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NGS testing for cardiomyopathy: Utility of adding RASopathy-associated genes

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Abstract

RASopathies include a group of syndromes caused by pathogenic germline variants in RAS-MAPK pathway genes and typically present with facial dysmorphology, cardiovascular disease, and musculoskeletal anomalies. Recently, variants in RASopathy-associated genes have been reported in individuals with apparently nonsyndromic cardiomyopathy, suggesting that subtle features may be overlooked. To determine the utility and burden of adding RASopathy-associated genes to cardiomyopathy panels, we tested 11 RASopathy-associated genes by next-generation sequencing (NGS), including NGS-based copy number variant assessment, in 1,111 individuals referred for genetic testing for hypertrophic cardiomyopathy (HCM) or dilated cardiomyopathy (DCM). Disease-causing variants were identified in 0.6% (four of 692) of individuals with HCM, including three missense variants in the *PTPN11*, *SOS1*, and *BRAF* genes. Overall, 36 variants of uncertain significance (VUSs) were identified, averaging ~3VUSs/100 cases. This study demonstrates that adding a subset of the RASopathy-associated genes to cardiomyopathy panels will increase clinical diagnoses without significantly increasing the number of VUSs/case.

Keywords

BRAF; cardio-facio-cutaneous (CFC) syndrome; dilated cardiomyopathy (DCM); hypertrophic cardiomyopathy (HCM); Noonan syndrome; *PTPN11*; RASopathy; *RAF1* gain; *SOS1*

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DISCLOSURE STATEMENT

The authors are currently or were previously employed by a non-profit, fee-for-service laboratory that offers genetic testing.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Gain-of-function germline variants in the genes encoding the RAS/mitogen-activated protein kinase (RAS-MAPK) pathway proteins cause a group of multisystem developmental disorders termed “RASopathies.” RASopathies include Noonan syndrome (NS; MIM# 163950), NS with multiple lentigines (NSML; previously referred to as LEOPARD syndrome; MIM# 151100), NS-like disorder with loose anagen hair (MIM# 607721), NS-like disorder (MIM# 613563), cardio-facio-cutaneous (MIM# 115150), and Costello syndrome (MIM# 218040) as well as recently emerging associations with the *MRAS*, *RRAS, SOS2*, and *LZTR1* genes (Aoki, Niihori, Inoue, & Matsubara, 2016; Gripp et al., 2016; Higgins et al., 2017; Rauen, 2013). Although each RASopathy has unique characteristics, they share many overlapping clinical features including cardiovascular disease, craniofacial dysmorphology, skeletal and cutaneous abnormalities, and cognitive impairment, each of which presents with varying expressivity. The incidence of cardiovascular disease in individuals with a RASopathy is ~80%, with the most common findings being pulmonic valve stenosis, early-onset hypertrophic cardiomyopathy (HCM), and atrial septal defect (Prendiville et al., 2014). Gene–phenotype correlations have identified a higher frequency of HCM (75%–85% and 56%–75%) in individuals with pathogenic variants in the *RAF1* and *RIT1* genes, respectively (Aoki et al., 2013; Gelb, Roberts, & Tartaglia, 2015; Yaoita et al., 2016). In addition, *RAF1* was recently associated with pediatric-onset dilated cardiomyopathy (DCM) (Dhandapany et al., 2014).

Establishing a diagnosis of a RASopathy disorder is important for appropriate clinical and reproductive management of patients, but can be challenging due to the variable expressivity as well as clinical overlap between the various RASopathies. On the mild end of the spectrum, affected individuals may present only with mild facial dysmorphology, a heart defect, and/or short stature. The facial features associated with RASopathies can be particularly subtle, especially at birth, and can transform with age, becoming less evident in adulthood (Roberts, Allanson, Tartaglia, & Gelb, 2013). Therefore, the clinical features of a RASopathy can be overlooked in mild cases, resulting in a diagnosis of isolated cardiomyopathy. While previous studies suggested that there may be no clinical utility in screening individuals with isolated HCM for variants for in RASopathy-associated genes, as either no variants or only variants of uncertain significance (VUS) were identified (Kaski et al., 2012; Roberts et al., 2005), a few individuals with apparently isolated HCM or DCM have been reported to harbor disease-causing *RAF1* variants (Dhandapany et al., 2014; Pandit et al., 2007). However, these studies were limited by the small cohort size and/or the number RASopathy-associated genes included in the analysis. In anticipation of increasing their clinical detection rates, some clinical laboratories have added a subset of the RASopathy-associated genes to their cardiomyopathy NGS panels, though there are no guidelines or recommendations on which genes or related diseases, if any, to add. To investigate the utility and burden of testing the RASopathy-associated genes in individuals referred for genetic testing of cardiomyopathy, we tested a cohort of 1,111 individuals referred for molecular genetic testing of HCM ($n = 692$) or DCM ($n = 419$) for disease-causing variants and VUSs in 11 RASopathy-associated genes.

All individuals were referred to the Laboratory for Molecular Medicine for diagnostic testing for inherited cardiomyopathy (see Supp. Table S1 for genes tested). Individuals with a clinical diagnosis of Wolff–Parkinson–White syndrome, amyloidosis, Pompe disease,

Fabry disease, other biochemical disorder, or a myopathy were excluded from this study due to having an expected, nonoverlapping genetic cause. Twelve individuals, who had features suggestive of a syndromic form of disease (described in Supp. Table S2), were retained. Overall, the cohort is 61% Caucasian and 57% male with 28% under the age of 19 (see Supp. Table S3 for more demographics). The cohort included 692 individuals with a clinical diagnosis or suspicion of HCM and 419 individuals with a clinical diagnosis or suspicion of DCM. Prior testing of cardiomyopathy-associated genes identified a disease-causing variant in 275 of individuals (DCM sub-cohort = 100, HCM sub-cohort = 175), which were included in the study to determine VUS detection rates. DNA samples, extracted from peripheral blood for each individual, were de-identified and tested via next-generation sequencing (NGS) for variants in 11 RASopathy-associated genes: *BRAF* (NM_004333.4; MIM# 164757), *CBL* (NM_005188.3; MIM# 165360), *HRAS* (NM_005343.2; MIM# 190020), *KRAS* (NM_004985.3; MIM# 190070), *MAP2K1* (NM_002755.3; MIM# 176872), *MAP2K2* (NM_030662.3; MIM# 601263), *NRAS* (NM_002524.4, MIM# 164790), *PTPN11* (NM_002834.4; MIM# 176876), *RAF1* (NM_002880.3; MIM# 164760), *SOS1* (NM_005633.3; MIM# 182530), and the only known pathogenic (c.4A>G (p.Ser2Gly)) variant in exon 2 of *SHOC2* (NM_007373.3; MIM# 602775). Copy number variants (CNVs) were identified via an NGS-based detection tool (VisCap) (Pugh et al., 2015). The clinical significance of the variants was classified based on ACMG/AMP standards (Richards et al., 2015) adapted for RASopathies by an expert panel of the Clinical Genome Resource (ClinGen) (Gelb et al., 2018). VUSs were further subcategorized into three categories: VUS-favor pathogenic when there is a suspicion of a pathogenic role but insufficient evidence to classify the variant as likely pathogenic, VUS-favor benign when the evidence suggests the variant does not contribute to disease but is insufficient to classify it as likely benign, and VUS when there is a lack of or conflicting evidence. Pathogenic variants, likely pathogenic variants, and CNVs were confirmed by either Sanger sequencing or droplet digital PCR as previously described (Ceyhan-Birsoy et al., 2016). The CNV in Case 5 was further analyzed by microarray analysis via SurePrint G3 Human comparative genomic hybridization (CGH) microarray 4 × 180K (AMDID 024409), run per the manufacturer's protocol (Agilent, Santa Clara, CA). CNV detection required a five-probe call with a minimum absolute average log2 ratio of 0.25. All variants identified were submitted to ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/). This study was approved by the Partners HealthCare Institutional Review Board.

Sequencing of 11 RASopathy-associated genes in the cohort identified three variants of clinical significance (i.e., pathogenic and likely pathogenic), two CNVs, and 36 VUSs/VUSs-favor benign. All identified variants of clinical significance have previously been associated with RASopathies. Four (0.6%) individuals in the HCM sub-cohort had a pathogenic or likely pathogenic missense variant in the *PTPN11* ($n = 2$), *SOS1* ($n = 1$), or *BRAF* ($n = 1$) gene, while one had a CNV of 3p25.2, including a partial gain of the *RAF1* gene (Table 1). No variants of clinical significance were identified in the DCM sub-cohort, but one individual had a CNV including a whole gene gain of *RAF1*. In addition, ~3% of individuals (DCM sub-cohort = 13, HCM sub-cohort = 23) had a VUS or VUS-favor benign (Supp. Table S4). No individual had more than one VUS in the RASopathy-associated genes, though some VUSs were identified in more than one person.

Four individuals with HCM carried variants of clinical significance. Cases 1 and 2 carried the pathogenic c.1528C>G (p.Gln510Glu; ClinVar ID: 40566) variant in *PTPN11*. This variant has been previously associated with NS and NSML presenting with severe HCM and progressive heart failure (Digilio et al., 2006; Faienza, Giordani, Ferraris, Bona, & Cavallo, 2009; Lehmann et al., 2009; Limongelli et al., 2008; Takahashi et al., 2005). At the time of testing, Case 1 was a 5-month-old male with a clinical diagnosis of concentric HCM and failure to thrive, who had also failed the newborn hearing screen and sequential hearing screens. Case 2 was a 15-year-old male with HCM and a clinical and genetic diagnosis of Klinefelter syndrome. As patients with Klinefelter syndrome typically have tall stature, gynecomastia, delayed puberty, and developmental delay, the combination of a RASopathy and Klinefelter syndrome may have masked the RASopathy features. Interestingly, the allelic fraction for the c.1528C>G variant in Case 2 was 38% (read depth at this position of 666) via NGS (Table 1), raising the possibly of mosaicism which could also contribute to the lack of apparent RASopathy features. The Sanger sequencing demonstrated an allelic fraction of 30% in the forward and 44% in the reverse (Supp. Figure S1). Due to the de-identified nature of this sample, additional tissues for possible mosaicism studies were unavailable. Case 3, a patient diagnosed with HCM at birth, carried the likely pathogenic c.1447A>C (p.Lys483Gln; ClinVar ID: 40369) variant in the *BRAF* gene. It is possible that the features of a RASopathy may have been overlooked in these individuals due to their young age or because they had another genetic disease, leading to a clinical diagnosis of isolated HCM. However, due to the deidentified nature of these samples and the limited clinical information, restricted to that on the requisition forms, this cannot be concluded at this time.

Case 4 presented with late onset of HCM at 82 years of age and carried the pathogenic c.1656G>C (p.Arg552Ser; ClinVar ID: 40682) variant in the *SOS1* gene. The allelic fraction for the c.1656G>C variant was 24% via NGS, suggestive of mosaicism. Sanger sequencing was consistent (Supp. Figure S2). To our knowledge, this is the first report of possible mosaicism for a disease-causing *SOS1* variant. Since the level of mosaicism often varies in different tissues, the mosaic state of this variant may have resulted in a presentation of nonsyndromic HCM. Alternatively, since the level of mosaicism for the *SOS1* variant in the heart muscle is unknown and the risk of HCM in individuals with *SOS1* variants is low, there may also be other factors contributing to the HCM in this individual that were not detected on the previously run HCM NGS panel.

Cases 5 and 6 carried CNVs that include the *RAF1* gene and were classified as VUSs. Copy number gains including the *RAF1* gene have been previously associated with cardiac phenotypes (Greenway et al., 2009; Lissewski, Kant, Stark, Schanze, & Zenker, 2015; Luo et al., 2012); although, those reported cardiac findings did not include cardiomyopathies. In addition, it is unclear if the *RAF1* gene in those CNVs is responsible for those cardiac phenotypes and, furthermore, current evidence suggests that these gains are not associated with a RASopathy phenotype (Lissewski et al., 2015). Case 5 was subsequently tested by array CGH (aCGH), which demonstrated that the gain of 3p25.2 (hg19, Chr3:12,630,854–12,777,380) included most of the *RAF1* gene (exons 1–12 on NM_002880.3) and extended into the neighboring *TMEM40* gene (Supp. Figure S3). A similar gain has been reported in an individual with tetralogy of fallot (Patient 419; Greenway et al., 2009), but whether this

CNV contributes to the cardiac finding in either individual (Patient 419 or Case 5) remains unknown.

The CNV in Case 6, who was diagnosed with infantile-onset DCM and left ventricular non-compaction cardiomyopathy (LVNC), included the entire *RAF1* gene. Interestingly, HCM- and DCM-associated *RAF1* variants have been reported to be functionally distinct, with the DCM-associated variants having reduced kinase activity compared to HCM-associated variants and unaltered residues critical to regulation (Dhandapany et al., 2014). An increase in copies of the entire *RAF1* gene could theoretically result in increased expression and possibly increased kinase activity, though reduced when compared with the kinase activity of HCM-associated variants. In addition to this gain, Case 6 also carried a pathogenic missense variant in *MYH7* (c.2146G>A (p.Gly716Arg) on NM_000257.3; ClinGen ID 14105), but whether it is sufficient to explain the early-onset DCM in this individual is unclear. The c.2146G>A variant in *MYH7* has previously been described in multiple families with HCM, but not DCM (Anan et al., 1994; Hwang et al., 1998). It may be possible that the *RAF1* gain modified disease in Case 6 leading to the infantile-onset of disease. However, because the functional impact of the *RAF1* gain has yet to be determined, its clinical significance remains uncertain.

Our results demonstrate the benefit of testing RASopathy-associated genes in individuals with apparently non-syndromic HCM, particularly infants. However, there appears little, if any, benefit to testing individuals with DCM. Even though the individuals discussed had a reported diagnosis of isolated HCM, it is likely that the other features of RASopathies were overlooked, especially in the infants. Since the clinical information presented here was limited to that provided on test requisition forms, the presence of other nondisclosed RASopathy-associated features in these individuals cannot be ruled out. However, since the clinicians only referred these individuals for molecular testing for inherited cardiomyopathies, and not RASopathy testing, the latter seems less likely. These data suggest that infants with apparently isolated HCM should be tested for RASopathy disorders. In addition, the older age of Case 4 (82 years of age) suggests that testing RASopathy-associated genes may also beneficial, though limited, for individuals with late-onset HCM as some of these individuals may be mosaic or have subtle RASopathy features that were overlooked. One limitation of this study is that we did not include all RASopathy-associated genes that have also been associated with HCM, such as *RIT1* and *MRAS*. Adding these additional genes to the cardiomyopathy NGS panel is expected to further increase the clinical utility of the panel.

While increasing the diagnostic yield is critical when designing multi-gene panels, the possible increase in inconclusive findings needs to be carefully examined. In our cohort, ~3% (36 out of 1,111) of cases had a VUS/VUS-favor benign in a RASopathy-associated gene, and no case had more than one VUS identified. Many of these VUSs could have been reclassified if familial studies were possible, further reducing the number of VUS/case. This study demonstrated that adding the 11 genes tested here increased the clinical utility by 0.6% and that this did not significantly add to the follow-up or cost associated with this test. While this small increase in VUSs would not be burdensome for the clinical laboratory,

other stakeholders, such as ordering providers and patients, must weigh in on the burden of these VUSs in the clinic.

While the overall ratio of cardiomyopathy patients who had disease-causing RASopathy variants was modest, the diagnosis of a RASopathy disorder dramatically changes the patient's clinical management compared to an individual with non-syndromic cardiomyopathy. Patients with RASopathies need to be followed with a routine evaluation of multiple systems throughout life, including complete physical and neurologic examination, ophthalmologic and hearing evaluation, coagulation screening, radiographic assessments, multidisciplinary developmental evaluation, and consider surveillance for malignancies in individuals with Costello syndrome, variants in *CBL*, or specific variants in *PTPN11* or *KRAS* known to be associated with myeloproliferative disorder/JMML (Rauen, 2013; Roberts et al., 2013; Villani et al., 2017). In addition, as many of the other RASopathy disorders are associated with a mild increase risk of cancer, increased awareness of possible malignancies and prompt assessment when suspicious clinical symptoms are present is justified (Villani et al., 2017). Testing of RASopathy genes in patients with cardiomyopathy may lead to improved diagnosis of individuals that are on the milder end of this phenotypic spectrum and would give critical information to guide their appropriate clinical and reproductive management.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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TABLE 1

RASopathy variants of potential clinical significance and copy number variants identified

	Clinical diagnosis									
Case	**Sex**	**Age**	**Cardiac features**	**Additional features**	**FH**	**Gene**	**Variant**	**Classification**	**AF**	**Reference**
SNVs										
1	M	4 mo	HCM	Failure to thrive and possible hearing loss	Unk	*PTPN11*	c.1528C > G (p.Gln510Glu)	Pathogenic	0.46	Digilio et al. (2006); Faienza et al. (2009); Lehmann et al. (2009); Limongelli et al. (2008); Takahashi et al. (2005)
2	M	15 yrs	HCM	Klinefelter syndrome	Unk	*PTPN11*	c.1528C > G(p.Gln510Glu)	Pathogenic	0.38	Digilio et al. (2006); Faienza et al. (2009); Lehmann et al. (2009); Limongelli et al. (2008); Takahashi et al. (2005)
3	F	1 mo	HCM		Unk	*BRAF*	c.1447A > C (p.Lys483Gln)	Likely pathogenic	0.5	Hakami et al. (2016)
4	F	82 yrs	HCM		Unk	*SOS1*	c.1656G >C (p.Arg552Ser)	Pathogenic	0.24	Beneteau et al. (2009); Slezak et al. (2010); Tartaglia et al. (2007); Zenker et al. (2007)
CNVs										
5	M	48 yrs	HCM		HCM	*RAF1/ TMEM40*	Partial gene gain	VUS	NA	Greenway et al. (2009)
6	M	1 yr	DCM/LVNC		Unk	*RAF1*	Whole gene gain	VUS	NA	Lissewski et al. (2015); Luo et al. (2012)

FH, family history; AF, allelic fraction via NGS; F, female; M, male; mo, month; yr, year; Unk, unknown; NA, not applicable; REF, references related to variant; *BRAF* NM_004333.4; *PTPN11* NM_002834.4; *SOS1* NM_005633.3.