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K-ras gene mutation status in colorectal cancer: comparative analysis of pyrosequencing and PCR-RFLP

MARIA DOBRE, MARIA COMĂNESCU, D. ARSENE, CRISTINA IOSIF, G. BUSSOLATI

Department of Pathology,
"Victor Babeş" National Institute for Research and Development in Pathology and Biomedical Sciences, Bucharest, Romania

Abstract

Background: In patients with high-stage colorectal carcinomas (CRC), anti-EGFR therapy is known to be effective only in cases with a wild-type K-ras gene status. Different procedures have been proposed for such evaluation. *Materials and Methods*: The mutation status of K-ras gene, codons 12, 13 and 61 was determined in 250 CRC cases using the pyrosequencing assay. In addition, we compared the performance of the pyrosequencing procedure with that of PCR-RFLP in a subset (*n*=100) of the CRC samples the latter only in codons 12 and 13. *Results*: Using pyrosequencing, 46.4% of the 250 CRC cases were found mutated. Most mutations were located in codon 12 (36.4% from all cases) and several were located in codon 61 (3.2%). All mutation identified by PCR-RFLP were confirmed by pyrosequencing and, in addition, one more mutated sample was identified in the subset of 100 samples. *Conclusions*: Both methods are highly specific and can profitably be used in the molecular diagnosis of colorectal cancer in order to establish the adequate therapy.

Keywords: colon cancer, K-ras mutation, pyrosequencing.

Introduction

In colorectal carcinoma (CRC), the third common cancer in men and the second in women in the world [1], the limited response to conventional therapies has led researchers around the world to seek potential prognostic and predictive factors. Therapy for this aggressive tumor has been improved by introducing targeted therapies with monoclonal antibodies such as anti-EGFR (cetuximab and panitumumab) and anti-VEGF (bevacizumab) [1, 2]. Bevacizumab is a recombinant humanized monoclonal antibody that binds to the vascular endothelial growth factor (VEGF), a pro-angiogenic cytokine and prevents the growth and maintenance of tumor blood vessels, while cetuximab and panitumumab bind to the epidermal growth factor receptor inhibiting EGFR phosphorylation [3, 4]. Interestingly, the response to anti-EGFR therapy is independent of the level of EGFR expression in CRC [4, 5].

Novel discoveries in the genetic component of the colorectal carcinogenesis brought a new perspective in approaching this pathology [6], since studies [7, 8] have shown that more than 50% of colorectal cancers show mutations in genes that encode proteins involved in the EGFR signaling pathway, most notably KRAS and BRAF. These data facilitated the selection of new therapeutic protocols.

The members of RAS family, Harvey-Ras (H-Ras), Kirsten-Ras (K-Ras) and N-Ras, are located on different chromosomes [9]. They build up a family of proto-oncogenes that encodes low molecular weight G-protein (21 kDa) [5, 10]. Ras proteins function as molecular switches and regulate critical cellular processes including mitosis, cell differentiation, apoptosis, gene expression, metabolism [1, 11]. K-Ras proteins are located on the internal surface of cell membranes and have a GTP-ase activity, playing an important role in cell division, cell differentiation and apoptosis [12, 13].

The ligand binding to the EGFR, as a transmembrane receptor, determines the cascade activation of transduction signals to the nucleus, first by phosphorylation of the intracellular tyrosine kinase domain of EGFR, which, in turn, causes a transitional activation of the Ras protein that phosphorylates other downstream proteins [14]. Mutational activation of K-Ras plays an important role in colorectal cancer progression [11], since it makes the downstream cascade proteins permanently switched on.

Approximately 40% of colorectal cancers present K-ras gene mutations [15]. These mutations are generally located at codons 12 and 13 in the majority of cases [16] and less frequently at other codons, like codon 61 or 64 [17]. These mutations may ultimately activate signal transduction pathways, including phosphatidylinositol 3-kinase cascade.

Point substitutions in codons 12 and 13, were validated as negative predictors of response to targeted therapies with anti-epidermal growth factor receptor antibodies [18]. Therefore, determining the KRAS mutational status of tumor samples has become an essential tool for managing patients with colorectal cancers.

Several procedures have been proposed for determining such mutational status in CRC samples but relative pros

and cons are still disputed. The aims of the present study was therefore to compare the performance of two detection methods (PCR-RFLP and pyrosequencing) in determining the status of K-ras mutation in a large series of CRC samples.

Materials and Methods

Tissue samples

The study included 250 tumor samples from patients with CRC aged between 19 and 76 years. Serial 5-μm sections from formalin-fixed, paraffin-embedded (FFPE) tissues were cut from each paraffin block. The first section was stained with Hematoxylin–Eosin and a histopathological diagnosis was established by a board-certified pathologist. All cases were classified and graded according to *World Health Organization* criteria [19].

In order to select the tumor area for DNA extraction, the sections were manually dissected, making sure that >80% of the test area was represented by tumoral tissue.

DNA extraction

All necessary procedures were taken for preventing genetic contamination. Genomic DNA from FFPE tissue sections was isolated using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocols. A number of 5–10 slides (50 μm) were cut from each case, according to sample volume. In order to extract DNA from tumor samples, sections were placed in two baths of xylene and ethanol to remove paraffin and were placed in SDS-containing lysis buffer and proteinase K (10 mg/mL) at 56^0C, up to complete lysis. After lysis, DNA was precipitated using ethanol and absorbed on the QIAamp silica membrane by centrifugation. After two washes, purified DNA was eluted in buffer AE or water. The DNA concentration and purity were measured spectrophotometrically at 260 nm and 280 nm with the NanoDrop ACTGene ASP-3700 USA.

Mutations in K-ras gene, codons 12 and 13 were detected by pyrosequencing (codons 12, 13, 61) and by PCR-RFLP (Polymerase Chain Reaction – Restriction Fragment Length Polymorphism).

Pyrosequencing

Pyrosequencing analysis was performed using CE-IVD marked PyroMark KRAS kit (QIAGEN, Hilden, Germany) according to the manufacture's protocols (Therascreen KRAS Pyro Kit Handbook, version 1, July 2011). For each sample, 10 ng of genomic DNA were used for analysis of mutations in codons 12 and 13 and another 10 ng DNA for mutations in codon 61.

10 μL of each PCR product were analyzed by pyrosequencing using PyroMark Gold Q96 reagents, Streptavidin Sepharose High Performance (GE Health-care Bio-Science AB, Uppsala, Sweden), a PyroMark Q24 instrument (QIAGEN, Hilden, Germany) and Pyro Mark Q24 1.0.6.3 software. Samples that failed the initial analysis or with an initial "check" status were subjected to a second analysis.

PCR-RFLP

For codon 12, we used the restriction enzyme MvaI (BstNI) (Fermentas). The PCR reactions contained 1X reaction buffer, 1.5 mM magnesium chloride, 0.2 mM deoxynucleotide triphosphates (dATP, dGTP, dCTP, dTTP), 1 mM of each primer, 1.5 units of PlatinumTaq DNA polymerase (Invitrogen, Brasil) and 500 ng of genomic isolated DNA in 25 μL total volume. The primers were synthesized by Invitrogen and the sequences used were according to data published by Kubrusly MS *et al.* [20]:

(sense)
ACTGAATATAAACTTGTGGTAGTTGGACCT,
(antisense)
TAATATGTCGACAAAACAAGATTTACCTC.

After amplification, fragments of 135 base pairs were incubated and then digested by MvaI. The absence of a mutation in codon 12 determines the cleavage with MvaI at one site, which no longer takes place when a mutation exists.

The wild-type fragment is cleaved in two fragments with sizes of 106 and 29 base pairs [20] and a mutant case has both alleles, normal and mutant.

PCR was performed in a thermocycler (Gene Amp PCR System 2f00, Applied Byosistems, Singapore) and each cycle was performed for denaturation at 94^0C for one minute, for annealing at 55^0C for one minute and 72^0C for 30 seconds for extension. The PCR comprised 45 cycles, followed by digestion with MvaI (10 U enzyme and 12-μL PCR product) overnight.

Electrophoresis was performed using 2% agarose gel, stained with ethidium bromide and photographed by ultraviolet transilluminator (BioImaging Systems Digi Doc, It System, Upland, USA).

For codon 13, we used the HaeIII (Fermentas) as restriction enzyme. The PCR reactions contained the same components except for the primers concentration (1.25 mM) and 50^0C annealing temperature. The sequences for primers for codon 13 were:

(sense)
5'-GTACTGGTGGAGTATTTGATAGTGTATTAA-3'
(antisense)
5'-GTATCGTCAAGGCACTCTTGCCTAGG-3' [21].

After amplification, 12.5 μL PCR products (159 base pairs) and 10 U HaeIII were incubated overnight. The wild-type allele were cleaved in three fragments of 85, 48 and 26 pair base while the mutant allele were cleaved in only two fragments of 85 and 74 bp. Electrophoresis was performed using 4% HR agarose gel.

Quality controls

The external control was assured on the base of the feedback of the quality of the PCR-RFLP system in the *2010 European KRAS External Quality Assessment Scheme* by *European Society of Pathology* and for pyrosequencing we participated in the *External Quality Assessment* by the *Italian Society of Pathology and Cytopathology* and both techniques were validated.

For PCR-RFLP, we used two DNA samples with known mutational status for K-ras gene codons 12 and 13: normal DNA (negative control – NC), mutant DNA (positive control – PC) and no template control (NTC) for monitoring potential contamination during the PCR

settings and amplification. For pyrosequencing, we included a sample with unmethylated control DNA, provided by the kit as a positive control for PCR, and sequencing reactions and a NTC in every run.

Setting the procedure for PCR-RFLP

In the run on the agarose gel, the image for a "wild" case (lacking K-ras mutations) shows two bands of 106 base pairs (bp) and 29 bp, while a mutant case shows three bands: one of 135 bp (mutant allele) and two of 106 bp, 29 bp (normal allele). We migrated PCR products (135 pb) and restriction fragments for each sample (Figure 1).

The image for a "wild" case shows three fragments of 85, 48 and 26 pb, while a mutant case four bands of 85 and 74 bp (mutant allele) and 85, 48 and 26 bp (normal allele) (Figure 2).

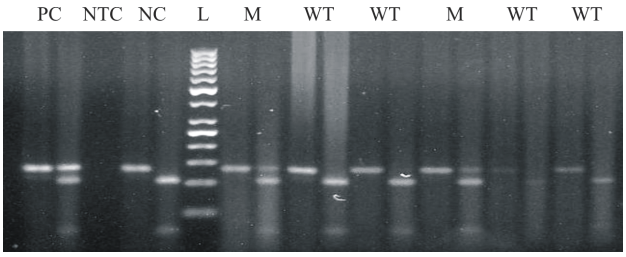


Figure 1 – ***Codon 12 gel electrophoresis of PCR products (band at 135 bp) and restriction fragments, agarose 2% (w/v). PC – Positive control (heterozygous, bands at 135, 106 and 29 bp), NC – Negative control (bands at 106 and 29 bp), NTC – No template control, L – DNA molecular weight markers (GeneRuler 50 pb DNA Ladder, Fermentas), WT – Wild type sample, M – Mutant sample.***

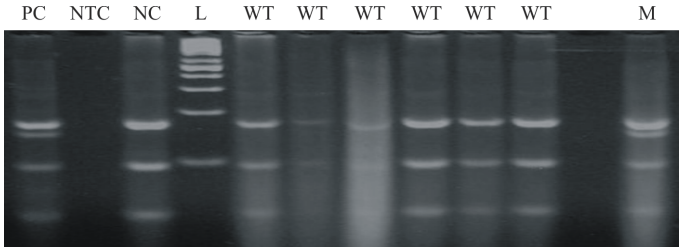


Figure 2 – ***Codon 13 gel electrophoresis of restriction fragments, agarose HR 4% (w/v). PC – Positive control (heterozygous, bands at 85, 76, 48 and 26 bp), NC – Negative control (bands at 85, 48 and 26 bp), NTC – No template control, L – DNA molecular weight markers (GeneRuler 50 pb DNA Ladder, Fermentas), WT – Wild type sample, M – Mutant sample.***

Results

All cases included in the study group had a histological diagnosis of adenocarcinoma of the colon.

The genomic DNA concentrations of the 250 specimens included in the study group were between 30 ng/μL–2466.2 ng/μL and 260 nm/280 nm ratio was between 1.63 and 2.18. Among the 250 patients, we were able to assess the mutation status for K-ras gene codons 12/13 and 61 in 248 (99.2%) cases by pyrosequencing. We identified K-ras mutations codons 12, 13 and 61 in 115 (46.4%) cases. The types of detected mutations and the ranges of allele quantification are showed in Table 1.

Table 1 – ***Types of K-ras mutations identified by pyrosequencing***

Codon	Mutation type	Nucleotide substitution	Amino acid substitution	Allele frequency [% units]
12 (GGT)	*Transitions*	GAT	G12D	7.2–78.8
		AGT	G12S	16.2–78.4
	Transversions	GTT	G12V	8.0–54.7
		TGT	G12C	8.4–56.5
		GCT	G12A	36.7–56.8
		CGT	G12R	29
13 (GGC)	*Transitions*	GGC	G13D	7.3–78.5
61 (CAA) reverse orientation (TTG)	*Transitions*	CGA	Q61R	12.7
	Transversions	CAC	Q61H	15.7
		CTA	Q61L	7.8–47.7
		CAT	Q61H	25.9
		GAA	Q61E	7.2

We identified seven variants of point mutations in codons 12/13 in 108 (43.2%) samples.

The representative pyrograms describing the identified mutations in codons 12/13 are illustrated in Figure 3.

As shown, most mutations were located in codon 12 (36.4% out of the total number of cases), while in codon 13 we identified 17 (6.8%) mutations and only eight (3.2%) mutations in codon 61 (Figure 4).

K-ras mutations in codon 61 were identified in eight (3.2%) cases, in five variants (Table 1). See Figure 5 for representative pyrograms describing mutations in codon 61.

The most common mutations were 12GAT (43.9%) and 12GTT (30.8%) in codon 12, while in codon 61, half of the mutations were 61CTA (CAA>CTA, substituted amino acid type Q61L). The results are depicted in Figures 5 and 6 for the distribution of type of mutations in codon 12 and 61, respectively.

Beside the identified mutations, one case had a double mutation, one in codon 12 (GGT>GAT) and one in codon 61 (CAA>CTA). We repeated the analysis and the results were the same.

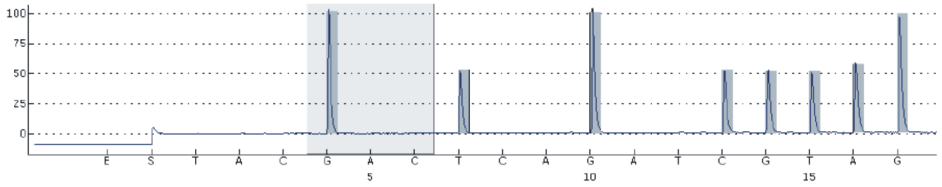
Comparative analysis of PCR-RFLP and pyrosequencing

We compared the results obtained with newer approach, pyrosequencing, with the ones obtained with the more classical method that is already used as a diagnostic tool in our laboratory (PCR-RFLP).

A subset of 100 successive cases was analyzed with both methods for a direct comparison. The samples were analyzed for mutations in codons 12 and 13 using both methods in a blind manner. Among the 100 cases, two of them did not amplified for codon 13 by PCR-RFLP and two cases failed the quality criteria by pyrosequencing, so we could compare the results for 96 cases. We identified 48 mutations by PCR-RFLP, 45 mutations in codon 12 and three mutations in codon 13. By pyrosequencing we detected 49 mutations, 46 mutations in codon 12 and three mutations in codon 13 (Figure 8). One case resulted as being wild type by PCR-RFLP and mutated in codon 12 (GGT>GAT) by pyrosequencing (Figure 9).

(a)

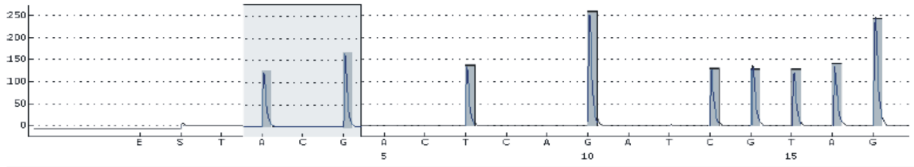
Well: B1
Assay: KRAS 12+13
Sample ID: 9
Note: 10 ng



Result	**WildType**
Quality	Passed

(b)

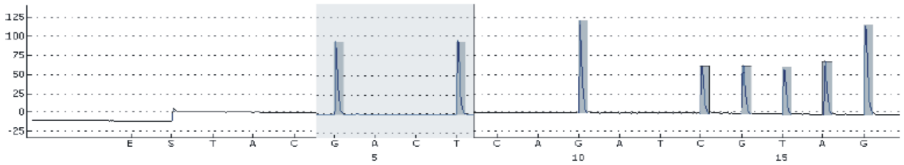
Well: C2
Assay: KRAS 12+13
Sample ID: 10
Note: 10ng



Result	**Mutation**
Frequency	78.4 % units (LOD: 1.9 % units)
Nucleotide Substitution	GGT>AGT
Amino Acid Substitution	G12S
Quality	Passed

(c)

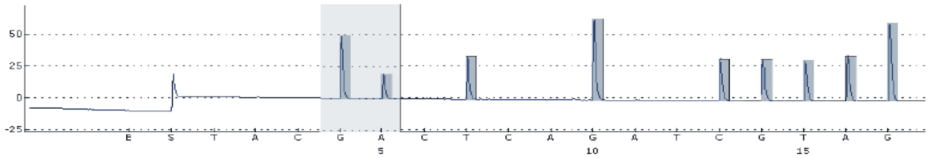
Well: B4
Assay: KRAS 12+13
Sample ID: 12
Note: 10ng



Result	**Mutation**
Frequency	52.3 % units (LOD: 1.0 % units)
Nucleotide Substitution	GGT>GTT
Amino Acid Substitution	G12V
Quality	Passed

(d)

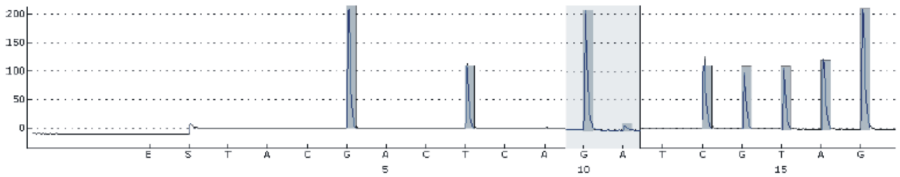
Well: A2
Assay: KRAS 12+13
Sample ID: 2
Note: 10 ng



Result	**Mutation**
Frequency	52.0 % units (LOD: 2.2 % units)
Nucleotide Substitution	GGT>GAT
Amino Acid Substitution	G12D
Quality	Passed

(e)

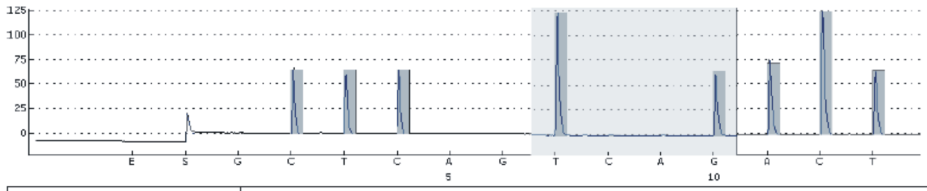
Well: B7
Assay: KRAS 12+13
Sample ID: 15
Note: 10ng



Result	**Mutation**
Frequency	6.5 % units (LOD: 1.9 % units)
Nucleotide Substitution	GGC>GAC
Amino Acid Substitution	G13D
Quality	Passed

Figure 3 – ***K-ras analysis by pyrosequencing. Representative pyrogram traces of samples with mutations in codon 12/13: (a) Wild type; (b) GGT>AGT; (c) GGT>GTT; (d) GGT>GAT; (e) GGC>GAC.***

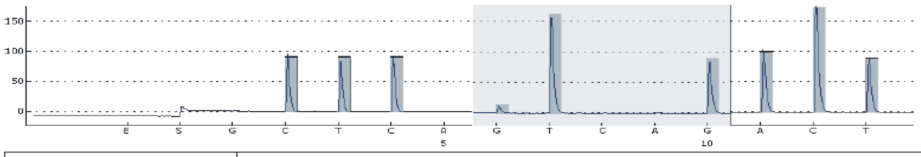
Well: B4
Assay: KRAS 61
Sample ID: 12
Note: 10ng



Result	WildType
Quality	Passed

(a)

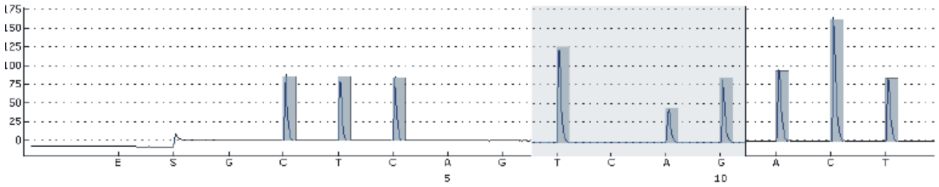
Well: C5
Assay: KRAS 61
Sample ID: 21
Note: 10ng



Result	Mutation
Frequency	15.7 % units (LOD: 2.8 % units)
Nucleotide Substitution	CAA>CAC
Amino Acid Substitution	Q61H
Quality	Passed

(b)

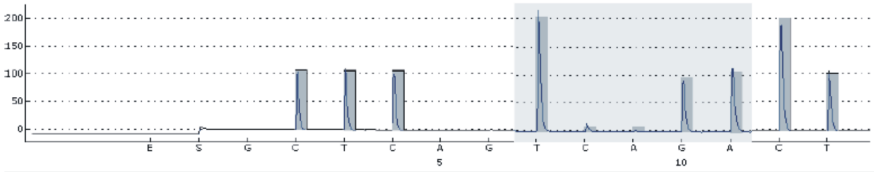
Well: B6
Assay: KRAS 61
Sample ID: 14
Note: 10ng



Result	Mutation
Frequency	47.7 % units (LOD: 3.1 % units)
Nucleotide Substitution	CAA>CTA
Amino Acid Substitution	Q61L
Quality	Passed

(c)

Well: B5
Assay: KRAS 61
Sample ID: 13
Note: 10ng



Result	Mutation
Frequency	7.2 % units (LOD: 3.1 % units)
Nucleotide Substitution	CAA>GAA
Amino Acid Substitution	Q61E
Quality	Passed

(d)

Figure 4 – ***K-ras analysis by pyrosequencing. Representative pyrogram traces of samples with mutations in codon 61: (a) Wild type; (b) CAA>CAC; (c) CAA>CTA; (d) CAA>GAA.***

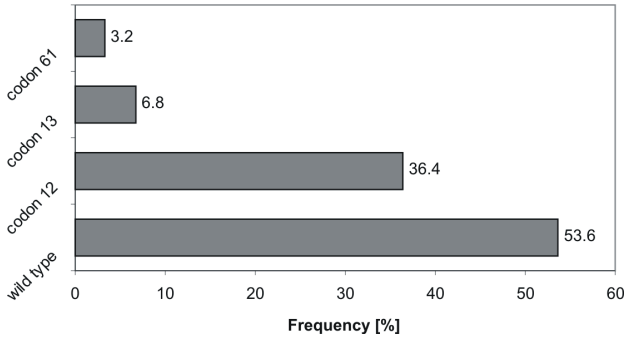


Figure 5 – ***Frequency of the detected K-ras mutations.***

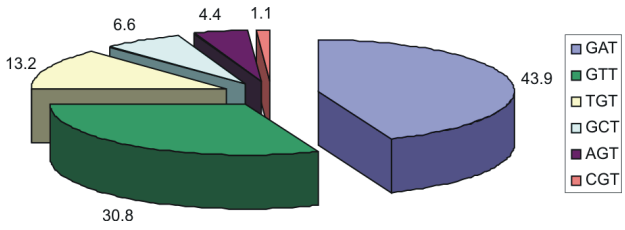


Figure 6 – ***Distribution of mutations detected in codon 12.***

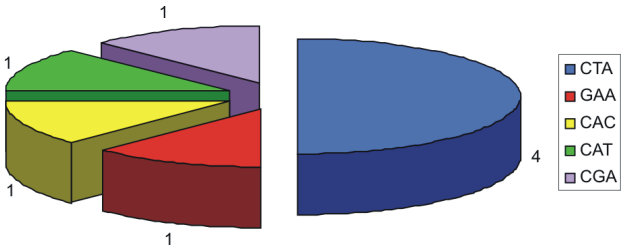


Figure 7 – ***Distribution of mutations detected in codon 61.***

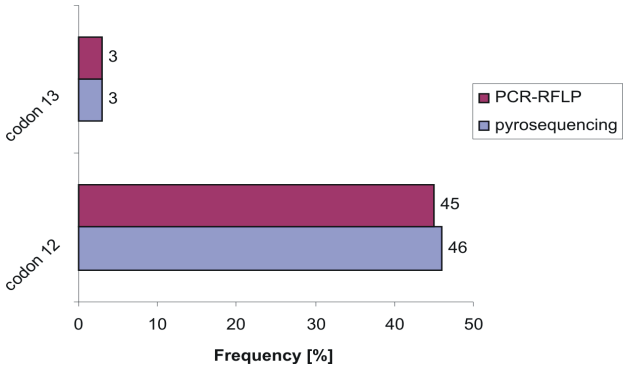
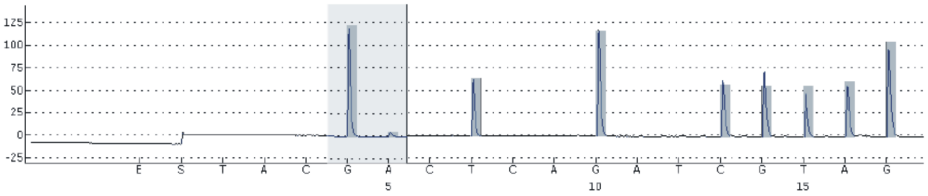


Figure 8 – ***Pyrosequencing vs. PCR-RFLP.***



Result	Mutation
Frequency	7.9 % units (LOD: 2.2 % units)
Nucleotide Substitution	GGT>GAT
Amino Acid Substitution	G12D
Quality	Passed

Figure 9 – ***Pyrogram trace of sample with mutations in codon 12 identified by pyrosequencing but not by PCR-RFLP.***

Discussion

Personalized, targeted therapies developed due to the advances in the understanding of tumorigenesis and the evolution of molecular and genetic tests. The analysis of the KRAS gene determines which patients will benefit from an anti-EGFR therapy, in present, KRAS being a commonly performed test in specialized pathology laboratories.

Our study involved 250 patients with histologically confirmed diagnosis of adenocarcinoma of the colon, with a ratio M:F of almost 1. There was no statistical correlation between gender and distribution or type of mutations determined.

In our study group of 250 patients, 46.4% (n=116) were found to be mutated in K-ras codons 12, 13 or 61 and the frequency and type of mutations were in accordance with other studies [1, 5, 22]. Moreover, the reliability of the adopted procedure was confirmed by two external quality controls.

In agreement with data of the literature [1], the highest frequency of K-ras mutation was detected in codon 12 (78.4%, n=91). At variance with some reported data, in codon 13 we found a mutation rate of 14.7% (17/116), while Bazan V *et al.* [12] found a mutation rate in codon 13 of 43%. As extensively illustrated by Brink M *et al.* [13], most mutations involve these two codons which in wild conditions are coding for two glycine amino acids. In codon 12, the most frequent mutation subtypes are 12 (GGT>GAT), carrying an amino acid substitution of glycine by aspartic acid (43.9%), and 12 (GGT>GTT), which involves a substitution by valine (30.8%). Oliveira C *et al.* [23] found only 2% mutations in codon 61 while we found 6.9%. From all type of mutation, the transversions were a bit higher than the transitions (51.7% *vs.* 48.3%) in codon 12 while in codon 61 the transitions were substantially higher than the transversions (87.5% *vs.*12.5%).

By pyrosequencing, in one case we detected a double mutation in codon 12 (GGT>GAT), with replacement of glycine with aspartic acid and in codon 61 (CAA>CTA), with replacement of glutamine with leucine. The frequency of codon 61 mutations is believed to be low and therefore this codon is less studied than codons 12 and 13. Interestingly, McLellan EA *et al.* [24] and Brink M *et al.* [13] found cases with double mutations, but only in codons 12 and 13.

In the comparative analysis of the two procedures employed out of the subset of 100 samples, we had to exclude two samples where the mutation status could not be analyzed because the DNA failed to amplify and by pyrosequencing the samples failed the quality criteria. A major bias affecting this type of analysis, to be carried out in formalin-fixed paraffin-embedded material is the low quality of extracted DNA. In fact, both pyrosequencing and PCR-RFLP are affected by nucleic acids degradation and DNA fragmentation produced by formalin fixation and degradation due to necrosis can reduce the success rate of amplification.

The number of samples with a codon 12 or 13 mutation determined using pyrosequencing was 49, while by PCR-RFLP we identified 48 cases. By pyrosequencing, in codon 12 were identified most of the mutations (93.9%) and a smaller number in codon 13 (6.1%). By PCR-RFLP, there was a similar distribution of the mutations, with 93.7% being located in codon 12 and 6.3% located in codon 13. One case resulted as being wild type by PCR-RFLP and mutated in codon 12 (GGT>GAT) by pyrosequencing. We could explain this difference by low frequencies of mutated allele (7.9%) (Figure 9).

Statistically, there was an almost perfect correlation between the two methods in the determination of the mutations in codon 12 (r=0.921), while for codon 13, the correlation was perfect from a statistical point of view (r=1, p<0.01), so sensitivity and specificity of the

pyrosequencing method qualifies it for the determination of the mutation status in FFPE CRC samples. There were no other correlation found between the epidemiological variables (sex, age) and K-ras mutations (Table 2).

Table 2 – ***Statistical correlation of the results obtained***

Correlations							
		Age	***PCR__RFLP_C12***	***Sex***	***PCR__RFLP_C13***	***PIROS_K12***	***PIROS_K13***
	Pearson correlation	1	.194*	-.083	.039	.142	.039
	Sig. (2-tailed)		.049	.404	.694	.153	.694
	N	103	103	103	103	103	103
PCR__RFLP_C12	*Pearson correlation*	.194*	1	.114	-.156	0.921**	-.156
	Sig. (2-tailed)	.049		.250	.117	.000	.117
	N	103	103	103	103	103	103
Sex	*Pearson correlation*	-.083	.114	1	-.153	.075	-.153
	Sig. (2-tailed)	.404	.250		.124	.452	.124
	N	103	103	103	103	103	103
PCR__RFLP_C13	*Pearson correlation*	.039	-.156	-.153	1	-.156	1.000**
	Sig. (2-tailed)	.694	.117	.124		.117	.000
	N	103	103	103	103	103	103
PIROS_K12	*Pearson correlation*	.142	.921**	.075	-.156	1	-.156
	Sig. (2-tailed)	.153	.000	.452	.117		.117
	N	103	103	103	103	103	103
PIROS_K13	*Pearson correlation*	.039	-.156	-.153	1.000**	-.156	1
	Sig. (2-tailed)	.694	.117	.124	.000	.117	
	N	103	103	103	103	103	103

*Correlation is significant at the 0.05 level (2-tailed). **Correlation is significant at the 0.01 level (2-tailed).

Conclusions

We observed a rate of 46.4% for mutations in K-ras gene, predominantly in codon 12. The G>A transitions and the G>T transversion are the most frequently mutations. Our results confirm that both methods are sensitive and can profitably be used in the molecular diagnosis of colorectal cancer in order to establish the adequate therapy. Out of the acquired experience, we feel inclined to recommend using PCR-RFLP method in cases where large tumor samples are available, with rich cell content and where the DNA concentration is higher than 100 ng/μL. In fact, in such conditions because the cost is lower. Pyrosequencing could be recommended mostly for small tumor samples (*i.e.*, fine needle biopsies) because in this method a smaller amount of DNA is used (20 ng for both reaction) or in samples with low tumoral cell content.

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Corresponding author
Maria Comănescu, MD, PhD, "Victor Babeş" National Institute for Research and Development in Pathology and Biomedical Sciences, 99–101 Independenţei Avenue, Sector 5, 050096 Bucharest, Romania; Phone/Fax +4021–319 27 34, e-mail: mariacomanescu@yahoo.com

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