THE
HUMAN VARIOME
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Variant Effect Prediction Training Course

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NUMed Johor, Malaysia

Concurrent Practical Session
ACMG Classification

Andreas Laner / Anna Benet-Pagès

Content

1. Background

The dramatic progress in sequence technology, lab automatization, and bio-IT data processing in the last decade have made high-throughput sequencing applications the standard method in molecular diagnostics. Especially since the development of benchtop NGS machines, almost every lab is able to create vast amounts of high-quality sequence data. However, there are still some important hurdles to overcome, especially the interpretation of sequence variants with a view to providing accurate clinical recommendations, a process that is considered a major bottleneck. Evaluating the pathogenicity of a variant is challenging given the plethora of types of genetic evidence that laboratories need to consider. Deciding how to weigh each type of evidence is difficult, and standards have been set. In 2015, the American College of Medical Genetics and Genomics (ACMG) and the Association for Molecular Pathology (AMP) published guidelines for the assessment of variants in genes associated with Mendelian diseases [1] (hereafter: ACMG-AMP guidelines). The goal of these guidelines is to establish standardized classification, annotation, interpretation, and reporting of sequence variants.

2. Aim of the Workshop

In this workshop, participants will be familiarized with the basic application of ACMG-AMP classification guidelines as well as with the limitations and pitfalls inherent in working with these guidelines on a daily basis. The following points will be addressed during the presentation:

- Familiarization with ACMG-AMP guidelines and their basic application
- Identification of classes of variants not covered by ACMG-AMP guidelines (e.g. somatic variants, pharmacogenomics, multigenic/complex disorders) or which must be considered cautiously (e.g. variants with low/moderate penetrance)
- Identification of top error-prone sources of information (e.g. ClinVar OMIM entries, old data sources, research submissions, disease areas, etc.) [5]
- Awareness of various possible errors in variant interpretation
- Awareness of the fact that a considerable number of inter-laboratory discrepancies in variant classification are the result of a lack of published internal data, special biology, and old or invalid data sources.[2, 3, 4, 5]

3. Outline

The workshop is divided in a practical part and in a short demonstration part.

Part A: Practical Variant Classification (75 min)

In the first part of the workshop, variants from real cases will be discussed collectively. The variants have been selected to represent ACMG-AMP categories that are known to be challenging in the classification process [2, 3, 4, 5]. These variants were sent to workshop participants in advance of the VEPTC (see cases 1 to 5 on the following pages). In order to focus discussion at the variant level, only one or two variants per case will be considered for the classification process.

To facilitate classification an overview of the ACMG Criteria are provided in this document in table format (see Chaper 5). The tables describe the criteria to classify pathogenic, benign and unclassified variants. The first table lists the criteria for pathogenic variants and the second table lists criteria for benign variants. The third table describes the rules for the combination of the criteria to classify the variant.

To make the workshop more interactive, participants were asked to collect evidence for and against pathogenicity for each case and to prepare possible questions and remarks in advance. During this practical section, differences, difficulties, and discrepancies in variant classification will be discussed for all five cases.

Furthermore, we encourage participants to **bring their own cases or variants** which can be discussed during or after the workshop, depending on the time.

Part B: Demonstration of inter-laboratory concordance in variant classification (15 min)

In the second part of the workshop recent publications reporting conflicting results regarding consistent variant classification using the ACMG-AMP guidelines will be discussed. Furthermore, the problem of discordant inter-laboratory variant classification will also be addressed.

4. Cases

Case 1

PATIENT: Male, 46 years of age.

PHENOTYPE: Mental retardation, hypophosphatemia and spinal deformity (osteopenia). Family history negative regarding mental retardation or skeletal abnormalities.

HPO-TERMS: Hypophosphatemia, Osteopenia

ANALYSED GENES: Clinical Exome-Kit (Illumina; 3.963 genes)

DETECTED VARIANT(S):

Gene (GRCh37/hg19)	Transcript	Variant (HGVS)	Zygosity	Ref	Alt	Chr. Position	dbSNP ID
SLC9A3R1	NM_004252.4	c.328C>G (p.Leu110Val)	heterozygous	C	G	chr17:72745313	rs35910969

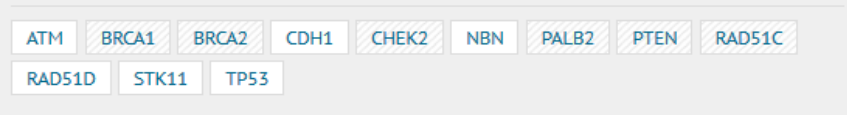
Notes:

Case 2:

PATIENT: Female, 38 years of age.

PHENOTYPE: Breast-cancer, mother and maternal aunt also affected by breast-cancer at age 45 and 59, respectively. Clinical suspicion of hereditary breast-cancer. No family segregation analysis performed.

ANALYSED GENES: 12



DETECTED VARIANT(S):

Gene (GRCh37/hg19)	Transcript	Variant (HGVS)	Zygosity	Ref	Alt	Chr. Position	dbSNP ID
CHEK2	NM_007194.3	c.470T>C (p.Ile157Thr)	heterozygous	A	G	chr22:29121087	rs17879961

Notes:

Case 3:

PATIENT: Male, 2 years of age.

PHENOTYPE: Childhood cardiomyopathy, poor feeding, and muscle hypotonia.

ANALYSED GENES: 23

AARS2 ACAD9 ACADM ACADS ACADVL AGK COX15 CPT1A CPT2
DSC2 DSP GLA JUP LAMP2 MTO1 RAF1 SCO2 SDHA
SLC22A5 SLC25A20 SLC25A3 TAZ TMEM70

DETECTED VARIANT(S):

Gene (GRCh37/hg19)	Transcript	Variant (HGVS)	Zygosity	Ref	Alt	Chr. Position	dbSNP ID
SLC22A5	NM_003060.3	c.1463G>A (p.Arg488His)	homozygous	G	A	chr5:131729380	rs28383481

Notes:

Case 4:

PATIENT: Male, 30 years of age

PHENOTYPE: Intracerebral hemorrhage at age 27. Positive family history of porencephaly, father deceased of haemorrhagic stroke.

ANALYSED GENES: 22

ABCC6 AMACR CACNA1C CBS CECR1 COL3A1 COL4A1 COL4A2
CST3 FBN1 FLNA GLA HTRA1 NOTCH3 OTC POLG SLC2A10
TGFB2 TGFBR1 TGFBR2 TREX1 TTR

DETECTED VARIANT(S):

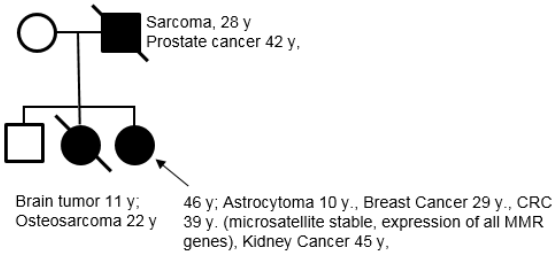
Gene (GRCh37/hg19)	Transcript	Variant (HGVS)	Zygosity	Ref	Alt	Chr. Position	dbSNP ID
COL4A1	NM_001845.5	c.3067G>A (p.Gly1023Arg)	heterozygous	C	T	chr13:110827696	-
COL4A1	NM_001845.5	c.3484G>A (p.Ala1162Thr)	heterozygous	C	T	chr13:110826268	rs778978790

Notes:

Case 5:

PATIENT: Female, 46 years of age.

PHENOTYPE: Astrocytoma at 10 years of age, breast cancer at 29 years of age, colorectal cancer at 39 years of age, kidney cancer at 45 years of age. Microsatellite stable with expression of all mismatch repair genes.



ANALYSED GENES: 94

AIP ALK APC ATM BAP1 BLM BMPR1A BRCA1 BRCA2 BRIP1
BUB1B CDC73 CDH1 CDK4 CDKN1C CDKN2A CEBPA CEP57
CHEK2 CYLD DDB2 DICER1 DIS3L2 EGFR EPCAM ERCC2 ERCC3
ERCC4 ERCC5 EXT1 EXT2 EZH2 FANCA FANCB FANCC FANCD2
FANCE FANCF FANCG FANCI FANCL FANCM FH FLCN GATA2
GPC3 HNF1A HRAS KIT MAX MEN1 MET MLH1 MSH2 MSH6
MUTYH NBN NF1 NF2 NSD1 PALB2 PHOX2B PMS1 PMS2
PRF1 PRKAR1A PTCH1 PTEN RAD51C RAD51D RB1 RECQL4 RET
RHBDF2 RUNX1 SBDS SDHAF2 SDHB SDHC SDHD SLX4 SMAD4
SMARCB1 STK11 SUFU TMEM127 TP53 TSC1 TSC2 VHL WRN
WT1 XPA XPC

DETECTED VARIANT(S):

Gene (GRCh37/hg19)	Transcript	Variant (HGVS)	Zygosity	Ref	Alt	Chr. Position	dbSNP ID
TP53	NM_000546.5	c.722C>T (p.Ser241Phe)	heterozygous	G	A	chr17:7577559	rs28934573
FH	NM_000143.3	c.1431_1433dup (p.Lys477dup)	heterozygous	-	TTT	chr1:241661227-241661228	rs367543046

Notes:

5. ACMG Tables

Criteria for classifying pathogenic variants (ACMG Standards and Guidelines)

Evidence of pathogenicity		Category
Very strong	PVS1	**Null variant (nonsense, frameshift, canonical ±1 or 2 splice sites, initiation codon, single or multiexon deletion) in a gene where LOF is a known mechanism of disease.** *Caveats:* • *Beware of genes where LOF is not a known disease mechanism (e.g., GFAP, MYH7)* • *Use caution interpreting LOF variants at the extreme 3′ end of a gene* • *Use caution with splice variants that are predicted to lead to exon skipping but leave the remainder of the protein intact* • *Use caution in the presence of multiple transcripts*
Strong	PS1	**Same amino acid change as a previously established pathogenic variant regardless of nucleotide change** • *Example: Val→Leu caused by either G>C or G>T in the same codon* • *Caveat: Beware of changes that impact splicing rather than at the amino acid/protein level*
	PS2	**De novo (both maternity and paternity confirmed) in a patient with the disease and no family history** *Note: Confirmation of paternity only is insufficient. Egg donation, surrogate motherhood, errors in embryo transfer, and so on, can contribute to non-maternity.*
	PS3	**Well-established in vitro or in vivo functional studies supportive of a damaging effect on the gene or gene product** *Note: Functional studies that have been validated and shown to be reproducible and robust in a clinical diagnostic laboratory setting are considered the most well established.*
	PS4	**The prevalence of the variant in affected individuals is significantly increased compared with the prevalence in controls** *Note 1: Relative risk or OR, as obtained from case-control studies, is >5.0, and the confidence interval around the estimate of relative risk or OR does not include 1.0.* *See the article for detailed guidance.* *Note 2: In instances of very rare variants where case-control studies may not reach statistical significance, the prior observation of the variant in multiple unrelated patients with the same phenotype, and its absence in controls, may be used as moderate level of evidence.*
	PM1	**Located in a mutational hot spot and/or critical and well-established functional domain (e.g., active site of an enzyme) without benign variation.**
	PM2	**Absent from controls (or at extremely low frequency if recessive) (Table 6) in Exome Sequencing Project, 1000 Genomes Project, or Exome Aggregation Consortium** • *Caveat: Population data for insertions/deletions may be poorly called by next-generation sequencing.*

Moderate	PM3	For recessive disorders, detected in trans with a pathogenic variant *Note: This requires testing of parents (or offspring) to determine phase.*
	PM4	Protein length changes as a result of in-frame deletions/insertions in a non-repeat region or stop-loss variants
	PM5	Novel missense change at an amino acid residue where a different missense change determined to be pathogenic has been seen before • *Example: Arg156His is pathogenic; now you observe Arg156Cys* • *Caveat: Beware of changes that impact splicing rather than at the amino acid/protein level.*
	PM6	Assumed de novo, but without confirmation of paternity and maternity

Supporting	PP1	Co segregation with disease in multiple affected family members in a gene definitively known to cause the disease *Note: May be used as stronger evidence with increasing segregation data*
	PP2	Missense variant in a gene that has a low rate of benign missense variation and in which missense variants are a common mechanism of disease
	PP3	Multiple lines of computational evidence support a deleterious effect on the gene or gene product (conservation, evolutionary, splicing impact, etc.) • *Caveat: Because many in-silico algorithms use the same or very similar input for their predictions, each algorithm should not be counted as an independent criterion. PP3 can be used only once in any evaluation of a variant.*
	PP4	Patient's phenotype or family history is highly specific for a disease with a single genetic etiology
	PP5	Reputable source recently reports variant as pathogenic, but the evidence is not available to the laboratory to perform an independent evaluation

Criteria for classifying benign variants (ACMG Standards and Guidelines)

Evidence of benign impact		Category
Stand alone	BA1	Allele frequency is >5% in Exome Sequencing Project, 1000 Genomes Project, or Exome Aggregation Consortium

Strong	BS1	Allele frequency is greater than expected for disorder (see Table 6)
	BS2	Observed in a healthy adult individual for a recessive (homozygous), dominant (heterozygous), or X-linked (hemizygous) disorder, with full penetrance expected at an early age
	BS3	Well-established in vitro or in vivo functional studies show no damaging effect on protein function or splicing
		Lack of segregation in affected members of a family

	BS4	• Caveat: The presence of phenocopies for common phenotypes (i.e., cancer, epilepsy) can mimic lack of segregation among affected individuals. Also, families may have more than one pathogenic variant contributing to an autosomal dominant disorder, further confounding an apparent lack of segregation.

Supporting	**BP1**	**Missense variant in a gene for which primarily truncating variants are known to cause disease**
	BP2	**Observed in trans with a pathogenic variant for a fully penetrant dominant gene/disorder or observed in cis with a pathogenic variant in any inheritance pattern**
	BP3	**In-frame deletions/insertions in a repetitive region without a known function**
	BP4	**Multiple lines of computational evidence suggest no impact on gene or gene product (conservation, evolutionary, splicing impact, etc.)** • Caveat: Because many in silico algorithms use the same or very similar input for their predictions, each algorithm cannot be counted as an independent criterion. BP4 can be used only once in any evaluation of a variant.
	BP5	**Variant found in a case with an alternate molecular basis for disease**
	BP6	**Reputable source recently reports variant as benign, but the evidence is not available to the laboratory to perform an independent evaluation**
	BP7	**A synonymous (silent) variant for which splicing prediction algorithms predict no impact to the splice consensus sequence nor the creation of a new splice site AND the nucleotide is not highly conserved**

Rules for combining criteria to classify sequence variants (ACMG Standards and Guidelines)

Pathogenic	**(i) 1 Very strong (PVS1) AND** *a) ≥1 Strong (PS1-PS4) OR* *b) ≥2 Moderate (PM1-PM6) OR* *c) 1 Moderate (PM1-PM6) and 1 supporting (PP1-PP5) OR* *d) ≥2 Supporting (PP1-PP5)*
	(ii) ≥2 Strong (PS1-PS4) OR
	(iii) 1 Strong (PS1-PS4) AND *a) ≥3 Moderate (PM1-PM6) OR* *b) 2 Moderate (PM1-PM6) AND ≥2 Supporting (PP1-PP5) OR* *c) 1 Moderate (PM1-PM6) AND ≥4 supporting (PP1-PP5)*

Likely	**(i) 1 Very strong (PVS1) AND 1 moderate (PM1-PM6) OR**
	(ii) 1 Strong (PS1-PS4) AND 1-2 moderate (PM1-PM6) OR
	(iii) 1 Strong (PS1-PS4) AND ≥2 supporting (PP1-PP5) OR

pathogenic	(iv) ≥3 Moderate (PM1-PM6) OR
	(v) 2 Moderate (PM1-PM6) AND ≥2 supporting (PP1-PP5) OR
	(vi) 1 Moderate (PM1-PM6) AND ≥4 supporting (PP1-PP5)

Benign	(i) 1 Stand-alone (BA1) OR
	(ii) ≥2 Strong (BS1-BS4)

Likely benign	(i) 1 Strong (BS1-BS4) and 1 supporting (BP1-BP7) OR
	(ii) ≥2 Supporting (BP1-BP7)

Uncertain significance	(i) Other criteria shown above are not met OR
	(ii) the criteria for benign and pathogenic are contradictory

6. Recommended Literature

(1) Richards et al.; Genet. Med. 17, 405–424, 2015

(2) Amendola et al.; Am J Hum Genet 98, 1067–1076, June 2, 2016

(3) Harrison et al.; Genet. Med. Mar 16 (PMID: 28301460)

(4) Pepin et al.; Genet Med. Jan; 18(1) 20-4 (PMID: 25834947)

(5) Yang et al.; Genet. Med. Jun 1 2017 (PMID: 28569743)

7. Databases

Population databases	Exome Aggregation Consortium http://exac.broadinstitute.org/
	Exome Variant Server http://evs.gs.washington.edu/EVS
	1000 Genomes Project http://browser.1000genomes.org
	dbSNP http://www.ncbi.nlm.nih.gov/snp

	dbVar https://www.ncbi.nlm.nih.gov/dbvar
	GnomAD http://gnomad.broadinstitute.org/
Disease databases	ClinVar http://www.ncbi.nlm.nih.gov/clinvar
	OMIM http://www.omim.org
	Human Gene Mutation Database http://www.hgmd.cf.ac.uk/ac/index.php
	Human Genome Variation Society http://www.hgvs.org/
	Leiden Open Variation Database http://www.lovd.nl
	DECIPHER http://decipher.sanger.ac.uk
Sequence databases	NCBI Genome http://www.ncbi.nlm.nih.gov/genome
	RefSeqGene http://www.ncbi.nlm.nih.gov/refseq/rsg
	Locus Reference Genomic (LRG) http://www.lrg-sequence.org
	MitoMap http://www.mitomap.org/MITOMAP/HumanMitoSeq
ClinGen	Sequence Variant Interpretation (SVI) Working Group https://www.clinicalgenome.org/working-groups/sequence-variant-interpretation/