Original Article

Prevalence and Clinical Implication of Double Mutations in Hypertrophic Cardiomyopathy

Revisiting the Gene-Dose Effect

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Background—Available data suggests that double mutations in patients with hypertrophic cardiomyopathy are not rare and are associated with a more severe phenotype. Most of this data, however, is based on noncontemporary variant classification.

Methods and Results—Clinical data of all hypertrophic cardiomyopathy patients with 2 rare genetic variants were retrospectively reviewed and compared with a group of patients with a single disease-causing variant. Furthermore, a literature search was performed for all studies with information on prevalence and outcome of patients with double mutations. Classification of genetic variants was reanalyzed according to current guidelines. In our cohort (n=1411), 9% of gene-positive patients had 2 rare variants in sarcomeric genes but only in 1 case (0.4%) were both variants classified as pathogenic. Patients with 2 rare variants had a trend toward younger age at presentation when compared with patients with a single mutation. All other clinical variables were similar. In data pooled from cohort studies in the literature, 8% of gene-positive patients were published to have double mutations. However, after reanalysis of reported variants, this prevalence diminished to 0.4%. All patients with 2 radical mutations in *MYBPC3* in the literature had severe disease with death or heart transplant during the first year of life. Data on other specific genotype–phenotype correlations were scarce.

Conclusions—Double mutations in patients with hypertrophic cardiomyopathy are much less common than previously estimated. With the exception of double radical *MYBPC3* mutations, there is little data to guide clinical decision making in cases with double mutations. **(*Circ Cardiovasc Genet*. 2017;10:e001685. DOI: 10.1161/CIRCGENETICS.116.001685.)**

Key Words: cardiomyopathy, hypertrophic ■ death, sudden, cardiac ■ genetics, medical ■ heart failure ■ mutation

As in many other inherited cardiac conditions, the discovery of the first disease-causing mutations in hypertrophic cardiomyopathy (HCM) brought hope that specific genotype–phenotype correlations will emerge and improve our ability to evaluate patients' prognosis. Indeed, early studies suggested an association between specific genetic variants or variants in specific genes and disease expression. Nevertheless, subsequent studies on larger cohorts failed to replicate many of these associations[1–3] casting doubt on the usefulness of genetic testing for prognostic evaluation. Although a specific genetic variant or its presence in a specific gene may not be predictive of risk, patients with any pathogenic or likely pathogenic (P/LP) variant seem to have worse clinical outcomes than those who are genotype negative.[2,4–6]

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The presence of more than one P/LP variant has also been reported to be associated with a more severe disease phenotype.[7,8] Specifically, an earlier disease onset, more severe left ventricular hypertrophy, higher prevalence of advanced heart failure,[9] and increased risk of sudden cardiac death (SCD)[10] have all been reported in patients with double mutations. These associations, however, are based on relatively small series and case reports and in most cases rely on genetic variant classification before the age of large exome databases. Recent studies suggest that some of the genes previously associated with HCM may not have a significant impact on disease expression[11] and that reassessment of variants according to new data results in reclassification in a substantial portion of cases.[12] Moreover, most recent guidelines[13] adopted a much stricter approach for variant classification and are likely to result in some variants previously classified as P/LP to be reclassified as variants of unknown significance (VUS). This impact can be expected to be especially great in double mutation cases where both variants would be required to satisfy guideline criteria and where cosegregation data may be clouded by the presence of multiple variants. We, therefore,

Received February 24, 2016; accepted February 7, 2017.

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The Data Supplement is available at http://circgenetics.ahajournals.org/lookup/suppl/doi:10.1161/CIRCGENETICS.116.001685/-/DC1.

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aimed to revisit the association between multiple disease-causing mutations and worsened outcome by analyzing the data from our large single-center HCM cohort and review of published literature.

Methods

Patient Selection

The study subjects were identified through the database of the multidisciplinary HCM clinic at the Toronto General Hospital. They included all patients >18 years of age who underwent genetic testing between January 2005 and June 2016. The clinical diagnosis of HCM was based on 2-dimensional echocardiographic or cardiovascular magnetic resonance findings of unexplained left ventricular hypertrophy with a maximal left ventricular wall thickness ≥15 mm. This study was approved by the Research Ethics Board of the University Health Network.

Genetic Testing

All index cases underwent genetic testing for HCM using a combination of commercially available panels. In 370 patients (26%), a 5-gene panel (*MYBPC3*, *MYH7*, *TNNT2*, *TNNI3*, and *TPM1*) was used, in 655 patients (46%), an 8-gene panel (with the addition of *ACTC1*, *MYL2*, and *MYL3*) was used, and in the remaining 386 patients (27%), a larger panel (19–51 genes) was used. All results were reviewed, and variants classified by a clinical genetics team and according to the current guidelines of the American College of Medical Genetics.[13]

Clinical Data Extraction

Electronic charts of patients with at least 1 P/LP variant a and second rare variant with a minor allele frequency (MAF) <0.1% in a gene encoding for a sarcomeric protein (*MYBPC3*, *MYH7*, *TNNT2*, *TNNI3*, *TPM1*, *ACTC1*, *MYL2*, and *MYL3*) were retrospectively reviewed. Arrhythmic adverse events were defined as SCD, resuscitated cardiac arrest, or appropriate therapy from an implanted cardioverter-defibrillator. End-stage HCM was defined as systolic left ventricular dysfunction (ejection fraction <50%) attributed to progression of HCM and in the absence of an alternative explanation.

Clinical variables of patients with 2 rare variants were compared with those of consecutive index cases with a single P/LP variant who underwent genetic testing between 2006 and 2012.

Literature Search

We systematically reviewed the literature for all publications describing phenotype in patients with 2 variants in genes encoding for sarcomere proteins. PubMed was searched up to June 2016 using the search terms double, compound, or homozygous each combined with mutation and either hypertrophic cardiomyopathy or HCM. References from the retrieved original articles and reviews were searched for missing studies. Studies not in the English language and those with missing data were excluded. Cases possibly included in more than one publication (eg, appearing in a case report and case series published by the same group) were counted only once.

Classification of Genetic Variants

All genetic variants in our cohort and those reported in the manuscripts uncovered by the literature search were reassessed and classified according to the current guidelines of the American College of Medical Genetics.[13] For this evaluation, we used MAF data derived from the Exome Aggregation Consortium and the Exome Variant Server. For in silico analysis of missense variants, PolyPhen2, SIFT, and MutationTaster were used. Cosegregation data were derived from families in our cohort, the original publications in the literature search, other publications including the variant in question, and data published on ClinVar and in accordance with the approach suggested by Jarvik et al[14] (Table I in the Data Supplement).

Statistical Analysis

Continuous variables are expressed as mean value±SD. Discrete variables are shown as percentages. Unpaired *t* test was used for comparison between continuous variables and Fisher exact test for categorical variables.

Results

Genetic Testing and Clinical Outcomes

Between January 2005 and June 2016, 1411 unrelated index cases underwent genetic testing for HCM. Of these, 272 cases (19%) had ≥1 P/LP variants identified, 60% of which were in *MYBPC3* (Figure). In 25 of these cases (9% of genotype-positive patients), a second rare variant with an MAF <0.1% in a gene encoding for a sarcomeric protein was found. However, only in 1 of these cases, both variants were classified as P/LP based on current guidelines (*MYBPC3* Gly853fs and *MYH7* Ala797Thr). This patient was diagnosed at the age of 21 years and underwent myectomy at the age of 35 years. An implanted cardioverter-defibrillator was implanted for primary prevention, but no appropriate therapies have been delivered during 15 years of follow-up. She gradually developed end-stage HCM with decline in systolic function and signs of heart failure toward the end of her 6th decade of life.

The clinical outcomes of the 24 cases with 1 P/LP variant and a second rare VUS are detailed in Table 1. When compared with 140 index cases who were found to have a single P/LP variant, the double-variant group had similar maximal wall thickness (22±6 versus 20±4; *P*=0.11) and a trend toward younger age at diagnosis (33±15 versus 40±14; *P*=0.09). Of note, in 10 of the 24 double-variant patients, compound heterozygosity with at least one nonsense, frameshift, or splice site variants was found. However, only in 3 of these cases, data were available to determine these 2 variants were in trans.

Systematic Review of Literature Results

The initial search resulted in a total of 362 studies after removing duplicates. Three additional studies were added after reviewing the references of the retrieved articles. After reviewing the titles and abstracts, 60 manuscripts underwent further review of the full-text version. After excluding studies because of missing data or possible overlap with other studies, 15 cohort studies and 25 case reports or series were included.

Cohort Studies

Fifteen cohort studies[7,15–28] provided data on the percentage of double mutations out of genotype-positive patients in HCM cohorts (Table 2). A panel including 8 to 10 genes was used for testing 33% of the patients included in these studies, 5 to 7 genes in 44%, and 2 to 4 genes in 23%. According to published data, 3% to 19% of gene-positive HCM index cases had 2 mutations. In 13 of these studies, the details of mutations were published and could be re-examined. All in all, there were 62 patients with double mutations according to published data in these 13 studies. In 13 of these patients (21% of published double mutation carriers), at least one of the 2 variants published was later found to have an MAF >0.1% in certain populations, and in 2 cases (3%), the MAF was >5%. In 45 cases (73%), at least one of the variants was reclassified as a VUS based on current guideline standards. Only in 3 of

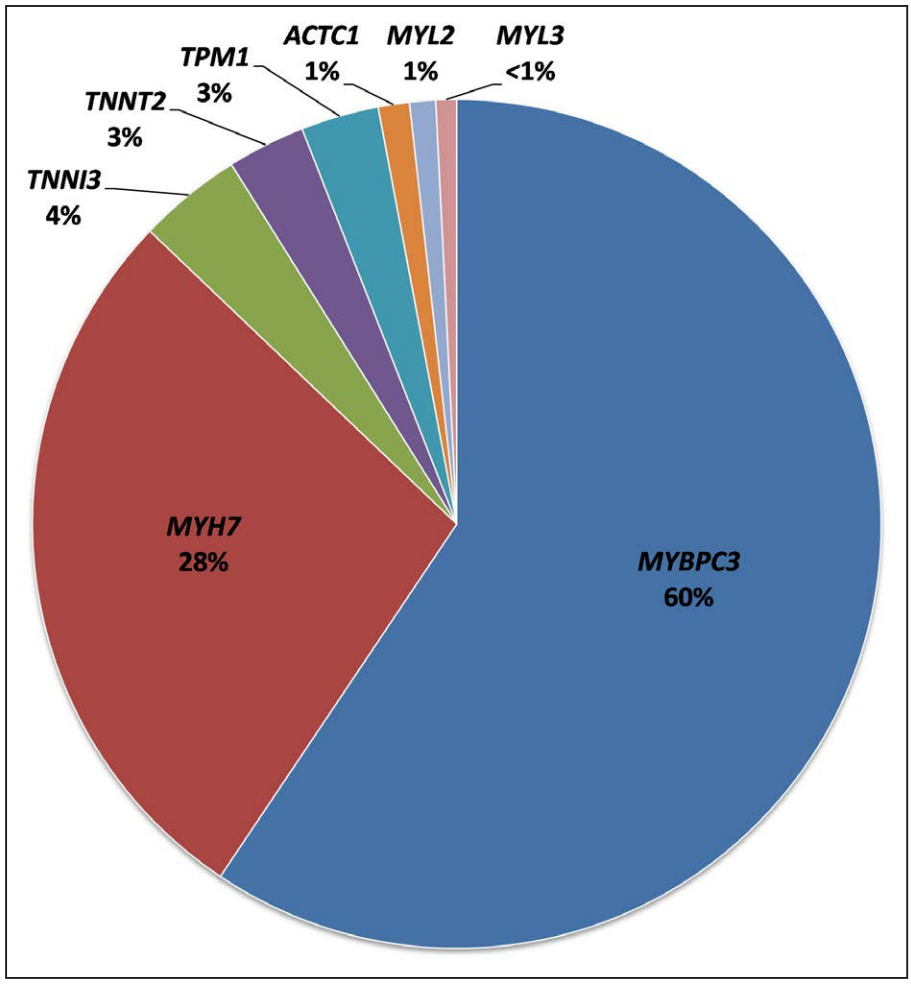

Figure. Distribution of mutations by genes. Distribution of pathogenic or likely pathogenic variants identified in 272 index cases.

62 published cases (5% of published double mutation carriers) were both variants classified as P/LP using current guidelines. In the 13 studies analyzed, there were 835 gene-positive patients. If these patients are pooled, only 0.4% of gene-positive patients would have double mutations according to the current analysis.

Table 1. Comparison Between Clinical Characteristic of Gene-Positive HCM Patients With and Without a Second Rare Variant of Unknown Significance

	Double-Variant Group (n=24)*	Single-Variant Group (n=140)	*P* Value
Age at diagnosis, y (median±SD)	33±15	40±14	0.09
Follow-up period, y (mean±SD)	17.7±16.7	15.2±38	0.84
MLVWT, mm (median±SD)	22±6	20±4	0.11
Arrhythmic end points, n (%)†	2 (8)	7 (5)	0.62
End-stage HCM, n (%)‡	2 (8)	2 (1)	0.1
Septal reduction, n (%)§	2 (8)	29 (21)	0.26
Atrial fibrillation, n (%)	3 (12.5)	36 (26)	0.2

HCM indicates hypertrophic cardiomyopathy; ICD, implanted cardioverter-defibrillator; LV, left ventricular; and MLVWT, maximal LV wall thickness.

*One pathogenic or likely pathogenic variant and a second rare variant of unknown significance in a gene encoding for a sarcmoeric protein (see Methods section).

†Defined as sudden cardiac death, resuscitated cardiac arrest, or appropriate ICD therapy.

‡Defined as a decline in LV ejection fraction to <50% attributed to progression of HCM and in the absence of an alternative explanation.

§Myectomy or alcohol septal ablation.

Table 2. Prevalence of Double Mutations* as Published in the Literature

Study	Country	N (Entire Cohort)	No. of Genes Analyzed	Gene Positive, %	Double Mutations, % (Out of Gene Positive According to Publication)
Richard et al[15]	France	197	9	63	6
Erdmann et al[16]	Germany	108	6	33	3
Van Driest et al[7]	United States	389	8	38	7
Ingles et al[17]	Australia	80	7	29	17
Girolami et al[18]	Italy	88	3	57	10
García-Castro et al[19]	Spain	120	5	27	6
Andersen et al[20]	Denmark	90	10	36	19
MIllat et al[28]	France	192	4	48	10
Kubo et al[21]	Japan	93	5	30	3
Bashyam et al[22]	India	55	2	35	11
Curila et al[23]	Czech Republic	100	4	40	10
Otsuka et al[24]	Japan	112	8	44	7
Liu et al[25]	China	136	3	27	11
Berge et al[26]	Norway	696	6	29	6
Chiou et al[27]	Taiwan	38	8	34	8
Pooled data†	...	2494	...	37	8

*Double heterozygote, compound heterozygote, or homozygote mutations.
†Note that some studies used limited genetic panels. See text for more details.

Case Reports and Series

Seventy-two cases with published double mutations were described in 25 publications,[29–53] including 20 cases from one large pedigree[29] (Table 3). Of these, 32 cases, including 20 from the large pedigree, had 2 variant classified as P/LP according to current American College of Medical Genetics guidelines. In 3 cases, 1 of the variants was later published as having an MAF >0.1%. In the remaining 36 cases, at least 1 variant would currently be classified as a VUS. In the Amish pedigree,[29] including 20 homozygote cases for a splice site variant in *MYBPC3*, all patients demonstrated an especially severe disease with diagnosis during the neonatal period and death or cardiac transplant during the first year of life. Similar severe disease expression was demonstrated in all of the 7 other cases who were homozygotes or compound heterozygotes for radical mutations (nonsense, frameshift, or splice site) in *MYBPC3*. Five cases from 3 families were found to have 2 P/LP variants according to current classification. These cases were all diagnosed during childhood or adolescence, and in 4 of them, serious adverse outcomes were published (3 cases of SCD and 1 heart transplant because of progression to end-stage HCM). In the remaining cases, in which at least 1 variant was classified as VUS, the phenotype was more variable as detailed in Table 3. Finally, we revisited the data on the presence of 2 disease-causing variants as a risk marker for adverse cardiac events. Only one study systematically evaluated double mutations as an independent risk marker for arrhythmic events, analyzing data of 18 patients with double mutations from 3 large centers. In 3 cases, SCD or resuscitated cardiac arrest occurred in the absence of any known risk factors for these end points.[10] Re-examining the variants published demonstrated that since the study's publication in 2012, one of the variants in 2 of these cases has been found to be relatively common in certain populations. The reported *MYBPC3* Gln998Glu variant was found in 9% of 1000 alleles in a Latino population and in 3% of 1336 alleles in an East Asian population. The *MYBPC3* Arg326Gln variant was found in 1.5% of 4954 Finnish alleles. In our literature search, 11 of the case reports found had arrhythmic end points (Table 3). In 8 of these cases, diagnosis was made during childhood/adolescence; in 2 of the cases, age of diagnosis was not published; and in 1 case, arrhythmias developed after progression to end-stage HCM. Maximal left ventricular wall thickness ranged from 17 to 38 with only 1 case with maximal left ventricular wall thickness >30 mm. Complete data on SCD risk factors in these cases was unavailable.

Double mutations as a risk factor for progression to end-stage HCM were systematically analyzed in 2 studies. Garcia-Pavia et al[54] analyzed data of 26 patients from 22 families who underwent heart transplantation for end-stage HCM. They found 3 patients from 2 families who were homozygotes for a P/LP variant. Biagini et al[9] found that 13 out of 156 patients (13%) with end-stage HCM had >1 pathogenic variant; however, the details of variants were not available for evaluation.

Discussion

In the current study, we looked in our large HCM cohort (n=1411) for patients with 2 P/LP variants using contemporary guidelines for variant classification.[13] In 25 patients (9% of gene-positive patients), 2 rare variants in genes encoding for sarcomeric proteins were identified. This is in the range of

Table 3. Hypertrophic Cardiomyopathy Cases With DMs Described in the Literature

Variant I	Variant II	DM Type	Index Case	Sex	Age at Symptom Onset	MLVWT	Remarks	Reference
MYBPC3 c.3330+2T>G*†	*MYBPC3* c.3330+2T>G*†	Hm	…	…	0–3 wk	NA	Neo 20 cases: 2 received heart transplant. 18 died <1 y	29
MYBPC3 c.2373dupG p.Trp792fs*†	*MYBPC3* c.2373dupG p.Trp792fs*†	Hm	Y	F	7 wk	NA	Neo death at 12 wk	30
MYBPC3 c.2827C>Tp. Arg943ter*†	*MYBPC3* c.2827C>Tp. Arg943ter*†	Hm	Y	F	6 wk	11	Neo death at 7 wk	30
MYBPC3 c.2373dupG p.Trp792fs*†	*MYBPC3* c.2827C>Tp. Arg943ter*†	CHt	Y	M	5 wk	12	Neo death at 12 wk	30
MYBPC3 c.2373dupG p.Trp792fs*†	*MYBPC3* c.2827C>Tp. Arg943ter*†	CHt	Y	M	4 wk	NA	Neo death at 12 wk	30
*MYBPC3*c.2373dupG p.Trp792fs*†	*MYBPC3*c.1624+1G>A*†	CHt	Y	F	2 d	17	Neo death at 5 wk	31
*MYBPC3*c.3288delA p.Glu1096fs*†	*MYBPC3* c. 2827C>T p.Arg943ter*†	CHt	Y	M	2 wk	NA	Neo biventricular hypertrophy. Death at 6 wk	31
MYBPC3 c.2414-1G>A*†	*MYBPC3* c.772G>A p.Glu258Lys (ss)*†	CHt	Y	F	Birth	NA	Neo+Dysmorphic features. Death at 4 wk	32
*MYBPC3*c.1624G>C p.Glu542Gln (ss)*†	*MYBPC3*c.1504C>T p.Arg502Trp*	CHt	Y	M	2	26	…	33
*MYBPC3*c.1624G>C p.Glu542Gln (ss)*†	*MYBPC3*c.1504C>T p.Arg502Trp*	CHt	N	M	5	17	Ar SCD (age not specified)	33
*TNNT2*c.328T>A p.Phe110Ile*	*TNNT2*c.388C>T p.Arg130Cys*	CHt	Y	F	12	NA	Ar rCA at 12 y	34
*TNNT2*c.328T>A p.Phe110Ile*	*TNNT2*c.388C>T p.Arg130Cys*	CHt	N	M	14	NA	Ar SCD at 14 y	34
*MYH7*c. 1207C>T p.Arg403Trp*§	*MYH7*c.1207C>T p.Arg403Trp§	Hm	NA	M	Adolescence	20	ES progression to end-stage HCM at 35 y and heart transplant at 39 y	35
*MYPBC3*c.2067+1G>A*†	*MYBPC3* c.2285T>A p.Val762Asp	CHt	Y	F	20¶	16	…	36
MYPBC3 c.2067+1G>A*†	*MYBPC3* c.2285T>A p.Val762Asp	CHt	N	M	52¶	16	ES developed end-stage HCM	36
*MYBPC3*c.3776delA p.Gln1269fs*†	*MYBPC3* c.3599T>C p.Leu1200Pro	CHt	Y	M	11 days	14	Neo+LVNC features. Death at 9 wk	37
MYBPC3 c.772G>A p.Glu258Lys (ss)*†	*MYBPC3* c.1321G>A p.Glu441Lys	CHt	Y	M	21	22	ES progression to end-stage HCM at 40 y	38
*MYBPC3*c.109G>T p.Gly37ter*†	*MYBPC3* c.478C>T p.Arg160Trp‡	CHt	NA	M	25	26	…	39
*MYBPC3*c.1504C>T p.Arg502Trp*	*MYPBC3* c.2573C>A p.Ser858Asn	CHt	Y	F	6	13	…	33
*MYBPC3*c.1504C>T p.Arg502Trp*	*MYBPC3* c.442G>Ap. Gly148Arg	CHt	N	F	41	9	…	33
*MYBPC3*c.1504C>T p.Arg502Trp*	*MYBPC3* c.442G>Ap. Gly148Arg	CHt	N	F	12	38	Ar VT	33
*MYBPC3*c.1504C>T p.Arg502Trp*	*MYBPC3* c.442G>Ap. Gly148Arg	CHt	Y	M	6	20	Ar CA at 10 y	33
*MYBPC3*c.1504C>T p.Arg502Trp*	*MYBPC3* c.442G>Ap. Gly148Arg	CHt	N	F	8	11	…	33
*MYPBC3*c.2459G>A p.Arg820Gln*	*MYBPC3* c.2285T>A p.Val762Asp	CHt	Y	F	16¶	11	…	36

(*Continued*)

Table 3. Continued

Variant I	Variant II	DM Type	Index Case	Sex	Age at Symptom Onset	MLVWT	Remarks	Reference
*MYH7*c.2609G>A p.Arg870His*	*MYH7* c.160C>T p.Arg54ter‖	CHt	Y	M	16	25	…	40
*MYH7*c.2155C>T p.Arg719Trp*	*MYH7* c.1046T>C p.Met349Thr	CHt	Y	M	6	18	Ar rCA at 6 y	41
*MYH7*c. 1207C>T p.Arg403Trp*	*MYH7* c.1358G>A p.Arg453His	CHt	Y	M	9	29	ES+RVH. AF at 21 y. End-stage HCM at 23 y.	42
*MYH7*c.1988G>A p.Arg663His*	*MYH7*c.632C>T p.Pro211Leu	CHt	Y	M	56	20	ES AF and progression to end-stage HCM at 76 y	43
*TNNI3*c.470C>T p.Ala157Val*	*TNNI3* c.235C>T p.Arg79Cys‡	CHt	Y	F	8	29	…	44
*TNNI3*c.470C>T p.Ala157Val*	*TNNI3* c.235C>T p.Arg79Cys‡	CHt	N	M	NA	28	…	44
*MYBPC3*c.1504C>T p.Arg502Trp*	*MYH7* c.1759G>A p.Asp587Asn	DHt	Y	F	1	20	Ar rCA at 13 y	33
*MYBPC3*c.1504C>T p.Arg502Trp*	*MYH7* c.1759G>A p.Asp587Asn	DHt	Y	F	18	18	…	33
*TNNT2*c.328T>A p.Phe110Ile*	*MYPBC3* c.2285T>A p.Val762Asp	DHt	Y	F	58¶	24	…	36
*TNNT2*c.328T>A p.Phe110Ile*	*MYPBC3* c.2285T>A p.Val762Asp	DHt	N	F	Phe(–)→	9	→Age at last clinical evaluation not specified	36
*MYH7*c.1988G>A p.Arg663His*	*CSRP3* c.50insGCAGATTTCTT p.Tyr18fs	DHt	Y	M	30	25	AF (age not specified)	45
*MYH7*c.1988G>A p.Arg663His*	*CSRP3* c.50insGCAGATTTCTT p.Tyr18fs	DHt	N	M	NA	21	AF (age not specified)	45
*MYH7*c.1988G>A p.Arg663His*	*CSRP3* c.50insGCAGATTTCTT p.Tyr18fs	DHt	N	F	Phe(–) at 19 y	NA	…	45
*MYBPC3*c.772+5G>A	*MYBPC3*c.772+5G>A	Hm	Y	F	34	24	…	46
*MYBPC3*c.772+5G>A	*MYBPC3*c.772+5G>A	Hm	N	M	34	26	ES progressed to end-stage HCM	46
*MYBPC3*c.1880C>T p.Ala627Val	*MYBPC3*c.1880C>T p.Ala627Val	Hm	Y	M	17	28	…	47
MYBPC3 c.1469G>T p.Gly490Val	*MYBPC3* c.1469G>T p.Gly490Val	Hm	Y	M	21	18	…	48
MYBPC3 c.1469G>T p.Gly490Val	*MYBPC3* c.1469G>T p.Gly490Val	Hm	N	M	19	15	…	48
*MYH7*c.619A>C p.Lys207Gln	*MYH7*c.619A>C p.Lys207Gln	Hm	Y	M	47	21	ES+Ar developed HF and AF+appropriate ICD interventions	43
*MYH7*c.2803G>A p.Glu935Lys	*MYH7*c.2803G>A p.Glu935Lys	Hm	Y	M	25	26	ES progressed to end-stage HCM and died of HF at 31 y	49
*MYH7*c.2803G>A p.Glu935Lys	*MYH7*c.2803G>A p.Glu935Lys	Hm	N	M	NA	26	Ar SCD at 34 y	49
MYH7 c.2605C>G p.Arg869Gly	*MYH7* c.2605C>G p.Arg869Gly	Hm	NA	M	17	17	ES AF at 17 y. Stroke at 28 y. Progressed to end-stage HCM at 33 y	50
MYH7 c.2605C>G p.Arg869Gly	*MYH7* c.2605C>G p.Arg869Gly	Hm	NA	F	12	38	ES AF at 17 y. Possible progression to end-stage HCM at 37 y	50

(*Continued*)

Table 3. Continued

Variant I	Variant II	DM Type	Index Case	Sex	Age at Symptom Onset	MLVWT	Remarks	Reference
*TNNI3*c.484C>T p.Arg162Trp	*TNNI3*c.484C>T p.Arg162Trp	Hm	Y	F	17	14	+LVNC features	51
*TNNI3*c.484C>T p.Arg162Trp	*TNNI3*c.484C>T p.Arg162Trp	Hm	N	M	15	25	Ar rCA at 15 y	51
*TNNT2*c.536C>T p.Ser179Phe	*TNNT2*c.536C>T p.Ser179Phe	Hm	N	M	NA	25	Ar+RVH. SCD at 17 y.	52
*MYL3*c.427G>A p.Glu143Lys	*MYL3*c.427G>A p.Glu143Lys	Hm	Y	M	11	25	…	53
*MYL3*c.427G>A p.Glu143Lys	*MYL3*c.427G>A p.Glu143Lys	Hm	N	M	12	NA	Died at 14 y during operation for removal of intracardiac thrombus	53
*MYBPC3*c.1807A>G p.Ile603Val	*MYBPC3* c.2429G>A p.Arg810His	CHt	NA	M	20	28	…	39

The minor allele frequencies of all variants in this table are detailed in Table II in the Data Supplement. Ar (arrhythmic end point [resuscitated cardiac arrest or sudden cardiac death]), ES (progression to end-stage HCM), and Neo (presentation during the neonatal period or early infancy) are variants classified according to current guidelines of the American College of Medical Genetics[15] as pathogenic or likely pathogenic—see Methods section for details. Underlined are radical mutations (nonsense, frameshift, or ss). Variants given in parentheses are variants with minor allele frequency> 0.1%. AF indicates atrial fibrillation; CHt, compound heterozygote; DHt, double heterozygote; DM, double mutation; HF, heart failure; Hm, homozygote; ICD, implanted cardioverter-defibrillator; LVNC, left ventricular noncompaction; Phe(–), phenotype negative; rCA, resuscitated cardiac arrest; RVH, right ventricular hypertrophy; SCD, sudden cardiac death; ss, splice site; and VUS, variant of unknown significance.

*Variants classified according to current guidelines of the American College of Medical Genetics15 as pathogenic or likely pathogenic. See the Methods section for details.

†Radical mutations (nonsense, frameshift, or splice site).

‡Variants with minor allele frequency >0.1%.

§This case was published as a triple mutation (homozygous *MYH7*+heterozygous *MYBPC3* mutations). However, the *MYBPC3* variant was later reclassified as benign because of presence in 10% of Finnish chromosomes included in the Exome Aggregation Consortium database.

¶Age at diagnosis.

‖A nonsense VUS can be expected to be a strong modifier in the presence of compound heterozygosity such as this.

previously published prevalence of double mutations in HCM cohorts (3% to 19%; Table 2) despite the fact that the majority of our cases included only 1 P/LP variant and a second VUS. In fact, only in a single case were both variants classified as P/LP, yielding a prevalence of double mutations of merely 0.4%. The probable reason for this low prevalence is the stricter criteria used in the current study. To investigate this, we reanalyzed all available variants published in double mutation cohort studies according to current American College of Medical Genetics guidelines. This analysis demonstrated that only 3 of 62 patients (5%) previously described as harboring double mutations would currently have both variants classified as P/LP. Regarding only these patients as having double mutations and pooling the data from the analyzed studies yielded a new prevalence of 0.4%, similar to that in our cohort. In 13 of these 62 patients (21%), at least 1 variant has been found in >0.1% of alleles in certain populations. Although such prevalence does not exclude these variants from having an impact on disease expression, it casts significant doubt on their pathogenicity, especially in the context of these patients harboring another P/LP variant. Taken together, these data suggest that the prevalence of double mutation carriers in HCM is much lower than that previously estimated.

These findings also call into question the validity of previously described genotype–phenotype correlations in relation to double mutations because a substantial number of previously published double mutation cases probably include at least 1 VUS. We, therefore, analyzed our patients with 1 P/LP variant and 1 VUS to evaluate whether a second rare variant, even if classified as a VUS, may be a marker of adverse prognosis. Although these patients had a trend toward younger age of diagnosis when compared with a group with single mutations, all other parameters were found to be similar (Table 1).

Finally, we analyzed the information gathered from our cohort and the case reports and series found in our literature search regarding patients with 2 variants classified as P/LP. Such an analysis is obviously limited by publication bias; however, several observations could tentatively be made as follows:

Double Mutations as a Risk Marker for SCD

The hypothesis that the presence of 2 disease-causing variants is an independent risk marker for SCD is mainly based on a single study that systematically evaluated all double mutation cases from 3 large centers. It included 3 cases of SCD or resuscitated cardiac arrest in the absence of any conventional risk markers.[10] Since this study's publication, however, one of the variants in 2 of the 3 cases were found to be relatively common in certain populations (MAF > 1.5%) and are, therefore, unlikely to be truly P/LP. Another 11 cases with double mutations and arrhythmic end points have been published in the literature; however, only in 3 were both variants classified as P/LP according to our current analysis (Table 3). Complete details on

SCD risk factors were not available in all cases, and, therefore, further analysis could not be performed. Accordingly, current data seems insufficient to support or refute the presence of a double mutation as an independent risk marker for SCD.

Compound/Homozygote Radical *MYBPC3* Mutations

All cases with 2 radical *MYBPC3* mutations (either homozygotes or compound heterozygotes) had an extremely severe phenotype with death of heart failure or heart transplant during the first year of life (Table 3). In these cases, no normal protein is produced, and little, if any, truncated proteins are expected to be incorporated into the sarcomere. Accordingly, severe phenotypic expression may be expected. Indeed, studies using *MYBPC3* knockout[55] or homozygote[56,57] mice have also demonstrated development of severe disease. It is, therefore, reasonable to conclude that double radical *MYBPC3* mutations are associated with a poor prognosis.

Of note, in both animal models[56,57] and some of the case reports with double radical *MYBPC3* mutations,[29–31] severe systolic dysfunction and ventricular dilatation were described as were signs of noncompaction.[30] Mechanisms responsible for this apparent phenotypic overlap with other cardiomyopathies are incompletely understood.

Other Double Mutations

All of the remaining 5 cases found in the literature with 2 P/LP variants according to analysis had a severe phenotype with presentation during infancy or childhood. In 4 of these cases, arrhythmic end points or progression to end-stage HCM occurred according to published data (Table 3). In the systematic analysis of our cohort, the single double mutation case was not mild as demonstrated by history of myectomy, development of atrial fibrillation, and progression to end-stage HCM in the 6th decade of life. Nevertheless, it was not as severe as the above-mentioned cases. Accordingly, these data lend support to the gene-dose effect in HCM but should be regarded with caution because of the paucity of information and possible publication bias.

Clinical Implication

Genotype–phenotype correlations may offer an opportunity to give more accurate information to patients on their prognosis and to tailor the patient's management accordingly. Unfortunately, the data presented in this study indicate that the evidence supporting the gene-dose effect in HCM is scarcer than previously thought. This is not to say that the gene-dose effect is not valid in HCM. Indeed, studies using animal models have clearly shown that specific double mutations are associated with a more severe phenotype.[56–58] Nevertheless, the combined effect of different double mutations is likely to be extremely variable and difficult to predict. Accordingly, using the presence of any double mutation as an independent prognostic marker should be done with caution and on a case-by-case basis. Extra caution may be warranted in double heterozygote cases as, except for the case from our cohort described here, only 7 other such cases were described in detail, and in none of these were both variants classified as P/LP according to the current analysis (Table 3).

The utilization of double mutations as a stand-alone marker for SCD risk stratification is of specific concern because this may be translated into implantation of implanted cardioverter-defibrillators for primary prevention. Although reanalysis of published data on this subject does not refute this specific genotype–phenotype correlation, it does demonstrate that there is minimal data to support it.

One exception may be the finding of double radical *MYBPC3* mutations because all cases published clearly had a severe phenotype. In these cases, the use of the genetic findings for prognostication was of limited value because the poor prognosis was clear at time of diagnosis. Nevertheless, such genetic information may be helpful for prenatal and preimplantation consult in rare cases of known HCM in both of the parents' families.

Finally, in our cohort, all P/LP variants were identified in 8 sarcomeric genes found in limited gene panels (Figure) despite the fact that more extended panels were used in 386 patients (27% of our cohort). This finding suggests that such extended panels have a limited incremental value. The previously held view that a significant minority of HCM patients harbor more than 1 mutation was used to support the utilization of larger panels in search of a second and perhaps third pathogenic variant. Our finding that, in fact, double mutations are rare undermines this view.

Limitations

Our clinic does not follow pediatric patients. Therefore, most severe cases with high mortality rates during childhood were under-represented in our cohort. Furthermore, for some of our patients and in some of the cohort studies, a limited gene panel was used. It is, therefore, possible that the prevalence of double mutations is slightly higher than that estimated in the current analysis. Nevertheless, because even limited panels are expected to identify the majority of mutations and because double mutation cases were rare even in cohort studies including pediatric cases, it is unlikely that these limitations have influenced significantly the overall conclusions.

Data on cosegregation was limited to that published. In some cases, cosegregation data may have been available to authors of the articles in our literature search but not for the current analysis. It is possible that some variants could have been classified as P/LP if such data were available. However, cosegregation data in families with multiple variants require screening of more family members than usual for conclusions regarding pathogenicity to be reached. Therefore, it is likely that such missing data are available for only few cases and is unlikely to have had a major impact on our study's conclusions.

The number of patients with 2 rare variants in our cohort was limited. It is possible that data derived from a larger cohort would uncover differences between this group and patients with a single rare variant. Therefore, our conclusion that a second rare variant (even if classified as a VUS) does not portend worse prognosis requires corroboration by larger studies.

Conclusions

The data from our cohort and reanalysis of published literature demonstrates that the prevalence of double mutations

in HCM is likely to be much lower than that previously estimated. It also shows that data supporting the association of double mutations with less favorable prognosis are limited. Therefore, the use of double mutations as a prognostic marker in HCM should be done with caution. Specifically, there is almost no data to support the use of such genetic information as a stand-alone indication for implanted cardioverter-defibrillator implantation for primary prevention. In the subset of patient with double radical *MYBPC3* mutations, available data do support an association with extremely severe disease manifestation. Such data may be considered for prenatal and preimplantation diagnoses.

Sources of Funding

Dr Fourey and Dr Adler were funded by the Richard and Edith Strauss Canada Foundation and the Halpern HCM Research Fund. Dr Siminovitch is funded by an Ontario Research Fund award (RE01-061), holds a Canada Research Chair award, and is the Sherman Family Chair in Genomic Medicine.

Disclosures

None.

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CLINICAL PERSPECTIVE

In the current study, we demonstrate that when using contemporary guidelines for variant classification, only 0.4% of gene-positive hypertrophic cardiomyopathy (HCM) patients have >1 pathogenic or likely pathogenic genetic variant. This is much lower than 3% to 19% prevalence of multiple mutations described in the literature. Reclassification of variants in previously published studies demonstrates that only a small minority (5%) would be classified as double mutation using current guidelines. Examining in this new light previously characterized genotype–phenotype associations demonstrated that the conclusions on worsened outcomes in HCM patients with double mutations are based on a limited amount of data. Although data are sufficient to support the validity of the gene-dose effect in HCM, the paucity of evidence calls for caution when translating this to the bedside. Specifically, there is virtually no evidence to support double mutations as an independent risk factor for sudden cardiac death. Similarly, there are almost no cases describing double heterozygote mutations in HCM, making conclusions on such cases difficult. One exception is the presence of compound heterozygosity or homozygosity with 2 radical variants (nonsense, frameshift, or splice site) in *MYBPC3* as such cases were repeatedly shown to have an extremely poor prognosis. These data may be used for prenatal or preimplantation consult in rare cases with HCM on both paternal and maternal sides of the family.

Circulation
Genomic and Precision Medicine

Prevalence and Clinical Implication of Double Mutations in Hypertrophic Cardiomyopathy: Revisiting the Gene-Dose Effect

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Circ Cardiovasc Genet. 2017;10:
doi: 10.1161/CIRCGENETICS.116.001685
Circulation: Cardiovascular Genetics is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Print ISSN: 1942-325X. Online ISSN: 1942-3268

Supplemental material

Supplemental table 1. Proposed cosegregation evidence to support each ACMG-AMP pathogenicity evidence level

	Single family	>1 family
Strong evidence	≤ 1/32	≤ 1/16
Moderate evidence	≤ 1/16	≤ 1/8
Supportive evidence	≤ 1/8	≤ 1/4

Probability of observed cosegregation if not pathogenic totaled over all families.

ACMG-AMP- American College of Medical Genetic and Genomics and Association of Molecular Pathology

Adapted with permission from Jarvik et al.[19]

Supplemental table 2. Minor allele frequency of genetic variants described in double mutation cases in the literature

Variant	Minor allele frequency
CSRP3 c.50insGCAGATTTCTT p.Tyr18fs	<0.0001
MYBPC3 c.109G>T p.Gly37ter	0
MYBPC3 c.442G>Ap.Gly148Arg	<0.0001
MYBPC3 c.478C>T p.Arg160Trp	0.0016 (0.049 in 700 Latino alleles)
MYBPC3 c.772G>A p.Glu258Lys	0
MYBPC3 c.772+5G>A	0
MYBPC3 c.1321G>A p.Glu441Lys	0.00016
MYBPC3 c.1504C>T p.Arg502Trp	<0.0001
MYBPC3 c.1469G>T p.Gly490Val	0
MYBPC3 c.1624G>C p.Glu542Gln	<0.0001 (0.00014 in 7284 African Alleles)
MYBPC3 c.1624+1G>A	<0.0001
MYBPC3 c.1807A>G p.Ile603Val	0
MYBPC3 c.1880C>T p.Ala627Val	0
MYPBC3 2067+1G>A	0
MYBPC3 c.2285T>A p.Val762Asp	0
MYBPC3 c.2373dupG p.Trp792fs	<0.0001
MYBPC3 c.2414-1G>A	0
MYBPC3 c.2429G>A p.Arg810His	<0.0001
MYPBC3 c.2459G>A p.Arg820Gln	<0.0001
MYPBC3 c.2573C>A p.Ser858Asn	0
MYBPC3 c.2827C>Tp.Arg943ter	<0.0001
MYBPC3 c.3288delA p.Glu1096fs	0
MYBPC3 c.3330+2T>G	0
MYBPC3 c.3599T>C p.Leu1200Pro	0
MYBPC3 c.3776delA p.Gln1269fs	0
MYH7 c.160C>T p.Arg54ter	0
MYH7 c.619A>C p.Lys207Gln	0
MYH7 c.632C>T p.Pro211Leu	<0.0001
MYH7 c.1046T>C p.Met349Thr	0
MYH7 c. 1207C>T p.Arg403Trp	0
MYH7 c.1358G>A p.Arg453His	0
MYH7 c.1759G>A p.Asp587Asn	0
MYH7 c.1988G>A p.Arg663His	<0.0001
MYH7 c.2155C>T p.Arg719Trp	0
MYH7 c.2605C>G p.Arg869Gly	0
MYH7 c.2609G>A p.Arg870His	0
MYH7 c.2803G>A p.Glu935Lys	0
MYL3 c.427G>A p.Glu143Lys	0
TNNI3 c.235C>T p.Arg79Cys	0.0004 (0.005 in 8052 East Asian alleles)
TNNI3 c.470C>T p.Ala157Val	0
TNNI3 c.484C>T p.Arg162Trp	<0.0001 (0.00012 in 8604 East Asian alleles)
TNNT2 c.328T>A p.Phe110Ile	0
TNNT2 c.388C>T p.Arg130Cys	0
TNNT2 c.536C>T p.Ser179Phe	0

Minor allele frequency according to the Exome Aggregation Consortium data.