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PAPER

Digital analysis of the expression levels of multiple colorectal cancer-related genes by multiplexed digital-PCR coupled with hydrogel bead-array†

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To digitally analyze expression levels of multiple genes in one reaction, we proposed a method termed as 'MDHB' (**M**ultiplexed **D**igital-PCR coupled with **H**ydrogel **B**ead-array). The template for bead-based emulsion PCR (emPCR) was prepared by reverse transcription using sequence-tagged primers. The beads recovered from emPCR were immobilized with hydrogel to form a single-bead layer on a chip, and then decoded by gene-specific probe hybridization and Cy3-dUTP based primer extension reaction. The specificity of probe hybridization was improved by using electrophoresis to remove mismatched probes on the bead's surface. The number of positive beads reflects the abundance of expressed genes; the expression levels of target genes were normalized to a housekeeping gene and expressed as the number ratio of green beads to red beads. The discrimination limit of MDHB is 0.1% (*i.e.*, one target molecule from 1000 background molecules), and the sensitivity of the method is below 100 cells when using the β-actin gene as the detection target. We have successfully employed MDHB to detect the relative expression levels of four colorectal cancer (CRC)-related genes (c-myc, COX-2, MMP7, and DPEP1) in 8 tissue samples and 9 stool samples from CRC patients, giving the detection rates of 100% and 77%, respectively. The results suggest that MDHB could be a potential tool for early non-invasive diagnosis of CRC.

Introduction

Colorectal cancer (CRC) is a kind of disease with high incidence and mortality rates, now becoming one of the leading causes of death in the Western world. As the mortality is closely related to the stage of the disease at the time of diagnosis, detection at an early stage is likely to result in an improved recovery rate; thus, the development of approaches enabling the early diagnosis of CRC is necessary.[1]

Gene expression analysis is widely used for CRC diagnosis as it could accurately reflect the progression of tumors at molecular levels.[2] As CRC progression is associated with the aberrant expression of multiple genes,[3] a method allowing the simultaneous analysis of expression levels of multiple genes in a single reaction is preferable. Quantitative PCR (qPCR) is routinely used for gene expression analysis because of its high sensitivity and specificity.[4] However, it is not suitable for multiplexed analysis in a single reaction because the number of fluorophores available for the gene-labeling is limited, resulting in a time-consuming and labor-intensive procedure to detect multiple targets. DNA microarray is a powerful technology for monitoring a panel of expressed genes in a single experiment.[5] Although thousands of genes can be detected at a time, the detection limit and quantitative performance are still a challenge for the early diagnosis of CRC, because transcripts of many CRC-related genes are often low in abundance at the early stage.

Recently, the BEAMing (**B**ead, **E**mulsion, **A**mplification, and **M**agnetics) technique was developed to quantify the percentage of mutants in clinical specimens for CRC diagnosis.[6] BEAMing is based on the digital amplification of rare mutated DNAs by bead-based emulsion PCR (emPCR) which transforms a population of DNA fragment into a population of beads coated with emPCR amplicons, followed by the digital counting of the amplified beads with a flow cytometer. In comparison to conventional analog methods, BEAMing is able to detect mutants at a very low level. The digital nature of BEAMing has made it attractive to study the gene expression analysis. However, BEAMing was proposed only for DNA analysis. In addition, only one gene can be analyzed in each reaction and an expensive flow cytometer is required for readout. Furthermore, each gene-specific probe should be labeled with fluorophores, causing a high cost if multiple targets were detected.

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Based on our previous work,[7] here we proposed an improved method termed as 'MDHB' (**M**ultiplexed **D**igital-PCR coupled with **H**ydrogel **B**ead-array) to quantify expression levels of multiple genes in a single assay without the use of a flow cytometer and multiple dye-labeled probes. Briefly, two major improvements were made in this study: first, we achieved multiplexed emPCR amplification by employing a pool of gene-specific primers together with a universal primer bound to the bead's surface; second, we realized a cost-efficient hydrogel bead-array for fluorescent detection by using label-free probes to specifically target the sequences of interest, and then performed the primer extension reaction to incorporate Cy3-dUTPs to indicate the probe–target duplex. The levels of target transcripts within a sample were measured by counting the amplified beads immobilized on a glass surface. We have successfully employed this method to analyze the expression levels of multiple CRC-related genes (c-myc, COX-2, MMP7, and DPEP1) normalized to a housekeeping gene (β-actin) in total RNA isolated from the tissue and stool samples of CRC patients, suggesting that MDHB should be a potential tool for the non-invasive diagnosis of CRC at an early stage.

Experimental

Reagents

N-Hydroxysuccinimide ester (NHS)-activated sepharose HP affinity column, dNTPs and Cy3-dUTP were purchased from Amersham Biosciences (Piscataway, NJ). SuperScript III Reverse Transcriptase RNase H^- was purchased from Invitrogen Inc. (Carlsbad, CA). *Taq* DNA polymerase and *exo*$^-$ Klenow Fragment were from Promega (Madison, WI). Therminator DNA polymerase was purchased from New England Biolabs (Berverly, MA). 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 available extra-pure grade. All solutions were prepared with deionized and sterilized water and were filtered through a 0.22 μm membrane before use.

Oligonucleotide sequences

The amine-modified primer (5′-NH_2-TTT TTT TTT TCC ATC TGT TGC GTG CGT GTC-3′) was synthesized by TaKaRa Biotechnology Co., Ltd (Dalian, China). All the other primers were synthesized by Invitrogen Inc. (Shanghai, China). The reverse-transcription (RT) primer was 5′-CCA TCT GTT GCG TGC GTG TCC GGT TTT TTT TTT TTT TTV N-3′ with a 23-bp tagged sequence at the 5′ end to supply one priming site for the universal emPCR primer. The gene-specific primers for emPCR amplification, the gene-specific probes for bead-decoding, and PCR primers for the preparation of ssDNA from PCR products were listed in Table 1.

Template preparation

Total RNAs were extracted from tissues and stool samples of CRC patients by using RNeasy Mini Kit (QIAGEN, Germany) and Stool RNA Kit (Omega-Biotek, USA) respectively, according to the manufacturer's instruction. All patients gave informed consent. The purity and concentration of the extracted RNA were determined by a UV-vis spectrophotometer (Naka Instruments, Japan). The first-strand cDNA was synthesized with the RT primer tagged with a universal sequence by SuperScript III Reverse Transcriptase, according to the manufacturer's instruction.

Bead-based single-molecule PCR in water-in-oil emulsions

To prepare the primer coated beads, first, the packed beads (sepharose, 30 μm, 10 mmol NHS sites/ml) from 1 ml of *N*-hydroxysuccinimide ester (NHS)-activated Sepharose HP affinity column were removed from the column and activated by 1 mM ice-cold HCl at 4 °C for 1 h. Then, the activated beads and amine-modified primers were incubated in the binding buffer (0.5 M NaCl, 0.2 M $NaHCO_3$, 10 μM amine-modified primer, pH 7.5) at 20 °C for 5 h.

Six hundred microlitres of emulsion oil were freshly prepared as literature described:[8] 40% (w/w) DC 5225C Formulation Aid, 30% (w/w) DC 749 Fluid and 30% (w/w) AR20 Silicone Oil. Then 300 μl of mock amplification mix (1 × Promega *Taq* Buffer, 2 mM $MgCl_2$, 0.1% molecular biology grade BSA and 0.01% Tween-80) was homogenized with 600 μl of emulsion oil by a magnetic microstir-bar at 1200 rpm in a 5 ml vial. The aqueous phase was prepared by adding about 10^6 primer-coated beads and cDNA synthesized from 50 ng of total RNA into the PCR reaction mix (1 × Promega *Taq* Buffer, 2 mM $MgCl_2$, 0.5 mM dNTP mixture, 0.125 U/ml *Taq* DNA polymerase, 0.1% molecular biology grade BSA, 0.01% Tween-80, 0.06 mM universal primer, and 0.6 mM each of the gene-specific primers). Then water-in-oil emulsions were prepared by a dropwise addition of 300 μl of the aqueous phase to the vial while keeping the emulsions stirred at 1500 rpm. After the addition, the emulsions were stirred for an additional 3 min. The average diameter of the emulsion particles was about 60 μm as determined by a microscope (VANOX, Olympus, Japan). We thus estimated that an emulsion formed under the conditions described above contains around 10^7 compartments.

The emulsions were aliquoted into 12 PCR-tubes with each of 100 μl. Amplification was then performed in the following program: preheating at 95 °C for 3 min, then 40 cycles (94 °C, 58 °C and 68 °C for 15 s, 30 s, 45 s each) for amplification and 10 cycles (94 °C and 58 °C for 15 s, 180 s each) for hybridization and extension. After amplification, the emulsions were broken by ethanol, and beads were finally recovered by filtering the emulsions through a 25 μm (in diameter) micropore membrane.

Preparation of hydrogel bead-array

Acryl-modified glass slides (25.4 mm × 76.2 mm) were prepared as described.[9] After getting ssDNA on the recovered beads, 15 ± 5% of the beads were re-suspended in 15 μl acrylamide monomer solution containing 3% (w/w) acrylamide monomer (acrylamide : bis-acrylamide = 29 : 1), 40% (w/w) glycerol, 1% (w/v) ammonium persulfate, and 0.4% (v/v) *N*,*N*,*N*′,*N*′-tetramethylethylenediamine. Then the beads solution were cast onto acryl-modified slides and immediately covered with a cover-slip (20 mm × 20 mm) under room temperature for 10 min to copolymerize. The thickness of the prepared gel was about 40 μm and the density of beads was 100–300 positive beads per mm^2. After the copolymerization, the cover-slip was carefully removed from the hydrogel.

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Table 1 Oligonucleotides used in the study

Gene symbol	Accession no.	Amplicon size	Primer sequences (5′–3′)
Gene-specific primers for emPCR			
β-actin	NM_001101	143 bp	GATGTATGAAGGCTTTTGGTC
c-myc	NM_002467	202 bp	CAGTTACACAGAATTTCAATCCTA
COX-2	NM_000963	176 bp	ACCTGGGAATTTGGGTTGT
MMP7	NM_002423	229 bp	TGTTCCTCCACTCCATTTAGC
DPEP1	NM_004413	235 bp	AGAGTCCCCTTTAGGGTTCC
Gene-specific probes for bead decoding			
β-actin	NM_001101	—	CTTACCTGTACACTGACTTG-Cy5
c-myc	NM_002467	—	CCTAGTATATAGTACCTAGTA
COX-2	NM_000963	—	AGTGCCTCAGACAAATG
MMP7	NM_002423	—	TATGGTGTGACTGTGTCT
DPEP1	NM_004413	—	TCCAGATGCCAGGAGCC
PCR primers for preparation of ssDNA from PCR products			
β-actin	NM_001101	361 bp	FW: CCATCTGTTGCGTGCGTGTC RW: GAAGTCCCTTGCCATCCT
c-myc	NM_002467	383 bp	FW: CCATCTGTTGCGTGCGTGTC RW: GATTTAGCCATAATGTAAACTGCC
COX-2	NM_000963	366 bp	FW: CCATCTGTTGCGTGCGTGTC RW: TTCTTTTGGGAAGAGGGAGA
MMP7	NM_002423	229 bp	FW: CCATCTGTTGCGTGCGTGTC RW: TGTTCCTCCACTCCATTTAGC
DPEP1	NM_004413	350 bp	FW: CCATCTGTTGCGTGCGTGTC RW: ACCCATTACGGCTACTCCTC

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Digital bead counting

The hybridization and extension reactions were performed to decode the hydrogel bead-array. The hybridization mix containing 10 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$, 10 mM NaCl, and 1 μM for each probe was lidded on the hydrogel bead-array and the hybridization was performed in a humid chamber at 50 °C for 1 h. After hybridization, the probe-hybridized slides were subjected to electrophoresis at 4 °C, under 60 V for 5 min in 1 × TBE buffer to remove the free and mismatched probes in the gel chip. Then, extension mix containing 1 × Klenow buffer, 36 U/ml Klenow fragment, 1 μM of each dATP, dGTP, dCTP, 0.5 μM dTTP and 0.5 μM Cy3-dUTP was lidded on the hybridized area of the hydrogel bead-array. The extension reaction was carried out in a humid chamber at 37 °C for 10 min. After extension, the primer-extended slides were subjected to electrophoresis under the conditions described above to remove the free dNTPs and non-incorporated Cy3-dUTPs. The beads were finally decoded as green (Cy3 dye) beads and red (Cy5 dye) beads by a scanner (LuxScanner, CapitalBio, China). The colored beads were counted by our home-made software Genepix pro 4.0. The expression level of CRC-related genes within a sample was normalized to a housekeeping gene and determined as the number ratio of green beads to red beads; to simplify the data expression, each ratio was multiplied by 10^4.

Results and discussion

Overview of MDHB

A schematic of MDHB was illustrated in Fig. 1, and includes three steps. (i) Template preparation. In conventional bead-based emPCR, a pair of universal primers was required for amplifying multiple targets; thus the targets should have universal priming regions in both ends. In the case of DNA analysis, these targets were prepared by coupling the DNA fragmentation with the ligation of two adaptors to the fragments, or by pre-amplification with sequence-tagged primers.[6,8] Unlike DNA, mRNA should be reverse-transcribed before PCR; hence the RT primer containing a universal tag sequence at its 5′-end is employed to generate a pool of first-strand cDNAs containing the tag sequence at the 5′-end, supplying a universal priming region for the emPCR primer immobilized on the bead's surface. (ii) Digital amplification. As the prepared cDNA template only contains one universal priming region, a group of primers specific to target genes (here 5 genes) was required for multiplexed emPCR amplification. To achieve single-molecule amplification in water-in-oil emulsions, both the templates and the primer-coated beads should be diluted to an extent that no more than one target molecule and one bead were present in each compartment. After PCR amplification, an amplified bead would be coated with amplicons originating from one target molecule. (iii) Bead counting. To number the amplified beads, the hydrogel was used to fix the beads and form a single-bead layer on a slide. The beads amplified from the housekeeping gene were decoded by hybridizing a 3′-Cy5-labeled probe to the bead-array; while the beads amplified from CRC-related genes were decoded by incorporating Cy3-dUTPs into the gene-specific probes (label-free), which were hybridized with the bead-array, through the primer extension reaction. As the 3′-end of the probe specific to housekeeping gene was labeled by Cy5, no Cy3-dUTPs would be incorporated. Therefore, the number of red (Cy5 dye) beads and the number of green (Cy3 dye) beads reflect the expression levels of housekeeping gene and CRC-related genes, respectively.

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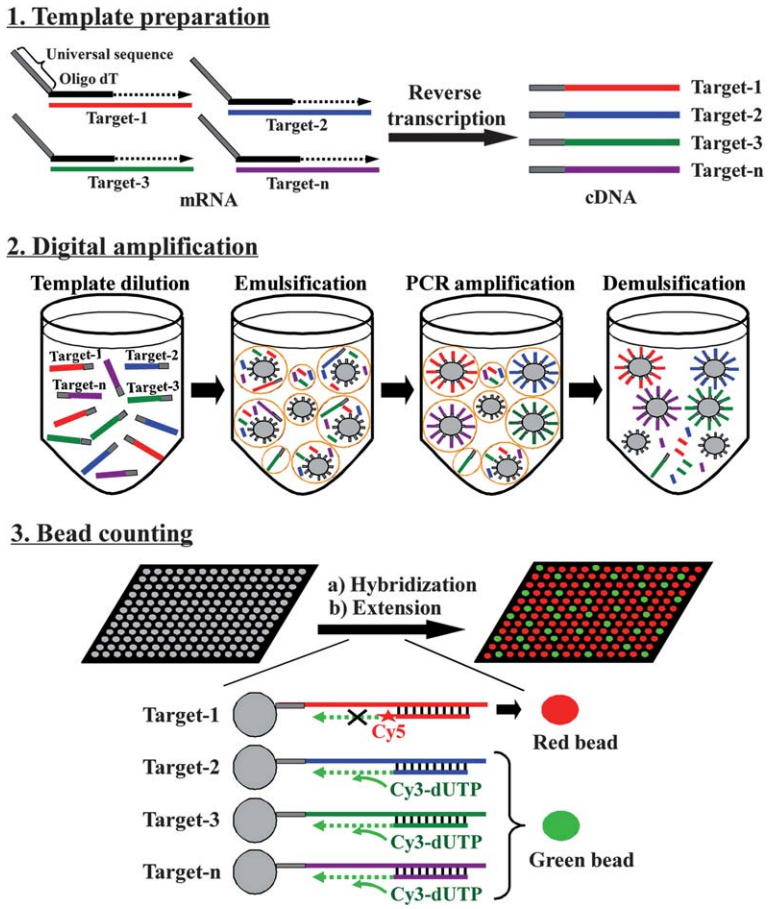

Fig. 1 Schematic illustration of MDHB. MDHB includes three steps: (1) template preparation by reverse transcription using a primer tagged with a universal sequence; (2) digital amplification by bead-based single-molecule emPCR; (3) bead counting through hydrogel bead-array. Target-1 represents the housekeeping gene as a quantitation reference, and target-2, -3, and -*n* represent CRC-related genes.

Evaluation of the extension reaction on amplicon-coated beads immobilized in hydrogel

As shown in Fig. 1, primer extension reaction was performed to incorporate Cy3-dUTPs into the strands coated on the surface of beads, which are immobilized in hydrogel. Our previous work showed that the 3-D porous structure of the hydrogel allows the probes to enter the inside of the gel for hybridization.[10] To investigate whether a polymerase can enter the hydrogel and effectively initiate the primer extension reaction, a sequencing strategy based on extension-quenching-extension was used.[11] In this process, Cy3-dNTPs (dATP, dCTP, dGTP and dTTP) was individually added according to the target sequence; the fluorescence is deactivated by hydrogen peroxide before the next extension step. The sequencing results in Fig. S-1† indicated that the primers annealed on beaded amplicons could successfully extend 21 bases; thus the 3-D porous structure of the hydrogel allows efficient polymerase extension reaction. Consequently, Cy3-dUTP can be used for labeling the amplicons on bead's surface.

Specificity of probe hybridization

In the proposed method, the specificity of the hybridization greatly affects the accuracy in the bead-decoding step. Previously, we employed electric stringency to remove the non-specific probes in polyacrylamide gel for SNP typing.[10] For evaluating the possibility of using electrophoresis to remove the mismatched probes on the bead's surface inside the hydrogel, gene-specific probes (c-myc, COX-2, MMP7, and DPEP1) were individually hybridized to the gel-immobilized beads coated with the amplicons of the β-actin gene; the mixture of the four probes and a negative control (no probe added) were also conducted for the hybridization; hence a total of six tests was performed. Results by only rinsing the hydrogel bead-array with water gave about 50% yellow beads (incorrect signals where both Cy5 and Cy3 are recorded); while only about 0.01% yellow beads were detected for all the six tests treated with electrophoresis (Table S-1†). As the fraction of falsely scored yellow beads is close to that of the negative control, the electrophoresis is much stringent to improve the specificity of probe hybridization on bead's surface inside of the hydrogel.

Template concentration suitable for single-molecule amplification

Single-molecule amplification is a prerequisite for an accurate quantitation in MDHB. In the present emPCR system, the number of microemulsion was estimated to be around 10^7; thus,

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in principle, the number of target templates for single-molecule emPCR should be less than 10^7 molecules. To investigate the effect of template concentration on single-molecule amplification, single-stranded PCR amplicons with 10^7 and 10^8 copies (Table S-2†), and first-strand cDNAs reverse-transcribed from 500 ng (equivalent to 10^7–10^8 copies of target cDNAs by qPCR) and 50 ng (equivalent to 10^6–10^7 copies of target cDNAs by qPCR) of total RNA were used as the templates for 5-plex emPCR, respectively. As shown in Fig. 2A and 2C, templates with 10^8 copies of ssDNAs and with cDNA from 500 ng of total RNA gave yellow beads, indicating that one microreactor accommodates more than one template molecule; while no yellow beads were observed when the template number is reduced by ten times (Fig. 2B and 2D). Therefore, to achieve single-molecule amplification, the maximal amount of templates for the present 5-plex emPCR system is around 10^7 copies. In the case of clinical samples, cDNA used for emPCR should be in an amount less than the transcripts from 50 ng of total RNA.

Accuracy

To investigate the accuracy of our method, comparison between MDHB and qPCR, a gold standard for quantitative gene expression analysis, was carried out through individually analyzing the expression levels of each of four CRC-related genes relative to the β-actin gene from the tumor tissue of a CRC patient. The MDHB scan-images of each of the four genes together with the panel of the four genes were shown in Fig. S-2.† Results of comparing MDHB with qPCR are shown in Fig. 3, and no obvious differences in the expression levels between these two methods were observed for either each of four genes or the panel of the four genes. For example, the relative expression levels of the total four genes in the CRC-patient tissue sample were 2776 by MDHB, close to the sum of 2817 calculated by qPCR. Therefore the accuracy of our digital assay is high, and can be used for the digital analysis of relative expression levels of multiple genes through simply performing serial hybridizations.

Quantitative performance

For evaluating the quantitative performance of MDHB, a serial dilution series of ssDNA (Table S-3†), artificially prepared by mixing the β-actin gene's amplicon with the pools of the four CRC-related gene's amplicons at the copy-number ratios of 1 : 1, 5 : 1, 25 : 1, 125 : 1, 1000 : 1, 3000 : 1, respectively, were used as the starting templates of multiplexed emPCR; the scan-images of MDHB were shown in Fig. S-3.† The number ratios of green beads to red beads in different templates were calculated and correlated with the expected values, giving a correlation coefficient as good as 0.999 (Fig. S-4†). As seen in Fig. 4A, the result from the template of 3000 : 1 is close to that from the control; thus the discrimination power of MDHB is as sensitive as 0.1% (one target molecule from 1000 background molecules). The data in Table S-3† also suggest that the sensitivity of MDHB is about 14 aM (5000 target molecules).

Because the tumor exfoliating cells are rare in feces, it is important to investigate the cell sensitivity of the MDHB assay. The expression level of the β-actin gene in a serial dilution series of SW480 cell lines was analyzed. As shown in Fig. 4B, there is no difference in the number of positive beads between a negative control and 10 cells, but the number of positive beads from the sample of 100 cells is significantly larger than that from the negative control, indicating that the cell sensitivity of MDHB is better than 100 cells (about 30 aM target transcripts in cDNAs). Since the abundance of the β-actin gene is much higher than that of cancer-related genes, the sensitivity of MDHB would be in the range of 1000 tumor cells. Normally, 0.2 g of a stool sample contains about 10^5 tumor exfoliating cells when a tumor size is 1 cm in diameter,[12] so the present assay is sensitive enough to detect the rare cancerous exfoliating cells in stool samples.

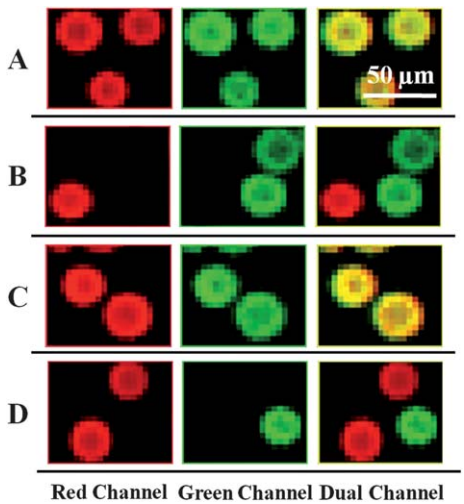

Fig. 2 Typical fluorescence images in different scan-channels (red, green, and dual) from the amplified beads of emPCR using the ssDNA of 10^8 (A) and 10^7 (B) copies, and with cDNAs synthesized from 500 ng of total RNA (C) and 50 ng of total RNA (D) as the template, respectively. Yellow beads shown in the dual channel suggest that the single molecular amplification was not successful (A and C); while red beads and green beads shown in the dual channel suggest that the single molecular amplification was successful (B and D).

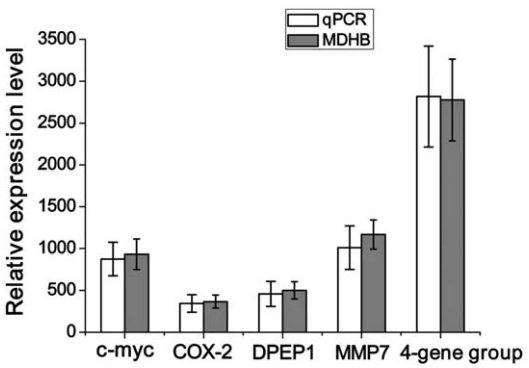

Fig. 3 Comparison of relative gene expression levels in the tumor tissue from a CRC patient between qPCR and MDHB ($n = 3$). The results from qPCR were calculated by dividing the copy number of the CRC-related gene with that of the housekeeping gene, and then were multiplied by 10^4. The 4-gene group means a panel of four CRC-related genes (c-myc, COX-2, MMP7, and DPEP1), and the relative expression levels of the group were obtained by summing the qPCR results from each of the four genes.

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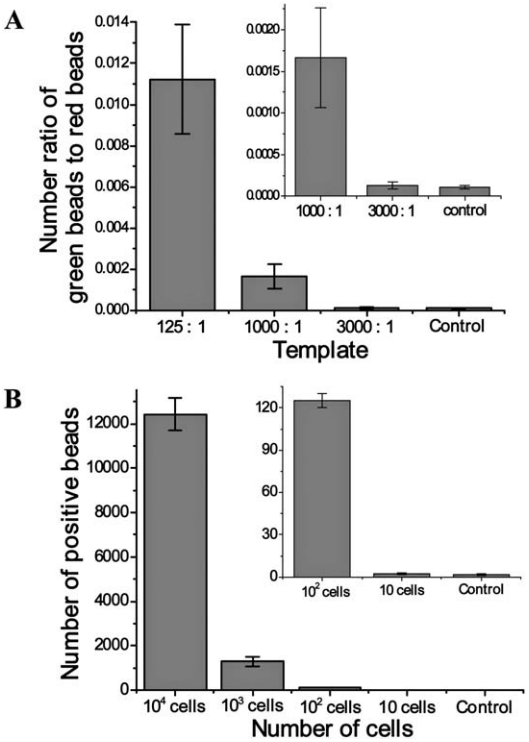

Fig. 4 Investigation of the quantitative performance of MDHB. (A) Discrimination power of MDHB with templates at different ratios of housekeeping gene (β-actin) to the target genes (c-myc, COX-2, MMP7, and DPEP1) ($n = 3$). (B) Sensitivity of MDHB using a serial dilution series of SW480 cell lines ($n = 3$). Insets are the enlarged view of the corresponding bars.

Digital analysis of tissue samples from CRC patients

To demonstrate the feasibility of MDHB in the detection of clinical specimens, the relative expression levels of four CRC-related genes in both tumor tissues and adjacent normal tissues from 8 CRC patients (Table S-4†) were detected. As shown in Fig. S-5,† using a panel of the four genes as a single diagnostic biomarker demonstrated a more marked difference than individual ones. The typical scan images for analyzing the group of four genes are shown in Fig. 5A, and the results of all 8 patients are illustrated in Fig. 5B, indicating that the relative expression levels of the total four CRC-related genes in tumor tissues are, on average, 7-fold higher than those in normal tissues; this significant difference ($P < 0.01$, by the Mann–Wallis test) allows an accurate diagnosis of CRC. Further study also demonstrated that the fold changes are closely correlated with Dukes' stages: for patients at early stages (Dukes' stages A and B), the expression level of the four genes is relatively low in tumor tissues (around 3–6-fold higher than that of the normal tissues); while for the patients in the late stages (Dukes' stages C and D), the fold change could go up to 7–14-fold. Therefore, employing this panel of CRC-related genes as a single diagnostic biomarker might be useful for monitoring the progression of tumors and a larger number of patients should be studied to further validate this conclusion.

To further prove the accuracy of our method, qPCR was used to detect the copy numbers of the above genes in the corresponding samples. As shown in Fig. S-5 and S-6,† the results from MDHB agree with those from qPCR, suggesting that MDHB is an accurate method for digital analysis of gene expression.

Digital analysis of stool samples from CRC patients

For early diagnosis of CRC, non-invasive detection is preferable. In contrast to other cancers, CRC could be non-invasively diagnosed if the tumor exfoliating cells in feces can be effectively detected. To employ MDHB for non-invasive detection, stool RNA was extracted from 13 CRC patients and 10 controls of healthy volunteers (Table S-4 and S-5†); but the stool RNAs from 9 patients and 7 healthy volunteers were successfully extracted. Fig. 6A showed the typical scan-images for analyzing the four CRC-related genes from a CRC patient and a healthy volunteer; it was observed that the number of green beads from the CRC patient is higher than that from the healthy volunteer. The comparison of the relative expression levels of the four genes in stool RNA between 9 cases and 7 negative controls were demonstrated in Fig. 6B, indicating that the specificity and the detection rate of MDHB for CRC diagnosis is 100% and 77%, respectively. It was also observed that the fold changes in stool samples decreased significantly compared with that in tissue samples. We believe that this is mainly caused by the increased proportion of background transcripts (housekeeping gene) deriving from normal exfoliating epithelial cells in the stool. Based on the detection results in Fig. 6B and the Dukes' stage of patients in Table S4, it is concluded that our method is promising in the early diagnosis of colorectal cancer because the detection rate of patients at the early stages (Dukes' stages A and B) is as high as 67% (4/6).

Currently, it is very challenging to efficiently extract tumor-specific RNA from a stool sample due to the rapid degradation of RNAs in feces and a large amount of background RNAs (such as bacterial RNA);[13,14] hence the key to the success of non-invasive diagnosis of CRC with MDHB is mostly dependent on the sampling process. An efficient extraction of tumor-specific RNA can be achieved when using tumor exfoliating cells, which are separated in advance from a stool sample by immuno-magnetic beads-based enriching technology,[14] as the starting material for RNA extraction. However, the high reagent cost and the labor-intensive steps limit its wide application in stool RNA extraction.

Conclusions

In summary, we have developed a sensitive and accurate assay for digitally detecting expression levels of multiple genes. The digital nature enables MDHB with a high accuracy and sensitivity as well as a wide dynamic range of relative quantification. The preparation of emPCR templates by sequence-tagged reverse-transcription is much simpler than that by sequence-tagged probe ligation.[15] Compared with the conventional detection platform based on the hybridization with fluorescence-labeled probes,[11,16] the proposed strategy is cost-effective because the gene-specific probes for the hybridization are dye-free.

Unlike qPCR, MDHB is able to digitally analyze the expression level of multiple target genes in one reaction, avoiding the troubles of preparing different standard curves for analyzing

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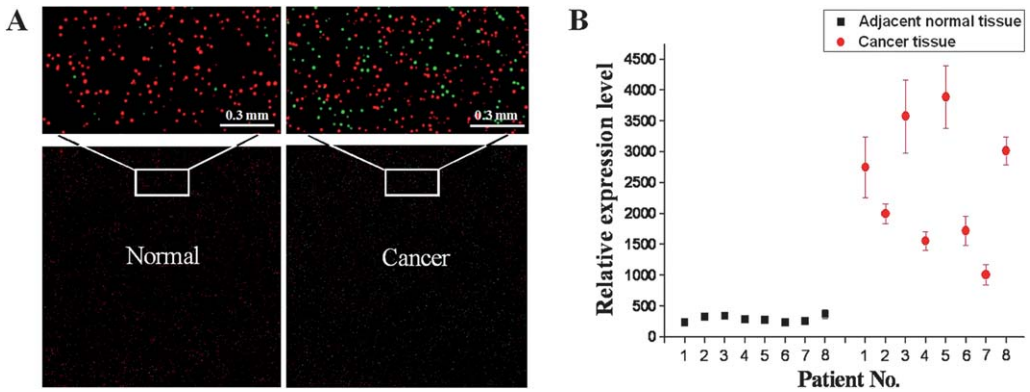

Fig. 5 Detection results of tissue samples with MDHB. (A) The typical scan images of MDHB for analyzing adjacent normal tissue and tumor tissue from a CRC patient. (B) The gene expression levels of total four CRC-related genes from the tissue samples of 8 CRC patients ($n = 3$).

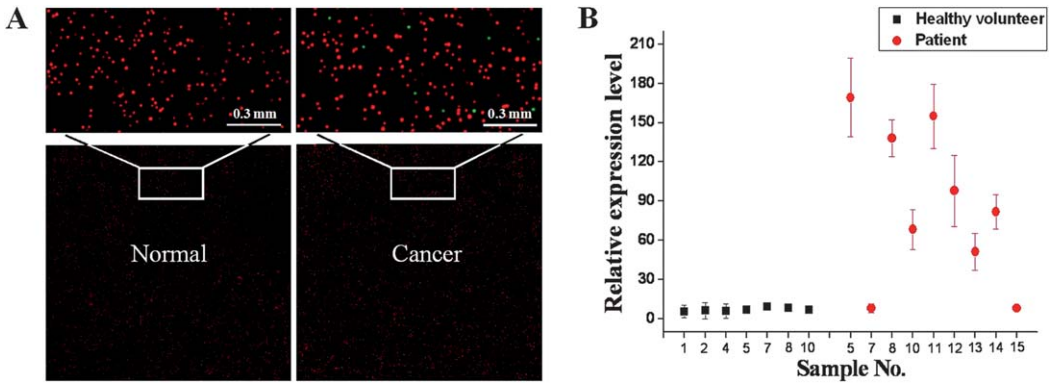

Fig. 6 Detection results of stool samples with MDHB. (A) The typical scan images of MDHB for analyzing stool samples from a healthy volunteer and a CRC patient. (B) The gene expression levels of total four CRC-related genes from stool samples of 7 healthy volunteers and 9 CRC patients ($n = 3$).

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different genes. Moreover, the combination of a group of biomarkers for diagnosis in MDHB might increase the detection rate, because the false negative results owing to the low expression level of a certain gene can be corrected by a panel of cancer-related genes. In principle, the detection rate could be further increased by increasing the size of the panel, but this is limited by the valuable biomarkers available. Although the sample size currently used for the evaluation is not large, the successful diagnosis of CRC using stool samples does give us hope of non-invasive diagnosis of CRC with MDHB.

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