1 *De novo* characterization of cell-free DNA fragmentation hotspots

2 boosts the power for early detection and localization of multi-

3 cancer

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26 Abstract

27 The global variation of cell-free DNA fragmentation patterns is a promising biomarker for
 28 cancer diagnosis. However, the characterization of its hotspots and aberrations in early-
 29 stage cancer at the fine-scale is still poorly understood. Here, we developed an approach to
 30 *de novo* characterize genome-wide cell-free DNA fragmentation hotspots by integrating both
 31 fragment coverage and size from whole-genome sequencing. These hotspots are highly
 32 enriched in regulatory elements, such as promoters, and hematopoietic-specific enhancers.
 33 Surprisingly, half of the high-confident hotspots are still largely protected by the nucleosome
 34 and located near repeats, named inaccessible hotspots, which suggests the unknown origin
 35 of cell-free DNA fragmentation. In early-stage cancer, we observed the increases of
 36 fragmentation level at these inaccessible hotspots from microsatellite repeats and the
 37 decreases of fragmentation level at accessible hotspots near promoter regions, mostly with
 38 the silenced biological processes from peripheral immune cells and enriched in CTCF
 39 insulators. We identified the fragmentation hotspots from 298 cancer samples across 8
 40 different cancer types (92% in stage I to III), 103 benign samples, and 247 healthy samples.
 41 The fine-scale fragmentation level at most variable hotspots showed cancer-specific
 42 fragmentation patterns across multiple cancer types and non-cancer controls. Therefore,
 43 with the fine-scale fragmentation signals alone in a machine learning model, we achieved
 44 42% to 93% sensitivity at 100% specificity in different early-stage cancer. In cancer positive
 45 cases, we further localized cancer to a small number of anatomic sites with a median of 85%
 46 accuracy. The results here highlight the significance to characterize the fine-scale cell-free
 47 DNA fragmentation hotspot as a novel molecular marker for the screening of early-stage
 48 cancer that requires both high sensitivity and ultra-high specificity.

51 Keywords

52 Cell-free DNA, Fragmentation hotspot, Whole-genome sequencing, Cancer screening,
 53 Cancer early detection, Cell free dna fraGmentation, CRAG

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60 Introduction

61 Routine screening in the average-risk population provides the opportunity to detect cancer
62 early and greatly reduce morbidity and mortality in cancer^{1,2}. Early identification of lethal
63 cancer versus non-lethal disease will minimize and better manage overdiagnosis³. Both
64 medical needs require non-invasive biomarkers with ultra-high specificity (>99%) and high
65 sensitivity at the same time, which, however, are still not available in the clinic.

66 Recent advances in circulating cell-free DNA (cfDNA) suggested a promising non-invasive
67 approach for cancer diagnosis by using the tumor-specific genetic and epigenetic alterations,
68 such as mutations, copy number variations, and DNA methylation^{4–9}. The number of tumor-
69 specific alterations identified by whole-genome sequencing (WGS), however, is small in
70 patients with early-stage cancer. Moreover, Clonal Hematopoiesis of Indeterminate Potential
71 (CHIP)-associated genetic variations are discovered in cfDNA, which are not specific to
72 cancer but a normal phenotype of aging¹⁰. Sequence degradation caused by sodium bisulfite
73 treatment for the measurement of DNA methylation will reduce the sensitivity of the test.

74 These limitations bring the challenges of using genetic and epigenetic variations for the early
75 diagnosis.

76 In contrast to the limited number of genetic alterations, the number of cfDNA fragments is
77 large in the circulation. The cfDNA fragmentation patterns, such as the fragment coverages
78 and sizes, are altered in cancer and their aberrations are not associated with CHIP^{11,12}.

79 Their derived patterns, such as nucleosome positions, patterns near transcription start sites,
80 ended position of cfDNA, and large-scale fragmentation changes at mega-base level, offer
81 extensive signals from the tumor, as well as possible alterations from immune cell deaths
82^{11,13–15}, which can significantly increase the sensitivity for cancer early detection. The *in vivo*
83 cfDNA fragmentation process during the cell death, however, is complicated and still not well
84 understood¹⁶. Moreover, sequence complexity, such as G+C% content and mappability, will
85 often cause the variations of fragment coverages and sizes^{17,18}. These challenges,
86 especially the unknown sources of fragmentation variations, will bring the potential noises for
87 cancer screening, which partially requires the ultra-high specificity.

88 To conquer these challenges, a genome-wide computational approach is needed to narrow
89 down the regions of interest with large signal-to-noise ratios across different pathological
90 conditions. Recent studies suggested that local nucleosome structure reduces the
91 fragmentation process, which also indicated the potential existence of cfDNA fragmentation
92 hotspots (lower coverage and smaller size) at the open chromatin regions^{13,19}. Nucleosome
93 positioning is still not well characterized at a variety of cell types across different pathological
94 conditions, while the variations of DNA accessibility at open chromatin regions have been
95 comprehensively profiled at many primary cell types across different physiological
96 conditions, including cancer and immune cells with nuanced activated and rest status^{20,21}.

97 Therefore, instead of identifying “fragmentation coldspots” at nucleosome protected regions,
98 we hypothesize that the characterization of fine-scale cfDNA fragmentation hotspots will not

only help us to better elucidate the unknown molecular mechanism of fragmentation patterns but also boost the power of cfDNA fragmentation for the identification of nuanced pathological conditions, such as early-stage cancer. Here, by integrating fragment coverage and size information, we developed a probabilistic based approach, named **C**ell **f**ree **d**NA **f**ra**G**mentation (CRAG), to *de novo* identify the cfDNA fragmentation hotspots genome-wide from cfDNA paired-end WGS data. We characterized the genome-wide distribution and novel unknown origin of these fragmentation hotspots. Finally, our results suggested that fragmentation signals alone from these regions can significantly boost the performance of screening and localization of early-stage cancer with high sensitivity at ultra-high specificity.

Results

CRAG: a probabilistic model to characterize the cell-free DNA fragmentation hotspots.

To integrate fragment coverage and size information, we developed an integrated fragmentation score (IFS) by weighting the coverage of each fragment based on the probability of its size in the overall distribution of the fragment size across the whole chromosome (Details in Methods). Since cfDNA in healthy individuals are mostly from hematopoietic cells, to understand the relationship between IFS and open chromatin regions, we generated the common open chromatin regions by the DNase-seq peaks shared across the major hematopoietic cell types in peripheral blood²². The distribution of IFS showed the expected depletion around the center of common open chromatin regions (Supplementary Figure 1a). We also compared the distribution with window protection score (WPS) and orientation-aware cfDNA fragmentation (OCF)^{13,23}, which were developed previously to integrate the coverage and size information but for different purposes (Supplementary Figure 1b-c). WPS around the common open chromatin regions showed a higher signal than that from the global background similar to that from nucleosome protected regions, which will bring the challenges to identify the fragmentation hotspots. To *de novo* characterize the fragmentation hotspot, we utilized a 200bp sliding window approach to scan the whole genome and built a background Poisson model for the local (+/- 5kb, +/-10kb) and global (whole chromosome) genomic regions (Details in Methods). After filtering the low mappability regions, we further calculated the significance level in each window and performed the multiple hypotheses correction. At the BH01 dataset, we identified 277,109 cfDNA fragmentation hotspots. The whole CRAG workflow is shown in Figure 1 and Supplementary Figure 2a. The IFS distribution, as well as the fragment coverage and size distribution, showed the expected depletion at the center of hotspots, which suggested that the model correctly captured the fragmentation hotspots (Supplementary Figure 2b). Since traditional double-stranded WGS cannot fully capture the small single-stranded fragments in open chromatin regions, we also plotted the distribution of fragment size from a single-stranded cfDNA WGS dataset in a healthy individual (IH01

from¹³). The patterns of fragment length around the hotspots are still similar (Supplementary Figure 2b).

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Cell-free DNA fragmentation hotspots are enriched in regulatory elements.

We next sought to understand the genomic distribution of these *de novo* fragmentation hotspots. The fragmentation hotspots covered the majority of the CpG island (CGI) promoters and CTCF motifs, but showed almost no enrichment in non-CGI promoters, 5' exon boundary, transcription terminated sites (TTS), and random genomic regions (Figure 2). DNase accessible signals from the major hematopoietic cell types in peripheral blood are enriched in hotspots as expected (Figure 3a,d, Supplementary Figure 3). We also observed the enrichment of active histone marks, such as H3K4me3 and H3K27ac, and the depletion of repressive or gene body relevant histone marks, such as H3K27me3, H3K9me3, and H3K36me3. The enhancer mark H3K4me1 from hematopoietic cell types but not other cell types showed enrichment in hotspots (Figure 3b,d, Supplementary Figure 3). To further understand the distribution of fragmentation hotspot in different chromatin states, we utilized the 15-states chromHMM segmentation result across different cell types from NIH Epigenome Roadmap Consortium (Figure 3c,d, Supplementary Figure 3)²². Similar to histone modification results, the hotspots showed depletion in non-regulatory regions but highest enrichment at active promoters (TssA and TssFlnk) and bivalent regions (TssBiv, BivFlnk, and EnhBiv) across almost all the cell types. While in cell-type-specific chromatin states, such as enhancer (Enh), the hotspots only showed the highest enrichment in hematopoietic cell types and liver that often release cfDNA to the blood in healthy individuals. The evolutionary conservation score (phastCons) in hotspots is also significantly higher than matched random regions (two-sided Mann–Whitney U test, p value < 2.2e⁻¹⁶, Supplementary Figure 4)²⁴.

To understand whether or not IFS in our model is the optimized way to integrate the fragment coverage and size information, we performed similar hotspot calling and chromatin states enrichment analysis by using WPS or OCF signals from the same cfDNA WGS dataset, IFS signals but with the Kolmogorov–Smirnov test (K-S test), and IFS signals in genomic DNA (gDNA) WGS obtained from 1000 genome project with merged similar sequencing depth (Supplementary Figure 5)²⁵. As expected, hotspots calling by WPS or OCF from cfDNA did not show enrichment in promoter regions and depletion in all the non-regulatory regions. Hotspot called by IFS from gDNA showed the enrichment in promoters across all cell types due to the coverage dropout at high G+C% content genomic regions near active promoters. However, the cell-type-specific enrichment of gDNA hotspots in enhancer states from hematopoietic cell types is not observed, which further suggested that the cell-type-specific enrichment of regulatory regions in hotspot from cfDNA WGS is not simply due to the technical bias, such as G+C% content in next-generation sequencing. We further explored the contributions of other possible technical factors, such as sequencing depth, on the performance of our hotspot calling. To quantify the performance of CRAG and

the minimum requirement of the sequencing depth, we created the balanced fragmentation-positive and fragmentation-negative regions that are randomly sampled from conserved activated chromatin states (TssA) and silenced chromatin states (Quies) (Detailed in Methods). We downsampled the effective fragments in BH01 from 1.2 billion to 10 million. The performance is saturated at ~0.9 F1-score with 400 million fragments. Even with 200 million fragments, we can still achieve reasonable performance (~0.8 F1-score) together with hematopoietic cell type specific enrichment in enhancer states (Supplementary Figure 6). Taken together, CRAG can *de novo* accurately identify the cfDNA fragmentation hotspots that are enriched in regulatory elements.

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Half of the cell-free DNA fragmentation hotspots are still protected by the local nucleosomes.

Previous studies indirectly suggested that cfDNA fragmentation hotspots are mostly co-localized with open chromatin regions from hematopoietic cell types in healthy individuals¹³. To test this hypothesis, we collected 523 public available open chromatin region datasets measured by DNase-seq or ATAC-seq across the liver and different hematopoietic cell types at rest or active status from NIH Epigenome Roadmap, ENCODE, BLUEPRINT, and other publications^{21,22,26–28}. To characterize the saturation point of the overlap with collected open chromatin regions, we subsampled the number of datasets and measured their overlaps with the fragmentation hotspots. Surprisingly, in the saturation point, half of the common fragmentation hotspots are not overlapped with existing open chromatin regions (Figure 4a, Supplementary Figure 7).

We next asked whether or not the lack of overlapping between open chromatin regions and fragmentation hotspots is due to the technical artifact in DNase-seq/ATAC-seq, such as the threshold choice of peak calling. In such a case, we should still be able to observe the residual accessibility level at the inaccessible hotspots that are not overlapped with any existing open chromatin regions. However, the normalized accessibility signals from the major hematopoietic cell types did not show any enrichment at the center of these inaccessible hotspots (Figure 4b). Even by using the GpC methyltransferase accessibility measured from NOME-seq that are not confounded by the coverage variations, the accessibility still showed significant differences between accessible and inaccessible hotspots (Supplementary Figure 8). Moreover, the neutrophil is one of the major cell types to contribute 40-60% cfDNA fragments. The direct measurement of DNA accessibility in neutrophil, however, is still not available. To mitigate this concern, we plotted the histone modification data from neutrophils around these inaccessible hotspots. The results did not suggest any potential open chromatin regions (Supplementary Figure 9). To avoid the possible fragmentation bias caused by k-mer differences, we normalized the IFS signal by k-mer composition (n=2) and still found similar fragmentation patterns before and after the correction (Supplementary Figure 10).

The distribution of cell-free DNA methylation levels from healthy individuals at the same

locations²⁹ suggested the intrinsic functional differences between accessible and inaccessible hotspots (Figure 4c). We further performed the motif enrichment analysis. The results showed that inaccessible hotspots are highly enriched with pioneer transcription factors, such as OCT (POU, Homeobox), which usually bind the nucleosome occupied regions (Figure 4d)³⁰, while accessible hotspots showed the highest enrichment in CTCF as expected.

These results suggested that the existence of the inaccessible hotspot is not due to the known technical artifact and shows the potential regulatory function. We next sought to answer the question of where these inaccessible hotspots located in the genome. Interestingly, 96% of the inaccessible hotspots are overlapped with the repeats, which is significantly higher than that of random regions with matched chromosome and length (71% overlapped, Hypergeometric test, $p < 2.2 \times 10^{-16}$) (Figure 4e). Even by using the most significant inaccessible hotspots (top 1% by p-value), the results are still largely the same (Supplementary Figure 11). We further analyzed the enrichment of these inaccessible hotspots at different types of repeat classes³¹. Highest enrichment is observed at the 5' end of microsatellite repeats (i.e., simple repeats) and low complexity regions (Figure 4e, Supplementary Figure 12). Interestingly, we also noticed the high enrichment of inaccessible hotspots right after the 3' end of transposable elements, which are not due to the mappability and G+C% bias (Supplementary Figure 13a-e). Moreover, the differences of DNA methylation at 3' end of Alu elements with or without overlapping the inaccessible hotspots indicated the potential functional association between fragmentation hotspots and transposable elements (Supplementary Figure 13f).

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241 **The variations of fine-scale fragmentation signals from the cell-free DNA** 242 **fragmentation hotspots boost the detection power for early-stage cancer.**

Further, we would like to characterize the aberrations of fine-scale fragmentation level at the cfDNA fragmentation hotspots in early-stage cancer. To obtain enough fragment number for the hotspot calling as that estimated above (Supplementary Figure 6b), we pooled the publicly available low-coverage cfDNA WGS (~1X/sample) from 90 patients with early-stage hepatocellular carcinoma (HCC, 85 of them are Barcelona Clinic Liver Cancer stage A, 5 of them are stage B) and 32 healthy individuals from the same study, respectively (Details in Methods)³². The volcano plot of the p-value (two-sample t-test) and z-score difference of IFS between HCC and healthy across all the fragmentation hotspots showed more fractions of hypo-fragmented hotspots in early-stage HCC (Figure 5a). Further, the unsupervised hierarchical clustering of the top 10,000 most variable hotspots showed a clear fragmentation dynamic between HCC and healthy (Figure 5b, Supplementary Figure 14-15). Therefore, we utilized the IFS from the cfDNA fragmentation hotspots to classify the HCC and healthy individuals by a linear Support Vector Machine (SVM) approach (Details in Methods). By 10-fold cross-validation, we observed a much higher classification performance (93% sensitivity at 100% specificity) than that by using copy number variations

(CNVs) with the same machine learning infrastructure and same data split (44% sensitivity at 100% specificity) and the previous publication by mitochondria DNA (mtDNA)³² (53% sensitivity at 100% specificity) (Figure 5c, Supplementary Table 2-3, Supplementary Figure 16).

We next asked why the IFS signal in fragmentation hotspots can boost the classification performance. Based on the z-score differences between HCC and healthy as well as the feature importance to the classification of the model, we split the hotspots that are significantly contributed to the classification into two groups: Class I (Hypo-fragmented in cancer) and Class II (Hyper-fragmented in cancer) (Figure 5d, Supplementary Table 4). The Class I hotspots are mostly accessible hotspots and significantly enriched in promoter regions, which suggested the potential silencing of genes with the decrease of fragmentation. While Class II hotspots are mostly inaccessible hotspots and overlapped with microsatellite repeats, which suggested the potential increases of fragmentation at microsatellite repeats in early-stage cancer (Figure 5e). We further checked the enrichment of motif and gene ontology biological processes at these two groups of hotspots. The results further suggested the differences of motif enrichment between two groups and the potential silencing of biological processes for the activation of peripheral immune cells that contributed most to the cfDNA composition, such as neutrophil and myeloid cells (Figure 5f-g, Supplementary Table 5).

Overdiagnosis is one of the major concerns for the screening of early-stage cancer. We next sought to explore whether or not the IFS signal from cfDNA fragmentation hotspot could also characterize the differences between early-stage HCC and non-malignant liver diseases. We identified the hotspots on the additional cfDNA WGS datasets from 67 patients with chronic HBV infection and 36 patients with HBV-associated liver cirrhosis in the same study³². PCA analysis of IFS signals across all the hotspots suggested a clear separation between early-stage HCC and non-malignant liver diseases, as well as healthy controls (Supplementary Figure 17a). To exclude the possible batch effect, we also performed PCA on IFS from matched random genomic regions in the same sample but did not observe a clear separation between groups of samples (Supplementary Figure 17b). Another possible artifact is our pooling strategy for the hotspot calling in low-coverage WGS data. The hotspot calling on the pooled group may enrich the regions with similar depletions in the genome without any biological meaningful indication. To avoid such an artifact, we randomly grouped the samples and called the hotspots from these random groups with the matched group sizes. The PCA results did not show any separations (Supplementary Figure 17c). We further selected the top 30,000 most variable peaks, performed the unsupervised hierarchical clustering, and observed the clear dynamics of the fragmentation patterns among early-stage HCC, HBV, Cirrhosis, and healthy controls (Supplementary Figure 18-19). Finally, by 10-fold cross validation, the linear SVM model showed a higher classification performance (83% sensitivity at 100% specificity) than other methods, such as CNVs and mtDNA (Supplementary Figure 20, Supplementary Table 6-7).

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299 **Screening and localization of multiple early-stage cancer types by the cell-free DNA** 300 **fragmentation hotspots.**

301 One of the biggest challenges for the screening of early-stage cancer in the average-risk
302 population is to obtain high sensitivity at ultra-high specificity (>99%) across different cancer
303 types, which is not available in clinics yet. To further validate our method in a more
304 comprehensive early-stage cancer dataset, we collected publicly available low-coverage
305 cfDNA WGS data (~1X/sample) from 208 patients across 7 different kinds of cancer (88% in
306 stage I-III, colon, breast, lung, gastric, bile duct, ovary, and pancreatic cancer) and matched
307 215 healthy controls in the same study ¹¹. We applied a similar strategy as that in the HCC
308 study above for the hotspots calling. Across 7 different types of cancer and healthy
309 conditions, the z-score of IFS signals in the most variable fragmentation hotspots showed
310 clear cancer-specific fragmentation patterns in both t-SNE visualization and unsupervised
311 hierarchical clustering (Figure 6a-b, Supplementary Figure 21, Details in Methods). The
312 fragmentation patterns alone at these hotspots can separate the cancer types very well. By
313 10-fold cross validation, the linear SVM model showed a consistent high classification
314 performance across different stages for its high sensitivity at ultra-high specificity (64%
315 sensitivity to 82% sensitivity at 100% specificity), which is significantly and consistently
316 higher in different stages than previous reported results by using large-scale fragmentation
317 pattern, copy number variations, and mtDNA from the same dataset ¹¹ (Figure 6c, Table 1).
318 For example, at 100% specificity, we achieved 93% sensitivity (95% CI: 85%-100%) in
319 gastric cancer, 88% sensitivity (95% CI: 76%-100%) in colorectal cancer and 81%
320 sensitivity (95% CI: 76%-91%) in breast cancer, which of these are poorly detected at ultra-
321 high specificity level by other liquid biopsy studies ^{5,8,9,11,33}. (Supplementary Figure 22-23,
322 Table 1, Supplementary Table 8). In the other types of cancer, the performance is largely
323 comparable to previous results ¹¹. We also tested the performance before GC bias
324 correction, the results are largely the same (Supplementary Figure 22). In addition, by the
325 same machine learning framework, the results of fragmentation hotspots are consistently
326 better (Supplementary Figure 22).

327 Another big challenge for the screening of early-stage cancer is to identify the cancer types
328 for the choices of most appropriate follow-up diagnosis and treatment. Here, we asked
329 whether or not we can identify the tissues-of-origin of cancer samples by using the fine-scale
330 fragmentation level alone. In the cancer positive samples identified above by machine
331 learning algorithm, without any clinical information about the patients, we further localized
332 the sources of cancer to one or two anatomic sites in a median of 85% of these patients
333 across five different cancer types, and 82.5% accuracy across six different cancer types.
334 Furthermore, we were able to localize the source of the positive test to a single organ in a
335 median of 65% of these patients across five different cancer types, and 56% accuracy
336 across six different cancer types, which is similar to previous reports using the combination
337 of mutation and protein ⁵ or DNA methylation ⁸, but consistently higher than that by using

large-scale fragmentation patterns from the same dataset¹¹ (Fig. 6d, Supplementary Table 9, Supplementary Figure 24) (Details in Methods). The prediction accuracy is varied with tumor type, from 70% (95%CI: 44%-96%) in ovarian cancer to 98% (95%CI: 94%-100%) in breast cancer (Fig. 6d and Supplementary Table 9), but significantly higher than random choices by the sample frequency in each cancer type (Supplementary Figure 25).

Discussions

In summary, we have developed a computational approach, named CRAG, to *de novo* identify the cfDNA fragmentation hotspots by integrating both fragment size and coverage information. The fragmentation patterns of cfDNA have long been associated with local nucleosome protection, which indicates the high overlap between fragmentation hotspots and open chromatin regions. Extreme G+C% bias and low mappability often leads to the drop out of fragment coverages and sometimes fragment sizes^{17,18}. Therefore, genomic regions with a higher fragmentation rate do not always mean open chromatin regions. After excluding these potential technical bias, our genome-wide analysis here found that half of the fragmentation hotspot regions are still protected by nucleosomes and mostly located at repeats, especially microsatellite and 3'end of potential functional transposable elements, which suggested the unknown origin of the cfDNA fragmentation process. At these inaccessible hotspots, we found the significant enrichment of pioneer transcription factor binding sites and increases of fragmentation level at the microsatellite repeats in early stage-cancer, which further indicated its functional importance for the classification of early-stage cancer. More importantly, the silencing of accessible hotspots is highly enriched at promoters and the biological processes for the activation of peripheral immune cells. A lot of recent efforts on cancer early detection, however, focused on how to enrich the circulating tumor DNA signals from tumor cells^{5,9}, which ignored the critical role of peripheral immune aberrations on cancer initiations³⁴. In addition, the CTCF motif is highly enriched at the silenced accessible hotspots, which indicated the potential three-dimensional chromatin organization changes in the initiation of early-stage cancer³⁵. Taken together, our results suggested that the fine-scale cfDNA fragmentation hotspot should be treated as a novel molecular marker rather than the representation of open chromatin regions. Previous efforts had been made to characterize the nucleosome-free regions by using the depletion of coverages from MNase-seq/ChIP-seq assay³⁶. The measurement of cfDNA fragmentation here, however, involves information from both fragment coverages and sizes. CRAG can be further improved by better integration of the fragment coverages and sizes, or even with more dimensions, such as the fragment orientation and endpoint. In addition, G+C% bias is known to affect the peak calling result in ChIP-seq/ATAC-seq³⁷. A better statistical model with the incorporation of GC normalization on both of the fragment coverage and sizes will potentially improve the performance of our method. PCR-free library preparation for WGS will also mitigate the concerns of GC bias and other sequencing

artifacts³⁸.

For the detection and localization of early-stage cancer, there are still several limitations in our study. First, due to the limited availability of public cfDNA WGS datasets from early-stage cancer patients, the classification performance here is evaluated by multi-fold cross-validation on a relatively small sample-size cohort in each cancer type, similar as other cfDNA WGS studies¹¹. Multiple independent large-scale prospective cohorts with similar cancer types will be a better way to assess the power of our approach for the diagnosis of early-stage cancer. Second, we pooled the low-coverage WGS samples from the same condition for the hotspot calling. If the number of samples is small in a condition, due to the random drop out of the fragment coverage and a large number of genomic windows in the genome, the number of false discovered hotspots without any biological interpretations will increase. Although our current strategy by filtering low mappability regions and correcting GC bias is helpful, imputation methods or more stringent statistical models are still needed to mitigate the potential missing data artifact. Third, the proportion of cancer types as well as the ratio between cancer and healthy is not an unbiased representation of the average-risk population in the US. The sensitivity and specificity here may not represent the true performance in the large-scale screening. Fourth, the proof-of-concept study on HCC here suggested the distinguished cfDNA fragmentation patterns between early-stage cancer and non-malignant liver disease controls. More cfDNA studies on non-malignant cancer, diseases and benign status are needed to minimize the overdiagnosis in the population-level screening. Lastly, in some cancer types, our fine-scale study here showed complementary classification performance compared with that in the previous large-scale fragmentation study at the same dataset¹¹. For example, our results on gastric, breast and colorectal cancer outperformed previous large-scale fragmentation studies, while at bile duct and lung cancer the result is reversed. Future combinations of the fragmentation patterns at multi-scales, as well as information from other modalities or clinical meta-data, may further improve the performance.

Our study here not only paves the road to further elucidate the molecular mechanism of cfDNA fragmentation but also lays the practical foundation of a highly sensitive and ultra-high specific blood test for multiple types of cancer on an existing matured high-throughput platform in a cost-effective way.

Methods

Data collection.

The details of public datasets used in this study are listed in Supplementary Table 1.

Preprocess of whole-genome sequencing data.

Adapter is trimmed by Trimmomatic (v0.36) in paired-end mode with following parameters:

416 ILLUMINACLIP:TruSeq3-PE.fa:2:30:10:2:keepBothReads MINLEN:36. After adapter
417 trimming, reads were aligned to the human genome (GRCh37, human_g1k_v37.fa) using
418 BWA-MEM 0.7.15³⁹ with default parameters. PCR-duplicate fragments were removed by
419 samblaster (v0.1.24)⁴⁰. Only high-quality autosomal reads (both ends uniquely mapped,
420 either ends with mapping quality score of 30 or greater, properly paired, and not a PCR
421 duplicate) were used for all of the downstream analysis.

422

423 **Preprocess of whole-genome bisulfite sequencing data.**

424 DNA methylation levels measured by WGBS in cfDNA from healthy individuals are obtained
425 from previous publications⁴¹. Single-end WGBS from cfDNA is processed by the following
426 internal pipeline. Based on FastQC result on the distribution of four nucleotides, the adapter
427 is trimmed by Trim Galore! (v0.6.0) with cutadapt (v2.1.0) and with parameters “--clip_R1 10”
428 and “--clip_R1 10 --three_prime_clip_R1 13” in different WGBS datasets according to the
429 distribution of base quality score along the sequencing cycle. After adapter trimming, reads
430 were aligned to the human genome (GRCh37, human_g1k_v37.fa) using Biscuit
431 (v0.3.10.20190402) with default parameters. PCR-duplicate reads were marked by samtools
432 (v1.9). Only high-quality reads (uniquely mapped, mapping quality score of 30 or greater,
433 and not a PCR duplicate) were used for all of the downstream analysis. Methylation level at
434 each CpG is called by Bis-SNP⁴².

435

436 **Identification of cfDNA fragmentation hotspots by CRAG.**

437 The goal of CRAG is to identify the genomic regions with lower fragment coverage and
438 smaller fragment size, i.e., low IFS value. A 200bp sliding window with 20 bp step was
439 applied to scan each chromosome (Only autosome). In the i_{th} window, all the n fragments
440 centered within the window were utilized to calculate IFS:

$$441 \quad C_i = n + \sum_{j=1}^n \frac{len_j}{S_{len}} S_{coverage} \quad (1)$$

442 len_j is the size of j_{th} fragment. S_{len} is the sum of fragment size across the whole

443 chromosome, $S_{coverage}$ is the sum of fragment number in the current chromosome. Based on
444 this equation, the sliding window with less fragments and shorter fragment size would have a
445 smaller IFS. The windows overlapped with dark regions or with average mappability scores
446 smaller than 0.9 were removed. Dark regions are defined by the merged DAC blacklist and
447 Duke Excluded from UCSC table browser. Mappability score was generated by the GEM
448 mappability program on the human reference genome (GRCh37, human_g1k_v37.fa,
449 45mer)⁴³. Poisson distribution was applied to test whether the IFS in the current sliding
450 window was significantly smaller than the local background (5kb and 10 kb) and global
451 background (the whole chromosome).

$$P(C_i) = f\left(\frac{C_i}{\lambda}\right) = e^{-\lambda} \sum_{k=0}^{C_i} \frac{\lambda^k}{k!} \quad (2)$$

452

453 Only the windows with p value smaller than a cut-off (p value $\leq 1.0e^{-05}$) in both the local
454 background and global background (the whole chromosome) were kept for further analysis.
455 Then the p value from the comparison with the global background was used for multiple
456 hypothesis correction (Benjamini and Hochberg method). Windows with false discovery rate
457 (FDR) more than 0.01 were filtered. Finally, the significant windows with distance less than
458 200bp to each other were merged as the hotspots.

459 To remove the possible bias caused by G+C% content, in each sliding window, the IFS and
460 mean G+C% content was calculated. Locally weighted smoothing linear regression (loess,
461 span = 0.75) was used to fit the GC content and IFS. The mean IFS score in each
462 chromosome was added back to the residual value after the correction. The hotspots were
463 called based on the corrected IFS in all the windows.

464 For the comparison, OCF or WPS of the cfDNA fragmentation in each window was also
465 utilized to call hotspots. OCF and WPS value could be negative, which is not suitable for the
466 poisson model. Thus, we used a two-sample Kolmogorov-Smirnov test to test whether WPS
467 (OCF) in the current sliding window was significantly lower or higher than the local
468 background and the whole chromosome.

469

470 **Cancer early detection by cfDNA fragmentation hotspots.**

471 To test whether our CRAG could capture disease-specific hotspots, we used the IFS in the
472 hotspots called in disease plasma samples (hepatic carcinoma, HBV, HBV induced Cirrhosis,
473 breast cancer, bile duct, colorectal cancer, gastric cancer, ovarian cancer and pancreatic
474 cancer) and healthy plasma samples to classify these samples. Here, we took liver cancer vs.
475 healthy samples classification as an example. Ten-fold cross validation was used in this work.
476 In each fold, at the training data set, all the liver cancer samples and healthy samples were
477 merged, respectively (for each coordinate in the whole genome, the IFS was set as the sum
478 of the IFS across all the samples at that genomic position in the same pathological condition).
479 Then, hotspots for liver cancer and healthy individuals were called separately. We kept the
480 top 30,000 most stable hotspots (ranked by the sum of variances in case and control group)
481 as the feature for the classification. In particular, when we call hotspots in the data set from
482 Cristiano et.al. paper ¹¹, since the sample size in the healthy condition is almost ten times
483 larger than single cancer type, we downsampled the healthy controls to the same size as the
484 cancer case in each classification (e.g. Breast cancer vs. Healthy). In addition, the hotspot
485 enrichment at enhancer chromatin states is better after the GC bias correction in Cristiano
486 et.al. data. Although IFS before and after GC bias correction have both been tested, IFS after
487 GC bias correction was shown in the main figure for the classification. Only genomic regions
488 at +/-100bp of the hotspot center were used to retrieve the IFS in each sample (the same
489 strategy was used in PCA and unsupervised clustering analysis). Then in each sample, the

IFS at the corresponding hotspot region was z-score transformed based on the mean and standard deviation in each chromosome at that particular sample. Finally, a support vector machine (SVM) classifier with linear kernel and default parameters (fitcsvm function at Matlab 2019b) was applied for the learning. At the testing dataset, z-score IFS score in each sample is calculated at the hotspot regions which were already identified at the training set in that particular fold. The average AUC and 95% Confidence Interval (95% CI) of the AUC was calculated based on the classification results at testing dataset across the 10 folds.

Tissues-of-origin prediction by cfDNA fragmentation hotspots.

Only samples diagnosed as cancer at 100% specificity by the method above are kept for the tissues-of-origin analysis. The saturation analysis of the fragment number needed for hotspot calling suggested that 400 million fragments are required to achieve the best performance (Supplementary Figure 6). Thus, pathological conditions with less than 400 million fragments in total after merging the samples diagnosed as cancer were not used in the tissues-of-origin analysis (e.g. lung cancer). Bile duct cancer is at the boundary condition with 380 million fragments. Therefore, we performed the analysis with/without bile duct cancer. In the 10-fold cross validation, similar as that for cancer early detection, hotspots for each cancer type in the training set were identified. z-score transformed IFS after GC bias correction in each sample was obtained as the feature. Since the sample size of breast cancer is much larger than that in the other cancer types, after the hotspot calling, we downsampled the breast cancer samples to the median sample size across all the cancer types. The centroid in each cancer type was then calculated by the z-score transformed IFS across all the hotspots in the training set. In the testing dataset, each sample was assigned to the top two candidate cancers based on their distance to the centroids in each cancer type identified at the training set. Distance was calculated by corr function with 'Type' of 'Spearman' at Matlab 2019b. To further narrow down the best candidate cancer type, several decision tree models (fitctree function at Matlab 2019b) are learned to identify the better candidate by the top 100,000 most stable hotspots in each possible pair of cancer types at the training set. Finally, between the top two candidates, we applied the corresponding decision tree model to further characterize the best candidate.

Saturation analysis of the fragment number needed for cfDNA fragmentation hotspots calling.

To benchmark the performance of CRAG on the identification of the known fragmentation hotspots, a group of fragmentation-positive regions (known hotspots) and fragmentation-negative regions were generated. For fragmentation-positive regions, we chose the CGI TSS that are overlapped with conserved TssA chromHMM states (15-state chromHMM) shared across the cell types in NIH Epigenome Roadmap. Regions that are -50bp to +150bp around these TSS were defined as the fragmentation-positive regions. For fragmentation-negative regions, we chose the same number of random genomic regions with the same size, G+C% content, mappability score from conserved Quies chromHMM states shared across the cell

530 types.

531 The high-quality fragments in BH01 were downsampled and then utilized to call hotspots by
532 CRAG. Based on their overlap with the benchmark regions, TP (true positive), FP (false
533 positive), TN (ture negative), FN (false negative) was calculated. F-score for the hotspots was
534 calculated as follow:

$$535 \quad F_{score} = 2 * \frac{Precision * Recall}{Precision + Recall} \quad (3)$$

536 in which, Precision and Recall were calculated using equation (4) and equation (5)
537 respectively:

$$538 \quad Precision = \frac{TP}{TP + FP} \quad (4)$$

$$539 \quad Recall = \frac{TP}{TP + FN} \quad (5)$$

540

541

542 **Enrichment of cfDNA fragmentation hotspots in chromHMM states and other** 543 **regulatory elements.**

544 The number of hotspots overlapped with each type of regulatory element was calculated. To
545 calculate its enrichment, after filtering out the dark regions and low mappability regions,
546 random genomic regions were generated with matched chromosomes and sizes. Fisher
547 exact test (two-tail) was performed to calculate the significance of enrichment.

548

549 **PCA analysis of cfDNA fragmentation hotspots across different diseases.**

550 The cfDNA fragmentation hotspots were called at each pathological condition similarly as
551 that in above “Cancer early detection” part. Principal Component Analysis (PCA) was
552 performed on the z-score transformed IFS across all the fragmentation hotspots (pca
553 function at Matlab 2019b).

554

555 **Unsupervised hierarchical clustering analysis of cfDNA fragmentation hotspots.**

556 The cfDNA fragmentation hotspots were called at each pathological condition similarly as
557 that in above “Cancer early detection” part. Top N most variable hotspots were kept for the
558 clustering (ranked by the variation across all the samples). Spearman's rank correlation was
559 used to evaluate the distance among the samples and Weighted average distance
560 (WPGMA, with 'weighted' as parameter in clustergram function at Matlab 2019b) was
561 applied together with linkage method. In samples from Cristiano et.al. paper¹¹, the similar
562 unsupervised hierarchical clustering strategy is applied. The number of healthy samples
563 here was downsampled to the median sample size (n=27) as the other cancer types. In
564 addition, one-way ANOVA (p value <= 0.01). was used to select the hotspots that showed
565 group specific fragmentation patterns. Top 5,000 hotspots in each group were finally used

(ranked by the z-score difference between the samples within the group and outside the group).

T-SNE visualization on multi-cancer and healthy.

t-SNE (tsne function at Matlab 2019b) was utilized for the dimensionality reduction and visualization of the fragmentation dynamics in the hotspots across multiple cancer and healthy conditions. Hotspots with a p-value ≤ 0.01 (one-way ANOVA) were used for the analysis. Distance similarity was calculated by Spearman correlation together with other default parameters (tsne function at Matlab 2019b).

Gene Ontology analysis of cfDNA fragmentation hotspots

Gene Ontology analysis of the cfDNA fragmentation hotspots was performed by GREAT (v4.04)⁴⁴. In this work, the GO Biological Process with FDR q-value of the binomial test ≤ 0.01 were selected. If more than 10 GO Biological Processes were significant, top ten of them were shown in the main figure.

Motif analysis of cfDNA fragmentation hotspots

Motif analysis of cfDNA fragmentation hotspots was performed using HOMER (v4.11) using command 'findMotifsGenome.pl hotspots_file hg19 output_file -size given'⁴⁵. Only the motifs with FDR q-value less than 0.01 were kept. When there were more than 10 motifs enriched, the top 10 of all the enriched motifs were shown in the figures.

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Availability of data and materials

CRAG is implemented in matlab. The source code is freely available on GitHub (<https://github.com/epifluidlab/CRAG.git>) under the MIT license for academic researchers.

603 The source code, readme files, hotspots location, intermediate analysis scripts, and
604 intermediate results are available at Zenodo.org (DOI: 10.5281/zenodo.3928546).

605

606 Authors' contributions

607 YL conceived the study. YL and XZ designed the methodological framework. XZ
608 implemented the methods. XZ and YL performed the data analysis. YL and XZ wrote the
609 manuscript together. All authors read and approved the final manuscript.

610

611 Competing interests

612 Not applicable.

613

614 Ethics approval

615 Although only using publicly available dataset, this research study was approved by
616 Cincinnati Children's Hospital Medical Center Institutional Review Board (#2019-0601) in
617 accordance with the Declaration of Helsinki.

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643 **Figures**

644

645 **Figure 1.** The schematic of CRAG approach

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647 **Figure 2.** The enrichment of cfDNA fragmentation hotspots at (a). Transcription Starting
648 Sites (TSS) overlapped with and without CpG island. (b). Exon boundary. (c). Transcription
649 Termination Sites (TTS). (d). CTCF transcription factor binding sites (no TSS within +/- 4 kb).
650 (e). Random genomics regions.

651

652 **Figure 3.** CfDNA fragmentation hotspots are enriched in gene-regulatory regions. The
653 enrichment of (a). DNase-seq and (b). Histone modification signals from hematopoietic cells
654 around the hotspots. (c). The enrichment of hotspots at different chromHMM states across
655 different cell types. (d). Genome browser track of cfDNA fragmentation hotspots.

656

657 **Figure 4.** Half of the cfDNA fragmentation hotspots are still protected by nucleosomes. (a).
658 Less than half of the cfDNA fragmentation hotspots are overlapped with existing 523 open
659 chromatin regions datasets from hematopoietic cells and liver. (b). DNase-seq signal from
660 major hematopoietic cell types around inaccessible and accessible cfDNA fragmentation
661 hotspots. (c). DNA methylation level at cell-free DNA around inaccessible and accessible
662 cfDNA fragmentation hotspots from healthy individuals (d). Motif enrichment around
663 inaccessible and accessible cfDNA fragmentation hotspots. (e). The distribution of the
664 inaccessible and accessible cfDNA fragmentation hotspots in different repeat elements.

665

666 **Figure 5.** The integrated fragmentation score (IFS) from cfDNA fragmentation hotspots can
667 boost the performance of cancer early detection. (a). Volcano plot of z-score differences and
668 p-value (two-way Mann–Whitney U test) for the aberration of IFS in cfDNA fragmentation
669 hotspots between early-stage HCC and healthy. (b). Unsupervised clustering on the Z-score
670 of IFS at the top 10,000 most variable cfDNA fragmentation hotspots called from HCC and
671 healthy samples. (c). Receiver operator characteristics (ROC) for the detection of early-
672 stage HCC by using IFS (after GC bias correction) from all the cfDNA fragmentation
673 hotspots (red), copy number variations (brown), and mitochondrial genome copy number
674 analysis (black). (d). Scatter plots of z-score differences and feature importance (coefficient
675 in linear SVM) split the cfDNA fragmentation hotspots into two groups: hypo-fragmented in
676 cancer (Class I) and hyper-fragmented in cancer (Class II). (e). The fraction of Class I and
677 Class II hotspots that are overlapped with existing open chromatin regions (accessible
678 hotspots) or microsatellite repeats, as well as their relative distance to the nearest TSS. (f)
679 The top 10 motif enrichment at Class I and Class II hotspots. (g). The top 10 enrichment of
680 Gene Ontology Biological Process at Class I and Class II hotspots.

681

Figure 6. The detection and location of multiple early-stage cancer. (a). t-SNE visualization on the Z-score of IFS (after GC bias correction) at the most variable cfDNA fragmentation hotspots (one-way ANOVA test with p value < 0.01) across multiple different early-stage cancer types and healthy conditions. (b). Unsupervised clustering on Z-score of IFS (after GC bias correction) at the top 40,000 most variable cfDNA fragmentation hotspots across multiple different early-stage cancer types and healthy conditions. (c). The sensitivity across different cancer stages at 100% specificity to distinguish cancer and healthy condition by using IFS (after GC bias correction) at cfDNA fragmentation hotspots. Error bars represent 95% confidence intervals. (d). Percentages of patients correctly classified by one of the two most likely types (sum of orange and blue bars) or the most likely type (blue bar). Error bars represent 95% confidence intervals.

Table 1. CRAG performance for cancer early detection.

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Figure 1

Figure 2

Figure 3

Figure 4

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Figure 6

Table 1: CRAG performance for cancer early detection

		Individuals analysed	100% specificity			95% specificity			85% specificity		
			Individuals detected	Sensitivity (%)	95% CI (%)	Individuals detected	Sensitivity (%)	95% CI (%)	Individuals detected	Sensitivity (%)	95% CI (%)
Cancer Type	Breast	54	44	0.8133	0.7168 - 0.9098	48	0.8867	0.7892 - 0.9842	51	0.9433	0.8865 - 1.0000
	Bile duct	26	11	0.4167	0.1819 - 0.6515	12	0.4500	0.2320 - 0.6680	14	0.5333	0.3166 - 0.7500
	Colorectal	27	24	0.8833	0.7636 - 1.0000	25	0.9167	0.8051 - 1.0000	25	0.9167	0.8051 - 1.0000
	Gastric	27	25	0.9333	0.8462 - 1.0000	25	0.9333	0.8462 - 1.0000	26	0.9667	0.9013 - 1.0000
	Lung	12	6	0.5000	0.2078 - 0.7922	8	0.6500	0.3560 - 0.9400	8	0.6500	0.3560 - 0.9400
	Ovarian	28	15	0.5500	0.3519 - 0.7451	23	0.8167	0.6592 - 0.9741	24	0.8500	0.7263 - 0.9737
	Pancreatic	34	19	0.5667	0.4251 - 0.7082	21	0.6333	0.4790 - 0.7877	22	0.6583	0.4889 - 0.8278
Cancer Stage	I	41	28	0.6350	0.4428 - 0.8272	34	0.8250	0.7046 - 0.9454	34	0.8250	0.7046 - 0.9454
	II	109	72	0.6667	0.5816 - 0.7518	79	0.7297	0.6503 - 0.8092	85	0.7813	0.7018 - 0.8607
	III	33	25	0.8200	0.6813 - 0.9587	28	0.8850	0.7796 - 0.9904	30	0.9350	0.8698 - 1.0000
	I-IV	205	141	0.6858	0.6098 - 0.7617	159	0.7742	0.7229 - 0.8255	167	0.8128	0.7596 - 0.8659