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Correlation Between Tumor-Specific Mutated and Methylated DNA in Colorectal Cancer

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abstract

PURPOSE Analysis of circulating tumor DNA (ctDNA) is a potential improvement in precision medicine. In colorectal cancer (CRC), somatic mutations such as *RAS* and *RAF* in the blood (mut-ctDNA) are investigated for prognostic and predictive purposes. However, they are only present in approximately 60% of patients. Recently, ctDNA has been detected in patients with *RAS*/*RAF* wild type (WT) by methylated ctDNA (meth-ctDNA). The aim of this study was to compare mutated DNA with methylated DNA in malignant and nonmalignant tissue and plasma from CRC cohorts to establish a universal biomarker for ctDNA in all patients with CRC.

MATERIALS AND METHODS Tissue (n = 170) and plasma (n = 147) samples were analyzed for *RAS*/*RAF* mutations and neuropeptide Y methylation by droplet digital polymerase chain reaction. Tissue originated from nonmalignant WT and *RAS*/*RAF*-mutated adenomas, tumor-adjacent colorectal tissue, and WT and *RAS*/*RAF*-mutated tumor tissue. Plasma samples represented healthy donors and localized and metastatic CRCs.

RESULTS The level of neuropeptide Y–methylated DNA in the tissue cohorts differed between nonmalignant and malignant/premalignant tissues with minimal overlap. Furthermore, meth-ctDNA was detected in plasma from 100% of patients with metastatic disease, compared with 67% of those with localized disease and 8% of healthy donors. Median fraction of meth-ctDNA in metastatic and localized cancers was 13.25% and 0.04%, respectively. Correlation between mut-ctDNA and meth-ctDNA was high (r = 0.77 and 0.80 in localized and metastatic settings, respectively).

CONCLUSION Mut-ctDNA is interchangeable with meth-ctDNA in patients with CRC. On the basis of our results, meth-ctDNA should be considered a universal biomarker in metastatic CRC, but additional investigations of clinical utility are warranted.

INTRODUCTION

Circulating tumor-specific DNA (ctDNA) is an attractive biomarker for obvious reasons, such as easily repeated access, better reflection of overall tumor biology, and timely correct tumor mutational status. It has the potential for improving precision medicine by early diagnosis of minimal residual disease and treatment monitoring. Consequently, the era is marked by enthusiasm, but it should be noted that its clinical utility, with few exceptions, remains to be proven.[1]

ctDNA can be determined by the presence of tumor-specific genetic or epigenetic alterations. The current analytic approaches to identifying ctDNA differ and thus hamper comparisons of studies. The main methods used are next-generation sequencing (NGS) and quantitative and digital polymerase chain reaction (PCR). NGS allows identification of mutations of interest and is a relatively broad approach applicable in the identification of mutational patterns and quantification of specific mutations, but this method is time and resource consuming. NGS requires complex equipment, and because interpretation of the results is not always straightforward, bioinformatic expertise is required. NGS of blood samples requires special attention. Different platforms may lead to contradictive results, as shown in a recent report, with only 60% agreement between analyses of the same mutations.[2] The second method relies on PCR with analysis of specific mutations. Compared with NGS, PCR, especially digital PCR, has a faster data turnaround time[3] and lower costs, but direct PCR is only applicable in the fraction of patients with known specific mutations.

In colorectal cancer (CRC), *RAS*/*RAF* mutations are of interest, because their presence in tumor tissue contraindicates treatment with monoclonal antibodies cetuximab and panitumumab. Multiplex technology allows for screening of 27 mutations in the same analysis,[4] but even so, only approximately 60% of patients present with a *RAS*/*RAF*-mutated tumor. In

ASSOCIATED CONTENT

Appendix

Author affiliations and support information (if applicable) appear at the end of this article.

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CONTEXT

Key Objective

Can circulating tumor-specific methylated DNA serve as a universal biomarker in colorectal cancer?

Knowledge Generated

Methylated DNA was tumor specific in different cohorts including tissue as well as plasma. High correlation was found between mutated and methylated DNA in both localized and metastatic disease.

Relevance

Circulating tumor-specific methylated DNA holds promise as a universal colorectal cancer marker that can be analyzed in a simple, highly reproducible approach.

most of these patients, the same tumor-specific mutation is detectable in ctDNA (mut-ctDNA). Therefore, an analysis based on *RAS*/*RAF* mutations in the blood is only applicable in a fraction of patients with CRC, even under optimal conditions. The situation calls for other mutations or tumor-specific epigenetic markers.

Aberrant methylation is an early event in carcinogenesis and occurs in almost all malignant tumors as methylation of promoter regions causing inactivation of genes. This, along with the stability of methylation changes, makes it a relevant biomarker for early diagnosis.[5] Analysis of abnormally methylated DNA in plasma (meth-ctDNA) has been proposed for screening,[6] and a test for CRC has been approved by the US Food and Drug Administration.[7] However, because of insufficient sensitivity and specificity, it has not been generally accepted. The *neuropeptide Y* gene (*NPY*) has been correlated with invasion and proliferation.[8,9] Exogenous NPY has been shown to influence the growth of malignant cholangiocarcinoma cells in vitro and inhibit invasion of CRC cells. In this disease, hypermethylation of the *NPY* promoter region has been correlated with inactivation of gene expression and therefore carcinogenesis, and currently, hypermethylation of the *NPY* gene is suggested as a blood-based biomarker.[10-12]

The purpose of our study was to compare mutated DNA with methylated DNA in malignant and nonmalignant colorectal tissue and plasma with the aim of determining a method applicable in all patients with CRC.

MATERIAL AND METHODS

Tissue Sampling

An experienced pathologist selected archival formalin-fixed paraffin-embedded (FFPE) tissue samples for analysis. The Danish Registry of Human Tissue Utilization was consulted before any tissue samples were used. Tumor slides were reviewed for selection of paraffin blocks with abundant tumor cells. Three 15-μm slices of FFPE tissue were subjected to 180 μL of incubation buffer and 20 μL of protein kinase K overnight at 70°C; 400 μL of lysis buffer was added to the DNA samples that were purified on the MAXWELL 16 LEV instrument using FFPE Plus LEV DNA Purification kit (Promega AS1135, Madison, WI) according to manufacturer recommendations. DNA was eluted in 50 μL of nuclease-free water and further diluted with nuclease-free water, if necessary.

Plasma Sampling and Purification

In EDTA tubes, 9-mL blood samples were collected from donors and different cohorts of patients. Plasma was isolated by centrifugation at 2,000 *g* for 10 minutes within 4 hours and stored at –80°C until use. Plasma was centrifuged again at 10,000 × *g* for 10 minutes before purification, and cysteine-rich polycomb-like protein 1 (CPP1) DNA fragments were added as exogenous internal control.[13] DNA from patients was purified from 2 × 2 or 2 × 4 mL of plasma on the QIAsymphony SP instrument using the QIAsymphony Circulating Nucleic Acid Kit (Qiagen, Hilden, Germany) according to manufacturer instructions. DNA from healthy donors was purified from 1 × 4 mL of plasma.

DNA was eluted in 60 μL of the supplied buffer and water added to 400 μL. Quantitative PCR was performed on 6 × 3 μL of eluate to quantify CPP1 and total cell-free DNA (gB2M) as previously described.[13] The remaining eluates were concentrated on an Amicon Ultra 0.5 centrifugal filter unit (Millipore, Billerica, MA) to 20 μL.

All participants provided written informed consent.

Mutation Analysis

Adenomas and tumor tissue samples were screened for *RAS*/*RAF* mutations as previously described.[14] Samples from adjacent tissue and plasma were analyzed for the specific mutation by droplet digital PCR in two wells. Positive controls for each mutation (gBlocks; IDT, Carolville, IA) or mutated fragments generated according to Spindler et al,[15] genomic donor DNA, and water were included in the analyses as controls. Mut-ctDNA in donors was not assessed because of limited material for analysis.

Methylation Analysis

Tumor-specific methylated DNA was defined as DNA with methylation of the *NPY* gene.[12] Before droplet digital PCR methylation analysis, bisulfite conversion of circulating and FFPE-isolated DNA was performed as recommended by the manufacturer (Zymo Research, Irvine, CA). Converted DNA was analyzed with a methylation-specific assay and control assay (albumin) using the BioRad Droplet Digital PCR System QX100. Water and a pool of lymphocyte DNA from cancer-free individuals were included in each round of analyses as negative controls; Universal Human Methylated Control DNA (Zymo Research) and EpiTech Control DNA (Qiagen) were included as positive controls. Samples were analyzed for tumor-specific methylated DNA according to the description in the Appendix.

To determine a positive tissue methylation analysis, a cutoff value was calculated. The NPY fractions were normally distributed after log transformation, and 95% CI was determined by back transformation. The upper limit of the 95% CI was 1.8%, and therefore, the cutoff value was set to 2%.

Limit of Blank in Plasma

Limit of blank (LOB) in the mutation analysis was determined by donor controls. Because fewer than two FAM-positive droplets were observed in all analyses of the genomic donor DNA controls and plasma DNA from healthy individuals as previously described,[16] plasma samples with more than two FAM-positive droplets in two wells were classified as positive.

LOB in the methylation analysis was defined as the quantity of NPY droplets counted in control DNA samples from a test cohort of healthy individuals (n = 50). The upper 95% CI was two droplets per reaction based on Poisson distribution. Samples were considered positive when the number of observed droplets was higher than the LOB value. LOB was validated in an independent cohort of 50 healthy donors age between 50 and 78 years (median age, 58.5 years). Eight percent (four of 50) had more droplets than the LOB value.

Statistical Analysis

Correlation was measured using the nonparametric Spearman rank test. Scatter plots were generated to illustrate correlation between levels of mut-ctDNA and meth-ctDNA. Statistical tests were performed using 2015 NCSS 10 statistical software (NCSS, Kaysville, UT).

RESULTS

Cohorts

A total of 170 tissue samples were collected from different cohorts of nonmalignant colorectal tissues (n = 25), *RAS/RAF* wild-type (WT) adenomas (n = 10), *RAS/RAF* mutated adenomas (n = 10), nonmalignant colorectal tissue adjacent to *RAS/RAF*-mutated tumors (n = 25), *RAS/RAF* WT tumors (n = 50), and *RAS/RAF*-mutated tumors (n = 50); 147 plasma samples were analyzed from healthy controls (n = 100), localized CRCs (n = 27), and metastatic CRCs (mCRCs; n = 20). Median age in these last two groups was 71 years (range, 46 to 81 years), different from that in the validation cohort of healthy donors ($P < .001$). Patients with mCRC had plasma analyzed both at baseline and after the first treatment cycle.

Tissue Methylation

In 25 nonneoplastic specimens, all but one were nonmethylated (4% were NPY positive). In contrast, all *RAS/RAF* WT adenomas harbored methylated NPY (10 [100%] of 10), and the same applied to the 10 *RAS/RAF*-mutated adenomas. In the samples adjacent to tumors, eight (32%) of 25 were methylated, but the positive samples had hypermethylated NPY frequencies just above the cutoff value (median value, 2.9%; range, 2.11% to 6.8%). None of these specimens were mutated, despite presence of a mutation in the tumor. All tumors except one (99%) were hypermethylated independent of mutation status in 50 *RAS/RAF*-mutated and 50 WT tumors. The non-hypermethylated tumor was also *RAS/RAF* WT. Figure 1 shows the results of the tissue NPY methylation analysis, with methylated NPY presented as actual fractions. It clearly shows that the level of NPY methylation was different in malignant and nonmalignant tissues. The range of NPY

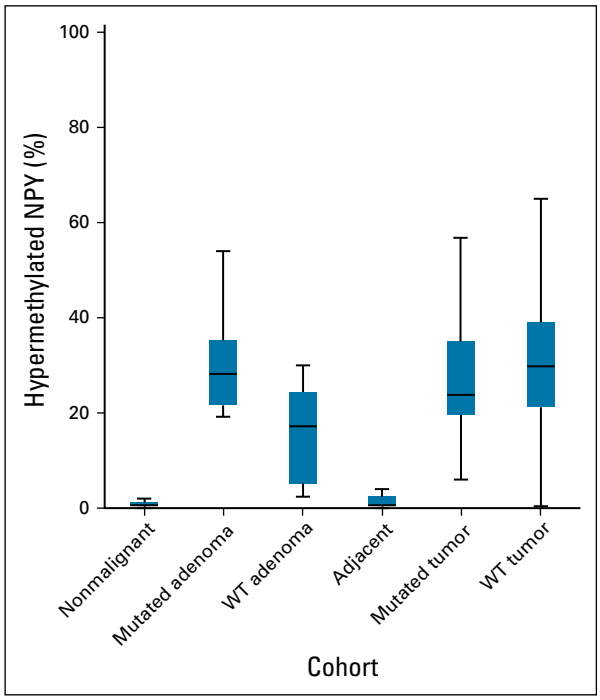

FIG 1. Level of methylated DNA in different tissue cohorts presented as actual frequencies in box-and-whisker plot. Level varies between nonmalignant colorectal tissue, adenomas, adjacent colorectal tissue, and tumor tissue. Medians, interquartile ranges (IQRs), and whiskers (1.5 × IQR) are shown. NPY, neuropeptide Y; WT, wild type.

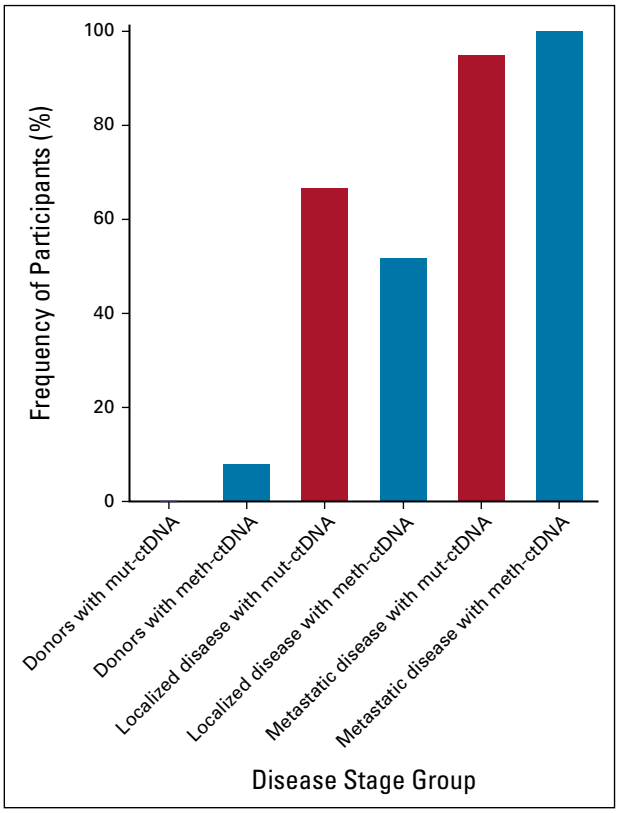

FIG 2. Frequency of patients with methylated and mutated circulating tumor DNA (ctDNA) divided into groups based on disease stage. Fifty healthy donors were only analyzed for methylated ctDNA (meth-ctDNA), and 20 healthy donors were analyzed for *KRAS G12D*–mutated ctDNA (mut-ctDNA). Plasma was drawn from patients with localized *RAS*/*RAF*-mutated tumors (n = 27) and metastatic *RAS*/*RAF*-mutated tumors (n = 20) and analyzed for the same specific mutations and methylation.

methylation in tumors did not overlap that of nonmalignant NPY methylation, except in three cases (3%).

Plasma Methylation

Figure 2 shows the plasma analysis of different cohorts. Samples from healthy donors showed that four (8%) of 50 had detectable meth-ctDNA above the LOB. No *KRAS G12D* mutations were detected in plasma from 20 healthy donors. Furthermore, the analysis showed that 67% (18 of 27) of patients with localized CRC had mut-ctDNA, compared with 52% (14 of 27) with meth-ctDNA. Medians were 0.3% (range, 0% to 4.3%) and 0.04% (range, 0% to 3.8%) for mut-ctDNA and meth-ctDNA, respectively. In the metastatic setting, 95% (19 of 20) had mut-ctDNA and 100% (20 of 20) had meth-ctDNA, with medians of 13.05% (range, 0% to 69.9%) and 13.25% (range, 0.27% to 40.7%), respectively.

TABLE 1. Spearman Rank Correlation Coefficient Between Mut-ctDNA and Meth-ctDNA in Different Stages of CRC and at Beginning of Treatment Course

Disease	*r*
Localized	0.77
Metastatic at baseline	0.80
Metastatic after one cycle	0.74

Abbreviations: CRC, colorectal cancer; meth-ctDNA, methylated circulating tumor DNA; mut-ctDNA, mutated circulating tumor DNA.

The Spearman rank correlation coefficients between mut-ctDNA and meth-ctDNA values are listed in Table 1. Correlation in localized CRC at baseline was $r = 0.77$ ($P < .001$); in the metastatic setting, it was $r = 0.80$ ($P < .001$). After the first cycle of treatment, it remained high at $r = 0.74$ ($P < .001$). Correlations are also illustrated in the scatter plots shown in Figures 3, 4A, and 4B, which represent correlation between meth-ctDNA and mut-ctDNA in the localized and metastatic settings at baseline and in the metastatic setting after the first treatment cycle, respectively.

DISCUSSION

Our study on tissue and plasma samples from donors and different stages of CRC indicates that meth-ctDNA correlated with mut-ctDNA across the cohorts and that meth-ctDNA was detectable in cases without activating DNA mutations. Furthermore, the levels of methylation in nonmalignant tissue and plasma clearly differed from their malignant counterparts. Therefore, meth-ctDNA may be a universal biomarker in CRC from the perspectives of detection and monitoring.

Key challenges in cancer management are early diagnosis and close monitoring during treatment and follow-up with minimal harm to the patients. Tumor markers have represented a field of major interest in this context, and detection of ctDNA in liquid biopsies has stimulated that interest. Liquid biopsies have several advantages over tissue specimens, including patient convenience. In the landscape of tumor markers, ctDNA represents a relatively new approach with the potential for progress in cancer management, because it contains the same molecular alterations as the corresponding tumor and potential metastases. Even so, clinical utility has been limited.

Recently, presence of ctDNA was confirmed in WT patients using epigenetic modifications such as hypermethylation.[17-19] Meth-ctDNA seems to represent tumor-specific DNA and is found in nearly all colorectal adenocarcinomas,[10] as opposed to ctDNA based on *RAS*/*RAF* mutations. It may be a major step forward, if mut-ctDNA can be replaced by meth-ctDNA in several clinical settings. Three recent studies have suggested that hypermethylation of the *NPY* gene may serve as a biomarker for monitoring the treatment of CRC,[10,11,20] but a detailed comparison of mutated and methylated DNA motivated our study.

The level of methylation varied among different cohorts with malignant and nonmalignant tissues, the latter in general being negative. Analysis of specimens with low levels of methylation raises several questions, but our cutoff value provided an

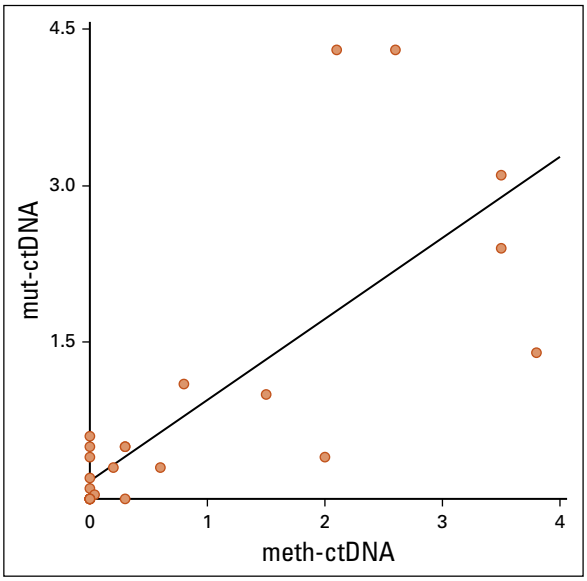

FIG 3. Scatter plot showing correlation of methylated circulating tumor DNA (ctDNA) and mutated ctDNA (mut-ctDNA) in localized disease.

almost complete separation of malignant and nonmalignant tissues based on the statistical variation of the observed frequencies in healthy individuals. All adenoma specimens were methylated independent of mutation status. Adjacent tissue showed NPY hypermethylation in one third of the samples, although they were just positive; none were mutated, despite *RAS*/*RAF* mutations in the nearby tumor. This is in agreement with a study by Roperch et al,[12] who analyzed 15 paired samples of tumor and adjacent tissue. NPY was detected in adjacent tissue but with a statistically significant difference in the cumulative mean between adjacent and tumor tissue. Methylation outcomes in precancerous tissue and tissue close to the tumor support the observation of a field effect.[21] This theory describes precancerous cells in proximity to the tumor cells as having some, however not all, of the genetic and epigenetic alterations as in the completely developed tumor. Furthermore methylation is an early event in carcinogenesis,[22] which may represent a potential universal biomarker.[23] All tumor tissue samples were hypermethylated irrespective of mutational status and to the same extent as *RAS*/*RAF*-mutated adenomas. This indicates that aberrant methylation takes place as an early part of cancer progression, which can predispose to additional genetic alterations.[24] Possibly, these epigenetic changes give an important and early window for therapeutic interventions in patients with CRC.

Plasma analyses showed that the frequency of patients with ctDNA varied depending on disease stage. We previously reported that mut-ctDNA was nondetectable in patients with adenoma.[25] In this study, no donors had meth-ctDNA, compared with patients with CRC. LOB of two positive droplets per reaction determined in a test cohort of 50 healthy donors seems in good agreement with previous results reporting an LOB of 3.30 on the basis of 17 control DNA samples without hypermethylated NPY.[11] The two self-reported healthy donor cohorts with 4% (two of 50) and 8% (four of 50) of patients having more than two positive droplets per reaction indicate good agreement. The latter represents an age interval relevant to CRC. Therefore, occurrence of aberrant methylation with increasing age does not seem to be a major problem. The false-positive rate may be acceptable for patients with advanced disease, and therefore, clinical application of meth-ctDNA in metastatic disease seems encouraging. However, application in detection of minimal residual disease requires further validation. In localized tumors, two thirds of patients harbored mut-ctDNA and meth-ctDNA, which is in accordance with current literature.[10] In the metastatic setting, all patients had meth-ctDNA, indicating its potential as a general biomarker. In agreement with our results, Garrigou et al[10] showed the level of meth-ctDNA to increase with increasing CRC stage by up to 80% in mCRC. Bachet et al[17] also found that 80% of 425 patients with mCRC had detectable ctDNA by analysis of both mut-ctDNA and meth-ctDNA. Similar results in mCRC were reported by Garlan et al,[20] who investigated ctDNA in 82 patients with

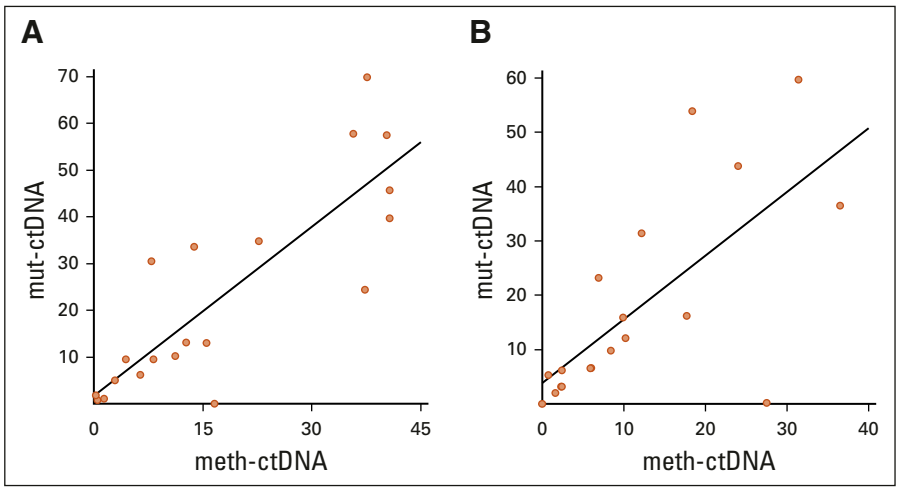

FIG 4. Scatter plots showing correlation of methylated circulating tumor DNA (ctDNA) and mutated ctDNA (mut-ctDNA) in metastatic colorectal cancer (A) at baseline and (B) after one cycle of treatment.

mCRC. They found that 77% had detectable ctDNA at baseline, assessed by mut-ctDNA and meth-ctDNA. Boeckx et al[11] reported that 87.5% of all baseline plasma samples were positive for NPY methylation in 24 patients with mCRC. Varying methods of analysis, which lack standardization throughout the field of research in ctDNA, are one obvious reason for the lower frequency of patients with detectable ctDNA compared with our findings.

Correlation between mut-ctDNA and meth-ctDNA was investigated to compare the two potential biomarkers. We found good correlation in all disease stages, with $r = 0.80$ in the metastatic setting, yielding the highest score. This is in agreement with previous studies concluding that methylated markers in ctDNA could replace tumor-specific mutations in the blood.[10] This conclusion was based on a correlation coefficient of $r = 0.94$ between mut-ctDNA (*KRAS*, *BRAF*, and *TP53*) and meth-ctDNA (*NPY*). The authors also found that meth-ctDNA allowed monitoring of patients with mCRC without knowing the mutational status of the tumor. We did not investigate the potential role of meth-ctDNA in monitoring WT patients, but we found a strong correlation between mut-ctDNA and meth-ctDNA after one cycle of treatment, which emphasizes the role of meth-ctDNA in treatment monitoring. In accordance with our results, Boeckx et al[11] found a correlation coefficient of $r = 0.86$ after the first cycle of treatment. Taken together, the current literature indicates high agreement between mut-ctDNA and meth-ctDNA,[10,11,20] further supported by our results.

A minor disadvantage in the methylation assays is the need for bisulfite conversion, which can degrade DNA and therefore requires additional material. However, the need for prior establishment of mutation status, as with mut-ctDNA, is bypassed, and therefore, meth-ctDNA is applicable in nearly all patients with CRC. It should be noted that meth-ctDNA is not relevant in the selection of patients for targeted treatment with epidermal growth factor receptor inhibitors.

In conclusion, to our knowledge, our study represents the first detailed comparison of methylated and mutated DNA in normal tissue as well as in localized and metastatic tumors. Included is also a detailed comparison of plasma samples in the corresponding cohorts. Our results show high agreement between methylated and mutated DNA and also demonstrate that methylated DNA is detectable independent of knowledge of tumor mutations. This indicates that hypermethylated NPY in plasma is applicable as a universal biomarker in all patients with mCRC, but further investigation of clinical utility is warranted.

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Collection and assembly of data: Caroline Brenner Thomsen, Rikke F. Andersen, Jan Lindebjerg, Torben F. Hansen, Lars Henrik Jensen
Data analysis and interpretation: All authors
Manuscript writing: All authors
Final approval of manuscript: All authors

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

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APPENDIX

Methylation Analysis

Concentrated DNA was bisulfite converted in a 50-μL reaction with EZ DNA Methylation Lightning Spin-Column Kit (Zymo Research, Irvine, CA) and eluated in 12 μL. The albumin/neuropeptide Y (NPY) duplex analysis was made in two wells with 5 μL of DNA per well in a 20-μL reaction. Droplet digital polymerase chain reaction (PCR) supermix for probes (no deoxyuridine triphosphate) and NPY/albumin assays were applied. Droplets were generated on the QX200 Automated Droplet Generator from BioRad (Hercules, CA). PCR was completed on the Veriti PCR device (Thermo Fisher Scientific, Waltham, MA). Step one was at 95°C for 10 minutes. Step two was 44 cycles at 95°C for 15 seconds and 56°C for 1 minute, with a 1.5°C per second ramp rate. Step three was at 98°C for 10 minutes. Droplets were counted via the QX100 Droplet Digital Reader from BioRad. Data analysis was performed with QuantaSoft (version 1.7.4; BioRad).

