Research Article

Cancer Prevention Research

Ultrasensitive Detection of Unknown Colon Cancer-Initiating Mutations Using the Example of the *Adenomatous Polyposis Coli* Gene

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Abstract

Detection of cancer precursors contributes to cancer prevention, for example, in the case of colorectal cancer. To record more patients early, ultrasensitive methods are required for the purpose of noninvasive precursor detection in body fluids. Our aim was to develop a method for enrichment and detection of known as well as unknown driver mutations in the *Adenomatous polyposis coli* (*APC*) gene. By coupled wild-type blocking (WTB) PCR and high-resolution melting (HRM), referred to as WTB-HRM, a minimum detection limit of 0.01% mutant in excess wild-type was achieved according to as little as 1 pg mutated DNA in the assay. The technique was applied to 80 tissue samples from patients with colorectal cancer ($n = 17$), adenomas ($n = 50$), serrated lesions ($n = 8$), and normal mucosa ($n = 5$). Any kind of known and unknown *APC* mutations (deletions, insertions, and base exchanges) being situated inside the mutation cluster region was distinguishable from wild-type DNA. Furthermore, by WTB-HRM, nearly twice as many carcinomas and 1.5 times more precursor lesions were identified to be mutated in *APC*, as compared with direct sequencing. By analyzing 31 associated stool DNA specimens all but one of the *APC* mutations could be recovered. Transferability of the WTB-HRM method to other genes was proven using the example of *KRAS* mutation analysis. In summary, WTB-HRM is a new approach for ultrasensitive detection of cancer-initiating mutations. In this sense, it appears especially applicable for noninvasive detection of colon cancer precursors in body fluids with excess wild-type DNA like stool. *Cancer Prev Res; 6(9); 898–907. ©2013 AACR.*

Introduction

Improving prevention and early detection of cancer is a defined goal of the World Health Organization based on the observation that removal of cancer precursor lesions has been shown to reduce cancer incidence, for example, in the case of colorectal cancer (1). In particular, there is a need to provide those people who refuse screening colonoscopy with highly sensitive alternative tests. The main problems in the attempt to establish molecular assays for noninvasive detection of early cancer stages or even of cancer precursors are the choice of the appropriate biomarkers, the high background of wild-type DNA in biologic specimens such as stool or other body fluids, and the difficulty to detect mutations of unknown type and position, for example, in genes with several possible mutation sites or large mutation cluster regions (MCR).

Mutations in *APC*, *KRAS*, or *BRAF* genes have been classified as colorectal cancer-initiating events and thus represent promising biomarkers for molecular detection of beginning cancer and its precursors (2–5). *APC* mutations have been described in early cancerous lesions as well as in cancer precursors like adenomas (6, 7) and even in aberrant crypt foci (8, 9). These mutations mimic an active Wnt signaling and are assumed to be initiating at least for traditional colon cancers developing along the adenoma-carcinoma sequence (10). *KRAS* and *BRAF* mutations, both resulting in a constitutively active Ras-Raf-MEK-MAPK signaling pathway, were described in earliest lesions of tumors following the so-called serrated path (3, 11, 12). *KRAS* mutations are furthermore present in some of the above-mentioned traditional tumors as well as in tumors combining features of both pathways in a so-called fusion pathway (13).

In addition, *APC* and *KRAS* are the most frequently mutated genes in sporadic colorectal cancer being affected in about 40% (*KRAS*) and up to 60% (*APC*) of patients with colorectal cancer, respectively (14–17). Commercially available kits for *KRAS* and *BRAF* mutation analysis (e.g., Signature *KRAS*/*BRAF* Mutations assay, Asuragen) possess an average detection limit of 1% mutated DNA in mixtures with wild-type DNA which is satisfactory for prognostic

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purposes rather than for early cancer screening, when taken into account that the ratio of mutated human DNA to total feces DNA is assumed to be in the range of 1:10,000–1:100,000 (18–20).

Low-level detection of unknown mutations is a formidable challenge. The paramount example is the *APC* gene. A large mutation cluster region of almost 1 kb being frequently hit during sporadic colon carcinogenesis has been defined in exon 15 of *APC* (21, 22). Surroundings of codons 1309, 1450, 1465, and 1556 represent mutational hotspots within the MCR (23, 24). From our recent work and from the literature, it is known that different mutation types (insertions, deletions, or base substitutions) can occur in one and the same mutational hotspot, aggravating the detection. For instance, the codon 1309 hotspot which contains repetitive sequences spanning codons 1306–1311, might be affected by a 5 bp deletion leading to a frameshift or by a G>T substitution resulting in a stop codon, whereas the codon 1556 hotspot (codons 1553–1556) most often exhibits a 1 bp insertion of an adenine but can also be affected by a G>T substitution resulting in a stop codon (4, 5). For this reason, detection methods using mutant-specific probes are inappropriate in this case and although *APC* is the most frequently mutated gene in colorectal cancer, there is no sensitive commercial test for *APC* mutation analysis available up to now.

We hypothesized that combined enrichment and ultrasensitive detection of carcinogenic driver mutations could represent a tool to sense not only more carcinomas but also more colorectal cancer precursors like advanced adenomas in body fluids. For this purpose, we developed a two-step method consisting of wild-type blocking (WTB)-PCR for suppressing wild-type DNA by use of locked nucleic acid (LNA) blocker molecules, followed by further enrichment and detection of mutations using high-resolution melting (HRM) analysis. The efficacy of this new approach, referred to as WTB-HRM, to detect low-level mutations in *APC* whose type or position inside the large MCR is not exactly predictable was compared with direct sequencing. The applicability on human colon tissue and feces analysis is shown and the transferability of the ultrasensitive method to other genes is proven using the example of *KRAS*.

Materials and Methods

Cell lines, human tissue, and feces

DNA from human colorectal cancer cell lines with known mutated or wild-type status of the examined genes was used as control for mutation analysis. For the analysis of the mutational hotspots around codons 1309 and 1556 of *APC* as well as in codon 1367 of *APC*, cell lines LS1034 (*APC* c.3927_3931del, p.E1309fs), Colo678 (*APC* c.4666_4667insA, p.T1556fs), and Caco-2 (*APC* c.4099C>T, p.Q1367*), respectively, served as positive controls. For *KRAS* codon 12 analysis, LS174T (*KRAS* c.35G>A, p.G12D) was applied. HCEC DNA was used as wild-type reference for *APC* as well as for *KRAS* analysis. Cells were purchased in 2009 from American Type Culture Collection (LS1034) and from DSMZ-German Collection of Microorganisms and Cell Cultures (Colo678) and were cultured according to protocols of the suppliers. LS174T, Caco-2, and HCEC were obtained as generous gifts from A. M. Otto (Technical University of Munich, Munich, Germany), R. Brigelius-Flohé [German Institute of Human Nutrition (DIfE), Nuthetal, Germany], and the Nestlé Ltd. Research Centre (Lausanne, Switzerland), respectively. For authentication, sanger sequencing was conducted to ensure the wild-type or mutated state of both, *APC* and *KRAS* genes, in every cell line. Furthermore, all cell lines were tested negative for mycoplasma infection within 6 months before use.

Eighty human colon tissue and 31 associated feces specimens from subjects that had undergone routine colonoscopy and histopathologic assessment had been obtained freshly frozen from the Maria Heimsuchung Caritas Hospital Pankow (Berlin, Germany). DNA from human specimens and from cell pellets was extracted within 2 weeks after harvesting by standard methods (QIAamp DNA Mini Kit and QIAamp DNA Stool Mini Kit, Qiagen). The *APC* mutation status of the colon tissues was known from a previous study (5) in which *APC* had been analyzed by direct sequencing. The study was carried out in compliance with the Declaration of Helsinki. Written informed consent was received from participants before inclusion in the study. Permission for the study was given by the Ethics Commission of the University of Potsdam, Germany (Decision 3/25 taken on November 03, 2008).

Site-directed mutagenesis and cloning

A positive control mutated in the *APC* codon 1465 mutation hotspot (*APC* c.4393_4394del, p.S1465fs) was generated by site-directed mutagenesis and subcloned into pGEM-T Easy vector (Promega). The frequent *APC* codon 1450 mutation (*APC* c.4348C>T, p.R1450*) was subcloned into pGEM-T Easy from a colon carcinoma previously analyzed for use as positive control.

Wild-type blocking-PCR

Design and calculation of melting conditions of LNA/DNA oligomers, referred to as LNA blockers, were conducted by using the online available LNA Oligo design tools (Exiqon). LNA blockers hybridized exclusively with wild-type DNA in the space of the respective mutational hotspots enclosed in corresponding gene segments. One LNA blocker for suppressing wild-type DNA amplification was included in every reaction (Supplementary Table S1). For *APC* analysis, the MCR was subdivided into four overlapping segments of 200 to 300 bp each that were analyzed separately (Supplementary Fig. S1). These segments are referred to as *APC1* (codons 1228–1328, including the codon 1309 mutational hotspot), *APC2* (codons 1328–1452, including the frequently mutated codon 1367), *APC3* [codons 1448–1509, including the codon 1450 mutational hotspot (*APC3.1*) and the frequently mutated codon 1465 (*APC3.2*)], and *APC4* (codons 1482–1568, including codon 1556 mutational hotspot). For *KRAS* analysis, a gene segment encompassing the complete exon 1 (with codons 12 and 13 mutational hotspots included) was amplified.

Oligonucleotide primers for both genes were the same as reported earlier (25). WTB-PCR reactions for *APC* analysis contained 1× AmpliTaq Reaction Buffer (Applied Biosystems), 4 mmol/L $MgCl_2$, 0.2 mmol/L of each of the 4 deoxynucleoside triphosphates (dNTP), 0.2 μmol/L of each primer, and 1 U/μL AmpliTaq DNA polymerase Stoffel Fragment (Applied Biosystems) in a 25 μL reaction volume. For *KRAS* amplification, 2.5 mmol/L $MgCl_2$ was used instead. Template amount was either 10 ng of control DNA or 50 ng of tissue DNA. WTB-PCR was conducted on a Mastercycler gradient (Eppendorf). *APC* amplification of tissue DNA was carried out for 5 minutes at 95°C followed by 18 cycles of 30 seconds at 95°C, 30 seconds at the respective annealing temperature Ta (Supplementary Table S1), 45 seconds at 72°C, and final extension was conducted for 10 minutes at 72°C. For *APC* amplification of fecal DNA, a touchdown-PCR protocol was run for 5 minutes at 95°C, 10 cycles of 30 seconds at 95°C, 30 seconds of the respective Ta (Supplementary Table S1) + 10°C, reduced by 1°C per cycle, and 40 seconds at 72°C. Subsequently, 15 cycles of 30 seconds at 95°C, 30 seconds at the appropriate Ta, and 45 seconds at 72°C were conducted, followed by a final extension for 10 minutes at 72°C. The *KRAS* protocol was as follows: 7 minutes at 94°C initial denaturation, 25 cycles of 94°C for 45 seconds, 79°C for 45 seconds, 58°C for 45 seconds, 72°C for 1 minute, and final extension at 72°C for 10 minutes. Amplicons obtained from each initial WTB-PCR reaction were diluted 10 times and used as template for subsequent HRM analysis.

High-resolution melting

For the purpose of further enrichment and detection of mutations in specimens previously amplified by WTB-PCR, HRM was carried out on a LightCycler 480 II instrument (Roche Diagnostics). All samples were examined in triplicates. Oligonucleotide primers used in the real-time amplification step associated with HRM (Supplementary Table S2) were seminested in relation to the WTB-PCR reaction (Supplementary Fig. S1). For *APC* analysis, the HRM reaction volume of 10 μL contained 1× LC480 HRM Master Mix (Roche Diagnostics), 1 μL of diluted template, 4 mmol/L $MgCl_2$, and 0.4 μmol/L of each PCR primer. For *KRAS* analysis, 2 μL of diluted template, 6.5 mmol/L $MgCl_2$, and 0.1 μmol/L of each PCR primer were used instead. *APC* amplification was conducted at 95°C for 10 minutes followed by 45 to 55 cycles of 95°C for 5 seconds, Ta (Supplementary Table S2) for 10 seconds, and 72°C for 10 seconds. The *KRAS* amplification protocol consisted of: 95°C for 5 minutes followed by 50 cycles of 95°C for 10 seconds, 58°C for 15 seconds, and 72°C for 10 seconds. A constant temperature increase of 4.4°C per second from 60°C to 95°C with an acquisition of fluorescence of 25 per second was directly attached. The LC 480 Gene Scanning Software Version 1.5.0 (Roche Diagnostics) was applied for HRM analysis. Normalized and temperature-adjusted melting curves of test samples and wild-type controls were compared and automatically grouped by the software. Curves of samples matching the wild-type were figured in the same color as the wild-type control, whereas mutated samples were assigned to mutant control or to further groups as depicted by different coloured melting curves. To validate LNA blocker efficiencies, the respective real-time PCR runs were analyzed by the Advanced Relative Quantification Software—Version 1.5.0 (Roche Diagnostics).

Control sequencing

WTB-HRM mutation analysis results were controlled by sequencing. HRM products were cleaned from a 2% agarose gel by use of Qiaquick Gel Extraction Kit (Qiagen) according to the manufacturer's protocol. Sanger sequencing of cleaned fragments was conducted by GATC Biotech. Sequencing results were analyzed by means of the DNAstar Lasergene 9 Core Suite software (DNASTAR).

Results

WTB-HRM is suitable to detect low-level gene mutations in large mutation cluster regions

The objective to provide a highly sensitive method applicable for the detection of unknown mutations in genes with more than a few possible mutation sites was pursued using the example of *APC* as an initially mutated gene in colorectal cancer. The intended mutation enrichment as a precondition for highly sensitive low-level mutation detection in biologic specimens could be achieved by the developed two-step method involving WTB-PCR upstream of HRM.

Four overlapping segments of the complete MCR of *APC* (*APC1-APC4*) were analyzed separately in presence of one LNA blocker in every analysis. In total, 5 different LNA blockers were used to hybridize wild-type *APC* in the region of mutational hotspots around codons 1309 (located inside *APC1*), 1367 (*APC2*), 1450 (*APC3.1*), 1465 (*APC3.2*), and 1556 (*APC4*). Specificity of every LNA blocker to suppress completely wild-type but not mutant *APC* amplification could be confirmed by conducting WTB-PCR on each *APC* segment (Fig. 1A). The respective blocking efficiency was apparent from the ratio of relative product formation in assays being run with and without added LNA blocker as determined by real-time quantitative PCR and depended on position and number of LNA molecules in the blocker as well as on sequence context (Table 1). The highest blocking efficiency was achieved by a blocker traversing the codon 1556 mutational hotspot in segment *APC4* suppressing wild-type amplification even by more than 7,000-fold compared with the amount of product being formed without use of LNA blocker. In contrast, amplification of control DNA, carrying mutations within *APC* mutational hotspots, was nearly unaffected in presence of the respective LNA blocker molecules (Table 1).

Detection limits for selected *APC* mutations, located in the space of the mutational hotspots, were determined by applying serial dilutions of mutated in wild-type control DNA to WTB-HRM. Exemplarily shown is the representative HRM curve chart of a 1 bp insertion (ins A) in segment *APC4* with a minimum detectable mutant level of 0.01% in the template composite according to a detection limit of 1 pg

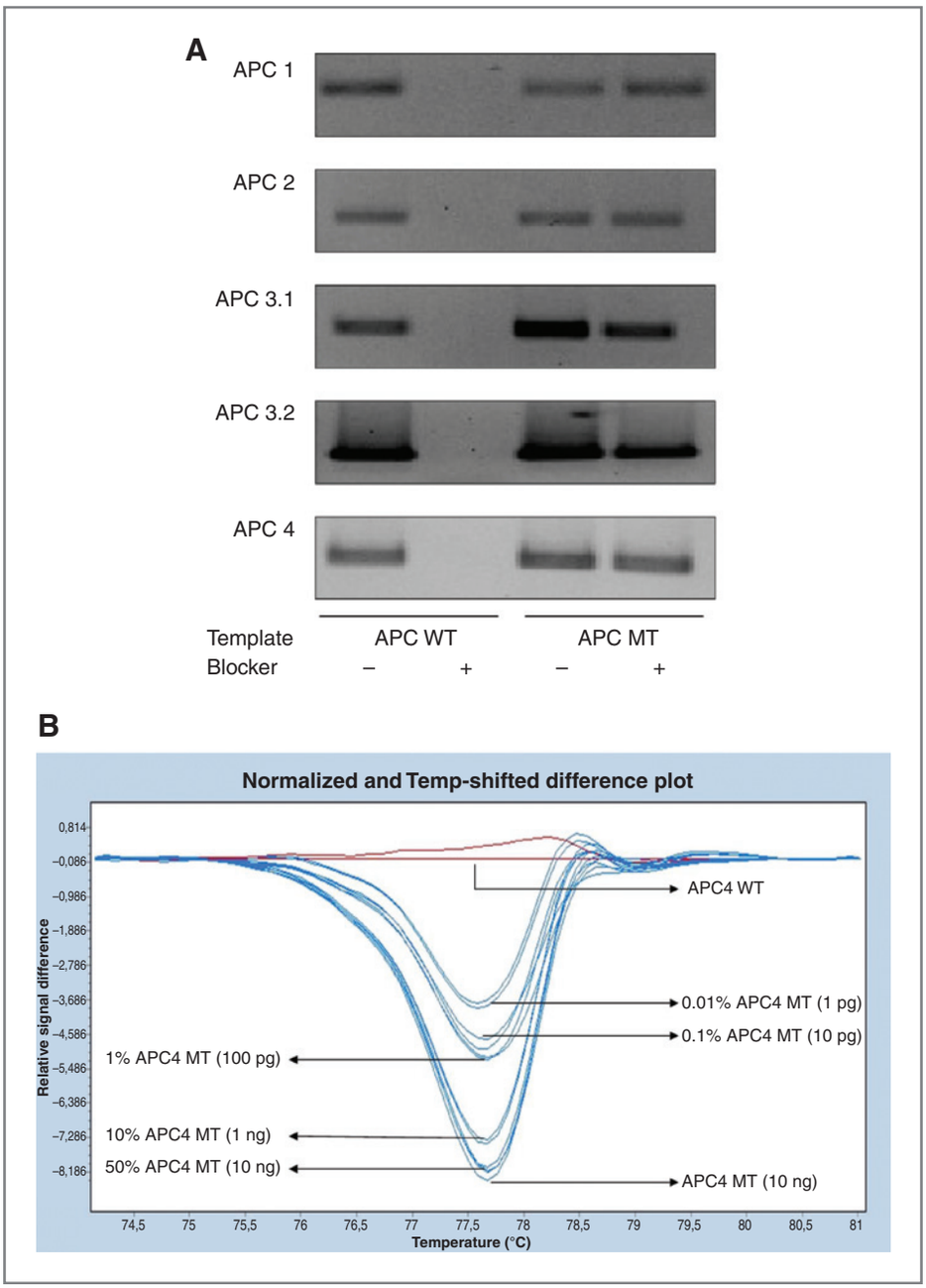

Figure 1. Analytic sensitivity of *APC* mutation analysis. A, specificity of LNA blockers to suppress amplification of wild-type (WT) but not of mutated (MT) *APC*. For each of the MCR segments (*APC1-APC4*), WTB-PCR was conducted on the corresponding control DNA in presence (+) or absence (–) of LNA blocker as indicated. Ten microliters of the PCR products were separated in a 2% agarose gel and visualized under UV light after ethidium bromide staining. B, detection limit of WTB-HRM for exemplary mutation *APC* c.4666_4667insA, p.T1556fs in segment *APC4*. The mutated control DNA (MT) was serially diluted (10 ng–1 pg) in 10 ng of wild-type DNA (WT) each and was subjected to WTB-HRM in presence of the LNA blocker hybridizing wild-type *APC* around codon 1556. The wild-type control was set as baseline. Percentage and template amount of mutated DNA in mixed samples are indicated.

mutated DNA in the assay (Fig. 1B). Regarding the remainder MCR segments, mutation detection limits differed between 1 pg and 100 pg mutated *APC* in the template composite, depending on the mutation type (Table 2).

Improvement in mutation enrichment and detection by using LNA blockers

The benefit of mutation enrichment using LNA blockers was verified by repeating *APC* analysis on mixed mutant and wild-type control DNA comparatively by conducting conventional PCR instead of WTB-PCR during the first step. By applying WTB-HRM on each of 4 overlapping segments (*APC1–APC4*) of the complete MCR, any deletions, insertions, and base substitutions contained in control DNA could be enriched and discriminated from wild-type DNA background in mixed samples. Related detection limits of each fragment analysis are summarized in Table 2. WTB-PCR improved mutation detection considerably compared with conventional PCR when coupled with HRM in each case. For 3 of 5 examined mutant controls, the detection limit could be even lowered by 5,000-fold when using WTB-PCR.

Table 1. Efficiency of LNA blockers used in WTB-PCR

APC Segment (hotspot enclosed)	Relative product formation (RFU)	
	WT/(WT+LNA blocker)	MT/(MT+LNA blocker)
APC1 (cd 1309)	732	1.23
APC2 (cd 1367)	315	0.92
APC3.1 (cd 1450)	442	0.85
APC3.2 (cd 1465)	977	1.00
APC4 (cd 1556)	7,109	1.00

NOTE: Indicated is the ratio of *APC* product formation without LNA blocker versus product formation in presence of blocker for wild-type (WT) and mutant (MT) *APC*, respectively.
Abbreviations: RFU, relative fluorescence units; cd, codon.

Detection of different mutation types in human colon tissue DNA

To test the feasibility of *APC* mutation analysis by WTB-HRM in human specimens, the method was applied to a total of 80 colon tissues with known mutation status due to previous direct sequencing. Exemplary HRM curve charts for *APC* segments 1 and 2 are shown in Fig. 2A and B, respectively. Segment *APC3* containing two different mutational hotspots was analyzed by the use of two separate LNA blockers *APC3.1* and *APC3.2* (Fig. 2C and D, respectively). A representative HRM analysis of segment *APC4* is shown in Fig. 2E. Tissues exhibiting wild-type *APC* clustered around the baseline, whereas HRM curves of specimens carrying an *APC* mutation within the enclosed mutational hotspot matched the positive control curve and peaked either below (Fig. 2A–C and E) or above (Fig. 2D) the baseline. Either kind of mutation, deletions, insertions, and substitutions were detectable.

Control sequencing of specimens displaying a melting curve different from both wild-type and mutant controls revealed further mutations that were detectable as well (Fig. 2A, B and E). For example, on analysis of segment *APC1*, a c.3925G>T, p.E1309* substitution was found within the codon 1309 mutational hotspot apart from the more common c.3927_3931del, p.E1309fs deletion at this site (Fig. 2A). In segment *APC4*, a 1 bp deletion (c.4666del, p.T1556fs) was clearly distinguishable from the c.4666_4667insA, p.T1556fs mutated tissue specimens and mutant control (Fig. 2E). Thus, WTB-HRM was able to detect different mutation types within *APC* mutational hotspots by using only one LNA blocker, hybridizing wild-type *APC* in the respective region of the gene.

In total, the MCR of *APC* was found to be mutated in 41 of the 80 (51%) analyzed colon tissues by use of WTB-HRM compared with 28 mutations (35%) detected by previous direct sequencing (Supplementary Table S3). Referred to histopathologic findings WTB-HRM detected mutations in 9 of 17 carcinoma, 27 of 50 adenoma, and 5 of 7 serrated lesions. No *APC* mutation was found in normal colon mucosa. All mutations previously detected by direct sequencing could be confirmed by WTB-HRM except for 2 adenoma samples (C80 and C91). However, WTB-HRM was vastly superior to direct sequencing detecting 9 instead of 5 mutations in carcinoma, 27 instead of 21 mutations in adenoma, and 5 instead of 2 mutations in serrated lesions. All mutations identified by means of aberrant melting curves could be confirmed by subsequent control sequencing of HRM products.

The analyses of colon tissues show that different types of *APC* mutations like insertions, deletions, and substitutions

Table 2. Mutation detection limits of conventional PCR/HRM and of WTB-HRM

Mutational hotspot of *APC* (MT control)[a]	Mutation detection limit in excess wild-type (pg)		Fold improvement of WTB-HRM versus conventional PCR/HRM
	Conventional PCR/HRM	WTB-HRM	
cd 1309 (c.3927_3931del, p.E1309fs)	5,000	100	50
cd 1367 (c.4099C>T, p.Q1367*)	1,000	10	100
cd 1450 (c.4348C>T, p.R1450*)	5,000	1	5,000
cd 1465 (c.4393_4394del, p.S1465fs)	5,000	1	5,000
cd 1556 (c.4666_4667insA, p.T1556fs)	5,000	1	5,000

NOTE: Indicated is the minimum amount of mutated (MT) DNA being detectable in mixed templates containing 10 ng of wild-type (WT) DNA each.
[a]Nomenclature of mutations according to guidelines of the Human Genome Variation Society.

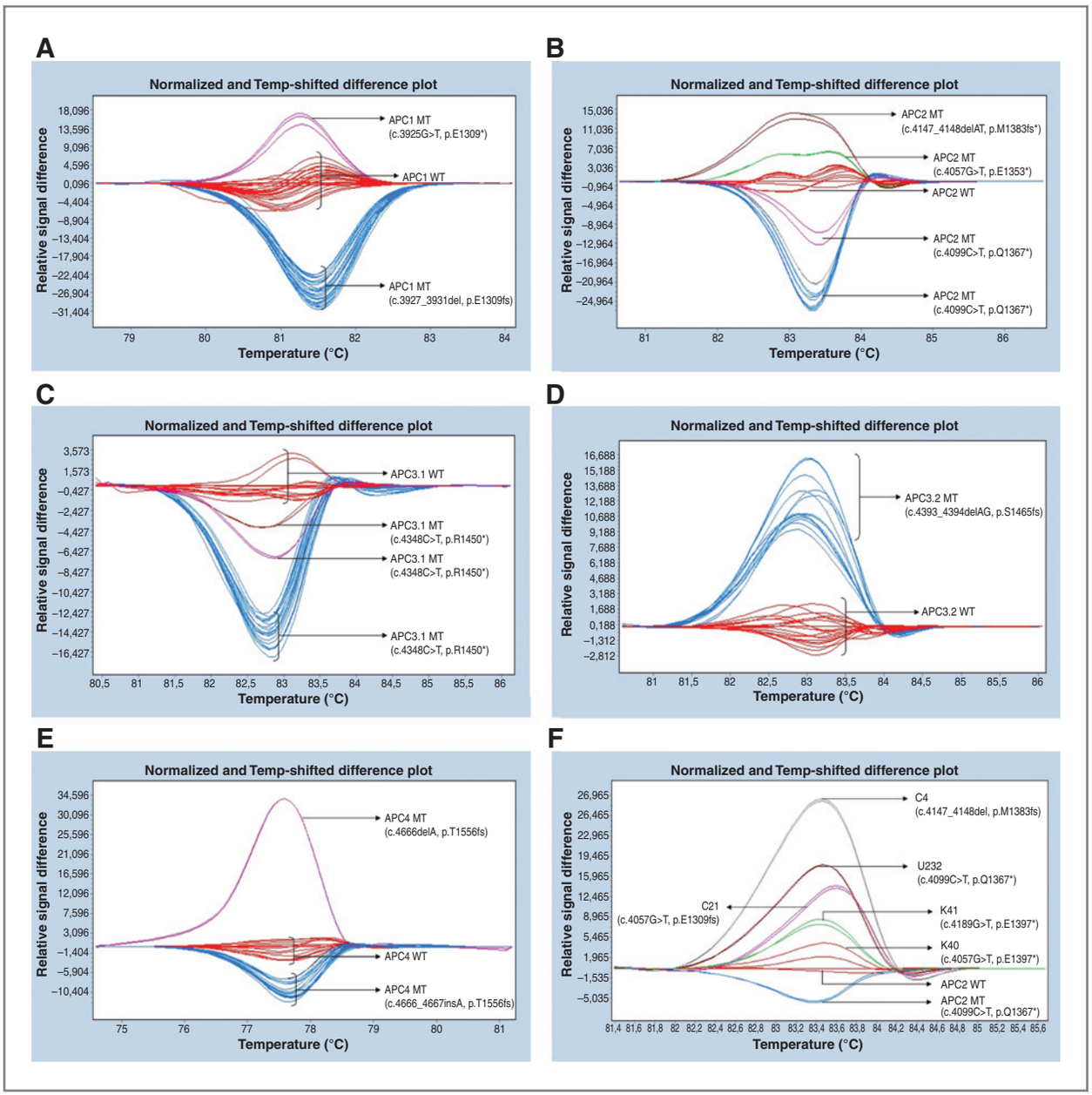

Figure 2. *APC* mutation screening of representative colon tissue specimens derived from patients with carcinomas or adenomas using WTB-HRM. Melting curves display the results for analysis of segments *APC1* (A), *APC2* (B), *APC3.1* (C), *APC3.2* (D), and *APC4* (E). F, exemplary analysis by WTB-HRM of colon tissues with unexpected mutations situated within segment *APC2*, but outside the LNA blocker-binding site which is covering the codon 1367 mutational hotspot. The WT control (HCEC) was set as baseline. Curves representing mutated tissues may proceed below or above the baseline. Results of control sequencing after HRM are shown in brackets.

being localized within mutational hotspots can clearly be distinguished from wild-type samples in a highly sensitive manner by conducting WTB-HRM.

Detection of unknown *APC* mutations situated outside mutational hotspots

On analysis of segment *APC2* in colon tissue specimens, two divergent mutations, c.4147_4148del, p.M1383fs and c.4057G>T, p.E1353*, were noticed besides the *APC* c.4099C>T, p.Q1367* mutant control (Fig. 2B). Both of these unexpectedly detected mutations affected different codons compared with the mutant control. On the basis of this finding, the putative detection of mutations being situated outside that DNA section, which is hybridized by the respective LNA blocker, was further examined for segment *APC2* as an example. A total of eight selected tissue

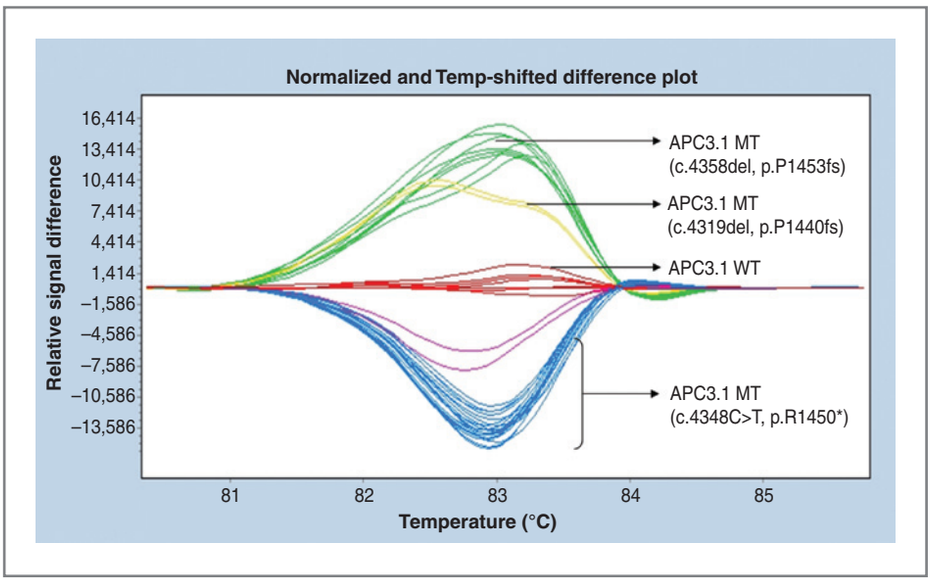

Figure 3. *APC* mutation screening of representative feces specimens derived from patients with colon carcinomas or adenomas using combined WTB-HRM. Melting curves display the results for exemplary analysis of segment *APC3.1*. The WT control (HCEC) was set as baseline. *APC* c.4348C>T, p.R1450* was used as MT control. Curves representing mutated feces samples may proceed below or above the baseline. Results of control sequencing after HRM are shown in brackets.

specimens with known different mutations (deletions and substitutions) situated in segment *APC2* but outside the LNA blocker-binding site around codon 1367 were subjected to WTB-HRM. All melting curves of these samples clearly differed from wild-type *APC2* curves as well as from curves of the control being mutated within the associated hotspot (Fig. 2F). In one case (patient no. K40), the software classified the melting curve as wild-type, whereas subsequent control sequencing of the corresponding HRM product clearly revealed the mutated state. Hence, WTB-HRM is principally able to detect different mutation types with unknown position being situated outside the LNA blocker-binding site.

Detection of *APC* mutations in feces DNA

The applicability of WTB-HRM for noninvasive stool screening was examined by analyzing the available corresponding fecal DNA of 22 patients (3 carcinoma, 17 adenoma, 2 serrated lesions) whose tissue analysis had been positive for *APC* mutations in one of the four investigated MCR sections. Furthermore, stool DNA of 9 patients with mutation-negative tissue (2 carcinoma, 3 adenoma, 1 serrated lesion, 3 normal) was analyzed. As exemplary depicted for segment *APC3.1*, WTB-HRM was able to detect *APC* mutations matching the mutant control as well as different mutations from fecal DNA (Fig. 3). In total, mutations could be recovered in stool DNA of all but one adenoma patient. By stool analysis of patients with mutation-negative tissues, the wild-type status for *APC* was confirmed (Supplementary Table S3).

Transferability of the method to other genes

Applying WTB-PCR on *KRAS* analysis, the associated LNA blocker was proven to suppress amplification of the wild-type control completely, whereas the positive control, carrying the frequent *KRAS* c.35G>A, p.G12D mutation, was still amplified in presence of the blocker (Fig. 4A). Real-time quantitative PCR revealed 1,700-fold stronger blocking of wild-type than of mutated *KRAS* by means of the respective LNA blocker (not shown).

When using serial dilutions of mutant in wild-type control DNA, WTB-HRM was able to discriminate a 0.05% *KRAS* c.35G>A, p.G12D mutant level from excess wild-type as can be clearly seen in the difference plot of melting curves (Fig. 4B). This is consistent with a detection limit of 5 pg mutated *KRAS* in the template composite. The curve shape of mutated *KRAS* at a level of 0.01% (1 pg) differed slightly from wild-type *KRAS* but was too close for being discriminated reliably (Fig. 4B).

To examine the applicability of the method on biologic specimens, a test set of 20 human colon tissues was analyzed for *KRAS* mutations by use of WTB-HRM. Samples mutated in *KRAS* could be clearly differentiated from wild-type control by their melting curves, whereas curve charts of samples exhibiting the wild-type shaped around the baseline (Fig. 4C). In total, five specimens of the test set were found to carry mutations in *KRAS* codon 12 which could be confirmed by subsequent control sequencing of HRM products (Supplementary Table S4).

Discussion

The data presented here show for the first time an ultrasensitive method for mutation analysis of the mutational cluster region of *APC* being able to detect mutations of unknown type and position in up to 10,000 times excess wild-type DNA. Furthermore, it is shown that by analyzing fecal DNA for cancer-initiating mutations, a sensitive noninvasive detection of colon cancer precursors becomes feasible. By the especially developed WTB-HRM method, a considerable enhancement of detection frequencies of carcinoma, adenoma, and serrated lesions, compared with direct sequencing, was achieved. The method was proven to

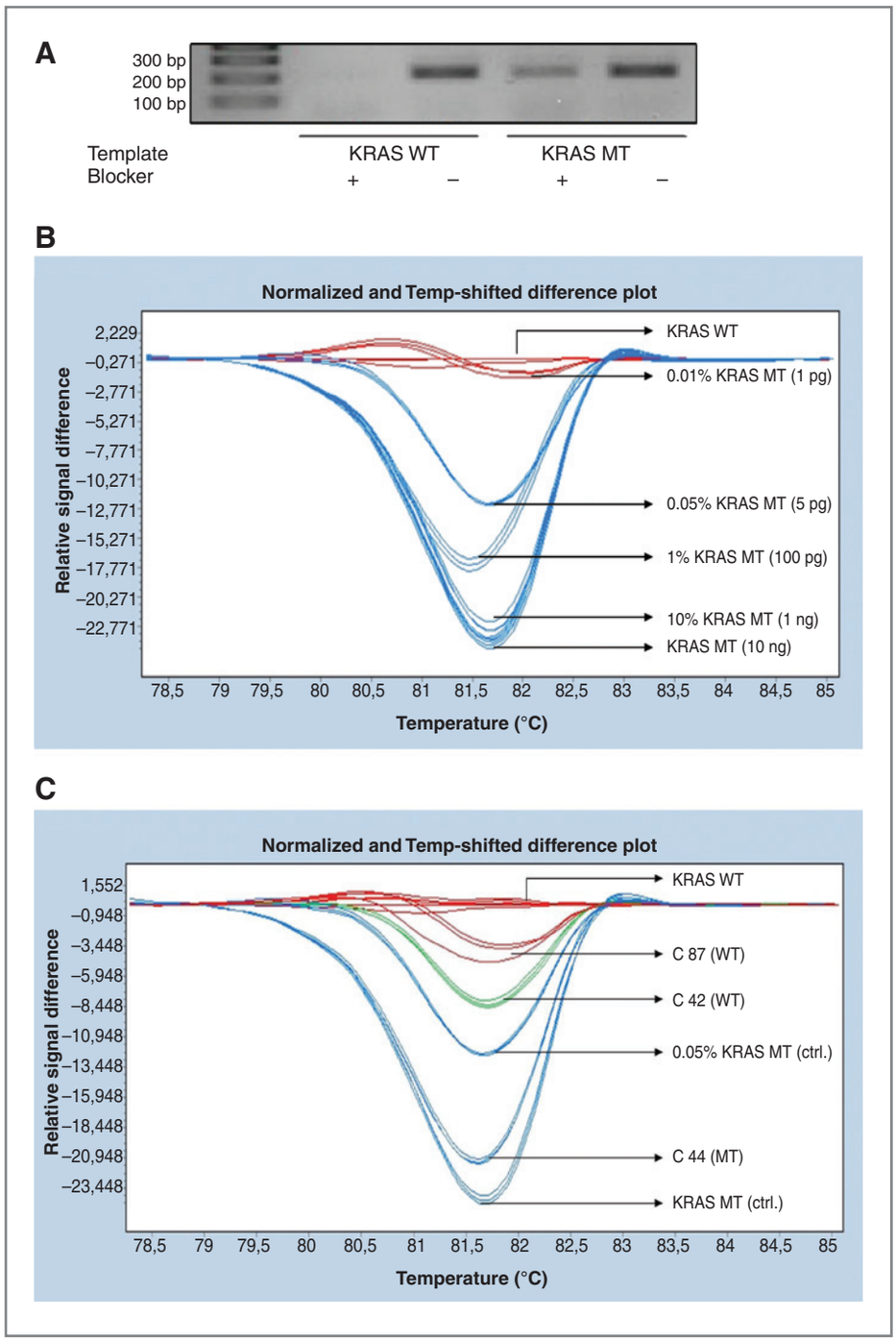

Figure 4. Analytic sensitivity of *KRAS* mutation analysis. A, complete suppression by LNA blocker of wild-type (WT) but not of mutated (MT) *KRAS* amplification. WTB-PCR was conducted on the respective control DNA in presence (+) or absence (–) of LNA blocker as indicated. Ten microliter of the PCR products were separated in a 2% agarose gel and visualized under UV light after ethidium bromide staining. B, detection limit of WTB-HRM for *KRAS* mutations. The MT control was serially diluted (10 ng–1 pg) in 10 ng WT DNA. The WT was set as baseline. Melting curves representing mutated DNA may proceed below or above the baseline. Percentage and amount of MT DNA in mixed templates as indicated. C, *KRAS* mutation screening of colon tissue specimens derived from patients with carcinomas or adenomas using WTB-HRM. The curve shape of the 0.05% MT level according to the detection limit was taken as reference for assessment of the mutated state of human colon samples. Results of control sequencing of HRM products are shown in brackets. Cellular genomic DNA of HCEC (*KRAS* WT) and LS174T (*KRAS* c.35G>A, p.G12D) was used as control, respectively.

detect not only those gene mutations expected at particular sites but also unknown mutations, even those being situated outside mutational hotspots, in excess wild-type DNA at a ratio present in body fluids like feces.

The extraordinary high sensitivity of WTB-HRM, being able to detect 0.01% (*APC*) and 0.05% (*KRAS*) mutant levels in excess wild-type, respectively, according to 1–5 pg of mutated DNA in the template composite, was achieved by a two-step protocol including mutation enrichment preceding the detection step. Suppression of wild-type DNA amplification by use of LNA blockers was mandatory for the excellent sensitivity of the method and was advantageously complemented by HRM.

Each of both techniques alone exhibits less sensitive detection limits. It was reported that LNA-based PCR clamping was able to detect 1% mutated *KRAS* in the template

composite (26). Regarding HRM, *KRAS* mutations at a ratio of 5% were necessary to be detectable (27, 28). A related method, the Digital Melt Curve (DMC) assay, was conducted as a two-step method enriching target genes by sequence-specific capture before combined digital PCR and HRM (29). By DMC, 0.1% levels of *KRAS* mutations and 0.6% levels of *APC* mutations were detected when applying 1,000 copies of mutant DNA (29). Deep-sequencing technology was reported to achieve a detection limit of 0.2% mutant DNA (30). In contrast, conventional PCR/HRM detected mutations only at a level of 2%–10%, whereas mutation enrichment by Co-amplification at lower denaturation temperature PCR (COLD-PCR) coupled with HRM resulted in a detection limit of 0.1%–1% mutant DNA as was shown using the example of the *TP53* gene (31). The aforementioned methods usually require nanogram amounts of mutated DNA. Altogether, these data support the need of an additional enrichment step to reach high sensitivity as was already postulated (32).

The extent of wild-type suppression by an LNA blocker is dependent on the number and position of LNA molecules contained as well as on the sequence context (33, 34). This explains why in this study the same substitution of C>T resulted in a different detection limit when either codon 1367 or codon 1450 of *APC* is affected. We tried just one LNA blocker for every mutational hotspot. Hence, there might be some scope for influencing blocker efficiency by ingenious design.

Detection of the frequent *APC* c.3927_3931del, p.E1309fs hotspot mutation exhibited the poorest detection limit because of its location within a repetitive sequence. A long LNA blocker for specific recognition of the wild-type was required in this case. As differences in melting temperatures of LNA oligomers decrease with increasing duplex length (34), the respective blocker was less effective in binding. This indicates that the detection of long deletions or insertions within repetitive sequences might be at the limits of the technique. Anyway, it was possible to detect said mutation not only in tissue but also in feces DNA from carcinoma as well as from patients with adenoma.

Two of the previously by direct sequencing detected mutations were not recovered by WTB-HRM analysis (samples no C80 and C91). In the case of sample number C91, this can be explained by the experimental design. For WTB-HRM, seminested PCR reactions were conducted. While the outer primers pairs for each segment produce overlapping amplicons, the inner primers leave small gaps between *APC* segments 1 and 2 as well as between segments 2 and 3. In the special case of sample number C91, the rare mutation *APC* c.4340del, p.Q1447fs was located outside the sequence which is amplified by use of the second primer pairs of segments 2 and 3. For a gapless analysis of this area, it would be necessary to move the primers a bit.

As seen by direct sequencing, sample no C80 contained a single-nucleotide polymorphism (SNP) affecting the third position of codon 1493. Although a substitution at this position has no effect the SNP should be principally detectable by means of WTB-HRM (like in sample no C92). However, the HRM chart of sample C80 differed only slightly from the wild-type curve. So it was classified as wild-type by the software. Resequencing of the HRM product revealed the coexistence of both possible nucleotides (G and A) in sample C80.

Because of its exceptionally low detection limit, the WTB-HRM method could facilitate noninvasive screening for early colorectal cancer as well as for precursor lesions by detecting cancer initiating gene mutations in body fluids like stool. In this context, it would be also interesting to answer the question whether those mutations indicating cancer precursor lesions can be detected at similar levels in plasma compared with stool. The data should be verified next by determining sensitivity and specificity of WTB-HRM in a representative contingent of patients. It can be expected that besides *APC* and *KRAS*, the technique is transferable to any other gene being mutated during carcinogenesis. Hence, WTB-HRM could be a valuable tool in cancer prevention.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: C. Gerecke, B. Scholtka
Development of methodology: C. Gerecke, C. Mascher, B. Kleuser, B. Scholtka
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C. Gerecke, U. Gottschalk, B. Scholtka
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C. Gerecke, C. Mascher, B. Kleuser, B. Scholtka
Writing, review, and/or revision of the manuscript: C. Gerecke, B. Kleuser, B. Scholtka
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): C. Gerecke, C. Mascher, B. Scholtka
Study supervision: B. Scholtka

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References

1. Mandel JS, Church TR, Bond JH, Ederer F, Geisser MS, Mongin SJ, et al. The effect of fecal occult-blood screening on the incidence of colorectal cancer. N Engl J Med 2000;343:1603–7.
2. Vogelstein B, Fearon ER, Hamilton SR, Kern SE, Preisinger AC, Leppert M, et al. Genetic alterations during colorectal-tumor development. N Engl J Med 1988;319:525–32.

3. Chan TL, Zhao W, Leung SY, Yuen ST. B-RAF and KRAS mutations in colorectal hyperplastic polyps and serrated adenomas. Cancer Res 2003;63:4878–81.
4. Scholtka B, Schneider M, Melcher R, Katzenberger T, Friedrich D, Berghof-Jäger K, et al. A gene marker panel covering the Wnt and the Ras-Raf-MEK-MAPK signalling pathways allows to detect gene mutations in 80% of early (UICC I) colon cancer stages in humans. Cancer Epidemiol 2009;33:123–9.
5. Schneider M, Scholtka B, Gottschalk U, Faiss S, Schatz D, Berghof-Jäger K, et al. Detection of up to 65% of precancerous lesions of the human colon and rectum by mutation analysis of APC, KRAS, B-Raf and CTNNB1. Cancers 2011;3:91–105.
6. Powell SM, Zilz N, Beazer-Barclay Y, Bryan TM, Hamilton SR, Thibodeau SN, et al. APC mutations occur early during colorectal tumorigenesis. Nature 1992;359:235–7.
7. Kinzler KW, Vogelstein B. Lessons from hereditary colorectal cancer. Cell 1996;87:159–70.
8. Smith AJ, Stern HS, Penner M, Hay K, Mitri A, Bapat BV, et al. Somatic APC and KRAS Codon 12 mutations in aberrant crypt foci from human colons. Cancer Res 1994;54:5527–30.
9. Yuan P, Sun MH, Zhang JS, Zhu XZ, Shi DR. APC and KRAS gene mutation in aberrant crypt foci of human colon. World J Gastroenterol 2001;7:352–6.
10. Fearon ER, Vogelstein B. A genetic model for colorectal tumorigenesis. Cell 1998;61:759–67.
11. Yang S, Farraye FA, Mack C, Posnik O, O'Brien MJ. B-RAF and KRAS mutations in hyperplastic polyps and serrated adenomas of the colorectum: relationship to histology and CpG island methylation status. Am J Surg Pathol 2004;28:1452–9.
12. Velho S, Moutinho C, Cirnes L, Albuquerque C, Hamelin R, Schmitt F, et al. BRAF, KRAS and PIK3CA mutations in colorectal serrated polyps and cancer: primary or secondary genetic events in colorectal carcinogenesis? BMC Cancer 2001;8:255–60.
13. Jass JR, Baker K, Zlobec I, Higuchi T, Barker M, Buchanan D, et al. Advanced colorectal polyps with the molecular and morphological features of serrated polyps and adenomas: concept of a 'fusion' pathway to colorectal cancer. Histopathology 2006;49:121–31.
14. Zhang B, Ougolkov A, Yamashita K, Takahashi Y, Mai M, Minamoto T. β-Catenin and ras oncogenes detect most human colorectal cancer. Clin Cancer Res 2003;9:3073–9.
15. Fransén K, Klintenäs M, Osterström A, Dimberg J, Monstein HJ, Söderkvist P. Mutation analysis of the BRAF, ARAF and RAF-1 genes in human colorectal adenocarcinomas. Carcinogenesis 2004;25:527–33.
16. Lüchtenborg M, Weijenberg MP, Wark PA, Saritas AM, Roemen GM, van Muijen GN, et al. Mutations in APC, CTNNB1 and KRAS genes and expression of hMLH1 in sporadic colorectal carcinomas from the Netherlands Cohort Study. BMC Cancer 2005;5:160–70.
17. Suraweera N, Robinson J, Volikos E, Guenther T, Talbot I, Tomlinson I, et al. Mutations within Wnt pathway genes in sporadic colorectal cancers and cell lines. Int J Cancer 2006;119:1837–42.
18. Ahlquist DA, Skoletsky JE, Boynton KA, Harrington JJ, Mahoney DW, Pierceall WE, et al. Colorectal cancer screening by detection of altered human DNA in stool: feasibility of a multitarget assay panel. Gastroenterology 2000;119:1219–27.
19. Traverso G, Shuber AP, Levin B, Johnson C, Olsson L, Schoetz DJ, et al. Detection of APC mutations in fecal DNA from patients with colorectal tumors. N Engl J Med 2002;346:311–20.
20. Klaassen CH, Jeunink MA, Prinsen CF, Ruers TJ, Tan AC, Strobbe LJ, et al. Quantification of human DNA in feces as a diagnostic test for the presence of colorectal cancer. Clin Chem 2003;49:1185–7.
21. Miyoshi Y, Nagase H, Ando H, Horii A, Ichii S, Nakatsuru S, et al. Somatic mutations of the APC gene in colorectal tumors: mutation cluster region in the APC gene. Hum Mol Genet 1992;1:229–33.
22. Cheadle JP, Krawaczak M, Thomas MW, Hodges AK, Al-Tasan N, Fleming N, et al. Different combinsations of biallelic APC mutatin confer different growth advantages in colorectal tumours. Cancer Res 2002;62:363–6.
23. Muniappan BP, Thilly WG. The DNA polymerase beta replication error spectrum in the adenomatous polyposis coli gene contains human colon tumor mutational hotspots. Cancer Res 2002;62:3271–5.
24. Rowan AJ, Lamlum H, Ilyas M, Wheeler J, Straub J, Papadopoulou A, et al. APC mutations in sporadic colorectal tumors: A mutational "hotspot" and interdependence of the "two hits". Proc Natl Acad Sci U S A 2000;97:3352–7.
25. Steinberg P, Scholtka B, inventors; Universitaet Potsdam, assignee. Method for conducting non-invasive early detection of colon cancer and/or of colon cancer precursor cells. United States Patent US 7,833,757 B2. 2010 Nov 16.
26. Efrati E, Elkin H, Peerless Y, Sabo E, Ben-Izhak O, Hershkovitz D. LNA-based PCR clamping enrichment assay for the identification of KRAS mutations. Cancer Biomark 2010/2011;8:89–94.
27. Do H, Dobrovic A. Limited copy number-high resolution melting (LCN-HRM) enables the detection and identification by sequencing of low-level mutations in cancer biopsies. Mol Cancer 2009;8:82.
28. Borràs E, Jurado I, Hernan I, Gamundi MJ, Dias M, Martí I, et al. Clinical pharmacogenomic testing of KRAS, BRAF and EGFR mutations by high resolution melting analysis and ultra-deep pyrosequencing. BMC Cancer 2011;11:406.
29. Zou H, Taylor WR, Harrington JJ, Hussain FT, Cao X, Loprinzi CL, et al. High detection rates of colorectal neoplasia by stool DNA testing with a novel digital melt curve assay. Gastroenterology 2009;136:459–70.
30. Thomas RK, Nickerson E, Simons JF, Jänne PA, Tengs T, Yuza Y, et al. Sensitive mutation detection in heterogeneous cancer specimens by massively parallel picoliter reactor sequencing. N Engl J Med 2006;350:2129–39.
31. Milbury CA, Li J, Makrigiorgos GM. COLD-PCR-enhanced high-resolution melting enables rapid and selective identification of low-level unknown mutations. Clin Chem 2009;55:2130–43.
32. Milbury CA, Li J, Makrigiorgos GM. PCR-based methods for the enrichment of minority alleles and mutations. Clin Chem 2009;55:632–40.
33. McTigue PM, Peterson RJ, Kahn JD. Sequence-dependent thermodynamic parameters for locked nucleic acid (LNA)-DNA duplex formation. Biochemistry 2004;43:5388–405.
34. You Y, Moreira BG, Behlke MA, Owczarzy R. Design of LNA probes that improve mismatch discrimination. Nucleic Acids Res 2006;34:e60.