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Original Article

GATA4 rs61277615, rs73203482, and rs35813172 in Newborns with Transposition of the Great Arteries

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Congenital heart disease is the most common malformative pathology in newborns, with a worldwide incidence at 0.4-5%. We investigated the possible relationship between variations in nucleotide sequences and specific cardiac malformations in the GATA-binding factor 4 (*GATA4*) exon 1 region by using Sanger sequencing. Forty-four newborns from a third-level neonatal intensive care unit who were diagnosed with nonsyndromic, ductal-dependent congenital heart disease (*i.e.*, transposition of the great arteries or ductal-dependent coarctation of the aorta) were enrolled. Their DNA was extracted using commercial methods and tested using the multiplex ligation-dependent probe amplification (MLPA) technique. The Sanger sequencing for *GATA4* exon 1 in the newborns' DNA identified rs61277615, rs73203482, and rs35813172 variants not reported in the ClinVar archive of human variations in newborns previously diagnosed with transposition of the great arteries (n = 5) and coarctation of the aorta (n = 1). The identification of these novel variants in newborns with transposition of the great arteries or ductal-dependent coarctation of the aorta may be the first step in determining the variants' contribution to the occurrence of congenital heart disease. However, these results may be inconclusive, since the observed variants within *GATA4* gene were not previously reported.

Key words: transposition of the great arteries, ductal-dependent coarctation of the aorta, *GATA4*, MLPA, Sanger sequencing

Congenital heart disease (CHD) is the most common malformative pathology described in newborns, with a variable incidence worldwide between 0.4% and 5% of live births [1-4]. For the design of plans for the prevention of CHD, it is crucial to first understand the mechanisms that underlie the inheritance of congenital heart malformations. Although the pathology of CHD has been studied intensively, it is estimated that a definite etiological diagnosis has been obtained in only 15% of cases [5].

Congenital heart malformations comprise a group of structural and functional anomalies that occur during cardiac embryogenesis. Using clinical criteria, CHD can be classified as life-threatening, clinically significant, or clinically non-significant [6]. The most frequent life-threatening CHDs diagnosed in newborns are (*i*) transposition of the great arteries (TGA) and (*ii*) coarctation of the aorta (CoA). Transposition of the great arteries is a congenital heart disorder character-

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ized by ventriculoarterial discordance. Frequently, the conditions of newborns diagnosed with TGA are associated with other structural heart defects, such as atrial or ventricular septal defects. These defects are crucial for newborns with TGA, and together with the ductus arteriosus, septal defects must ensure efficient mixing until the time of corrective surgery, since complete parallel circulation circuits are incompatible with life.

This pathology can be categorized into (*i*) simple TGA, which is characterized by ventriculoarterial discordance and is associated with a patent foramen ovale and persistent arterial duct, and (*ii*) the complex forms of TGA that are associated with wide ventricular septal defects, obstruction of the ejection tracts, and/or anomalies of the aortic arch. TGA has been identified in patients with genetic disorders characterized by defects in the establishment of laterality or the development of the heart outflow tract. Although the relationship between a certain genetic condition and the occurrence of ventriculoarterial discordance has been demonstrated in experimental conditions, the etiology of this malformative complex remains poorly understood (https://www.cdc.gov/ncbddd/heartdefects/d-tga.html, accessed Feb. 22, 2022) [7, 8].

Coarctation of the aorta represents a narrowing of the aortic paths, frequently located beyond the left subclavian artery. It may be identified as a single cardiac malformation or in association with other defects, frequently with bicuspid aortic valve or ventricular septal defect [9]. Depending on the location of the aortic narrowing compared to the insertion of the arterial channel, CoA can be divided into preductal, ductal or postductal CoA.

The involvement of several genes in cardiac development and specification has been described; *GATA4* (GATA-binding factor 4), *HAND2* (heart and neural crest derivatives expressed 2), *NKX2.5* (NK2 homeobox 5), and *MYOCD* (myocardin) [10] are the most frequently identified genes in this context. *GATA4* gene is involved in embryogenesis and in cardiac and testes development as a transcription factor. Its mutations have been directly linked to the occurrence of CHD [11], and the complete loss of *GATA4* and *GATA6* in animal models resulted in acardia [12].

The main objective of the present study was to investigate the possible relationship between variations in nucleotide sequences and specific cardiac malformations in newborns with TGA or CoA. The second aim was to investigate the *GATA4* exon 1 region using a personalized application of Sanger sequencing in patients with suspected modifications in this region, and then extend the investigation to the whole study group.

Patients and Methods

We enrolled 44 newborns diagnosed with nonsyndromic, ductal-dependent CHD from the third-level neonatal intensive care unit in Târgu Mureș Emergency Clinical County Hospital, Târgu Mureș, Romania. The newborns had been diagnosed with TGA (n = 21) or ductal-dependent of the aorta (n = 23; no other associated structural defect [n = 19], and complex coarctations with associated ventricular septal defect [n = 4]). Written informed consent was obtained from the parents or the legal guardians of the newborns enrolled. Study approval was obtained from the local ethics committees of the Târgu Mureș Emergency Clinical County Hospital, Târgu Mureș, Romania (approval no. 20695/23.09.2015) as well as the "George Emil Palade" University of Medicine, Pharmacy, Science, and Technology, Târgu Mureș, Romania (approval no. 128/21.10.2016), and it was conducted in accord with the Helsinki Declaration.

The inclusion criteria consisted of newborns diagnosed with simple TGA or ductal-dependent coarctation of the aorta. We excluded newborns with other ductal or nonductal-dependent heart defects, any type of congenital malformation (be it a specific syndrome-like phenotype or not, or relatives of the patients with a 1st or 2nd degree congenital heart defect), and newborns from mothers with specific pathology such as gestational diabetes, pregestational diabetes, autoimmune diseases, or metabolic diseases.

For each newborn, a study sheet was drawn up in which we collected the heredo-collateral antecedents, the maternal pathology before the current pregnancy, information about the pregnancy (evolution, degree of dispensation, possible treatment followed, parity), and data about the birth and the postpartum evolution of the patient (data not shown).

From every newborn enrolled, 2 mL of blood was collected on etilendiaminotetraacetic acid (EDTA), and the DNA was extracted using the PureLink Genomic DNA Mini Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instruc-

tions. Initially, samples were tested using the multiplex ligation-dependent probe amplification (MLPA) technique [13], with the SALSA MLPA P311 Probemix, and the result for all of the samples was negative.

The newborns' DNA was also amplified by polymerase chain reaction (PCR) using DreamTaq PCR Master Mix (2X) (Thermo Fisher Scientific) according to an *in-house* protocol, developed specifically for the primers we used (specific for the *GATA4* exon 1 region). A 10-µL subsample of the resulted amplicons was tested by gel electrophoresis, in 2% agarose, and the presence of an approx. 580-nucleotide fragment was observed in all of the samples.

The amplicons were prepared for Sanger sequencing using the BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher) and later purified with the BigDye XTerminator Purification Kit (Thermo Fisher). Capillary electrophoresis was performed on a 24-capillary 3500xL Dx Genetic Analyzer, using POP-7 (Thermo Fisher) polymer, under standard migration settings. We processed the electronic files that were generated, aligned to the reference sequence (GATA4 NM_002052.5) (https://www.ncbi.nlm.nih.gov/genbank/, accessed Feb. 10, 2021), and interpreted the data using the Variant Reporter ver. 1.1 program (Applied Biosystems, Foster City, CA, USA) software.

The sequence of interest for Sanger sequencing was represented by the following 162-nucleotide-long sequence:
GGGACTTGGAGGCGGCCGGCGCAGGGGCCGCG
AGAGGCTTCGTCGCCGCTGCAGCTCCGGGGGC
TCCCAGGGGAGCGTGCGCGGAACCTCCAGGCC
CAGCAGGTAGGGCTTTTTTCTTCCCTTTCTTTG
CTCCTTCCCGCGGTCCCCCAAACTCGGAGCTTC.
This sequence includes both the first exon of *GATA4*, according to GenBank (https://www.ncbi.nlm.nih.gov/genbank/, accessed Feb. 10, 2021) genetic database, ver. GRCh38.hg38, transcript_id NM_002052.5, as well as the MLPA region (https://www.mrcholland.com/product/P311/3024?, accessed Feb. 10, 2021) previously investigated.

For the design of sequencing primers we used Primer3 web ver. 4.1.0 [14, 15] software to create *de novo* oligonucleotide sequences, according to the following criteria: length 18-23 nucleotides, annealing temperature 57°-62°C, 50% optimum CG content, and amplicon length 250-800 nucleotides. The amplicons generated were verified with the GeneTools SNPCheck ver. 3 program (https://genetools.org/SNPCheck/snpcheck.htm, accessed Feb. 10, 2021) for the presence of single nucleotide polymorphisms (SNPs) within the annealing regions, as well as for the complete or partial annealing with other human genomic regions, using data available in the Single Nucleotide Polymorphism Database (dbSNP) (https://genetools.org/SNPCheck/snpcheck.htm, accessed Feb. 10, 2021) and the Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed Feb. 10, 2021). The most appropriate primers from among all the generated primers were chosen for sequencing.

These primers were then run on an *in silico* PCR tool, *i.e.*, the UCSC In-Silico PCR tool (https://genome.ucsc.edu/, accessed Feb. 10, 2021), using the GRCh38.hg38 human genome version. A 578-nucleotide-long fragment was obtained that included *GATA4* exon 1, both its intron-exon junctions, and a small part of the introns.

Results

We identified three variants not reported in the ClinVar archive of human variations (https://www.ncbi.nlm.nih.gov/clinvar/, accessed Apr. 25, 2021), *i.e.*, rs61277615, rs73203482, and rs35813172, in six (13.6%) unrelated Caucasian newborns diagnosed with TGA (n = 5) and CoA (n = 1; simple coarctation, without any other associated heart defect).

The results and the the underlying cardiac defects are summarized in Table 1.

Discussion

The main physiopathological processes involved in the occurrence of TGA are an inappropriate differentiation of cardiomyocyte and/or ciliary cells, defective activity in a left-right gene, altered heart field development, and deficiency of outflow tract remodeling [7]. Experimental studies have demonstrated the role of retinoic acid or retinoic acid inhibitors in inducing TGA in mice. The monogenic inheritance in families with TGA or congenitally corrected TGA has also been confirmed [16]. Unolt *et al.* demonstrated a relationship between TGA and heterotaxy by identifying mutations in laterality genes such as *NODAL* (nodal growth differentiation factor) and *ZIC3* (zinc family member 3) [16].

According to Rakhmanov *et al.*, non-syndromic CoA

Table 1 Variants identified during Sanger sequencing

Patient	Gene variant	Chromosomal position	Nucleotide variation	Congenital heart defect
1.	rs61277615	chr8:11704219 (GRCh38.p12)	C>T	TGA
	rs73203482	chr8:11704309 (GRCh38.p12)	G>A	
	rs35813172	chr8:11704474 (GRCh38.p12)		
2.	rs61277615	chr8:11704219 (GRCh38.p12)	C>T	TGA
	rs73203482	chr8:11704309 (GRCh38.p12)	G>A	
	rs35813172	chr8:11704474 (GRCh38.p12)	T>G	
3.	rs61277615	chr8:11704219 (GRCh38.p12)	C>T	TGA
	rs73203482	chr8:11704309 (GRCh38.p12)	G>A	
	rs35813172	chr8:11704474 (GRCh38.p12)	T>G	
4.	rs61277615	chr8:11704219 (GRCh38.p12)	C>T	TGA
	rs35813172	chr8:11704474 (GRCh38.p12)	T>G	
5.	rs35813172	chr8:11704474 (GRCh38.p12)	T>G	TGA
6.	rs61277615	chr8:11704219 (GRCh38.p12)	C>T	CoA
	rs73203482	chr8:11704309 (GRCh38.p12)	G>A	
	rs35813172	chr8:11704474 (GRCh38.p12)	T>G	

is genetically characterized by autosomal dominant inheritance with high penetrance and variable expressivity. The most common genes involved in isolated CoA are *NOTCH1* (Notch receptor 1 - OMIM gene 190198) and *MCTP2* (multiple C2 and transmembrane domain containing 2 - OMIM gene 616297) [17].

The involvement of mutations within transcription factors in the occurrence of heart defects is a topic of intense debate. Numerous studies have investigated the relationship between mutations at the *GATA4* level and the appearance of a specific heart defect, mainly in syndromic cardiac septal defects. The main research directions are oriented toward the *GATA4* gene and T-box transcription factor. GATA4 protein is a member of a family of zinc finger transcription factors that has roles in embryogenesis, cardiac differentiation, and testes development. These family of zinc finger transcription factors are the promoter of a number of genes. The activity of GATA4 protein is closely related to other transcription factors with which it interacts, namely *NKX2-5* (NK2 homeobox 5) and *TBX5* (T-box transcription factor 5). *GATA4* haploinsufficiency causes the occurrence of heart defects, typically in the form of ventricular or atrial septal defects. However, heterozygous *GATA4* mutations can be associated with more complex heart defects or with testicular dysgenesis [18].

In a Europe-based study, control patients and patients with a bicuspid aortic valve were enrolled, and *GATA4*, *GATA5*, and *GATA6* exon sequencing was performed. The sequencing revealed the occurrence of four rare variants in the study group but not the controls: *GATA4* p.Cys274= (rs55980825), p.His302= (rs201516339), *GATA5* p.Arg202Gln (rs782614097), and *GATA6* p.Asn458= (rs143026087) [19]. We identified none of these variants in the present patients, even though some of the patients had CoA, a condition associated with the bicuspid aortic valve. The disparity in these past and present results may be due to different enrollment strategies, since we did not use stand-alone bicuspid aortic valve as a study inclusion criterion.

Patients with an aortic bicuspid valve were enrolled in a study by Montes *et al.* based on *GATA4*, *GATA5*, and *GATA6* exon sequencing. Four rare variants were identified in the patients: *GATA4* p.Cys274= (rs55980825), p.His302= (rs201516339), *GATA5* p.Arg202Gln (rs782614097), and *GATA6* p.Asn458= (rs143026087) [19]. Although coarctation is frequently associated with aortic bicuspid valve, the gene variants identified in the Montes study were not detected by our present analyses. These different results may also be due to the fact that as an independent pathology, bicuspid aortic valve was not an inclusion criterion in our study.

Septal defects with familial aggregation have been described, usually with an autosomal dominant pattern of inheritance, but their precise prevalence remains unknown. The involvement of *GATA4* mutations in

unrelated individuals affected by conotruncal or other heart defects is still undergoing research. Butler *et al.* enrolled 357 unrelated patients with various congenital heart defects, and they observed *GATA4* variants only in the patients with atrial or ventricular septal defects. Their study indicated the existence of two mutations that caused protein changes, namely A411V (p.Ala412Val/rs55633527) and D425N (p.Asp426Asn/rs56208331), plus two possible pathogenic variants that caused G69D (p.Gly69Val/rs938776629) and P163R (p.Pro163Arg/rs540578824) changes [20]. In a sequencing study that focused only on the first exon of *GATA4*, Shaker *et al.* recruited patients with nonsyndromic congenital heart defects, the most prevalent condition being isolated septal defects. They were able to identify a sequence variant that caused a protein change, P193H, in patients with septal defects, while the control group lacked the same variant [21]. None of the variants described by Butler *et al.* and Shaker *et al.* were present in our study population. This is most likely because our study included only nonsyndromic, isolated congenital heart defects, while minor cardiac defects such as septal defects were excluded.

A study that focused on sequencing variants at the *GATA4* level enrolled patients with atrial and ventricular septal defects and single ventricle defects or Tetralogy of Fallot. Three variants were identified: rs61277615 in the gene promoter, the intronic variant rs4841587, and the splicing variant rs73203482, which were predicted to be pathogenic when analyzed *in silico* [22]. Two of these three variants, specifically rs73203482 and rs61277615, were also detected in our study population. rs35813172 is a single nucleotide variation (SNV) determined by a replacement of T with G in the chr8: 11704474 (GRCh38.p12) position. In the general population, this variant has had largely different reported frequencies, from 0.069 in a Vietnamese population to 0.5 in a Siberian population, with no clinical significance described in the ClinVar archive (https://www.ncbi.nlm.nih.gov/clinvar/, accessed Apr. 25, 2021).

Wong *et al.* identified four variants in their study of patients with non-compaction cardiomyopathy: rs61277615 and rs73203482 in *GATA4*, rs2277923 in *NKX2.5*, and rs17837976 in *CDC42* [23]. Both variants in *GATA4* were also identified in the present patients, even though our study did not include any patients with non-compaction cardiomyopathy; however, this may indicate that both variants may play a role in abnormal cardiac development during embryogenesis.

In an *in silico* study, Osman *et al.* performed a computer-based analysis of 18,598 variants in the *GATA4* gene and identified rs61277615 as responsible for influencing gene expression, impact splicing, and the regulation of alternative splicing [24].

An investigation by Blue *et al.* was based on the sequencing of the entire genome of 100 patients diagnosed with TGA. Although >50 variants were identified, no definite correlation could be made between the identified gene variants and the described structural defects. Blue *et al.* attempted to clarify the correlations among heart malformation, laterality defect, and neurological disability. From a genetic point of view, the results obtained by Blue *et al.* confirm that TGA is a polygenic pathology with multifactorial influences. The use of polygenic risk scores may present an opportunity to establish the etiology of this pathology [25].

In light of the existing reports, it has been speculated that the simultaneous presence of the three variants in newborns with TGA or CoA may be viewed as a risk factor for CHD development, since the SNPs may also affect gene function or be pathogenic, at least when *in silico* models are used [22, 24].

Our finding that six of 44 newborns presented the GATA4 variations represents a starting point in our future research. The importance of these variants will be better understood as we continue enrolling patients to investigate a larger cohort, but at the same time we will focus on animal models to test this finding and investigate the variants in daily clinical practice.

A possible limitation of this study is the relatively small sample size, but these patients represented a highly selected sub-population of newborns diagnosed with CHD in our neonatology clinics. Another limitation may be the lack of knowledge regarding the identified variants, since our comparison of these results with those of other studies did not reveal strong evidence confirming the pathogenic involvement of these genetic conditions in the occurrence of congenital heart disease.

In conclusion, our present findings may be considered inconclusive at the moment since we observed three variants of the *GATA4* gene not previously reported. The further identification of these novel variants in newborns with TGA and ductal-dependent CoA may be the first step in proving the variants' contribu-

tion to the occurrence of congenital heart disease. It is necessary to extend this research to determine the exact proportions of these variants in general populations as well as in the patients' relatives in order to confirm variant segregation.

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