Elucidating the Pathogenicity of Rare Missense Variants with Statistically-Validated *In vitro* Functional Studies

Introduction

Effectively managing patients that receive inconclusive genetic testing results remains one of the largest challenges facing healthcare institutions today. With all the clinical information that we've acquired in the past century, we're still left with far more questions than answers when it comes to understanding how our genes affect our health. More relevant than the exponentially decreasing costs of human genome sequencing[1] is the exponentially increasing amount of genetic data being produced, which is rapidly outpacing the amount of clinically-usable information required to make sense of it all.

The lack of reliable clinical information leads to an increased potential for patient mismanagement. Faced with potential lawsuits and wary of a negative public perception, many institutions have become more conservative when relaying results and recommending management plans based on genetic testing results.

In lieu of available clinical information, healthcare professionals rely on various research-based and computational evidence to determine the impact that rare genetic variations have on the development of genetic disease. One of the most widely-used variant classification systems, the 2015 ACMG-AMP Guidelines (created by a joint workgroup of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology)[2] enables clinicians to weigh specific pieces of evidence to determine a more accurate classification.

While it's been shown that this weighted system can enable greater accuracy and consistency of variant classification across clinical institutions[3], several drawbacks still exist when applying evidence, including:

(1) *In silico* (computational) predictions can conflict with each other or with separate strong, validated evidence, which may result in the downgrade of otherwise certain classifications of pathogenicity; and
(2) A general lack of confidence in the validity of particular *in vitro* functional studies can prevent otherwise strong, accurate evidence from being utilized when assessing variants

As a result, variants with indeterminable clinical significance in even well-understood disease genes like BRCA1 mar the genetic testing landscape[4], leaving far too many undiagnosed and mismanaged patients in our hospitals. Only by standardizing when and how *in silico* predictions and *in vitro* functional evidence are applied can we garner a clearer, more accurate picture of the clinical consequences of genetic variants.

Assessing Multiple Lines of Conflicting Computational Evidence

Widely considered low-weight evidence amongst medical geneticists, commonly-used *in silico* prediction tools like PolyPhen-2[5] and SIFT[6] are often contradictory amongst themselves and other non-computational evidence.

To understand how inconsistencies in the predictions between SIFT and PolyPhen affect classification, we analyzed 68 missense variants in the RING functional domain of the BRCA1 tumor suppressor gene (Table 1). Missense variation in the BRCA1 RING domain, a highly-conserved region whose activity is critical for tumor suppression and the proper regulation of DNA repair, may result in reduced protein function and an increased risk of developing breast or ovarian cancer[7,8].

Each variant assessed has been previously reported in ClinVar[9] as a variant of uncertain significance (VUS) or classified as benign, likely benign, likely pathogenic, or pathogenic (B/LB/LP/P). Of the 24 variants reported as B/LB/LP/P, both SIFT and PolyPhen predictions agree and are consistent with the indicated classification in 87.5% (21/24) of variants. Meanwhile, of the 44 variants reported as VUS, both SIFT and PolyPhen scores are in agreement for only 25/44 (56.8%) variants. This suggests that up to half of the variants with uncertain clinical significance detected during breast and ovarian cancer testing may not have reliable computational information to use as evidence for interpretation.

The ACMG-AMP guidelines warn that “it is important not to overestimate computational evidence, particularly given that different algorithms may rely on the same (or similar) data to support predictions and most algorithms have not been validated against well-established pathogenic variants.”[2] And while *in vitro* functional studies are considered more predictive of a variant’s impact in a clinical case and are weighted higher in the ACMG-AMP classification framework, the prevalence of dissimilar, unvalidated research methodologies used across laboratories has created a bottleneck between research utility and clinical adoption. As a result, it’s unknown just how much of the functional data published on rare variants can be relied on for clinical application.

	B / LB / LP / P	VUS	All
SIFT / PolyPhen Agreement	21/24 (87.5%)	25/44 (56.8%)	46/68 (67.6%)

Table 1. Agreement between the SIFT and PolyPhen-2 *in silico* predictions of variant impact in the RING functional domain of the BRCA1 tumor suppressor gene.

Establishing Valid *In vitro* Functional Evidence for Variant Classification

Observing a variant's impact in representative *in vitro* models can provide a clear picture of its role in disease pathogenesis. Unfortunately, few of the rare variants that arise during clinical testing have been characterized with *in vitro* research studies, and the prevalence of dissimilar research techniques across institutions means much of those data might be unreliable. While hundreds of labs generate data from countless functional assays, the lack of standardized data acquisition, processing, and reporting has created a bottleneck between research utility and clinical adoption.

> "Sites agreed that, at a minimum, the assay must be validated with known pathogenic and benign variants and the output of the assay must have an established mechanistic relevance to the associated phenotype."
> Amendola, et al. 2016

To address this need, Ranomics has developed a high-throughput variant synthesis and analysis platform to generate, screen, and determine the functional impact of thousands of missense mutations in well-known disease genes.

By introducing individual missense mutations into the coding sequence of an indicated gene, Ranomics can systematically probe the biological function of every resulting protein variation, even for variants that have not yet been seen in either a research or clinical setting. The function of each variant is then assessed using well-established *in vitro* assays that model the phenotype of an indicated disease or the biochemical activity of the native protein (Table 2). Based on their functional activity, each variant is benchmarked and scored according to a "loss-of-function" cutoff scale calculated between clinically validated benign and pathogenic missense variants of the same gene.

Protein (domain)	Functional assay type	Functional assay description	References
BRCA1 (RING domain)	Biochemical function	Dysfunctional BRCA1 demonstrates decreased ligase activity and weaker protein-protein interactions in its heterodimer with the BARD1 protein	10
BRCA1 (BRCT domain)	Phenotype recapitulation	Cells expressing dysfunctional variants in the BRCA1 BRCT domain grow faster than those with normally functioning variants in the BRCA1 BRCT domain	11
p53	Gene expression	Missense mutations in *TP53* disrupt the ability of p53 to bind to DNA and activate downstream transcription	12, 13, 14

Table 2. Descriptions of Ranomics high-throughput *in vitro* functional assays.

Using the Ranomics variant analysis and synthesis platform, every missense variant in the BRCA1 RING domain was synthesized and assessed *in vitro* for its respective impact on BRCA1 biochemical function. Of the 24 variants reported as B/LB/LP/P in ClinVar, the Ranomics BRCA1 RING functional study accurately predicted the classification of 21 variants (87.5%; three variants designated as "functional" by the Ranomics assay are reported as pathogenic in ClinVar).

Because each variant is designated "functional" or "not functional", Ranomics evidence can be applied to the BS3 ("well-established functional studies show no deleterious effect") or PS3 ("well-established in functional studies show a deleterious effect") criteria of the ACMP-AMP guidelines, respectively.

Assessing Conflicting Computational and Functional Evidence

In a recent study by the Clinical Sequencing Exploratory Research (CSER) consortium, the group uncovered a flaw in the way several institutions applied the ACMG-AMP guidelines to their classifications-- while the guidelines suggest classifying a variant as a VUS when separate criteria produce conflicting pathogenic and benign evidence, "some laboratories allowed one line of conflicting benign evidence of only a supporting level (e.g., computational predictions) to override otherwise strong evidence of pathogenicity."[3]

To determine how conflicting computational and functional evidence affect the outcome of a variant classification using the ACMG-AMP guidelines, we investigated nine variants of the BRCA1 RING domain (Table 3). Each variant has been determined to have normal protein function by Ranomics *in vitro* studies (corresponding to the BS3 criterion), and "damaging" by both PolyPhen and SIFT (corresponding to the PP3 criterion).

To ensure that lines of evidence were properly applied according to the ACMG-AMP guidelines, we utilized the ACMG variant scoring and classification module in the Fabric Genomics (formerly Omicia) Opal™ Clinical interpretation platform. Opal Clinical provides reviewers with a stepwise, guided assessment of each of the 28 ACMG-AMP criteria. As criteria evaluation progresses, Opal Clinical calculates an inferred classification based on the rules defined for combining criteria[2], which can be manually overridden by the interpreter.

> "All sites agreed that computational predictions of missense or splice variants are well known to have reduced accuracy and therefore should not be used to override other strong evidence."
> Amendola, et al. 2016

With all known evidence supporting the ACMG-AMP criteria applied in the absence of computational and functional evidence, the Opal Clinical system inferred a classification of VUS for each of the nine *BRCA1* variants, concordant with the reported ClinVar classifications. Since the Ranomics BRCA1-RING *in vitro* assay determined each variant to be functional, Opal Clinical derived a classification of likely benign with the BS3 criterion applied.

Because the ACMG-AMP guidelines are fully integrated within the Fabric Genomics clinical interpretation workflow, we did not have to ignore or manually override the *in silico* prediction criteria. Given the low weight that the ACMG-AMP guidelines place on computational evidence, it is unsurprising that the application of PolyPhen/SIFT "damaging" predictions (PP3) failed to downgrade any variant's classification either with Ranomics functional evidence applied (from likely benign to VUS) or without (from VUS to likely pathogenic).

BRCA1 Nucleotide change	BRCA1 Protein change	Classification -PP3, -BS3 [a]	Classification +PP3, -BS3 [b]	Classification -PP3, +BS3 [c]	Classification +PP3, +BS3 [d]
c.11C>T	p.Ser4Phe	VUS	VUS	Likely benign	Likely benign
c.154C>T	p.Leu52Phe	VUS	VUS	Likely benign	Likely benign
c.189A>T	p.Leu63Phe	VUS	VUS	Likely benign	Likely benign
c.19C>T	p.Arg7Cys	VUS	VUS	Likely benign	Likely benign
c.216C>A	p.Ser72Arg	VUS	VUS	Likely benign	Likely benign
c.230C>T	p.Thr77Met	VUS	VUS	Likely benign	Likely benign
c.32T>C	p.Val11Ala	VUS	VUS	Likely benign	Likely benign
c.60A>C	p.Lys20Asn	VUS	VUS	Likely benign	Likely benign
c.66A>C	p.Leu22Phe	VUS	VUS	Likely benign	Likely benign

Table 3. Inferred classifications of variants located in the RING domain of BRCA1. SIFT and PolyPhen both predict that each variant is damaging. Ranomics *in vitro* studies have determined each variant to produce normally functioning proteins. Classifications were determined (a) without SIFT/PolyPhen *in silico* predictions and without Ranomics *in vitro* functional evidence (-PP3, -BS3); (b) with SIFT/PolyPhen *in silico* predictions and without Ranomics *in vitro* functional evidence (+PP3, -BS3); (c) without SIFT/PolyPhen *in silico* predictions and with Ranomics *in vitro* functional evidence (-PP3, +BS3); (d) with SIFT/PolyPhen *in silico* predictions and Ranomics *in vitro* functional evidence (+PP3, +BS3).

Conclusion

In far too many cases, the clinical evidence required to accurately determine the pathogenicity of genetic variants remains unavailable to the clinicians interpreting genetic testing results. Emerging algorithmic predictions have proven merely speculative-- if not unreliable-- and given how little influence computational evidence has in determining the pathogenicity of variants, it's imperative that stronger, more dependable sources of evidence are found and applied.

In the absence of real world clinical information, utilizing well-established *in vitro* functional evidence remains the most efficient, feasible option to apply strong evidence towards variant classification.

While the reliance on functional evidence remains low partially due to skepticism of the validity of the *in vitro* assays themselves[3], continuing efforts to standardize the acquisition, processing, and reporting of data from *in vitro* functional studies are clearing the bottleneck between research application and clinical acceptance. Furthermore, quantifying and statistically validating the observed impact that a variant has on protein function in a biological system can provide a more accurate picture than a purely *in silico* prediction.

By determining the functional impact of every variant in known disease-causing genes, we can equip healthcare organizations with the clinically-applicable information they need to accurately assess the pathogenicity of novel and rare variants.

About Ranomics

Ranomics is committed to eliminating uncertainty from genetic testing. We're building the world's first comprehensive knowledgebase to help healthcare organizations understand the impact that missense genetic variation has on biological function and disease development. Our proprietary high-throughput variant synthesis and analysis platform was developed to generate, screen, and determine the functional impact of thousands of missense mutations that have never been reported in well-established disease genes like BRCA1 and TP53. Every functional assay has been standardized and automated to provide clinical labs with reliable, reproducible functional evidence that can be applied to standardized variant classification frameworks like the ACMG-AMP guidelines.

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