SHORT REPORT

Somatic NKX2-5 mutations as a novel mechanism of disease in complex congenital heart disease

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NKX2–5 is a pivotal transcription factor in heart development. Previous studies on lymphocytic DNA provided evidence of familial NKX2–5 gene mutations in cardiac malformations. Common mutations are rare in unrelated families. We analysed, by direct sequencing, the gene encoding NKX2–5 in the diseased heart tissues of 68 patients with complex congenital heart disease, focussing particularly on atrial, ventricular, and atrioventricular septal defects. We identified 35 non-synonymous NKX2–5 mutations in the diseased heart tissues of patients. These mutations were mainly absent in normal, for example, unaffected, heart tissue of the same patient, indicating the somatic nature and mosaicism of mutations. We also observed multiple mutations and multiple haplotypes, as well as mutations in Down's syndrome patients with cardiac malformations. Taken collectively, the above results suggest the somatic nature of NKX2–5 mutations associated with complex cardiac malformations. Somatic mutations in transcription factor genes of cardiac progenitor cells provide a novel mechanism of disease.

NKX2–5 is a homeodomain containing transcription factor crucial to mammalian heart development.[1] Lack of NKX2–5 in animal models results in lethality due to impaired cardiac looping. Genetic analysis of lymphocytic DNA identified germline mutations in the human NKX2–5 gene which are associated with cardiac anomalies, but most families have single and unique mutations.[2–10] Germline NKX2–5 mutations are rare among sporadic cases of congenital heart disease (CHD), genotype–phenotype correlation is difficult, and mechanisms leading to complex CHD remain unclear.

Next to inherited mutations, the importance of somatic mutations during early embryogenesis is now being recognised, but rarely studied.[11 12] Disease associated or disease causing somatic mutations are undetected by genetic analysis of lymphocytic DNA alone, and mosaicism may reduce the likelihood of detection in the affected tissue. Therefore, mutations present in cardiac tissues may be absent or sporadic in blood samples of the same individual. Unlike previous studies, we searched for mutations in the diseased tissues of 68 explanted hearts of patients with definitive atrial (ASD), ventricular (VSD), and atrioventricular (AVSD) septal defects. These patients, however, had complex cardiac malformations and died mostly in early infancy (fig 1A and B).

The human NKX2–5 gene maps to chromosome 5q34 and consists of two exons encoding a protein of 324 amino acids. The homeodomain (HD) of NKX2–5, which lies within exon 2, consists of three α helices. Helix 3 is important for DNA binding specificity.[13] Other conserved regions of NKX2–5 are the TN domain and NK2 specific domain (NK2-SD). Using validated PCR primers,[3 5] we amplified fragments from the two exons (fig 1C). Mutations were detected by direct sequencing of both DNA strands and confirmed by PCR-RFLP or cloning of the sequenced fragments, allowing separation of the two variant alleles in a heterozygous locus (fig 1D).

We identified two NCBI dbSNPs (rs2277923 and rs703752) and 35 non-synonymous NKX2–5 mutations in diseased heart tissues of patients with complex cardiac malformations (table 1). Three of these mutations (Arg25Cys, Thr178Met, and Ala219Val) are known. We detected 29 mutations in VSD patients, 12 in ASD, and 14 in AVSD. We found one mutation in the TN domain (Asn19Ser) and five mutations (Lys183Glu, Gln187Ter, Lys192Thr, Lys192Arg, Lys194Arg) in the third helix of HD (table 1). We observed only heterozygous genotypes based on the sequence electropherograms. As determined by haplotype analysis, this heterozygosity may simply be a reflection of genotypes of mixed wild type and mutant cells. Therefore, the occurrence of homozygous genotypes in analysed diseased heart tissues cannot be excluded, although germline homozygous mutations in animal models are lethal. Further, somatically occurring mutations as seen in cancerous diseases result in loss of heterozygosity. Again, such loss of heterozygosity may result in cardiac anomalies as seen in our study. The 35 mutations were absent in lymphocytic DNA of 50 healthy individuals and in DNA from six frozen normal hearts.

The explanted hearts were formalin fixed more than 40 years ago, but analysis of NKX2–5 and five other cardiac transcription factor genes (46 709 nt) in 10 normal hearts from the same collection of formalin fixed hearts basically revealed no sequence alterations except dbSNPs, showing the reliability of using valuable archival material. We found a non-synonymous NKX2–5 substitution (T984A, Cys270Ser) in two individuals who died as babies, but this mutation was different from those in patients with CHD. This sequence alteration may just be a polymorphism.

The NKX2–5 mutations were detected in heart tissues sampled within the septal defects of the hearts. To determine whether these mutations were also present in normal heart tissues (such as in patients with VSD, where tissue samples were taken from unaffected atria), we compared 27 distinct mutations in normal and diseased heart tissues of the same patients (table 1). We analysed a total of 92 samples, 146 PCR fragments, and 77 598 nucleotides and found mutations mainly absent in normal heart tissues. The absence of mutations in matched normal heart tissue of the same patient indicates the somatic nature and mosaicism of NKX2–5 mutations.

AVSD is highly associated with Down's syndrome (trisomy 21)[14] and some of our patient cohorts had Down's syndrome.

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Abbreviations: ASD, atrial septal defect; AVSD, atrioventricular septal defect; CHD, congenital heart disease; HD, homeodomain; VSD, ventricular septal defect
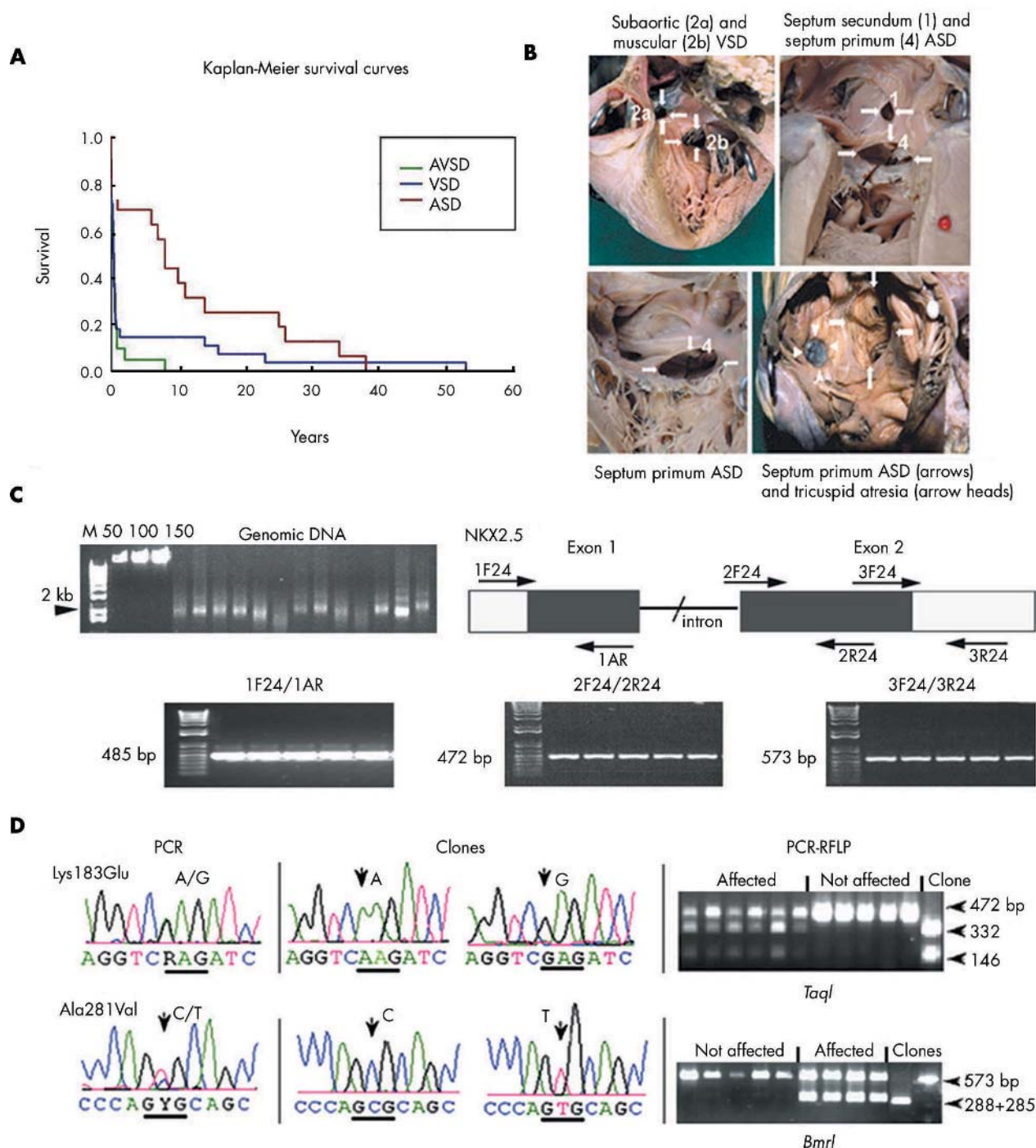
Figure 1 NKX2–5 mutation analysis in complex CHD. (A) Kaplan-Meier survival curves showing AVSD and VSD patients with lower life expectancy than ASD patients. (B) Examples of cardiac malformations in patients. (C) Three NKX2–5 fragments were amplified from genomic DNA (size of about 2 kb) isolated from archival cardiac tissues using three known primer pairs: 1F24/1AR (485 bp), 2F/2R24 (472 bp), and 3F/3R24 (573 bp). About 20–50 ng genomic DNA was used for PCR reaction. M (kb ladder): 50, 100, 150 ng (λ DNA standard). (D) Detection of mutations by direct sequencing, verification by PCR-RFLP, or cloning of fragments with heterozygous loci, separating the two variant alleles.

Analysis of 18 AVSD patients with Down's syndrome for NKX2–5 mutations found no difference in the mutation spectrum between syndromic and non-syndromic AVSD patients (tables 1 and 2). The occurrence of both trisomy 21 and NKX2–5 mutations suggests genomic chaos in the heart tissues of patients with complex CHD. We also detected common mutations among unrelated patients and some mutations were specific to ventricular or atrioventricular septal defects (table 2). We found common mutations such

as A232G (Asn19Ser), A723G (Lys183Glu), C1012T (Ser279Phe), C1018T (Ala281Val), and A1072G (Asp299Gly) (table 2). We also found multiple mutations in 57 patients; 66% had five or more mutations. The range of multiple mutations per patient was 2–14, 2–7, and 2–10 in VSD, ASD, and AVSD patients, respectively (table 2).

The presence of multiple gene mutations in a patient is an unlikely occurrence in inherited mutations, but multiple mutations are frequently observed in high capacity cell

Table 1 Summary of NKX2-5 mutations

Nucleotide change (NKX2–5 NM004387)	Nucleotide reference (coding region)	Amino acid change	Location	Number of positive hearts (n = 68)	Mutations compared in normal v diseased heart tissues	Mutations detected in both Down's and non-Down's syndrome AVSD patients	PCR-RFLP assay
Non-synonymous							
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			Hhal
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	5		×	
516 T→C	340	114 Cys→Arg	Exon 2	22	×	×	
529 A→G	353	118 Lys→Arg	Exon 2	5	×		
547 A→G	371	124 Lys→Arg	Exon 2	6	×		
553 A→T	377	126 Glu→Val	Exon 2	11	×	×	
573 C→T	397	133 Pro→Ser	Exon 2	6	×		
579 G→A	403	135 Ala→Thr	Exon 2	9	×	×	Aval
607 T→C	431	144 Leu→Pro	Exon 2	11	×	×	
709 C→T	533	178 Thr→Met	Exon 2, HD	5	×		NlaIII
723 A→G	547	183 Lys→Glu	Exon 2, HD, third helix	29	×	×	TaqI
735 C→T	559	187 Gln→Ter	Exon 2, HD, third helix	6	×		Xbal
751 A→C	575	192 Lys→Thr	Exon 2, HD, third helix	6	×		Tail
751 A→G	575	192 Lys→Arg	Exon 2, HD, third helix	2	×		Bfml
757 A→G	581	194 Lys→Arg	Exon 2, HD, third helix	2	×		Bsgl
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	23	×	×	
1018 C→T	842	281 Ala→Val	Exon 2	34	×	×	Bmrl
1033 C→T	857	286 Ala→Val	Exon 2	22	×	×	
1056 A→C	880	294 Asn→His	Exon 2	14	×	×	
1072 A→G	896	299 Asp→Gly	Exon 2	36	×	×	
1089 A→G	913	305 Ser→Gly	Exon 2	1	×		
1134 G→A	958	320 Gly→Ser	Exon 2	17	×		
1141 G→A	965	322 Arg→Gln	Exon 2	2	×		
1149 T→C	973	Stop→Gln	Exon 2	12	×	×	
dbSNPs							
239 A→G	63	21 Glu	Exon 1, rs2277923	8AA: 55 AG			Bpml
1212 T→G			Exon 2, rs703752	13 GG: 53 GT			

division such as in cancer.[15] For example, mutational analysis of the p53 tumour suppressor gene in human mammary carcinomas evidenced up to six mutations in this gene.[16] It is, however, uncommon to find carriers of multiple mutations within one gene, as described here. We observed, on average, five mutations in VSD and AVSD subjects, and three mutations in ASD subjects. Moreover, nine of 29 VSD subjects, nine of 23 AVSD subjects, and three of 16 ASD subjects had more than five mutations. Nonetheless, recent genetic studies also demonstrate the occurrence of multiple mutations in human genes including the genes coding for butyrylcholinesterase[17] and hALG6, which codes for a glucosyltransferase.[18] In both genes, three mutations per patient were identified. In our study, it remains unclear whether multiple NKX2–5 mutations are a result of primary or secondary events.

We cloned PCR products containing heterozygous loci, as seen on sequence electropherograms. In most cases, a locus was scored heterozygous when the ratio of the second peak to the highest peak was at least 50%. After cloning, we sequenced random clones, and scored only those loci previously identified as heterozygous after direct sequencing of the PCR product. Surprisingly, we obtained more than the two expected haplotypes in one individual, and identified clones carrying all the reference alleles only, mutated alleles only, or a combination of both (table 3). In most analysed clones, both alleles were affected. A likely explanation for multiple haplotypes would be a mixed population of cardiomyocytes carrying different mutations or de novo chromosomal rearrangements and gene duplications in the heart tissues of patients affected by various mutations. Heart defects are observed following chromosomal abnormalities including chromosome 5.[19] It will be the subject of future research to determine the origin of these multiple haplotypes.

Our patient cohort of rare and complex cardiac malformations had non-synonymous mutations in other cardiac transcription factor genes including GATA4, HAND1, and TBX5, although mutation frequencies varied considerably (data not shown). Simultaneous occurrence of multiple mutations in several genes is uncommon, but may be explained by the sheer complexity of the observed malformations, with patients carrying multiple defects in the heart. Somatically occurring mutations may affect various cell types at different stages of organogenesis, with different transcription factor programs taking place. These patients, however, did not carry mutations in the sequences affecting binding regions of MEF2C, an upstream determinant of cardiac development.[20] NKX2–5, HAND1, and MEF2C are all located

Table 2 Mutational analysis of individual hearts

No	Patient	Age	DS	Total
1	C06VSD	1 h		5
2	C10VSD	Stillborn		5
3	C19VSD	3 months		4
4	C58VSD	18 days		3
5	D01VSD	Aborted		5
6	D03VSD	2 months		10
7	E03VSD	6 months	Yes	5
8	E04VSD	6 weeks	Yes	7
9	E08VSD*	53 years		0
10	E10VSD	6 months		9
11	E11VSD	Newborn		4
12	E13VSD	16 years		2
13	E15VSD	8 months		4
14	E16VSD	3 months		8
15	E20VSD	2 days		7
16	E22VSD	4 months		3
17	E23VSD	22 days	Yes	1
18	E26VSD	6 months		7
19	E27VSD	5 months		10
20	E28VSD	5 months		7
21	E29VSD	8 weeks		2
22	E31VSD	5 months		10
23	E33VSD	14 years		2
24	E34VSD	3 months		4
25	E35VSD	4½ months		13
26	E39VSD	9 months		14
27	E40VSD	15 months		5
28	E41VSD	23 years		1
29	E49VSD	Newborn		1
	Total			

No	Patient	Age	Total
1	C09ASD*	26 years	2
2	C39ASD	25 years	2
3	C45ASD	6 days	1
4	C46ASD	34 years	2
5	C64ASD	11 month	3
6	C75ASD	2 months	0
7	D30ASD*	11 years	2
8	D33ASD	8 years	6
9	F17ASD	4½ h	3
10	F19ASD	8 years	4
11	F20ASD	10 years	5
12	F24ASD	6 years	4
13	F27ASD	7 years	7
14	F29ASD	14 years	6
15	F30ASD	1 day	6
16	F32ASD	38 years	4
	Total		2

Mutation columns (left to right): DS, T196C, A232G, C249T, T309C, T327C, T382C, C406T, T516A, T516C, A529G, A547G, A553T, C573T, G579A, T607C, C709T, A723G, C735T, A751C, A751G, A757G, T790A, C832T, G852A, T918C, T1011C, C1012T, C1018T, C1033T, A1056C, A1072G, A1089G, G1134A, G1141A, T1149C, Total

Table 2 Continued

No	Patient	Age	DS	T196C	A232G	C249T	T309C	T327C	T382C	C406T	T516A	T516C	A529G	A547G	A553T	C573T	G579A	T607C	C709T	A723G	C735T	A751G	A751C	A757G	T790A	C832T	G852A	T918C	T1011C	C1012T	C1018T	C1033T	A1056C	A1072G	A1089G	G1134A	G1141A	T1149C	Total
1	C43	AVSD 10 months																		▲																			3
2	C70/2	AVSD 8 months	Yes														▲			▲											▲								4
3	C71	AVSD 4 days	Yes							▲	▲	▲		▲		▲		▲		▲											▲							6
4	E05	AVSD 16 weeks								▲	▲	▲		▲						▲											▲							4
5	E36	AVSD 3½ months	Yes													▲	▲			▲																			2
6	E43	AVSD 4½ months														▲	▲	▲		▲									▲	▲	▲							6
7	F00	AVSD 6 weeks	Yes ▲				▲													▲			▲							▲	▲	▲							5
8	F02	AVSD 10 months																		▲			▲								▲	▲						3
9	F03	AVSD 1 month	Yes																	▲									▲		▲	▲	▲		▲			5
10	F04	AVSD 11 weeks	Yes																	▲									▲	▲	▲	▲	▲		▲			5
11	F05	AVSD 10 weeks	Yes																	▲									▲		▲	▲	▲		▲			5
12	F08	AVSD 5 months	Yes															▲		▲									▲	▲		▲	▲					5
13	F11	AVSD 7 months	Yes								▲	▲		▲		▲	▲	▲		▲									▲	▲	▲	▲	▲		▲			8
14	F12	AVSD 6 months									▲	▲		▲		▲	▲	▲		▲			▲						▲	▲	▲	▲	▲					8
15	F13	AVSD 25 months	Yes								▲	▲		▲		▲	▲	▲		▲									▲	▲	▲	▲	▲		▲		▲	7
16	F14	AVSD 1 month	Yes														▲			▲			▲						▲	▲	▲	▲	▲		▲		▲	10
17	F14a	AVSD 5 weeks	Yes																	▲									▲	▲	▲	▲	▲				▲	5
18	F15	AVSD 5 days																		▲									▲	▲	▲	▲	▲				▲	5
19	F18	AVSD 7 months	Yes								▲	▲				▲	▲	▲		▲									▲	▲	▲	▲	▲		▲		▲	7
20	F28	AVSD 8 years																		▲									▲	▲	▲	▲	▲				▲	7
21	F31	AVSD 8 days	Yes								▲	▲		▲		▲	▲	▲		▲									▲	▲	▲	▲	▲				▲	7
22	F38	AVSD 3 months																		▲									▲	▲	▲	▲	▲				▲	5
23	F40	AVSD 10 months																		▲									▲	▲	▲	▲	▲		▲		▲	5
	Total		1	0	0	0	0	0	2	8	0	0	5	0	8	8	8	0	22	0	0	0	0	0	0	0	0	13	5	8	14	21	0	5	0	7		

*Not all NKX2-5 fragments can be amplified; DS, Down's syndrome.

Table 3 Multiple NKX2–5 haplotypes in complex cardiac malformations

Patients	Mutations											Total
	T516C	A529G	A547G	A553T	C573T	C709T	C735T	A751C	A751G	A757G	C832T	
E04VSD												2
PCR		A/G		A/T					A/G	A/G		
Clone 1		A		A					A	A		Haplotype 1, all reference alleles
Clone 2		G		T					G	G		Haplotype 2, all mutant alleles
Clone 3		G		T					G	G		
Clone 4		G		T					G	G		
Clone 5		G		T					G	G		
Clone 6		G		T					G	G		
E10VSD												3
PCR		A/G		A/T					A/G	A/G		
Clone 1		A		A					A	A		Haplotype 1, all reference alleles
Clone 2		A		A					A	A		
Clone 3		A		T					G	G		Haplotype 2 ,
Clone 4		G		T					G	G		Haplotype 3, all mutant alleles
Clone 5		G		T					G	G		
Clone 6		G		T					G	G		
E03VSD												3
PCR		A/G			C/T			A/C			C/T	
Clone 1		A			C			A			C	Haplotype 1, all reference alleles
Clone 2		A			C			A			C	
Clone 3		G			C			C			T	Haplotype 2
Clone 4		G			T			C			T	Haplotype 3, all mutant alleles
D03VSD												3
PCR		A/G			C/T			A/C			C/T	
Clone 1		G			C			A			C	Haplotype 1, all reference alleles
Clone 2		A			C			A			T	Haplotype 2
Clone 3		G			T			C			T	Haplotype 3, all mutant alleles
E31VSD												4
PCR	T/C				C/T		C/T					
Clone 1	T				C		C					Haplotype 1, all reference alleles
Clone 2	T				C		C					
Clone 3	T				C		C					
Clone 4	T				C		T					Haplotype 2
Clone 5	T				C		T					
Clone 6	T				C		T					
Clone 7	T				C		T					
Clone 8	T				T		C					Haplotype 3
Clone 9	C				T		C					Haplotype 4
Clone 10	C				T		C					
E35VSD												5
PCR					C/t	C/t	C/t		A/g	A/g		
Clone 1					C	C	C		A	A		Haplotype 1, all reference alleles
Clone 2					C	C	C		A	A		
Clone 3					C	C	C		A	A		
Clone 4					C	C	T		A	A		Haplotype 2
Clone 5					C	C	C		G	G		Haplotype 3
Clone 6					T	C	T		A	A		Haplotype 4
Clone 7					T	C	T		A	A		
Clone 8					T	T	C		A	A		Haplotype 5
E39VSD												7
PCR	T/C	A/g	A/g	A/t	C/t	C/t	C/t	A/c/g	A/c/g	A/g	C/t	
Clone 1	C	A	A	A	C	C	C	A		A	C	Haplotype 1
Clone 2	T	A	G	A	C	C	C	A		A	C	Haplotype 2
Clone 3	C	G	A	T	C	T	C	A		A	T	Haplotype 3
Clone 6	T	A	A	A	C	C	C	C		A	T	Haplotype 4
Clone 5	T	A	A	T	C	T	C	C		A	C	Haplotype 5
Clone 4	T	A	G	A	C	T	C	C		A	T	Haplotype 6
Clone 7	T	A	A	A	C	C	C		G	G	C	Haplotype 7
Clone 8	T	A	A	A	C	C	C		G	G	C	

*(C/T), ratio of second highest peak to highest is 50%; (C/t) ratio of second highest peak to highest is 30%. Bold in nucleotides indicates variant alleles; bold in haplotypes indicates the reference allele.

on chromosome 5: 5q34, 5q32, and 5q14, respectively, but differ profoundly in the spectrum of mutation. Deleterious mutations in MEF2C would most likely lead to early termination of pregnancy, for example, intrauterine resorption or spontaneous abortion due to its pivotal function in the trans-differentiation process during organogenesis of the heart.

We also analysed lymphocytic DNA in seven families and four unrelated patients with CHD, but did not identify

NKX2–5 mutations, except for one synonymous substitution in an unaffected family member. Further sequencing of nine cardiac transcription factor genes evidenced only dbSNPs. Thus, analysis of lymphocytic DNA may not reveal the genetic aberrations which are associated with CHD. In conclusion, our results demonstrate absence of mutations in matched normal heart tissues of the same patients' hearts, multiple mutations as well as multiple haplotypes in patients. These observations strongly suggest that NKX2–5 mutations occur somatically and that such somatic mutations are associated with complex CHD. These mutations probably resulted from postzygotic errors in cell division, particularly in cardiac progenitors cells. Presumably, various cell types at different stages of organogenesis with different transcription factor programs in place are affected by somatic mutations. Determining the onset of somatic mutations, effects of multiple mutations, and breakdown of transcription factor crosstalks resulting from genomic instability in cardiac cells, will be an interesting avenue to explore in future stem cell research.

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