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Detection of concurrent hematologic malignancies in solid tumor NGS testing may cause false-positive results

CAP TODAY *and the Association for Molecular Pathology have teamed up to bring molecular case reports to* CAP TODAY *readers. AMP members write the reports using clinical cases from their own practices that show molecular testing's important role in diagnosis, prognosis, and treatment. The following report comes from Weill Cornell Medicine. If you would like to submit a case report, please send an email to the AMP at* amp@amp.org. *For more information about the AMP and all previously published case reports, visit* www.amp.org.

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Next-generation sequencing is becoming the standard of care in the diagnostic workup of lung adenocarcinoma and other solid tumors. This technology leverages massively parallel sequencing to interrogate multiple genes of interest in a single test. The results of NGS have important implications for patient care, providing diagnostic, prognostic, and predictive information.

False-positive NGS results may arise due to multiple scenarios, for example, misidentification of a germline finding as a somatic finding. These false-positive results may lead to misinterpretation or inappropriate use of the NGS results and have serious clinical impact. Here, we discuss a case of NGS solid tumor testing revealing mutations from the patient's concurrent hematologic malignancy.

Case. An 82-year-old female with chronic myelomonocytic leukemia (CMML) and a remote history of tobacco smoking presented to the emergency department with dyspnea and cough for several weeks that had failed to improve with antibiotics. A chest x-ray and subsequent CT scan without contrast were performed, revealing a 2-cm right upper lobe spiculated lesion. Lung adenocarcinoma was diagnosed on fine-needle aspiration of the nodule, leading to a lobectomy resection of the patient's right upper lobe. Histologic evaluation of the resection specimen revealed a poorly differentiated pleomorphic carcinoma consisting of adenocarcinoma and giant cell carcinoma with visceral pleura involvement and lymphatic invasion (**Fig. 1**).

Targeted next-generation sequencing was performed following microdissection of formalin-fixed, paraffin-embedded tissue from the FNA cell block and resection specimens using the ion semiconductor-based sequencing platform Ion Torrent Personal Genome Machine (Thermo Fisher Scientific) with the Ion AmpliSeq Cancer Hotspot Panel v2 (Thermo Fisher Scientific). The panel concurrently interrogates 2,800 hotspots/variants with 207 amplicons in 50 cancer-related genes. Sequence data analysis and variant calling were performed with Torrent Suite Software 5.0 (Thermo Fisher Scientific).

The cytology specimen had a visually estimated tumor cellularity (or neoplastic content) of 25 percent, while the surgical pathology resection specimen had a higher tumor cellularity of 70 percent. Sequencing of the lung cancer specimens revealed multiple concurrent mutations as follows (**Table 1,** page 2):

- c.34G>T missense mutation (NM_004985, p.Gly12Cys) in the

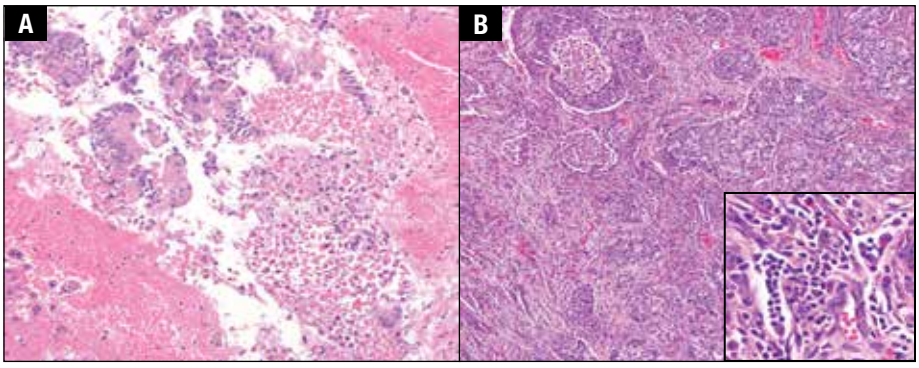

Fig. 1. A. Fine-needle aspiration cell block of the lung nodule. **B.** Low-power view of the lung nodule resection specimen. Inset shows numerous leukocytes packing intratumoral blood vessels on high-power view.

KRAS gene with 22 percent variant allele frequency (VAF) in the FNA and 38 percent in the resection;

- c.35G>A missense mutation (NM_004985, p.Gly12Asp) in the *KRAS* gene with three percent VAF in the FNA and six percent in the resection;
- c.35G>A missense mutation (NM_002524, p.Gly12Asp) in the *NRAS* gene with 15 percent VAF in the FNA and three percent in the resection; and
- c.52delA frameshift deletion (NM_000546, p.Thr18Hisfs*26) in the *TP53* gene with 23 percent VAF in the FNA and 41 percent in the resection.

Due to the finding of multiple concurrent mutations in cancer driver genes (*KRAS* and *NRAS*) on NGS testing of the lung adenocarcinoma, the patient's clinical history regarding her CMML was investigated further. The patient had a reported history of long-standing mild monocytosis since 2009 and elevated hemoglobin and hematocrit since 2013, which were being followed by a hematologist. However, the patient refused to undergo a bone marrow aspiration. In 2015, when the patient was 80 years old, a follow-up complete blood count revealed leukocytosis ($14.2 \times 10^3/\mu L$ [ref: $3.4–11.2 \times 10^3$]) with 15.4 percent monocytes (ref: 2.0–11.0 percent). The patient then agreed to a bone marrow aspiration, which revealed monocytosis without an increase in blasts or dysplasia. Cytogenetics showed a normal female karyotype. A limited, targeted, amplicon-based NGS panel for myeloproliferative neoplasms interrogating five genes was performed at Genoptix (Carlsbad, Calif.) on a peripheral blood sample, which was negative for mutations in *JAK2, MPL, CALR, CSF3R,* and *SETBP1*. Because cytogenetics and the limited MPN panel failed to reveal an acquired clonal cytogenetic or molecular genetic abnormality, a full myeloid molecular profile using amplicon-based NGS technology interrogating clinically relevant regions of 44 genes was additionally performed (Genoptix) and revealed the following (**Table 1**):

- c.2278C>T nonsense mutation (NM_015338, p.Gln760*) in the *ASXL1* gene with a VAF of 43 percent; and
- c.35G>A missense mutation (NM_002524, p.Gly12Asp) in the *NRAS* gene with a VAF of 34 percent.

The patient was then followed clinically for her CMML and did not receive any therapy or further treatment. She had an absolute monocytosis of $1.6 \times 10^3/\mu L$ (ref: $0.2–0.9 \times 10^3/\mu L$) one week prior to the FNA and $2.6 \times 10^3/\mu L$ the day of the right upper lobectomy.

We speculated that additional mutations may have been present on the peripheral blood sample for the CMML workup but not reported in the official Genoptix report. The Genoptix pathologist responsible for interpreting the patient's case was contacted to determine if variants were identified in the sample that were not reported. It was verbally confirmed that an additional mutation was present, a c.35G>A missense mutation (NM_004985, p.Gly12Asp) in the *KRAS* gene with a VAF of three percent (below their laboratory minimum quality control metric of VAF for reporting, five percent). The *TP53* gene is included on the Genoptix myeloid molecular profile panel; however, no *TP53* variants were identified in the patient's sample. Based on the findings in the patient's next-generation sequencing results from the lung adenocarcinoma, the patient was not eligible for targeted tyrosine kinase inhibitor therapy and received standard chemotherapy with carboplatin and pemetrexed.

Table 1. Summary of variants identified on NGS analysis of solid tumor and peripheral blood specimens

Specimen	Fine-needle aspiration	Lobectomy	Peripheral blood
Genetic mutations (VAF %)	*KRAS* G12C (22%)	*KRAS* G12C (38%)	—
	TP53 T18Hfs*26 (23%)	*TP53* T18Hfs*26 (41%)	—
	NRAS G12D (15%)	*NRAS* G12D (3%)	*NRAS* G12D (34%)
	KRAS G12D (3%)	*KRAS* G12D (6%)	*KRAS* G12D (3%)
	No data	No data	*ASXL1* Q760* (43%)

Discussion. *NRAS* and *KRAS* are members of the *RAS* family of oncogenes. Activating point mutations in these genes have been reported in a variety of tumors, including non-small cell lung cancer and hematological malignancies, mostly concentrating in codons 12, 13, and 61. *RAS* mutations in lung adenocarcinoma are usually mutually exclusive of other oncogenic driver aberrations including *EGFR, BRAF, ERBB2,* and *ALK* and *ROS1* rearrangements, although coexistence has been rarely reported.[1] Because *NRAS* and *KRAS* are oncogenes, single activating heterozygous mutations ("one hit") are sufficient for conferring a selective advantage, and therefore cancers with two concurrent mutations are rare. Multiple concurrent mutations in *KRAS* are reported only in approximately two percent of mutated colorectal carcinomas[2] and have also been commonly reported in pancreatic cancer.[3] Double *KRAS* mutations in lung adenocarcinoma are exceedingly rare, although they have been reported in one case.[4]

While *NRAS* mutations are rare in lung adenocarcinoma and tend to be codon 61 mutations (approximately one percent),[5] *KRAS* mutations are common in lung cancer (approximately 25–30 percent of cases).[5,6] *KRAS* variants in lung adenocarcinoma are usually in codons 12 and 13

and are less likely to be in codon 61.[5,6] *NRAS* mutations are more commonly identified in lung adenocarcinoma in current and former smokers.[7] *KRAS* mutations also define a distinct molecular subset of lung adenocarcinoma. While *KRAS* mutations are found in former and current smokers and never smokers, they are rarer in never smokers and are also less common in patients of East Asian descent.[8,9] Importantly, *KRAS* mutations have been reported as an indicator of resistance and poor survival in patients with non-small cell lung carcinoma treated with EGFR-tyrosine kinase inhibitors.[10,11] The prognostic as well as predictive role of *KRAS* mutations continues to be studied in lung adenocarcinoma. Although various attempts at inhibiting *KRAS* have been made, there is no established therapy specific for this large subpopulation of lung cancer patients.

Genetic mutations are common in CMML and are seen in greater than 90 percent of cases.[12] *RAS* mutations are highly prevalent and are seen in 20–30 percent of CMML, with *NRAS* mutations more common than *KRAS*.[13] *RAS* mutations in CMML have been associated with features of cell proliferation and monocytosis and with shorter survival, although some multivariate models have not substantiated that *RAS* mutations confer inferior outcome.[14–17] *RAS* variants in CMML are often associated with a myeloproliferative phenotype rather than a myelodysplastic phenotype.[16]

Interestingly, it is thought that the initial driver mutation in CMML is likely to be a mutation in *TET* or *ASXL1* and then subsequent secondary mutations, including *RAS* mutations, may allow clonal subsets to progress.[18] The patient in the case presented here had a nonsense mutation in *ASXL1* identified at a notably higher VAF than the *RAS* variants identified in the same specimen, which probably indicates that the *RAS*-mutated clones were subpopulations of the larger *ASXL1* CMML population. Genetic heterogeneity due to different clonal subpopulations is a well-recognized phenomenon.[19] In most cases of CMML, clonal architecture is mostly linear, but split architecture with several branches arising from the same ancestor have been observed.[19]

Despite being found on NGS testing of the lung FNA and resection specimens, the likelihood of two *KRAS* variants and an *NRAS* variant occurring as concurrent somatic alterations in the lung adenocarcinoma is exceedingly low. Most likely, the *NRAS* G12D and *KRAS* G12D are somatic mutations in the patient's CMML, while the *KRAS* G12C and *TP53* variants are truly from the lung adenocarcinoma. The *NRAS* G12D was found at a higher VAF in the peripheral blood testing (Genoptix) than the solid tumor testing, and *NRAS* mutations are more common in CMML than lung adenocarcinoma. Additionally, the *NRAS* G12D has a higher VAF in the FNA cell block than the lobectomy resection specimen. This correlates with the observance that the FNA had significantly lower adenocarcinoma tumor cellularity (25 percent versus 70 percent), with contaminating peripheral blood (along with the patient's CMML) constituting much of the non-adenocarcinoma cells. While the VAF of the *KRAS* G12D was slightly higher in the lung resection specimen than the verbally reported VAF for the same mutation in the peripheral blood, this result can be explained by proliferation of the *KRAS*-mutated CMML subclone between the time of the peripheral blood analysis and the lung cancer resection. The *NRAS* G12D and *KRAS* G12D therefore are thought to most likely represent false-positives in the solid tumor NGS testing. These findings could have been further confirmed with analysis of *ASXL1* in the solid tumor specimens; however, this gene is not included in the 50-gene panel used in this case.

In this case, the multiple *RAS* mutations were a sign to further investigate the patient's clinical history, but this finding may not always be present. In cases in which an expected variant is identified in a particular tumor type—for example, a single *KRAS* mutation in a lung adenocarcinoma—a false-positive result would not be as obvious. False-positives may have important clinical significance; therefore, inconsistent or unlikely results on NGS testing always warrant investigation of the patient's history and prior molecular results. Ordering clinicians and pathologists can mitigate the possibility of false-positives owing to secondary malignancies on NGS testing by alerting molecular pathology laboratories to patients' hematologic diagnoses and prior molecular testing, when applicable. □

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Test yourself

Here are three questions taken from the case report. Answers are online now at www.amp.org/casereports *and will be published next month in* CAP TODAY.

1. *KRAS* mutations in lung adenocarcinoma are most likely to be found in which codon(s)?
a. 12 and 13
b. 13 and 61
c. 12 and 61
d. 61

2. *KRAS* mutations are usually mutually exclusive of which other mutations?
a. *BRAF*
b. *EGFR*
c. *ALK*
d. All of the above

3. *RAS* mutations are found in approximately what percentage of CMML?
a. 50–60 percent
b. 20–30 percent
c. 5–10 percent