Up close on clonal hematopoiesis in cfDNA testing

Amy Carpenter Aquino

October 2021—Clonal hematopoiesis is a significant biological phenomenon and denotes presence of mutations in bone marrow stem cells in the absence of a hematologic malignancy. Prevalence rises with aging and certain therapeutic or environmental exposures. CH has been associated with increased risk for leukemias, cardiovascular disease, and mortality.

Dr. Razavi

A study presented by Pedram Razavi, MD, PhD, a medical oncologist and physician-scientist at Memorial Sloan Kettering Cancer Center, in an AMP session last year, revealed that CH is far more prevalent in patients with cancer as well as in healthy individuals than previously thought, making it a technical pitfall in cell-free DNA testing for detection of circulating tumor DNA (Razavi P, et al. *Nat Med.* 2019;25[12]:1928–1937).

Sequencing at high depth can identify the majority of the cfDNA clonal hematopoiesis mutations but not all.

In the study (**Fig. 1**), which used matched cfDNA-WBC sequencing, CH was not found to be limited to the older patients. “Almost all the cancer patients and the healthy individuals had some level of detectable CH in the white cells and in the cell-free DNA,” regardless of age, Dr. Razavi said. The number of CH variants in an individual was found to be associated with age, but it was highly variable, he said, “with many of the young patients also having high levels of CH.”

Dr. Razavi and colleagues sought to define the technical feasibility of a high-intensity sequencing assay of cfDNA and matched white blood cell DNA covering a large genomic region in their prospective study of 124 patients with metastatic cancer (with matched tumor tissue biopsies) and 47 noncancer controls. “At the time of progression or de novo metastatic diagnosis, we collected cell-free DNA and tissue at very short time intervals without any intervening therapy,” Dr. Razavi said.

The cfDNA and white blood cells were sequenced to a minimum target depth of 60,000×. “We spent a lot of time on the joint calling, noise canceling, and noise reduction modeling,” he said, using a machine-learning–based error model. For tumor sequencing, “we used our well-validated MSK-IMPACT assay that includes tumor and normal sequencing of 410 genes.”

A comparison of raw and collapsed sequence depth across the patients with cancer and the noncancer controls showed similar depth of sequencing, he said. The depth—about 70,000× for cfDNA and WBCs—“as expected, was associated with input DNA, with the input DNA being around 12 to 75,” and 75 the maximum DNA input for both, Dr. Razavi said.

After implementing the error correction and joint cfDNA and WBC variant calling, the team categorized the cfDNA fragments into five variant groups. If they were germline, no further analysis was done. If they were biopsy matched, it meant they were found by MSK-IMPACT and reported in the clinical MSK-IMPACT report. The biopsy subthreshold category was for the mutations found in the binary alignment map files of the tumor MSK-IMPACT (≥3 reads) but that did not pass the threshold for clinical mutation calling. The WBC-matched mutations were those found in the white cells that were not germline or tumor-derived (biopsy matched or subthreshold). And variants of

unknown source were none of the above and could not be matched.

“The assay showed a high sensitivity and a low false-positive rate,” with performance of the cfDNA assay comparable to that of Droplet Digital PCR, and high reproducibility in independent biological replicates, Dr. Razavi said.

“As expected, the majority of the tumor-derived mutations were found in cell-free DNA,” he said, “and we’ve been able in most of the cases to find at least one tumor-derived mutation in the cell-free DNA. And the patterns of mutation followed what we expect for the specific cancer type.”

The surprise was that the 10 hypermutated samples accounted for 60 percent of all cfDNA mutations and 75 percent of biopsy subthreshold mutations and variants of unknown source across the entire cohort (**Fig. 2**). “In the other 114 patients,” Dr. Razavi said, “the match between the tumor and cell-free DNA was very good.”

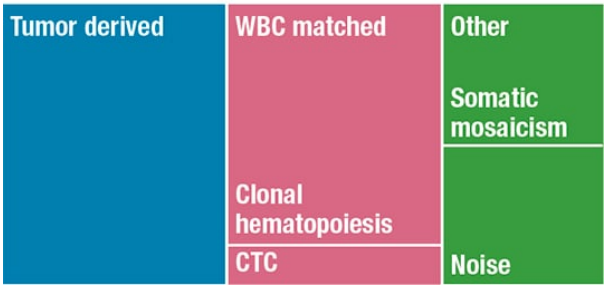
He and his colleagues analyzed the mutational signature on the 10 hypermutated samples to show the utility of the assay for such an analysis. “We found what we expected in most of the breast cases” (n = 5): an APOBEC mutational signature, known to amplify tumor heterogeneity and subclonal diversity. One of the three hypermutated prostate cancer cases displayed the same; another had a dominant MMR signature. “In lung cases, it was either smoking or aging,” he said.

Another surprise: The vast majority of somatic cfDNA mutations in controls and nonhypermutated cancer patients were WBC matched.

Fig. 1

Study aims

- Develop a high-intensity sequencing approach for plasma cfDNA (ultra-deep sequencing with broad genomic coverage) to identify tumor-derived mutations.
- Identify the major sources of somatic mutations in cfDNA of patients with cancer.



“We expected most of the somatic mutations in the healthy individuals to come from the white blood cells and originate from clonal hematopoiesis,” Dr. Razavi said, “but in cancer patients we were surprised to see more than 50 percent of the mutations we found in nonhypermutated cases were also found in the white cells.”

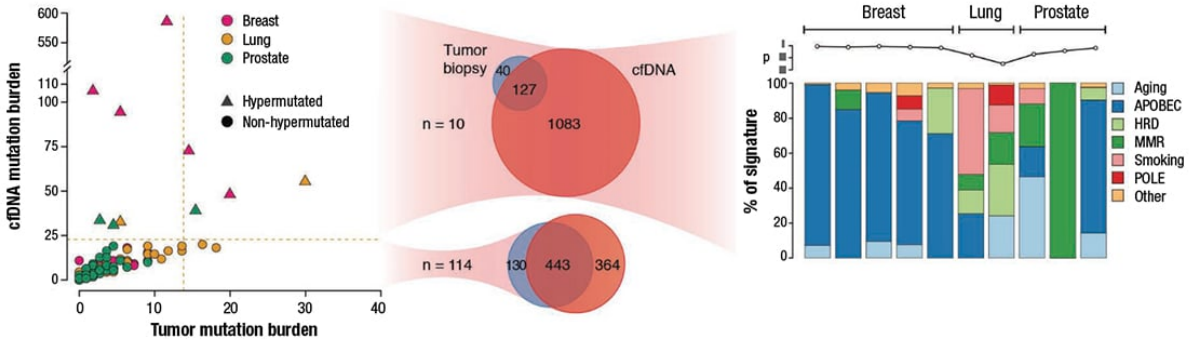
The WBC-matched mutation burden was not associated with the tumor-matched mutational burden, “telling us this is probably not a sequencing noise,” Dr. Razavi said. “That prompted us to think that this is most likely coming from clonal hematopoiesis.”

Bone marrow stem cells accumulate mutations as they replicate; some mutations are passenger mutations and don’t result in clonal expansion but nevertheless exist in the stem cell and daughter population coming from that stem cell. Some mutations result in expansion and selection of the subclone. “In cancer patients, this is even more important because we impose bottlenecks by giving patients cytotoxic therapies,” Dr. Razavi said. Some of these mutations result in resistance to therapy and expansion of these subclones further on, after each therapy cycle. “That’s why we probably see more clonal hematopoiesis in cancer patients, and some of the treatments we give probably induce hematopoiesis in the bone marrow” (Bowman RL, et al. *Cell Stem Cell.* 2018;22[2]:157–170).

The frequency of clonal hematopoiesis increases with age and starts peaking at age 60 to 65, Dr. Razavi said. Traditional assays using whole exome sequencing with a lower depth of sequencing have been able to find clonal hematopoiesis at levels of five to 10 percent in the WBCs. MSK's high-depth sequencing using the MSK-IMPACT assay showed clonal hematopoiesis can be found at the younger ages and in a larger proportion of patients, Dr. Razavi said.

Fig. 2

10 hypermutated samples accounted for 60% of all cfDNA mutations and 75% of biopsy subthreshold mutations and VUSO across the entire cohort



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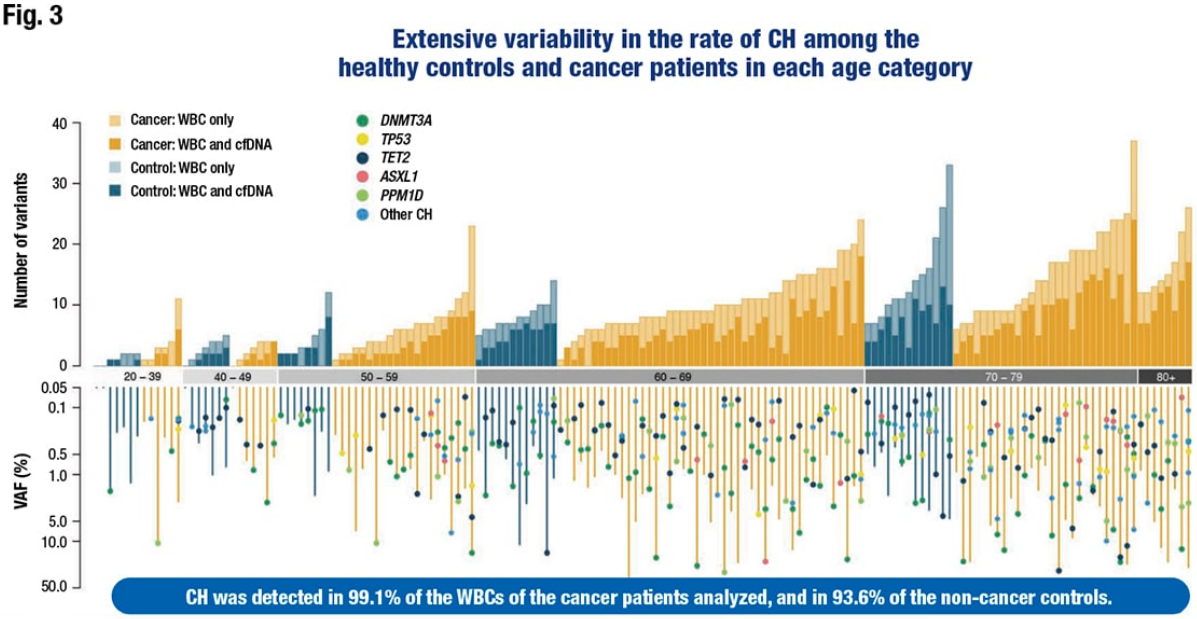
The MSK data revealed that the number of WBC-matched variants in the cfDNA was strongly associated with age, he said. This was not the case with the biopsy-matched or biopsy-subthreshold mutations. "Some of the variants of unknown source also seemed to be a weak association with age, indicating that some of the VUSOs may also be coming from clonal hematopoiesis," he said.

The mutations followed expectations. The majority of the WBC-matched mutations involved the canonical clonal hematopoiesis genes, such as *DNMT3A, TET2, PPM1D,* and *TP53*. "The list was long," Dr. Razavi said, and notably many known cancer-driver mutations and even some pathogenic actionable mutations were found to originate from CH.

An overall strong association of CH with age was found, as was an increased number of mutations by age, though there was extensive variability in the rate of CH among healthy controls and cancer patients in each age category (**Fig. 3**). High levels of CH were seen in some of the younger patients, "something that hasn't been reported to this extent before," Dr. Razavi said.

Fig. 3



Some of the younger patients in the cohort had high allele frequencies of the clonal hematopoiesis in their WBCs. Most of the mutations with the high allele frequencies, as expected, were canonical CH genes, indicating that those mutations are clones that expanded, Dr. Razavi said.

Consistent with previous reports, CH mutations both in WBCs and cfDNA were found to be strongly associated with prior treatment for cancer as some can result in resistance to treatment and may expand post-therapy (Hsu JI, et al. *Cell Stem Cell.* 2018;23[5]:700–713.e6). "We found a strong indication with truncating *PPM1D* mutations being much more prevalent in cancer patients who received chemotherapy or radiation," compared with cancer patients who received no treatment, Dr. Razavi said. Most of these *PPM1D* mutations were focused in the C-terminus of the protein, and *PPM1D* was the only gene for which all the mutations clustered in one position and one region of the genome.

"This is very much consistent with more recent work showing *PPM1D* C-terminus mutations result in stabilization of the protein," he said, "and that protein inhibits *TP53* and prevents apoptosis and results in resistance to certain chemotherapies."

Dr. Razavi said the group's findings were consistent overall with previous studies in patients who do not have cancer. In a study of somatic mutations in the cfDNA of 259 healthy individuals, the authors identified clonal hematopoiesis in 60 percent of the subjects using two panels of 508 or 599 cancer-related genes with a depth of sequencing of 6200×. White cell sequencing was done to a depth of about 400×, lower than the sequencing depth of the MSK study. "Even in healthy individuals, most of the mutations involved the known canonical clonal hematopoiesis genes," Dr. Razavi said (Liu J, et al. *Ann Oncol.* 2019;30[3]:464–470). The authors wrote, "Hematopoietic clone-derived mutations, including 'driver mutations' and 'passenger mutations,' are prevalent in the cfDNA of both healthy individuals and cancer patients and may be a potential source of false positives in the liquid biopsy."

This prompted Dr. Razavi's group to look at its data to determine the optimal WBC sequencing depth to find CH mutations in the cfDNA, he said. In looking at cfDNA variants and the variants that have been matched to the WBCs, the majority of mutations found with a variant allele frequency of less than one percent were WBC matched, "telling us this is a significant phenomenon," he said. In looking at the mutations in the WBCs that were matched to cfDNA, "we see the same phenomenon, telling us the deep white cell sequencing is required to identify many of these CH cell-free DNA mutations found in the blood." (**Fig. 4**).

In the MSK study, a small proportion of the variants of unknown source were detected in the control and cancer nonhypermutated groups. As expected, the vast majority (78 percent) of such variants were detected in the 10 hypermutated cancer samples, “telling us that most likely these are the mutations that are coming from the tumor,” Dr. Razavi said. In the control and nonhypermutated groups, the genes mutated in these variants are mainly clonal hematopoiesis mutations, “telling us there is still residual CH in the variant of unknown source.”

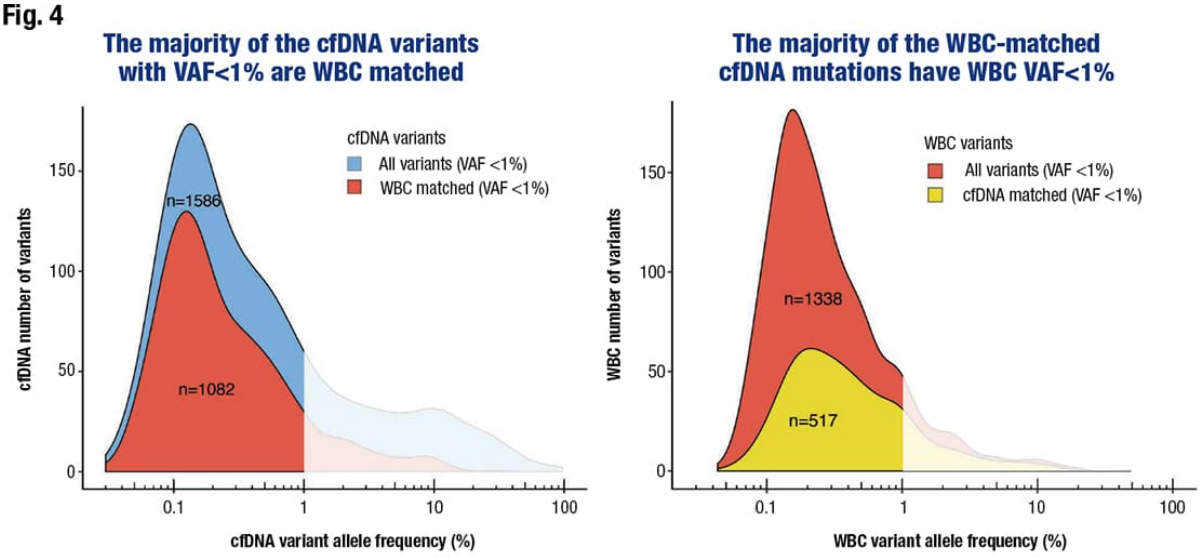
Dr. Razavi and colleagues found a strong correlation between gene size and the number of variants of unknown source in the hypermutated samples, indicating “that most of the mutations in the hypermutated cases are likely coming from subclonal mutations from different sites of the tumor.”

In looking at the distribution of mutations, they found the majority of variants of unknown source in patients with cancer mirror the mutations that are biopsy subthreshold or biopsy matched, indicating most are coming from tumor heterogeneity. “But some follow the patterns of the white-cell–matched mutations, again telling us some of the residual variants of unknown source are also coming from clonal hematopoiesis.”

Somatic clonal expansion and somatic mosaicisms are another source of mutation about which there was concern. “Recent work has shown you can find clonal expansion in the normal tissue,” Dr. Razavi said, with most mutations coming from skin, especially if exposed to the sun, or the lungs or esophagus (Yizhak K, et al. *Science*. 2019;364[6444]:eaaw0726). He and his group didn’t see this. “We expect this to be a smaller contributor to the source of cell-free DNA in cancer patients and maybe in the healthy individuals,” Dr. Razavi said. “Most of the mutations are coming from the CH.”

In the MSK study, CH variants accounted for the majority of the cfDNA variants with low variant allele frequencies, and that has implications in testing for measurable residual disease or early cancer detection, Dr. Razavi said in summing up. “When the levels of the tumor-driver mutations are very low or when we expand the panel and look at the wider part of the genome, if you don’t take into account these factors, many of the mutations that we find in the cell-free DNA may actually originate from the clonal hematopoiesis.”

Fig. 4



CH sequencing at high depth can help. “We also think that addition of in silico CH filtering based on the type of variant, fragment size, and genomic content, combined with machine-learning algorithms that tell us where the site is, can also help with filtering out some of the CH variants,” Dr. Razavi said. “But in silico approaches are not by themselves enough to filter out CH, especially when we look at the large gene panel. And white cell sequencing at high depth probably at this point is the only solution.”

High-intensity cfDNA sequencing assays, with their high genomic footprint and high sensitivity, can be used, he said, “to assess the tumor mutational burden and assign mutational signatures and also provide the better, broader landscape of the genomic alterations that can be used to assess the tumor heterogeneity and also clonal

evolution by time.”

In the view of Dr. Razavi and colleagues, fixed panels by themselves are likely to have limited utility in early-stage disease as a standalone test. And if they are used, he said, they need to be combined with other approaches to improve the sensitivity and specificity of these types of assays.

Amy Carpenter Aquino is CAP TODAY senior editor.