

Quantifying *MLH1* promoter methylation via liquid biopsy

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Introduction

MLH1 is one of the primary genes involved in mismatch repair. Promoter methylation of *MLH1* is known to cause abnormally low expression of *MLH1*, which can result in deficient mismatch repair and microsatellite instability. While tissue-based assays for *MLH1* promoter methylation are commercially available, using liquid biopsy to measure *MLH1* promoter methylation is a non-invasive approach that allows for longitudinal monitoring and may be more representative of a patient's cancer compared to a limited sample from a tumor biopsy.

We quantified *MLH1* promoter methylation as part of the Northstar Response assay. We performed analytical testing of *MLH1* promoter methylation measurements in clinical plasma samples with tissue results as ground truth. *MLH1* promoter methylation was also compared with microsatellite instability calls in replicate plasmas. Finally, we present case studies of patients with detected *MLH1* promoter methylation.

Methods

- Plasma and buffy coat samples were processed using Northstar Response, a methylation-based liquid biopsy assay for treatment monitoring, in BillionToOne's CLIA / CAP certified laboratory.
- Northstar Response leverages next-generation sequencing and QCT technology to accurately and precisely count the number of methylated molecules at each targeted location¹. The number of methylated molecules is summed across hypermethylated regions in cancer and normalized to input mass to calculate a Tumor Methylation Score. Only molecules where all CpG sites in the insert region were methylated were counted as being methylated.
- The number of *MLH1* promoter methylated molecules were measured using a single amplicon in the promoter region of *MLH1*. Any non-zero amount of *MLH1* promoter methylation was considered as having detected methylation.
- MLH1* promoter methylation quantification was analytically tested with contrived samples of 30 ng input mass made of universally methylated DNA at varying methylation fractions diluted in genomic DNA from buffy coat pooled across subjects.
- Tumor methylation sequencing and IHC results were queried from the electronic medical record for 21 patients, each with at least one sample with detected *MLH1* promoter methylation.
- MLH1* promoter methylation status was also compared with microsatellite instability calls from Northstar Select², a comprehensive genomic profiling liquid biopsy assay, run on plasma from the same collection time point. Samples with non-zero *MLH1* promoter methylation in the corresponding buffy coat were excluded from analysis.

Results

Linearity

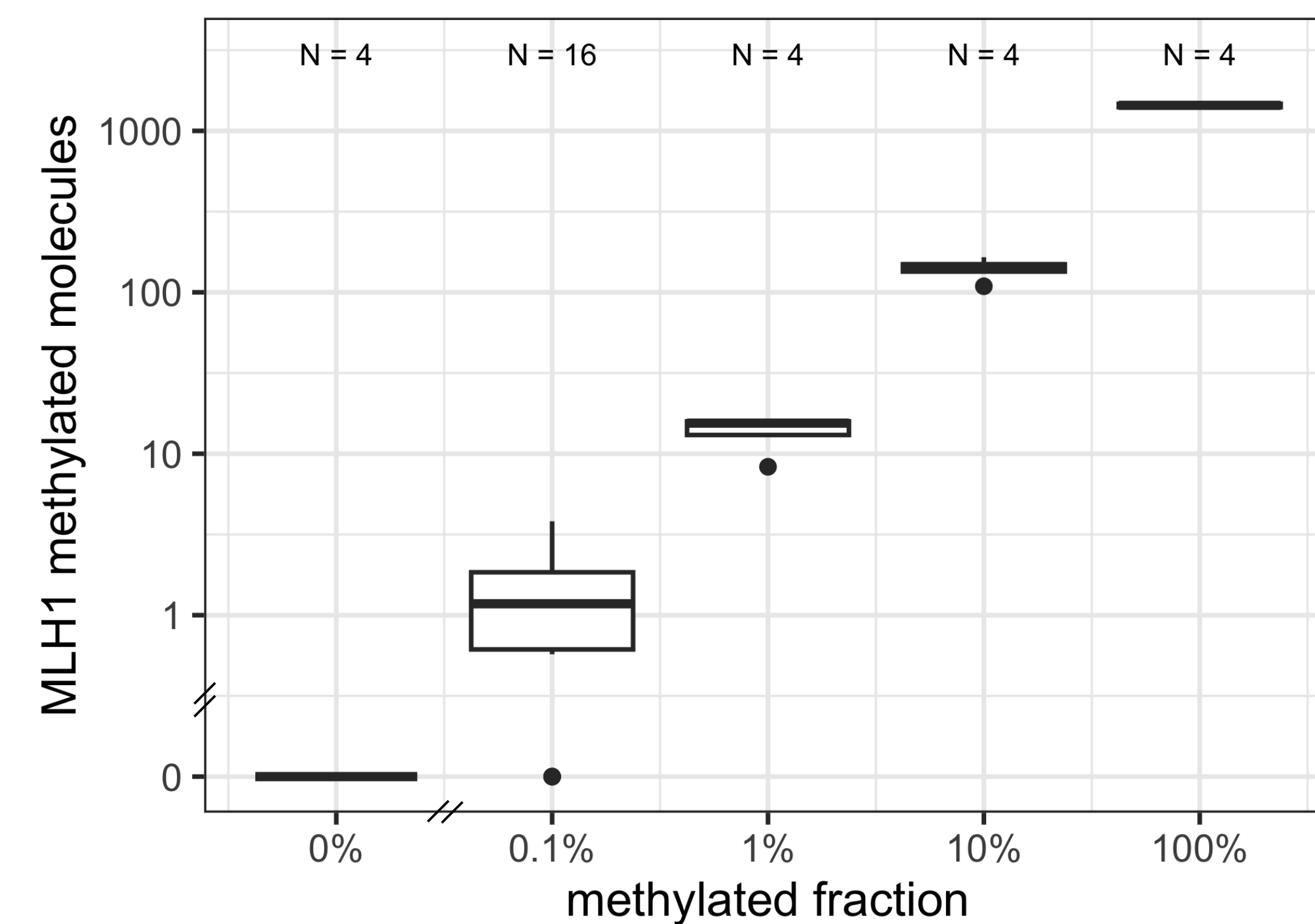


Figure 1: Linearity in *MLH1* promoter methylation in contrived samples across a range of methylation fractions (log-log $R^2 = 0.91$). The limit of detection is between 0.1% and 1%, with 13 out of 16 replicates with detected *MLH1* promoter methylation at 0.1% methylation fraction.

Limit of blank

- 0 out of 335 samples from healthy subjects had *MLH1* promoter methylation.

Prevalence in clinical samples

- 0.76% of samples (78 / 10,254) had *MLH1* promoter methylation detected on Northstar Response.
- Among colorectal cancer patients, 1.6% of samples (24 / 1,517) had *MLH1* promoter methylation detected on Northstar Response.

MLH1 promoter methylation testing

- 5 out of 5 patients with *MLH1* promoter methylation in tissue had *MLH1* promoter methylation detected in at least one sample on Northstar Response.

MLH1 immunohistochemistry

- Out of 10 patients with detected *MLH1* promoter methylation on Northstar Response at any time point and had *MLH1* IHC tissue results, 8 patients showed loss of *MLH1* expression.

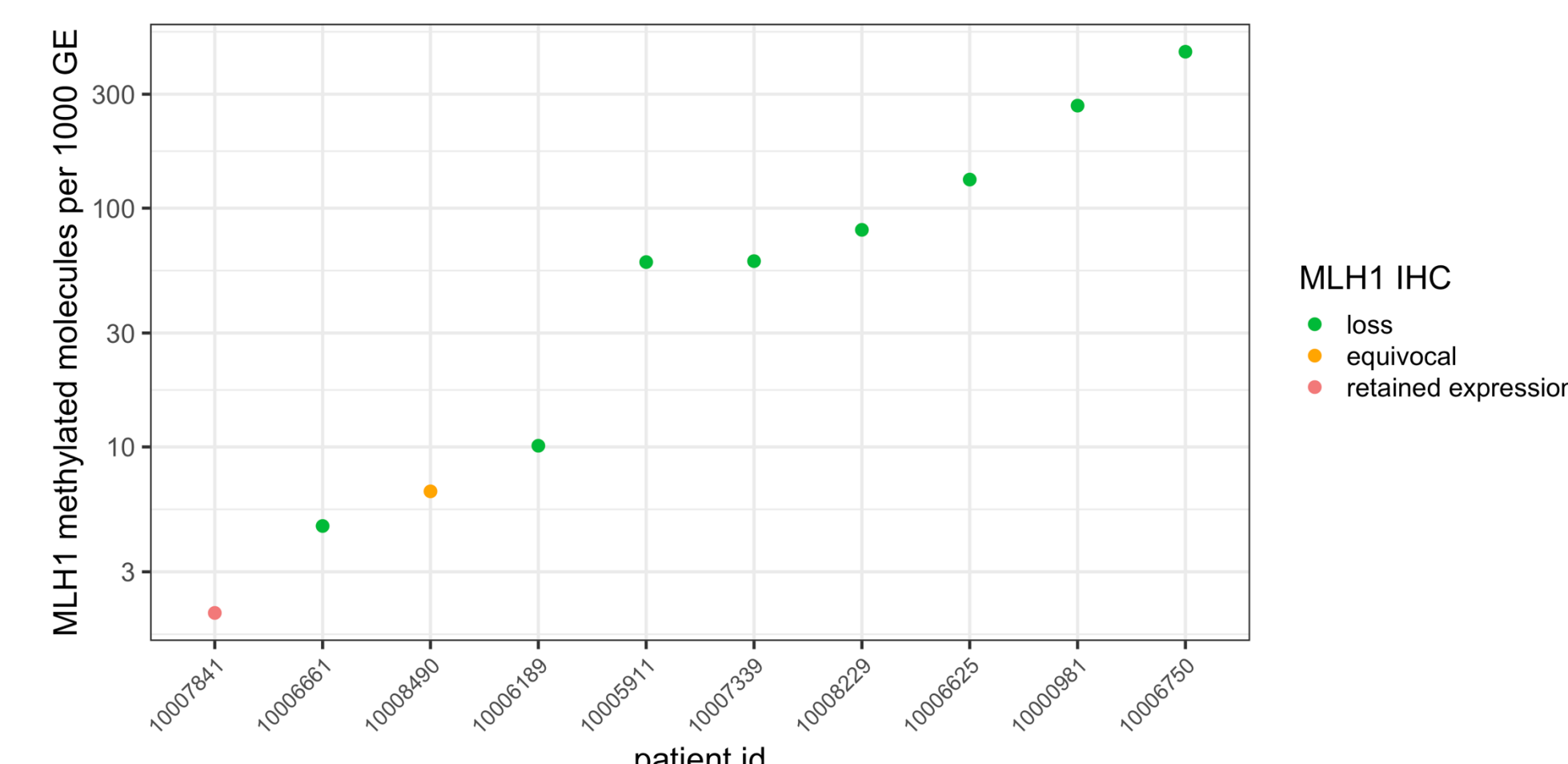


Figure 2: Largest amount of *MLH1* promoter methylation measured over all time points for each patient with reported *MLH1* IHC tissue results. Patients with equivocal or retained *MLH1* expression tend to have fewer *MLH1* promoter methylation molecules detected on Response.

Comparison with microsatellite instability

	all cancers		colorectal cancers	
	MSI-high	MS-stable	MSI-high	MS-stable
<i>MLH1</i> promoter methylation detected	28	26	13	3
No <i>MLH1</i> promoter methylation	31	5,314	5	763

Table 1: *MLH1* promoter methylation compared with microsatellite instability. *MLH1* promoter methylation was detected in 47% of MSI-high samples across all cancer types and 72% of MSI-high colorectal cancer samples.

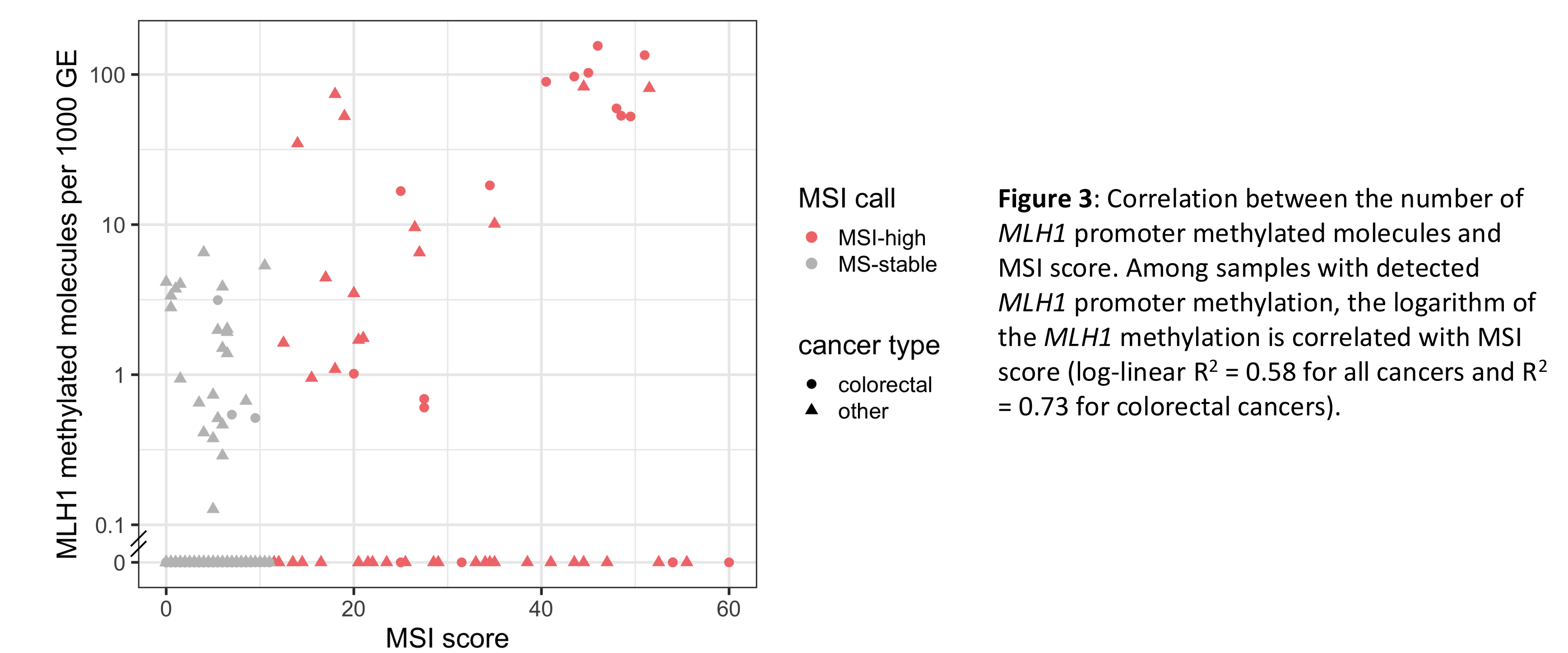


Figure 3: Correlation between the number of *MLH1* promoter methylated molecules and MSI score. Among samples with detected *MLH1* promoter methylation, the logarithm of the *MLH1* methylation is correlated with MSI score (log-linear $R^2 = 0.58$ for all cancers and $R^2 = 0.73$ for colorectal cancers).

Case Studies

48 y.o. female with colorectal adenocarcinoma

Tx: encorafenib + cetuximab initiated 7/12/2024, previously on pembrolizumab

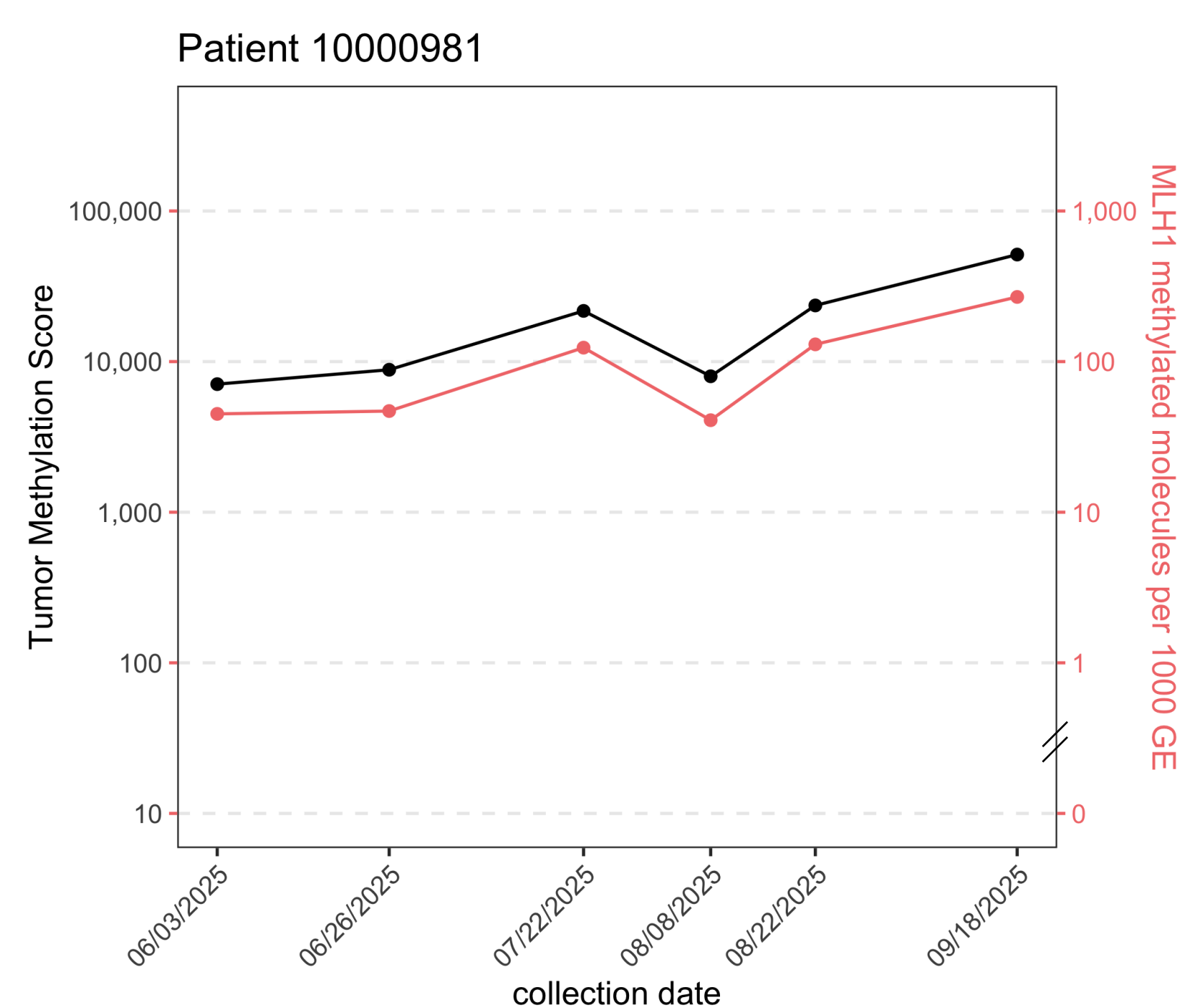


Figure 4: Tumor Methylation Score and *MLH1* promoter methylation over time for Patient 10000981. MSI-high was detected on 08/22/2025 and in three previous time points before June 2025. *MLH1* promoter methylation levels track closely with Tumor Methylation Score. Imaging in September 2025 indicates progression in multiple organ sites.

77 y.o. female with GEJ adenocarcinoma

Tx: pembrolizumab initiated 5/5/2025, discontinued 9/8/2025 and placed on chemotherapy

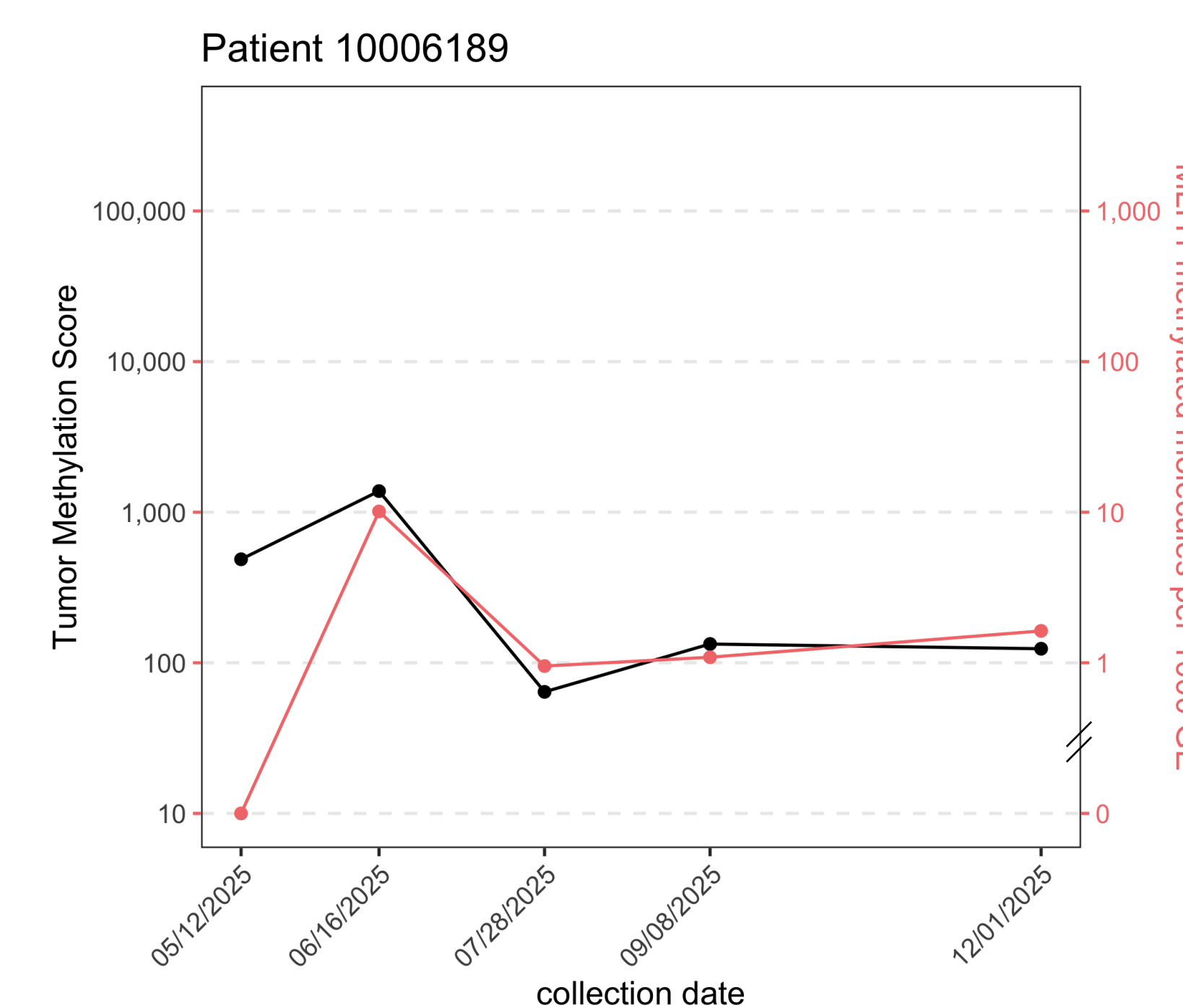


Figure 5: Tumor Methylation Score and *MLH1* promoter methylation over time for Patient 10006189. At the baseline time point, liquid biopsy did not detect *MLH1* methylation or MSI-high. However, both *MLH1* promoter methylation and MSI-high were detected in all subsequent time points. The patient responded very well to immunotherapy and is stable on chemotherapy.

77 y.o. female with gastric adenocarcinoma

Tx: FOLFOX + nivolumab + anti-CLDN18.2 bispecific antibody

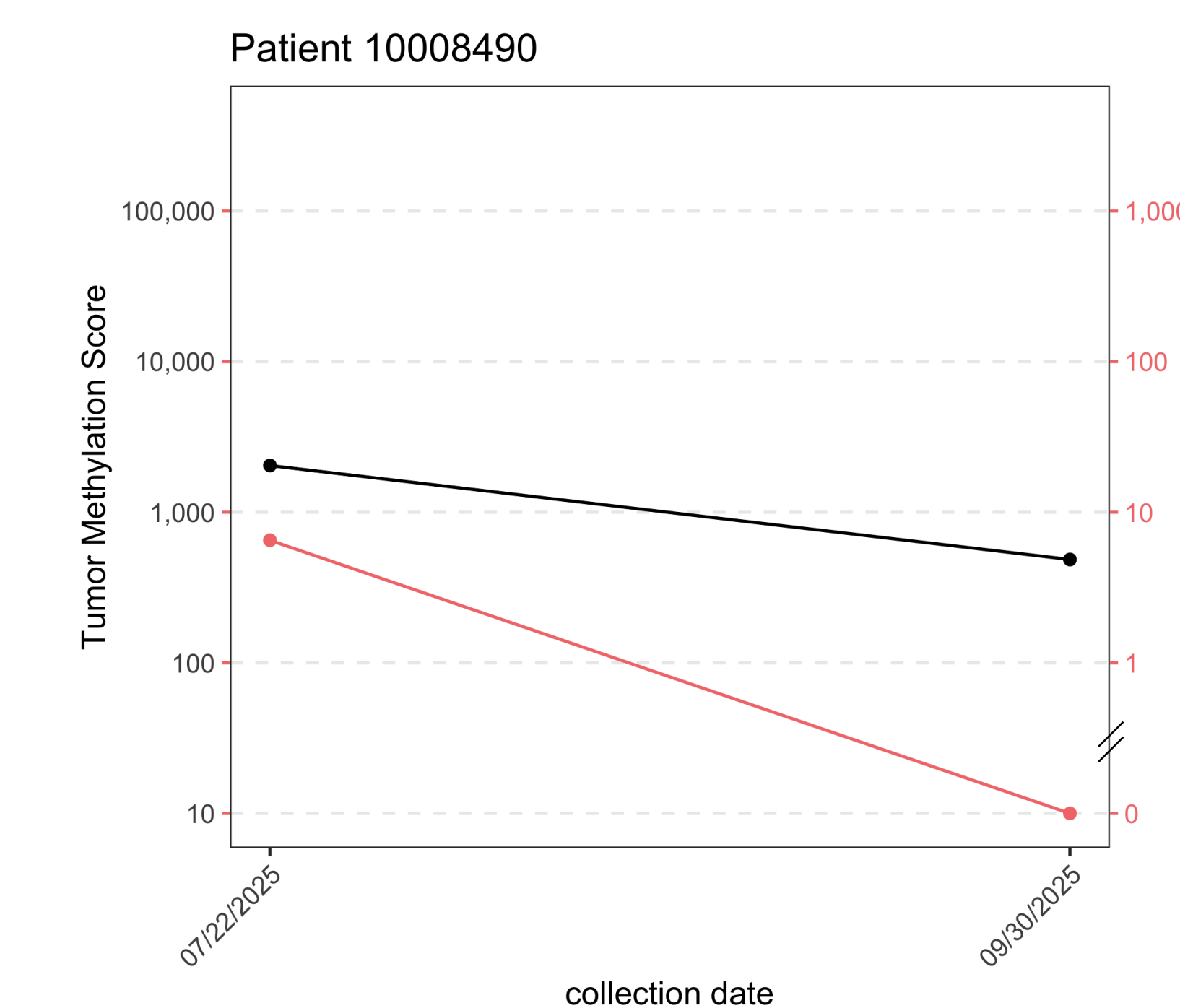


Figure 6: Tumor Methylation Score and *MLH1* promoter methylation over time for Patient 10008490. *MLH1* promoter methylation and MSI-high were detected at the baseline time point, with both *MLH1* promoter methylation and MSI-high becoming undetectable in the subsequent time point, in correspondence with a 4x decrease in Tumor Methylation Score. The patient responded to therapy.

Discussion

- Quantification of *MLH1* promoter methylation with Northstar Response is accurate and precise in analytical testing.
- MLH1* promoter methylation shows promising concordance with tissue methylation sequencing and IHC ground truths in a limited sample set.
- MLH1* promoter methylation was detected in a significant portion but certainly not all MSI-high samples in all cancer types and colorectal cancers, in concordance with reported literature³. Thus, *MLH1* promoter methylation adds partially overlapping yet complementary information to somatic mutation assays for understanding a cancer's mismatch repair status.
- In the three case studies, the *MLH1* promoter methylation tracks closely with overall ctDNA abundance as measured by Tumor Methylation Score.
- Additional clinical validation could help validate *MLH1* promoter methylation from liquid biopsy as a stand-alone biomarker for immunotherapy.

References

- Ye et al. Molecular counting enables accurate and precise quantification of methylated ctDNA for tumor-naïve cancer therapy response monitoring. *Sci Rep* 2025
- Bower et al. Validation of a liquid biopsy assay with increased sensitivity for clinical comprehensive genomic profiling. *J Liq Biopsy* 2025
- Poynter et al. Molecular Characterization of MSI-H Colorectal Cancer by *MLH1* Promoter Methylation, Immunohistochemistry, and Mismatch Repair Germline Mutation Screening. *Cancer Epidemiol Biomarkers Prev* 2008

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