VUS in NGS myeloid panel for CIN adult patients

Grigoris Tsaknakis - Helen A. Papadaki
Gene Panel Design

<table>
<thead>
<tr>
<th>ASXL1</th>
<th>ATM</th>
<th>BRAF</th>
<th>BTK</th>
<th>CALR</th>
<th>CBL</th>
<th>CEBPA</th>
<th>CSF3R</th>
<th>CXCR4</th>
<th>DNMT3A</th>
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<tbody>
<tr>
<td>ETV6</td>
<td>EZH2</td>
<td>FBXW7</td>
<td>FLT3</td>
<td>IDH1</td>
<td>IDH2</td>
<td>IKZF2</td>
<td>IKZF3</td>
<td>IKZF1</td>
<td>JAK1</td>
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<tr>
<td>JAK2</td>
<td>MPL</td>
<td>MYD88</td>
<td>NOTCH1</td>
<td>NPM1</td>
<td>NRAS</td>
<td>PAX5</td>
<td>PLCG2</td>
<td>RHOA</td>
<td>RUNX1</td>
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<tr>
<td>SETBP1</td>
<td>SF3B1</td>
<td>SRSF2</td>
<td>STAG2</td>
<td>TET2</td>
<td>TP53</td>
<td>U2AF1</td>
<td>ZRSR2</td>
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</tbody>
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Ion Ampliseq™ technology (Thermo) is used to amplify the whole coding sequence of the above 38 genes (Amplicon-based sequencing).

The design is covered by 1360 amplicons, distributed in two primer pools and requires 30 ng of template DNA per pool (total input 60ng). Target coverage = 98.68%, Defined Threshold >500 read – depth, >5% Variant Allele Frequency

Custom-made panel used from Hellenic Precision Medicine Network in Oncology to assess the mutational profile of patients with hematological malignancies (both myeloid and lymphoid)
What are Variants of Undetermined Significance (VUS) (I)?

- In 2015, the ACMG/AMP published clinical guidelines on the genetic classification of variants based on several criteria such as *in silico* predictions, global population frequencies and functional *in vivo/in vitro* analysis and published data.

- A five-tier classification system (pathogenic, likely pathogenic, uncertain significance, likely benign, benign) is typically used to interpret variation at the DNA sequence or chromosome level and assess the oncogenic potential.

- Variants with insufficient or conflicting evidence supporting disease association, such that they cannot be classified as 'pathogenic/likely pathogenic', nor as 'benign/likely benign', are VUSs.

- The presence of a VUS, even in a relevant gene or chromosome, does not confirm a genetic diagnosis.
What are Variants of Undetermined Significance (VUS) (II)?

- VUS are mainly missense or synonymous substitutions, substitutions of biochemically similar residues, or in-frame insertions/deletions.

- They may be found in non-coding regions, at less conserved residues, at splicing boundaries, or in less functionally relevant domains compared to true pathological variants.

- Thus, the impact of such VUS on the proteins and their functions are more difficult to uncover, compared to nonsense mutations. This explains the scarcity and complexity of in vitro assays, but strengthens the need for experimental solutions when dealing with VUS.

- VUS are noteworthy because they can unveil peculiar genetic and protein alterations involved in biochemical processes, although they are not always informative for clinical purposes.
Variants of Undetermined Significance (VUS) in myeloid genes and clonal hematopoiesis

- **a. Oncogenic**
  - Known oncogenic variants previously reported in the literature;
  - Novel recurrent variants (n ≥ 2) that cluster with known somatic variants in well characterised myeloid driver genes;
  - Truncating variants (nonsense mutations, essential splice mutations or frameshift indels) in genes implicated in myeloid malignancies through acquisition of loss of function mutations.

- **b. Possible oncogenic**
  - Previously unreported variants that cluster (±3aa) with known oncogenic variants in COSMIC.

- **c. Unknown**
  - Variants identified outside the range of frequent driver variants in genes with known oncogenic variants;
  - Variants (even if recurrent) in genes whose role in myeloid disease is not yet established.
VUS in adult CIN patients: some interesting cases
PHF6 G93C

- 1 individual in the study has the variant at 2.49% VAF
- Patient presenting mild neutropenia
- PHF6, a chromatin binding protein, is frequently altered by mutation and deletion in a range of hematologic malignancies, including acute myeloid leukemia and T-cell acute lymphoblastic leukemia.
- Pathogenic mutations in PHF6 have been shown to be truncating (nonsense, frameshift, or splice site) (throughout the coding region) or missense in the PHD2 domain
- ACMG classification: Likely Pathogenic
- Position conserved, Pathogenic computational verdict based on 13 pathogenic predictions vs 5 benign predictions
- Never reported variant in gnomAD
IKZF1 D120G

- 1 individual in the study has the variant at 2.45% VAF

- Patient presenting mild neutropenia

- IKZF1 encodes a transcription factor involved in lymphocyte development. Loss-of-function alterations of IKZF1 are frequently found in B-cell acute lymphocytic leukemia.

- Pathogenic mutations in IKZF1 have been shown to be truncating (nonsense, frameshift, or splice site)

- ACMG classification: Likely Pathogenic
  - Position strongly conserved. Pathogenic computational verdict based on 9 pathogenic predictions vs 2 benign predictions
  - Never reported variant in gnomAD
TET2 T1980I

- 2 different individuals in the study have the same variant at ~50% VAF (2 out of 200 patients tested)
- Both patients presenting mild neutropenia
- TET2, a tumor suppressor and DNA demethylase, is frequently mutated in hematologic malignancies
- Pathogenic TET2 missense mutations have been reported in the catalytic domain (codons 1134-1444 and 1842-1921), whereas only truncating mutations are distributed throughout the coding region
- ACMG classification: Variant of Uncertain Significance
- Position conserved, Pathogenic computational verdict based on 8 pathogenic predictions vs 3 benign predictions
- Very rare variant in gnomAD
BCORL1 E1693K

- 2 different individuals in the study have the same variant at >40% VAF (2 out of 200 patients tested)

- Both patients presenting mild neutropenia

- BCORL1, a transcriptional repressor, is recurrently mutated in hematopoietic malignancies, astrocytomas, and intracranial germ cell tumors.

- Pathogenic mutations in BCORL1 have been shown to be truncating (nonsense, frameshift, or splice site)

- ACMG classification: Variant of Uncertain Significance
  - Position conserved, Pathogenic computational verdict based on 8 pathogenic predictions vs 3 benign predictions
  - Very rare variant in gnomAD
SETBP1 Asp949Asn

- 2 different individuals (siblings) in the study have the same variant at 50% VAF
- Both patients presenting mild neutropenia
- SETBP1, an epigenetic remodeling protein, is frequently altered by mutation in a range of hematopoietic malignancies.
- Pathogenic mutations in SETBP1 have been shown to be missense in codons 855-880
- ACMG classification: Variant of Uncertain Significance
- Position conserved, Pathogenic computational verdict based on 12 pathogenic predictions vs 8 benign predictions
- Very rare variant in gnomAD
SF3B1 Thr56Ile

- 1 individual in the study has the variant at 50% VAF

- Patient presenting mild neutropenia

- SF3B1, a component of the spliceosome complex, is frequently mutated in hematologic malignancies. Mutations in SF3B1 lead to altered gene expression and aberrant alternative splicing

- Pathogenic mutations in SF3B1 have been shown to be missense in codons E622, Y623, R625, N626, H662, T663, K666, K700E, I704, G740, G742, D781

- ACMG classification: Likely pathogenic
  - Position conserved, Pathogenic computational verdict based on 16 pathogenic predictions vs 3 benign predictions
  - Never reported variant in gnomAD:
CXCR4 R235C

- 1 individual in the study has the variant at 50% VAF
- Patient presenting mild neutropenia

CXCR4, a chemokine receptor, is altered in various solid and hematologic malignancies including lymphoplasmacytic lymphoma. Germline mutations in CXCR4, including activating C-terminal truncating mutations, cause WHIM syndrome, an immunodeficiency disorder characterized by neutropenia.

- ACMG classification: Variant of Uncertain Significance
- Position conserved, Pathogenic computational verdict based on 13 pathogenic predictions vs 5 benign predictions
- Very rare variant in gnomAD
Conclusions/Question arising

- Variant at 50% VAF need paired analysis with constitutional reference tissue to determine the germline nature of the mutation.

- Functional testing is required

- Which set of predefined criteria should be met to select VUSs for further studies?