

ONLINE MUTATION REPORT

GATA4 zinc finger mutations as a molecular rationale for septation defects of the human heart

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J Med Genet 2005;42:e32 (<http://www.jmedgenet.com/cgi/content/full/42/5/e32>). doi: 10.1136/jmg.2004.025395

GATA4 is localised on human chromosome 8p23.1-p22 and codes for a zinc finger transcription factor. Mice lacking Gata4 suffer from defective ventral morphogenesis and heart tube formation.^{1,2} This factor therefore targets various genes important for heart development and basic cardiac function. Recently, three GATA4 mutations have been detected in families with congenital heart disease.^{3,4} A mutation affecting a residue next to the C-terminal zinc finger has been identified in family members with atrial septal defects, and resulted in the disruption of a physical interaction between Gata4 and TBX5.³ There is growing evidence that the two zinc fingers of Gata4 display distinct functions so as to facilitate protein–DNA and protein–protein interactions.

To further our understanding of the specific role of GATA4 in human congenital heart disease, we searched for mutations in the sequences coding for the zinc fingers in diseased heart tissues of 68 patients with complex heart malformations, encompassing atrial (ASD), ventricular (VSD), and atrioventricular septal defects (AVSD). These patients mostly died in early infancy as a result of the severity of the cardiac abnormalities. We isolated genomic DNA from the formalin fixed malformed hearts, amplified exons 3 and 4 from DNA fragments of about 2 kb, and carried out double strand direct sequencing. Mutations were confirmed by polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) or cloning and subsequent sequencing to separate alleles (fig 1A–F).

We identified 23 mutations, of which several are located in the zinc fingers and basic regions of GATA4 (table 1). Except for three, the genotypes observed in our collection of 68 hearts were mostly heterozygous, as seen on sequence electropherograms. Some patients had two to four mutations, including four with mutations in both zinc fingers of GATA4. Cloning of a fragment from a patient heterozygous for two zinc N-finger mutations showed location on different alleles. Additional cloning of a fragment heterozygous for three non-synonymous mutations (c.631T→C, c.687G→T, c.731A→G) gave three haplotypes after sequencing of four clones—that is, one clone with all three reference alleles (c.631T, c.687G, c.731A), two clones with one mutant allele (c.631T, c.687T, c.731A), and one clone with two mutant alleles (c.631C, c.687G, c.731G). These mutations were absent in DNA isolated from 10 normal formalin fixed hearts, five frozen normal hearts, and 50 blood samples from unrelated healthy individuals used as controls.

COMMENT

Although our heart collection is basically homogeneous with respect to septation defects, we found 21 hearts that did not carry mutations in the zinc fingers and adjacent amino acids. This suggests that additional molecular changes result in a similar disease phenotype. It is of importance that mutations were absent in unaffected tissues of the same heart (table 1), and this agrees well with our findings on NKX2-5.^{5,6} We

Key points

- GATA4 is a zinc finger transcription factor and master regulator of heart development.
- A zinc finger mutation was identified which affects a zinc coordinating cysteine in the C-terminal finger and which is strongly associated with ventricular septal defects.
- GATA4 zinc finger mutations were shown to affect DNA binding, contacts on zinc ion, and protein secondary structure.
- Impaired GATA4 interaction with the third helix of the homeodomain of NKX2-5 results in septation defects of the human heart.

therefore show that GATA4 zinc finger mutations also occur somatically, most probably from cell division errors during early embryogenesis. Notably, among the mutations is c.874T→C (Cys292Arg), which is located in the C-terminal zinc finger, affects one of the four zinc coordinating cysteines, and is predicted to disrupt the secondary structure of the C-finger (fig 1G). This mutation was detected in 19 of 29 VSDs, six of 16 ASDs, and six of 23 AVSDs. Many affected VSDs had a subaortic VSD with overriding aorta together with other cardiac anomalies. For instance, patient D03VSD had a subaortic/subpulmonic VSD with overriding aorta, a patent foramen ovale, a patent ductus arteriosus, and a double outlet left ventricle.

Gata4 regulates expression of genes through its zinc fingers. The N-terminal zinc finger interacts with Fog2, and replacement of an amino acid (mouse Val217Gly) in the N-finger impairs physical interactions.⁷ Mutant mice died at E12.5 and showed semilunar cardiac valve defects and a double outlet right ventricle. We found six mutations in the N-finger of GATA4, including a homozygous deletion (677delC) that would lead to a frameshift mutation affecting critical residues. The patient with this mutation had subaortic VSD, septum primum defect (15 mm), patent foramen ovale, patent ductus arteriosus, and a common atrioventricular canal. Disruption of GATA4–FOG2 interaction most probably contributed to the cardiac anomalies of this patient. Further, four of the mutations that we found in the N-finger are highly conserved amino acids and may also affect DNA binding or protein–protein interactions of GATA4.

The C-terminal zinc finger is required for the many interactions of Gata4 including Nf-At3⁸ and Nkx2-5^{9–12} resulting in transcriptional synergy of regulated genes. Gata4–Nkx2-5 interaction involves the third helix (homeodomain) of Nkx2-5 and the C-finger of Gata4. Mutagenesis experiments involving the zinc coordinating

Abbreviations: ASD, atrial septal defect; AVSD, atrioventricular septal defect; VSD, ventricular septal defect

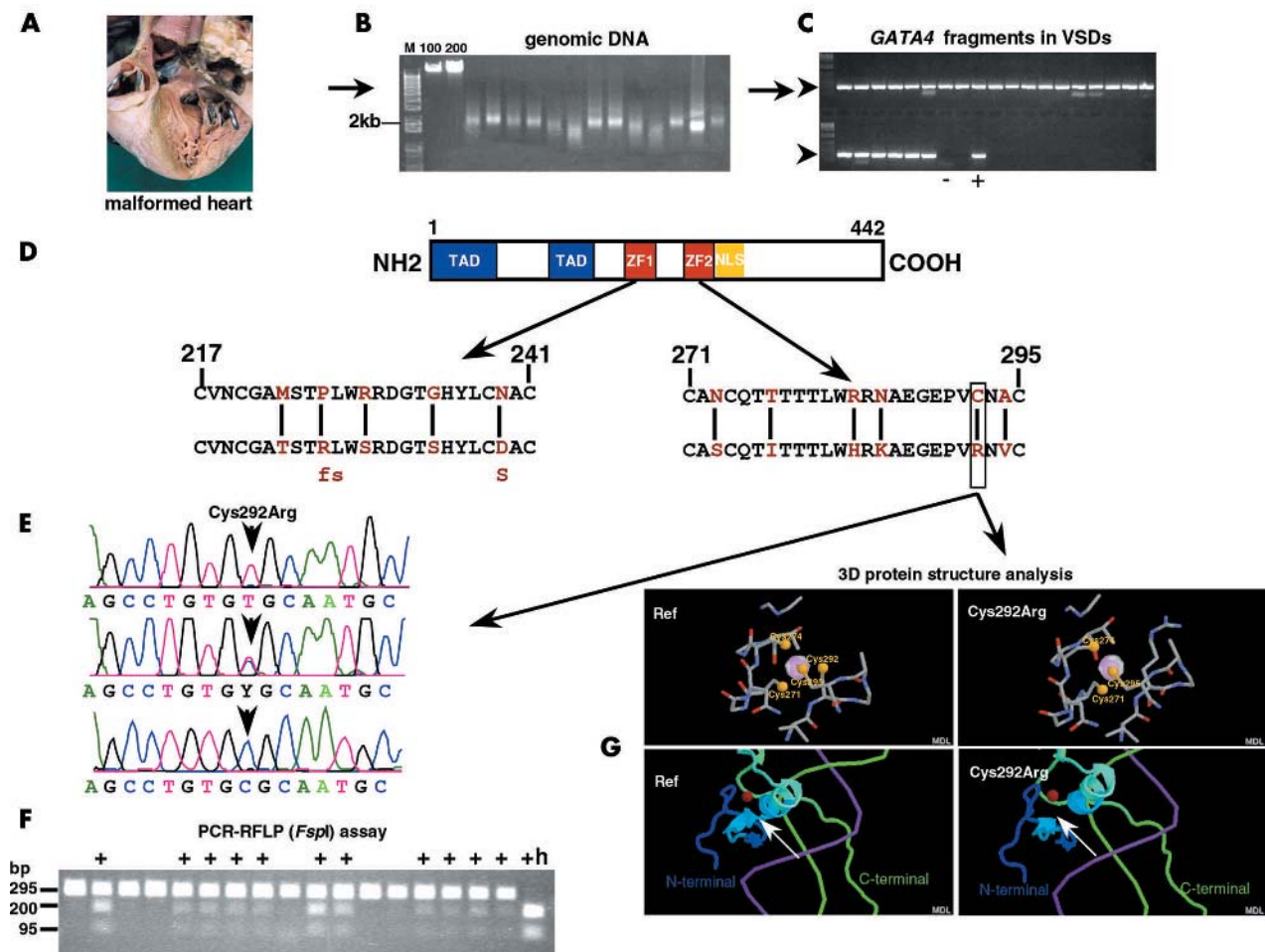


Figure 1 Analysis of GATA4 mutations in malformed human hearts. (A) DNA was isolated from the affected tissues of 68 formalin fixed hearts with complex cardiac malformations. (B) Genomic DNA was about 2 kb and 50 ng was used for polymerase chain reaction (PCR). (C) Exons 3 and 4 of GATA4 were amplified to analyse mutations affecting the two zinc fingers; arrows show exon 4 fragments; -/+ depicts negative and positive controls, respectively. (D) The domains of GATA4 and location of identified mutations in the two zinc fingers, especially Cys292Arg which affects one of the four zinc coordinating cysteines in the C-terminal finger and prevalent in hearts with subaortic ventricular septal defects with overriding aorta (E) Sequence analysis of Cys292Arg, showing homozygous (for T or C) and heterozygous genotypes (Y). (F) PCR-RFLP (restriction fragment length polymorphism) assay for Cys292Arg, which creates a site for *FspI*; +h denotes a homozygous genotype for the mutation. (G) The effect of Cys292Arg on protein structure was determined using the C-terminal domain of chicken Gata1 bound to DNA as a model (PDB id: 2gat), Swiss-PdbViewer (<http://expasy.org/spdbv/>) and Protein Explorer (<http://proteinexplorer.org>). Left panels are the reference structures; the right panels are with cysteine in position 292 mutated to arginine. Upper panels show important residues near the ligand zinc, particularly sulphur atoms (yellow) of the four coordinating cysteines. Lower panels show the ribbon structure and Cys292Arg disrupts the secondary structure (arrow).

cysteines abolished DNA binding, binding ability to Nkx2-5 and synergy.^{9, 10} Our mutation Cys292Arg will certainly have the same outcome to result in loss of NKX2-5–GATA4 protein–protein interaction, and this was highly associated with septation defects in our patient cohort. This mutation was prevalent in VSDs as compared to ASDs and AVSDs.

We detected further five C-finger mutations in AVSDs, of which three were also highly conserved residues. Using the C-terminal domain of chicken Gata1 bound to DNA as a template (see PDB id: 2gat), Swiss-PdbViewer (<http://expasy.org/spdbv/>) and Protein Explorer (<http://proteinexplorer.org>), we located homologous residues and predicted the consequence of mutations on DNA binding, contacts on zinc, and protein secondary structure. Two of the five mutations would affect 19Arg and 30Ala, which are in close contact with the DNA. Indeed, mutations Arg283His and Ala294Val would weaken but not abolish DNA contact, although it has been shown, that mutants on the C-finger with intact DNA binding ability may still abolish Gata4–Nkx2-5 interaction and synergy.¹⁰ Further, mutation Ala294Val will disrupt the secondary structure similar to Cys292Arg (fig.1G).

Next to Nkx2-5, the Gata4 C-finger also interacts with the basic helix-loop-helix (bHLH) domain of dHand to activate synergistically the expression of heart specific genes such as atrial natriuretic factor (ANF), the brain type natriuretic peptide (BNP) and the α -myosin heavy chain (α MHC).¹³ The residues WRR (mouse, 281–283 WRR) are critical for the interactions with dHand, which in turn are crucial for Gata4 interaction with a coactivator, p300.¹⁴ We found one AVSD patient with the mutation Arg283His, which affects one of these critical residues. This patient had subaortic VSD with overriding aorta, septum primum defect, patent foramen ovale, and a common atrioventricular canal. The Gata4 C-finger likewise interacts with the DNA binding domains of Mef2 proteins to activate ANF, BNP, α MHC, and α cardiac actin (α CA) synergistically.¹⁵ A point mutation in one of the zinc coordinating cysteines (mouse, Cys273Gly) that had abolished Gata4 DNA binding and Gata4–Nkx2-5 physical interaction⁹ likewise destroyed Gata4–Mef2 synergy. Moreover, it has been shown that the C-finger and adjacent basic domain of Gata4 are required for interaction with Smad proteins in the activation of Nkx2-5.¹⁶ A double mutant (mouse

Table 1 Summary of GATA4 mutations

Nucleotide change	Amino acid change	Location	Positive hearts (VSD)	Positive hearts (ASD)	Positive hearts (AVSD)	PCR-RFLP assay	Matched healthy heart tissue
c.622T→C	Phe208 Leu	exon 3	1				E22VSD, absent
c.631T→C	Phe211 Leu	exon 3	1		1		
c.640G→A	Gly214Ser	exon 3	1				
c.668T→C	Met223Thr	exon 3, 1st zinc finger	1			TaqI	E27VSD, absent
c.677delC	Pro226fs	exon 3, 1st zinc finger			1*		F08AVSD, absent
c.687G→T	Arg229Ser	exon 3, 1st zinc finger	1	1	1	TaqI	E27VSD, absent
c.700G→A	Gly234Ser	exon 3, 1st zinc finger			1	HinfI	C70/2AVSD, absent
c.715A→G	Asn239Asp	exon 3, 1st zinc finger	1				
c.716A→G	Asn239Ser	exon 3, 1st zinc finger	1				
c.731A→G	Tyr244Cys	exon 3, basic region	1		1		
c.743A→G	Asn248Ser	exon 3, basic region		1	1	BsrBI	F02AVSD, absent
c.755G→C	Arg252Pro	exon 3, basic region			1		C70/2AVSD, absent
c.764T→C	Ile255Thr	exon 3, basic region		1			
c.779G→A	Arg260Gln	exon 3, basic region	1			MspAII	
c.782T→C	Leu261Pro	exon 3, basic region	1	1			
c.796C→T	Arg266Ter	exon 4			1*	MspAII	F12AVSD, absent
c.818A→G	Asn273Ser	exon 4, 2nd zinc finger			1	AluI	F28AVSD, absent
c.830C→T	Thr277Ile	exon 4, 2nd zinc finger			1	HphI	
c.848G→A	Arg283His	exon 4, 2nd zinc finger			1	HhaI	E43AVSD, absent
c.855T→G	Asn285Lys	exon 4, 2nd zinc finger			1	BseMI	
c.874T→C	Cys292Arg	exon 4, 2nd zinc finger	19*	6	6	FspI	in 20 matches, absent
c.881C→T	Ala294Val	exon 4, 2nd zinc finger		1			
c.905A→G	His302Arg	exon 4, basic region, nls			1	SacII	

*Homozygous genotypes observed.

+1 in nucleotide numbering is A of start codon ATG.
nls, nuclear localisation signal.

Cys270Gly/Cys273Gly) in the C-finger of Gata4 abolished binding and disrupted interaction with Smad factors.

There is much evidence that Gata4 participates in combinatorial interactions in regulating gene expression of cardiac genes. Crucial for these interactions are the zinc fingers, especially the C-terminal zinc finger. Recently, zinc deficiency in rats was shown to result in impaired Gata4 expression and complex cardiac anomalies.¹⁷ Here we report zinc finger mutations, one of which disrupts zinc coordination and was highly associated with ventricular septal heart defects. Further, we identified in the same cohort of mutations in the binding domains of NKX2-5^{5,6} and TBX5.¹⁸ It will therefore be interesting to determine whether these mutations will disrupt GATA4–NKX2-5 or GATA4–TBX5 combinatorial interactions to contribute to complex cardiac malformations.

ACKNOWLEDGEMENTS

We wish to acknowledge the technical support of Si-Hyen Cho, Annika Roskowitz, and Andreas Hiemisch as well as the financial support of the Lower Saxony Ministry of Science and Culture to JB.

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Competing interests: none declared

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