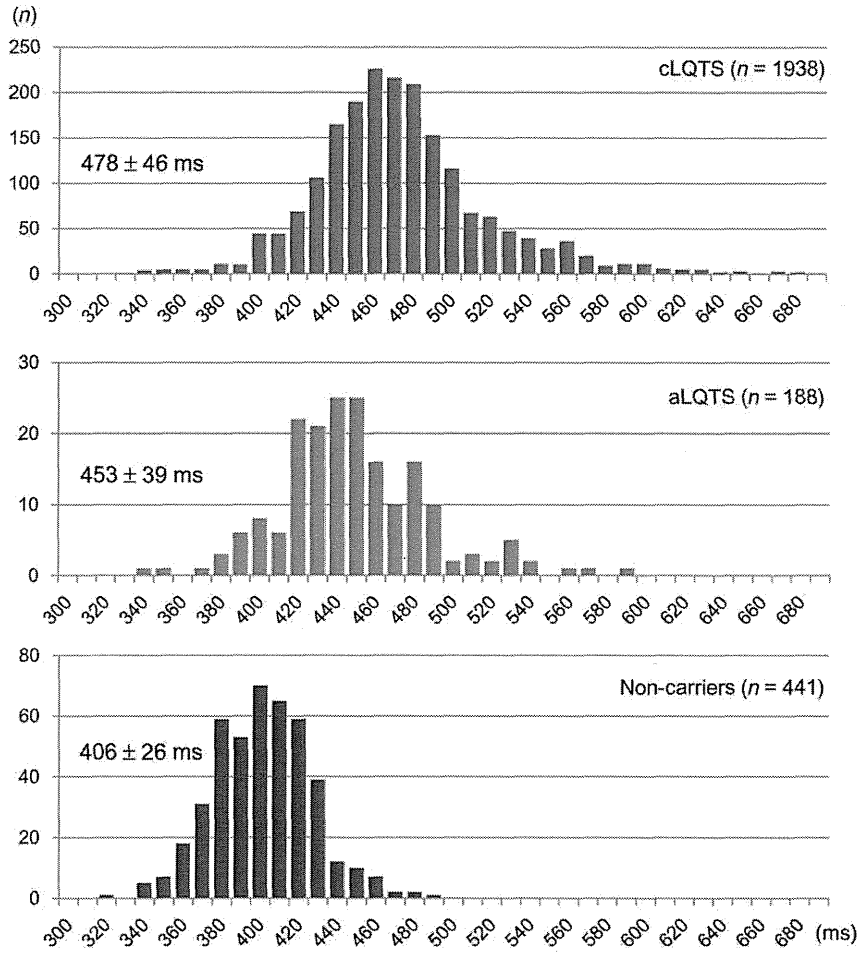

Figure 1 Distribution of QTc interval in genotyped congenital long QT syndrome, acquired long QT syndrome, and non-carriers.

cardiac events with prolonged QTc of 628 and 678 ms, respectively, while taking terfenadine or probucol; they had not been diagnosed as 'cLQTS' prior to these cardiac events.[9,19]

Mutations in acquired long QT syndrome and congenital long QT syndrome

To assess the relative frequencies of the LQTS genetic subtypes, we focused on single mutation carriers only, after exclusion of the 55 (2 aLQTS and 53 cLQTS) compound heterozygous MCs. Distribution of the LQTS genetic subtypes differed significantly between aLQTS and cLQTS (*Figure 3A*). Among the 957 cLQTS MCs, *KCNQ1* and *KCNH2* mutations were found in 477 (50% [95% CI 47–53%]) and 397 (41% [95% CI 38–45%]) patients, respectively. In contrast, among the 51 aLQTS individuals, carriers of *KCNQ1* mutations (n = 15, 29% [95% CI 17–44%]) were significantly less frequent than *KCNH2* MCs (n = 30, 59% [95% CI 44–72%]) compared with cLQTS ($P < 0.01$). The larger proportion of *KCNH2* MCs observed in the aLQTS cohort compared with cLQTS was independent of specific ethnic background, as it was comparable in the Japanese and Caucasian subsets [22/35 (63%) and 8/16 (50%), respectively]. It was also present both in the drug-induced LQTS and in the 'other' cause-aLQTS subset (*Table 1*). This overrepresentation of the LQT2 subtype with respect to cLQTS was more evident in true aLQTS than in the unmasked cLQTS subtype in which the percentages of *KCNQ1* (38%) and *KCNH2* (54%) MCs were intermediate between true aLQTS and cLQTS MCs (*Figure 3B*).

Interestingly, despite the small number of MCs in each genetic subgroup, *KCNE1/KCNE2* mutations seemed more frequent in aLQTS than in cLQTS (5.9% [95% CI 1.2–16%] vs. 1.7% [95% CI 1–2.7%], $P = 0.07$).

The common variant *KCNQ1*-D85N was found in 10/188 (5%) heterozygous subjects, corresponding to a minor allele frequency of 2.7%. Five of these were also carriers of *KCNH2* mutations. No significant difference was observed between aLQTS subjects with or without the D85N variant with respect to all demographic and

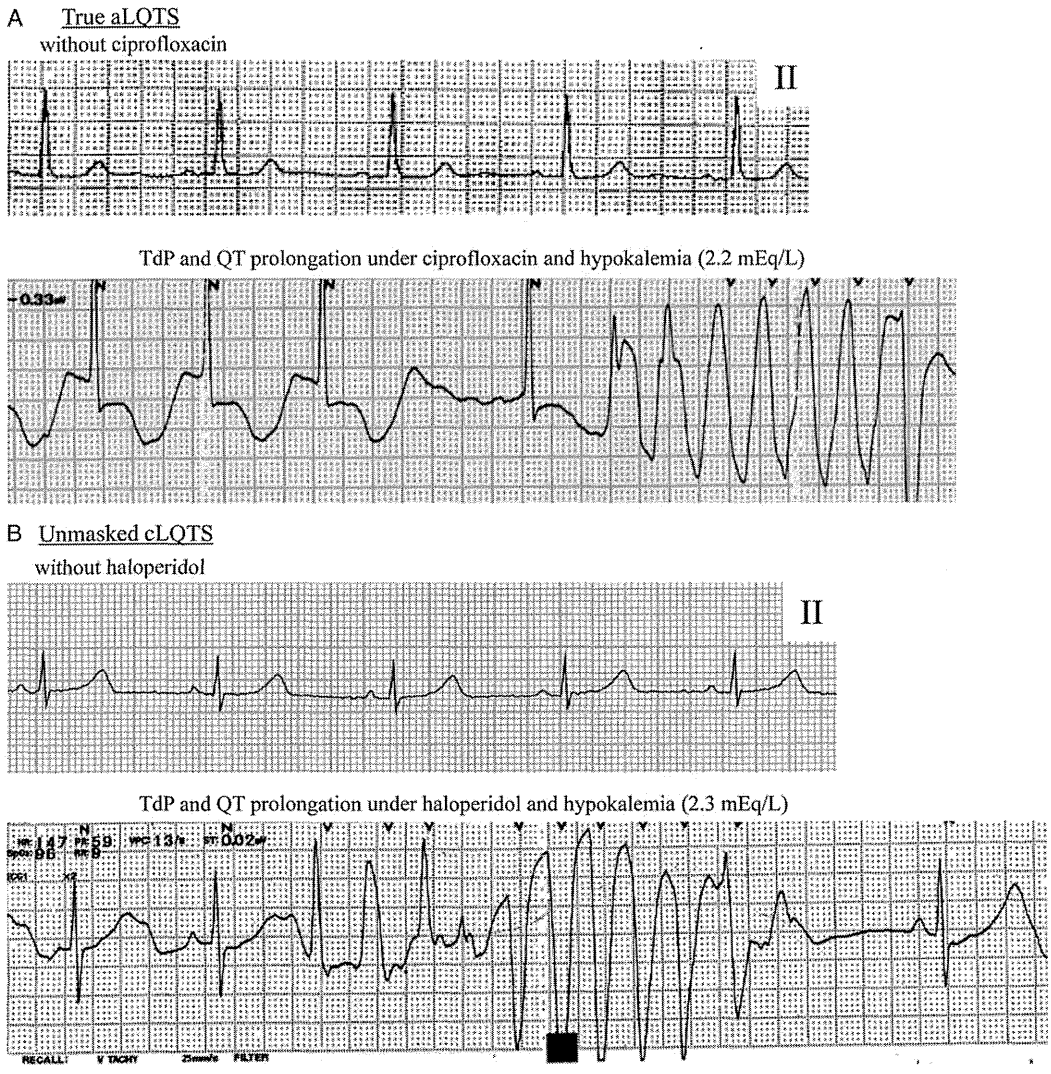

Figure 2 Electrocardiograms of true acquired long QT syndrome and unmasked congenital long QT syndrome. (*A*) A representative case with true long QT syndrome. This 73-year-old female was admitted for the treatment of bacterial pneumonia. Her basal QTc was normal (396 ms, upper panel). Torsades de Pointes and severe QT prolongation appeared 3 days after receiving ciprofloxacin (lower panel) and led to ventricular fibrillation. After cardioversion, the ECG showed marked QT prolongation (613 ms) and atrial fibrillation with hypokalaemia (2.2 mEq/L). (*B*) A representative case with unmasked congenital long QT syndrome. This 52-year-old alcoholic male was treated with haloperidol i.v. (6 mg) for withdrawal symptoms. Though his control QTc interval was prolonged but not remarkably (466 ms), a monitoring ECG showed Torsades de Pointes and marked QTc prolongation (624 ms) after haloperidol accompanied by hypokalaemia (2.3 mEq/L). Genetic analysis revealed a *KCNH2* mutation (R948S) located in the C-terminus.

clinical variables measured, although D85N carriers trended towards a longer control QTc (475 ± 38 vs. 452 ± 39 ms, $P = 0.067$) compared with non-carriers.

A score to predict genetic status

Using a multivariable logistic regression model, age at exposure to proarrhythmic triggers, control QTc measured in the absence of these factors, and a history of symptoms at the time of the critical event were significant and independent predictors for carriers of disease-causing mutations (*Table 3*). Their combination in a summative score ranging from 0 to 3 points allowed identification of individuals in whom genetic screening is more or less likely to reveal an LQTS mutation. The discriminatory power of the score was relatively good (AUC, 0.72, 95% CI 0.64–0.80, $P < 0.001$). As shown

Table 2 Prevalence of mutation carriers according to baseline features of the 188 aLQTS subjects

Demographic/clinical variable	Mutation carriers (n = 55)	P-value
Ethnicity		
Blacks	0/2	0.09
Caucasians	16/39 (41)	
Japanese	37/147 (25)	
Age of onset		
<40 years	20/49 (41)	0.03
≥40 years	33/139 (24)	
Sex		
Males	11/48 (23)	0.46
Females	42/140 (30)	
Secondary factors		
Drug	19/81 (23)	0.36
Hypokalaemia	17/42 (40)	
Bradycardia	5/17 (29)	
Combination	11/43 (26)	
Other	1/5 (20)	
Control QTc		
True aLQTS	26/112 (23)	0.07
Unmasked LQTS	27/76 (36)	
Cardiac symptoms		
Symptomatic	52/162 (32)	0.002
Asymptomatic	1/26 (4)	

Numbers are *n/N* (%).
LQTS, long QT syndrome; aLQTS, acquired long QT syndrome.

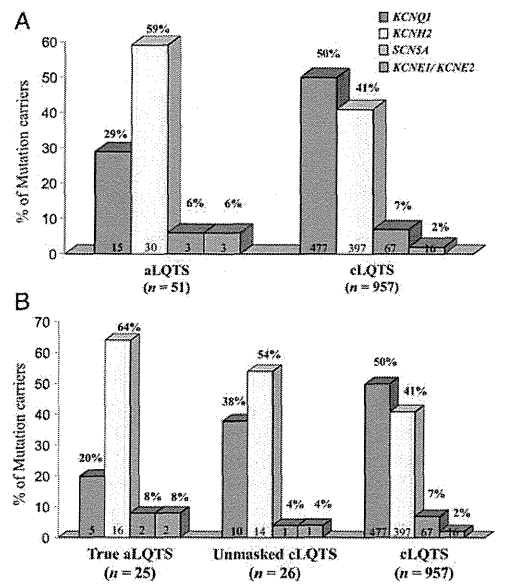

Figure 3 Distribution of genetic subtypes in acquired long QT syndrome and congenital long QT syndrome. All acquired long QT syndrome mutation carriers are shown in *A*; they are then subdivided in *B* as 'true acquired long QT syndrome' or 'unmasked congenital long QT syndrome', according to the study definitions.

Table 3 Predictors of the probability of being mutation carriers (MCs) in aLQTS

	OR (95% CI)	P-value
Age, <40 vs. ≥40 years	2.5 (1.2–5.3)	0.020
QTc, >440 vs. ≤440 ms	5.2 (2.2–12.2)	<0.001
Clinical status, symptomatic vs. asymptomatic	10.6 (1.3–83.5)	0.025

LQTS, long QT syndrome; aLQTS, acquired long QT syndrome.

in *Figure 4*, the proportion of aLQTS subjects with a positive genetic result increased linearly (P for trend < 0.001) from 0 to 63% (from 0 to 3 points), where all three factors conferring a higher probability (age ≤40 years, symptoms, and QTc >440 ms) were present. Of the aLQTS patients carrying a mutation, 89% (47 of 53) had a score of 2 or 3 points; from a different and broader perspective, of all the 188 aLQTS subjects none with a score of 0 and only 6 (3%) with a score of 1 had a mutation. The same significant pattern was present when examining separately Japanese and Caucasians (*Figure 5*).

When in a sort of sensitivity analysis we used a 'weighted' score by assigning 1 point to age <40 years, 2 points to QTc >440 ms, and 3 points to symptomatic status, the pattern was confirmed (see Supplementary material online, *Figure S1*).

Discussion

The present multicentre study focused on the genetic basis of aLQTS and provides four major findings: (i) the QTc measured in the absence of triggering factors of aLQTS cases is shorter than that of cLQTS patients, but is significantly longer than that of individuals without cLQTS; (ii) 28% of aLQTS subjects have mutations in cLQTS genes; (iii) at variance with cLQTS, the most prevalent mutations in aLQTS are on the *KCNH2* gene; and (iv) the QTc measured in the absence of triggers, together with simple clinical parameters, allows identification of aLQTS subjects more likely to be carriers of LQTS mutations and for whom, therefore, genetic screening is warranted.

Even though QTc returns to the normal range once QT-prolonging factors are removed,[20] previous reports observed that QTc sometimes remains prolonged even after withdrawal of culprit drugs[21] or the correction of serum electrolytes.[3] Our study cohort showed that the 'off' trigger QTc of aLQTS was indeed significantly longer than that of unaffected family members of cLQTS patients, in agreement with the concept that aLQTS could represent at least in part a latent genetic predisposition.[8] Our study now demonstrates in a large cohort of aLQTS cases that ~30% of these subjects have an

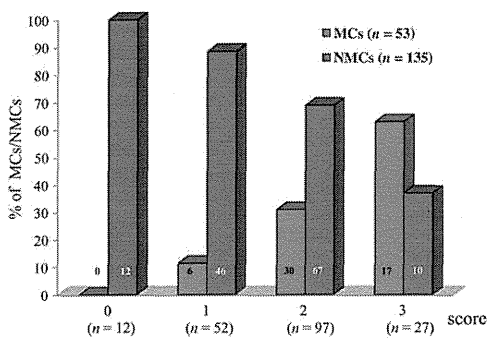

Figure 4 Proportions of mutation carriers/non-mutation carriers among the 188 patients according to increasing score values. Score 0 = age ≥40 years + asymptomatic + QTc ≤440 ms; score 3 = age <40 years + symptomatic + QTc >440 ms; Scores 1 and 2 represent the presence of one or of two factors. The number of mutation carriers increases with increasing score values (from 0 in the group with Score 0) and indicates that 89% of mutation carriers (47 of 53) are found within the Scores 2 and 3. Conversely, among the 52 patients with a score of 1, which represent 28% of the entire population, there were six mutation carriers; this means that while within the group with a score of 1, there is an 11% of mutation carriers, when looking at the entire population this percentage drops to 3%. This would be the percentage of mutation carriers missed if genetic screening would be limited to the groups with a score of 2 and 3.

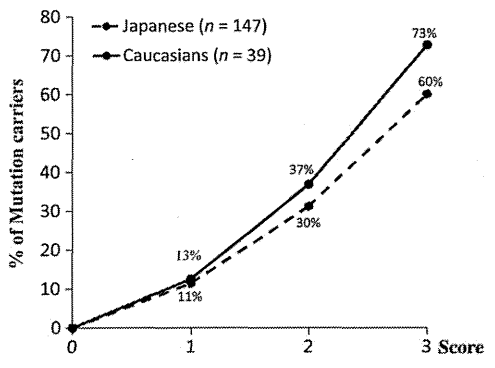

Figure 5 The percentages of mutation carriers, within each value of the probability score, are similar between Japanese and Caucasian acquired long QT syndrome subjects. The figures of 11 and 13% for a score of 1 correspond approximately at a 3% prevalence of mutation carriers on the total population of acquired long QT syndrome.

underlying mutation in one of the major LQTS-related genes. Even among the 'true aLQTS', i.e. those with normal QT interval outside the triggering episode, 23% had cLQTS-causing mutations and this percentage is much higher than the ~6% reported previously.[11,22] We did consider the possibility that this greater prevalence, which was independent of ethnic background, might be due to the fact that our cohort included subjects whose aLQTS was caused not only by drugs, but also by hypokalaemia or bradycardia. However, this was not the case as the percentage of MCs among the drug-induced aLQTS was already high (23%) and the addition of non-drug-induced MCs increased this percentage.

The percentages of LQT1 and LQT2 patients in cLQTS are either similar or show a predominance of LQT1 mutations,[23,24] which was also the case in the present study. In contrast, in the 'true aLQTS', we observed a significantly lower frequency of LQT1- than LQT2-causing mutations. Even though it is unclear why *KCNH2* mutations are more frequently found in aLQTS, it may be relevant that most of these mutations are located in the non-pore regions, usually associated with rather benign phenotypes.[25] There was also a tendency for mutations in *KCNE1* and *KCNE2* to occur more frequently in aLQTS than in cLQTS. A possible explanation is that β-subunits are accessory proteins which modulate the function of the main α-subunits of the cardiac potassium channels and their altered channel dysfunctions are often benign and not sufficient *per se* to cause the full blown manifestations of cLQTS. Such considerations are clinically important because, in the absence of triggering factors, most of these mutations remain silent and the subjects appear normal.

No mutations were detected in two of three aLQTS patients. This does not necessarily rule out a genetic component, which may yet be identified. A number of rare or common polymorphisms could induce cumulative effects on QTc prolongation,[26] and even clinical phenotypes of long QT syndrome,[12] such as *KCNH2*-K897T,[27,28] *SCN5A*-S1103Y,[29] *KCNE1*-D85N,[17,18,28] and *NOS1AP*[30] variants. It is therefore tempting to speculate that the synergistic association of several functional polymorphisms could contribute to the genetic background predisposing to aLQTS. The D85N polymorphism, often associated with both cLQTS and aLQTS,[17,18,30] was not found in the present study to play any significant role despite a trend for longer QTc among the carriers.

The present findings go beyond clarification of the genetic basis for aLQTS because our analysis shows that a score incorporating simple parameters such as QTc, age, and presence/absence of symptoms may provide a useful guide for the clinician, assisting his/her decision-making and allowing cost-effective genetic testing.

Limitations

Genes responsible for the rarer variants of LQTS[12] were not analysed. Although their probability to cause cLQTS is very low (<1%), we cannot exclude the possibility that some genotype-negative aLQTS patients may have mutations in these genes or in others not yet identified. Our score lacks external or internal cross-validation and therefore it should be considered as a preliminary tool. However, the three variables used are well-known risk factors for arrhythmias in cLQTS, and their dichotomization was performed at traditional clinically based LQTS cut-offs, thus limiting the potential bias.

Conclusion

In clinical practice, greater attention should be paid to even mild prolongation of the QT interval because this could represent a signal of potential risk of manifestation of aLQTS if exposed to the

appropriate triggers. Some individuals initially labelled as aLQTS are diagnosed as cLQTS ('unmasked cLQTS') following the lack of QT normalization after removal of the trigger, and should be treated as such. Evidence of a genetic predisposition often present in aLQTS justifies and recommends a genetic study in all patients with a score of 2 or 3, where one finds almost 90% of the mutation carriers. While screening those with a score of 0 is unlikely to be useful, not to screen those with 1 point would miss only 3% of the subjects carrying a mutation within the entire aLQTS population and would be more debatable. Having identified the predisposing mutation in the proband, 'cascade screening' in their families will rapidly and inexpensively identify additional mutation carriers and prevent avoidable risks of life-threatening arrhythmias.

Supplementary material

Supplementary material is available at *European Heart Journal* online.

Authors' contributions

C.S., Y.M.: performed statistical analysis; P.J.S., H.I., L.C., W.S., M.H.: handled funding and supervision; H.I., L.C., T.A., I.D., V.F., K.H., T.N., S.O., T.M., J.W., K.H., E.M., F.D., M.P., M.Y., M.B., W.S., P.G., P.J.S., M.H.: acquired the data; P.J.S., L.C., C.S., P.G., M.H., H.I.: conceived and designed the research; P.J.S., H.I., C.S., M.H.: drafted the manuscript; P.J.S., L.C., C.S., M.H., H.I.: made critical revision of the manuscript for key intellectual content.

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Conflict of interest: none declared.

References

1. Roden DM. Drug-induced prolongation of the QT interval. *N Engl J Med* 2004;**350**:1013–1022.
2. Wysowski DK, Bacsanyi J. Cisapride and fatal arrhythmia. *N Engl J Med* 1996;**335**:290–291.
3. Berthet M, Denjoy I, Donger C, Demay L, Hammoude H, Klug D, Schulze-Bahr E, Richard P, Funke H, Schwartz K, Coumel P, Hainque B, Guicheney P. C-terminal HERG mutations: the role of hypokalemia and a KCNQ1-associated mutation in cardiac event occurrence. *Circulation* 1999;**99**:1464–1470.
4. Kurita T, Ohe T, Marui N, Aihara N, Takaki H, Kamakura S, Matsuhisa M, Shimomura K. Bradycardia-induced abnormal QT prolongation in patients with complete atrioventricular block with Torsades de Pointes. *Am J Cardiol* 1992;**69**:628–633.
5. Food and Drug Administration HHS. International Conference on Harmonisation; guidance on S7B Nonclinical Evaluation of the Potential for Delayed Ventricular Repolarization (QT Interval Prolongation) by Human Pharmaceuticals; availability. Notice. *Fed Regist* 2005;**70**:61133–61134.
6. Food and Drug Administration HHS. International Conference on Harmonisation; guidance on E14 Clinical Evaluation of QT/QTc Interval Prolongation and Proarrhythmic Potential for Non-Antiarrhythmic Drugs; availability. Notice. *Fed Regist* 2005;**70**:61134–61135.
7. Darpo B, Garnett C, Benson CT, Keirns J, Leishman D, Malik M, Mehrotra N, Prasad K, Riley S, Rodriguez I, Sager P, Sarapa N, Wallis R. Cardiac Safety Research Consortium: can the thorough QT/QTc study be replaced by early QT assessment in routine clinical pharmacology studies? Scientific update and a research proposal for a path forward. *Am Heart J* 2014;**168**:262–272.
8. Moss AJ, Schwartz PJ. Delayed repolarization (QT or QTU prolongation) and malignant ventricular arrhythmias. *Mod Concepts Cardiovasc Dis* 1982;**51**:85–90.
9. Donger C, Denjoy I, Berthet M, Neyroud N, Cruaud C, Bennaceur M, Chivoret G, Schwartz K, Coumel P, Guicheney P. KVLQT1 C-terminal missense mutation causes a forme fruste long-QT syndrome. *Circulation* 1997;**96**:2778–2781.
10. Makita N, Horie M, Nakamura T, Ai T, Sasaki K, Yokoi H, Sakurai M, Sakuma I, Otani H, Sawa H, Kitabatake A. Drug-induced long-QT syndrome associated with a subclinical SCN5A mutation. *Circulation* 2002;**106**:1269–1274.
11. Yang P, Kanki H, Drolet B, Yang T, Wei J, Viswanathan PC, Hohnloser SH, Shimizu W, Schwartz PJ, Stanton M, Murray KT, Norris K, George AL, Roden DM. Allelic variants in long-QT disease genes in patients with drug-associated Torsades de Pointes. *Circulation* 2002;**105**:1943–1948.
12. Schwartz PJ, Ackerman MJ, George AL, Wilde AA. Impact of genetics on the clinical management of channelopathies. *J Am Coll Cardiol* 2013;**62**:169–180.
13. Bazett HC. An analysis of the time relations of electrocardiograms. *Heart* 1920;**7**:353–370.
14. Richards S, Aziz N, Bale S, Bick D, Das S, Gastier-Foster J, Grody WW, Hegde M, Lyon E, Spector E, Voelkerding K, Rehm HL, Committee ALQA. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. *Genet Med* 2015;**17**:405–424.
15. Kapa S, Tester DJ, Salisbury BA, Harris-Kerr C, Pungliya MS, Alders M, Wilde AA, Ackerman MJ. Genetic testing for long-QT syndrome: distinguishing pathogenic mutations from benign variants. *Circulation* 2009;**120**:1752–1760.
16. Giudicessi JR, Kapplinger JD, Tester DJ, Alders M, Salisbury BA, Wilde AA, Ackerman MJ. Phylogenetic and physicochemical analyses enhance the classification of rare nonsynonymous single nucleotide variants in type 1 and 2 long-QT syndrome. *Circ Cardiovasc Genet* 2012;**5**:519–528.
17. Nishio Y, Makiyama T, Itoh H, Sakaguchi T, Ohno S, Gong YZ, Yamamoto S, Ozawa T, Ding WG, Toyoda F, Kawamura M, Akao M, Matsuura H, Kimura T, Kita T, Horie M. D85N, a KCNE1 polymorphism, is a disease-causing gene variant in long QT syndrome. *J Am Coll Cardiol* 2009;**54**:812–819.
18. Kääb S, Crawford DC, Sinner MF, Behr ER, Kannankeril PJ, Wilde AA, Bezzina CR, Schulze-Bahr E, Guicheney P, Bishopric NH, Myerburg RJ, Schott JJ, Pfeufer A, Beckmann BM, Martens E, Zhang T, Stallmeyer B, Zumhagen S, Denjoy I, Bardai A, Van Gelder IC, Jamshidi Y, Dalageorgou C, Marshall V, Jeffery S, Shakir S, Camm AJ, Steinbeck G, Perz S, Lichtner P, Meitinger T, Peters A, Wichmann HE, Ingram C, Bradford Y, Carter S, Norris K, Ritchie MD, George AL, Roden DM. A large candidate gene survey identifies the KCNE1 D85N polymorphism as a possible modulator of drug-induced torsades de pointes. *Circ Cardiovasc Genet* 2012;**5**:91–99.
19. Hayashi K, Shimizu M, Ino H, Yamaguchi M, Terai H, Hoshi N, Higashida H, Terashima N, Uno Y, Kanaya H, Mabuchi H. Probucol aggravates long QT syndrome associated with a novel missense mutation M124T in the N-terminus of HERG. *Clin Sci (Lond)* 2004;**107**:175–182.
20. Roden DM, Viswanathan PC. Genetics of acquired long QT syndrome. *J Clin Invest* 2005;**115**:2025–2032.
21. Itoh H, Sakaguchi T, Ding WG, Watanabe E, Watanabe I, Nishio Y, Makiyama T, Ohno S, Akao M, Higashi Y, Zenda N, Kubota T, Mori C, Okajima K, Haruna T, Miyamoto A, Kawamura M, Ishida K, Nagaoka I, Oka Y, Nakazawa Y, Yao T, Jo H, Sugimoto Y, Ashihara T, Hayashi H, Ito M, Imoto K, Matsuura H, Horie M. Latent genetic backgrounds and molecular pathogenesis in drug-induced long-QT syndrome. *Circ Arrhythm Electrophysiol* 2009;**2**:511–523.
22. Behr ER, Ritchie MD, Tanaka T, Kääb S, Crawford DC, Nicoletti P, Floratos A, Sinner MF, Kannankeril PJ, Wilde AA, Bezzina CR, Schulze-Bahr E, Zumhagen S, Guicheney P, Bishopric NH, Marshall V, Shakir S, Dalageorgou C, Bevan S, Jamshidi Y, Bastiaenen R, Myerburg RJ, Schott JJ, Camm AJ, Steinbeck G, Norris K, Altman RB, Tatonetti NP, Jeffery S, Kubo M, Nakamura Y, Shen Y, George AL, Roden DM. Genome wide analysis of drug-induced Torsades de Pointes: lack of common variants with large effect sizes. *PLoS One* 2013;**8**:e78511.

23. Priori SG, Schwartz PJ, Napolitano C, Bloise R, Ronchetti E, Grillo M, Vicentini A, Spazzolini C, Nastoli J, Bottelli G, Folli R, Cappelletti D. Risk stratification in the long-QT syndrome. *N Engl J Med* 2003;**348**:1866–1874.
24. Tester DJ, Will ML, Haglund CM, Ackerman MJ. Compendium of cardiac channel mutations in 541 consecutive unrelated patients referred for long QT syndrome genetic testing. *Heart Rhythm* 2005;**2**:507–517.
25. Shimizu W, Moss AJ, Wilde AA, Towbin JA, Ackerman MJ, January CT, Tester DJ, Zareba W, Robinson JL, Qi M, Vincent GM, Kaufman ES, Hofman N, Noda T, Kamakura S, Miyamoto Y, Shah S, Amin V, Goldenberg I, Andrews ML, McNitt S. Genotype-phenotype aspects of type 2 long QT syndrome. *J Am Coll Cardiol* 2009;**54**:2052–2062.
26. Newton-Cheh C, Eijgelsheim M, Rice KM, de Bakker PI, Yin X, Estrada K, Bis JC, Marciante K, Rivadeneira F, Noseworthy PA, Sotoodehnia N, Smith NL, Rotter JI, Kors JA, Witteman JC, Hofman A, Heckbert SR, O'Donnell CJ, Uitterlinden AG, Psaty BM, Lumley T, Larson MG, Stricker BH. Common variants at ten loci influence QT interval duration in the QTGEN Study. *Nat Genet* 2009;**41**:399–406.
27. Crotti L, Lundquist AL, Insolia R, Pedrazzini M, Ferrandi C, De Ferrari GM, Vicentini A, Yang P, Roden DM, George AL, Schwartz PJ. KCNH2-K897T is a genetic modifier of latent congenital long-QT syndrome. *Circulation* 2005;**112**:1251–1258.
28. Gouas L, Nicaud V, Berthet M, Forhan A, Tiret L, Balkau B, Guicheney P, Group DE-SIRS. Association of KCNQ1, KCNE1, KCNH2 and SCN5A polymorphisms with QTc interval length in a healthy population. *Eur J Hum Genet* 2005;**13**:1213–1222.
29. Van Norstrand DW, Tester DJ, Ackerman MJ. Overrepresentation of the proarrhythmic, sudden death predisposing sodium channel polymorphism S1103Y in a population-based cohort of African-American sudden infant death syndrome. *Heart Rhythm* 2008;**5**:712–715.
30. Crotti L, Monti MC, Insolia R, Peljto A, Goosen A, Brink PA, Greenberg DA, Schwartz PJ, George AL. NOS1AP is a genetic modifier of the long-QT syndrome. *Circulation* 2009;**120**:1657–1663.

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Original article

Whole exome sequencing combined with integrated variant annotation prediction identifies a causative myosin essential light chain variant in hypertrophic cardiomyopathy

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ABSTRACT

Background: The development of candidate gene approaches to enable molecular diagnosis of hypertrophic cardiomyopathy (HCM) has required extensive and prolonged efforts. Whole exome sequencing (WES) technologies have already accelerated genetic studies of Mendelian disorders, yielding approximately 30% diagnostic success. As a result, there is great interest in extending the use of WES to any of Mendelian diseases. This study investigated the potential of WES for molecular diagnosis of HCM.

Methods: WES was performed on seven relatives from a large HCM family with a clear HCM phenotype (five clinically affected and two unaffected) in the Kanazawa University Hypertrophic Cardiomyopathy Registry. Serial bioinformatics filtering methods as well as using combined annotation dependent depletion (CADD) score and high heart expression (HHE) gene data were applied to detect the causative variant. Moreover, additional carriers of the variant were investigated in the HCM registry, and clinical characteristics harboring the variant were collected and evaluated.

Results: WES detected 60020 rare variants in the large HCM family. Of those, 3439 were missense, nonsense, splice-site, or frameshift variants. After genotype–phenotype matching, 13 putative variants remained. Using CADD score and HHE gene data, the number of candidates was reduced to one, a variant in the myosin essential light chain (MYL3, NM_000258.2:c.281G>A, p.Arg94His) that was shared by the five affected subjects. Additional screening of the HCM registry (n = 600) identified two more subjects with this variant. Serial assessments of the variant carriers revealed the following phenotypic characteristics: (1) disease-penetrance of 88%; (2) all clinically affected carriers exhibited asymmetric septal hypertrophy with a substantial maximum left ventricular wall thickness of 18 ± 3 mm without any obstruction.

Conclusions: WES combined with CADD score and HHE gene data may be useful even in HCM. Furthermore, the MYL3 Arg94His variant was associated with high disease penetrance and substantial interventricular septal hypertrophy.

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Introduction

Hypertrophic cardiomyopathy (HCM) is a genetically heterogeneous myocardial disorder with various morphological, functional, and clinical characteristics [1–5]. Familial HCM, which has a prevalence of up to 1 in 500 individuals, is one of the most common autosomal dominant inherited disorders [6]. Myosin heavy chain (MYH7, OMIM#: 160760), myosin binding protein C (MYBPC3, OMIM#: 600958), troponin T (TNNT2, OMIM#: 191045), tropomyosin (TPM1, OMIM#: 191010), and troponin I (TNNI3, OMIM#: 191044) have been reported to be the main causal genes [2–4,7–11]. To date, candidate-gene approaches using traditional Sanger sequencing have enabled the identification of causative variants in approximately 50% of HCM patients [8]. However, because these techniques are onerous and time-consuming, motivating the researchers to perform target re-sequencing with next generation sequencing (NGS) as well as whole exome sequencing (WES) which is reported to have a success rate of nearly 30% in identifying disease-causing variants in patients whose causal variants have not been detected [12]. Although several other reports have demonstrated the efficacy of target re-sequencing for sporadic HCM patients [13,14], it remains unclear whether WES enables comprehensive identification of causative variants in an exome-wide manner. This study investigated the effectiveness of WES with bioinformatics for molecular diagnosis for HCM.

Materials and methods

Study population

All subjects were enrolled through the Kanazawa University Hypertrophic Cardiomyopathy Registry in Kanazawa, Japan. The largest HCM family in the registry, whose causative variant had not been detected by traditional direct sequencing, was first investigated in this study. HCM was diagnosed according to the 2011 version of the guideline of the American College of Cardiology Foundation/American Heart Association [1]. In brief, subjects with a maximal left ventricular wall ≥13 mm (involving asymmetric septal hypertrophy) without extra-cardiac or metabolic findings based on echocardiography were diagnosed with HCM. Individuals were classified as HCM-affected in the case diagnosed with HCM, or HCM-unaffected in the case of either a non-HCM phenotype or known diseases such as hypertensive heart disease. Detailed clinical data, including family history, age, and symptoms at evaluation, presence of hypertension, physical examination, New York Heart Association (NYHA) classification, electrocardiogram (ECG) and echocardiographic findings were collected for each subject. Hypertrophic obstructive cardiomyopathy (HOCM) was defined as HCM with left ventricular outflow tract (LVOT) obstruction and a peak instantaneous LVOT pressure gradient of >30 mmHg. Maximum left ventricular wall thickness (MWT) was defined as the greatest thickness within the chamber. Other echocardiographic parameters were evaluated using the recommendation of the American Society of Echocardiography [15]. Hypertension was defined as when a patient's systolic blood pressure (BP) was ≥140 mmHg and/or its diastolic BP was ≥90 mmHg. Pathological Q waves were defined as follows based on previous studies: Q wave >1/4 of the ensuing R wave in depth and/or >40 ms in duration in at least two leads except aVR [16]. T waves >10 mm in depth in any leads were defined as giant negative T waves (GNTW) [17]. The Ethics Committee for Medical Research at our institution approved the study protocol, and all subjects provided written informed consent.

Exome sequencing

Genomic DNA was isolated from peripheral white blood cells of all subjects using a standard DNA extraction protocol. DNA was pooled, selected size, ligated to sequencing adapters, and amplified to enrich for targets to be sequenced by the Agilent SureSelect^{XT} Target Enrichment System (Agilent Technologies Inc., Santa Clara, CA, USA). Exome capture was performed with the Agilent SureSelect^{XT} Human All Exon 50 Mb Kit (Agilent Technologies Inc.). Exome enriched products were sequenced using the Illumina HiSeq 2000 by Takara Bio Inc., Shiga, Japan. One sample was sequenced per lane to obtain an average theoretical depth of 80x, using 2x100 bp sequencing.

Bioinformatics

For the samples, paired-end reads were aligned using the Burrows-Wheeler Aligner on the human reference genome build hg19 using quality score calibration, soft clipping, and adapter trimming. Following the exclusion of PCR duplicate reads using the Picard, insertions/deletions and single-nucleotide polymorphisms (SNP) were called using the Genome Analysis Toolkit (GATK) [18,19]. Variants (SNP/indels) were filtered on the basis of the Phred scaled genotype quality score. Re-alignment was performed and the calling algorithm merged the output of GATK Unified Genotyper. All samples were annotated using SnpEff [20] (version 3.6) to classify variants (*e.g.* missense, stop gain/loss, splice-site variant, synonymous, intronic, insertions/deletions).

In addition to the standard variant quality controls, six independent filters were applied to facilitate detection of causal variants among the enrolled HCM families. Variants were filtered by: (1) minor allele frequency (MAF) >1% in Asian population; (2) benign, as predicted by SnpEff; (3) genotype–phenotype unmatched under the assumption of complete penetrance without phenocopies; (4) registered in the SNP Database (dbSNP137); (5) combined annotation dependent depletion (CADD) score <10; and (6) low heart expression of the genes, less than the top quartile.

The frequency filter adopted the allele frequency estimates from the Asian cohort of the 1000 Genomes Project database [21], and we used a MAF 1% as the cut-off. Genotype–phenotype matching was defined as narrowing the variants at which affected subjects had ≥1 alternative allele(s) and unaffected subjects had no alternative allele. As a functional filtering method, variants not registered in NCBI dbSNP137 were considered to be candidate variants. Prediction of *in silico* pathogenicity for novel missense variants was performed using the CADD prediction software (version 1.0), which objectively integrates many diverse annotations into a single measure (C-score) for each variant [22]. A variant was predicted to be pathogenic if the scaled C-score calculated by the software was above 10, a score indicative of the variant being within the top 10% of deleteriousness substitutions. Candidate variants were evaluated if the gene associated with each variant was directly involved in the myocardium or worsening cardiac function by using the high heart expression (HHE) gene data as previously reported [23]. In brief, HHE genes were defined as the top quartile [>40 reads per million mapped reads (rpm)] of 16,599 human-mouse orthologous gene expressions generated by RNA sequencing data of mice hearts at embryonic day 14.5.

After the evaluation, Sanger sequencing method was performed to confirm the putative variant identified by the bioinformatics analysis in the tested subjects and other relatives.

Additional screening for HCM registry

In addition, restriction fragment length polymorphism (RFLP) was performed on HCM probands listed in our registry to identify other subjects harboring the variant. In brief, restriction enzyme (*Van91I*) was added to each DNA sample and incubated for 3 h, which allowed the enzyme to cut at the recognition site (in this case, the myosin essential light chain [(MYL3, OMIM#: 160790),

Table 1
Clinical features of carriers harboring the MYL3 (Arg94His) variant in HCM-F18 and HCM-F189 families.

Pedigree ID	Gender	Age[a]	HT	LVOT obstruction	Symptoms	NYHA	ECG findings	QW	GNTW	LAD	MWT	IVS	PW	LVDd	LVDs	LVEF
HCM-F18																
III:3	Male	74	–	–	–	I	1°AV-block, CLBBB	–	–	47	19	19	8	55	33	61
III:6 (proband)	Female	60	–	–	–	I	AF, LVH	–	–	61	20	20	10	38	25	64
III:9	Female	58	–	–	–	I	LVH	–	–	28	23	23	11	38	23	71
III:12	Male	51	–	–	Syncope	I	1°AV-block, NSVT	–	–	53	18	18	11	48	33	59
IV:6	Female	37	–	–	–	I	–	–	–	18	10	10	9	38	23	65
IV:11	Female	24	–	–	–	I	IRBBB	–	–	30	15	15	11	39	21	72
HCM-F189																
II:2	Male	75	–	–	–	I	LVH	–	+	35	18	18	12	43	26	65
III:5	Male	41	–	–	–	I	LVH	–	–	28	13	13	10	43	30	52

AF, atrial fibrillation; ASH, asymmetric septal hypertrophy; CLBBB, complete left bundle branch block; ECG, echocardiography; GNTW, giant negative T wave; HT, hypertension; IRBBB, incomplete right bundle branch block; IVS, interventricular septum diameter; LAD, left atrial dimension; LVDd, left ventricular diastolic diameter; LVDs, left ventricular systolic diameter; LVEF, left ventricular ejection fraction; LVH, left ventricular hypertrophy; LVOT, left ventricular outflow tract; MWT, max wall thickness; NSVT, non-sustained ventricular tachycardia; NYHA, New York Heart Association heart failure classification; PW, posterior wall diameter; QW, Q wave.
[a] Age at examination.

NM_000258.2:c.281G>A variant site]). The DNA fragments were separated by size using gel electrophoresis to allow confirmation of cutting of the sample DNAs. To investigate the ancestral origin of the variant, haplotype analysis was performed by using six microsatellite markers (short tandem repeats $[(CA)_n$: D3S3687, D3S3678, D3S3647, D3S3582, D3S3640, D3S1568] flanking the variant gene) [24,25]. Clinical characteristics of variant carriers were documented for phenotype evaluation of the variant.

Results

Characteristics of HCM-F18 family members

Clinical characteristics of HCM-F18 family members are shown in Table 1, and a representative case (III:6, proband) is presented in Fig. 1. The subject was referred to our hospital because of an abnormal ECG finding detected by a local hospital. ECG showed atrial fibrillation with left ventricular hypertrophy pattern, chest X-rays displayed an increase in cardiothoracic ratio, and echocardiography revealed an apparent interventricular septal hypertrophy without LVOT obstruction. After excluding other cardiac diseases, the subject was diagnosed with HCM.

Although the HCM-F18 proband and her family members had been clinically observed for years, traditional Sanger sequencing failed to identify the apparent causative variants. The HCM-affected family members had a typical clinical phenotype consistent with HCM, exhibiting interventricular septal hypertrophy and asymmetric septal hypertrophy. The affected subjects comprised two males and three females with an age range at enrollment of between 24 and 74 years. The HCM-unaffected subjects were a 67-year-old female diagnosed with hypertensive heart disease and a 48-year-old female without any cardiac disease.

Exome sequencing and bioinformatics analyses

DNA samples from seven members of the HCM-F18 family, five clinically HCM-affected subjects, and two unaffected subjects were analyzed using WES (Fig. 2) followed by bioinformatics filtering methods (Table 2). The mean sequencing depth for the seven subjects was 56.1x, 60.0x, 54.8x, 58.7x, 58.2x, 63.7x, and 66.0x per base across the whole exome, for samples III:2 (unaffected), III:3 (affected), III:6 (affected), III:9 (affected), III:12 (affected), IV:7 (unaffected), and IV:11 (affected), respectively. Percentages of on-target reads were 78.6% (III:2), 78.0% (III:3), 77.3% (III:6), 79.4% (III:9), 77.2% (III:12), 78.7% (IV:7), and 77.0% (IV:11), respectively, while coverage rates of target coding lesions (20x) were 89.3% (III:2), 90.1% (III:3), 89.0% (III:6), 90.8% (III:9), 89.7% (III:12), 91.7% (IV:7), and 92.0% (IV:11), respectively.

Subsequent to WES, bioinformatics analysis and genotype–phenotype matching following exome sequencing were performed for family HCM-F18 to identify causative variants found in all five HCM-affected subjects but not in the two unaffected subjects (Table 2). The number of aligned variants in the seven subjects with

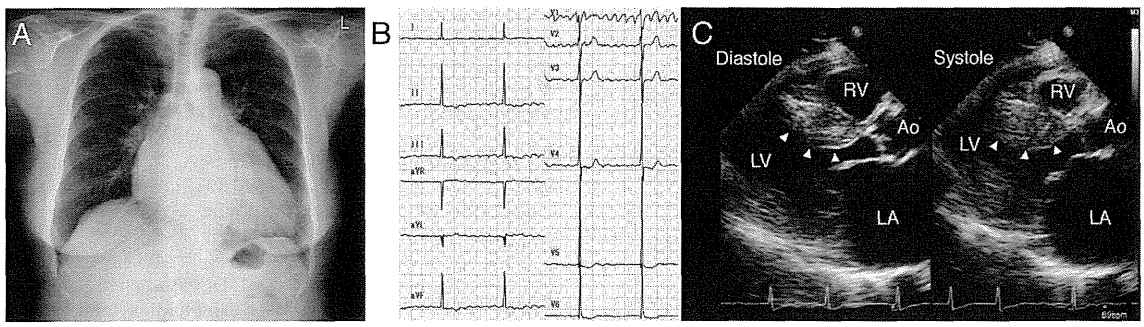

Fig. 1. A representative case (proband) in hypertrophic cardiomyopathy (HCM)-F18 family. Clinical images of the proband (III:6 in Table 1) in HCM-F18 family are shown: (A) chest X-ray; (B) electrocardiogram; (C) echocardiography of longitudinal views. Chest X-ray showed that the cardiothoracic ratio was increased (61%); electrocardiogram showed atrial fibrillation with left ventricular hypertrophy pattern; and echocardiography presented normal ejection fraction and an apparent interventricular septal hypertrophy without left ventricular outflow tract obstruction (arrow head). Ao, aorta; LA, left atrium; LV, left ventricle; RV, right ventricle.

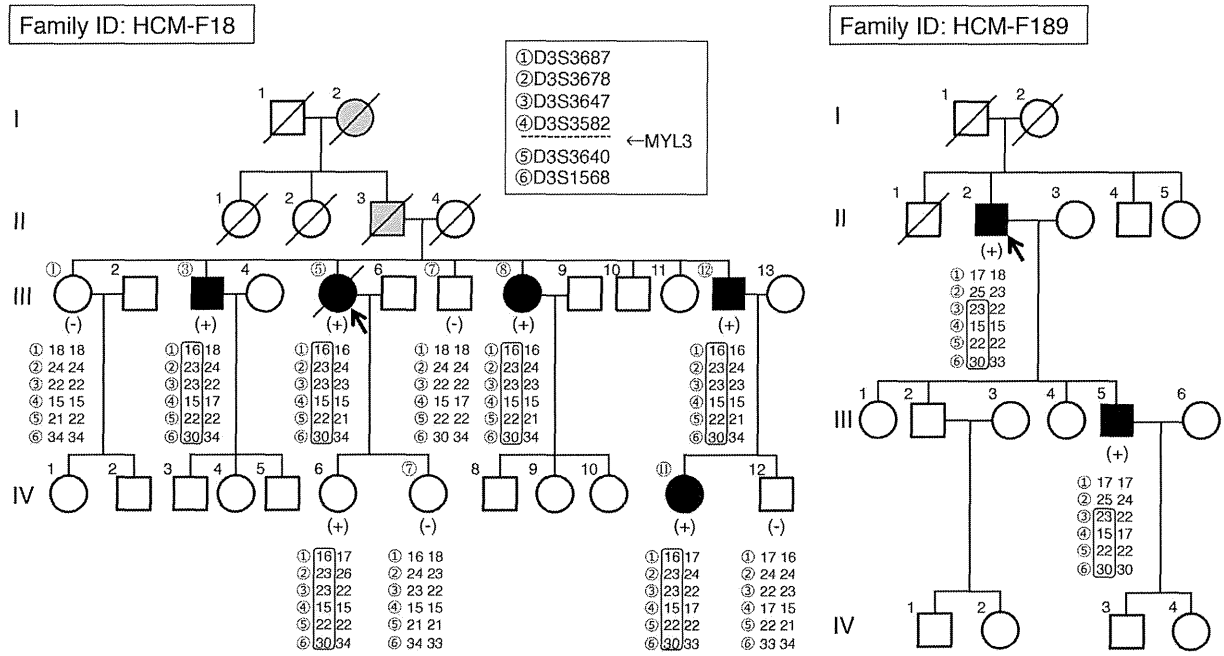

Fig. 2. Pedigrees of families: (A) hypertrophic cardiomyopathy (HCM)-F18 and (B) HCM-F189. The pedigrees of HCM families with the myosin essential light chain (MYL3) variant (Arg94His) are shown. Arrows indicate probands. Squares represent male subjects; circles represent female subjects. Diagonal lines mark deceased individuals. Solid symbols denote HCM. Open symbols represent unaffected individuals or individuals with no data available for analysis. Gray symbols represent obligate carriers. Plus "(+)" and minus "(−)" signs indicate presence or absence of the Arg94His variant confirmed by direct sequencing, respectively. Encircled numbers indicate subjects investigated by whole exome sequencing. Haplotypes (the number of $[CA]_n$ repeat) are listed for six microsatellite markers ([1] D3S3687 – [2] D3S3678 – [3] D3S3647 – [4] D3S3582 – [MYL3] – [5] D3S3640 – [6] D3S1568) from top to bottom. Boxed haplotypes demonstrate affected haplotypes. Affected haplotypes share same numbers of $[CA]_n$ repeat from [3] D3S3647 to [6] D3S1568 in both families, indicating that the MYL3 Arg94His variant originated from the same ancestor.

passing quality filtering was 243359. Among those, 60200 were considered to be rare variants (MAF <1%) using the Asian cohort in 1000 Genome Project [21]. Focusing on missense, nonsense, splice-site, and frameshift variants, 3439 variants were detected. Genotype–phenotype matching enabled putative variants to narrow the number to 13. After excluding variants registered in dbSNP137, six putative variants remained (Table 3). To evaluate these variants, CADD prediction software was used to calculate each scaled C-score. Five out of six variants were predicted to be damaging (scaled C-score >10), indicating that these were potentially deleterious variants. Among the candidate variants, myosin essential light chain (MYL3, c.281G>A, p.Arg94His) was the only gene that belonged to the HHE genes and was directly associated with the myocardium (ventricular muscle). Although this MYL3 Arg94His variant was not listed in the NHLBI ESP exome variant server [26], the Human Genetic Variation Database [27], or the Exome Aggregation Consortium database [28], the variant was reported to be a possibly damaging variant in the Human Gene Mutation Database [29]. Sanger sequencing for the 10 HCM-F18 family members validated the variant in all the affected subjects and resulted in the detection of one additional variant carrier without an apparent HCM phenotype (Figs. 2 and 3).

Table 2
Algorithm of bioinformatics filtering methods.

Total aligned variants in seven subjects	257452
After QC	243359
↓	
Filtering methods	
MAF <1% in the 1000 Genome Project (Asian cohort)	60020
↓	
Missense, nonsense, splice-site or frameshift variants	3439
↓	
Genotype–phenotype matching	13
↓	
Remove dbSNP137 registered variants	6
↓	
CADD score >10	5
↓	
High heart expression gene	1

The number of variants was decreased according to each filtering step. Finally, 1 variant, MYL3 c.281G>A was thought to be the causative variant.
CADD, combined annotation dependent depletion; MAF, minor allele frequency; QC, quality control; dbSNP, the Single Nucleotide Polymorphism Database.

Phenotype evaluation of MYL3 (c.281G>A, p.Arg94His) variant

All 600 familial or sporadic HCM probands listed in the registry were screened for the MYL3 (c.281G>A, p.Arg94His) variant by RFLP analysis using the *Van91I* restriction enzyme. The Arg94His variant was identified in one additional proband (HCM-F189) and Sanger sequencing confirmed that two additional subjects had the MYL3 Arg94His variant (Fig. 2). Moreover, haplotype analysis of family HCM-F18 and family HCM-F189 using six microsatellites in the neighborhood revealed that the MYL3 Arg94His variant originated from the same ancestor (Fig. 2).

The clinical characteristics of the eight MYL3 Arg94His variant carriers are summarized in Table 1. Disease-penetrance was 88%, and all clinically affected carriers exhibited asymmetrical septal hypertrophy with a substantial maximal left ventricular wall thickness of 18 ± 3 mm. Left ventricular systolic dysfunction (ejection fraction <50%) was not observed. Abnormal ECG findings were observed in seven carriers with an apparent HCM phenotype.

Table 3
Candidate variants after filtering methods in HCM-F18.

Gene	Function	Chr.	Exon	Position (build 37)	NM #	Amino acid	SIFT	PolyPhen-2	MutationTaster2	CADD score	HHE gene
HMGB4	High Mobility Group Box 4	1	2	34329932	145205	E47A	0.01	0.999	DC	24.5	–
HHATL	Hedgehog Acyltransferase-Like	3	9	42738359	20707	R341C	0	1	DC	18.7	–
MYL3	**Myosin Light Chain 3, Ventricular, Skeletal**	**3**	**3**	**46902192**	**258**	**R94H**	**0.04**	**0.008**	**DC**	**16.63**	+
NIPAL4	NIPA-Like Domain Containing 4	5	1	156887258	1172292	R39Q	0.25	0.002	Poly	19.5	–
PLAU	Plasminogen Activator, Urinary	10	10	75676220	1145031	R381L	0.11	0.009	Poly	9.587	–
LIPM	Lipase, Family member M	10	4	90574372	1128215	G184S	0.01	0.981	DC	35	–

The six variants in six genes filtered by bioinformatics methods are present in five HCM-affected subjects and are absent in two unaffected subjects. Of those, 5 variants had the CADD score more than 10, and MYL3 gene was the only gene that highly expressed in mice hearts.
SIFT scores (ranges from 0 to 1) were calculated by SIFT version 5.2.2, and the SIFT score less than 0.05 was considered as deleterious. PolyPhen-2 scores (ranges from 0 to 1) were calculated by PolyPhen-2 version 2.2.2, and the PolyPhen-2 score more than 0.85 was considered as probably damaging. The six variants were also evaluated by MutationTaster2 (classifying variants as "Disease causing" or "Polymorphism").
CADD, combined annotation dependent depletion; Chr, chromosome; DC, disease causing; HCM, hypertrophic cardiomyopathy; HHE, high heart expression; Poly, polymorphism; PolyPhen, Polymorphism Phenotyping v2 score; Position, nucleotide position; SIFT, Sorting Intolerant From Tolerant score.
Bold highlights the causative variant.

Three subjects had left ventricular hypertrophy, two had first-degree atrioventricular block, two had bundle branch block, and one had atrial fibrillation with an enlarged left atrium. GNTW was observed in one subject, but pathological Q wave was not observed.

Clinical follow-up data were available for four of eight subjects. Although they suffered from neither cardiac death nor progression of left ventricular systolic dysfunction during >10 years follow-up, one subject (HCM-F18; III:12) received an implantable cardioverter defibrillator (ICD) because of syncope with frequent non-sustained ventricular tachycardia. Another subject (HCM-F18; III:6) died from non-cardiac causes (postoperative infection after an orthopedic surgery).

Discussion

In this study, WES was demonstrated to be an effective tool, even in HCM. Furthermore, we determined the MYL3 Arg94His variant was newly identified as a variant associated with high disease penetrance and substantial degree of interventricular septal hypertrophy for the first time. This is the first study to validate the MYL3 Arg94His as a causative variant of familial HCM using WES.

Since the application of NGS methods such as WES and target re-sequencing for clinical genetics, the number of bioinformatics approaches for detecting the causative variant in patients in

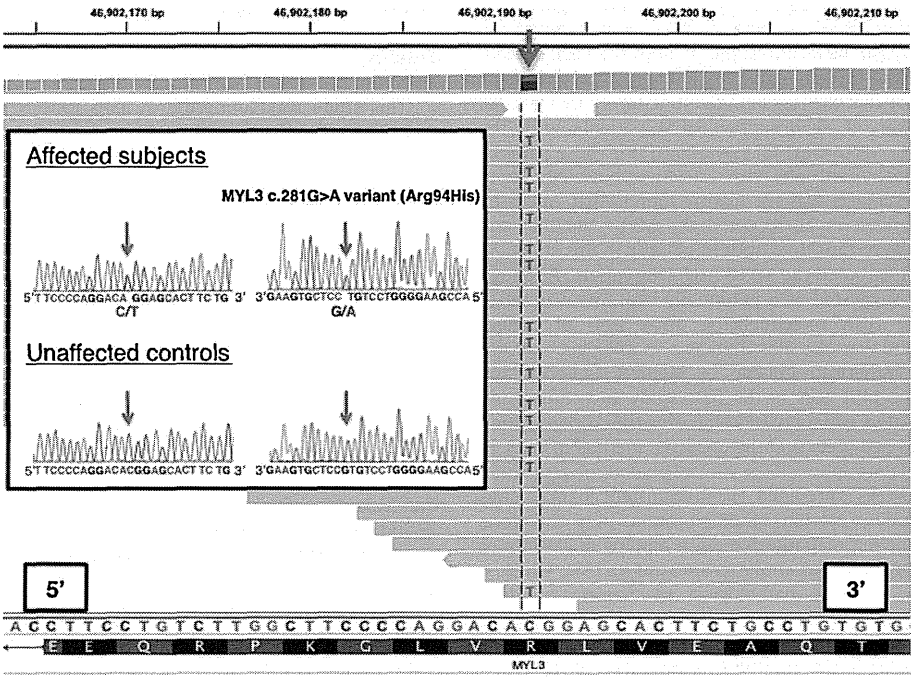

Fig. 3. NGS read alignment and the Sanger sequencing validation in the MYL3 gene at exon 3. Alignment reads are visualized by the Integrative Genomics Viewer (IGV) version 2.3. The red arrow in IGV indicates the p.Arg94His variant position in the MYL3 gene at exon 3 (5′ → 3′ complementary strand). The red arrow in Sanger sequencing data of affected subjects also shows the same variant at the same position.

cardiomyopathy has gradually increased [30,31]. This is because the traditional direct sequencing methods have only been able to identify approximately 50% of the causative variants in patients with cardiomyopathy [8]. Several clinical studies have demonstrated that WES is effective for molecular diagnosis of cardiomyopathy [30,31], providing support for the notion that WES may be useful in diverse ethnic settings.

In this study, two novel filtering schemas were adopted to enable causative variants to be distinguished from thousands of other incidental variants detected *via* WES. The CADD C-score [22] is a highly combined damaging score for each variant that is generated by 63 distinct annotation tools including SIFT [32] and PolyPhen [33]. This means that we can obtain the comprehensive damaging scores of each candidate variant even though each annotation tool shows discordant results. For example, the MYL3 Arg94His variant was first predicted as deleterious by SIFT and disease-causing by MutationTaster2 [34], but was considered as benign by PolyPhen-2 (Table 3). However, the CADD C-score of the variant was 16.63, clearly predicting that the MYL3 Arg94His as deleterious. Furthermore, prediction of the pathogenicity of human variants with the CADD C-score is more accurate than that of any other *in silico* single annotation tool [22]. Actually, the usefulness of CADD C-score for detecting causative variants in patients with Tangier disease and long QT syndrome were previously reported [35,36]. In terms of HHE [23], Zaidi et al. demonstrated that HHE genes had a higher frequency of protein-alternating *de novo* variants than genes with low heart expression (less than top quartile) in patients with congenital heart diseases [23]. In the present study, the combination of the CADD scaled C-score and the heart expression-oriented filtering schema successfully narrowed down the number of causative variants to a single variant, the MYL3 Arg94His variant, suggesting the usefulness of this schema even in heterogeneous diseases such as HCM.

Completion of the final filtering method (Table 2) indicated that MYL3 was the only gene that was directly associated with ventricular myocardium. MYL3 encodes the essential light chain, an important component of sarcomere, which wraps around the lever arm of the myosin head, and supports both the neck domain and the lever arm with myosin regulatory light chain [37]. Previous reports demonstrate that the loss of MYL3 function causes familial HCM [38–43]. Olson et al. described HCM patients with the autosomal recessive MYL3 Glu143Lys variant [38]. These patients exhibited mid-cavity hypertrophy with restrictive physiology, but in none of our HCM-subjects was the feature found. Also, Andersen et al. and Kazmierczak et al. reported the MYL3 Val79Ile and Ala57Gly variants were causative in HCM [41,43]. In this study, all subjects with HCM harbored the MYL3 Arg94His variant. Although the Arg94His was suggested as a potentially causative variant of sporadic HCM by DNA re-sequencing array [44] or Sanger sequencing [45], and was also classified as a "pathogenic" variant according to the American College of Medical Genetics and Genomics guideline [46], this is the first study to confirm and support it as a causative variant of HCM by using the co-segregation pattern in the HCM pedigrees and the unbiased method of WES. In addition, a detailed description of the clinical phenotype associated with the MYL3 Arg94His is provided. The myosin light chain belongs to the EF-hand family of calcium-binding proteins [47]. Therefore, loss-of EF-hand function may interfere with binding of calcium or magnesium causing malfunction of myosin kinetics [37], which is thought to be a potential pathological mechanism of HCM development [42]. The MYL3 causative variants are primarily located in exons 3 and 4, which encode the EF-hand 2 domain [37], and the Arg94 residue is also located in exon 3. Use of the CADD scaled C-score [22] also predicted this to be a damaging variant site. The MYL3 Arg94His variant is expected to cause crucial damage to EF-hand function, myosin kinetics, and its structure, and eventually hypertrophic changes in the myocardium, which may underlie the high disease penetrance and substantial interventricular septal hypertrophy observed in this study (Table 1). However, due to the small number of subjects harboring this variant, it remains unclear whether screening of families with HCM for the MYL3 Arg94His variant will be useful in patient management. Further study is needed to clarify the long-term clinical courses in HCM patients with the MYL3 Arg94His variant.

Study limitations

This study has some limitations. WES was performed with protein-coding regions only, which potentially overlooked other intergenic variants that influenced the specific phenotype. HCM is a heterogeneous disorder in terms of both clinical characteristics and age of onset, limiting the accuracy of the phenotype modeling. In this study, we could accurately find out one causative variant for HCM, but it might be controversial whether a 48-year-old woman without apparent HCM phenotype could be classified as an unaffected HCM subject. Although CADD enabled more precise prediction of *in silico* damaging score than other software, the optimal cut-off value for detection of deleterious variants with CADD scaled C-scores is debatable. HHE genes were generated only by known human-mouse orthologues, which might rule-out potential human-specific genes that were highly expressed in human heart. In addition, clinical information was not available for some family members, which may have affected the penetrance and clinical characteristics of HCM with MYL3 Arg94His variant.

Conclusion

Together with CADD score and HHE gene data, WES facilitated successful identification of the one causative variant, the MYL3 Arg94His, even in HCM. Furthermore, the MYL3 Arg94His variant was associated with high disease penetrance and substantial ventricular hypertrophy.

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Conflict of interest

The authors declare no conflict of interest.

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References

[1] Gersh BJ, Maron BJ, Bonow RO, Dearani JA, Fifer MA, Link MS, Naidu SS, Nishimura RA, Ommen SR, Rakowski H, Seidman CE, Towbin JA, Udelson JE, Yancy CW, American College of Cardiology Foundation/American Heart Association Task Force on Practice G. 2011 ACCF/AHA Guideline for the Diagnosis and Treatment of Hypertrophic Cardiomyopathy: a report of the American

College of Cardiology Foundation/American Heart Association Task Force on Practice Guidelines. Developed in collaboration with the American Association for Thoracic Surgery, American Society of Echocardiography, American Society of Nuclear Cardiology, Heart Failure Society of America, Heart Rhythm Society, Society for Cardiovascular Angiography and Interventions, and Society of Thoracic Surgeons. J Am Coll Cardiol 2011;58:e212–60.
[2] Maron BJ, Maron MS. Hypertrophic cardiomyopathy. Lancet 2013;381: 242–55.
[3] Fujino N, Konno T, Hayashi K, Hodatsu A, Fujita T, Tsuda T, Nagata Y, Kawashiri MA, Ino H, Yamagishi M. Impact of systolic dysfunction in genotyped hypertrophic cardiomyopathy. Clin Cardiol 2013;36:160–5.
[4] Kawashiri MA, Hayashi K, Konno T, Fujino N, Ino H, Yamagishi M. Current perspectives in genetic cardiovascular disorders: from basic to clinical aspects. Heart Vessels 2014;29:129–41.
[5] Fujita T, Konno T, Yokawa J, Masuta E, Nagata Y, Fujino N, Funada A, Hodatsu A, Kawashiri MA, Yamagishi M, Hayashi K. Increased extent of myocardial fibrosis in genotyped hypertrophic cardiomyopathy with ventricular tachyarrhythmias. J Cardiol 2015;66:63–8.
[6] Maron BJ, Gardin JM, Flack JM, Gidding SS, Kurosaki TT, Bild DE. Prevalence of hypertrophic cardiomyopathy in a general population of young adults. Echocardiographic analysis of 4111 subjects in the CARDIA Study. Coronary Artery Risk Development in (Young) Adults. Circulation 1995;92: 785–9.
[7] Kimura A, Harada H, Park JE, Nishi H, Satoh M, Takahashi M, Hiroi S, Sasaoka T, Ohbuchi N, Nakamura T, Koyanagi T, Hwang TH, Choo JA, Chung KS, Hasegawa A, et al. Mutations in the cardiac troponin I gene associated with hypertrophic cardiomyopathy. Nat Genet 1997;16:379–82.
[8] Maron BJ, Maron MS, Semsarian C. Genetics of hypertrophic cardiomyopathy after 20 years: clinical perspectives. J Am Coll Cardiol 2012;60:705–15.
[9] Lopes LR, Rahman MS, Elliott PM. A systematic review and meta-analysis of genotype–phenotype associations in patients with hypertrophic cardiomyopathy caused by sarcomeric protein mutations. Heart 2013;99:1800–11.
[10] Kokado H, Shimizu M, Yoshio H, Ino H, Okeie K, Emoto Y, Matsuyama T, Yamaguchi M, Yasuda T, Fujino N, Ito H, Mabuchi H. Clinical features of hypertrophic cardiomyopathy caused by a Lys183 deletion mutation in the cardiac troponin I gene. Circulation 2000;102:663–9.
[11] Terauchi Y, Kubo T, Baba Y, Hirota T, Tanioka K, Yamasaki N, Furuno T, Kitaoka H. Gender differences in the clinical features of hypertrophic cardiomyopathy caused by cardiac myosin-binding protein C gene mutations. J Cardiol 2015;65:423–8.
[12] Stitziel NO, Peloso GM, Abifadel M, Cefalu AB, Fouchier S, Motazacker MM, Tada H, Larach DB, Awan Z, Haller JF, Pullinger CR, Varret M, Rabes JP, Noto D, Tarugi P, et al. Exome sequencing in suspected monogenic dyslipidemias. Circ Cardiovasc Genet 2015;8:343–50.
[13] Lopes LR, Zekavati A, Syrris P, Hubank M, Giambartolomei C, Dalageorgou C, Jenkins S, McKenna W, Uk10k C, Plagnol V, Elliott PM. Genetic complexity in hypertrophic cardiomyopathy revealed by high-throughput sequencing. J Med Genet 2013;50:228–39.
[14] Gomez J, Reguero JR, Moris C, Martin M, Alvarez V, Alonso B, Iglesias S, Coto E. Mutation analysis of the main hypertrophic cardiomyopathy genes using multiplex amplification and semiconductor next-generation sequencing. Circ J 2014;78:2963–71.
[15] Lang RM, Bierig M, Devereux RB, Flachskampf FA, Foster E, Pellikka PA, Picard MH, Roman MJ, Seward J, Shanewise JS, Solomon SD, Spencer KT, Sutton MS, Stewart WJ, Chamber Quantification Writing G. et al. Recommendations for chamber quantification: a report from the American Society of Echocardiography's Guidelines and Standards Committee and the Chamber Quantification Writing Group, developed in conjunction with the European Association of Echocardiography, a branch of the European Society of Cardiology. J Am Soc Echocardiogr 2005;18:1440–63.
[16] Konno T, Shimizu M, Ino H, Yamaguchi M, Terai H, Uchiyama K, Oe K, Mabuchi T, Kaneda T, Mabuchi H. Diagnostic value of abnormal Q waves for identification of preclinical carriers of hypertrophic cardiomyopathy based on a molecular genetic diagnosis. Eur Heart J 2004;25:246–51.
[17] Konno T, Fujino N, Hayashi K, Uchiyama K, Masuta E, Katoh H, Sakamoto Y, Tsubokawa T, Ino H, Yamagishi M. Differences in the diagnostic value of various criteria of negative T waves for hypertrophic cardiomyopathy based on a molecular genetic diagnosis. Clin Sci 2007;112:577–82.
[18] McKenna A, Hanna M, Banks E, Sivachenko A, Cibulskis K, Kernytsky A, Garimella K, Altshuler D, Gabriel S, Daly M, DePristo MA. The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. Genome Res 2010;20:1297–303.
[19] DePristo MA, Banks E, Poplin R, Garimella KV, Maguire JR, Hartl C, Philippakis AA, del Angel G, Rivas MA, Hanna M, McKenna A, Fennell TJ, Kernytsky AM, Sivachenko AY, Cibulskis K, et al. A framework for variation discovery and genotyping using next-generation DNA sequencing data. Nat Genet 2011;43:491–8.
[20] Cingolani P, Platts A, Wang le L, Coon M, Nguyen T, Wang L, Land SJ, Lu X, Ruden DM. A program for annotating and predicting the effects of single nucleotide polymorphisms, SnpEff: SNPs in the genome of *Drosophila melanogaster* strain w1118; iso-2; iso-3. Fly 2012;6:80–92.
[21] 1000 Genomes Project Consortium, Abecasis GR, Altshuler D, Auton A, Brooks LD, Durbin RM, Gibbs RA, Hurles ME, McVean GA. A map of human genome variation from population-scale sequencing. Nature 2010;467:1061–73.
[22] Kircher M, Witten DM, Jain P, O'Roak BJ, Cooper GM, Shendure J. A general framework for estimating the relative pathogenicity of human genetic variants. Nat Genet 2014;46:310–5.
[23] Zaidi S, Choi M, Wakimoto H, Ma L, Jiang J, Overton JD, Romano-Adesman A, Bjornson RD, Breitbart RE, Brown KK, Carriero NJ, Cheung YH, Deanfield J, DePalma S, Fakhro KA, et al. De novo mutations in histone-modifying genes in congenital heart disease. Nature 2013;498:220–3.
[24] Sakata K, Shimizu M, Ino H, Yamaguchi M, Terai H, Fujino N, Hayashi K, Kaneda T, Inoue M, Oda Y, Fujita T, Kaku B, Kanaya H, Mabuchi H. High incidence of sudden cardiac death with conduction disturbances and atrial cardiomyopathy caused by a nonsense mutation in the STA gene. Circulation 2005;111:3352–8.
[25] Thierfelder L, Watkins H, MacRae C, Lamas R, McKenna W, Vosberg HP, Seidman JG, Seidman CE. Alpha-tropomyosin and cardiac troponin T mutations cause familial hypertrophic cardiomyopathy: a disease of the sarcomere. Cell 1994;77:701–12.
[26] NHLBI Exome Sequencing Project (ESP). Exome Variant Server, http://evs.gs.washington.edu/EVS/.
[27] Japanese genetic variation consortium. A reference database of genetic variations in Japanese population, http://www.genome.med.kyoto-u.ac.jp/SnpDB.
[28] The Exome Aggregation Consortium (ExAC), http://exac.broadinstitute.org.
[29] Human Gene Mutation Database (HGMD), http://www.hgmd.cf.ac.uk.
[30] Campbell N, Sinagra G, Jones KL, Slavov D, Gowan K, Merlo M, Carniel E, Fain PR, Aragona P, Di Lenarda A, Mestroni L, Taylor MR. Whole exome sequencing identifies a troponin T mutation hot spot in familial dilated cardiomyopathy. PLOS ONE 2013;8:e78104.
[31] Guo Q, Xu Y, Wang X, Guo Y, Xu R, Sun K, Chen S. Exome sequencing identifies a novel MYH7 p.G407C mutation responsible for familial hypertrophic cardiomyopathy. DNA Cell Biol 2014;33:699–704.
[32] Ng PC, Henikoff S. SIFT: predicting amino acid changes that affect protein function. Nucleic Acids Res 2003;31:3812–4.
[33] Adzhubei IA, Schmidt S, Peshkin L, Ramensky VE, Gerasimova A, Bork P, Kondrashov AS, Sunyaev SR. A method and server for predicting damaging missense mutations. Nat Methods 2010;7:248–9.
[34] Schwarz JM, Cooper DN, Schuelke M, Seelow D. MutationTaster2: mutation prediction for the deep-sequencing age. Nat Methods 2014;11:361–2.
[35] Tada H, Kawashiri MA, Nohara A, Saito R, Tanaka Y, Nomura A, Konno T, Sakata K, Fujino N, Takamura T, Inazu A, Mabuchi H, Yamagishi M, Hayashi K. Whole exome sequencing combined with integrated variant annotation prediction identifies asymptomatic Tangier disease with compound heterozygous mutations in ABCA1 gene. Atherosclerosis 2015;240:324–9.
[36] Hayashi K, Konno T, Tada H, Tani S, Liu L, Fujino N, Nohara A, Hodatsu A, Tsuda T, Tanaka Y, Kawashiri MA, Ino H, Makita N, Yamagishi M. Functional characterization of rare variants implicated in susceptibility to lone atrial fibrillation. Circ Arrhythm Electrophysiol 2015 [Epub ahead of print].
[37] Moore JR, Leinwand L, Warshaw DM. Understanding cardiomyopathy phenotypes based on the functional impact of mutations in the myosin motor. Circ Res 2012;111:375–85.
[38] Olson TM, Karst ML, Whitby FG, Driscoll DJ. Myosin light chain mutation causes autosomal recessive cardiomyopathy with mid-cavitary hypertrophy and restrictive physiology. Circulation 2002;105:2337–40.
[39] Sanbe A, Nelson D, Gulick J, Setser E, Osinska H, Wang X, Hewett TE, Klevitsky R, Hayes E, Warshaw DM, Robbins J. In vivo analysis of an essential myosin light chain mutation linked to familial hypertrophic cardiomyopathy. Circ Res 2000;87:296–302.
[40] Lee W, Hwang TH, Kimura A, Park SW, Satoh M, Nishi H, Harada H, Toyama J, Park JE. Different expressivity of a ventricular essential myosin light chain gene Ala57Gly mutation in familial hypertrophic cardiomyopathy. Am Heart J 2001;141:184–9.
[41] Andersen PS, Hedley PL, Page SP, Syrris P, Moolman-Smook JC, McKenna WJ, Elliott PM, Christiansen M. A novel Myosin essential light chain mutation causes hypertrophic cardiomyopathy with late onset and low expressivity. Biochem Res Int 2012;2012:685108.
[42] Lossie J, Ushakov DS, Ferenczi MA, Werner S, Keller S, Haase H, Morano I. Mutations of ventricular essential myosin light chain disturb myosin binding and sarcomeric sorting. Cardiovasc Res 2012;93:390–6.
[43] Kazmierczak K, Paulino EC, Huang W, Muthu P, Liang J, Yuan CC, Rojas AI, Hare JM, Szczesna-Cordary D. Discrete effects of A57G-myosin essential light chain mutation associated with familial hypertrophic cardiomyopathy. Am J Physiol Heart Circ Physiol 2013;305:H575–89.
[44] Fokstuen S, Lyle R, Munoz A, Gehrig C, Lerch R, Perrot A, Osterziel KJ, Geier C, Beghetti M, Mach F, Sztajzel J, Sigwart U, Antonarakis SE, Blouin JL. A DNA resequencing array for pathogenic mutation detection in hypertrophic cardiomyopathy. Hum Mutat 2008;29:879–85.
[45] Zou Y, Wang J, Liu X, Wang Y, Chen Y, Sun K, Gao S, Zhang C, Wang Z, Zhang Y, Feng X, Song Y, Wu Y, Zhang H, Jia L, et al. Multiple gene mutations, not the type of mutation, are the modifier of left ventricle hypertrophy in patients with hypertrophic cardiomyopathy. Mol Biol Rep 2013;40:3969–76.
[46] Richards S, Aziz N, Bale S, Bick D, Das S, Gastier-Foster J, Grody WW, Hegde M, Lyon E, Spector E, Voelkerding K, Rehm HL. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. Genet Med 2015;17:405–23.
[47] Collins JH. Homology of myosin DTNB light chain with alkali light chains, troponin C and parvalbumin. Nature 1976;259:699–700.