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Genetic analysis of *KRAS* mutation status in metastatic colorectal cancer patients

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Colorectal carcinoma (CRC) represents a serious problem worldwide: in the Slovak republic are diagnosed about 2600 new CRC cases annually and its incidence is increasing. Colorectal cancer patients may succumb to the disease because of local recurrence or local formation of metastasis. Therefore, it is necessary to modulate therapeutic algorithm with new methods, leading to early diagnostic of CRC or changing the existing therapeutic procedures.

Recent progresses have been made in understanding of EGFR pathway involved in CRC carcinogenesis, especially the role of Ras protein. Mutations in *KRAS* oncogene are frequently found in human cancers, particularly colorectal, pancreatic, billiary tract and lung tumors. The presence of the *KRAS* mutations in metastatic colorectal cancer patients correlates with lack of response to the certain epidemal growth factor receptor (EGFR) inhibitor therapies, such as Panitumumab and Cetuximab. Consequently, screening for *KRAS* mutations status may be used as a prognostic marker, because the CRC patients with *KRAS* positive tumors have a worse prognosis.

The aim of our study was to establish the methods for rapid and sensitive detection of *KRAS* mutation status in formalin fixed paraffin embedded (FFPE) tissues DNA. We applied Real Time PCR analysis (TheraScreen KRAS Mutation Test Kit) and sequencing analysis (optimised for the analysis of FFPE tissues) to detect somatic mutations in codon 12 and 13 of *KRAS* gene. Both methods were used concurrently in the panel of DNA isolated from 25 colorectal FFPE tissues tumor. The positive or negative results from all 25 samples were identified by both methods independently. The *KRAS* mutations were presented in 8 of 25 patients (32%). Our results demonstrate that the Real Time PCR analysis can be used for detection of somatic *KRAS* mutations in FFPE clinical samples. However, we also recognize that the sequencing analysis of approximately 200bp amplicons may be used for *KRAS* mutations status screening, but with care of method sensitivity.

Key words: KRAS mutation, genetic analysis, colorectal cancer, metastasis

Introduction

Colorectal cancer is one of the best-studied system of multistage human carcinogenesis [1]. *RAS* genes encode the membrane-attached small guanine triphosphate-bound proteins that play a key role in signal transduction of extracellular mitogenic signals (growth factors) to the nucleus [mitogen- activated protein kinase (MAPK) signaling pathway]. A number of *RAS* gene mutations have been identified that result in constitutive activation of Ras protein [2].

KRAS gene is a member of the *RAS* gene family and *KRAS* mutations (mainly at codons 12 and 13) are found in all types of human tumors (Table 1). Approximately 30-40% of CRC tumors appear to have these types of mutations [3]. *KRAS* mutation testing is important, because *KRAS* mutations are significantly associated with absence of responsiveness to EGFR inhibitors. Thus, the mutational status of *KRAS* gene can provide important information for stratification of CRC patients to receive molecularly targeted treatment with tyrosine kinase inhibitors.

The most widely applied method for mutation screening is *direct sequencing*. To be successful, the sequencing method-

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Table 1. Prevalence of *KRAS* mutations in selected human tumours

Cancer type	Frequency of KRAS mutations
Biliary tract	33%
Bladder	4%
Breast	4%
Cervix	9%
Colon	32%
Endometrial	15%
Kidney	1%
Liver	8%
Lung	19%
Melanoma	2%
Myeloid leukaemia	5%
Ovarian	17%
Pancreas	60%
Thyroid	2%

The mutation data was obtained from the Sanger Institute Catalogue of Somatic Mutations in Cancer web site

ology requires a sufficient amount of tumor material of relatively good quality, which is difficult to obtain from tumor materials usually fixed in formalin. Formalin fixation followed by paraffin embedding of tissue specimens is widly used preservation method, but the irreversible damage that occur in the DNA may provide a block to the DNA polymerase activity [4]. The relatively low sensitivity of sequencing analysis in somatic mutation detection [5] is a particular problem because tumor material often contains only a small proportion of neoplastic cells. Therefore is it necessary to use other approaches for detection of *KRAS* mutation status in tumors [6].

Real Time PCR analysis has enormous potential for the detection of DNA sequence changes. ARMS (Amplification Refractory Mutation System) and Scorpion assay (TheraScreen DxS Mutation TestKit) is a highly sensitive and selective method and can detect approximately 1% of mutants in a background of wild-type genomic DNA (http://www.dxsgenotyping.com). This method is able to work with tumor material of relatively low quality, even when only a few amplifiable copies of DNA are presented in the sample.

The aim of this study is to describe the possible approaches for mutational screening of *KRAS* gene in FFPE tissues and to introduce the mutation screening of the *KRAS* gene using the Real Time PCR analysis at our department and so to expand the molecular diagnoses already used in clinical practice in Slovak Republic [7-11]. We also suggested the possible effective screening strategies, with priority to the sensitivity and the economic costs.

Materials and methods

Patients and samples. This preliminary study involved 25 metastatic colorectal cancer patients operated and treated at St. Elizabeth Cancer Institute in Bratislava. Tumor-rich areas were marked by the pathologist to ensure that the maximum amount of tumor material was collected for the genetic testing. Written informed consent was obtained from all patients or families prior to testing.

DNA extraction from formalin-fixed paraffin-embedded (FFPE) tissue. Genomic DNA form 10 μm thickness was extracted using the RecoverAll ™ Total Nucleic Acid Isolation kit (Ambion, Austin, USA) according to the manufacturer's protocol and was kept at 4°C before use.

DNA sequencing. Samples were sequenced for *KRAS* exon 2 mutations using a 215 bp amplicon (primer sequences are available upon request). The PCR reaction contained 10 pmol each primer, 12,5 μl AmpliTaq Gold PCR Master Mix (Applied Biosystems, Foster City, USA) and 50 ng DNA in a final reaction volume of 25 μl. The following cycling conditions were used for PCRs: initial denaturation at 95°C for 7 minutes; 35 cycles: 95°C for 30 seconds, 55°C for 35 second, 72°C for 45 seconds, followed by one cycles of 72°C for 7 minutes. PCR products were purified by Exonuclease I and SAP (USB Corporation, Cleveland, USA) and subsequently sequenced in both directions using BigDye Terminator v.1.1. cycle sequencing kit (Applied Biosystems, FosterCity, USA). The sequencing products were purified by SigmaSpin Sequencing Reaction Clen-Up Post Reaction Purification Columns (Sigma-Aldrich, Missouri, USA) before running on a 3130 Genetic Analyser (Applied Biosystems). The sequencing data were independently analysed by two scientists. The identified DNA changes were compared to reference sequences of KRAS gene at http://www.ensembl.org. Each mutation was confirmed by sequencing a second independent PCR reaction.

Real Time PCR analysis. Samples were analysed by TheraScreen DxS Mutation TestKit (DxS Ltd, Manchester,UK) according to the manufacturer's protocol and using 7500 Real Time PCR System (Applied Biosystems). Briefly, this kit combines two technologies, ARMS assay and Scorpions probes to detect mutations in real-time PCR reactions. This method is highly selective and can detect approximately 1% of mutants in background of wild–type genomic DNA, but it is necessary to have approximately 20 ng of DNA. Eight assays are supplied for each sample. The control assay (region of *KRAS* exon 4), labeled with FAM, is used to assess the total DNA in a sample. The mutation assays (12Ala, 12Asp, 12Arg, 12Cys, 12Ser, 12Val, 13Asp) are also labeled with FAM. They contain one Scorpion plus one ARMS primer for discrimination between the wild type DNA and the mutant DNA detected by that assay. For each sample, the control and mutation assay must be analysed on the same PCR run, to avoid run to run variations in the threshold setting. The Real Time PCR for one assay (control assay or one of mutations assays) contained 19,8 μl Reaction Mix and 0,2 μl Taq DNA polymerase and a 20ng of DNA in a final reaction volume of 25 μl. The following cycling conditions were used for *Absolute quantification* of real-time PCRs : stage I. 95°C for 4 minutes; stage II. 40 cycles of 95°C for 30 seconds and at 60°C for 1 minute (at this moment is measured the light emitted from dye for all mutation assays). The Real Time PCR data were independently analysed by two scientists

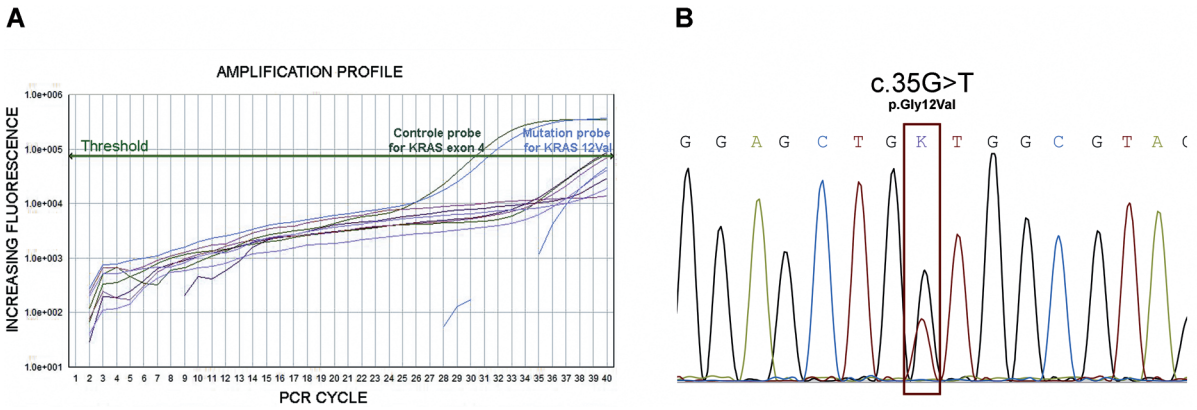

Fig. 1. Somatic mutation c.35G>T (p.Gly12Val) found in *KRAS* gene in this study: (A) Real Time PCR plot; (B) Sequencing chromatogam (forward sequence).

according to the manufacturer's protocol. Each mutation was confirmed by a second independent PCR reaction.

Results and Discussion

The advent of molecular targeted therapy is shifting the paradigm of management of cancer patients from generalised chemotherapy and/or radiotherapy to personalised treatments with better efficiency and lower toxicity. An example of this is the development of *KRAS* mutation status testing in patients' tumors in order to decide about the appropriate treatment [6, 12-17]. The optimal use of EGFR-targeted therapies requires accurate *KRAS* mutation testing with specific guideline recommendations and a European quality assurance program [18].

In the presented study, we have analyzed *KRAS* mutations in tomours from 25 metastatic colorectal cancer patients. All 25 samples were tested concurrently by direct DNA sequencing and Real Time PCR analysis for *KRAS* exon 2 mutations. Mutations were detected in 8 tumors (32%) at codon 12 or 13 of *KRAS* gene and are summarized in Table 2. The spectrum of mutation was as follows: c.35G>C (n=1), c.35G>A (n=3), c.34G>A (n=1), c.35G>T (n=3). Neither of screened tumours contained double mutations in codons 12 or 13. All mutations identified by sequencing were correctly identified also by Real Time PCR analysis. The Real Time PCR plot for *KRAS* mutations and sequencing traces of positive samples are showed in Figure 1.

Direct sequencing is the standard method for mutation detection, but it has a limited sensitivity and requires sufficient amount of tumor material of relatively good quality. These limitations have prompted the development of alternative methods for routine clinical testing which may be more practically for diagnostic somatic mutations. According to our experiences, we suggested the following possible screening strategies for *KRAS* mutation status in tumors comprises two stages: (1) sequencing analysis; (2) Real Time PCR analysis. Sequencing analysis of approximately 200bp amplicons can be used as a first step for mutation detection. Samples not convenient for sequencing analysis (due to relatively low quality of DNA) and also samples without detected mutation by DNA sequencing could be screened by Real Time PCR analysis since this method is highly sensitive and is able to work also with material of relatively low quality. Where sequencing analysis requires that the mutated material represented at least 20% of the sample, Real Time PCR (TheraScreen DxS

Table 2. Summary of results from 25 CRC cancers screened for mutations in *KRAS* gene

Patient	Real-time PCR/ TheraScreen KRAS Mutation Test Kit	Sequencing	AA change
1	wt	wt	-
2	wt	wt	-
3	wt	wt	-
4	wt	wt	-
5	c.34G>A	c.34G>A	p.Gly12Ser
6	c.35G>A	c.35G>A	p.Gly12Asp
7	c.35G>T	c.35G>T	p.Gly12Val
8	wt	wt	-
9	c.35G>A	c.35G>A	p.Gly12Asp
10	wt	wt	-
11	c.35G>T	c.35G>T	p.Gly12Val
12	wt	wt	-
13	c.35G>A	c.35G>A	p.Gly12Asp
14	wt	wt	-
15	wt	wt	-
16	wt	wt	-
17	wt	wt	-
18	wt	wt	-
19	c.35G>C	c.35G>C	p.Gly12Ala
20	wt	wt	-
21	wt	wt	-
22	c.35G>T	c.35G>T	p.Gly12Val
23	wt	wt	-
24	wt	wt	-
25	wt	wt	-

Wt-wild – type, **AA change**-amino acid change

Mutation TestKit) can detect approximately 1% of mutants in background of wild–type genomic DNA.

During last years, testing of several biomarkers was established in clinical practice around the world, with the aim of reducing cancer mortality or predicting the survival of patients. The ideal biomarker should identify responders and patients with improved survival with high sensitivity and specificity. However, the *KRAS* status of tumor may still fall short in this respect, as not all wild-type patients respond or have improved survival and some mutant patients experience long-term survival disease control [14].

In summary, 8 of 25 (32%) tumors analysed in this study showed somatic mutation in the exon 2 of *KRAS* gene. Taken together, this evidence suggests that most mutations in Slovak metastatic colorectal patients are p.Gly12Asp and p.Gly12Val. However, reported were also other types of somatic mutations e.g. in codon 61, which results in constitutive activation of Ras protein [19-21], this particular mutation is not detectable by our primers for DNA sequencing analysis and by TheraScreen DxS Mutation TestKit. In order to eliminate the possibility that this type of mutation could escape to the detection, we are currently developing analysis, which may detect mutation in codon 61. Taken together, we have established fast, efficient and reproducible screening methods for *KRAS* mutations detection by which also degraded DNA from FFPE tissues can be tested. To our knowledge is this the first study in Slovakia in which somatic mutations in *KRAS* gene were analysed for possible different strategy in treatment of metastatic colorectal patients.

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