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Robust Discovery of Candidate DNA Methylation Cancer Drivers

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The field of cancer genomics has been empowered by increasingly sophisticated inference tools to distinguish driver mutations from the vastly greater number of passenger mutations. Epigenetic alterations such as promoter DNA hypermethylation have been shown to drive cancer through inactivation of tumor suppressor genes (TSGs), but growing malignant populations also accrue pervasive stochastic epigenetic changes in DNA methylation (DNAme), most of which likely carry little functional impact. Unlike with somatic mutations, we have limited ability to robustly differentiate driver DNAme changes (DNAme drivers) from stochastic, passenger DNAme changes. To address this challenge, we developed MethSig, a statistical inference framework that accounts for the varying stochastic hypermethylation rates across the genome and between samples. MethSig estimates expected background DNAme changes, thereby allowing the identification of epigenetically disrupted loci, where observed hypermethylation significantly exceeds expectation, potentially reflecting positive selection (Fig. 1a).

We applied MethSig to reduced representation bisulfite sequencing (RRBS) data of chronic lymphocytic leukemia (CLL) cohorts, which include 304 CLLs collected in a prospective clinical trial (CLL8) and 103 CLLs in a previously published study (CLL-DFCI, Landau *et al*., 2014), as well as other malignancies where RRBS data is available, including ductal carcinoma in situ (Abba *et al*., 2015) and multiple myeloma. Area under the receiver operating characteristic curve (AUROC) was used to evaluate sensitivity and specificity of methods in the inference of likely DNAme drivers. We identified two key features that are likely to be strongly associated with true candidate DNAme drivers: gene silencing in relation to promoter hypermethylation and association with clinical outcome. MethSig qualitatively improved ROC across those clinical and biological read outs (0.955 of MethSig, 95% confidence interval [CI] 0.945 - 0.965, versus 0.703 of benchmarked methods, 95% CI 0.669 - 0.737, Fig. 1b used CLL8 as an example).

We identified 189 candidate DNAme drivers in CLL, which include known TSGs, and are enriched in genes hypermethylated or inactivated across cancer types. To further validate MethSig's inferences, selected CLL candidate DNAme drivers (*DUSP22*, *RPRM*) underwent CRISPR/Cas9 knockout (KO) in CLL cells and stable KO clones were generated through single-cell cloning to eliminate genetic heterogeneity effect. The *RPRM* and *DUSP22* KO clones showed faster growth without treatment (Fig. 1c) and superior fitness in ibrutinib/fludarabine treatment compared with controls (Fig. 1d). Notably, we observed a gene dose effect in the *RPRM* KO clones (Fig. 1c-d, greater growth of the bi-allelic compared to mono-allelic KO).

Elastic net regression with a Cox proportional hazards model was used to evaluate DNAme drivers' contribution to the prediction of failure-free survival after treatment (FFS; failure defined as retreatment

or death) and a rigorous training (CLL8) and validation (an independent cohort CLL-DFCI) cohort study design was implemented to safeguard from overfitting and poor generalizability. DNAme drivers were found to be associated with shorter FFS in independent CLL cohorts (Fig. 1e-f). A regression model including established CLL risk indicators (IGHV unmutated status, *del*[17p] or *TP53* mutation) showed an adjusted hazard ratio of 2.3 (95% CI 1.6 - 3.3, $P = 2 \times 10^{-6}$) in CLL8 cohort and 3.2 (95% CI 1.2 - 8.8, P = 0.02) in CLL-DFCI cohort for patients with high risk. Application of MethSig to CLL relapsed after chemoimmunotherapy further identified relapse-specific DNAme drivers, enriched in TP53 targets as well as DNA damage pathway, which indicates that CLL relapse after chemotherapy may follow an alternative path compared to CLL progression in the absence of therapy, offering novel insights for therapeutic strategies to address drug-resistant or relapsed cancer.

Collectively, our data support a novel framework for the analysis of DNAme changes in cancer to specifically identify DNAme drivers of disease progression and relapse, empowering the discovery of epigenetic mechanisms that enhance cancer cell fitness. This work addressed a central gap between

cancer epigenetics and cancer genetics, where such tools have had a transformative impact in precision oncology and cancer gene discovery.

Figure 1

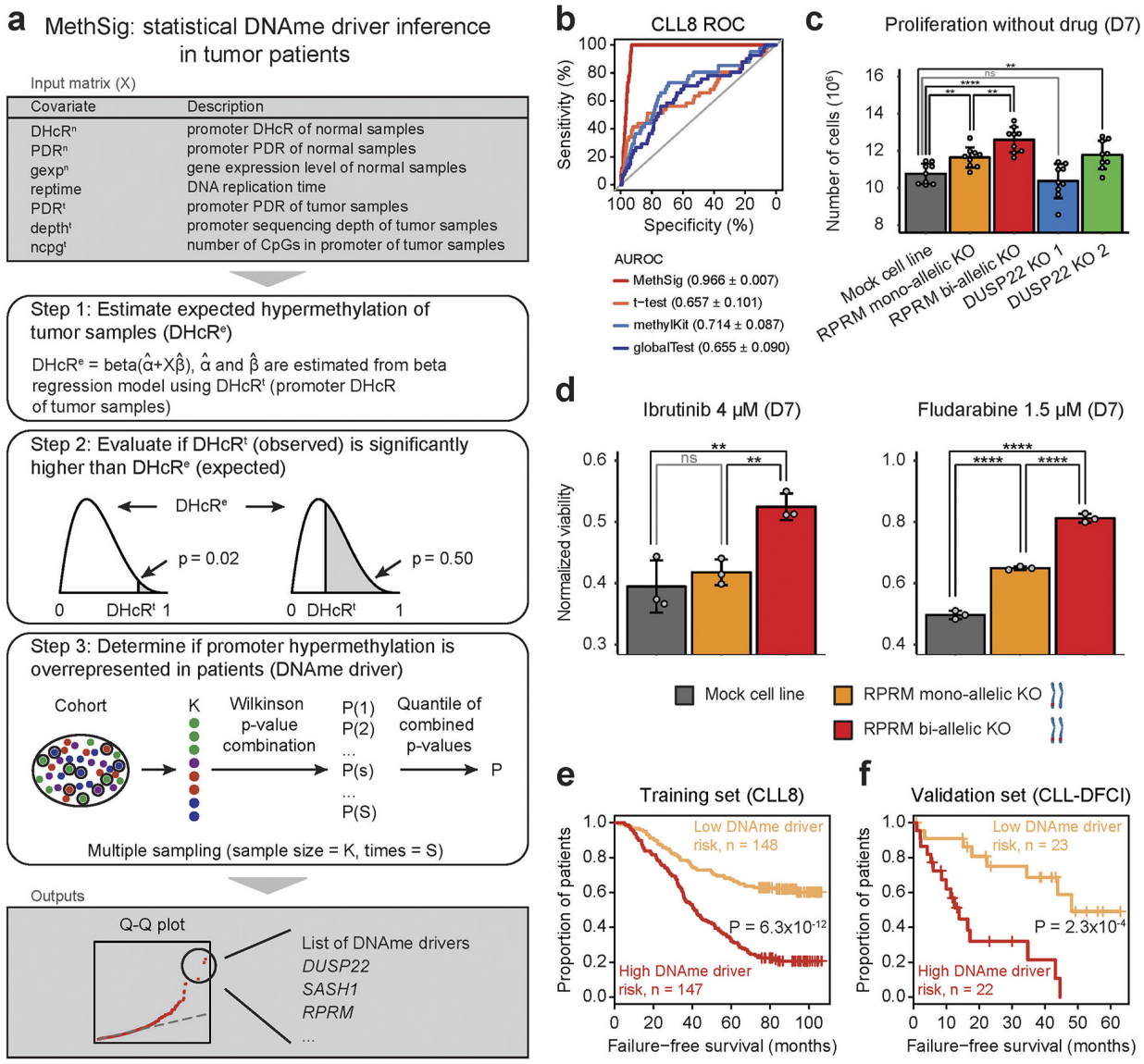

Figure 1. Robust Discovery of Candidate DNA Methylation Cancer Drivers. (a) An overview of the MethSig statistical inference model. Promoter hypermethylation was measured using differentially hypermethylated cytosine ratio (DHcR), defined as the ratio of hypermethylated cytosines (HCs) to the total number of CpGs profiled in promoters. HCs of each sample were defined as CpGs at which DNAme is significantly higher than the average DNAme of tissue-matched control samples (Chi-squared test, FDR = 20%). Methylation heterogeneity was defined by promoter proportion of discordant reads (PDR). n ~ control sample; t ~ tumor; e ~ expected. (b) ROC of MethSig, t-test, methylKit and globalTest in the inference of likely DNAme drivers in CLL8. True positives were defined as genes where promoter hypermethylation is significantly associated with FFS (Benjamini-Hochberg FDR Q < 0.25) and other genes were true negatives. (c) CellTiter-Glo Viability assay of 2 single cell *RPRM* KO clones, 2 single cell *DUSP22* KO clones and a control cell line (cf method) after 7 days of growth without any drug. Nine replicates were performed for each cell line. Data are presented as mean ± s.d. Statistical analysis was performed by one-way ANOVA: ****P < 0.0001; **P < 0.01; ns, not significant. (d) CellTiter-Glo Viability assay after 7 days of exposure to ibrutinib or fludarabine of *RPRM* KO clones. Triplicates were performed for each condition. Data are presented as mean ± s.d. Statistical analysis was performed by one-way ANOVA: ****P < 0.0001; ***P < 0.001; **P < 0.01; *P < 0.05; ns, not significant. (e) A Kaplan-Meier plot showing FFS in CLLs with higher versus lower risk in the training cohort (CLL8). (f) A Kaplan-Meier plot showing FFS in CLLs with higher versus lower risk in the independent validation cohort (CLL-DFCI). In e-f, statistical analysis was performed with log-rank test.

Disclosures

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