Cardiology in the Young

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Individual and joint effects of genetic polymorphisms in microRNA-machinery genes on congenital heart disease susceptibility

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Original Article

Cite this article: Borghini A, Vecoli C, Mercuri A, Turchi S, and Andreassi MG (2021) Individual and joint effects of genetic polymorphisms in microRNA-machinery genes on congenital heart disease susceptibility. *Cardiology in the Young* **31**: 965–968. doi: 10.1017/S1047951120004874

Received: 21 October 2020
Revised: 6 December 2020
Accepted: 13 December 2020
First published online: 11 January 2021

Keywords:
Congenital heart disease; single-nucleotide polymorphism; *DICER*; *DROSHA*; *XPO5*

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Abstract

Single-nucleotide polymorphisms in miRNA-machinery genes may alter the biogenesis of miRNAs affecting disease susceptibility. In this case–control study, we aimed to evaluate the impact of three single-nucleotide polymorphisms (*DICER* rs1057035, *DROSHA* rs10719, and *XPO5* rs11077) and their combined effect in a genetic risk score model on congenital heart disease (CHD) risk. A total of 639 participants was recruited, including 125 patients with CHD (65 males; age 9.2 ± 10 years) and 514 healthy controls (289 males; age 15.8 ± 18 years). Genotyping of polymorphisms in miRNA-machinery genes was performed using a TaqMan®SNP genotyping assay. A genetic risk score was calculated by summing the number of risk alleles of selected single-nucleotide polymorphisms. There was a significantly increased risk of CHD in patients with *XPO5* rs11077 CC genotype as compared to AC heterozygote and AA homozygote patients ($OR_{adjusted} = 1.7$; 95% CI: 1.1–2.8; $p = 0.018$). A clear tendency to significance was also found for *DROSHA* rs10719 AA genotype and CHD risk for both codominant and recessive models ($OR_{adjusted} = 1.8$; 95% CI: 0.91–3.8; $p = 0.09$ and $OR_{adjusted} = 1.9$; 95% CI: 0.92–4; $p = 0.08$, respectively). The resulting genetic risk score predicted a 1.73 risk for CHD per risk allele (95% CI: 1.2–2.5; $p = 0.002$). Subjects in the top tertile of genetic risk score were estimated to have more than three-fold increased risk of CHD compared with those in the bottom tertile ($OR_{adjusted} = 3.52$; 95% CI: 1.4–9; $p = 0.009$). Our findings show that the genetic variants in miRNA-machinery genes might participate in the development of CHD.

Congenital heart diseases (CHDs) are the most common congenital anomalies worldwide and comprise a broad spectrum of malformations that arise during cardiac development.[1] Although 20% of CHD incidence can be attributed to genetic syndromes, teratogens, or maternal diabetes, substantial uncertainty persists regarding risk factors for the remaining 80% of non-syndromic cases.[2]

The genetic and epigenetic changes are being increasingly acknowledged as key factors in the development and progression of CHDs.[3] In particular, the posttranscriptional regulation of gene expression by miRNAs in the complex process of cardiogenesis may be crucial for CHD prevention.[4] Nevertheless, profiling of specific miRNAs in human CHD poses some inherent challenges, due to the complex aetiology of disease and the high genetic heterogeneity between CHD patients.[4]

A novel approach has recently emerged from the study of genetic variants within miRNA-machinery genes, key factors involved in the biogenesis of miRNAs.[5] Such variants are likely to affect the maturation and function of miRNAs, possibly leading to the overall suppression of miRNA output with crucial effects in biological functions and disease risk.[5,6]

Recent studies have investigated the role of these single-nucleotide polymorphisms in the development and progression of different types of cancers[7,8] as well as their influence on the risk of stroke[9] and coronary artery disease.[10] Conversely, the association between genetic variants in miRNA-machinery genes and CHD susceptibility has not been investigated yet.

Therefore, the purpose of this study was to investigate the individual impact of single-nucleotide polymorphisms in *DICER* (rs1057035), *DROSHA* (rs10719), *and XPO5* (rs11077) and their combined effect in a genetic risk score on the risk of CHD.

Materials and methods

Study population

This study included a total of 125 patients (65 males; age 9.2 ± 10 years) with a diagnosis of isolated non-syndromic CHD and without a familial history, including ventricular septal defect, atrial septal defect, tetralogy of Fallot, patent ductus arteriosus, coarctation of the aorta, and transposition of great arteries. The diagnosis was confirmed by one or more of the following

procedures: echocardiography, cardiac catheterization, and/or surgical intervention.[11] A control group of 514 healthy controls (289 males; age 15.8 ± 18 years) without any congenital malformations was also enrolled. A sample of venous blood was collected from adult participants, whereas a cord blood sample was collected from newborns (both CHDs and controls). This study was conducted with an informed consent of every participant subject/parent and was approved by the local Ethical Research Committee.

Genotyping assays

Genomic DNA was extracted by using QIAamp DNA Blood Mini Kit (Qiagen) according to the manufacturer's instructions. DNA concentration and quality were assessed by a NanoDrop Lite Spectrophotometer (Thermo Scientific, Waltham, USA), and an absorbance ratio at both 260 and 280 nm (A260/A280) greater than 1.6 was considered suitable for the subsequent analysis.

Allelic discriminations for the *DICER* rs1057035, *DROSHA* rs10719, and *XPO5* rs11077 genetic variants were completed by quantitative real-time PCR on CFX RT-PCR System (Bio-Rad) TaqMan® SNP Genotyping assays (Applied Biosystems, USA). Negative and positive controls were included as a quality control measure. Genotyping results were analysed by allelic discrimination assay of the CFX Manager® software (Bio-Rad).

Statistical analysis

Statistical analysis of the data was conducted with the StatView statistical package, version 5.0.1 (Abacus Concepts, Berkeley, CA, USA). Values are presented as mean ± standard deviation (SD) or percent. Comparisons of normally distributed variables between groups were performed by using unpaired t-tests. Chi-square test was used to test for deviation from Hardy–Weinberg equilibrium and to compare allelic and genotypic frequencies between groups. The most significant test among the different genetic models (codominant, dominant, and recessive) was used to determine the statistical significance of each single-nucleotide polymorphism. Multivariate logistic regression analysis was used to estimate odds ratios (ORs) and 95% confidence intervals (CIs) for the association of the polymorphisms in microRNA-machinery genes and CHD risk.

High-risk alleles were used to generate a genetic risk score using an unweighted approach. Genetic risk score was calculated by the following method: the genotypes were coded as 0 for both homozygous for low-risk allele and heterozygous, and as 1 for homozygous for the high-risk allele. A summation term was then created by adding the risk allele count for each participant. The genetic risk score was modelled as a continuous variable and divided into three groups. Values of $p < 0.05$ were considered statistically significant.

Results

Demographic and genetic characteristics of the study participants are summarised in Table 1. There was no statistically significant difference between cases and controls in terms of gender ($p = 0.4$), whereas a significant difference was found for age ($p = 0.0001$).

Minor allele frequencies (MAF) of *DICER* rs1057035, *DROSHA* rs10719, and *XPO5* rs11077 were comparable with MAF reported in the public database for European population (i.e., Ensembl Genome Browser). The observed genotype frequency of the three single-nucleotide polymorphisms in both groups was in agreement with that expected under the Hardy–Weinberg equilibrium ($p > 0.05$; Table 1). No difference was observed in the distribution of genotypes between CHD patients and controls.

Table 1. Demographic and genetic characteristics between healthy controls and CHD patients

	Healthy control n = 514	CHD patients n = 125	p value
Mean ± SD age, yrs	15.8 ± 18	9.2 ± 10	0.0001
Gender, male n (%)	289 (56)	65 (52)	0.4
DICER rs1057035 T>C genotype, n (%)			
TT	251 (49)	65 (52)	0.5
TC	223 (43)	48 (38)	
CC	40 (8)	12 (10)	
HWE-p	0.3	0.5	
DROSHA rs10719 G>A genotype, n (%)			
GG	307 (60)	70 (56)	0.3
GA	177 (34)	43 (34)	
AA	30 (6)	12 (10)	
HWE-p	0.5	0.2	
XPO5 rs11077 A>C genotype, n (%)			
AA	160 (31)	37 (30)	0.1
AC	254 (49)	54 (43)	
CC	100 (20)	34 (27)	
HWE-p	0.9	0.1	

HWE: Hardy–Weinberg equilibrium

Table 2 summarises the associations of the three single-nucleotide polymorphisms in miRNA-machinery genes and CHD susceptibility under different genetic models. After adjustment for age and gender, the *XPO5* rs11077CC genotype was significantly associated with increased risk for CHD ($OR_{adjusted} = 1.7$; 95% CI: 1.1–2.8; $p = 0.018$) in the recessive model. A tendency towards increased risk was observed in carriers of *XPO5* rs11077 CC genotype as compared to AC heterozygote and AA homozygotes ($OR_{adjusted} = 1.69$; 95% CI: 0.98–2.9; $p = 0.057$).

Although not statistically significant, a trend close to significance was also found for *DROSHA* rs10719 AA genotype and CHD risk in both codominant ($OR_{adjusted} = 1.9$; 95% CI: 0.92–4; $p = 0.08$) and recessive models ($OR_{adjusted} = 1.8$; 95% CI: 0.91–3.8; $p = 0.09$).

The integrated effect of the three single-nucleotide polymorphisms was further evaluated by calculating a genetic risk score representing the sum of the high-risk alleles for each variant, which resulted in a score ranging from 0 to 2. The resulting genetic risk score was significantly associated with CHD susceptibility, with each allele associated with a 1.73 (95% CI: 1.2–2.5; $p = 0.002$).

Splitting the risk score in tertiles (I tertile with a score of 0; II tertile with a score of 1; III tertile with a score of 2), subjects in the top tertile of genetic risk score were estimated to have more than three-fold increased risk of CHD compared with those in the bottom tertile ($OR_{adjusted} = 3.5$; 95% CI: 1.4–9; $p = 0.009$). Patients in the second tertile of the genetic risk score had a 1.62-fold increased risk compared with those in the I tertile ($OR_{adjusted} = 1.62$; 95% CI: 1.05–2.5; $p = 0.03$).

https://doi.org/10.1017/S1047951120004874 Published online by Cambridge University Press

Table 2. Association of *DICER*, *DROSHA* and *XPO5* polymorphisms and CHD susceptibility under different genetic models

SNP	Genotype	OR(95% CI)*	p value
DICER rs1057035			
Codominant	TT	1	
	TC	0.83 (0.55–1.3)	0.4
	CC	1.35 (0.66–2.8)	0.4
Dominant	TT	1	
	TC + CC	0.9 (0.6–1.3)	0.6
Recessive	TT + TC	1	
	CC	1.47 (0.73–2.9)	0.27
DROSHA rs10719			
Codominant	GG	1	
	GA	1.09 (0.7–1.7)	0.7
	AA	1.9 (0.92–4)	0.08
Dominant	GG	1	
	GA + AA	1.2 (0.8–1.8)	0.4
Recessive	GG + GA	1	
	AA	1.8 (0.91–3.8)	0.09
XPO5 rs11077			
Codominant	AA	1	
	AC	0.95 (0.59–1.5)	0.8
	CC	1.69 (0.98–2.9)	0.057
Dominant	AA	1	
	AC + CC	1.1 (0.7–1.8)	0.5
Recessive	AA + AC	1	
	CC	1.7 (1.1–2.8)	0.018

*Adjusted for age and gender

Discussion

The present study is the first to investigate the impact of genetic variants in miRNA-machinery genes on CHD susceptibility. Our findings demonstrated that the XPO5 rs11077 CC genotype was significantly associated with an increased CHD risk. A clear tendency was also found for *DROSHA* rs10719 AA genotype, although the association did not reach statistical significance. Interestingly, the analysis of the joint effect of single-nucleotide polymorphisms in miRNA-machinery genes showed that higher scores were associated with increased risk of CHD, supporting the utility of a genetic risk score to provide a stronger predictive value than any single variant.

Accumulating evidence has revealed the highly complicated regulatory networks governing heart development, underscoring the importance of genetic and epigenetic factors in CHD development. Although various forms of CHD have been associated with altered expression of miRNAs, their causal role in the pathogenesis of CHD remains to be elucidated.[4]

Our analysis focused on potentially functional single-nucleotide polymorphisms located in genes involved in the biogenesis and processing of miRNAs. In general, miRNAs are formed in a multi-step biological process involving proteins and protein complexes, including DICER, DROSHA, and XPO5.[12] The presence of polymorphic variants in the core components of miRNA-machinery genes may impair or enhance miRNA processing efficiency or function, resulting in altered levels of mature miRNAs with potential deleterious effects.[5,6]

XPO5 is a protein responsible for the transport of pre-miRNAs between the nucleus and cytoplasm. The overexpression of *XPO5* results in enhanced miRNA activity,[13] whereas the loss of *XPO5* leads to reduced expression and function of pre-miRNAs.[14]

Among the genetic variants in *XPO5*, rs11077 has received the most attention. Located in the 3'-UTR, the A > C substitution may modify the expression of *XPO5* and, consequently, alter the expression of miRNAs.[7,15] The rs11077 C allele has been associated with an increased risk of developing cancer[15] and venous thromboembolism,[16] but it also appears to decrease the risk of coronary artery disease.[10]

To our knowledge, no previous studies associating these specific single-nucleotide polymorphisms with CHD are available. In our work, this polymorphic variant resulted associated with an increased risk of disease revealing a potential role of *XPO5* miRNA-machinery gene in CHD susceptibility. An increased risk of disease was also found for the *DROSHA* rs10719 AA genotype, although the results did not reach statistical significance.

DROSHA is an RNAse III enzyme, which mediates the processing of pri-miRNAs into pre-miRNAs inside the nucleus. In a previous in vitro functional investigation, a reduction in miRNA processing efficacy induced by the knockdown of *DROSHA* was found to reduce the levels of mature forms of tumour-suppressive miRNAs.[17] In addition, the loss of *DROSHA* leads to vascular smooth muscle cells disorder followed by hypoplastic blood vessel walls, cardiomyopathy, and embryonic lethality in mice.[18]

The 3′-UTR rs10719 polymorphism alters DROSHA expression levels.[19] It has been associated with different kinds of cancers[20] as well as recurrent implantation failure.[21] Interestingly, these single-nucleotide polymorphisms may affect the risk of non-syndromic orofacial clefts which represent major craniofacial congenital abnormalities characterised by unsuccessful fusion of normal facial processes.[22]

Previous studies have already proven the important role of DICER in the craniofacial development.[23] DICER is essential for the survival of the tissues derived from neural crest cells which exerts an essential influence on the formation of a majority of embryo tissues, including craniomaxillofacial structures.[24]

Deletion of cardiac *DICER* also results in defective heart morphogenesis and embryonic lethality.[25] Cardiac outflow defects were observed in mice with a conditional knockout of the *DICER* gene in the developing heart. In particular, embryos lacking *DICER* displayed a highly penetrant phenotype of double outlet right ventricle with a concurrent ventricular septal defect.[26] Nevertheless, the analysis of *DICER* mutations in sporadic and familial cases did not appear to play a major role in the transposition of the great arteries.[27]

DICER is an enzyme responsible for the cleavage of pre-miRNAs into their mature form. The rs1057035 T>C polymorphism is located within 3′-UTR and the C allele may lead to reduced expression levels of the enzyme as compared to the T allele.[28]

To date, conflicting results remain about the association between *DICER* rs1057035 and cancer risk in Caucasian populations,[29] whereas it seems to be involved in coronary artery disease.[5] In our study, no significant association was observed between this genetic variant and CHD susceptibility.

To overcome the limited effects of individual risk variants, we further constructed a genetic risk score by summing the risk alleles of the selected variants present in each study participant. The

https://doi.org/10.1017/S1047951120004874 Published online by Cambridge University Press

genetic risk score approach allows combining multiple loci with low-modest effects into a global risk score facilitating the identification of high-risk patients.

Interestingly, in the analysis of the combined effects of single-nucleotide polymorphisms in miRNA-machinery genes, the resultant genetic risk score predicted a 1.73 risk for CHD per risk allele. In addition, individuals in the top tertile score had a three-fold higher CHD risk, thus supporting the utility of the genetic risk score approach to increase understanding of the genetic basis of CHD.

The results of this case–control study should be approached and interpreted bearing in mind some limitations. Firstly, the number of enrolled patients and controls is limited. This does not allow to focus on the impact of the single-nucleotide polymorphisms on different types of CHD. Secondly, the study did not evaluate the relationship between other genetic variants in miRNA-machinery genes and CHD risk. Finally, the molecular mechanisms underlying the impact of these polymorphisms in CHD have not been investigated.

In conclusion, this study is the first to identify an association between the 3′-UTR XPO5 rs11077 polymorphism and CHD susceptibility. Additionally, our findings indicate that the combined analysis of single-nucleotide polymorphisms in miRNA-machinery genes might participate in the development of CHD.

Further studies are necessary to validate our results in larger populations and other ethnic groups. Experimental studies should also be conducted to understand the biological relevance of *DICER* rs1057035, *DROSHA* rs10719, and *XPO5* rs11077 polymorphisms in the pathogenesis of cardiac defects. The characterization of these variants and the identification of their functional impact may provide a good starting point for the development of individually tailored miRNA-based therapy. Therapeutic modulation of miRNA expression during cardiac malformations by either inhibition by anti-miRNAs or overexpression by miRNA-mimics may represent a promising approach to enhance clinical outcomes in CHD patients.

Acknowledgements. We thank all the individuals who participated in this study. We are very grateful to all clinical staff of the Pediatric Cardiology (Fondazione Toscana "G. Monasterio", Massa, Italy) and of Maternity and Pediatric Units (ASL 01, Massa and Carrara, Italy) for kindly support and assistance.

Financial support. This research received no specific grant from any funding agency, commercial or not-for-profit sectors.

Conflicts of interest. None.

Ethical standards. The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national guidelines on human experimentation and with the Helsinki Declaration of 1975, as revised in 2008, and has been approved by the local Ethical Research Committee

References

1. Triedman JK, Newburger JW. Trends in congenital heart disease: the next decade. Circulation 2016; 133: 2716–2733.
2. Blue GM, Kirk EP, Sholler GF, Harvey RP, Winlaw DS. Congenital heart disease: current knowledge about causes and inheritance. Med J Aust 2012; 197: 155–159.
3. Vecoli C, Pulignani S, Foffa I, Andreassi MG. Congenital heart disease: the crossroads of genetics, epigenetics and environment. Curr Genomics 2014; 15: 390–399.
4. Pulignani S, Andreassi MG. MicroRNAs and congenital heart disease: where are we now? Rev Esp Cardiol (Engl Ed) 2019; 72: 7–9.
5. Borghini A, Andreassi MG. Genetic polymorphisms offer insight into the causal role of microRNA in coronary artery disease. Atherosclerosis 2018; 269: 63–70.
6. Cammaerts S, Strazisar M, De Rijk P, Del Favero J. Genetic variants in microRNA genes: impact on microRNA expression, function, and disease. Front Genet 2015; 21: 186.
7. Guo Z, Shu Y, Zhou H, Zhang W. Identification of diagnostic and prognostic biomarkers for cancer: focusing on genetic variations in microRNA regulatory pathways. Mol Med Rep 2016; 13: 1943–1952.
8. Ryan BM. microRNAs in cancer susceptibility. Adv Cancer Res 2017; 135: 151–171.
9. Kim JO, Bae J, Kim J, et al. Association of microrna biogenesis genes polymorphisms with ischemic stroke susceptibility and post-stroke mortality. J Stroke 2018; 20: 110–121.
10. Borghini A, Pulignani S, Mercuri A, et al. Influence of genetic polymorphisms in DICER and XPO5 genes on the risk of coronary artery disease and circulating levels of vascular miRNAs. Thromb Res 2019; 180: 32–36.
11. Pulignani S, Vecoli C, Borghini A, Foffa I, Ait-Alì L, Andreassi MG. Targeted next-generation sequencing in patients with non-syndromic congenital heart disease. Pediatr Cardiol 2018; 39: 682–689.
12. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism and function. Cell 2004; 116: 281–297.
13. Yi R, Doehle BP, Qin Y, Macara IG, Cullen BR. Overexpression of exportin 5 enhances RNA interference mediated by short hairpin RNAs and microRNAs. RNA 2005; 11: 220–226.
14. Zeng Y, Cullen BR. Structural requirements for pre-microRNA binding and nuclear export by Exportin 5. Nucleic Acids Res 2004; 32: 4776–4785.
15. Shao Y, Shen Y, Zhao L, Guo X, Niu C, Liu F. Association of microRNA biosynthesis genes XPO5 and RAN polymorphisms with cancer susceptibility: Bayesian hierarchical meta-analysis. J Cancer 2020; 11: 2181–2191.
16. Ko EJ, Kim EJ, Kim JO, et al. Analysis of the association between microRNA biogenesis gene polymorphisms and venous thromboembolism in Koreans. Int J Mol Sci 2019; 20: 15.
17. Noh H, Hong S, Dong Z, Pan ZK, Jing Q, Huang S. Impaired MicroRNA processing facilitates breast cancer cell invasion by upregulating Urokinase-Type plasminogen activator expression. Genes Cancer 2011; 201: 140–150.
18. Fan P, Chen Z, Tian P, et al. miRNA biogenesis enzyme Drosha is required for vascular smooth muscle cell survival. PLoS One 2013; 18: e60888.
19. Saeki M, Watanabe M, Inoue N, et al. DICER and DROSHA gene expression and polymorphisms in autoimmune thyroid diseases. Autoimmunity 2016; 49: 514–522.
20. Wen J, Lv Z, Ding H, Fang X, Sun M. Association of miRNA biosynthesis genes DROSHA and DGCR8 polymorphisms with cancer susceptibility: a systematic review and meta-analysis. Biosci Rep 2018; 38: 3.
21. Lee AE, Ryu CS, Kim JO, et al. Association between microRNA machinery gene polymorphisms and recurrent implantation failure. Exp Ther Med 2020; 4: 3113–3123.
22. Xu M, Ma L, Lou S, et al. Genetic variants of microRNA processing genes and risk of non-syndromic orofacial clefts. Oral Dis 2018; 24: 422–428.
23. Barritt LC, Miller JM, Scheetz LR, et al. Conditional deletion of the human ortholog gene Dicer1 in Pax2-Cre expression domain impairs orofacial development. Indian J Hum Genet 2012; 18: 310–319.
24. Zehir A, Hua LL, Maska EL, Morikawa Y, Cserjesi P. Dicer is required for survival of differentiating neural crest cells. Dev Biol 2010; 340: 459–467.
25. Zhao Y, Ransom JF, Li A, et al. Dysregulation of cardiogenesis, cardiac conduction, and cell cycle in mice lacking miRNA-1–2. Cell 2007; 129: 303–317.
26. Saxena A, Tabin CJ. miRNA-processing enzyme Dicer is necessary for cardiac outflow tract alignment and chamber septation. Proc Natl Acad Sci USA 2010; 107: 87–91.
27. Sabbaghian N, Digilio MC, Blue GM, Revil T, Winlaw DS, Foulkes WD. Analysis of DICER1 in familial and sporadic cases of transposition of the great arteries. Congenit Heart Dis 2018; 13: 401–406.
28. Ma H, Yuan H, Yuan Z, et al. Genetic variations in key microRNA processing genes and risk of head and neck cancer: a case-control study in Chinese population. PLoS One 2012; 7: e47544.
29. Yu YY, Kuang D, Yin XX. Association between the DICER rs1057035 polymorphism and cancer risk: evidence from a meta-analysis of 1,2675 individuals. Asian Pac J Cancer Prev 2015; 16: 119–124.

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