The broad use of next-generation sequencing and microarray platforms in research and clinical laboratories has led to an increasing appreciation of the role of germline mutations in genes involved in hematopoiesis and lineage differentiation that contribute to myeloid neoplasms. Despite implementation of the American College of Medical Genetics and Genomics 2015 guidelines for sequence variant interpretation, the number of variants deposited in ClinVar, a genomic repository of genotype and phenotype data, and classified as having uncertain significance or being discordantly classified among clinical laboratories remains elevated and contributes to indeterminate or inconsistent patient care. In 2018, the American Society of Hematology and the Clinical Genome Resource co-sponsored the Myeloid Malignancy Variant Curation Expert Panel to develop rules for classifying gene variants associated with germline predisposition to myeloid neoplasia. Herein, we demonstrate application of our rules developed for the RUNX1 gene to variants in six examples to show how we would classify them within the proposed framework.

How I curate: applying American Society of Hematology-Clinical Genome Resource Myeloid Malignancy Variant Curation Expert Panel rules for RUNX1 variant curation for germline predisposition to myeloid malignancies

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ABSTRACT

The broad use of next-generation sequencing and microarray platforms in research and clinical laboratories has led to an increasing appreciation of the role of germline mutations in genes involved in hematopoiesis and lineage differentiation that contribute to myeloid neoplasms. Despite implementation of the American College of Medical Genetics and Genomics and Association for Molecular Pathology 2015 guidelines for sequence variant interpretation, the number of variants deposited in ClinVar, a genomic repository of genotype and phenotype data, and classified as having uncertain significance or being discordantly classified among clinical laboratories remains elevated and contributes to indeterminate or inconsistent patient care. In 2018, the American Society of Hematology and the Clinical Genome Resource co-sponsored the Myeloid Malignancy Variant Curation Expert Panel to develop rules for classifying gene variants associated with germline predisposition to myeloid neoplasia. Herein, we demonstrate application of our rules developed for the RUNX1 gene to variants in six examples to show how we would classify them within the proposed framework.

Introduction

Germline mutations in genes involved in hematopoiesis and lineage differentiation predispose patients to myeloid neoplasia with or without thrombocytopenia. The broad adoption of next-generation sequencing and microarrays in the clinical laboratory has expanded our knowledge of germline contribution to myeloid neoplasia. Drazer et al. reported that in six of 24 patients with myeloid neoplasia, presumed somatic variants in DDX41, GATA2 and TP53 were of germline origin.1 Similarly, Churpek et al. showed that 29% of acute myeloid leukemia (AML)/myelodysplastic syndrome (MDS) kindreds with a positive family history carried a variant in one of 12 genes associated with germline predisposition to hematopoietic malignancies, including FANCA, GATA2, RUNX1, and SBDS.2 To date, more than 65 genes have been associated with a predisposition to hematologic malignancies.3 Recognizing the contribution of germline variation toward myeloid neoplasia, the ‘WHO classification of Tumors of Hematopoietic and
Lymphoid Tissues’ incorporated the classification of myeloid neoplasia with germline predisposition in their 2016 revised edition.3,5 In parallel, clinical laboratories are increasingly offering broad next-generation sequencing-based tests for patients with myeloid neoplasia for somatic testing, and will readily detect germline variants, if present in a patient. While there is increased clinical awareness of the potential for these germline variants to contribute to a patient’s disease, there are often insufficient data in the literature to definitively classify whether a detected variant is contributing to the patient’s phenotype.5,7 For example, familial platelet disorder with predisposition to AML (FPD/AML) is an autosomal dominant disorder in which germline mutations in RUNX1 result in thrombocytopenia, platelet functional and/or ultrastructural defects, and/or susceptibility to hematologic malignancies commonly including MDS, AML, and other malignancies4-11 (Table 1). ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/) is a database repository of clinically actionable genomic variants that currently lists 325 germline RUNX1 variants deposited by clinical laboratories. More than half of these variants are currently reported as being of uncertain significance.

Worldwide, most clinical laboratories follow the 2015 American College of Medical Genetics and Genomics (ACMG) and the Association for Molecular Pathology (AMP) guidelines for sequence variant interpretation.14 In this framework, germline variants are classified using a five-tier system: benign (BEN), likely benign (LBEN), variant of uncertain significance (VUS), likely pathogenic (LPATH) and pathogenic (PATH). During sequence variant interpretation, laboratories systematically review the supporting criteria of a genomic variant, such as: minor allele frequencies (MAF), computational predictions, functional experiments and segregation with disease in order to determine the five-tier classification.15-16

Although the ACMG/AMP guidelines provide a comprehensive framework for sequence variant interpretation, the high rate of VUS and curation discrepancies continue to be an impediment to accurate clinical annotation and interpretation of genomic variants.6,5 To encourage genomic and phenotypic data sharing, and engage experts in consensus-driven variant interpretation, the Clinical Genome Resource (ClinGen) convened Variant Curation Expert Panels (VCEP) to develop gene- and disease-specific modifications of the original guidelines and provide expert-reviewed variant classification for depositing into ClinVar (Online Supplementary Figure S1).7 In 2018, the American Society of Hematology (ASH) sponsored a ClinGen Myeloid Malignancy Variant Curation Expert Panel (MM-VCEP), composed of 34 international members, who started working on gene- and disease-specific rules for RUNX1 as the first of several genes conferring predisposition to myeloid malignancies (Online Supplementary Figure S1A). After designing, modifying and testing the preliminary RUNX1 rules on 52 pilot variants, which improved classification in 33% VUS or variants with conflicting interpretations (CONF), MM-VCEP-specified ACMG/AMP rules were approved by the ClinGen oversight committee and efforts to curate variants to ClinVar using the Variant Curation Interface have commenced (Online Supplementary Figure S1B).16 This pilot effort resulted in one variant being upgraded to PATH, two variants being upgraded to LPATH, and three variants being downgraded to LBEN. ClinGen’s website contains the MM-VCEP variant classification recommendations and any subsequent modifications to these codes over time (https://www.clinicalgenome.org/affiliation/10354/).

Herein, we demonstrate the application of RUNX1-specific rules (Table 2) to classify nine representative RUNX1 variants in six examples (Table 3) while reviewing phenotypic criteria for FPD/AML and summarizing molecular and functional roles of RUNX1.

**Example 1. Early nonsense variants, (p.Arg204Ter) (PATH with PVS1, PM2, PS4, supporting, and PP1)**

A 50-year old female with new pancytopenia was referred to a hematology service. A bone marrow biopsy showed hypocellularity with severe trilineage dysplasia and 12% blasts, diagnostic of MDS with excess blasts (MDS-EB2). Further investigation showed pathogenic variants in RUNX1 (NM_001754.c.610C>T, (p.Arg204Ter)), BCOR and ASXL1 with a normal karyotype. The medical history was positive for thrombocytopenia (baseline 70-120x10^9/L) and a propensity to excessive bleeding after tooth extractions. The family history was positive for two sons with persistent thrombocytopenia (baseline 50-100x10^9/L) not otherwise explained and a granddaughter with thrombocytopenia and MDS with monosomy 7 (Figure 1). During the initial assessment, an increase in lactate dehydrogenase and the peripheral blast count were noted. A second marrow biopsy confirmed transformation into AML with 40% blasts. The patient underwent induction chemotherapy without achieving remission and clofarabine bridging for unrelated stem cell transplantation. During conditioning, the patient developed sepsis with Gram-negative bacteria and died shortly afterwards. Since she had a remarkable personal and family history pointing towards a germline predisposition syndrome, a skin biopsy was performed at the time of the diagnosis of MDS, and DNA testing from cultured skin

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**Table 1. Clinical phenotypes of RUNX1 familial platelet disorder and hereditary malignancies.**

| Clinical and laboratory features | Details | Life-time risk |
|---------------------------------|---------|---------------|
| Hematologic malignancy          | Commonly AML or MDS; less frequently T-ALL and rarely mixed MPN/MDS such as CMML, as well as B-ALL and hairy cell leukemia | ~44% |
| Thrombocytopenia                | Mild to moderate thrombocytopenia with normal platelet size, in the absence of other causes | Most patients |
| Platelet functional and/or ultrastructural defects | Includes impaired platelet aggregation (particularly in response to collagen and epinephrine) and platelet alpha or dense granule secretion defects | Not known |

Adapted from Table 2 from Luo and Feurstein, et al.5 AML: acute myeloid leukemia; ALL: acute lymphoblastic leukemia; MPN: myeloproliferative neoplasms; MDS: myelodysplastic syndrome; CMML: Chronic myelomonocytic leukemia.
Table 2. Clinical Genome Resource Myeloid Malignancy Variant Curation Expert Panel-approved rules for *RUNX1* variant interpretation.

| ACMG/AMP CC | Specification | Stand alone | Very strong | Strong | Moderate | Supporting | Comments |
|-------------|---------------|-------------|-------------|--------|----------|------------|----------|
| **BA1**     | Allele frequency is >5% in ESP, 1000G, or ExAC. | Disease-specific | MAF ≥ 0.0015 (0.15%) | na     | na       | na         | (1) The variant is present in any general continental population dataset with a minimum number of 2,000 alleles and variant present in ≥5 alleles. |
| **BS1**     | Allele frequency is greater than expected for disorder | Disease-specific | na         | na     | na       | na         | (1) The variant is present in any general continental population dataset with a minimum number of 2,000 alleles and variant present in ≥5 alleles. (2) Variant can be classified as likely benign based on BS1 alone if there is no contradictory evidence supporting pathogenicity. |
| **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. | na | na | na | na | (1) FPD/AML patients display incomplete penetrance and the average age of onset of hematologic malignancies is 33 years. |
| **BS3**     | Well-established *in vitro* or *in vivo* functional studies show no damaging effect on protein function or splicing. | Gene-specific, strength | na         | na     | (1) Transactivation assays demonstrating normal transactivation (80-115% of wt) AND (2) data from a secondary assay demonstrating normal function. | na | See PS3 (1) and (2) |
| **BS4**     | Lack of segregation in affected members of a family. | General rec | na         | Applied when seen in ≥2 informative meioses. | na | na | This code should only be applied for genotype-positive, phenotype-negative (with sufficient laboratory evidence) family members. |
| **BP1**     | Missense variant in a gene for which primarily truncating variants are known to cause disease. | na | na | na | na | na | FPD/AML is caused by both pathogenic missense and truncating variants. |
| **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. | General rec | na | na | na | Per original ACMG/AMP guidelines. | BP2 can also be applied if the variant is detected in a homozygous state. |
| **BP3**     | In-frame deletions/insertions in a repetitive region without a known function. | na | | | | | *RUNX1* does not contain a repetitive region without known function. |
| **BP4**     | Multiple lines of computational evidence suggest no impact on gene or gene product. | General rec | na | na | na | Per original ACMG/AMP guidelines. | BP4 should be applied for missense variants if all of the following apply: (1) REVEL score <0.15, (2) SSF and MES predict either an increase in the canonical splice |

continued on the next page
BP5  Variant found in a case with an alternate molecular basis for disease.

BP6  Reputable source recently reported variants as benign, but data are not available for laboratories to perform independent evaluations.

BP7  A synonymous 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.

PVS1  Null variant in a gene for which LOF is a known mechanism of disease.

PS1  Same AA change as a previously established pathogenic variant regardless of nucleotide change.

PS2  De novo (maternity and paternity confirmed) in a patient with the disease and no family history.
| ACMG/AMP CC | Original ACMG/AMP rule summary | Specification | Stand alone | Very strong | Strong | Moderate | Supporting | Comments |
|------------|--------------------------------|---------------|-------------|-------------|--------|----------|------------|----------|
| PS3        | Well-established/ in vitro or in vivo functional studies supportive of a damaging effect. | Gene-specific, strength | na | na | Transactivation assays demonstrating altered transactivation (<20% of wt, and/or reduced to levels similar to well-established pathogenic variants such as Arg201Gln or Arg166Gln) AND data from a secondary assay demonstrating altered function. PS3 cannot be applied if the variant meets PVS1. If the variant meets criteria for PVS1_strong and PS3, we recommend either applying PVS1_strong or upgrading PVS1_strong to PVS1 without applying PS3. | Transactivation assays demonstrating altered transactivation (<20% of wt, and/or reduced to levels similar to well-established pathogenic variants such as Arg201Gln or Arg166Gln) OR ≥2 secondary assays demonstrating altered function. | Transactivation assays demonstrating enhanced transactivation (>115% of wt). | | (1) Transactivation assays should include wt and known pathogenic controls, as well as co-expression with CBFβ. Promoter sequences of CSFIR (M-CSFR), PF4, C-FMS and GZMB, containing consensus RUNX1 binding sites have been used for transactivation assays. (2) The following secondary assays have been performed: EMSA and yeast hybrid assays (decreased DNA-binding affinity), co-IP, FRET and affinity assays (diminished heterodimerization ability with CBFβ), IF and WB with cell fractionation (abnormal cellular localization), colony-forming assays (reduced colony-forming potential), xenotransplantation experiments (abnormal function of mutant RUNX1 in vivo). (3) PS3 can also be applied for evidence of very low or abnormal mRNA/protein expression of the variant allele as a functional consequence of a null variant or incorrect mRNA/protein products. |
| PS4        | The prevalence of the variant in affected individuals is significantly increased compared to the prevalence in controls. | Disease-specific, strength | na | na | ≥ 4 probands meeting RUNX1-phenotypic criteria. | 2-3 probands meeting RUNX1-phenotypic criteria. | 1 proband meeting RUNX1-phenotypic criteria. | The affected individual has to fit at least one of the RUNX1-phenotypic criteria AND variant has to be either absent from gnomAD (overall population) or only present once. |
| PM1        | Located in a mutational hot spot and/or critical and well-established functional domain without benign variation. | Gene-specific, strength | na | na | na | Variant affecting one of the following 13 hotspot residues: Arg107, Lys110, Ala119, Arg162, Arg166, Ser167, Arg169, Gly170, Lys194, Thr196, Asp198, Arg201, | Variant affecting one of the other AA residues 105-204 within the RHD. | The RHD (AA 77-204) has been established to be a highly conserved DNA-binding domain without any benign variation in ClinVar. No germline pathogenic variants have been reported in residues in the region (AA 77-104) to date. The AA range under PM1_supporting may be expanded in the future to other parts of the protein if more evidence emerges. | | continued on the next page |
| ACMG/AMP CC | Original ACMG/AMP rule summary | Specification | Stand alone | Very strong | Strong | Moderate | Supporting | Comments |
|-------------|--------------------------------|---------------|-------------|-------------|--------|----------|------------|----------|
| PM2         | Absent from controls.          | General rec   | na          | na          | na     | na       | Per original ACMG/AMP guidelines. | na       | Variant must be completely absent from all population databases. The mean coverage of RUNX1 in the population database used should be at least 20x. |
| PM3         | For recessive disorders,       | na            |             |             |        |          | FPD/AML is inherited in an autosomal dominant manner. |
|             | detected in trans with a       |               |             |             |        |          |                                        |
|             | pathogenic variant.            |               |             |             |        |          | see PM1 |
| PM4         | Protein length changes due     | Gene-specific,| na          | na          | na     | na       | In-frame deletion/insertion         | Other     | see PM1 |
|             | to in-frame deletions/insertions in a non-repeat region or stop-loss variants. | strength      |             |             |        | deletion/insertion impacting at least one of the 13 hotspot residues Arg107, Lys110, Ala134, Arg162, Arg166, Ser167, Arg169, Gly170, Lys194, Thr196, Asp198, Arg201, Arg204 within the RHD. |
| PM5         | Missense change at AA residue  | Strength      | na          | na          | na     |          | Missense change at the same residue where ≥2 different missense changes have previously been determined to be pathogenic. PM5_strong cannot be applied together with PM1. | Missense change at the same residue where a different missense change has previously been determined to be pathogenic. | see PSI |
|             | where a different missense     |               |             |             |        |          | see PS1 |
|             | change determined to be         |               |             |             |        |          | see PS2 |
|             | pathogenic has been seen before. |             |             |             |        |          |                                        |
| PM6         | Assumed de novo (but without confirmation of maternity and paternity) in a patient with the disease and no family history. | Disease-specific, strength | na          | na          | na     |          | ≥4 assumed de novo occurrences (without confirmation of maternity and paternity) in patients with the RUNXI- | 2 or 3 assumed de novo occurrences (without confirmation of maternity and paternity) in patients with the RUNXI- | continued on the next page |
| ACMG/AMP CC | Specification | Stand alone | Very strong | Strong | Moderate | Supporting | Comments |
|-------------|---------------|-------------|-------------|--------|----------|------------|----------|
| PP1         | Co-segregation with disease in multiple affected family members; specific, strength | na          | na          | ≥ 7 meioses observed within one or across multiple families. | 5 or 6 meioses observed within one or across multiple families. | 3 or 4 meioses observed within one or across multiple families. | (1) Affected individuals show at least one of the RUNX1-specific phenotypic criteria. (2) Only genotype and phenotype positive individuals and obligate carriers are counted. (3) Demonstration of co-segregation in multiple families is not required since many RUNX1 variants are unique and only occur in one family. |
| PP2         | Missense variant in a gene that has a low rate of benign missense variation and where missense variants are a common mechanism of disease. | na          | na          | na     | na       | na         | Missense constraint z-score for RUNX1 is < 3.09. |
| PP3         | Multiple lines of computational evidence support a deleterious effect on the gene or gene product. | General rec | na          | na     | na       | Per original ACMG/AMP guidelines. | (1) PP3 should be applied for missense variants with a REVEL score of > 0.75. (2) PP3 should be applied for missense or synonymous variants if the variant alters the last three bases of an exon preceding a donor splice site or the first three bases of an exon following a splice acceptor site and the predicted decrease in the score of the canonical splice site (measured by both MES and SSF) is at least 75% regardless of the predicted creation/presence of a putative cryptic splice site. (3) PP3 should also be applied for intronic variants (in introns 4-8) located in reference to exons at positions +3 to +5 for splice donor sites or -3 to -5 for splice acceptor sites for which the predicted decrease in the score is at least 75% (measured by both MES and SSF) regardless of the predicted creation/presence of a putative cryptic splice site. (4) PP3 cannot be applied for canonical splice site variants. |
| PP4         | Patient's phenotype or family history is highly specific for a disease with a single genetic etiology. | na          | na          | na     | na       | na         | FPD/AML does not exhibit a highly specific phenotype and there is substantial genetic heterogeneity. |
| PP5         | Reputable source recently reports variant as pathogenic, but the evidence is not available to the laboratory to perform an independent analysis. | na          | na          | na     | na       | na         | According to SVI recommendations. |

Adapted from Table 1 of Luo and Feurstein et al. ACMG: American College of Medical Genetics; AMP: Association for Molecular Pathology; CC: criteria code; ESP: Exome Sequencing Project; 1000G: 1000 Genome project; ExAC: Exome Aggregation Consortium; MAF: minor allele frequency; na: not applicable; FPD/AML: familial platelet disorder with predisposition to acute myeloid leukemia; rec: recommendation; SSF: splice site finder; MES: MaxEntScan; SVI: ClinGen Sequence Variant Interpretation Working Group; LOF: loss-of-function; SNV: single-nucleotide variant; CNV: copy number variant; NMD: nonsense-mediated decay; AA: amino acid; MM: Malignancy; LOVD: Leiden Open Variation Database; Panel: wildtype; EMSA: electrophoretic mobility shift assay; IP: immunoprecipitation; FRET: fluorescence resonance energy transfer; IF: immunofluorescence; WB: western blot; gnomAD: Genome Aggregation Database; RHD: Runt homology domain.
fibroblasts later confirmed the germline, nonsense RUNX1 variant. Her two sons and granddaughter also tested positive for the RUNX1 variant (Figure 1).

Similar to this case, most patients with FPD/AML will have a characteristic phenotype (Table 1) including mild to moderate thrombocytopenia with normal platelet size, platelet α or dense granule secretion defects and impaired platelet aggregation, particularly in response to collagen and epinephrine as well as a predisposition to hematologic malignancies. Although there is variability in disease onset in FPD/AML, development of a hematologic malignancy is common with a lifetime risk of ~44%: AML and MDS are common, other malignancies occur less frequently (Table 1).19-23 FPD/AML has a high but incomplete

| Example No. | RUNX1 variant NM_001754 (isoform C) | ClinVar Assertion | Criteria | MM-VCEP classification |
|-------------|--------------------------------------|------------------|----------|-------------------------|
| 1           | c.610C>T (p.Arg204Ter)               | PATH             | PV1, PM2, PS4, supporting, PP1 | PATH |
| 2           | c.314A>C p.(His105Pro)               | VUS              | PM2, PP3, PS4, supporting, PM1, supporting, PM5, supporting | LPATH |
|             | c.315C>A p.(His105Gln)               |                  |          |                         |
| 3           | c.253C>A a.(His84Asn)                | CONF: wildtype   | BS1, BS3, PP3 | LBEN |
|             |                                      |                   |          |                         |
| 4           | c.508+3delA                          | PATH             | PS3, PP1_strong, PM2, PP3, PS4, supporting | PATH |
|             | c.444C>T p.(Thr148=)                 | Illumina: VUS    | BP4, BP7 | LBEN |
|             | c.1257G>A p.(Val419=)                | VUS              |          |                         |
| 5           | Copy number variant, deletion of exon 2 | PS4, PP1_strong, PM2, PV1_moderate | PATH |
| 6           | c.1118C>A a.(p.Ser373Ter)            | Path             | PS1_strong, PM2, PS4, supporting | LPATH |

The five-tier ClinVar classification: PATH (pathogenic), LPATH (likely pathogenic), VUS (variant of uncertain significance), LBEN (likely benign), BEN (benign); CONF (conflicting interpretations in ClinVar); criteria from Luo and Feurstein et al.

Table 3. Summary of RUNX1 variant examples with application of the Myeloid Malignancy Variant Curation Expert Panel criteria.

RUNX1 NM_001754:c.610C>T, (p.Arg204Ter)

Figure 1. Family pedigree of a patient with acute myeloid leukemia, example 1. Germline RUNX1 NM_001754:c.610C>T, (p.Arg204Ter) showing typical autosomal dominant inheritance with other first and second-degree relatives with thrombocytopenia and/or myeloid malignancy (acute myeloid leukemia and myelodysplastic syndrome), mut: mutated; MDS: myelodysplastic syndrome; sAML: secondary acute myeloid leukemia; +: wildtype.
Figure 2. Overview of RUNX1 protein domains, ClinVar-deposited and MM-VCEP curated pilot and COSMIC variants. (A) Schematic of the RUNX1 protein showing its key domains, including the Runt homology domain (RHD); annotation according to MM-VCEP is based on RUNX1 transcript isoform C (NM_001754). (B) Schematic of ClinVar-deposited RUNX1 variants in a one-dimensional line plot showing the current ClinVar five-tier classification; data plotted using proteinpaint from St. Jude.org with data from ClinVar (accessed 6/2019); RHD (purple), and ID (green) are highlighted; numbers indicate amino acid; dotted lines indicate exon boundaries. Some ClinVar-deposited variants (in 5’ and 3’ untranslated regions and large deletions) are not shown. Lollipops representing the variants are colored as follows: PATH (red), LPATH (orange), VUS (grey), LBEN (blue), BEN (purple), CONF (green). MM-VCEP pilot variants are shown in the third row. (continued on next page)
(continued from previous page) (C) Three-dimensional structure of the RHD of RUNX1 (blue) complexed to CBFβ (pink) and DNA (orange). ClinVar RUNX1 variants in this domain (codons 81 to 204 only) are shown using PyMOL, version 2.3.0, as follows: yellow = VUS (n=20), pink = CONF (n=2), orange = LPATH (n=3), red = PATH (n=5); BEN and LBEN variants (n=0). Non-missense variants are not shown. (D) One-dimensional line plot of somatic variants deposited with the Catalog of Somatic Mutations in Cancer (COSMIC: data release 3 Nov. 2018, version 87; https://cancer.sanger.ac.uk/cosmic). MM-VCEP: Myeloid Malignancy Variant Curation Expert Panel; PATH: pathogenic; LPATH: likely pathogenic; VUS: variant of uncertain significance; LBEN: likely benign; BEN: benign; CONF: conflicting interpretations in ClinVar.

![Diagram of RUNX1 variant curation](https://cancer.sanger.ac.uk/cosmic)
penetrance, with several affected individuals reported to have normal platelet counts or function.\textsuperscript{12,24} The nonsense mutation in this patient (p.Arg204Ter) is predicted to lead to nonsense-mediated decay of the RUNX1 mRNA transcript. RUNX1 is located on the long arm of chromosome 21 and is translated into three major isoforms, designated RUNX1A, RUNX1B, and RUNX1C, by using two different promoters and alternative splicing. All transcripts are expressed during hematopoietic differentiation and/or maintenance of normal bone marrow function.\textsuperscript{25-32} For variant annotation, the MM-VCEP utilizes the longest isoform, RUNX1C (NM_001754), as the default transcript, which includes all key domains such as the 128 amino acid (AA) long RUNT homology domain for DNA binding (RHD, AA 77-204), transactivation domain (TAD), inhibitory domain (ID) and the transcriptional repressor binding motif (VWRPY) and is most often used by clinical laboratories for RUNX1 variant curation (Figure 2D). Germeline variants have been reported throughout the gene in ClinVar with the majority currently classified as VUS (Figure 2B). The RUNX1 protein heterodimerizes through its RHD with CBFβ to form a master hematopoietic transcription factor (Figure 2C), which is essential for proliferation and differentiation of hematopoietic stem and progenitor cells, especially in the case of megakaryocytic differentiation.\textsuperscript{33-35} Somatic mutations commonly occur in RUNX1 (Figure 2D). According to RUNX1-specific criteria (Table 2),\textsuperscript{13} the MM-VCEP applied the following codes (Table 3): PVs1 (nonsense variant predicted to undergo nonsense-mediated decay), PM2 (absence in all population databases), PS4\_supporting (one proband meeting at least one of the RUNX1 phenotype criteria), PP1 (co-segregation with disease in the family, three meioses) and arrived at a consensus classification of PATH.

**Example 2. Missense variants, p.(His105Pro) (PATH with PM2, PP3, PS4\_supporting, PM1\_supporting, and PM5\_supporting) and p.(His105Gln) (PATH with PS3, PM2, PP3, and PM1\_supporting)**

Missense mutations in RUNX1 commonly occur in the RHD in somatic and germline contexts.\textsuperscript{36-38} Of 325 RUNX1 ClinVar variants 122 (37.5\%) are missense, and currently in ClinVar, none in the RHD has been classified as BEN or LBEN (Figure 2C). When a novel missense variant is identified which has not been established as PATH or LPATH, it can be difficult to know whether the given change will affect protein function and explain the patient’s phenotype. For example, two RUNX1 missense variants in the RHD (NM_001754:c.314A>C, p.(His105Pro); and NM_001754:c.315C>A, p.(His105Gln)) were considered during the pilot variant analysis. The former was initially classified as a VUS in ClinVar (Figure 2B), but subsequently revised to LPATH upon MM-VCEP review (Figures 2B, 5A and 4). The conclusion of the LPATH assertion is based on the codes applied for this variant: PM2, PP3, PS4\_supporting, PM1\_supporting, and PM5\_supporting (Table 3). Since the variant is completely absent from population databases, the MAF code PM2 is applied. For in-silico evaluation of missense variants, the MM-VCEP recommends using REVEL, a meta-predictor that combines 13 individual tools with high sensitivity and specificity, which has demonstrated the highest performance compared with individual tools or other ensemble methods.\textsuperscript{39-41} The computational prediction code PP3 is applicable to the p.His105Pro variant due to a high REVEL score of 0.953 (MM-VCEP defined >0.75 as the cutoff). The ClinVar submitter (SCV00087773.1) provided us with the patient’s clinical data from their laboratory and the proband met at least one of the RUNX1 phenotype criteria (Table 1) which qualified for PS4\_supporting. This example emphasizes the critical value of sharing internal laboratory data. There is only one meiosis in this family which is lower than the three required for the segregation code PP1. The MM-VCEP defined 15 residues in the RHD as the mutational hotspots for the PM1 code. In addition, variants in other parts of the RHD (AA 105-204) can have a reduced strength-level resulting in application of PM1\_supporting. The last code PM5\_supporting is applied on the p.(His105Pro) variant, because a different missense change p.(His105Gln) at the same residue has been classified as LPATH by the MM-VCEP (Table 3).

The codes PM2, PP3, PM1\_supporting are also applicable to the p.(His105Gln) variant for the same reasons described. Furthermore, a strong pathogenic code PS3 is applied which contributes a significant weight to the final assertion. Transactivation assays of the p.(His105Gln) variant demonstrate altered transactivation (<20\% of wildtype) and secondary assays also indicate altered DNA binding and functional consequences in a mouse model.\textsuperscript{42,43} Missense mutations in RUNX1 have been classified as LPATH by the MM-VCEP (Table 3).

**Example 3. Missense variant, p.His85Asn (LBEN with BS1, BS3, and PP3)**

RUNX1 NM_001754:c.253C>A, p.(His85Asn) is a missense mutation located within the RHD, but not within the mutational hotspot region (AA 105-204), with conflicting interpretations of pathogenicity in ClinVar (Figure 2B, C). Specifically, this variant had three submissions in ClinVar with two being PATH (submitted in 2002) and one being a VUS (submitted in 2018). The two 2002 submissions are from OMIM, which cited evidence from individual literature sources without a systematic curation process. Osato et al. reported an adult patient with AML carrying this variant.\textsuperscript{44} However, the germline nature of the variant was not definitively determined. This variant has also been reported in an infant diagnosed with transient myeloproliferative disorder and Down syndrome whose phenotype does not meet any of the RUNX1 phenotype criteria.\textsuperscript{45} After analysis and curation by the MM-VCEP using the RUNX1\_specific classification rules,\textsuperscript{13} this variant was re-classified as LBEN, meeting codes BS1 and BS3, despite meeting PP3 (Table 3). According to the penetrance, prevalence and genetic and allelic heterogeneity of RUNX1, MM-VCEP refined the RUNX1 specific MAF threshold for application of BS1 to 0.00015 (0.015\%). The highest MAF of the p.His85Asn variant is 0.00043 (8 out of 18,768 alleles) from the East Asian(subpopulation in the Genome Aggregation Database (gnomAD) which is higher than the RUNX1-
specific BS1 cutoff. Experimental studies have shown that this missense change displays normal transactivation activities (80-114% of wildtype) and does not affect DNA binding, heterodimerization with CBFβ or subcellular localization of the RUNX1 protein.\textsuperscript{44,46} Therefore, the strong benign functional evidence code, BS3, is applied. Although this variant disrupts KMT2A binding, which impairs proper H3K4 histone methylation, this is not a qualified functional assay based on the MM-VCEP \textit{RUNX1}-specific PS3/BS3 rule. Moreover, another well-established BEN variant p.(Leu56Ser) also impairs KMT2A binding.\textsuperscript{46} Likely due to the location of the p.(His85Asn) variant within the RHD (Figure 2B), the REVEL score (0.852) of this variant is higher than the MM-VCEP defined 0.75 cutoff,\textsuperscript{18} which results in the variant meeting a conflicting PF3 code. However, combining the BS1, BS3 and PP3 codes, a final assertion of LBEN is made based on a Bayesian classification framework.\textsuperscript{15} Given that His85 is located away from binding interfaces in the three-dimensional structure (Figure 3B), it seems reasonable that variants at this position are LBEN.

This example highlights the value of functional studies in the context of the MM-VCEP variant curation and shows that substantive corrections of variant annotation may occur upon application of ClinGen MM-VCEP rules.\textsuperscript{18} Implicit in this process is the expectation that as knowledge about FPD/AML improves with more functional or family data becoming available, the MM-VCEP rules are subject to revision so that annotation of clinical variants will become more accurate (Figure 4). ClinVar variant classifications such as VUS or those with conflicting interpretations may thus evolve to more diagnostic certainty.\textsuperscript{47,48}

**Example 4. Synonymous/intronic/non-coding variants, c.508+3delA (PATH with PS3, PP1\_strong, PM2, PP3, PS4\_supporting), p.Thr148= (LBEN with BP4, BP7) and p.Val419= (VUS with no codes)**

RUNX1 variants affecting canonical splice positions ±1 or 2 at intron-exon boundaries are expected to disrupt

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**Figure 3. Three-dimensional structure of RUNX1 missense variants His105 and His85 considered as examples 2 and 3.** (A) RUNX1 His105 is important functionally due to its location and thus involvement in DNA binding and close interaction with Thr196 by hydrogen bonding. Thr196 is a hotspot residue known to be critical. This structure-function relationship further supports classification of His105 variants as likely pathogenic (LPATH). (B) RUNX1 His85 is located close to the start of the Runt homology domain in a linker region, and is located far from the DNA binding surface. It is not involved in the core \(\beta\)-barrel structure and does not show any interactions, further supporting its classification as likely benign (LBEN). Structure of RUNX1 complexed to DNA and CBF\(\beta\) (https://www.rcsb.org/structure/1H9D)\textsuperscript{82} and plotted using PyMOL version 2.3.0.
splicing, leading to protein dysfunction (see Online Supplementary Table S3 of reference by Luo and Feurstein18). All of the three canonical splicing site variants in the pilot set were classified as PATH or LPATH. More challenging, however, is the consideration of synonymous/intronic/non-coding variants which may result in cryptic splice site activation, and/or enhancement or repression of adjacent canonical splice sites. For example, the intronic NM_001754:c.508+3delA variant has been reported in a single family with disease segregation (8 meioses, PP1_strong). Several family members were diagnosed with thrombocytopenia, aspirin-like platelet aggregation defects, and dense granule abnormalities.29 This variant is absent from population databases (PM2) and both splicing predictors (MaxEntScan and Splice-SiteFinder)30,31 predict a significant decrease in the score of the canonical splice site (PS3), using RNA derived from two affected family members, were performed and indicate the creation of a novel cryptic splice site 23 nucleotides upstream of the normal splice site resulting in a frameshift p.(Arg162fs*177), and the transcript is predicted to undergo nonsense-mediated decay.49 Combining all of these codes, a final assertion of PATH is given by the MM-VCEP (Table 3).

BP7 is a benign code specifically designed to evaluate synonymous/intronic/non-coding variants in the ACMG/AMP framework. BP7 can be applied if computational evidence suggests no impact on splicing, and the nucleotide is not conserved. The ClinVar variant with conflicting interpretations in ClinVar, NM_001754:c.444C>T, p.(Thr148=), has been classified as LBEN by the MM-VCEP using BP7 and the benign in silico prediction code, BP4 (Table 3). This nucleotide change is predicted to have no impact on splicing and is also not conserved (phyloPscore: -4.3832, below the MM-VCEP-specified threshold of <0.1). Clinical data from seven individuals with this variant were acquired from the original ClinVar submitter (SCV000761123.1) and revealed that none of the probands met any of the RUNX1 phenotypic criteria.18 Currently, only two RUNX1 variants have been reported to display an abnormal splicing effect as demonstrated by RNA assays.11,14 The potential effects of other splicing variants rely solely on in silico predictions. Although there is robust effort in consideration of algorithms to predict the effects of splicing variants, these algorithms require further evaluation. Indeed, we know of only limited experimental data within the RUNX1 gene specifically to test these tools. Accordingly, the synonymous variant, NM_001754:c.1257G>A, p.(Val419=) is predicted to create alternative splice acceptor sites, but is not expected to abolish any existing consensus sites, as it is too far away from either end of the exon. Due to this in silico prediction result, none of the PS3/BP4 and BP7 codes can be assigned, and the classification of this variant remains a VUS. Further resolution of the significance of this variant could be obtained through parental testing, and/or RNA-sequencing data.

**Example 5. Copy number variants, deletion of exon 2 (PATH with PM2_moderate, PM2, PS4, and PP1_strong)**

Not infrequently, patients with FPD/AML have been reported to have copy number variants resulting in intragenic deletions of RUNX1.25 As part of our pilot cohort, we evaluated several probands with copy number variants which at a minimum include RUNX1 exon 2 deletion; data from two cases are shown (Figure 5). The analysis of copy number variants by using next-generation sequencing and/or single nucleotide polymorphism microarrays is particularly challenging because the breakpoints are often not captured in the sequenced regions or the microarray resolution defines only a range for the chromosomal location of the breakpoint, respectively, and thus the nucleotide level breakpoint may remain unknown. It can, therefore, be difficult to know the effect of partial gene/exon deletions, such as if the deletion is in- or out-of-frame, the latter of which may also lead to the introduction of a premature stop codon. Nevertheless, partial or whole gene deletion of RUNX1 is expected to result in haploinsufficiency of the RUNX1 protein. Although MM-VCEP rules did not include recommendations for the formal classification of copy number variants,13 several points should be noted. First, evaluation of the reference Database of Genomic Variants (http://dgv.tcag.ca/dgv/app/home) shows that copy number variants affecting RUNX1 do not appear to be frequent.13 Second, annotation of the specific breakpoints of these intragenic deletions may not always be possible, given that whole genome sequencing is not typically performed. Since contiguous exon deletion is a common pathogenic disease mechanism, it is imperative that laboratories performing germline testing for RUNX1 use concurrent microarray testing, develop appropriate next-generation sequencing bioinformatics pipelines, or use alternate molecular techniques, such as quantitative polymerase chain reaction and multiplex ligation-dependent probe amplification, to screen for and exclude copy loss,
as sequencing for single nucleotide variants and indels alone is insufficient for comprehensive germline evaluation. For the two cases herein (Figure 5), although we do not know the specific breakpoints for each, the common deletion of at least exon 2 allows us to apply the following codes: PVS1\_moderate, PM2, PS4 (4 probands: 3 with chronic thrombocytopenia and 1 with AML), PP1\_strong (7 meioses) to arrive at a PATH classification (Table 3).

**Example 6. Late truncation variant, (p.Ser373Ter) (LPATH with PVS1\_strong, PM2, PS4\_supporting)**

A 14-year old male with a non-contributory family history presented with malaise, poor appetite, night sweats, and intermittent fever of about 1-month duration, thrombocytopenia (27x10^9/L), and subsequent bone marrow biopsy showed AML (PS4\_supporting). After whole genome and exome sequencing on paired tumor and germline samples, along with RNA-sequencing (directed for recurrent fusion identification), his leukemia sample was shown to be RUNX1\_mutated (NM\_001754:c.1118C>A, (p.Ser373Ter)), hypodiploid with Y chromosome loss, without chromosomal fusions, and loss of heterozygosity, but had additional mutations including an intragenic heterozygous deletion of one copy of exon 7 of PALB2 (Table 4).

Similar to Example 1, this RUNX1 (p.Ser373Ter) variant is a nonsense mutation; however, it is not predicted to undergo nonsense-mediated decay, but rather is expected to generate a truncated protein without part of the TAD, ID and the VWRY motif (Figure 2A). From a computational and predictive perspective, a PVS1\_strong code is assigned following the PVS1 decision tree for null or truncating variants in RUNX1. The variant is absent from the gnomAD and other population databases (with confirmed >20x sequencing coverage at this position in gnomAD). Given the variant’s absence from population databases and adequate sequencing coverage of the region, a PM2 code is assigned. Although no additional evidence for the other categories (functional, segregation, de novo and allelic data) are available, this variant can be classified as LPATH (PVS1\_strong, PM2, PS4\_supporting). It is of interest to note that the somatic alterations reported in the diagnostic leukemia sample included partial tandem duplication of KMT2A and a single nucleotide varia-
tion in PHF6. Alterations in these two genes have been reported as cooperating events seen in leukemias from patients with germline RUNX1 mutations.37,53 Importantly, if consideration is given to the mutations found in the leukemic cells in isolation, one cannot determine the germline or somatic origin of the variants reported. This is the case for most of the ‘tumor-only’ analyses being performed in many clinical laboratories. Without paired analysis of true germline tissue (e.g. cultured skin fibroblasts), such studies cannot definitively identify germline variants. In this case, the KMT2A partial tandem duplication and single nucleotide variation in PHF6 and RUNX1 could be tumor-drivers in the AML. However, given the sequencing data, including the variant allele frequency, both the RUNX1 mutation and the PALB2 exon 7 intragenic deletion could be germline variants. A detected variant allele frequency approaching 50% or 100% in the tumor may indicate potential germline origin with either an intact wildtype allele or loss of heterozygosity, respectively. However, a high variant allele frequency cannot reliably serve as a proxy for testing of a true germline source. Therefore, if there is concern that a variant could be constitutional, testing of true germline material is critical.1

Discussion

Kindreds with FPD/AML were first reported by Luddy et al. in 197854 and phenotypically well-described as having a bleeding diathesis and myeloid neoplasia by Dowton et al. in 1985.55,56 Subsequent linkage analysis identified RUNX1 as the candidate gene at chromosome 21q22,56 and mutations were detected in FPD/AML families in 1999.11 Since these initial early reports, routine clinical testing for RUNX1 gene mutations is now commonplace for the evaluation of somatic and germline disease in patients with myeloid neoplasms and thrombocytopenia.

In general, RUNX1 variants include single nucleotide variations and indels, such as nonsense, frameshift, and splice site variants, and copy number variations such as whole-gene and intragenic deletions. RUNX1 is also frequently mutated somatically in AML and often the partner of various translocations resulting in gene fusions, such as t(8;21)(q22;q22) RUNX1-\(\text{RUNX1T1}\).57,58 To date, fusions of RUNX1 have not been reported in the germline context, and most germline RUNX1 variants are unique,11 although some have been rarely seen in unrelated families. Given the limited data on rare variants, the clinical annotation of new variants remains challenging. The MM-VCEP was convened by ASH/ClinGen (Online Supplementary Figure S1) to develop rules for curating gene variant causing predisposition to myeloid neoplasia (Table 2). In this review, we describe the classification of six variant examples (Table 3) using the gene- and disease-specific rule modifications of the original ACMG/AMP 2015 framework.14 Several points should be made.

First, it is critical to ensure that genomic testing intended to assess a germline predisposition is performed on a definitive germline sample because malignant hematologic diseases involve the peripheral blood and bone marrow, and somatic variants in these diseases can confound variant interpretation if an inappropriate sample is used. Here, in keeping with our MM-VCEP rules, cultured skin fibroblasts (gold standard, albeit invasive, costly, and time-consuming), cultured bone marrow mesenchymal stromal cells or DNA from hair roots are appropriate sources.59,60 Alternatively, confirmation of the germline nature of a variant can be achieved by demonstrating its presence in two or more related individuals. The possibility of sample contamination by malignant cells is significant and consequently, peripheral blood, bone marrow, saliva, buccal swabs, DNA from paraffin blocks and even fingernails, which can contain monocytes, are inappropriate samples for germline testing. In some institutions, laboratories may accept T cells, enriched via flow cytometry sorting or column-based magnetic cell separation, as a germline sample for testing. It is important to recognize that some somatic alterations may occur early in hematopoietic stem and progenitor cells with multilineage potential to differentiate into T cells,41 as recent single-cell studies have confirmed.42-45 Thus, if T cells are used, the possibility that a detected variant may be somatic should still be considered. Once a variant is confirmed to be germline in a proband, however, additional testing for the known variant in related family members can be performed on any tissue source.

Second, we should keep an open mind about disease-causing alleles and the type of variants that may be seen and thus, we advocate for a broad testing approach. For example, in some laboratories, non-coding variants are automatically filtered as part of bioinformatic pipelines and may thus be omitted from subsequent review and interpretation. Recently, however, synonymous variants66 in the GATA2 gene, another gene predisposing to myeloid malignancy, were reported in addition to the known pathogenic deep intronic variants of an enhancer region of GATA2.67 In ANKRD26, variants of the 5’ untranslated region cause disease.68,69 Furthermore, copy number alterations may not be assessed in somatic tumor testing panels. As diagnosticians, it is important to think broadly when analyzing genomic information for germline pathogenic variants. Given that these are rare diseases, we should not inadvertently exclude disease mechanisms and/or specific classes of mutations. For example, in case 4, some variants may remain as VUS until additional functional or familial segregation data become available for reclassification.40,41

Third, definitive annotation of variants by one institution will likely remain challenging. However, consistent application of MM-VCEP rules with ClinVar data deposition and thus inter-laboratory correspondence can significantly improve the accuracy and consistency of variant curation. In this regard, examples 2, 3, and 5 show how leveraging shared genomic and phenotypic data can be helpful to clarify VUS. We therefore advocate that clinical variant data be deposited into ClinVar. Specifically, laboratories offering germline testing should modify their test requisition forms to indicate that de-identified phenotype and variant data will be deposited into ClinVar as part of ongoing quality assurance and improvement efforts (https://www.clinicalgenome.org/share-your-data/laboratories/).70,71 Additional details of the ClinVar deposition process are included in Online Supplementary Figure S1.

Fourth, RUNX1 variant curation will improve as more is understood about the disease and gene through functional and family studies. Currently, variant annotation remains a challenging task, because of limited data for
determining the functional effect of a given variant change, despite methods of engineering variants for functional assessment.\textsuperscript{72,74} Early studies in Speck’s laboratory on RUNX1 showed the significance of key residues in the RHD of RUNX1 by performing alanine scanning mutagenesis.\textsuperscript{75,76} However, these early approaches are limited in that not every combination of nucleotide change was explored. By contrast, recent high-throughput functional genomic methods,\textsuperscript{77} known as deep mutational scanning, utilize large-scale approaches to mutate every nucleotide of a gene, permitting one to test the functional consequence of all single nucleotide variations. This has, for example, been recently demonstrated for \textit{BRCA1}.\textsuperscript{78} Additionally, systematic mutagenesis of PTEN has provided a wealth of functional data to inform the classification of \textit{PTEN} variants,\textsuperscript{79} in conjunction with published rules developed by the PTEN-VCEP.\textsuperscript{80} In the future, focused functional assays targeting specific VUS\textsuperscript{81} and deep mutational scanning of genes should contribute to variant curation to resolve VUS.

Fifth, while functional testing of every given genomic variant is possible, it can be costly and difficult to do for every clinically significant gene. In this regard, family studies can aid in the classification of VUS. By systematically evaluating disease segregation in family members with paired genotyping for a known variant, accurate classification of a given variant can be achieved. For example, a recent study showed that this family-based method for variant classification can resolve a VUS classification more frequently than other traditional approaches can.\textsuperscript{82} For rare diseases, such as FPD/AML, detailed pedigree and segregation analyses can be incredibly informative, and clinicians should be encouraged to test family members when possible, seeking help from local genetic counselors and/or geneticists as needed.\textsuperscript{83} Hematologists and oncologists need to consistently take a detailed family and genetic history.

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\section*{Summary}

\textit{RUNX1} germline mutations associated with FPD/AML are key events in myeloid neoplasms, thrombocytopenia and leukemogenesis and represent a model of a germline gene disorder with pathogenic variants predisposing to myeloid and (to a lesser extent) lymphoid malignancies.\textsuperscript{56} Providing an accurate clinical and pathologic variant interpretation for genomic variants detected in routine laboratory testing will remain critical for the provision of appropriate clinical care, including genetic counseling for the index patient and their at-risk relatives and donor-selection, in some cases benefiting from stem cell transplantation.

The ClinGen MM-VCEP variant interpretation process requires a detailed understanding of the biological and functional properties of \textit{RUNX1} and disease phenotype. Here, we demonstrate the process for sequence variant interpretation of six variant examples. By introducing and thus standardizing genomic variant interpretation, we hope to improve patients’ care, identify VUS that may benefit from directed research and encourage sharing of internal laboratory data to resolve uncertainty. In doing so, the MM-VCEP rules may ensure optimal insurance coverage for appropriate genomic testing and screening of family members, and ensure appropriate reimbursement for clinical laboratories. Overall, the ASH/ClinGen collaboration resulting in the first set of modified criteria for germline \textit{RUNX1} variants should improve clinical care and recommendations for FPD/AML patients.
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