

## LNA clamped PCR: A specific method for detection of Ki-ras gene mutations in patients with sporadic colorectal carcinomas

Beránek M.<sup>1</sup>, Jandík P.<sup>2</sup>, Šácha M.<sup>3</sup>, Rajman M.<sup>3</sup>, Sákra L.<sup>3</sup>, Šturm F.<sup>4</sup>, Soudková E.<sup>4</sup>, Živný P.<sup>1</sup>, Havlíček K.<sup>3</sup>

<sup>1</sup>*Institute of Clinical Biochemistry and Diagnostics, Faculty of Medicine Hradec Králové, Charles University Prague and University Hospital Hradec Králové*

<sup>2</sup>*Department of Surgery, Faculty of Medicine Hradec Králové, Charles University Prague and University Hospital Hradec Králové*

<sup>3</sup>*Department of General Surgery, Regional Hospital Pardubice*

<sup>4</sup>*Department of Clinical Biochemistry, Regional Hospital Pardubice*

### SUMMARY

**Objective:** The aim of this study is to demonstrate possibility of detection of Ki-ras gene mutations using PCR clamped with a synthetic nucleotide analogue, locked nucleic acid (LNA).

**Setting:** Institute of Clinical Biochemistry and Diagnostics, Faculty of Medicine Hradec Králové, Charles University Prague and University Hospital Hradec Králové; Department of Surgery, Faculty of Medicine Hradec Králové, Charles University Prague and University Hospital Hradec Králové; Department of General Surgery, Regional Hospital Pardubice; Department of Clinical Biochemistry, Regional Hospital Pardubice.

**Material and Methods:** We examined seventy-three colorectal carcinomas (CRC) and plasma samples of twenty CRC patients for the presence of Ki-ras gene mutations in codon 12. PCR reactions were performed in Roche LightCycler System with hybridization probes and clamping LNA molecules.

**Results:** Selective amplification of mutant gene sequences revealed Ki-ras gene changes in 22 cases (30%) of CRC. DNA sequencing confirmed GGT-GAT transition in 13 patients, GTT transversion in 8 patients, and AGT transition in one case. In our small experimental group of plasma DNA samples, however, we did not find any mutation in the Ki-ras gene. Most examined carcinomas were classified T2N0M0-T3N0M0, one carcinoma was T3N1M0. The detection limit of LNA-clamped PCR was about 2ng.

**Conclusion:** We consider LNA-clamped real-time PCR to be a fast alternative method for specific analysis of the Ki-ras gene in colorectal carcinomas. The reliability determined in CRC tissues was comparable with the enriched PCR/RFLP and nucleotide sequencing.

**Key words:** Ki-ras gene, point mutations, LNA, DNA, PCR, colorectal carcinoma, plasma.

### Introduction

The incidence of colorectal carcinoma (CRC) in the Czech Republic is about 95/100,000 in men and 63/100,000 in women [1]. Sporadic CRCs develop from benign colon adenomas without any signs of Mendelian inheritance. A multifactorial and polygenic background of colorectal carcinogenesis includes multiple genetic changes in tens of proto-oncogenes, tumour suppressor genes and mismatch repair genes [2, 3].

Somatic mutations in Kirsten-ras-2 gene (Ki-ras gene; location 12p12.1) were found in 30–50 percent of CRC cases and are considered a crucial step in malignant transformation of colon adenomas to adenocarcinomas. Specific mutations in codon 12 (exon I) account for 80–90% of Ki-ras gene alterations [2, 4].

A lot of molecular techniques for detection of genetic changes in the Ki-ras gene have been published previously, however, somatic mosaicism present in earlier steps of tumourigenesis has predestined development of more specific experimental approaches. One of them includes extraction and separation of

mutant gene sequences from the correct wild-type ones followed by PCR amplification of the gene fragments contained in the supernatant. The removal is performed using solid surface-linked oligonucleotides complementary towards the wild-type allele of the Ki-ras gene [5]. The second approach is based on extraction of DNA without a separation step but with more specific amplification of mutant fragments. The protocol includes two-step PCR with pre-digestion of the wild-type gene sequences before the second step by restriction endonucleases [6, 7]. Both the described processes are very time consuming.

A novel analytical approach for specific amplification of mutant fragments using blocking (clamping) wild-type sequences has been published. For the clamping the authors used synthetic DNA analogues (peptide nucleic acids, PNA) with a higher affinity towards complementary nucleic chains than their corresponding natural forms [8]. The aim of this study is to demonstrate possibility of detection of Ki-ras gene mutations using PCR clamped with a newer synthetic nucleotide analogue, locked nucleic acid (LNA).

## Material and Methods

After institutional approval we examined fresh specimens of primary colorectal carcinomas of 73 patients who underwent colon or rectum surgery at the Charles University Hospital Hradec Králové or Regional Hospital Pardubice (40 men and 33 women, mean age 63 years, range 42–88 years). Histological grading and staging according to the Dukes' and TNM classifications characterized each CRC. The specimens were prepared from the central region of obtained tumour tissues and stored at  $-70^{\circ}\text{C}$  prior to analysis. Collections and all described analytical procedures were performed with informed consent of the patients. DNA was extracted by the standard phenol/chloroform procedure after digestion of biological specimens with proteinase K at  $56^{\circ}\text{C}$  for 16 hours.

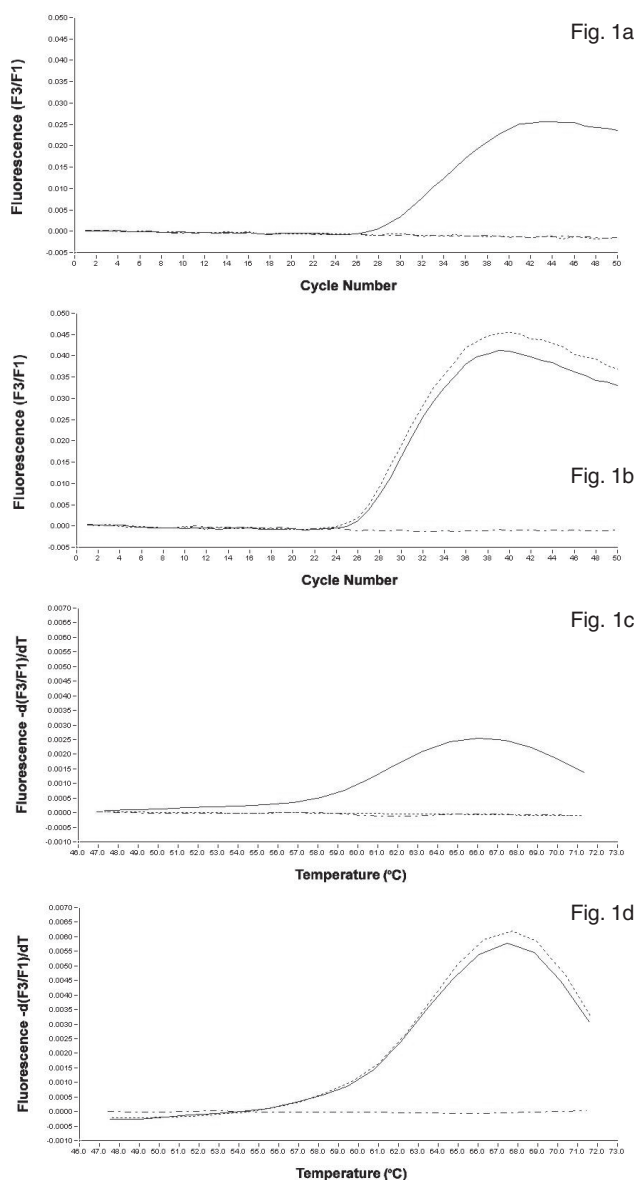
Plasma samples (900  $\mu\text{l}$ ) were prepared from peripheral venous blood (before and after carcinoma surgery) and from portal vena (during surgery) of twenty CRC patients with their informed consent as above. DNA was extracted using commercial microcolumns (QIAamp Blood Mini Kit, Qiagen, Hilden, Germany) [9].

PCR reactions were performed in Roche LightCycler System (Roche Diagnostics, Germany) in glass capillaries (10  $\mu\text{l}$ ). Each capillary contained 60 ng of DNA, 1  $\mu\text{l}$  of the LightCycler DNA Master Hybridization Mixture (Roche), 5mM  $\text{MgCl}_2$ , 0.3  $\mu\text{M}$  anchor and sensor probes, 0.1  $\mu\text{M}$  LNA oligomers, and 0.5 mM each of two primers. After initial denaturation (10 min at  $95^{\circ}\text{C}$ ), real-time PCR was run for 45 cycles consisting of 5 sec denaturation at  $95^{\circ}\text{C}$ , 10 sec annealing at  $60^{\circ}\text{C}$ , and 7 sec elongation at  $72^{\circ}\text{C}$ . Both primers (5'-AGG CCT GCT GAA AAT GAC TG-3'; 5'-GGT CCT GCA CCA GTA ATA TGC A-3'), probes (anchor: 5'-CGT CCA CAA AAT GAT TCT GAA TTA GCT GTA TCG TCA AGG CAC T-FL-3'; sensor: 5'-LC RED705-TTG CCT ACG CCA CAA GCT CCA A-PH-3'), and LNA oligomer (5'-CCT ACG CCA CCA GCT CC-PH-3') were synthesized in TIB MOLBIOL, Berlin, Germany according to the original design of Chen et al. [10]. As wild-type controls we used biopsy specimens of morphologically normal colonic mucosa from subjects without colorectal tumours. Performing PCR without LNA molecules in the mixture we checked amplifiability of our DNA extracts. The enriched PCR/RFLP method [6] and nucleotide sequencing with ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit and ABI PRISM 310 Genetic Analyser (Applied Biosystems, Foster City, USA) were performed to confirm and identify the type of mutations.

## Results and Discussion

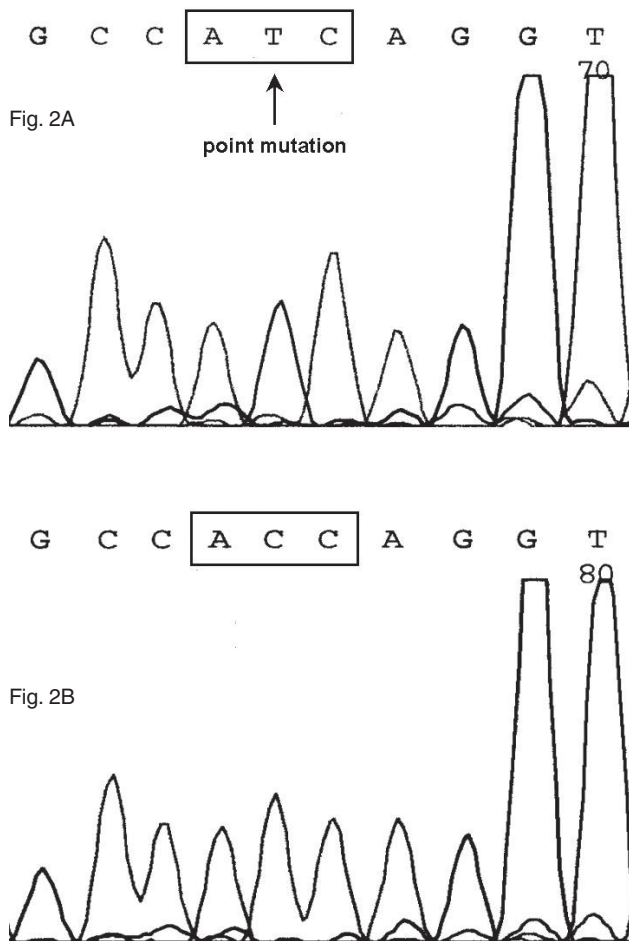
Real-time PCR without the presence of LNA successfully amplified PCR products from all tumour and plasma DNA extracts. Selective amplification of mutant sequences after addition of clamping LNA molecules into reaction mixtures revealed Ki-ras gene changes in 22 cases (30 %) of CRC. Typical amplification and melting

curves for mutant and unaffected Ki-ras genes are demonstrated in Fig. 1. Both enriched PCR/RFLP and DNA sequencing confirmed the presence of mutations (Fig. 2). Thus, all three methods provided the same analytical results. Despite we used the sensor probe primarily designed for the cysteine-specific coding sequence (GGT $\rightarrow$ TGT); real-time PCR detection of substitutions GAT (code for aspartate), AGT (serine), and GTT (valine) in codon 12 is also possible [10]. Confirmatory DNA sequencing revealed GAT transition in 13 patients, GTT transversion in 8 patients, and AGT transition in one case. Twenty-one of the mutated CRC specimens (95 %) carried a genetic change in the second position of codon 12. The GTT transversion implicates a poor prognosis of CRC, and a higher risk of recurrence and death [11]. Conversely, the GAT transition was predominantly found in earlier grades of CRC [12].



**Fig. 1.** Amplification of mutant (full line) and wild-type (dashed line) alleles in Ki-ras gene codon 12; negative control (water) was included (dashed and dotted line)

Real-time PCR with (1a) or without (1b) clamping LNA molecules; melting curve analysis with (1c) or without (1d) LNA in the PCR mixture.



**Fig. 2.** Sequencing data (antisense chain) on mutant (2A) and unaffected (2B) Ki-ras gene alleles. The frame marks Ki-ras gene codon 12. The C→T point mutation (corresponding with G→A genetic change in the sense chain) in the second position of the codon is indicated by the up-arrow.

What is the genetic background of the remaining fifty-one Ki-ras-negative patients? There are several hypothetical answers. The first of them considers existence of other mutant sites present outside of the Ki-ras PCR product sequence. However, such genetic events are quite rare in sporadic colorectal carcinomas. Taken together, codons 12 and 13 make over 90% of all mutations obtained in the Ki-ras gene. The changes at codon 61 are found only at about 2% of cancer patients [13]. Moreover, single-point mutations except the mentioned critical “hot spots” probably do not lead to significant alterations in the tertiary structure of the highly conserved N-terminal domain of the coded p21<sup>ras</sup> protein, and do not convert the normal Ki-ras gene into the oncogene form.

The second possible answer takes into account incorporation of p21<sup>ras</sup> protein into important intracellular signal cascades where structural changes appearing in other participating proteins downstream or upstream of p21<sup>ras</sup> could form the similar clinical manifestation like Ki-ras gene mutations. The potential “risk targets” might be genes for Raf protein kinase activating several transcription factors (e. g., Fos, Jun) through the mitogen-activating protein kinase (MAPK) cascade, phosphatidylinositol 3 kinase, protein kinase C, cera-

mid-activated protein kinase, neurofibromin, or genes for molecular chaperons hsp90 or p50. Overproduction of transcription factors would consequently affect the expression of genes included in the control of cell proliferation or differentiation.

The third possible answer combines results of *in vitro* and clinical experiments dealing with biochemical cooperation between p21<sup>ras</sup> protein and products of other oncogenes or tumour suppressor genes in malignant transformation of colon cells. It has been recognized that the development of colonic adenomatous polyps and sporadic carcinomas involves activation of many proto-oncogenes (e. g., myc, src, myb, erb-2, neu, trk, etc.), loss or inactivation of tumour-suppressor genes (p53, APC, MCC, DCC, DPC4, etc.), and alterations in the DNA mismatch repair genes (hMSH2, hMLH1). A number of different mechanisms in a complex and not fully understood fashion alter these genes and encoded proteins resulting in abnormal cell growth, division, communication, and suppression of normal differentiation of cells.

The second part of the study dealt with finding circulating cancer cells in the blood of Ki-ras-mutation-positive CRC subjects. Published papers reported that the frequency of mutations in plasma of CRC patients closely corresponded with the data on tumours [14, 15, 16]. In our small experimental group of plasma DNA samples, however, we did not find any mutation in the Ki-ras gene. We explain that difference by the fact that most our carcinomas were classified T2N0M0-T3N0M0. One CRC mutated in the Ki-ras gene was T3N1M0. The sensitivity of LNA-clamped PCR was determined using serial dilutions of mutant DNA samples received from CRC. The limit of detection was about 2ng. Thus, DNA controls containing 3–5ng of mutant alleles inserted into each analytical series allow us to exclude low sensitivity as a factor causing the negativity of the plasma DNA samples.

In agreement with the paper that used PNA synthetic oligomers in combination with LightCycler hybridization probes for detection of Ki-ras mutations in malignant biliary tumours [10], we consider LNA-clamped PCR to be a fast alternative method for specific analysis of the Ki-ras gene in colorectal carcinomas. The reliability determined in CRC tissues was comparable with the enriched PCR/RFLP and nucleotide sequencing. Because of the low limit of detection, the method can be used for early diagnostics of the genetic changes in tumour tissue, stool, or plasma of CRC patients. DNA sequencing remains a gold standard for confirmation and more detail characterization of the found mutations.

## References

1. Zdravotnická ročenka České republiky 2004. ÚZIS : Praha 2005, s. 56–58.
2. Breivik, J., Lothe, R.A., Meling, G. I., Rognum, T. O., Borresen-Dale, A. L., Gaudernack, G. Different genetic pathways to proximal and distal colorectal cancer influenced by sex-related factors. *Int. J. Cancer*, 1997, 74, p. 664–669.

3. **Bohr, V. A.** DNA repair fine structure and its relations to genomic instability. *Carcinogenesis*, 1995, 16, p. 2885–2892.
4. **Vogelstein, B., Fearon, E. R., Hamilton, S. R. et al.** Genetic alteration during colorectal-tumor development. *N. Engl. J. Med.*, 1988, 319, p. 525–532.
5. **Nollau, P., Fischer, C., Tschentscher, P., Wagener, C.** Enrichment of mutant alleles by chromatographic removal of wild type alleles: a new principle for the detection of alleles with unknown point mutations at excess of wild type alleles. *Clin. Chem. Lab. Med.*, 1999, 37, p. 877–881.
6. **Beránek, M., Bureš, J., Palička, V., Jandík, P., Langr, F., Nejedlá, E.** A relationship between K-ras gene mutations and some clinical and histologic variables in patients with primary colorectal carcinoma. *Clin. Chem. Lab. Med.*, 1999, 37, p. 723–727.
7. **Beránek, M., Jandík, P., Bureš, J., Rejchrt, S., Dědič, K., Palička, V.** Occurrence of point mutations in codon 13 of the K-ras gene in colorectal tumors. *Klin. Biochem. Metab.*, 2002, 10, s. 146–150.
8. **Thiede, C., Bayerdorffer, E., Blasczyk, R., Wittig, B., Neubauer, A.** Simple and sensitive detection of mutations in the ras proto-oncogenes using PNA-mediated PCR clamping. *Nucleic Acids Res.*, 1996, 24, p. 983–984.
9. **Beránek, M., Vlčková, J., Hypiusová, V., Živný, P., Palička, V.** Comparison of various methods used for extraction of cell-free genomic DNA from human plasma. *Klin. Biochem. Metab.*, 2006, 14, s. 221–224.
10. **Chen, C. Y., Shiesh, S. C., Wu, S. J.** Rapid detection of K-ras mutations in bile by peptide nucleic acid-mediated PCR clamping and melting curve analysis: comparison with restriction fragment length polymorphism analysis. *Clin. Chem.*, 2004, 50, p. 481–489.
11. **Andreyev, H. J. N., Norman, A. R., Cunningham, D. et al.** Kirsten ras mutations in patients with colorectal cancer: the RASCAL II study. *Brit. J. Cancer*, 2001, 85, p. 692–696.
12. **Moerkerk, P., Arends, J. W., van Driel, M., de Bruine, A., de Goeij, A., Ten Kate, J.** Type and number of Ki-ras point mutations relate to stage of human colorectal cancer. *Cancer Res.*, 1994, 54, p. 3376–3378.
13. **Morris, R. G., Curtis, L. J., Romanowski, P. et al.** Ki-ras mutations in adenomas: a characteristic of cancer-bearing colorectal mucosa. *J. Pathol.*, 1996, 180, p. 357–363.
14. **Kopreski, M. S., Benko, F. A., Kwee, C. et al.** Detection of mutant K-ras DNA in plasma or serum of patients with colorectal cancer. *Brit. J. Cancer*, 1997, 76, p. 1293–1299.
15. **De Kok, J. B., Van Solinge, W. W., Ruers, T. J. M. et al.** Detection of tumour DNA in serum of colorectal cancer patients. *Scand. J. Clin. Lab. Invest.*, 1997, 57, p. 601–604.
16. **Ryan, B. M., Lefort, F., McManus, R. et al.** A prospective study of circulating mutant KRAS2 in the serum of patients with colorectal neoplasia: strong prognostic indicator in post-operative follow up. *Gut*, 2003, 52, p. 101–108.

### Acknowledgement

We wish to express our thanks to Dr. Jana Klánová and Dr. Dana Bastlová, Laboratory of Plant Molecular Physiology, Masaryk University, Brno, Czech Republic for DNA sequencing and Dr. Olfert Landt, TIB MOLBIOL, Berlin, Germany for synthesis of probes, and for excellent technical assistance.

The project was supported by the Grant Agency of Czech Ministry of Health, NR-7884-3.

Do redakce došlo 20. 3. 2006.

Adresa pro korespondenci:

PharmDr. Martin Beránek, Ph.D.

Ústav klinické biochemie a diagnostiky LF UK a FN

Sokolská 581

500 05 Hradec Králové

e-mail: beranek@lfhk.cuni.cz