Clinical Chemistry 48:3
428–435 (2002)

Molecular Diagnostics and Genetics

Diagnostic Biochip Array for Fast and Sensitive Detection of K-*ras* Mutations in Stool

Lothar Prix, Peter Uciechowski, Beatrix Böckmann, Michael Giesing, and Andreas J. Schuetz*

Background: **Tumor cells that shed into stool are attractive targets for molecular screening and early detection of colon or pancreatic malignancies. We developed a diagnostic test to screen for 10 of the most common mutations of codons 12 and 13 of the K-*ras* gene by hybridization to a new biochip array.**

Methods: **DNA was isolated from 26 stool samples by column-based extraction from 9 cell lines. Peptide nucleic acid (PNA)-mediated PCR clamping was used for mutant-specific amplification. We used a biochip, consisting of a small plastic support with covalently immobilized 13mer oligonucleotides. The read out of the biochip was done by confocal time-resolved laser scanning. Hybridization, scanning, and data evaluation could be performed in <2 h.**

Results: **Approximately 80 ng of DNA was obtained from 200-mg stool samples. No inhibition of the PCR by remaining impurities from stool was observed. Mutation detection was possible in 1000-fold excess of wild-type sequence. Discrimination ratios between the mutations were >19 as demonstrated by hybridization with tumor cell line DNA. Stool samples (n = 26) were analyzed in parallel with PNA-PCR, restriction assay for K-*ras* codon 12 mutations, sequencing, and hybridization to the biochip. Nine mutations were found by hybridization, all confirmed by sequencing. PNA-PCR alone leads to an overestimation of mutations because suppression of the wild type is not effective enough with high concentrations of wild-type DNA. The restriction assay found only four mutations.**

Conclusions: **The K-*ras* biochip is well suited for fast mutation detection from stool in colorectal cancer screening.**

Institut für Molekulare NanoTechnologie, Berghäuser Strasse 295, 45659 Recklinghausen, Germany.

*Author for correspondence. Fax 49-0-2361-3000-142; e-mail a.schuetz@imnt.de.

Received September 24, 2001; accepted December 20, 2001.

Colorectal cancer is one of the most common forms of cancer. In Western Europe it is the second most frequent cause of cancer death *(1)*. It is, like many other tumors, curable if detected in an early stage. The prediagnostic classification is "high risk" in <25% of patients *(2)*, creating a high demand for screening tools for early detection *(3)*. The most widespread test is the fecal occult blood test, although its sensitivity and specificity are poor. Colonoscopy is sensitive because the whole colon can be visualized, but the costs and inconvenience for the patients are high *(4)*. The shedding of tumor cells into stool provides an attractive basis for noninvasive molecular screening for colorectal and even pancreatic cancer *(5, 6)*.

Mutations of the K-*ras* oncogene are found in >40% of colorectal tumors and in >80% of pancreatic tumors. *ras* protooncogenes code for a family of 21-kDa GTPases involved in signal transduction. Oncogenic forms of K-*ras* are permanently activated proteins, resulting from point mutations *(7)*. In contrast to other genes involved in tumorigenesis, mutations in the K-*ras* gene occur almost exclusively in codons 12, 13, and 61 (>90% of them in codon 12 and 13), and the mutational events occur in early stages of colorectal tumorigenesis.

Although there are many techniques available for the detection of K-*ras* mutations *(8)*, most of them show low sensitivity if applied to DNA extracted from feces. One problem is the heterogeneity of the stool: >95% of the isolated DNA is derived from nonhuman sources, and within the human DNA, only 0.5–5% will show a mutation in the *ras* gene *(1)*. PCR inhibitors and degrading enzymes further complicate the DNA isolation and sample preparation and often can lead to poor assay sensitivities *(9)*. Simple extraction procedures, as well as sensitive and specific amplification and detection techniques, are needed to make the molecular screening of stool samples available for routine diagnosis *(10)*.

Here we describe an analytical platform with very high sensitivity and specificity for the detection of K-*ras* muta-

[1] Nonstandard abbreviations: PNA, peptide nucleic acid; PM, perfect match; and MM, mismatch.

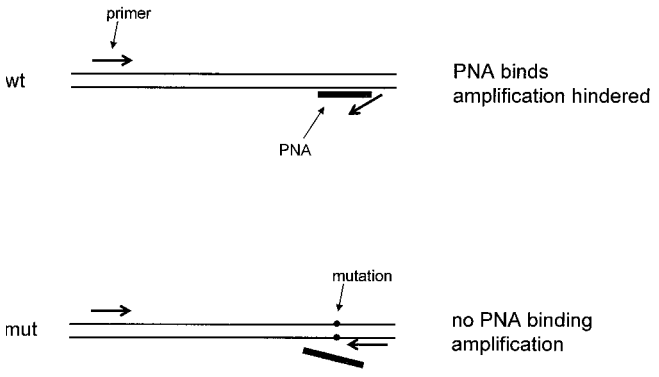

Fig. 1. Principle of PNA-mediated PCR clamping.

Addition of a 15mer PNA, which overlaps codons 12 and 13 of K-*ras*, to the PCR reaction forms a perfect-matched duplex with wild-type chromosomal DNA. As a result, annealing of the downstream primer is impaired and the amplification is blocked. Amplification proceeds if a mutation destabilizes the PNA-DNA duplex.

tions in codons 12 and 13 from stool samples by hybridization to a diagnostic biochip array after mutant-enriched amplification. The amplification is derived from a slightly modified protocol for peptide nucleic acid (PNA)[1]-mediated PCR clamping described by Thiede et al. *(11)*. Because of their chemical difference, PNA/DNA hybrids have a higher thermal stability and are more destabilized by single base-pair mismatches than DNA/DNA hybrids. In the case of mutant alleles, the melting temperature of the PNA/DNA hybrids is reduced, allowing the overlapping 23mer primer to inhibit PNA binding, leading to preferential annealing and amplification of mutant sequences (Fig. 1).

During the design of the biochip and the optimization of the hybridization protocol, special importance was attended to the effectiveness of the extraction procedure, discrimination between single mutations, sensitivity, and reproducibility.

Materials and Methods

STOOL SAMPLES AND DNA ISOLATION

We used 26 stool samples from patients who had colorectal cancer (n = 7), who were suspected of having colorectal cancer (n = 15), or who were at risk for colorectal cancer (n = 4). Tumors of five patients with colorectal cancer were staged according to the Dukes classification (three stage B; two stage D).

Stool samples were frozen immediately at −20 °C or were processed as soon as possible. DNA was extracted from 200 mg of stool material by a column-based method (QIAamp® DNA Stool Mini Test Kit; Qiagen). Purified DNA was suspended in 200 μL of 10 mmol/L Tris-HCl (pH 7.4)–1 mmol/L EDTA buffer.

TUMOR CELL LINES

For each possible mutation implemented on the biochip, a different tumor cell line was used. The cell lines are listed in Table 1. All cell lines were purchased from ATCC and grown according to the supplier's instructions. DNA isolation was performed with the QIAamp DNA Mini Kit (Qiagen), and the yield was quantified by ultraviolet spectrometry (260/280 nm). For the mutations Ile12 and Leu12, no cancer cell lines were available. PCR products containing this mutation were generated by site-directed mutagenesis with a method known as "splicing by overlap extension" *(12)*. Using DNA from SW480 cells as template, we introduced mutations coding for Ile12 and Leu12 by two-step PCR amplification with proofreading PfuTurbo-DNA-polymerase (Stratagene). Nonmutagenic outer primers were used: 5′-GTA CTG GTG GAG TAT

Table 1. Capture probes used and cell lines harboring corresponding mutations.

No.	Mutation (codon)	Cell line	Capture probe[a]
1	Wild type (Gly12/13)	Colo320	CT**GGTGGC**GTAGG
2	Ala12	SW1116	AGCTG**C**TGGCGTA
3	Arg12	H157	AGCT**C**GTGGCGTA
4	Asp12	LS174T	AGCTG**A**TGGCGTA
5	Asp13	DLD1	TGGTG**A**CGTAGGC
6	Cys12	MIA Paca2	AGCT**T**GTGGCGTA
7	Cys13	H1355	TGGT**T**GCGTAGGC
8	Ile12	Not available[b]	GAGCT**AT**TGGCGT
9	Leu12	Not available[b]	GAGCT**CT**TGGCGT
10	Ser12	A549	AGCT**A**GTGGCGTA
11	Val12	SW480	AGCTG**T**TGGCGTA
12	(HLA-)DRA PM	All	GGAGACGGTCTGG
13	(HLA-)DRA MM		GGAGACG**C**TCTGG
14	PCR control[c]	All	AAGGCCTGCTGAA
15	Hybridization control PM	Synthetic target	ACAGAAACGAGCG
16	Hybridization control MM		ACAGAAAC**C**AGCG

[a] Mutated bases are underlined. For the wild type, codons 12 and 13 are indicated.

[b] PCR products for these mutations were generated according to the *Materials and Methods* section.

[c] Capture probe located upstream of codons 12 and 13 served as universal hybridization control for K-*ras* amplicon irrespective of mutation status.

TTG ATA GTG-3′ (upstream); 5′-TGT ATC AAA GAA TGG TCC TGC ACC-3′ (downstream). Mutations were introduced by PCR amplification with nonmutagenic outer-upstream primer and the downstream primers 5′-TTG CCT ACG CCA ATA GCT CCA ACT-3′ (Ile12) and 5′-TTG CCT ACG CCA AGA GCT CCA ACT-3′ (Leu12), respectively. Corresponding overlapping mutated downstream amplicons were generated with the upstream primers 5′-AGT TGG AGC TAT TGG CGT AGG CAA-3′ (Ile12) or 5′-AGT TGG AGC TCT TGG CGT AGG CAA-3′ (Leu12) and the nonmutagenic outer-downstream primer. The four products of the first PCR steps were purified by agarose gel electrophoresis, and each of the corresponding two products was spliced together by a second PCR step with the nonmutagenic outer primers. The nucleotide sequence of the entire amplified region was verified by sequencing.

MUTANT-ENRICHED DUPLEX PCR

Sequences of primers used for K-*ras* amplification and PCR conditions were as described by Thiede et al. *(11)*. Primers specific to K-*ras* and PNA binding to the wild-type sequences of K-ras codons 12 and 13 were purchased from TIB Molbiol. Because of the low amounts of human DNA in feces, the reported protocol was modified with 45 cycles instead of 28 cycles. For an internal control for the extraction efficiency, the PCR was performed as duplex with K-*ras* and *HLA-DRA*. The primer sequences for *HLA-DRA* were 5′-GGC CGA GTT CTA TCT GAA TCC TGA-3′ (upstream) and 5′-GAG CGC TTT GTC ATG ATT TCC AGG-3′ (downstream). Because the amplification efficiency for *HLA* is higher, which is attributable to the absence of the PNA inhibitor, the primer concentration for the *HLA* primers was reduced to a final concentration of 0.1 μM compared to 0.25 μM for the K-*ras* primers. All downstream primers were Cy5-labeled at the 5′-position. The 157-bp K-*ras* product and the 204-bp *HLA* product were electrophoresed and visualized after ethidium bromide staining of the agarose gel.

SEQUENCING

The DNA mixtures were amplified by PNA-mediated clamping-PCR over 35 cycles for the tumor cell lines and 45 cycles for the stool samples. The amplification products were resolved on a 1.5% agarose gel, excised from the gel, and extracted with a gel extraction method (Qiagen).

The purified PCR products were sequenced on an ABI377 sequencer (PE Biosystems) with a BigDye Terminator cycle sequencing method (PE Biosystems) according to the manufacturer's protocol. In each sequencing reaction, 3 pmol of primer C12us67 (5′-CAT TAT TTT TAT TAT AAG GCC TGC-3′) was used.

RESTRICTION ASSAY

The restriction site mutant-specific PCR conditions, as well as primer nucleotide sequences for K-*ras* codon 12, were described previously *(13)*. The PCR products were generated by nonmutagenic or mutagenic primers with natural or artificial restriction sites. We modified the protocols by designing fluorescence-labeled primers for K-*ras* amplifications (TIB Molbiol). A 5-μL aliquot of the purified DNA was amplified by PCR (total volume, 50 μL) in a thermal cycler (40 cycles). The PCR products were then loaded on a 2% agarose gel, electrophoresed, and visualized by ethidium bromide staining. After digestion of the PCR products, the sizes of the fragments were measured by capillary electrophoresis (ABI PrismGenetic Analyzer 310), and data were analyzed with the Gene-Scan software (PE Biosystems). The DNA extracts of individual human fecal specimens were each analyzed in the PCR reactions with positive and negative internal controls.

K-*ras* BIOCHIPS

Capture probes (13mer) for the 10 most common mutations of K-*ras* codons 12 and 13 were immobilized on the surface of a new cycloolefin polymer with optical transparency and background fluorescence comparable to Pyrex glass as described previously *(14)*. An additional 13mer oligonucleotide, starting at position 81 of the K-*ras* gene, was immobilized for monitoring the success of the mutant-specific PCR reaction. To control the extraction efficiency of DNA from stool, two capture oligonucleotides from *HLA-DRA* were designed as perfect-match (PM) and mismatch (MM) probes. Stringency was controlled by synthetic PM and MM probes (HyCtrl; hybridization control). The sequences of all capture probes are listed in Table 1. In terms of the design process, special attention was paid to equalized melting temperatures, following a new nearest-neighbor algorithm *(15)*. Each capture probe was spotted in duplicate. The spot diameter was ~150 μm, and the spot-to-spot distance was ~400 μm.

HYBRIDIZATION

Hybridization was carried out in 6× saline-sodium phosphate-EDTA (Sigma) containing 1 mL/L Tween 20 (molecular biology grade; Sigma) in a 50-μL sample size containing 12.5 μL of the PCR product and the synthetic, 5′-Cy5-labeled hybridization control target (5′-A AGT CGC TCG TTT CTG TCA AGA-3′) at a 0.3 nM concentration. The mixture was heated at 95 °C for 3 min and cooled on ice for 1 min. The hybridization mixture (25 μL) was transferred to the biochip immediately and incubated at 37 °C for 1 h in a humidified chamber. After that the chip was rinsed with 1 mL of 6× saline-sodium phosphate-EDTA containing 1 mL/L Tween 20 and scanned, it was covered with 6× saline-sodium phosphate-EDTA under a microscope cover slip without further washing steps.

SCANNING OF BIOCHIPS AND DATA ANALYSIS

For the measurement of the biochip, a proprietary confocal laser scanner was used in time-resolved scanning

Table 2. Quality criteria for the interpretation of the biochip data.[a]

Diagnosis	Quality criteria	Allowed values
Enough material	[DRA-PM]/[Hybridization control PM]	>0.1
Measurement valid	Discrimination hybridization control	>20
	Discrimination *HLA-DRA*	>10
Mutation present	[Found mutation]/[Highest other mutation]	>3
	[Found mutation]/[Background]	>5
	[PCR control]/[Found mutation]	0.2–5
	[Found mutation]/[Hybridization control PM]	>0.1

[a] Brackets indicate values that are the mean fluorescence values of two replicate spots (background corrected).

mode *(14)*. Images were scanned with 20-μm resolution and 16-bit depth. Image analysis was performed with the ImageQuant 5.0 software program (Molecular Dynamics). Further analysis of the raw data, determination of discrimination rates, and checking of the controls were performed automatically with a specially developed, database-connected algorithm. To detect a single mutation in stool samples, we had to fulfill the quality criteria listed in Table 2. These criteria were determined empirically by experience gained during the evaluation phase. The discrimination ratio was defined as PM signal/MM signal.

Results

QUALITY OF ISOLATED DNA

The DNA yields from 56 independent isolations with the QIAamp stool analysis method are high (Fig. 2), and 200 mg of stool is sufficient for PCR amplification. Typical DNA amounts were 4–48 μg, corresponding to 100–1200 ng of DNA as starting material for the PCR. Significant inhibition of the PCR reaction by remaining impurities was not observed, as indicated by our successful amplification of *HLA-DRA.*

MUTANT-ENRICHED AMPLIFICATION

Only a minor part of the DNA isolated from stool is of human origin. To control the effectiveness of the DNA-extraction procedure, a human control gene, *HLA-DRA*, was coamplified in a duplex PCR protocol. A missing amplification of the internal control *HLA-DRA* was taken as an indication that not enough human DNA was extracted or originally present in the stool sample or that PCR-inhibiting substances were present.

The sensitivity of the amplification specific to the K-*ras* mutant was examined by a dilution series of DNA extracted from SW480 cells. A positive hybridization signal was obtained with 45 PCR cycles, even with 10 pg of DNA as the starting material. This amount corresponds to approximately one to two tumor cells. Although PNA-PCR alone can detect this low sample amount, false-positive PCR signals are obtained with higher DNA concentrations or increased cycle numbers. This effect is demonstrated in Fig. 3. With only 35 cycles in the amplification process, wild-type amplification was successfully suppressed by the addition of PNA and only the mutant alleles were amplified. When 45 cycles were applied, the suppression of the wild-type allele by PNA was insufficient, and false-positive signals were obtained. The additional hybridization to the K-*ras* biochip, on the other hand, allowed the precise classification of the present mutation even in the presence of an excess of wild-type DNA.

The hybridization signals after duplex PCR and the hybridization of 0.1 ng of SW480 cell line DNA mixed with 100 ng of wild-type DNA (cell line Colo320) are shown in Fig. 4. The unambiguous identification of the single mutation was also possible even in this 1000-fold excess of wild-type DNA for all other cell lines.

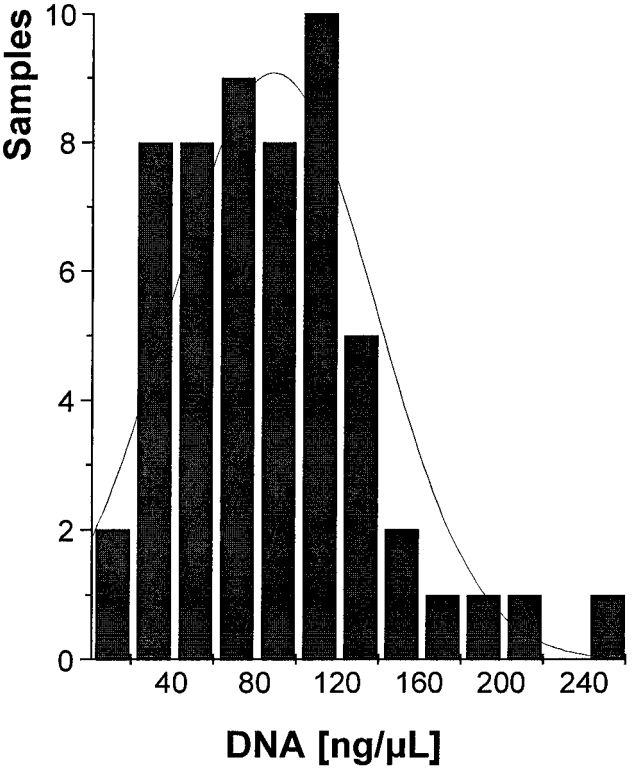

Fig. 2. DNA extraction efficiency from stool using the QIAamp stool extraction method.

From 56 samples, 200-mg fractions were taken for DNA isolation, from which 200 μL of DNA solution was obtained. DNA concentration was determined by ultraviolet measurements (260 nm).

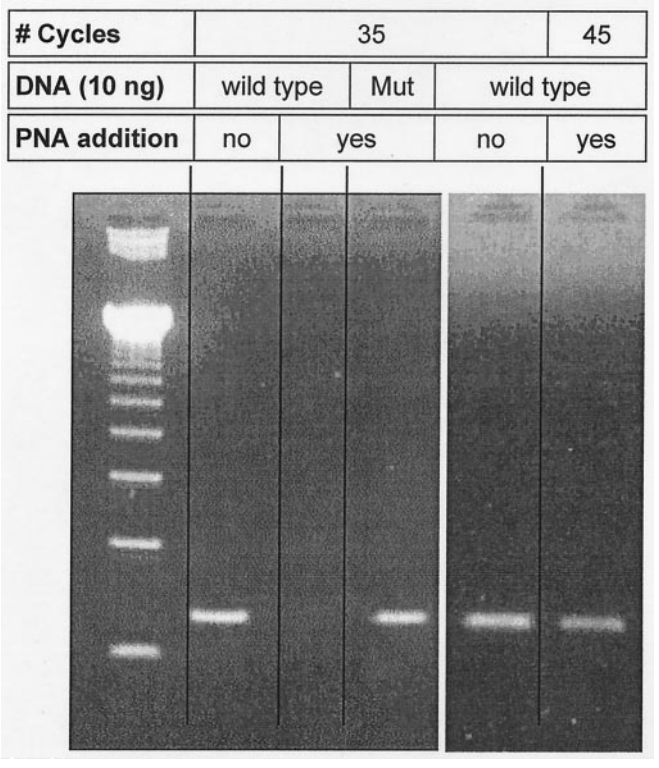

Fig. 3. Wild-type suppression by the addition of PNA to the PCR reaction.

Ten nanograms of either SW480 (Val12 mutation) or Colo320 (wild-type) DNA was used. With 35 cycles, wild-type DNA is effectively suppressed by the addition of PNA and only mutated DNA is amplified. With 45 cycles, wild-type is amplified even with the addition of PNA.

BIOCHIP VALIDATION WITH TUMOR CELL LINES

For a precise determination of all possible mutations within codons 12 and 13, a high discrimination ratio for a single target sequence with all other capture probes is necessary. The discrimination ratio for one target is defined as the quotient of the hybridization signal of the specific capture probe to the hybridization signal of the nonspecific capture probe. To analyze the discrimination ratio of each possible target sequence with all immobilized capture probes, tumor cell lines with known mutations have been used (Table 1). After mutation-enriched amplification of 10 ng of DNA with 35 PCR cycles, the amplicons were hybridized to the biochip for 1 h at 37 °C. The results can be seen in Fig. 5. High discrimination ratios for all cell lines and the synthetic targets were achieved. The lowest discrimination ratio between the mutations of 19.2 was observed for the cell line LS174T bearing the Asp12 mutation. The Mia Paca2 cell line (Cys12 mutation) showed low discrimination to the wild-type capture probe (12.8). All other discrimination ratios were >20.

EVALUATION OF THE BIOCHIP FOR DIAGNOSTIC APPLICABILITY TO STOOL SAMPLES

To determine the diagnostic applicability of the new biochip platform, 26 stool samples were analyzed in parallel by biochip hybridization, mutant-enriched PNA-PCR, restriction-based identification of codon 12 mutations, and sequencing. DNA was extracted from 200-mg stool samples. The results are summarized in Table 3. Twelve samples positive for K-*ras* were found by mutant-enriched PNA-PCR. After hybridization to the biochip, only 9 samples were found to have a mutation (8 Val12, 1 Asp12). All of the mutations found by the K-*ras* biochip were confirmed by sequencing. Only two of the found mutations (one Val12, one Asp12) belonged to patients with colorectal cancer; the others were from patients suspected of having colorectal cancer. Although a capture probe for the wild type is implemented on the biochip, wild-type DNA is not detected. This phenomenon can be explained by the presence of excess PNA in the PCR reaction used for hybridization. Because the PNA is complementary to the wild-type capture probe, its binding blocks further hybridization to the wild-type capture

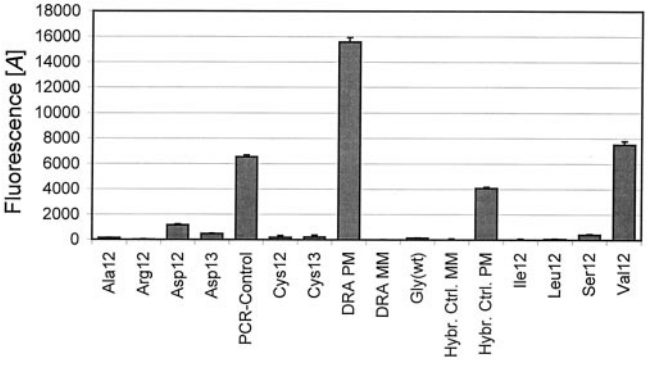

Fig. 4. Biochip data after hybridization of the PNA-PCR product of a 1:1000 mixture of SW480 and Colo320 cell lines.

The total amount of DNA was 100 ng. Shown are the mean (1 SD) values of the two spots for each capture probe. CVs are <7%. All of the criteria in Table 2 were reached. Identification of the Val12 mutation is unambiguous. *DRA*, *HLA-DRA* (DNA extraction control); *HyCtrl*, hybridization control (synthetic target).

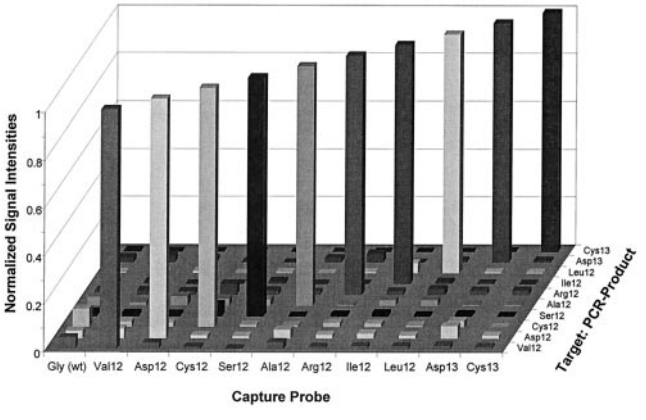

Fig. 5. Discrimination ratios of all possible targets with the immobilized capture probes.

Products of PNA-PCR of all cell lines from Table 1 were used. No *HLA-DRA* primers were used in these experiments. The signals (n = 2) were normalized to the highest signal (mutation) for each sample. CVs were <7% (not shown). Discrimination ratios were >20 for all mutations except Asp12 (19.2).

Table 3. Summary of data from 26 stool samples.

Sample	*Ras*-PCR[a]	HLA-PCR[a]	Biochip	Sequencing	Restriction assay
1	+[b]	+	Val12	Val12	+
2	+	+	Val12	Val12	−
3	+	+	Val12	Val12	−
4	+	+	Val12	Val12	+
5	+	+	Asp12	Asp12	−
6	+	+	Val12	Val12	+
7	+	+	Val12	Val12	−
8	+	+	−	Gly12 (wt)	−
9	+	+	Val12	Val12	−
10	+	+	Val12	Val12	+
11	+	+	−	Gly12 (wt)	−
12	+	+	−	Gly12 (wt)	−
13–26	−	+	−	ND	−

[a] Corresponding line in agarose gel after mutant-enriched duplex PCR (PNA-PCR).

[b] +, positive; −, negative; wt, wild type; ND, not done.

probe. Separation of the PNA after PCR allows the detection of wild-type alleles by hybridization. However, removal of the PNA from the hybridization solution is not recommended because higher specificity was observed when PNA was included in the hybridization buffer (Fig. 6). This improvement may be explained by the competitive effect that has already been described for DNA oligonucleotides *(16)*. We are currently using a model system in our laboratory to perform detailed investigations of the impact of PNA on hybridization specificity.

Only four samples were identified as positive for codon 12 mutations by the restriction assay; all of them could be confirmed by sequencing and hybridization.

The reliability of the single methods could be depicted by calculating the ratio of sensitivity to specificity. Results of PNA-PCR product sequencing were set to 100% for sensitivity and specificity. Ratios >1, as obtained by the PNA-PCR, indicate the possibility for overestimation and/or false-positive results. Lower values, as obtained with the restriction assay, indicate false-negative results. Only the results obtained by the biochip are as sensitive and specific as sequencing.

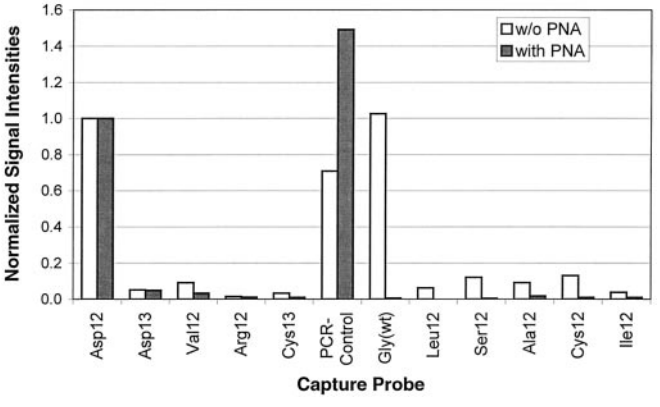

Fig. 6. Improvement of specificity by the addition of PNA to the hybridization solution.

K-*ras* was amplified (with PCR as described in the text, but without PNA) from the cell line LS174T (heterozygous for Asp12). The products were hybridized on biochips as described in the text with and without the addition of PNA (2 μM) to the hybridization buffer. Detection of wild-type DNA was not possible in the presence of PNA because the probe Gly was blocked. However, higher unspecific signals were observed in the absence of PNA.

Discussion

The results show that it is possible to detect K-*ras* mutations in human stool. With all applied methods, mutations were detected successfully, indicating the quality of the isolated DNA. From 200-mg stool samples, an average of ~20 μg DNA was obtained. If 5% of the DNA was of human origin and 1% of the human DNA was derived from tumor cells, only 0.2 ng of the 450 ng used in the PCR was tumor DNA. DNA amounts of the same range were applied for the experiments using tumor cell lines. Although other protocols for DNA isolation from human feces are available *(17, 18)*, the attraction of the introduced isolation procedure is its fast and simple handling, the applicability to low sample amounts, and the high DNA yields. In addition, the amplification of the *HLA-DRA* control sequence was successful with all samples, indicating the reliable removal of PCR inhibitors.

Sidransky et al. *(5)* reported that epithelial and tumor cells shed from the colon mucosa are located on the surface of the stool. One of the problems in detecting mutations in stool samples from patients is the heterogeneous distribution of human cells or DNA in the material. This is caused by the transfer of stool samples into tubes, which destroys their former structure and leads to heterogeneous mixtures. Therefore, we always took 200-mg fractions from at least three different locations of each specimen. This appears to be important because we could identify K-*ras*, and additionally *p53* mutations (data for *p53* not shown), in one fraction, but not in the others of the same colorectal cancer patient. The *HLA* control locus could be amplified from all fractions of such cases, indicating no differences in the presence of potential PCR inhibitors.

Special requirements have to be fulfilled for a routine diagnostic tool: high specificity and sensitivity, reproducibility, and often underestimated, an easy, fast, and reliable protocol. Some concepts use one specific primer for each investigated point mutation *(19)*. In this case, one PCR reaction has to be performed for each mutation. Moreover, cycle numbers and annealing temperatures differ, so this approach is often limited by economic restraints. High sensitivity is reached by the Point-EXACCT test *(20)*, but several reaction steps (amplification, digestion, hybridization, ligation, washing) and a separate reaction vessel or microtiterplate well are needed for each investigated point mutation *(21)*. Array-based approaches such as the introduced K-*ras* biochip have the advantage that all investigated probes, including controls, can be implemented on the same surface within one reaction vessel, and all capture probes are incubated with

the same sample at the same time. The applied protocol is fast and easy to perform because no clean up is necessary after the amplification step and no special washing steps after hybridization are required to adopt stringency.

Lopez-Crapez et al. *(22)* describe a DNA chip for the detection of K-*ras* mutations in solid tumors. This chip is able to detect K-*ras* mutations of codon 12 even in a 10-fold excess of wild-type sequence. For the detection of K-*ras* mutations from human feces, more sensitive platforms are needed because the ratio of mutant to wild type is 10- to 100-fold higher.

For diagnostic application, the only information needed is whether there is a mutation. A two-parameter analysis would be sufficient for this purpose, and one could object that the use of a biochip is, therefore, somewhat oversized. On the other hand, PNA-PCR or the restriction assay alone do not demonstrate the required sensitivity and specificity. If the wild-type DNA concentration in the investigated sample is high, false-positive results are obtained in the PNA-PCR, as demonstrated by the dilution experiments with tumor cell lines. To obtain valid values, further analysis is unavoidable. Here hybridization to a biochip is faster and easier to handle than sequencing. Hybridization, scanning, and data analysis can be performed in <2 h.

Eight of the nine mutations found in our study were Val12, and the other was Asp12. Val12 is the most frequent mutation in colorectal cancer *(7)*, followed by Asp12. The ratio of Val12/Asp12 is only 1.1–2 in the literature *(7)*, but the sample number in our study was too small to provide representative data of mutation frequency. For other malignancies, different mutation frequencies have been reported *(7*, *23*, *24)*. Although large differences in mutation frequencies are found in different ethnic populations and geographic regions *(25*, *26)*, even dietary intake was shown to have an influence on the mutation pattern *(27)*.

The prognostic value of K-*ras* mutations is controversial. Neubauer et al. *(23)* found increased survival in patients with acute myeloid leukemia having a *ras* mutation; Kawesha et al. *(24)* found increased survival of pancreatic ductal adenocarcinoma patients with only tumors bearing Val12, Ser12, or Asp13 mutations. In contrast, Samowitz et al. *(25)* found decreased survival rates for colon cancer patient with an Asp13 mutation. A reliable prognosis, therefore, may not be possible by testing for a single molecular marker.

We chose 13mer capture probes to achieve optimum discrimination among the single mutations. The position of the capture probes was shifted for 1–2 bases up- or downstream to adapt the melting temperatures and to optimize discrimination ratios. With the 13mer capture probes, a 1-h hybridization time at 37 °C provides sufficient stringency without further washing steps. The measured discrimination ratios appear excellent for a DNA biochip array. Such 13mer capture probes will not be suited for applications with more complex samples, such as whole transcriptomes or genomes, which will require longer capture probes (20- to 60mers) to avoid cross-hybridization. However, the use of longer capture probes decreases stringency, and more stringent washing steps are required. Discrimination ratios also decrease with decreasing target copy numbers, which is also true for stool samples. Therefore, we established the criteria listed in Table 2 where the positive mutation signal has to be fivefold above the background.

There is wide unanimity that the finding of K-*ras* mutations is a marker for colorectal and pancreatic cancer or cancer prestages *(1*, *3*, *6*, *28)*. On the other hand, it is clear that the detection of one marker alone, as a single or even double mutation in one gene, is not sensitive enough to detect all cases of these malignancies. Further studies like the large NCI-funded study announced in the US, will show if a reduced panel of markers, such as mutations in *p53* or APC or BAT-26 microsatellite instabilities, are sufficient to detect most cases of colorectal cancer at an early stage *(29)*. For additional markers, fast and reliable tests with sufficient diagnostic power have to be developed to reach high sensitivity in colorectal cancer screening. For K-*ras*, the introduced biochip offers the possibility to detect ~40–50% of colorectal cancers with high sensitivity and to avoid false-positive results.

We thank Daniela Meermann for excellent technical assistance.

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