A Novel \textit{K-ras} Mutation in Colorectal Cancer.  
A Case Report and Literature Review 

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Abstract. Background: Activating mutations in the \textit{K-ras} oncogene mainly occur in codons 12 and 13 and may be predictive of response to drugs directly linked to the \textit{K-ras} signaling pathway, such as panitumumab and cetuximab. Materials and Methods: \textit{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 \textit{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 \textit{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.

\textit{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 \textit{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 \textit{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 \textit{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 \textit{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 \textit{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 then 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 \textit{et al.} identified a novel G to T transversion \textit{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 \textit{K-ras} activity (22). This mutation occurred at an early stage of tumor development, like mutations at codons 12, 13 and 61 (1).
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 hemicolecctiony 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)
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 \( K\text{ras} \) 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 \( K\text{ras} \) 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. The mutation was named according to recommendations of the Nomenclature System for Human Gene Mutations. The GenBank mRNA sequence (M54968) of \( K\text{ras} \) was used as a reference.

![Sequence analysis of the \( K\text{ras} \) 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 \( K\text{ras} \) was used as a reference.](image)

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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 \( B\text{RAF} \) 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 disseminaton to main lymph node regions. In view of the use of chemotherapy disease, relapsed with multiple cancer ascending colon who, after 5 years from completion of adjuvant We report a case of a patient with an adenocarcinoma of the peripheral venous blood.

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. 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 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 mononucleotidic 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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