Poster
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Detection of tumor-associated gene inactivation in clinical blood draw via cell-free DNA methylation profiling

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Abstract

The ability to detect genome-wide epigenetic changes, such as DNA methylation, has expanded translational applications in oncology settings. Because these changes occur early in carcinogenesis, they can be used for early cancer detection when genomic technologies fall short due to lower sensitivity, and in the early and late-stage cancer setting for minimal residual disease detection, disease monitoring and therapy selection[1-2].

In this analysis, we demonstrate our highly sensitive targeted assay simultaneously captures both genomic alterations and methylation signatures in cell-free DNA (cfDNA). Our assay can detect differential methylation that classifies cancer from healthy donors, as well as the quantification of promoter methylation.

Methods

To capture tumor-associated methylated cfDNA signals, we developed a custom assay on a broad genomic panel (~15.2Mb) targeting unmethylated regions in plasma cfDNA from healthy individuals. This panel covers promoter regions of ~12,000 genes, including well-known tumor suppressor genes (TSGs)[7] e.g., *PTEN, TP53* and homologous recombination and repair (HRR) genes, e.g., *BRCA1, RAD51*.

For each sample that runs through our assay, with the pre-defined promoter regions of each covered gene, we calculate methylation scores for each gene and this scores is the basis for promoter hypermethylation calls. We first trained and evaluated the specificity of our model on blood samples from 131 cancer-free donors. We then tested the performance on a validation dataset of blood samples from 559 stage IV cancer patients (203 lung cancer, 146 breast cancer, 151 bladder cancer, 32 colorectal cancer (CRC) and 27 other cancer types) and 2,612 self-reported cancer-free donors.

Conclusions

We demonstrate that our assay can accurately detect cancer-driven DNA methylation across the genome in clinical plasma samples:

- In selected TSG and HRR genes, our promoter hypermethylation calling method has a >99% specificity on an independent test dataset.
- With *in-silico* titration, we observed 0.1%-0.65% LoD for tested genes.
- Our promoter hypermethylation calls are largely consistent with previous studies from publicly available databases the public data.
- In an expanded analysis, we demonstrate that our promoter methylation calling method can also provide orthogonal information that may be relevant in therapeutic selection.

References

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6. Bettstetter, Marcus, et al. "Distinction of hereditary nonpolyposis colorectal cancer and sporadic microsatellite-unstable colorectal cancer through quantification of MLH1 methylation by real-time PCR." *Clinical Cancer Research* 13.11 (2007): 3221-3228.
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Results

Promoter methylations in the training and the test dataset

In blood samples from 131 cancer-free donors, we established the thresholds of promoter methylation calls. We focus our analysis on 88 well-known TSGs and HRR genes.

Under our established thresholds, 12 promoter methylation calls (in at least one of the 88 genes) was observed across all 131 training samples (Figure 1, left panel). In the test dataset of 2,612 cancer-free donors, we observed a total of 317 promoter methylation calls in these 88 genes in 209 samples with call level specificity of 99.86% (Figure 1, right panel) and sample level specificity of 92%. In the test dataset of 559 late-stage cancer patients, 334 (60%) were called with at least one promoter methylation in these genes. Among the samples positive for promoter methylation calls, the median number of promoter methylation calls per samples was 3 (Figure 1, middle panel).

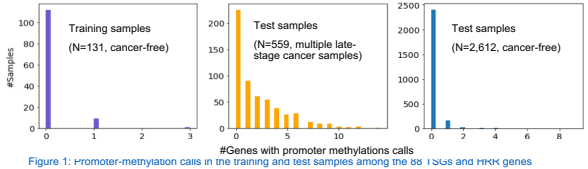

Figure 1: Promoter-methylation calls in the training and test samples among the 88 TSGs and HRR genes

Cancer-free donors with methylation calls

We manually examined the distribution of methylation signals in cancer patients and cancer-free donors from the test dataset. In the cancer patients with methylation calls, we observed signals mostly within CpG clusters. In most cancer-free donors, we observed no signals associated with methylation: e.g., in *MLH1* promoter region, we only observed very weak methylation signals in 3 out of total 2,743 healthy donors from the training set and the test set together.

To further investigate the potential false positives (FP), we examined the cancer-prediction scores for the cancer-free donor samples that have more than one methylation call (See our poster #5189 "Accurate epigenomic estimates of circulating tumor fraction in large-scale clinical data" for details). We found 40% had high cancer-prediction scores (>10 times of standard deviation than the cancer/cancer-free cutoff, Figure 2). As the "cancer-free" donors are self-reported, it was possible that these individuals were not in fact cancer-free.

For the samples with low cancer-prediction scores, we also found likely true methylations for a few TSGs or HRR genes, e.g. *BRCA1*. Publications indicate that promoter methylations may happen sporadically in the general population at a very low rate[3].

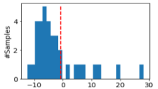

Figure 2: Cancer-prediction scores of cancer-free samples that have >1 methylation call

Red dashed line shows cancer/cancer-free cutoff

Gene	#Samples (%)
FAT1	56 (2.1%)
RAD51C	41 (1.6%)
IFNL2	35 (1.3%)
BRCA1	30 (1.1%)
CDH1	24 (0.92%)

Table 1: Genes that were called most often for promoter methylation in cancer-free donors

Preliminary analysis on Limit of Detection (LoD)

We calculated LoD of our detection method by *in-silico* mixing sequencing reads of KM12 cell line and cancer-free donors at the level of 0.05% to 1%. We calculated LoD for four of the cancer-related genes that were called as promoter methylation in KM12 (Figure 3).

LoD for each gene is affected by both methylation levels and background noise in the healthy population. *MLH1*, with full methylation in KM12 (validated by orthogonal experiments) and a very low methylation rate in the general population, has the lowest estimated LoD (0.095%). LoD for other three genes ranges between 0.3% and 0.65% (Table 2).

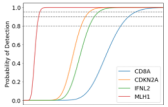

Figure 3: LoD probit of selected genes from KM12

Grey dashed line shows probability of 0.8, 0.85 and 0.95, respectively

Gene	Estimated LoD
MLH1	0.095%
CDKN2A	0.38%
IFNL2	0.43%
CD8A	0.64%

Table 2: *In-silico* LoD estimate of selected genes from KM12

Prevalence of promoter methylation

To further validate the promoter hypermethylation calls and to validate our findings, we examined the TSGs and HRR genes in cancer samples from the Cancer Genome Atlas (TCGA) database[8]. We focused our analysis on 2,380 primary tumor cancer samples with available methylation data from Illumina 450K array. For each gene, we calculated its prevalence (percentage of samples observed with promoter methylation) in both TCGA data and our test dataset (Figure 4).

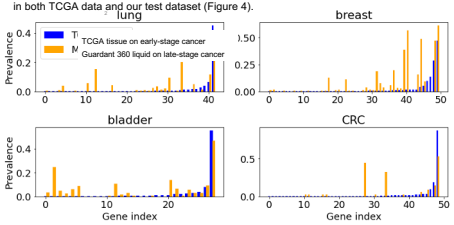

Figure 4: Prevalence of promoter methylation of TSGs and HRD genes in our test dataset (total N=532) and in TCGA public data (total N=2,380). Limited to genes with promoter methylation in TCGA.

For most genes, we observed similar prevalence between TCGA and our methylation detection calls. It is worthwhile to note that samples in TCGA are tissue samples and many of them are still in early-stage disease, but our test dataset is plasma cfDNA from late-stage patients.

We additionally examined the two genes (*CD8A* and *CDKN2A*) with high prevalence in our CRC samples but not in TCGA CRC data. The *CD8A* promoter region was covered by five array probes; two show >0.5 prevalence but the other three array probes showed no methylation signals. Similarly in *CDKN2A*, one out of five array probes showed a prevalence of 0.1 but the remainder had very low levels of methylation signals.

MLH1 promoter hypermethylation

To demonstrate the potential clinical relevance of our assay, we further summarized promoter methylation in *MLH1*. Previous studies have shown that 54%-100% of CRC patients with microsatellite instability (MSI-H) tumors harbor *MLH1* promoter methylation[4,6].

Among 1,966 CRC patients, we detected significantly higher *MLH1* promoter methylation in MSI-H group (Fisher's p<0.05), compared to patients with microsatellite stable (MSS) tumors and the cancer-free population (Figure 5).

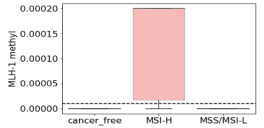

Figure 5: *MLH1* promoter methylation in cancer-free donors and CRC patients (MSI-H and MSS)

Furthermore, we tested the prevalence of *BRAF* V600E, a common driver mutation for CRC[5]. We identified strong co-occurrence of *MLH1* promoter hypermethylation and *BRAF* V600E (Table 4), consistent with previous findings.

	BRAF WT	*BRAF* V600E
MLH1 Promoter Hypermethylation Detected	118 (6.2%)	34 (61%)
MLH1 Promoter Hypermethylation Not Detected	1792 (93.8%)	22 (39%)
Total	1910	56

Table 4: Calls of *MLH1* promoter methylation and *BRAF* V600E in CRC patients