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

Valentina Guida\textsuperscript{a}, Francesca Lepr\textsuperscript{a}, Raymon Vijzelaar\textsuperscript{b}, Andrea De Zorzi\textsuperscript{c}, Paolo Versacci\textsuperscript{d}, Maria Cristina Digilio\textsuperscript{c}, Bruno Marino\textsuperscript{d}, Alessandro De Luca\textsuperscript{a,*} and Bruno Dallapiccola\textsuperscript{c}

\textsuperscript{a}Mendel Laboratory, Casa Sollievo della Sofferenza Hospital, IRCCS, San Giovanni Rotondo, Rome, Italy
\textsuperscript{b}MRC Holland, Amsterdam, The Netherlands
\textsuperscript{c}Bambino Gesù Children Hospital, IRCCS, Rome, Italy
\textsuperscript{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 arteriosus, 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].
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 doubly 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 dysmorphism, microcephaly, mental retardation, and CHDs, prevalently AVCD, ToF, doubly 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, suggesting that GATA4 is a dosage-sensitive gene with variable penetrance.

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 trans-thoracic 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 FDTF1 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-
| Study population | Nucleotide change | Amino acid substitution | Type of mutation | Phenotype | Study |
|------------------|------------------|-------------------------|------------------|-----------|-------|
| Familial septal defects (2 families) | 886G>A | G296S | Missense | ASD±VSD, AVCD, PS (1 familial case) | Garg et al. 2003 [4] |
| | 1075delG | E359RfsX44 | Deletion (out-of-frame) | ASD (1 familial case) | |
| Familial ASD (16 families) | 1075delG | E359RfsX44 | Deletion (out-of-frame) | ASD (1 familial case) | Hiyama-Yamada et al. 2005 [5] |
| | 155C>T | S52F | Missense | ASD (1 familial case) | |
| 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] |
| ASDCD (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. | V267M | Missense | 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 | G93A | Missense | ASD (1 sporadic case) | Tomita-Mitchell et al. 2007 [11] |
| | 946C>G | Q316S | Missense | ASD/VSD (1 case with family history unknown) | |
| | 1232C>T | A411V | Missense | VSD (1 sporadic case) | |
| | 1273G>A | D423N | Missense | ASD (1 sporadic case) | |
| | 487C>T | P163S | Missense | AVCD (1 sporadic case) | Ragajopal et al. 2007 [8] |
| | 886G>T | G296C | Missense | ASD+PS (1 familial case) | |
| | 1037C>T | A346V | Missense | AVCD (1 sporadic case) | |
| | 1207C>A | L403M | Missense | Hypoplastic RV (1 sporadic case) | |
| Study population                                      | Nucleotide change | Amino acid substitution | Type of mutation | Phenotype                  | Study               |
|------------------------------------------------------|-------------------|-------------------------|------------------|---------------------------|---------------------|
| Largely sporadic CHDs (205 probands: 110 ASD, 95 CHDs) |                   |                         |                  |                           | Posch et al. 2007 [37] |
|                                                      | 1232C>T           | A411V                   | Missense         | ASD/PAPVC (1 sporadic case) |                     |
|                                                      |                   |                         |                  |                           |                     |
| Largely sporadic CHDs (486 probands: 319 VSD, 37 ASD, 11 AVCD, 13 TGA, 7 PDA, 2 PA, 3 PS, 2 IAA, 2 DORV, 5 other) | 17C>T             | A6V                    | Missense         | VSD (1 sporadic case)   | Zhang et al. 2008 [9] |
|                                                      |                   |                         |                  |                           |                     |
|                                                      | 136L,138delTCC    | 46delS                  | Deletion (in-frame) | VSD (1 sporadic case)   |                     |
|                                                      |                   |                         |                  |                           |                     |
|                                                      | 35L,55insGCC      | 11L,119insA             | Insertion (in-frame) | ToF (1 sporadic case)   |                     |
|                                                      |                   |                         |                  |                           |                     |
|                                                      | 37L,375insTGCCGC  | 125L,126insAA           | Insertion (in-frame) | VSD (1 sporadic case)   |                     |
|                                                      |                   |                         |                  |                           |                     |
|                                                      | 487C>T            | P163S                   | Missense         | VSD (1 sporadic case)   |                     |
|                                                      |                   |                         |                  |                           |                     |
|                                                      | 1075G>A           | E359K                   | Missense         | AVCD (1 sporadic case)  |                     |
|                                                      |                   |                         |                  |                           |                     |
|                                                      | 1220C>A           | P407Q                   | Missense         | VSD (2 cases in a family) |                     |
|                                                      |                   |                         |                  |                           |                     |
|                                                      | 1286C>C           | S429T                   | Missense         | ToF (1 sporadic case)   |                     |
|                                                      |                   |                         |                  |                           |                     |
|                                                      | 1325C>T           | A442V                   | Missense         | VSD (1 sporadic case)   |                     |
|                                                      |                   |                         |                  |                           |                     |
| CHDs patients (62 probands: 27 VSD, 14 ASD, 7 ToF, 2 TAPVC, 2 AVCD, 5 PDA, 3 PS, 1 TGA, 1 DORV) | 1220C>A           | P407Q                   | Missense         | ToF (1 sporadic case)   | Zhang et al. 2009 [12] |
|                                                      |                   |                         |                  |                           |                     |
|                                                      | 1273G>A           | D425N                   | Missense         | VSD (1 sporadic case)   |                     |
|                                                      |                   |                         |                  |                           |                     |
| Sporadic CHDs (104 probands: 76 ASD, 28 ToF)        | 34L,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 [11] 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 CHD patients 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.

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