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Quantifying evidence toward pathogenicity for rare phenotypes: The case of succinate dehydrogenase genes, SDHB and SDHD

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ABSTRACT

Purpose: The weight of the evidence to attach to observation of a novel rare missense variant in SDHB or SDHD in individuals with the rare neuroendocrine tumors, pheochromocytomas and paragangliomas (PCC/PGL), is uncertain.

Methods: We compared the frequency of SDHB and SDHD very rare missense variants (VRMVs) in 6328 and 5847 cases of PCC/PGL, respectively, with that of population controls to generate a pan-gene VRMV likelihood ratio (LR). Via windowing analysis, we measured regional enrichments of VRMVs to calculate the domain-specific VRMV-LR (DS-VRMV-LR). We also calculated subphenotypic LRs for variant pathogenicity for various clinical, histologic, and molecular features.

Results: We estimated the pan-gene VRMV-LR to be 76.2 (54.8-105.9) for SDHB and 14.8 (8.7-25.0) for SDHD. Clustering analysis revealed an SDHB enriched region (αα177-260, P = .001) for which the DS-VRMV-LR was 127.2 (64.9-249.4) and an SDHD enriched region (αα70-114, P = .000003) for which the DS-VRMV-LR was 33.9 (14.8-77.8). Subphenotypic LRs exceeded 6 for invasive disease (SDHB), head-and-neck disease (SDHD), family history of PCC/PGL, loss of SDHB staining on immunohistochemistry, and succinate-to-fumarate ratio >97 (SDHB, SDHD).

Conclusion: Using methodology generalizable to other gene-phenotype dyads, the LRs relating to rarity and phenotypic specificity for a single observation in PCC/PGL of a SDHB/SDHD VRMV can afford substantial evidence toward pathogenicity.

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**Introduction**

Clinical genomic analysis is typically undertaken with the aim of identifying an underlying monogenic cause in a patient with suggestive clinical features. For any genomic variant identified, a variety of evidence types are integrated to assess the likelihood of the variant being pathogenic. In 2015, the American College of Medical Genetics (ACMG) and the Association of Molecular Pathology (AMP) published a framework prescribing how these disparate evidence elements should be combined by diagnostic laboratories for classification of a newly identified genomic variant.\(^1\) They defined 4 strengths for the evidence elements, namely supporting, moderate, strong, and very strong, which could be combined in the classification categories of pathogenic, likely pathogenic, likely benign, and benign. However, inclusion and strength of evidence elements often differ between diagnostic laboratories and produce discrepant classifications.\(^2\)

Pheochromocytomas and paragangliomas (PCC/PGL) are neuroendocrine tumors of the adrenal medulla and autonomic nervous system with an estimated frequency of 1 in 4000 and 1 in 16,000 respectively.\(^3\)–\(^6\) Head-and-neck paragangliomas (eg, chemodectoma, glomus jugulare) are derived from parasympathetic ganglia. Inherited predisposition to PCC/PGL is associated with constitutional pathogenic variants (PVs) in >15 genes, including SDHA, SDHAF2, SDHB, SDHC, SDHD, VHL, FH, MAX, TMEM127, RET, MEN1, and NF1.\(^4,\)\(^7\) Among the Mendelian genes, the pattern of disease transmission followed by SDHB is the phenotype SDHx followed by SDHD.\(^4,\)\(^8\) Associations with subphenotypes of head-and-neck paragangliomas, namely multiple, familial, and/or young-onset disease, have been reported with underlying germline PVs in SDHA, SDHB, SDHC or SDHD (SDHx) and with metastatic disease for SDHB PVs.\(^9\) The SDH proteins together form the succinate dehydrogenase enzymatic complex or mitochondrial complex II, disruption of which by PVs of any of the SDHx components may cause loss of SDHB expression in tumor material.\(^10\) The SDH succinate-to-fumarate ratio (SSFR) in the tumor has also been associated with underlying PVs in SDHx.\(^11\) Other tumors have been associated with PVs in SDHx but with much lower relative risks; these include wild-type gastrointestinal stromal tumors, SDH-deficient renal cell carcinoma, and pituitary adenomas.\(^10\) In the case of SDHD, MAX, and SDHAF2, disease is typically only manifested when PVs are transmitted paternally.\(^4,\)\(^10\) For the other SDHx genes, the pattern of disease transmission follows the normal autosomal dominant model of inheritance.

As per the classical Knudson 2-hit model of loss-of-function, protein-truncating variants in SDHB/SDHD are typically pathogenic.\(^9\) Interpretation and classification of missense variants is more challenging. On encountering a patient with PCC/PGL and a rare missense variant in SDHx, evidence of pathogenicity could be inferred from (1) the very observation in an individual with the relevant rare PCC/PGL phenotype of a rare variant in an associated gene (PP2 in the ACMG/AMP framework), (2) location of that variant within a sub-region of the gene particularly associated with pathogenicity (PM1), and (3) subphenotypic features particularly associated with PVs in the SDHx genes, eg, invasive disease or loss of SDHB staining on immunohistochemistry (IHC) (PP4). We demonstrate generalizable quantitative approaches and requisite data sets from which likelihood ratios (LRs) can be calculated for each of these elements using the genes SDHB/SDHD, the phenotype PCC/PGL, and missense variation as our exemplar gene/phenotype/variant-class paradigm.\(^12\)

**Materials and Methods**

**Assembly of group clinical and laboratory experts for the gene-phenotype paradigm**

Via our national United Kingdom multidisciplinary network Cancer Variant Interpretation Group UK (CanVIG-UK), we identified from the 2 United Kingdom genetics centers the lead diagnostic laboratory scientists, clinical geneticists, and endocrinologists for PCC/PGL to assemble the CanVIG-UK SDHx expert group, who guided sourcing of case data and focused survey of the literature.\(^13\)

**Assembly of case variant data**

For the case control analyses, we were able to identify only 1 data series providing the frequency for individual SDHB/SDHD variants, fully stratified by ethnicity, ascertained from a full SDHx gene analysis in a PCC/PGL series unselected for subphenotypes, which comprised 179 cases of PCC/PGL recruited to The Cancer Genome Atlas data set.\(^14\) We obtained summary-level per-variant frequencies for 4 additional PCC/PGL series from clinical testing; these series were all of predominantly White (Western European) ethnicity, but detailed/individual-level ethnicity data were unavailable.

The Birmingham and Leeds data sets comprised per-variant summary results from unrelated probands with PCC/PGL referred from United Kingdom clinical genetics and endocrinology centers to West Midlands Regional Genomic Laboratory Hub (2000-2020) and Yorkshire and North East Genomic Laboratory Hub (2015-2020), respectively, comprising clinical testing (single gene/gene panel including dosage analysis) of SDHB and SDHD for 3044 and 2565 patients (Birmingham) and 215 and 215 patients (Leeds), respectively. The Ambry data set comprised per-variant summary results from clinical testing for SDHx undertaken at Ambry Genetics of 1338 PCC/PGL cases referred from the US clinical genetics and endocrinology centers from 2012-2020. The French data set comprised per-variant summary results from single gene/gene panel testing.
of SDHB and SDHD for 1552 and 1550 patients, respectively, French PCC/PGL cases accrued 2001-2010, as previously described by Buffet et al. In total, 6328 and 5847 unrelated PCC/PGL probands were available for analysis for SDHB and SDHD, respectively. For SDHB, we identified in total 308 PVs predicted to truncate the protein and 315 missense variants classified in ClinVar as (likely) pathogenic. For SDHD, there were 155 protein-truncating PVs and 139 (likely) pathogenic missense variants (of which 116 were c.242C>T p.Pro81Leu). These classifications for missense variants are based on ClinVar (≥1 star, pathogenic/likely pathogenic) and for truncating variants on classification using ACMG criteria performed by a diagnostic clinical scientist.

For subphenotype analyses, we were able to access individual-level clinical phenotype data for a subset of 709 of the Birmingham probands, including (1) tumor location (head-and-neck/thoraco-abdominal), (2) tumor behavior (invasive/noninvasive), (3) tumor number (multiple/single), (4) family history (familial/isolated), and (5) age at diagnosis, as previously described by Andrews et al and Ricketts et al. Data on the relevant molecular subphenotypes, namely SDHB IHC and SSFR, were unavailable for any of our case series and thus were instead derived from the literature. We identified suitable IHC data, stratified by SDHx variant type, generated by Van Nederweert et al for 175 PCC/PGL cases with known germline SDHx status (retrospective series) and 45 PCC/PGL cases in whom SDHx germline testing was performed subsequently (prospective series). Two different commercial primary antibodies against SDHB (mouse monoclonal clone 21A11 and rabbit polyclonal HPA00286) were used to perform IHC. We identified suitable SSFR data, stratified by SDHx variant type, generated by Richter et al for 210 PCC/PGL cases (69 with PVs, 14 with variants of uncertain significance, and 127 with wild-type SDHx). Metabolites were measured using liquid chromatography-mass spectrometry, and variant classification was conducted according to ACMG/AMP guidelines.

Assembly of control data series

For the control comparison group, we made use of the publicly available gnomAD v2.1.1 (noncancer) data set: exome data from 118,479 individuals recruited via studies of common complex diseases, such as hypertension and type 2 diabetes (from which cancer-related series were excluded). The gnomAD v2.1.1 data set comprised 51,377 non-Finnish Europeans (NFEs), 10,816 Finnish Europeans, 17,130 Latino/African-Americans, 15,263 South Asians, 8846 East Asians, 4786 Ashkenazi Jewish, and 2810 others. To extend representation of rarer ethnicities, we also utilized the 1000 Genomes Project (1000GP) phase 3 data comprising 2504 individuals from 26 subpopulations, recognizing that some overlap between the 1000GP and gnomAD populations is reported.

Calculation of predicted maximum tolerated allele frequency

We calculated a predicted maximum tolerated allele frequency (MTAF\_pred) for pathogenicity for a newly identified missense variant in PCC/PGL for each of SDHB and SDHD on the basis of the methods described by Whiffin et al as follows: MTAF\_pred = disease prevalence × maximum allelic contribution × 1/penetrance, where maximum allelic contribution = genetic heterogeneity × allelic heterogeneity. MTAF\_pred represents the estimated allele frequency in the population above which a newly identified very rare missense variant (VRMV) is not plausibly pathogenic. We sought guidance from the CanVIG-UK SDH expert group to ensure best estimation of the constituent parameters underpinning the MTAF\_pred estimation.

Disease prevalence

Because PCC/PGL is typically a time-limited condition resolved by surgery, we used lifetime risk to approximate the disease prevalence for this analysis. Estimates of the frequency in the population of PCC/PGL vary widely. For example, the estimated PCC/PGL incidence in the Netherlands was 0.04 to 0.21 per 100,000 person-years (equating to an approximate lifetime risk of ~1 in 6000 to 1 in 31,000), whereas the estimated PCC/PGL incidence in the United States was 500 to 1600 cases per year (equating to an approximate lifetime risk of ~1 in 2500 to 1 in 8000). We used a widely-cited cancer registry–derived estimate of lifetime risk for pheochromocytoma from Pecak et al of 1 in 4500 with a frequency of paraganglioma estimated to be 4-fold less common (1 in 18,000), totaling a combined lifetime incidence of 1 in 3600.

Penetrance

We used estimates of penetrance from the study by Andrews et al, which comprised prospective follow-ups of 371 and 67 unaffected SDHB and SDHD PV-positive probands, respectively, ascertained on account of an affected index case in the family, the largest series we could identify. Penetrance to age 60 and 80 for nonprobands was estimated for SDHB to be 22% and 39%, respectively. For SDHD, penetrance in probands for paternally-inherited SDHD PVs was estimated to be 50% to age 60. Applying to SDHB the proportionate age-related penetrance of SDHD, we thus predicted a penetrance to age 80 for paternally-inherited PVs of 88.6%. At the population-level, assuming absence of sex-selection in transmission of pathogenic SDHB alleles, we thus predicted an overall penetrance for SDHD to age 80 of approximately 44%.

Genetic heterogeneity

We used data from our amalgamated series to estimate genetic heterogeneity. The frequency in our PCC/PGL cases of missense (likely pathogenic/PVs) was 315 of 6328 (4.9%) for SDHB and 139 of 5847 (2.3%) for SDHD.
Allelic heterogeneity

Because these are well-characterized genes for which extensive clinical testing has been performed, it is likely that majorly recurrent variants have been identified, and thus, we estimated conservatively that any newly identified variant of standard penetrance is unlikely to constitute >10% of the total missense PVs.19

Using parameter estimates for disease frequency (1 in 3600), penetrance (SDHB: 0.39, SDHD: 0.44), genetic heterogeneity for missense variants (0.049 for SDHB, 0.023 for SDHD), and allelic heterogeneity (0.1), we estimated the MTAF\textsubscript{pred} to be $1.7 \times 10^{-6}$ for SDHB and $7.3 \times 10^{-7}$ for SDHD.19 Assuming a Poisson distribution, adequate coverage, and estimates based on the lower 95th confidence interval, the MTAF\textsubscript{pred} (VRMV threshold) for each of SDHB and SDHD is compatible with the observation of a maximum of 1 allele in gnomAD v2.1.1 (noncancer) NFE series. We also performed a modified PG-VRMV-LR estimation in which established pathogenic VRMVs observed recurrently in the case series were excluded. We defined these as variants classified in ClinVar as pathogenic/likely pathogenic and observed in more than 8 probands in our series. This rather conservative threshold, derived from visual inspection of the frequency distribution, equates to a variant present in >1 in 800 probands or constituting in our series >5% of all VRMVs.

Calculation of domain-specific VRMV-LRs

Using the windowing method described by Walsh et al,23 we performed a clustering algorithm to examineagnostically regional enrichment of VRMVs reported in cases vs VRMVs reported in controls.

Calculation of subphenotype LRs

Clinical subphenotypic data captured at ascertainment for (1) tumor location (head-and-neck/thoraco-abdominal), (2) tumor behavior (invasive/noninvasive), (3) tumor number (multiple/single), and (4) family history of PCC/PGL (familial/isolated) were used to generate positive and negative LRs. All cases with a variant of uncertain significance in any SDHx gene were excluded from the wild-type SDHx group. Age was excluded from the multivariable analysis because on visual inspection, there was a complex relationship between PV status and age, not well captured by categorical groupings. We quantified and adjusted for colinearity among subphenotypic variables using univariable and multivariable logistic regressions. For the univariable analysis, all individuals with data for that parameter were included. For the multivariable analysis, only those with complete data on all clinical subphenotypes were included.

Using the combined van Nederveen et al16 series, positive and negative LRs for PVs vs wild-type SDHx/untested samples were calculated for loss on IHC. Using the Richter et al11 series, the positive and negative LRs for PVs vs wild-type SDHx were calculated for SSFR >97 and SSFR ≤97. For all subphenotypes, data are presented for missense PVs and all PVs.
Results

The proportion of individuals for whom a VRMV was identified was 366 of 6328 in the PCC/PGL probands, 39 of 51,377 in the SDHB controls, 37 of 5847 in the PCC/PGL probands, and 22 of 51,377 in the SDHD controls. We calculated the PG-VRMV-LR to be 76.2 (54.8-105.9) for SDHB and 14.8 (8.7-25.0) for SDHD. PG-VRMV-LRs were broadly consistent when analyzed for the 5 case series individually. These frequencies do not include recurrent founder PVs observed in controls at a frequency exceeding the MTAF pred threshold, namely SDHB c.286G>A p.Gly96Ser (frequency of 17 in cases and 2 in v2.1.1 [non-cancer]NFE), SDHB c.688C>T p.Arg230Cys (frequency of 10 in cases and 2 in v2.1.1 [non-cancer]NFE), SDHD c.725G>A p.Arg242His (frequency of 19 in cases and 3 in v2.1.1 [non-cancer]NFE), and SDHD c.242C>T p.Pro81Leu (frequency of 116 in cases and 4 in v2.1.1 [non-cancer]NFE).

However, although observed at sufficiently low frequency in controls to constitute a VRMV, a number of variants were observed in multiple independent probands and are well documented in ClinVar as (likely) pathogenic. On removal of these recurrent-pathogenic-VRMVs, the frequencies reduced to 156 in 6118 probands for SDHB and 37 in 5847 for SDHD, thus downadjusting the PG-VRMV-LR to 34.6 (24.3-49.2) for SDHB and 14.8 (8.7-25.0) for SDHD (Table 1, Supplemental Tables 2 and 3).

From the clustering analysis, we identified a region comprising 30% of the coding region of SDHB (aa 177-260) enriched for VRMVs in cases when compared with controls (P = .001). This generated domain-specific VRMV-LR (DS-VRMV-LR) of 127.2 (64.9-249.4) for variants within the region and DS-VRMV-LR of 60.9 (41.6-89.0) for those outside the region. For SDHD, there was also a cluster region (aa 70-114, 28% of coding region, P = .000003) such that DS-VRMV-LR was 33.9 (14.8-77.8) inside and 5.9 (2.6-13.0) outside of that region. Excluding the recurrent-pathogenic-VRMVs reduced the hot-DS-VRMV-LR to 59.7 (28.5-125.2) and the cold-DS-VRMV-LR to 28.2 (18.8-42.4) for SDHB; for SDHD, the DS-VRMV-LRs were unchanged (Figure 1).

Based on PV-positive vs wild-type SDHx case-only adjusted comparisons, invasive disease was predictive for SDHB missense PV status when compared with wild-type SDHx status (subphenotypic LR [SP-LR] = 6.5 [3.9-10.7]). Both head-and-neck disease (SP-LR = 10.6 [8.8-12.7]) and multiple tumors (SP-LR = 9.5 [5.3-17.1]) were predictive of SDHD missense PV status when compared with wild-type SDH wild-type. Family history of at least 1 affected first degree relative was highly predictive of missense PVs in SDHB (SP-LR = 18.7 [8.7-40.0]) and SDHD (SP-LR = 54.4 [25.6-115.5]) when compared with wild-type status (Table 2, Supplemental Tables 4 and 5). In a univariable analysis, loss of SDHB staining on IHC was strongly predictive of a PV in both SDHB (SP-LR = 17.9 [14.7-21.8]) and SDHD (SP-LR = 18.1 [16.6-19.8]) when compared with wild-type SDHx status (Table 2, Supplemental Table 6). SSFR >97 was also strongly predictive of PVs in SDHB (SP-LR = 108.9 [92.9-127.6]) and SDHD (SP-LR = 93.1 [78.3-110.8]) when compared with wild-type SDHx (Table 2, Supplemental Table 7). In Supplemental Table 8, some hypothetical variant scenarios are presented to illustrate a combination of these LRs under the points-based Bayesian adaptation of the ACMG variant classification framework.

Discussion

Before evolution of the ACMG/AMP framework, assignment of a variant as pathogenic was frequently based primarily on observation thereof in an individual with the correct phenotype (along with the absence on sequencing of a few hundred control chromosomes). However, this adage led to erroneous classification of many innocuous variants as pathogenic on account of (1) insufficient size of the population/control data series for confirmation of requisite rarity and/or (2) application in the context of nonspecific phenotypes such as familial breast cancer.

The notion of phenotypic specificity is not simple. For a given gene/phenotype/variant-class scenario, phenotypic specificity encompasses (1) rarity of the clinical phenotype in the general population, (2) how much of the phenotype is...
Figure 1  **Variant position schematic.** Lollipop showing the position of *SDHB* and *SDHD* variants in 51,377 controls and 6328 and 5847 cases (of phaeochromocytoma and paraganglioma), respectively. Variants identified in cases are represented by red circles and those in controls are represented by yellow circles with proportional representation for variants identified in both. Exon-exon boundaries are shown with a dashed line. Protein domains are represented by colored blocks. Variant cluster regions (CR), as defined using a custom clustering algorithm (see methods), are shown as black rectangles below each protein (*P* < .004). Fer_2_3, 2Fe-2S iron-sulfur cluster binding domain (red); Fer4_17, 4Fe-4S dicluster domain (purple); CybS, succinate dehydrogenase cytochrome B small subunit (blue). Escore interspecies conservation is presented.
attributable to that gene, (3) the level of enrichment of gene variants of a particular class in that phenotype (i.e., penetrance), and (4) how noisy the gene is for innocuous variants of that variant-class, and there may in addition be (5) regional variation for pathogenic vs innocuous variants of that variant-class and (6) gene-specific subphenotypic features that are particularly associated with pathogenicity.

Using SDHB/SDHD, PCC/PGL, and missense variants as our gene/phenotype/variant-class exemplar, we have demonstrated that quantitation of these LRs encompass (1) identification of a rare missense variant in an individual with the correct rare phenotype in a gene variably constrained for those variants (ACMG/AMP criterion: PP2), (2) enrichment for rare variants in cases compared with controls within specific gene regions (PM1), and (3) presence of macroscopic or molecular subphenotypic features particularly associated with germline PVs in a specific gene (PP4).

These analyses demonstrate a substantial PG-VRMV-LR for SDHB in particular, which is quite striking even after removal of the recurrently-reported pathogenic VRMVs. It

| Table 2  | Case-only subphenotype analyses |
|----------|---------------------------------|
| Genotype | Count | LR Univariable | LR Multivariable |
| Tumor location: HNPGL vs PCC/PGL w/o HN |
| SDHB missense PV | HNPGL | 37 | 1.77 (1.31-2.40) | 2.43 (1.80-3.29) |
| | PCC/PGL w/o HN | 55 | 0.77 (0.65-0.92) | 0.56 (0.47-0.67) |
| | SDHD missense PV | HNPGL | 31 | 4.26 (3.54-5.13) | 10.56 (8.78-12.72) |
| | | PCC/PGL w/o HN | 1 | 0.04 (0.01-0.28) | 0.02 (0.00-0.11) |
| | SDHx wild-type | HNPGL | 97 | 0.04 (0.01-0.28) | 0.02 (0.00-0.11) |
| | | PCC/PGL w/o HN | 330 | 0.04 (0.01-0.28) | 0.02 (0.00-0.11) |
| Tumor behavior: invasive vs noninvasive |
| SDHB missense PV | Invasive | 24 | 4.27 (2.57-7.08) | 6.46 (3.89-10.72) |
| | Noninvasive | 66 | 0.78 (0.69-0.89) | 0.52 (0.46-0.59) |
| SDHD missense PV | Invasive | 3 | 1.50 (0.48-4.69) | 1.15 (0.37-3.60) |
| | Noninvasive | 29 | 0.97 (0.86-1.08) | 1.27 (1.13-1.42) |
| SDHx wild-type | Invasive | 26 | 0.78 (0.64-0.95) | 0.56 (0.46-0.67) |
| | Noninvasive | 390 | 0.78 (0.64-0.95) | 0.56 (0.46-0.67) |
| Tumor number: multiple vs solitary |
| SDHB missense PV | Multiple | 12 | 3.04 (1.52-6.08) | 2.38 (1.19-4.77) |
| | Solitary | 80 | 0.91 (0.84-0.99) | 1.15 (1.06-1.25) |
| SDHD missense PV | Multiple | 15 | 10.91 (6.09-19.55) | 9.53 (5.32-17.07) |
| | Solitary | 17 | 0.56 (0.40-0.77) | 0.67 (0.48-0.92) |
| SDHx wild-type | Multiple | 18 | 0.91 (0.84-0.99) | 0.70 (0.60-0.81) |
| | Solitary | 401 | 0.91 (0.84-0.99) | 0.70 (0.60-0.81) |
| Family history: familial vs isolated |
| SDHB missense PV | Familial | 24 | 15.95 (7.45-34.16) | 18.68 (8.72-40.01) |
| | Isolated | 52 | 0.70 (0.60-0.81) | 0.70 (0.60-0.81) |
| SDHD missense PV | Familial | 16 | 29.93 (14.08-63.61) | 54.36 (25.57-115.54) |
| | Isolated | 11 | 0.42 (0.26-0.66) | 0.34 (0.21-0.53) |
| SDHx wild-type | Familial | 8 | 0.70 (0.60-0.81) | 0.70 (0.60-0.81) |
| | Isolated | 396 | 0.70 (0.60-0.81) | 0.70 (0.60-0.81) |
| IHC: IHC negative (abN) vs IHC positive (normal) |
| SDHB missense PV | IHC negative | 21 | 17.9 (14.7-21.8) | 18.68 (8.72-40.01) |
| | IHC positive | 0 | 0.023 (0.001-0.396) | 0.023 (0.001-0.396) |
| SDHD missense PV | IHC negative | 53 | 18.1 (16.6-19.8) | 18.68 (8.72-40.01) |
| | IHC positive | 0 | 0.010 (0.001-0.171) | 0.010 (0.001-0.171) |
| SDHx wild-type | IHC negative | 6 | 0.023 (0.001-0.396) | 0.023 (0.001-0.396) |
| | IHC positive | 112 | 0.010 (0.001-0.171) | 0.010 (0.001-0.171) |
| SDH Succinate: Fumarate Ratio: High (>97) vs Low (≤97) |
| SDHB missense PV | SSFR >97 | 12 | 108.9 (92.9-127.6) | 108.9 (92.9-127.6) |
| | SSFR ≤97 | 2 | 0.14 (0.014-1.49) | 0.14 (0.014-1.49) |
| SDHD missense PV | SSFR >97 | 11 | 93.1 (78.3-110.8) | 93.1 (78.3-110.8) |
| | SSFR ≤97 | 4 | 0.27 (0.03-2.28) | 0.27 (0.03-2.28) |
| SDHx wild-type | SSFR >97 | 1 | 0.27 (0.03-2.28) | 0.27 (0.03-2.28) |
| | SSFR ≤97 | 126 | 0.27 (0.03-2.28) | 0.27 (0.03-2.28) |

Analysis of clinical subphenotypic features in 206 SDHB PV-positive, 66 SDHD PV-positive, and 427 SDHx wild-type cases of PCC/PGL. Analysis of SDHB IHC staining in 21 SDHB PV-positive, 53 SDHD PV-positive, and 118 SDH wild-type/untested cases. Analysis of SDH SSFR in 14 SDHB PV-positive, 15 SDHD PV-positive, and 127 SDH wild-type cases of PCC/PGL.

HNPGL, head-and-neck paraganglioma; IHC, immunohistochemistry; PCC/PGL, pheochromocytoma and paraganglioma; PV, pathogenic variant; SSFR, succinate-to-fumarate ratio; w/o HN, not in head or neck.
is plausible that for other gene/phenotype/variant-class scenarios in which the gene is constrained and/or the phenotype is rare, the PG-VRMV-LR may be equivalently substantial and we may currently be underscoring evidence afforded by a single observation of a very rare variant in the relevant phenotype. Wide variation in the etiologic fraction, a similar metric, has been demonstrated for genes associated with cardiomyopathies.24

These analyses also exemplify the potential clinical utility of formal quantitation of LRs for subphenotypic features, in this case abnormality on IHC, high SSFR, head-and-neck disease, invasive disease, multiplex tumors, and familial disease. Although we were only able to undertake multivariable regression to adjust for collinearity between clinical subphenotypic features, collinearity between the clinical and molecular features would not be predicted a priori. Stringent technical validation would be a prerequisite for inclusion of locally-generated laboratory data: although methodology and quality assurance for IHC is well explored, there is no international reference method for SSFR, and assay thresholds may be influenced by tumor input material. The corresponding negative LR should be applied when the absence of the subphenotypic feature has high specificity for wild-type status (eg, absence of head-and-neck disease for SDHD). Currently, the ACMG framework lacks formal designation of a negative-phenotype evidence item.

As demonstrated by Tavtigian et al,25 the ACMG/AMP categorical evidence strengths can be converted to LRs (supportive LR = 2.08, moderate LR = 4.33, strong LR = 18.8, and very strong LR = 350). Thus, the 3 types of evidence items we have described can be combined together with other relevant LRs in a Bayesian framework in which the posterior probability is a function of the prior probability and the product of relevant LRs ([LR]a × [LR]b × [LR]c × [LR]d × [LR]e). The application of SDHB/SDHD—PCC/PGL cases illustrates a number of challenges and limitations in the methods presented. First, critical to the PG-VRMV-LR is conversion of the MTAFpred to a filtering allele count for a given control data set. Even the largest subpopulation of the cancer-free gnomAD series (NFE) provides poor precision at low values of MTAFpred. Observation of 1 in 51,377 in the NFE group is consistent with the underlying frequency of 4.93 × 10−7 to 1.084 × 10−4 (95% confidence interval of binomial distribution). Accordingly, we may be overestimating the frequency of VRMVs in controls (and thus underestimating the PG-VRMV-LR). As illustrated in our sensitivity analyses (Supplemental Table 1), access to larger control series for our VRMV case control comparison will improve the precision by which we filter for MTAFpred.

Second, limited size of non-NFE control series precludes accurate filtering of variants in non-NFE populations. Variants with a true frequency above the MTAFpred may still be sufficiently rare to be absent in these modest-sized control series. Although the case series used were predominantly Western European, in the non-White minority, we may be erroneously including as VRMVs (1) variants common in ethnic groups entirely unrepresented in gnomAD or 1000GP or (2) variants absent in the respective partition of gnomAD/1000GP but at a frequency exceeding the MTAFpred for the relevant ethnicity group. Erroneous inclusion of such variants as VRMVs in the case series may result in overestimation of the PG-VRMV-LRs. Furthermore, because of limited control and case data, we have had to develop parameters and apply them to the same data set. Access to additional independent data sets would allow testing of reproducibility.

Third, for most rare phenotypes, parameter estimates for population frequency, lifetime penetrance, and genetic heterogeneity vary widely and may be subject to substantial ascertainment bias. For pleomorphic syndromic phenotypes, it is only feasible to estimate MTAFpred by pulling out a specific component of the syndrome and estimating the frequency, penetrance, and genetic heterogeneity for this component (eg, type 2 renal papillary cancer for the FH gene [hereditary leiomyomatosis and renal cell carcinoma] or medullary thyroid cancer for the RET gene [multiple endocrine neoplasia type 2]).27

Fourth, a specific case definition (ascertainment framework) is required to which the VRMV-LR is applicable. In practice, eligibility for clinical gene testing likely varies in space and time, rendering it challenging to precisely pinpoint the framework for ascertainment and case inclusion.

Fifth, the VRMV-LR metrics are based on and applicable only to observed variants that themselves are very rare (ie, are observed in the control population at frequencies below the MTAFpred). Variants that are disease-associated but of lower penetrance will likely occur in the population but at frequencies above the MTAFpred. Such variants would not have been included in the VRMV case control analyses, and the VRMV-LR metric would not be applicable to them.

Thus, although our parameter estimates were deliberately conservative and the limited size of NFE control data may have caused underestimation of PG-VRMV-LR, failure to guarantee full exclusion of VRMVs in non-NFE cases would support a conservative translation of our estimates of PG-VRMV-LRs to evidence strengths for clinical variant classification.12

Although we used SDHB/SDHD, PCC/PGL, and missense variation as our exemplar gene/phenotype/variant-class paradigm, the principles, requisite data sets, and methodologies illustrated here are universally applicable to any other gene/phenotype/variant-class scenario. We propose that adoption of the methodologies illustrated for other rare Mendelian cancer syndromes would improve consistency and accuracy of quantitative estimation of the rare variant/rare phenotype phenomenon (PG-VRMV-LR for PP2), of the variant location in the right hot-spot (DS-VRMV-LR for PM1), and of the quantitative evaluation of subphenotypes (SP-LR for PP4).
Data Availability

The publicly available data analyzed are available as per the references/URLs provided. Any materials and data developed during this study will be made available upon request from the corresponding author.

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Ethics Declaration

The human variant data used were all de-identified. The data used for frequency analyses were provided in summary form only; all these data were wholly de-identified, and thus, institutional review board approval was not required. Collection and analysis of individual-level subphenotypic data were approved by the South Birmingham Research Ethics Committee as per the previous publications describing these data.9,15

Conflict of Interest

The authors declare no conflict of interest.

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Additional Information

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