CLINICAL IMPLICATIONS OF BASIC RESEARCH

Elizabeth G. Phimister, Ph.D., *Editor*

The Potency of a *KRAS* Silent Variant

Miriam Molina-Arcas, Ph.D., and Julian Downward, Ph.D.

The RAS family of closely related oncogenes (*KRAS*, *NRAS*, and *HRAS*) are the most frequently mutated drivers of cancer formation. Although the RAS genes were discovered as human oncogenes 40 years ago, the RAS proteins have proved to be challenging targets in drug discovery: the first direct inhibitor of a RAS protein, sotorasib, was only approved for clinical use in 2021. Sotorasib is a selective inhibitor that is changing clinical practice with regard to the treatment of patients whose tumors have the KRAS G12C mutation, which occurs in codon 12 and results in the substitution of a glycine with a cysteine. The KRAS G12C mutation is induced by smoking and is responsible for about 12% of lung adenocarcinomas.[1] However, this mutant form of KRAS is found in only a small fraction of RAS-mutant cancers. Progress has been made in the development of other mutant-specific KRAS inhibitors, most notably in targeting the most common form of mutant KRAS (KRAS G12D mutation) and in developing pan-inhibitors and pan-degraders of RAS oncoproteins in general. Inhibitors that specifically target mutated oncoproteins are likely to be associated with comparatively less toxicity than pan-inhibitors because they do not affect the normal functions of wild-type RAS proteins throughout the body. Regardless, there remains a pressing need for new strategies that target mutant RAS proteins in cancer.

Of interest, then, is a recent report by Kobayashi and colleagues that describes a new, mutant-specific strategy to treat patients with tumors that harbor mutations in codon 61 of RAS-family oncogenes.[2] (Codon 61 is a hotspot; oncogenic mutations occur more frequently in this codon than in most other codons in *KRAS*.) The authors did not set out to identify new therapeutic strategies to treat patients with RAS-mutant tumors. Rather, they were studying the mechanisms of resistance to osimertinib in lung cancer cells. Osimertinib, a drug that inhibits the EGF receptor tyrosine kinase (a key signal transducer of the EGF receptor), is used in the treatment of patients with non–small-cell lung cancer in which mutant, overactive EGFR is present. In order to mimic some of the acquired mutations that can drive resistance to osimertinib, the investigators introduced mutations into *KRAS* in a cell line that had mutant, constitutively active EGFR. They were surprised to find that a strongly oncogenic form of KRAS with mutant Q61K that should have caused resistance, failed to do so efficiently. However, rare cell clones of the KRAS Q61K mutation that also had a serendipitous second, silent mutation in a proximally adjacent codon (G60, encoding glycine) did have resistance to osimertinib. Silent mutations are often ignored, because they only change one or two nucleotides in the DNA sequence without changing the amino acid sequence of the encoded protein and thus are not expected to alter the protein function. But Kobayashi et al. decided to investigate why the presence of a coincident silent mutation, KRAS G60G, was required in order for the KRAS Q61K mutation to confer resistance to drug therapy.

Interrogation of cancer genome-sequence databases showed that almost all tumors with *KRAS* Q61K mutations also contained a silent mutation in *KRAS* G60G, a finding that suggests that the oncogenic effect of the KRAS Q61K mutation is dependent on the presence of the silent mutation in the adjacent codon. To identify the mechanism of this dependency, Kobayashi et al. examined the *KRAS* messenger RNA (mRNA) from the cell lines that they had generated. They

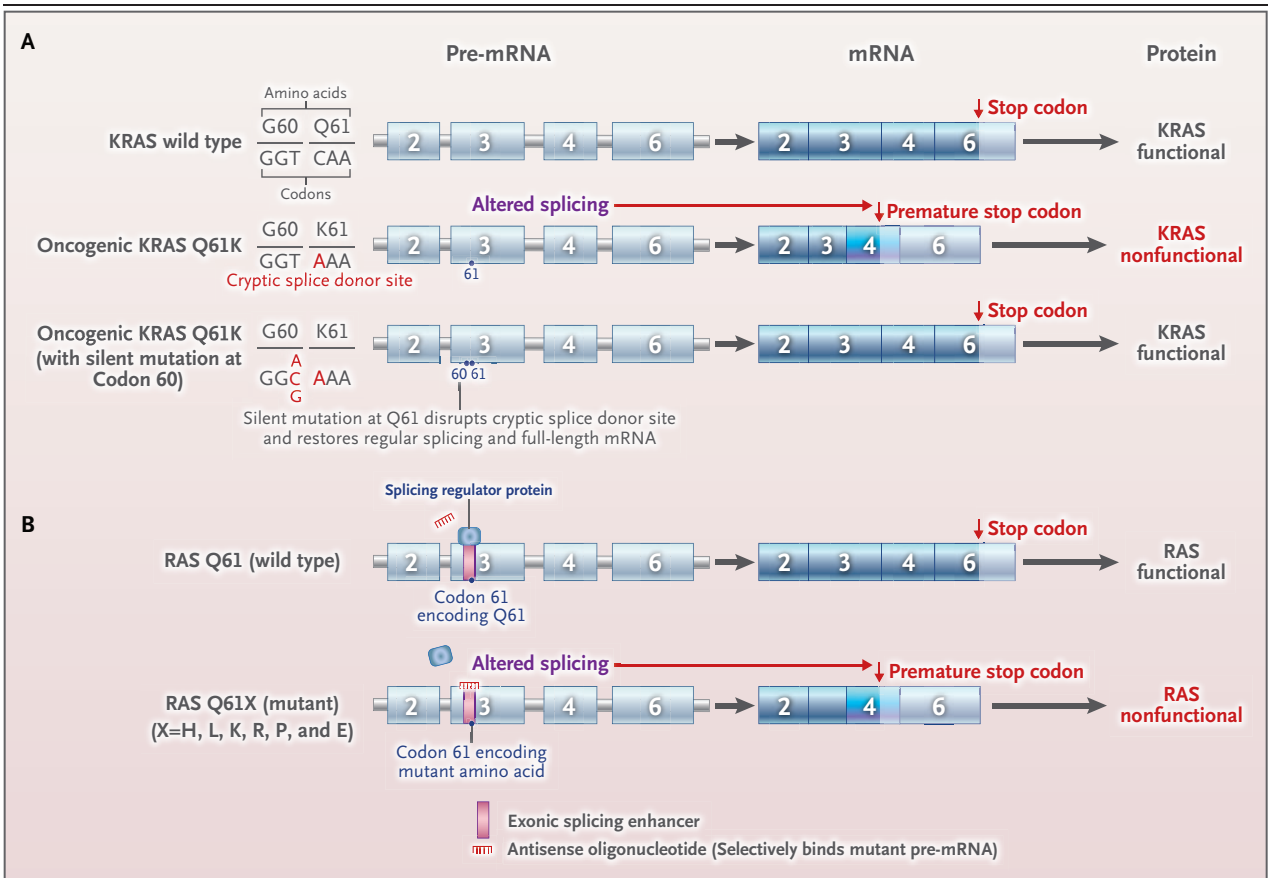

Figure 1. Mimicking Aberrant Splicing Induced by Q61K Mutation with Antisense Oligonucleotides.

Kobayashi et al. recently reported that the oncogenic effect of an activating variant (the KRAS Q61K mutation) was dependent on a second, silent variant, G60, in *KRAS*.[2] The mutation resulting in KRAS Q61K produces aberrant RNA splicing in exon 3, which results in a frameshift (in exon 4) and introduction of an early stop codon (Panel A). Co-occurrence of a silent mutation in G60 disrupts the cryptic splice donor site that was introduced by Q61K, producing a normal full-length KRAS protein. In wild-type RAS, serine- and arginine-rich (SR) splicing regulator proteins bind to the exonic splicing enhancer (ESE) motif in exon 3, which enhances exon inclusion (Panel B). Mutant-selective antisense oligonucleotides that bind the ESE motif compete with SR proteins to cause aberrant splicing of exon 3 and nonfunctional RAS protein.

found that the mutation that resulted in KRAS Q61K also caused a change in the generation (splicing) of *KRAS* mRNA, such that the mature mRNA — and thus the protein — was truncated and nonfunctional. In other words, the mutation both created an oncogenic version of the protein and resulted in an unstable mRNA (and thus no — or greatly reduced levels of — mutant protein). However, the aberrant RNA splicing was prevented when the silent mutation in codon 60 was introduced, which resulted in the production of a full-length functional KRAS protein with both G60G and Q61K mutants (Fig. 1A).

Building on this knowledge, Kobayashi et al. proceeded to explore a therapeutic strategy that might be applicable to all tumors that have mutations in codon 61 of any RAS gene. The third exon (a block of DNA that encodes amino acids) of *KRAS*, *NRAS*, and *HRAS* is enriched in DNA motifs that bind specific proteins (splicing regulators) that promote the inclusion of exon 3 in the mRNA. The blocking of these motifs could lead to the disruption of normal splicing, the exclusion of exon 3, and the generation of a truncated, nonfunctional RAS protein. To test this possibility, the authors designed antisense oligonucleotides that block the motifs of the RAS Q61 mutant mRNA but do not block those

of the wild-type mRNA. The growth of cell lines that were dependent on RAS oncogene expression was inhibited when the cell lines were treated with the antisense oligonucleotides, thus validating the strategy. Similarly, the growth of KRAS Q61 mutant cancer cell lines as xenografts in mice was inhibited after intratumoral injection of one of these oligonucleotides (Fig. 1B).

The potential to target the regulation of splicing in exon 3 of RAS oncogenes as a therapeutic strategy in cancer depends on several factors. First, how frequent are exon 3 mutations in RAS oncogenes? Although *KRAS* Q61K mutations account for less than 0.5% of all mutations in *KRAS*, other Q61 mutations, such as mutations to histidine or arginine, are more common, although still less than 5% of the total.[3] In *HRAS*, Q61 mutations are more common (about 37% of the total), but the overall percentage of *HRAS* mutation in cancer is low (1.3%). *NRAS* is probably the most attractive target for regulation of splicing, because 63% of mutations in *NRAS* occur at codon 61, and these mutations are particularly common in melanoma and in myeloid cancers.

Second, how feasible would it be for oligonucleotides that disrupt the splicing machinery to be introduced into tumors? Effective delivery of oligonucleotide-based drugs in the clinic has been extremely challenging, and many clinical trials have failed, including a trial of a KRAS-targeting antisense oligonucleotide.[4] Effective delivery can be challenging in the lab, as well. Kobayashi et al. observed no significant change in the volume of established tumors in mice when the oligonucleotide was delivered intravenously. On the other hand, outside the context of cancer treatment, antisense oligonucleotides have been approved for the treatment of some genetic diseases, such as Duchenne's muscular dystrophy.[5] In most of these diseases, the partial modulation of target-protein function in a relatively small number of cells can produce a clinical benefit. However, previous outcomes with the use of inhibitors of the RAS pathway indicate that strong inhibition in almost all tumor cells is necessary in order for a clinical benefit to result. Although oligonucleotide drug technology is advancing rapidly, the therapeutic use of oligonucleotides for the suppression of oncogenic KRAS signaling remains theoretical.

Although clinical applications of this work are distant, the prospect of the RNA splicing machinery as a target in the treatment of RAS-mutant cancers should not be dismissed. Indeed, the development of small-molecule–specific inhibitors of splicing in exon 3 of RAS oncogenes is a candidate strategy for the growing portfolio of approaches that are being pursued to target cancers with oncogenic RAS. Finally, this study is an example of the importance of noting and having the resources to investigate unexpected findings, a point that should resonate with all scientists and funders of research.

Disclosure forms provided by the authors are available with the full text of this article at NEJM.org.

From the Francis Crick Institute, London.

1. Molina-Arcas M, Samani A, Downward J. Drugging the undruggable: advances on RAS targeting in cancer. Genes (Basel) 2021;12:899.
2. Kobayashi Y, Chhoeu C, Li J, et al. Silent mutations reveal therapeutic vulnerability in RAS Q61 cancers. Nature 2022;603:335-42.
3. Hobbs GA, Der CJ, Rossman KL. RAS isoforms and mutations in cancer at a glance. J Cell Sci 2016;129:1287-92.
4. Ross SJ, Revenko AS, Hanson LL, et al. Targeting KRAS-dependent tumors with AZD4785, a high-affinity therapeutic antisense oligonucleotide inhibitor of KRAS. Sci Transl Med 2017;9(394):eaal5253.
5. Dhuri K, Bechtold C, Quijano E, et al. Antisense oligonucleotides: an emerging area in drug discovery and development. J Clin Med 2020;9:2004.

DOI: 10.1056/NEJMcibr2202981