Prevalence of Sequence Variants in the RAS-Mitogen Activated Protein Kinase Signaling Pathway in Pre-Adolescent Children With Hypertrophic Cardiomyopathy

Juan Pablo Kaski, MD(Res); Petros Syrris, PhD; Adam Shaw, MD; Krisztina Zuborne Alapi, MSc; Viviana Cordeddu, MSc; Maria Teresa Tome Esteban, PhD; Sharon Jenkins, MSc; Michael Ashworth, MD; Peter Hammond, PhD; Marco Tartaglia, PhD; William J. McKenna, MD; Perry M. Elliott, MD

Background—Most cases of apparently idiopathic hypertrophic cardiomyopathy (HCM) in children are caused by mutations in cardiac sarcomere protein genes. HCM also commonly occurs as an associated feature in some patients with disorders caused by mutations in genes encoding components of the RAS-mitogen activated protein kinase (MAPK) signaling pathway. Although diagnosis of these disorders is based on typical phenotypic features, the dysmorphic manifestations can be subtle and therefore overlooked. The aim of this study was to determine the prevalence of mutations in RAS-MAPK genes in preadolescent children with idiopathic HCM.

Methods and Results—Seventy-eight patients diagnosed with apparently nonsyndromic HCM aged ≤13 years underwent clinical and genetic evaluation. The entire protein coding sequence of 9 genes implicated in Noonan syndrome and related conditions (PTPN11, SOS1, HRAS, KRAS, NRAS, BRAF, RAF1, MAP2K1, and MAP2K2), together with CBL (exons 8 and 9) and SHOC2 (4A>G), were screened for mutations. Five probands (6.4%) carried novel sequence variants in SOS1 (2 individuals), BRAF, MAP2K1, and MAP2K2. Structural and molecular data suggest that these variants may have functional significance. Nine cardiac sarcomere protein genes were screened also; 2 individuals also had mutations in MYBPC.

Conclusions—This study reports novel and potentially pathogenic sequence variants in genes of the RAS-MAPK pathway, suggesting that genetic lesions promoting signaling dysregulation through RAS contribute to disease pathogenesis or progression in children with HCM. (Circ Cardiovasc Genet. 2012;5:317-326.)

Key Words: cardiomyopathy ■ genetics ■ signal transduction ■ rasopathy ■ syndrome

Hypertrophic cardiomyopathy (HCM) is defined by the presence of left ventricular (LV) hypertrophy in the absence of abnormal loading conditions.1 Numerous studies have shown that, in adolescents and adults, most cases are caused by mutations in genes encoding proteins of the cardiac sarcomere. Recent studies have shown that HCM in children is also caused by mutations in sarcomere protein genes in over one half of cases.2 In less than 10% of children, LV hypertrophy is caused by inborn errors of metabolism, neuromuscular disease, or malformation syndromes, respectively;4–6 the cause in the remaining cases is unknown.

Clinical Perspective on p 326

Noonan syndrome (NS) is a developmental disorder characterized by facial dysmoria, postnataally reduced growth, skeletal malformations, webbing of the neck, and variable cognitive deficits.7–9 Cardiac manifestations, most commonly pulmonary valve stenosis and HCM,8,10 are present in up to 90% of affected individuals. Approximately 50% of cases of NS are caused by activating mutations in the PTPN11 gene, which encodes the SHP2 protein tyrosine phosphatase that plays a critical role in the RAS-mitogen activated protein kinase (MAPK) pathway.11 Recently, mutations in other genes encoding proteins of the RAS-MAPK signaling cas-
caused have been found to cause NS (SOS1, KRAS, NRAS, RAF1, and BRAF),13–18 and related disorders such as LEOPARD syndrome (PTPN1119,20 and RAF121), Noonan-like syndrome with loose anagen hair (SHOC222), “CBL mutation-associated” syndrome (CBL23), Costello syndrome (HRAS24), and cardiofaciocutaneous (CFC) syndrome (KRAS,25 BRAF,24,25 MAP2K1,24 and MAP2K224). Based on the shared pathogenetic mechanism and clinical overlap, these diseases are now grouped in a single family of disorders termed “RASopathies.”26

The diagnosis of most RASopathies is based on typical dysmorphic features. However, these can be subtle, particularly in early infancy, or can become less evident in adulthood.27 We therefore hypothesized that incomplete phenotypic expression of these disorders might cause apparently nonsyndromic LV hypertrophy. In support of this, mutations in a subset of these disease genes occasionally have been reported in subjects with seemingly isolated heart disease,13,28 and there are data demonstrating a role of RAS-MAPK signaling in controlling cardiomyocyte survival, growth, and hypertrophy.29 The aim of this study was to determine the frequency of potentially pathogenic RAS-MAPK gene variants in preadolescent children with idiopathic HCM.

Methods

Patients

The study cohort comprised patients first diagnosed with HCM at an age of ≤13 years that were evaluated by the same team in dedicated HCM clinics at St. George’s Hospital (1989–2003), The Heart Hospital (2003–2007), and Great Ormond St. Hospital (1989–2007), London, United Kingdom. These patients have previously been reported;21 1 patient included in the previous study is excluded from the present study as there was no further DNA available and the patient was no longer under follow-up at our institution. All patients fulfilled conventional diagnostic criteria for HCM (LV hypertrophy more than 2 standard deviations corrected for body surface area, or above the upper limit of normal for age),30 and were not considered to have phenotypic features of NS or other RASopathies by the examining cardiologists.

Patients underwent noninvasive assessment, including clinical history, physical examination, 12-lead ECG, and 2-dimensional, M-mode, and Doppler echocardiography. Data collected at the initial visit and during follow-up outpatient consultations were entered into a relational database. Left atrial and LV internal dimensions and maximal LV wall thicknesses were determined using standard 2-dimensional and M-mode echocardiographic views, as previously described.31 Resting LV outflow tract gradients were determined using continuous wave Doppler echocardiography.31 Where patients’ height and weight were available (n=65), echocardiographic parameters were expressed as a deviation from the body surface area-corrected mean (z-score) based on previously published normal values in our laboratory.32

Three patients (Pt 2, Pt 3, and Pt 4) and, in the case of Pt 2 and Pt 4, their first-degree relatives, retrospectively underwent detailed evaluation by a dysmorphologist with experience in NS and related disorders (AS). In addition, 2 probands (Pt 2 and Pt 4) underwent 3-dimensional analysis of facial morphology following the identification of the SOS1 variants, as previously described.33

Table 1. Clinical Characteristics at Initial Evaluation

<table>
<thead>
<tr>
<th>Component</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at 1st evaluation, y</td>
<td>9.9 (6.0–12.9)</td>
</tr>
<tr>
<td>Male/Female</td>
<td>48/30 (61.5/38.5)</td>
</tr>
<tr>
<td>Symptoms at diagnosis</td>
<td>26 (33.3)</td>
</tr>
<tr>
<td>Dyspnea</td>
<td>13 (16.7)</td>
</tr>
<tr>
<td>Chest pain</td>
<td>11 (14.1)</td>
</tr>
<tr>
<td>Syncope</td>
<td>3 (3.8)</td>
</tr>
<tr>
<td>Pre-syncope</td>
<td>4 (5.1)</td>
</tr>
<tr>
<td>Palpitation</td>
<td>8 (10.3)</td>
</tr>
<tr>
<td>FHz HCM</td>
<td>47 (60.3)</td>
</tr>
<tr>
<td>FHz SCD</td>
<td>28 (35.9)</td>
</tr>
<tr>
<td>Echocardiography</td>
<td></td>
</tr>
<tr>
<td>MLVWT (mm)</td>
<td>17 (11–23)</td>
</tr>
<tr>
<td>MLVWT z-score</td>
<td>+14.2 (+6.8–22.0)</td>
</tr>
<tr>
<td>LVEDD (mm)</td>
<td>36 (30–40)</td>
</tr>
<tr>
<td>LVEDD z-score</td>
<td>−1.7 (−2.8–0.8)</td>
</tr>
<tr>
<td>LVESD (mm)</td>
<td>19 (15–22)</td>
</tr>
<tr>
<td>LVESD z-score</td>
<td>−3.0 (−4.7–1.5)</td>
</tr>
<tr>
<td>FS (%)</td>
<td>45 (37–54)</td>
</tr>
<tr>
<td>LA dimension (mm)</td>
<td>33 (27–38)</td>
</tr>
<tr>
<td>LA dimension z-score</td>
<td>+2.1 (+1.0–4.1)</td>
</tr>
<tr>
<td>LVOT gradient (mm Hg)</td>
<td>10 (6–30)</td>
</tr>
<tr>
<td>Pattern of LV hypertrophy</td>
<td></td>
</tr>
<tr>
<td>ASH</td>
<td>62 (79.5)</td>
</tr>
<tr>
<td>Concentric</td>
<td>11 (14.1)</td>
</tr>
<tr>
<td>Eccentric</td>
<td>5 (6.4)</td>
</tr>
</tbody>
</table>

N=78.

Data are presented as No. (%) of patients or median value (interquartile range).

FHz indicates family history; HCM, hypertrophic cardiomyopathy; SCD, sudden cardiac death; MLVWT, maximal left ventricular wall thickness; LVEDD, left ventricular end-diastolic dimension; LVESD, left ventricular end-systolic dimension; FS, fractional shortening; LA, left atrium; LVOT, left ventricular outflow tract; LV, left ventricular; ASH, asymmetric septal hypertrophy.

DNA Extraction and Mutation Analysis

Genomic DNA from HCM patients and family members was extracted from whole blood samples using a QIAamp DNA Mini kit (Qiagen). Standard polymerase chain reaction protocols (AmpliTaq Gold, Applied Biosystems) were used to amplify the entire protein-coding sequence of 9 genes encoding key RAS-MAPK pathway components: PTPN11, SOS1, HRAS, KRAS, NRAS, BRAF, RAF1, MAP2K1, and MAP2K2, as well as the mutational hotspots of CBL (exons 8 and 9, and intronic flanking stretches) and SHOC2 (5’-terminal portion of the first coding exon). Polymerase chain reaction conditions and primer sequences are available on request. CBL and SHOC2 amplified genomic fragments were scanned for mutations by denaturing high-performance liquid chromatography, as previously reported.21,22 Variant elution profiles were reamplified and sequenced bidirectionally on an ABI 3130 genetic analyzer using BigDye Terminator chemistry v3.1 (Applied Biosystems). For the remaining disease genes, mutational analysis was performed by direct sequencing of the amplified products. To exclude that the novel sequence variants found in HCM patients were disease-unrelated polymorphisms occurring in the population, we screened a cohort of 200 control samples (unrelated healthy volunteers) using the same methodology. The likelihood of pathogenic effect of sequence variants in RAS-MAPK pathway genes was determined by 4 in silico prediction methods: the Grantham score,34 PolyPhen,35 PolyPhen-2,35 and SIFT.36 The identified sequence variants were checked against the NCBI dbSNP database (http://www.ncbi.nlm.nih.gov/snp) and the 1000 genomes database (www.1000genomes.org) to determine whether they were known genetic variants. The Human Gene Mutation Database (http://www.hgmd.cf.ac.uk) was accessed to establish whether these variants were reported previously as pathogenic mutations. Finally, a literature review was performed.
Figure 1. Mutations in RAS/mitogen activated protein kinase (MAPK) pathway genes identified in 5 patients with apparently idiopathic HCM. A, BRAF c.107C>G, S36C. B, MAP2K2 c.238G>A, A80T. C, SOS1 c.1870G>T, V624F. D, SOS1 c.3286T>A, S1096T. E, MAP2K1 c.144_145delGCinsA, K48fsX63. Protein sequences were accessed on Ensembl (http://www.ensembl.org/) and aligned using CLUSTAL W multiple sequence alignment (http://www.ebi.ac.uk/Tools/msa/clustalw2/). *Sequence of orthologue at this position not present on Ensembl database. **No sequence alignment between human and fruitfly orthologues at these positions.
to determine whether the identified sequence variants had been associated with disorders involving genes coding for components of the RAS-MAPK signaling pathway.

Ethics
The study was approved by the local research ethics committee and informed written consent was obtained for genetic testing from all participants aged ≥16 years or from the parents of those aged <16 years. The investigation conforms to the principles outlined in the Declaration of Helsinki. Written informed consent was obtained from patients and their parents for publication of photographs.

Statistical Analysis
SPSS (v18.0 for Mac) was used for all statistical analyses. Normally distributed data are expressed as mean (95% CI); non-normally distributed data are expressed as median (interquartile range [IQR]).

Results
Clinical Characteristics
The study cohort comprised 78 patients. The clinical characteristics of the study population at the initial evaluation are shown in Table 1. The median age at diagnosis was 8.3 years [IQR 1.9 to 11.0 years; mean (±SD) 6.9±4.8 years]. Seventeen patients (21.8%) were diagnosed in infancy (under the age of 1 year). Ninety percent were of European ancestry; 47 patients, at least 1 first-degree relative was affected; in the remaining 6 patients, a second-degree relative was affected, but the probands’ parents had normal ECGs and echocardiograms, and were likely to represent obligate carriers. The baseline characteristics of the study cohort have been reported previously.2

Prevalence of Sarcomere Protein Gene Mutations
Mutations in cardiac sarcomere protein genes were found in 42 patients (53.8%), including 5 patients with 2 sarcomeric mutations. Mutations were most common in MYH7 (54.8% of cases) and MYBPC3 (35.7%); mutations in MYL3, TNNT2, TNN13, and ACTC were found in less than 5% of cases, respectively. These data are reported in detail elsewhere.2

Analysis of RAS/Mitogen Activated Protein Kinase Pathway Genes
Five probands (6.4%) carried novel sequence variants in BRAF, MAP2K1, MAP2K2, and SOS1 (2 individuals). Patient Pt 3 carried a novel c.107C>G transversion (S36C) in exon 1 of BRAF. Patient Pt 1 was heterozygous for a c.238G>A (A80T) sequence change in exon 2 of MAP2K2. Patients Pt 4 and Pt 2 had mutations in SOS1: c.1870G>T (V624F) in exon 11 and c.3286T>A (S1096T) in exon 20, respectively. A deletion-insertion (c.144_145delGCinsA) in exon 2 of MAP2K1 was detected in patient Pt 5. Two patients (Pt 1 and Pt 5) exhibited a concomitant heterozygous mutation in the MYBPC3 sarcomere protein gene.2 No sequence variations were identified in PTPN11, HRAS, KRAS, NRAS, RAF1, CBL, or SHOC2.

The 5 sequence variants occurred in coding regions of the respective genes that are highly conserved among vertebrate orthologs (Figure 1), and were absent in DNA from 200 unrelated population-matched control samples. The results of the prediction software analysis for the 5 sequence variants are shown in Table 2, suggesting that at least 2 of the variants may be pathogenic.

The MAP2K1 lesion affected a region that represents a mutational hot spot in CFC syndrome.38 MAP2K1/2

### Table 2. Likelihood of Pathogenic Effect of Missense Sequence Variants in RAS-Mitogen Activated Protein Kinase Pathway Genes as Determined by Four In Silico Prediction Methods

<table>
<thead>
<tr>
<th>Gene-Sequence Variant</th>
<th>Coding Effect</th>
<th>Grantham Score</th>
<th>PolyPhen Prediction (Score)</th>
<th>PolyPhen-2 Prediction (Score)</th>
<th>SIFT Prediction (Score)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BRAF c.107C&gt;G</td>
<td>S36C</td>
<td>112</td>
<td>Benign (1.335)</td>
<td>Benign (0.180)</td>
<td>Not tolerated; affects protein function (0.00)</td>
</tr>
<tr>
<td>MAP2K2 c.238G&gt;A</td>
<td>A80T</td>
<td>58</td>
<td>Benign (1.106)</td>
<td>Possibly damaging (0.809)</td>
<td>Tolerated (0.33)</td>
</tr>
<tr>
<td>SOS1 c.1870G&gt;T</td>
<td>V624F</td>
<td>50</td>
<td>Possibly damaging (1.880)</td>
<td>Possibly damaging (0.894)</td>
<td>Tolerated (0.23)</td>
</tr>
<tr>
<td>SOS1 c.3286T&gt;A</td>
<td>S1096T</td>
<td>58</td>
<td>Benign (1.098)</td>
<td>Possibly damaging (0.808)</td>
<td>Not tolerated; affects protein function (0.05)</td>
</tr>
</tbody>
</table>

### Table 3. Clinical and Genetic Characteristics of Five Patients With Mutations in RAS/Mitogen Activated Protein Kinase Pathway Genes

<table>
<thead>
<tr>
<th>Patient (Gender)</th>
<th>RAS-MAPK Pathway Sequence Variant</th>
<th>Sarcomeric Mutation</th>
<th>Age at Dx</th>
<th>FHx</th>
<th>FHx</th>
<th>Height at First Evaluation (Centile)</th>
<th>Height at Last Follow-Up (Centile)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pt 1 (F)</td>
<td>MAP2K2 A80T</td>
<td>MYBPC3 Y1171C</td>
<td>8 Y</td>
<td>No</td>
<td>No</td>
<td>143 cm (75th)</td>
<td>167 (75th)</td>
</tr>
<tr>
<td>Pt 2 (F)</td>
<td>SOS1 S1096T</td>
<td>. . .</td>
<td>5 Y</td>
<td>Yes</td>
<td>Yes</td>
<td>116 cm (91st)</td>
<td>120 cm (91st)</td>
</tr>
<tr>
<td>Pt 3 (M)</td>
<td>BRAF S36C</td>
<td>. . .</td>
<td>5 Y</td>
<td>Yes</td>
<td>Yes</td>
<td>178 cm (50th–75th)</td>
<td>. . .</td>
</tr>
<tr>
<td>Pt 4 (F)</td>
<td>SOS1 V624F</td>
<td>. . .</td>
<td>10 Y</td>
<td>No</td>
<td>No</td>
<td>136 cm (9th–25th)</td>
<td>150 cm (0.4th–2nd)</td>
</tr>
<tr>
<td>Pt 5 (M)</td>
<td>MAP2K1 K488xX63</td>
<td>MYBPC3 R502W</td>
<td>3 mol/L</td>
<td>Yes</td>
<td>Yes</td>
<td>103 cm (25th–50th)</td>
<td>167 cm (25th)</td>
</tr>
</tbody>
</table>

Age is expressed in years (Y) or months (M). MAPK indicates mitogen activated protein kinase; Dx: diagnosis; FHx, family history; HCM, hypertrophic cardiomyopathy; SCD, sudden cardiac death; MLVWT, maximal left ventricular wall thickness; LVEDD, left ventricular end-diastolic dimension; LVESD, left ventricular end-systolic dimension; LA, left atrium; LVOt, left ventricular outflow tract; LWH, left ventricular hypertrophy; ASH, asymmetric septal hypertrophy; RVH, right ventricular hypertrophy.

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Table 3. Continued

<table>
<thead>
<tr>
<th>Cardiac Symptoms at Dx</th>
<th>MLVWT (Z-Score)</th>
<th>LVEDD (Z-Score)</th>
<th>LVEDS (Z-Score)</th>
<th>LA Size (Z-Score)</th>
<th>LVOT Gradient</th>
<th>Pattern of LVH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absent; incidental finding</td>
<td>32 mm (z+31.9)</td>
<td>33 mm (z−3.2)</td>
<td>18 mm (z−3.9)</td>
<td>25 mm (z−0.8)</td>
<td>10 mm Hg</td>
<td>ASH</td>
</tr>
<tr>
<td>Chest pain</td>
<td>9 mm (z+4.4)</td>
<td>31 mm (z−1.8)</td>
<td>14 mm (z−4.1)</td>
<td>26 mm (z+0.9)</td>
<td>7 mm Hg</td>
<td>Concentric+RVH</td>
</tr>
<tr>
<td>Absent; family screening</td>
<td>17 mm (z+14.2)</td>
<td>30 mm (z−2.6)</td>
<td>16 mm (z−3.4)</td>
<td>. . .</td>
<td>11 mm Hg</td>
<td>ASH</td>
</tr>
<tr>
<td>Absent; incidental finding</td>
<td>24 mm (z+22.0)</td>
<td>37 mm (z−1.6)</td>
<td>27 mm (z+0.1)</td>
<td>49 mm (z+8.0)</td>
<td>61 mm Hg</td>
<td>ASH+RVH</td>
</tr>
<tr>
<td>Absent; incidental finding</td>
<td>10 mm (z+6.2)</td>
<td>30 mm (z−0.9)</td>
<td>20 mm (z−0.4)</td>
<td>30 mm (z+3.2)</td>
<td>7 mm Hg</td>
<td>ASH+RVH</td>
</tr>
</tbody>
</table>

(mitogen-activated protein kinase-1/2) code for MEK1/2. MAPK that phosphorylate and activate ERK1/2, the most downstream effectors on the RAS-MAPK pathway. The sequence variant identified in this study is predicted to result in a frameshift and premature termination of translation of the kinase at amino acid position 63 (K48fsX63). Because the kinase domain of the protein is encoded by residues 68 to 361, this truncating mutant is predicted to be catalytically dead. Two previously unreported sequence variants were identified in SOS1 and affected residues located in different functional domains of the guanine nucleotide exchange factor. Son of sevenless (SOS) proteins, encoded by SOS1, are guanine nucleotide exchange factors that increase guanine diphosphate phosphorylation, resulting in an increase in RAS in the active guanine triphosphate-bound form. Val624 is located within the RAS exchanger motif, in a region close to the allosteric RAS binding site and controlling SOS1 activation,40 and whose conformation is essential for both RAS binding to the active site and nucleotide exchange.40 Among these, a single mutation affecting the adjacent Phe623 has been reported. This residue is placed at the interface with the CDC25 domain and is part of an extended hydrophobic groove of the RAS exchanger motif domain that accommodates the side chains of 2 hydrophobic residues (Ile956 and Phe958) of the helical hairpin of the CDC25 domain. Such a hydrophobic interaction has been demonstrated to be important for the correct orientation of the helical hairpin, and for SOS1’s catalytic activity.41 Based on these observations, it can be hypothesized that the V624F substitution might perturb the catalytic activity of the CDC25 domain by altering either on RAS binding at the catalytic site, on the domain’s catalytic efficiency, or on allosteric control.42

The second SOS1 variant, S1096T, falls within a region for which a number of unclassified variants or apparently disease unrelated polymorphisms have been reported.42 None of the amino acid changes affecting this region definitively has linked causatively to NS or a related trait. These considerations and the observation that the identified change is conservative do not provide any evidence for a perturbing effect of this change on protein function. This change may represent a reduced penetrance allele, but further functional studies to confirm this are required.

The BRAF protein is a MAPK kinase, which phosphorylates and activates MEK1 or MEK2. The BRAF missense change predicting the S36C amino acid substitution affects a low complexity region that is far from the 3 major functional domains of RAF family proteins. The region has not been involved in RASopathies or human cancers so far. Similarly, the mutated MAP2K2 residue, Ala80, is relatively distant from the 2 mutational hot spots (L46 to A62 and P128 to Y134) occurring in the MAP2K2 paralogs.38 Biochemical studies are needed to determine the effects of these amino acid substitutions on protein function and signal flow through the MAPK cascade.

Clinical Features of Probands With RAS-Mitogen Activated Protein Kinase Pathway Gene Variants and Their Relatives

The baseline clinical data for the 5 patients in whom SOS1, BRAF, MAP2K1, and MAP2K2 gene pathway variants were identified are presented in Table 3. There were no statistically significant differences in clinical and echocardiographic features or outcome between those patients with RAS-MAPK pathway variants and those with sarcomeric mutations or no mutations (data not shown). None of the patients had evidence of RAS polyvalvulopathy. All 5 patients had normal renal, liver, and metabolic blood tests. No obvious dysmorphic facial features were identified in any of these 5 patients by the examining clinician. Furthermore, there was no known antenatal history of polyhydramnios, increased nuchal thickness, high birth weight, or postnatal growth retardation. One patient (Pt 4) had scoliosis and her height was between the 9th and 25th percentiles at initial evaluation, aged 10 years; her height at 17 years fell between the 0.4th and 2nd percentiles. Detailed evaluation by a dysmorphologist with experience of NS (AS) suggested subtle facial features consistent with NS. Three-dimensional analysis of facial morphology, however, showed a large face compared with most individuals with NS and no ptosis or hypoplasia of the lower face and mandible typically seen in individuals with NS.39 The lower periorbits appeared rather prominent (Figure 2A). Compared with normal controls and a group of NS controls (age and sex-matched), the facial morphology of individual Pt 4 is an
Outlier to both groups, but closer to the normal controls. Her parents are consanguineous. The c.1870G>T change in SOS1 also was identified in the proband’s mother and 1 sister; both had normal cardiovascular investigations and had no dysmorphic features (evaluated by AS). Of note, 2 other siblings of the proband, neither of whom carried the SOS1 sequence variant, had scoliosis and short stature, requiring growth hormone treatment, but no cardiac manifestations (Figure 3A). This suggests that the short stature and scoliosis in the proband is unlikely to be related to the SOS1 change.

One patient (Pt 2) had mild concentric LV hypertrophy at diagnosis, aged 5 years, which progressed rapidly over 3 years, becoming severe (maximal LV wall thickness 28 mm, z-score +2.7), with intractable symptoms related to LV outflow tract obstruction. She underwent a surgical myectomy, and was found postoperatively to have multiple accessory pathways for which she subsequently underwent radiofrequency ablations. Her mother also had HCM, Wolff-Parkinson-White with multiple pathways, and retinitis pigmentosa, and also was found to harbor the c.3286T>A substitution in the SOS1 gene. The change was absent from the proband’s brother and maternal grandfather, both of whom had normal cardiovascular investigations, but was present in the maternal grandmother, who also had no phenotypic evidence of HCM (Figure 3B). Detailed evaluation (AS) revealed no features suggestive of NS or related disorders in either the proband (Figure 4A) or her mother. Three-dimensional analysis of facial morphology of 2 individuals with SOS1 sequence variants. A, Patient Pt 4. B, Patient Pt 2. Each figure shows the closest mean classification of groups of female controls and individuals with Noonan syndrome (NS) whose mean age matches that of the probands. On the horizontal axis the mean control and NS faces are normalized to –1 and +1 respectively. The vertical axis reflects difference and hence outlier status compared with the means. The color heat maps show where the NS mean and probands’ faces are augmented (blue), the same (green), and diminished (red) relative to the normal surface of the mean control (shown green). All faces shown are to the same relative scale.

Figure 2. Three-dimensional analysis of facial morphology of 2 individuals with SOS1 sequence variants. A, Patient Pt 4. B, Patient Pt 2. Each figure shows the closest mean classification of groups of female controls and individuals with Noonan syndrome (NS) whose mean age matches that of the probands. On the horizontal axis the mean control and NS faces are normalized to –1 and +1 respectively. The vertical axis reflects difference and hence outlier status compared with the means. The color heat maps show where the NS mean and probands’ faces are augmented (blue), the same (green), and diminished (red) relative to the normal surface of the mean control (shown green). All faces shown are to the same relative scale.

Figure 3. Pedigrees of 5 families in which RAS-mitogen activated protein kinase (MAPK) pathway sequence variants were identified. A, Pt 4 (SOS1 V624F). B, Pt 2 (SOS1 S1096T). C, Pt 3 (BRAF S36C). D, Pt 1 (MAP2K2 A80T). E, Pt 5 (MAP2K1 K48fsX63). Black symbols represent individuals with a hypertrophic cardiomyopathy (HCM) phenotype; white symbols with N’ represent unaffected individuals; arrow denotes proband; + represents individuals with RAS-MAPK sequence variants; — represents individuals genetically screened and not found to harbor the RAS-MAPK variants; * represents individuals with coexisting MYBPC3 mutation; ‘represents individuals with short stature requiring growth hormone treatment.
dimensional analysis of facial morphology demonstrated midfacial hypoplasia and a prominent glabella, but no ptosis or lower facial and mandibular hypoplasia, and a large face compared with NS controls (Figure 2B). Compared with the means of normal and NS age and sex-matched controls, individual Pt 2 is classified as being more like the normal control mean. Screening for mitochondrial DNA mutations, mutations in the \textit{LAMP2} gene associated with Danon syndrome, and muscle biopsy for mitochondrial evaluation were all negative in the proband.

One individual (Pt 3) was diagnosed with HCM with asymmetrical septal hypertrophy morphology at the age of 5 years as a result of family screening, his twin brother having been diagnosed with HCM in infancy. An older brother was subsequently diagnosed aged 12 years. Both brothers had undergone surgical septal myectomies at the ages of 8 and 13 years, respectively, and 1 subsequently died suddenly aged 10 years. Histopathologic analysis of the myectomy specimens in both showed features typical of HCM. Their mother had normal cardiovascular investigations, and the c.107C>G change in \textit{BRAF} found in the proband was absent from her blood. Their father had died from cancer and had not had clinical cardiovascular screening in life, and the remaining siblings refused clinical or genetic screening (Figure 3C). Detailed retrospective evaluation of the proband (AS) revealed no features of NS or related disorders (Figure 4B).

No blood was available for analysis on any first-degree relatives of the remaining 2 probands. One proband (Pt 1) was no longer followed up at our institution and could not be contacted (Figure 3D); the other (Pt 5) refused to undergo retrospective dysmorphology evaluation (Figure 3E). The first-degree relatives of these 2 individuals had undergone clinical screening for HCM at the time of diagnosis of the respective probands (over 10 years previously in both cases), and no phenotypic features had been identified.

\textbf{Discussion}

This study suggests that genes implicated in malformation syndromes such as NS, Noonan syndrome with multiple
Mechanism of Disease of RAS-Mitogen Activated Protein Kinase Pathway Mutations
The RAS-MAPK pathway transduces extracellular signals to the intracellular environment. Activation of RAS proteins by growth factors to receptor tyrosine kinases, G-protein coupled receptors, extracellular matrix receptors, and cytokine receptors results in downstream activation of a number of intracellular cascades, including the RAF-mediated MAPK pathway. This in turn results in effects on a large number of downstream cytosolic and nuclear molecules, including protein kinases and transcription factors that regulate cellular functions, such as cell cycle progression, cellular differentiation, and the control of cell growth. Therefore, mutations in genes that encode any of the components of this complex and ubiquitous pathway could be implicated in the development of cardiomyocyte hypertrophy.

Hypertrophic Cardiomyopathy in the RASopathies
All of the RASopathies have unique phenotypic characteristics, but also share a number of features, including facial dysmorphism, musculoskeletal, cardiac, ocular, and cutaneous abnormalities, and an increased risk of developing cancer. LV hypertrophy is also a frequent finding, but current data suggest that it is more common with particular mutations. For example, mutations in PTPN11 are reported in 59% of familial and 37% of sporadic cases of NS, but patients with NS that have HCM are much more likely to have mutations in RAF1. The prevalence of HCM in individuals with mutations in other genes encoding components of the RAS-MAPK pathway is highly variable, ranging from <10% to >80%.33,44

Role of RAS-Mitogen Activated Protein Kinase Gene Mutations in Nonsyndromic Hypertrophic Cardiomyopathy
Hitherto, few studies have examined the prevalence of RAS-MAPK gene mutations in patients with nonsyndromic HCM. Two studies have shown that PTPN11 mutations rarely if ever cause isolated HCM,45,46 and there is a single case report of a de novo RAF1 mutation in a child with apparently isolated HCM.13 PTPN11 mutations have been shown to be rarely associated with isolated congenital heart disease.28

The findings in this study suggest that both PTPN11 and RAF1 mutations are an unlikely cause of nonsyndromic HCM. While each of the 5 novel sequence variants identified in this study occurred in highly conserved coding regions and were absent from control samples, in the absence of functional studies, we cannot be sure that all 5 are pathogenic; however, at least 2 (c.144_145delGCinsA in exon 2 of MAP2K1 and V624F in SOS1) are predicted to have significant effects on gene function or occur in regions where pathogenic mutations have been reported previously in patients with NS. The finding in 2 patients of additional mutations in the MYBPC3 gene raises the possibility that even if nonpathogenic, variants in the RAS-MAPK pathway may act as genetic modifiers for sarcomeric mutations, although this needs to be explored with functional studies.

For the 3 families in which there is some segregation data, the results suggest that, if the sequence variants identified are indeed pathogenic, there is incomplete penetrance. The possibility of variable penetrance of RAS-MAPK variants in families with NS has been described previously.47

Clinical Phenotype
The primary aim of this study was to determine the frequency of RAS-MAPK gene mutations in a cohort of children without syndromic features. However, as the initial clinical evaluation was performed by experienced pediatric cardiologists and not clinical geneticists, it is possible that subtle somatic manifestations were missed. Retrospective assessment by an experienced dysmorphologist in 3 patients and with additional 3-dimensional facial photography in 2 of the putative mutation carriers did not demonstrate features of NS in these patients. This suggests that mutations in the RAS-MAPK pathway could play a role in the pathogenesis of apparently idiopathic, nonsyndromic HCM.

Limitations
A limitation of this study is the lack of complete segregation data from the first-degree relatives of those individuals found to harbor RAS-MAPK sequence variations. In addition, as all the mutations identified are novel, expression and functional studies are required to determine their pathogenicity. Furthermore, detailed dysmorphology assessment and 3D facial photography was not possible in all probands, raising the possibility that some may have had syndromic features. However, initial evaluation by the examining physicians did not suggest any dysmorphism, and there were no antenatal features suggestive of NS or related disorders in any of the probands. The incomplete clinical, genetic, and functional data in this study preclude robust conclusions about genotype-phenotype correlations. However, the findings are consistent with the hypothesis that genes involved in the RAS-MAPK pathway are implicated in the pathogenesis of nonsyndromic HCM, and further studies to evaluate this are warranted.

Conclusions
This study identifies novel and potentially pathogenic sequence variants in children with nonsyndromic HCM. These findings have important implications for the evaluation and management of children with HCM, and may provide an insight into the pathogenesis of apparently idiopathic and sarcomeric HCM. Further functional, biochemical, and expression studies are required to evaluate the pathogenic significance of the sequence variants identified, and their possible interaction with sarcomere protein genes.

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insights on pathogenic effects, and genotype-phenotype correlations. 


**CLINICAL PERSPECTIVE**

Hypertrophic cardiomyopathy (HCM) in children can be associated with malformation developmental syndromes, including Noonan syndrome (NS). NS and other related conditions are caused by mutations in genes encoding proteins of the RAS-mitogen activated protein kinase (MAPK) signaling pathway. Although diagnosis of these disorders is based on typical phenotypic features, the dysmorphic manifestations can be subtle and therefore overlooked. This study aimed to determine the prevalence of mutations in RAS-MAPK genes in 78 pre-adolescent children with apparently idiopathic HCM. Five probands (6.4%) carried novel sequence variants in 4 key genes of the RAS-MAPK pathway. Structural and molecular data suggest that these variants may have functional significance. Nine cardiac sarcomere protein genes were also screened; 2 individuals also had mutations in MYBPC. These findings suggest that genetic lesions promoting signaling dysregulation through RAS contribute to disease pathogenesis or progression in children with idiopathic and sarcomeric HCM. In particular, the results raise the possibility that some cases of HCM may represent incomplete phenotypic expression of RAS-MAPK disease. This has important implications for the evaluation and management of children with HCM, and further studies are needed to assess the possible interaction between sarcomere protein and RAS-MAPK genes.
Prevalence of Sequence Variants in the RAS-Mitogen Activated Protein Kinase Signaling Pathway in Pre-Adolescent Children With Hypertrophic Cardiomyopathy
Juan Pablo Kaski, Petros Syrris, Adam Shaw, Krisztina Zuborne Alapi, Viviana Cordeddu, Maria Teresa Tome Esteban, Sharon Jenkins, Michael Ashworth, Peter Hammond, Marco Tartaglia, William J. McKenna and Perry M. Elliott

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