A Next-Generation Sequencing Approach to Identify Gene Mutations in Early- and Late-Onset Hypertrophic Cardiomyopathy Patients of an Italian Cohort

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Academic Editor: William Chi-shing Cho

Received: 16 June 2016; Accepted: 22 July 2016; Published: 30 July 2016

Abstract: Sequencing of sarcomere protein genes in patients fulfilling the clinical diagnostic criteria for hypertrophic cardiomyopathy (HCM) identifies a disease-causing mutation in 35% to 60% of cases. Age at diagnosis and family history may increase the yield of mutations screening. In order to assess whether Next-Generation Sequencing (NGS) may fulfill the molecular diagnostic needs in HCM, we included 17 HCM-related genes in a sequencing panel run on PGM IonTorrent. We selected 70 HCM patients, 35 with early (<25 years) and 35 with late (≥65 years) diagnosis of disease onset. All samples had a 98.6% average of target regions, with coverage higher than 20× (mean coverage 620×). We identified 41 different mutations (seven of them novel) in nine genes: MYBPC3 (17/41 = 41%); MYH7 (10/41 = 24%); TNNT2, CAV3 and MYH6 (3/41 = 7.5% each); TNNI3 (2/41 = 5%); GLA, MYL2, and MYL3 (1/41=2.5% each). Mutation detection rate was 30/35 (85.7%) in early-onset and 8/35 (22.9%) in late-onset HCM patients, respectively (p < 0.0001). The overall detection rate for patients with positive family history was 84%, and 90.5% in patients with early disease onset. In our study NGS revealed higher mutations yield in patients with early onset and with a family history of HCM. Appropriate patient selection can increase the yield of genetic testing and make diagnostic testing cost-effective.

Keywords: genetics; gene variants; hypertrophic cardiomyopathy; next-generation sequencing

1. Introduction

Hypertrophic cardiomyopathy (HCM) is a common genetic cardiac disease that affects one out of 500 individuals from the general population [1]. It is a clinically variable and genetically heterogeneous disease. In fact, more than 20 genes were related with HCM and a total number of about 1400 distinct mutations were identified in affected patients [2]. The most frequently encountered mutations fall within myosin heavy chain 7 (MYH7) and myosin binding protein C (MBPC3) [3,4]. Sequencing of
sarcomere protein genes in patients fulfilling clinical diagnostic criteria identifies a disease-causing mutation in only 35% to 60% of cases [5–8]. Identification of an HCM-causing mutation is an important step in the disease’s clinical management, not only to better support the clinical diagnosis in the proband but also to either exclude or confirm the presence of disease-causing mutations in other family members.

Considering the extreme genetic heterogeneity of the disease and the cost of genetic testing, several attempts were made to identify the clinical predictors of an underlying mutation [9–11]. In a large study of HCM patients genotyped for mutations in nine genes, the presence of a set of five clinical markers, including age at diagnosis <45 years, accounted for an 80% likelihood of positive genetic testing [11].

In addition, more reliable, precise, and possibly not time-consuming molecular diagnostic approaches are needed. In this regard, Next-Generation Sequencing (NGS), which has already been applied for the diagnosis of hereditary cardiovascular conditions as well as of other diseases [12–16], may represent a suitable tool. Targeted gene panels were shown to generate results with analytical quality identical to Sanger sequencing, and to have the advantage of being faster and cheaper with better coverage and sensitivity than that used in more expanded analyses.

The purpose of the present study was to analyse the yield of NGS applied to the genetic screening of a well-phenotyped Italian HCM cohort, composed of patients with both early- and late-onset diagnosis, also including patients with positive family history, and to explore the ability of NGS to accomplish the molecular diagnostic needs in clinical practice.

2. Results

2.1. Description of Study Population

The clinical characteristics of patients enrolled in the study are shown in Table 1A. The patients were divided into two subgroups of 35 patients each, depending on the age at HCM diagnosis: the early-onset (EO) group with a mean age at diagnosis of 18.6 ± 8.5 years and the late-onset (LO) group with a mean age at diagnosis of 70.4 ± 4.8 years. The number of patients with a positive family history for HCM was significantly higher in the EO group (p = 0.0001) (Table 1B). Thirty-four patients were women and 36 were men. The sex distribution of patients was different in the two subgroups, with more males in the EO group (p = 0.0001). The left atrium size was significantly different in the two groups (p = 0.0001), with LO patients more frequently exhibiting left atrial enlargement. The obstructive form of HCM was less frequently observed in the EO as compared to the LO group (p = 0.03). Evolution of the disease towards end stage (left ventricular ejection fraction <50%) was observed only in the EO group. None of the other clinical features considered in the study was significantly different between the two groups.
Table 1. (A) Clinical characteristics of HCM patients with early or late onset of disease; (B) Familial vs. sporadic HCM.

(A)

| Variables                      | Early-Onset n = 36 | Late-Onset n = 35 | p    |
|--------------------------------|--------------------|-------------------|------|
| Age at diagnosis (years)       | 18.6 ± 8.5         | 70.4 ± 4.8        | 0.0001 |
| Male                           | 27 (77.1)          | 9 (25.7)          | 0.0001 |
| LV obstruction                 | 14 (40)            | 24 (68.6)         | 0.03  |
| Family history of HCM          | 21 (60)            | 4 (11.4)          | 0.0001 |
| NYHA functional class I        | 24 (68.6)          | 4 (11.4)          |      |
| II                             | 9 (25.7)           | 25 (71.4)         | 0.0001 |
| III                            | 2 (5.7)            | 6 (17.1)          |      |
| Unexplained syncope            | 5 (14.3)           | 6 (17.1)          | 1    |
| Non sustained ventricular      | 6 (24)             | 5 (22.7)          | 1    |
| atrial tachycardia             |                    |                   |      |
| Left atrial dimension (mm)     | 39.3 ± 6.2         | 45 ± 4.5          | 0.0001 |
| Maximal LV wall thickness (mm) | 21.4 ± 6.2         | 18.7 ± 2.6        | 0.02  |
| Late gadolininium enhancement  | 24/29 (82.8)       | 9/19 (47.4)       | 0.01  |
| Atrial fibrillation            | 11 (31.4)          | 10 (28.6)         | 1    |
| End stage disease              | 4 (11.4)           | 0 (0)             | 0.11  |
| Myectomy                       | 2 (5.7)            | 0 (0)             | 0.49  |
| ICD implantation               | 12 (34.3)          | 2 (5.7)           | 0.006 |
| Death                          | 0 (0)              | 1 (2.9)           | 1    |

(B)

| Patients                      | All n = 70 | EO n = 35 | LO n = 35 | p    |
|--------------------------------|------------|-----------|-----------|------|
| Familial HCM                  | 25 (36)    | 21 (60)   | 4 (14.4)  | 0.0001 |
| Sporadic HCM                  | 45 (64)    | 14 (40)   | 31 (88.6) | 0.0001 |

In (A): Continuous variables are expressed as mean ± SD. Qualitative variable are expressed as n (%). HCM: hypertrophic cardiomyopathy; NYHA: New York Functional Class; LV: left ventricular; ICD: implantable cardioverter defibrillator; In (B): Variable are expressed as n (%); EO: early-onset; LO: late-onset.

2.2. Sequencing

The coding region of each of the 17 HCM phenotype causative genes included in the HCM panel was sequenced on Personal Genome Machine (PGM) Ion Torrent sequencer. The 17 genes included in the HCM panel used for this analysis are shown in Table 2. Sequencing produced an average of 240,000 reads per patient; the mean read length was 130 bp; the average read depth per sample was 620 × with a mean percentage of reads on target of 93.77%; the mean percentage of regions of interest (ROI) covered at least by 20 × was 98.6%, and that covered at least by 100 × was 94.7%. Details of the sequencing metrics for each patient are reported in Table 3.

Two hundred eighty-two variants were identified within the 17 genes analysed: two were ins/del, 175 were intronic, 37 missense, 59 synonymous, five splicing, and four stop mutations. After filtration, 41 variants with a possible clinical effect were selected and confirmed by Sanger sequencing (data not shown). These variants were located in nine of the 17 genes: MYBPC3 (17/41 = 41%); MYH7 (10/41 = 24%); troponin T2 (TNNT2), caveolin 3 (CAV3), and myosin heavy chain 6 (MYH6) (3/41 = 7.5% each); troponin I 3 (TNNI3) (2/41 = 4.8%); and galactosidase alpha (GLA), myosin light chain 2 (MYL2), and myosin light chain 3 (MYL3) (1/41 = 2.5% each). Thirty-four were known variants, whereas seven were novel. Out of the seven new missense mutations, four had uncertain significance, two were likely pathogenic, and one was likely benign. Considering the 34 known variants, 15 were known to have pathogenic effect, six were likely pathogenic, one was likely benign, and 12 were known registered variants but with unknown clinical significance (Table 4). Mutations in sarcomeric genes accounted for 90% of all identified mutations, with MYBPC3 and MYH7 alone accounting for 65% of all mutations. Considering only mutations in MYBPC3, eight missense mutations and nine truncating mutations were identified (Table 4).
Table 2. Metrics of the 17 genes included into the HCM panel.

| #No. | Gene Name | Ref Seq NCBI | Genomic Location (hg19) | Description | Amplicons | Coverage (%) | Target (bp) | Missed (bp) |
|------|------------|--------------|-------------------------|-------------|-----------|--------------|-------------|-------------|
| 1    | MYBPC3     | NM_000256    | chr11:47352958-47374253 | myosin binding protein C, cardiac | 53        | 100          | 5458        | 105         |
| 2    | MYH7       | NM_000257    | chr14:23881948-23904870 | myosin, heavy chain 7, cardiac muscle, β | 67        | 98           | 7746        | 231         |
| 3    | TPM1       | NM_00101805  | chr15:63334838-63364111 | tropomyosin 1 α chain isoform 7 | 23        | 99.91        | 2245        | 2           |
| 4    | TNNI2      | NM_001001430 | chr1:201328143-201346803 | troponin I type 2, cardiac isoform 1 | 20        | 100          | 2357        | 0           |
| 5    | TNN13      | NM_000363    | chr19:55663137-55669100 | troponin I, cardiac | 10        | 99.9         | 989         | 1           |
| 6    | MYL2       | NM_000432    | chr12:111348626-111358404 | slow cardiac myosin regulatory light chain 2 | 9         | 84.8         | 858         | 46          |
| 7    | MYL3       | NM_000258    | chr3:46899357-46904973 | slow skeletal ventricular myosin alkali light | 9         | 94.6         | 894         | 136         |
| 8    | ACTC1      | NM_005159    | chr5:35080297-35087927 | cardiac muscle α actin 1 proprotein | 13        | 100          | 1440        | 0           |
| 9    | LAMP2      | NM_002294    | chrX:119560004-119603204 | lysosomal-associated membrane protein 2 isoform | 21        | 100          | 2077        | 0           |
| 10   | PRKAG2     | NM_016203    | chr7:151253203-151574316 | AMP-activated protein kinase γ 2 subunit | 26        | 84.3         | 2713        | 426         |
| 11   | GLA        | NM_000169    | chrX:100652779-100663001 | α-galactosidase A precursor | 14        | 100          | 1647        | 0           |
| 12   | MYH6       | NM_002471    | chr14:23851199-23877482 | myosin heavy chain 6 | 66        | 94.52        | 7707        | 422         |
| 13   | TNNC1      | NM_000326    | chr3:52485108-52488057 | tropomyosin C, slow | 8         | 98.2         | 792         | 14          |
| 14   | CSRP3      | NM_003476    | chr11:19203578-19223589 | cysteine and glycine-rich protein 3 | 8         | 100          | 840         | 0           |
| 15   | PLN        | NM_002667    | chr6:11886944-118881586 | phospholamban | 2         | 100          | 210         | 0           |
| 16   | TCAP       | NM_003673    | chr17:37821599-37822806 | telethonin | 5         | 100          | 606         | 0           |
| 17   | CAV3       | NM_033337    | chr3:8775486-8788451 | Homo sapiens caveolin 3 (CAV3), transcript variant 1, mRNA. | 4         | 100          | 558         | 0           |

Gene symbols: TPM1: tropomyosin 1; ACTC1: actin, α, cardiac muscle 1; LAMP2: lysosomal associated membrane protein 2; PRKAG2: protein kinase AMP-activated non-catalytic subunit γ 2; TNNC1: troponin C 1; CSRP3: cysteine and glycine-rich protein 3; PLN: phospholamban; TCAP: telethonin.
Table 3. Patient sequencing metrics.

| Patients | Mapped Reads | Reads on Target (%) | Uniformity (%) | ROI MEAN COVERAGE | ROI ≥ 20 × (%) | n of Amplicons < 20 × | ROI ≥ 100 × (%) | n of Amplicons < 100 × |
|----------|--------------|---------------------|----------------|------------------|----------------|----------------------|-----------------|------------------------|
| EO1      | 178,727      | 92.13               | 93.95          | 459.94           | 98.60          | 5                    | 94.97           | 18                     |
| EO2      | 178,731      | 90.57               | 94.83          | 452.19           | 98.88          | 4                    | 95.53           | 16                     |
| EO3      | 72,440       | 91.78               | 93.75          | 185.71           | 96.93          | 11                   | 83.52           | 59                     |
| EO4      | 247,711      | 90.70               | 93.90          | 627.61           | 99.44          | 2                    | 96.09           | 14                     |
| EO5      | 111,232      | 91.03               | 93.57          | 282.82           | 97.21          | 10                   | 91.62           | 30                     |
| EO6      | 280,419      | 93.08               | 94.15          | 729.08           | 99.16          | 3                    | 96.09           | 14                     |
| EO7      | 623,594      | 92.53               | 93.81          | 1611.77          | 99.44          | 2                    | 98.32           | 6                      |
| EO8      | 561,715      | 97.46               | 92.18          | 1529.12          | 99.44          | 2                    | 97.49           | 9                      |
| EO9      | 77,846       | 93.36               | 93.83          | 203.00           | 96.93          | 11                   | 86.87           | 47                     |
| EO10     | 381,796      | 96.33               | 93.71          | 1027.32          | 99.44          | 2                    | 97.21           | 10                     |
| EO11     | 311,688      | 93.28               | 93.70          | 812.08           | 99.44          | 2                    | 96.09           | 14                     |
| EO12     | 239,783      | 93.00               | 94.15          | 622.93           | 98.88          | 4                    | 95.53           | 16                     |
| EO13     | 276,453      | 93.48               | 94.44          | 721.90           | 99.44          | 2                    | 96.09           | 14                     |
| EO14     | 215,672      | 93.30               | 94.53          | 562.09           | 99.44          | 2                    | 95.81           | 15                     |
| EO15     | 465,323      | 94.73               | 93.01          | 1231.34          | 99.44          | 2                    | 96.65           | 12                     |
| EO16     | 465,619      | 97.25               | 92.65          | 1264.84          | 99.44          | 2                    | 96.93           | 11                     |
| EO17     | 441,220      | 95.42               | 92.96          | 1176.05          | 99.72          | 1                    | 97.49           | 9                      |
| EO18     | 192,373      | 98.07               | 91.50          | 526.97           | 98.60          | 5                    | 94.13           | 21                     |
| EO19     | 313,968      | 95.80               | 93.72          | 840.19           | 99.16          | 3                    | 96.65           | 12                     |
| EO20     | 192,211      | 95.35               | 94.02          | 517.24           | 98.60          | 5                    | 95.81           | 15                     |
| EO21     | 196,251      | 95.05               | 94.02          | 521.07           | 98.88          | 4                    | 95.81           | 15                     |
| EO22     | 303,435      | 96.01               | 93.55          | 813.79           | 98.88          | 4                    | 96.65           | 12                     |
| EO23     | 322,467      | 94.14               | 92.17          | 847.94           | 98.88          | 4                    | 95.81           | 15                     |
| EO24     | 253,552      | 95.97               | 91.35          | 679.21           | 98.88          | 4                    | 95.53           | 16                     |
| EO25     | 188,696      | 95.33               | 93.96          | 502.45           | 98.60          | 5                    | 95.53           | 16                     |
| EO26     | 182,956      | 94.99               | 92.88          | 485.47           | 98.88          | 4                    | 94.13           | 21                     |
| EO27     | 191,880      | 94.62               | 93.58          | 507.12           | 98.88          | 4                    | 94.97           | 18                     |
| EO28     | 228,313      | 92.89               | 93.22          | 592.43           | 98.88          | 4                    | 94.69           | 19                     |
| EO29     | 199,442      | 98.07               | 92.15          | 546.33           | 98.32          | 6                    | 94.97           | 18                     |
| EO30     | 190,915      | 97.24               | 92.66          | 518.58           | 98.04          | 7                    | 94.69           | 19                     |
| EO31     | 161,793      | 95.49               | 92.19          | 431.54           | 97.77          | 8                    | 93.30           | 24                     |
| EO32     | 245,414      | 89.57               | 93.45          | 613.99           | 98.52          | 6                    | 95.81           | 15                     |
| EO33     | 205,076      | 95.46               | 85.54          | 546.83           | 96.65          | 12                   | 89.39           | 39                     |
| EO34     | 210,900      | 97.24               | 93.66          | 572.83           | 98.60          | 5                    | 95.25           | 17                     |
| EO35     | 147,306      | 97.24               | 92.62          | 402.14           | 98.32          | 6                    | 94.13           | 21                     |
| LO1      | 178,290      | 90.77               | 93.68          | 321.94           | 97.77          | 8                    | 91.90           | 29                     |
| LO2      | 205,008      | 93.84               | 93.97          | 537.36           | 99.16          | 3                    | 96.09           | 14                     |
| LO3      | 159,828      | 93.15               | 93.80          | 502.12           | 98.88          | 4                    | 95.53           | 16                     |
| LO4      | 193,973      | 93.90               | 94.09          | 1082.97          | 99.44          | 2                    | 96.37           | 13                     |
| LO5      | 191,160      | 93.72               | 93.21          | 1097.36          | 99.16          | 3                    | 96.65           | 12                     |
| LO6      | 177,316      | 94.10               | 93.42          | 931.78           | 99.44          | 2                    | 96.37           | 13                     |
| LO7      | 238,812      | 94.23               | 93.77          | 593.70           | 97.77          | 8                    | 92.18           | 28                     |
| LO8      | 158,483      | 93.67               | 93.01          | 708.10           | 99.44          | 2                    | 97.49           | 9                      |
Table 3. Cont.

| Patients | Mapped Reads | Reads on Target (%) | Uniformity (%) | ROI MEAN COVERAGE | ROI $\geq 20 \times$ (%) | $n$ of Amplicons < 20× | ROI $\geq 100 \times$ (%) | $n$ of Amplicons < 100× |
|----------|-------------|---------------------|----------------|-------------------|--------------------------|--------------------------|---------------------------|--------------------------|
| LO9      | 213,370     | 93.89               | 94.34          | 861.05            | 99.44                    | 2                        | 97.21                     | 10                       |
| LO10     | 190,285     | 94.47               | 93.70          | 415.86            | 98.32                    | 6                        | 94.11                     | 20                       |
| LO11     | 182,160     | 93.99               | 94.02          | 505.35            | 98.32                    | 6                        | 94.97                     | 18                       |
| LO12     | 213,052     | 92.51               | 94.69          | 532.23            | 98.88                    | 4                        | 94.97                     | 18                       |
| LO13     | 249,591     | 93.48               | 93.92          | 304.45            | 96.93                    | 11                       | 90.78                     | 33                       |
| LO14     | 195,422     | 94.82               | 94.22          | 378.77            | 98.04                    | 7                        | 92.74                     | 26                       |
| LO15     | 201,815     | 95.14               | 93.81          | 717.45            | 98.60                    | 5                        | 95.53                     | 16                       |
| LO16     | 400,156     | 98.18               | 90.79          | 500.41            | 98.60                    | 5                        | 94.41                     | 20                       |
| LO17     | 274,868     | 95.75               | 91.07          | 423.42            | 98.04                    | 7                        | 92.74                     | 26                       |
| LO18     | 158,695     | 92.07               | 94.09          | 457.87            | 98.32                    | 6                        | 94.97                     | 18                       |
| LO19     | 195,752     | 89.66               | 93.66          | 206.68            | 96.65                    | 12                       | 86.03                     | 50                       |
| LO20     | 83,846      | 88.25               | 93.28          | 490.24            | 98.32                    | 6                        | 94.13                     | 21                       |
| LO21     | 179,015     | 91.57               | 94.00          | 408.12            | 98.60                    | 5                        | 94.69                     | 19                       |
| LO22     | 170,180     | 89.07               | 93.89          | 735.17            | 98.60                    | 5                        | 94.97                     | 18                       |
| LO23     | 161,290     | 90.82               | 93.26          | 680.84            | 99.16                    | 3                        | 96.09                     | 14                       |
| LO24     | 281,769     | 92.16               | 93.07          | 536.34            | 98.60                    | 5                        | 95.33                     | 16                       |
| LO25     | 145,219     | 93.37               | 93.76          | 517.60            | 99.16                    | 3                        | 95.81                     | 15                       |
| LO26     | 390,025     | 97.57               | 92.65          | 508.78            | 98.88                    | 4                        | 95.53                     | 16                       |
| LO27     | 124,848     | 87.30               | 93.29          | 651.69            | 99.16                    | 3                        | 95.25                     | 17                       |
| LO28     | 214,031     | 89.02               | 93.84          | 550.53            | 99.16                    | 3                        | 96.37                     | 13                       |
| LO29     | 203,428     | 88.93               | 94.12          | 479.56            | 98.88                    | 4                        | 95.53                     | 16                       |
| LO30     | 127,496     | 90.40               | 93.91          | 452.05            | 98.32                    | 6                        | 94.69                     | 19                       |
| LO31     | 25,524      | 95.49               | 93.62          | 409.17            | 98.04                    | 7                        | 93.02                     | 25                       |
| LO32     | 329,472     | 93.56               | 94.58          | 559.60            | 99.16                    | 3                        | 96.09                     | 14                       |
| LO33     | 265,135     | 95.60               | 93.94          | 414.68            | 98.32                    | 6                        | 94.13                     | 21                       |
| LO34     | 21,736      | 97.78               | 89.25          | 628.59            | 98.88                    | 4                        | 95.81                     | 15                       |
| LO35     | 352,805     | 94.55               | 92.69          | 466.05            | 97.77                    | 8                        | 94.69                     | 19                       |
Table 4. Mutations detected per gene.

| Gene ID | Chrom | Position | Exon | DNA Change | Protein Change | Mutation Type | dbSNP | GMAF | SIFT | POLYPHEN | PROVEAN (cutoff = 2.5) | Clinical Significance |
|--------|-------|----------|------|------------|----------------|---------------|-------|------|------|----------|------------------------|---------------------|
| CAV3   | chr3  | 8787313  | 2    | c.216C>G   | Cys72Trp       | MISSENSE      | rs116840776 | yes  | 0.00100 (G) deleterious 0 | probably damaging 0.999 possibly damaging 0.537 benign 0.07 deleterious 6.167 | known/uncertain significance |
|        | chr3  | 8787330  | 2    | c.233C>T   | Thr78Met       | MISSENSE      | rs72546668  | yes  | 0.00200 (T) tolerated 0.05 | probably damaging 0.999 possibly damaging 0.537 benign 0.07 deleterious 6.167 | known/uncertain significance |
|        | chr3  | 8787497  | 2    | c.400G>T   | Ala134Ser     | MISSENSE      | rs72546668  | yes  | 0.00200 (T) tolerated 0.05 | probably damaging 0.999 possibly damaging 0.537 benign 0.07 deleterious 6.167 | known/uncertain significance |
| GLA    | chrX  | 10063420 | 6    | c.937G>T   | Asp313Tyr     | MISSENSE      | rs28935490  | yes  | 0.0021 (A) deleterious 0 | probably damaging 0.952 deleterious 3.183 | known/uncertain significance |
| MYBPC3 | chr11 | 47371426 | 5    | c.553A>T   | Lys185Ter     | STOP          | rs375607980 | yes  | 0.00200 (T) tolerated 0.44 | benign 0.132 Neutral 0.418 deleterious 6.167 | known/pathogenic known/likely benign known/uncertain significance |
|        | chr11 | 47371414 | 5    | c.565G>A   | Val189Ile     | MISSENSE      | rs11570052  | yes  | 0.00008 (G) deleterious 0 | probably damaging 0.994 deleterious 8.043 deleterious 6.167 deleterious 3.183 | known/pathogenic known/uncertain significance |
|        | chr11 | 47365154 | 13   | c.1112C>G  | Pro371Arg     | MISSENSE      | rs397515887 | yes  | 0.00008 (G) deleterious 0 | probably damaging 0.994 deleterious 8.043 deleterious 6.167 deleterious 3.183 | known/pathogenic known/uncertain significance |
|        | chr11 | 47365147 | 13   | c.1120C>T  | Gln374Ter     | STOP          | rs730880635 | yes  | 0.00008 (G) deleterious 0 | probably damaging 0.994 deleterious 8.043 deleterious 6.167 deleterious 3.183 | known/pathogenic known/uncertain significance |
|        | chr11 | 47364429 | 15   | c.1409G>A  | Arg470Gln     | MISSENSE      | rs397515887 | yes  | 0.00008 (G) deleterious 0 | probably damaging 0.994 deleterious 8.043 deleterious 6.167 deleterious 3.183 | known/pathogenic known/uncertain significance |
|        | chr11 | 47364270 | 16   | c.1483C>T  | Arg495Trp     | MISSENSE      | rs397515905 | yes  | 0.00008 (G) deleterious 0 | probably damaging 0.994 deleterious 8.043 deleterious 6.167 deleterious 3.183 | known/pathogenic known/uncertain significance |
|        | chr11 | 47364162 | 16   | c.1591G>C  | Gly531Arg     | MISSENSE      | rs397515912 | yes  | 0.00008 (G) deleterious 0 | probably damaging 0.994 deleterious 8.043 deleterious 6.167 deleterious 3.183 | known/pathogenic known/uncertain significance |
|        | chr11 | 47364129 | 16   | c.1624G>C  | Glu542Gln     | MISSENSE      | rs397515912 | yes  | 0.00008 (G) deleterious 0 | probably damaging 0.994 deleterious 8.043 deleterious 6.167 deleterious 3.183 | known/pathogenic known/uncertain significance |
|        | chr11 | 47360071 | 22   | c.2306G>A  | Asp770Asn     | MISSENSE      | rs36211723  | yes  | 0.00008 (G) deleterious 0 | probably damaging 0.994 deleterious 8.043 deleterious 6.167 deleterious 3.183 | known/pathogenic known/uncertain significance |
|        | chr11 | 47359347 | 23   | c.2309-2A>G | Asp770Asn     | MISSENSE      | rs36211723  | yes  | 0.00008 (G) deleterious 0 | probably damaging 0.994 deleterious 8.043 deleterious 6.167 deleterious 3.183 | known/pathogenic known/uncertain significance |
|        | chr11 | 47359115 | 24   | c.2429G>A  | Arg810His     | MISSENSE      | rs375675796 | yes  | 0.00008 (G) deleterious 0 | probably damaging 0.994 deleterious 8.043 deleterious 6.167 deleterious 3.183 | known/pathogenic known/uncertain significance |
|        | chr11 | 47359085 | 24   | c.2459G>A  | Arg820Gln     | MISSENSE      | rs3856655  | yes  | 0.00008 (G) deleterious 0 | probably damaging 0.994 deleterious 8.043 deleterious 6.167 deleterious 3.183 | known/pathogenic known/uncertain significance |
|        | chr11 | 47356592 | 26   | c.2905+1G>A | Arg820Gln     | MISSENSE      | rs397515905 | yes  | 0.00008 (G) deleterious 0 | probably damaging 0.994 deleterious 8.043 deleterious 6.167 deleterious 3.183 | known/pathogenic known/uncertain significance |
|        | chr11 | 47355264 | 28   | c.3034C>T  | Gln1012Ter    | STOP          | rs720800586 | yes  | 0.00008 (G) deleterious 0 | probably damaging 0.994 deleterious 8.043 deleterious 6.167 deleterious 3.183 | known/pathogenic known/uncertain significance |
|        | chr11 | 47354882 | 29   | c.3192_3193insC | Lys1065Gln    | INS           | rs397516007 | yes  | 0.00008 (G) deleterious 0 | probably damaging 0.994 deleterious 8.043 deleterious 6.167 deleterious 3.183 | known/pathogenic known/uncertain significance |
|        | chr11 | 47353801 | 32   | c.3636T>G  | Ile1212Met    | MISSENSE      | rs28916470  | yes  | 0.00008 (G) deleterious 0 | probably damaging 0.994 deleterious 8.043 deleterious 6.167 deleterious 3.183 | known/pathogenic known/uncertain significance |
|        | chr11 | 47353662 | 32   | c.3775C>T  | Glu1259Ter    | STOP          | rs73080605 | yes  | 0.00008 (G) deleterious 0 | probably damaging 0.994 deleterious 8.043 deleterious 6.167 deleterious 3.183 | known/pathogenic known/uncertain significance |
Table 4. Cont.

| Gene ID | Chrom | Position | Exon | DNA Change | Protein Change | Mutation Type | dbSNP | Prev. Rep. | GMAF | SIFT | POLYPHEN | PROVEAN (cutoff = -2.5) | Clinical Significance |
|---------|-------|----------|------|------------|----------------|---------------|-------|------------|------|------|-----------|------------------------|----------------------|
| MYH7    | chr14 | 23900850 | 8    | c.676G>A   | Ala226Thr      | MISSENSE      |       |            | deleterious 0 | probably damaging 0.985 | neutral −1.757      | new/uncertain significance |
|         |       |          |      |            |                |               |       |            |       |      |           |                        |                      |
|         | chr14 | 23896866 | 16   | c.1816G>A  | Val606Met      | MISSENSE      |       |            | yes 0.0008(T) | probably damaging 0.995 | deleterious −3.728     | known/pathogenic         |
|         | chr14 | 23896042 | 18   | c.1998G>A  | Arg663His      | MISSENSE      |       |            | yes 0.0008(T) | probably damaging 0.995 | deleterious −3.728     | known/pathogenic         |
|         | chr14 | 23895189 | 19   | c.2146G>C  | Gly716Arg      | MISSENSE      |       |            | deleterious 0.01 | deleterious −3.728     | new/likely pathogenic    |
|         |         |                      |      |            |                |               |       |            |       |      |           |                        |                      |
|         | MYH7   | chr14 | 23894116 | 22    | delAAG       | Lys847del     | DEL           |       |            | 0.00020(A) | deleterious 0.03         | deleterious −6.180     | new/uncertain significance |
|         |         |                      |      |            |                |               |       |            |       |      |           |                        |                      |
|         |         |                      |      |            |                |               |       |            |       |      |           |                        |                      |
|         | chr14 | 23893234 | 23   | c.2804A>T  | Glu935Val      | MISSENSE      |       |            | yes 0.0008(T) | deleterious 0.03         | deleterious −6.180     | new/uncertain significance |
|         |         |                      |      |            |                |               |       |            |       |      |           |                        |                      |
|         |         |                      |      |            |                |               |       |            |       |      |           |                        |                      |
|         | chr14 | 23889413 | 27   | c.3367G>C  | Glu1129Gln     | MISSENSE      |       |            | deleterious 0.01 | deleterious −6.180     | new/uncertain significance |
|         |         |                      |      |            |                |               |       |            |       |      |           |                        |                      |
|         |         |                      |      |            |                |               |       |            |       |      |           |                        |                      |
|         | chr14 | 23887615 | 30   | c.3973G>A  | Ala1325Thr    | MISSENSE/SPLICING |        |            | deleterious 0.02 | deleterious −6.180     | new/uncertain significance |
|         |         |                      |      |            |                |               |       |            |       |      |           |                        |                      |
|         |         |                      |      |            |                |               |       |            |       |      |           |                        |                      |
|         | chr1  | 201334751 | 9    | c.281G>C   | Arg94Thr      | MISSENSE      |       |            | deleterious 0 | deleterious −5.588     | new/uncertain significance |
|         | TNNT2  | chr1  | 201330414 | 14   | c.794A>T    | Lys265Ile     | MISSENSE      |       |            | deleterious 0 | deleterious −6.86     | known/uncertain significance |
|         |         |                      |      |            |                |               |       |            |       |      |           |                        |                      |
|         |         |                      |      |            |                |               |       |            |       |      |           |                        |                      |
|         | MYH6   | chr14 | 23873951 | 7     | c.611G>A    | Arg204His     | MISSENSE      |       |            | tolerated 0.05 | deleterious −6.86     | known/uncertain significance |
|         |         |                      |      |            |                |               |       |            |       |      |           |                        |                      |
|         |         |                      |      |            |                |               |       |            |       |      |           |                        |                      |
|         | MYL2   | chr12 | 11135901 | 6     | c.401A>C    | Glu134Ala     | MISSENSE      |       |            | deleterious 0.01 | deleterious −6.180     | known/likely pathogenic |
|         |         |                      |      |            |                |               |       |            |       |      |           |                        |                      |
|         | MYL3   | chr3  | 46902303 | 3     | c.170C>A    | Ala57Asp      | MISSENSE      |       |            | deleterious 0 | deleterious −5.236     | known/uncertain significance |
|         |         |                      |      |            |                |               |       |            |       |      |           |                        |                      |
|         |         |                      |      |            |                |               |       |            |       |      |           |                        |                      |
|         | TNNI3  | chr19 | 55665561 | 6     | c.385C>G    | Thr128Ser     | MISSENSE      |       |            | tolerated 0.186 | deleterious −6.180     | new/likely benign         |
|         |         |                      |      |            |                |               |       |            |       |      |           |                        |                      |
|         |         |                      |      |            |                |               |       |            |       |      |           |                        |                      |

Prev. Rep.: previously reported; GMAF: Global minor allele frequency; Software prediction programs used for sequence variant interpretation: SIFT: Evolutionary conservation; POLYPHEN: Protein structure/function and evolutionary conservation; PROVEAN: Alignment and measurement of similarity between variant sequence and protein sequence homolog.
2.3. Group Comparison after Sequencing

The mutation detection rate was 85.7% (30/35) in the EO group and 22.9% (8/35) in the LO group. The number of patients in which the molecular screening allowed the identification of at least one mutation was significantly different in the two groups of patients with different age at diagnosis ($p < 0.0001$). The overall detection rate, regardless of the age of onset, was 54.3% (38/70). The NGS analysis confirmed the known mutational status of the 22 controls (seven positive and 15 negative) included in this study. Mutations identified in each patient are listed in Table 5. Considering only patients with positive family history, the detection rate was 88% (22/25), ranging from 75% (3/4) in the LO group to 90.5% (19/21) in the EO group. Considering sporadic cases only, the overall detection rate was 35.5%, with a significant difference between EO (11/14, 78.6%) and LO (5/31, 16%), $p < 0.0002$.

In the EO group, patients EO13 and EO33 carried three different mutations in MYBPC3. One of them was clinically characterized by an unfavourable course with evolution to end stage disease. Four patients carried two different mutations: EO23 carried two mutations in MYH7, whereas EO6, EO11, and EO21 carried two mutations in two different genes (Table 5). In the LO group, only two patients, LO8 and LO17, harboured two mutations in different genes (Table 5).

The distribution of the identified gene mutations was similar between the two groups with the exceptions of MYH6 and TNNT2. In fact, mutations in MYH6 were identified in the LO group only, whereas mutations in TNNT2 were identified in the EO group only.
| Patient ID | Familiarity | Gene ID | Exon | DNA Change | Protein Change | Mutation Type | Clinical Significance | dbSNP | Previously Reported | Coverage | Allele Coverage |
|------------|------------|---------|------|------------|----------------|---------------|----------------------|--------|--------------------|----------|------------------|
| EO1        | yes        | MYBPC3  | 5    | c.553A>T   | Lys185Ter      | STOP          | known/pathogenic     | rs375607980 | yes                | 384      | 202              |
| EO2        | yes        | MYH7    | 19   | c.2156G>A   | Arg719Gln      | known/pathogenic | known/pathogenic   | rs121913641 | yes                | 399      | 204              |
| EO3        |           | CAV3    | 2    | c.233C>T    | Thr78Met       | known/pathogenic | known/uncertain     | rs72546668  | yes                | 124      | 57               |
| EO4        |           | MYBPC3  | 23   | c.2309-2A>G | SPLICING       | known/pathogenic | known/pathogenic   | rs111729952 | yes                | 400      | 186              |
| EO5        |           | MYH7    | 16   | c.1816G>A   | Val606Met      | known/pathogenic | new/uncertain       | rs121913627 | yes                | 383      | 204              |
| EO6        | yes        | MYH7    | 8    | c.676G>A    | Ala226Thr      | new/uncertain   | known/uncertain     | rs375607980 | yes                | 399      | 208              |
| EO7        | yes        | GLA      | 6    | c.937G>T    | Asp313Tyr      | MISSENSE       | known/uncertain     | rs28935490  | yes                | 399      | 183              |
| EO8        | yes        | MYBPC3  | 28   | c.3034C>T   | Gly1012Tyr     | SPLICING       | known/pathogenic   | rs730880586 | yes                | 397      | 194              |
| EO9        | yes        | MYH7    | 23   | c.2309-2A>G | SPLICING       | known/pathogenic | known/likely       | rs111729952 | yes                | 398      | 204              |
| EO10       | yes        | MYBPC3  | 16   | c.1816G>A   | Val606Met      | known/pathogenic | known/likely       | rs121913638 | yes                | 354      | 169              |
| EO11       | yes        | CAV3    | 2    | c.216C>G    | Cys72Trp       | MISSENSE       | known/uncertain     | rs397515905 | yes                | 400      | 259              |
| EO12       | yes        | MYBPC3  | 23   | c.2309-2A>G | SPLICING       | known/likely    | known/uncertain     | rs111729952 | yes                | 399      | 185              |
| EO13       | yes        | MYBPC3  | 32   | c.3636T>G   | Ile1212Met     | MISSENSE       | known/likely        | rs111729952 | yes                | 399      | 201              |
| EO14       |           | CAV3    | 2    | c.233C>T    | Thr78Met       | MISSENSE       | known/uncertain     | rs72546668  | yes                | 399      | 214              |
| EO15       | yes        | MYBPC3  | 13   | c.1120C>G   | Tyr374Ter      | SPLICING       | known/pathogenic   | rs36211723  | yes                | 399      | 195              |
| EO16       | yes        | MYBPC3  | 32   | c.3775C>T   | Gin1259Ter     | SPLICING       | known/pathogenic   | rs730880605 | yes                | 398      | 204              |
| EO17       | yes        | MYL2    | 6    | c.401A>C    | Gln134Ala      | MISSENSE       | known/likely       | rs143139258 | yes                | 398      | 191              |
| EO18       | yes        | MYH7    | 22   | c.2543_2545 | Lys847del      | DELETION       | known/pathogenic   | rs11570052  | yes                | 309      | 253              |
| EO19       |           | CAV3    | 2    | c.400G>T    | Ala134Ser      | MISSENSE       | new/uncertain       | rs11570052  | yes                | 391      | 194              |
| EO20       | yes        | MYH7    | 30   | c.3973G>A   | Ala1325Thr     | MISSENSE       | known/uncertain     | rs730880761 | yes                | 400      | 176              |
| EO21       | yes        | MYH7    | 23   | c.2804A>T   | Glu935Val      | MISSENSE       | known/pathogenic   | rs730880761 | yes                | 400      | 206              |
| Patient ID | Familiarity | Gene ID | Exon | DNA Change | Protein Change | Mutation Type | Clinical Significance | dbSNP | Previously Reported | Coverage | Allele Coverage |
|-----------|------------|---------|------|------------|----------------|---------------|-----------------------|-------|-------------------|----------|------------------|
| EO25      | yes        | MYBPC3  | 15   | c.1409G>A  | Arg470Gln      | MISSENSE      | known/uncertain significance | yes   | 293               | 130      |                  |
| EO26      | yes        | TNNT2   | 16   | c.853C>T   | Arg285Cys      | MISSENSE      | known/likely pathogenic | rs121964857 | yes             | 323      | 167              |
| EO27      | yes        | TNNT2   | 14   | c.794A>T   | Lys265Ile      | MISSENSE      | known/uncertain significance | rs397516482 | yes             | 395      | 193              |
| EO29      | yes        | MYBPC3  | 24   | c.2429G>A  | Arg810His      | MISSENSE      | known/likely pathogenic | rs375675976 | yes             | 400      | 148              |
| EO30      | yes        | TNNI3   | 6    | c.431T>A   | Leu144Gln      | MISSENSE      | known/likely pathogenic | rs121917760 | yes             | 398      | 227              |
| EO31      | yes        | MYBPC3  | 5    | c.565G>A   | Val189Ile      | MISSENSE      | known/likely pathogenic | rs11570052  | yes             | 312      | 152              |
| EO32      | yes        | MYH7    | 18   | c.1988G>A  | Arg663His      | MISSENSE      | known/likely pathogenic | rs37189076  | yes             | 400      | 211              |
| EO33      | yes        | MYBPC3  | 16   | c.1591G>C  | Gly531Arg      | MISSENSE      | known/likely pathogenic | rs397519512 | yes             | 400      | 212              |
|            |            |         | 13   | c.1112C>G  | Pro371Arg      | MISSENSE      | known/uncertain significance | rs397515887 | yes             | 235      | 87               |
| EO34      | yes        | TNNT2   | 9    | c.281G>C   | Arg94Thr       | MISSENSE      | known/uncertain significance | rs397516452 | yes             | 400      | 196              |
| EO35      | yes        | MYBPC3  | 26   | c.2905+1G>A| ex26           | SPlicing      | known/pathogenic         | rs397515991 | Yes             | 296      | 139              |
| LO1       | Yes        | MYH7    | 25   | c.3133C>T  | Arg1045Cys     | MISSENSE      | known/uncertain significance | rs45611033 | yes             | 213      | 113              |
| LO4       | MYH7       | 27     | c.3367G>C | Glu1123Gln | MISSENSE      | new/uncertain significance |                   |       | 400               | 220      |                  |
| LO6       | MYH6       | 20     | c.2425G>T | Arg809Cys | MISSENSE      | new/likely pathogenic |                   |       |                   | 299      | 139              |
| LO8       | Yes        | MYBPC3  | 16   | c.1624G>C  | Glu542Gln      | MISSENSE/SPLICING | known/pathogenic | rs121909374 | yes             | 353      | 188              |
| LO13      | Yes        | TNNI3   | 6    | c.385C>G   | Thr128Ser      | MISSENSE      | new/likely benign         | rs121913627 | yes             | 383      | 204              |
| LO14      | Yes        | MYH6    | 16   | c.5519A>G  | Lys1840Arg     | MISSENSE      | known/uncertain significance | rs373629059 | yes             | 399      | 196              |
| LO16      | MYBPC3     | 24     | c.2459G>A | Arg820Gln | MISSENSE      | known/likely pathogenic | rs2856655   | yes             | 400      | 213              |
| LO17      | MYH6       | 7      | c.611G>A  | Arg204His   | MISSENSE      | known/uncertain significance | rs200623022 | yes             | 398      | 201              |
| MYL3      |            | 3      | c.170C>A  | Ala57Asp    | MISSENSE      | known/uncertain significance | rs139794067 | yes             | 398      | 168              |

dbSNP: database single nucleotide polymorphisms (www.ncbi.nlm.nih.gov/SNP).
3. Discussion

This report describes the results of a genetic screening obtained through NGS approach in an Italian population of unrelated and clinically well characterized HCM cases, divided into two groups according to age at diagnosis. Our population included a good percentage of patients with a family history of HCM. As expected, the prevalence of familial forms was higher in the EO group, whereas the prevalence of sporadic forms was higher in the LO group.

The key finding of our investigation was the higher yield of mutation detection rate in the EO group and in patients with a family history of disease, with 90.5% of cases carrying an identified mutation. The overall yield of genetic testing was close to 50%, and, as previously reported in the literature [4,7–9,11], mutations in MYBPC3 and MYH7 accounted for about 65% of all variants. Other mutations were found in six additional sarcomeric genes (TNNT2, CAV3, MYH6, TNNI3, MYL2, and MYL3) and in one non-sarcomeric gene (GLA). Approximately a quarter of all variants were novel, most of them belonging to MYH7. The pathogenicity of novel mutations was verified through appropriate software for analysis.

HCM is a disease characterized by a relevant heterogeneity of both morphological and clinical features. For this reason, despite the growing knowledge on its genetic basis, the establishment of a more precise genotype–phenotype correlation has been difficult to achieve.

The main original aspect of our investigation was to test through NGS a wide range of HCM-causing genes (14 sarcomeric and three non-sarcomeric) while comparing the extreme ages of disease onset and evaluating the impact of familial occurrence of the disease even in patients with late diagnosis. Due to the small sample size of the population, our study could not address the issue of a relationship between genetic variants and phenotypic characteristics of different HCM onset patients. Notably, the presence of double and triple mutations was detected mostly among younger patients, and one of them showed a more severe form of the disease.

The different rate of pathogenic mutations found in HCM patients with early and late onset of the disease was consistent with the literature [17–19], confirming that some mutations can be found mainly in young HCM patients (TNNT2) whereas other mutations are detected exclusively in the elderly (MYH6) [17–19].

In our study, a majority of patients with young age at diagnosis had a positive genetic testing (80% of cases), four-fold higher than that of the elderly and sporadic HCM cases. These data, together with previous observations, reinforce the concept that age at HCM diagnosis is a powerful predictor of positive genetic testing [11,17–19]. We also support the notion that family history of HCM has a key role in appropriately addressing the genetic test. In fact, among HCM patients with a late diagnosis, those with a family history of the disease had a higher rate of mutation detection (75%).

We used an expanded panel of 17 genes in the attempt to improve the mutation detection rate. With this approach we mostly confirmed the type of mutations and the mutation distribution already described in the literature for HCM. In particular, the most frequent sarcomeric gene mutations, namely those in MYBPC3 and MYH7, accounted for the majority of the positive findings. Moreover, six of the seven novel mutations identified in our patients were in the main sarcomeric genes (three in MYH7, one in MYH6, one in MYBPC3, and one in TNNI3). In this regard, the limitations of using a wide diagnostic panel for HCM genetic testing have been recently highlighted in one of the largest clinical genetic studies ever reported for HCM [20]. Consistently, a panel designed only for the main HCM genes (n = 9), was able to successfully screen a large cohort of HCM patients [21]. Our findings support the choice of a limited, well-selected panel of HCM genes as the best tool for diagnostic purposes.

4. Materials and Methods

4.1. Patient Selection

Seventy patients with clinical diagnosis of HCM were included in the study. We selected 35 patients with early diagnosis of the disease (<25 years, EO-early onset) and 35 patients with
a late diagnosis (≥65 years, LO-late onset). All patients underwent a cardiologic evaluation as well as genetic counselling. Clinical data for each patient included a detailed personal and family history and a thorough scrutiny of the age at which HCM was first diagnosed. Both electrophysiologic and echocardiographic examinations were performed at the time of inclusion into the study. The echocardiographic parameters included both structural measurements and resting LV outflow tract gradients derived from the continuous-wave Doppler velocities. The clinical diagnosis of HCM was based on the echocardiographic demonstration of a hypertrophied and not dilated left ventricle (wall thickness >15 mm in adults, or the equivalent wall thickness relative to body surface area in children) in the absence of another cardiac or systemic disease that could produce comparable left ventricular hypertrophy [22,23].

The mutational status for MYH7, MYBPC3, TNNI3, TNNT2, TPM1, and MYL2 genes was already known in 22/70 patients (8 EO and 14 LO patients). All coding exons (+/−20 bp) of the six genes were previously analysed by Sanger sequencing. The 22 samples were included in our study as positive and negative controls for the six genes also present in our NGS panel. The seven positive controls carried mutations in MYBPC3 (EO7, EO29, EO35), MYH7 (LO13), TNNI3 (EO30), and MYL2 (EO20). The 15 negative controls for the six genes were: EO10, EO15, LO5, LO6, LO9, LO12, LO19, LO21, LO22, LO25, LO27, LO28, LO29, LO32, and LO33.

This study was conducted in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards (The approval identification number: 42 of 28 September 2007). A signed informed consent for blood sampling was obtained from all patients included in the study.

4.2. DNA Extraction and Quantification

Genomic DNA was extracted from peripheral whole blood using a commercially available kit (Invitrogen, Milan, Italy), and then quantified using Qubit dsDNA HS Assay Kit on Qubit 2.0 Fluorometer (Invitrogen).

4.3. Sequencing

Seventeen genes known to be causative of HCM phenotype were selected for targeted sequencing (Table 2). A custom panel for coding DNA (+/−25 bp of intronic flanking regions) analysis of selected genes was designed online using Ion AmpliSeq Designer 2.0.3 (https://www.ampliseq.com/browse.action) [24]. The final custom panel was composed of 358 amplicons divided into two primer pools for a total of 61.89 kb of DNA. The panel covered 96.47% of regions of interest (ROI). Libraries were prepared using Ion AmpliSeq Library Kit v2.0 (Life Technologies, Carlsbad, CA, USA), according to the manufacturer’s instructions. One of 16 barcodes of the Ion Xpress Barcode Adapters1-16 Kit (Thermo Fisher Scientific Life Sciences Solutions, Carlsbad, CA, USA) was added to each sample. Libraries were quantified with Qubit dsDNA HS Assay Kit on Qubit 2.0 Fluorometer (Molecular Probes, Eugene, OR, USA) and equimolar amounts of each library were used to prepare template for clonal amplification. Emulsion PCR with Ion PGM Template OT2 200 Kit (Life Technologies, Carlsbad, CA, USA) was performed on OneTouch2 Systems (Life Technologies, Carlsbad, CA, USA). Templates were enriched using Ion OneTouch ES (Life Technologies, Carlsbad, CA, USA) and prepared for 316v2 chip loading (Life Technologies, Carlsbad, CA, USA). Groups from 12 to 16 sample libraries were sequenced on each chip. Sequencing runs were performed on Ion Torrent Personal Genome Machine (PGM, Life Technologies) using Ion PGM Sequencing 200 Kit v2, according to the manufacturer’s instructions.

4.4. Alignment

Data analysis was performed using the Torrent Suite Software v.4.0.2. (Life Technologies, Carlsbad, CA, USA). Reads were aligned to human reference genome hg19 from UCSC Genome Browser [25]
and to a designed bed file from Ion AmpliSeq Designer results. Alignments were visually verified with Integrative Genomics Viewer IGV v.2.3, Broad Institute [26].

4.5. Coverage Analysis

The average read depth and the percentage of reads that mapped on ROI out of the total number of reads (reads on target) was calculated using Coverage Analysis plug-in (Life Technologies, Carlsbad, CA, USA). For each sample the percentage of ROI covered by at least 100× and 20× using amplicon coverage matrix file was calculated.

4.6. Variant Analysis

Variant calling was performed with Variant Caller plug-in configured with germ line-low stringency parameters. Variants were annotated using Ion Reporter 4.0 software (Carlsbad, CA, USA) [27]. Common single nucleotide variants (minor allele frequency MAF>5%, source 1000 Genomes), exonic synonymous variants, and intronic variants were removed from the analysis, while exonic non-synonymous, splice-site, and loss-of-function variants were analysed. The novel variants were analysed by means of three types of prediction software (SIFT, POLYPHEN, and PROVEAN) and classified based on the concordance of the prediction between the three types: “likely pathogenic,” “likely benign” (3/3 concordance), or “uncertain significance” (2/3 concordance).

4.7. Variant Validation

The identified variants were validated by Sanger sequencing using standard protocols. Specific primers were designed for the analysis. Polymerase Chain Reaction (PCR) products were directly sequenced by using the BigDye Terminator v3.1 Cycle Sequencing Kit (Life Technologies Corporation, Carlsbad, CA, USA). Sample analysis was performed on an ABI PRISM 3130xl Genetic Analyser (Applied Biosystems, Carlsbad, CA, USA).

4.8. Statistical Analysis

Statistical analysis was performed with SPSS statistical software (SPSS Inc., Chicago, IL, USA, version 17.0). Continuous variables are expressed as mean±SD. Comparisons between the two groups were performed using a Student’s t-test. The association between the mutational status and the clinical features of the two patient groups was evaluated using Chi-square and Fisher’s exact tests. A p value was considered statistically significant when <0.05.

5. Conclusions

In summary, through NGS, we were able to detect pathogenic mutations responsible for HCM, particularly in patients with early onset of the disease and in those with a family history of HCM. Our findings document the suitability of a novel molecular diagnostic strategy for clinical purposes and the important role of appropriate patient selection in making genetic molecular testing more cost-effective.

Acknowledgments: This work was supported by a 5% grant (Ricerca Corrente) from the Italian Ministry of Health to Massimo Volpe and Speranza Rubattu. The funding sources had no involvement in the study design, in the collection, analyses, and interpretation of data; in the writing of the report; and in the decision to submit the article for publication.

Author Contributions: Speranza Rubattu and Camillo Autore conceived and designed the study. Beatrice Maria Musumeci, Erika Pagannone, Ermelinda Pennacchini, and Pietro Francia collected the study population. Cristina Bozzao, Maria Piane, Camilla Savio, and Aldo Germani performed the genetic analyses. Speranza Rubattu and Camillo Autore drafted and Luciana Chessa and Massimo Volpe finalized the manuscript. All authors closely interpreted all the results, reviewed, and approved the final version of the paper.

Conflicts of Interest: The authors declare no conflict of interest.
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