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PEARLS OF LABORATORY MEDICINE

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TITLE: Variants of Uncertain Significance

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Slide 1:
Hello, my name is John R. Mills. I am a clinical molecular genetics fellow at Mayo Clinic. Welcome to this Pearl of Laboratory Medicine on "Variants of Uncertain Significance."

Before we get started, I'd like to encourage you to watch Dr. Lora Bean's Pearl on "DNA Sequence Nomenclature and Variant Interpretation" (available at www.traineecouncil.org), as it provides complementary information to this Pearl.

Slide 2:
As many of you are aware, genetic testing is a rapidly growing area of laboratory medicine. This had been driven by a couple of different factors. The rapidly declining cost of DNA sequencing and the advent of next-generation sequencing have enabled researchers to perform large scale discovery-based sequencing that has uncovered the genetic basis for a variety of disorders. The number of studies aimed at profiling cancers has also exploded, leading to a much better understanding of the molecular basis of cancer development as well as treatment responses. Therefore, there are a growing number of disorders that can be diagnosed molecularly and the number of genes and variants that are clinically actionable has grown. More genes are being sequenced clinically, and more tests are being offered. The end result is a huge increase in the discovery of new variants.

Slide 3:
Given the dramatic growth in the discovery of genetic variants, there has been growing concern about how to properly classify these variants in a standardized and meaningful way so that healthcare providers can properly interpret these variants. Due to both the expanding role of sequencing in clinical practice and the increased complexity of analysis, the American College of Medical Genetics and Genomics (ACMG), the Association of Molecular Pathology (AMP), and the College of American Pathologists (CAP) have put forward efforts to standardize how findings are reported and communicated in order to properly direct follow-up care. The recent ACMG standards and guidelines recommendation published in May of 2015 proposes an updated variant classification system for interpretation of variants.

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The updated ACMG classification system is a 5-tier approach that classifies variants using the following modifiers: “pathogenic,” “likely pathogenic,” “uncertain significance,” “likely benign,” and “benign.” Of important note, this classification system is intended for classifying variants associated with monogenic mendelian inheritance. Although many of the same principles included here could be useful for non-mendelian inheritance, this classification system was not intended for the classification of somatic variants, mitochondrial DNA variants, nor complex multigenic disorders.

In order to classify a variant as “pathogenic,” we need to have strong evidence that heavily favors pathogenicity. When a variant is classified as “pathogenic,” generally, targeted testing will be offered to at-risk family members; and if medically actionable, there may be changes in medical management (i.e. regular screenings). “Pathogenic” variants should be included in the results report.

“Likely pathogenic” variants are those that have consistent evidence that favors pathogenicity but is more limited. “Likely pathogenic” variants should be treated similarly to “pathogenic” variants – meaning there may be potential changes in medical management and targeted testing should be offered to at-risk family members. These also should be reported.

“Likely benign” variants are those with strong evidence arguing against pathogenicity. Typically, there would be no reason to offer targeted testing for family members and generally “likely benign” variants are not included in result reports. There are some exceptions, such as pseudodeficiency alleles that alter the protein product or change the gene's expression without causing disease. This may result in an abnormal biochemical test results due to an inability of the gene to metabolize exogenous substrates that maybe used in the in vitro reaction. However, the gene product resulting from the pseudo deficiency allele may retain full activity for its endogenous substrate. In these situations, reporting such variants can explain abnormal testing results that may be of concern.

“Benign” variants are treated similarly to “likely benign” variants with the distinction that there is very strong evidence that the variant has no clinical impact on the gene or gene product function.

The last category, which is the most problematic, is the variants of “uncertain significance.” These are variants where there is limited evidence to favor either benign or pathogenic. Alternatively, there could be conflicting evidence regarding pathogenicity. Typically, a variant of “uncertain significance” is included in the results report. Next, we will discuss the different types of evidence used to classify variants and then focus the remainder of this Pearl on understanding the impacts of a variant of “uncertain significance.”

Slide 5:
The next two slides highlight the common sources of evidence that are used to establish pathogenicity as recommended in the 2015 ACMG recommendations.

In the right column, I have highlighted the relative strength of each type of evidence. Typically, one of the strongest pieces of evidence supporting pathogenicity is a variant that causes a

drastic change in the gene product where disruption of the gene of interest is a known mechanism of disease. These variants include nonsense changes leading to a premature stop codon, variants that induce frameshifts that often result in a severe protein truncation, and variants affecting canonical splice sites. Such variants are generally sufficient to suggest a pathogenic effect in the absence of conflicting evidence. Variants that cause the same amino acid change as that of a well-established pathogenic variant strongly favor pathogenicity. Functional studies that demonstrate the variant is detrimental to the normal function of the gene product strongly favor pathogenicitiy. However, it's important to remember this still requires an objective review of the data that indicates a functional defect. Critical review of the primary data can often uncover poorly designed and controlled functional studies. If the change is *de novo*, meaning it is not found in unaffected biological parents and is found in the affected individual, this supports pathogenicity. In large population-based genetic association case-control studies, if the variant is strongly enriched in the affected population, this can strongly favor pathogenicity. It's important to consider the number of individuals in these studies and to confirm the control population used was appropriate.

Slide 6

Other forms of evidence supporting pathogenicity that carry less weight include whether the variant is located in a functionally important area of the gene product, whether the variant has been proven to be absent or at very low frequency in control populations, whether the variant occurs in trans in a recessive condition along with a known pathogenic variant, and whether the variant results in an amino acid change that is known to support disease when it is altered.

The lowest tier of evidence that supports pathogenicity includes *in silico* predictions indicating the variant interferes with normal protein function. There are a couple of reasons why using these *in silico* programs must be considered cautiously. First, the sensitivities of these programs are limited to around 60-70% and they are prone to make false-positive calls at a rate of ~20%. In addition, different algorithms often produce conflicting results. The predictability of this varies greatly from protein to protein.

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The next two slides highlight the evidence that can be used to support that a variant is benign. Generally, if the allele frequency is greater than 5% in one of the large control population cohorts that are publicly available (which include the Exome Sequencing Project, the 1000 Genomes Project, or the Exome Aggregation Consortium), this is considered strong evidence that the variant is benign. Using the disease prevalence, we can calculate the expected frequency of the disorder in the population; if the allele frequency of a particular variant is much greater than the frequency of the disorder, this would favor the variant being benign. If the variant is observed in a healthy adult where if pathogenic it would be expected to cause disease, such as where full-penetrance is anticipated, this would be strong evidence to the benign nature of the variant. Similarly, if the variant does not segregate with disease in affected families, this would favor non-pathogenicity. Strong functional data indicating no detrimental effects on the function of the gene or gene product would also be suggestive of a benign variant.

Slide 8:
This table lists a variety of supporting evidence that, alone, would have limited impact on the variant classification but in combination, could impact the classification.

Slide 9:
When a variant is reviewed, we can distribute each piece of evidence into two separate bins; those that support pathogenicity and those that favor the variant being benign. ACMG guidelines propose a complex method for incorporating the evidence into a final classification decision. This is beyond the scope of this Pearl but I would encourage you to read Reference #1 by Richards et al to learn more.

The value in the ACMG guidelines is they provide a detailed and objective mechanism to classify variants in a reproducible manner. The criteria tend to be more stringent than how many laboratories are currently classifying variants. Therefore, more variants are likely to be classified as variants of "uncertain significance." Ultimately, if there is limited evidence to swing the scale in either direction or if there is conflicting evidence, the variant will be classified as a variant of "uncertain significance."

Slide 10:
Here is an example of a variant which we classified as a variant of "uncertain significance" in our laboratory. In this particular case, a variant was found in the *PRSS1* gene, which encodes cationic trypsinogen, the major precursor protein of trypsin expressed by the pancreas. This gene is strongly implicated in hereditary pancreatitis and there are several well-documented pathogenic variants that lead to inappropriate activation of trypsin. For this clinical case, testing had been ordered due to a long-standing history of idiopathic pancreatitis. Sequencing identified a missense mutation (c.623G>C), at cDNA position 623 leading to an amino acid change from Glycine to Alanine at codon 208. Because this is a missense change, we can't favor either a benign or pathogenic variant without additional evidence.

Review of the literature found that in one family study, the variant was found in multiple unaffected family members and did not segregate with disease. However, in a separate study, the variant co-segregated with only affected family members. These two pieces of literature directly contradicted each other and it was not possible to discern if one study was incorrect or to explain why they might be in disagreement.

Functional studies were performed on the variant. However, these indicated the variant had limited impact on the protein function as it relates to pancreatitis. We reviewed several allele frequency databases and found that in certain Asian populations, the variant occurred at a frequency greater than 1%, which favors this being a benign variant. A case control study indicated the variant was strongly enriched in the affected population with an odds ratio 15, which supports pathogenicity. Two of three *in silico* programs predicted the variant to be damaging.

In this example, we have to closely evaluate the evidence and decide if there is sufficient information to favor pathogenic or benign. In this case, because we deemed the evidence to be contradictory, we called this a variant of "uncertain significance." Although a variant of "uncertain

significance" classification occurs more frequently due to lack of evidence, this particular case highlights the challenges of interpreting conflicting sets of evidence, why it is common to encounter variants of "uncertain significance," and why the lack of standardization in how laboratories classify variants can be troublesome.

Slide 11:
Now we will begin to focus more closely on variants of "uncertain significance." Studies of large cohorts of diverse patients who have had clinical genetics testing have suggested a majority of variants selected for review will be categorized as variants of "uncertain significance." This is largely due to the limited information about many of the genes that are being sequenced clinically. Therefore, it is natural that as more genes are sequenced, the more variants of "uncertain significance" we are expected to encounter. As a gene first enters the clinical realm, it is likely there will be limited information available for that gene. Often, in large disease-focused panels, a gene may be included based on a couple of studies that only looked at a handful of variants. On average, the vast majority of variants that will be subsequently found, at least in the early phases of clinical testing, may be of "uncertain significance." There likely won't be sufficient evidence to support an informative classification in many cases.

Slide 12:
As laboratories transition to more comprehensive sequencing approaches moving away from limited targeted sequencing and towards whole exome or genome sequencing, the number of variants of "uncertain significance" is expected to increase substantially. This is determined largely by the number of genes being interrogated and the extent of our knowledge of the genes being sequenced. The number of potential variants of "uncertain significance" found by whole exome sequence can approach the thousands. This is prohibitive both from the turn-around-time perspective and the cost of variant review.

Slide 13:
The question then becomes, "How will we handle all of these variants?" A broad estimate from exome sequencing would anticipate finding, on average, 20,000 variants, of which a majority would be of "uncertain significance." Therefore, to reduce this workload, bioinformatic approaches are used to reduce the number of variants that require review.

Several approaches can be used to accomplish this. Quality metrics can be used to remove less reliable variant calls, thereby focusing on only high quality calls. Using the sequence obtained from the affected individual as well as that from the biological parents, variants can be filtered out that do not fit the expected mode of disease inheritance. Another approach is to apply an allele frequency cut-off; by increasing the stringency of the cut-offs, the number of variants identified for review can be decreased, and because the current use of exome sequencing is generally aimed at diagnosing rarer conditions, a lower allele frequency cut-off can be tolerated without having a dramatic impact on the diagnostic sensitivity. Once the list of variants is narrowed, the remaining variants will still need to be prioritized for review. Generally, primary consideration is to look at those anticipated to have more dramatic impact on the gene product.

Slide 14:
In the current state of clinical genetics, the reality is that it is possible that variants of "uncertain significance" may be the only reportable finding. It is, therefore, important to prepare the patient for this possibility prior to testing. The patient should receive some pretest genetic counseling where the patient can be informed of the possibility of uncertain testing results and has the opportunity to discuss this with a healthcare provider. In the case that a variant of "uncertain significance" is found, it is important that all parties understand the uncertainty associated with a variant of "uncertain significance." There are plenty of cases reported in the literature where a variant of "uncertain significance" is interpreted as a definitive pathogenic result and medical action is taken inappropriately only to find the variant is later reclassified as a benign finding as new information is garnered.

In general, targeted testing for a variant of "uncertain significance" in family members should not be offered except in the setting of trying to clarify the meaning of a variant of "uncertain significance" as part of reclassification efforts. Co-segregation of a variant of "uncertain significance" in either affected or unaffected family members could provide evidence to reclassify the variant of "uncertain significance."

Reclassification is not uncommon in laboratories. In the early phases of clinical testing, a variant may be classified as of "uncertain significance;" later on, however, the same variant may be encountered and a subsequent review may uncover new literature that may be sufficient to reclassify the variant. If the laboratory reclassifies the variant, the laboratory may seek to notify the healthcare provider of the change. Whether follow-up on a variant of "uncertain significance" is the responsibility of the laboratory, the healthcare provider, or the patient remains an area of debate. Currently, not all laboratories provide the same level of follow-up on reclassifications.

Slide 15:
There are several drawbacks to a variant of "uncertain significance" classification. A variant of "uncertain significance" classification is not a definitive result and fails to provide closure. While a variant of "uncertain significance" should be interpreted as non-actionable, there is some risk in reporting these results. They could be interpreted as actionable leading to inappropriate treatment decisions. For example, in hereditary cancer screening, the presence of a variant of "uncertain significance" in a gene associated with a devastating early onset cancer syndrome could have significant psychosocial impacts. Lastly, while it is recommended that a variant of "uncertain significance" should not trigger targeted testing in family members outside of reclassification efforts, lack of understanding and poorly presented laboratory results can be confusing to interpret, which can lead to unnecessary testing.

Slide 16:
Another question that comes up is, "How can clinical genetic testing laboratories improve classification such that a larger portion of variants can be classified as either benign or pathogenic?" Currently, one of the best solutions is the creation of well-maintained, critically-reviewed databases of documented genotype-phenotype relationships. This can improve the speed at which variants are classified, improve standardization, and centralize supporting evidence into a single location.

One of the success stories of this approach relates to *BRCA1/2* testing. Early on in *BRCA1/2* testing, 4 of every 10 reported variants were classified as of "uncertain significance." At this point, Myriad Genetics began building a proprietary database of variants from patient testing. This initially helped reduce the number of *BRCA1/2* variants reported by Myriad classified as variants of "uncertain significance" down to 13% and more recently, now that upwards of 1 million patients have been tested, this number is purported to be a mere 2% - a much lower rate than other laboratories. This has created controversy as this is a powerful resource that greatly benefits patient care; however, Myriad has not indicated that they intend to make their well-annotated database public.

Another demonstration of the power of variant databases is from the public database provided by the International Society for Gastrointestinal Hereditary Tumours (InSiGHT). InSiGHT created a central repository for variant classification for *MLH1, MSH2, MSH6* and *PMS2* and reexamined classification of their database, which led to reclassification of ~25% of variants, including a large number of variants of "uncertain significance." All the variants in this database were reviewed by an expert panel, making for a trusted resource.

Slide 17:

The clinical genetics community and the NIH broadly appreciated the need to establish a centralized and standardized public database of variants that is built upon high quality data that provides consistent interpretation of variants. Subsequently, the NIH funded the ClinGen Project, which is dedicated to "building an authoritative central resource that defines the clinical relevance of genomic variants for use in precision medicine and research". ClinGen has been supporting the expansion of ClinVar, an open access archive of data documenting the relationships between genetic variations and phenotypes that provide supporting evidence. This can be found at clinicalgenome.org. ClinVar accepts variant classification data from a variety of submitters, including basic research groups, clinical laboratories, expert groups, other pre-existing disease-specific databases, as well as patient registries.

Depending on the quality of the submission, the completeness of the provided evidence, and the number of submissions in agreement, different levels of support are provided for a given variant classification. A star-based system is utilized with variant classifications recommended by practice guidelines given the highest level of support.

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While the concept of a public database for variant classification is promising and almost universally agreed upon as a long-term solution for the challenges of variant classification, currently, database entries must be viewed cautiously, particularly with lower levels of support. Submitters aren't required to follow specific guidelines for how variants are classified prior to upload into the public database. There is still significant variation across institutions in regards to how variants are classified. Furthermore, many submissions lack complete documentation of the evidence used to justify classifications. The frequency and numbers of submissions in the database is limited and many variants are single entries. There are cases of submission retraction due to inappropriate classifications. This can have a dangerous impact on patient care and thus, public databases only provide a small piece to the puzzle for now.

Slide 19:
In summary, it is important to note that classification of variants is a costly component of genetic testing and requires standardization to optimize value. In the short term, as new disorders are uncovered and new genes are sequenced, there will be an increase in the number of variants of "uncertain significance." Finding a variant of "uncertain significance" doesn't provide a solution to the problem the healthcare provider and/or patient was hoping to solve with genetic testing. Healthcare providers should interpret variants of "uncertain significance" cautiously and seek advice from the laboratory or a genetic counselor if there are any concerns with how to handle a variant of "uncertain significance."

Slide 20: References

Slide 21: Disclosures

Slide 22: Thank You from www.TraineeCouncil.org
Thank you for joining me on this Pearl of Laboratory Medicine on "Variants of Uncertain Significance."