An oligonucleotide-tagged microarray for routine diagnostics of colon cancer by genotyping *KRAS* mutations

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Received January 20, 2014; Accepted May 14, 2014

DOI: 10.3892/ijo.2014.2541

Abstract. Colorectal cancer (CRC) is one of the most prevalent types of cancer, causing significant morbidity and mortality worldwide. CRC is curable if diagnosed at an early stage. Mutations in the oncogene *KRAS* play a critical role in early development of CRC. Detection of activated *KRAS* is of diagnostic and therapeutic importance. In this study, *KRAS* gene fragments containing mutations in codon 12 were amplified by multiplex PCR using a 5'-Cy5-labeled reverse primer in combination with 3'-mutation-specific forward primers that were linked with four unique nucleotide-sequence tags at the 5'-end. The Cy5-labeled reverse primer was extended under PCR amplification to the 5'-end of the mutation-specific forward primers and thus included the complimentary sequence of the tag. PCR products were hybridized to tag-probes immobilized on various substrates and detected by a scanner. Our results indicate that all mutations at codon 12 of *KRAS* derived from cancer cells and clinical samples could be unambiguously detected. *KRAS* mutations were accurately detected when the mutant DNA was present only in 10% of the starting mixed materials including wild-type genomic DNA, which was isolated from either cancer cells or spiked fecal samples. The immobilized tag-probes were stable under multiple thermal cycling treatments, allowing re-use of the tag-microarray and further optimization to solid PCR. Our results demonstrated that a novel oligonucleotide-tagged microarray system has been developed which would be suitable to be used for detection of *KRAS* mutations and clinical diagnosis of CRC.

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Key words: colon cancer, *KRAS*, mutation, microarray, hybridization, diagnostics

Introduction

Colorectal cancer (CRC) is one of the most frequent cancers in Western countries, accounting for more than 10% of all cancer deaths (1). Survival of CRC has been shown to be highly dependent on the stage of the disease at the time of diagnosis (2,3). Several methods of colorectal neoplasm screening are currently available, including colonoscopy (4), barium enema (BE) (5) and fecal occult blood testing (FOBT) (6,7). These methods have several disadvantages for population-based screening, which include invasiveness, relative high cost, frequent false-positive results or the requirement of expert endoscopists. For example, colonoscopy, which remains the gold standard for identification of neoplasia (8), is unsuited for mass screening due to its invasiveness and requirement of expert endoscopists. FOBT is widely used as an initial screening method for colorectal tumors, however, it is not a robust assay because false-positive results are frequent (9). An ideal CRC-screening method, which has a high sensitivity and specificity for the target pathology at a curable stage, is therefore needed.

The Kirsten RAS (*KRAS*) is the most frequently mutated proto-oncogene that is critical for tumor progression. Activating mutations of *KRAS* in colorectal tumorigenesis are thought to alter GTPase activity, leading to unregulated cellular proliferation and malignant transformation (10-12). *KRAS* mutations occur early in the colon tumorigenesis pathway, hence, detection of *KRAS* mutations would be beneficial for early diagnosis, prognosis and evaluation of a therapeutic outcome in cancer treatment (13,14). *KRAS* is an effector molecule of epidermal growth factor receptor (EGFR), which is a key target of therapeutic strategies designed to treat metastatic CRC (15). Patients harboring *KRAS* mutations in codon 12 or 13 usually do not derive benefit from anti-EGFR treatment (16,17). However, recent studies indicate that patients who have *KRAS* condon 12- or *KRAS* 13-mutated tumors can respond to anti-EGFR treatment, and the survival of these patients with *KRAS* mutations correlates with anti-EGFR therapy in some cases (18-20). The American Society of

Clinical Oncology has recommended recently that all patients with metastatic colorectal carcinoma should have their tumor tested for *KRAS* mutations before anti-EGFR therapy with cetuximab or panitumumab (21).

Various methods have been devised to identify *KRAS* gene mutations, such as direct DNA sequencing (22), mutant enriched polymerase chain reaction (PCR) (23), peptide nucleic acid (PNA)-based PCR (24), restriction endonuclease-mediated selective (REMS)-PCR (25), PCR-restriction fragment length polymorphism (PCR-RFLP) (26), mutation tube assay (MUTA) test (27). There are a number of disadvantages in these methods, such as they are not convenient for use in clinical laboratories owing to multiple procedural manipulations that are laborious, time-consuming and cost-ineffective. A PamChip microarray system was reported previously by Maekawa *et al* (28), however, target regions of different genotypes were amplified separately in this approach, which is inefficient. Recently, a quantitative method termed allele-specific competitive blocker PCR (ACB-PCR) has been described for detecting *KRAS* gene mutations (29). Nevertheless, this method may need optimization in order to be applied in clinical screening, because multiple procedures are required to use this approach.

To establish a reliable technique suitable for detection of *KRAS* mutations for colon cancer screening, in this study we have developed an oligonucleotide-tagged microarray. Using this approach, we were able to detect *KRAS* codon 12 mutations in cancer cell lines and clinical samples. The optimized operating conditions permitted successful detection of homozygous as well as heterozygous DNA samples. Our results showed that 10% of mutant DNA could be detected in the WT background, suggesting that the tag-microarray-based method is suitable for CRC routine diagnosis.

Materials and methods

Oligonucleotides and cell lines. Oligonucleotides used in this study are summarized in Table I. All cell lines listed in Table II were maintained in our laboratory (National Veterinary Institute, Technical University of Denmark) or kindly provided by Mogens Kruhøffer (Aarhus University Hospital, Denmark). Cell lines were classified into four groups based on the sequence of codon 12 of *KRAS* gene: wild-type sequence (GGT) was designated 'WT', GTT was designated 'M1T1', GAT was designated 'M2T2', AGT was designated 'M3T3' (Table II). Cell lines HT29 and CALU-1 were maintained in McCoy's medium (HyClone); SW480, SW620 and A549 cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen); all other cell lines were grown in RPMI-1640 medium (Invitrogen). All media used for cell culture were supplemented with 10% fetal calf serum (Invitrogen). Cells were cultured at 37˚C in a humidified 5% CO_2 atmosphere. Four unique tags were selected based on published data (30) after analysis with regard to cross-priming, melting temperature, percentage of G + C contain, and possible secondary structure. Four single nucleotide polymorphism (SNP)-specific forward primers specific to four genotypes were designed. One of the four tags was linked to the 5'-end of each of these primers, respectively, to form forward chimeric tagged-primers (Table I and Fig. 1A). Four oligonucleotide probes were modified at the 5'-end with a poly (T) 10 - poly (C) 10 - probe binding tail (TC tail) to facilitate the attachment to the solid substrate, as previously described (31,32). One of the four unique tag sequences was introduced into the 3'-end of each probe (Table I). The reverse primer (Cy5-K-ras-RP2) was labeled with Cy5 fluorescent dye to facilitate detection (Table I). Oligonucleotides were synthesized at DNA Technology A/S (Arhus, Denmark).

Polymerase chain reaction (PCR) and multiplex PCR. Genomic DNA from all cell lines (Table II) was isolated using QIAamp DNA Mini kit (Qiagen, Hilden, Germany). A 111-bp fragment encompassing codon 12 of *KRAS* gene was amplified by PCR using primers KRASFP1 and KRASRP2 (Table I). PCR was performed in a 20 μl reaction mixture containing 10 μl HotStarTaq Plus Master Mix buffer (Qiagen), 2.5 mM $MgCl_2$, 20 μM forward and reverse primers and 10 ng extracted DNA. The reaction mixtures were subjected to amplification on PTC-200 Peltier Thermal Cycler (MJ Research, Watertown, NY, USA) with initial step at 95˚C for 5 min, followed by 30 cycles with denaturation at 95˚C for 40 sec, annealing at 55˚C for 40 sec and extension at 72˚C for 30 sec. Final extension was at 72˚C for 10 min. The PCR products were purified by QIAquick PCR purification kit (Qiagen) and were sequenced directly to determine the altered nucleotide of *KRAS* gene in codon 12. Equal amounts of four forward 'tag-primers' (Table I) were mixed thoroughly and used for multiplex PCR. The multiplex PCR was carried out in a total volume of 20 μl containing the following: 10 μl HotStarTag Plus Master Mix, 2.5 mM $MgCl_2$, 50 pg purified PCR product, 0.05 pM of mixed forward primer and 2.5 pM of reverse primer Cy5-K-ras-RP2 (Table II). PCR was performed with initial step at 95˚C for 5 min, followed by 30 cycles with denaturation at 95˚C for 40 sec, annealing at 68˚C for 40 sec and extension at 72˚C for 50 sec. Final extension at 72˚C for 10 min.

Preparation of the tag-microarray and hybridization. Topas plastic microscope slides (Microfluidic, Germany) were treated with 95% ethanol for 1 h and then rinsed with sterile water and dried at room temperature before use. Super Frost glass microscope slides were purchased from Menzel (Braunschweig, Germany). GAPS-II coated glass slides were purchased from Corning (NY, USA). Four DNA probes (Table I) modified at the 5'-end with TC tail (31) were diluted in 150 mM sodium phosphate buffer (pH 8.5) to a final concentration of 50 pM, and deposited in a volume of 10 nl/spot onto the slides using Q-Array robot spotter (Genetix, UK).

The DNA probes were linked to the solid support using a simple one-step method previously described (31,32). After UV irradiation, the slides were washed with agitation in filtered 0.2% (w/v) sodium dodecyl sulfate (SDS) for 5 min followed by 0.1X saline-sodium citrate (SSC, 300 mM NaCl, 30 mM sodium citrate, pH 7.0) for 5 min and then spin-dried.

Cy5-labeled multiplex PCR products (without any additional post-PCR manipulation steps) were mixed with PerfectHyb Plus hybridization buffer (Sigma). Single strand DNA was obtained by boiling the mixture for 5 min followed by incubation in ice-water for 2 min. The solution was

Table I. Primers and probes used in this study.

Name	Oligonucleotide sequences (5'→3')[a]	Tm (°C)
KRASFP1	ATGACTGAATATAAACTTGT	35.0
KRASRP2	CTCTATTGTTGGATCATATT	38.1
Tag-K-ras-WT	ggttctgttcttcgttgacatgaggTGTGGTAGTTGGAGCT**GG**	76.7
Tag-K-ras-M1T1	gcagaactgatgagcgatccgaata TGTGGTAGTTGGAGCT**GT**	76.2
Tag-K-ras-M2T2	aatgatgctctgcgtgatgatgttg TTGTGGTAGTTGGAGCT**GA**	77.6
Tag-K-ras M3T3	gcggaacggtcagagagattgatgt AACTTGTGGTAGTTGGAGCT**A**	76.0
Tag-T10C10-WT	*TTTTTTTTTTCCCCCCCCCC*ggttctgttcttcgttgacatgagg	81.0
Tag-T10C10-M1T1	*TTTTTTTTTTCCCCCCCCCC*gcagaactgatgagcgatccgaata	81.8
Tag-T10C10-M2T2	*TTTTTTTTTTCCCCCCCCCC*aatgatgctctgcgtgatgatgttg	81.7
Tag-T10C10-M3T3	*TTTTTTTTTTCCCCCCCCCC*gcggaacggtcagagagattgatgt	82.5
Cy5-Tag-WT	Cy5-ggttctgttcttcgttgacatgagg	57.8
Cy5-Tag-M1T1	Cy5-gcagaactgatgagcgatccgaata	61.1
Cy5-Tag-M2T2	Cy5-aatgatgctctgcgtgatgatgttg	59.9
Cy5-Tag-M3T3	Cy5-gcggaacggtcagagagattgatgt	61.4
Anti-Tag-Prob-WT	*TTTTTTTTTTCCCCCCCCCCCC*TCATGTCAACGAAGAACAGAACC	69.4
Anti-Tag-Prob-M1T1	*TTTTTTTTTTCCCCCCCCCCCC*TATTCGGATCGCTCATCAGTTCTGC	69.1
Anti-Tag-Prob-M2T2	*TTTTTTTTTTCCCCCCCCCCCC*AACATCATCACGCAGAGCATCATT	69.4
Anti-Tag-Prob-M3T3	*TTTTTTTTTTCCCCCCCCCCCC*ACATCAATCTCTCTGACCGTTCCGC	69.5
Anti-P2-T10C10	*TTTTTTTTTTCCCCCCCCCC*TTATTCAGAATCATTTTGTGGACGAA TATGATCCAACAATAGAG	81.7
Cy5-K-ras-RP2	Cy5-CTCTATTGTTGGATCATATTCGTCCACAAAATGATTCTGAATTAG	69.6

[a]The unique tag sequences are shown in lowercase; the probe-binding poly (T)10-poly (C)10 tail at the 5'-end is shown in italic; the SNP-specific nucleotides are shown in bold.

Table II. Cell lines used in this study.

Cell line	Histology	Mutation	Sequence type	Genotype
HT29	Human colon adenocarcinoma	GGT	Homozygous	WT
PC3	Human prostate adenocarcinoma	GGT	Homozygous	WT
LNCaP	Human prostate carcinoma	GGT	Homozygous	WT
Colon205	Human colon adenocarcinoma	GGT	Homozygous	WT
MCF-7	Human breast carcinoma	GGT	Homozygous	WT
SW480	Human colon adenocarcinoma	GGT→G**T**T[a]	Homozygous	M1T1
SW620	Human colon adenocarcinoma	GGT→G**T**T[a]	Homozygous	M1T1
LS174T	Human colon adenocarcinoma	GGT→G**A**T[a]	Heterozygous	M2T2
A427	Human lung adenocarcinoma	GGT→G**A**T[a]	Heterozygous	M2T2
A549	Human lung adenocarcinoma	GGT→**A**GT[a]	Homozygous	M3T3
CALU-1	Human lung adenocarcinoma	GGT→**A**GT[a]	Homozygous	M3T3

[a]Mutant nucleotide in codon 12 is bold and underlined.

transferred immediately onto the surface of the slide. The microarrays were hybridized under a glass coverslip for 1 h at 37˚C in a humidified chamber. Slides were washed at room temperature in filtered 0.5 x SDS + 0.1% SSC for 10 min with agitation, rinsed in water and spin-dried. The microarrays were scanned in a LaVision scanner (LaVision BioTech, Germany) using appropriate laser power and exposure time suggested by the manufacturer. The fluorescent intensities of the spots were quantified using Fips BioAnalyzer 4F/4S software.

Sensitivity of the tag-microarray assay. Genomic DNA extracted from CALU-1 (M3T3) was mixed with either Colon205 DNA (WT) or DNA extracted from feces of a healthy donor at the indicated ratios. The mixed genomic DNA was used as a template for PCR and hybridization.

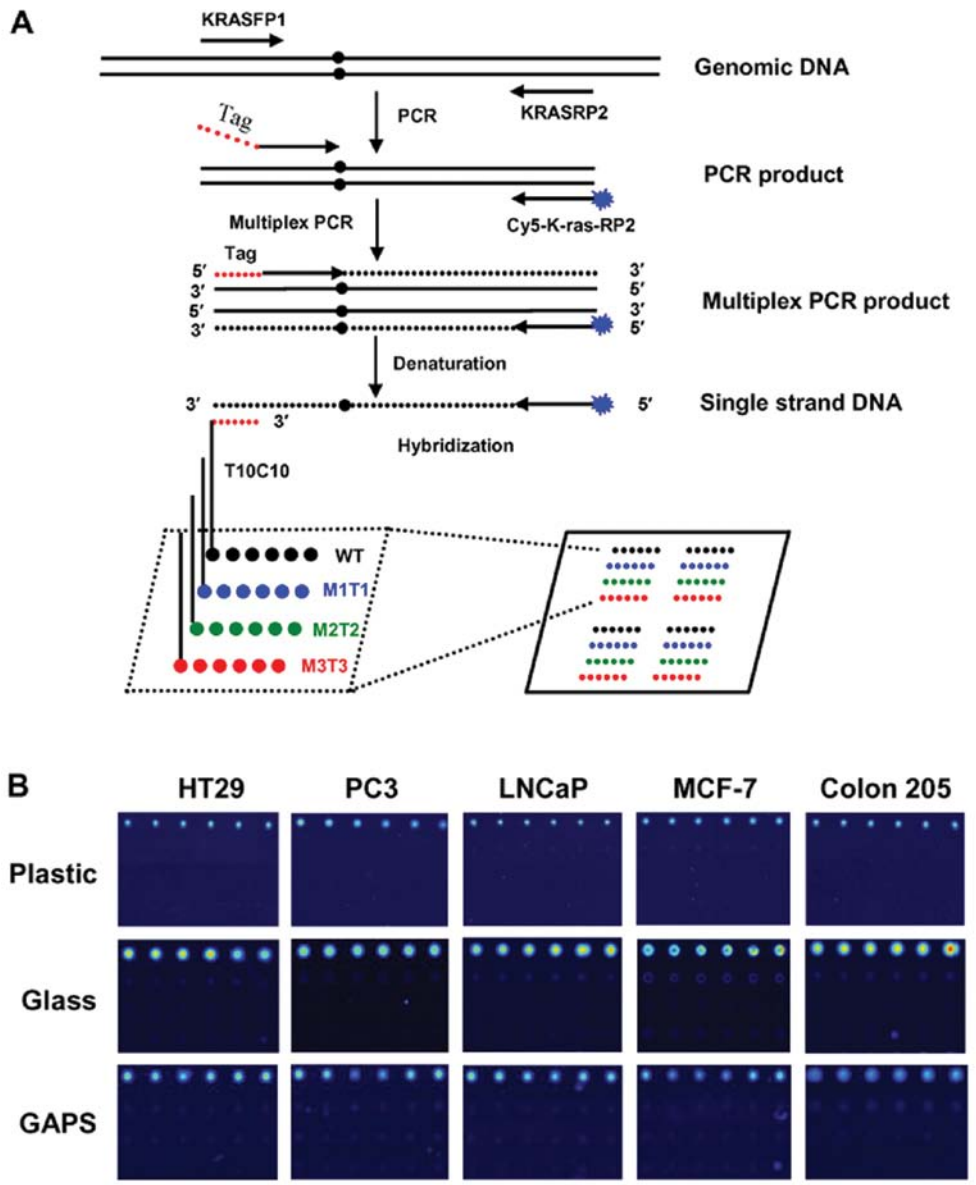

Figure 1. Schematic representation of the tag-microarray technique and detection of the WT genotypes. (A) Each slide was spotted with two array areas, each of which was used for hybridization with one multiplex PCR product. Four kinds of probes, marked with black, blue, green and red for WT, M1T1, M2T2 and M3T3 genotypes, respectively, were spotted robotically in each area and each probe was spotted in a row of six identical spots. Four identical arrays were formed in one area. Fragments spanning the K-RAS codon 12 ORF were amplified followed by multiplex PCR using a shared Cy5-labeled reverse primer and forward hybrid primers which each contains a unique tag sequence and a locus-specific sequence. The labeled reverse primer was extended to the 5'-end of the mutation-specific forward primer and was incorporated into the complementary sequence of the respective tag (anti-tag). The multiplex PCR product was hybridized to the immobilized tag probes. To enable immobilization using our simple UV method, the probes were modified in the 5'-end with poly (T) 10 - poly (C) 10 (31,32). The presence of Cy5 fluorescence allowed for the detection of specific hybridization. (B) Representative fluorescence images obtained from hybridization of WT targets of multiplex PCR product with probes immobilized on different substrates (plastic, glass, GAPS-II) are shown.

Genotyping of clinical tumor samples. Twenty-eight DNA samples from patients with tumors were used as template for PCR and hybridization as described above to determine the *KRAS* codon 12 mutations, which were further verified by direct DNA sequencing.

Thermal cycling stability and re-use of slide. Hybridization was performed as described above using WT DNA as a target. To remove the hybridized oligonucleotide DNA target, the arrays were boiled in distilled water for 5 min. SW480 DNA (M1T1) was used for hybridization utilizing the same array. Further boiling and hybridization were performed using the same arrayed slide and A549 DNA (M3T3) as the target. Before and after hybridization, the DNA arrays were scanned for Cy5 fluorescence.

Results

Tag-microarray design. To develop a robust, affordable, high-throughput method for the detection of *KRAS* mutations, a tag-microarray-based genotyping protocol, as shown schematically in Fig. 1A, was developed. A 111-bp fragment

encompassing the SNP region of *KRAS* codon 12 was amplified. The obtained PCR product was used as a template for nested multiplex PCR. In the multiplex PCR, the forward primers were chimeric tag-primers consisting of one of the four unique tags, linked at 3'-end with allele-specific sequences corresponding to WT and three mutant genotypes (M1T1, M2T2, M3T3, see Tables I and II). These primers were used in pair with a 5'-Cy5-labeled reverse primer (Cy5-K-ras-RP2, see Table I). The PCR products were hybridized to the tag probes immobilized on solid substrates (Fig. 1A).

The selection of the tag is an important step because the tag sequence significantly affects efficiency and specificity of hybridization (29,33-35). To minimize cross-hybridization, we first identified tag sequences that were predicted to have minimal cross-priming and unlikely to produce secondary structure. The selected four tags were tested experimentally for cross-hybridization by labeling all four tags with Cy5 fluorescent dye and hybridizing them respectively to a microarray containing four anti-tag probes (Table I). Strong fluorescent signal was reproducibly observed when Cy5-labeled tag target was hybridized with its complementary probe, whereas no signal was observed in random-combination groups (data not shown).

Detection of single-base variations of the KRAS gene of cancer cells. To assess the usefulness of the tag-microarray approach for detection of SNPs in genomic DNA, we examined DNA samples from various cancer cell lines carrying WT or different mutations in *KRAS* codon 12 (27,29,36,37). The specificity of the tag-microarray protocol described here relies largely on the performance of multiplex PCR, because the forward 'tag-primers' are allele-specific for the mutations of *KRAS*. We established an effective PCR amplification system in which no PCR amplicon was observed in gel-electrophoresis when one forward 'tag-primer' was omitted and its corresponding genotype DNA template was used in multiplex PCR which was accomplished based essentially on the same conditions described above (data not shown). DNA isolated from each cancer cell line (Table II) was used as template to obtain PCR amplicons containing codon 12. The PCR amplicons were purified and sequenced. The sequencing results revealed that there were five WT genotypes (HT29, PC3, LNCaP, MCF-7, Colon205), four homozygous mutants (SW480, SW620, A549, CALU-1), and two heterozygous mutants (LS174T, A427) (Table II). These results were consistent with data previously reported (2,14,22,23,27,38). A multiplex PCR was subsequently performed and the PCR products were used directly for the tag-microarray hybridization. Representative images obtained from WT samples were shown in Fig. 1B. As expected, specific signals were readily observed in all WT hybridizations in repeated experiments (Fig. 1B), indicating that DNA isolated from WT cell lines was hybridized specifically to its complementary probe immobilized on all substrates (Topas plastic, glass and GAPS-II coated slides). No cross-hybridization was detected in any WT hybridizations (Fig. 1B), demonstrating specificity of the hybridization. It should be noted that the size of the spot on plastic surface was slightly smaller than that on the surface of glass slide or GAPS-II coated slide (Fig. 1B, compare top panel and middle or bottom panel). This likely resulted from the different physical characteristics (surface tension) between plastic and glass substrates, rather than from hybridization. Relative fluorescence intensity value was calculated by dividing the value of intensity of fluorescence obtained from each spot to that obtained from background. The final relative value for each probe was determined as the average value obtained from six spots in three independent experiments. The results showed that higher averaged relative fluorescent signals were detected in normal glass slide, compared to those obtained in Topas plastic slide or GAPS-II coated slide (data not shown). However, overall, the intensity of fluorescence measured on different substrates was not significantly different.

Next, we analyzed all mutant DNAs extracted from cancer cell lines using procedures as described above. The results indicated that all mutant genotypes were assigned correctly (Fig. 2). Fluorescent signal was consistently detected at the expected genotyped sites in repeated experiments (Fig. 2), indicating that mutations in codon 12 could be distinguished from each other using our tag-microarray approach. The sequencing results showed that LS174 and A427 cells contain a WT allele in addition to the mutated genotype in *KRAS* gene (data not shown). Consistently, fluorescent signals were detected in both WT and M2T2 probe areas on all slides where the genomic DNAs extracted from LS174T and A427 cells were examined (Fig. 2, M2T2). The intensity of fluorescence between WT and M2T2 genotyped sites was equivalent within the margin of error for LS174T and A427 (Fig. 2), suggesting that the ratio between M2T2 and WT genotypes is 50% as expected in a heterozygous sample. The relative fluorescent intensity of all mutant DNAs hybridized to all immobilized probes was determined, and are shown in Fig. 2. Overall, the calculated values correlated well with the observed images with respect to the intensity of signal (Fig. 2). The presence of WT and mutations in different allele argued that it is of importance to include four primer sets (WT, M1T1, M2T2 and M3T3) in a multiplex PCR. Moreover, targeting both WT and all three mutation possibilities in a multiplex PCR allowed the assay to be self-controlled on a hybridization slide, as at least one set of the probes would be detected as positive, demonstrating the assay was successful. Thus, we did not test other primer mixes, e.g., with less primer sets, against a specific template. In sum, our data indicated that all genotypes of the cells tested were accurately discriminated by using the tag-microarray hybridization technique.

Sensitivity of the method. To quantify the detection limit of the tag-microarray method in a background of WT DNA, CALU-1 (M3T3) cell genomic DNA was mixed with varying quantities of WT genomic DNA from Colon205 cells (Fig. 3A) or fecal samples (Fig. 3B) collected from a healthy donor to generate spiked DNA samples. Fragment containing *KRAS* codon 12 was amplified using the mixed DNA sample as template, and hybridization was performed using normal glass slides, as described above. The results revealed that the *KRAS* mutant sequence was detectable when it was present only in 10% or more of the starting mixed materials (Fig. 3, proportion of DNA 90:10), demonstrating that the tag-microarray assay was sensitive. Overall, the intensity of the signal observed in both WT and mutant DNA samples was dose-dependent (Fig. 3).

Clinical validation. To evaluate the applicability of the tag-microarray approach, 28 tumor DNAs from clinical patients

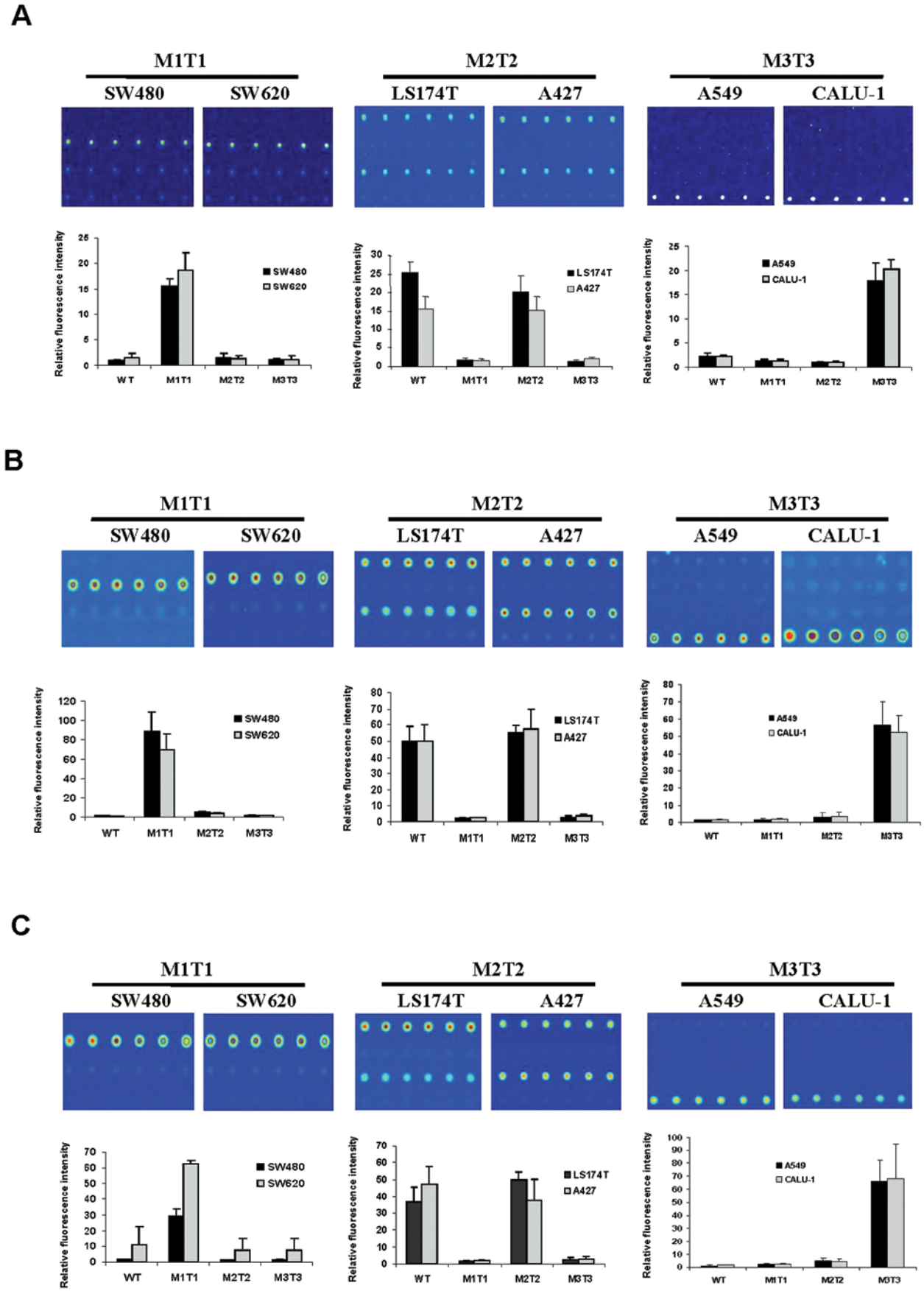

Figure 2. Representative fluorescence images of mutant target hybridization and quantitative analysis of intensity of fluorescent signal. Mutant targets (SW480, SW620, LS174T, A427, A549, CALU-1) of multiplex PCR product were hybridized with poly (T) 10-poly (C) 10-modified probes immobilized on (A) Topas plastic slide, (B) normal glass slide or (C) GAPS-II coated glass slide. PCR and hybridization were performed as described in Materials and methods. The intensity of fluorescent signal of each hybridization was obtained based on the averaged value of the six spots as shown in Fig. 1A. Data were normalized by dividing the averaged value of fluorescent signal of each spot to that of its corresponding background. The values of relative intensity represent an average of the results from three independent experiments.

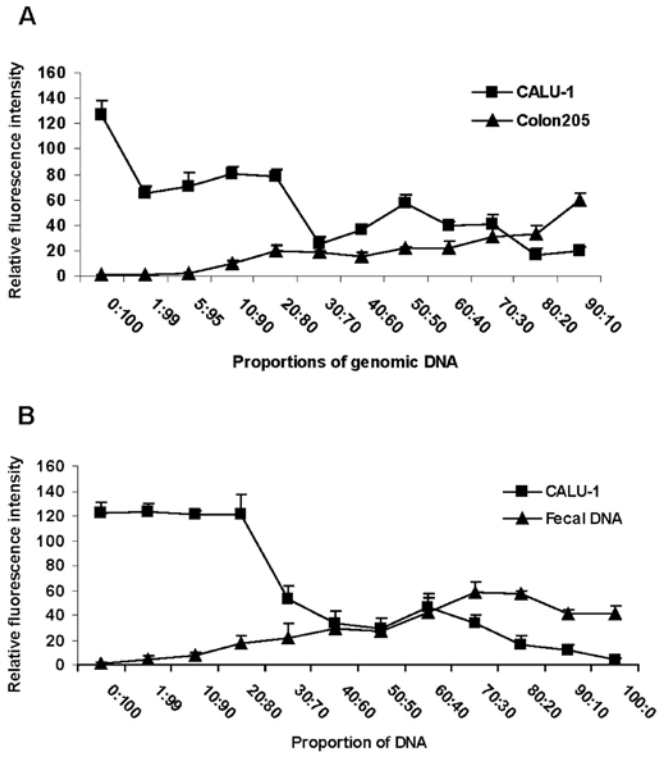

Figure 3. Sensitivity of the tag-microarray method. DNA extracted from M3T3 mutant cell (CALU-1) was mixed in different ratios with (A) WT DNA extracted from Colon205 cell or (B) fecal material from a healthy donor. The mixed DNAs were used for multiplex PCR and subsequent hybridization. The relative intensity of fluorescent signal was obtained as described in Fig. 2. The values of relative intensity represent an average of the results from three independent experiments. The lowest percent of the mutant DNA that was detectable represented the detection limit.

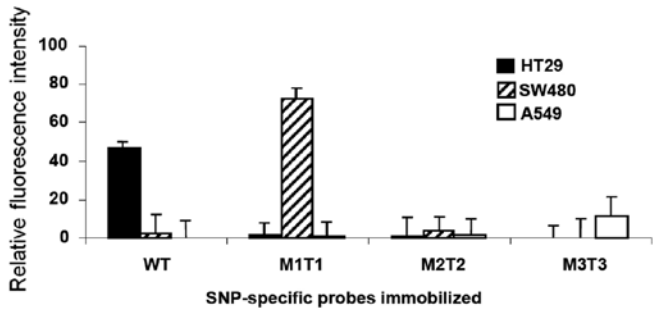

Figure 4. Stability of the immobilized probes and re-use of spotted slide. Probes were spotted on a normal glass slide and then hybridized sequently with H29 (WT), SW480 (M1T1) and A549 (M3T3) targets after boiling in distilled water for 5 min before and after each hybridization. The intensity of fluorescent signal produced in each hybridization was calculated as described in Fig. 2. The values of relative intensity representing an average of the results from three independent experiments were shown.

were analyzed using this technique. As shown in Table III, 20 (75%) of 28 samples were detected as WT and seven (25%) of those samples were detected as mutant. All detections were confirmed by direct sequencing. Of seven detected *KRAS* mutations, two (28.6%) were identified as M1T1 (GGT→GTT); four (57.1%) were identified as M2T2 (GGT→GAT); one (14.3%) was determined as M3T3 (GGT→AGT) (Table III). As verified by a comparison with direct sequencing, all the WT and mutant samples were correctly identified, suggesting that the tag-microarray detection technique performed well.

Table III. Clinical validation of the method.

		Mutants		
Genotype	WT	M1T1	M2T2	M3T3
Percent	(21/28, 75%)	2/7 (28.6%)	4/7 (57.1%)	1/7 (14.3%)

Stability of the attached probes and re-use of slide. Finally, we investigated the stability of immobilization of probes on the solid surfaces and examined whether the normal glass slides were reusable. We performed hybridization-boiling-hybridization cycles. Following a successful hybridization with significant fluorescence read, the 5-min boiling led to a background signal (data not shown), indicating that the hybridized targets were completely removed by the boiling. Re-hybridization of the boiled slides with HT29 target re-gained a specific signal, indicating the probes are stably attached and recognized by its target sequence after hybridization-boiling cycle. Similar results were observed for SW480 and A549 targets, and that an additional 15 min of boiling did not result in significant loss of the signal (data not shown). These results suggested that the immobilized probes were stable on the solid phase and the slide could be re-used after a thermo treatment. The corresponding value of signal for each target in repeated experiments is shown in Fig. 4.

Discussion

Currently, great attention is being paid to the detection of genetic variation in the development of human diseases such as cancer. Identification of mutations of host specific cellular gene is of considerable importance for the diagnosis of serious diseases such as CRC. DNA microarray has emerged as a promising tool for large-scale genetic analysis (39,40). The World Health Organization (WHO) estimates that there are over 940,000 CRC cases annually worldwide, with almost 500,000 deaths (41). Early diagnosis of CRC plays an important role in the minimization of death from CRC. We report here a powerful technique for accurate screening of mutations of *KRAS* for the early and fast diagnosis of CRC.

KRAS codon 12 was selected for this study since previous investigations revealed that activating mutations of the *KRAS* gene occurred in more than 90% of CRC cases, and most mutations are restricted to codon 12 (2,23,36,37,42). The codon 12 mutations are therefore an ideal biomarker for early detection of CRC. Previous study showed that the *KRAS* codon 12 GGT→GAT mutation was the most common mutation in the mucosa of colon cancer patients (28). Our result supported this idea, as 57.1% of clinical patient samples with *KRAS* mutations were identified as GGT→GAT mutations. Cross-hybridization remains one of the significant limitations of microarray system. We addressed this problem by linking a unique tag sequence at the 5'-end of forward primers that were used for multiplex PCR. The Cy5-fluorescence could be detected only in hybridization in which an entire multiplex

PCR fragment is present (Fig. 1A). Thus, the presence of the unique tag allows high ability of discrimination upon hybridization on the microarray and greatly minimizes the risk of false-positive results due to cross-hybridization, as evidenced by the findings in this study that all types of mutation in cells and clinical samples could be unambiguously discriminated. In contrast, attempt of genotyping by PCR with non-tagged primers was not successful, as nonspecific signal was readily observed due to cross-hybridization (data not shown).

Activated *KRAS* mutants in feces are more clinically significant than those in other samples (3,25,43). However, clinical specimens such as feces often contain only a small percentage of mutant cells in a large background of normal cells. Thus, assays to detect mutations leading to cancer need to be sensitive under such conditions (36). Hence, we investigated the detection limit of the tag-microarray method by mixing mutant cellular DNA (M3T3) with varying amount of DNA either from WT cell or from feces of a healthy donor (Fig. 3). To our knowledge, this attempt has not been reported previously. We sought to mimic the clinical sample by doing so, as usually patient sample is of poor quality rather than a sample which is pure like a cell line. Without any step of mutant DNA enrichment, an accurate mutation detection limit of 10% mutant DNA in the mixed cell-cell or cell-fecal DNA materials was reproducibly obtained using our technique (Fig. 3), indicating this approach could be used for clinical samples containing a small fraction of mutant DNA. The successful detection of clinical tumor samples confirmed our expectations and strongly verified the applicability of this approach in practice. We are currently testing additional clinical samples employing this assay to further validate the performance of this method. Overall, the sensitivity and accuracy of this method were satisfactory and comparable to the best results described by other groups (25,27,43). Using the tag-microarray method, the examination of clinical samples, from DNA isolation to the eventual result interpretation, can be finished within one working day, thus, our assay greatly shortens the hand-on time and time to results, compared to the method of direct sequencing which normally takes 2-3 working days. Our technique has additional advantage over other existing methods, such as it is ideal for large-scale screening of patient samples at an early stage.

It is desirable to lower the cost of genotyping as much as possible to obtain more benefits. Toward this direction, we used different solid substrates for spotting. We found that the quality of signal and reproducibility of hybridization were higher when a normal glass slide was used, compared to that of Topas plastic slide or GAPS-II coated glass slide. Moreover, the regular glass material is cheaper and easier to use compared to other two materials tested. Thus, we suggest using the regular glass slide as a substrate for further research and practical applications. It should also be noted that the probes immobilized on the solid surface could undergo treatment of high temperature, indicating that it is feasible to remove the hybridized target DNA from the surface of the substrate without disturbing the immobilized probes. Consequently, new DNA samples can be examined using the same glass slide as well as the same immobilized probes. The re-use of the slide and probes makes our approach inexpensive in practice. In addition, the property of thermo-resistance of the immbolization allows the possibility of developing a solid PCR platform. Experiments are underway to establish a solid-PCR system based on the tag-microarray approach.

Looking solely at the common mutations in a single codon, as we did, does not rule out other mutations at the same codon or other codons, such as 13, 59, 61 or 146 in the case of *KRAS* (22,28,38,44). In addition, detection of *KRAS* codon 12 mutations does not cover the possibilities of all CRC cases, as it was reported that *KRAS* mutations are present in 30-40%, but not 100%, of colorectal carcinoma cases (45). Nevertheless, our method is flexible, as in theory multiple mutations in different codons can be amplified and detected simultaneously using the technique described here. In addition to the four mutations in the 'hotspot' codon 12 described here, detection of more mutations in other codons, such as codon 13, 59 and/or 61, is underway using the tag-microarray technique. The tag-microarray approach described here is highly specific, noninvasive, cost-effective, and should provide an alternative for direct sequencing for *KRAS* mutation detection in colon cancer patients, particularly if used in combination with FOBT or other methods for early and mass detecting genetic abnormalities. We stress the importance of our tag-microarray method as an innovative technique that can be used for routine diagnosis of CRC in clinical practice.

Acknowledgements

We thank Dr Mogens Kruhøffer for providing cancer cell lines and clinical DNA samples, Dr Kurt J. Handberg for providing cell culture facilities, Jonas Høgberg for expert technical assistance. This study was supported by the Danish Council for Independent Research; Technology and Production Sciences (FTP) (grant no. 274-05-0017). The funders had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript.

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