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# Genome-wide Cell-free DNA (cfDNA) Methylation Signatures and Effect on Tissue of Origin (TOO) Performance

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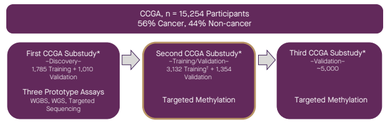
## BACKGROUND

- In order to provide a clinical benefit to patients, an effective multi-cancer detection tool should detect clinically significant cancers across stages with very high specificity and localize cancer to its tissue of origin (TOO).[1,2]
- Multi-cancer detection across stages was demonstrated at 98% specificity in a pre-specified case-control substudy from the Circulating Cell-free Genome Atlas (CCGA) study (NCT02889978).
  - This prospective, multi-center, longitudinal, observational study for the development of a noninvasive blood-based assay for cancer detection includes a discovery[3], training/validation, and validation phase (Figure 1).
- Here, we report multi-cancer detection and TOO determination from initial analyses of an optimized targeted methylation assay in 2,301 participants from the second CCGA substudy.
  - Prior methylation-based approaches may be limited by the number of captured CpGs; previous array-based studies captured <2% of genomic CpGs.[4]
  - Sequencing to identify cancer-specific methylation patterns allows genome-wide fragment-level analysis.
- Importantly, we show how a methylation database that interrogated genome-wide fragment-level methylation patterns across 789 cancer cell methylomes representing 20 tumor types (97% of SEER cancer incidence) improved performance of this multi-cancer test.

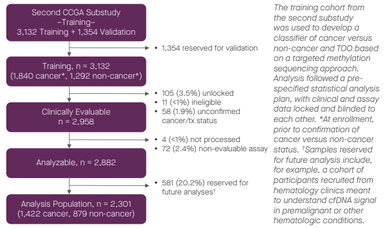
Figure 1. The CCGA Study

A. CCGA Divided into Three Substudies




*Includes participant samples used in evaluating the classifiers; approximately 2,700 participants not included in evaluating classifiers (eg, used as tissue references or in panel design) not represented. †Reported here. WGBS, whole-genome bisulfite sequencing; WGS, whole-genome sequencing.

B. Detail of Training Cohort from Second Substudy




*The training cohort from the second substudy was used to develop a classifier of cancer versus non-cancer and TOO based on a targeted methylation sequencing approach. Analysis followed a pre-specified statistical analysis plan, with clinical and assay data locked and blinded to each other. *At enrollment, prior to confirmation of cancer versus non-cancer status. †Samples reserved for future analysis include, for example, a cohort of participants recruited from hematology clinics meant to understand cfDNA signal in premalignant or other hematologic conditions.*

## METHODS

### CCGA Study

- 15,254 participants have been enrolled (56% cancer, 44% non-cancer).
- The training phase (reported here) of this second pre-specified sub-study included prospectively collected blood samples (N=3,132) from 1,840 participants (pts) with newly diagnosed, untreated cancer (>20 tumor types, all stages) and 1,292 participants with no cancer diagnosis (Figure 1).
- Plasma cfDNA was subjected to a bisulfite sequencing assay targeting the most informative regions of the methylome, as identified from a unique methylation database (see below) and prior prototype whole-genome and targeted sequencing assays[3] to identify cancer- and tissue-defining methylation signal.

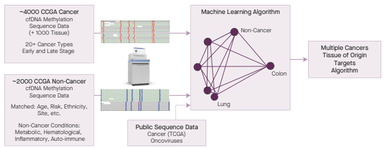
### Methylation Database

- Genomic DNA from formalin-fixed, paraffin-embedded (FFPE) tumor tissues and isolated cells from tumors was subjected to whole-genome bisulfite sequencing to generate a large database of cancer-defining methylation signals for use in panel design and in training to optimize performance.
- How the methylation database contributes to target selection is indicated in Figure 2.

### Classification

- Fragment methylation states were treated as being drawn from a mixture of latent methylation patterns.
- Observed fragments were assigned a relative probability of originating from cancer.
- Similarly, for TOO, observed fragments were assigned a relative probability of originating from a particular tissue.
- Fragments characteristic of cancer and TOO were combined across targeted regions to classify cancer versus non-cancer and identify TOO.
  - For binary cancer classification, clinical sensitivity was estimated at 99% specificity.
  - For TOO, two independent models, one with and one without the methylation database, were fitted; reported TOO results reflect percent agreement between predicted and true TOO among cases classified as cancer at 99% specificity.

Figure 2. Methylation Database




*A large methylation sequence database of cancer and non-cancer was generated to enable target selection for a single test able to classify multiple cancers at high specificity and identify TOO.*

## RESULTS

### Assay Selection

- The first pre-specified substudy[3] identified methylation patterns as most informative, and least subject to confounding singal from clonal hematopoiesis.[5,6]
  - The targeted assay was comparable to WGBS only when the targeted assay accounted for CH (WGBS vs targeted w/CH: p=0.61; WGBS vs targeted w/out CH: p<0.001); WGBS outperformed WGS regardless of whether the assay accounted for CH (p<0.001 for each comparison).
  - The WGBS assay also outperformed the targeted (p<0.001) and WGS (p<0.001) assays in TOO accuracy.

### Participant Demographics

- Overall, the cancer and non-cancer groups were comparable (Table 1).
  - Participants with lung cancer tended to be slightly older, and more were ever-smokers.
- A broad range of stages were represented in participants with cancer (Table 1).

Table 1. Participant Demographics and Stage Distribution

| | Cancer* | Non-cancer |
|---|---|---|
| Total | 1,422 | 879 |
| Age, Mean ± SD | 62.0 ± 11.8 | 54.2 ± 13.6 |
| Age Group, ≥ 50 yrs, n (%) | 1,220 (85.8) | 576 (65.5) |
| Sex, Female, n (%) | 712 (50.1) | 583 (66.3) |
| Race/Ethnicity, n (%) | | |
| White, Non-Hispanic | 1,174 (82.6) | 713 (81.1) |
| African American | 97 (6.8) | 67 (7.6) |
| Hispanic, Asian, Other | 151 (10.6) | 99 (11.3) |
| Never-smoker, n (%)† | 633 (45.3) | 495 (57.1) |
| Body Mass Index, Normal/Underweight, n (%)‡ | 381 (26.8) | 216 (24.6) |
| Dx by Screening, n (%) | 350 (24.6) | - |
| Clinical Stage, n (%)§ | | |
| I | 398 (28.0) | - |
| II | 366 (25.7) | - |
| III | 290 (20.4) | - |
| IV | 327 (23.0) | - |
| Non-informative/Missing¶ | 41 (2.9) | - |

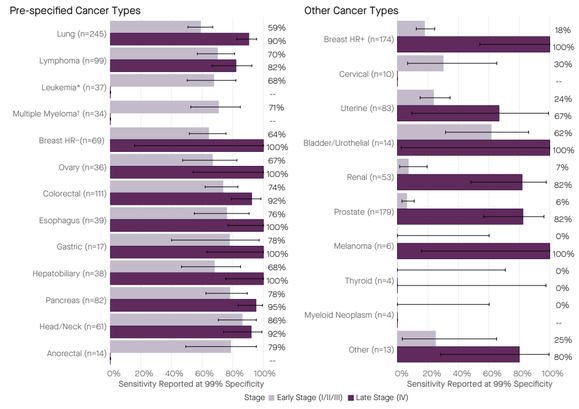
*Cancer and non-cancer groups were comparable with respect to age, race, sex, and body mass index. *Includes anorectal, bladder, brain, breast, cervical, colorectal, esophageal, gastric, head and neck, hepatobiliary, lung, lymphoid neoplasm (chronic lymphocytic leukemia, hairy cell leukemia, lymphoma), multiple myeloma, myeloid neoplasm (chronic myeloid leukemia), ovarian, pancreatic, prostate, renal, sarcoma, and uterine cancers. †Excludes 38 participants missing smoking status information. ‡Excludes two participants missing BMI values. §Invasive cancer only. ¶Staging information not available.*

### Sensitivity

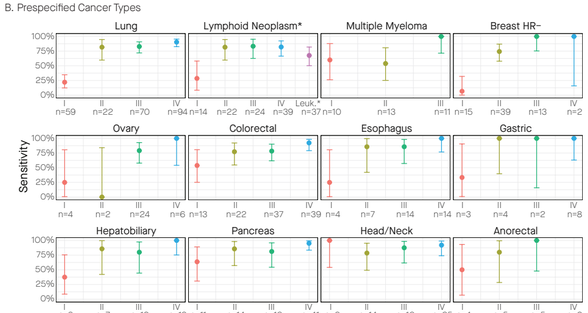
- Sensitivity was estimated at 99% specificity (Figure 3).
  - Demographic information alone (baseline modeling) classified <5% of participants correctly.
- **Overall sensitivity was 76.1% (95% CI: 73.1–78.9%)** in a pre-specified list of clinically significant cancers (anorectal, breast [HR-negative], colorectal, esophageal, gastric, head and neck, hepatobiliary, lung, lymphoid neoplasm [chronic lymphocytic leukemia, hairy cell leukemia, lymphoma], multiple myeloma, ovarian, pancreatic).
  - **Sensitivity was 68.8% (95% CI: 64.8–72.6%) in early stage (I–III) cancers in this pre-specified cohort.**
- Overall sensitivity was 55.1% (95% CI: 52.5–57.7%) across all cancer types and stages.
  - In early stage (I–III) cancers, sensitivity was 43.8% across all cancer types in the sub-study (95% CI: 40.7–46.8%).

Figure 3. Sensitivity is Reported at 99% Specificity

A. Stages I–III vs Stage IV




B. Prespecified Cancer Types

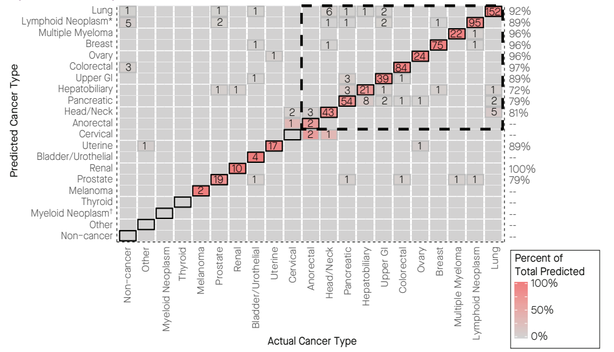



*A. Left panel: pre-specified list of cancers. Right panel: other cancers included in the study. 95% confidence intervals are indicated. *Leukemia includes chronic lymphocytic leukemia and hairy cell leukemia (unstaged). †Myeloid neoplasm includes chronic myeloid leukemia. B. Sensitivity by individual stage for the pre-specified cancers is reported at 99% specificity. 95% confidence intervals are indicated. *Lymphoid Neoplasm includes lymphoma, chronic lymphocytic leukemia, and hairy cell leukemia (unstaged).*

### Tissue of Origin

- Classifier performance was higher with the methylation database versus without.
  - Of 1,422 total samples, 784 with vs. 763 without were called cancer, 735 vs. 716 returned a TOO result, and 663 vs. 642, respectively, were correctly localized (p=0.0066, Stuart-Maxwell test).
- The assay assigned a TOO in 93.8% (735/784) of cases, with the methylation database.
  - **90.2% (663/735) of these TOO predictions were correct** (Figure 4).
  - This was consistent regardless of stage (stage I–III predictions: 89.9% [384/427]; stage IV predictions: 90.1% [255/283]).

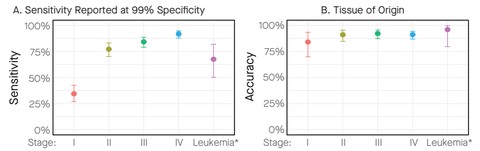
Figure 4. Tissue of Origin Performance




*Agreement between the true (x-axis) and predicted (y-axis) TOO per sample using the TOO classifier with the methylation database in stage I-IV samples. Color corresponds to the proportion of predicted TOO (y-axis) which were correct (x-axis), as indicated to the right of the plot. Percent correct predictions from the total predictions for each cancer type (n>5) is indicated to the right of the plot. Numbers in each box represent the total number of calls. *Lymphoid neoplasm includes chronic lymphocytic leukemia, hairy cell leukemia, lymphoma. †Myeloid neoplasm includes chronic myelocytic leukemia. Numbers in each box represent the total number of calls.*

- An effective multi-cancer test ideally should simultaneously detect clinically significant cancers across stages with very high specificity (and thus would have a single fixed, low false positive rate), and accurately determine TOO.
- To demonstrate the potential of this approach, simultaneous detection (sensitivity reported at 99% specificity) and TOO determination for the pre-specified list of cancer types, in aggregate, at individual stages, is displayed in Figure 5.

Figure 5. Detection and TOO Determination by Stage




*Sensitivity (reported at 99% specificity, top panel) and tissue of origin (bottom panel) for the pre-specified list of cancers is reported by individual stage. Numbers in each stage are indicated. *Leukemia includes chronic lymphocytic leukemia and hairy cell leukemia (unstaged).*

## CONCLUSIONS

- Initial results from the ongoing second sub-study of CCGA showed targeted methylation simultaneously detected multiple cancer types, at early stages, at a specificity (99%) appropriate for population screening.
  - Detection of multiple cancers was achieved with a single, fixed, low false positive rate.
- This approach also accurately localized the TOO, which could streamline subsequent diagnostic work-up.
- Incorporating data from a large methylation database improved performance.
- Targeted methylation was selected for further development in preparation for clinical validity studies.
  - This was based on methylation (WGBS) outperforming WGS and targeted sequencing approaches (for details of WGBS cancer detection and survival, please see Poster 1545).
- Together, these findings support the potential clinical applicability of this targeted methylation approach as a multi-cancer detection test for numerous clinically significant cancer types.

**References:** 1. Medicine I of, Council NR. Fulfilling the Potential of Cancer Prevention and Early Detection. (Curry SJ, Byers T, Hewitt M, eds.). Washington, DC: The National Academies Press; 2003. https://www.nap.edu/catalog/10863/fulfilling-the-potential-of-cancer-prevention-and-early-detection. 2. Aravanis AM, et al. *Cell*. 2017;168(4):571-574. doi:10.1016/j.cell.2017.01.030. 3. Liu MC, et al. *Annals of Oncology*. 29(suppl_8):viii14-viii57. doi:10.1093/annonc/mdy289. 4. The Cancer Genome Atlas Research Network. Comprehensive genomic characterization defines human glioblastoma genes and core pathways. *Nature*. 2008;455(7216):1061-1068. doi:10.1038/nature07385. 5. Hu Y, et al. *Clinical Cancer Research*. 2018;24(18):4437-4443. doi:10.1158/1078-0432.CCR-18-0143. 6. Adalsteinsson VA, et al. *Nature Communications*. 2017;8(1). doi:10.1038/s41467-017-00965-y

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