Genetic analysis of the Irx4 gene in hypertrophic cardiomyopathy

Hipertrofik kardiyomiyopatide Irx4 geni analizi

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Objectives: The Irx4 gene is predominantly expressed in cardiac ventricles. It has been demonstrated in animal studies that disruption of the Irx4 gene caused inhibition of chamber-specific expression of myosin heavy chain genes, resulting in abnormal ventricular gene expression and cardiac hypertrophy. In this study, we aimed to investigate a possible association between mutations in the Irx4 gene and hypertrophic cardiomyopathy (HC).

Study design: The study included 68 patients (32 females, 36 males; mean age 49 years; range 17 to 74 years) with HC and 67 healthy controls (33 females, 34 males; mean age 45 years; range 20 to 88 years). All the patients were evaluated with a detailed history, physical examination, 12-lead electrocardiography, and transthoracic echocardiography. DNA samples of all the subjects were extracted. Genomic DNA fragments were amplified by polymerase chain reaction and screened by single-strand conformation polymorphism analysis. DNA sequences were determined through an automated sequencing system.

Results: All exons in the Irx4 gene were examined. No mutations were detected associated with HC. Four polymorphisms were identified including G355>A, A381>G, G1203>A, and C1431>T. Compared with patients having the GA and GG genotyes, patients with the AA genotype of A381>G polymorphism were found to have a higher maximal left ventricle outflow tract gradient (p=0.03), prolonged corrected QT dispersion (p=0.05), and albeit not statistically significant, increased septal thickness (p=0.07).

Conclusion: This is the first human study investigating the association between the Irx4 gene and HC. Polymorphism A381>G of the Irx4 gene may have a modifier effect on septal thickness, resulting in increased corrected QT dispersion and higher outflow gradients.

Key words: Cardiomyopathy, hypertrophic/genetics; genetic screening; genotype; polymorphism, genetic.

Amaç: Irx4 geni temel olarak kardiyak ventriküllerde eksprese olmaktadır. Hayvan çalışmalarında, Irx4 geninde meydana gelen hasarlanmanın miyozin ağır zincir gen ekspresyonunu bozarak, ventrikül gen ekspresyonunda anormalliğe yol açtığı ve kardiyak hipertrofiye neden olduğu gösterilmiştir. Bu çalışmada, Irx4 geni mutasyonları ile hipertrofik kardiyomiyopati (HK) arasında olası bir ilişkinin araştırılması amaçlandı.

Çalışma planı: Çalışmaya HK tanısı konan 68 hasta (32 kadın, 36 erkek; ort. yaş 49; dağılım 17-74) ve 67 sağlıklı kontrol (33 kadın, 34 erkek; ort. yaş 45; dağılım 20-88) alındı. Tüm hastalar ayrıntılı öykü, fizik muayene, 12 derivasyonlu elektrokardiyografi ve transtorasik ekokardiyografi ile değerlendirildi. Tüm olguların DNA örnekleri çıkarıldı. Genomik DNA fragmanları polimeraz zincir reaksiyonuyla amplifiye edildikten sonra SSCP (single-strand conformation polymorphism) analiziyle tarandı. DNA dizinleri otomatik DNA dizileme sistemiyle belirlendi.

Bulgular: Irx4 geninin tüm ekzonları tarandı, HP ile ilişkili olabilecek bir mutasyona rastlanmadı. Bununla birlikte, dört polimorfizm (G355>A, A381>G, G1203>A ve C1431>T) saptandı. GA ve GG genotipli hastalarla karşılaştırıldığında, A381>G polimorfizminin AA genotipini taşıyan hastalarda sol ventrikül çıkış yolu gradiyentinin daha yüksek (p=0.03), düzeltilmiş QT dispersiyonunun daha uzun (p=0.05) ve istatistiksel olarak anlamlı olmamakla birlikte, interventriküler septum kalınlığının daha fazla (p=0.07) olduğu görüldü.

Sonuç: Bu çalışma, insanda Irx4 geni ile HK arasındaki ilişkiyi araştıran ilk çalışmadır. Irx4 geninde belirlenen A381>G polimorfizminin septal kalınlık artışı üzerine modifiye edici etkisi olabilir; bu durum da düzeltilmiş QT dispersiyonunda ve çıkış yolu gradiyentinde artışa yol açabilir.

Anahtar sözcükler: Kardiyomiyopati, hipertrofik/genetik; genetik tarama; genotip; polimorfizm, genetik.

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Hypertrophic cardiomyopathy (HC) is inherited as a Mendelian autosomal dominant trait and is caused by more than 200 different mutations in genes, each encoding protein components of the cardiac sarcomere.[1,2] The disease is genetically and clinically heterogeneous. The severity and the onset age of the disease change depending on mutation types.[3] Mutations in the known contractile protein genes are not found in one-third of the HC cases.

Molecular studies of HC patients without sarcomeric protein mutations revealed other genetic causes of cardiac hypertrophy, such as mutations in the regulatory γ2 subunit of AMP-activated protein kinase,[4-6] and lysosome-associated membrane protein 2.[7] Recently, mutations in the muscle LIM protein gene have been proposed to cause HC.[8]

The Irx4 protein (Iroquois family homeobox protein 4) is predominantly expressed in the cardiac ventricles and is a necessary mediator of ventricular differentiation during cardiac development, including the adulthood period.[9-13] No mutation screening has been performed on the human Irx4 gene which is localized in chromosome 5p15.3.[10] In animal studies, the absence of the Irx4 protein caused inhibition of chamber-specific expression of myosin heavy chain genes which led to abnormal ventricular gene expression, resulting in adult-onset cardiac hypertrophy.[9,11] Considering that the absence of genes under the control of Irx4 caused cardiomyopathy in mice, we presumed that it might have a similar effect in humans. Therefore, this study was designed to investigate the relationship between possible mutations in protein coding region of the human Irx4 gene and HC.

PATIENTS AND METHODS

Study sample. The study was approved by the local ethics committee and each participant gave written informed consent after appropriate genetic counseling. The study consisted of 68 patients (32 females, 36 males; mean age 49±17 years; range 17 to 74 years) with HC. All the patients were evaluated with a detailed history, physical examination, 12-lead electrocardiography, and transthoracic echocardiography. Age- and gender-matched 67 healthy subjects (33 females, 34 males; mean age 45±14 years; range 20 to 88 years) with normal transthoracic echocardiograms were also included as controls. The diagnosis of HC was based on the demonstration of a hypertrophied, nondilated left ventricle (wall thickness of at least 15 mm) by two-dimensional echocardiography, in the absence of other cardiac or systemic diseases that might produce hypertrophy of similar degree.[14] The ECG recordings were obtained with a paper speed of 50 mm/sec at normal filtering. The QT interval was defined as the interval between the beginning of the QRS complex and the end of the T wave. Three consecutive cycles were manually measured in each of the standard 12 leads, and a mean value was calculated from these three measurements. The QT interval was then corrected (QTc) using Bazett's formula. The corrected dispersion of QT intervals was defined as the difference between the maximum and minimum of the corrected QT interval which could be measured in any of the 12 ECG leads.

Genetics analysis. DNA was isolated from whole blood using standard procedures. For mutation screening, the Irx4 gene was subdivided into seven overlapping

Table 1. Primers used for the amplification of Irx4 gene fragments and parameters of the PCR and SSCP analysis

Oligonucleotides (5'→ 3')	Strand[a]	Exons	Fragment size (bp)	Nucleotide numbering	Annealing Tm (°C)	AC (%)/ AG (%)[b]	Temperature (°C)/ Voltage/ Run time
CCGCTGCTCACTTTGTTA	S	Exon1	320	5'UTR -64~-46	58	12/10	10/ 600/ 3 hr 30 min
AGTCGTAGGTCGGACACT	A			IVS1 +92~+74			
CCTCTCTCTGCAGTTCTT	S	Exon2	325	IVS1 -13~E2 +5	60	12/10	10/ 600/ 3 hr 30 min
AAGTGGGCTTGGCAAACT	A			IVS2 +60~+42			
AGAGAGGCTGGAGTGGGGTTG	S	Exon3	263	IVS2 -63~-42	56	10/10	14/ 600/ 3 hr
AGAAAGGGGGCATGATGGTGG	A			IVS3 +91~+70			
CATTGCAGAGCCTGTCTCCAG	S	Exon4	372	IVS3 -21~-1	60	12/10	14/ 500/ 3 hr 30 min
CAGTGACAACCTCCCAACCCA	A			IVS4 +22~+1			
GTCTGATTCACCTACCAACCC	S	Exon5	368	IVS4 -55~-34	60	8/15	20/ 500/ 3 hr
TTGGCCTCGCAGACCTGA	A			E5 +313~+29			
TGGACGAGGACCTGGAGA	S	Exon5	370	E5 +210~+228	60	8/15	20/ 400/ 5 hr
CACCCAGTTTCTAGAACTGGTT	A			E5 +580~+558			
TGGACAGGAACCAGGACT	S	Exon5	380	E5 +533~+551	56	10/10	20/ 400/ 3 hr
AGTGAAAAGAGTCGGCGC	A			3'UTR +67~+49			

[a]S indicates the forward primer with the sequence from the sense strand, A indicates the reverse primer with the sequence from the antisense strand.
[b]AC: Acrylamide concentration; AG: Addition of glycerol; PCR: Polymerase chain reaction; SSCP: Single-strand conformation polymorphism.

Table 2. Clinical and echocardiographic characteristics of patients with hypertrophic cardiomyopathy

	n	%	Mean±SD	Range
Age (years)			49±17	17-74
Male gender	36	52.9		
Family history				
Hypertrophic cardiomyopathy	16	23.5		
Sudden death	15	22.1		
Clinical status				
Symptomatic	43	63.2		
Asymptomatic	25	36.8		
Left ventricle				
End-systolic diameter (cm)			2.4±0.5	
End-diastolic diameter (cm)			4.3±0.6	
Maximal wall thickness (cm)			2.5±0.6	
Ejection fraction (%)			75±8.4	51-93
Left atrium size (cm)			4.6±0.7	2.9-6.5
Patients with resting gradient >30 mmHg	29	42.7		
QRS duration (msec)			120±24	
QT dispersion (msec)			72±24	
Corrected QT dispersion (msec)			80±26	

fragments including the intron-exon boundaries and the coding regions. Primers were designed from flanking intronic sequences for all exons of the Irx4 gene (GenBank accession number: AY335941). Genomic DNA fragments were amplified by polymerase chain reaction (PCR) and screened by single-strand conformation polymorphism (SSCP) analysis (Table 1). Amplified fragments were purified with the QIAquick PCR purification kit (Qiagen GmbH, Hilden, Germany). PCR fragments with differential migration were characterized by DNA sequencing in the patients and controls. DNA sequences were determined through an automated DNA sequencing system (Genosphere Biotechnologies, Paris, France). In addition, the G355>A and G1203>A polymorphisms detected in Irx4 gene fragments were confirmed by digestion with Msp A1I and Sac II restriction enzymes, respectively.

Statistical analysis. Genotypic and allelic distributions were compared using the chi-square test. Continuous variables were compared with a two-tailed t-test and expressed as means ± standard deviation (SD), while categorical variables were compared using the chi-square test. A two-tailed *p* value of less than 0.05 was considered statistically significant. All statistical analyses were performed using the SPSS software (Windows, version 10.0).

RESULTS

The clinical, electrocardiographic, and echocardiographic characteristics of the patients with HC are shown in Table 2. Sixty-four patients had asymmetric septal hypertrophy (the ratio of end-diastolic septal to left ventricular posterior wall thickness ≥1.5), and four patients had apical hypertrophy. Basal left ventricular outflow obstruction (gradient ≥30 mmHg) was present in 29 patients (42.7%). All control subjects had normal transthoracic echocardiograms.

Four polymorphisms were identified in the Irx4 gene, namely, G355>A, A381>G, G1203>A, and C1431>T. The location of these single nucleotide polymorphisms (SNPs) in relation to genomic structure of the Irx4 gene is shown in Fig. 1. In the public database (dbSNP), nine polymorphisms are present in the protein coding region of the Irx4 gene, including these four polymorphisms detected in our study: rs2232376 (G355>A), rs4975753 (A381>G), rs1689717 (G1203>A), and rs2279589 (C1431>T). The G-to-A transition at nucleotide 355 (G355>A) in exon 3 resulted in substitution of alanine for threonine at residue 119 (Ala119Thr) of the Irx4 protein. The other polymorphisms did not cause residue change in the protein (Pro127Pro, Ala401Ala, and Gly477Gly).

Comparison of genotypic and allelic frequencies of each polymorphism between patients and controls is shown in Table 3. Allelic distributions did not differ significantly between patients and controls.

Compared to patients with GA and GG genotypes, the AA genotype of A381>G polymorphism was found to be associated with a higher maximal left ventricle outflow tract gradient (48±53 mmHg *vs* 24±35 mmHg; p=0.03), prolonged corrected QT dispersion (79±21

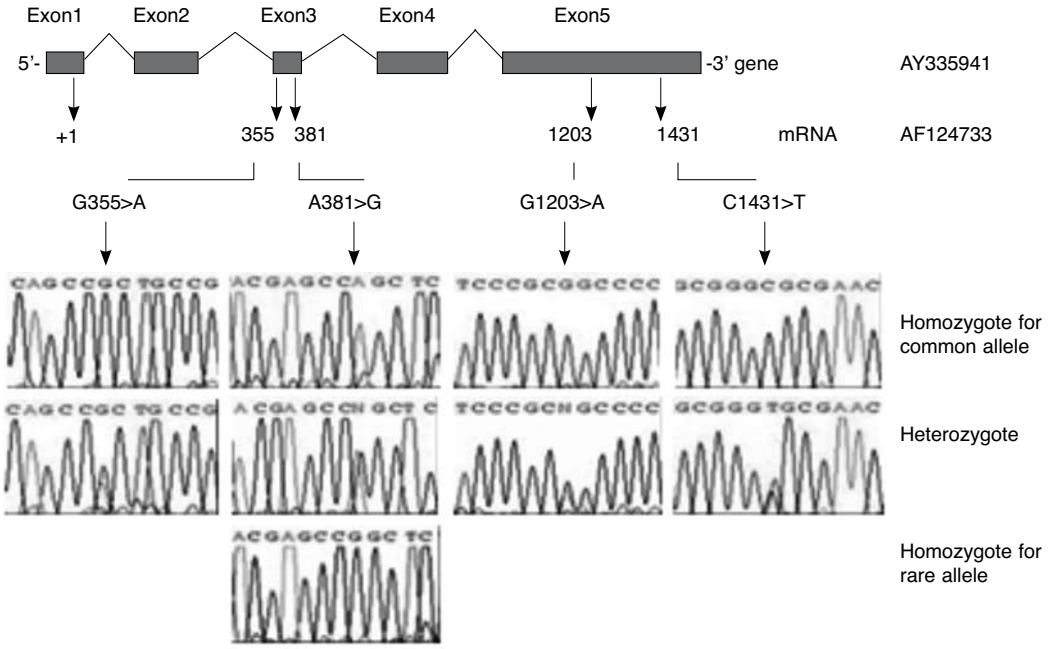

Figure 1. Schematic structure of the human Irx4 gene showing the localization of four polymorphisms and associated DNA sequencing results. Polymorphisms are counted downstream from the ATG triplet (A=+1) coding for the start methionine.

msec *vs* 63±18 msec; p=0.05), and albeit statistically insignificant, increased septal thickness (2.6±0.7 cm *vs* 2.2±0.5 cm; p=0.07).

DISCUSSION

For the first time, we examined all exons in the human Irx4 gene and identified four polymorphisms (G355>A, A381>G, G1203>A, and C1431>T), but we could not find any mutations in the Irx4 gene associated with HC.

Modifier genes are neither necessary nor sufficient to cause HC, but they have a significant effect on the severity of the disease.[15] Recent reviews have pointed out that the influence of modifier genes and environmental factors on the phenotypic expression of HC limit the utility of, and drawing conclusions from genotype-phenotype correlation studies.[3,15] On the other hand, some modifier genes such as A/G 18443 polymorphism in the myosin binding protein C (MyBP-C3) gene and

Table 3. Genotype and allele frequencies for polymorphisms of the Irx4 gene in patients and controls

		Patient group		Control group		
	Genotypes	n	%	n	%	*p*
G355>A	GG	41	60.3	45	67.2	
	AG	21	30.9	19	28.4	0.528
	AA	6	8.8	3	4.5	
G/A allele		0.76/0.24		0.81/0.19		
A381>G	AA	33	48.5	31	46.3	
(n=67)	AG	27	39.7	28	41.8	0.929
	GG	7	10.3	8	11.9	
A/G allele		0.69/0.31		0.67/0.33		
G1203>A	GG	55	80.9	50	74.6	
	GA	13	19.1	17	25.4	0.382
G/A allele		0.905/0.095		0.873/0.127		
C1431>T	CC	54	79.4	52	77.6	
	CT	14	20.6	15	22.4	0.799
C/T allele		0.897/0.103		0.888/0.112		

an insertion/deletion polymorphism in the angiotensin-1 converting enzyme (ACE-1) gene were found to be associated with the clinical phenotype of HC.[16-18] Although over 29 diverse mutations in MyBP-C3 account for approximately 20% to 25% of all HC cases,[3] it was found that the A/G 18443 polymorphism of MyBP-C3 was also a genetic modifier for the development of left ventricular wall thickness in patients with HC.[16]

In our study, no mutations were detected associated with HC, but compared with the GA and GG genotypes, the AA genotype of A381>G polymorphism was found to be associated with a higher maximal left ventricle outflow tract gradient, a prolonged corrected QT dispersion, and albeit statistically insignificant, increased septal thickness in patients with HC. Thus, the A381>G polymorphism of the Irx4 gene may have a modifier effect on septal thickness resulting in a prolonged corrected QT dispersion and higher outflow gradients, but these data need to be validated by further large scale studies.

In our study, mutation screening included the intron-exon boundaries and the coding regions of the Irx4 gene, but not other sites such as introns, and regulatory elements. Possible polymorphisms or mutations of these sites might be the genetic modifier in the development of cardiac hypertrophy because animal studies demonstrated that the absence of the Irx4 protein caused adult-onset cardiac hypertrophy.[9,11] However, this needs to be substantiated by further studies that include whole gene screening and expression pattern analysis.

Our sample size may not be large enough to detect mutations in the Irx4 gene; therefore, further studies with broader patient populations are necessary. It may also be of value to analyze the Irx4 gene in patients without sarcomere gene mutations.

This is the first human study investigating the association between possible mutations in the Irx4 gene and HC. Four polymorphisms were identified within the Irx4 gene. The AA genotype of A381>G polymorphism was found to be associated with a higher maximal left ventricle outflow tract gradient and prolonged corrected QT dispersion.

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