Genetic association analyses highlight biological pathways underlying mitral valve prolapse

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Nonsyndromic mitral valve prolapse (MVP) is a common degenerative cardiac valvulopathy of unknown etiology that predisposes to mitral regurgitation, heart failure and sudden death1. Previous family and pathophysiological studies suggest a complex pattern of inheritance2–5. We performed a meta-analysis of 2 genome-wide association studies in 1,412 MVP cases and 2,439 controls. We identified 6 loci, which we replicated in 1,422 cases and 6,779 controls, and provide functional evidence for candidate genes. We highlight LMCD1 (LM and cysteine-rich domains 1), which encodes a transcription factor and for which morpholino knockdown of the ortholog in zebrafish resulted in atrioventricular valve regurgitation. A similar zebrafish phenotype was obtained with knockdown of the ortholog of TNS1, which encodes tensin 1, a focal adhesion protein involved in cytoskeleton organization. We also showed expression of tensin 1 during valve morphogenesis and describe enlarged posterior mitral leaflets in Tnst−/− mice. This study identifies the first risk loci for MVP and suggests new mechanisms involved in mitral valve regurgitation, the most common indication for mitral valve repair7.

The prevalence of nonsyndromic MVP has been estimated as 2.4% in the general population8. Familial aggregation9–10, presence in rare connective-tissue syndromes11 and the identification of four linked loci2–5 indicate genetic heterogeneity for MVP. Additional factors, such as age- and sex-dependent penetrance, with a possible association with myocardial structural and functional abnormalities, suggest additional genetic complexity12. To identify susceptibility loci for MVP, we conducted an initial discovery meta-analysis on 2 independent French genome-wide association studies (GWAS) that included 1,412 MVP cases and 2,439 controls (Supplementary Table 1), all of European ancestry, for ~4.8 million genotyped or imputed common (minor allele frequency (MAF) > 0.1) SNPs (Fig. 1 and Supplementary Fig. 1). Three loci showed genome-wide significant associations with MVP (P < 5 × 10−8) (Table 1). The strongest association (rs12465515: odds ratio (OR) = 1.33, P = 1.08 × 10−9) was observed at 2q35 in a ~424-kb gene desert region where the nearest upstream genes are TNP1, IGFBP5 and IGFBP2 and the nearest downstream genes are DIRC3 and TNS1 (Table 1). The two other genome-wide significant loci were at 17p13 (lead SNP rs216205: OR = 1.35, P = 3.02 × 10−8) and at 22q12 (rs17767392: OR = 1.23, P = 2.27 × 10−8) (Table 1).

We genotyped or imputed a total of 47 SNPs (23 loci). An intermediate meta-analysis including the discovery set and follow-up sets 1 and 2 (nCases = 2,312 and nControls = 8,296) identified a subset of 24 SNPs (15 loci) with significant associations with MVP (P < 0.01), which were genotyped or imputed in 2 additional case-control studies from Canada and France (sets 3 and 4, respectively; Supplementary Fig. 1). In the global meta-analysis that included 2,864 cases and 9,218 controls, 3 additional loci associated with MVP at the genomic level (Table 1 and Supplementary Table 2). The overall strongest association was observed at 3p13 for rs171408, which maps in an intron of LMCD1 (OR = 1.32, P = 1.29 × 10−11; Table 1). Two additional signals were identified, one at 21q22 near CBR1 and SETD4 (rs62229266: OR = 1.22, P = 1.18 × 10−8) and the other at 14q24 near SIPA1L1 and PCNX (rs17767392: OR = 1.23, P = 2.27 × 10−8; Table 1). We also confirmed the three genome-wide significant signals identified in

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the discovery samples with lead SNPs rs12465515 near IGFBP5 and TNS1 (OR = 1.25, \( P = 3.11 \times 10^{-11} \)), rs11705555 near PITPNB and MNI (OR = 1.23, \( P = 1.39 \times 10^{-5} \)) and rs216205 in SMG6 (OR = 1.24, \( P = 1.46 \times 10^{-8} \)). Overall, we observed consistency in the direction of effect as well as nominally significant association in the follow-up meta-analysis and did not detect significant heterogeneity \((P > 0.05)\) among the case-control studies (Table 1).

Many individuals in the general population with MVP show few clinical symptoms, if any\(^1\). Nonetheless, a substantial subset of these individuals are at risk of heart failure and cardiac-related death, and MVP is the most common cause of isolated mitral regurgitation requiring surgical repair\(^7\). To investigate whether the confirmed MVP risk alleles are more prevalent among more severely affected individuals who require valve repair or replacement, we analyzed 1,680 French MVP cases who underwent surgical intervention and compared them to 3,259 French controls (Supplementary Table 3). We did not find a stronger effect for any of the MVP risk alleles, except for a slight increase in the frequency of the risk-associated allele of rs11705555 at the PITPNB-MNI locus \((OR = 1.31, 95\% \text{ confidence interval} \ (CI) = 1.19–1.44, \ P = 1.88 \times 10^{-8})\). Overall, our findings support the idea that MVP is under significant genetic control with susceptibility loci of relatively homogeneous effect sizes \((OR \text{ from } 1.22 \text{ to } 1.33)\).

The MVP-associated loci implicate four intergenic (IGFBP5-TNS1, SETD4-CBR1, PITPNB-MNI and PCNX-SIPA1L1) and two intronic (LMCD1 and SMG6) regions (Supplementary Table 4). From an initial list of 53 genes \((\text{located within } 500 \text{ kb to } 1 \text{ Mb of the lead SNP})\) we identified candidate genes at each locus on the basis of proximity to the sentinel SNP, expression level in the heart, presence of expression quantitative trait locus \((\text{eQTL})\) signal in the publically available Gene-Tissue Expression \((\text{GTEx})\) database, proximity to previously identified GWAS signals for cardiovascular traits and a biological link with mitral valve or general cardiac development (see Supplementary Table 5 and the Supplementary Note). We limited our analysis to eight genes at the three human loci: igfbp2a, igfbp2b, igfbp5a, igfbp5b and tns1 at the 2q35 locus; lmc1 at the 3p13 locus; and smg6 and sgsm2 at the 17p13 locus.

At 2q35, rs12465515 lies within a large intergenic region, and TNS1 and IGFBP5 were identified as the best two candidate genes (Supplementary Table 4). TNS1 maps 750 kb downstream of the associated signal at the 2q35 locus (Fig. 2). Tensin 1 protein localizes to focal adhesions and interacts with actin \((\text{as does filamin A})\), and variants can cause a rare X-linked form of MVP\(^13\). Tensin 1 interacts with the cytoplasmic tails of integrins to anchor stress fibers and has an important role in determining the metastatic capacities of cancer cells\(^14\). IGFBP5, which encodes insulin-like growth factor–binding protein 5 \((\text{IGFBP5})\), is known to modulate muscle differentiation and mediate high glucose–induced profibrotic effects in cardiac fibroblasts\(^15\). IGFBP5 has also been demonstrated to modulate the migration and adhesion of cancer cells\(^15\) and could potentially be at play in valve development and valvular interstitial cell integrity.

We observed only faint nonspecific staining for Igfbp5 in the valves of developing and adult mice (Supplementary Fig. 2). In contrast, mouse IHC data showed sustained expression of tensin 1 during valve morphogenesis, with expression stronger along the atrial aspect of the forming leaflet (Fig. 3a). We also found that tensin 1 expression was maintained during adulthood and was localized to endothelial and valvular interstitial cells (Fig. 3a). Hematoxylin and eosin (H&E) histological staining in 9-month–old Tns1\(^{-/-}\) mice showed enlarged posterior mitral leaflets as compared to those in wild-type littermates (Fig. 3b). In addition, valves from a Tns1\(^{-/-}\) mouse showed evidence of myxomatous degeneration, as indicated by increased proteoglycan content and loss of normal matrix stratification, demonstrated by the accumulation of proteoglycan in the valves (Fig. 3c). Preliminary echographic exploration of Tns1\(^{-/-}\) mice \((n = 2)\) showed slight leaflet displacement \((0.4 \text{ mm})\) as compared to the leaflets in wild-type mice \((0.1 \text{ mm})\), consistent with larger leaflets but no mitral regurgitation (Supplementary Fig. 3) and indicating subtle anomalies of the mitral valve that warrant future confirmation.

In zebrafish experiments (Fig. 2b–e), a significant increase in the incidence of atrioventricular \((\text{AV})\) regurgitation was observed in tns1 knockout animals as compared to control animals for both morpholinos \((3 \text{-fold} \ (P = 0.02)\) and 1.1-fold \((P = 0.01)\) increases; Supplementary Videos 1 and 2) but not in animals with simultaneous knockdown of the igfbp2 and igfbp5 isoforms (Fig. 2b). In situ hybridization identified high tns1 expression throughout the developing heart, and knockdown of this gene diminished the aggregation of endothelial cells at the developing valve (Fig. 2c and Supplementary Fig. 4). Furthermore, although notch1b expression remained localized...
Table 1. Genome-wide significant associations of sNPs with MVP

| Locus | SNP | Frequency | OR (95% CI) | P (Heterogeneity) | Heterogeneity value | P (value) |
|-------|-----|-----------|-------------|-------------------|---------------------|-----------|
| IGFBP5 | rs112465515 | 0.34 | 1.39 (1.23–1.56) | 0.02 | 8.0×10^{-8} | 0.017 |
| LMCD1 | rs1714198 | 0.22 | 1.22 (1.2–1.47) | 0.02 | 3.0×10^{-7} | 0.004 |
| SNMG6 | rs11767392 | 0.25 | 1.14 (1.1–1.35) | 0.02 | 4.0×10^{-7} | 0.009 |
| CBR1 | rs132292668 | 0.02 | 2.12 (1.4–2.6) | 0.02 | 3.0×10^{-7} | 0.004 |

*Note:* The OR values were calculated using a logistic regression model adjusted for age, sex, and center. The significance threshold for genome-wide significance was set at P < 5.0×10^{-8}.
led to an abnormal valvular phenotype (Supplementary Fig. 7). Additional candidate genes need to be explored at 17p13, which has largely been elusive. The first molecular pathways implicated in MVP were described on the basis of observations of disease in individuals with Marfan or Ehlers-Danlos syndrome, findings that highlighted the importance of extracellular matrix composition, the transforming growth factor (TGF)-β growth factor pathway and valve cell proliferation and differentiation. Several mechanisms describing structural changes as a basis for MVP have also been proposed, including enlargement and flattening of the mitral annulus that can impose additional stresses on genetically susceptible valves and chordae. In this first GWAS of nonsyndromic MVP, we have identified several susceptibility loci that support the concept that genetic variants affecting the expression of proteins during valve development can progressively affect mitral valve function into adult life, as was recently shown for filamin A. In particular, we provide genetic and functional evidence that TNS1 and LMCD1, both implicated in cell proliferation and migration, contribute to mitral valve degeneration.

Despite the widespread prevalence of MVP, its molecular basis has largely been elusive. The first molecular pathways implicated in MVP were described on the basis of observations of disease in individuals with Marfan or Ehlers-Danlos syndrome, findings that highlighted the importance of extracellular matrix composition, the transforming growth factor (TGF)-β growth factor pathway and valve cell proliferation and differentiation. Several mechanisms describing structural changes as a basis for MVP have also been proposed, including enlargement and flattening of the mitral annulus that can impose additional stresses on genetically susceptible valves and chordae. In this first GWAS of nonsyndromic MVP, we have identified several susceptibility loci that support the concept that genetic variants affecting the expression of proteins during valve development can progressively affect mitral valve function into adult life, as was recently shown for filamin A. In particular, we provide genetic and functional evidence that TNS1 and LMCD1, both implicated in cell proliferation and migration, contribute to mitral valve degeneration.

Figure 2 Cardiac regurgitation in zebrafish embryos with morpholino-mediated knockdown of orthologs to the candidate genes at 2q35 in human. (a) Genomic context of the association signal observed in the GWAS meta-analysis. The regional association plot was generated using LocusZoom and displays surrounding genes, with TNS1, IGFBP2 and IGFBP5 identified as the best potential candidates at this locus. (b) Mitral regurgitation observed at 72 hours post-fertilization (h.p.f.) in zebrafish embryos after morpholino-mediated knockdown. All results are presented as fold change compared to clutchmate controls. n is the number of biological replicates per morpholino. *P < 0.05. (c) Two-dimensional projections of z-series images stacks taken on hearts excised from a control zebrafish (injected with nontargeting morpholino) and one with tns1 knockdown. Green denotes EGFP expression (driven by the flk promoter), a marker of the endothelium. Red staining indicates the distribution of F-actin, which is highly expressed in the functional myocardium. A, atrium; V, ventricle. White arrows indicate the AV canal. Scale bar, 50 μm. (d) Antisense probe for bmp4 labels the valve and surrounding myocardium in a control embryo and two with tns1 knockdown. Scale bar, 50 μm. (e) Brightfield micrographs displaying the gross morphology of zebrafish morphants at 72 h.p.f. The body axis length of morpholino-injected fish is slightly reduced compared to that of wild-type control fish (injected with nontargeting morpholino). Scale bar, 1 mm.

Figure 3 Tensin 1 expression in developing mouse valves and the knockout phenotype at 9 months. (a) Tensin 1 expression in the developing mouse heart. IHC was performed for tensin 1 (red) at E13.5 (complete EMT), E17.5 (valve sculpting and elongation) and 9 months of age. MF20 (green) labels myocytes; Hoechst (blue) labels nuclei. Scale bars, 200 μm. (b) Tns1 knockout mice exhibit enlarged mitral leaflets. H&E histological staining was performed on wild-type (Tns1+/+) and knockout (Tns1−/−) mice. Scale bars, 200 μm. (c) Tns1 knockout mice exhibit myxomatous mitral leaflets. IHC for collagen (red) and proteoglycans (green) shows failure of normal matrix stratification and expansion of proteoglycan expression in Tns1−/− mitral leaflets, indicative of a myxomatous phenotype. Scale bars, 200 μm. AL, anterior leaflet; PL, posterior leaflet; LV, left ventricle; IVS, interventricular septum.
METHODS

Methods and any associated references are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

C.D., N.B.-N. and X.J. organized and designed the genome-wide association study and the manuscript preparation. A.A.H., R.A.L., S.A.S., H.L.M., P.P., J.S. and L.F.-F. organized and coordinated the network efforts and recruitment of patients. R.D., V.P., T.L.T., F.K., P.P. and Y.B. participated in the recruitment of patients and the interpretation of the echocardiograms. J.-S. and X.J. coordinated the collection of patient samples. M.P. and S.L. managed the collection of patient samples and performed DNA extraction and genotyping. C.D. and N.B.-N. conceptualized the statistical analyses. C.D., M.-H.C. and F.S. performed the statistical analyses. D.J.M. conceptualized and supervised the zebrafish experiments. N.T. performed zebrafish experiments, interpreted the data and wrote parts of the manuscript. P.T.E. and E.D. participated in zebrafish experiments and interpretation of data. R.R.M. and R.A.N. conceptualized and supervised the mouse studies and interpreted the data. K.T. performed immunohistochemistry staining of mouse organs. S.H.L. generated the mice. F.N.D., E.I.B., D.Z., M.L., S.H., R. Roussel, F.B., R. Redon, P.F. and R.S.V. conducted the epidemiological studies in control cohorts and/or contributed samples to the GWAS and/or follow-up genotyping. P.B. supervised valve tissue DNA extraction. S.A.S. contributed patient samples and organized follow-up genotyping and interpreted the GWAS and follow-up data. C.D., N.B.-N. and X.J. wrote the manuscript. F.N.D., R.A.N., D.J.M., S.A.S., R.A.L., J.-S. and A.A.H. edited the manuscript, and all authors approved its content.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Leduq Transatlantic MITRAL Network. The majority of patients were recruited as a major project of the Leduq MITRAL Network, a transatlantic consortium with basic-science and clinical investigators from 10 clinical and research centers investigating the physiopathology of mitral valve disease. Six centers recruited MVP cases: MVP-Nantes and MVP-France for the initial GWAS effort; MVP-USA, the Framingham Heart Study (FHS) and Prolapso Mitral en Centros Españoles (PROMESA) at the Centro Nacional de Investigaciones Cardiovasculares (CNIC) for initial replication; and HEGP—Surgical Cases with QCCMRc data sets for the last replication stage. Cases were compared with controls (FHS, PROMESA-CNlC and QCCMRc) or the general population (D.E.S.I.R. for the initial GWAS and one replication stage, SU.VI.MAX for the GWAS) (Supplementary Fig. 1).

Case recruitment criteria. Our consensus inclusion criteria included adult patients (≥18 years of age) with idiopathic MVP if they presented displacement into the left atrium of any part of the mitral valve leaflet(s) ≥2 mm beyond a line connecting the annular hinge points on the parasternal long-axis view of the left ventricle by two-dimensional echocardiography8,29. We also included patients who had undergone previous surgeries for pure severe mitral regurgitation due to MVP, supported by an operative report and written confirmation of the diagnosis by the surgeon (MVP-France, MVP-Nantes and Surgery Cases). All cases were validated by a local, experienced team of cardiologists on the basis of clinical and echocardiography records. Recruitments excluded patients with MVP associated with other heart disease (coronary artery disease or known syndromes (such as Marfan and Ehlers-Danlos). Local ethics committees approved all studies, and all patients and controls provided written informed consent. Recruitment procedures for DNA collection are detailed for each cohort in the Supplementary Note.

GWAS genotyping and quality control. Genotyping of the discovery cohorts was performed independently on different genetic platforms that included ethics committees approved all studies, and all patients and controls provided disease) or known syndromes (such as Marfan and Ehlers-Danlos). Local recruitment criteria. Our consensus inclusion criteria included adult patients (≥18 years of age) with idiopathic MVP if they presented displacement into the left atrium of any part of the mitral valve leaflet(s) ≥2 mm beyond a line connecting the annular hinge points on the parasternal long-axis view of the left ventricle by two-dimensional echocardiography8,29. We also included patients who had undergone previous surgeries for pure severe mitral regurgitation due to MVP, supported by an operative report and written confirmation of the diagnosis by the surgeon (MVP-France, MVP-Nantes and Surgery Cases). All cases were validated by a local, experienced team of cardiologists on the basis of clinical and echocardiography records. Recruitments excluded patients with MVP associated with other heart disease (coronary artery disease or known syndromes (such as Marfan and Ehlers-Danlos). Local ethics committees approved all studies, and all patients and controls provided written informed consent. Recruitment procedures for DNA collection are detailed for each cohort in the Supplementary Note.

GWAS genotyping and quality control. Genotyping of the discovery cohorts was performed independently on different genetic platforms that included standard quality control measures of genotyping and data acquisition from diverse high-density genotyping arrays (Supplementary Table 1). We excluded participants with genotype call rates <97% and individual heterozygosity levels <10,000 (determined as the outlier limit after visual inspection). We excluded SNPs that had a MAF <0.1, had a call rate <95%, were monomorphic or had an exact Hardy-Weinberg equilibrium P value of <0.0001 in controls and <1 × 10^−7 in demographically homogenous cases to exclude SNPs that showed very large deviations.

Imputation. To complement the directly genotyped SNPs, we performed large-scale imputation in the four discovery cohorts. First, genotyped SNPs in cases and controls were phased using the SHAPE-IT (v1) program30. Then, the imputation of 4.8 million common SNPs (MAF > 0.1 in 1000 Genomes Project Europeans, proper-info > 0.4) was carried out using IMPUTE2 (v2)31 in −7-Mb chunks. The reference panel used was the Phase I integrated variant set release (v3) in NCBI Build 37 (hg19). We used similar procedures to impute non-genotyped SNPs in the FHS replication cohort with MACH software (0.3 r2_hat)32.

Direct genotyping in the replication sets. MGH cases from follow-up set 1 and all cases and controls from set 2 were genotyped at the Massachusetts General Hospital PNGU Core Lab using the Sequenom iPLEX Gold application and MassARRAY system. Follow-up sets 3 and 4 were genotyped at the LGC genomics company using KASP genotyping chemistry. We excluded samples for nine individuals that failed genotyping for all SNPs and had SNPs with call rates <0.90. No SNP deviated from Hardy-Weinberg equilibrium (P > 0.05).

Demographic analyses. The ancestry of the patients was assessed using a multidimensional scaling technique implemented in PLINK33. SNPs were selected for short-range linkage disequilibrium (LD) independence (r2 > 0.2). The multidimensional scaling method was applied to the identity-by-state matrix, and we excluded outliers on the first two components (Supplementary Fig. 8) using an expectation maximization–fitted Gaussian mixture clustering method implemented in the R package M-CLUST, assuming one cluster and noise (Supplementary Note).

Genome-wide and replication association with MVP status. We applied a logistic regression (additive) model as implemented in SNPTEST13 to test the association with MVP in the GWAS discovery set with the first five principle components included as covariates. We also used SNPEST and/or logistic regression on allele dosage in replication sets when cases and/or controls were imputed for genotypes (FHS in set 1 and D.E.S.I.R. 2 in set 4; Supplementary Fig. 1). Genetic relatedness was also taken into account when appropriate (FHS cohort). For directly genotyped case-control studies (sets 2 and 3), we used logistic regression as implemented in PLINK.

For the GWAS meta-analysis, we applied the inverse normal strategy34. Because the number of controls greatly exceeded the number of cases in all studies, we used the effective sample size as advised in METAL software35; w = 4/(1/n_cases + 1/n_controls).

Regional association plots for 2q35, 3p13 and 17p13 were created using LocusZoom36.

Protein detection in mouse embryos and adult hearts. Standard histological and IHC procedures were used, as previously described37. For all immunohistochemistry experiments, 5-min antigen retrieval was performed with Vectastain in a pressure cooker (Cusinart). The antibodies used for immunological experiments were to tensin 1 (Novus, NB100-41087) and myosin heavy chain (Developmental Hybridoma Banks, MF20). Primary antibodies were used for IHC at a 1:100 dilution; Hoechst 33342 (nuclear stain) was used at a 1:10,000 dilution. Appropriate secondary antibodies were used for detection.

Histology and expression studies were performed in adult (9-month-old) wild-type (Tns1+/+) and knockout (Tns1−/−) mouse hearts. For histology, adult (9-month-old) hearts were processed for H&E staining and IHC, as previously described37. For all analyses, male mice were used (n = 3 for each genotype; deemed sufficient as studies were a qualitative assessment of expression patterns and did not involve quantifiable metrics). The antibodies used for IHC were to hyaluronan-binding protein (HABP) to stain proteoglycans (1:100 dilution; EMD Millipore, 385911) and collagen I (1:100 dilution; MBio, 203002); Hoechst was used to stain nuclei (1:10,000 dilution; Invitrogen). All mouse experiments were performed under protocols approved by the Institutional Animal Care and Use Committee at the Medical University of South Carolina. Prior to cardiac resection, mice were sacrificed in accordance with the Guide for the Care and Use of Laboratory Animals.

Zebrafish experiments. Zebrafish experiments were performed in accordance with approved Institutional Animal Care and Use Committee (IACUC) protocols. TuAB zebrafish strains were reared according to standard techniques. Minimal effective doses of antiens fibronoligo oligonucleotides were injected at the single-cell stage, and morpholino-injected animals were compared to controls injected with nontargeting morpholino. The oligonucleotide sequences are provided in Supplementary Table 6. Embryos were scored for the presence of AV regurgitation at 72 h.p.f. using high-speed videography. Semi-quantitative PCR was used to demonstrate the efficacy of morpholino knockdown (Supplementary Fig. 9). In situ hybridizations for tissue-specific expression of lmcd1, tns1, bmp4 and notch1b were performed as described38. To visualize the localization of the developing cardiac cushions, ftk-EGFP reporter fish were microinjected with morpholinos to tns1 or lmcd1. After manual excision of the heart at 72 h.p.f., the hearts were counterstained with rhodamine-labeled phallolidin and mounted using Vectashield. Confocal micrographs were acquired on a Zeiss 510 LSM microscope with a 20× air lens. Final images represent two-dimensional projections of a z series (ImageJ).

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