Fragment Size Analysis May Distinguish Clonal Hematopoiesis from Tumor-Derived Mutations in Cell-Free DNA

To the Editor:

Noninvasive detection of somatic, solid tumor-derived mutations in the blood is an important clinical and investigative tool. However, analysis of cell-free DNA (cfDNA) for somatic mutations can be confounded by the presence of mutations that are not of tumor origin. These include germline alterations, mutations from clonal events in nonneoplastic tissue, and artifacts from the sequencing process *(1)*. The most abundant set of clonal mutations is derived from the hematopoietic system and these may be mistaken for tumor mutations since similar genetic alterations may be present in both *(2)*. One strategy to determine whether mutations stem from this process, termed "clonal hematopoiesis" (CH), or from the tumor is to sequence matched white blood cells. However, cfDNA sequencing is frequently not paired with a matched blood control. Multiple studies have shown that tumor-derived cfDNA consists on average of shorter fragments than cfDNA derived from white blood cells *(3)*. We therefore hypothesized that the size profile of fragments bearing CH mutations would be more similar to the profile of normal white blood cells than to the profile of circulating tumor DNA, and that this difference may allow discrimination between the 2 types of mutation in cell-free DNA.

To test this hypothesis, we studied 44 patients with solid tumors (including prostate, bladder, breast, melanoma, and lung cancers) with CH mutations previously identified by matched tumor: normal analysis using our institutional FDA-authorized clinical test, Memorial Sloan Kettering-Integrated Mutation Profiling of Actionable Cancer Targets (MSK-IMPACT, Memorial Sloan Kettering- Integrated Mutation Profiling of Actionable Cancer Targets), then analyzed the matched plasma cfDNA collected from these patients. The protocol was approved by Memorial Sloan Kettering Cancer Center institutional review board and informed consent was obtained from all patients. Blood samples were processed to extract cfDNA *(4)*, and subjected to the MSK-IMPACT hybridization-capture protocol as described except modified to adjust the adapter concentration to 4.5 μM *(5)*. Captured DNA libraries were sequenced on a HiSeq 4000 with PE100 reads to a mean of 646× coverage per sample, demultiplexed and aligned *(5)*. CH-derived and tumor-derived nonsynonymous mutations from the tumor: normal MSK-IMPACT data were genotyped in the matched cfDNA.

In the cohort, 38 patients had 69 CH-derived mutations and 42 patients had 349 tumor-derived mutations. We detected a total of 63 CH-derived mutations (variant allele frequency (VAF)) median 3.85%, range 0.1–39.3%) and 169 tumor-derived mutations (VAF median 4%, range 0.1–80%) in the matched cfDNA. Fragments bearing either tumor-derived mutations or CH-derived mutations were extracted from aligned files, resulting in 13 353 CH mutant reads, 25 373 tumor mutant reads, and 429 769 wild-type reads, aggregated across multiple loci in each group. Fragment lengths were extracted in the range of 1–720 bp, tallied, and counts were normalized into proportions. We then computed the difference between fragment length proportions of tumor-derived and CH fragments to highlight regions of differential enrichment, which approximately follow the ∼160 bp periodic nucleosomal pattern. This allowed us to define 2 predominantly tumor-specific regions (127–141 bp and 272–292 bp, inclusive) and 2 CH-specific regions (173–191 bp and 346–361 bp, inclusive), consistent with the hypothesis that fragments of tumor origin are shorter compared to cfDNA from noncancer cells (Fig. 1). For each mutation, whether tumor or CH, we computed the proportion of fragments falling in the 2 tumor regions out of all fragments falling in the 4 selected regions, and we performed classification by considering all mutations with fewer than 4 supporting reads across the selected regions were removed. Classification based on this simple statistic achieved an area under the curve (AUC) of 0.74. However, performance improved when we considered mutations with at least 20 supporting reads (AUC 0.8089), because estimation of the statistic from few reads was inaccurate. Doing so reduced the number of mutations to 125 from 232 (54%); of these, 35 were CH mutations. As the threshold was increased further, performance on this dataset plateaued.

As a proof-of-concept, our data indicate that tumor-derived cfDNA presents a shorter fragment size distribution than CH-derived cfDNA. This supports a strategy to distinguish CH-derived mutations from tumor-derived mutation in cfDNA. Incorporating additional information such as patient age may further improve prediction accuracy. Larger datasets will be needed to refine the definition of the regions of interest, the statistic used for classification, and the read threshold. Finally, the predictive performance of this approach will need to be evaluated in independent datasets.

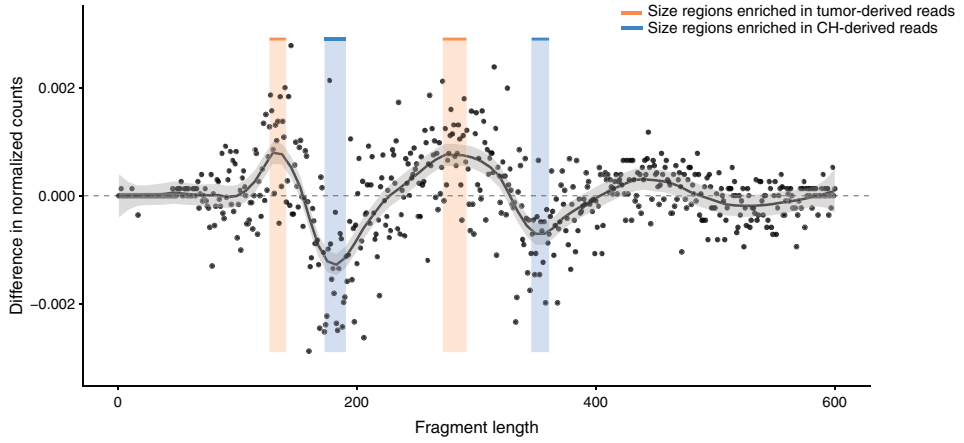

Fig. 1. Fragment size analysis of reads bearing mutations derived from tumor and CH in plasma cell-free DNA. Relative enrichment between tumor (positive values) and clonal hematopoiesis (CH) fragments (negative values), obtained by subtracting the normalized CH size profile from the normalized tumor profile. Shown in black is a LOESS fit. Colored areas denote the selected regions (orange for tumor, blue for CH).

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Prevalence of Suspected Direct Oral Anticoagulant Interference on Thrombophilia Testing at a US National Reference Laboratory

To the Editor:

The introduction of new direct oral anticoagulants (DOACs) has brought new challenges to the laboratory when assessing thrombophilia risk. These challenges include drug-associated interference with clot-based assays, which can lead to false-positive or -negative results (depending on drug concentration) *(1–5)*. From a thrombophilia testing perspective, assays for which this has become particularly problematic include clotting assays for proteins C and S, and lupus anticoagulant (LA). However, little is known about the prevalence this interference on thrombophilia testing in the United States. To estimate the prevalence of suspected interference with thrombophilia tests by DOACs, we undertook a retrospective study that examined potential interference before and after DOACs were introduced to the US market.

We conducted a retrospective review of venous thrombosis panel test results obtained from January 2008 to December 2018 at our reference coagulation laboratory. During this time, there was no change in thrombophilia testing performed at our laboratory. To identify potential DOAC interference among patients with thrombophilia, our analysis included samples with both a protein C activity test and a dilute Russell Viper Venom test (dRVVT), a LA test. This combination was based on prior investigation of samples submitted for thrombophilia testing from patients on DOACs. We could not evaluate protein S activity because our venous thrombosis panel includes free and total protein S antigen assays (used for the assessment of protein S deficiency) that are not subject to DOAC interference. Similarly, we did not evaluate antithrombin activity because our assay uses an anti-IIa method that is not significantly affected by DOACs with anti-Xa activity *(1, 3)*. DOAC interference was suspected when samples had increased protein C activity (>200%) with strong interference suspected when both protein C activity was increased (>200%) and dRVVT testing demonstrated an inhibitor pattern. The annual percentage of tests with strongly suspected DOAC interference was compared to that of samples with suspected DOAC interference from 2008 to 2018. (Fig. 1).

The proportion of samples with suspected DOAC interference began to increase annually from 2010 (0.66%), peaking in 2014 (15.12%), which corresponded to a 24.8-fold increase compared to 2009 (0.61%). These increases corresponded to the introduction of the major DOACs to the US market (dabigatran, 2010; rivaroxaban, 2011; apixaban, 2012). This suspected DOAC interference decreased from 2014 to 2018 but appeared to stabilize from 2016 to 2018 (range 7.22–8.14%).

The proportion of samples with strongly suspected DOAC interference increased beginning in 2011 (0.52 vs. 0.07% to 0.27% in 2008–2010) and peaked in 2015 (5.07%)—an approximately 18.7-fold increase from the highest pre-DOAC (before 2010) baseline percentage of 0.27%.

Educational efforts to make clinicians aware of DOAC interference and improve interpretation of abnormal testing at our laboratory included the following: (*a*) creating easily accessible, web-based documents that include frequently asked questions (FAQs), (*b*) noting the possibility of DOAC interference in interpretative comments that accompany thrombophilia test results, and (*c*) discussing such interference in requested coagulation consultations. In addition, during this time period, publication of laboratory studies from a variety of authors *(1–5)* likely helped to decrease this type of interference in thrombophilia testing. Although these efforts may have resulted in the decrease from 2015 to 2016 in the