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ClinGen's RASopathy Expert Panel Consensus Methods for Variant Interpretation

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Abstract

Purpose—Standardized and accurate variant assessment is essential for effective medical care. To that end, Clinical Genome (ClinGen) Resource clinical domain working groups (CDWG) are systematically reviewing disease-associated genes for sufficient evidence to support disease causality and creating disease-specific specifications of ACMG-AMP guidelines for consistent and accurate variant classification.

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Disclosures

Many authors are clinical service providers and are employed by laboratories that offer fee-based clinical sequencing. This employment is noted in the author affiliations.

Conflict of Interest

The authors declare no additional conflicts of interest beyond their employment affiliation.

Supplementary information is available at the *Genetics in Medicine* website.

Methods—The ClinGen RASopathy CDWG established an expert panel (EP) to curate gene information and generate gene and disease-specific specifications to ACMG-AMP variant classification framework. These specifications were tested by classifying 37 exemplar pathogenic variants plus an additional 66 variants in ClinVar distributed across nine RASopathy genes.

Results—RASopathy-related specifications were applied to sixteen ACMG-AMP criteria, with five also having adjustable strength with availability of additional evidence. Another five criteria were deemed not applicable. Key adjustments to minor allele frequency thresholds, multiple *de novo* occurrence events and/or segregation, and strength adjustments impacted 60% of variant classifications. Unpublished case-level data from participating laboratories impacted 45% of classifications supporting the need for data sharing.

Conclusions—RAS-specific ACMG-AMP specifications optimized the utility of available clinical evidence and Ras/MAPK pathway-specific characteristics to consistently classify RASopathy-associated variants. These specifications highlight how grouping genes by shared features promotes rapid multi-genic variant assessment without sacrificing specificity and accuracy.

Keywords

RASopathy; ClinGen; variant interpretation; Ras/MAPK; Noonan

INTRODUCTION

With recent advances in sequencing technologies, generating genetic data is rapidly becoming cheaper, faster, and utilized across both clinical and research laboratories; however, clinical interpretation of variation remains subjective and complex, limiting accuracy and consistency. Interpretations can differ based on inter-laboratory classification rules, access to unique case-level data, and other evidence.[1, 2] Funded by the National Institutes of Health, the Clinical Genome Resource (ClinGen; www.clinicalgenome.org) aims to elucidate, standardize, and archive clinical genetic knowledge and relevance of genetic variation for public use. A major standardization effort was the joint release of variant interpretation guidelines by the American College of Medical Genetics and Genomics (ACMG) and the Association of Molecular Pathology (AMP) in 2015.[3] These ACMG-AMP guidelines outline an evidence-based, quantitative framework to inform variant classification, but evaluation of its across-user reliability highlights the need for guidance and expert judgment in its application.[2] More recently, clinical laboratories resolving variant discrepancies estimated ~13% of variant classification differences remained discordant due to differing use of these guidelines.[1] In addition, differing allele frequency thresholds, which inform benign classifications, impact 9% of initial discrepancies. Thus, additional guideline specificity remains essential to providing accurate and consistent variant interpretations necessary for effective medical care. To improve specificity, ClinGen disease-specific working groups are systematically reviewing disease-associated genes for key evidence that supports disease causality and then creating disease-specific specifications of ACMG-AMP guidelines for variant classification.[4]

The ClinGen RASopathies Expert Panel (RAS EP) focuses on providing disease-specific recommendations for the genetically heterogeneous disorders caused by pathogenic variants in genes within the Ras/mitogen-activated protein kinase (Ras/MAPK) pathway. These phenotypically-related disorders, collectively termed RASopathies, include Noonan syndrome (NS), Noonan syndrome with multiple lentigines (NSML, formerly known as LEOPARD syndrome), Costello syndrome, cardio-facio-cutaneous (CFC) syndrome, and Noonan-like syndromes resulting from pathogenic variants in *BRAF, CBL, HRAS, KRAS, LZTR1, NF1, NRAS, MAP2K1, MAP2K2, PTPN11, RAF1, RIT1, SHOC2, SOS1, SOS2, and others*.[5-9] Most pathogenic variants produce an abnormal gain-of-function (GOF) effect that dysregulates Ras/MAPK pathway signaling and are inherited in an autosomal dominant manner with complete penetrance and variable expressivity; however, some genes (e.g., *NF1*, *LZTR1*) have been associated with loss-of-function (LOF)[10, 11] and/or autosomal recessive inheritance (unpublished data). NS, the most common of the RASopathies, is estimated to affect 1:1000 to 1:2500 individuals and has multi-system involvement with principal features including characteristic facial anomalies with hypertelorism and downslanted palpebral fissures, variable intellectual disabilities, skeletal involvement (*e.g.*, pectus deformity and short stature), and cardiovascular abnormalities.[12] Cardiovascular defects, observed in roughly 50-80% of NS individuals, include pulmonary valve stenosis, hypertrophic cardiomyopathy, and other congenital heart defects usually presenting early in life.[13] Neurodevelopment varies substantially from normal to ~25% of affected individuals having learning disabilities.[14] NS affects the ectodermal, hematopoietic, lymphatic, gastrointestinal and genitourinary systems, and entails a predisposition to certain malignancies. Other RASopathies share these overlapping phenotypical features with varying severity and expressivity.

To date, more than 35 clinical laboratories across many countries offer multi-gene, next generation sequencing (NGS)-based genetic testing panels for the RASopathies (www.genetests.org; www.ncbi.nlm.nih.gov/gtr/). Some genes have established hotspots (*e.g.*, missense defects altering Asn^{308} in *PTPN11* or Ser^{257} in *RAF1*); however, many NS-causing variants have limited observations in affected individuals. Although these genes are highly evolutionarily conserved, missense variants are observed in general population cohorts (*e.g.*, Exome Aggregation Consortium (ExAC; http://exac.broadinstitute.org/)).[15] Most observed variation in these cohorts is benign as the frequencies are higher than the general population prevalence of NS; however, observations of ultra-rare missense variation remains confounded as affected individuals with milder phenotypes and unaware of an existing genetic condition may be included.

Familial testing is informative for classification when a variant is inherited from a well-phenotyped unaffected parent, segregates with disease in affected family members, or occurs *de novo* in sporadic cases, which is estimated to occur in ~50% of NS cases[12]. The predicted effect of a novel variant on protein function influences the likelihood that an ultra-rare variant is disease causing. Missense variants underlying NS have GOF (i.e., hypermorphic or neomorphic) effects on Ras/MAPK signaling, and thus can be assessed through functional analysis of the Ras pathway using activation/inactivation mechanisms and/or catalytic functions.[5] On the other hand, *in silico* informatic approaches predicting tolerance of amino acid substitutions depend highly on evolutionary conservation and, given Ras pathway

proteins are highly conserved, most variation is deemed not tolerated, thus limiting the specificity of those algorithms for the RASopathies.

Here, we report the RAS Expert Panel's (EP's) results in curating gene and Ras/MAPK pathway-specific information and generating ACMG-AMP disease specific guidelines for assessing variation associated with GOF effects in RASopathy genes.

MATERIALS AND METHODS

ClinGen RASopathy Expert Panel

The RAS EP membership represents a diverse range of expertise and qualifications including medical geneticists, research scientists, and clinical laboratory diagnosticians. Additionally, representatives from clinical diagnostic laboratories contribute to curating case-level data, variant classifications, and other RAS EP projects. Members divided into smaller teams to support gene groups with similar structure and/or function. The EP members and support team span national and international institutions in three countries (USA, France, and Germany) and welcome public participation. All RAS EP members were subjected to disclosure of potential conflicts of interest and are required to maintain disclosure with ClinGen.

Data Sources for Variant Classification

Genes and variants were curated using publically available data sources and *in silico* prediction algorithms listed in Table S1. Variants were prioritized for classification due to well-established pathogenic classifications or presence in ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/). A variant's filtering allele frequency (FAF), representing its statistically corrected population frequency, was obtained from ExAC[15, 16]. NHLBI Exome Sequencing Project Exome Variant Server (ESP EVS) was used rarely for frequencies of insertion/deletion variants.[17] Clinical and research laboratories submitted internal data for review during variant interpretations or their data was extrapolated from ClinVar. Published data were obtained from relevant manuscripts.

Gene Parameters

RASopathy-associated genes and conditions in the literature are listed in Table S2.[5-9] RAS EP gene-specific teams focused on defining parameters beneficial to variant classification of GOF effects for *BRAF, HRAS, KRAS, MAP2K1, MAP2K2, PTPN11, RAF1, SHOC2*, and *SOS1*. Assessed parameters include approved HUGO Gene Nomenclature Committee (HGNC) symbol, primary clinically relevant transcript, and associated RASopathy conditions. Dosage sensitivity available from the ClinGen Dosage Sensitivity Map was reviewed and accepted by the RAS EP. Additional curated information included identification of key functional domains, functional assays, and animal models. Gene parameters were recorded in Table S6. Application of ClinGen's gene-disease validity framework[18] to define the strength of evidence for the association between each RASopathy gene and condition, in which at least one claim was made, is in progress.

Specification of ACMG-AMP Criteria

Each ACMG-AMP criterion[3] was addressed for potential disease or gene-specific specifications based on RAS EP expertise in evaluating variants in these genes and relative to the pathogenicity, incidence, and GOF disease mechanism of the RASopathies. NS was utilized as the representative disorder given it is the most common and well-studied disorder. Criteria specifications included gene or disease-specific specifications, strength adjustments for surplus evidence, and judgement of criteria not applicable to the RASopathies (summarized in Table 1). Remaining criteria were used as recommended.

The RAS EP collaboratively engaged with ClinGen initiatives and WGs, including the Sequence Variant Interpretation (SVI) WG and the Cardiomyopathy WG, to share and review specifications in order to maintain consistency and integrity of ACMG-AMP criteria application.

Specification Validation and Variant Classification

The EP performed retrospective analysis of 37 exemplar pathogenic variants distributed across the aforementioned genes to highlight evidence requirements supporting pathogenicity. Classification of an additional 66 ClinVar variants validated final ACMG-AMP specifications.

Minor allele frequency (MAF) thresholds were calculated as described in the Results section (see Table S3) and validated by evaluating the general population frequency of common pathogenic variants and/or hotspot positions. Briefly, over 5000 diagnostic cases with reported pathogenic variants in the GeneDx (Gaithersburg, Maryland) internal database were compiled, and variants were grouped by the codon they altered (data not shown). The top three codons with pathogenic variation in each gene were evaluated for allele frequencies in three large population cohorts: ExAC, ESP EVS, and 1000 Genomes[15, 17, 19] in order to confirm benign allele frequency thresholds were not attained.

The RAS EP classified variants grouped into three categories: 1.) well-established pathogenic variants (n=37), 2.) variants with consistent (concordant) classifications in ClinVar across clinical laboratories (n=28), and 3.) variants with inconsistent (discrepant) classifications in ClinVar (n=38). First, each gene-specific team reviewed well-established pathogenic variants, typically with excessive functional and/or case-level data to determine criteria with abundant evidence supporting pathogenicity. Variants were re-reviewed using criteria strength specifications to ensure pathogenicity was retained in the absence of standard strong criteria. Any issues with criteria application or ambiguous data were reviewed by the RAS EP to improve specificity. Next, group 2 and 3 variants were used to validate proposed specifications. Final minor criteria adjustments occurred as needed based on expertise judgment of potentially unexpected classifications. The resultant approved specifications are reported herein.

For each variant, publicly available information and case-level data were collected through clinical and research laboratory contributions. Laboratories were encouraged to provide preliminary criteria assessments based on their available information. Gene-specific teams reviewed the cumulative data and relevant application of the modified ACMG-AMP criteria

for accuracy. If the team unanimously approved of criteria usage, then criteria were combined per ACMG-AMP rules for final variant class. If criteria application was unclear or contested, the team presented evidence to the RAS EP for review. If needed, official polling of criteria application or variant classification was completed. Final applied criteria and approved classifications required complete consensus by gene-specific teams or an 80% quorum vote by the RAS EP. If these conditions were not met, the RAS EP deferred assignment of an official classification.

RESULTS

ACMG-AMP criteria were established as general guidelines for interpreting variants for Mendelian disorders, so these broad and highly conservative criteria inevitably created disparities in their application. Reviewing ACMG-AMP guidelines in the context of a specific group of genes like those involved in the RASopathies revealed two distinct approaches for solving potentially ambiguous usage. First, initial specifications established a basic infrastructure for criteria specification relative to any related or unrelated genes that share the same inheritance pattern (*e.g.*, autosomal dominant), general prevalence (*e.g.*, rare), and disease mechanism (*e.g.*, GOF). This approach provided significant utility in binning groups of genes sharing common underlying genetic characteristics for rapid variant classification in large data sets. Second, unique specifications including additional disease and/or gene-specific assessments further refined classification precision when genes share similar features like overlapping functionality within a cellular process, signaling pathway, or protein structure. Utilizing either or both of these approaches can rapidly streamline interpretation workflows when genes, such as those involved in the RASopathies, are analyzed together in a clinical setting.

The RAS EP reviewed each ACMG-AMP criteria for its applicability to the RASopathies. Specifications (or lack thereof) were categorized into five major areas: 1.) not applicable criteria, 2.) no changes, 3.) disease-specific, 4.) gene-specific, and 5.) strength adjustable with extra evidence. Disease-specific ACMG-AMP specifications were adjusted relative to inheritance, prevalence, and GOF disease mechanism, while other criteria were deemed not applicable to the RASopathies. Gene-specific specifications highlighted criteria that uniquely apply based on the Ras/MAPK pathway and protein characteristics. For strength, the RAS EP recognized that certain criteria such as *de novo* occurrences in affected individuals were crucial evidence supporting pathogenicity, and, as these events accumulated, the likelihood a variant was pathogenic increased. Summarized RAS EP ACMG-AMP pathogenic and benign criteria specifications are listed in Table 1.

ACMG-AMP Specifications and Gene Curation

Gene curation produced key information relevant to ACMG-AMP specifications and variant classification. All curated information discussed henceforth is summarized within Table S6.

Based on the GOF disease mechanism and highly conserved nature of these genes, some disease- and gene-specific adjustments were assessed quickly. Supporting criterion PP2 (genes with a low rate of benign missense variation) was considered applicable to all genes. Supporting benign criterion BP1 (missense variant in a gene where only LOF cause disease)

is not applicable given GOF variants are typically missense; however, in keeping with its rationale, we redefined BP1 to LOF variants in a gene where only GOF cause disease. The BP7 definition related to synonymous variants was expanded to include non-canonical intronic and regulatory variants, which are similarly correlated in likelihood of disease causality.

Other criteria were deemed not applicable. PM3 and PP4 are not applicable due to the RASopathies being a genetically heterogeneous group of autosomal dominant disorders. Additionally, a reputable database of RASopathy variant interpretations without supporting evidence does not exist; therefore, criteria PP5 and BP6 are not applicable.

Upon reviewing dosage sensitivity data, PVS1 (related to LOF variants) was also judged not applicable when assessing pathogenicity for RASopathies due to GOF effects. *PTPN11* was the only gene with sufficient evidence supporting haploinsufficiency; however, this is associated with autosomal dominant metachondromatosis. Predicted LOF or null alleles in *PTPN11* should be assessed using unmodified ACMG-AMP criteria. No gene had sufficient evidence supporting triplosensitivity, so criteria were not adjusted for this mechanism.

Gene-specific specifications were based on five subgroups sharing similar function and/or structure: 1.) *PTPN11*, 2.) *BRAF/RAF1*, 3.) *HRAS/KRAS/NRAS*, 4.) *MAP2K1/MAP2K2*, and 5.) *SOS1/SOS2*. Genes within these groups often share analogous residues, thus a known functional residue in one gene is equivalent in function to other genes within that subgroup. Using this logic, any pathogenic variant in one gene should have analogous pathogenic residues in other subgroup genes. ACMG-AMP criteria utilizing this logic are PS1 and PM5. Additionally, PM1 usage (*i.e.*, mutational hotspots and/or critical and well-established functional domains) was explicitly defined for each gene and/or subgroup.

Appropriate functional assays for assessing pathogenicity primarily measured Ras/MAPK pathway dysregulation through increased phosphorylation of ERK or MEK. Most approved animal models exhibited dysmorphic or craniofacial anomalies and a cardiac phenotype. Detailed guidance for approved functional assays for application of PS3 is described in supplemental Table S6.

Disease-Specific and Strength Specifications of ACMG-AMP Classification Criteria

Assessments of allele frequencies (BA1, BS1, and PM2)—The standard ACMG-AMP threshold for applying BA1 was set at a highly conservative value of 5%. BS1 has a standard definition that the MAF is greater than expected for the disorder; however, defining expected remains subjective when factoring in genetic and allelic heterogeneity, penetrance, and other contributions. We established BA1, BS1, and PM2 by evaluating the generally accepted 1:1000 to 1:2500 prevalence range of NS[12] and validated these thresholds by retrospective review of MAFs of known pathogenic variants. This prevalence range is an estimate of the true prevalence and observed prevalence, respectively, where true prevalence reflects the assumption that NS is often considered underdiagnosed or unascertained in the general population. To address this potential ascertainment bias, MAFs were assessed over varying values of bias (Tables S3A and S3B). Ultimately, the estimated true prevalence of 1:1000 at full ascertainment was equivalent to 40% ascertainment bias in the observed

prevalence of 1:2500. The RAS EP agreed that the likelihood of 60% of unascertained affected individuals in the population was dubious. Thus, the extremely conservative prevalence of 1:1000 at 100% ascertainment was approved to calculate the stand-alone MAF (BA1) as 0.0005 for these autosomal dominant, fully penetrant disorders.

The genetic and allelic heterogeneity of NS afforded additional means to adjust MAF thresholds for BS1. We refined BS1 MAF by incorporating gene contributions to NS. Pathogenic variants in *PTPN11* were estimated as the highest contributor to NS causing ~50% of all cases; thus, at a prevalence of 1:1000 with full ascertainment, the MAF of all *PTPN11* pathogenic alleles is estimated to be 0.00025. Given this extremely low threshold, it is neither beneficial nor necessary to further refine individual or allelic contribution values for each gene. Therefore, 0.00025 was established as BS1 MAF threshold for all genes.

To validate MAFs, the RAS EP retrospective reviewed the prevalence of the most common pathogenic variants observed in clinical testing (Table S5). Given that there are known hotspot codons, the EP evaluated the combined MAF at a given codon versus independent allelic substitutions to ensure thresholds were conservative. At most one pathogenic allele at a given codon was observed in any population cohort with a minimum of 1000 individuals, [15, 17, 19] thus validating these MAF thresholds while recognizing that an occasional individual could be undiagnosed in the general population. Furthermore, a variant, *PTPN11* p.Arg265Gln, presenting in patients with likely unrecognized mild phenotypic features[20] also fell below these conservative thresholds. These assessments concluded that a variant should be completely absent from large population cohorts for PM2 usage.

Observation of multiple cases of de novo events (PS2 and PM6)—Strength of *de novo* case evidence corresponds to whether parentage is confirmed (PS2) or presumed (PM6). Knowing >50% of NS cases are sporadic, multiple cases may be observed, some with and some without parental confirmation, making it difficult to navigate when to apply PS2 and/or PM6. Therefore, we developed a series of criteria across PS2 and PM6 to denote various combinations of independent *de novo* occurrences, with and without parental data, guiding how to modulate the strength of evidence. For example, documentation of three affected individuals with presumed *de novo* events (PM6) should equal strong criteria, as it is highly unlikely that all cases have misattributed parentage. Very strong evidence of pathogenicity is supported with at least two confirmed cases or having one confirmed case in conjunction with two presumed. Dual application of both unmodified PS2 and PM6 is only acceptable with a singleton occurrence in each category.

Increased prevalence of variant in probands versus controls (PS4)—Criteria PS4 designates that variants with significantly higher prevalence in cases versus controls is strong evidence. Due to a potential lack of historical case-control studies, an added caveat allows for application of moderate evidence in instances where very rare variants were observed in multiple unrelated individuals with the same phenotype but absent from large population cohorts such as ExAC. Following the spirit of this caveat and given the inability to use PP4 due to genetic heterogeneity, the EP defined PS4 as an observation of at least five unrelated probands with similar phenotype as sufficient. Moderate and supporting criteria usage was defined as three to four and one to two unrelated probands, respectively. Every

effort should be made to ensure that probands assessed from the literature are unique cases with a valid and relevant phenotype and no other reportable or potentially pathogenic variants are observed.

Observation of multiple segregations of phenotype in affected family members (PP1)—Criterion PP1 allocates supporting evidence when a variant co-segregates with disease and allows increasing strength with increasing co-segregations. The RAS EP emulated the statistical approach of the ClinGen cardiomyopathy (CMP) MYH7 EP (*in press*), which specified three levels of evidence using autosomal dominant likelihood ratios of 10 (3 meioses, LOD 0.9), 30 (5 meioses, LOD 1.5), and 100 (7 meioses, LOD 2.1) to count as supporting, moderate, and strong evidence, respectively. For application, the variant must be absent from large populations (*i.e.*, meet PM2 requirements). The EP recommends segregation observations in at least two separate families to decrease the likelihood that the identified variant is in linkage disequilibrium with an unidentified, truly causative variant or have additional evidence supporting the variant as being causative (*e.g.*, *de novo* occurrences or functional studies) and not the locus. This issue is being further defined by the ClinGen SVI WG.

Performance of the RAS EP ACMG-AMP Specifications in Variant Classification

Over 100 variants were classified using the modified RAS EP ACMG-AMP criteria presented here. These variants fell into three categories: 1.) well-established pathogenic variants (n=37), 2.) variants with consistent (concordant) classifications in ClinVar by clinical laboratories (n=28), and 3.) variants with inconsistent (discrepant) classifications in ClinVar (n=38).

Well-established pathogenic variants (Group 1) achieved a pathogenic classification without using modified criteria; however, additional evidence available for modified criteria usage was noted and compared. The typical evidence supporting pathogenicity included PS2 (35%), and PS3 (81%), PM1 (76%), PM2 (97%), and PM6 (70%). Other criteria applied include PS1 (3%) and PP1 (14%). Given that PVS1 is not applicable to these genes, one strong criterion is required to classify a variant as pathogenic. Thus, if functional studies were unavailable, 54% (20/37) of these well-established pathogenic variants would not reach a pathogenic classification using standard ACMG-AMP criteria; however, use of strength specifications would recoup pathogenicity for 45% (9/20). This reinforced our strength specifications with additional criteria evidence.

ClinVar variants with concordant (n=28) or discordant (n=38) calls were compared relative to use of specified or unspecified criteria. Adjustment of MAF thresholds automatically classified 41% (27/66) of variants into the benign spectrum (Figure 1). These thresholds impact resolution of 37% (14/38) of discrepant ClinVar variants. No variant meeting BA1 or BS1 had conflicting evidence supporting pathogenicity. PM1 for curated functional domains and hotspots applied to 15% of variants. Multiple variants had additional evidence to support usage of strength-modified ACMG-AMP criteria (Figure 2). PS2 and PM6 for *de novo* occurrences and PS4 for probands were the most frequent criteria with additional evidence, and this underscores the importance of case level data in variant classification.

Approximately 27% of variants had multiple cases presumed *de novo* (PM6) and 23% had at least three probands (PS4). Interestingly, almost 60% (39/66) of variants had unique case level data contributed by clinical or research laboratories not reported in the literature (data not shown), supporting the crucial need for public data sharing in databases such as ClinVar.

Figure 3 compares original ClinVar classifications grouped by concordant tiers (Path/LPath, uncertain significance (VUS), Likely Benign (LBen)/Benign (Ben)) or discordant tiers (Path/LPath versus VUS, LBen/Ben versus VUS) to final RAS EP classifications. Of the final Path/LPath calls, 6/21 (~29%) of discordant or VUS ClinVar classifications upgraded to Path/LPath. Five variants specifically relied on strength-modified pathogenic criteria use for upgrading from VUS>LPath (n=1) or LPath>PATH (n=4). On the benign spectrum, 22/66 (~33%) of discordant or VUS classifications were deemed Ben/LBen. Interestingly, 73% (27/37) of benign spectrum variants met modified BA1 or BS1 MAF thresholds. The EP deemed that ~8% (5/66) of variants with either pathogenic or benign spectrum classifications in ClinVar lacked sufficient evidence for classification.

Limitations

The RAS EP acknowledges that these criteria are generally conservative to minimize false positive interpretations and further refinement over time may be necessary. Proband counts and (non-) segregations rely on well-phenotyped individuals; clinical labs must rely on notes by clinical providers to use these rules. Often clinical notes are lacking, and phenotypes provided on requisition forms may be inaccurate; therefore, the proband and segregation counts recommended here are also conservative. These specifications do not explicitly address small in-frame deletions or insertions due to exonic (or rarely intronic) variations that may have GOF effects. The PM4 criterion as written supplies moderate evidence for these variants.

Conclusions and Future Directions

The RAS EP presents a model of ACMG-AMP adaptation that can serve as a common framework for rare, autosomal dominant disorders. These RAS EP specifications highlight how grouping genes under a common phenotype, disease mechanism, and gene functionality allow for rapid multi-genic variant assessment without sacrificing specificity and accuracy. Despite a highly conservative approach, MAF assessments had the greatest impact by instantly classifying over 40% of variants to likely benign or benign. Combining similar gene group approaches, as typically seen in clinical testing panels, and available incidence values from the literature or other reporting sources, such as Orphanet (www.orpha.net), would provide the necessary information for automating NGS pipelines to rapidly classify variants with MAF <5% in global populations. Harmonizing ACMG-AMP criteria usage across disease groupings will increase consistency and accuracy of variant interpretations, thus improving clinical utility and management of patient care. In the future, the RAS EP will evaluate variants in ClinVar and their evidence in order to refine classifications of variants, especially those with uncertain significance, or resolve variants with discrepant classifications. Additionally, the RAS EP will assess validity of new disease-causing genes and provide expertise in order to improve the understanding of the Ras/MAPK genes and their related RASopathy conditions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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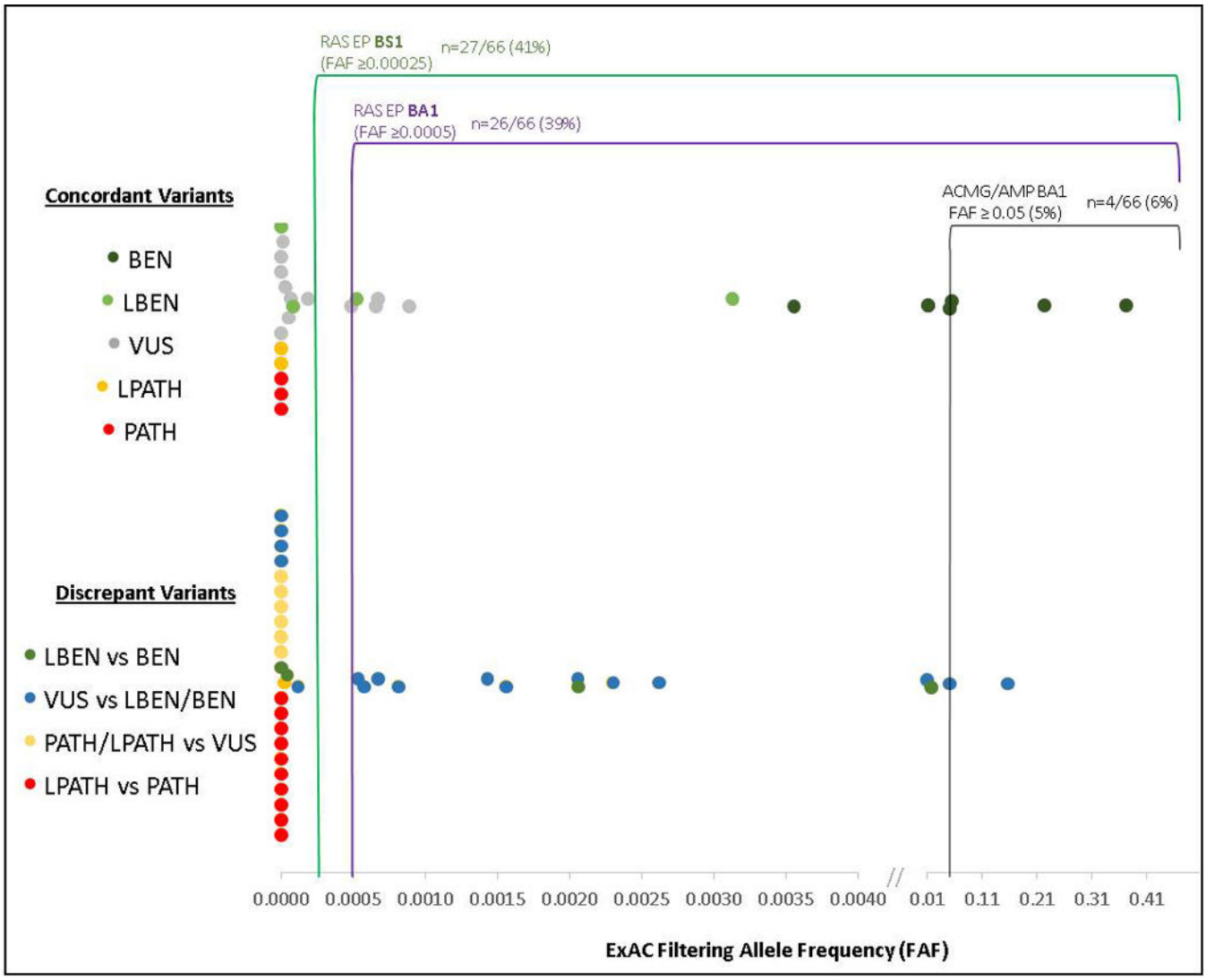

Figure 1. Effect of Adjusted Allele Frequency Criteria on Variants from ClinVar

Variants with either concordant or discrepant classifications were assessed for their frequency in the general population. An additional 32% of variants met the RAS EP adjusted frequency threshold for BA1 versus the standard ACMG-AMP BA1 Threshold. One variant met the RAS EP adjusted frequency for BS1. Data points are colored by the ClinVar classification or discrepancy category of the variant. (Ben: benign, LBen: likely benign, VUS: uncertain significance, LPath: likely pathogenic, Path: pathogenic)

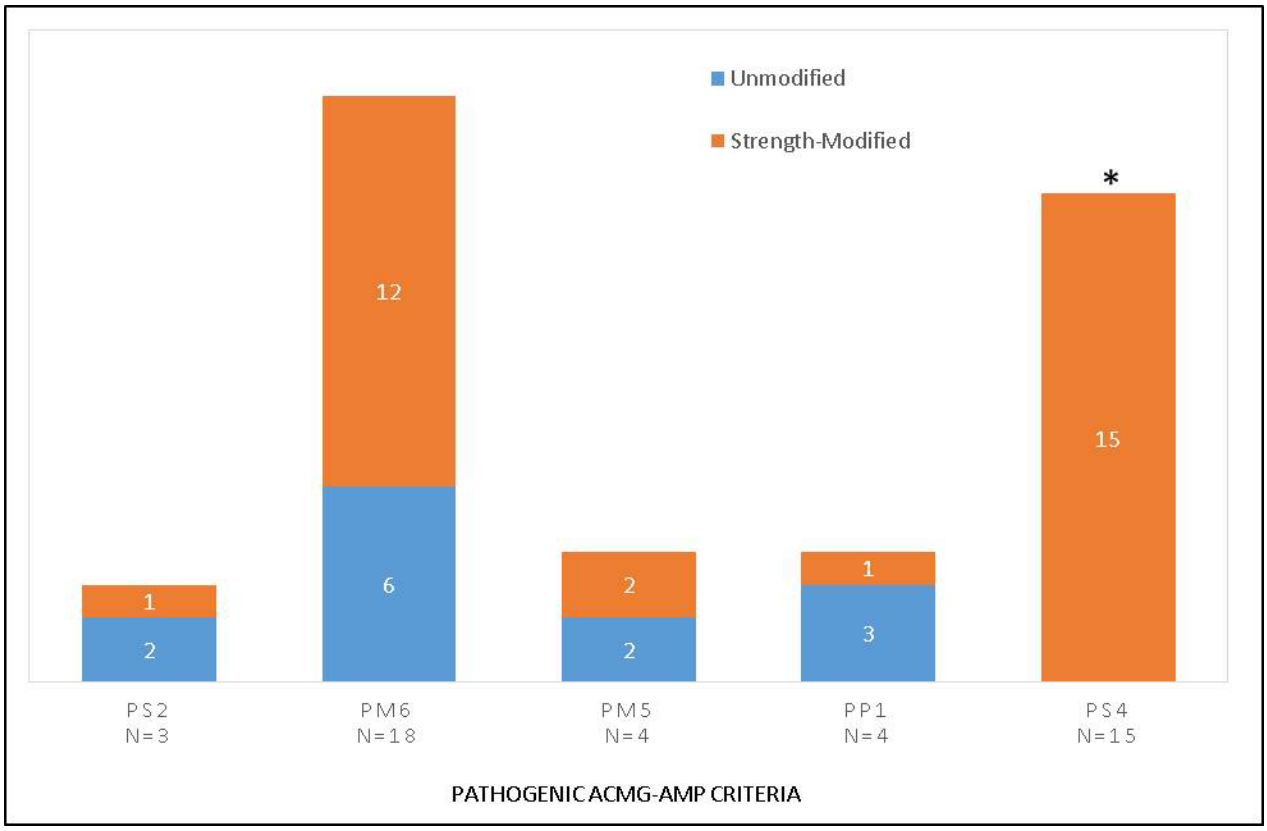

Figure 2. Assessment of usage of unmodified versus strength-modified pathogenic ACMG-AMP criteria in RAS EP classifications of variants

Typically, most variants had additional evidence to achieve higher strength specifications beyond the standard ACMG-AMP definitions. Note that all modified criteria increase in strength with additional evidence except PS4 (*) given it begins at the strong category. No variant met the PS4 threshold of at least five occurrences due to the requirement of extensive phenotypic data.

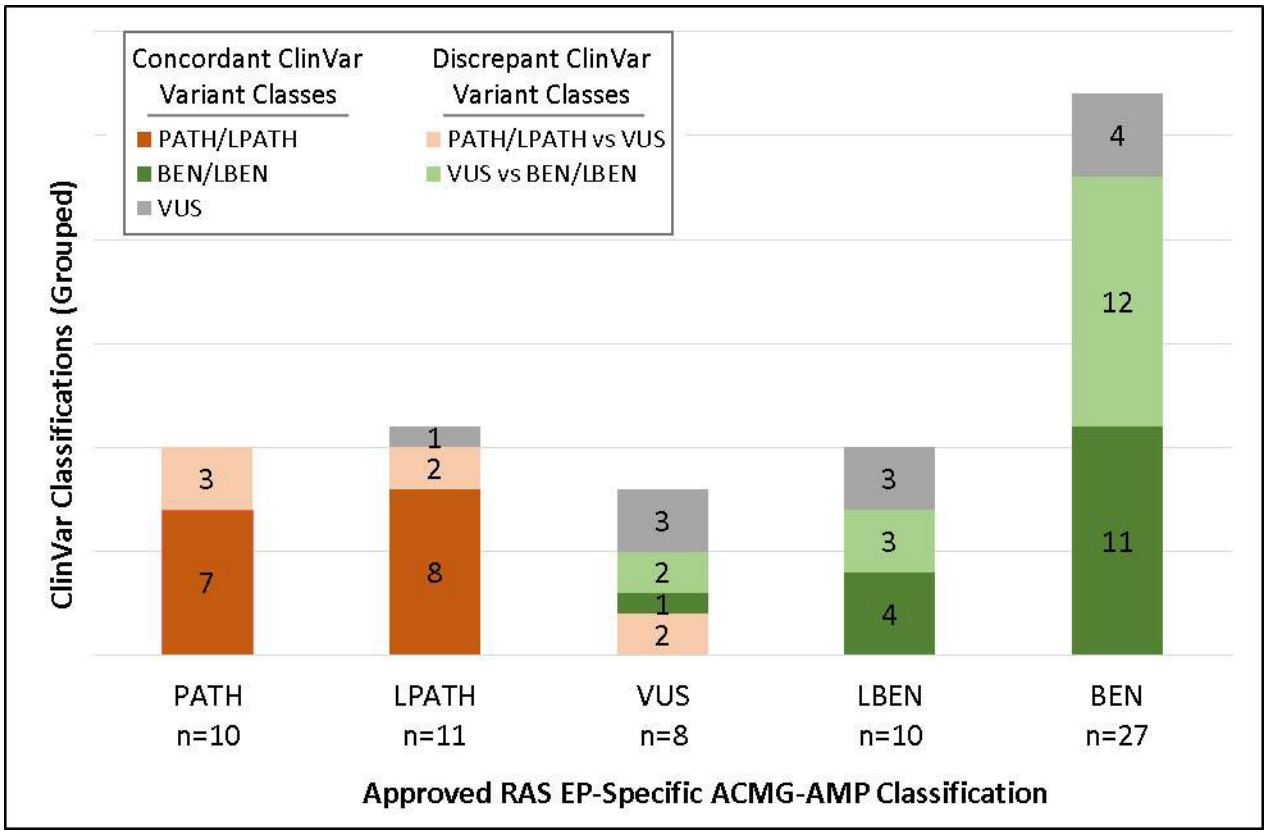

Figure 3. Comparison of Approved RAS EP Specific ACMG-AMP Variant Classifications to ClinVar Variant Classifications

Prior to determining concordance, ClinVar classes were grouped into three categories: 1.) pathogenic (Path) and likely pathogenic (LPath), 2.) benign (Ben) and likely benign (LBen), and 3.) VUS. Variant classifications were considered discordant if clinical laboratory submissions did not group into the same category. These grouped ClinVar classifications were compared to the classifications determined by using the RAS EP-Specific ACMG-AMP specifications.

Table 1

Summary of the Pathogenic and Benign ACMG-AMP Modified Criteria for the RASopathies

This table summarizes all criteria and their evidence requirements for strength modifications. RASopathy Expert Panel Specifications include: Gene-Specific: Criteria specified with clear definitions for gene-specific usage; Disease-Specific: Disease-specific specifications or criteria that can apply for same amino acid or position in highly analogous gene groupings; Strength: Increasing or decreasing strength of criteria based on accumulation of evidence; N/A: is not applicable to the RASopathies; None: no changes made to existing criteria definitions.

				Evidence Requirements for Strength Specifications			
PATHOGENIC CRITERIA	**OFFICIAL ACMG CRITERIA [Richards et al. 2015]**	**RASopathy Expert Panel Specification**	***Comments on Usage**	**VERY STRONG**	**STRONG**	**MODERATE**	**SUPPORTING**
PVS1	Null variant (nonsense, frameshift, canonical +/ − 1 or 2 splice sites, initiation codon, single or multi-exon deletion) in a gene where loss of function (LOF) is a known mechanism of disease	Not applicable	Loss of function (LOF) and/or haploinsufficiency has not been clearly identified as disease mechanisms for these genes relative to the RASopathy spectrum phenotype, therefore in general this rule is not applicable. Note that PTPN11 is currently the only gene with a confirmed association to another non-RASopathy disorder due to LOF alleles. Variants in PTPN11 or other genes with predicted LOF should not be evaluated by these RASoathy specific criteria, but should defer to non-adjusted criteria. Given that some historical LOF variants (e.g. canonical splice sites) could potentially result in a gain of function, users should assess using these criteria and non-adjusted criteria to identify the highest likelihood of	Not applicable			

				Evidence Requirements for Strength Specifications			
PATHOGENIC CRITERIA	**OFFICIAL ACMG CRITERIA [Richards et al. 2015]**	**RASopathy Expert Panel Specification**	***Comments on Usage**	**VERY STRONG**	**STRONG**	**MODERATE**	**SUPPORTING**
			pathogenicity for all associated diseases.				
PS1	Same amino acid change as a previously established pathogenic variant regardless of nucleotide change.	Gene-Specific	Previously established variant must be established as pathogenic per these criteria for germline RASopathy variants.		Can also be applied for the same analogous residue positions/regions in highly analogous groupings: Group 2: BRAF, RAF1 Group 3: HRAS, KRAS, NRAS Group 4: MAP2K1, MAP2K2 Group 5: SOS1, SOS2		
PS2/PM6	PS2: De novo (both maternity and paternity confirmed) in a patient with the disease and no family history PM6: Assumed de novo, but without confirmation of paternity and maternity	Strength	*Dual application of both unmodified PS2 and PM6 is only acceptable with a singleton occurrence in each category.	≥2 occurrences with parental confirmation OR 3 occurrences, at least 1 with parental confirmation (PS2_Very Strong) OR ≥4 occurrences with or without confirmation (PM6_Very Strong)	1 occurrence with parental confirmation (PS2)* or 2–3 occurrences without confirmation (PM6_Strong)	1 occurrence without confirmation (PM6)*	
PS3	Well-established in vitro or in vivo functional studies strongly supportive of a damaging effect on gene or gene product.	Gene-Specific	See approved functional studies in Supplemental Table		Follows primary definition of PS3		
PS4	The prevalence of the variant in affected individuals is significantly	Disease-Specific	Due to variable expressivity and severity, individuals must be well-phenotyped.		≥5 independent occurrences	3–4 independent occurrences (PS4_Moderate)	1–2 independent occurrences (PS4_Supporting)

				Evidence Requirements for Strength Specifications			
PATHOGENIC CRITERIA	**OFFICIAL ACMG CRITERIA [Richards et al. 2015]**	**RASopathy Expert Panel Specification**	***Comments on Usage**	**VERY STRONG**	**STRONG**	**MODERATE**	**SUPPORTING**
	increased compared to the prevalence in controls.						
PM1	Located in a mutational hot spot and/or critical and well-established functional domain (e.g. active site of an enzyme) without benign variation	Gene-Specific				Can also be applied for the same analogous residue positions/regions in highly analogous groupings: Group 2: BRAF, RAF1 Group 3: HRAS, KRAS, NRAS Group 4: MAP2K1, MAP2K2 Group 5: SOS1, SOS2	
PM2	Absent from controls (or at extremely low frequency if recessive) in Exome Sequencing Project, 1000 Genomes or ExAC	Disease-Specific				The variant must be completely absent from all population databases.	
PM3	For recessive disorders, the variant is detected in trans with a pathogenic variant.	Not applicable	RASopathies are historically autosomal dominant disorders.			Not applicable	
PM4	Protein length changes as a result of in-frame deletions/ insertions in a nonrepeat region or stop-loss variants	None				Follows primary definition of PM4	

				Evidence Requirements for Strength Specifications			
PATHOGENIC CRITERIA	**OFFICIAL ACMG CRITERIA [Richards et al. 2015]**	**RASopathy Expert Panel Specification**	***Comments on Usage**	**VERY STRONG**	**STRONG**	**MODERATE**	**SUPPORTING**
PM5	Novel missense change at an amino acid residue where a different missense change determined to be pathogenic has been seen before	Gene-Specific	Previously established variant(s) must be established as pathogenic per these criteria for germline RASopathy variants. Amino acid changes of variants should be concordant with pathogenicity based on how conservative or non-conservative (within the context of amino acid chain groupings) the residue change is relative to the known pathogenic residue changes. This rule should not be used as independent criteria for calculating pathogenicity in conjunction with PM1 if the amino acid residue being interrogated is explicitly designated as a “mutational hot-spot”.		≥2 different pathogenic missense changes (PM5_Strong)	Can also be applied for the same analogous residue positions/regions in highly analogous groupings: Group 2: BRAF, RAF1 Group 3: HRAS, KRAS, NRAS Group 4: MAP2K1, MAP2K2 Group 5: SOS1, SOS2	
PP1	Co-segregation with disease in multiple affected family members in a gene definitively known to cause the disease	Disease-Specific	Due to variable expressivity and severity, individuals must be well-phenotyped.		≥7 meioses (PP1_Strong)	5–6 meioses (PP1_moderate)	3–4 meioses
PP2	Missense variant in a gene that has a low rate of benign	Gene-Specific					Follows primary of definition of PP2

				Evidence Requirements for Strength Specifications			
PATHOGENIC CRITERIA	**OFFICIAL ACMG CRITERIA [Richards et al. 2015]**	**RASopathy Expert Panel Specification**	***Comments on Usage**	**VERY STRONG**	**STRONG**	**MODERATE**	**SUPPORTING**
	missense variation and in which missense variants are a common mechanism of disease						
PP3	Multiple lines of computational evidence support a deleterious effect on the gene or gene product (conservation, evolutionary, splicing impact, etc.)	None					Follows of primary definition of PP3
PP4	Patient's phenotype or family history is highly specific for a disease with a single genetic etiology.	Not applicable	The RASopathies are a genetically heterogenous group of disorders. Use PS4 for proband counting options.				Not applicable
PP5	Reputable source recently reports variant as pathogenic but the evidence is not available to the laboratory to perform an independent evaluation .	Not applicable	Currently, there are no resources that are acceptable for this criterion; however, additional groups are working on policies regarding use of somatic variation for germline disorders. Once these policies are established, the RAS EP will consider the use of other external resources (e.g. COSMIC database).				Not applicable

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				Evidence Requirements for Strength Specifications		
BENIGN CRITERIA	**OFFICIAL ACMG CRITERIA [Richards et al. 2015]**	**RASopathy Expert Panel Specification**	***Comments on Usage**	**STAND-ALONE**	**STRONG**	**SUPPORTING**
BA1/BS1	Allele frequency is above 5% In Exome Sequencing Project, 1000 Genomes, or ExAC Allele frequency is greater than expected for disorder	Disease-specific	BS1 is sufficient as stand-alone for likely benign classification in the absence of contradictory pathogenic evidence.	ExAC filtering allele frequency ≥0.0005 (BA1)	ExAC filtering allele frequency ≥0.00025. Based on disease prevalence of 1:1000 (BS1)	
BS2	Observed in a healthy adult individual for a recessive (homozygous), dominant (heterozygous), or X-linked (hemizygous) disorder with full penetrance expected at an early age	Disease-specific	Due to variable expressivity and severity, population data should not be used for this criteria. Individuals must be well-phenotyped.		≥3 well phenotyped individuals.	
BS3	Well-established in vitro or in vivo functional studies show no damaging effect on protein function or splicing	Gene-specific			Follows primary definition of BS3	
BS4	Lack of segregation in affected members of a family	Disease-specific	Due to variable expressivity and severity, individuals must be well-phenotyped.		≥1 meiosis	
BP1	Missense variant in a gene for which primarily truncating variants are known to cause disease	Disease-specific				CONTRAINDICATION: Truncating variant (nonsense, frameshift, affects canonical splice sites, initiation codon, entire gene or multi exon deletion) when only known disease mechanism for gene is gain-of-function.
BP2	Observed in trans with a pathogenic variant for a fully penetrant dominant gene/disorder or observed in cis with a pathogenic variant in any inheritance pattern	None				Follows primary definition of BP2
BP3	In-frame deletions/insertions in a repetitive region without a known function	None				Follows primary definition of BP3
BP4	Multiple lines of computational evidence suggest no impact on gene or gene product (conservation, evolutionary, splicing impact, etc.)	None				Follows primary definition of BP4
BP5	Variant found in a case with an alternate molecular basis for disease	None				Follows primary definition of BP5
BP6	Reputable source recently reports variant as benign but the evidence is	Not applicable	Currently, there are no resources that are acceptable for this criterion; however, additional			Not applicable

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				Evidence Requirements for Strength Specifications		
BENIGN CRITERIA	**OFFICIAL ACMG CRITERIA [Richards et al. 2015]**	**RASopathy Expert Panel Specification**	***Comments on Usage**	**STAND-ALONE**	**STRONG**	**SUPPORTING**
	not available to the laboratory to perform an independent evaluation		groups are working on policies regarding use of somatic variation for germline disorders. Once these policies are established, the RAS EP will consider the use of other external resources (e.g. COSMIC database).			
BP7	A synonymous (silent) variant for which splicing prediction algorithms predict no impact to the splice consensus sequence nor the creation of a new splice site AND the nucleotide is not highly conserved	Disease-specific	Also applicable for intronic or non-coding variants and can be used in conjunction with BP4.			