ORIGINAL PAPER

RJME
Romanian Journal of Morphology & Embryology
http://www.rjme.ro/

KRAS gene mutations – prognostic factor in colorectal cancer?

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Abstract

The colorectal cancer (CRC) modern therapy is using adjuvant and neoadjuvant companion therapeutic agents, part of them having an anti-angiogenic action. Their benefic effect can be annulated by some gene mutations, which are interfering in signal transduction pathways. One of the more frequent activating mutations is occurring in the KRAS gene. We assessed the KRAS mutations by two molecular methods, in a group of patients with a follow-up until 144 months, aiming to establish eventual correlations between the presence of mutations and the evolution of patients. We tried to appreciate the prognostic value of these mutations. A retrospective study was conducted on 74 patients treated by radical surgery; the surgical specimens were analyzed macroscopically and the histopathological type and degree were established. PCR–RFLP (polymerase chain reaction–restriction fragment length polymorphism) and pyrosequencing were performed on paraffin-embedded tumor specimens. Statistical analysis showed significant differences in survival between patients with wild type gene and patients with mutation in codon 13; the same results were also obtained regarding TNM I, II stages or Dukes type A and B cases. However, for the patients in stage IV pTNM, the evolution was slightly better in association with a KRAS mutation than in wild type cases.

Keywords: colorectal cancer, KRAS gene, prognostic, gene mutation, targeted therapy.

Introduction

Colorectal cancer ranks third in frequency among new cases of diagnosed malignancies 1.4 million new cases (9.7% of all malignancies), after lung cancer and breast cancer. Currently, it is the most common gastrointestinal malignancy and represents 13% of all malignancies. In 2012, colorectal cancer was the fourth cause of cancer death in the world, after lung, liver and stomach [1].

The development of colorectal cancer (CRC) is a multi-step process encompassing accumulation of several genetic alterations, including chromosomal abnormalities, gene mutations, and epigenetic changes, involving several genes that regulate proliferation, differentiation, apoptosis, and angiogenesis [2, 3].

Carcinogenesis in sporadic CRC is depending of various factors and pathways, like down regulation of tumor suppressor genes, mismatch repair genes and activation of oncogenes such as KRAS.

KRAS is a proto-oncogene located on the short arm of chromosome 12, encodes the protein KRAS, a GTP-ase involved in cell division, differentiation and apoptosis [4]. This protein is part of the RAS/MAPK pathway, which is activated by the epidermal growth factor receptor (EGFR).

A single nucleotide substitution in the KRAS oncogene at amino acid residues 12, 13, and 61, triggers constitutive activation of KRAS, resulting in continuous transmission of signals to the nucleus [4] through RAS/MAPK pathway [5, 6].

Activating KRAS mutations are point mutations mostly affecting amino acid residues all of which decrease the intrinsic KRAS and GTPase activating protein, promoted GTP hydrolysis, resulting in constitutive KRAS activation [5, 6].

While some studies on CRC patients proposed that KRAS gene mutations represent a risk factor for reduced overall survival, other studies have found no correlations [7].

The KRAS oncogene is currently the most relevant molecular biomarker that predicts the response to EGFR-targeted therapy in CRC. Metastatic CRC patients with tumors harboring a KRAS mutation are resistant to treatment with anti-EGFR antibodies, showing lower response rates, decreased progression free survival, and overall survival compared with patients with KRAS wild-type tumor [8, 9].

Aiming to find a correlation between KRAS mutations status and the overall survival of CRC patients, we investi-

gated several clinical (TNM stage, relapsing time) and pathological parameters (histopathological type, mucus production, differentiation degree), in CRC patients treated with conventional (5-FU – 5-Fluorouracil, FOLFOX), radiotherapy or targeted therapies (Avastin, Bevacizumab).

Materials and Methods

The study group consisted of 74 patients with colorectal adenocarcinoma who underwent surgical resection without any immediate postoperative mortality at the Clinic of General and Esophageal Surgery, "Sf. Maria" Clinical Hospital, Bucharest, Romania. The retrospective analysis included patients diagnosed, treated and followed up between six and 144 months, during 2002–2013, in the mentioned hospital. Based on echographic and endoscopic results, surgery was performed in 73 patients. Surgical specimens were analyzed regarding tumor location, peritumoral invasion, resection limits and locoregional lymph nodes status. Histological type and differentiation, mucinous character, perineural and intravascular invasion, the budding and deepness of parietal invasion, the resection microscopic limits, lymph node invasion were assessed by microscopic investigations. The TNM and pTNM stages were established for every patient and therapeutic protocols were specifically implemented. Patients' follow-up comprised clinical/imaging investigations (clinical examination, endoscopy, CT), and biological parameters (tumor markers). Paraffin-embedded surgical samples were used for determining KRAS gene status by molecular biology methods.

Tissue samples

Serial 5–10 slides with 5 μm sections from all buffered formalin-fixed, paraffin-embedded (FFPE) blocks were cut. The histopathological diagnosis was performed on the first section stained with Hematoxylin–Eosin (HE).

DNA extraction

Genomic DNA was isolated according to the manufacturer's protocols with QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). For deparaffinization, tumor sections were placed in two baths of xylene and two baths of ethanol and thereafter they were placed in lysis buffer with proteinase K (10 mg/mL) at 56^0C. DNA was precipitated using ethanol and was fixated on the QIAamp silica membrane by centrifugation. Two washes were performed and the purified DNA was eluted in buffer AE. The DNA quality and quantity were determined spectrophotometrically at 230, 260 and 280 nm using UV–VIS spectrophotometer (ASP–3700; ACT Gene, Piscataway, NJ, USA).

KRAS gene mutations were detected by pyrosequencing (codons 12, 13, 61) and by PCR–RFLP (polymerase chain reaction–restriction fragment length polymorphism) (codons 12 and 13).

Pyrosequencing

For pyrosequencing analysis, we used the CE-IVD marked PyroMark KRAS kit (QIAGEN, Hilden, Germany) according to the producer protocols (Therascreen KRAS Pyro Kit Handbook, version 1, July 2011). From each sample, 10 ng DNA were amplified for determining mutations status in codons 12 and 13, and another 10 ng DNA for codon 61. Pyrosequencing was performed using 10 μL of each PCR product with PyroMark Gold Q96 reagents, Streptavidin Sepharose High Performance (GE Healthcare Bio-Science AB, Uppsala, Sweden), in the PyroMark Q24 instrument (QIAGEN, Hilden, Germany). The results were analyzed using PyroMark Q24 1.0.6.3 software (QIAGEN, Hilden, Germany, version 2.0.6).

PCR–RFLP

KRAS gene mutations (codons 12 and 13) were detected by PCR–RFLP. For codon 12, we used 25 μL PCR mix containing 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, Brazil) and 500 ng of genomic DNA. After amplification, PCR products (135 base pairs) were incubated overnight at 37^0C with MvaI (Fermentas Inc.). The mutation in codon 12 cancels the restriction situs for MvaI since the wild type fragment will be cleaved in two fragments with 106 and 29 base pairs; whilst the mutant case will remain uncut. Mutated cases presented both alleles (wild type and mutated).

For codon 13, the PCR reactions contained the same components except for the primers sequences and their concentration (1.25 mM). The wild-type allele was cleaved by the restriction enzyme HaeIII (Fermentas Inc.) in three fragments of 85, 48 and 26 base pairs, while the mutant allele was cleaved in two fragments of 85 and 74 bp.

PCR was performed in the Gene Amp PCR System 9700 (Applied Biosystems, Singapore). Electrophoresis was performed using 2% agarose gel for codon 12 and 4% HR (high-resolution) agarose gel for codon 13. The agarose gels were stained with ethidium bromide and photographed by ultraviolet transilluminator (BioImaging Systems DigiDoc-It System, Upland, USA).

The quality control of the PCR–RFLP method included two DNA samples with known KRAS gene mutational status for codons 12 and 13: wild type DNA (negative control – NC), mutant DNA (positive control – PC) and no template control (NTC) as PCR settings and amplification control. We used unmethylated control DNA, provided by the kit, as a positive control for PCR, and a NTC for monitoring every run by pyrosequencing.

Statistical analysis

The prognostic value of KRAS mutations in colorectal cancer was evaluated using survival analysis. Survival curves were determined by using the Kaplan–Meier method and were analyzed by using the *log*-rank test (Mantel–Cox) and Breslow (generalized Wilcoxon).

Possible associations between KRAS mutation and clinicopathological parameters of colorectal cancer were investigated using the Likelihood Ratio or Fisher's exact test. All analyses were conducted using SPSS (version 15.0). Significance for all statistics was recorded if $p<0.05$ (two-tailed).

Results

After the surgical procedure, 74 patients were enrolled in the study group in base of including and excluding criteria (age, tumor location, histological parameters, type

of treatment). Tumor paraffin blocks were collected for KRAS mutations status analysis.

The location of the primary tumor was: right colon in 17 patients, left colon in 39 patients and rectum in 20 patients; among these, two patients had metachronous tumors. The tumor was a conventional adenocarcinoma form in 55 patients, and mucus-secreting adenocarcinoma in 21 patients.

Regarding tumor grading, the following types were assessed: G1 – three patients, G2 – 64 patients and G3 – nine patients. TNM staging was: stage I – 18 patients, stage II – 24 patients, stage III – 26 patients and stage IV – six patients.

Adjuvant therapy was applied in 70 patients, consisting of chemotherapy (5-FU in 48 patients, FOLFOX in 19 patients and Bevacizumab in three patients) or radiotherapy – 11 patients.

Patients' follow-up by clinical, imaging and laboratory monitoring was performed for six months to 144 months, during 2002–2013. Recurrence developed in 27 patients – 11 patients with loco-regional recurrence, 13 patients with distant metastasis, and three patients with both local recurrence and distant metastases, leading to the continuation of therapy. Survival at three years was 89.2% and 75.6% at five years. Patients were followed up until 31.12.2013 and survival at the end of follow-up was 72.5%.

All cases were analyzed by the PCR–RFLP method for KRAS mutations, identifying 34 cases with this mutation: 27 cases with codon 12 mutation, six cases with codon 13 mutation, one patient with a double mutation of both codon 12 and codon 13 (Figure 1).

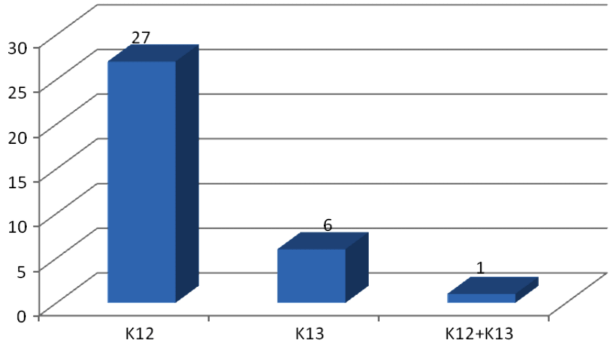


Figure 1 – ***The distribution of KRAS gene mutation in codons 12 and 13.***

Using the pyrosequencing method, 58 cases were examined. Codon 12 mutations were identified in 17 cases, and wild type in the rest of them. The types of substitutions identified in KRAS gene, codon 12 were: 11 cases with G→A transition and six cases with G→T transversion (five cases GGT>GTT substitution and one case with GGT>TGT substitution) (Figures 2–5).

The mutation in codon 13 was GGC>GAC substitution and it was identified in six cases, the rest cases were wild type (Figure 6).

All cases were wild type in codon 61, as assessed by pyrosequencing (Figure 7).

Survival analysis shows significant differences between patients with wild type gene and with mutation in codon 13 (Figure 8), therefore, KRAS gene mutation in codon 13 could be considered a poor prognostic factor in patients from the study (Table 1, $p<0.013$).

Table 1 – ***Test of equality of survival distributions for the different levels of codon 13***

Case processing summary				
Codon 13	***Total No.***	***No. of events***	***Censored (survival)***	
			No.	*Percent*
Yes	7	5	2	28.6%
No	68	16	52	76.5%
Overall	75	21	54	72.0%

Overall comparisons			
Tests	***Chi-square***	***df***	***Sig. (p-value)***
Log-rank (Mantel–Cox)	6.230	1	0.013
Breslow (generalized Wilcoxon)	5.815	1	0.016

Statistical analysis of survival in TNM stages

Stage I

Of the 18 patients in TNM stage I, 14 patients with wild type KRAS gene had a 100% survival rate (Table 2) The factor showing significant association with poor survival was mutation in codon 13; so, the codon 13 mutation may be considered a bad prognostic factor in TNM stage I patients (p=0.000).

Table 2 – ***Test of equality of survival distributions for the different levels of codon 13 in stage I***

Case processing summary[a]				
Codon 13	***Total No.***	***No. of events (deaths)***	***Censored (survival)***	
			No.	*Percent*
Yes	4	3	1	25.0%
No	14	0	14	100.0%
Overall	18	3	15	83.3%

Overall comparisons[a]			
Tests	***Chi-square***	***df***	***Sig. (p-value)***
Log-rank (Mantel–Cox)	13.307	1	0.000
Breslow (generalized Wilcoxon)	12.792	1	0.000

[a]TNM staging_Simpl = I.

Stage II

Of the 24 patients in TNM stage II, we identified one patient with mutation in codon 13 and he died. The statistical analysis revealed significant survival differences depending on mutational status in codon 13 (Table 3) and this mutation could be considered poor prognostic factor in patients in TNM stage II (*log*-rank test, $p<0.001$).

Table 3 – ***Test of equality of survival distributions for the different levels of codon 13 in stage II***

Case processing summary[a]				
Codon 13	***Total No.***	***No. of events (deaths)***	***Censored (survival)***	
			No.	*Percent*
Yes	1	1	0	0.0%
No	23	3	20	87.0%
Overall	24	4	20	83.3%

Overall comparisons[a]			
Tests	***Chi-square***	***df***	***Sig. (p-value)***
Log-rank (Mantel–Cox)	10.267	1	0.001
Breslow (generalized Wilcoxon)	9.800	1	0.002

[a]TNM staging_Simpl = II.

Stage III

We found no statistically significant differences between the survival curves or occurrence of relapse, according to KRAS gene mutation status in both codons, for the 26 patients in TNM stage III.

Stage IV

We analyzed six patients TNM stage IV: three patients with wild-type KRAS gene (one patient with bone metastases and two with peritoneal carcinomatosis) and three patients with codon 12 mutations (one with single brain metastasis and two with liver metastases). Surprisingly, statistical analysis showed a better survival in patients with mutation (*log*-rank test, $p<0.025$) (Table 4). Since the reduced number of the cases could be a cause of these statistical results, it has also to mention that the patients were differently treated with antiangiogenic agents, or had various localization and types of metastases.

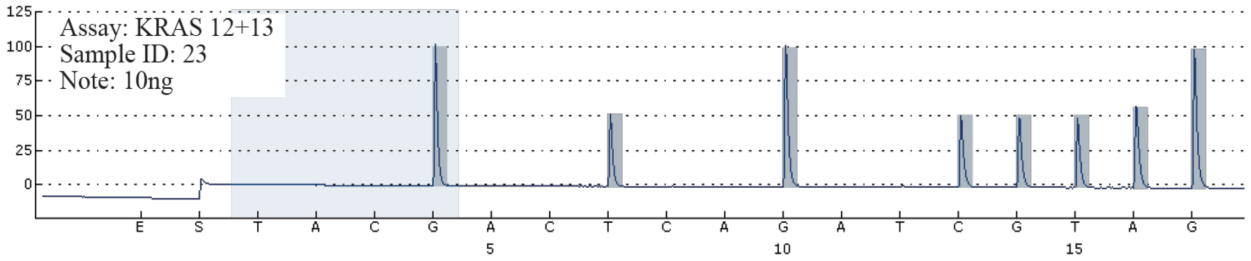
In patients in A and B Dukes stages, we have found differences in survival of patients with mutation in codon 13 comparing with patients with wild type KRAS gene (stage A – $p<0.046$; stage B – $p<0.001$).

Table 4 – ***Test of equality of survival distributions for the different levels of codon 12 in stage IV***

Case processing summary[a]				
Codon 12	***Total No.***	***No. of events (deaths)***	***Censored (survival)***	
			No.	*Percent*
Yes	3	0	3	100.0%
No	3	3	0	0.0%
Overall	6	3	3	50.0%

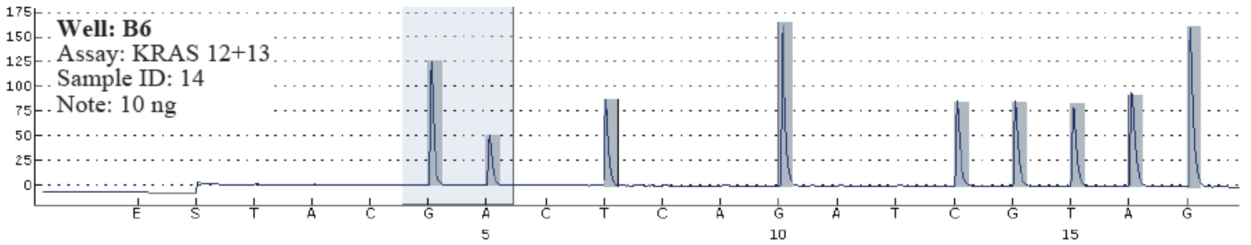
Overall comparisons[a]			
Tests	***Chi-square***	***df***	***Sig. (p-value)***
Log-rank (Mantel–Cox)	5.052	1	0.025
Breslow (generalized Wilcoxon)	4.500	1	0.034

[a]TNM staging_Simpl = IV.



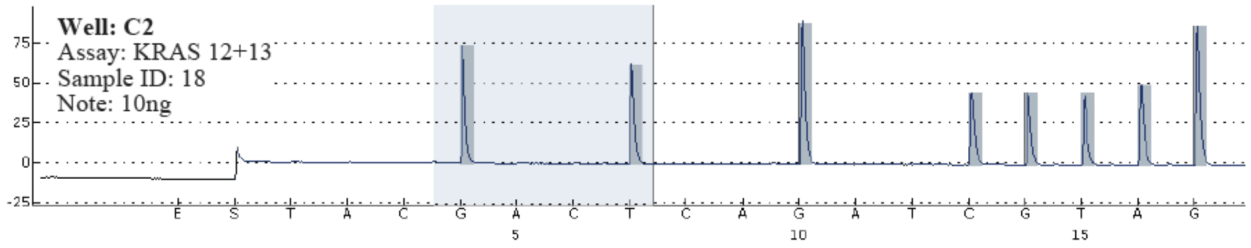
Result	**WildType**
Quality	Passed

Figure 2 – ***Example of wild type – codons 12, 13.***



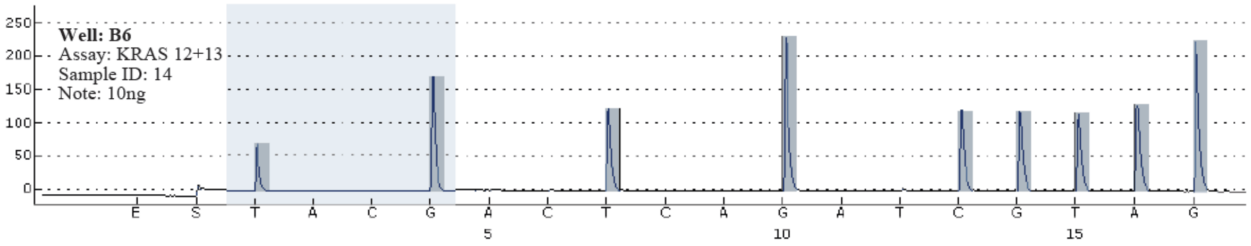
Result	**Mutation**
Frequency	52.5 % units (LOD: 2.2 % units)
Nucleotide Substitution	GGT>GAT
Amino Acid Substitution	G12D
Quality	Passed

Figure 3 – ***Example of mutation in codon 12 (GGT>GAT).***



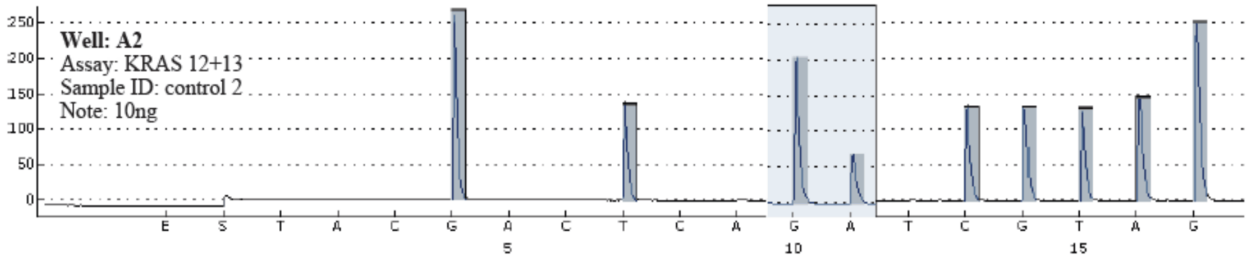
Result	**Mutation**
Frequency	37.7 % units (LOD: 1.0 % units)
Nucleotide Substitution	GGT>GTT
Amino Acid Substitution	G12V
Quality	Passed

Figure 4 – ***Example of mutation in codon 12 (GGT>GTT).***



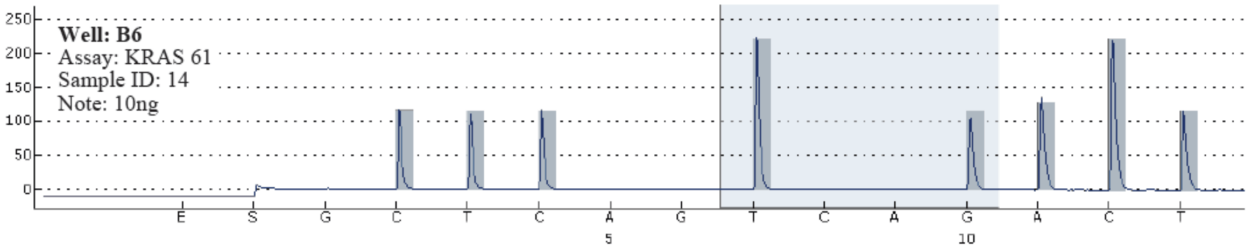
Result	Mutation
Frequency	56.5 % units (LOD: 2.1 % units)
Nucleotide Substitution	GGT>TGT
Amino Acid Substitution	G12C
Quality	Passed

Figure 5 – ***Example of mutation in codon 12 (GGT>TGT).***



Result	Mutation
Frequency	45.8 % units (LOD: 1.9 % units)
Nucleotide Substitution	GGC>GAC
Amino Acid Substitution	G13D
Quality	Passed

Figure 6 – ***Example of mutation in codon 13 (GGC>GAC).***



Result	WildType
Quality	Passed

Figure 7 – ***Example of wild type – codon 61.***

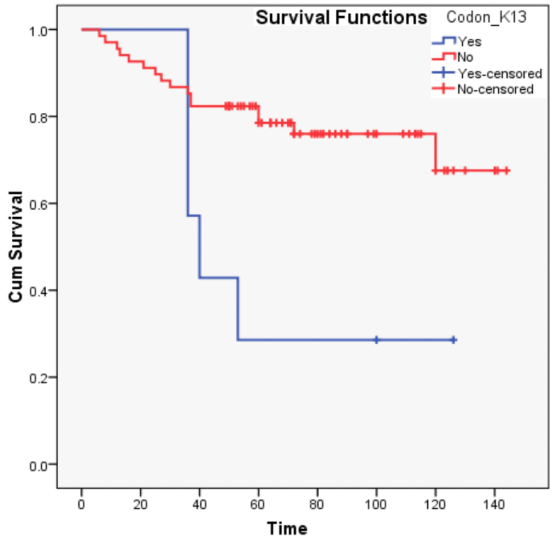


Figure 8 – ***Survival distributions for the different levels of codon 13.***

Discussion

The prognosis of CRC is affected by the mutational status of several genes, as including KRAS. Tumorigenesis and tumor progression in CRC result from multiple genetic and epigenetic abnormalities, including defective DNA mismatch repair and mutation of KRAS, NRAS, BRAF, PI3K, PIK3CA and p53 [10–12].

These (epi)genetic changes may affect the survival of CRC patients. In this study, we focused on the status of KRAS mutation and correlated it to patient's survival.

In the current study group, the frequency of KRAS gene mutations was 45.3%, which is comparable with results of other [13–19].

Data from the literature suggests that the KRAS mutation is mainly present in codon 12 and 13 (95% of all mutations); other mutations in codons 61, 146, 154 appear rarely, about 5% of all the mutations. In codon 12, the most frequent mutation was G12V and G12D, and G13D in codon 13 [20].

In our study group by pyrosequencing we assessed mutations in codons 12, 13, 61. Mutations in codon 12 accounted for 79.3% of the mutations, the rest of them being in codon 13; there were no mutations identified in codon 61. Regarding the types of substitutions, we have found: in the codon 12 – G12D in 64.7% cases, G12V in 29.4% cases, and G12C in 5.9% cases; in the codon 13, we have found G13D mutation in 5/5 cases.

Some associations were found in the literature, regarding the codon 12 and 13 located mutations: mucinous character associated with codon 12 mutations, while non-mucinous, but more aggressive with rapidly metastatic spreading associated with codon 13 mutations [21–23].

Our results were not entirely in accord with those of other reports, as we found that only nine out of 21 (43%) mucinous adenocarcinomas presented mutations in codon 12 in respect to 57% found in the literature [22]. For the non-mucinous adenocarcinoma, our results showed 17 (32%) cases with codon 12 mutations, out of 53 cases. Only 7.5% from non-mucinous adenocarcinomas presented mutation in codon 13, while other studies showed 24%.

In terms of aggressiveness and prognostic evaluation, nine patients had histological grade type G3, but no one had mutation in codon 13; from the 29 patients with lymph node metastases, only one had mutation in codon 13.

Interest in the KRAS gene mutations is both prognostic and predictive; KRAS gene mutations is the most widely studied biomarker and most promising in what concerns the treatment strategies in CRC.

Most studies suggest a prognostic value for the KRAS gene status in CRC [24]. In the study group, mutated KRAS could be a poor prognostic factor when it is appearing in codon 13. Several clinical trials studied the predictive and prognostic value of KRAS mutations in CRC, but the results were contradictory. Clinical trials as RASCAL conducted on 2721 patients found a significant association of KRAS mutations with the overall survival and relapse [24], while other study [25] observed no correlation between KRAS mutations and patients' prognostic. Similar results with this second type of study we obtained in our study group for TNM stages, II and III.

However, multivariate analysis by Bazan *et al.*, revealed that KRAS codon 13 mutations, but not other mutations, were independently related to the risk of relapse or death in a consecutive series of 160 untreated patients (median of follow-up period = 71 months), who underwent resective surgery for primary CRC [22]. Consistent with this study, Yokota *et al.*, examined 229 patients with advanced and recurrent CRC who were treated with systemic chemotherapy, and demonstrated that the OS (overall survival) for patients with KRAS 13 mutations was significantly worse than for those who had wild-type KRAS and wild-type BRAF, whereas KRAS 12 mutation did not affect patient OS [26].

Furthermore, KRAS/BRAF genotype was analyzed in a large subgroup of 845 patients with metastatic CRCs who received FOLFIRI and FOLFOX chemotherapy with or without cetuximab as the first-line treatment in the CRYSTAL and OPUS studies, respectively [27]. The results revealed that KRAS 13D mutations are associated with poor prognosis as our results reported, although patient selection was randomly made and treatment was different.

All mentioned studies revealed that stage III patients with KRAS mutations displayed significantly worse disease-free survival than those with wild-type KRAS [28–30], which might be partially explained by the impact of either codon 12 or codon 13 mutations on prognosis, but the results are different from our study which did not obtained any significant association for this stage.

In our study group, in patients in stages I and II TNM, KRAS mutation in codon 13 was correlated with statistically significant lower survival and there were no proof that mutation in codon 12 would have a prognostic role. There are no studies in literature regarding the prognostic role of KRAS mutations in TNM stage I colorectal cancer.

RASCALII study showed that mutation in codon 12 is aggressive in patients with CRC Dukes stage C [31], and is associated with an increase of about 50% in the risk of relapse or death in this patient group. Unlike the results of this study, we have found no prognostic differences for KRAS mutations in codon 12. From the 26 patients in Dukes stage C, seven had mutations in codon 12 and 19 patients respectively, wild type. We recorded 12/26 (53.8%) deaths, 3/7 (57.1%) of patients with mutations and 9/19 (52.6%) of patients with wild type.

In the study group, in patients with Dukes stages A and B, we found that mutations in codon 13 are an unfavorable prognostic factor, but we cannot relate to similar data from the literature.

Taken together, differences in KRAS mutations at codons 12 and 13 may result in different biological, biochemical, and functional consequences that could influence the prognosis of CRC [27]. Larger studies are required to confirm whether a specific KRAS mutation might lead to a clinically relevant prognostic effect in patients with CRC.

Identification of KRAS gene mutation and testing the types of mutation confers a new opening for personalized medicine and its use as a new biomarker. The targeted therapy is usually approved to be used as second- or third-line therapy [32]. Applying personalized treatment not only benefits the patient by reducing side effects and improving survival, but also the health care system by lowering costs. In the future, personalized treatment will mean the application of therapeutic protocols tailored as much to the individual, including minimally invasive surgery combined with PCT and monoclonal antibodies.

Conclusions

According to statistical analysis, the type of KRAS mutations may have prognostic implications in patients with CRC. Mutations in codon 13 are associated with statistically significant reduced survival, regardless of TNM stages, and histological types. KRAS gene mutations do not appear as a poor prognostic factor in patients with TNM stage IV CRC as showed our study.

Conflict of interests

The authors declare that they have no conflict of interests.

Acknowledgments

This work was supported by the strategic grant POSDRU/159/1.5/S/133391 and POSCCE 173/2010.

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Received: September 20, 2014

Accepted: August 15, 2015