

A Novel *K-ras* Mutation in Colorectal Cancer. A Case Report and Literature Review

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Abstract. *Background:* Activating mutations in the *K-ras* oncogene mainly occur in codons 12 and 13 and may be predictive of response to drugs directly linked to the *K-ras* signaling pathway, such as panitumumab and cetuximab. *Materials and Methods:* *K-ras* analysis was carried out on DNA extracted from paraffin-embedded tumor samples after microdissection. Exons 1 and 2 were amplified by PCR and then sequenced. *Results:* A never-reported *K-ras* mutation (CAG>TAG) determining a premature stop signal at codon 22 (Gln22Stop) was found in a patient with metastatic colorectal cancer. *BRAF* and *p53* were not found to be modified and microsatellite instability was not present. The patient, however, was found to be unresponsive to an anti-EGFR MAb treatment. *Conclusion:* This study is the first report of a novel *K-ras* truncating mutation in a patient with metastatic colorectal cancer and is also suggestive for the evaluation of alternative pathways to better identify individuals who are likely to benefit from targeted therapies.

K-ras proto-oncogene is frequently mutated in colorectal cancer and has been associated with colorectal cancer initiation and progression (1-2). Approximately 90% of the activating mutations, determining single amino acid substitution in the GTPase pocket that leads to a block of the GTP hydrolytic activity of *K-ras*-p21 protein, are found in codons 12 (GGT) and 13 (GGC) of exon 1 and nearly 5% in codon 61 (CAA) located in exon 2 (3-4). The most frequently observed types of mutations are G>A transitions

(5) and G>T transversions (6) and these alterations were found to be associated with gender and sub-localization of the tumor (7).

To date, frequencies and specific types of activating *K-ras* point mutations in colorectal cancer have been investigated in several studies including a selected although limited population of patients (1, 2, 8, 9). At the same time, other studies have analysed the frequency of the type of *K-ras* point mutation in colorectal cancer in a large series of unselected, incident colorectal cancer patients from various countries, *i.e.*, UK (10-11), former Yugoslavia (6), Czech Republic (12), Norway (7), Switzerland (13), Mexico (5), USA (14) and the Netherlands (15-18). All studies, except that performed in former Yugoslavia (6), have identified the G>A transition in codon 12 as the most frequently found type of *K-ras* mutation. Moreover, the majority, including the most recently published studies, reported only the presence of classical somatic mutations in codons 12 and 13 (19-20), while few other authors reported cases presenting mutations in other codons, even without mutations in codons 12 and 13. As an example, in the large Netherlands Cohort Study, 37% mutations were found in codons 12 and 13, but 6.6% were found in codons 8, 9, 10, 15, 16, 19, 20 or 25 (18). Moreover, Rouleau *et al.* reported a mutation in codon 19 in two patients, although no polymorphism has been described in this codon. In these two cases, the intensity of the mutated allele in the sequence electropherogram was less than 30% of the normal allele. It should be emphasized, however, that the intensity of mutated and normal allele in heterozygous polymorphism should have been similar (21).

A recent study by Akagi *et al.* identified a novel G to T transversion *K-ras* mutation at the third position of codon 19 in colon adenocarcinoma, determining a substitution of phenylalanine (TTT) for leucine (TTG) and, moreover an oncogenic *K-ras* activity (22). This mutation occurred at an early stage of tumor development, like mutations at codons 12, 13 and 61 (1).

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Mutations located at codons 15, 16, 18 and 31 were detected in few cases of adrenocortical tumors while they were not found in other tissues, strongly suggesting that the presence of these hot spots was highly related to adrenocortical tumors (23).

A study performed on 126 primary colorectal cancer tissues and 40 colorectal cancer cell lines identified a G to A transition, determining 146 missense substitutions (Ala146Thr) as a new type of recurrent somatic mutation involved in approximately 4% of colorectal cancer cases (24).

A more recent study by Wójcik *et al.*, reported a 15-bp insertion in exon 2 resulting in tandem duplication of codons 62-66 with a presumably functional addition of 5 amino acids (25).

The evaluation of *K-ras* mutations at codon 22 has been also performed (27, 28). C to A transversion substituting lysine (AAG) for normal glutamine (CAG) at codon 22 of *K-ras* was found in colon cancer (26-27), while Miyakura *et al.* presented, for the first time, point mutations of the *K-ras* oncogene at codons 22 (arginine for glutamine) and codon 12 (a substitution of serine for glycine) occurring concurrently in the same allele (28).

The majority of *K-ras* mutations and polymorphisms reported in the literature are available on the websites www.sanger.ac.uk/genetics/CGP/cosmic/ and dbSNP <http://www.ncbi.nlm.nih.gov/SNP>, respectively. The relatively low frequency of mutations outside of classical hot spots may be due to the fact that most clinical studies, however, limited the test to *K-ras* codons 12 and 13. Indeed to date, only two *K-ras* mutation test kits are commercially available and have met the essential requirements of the relevant European Directives (CEMark) for diagnostic use in the European Union. In both cases, only the 7 key mutations in the *K-ras* gene, at codons 12 and 13, are detectable (29).

K-ras mutational status has recently been proven to be highly predictive of the activity of two monoclonal antibodies directed against epidermal growth factor receptor (EGFR) (cetuximab and panitumumab) so that the exclusion of the use such drugs in the presence of mutated *K-ras* is becoming a standard in clinical practice (29). According to the emerging needs to tailor the treatment on the basis of mutational analysis, additional *K-ras*-activating mutations should be considered for predicting treatment response. Here we report, for the first time, a point mutation of codon 22 determining a premature stop signal and the synthesis of a truncating form of *K-ras*-p21 protein in a subpopulation of cells from a human adenocarcinoma of the ascending colon.

Case Report

A 43-year-old female was diagnosed with an adenocarcinoma of the ascending colon following investigations for increasing abdominal pain in the right lower quadrant associated with diarrhea. Baseline computed tomography (CT) scan revealed no

distant cancer disseminations and the patient underwent radical right hemicolectomy two weeks after diagnosis. The histological evaluation showed a pT3 pN0 (0/23) moderately differentiated mucinous adenocarcinoma and, after recovery from surgery, the patient received 6-month adjuvant chemotherapy, with an oxaliplatin/leucovorin/fluorouracil-based regimen. After 5 years from completion of adjuvant chemotherapy disease relapsed with multiple cancer dissemination to main lymph node regions (inguinal bilateral, right axillar and supraclavicular, retroperitoneal and mesenteric lymphonodal stations). A first-line bevacizumab/irinotecan/fluorouracil-based chemotherapy was started with a RECIST-defined stable disease at the repeated CT scan after three months of therapy and a progression of disease after six months. Because of the poor activity of the bevacizumab/irinotecan/fluorouracil combination and the lymphoid-specific cancer dissemination sparing the usual metastatic sites of liver and lungs, a possible differential diagnosis with lymphoma was raised and an inguinal lymph node biopsy was performed to address the histological nature of the metastatic disease. Histological examination revealed lymph node tissue infiltration from adenocarcinoma cells in keeping with metastatic cancer of colorectal origin.

In order to use an anti-EGFR monoclonal antibody (panitumumab)-containing second-line regimen, it was decided to assess EGFR immunohistochemical expression and *K-ras* gene mutational status.

Paraffin-embedded sections were collected on microscope slides. Tumor and tumor-free areas were identified within 15 µm-thick deparaffinized sections lightly counterstained with hematoxylin and microdissected by gentle scraping with sterile scalpels into 1.5 ml polypropylene vials, using a hematoxylin and eosin-stained step section from the same block as a guide. Areas representative of colorectal tumor were identified and the sample was microdissected into three parts (Figure 1).

DNA extraction from each microdissected area was performed as reported elsewhere (30).

Exons 1 and 2 of *K-ras* were individually amplified using a seminested amplification protocol (31). DNA extractions and set-up of PCR reactions were performed in a laboratory distinct from that in which amplified DNAs are manipulated. Sequencing reactions were performed using Big Dye Terminator (Applied Biosystem, Foster City, CA, USA), and run on an ABI 3130 Genetic Analyzer (Applied Biosystem). All sequencing analyses were carried out on both strands in order to exclude pre-analytical and analytical errors and were repeated on PCR products obtained from new nucleic acid extractions.

Furthermore, DNA samples were subjected to mutational analysis of *p53* (exons 5, 6, 7, 8 and 9) and *BRAF* (exon 15) performed by PCR and direct sequencing, as described above. Primers and PCR conditions are available upon request. To characterize genomic instability (MSI, microsatellite instability)

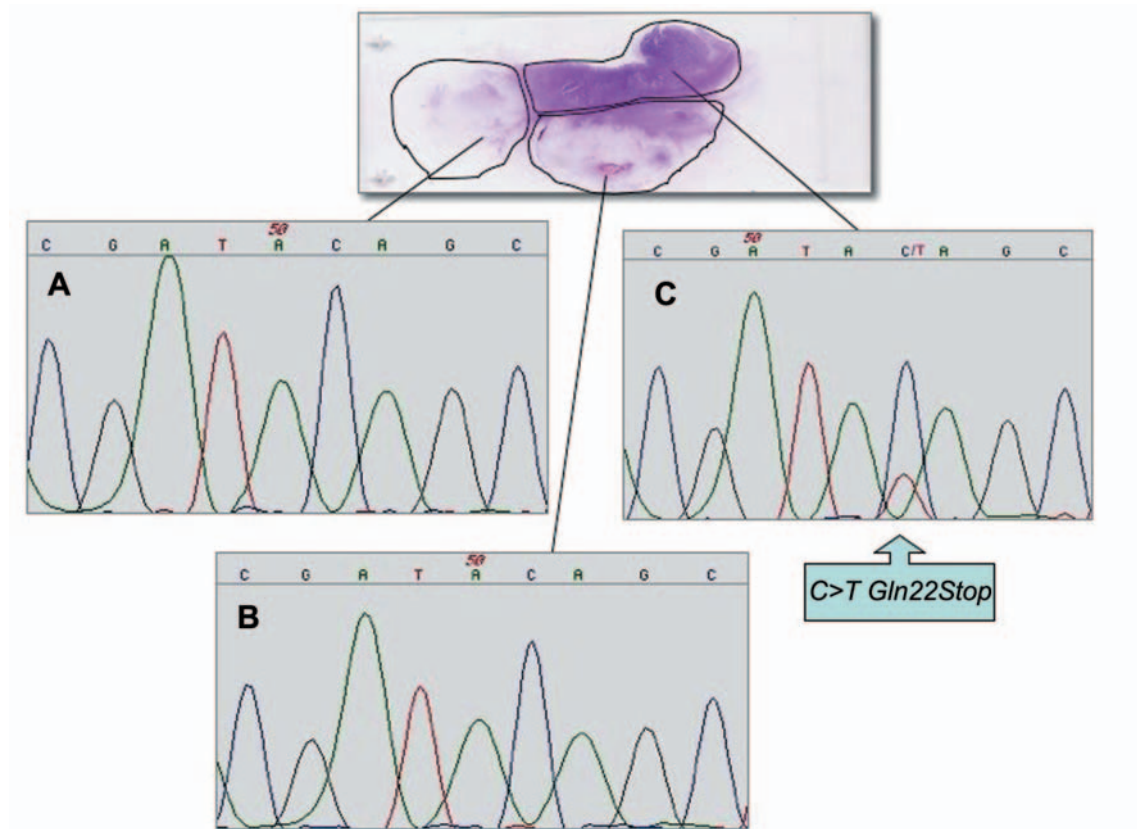


Figure 1. Sequence analysis of the *K-ras* exon 1 performed on DNA extracted from each microdissected area of paraffin-embedded tumor sections. Wild-type sequence (panels A and B) and C to T transitions (panel C) that determine a premature stop signal at codon 22 (Gln22Stop) in one of the three microdissected areas. The mutation was named according to recommendations of the Nomenclature System for Human Gene Mutations. The GenBank mRNA sequence (M54968) of *K-ras* was used as a reference.

DNA from the blood of the same patient and from microdissected tumoral areas was analyzed using microsatellite markers mononucleotide repeats, BAT26 and BAT40, known to be the most sensitive markers of MSI status and widely regarded as sufficient for the identification of colorectal cancer with mismatch repair (MMR) defect. Primers and PCR conditions used for amplifications were as described (32).

All analyses were confirmed in duplicate experiments, using independently extracted DNA samples.

A further genetic characterization of other molecular markers was not feasible due to insufficiency of extracted nucleic acids and the paraffin tissue.

Results

EGFR immunohistochemical expression in the lymph node biopsy material was mild (score: 1+ in 10% of tumor cells). The mutational analysis for *K-ras* exons 1 and 2 was performed by PCR and direct sequencing using somatic DNA extracted from paraffin-embedded tumoral tissue. Figure 1 shows sequencing results, and the arrow indicates the point

mutation at the first letter of *K-ras* codon 22, which is a C to T transitions that determines a premature stop signal at codon 22 (Gln22Stop) in one of the three microdissected areas. Regarding the detected *K-ras* variant, the intensity of this mutated band was about 35% of that of the normal band (Figure 1). Tumor morphological examination showed a heterogeneous spreading of cancer cells within the same tumor. Therefore it was hypothesized that this heterozygous mutation could not be present in all cancerous cells. For this reason, after a morphological reassessment by an independent pathologist, the microdissected area containing the mutation was further microdissected into three microareas using a new histological slide in order to identify Gln22Stop. Several experiments, however, did not reveal the presence of the sequence variant.

The original embedded samples were then subjected to mutational analysis of exons 5-9 of the *p53* gene, which encodes the DNA-binding region where cancer-associated mutations most frequently occur (31), and exon 15 of the *BRAF* gene, containing the polymorphisms Val600Glu, recently identified as genetic determinants of primary resistance to EGFR-targeted therapies in colorectal cancer (33).

No mutations were found in either gene, and MSI status assessed by the analysis of the microsatellite loci BAT 26 and BAT 40, did not show constitutional alterations in the allele pattern of tumor samples compared with that of peripheral venous blood.

Discussion

We report a case of a patient with an adenocarcinoma of the ascending colon who, after 5 years from completion of adjuvant chemotherapy disease, relapsed with multiple cancer dissemination to main lymph node regions. In view of the use of an anti-EGFR monoclonal antibody (panitumumab)-containing second-line regimen (34), EGFR immunohistochemical expression in the lymph node biopsy material and *K-ras* gene mutational status in the primary colorectal tumor were performed (19). EGFR expression was mild in 10% of tumor cells (1+) whilst *K-ras* gene sequencing obtained different results according to the region of the tumor tissue analysed. In fact, the tumor sample was microdissected into three parts and two of them revealed wild-type *K-ras* gene, while in the third microdissected area, a point substitution in the codon 22 region (CAG>TAG; Gln22Stop) was found, determining an early STOP signal and encoding a truncated form of p21 protein. The patient was then put on a panitumumab/capecitabine-containing regimen but a rapid clinical worsening was observed and the patient eventually died after 4 months from the beginning of second-line therapy.

Several preclinical (35) and clinical (36) studies have previously shown that the presence of mutated *K-ras* alleles is an independent predictive marker of anti-EGFR mAb resistance. On the basis of these results, the European Union drug regulatory body, the European Medicines Agency, has approved the use of panitumumab only for patients with metastatic colorectal cancer whose tumors display wild-type *K-ras* (29): cetuximab and panitumumab have shown efficacy in approximately 10% to 20% of patients with metastatic colorectal cancer. In particular, the role of oncogenic *K-ras* has been extensively analyzed, leading to the conclusion that the occurrence of *K-ras* mutations represents a predictive parameter of anti-EGFR mAb resistance (36).

The most frequent *K-ras* gene alterations are detected in codons 12 and 13 and minor proportion in codons 61 and 146. Some studies, although limited in number, described mutations identified at codons other than those generally described (33). This novel type of *K-ras* mutation might be more frequent than we presently know, probably because current conventional analytical techniques, such as mutant-allele specific amplification, have focused exclusively on the identification of conventional mutations (29). Due to the low frequency of these rare or sporadic mutations, their clinical relevance in colorectal cancer is still unclear.

Similarly, mutations in codon 22 are described in a limited

number of cases. Previous studies have identified a C to A transversion substituting lysine (AAG) for normal glutamine (CAG) at the 22nd codon of the *K-ras* gene in a primary colon cancer. The biological activity of the mutation, tested by *in vivo* studies, suggested that this variant could be advantageous for *in vivo* tumor growth. Moreover, it is of particular interest that the 22nd amino acid residue of RASp21 is located between the GTPase domain and the effector domain (26, 27). Another study performed on a differentiated adenocarcinoma reported point mutations which caused substitutions of serine for glycine (GGT to AGT) at codon 12 and arginine for glutamine (CAG to CGG) at codon 22 concurrently in the same allele (28).

Our patient presented a never before reported premature stop signal at *K-ras* codon 22 determining the synthesis of a completely inactive truncated *K-ras* protein. To our knowledge, there are no reports that describe nonsense mutations of *K-ras* gene resulting in a premature stop signal. Only two studies described a G deletion mutation from the contiguous G base pairs located between codons 68 and 69 (AGG GAC) (37) and a single nucleotide deletion of one G in codon 12 of *K-ras* (38) both in somatic nucleic acid recovered from lung carcinomas. These mutations cause frameshift resulting in a stop codon several bases downstream; the resulting short truncated peptides probably have no apparently transforming activity because most of the carboxy part of the protein cannot be synthesized.

In our sample, the mutation detected only in a microdissected paraffin-embedded tissue area was not found in other tumoral areas of the slide. The mutated allele showed a lower intensity as compared to wild-type, suggesting the presence of the sequence variant in a small subclone of tumor cells. However, the mutational analysis conducted on more microareas obtained by a further microdissection of the tumoral area in question, did not reveal the presence of mutations, suggesting the presence of a cellular clone which was not identified during the review of the slide.

This observation corroborates the heterogeneity of a cancer population and also confirms previously published data demonstrating that intraneoplastic diversity could determine the genesis of potential metastatic clones and explain the presence of drug-resistant tumors (39, 40).

Heterogeneous multiple *K-ras* gene alterations in the same tumor have been documented, suggesting that different cellular clones arising from the same original transformed cell may harbour different genetic disruption of the same gene (28, 41). How these mutational clonality of *K-ras* may overall influence cancer growth and resistance to anti-EGFR drugs is unknown.

To date, *K-ras* mutational tests commonly provide an on/off result (*i.e.* wild-type or mutated), without considering the relative percentage of tumor cells in the entire tumor mass harbouring wild-type, mutated, or other type of *K-ras* gene disruptions.

K-ras mutations, however, only account for 30% to 40% of nonresponsive patients. Therefore, the identification of additional genetic determinants of primary resistance to EGFR-targeted therapies in colorectal cancer would be of primary importance. Although the mechanism of action is still under study (35), genetic and biochemical evidence indicates that *BRAF* is the principal downstream effector of *K-ras* (42, 43). Because *BRAF* mutations are linked to MSI, a condition generally associated with better prognosis and resistance to standard chemotherapy (44), we also investigated MSI in our patient. Neither alteration for the *BRAF* gene V600E polymorphism nor genomic instability tested with mononucleotide markers BAT26 and BAT40 were evident.

The presence of an inactivating *K-ras* mutation in a subclone of the cancer population does not seem to be the cause of the unresponsive EGFR-related treatment performed in this case. Other related *K-ras* pathways evaluated did not show any alteration. Therefore, other mechanisms should be considered for this specific case. One hypothesis which might explain the poor response to panitumumab is that tumor cells with a minimal inactivation of *K-ras* may develop alternative pathways to trigger downstream signals, thus escaping anti-EGFR tumor control in a similar fashion to cells that express constitutively activated *K-ras*.

In conclusion, although *K-ras* mutational analysis still remains the most important predictive parameter for EGFR-related therapies, further studies, considering other genes and pathways, should be performed in order to improve the predictivity of targeted therapies.

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References

- 1 Fearon ER and Vogelstein B: A genetic model for colorectal tumorigenesis. *Cell* 61: 759-767, 1990.
- 2 Vogelstein B, Fearon ER, Hamilton SR, Kern SE, Preisinger AC, Leppert M, Nakamura Y, White R, Smits AM and Bos JL: Genetic alterations during colorectal-tumor development. *N Engl J Med* 319: 525-532, 1988.
- 3 Andreyev HJ, Norman AR, Cunningham D, Oates JR and Clarke PA: *Kirsten ras* mutations in patients with colorectal cancer: the multicenter 'RASCAL' study. *J Natl Cancer Inst* 90: 675-684, 1998.
- 4 Bos JL, Fearon ER, Hamilton SR, Verlaan-de Vries M, van Boom JH, van der Eb AJ and Vogelstein B: Prevalence of *ras* gene mutations in human colorectal cancers. *Nature* 327: 293-297, 1987.
- 5 Martinez-Garza SG, Nunez-Salazar A, Calderon-Garciduenas AL, Bosques-Padilla FJ, Niderhauser-Garcia A and Barrera-Saldana HA: Frequency and clinicopathology associations of *K-ras* mutations in colorectal cancer in a northeast Mexican population. *Dig Dis* 17: 225-229, 1999.
- 6 Urosecic N, Krtolica K, Skaro-Milic A, Knezevic-Usaj S and Dujic A: Prevalence of G-to-T transversions among *K-ras* oncogene mutations in human colorectal tumors in Yugoslavia. *Int J Cancer* 54: 249-254, 1993.
- 7 Breivik J, Meling GI, Spurkland A, Rognum TO and Gaudernack G: *K-ras* mutation in colorectal cancer: relations to patient age, sex and tumor location. *Br J Cancer* 69: 367-371, 1994.
- 8 Bos JL: *Ras* oncogenes in human cancer: a review. [published erratum appears in *Cancer Res*. 1990; 50: 1352]. *Cancer Res* 49: 4682-4689, 1989.
- 9 Baisse B, Bouzourene H, Saraga EP, Bosman FT and Benhattar J: Intratumor genetic heterogeneity in advanced human colorectal adenocarcinoma. *Int J Cancer* 93: 346-352, 2001.
- 10 Hughes R, Cross AJ, Pollock JR and Bingham S: Dose-dependent effect of dietary meat on endogenous colonic *N*-nitrosation. *Carcinogenesis* 22: 199-202, 2001.
- 11 Bingham SA, Pignatelli B, Pollock JR, Ellul A, Malaveille C, Gross G, Runswick S, Cummings JH and O'Neill IK: Does increased endogenous formation of *N*-nitroso compounds in the human colon explain the association between red meat and colon cancer? *Carcinogenesis* 17: 515-523, 1996.
- 12 Beranek M, Bures J, Palicka V, Jandik P, Langr F and Nejedly E: A relationship between *K-ras* gene mutations and some clinical and histological variables in patients with primary colorectal carcinoma. *Clin Chem Lab Med* 37: 723-727, 1999.
- 13 Cerottini JP, Caplin S, Saraga E, Givel JC and Benhattar J: The type of *K-ras* mutation determines prognosis in colorectal cancer. *Am J Surg* 175: 198-202, 1998.
- 14 Samowitz WS, Curtin K, Schaffer D, Robertson M, Leppert M and Slattey ML: Relationship of *Ki-ras* mutations in colon cancers to tumor location, stage and survival: a population-based study. *Cancer Epidemiol Biomarkers Prev* 9: 1193-1197, 2000.
- 15 Moerkerk P, Arends JW, van Driel M, de Bruine A, de Goeij A and ten Kate J: Type and number of *Ki-ras* point mutations relate to stage of human colorectal cancer. *Cancer Res* 54: 3376-3378, 1994.
- 16 Span M, Moerkerk PT, De Goeij AF and Arends JW: A detailed analysis of *K-ras* point mutations in relation to tumor progression and survival in colorectal cancer patients. *Int J Cancer* 69: 241-245, 1996.
- 17 Kampman E, Voskuil DW, van Kraats AA, Balder HF, van Muijen GN, Goldbohm RA and van't Veer P: Animal products and *K-ras* codon 12 and 13 mutations in colon carcinomas. *Carcinogenesis* 21: 307-309, 2000.
- 18 Brink M, de Goeij AFPM, Weijenberg MP, Roemen GMJM, Lentjes MHFM, Pachen MMM, Smits KM, de Bruine AP, Goldbohm RA and van den Brandt PA: *K-ras* oncogene mutations in sporadic colorectal cancer in the Netherlands Cohort Study. *Carcinogenesis* 24(4): 703-710, 2003.
- 19 Amado RG, Wolf M, Peeters M, Van Cutsem E, Siena S, Freeman DJ, Juan T, Sikorski R, Suggs S, Radinsky R, Patterson SD and Chang DD: Wild-type *K-RAS* is required for panitumumab efficacy in patients with metastatic colorectal cancer. *J Clin Oncol* 26: 1626-1634, 2008.

- 20 Lievre A, Bachet JB, Boige V, Cayre A, Le Corre D, Buc E, Ychou M, Bouche O, Landi B, Louvet C, Andre T, Bibeau F, Diebold MD, Rougier P, Ducreux M, Tomasic G, Emile JF, Penault-Llorca F and Laurent-Puig P: *K-RAS* mutations as an independent prognostic factor in patients with advanced colorectal cancer treated with cetuximab. *J Clin Oncol* 26: 374-379, 2008.
- 21 Rouleau E, Spyrtatos F, Dieumegard B, Guinebretière JM, Lidereau R and Bièche I: *K-RAS* mutation status in colorectal cancer to predict response to EGFR targeted therapies: the need for a more precise definition. *Br J Cancer* 99(12): 2100, 2008.
- 22 Akagi K, Uchibori R, Yamaguchi K, Kurosawa K, Tanaka Y and Koza T: Characterization of a novel oncogenic *K-ras* mutation in colon cancer. *Biochem Biophys Res Commun* 352: 728-732, 2007.
- 23 Lin SR, Tsai JH, Yang YC and Lee SC: Mutations of *K-ras* oncogene in human adrenal tumors in Taiwan. *Br J Cancer* 77: 1060-1065, 1998.
- 24 Edkins S, O'Meara S, Parker A, Stevens C, Reis M, Jones S, Greenman C, Davies H, Dalgliesh G, Forbes S, Hunter C, Smith R, Stephens P, Goldstraw P, Nicholson A, Chan TL, Velculescu VE, Yuen ST, Leung SY, Stratton MR and Futreal PA: Recurrent *K-RAS* codon 146 mutations in human colorectal cancer. *Cancer Biol Ther* 5(8): 928-932, 2006.
- 25 Wójcik P, Kulig J, Okoń K, Zazula M, Moździoch I, Niepsuj A and Stachura J: *K-RAS* mutation profile in colorectal carcinoma and novel mutation-internal tandem duplication in *K-RAS*. *Pol J Pathol* 59(2): 93-96, 2008.
- 26 Tsukuda K, Tanino M, Soga H, Shimizu N and Shimizu K: A novel activating mutation of the *K-ras* gene in human primary colon adenocarcinoma. *Biochem Biophys Res Commun* 278: 653-658, 2000.
- 27 Yuen ST, Davies H, Chan TL, Ho JW, Bignell GR, Cox C, Stephens P, Edkins S, Tsui WW, Chan AS, Futreal PA, Stratton MR, Wooster R and Leung SY: Similarity of the phenotypic patterns associated with *BRAF* and *K-RAS* mutations in colorectal neoplasia. *Cancer Res* 62(22): 6451-6455, 2002.
- 28 Miyakura Y, Sugano K, Fukayama N, Konishi F and Nagai H: Concurrent mutations of *K-ras* oncogene at codons 12 and 22 in colon cancer. *Jpn J Clin Oncol* 32(6): 219-221, 2002.
- 29 van Krieken JH, Jung A, Kirchner T, Carneiro F, Seruca R, Bosman FT, Quirke P, Fléjou JF, Plato Hansen T, de Hertogh G, Jares P, Langner C, Hoefler G, Ligtenberg M, Tiniakos D, Tejpar S, Bevilacqua G and Ensari A: *K-RAS* mutation testing for predicting response to anti-EGFR therapy for colorectal carcinoma: proposal for an European quality assurance program. *Virchows Arch* 453(5): 417-423, 2008.
- 30 Palmirotta R, Matera S, Curia MC, Aceto G, el Zhobi B, Verginelli F, Guadagni F, Casale V, Stigliano V, Messerini L, Mariani-Costantini R, Battista P and Cama A: Correlations between phenotype and microsatellite instability in HNPCC: implications for genetic testing. *Fam Cancer* 3(2): 117-121, 2004.
- 31 Catalano T, Curia MC, Aceto G, Verginelli F, Cascinu S, Cama A, Mariani-Costantini R, Teti D and Battista P: Mutations in the *p53* and *Ki-ras* genes, microsatellite instability and site of tumor origin in colorectal cancer. *Oncol Rep* 14: 625-631, 2005.
- 32 Ottini L, Falchetti M, D'Amico C, Amorosi A, Saieva C, Masala G, Frati L, Cama A, Palli D and Mariani-Costantini R: Mutations at coding mononucleotide repeats in gastric cancer with the microsatellite mutator phenotype. *Oncogene* 16(21): 2767-2772, 1998.
- 33 Di Nicolantonio F, Martini M, Molinari F, Sartore-Bianchi A, Arena S, Saletti P, De Dosso S, Mazzucchelli L, Frattini M, Siena S and Bardelli A: Wild-type *BRAF* is required for response to panitumumab or cetuximab in metastatic colorectal cancer. *J Clin Oncol* 26(35): 5705-5712, 2008.
- 34 Van Cutsem E, Peeters M, Siena S, Humblet Y, Hendlisz A, Neyns B, Canon JL, Van Laethem JL, Maurel J, Richardson G, Wolf M and Amado RG: Open-label phase III trial of panitumumab plus best supportive care compared with best supportive care alone in patients with chemotherapy-refractory metastatic colorectal cancer. *J Clin Oncol* 25(13): 1658-1664, 2007.
- 35 Benvenuti S, Sartore-Bianchi A, Di Nicolantonio F, Zanon C, Moroni M, Veronese S, Siena S and Bardelli A: Oncogenic activation of the RAS/RAF signaling pathway impairs the response of metastatic colorectal cancers to anti-epidermal growth factor receptor antibody therapies. *Cancer Res* 67: 2643-2648, 2007.
- 36 Lièvre A, Bachet JB, Le Corre D, Boige V, Landi B, Emile JF, Côté JF, Tomasic G, Penna C, Ducreux M, Rougier P, Penault-Llorca F and Laurent-Puig P: *K-RAS* mutation status is predictive of response to cetuximab therapy in colorectal cancer. *Cancer Res* 66: 3992-3995, 2006.
- 37 Wang YC, Lee HS, Chen SK, Yang SC and Chen CY: Analysis of *K-ras* gene mutations in lung carcinomas: correlation with gender, histological subtypes, and clinical outcome. *Cancer Res Clin Oncol* 124: 517-522, 1998.
- 38 Vachtenheim J, Horáková I, Novotná H, Opáalka P and Roubková H: Mutations of *K-ras* oncogene and absence of H-ras mutations in squamous cell carcinomas of the lung. *Clin Cancer Res* 1: 359-365, 1995.
- 39 Dexter DL and Leith JT: Tumor heterogeneity and drug resistance. *J Clin Oncol* 4(2): 244-257, 1986.
- 40 Sweeney C, Boucher KM, Samowitz WS, Wolff RK, Albertsen H, Curtin K, Caan BJ and Slatery ML: Oncogenetic tree model of somatic mutations and DNA methylation in colon tumors. *Genes Chromosomes Cancer* 48(1): 1-9, 2009.
- 41 Kimura K, Nagasaka T, Hoshizima N, Sasamoto H, Notohara K, Takeda M, Kominami K, Iishii T, Tanaka N and Matsubara N: No duplicate *K-RAS* mutation is identified on the same allele in gastric or colorectal cancer cells with multiple *K-RAS* mutations. *J Int Med Res* 35(4): 450-457, 2007.
- 42 Yan J, Roy S, Apolloni A, Lane A and Hancock JF: Ras isoforms vary in their ability to activate Raf-1 and phosphoinositide 3-kinase. *J Biol Chem* 273: 24052-24056, 1998.
- 43 Zhang BH, Guan KL: Activation of B-Raf kinase requires phosphorylation of the conserved residues Thr598 and Ser601. *EMBO J* 19: 5429-5439, 2000.
- 44 Locker GY, Hamilton S, Harris J, Jessup JM, Kemeny N, Macdonald JS, Somerfield MR, Hayes DF and Bast RC Jr; ASCO: ASCO 2006 update of recommendations for the use of tumor markers in gastrointestinal cancer. *J Clin Oncol* 24: 5313-5327, 2006.

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