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SHORT REPORT

Functional evidence of mTORβ splice variant involvement in the pathogenesis of congenital heart defects

Mattia Gentile[1] | Carlotta Ranieri[2] | Daria C. Loconte[2] | Emanuela Ponzi[1] | Romina Ficarella[1] | Paolo Volpe[3] | Gabriele Scalzo[4] | Martina Lepore Signorile[5] | Valentina Grossi[5] | Angela Cordella[6] | Giovanna Maria Ventola[6] | Francesco C. Susca[1] | Antonella Turchiano[2] | Cristiano Simone[2,5] | Nicoletta Resta[2]

[1]Department of Human Reproductive Medicine, Medical Genetics Unit, ASL Bari, Bari, Italy

[2]Department of Biomedical Sciences and Human Oncology (DIMO), Medical Genetics, University of Bari "Aldo Moro", Bari, Italy

[3]Department of Human Reproductive Medicine, Fetal Medicine Unit, ASL Bari, Bari, Italy

[4]Department of Pediatric Sciences, Pediatric Cardiac Surgery Unit, Giovanni XXIII Pediatric Hospital, Bari, Italy

[5]Medical Genetics, National Institute of Gastroenterology "S. de Bellis" Research Hospital, Castellana Grotte, Bari, Italy

[6]Genomix4Life SRL, Via S. Allende 84081, Baronissi, Italy

Correspondence
Mattia Gentile, Department of Human Reproductive Medicine, Medical Genetics Unit, ASL Bari, via Di Venere s.n. 70131 Bari, Italy.
Email: mattia.gentile@asl.bari.it

Nicoletta Resta, Department of Biomedical Sciences and Human Oncology (DIMO), Medical Genetics, University of Bari "Aldo Moro," Policlinico di Bari, Piazza G. Cesare, 11 Bari, Italy.
Email: nicoletta.resta@uniba.it

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Universita degli Studi di Bari Aldo Moro

Abstract

mTOR dysregulation has been described in pathological conditions, such as cardiovascular and overgrowth disorders. Here we report on the first case of a patient with a complex congenital heart disease and an interstitial duplication in the short arm of chromosome 1, encompassing part of the mTOR gene. Our results suggest that an intragenic mTOR microduplication might play a role in the pathogenesis of non-syndromic congenital heart defects (CHDs) due to an upregulation of mTOR/Rictor and consequently an increased phosphorylation of PI3K/AKT and MEK/ERK signaling pathways in patient-derived amniocytes. This is the first report which shows a causative role of intragenic mTOR microduplication in the etiology of an isolated complex CHD.

KEYWORDS
CNV, congenital heart defects, human malformations, mTOR

1 | INTRODUCTION

mTOR is a serine/threonine protein kinase forming the catalytic subunit of two distinct enzymatic complexes: mTOR Complex 1 (mTORC1) and 2 (mTORC2). In recent years, extensive research has established that mTOR signaling plays a central role in many fundamental cellular processes such as cell proliferation, survival, metabolism and autophagy.[1]

The involvement of the mTOR pathway in cardiovascular physiology as well as the role its dysregulation plays in cardiovascular pathology have been experimentally demonstrated in several animal models. Studies conducted on mouse models have shown that modulation by mTORC1 and mTORC2 signaling in the heart is fundamental to preserve cardiovascular integrity and function in both prenatal and postnatal stages, underlining the importance of the mTOR pathway in the development of the cardiovascular system in the embryo.[2,3] The evidence of embryonic lethality and marked cardiovascular anomalies in Rictor−/− mice substantiate the central role of mTORC2.[4]

Mattia Gentile and Carlotta Ranieri contributed equally to this study.

So far, there is no evidence linking mTOR constitutional mutations or rearrangements to the etiology of congenital heart defects (CHDs). This is the first report which shows a causative role of an intragenic mTOR microduplication in the etiology of coarctation of the aorta and aortic arch hypoplasia.

2 | PATIENTS AND METHODS

2.1 | Clinical report

The pregnancy was uneventful for up to 21 + 6 weeks, when fetal echocardiography showed prevalence of the right cardiac sections compared to the left ones and prevalence of the pulmonary artery compared to the ascending aorta. The ductal arch appeared significantly larger than the aortic arch. Such evidence raised the suspicion of aortic coarctation.

At 22 + 5 weeks, the pregnant woman underwent amniocentesis. The karyotype was normal (46,XX), while array-CGH analysis revealed an interstitial duplication in the short arm of chromosome 1 (1p36.22), encompassing part of the mTOR gene. Segregation analysis established that the variant was de novo. After genetic and fetal medicine counseling, the parents opted to continue the pregnancy.

The neonate was a female, born at 37 + 5 weeks by vaginal delivery, with a weight of 2640 g (25–50th percentile); the APGAR score was 10/10. At 8 days of life, her weight was 2700 g (3–10th percentile), and length was 47 cm (3–10th percentile).

Postnatal echocardiography confirmed the prenatal findings, showing coarctation of the aortic isthmus, hypoplasia of the transverse part of the aortic arch, and subaortic interventricular defect. At 10 days of life, the baby underwent aortic arch repair and ventricular septal defect closure under cardiopulmonary bypass with anterograde cerebral perfusion. At 1 month of life, she presented left ventricular pseudoaneurysm (LV-PSA), a rare complication of the cardiac surgical intervention, and she was transferred to the Pediatric Cardiology Unit to undergo surgical repair. At 2 months of life, she was returned to the NICU. During hospitalization, her growth was regular with a weight of 3510 g, a length of 56 cm and a HC of 37 cm. Her auditory brainstem response was bilaterally normal.

At the last examination (10 months), her height was 67.5 cm (10–25th centile), weight 7200 g (< 5th centile), and HC 43.5 cm (10–25th centile). Her psychomotor development was normal: she was able to stand with support and to say her first short words (mom, dad). We noticed a normal cranium, thin and arched eyebrows, short palpebral fissures with epicanthal folds, smooth philtrum, wide nasal bridge, full cheeks, thin upper and lower lips, and small, but regular earlobes. No hyperpigmented (café-au-lait) spots and/or asymmetry of the limbs were present. The parents refused to perform further analyses (e.g., magnetic resonance imaging) in the absence of specific clinical indications.

2.2 | Genetic studies and immunoblotting analysis

Array CGH analysis was performed using the CytoSure™ ISCA v2 kit, composed of 180-mer oligonucleotides spaced at about 25 Kb density across the genome, and data were analyzed using CytoSureTM Interpret Software (Oxford Gene Technology).

Immunoblotting analyses were performed according to the instructions of Cell Signaling Technology (Beverly, USA).

Next generation sequencing experiments were performed by Genomix4life S.R.L. (Baronissi, Salerno, Italy), using an Illumina NextSeq 500 System (Illumina).

Detailed protocols are in the Supplementary File.

3 | RESULTS

Array CGH analysis on cells derived from amniotic fluid identified a de novo interstitial duplication in the short arm of the chromosome 1 arr[hg19] 1p36.22(11258825–11 318 811_36088096)x3, sized 59.99 Kb and encompassing part of the mTOR gene (from exon 2 to exon 28 of the genomic sequence) (Figure 1(A)).The type of rearrangement and its de novo origin, associated with the ultrasound features, induced us to further explore the underlying pathogenic mechanism. There were no other pathogenic/likely pathogenic CNVs or CNVs of uncertain significance noted.

We performed immunoblotting analysis on the cells cultured from the mother's amniotic fluid and on the cells obtained from a control patient at a similar gestational age, in order to detect the presence of mTOR isoforms potentially produced by the de novo intragenic microduplication. This analysis revealed the full-length mTOR isoform (mTORα) and the presence of an additional shorter mTOR isoform only on patient-derived cells, probably originating from aberrant or alternative splicing as a consequence of intragenic rearrangement (Figure 1(B)).

To determine the consequences of the de novo microduplication in the mTOR gene, we profiled the protein-coding transcriptome of the control and patient-derived cells. A non-standard RNA-sequencing analysis was performed on our two samples, to identify the genes and the isoforms that were involved. Data obtained from two independent experiments highlighted a reduction in the full-length mTOR transcript (ENST00000361445.8/MTOR-201) and a moderate increase in the mTORβ splice variant (ENST00000376838.5/MTOR-202) in patient-derived cells. Moreover, the results confirmed a moderate decrease in the mTOR shortest transcript (ENST00000455339.1/MTOR-203) that encodes a 161-amino acid protein containing only the catalytic domain and in one LncRNA (ENST00000495435.1/MTOR-207) the meaning of which is not clear (Supplementary Table 1).

These findings prompted us to extend our analysis by investigating the mTOR cascade (Figure 2(A)). The patient's amniotic cells showed increased levels of phosphorylated AKT residue (Ser473), which is targeted by the mTORC2 complex (mTOR/Rictor) in a PI3K-dependent manner, and increased levels of another AKT residue (Thr308), which is targeted by PI3K directly or indirectly (through PDK1). The cascade effect of phosphorylation events was confirmed by the increased levels of the pAKT1S1 residue (Thr246) and its downstream target, pRPS6KB1 (Ser371) (Figure 2(B)). Our analysis revealed that mTOR/Rictor was significantly upregulated in our

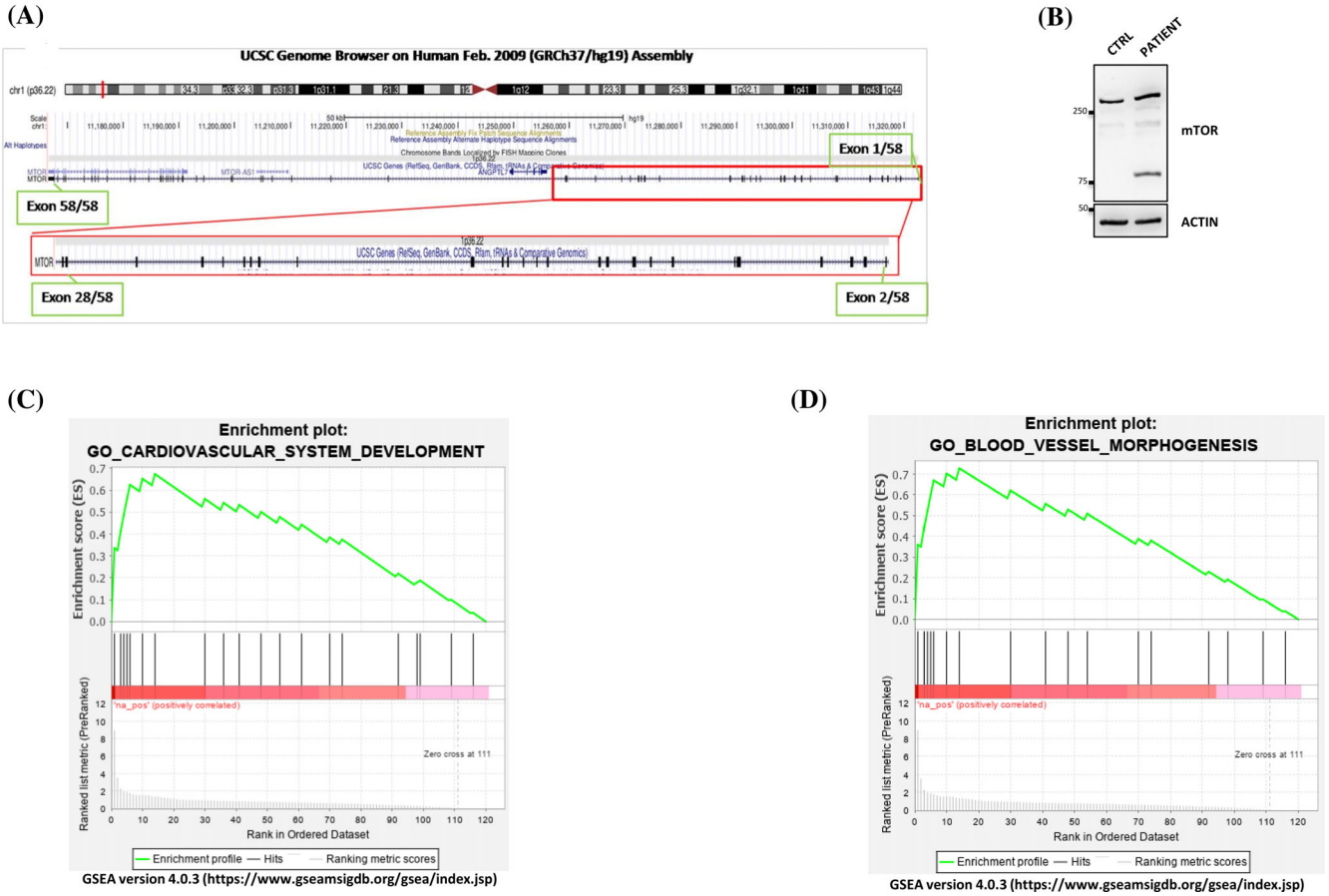

FIGURE 1 (A) Overview of the 1p36.22 microduplication region in our case. arr[GRCh37]1p36.22(chr1:11258825–11 318 811). (B) Immunoblotting analysis of mTOR protein levels in patient- versus control-derived amniotic cells. Actin was used as a loading control. The presented results are representative of at least three independent experiments. (C)-(D) GSEA enrichment plot (GSEA version 4.0.3 (https://www.gsea-msigdb.org/gsea/index.jsp): "GO_CARDIOVASCULAR_SYSTEM_DEVELOPMENT" and "GO_BLOOD_VESSEL_MORPHOGENESIS", respectively: a graphical view of the enrichment score for a gene set. The enrichment score (ES), which reflects the degree to which a gene set is overrepresented at the top or bottom of a ranked list of genes [Colour figure can be viewed at wileyonlinelibrary.com]

patient's amniotic cells; indeed, our immunoblotting analysis showed an increased level of Rictor as compared to that of the control-derived amniocytes. As shown in Figure 2(C), our immunoblotting analysis revealed a strong upregulation of the MEK–ERK signaling pathway with increased levels of phosphorylated MEK and more significant increased levels of phosphorylated ERK in patient-derived cells.

To investigate these findings, we performed a differential analysis of RNA sequencing data that showed lack of expression of AKT2 isoform ENST00000486368.6/AKT2-219 with TPM mean value equal to 0.01 and 18.18 in patient- and control-derived cells, respectively and PIK3R1isoform ENST00000336483.9/PIK3R1–202 with TPM mean value equal to 0.21 and 50.93 in patient- and control-derived cells, respectively, with a probability ≥0.85 (Supplementary Table 2). PIK3R1 encodes for three out of the five different regulatory subunits that associate with p110αcatalytic isoform with a fundamental role in the PI3K signaling network where mTOR is one of the principal downstream effectors.

In fact, two Gene Set Enrichment Analysis (GSEA) analyses were performed, one on mTOR pathway genes (Supplementary Table 3A) and one on differentially-expressed genes (Supplementary Table 3B). Results showed enrichment in different pathways (Supplementary Table 3A-B). We focused on the "GO_CARDIOVASCULAR_SYSTEM_DEVELOPMENT" pathway and the "GO_BLOOD_VESSEL_MORPHOGENESIS" pathway including the results obtained from the mTOR pathway gene GSEA analysis (see enrichment plots [Figure 1(C),(D)]). These pathways had a significant nominal p-value (NOM p-value) but not a significant FDR-q-value (Supplementary Table 3A) and share several genes due to an overlapping of the genes belonging to them that is already present in the MSigDB database (version 7.1; see Supplementary Table 4 and the Venn Diagram in Supplementary Figure 1). In particular, the blood vessel morphogenesis and the cardiovascular pathway share 686 genes, which are all genes belonging to blood vessels morphogenesis and included in the cardiovascular pathway.

4 | DISCUSSION

We describe here the case of a female infant aged 10 months at our last examination, with a phenotype characterized by the absence of

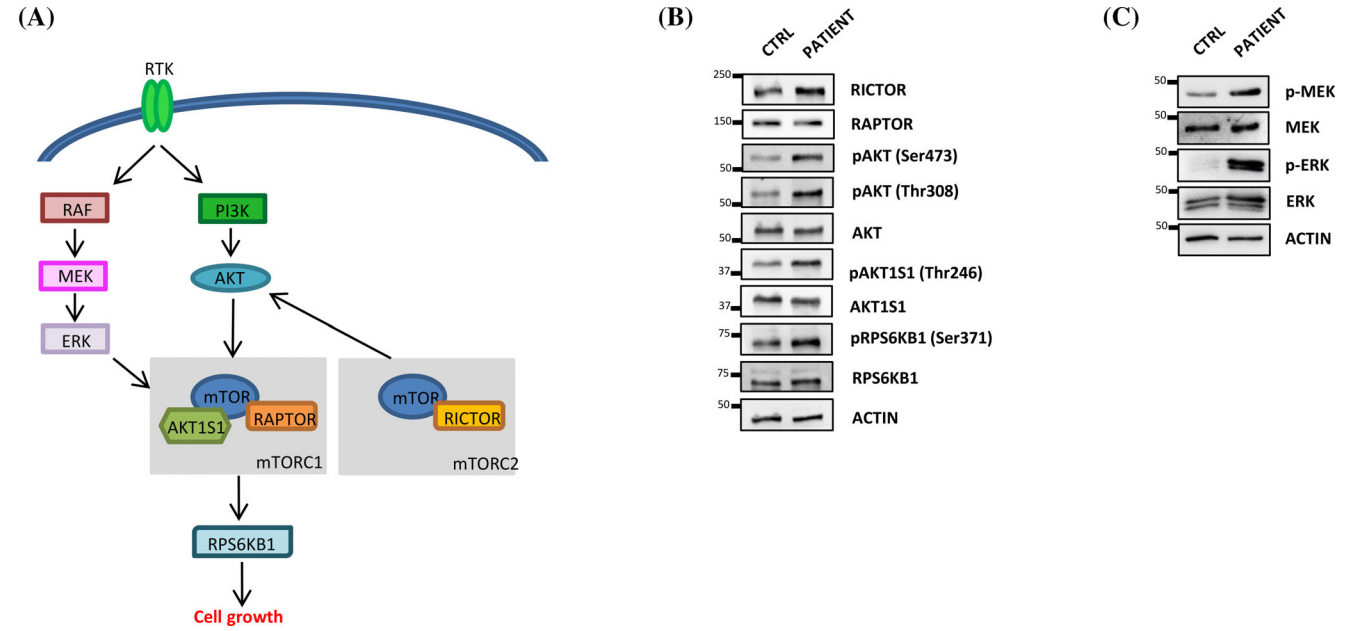

FIGURE 2 (A) Rapresentative figure of mTOR pathway. (B)-(C) Immunoblot analysis was performed to evaluate PI3K/AKT/mTOR (B) and MEK/ERK (C) pathway. (B) RICTOR, RAPTOR, pAKT (Ser473), pAKT (Thr308), total AKT, pAKT1S1 (Thr246), total AKT1S1, pRPS6KB1 (Ser371), total RPS6KB1 were assessed by immunoblot in patient- vs. control-derived amniocytes. (C) Protein levels of pMEK, total MEK, pERK, total ERK. Actin was used as a loading control. The presented results are representative of at least three independent experiments [Colour figure can be viewed at wileyonlinelibrary.com]

significantly dysmorphic facial features, and major cardiac malformation, consisting of aortic isthmus coarctation and aortic arch hypoplasia, prevalence of the right sections with functional tricuspid insufficiency and subaortic ventricular septal defect. The nosology of these malformations is also controversial, as they cannot be included in the classic conotruncal anomalies, a group of defects in which the development of the secondary cardiac field is affected by abnormal migration of the ectomesenchymal tissue from the neuronal crest.

Our case is the first report of an isolated CHD associated with a de novo interstitial duplication of approximately 60 Kb in region 1p36.22 encompassing from exon 2 to exon 28 of the mTOR gene which caused mTOR hyperactivation.

Several authors have investigated the role of the mTOR pathways in human malformations. mTOR brain somatic mutations are a major cause of focal malformations of the cortical development. The mechanism has been extensively investigated and appears to be primarily attributable to impaired neuronal ciliogenesis which causes defective neuronal migration and cortical dyslamination.[5] Ciliogenesis and the mTOR pathway could play a relevant, perhaps even greater, role in human CHDs. Cilia are structurally present in the second heart field and are required for Hedgehog signaling, which supports a possible role of the mTOR pathway in heart development.[6]

As reviewed by Casar Tena et al,[7] the mTOR pathway is also implicated in left–right (LR) asymmetry. A precise amount of TORC1 activity seems necessary to develop the correct asymmetry: hyperactivation of TORC1 alters ciliary length and causes situs anomalies in the zebrafish model.[8] A recent study[9] has clearly demonstrated that mTOR dysregulation induces cilia elongation, disturbing LR patterning. In other experimental studies, mTOR dysregulation seems to impair neural crest cells development and/or migration.[10]

In short, the first steps of cardiac morphogenesis seem strictly influenced by the genetic and pathogenic mechanisms of laterality development. Increasing evidence in the literature indicates that some isolated CHDs, especially those with ventricular L-loop and those with major distortion of ventricles and great arteries, could also be considered as lateralization defects.[11] Burnicka-Turek et al,[12] using the model of atrioventricular septal defect, suggested that cilia gene mutations could contribute to syndromic and isolated cardiac defects by multiple mechanisms.

There is enough evidence to consider that the hyperactivation of mTOR could have caused the pattern of cardiac malformations in our patient. Based on the literature, we hypothesize that mTOR dysregulation could have caused this malformation by disturbing ciliary functions in the first steps of cardiac embryology.

Finally, we would like to add a short comment on our patient's prognosis. One month after the surgery she had a LV-PSA, a rare complication of the cardiac surgical intervention. Zou et al,[13] reported AKT2 plays a key role in protecting the aortic wall. We can speculate that the significant decrease in the mTOR pathway specific transcripts such as AKT2 and PIK3R1 could have contributed to this life-threatening complication.

Our study has several limitations. The mTOR pathway dysregulation found in amniocytes might not affect cardiomyocytes exactly in the same way, so it is difficult to predict what effects mTOR dysregulation would have on the maintenance of postnatal cardiac structure and function.[4] So far, studies aimed at defining the role of

mTOR signaling in the cardiovascular system were performed on animal models rather than cells derived from patients with a constitutional genetic anomaly. Moreover, we cannot exclude long-term effects in our patient during her lifetime. This mTOR microduplication could be responsible not only for the heart defect but also for late-onset neurodevelopmental disorders.[14]

Despite these limitations, our results suggest that a dysregulated mTOR pathway resulting from an intragenic microduplication might play a key role in the pathogenesis of non-syndromic CHDs. This adds new insights to the plethora of functions that may be ascribed to the mTOR signaling network and opens the way for future targeted therapies for non-syndromic patients.

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CONFLICT OF INTEREST

The authors declare no potential conflict of interest.

PEER REVIEW

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DATA AVAILABILITY STATEMENT

Data are available on request.

ETHICS STATEMENT

Written informed consent to perform genetic testing and further studies was obtained from the family using a form approved by the competent Ethics Committee, in line with the principles of the Declaration of Helsinki and any other applicable local ethical and legal requirements. The parents did not consent to publishing photos of the patient.

ORCID

Mattia Gentile https://orcid.org/0000-0002-5299-6036
Nicoletta Resta https://orcid.org/0000-0001-8640-5532

REFERENCES

1. Saxton RA, Sabatini DM. mTOR signaling in growth, metabolism, and disease. *Cell*. 2017;168:960-976.
2. Zhang D, Contu R, Latronico MVG, et al. MTORC1 regulates cardiac function and myocyte survival through 4E-BP1 inhibition in mice. *J Clin Invest*. 2010;120:2805-2816.
3. Zhu Y, Pires KMP, Whitehead KJ, et al. Mechanistic target of rapamycin (Mtor) is essential for murine embryonic heart development and growth. *PLoS One*. 2013;8:e54221.
4. Sciarretta S, Forte M, Frati G, Sadoshima J. New insights into the role of mtor signaling in the cardiovascular system. *Circ Res*. 2018;122:489-505.
5. Sapir T, Barakat TS, Paredes MF, et al. Building bridges between the clinic and the laboratory: a meeting review–brain malformations: a roadmap for future research. *Front Cell Neurosci*. 2019;13:434. https://doi.org/10.3389/fncel.2019.00434.
6. Dyer LA, Kirby ML. The role of secondary heart field in cardiac development. *Dev Biol*. 2009;336:137-144.
7. Casar Tena T, Burkhalter MD, Philipp M. Left-right asymmetry in the light of TOR: an update on what we know so far. *Biol Cell*. 2015;107:306-318.
8. Chaudhry B, Henderson D, Anderson R. Double-outlet right ventricle is not hypoplastic left heart syndrome. *Nat Genet*. 2019;51:198.
9. Burkhalter MD, Sridhar A, Sampaio P, et al. Imbalanced mitochondrial function provokes heterotaxy via aberrant ciliogenesis. *J Clin Invest*. 2019;129:2841-2855.
10. Nie X, Zheng J, Ricupero CL, He L, Jiao K, Mao JJ. mTOR acts as a pivotal signaling hub for neural crest cells during craniofacial development. *PLoS Genet*. 2018;14:e1007491.
11. Versacci P, Pugnaloni F, Digilio MC, et al. Some isolated cardiac malformations can be related to laterality defects. *J Cardiovasc Dev Dis*. 2018;5:24. https://doi.org/10.3390/jcdd5020024.
12. Burnicka-Turek O, Steimle JD, Huang W, et al. Cilia gene mutations cause atrioventricular septal defects by multiple mechanisms. *Hum Mol Genet*. 2016;25:3011-3028.
13. Zou S, Ren P, Zhang L, et al. AKT2 promotes bone marrow cell-mediated aortic protection in mice. *Ann Thorac Surg*. 2016;101:2085-2096.
14. Gordo G, Tenorio J, Arias P, et al. mTOR mutations in smith-Kingsmore syndrome: four additional patients and a review. *Clin Genet*. 2018;93:762-775.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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