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# CORRESPONDENCE

## Identification of Germline Variants in Tumor Genomic Sequencing Analysis

### To the Editor-in-Chief:

Nathan D. Montgomery,*† Sara R. Selitsky,† Nirali M. Patel,*† D. Neil Hayes,† Joel S. Parker,† and Karen E. Weck*†

*From the Department of Pathology and Laboratory Medicine* and the Lineberger Comprehensive Cancer Center,† The University of North Carolina School of Medicine, Chapel Hill, North Carolina*

Recently, the Association for Molecular Pathology (AMP), the American Society of Clinical Oncology (ASCO), and the College of American Pathologists (CAP) published consensus guidelines in *The Journal of Molecular Diagnostics* to standardize variant interpretation and reporting in oncology specimens.[1] This group's recommendations included guidance related to a dilemma commonly encountered in clinical practice—accurately distinguishing somatic mutations that arose in a patient's tumor from inherited germline variants.

Distinguishing somatic from germline variants is important for several reasons. First, most germline sequence variants will be clinically benign, and these should not be included in clinical reports of actionable somatic driver mutations. Second, however, some rare germline variants are pathogenic and may be associated with an inherited disorder, including familial cancer predisposition. It is important to recognize these variants as inherited to ensure appropriate management not only of the patient but also of family members. Moreover, even when germline variants are not associated with a high-penetrance cancer susceptibility syndrome, they may be functionally and even clinically significant. Finally, accurately recognizing that a sequence variant is of somatic origin may provide evidence of a clonal process, which can be diagnostically important in some neoplastic conditions, such as myeloid neoplasia.[2]

The task of distinguishing somatic and germline variants is made more difficult in tumor-only sequencing protocols. These workflows involve identification of mutations in neoplastic tissue without comparison to a normal sample from the patient. Many clinical laboratories have adopted such protocols for practical reasons and/or to avoid the substantial cost of sequencing paired normal specimens. However, tumor-only workflows carry some risk of misclassifying somatic and germline variants.

The ASCO/AMP/CAP guidelines make several recommendations to assist recognition of germline variants in tumor-only protocols.[1] Chief among these, the guidelines indicate that the main criterion for germline designation is variant allele fraction (VAF), which the authors note is expected to be near 50% or 100% for heterozygous and homozygous germline alleles, respectively, that are present at diploid copy number in all cells in a specimen. However, performance metrics of this recommendation have not been established. Moreover, as the authors of the guidelines acknowledge, there are certain limitations to using VAF as a screen for germline variants. For instance, somatic mutations may also be present near 50% or 100% VAF in tissue with high tumor burden, and germline variants may deviate from 50% or 100% VAF because of chromosomal aneuploidy or other changes in gene copy number, both of which are common in tumors.

We examined the usefulness of the AMP/ASCO/CAP guideline for identification of germline variants in a cohort of 1310 cancer patients with paired sequencing of >200 genes in both a tumor and a normal sample (peripheral blood or tissue) as part of a large next-generation sequencing research program at our institution (LCCC1108/UNCSeq, NCT01457196).[3] A diverse group of neoplasms was represented in this cohort. Common sites of tumor origin included gynecologic tract ($n = 402$), breast ($n = 212$), head and neck ($n = 203$), gastrointestinal tract organs ($n = 115$), nervous system/brain ($n = 95$),

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Address correspondence to Karen E. Weck, M.D., Department of Pathology and Laboratory Medicine, The University of North Carolina School of Medicine, Campus Box 7525, Chapel Hill, NC 27599-7525. E-mail: kweck@unc.edu.

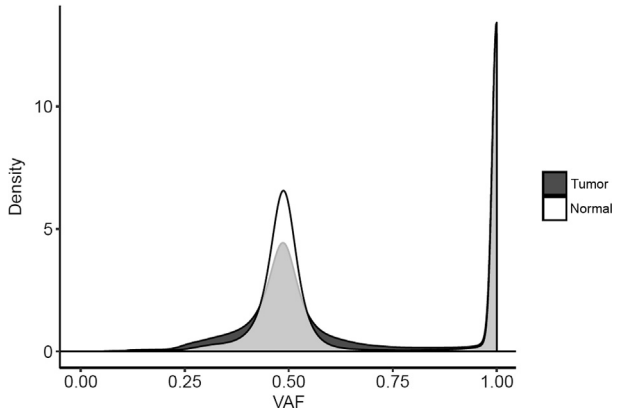


**Figure 1** Density plots of allele fraction for germline variants in tumor and paired normal samples. Density plots are shown for the observed allele fraction of germline variants measured in tumor and paired normal samples from 1310 subjects. **Light gray regions** represent the area encompassed under both curves. For this analysis, variants detected in the paired normal sample were defined as germline variants. However, to be included in the study, variants were required to have a read depth of ≥100× in both tumor and normal samples. Approximately 6.8 million variants met these inclusion criteria. Somatic variants present only in the tumor sample are not included in this plot. VAF, variant allele fraction.

genitourinary system ($n = 94$), hematologic/lymphoid ($n = 57$), soft tissue ($n = 46$), skin ($n = 30$), and lung ($n = 28$).

Analysis was restricted to regions with 100× or deeper coverage in both the tumor and normal samples. Within these regions, a total of 6.8 million variants were identified in the normal samples as candidate germline variants. Density plots of observed VAFs for these variants are shown in Figure 1. As expected, measured VAF of germline variants in normal tissue showed a bimodal distribution, with peaks centered at 50% and 100%. Although a bimodal pattern was also observed for germline variants in tumor samples, the Gaussian distribution was broader around the 50% VAF peak, indicating a wider range of VAFs for these variants within the tumor samples (Figure 1).

To further explore this observation, VAFs of variants detected in normal samples were plotted against VAFs of the same variants in tumor samples (tumor VAF) (Figure 2). As expected, germline variants were densest in both normal and tumor VAFs at around 50% and 100%. In the tumor sample, 46% of all germline variants were present at VAFs between 40% and 60%, as expected for a heterozygous allele, and 35% of all germline variants were present at a VAF of >95%. However, 19% of candidate germline variants exhibited a VAF in the tumor sample outside of the ranges suggested by the AMP/ASCO/CAP guidelines. This deviation was particularly striking for heterozygous germline variants, many of which showed considerable scatter away from 50% VAF in the tumor sample (Figure 2). Specifically, 22% of germline variants that were detected at a VAF between 40% and 60% in the normal sample (representing heterozygous alleles) had a VAF outside of this range in the paired tumor samples (10% with a VAF of >60% and 11% with a VAF of <40%) (Figure 2).

Next, the percentage of all variants with a tumor VAF between 40% and 60% that were germline versus somatic in origin was determined. Of a total of 3.1 million variants with a tumor VAF between 40% and 60%, most (96%) were confirmed as germline by presence in the normal sample (data not shown).

Our observations indicate that the VAF of a substantial percentage of germline variants will deviate markedly from 50% or 100% in tumor samples. Although performance metrics are likely to be affected by several variables, including tumor type and tumor percentage, these data provide an informative first estimate of the sensitivity and specificity of the AMP/ASCO/CAP guideline for identification of germline variants in a cohort of patients with diverse tumors. As such, our observations have important implications for variant interpretation in tumor-only platforms.

In our cohort, VAF discordance between normal and tumor samples was most prominent for heterozygous alleles. In a subset of these cases, the finding of a VAF of <40% or >60% in tumor may indicate loss of heterozygosity of a pathogenic germline variant, a possibility worthy of future study. However, given that such discordance was common in our cohort, it may be more often the case that changes in VAF in tumor are passenger effects of an unstable genome.

Ultimately, there is no true substitute to tumor-normal pairs in the accurate distinction of germline from somatic variants. However, given persistent reimbursement issues in this space,[4] the additional expense of paired normal

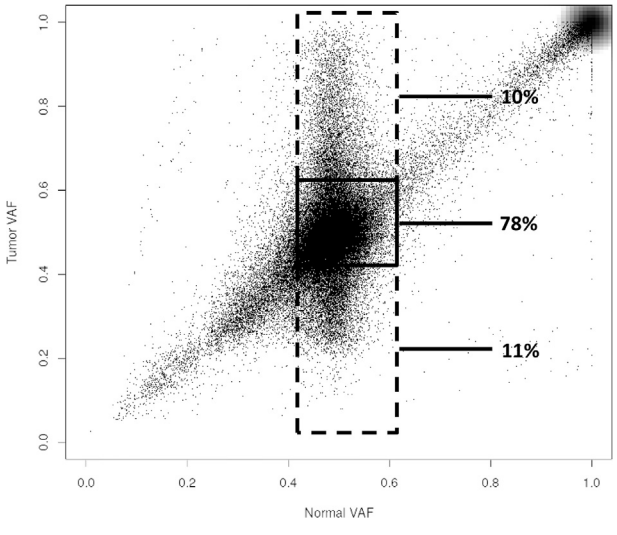


**Figure 2** Comparison of variant allele fraction (VAF) of germline variants in tumor and paired normal samples. Observed VAF for germline variants in normal samples (*x* axis) and tumor samples (*y* axis). Results from a random sample of 100,000 variants (of 6.8 million total variants) are plotted for visualization. Among variants at 40% to 60% VAF in the normal sample, 78% are also detected at 40% to 60% VAF in tumor (**boxed area**), 10% were detected at >60% VAF in tumor (**top dashed boxed area**), and 11% were detected at <40% VAF in tumor (**bottom dashed boxed area**).

sequencing may prove cost prohibitive for many laboratories. Although the AMP/ASCO/CAP recommendations provide helpful guidance to such laboratories, reporting should emphasize the inherent inability of definitively distinguishing germline and somatic variants in tumor-only sequencing assays. If suspicion for a germline-inherited variant is present, follow-up testing of a normal sample should be recommended.

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## Authors' Reply


Marilyn M. Li,[*†] Michael Datto,[*‡] Eric J. Duncavage,[*§] Shashikant Kulkarni,[*¶] Neal I. Lindeman,[*‖] Somak Roy,[*,**] Apostolia M. Tsimberidou,[*††] Cindy L. Vnencak-Jones,[*‡‡] Daynna J. Wolff,[*§§] Anas Younes,[*¶¶] and Marina N. Nikiforova[*,**]

*From the Interpretation of Sequence Variants in Somatic Conditions Working Group of the Clinical Practice Committee,* Association for Molecular Pathology, Bethesda, Maryland; the Department of Pathology and Laboratory Medicine,† Division of Genomic Diagnostics, the Children's Hospital of Philadelphia, University of Pennsylvania Perelman School of Medicine, Philadelphia, Pennsylvania; the Duke University School of Medicine,‡ Durham, North Carolina; the Department of Pathology and Immunology,§ Washington University School of Medicine, St. Louis, Missouri; Baylor Genetics,¶ Houston, Texas; the Brigham and Women's Hospital,‖ Harvard Medical School, Boston, Massachusetts; the University of Pittsburgh Medical Center,** Pittsburgh, Pennsylvania; the Department of Investigational Cancer Therapeutics,†† University of Texas MD Anderson Cancer Center, Houston, Texas; the Department of Pathology, Microbiology and Immunology,‡‡ Vanderbilt University Medical Center, Nashville, Tennessee; the Department of Pathology and Laboratory Medicine,§§ Medical University of South Carolina, Charleston, South Carolina; and the Memorial Sloan Kettering Cancer Center,¶¶ New York, New York*


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Overall, the experience of Montgomery et al[1] supports several of the AMP/ASCO/CAP guideline variant interpretation and reporting recommendations: i) When a pathogenic germline variant is suspected during tumor-only testing, confirmation of the variant with a normal tissue sample, along with appropriate genetic counseling, should be recommended. For definitive classification of germline status, sequencing of nontumor (ie, uninvolved healthy) tissue should be performed. ii) Concurrent analysis of a paired germline sample is desirable because it clarifies interpretation. However, it is not always practical and should not be required. iii) When paired germline samples are not used, next-generation sequencing analysis on tumor only does not distinguish germline and somatic variants, and sequencing results may contain both findings. In this case, findings can be reported with a disclaimer that the next-generation sequencing test used does not allow definitive differentiation between germline and somatic variants. The test reports should include a statement addressing the manner in which the distinction between somatic and germline alterations is made and indications of remaining uncertainty, where appropriate.

The AMP Working Group believes the difficulties in extrapolating germline variants from tumor-only sequencing presented in the letter are understood by clinical laboratories performing somatic sequencing. However, it is extremely important for laboratories doing tumor-only sequencing to acknowledge in the report that one of the limitations of their assay will be the inability to definitively differentiate germline variants from somatic ones.

The letter by Montgomery et al also reinforces the pivotal role of the molecular professional in providing variant interpretation in both somatic and germline settings. It is within the scope of the molecular professional's medical practice to use his or her considered judgment regarding the

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Address correspondence to Marilyn M. Li, M.D., Department of Pathology and Laboratory Medicine, Children's Hospital of Philadelphia, University of Pennsylvania Perelman School of Medicine, 3615 Civic Center Blvd, ARC 716i, Philadelphia, PA 19104. E-mail: lim5@email.chop.edu.