Disease Markers 28 (2010) 287–292
DOI 10.3233/DMA-2010-0703
IOS Press

Multiplex ligation-dependent probe amplification analysis of *GATA4* gene copy number variations in patients with isolated congenital heart disease

Valentina Guida[a], Francesca Lepri[a], Raymon Vijzelaar[b], Andrea De Zorzi[c], Paolo Versacci[d], Maria Cristina Digilio[c], Bruno Marino[d], Alessandro De Luca[a,*] and Bruno Dallapiccola[c]
[a]*Mendel Laboratory, Casa Sollievo della Sofferenza Hospital, IRCCS, San Giovanni Rotondo, Rome, Italy*
[b]*MRC Holland, Amsterdam, The Netherlands*
[c]*Bambino Gesù Children Hospital, IRCCS, Rome, Italy*
[d]*Division of Pediatric Cardiology, Department of Pediatrics, "Sapienza" University, Rome, Italy*

Abstract. *GATA4* mutations are found in patients with different isolated congenital heart defects (CHDs), mostly cardiac septal defects and tetralogy of Fallot. In addition, *GATA4* is supposed to be the responsible gene for the CHDs in the chromosomal 8p23 deletion syndrome, which is recognized as a malformation syndrome with clinical symptoms of facial anomalies, microcephaly, mental retardation, and congenital heart defects. Thus far, no study has been carried out to investigate the role of *GATA4* copy number variations (CNVs) in non-syndromic CHDs. To explore the possible occurrence of *GATA4* gene CNVs in isolated CHDs, we analyzed by multiplex ligation-dependent probe amplification (MLPA) a cohort of 161 non-syndromic patients with cardiac anomalies previously associated with *GATA4* gene mutations. The patients were mutation-negative for *GATA4*, *NKX2.5*, and *FOG2* genes after screening with denaturing high performance liquid chromatography. MLPA analysis revealed that normalized MLPA signals were all found within the normal range values for all exons in all patients, excluding a major contribution of *GATA4* gene CNVs in CHD pathogenesis.

Keywords: CHD, MLPA, GATA4, CNV

1. Introduction

Congenital heart defects (CHDs) are the most common type of birth anomalies affecting nearly 1% of all live births [1]. Although CHDs may occur in association with other birth defects as part of a syndrome, they are often found as an isolated anomaly. Studies of animal models have demonstrated that genetic factors contribute significantly to the etiology of CHDs, but only a few genes involved in isolated human CHDs have been identified so far [2,3]. Mutations in the cardiac transcription factor *GATA4* have been identified as a cause of isolated CHD in a subset of individuals. Most of *GATA4* mutations have been found in patients with familial cardiac septal defects [4–9]. Nevertheless, mutations in the *GATA4* gene have also been associated with other CHD phenotypes, such as atrioventricular canal defect (AVCD), tetralogy of Fallot (ToF), patent ductus ateriosus, pulmonary stenosis, and hypoplastic right ventricle [4,8–13]. Furthermore, somatic *GATA4* mutations have been found in formalin-fixed heart tissues from CHD patients with septal defects and AVCD [14].

*Corresponding author: A. De Luca, Istituto CSS-Mendel, Viale Regina Margherita 261, 00198, Roma, Italia. Tel.: +39 06 4416 0533; Fax: +39 06 4416 0548; E-mail: a.deluca@css-mendel.it.

ISSN 0278-0240/10/$27.50

GATA4 gene encodes a zinc finger transcription factor that is involved in the regulation of a number of genes important in heart development [15]. Mice with targeted mutations in *GATA4* suffer from defective ventral morphogenesis and heart tube formation [16,17]. Mouse embryos with hypomorphic *GATA4* mutations present various cardiac malformations, including hypoplasia of the compact myocardium, AVCD, and double outlet right ventricle [18,19]. Accordingly, *GATA4* is supposed to be the gene responsible for the cardiac anomalies in the chromosomal 8p23 deletion syndrome, which is recognized as a malformation disorder with facial dysmorphisms, microcephaly, mental retardation, and CHDs, prevalently AVCD, ToF, double outlet right ventricle, and atrial septal defect (ASD) [20–23]. Recently, an atypical small interstitial deletion of 8p23 that includes the *GATA4* gene was also described in two unrelated patients showing Ebstein anomaly associated with septal defects [24]. Furthermore, duplications of *GATA4* gene have been observed in normal as well as in syndromic patients with and without heart defects, suggesting that *GATA4* is a dosage-sensitive gene with variable penetrance [25–28].

A recent study predicted that at least 10% of sporadic non-syndromic cases of tetralogy of Fallot, one of the most common CHDs, result from *de novo* copy number variations (CNVs) [29]. To this date, no study has investigated the role of *GATA4* gene CNVs in non-syndromic CHDs. Hence, to explore the possible occurrence of these *GATA4* gene lesions in non-syndromic CHDs we developed a multiplex ligation-dependent probe amplification (MLPA) [30] for this gene. We used this new assay to analyze a cohort of non-syndromic patients with anatomic types of cardiac defects previously associated with *GATA4* gene mutations. This cohort was mutation-negative for *GATA4* [4–11,13,14], as well as for the *NKX2.5* [5,7,31–33] and *FOG2* [34] genes, previously related to cardiac septal defects and/or conotruncal anomalies.

2. Materials and methods

The study cohort included 161 non-syndromic subjects, comprising 33 patients affected by ASD, 40 with AVCD, 80 with ToF and 8 with Ebstein anomaly. Patients had been recruited at the "Bambino Gesù" Hospital, and at the Pediatric Cardiology Unit of "Policlinico Umberto I" Hospital in Rome, during the years 1995–2004. Association with extracardiac anomalies was excluded in all subjects by complete physical evaluation for phenotypic anomalies, neuropsychological evaluation, anthropometric measurement, renal ultrasonography and radiological studies. Patients' clinical assessment included family history evaluation. Cardiac assessment consisted in a preoperative chest X-ray film, 12-lead electrocardiograms, and 2-dimensional transthoracic echocardiography with color-flow Doppler. The study was conducted in accordance with the Declaration of Helsinki and blood samples were obtained after informed consent had been given. The protocol was approved by the Institutional Review Board of the participating Institutions.

Genomic DNA was isolated from peripheral blood leukocytes using standard procedures. Point mutations and other subtle lesions in *GATA4*, *NKX2.5*, and *FOG2* genes had been previously excluded by denaturing high performance liquid chromatography. MLPA analysis was performed by using the newly designed SALSA P234-MLPA kit (MRC-Holland, Amsterdam, The Netherlands) according to the manufacturer's recommendations. The P234 GATA4 kit contains probes for each of the 7 exons of *GATA4*. In addition, it contains several probes upstream and downstream of *GATA4*, including probes for *BLK* and *FDFT1* genes, and probes for 5 of the exons of *GATA3*. MLPA products were analyzed using an ABI PRISM 3130 automated sequencer (Applied Biosystem, Foster City, CA). MLPA data were collected by using Gene Mapper software (Applied Biosystem) and subsequently analyzed by Coffalyzer software (MRC-Holland). Four unrelated control DNA samples were included as reference population together with the DNA of a patient hemizygous for an interstitial 4 Mb deletion at 8p23.1 encompassing the *GATA4* gene, which was used as positive deletion control. Based on the results obtained on the control group, observed values falling within the range of 0.7–1.3 were considered to have two gene copies.

Confidence intervals for proportions of CNVs were calculated by means of VassarStats software (http://faculty.vassar.edu/lowry/prop1.html) using the Newcombe-Wilson method including continuity correction [35,36].

3. Results and discussion

MLPA analysis revealed that normalized MLPA signals were all found within the normal range values for all exons in all subjects (Newcombe–Wilson score method; $k = 0$, $n = 161$, proportion $= 0/161$, $z = 1.960$, 95% confidence interval $= 0.000$–0.029) [35,36], ex-

Table 1

Summary of *GATA4* mutation screening studies on isolated CHDs

Study population	Nucleotide change	Amino acid substitution	Type of mutation	Phentoype	Study
Familial septal defects (2 families)	886G>A 1075delG	G296S E359RfsX44	Missense Deletion (out-of-frame)	ASD±VSD, AVCD,PS (1 familial case) ASD (1 familial case)	Garg et al. 2003 [4]
Familial ASD (16 families)	1075delG 155C>T	E359RfsX44 S52F	Deletion (out-of-frame) Missense	ASD (1 familial case) ASD (1 familial case)	Hiaryama-Yamada et al. 2005 [5]
Familial ASD (1 family)	1074delC	S358RfsX45	Deletion (out-of-frame)	ASD±PS (1 familial case)	Okubo et al. 2004 [6]
ASD (29 probands: 16 families; 13 sporadic cases)	886G>A	G296S	Missense	ASD±PS (2 familial cases)	Sarkozy et al. 2005 [7]
AVCD (35 probands: 9 families; 26 sporadic cases)	None	None	–	–	Sarkozy et al. 2005 [39]
Largely sporadic CHDs (94 probands: 30 VSD, 18 PS, 15 PDA, 12 ASD, 8 TA, 6 TGA, and 5 CoA)	648C>G	E216D	Missense	ToF (2 sporadic cases)	Nemer et al. 2006 [10]
Largely sporadic CHD (99 probands: 36 VSD, 4 ASD, 11 ToF, AVCD 1, 47 other)	None	None	–	–	Zhang et al. 2006 [40]
Sporadic CHDs (31 sporadic cases)	N.a. N.a.	V267M V380M	Missense Missense	CHD (1 sporadic case) CHD (1 sporadic case)	Tang et al. 2006 [13]
Sporadic CHDs (135 probands: 24 septal defects, 39 LSD, 17 RSD, 19 CTD, 16 complex CHDs, 7 AVCD, 13 other), familial CHDs (22 probands: 8 septal defects, 6 LSD, 1 RSD, 1 ToF, 3 complex CHD, 3 other)	None	None	–	–	Schluterman et al. 2007 [41]
Largely sporadic CHDs (628 probands: 122 ASD, 137 VSD, 201 ToF, 76 TGA, 45 DORV, 20 TA, 11 IAA, 10 CCTGA, 6 other)	278G>C 946C>G 1232C>T 1273G>A	G93A Q316S A411V D425N	Missense Missense Missense Missense	ASD (1 sporadic case) ASD/VSD (1 case with family history unknown) VSD (1 sporadic case) ASD (1 sporadic case) ToF (1 possible familial case)	Tomita-Mitchell et al. 2007 [11]
Largely sporadic CHDs (107 probands: 42 AVCD, 9 DILV, 8 ASD/VSD, 48 Cardiomyopathy)	487C>T 886G>T 1037C>T 1207C>A	P163S G296C A346V L403M	Missense Missense Missense Missense	AVCD (1 sporadic case) ASD+PS (1 familial case) AVCD (1 sporadic case) Hypoplastic RV (1 sporadic case)	Ragajopal et al. 2007 [8]

Table 1, continued

Study population	Nucleotide change	Amino acid substitution	Type of mutation	Phentoype	Study
Largely sporadic CHDs (205 probands: 110 ASD, 95 CHDs)	1232C>T	A411V	Misssense	ASD/PAPVC (1 sporadic case)	Posch et al. 2007 [37]
Largely sporadic CHDs (486 probands: 319 VSD, 37 ASD, 64 ToF, 21 TAPVC, 11 AVCD, 13 TGA, 7 PDA, 2 PA, 3 PS, 2 IAA, 2 DORV, 5 other)	17C>T 136_138delTCC 354_355insGCC 374_375insTGCCGC 487C>T 1075G>A 1220C>A 1286G>C 1325C>T	A6V 46delS 118_119insA 125_126insAA P163S E359K P407Q S429T A442V	Missense Deletion (in-frame) Insertion (in-frame) Insertion (in-frame) Missense Missense Missense Missense Missense	VSD (1 sporadic case) VSD (1 sporadic case) ToF (1 sporadic case) VSD (1 sporadic case) VSD (1 sporadic case) AVCD (1 sporadic case) VSD (2 cases in a family) ToF (1 sporadic case) VSD (1 sporadic case) VSD (2 sporadic cases)	Zhang et al. 2008 [9]
CHDs patients (62 probands: 27 VSD, 14 ASD, 7 ToF, 2 TAPVC, 2 AVCD, 5 PDA, 3 PS, 1 TGA, 1 DORV)	1220C>A 1273G>A	P407Q D425N	Missense Missense	ToF (1 sporadic case) VSD (1 sporadic case)	Zhang et al. 2009 [12]
Sporadic CHDs (104 probands: 76 ASD, 28 ToF)	341_342insA	T114TfsX95	Insertion (out-of-frame)	ASD (1 sporadic case)	Hamanoue et al. 2009 [38]
Familial TGA (7 families)	None	None	–	–	De luca et al. 2009 [42]

ASD, atrial septal defect; VSD, ventricular septal defect; AVCD, atrioventricular canal defect; PS, pulmonic stenosis; PDA, patent ductus arteriosus; TA, truncus arteriosus; TGA, transposition of the great arteries; CoA, coarctation of the aorta; DORV, double outlet right ventricle; ToF, tetralogy of Fallot; N.a., Not available; IAA, interrupted aortic arch; CCTGA, congenitally corrected transposition of the great arteries; DILV, double inlet left ventricle; RV, right ventricle; LVOTO, left ventricular outflow tract obstruction; LSD, left-sided defect; RSD, right-sided defect; CTD, conotruncal heart defect; PA, pulmonary atresia; PAPVC, partial anomalous pulmonary venous connection; TAPVC, total anomalous pulmonary venous connection.

cluding a major contribution of *GATA4* gene CNVs in the pathogenesis of the investigated CHDs.

Table 1 summarizes the many mutation screening studies that have been performed relevant to *GATA4* gene [4]. So far, 24 different *GATA4* germline mutations including 18 missense mutations [4,5,7–13,37], 3 deletion mutations [4–6,9], and 3 insertion mutations [9,38] have been identified in different CHD patients. Among them, 6 mutations were associated with familial CHD [4–9]. Furthermore, 23 somatic *GATA4* mutations were found in 68 formalin-fixed heart tissues from CHD patients [14] including one deletion and 22 missense mutations. Evidence from these studies supports a major role of *GATA4* in human cardiac morphogenesis. Nevertheless, results from *GATA4* gene analysis are difficult to interpret owing to the small percentage of CHDs due to *GATA4* mutations. Tomita-Mitchell and Colleagues investigated *GATA4* mutations in 628 patients with septal or conotruncal defects [11]. Four missense mutations were observed in 2 of 122 patients with ASD (1.6%), 2 of 137 patients with ventricular septal defect (VSD) (1.5%), and 1 of 201 patients with ToF (0.5%), with an overall prevalence rate of 0.8%. More recently, Zhang and Colleagues replicated this analysis in a cohort of 486 CHD Chinese patients, which included more VSD and less ASD cases compared to previous study. Six missense mutations, 2 small insertions, and 1 small deletion were found in 9 of 319 patients with VSD (2.8%), 2 of 64 patients with ToF (3.1%), and 1 of 11 patients with AVCD (9.1%), with an overall *GATA4* mutation prevalence rate of 2.5%. Considering the overall prevalence of *GATA4* mutations in CHDs, the existence of very rare CNVs of *GATA4* gene in CHDs cannot be excluded with certainty. In addition, none of our patients was affected by VSD. Thus, it is still possible that *GATA4* germ-line CNVs may occur at a significant frequency among VSD cases or other specific subgroups of CHD phenotypes excluded from the present analysis. However, the absence of CNVs in *GATA4* gene in more than 160 patients with cardiac anomalies previously associated with *GATA4* gene mutations, who were also mutation-negative for *GATA4*, *NKX2.5*, and *FOG2* genes, strongly suggests that *GATA4* CNVs do not represent a common cause of CHD, at least in our cohort.

Acknowledgments

The authors wish to thank the patients who participated in this research. This research was supported by the Italian Ministry of Health grant RF2009 and RC2009, and Italy-USA Program on Rare Diseases (Istituto Superiore di Sanità).

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