Genomics

ROS1 and ALK Fusions in Colorectal Cancer, with Evidence of Intratumoral Heterogeneity for Molecular Drivers

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Abstract

Activated anaplastic lymphoma kinase (ALK) and ROS1 tyrosine kinases, through gene fusions, have been found in lung adenocarcinomas and are highly sensitive to selective kinase inhibitors. This study aimed at identifying the presence of these rearrangements in human colorectal adenocarcinoma specimens using a 4-target, 4-color break-apart FISH assay to simultaneously determine the genomic status of ALK and ROS1. Among the clinical colorectal cancer specimens analyzed, rearrangement-positive cases for both ALK and ROS1 were observed. The fusion partner for ALK was identified as EML4 and the fusion partner for one of the ROS1-positive cases was SLC34A2. The partner for the other ROS1-positive case remains to be identified. A small fraction of specimens presented duplicated or clustered copies of native ALK and ROS1. In addition, rearrangements were detected in samples that also harbored KRAS and BRAF mutations in two of the three cases. Interestingly, the ALK-positive specimen displayed marked intratumoral heterogeneity and rearrangement was also identified in regions of high-grade dysplasia. Despite the additional oncogenic events and tumor heterogeneity observed, elucidation of the first cases of ROS1 rearrangements and confirmation of ALK rearrangements support further evaluation of these genomic fusions as potential therapeutic targets in colorectal cancer.

Implications: ROS1 and ALK fusions occur in colorectal cancer and may have substantial impact in therapy selection. Mol Cancer Res; 12(1); 111–8. ©2013 AACR.

Introduction

Activation of protooncogenes by genomic rearrangements resulting in the fusion of two unrelated genes was identified in leukemias and lymphomas decades ago, and is an extensively explored mechanism of tumorigenesis as well as a basis for the classification of hematopoietic neoplasms (1, 2). More recently, similar phenomena have been identified in a variety of solid tumors. Among these, rearrangement of the anaplastic lymphoma kinase (ALK) gene, originally identified in association with anaplastic large cell lymphoma (3), has been implicated in lung adenocarcinoma. Activation of ALK through gene fusions in lung cancer has been reported in approximately 5% of unselected lung adenocarcinomas, with increasing incidence when some clinicopathologic selection criteria are applied (4–17). The importance of this molecular diagnosis is that it predicts benefit from targeted kinase inhibitors. Patients with advanced ALK+ lung cancers, when treated with ALK inhibitors (e.g., crizotinib), have shown dramatic clinical response (18). The v-ros avian UR2 sarcoma virus oncogene homolog (ROS1) encodes a tyrosine kinase that shares significant homology with ALK and is activated by fusion events in 1.2% to 2.6% of lung cancer. Crizotinib is also clinically effective in patients with lung cancer harboring these ROS1 rearrangements (19–21). ROS1 gene fusions have also been found in many other tumor types beyond lung cancer (22).

Colorectal cancer is a major cause of cancer-related deaths worldwide. However, because existing therapies can be toxic, more specific therapeutic regimens such as targeted agents have been sought to improve the outcomes and quality of life of patients with colorectal cancer. Efforts to identify alterations that could predict benefit from a targeted therapy approach in colorectal cancer have proved difficult. Although KRAS mutation analysis is an accepted molecular approach in colorectal cancer, unlike the demonstration of EGFR mutation or ALK rearrangement in non–small cell...
lung cancer (NSCLC), which are used to select patients for targeted therapies. KRAS mutational status is instead used to exclude patients unlikely to benefit from monoclonal anti-EGFR therapy. Descriptions of fusion events such as ALK fusions in colorectal cancer have been rare as summarized in Table 1. In studies using reverse transcriptase PCR (RT-PCR) for EML4–ALK fusions, no ALK rearrangements were found among 48 cases (8) and 96 cases (23) of colorectal cancer tested. ALK rearrangements were also not found by FISH in 12 colorectal neuroendocrine carcinoma cases (24), but ALK gene copy gain or amplification were found in 26 of 756 colorectal carcinoma cases (25). On the other hand, EML4–ALK gene fusions were detected in 2 of 83 (2.4%) colorectal cancer specimens through exon array profiling (9), the PRKAR1A–ALK fusion was found in colorectal cancer by full exome sequencing (26), and the C2orf44–ALK fusion was found in 1 of 40 (2.5%) colorectal cancer specimens tested by next generation sequencing (27). In this case, the in-frame fusion C2orf44–ALK resulted from a 5 megabase tandem duplication. The authors reported an approximately 90-fold increase in 3’ALK expression, suggesting that the C2orf44–ALK fusion transcript resulted in ALK kinase overexpression. On the basis of the presence of ALK rearrangements in colorectal cancer, and due to the extensive homology between ALK and ROS1, we hypothesized that ROS1 genes may also be activated by gene fusions in colorectal cancer. Although there has been no report of ROS1 activation in colorectal cancer to date, Lee and colleagues (28) recently reported 23 of 49% gastric adenocarcinoma cases (4.6%) with a high level of ROS1 expression by immunohistochemistry. Of these 23 cases, 3 were positive for gene rearrangement by FISH break-apart, 2 of which were found to present the SLC34A2–ROS1 (S4-R32) fusion by RT-PCR (28). In addition, in 2011, Gu and colleagues (29) reported 2 of 23 cases of cholangiocarcinoma that were positive for the GOPC (FIG)–ROS1 gene fusion using phosphotyrosine signaling profiling (mass spectrometry) followed by S’RACE.

Overall, these findings suggest that an unrecognized subset of colorectal cancer may harbor genetic alterations predicting response to crizotinib and other targeted therapies. We herein analyzed the frequency of predicting response to crizotinib and other targeted therapies in a subset of colorectal cancer may harbor genetic alterations predicting response to crizotinib and other targeted therapies. We herein analyzed the frequency of predicting response to crizotinib and other targeted therapies in a subset of colorectal cancer specimens from patients enrolled in the Australian Gastrointestinal Trials Group Randomized Phase III MAX

Materials and Methods

Patients and tissue microarrays

The tissue microarray (TMA) was prepared using formalin-fixed paraffin-embedded (FFPE) colorectal cancer tissue specimens from 268 patients enrolled in the Australian Gastrointestinal Trials Group Randomized Phase III MAX

Table 1. Summary of published reports evaluating ALK and ROS1 rearrangements in tumors of the gastrointestinal system

<table>
<thead>
<tr>
<th>Gene</th>
<th>Reference</th>
<th>Tumor type</th>
<th>Technology</th>
<th># Tested</th>
<th># Positive</th>
<th>Fusion partner</th>
<th>Fusion partner a</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALK</td>
<td>Fukuyoshi et al. (23)</td>
<td>Colon cancer</td>
<td>RT-PCR for EML4</td>
<td>48</td>
<td>0</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Takeuchi et al. (8)</td>
<td>Colon cancer</td>
<td>RT-PCR for EML4</td>
<td>48</td>
<td>0</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Lin et al. (9)</td>
<td>Colorectal cancer</td>
<td>Exon-array profiling</td>
<td>63</td>
<td>2</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Karkouche et al. (24)</td>
<td>Colorectal cancer</td>
<td>FISH</td>
<td>12</td>
<td>0</td>
<td>C2orf44 E20 and E21</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Lipson et al. (27)</td>
<td>Colorectal cancer</td>
<td>Next-generation sequencing</td>
<td>40</td>
<td>1</td>
<td>ROS1</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Bavi et al. (25)</td>
<td>Colorectal cancer</td>
<td>FISH</td>
<td>756</td>
<td>0</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Eddy et al. (26)</td>
<td>Full exome sequencing</td>
<td>NAb</td>
<td>NAb</td>
<td>NAb</td>
<td>NAb</td>
<td>NAb</td>
</tr>
<tr>
<td></td>
<td>Lee et al. (28)</td>
<td>Gastric adenocarcinoma</td>
<td>FISH then RT-PCR for EML4</td>
<td>495</td>
<td>3</td>
<td>GOPC (FIG)</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>Gu et al. (29)</td>
<td>Cholangiocarcinoma</td>
<td>Phosphotyrosine signaling profiling (mass spectrometry) followed by S’RACE</td>
<td>3</td>
<td>2</td>
<td>GOPC (FIG)</td>
<td>23</td>
</tr>
</tbody>
</table>

Abbreviation: NA, not applicable. E, exon of the partner gene fused to ALK or ROS1. aNA, data not available.
(Mitomycin, Avastin, Xeloda) Study (30), including all patients with adequate tissue available. These patients are representative (clinicopathologic characteristics) of the MAX phase III clinical trial population, as previously reported (31). All patients had histologically confirmed colorectal adenocarcinoma. Three tumor tissue cores per patient were distributed in 10 blocks, making up a 12 \times 8 \text{ grid of cores on each slide. Thirty-nine of the patients were duplicated, and 3 were triplicated in the TMA for quality control. Institutional Review Board–approved informed consent was obtained by the MAX trial investigators for biomarker evaluation. Additional slides from the original pathology blocks of the positive samples were also made available for PCR and investigation of intratumoral heterogeneity.

**FISH assays and analyses**

The TMA slides were subjected to a FISH assay using a novel 4-color, 4-target ALK/ROS1 break-apart probe (Abbott Molecular) developed to determine genomic status of ALK and ROS1 in the same cells. ALK gene sequences were labeled in SpectrumRed (5′/ALK) and SpectrumGreen (5′/ALK); fused 5′/5′ ALK signals were classified as normal, whereas split 5′–3′ ALK by >2 diameters of the signal, and single 5′ALK were classified as abnormal (32). Of note, 3′ALK doublets–single green (red-green-red or red-red-green) was expected as the FISH pattern for the C2orf44–ALK fusion (27), thus this pattern was considered positive atypical. ROS1 gene sequences were labeled in SpectrumAqua (5′/ROS1) and SpectrumGold (5′/ROS1); fused 5′–3′ ROS1 signals were classified as normal, whereas split 5′–3′ ROS1 signals by >1 signal diameter, and single 5′ROS1 or single 3′ROS1 were classified as abnormal. For each patient, at least 50 tumor cells in two cores were scored. Variant patterns, such as the appearance of doublets or pairs, were annotated. A signal doublet was defined as the presence of two copies of the signal for a given target placed adjacently, that is, separated by \( \leq \)1 diameter of the average signal diameter; paired signals were defined as two fusion signals placed adjacently but separated by 1 to 2 diameters of the average signal. Doublet and paired signals were observed in hybridizations with both ALK and ROS1 probes and, when present in >10% of cells, the specimen was classified as atypical. Some specimens displayed both fusion signal doublets and pairs, in which case they were included in the doublet category.

Using the same ALK/ROS1 probe described above, additional FFPE slides of the resection blocks for the ALK+ patient were investigated for intratumoral heterogeneity. The FISH assays were performed using the Zymed SpotLight Tissue Pretreatment Kit (Invitrogen) in the TMAs and the Vysis Paraffin Pretreatment IV and Post-Hybridization Wash Buffer Kit (Abbott Molecular) in the sections, per the manufacturer’s instructions. Analysis was performed using interference filters set for blue (4′,6-diamidino-2-phenylindole), green fluorescein isothiocyanate (FITC), red (Texas Red), turquoise (aqua), and yellow (gold). Monochromatic images were acquired for each interference filter and merged using the CytoVision application (Leica Microsystems).

**RT-PCR**

To identify the fusion partner for ALK and ROS1, RT-PCR was carried out as described using the SuperScript III First-Strand Synthesis System (Invitrogen) with previously published ALK and ROS1 primers; the ALK primer was located in exon 20 (ALK Rev20; ref. 13), whereas the ROS1 primer was located in exon 34 (ROS1 E34R; ref. 33). First-strand synthesis was carried out as above followed by a 20-minute RNaseH digestion at 37°C. Individual PCR reactions were carried out to amplify either EML4–ALK or C2orf44–ALK, using previously published primers for exon 6, exon 13, and exon 18 of EML4 and exon 20 of ALK (ALK Sanders R20 (ref. 13) and an in-house primer for C2orf44 (C2orf441)). Likewise, individual PCR reactions were carried out to amplify either SLC34A2–ROSI1, CD74–ROSI1, or SDC4–ROSI1, or SDC4–E34F, and ROS1:E34F; ref. 20) along with a primer to SDC4 of our design (SDC4–E2F; ref. 33). PCR conditions for detecting the ALK and ROS1 fusion partners included an initial denaturation at 95°C for 5 minutes followed by 10 cycles of touchdown PCR and 30 cycles of PCR. PCR products were resolved on a 2% agarose gel. Positive PCR products were excised from agarose gel, purified (Wizard SV Gel and PCR Clean Up Kit; Promega), and sequenced. All primer sequences are listed in Supplementary Table S1.

**Microdissection and mutation analysis**

**KRAS** and **BRAF** mutational analysis was performed initially on colorectal cancer specimens used in the TMA using high-resolution melting point (HRM) PCR as previously reported (34). Subsequently for specimens in which ALK or ROS1 rearrangements were identified with KRAS or BRAF mutations, tumor areas were identified and areas for differential microdissection were mapped on the basis of the parallel hematoxylin and eosin (H&E)-stained slide. Of note, 4 μm sections were deparaffinized, hematoxylin counterstained and microdissected by scalpel point under microdissecting microscope. Microdissected material was washed with 70% ethanol, air dried, and resuspended in lysis buffer and DNA extracted (Qiagen QIAamp DSP DNA FFPE Tissue Kit; #60404) using manual extraction with elution into 30 μL of elution buffer.

For first round mutational analysis, DNA samples from selectively microdissected areas were PCR amplified with primers flanking KRAS exon 2 as previously described (35), followed by Sanger DNA sequencing. Positive samples for mutation by Sanger sequencing were not further evaluated, negative samples were evaluated by HRM curve to achieve higher analytical sensitivity. Briefly, DNA samples from selectively microdissected areas were PCR amplified with primers flanking KRAS exon 2 on the Roche LightCycler 480 using the Roche LC480 High Resolution Melting Master Kit (#04909631001). Resulting real-time PCR curves were evaluated for perturbations in the melting curve profiles with appropriate controls. The HRM assay was estimated to have an analytical sensitivity of approximately 5% based on dilution studies.
Results

Demonstration of ALK and ROS1 fusions in colorectal cancer

Of the 268 patient specimens originally included in the TMA, 236 had evaluable FISH results defined as at least 50 tumor cells in two cores. The cutoff threshold for positivity was identified as ≥15% of cells displaying patterns compatible with rearrangement, based on the distribution of relevant patterns in the cohort with application of evaluation of mean + 3 × SD of signal and beta inverse function (data not shown). Two cases (0.8%) demonstrated FISH patterns consistent with ROS1 rearrangement, predominantly single 3' ROS1 signals (Fig. 1A and B). One case (0.4%) demonstrated a pattern consistent with ALK rearrangement, and it also had predominantly single 3' ALK signals (Fig. 1C). The atypical pattern 3' ALK doublets (3'5'/3' ALK) associated with the C2orf44–ALK fusion were identified in 7 cases (3%), subjected later to RT-PCR testing. Other signal variants were identified for both ALK and ROS1, including 25 cases with 3'/5' fusion ALK doublets (10.6%), and 12 cases each (5.1%) with 3' ROS1 doublets (3'/5'/3' ROS1) and 3'/5' fusion doublets for ROS1.

In patient #406 (ALK-positive), the primary tumor site was the rectum with metastases of lymph nodes and lung. In patient #38 (ROS1-positive), the primary tumor site was the ascending colon with metastases in lymph nodes and liver. In patient #100 (ROS1-positive), the primary tumor site was the rectum and the sigmoid colon with metastasis in the lung. These patients were, respectively, 84 (female), 78 (male), and 69 (female) years old at the time at which their metastatic disease was diagnosed.

Original pathology blocks from the identified cases were evaluated by RT-PCR to further verify the presence of fusion events. RT-PCR spanning previously published ROS1 breakpoints paired with specific primers for known fusion partners of ROS1 was employed. Of the 2 cases demonstrating FISH patterns consistent with ROS1 fusion events, one was confirmed by RT-PCR to harbor an SLC34A2–ROS1 fusion (exons 4 and 34, respectively; Fig. 1D), whereas the second case was negative for all known fusion partners of ROS1. Similarly, RT-PCR assays spanning previously published ALK breakpoints paired with specific primers for known fusion partners of ALK were employed. The case identified as consistent with ALK rearrangement demonstrated the presence of an EML4–ALK rearrangement (exons 6 and 20, respectively; Fig. 1E). The 7 cases identified as atypical with 3' ALK doublets (3'/5'/3' ALK fusion) were all negative for known fusion partners of ALK, including EML4.

Figure 1. A and B, FISH images showing ROS1 rearrangement in two specimens as demonstrated by single 3' ROS1 (aqua) signals; C, FISH image demonstrating ALK rearrangement based on single 3' ALK (red) signals; D, sequencing of RT-PCR product from the sample depicted in A confirming ROS1 fusion with SLC34A2; E, sequencing of RT-PCR product from the sample depicted in C confirming EML4–ALK fusion.
Specimens with other variants were also tested, when available, by RT-PCR and no fusion was detected.

Of note, the specimens with SLC34A2–ROS1 fusion and the ALK<sup>+</sup> case were previously classified as positive for BRAF [c.1799T>A (p.V600E)] and KRAS [c.35G>C (p.G12A)] mutations, respectively, during routine clinical testing.

**Identification of intratumoral heterogeneity**

In the case identified with ALK rearrangement, three tissue cores containing tumor were subjected to analysis; however, only two of the three cores demonstrated the finding of ALK rearrangement by FISH. Pathologic evaluation confirmed the presence of tumor in the core negative for ALK rearrangement, and confirmed morphology of the tumor compatible with the other two tissue cores. This finding was suggestive of intratumoral heterogeneity, which was further explored by evaluation of two tissue blocks from the source material. Multiple tissue areas from each of two tumor blocks were selected for additional FISH evaluation. Areas were marked on a parallel H&E-stained section, and each region was separately evaluated for the presence of ALK rearrangement by FISH. Analysis of one block demonstrated a marked separation between areas of tumor that were positive and negative for ALK rearrangement by FISH (Fig. 2A). Analysis of the second block demonstrated multiple areas of tumor with positive and negative patterns for ALK rearrangement in a more interposed distribution (Fig. 2B). Histologic evaluation demonstrated that some of the tissue areas identified as positive for ALK rearrangement were pathologically best classified as high-grade dysplasia (Fig. 2C and D).

Given that the specimen with heterogeneity for ALK rearrangement was classified as KRAS-positive during routine clinical analysis, we sought to determine whether the ALK status overlapped with KRAS status within subregions of the tumor. Areas of tumor that were parallel to those evaluated by FISH were separately microdissected and KRAS mutation status ascertained by Sanger sequencing and, when negative, also by HRM curve analysis. These analyses demonstrated that some areas of the tumor retained positivity for KRAS mutation (Fig. 3A), whereas other regions showed all four possible combinations of ALK/KRAS status (Fig. 3B). The finding of intratumoral heterogeneity with respect to both ALK and KRAS alterations also led to the question of whether such heterogeneity was observed in either case with ROS1 rearrangement. However, no evidence of intratumoral heterogeneity with respect to ROS1 rearrangement was identified, therefore further analysis of intratumoral heterogeneity BRAF mutation in the ROS1<sup>+</sup> case was not pursued.

**Discussion**

Previous reports of gene fusions involving ALK in colorectal cancer indicate that these events are rare, and our findings are consistent with reported studies demonstrating a low but detectable rate of ALK rearrangement in colorectal cancer. In addition, this study is the first to demonstrate a similarly low but detectable rate of ROS1 rearrangement in colorectal cancer. The demonstration of a fusion product by RT-PCR in 2 of the 3 rearrangement-positive cases confirms the FISH findings and serves to underscore the importance of further characterization of these fusion events in colorectal cancer. The absence of a detectable fusion product in the third case (ROS1-positive) is likely attributable to an unknown fusion partner. These findings have potentially significant therapeutic implications, as identification of these
rearrangements may open the possibility for targeted therapy.

Of particular note, 2 of the 3 cases positive for fusion events were found in concert with oncogene point mutation events (KRAS and BRAF). This is in contrast with the predominant findings in NSCLC, which show that concurrent "driver" mutations such as EGFR mutation and ALK rearrangements may occur but are uncommon (36–39).

This result is of particular clinical relevance, as attempts to identify colorectal cancer cases harboring these fusion events cannot benefit from an enrichment strategy in which patients with KRAS or BRAF mutations are excluded from further testing.

A surprising result in this study was the demonstration of marked intratumoral heterogeneity for both KRAS mutation and ALK rearrangement status. Moreover, the identification of all four combinations of KRAS and ALK status throughout the specimen was particularly unexpected, as was the identification of a region of high-grade dysplasia harboring both molecular alterations. Multiple studies have indicated that not only is KRAS mutation an early event in colorectal carcinogenesis, but it shows a very low discordance rate between primary tumor and corresponding metastasis (40, 41). These findings often support the notion that KRAS mutation is both homogeneously distributed and required for tumor perpetuation. However, recent studies have demonstrated that marked intratumoral heterogeneity does exist (42). Importantly, the current study was performed retrospectively, and none of the 3 patients identified with fusion events was treated with targeted therapy agents specific to those fusion products before death.

High-grade dysplasia is the precursor lesion to invasive carcinoma in the lower gastrointestinal tract, and the identification of a region of high-grade dysplasia harboring both KRAS mutation and ALK rearrangement is intriguing and creates the basis for several hypotheses explaining mechanisms by which all four combinations of KRAS and ALK status might exist through clonal evolution (Fig. 3). In each of these hypotheses, the originating cell is negative for both alterations, and a gain of one alteration is the first step. One possibility is that the gain of alteration is stepwise (Fig. 4A and B), in which either KRAS or ALK is sequentially gained in the neoplastic population. In order for this "sequential gain" hypothesis to then yield a fourth species, the population must, by definition, undergo a "loss event." Alternatively, the gains of alterations could first be in parallel, in which separate populations of cells independently gain either KRAS mutation or ALK rearrangement (Fig. 4C). This "separate gain" hypothesis would then require that a second event occur to generate a fourth species. Finally, the possibility that the technology used to interrogate KRAS mutation...
status and ALK rearrangement is not sufficiently sensitive to determine whether the alterations actually occur in the same cells is a consideration, and gives rise to a "separate clones" hypothesis (Fig. 4D). Among these possibilities, we regard the separate gain hypothesis and the sequential gain hypothesis with ALK rearrangement occurring before KRAS mutation to be the least likely of these events, largely because of the substantial volume of data demonstrating KRAS mutation commonly occurring in adenomatous lesions.

These potential hypotheses about the genesis of the observed spectrum of subspecies in this heterogeneous lesion have several putative functional considerations. One possibility is that KRAS mutation is a "driver" and ALK rearrangement observed functionally acts as a "passenger." This explanation does not sufficiently explain the finding of ALK/KRAS regions. Similarly, the ALK rearrangement may be a modulator of tumor growth, which is also difficult to reconcile with the finding of ALK/KRAS regions. Another possibility, best hypothesized in the "separate clones" explanation is that KRAS and ALK represent dual drivers with subclonal evolution. This hypothesis is best considered in the context of underlying genomic instability, which could fuel random events expressed as subclonal heterogeneity.

The findings of this study have several specific implications with regard to future analysis of colorectal carcinoma. These data strongly support further evaluation of colorectal carcinoma for fusion events in ALK and ROS1, and further suggest the possibility that these events may serve as targets for therapy in colorectal cancer. As mentioned, the overlap of these fusions with both KRAS and BRAF mutations is a potential confounding factor, as not only does it impact approaches to screening, but these alterations may also modulate responsiveness to targeted therapy agents. On the basis of our findings, it may be challenging to identify a substantial number of patients with a uniform molecular profile with regard to fusion events and mutation status. The screening process itself may be impacted by these findings, which suggest that multiple regions of tumor may need to be evaluated.

Furthermore, these findings highlight the technological hurdles involved in the evaluation of tumor heterogeneity, and underscore the importance of methodologies to evaluate mutation status on a single-cell in situ basis.

Disclosure of Potential Conflicts of Interest

D.L. Aisner has honoraria from speakers' bureau from Abbott Molecular and is a consultant/advisory board member of Boehringer Ingelheim. R.C. Doebele has received commercial research grant and is a consultant/advisory board member of Pfizer, and also has honoraria from speakers' bureau from Abbott Molecular. A.J. Weickhardt has honoraria from speakers' bureau from Pfizer. M. Varella-Garcia has received commercial research grant and honoraria from speaker's bureau from Abbott Molecular. No potential conflicts of interest were disclosed by the other authors.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): D.L. Aisner, A.T. Le, N. Tehburt, R.C. Doebele, A.J. Weickhardt, M. Varella-Garcia, T.T. Nguyen

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Study supervision: N. Tehburt, R.C. Doebele, A.J. Weickhardt, M. Varella-Garcia

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