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ACMG STATEMENT

Incidental detection of acquired variants in germline genetic and genomic testing: a points to consider statement of the American College of Medical Genetics and Genomics (ACMG)

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INTRODUCTION

With recent advances in DNA sequencing technology, it is now possible to begin to appreciate the full scope of DNA variation that arises over the course of an individual's lifetime.[1,2] Our understanding of how the human genome changes over time and in response to external exposures is growing with the improved availability of next-generation sequencing (NGS) based testing, including exome/genome sequencing of large patient cohorts. Clinical laboratories employing NGS-based methodologies can detect many types of DNA sequence variation including those that are present at a reduced variant allele fraction (VAF) (i.e., less than the 50% expected for a heterozygous germline finding). The vast majority of clinical molecular genetic testing for constitutional disorders is currently performed on DNA isolated from white blood cells from either a blood or saliva sample, which is generally considered to be representative of an individual's germline genome. However, DNA isolated from these samples may also include somatic variation, due to either postzygotic variation or variation unique to the hematopoietic cell lineage of the developed child or adult. In the context of genetic testing for heritable conditions, the detection and reporting of these variants may lead to the misdiagnosis of a Mendelian disorder.[3] In addition, somatic variation has sometimes been misattributed as germline variation and included in genomic databases of healthy populations, such as gnomAD.[4] The common use of these databases as a source to filter out DNA variants that are likely to be benign based on population allele frequencies may then lead to the misclassification of a disease-related variant and subsequently, a missed diagnosis of a Mendelian disorder.[5]

NGS quality metrics, including VAFs below 50% and particularly below 30%, can suggest the possibility of somatic variant detection. In these cases, the presence of a somatic DNA variant in a sample from which isolated DNA is expected to be of germline origin may lead to a misinterpretation of a germline Mendelian disorder risk when the result is actually due to acquired somatic variation. However, low VAFs can also have other technical (e.g., preferential allele amplification, DNA sequence context) or biological explanations, including somatic and gonadosomatic mosaicism, hematologic malignancy, and aberrant clonal expansion, etc. Recent reports have identified clonal hematopoiesis of indeterminate potential, sometimes referred to as CHIP, as a common explanation for these findings and have identified specific genes at high risk for detection of acquired variants in this setting, including *ASXL1* (MIM 612990), *DNMT3A* (MIM 602769), and *TP53* (MIM 191170).

Current clinical laboratory practices vary widely in how DNA variants are further evaluated for suspicion of somatic origin. Given what is now understood regarding the prevalence of somatic variation in the population, and in particular, age-related clonal hematopoiesis, nearly all molecular genetic testing laboratories offering NGS testing for constitutional disorders are likely to encounter acquired variants in both their private data and in the public databases. Professional recommendations are needed to help standardize laboratory practices for identifying, investigating, and interpreting variants that are at an increased

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risk for being of true hematopoietic origin. These points to consider will provide guidance for laboratories, focused on NGS testing for constitutional (Mendelian) disorders, to identify and investigate potential somatic or acquired variants as well as recommendations for ancillary testing and options for clinical diagnosis.

METHODS

This points to consider statement was informed by a review of the literature and current guidelines. Resources consulted included PubMed and relevant American College of Medical Genetics and Genomics (ACMG), Association for Molecular Pathology (AMP), College of American Pathologists (CAP), and American Society of Clinical Oncology (ASCO) guidelines. The workgroup members also used their expert opinion and empirical data to inform their recommendations. Any conflicts of interests for workgroup members are listed at the end of the paper. The ACMG Laboratory Quality Assurance Committee reviewed the document providing further input on the content, and a final draft was presented to the ACMG Board of Directors for review and approval to post on the ACMG website for member comment. Upon posting to the ACMG website, an email and link were sent to all ACMG members inviting participation in the 30-day open comment process. All members' comments and additional evidence received were assessed by the authors, and these recommendations were incorporated into the document as deemed appropriate. Member comments and author responses were reviewed by representatives of the ACMG Laboratory Quality Assurance Committee and the ACMG Board of Directors. The final document was approved for publication by the ACMG Board of Directors.

GENERAL POINTS TO CONSIDER

- Detection of aberrant clones in genetic testing has now been well described in the literature.[6–8]
- Most commonly, these abnormal clones are derived from precursor cells of hematopoietic origin.
    - The variants identified in these cases are acquired and limited to a single cellular lineage.
- The risk increases with older age, exposure to chemotherapy, radiation, and tobacco, and the presence of malignant or premalignant conditions (e.g., myelodysplastic syndrome, chronic lymphocytic leukemia [CLL]).
- The association between aberrant clonal expansion (ACE) and/or CHIP and the risk for development of hematopoietic malignancy or premalignancy remains an active area of investigation.[3]
- The spectrum of genes most commonly impacted by ACE/CHIP has been well described,[8] and some of these genes overlap significantly with those available for clinical genetic testing for hereditary cancer predisposition and other Mendelian disorders (Table 1).
- In addition to variation at the level of the nucleotide sequence, somatic copy-number variants can be identified when genetic testing includes copy-number variant detection.
    - These may include age-related changes, such as loss or gain of the sex chromosomes in blood and bone marrow, as well as other changes such as trisomy 8, monosomy 5/deletion 5q, and monosomy 7/deletion 7q, which are common findings in aberrant hematopoietic clones and are potentially related to an underlying condition.
- The origin (acquired or heritable) of reported genetic variants can, in some circumstances, be clarified by testing additional tissue types (e.g., cultured fibroblasts) or by identifying the variant in other family members (transmission testing).
    - Laboratories should consider recommending and/or offering these supplementary tests when a reported variant is suspicious for an acquired origin during genetic testing for hereditary disorders.

POINTS TO CONSIDER FOR TESTING LABORATORIES

Sample type

- Blood is the most common sample type accepted for germline genetic testing, and the vast majority of DNA isolated from blood derives from peripheral white blood cells (of hematopoietic origin).
- DNA isolated from saliva and/or buccal swab samples can also be predominantly of hematopoietic origin, when isolated using standard methodologies, and the proportion of hematopoietic cell contribution varies widely, from 10% to 96% in pediatric and adult populations. This wide range may depend, in part, on the effectiveness of the user to collect a sufficient component of buccal epithelial cells.[9–12]
- Other sample types including cultured fibroblasts, typically derived from a skin biopsy, or other available nonblood tissues should be considered to limit the risk of detecting aberrant hematopoietic clones or to follow-up a result based on DNA isolated from blood or saliva.
    - DNA derived from some tissue types, such as lymph node, spleen, or bone marrow, are also of hematopoietic origin and are not distinct from blood related findings.
    - Other solid tissue (fresh-frozen or formalin-fixed paraffin embedded) specimens may also have significant lymphocytic infiltrate present and extracted DNA may have an unexpectedly high lymphocytic origin.
    - Additionally, genetic testing performed on solid tissue may be subject to other somatic contamination ("field effects") from adjacent malignant tissue.[13]
    - Nontraditional DNA sources such as hair follicles and fingernails may represent alternative noninvasive sources of DNA that could be used for germline confirmation. However, testing options may be limited by the quantity of available DNA from these sources.[10,14]

Test ordering

- Laboratories should routinely ask for clinical information as part of their test requisition, including any personal history of a hematologic condition.
    - Blood, saliva, lymph nodes, and other samples of hematologic or lymphatic system origin are not likely to be acceptable specimen types in cases where there is a history of active or overt hematologic malignancy.
    - Where no other reasonable sample type is available, a laboratory director could use their discretion to accept such samples, provided the ordering clinician consents to the potential risks of identifying a variant of indeterminate origin (somatic vs. germline).
        - Note that CLL is one of the most underappreciated diagnoses known to interfere with germline genetic testing, as active treatment is often deferred, and these samples should be accepted with caution and the consent of the ordering clinician.
- Laboratories should routinely ask about a history of allogeneic bone marrow, peripheral stem cell, or other transplant, as these pose a substantial risk to the integrity of the sample and validity of the data as it relates to an individual's germline DNA.
- Laboratories should have a policy concerning the acceptability of specimens submitted following a blood transfusion.

Table 1. Most commonly altered genes in clonal hematopoiesis.

Gene	Mendelian disorder (OMIM)	Variant type(s) causing Mendelian disorder	Mechanism of disease	Short description
DNMT3A	Tatton–Brown–Rahman syndrome (615879)	Missense/premature truncation	Uncertain	Autosomal dominant neurodevelopmental growth disorder
TET2	None reported			
ASXL1	Bohring–Opitz syndrome (605039)	Premature truncation	Haploinsufficiency	Autosomal dominant neurodevelopmental growth disorder
TP53	Li–Fraumeni syndrome (151623)	Missense/premature truncation	Haploinsufficiency/dominant negative	Hereditary cancer predisposition, often childhood onset
JAK2	Thrombocythemia (614521)	Missense	Gain of function	Autosomal dominant risks for thrombosis, cerebrovascular events and myocardial infarction
SF3B1	None reported			
GNB1	Autosomal dominant mental retardation 42 (616973)	Missense	Disruption of protein–protein interactions	Autosomal dominant intellectual disability disorder
CBL	Noonan syndrome with or without juvenile myelomonocytic leukemia (613563)	Missense	Haploinsufficiency/dominant negative	Autosomal dominant neurodevelopmental growth disorder
SRSF2	None reported			
PPM1D	Jansen–De Vries syndrome (617450)	3′ Premature truncating (loss of nuclear localization signal)	Loss of nuclear localization signal	Autosomal dominant neurodevelopmental growth disorder
GNAS	McCune–Albright syndrome (174800)/ Pseudohypoparathyroidism (612463)	Missense/premature truncation	Loss of function	Autosomal dominant disorder including short stature, obesity, skeletal anomalies, and hormone resistance
BRCC3	None reported			
CREBBP	Rubinstein–Taybi syndrome (180849)	Premature truncation	Loss of function	Autosomal dominant neurodevelopmental growth disorder
NRAS	Noonan syndrome (613224)	Missense	Gain of function	Autosomal dominant neurodevelopmental growth disorder
RAD21	Cornelia de Lange syndrome (614701)	Missense	Loss of function	Autosomal dominant multisystem neurodevelopmental disorder (cohesinopathy)
SETDB1	None reported			
U2AF1	None reported			
SETD2	Luscan–Lumish syndrome (616831)	Missense/premature truncation	Loss of function	Autosomal dominant neurodevelopmental growth disorder
CHEK2	CHEK2-related cancer susceptibility (114480)	Missense/premature truncation	Loss of function	Autosomal dominant hereditary cancer predisposition
ATM	Ataxia–telangiectasia syndrome (208900)/ ATM-related cancer susceptibility (114480)	Missense/premature truncation	Loss of function	Autosomal recessive progressive cerebellar ataxia; autosomal dominant hereditary cancer predisposition
NF1	Neurofibromatosis, type 1 (613113)	Missense/premature truncation	Loss of function	Autosomal dominant neurodevelopmental growth disorder

- In studies of cytogenetic testing following transfusion of irradiated, leukodepleted, packed red blood cells, no donor chromosomes were found in downstream analysis, suggesting that there is limited evidence to support delaying genetic testing following transfusion.[15,16]
- The phenomenon of transfusion-associated microchimerism has been previously described in the setting of posttraumatic patients; however, a recent study that used single-nucleotide polymorphism (SNP) genotyping to quantify donor alleles suggests that with more modern methods for leukodepletion, this complication is rare.[17]

- It has been suggested that routine review of past or present test results, such as a complete blood count, peripheral smear, and flow cytometry, may help identify previously affected individuals at risk for the presence of clonal expansion, but there are limited data to support this hypothesis, and therefore the utility of such review is limited and may be cost prohibitive.
  - Additional assessment of such hematologic indices and/or laboratory review of such lab test results may not obviate the risk of aberrant clonal expansion.
  - Furthermore, these assays require specific clinical expertise for interpretation and should be reviewed by trained personnel. Such individuals may not work within a cytogenetic or molecular genetic testing laboratory.
  - At this time, clinical history remains the best predictor; often germline genetic testing results are the first indicator of clonal expansion in the blood.

Test performance and data analysis

- NGS-based assays are exquisitely sensitive and can often identify allelic fractions as low as 5%.[18]
  - Heterozygous germline variants are expected to be present in one of two autosomal copies, with a normal distribution of allelic fractions centered on 50%.
    - However, there are both technical and biological reasons why these may be skewed at the time of testing, even for true germline variants.
  - In contrast, acquired variants could be present at any proportion (0–100%), but are most commonly recognized at lower levels in the absence of overt hematologic malignancy.
  - Therefore, a lower allelic fraction can be suggestive of a variant being acquired, and somatic in origin, but does not provide conclusive data.
  - In cases of a reduced allelic fraction, there also remains the possibility of postzygotic mosaicism, i.e., a variant that was acquired early on in embryonic development and is therefore present in multiple, but not all, cellular lineages, resulting in the reduced allelic fraction. This may include the germ cells in cases of gonadosomatic mosaicism. *The implications for familial risk and genetic diagnosis are substantially different in these cases compared with clonal expansion of exclusively hematopoietic origin.*

- Laboratories who perform NGS-based genetic testing assays, as well as other methodologies sensitive to the detection of low-level genetic variation, should be aware of the possibility that some of the DNA variants detected may not be of germline origin, regardless of sample type.
  - Laboratories should develop quality metrics or thresholds to alert them to the presence of an acquired variant of somatic origin. Some examples might include:
    - VAF < 30%
    - Significant phenotype–genotype mismatch
      - *ASXL1* pathogenic variant in an elderly male with cardiovascular disease.
      - *TP53* likely pathogenic variant in a healthy adult without a personal or family history of cancer.
      - Among other factors, penetrance and age of onset should be considered when performing this comparison.
    - Consideration of the risk of postzygotic mosaicism, when known for a genetic syndrome
      - Mosaic or segmental neurofibromatosis type I (NF1) is relatively common, observed in up to 10% of cases,[19] and in these cases, pathogenic variants will frequently impact more than one cellular lineage (i.e., skin but not blood).
  - Laboratories should consider validating methods of DNA extraction from additional tissue types, outside of blood/saliva/buccal, to facilitate further discernment of the origin of reported variants.

- Laboratory resources, such as population databases (e.g., gnomAD) used for filtering by variant allele frequency, are also likely to harbor acquired variants.
  - The risk is that the presence of acquired variants can artificially inflate allele frequencies and lead to filtering and/or misclassification of variants as benign or likely benign, based on recommended thresholds (BA1, BS1, BS2).[20,21]
  - Unexpected skewing in the distribution of the age of the individuals with the variant may help to flag these variants (i.e., if a variant has only been observed in older individuals that may help identify it as a potential somatic variant).

Variant interpretation and reporting

- Prior to reporting, each DNA variant is assessed for pathogenicity and clinical significance.
- This process includes a review of genotype–phenotype correlation.
  - Discordance should alert the laboratory to the possible presence of an acquired or somatic variant, and a potential challenge to a valid molecular diagnosis.
  - This analysis may be confounded due to concerns of reduced penetrance, de novo events, incidence of postzygotic mosaicism, etc.

- When a variant detected through germline genetic testing is suspected to be acquired, rather than of germline origin, this should be communicated to the ordering provider on the final report. In addition, laboratories should:
  - Avoid the use of the term de novo in reporting these variants, as acquired or somatic variants are, by definition, absent from biologic parents. De novo variants are also typically associated with a risk, albeit low, of recurrence in future pregnancies, which would not be true for a somatic variant that is confined to cells of the hematopoietic lineage.
    - Rates of gonadosomatic mosaicism remain largely unknown but might be extrapolated from reports in trio exome sequencing where a limited number of variant reads have been identified in an unaffected parent of a heterozygous child.[22]
  - Consider reanalysis with an orthogonal methodology to reduce the risk of a result due to technical reasons.

- Recommend appropriate follow-up testing on the test report, which may include the following:
  - Acceptance of ancillary tissues to discern germline status (labs could consider including these tests as part of their germline genetic test offerings to help clarify results).
  - Site-specific testing of offspring, or other first-degree relatives, for evidence of variant transmission, not for risk assessment (cascade testing).

- The risks related to the misapplication of a molecular diagnosis in the setting of somatic variation vary according to the gene/condition, and related management and laboratory policies should reflect the level of risk.
  - Clinical laboratories could prioritize a list of genes/conditions at risk for acquired variant detection where there is a major risk of clinical impact.

Clinical interpretation and diagnosis

- Laboratory genetic and genomic testing has an important role in patient care and is intended to support but not replace a clinical genetic evaluation and diagnosis.
- When genetic testing results appear inconsistent with the clinical phenotype, acquired variant detection may be an explanation, particularly in older individuals.
  - Table 1 highlights the genes where this is most likely to occur.
- When somatic or acquired origin is suspected or confirmed, clinical management may be adjusted appropriately to avoid unnecessary treatment or intervention or further evaluate the source of the variant.
  - Additional clinical investigations (lab testing, imaging, specialty referral, etc.) may be warranted to further investigate a suspicious finding and differentiate age-related changes from a possible underlying malignancy or other condition.

ILLUSTRATIVE CASE EXAMPLES

Case 1

A 43-year-old female had a personal history of breast cancer at age 42 and reported a family history of prostate cancer in her father at age 65 and brain cancer in her mother at age 62. Multigene panel testing included 20 genes associated with increased hereditary cancer risks. The results of testing DNA isolated from a blood specimen detected a likely pathogenic variant in the *NF1* gene (OMIM 613113), which would be consistent with a diagnosis of type I neurofibromatosis (NF1). The variant was identified at an allele fraction of 20%, and the ordering clinician was contacted to request a full clinical evaluation for signs and symptoms of NF1 and an alternate specimen type for assessment. Subsequent testing on cultured skin fibroblast cells also detected the *NF1* likely pathogenic variant. These results are most likely consistent with postzygotic mosaicism (segmental NF1), and the patient should be counseled and subsequently managed based on the recommendations for this disorder.[23]

Case 2

A 68-year-old female had a personal history of ER/PR+ breast cancer and reported a family history of a paternal half-sister with breast cancer in her 40s. Multigene panel testing included 30 genes associated with increased hereditary cancer risks. The results of testing on a blood specimen detected a pathogenic variant in the *TP53* gene, which would be consistent with a molecular diagnosis of Li–Fraumeni syndrome (OMIM 151623). The variant was identified at an allele fraction of 10%, and the ordering clinician was contacted to request an alternate specimen type for assessment. Subsequent testing on cultured skin fibroblast cells did not detect the *TP53* pathogenic variant. These results are most likely to be consistent with an age-related clonal expansion limited to the hematopoietic lineage; however, though unlikely, the remote possibility of postzygotic mosaicism, and even gonadosomatic mosaicism, cannot be entirely excluded.

Case 3

A 32-year-old female had a personal history of colon cancer and no reported family history of cancer. Multigene panel testing included 25 genes associated with increased hereditary cancer risks. The results of testing on a blood specimen detected a pathogenic variant in the *TP53* gene, which would be consistent with a molecular diagnosis of Li–Fraumeni syndrome (OMIM 151623). The variant was identified at an allele fraction of 20%, and the ordering clinician was contacted to request an alternate specimen type for assessment. Subsequent testing identified the pathogenic *TP53* variant at a reduced allele fraction in saliva, rectal tumor tissue, and cultured fibroblast cells from the skin. These results are most likely to be consistent with postzygotic mosaicism, as the variant was confirmed in multiple tissue types. Notably, the data from cultured fibroblasts provide substantial additional support for this diagnosis, given that the presence of the variant in rectal tumor tissue may be attributable to an inflammatory component. Cascade testing performed in her 5-year-old son identified him as heterozygous for this pathogenic variant, definitively confirming the diagnosis of Li–Fraumeni syndrome in this family.

Case 4

A 6-month-old female was admitted to a children's hospital for failure to thrive with developmental delay, abnormal tone and posturing, and dysmorphic features. Trio exome sequencing was requested, which identified a de novo variant in the *ASXL1* gene that expected to result in premature truncation. Loss-of-function variants in the *ASXL1* gene are well described in Bohring–Opitz syndrome (OMIM 605039), a severe neurodevelopmental disorder with a significant number of overlapping features with this patient.[24,25] However, in the case-based laboratory analysis, this variant was deprioritized and/or filtered from the candidate list due to the large number of loss-of-function variants reported in this gene in the gnomAD database.[5] However, this variant was flagged for re-review due to significant clinical overlap of this gene with the presenting phenotype. Further manual review of the data in gnomAD confirmed that these loss-of-function variants are likely to be somatic, as they were primarily identified in older individuals. Therefore, the results in this female infant are most likely to be consistent with a diagnosis of Bohring–Opitz syndrome.

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COMPETING INTERESTS

E.C.C., C.A., J.L.D., M.P., and H.V.R. all serve as directors in clinical laboratories that perform a breadth of genetic and genomic analyses on a fee-for-service basis. E.C.C. and M.P. are part- and full-time employees of Ambry Genetics, a wholly owned subsidiary of Konika Minolta. J.N.W. serves on the speakers' bureau for AstraZeneca.

ADDITIONAL INFORMATION

Correspondence and requests for materials should be addressed to ACMG