Analysis of Circulating Tumor DNA to Confirm Somatic KRAS Mutations

KRAS mutations have clearly emerged as a pharmacogenomic marker that can predict which metastatic colorectal cancers will be resistant to treatment with antibodies that inhibit the epidermal growth factor receptor (EGFR)\(^1,2\). The evaluation of patients for mutations in KRAS is rapidly becoming part of routine practice in clinical oncology and so far has relied mostly on formalin-fixed paraffin-embedded (FFPE) tumor tissue. Accurate KRAS testing is critical because it determines which patients may benefit from anti-EGFR therapy. However, the selection of specimens with a sufficient number of tumor cells, possible genetic heterogeneity between different tumor sites (eg, between primary tumor and metastases), the quality of extracted DNA, and different detection methods for KRAS mutations can interfere with accurate analysis. In addition, formalin fixation often indiscriminately and irreversibly damages DNA, increasing sample-to-sample variability and decreasing the amount of DNA available for molecular analysis. A recent article by Tol et al.\(^3\), on the effects of KRAS mutations on first-line therapy of colorectal cancer patients with anti-EGFR therapies, highlights this issue. Eight patients had to be excluded from the study because of the discordance in the mutation status of KRAS as assessed by two independent sequencing methods, both performed on FFPE sections of tumor tissue.

As an additional example, we would like to report the case of a 58-year-old man with metastatic colorectal cancer whose tumor was being evaluated for mutations in KRAS. Inadvertently, testing was performed by two independent laboratories and revealed two different results. In both laboratories, tissue sections were reviewed by a pathologist, DNA was purified from the malignant areas of microdissected tumor specimens, a region of exon 2 from the KRAS [GenBank accession No. NM_004985.3] gene was amplified by polymerase chain reaction (PCR) and analyzed for the presence of mutations at codons 12 and 13. The first laboratory reported the presence of only wild-type KRAS by melting curve analysis. The second laboratory detected a 35G>T mutation, which causes a glycine to valine substitution at codon 12 of KRAS (G12V), using single-nucleotide primer extension analysis.

To resolve these discordant findings, we tested DNA from this patient’s plasma for KRAS mutations using a highly sensitive technique termed “BEAMing,” which was named after components of this method (Beads, Emulsification, Amplification, and Magnetics), as previously described\(^4\). This method uses standard laboratory tools and reagents to create a water-in-oil emulsion wherein each aqueous microdroplet houses an individual fragment of DNA bound to a bead. This setting allows billions of compartmentalized PCRs to be performed in parallel in a single test tube. The products of these reactions coat each bead with thousands of copies of DNA fragments that are identical to the single DNA molecule originally present. In this case, the result is millions of beads coated entirely with either KRAS mutant or KRAS wild-type DNA. To distinguish mutant from wild-type coated beads, allele-specific fluorescent probes complementary to the known wild-type or mutant sequences of KRAS are simultaneously added to the beads for hybridization. The beads are then assessed via flow cytometry to detect rare mutant DNA molecules among a much larger number of normal DNA molecules\(^5\). BEAMing is a digital assay that is able to count the frequency of mutant- or wild-type–specific probes, and quantified by flow cytometry. The histograms show results from plasma samples with and without the mutation, in which Cy5-tagged beads are coated with mutant KRAS DNA fragments (red) and Cy3-tagged beads are coated with wild-type KRAS DNA fragments (green). The Cy5-labeled probe identifies a G to T mutation at nucleotide 35 in the KRAS gene, corresponding to a glycine to valine substitution at amino acid 12 of KRAS (G12V).

Figure 1. Flow cytometry histogram from a test of circulating tumor DNA (from plasma) for the presence of a KRAS mutation. DNA was purified from 2 mL of plasma from a patient with metastatic colorectal cancer (A) and from a control subject without cancer (B) and was assessed for the KRAS 35G>T mutation by BEAMing, a technique in which individual DNAs are attached to beads which are subjected to compartmentalized polymerase chain reaction amplification, hybridized to fluorescent mutant- or wild-type–specific probes, and quantified by flow cytometry. The histograms show results from plasma samples with and without the mutation, in which Cy5-tagged beads are coated with mutant KRAS DNA fragments (red) and Cy3-tagged beads are coated with wild-type KRAS DNA fragments (green). The Cy5-labeled probe identifies a G to T mutation at nucleotide 35 in the KRAS gene, corresponding to a glycine to valine substitution at amino acid 12 of KRAS (G12V).
individual DNA fragments in a sample, but the sensitivity can be limited by sequence errors introduced by DNA polymerase during PCR. As previously defined in DNA from patients without cancer, the false-positive rate for \textit{KRAS} mutations ranges between 0.0061\% and 0.00023\% (4,6).

In this case, DNA fragments from 2 mL of plasma, instead of from paraffin-embedded sections, were screened for known mutations in codons 12 and 13 of \textit{KRAS}. A mutation (35G>T) was detected that matched the reported G12V mutation (Figure 1). The detected mutation represented 0.3\% of the DNA from the 2 mL of plasma analyzed, which was substantially higher than the background levels noted above. No other mutations in \textit{KRAS} had been detected in the patient’s sample or in the DNA from patients without cancer. Serial dilutions of tumor DNA that contained the \textit{KRAS} 35G>T mutation with normal DNA that did not contain the mutation revealed that the mutation could still be detected when it was present only 0.01\% as frequently as the wild-type allele (Supplementary Figure, available online).

This case illustrates the limited sensitivity of standard methods to detect mutations in DNA from FFPE specimens. By contrast, highly sensitive methods like BEAMing can be performed on other clinical specimens, such as plasma, precluding the possibility of sampling of nonmutation-bearing portions of the tumor. Testing of circulating tumor DNA in peripheral blood to screen for mutations resident in the parent tumor is unencumbered by many of the factors that limit testing of FFPE-derived specimens. Blood is easily accessible, not prone to selection bias, and provides a continuous source of DNA. Accordingly, tests for circulating tumor DNA are able to screen for mutations present at the time of treatment unlike tests that rely on archived tissue samples that were acquired previously (7). The fact that circulating tumor DNA is detectable in all patients with metastatic colorectal cancers makes a blood test for detecting somatic \textit{KRAS} mutations a promising approach for companion diagnostics (4).

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