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Genetic analysis of rare coding mutations of *CELSR1–3* in congenital heart and neural tube defects in Chinese people

Xiaojin Qiao*†[1], Yahui Liu*†[1], Peiqiang Li*†, Zhongzhong Chen*†, Huili Li‡, Xueyan Yang*†, Richard H. Finnell†§, Zhangmin Yang¶, Ting Zhang‡, Bin Qiao‖, Yufang Zheng*†**†† and Hongyan Wang*,**

*State Key Laboratory of Genetic Engineering, MOE Key Laboratory of Contemporary Anthropology, Fudan University, Shanghai 200438, China
†Collaborative Innovation Centre for Genetics & Development, School of Life Sciences, Fudan University, Shanghai 200438, China
‡Capital Institute of Paediatrics, Beijing 100020, China
§Dell Pediatric Research Institute, Department of Pediatrics, Dell Medical School at the University of Texas, Austin, U.S.A.
¶Department of Biochemistry and Molecular Biology, College of life Sciences, Shaanxi Normal University, Xi'an 710062, China
‖Institute of Cardiovascular Disease, General Hospital of Jinan Military Region, Jinan 250022, China
**Key Lab of Reproduction Regulation of NPFPC in SIPPR, Institute of Reproduction & Development in Obstetrics & Gynaecology Hospital, Fudan University, Shanghai, 200433, China
††Institute of Developmental Biology & Molecular Medicine, Fudan University, Shanghai 200433, China

Abstract

The planar cell polarity (PCP) pathway is critical for proper embryonic development of the neural tube and heart. Mutations in these genes have previously been implicated in the pathogenesis of neural tube defects (NTDs), but not in congenital heart defects (CHDs) in humans. We systematically identified the mutation patterns of *CELSR1–3*, one family of the core PCP genes, in human cohorts composed of 352 individuals with NTDs, 412 with CHDs and matched controls. A total of 72 disease-specific, rare, novel, coding mutations were identified, of which 37 were identified in patients with CHDs and 36 in patients with NTDs. Most of these mutations differed between the two cohorts, because only one novel missense mutation in *CELSR1* (c.2609G>A p.P870L) was identified in both NTD and CHD patients. Both *in vivo* and *in vitro* assays revealed that *CELSR1* P870L is a gain-of-function mutation. It up-regulates not only the PCP pathway, but also canonical WNT signalling in cells, and also induces both NTDs and CHDs in zebrafish embryos. As almost equal numbers of mutations were identified in each cohort, our results provided the first evidence that mutations in *CELSR* genes are as likely to be associated with CHDs as with NTDs, although the specific mutations differ between the two cohorts. Such differences in mutation panels suggested that CELSRs [cadherin, EGF (epidermal growth factor), LAG (laminin A G-type repeat), seven-pass receptors)] might be regulated differently during the development of these two organ systems.

Key words: *CELSR1–3*, congenital heart defects (CHDs), neural tube defects (NTDs), PCP pathway.

INTRODUCTION

Birth defects are a major cause of morbidity and mortality in newborn infants worldwide [1]. Neural tube defects (NTDs) and congenital heart defects (CHDs) are among the most common human structural congenital malformations. NTDs, including craniorachischisis, anencephaly and spina bifida, are severe defects of the brain and spinal cord resulting from a failure of neural tube closure (NTC), and occur with a prevalence of around 1–2 infants per 1000 births [2–4]. CHDs are the most common congenital anomaly in newborn infants with a prevalence of approximately 8–9 per 1000 live births worldwide [5,6]. CHDs are complicated disorders including a large set of structural and functional deficits, the most obvious defects being ventricular septal defects (VSDs) and conotruncal defects [7]. Although NTDs and CHDs are different disorders, they share several genetic and developmental features. First, both classes of defects are severe disorders that occur mostly sporadically [8,9]. Second, both the neural tube

Abbreviations: CHD, congenital heart defect; CELSR, cadherin, EGF, LAG, seven-pass G-type receptor; EGF, epidermal growth factor; ExAC, Exome Aggregation Consortium; LAG, laminin A G-type repeat; MAF, major allele frequency; MDCK, Madin–Darby canine kidney; MO, morpholino-modified oligonucleotide; NTC, neural tube closure; NTD, neural tube defect; NS-CHD, non-syndromic CHD; PCP, planar cell polarity; PFO, patent foramen ovale; pRLCMV, *Renilla*-luciferase plasmid; PTV, protein-truncating variant; S-CHD, syndromic CHD; SHH, sonic hedgehog; SIFT, sorting intolerant from tolerant; SNP, single nucleotide polymorphism; SNV, single nucleotide variant

Correspondence: Hongyan Wang (email wanghy@fudan.edu.cn) and Yufang Zheng (email zhengyf@fudan.edu.cn).

[1] These two authors contributed equally to this work.

and the heart tube arise during the first trimester, resulting in an embryologically critical period that partially overlaps for the development of NTDs and CHDs [7]. Third, folic acid supplementation has been shown to effectively prevent both defects [7]. Last, but not least, many key morphogenetic signalling pathways have been shown to be involved in both birth defects, including: Wnt, planar cell polarity (PCP), sonic hedgehog (SHH) and Notch pathways [3,10,11,12]. Most of the evidence for the involvement of these morphogenetic pathway genes came from animal models with knockout or mutated genes, e.g. a large forward screen using echocardiography on C57BL/6J fetal mice that had undergone chemical mutagenesis identified 34 cilia-related genes contributing to CHDs [13]. However, it is unclear how mutations in these pathways contribute differently to the development of these two congenital defects in human populations.

One of those key morphogenetic pathways, the PCP pathway, is important for the organization of cell sheets in the tissue plane, so it is essential for multiple developmental processes including: cellular orientation, directional migration and convergent extension during NTC and heart morphogenesis [14–16]. The 'core' PCP genes include: *VANGL1*, *VANGL2* (Van Gogh homologues), *CELSR1*, *CELSR2*, *CELSR3* (Starry night homologues), *DVL1*, *DVL2*, *DVL3* (Dishevelled homologues), *PRICKLE1*, *PRICKLE2* (Prickle homologues) and ANKRD6 (Diego homologue), which are highly conserved from invertebrates to vertebrates [15,16]. Genetic modification of PCP genes, including *Celsr1*, *Vangl2*, *Dvl1/2*, *Fz3/6* and *Ptk7*, in mouse models resulted in severe NTDs, such as craniorachischisis, implicating the essential role of core PCP genes in NTC [15,17]. The mouse models also revealed that PCP signalling is critical for heart development, because several PCP gene-knockout mouse models also expressed CHD phenotypes, including *Vangl2*, *Dvl2/3*, *Ptk7*, *Fz1/2*, *Fz2/7* and *Fuz* [18–21]. Those models provide evidence that PCP signalling is involved in establishing the early heart tube, polarizing cardiomyocytes, remodelling the cardiac outflow tract and playing crucial roles in the directed migration of cardiac crest cells [20,22,23].

Largely based on mouse model studies, we hypothesized that mutations in PCP genes could also be associated with human NTDs and CHDs. Our own work and that of others clearly demonstrated that alterations in PCP genes contribute to the aetiology of human NTDs. Mutations in PCP genes, including *VANGL2* [24,25], *SEC24B* [26] and *CELSR1* [27–29], have been identified in patients with NTDs. However, there is no report of an association between PCP genes and an increased risk for CHDs in humans to date [20].

In the present study we focused on one set of the core PCP genes, the *CELSRs* [cadherin, epidermal growth factor (EGF), laminin A G-type repeats (LAG), seven-pass G-type receptors]. *CELSRs* are a special subgroup of adhesion G-protein-coupled receptors that function in contact-mediated homophilic communication. They were first discovered in *Drosophila* sp. as *flamingo (fmi)* for regulating PCP signalling [30], and three homologues named *CELSR1–3* were identified in mammals. For the most part, CELSRs form haemophilic dimers on adjacent cells to mediate PCP signalling, but they also interact with other Wnt receptors such as Frizzled (FZ) and Vang(l)s [31,32].

Recent studies have supported rare and new mutations making a significant contribution to the population prevalence of severe diseases such as NTDs and CHDs, which have an impact on mortality and reproductive fitness [5,33,34], e.g. a large exome-sequencing study of 1213 CHD trios also showed that new protein-damaging mutations in genes, highly expressed in the developing heart and brain, are associated with both CHDs and neural developmental disorders [35]. Another whole-exome-sequencing study on syndromic and non-syndromic CHD (S-CHD and NS-CHD) patients not only strongly supported the finding that new protein-truncating variants (PTVs) in known CHD-associated genes are enriched in S-CHDs, but also showed that rare inherited PTVs from unaffected parents in CHD-associated genes contribute significantly to NS-CHD [36]. For a better understanding of the association between *CELSR1–3* gene variants and the risk of NTDs and CHDs in human cohorts, we sequenced all exons of *CELSR* family genes (*CELSR1*, *CELSR2*, *CELSR3*) for an NTD cohort with 352 cases and 224 matched controls, and a CHD cohort with 412 cases and 213 matched controls. As we do not have biological samples from the parents in our cohorts to identify new mutations in the patients, we focused on those coding mutations that are rare, especially novel mutations not previously reported in 1000 Genomes Project, dbSNP or Exome Aggregation Consortium (ExAC) databases (east Asian). As a result, a total of 72 disease-specific, rare, novel, coding mutations were identified among the three *CELSR* genes in our cohorts, of which 42 are non-synonymous mutations that cause amino acid changes. It is interesting that almost equal numbers of novel coding mutations were identified in the CHD and NTD cohorts, with only one overlapping mutation, *CELSR1* c.2609G>A p.P870L. Our *in vitro* and *in vivo* functional analyses showed that *CELSR1* c.2609G>A p.P870L is a gain-of-function mutation, with the ability to increase not only PCP but also canonical Wnt signalling. Our results provided the first evidence that mutations of *CELSRs* are probably associated with CHDs, but with a different panel of mutations from those in NTDs. Such a difference in mutation panels could suggest that *CELSRs* might be regulated differently or have had different interaction partners during the development of the two organ systems.

MATERIALS AND METHODS

Human cases

Blood samples from 412 CHD patients (mean age 2.9±2.7 years, 55.6% male) were collected from the Cardiovascular Disease Institute of Jinan Military Command between March 2010 and March 2012. Sporadic CHD cases were diagnosed on the basis of echocardiography, with some diagnoses further confirmed surgically. Patients who had clinical features of developmental anomalies were excluded. Patients were also excluded if they had a positive family history of CHD in a first-degree relative, maternal diabetes mellitus, maternal exposure to known teratogens or any therapeutic drugs during gestation. All the CHD cases were classified according to previously described methodology (detailed diagnosis information on the patients is shown in Table 1) [37]. The 213 controls (mean age 7.1±3.7 years, 49.8% male)

Table 1 Demographic characteristics in CHD cases and controls

Variable	Case (%)	Controls (%)
Sequencing group	412	213
Region		Shandong Province
Age: years (mean±S.D.)	2.9±2.7	7.1±3.7
Gender		
Male [(no. (%)]	229 (55.6)	106 (49.8)
Female [(no. (%)]	183 (44.4)	107 (50.2)
CHD classification		
Septation defects [(no. (%)]	136 (33.0)	
Conotruncal defects [(no. (%)]	139 (33.7)	
RVOTO [(no. (%)]	44 (10.7)	
PDA defect [(no. (%)]	17 (4.1)	
LVOTO defects [(no. (%)]	19 (4.6)	
AVSD [(no. (%)]	20 (5.3)	
APVR defects [(no. (%)]	11 (2.7)	
Others [(no. (%)]	26 (6.3)	

APVR, anomalous pulmonary venous return; AVSD, atrioventricular septal defect; LVOTO, left ventricular outflow tract obstruction; PDA, patent ductus arteriosus; RVOTO, right ventricular outflow tract obstruction.
'Others' includes CHDs with other defects such as heterotaxy.

Table 2 Demographic characteristics in NTD cohort

Variable	Case (%)	Controls (%)
Sequencing Group	352	224
Region		
Tianjin	25	
Suzhou	14	
Liaoning	28	
Heilongjiang	15	
Shanxi	270	192
Shanghai		32
Age: weeks/years (mean ± SD)		
Tianjin	3.13 ± 3.4 y	
Jiangsu	N.D.	
Liaoning	26.7 ± 8.1 w	
	3.2 ± 4.5 y	
Heilongjiang	N.D.	
Shanxi	23.4 ± 6.4 w	20.0 ± 3.2 w
Shanghai		18.0 y
Gender		
Male	134(38.1%)	91(40.6%)
Female	181(51.4%)	132(58.9%)
Unknown	37(10.5%)	1(0.5%)
CRS	26 (7.4%)	
EC	73 (20.7%)	
AE	74 (21.0%)	
EX	3 (0.85%)	
SB	255 (72.4%)	
NTD	1 (0.2%)	

CRS, craniorachischisis; **EC**, encephalocele; **AE**, anencephaly; **EX**, exencephaly; **SB**, spina bifida

were ethnically and gender-matched, unrelated healthy volunteers recruited from the same geographical area (Table 1). Controls with any congenital anomalies or cardiac disease were excluded.

NTD samples were collected from either aborted fetuses or children with spina bifida aged <10 years from the 1990s to the 2010s. A total of 352 NTD samples (38.1% male, 51.4% female, 10.5% unknown) were collected from Shanxi Province (270), Liaoning (28), Heilongjiang (15), Suzhou (14) and Tianjin (25) (Table 2). Of the 352 cases 309 were tissue samples from aborted fetuses with severe rostral NTDs, such as craniorachischisis or anencephaly. The remaining 43 cases represented children aged <10 years with spina bifida, from whom blood samples were collected. The 224 controls (40.6% male, 58.9% female, 0.5% unknown) were unrelated healthy volunteers recruited from Shanxi Province (192 tissue samples of aborted fetuses from unrelated healthy volunteers) and Shanghai (32 blood samples from healthy college freshman students) who were ethnically and gender matched (Table 2).

The studies described in the present paper were conducted in accordance with the Declaration of Helsinki. Protocols were reviewed and approved by the Ethics Committee of the School of Life Sciences, Fudan University and local ethics committees before the start of the present study. Written informed consent from the parents or guardians of the children was obtained.

Approximately 2 ml of peripheral blood or 50 μg of tissue samples was collected from each test case. Genomic DNA of each test case was isolated from peripheral blood or tissue samples, using conventional reagents, and quantified using a NanoDrop2000 (Thermo Scientific).

DNA sequencing, genotyping and data analysis

The genomic structures of human *CELSR1–3* genes were determined using NCBI GenBank (NM_014246, NM_001408 and NM_001407). The 5′-UTR, 3′-UTR and coding regions in *CELSR1–3* were detected by next-generation sequencing. Genomic DNA-fragment libraries and target enrichment using a probe specific for *CELSR1–3* were performed using the Agilent SureSelect XT Custom enrichment system with some modifications, which was carried out at GBP Biotechnology. In brief, 1 μg of genomic DNA was subjected to ultrasonic energy fragmentation with the aid of a Covaris S2 instrument. The average size of genomic DNA fragments was approximately 250 bp. Each fragmented genomic DNA library was ligated with an indexed adapter, as previously described [38]. The ligated libraries were gel purified and, in turn, PCR amplified (Phusion, Thermo Scientific). The quality and quantity of the prepared libraries were assessed using an Agilent 2100 Bioanalyzer. Each of the 48 individual libraries was equivalently pooled and then hybridized to RNA library baits, and the targeted sequences were purified and amplified (Herculase II fusion, Stratagene). Sequencing was performed on an Illumina HiSeq2000 DNA sequencer (version 3). The fastq files that were obtained were mapped to a hg19 reference sequence using Burrows–Wheeler Aligner's Smith–Waterman Alignment (BWA-SW) [39], and the resulting SAM files were converted to BAM files, followed by sorting and removal of duplicates using SAMtools [40]. Combined variant call-

ing was conducted on the resulting BAM files using SAMtools pileup, followed by VarScan [41]. Variants [single nucleotide variants (SNVs) and Indels] were annotated with information from Ensembl release 79 using the Variant Effect Predictor [42], based on the hg19/GRCh37 database.

To confirm the genotyping results from next-generation sequencing, 21 randomly selected, missense mutations in 28 CHD cases, and all exons of *CELSR1* within 176 randomly selected NTD samples, were amplified by PCR and investigated using Sanger DNA sequencing. When compared with the next-generation data, the results were 98.04% concordant. The primers used in the PCR and DNA-sequencing reactions are listed in Supplementary Tables 3 and 4.

All confirmed, case-specific, coding, single nucleotide polymorphisms (SNPs) were further annotated as 'known' or 'novel', depending on whether they had been previously reported in dbSNP (version 137) (http://www.ncbi.nlm.nih.gov/projects/SNP/index.html), the 1000 Genomes Project [43] or ExAC (Cambridge, MA: http://exac.broadinstitute.org) [44]. All missense mutations were also evaluated using SIFT (sorting intolerant from tolerant: http://sift.jcvi.org/) [45,46] and PolyPhen-2 (Polymorphism Phenotyping v2: http://genetics.bwh.harvard.edu/pph/) for potentially damaging mutations [47].

Plasmids

Myc-tagged human *CELSR1* was purchased from Origene Technologies (RC211652). We then performed the site-directed mutation *CELSR1* P870L with primers (forward: 5′-GACAACGGCATCCTGCAGAAATCAGAC-3′; reverse: 5′-GTCTGATTTCTGCAGGATGCCGTTGTC-3′). The *CELSR1* cDNA and *CELSR1* P870L cDNA were subcloned into the pCMV6-AC-GFP vector between the SgfI and HindIII restriction sites. The mutation in the *CELSR1* P870L plasmid was validated by DNA sequencing with primers 5′-ACAGGCCTGTC TTTCAGAGC-3′ and 5′-TGTACACGGCCACATTCTCC-3′. The pFR-luciferase reporter construct was obtained from Stratagene. The Topflash-luciferase reporter construct was a gift from Dr Tao P. Zhong at Fudan University.

Zebrafish injection

Studies were undertaken using AB strain zebrafish (*Danio rerio*) under standard conditions at 28 °C. All the *CELSR1* plasmids were diluted in RNA-free water. A mixture (2.3 nl) of 0.3 mM zcelsr1a-MO (MO: morpholino or antisense morpholino-modified oligonucleotides) and 0.3 mM zcelsr1b-MO (antisense to zebrafish *celsr1a* and *celsr1b* start-codon region) were injected into each one- to two-cell stage zebrafish embryo. Zebrafish celsr1 morpholinos were purchased from Gene Tools and the sequences are: zcelsr1a: 5′-ATGGTGTAAAACTCCGCAAACAGGC-3′; zcelsr1b: 5′-ATGGCTCACGCGCATCTCATTGGGA-3′. In the rescue (add-back) study, zcelsr1-MOs were co-injected with either *CELSR1*-WT or *CELSR1*-MUT, or empty vector plasmids into zebrafish embryos at the one- to two-cell stage. From each plasmid 2.3 nl was injected at a concentration of 25 ng/μl, 50 ng/μl or 100 ng/μl, resulting in injection of 57.5 pg, 115 pg or 230 pg at each concentration. In the over-expression study, each plasmid was individually injected into each one- to two-cell zebrafish embryo at the same dosage. Then 48 h post-injection, photos were taken of the harvested embryos and their morphology was grossly analysed. All statistical tests were two tailed with $P < 0.05$ as the significance level.

Cell culture, transfection and dual-luciferase reporter assay

Wnt3a mouse fibroblast cell line (ATCC CRL-2647) was kindly provided by Dr Xiliang Zha at Fudan University. MDCK (Madin–Darby canine kidney) cells were seeded in 24-well cell culture plates and incubated until they reached 80% confluence. The cells from each well were transfected with 300 ng of Firefly-luciferase reporter (pFR or Topflash), 300 ng of *CELSR1*-WT/*CELSR1*-MUT/empty vector plasmids and 10 ng of *Renilla*-luciferase plasmid (pRLCMV) as an internal control, using Lipofectamine 3000 (Invitrogen). The transfected cells were cultured in high-glucose Dulbecco's modified Eagle medium (Gibco) with 5% FBS. For the pFR-luciferase assay, the cells were lysed and assayed for luciferase activity 36 h post-transfection. For the Topflash-luciferase assay, 24 h after transfection the cells were treated with either Wnt3a conditional medium or control medium for another 48 h, then they were lysed and assayed for luciferase activity. Both *Renilla*- and Firefly-luciferase activities were analysed using the dual-luciferase reporter assay system (Promega), as described previously [48]. Luciferase activity was corrected for transfection efficiency (pFR/pRLCMV or Topflash/pRLCMV) and extraction yield (via total protein assay). Four independent repeats were performed and all statistical tests were two tailed with $P < 0.05$ as the significance level.

RESULTS

Identification of *CELSR1–3* variants in two cohorts

A Chinese CHD and a Chinese NTD cohort were used in this study. Due to temporal and geographical differences, each cohort contained its own matched control group. The detailed information on our cohorts is provided in Tables 1 and 2 for the CHD and NTD cohorts, respectively. The 5′-UTR, 3′-UTR and coding regions of *CELSR1–3* genes were sequenced in all samples and controls. In our cohorts, there are more male patients affected in the CHD cohort and more female patients in the NTD cohort (see Tables 1 and 2). Such significant gender-based differences in the prevalence of the malformations was not unexpected, because it has been observed in several previous reports in different populations [49–51], suggesting that the gender effect in these two birth defects is not affected by population differences.

As both NTDs and CHDs are severe disorders that have an impact on mortality and reproductive fitness, a very large negative selection eliminates mutations from the human population. Therefore, it is most likely that rare and new mutations make a significant contribution to the population prevalence of these defects, especially for sporadic cases [5,33,34]. Based on this hypothesis, we filtered out the SNVs that have a major allele frequency (MAF) <1% as rare SNVs. There is a total of 184 and 181 rare SNVs in the *CELSR1–3* genes identified in our CHD

and NTD cohorts, respectively (Figure 1A). Within them, 103 and 95 are case-specific rare SNVs in the CHD and NTD cohorts, respectively (Figure 1A). Within these case-specific rare SNVs, there are 79 and 87 case-specific rare, coding mutations in CHD and NTD cohorts, respectively (Figure 1A).

We further filtered out all the novel coding SNVs that did not exist in the dbSNP137, 1000 Genomes or ExAC (eastern Asian only) databases, leaving 57 and 59 variants that were identified in the CHD and NTD cohorts, respectively. Of these, 37 in CHDs and 36 in NTDs are case-specific novel, coding variants (Figure 1A). Within these case-specific novel, coding variants, 21 missense mutations and 16 synonymous mutations were identified in the CHD cohort (Figures 1B and 1C, Table 3 and see Supplementary Table S1), although 22 missense mutations and 14 synonymous mutations were identified in the NTD cohort (Figures 1B and 1C, Table 4 and see Supplementary Table S2). Through all the filtering processes, there are comparable numbers of SNVs identified in the *CELSR1–3* genes in each category between our CHD and NTD cohorts. It is interesting that most of those novel mutations differ between CHD and NTD cohorts. Only one case-specific novel SNV was shared by the two cohorts, and this is a missense mutation in *CELSR1* (*CELSR1* c.2609G>A p.P870L) (Figure 1B).

CELSR1 P870L is a gain-of-function mutation in zebrafish

The only case-specific novel, coding SNV identified in both diseases is *CELSR1* c.2609G>A p.P870L, which is located in the sixth cadherin tandem repeat domain. It was identified in a 17-week-old female fetus, with both anencephaly and myelomeningocele, and a 3-year-old CHD patient who presented with a patent foramen ovale (PFO) and a VSD (Tables 3 and 4).

Given the fact that this *CELSR1* mutation was the only one detected in both cohorts, we thought that this specific variant deserved further investigation. To investigate the function of *CELSR1* p.P870L (*CELSR1*-MUT), we initially explored its potential in the *in vivo* pathogenetic effect of *CELSR1*-MUT in zebrafish. It has previously been shown that *celsr1a* and *celsr1b* are highly expressed during neural tube convergence/extension stages in zebrafish [52–54]. Antisense morpholino-modified oligonucleotides (MOs) for both zebrafish *celsr1* isoforms (*zcelsr1a* and *zcelsr1b*) were injected together into one- to two-cell zebrafish embryos. Then, 48 h post-injection, both NTD and CHD phenotypes were observed in these *zcelsr1* knock-down fish (Figures 2A and 2B). Of the MO-injected fish, 70.6% showed shortening of the anterior–posterior axis, which is probably due to convergence extension defects of the neural tube (Figures 2A and 2C). Approximately 80% of the embryos exhibiting an NTD phenotype also had an enlarged pericardium (a CHD phenotype), which accounted for 56.6% of the total MO-injected fish (Figures 2B and 2D).

We also tested whether human CELSR1 protein could rescue these defects when added back to those knock-down embryos. We co-injected embryos with a mixture of antisense MOs and with either human *CELSR1*-WT or *CELSR1*-MUT plasmid. The response frequency of both NTDs and CHDs caused by MO knock-down was significantly reduced by both *CELSR1*-WT and -MUT in a dosage-dependent manner (Figures 2C and 2D). It is interesting that *CELSR1*-MUT demonstrated a significantly more robust ability to rescue the normal phenotype than *CELSR*-WT for both phenotypes at the same concentrations (Figures 2C and 2D). For example, 115 pg of *CELSR1*-MUT co-injected with MOs could reduce the NTD prevalence from 70.6% to 37.1%, whereas *CELSR1*-WT co-injected with MOs could reduce the NTD response frequency only from 70.6% to 53.9% (Figure 2C). Similar rescue effects were observed for the pericardial defects, because a co-injection of 115 pg of *CELSR1*-MUT could reduce the CHD prevalence from 56.6% to 23.1%, whereas a *CELSR1*-WT co-injection could reduce the CHD response frequency only from 56.6% to 33.3% (Figure 2D). These results suggest that *CELSR1*-MUT is a gain-of-function mutation *in vivo*.

A similar gain-of-function effect was also observed in an over-expression experiment when *CELSR1*-WT and -MUT were injected alone into zebrafish embryos. A significantly higher response frequency of NTDs and CHDs was observed in zebrafish embryos injected with *CELSR1*-MUT compared with those injected with *CELSR1*-WT at the same concentration (79.7% vs 55.5% NTDs and 68.4% vs 46.6% CHDs) (Figures 2E and 2F). Our results showed that hyperactivity of *CELSR1*, by either mutation or over-expression, could lead to both NTDs and CHDs in zebrafish.

Luciferase reporter assay showed that *CELSR1* P870L increases not only PCP but also Wnt signalling

To explore the functional effect of the *CELSR1* P870L mutation, we examined the effect of *CELSR1* on the PCP pathway using a JNK-responsive, reporter pFR-luciferase co-transfected with either *CELSR1*-WT or *CELSR1*-MUT into MDCK cells. The results showed that, although *CELSR1*-WT activated the PCP pathway in MDCK cells, the *CELSR1*-MUT actually resulted in significantly higher activation of the PCP pathway (Figure 3A).

As *CELSR1* interacts not only with PCP receptors but also with Wnt receptors, we additionally examined the effect of *CELSR1*-MUT on canonical Wnt signalling using the Topflash-luciferase reporter, which is a β-catenin-responsive reporter. When *CELSR1*-WT or *CELSR1*-MUT was over-expressed in MDCK cells, both *CELSR1*-WT and *CELSR1*-MUT could activate the Wnt pathway under Wnt3a stimulation (Figure 3B). It is interesting that *CELSR1*-MUT had a much stronger effect in promoting canonical Wnt signalling, because this mutant protein could significantly activate the Wnt pathway even without the Wnt3a ligand (Figure 3B). Consistent with the *in vivo* data, these *in vitro* data strongly suggested that *CELSR1*-MUT is a gain-of-function mutation.

DISCUSSION

Previous studies have shown that *CELSR1* is more strongly associated with NTDs than *CELSR2* and *CELSR3,* given that *Celsr2* and *Celsr3* mutant mice lacked an NTD phenotype apart from an occasional hydrocephalus [31,55]. There are several

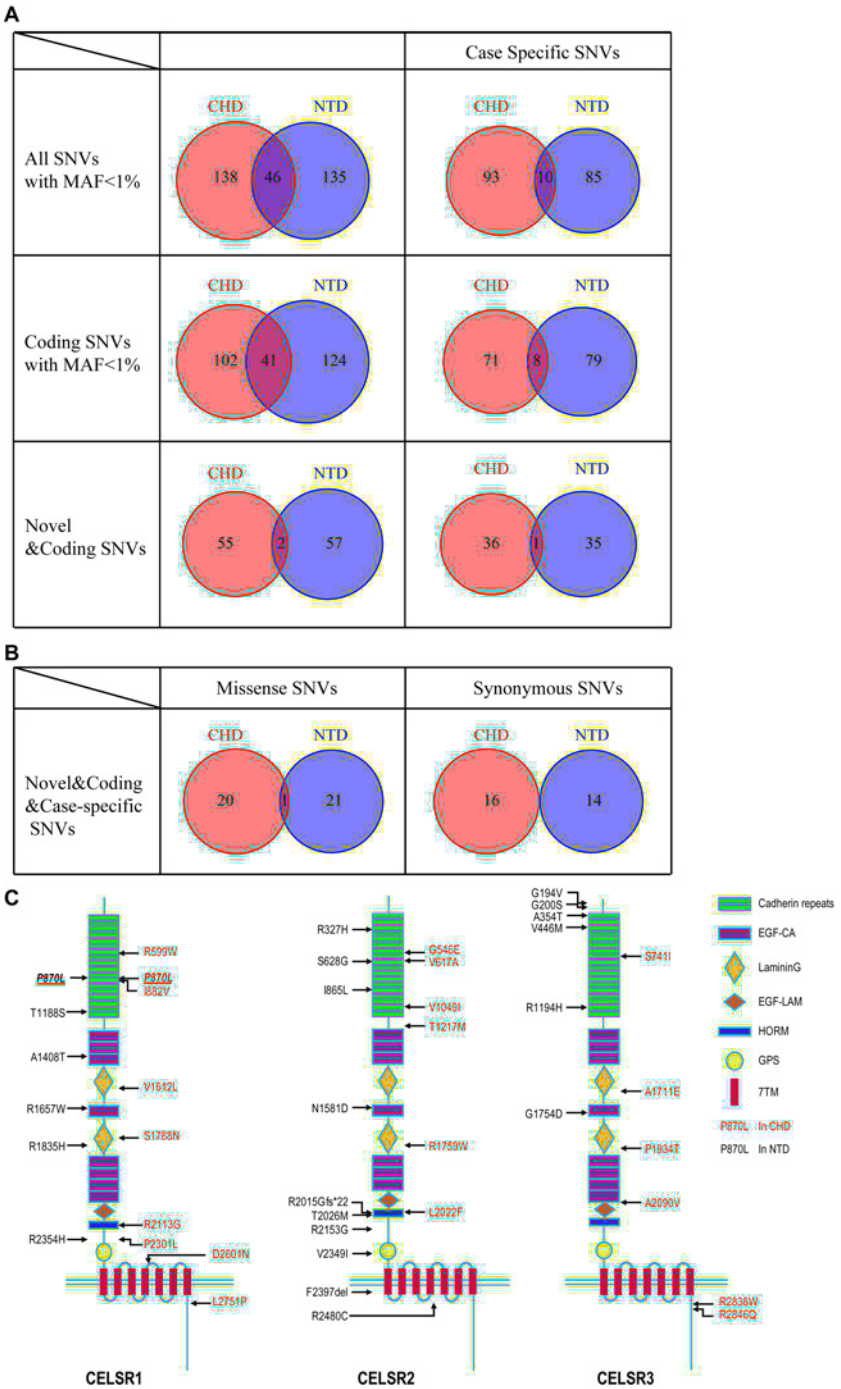

Figure 1 **Comparison of mutations identified in NTD and CHD cohorts**

(**A**) Four variant categories were identified for detailed analyses. These are SNVs with MAF <1%, novel SNVs, coding SNVs with MAF <1% and novel coding SNVs. The numbers of SNVs for each category that is identified in CHD or NTD cohorts are listed in the left column. Within each category, the numbers of case-specific SNVs are listed on the right column. (**B**) The non-synonymous and synonymous SNVs within novel/coding/case-specific SNVs in two cohorts. (**C**) Illustration of CELSR1–3 protein structure with all novel/case-specific/(missense+frameshift) SNVs identified in NTD and CHD cohorts. Missense SNVs identified in NTD or CHD patients are labelled in black and red letters, respectively.

Table 3 **Novel non-synonymous mutations of *CELSR1-3* detected in CHDs but not in controls**
[a]MAF from ExAC database. [b]Shared variants between CHD and NTD cohorts.

Gene	Nucleotide change	Amino acid change	Case no.	Domain	SIFT	PolyPhen	Sex	Phenotype	MAF in CHD	MAF in east Asian[a]
CELSR1	c.1795G>A	p.Arg599Trp	1	Cadherin_repeat	0.01	0.826	M	VSD, ASD, PDA, PFO	0.000084	Not reported
CELSR1	**c.2609G>A**	**p.Pro870Leu[b]**	**1**	**Cadherin_repeat**	**0**	**1**	**M**	**PMVSD, PFO**	**0.00089**	0
CELSR1	c.2644T>C	p.Ile882Val	2	Cadherin_repeat	1	0.032	F	ASD	0.00161	Not reported
							F	ASD, PDA		
CELSR1	c.4834C>A	p.Val1612Leu	1	Laminin G	0.01	0.959	M	DORV, PS, MPVSD	0.000828	Not reported
CELSR1	c.5363C>T	p.Ser1788Asn	1	Laminin G	0.43	0.02	F	TOF	0.000816	Not reported
CELSR1	c.6337T>C	p.Arg2113Gly	1	HormR	0.06	0	F	VSD, PFO, PH	0.000808	Not reported
CELSR1	c.6902G>A	p.Pro2301Leu	2	/	0.38	0.01	F	VSD, ASD	0.00289	0
							M	TOF, ASD, PDA		
CELSR1	c.7801C>T	p.Asp2601Asn	1	7TM	0.33	1	F	TOF, PA	0.000821	0
CELSR1	c.8252A>G	p.Leu2751Pro	3	Cytotail	0.06	0.967	M	TECD	0.003632	Not reported
							F	VSD, ASD		
							F	DORV, PS		
CELSR2	c.1637G>A	p.Gly546Glu	1	Cadherin_repeat	0.25	0.003	F	PDA, MI	0.000805	Not reported
CELSR2	c.1850T>C	p.Val617Ala	1	Cadherin_repeat	0.19	0.003	M	PMVSD, PFO	0.000808	Not reported
CELSR2	c.3145G>A	p.Val1049Ile	1	Cadherin_repeat	0.8	0.008	M	TECD	0.000806	Not reported
CELSR2	c.3650C>T	p.Thr1217Met	2	/	0.08	0.073	F	AS	0.001658	0
							F	TA, SV, PH		
CELSR2	c.5275C>T	p.Arg1759Trp	1	Laminin G	0.01	1	F	PS, ASD	0.000885	0
CELSR2	c.6064C>T	p.Leu2022Phe	2	HormR	0.02	1	M	Ebstein's anomaly, TI	0.001613	0
							M	VSD, SASD, PDA		
CELSR3	c.2222C>A	p.Ser741Ile	1	Cadherin_repeat	0.05	0.998	M	TOF	0.000809	Not reported
CELSR3	c.5132G>T	p.Ala1711Glu	1	Laminin G	0.11	0.991	M	VSD	0.000821	Not reported
CELSR3	c.5800G>T	p.Pro1934Thr	1	Laminin G	0.31	0.23	M	TOF	0.001385	Not reported
CELSR3	c.6269G>A	p.Ala2090Val	1	EGF_Lam	0.03	0.627	F	TAPVC, ASD	0.000911	Not reported
CELSR3	c.8512G>A	p.Arg2838Trp	1	Cytotail	0.02	0.999	F	VSD, ASD, PDA, PFO	0.00098	Not reported
CELSR3	c.8537C>T	p.Arg2846Gln	1	Cytotail	0.13	0.453	M	PMVSD		0

AS, aortic stenosis; ASD, atrial septal defect; DORV, double outlet right ventricle; MI, mitral insufficiency; PA, pulmonary atresia; PDA, patent ductus arteriosus; PMVSD, perimembranous VSD; PAPVD, partial anomalous pulmonary venous drainage; PECD, partial endocardial defect; PH, pulmonary hypertension; PS, pulmonary stenosis; MPVSD, muscular portion VSD; SASD, secundum atrial septal defect; SV, single ventricle; TA, tricuspid atresia; TAPVC, total anomalous pulmonary venous connection; TECD, transitional endocardial defect; TI, tricuspid incompetence; TOF, tetralogy of Fallot.
The Bold is the mutant identified in both cohorts.

Table 4 Novel non-synonymous mutations of *CELSR1–3* detected in NTDs but not in controls

Gene	Nucleotide change	Amino acid change	Case no.	Domain	SIFT	PolyPhen	Sex	Phenotype	MAF in NTDs	MAF in east Asian[a]
CELSR1	**c.2609G>A**	**p.Pro870Leu[b]**	**1**	**Cadherin_repeat**	**0**	**1**	**F**	**CRS**	**0.00089**	**0**
CELSR1	c.3562T>A	p.Thr1188Ser	1	Cadherin_repeat	0.41	0.036	M	SB	0.00098	Not reported
CELSR1	c.4222C>T	p.Ala1408Thr	1	EGF_CA	0.65	0.101	M	SB	0.00134	Not reported
CELSR1	c.4969G>A	p.Arg1657Trp	1	EGF_CA	0.09	0.002	ND	SB	0.00019	0
CELSR1	c.5504C>T	p.Arg1835His	1	/	0.34	0.002	F	AE, SB	0.00092	0
CELSR2	c.980G>A	p.Arg327His	1	Cadherin_repeat	0.31	0.057	M	SB	0.00089	Not reported
CELSR2	c.1882A>G	p.Ser628Gly	1	Cadherin_repeat	0.32	0.999	F	AE, SB	0.00089	Not reported
CELSR2	c.2593A>C	p.Ile865Leu	1	Cadherin_repeat	0.62	0.012	M	AE, SB	0.00090	Not reported
CELSR2	c.4741A>G	p.Asn1581Asp	1	EGF_CA	0.18	0.01	F	SB	0.0009	Not reported
CELSR2	c.6077C>T	p.Thr2026Met	1	HormR	0	1	M	SB	0.0009	0
CELSR2	c.6457C>G	p.Arg2153Gly	1	GAIN	0.12	0.981	M	EC	0.00095	Not reported
CELSR2	c.7045G>A	p.Val2349Ile	1	GPS	0.39	0.094	M	AE, SB	0.00090	0
CELSR2	c.7438C>T	p.Arg2480Cys	1	7TM	0	1	M	SB	0.0009	Not reported
CELSR2	c.6043_6044	p.Arg2015Gly fs*22	1	HormR	–	–	F	CRS	0.0009	Not reported
CELSR2	c.7182_7184	p.Phe2397Leu fs*584	1	7TM	–	–	M	SB	0.0009	Not reported
CELSR3	c.581C>A	p.Gl**y19**4Val	1	Signal peptide	0.02	0.002	F	AE, SB	0.0009	Not reported
CELSR3	c.598C>T	p.Gly200Ser	1	/	0.66	0	M	SB	0.0009	0
CELSR3	c.1060C>T	p.Ala354Thr	1	Cadherin_repeat	0.57	0.003	F	SB	0.0013	Not reported
CELSR3	c.1336C>T	p.Val446Met	1	Cadherin_repeat	0.05	0.786	M	CRS	0.0036	Not reported
CELSR3	c.5261C>T	p.Gly1754Asp	1	EGF_CA	0	1	M	CRS	0.0009	Not reported
CELSR3	c.8587C>T	p.Ala2863Thr	1	Cytotail	0.43	0.555	N.D.	SB	0.00092	0

AE, anencephaly, CRS, craniorachischisis; EX, exencephaly; EC, encephalocele; HD, hydranencephaly; ND, not determined; SB, spina bifida.
[a]MAF from ExAC database.
[b]Shared variants between CHD and NTD cohorts.
The Bold is the mutant identified in both cohorts.

previous reports linking *CELSR1* non-synonymous mutations to human NTDs [27–29]. Although one previous study reported that rs646776 near *CELSR2* is associated with early onset myocardial infarction [56], no association between mutations in *CELSR1–3* and CHDs has been reported so far. In the present study, we evaluated *CELSR1–3* mutations in two separate CHD and NTD cohorts, with comparable numbers of cases and controls. We identified almost equal numbers of variants between the two cohorts throughout all the filtration processes involving both rare SNVs and novel coding SNVs. In the end, a total of 72 case-specific novel mutations in *CELSR1–3* were identified (37 in CHDs and 36 in NTDs). These results indicate that *CELSR1–3* probably make comparable contributions to the aetiology of NTDs and CHDs. Therefore, our data provide evidence that mutations in *CELSRs* may be associated with increased risk not only for NTDs but also for CHDs in humans. To our knowledge, this is the first report of *CELSR* mutations associated with human CHDs.

Most of the mutations that we identified in the present study differ between the two malformation cohorts. It is possible that factors, including differences in location and age groups, may contribute to the observed differences in distinct *CELSR* mutations. Alternatively, different selective pressures were present for the two developmental systems, and *CELSRs* might interact with different molecules during the development of these individual organ systems in humans. Future study of the molecular control of *CELSR* gene functions and whole-genome

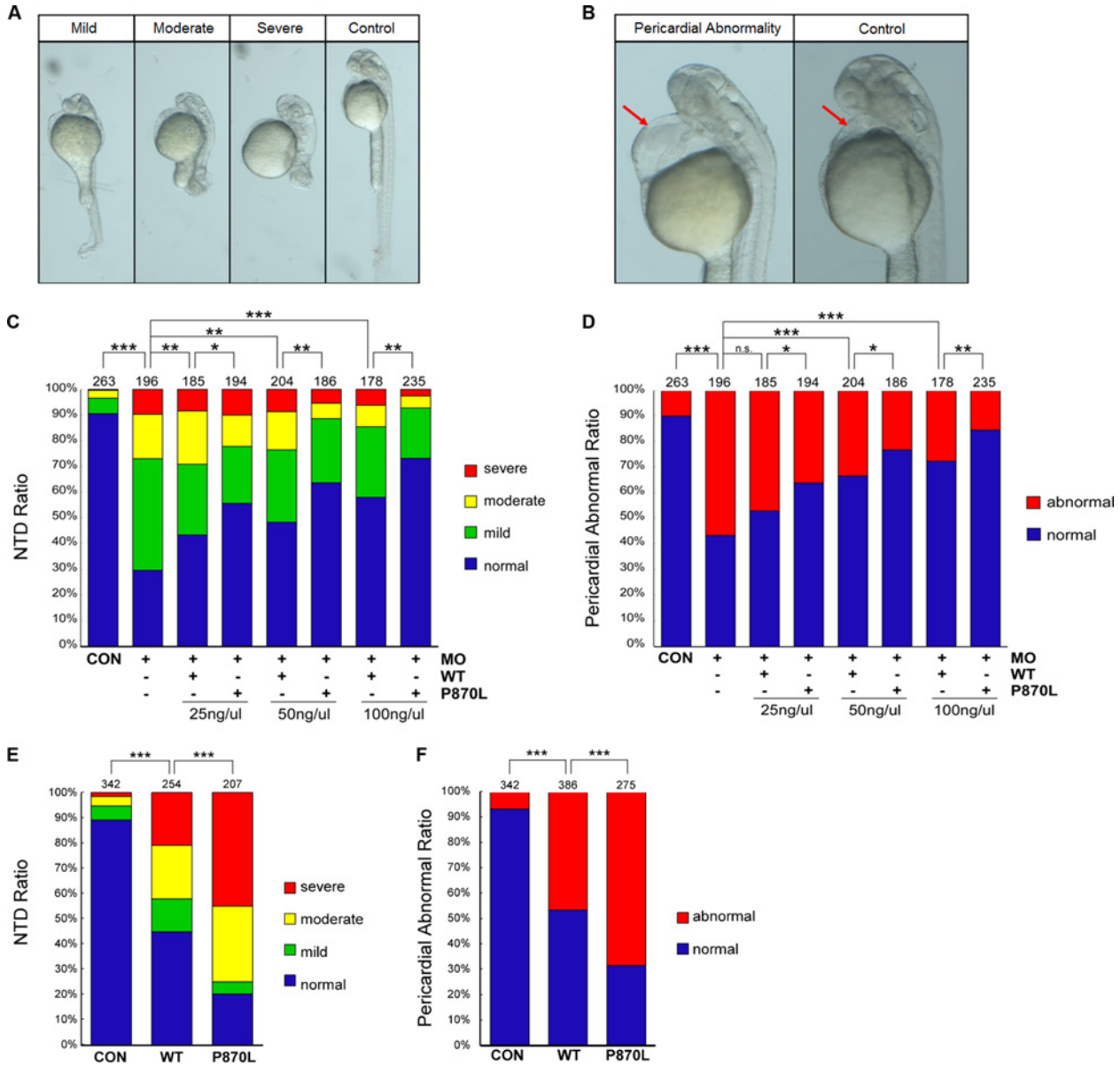

Figure 2 **Human CELSR1-MUT (p.P870L) caused severe NTD and CHD phenotypes in zebrafish**

Two types of abnormalities, (**A**) NTDs and (**B**) pericardial abnormalities, were observed in zebrafish embryos after MO (0.3 mM *zcelsr1a*-MO and 0.3 mM *zcelsr1b*-MO) injection. The severity of the NTD was determined based on the length and curve of the neural tube, as shown in (A). Both *CELSR1*-WT and *CELSR1*-MUT could rescue the NTDs and pericardial defects in zebrafish embryos when co-injected with antisense MOs. Approximately 2.3 nl of 25 ng/μl, 50 ng/μl and 100 ng/μl of each of the *CELSR1* plasmids was used. (**C**, **D**) The response frequencies of NTDs and pericardial defects. Over-expression of *CELSR1*-WT and -MUT (p.P870L) alone also induced (**E**) NTDs and (**F**) pericardial abnormalities. Plasmids (115 pg) were injected into each zebrafish embryo. The number above each bar is the total number of embryos examined under each experimental condition. *P* was calculated by χ^2 analysis. $***P < 0.001$, $**P < 0.01$, $*P < 0.05$. Red arrows (**B**) indicate the paricardial cavity of zebrafish embryoes.

sequencing studies, with larger sample sizes and parent samples, will certainly provide more information about the relationship between mutations in the *CELSR* genes and the risk for NTDs and CHDs in humans.

The single novel *CELSR1* mutation (c.2609G>A p.P870L) observed in both NTD and CHD patients led to both defects in the zebrafish model. Therefore, it is very likely that the gain-of-function mutation *CELSR1* P870L also affects both neural tube and heart development in human embryos. The different phenotypes in the two patients again suggested that molecular regulation of *CELSR1* gene function may differ in humans. There could have been different genetic, epigenetic or environmental factors that interacted with the same *CELSR1* during the development of these two early embryonic systems in humans. *CELSR1* P870L mutation leads to hyperactivity of *CELSR1*. The interaction of genetic elements or environmental factors with this hyperactive

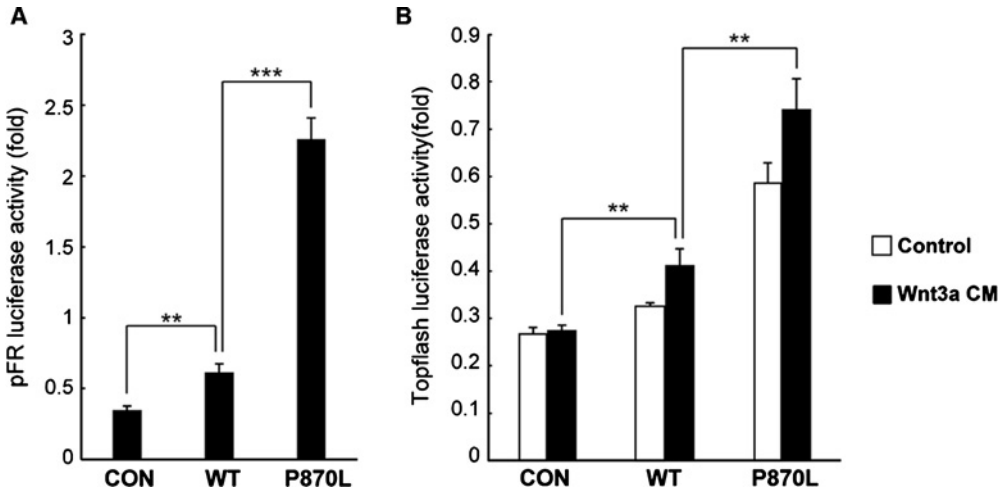

Figure 3 CELSR1-MUT (p.P870L) could increase both PCP and WNT signalling

CELSR-WT, CELSR-MUT and an empty vector (as control) were co-transfected with either (**A**) PCP signalling reporter, JNK-responsive, pFR-luciferase reporter or (**B**) Wnt signalling reporter, β-catenin-responsive, Topflash-luciferase reporter into MDCK cells, respectively. After 48 h, cells were collected and lysed for luciferase assay. In (B) the Wnt signalling assay, the cells were treated with either control medium or Wnt3a conditional medium 36 h after transfection and incubated for another 12 h before harvesting the cells for a luciferase assay. $***P < 0.001$, $**P < 0.01$,$*P < 0.05$.

CELSR1 may determine the outcome. An interesting observation is that *CELSR1* P870L not only increased PCP signalling, but also induced canonical Wnt signalling. This has not been reported before because previous studies have shown only that *CELSR1* acts homophilically to transmit intercellular PCP signals [31,57]. However, as *CELSR1* interacts not only with other core PCP proteins such as VANGL2 but also with the Wnt receptor FRIZZLED [32], it is possible that *CELSR1* P870L could increase its interaction with FRIZZLED to activate a canonical Wnt pathway even without the ligand Wnt3a. Therefore, such cross-talk with other signalling pathways might explain such a 'syndromic' effect of this mutant. Other variants in the PCP pathway that compromise cross-talk with canonical Wnt signalling or other morphogenetic pathways might be involved in determining the risk for NTDs and CHDs in humans. It is interesting that a similar phenomenon was observed in a mouse model of another PCP gene, the PCP effector *Fuz*, which is also involved in cilia, and the SHH pathway, in which the knockout mice presented with both NTD and CHD phenotypes [18]. It is highly likely that FUZ plays an important role in the aetiology of human NTDs and CHDs. Besides *Fuz*, it is possible that those PCP mutants, such as *Vangl2* and *Ptk7*, that had both NTD and CHD phenotypes in knockout mice models [18–20] would be involved in the aetiology of both NTDs and CHDs in humans. Future investigations should focus on mutations that can compromise cross-talk of different developmental pathways, which could provide some insight into these severe birth defects.

In conclusion, we systematically identified a set of rare novel mutations in *CELSR1–3* genes in human CHD and NTD patients that have the potential to be used diagnostically. Our data support our hypothesis that core PCP gene *CELSR1–3* mutations are related to both NTDs and CHDs in humans. The shared mutation, *CELSR1* P870L, has the potential to help us have a better understanding of the developmental overlap between NTDs and CHDs. Our data suggest that mutations in PCP genes, especially hyperactive mutations such as *CELSR1* P870L, are likely to be associated with both NTDs and CHDs in humans. Further understanding of the regulations and cross-talk between PCP and other signalling pathways, such as the canonical Wnt pathway, during early embryonic development, is critical for identifying how best to prevent these preventable birth defects.

CLINICAL PERSPECTIVES

- Our study provided first evidence that mutations in *CELSR* genes are as likely to be associated with CHDs as with NTDs. Our results accentuated the importance of genetic variants in PCP genes in the aetiology of both NTDs and CHDs.
- Distinct mutation patterns between two cohorts suggest that different regulations/factors contribute to different phenotypes.
- The novel missense mutation of CELSR1 (c.2609G>A p.P870L), which was identified in both NTD and CHD patients, is a gain-of-function mutation *in vivo* and *in vitro*. It could cause both NTD and CHD in zebrafish embryos and up-regulate not only PCP but also canonical WNT signalling in cells. Such gain-of-function mutation suggested that hyperactive CELSR1 could disrupt the development of both neural tube and heart.
- Mutations compromise crosstalk of different developmental pathways will provide some insight on those severe birth defects.

AUTHOR CONTRIBUTION

H. Wang and Y. Zheng designed the study and Y. Zheng, H. Wang and R. Finnell prepared the manuscript. X. Qiao and Y. Liu

performed lab experiments. X. Qiao, P. Li, H. Li, B. Qiao, T. Zhang and X. Yang participated in the recruitment of patients and sequencing confirmation. P. Li, H. Li, X. Yang and Z. Chen performed the statistical analysis. All authors reviewed the manuscript and contributed to its scientific content. X. Qiao and Y. Liu contributed equally to this paper.

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