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Promoter Methylation as a Cancer Biomarker: Insights From ctDNA-Based Targeted Methylation Data

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INTRODUCTION

- Promoter methylation is a hallmark of cancer and is a promising biomarker for early cancer detection, prognostication, treatment guidance, and response monitoring.[1]
- GRAIL's circulating tumor DNA (ctDNA)-based targeted methylation platform is a robust and scalable biopsy-free assay that was optimized to detect cancer and distinguish between methylation patterns of different cancer signal origins.[2]
- Previously, we used GRAIL's ctDNA-based targeted methylation assay to sensitively detect and quantify *BRCA1* promoter methylation from a plasma sample.[3]
- To further explore the utility of GRAIL's ctDNA-based assay in detecting gene promoter methylation, we selected four genes with previously reported associations between promoter methylation and cancer for analysis in the Circulating Cell-free Genome Atlas (CCGA) cohort.
    - ***EYA4*** has been implicated as a tumor regulatory gene[4] and hypermethylation of its promoter has been proposed as a prognostic marker in HCC, ICC and CRC.[5,6,7]
    - ***BNIP3*** promoter methylation is associated with chemoresistance and worse survival in pancreatic and colorectal cancers and is a potential therapeutic target.[8,9]
    - ***SFRP1*** promoter methylation was shown to be associated with treatment resistance and progression in lung, colorectal, and gastric cancers.[10]
    - ***ASCL1*** promoter methylation is associated with cancer risk and severity in patients with human papillomavirus (HPV) infection.[11]

OBJECTIVE

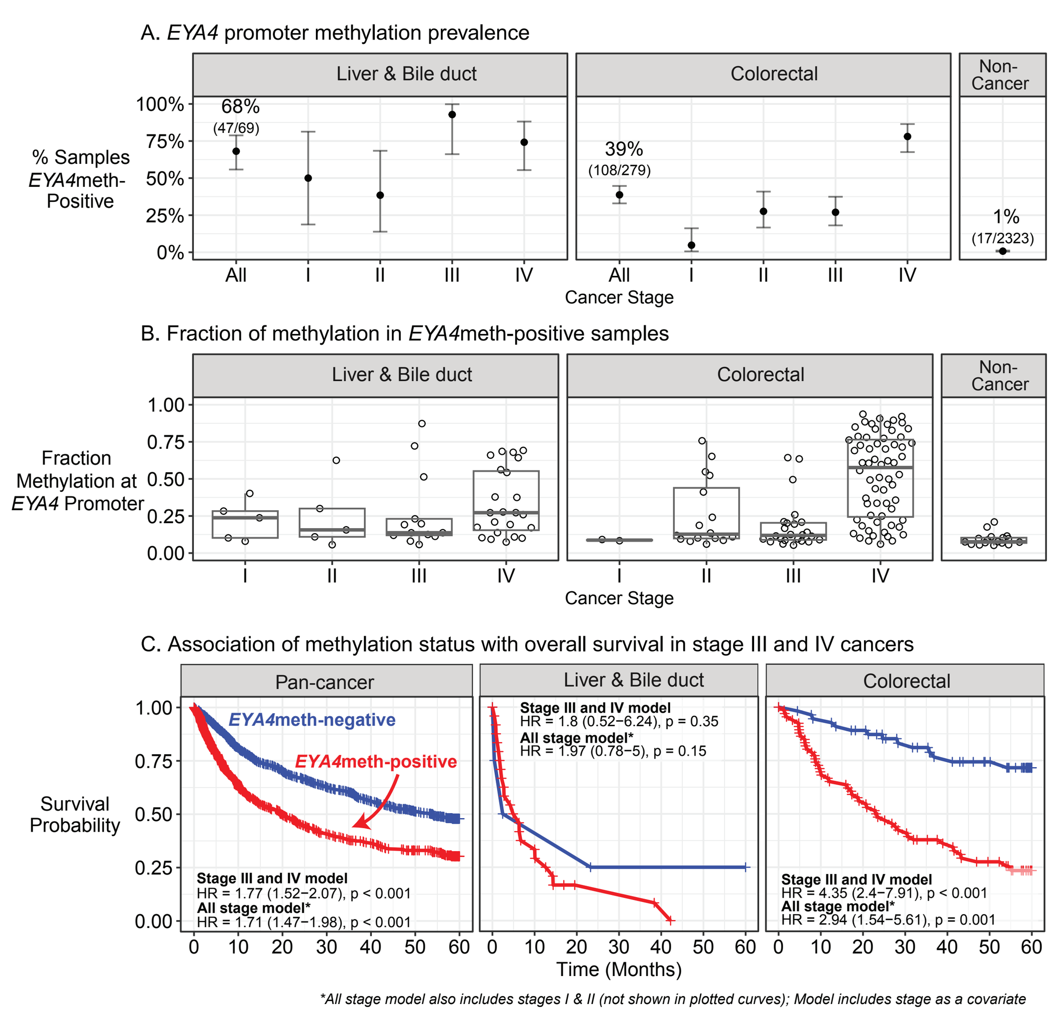
- Demonstrate the utility of GRAIL's ctDNA-based targeted methylation assay in detecting promoter methylation signals.
- Evaluate gene-specific promoter methylation data in the CCGA cohort using four genes (*EYA4, BNIP3, SFRP1, ASCL1*) previously implicated in multiple cancer types as a proof-of-concept.

KEY RESULTS: CHARACTERIZING CANCER-ASSOCIATED PROMOTER METHYLATION SIGNALS IN A LARGE PAN-CANCER COHORT USING ctDNA-BASED TARGETED METHYLATION DATA

EYA4 Promoter Methylation Prevalence and Association With Overall Survival

- *EYA4* promoter methylation was prevalent in liver and bile duct (68%, 47/69, 95% CI: 56%–79%) and colorectal cancers (39%, 108/279, 95% CI: 32%–44%), compared to 1% prevalence in the non-cancer group (**Figure 3A**).
- Significant association of *EYA4* promoter methylation with survival was observed in colorectal cancer (HR=2.94, p=0.001) and in the pan-cancer setting (HR=1.71, p<0.001) (**Figure 3C**).

Figure 3. *EYA4* Promoter Methylation Prevalence and Association With Overall Survival

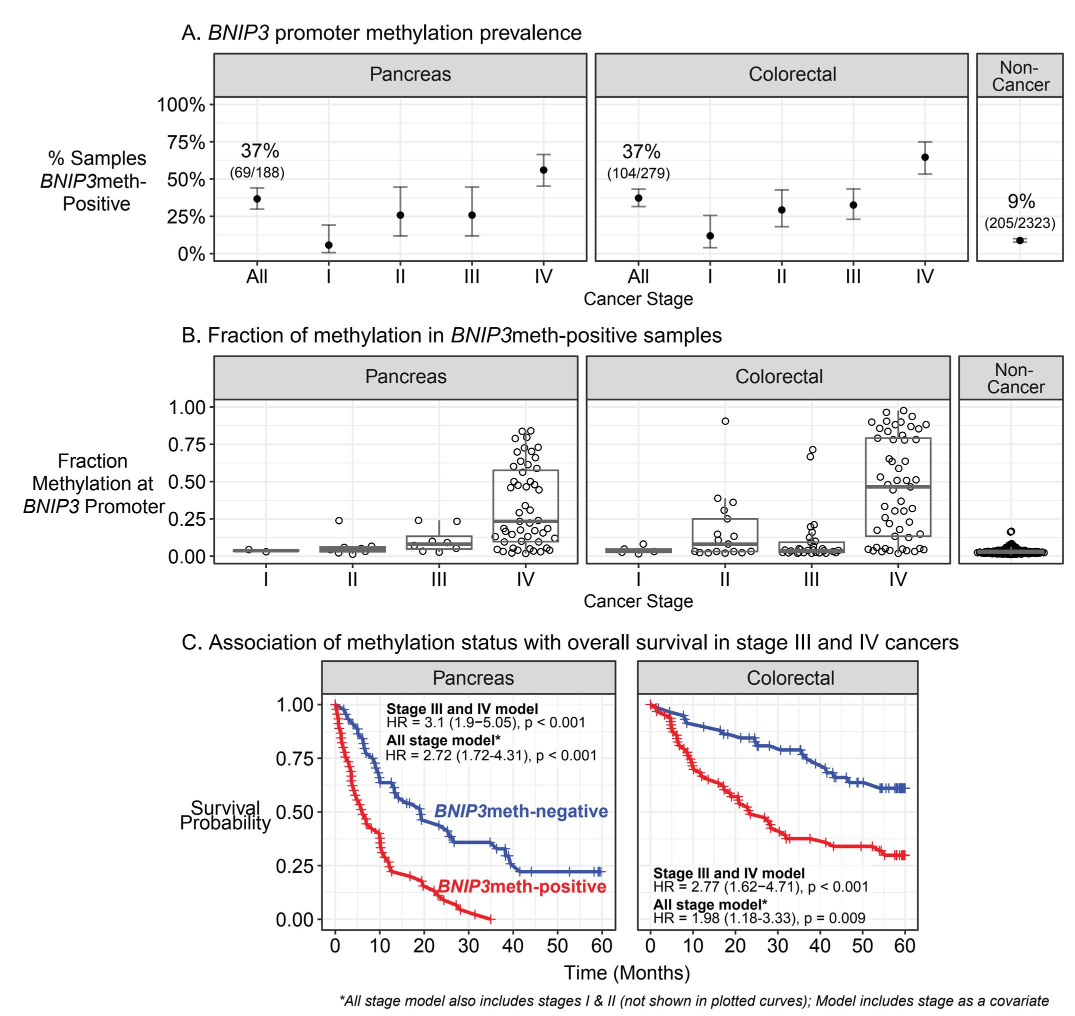


(A) Percentage of samples exhibiting EYA4 promoter methylation across cancer stages. Error bars indicate the 95% binomial confidence interval. Overall prevalence and sample size are shown. (B) Boxplots of the fraction of methylated fragments at the EYA4 promoter in EYA4meth-positive samples, separated by cancer stage. (C) Kaplan-Meier survival curves comparing EYA4meth-positive and -negative patients from stages III and IV. Hazard ratios and associated p-values from a Cox proportional hazards model are reported for stages III and IV combined and, separately, for all cancer stages (adjusting for stage).

BNIP3 Promoter Methylation Prevalence and Association With Overall Survival

- *BNIP3* promoter methylation, reportedly associated with chemoresistance, was prevalent in colorectal cancer (37%, 104/279, 95% CI: 32%–43%) and in pancreatic cancer (37%, 69/188, 95% CI: 30%–44%), compared to 9% prevalence in the non-cancer group (**Figure 4A**).
- *BNIP3* promoter methylation was significantly associated with worse survival among patients with pancreatic cancer (HR=2.72, p<0.001) and colorectal cancer (HR=1.98, p=0.009) (**Figure 4C**).

Figure 4. *BNIP3* Promoter Methylation Prevalence and Association With Overall Survival

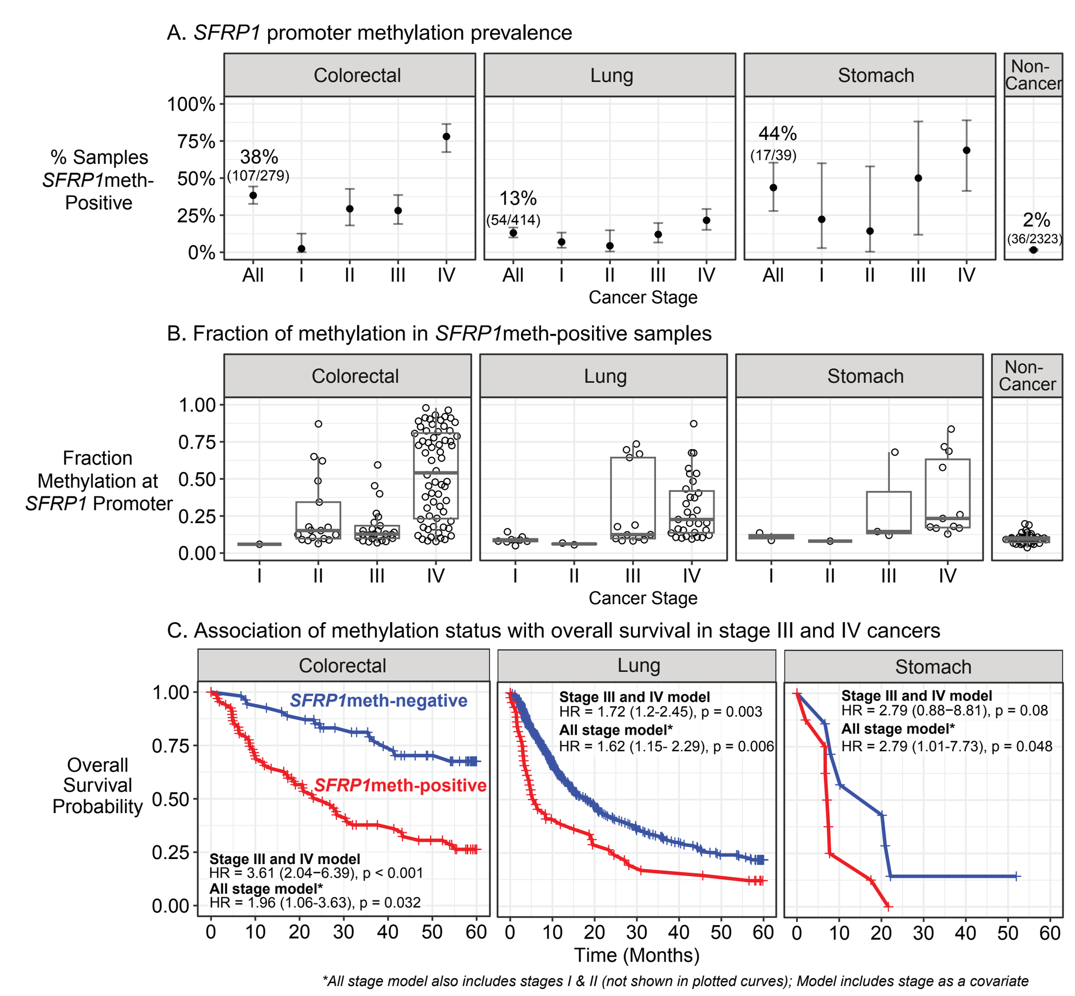


(A) Percentage of samples exhibiting BNIP3 promoter methylation across cancer stages. Error bars indicate the 95% binomial confidence interval. Overall prevalence and sample size are shown. (B) Boxplots of the fraction of methylated fragments at the BNIP3 promoter in BNIP3meth-positive samples, separated by cancer stage. (C) Kaplan-Meier survival curves comparing BNIP3meth-positive and -negative patients from stages III and IV. Hazard ratios and associated p-values from a Cox proportional hazards model are reported for stages III and IV combined and, separately, for all cancer stages (adjusting for stage).

SFRP1 Promoter Methylation Prevalence and Association With Overall Survival

- *SFRP1* promoter methylation was prevalent among patients with colorectal (38%, 95% CI: 33–44%), lung (13%, 95% CI: 10–17%), and stomach (44%, 95% CI: 28–60%) cancers, compared to 2% prevalence in the non-cancer group (**Figure 5A**).
- *SFRP1* promoter methylation was significantly associated with poorer survival in colorectal (HR=1.96, p=0.032), lung (HR=1.62, p=0.006), and stomach cancers (HR=2.79, p=0.048) (**Figure 5C**).

Figure 5. *SFRP1* Promoter Methylation Prevalence and Association With Overall Survival

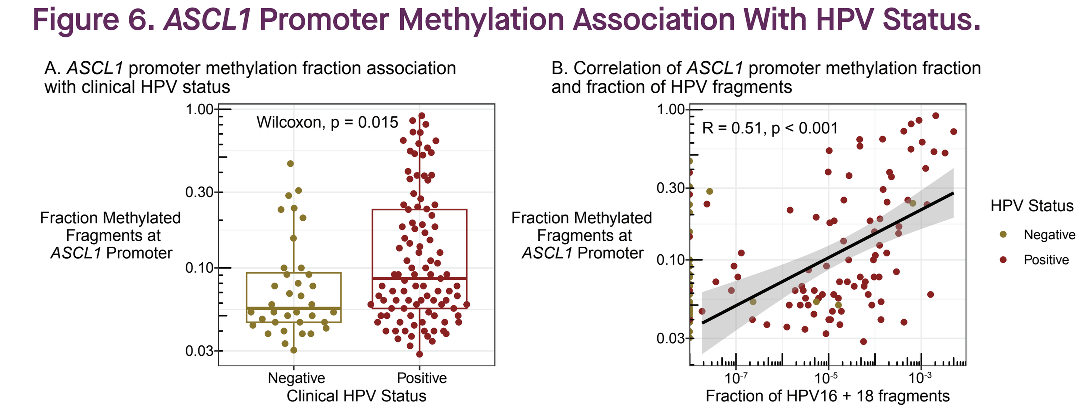


(A) Percentage of samples exhibiting SFRP1 promoter methylation across cancer stages. Error bars indicate the 95% binomial confidence interval. Overall prevalence and sample size are shown. (B) Boxplots of the fraction of methylated fragments at the SFRP1 promoter in SFRP1meth-positive samples, separated by cancer stage. (C) Kaplan-Meier survival curves comparing SFRP1meth-positive and -negative patients from stages III and IV. Hazard ratios and associated p-values from a Cox proportional hazards model are reported for stages III and IV combined and, separately, for all cancer stages (adjusting for stage).

ASCL1 Promoter Methylation Association With HPV Status

- *ASCL1* promoter methylation correlated with clinical HPV status of HPV-associated cancers, with higher fraction of methylated fragments in HPV-positive head and neck and cervical cancers (p=0.015) (**Figure 6**).

Figure 6. *ASCL1* Promoter Methylation Association With HPV Status.



(A) Boxplots of the fraction of methylated fragments at the ASCL1 promoter by clinical HPV status. A Wilcoxon p-value assesses the significance of the difference. (B) Fraction of methylated fragments at the ASCL1 promoter (y-axis; log_{10}-transformed) as a function of the fraction of fragments attributed to HPV16/18 (x-axis; log_{10}-transformed), with the Pearson correlation indicated. One is added to the numerator of the fraction of methylated fragments prior to the log transformation.

CONCLUSIONS

- This proof-of-concept study underscores the potential of GRAIL's ctDNA-based targeted methylation assay for detecting clinically informative promoter methylation signals in a plasma sample.
- Using contrived samples with known global methylation fractions, we demonstrate linear and sensitive quantification of gene promoter methylation.
- Promoter methylation increases with cancer stage and may reflect overall ctDNA shedding by the tumor, which could be incorporated into future models.
- This study demonstrates the value of GRAIL's large and well-annotated datasets in studying promoter methylation signals across diverse cancer types and patient subgroups. These insights could support indication prioritization and patient selection in clinical settings.
- Further validation and investigation in therapeutic settings are necessary to fully leverage promoter methylation as a biomarker in precision oncology.

METHODS

Selection of Promoter Methylation Signals for Analysis

- A comprehensive literature review was conducted to identify genes with promoter methylation proposed as a cancer biomarker, prioritizing those implicated in multiple cancer types across several studies.
- As a proof-of-concept, four genes (*EYA4, SFRP1, BNIP3, ASCL1*) were evaluated using the ctDNA targeted methylation data from the CCGA study (NCT02889978).[2]

Approach for Assessing Gene Promoter Methylation in Cell-Free DNA

- Fragments mapping to the promoter region of interest were analyzed. A fragment was considered methylated if the number of methylated CpGs on the fragment exceeded that of background noise (**Figure 1**).
- Samples were considered positive for methylation at a given promoter if the number of methylated fragments exceeded a threshold (n) optimized to maximize sensitivity and minimize false positives in contrived samples with known global methylation fractions. The contrived samples were also utilized to establish the sensitivity and linearity of the approach (**Figure 2**).

Figure 1. Approach for Quantifying Methylation at a Given Gene Promoter Region.

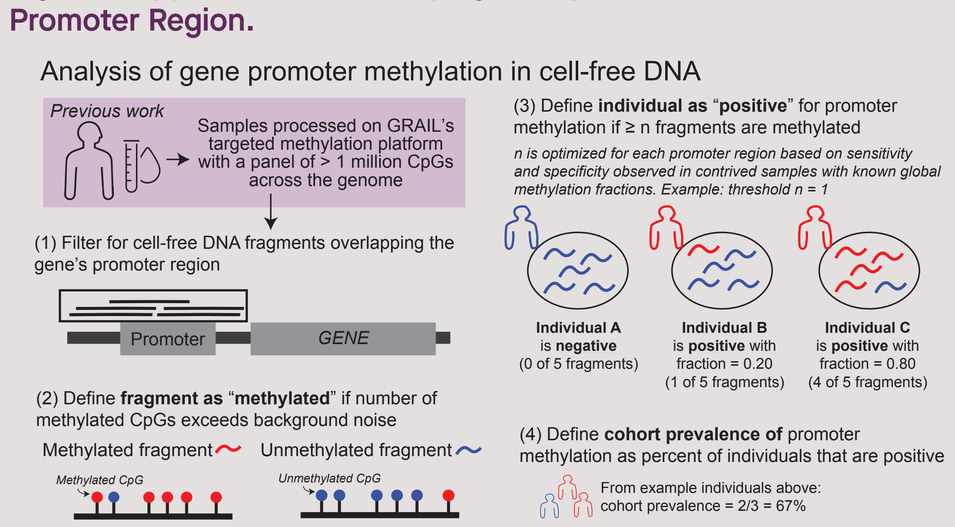
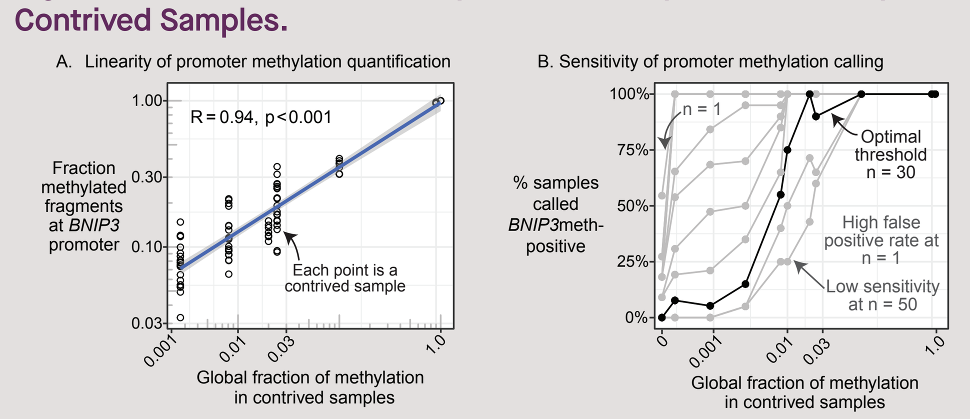


Figure 2. *BNIP3* Promoter Methylation Linearity and Sensitivity in Contrived Samples.



(A) BNIP3 promoter methylation fraction (y-axis) is plotted against the expected global (genome-wide) methylation fractions of contrived samples (x-axis). A linear regression line is fit to the data and the Pearson correlation is shown. (B) For different thresholds between n=1 and n=50, sensitivity (% of samples with BNIP3 promoter methylation exceeding the set threshold) is shown across expected methylation fractions. The "optimal threshold", in this case n=30, is selected based on maximizing sensitivity and minimizing false positives. This threshold is then used to calculate cohort prevalence of promoter methylation.

- Our analysis included 6127 CCGA cancer samples, spanning 21 cancer types, and 2323 non-cancer samples. We estimated the fraction of methylated fragments in each promoter region for all samples and the prevalence of promoter methylation for each cancer type. All cancer types are included under the label "Pan-cancer": Anus, Bladder, Breast, Colon/Rectum, Cervix, Esophagus, Gallbladder, Head and Neck, Kidney, Liver/Bile-duct, Lymphoid Leukemia, Lymphoma, Lung, Melanoma, Ovary, Pancreas, Plasma Cell Neoplasm, Prostate, Sarcoma, Stomach, Thyroid, Uterus, Urothelial Tract.
- On a subset of samples for which 5-year survival status was available, a Cox proportional hazards model was employed to evaluate the association of survival and promoter methylation. The "Stage III and IV model" includes only stage III and IV patients, where most survival events are concentrated. This model corresponds to the data plotted in the Kaplain-Meier curves. A second model ("All stage model") includes stages I, II, III, and IV, with stage as a covariate. Hazard ratios (HR) are reported with the 95% confidence interval in parentheses and the associated p-value.
- For *ASCL1*, promoter methylation is compared to clinical HPV status and fraction of HPV fragments, which is the number of fragments aligning to the HPV16 or HPV18 genome over the total number of fragments surveyed.[12]

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Disclosures

MLA, EM, RR, and CZ are current or former employees of GRAIL, Inc., with equity in the company.

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