Pathol. Oncol. Res. (2015) 21:399–404
DOI 10.1007/s12253-014-9834-2

RESEARCH

Changes of KRAS Exon 2 Codon 12/13 Mutation Status in Recurrent Colorectal Cancer

Ottó Dócs · Ferenc Fazakas · Nóra Lugosiné Horváth · László Tóth · Csilla András · Zsolt Horváth · Gábor Méhes

Received: 9 April 2014 / Accepted: 13 August 2014 / Published online: 24 September 2014

Abstract Colorectal cancer (CRC) is a heterogeneous disease presenting with a wide spectrum of morphological and molecular characteristics sometimes even within the same patient. To understand the mechanisms of oscillations in the KRAS status we evaluated the collective of CRC patients tested using allele-specific PCR and Sanger-sequencing. Mutant KRAS allele was observed in 43.3 % of cases. Repeated analysis of KRAS status in recurrent tumors or metastases was performed in 18/665 cases and a total of 6 cases with different KRAS status was found. In three cases the histological pattern of the tumor was identical. In one patient different histology and molecular status was seen between the primary and the recurrent tumor samples. In two further cases localization, histological type and KRAS mutational status all supported the occurrence of synchron/metachron colorectal tumors. In conclusion, both the progression of the original disease but also multiple tumor formation may contribute to mutation status differences during the course of colorectal carcinoma.

Keywords KRAS · Colorectal carcinoma · Heterogeneity · Multiple tumors

O. Dócs · F. Fazakas · N. L. Horváth · L. Tóth · G. Méhes (✉)
Department of Pathology, Medical Center, University of Debrecen, Nagyerdei krt. 98, H-4032 Debrecen, Hungary
e-mail: gabor.mehes@med.unideb.hu

G. Méhes
e-mail: gabor.mehes@dote.hu

C. András · Z. Horváth
Department of Oncology, Medical Center, University of Debrecen, Nagyerdei krt. 98, H-4032 Debrecen, Hungary

Introduction

Molecular testing of EGFR (Epidermal growth factor receptor) and its downstream molecule, the KRAS (Kirsten rat sarcoma viral oncogene homolog) genes are used as predictive factors of TKI therapy. EGFR is a transmembrane tyrosine kinase receptor triggering RAS-RAF-MAPK and PI3K-PTEN-AKT signaling pathways [1]. By these signal transductors the growth signal from EGFR molecule is transduced to the nucleus stimulating cell proliferation and inhibiting apoptosis. Oncogenic mutations of KRAS gene are observed in 30–50 % of metastatic colorectal cancer [2, 3] comprising 86 % of all RAS family member mutations [4]. Tanaka et al. reported that *KRAS* mutation was an independent factor associated with prognosis in a multivariate analysis [5]. In The Kirsten Ras In-Colorectal-Cancer Collaborative Groups (RASCAL) multivariate analyses, the presence of *KRAS* mutation was significantly associated with poorer prognosis [6]. Lievre et al. first reported the link between *KRAS* gene mutation and decreased response to anti-EGFR agents [7]. The analysis of the most frequent activating mutations in exon 2 of the KRAS gene (codon 12 and 13 mutations) became a routine procedure in colorectal cancer diagnostics which was recently extended with the less frequent exon 3 and 4 as well as NRAS exon 2, 3 and 4 mutation testing. The success of an anti-EGFR treatment necessitates the exclusion of dominant RAS mutations which trigger the signaling pathway independent from the EGFR tyrosin kinase activity (Fig. 1). RAS mutation testing is done in the majority of primary CRC cases from DNA isolated from the FFPE tumor samples right after histology.

Despite of reports on the intratumoral mutational heterogeneity and potential changes in the KRAS status during the course of the disease it is generally believed that KRAS mutations occur early during the process of neoplastic transformation and by that it is a constant genetic abnormality [8,

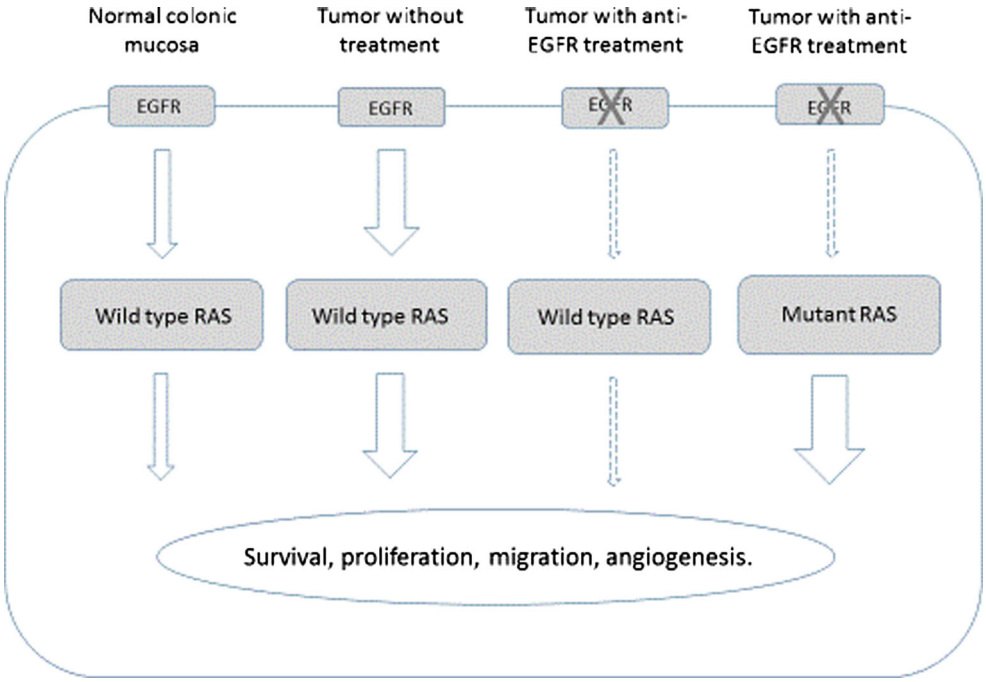

Fig. 1 The influence of mutational status of RAS on the anti-EGFR treatment efficacy in metastatic colorectal adenocarcinoma

9]. Primarily financial aspects can explain why serial reanalysis of the KRAS mutation status in disease recurrence is rarely done. During the routine testing of a large number of colorectal carcinoma samples in the last 5 years we were continuously interested in the reproducibility of the KRAS status in recidive tumor samples obtained from the same patient in different time points of the disease and we also looked for potential biological explanations in the background of the changes demonstrated. After the exclusion of technical problems the role of mutational status heterogeneity and the opportunities for multiple tumor development were addressed in particular.

Patients and Methods

Patients and Samples

665 samples from 645 patients suffering from colorectal adenocarcinoma were retrospectively evaluated for complete clinicopathologic characteristics including age, gender, tumor localization, histological type, grade, TNM stage, KRAS mutation status and KRAS genotypes. All patients had undergone primary tumor surgery for diagnostic and therapeutic purposes. The samples were fixed in neutral-buffered formalin and embedded in paraffin as usual. Sections were stained with H&E and examined by dedicated pathologists. Samples were collected according to the rules and regulations of the Clinical Center at University of Debrecen with the approval of the local ethical committee (file number: RKEB/IKEB 3856–2013).

DNA Isolation and KRAS Mutation Analysis

Samples with tumor area ~1 cm^2 were selected for KRAS mutation analysis. DNA isolation was carried out from a 1x5μm thick FFPE slides after xylene deparaffination using a commercially available kit (Quick Gene DNA Tissue Kit, KURABO, Japan) according to manufacturer's instructions (OD260/OD280=1.5–2.1).

KRAS exon 2 codon 12–13 mutation analysis was performed by a PNA clamped RT-PCR assay based on the direct blocking of the wild type allele of examined exons. For each sample two PCR reactions (unclamped and PNA-clamped) were run. For PCR clamping 600 nM PNA was added to PCR mixture. PCR was performed on a 7300 Real Time PCR System for 45 cycles followed by a 10 min elongation step at 72 °C. The samples were considered mutant when amplification of the clamped reaction occurred as follows: $\Delta Ct_{(clamped\text{-}unclamped)} < 10$ (e.g. 0.1 % mutant allele frequency) and $Ct_{(clamped)} < 40$. ROC analysis of the diagnostic setup showed that the chance for false positivity is the least when $\Delta Ct < 5.26$ (mutant allele frequency >2.6). Sanger sequencing of PCR products was performed in all cases with ΔCt higher than 5.26 on a 310 Genetic Analyzer (Applied Biosystem) using the forward PCR primer and Big Dye Terminator chemistry.

Statistical Analysis

Molecular results together with the clinicopathologic data were statistically analyzed using median±SD, average, Students *t*-test and compared with literature data.

Results

Clinicopathologic Characteristics of KRAS Tested Colorectal Adenocarcinomas

Results of altogether 665 KRAS analyses were evaluated. 427 patients were males (64.2 %), the median age of patients was 60 years (range 19–86). Detailed clinicopathologic data including primary tumor localization were available from 572 in-house cases. The vast majority of samples had origin from the recto-sigmoid region (rectum: n=276, 48.3 %; sigma: n=129, 22.5 %). 83.2 % of primary tumors were identified as pT3-T4 (pT3: 63.8 %; pT4: 19.5 %). Nodal status was observed as follows: pN0: 35.0 %; pN1: 44.5 %; pN2: 20.5 %. In 75.6 % of the cases histological grade 1–2, in 24.4 % grade 3–4 was described. Mutant KRAS allele was observed in 43.3 % of the cases (42.1 % in males; 45.4 % in females). G12D, G12V and G13D were the most frequent genotypes.

Analysis of Cases With Multiple KRAS Examinations

In 18/665 cases multiple KRAS analyses were performed from at least two sequential samples obtained at different times from the same patient. In 6/18 cases different KRAS status and/or genotype was observed. All samples were reexamined for KRAS mutation including sequencing of the exon 2 codon 12/13 region to exclude analytical problems. Histological types were also revalidated and all histological and molecular features were summarized (Table I). In order to understand the nature of the observed molecular differences all cases were individually analyzed and interpreted.

Patient 1 was a 61-year-old male with tubulovillous primary tumor (Grade 2, Stage pT3 pN1 pM1) of the transversal colon and wild type KRAS. 18 months after 1st line FOLFOX therapy hepatic metastases with identical histology occurred showing a KRAS exon 2 mutation at G12C with a mutation rate of 25 %. Repeated analysis of the primary lesion excluded the presence of G12C mutated subclones. Mutant KRAS status indicated a second line bevacizumab+FOLFIRI therapy.

Patient 2 was a 56-year-old female with a rectum tumor presenting with a conventional tubular histology (Grade 2, Stage pT2 pN0 pM1). The initial biopsy repeatedly showed a wild type KRAS status, but the surgically resected primary tumor showed a G12V mutant KRAS genotype (tumor cell content – 90 %, mutation rate – 25 %) in the presence of identical histological architecture. She received bevacizumab as 1st line treatment and had a stable disease for 23 months when a recurrent rectal tumor with tubular architecture was observed. As 2nd line therapy she received the FOLFIRI protocol. After another 20 months stable disease predominantly tubulovillous metastases of the thyroid gland occurred which proved to be of colorectal adenocarcinoma origin (CK20+, CDX2+, TTF1-). The KRAS status of the thyroid metastasis was mutant (G12V) that was identical with that of the primary tumor resected 2.5 years before.

Patient 3 was a 66-year-old male diagnosed with a primary tubular adenocarcinoma (Grade 4, Stage pT3 pN0 pM0) of the right colon (hepatic flexure) showing mutant KRAS with G13D genotype (12 % mutation rate). He received bevacizumab+FOLFIRI first line therapy and 5FU as second line therapy. After 9 months of stable disease a hepatic metastasis was discovered. The biopsy presented with a tubular histological type and with a wild type KRAS status. Targeted anti-EGFR therapy was, however, not applied due to the rapid progression of the process.

Patient 4 was a 51-year-old male treated for Crohn's disease for 11 years before a primary tumor in the rectosigmoid localization and tubular histology (Grade 1, Stage pT1 pN0) was diagnosed. Molecular testing resulted in a wild type KRAS status. After 28 months following hemicolectomy and a stable disease a tumor in the caecum – far from the surgical resection line – was observed. Histologically it showed primarily tubulovillous pattern (Grade 1, stage pTis pN0) and a mutant KRAS status was stated with the G12C genotype. The patient underwent a second surgery which was followed by a combined bevacizumab+FOLFIRI chemotherapy. After 13 months a tumor mass in retroperitoneal space was discovered by the control CT scan. The guided needle biopsy presented a metastatic mucinous adenocarcinoma with colon origin and a wild-type KRAS status was stated. The examination of the surgically removed tumor mass confirmed the mucinous histological type, but molecular typing of this metastatic tumor sample revealed a different mutation at G12V (Fig. 2)

Patient 5 was a 59-year-old male with two synchronously growing tumors, one in the sigmoid colon (Grade 2, Stage ypT3 ypNx) and one in the rectum (Grade 2, Stage ypT2 ypNx), both similarly showing tubular tissue architecture. However, the sigma tumor proved to be KRAS mutant with G12C genotype (mutation rate 12 %), while the rectal one was KRAS wild type. Both tumors were dissected and the patient was treated by first line FOLFIRI followed by second line FOLFOX and third line anti-EGFR chemotherapy (panitumumab). 6 months

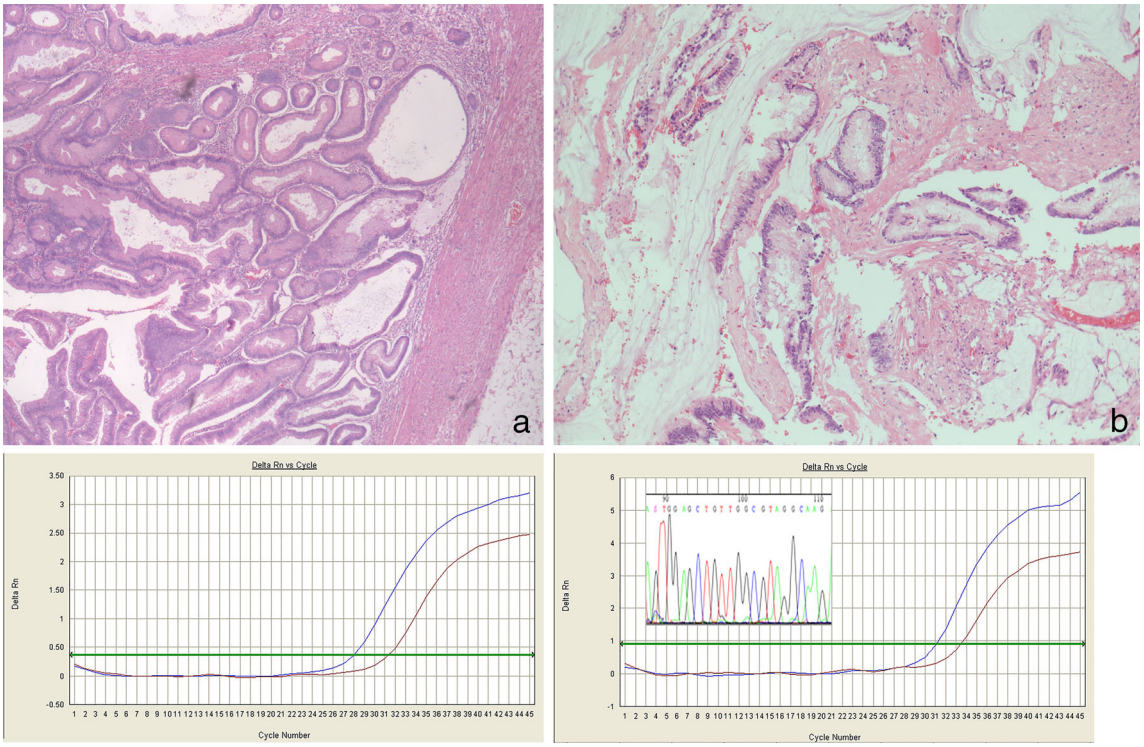

Fig. 2 Tubular histologic type and wild type KRAS PCR-result of the primary adenocarcinoma of the sigma **a** compared to the mucinous histologic pattern featuring mutant KRAS genotype of the retroperitoneal metastatic tumor **b** from the same patient (case no. 4) (HE; 10× magnification). The time span between the two tumor samples was 41 months. KRAS exon two mutant allele specific PCR was done from the FFPE tumor material displayed in the figure

after the detection of the double primary tumors a hepatic metastasis with tubular histology occurred. The metastasis showed a wild type KRAS status.

Patient 6 was a 52-year-old male presenting with a tubular rectal tumor (Grade 4, Stage pT3 pN0) and with a mutant KRAS status (G12D genotype, 12 % mutation rate). After 42 months of first line FOLFIRI and second line FOLFOX therapy a second tumor of the rectum was observed. The histological type proved to be mucinous adenocarcinoma and a wild type KRAS was demonstrated. Panitumumab monotherapy for seven cycles was started which resulted in at least 4.5 months stable disease. Treatment was then intensified by the administration of capecitabine.

Discussion

Our results also indicate that colorectal cancer is a heterogeneous disease both in its histopathologic appearance and in its molecular pathologic composition. However, internal variations of the genetic features remain mostly uncovered for the routine oncological practice. In our collection of almost 700 colorectal adenocarcinomas tested between 2008 and 2012 we identified six cases with differences in the KRAS mutational status during disease progression and serial testing. In the frame of this study all samples were repeatedly evaluated for exon 2 codon 12/13 mutations and carefully evaluated to exclude technical artifacts. In addition, detailed histological revision was also performed to identify morphological specialities between samples obtained at different time points. Following the summary of all available data we concluded that differences in KRAS status occured due to different biological mechanisms.

Cases 1–3 showed different KRAS status in the primary and metastatic/recidive tumors while no definitive change in the histological type during progression was observed. The mutation rates determined by allele specific PCR amplification (Table 1.) suggested mutational heterogeneity within the same tumor potentially enabling the persistence and dissemination of genotypically different tumor subclones during the progression of the disease. In this model true tumor heterogeneity may explain differences in the mutational status during the course of the disease.

Table 1 Summary of cases showing differences in KRAS exon two mutation status in sequentially obtained from follow-up tumor samples. Histopathological and KRAS genotype differences between primary tumors and their recidive/metastatic lesions were evaluated. M-male; F-female; NA-data not available

Case #	Gender (age)	Anatomic site of tumor	Time between tumors (months)	Histologic type (grade)	KRAS genotype	Mutation rate	Tumor cell content
The same histologic type but different genotype of tumor							
1	M (61)	Colon transversum	18	Tubulovillous (G2)	Wild type	–	30 %
		Liver met		Tubulovillous	G12C	25 %	10 %
		Lung met		Tubulovillous	Mutant	NA	50 %
2	F (56)	Rectum biopsy	23	Tubular	Wild type	–	40 %
		Rectum		Tubular (G2)	G12V	25 %	90 %
		Rectum		Tubular	Wild type	–	NA
		Tyroid gland	20	Villous	G12V	75 %	10 %
3	M (66)	Flexura hepatica	9	Villous (G4)	G13D	12 %	75 %
		Liver		Tubular	Wild type	–	50 %
Differencies between synchronous/metachronous tumors and their metastase							
4	M (51)	Rectosigma	28	Tubular (G1)	Wild type	–	10 %
		Caecum		Tubulovillous (G1)	G12C	20 %	40 %
		Retroperitoneum biopsy		Mucinous	Wild type	–	10 %
		Retroperitoneum	13	Mucinous	G12V	15 %	30 %
5	M (59)	Sigma	0	Tubular (G2)	G12D	12 %	5 %
		Rectum		Tubular (G2)	Wild type	–	5 %
		Liver	6	Glandular	Wild type	–	5 %
6	M (52)	Rectum	42	Tubular (G3)	G12D	12 %	10 %
		Rectum		Mucinous	Wild type	–	20 %

An alternative mechanism could be suggested when molecular heterogeneity is associated with significant differences in the histological architecture. Moreover, distant anatomical locations within the large intestine further indicate to the coexistence of independent tumors. In cases 4–6 described here tissue changes occurred in a timely and spatially separated manner. Case 4 presented with two obviously independent colon cancers (rectum and cecum) and a retroperitoneal metastasis which was difficult to associate with any of the primaries due to phenotypic and genotypic deviations. In case 5 a G3, pT3 pN0 stage tumor was first observed which was followed by a histologically and genetically different second colon tumor occurring 42 months later. Finally, the synchronously growing colon carcinomas and distant metastases demonstrated in case 6 also proved to be different both at histological and at the molecular level.

Current data about the molecular heterogeneity in colorectal cancer are controversial. Oltedal and coworkers demonstrated variable distribution of KRAS codon 12 and 13 mutations between primary tumor and corresponding sentinel lymph node metastases in 20 % of cases [10]. Knijn and coworkers observed a high concordance (96.4 %) between the KRAS mutation status of colorectal cancers and their corresponding liver metastases, meaning 5.6 % discrepancies for any reason. Inspite of this, they suggested that both primary tumors and liver metastases could be used for KRAS mutation analysis [11]. Kimura et al. described intratumoral heterogeneity in 7 % of CRC representing different single mutations [12]. Losi et al. observed intratumoral heterogeneity in 60 % of early CRCs with no predominant KRAS mutant clones, however, advanced stages were found to become homogeneous for KRAS in 80 % due to clonal progression [8]. Gash et al. demonstrated considerable intra- and inter-patient heterogeneity of EGFR expression and genetic alterations in EGFR, KRAS, and PIK3CA, serving with explanation for the variable response rates to EGFR inhibitors in patients with CRC [13].

During the progression of malignancies new mutations are acquired contributing to the development of a more aggressive and metastatic phenotype through clonal evolution [14]. These phenotypic and molecular characteristics enable the accurate analysis of tumor development in individual cases. In the light of the cited observations and also of our experience, the significance of multiple tumor formation in CRC is underestimated. Tziris and coworkers described synchronous and metachronous adenocarcinomas of the large intestine in 12/268 (4.3 %) cases. Metachronous cancers were found to be more frequent and more often localized in the rectum [15].

Although the occurrence of synchronous/metachronous colorectal tumors is clinically relevant, a detailed and systemic comparative analysis considering the KRAS status is still missing.

The method based on PNA blocking of wild type allele and PCR amplification of mutant sequences allows quantitative determination of the mutant alleles within a tumor mass. In general, mutations are allowed to be safely detected at a concentration of 2 %, which could be also proven by direct sequencing in all cases in our practice. However, the exact nature and biological significance of activating mutations at this low frequency is unclear. Due to some technological limitations and the admixing of non-neoplastic component true intratumoral heterogeneity mutation rates of 5 % or less should be evaluated with caution. Heterogeneity on the other hand may evolve for many biological reasons. It may occur due to early clonal evolution, but passive biological mechanisms, e.g. chromosomal aneusomies, allelic losses, etc. may also significantly influence mutant allele density. The exact intratumoral distribution of the proposed genotypic heterogeneity is neither really known nor understood. There might be differences between well and poorly differentiated areas, but also between the central and the peripherial/invasive zones of the tumor. Routinely performed molecular analyses were developed to represent the mutational status in general but they do not really focus on the KRAS mutational spectrum occurring at the tissue or cellular level.

In summary, multiple biological reasons may contribute to cause alterations in KRAS mutational status in colorectal cancer when serially tested. The case can be even more complex considering multiple clinically relevant mutations, such as those of the extended RAS gene family. For the above mentioned reasons it seems to be increasingly relevant to perform molecular testing in any new lesions in colorectal carcinoma, especially when obvious pathoanatomical differences are stated.

Acknowledgments The publication and scientific work was supported by the TÁMOP-4.2.2.A-11/1/KOV-2012-0045 project and the MTA-DE Vascular Biology, Thrombosis and Hemostasis Research Group (vascular biology – 11003) of the Hungarian Academy of Sciences.

References

1. Baselga J (2001) The EGFR as a target for anticancer therapy – focus on cetuximab. Eur J Cancer 37:16–22
2. Vogelstein B, Fearon ER, Hamilton SR, Kern SE, Preisinger AC, Leppert M, Nakamura Y, White R, Smits AM, Bos JL (1988) Genetic alterations during colorectal-tumor development. N Engl J Med 319(9):525–532
3. Vaughn CP, Zobell SD, Furtado LV, Baker CL, Samowitz WS (2011) Frequency of KRAS, BRAF, and NRAS mutations in colorectal cancer. Gene Chromosom Cancer 50(5):307–312
4. Bamford S, Dawson E, Forbes S, Clements J, Pettett R, Dogan A, Flanagan A, Teague J, Futreal PA, Stratton MR, Wooster R (2004) The COSMIC (catalogue of somatic mutations in cancer) database and website. Br J Cancer 91(2):355–358
5. Tanaka M, Omura K, Watanabe Y, Oda Y, Nakanishi I (1994) Prognostic factors of colorectal cancer: K-ras mutation, overexpression of the p53 protein, and cell proliferative activity. J Surg Oncol 57(1):57–64
6. Andreyev HJ, Norman AR, Cunningham D, Oates JR, Clarke PA (1998) Kirsten ras mutations in patients with colorectal cancer: the multicenter "RASCAL" study. J Natl Cancer Inst 90(9):675–684
7. Lievre A, Bachet JB, Le Corre D, Boige V, Landi B, Emile JF, Cote JF, Tomasic G, Penna C, Ducreux M, Rougier P, Penault-Llorca F, Laurent-Puig P (2006) KRAS mutation status is predictive of response to cetuximab therapy in colorectal cancer. Cancer Res 66(8): 3992–3995
8. Losi L, Baisse B, Bouzourene H, Benhattar J (2005) Evolution of intratumoral genetic heterogeneity during colorectal cancer progression. Carcinogenesis 26(5):916–922
9. Velho S, Oliveira C, Seruca R (2009) KRAS mutations and anti-epidermal growth factor receptor therapy in colorectal cancer with lymph node metastases. J Clin Oncol 27(1):158–159, author reply 159
10. Oltedal S, Aasprong OG, Moller JH, Korner H, Gilje B, Tjensvoll K, Birkemeyer EM, Heikkila R, Smaaland R, Nordgard O (2011) Heterogeneous distribution of K-ras mutations in primary colon carcinomas: implications for EGFR-directed therapy. Int J Color Dis 26(10):1271–1277
11. Knijn N, Mekenkamp LJ, Klomp M, Vink-Borger ME, Tol J, Teerenstra S, Meijer JW, Tebar M, Riemersma S, van Krieken JH, Punt CJ, Nagtegaal ID (2011) KRAS mutation analysis: a comparison between primary tumours and matched liver metastases in 305 colorectal cancer patients. Br J Cancer 104(6):1020–1026
12. Kimura K, Nagasaka T, Hoshizima N, Sasamoto H, Notohara K, Takeda M, Kominami K, Iishii T, Tanaka N, Matsubara N (2007) No duplicate KRAS mutation is identified on the same allele in gastric or colorectal cancer cells with multiple KRAS mutations. J Int Med Res 35(4):450–457
13. Gasch C, Bauernhofer T, Pichler M, Langer-Freitag S, Reeh M, Seifert AM, Mauermann O, Izbicki JR, Pantel K, Riethdorf S (2013) Heterogeneity of epidermal growth factor receptor status and mutations of KRAS/PIK3CA in circulating tumor cells of patients with colorectal cancer. Clin Chem 59(1):252–260
14. Coghlin C, Murray GI (2010) Current and emerging concepts in tumour metastasis. J Pathol 222(1):1–15
15. Tziris N, Dokmetzioglou J, Giannoulis K, Kesisoglou I, Sapalidis K, Kotidis E, Gambros O (2008) Synchronous and metachronous adenocarcinomas of the large intestine. Hippokratia 12(3):150–152