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Digital Detection of Multiple Minority Mutants in Stool DNA for Noninvasive Colorectal Cancer Diagnosis

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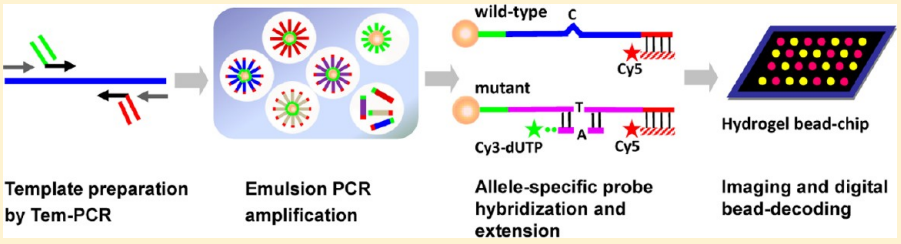
S *Supporting Information*



ABSTRACT: Somatic mutations in stool DNA are quite specific to colorectal cancer (CRC), but a method being able to detect the extraordinarily low amounts of mutants is challengeable in sensitivity. We proposed a hydrogel bead-array to digitally count CRC-specific mutants in stool at a low cost. At first, multiplex amplification of targets containing multiple mutation loci of interest is carried out by a target enriched multiplex PCR (Tem-PCR), yielding the templates qualified for emulsion PCR (emPCR). Then, after immobilizing the beads from emPCR on a glass surface, the incorporation of Cy3-dUTP into the mutant-specific probes, which are specifically hybridized with the amplified beads from emPCR, is used to color the beads coated with mutants. As all amplified beads are hybridized with the Cy5-labeled universal probe, a mutation rate is readily obtained by digitally counting the beads with different colors (yellow and red). A high specificity of the method is achieved by removing the mismatched probes in a bead-array with electrophoresis. The approach has been used to simultaneously detect 8 mutation loci within the APC, TP53, and KRAS genes in stools from eight CRC patients, and 50% of CRC patients were positively diagnosed; therefore, our method can be a potential tool for the noninvasive diagnosis of CRC.

Colorectal cancer (CRC) is one of the most malignant cancers in the gastrointestinal tract, causing numerous deaths each year in the world, but can be effectively treated by surgical excision if detected at an early stage. Conventionally, CRC screening tests include colonoscopy and fecal occult blood test (FOBT).[1−3] However, colonoscopy is invasive[4] and FOBT is less sensitive;[5] these tools are thus inadequate for the early diagnosis. Therefore, the development of approaches enabling the early diagnosis of CRC is necessary.

Stool-based molecular testing is a newly developed technique for noninvasive detection of CRC and can be employed for screening CRC at the early stage.[6,7] The typical fecal markers include mRNAs (abnormal gene expression) and mutant DNA molecules. Although it is sensitive to detect CRC by measuring the expression levels of CRC-related genes,[8−10] mRNA molecules are easily degraded during stool storage and RNA extraction; thus, the requirement of the use of fresh stool and the effective extraction greatly limits the clinical applications of mRNA molecules as the starting material for the early detection. On the other hand, mutant DNA molecules are relatively stable in stool,[11] so they become the most suitable biomarkers in molecular diagnosis of CRC.[12] Several studies have shown that mutant DNA is quite specific to CRC and can be detected in stool, urine, and blood of CRC patients.[12−15] However, as the amount of mutant DNA is extraordinarily low at the early cancer stage,[16,17] the method being able to detect minority mutants in a large amount of background molecules (wild-type DNA) is challengeable in sensitivity.

Currently, BEAMing technology that converts single DNA molecules to single beads containing tens of thousands of exact copies of one template through water-in-oil emulsion PCR was developed for detecting somatic mutations at an ultra low level.[12,17] BEAMing technology dramatically increases the sensitivity in the discrimination of mutants from wild-type DNA. Although BEAMing was a powerful digital assay for mutation analysis, an expensive instrument, a flow cytometer, was required for the readout and only one mutation could be analyzed in a single experiment. Furthermore, each mutant-

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Table 1. Primers Used for Tem-PCR

genes	primer sequences[a]
APC	F_o: 5′-AACCAAGAAACAATACAGAC-3′
	R_o: 5′-GAAGAACCTGGACCCTC-3′
	F_i: 5′-TTTTCAGCAACTTGGCATTGTAATCAGACGACACAGGAA-3′
	R_i: 5′-TACTATCCTTCCCAGCTCCTGCTAAACATGAGTGGGGTCT-3′
TP53	F_o: 5′-GGTGGTTGGGAGTAGATGG-3′
	R_o: 5′-AGAGGCAAGGAAAGGTGAT-3′
	F_i: 5′-TACTATCCTTCCCAGCTCCTGCTTCTCTTTTCCTATCCTG-3′
	R_i: 5′-TTTTCAGCAACTTGGCATGCTTACCTCGCTTAGTGCT-3′
KRAS	F_o: 5′-TTTGAGAGCCTTTAGCCG-3′
	R_o: 5′- ACTCCCAAGGAAAGTAAAG-3′
	F_i: 5′-TTTTCAGCAACTTGGCATGGTGGAGTATTTGATAGTGTA-3′
	R_i: 5′-TACTATCCTTCCCAGCTCCTGATTTACCTCTATTGTTGGAT-3′
	F_s: 5′-TTTTCAGCAACTTGGCAT-3′
	R_s: 5′-TACTATCCTTCCCAGCTCCT-3′

[a] F_o, R_o, F_i, and R_i were gene-specific primers; F_s and R_s were universal primers.

specific probe should be labeled with fluorophores, resulting in a high cost when detecting multiple mutations.

On the basis of our previous work,[18] we here propose a novel method to digitally detect CRC-specific mutant DNA in stool at a low cost by improving the following three points. First, target enriched multiplex PCR (Tem-PCR),[19] instead of conventional PCR, was used to amplify multiple targets in a single tube for preparing templates with universal ends. Second, the incorporation of dye-labeled dUTP into the mutant-specific probes, instead of conventional dye-labeled probes, was used to label multiple mutants. Third, a hydrogel bead-array, instead of a flow cytometer, was used to decode beads coated with amplicons originating from single target molecules. We have successfully applied this method to CRC diagnosis by detecting a panel of 8 mutation loci within three genes (APC, TP53, and KRAS) in stool samples from CRC patients. The results suggest that our method has potential for realizing the noninvasive diagnosis of CRC and can help us design a clinically applicable DNA-based test in the future.

EXPERIMENTAL SECTION

Reagents and Cell Lines. *N*-Hydroxysuccinimide ester (NHS)-activated sepharose HP affinity column, dNTPs, and Cy3-dUTP were purchased from Amersham Biosciences (Piscataway, NJ). *Taq* DNA polymerase and exo$^-$ Klenow Fragment were from Promega (Madison, WI). DC 5225C Formulation Aid and DC 749 Fluid were purchased from Dow Chemical Co. (Midland, MI). Ar20 Silicone Oil was obtained from Sigma (St. Louis, MO). Other chemicals were of a commercially extra-pure grade. All solutions were prepared with deionized and sterilized water and were filtered by a 0.22 μm membrane before use.

Three types of cell lines, including SW480, CaCO, and LOVO, were used in the experiments. In SW480 cell lines, 4012C > T in the APC gene and 818G > A in the TP53 gene are mutants. In CaCO cell lines, 4099C > T in the APC gene is mutant, and in LOVO cell lines, 35G > T in the KRAS gene is mutant.

Clinical Specimens. For the study, specimens from CRC patients were obtained from Jiangsu Cancer Hospital (Nanjing, China). Patient details were summarized in Table S-1 in the Supporting Information. All stools were collected before colonoscopy or surgery. None of the CRC patients had undergone chemotherapy or radiotherapy before stool collection. A plastic bucket device was used to collect whole stool. Samples were immediately extracted or stored at −80 °C.

DNA Extraction. Genomic DNA from tissues was isolated by sodium dodecyl sulfate/proteinase K digestion, phenol−chloroform extraction, and ethanol precipitation. Stool was homogenized in ASL buffer and extracted with a QIAamp DNA Stool Mini Kit (Qiagen, Germany).

Initial Amplification by Tem-PCR. The initial amplification was performed in 25 μL PCR reactions, containing 1× TaKaRa *Taq* buffer, 1 U of *Taq* DNA polymerase, 1.5 mM $MgCl_2$, 0.2 mM of each deoxynucleoside triphosphate (dNTP), 0.04 μM of each gene-specific primers (F_0, R_0, F_i, and R_i in Table 1), 0.4 μM of universal primers (F_s and R_s in Table 1), and 50 ng of genomic DNA. Amplification was carried out using a five-stage cycling program: (i) hot start (15 min at 95 °C), (ii) enrichment stage (10 cycles, with 1 cycle consisting of 30 s at 94 °C, 1 min 30 s at 60 °C, and 1 min at 72 °C), (iii) tagging stage (10 cycles, with 1 cycle consisting of 30 s at 94 °C and 1 min 30 s at 72 °C), (iv) amplification stage (35 cycles, with 1 cycle consisting of 20 s at 94 °C, 20 s at 55 °C, and 20 s at 72 °C), and (v) extension stage (3 min at 72 °C). The Tem-PCR products were purified using QIAquick PCR Purification Kit (QIAGEN, Germany). The concentration of the purified PCR products was determined by a UV−vis spectrophotometer (Naka Instruments, Japan).

After Tem-PCR, there are two strands available for the immobilization to the beads during emulsion PCR. If the mutation locus of interest is close to the beaded end of a strand, the efficiency of subsequent extension reaction from an allele-specific probe may be greatly reduced. To address this issue, a simple way is to use the complementary strand as the template of extension reaction. This can be readily achieved by exchanging the 5′-terminal regions (the sequence tags for priming the universal primers F_s and R_s in Tem-PCR) between the primers F_i and R_i. As shown in Table 1, the 5′-terminal tags of the primers F_i and R_i used for amplifying the TP53 gene were exchanged.

Bead-Based Emulsion PCR. First, packed beads (10 μmol NHS sites ml^{-1}) from a *N*-hydroxysuccinimide ester (NHS)-activated Sepharose HP affinity column (1 mL) were activated by 1 mM ice-cold HCl at 4 °C for 1 h. Then, the activated beads and amine-modified primer (5′-NH_2-TTTTTTTTTTTTTTCAGCAACTTGGCAT-3′) were incu-

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bated in binding buffer (0.5 M NaCl, 0.2 M $NaHCO_3$, pH 7.5) at 20 °C for 5 h.

Three hundred and seventy five milliliters of emulsion oil were freshly prepared by 40% (w/w) DC 5225C Formulation Acid, 30% (w/w) DC 749 Fluid, and 30% (w/w) AR 20 Silicone Oil. Then, 225 μL of mock amplification mix (1× Promega *Taq* Buffer, 2 mM $MgCl_2$, 0.1% BSA, and 0.01% Tween-80) was homogenized with emulsion oil to make the emulsion more stable by forming extremely small emulsions. Primer-coated beads, templates, and PCR reaction (1× Promega Taq Buffer, 2 mM $MgCl_2$, 0.5 mM dNTP mixture, 0.1 U/μL Taq DNA polymerase, 0.06 mM forward primer (5′-TTTTCAGCAACTTGGCAT-3′), 0.6 mM reverse primer (5′-TACTATCCTTCCCAGCTCCT-3′), 0.1% BSA, and 0.01% Tween-80) were then added into emulsified material and mixed well. Amplification was carried out using the following cycling conditions: 94 °C for 4 min; 40 cycles of 94 °C for 30 s, 58 °C for 60 s, 68 °C for 45 s; 13 cycles of 94 °C for 30 s, 58 °C for 4 min.

Preparation of Hydrogel Bead-Array and Digital Analysis. After completion of the PCR program, the emulsion was broken and beads were recovered by expelling the emulsion through a 25 μm (in diameter) micropore membrane. Single-stranded DNA (ssDNA) was prepared by treating the beads with 0.1 mM NaOH for 2 min followed by two washes with ddH_2O. Acryl-modified glass slides (25.4 mm × 76.2 mm) were prepared as described.[20]

The hydrogel bead-array was prepared by adding the ssDNA-immobilized beads together with acrylamide monomer solution containing 3% (w/w) acrylamidemonomer (acrylamide: bis-acrylamide = 29: 1), 40% (w/w) glycerol, 1% (w/v) ammonium persulfate (APS), and 0.4% (v/v) *N,N,N′,N′*-tetramethylethylenediamine (TEMED) on a chip. Then, the bead solution was cast onto acryl-modified slides and immediately covered with a coverslip (20 mm × 20 mm) under room temperature for a 10 min copolymerization.

After the copolymerization, the coverslip was carefully removed from the hydrogel, and a universal fluorescence probe labeled with Cy5 and mutant-specific probes (Table 2) were hybridized in polyacrylamide gel at 40 °C for 2 h. To remove the free and mismatched probes in the gel chip, the probe-hybridized slides were subjected to electrophoresis at 4 °C under 50 V for 5 min in 1× TBE buffer. Then, extension was performed in gel with reaction mix containing 0.1 M Tris-Ac (pH7.7), 2 mM EDTA, 10 mM $Mg(Ac)_2$, 0.4 mg/mL PVP, 0.1% BSA, 1 mM DTT, 18 U/mL Klenow DNA polymerase, 0.5 mM Cy3-dUTP, 0.5 mM dTTP, and 1 mM of each dATP, dGTP, and dCTP. The extension reaction was carried out at 37 °C for 10 min. After the extension, the primer-extended slides were subjected to electrophoresis under the conditions described above to remove the free dNTPs and non-incorporated Cy3-dUTP. At last, the image of an array was obtained from the scanner (LuxScanner, CapitalBio, China).

Table 2. Probes Specific to Mutants

genes	mutation (codon)	probe sequences
APC	3925 G > A (1309)	5′-TCCAATCTTTTTTTTTAT-3′
	4012 C > T (1338)	5′-AACCCTACAGTCT-3′
	4067 C > T (1356)	5′-CGCTCCTAAAGAAA-3′
	4099 C > T (1367)	5′-GTGTCTAAGCACC-3′
TP53	814 G > A (272)	5′-TGAGATGCGTGTT-3′
	818 G > A (273)	5′-AGGTGCATGTTTG-3′
KRAS	35 G > T (12)	5′-TACGCCAACAGC-3′
	38 G > A (13)	5′-TACGTCACCAGC-3′
universal fluorescence probe		5′-TATCCTTCCCAGC-Cy5-3′

To count the color beads on an array, the scanning image of the bead array was first input into our homemade software Genepix pro 4.0. Then, the image of each channel (red and green) was converted into a gray-graded image. A threshold value, which was used to discrimnate a signal from a noise, was obtained by calculating the average intensity of each pixel on the image. Finally, signal dots were recognized and validated according to the threshold value and the size range (20−60 μm) as well as the shape (round) of the bead used in emPCR.

RESULTS AND DISCUSSION

Principle. Aiming at simultaneously analyzing multiple somatic mutations in various genes, we have combined a target enriched multiplex PCR (termed as Tem-PCR)[19] with bead-based emulsion PCR (emPCR)[17] for multiplex single-molecule amplification. The principle of our assay is showed in Figure 1, including three main steps. At first, use Tem-PCR to amplify multiple gene targets including all of the mutations of interest. In Tem-PCR, there are six primers, two outer primers (F_{on}, R_{on}), two inner primers (F_{in}, R_{in}), and one pair of universal primers (F_s, R_s). The universal primer pair (F_s, R_s) has the same sequence as the 5′-ends of two inner primers (F_{in}, R_{in}). As the concentration of the universal primer pair is much higher than that of other primers, all targets are tagged with the universal sequences of F_s and R_s; thus, the amplicons from Tem-PCR can be directly used as the template subsequent for multiplexed emPCR. Second, use emPCR to prepare amplicon-coated beads. In emPCR, only two primers (F_s and R_s) are required, and one of them is immobilized on the bead surface. Because both beads and amplicons from Tem-PCR are extensively diluted to an extent that no more than one target molecule and one bead are present in each compartment, amplification in water-in-oil emulsions generates beaded amplicons exactly originating from one target molecule. After emPCR, amplified beads are collected by breaking the emulsions. Finally, use a hydrogel bead-array, instead of conventionally a flow cytometer, to genotype the amplicons coated on each bead surface. To image the amplified beads, a universal probe labeled with Cy5 is used to hybridize the 3′end of the beaded amplicons. If a bead is coated with amplicons, the bead will be in a red color (Cy5). To identify the beads coated with mutants, a set of dye-free mutant-specific probes are also added into the gel array together with the universal probe. As the mutant-specific probes only have a single base difference from the wild-type amplicons, the specificity of probe hybridization is not always perfect. To increase the specificity, the slide with a hydrogel bead-array is subject to electrophoresis under a stringent condition whereby the mismatched probes can be efficiently removed by the electric force. To color the beads coated with mutant DNA, Cy3-dUTP and dNTPs are added into the slide for extending the perfectly matched mutant-specific probes, which are hybridized with beaded amplicons. After the electrophoresis treatment of the side, the beads coated with mutant amplicons show yellow color (Cy3 + Cy5) due to the incorporation of Cy3-dUTP and the hybridization of the universal probe labeled with Cy5, while the beads coated with wild-type amplicons only show red color because no extension reaction occurs on the bead surfaces. To block the extension reaction from the universal probe, Cy5 is labeled at the 3′-end

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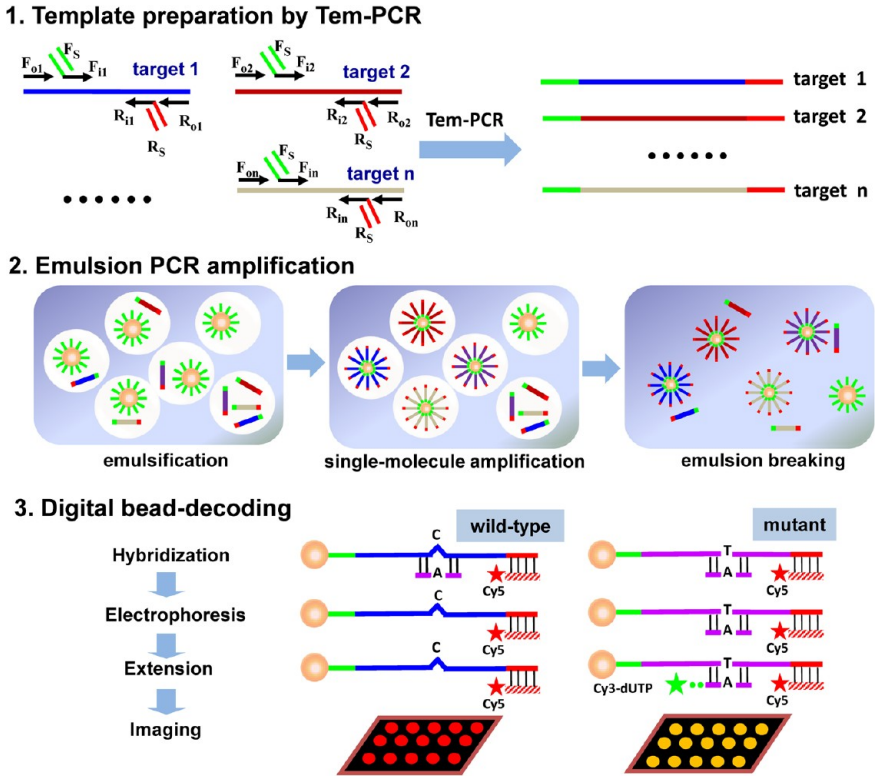

Figure 1. The schematic illustration of the assay for digitally detecting multiple mutants in stool. The assay includes three steps: (1) Tem-PCR for preparing universal sequence-tagged templates containing all mutation sites of interest; (2) emPCR for preparing beads coated with amplicons generated from a single molecule of Tem-PCR products. (3) Digital bead-decoding by hybridizing a universal Cy5-labeled probe and multiple mutant-specific probes with the amplicons coated on the beads which are fixed on a slide surface by hydrogel, followed by the extension reaction to incorporate Cy3-labled dUTP into the mutant-specific probes. If a bead displays a yellow color, the amplicons coated on the bead should be amplified from a mutant template, while a red bead means a wild-type template.

of the probe. In this assay, the specificity is dependent on the mutant-specific probe hybridization. The incorporated Cy3-dUTP is a universal reporter for all mutants; thus, the cost of the assay is dramatically decreased due to no need of dye-labeled mutant-specific probes for decoding the beads coated with mutants.

Tem-PCR for Preparing Templates of emPCR. Tem-PCR uses nested gene-specific primers at an extremely low concentration to enrich the specific targets during initial PCR cycles and relies on a pair of universal primers at a high concentration to achieve exponential amplification.[19] In our Tem-PCR, three genes (APC, TP53, and KRAS) containing 8 mutations were simultaneously amplified by 3-plex Tem-PCR in a single reaction using a universal primer pair and 3 sets of gene-specific nested primers. To investigate the accuracy of Tem-PCR, the three genes were also amplified by conventional PCR, respectively. The electrophorogram of both PCR amplicons is shown in Figure S-1 in the Supporting Information, indicating that Tem-PCR yielded three distinct bands which also occurred in three individual conventional PCR. Most importantly, no dimers and nonspecific bands are found in the figure, indicating that Tem-PCR can yield the same amplicons as conventional PCR, although multiple nested primers exists in a single reaction; thus, Tem-PCR is a convenient and effective way for multiplex amplification of different targets. Through Tem-PCR, all of the amplicons containing potential mutation sites of interest were simultaneously fragmented, enriched, amplified, and tagged with universal sequences; the amplicons could, hence, be served as templates for subsequent emPCR.

Decoding Amplified Beads in Hydrogel by Combining Probe Hybridization with Cy3-dUTP Mediated Extension Reaction. To discriminate beads coated with mutant amplicons from beads coated with wild-type amplicons, hybridization and extension of mutant-specific probes were employed; however, a potential issue is the false-positive results caused by the extension reaction from the nonspecifically hybridized probes. To verify the specificity of the method, here, we used a dye-free mutant-specific probe (4012 C > T) to capture the amplicons of the APC gene on beads at first and, then, incorporated Cy3-dUTP into these hybridized probes through polymerase extension reaction. As the specificity is mainly dependent on the probe hybridization, it is necessary to remove any mismatched probes on the bead surface before the extension reaction. We used electrophoresis, which is sensitive to one base difference in DNA duplex,[21] to treat the glass slide with hydrogel bead-array for increasing the specificity. To investigate whether the electrophoresis could discriminate single-base mismatches perfectly, two hydrogel slides, containing beads coated with wild-type amplicons and mutant amplicons, respectively, were hybridized with the mutant-specific probes, followed by treating the slides with and without electrophoresis under a stringent condition. As can be seen in Figure 2A,B, beads in both slides show green when the slide was not treated with electrophoresis. In contrast, no green signal was observed for the beads coated with wild-type amplicons after the electrophoresis (Figure 2C), but the signal

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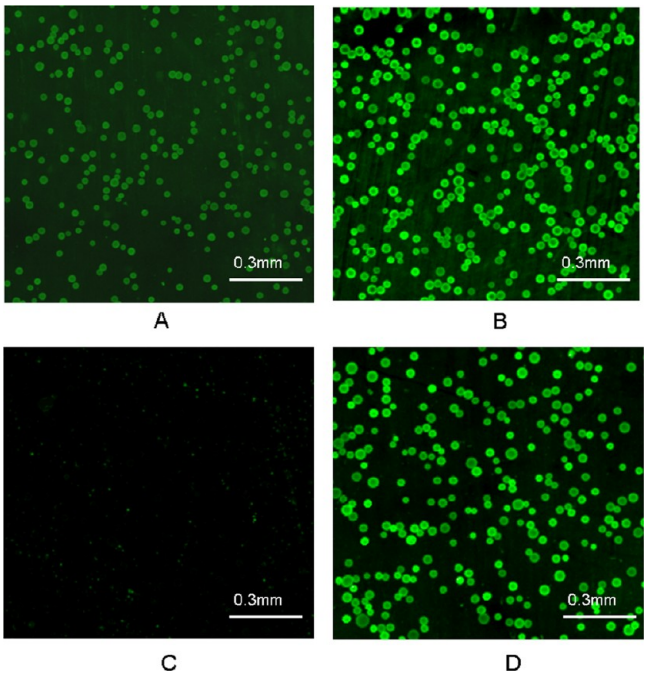

Figure 2. Images from hydrogel slides between wild-type amplicon-immobilized beads (A, C) and mutated amplicon-immobilized beads (B, D) after treating the slides with electrophoresis (C, D) and without electrophoresis (A, B). Amplicons of both wild-type DNA and mutants are from the APC gene and include the 4012C > T mutation site. The bead-array slides were hybridized with the 4012C > T mutant-specific probe at first and, then, treated by electrophoresis. After electrophoresis, the extension reaction by Klenow, dNTPs, and Cy3-dUTP in hydrogel was performed before scanning the slides.

remained the same for the beads coated with mutant amplicons (Figure 2D), indicating that the mismatched probes were successfully removed by electrophoresis, and the mutant-specific probes were extended only on the beads coated with mutant amplicons.

Further research (data not showed) also demonstrated that electrophoresis at a single voltage (50 V) is sufficient to simultaneously remove all types of mutant-specific probes hybridized with the corresponding wild-type amplicons from Tem-PCR. To obtain a somatic mutation rate in a given sample, it is necessary to count the number of beads coated with amplicons of both wild-type DNA and mutants. After emPCR, some beads coated with amplicons, but some did not. To digitally count beads coated with amplicons, a hydrogel was used to fix a single layer of the beads from emPCR on the glass slides. To recognize all beads coated with amplicons in the hydrogel, an amplicon-universal probe labeled with Cy5 at its 3′-end was also added into the gel together with the mutant-specific probes to capture the beads coated with amplicons; thus, the beads coated with amplicons should be red in the gel and would become yellow if the coated amplicons were of mutants. As shown in Figure 3A, beads coated with amplicons only originating from wild-type DNA templates exhibit red color, and the beads coated with mutants show yellow (Figure 3B), while both red and yellow beads are observed in the sample containing a small amount of mutants (Figure 3C). Therefore, it is easy to calculate the mutation rate in a sample by counting the number of colored beads.

Accuracy Evaluation. The above results have demonstrated that a perfect discrimination of single-base mismatch can be achieved by electrophoresis, causing a high specificity of hybridization and extension in hydrogel bead-array. To accurately detect the mutation rate of multiple mutation loci in a sample, it is necessary to investigate whether mutant-coated beads can be accurately recognized. As a proof-of-concept, four types of mutant DNA fragments (4012C > T and 4099C > T in the APC gene, 35G > T in the KRAS gene, and 818G > A in the TP53 gene), which were prepared by individual Tem-PCR, were equally pooled as the template of emPCR. The microbeads yielded from emPCR were divided into five aliquots, each of which was immobilized on a glass slide by hydrogel, producing five bead-array slides. Four of them were individually hybridized with the corresponding mutant-specific probes, and one of them was hybridized with the mix of the four types of mutant-specific probes. To image the beads coated with amplicons, the universal Cy5-labeled probe was also included for the hybridization. After extension reaction with Cy3-dUTP, the slides were imaged as shown in Figure S-2 in the Supporting Information. By counting the yellow beads in Figures S-2A, Supporting Information, and 2B,C,D with the software (Genepix pro 4.0), we found that the yellow beads are uniquely corresponding to the mutant-specific probes, and the red beads show yellow only in one of the four images (A, B, C, D in Figure S-2 in the Supporting Information), indicating that the multiple probes did not cause any cross-hybridization between amplified beads; thus, false-positive results should not occur in the assay. Because no red bead appears in Figure S-2E,

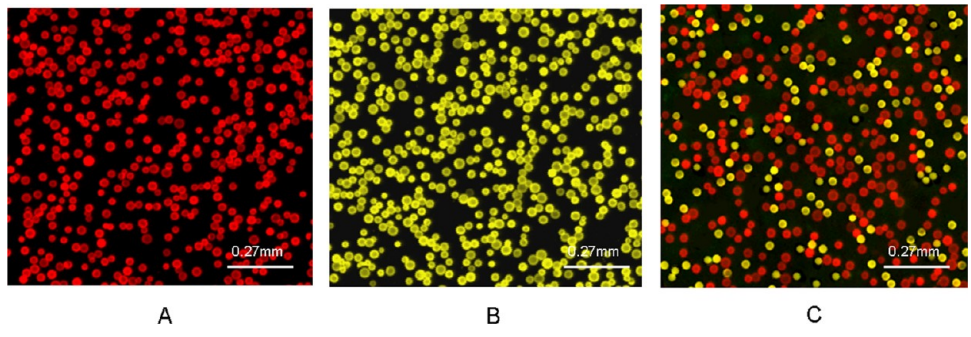

Figure 3. The images by scanning the hydrogel chips immobilized with beaded amplicons originating from wild-type DNA templates (A), mutants (B), and mix of wild-type DNA templates and mutants (C). Amplicons of both wild-type DNA and mutants are from the APC gene and include the 4012C > T mutation site. The bead-array slides were hybridized with the Cy5-labeled universal probe and the 4012C > T mutant-specific probe at first and, then, treated by electrophoresis. After electrophoresis, the extension reaction by Klenow, dNTPs, and Cy3-dUTP in hydrogel was performed before scanning the slides.

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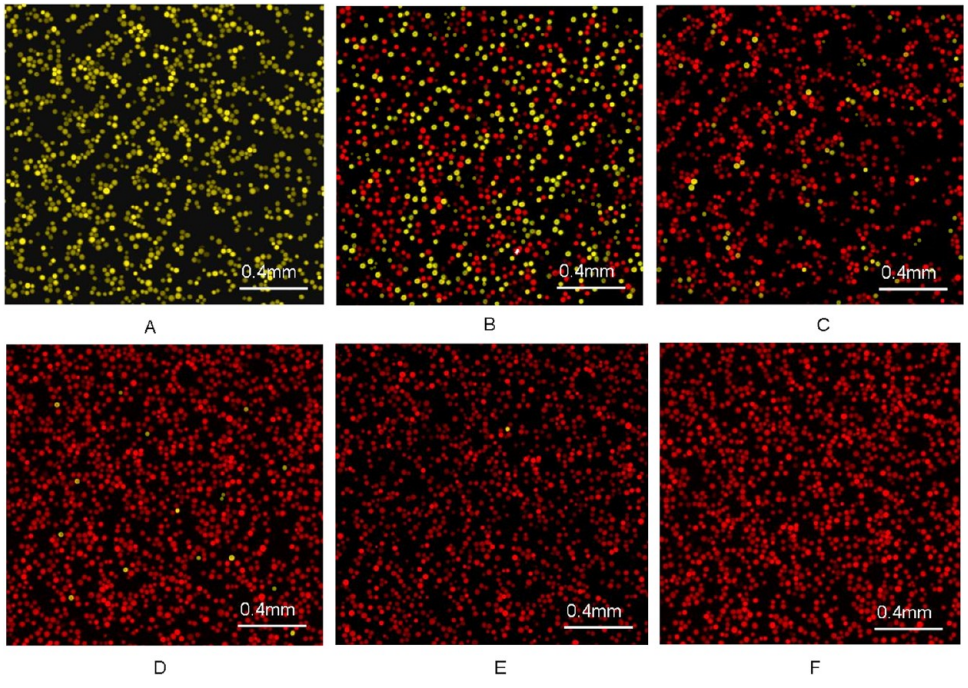

Figure 4. The images by scanning the gel-chips immobilized with beads from emulsion-PCR using the wild-type DNA spiked with 100% (A), 50% (B), 10% (C), 1% (D), 0.1% (E), and 0% (F) mutant DNA as templates.

Supporting Information (whole yellow beads), all amplified beads were captured by the mutant-specific probes; hence, we should not worry about false-negative results in the assay.

By counting the color beads on the chip (part of images can be seen in Figure S-2 in the Supporting Information), the mutation rates of 4012C > T and 4099C > T in the APC gene, 35G > T in the KRAS gene, and 818G > A in the TP53 gene are 24.7%, 25.3%, 23.9%, and 24.1%, respectively, which are very close to the expected value of 25%. The results indicated that the total mutation rate of multiple mutation sites in a sample can be accurately detected just by counting the ratio of yellow beads to the color beads on the chip.

Detection Limit. As the number of mutant DNA molecules is much lower than that of wild-type at the early cancer stage, it is necessary to investigate the detection limit of mutants in the presence of a large amount of wild-type DNA molecules. As a proof-of-concept, the mutation locus of 4012C > T in the APC gene was taken as an example, and the wild-type DNA and the corresponding mutant were individually prepared by Tem-PCR at first. Then, templates for emPCR were artificially prepared by spiking different amounts of the mutant into the wild-type DNA, allowing the templates to have the mutation rates of 50%, 10%, 1%, and 0.1%, respectively. After emPCR, the beads coated with emPCR products were immobilized onto glass slides by hydrogel. The typical images of the yielded bead-arrays including a positive control (100% mutant) and a negative control (0% mutant) are shown in Figure 4. By counting the color beads on the chip, the mutant percentages from the four templates were determined as 48.1 ± 2% (n = 3), 11.5 ± 0.7% (n = 3), 1.2 ± 0.06% (n = 3), and 0.13 ± 0.005% (n = 3), respectively; these values are very close to the expected values of 50%, 10%, 1%, and 0.1%. The mutant percentage detected from the negative control (0% mutant) is 0.02%; hence, the proposed method is accurate enough for determining as low as 0.1% mutant in a wild-type DNA background. The images from two controls indicate that the assay is very specific and accurate. The relatively large error at low mutant levels is believed to originate from the restriction of the scanning area (a standard size of a glass-slide) and the error in the counting method (the threshold for recognizing a bead). The sensitivity would be improved by increasing the scanning area.

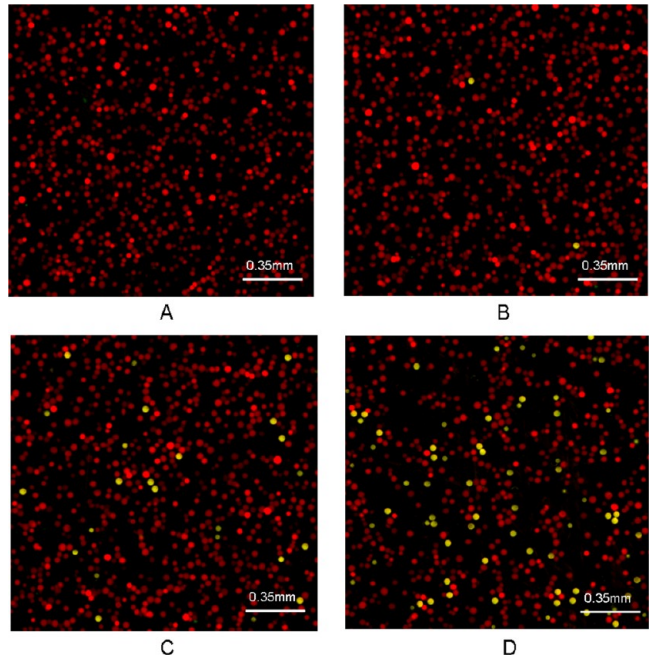

Figure 5. Images of bead-arrays originating from simulated stool samples prepared by spiking 10^2 (A), 10^3 (B), 10^4 (C), and 10^5 (D) SW480 cells into 0.4 g of stool from a healthy person.

Because the exfoliated tumor-cells are rare in stool, it is important to investigate the cell sensitivity of the assay. The stool with positive mutants were simulated by spiking a serial

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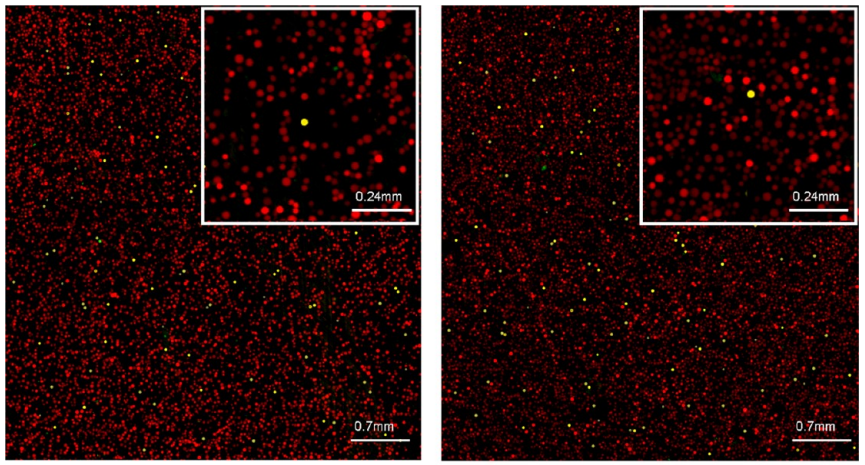

Figure 6. The typical images of bead-arrays originating from tumor tissue (A) and stool (B) from the CRC patient with the number of 203901 (Table S-3 in the Supporting Information).

dilution of SW480 cells into 0.4 g of a stool sample from a healthy person, achieving the stool samples with 10^2, 10^3, 10^4, and 10^5 SW480 cells, respectively. As SW480 cells are mutated at 4012C > T in the APC gene, this mutation locus was used as an example for the investigation. After extracting the DNA in stool, Tem-PCR and emPCR were performed. The images of bead-arrays from various amounts of cells are shown in Figure 5, indicating that the assay is able to detect yellow beads from the stool sample containing 10^3 SW480 cells; thus, the cell sensitivity of our method is around 10^3 tumor cells in 0.4 g of stool. Usually, more than half of the cells are lost after the extraction by the commercialized kit, so this sensitivity should be further increased if no loss of cells occurs during stool DNA extraction.

Analysis of Clinical Stool Samples. To evaluate the feasibility of the assay in noninvasive detection of clinical samples, stools from 10 CRC patients were employed for the investigation. As no DNA was obtained from 2 samples, we only detected the somatic mutations in 8 stool samples. The mutation rate was calculated from a panel of 8 mutation loci, including 3925 G > A, 4012C > T, 4067 C > T, and 4099C > T in the APC gene; 35G > T and 38 G > A in the KRAS gene; 814 G > A and 818G > A in the TP53 gene. The pathological information of the patients is listed in Table S-2 in the Supporting Information. After the detection, stools from 4 patients were detected to be mutant-positive. The positive results from patients (No. 203672 and No.203317 in Table S-2 in the Supporting Information) with early Dukes' stage (stage B) of colorectal cancers suggest that our method is promising in the early diagnosis of colorectal cancer. Although only 50% CRC patients were positively diagnosed, we believe that the sensitivity could be increased if more hot-mutation loci were employed for the detection.

To further look for whether the results from stool are consistent with that from tumor tissues, it is necessary to simultaneously detect the mutants from the corresponding tumor tissue. As not all stool samples have paired tumor tissue, we only detected mutation rates in the 4 paired tumor tissues. As shown in Table S-3 in the Supporting Information and Figure 6, the results from tumor-tissue samples are consistent with those from stool samples, indicating that stool can be used as starting material for noninvasive CRC diagnosis.

CONCLUSIONS

In this study, we have successfully demonstrated a simple and powerful platform based on hydrogel bead-array for noninvasive CRC diagnosis. As a perfect discrimination of single-base mismatch is achieved by combining electrophoresis with beads fixed on gel-chip, the assay is highly specific; thus, minority mutants can be digitally detected in the presence of a large amount of wild-type DNA molecules. In addition, the use of Cy3-dUTP for extending the mutant-specific probes, which is hybridized with the amplicon-coated beads fixed in hydrogel, allows the assay to be cost-effective. As Tem-PCR is an ideal tool for multiplex amplification of multiple targets with universal ends, it is very convenient to simultaneously prepare emPCR templates with multiple mutation loci of interest. It is expected that the sensitivity could be significantly increased if we employ more hot mutation loci in various genes as detection targets.

Here, we only performed a proof-of-concept study using very limited stool samples. To further test the method proposed here, a large number of samples from controls and patients with different stages of colorectal tumors are needed. Besides, we find that qualified genomic DNA can not be effectively extracted from every stool sample; hence, an improved version of the kit for efficiently extracting qualified genomic DNA from stool holds a great interest for further study. As only stool is used for the detection, we believe that our method should be a good tool for the noninvasive screening of CRC patients.

ASSOCIATED CONTENT

Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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