ONLINE MUTATION REPORT

Spectrum of atrial septal defects associated with mutations of NKX2.5 and GATA4 transcription factors

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Atrial septal defect (ASD) is a common cardiovascular malformation, affecting over 1 in 1000 live births, accounting for 10% of congenital heart defects (CHD).[1] ASD refers to a communication between the right and left atria, anatomically classified into the deficient atrial septum structure. ASD ostium secundum (ASDos) is the prevalent defect, representing 85% of all ASDs.[2] ASD may be isolated or associated with other CHDs, such as pulmonary valve stenosis (PVS), ventricular septal defect (VSD), or conduction defects. In addition, persistent left to right blood shunt may result in atrial and ventricular dysfunctions and atrial arrhythmias, in the absence of surgical or catheter based repair.

The atrial septum is one of the cardiac structures most sensitive to environmental or genetic factors. Several lines of evidence have highlighted a role for different proteins and transcription factors in the septogenesis process;[3] however, only two genes, encoding for the transcription factors NKX2.5 and GATA4, have been implicated so far in non-syndromic ASDs.[4 5] Clinical and molecular analyses have shown that mutations in these two genes are responsible for ASDs in association with distinct cardiac features.[4–15] Mutations in *NKX2.5*, a member of the NK-2 class of homeobox genes, have been described in autosomal dominant ASDos with progressive atrioventricular (AV) block, and in 1–4% of sporadic ASD patients.[4 6–12] Most of these mutations occur within the homeodomain, a critical protein domain that interacts specifically with DNA, and are associated with conduction anomalies. Low penetrance *NKX2.5* gene mutations, mainly outside the homeodomain, have been found in 5% of patients with tetralogy of Fallot, and in a number of individuals with other CHDs and normal conduction.[4 6 9–13] Recently, heterozygous mutations in the *GATA4* zinc finger transcription factor gene have been identified in three families with autosomal dominant ASDos and normal conduction.[5 14] Individuals with *GATA4* mutations may also show concurrent CHDs, in particular PVS.

In this study, we screened the *NKX2.5* and *GATA4* genes in a cohort of Italian families and sporadic patients affected by non-syndromic ASDs, in order to assess the prevalence of these mutations and appraise any genotype–phenotype correlations.

Key points

- Heterozygous mutations in the *NKX2.5* and *GATA4* genes have been detected in patients with atrial septal defect (ASD) and other congenital heart defects.
- In order to assess the mutations prevalence and appraise any genotype–phenotype correlations, we screened *NKX2.5* and *GATA4* in a cohort of patients with isolated ASD or ASD associated with concurrent heart defects.
- *NKX2.5* and *GATA4* coding regions were screened by direct sequencing in 16 familial and 13 sporadic ASD patients.
- We identified one previously reported *GATA4* and three novel *NKX2.5* mutations. The *NKX2.5* mutations (one missense and two frameshifts) were detected in two families and one sporadic patient. ASD was detected in all affected individuals, and adults also showed conduction anomalies. In addition, multiple muscular ventricular septal defects and non-compaction of the left ventricular myocardium were detected in one kindred. The Gly296Ser *GATA4* mutation was found in two families segregating ASD and pulmonary valve stenosis (PVS) with normal conduction.
- These results confirm that *NKX2.5* and *GATA4* mutations are major causes of different patterns of ASDs, mostly in familial cases. While associated conduction defects are strongly suggestive for *NKX2.5* mutations, PVS with normal conduction should require *GATA4* gene screening first.

METHODS

Subjects

Patients were recruited at the Pediatric Cardiology Unit of the Bambino Gesù Hospital and Policlinico Umberto I Hospital in Rome, Italy. Sporadic patients were enrolled from July 2003 to April 2004, while families were selected from a cohort of patients recruited retrospectively for genetic studies on CHDs from 1995 to 2004. Clinical assessment included complete physical evaluation of dysmorphisms and malformations, anthropometric measurements, renal ultrasonography, and radiological studies. Cardiac evaluation included preoperative chest *x* ray film, 12 lead electrocardiogram, and two dimensional transthoracic echocardiography with colour flow Doppler. All patients had a classic form of ASDos, with a defect diameter of >5 mm. Family history was assessed in all cases. The patients' parents and available family members were investigated for cardiac and extracardiac anomalies. After written informed consent, blood samples were obtained from all available affected individuals and unaffected parents, when possible. Karyotype analysis was performed in all patients. Criteria for patient exclusion were: major CHDs such as single ventricle and conotruncal anomalies, distinct syndromic associations, and identified chromosomal anomaly including 22q11 deletion. Sporadic and familial index patients were included in the mutation screening. Following mutation identification, molecular analysis was also performed in affected and unaffected relatives.

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Molecular analysis

The *NKX2.5* and *GATA4* gene coding regions and exon–intron boundaries, including about 50 bp upstream and downstream from the exons, were amplified from genomic DNA by PCR using standard techniques. The PCR products were sequenced by the Big Dye Terminator ABI Prism sequencing kit (Applied Biosystems, Foster City, CA, USA), and run on an ABI Prism 3100 Genetic Analyzer automated sequencer (Applied Biosystems).

RESULTS

The ASD study group included 16 families (Fam.01–16) with multiple affected members and 13 unrelated sporadic individuals (Sp.01–13). Familial index patients were six males and 10 females, with an age ranging between 0.9 and 17 years. In nine families, ASDos segregated as an isolated anomaly. AV conduction defects, VSD, PVS, left ventricular myocardium noncompaction (LVMN), atrioventricular canal defect (AVCD), anomalous pulmonary venous return (APVN), and mitral valve prolapse (MVP) were observed as concurrent CHDs in the remaining families. There were six male and seven female sporadic patients, whose ages ranged between 3 and 35 years. Four of these patients presented concurrent cardiac defects: AV block in Sp.01, VSD plus PVS in Sp.04, PVS in Sp.05, and supraventricular tachycardia in Sp.06. *NKX2.5* or *GATA4* sequence variations were detected in five of the 29 index patients (17%) (table 1). Detailed pedigrees of familial and sporadic patients with mutation are shown in fig 1.

NKX2.5 mutations

None of the detected *NKX2.5* mutations has been previously reported. Two DNA sequence variations involved amino acid changes in the homeodomain and one involved a change in the C terminus of the protein. Two mutations are predicted to cause premature terminations of translation. The first, a heterozygous 1 bp insertion in the homeodomain (498-499insC), results in a frameshift and a premature stop codon at amino acid 250, with a 75 amino acid deletion at the C terminal. The second is a heterozygous 2 bp deletion (605-606delTG), also causing an identical premature termination of translation. The heterozygous 554G→T mutation results in a Trp185Leu codon change affecting the homeodomain. We regard these DNA sequence variations as pathogenic mutations, because of their co-segregation with the disease in the familial cases, the truncating nature of the mutation or the involvement of a highly conserved amino acid (Trp185), and the negative results from 100 normal chromosomes from a random control population.

The 605-606delTG mutation was found in three members of family 1. The proband (IV:1), at the age of 6 years, manifested ASDos with second degree AV block. Her mother (III:3) was affected by ASDos, complete AV block, and VSD. Individual II:3, at the age of 65 years, showed ASDos with

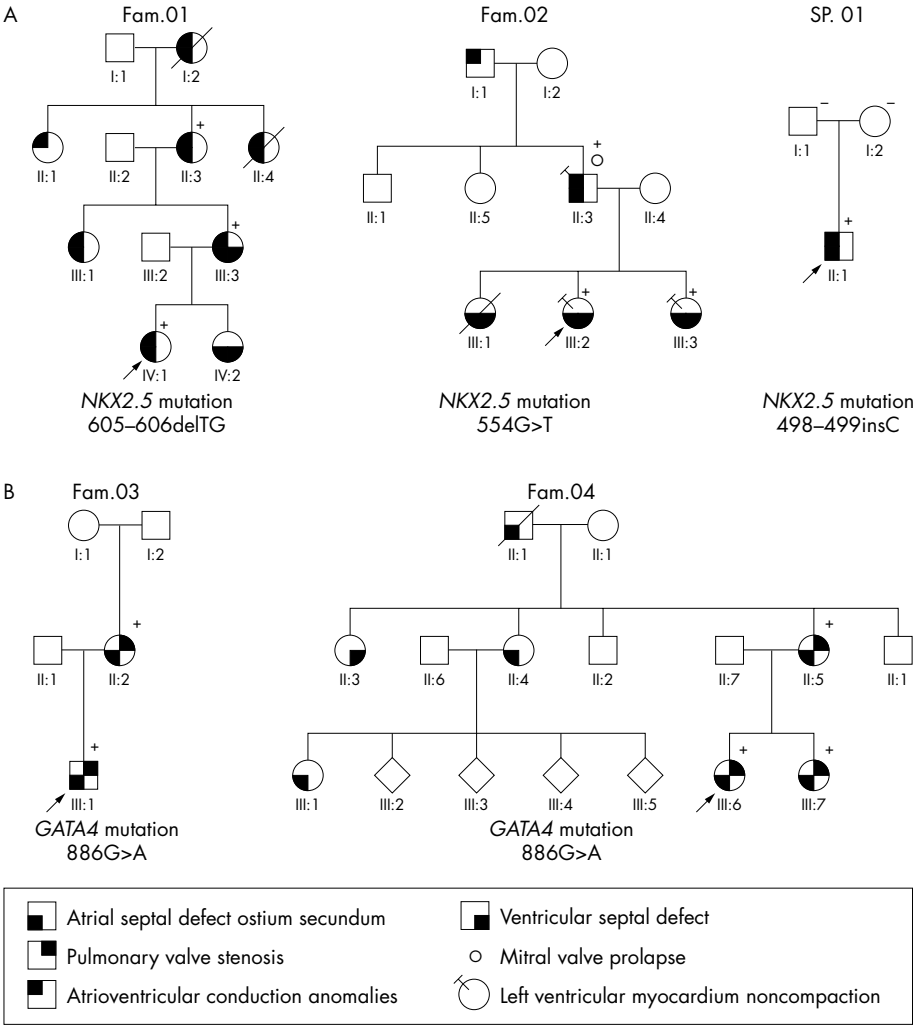

Figure 1 Pedigrees of patients with *NKX2.5* (A) or *GATA4* (B) mutations. Different symbols indicate distinct cardiac defects; +/− symbol represents presence/absence of mutation. Arrows indicate index patients. The mutation found in each family is specified under the pedigree.

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Table 1 Clinical and molecular characteristics of familial and sporadic patients

Mutation	Familial patients	Sporadic patients	Cardiac defects: ASDos	VSD	PVS	AV block	Other
NKX2.5	2	1	3	2*	0	3	1 LVMN; 1 MVP
GATA4	2	0	2	1	2	0	
Negative	12	12	24	2	3	0	1 AVCD; 1 APVR, 1 SVT
Total	16	13	29	5	5	3	5

*One familial case with multiple muscular ventricular septal defects. ASDos, atrial septal defect ostium secundum; VSD, ventricular septal defect; PVS, pulmonary valve stenosis; AV, atrioventricular; LVMN, left ventricular myocardial noncompaction; MVP, mitral valve prolapse; AVCD, atrioventricular canal defect; APVR, anomalous pulmonary venous return; SVT, supraventricular tachycardia.

ECG evidences of AV block. Individuals II:1, III:1, and IV:2 were not available for molecular testing. While subject II:1 had a history of isolated rhythm anomalies, individuals III:1 and IV:2 were affected by ASDos, in association with rhythm disturbances in III:1 and VSD in IV:2, respectively (fig 1A).

The 554G→T mutation was identified in three affected members of family 2. The proband (III:2), a 9 month old girl, and her younger sister (III:3) had ASDos, associated with multiple muscular VSDs (''Swiss cheese'' VSD). Their father (II:3), who was affected by ASDos with complete AV block and MVP, had undergone a surgical repair and a pacemaker implantation. Both affected father and daughters showed echocardiographic findings of LVMN. The paternal grandfather (I:1), who was not genotyped, had a history of rhythm disturbances and pacemaker implantation. Individual III:1 was diagnosed in infancy with ASDos and multiple muscular VSDs, and died of severe heart failure at 8 months of age. Blood samples were not available from individuals II:1 and II:5, who were reported as clinically normal (fig 1A).

The 498-499insC frameshift de novo mutation was detected in patient Sp.01 (individual II:1), who was diagnosed with ASDos and a second degree AV block in early infancy. The ASD was corrected at the age of 6 years (fig 1A).

GATA4 mutations

The 886G→A (Gly296Ser) *GATA4* mutation, previously reported by Garg *et al*, was identified in two kindreds.[5] This mutation segregated in two members of family 3 (II:2 and III:1), both affected by ASDos and PVS, with a right ventricle to pulmonary artery gradient >40 mmHg, which required a surgical repair. In individual III:1, hypospadia was diagnosed in infancy. Parents of individual I:1 were not blood sampled, but they were reported as clinically normal (fig 1B).

The 886G→A mutation was also identified in individuals II:5, III:6, and III:7 of family 4, all of whom were affected by ASDos and PVS with a right ventricle to pulmonary artery gradient of 50 mmHg, which was surgically corrected at the age of 25 years in II:5 and at the age of 1 year in III:7. Blood samples were not available from other affected members (II:3, II:4, and III:1), who had a history of a ASD or VSD (details unavailable). ASDos was echographically diagnosed in the deceased family member I:1 (fig 1B).

DISCUSSION

Functional haploinsufficiency of *NKX2.5* and *GATA4* genes represents an important cause of non syndromic ASDs. In the present study we identified one recurrent *GATA4* and three novel *NKX2.5* mutations, associated with different types of CHDs, including ASDs.

The most consistent CHDs associated with the *NKX2.5* mutations reported here were ASDos, AV block, and VSD. Previous studies have shown that missense mutations within the gene homeodomain (in particular those located in the third helix, such as the Trp185Leu change identified in family 2) reduce DNA binding and transcriptional activation.[16] These mutations result in fully penetrant ASD and AV block.[4 6] In two subjects, Trp185Leu was also associated with multiple muscular VSDs, a feature previously reported only in a family segregating the Tyr259ter mutation.[6] LVMN, diagnosed in all subjects with Trp185Leu mutation, has been previously reported in association with *NKX2.5* haploinsufficiency only once, in a patient with 5q deletion.[17] The cardiac features shown by the affected members of family 2 reveal a pathogenetic link between multiple muscular VSD and LVMN. Previous studies have shown that mice with a ventricular restricted knockout of the *Nkx2.5* gene exhibit echocardiographic and histological evidence of LVMN, and analogous myocardial findings have been also reported in the hypomorphic Gata4 murine model and in the Tbx5 deficient mice.[15 18 19] It is notable that haploinsufficiency of the TBX5 transcription factor in humans is responsible for septal and conduction defects in Holt-Oram syndrome.[20 21] This clinical and molecular evidence supports the functional connection between NKX2.5, GATA4, and TBX5 in cardiogenesis and cardiac diseases.[22 23]

Mutations at the C terminal cause protein truncation after the homeodomain, and result in hypomorphic mutant proteins, with a normal monomeric DNA binding ability, and reduced transcriptional activity.[16] Individuals with truncating mutations after the homeodomain manifest CHDs overlapping those found in individuals with mutations inside the homeodomain.[4 6 7] In fact, the 605-606delTG and 498-499insC mutations resulted in ASD and AV block, associated with VSD in two members of family 2. Interestingly, these mutations, as well as the 701-702insTCCCT mutation and the two nonsense 886C→A and 901C→A mutations,[6 8 12] predict analogous truncated proteins (missing ~ 250 amino acids), arguing for an association between these mutations and ASD with AV block. It is noteworthy that in the present cohort a number of young individuals with *NKX2.5* mutations had normal cardiac conduction. Heart restricted knockout *Nkx2.5* mice manifest defects in AV nodal and working myocyte cell lineages, chamber morphogenesis, maturation, and specification, with gradual nodal degeneration and cell dropout.[19] Progressive AV nodal degeneration has also been documented in individuals with *NKX2.5* mutations, supporting the role of *NKX2.5* in the maintenance and maturation of human AV nodal myocytes.[19] Accordingly, AV block was invariably present in our series of adults with *NKX2.5* mutations, supporting a role of this protein in preserving integrity of the conduction system.

Affected individuals, segregating the Gly296Ser *GATA4* mutation (families 3 and 4), manifested ASDos associated with PVS, in the absence of conduction defects. This observation corroborates previous evidences for an association between *GATA4* mutations and ASD with semilunar

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valve malformations.[5 14 15] In particular, the Gly296Ser mutation, previously reported in one kindred, resulted in full penetrant autosomal dominant ASD and PVS with normal conduction.[5] It has been demonstrated that *GATA4*, which is expressed throughout the developing heart, is essential for heart formation, through interaction with other transcription factors including NKX2.5.[22 24 25] The Gly296Ser mutation affects a highly conserved amino acid localised between the C terminal zinc finger and the nuclear localising signal. This, in turn, not only changes the DNA binding affinity and the transactivation of downstream targets, but also disrupts the interaction between GATA4 and TBX5.[5] While *TBX5* mutations lead to syndromic ASD with conduction defect,[20 21] *GATA4* mutations also cause malformations of the pulmonary outflow tract (including PVS) and extracardiac abnormalities, supporting its role in the morphogenesis of different structures. The same conclusions have also been drawn for the *GATA4* gene haploinsufficiency. Distal 8p deletions in humans are characterised by a wide spectrum of CHDs, microcephaly, other physical anomalies, and mental retardation.[26] The contribution of *GATA4* to the 8p cardiac phenotype has been postulated,[27] although other evidence has mapped *GATA4* outside the critical region for this condition.[28] Heterozygous *GATA4* mutations cause distinct cardiac defects, such as ASD, PVS, AVCD, and dextrocardia, which are also features of 8p syndrome.[5 14 26] Interestingly, one of the patients with Gly296Ser mutation (individual II:1, family 3) also had hypospadia, a defect found in six of seven previously described 8p patients.[26] However, no patient with a *GATA4* mutation manifested additional extracardiac malformations or mental retardation, arguing for a role of GATA4 haploinsufficiency in CHDs and, possibly, genital anomalies, in agreement with its expression pattern.[29]

While *NKX2.5* and *GATA4* mutations may both result in ASDos and VSD, involvement of each of these two genes causes some consistent difference in the pattern of cardiac defects, notably in the conduction system and the semilunar valves. Conduction anomalies do not associate with *GATA4* mutations, while *NKX2.5* haploinsufficiency may also affect the semilunar valves but is generally found in pulmonary valve atresia and tetralogy of Fallot.[13] Intrafamilial variability appears to be age related. In fact, conduction defects, although not detected in infancy, can manifest with increasing age, while spontaneous healing of small VSDs results in an intact septum during adulthood.

We detected *GATA4* mutations only in familial cases, while *NKX2.5* mutations were also identified in sporadic individuals. Analysis of families with mutations supports full penetrance, although the unavailability of some unaffected family members in kindreds with mutations weakens this conclusion.

A prevalence of about 1–4% of *NKX2.5* mutations in sporadic patients with ASDs has been estimated from three previous studies.[8 11 12] No consistent figure is available on the prevalence of mutations in the familial cases, although a single study has reported one unique mutated family of 13 analysed kindreds (8%).[11] The prevalence of *NKX2.5* mutations in the personal series of sporadic cases (1/13; 8%), even if not significant because of the low patient numbers, is consistent with the reported low mutation rate in sporadic ASDs. In contrast, although our familial cohort is also not large, the figure of 2/16 mutations (12.5%) in our pedigrees suggests that the *NKX2.5* mutations could be a major cause of familial ASDs. Notably, we found *NKX2.5* mutations in all families and sporadic patients with AV block. We detected *GATA4* mutations in two of 16 families (12.5%), including two of five individuals with semilunar valves malformations. Thus, *GATA4* seems to be an important gene involved in this type of ASD. Genetic heterogeneity of ASDs was proved by an inability to detect mutations in 83% of our patients. However, the contribution of genes other than *NKX2.5* and *GATA4* in ASD pathogenesis appears likely, particularly in the absence of conduction defects.

In conclusion, the present results corroborate functional links between the transcription factors NKX2.5, GATA4, and TBX5 in cardiac morphogenesis, and confirm that *NKX2.5* and *GATA4* gene mutations cause distinct patterns of ASDs. The frequency of *NKX2.5* mutations in familial ASD with AV block recommends molecular testing in this subtype of defects, while *GATA4* screening is indicated in familial ASD associated with PVS and normal cardiac conduction. Therefore, molecular diagnosis should assist with the clinical management of ASD and improve genetic counselling in these families.

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