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Original Article

Investigation of the *MYH11* gene in sporadic patients with an isolated persistently patent arterial duct

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Abstract Persistent patency of the arterial duct is one of the most common congenital cardiac malformations. We recently showed that mutations in the *MYH11* gene result in a disease combining familial thoracic aortic aneurysm and dissection, along with patency of the arterial duct. It is also known that the smooth muscle myosin heavy chain is involved in the physiological closure of the arterial duct. With this in mind, we investigated whether the *MYH11* gene was a susceptibility gene for sporadic occurrence of isolated persistent patency of the arterial duct. We sequenced the entire coding sequence of the *MYH11* gene in 60 Caucasian children with persistent patency born after 36 weeks of gestation. The frequencies of rare genetic variants, and single nucleotide polymorphisms, were compared with 192 normal controls. Two possible functional missense mutations were found in two affected individuals. Another rare variant, specifically p.Arg1535Gln, and two coding polymorphisms, namely p.Ala1234Thr and p.Val1289Ala, had allele frequencies similar to those in controls. Haplotype analysis after estimating linkage disequilibrium was carried out using six polymorphisms. Individual genotypes were distributed similarly among cases and controls. Only one of the seven major haplotypes was significantly less frequent among cases, at 0.07, than among controls, when the figure was 0.22 (OR 0.23 [0.08–0.27]). Our findings suggest that the *MYH11* gene is involved in only rare instances when persistent patency of the arterial duct occurs in sporadic fashion.

Keywords: Congenital heart disease; polymorphism; genetics; myosin

THE PERSISTENT PATENCY OF THE ARTERIAL DUCT occurs in about one in every 2000 births. As such, it is the second most common congenital cardiac malformation.[1] Although generally regarded as a sporadic abnormality, genetic risk factors may play a role, and recurrence is observed among 5% of siblings of affected subjects.[2,3] Persistent patency is also part of rare syndromic defects, such as the Char syndrome,[4,5] and a new heart-hand syndrome comprising patency of the duct, an aortic valve with 2 leaflets, hypoplasia of the 5th metacarpal, and brachydactyly.[6] A recessive form of non-syndromic smooth muscle myosin heavy chain has also been reported in the Iranian population.[7] Homozygosity mapping in 21 unrelated consanguineous kindreds led to the identification of a susceptibility locus on chromosome *12q24*.

More recently, investigations have been made of American and French kindreds with familial aneurysm and dissection of the thoracic aorta, and persistent patency of the arterial duct.[8,9] In these

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families, genome-wide linkage analysis mapped the causal locus to *16p12.2-p13.13*,[10] and mutations in the *MYH11* gene were eventually identified.[11] This gene encodes the smooth muscle myosin heavy chain, a major contractile protein of smooth muscle cells. It is known that differentiation of smooth muscle cells, constriction, migration, and apoptosis all play a major role in physiological closure of the arterial duct.[12–14] Differences in levels of smooth muscle myosin heavy chain between the arterial duct and aorta have also been reported towards the end of fetal development.[13,14] *MYH11* is thus a legitimate candidate gene for variations potentially favouring persistent patency of the arterial duct.

Methods

Patients

We recruited patients from the Paediatric Cardiologic Department of Necker-Enfants Malades Hospital. In total, we had treated 84 patients with isolated persistent patency of the arterial duct in the three years preceding the study, and these were the patients available for our genetic analysis. We selected 60 of these children, based on them having isolated persistent patency of the arterial duct with no other abnormality, the duct continuing to be patent after one year of age in 3, or having required endovascular closure in 50, and surgical closure in the remaining 7. All were delivered after 36 weeks of gestation in the absence of maternal infection with rubella or any of the other known factors that might have favoured persistent patency. All patients were Caucasian, and written informed consent for genetic analysis was obtained from their parents. Our study was approved by the ethics committee of the Necker Hospital.

MYH11 sequencing

Direct bidirectional sequencing of the 42 exons of the human *MYH11* gene, which encodes smooth muscle myosin heavy chain 11 isoform SM2 (MIM 160745), was carried out for all the 60 cases, using the Sanger method (BigDye Terminator cycle sequencing kit, Applied Biosystems, CA, USA). Sequencher® 4.0.5 (Gene Codes Corporation, Michigan, USA) was used for sequence analysis and alignment.

We also used this method to check for the presence of rare genetic variants or common polymorphisms in our control population. The primers used for direct sequencing of the *MYH11* gene have been described elsewhere.[11] The control group consisted of 192 normal, healthy, Caucasian volunteers from the general population.

Coiled-coil region prediction

We used Coils software[15] to predict residue positions in the heptad repeat pattern and the probability of coiled coil formation. The wildtype smooth muscle myosin heavy chain sequence was entered under the following configurations: matrix MTK, window width 21, weighted.

Sequence alignment

We used ClustalW to align the amino-acid sequences of several type II myosin heavy chains.[16]

Statistics

Quantitative values are expressed as means plus or minus standard deviations. We used non-parametric Chi-squared tests, as implemented in StatView 5.0 software (SAS Institute Inc, NC, USA), to compare groups. Linkage disequilibrium and haplotype analyses were carried out with THESIAS software (www.genecanvas.org), based on the SEM algorithm.[17] Linkage disequilibrium was deduced from the estimated haplotype frequencies, and its extent was expressed in terms of D', this being the ratio of the nonstandardized coefficient to its maximal and minimal values. Haplotype effects are expressed as odds ratios with 95% confidence intervals (ORs; 95% CI). Effects associated with rare haplotypes, considered as those having a frequency of less than 0.03, were not estimated, but instead were fixed at zero.

Results

Characteristics of the patients

The characteristics of the 60 selected cases are summarized in Table 1. In line with the criterions for selection, none of the patients was born before term, with the mean gestational age at birth being 39.9 plus or minus 1 week, and this maturity at delivery was reflected in the mean birth weight, of 3518 plus or minus 624 grams. Diagnosis in our department occurred quite late, at a mean of 4 years,

Table 1. General characteristics of the patients.

Number of patients	60
Female (%)	35 (58%)
Male (%)	25 (42%)
Birth weight (grams) (mean ± SD)	3518 ± 624
Age at diagnosis (years) (mean ± SD)	2.75 ± 3.6
Age at closure (years) (mean ± SD)	4 ± 4
Closure by surgery	7 (12%)
Closure by catheterization	50 (83%)
Patency	3 (5%)
Familial history	0

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because most of the children were referred to us for the treatment of congenital cardiac malformations. Indeed, 50 of the patients required transcatheter, and 7 surgical, closure.

Rare MYH11 genetic variants

We detected 5 non-synonymous variants (Table 2) among these 60 cases. We also found 2 rare heterozygous missense mutations, absent from the control group, in 2 affected individuals (Fig. 1). The first, p.Arg669Cys, was located in a conserved motif of the smooth muscle myosin heavy chain also present in the human cardiac myosin heavy chain *MYH7* and the chicken skeletal myosin heavy chain. The corresponding residue is located on the surface of the molecule in all structural states characterized to date, and could contribute to actin binding. The second mutation, p.Glu1290Gln, corresponded to an amino acid in the rod region of the smooth muscle myosin heavy chain. This mutation was present in the sequence of smooth muscle myosin heavy chains from different species, but not in other myosin heavy chains (Fig. 1). Its position "*b*" in the heptad repeat of the coiled-coil tail suggests a possible effect on the arrangement of the smooth muscle myosin heavy chain tail. This mutation did not significantly affect the probability of coiled coil formation, as predicted by Coils software (data not shown).

Another rare missense variant, namely p.Arg-1535Gln, was observed in 2 children with persistently patent arterial ducts. This mutation was also located in a region conserved between humans, mice, rabbits and chickens. It was also found in two controls, providing evidence that this variant plays no substantial role in promoting persistent patency.

Common MYH11 polymorphisms

We identified 2 coding polymorphisms, p.Ala-1234Thr and p.Val1289Ala, with similar allelic frequencies in cases and controls (0.27 versus 0.25 and 0.05 versus 0.06, both ns). We found 5 non-synonymous variants, and 23 synonymous polymorphisms in the coding region, 14 of which were already present in public databases (http://www.ncbi.nlm.nih.gov/SNP), and 9 of which had never before been described (see supplementary Table 1). We also identified 38 diallelic polymorphisms in the non-coding sequences explored during the analysis of intron-exon boundaries, 21 of which had not been described before. We also identified several new intronic variations in the vicinity of splicing sites (see supplementary Table 1). None of these variations affected the probability of splicing, as predicted with NetGene 2 software[18,19] (data not shown).

Table 2. New *MYH11* variants and polymorphisms found in the sporadic cases of persistent patency of the arterial duct (PAD).

					PAD					Control				
Function	DbSNP reference	Region	Nucleotide change	Predicted residue change	Number	AA	Aa	aa	Allelic frequency	Number	AA	Aa	Aa	Allelic frequency	p
Non-synonym		exon_16	c.2005C > T	p.Arg669Cys	59	58	1	0	0.006	192	192	0	0	0	>0.05
	rs16967494	exon_28	*c.3700G > A	p.Ala1234Thr	60	33	21	6	0.275	189	106	70	13	0.254	>0.05
	rs16967510	exon_29	c.3866T > C	p.Val1289Ala	58	52	6	0	0.052	171	151	20	0	0.058	>0.05
		exon_29	c.3868G > A	p.Glu1290Gln	60	59	1	0	0.006	181	181	0	0	0	>0.05
		exon_33	c.4604G > A	p.Arg1535Gln	60	58	2	0	0.012	173	171	2	0	0.006	>0.05
Synonym	rs2272554	exon_14	*c.1743T > C		59	26	20	13	0.390	171	55	92	24	0.409	>0.05
	rs12907	exon_30	c.3967C > T		58	55	3	0	0.026	171	162	9	0	0.026	>0.05
	rs2075511	exon_31	*c.4242T > G		60	19	25	16	0.475	175	39	92	44	0.514	>0.05
	rs1050162	exon_38	*c.5439G > A		60	19	30	11	0.433	159	40	78	41	0.503	>0.05
Intronic		intron_16	*c.2058 + 30C > T		60	46	13	1	0.125	152	96	48	8	0.211	>0.05
	rs11130	intron_31	*c.4117 − 44C > T		60	21	22	17	0.467	174	36	92	46	0.529	>0.05

*Single nucleotide polymorphism (SNP) used for haplotype frequency analysis. Abbreviations: AA: homozygous wild-type; Aa: heterozygous variant; aa: homozygous variant.

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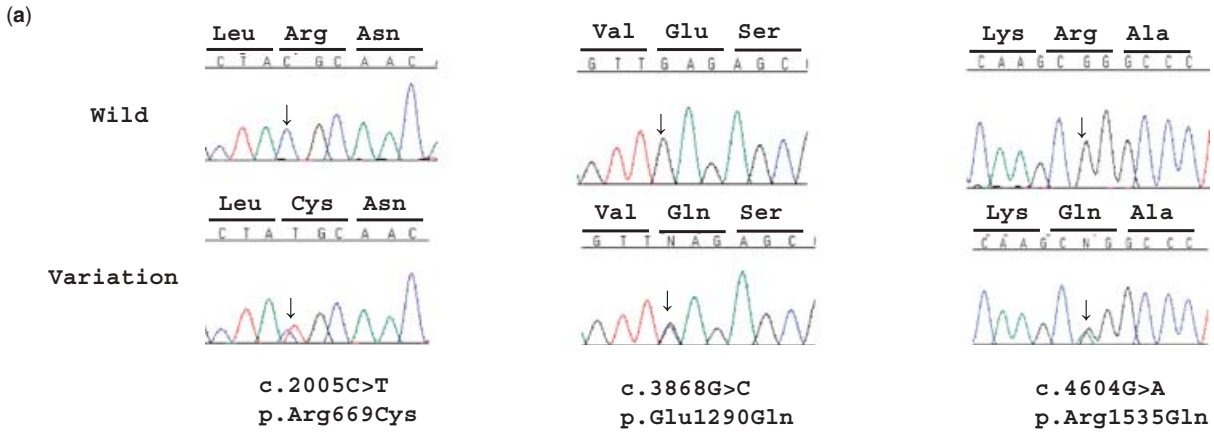

(b)

	R669C	A1234T	V1289A/E1290Q	R1535Q
Chicken SM-MHC	KLMTTL**R**NTNPNF	TLEKDN**A**DLANEI	HKLQIE**VE**NVTSLL	ELEKSK**R**TLEQQV
Rabbit SM-MHC	KLMTTL**R**NTTPNF	TLEKEN**A**DLAGEL	HKLQNE**VE**SVTGML	ELEKSK**R**ALETQM
Mouse SM-MHC	KLMATL**R**NTTANF	TLEKEN**A**DLAGEL	HKLQNE**VE**SVTGML	ELEKSK**R**ALETQM
Human SM-MHC	KLMTTL**R**NTTPNF	TLEKEN**A**DLAGEL	HKLQNE**VE**SVTGML	ELEKSK**R**ALETQM
Human NM-MHC	KLMATL**R**NTNPNF	TLENER**G**ELANEV	TKLQVE**LD**NVTGLL	ELEKSK**R**ALEQQV
Human Cardiac MHC	KLMTNL**R**STHPHF	EFKLEL**D**DVTSNM	AKLQTE**NG**ELSRQL	ELEKVR**K**QLEAEK
Chicken Skeletal MHC	NLMTNL**R**STHPHF	ELKMEI**D**DLASNM	ARLQTE**SG**EYSRQV	ELEKVK**K**QIEQEK

Figure 1.
*Description of the five non-synonymous variants found in the cases of persistent patency of the arterial duct. (**a**) Chromatography of the three new non synonymous variants. (**b**) Alignment of the human SM-MHC with other isoforms of type II MHC in the regions affected by the non synonymous variants. The residues concerned are shown in bold.*

Table 3. Pairwise analysis of linkage disequilibrium between the six polymorphisms of the *MYH11* gene in the 150 pooled samples.

	c.1743T > C	c.2058 + 30C > T	c.3700G > A	c.4117 − 44C > T	c.4242T > G	c.5439G > A
c.1743T > C.	•	−0.93	0.74	0.79	0.79	−0.73
c.2058 + 30C. > T		•	−0.84	−0.82	−0.82	0.81
c.3700G > A			•	0.89	0.86	−0.91
c.4117 – 44C. > T				•	0.96	−0.77
c.4242T > G					•	−0.79
c.5439G > A						•

Association study: single polymorphism and haplotype analysis

We selected 6 polymorphisms, 1 non-synonymous, 3 synonymous, and 2 intronic, based on their allelic frequency being greater than 0.10, and on the absence of complete linkage disequilibrium among the 60 cases. The 192 individuals in the control group were genotyped for these polymorphisms, by the same direct sequencing method used in the affected group. In some cases, genotyping was difficult due to poor-quality samples. This resulted in only 300 chromosomes from 150 normal individuals being genotyped without ambiguity for all six polymorphisms.

Individual comparisons of genotypic frequencies showed no significant difference between cases and controls. Significant linkage disequilibrium was found between the various polymorphisms, with all D' values significantly different from zero (Table 3). These six polymorphisms generated seven major haplotypes (Table 4). The global haplotype distribution differed significantly between cases and controls ($\chi^2 = 14.32$ with 6 d.f.; $p = 0.026$). This difference was mainly due to the lower frequency in cases than in controls (0.072 versus 0.220) of the only haplotype carrying the c2058 + 30T allele (TTGTGA). In logistic regression analysis, the odds ratio for patent arterial duct being associated with this haplotype was estimated at 0.231 [95% CI 0.079–0.675] ($p = 0.007$).

Discussion

This is the first study to investigate the possible relationship between variations in the *MYH11* gene

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Table 4. Main haplotype frequencies of the *MYH11* gene, as a function of case as opposed to control state, in the whole sample and estimation of haplotypic odds ratios, amongst the 150 samples.

Haplotypes						Haplotype frequency		Haplotypic odds ratio [95% CI]
c.1743T>C	c.2058+30C>T	c.3700G>A	c.4117−44C>T	c.4242T>G	c.5439G>A	Cases	Controls	
C	C	G	C	T	G	0.088	0.090	0.670 [0.260–1.725]
C	C	G	C	T	A	0.045	0.029	1.083 [0.202–5.802]
C	C	A	C	T	G	0.190	0.226	0.639 [0.309–1.322]
T	C	G	C	T	G	0.085	0.079	0.757 [0.280–2.042]
T	C	G	T	G	G	0.037	0.025	1.040 [0.208–5.196]
T	C	G	T	G	A	0.289	0.239	1*
T	T	G	T	G	A	0.072	0.220	0.231 [0.079–0.675]

*Haplotype used as the reference haplotype.

and the sporadic occurrence of persistent patency of the arterial duct. Overall, our results show that the *MYH11* gene does not play a substantial role in such apparently sporadic cases. We identified four subjects (6.7%) with three rare missense mutations, R669C, E1290Q, and R1535Q, that might affect the structure of the smooth muscle myosin heavy chain tail or interaction with actin, thereby potentially causing problems with closure of the arterial duct. Only two of these mutations were not also found in normal volunteers, reducing to two (3%) the number of cases with unique missense variants. The smooth muscle myosin molecule is composed of two heavy chains, two essential light chains and two regulatory light chains. It assembles in dimers with two N-terminal globular heads housing the ATP- and actin-binding sites, and one coiled-coil rod assembled from two α-helical C-terminal tails. In one of the two variants, R669C, the mutation affected the globular head of the smooth muscle myosin heavy chain, whereas in the other, E1290Q, the rod domain was affected.

The myosin head is a motor domain containing binding sites for ATP and actin. Hydrolysis of ATP brings the myosin head into contact with actin filaments, allowing the thin filament of actin and the thick filament of myosin to slide past each other, resulting in generation of force in the myocytes and/or contraction.[20,21] The R669 residue is located in a highly conserved segment within a long α helix in the globular head, and directly toward the proposed actin binding region.[22,23] The structural role of this residue has not been identified,[24] but it could contribute to binding of actin, either in the state of rigour, or as a contributor to one of the weak binding interactions. The mutations affecting the corresponding residue in the human cardiac myosin heavy chain, R663 H and R663C, have been associated with familial hypertrophic cardiomyopathy.[25,26] Based on these evidences, the substitution of R669C could change the binding constant to actin or actin activation, and thus influence the contractile properties of the molecule.

The rod region of myosin consists of a series of heptad repeats (*a, b, c, d, e, f, g*), resulting in 28-residue periodicity, with alternate bands of positive and negative charges essential for rod assembly.[24,27] The residues at position *a* and *d* are usually hydrophobic, and this property is critical for the association of two alpha-helical myosin heavy chain tail regions to form the coiled-coil rod. The other residues at positions e, b, f, c and g participate in the interaction with other myosin rods to form myosin thick filaments. They are usually hydrophilic, and contribute to the periodic alternation of charge. Four non-synonymous variants were found

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in this region. Of these, the p.Glu1290Gln variant was found in only one child with persistent patency. This glutamine residue is conserved among smooth muscle myosin heavy chain proteins from different species, but not in cardiac or skeletal muscle myosin heavy chain. Its position "*b*" in the heptad repeat of the coiled-coil tail indicates that it may affect the interaction of smooth muscle myosin heavy chain rods to assembly thick filaments, but not the coiled-coil formation as indicated by the Coils prediction. The negatively charged glutamine replaced by a non-charged glutamic acid may change the periodicity of the rod and interfere with thick filament formation,[24] or influence the proper alignment or registration of dimers into filaments as showed for similar *MYH9* mutations In this study by Franke et al.,[28] change of charged pattern in the myosin rod was showed to form paracrystals with normal periodicity, but the mixed wildtype and mutant paracrystals were narrower than the wildtype ones. Further structural data is needed to draw firm conclusions on the functional effects of our mutation.

The other three variants, p.Ala1234Thr, p.Val1289Ala, p.Arg1535Gln, all involved the smooth muscle myosin heavy chain rod region. Based on Coils prediction, the affected residues correspond to positions b, a, and b, respectively, and the mutations do not significantly affect the probability of coiled coil formation. All three variants were observed in controls with a similarly low, for p.Arg1535Gln and p.Val1289Ala, or high, for p.Ala1234Thr, frequency. They should therefore be considered not as mutations, but rather as genetic rare variants or polymorphisms.

Our analysis of the six informative polymorphisms in this series of cases and 150 Caucasian controls suggested, therefore, that the *MYH11* gene does not have a major effect on producing persistent patency of the arterial duct. A significant, but mild overall effect was observed, but this effect resulted mostly from a difference in the frequency of one particular haplotype between cases and controls, at 0.072 versus 0.220. This haplotype carries the c2058 + 30T allele, corresponding to intron 16 of the *MYH11* gene. As this C > T transition at position +30 after the splicing donor site is not predicted to affect the splicing of exon 16, we can only suggest that it is part of a haplotype containing another variant affecting expression of the *MYH11* gene or production of protein. The protective effect of this variant against persistent patency is difficult to interpret because we were expecting to find a deleterious risk factor. The replication of these findings in an independent sample is required before we can conclude that *MYH11* is a gene involved in producing the sporadic variant of persistent patency of the arterial duct.

Although genes might play a role in the occurrence of isolated persistent patency, our preliminary analysis does not demonstrate that the human *MYH11* gene is a strong susceptibility factor for such condition. It remains possible that rare missense variants of *MYH11* may account for a small percentage of cases.

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