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Short report

Search of somatic *GATA4* and *NKX2.5* gene mutations in sporadic septal heart defects

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ABSTRACT

High prevalence of somatic mutations in the cardiac transcription factor genes *NKX2.5* and *GATA4* have been reported in the affected cardiovascular tissue of patients with isolated cardiac septal defects, suggesting a role of somatic mutations in the pathogenesis of these congenital heart defects (CHDs). However, all somatic mutations have been identified in DNA extracted from an archive of formalin-fixed cardiac tissues. In the present study, to address the hypothesis that somatic mutations are important in isolated CHDs, we analyzed the *GATA4* and *NKX2.5* genes in the fresh-frozen pathologic cardiac tissue specimen and corresponding non-diseased tissue obtained from a series of 62 CHD patients, including 35 patients with cardiac septal defects and 27 with other cardiac anomalies. We identified one variant and two common polymorphisms in the *NKX2.5* gene, and six variants and two common polymorphisms in the *GATA4* gene. All identified variants were seen in both the fresh-frozen pathologic cardiac tissue and the corresponding non-diseased tissue, which indicates that they all were constitutional variants. The present study has identified *NKX2.5* and *GATA4* constitutional variants in our CHD cohort, but was unable to replicate the previously published findings of high prevalence of somatically derived sequence mutations in patients with cardiac septal defects using fresh-frozen cardiac tissues rather than formalin-fixed tissues.

1. Introduction

Somatic mosaicism refers to the condition in which a mutation arises after fertilization such that only a subset of cells or tissues harbors the defect. Aside from occupying a major role in the pathogenesis of many cancers, somatic mosaicism has been shown to underlie some cases of certain genetic disorders [1]. This genetic mechanism has been raised theoretically as relevant for congenital heart defects (CHDs), particularly when isolated, and over the last past years the first somatic mutations confined to affected cardiovascular tissue have been reported. Somatic mutations have been found in *GJA1* [2], *NKX2.5* [3], *TBX5* [4], *GATA4* [5,6], *HEY2* [7] and *HAND1* [8,9] genes. Reamon-Buettner and Borlak have reported the majority of somatic mutations in CHDs, including mutations in transcription factors *NKX2.5*, *TBX5*, *GATA4*, *HEY2* and *HAND1* [3–11]. In particular, *NKX2.5* somatic mutations were identified in almost all human hearts with septal heart defects (*i.e.* 66 of 68 formalin-fixed hearts examined by Reamon-Buettner and Borlak had a somatic sequence variant) [3,11]. Moreover, in several patients different mutations in the same gene with cumulative down-regulation of transcription were reported (up to 14 mutations in patients with ventricular septal defects [VSD]) [3,6,11]. These somatic sequence variants were identified in DNA extracted from the University of Leipzig (Germany) collection of malformed hearts,

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an archive of formalin-fixed tissues stored for more than 20 years [3–7,10,11]. Although only a limited number of studies have been performed, the abundance of somatic mutations detected by Reamon-Buettner and Borlak does not fit with the limited number of mutations in other studies. No *NKX2.5* gene mutations could be found in cardiac tissue from patients with bicuspid aortic valve (BAV) and associated aneurysm [12], and no somatic 22q11.2 deletions could be identified in heart tissue from patients with conotruncal heart defects without germline 22q11.2 deletion [13]. Recently, no evidence for somatic *NKX2.5* mutations was found in 28 fresh-frozen cardiac tissues taken near the septal defect of patients with atrial septal defect (ASD), VSD and atrioventricular septal defect (AVSD) [14]. Therefore, the role of somatic mutations in CHDs awaits further confirmation.

In the present study, we have attempted to address the hypothesis that somatic mosaicism is important in isolated CHDs analyzing for mutations the *GATA4* and *NKX2.5* genes in the fresh-frozen tissue obtained from a series of 62 CHD patients who underwent cardiac surgery. Of these, 35 were affected by cardiac septal defects, and 27 by various CHDs previously related to rare mutations in either *NKX2.5* or *GATA4* gene [15–17].

2. Materials and methods

From March 2008 to December 2009, 48 patients (31 females, 17 males) were evaluated and enrolled in the cardiology consultation of the Abood Shaio Foundation, San Ignacio Hospital, Bogotá, Colombia. Fourteen additional patients were accrued at the Monaldi Hospital, Second University of Naples, Naples, Italy (4 females, 10 males) (Table 1 and Table S1). In all patients, the cardiac diagnosis was confirmed by one or more of the following: echocardiography, cardiac catheterization, surgical intervention, and/or autopsy. Patients with major extracardiac malformations and/or with facial dysmorphisms in the setting of chromosome anomalies, mendelian or unknown syndromes or associations, and patients with 22q11.2 microdeletion were excluded from the study. All patients were sporadic. From all patients we collected either peripheral blood or a sample of non-affected cardiac tissue and a cardiac biopsy of approximately 0.5–1.5 mg located within or in close proximity (1–3 mm) of the cardiac defect. Tissue samples were acquired at the time of operation, immediately snap frozen in liquid nitrogen and then stored in a freezer at −80 °C. Blood samples were collected before transfusion. Written informed consent was obtained for all patients. Genomic DNA was isolated from pathologic cardiac tissue, blood and non-diseased cardiac tissue using standard procedures.

Table 1
Diagnoses of screened study population with congenital heart disease.

Cardiac diagnosis	Number of patients
Septation defects	(35)
A trial septal defects	11
Ostium primum (4)	
Ostium secundum (7)	
Ventricular septal defects	18
Perimembranous (9)	
Muscular (7)	
Supracristal (2)	
Atrioventricular septal defects	6
Complete AVCD (3)	
Partial AVCD (3)	
Left-sided defects	(19)
Coarctation of the aorta	14
Mitral insufficiency	1
AS or sub AS	2
Subaortic membrane	1
Bicuspid aortic valve	1
Conotruncal defects	(5)
Tetralogy of Fallot	4
Truncus arteriosus	1
Other	(3)
Transposition of the great arteries	1
Total anomalous venous return	1
Corrected transposition of the great arteries	1

AVCD, Atrioventricular canal defect; AS, Aortic stenosis.

The entire coding regions, including exon–intron boundaries, of the *GATA4* and *NKX2.5* genes were PCR amplified and analyzed for mutations by means of denaturing high performance liquid chromatography (DHPLC), using a WAVE system (Transgenomic, Omaha, NE), except for *NKX2.5* exon 1.1 PCR fragment, which contains the frequent single nucleotide polymorphism 63A→G (Glu21Glu) (refSNP: rs2277923) and was analyzed by direct sequencing. Primers were designed using Primer3 (v. 0.4.0) software (http://frodo.wi.mit.edu/primer3/) based on the cDNA sequences available in GenBank (*NKX2.5* [NM_004387.3], *GATA4* [NM_002052.3]) and the corresponding genomic regions. Primer pair sequences, as well as PCR and DHPLC analysis settings are available in Table S2. PCR products showing an abnormal DHPLC profile were re-amplified starting from the original DNA sample and processed through PCR purification columns (Qiagen, Hilden, Germany) and sequenced using the ABI BigDye Terminator Sequencing Kit v.1.1 (Applied Biosystems, Foster City, CA) and an ABI 3130 Genetic Analyzer (Applied Biosystems). Samples showing DNA base changes were PCR amplified and sequenced twice. The NCBI HomoloGene tool (http://www.ncbi.nlm.nih.gov/sites/entrez?Db=homologene&Cmd=Retrieve&list_uids=4117) was used to analyze the level of conservation of sequence variants in orthologous genes.

3. Results

NKX2.5 and *GATA4* exons and exon–intron boundaries were screened for mutations by DHPLC analysis or direct sequencing in 62 individuals with ASDs ($n = 11$), VSDs ($n = 18$), AVSDs ($n = 6$), left-sided lesions ($n = 19$), conotruncal defects ($n = 5$), and other cardiac anomalies ($n = 3$). To determine if any of the sequence variants identified in the affected tissue was germline or somatic, in addition to the pathologic cardiac tissue DNA specimen, blood sample or phenotypically normal tissue was also screened from each patient. Direct sequencing (exon 1.1) and DHPLC scanning (exons 1.2, 2.1 and 2.2) of *NKX2.5* gene in the pathologic cardiac tissue DNA specimens allowed the identification of 3 different sequence substitutions in 34 patients, including a known non-synonymous variant (c.73C→T; p.Arg25Cys) [15,16,18–22], and two polymorphisms, one synonymous (63A→G, refSNP: rs2277923) and one located in the 3′ untranslated region (UTR) of the gene (c.*61G→T, refSNP: rs703752). Eight separate *GATA4* sequence substitutions were found in 9 unrelated patients, including 6 novel variants (one non-synonymous [c.470C→G, p.Ser157Cys], one synonymous [c.543C→T, p.Ala181Ala], one in the 5′UTR [c.-193G→C], and three intronic [c.IVS2+23C→T; c.IVS3-14T→C; c.IVS5-20G→A]), and two polymorphisms (c.1129A→G [p.Ser377Gly], refSNP: rs3729856; c.1138G→A [p.Val380Met] [23]). Details of the specific sequence variants and cardiac phenotype of mutation-positive patients are summarized in Table 2. Importantly, all nucleotide variants found in the pathologic tissue were also present in the blood sample (or unaffected tissue) taken from the same patient, which demonstrates that they all were constitutional variants (Tables S3, S4a and S4b). Except for intronic variant c.IVS2+23C→T which was identified in a patient from the Italian cohort and was confirmed to be absent in 200 population-matched controls (Italians of Caucasian origin), all other variants were detected in patients from Colombia. Unfortunately, population-matched controls were unavailable to increase the evidence for pathogenicity of sequence variants identified in this population.

Table 2
Sequence variations identified in CHD population.

Gene	Nucleotide change	Amino acid change	Cardiac phenotype (number of patients)	Type	Status (refSNP)
NKX2.5	c.63A→G	p.Glu21Glu	VSD (11), ASD (4), CoA (8), TOF (2), AS (2), AVSD (1), BAV (1), TGA (1), SM (1), TAVR (1), PS (1), and TA (1)	Silent polymorphism	[15] (rs2277923)
	c.73C→T	p.Arg25Cys	CoA (2)	Non-synonymous variant	[15,16,18–22]
	c.*61G→T	–	VSD (4), ASD (5), CoA (7), AVSD (3), MI (1)	3′UTR polymorphism	(rs703752)
GATA4	c.-193G→C	–	CoA (1)	5′UTR variant	Unreported
	c.470C→G	p.Ser157Cys	VSD (1)	Non-synonymous variant	Unreported
	c.543C→T	p.Ala181Ala	TGA (1)	Synonymous variant	Unreported
	c.IVS2+23C→T	–	VSD (1)	Intronic variant	Unreported
	c.IVS3-14T→C	–	VSD (1)	Intronic variant	Unreported
	c.1129A→G	p.Ser377Gly	VSD (1), CoA (1)	Non-synonymous polymorphism	[23] (rs3729856)
	c.1138G→A	p.Val380Met	AVSD (1)	Non-synonymous polymorphism	[23]
	c.IVS5-20G→A	–	VSD (1)	Intronic variant	Unreported

4. Discussion

In a series of relevant papers, Reamon-Buettner and Borlak have attempted to address the hypothesis that somatic mosaicism is important in isolated CHD using the Leipzig heart collection, which includes 68 formalin-fixed hearts of patients with ASDs, VSDs, and AVSDs, some in the context of complex CHD, which were collected between 1954 and 1982 [3–6,9,10]. The authors screened multiple transcription factors known to be relevant for cardiac development, such as *NKX2.5*, *TBX5*, *GATA4*, *HEY2* and *HAND1* for mutations, and found many in diseased tissue [3–6,9,10]. Some patients carried multiple missense mutations, which were mainly absent in unaffected cardiac tissue of the same patients. Some of the mutant proteins were expressed in yeast and found to have variable degrees of decreased or absent transcriptional activity. Moreover, the presence of multiple mutations was found to synergistically reduce transactivation capacity of *NKX2.5*, suggesting a gene-dosage effect.

In the present study, we aimed to corroborate these important findings by investigating *GATA4* and *NKX2.5* mutations in diseased tissues from patients with non-syndromic CHD. Our study cohort included cardiac septal defects as well as other heart anomalies previously related to *GATA4* or *NKX2.5* germline and somatic mutations [16,17,24]. Differently from Reamon-Buettner and Borlak, who used DNA isolated from archival tissues, we used DNA obtained from fresh-frozen pathologic cardiac tissue specimens and compared results obtained in pathologic tissues with corresponding blood samples or non-diseased tissue. Contrary to previous studies performed on formalin-fixed tissues, present mutation survey identified no *NKX2.5* or *GATA4* somatic mutation in any of the fresh-frozen cardiac septal defects, as well as in any of the other CHDs examined. A possible explanation for the absence of somatic mutations in the present cohort could be the wide range and small number of CHD cases examined. In fact, we cannot exclude that somatic mutations may rarely contribute to CHDs, especially given the low prevalence of *NKX2.5* and *GATA4* mutations in the CHD population [16,17,24]. Nevertheless, absence of somatic mutations in all fresh-frozen septal defects currently examined visibly contrasts with the “huge” number of somatic mutations previously detected by Reamon-Buettner and Borlak in formalin-fixed hearts with cardiac septal defects. Precisely, they reported the identification of *NKX2.5* somatic mutations in more than 95% of patients with septal defects (66 of 68 hearts had a sequence variant) [3,11]. Moreover, most of their patients had multiple somatic mutations, with formalin-fixed VSD samples carrying up to 14 mutations. Hence, complete absence of somatic mutations in any of the 35 fresh-frozen septal defects examined here, namely 11 ASDs, 18 VSDs, and 6 AVSDs, is remarkable. Moreover, our data are consistent with recent results of Draus and colleagues, who did not find any *NKX2.5* somatic mutation in a series of 28 fresh-frozen cardiac tissues from patients with septal defects, namely 13 ASDs, 5 VSDs, and 10 AVSDs [14]. Taken together, present and previous study have excluded somatic mutations in the fresh-frozen pathologic tissue of a total of 63 cardiac septal defects (24 ASDs, 23 VSDs, and 16 AVSDs), a cohort well-comparable with the 68 formalin-fixed hearts examined by Reamon-Buettner and Borlak (16 ASDs, 29 VSDs, and 23 AVSDs) [3,5,6,11]. Given that formalin can cause random base damage and affect polymerase chain reaction fidelity [25], it has been previously hypothesized that the poor DNA quality from the formalin-fixed tissue used by Reamon-Buettner and Borlak could have accounted for the high amount of observed somatic mutations [14]. To strengthen this suggestion Draus and colleagues also looked into available mutational spectra databases and pointed out that somatic mutational spectra from the Reamon-Buettner and Borlak studies were significantly different from the germline *NKX2.5* mutations, as well as from inherited disease associated mutations, tumor derived somatic mutations, and mitochondrial mutations [14]. We consider additional explanations for this discrepancy highly unlikely, including the possibility that some mosaic confined to a few cardiac cells could undergo undetected using DHPLC analysis, or even that we screened a non appropriate cardiac tissue specimen. It should be considered that data obtained by previous studies were acquired using conventional sequencing, and compared to sequencing DHPLC is a more robust technique in detecting low level mosaicism [26]. Moreover, all tissues used in the present analysis, similarly to previous studies, were sampled within or very close to the cardiac defects, excluding that differences in the sampling criteria between the studies might have accounted for the discrepancy in the results.

Of note, although we found no evidence of somatic mutations, we identified one variant and two polymorphisms in the *NKX2.5* gene, and six variants and two polymorphisms in the *GATA4* gene, proving that our mutation scanning protocol is enough sensitive to identify sequence variations in these genes. It might be possible that some of these variants represent non-random changes. However, neither family members nor ethnically matched controls were available to increase the evidence for pathogenicity of these sequence changes.

5. Conclusions

The present study has identified constitutional *NKX2.5* and *GATA4* mutations in our CHD cohort, but was unable to replicate the previously published findings of somatically derived sequence variants in patients with cardiac septal defects using fresh-frozen cardiac tissue rather than formalin-fixed tissues. Given the low frequencies of mutations in all the genes known to date that can be associated with CHD, and the complex interactions and signaling pathways involved in heart formation, it remains possible, and indeed likely that most CHDs have a multifactorial etiology. Further studies are warranted to unravel the genetic basis of cardiac defects.

Disclosure statement

The authors declare no conflict of interest.

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Appendix. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.ejmg.2011.01.004.

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