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Sensitive digital quantification of DNA methylation in clinical samples

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Abstract

Abnormally methylated genes are increasingly being used as cancer biomarkers ^{1, 2}. For clinical applications, it is important to precisely determine the number of methylated molecules in the analyzed sample. We here describe a digital approach that can enumerate one methylated molecule out of ~5000 unmethylated molecules. Individual DNA fragments can be amplified and analyzed either by flow cytometry or next generation sequencing instruments. Using methylated vimentin as a biomarker, we tested 191 plasma samples and detected cancer cases with 59% sensitivity (95% CI, 48%–70%) and 93% specificity (95% CI, 86%–97%). Using the same assay, we analyzed 80 stool samples and demonstrated 45% sensitivity for detecting colorectal adenomas (23%–68%), 41%

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AUTHOR CONTRIBUTIONS

M.L., S.D.M., S.G., K.W.K. and B.V. designed the project. M.L. developed the Methyl-BEAMing assay and performed the experiments on plasma and fecal DNA. N.P. M.L. and Y.H. performed the Solexa sequencing experiments. H.M. performed the MSP assay. K.D. purified fecal DNA. S.G. provided statistical analysis. H.J., L.D., N.C.B., S.L., N.A. and K.S. collected clinical samples. W.D.C., S.Z., V.E.V, F.D. and B.L. made intellectual contributions to the project. B.V. M.L. and S.D.M. wrote the manuscript.

COMPETING INTERESTS STATEMENT

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sensitivity for detecting cancer (21%–64%), and 95% specificity (82%–99%). This digital quantification of rare methylation events should be applicable to diagnostic evaluations of clinical samples, to preclinical assessments of new epigenetic biomarkers, and to quantitative analyses of epigenetic biology.

In humans, DNA methylation is largely restricted to cytosines within 5'-CpG dinucleotides. This covalent modification of DNA functions as an important mediator of gene regulation and, together with covalent modifications of histone proteins, forms the cornerstone for the burgeoning field of epigenetics. Though cancers are globally hypomethylated³, specific regions of genes have been shown to be hypermethylated in association with transcription silencing⁴. In addition to its implications for gene regulation, DNA methylation is providing a new generation of cancer biomarkers⁵. Though mutant sequences provide exquisitely specific biomarkers of this class^{6, 7}, their utility is compromised by their heterogeneity: the same gene can be mutationally inactivated through many different mechanisms or mutated at many different positions. In contrast, DNA hypermethylation in cancers often affects identical residues in the regulatory regions of particular genes, providing major advantages in biomarker test design. Accordingly, many studies have employed DNA methylation of specific genes for diagnostics development^{2, 4, 5, 8}. Such diagnostic tests can in principle be used for early detection of cancers, for assessing prognosis, and for determining the effects of therapy or detecting residual disease.

The majority of diagnostic tests based on DNA methylation have employed bisulfite to convert cytosine residues to uracils. This conversion alters the sequence of DNA⁹, providing an opportunity to assess DNA methylation with allele-specific PCR, restriction digestion or specific hybridization probes^{10–14}. However, in many important diagnostic scenarios, DNA from the cancer represents only a small fraction of the total DNA in the clinical sample. Such scenarios include the use of DNA from plasma, serum, urine, feces, or sputum for early diagnosis or therapeutic monitoring and the use of DNA from surgical margins or lymph nodes to monitor the extent of disease¹. For such purposes, there are numerous advantages of using digital approaches¹⁵. Digital approaches involve the counting of methylated and unmethylated fragments, one-by-one, thereby dramatically increasing the signal-to-noise ratio of the assay. In this work, we present a technology, called “Methyl-BEAMing”, for direct digital quantification of DNA methylation at specific sites that is readily applicable to clinical samples, even when the fraction of methylated fragments in such samples is minute. We demonstrate this approach by applying Methyl-BEAMing to detect cancer-derived methylation of vimentin gene DNA in plasma and fecal DNA from colon cancer patients.

DNA in clinical samples such as plasma has already been degraded to small size by nucleases and is present at only a few nanograms per milliliter⁶. Conventional methods for bisulfite conversion further degrade DNA and are difficult to implement with samples containing only small amounts of DNA because of cumulative losses during the procedure^{16, 17}. After much optimization, we identified conditions that resulted in nearly complete (99.4%; 95% CI, 98.6%–99.8%) conversion of dC to dU residues while preserving the majority of the DNA (detailed in Experimental Methods).

The exon 1 region of the vimentin gene has been shown to be hypermethylated in colorectal cancers when compared to normal colorectal mucosae and other normal tissues^{18, 19}. Moreover, this difference is the basis for the only commercially available diagnostic test based on DNA methylation (ColoSure, LabCorp). To assess the dynamic range and accuracy of Methyl-BEAMing, we designed primers to amplify ~100 bp of vimentin exon-1 that contains the 5'-CpG sites known to be methylated in cancer cells. Amplicons of ~100 bp were chosen to accommodate the small size of circulating DNA molecules^{6, 20}. The PCR primers were designed to amplify bisulfited converted products derived from both methylated and

unmethylated vimentin templates. The methylation status of these amplicons was then digitally enumerated by BEAMing (Beads, Emulsion, Amplification and Magnetics)²¹.

In BEAMing, PCR amplification of individual DNA molecules takes place within aqueous nanocompartments suspended in a continuous oil phase (Fig. 1a & Supplementary Fig. 1 online). Each aqueous nanocompartment contains the DNA polymerase, cofactors, and dNTP's required for PCR. When a compartment contains a single DNA template molecule as well as a bead, the PCR product within the compartment becomes bound to the bead. Each bead thereby ends up with thousands of identical copies of the template within its nanocompartment a process similar to that resulting from cloning an individual DNA fragment into a plasmid vector to form a bacterial colony. After PCR, the beads are collected by breaking the emulsion, and their status is individually assessed by incubation with fluorescent hybridization probes. In Methyl-BEAMing, the status of harvested beads is interrogated by fluorescent probes that specifically hybridize to either methylated or unmethylated derived sequences, with flow cytometry providing an accurate enumeration of the fraction of original template molecules that were methylated or unmethylated within the queried sequence (examples in Fig. 1b).

We first tested Methyl-BEAMing on mixtures of templates representing DNA from peripheral blood lymphocytes (unmethylated vimentin) and a colorectal cancer cell line (fully methylated vimentin). We found that the fraction of beads containing methylated vimentin sequences was directly proportional to the fraction of methylated input DNA ($R^2=0.99$, Supplementary Fig. 2 online). Moreover, Methyl-BEAMing could accurately detect methylated vimentin DNA in a mixture that contained only a single copy of methylated vimentin sequences admixed with 1000 copies of unmethylated vimentin sequences (i.e., 0.1%). In contrast, in parallel assays, a previously optimized methylation-specific PCR assay did not detect methylated templates when the fraction of methylated fragments was <6.2% (Supplementary Table 1 online). Methyl-BEAMing thus enabled accurate detection of a single copy of methylated vimentin sequences in a mixture, and enhanced overall technical sensitivity for detecting methylated vimentin exon 1 DNA by at least 62-fold.

Some of the most important uses of cancer biomarkers involve the assessment of circulating molecules, either for early detection or disease-monitoring following therapy. The ability to detect and count a single molecule of methylated DNA suggested Methyl-BEAMing could be applied for this purpose. To determine the sensitivity and specificity of Methyl-BEAMing in plasma samples, we evaluated 191 samples, 81 from patients with colorectal cancer and 110 from age- and sex-matched controls. The total amount of DNA in the plasma was somewhat higher in patients with cancer than in the cancer-free controls, as expected² (Supplementary Fig. 3 online). The average fraction of methylated vimentin fragments proved very low in the normal controls, with a mean of 0.026%, but gradually increased with cancer stage up to a mean of 4.4% in the most advanced disease (Duke's D; Table 1). The theoretical limit of detection in any digital assay is one event, i.e., in the current study, the limit of detection was one methylated vimentin fragment in the 2 ml plasma that was assayed for each patient. Using this cutoff, the sensitivity of Methyl-BEAMing was 59% (95% CI, 48%–70%) in cancer patients, and the specificity was 93% (95% CI, 86%–97%; eight of the 110 normal samples contained 1 methylated vimentin fragment per 2 ml plasma, Table 1, Fig. 2a and Supplementary Table 2 online). As expected, the absolute number of methylated vimentin fragments in plasma increased with tumor stage, ranging from a mean of 1.8 in Duke's A patients with detectable circulating methylated vimentin to 1200 in Duke's D patients (Table 1 and Fig. 2b). Importantly, the sensitivity for detecting colorectal cancer was 52% (95% CI, 37%–68%, 23 of 44 cases) in the patients with Duke's A and B cancers, most of whom were likely to be curable by conventional surgery ($P = 3 \times 10^{-9}$ for distinguishing Duke's A and B cancers from normal; Table 1 and Fig. 2a, b). For 43 of these Duke's A and B cases, preoperative serum CEA values had also been determined. Only 6 of these cases (14%, 95% CI, 5%–28%) had

CEA values exceeding the upper limit of normal (5 ng/ml). Thus Methyl-BEAMing provided a substantial increase in the ability to detect curable early stage colon cancers via assay from blood (52% versus 14%, Supplementary Fig. 4 online, $P = 2 \times 10^{-4}$). Serum CEA values were also available for 74 members of our normal control cohort, of whom 7 had values above >5 ng/ml, corresponding to a CEA specificity of 91% (95% CI, 81%–96%). The Area under the Receiver Operating Characteristic (AUC) curve for Methyl-BEAMing was 0.81, varying from 0.67 to 0.95 among the four cancer stages while the corresponding values for CEA was 0.63, varying from 0.42 to 0.97 in the different stages (Fig. 2c, d).

Values for circulating methylated vimentin fragments and CEA were additionally determined from matched pre- and post-surgical blood samples of three colon cancer patients (Supplementary Table 3 online). Patient 1 presented with recurrent metastatic disease in the liver. At this time, the CEA was elevated to ~2 times the normal range while circulating methylated vimentin DNA was elevated by >100-fold above the normal threshold. The patient underwent resection of his liver metastasis but was later found to have a residual solitary lung metastasis. A month following surgery, the CEA had returned to normal and methylated vimentin DNA in the circulation had fallen 20-fold. However, an abnormal level of methylated vimentin DNA continued to be detectable (7.2 molecules per 2 ml plasma), consistent with this patient's residual disease.

Patient 2 presented with recurrent metastatic disease in the liver with CEA elevated 1.5-fold and circulating methylated vimentin DNA elevated 800-fold over the normal range. One month following liver resection and radiofrequency ablation, the CEA returned to normal. Methylated vimentin levels fell 2.5-fold, but was still elevated (320 molecules per 2 ml of plasma). One month following these results, PET and CT imaging confirmed the presence of remaining active metastatic disease in both the lung and the liver.

Patient 3 presented with a cancer in the sigmoid colon and with synchronous metastatic disease in the liver. The CEA was within the normal range, but circulating methylated vimentin DNA was elevated over 440 fold. The patient underwent resection of his primary colon cancer and partial resection of his liver metastasis, and was started on chemotherapy for palliation of his residual disease. Measurement of circulating methylated vimentin DNA showed that it had fallen 440-fold, but still remained detectable, consistent with this patient's residual disease.

The near disappearance of circulating methylated vimentin DNA following these patients' surgeries suggests that these circulating methylated DNAs were derived directly from the malignant tumors. To further support this interpretation, we compared in the plasma of two colorectal cancer patients the fraction of methylated vimentin molecules (determined by Methyl-BEAMing) versus the fraction of mutant molecules (APC G4189T and PIK3CA G1624A) determined by standard BEAMing²². In these patients, the mutations were somatic and therefore exclusively derived from the patients' cancer cells. The fraction of methylated vimentin templates in the first sample was 13.6%, similar to the fraction of mutated APC (17.5%). In the second patient's plasma, methylated vimentin represented 3.5% of the total vimentin templates while mutated PIK3CA represented 3.0% of the PIK3CA templates.

Next, we used vimentin Methyl-BEAMing to screen stool samples (4 g) from 80 individuals, including 38 normal individuals, 20 patients with clinically significant adenomas (defined as 1centimeter in diameter), and 22 colorectal cancer patients of various stages. The concentration of vimentin DNA fragments in stool varied widely (Supplementary Fig. 5 online). Though there were no reproducible differences between the concentrations of total vimentin DNA fragments in the three patient groups, there was a substantial difference in the fraction of methylated fragments. This fraction increased from a mean of 0.96% in normal individuals to 3.8% and 7.3% in patients with adenomas and carcinomas, respectively (Table 1 and Fig. 3a,

b, c). Using a threshold of 2% methylation, 45% (95% CI, 23%–68%) and 41% (95% CI, 21%–64%) of the patients with adenomas and carcinomas, respectively, scored positive in the Methyl-BEAMing assay, while only 5% (95% CI, 0.6%–18%) of the samples from normal individuals did so ($P = 5.5 \times 10^{-4}$ and $P = 1.1 \times 10^{-3}$ for the difference between normal individuals and those with adenomas and cancers, respectively) (Table 1, Fig. 3a, b, c and Supplementary Table 4 online). In fecal DNA, Methyl-BEAMing preserved sensitivity for cancer detection, but markedly increased specificity compared to MSP detection of methylated vimentin¹⁹. The corresponding areas under the ROC curves for Methyl-BEAMing analysis of adenomas and carcinomas were 0.69 and 0.62, respectively (Fig. 3d).

Sequencing-by-synthesis (SBS) instruments provide an alternative way to digitally assess DNA methylation. The methods we developed for bisulfite conversion and recovery of DNA from clinical samples can also be applied to DNA prepared for such instruments. Though the cost and throughput of SBS limits the use of this method in the clinic, it provided a method of validating the accuracy of Methyl-BEAMing. For this purpose, we evaluated five samples with low and high fractions of methylated fragments on an Illumina Genome Analyzer sequencing instrument. Following bisulfite conversion and PCR amplification (Fig. 1a, Supplementary Fig. 1 online), the PCR products were ligated to adapters and sequenced using a standard Illumina protocol. The 36-base region sequenced encompassed the five core CpG's that were assessed with the hybridization probes employed in Methyl-BEAMing. Through the analysis of C residues not present within CpG motifs from normal lymphocyte DNA, we found that the bisulfite conversion efficiency used for Methyl-BEAMing was 99.4% (95% CI, 98.6%–99.8%). In this sample, the fraction of sequenced fragments in which four or five of the five core CpG sites were methylated was 0.015%. Given the high efficiency of the bisulfite conversion step, this low level of background methylation across multiple CpG sites likely arises from a low but real level of vimentin exon-1 methylation in lymphocytes ($P < 0.0001$, Binomial Distribution). Analysis of this sample by Methyl-BEAMing determined that the methylated DNA fraction was 0.018%. Thus, in a sample with an extremely low content of methylated DNA sequences, enumeration of methylation by either next generation sequencing or by Methyl-BEAMing gave essentially the same result. Likewise, in a sample from fecal DNA with a high degree of methylation the fraction of methylated fragments determined by either sequencing or Methyl-BEAMing was similar (11.3% vs. 10.8%, respectively, Supplementary Table 5 online). This was substantiated in three other samples, one with a low level of methylation and two with relatively high levels. These data demonstrate that Methyl-BEAMing provides the same precision for enumerating methylated DNA molecules as does next generation sequencing across a wide range of methylated DNA inputs.

The results described above demonstrate that digital techniques such as Methyl-BEAMing enable the quantitative assessment of DNA methylation in clinical samples. One of the most important applications of Methyl-BEAMing is in early diagnosis. In this study, 59% (95% CI, 48%–70%) of patients with colorectal cancer could be detected by a plasma-based Methyl-BEAMing assay. This sensitivity is close to the maximal detectable with the vimentin biomarker, which has been shown to be methylated in 53–83% of colorectal cancers¹⁹. The fact that 52% (95% CI, 37%–68%) of presumably curable Duke's A and B stage cancers could be detected by this method, in a cohort in which only 14% (95% CI, 0.6%–18%) of cancers could be detected by CEA, was particularly encouraging. Equally encouraging was the observation that 45% (95% CI, 23%–68%) of patients with clinically significant pre-malignancies (advanced adenomas) could be detected by Methyl-BEAMing of fecal DNA. Patients with these lesions would generally be treated by endoscopic excision without abdominal surgery. The 45% detection rate of advanced adenomas by Methyl-BEAMing observed in this study exceeds the 25%–27% detection rate reported for comparably specific immunochemical fecal occult blood tests (specificity 93%–97%)²³. In screening an asymptomatic cohort of adults over age 50, the prevalence of colon adenomas 1 centimeter

and of colon cancer is expected to be 6.0% and 0.5–1.0%, respectively^{24, 25}. Detection of 45% (95% CI, 23%–68%) of adenomas and 41% (95% CI, 21%–64%) of colon cancers by Methyl-BEAMing of stool DNA, at 95% (95% CI, 82%–99.4%) specificity, would translate into a positive predictive value of 40% for an individual with a positive test harboring an advanced colorectal adenoma or cancer. This value exceeds that achieved in many studies of mammography, a cancer screening test of established clinical value in reducing mortality²⁶. Moreover, Methyl-BEAMing represents a flexible platform. Applying Methyl-BEAMing to a panel of suitable markers would increase sensitivity for colon cancer detection even further. With identification of suitable markers, the technique could be applied for early detection of other malignancies. Last, one could also imagine application of Methyl-BEAMing to prenatal testing, for example for Down's syndrome, based on enumeration of circulating chromosome 21 derived sequences that are specifically methylated in the fetus.

METHODS

Methods and any associated references are available in the online version of the paper at <http://www.nature.com/naturebiotechnology/>.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Schematic and examples of digital quantification of rare DNA methylation in clinical samples. (a) Schematic. (b) Representative results of Methyl-BEAMing obtained with flow cytometry. The number of beads representing methylated, unmethylated, mixture, and virgin beads are indicated in the top right corner of each box.

Figure 2.

Vimentin Methyl-BEAMing of plasma from colorectal cancer patients. **(a)** The number of methylated vimentin fragments in 2 ml plasma from normal, age and sex-matched control patients. **(b)** The number of methylated vimentin fragments in 2 ml plasma from colorectal cancer patients of various stages. **(c)** Receiver operating characteristic (ROC) curves based on the number of methylated vimentin fragments for the indicated classes of samples. **(d)** ROC curves based on CEA level for the indicated classes of samples. The dotted lines in **(a)** **(b)** **(c)** represent 1 methylated fragment per 2 ml plasma DNA. The dotted line in **(d)** represents 5 ng/ml CEA level.

Figure 3.

Vimentin Methyl-BEAMing of fecal DNA from colorectal tumor patients. **(a)** The fraction of methylated vimentin fragments in 4 g feces from normal, age and sex-matched control patients. **(b)** The fraction of methylated vimentin fragments in 4 g feces from patients with colorectal adenomas **(c)** The fraction of methylated vimentin fragments in 4 g feces from patients with colorectal carcinomas (Duke's A or B red; Duke's C or D, blue). The dotted line represents 2% methylated fragments. **(d)** Receiver operating characteristic (ROC) curves based on the fraction of methylated vimentin fragments for the indicated classes of samples.

Table 1

Methylated vimentin DNA fragments in patient samples

Patient Type	Sample Type	Number of Patients	Total vimentin fragments (methylated plus unmethylated) (mean $\pm$ SEM)	Fraction of methylated fragments (mean $\pm$ SEM)	# of vimentin methylated fragments in 2 ml plasma (mean $\pm$ SEM)	# of samples with ≥ 1 methylated fragments in 2 ml plasma (%; 95% CI)	P-values* (cancer vs normal)
Normal	Plasma	110	3170 $\pm$ 380	0.026% $\pm$ 0.013%	0.60 $\pm$ 0.27	8 (7%, 3%–14%)	
Colorectal cancers (all stages)	Plasma	81	8240 $\pm$ 1180	1.4% $\pm$ 0.40%	335 $\pm$ 177	48 (59%, 48%–70%)	3.2E-15
Duke's A	Plasma	22	5830 $\pm$ 781	0.052% $\pm$ 0.025%	1.8 $\pm$ 0.54	11 (50%, 28%–72%)	7.5E-06
Duke's B	Plasma	22	4560 $\pm$ 458	0.31% $\pm$ 0.23%	8.9 $\pm$ 5.3	12 (55%, 32%–76%)	1.2E-06
Duke's C	Plasma	15	6530 $\pm$ 632	0.76% $\pm$ 0.65%	38.4 $\pm$ 31.6	6 (40%, 16%–68%)	0.0019
Duke's D	Plasma	22	15,500 $\pm$ 4270	4.4% $\pm$ 1.2%	1200 $\pm$ 626	19 (86%, 65%–97%)	6.7E-14

Patient type	Sample Type	Number of Patients	Total vimentin fragment (methylated plus unmethylated) (mean $\pm$ SEM)	Fraction of methylated fragments (mean $\pm$ SEM)	# of samples with $\geq 2.0\%$ methylated fragments in 4 g stool (%; 95% CI)	P-values* (Adenoma or cancer vs normal)
Normal	Feces	38	47300 $\pm$ 16700	0.96% $\pm$ 0.25%	2 (5.3%, 0.6%–18%)	
Adenoma	Feces	20	69600 $\pm$ 45200	3.8% $\pm$ 0.86%	9 (45%, 23%–68%)	0.00055
Colorectal cancer (mixed stages)	Feces	22	236000 $\pm$ 66600	7.3% $\pm$ 3.3%	9 (41%, 21%–64%)	0.0011

*Two tail p-values were calculated using Fisher's exact test.