Sequence variations of NKX2-5 and HAND1 genes in patients with atrial isomerism

Atriyal izomerizmli hastalarda NKX2-5 ve HAND1 genlerindeki dizi farklılıkları

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ABSTRACT

Objective: Atrial isomerism is a congenital disorder, which is characterized by lateralization defects in normally asymmetrical developing organs like the heart. Atrial isomerism is supposed to be caused by molecular defects during early development. The NKX2-5 is a cardiac specific transcription factor, which initiates and regulates downstream transcriptional cascades of cardiogenesis. The HAND1 is another transcription factor expressed in the heart, and it is characterized by an asymmetrical pattern of expression. In this study, we aimed to test whether mutations in NKX2-5 and HAND1 genes play a role in the etiology of atrial isomerism.
Methods: This case-control study consisted of 70 patients who underwent surgical treatment for congenital heart defects including atrial isomerism, 80 healthy subjects (HAND1 gene) and 40 healthy subjects (NKX2-5 gene). All exons and exon-intron boundaries of NKX2-5 and HAND1 genes were analyzed by SSCP, and suspected samples were sequenced for mutation analysis. Digestion with appropriate restriction enzymes was performed for analysis of known mutations and polymorphisms. The frequencies of the alleles and the genotypes were compared among patient and control groups using the Chi-square and the Fisher tests when appropriate.
Results: In intronic region of HAND1 gene, we identified a C>G substitution both in patients and controls. Frequency of mutant allele (11, 42%) was found higher (p=0.046) in patient group than that of the control group (2.5%). Association between atrial isomerism and genotypes with mutant allele was found borderline significant (p=0.054). In NKX2-5 gene, we identified heterozygous Q170X (Gln170ter) mutation in one patient. We did not found any correlation between defined sequence variations and clinical properties of the patients.
Conclusion: Our results suggest that mutations or sequence variations in HAND1 or NKX2-5 genes may play role in etiology or pathogenesis of atrial isomerism. *(Anadolu Kardiyol Derg 2011; 11: 319-28)*
Key words: Isomerism, NKX2-5, HAND1, mutation

ÖZET

Amaç: Atriyal izomerizm, kalp gibi normalde asimetrik olan organlardaki lateralizasyon defektleriyle karakterize olan doğumsal bir anomalidir. Atriyal izomerizmin, erken gelişim sırasındaki moleküler defektler nedeniyle oluştuğu düşünülmektedir. NKX2-5, kardiyogenezi başlatan ve kardiyogenezin sonraki transkripsiyonel olaylarını düzenleyen kalbe özgü bir transkripsiyon faktörüdür. HAND1 ise kalpte ekspres olan, asimetrik ekspresyon paterni ile karakterize başka bir transkripsiyon faktörüdür. Çalışmamızda, NKX2-5 ve HAND1 genlerindeki mutasyonların atriyal izomerizm etiyolojisinde rol oynayıp oynamadığını anlamak için, atriyal izomerizm hastalarında bu iki geni incelemeyi amaçladık.
Yöntemler: Bu vaka-kontrol çalışması atriyal izomerizm tanısı alan veya atriyal izomerizm nedeniyle cerrahi tedavi gören 70 hasta ve HAND1 için 80, NKX2-5 için, 40 sağlıklı kontrolden oluşturulmuştur. NKX2-5 ve HAND1 genlerinin tüm ekzonları ve ekzon-intron sınırları SSCP ile analiz edilmiş, şüpheli örnekler mutasyon analizi için dizilenmiştir. Bilinen polimorfizmler ve mutasyonlar için uygun restriksiyon enzimi ile enzim kesimi uygulanmıştır. Hasta ve kontrol grupları arasındaki allel ve genotip sıklıklarını Ki-kare ve Fisher testleri kullanılarak karşılaştırıldı.
Bulgular: Kontrol ve hastalarda, HAND1 geninin intronik bölgesinde bir C>G dönüşümü tanımladık. Mutant allelin hasta grubundaki sıklığı (%11.42) kontrol grubundakinden (%2.5) daha yüksek bulundu (p=0.046). Mutant allel taşıyan genotipler ile atriyal izomerizm arasındaki bağlantı sınırda anlamlı bulundu (p=0.054). NKX2-5 geninde, bir hastada heterozigot durumda Q170X (Gln170ter) mutasyonu tanımladık. Tanımlanan dizi farklılıkları ile hastaların klinik özellikleri arasında herhangi bir ilişki bulunmadı.
Sonuç: Sonuçlarımız, HAND1 veya NKX2-5 genlerindeki mutasyon veya dizi farklılıklarının, atriyal izomerizm etiyolojisi veya patogenezinde rol oynayabileceğini akla getirmektedir. *(Anadolu Kardiyol Derg 2011; 11: 319-28)*
Anahtar kelimeler: İzomerizm, NKX2-5, HAND1, mutasyon

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Introduction

Atrial isomerism is a disorder characterized by failure of body asymmetry. In addition to heart, other asymmetric organs like spleen and lungs are also affected in atrial isomerism. Depending on absence or presence of the spleen, atrial isomerism is classified as asplenic (or right) and polysplenic (or left) atrial isomerism (1, 2). Although many organs are subject to disordered body asymmetry, structural anomalies of the heart and great arteries are the most serious problems of the patients with atrial isomerism (1-3). Patients with right atrial isomerism have also frequently some anomalies like complete atrioventricular canal defect, transposition of the great arteries and double outlet right ventricle. On the other hand, patients with left atrial isomerism have defects in the intrahepatic portion of the inferior vena cava and heart block (4). Because of serious clinical problems, patients with atrial isomerism need surgical treatment.

The cause of atrial isomerism remains largely unknown. However, many studies suggest that any molecular defect during the early embryogenesis might be responsible for disruption of body asymmetry. Many transcription factors and secreted molecules are known to be involved in determination of left-right axis of the body (5-9). Asymmetric distributions of secreted factors and asymmetric expression of transcription factors guide the asymmetric development of the organs in digestive, circulatory and respiratory systems. Nodal is one of these asymmetrically distributed secreted factors and Pitx2 is one of these asymmetrically expressed transcription factors (10, 11). Some studies have demonstrated that human orthologue of the Nodal signaling genes, ACVR2B (12), LEFTYA (13) and CFC1 (14), were mutated in patients with heterotaxia. However, the etiology in most of the patients with laterality defects (heterotaxia syndromes) is thought to be chromosomal or polygenic-multifactorial, rather than monogenic.

Although asymmetry at molecular level is determined before organogenesis, morphological asymmetry is seen at a later stage of the embryogenesis. Morphologically first asymmetric event during embryogenesis is looping of the heart tube (15). Looping of the heart tube is one of the main stages of the cardiogenesis like primitive/linear heart tube formation, chambering and septation. Each one of those stages is characterized by specific molecular events (16). Main regulatory steps of heart development are downstream events of transcription factors like NKX2-5, HAND1, HAND2, SRF and GATA4 (16-18).

The NKX2-5 (NK type homeobox) is a NK-2 homeobox containing transcription factor which is required for heart development. As an earliest known marker of myocardial progenitor cells in all species, NKX2-5 is the main regulatory factor of early cardiac development (19-21). Interacting with other transcription factors like GATA4 and TBX5, NKX2-5 modulates expression of the genes which are required for morphogenesis and function of the heart (22, 23). Furthermore, there is evidence that NKX2-5 may be important during post natal life as well (24).

Human NKX2-5 gene is localized on chromosome 5q34 and consists of two exons which encode a 324 amino acid protein. Mutations in human NKX2-5 gene were shown to be responsible for >4% patients of tetralogy of Fallot (TOF) (25). Mutations of NKX2-5 gene were also found in patients with non-syndromic congenital heart disease (26) and in patients with congenital cardiac septal defects (27).

HAND1 (Heart and neural crest derivatives expressed 1, also known as eHand) is a basic helix-loop-helix transcription factor which is expressed at high level in the embryonic heart. HAND1 is one of asymmetrically expressed transcription factors during embryogenesis (28-30).

Although HAND1 is known to be essential for normal chamber formation, the main role and the target genes of the HAND1 are not known yet. However, many studies have shown that HAND1 may play role in regulating the balance of proliferation and differentiation in the myocardium of the ventricle and outflow tract (31). Absence of HAND1 in mice results in embryonic lethality, and HAND1 mutant mice have many cardiac anomalies like defective chamber formation, failed cardiac looping and impaired ventricular development (32). These findings suggest that this gene may play role in pathogenesis of congenital heart diseases. The finding that HAND1 mRNA levels were elevated in left ventricular biopsies from patients with hypertrophic cardiomyopathy, TOF (33), and cardiomyopathies (34) supports this idea.

Since asymmetric expression of HAND1 is known to be controlled by NKX2-5 during murine heart development (35), NKX2-5 gene plays an indirect role in the asymmetry of the heart. Due to their critical role in asymmetric heart development, NKX2-5 and HAND1 seem to be candidate genes for atrial isomerism.

To investigate whether NKX2-5 and HAND1 genes play role in the pathogenesis of disorder of heart symmetry, we analyzed NKX2-5 and HAND1 genes of patients with atrial isomerism. These two genes were analyzed for the first time in this study, concerning their possible role in atrial isomerism.

Methods

Patients

This study included 70 patients diagnosed with, or underwent surgical treatment for laterality defect/isomerism in Department of Cardiovascular Surgery, Institute of Cardiology İstanbul University. Patient group had normal karyotypes and free of consanguinity.

Control group was composed of healthy volunteers without family history for any cardiac or inherited disease. Control group included 80 healthy subjects for HAND1 and 40 healthy subjects for NKX2-5.

This case-control study was approved by the local Ethics Committee and each participant gave written informed consent after appropriate genetic counseling.

DNA isolation

DNA was yielded from peripheral blood of both patients and healthy subjects by using standard ammonium acetate method (36).

Polymerase chain reaction (PCR)

To yield specific DNA material for further genetic analyses, we used standard PCR technique (37). Genomic regions of HAND1 and NKX2-5 genes from DNA samples of patients and controls were amplified using primers listed in Table 1. PCR reactions was carried out in 25 ml volume containing 10x PCR buffer (50 mM KCl; 10 mM Tris-HCl; 1.5 mM $MgCl_2$), 2 mM $MgCl_2$, 0.8 µM each of primer, 200 µM dNTP mix, 1% DMSO, 0.5 U Taq DNA polymerase and 50 ng genomic DNA. Taq DNA polymerase was obtained from Roche (MBI Fermentas, Hanover, MD). PCR amplification was carried out in a DNA Thermal Cycler (MJ Research Techne, Berlin, Germany).

Amplification conditions were as follows

Initial denaturation at 95°C for 5 min followed by 30 cycles of denaturation at 95°C for 60 sec, annealing at 64°C for 60 sec, extension at 72°C for 60 sec with a final extension at 72°C for 10 min for HAND1.

Initial denaturation at 95°C for 5 min followed by 30 cycles of denaturation at 94°C for 10 sec, annealing at 65°C for 30 sec, extension at 68°C for 2 min with a final extension at 68°C for 10 min. for NKX2-5.

Restriction fragment length polymorphism (RFLP)

For the genotyping of known mutations in the NKX2-5 gene, PCR products were digested with appropriate restriction enzymes listed in Table 2. In the case of CM993125 (C>T), CM980448 (C>T) or CM980449 (C>T) mutations, mutant allel causes occurrence of a digestion site in PCR product for the restriction enzymes BsgI, BfaI and Hsp92II, respectively. In the case of CM993127 (C>A), CM993128 (C>G) or CM993130 (C>A) mutations, mutant allele causes disappearance of a preexisting digestion site in PCR product for the restriction enzymes Msp1, Msp2 and Mae3, respectively.

Single strand conformation polymorphism (SSCP)

For the determination of unknown mutations or Single Nucleotide Polymorphisms (SNPs) in NKX2-5 and HAND1 genes, we used SSCP analysis (38). SSCP analysis was performed using non-denaturing polyacrylamide gels on the Owl Separation Systems (Thermo Scientific, Rochester, NY, USA).

For SSCP detection, a volume of 2 µl PCR product was transferred into an Eppendorf tube, mixed with 5 µl gel loading solution containing 98% formamide, 0.025% bromophenol blue, 0.025% xylene cyanol, 20 mmol/l EDTA (pH 8.0) and 10% glycerol. The mixture was centrifuged and denatured at 98°C for 10 min, then chilled on ice for 5 min and loaded on 12% polyacrylamide gels (acrylamide:bisacrylamide=99:1). Electrophoresis was per-

Table 1. Sequences of primers used in the study and expected fragment sizes of amplified regions

Primer	Primer sequence	Expected amplification fragment size (bp)
	Primers for HAND1 Gene *	
H1F	ACGAACCCTTCCTCTTCGGTC	266
H1R	GCTGTTAATGCTCTCAGTGCG	
H2F	AAGATCAAGACTCTGCGCCTA	239
H2R	CAACACAGCCTCCTTCGACTA	
	Primers for NKX2-5 Gene **	
N1F	GTCCCGCCTCTCCTGCCCCTTGTG	582
N1R	TCCTCCTCCTGGCCCTGAGTTTCT	
N2F	TGGGCGCTCCAGGCAGGACACAGT	472
N2R	GCTTGCCATCGCGCACCAGCACTG	
N3F	GTTCCAGAACCGGCGCTACAAGTG	724
N3R	GCGTGCCCGAGCTCAGTCCCAGTT	
	Primers for NKX2-5 Gene ***	
N1F	GTCCCGCCTCTCCTGCCCCTTGTG	241
N1FR	GCTGCTGTTCCAGGTTTAGGATGT	
N1RF	ACGCCCTTCTCAGTCAAAGACATC	384
N1R	TCCTCCTCCTGGCCCTGAGTTTCT	
N2F	TGGGCGCTCCAGGCAGGACACAGT	277
N2FR	ACAGGTACCGCTGCTGCTTGAA	
N2RF	GGAGAAGACAGAGGCGGACAAC	320
N2R	GCTTGCCATCGCGCACCAGCACTG	
N3F	GTTCCAGAACCGGCGCTACAAGTG	334
N3FR	GCCGAAGTTCACGAAGTTGTTGT	
N3RF	CCGCCAACAACAACTTCGTGA	419
N3R	GCGTGCCCGAGCTCAGTCCCAGTT	

*Primers used for both SSCP and RFLP analysis
**Primers used for RFLP analysis
***Primers used for SSCP analysis

Table 2. Known NKX2-5 mutations examined in the study, and restriction enzymes used for their determination

HGMD* accession number	Base substitution	Codon change**	Aminoacid change***	Restriction enzyme
CM993125	C>T	AGC-AGT	Arg-Cys	BsgI
CM993127	C>A	AAC-AAA	Asn-Lys	MspI
CM 993128	C>G	CGG-GGG	Arg-Gly	MspI
CM980448	C>T	CAG-TAG	Gln-termination	BfaI
CM980449	C>T	ACG-ATG	Thr-Met	Hsp92II
CM993130	C>A	TAC-TAA	Tyr-termination	MaeIII

*Human Genome Mutation Database
**Changed base in codon is shown as bold character
***Arg - arginine, Cys - cysteine, Gln - glutamine, Gly - glycine, Lys - lysine, Met - methionine, Thr - threonine, Tyr - tyrosine

formed in Tris borate (pH 8.3-EDTA buffer at 600 V/cm at 14°C). After electrophoresis, the DNA fragments in the gels were visualized by silver-staining method using standard protocols. All chemicals used in gel electrophoresis and SSCP analysis were obtained from Sigma-Aldrich (Stockholm, Sweden), Merck (Darmstadt, Germany), and AppliChem GmbH (Darmstadt, Germany). Samples with different SSCP patterns were sequenced by commercial sequencing service, Iontek.

Statistical analysis

SPSS 10.0 (SPSS, Inc., Chicago, IL, USA) for Windows and the Microsoft Excel were used for statistical analysis. The frequencies of the alleles and genotypes were compared among patients and control groups using the Chi-square and the Fisher tests when appropriate. Statistical significance was taken as p<0.05.

Results

Patients

Clinical properties of the patients are presented in Table 3.

HAND1 gene

We did not found any genetic variation in the first exon of HAND1 gene. In intronic region, 52 bp downstream of exon-inron boundary of the HAND1 gene, we found one-base substitution (IVS1,+52C>G) both in patients and in controls (Fig. 1). This SNP was found in 8 patients and 2 controls (4 patients as homozygous, 4 patients and 2 controls as heterozygous). Both genotypes (CG and GG) of mutant allele (G) were found higher in patient group than in control group with a borderline significance (p=0.054) (Table 4). Mutant allele trait of patient group was found significantly higher than that of control group (p=0.046) (Table 4).

Clinical properties of the patients (no: 16, 22, 30 and 31 in Table 3) with heterozygous genotype (CG) and the patients (no: 12, 23, 27 and 63 in Table 3) with homozygous genotype (GG) for IVS1,+52C>G in HAND1 gene have no common pathology (Table 5).

NKX2-5 gene

In the first exon of NKX2-5 gene, we did not found any polymorphism by SSCP. However, we found that one patient (no: 58 in Table 3) had BfaI site polymorphism (Fig. 2) which leads to termination in protein sequence at position 170 (Gln170ter mutation). We did not found any variations in sites for BsgI, MspI, Hsp92II and Mae3.

Discussion

The aim of our study was to investigate whether the patients with atrial isomerism had any mutation in their HAND1 or NKX2-5 genes. Though we did not find any mutation in HAND1 gene, we found a one-base substitution in HAND1 intron, and we demonstrated a correlation between this genetic variation and the atrial isomerism. In NKX2-5 gene, we found a mutation, which was found in other congenital heart diseases before. However,

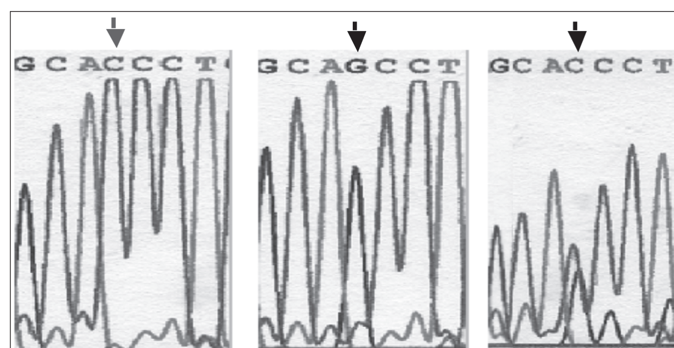

Figure 1. Sequence analysis of HAND1 intronic region from three samples. Homozygous for C allele (left), homozygous for G allele (middle) and heterozygous (right)

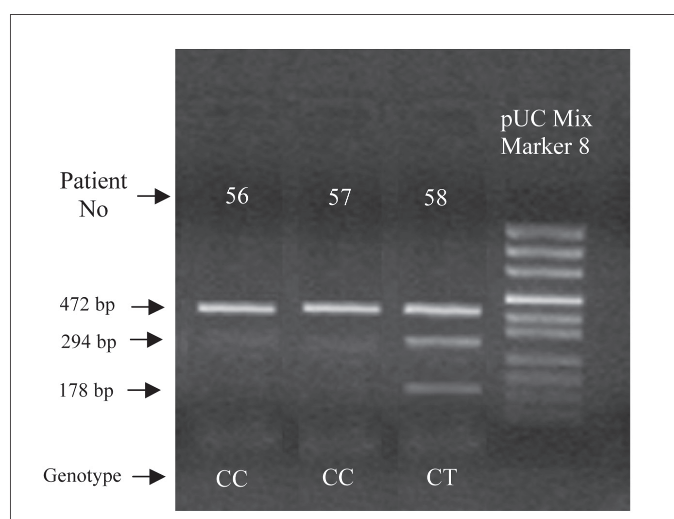

Figure 2. Agarose gel image of 472bp-PCR product after BfaI restriction enzyme digestion. Digested (294bp and 178bp) and undigested (472bp) fragments in line 3, indicate that the sample (Patient no: 58) is heterozygous for the mutant allele (T)

this mutation in the NKX2-5 was shown to be involved in atrial isomerism for the first time in this study.

The heart is developed from the cardiac mesenchyma, which is characterized by the expression of cardiac specific transcription factor NKX2-5 (21, 39, 40). Although, heart is the first organ, which demonstrates asymmetric morphological pattern during embryogenesis, asymmetric pattern at molecular level is defined at earlier stage of the embryogenesis (7, 10). Molecular asymmetry during embryogenesis leads to morphological asymmetry at later stages. Switch from molecular asymmetry to morphological asymmetry is known as lateralization (15-18). Lateralization during the embryogenesis is provided by asymmetric distribution of some secreted factors and asymmetric expression of some transcription factors (5-7). Secreted factors like Nodal and Lefty, and transcription factors like PITX2 are supposed to initiate an asymmetric activation of cascade of many other transcription factors (10, 41, 42). One of those asymmetrically expressed transcription factor is HAND1. Together with cardiac specific transcription factor NKX2-5, HAND1 is known to regulate cardiac ventricle formation (43, 44).

Table 3. Pathological properties (Cardiac phenotype) evaluated in study subjects with atrial isomerism (*)

Patient no	Cardiac phenotype **																																																	
	LAI	LC	MC	DC	RAI	ASI	ASS	SA	SV	SAVV	AVVi	RAVVA	RAVVi	LAVVA	ARAVC	ALAVC	PA	CAVSD	AVSD	IASD	ASD	IVSD	VSD	TOF	PS	CSLPAO	CSRPAO	SAS	hLV	hRV	AVD	VAD	DILV	DIRV	DORV	TGA	cTGA	ASVR	TAPVR	PAPVR	dSVC	LPSVC	PDA	hPA	PH	EA	TI	MI	AI	TOTAL
1				X					X						X		X				X																						X							6
2	X			X				X										X				X																												6
3		X				X																															X										X			4
4	X			X							X							X							X										X															6
5	X			X																					X				X							X														5
6					X			X	X	X							X																										X							6
7	X										X							X							X					X																				5
8				X		X					X							X							X										X														X	7
9			X			X											X						X														X													5
10	X							X																																					X			X		4
11				X		X																			X								X		X															5
12		X				X										X																																		3
13				X																			X					X																						4
14					X																				X					X					X															4
15						X			X					X											X		X														X									6
16	X																		X		X				X																									4
17				X			X	X	X	X	X														X										X															8
18				X	X																									X															X					4
19				X											X						X							X																						4
20	X																	X							X																									3
21		X			X													X							X							X																		5
22	X																							X																X										3
23					X					X	X						X				X													X																6
24					X													X																X	X					X										5
25				X																			X									X											X							4
26					X												X	X																				X			X									5
27			X			X																	X														X													4
28	X														X						X		X		X																									5
29						X																															X											X		4
30					X													X																X	X					X										5
31				X													X																	X							X									4
32					X			X	X	X							X						X																X				X							8
33					X													X																X	X					X										5
34				X													X																	X							X									4
35	X			X				X		X	X														X										X						X									8

Table 3. (continued)

Cardiac phenotype **

Patient no	LAI	LC	MC	DC	RAI	ASI	ASS	SA	SV	SAVV	AVVI	RAVVA	RAVVI	LAVVA	ARAVC	ALAVC	PA	CAVSD	AVSD	IASD	ASD	IVSD	VSD	TOF	PS	CSLPAO	CSRPAO	SAS	hLV	hRV	AVD	VAD	DILV	DIRV	DORV	TGA	cTGA	ASVR	TAPVR	PAPVR	dSVC	LPSVC	PDA	hPA	PH	EA	TI	MI	AI	TOTAL
36		■			■													■							■							■																		5
37				■			■																■																						■					4
38				■			■																■																						■					4
39	■							■	■	■	■														■																■	■								8
40				■													■				■		■								■																			5
41	■							■		■					■								■		■																									6
42				■																					■																									2
43					■												■		■																							■								4
44			■																				■		■												■													4
45				■								■					■																																	3
46			■																				■		■												■													4
47	■							■		■					■								■		■																									6
48					■						■						■	■																							■		■							6
49				■																					■								■																	3
50					■												■	■																	■						■									5
51		■			■				■	■										■					■																■									7
52		■											■			■					■				■							■																		6
53				■	■				■	■											■																				■									6
54				■	■			■	■																																									5
55					■												■	■																	■				■											5
56					■						■							■																							■									4
57				■																	■		■		■												■													5
58	■							■	■														■		■				■						■						■									8
59				■		■																	■		■										■										■					6
60	■	■							■	■															■				■						■															7
61		■																							■						■				■											■				5
62	■								■					■			■				■																						■	■						7
63				■	■						■						■	■								■																								6
64					■													■							■																									3
65						■																	■		■												■										■	■		6
66				■	■													■							■										■				■		■									7
67				■	■													■							■										■						■									6
68				■		■								■																																				3
69				■							■														■					■					■															5
70				■	■			■	■	■							■																																	6
Total	17	8	4	31	24	11	3	12	13	12	11	1	1	3	5	2	17	19	2	1	10	1	17	1	35	1	1	2	3	4	2	4	2	6	18	1	8	1	3	4	14	2	6	1	5	1	2	3	1	
Perentage	11.9	5.6	2.8	21.7	16.8	7.7	2.1	8.4	9.1	8.4	7.7	0.7	0.7	2.1	3.5	1.4	11.9	13.3	1.4	0.7	7	0.7	11.9	0.7	24.5	0.7	0.7	1.4	2.1	2.8	1.4	2.8	1.4	4.2	12.6	0.7	5.6	0.7	2.1	2.8	9.8	1.4	4.2	0.7	3.5	0.7	1.4	2.1	0.7	

* Filled boxes indicate the cardiac pathology carried by the patient

** AI- aortic insufficiency, ALAVC- absence of left atrio-ventricular connection, ARAVC- absence of right atrio-ventricular connection, ASD- atrial septal defect, ASI- atrial situs inversus, ASS- atrial situs solitus, ASVR- abnormal systemic venous return, AVD- atrio-ventricular discordance, AVSD- atrio-ventricular septal defect, AVVI- atrio-ventricular valve insufficiency, CAVSD- complete atrio-ventricular septal defect, CSLPAO- critical stenosis of left pulmonary artery ostium, CSRPAO- critical stenosis of right pulmonary artery ostium, cTGA- corrected transposition of great arteries, DC- dextrocardia, DILV- double inlet left ventricle, DIRV- double inlet right ventricle DORV- double outlet right ventricle, DSVC- double superior vena cava, EA- Ebstein anomaly, hLV- hypoplastic left ventricle, hPA- hypoplastic pulmonary arteries, hRV- hypoplastic right ventricle, LAI- left atrial isomerism, IASD- large atrial septal defect, LAVVA- left atrioventricular valve atresia, LC- levocardia, LPSVC- left persistent superior vena cava, IVSD- large ventricular septal defect, MC- mesocardia, MI- mitral insufficiency, PA- pulmonary atresia, PAPVR- partial anomalous pulmonary venous return, PDA- patent ductus arteriosus, PH- pulmonary hypertension, PS- pulmonary stenosis, RAI- right atrial isomerism, RAVVA- right atrioventricular valve atresia, RAVVI- right atrioventricular valve insufficiency, SA- single atrium, SAS- subaortic stenosis, SAVV- single atrio-ventricular valve, SV- single ventricle, TAPVR- total anomalous pulmonary venous return, TGA- transposition of great arteries, TI- tricuspid insufficiency, TOF- tetralogy of Fallot, VAD- ventriculo-arterial discordance, VSD- ventricular septal defect

Disruptions of the asymmetric development are supposed to cause heterotaxy syndromes (45). Since the heart is one of the asymmetrically situated organs, manifestations of heterotaxy syndromes include complicated heart defects. Therefore, patients with heterotaxy syndromes are subject to cardiac surgery.

Table 4. Comparison of genotypes and mutant allele carriers for IVS1,+52C>G substitution in HAND1 gene

Groups	Genotype			Mutant Allele	
	CC	CG	GG	CC	CG+GG
Controls (n=80)	78	2	0	78	2
Patients (n=70)	62	4	4	62	8
p	0.054*			0.046 **	

*Pearson Chi-square test
**Fisher's exact test

With or without complicated heart defects, heterotaxy syndromes are characterized by absence of one side and duplication of other side of the heart. Depending on which side of atrium is duplicated in the heart, heterotaxy syndromes are classified as right or left atrial isomerism (1-3). The term of atrial isomerism describes a congenital disorder (of lateralization) which is characterized by symmetric development of normally asymmetric cardiac atria and organ systems.

Molecular mechanism responsible for atrial isomerism or other laterality defects is not known yet. However, many studies suggest responsibility of developmental genes in such laterality defects (45-48).

Studies in model organisms have revealed complex genetic pathways in asymmetric development of vertebrates (5-9). These results suggest that several genes may be involved in the patho-

Table 5. Comparison of mutant allele (G) carriers for HAND1 +52C>G substitution, with respect to patients' clinical properties

Pathology	LAI (n=17)	LC (n=8)	MC (n=4)	DC (n=31)	RAI (n=24)	ASI (n=11)	ASS (n=3)	SA (n=12)	SV (n=13)	SAVV (n=12)
CG+GG (n=8)	2	1	1	2	3	2	0	0	0	1
CC (n=62)	15	7	3	29	21	9	3	12	13	11
*p	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Pathology	**AVVI (n=11)**	**RAVVA (n=1)**	**RAVVI (n=1)**	**LAVVA (n=3)**	**ARAVC (n=5)**	**ALAVC (n=2)**	**PA (n=17)**	**CAVSD (n=19)**	**AVSD (n=2)**	**IASD (n=1)**
CG+GG (n=8)	2	0	0	0	0	1	3	2	1	0
CC (n=62)	9	1	1	3	5	1	14	17	1	1
*p	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Pathology	**ASD (n=10)**	**IVSD (n=1)**	**VSD (n=17)**	**TOF (n=1)**	**PS (n=35)**	**CSLPAO (n=1)**	**CSRPAO (n=1)**	**SAS (n=2)**	**hRV (n=4)**	
CG+GG (n=8)	2	0	1	1	1	1	0	0	0	
CC (n=62)	8	1	16	0	34	0	1	2	4	
*p	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	
Pathology	**hLV (n=3)**	**AVD (n=2)**	**VAD (n=4)**	**DILV (n=2)**	**DIRV (n=6)**	**DORV (n=18)**	**TGA (n=1)**	**cTGA (n=8)**	**ASVR (n=1)**	**TAPVR (n=3)**
CG+GG (n=8)	0	0	0	0	3	1	0	1	0	0
CC (n=62)	3	2	4	2	3	17	1	7	1	3
*p	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Pathology	**PAPVR (n=4)**	**dSVC (n=14)**	**LPSVC (n=2)**	**PDA (n=6)**	**hPA (n=1)**	**PH (n=5)**	**EA (n=1)**	**TI (n=2)**	**MI (n=3)**	**AI (n=1)**
CG+GG (n=8)	2	1	0	0	0	0	0	0	0	0
CC (n=62)	2	13	2	6	1	5	1	2	3	1
*p	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

*Fisher's exact test

AI - aortic insufficiency, ALAVC - absence of left atrio-ventricular connection, ARAVC - absence of right atrio-ventricular connection, ASD - atrial septal defect, ASI - atrial situs inversus, ASS - atrial situs solitus, ASVR - abnormal systemic venous return, AVD - atrio-ventricular discordance, AVSD - atrio-ventricular septal defect, AVVI - atrio-ventricular valve insufficiency, CAVSD - complete atrio-ventricular septal defect, CSLPAO - critical stenosis of left pulmonary artery ostium, CSRPAO - critical stenosis of right pulmonary artery ostium, cTGA - corrected transposition of great arteries, DC - dextrocardia, DILV - double inlet left ventricle, DIRV - double inlet right ventricle DORV - double outlet right ventricle, DSVC - double superior vena cava, EA - Ebstein anomaly, hLV - hypoplastic left ventricle, hPA - hypoplastic pulmonary arteries, hRV - hypoplastic right ventricle, LAI - left atrial isomerism, IASD - large atrial septal defect, LAVVA - left atrioventricular valve atresia, LC - levocardia, LPSVC - left persistent superior vena cava, IVSD - large ventricular septal defect, MC - mesocardia, MI - mitral insufficiency, n.s. - not significant, PA - pulmonary atresia, PAPVR - partial anomalous pulmonary venous return, PDA - patent ductus arteriosus, PH - pulmonary hypertension, PS - pulmonary stenosis, RAI - right atrial isomerism, RAVVA - right atrioventricular valve atresia, RAVVI - right atrioventricular valve insufficiency, SA - single atrium, SAS - subaortic stenosis, SAVV - single atrio-ventricular valve, SV - single ventricle, TAPVR - total anomalous pulmonary venous return, TGA - transposition of great arteries, TI - tricuspid insufficiency, TOF - tetralogy of Fallot, VAD - ventriculo-arterial discordance, VSD - ventricular septal defect

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genesis of heterotaxy syndromes. Although, many studies have identified mutations in a small number of individuals (12-14), mutations in genes affected in most patients remain unknown.

Due to both asymmetric expression of HAND1 and cardiac specificity of NKX2-5, these two genes seem like to be candidate genes for atrial isomerism. In this purpose, we aimed to analyze NKX2-5, which is specifically expressed in cardiac tissue and, an asymmetrically expressed gene HAND1, in patients with atrial isomerism.

HAND1 mutations were found in patients with septation defects (49) and hypoplastic hearts (50) previously. We have found IVS1,+52C>G (NM_004821.1:c.543+52C>G; NT_029289.10:g.1501991-0C>G) substitution in HAND1 gene. Although this base substitution did not cause any change in protein sequence, it was found associated with atrial isomerism. While two controls (n=80) had C/G genotype in this locus, 4 patients (n=70) had C/G genotype and 4 patients had G/G genotype. There was not found G/G genotype for this locus in control group (Table 4). Statistical analysis of allele and genotype distribution between the patient and control groups suggests an association between atrial isomerism and the G allele (Table 4). Presence of G allele at this locus seemed to be a risk factor for atrial isomerism.

A higher incidence of the G allele, and the presence of G allele homozygosity in patients with atrial isomerism suggest the possibility that this intronic substitution may affect gene function. Since it is localized in the intronic region, this variation might exhibit its pathological phenotype by affecting RNA processing. However, the molecular mechanism responsible for this "allele-disease association" needs to be elucidated by further studies. In addition to molecular mechanism responsible for "allele-disease association", this intronic substitution needs to be tested in other cardiac diseases, as well.

During our NKX2-5 gene screening, we found one patient heterozygous for Gln170ter (Q170X) mutation (Fig. 2). This mutation has been identified earlier by Schott in patients with congenital heart disease (51). Gln170ter mutation is known to cause termination of translation just after helix 3 of NK homeodomain, thereby deleting the COOH-terminal NK domain (51). Therefore, this mutation is supposed to influence cardiac development through NKX2-5 target genes.

While common pathology of the patients carrying Gln170ter mutation (51) reported to date included atrial septal defects (ASD) and atrio-ventricular conduction defects, our patient had only ASD from these common findings. In addition to ASD, pathology of our patient included left atrial isomerism, DORV (double outlet right ventricle) hypoplastic left ventricle, pulmonary stenosis and double vena cava superior, as well (Patient no:58 in Table 3).

Although NKX2-5 gene was not studied in patients with atrial isomerism to date, several mutations in this gene have been detected in many other heart diseases like TOF (25), non-syndromic congenital heart diseases (26, 51, 52), atrial septal defects (27, 48) and recently patent foramen ovale (53). All these studies suggest that mutations of NKX2-5 gene seem like to be responsible for various congenital heart diseases. Responsibility of NKX2-5 mutations in various congenital heart diseases may be explained by different locations of the mutations. However, genotype-phenotype correlation of NKX2-5 mutations in cardiac diseases is not clear. There are numerous controversial studies on this issue. While some of these studies supported the genotype-phenotype correlation for NKX2-5 mutations, others impaired this correlation.

Schott et al. (51) have reported that heterozygous mutations in the NKX2-5 transcription factor are among the first evidence of a genetic cause for congenital heart disease in humans and most reported NKX2-5 mutations were found in the homeodomain, and were associated with cardiac conduction anomalies.

McElhinney et al. (52) demonstrated that NKX2-5 mutations occur in a small percentage of patients with various congenital heart diseases. Most of the mutations identified in that study were missense, outside the domain, and not associated with atrioventricular block. These findings suggest that NKX2-5 mutations in non-homeodomain regions may be important in the development of human structural cardiac defects.

Goldmuntz et al. (25) found that NKX2-5 mutation is present in >%4 of tetralogy of Fallot patients. Mutations identified in that study mapped outside of the domain, were not associated with atrioventricular conduction disturbances, and were not fully penetrant, in contrast to the mutations previously reported that impair homeodomain function.

On the other hand, Posch et al. (54) suggested that mutations in NKX2-5, GATA4, CRELD1 and BMP4 are infrequently found in patients with congenital cardiac septal defects.

Similarly, Hirayama-Yamada et al. (55) have not found clear genotype-phenotype correlation among 10 mutations located in NK homeodomain in familial atrial septal defect.

Since our findings are not sufficient for an assertion on genotype-phenotype correlation, we did not conclude any correlation.

More importantly, many studies have demonstrated that somatic mutations play crucial role in congenital heart diseases (56, 57). Reamon-Buettner et al. (56) have found somatic NKX2-5 sequence variants by direct sequencing in >95% of human hearts (n=68) with septal defects. These sequence variants were primarily identified within malformed regions and not in unaffected regions taken from the same heart. These data suggest that somatic sequence variants occur with high frequency and are etiologic in cardiac malformations.

These studies, which demonstrated involvement of somatic sequence variations in cardiac diseases, are particularly remarkable since most studies are performed in DNA from peripheral blood. Considering the possibility of somatic sequence variation, peripheral DNA sampling seems like to be major limitation of genetic studies. Therefore, tissue DNA sampling is recommended for further studies.

However, atrial isomerism that we have handled in this study is a heart disorder included to heterotaxy syndromes. As distinct from septal defects, heterotaxy syndromes are disorders in

which, rather than restricted region of the heart, all body is affected. Therefore, somatic mutations, if implicated in atrial isomerism as well, are expected to have occurred in earlier stages of embryogenesis, and to affect wider body regions including the heart. Alternatively, in case of somatic mosaicism covering Hensen's node, which determines Nodal flow and lateralization during early embryogenesis (58, 59), it is possible that heterotaxy syndrome develops without mutations detectable in blood or heart tissue.

Implication and importance of somatic mutations in atrial isomerism need to be clarified by further studies. In this study, at least germline mutations in HAND1 and NKX2-5 genes were shown to have implication in atrial isomerism.

Study limitations

Since the heart development requires complex interactions between genes, there are many candidate genes for congenital heart diseases like atrial isomerism. However, only two of those candidate genes were included in this study.

We analyzed the DNA samples isolated from the blood of the patients. Therefore, possible tissue-restricted mutations were omitted due to the requirement of heart tissue sampling.

Conclusion

Due to cardiac specific expression of NKX2-5 gene and asymmetrical expression of HAND1 gene, we screened these genes in patients with atrial isomerism. We have found a nonsense mutation (Gln170ter) in NKX2-5 gene of a patient and one intronic variation in HAND1 gene of eight patients. Though, Gln170ter mutation has been shown to cause cardiac diseases before, an intronic variation in the HAND1 gene was shown to be related to atrial isomerism first time.

In conclusion, we have demonstrated for the first time that sequences variations in NKX2-5 gene and HAND1 gene may have importance in etiology or pathogenesis of atrial isomerism. Although, there was not found any genotype-phenotype correlation for our patients in this study, defined base substitutions in HAND1 and NKX2-5 genes seem like to be pathology-related variations.

Conflict of interest: None declared.

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