Novel *NKX2–5* Mutations in Diseased Heart Tissues of Patients with Cardiac Malformations

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NKX2–5 is a homeodomain-containing transcription factor important in cardiac development. Familial mutations in the *NKX2–5* gene are associated with cardiac abnormalities, but mutations are rare in sporadic cases. We studied the pathology and molecular genetics of *NKX2–5* in diseased heart tissues of 68 patients with complex congenital heart disease (CHD), particularly atrial (ASD), ventricular (VSD), and atrioventricular septal defects (AVSD). We also studied DNA extracted from 16 normal hearts, as well as lymphocytic DNA from 50 healthy volunteers, 7 families, and 4 unrelated individuals with CHD. Direct sequencing revealed 53 *NKX2–5* mutations in the diseased heart tissues, including nonsynonymous substitutions in the homeodomain of *NKX2–5*. We found common mutations among unrelated patients, but certain mutations were specific to VSDs and AVSDs. Many patients had multiple *NKX2–5* mutations, up to 14 nonsynonymous mutations per patient in VSDs. Importantly, these nonsynonymous mutations were mainly absent in normal heart tissues of the same CHD patients, thus indicating somatic origin and mosaicism of mutations. Further, observed mutations were completely absent in normal hearts and lymphocytic DNA of healthy individuals. Our findings provide new insights for somatic *NKX2–5* mutations to be of importance in congenital heart disease. (Am J Pathol 2004, 164:2117–2125)

Nkx2–5 is a transcription factor which contains a highly conserved 60-amino-acid homeodomain.[1] This homeodomain (HD) interacts with DNA through a helix-turn-helix DNA-binding motif of three α helices, with helix 3 providing binding specificity. Nkx2–5 contains additional conserved regions, the TN domain and the NK2-specific domain (NK2-SD). Functions of these two conserved domains are not known, but NK2-SD may serve as a trans-activation regulator of Nk-2 family members.[2] The human

NKX2–5 (*CSX1*) gene maps to chromosome 5q34 and consists of two exons that encode a 324-amino-acid protein.[3,4]

Nkx2–5 occupies a crucial position in the hierarchy of cardiac determinants[5] and this factor is important to heart development in many organisms including zebra fish, frog, chick, mouse, and human.[6] In knockout mice, lack of Nkx2–5 is lethal or results in impaired cardiac development, thus demonstrating an essential role for Nkx2–5 in normal heart morphogenesis and function.[7–10] Mutations in the human *NKX2–5* gene are associated with cardiac anomalies,[11–18] but the molecular mechanisms are still unclear. Familial cases studied have different mutations and no mutation can be associated with a specific clinical phenotype.

The high incidence of severe congenital heart disease (CHD), which is about 3/1000 live births[19] necessitates an understanding of the mechanism of disease. We therefore searched for disease-associated *NKX2–5* mutations in the heart tissues of patients with cardiac malformations. We identified 53 *NKX2–5* mutations in diseased heart tissues and found common mutations in unrelated patients. Certain mutations were specific to ventricular and atrioventricular septal defects. Notably, in matched normal heart tissues of same patients (such as in patients with VSD, tissue samples were taken from unaffected atria), mutations detected in diseased tissues were mainly absent, indicating somatic origin and mosaicism of mutations. Besides germline mutations, our findings are suggestive for *NKX2–5* somatic mutations to cause congenital heart disease as well.

Materials and Methods

Materials

Sixty-eight formalin-fixed hearts from unrelated patients with cardiac malformations were obtained from the Institute of Anatomy, University of Leipzig, Germany. The hearts were collected by Dr. F. Spreer from 1954 to 1982

at the Institute of Pathology, University of Leipzig and District Hospital Borna, Germany. We classified them primarily by their septal defects using sequential segmental analysis.[20] Most of the patients died in early infancy. As control, we analyzed DNA from *n* = 16 hearts, eg, 10 formalin-fixed heart tissues collected from 1955 to 1963, in which 7 individuals died at birth or early infancy, and 6 frozen normal heart tissues. We also analyzed blood samples of 4 unrelated individuals and 7 families with CHD (8 family members with cardiac malformations), as well as blood samples of 50 unrelated healthy individuals. Defects in blood samples with CHD included ASD, VSD, hypoplastic left heart syndrome (HLHS), transposition of the great arteries (TGA), subpulmonary stenosis (SPS), and heterotaxy.

DNA Isolation and Amplification of NKX2–5 Fragments from Formalin-Fixed Tissues

Excess formalin in fixed tissues was removed with 1X GTE[21] and genomic DNA was isolated with NucleoSpin-Tissue Kit (Macherey-Nagel, Düren, Germany). The quality and quantity of isolated genomic DNA were checked on 1% agarose gel using known lambda DNA concentration (Amersham, Freiburg, Germany). The primer sequences for the amplification of *NKX2–5* fragments are known.[12,13] A PCR reaction consisted of 20 to 50 ng of genomic DNA, 12.5 μl of 2X Hot StarTaq Master Mix Kit (Qiagen, Hilden, Germany), 5 μl of 5X Q Solution (Qiagen), 1 μl (10 pmol/μl) of each primer pair, to a volume of 25 μl with distilled water. Except for a 10-minute final extension at 68°C, the PCR conditions were carried out as earlier described.[12] PCR reactions were done on Biometra thermocyclers (Biometra, Göffingen, Germany) and the PCR products were analyzed on 1% agarose gels. A negative control was always included in PCR experiments.

Detection and Confirmation of Mutations

Mutations were analyzed by double-strand direct sequencing using *NKX2–5* specific primers. PCR fragments were purified with QIAquick PCR Purification Kit (Qiagen), subjected to cycle sequencing using BigDyeTerminator v3.1 Kit and injected to ABI 3100 Genetic Analyzer (Applied Biosystems, Darmstadt, Germany). Sequences were analyzed using SeqMan (DNASTAR, Madison, WI). For uniformity of numbering of nucleotide changes in both coding and untranslated regions, we used the reference sequence NM004387 throughout the text. Corresponding numbering of *NKX2–5* mutations in the coding region starts with A of start codon ATG.

We confirmed mutations by digestion with restriction enzymes (PCR-RFLP). If restriction assay was not possible, we cloned the PCR fragments with heterozygous genotypes, and re-sequenced the clones, allowing the detection of two different alleles. PCR fragments were cloned into TOPO TA Cloning Kit for Sequencing (Invitrogen, Karlsruhe, Germany). Plasmid DNA was isolated using QIAprep Spin Miniprep Kit (Qiagen) and inserts

were analyzed by digestion with *Eco*RI (New England Biolabs, Frankfurt, Germany).

We compared nonsynonymous mutations in normal and diseased heart tissues of the same patient (such as in a patient with VSD, tissue samples were taken from an unaffected atrium). We analyzed 23 mutation loci in both normal and diseased heart tissues in 27 patients with VSDs, 2 mutation loci in 6 ASDs, and 7 mutation loci in 13 AVSDs. For this comparison, we analyzed a total of 92 samples, 146 PCR fragments, and 77,598 nucleotides.

Results

Cardiac Malformations of Patients

The explanted hearts with CHD were classified primarily by their septal defects (Figure 1, A–F). Sequential segment analysis resulted in 29 VSDs, 16 ASDs, and 23 AVSDs. Four patients within VSDs and 14 patients within AVSDs had Down syndrome. We also examined these patients to determine whether cardiac defects associated with Down syndrome would be positive for *NKX2–5* mutations.

Cardiac malformations in VSDs consisted of subaortic (76%) and muscular VSD (28%) (Table 1). Many patients were affected by patent foramen ovale (79%) and patent ductus arteriosus (28%). Several patients had further cardiac defects. For example, patient E39 had muscular and subaortic VSD with overriding aorta and persistent left superior vena cava draining into coronary sinus. The most prevalent septal anomaly in ASDs was septum secundum (88%), followed by septum primum (13%) and septum sinus (13%) (Table 1). Additionally, 12 other cardiac defects were present in ASDs and all patients but one had multiple defects. For example, patient C75 had septum secundum and septum primum defects, patent foramen ovale, patent ductus arteriosus, preductal aortic isthmus stenosis, mitral valve stenosis, hypoplastic left ventricle, and left isomerism. All 23 patients diagnosed as AVSD had subaortic VSD, primum septum defect and a common atrioventricular canal (Table 1). Patients had patent foramen ovale (70%) and patent ductus arteriosus (39%). Except for one, all patients had a minimum of four defects. For example, patient F13 had subaortic VSD, septum primum defect, patent foramen ovale, aortic stenosis, and pulmonary stenosis.

Amplification of NKX2–5 Fragments from Formalin-Fixed Tissues

Using known primer sequences, we amplified *NKX2–5* fragments from genomic DNA isolated from formalin-fixed heart tissues (Figure 2A). DNA yield from 25 mg of tissue ranged from 0.5 to 1.0 μg, with an average size of about 2 kb (Figure 2B). We were able to amplify all three fragments in the two exons of *NKX2–5* except in two patients. We obtained a 489-bp fragment with primers 1F24 and 1AR (Figure 2C), 472-bp fragment with 2F24/2R24 (Figure 2D), and 573-bp fragment with 3F24/3R24 (Figure 2E).
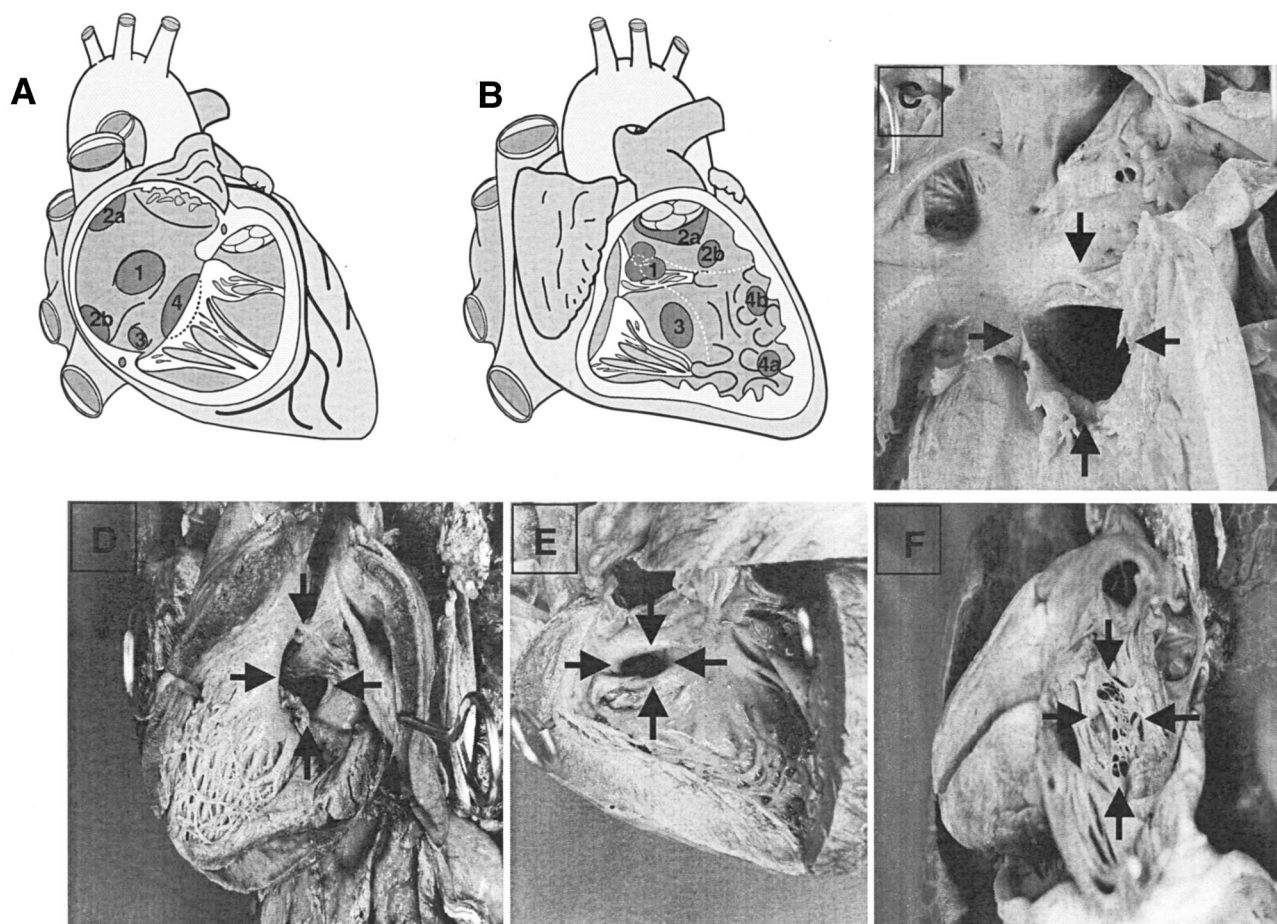
Figure 1. Classification of cardiac malformations in patients. **A:** Diagram showing classification of defects with interatrial shunt. **1**, ASD at fossa ovalis (ostium secundum type); **2a**, ASD at superior vena cava ostium (sinus venosus type); **2b**, ASD at inferior vena cava ostium (sinus venosus type); **3**, ASD at coronary sinus ostium (sinus coronarius type); **4**, atrioventricular septum defect (ostium primum type). **B:** Diagram showing classification of defects with interventricular shunt. Inlet, trabecular, and infundibular septal components are separated by **dotted lines**. **1**, VSD at membranous septum, subaortic (perimembranous septal defect); **2a**, VSD of outlet septum, subarterial, subvalvar; **2b**, VSD of outlet septum not related to arterial valves; **3**, VSD of inlet septum (central muscular septal defect); **4a**, VSD of apical septum, central (centroapical muscular septal defect); **4b**, VSD of apical septum, marginal. **C, D:** Large atrioventricular septal defects (**arrows**). **E:** Muscular ventricular septal defect (**arrows**). **F:** Arrows, atrial septal defect (ostium secundum type).

Detection of NKX2–5 Mutations

We identified by direct sequencing 53 mutations in the diseased heart tissues of patients consisting of 35 nonsynonymous, 13 synonymous, and 3′-UTR and 2 intronic (Table 2). Three mutations (Arg25Cys, Thr178Met and Ala219Val) are known. We found 4 patients in VSDs who were positive for both Thr178Met and Ala219Val. We also detected NCBI dbSNPs rs2277923 (A239G, Glu21Glu), and rs703752 (T1212G) in patients (Table 2). Only heterozygous genotypes were obtained. We confirmed mutations by PCR-RFLP assays or by cloning fragments with heterozygous loci, allowing detection of two different alleles (Figure 3, A and B). We obtained in normal lymphocytic DNA, the genotypic frequency 22 AA:17 AG: 6 GG for dbSNP rs2277923 in 45 samples; and for dbSNP rs703752, the frequency 19 GG: 20 GT:11 TT, in 50 samples. Except for these two dbSNPs, which were readily detected in all analyzed material, the 53 nucleotide changes were absent in DNA from six frozen, normal heart tissues and 50 blood samples of healthy individuals. We found 3 nucleotide alterations (G833A, T984A,

A1205T) in the 10 formalin-fixed, normal hearts, but these were different from those of patients with CHD. A synonymous change G833A (219 Ala) was found in 5, while T984A (Cys270Ser) and A1205T (3′-UTR) were present in 2 individuals. These 2 individuals, who were compound heterozygous for T984A (Cys270Ser) and A1205T (3′-UTR), died in early infancy. The normal formalin-fixed hearts were basically absent of mutations.

Furthermore, we did not detect any *NKX2–5* mutations in blood samples of patients with CHD except for a synonymous nucleotide change (G719A, 181 Gln) in an unaffected family member. This substitution was confirmed by *Bsp*MI restriction assay.

Together with the three known mutations, we obtained 35 nonsynonymous mutations; distributed as 29 in VSDs, 12 in ASDs, and 12 in AVSDs. Certain mutations were specific for ventricular and atrioventricular septal defects. Most patients had multiple nonsynonymous mutations, with VSDs carrying up to 14 mutations per patient (Figure 4A). We identified 6 nonsynonymous mutations in the homeodomain, and 5 would affect the third helix of the

Table 1. Cardiac Malformations of Patients Analyzed for *NKX2-5* Mutations

No.	Cardiac malformations	VSD group (29 patients)	ASD group (16 patients)	AVSD group (23 patients)
1	Subaortic VSD	22		23
2	Subaortic VSD with overriding aorta	21		6
3	Muscular VSD	8		
4	Septum primum defect		2	23
5	Septum secundum defect		14	1
6	Septum sinus defect		2	
7	Common atrioventricular canal			23
8	Overriding common atrioventricular valve			8
9	Overriding common quadricuspid atrioventricular valve			6
10	Patent foramen ovale	23	6	16
11	Patent ductus arteriosus	8	4	9
12	Aortic stenosis	1	1	2
13	Preductal aortic isthmus stenosis	3	3	3
14	Pulmonary stenosis		3	1
15	Mitral valve stenosis		1	
16	Partial anomalous venous return		1	
17	Double-outlet left ventricle	1		
18	Double-outlet right ventricle	1		
19	Persistent left superior vena cava draining into coronary sinus	2	2	
20	Hypoplastic left heart	1		
21	Hypoplastic left ventricle		3	3
22	Hypoplastic right ventricle		1	
23	Hypoplastic ascending aorta	2		2
24	Total anomalous pulmonary venous return		1	3
25	Isomerism of right atrial appendages	1		
26	Bicuspid right atrioventricular valve		1	
27	Bicuspid aortic valve		1	

homeodomain. Prevalent nonsynonymous mutations in VSDs were A232G (Asn19Ser) and C1018T (Ala281Val) with frequencies 64% and 86%, respectively. Prevalent nonsynonymous mutations in AVSDs were A723G (Lys183Glu) and A1072G (Asp299Gly) with frequencies 96% and 91%, respectively. The nonsynonymous *NKX2–5* mutations were identified in diseased heart tissues of patients, but not in matched normal tissues of the same patients' hearts (eg, patients with VSD, tissue samples were taken from unaffected atria). Except for one (C1018T, Ala281Val), none of the mutations was detected in normal heart tissue after comparing normal *versus*

Figure 2. Amplification of *NKX2–5* fragments from formalin-fixed tissues. **A:** Location of PCR primers on the genomic sequence of *NKX2.5*. **B:** Quality and concentration of genomic DNA isolated from formalin-fixed heart tissues in 1% ethidium bromide gel, M (kb plus ladder); 50, 100, 150 ng (lambda DNA standard). **C:** Amplified 1F24/1AR fragments (489 bp) in VSDs, ASDs, AVSDs (2 failed PCR). **D:** 2F/2R24 fragments (472 bp) in 29 VSDs (1 failed PCR). **E:** 3F/3R24 fragments (573 bp) in 29 VSDs (2 failed PCR).

disease cardiac tissues of 27 distinct mutation loci (Figure 4B). Furthermore, mutation spectrum did not differ basically in AVSD patients with and without Down syndrome (Figure 4C).

Cluster Analysis of Mutations

We used a mathematical algorithm to allow clustering of mutations by response.[22] Clustering by response tests all possible combinations among all patients and mutations and reveals the best constellation ranked by statistical significance. We asked two questions: 1) which among the prevalent mutations would be highly associated with Down syndrome; 2) which prevalent mutations would be of significance in predicting septal defects, independently of Down syndrome. For the first question, the test found Lys183Glu highly associated with Down syndrome. For the second question, we excluded the 18 patients with Down syndrome and used prevalent nonsynonymous mutations as predictors. These predictors included Asn19Ser, Lys183Glu, Ser279Phe, Ala281Val and Asp299Gly. Permutation tests gave a *P* value of < 0.001, which shows that these five mutations are predictive of septal defects. Two mutations eg, Asn19Ser and Lys183Glu were selected as highly predictive (*P* value 6.07×10^{-9}) This constellation predicts 100% VSD if Asn19Ser is present, but Lys183Glu is absent (Table 3).
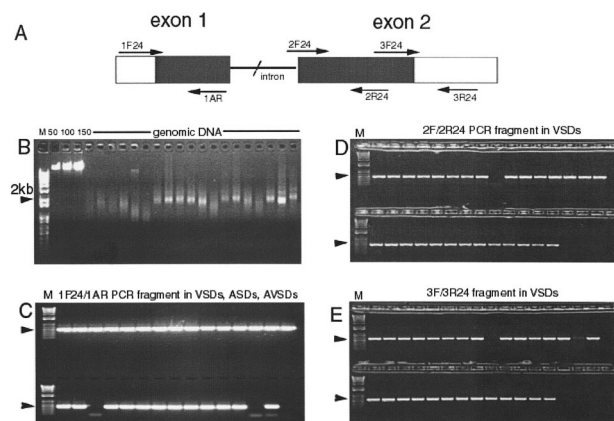
Discussion

Genetic analysis of *NKX2–5* in diseased heart tissues of 68 unrelated patients with primarily septal defects,

Table 2. *NKX2-5* Mutation Summary

Nucleotide change (*NKX2-5* NM004387)	Nucleotide reference (coding region)	Amino acid change	Location	No. of positive patients (VSD)	No. of positive patients (ASD)	No. of positive patients (AVSD)	PCR-RFLP assay
Nonsynonymous							
196 T→C	20	7 Leu→Pro	Exon 1			1	
232 A→G	56	19 Asn→Ser	Exon 1, TN domain	18			
249 C→T	73	25 Arg→Cys	Exon 1	1			*Hha*I, create site
309 T→C	133	45 Ser→Pro	Exon 1	1			
327 T→C	151	51 Phe→Leu	Exon 1	1			
382 T→C	206	69 Leu→Pro	Exon 1	1			
406 C→T	230	77 Pro→Leu	Exon 1	1			
516 T→A	340	114 Cys→Ser	Exon 2		3	2	
516 T→C	340	114 Cys→Arg	Exon 2	8	6	8	
529 A→G	353	118 Lys→Arg	Exon 2	4	1		
547 A→G	371	124 Lys→Arg	Exon 2	6			
553 A→T	377	126 Glu→Val	Exon 2	4	2	5	
573 C→T	397	133 Pro→Ser	Exon 2	6			
579 G→A	403	135 Ala→Thr	Exon 2		1	8	*Ava*I, abolish site
607 T→C	431	144 Leu→Pro	Exon 2		3	8	
709 C→T	533	178 Thr→Met	Exon 2, HD	5			*Nla*III, create site
723 A→G	547	183 Lys→Glu	Exon 2, HD, 3rd helix		7	22	*Taq*I, create site
735 C→T	559	187 Gln→Ter	Exon 2, HD, 3rd helix	6			*Xba*I, create site
751 A→C	575	192 Lys→Thr	Exon 2, HD, 3rd helix	6			*Tai*I, create site
751 A→G	575	192 Lys→Arg	Exon 2, HD, 3rd helix	2			*Bfm*I, create site
757 A→G	581	194 Lys→Arg	Exon 2, HD, 3rd helix	2			*Bsg*I, create site
790 T→A	614	205 Val→Glu	Exon 2	6			
832 C→T	656	219 Ala→Val	Exon 2, NK2-SD	10			
852 G→A	676	226 Asp→Asn	Exon 2	2			
918 T→C	742	248 Tyr→His	Exon 2	5			
1011 T→C	835	279 Ser→Pro	Exon 2	3			
1012 C→T	836	279 Ser→Phe	Exon 2	11		13	
1018 C→T	842	281 Ala→Val	Exon 2	24	5	5	*Bmr*I, create site
1033 C→T	857	286 Ala→Val	Exon 2	3	11	8	
1056 A→C	880	294 Asn→His	Exon 2			14	
1072 A→G	896	299 Asp→Gly	Exon 2	9	6	21	
1089 A→G	913	305 Ser→Gly	Exon 2	1			
1134 G→A	958	320 Gly→Ser	Exon 2	8	4	5	
1141 G→A	965	322 Arg→Gln	Exon 2	2			
1149 T→C	973	Stop→Gln	Exon 2	3	2	7	
Synonymous							
239 A→G	63	21 Glu	Exon 1, dbSNP	2AA: 26 AG	12 AG	6AA: 17 AG	*Bpm*I, create site
560 C→T	384	128 Asp	Exon 2		6	19	
614 G→T	438	146 Ser	Exon 2		3	10	
629 T→C	453	151 Tyr	Exon 2, HD	22	5	1	*Acc*I, create site
677 A→G	501	167 Glu	Exon 2, HD	2			
680 C→T	504	168 Arg	Exon 2, HD	5			
704 A→G	528	176 Lys	Exon 2, HD		6	18	
779 T→C	603	201 Thr	Exon 2	2			
902 C→G	726	242 Gly	Exon 2	10			
995 T→C	819	273 Ala	Exon 2	28	4	6	
1034 C→T	858	286 Ala	Exon 2		2	17	
1079 T→C	903	301 Asn	Exon 2	1			
1118 A→G	942	314 Gly	Exon 2	4	6	8	
1142 A→G	966	322 Arg	Exon 2	28	1	1	*Msp*I, create site
Untranslated region							
113 C→T			Exon 1, 5′ UTR	1			
141 C→T			Exon 1, 5′ UTR	1			
1156 G→A			Exon 2, 3′ UTR	13	13	23	
1212 T→G			Exon 2, 3′ UTR, dbSNP	28 GT	1GG: 14 GT	12 GG: 11 GT	
Intronic region							
−24 IVS C→A			Intron	28	4	1	
−11 IVS T→C			Intron		1		

Figure 3. Detection and confirmation of *NKX2–5* mutations. Heterozygous loci as detected by direct sequencing (electropherograms) are confirmed by PCR-RFLP assay or cloning and re-sequencing of clones allowing detection of two alleles. **A:** Analysis of two patients (E10VSD, F04AVSD) who are compound heterozygous for mutations in the third helix of the homeodomain. *Bfm*I and *Bsg*I are PCR-RFLP assays for Lys192Arg and Lys194Arg, respectively; while *Taq*I is for Lys183Glu. **B:** Analysis of a patient (D03VSD) showing multiple known mutations and more than two haplotypes. Clone 1: C/A/C (all reference alleles); clone 2: C/A/T (recombinant type); clone 3: T/C/T (all mutant alleles).

yielded 53 mutations and two known single nucleotide polymorphisms (NCBI dbSNPs). Among these were 35 single nucleotide changes that would result in amino acid change (nonsynonymous mutations). We identified three known mutations, namely Arg25Cys, Thr178Met, and Ala219Val in VSDs. Mutations Arg25Cys and Ala219Val were detected previously in patients with tetralogy of Fallot (TOF).[12,13,18] Detection of Arg25Cys and Ala219Val in VSDs is not surprising, as ventricular septal defect is one of the four complications of TOF. Among familial *NKX2–5* mutations, Thr178Met was detected in unrelated families with ASD.[11,17] All other known mutations are uncommon and specific to single families. We show here that Thr178Met affects ventricular septal defects as well and found four patients in VSDs who were compound heterozygous for Thr178Met and Ala219Val.

Six of the new nonsynonymous mutations are located in highly conserved regions. Mutation Asn19Ser is in the TN domain at the NH_2 terminus. The function of this domain remains unclear, but the change from asparagine to serine results in change of amino acid charge and may affect binding properties of NKX2–5. Mutations Lys183Glu, Glu187Ter, Lys192Arg, Lys192Thr, and Lys194Arg are located in the third helix of HD, which is

important for DNA binding specificity. These mutations may cause cardiac defects through reduced DNA affinity or affect transactivation activities.[23,24] Further studies will be undertaken to clarify the exact molecular mechanisms by which these mutations lead to septal defects.

Independent of their location, clustering by response analysis of *NKX2–5* mutations found Asn19Ser and Lys183Glu as highly predictive mutations. Cluster analysis also found that Ser279Pro, Ala281Val, and Asp299Gly predictive of septal defects. How these three mutations will effect cardiac malformations needs further study, because these are located in the COOH terminus of NKX2–5. Whether they cause cardiac malformations through gain-of-function activities or through other mechanisms is unclear. Different results were obtained in carriers of COOH terminus mutations.[25,26] The applied genetic algorithm in cluster analysis assumes correlation between primary mutations and disease phenotype. It will, therefore, be interesting to explore further the role of primary mutations in predisposing mutant cardiac tissue to secondary somatic mutations.

While all previous reports on *NKX2–5* mutations were based on lymphocytic DNA and mutations are of germline in nature,[11–18] our genetic analysis was based on
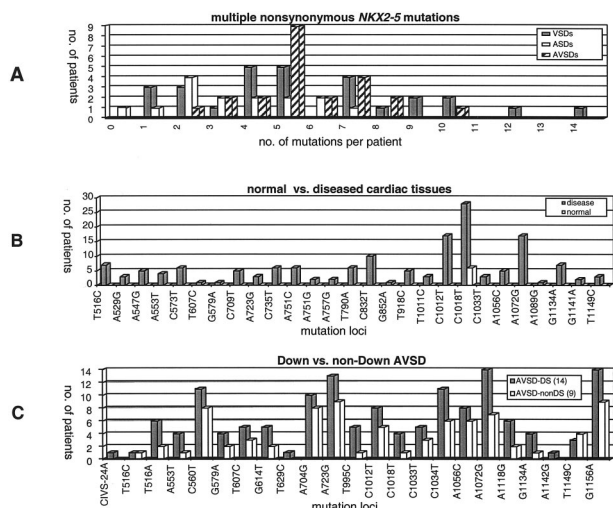
Figure 4. Evidence of somatic nature of identified *NKX2–5* mutations in diseased heart tissues. **A:** Multiple nonsynonymous mutations in VSDs, ASDs, and AVSDs. **B:** Mutations are mainly absent in matched normal and diseased cardiac tissues of the same patient. **C:** No difference in *NKX2–5* mutation spectrum in Down and non-Down syndrome AVSD.

DNA from diseased cardiac tissues of unrelated patients. These earlier studies found familial and private mutations in patients, and mutations were rare in sporadic cases. We obtained a higher rate of mutations among unrelated patients, which may be explained by complex cardiac malformations in patients and somatic nature of mutations. Several lines of evidence suggest that the *NKX2–5* mutations may be of somatic nature, originating most likely from cell division errors during early embryogenesis. For instance, we observed multiple nonsynonymous mutations in the same patient, which is an unlikely event in inherited mutations. Multiple mutations are well known in cancer,[27] but the occurrence of more than two inherited gene mutations in a patient is rare.[28] We found that mutations in diseased heart tissues were mainly absent in matched normal heart tissue (ie, patients with VSD, tissue samples were taken from unaffected atria), indicating somatic nature and mosaicism of mutations.

We observed multiple haplotypes in a patient. Cloning of *NKX2–5* fragments containing several heterozygous mutations yielded more than the two expected haplotypes in a patient, including clones with all of the mutant alleles. We obtained three haplotypes in each two patients who were compound heterozygous for known mutations Thr178Met and Ala219Val. Similar results were found in a patient (D03VSD) who had three known mutations (Figure 3B). The presence of multiple haplotypes suggests mixed population of cells or *de novo* chromosomal rearrangements or duplications affecting *NKX2–5* in heart tissues of patients. Congenital heart defects (VSD, TOF) have been observed in trisomy 5 mosaicism[29] or tandem duplication mosaicism on the long arm (5q13–33) of chromosome 5.[30]

We did not find any *NKX2–5* mutations after screening of lymphocytic DNA of families and unrelated patients with CHD except dbSNPs and a synonymous substitution in an unaffected family member. Analysis of lymphocytic DNA alone does not reveal chromosomal aberrations occurring in cardiac tissues, as has been found previously.[31,32] It is of considerable importance that we did not observe any difference in *NKX2–5* mutation spectrum in AVSD Down and non-Down syndrome. This result was unexpected, since atrioventricular septal defect is the most common feature in Down syndrome (trisomy 21).[33] The existence of both *NKX2–5* mutations (chromosome 5) and Down syndrome in a patient indicates further genomic chaos in the diseased heart tissues of patients with CHD.

Although there are reservations in the use of formalin-fixed tissues,[34,35] our data show that DNA sequence is preserved after more than 40 years of formalin storage. Indeed we demonstrate reliability in applying a well-developed assay for isolating DNA from long-term formalin-fixed tissue. Our study benefits from well-characterized hearts with distinct and definitively determined malformations. We, therefore, overcome the limitation of using surrogate tissue DNA (such as lymphocytic DNA) to correlate phenotype to genotype. Archival material is therefore valuable and should be considered for genetic studies.

The original aim of this study initiated 40 years ago was to improve an understanding of the anatomy of complex malformed hearts. We, therefore, have limited information on the pedigrees of our cohorts. In the case of AVSDs, patient F08 had a prematurely aborted sibling and a further sibling who died 3 months after birth; patient F15 had a prematurely aborted sibling and, patient F17 had a twin sister who died shortly after birth. In addition, patient E27 in VSDs had a prematurely aborted sister. The scarce information on family history is supportive of possible chromosomal abnormalities in patients.

We found three nucleotide changes in formalin-fixed normal hearts, including a nonsynonymous mutation (Cys270Ser) in two patients who died in early infancy.

Table 3. Cluster Analysis of *NKX2-5* Nonsynonymous Mutations in Complex CHD without Down Syndrome

Cluster		Predictors		ASD	VSD	AVSD	Total
		Asn19Ser	Lys183Glu				
1	1 0	+	−	0 (0.0%)	17 (100.0%)	0 (0.0%)	17 (34.0%)
2	1 1	+	+	7 (43.8%)	0 (0.0%)	9 (56.4%)	16 (32.0%)
	0 1	+	−				
3	0 0	−	−	9 (52.9%)	8 (47.2%)	0 (0.0%)	17 (34.0%)
Total				16 (32.0%)	25 (50.0%)	9 (18.0%)	50

Selected predictors: Asn19Ser, Lys183Glu. Predictors used: Asn19Ser, Lys183Glu, Ser279Phe, Ala281Val, Asp299Gly.
Cluster 1, VSD: to obtain 100% VSD, Asn19Ser present, but Lys183Glu absent.
χ^2: 4.411443e+001.
P value: 6.074074e-009.

Nonsynonymous *NKX2–5* mutations have been observed in normal population (NCBI dbSNPs rs3729754, rs3729938). Cys270Ser has not been found in diseased heart tissues of patients with CHD and thus, this alteration may simply reflect genetic polymorphism. We also detected the germline mutation Arg25Cys, which was reported previously in patients with CHD,[12,13,18] but was later detected in control population[13] or in unaffected family members.[17] It is, therefore, not impossible to have nonsynonymous *NKX2–5* mutations in disease-free individuals. Further, comparison of mutations in normal versus diseased heart tissues shows that mutations may differ in different regions of the same heart. After analysis of 27 distinct nonsynonymous mutation loci in normal and diseased heart tissues, only one (C1018T) was detected in both tissues of some patients. Such mutation may simply have occurred during early embryogenesis.

The diseased heart tissues may, in turn, contain a mixture of cells carrying different mutations. We cannot discount the possibility of not detecting all types of *NKX2–5* mutations present within the diseased heart tissues. Also, we cannot determine with certainty disease-causing mutations from disease-associated mutations, although specific patterns of mutation loci and frequency of occurrence are linked to patient phenotype (see clustering by response analysis in Table 3, which reports a statistically significant relationship between various constellations of mutations and disease phenotype).

The process of cardiac development is complex, and NKX2–5 participates in protein-protein interaction with other transcription factors. New studies are underway to obtain in-depth information on the role of mutations of other transcription factors in the network of NKX2–5 protein-protein interactions. Perhaps failure of cardiac transcription factor networks resulting from genetic instability in cardiac cells is a plausible pathway to congenital heart disease. We show for the first time that malformed hearts are affected by multiple somatic *NKX2–5* mutations. It will be of interest to investigate further whether a single transcription factor mutation leads to genomic instability or whether several transcription factors are affected simultaneously to give rise to genomic chaos. Lastly, a prospective analysis of CHD patients is needed to compare cardiac and non-cardiac tissues to confirm our findings.

Acknowledgments

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