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How human genetic context can inform pathogenicity classification: *FGFR1* variation in idiopathic hypogonadotropic hypogonadism

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Abstract

Precision medicine requires precise genetic variant interpretation, yet many disease-associated genes have unresolved variants of unknown significance (VUS). We analyzed variants in a well-studied gene, *FGFR1*, a common cause of Idiopathic Hypogonadotropic Hypogonadism (IHH) and examined whether regional genetic enrichment of missense variants could improve variant classification. *FGFR1* rare sequence variants (RSVs) were examined in a large cohort to (i) define regional genetic enrichment, (ii) determine pathogenicity based on the American College of Medical Genetics/Association for Molecular Pathology (ACMG/AMP) variant classification framework, and (iii) characterize the phenotype of *FGFR1* variant carriers by variant classification. A total of 143 *FGFR1* RSVs were identified in 175 IHH probands (n = 95 missense, n = 48 protein-truncating variants). *FGFR1* missense RSVs showed regional enrichment across biologically well-defined domains: D1, D2, D3, and TK domains and linker regions (D2–D3, TM–TK). Using these defined regions of enrichment to augment the ACMG/AMP classification reclassifies 37% (20/54) of *FGFR1* missense VUS as pathogenic or likely pathogenic (PLP). Non-proband carriers of *FGFR1* missense VUS variants that were reclassified as PLP were more likely to express IHH or IHH-associated phenotypes [anosmia or delayed puberty] than non-proband carriers of *FGFR1* missense variants that remained as VUS (76.9% vs 34.7%, p = 0.035). Using the largest cohort of *FGFR1* variant carriers, we show that integration of regional genetic enrichment as moderate evidence for pathogenicity improves the classification of VUS and that reclassified variants correlated with phenotypic expressivity. The addition of regional genetic enrichment to the ACMG/AMP guidelines may improve clinical variant interpretation.

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Conflict of interest The authors have no relevant financial or non-financial interests to disclose.

Ethical approval This study was performed in line with the principles of the Declaration of Helsinki. This research was reviewed and approved by the Massachusetts General Hospital Partners Institutional Review Board (ClinicalTrials.gov no. NCT00494169; registered 29 June 2007).

Consent to participate Written informed consent was obtained from all individual participants included in the study.

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Introduction

When a gene is implicated in human disease, precision medicine seeks to translate this discovery into clinical medicine. The American College of Medical Genetics /Association for Molecular Pathology (ACMG/AMP) provides standard guidelines for classifying variants in disease-implicated genes from benign to pathogenic (Richards et al. 2015). The goal is to empower clinicians and genetic counselors to provide accurate and informative information to patients. Practically, most of the rare variation, especially missense variation, in disease-implicated genes are classified as variants of unknown significance (Shirts et al. 2016; Burke et al. 2022; Cangiano et al. 2021) (VUS). Improving the ability to classify a variant as benign or pathogenic rather than VUS, enhances the ability to counsel patients with these variants (Burke et al. 2022).

The ACMG/AMP guidelines for classification sum multiple sources of information, including functional, computational, and population data to support the grading of a variant (Richards et al. 2015). Functional data does not exist for every variant and designing and conducting experiments for high throughput screening of every new rare or hitherto unreported missense variant across the coding genome is challenging (Burke et al. 2022). In addition, population data does not work for the $N = 1$ problem of ultra-rare variation in a rare disease: there are no other individuals with these variants and individual variant-based assessment is hence not feasible. To help resolve variant interpretation relating to VUS, we leveraged a large cohort of genetically characterized probands with Idiopathic Hypogonadotropic Hypogonadism (IHH, MIM147950) and rare or ultra-rare variation in *FGFR1* to examine if aggregated domain-based population-level genetic data could improve ACMG/AMP variant classification guidelines.

FGFR1 is a prototypical candidate to examine the use of ACMG/AMP variant classification guidelines and to apply domain-based genetic enrichment. *FGFR1* is the largest single contributing cause of IHH, has well-studied domains, has a significant amount of generated in vitro data, and is frequently inherited (Pitteloud et al. 2006; Lansdon et al. 2017; Dode et al. 2003; Belov and Mohammadi 2013; Topaloglu 2017). After applying ACMG/AMP variant classification guidelines based on known data, we examined whether population data of domain-based enrichment could improve the classification of rare missense variants in *FGFR1*. Given that *FGFR1* variants are commonly inherited we examined the phenotype of non-probands who inherited rare *FGFR1* variants by ACMG/AMP classification to determine the impact of augmenting ACMG/AMP variant classifications with domain-based enrichment data. Our findings lend credence to incorporating domain-based enrichment to improve ACMG/AMP classification and based on this information, we offer suggestions for its application to the ACMG/AMP variant classification criteria.

Materials and methods

This research was reviewed and approved by the Massachusetts General Hospital Partners Institutional Review Board. All subjects provided written informed consent (ClinicalTrials.gov no. NCT00494169; registered 29 June 2007).

Patient cohort

The cohort contains probands with IHH (n = 1461) as well as individuals with related phenotypes (i.e., isolated anosmia, constitutional delay of puberty) (n = 510) and family members (n = 1505). Idiopathic hypogonadotropic hypogonadism (IHH) was defined as previously published (Kallmann et al. 1944; Balasubramanian and Crowley 2011). In brief, IHH was defined as: i) absent/incomplete sexual maturation; (ii) serum testosterone levels of < 100 ng/dL in men or estradiol < 20 pg/mL in women in the setting of low or normal levels of serum gonadotropins; iii) no functional reason for hypogonadism. Olfactory function (i.e., determination of normosmic hypogonadotropic hypogonadism [nIHH] and Kallmann Syndrome [KS]) was determined by the University of Pennsylvania Smell Identification Test (UPSIT) or by self-reported inability to smell—as self-report has been previously been shown to accurately identify complete anosmia] (Doty et al. 1984, 1996; Lewkowitz-Shpuntoff et al. 2012). Isolated anosmia (without IHH) was defined by UPSIT or by self-report of inability to smell and no evidence for IHH. Constitutional delay of puberty (CDP) was defined as having late-onset pubertal development by physician report and having evidence of spontaneous progression through puberty before age 18. Phenotypic information was obtained from clinical evaluation of the proband or family member in the clinic practice of the Massachusetts General Hospital Reproductive Endocrine Unit, review of medical records from referring physicians, or detailed questionnaires on overall health and reproductive history completed by study subjects.

Genotyping

The Massachusetts General Hospital Reproductive Endocrine Unit Cohort (1971 probands, 1505 family members) have been screened for variants in *FGFR1* using either whole-exome or traditional Sanger sequencing.

Whole-exome sequencing (WES)—WES was performed on the Broad Institute Genomics Platform and processed using GATK best practices (McKenna et al. 2010). The jointly called VCF files were then analyzed using the integrative database framework, GEMINI v.0.19.1. Variant annotation was noted with the Ensembl Variant Effect Predictor (VEP) against the GRC37/hg19 reference Human Genome. Rare sequence variants (RSVs) for both homozygous and heterozygous RSVs were required to be either: non-synonymous variants (stop gain, frameshift, or missense) or canonical splice altering variants (± 2 bp from exon–intron boundaries); and have minor allele frequencies (MAF) of < 0.1% in the genome Aggregation Database (gnomAD) browser in all ethnic groups. All RSVs identified by WES were confirmed using bi-directional Sanger sequencing.

Sanger sequencing—Sanger sequencing was performed by the Center for Computational and Integrative Biology (CCIB) DNA Core Facility at Massachusetts General Hospital (Cambridge, MA). All *FGFR1* RSVs are reported on transcript ENST00000447712.6 (RefSeq NM_023110.2) which codes for the 822 amino acid FGFR1 protein.

Variant classification

All variants were classified as pathogenic/likely pathogenic (PLP), uncertain significance (VUS), or benign/likely benign (BLB) according to the ACMG guidelines. Prediction RSVs impact on protein function utilized PolyPhen2 and SIFT (Ng and Henikoff 2003; Adzhubei et al. 2013). Variant frequency was classified according to gnomAD. After assigning variant classification, the computational prediction tool REVEL was used to assess for differences between variant classes (Ioannidis et al. 2016; Tian et al. 2019).

Defining regional enrichment

Comparison of the genetic burden of variants in the IHH cohort compared to population controls (gnomAD) (i.e., "burden testing") was performed as previously described (Guo et al. 2018). *FGFR1* RSVs domain locations were determined according to UniProt (https://www.uniprot.org/). Domain-specific burden testing was also performed using Fisher's exact test on RSVs in the gnomAD and IHH cohort. *FGFR1* domains or regions significantly over-represented in IHH cases, compared to gnomAD controls, were classified as enriched. Domains or regions not reaching statistical significance were classified as un-enriched.

Assessment of ACMG classifications using non-proband family members

In this study, we included all family members who shared the proband's *FGFR1* RSV and classified their phenotypes as (IHH, isolated anosmia, CDP, anosmia, and CDP, unaffected (i.e., normal timing and pace of puberty with intact sense of smell), or unknown (not enough phenotypic information). We did not include probands due to referral bias. We calculated the percentage of non-probands with an *FGFR1* RSV who were diagnosed with an *FGFR1*-related phenotype defined above by the ACMG category (with or without the use of moderate evidence provided by regional genetic enrichment).

Statistical methods

Fisher exact test was used for burden testing with adjustment using Bonferroni correction. REVEL variant scores were not normally distributed; therefore, the non-parametric Kruskal–Wallis test with Dunn's post-test was used in GraphPad Prism. Chi-square or Fisher exact was used in phenotype analyses as appropriate. The p values reported are adjusted and a p value of 0.05 was considered significant.

Results

FGFR1 RSVs are enriched in IHH probands compared to population controls

In total, 175/1461 (12%) IHH probands and 139 family members were found to harbor 143 *FGFR1* RSVs (n = 95 missense, n = 48 protein-truncating, PTV—Supplementary Tables 1, 2) including 40 missense mutations not reported in prior literature. Confirming prior assessments in smaller cohorts, gene-based burden testing for both missense and PTV variants revealed significant enrichment (p = 1.4E–62 and 7.2E–84, respectively) in IHH probands (Supplementary Table 3) compared to gnomAD controls adjusted for coverage (Guo et al. 2018). Compared to gnomAD controls, missense RSVs in IHH probands were enriched in the majority of defined domains/regions: D1 domain (p = 6.0E–4), D2 domain

(p = 3.7E–19), linker (D2–D3) (p = 1.4E–11), D3 domain (p = 4.1E–16), linker (TM–TK) (p = 1.2E–02), and TK domain (p = 2.6E–34) (Fig. 1). Strikingly, the enrichment in the D1 domain was driven by all the variants located in the C terminal end of D1 (aa 69–117, p = 6.0E–10), which suggests sub-domain level enrichment. This is consistent with the published data by other groups that found 85% of variants in D1 reported in IHH individuals were also found in C terminal end of D1 (n = 17/20, Supplementary Table 4). Based on this analysis, 93% of missense variants in our cohort (n = 88/95) fall within an enriched region.

Integration of regional genetic enrichment to ACMG classification improves pathogenicity prediction

ACMG/AMP standards and guidelines for the interpretation of sequence variants are used nationwide by clinical genetics laboratories to assess the pathogenicity of variants (Richards et al. 2015). In this study, the following categories were not used to classify missense variants as they were not relevant to *FGFR1*-related IHH or missense variation: recessive disease categories (BP1 and PM3), PTVs and in-frame indels categories (BP3, PM4, PVS1), categories that do not account for oligogenicity reported previously in IHH (BP1, BP2 and BP5) (Table 1). Of the total 95 missense RSVs, 40 were classified as pathogenic/likely pathogenic (PLP), 54 were deemed variants of uncertain significance (VUS), and only 1 was classified as benign (Supplementary Table 2; Fig. 2A). Thus, the ACMG classification was not able to resolve the pathogenicity for a majority (57%) of IHH-associated *FGFR1* missense variants.

FGFR1-associated IHH is characterized by autosomal dominant inheritance of ultrarare (< 0.1%), missense and PTVs variants with variable expressivity (IHH, anosmia and constitutional delay of puberty) resulting in several uninformative ACMG/AMP guidelines (Table 1). BA1/BS1, and BS2 which examine observations or minor allele frequencies in controls inconsistent with disease do not distinguish between gnomAD and IHH cohorts of *FGFR1* variants; with few exceptions, *FGFR1* variants are ultrarare (< 0.1%). Ultra-rare variants typically are single observations either in gnomAD or IHH, preventing a population level data informed variant-based assessment of pathogenicity (PS4). As such, the only population level data category of ACMG/AMP that can be used is PM2—“absent in population databases”. Given the known variable expressivity, it is reasonable to assume that gnomAD could have individuals with anosmia or CDP (2.5% of the population by statistical definition); therefore, we have developed an aggregated population level metric that can help inform pathogenicity at a regional or domain rather than variant level.

Domain-based assessments are used to assign variant pathogenicity based on functional data in two different ways. First, the PM1 category requires well-studied functional domain without benign variation; however, current functional data does not align with updated recommendations which suggests modeling population-based variants and case-based variants in a well-validated assay specific to disease mechanism (Brnich et al. 2019; Gelman et al. 2019). As a result, PM1 cannot be used at this time to define domains of *FGFR1*, as there is not enough data on population-based variants, especially considering the number of missense variants found in gnomAD (n = 334) across all domains of the protein (Fig. 3; Supplementary Tables 5, 6). Second, the PP2 functional category defined

as missense variants in a gene with "low rate of benign missense variants and pathogenic missenses common" could apply to the entire *FGFR1* protein based on published *in-vitro* work. However, population level data demonstrates regional protein constraint suggesting parts of the protein are under no selective pressure and may not harbor uniformly deleterious alleles (Samocha et al. 2017). Given the scope of uniformly modeling all current known *FGFR1* variants (95 in this cohort, 134 in 81 other publications, and 334 found in gnomAD), we applied a regional enrichment population level metric to inform variant pathogenicity.

We integrated regional genetic enrichment as "moderate evidence" in support of pathogenicity into the ACMG/AMP standards and guidelines. This assertion is qualitatively similar to the use of variant-level data from in vitro studies to define critical functional domains (regions) that are considered to have "moderate evidence" in support of pathogenicity and has previously been adapted by ClinGen's Inherited Cardiomyopathy Expert Panel (Kelly et al. 2018). With this reclassification, 37% (20/54) of VUS were assigned PLP (n = 18) or BLB (n = 2; Fig. 2B, Supplementary Table 2). The use of regional genetic enrichment in this case increased the likelihood of an *FGFR1* missense variant being labeled as PLP. *FGFR1* missense VUS reclassified as likely pathogenic or pathogenic (PLP) were more damaging by REVEL, a composite protein prediction program used to assign variant pathogenicity than the *FGFR1* missense that remained assigned as VUS (Fig. 3) (Tian et al. 2019). As expected, based on this finding, 83% of *FGFR1* variants previously reported in other IHH cohorts (n = 111/134) occurred in these enriched regions (Supplementary Tables 4, 6).

We compared our regional genetic enrichment assessment to the ClinGen's inherited cardiomyopathy expert panel assessment called the etiological Fraction (Walsh et al. 2019) (EF; Supplementary Table 3). Our proposed regional genetic enrichment was largely concordant with the EF method (90% match when grouped into 3 categories: BLB, VUS, and PLP, n = 86/95 variants). Much of the discordance is due to six variants in the D2 region that would be re-classified from VUS to LP due to strong evidence for pathogenicity based on the Etiological Fraction as compared to moderate evidence by regional genetic enrichment. Additional comparisons of regional enrichment and Etiological Fraction across diseases are needed to determine which has the best broad applicability.

Integration of regional genetic enrichment to ACMG classification corroborates with phenotypic expressivity in non-proband carriers of *FGFR1* variants

To further corroborate the relevance of regional genetic enrichment in improving pathogenicity prediction and phenotypic consequence, we examined whether the phenotypic expressivity differed between VUS variants reclassified as PLP vs. those that remained VUS after application of regional genetic enrichment (Supplementary Tables 1, 2, and 3). Amongst non-proband *FGFR1* variant carriers, those harboring missense VUS reclassified as PLP were more likely to be associated with IHH or IHH-related phenotypes (CDP, and or anosmia; n = 10/13, 76.9%) than non-proband carriers of *FGFR1* variants that remained as VUS (34.7%, n = 8/23) (p = 0.035; Fisher's Exact Test). Integration of regional genetic enrichment did not change the proportion of non-proband carriers of PLP missense variants with IHH or IHH-related phenotypes (ACMG 71%, n = 36/49; ACMG with missense

regional enrichment 72.5%, $n = 45/62$). Of the 6 missense variants reclassified as PLP by EF, but not by regional genetic enrichment, none of the non-proband carriers had IHH ($n = 0/5$). In comparison, 46.2% of non-proband carriers of missense variants reclassified by regional genetic enrichment had IHH ($n = 6/13$). There were too few observations of the BLB variants in the IHH cohort; however, the two non-probands carriers of *FGFR1* BLB missense variants, based on regional enrichment, did not have IHH, CDP, or anosmia.

Discussion

Accurate interpretation of genetic variants is critical for genetic discovery, clinical care, and genetic counseling of patients with rare disorders. With regard to the IHH phenotype, *FGFR1* has been a well-studied gene with in vitro studies examining D2, D3, and TK regions. However, the majority of missense variants are still graded as VUS (Pitteloud et al. 2006; Kim et al. 2008; Villanueva et al. 2015). In this study, we utilized a large IHH cohort and by juxtaposing variant information from cases alongside population level data, we show that incorporation of domain-based enrichment information is able to augment ACMG/AMP assessment of pathogenicity of VUS. Approximately one-third of VUS were able to be reclassified to either PLP or benign variants following domain-based information and through phenotypic evaluation of non-probands with *FGFR1* variants, reclassified PLP variants were shown to be more likely to be associated with phenotypic expressivity of IHH and IHH-related phenotypes.

Adaptations and customization of the ACMG/AMP criteria have been occurring at the disease level by Clinical Genome Resource Variant Curation Expert Panels (VCEP) but to-date, such VCEPs have not been instituted for the IHH disease phenotype. In this report, we applied the standard ACMG/AMP criteria to *FGFR1* gene variants that account for 12% of the genetic etiology of IHH. A significant proportion of *FGFR1* missense variants (~ 56%) were deemed as VUS by standard criteria. By utilizing population level data, we applied a simple domain-enrichment-based metric to standard ACMG/AMP guidelines and were able to resolve 37% of the VUS determinations. These findings were in keeping with other specialized disease curation variant panels that reclassified a similar number of VUS across a range of disease entities (hypercholesteremia, hereditary gastric cancer, hearing loss and platelet disorders), such that, in both cases, approximately only one-third of variants remain classified as having unknown significance (Lee et al. 2018; Patel et al. 2021; Chora et al. 2022; Ross et al. 2021).

The use of population data and case/control data to establish critical protein domains as done in this report is further supported by work in the field of inherited cardiomyopathies done by members of ClinGen's Inherited Cardiomyopathy Expert Panel (Walsh et al. 2019). In this prior report, Walsh et al. proposed the use of a high prior likelihood of pathogenicity, as defined by an etiological fraction, based on gene, variant class, and variant location within the gene/protein (Walsh et al. 2019). In this new system, any gene or region with an etiological fraction greater than 0.8 would be considered in the assignment of pathogenicity with increasing levels of evidence as the etiological fracture approaches. In this study, we also examined the *FGFR1* etiological fraction and given the ultra-rarity of *FGFR1* variants in the general population, noted a high EF for variants across the whole protein

(0.91). The assessment of regional EF was mostly similar to the findings with our domain-based regional enrichment data, with the exception of Linker TM–TK and D2 regions. However, the variants re-coded as PLP using the strong evidence criteria in the EF graded scoring system (supporting, moderate or strong evidence based on EF score) appear to have lower expressivity in non-proband variant carriers than variants reclassified by moderate evidence. This suggests using strong evidence criteria for population data may overcall the pathogenicity of variants. We propose a more conservative addition to the ACMG/AMP guidelines which is the use of regional genetic enrichment, but not gene-level enrichment, as moderate evidence in support of pathogenicity. Future research is needed to understand how these two metrics perform for assessment of pathogenicity of missense variants performs across diseases.

While this study examined case-population variant data comparisons looking for enrichment of variants in specific domains, prior approaches have used human population data to identify highly conserved regions of the genome, where there is less, or depletion of variation seen in humans than expected. Indeed, *FGFR1* has been reported to have regional genetic constraint/depletion of variants across the gene that partially overlaps with the regional enrichment noted in this study (Samocha et al. 2017). However, in the constraint-based assessment regional constraint was not based on domains and the reported constraint regions overlaps with domains of *FGFR1,* where we did not find any enrichment of variation in IHH cases. This study finding suggests that domain-based enrichment assessments using disease populations may provide more accurate information about variant pathogenicity compared to more general constraint-based analyses. Indeed, other authors have advised caution in the consideration of entire genes being constrained in the ACMG/AMP criteria (Richards et al. 2015; Harrison et al. 2019).

Limitations

This study has some limitations. There are concerns about the limitations of burden testing based on cohort size, matching, and ethnicity. Given the rarity of IHH, even with the largest published cohort, further refinement of the study findings could come from even larger cohorts. Given very rare/private mutations have a similar distribution across ethnic spectrums, particularly in non-African populations that have not experienced severe population bottlenecks, IHH cases were not subdivided by ethnic group for burden testing (Lek et al. 2016; O'Connor et al. 2013; Genomes Project C et al. 2015). Further refinements of burden testing methods could further improve the use of domain-based burden in the assessment of variant pathogenicity. Even with the limitations of the current methods, the reclassified variant's behavior in non-probands supports the use of domain-based enrichment.

Conclusions

The incorporation of domain-based enrichment as "moderate evidence" into the ACMG/AMP guidelines for variant classification significantly reduced the number of VUS in *FGFR1*. Those reclassified variants exhibited behavior consistent with their new classification: non-probands with *FGFR1* missense variants reclassified as PLP were as

likely to be affected as non-probands with PLP *FGFR1* missense variants by ACMG/AMP criteria. Based on this data, updating the ACMG standards may improve the accuracy of genetic counseling for patients with *FGFR1* variants. This extends the support for updating ACMG standards with an assessment of regional enrichment and suggests this may help with variant classification across genes and diseases. Future research is needed to determine the best broadly applicable method to assess regional enrichment across diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data Availability

Confirmed sequencing data for variants in this manuscript are deposited into ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/, submissions SCV003932463–SCV003932605). Data generated or analyzed during this study are included in this published article and its supplementary information files. Additional data sets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Fig. 1.

A Schematic domain diagram for FGFR1 protein; **B** enrichment of missense mutations, bold black line represents significant enrichment in IHH cohort compared to gnomAD (adjusted $p < 0.05$)

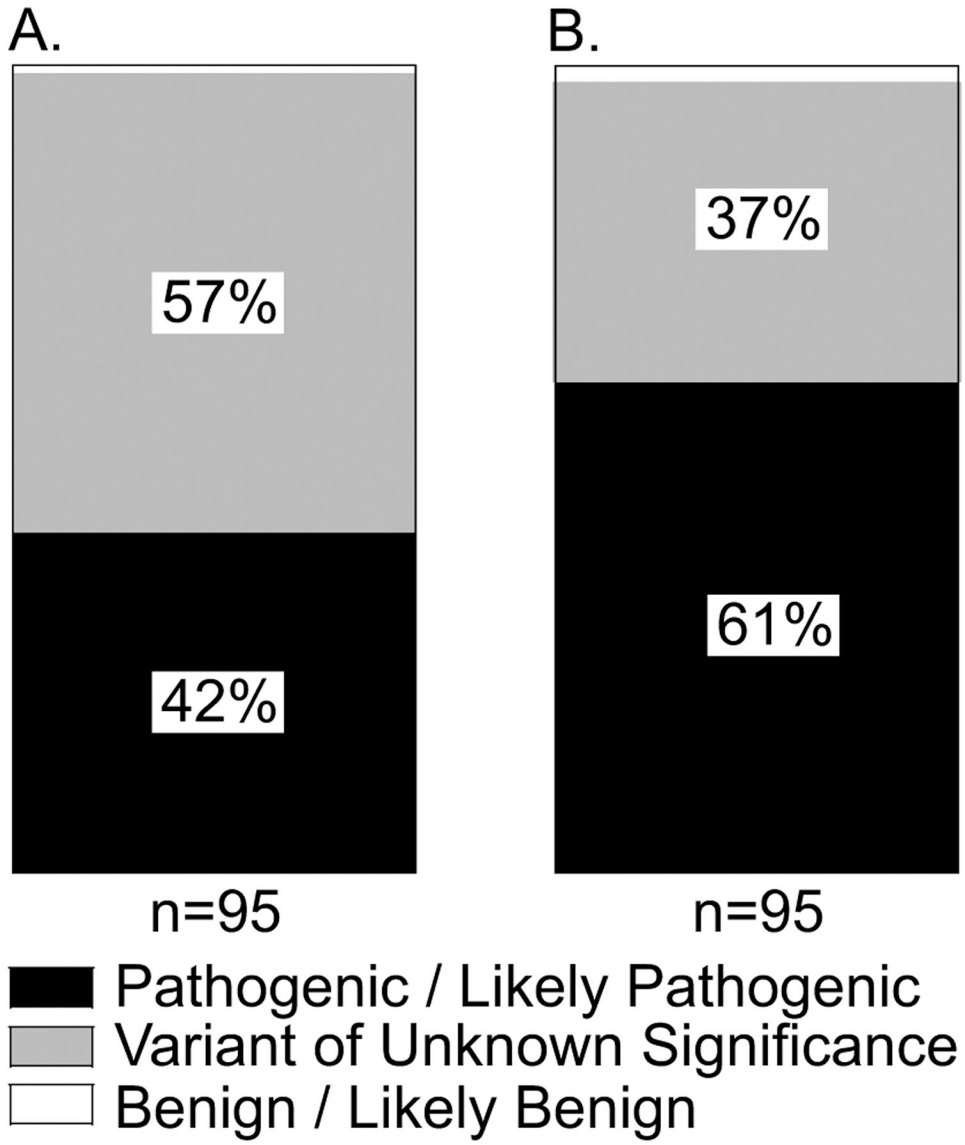

Fig. 2.
FGFR1 missense variants (*n* = 95). **A** ACMG classification. **B** ACMG classification using missense regional enrichment

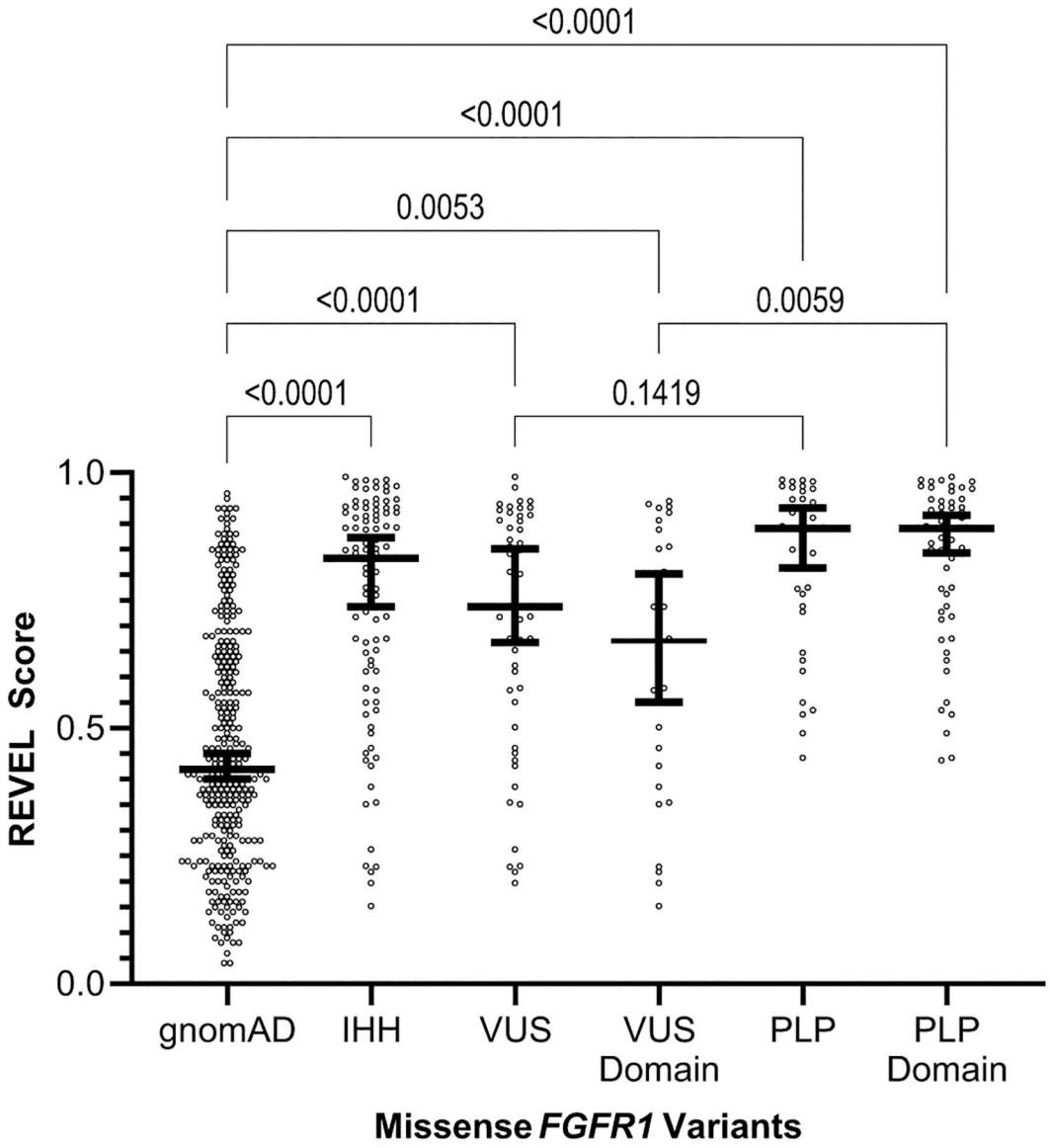

Fig. 3.

FGFR1 missense variants (n = 95). REVEL scores for recategorization of VUS by ACMG criteria VUS, and PLP: missense variants categorized as variant of unknown significance (VUS) or pathogenic or likely pathogenic (PLP). VUS domain and PLP domain: missense variants categorized using ACMG/AMP guidelines and domain-based burden

Table 1

Use of ACMG criteria in assessment of *FGFR1* missense variants found in hypogonadotropic hypogonadism

Category	Domain	Defzinition	Assessment
PM2	Population Data	Absent in population databases	Applicable without modification
BP4	Computational Data	Multiple lines of computation evidence suggest no impact on gene/gene product	Applicable without modification
BP7	Computational Data	Silent variant with non-predicted splice impact	Applicable without modification
PP3	Computational Data	Multiple lines of computation evidence support a deleterious effect on the gene/gene product	Applicable without modification
PM5	Computational Data	Novel missense change at an amino acid residue, where a difference pathogenic variant has been seen before	Applicable without modification
PS1	Computational Data	Same AA change as an established pathogenic variant	Applicable without modification
BS3	Functional Data	Well-studied functional studies show no deleterious effect	Applicable without modification
PP2	Functional Data	Missense in gene with low rate of benign missense variants and pathogenic missenses common	Recommend using PM1 with population level data instead
PM1	Functional Data	Mutational hot spot or well-studied functional domain without benign variation	FGFR1 has multiple well-studied functional domains; however, all have predicted benign missense variants found in population database. We recommend using PM1 to allow for domain-based enrichment assessment based on population data
PS3	Functional Data	Well-established functional studies show a deleterious effect	Applicable without modification
BS4	Segregation Data	Non-segregation with disease	Not applicable to FGFR1 as a disease with variable penetrance
PP1	Segregation Data	Co-segregation with disease in multiple affected family members	Applicable without modification
PM6	De Novo Data	De novo without or without paternity or maternity confirmed	Applicable without modification
BP1	Allelic Data	Observed in trans with a dominant variant (BP2)	Not applicable given oligogenic basis of IHH as a disease
BP2	Allelic Data	Observed in cis with a dominant variant (BP2)	Not applicable given oligogenic basis of IHH as a disease
BP6	Other	Reputable source without shared data = benign	Recommend not using; did not use
PP5	Other	Reputable source without shared data = pathogenic	Recommend not using; did not use
BP5	Other	Found in case with alternative cause	Not applicable given oligogenic basis of IHH as a disease
PP4	Other	Patient phenotype or FH highly specific for gene	Applicable without modification for phenotypes consistent with allelic disorder (Hartsfield, split-hand foot, cleft lip, or cleft palate)