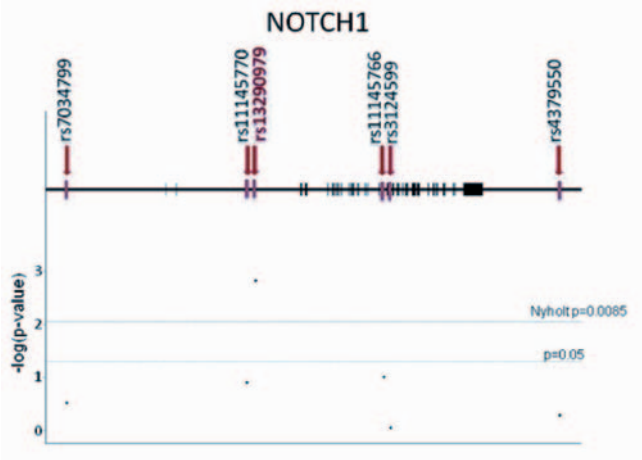

Figure 3: Genetic association of common SNPs in the NOTCH1 gene with AS. The upper part of the figure shows the intron-exon structure of the gene and the localization of the genotyped SNPs. The lower part of the figure shows the genetic association results. The x-axis shows the localization of the SNPs relative to the upper part of the figure. The y-axis shows the p-values on a -log10 scale. The horizontal lines represent p-value thresholds of 0.05 and 0.0085.

A common SNP is associated with the disease

A common polymorphism (rs13290979) in intron 2 of the NOTCH1 gene was significantly associated with AS (Fig. 3; Table III). The minor allele frequency was 32.6% in cases and 37.7% in controls (p = 0.0034). The risk allele is the common allele (common:rare allele counts were 601:291 in cases and 3999:2417 in controls) with an odds ratio of 1.25 (95% confidence interval 1.08-1.45). The association remained significant after correction for multiple testing. The estimated population-attributable risk was 14.3%. A genomic inflation factor of 2.9 was calculated using AIMs between cases and controls. After accounting for population stratification, the association between rs13290979 and AS was no longer significant (p = 0.088). Among cases, no association was found between rs13290979 and disease severity. The AVA, the mean transvalvular gradient, and age at surgery were not different between genotyping groups (Fig.4A). A search was made for SNPs in LD ± 100 kilobases upstream and downstream of rs13290979 using the 1000 genomes (19) genotyping dataset. The SNP rs13290840 also located in the second intron of NOTCH1 was in moderate LD (r^2 = 0.759) with rs13290979. The other five common variants tested in the cases-controls cohort were not significantly associated with AS (Fig. 3; Table III).

Three rare variants were found in patients with AS

Among the cases, 33 carried one single rare mutation. The mutations R1107X, R1279H and V2285I were found in one, 18 and 14 patients, respectively. The single carrier of the R1107X variant was confirmed by sequencing (Fig. 5). Despite the presence of these variants in the cohort, there was no difference in disease severity or age of surgery between AS patients with or without these mutations (Fig. 4B). The other mutations, T596M, R938Q or H1504del, were not found among cases.

Discussion

The results of a previous study suggested that rare mutations in the NOTCH1 gene caused aortic valve diseases (7). The frequency of these mutations in the general population and in patients with valve diseases is unclear, and it is also unclear whether common polymorphisms in the NOTCH1 gene confer susceptibility to AS. In the current study, rare mutations (previously) associated with AS and common polymorphisms in NOTCH1 were typed in a cohort of 457 patients with severe AS, and an association between a common SNP in the NOTCH1 gene and AS was identified that will require further validation in an independent cohort. Multiple rare variants were also detected in the case population, including one carrier of the R1107X mutation.

Table III: Characteristics of common SNPs tested for association.

SNP	Call rate	MAF*	HW p-value*	Frequency cases	Frequency controls	Association p-value	p-value genomic control	Risk allele	OR	95% CI
rs4379550	99.97	0.4333	0.0593	0.442	0.432	0.5942	0.7557	G	1.039	0.903-1.197
rs3124599	99.95	0.0514	0.6100	0.053	0.051	0.8548	0.9149	A	1.03	0.752-1.41
rs11145766	98.03	0.1088	0.7959	0.128	0.106	0.0509	0.2542	A	1.235	0.999-1.527
rs13290979	99.89	0.3706	0.3385	0.326	0.377	**0.0034**	0.0876	**A**	**1.248**	1.076-1.449
rs11145770	99.70	0.3591	0.7192	0.343	0.361	0.2873	0.5344	G	1.083	0.935-1.255
rs7034799	99.75	0.0554	1.0000	0.051	0.056	0.5043	0.6966	A	1.114	0.811-1.532

*Calculated from combined cases and controls.
CI: Confidence interval; HW: Hardy-Weinberg; MAF: Minor allele frequency; OR: Odds ratio.

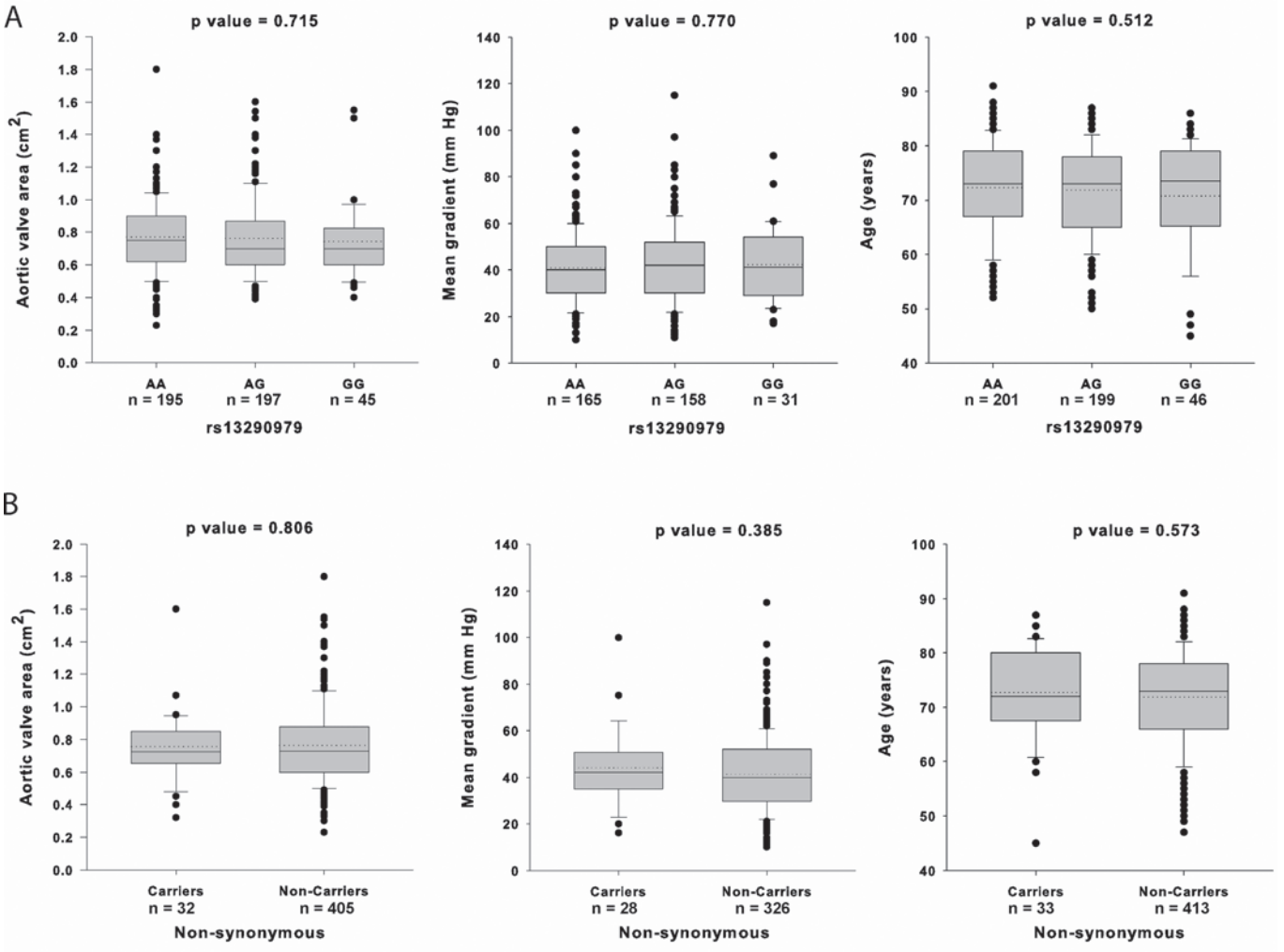

Figure 4: Disease severity assessed by aortic valve area, mean transvalvular gradient and age at surgery. A) Association between disease severity and the common SNP rs13290979. Box plots show the median, 25th, and 75th percentiles. The dotted line inside the box represents the mean. Whiskers above and below the box indicate the 90th and 10th percentiles. B) Association between disease severity and carrier status for rare non-synonymous SNPs (rs41309764, rs61751489, and rs61751543). Patients included those for which clinical and genotypic data were available.

In previous NOTCH1 gene association studies, rare variants were thought to have a role in the pathobiology of AS (7,8). Garg et al. (7) reported a nonsense mutation to cause developmental valves anomalies and severe valve calcification when they described a five-generation family of European-American descent with 11 cases of congenital heart disease (nine with aortic valve disease). Members of this family had either bicuspid or tricuspid valves; seven developed calcific AS and four of them underwent AVR. Every affected family member carried the mutation R1107X, while unaffected members were non-carriers, which suggested an autosomal-dominant mutation with a complete penetrance (7).

Interestingly, and for the first time since Garg et al., one male patient was identified in the present study who carried this mutation. The patient was aged 58 years at surgery, and had grade 3 calcification based on the Warren and Yong scoring system (20) and light fibrosis. He had an indexed AVA of 0.47 cm^2/m^2, a waist circumference of 107.4 cm, and a body mass index of 29.5 kg/m^2. This individual had multiple cardiovascular risk factors: he suffered from metabolic syndrome, dyslipidemia, hypertension, and CAD, and was receiving angiotensin-converting enzyme inhibitor and statin medications. He had undergone AVR for severe AS at a young age, which suggested that the presence of this variation might be associated with the premature development of AS. No family history of calcific aortic valve stenosis was found in the patient's medical chart. No DNA or echocardiography examinations for relatives of this patient were available, and there was no family history of AS.

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Nevertheless, the results confirmed that this mutation is found in the French Canadian population and may be more widely spread than previously suggested.

In the present study, suggestive evidence was provided that a common polymorphism in the NOTCH1 gene is associated with AS. Although the odds ratio (1.25) of this polymorphism is modest, the population-attributable risk indicates that more than 14.3% of AS cases could be attributed to this common variant. This polymorphism is in moderate LD with a second variation in the same intron. The genotyping of the latter variation in a cohort of AS patients and controls will be needed to determine if it is also associated with the disease. Accordingly, in addition to several rare penetrant variants, there is a common risk allele that seems to modulate the risk of suffering from AS. The present data do not support the concept that multiple rare variants collectively contribute to disease severity among AS cases. However, when combined with a previous report (7), the present study results suggests that the NOTCH1 locus contains multiple polymorphisms covering a range of allele frequency and penetrance associated with AS. Functional studies will be required to prove causality of the common rs13290979 polymorphism.

Study limitations

Validation in independent populations will be required. Although three known and potentially deleterious mutations were detected in patients with AS, the frequency of these rare mutations will need to be evaluated in large populations with and without valve diseases in order to demonstrate their clinical relevance. Finally, a set of control subjects was used which is publically available to perform the case-control analysis, and detailed phenotyping of the cardiovascular system and other traits of interest are not available in this control group. One consequence of such a strategy relates to the potential for misclassification: a proportion of the controls is likely to have AS and some others will develop this disease in the future. However, the effect of this misclassification in the control population on statistical power was shown to be minimal unless the extent of misclassification bias is substantial. For example, if 5% of controls were to meet the definition of cases, the loss of power is approximately the same as that due to a reduction in sample size by 10% (21). In the general population, the prevalence of all valve diseases is estimated at 2.5% (22). Accordingly, the effect of misclassification is likely to be low in the proposed control group.

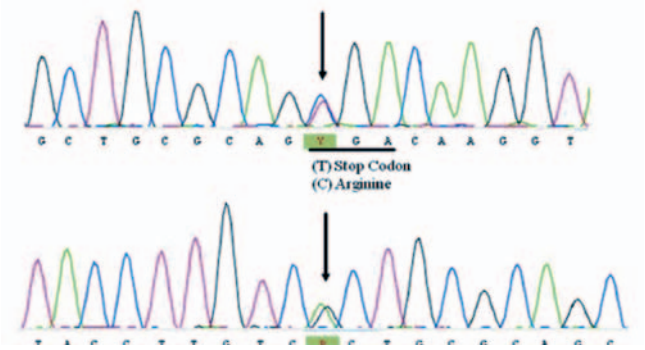

Figure 5: Sequence chromatogram of the single heterozygote subject carrying the R1107X variant. The sequence was obtained on both strands (top and bottom panels). R = A or G.

In conclusion, the results of the present study further support NOTCH1 as a potentially important gene involved in AS. An autosomal-dominant mutation with complete penetrance (R1107X) originally identified in a single pedigree was found in one patient with AS. The clinical condition and age of this patient at surgery support this mutation as being penetrant. Although additional rare variants were found, they were not associated with disease severity among cases. The frequency of these non-synonymous sequence variants in healthy controls is required. Finally, some evidence was found that a common variant in the NOTCH1 gene was associated with AS, suggesting that more subtle but frequent polymorphisms at this locus might predispose to the disease. Replication in an independent cohort is needed.

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