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CLINICAL STUDY

Detection of point mutations in *KRAS* oncogene by real-time PCR-based genotyping assay in GIT diseases

Ugorcakova J[1], Hlavaty T[2], Novotna T[3], Bukovska G[1]

Institute of Molecular Biology, Slovak Academy of Sciences, Bratislava, Slovakia. **jana.ugorcakova@savba.sk**

Abstract: *Objectives:* The determination of gene mutations is important for the diagnosis and prognosis of various gastrointestinal cancers. The aim of our study was to develop a new procedure for the analysis of *KRAS* gene mutation by application of the real-time PCR method.
Background: The detection process requires discriminate trace amount of mutant allele in a large excess of wild-type DNA in various samples.
Methods: The real-time PCR based technique using hybridization probes for five most frequently *KRAS* codon 12 mutations and WT specific peptide nucleic acid (PNA) was performed. Our multiplex detection system was tested in various DNA samples (tissue, bile, pancreatic juice) of patients with different diagnoses of gastrointestinal tract disease obtained by endoscopy and ERCP.
Results: We designed and optimized the real-time PCR conditions and tested various amount of PNA in PCR reaction to suppress amplification of the wild-type DNA. We determined the interassay variability of the melting temperatures and the results of mutation testing were confirmed by DNA sequencing with the 100 % accuracy. Incidence of searched mutations was 67.5 % in cohort of 40 patients; for $KRAS^{G12D}$ it was in 44.4 %, $KRAS^{G12V}$ in 22.2 %, $KRAS^{G12S}$ in 14.8 %, $KRAS^{G12A}$ in 14.8 % and $KRAS^{G12C}$ in 3.8 %. The sensitivity of the assays is $1x10^{-5}$.
Conclusions: Advantages of this technique are rapidity, accuracy and it is generally easy to perform. This method can be adapted for synchronic detection of multiple mutations and after readjustment by other type mutation of *KRAS* gene may serve as useful clinical tool for analyzing point mutations in various clinical samples *(Tab. 3, Fig. 3, Ref. 42)*. Full Text in PDF *www.elis.sk.*
Key words: colorectal carcinoma, *KRAS*, codon 12 mutation, pancreatic carcinoma, real-time PCR.

The *RAS* genes encode a family of GTPases that act as the signal switch molecules for many important cellular processes. RAS proteins, which play a key role in cell growth, apoptosis and differentiation, are low molecular weight (21 kD) GTPases, which cycle between the GDP-bound (inactive) and the GTP-bound (active) states at the plasma membrane. Genetic alterations of *KRAS* oncogene have a crucial role in pancreatic and colorectal carcinogenesis (1, 2, 3). The prevalence of *KRAS* mutations are in pancreatic carcinoma 75–100 %, in colorectal carcinoma (CRC) 40–50 %, in lung carcinoma 15–40 %. *KRAS* point mutations mostly occur in codons 12, 13 and 61. The most frequent type of *KRAS* mutations in CRC are GGT>GAT ($KRAS^{G12D}$, Asp) in 37 to 54 %, GGT>GTT ($KRAS^{G12V}$, Val) in 28 to 30 %, GGT>AGT ($KRAS^{G12S}$, Ser) in 11 to 15 %, GGT>TGT ($KRAS^{G12C}$, Cys) in 7 to 20 %, GGT>GCT ($KRAS^{G12A}$, Ala) in 4 to 10 %, GGT>CGT ($KRAS^{G12R}$, Arg) in 2 to 7 %, (4, 5). Recent evidences suggest that different mutations in *KRAS* have different biological consequences *in vivo* (6). In addition, although $KRAS^{G12D}$ seems to be more frequent compared to $KRAS^{G12V}$ in colon cancer and has been associated with more aggressive colorectal carcinomas and higher mortality than other codon 12 or 13 mutations (7, 8, 9). It follows that the type of *KRAS* mutations can influence the survival rate of CRC patients.

Despite of some problems with sampling (tissue availability, tissue release and shipment, and results feedback) it was shown that the *KRAS* mutation screening is clinically relevant for detection of CRC and pancreatic cancer and for treatment response (10).

KRAS oncogene was described more than 25 years ago. The development of effective tools for early detection could play a key role in reducing the mortality of this disease. This type of mutation is detected in early stadium where only few evident symptoms are present and therefore more hope for curability exists (11). There are various mostly PCR-based techniques for detection of *KRAS* gene point mutation. The problem of using somatic mutations as markers of malignancy is minimal amounts of mutant DNA in a large excess of wild-type DNA containing in clinical samples. Variety of technologies based on allele discrimination strategies have been applied in the identification of

[1]Institute of Molecular Biology, Slovak Academy of Sciences, Bratislava, Slovakia, [2]Department of Internal Medicine, Faculty Hospital Ruzinov, Comenius University, Bratislava, Slovakia, and [3]Department of Gastroenterology, Faculty Hospital St. Cyril and Method's, Comenius University, Bratislava, Slovakia

Address for correspondence: J. Ugorcakova, Institute of Molecular Biology, Slovak Academy of Sciences, Dubravska cesta 21, SK-845 51 Bratislava, Slovakia.
Phone: +421 2 59307440

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point mutations, such as oligonucleotide ligation (12, 13), PCR enriched enzymatic cleavage (14, 15, 16), allele-specific oligonucleotide hybridization (17) and integration of allele-specific oligonucleotide ligation assay (OLA) with magnetic beads based on electrochemiluminescence (18). Pyrosequencing methods based on chemiluminescence (19, 20) and primer extension (21) was also described. However, each of these methods has some disadvantages. Many of them are either technically very complicated, expensive or time consuming and thus they are not suitable for routine clinical diagnostic process.

Real-time PCR has attractive features for tumor profiling in the clinical laboratory (22). The novel method was introduced recently for genotyping of *KRAS* gene (23, 24, 25, 26). The are several advantages of real-time PCR techniques: PCR is not influenced by non-specific amplification**,** there is no post-PCR processing of products (high throughput, low contamination risk), amplification can be monitored in real-time, ultra-rapid cycling (30 minutes to 2 hours), confirmation of specific amplification by melting point analysis. These methods are more specific, sensitive and reproducible, not much more expensive than conventional PCR (except equipment cost). The mutation-sensitive hybridization profile of peptide nucleic acids (PNAs) has been exploited to design PCR-clamping protocols (27). PNAs are non-extendable oligonucleotides, in which the ribose-phosphate backbone is replaced by 2-aminoethyl glycine units linked by amide bonds. PNA oligomers suppress amplification of the wild-type sequence confined by pair DNA oligonucleotide primers (competitive clamping) because they are no substrate for DNA polymerases. For this reason, all alleles with single base changes can be easily distinguished from wild-type by melting peak analysis. The PNA-clamped probe assay is more sensitive than direct sequencing with the ability to detect mutations in samples containing less than 1 % mutant alleles (28).

In our study we present a novel real-time PCR method that is useful to detect simultaneously defined *KRAS* mutations in various types of clinical samples. In preliminary study, we found mutations in samples of patients with different GIT diagnosis. In our assay we tested only 5 the most frequent codon 12 mutation of *KRAS* gene. We have improved an assay of hybridization probes by combination of wild-type PNA 15 oligomer to achieve more sensitivity and specificity of detection.

Tab. 1. Patients and incidence of KRAS mutations in DNA samples.

Patient N°	Age	Sex	Dg	Type of sample	Mu
1	71	M	CPo	T	C
2	47	F	CPo	T	-
3	76	M	CPo	T	D
4	80	F	CRC	T	A
5	62	M	BTC	Pj	S
6	57	F	BDS	Pj	A
7	60	M	PAC	B	-
8	47	P	PC	B	D
9	57	M	PAC	B	-
10	66	M	BTC	B	D
11	58	M	PC	B	D
12	74	M	BTC	B	-
13	47	M	BTC	B	D
14	62	M	PC	B	-
15	65	M	PAC	B	V
16	74	F	BDS	B	V
17	83	F	PA	B	V
18	57	F	BDS	B	-
19	65	M	BTC	B	D
20	81	F	BDS	B	-
21	38	F	BDS	B	D
22	59	M	PAC	B	-
23	52	M	PAC	B	D
24	58	F	PAC	B	V
25	54	M	PC	B	D
26	59	M	PA	B	S
27	52	F	PAC	B	D
28	28	M	PC	B	D
29	59	M	CRC	T	A
30	77	F	CRC	T	-
31	36	M	PA	B	S
32	63	M	PAC	B	V
33	54	M	BDS	B	-
34	70	F	BTC	B	S
35	70	M	BDS	B	-
36	73	F	BDS	B	-
37	64	M	BTC	B	D
38	71	F	CRC	T	A
39	60	M	CRC	T	-
40	49	F	PC	T	V

M – male, F- female, Dg – diagnose, CPo – Colon polyp, CRC – Colorectal carcinoma, BTC – Biliary tract carcinoma, BDS – Biliary duct stenosis, PAC – Pancteatitis chronica, PC – Pancreatic carcinoma, PA – Papillary adenoma, T – tissue, Pj – pancreatic juice, B –bile, Mu – mutation in KRAS codon 12, A – KRASG12A (GGT>GCT), D – KRASG12D (GGT>GAT), V – KRASG12V (GGT>GTT), S – KRASG12S (GGT>AGT), C – KRASG12C (GGT>TGT)

Tab. 2. DNA sequences of primers, PNA oligomer and probes for detecting KRAS mutations.

Reporter Name	Sequence	Reporter Dye
Asp Sensor	705-TTGCCTACGCC **ATC**AGCTCCAA-**p**	Red 705 **p**-phosphate
Val Sensor	705-TTGCCTACGCC **AAC**AGCTCCAA-p	Red 705 **p**-phosphate
Ala Sensor	705-TTGCCTACGCC **AGC**AGCTCCAA-p	Red 705 **p**-phosphate
Cys Sensor	705-TTGCCTACGCC **ACA**AGCTCCAA-p	Red 705 **p**-phosphate
Ser Sensor	705-TTGCCTACGCC **ACT**AGCTCCAA-p	Red 705 **p**-phosphate
Gly wt	640-TGCCTACGCC ACCAGCTCCAA-p	Red 640 **p**-phosphate
Anchor KRAS	CGTCCACAAAATGATTCTGAATTAGCTGT ATCGTCAAGGCACT-**f**	**f** –fluorscein
PNA KRAS	TAC**GCCA**CCAGCTCC-**p**	
KRAS-for	AAGGCCTGCTGAAAATGACTG	
KRAS-rev	GGTCCTGCACCAGTAATATGCA	
WT-sequence	GTTGGAGCT**GGTGGC**GTAGGCAAG Genbank accession no. K01519	

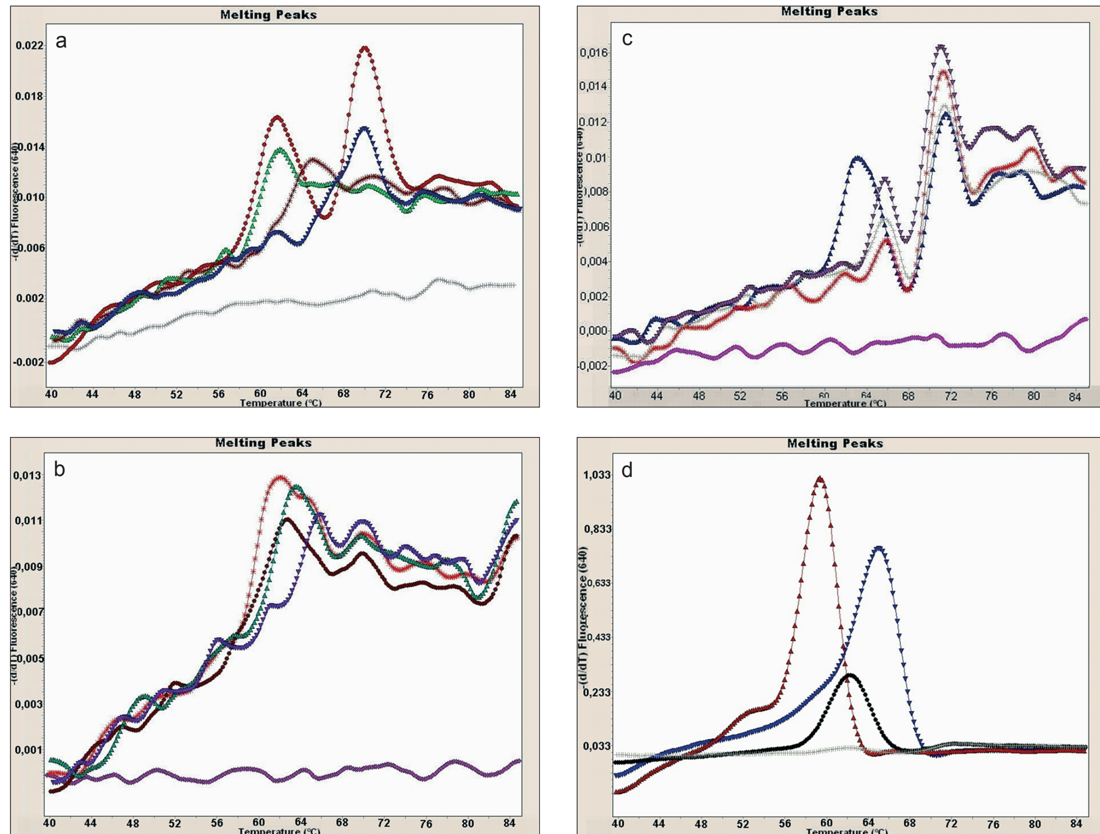

Fig. 1. Melting curves for the detection of *KRAS* mutation.
A. Melting peaks for mutation KRAS^{G12V} (GTT, ▲triangles – homozygous – sample N°16, ●circles – heterozygous – sample N°17), KRAS^{G12D} (GAT, ✷stars – sample N°8), without mutation (GGT, ▼down triangles – sample N°14), NTC (+cross – non template control) without of PNA in reaction.
B. Peak with ▲triangles represents sample N°29 with mutation KRAS^{G12A} (GCT), ▼down triangles – sample N°5, ✷stars – sample N°31, +cross – sample N°34 all with mutation KRAS^{G12S} (AGT) respectively, ♦diamonds – NTC, concentration of PNA 0.05 μM.
C. Peak with ✷stars represents sample N°15 with mutation KRAS^{G12V}, ●circles – sample N°6 with mutation KRAS^{G12A}, ▲triangles – sample N°25 with mutation KRAS^{G12D}, ▼down triangles – sample N°26 with mutation KRAS^{G12S}, ♦diamonds – NTC, concentration of PNA 0.15 μM.
D. Melting peaks for KRAS^{G12C} (TGT, ▲triangles – sample N°1), KRAS^{G12D} (GAT, ▼down triangles – sample N°3), KRAS^{G12V} (GTT, ●circles – DNA from cell line SW480), NTC (+ cross – non template control), concentration of PNA 2.5 μM.

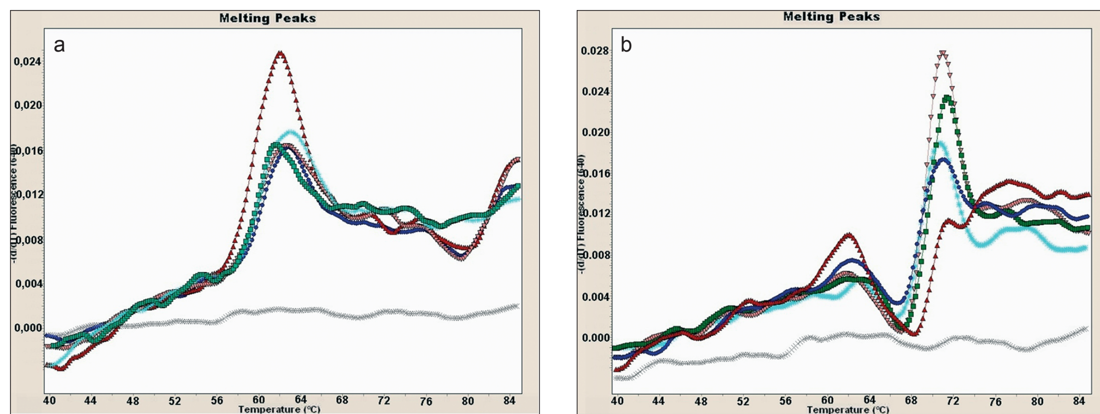

Fig. 2. Comparison of melting curve analysis of samples with *KRAS* gene mutation.
A. with PNA (1.5 μM) in reaction
B. without PNA in reaction
Peaks with ▲triangles and ■rectangles represent samples N°32 and N°40 respectively which contain mutation in KRAS^{G12V}, peaks with ✷stars, ▼down triangles and ●circles represent samples N°10, N°11 and N° 28 respectively which contain mutation KRAS^{G12D}, NTC (x diag cross – non template control).

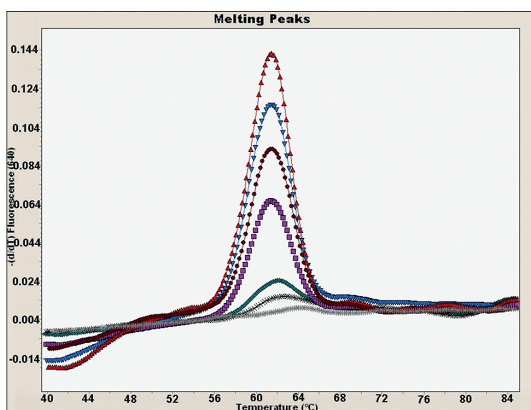

Fig. 3. Assay sensitivity for detection of *KRAS* mutation in a large excess of wild-type DNA.
Peak with ▲triangles represents only mutant DNA, with ▼down triangles represents ratio of 1:10, ●circles – 1:100, ■rectangles – 1:1000, ♦diamond – 1:10000, × diag cross – 1:100000, ✳stars – NTC. Under the optimal conditions using 12 Val Sensor as the probe and PNA (2.5 μM) as the blocker of WT amplification.

Methods

Clinical samples. Samples were obtained with the patients' permissions after biopsy, endoscopically and by ERCP from 40 individuals: 4 with colorectal carcinoma (CRC), 3 colon polyp (CPo), 5 pancreatic cancer (PC), 8 chronic pancreatitis (CP), 9 biliary duct stenosis (BDS), 8 biliary tract carcinoma (BTC), 3 papillary adenoma (PA) (Tab. 1). DNA from cell line with *KRAS* gene codon 12 mutation SW480 (G12V) from DSMZ (Braunschweig, Germany) were used as the positive control. DNA from normal human lymphocytes without mutation was used as the negative control.

Nucleic acid extraction. Genomic DNA was extracted from fresh frozen tumor tissue and from body fluids (bile, pancreatic juice, blood) using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations.

Real-time PCR method of hybridization probes. The method is based on two adjacent probes. The sequence of the primers, probes and the PNA oligomer (TIB MOLBIOL, Berlin, Germany) are listed in Table 2. The amplification of template DNA (50-100ng) was performed in LightCycler 1.5 Instrument (Roche Applied Science, Mannheim, Germany). Obtained fluorescence data were analyzed using the LightCycler software with "Tm Calling" Analysis mode (software version 4.1, Roche Diagnostics).

Sequence analysis. Detection of mutation was confirmed by direct sequencing of PCR amplicons after PNA-probe assay by using of primers KRAS-for and KRAS-rev (Tab. 2). Sequences were aligned with previously published sequences [The National Center for Biotechnology Information (NCBI) GenBank accession No. M54968].

Assay sensitivity. We tested assay's limits for detection of the trace amount of mutant allele in a large excess of wild-type DNA. Mutant DNA from the homozygous GTT mutant cell line SW480 (MT) was serially diluted with wild-type genomic DNA [wild-type *KRAS* codon 12 DNA (WT) originated from normal human lymphocytes] to get decreasing ratios of mutant-to-total DNA. 1 μg of WT DNA was diluted with 100 ng, 10 ng, 1 ng, 100 pg, 10 pg of MT DNA. 100 ng of total DNA from each of the diluted samples were used as a template to establish the detection threshold of the method (Fig. 3). The experiment was repeated 3 times.

Results

We used the method of real-time PCR and the Light-Cycler software for the detection of *KRAS* alterations by the determination of amplicon T_m properties depending upon duplex formation and sequence variance. This method requires a specific anchor probes and results in generation of a different T_m specific for each mutation. We designed probes adjacent to codon 12 for the most common mutations found in GIT malignancies [GAT ($KRAS^{G12D}$ – Asp), GTT ($KRAS^{G12V}$ – Val), GCT ($KRAS^{G12A}$ – Ala), TGT ($KRAS^{G12C}$ – Cys) and AGT ($KRAS^{G12S}$ – Ser)].

Analysis of the sensitivity. To optimize the PCR conditions we used DNA from cell line SW480 (*KRAS* codon 12 homozygous GTT). We tested various amount of PNA in PCR reaction to suppress amplification of the wild-type DNA (Figs. 1, 2). Lower concentration was inefficient – 0.05 and 0.15 μM PNA and resulted in a minimal decrease of the wild-type signal (Fig. 1B, 1C). As is shown on the Figure 2, where is comparison of melting curve analysis of the equal samples with *KRAS* gene mutation - with PNA (1.5 μM) in reaction (Fig. 2A) and without PNA in reaction (Fig. 2B), peaks for particular mutation are more expressive. Optimal concentration of PNA which resulted in complete suppression of wild-type signal was 2.5 μM (Fig. 1D), while 1.5 and 2.0 μM of PNA in reaction suppressed significantly amplification of wild-type allele. Genomic mutant DNA SW480 (MT) was serially diluted into wild-type *KRAS* codon 12 DNA (WT) isolated from normal human lymphocytes to get decreasing ratios of MT : WT DNA (1:10, 1:100, 1:1000, 1:10 000, 1:10 000). Under the optimal condition (using 12 Val Sensor as the probe and 2.5 μM PNA as the blocker of WT amplification) in the assay, the signal from as few as 1 pg mutant DNA in presence of 100 ng wild-type genomic DNA was detected. As is shown in the Figure 3, by using this assay we can detect one mutated cell in access of 10^5 healthy cells.

Genotyping of KRAS mutation in various samples of GIT malignancies. For the testing of our method, we used various clinical samples. Cohort of 40 patients with different diagnosis of GIT disease was examined for the presence of *KRAS* gene codon 12 mutations. Genotyping was carried on the base of melting temperatures (T_m). As is shown in the Tab. 1, the incidence of searched mutations was 27/40 (67.5 %). We established the point mutation in 3 of 6 (50 %) samples from colorectal carcinoma, 2 of 3 (66.7 %) samples from colon polyp, 5 of 6 (83.3 %) samples from pancreatic carcinoma, 5 of 8 (62.5 %) samples from chronic pancreatitis, 6 of 7 (85.7 %) samples from biliary tract carcinoma, 3 of 8 (25 %) samples from biliary duct stenosis and 3 of 3 (100 %) samples from papillary adenoma (Tab. 3).

Tab. 3. Incidence of KRAS mutations in samples of different GIT diagnoses.

CRC		CPo		PC		PAC		BTC		BDS		PA	
N°	Mu	N°	Mu	N°	Mu	N°	Mu	N°	Mu	N°	Mu	N°	Mu
4	A	1	C	8	D	7	-	5	S	6	A	31	S
29	A	2	-	11	D	9	-	10	D	16	V	17	V
30	-	3	D	14	-	15	V	12	-	18	-	26	S
38	A			25	D	23	D	13	D	20	-		
39	-			28	D	22	-	19	D	21	D		
				40	V	24	V	34	S	33	-		
						27	D	37	D	35	-		
						32	V			36	-		

We determined the interassay variability of the melting temperatures. For *KRAS*^{G12C}, *KRAS*^{G12V}, *KRAS*^{G12A}, *KRAS*^{G12D}, *KRAS*^{G12S} and wild-type the variability was 59.0 °C±0.8 (n=20), 61.5 °C±0.8 (n=30), 63.0 °C±0.8 (n=15), 64.0 °C±0.8 (n=30), 65.8 °C±0.8 (n=30) and 71.5 °C±0.8 (n=30) respectively (Fig. 1, 2). Therefore this allows precise discrimination of wild-type and mutant DNA by melting point analysis.

Incidence of the particular type of mutations in our samples was: *KRAS*^{G12D} in 12 (44.4 %), *KRAS*^{G12V} in 6 (22.2 %), *KRAS*^{G12S} in 4 (14.8 %), *KRAS*^{G12A} in 4 (14.8 %), *KRAS*^{G12C} in 1 (3.8 %) from all of 27 founded mutations at codon 12 of the *KRAS* gene. Consequently, the results of mutation testing were confirmed by DNA sequencing of the corresponding fragment of the *KRAS* gene and the identification of the mutation status of the sample was confirmed with the 100 % accuracy.

Discussion

The aim of our work was to develop a sensitive procedure for detection of *KRAS* mutation in codon 12 in various types of clinical samples. In the present study, we tested the multiplex real-time PCR technique using the five hybridization probes labeled with an acceptor fluorochrome (at the 3' end) and probe labeled with a donor fluorochrome (LightCycler system). In general, the proportion of malignant or premalignant to normal cells in clinical samples is extremely low. To increase the detection of a minimal amount of mutant DNA we used the peptide-nucleic-acid (PNA) oligomers. In contrast to work of Däbritz et al (31), where was a wild-type PNA (17-mer) and mutant-specific fluorescent-labeled hybridization probe for one mutation used, we used the 15-mer wild-type PNA and five mutant-specific fluorescent-labeled hybridization probes, which include more frequent codon 12 *KRAS* mutation. For this reason, all alleles with single base changes can be easily distinguished from wild-type by melting peak analysis. Recently several studies were done (29), (30), (28), in which was a similar PCR technique used with fluorescent hybridization probes and competing peptide nucleic acid oligomers to detect *KRAS* mutations. Detection and discrimination of present mutation from the wild-type allele was determined by the melting point temperature (T_m) shift. We could assign the particular mutation only approximately, because the melting points (T_m) were in close vicinity in our case. In spite of this incidence, the particular type of mutations in our samples corresponds with findings in literature (4). The determination of difference between samples with and without mutation is undoubted (Fig. 1). The sequencing results showed 100 % concordance with genotyping by our method.

Sampling is very important for objective estimation of the point mutation. The most frequent source of sample for analysis is i.e. paraffin embedded tissue. It was shown, that it is useful as well to collect blood samples before treatment and to combine blood tests with others (31). Also, the testing for the presence of mutant *KRAS* in stool has been proposed for the detection of pancreatic and colorectal carcinoma (32, 33). Some studies mentioned other samples like bile or pancreatic juice (34, 13, 29). Detection of *KRAS* mutation in pancreatic juice alone is considered insufficient for discriminating between pancreatic cancer and benign diseases (35). In our work we used nine samples obtained after colonoscopy and other 31 were originated from ERCP examination. We confirmed that also bile and pancreatic juice are suitable as samples for genotyping. The frequency of mutations observed in our samples (Tab. 3) was similar to the frequency determined by other researchers (5). The advantage of our assay was that the detection of mutation was accomplished in a single tube on LightCycler without having to go through several laborious procedures including electrophoresis, hybridization and enzymatic reaction.

Numbers of potential biomarkers including mutational activation of *KRAS* oncogenes are continually investigated as predictors of therapy response. Moreover, *KRAS* mutation plays an important role in the response rate of anti-EGFR antibodies treatment in patients with metastatic colorectal cancer (36, 37, 38, 10). Several recent studies have shown that patients with *KRAS* mutations in codons 12 or 13 in metastatic tumors do not benefit from anti–EGFR therapy (with cetuximab or panitumumab) (39, 40, 41, 42). In many case it can help to prevent from death of patient.

Finally, we can conclude that this method can be adapted for synchronic detection of multiple mutations and after readjustment by other type mutations may be a useful clinical tool for analyzing *KRAS* point mutation in various clinical samples. Identification of *KRAS* mutations is another powerful tool in CRC and pancreatic cancer diagnostic and this method shows a great promise as a molecular biomarker to assign chemopreventive strategies.

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