Development of a Liquid Biopsy Based Purely Quantitative Digital Droplet PCR Assay for Detection of *MLH1* Promoter Methylation in Colorectal Cancer Patients

Danyi Wang, PhD[1,2#], Dennis O'Rourke, BS[1,2#], Jorge F Sanchez-Garcia, PhD [1,2], Ti Cai, PhD [1,2], Juergen Scheuenpflug, PhD[1,3] and Zheng Feng, PhD, MD[1,2*]

1. Global Clinical Biomarkers and Companion Diagnostics, Global Early Development
2. EMD Serono Research and Development Institute, Billerica, MA, USA
3. Merck Biopharma; Merck KGaA, Darmstadt, Germany

Co-first author
* Corresponding author

Keywords: *MutL Homolog 1*, Circulating tumor DNA, Methylation, Methylation sensitive restriction enzyme, Droplet digital PCR

Abstract

Background

MutL Homolog 1 (*MLH1*) promotor methylation is associated with microsatellite instability high colorectal cancer (CRC). The strong correlation between methylation status and cancer development and progression has led to a growing interest in the use of methylation markers in circulating tumor DNA (ctDNA) for early cancer detection and longitudinal monitoring. As cancer-specific DNA methylation changes in body fluids are limited, it is particularly challenging to develop clinically applicable liquid biopsy methodologies with high sensitivity and specificity. The purpose of this study was to develop a fit-for-purpose methylation sensitive restriction enzyme (MSRE) based digital droplet PCR (ddPCR) assay to examine *MLH1* promoter methylation in ctDNA in advanced CRC.

Methods

Primers and probes were designed to amplify CpG sites of the *MLH1* promoter. Methylated and unmethylated control genomic DNA were sheared to mimic ctDNA and subjected to MSRE *HpaII* digestion. Plasma samples from 20 healthy donors and 28 CRC patients were analyzed with the optimized MSRE procedure using ddPCR.

Results

Using methylated and unmethylated controls, we optimized the conditions for *HpaII* enzyme digestion to ensure complete digestion and avoid false positives. Based on the results from the ddPCR assay using 1ng circulating cell-free DNA (cfDNA) input from healthy donors or CRC samples, ROC curves were generated with an area under the curve (AUC) value of 0.965. The optimal assay sensitivity and specificity was achieved when 8 positive droplets were used as acceptance criteria (78% sensitivity and 100% specificity). A tiered-based cutoff (20%, 50%, 80% percentile based) was applied to distinguish CRC samples with different methylation level.

Conclusions

Our study demonstrated that the liquid biopsy assay for *MLH1* promoter methylation detection using purely quantitative ddPCR is a simple and ultrasensitive procedure that provides reliable methylation detection in ctDNA. The MSRE ddPCR approach can also be applied to other genes of interest where methylation patterns could reveal clinically relevant information for future clinical biomarker and/or companion diagnostic development.

Background

Methylation changes are present in a variety of cancers, and occur early in carcinogenesis, typically repressing the expression of tumor suppressor genes. Acquired promoter hypermethylation often occurs with global hypermethylation of gene promoters known as CpG island methylator phenotype [1]. These epigenetic changes are highly pervasive across a tumor type and can be a very consistent feature of cancer in contrast to mutations, which typically occur at a wide range of sites. *MLH1* promoter hypermethylation is an important event, silencing the *MLH1* gene expression and preventing the formation of MLH1 protein and normal activation of the DNA repair gene. *MLH1* promoter methylation in sporadic tumors causes high levels of microsatellite instability (MSI). Approximately 15% of CRC cases

are MSI-High (MSI-H); 3% of which include hereditary polyposis colorectal cancer (HNPCC) or Lynch syndrome and sporadic MSI-H CRC, typically caused by somatic methylation of the *MLH1* gene promoter, making up the remaining 12% of cases. [2, 3]. Specifically, within gastric cancer, methylation of CpG islands in the MLH1 promoter region is frequent resulting in MSI and MLH1 inactivation. [4][5-7]. Emerging evidence demonstrated that detection of DNA methylation-based biomarkers could be employed for diagnosis, indicating prognosis and predicting response to therapy [8-10]. Therefore, the assessment of *MLH1* promoter methylation status will add essential clinical utility values across the cancer types.

DNA methylation analysis is not only limited to tissue specimens, but also can be readily extended to be detected in cfDNA/ctDNA, which enables a non-invasive clinical solution. Importantly, the robust and common nature of DNA methylation aberrations in cancer, and the stability of cell-free DNA in body fluids are attractive properties for biomarker development. Recently, MSI status could be determined using blood ctDNA test with comparable performance as tissue biopsies-based test [11], potentially providing an alternative pan cancer based approach to monitor patient's response to therapies.

One of the critical challenges to establishing a DNA methylation assay with clinical usefulness, is identifying the proper region of interest. The investigated region should ideally fulfill the following criteria: first, the region should be unmethylated in normal cases and methylated only in cancer cases; and second, the methylation levels of this region should clearly allow the classification of two distinct populations (for example high versus low or cancer versus healthy). As methylation markers mainly depend on the methylation level of individual CpG sites, the assay sensitivity is limited by the technical difficulties in measuring single-CpG methylation.

Current approaches to methylation analysis depend on bisulfite sequencing, which may cause a degree of DNA degradation and reduce assay reproducibility. Whole-genome bisulfite Sequencing

(WGBS) provides complete methylation information, however this approach is not cost-efficient and has no clinical application path. Subsequent optimized approaches such as reduced-representation bisulfite sequencing (RRBS-Seq) and methylated CpG tandems amplification and sequencing (MCTA-seq) aim at capturing CpG-enriched cfDNA fragments, which would lead to loss of some critical DNA methylation sites [12]. Therefore, it is essential to develop a cost-efficient ctDNA methylation analysis approach with improved sensitivity and specificity which could be implemented in a clinical setting. In this study, we developed a methylation sensitive restriction enzyme (MSRE) digested assay to absolutely quantify the *MLH1* promoter methylation in ctDNA using highly sensitive ddPCR in advanced stage CRC samples.

Materials and Methods

Sample preparation

Preparation of sheared methylated/unmethlated DNA: EpiScope® Unmethylated HCT116 DKO gDNA and EpiScope® Methylated HCT116 gDNA reference cell-line DNA samples (catalog #3521, #3522 Takara Bio, Moutainview CA) were sheared to approximately 180 bp by acoustic shearing to mimic fragmented cfDNA using a Covaris Ultrasonicator (Covaris, Woburn MA). DNA size distribution of sheared DNA was tested using Agilent 2100 Bioanalyzer High Sensitivity DNA Kit (catalog #5067-4626, Agilent Technologies, Santa Clara CA) and quantified by Qubit dsDNA High Sensitivity kit (catalog #Q32854, ThermoFisher Scientific, Waltham MA). Fully methylated and unmethylated DNA were mixed to obtain the following ratios of methylation: 0%, 10%, 25%, 50%, 75%, 100%. Standard curves with known methylation ratios were included in the assay to deduce the methylation level of tumor samples.

Sample acquisition and cfDNA extraction: Healthy donor (n= 20, median age 44 with range 22-71) and advanced colorectal cancer samples (n= 28 (stage IIIB: 21, IIIC: 4, IV:3), median age 63 with range 32-77)

used in this study were procured from BioIVT. The written informed consent was received from all subjects used in this study and all samples were collected under IRB approved protocols, as defined by the specimen provider BioIVT. Ethnical approval was obtained from BioIVT ethics committee. This study was conducted under the guidelines put forth into the Declaration of Helsinki. No clinical treatment was applied to the CRC patients before blood collection. Specifically, blood samples were collected using blood collection tubes containing EDTA (BD Vacutainer®). Plasma isolation was performed within 4 hours of blood collection using a double spin protocol. Plasma nucleic acid was extracted using QIAamp MinElute ccfDNA Kit (catalog #55204, Qiagen, Valencia CA) according to manufacturer's instructions. Approximately 5mL EDTA plasma was used for the extraction of cfDNA from normal patient samples. CRC plasma samples ranged in volume from 1-4mL and were brought up to 5mL each by adding PBS to necessary samples for uniform extraction and to avoid freeze/thaw cycles. Purified nucleic acids were collected in 35µl elution buffer for both normal samples and CRC samples. Two microliters were used for quantification of extracted cfDNA using Qubit and Bioanalyzer.

Methylation sensitive restriction enzyme (MSRE) digestion

MSREs cleave DNA at specific unmethylated-cytosine residues and DNA is then amplified by PCR following digestion. As unmethylated DNA is digested by the MSRE reaction, the only amplification products detected are methylated DNA. Here, a MSRE protocol was optimized for selected *MLH1* promoter region by *HpaII* enzymesTo ensure ful digestion of the DNA before PCR amplification to generate accurate data, different *HpaII* enzymes were tested at various concentrations, and with various incubation times. First, two *HpaII* enzymes were identified and tested for complete digestion. Methylated DNA standards and unmethylated DNA standards were treated with each enzyme per manufacturer's recommendation as well as run in mock digestion reactions where no enzyme was added. These reactions were tested in qPCR and the calculated percent methylation was determined.

The percent methylation was calculated by subtracting the mean Cq values of the MSRE digested templates from the corresponding mock digest (ΔCq). The methylation level was then calculated using the formula;

Methylation level = (2^-ΔCq)*100

Ultimately, we determined the ThermoFisher *HpaII* enzyme (catalog #ER0511 Waltham, MA USA) was the better of the two enzymes (data not shown). Next, we tested different incubation times (2-16 hours) and concentrations (2µL/reaction and 0.5µL/reaction) to ensure complete digest of the unmethylated samples. Using the same calculations as above, we determined a 3hour incubation with 2µL of enzyme per reaction was sufficient for complete digestion and extending the incubation time did not significantly improve digestion. Our final MSRE reaction contained: 2µl 10 × Tango Buffer 3, 2µl *HpaII* enzyme and 5ng DNA in total 20µl final volume. Mock reactions contained 2µl 10 × Tango Buffer 3 and 2µl HpaII enzyme. Reactions were incubated at 37°C for 3 hours and subsequently heat inactivated for 20 min at 65°C. The resulting, 0.25 ng/µL digested DNA was stored at -20°C for subsequent analysis.

Primers and probes design

Beta-Actin (*ACTB*) and Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) genes were used as the endogenous reference genes for qPCR and acquired from ThermoFisher (ACTB Taqman Assay ID: Hs01060665_g1, GAPDH Taqman Assay ID: Hs02786624_g1, FAM-MGB). Primers and probe (Forward: 5'-GATGAGGCGGCGACAGA reverse: 3'-AGAAGCAAGATGGAAGTCGAC, probe: 5'-ACCAAATAACGCTGGGTC) of *MLH1* gene promoter were designed to specially amplify the CpG sites and to be located up-stream of exon one (NG_007109.2 region: 4,591 -4,710), which was interpreted as the promoter regions of the *MLH1* gene using ABI Primer Express version 3.0 (Thermofisher, Waltham MA). The primers and probe were supplied by ABI and optimized for the present study. Briefly, qPCR using FastSYBR Green Master Mix (cat # 4385610, ThermoFisher, Waltham MA) was used to check for

nonspecific product formation by dissociation-curve while qPCR using TaqMan Universal Master Mix II (Cat # 4440038, ThermoFisher, Waltham, MA) was used to test the probes and determine the qPCR efficiency.

Real-Time PCR (qPCR)

qPCR was performed in accordance with the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines [13]. The reaction volume was set as 20µL and each reaction contained 1× Taqman Universal PCR mastermix (cat # 4304437, Life Technologies, Carlsbad CA), 900nM final concentration forward and reverse primers, 200nM Probes and 6ng template DNA. At least one PCR No Template Control (NTC) was run for each assay as controls on all plates. All qPCR analyses were performed in triplicate on a real time PCR system (Model 7500 Fast, Thermo Fisher Scientific®). qPCR thermal cycling conditions were as follows: 95°C for 10min, followed by 40 cycles of 95°C for 15s, 60°C for 1 min. The SDS software v2.4 (Life Technologies) was used to calculate the quantification cycle (Cq) value. Invalid PCR results indicated by high cycle threshold (CT) values > 33 were omitted from the analysis.

Droplet digital PCR (ddPCR)

ddPCR was performed in accordance with the Minimum Information for Publication of Digital Quantitative PCR Experiments (digital MIQE) guidelines [14] using Bio-Rad QX200 droplet digital system (Bio-Rad, Hercules, CA, USA). Droplets were generated in the QX200 automated droplet generator, amplified on an Applied Biosystems VeritiDx thermal cycler using standard ddPCR cycling conditions with an anneal/extension temperature of 55°C and read using the QX200 droplet reader (Bio-Rad). The analysis was performed using QuantaSoft software (v1.6.6.0320, Bio-Rad, Pleasanton, CA) as follows. Positive droplets, containing amplification products, were discriminated from negative droplets without

amplification products by applying a fluorescence amplitude threshold. The threshold was set manually, using the 1D amplitude chart. To assess the specificity of the ddPCR assay, initial experiments were conducted using 100% methylated DNA and 100% unmethylated DNA.

Data analysis

Values are expressed as mean ± SD, unless otherwise stated. Differences between groups were tested using t test, Mann-Whitney U test, or ANOVA, where appropriate.

Assay performance parameters (sensitivity, specificity and ROC curves) were calculated using the 28 cancer samples and 20 healthy donors. To determine the optimal DNA input amount of the qPCR assay, the standard DNA sample was first prepared as 100 ng μL^{-1} and then was gradient diluted from 100ng μL^{-1} to 1ng μL^{-1}. For the optimal input amount of the ddPCR assay, the DNA sample was gradient diluted from 6ng μL^{-1} to 0.094ng μL^{-1}. Each assay was conducted in at least three independent runs with four replicates in a run. The limit of detection (LOD) is defined as the lowest detectable percentage of methylation above background and was determined in the ddPCR assay by using the methylation spike-in samples. The linear regression analyses were performed to obtain the correlation coefficients coefficient of determination (R^2) and the LOD. Receiver operating characteristic (ROC) curves were calculated to establish the cutoffs or threshold methylation level of *MLH1* promoter using the Web-based calculator for ROC curves from online tool http://www.rad.jhmi.edu/jeng/javarad/roc/JROCFITi.html which calculates the cutoff based on the value corresponding with the highest average of sensitivity and specificity. Area under the curve (AUC) indicates the value of the test. Correlation between variables was tested using Spearman's rank correlation. The Bonferroni correction was used in case of multiple pairwise comparisons.

Results

Assay performance

FastSYBR Green PCR revealed a single peak of amplified product following melt curve analysis and TaqMan qPCR efficiency of 94.98% (**Figure 1a**) with an input range of 100ng-3.125ng. The qPCR assay showed very good linearity >0.99 indicating good performance of the assay across this range of input. The linearity of the ddPCR assay was determined by titrating the input of sheared methylated control DNA and sheared unmethylated control DNA from 6ng to 0.096ng and measuring the number of positive droplets detected. In ddPCR assay, using sheared synthetic DNA, *MLH1* methylation was able to be detected using as low as 0.096ng of DNA (**Figure 1b**).

To determine the optimal input amount for this assay, a titration of input amounts was tested from 4ng DNA to 0.125ng DNA in two-fold dilutions. Four different conditions were tested including methylated DNA treated with *HpaII* enzyme, methylated DNA untreated, unmethylated DNA treated with *HpaII* enzyme, and unmethylated DNA untreated. With 0.125-4ng input of synthetic DNA, *MLH1* methylation levels detected in the methylated treated, methylated untreated, and unmethylated untreated sample remained fairly constant, the unmethylated enzyme treated sample showed a significant reduction in signal (P <0.001, **Figure 2**). To achieve the optimal signal/noise ratio and operational/practical advantage, 1ng was selected as the optimal input for further analysis.

In terms of the percent methylation, the LOD of this assay was determined by spiking different percentages of methylated DNA into normal plasma, extracting cfDNA and then testing 1ng of DNA in ddPCR to detect *MLH1* methylation levels. While the entire titration showed a good linear relationship overall (R^2 = 0.985, **Figure 3**), levels below 10% methylation displayed a weak linear relationship (R^2= 0.427) when examined alone. Therefore, we can consider 10% methylation, or approximately 8 positive droplets, as the LOD for the ddPCR assay.

Testing *MLH1* methylation in healthy controls and CRC patient samples

We tested 28 advanced stage CRC cancer samples and 20 normal healthy samples in ddPCR assay to assess the level of *MLH1* methylation in each group (**Figure 4**). Except for one sample that failed due to insufficient amount of cfDNA, the rest of samples returned quality ddPCR results. First, both groups when untreated showed a significant increase in positive droplets over the treated samples (P <0.001). Normal control samples without enzyme treatment yielded 86.45 $\pm$ 41.45 positive droplets for *MLH1* while cancer samples without enzyme treatment yielded an average of 117.39 $\pm$ 43.60 positive droplets for *MLH1* (P > 0.05). Normal healthy samples treated with *HpaII* enzyme yielded 1.63 $\pm$ 1.84 positive droplets indicating that the majority of *MLH1* in normal samples is unmethylated. In contrast the cancer samples treated with *HpaII* enzyme yielded an average of 15.91 $\pm$ 9.76 positive droplets suggesting higher methylation levels in the cancer population. A t-test of the cancer and healthy volunteers' samples was shown to be a statistically significant difference (P < 0.001).

The data generated here could be used to create a receiver operating characteristic (ROC) curve to differentiate cancer from normal patients with an area under the curve (AUC) of 0.965. The positive droplet count for the normal and CRC patients could then be converted to percent methylation using the equation of the line of best fit from the methylation spike-in standard curve (**Figure 5**a). In our cohort all normal patient samples fell below the limit of detection and 21/27 (77.8%) of cancer patients were above this threshold.

By correlating promoter methylation status with age, an increase of methylation levels of *MLH1* with aging observed in CRC samples (R = 0.21, P = 0.29) (Figure 6a). Females showed higher *MLH1* promoter methylation levels with respect to males (P = 0.23) (Fig. 6b).

Cut-off value determination strategies

Based on the results of the ddPCR assay, we set up the cut-off values of *MLH1* promoter methylation for discriminating the healthy cases and the malignant cases. Data showed that the optimal sensitivity and

specificity was displayed by the assay when using 5 positive droplets as cutoff value (88% sensitivity and 95% specificity). However, this cut-off was below the reported LOD of the assay. Therefore, the LOD, 8 positive droplets was used as an optimal cut-off to distinguish healthy from CRC patients (78% sensitivity and 100% specificity, **table 1**).

A tiered-based (20^{th}, 50^{th}, 80^{th} percentile) cutoff was applied to distinguish CRC samples with different methylation level by using the spike-in normal plasma data. A cut-off at the 80^{th} percentile could identify those patients with over *40% MLH1* methylation, those samples falling in between the 50^{th} percentile and 80^{th} would be identified as 25-40% *MLH1* methylation and those samples below the 20^{th} percentile could be considered as 'low' *MLH1* methylation (<10% methylation) (**Figure 5b**). However, further clinical analysis of samples would be needed to elucidate the optimal percentiles of the cut-off to ensure a meaningful analysis.

Discussion

Given the stability of DNA methylation in body fluids, cfDNA methylation markers using plasma samples are a promising matrix for non-invasive clinical biomarker implementation [15]. In addition, global hypomethylation of the human genome paired with CpG islands of hypermethylation, allow for a minimal number of loci to be targeted and yet have sufficient test coverage [16]. These features make cancer-associated DNA methylation changes an attractive source for clinical biomarker discovery[17, 18]. Therefore, targeting DNA methylation changes as cancer biomarkers hold potential for detection and minimal residual disease. However, for such assays to be adopted into a clinical setting, high sensitivity and specificity need to be demonstrated. In many cases it is crucial for the assay to detect only a few cancer specific DNA methylation changes in a large background of normal DNA methylation

patterns. We developed a MSRE digested ddPCR assay to quantitate the methylation of *MLH1* promoter in cfDNA for detection of low concentrations of methylated DNA fragments in advanced stage CRC.

While many other ctDNA methylation biomarkers for cancer have been reported [9, 19-21], these mainly depend on the methylation level of individual CpG sites, which limits the detection sensitivity. Until now, only one blood-based assay that detects *SEPT9* gene methylation was approved by the U.S. Food and Drug Administration for CRC screening with a sensitivity of ~70% and a specificity of ~80% or above [22]. Our assay tests the methylation level of the *MLH1* promoter region and offer a cost efficient, clinical applicable alternative approach compared to bisulfite-treatment approaches. A MSRE assay followed by ddPCR achieves absolute quantification of the degree of methylation of the DNA with minimal (1ng) cfDNA input and the assay does not rely on a standard calibration curve. Using the control DNA samples, we have proven that the LOD of the ddPCR assay is supersensitive, with 25-fold improvement compared to the conventional qPCR-based assay. Based on the results of the ddPCR assay, ROC curve produced from the data gave an AUC of 0.965 indicating the assay does a good job of differentiating normal samples from cancer samples.

Moreover, we applied comprehensive approaches to determine the cut-off value for potential clinical applications. First, a statistical cut-off of 8 positive droplets with optimal sensitivity and specificity (78% sensitivity and 100% specificity) of the assay for discriminating healthy volunteers from CRC patients was determined. Second, a tiered cut-off was applied to distinguish CRC samples with different methylation levels (20%, 50%, 80%). Coppede et al. demonstrated that *MLH1* promoter level in CRC tissue is highly correlated with MLH1 protein expression using immunostaining [23].Their results showed that in the vast majority of cases (80%) if promoter methylation was present, MLH1 immunostaining was negative, and, in contrast, in 93% of cases with 0% promoter methylation MLH1 immunostaining was positive. In the 20% of cases with both positive MLH1 promoter methylation and positive immunostaining, the overall promoter methylation level was low [23]. Therefore, it is

reasonable to speculate that the tiered *MLH1* methylation level is linked to MLH1 protein expression and could be further developed as a complementary assay of IHC tests for mismatch repair deficient (dMMR) to identify the MSI-H or dMMR tumor status.

Our analysis revealed an increased ctDNA *MLH1* promoter methylation in plasma with aging, as well as gender differences with females showing higher hMLH1 promoter methylation than males in CRC patients (**Figure 6**). Although there is no statistical significance due to the small sample size, our results showed the similar trend observed in CRC tissue as previous reported [23]. These findings provide evidence that *MLH1* promoter methylation signals could be captured in plasma. Recent publications have established an association between the epigenetic alterations found in primary tumor specimens and in plasma, suggesting epigenetic biomarkers from liquid biopsy can be implemented as surrogate tumor biomarkers [24-28].

The tissue based pooled frequency of *MLH1* promoter methylation in CRC is reported as ~18% in sporadic CRC [29, 30]. DNA methylation of *MLH1* has been found in more than 80% of sporadic CRC with MSI and predict the mutation load. Thus, the assessment of *MLH1* promoter methylation status will add essential diagnostic and prognostic information with clinical relevance. In our study, a theoretical cut-off methylation levels based on real-world data was set at ~24 positive ddPCR droplets (equivalent to 40% methylation in **Figure 5b**) in CRC plasma samples. As the current study is relatively small, proper validation using a different cohort with a larger sample size is crucial. Comprehensive and fit-for-purpose cut-off determination strategies need to be considered to fulfill the different project goals and clinical application specific requirements.

Conclusion

The developed liquid biopsy assay for detecting *MLH1* promoter methylation by ddPCR is a convenient and cost-efficient approach which could be easily transferred to a clinical setting. The simple MSRE ddPCR procedure and high sensitivity of the assay provides a reliable ctDNA methylation biomarker assay to reflect single or multiple DNA methylation changes originating in tumor cells. Furthermore, this approach can be applied to other genes of interest, or cancer indications, where methylation patterns could reveal crucial clinically relevant information for future clinical biomarker and/or companion diagnostic development and implementation.

Declarations

Ethics approval and consent to participate

The written informed consent was received from all subjects used in this study and all samples were collected under IRB approved protocols, as defined by the specimen provider BioIVT. Ethnical approval was obtained from BioIVT ethics committee.

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

Funding

This project was supported by the EMD Serono, Merck KGaA, Darmstadt, Germany

Author Contributions

DW contributed in the planning and execution of experiments, data analysis and writing of article.

DO conducted in house experiments, initial data analysis, and writing of article.

JSG contributed to data analysis.

TC provided scientific expertise in study planning and manuscript drafting.

JS provided scientific expertise.

ZF provided scientific expertise, advised in experimental planning and data analysis as well as substantial contribution to composing the manuscript.

Acknowledgements

The authors would like to acknowledge Waldemar Miller and Xiaoyan Yin for their biostatistical analysis and guidance.

References

1. Irizarry RA, Ladd-Acosta C, Wen B, Wu Z, Montano C, Onyango P, Cui H, Gabo K, Rongione M, Webster M *et al*: **The human colon cancer methylome shows similar hypo- and hypermethylation at conserved tissue-specific CpG island shores**. *Nat Genet* 2009, **41**(2):178-186.
2. Chen W, Swanson BJ, Frankel WL: **Molecular genetics of microsatellite-unstable colorectal cancer for pathologists**. *Diagnostic pathology* 2017, **12**(1):24.
3. Boland CR, Goel A: **Microsatellite instability in colorectal cancer**. *Gastroenterology* 2010, **138**(6):2073-2087.e2073.
4. Yamamoto H, Imai K: **Microsatellite instability: an update**. *Archives of toxicology* 2015, **89**(6):899-921.
5. Liu L, Yang X: **Implication of Reprimo and hMLH1 gene methylation in early diagnosis of gastric carcinoma**. *International journal of clinical and experimental pathology* 2015, **8**(11):14977-14982.
6. Cabel L, Proudhon C, Romano E, Girard N, Lantz O, Stern MH, Pierga JY, Bidard FC: **Clinical potential of circulating tumour DNA in patients receiving anticancer immunotherapy**. *Nature reviews Clinical oncology* 2018, **15**(10):639-650.
7. Hu G, Qin L, Zhang X, Ye G, Huang T: **Epigenetic Silencing of the MLH1 Promoter in Relation to the Development of Gastric Cancer and its use as a Biomarker for Patients with Microsatellite Instability: a Systematic Analysis**. *Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology* 2018, **45**(1):148-162.
8. Gai W, Sun K: **Epigenetic Biomarkers in Cell-Free DNA and Applications in Liquid Biopsy**. *Genes* 2019, **10**(1).

9. Warton K, Mahon KL, Samimi G: **Methylated circulating tumor DNA in blood: power in cancer prognosis and response**. *Endocr Relat Cancer* 2016, **23**(3):R157-171.
10. Lissa D, Robles AI: **Methylation analyses in liquid biopsy**. *Translational lung cancer research* 2016, **5**(5):492-504.
11. Willis J, Lefterova MI, Artyomenko A, Kasi PM, Nakamura Y, Mody K, Catenacci DVT, Fakih M, Barbacioru C, Zhao J *et al*: **Validation of Microsatellite Instability Detection Using a Comprehensive Plasma-Based Genotyping Panel**. *Clin Cancer Res* 2019, **25**(23):7035-7045.
12. Liu X, Ren J, Luo N, Guo H, Zheng Y, Li J, Tang F, Wen L, Peng J: **Comprehensive DNA methylation analysis of tissue of origin of plasma cell-free DNA by methylated CpG tandem amplification and sequencing (MCTA-Seq)**. *Clin Epigenetics* 2019, **11**(1):93.
13. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL *et al*: **The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments**. *Clin Chem* 2009, **55**(4):611-622.
14. Huggett JF, Foy CA, Benes V, Emslie K, Garson JA, Haynes R, Hellemans J, Kubista M, Mueller RD, Nolan T *et al*: **The digital MIQE guidelines: Minimum Information for Publication of Quantitative Digital PCR Experiments**. *Clin Chem* 2013, **59**(6):892-902.
15. Galanopoulos M, Tsoukalas N, Papanikolaou IS, Tolia M, Gazouli M, Mantzaris GJ: **Abnormal DNA methylation as a cell-free circulating DNA biomarker for colorectal cancer detection: A review of literature**. *World J Gastrointest Oncol* 2017, **9**(4):142-152.
16. Pfeifer GP: **Defining Driver DNA Methylation Changes in Human Cancer**. *International journal of molecular sciences* 2018, **19**(4).
17. Locke WJ, Guanzon D, Ma C, Liew YJ, Duesing KR, Fung KYC, Ross JP: **DNA Methylation Cancer Biomarkers: Translation to the Clinic**. *Front Genet* 2019, **10**:1150.
18. Taryma-Leśniak O, Sokolowska KE, Wojdacz TK: **Current status of development of methylation biomarkers for in vitro diagnostic IVD applications**. *Clin Epigenetics* 2020, **12**(1):100.
19. Leygo C, Williams M, Jin HC, Chan MWY, Chu WK, Grusch M, Cheng YY: **DNA Methylation as a Noninvasive Epigenetic Biomarker for the Detection of Cancer**. *Disease markers* 2017, **2017**:3726595.
20. Dong L, Ren H: **Blood-based DNA Methylation Biomarkers for Early Detection of Colorectal Cancer**. *Journal of proteomics & bioinformatics* 2018, **11**(6):120-126.
21. Nunes SP, Moreira-Barbosa C, Salta S, Palma de Sousa S, Pousa I, Oliveira J, Soares M, Rego L, Dias T, Rodrigues J *et al*: **Cell-Free DNA Methylation of Selected Genes Allows for Early Detection of the Major Cancers in Women**. *Cancers (Basel)* 2018, **10**(10).
22. Semaan A, van Ellen A, Meller S, Bergheim D, Branchi V, Lingohr P, Goltz D, Kalff JC, Kristiansen G, Matthaei H *et al*: **SEPT9 and SHOX2 DNA methylation status and its utility in the diagnosis of colonic adenomas and colorectal adenocarcinomas**. *Clin Epigenetics* 2016, **8**:100.
23. Coppedè F, Migheli F, Lopomo A, Failli A, Legitimo A, Consolini R, Fontanini G, Sensi E, Servadio A, Seccia M *et al*: **Gene promoter methylation in colorectal cancer and healthy adjacent mucosa specimens: correlation with physiological and pathological characteristics, and with biomarkers of one-carbon metabolism**. *Epigenetics* 2014, **9**(4):621-633.
24. Li W, Zhang X, Lu X, You L, Song Y, Luo Z, Zhang J, Nie J, Zheng W, Xu D *et al*: **5-Hydroxymethylcytosine signatures in circulating cell-free DNA as diagnostic biomarkers for human cancers**. *Cell research* 2017, **27**(10):1243-1257.
25. Chan KC, Jiang P, Chan CW, Sun K, Wong J, Hui EP, Chan SL, Chan WC, Hui DS, Ng SS *et al*: **Noninvasive detection of cancer-associated genome-wide hypomethylation and copy number aberrations by plasma DNA bisulfite sequencing**. *Proc Natl Acad Sci U S A* 2013, **110**(47):18761-18768.

26. Guo S, Diep D, Plongthongkum N, Fung HL, Zhang K, Zhang K: **Identification of methylation haplotype blocks aids in deconvolution of heterogeneous tissue samples and tumor tissue-of-origin mapping from plasma DNA**. *Nat Genet* 2017, **49**(4):635-642.
27. Schwarzenbach H, Hoon DS, Pantel K: **Cell-free nucleic acids as biomarkers in cancer patients**. *Nat Rev Cancer* 2011, **11**(6):426-437.
28. Krishnamurthy N, Spencer E, Torkamani A, Nicholson L: **Liquid Biopsies for Cancer: Coming to a Patient near You**. *J Clin Med* 2017, **6**(1).
29. Li X, Yao X, Wang Y, Hu F, Wang F, Jiang L, Liu Y, Wang D, Sun G, Zhao Y: **MLH1 promoter methylation frequency in colorectal cancer patients and related clinicopathological and molecular features**. *PLoS One* 2013, **8**(3):e59064.
30. Shen S CX, Li H, Sun L, Yuan Y: **MLH1 Promoter Methylation and Prediction/Prognosis of Gastric Cancer: A Systematic Review and Meta and Bioinformatic Analysis**. *J Cancer* 2018, **9**((11)):1932–1942.

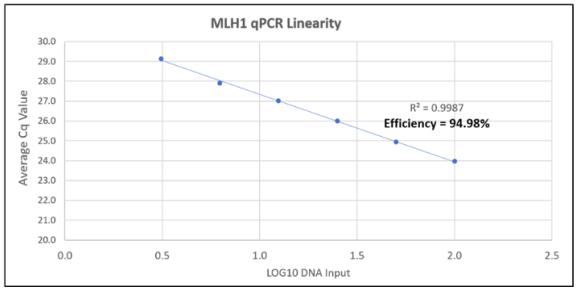
Table 1. Summary of sensitivity and specificity calculations at different positive droplets cut off.

CUTOFF	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Sensitivity	0.96	0.96	0.93	0.93	**0.93***	0.93	0.89	**0.78#**	0.78	0.70	0.67	0.67	0.52	0.48	0.44	0.44	0.44	0.41	0.26	0.26
Specificity	0.50	0.60	0.85	0.90	**0.95***	0.95	0.95	**1.00#**	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
ACCURACY	0.77	0.81	0.89	0.91	0.94	0.94	0.91	0.87	0.87	0.83	0.81	0.81	0.72	0.70	0.68	0.68	0.68	0.66	0.57	0.57
PPV	0.72	0.76	0.89	0.93	0.96	0.96	0.96	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00

* The optimal sensitivity and specificity were displayed at 5 positive droplets as cutoff value (88% sensitivity and 95% specificity).

As 5 positive droplets was below the reported LOD, 8 positive droplets were used as an exploratory cut-off to distinguish healthy from CRC patients (78% sensitivity and 100% specificity).

a.



b.

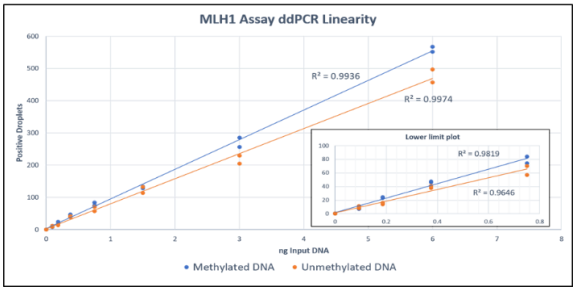


Figure 1. **Titration of input amount using synthetic DNA to determine minimum input for qPCR and ddPCR. a.** Linearity of MLH1 qPCR assay showed very good linearity 0.9987 and efficiency 94.98% with an input range of 100ng-3.125ng control DNA. **b.** Comparative analysis of MLH1 promotor methylation using ddPCR demonstrated the ability to detect MLH1 copies in this assay at very low input level (<1ng equivalent input). As low as 0.096ng of MLH1 methylation DNA could be detected by ddPCR.

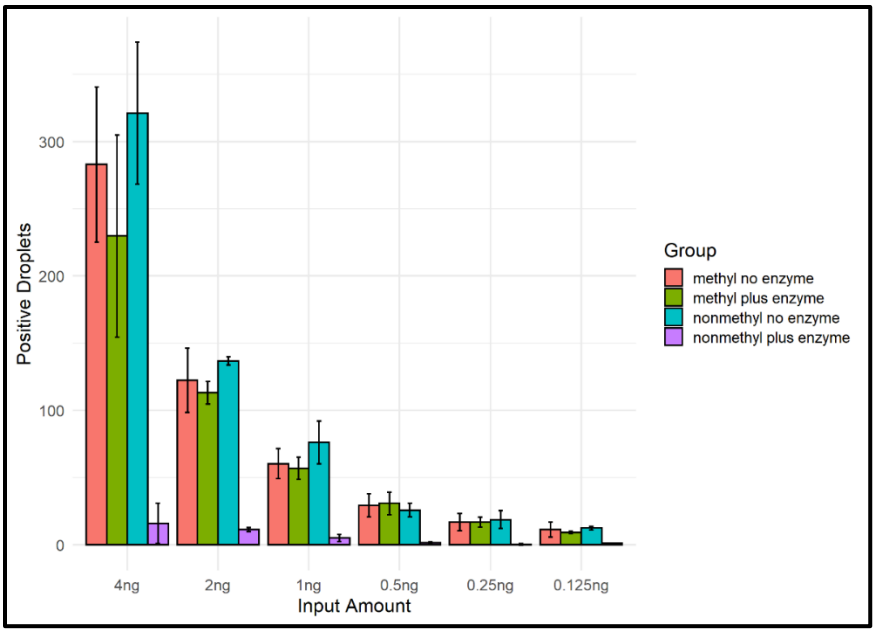


Figure 2. Sample input optimization for *MLH1* promoter methylation ddPCR assay. With 0.125-4ng input of synthetic DNA, *MLH1* methylation levels detected in the methylated treated, methylated untreated, and unmethylated untreated sample remained fairly constant, the unmethylated enzyme treated sample showed a significant reduction in signal (*P* <0.001). To achieve the optimal signal/noise ratio and operational/practical advantage, 1ng was selected as the optimal input for further analysis.

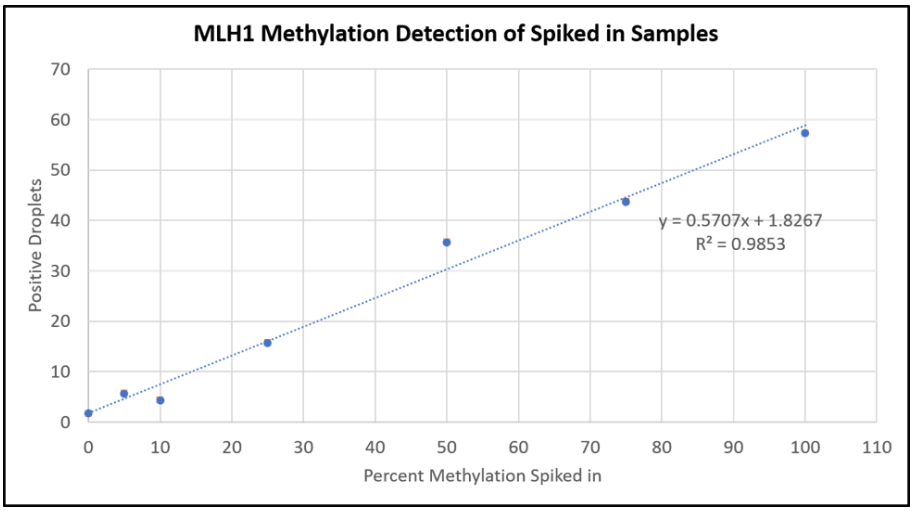


Figure 3. *MLH1* methylation detection of spike in normal plasma sample. Standard curve generated by spiking in 50ng of DNA with known percentage of methylation into normal plasma, extracting cfDNA from the sample and testing in ddPCR. The normal samples had a measured baseline level of 0 positive droplets. The standard curve gave a R^2 value of 0.985 and the equation of best can be used to relate positive droplet count to percent methylation.

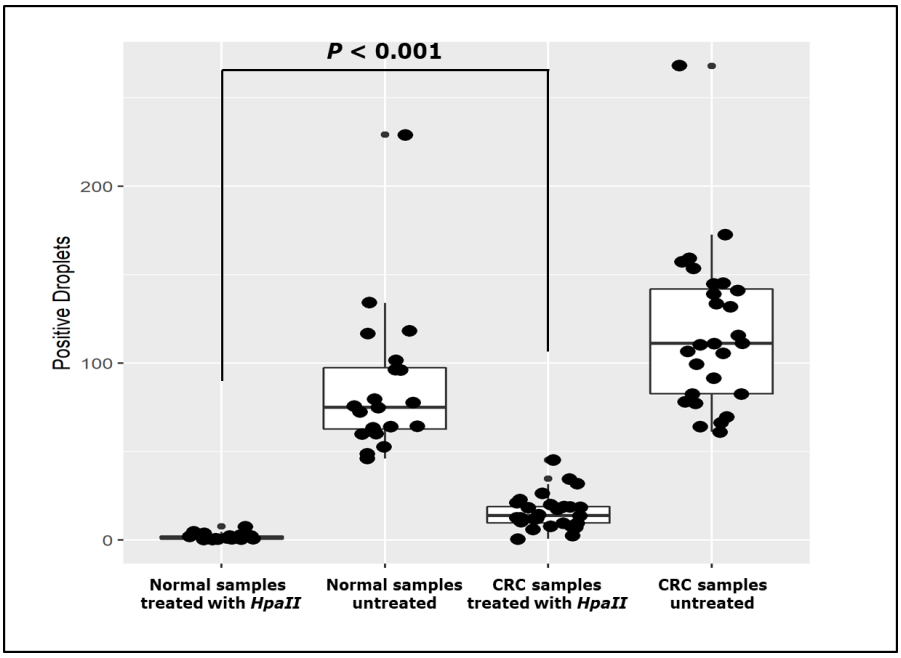


Figure 4: Distribution of detected positive droplets for *MLH1* methylation in Normal and CRC Samples. With 1ng input cfDNA, 20 normal and 28 CRC samples were treated or untreated with *HpaII* enzyme. In both populations the untreated samples showed higher positive droplets than the treated samples. Compared to normal samples, significant higher methylation levels in the cancer population (*P <0.001*).

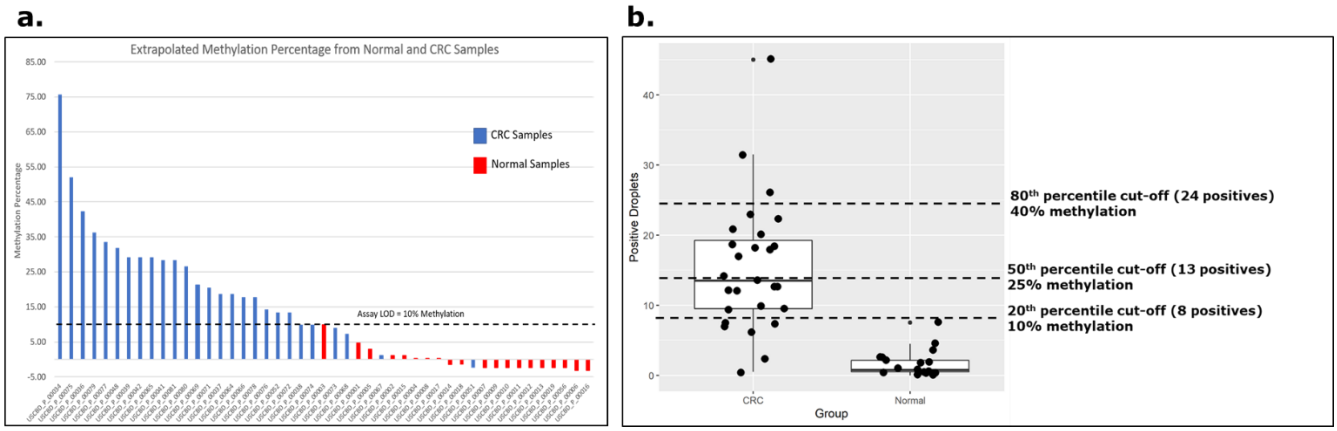


Figure 5: Determining an optimal cut-off to distinguish healthy from CRC patients. a. Based on ddPCR LOD at 10% methylation (approximately 8 positive droplets), all 20 normal patient samples fell below the limit of detection and 21/27 (77.8%) of cancer patients were above this threshold. **b**. A tiered-based cutoff (20^{th}, 50^{th}, 80^{th} percentile based) to distinguish "methylation high" CRC samples from "methylation low" CRC samples.

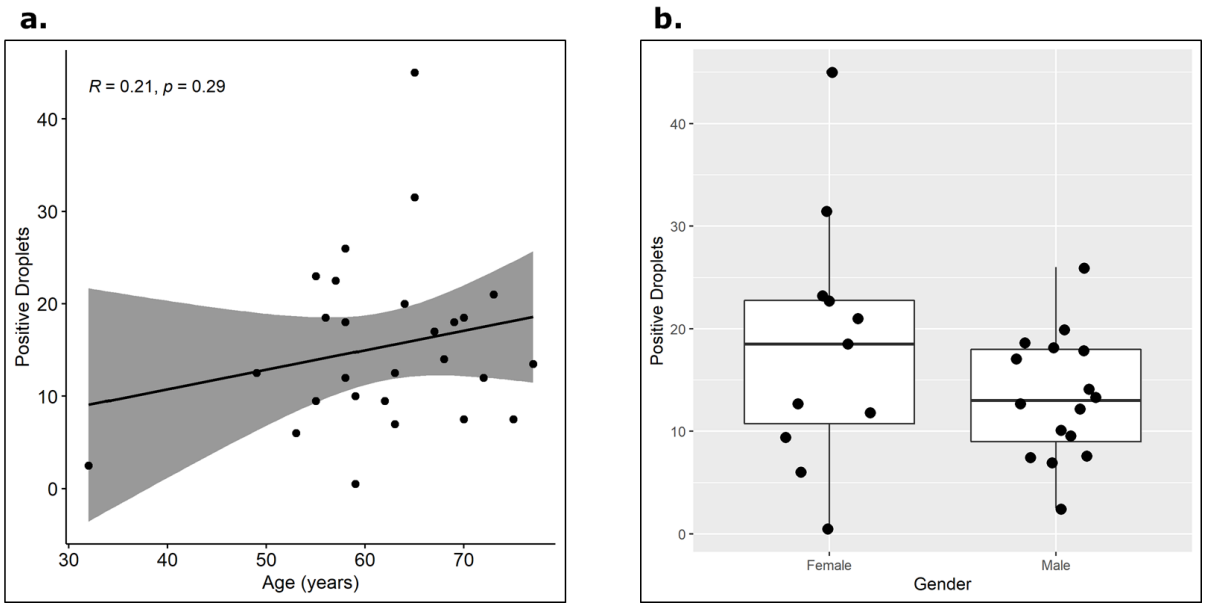


Figure 6: Assessment of the contribution of age (a) and gender (b) with respect to *MLH1* promoter methylation in CRC plasma samples.