Liquid biopsy

By Tim Fulmer, Senior Writer

A Cancer Research UK team has developed a diagnostic platform that uses tumor-derived circulating DNA to noninvasively monitor a patient's response to therapy.¹ The researchers are now moving the technology into a hospital setting, where they will test its ability to diagnose ovarian cancer earlier than standard pelvic scans.

In cancer patients, 1%–10% of circulating or cell-free DNA (cfDNA) derives from tumor cells,² including fragments of mutated oncogenes such as *RAS*, *p53* and *phosphoinositide 3-kinase (PI3K)*.^{3–6}

Those findings have suggested it might be possible to develop a diagnostic assay that detects tumor-specific genes and gene mutations in the serum. A so-called liquid biopsy could help monitor treatment response and relapse in cases in which multiple tissue biopsies are impossible or not recommended.

The challenge has been designing assays that are sufficiently sensitive to detect low levels of rare cfDNA cancer mutants among the much higher background levels of wild-type cfDNA in circulation.⁷ Consequently, most liquid biopsy approaches have focused on detecting circulating levels of a single high-frequency mutation in a single oncogene, for example, the V600E mutation in *BRAF*.⁸ That mutation is found in 31% of primary melanoma and 57% of metastatic melanoma tumors.

The Cancer Research UK team, led by James Brenton and Nitzan Rosenfeld, hypothesized that it might be possible to harness the sensitivity of second-generation DNA sequencing to develop a liquid biopsy platform that detected both abundant and rare cancer mutations in the cfDNA of patients with cancer.

Brenton is a researcher in the Functional Genomics of Ovarian Cancer Laboratory at Cancer Research UK's **Cambridge Research Institute**. Rosenfeld is a researcher in the Molecular and Computational Diagnostics Laboratory at the Cambridge Research Institute.

The team first developed a method called tagged-amplicon deep sequencing (TAm-Seq), which allowed for the two-step amplification and deep sequencing of genomic regions spanning thousands of DNA base pairs. The group then designed a set of 48 primers to amplify genomic sequences covering the exons of several oncogenes, including *p53*, *PTEN (MMAC1; TEPI)*, *epidermal growth factor receptor (EGFR)*, *BRAF*, *K-Ras* and *PI3K*.

To validate the method, the team used TAm-Seq and the primers to amplify and sequence DNA extracted from 47 ovarian tumor samples and compared the results with data from first-generation Sanger sequencing. TAm-Seq identified 43 mutations, whereas Sanger sequencing identified 40 mutations.

TAm-Seq was next used to identify mutations in the cfDNA of patients with high-grade serous ovarian cancer (HGSOC), which is

strongly associated with *p53* mutations.⁹ In two cohorts totaling 44 patients with HGSOC, TAm-Seq identified *p53* mutations at frequencies varying from 4%–54%, and in one patient identified an additional mutation in *EGFR*. The frequencies were confirmed by digital PCR.

TAm-Seq was then applied to three potential clinical scenarios.

First, TAm-Seq monitored the frequencies of circulating *p53* mutants in two patients with relapsed HGSOC during treatment and follow-up. In those patients, the *p53*-mutant frequencies reflected the clinical course of disease, showing decreases during treatment and increases paralleling disease progression.

Next, TAm-Seq monitored 10 mutations in a single patient with metastatic breast cancer over the course of 497 days before and after treatment. All 10 mutations showed a common decline in frequency at the start of therapy and increases in frequency after termination of therapy.

Finally, TAm-Seq analyzed samples from a patient with two primary cancers—bowel and ovarian. In tissue collected at surgical resection, both ovarian cancer- and colorectal cancer-associated mutations were detected. When a pelvic mass of uncertain origin was identified five years later, only the ovarian cancer-associated mutations were detected in the plasma, showing that TAm-Seq was robust enough to identify which cancer type was responsible for the relapse.

The results were published in *Science Translational Medicine*.

More validation in more patients

The accuracy and sensitivity of TAm-Seq now need to be validated against established platforms and assessed in a wider population of patients with cancer.

“Second-generation deep sequencing methods like the one developed for the liquid biopsy need to be routinely validated against one or more independent platforms to rigorously determine their rates of false positives and false negatives,” said Charles Cantor, CSO of **Sequenom Inc.**

“In this case, an ideal independent platform might be mass spectrometry, which can be multiplexed much the same way second-generation sequencing methods are,” said Cantor. “While the authors do a fine job using digital PCR to validate their method in the paper, that may not be the best choice of validation platform moving forward since it does not multiplex well and may have difficulty validating many mutations efficiently.”

Sequenom's SEQuReDx platform detects and analyzes cell-free fetal nucleic acids in the mother's serum to prenatally diagnose chromosomal abnormalities associated with genetic disorders such as Down syndrome.¹⁰

Cantor also thinks improving the sensitivity of TAm-Seq will be necessary to ensure broad clinical applicability. “In the paper, they detect

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mutations down to about a 2% frequency. However, many clinically relevant diagnostic mutations occur orders of magnitude below that. For example, in some leukemias, a clinically actionable mutation can occur at a rate of 1 in 1,000," he said.

Even in ovarian cancer, Stanley Kaye, professor of medical oncology at Cancer Research UK, had questions about applicability. He noted that the paper mainly addressed the high-grade serous subtype and its associated *p53* mutation. "I would like to know if the method can detect clinically relevant mutations occurring in other ovarian cancers at lower frequencies than the *p53* mutation," he said.

The researchers should now apply the liquid biopsy "to low-grade serous ovarian cancer, which is associated with *BRAF/NRAS* mutations, as well as endometrioid and clear cell ovarian cancers, which are associated with mutations in the *AKT/PI3K* pathway," said Kaye.

Kaye was not an author on the paper. In 2011, Kaye and colleagues published data in *Gynecologic Oncology* showing that high levels of detectable circulating tumor cells were associated with risk for disease progression and death in patients with relapsed ovarian cancer.¹¹

However, Kaye noted that "on average there are too few detectable circulating tumor cells in ovarian cancer using the current technology to make it a promising prognostic approach. We are now studying circulating tumor cells in breast and prostate cancer, where their numbers are greater than in ovarian cancer."

Analysis of circulating tumor cells and circulating cell-free DNA "provide different indications of what is occurring in cancer patients," said David Hoon. "Circulating tumor cells measure the actual metastatic spreading by the tumor, whereas cell-free DNA cannot always be interpreted that way."

Nonetheless, combining analyses of circulating tumor cells and circulating cell-free DNA could have synergistic value in predicting disease outcome, said Hoon, who is chief of scientific intelligence and director of molecular oncology at the **John Wayne Cancer Institute**. He is developing cell-free DNA-based diagnostics for melanoma and breast, prostate and other cancers.^{8,12,13}

In 2006, Hoon and colleagues published data in *Cancer Research* that melanoma patients with detectable levels of both circulating tumor cells and methylated cell-free DNA had a poorer response to therapy than patients who had detectable levels of only one of the markers.¹⁴

Getting real

"We are now moving our liquid biopsy into a real-world hospital setting," Brenton told *SciBX*. "We initially plan to use it early in the diagnostic pathway, to diagnose low-bulk ovarian tumors prior to a pelvic scan. We

will also use the liquid biopsy to monitor treatment response, and we plan to study mutations in other genes besides *p53*."

Compared with tissue biopsy, the liquid biopsy has at least three advantages, said Brenton. "It's noninvasive, it's able to monitor multiple tumor-associated mutations through time without requiring serial tissue biopsies, and it's relatively cheap." He estimated a single liquid biopsy would cost around £20–£30 (\$31–\$47) compared with the £800–£1,000 (\$1,248–\$1,560) typically charged for a single tissue biopsy.

Kaye does not expect the team's platform to replace conventional diagnostic biopsies. "However, the liquid biopsy could be very useful after the initial diagnosis, allowing the clinician to follow treatment response and disease progression without doing additional tissue biopsies," he said.

Rosenfeld said the team will need to find a balance between breadth and depth of cancer genome coverage and speed of throughput.

The findings and the platform described in the paper are not covered by patents.

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