Assay design and optimization of mutant-enriched PCR based method for detection of K-ras gene mutations in pancreatic carcinoma*

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The aim of our work was to develop a fast, reliable and sensitive PCR method to detect K-*ras* mutations in various clinical samples. There is a need for an unimpeachable method for early diagnosis and/or screening of pancreatic cancer (PC).

We optimized and subsequently analyzed four methods based on mutant-enriched PCR for the sensitivity, cost and time expense. Using the selected optimal method we examined codon 12 K-*ras* mutations in a study population of 59 patients with upper GIT malignancies. Reliability of the genotyping was confirmed by sequencing.

By using the best of our modified mutant-enriched PCR methods we achieved sensitivity of $1:1x10^5$. Further studies are necessary to determine the optimal biological material sampling in PC.

Key words: K-ras mutation, codom 12, pancreatic cancer, early detection, mutant-enriched PCR

Cancer of the exocrine pancreas is the fifth leading cause of cancer death in the Western population with an average survival of about 5 months and a five-year survival rate less than 5% [1]. Radical pancreas resection remains the only potentially curative therapeutic modality. However, only 15–20% of patients are diagnosed in an early stage when an attempt for a curative resection can be considered. Current post-resection 5-year survival rate is approximately 20% [2]. Conventional tumor markers, such as CA 19-9, lack sensitivity in early stages and are usually used only to confirm late stage of the disease or for post-surgical follow-up. In order to improve the bad prognosis of pancreatic cancer (PC), there is an urgent need for sensitive diagnostic and/or screening methods for detection of early stages of PC [3].

Pancreatic cancer is a multi-stage process resulting from the accumulation of genetic changes in the somatic DNA of normal cells. The accumulation of mutations in the oncogenes K-*ras, HER2/neu, AKT2* and the tumor-suppressor genes *p16, TP53, MADH4,* and *BRCA2* leads to a profound disturbance of the cell cycle regulation and continuous growth [4].

Three well defined K-*ras* gene mutations (at codon 12 and less frequently 13 and 61) are detected in 80–90% of pancreatic carcinomas [5–7]. Moreover, these mutations are observed in early stages of the pancreatic carcinogenesis, i.e. in ductal changes that are thought to be precursor lesions of PC [8–10]. On the contrary, the patients with chronic pancreatitis do not have K-*ras* mutations [11]. This makes the K-*ras* oncogenes a very promising candidate genetic marker for detection of early pancreatic cancer or for differential diagnosis of unclear pancreatic lesions.

The K-*ras* oncogene is one of the three members of the human *ras* gene family that code for the highly related 21-kDa proteins with guanosine triphosphatase (GTPase) activity. *Ras* genes are the most frequently mutated oncogenes detected in human malignancies [12]. Owing to an associated inappropriate stimulation signal, the mutant *ras* transmits a continual growth signal to the nucleus [13].

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Abbreviations: PC – pancreatic cancer, WT – wild type, MT – mutant type, ME PCR – mutant-enriched PCR, REMS – restriction endonuclease-mediated selective, SAMA – stencil-aided mutation analysis, SSCP – single-strand conformational polymorphism, PHFA – preferential homoduplex formation assay, ELMA – enzyme-linked mini-sequence assay, MASA – mutant allele-specific amplification

An optimal method for detection of K-*ras* mutations should be sensitive enough to detect even minute amounts of mutated genes in examined biological material in the presence of vast majority of healthy copies of the gene. It should be also technically simple, fast and possibly inexpensive to allow for cost effectiveness. First techniques to detect mutations in the K-*ras* oncogene were described more than 20 years ago [14]. Since then a number of various, mostly PCR-based techniques have been developed to detect mutated K-*ras* gene codon 12 [15–21]. Many of these methods are, however, either technically very complicated, expensive or time consuming and are thus so far not suitable for routine clinical diagnostic process.

The aim of our study was to develop a simple, fast and sensitive method for detection of K-*ras* codon 12 mutations suitable for a routine clinical use. We adapted and optimized four known sensitive and potentially inexpensive methods and compared them in terms of their sensitivity, expensiveness and time consumption.

In the next step we were interested in the presence of K-*ras* codon 12 mutations in tumors of the upper GI tract that might shed malignant cells into the duodenal fluid and tested the method of K-*ras* detection that came up as the most appropriate one.

Material and methods

Positive control. The positive control for codon 12 mutation of K-*ras* gene was obtained from several fresh tumor samples from patients with colorectal carcinoma. DNA was extracted from 25 mg of tumor tissue which was previously frozen in a liquid nitrogen. DNA was isolated using the QIAamp DNA Mini Kit according to the manufacturer's recommendations. Mutation on gcodon 12 (GGT → GAT) was confirmed by sequencing.

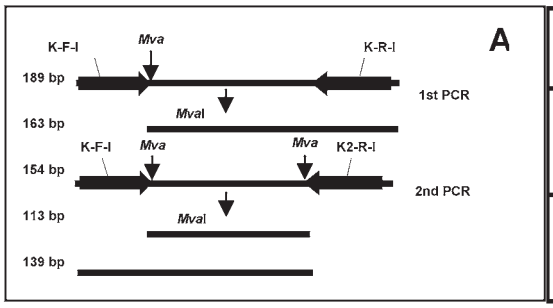
Detection of K-ras mutation. As described in detail below, we optimized four potentially fast and reliable PCR methods for detection of the K-*ras* gene mutation at the codon 12 position. All methods are based on a known technique of mutant-enriched PCR. The PCR primers were designed for the exon 1 of the codon 12 (Fig. 1). The forward primer used in all four methods was designed to introduce a base substitution creating a *Mva*I (or *Bst*OI) recognition site (5'CCTGG 3') (Fig. 1). This enabled to selectively cleave the healthy allele and enrich the mixture with mutated allele, if this one was present in the analyzed sample. All synthetic oligonucleotides used were purchased from Invitrogen Life Technologies.

Method I. Two rounds of PCR amplification were applied, each followed by a digestion with the restriction endonuclease *Mva*I as depicted in Figure 1. Fifty nanogram of template DNA was added to 50 µl of a reaction mixture containing 1x PCR buffer [50 mmol.l^{-1} KCl, 10 mmol.l^{-1} Tris-HCl (pH 8.3), 1.5% (vol/vol) Triton X-100], 2 mmol.l^{-1} $MgCl_2$, 0.2 mmol.l^{-1} concentrations of each deoxynucleotide triphosphate, 50 pmol of each primer, and 1 U of *Taq* DNA polymerase (Promega, Madison, Wis.). PCR cycle conditions were 95 °C for 5 min, 30 cycles at [94 °C for 30 s, 55 °C for 1 min, and 72 °C for 1 min], 72 °C for 4 min were performed in a Biometra Thermocycler. After each PCR reaction, 10 µl of the final product was digested for 3 hours with the restriction endonuclease *Mva*I (Fermentas UAB, Lithuania) at 37 °C according to the manufacturer's instruction. After the first PCR reaction the *Mva*I cleaves the healthy allele of codon 12 i.e. containing the GGT sequence. The reaction mix is thus enriched by an uncleaved allele containing a mutation at codon 12 after the first PCR round (Fig. 1. part A). Five µl of the reaction mixture was added to the second PCR reaction which was run under the same condition as in the first PCR. After the second round of PCR, another digestion with *Mva*I differentiates the mutant (139-bp band) from the wild-type allele (113-bp band) (Fig. 2).

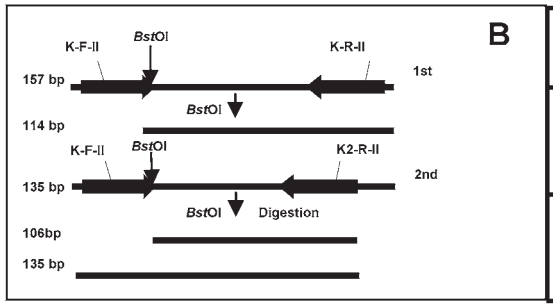
Method II. This method is principally similar to method I but in this case different primer sets, reaction conditions and restrictions enzyme were tested. (Fig. 1, part B). The first PCR round was reduced to 15 cycles, whereas the number of cycles of the second PCR was 30. After each round of PCR reaction, 10 µl of the final product was digested for 3 hours with a restriction endonuclease *Bst*OI (Promega) at 60 °C. *Bst*OI cleaves the healthy allele at two sites, whereas mutated allele at just one. In this method the presence of mutant allele shows 135 base pair band and the wild-type allele 106 base pair band (Fig. 2).

Method III. This method was only one-round PCR with subsequent endonuclease restriction. (Fig. 1 part C). Fifty pmol.l^{-1} of each primer was put into 50 µl reaction mixture which contains 1x PCR buffer [50 mmol.l^{-1} KCl, 10 mmol.l^{-1} Tris-HCl (pH 8.3), 1.5% (vol/vol) Triton X-100, 4 mmol.l^{-1} $MgCl_2$, 20 mmol.l^{-1} concentrations of each dNTP and 1 U of *Taq* DNA polymerase (Fermentas, Lithuania)]. The PCR cycle conditions were 95 °C for 5 min, 40 cycles [94 °C for 30 s, 56 °C for 1 min, and 72 °C for 30 s] 72 °C for 4 min. The product of PCR was consequently digested 3 h with *Mva*I at 37 °C (or *Bst*OI at 60 °C) and visualized on a gel electrophoresis. Healthy allele is cleaved and gives a 77 base-pair fragment. Mutated allele is not cleaved and gives a 110 base-pair fragment (Fig. 2).

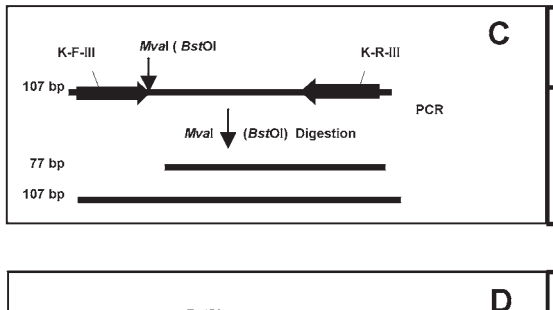
Method IV. In this one-step method the PCR mixture was enriched by mutated alleles after each PCR cycle due to the presence of the thermo-stable restriction enzyme in the PCR mixture cleaving the healthy allele directly during the PCR reaction. (Fig. 1 part D): 40 pmol.l^{-1} of first two-diagnostic primers (produce products 81 bp only if mutation at codon 12 is present), 20 pmol.l^{-1} PCR second two control primers (produced products 215 bp from exon 4b of K-*ras*) and 2 pmol.l^{-1} enzyme control primers (produced products 130 bp from exon 3 of K-*ras*) (Fig. 2). The PCR reactions were carried out in a final reaction volume of 25 µl. Reaction mixture contained 1 mmol.l^{-1} DTT; each dNTP at 50 mmol.l^{-1}, and 40 U of *Bst*OI in 100 mmol.l^{-1} NaCl, 50 mmol.l^{-1} Tris-HCl (pH



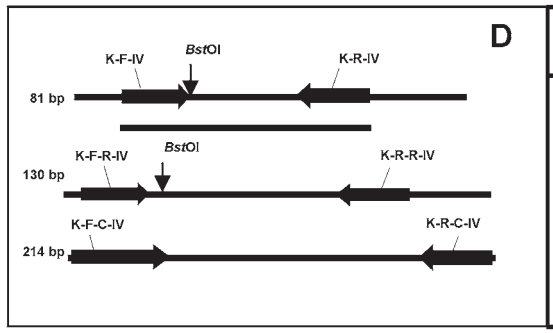
Round of PCR	Name of primer	Primer sequence (5´ – 3´)	Annealing temperature [°C]	Restriction endo-nuclease
1st	K-F-I	GAATATAAACTTGTGGTAGTTGGACCT	55	*Mva*I
	K-R-I	TCATGAAAATGGTCAGAGAAACC		
2nd	K-F-I	GAATATAAACTTGTGGTAGTTGGACCT		
	K2-R-I	TCAAAGAATGGTCCTGACC		



Round of PCR	Name of primer	Primer sequence (5´ – 3´)	Annealing temperature [°C]	Restriction endo-nuclease
1st	K-F-II	ACTGAATATAAACTTGTGGTAGTTGGACCT	56	*Bst*OI
	K-R-II	TCAAAGAATGGTCCTGGACC		
2nd	K-F-II	ACTGAATATAAACTTGTGGTAGTTGGACCT		
	K2-R-II	TAATATGCATATTAAAACAAGATTTACCTC		



Round of PCR	Name of primer	Primer sequence (5´ – 3´)	Annealing temperature [°C]	Restriction endo-nuclease
1	K-F-III	GACTGAATATAAACTTGTGGTAGTTGGACCT	56	*Mva*I (*Bst*OI)
	K-R-III	CCTCTATTGTTGGATCATATTCGTCC		



Round of PCR	Name of primer	Primer sequence (5´ – 3´)	Annealing temperature [°C]	Restriction endo-nuclease
1	K-F-IV	ATAAACTTGTGGTAGTTGGACCT	58	*Bst*OI
	K-R-IV	CGTCCACAAAATGATTCTGA		
	K-F-R-IV	GTACACATGAAGCCATCGTATA		
	K-R-R-IV	CCACTTGTACTAGTATGCCTTAAG		
	K-F-C-IV	CTAGAACAGTAGACACAAACCA		
	K-R-C-IV	GATTTTGCAGAAAACAGATC		

Figure 1. Schema of used methods and appropriate primers. A – method I, B – method II, C – method III, D – method IV. A: Sequence DNA of primers used in method I. Forvard primer K-F-I creates recognition site for restriction endonuclease *Mva*I (or *Bst*OI).

8.3), 4 mmol.l^{-1} $MgCl_2$. Reaction conditions were 94 °C, 2 min, 35 cycles (58 °C for 1 min, 92 °C for 20 s).

Gel electrophoresis. Final products after PCR (in Method IV), after digestion (in Method III) and after second digestion (in Method I and II) were visualized by electrophoresis on an agarose gel (2% TypeII, Sigma, 1% Type IX Low Gelting Temperature, Sigma) stained with ethidium bromide (0.5 mg.ml^{-1}) and photographed on UV transilluminator using ULTRA.LUM Panasonic CCD Camera.

Sequence determination of PCR amplicons. In several experiments, as described in the results, the PCR based detection of mutation was confirmed by a direct sequencing of exon 1 of the K-*ras* gene. PCR amplicons for sequencing were purified using the QIAquick PCR Purification Kit (QIAGEN, GmbH, Germany). DNA was performed using the ABI PRISM BigDye^R Terminator v3.1 Cycle Sequencing Kit (Perkin-Elmer, Applied Biosystems, Foster City, Calif., USA) with the Genetic Analyzer ABI 3100 DNA sequencer.

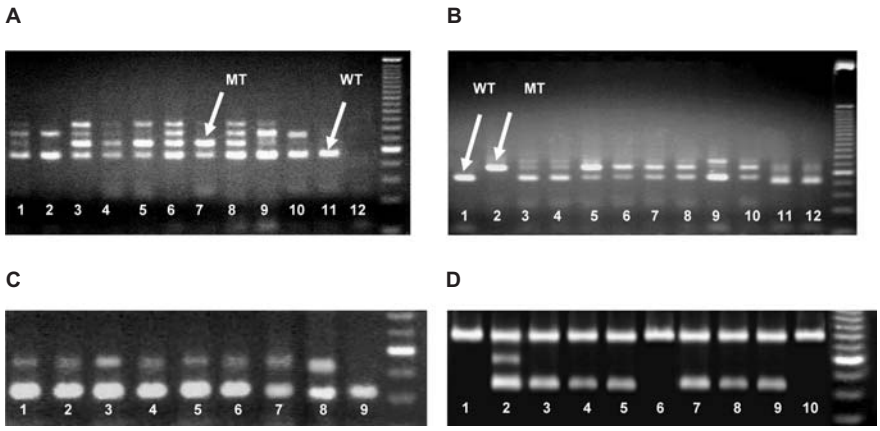


Figure 2. Agarose gel electrophoresis (3%) by using: A) method I after 2nd round of PCR and cleaving with *Mva*I. Lines: 1, 3–9 indicate presence of mutation in DNA samles; 2, 10, samples without mutation, 11 – positive control of WT, 12 – negative control (no DNA template). 139 bp indicate fragment with mutation, 113 bp indicate fragment without mutation. B) method II after 2nd round of PCR and cleaving with *Bst*OI. Lines: 1 – negative control sample of WT, 2 – control sample of MT, 3–8, 10–12 indicate presence of mutation in DNA samles; 9 – sample without mutation, 135 bp indicate non cleaved fragment with mutation, 106 bp indicate fragment without mutation. C) method III after PCR and cleaving with *Mva*I. Lines: 1–7 indicate presence of mutation in DNA samles; 8 – control sample with mutation (MT - 109 bp fragment), 9 – control sample without mutation (WT – 77 bp fragment). D) method IV. Lines: 1, 2 – control DNA without mutation (WT), + with BstOI, – without *Bst*OI; lines: 3, 4, 5, 7, 8, 9 indicate presence of mutation in DNA samles (81 bp fragment); 6, 10 – samples without mutation.

Amplified DNA fragments were sequenced in both orientations using 1 pmol.μl^{-1} of the appropriate oligonucleotide primers. The sequence data were analyzed with the Sequencing Analysis 3.1 and Sequencing Navigator 1.0.1 programs included in the ABI PRISM software package (Perkin-Elmer, Applied Biosystems). Sequence comparison was subsequently carried out using the BLAST 2.1 software program and the GenBank database. For the design of primers the sequence of Human cellular c-Ki-ras 2 proto-oncogene (K-*ras*, GeneID: 3845), exon 1 of gene was used.

Comparison of the methods for sensitivity and duration. As the optimal detection method has to be highly sensitive, and fast, we tested all methods for these parameters.

Testing the sensitivity. We tested all four methods for the sensitivity to detect mutated K-*ras* codon 12 allele in redundancy of healthy alleles. Serial dilutions of mutant alleles in excess of healthy alleles were prepared from positive (MT) and negative (WT) stock samples. The stock samples were amplified from DNA extracted from positive controls and from blood of healthy volunteers using following primers: Forward primers:
WKF 5'ATGACTGAATATAAACTTGTGG 3', MKF 5'AAACTTGTGGTAGTTGGAGCT GAT 3' and reverse primer: KR-I 5'TCATGAAAATGGTCAGAGAAACC 3'. We amplified the fragments, measured exact concentration of DNA and verified the sequence of codon 12 of K-*ras* gene by sequencing as described above. (GGT – healthy allele, GAT – mutated allele). Serial dilutions consisted of mixing one aliquot mutated DNA to serial multiple of healthy DNA (without mutation) (1:10–1:10^8). In the second step, the dilutions were precised to 1:2x10^4, 1:3x10^4, 1:4x10^4, 1:5x10^4, 1:6x10^4, 1:7x10^4, 1:8x10^4, 1:9x10^4, 1:10^5. Fifty ng of DNA from each of the prepared diluted samples was then a template in every analyzed method to establish its detection threshold. The experiment was repeated 3 times and the average achieved sensitivity was calculated.

Analysing the duration. To calculate the minimal duration of all four methods we considered the duration of all steps in particular protocol what usually included the isolation of DNA from the biological material, time spent with preparing and completing the PCR reactions, duration of restriction step and gel electrophoresis. Duration of each step was measured and final duration was calculated.

Testing the optimal method on a study population of patients with malignancies. To test the method selected as optimal for the use in routine clinical practice we analyzed tumor samples from 59 patients (29 males and 30 females, average age 69 years) with malignancies of the upper gastrointestinal tract. The study group included 17 patients with pancreas carcinoma, 4 patients with carcinoma of the duodenal ampulla, 10 patients with carcinoma of the biliary tract, 10 patients with carcinoma of the gallbladder and 18 patients with gastric carcinoma. All patients were diagnosed in one centre (Dept of Internal Medicine I, University Hospital, Bratislava) over the past 5 years. Control cohort consisted of mucosal biopsy samples from 20 patients with uncomplicated peptic ulcer. The clinical diagnosis was made by an experienced gastroenterologist (ID). All patients with malignancies were operated on and the diagnosis was confirmed from the tissue samples by an experienced pathologist (PB). Biopsy samples were evaluated also by an experienced pathologist (PB).

DNA was extracted from paraffin embedded tissue/biopsy samples using the QIAamp spin columns (QIAamp DNA Mini Kit, Qiagen, Hilden, Germany) according to the manufacturer's instruction. In brief, not more than 25 mg of paraffin-embedded tissue was placed into a 2 ml microcentrifuge tube. Removal of paraffin was ensured by adding and mixing 1200 µl of xylene for 5 min. After a centrifugation at 13 000 rpm for 5 min at a room temperature, the supernatant was carefully removed and 1200 µl of ethanol (96–100%) was added to the pellet. The mixture was centrifuged at

13 000 rpm for 5 min at a room temperature. In the next step ethanol was carefully removed. To remove residual xylene completely, the procedure was repeated once again. The open microcentrifuge tube was incubated at 37 °C for 15 min until ethanol evaporation. The lysis with the proteinase K was performed for 3 hours at 56 °C. DNA was then extracted with the QIAamp spin columns according to the manufacturer's instruction. Concentration of the resulting DNA was measured. Method I was used to detect the presence of mutation in the codon 12 of K-*ras* gene and the result of genotyping was confirmed by sequencing on ABI 3100 DNA sequencer as described above.

Results

Sensitivity analysis. Serial dilutions of mutant DNA in excess of healthy alleles were used for testing the sensitivity of these methods. Method I showed to be the most sensitive one and using it we were able to detect 1 mutant allele in a presence of $1x10^5$ healthy alleles in all three repeated experiments. The sensitivity of method II and III was $1:5x10^4$, and, $1:1x10^4$, respectively and could be repeated consistently in all three experiments. The sensitivity of method IV was $1:8x10^4$ in the first experiment, however in the repeated experiments the results were inconsistent. This method showed to be not reliable and even in several low dilutions we missed the positive result although even at high dilutions it was positive again. This unreliability is probably related to sensitivity of the restriction enzyme *Bst*OI to reaction conditions. However this unpredictable outcome makes this method unreliable for the clinical use. Comparison of the sensitivity of the four methods is summarized in Table 2.

Duration analysis. Average duration for performing particular method is summarized in Table 1. The most time consuming part of the protocol is the isolation of DNA. Standardized commercial isolation kits enable simple procedures, simultaneous processing of multiple samples, and give standard yields of pure DNA for direct amplification. Two-round PCR methods with consecutive restriction were more time consuming. Duration of PCR including preparation of reaction mix was 2.5 hours, duration of one restriction step was 3 hours. In method IV the restriction endonuclease *Bst*OI was present directly in the PCR reaction. Average duration of the electrophoresis was 1 hour. Taken together, methods I and II took approximately 17 hours to perform and 2 working days were needed. Method III can be accomplished in approximately 11.5 hours and can be done in one and half day. Finally the fastest method was the method IV which can be performed in 8.5 hours i.e. completely within one working day.

Incidence of K-ras mutation from upper GIT malignancies. Using method I, which seems to have optimal parameters for clinical use we examined a cohort of 59 patients with upper GIT tumors for the presence of codon 12 mutations of K-*ras* gene. We diagnosed a K-*ras* codon 12 mutation in 14 of 17 (82.4%) samples from pancreatic carcinoma, in 3 of 4 (75%) samples from duodenal ampullary carcinoma, in 8 of 10 (80%) samples from biliary tract carcinoma, in 3 of 10 (30%) samples from gall bladder carcinoma and in 10 of 18 (55.6%) samples from gastric tumors. K-*ras* mutation was not detected in any of the samples obtained from control cohort with peptic ulcer (Fig. 3).

The results of mutation testing were consequently confirmed by an independent sequencing of the whole exon 1 of the K-*ras* gene, as shown in Figures 4, 5 and 6. The sequencing results showed a 100% concordance with genotyping using ME PCR base method I in identification of the mutation status of the sample and confirmed thus the 100% accuracy of method I in the diagnosis of K-*ras* codon 12 mutations.

Table 1. Duration of four analyzed methods

Type of analysis	Method I	Method II	Method III	Method IV
	Duartion of analysis [h]			
Isolation of DNA	5	5	5	5
1st PCR	2.5	2.5	2.5	2.5
Restriction	3	3	3	–
2nd PCR	2.5	2.5	–	–
Restriction	3	3	–	–
Electrophoresis	1	1	1	1
Summary	17	17	11.5	8.5

Table 2. Final summarization of testing methods

Characteristics	Method I	Method II	Method III	Method IV
Sensitivity (detection of mutant DNA in excess of healthy DNA)	$1 : 1\times10^5$	$1 : 5\times10^4$	$1 : 1\times10^4$	$1 : 8\times10^4$
Duration of analysis [h]	17	17	11.5	8.5

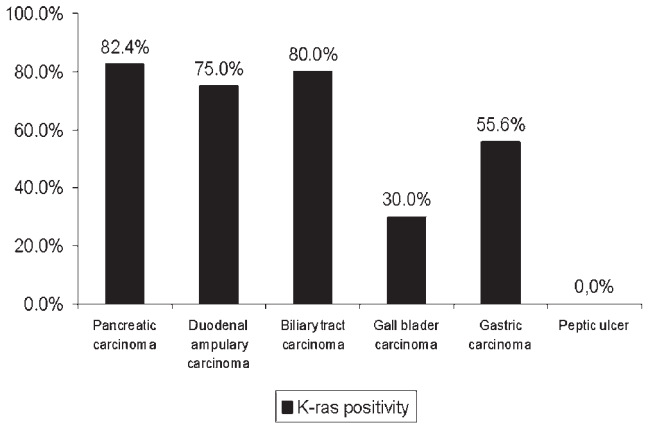


Figure 3. Prevalence of K-*ras* mutations among the studied malignancies of the upper gastrointestinal tract.

Discussion

Detection of early stages of malignant tumours by molecular genetic analyses holds promise in clinical oncology. More effective screening techniques are urgently needed to improve poor prognosis of the disease [3].

Point mutations in K-*ras* are particularly helpful in the diagnosis of several gastrointestinal malignancies and pancreatic carcinoma for several reasons. Mutations in this oncogene are usually limited to just one codon. Point mutations are sufficiently frequent in pancreatic adenocarcinoma suggesting that this gene may be a sensitive marker for the diagnosis of pancreatic carcinoma and finally, mutations in the K-*ras* oncogene are present in pancreatic carcinoma *in situ* which makes early detection of the tumor possible, thereby increasing possibility of cure.

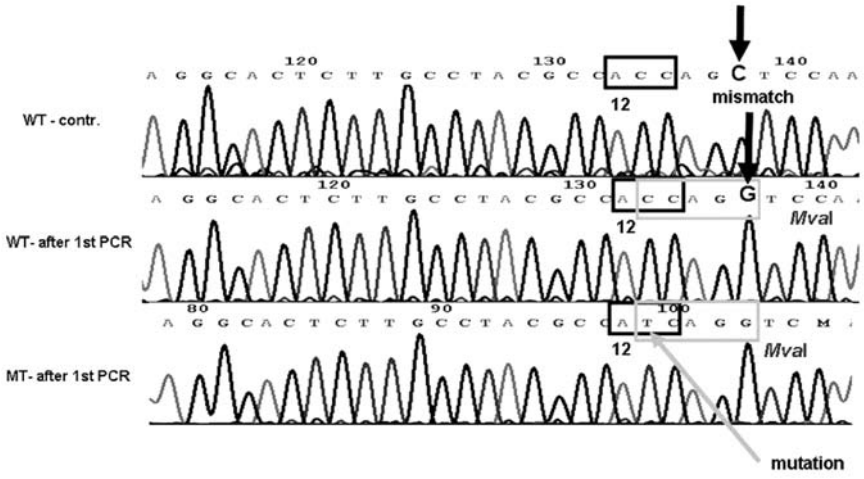


Figure 4. DNA sequence analysis of WT and MT samples. Creation of suitable sequence for restriction endonuclease *Mva*I after 1st round of PCR. In black rectangle is codon 12, in grey rectangle is restriction site for *Mva*I.

The aim of our work was to develop a sensitive, reliable, fast, and inexpensive PCR technique to detect the K-*ras* codon 12 mutations, which would be suitable for a routine clinical use. Various methods for the detection of mutant K-*ras* alleles in the presence of an excess of healthy alleles have been described so far. Detection of mutations is achieved by e.g.: "allele"-specific hybridization, utilization of restriction enzyme during or after PCR reaction, by SSCP (single strand conformational polymorphism) or assessment of primary nucleotide sequence. Detection of rare mutant alleles can also be achieved using so called mutant enriched PCR (ME PCR), which requires either multiple rounds of PCR with intermittent selective restrictions of healthy alleles or using restriction enzyme during the PCR reaction, i.e. so called restriction endonuclease-mediated selective (REMS)-PCR, which achieves the same result in single round of PCR [15–17]. The SAMA ("stencil-aided mutation analysis") pre-PCR method has been developed on a similar basis [22]. Another method achieves an enrichment of mutant alleles by removing of the wild-type alleles by differential hybridization to complementary oligonucleotides [23]. Another methods like PCR-SSCP (single-strand conformational polymorphism) [24], dot blot hybridization and immunohistochemical analysis have been described, as well [25]. Two types of quantitative assay kits for K-*ras* mutations, PCR-preferential homoduplex formation assay (PHFA) and enriched PCR and enzyme-linked mini-sequence assay (ELMA) were recently developed [26]. However, due to complexity and/or high costs these methods are so far not very suitable for routine clinical use. Due to relative simplicity and reported good sensitivity we selected the ME PCR approach as the most promising one. We subsequently designed and optimized four PCR techniques based on the principle of

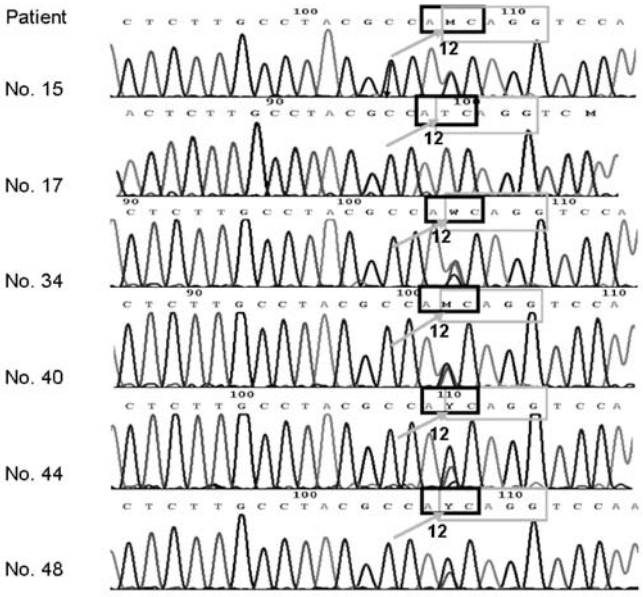


Figure 5. Results of the reverse sequencing of the 2nd round PCR fragments. Arrows indicate mutation at position 2 of the K-*ras* codon 12. In rectangles are codon 12 and created sequence for restriction endonuclease *Mva*I, respectively. Aminoacid glycine was changed in patient No.15 and 34 to methionine, No. 18 to threonin, No. 34 to tryptophan, No. 44 and 45 to tyrosine. In black rectangle is codon 12, in grey rectangle is restriction site for MvaI. (Y = A, T; M = A, C; W = A, T).

mutant enrichment during the detection process [27]. We tested two various endonucleases and several various protocols of ME PCR, as well as the REMS PCR approach. The novelty of the optimized method I is that we designed the primers in such a way that two restriction sites in the wild

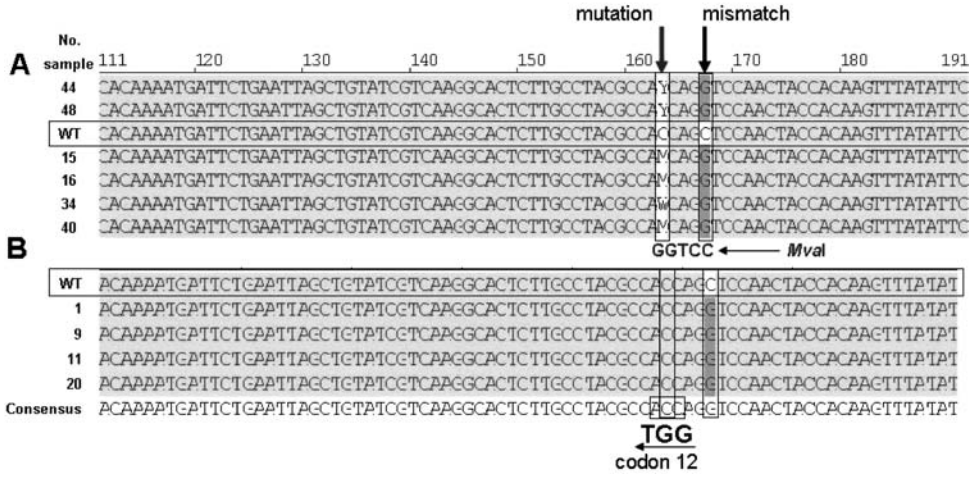


Figure 6. Alignment of sequences of samples after 2nd round PCR. A – samples with mutation – white column (reverse seq.), B – samples without mutation. In horizontal rectangle is WT sequence. (Y = A, T; M = A, C; W = A, T)

type allele occur, secondly we incorporated two very intensive restriction steps into the protocol with 3 IU of restriction enzyme per examination. With this method we were able to detect point mutations of K-*ras* codon 12 with a sensitivity of $1{:}1x10^{-5}$.

If only one restriction site contains primer for second round of PCR as was used in method II, sensitivity decreased by a factor of 10. Comparing these two methods, elimination of healthy fragments (mutant enrichment) seems to be less effective and may be explanation for lower sensitivity. This method is due to the higher cost of *Bst*OI also a bit more expensive and offers thus no advantage over method I. Some investigators have used one-step ME PCR method to detect K-*ras* mutations [28, 18]. Our method III was also a one-step PCR amplification. Compared to method I the detection of mutant alleles was about 5 times less sensitive. The conclusion was that just one round of mutant enrichment is not satisfactory if high sensitivities are desired.

Method IV was based on the REMS-PCR and was very promising due to a very fast and inexpensive protocol [17]. REMS-PCR systems contain primers for amplification of both diagnostic and control amplicons, the presence or absence of which confirms specific sequences at the diagnostic locus and efficient assay function, respectively. Development of REMS-PCR protocols requires identification of enzymes and specific buffer conditions that are capable of sustaining enzymatic activity despite repeated cycles of thermal denaturation. Although initially the sensitivity was promising, when we repeated the experiments with serial dilutions, the results were inconsistent showing an unpredictable and unreliable detection of K-*ras* mutations. The explanation is probably the incomplete *Bst*OI digestion during the PCR cycles [16].

Method I showed a sensitivity of detecting 1 mutant allele in a background of 10^5 wild-type alleles which is excellent, compared to previous reports [29, 30, 21]. Having this high sensitivity gives rise to the possible mutagenesis during the PCR itself, because of incorrect reading of nucleotides during the amplification process [31]. It is therefore imperative to use high fidelity polymerase if extreme sensitivities are desired. Even higher sensitivity (10^7) was achieved by FERNANDEZ-VEGA et al [32] by using a method based on mutant allele-specific amplification (MASA).

To test the accuracy of method I we performed analysis of codon 12 K-*ras* status in 59 samples on presence mutation in gene K-*ras* codon 12. Mutations were present in 14 of 17 (82.4%) cases with PC what is in accordance with results published previously and this finding has been extensively discussed in the literature [7, 33–36]. Moreover, we were able to detect mutations in codon 12 also in 80% of samples from biliary tract carcinoma, in 30% from gallbladder carcinoma and in 55.6% from gastric carcinomas. All these tumors might shed malignant cells into the duodenal fluid, to collected pancreatic fluid, blood as well as stool, what needs to be considered by evaluating the specificity of particular sampling method for the diagnosis of pancreatic cancer [37–39].

What is the clinical utilization of our modified ME PCR method? First of all very sensitive detection of the K-*ras* mutations enables an early diagnosis of pancreatic cancer in various biological samples such as duodenal or pancreatic fluid, fine-needle aspirates, blood or even stool [4, 19, 40–42]. Another area of the use is the differential diagnosis of unclear pancreatic lesions or differentiating between chronic pancreatitis and pancreatic carcinoma. It would be possible to detect micrometastases from dissected lymph nodes with potential prognostic value. K-*ras* mutations detection can be used as screening tests that detect exfoliated tumor cells or soluble tumor DNA in body fluids such as blood, stools (colorectal and pancreatic cancer), or sputum (lung cancer); and in tests that may assist clinicians in tailoring therapy. Some of these applications require detection of small numbers of tumor cells present in a 10^3–10^5-fold excess of healthy cells [28, 32]. The sensitivity of our modified mutant enriched PCR method would meet even the hardest requirements on a reliable diagnostic tool.

So far, the attempts for sensitive method for detection of PC gave only partially satisfying results. Even in PC with proven *K-ras* codon 12 mutations, detection sensitivity from the duodenal fluid reached only about 25%, being mostly

positive only in the periampullary carcinomas [43]. Sensitivity rises to 55–100% if pure pancreatic juice is collected [44–47]. Detecting K-*ras* from stool is technically very difficult although sensitivity of 57% [48] and from 42% to 48% [49] has been reported. Detecting K-*ras* from blood is even less sensitive [50, 51]. Rather low sensitivity for detection of K-*ras* mutations from various biological materials even in K-*ras* positive tumors can have several reasons. First, the malignant cell might not be shed into the particular material or alternatively the detection technique is not sensitive enough. It is also possible that DNA from malignant cells is cleaved e.g. by the bile acids or bacterial endonucleases. Improving the sensitivity of detecting K-*ras* mutations is an extremely important issue and further studies are necessary to tackle it [52].

To conclude, we optimized the mutant enriched PCR method for extremely sensitive detection of K-*ras* codon 12 mutations in an excess of healthy alleles. Compared to other methods it is sensitive, reliable, inexpensive and can be performed relatively fast. Further studies are necessary to determine optimal biological material sampling for the diagnosis of pancreatic cancer.

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