

Concurrent mutation in exons 1 and 2 of the K-ras oncogene in colorectal cancer

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Abstract: The K-ras gene is frequently mutated in colorectal cancer and has been associated with tumor initiation and progression; approximately 90% of the activating mutations are found in codons 12 and 13 of exon 1 and just under 5% in codon 61 located in exon 2. These mutations determine single aminoacidic substitutions in the GTPase pocket leading to a block of the GTP hydrolytic activity of the K-ras p21 protein, and therefore to its constitutive activation. Point mutations in sites of the K-ras gene, other than codons 12, 13 and 61, and other types of genetic alterations, may occur in a minority of cases, such as in the less frequent cases of double mutations in the K-ras gene. However, all mutations in this gene, even those which occur in non-canonical sites or double mutations, are relevant oncogenic alterations in colorectal cancer and may underlie K-ras pathway hyperactivation. In the present study, we report the case of a patient with colorectal cancer presenting a concurrent point mutation in exons 1 and 2 of the K-ras gene, a GGT to TGT substitution (Glycine to Cysteine) at codon 12, and a GAC to AAC substitution (Aspartic Acid to Asparagine) at codon 57. In addition, we found in the same patient's sample a silent polymorphism at codon 11 (Ala11Ala) of exon 1. (*Folia Histochemica et Cytobiologica* 2011; Vol. 49, No. 4, pp. 729–733)

Key words: colorectal cancer, K-ras, double mutation

Introduction

The human ras gene family is one of the potential targets for mutational changes that play a role in human tumorigenesis. Members of the ras family of genes (K-ras, H-ras and N-ras) encode for a 21 kDa protein (p21) located on the inner surface of the plasma membrane with a farnesyl molecule attached to its carboxy-terminus [1]. K-ras is a proto-oncogene located on chromosome 12p12.1 and is activated by point mutations that occur at the critical hot-spot

coding sequences [1]. The K-ras gene is frequently mutated in colorectal cancer and it has been associated with colorectal cancer initiation and progression. Once acquired, K-ras mutations are preserved throughout the natural history of tumor development and are excellent targets for diagnostic testing. These mutations determine the constitutive activation of the K-ras protein mainly thorough abrogation of its GTP-hydrolytic function. A permanently activated K-ras pathway escapes from the control of anti-epidermal growth factor (EGFR) agents which conversely exert anticancer effects by inhibiting a non-mutated (wild-type) physiologically competent EGFR/K-ras signal [1]. K-ras mutational status has recently been demonstrated to be highly predictive of the activity of two monoclonal antibodies linked with EGFR (cetuximab and panitumumab); therefore the exclusion of such

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drugs in the presence of mutated K-ras is becoming a standard in clinical practice [1]. Approximately 90% of the activating mutations leading to a block of the GTP hydrolytic activity of the 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 [2]. The most frequently observed types of mutations are G > A transitions [3], and G > T transversions [4]. These alterations have been found associated with gender and sub-localization of the tumor [5]. Other authors have reported clinical cases presenting sequence variants in other codons, even without mutations in codons 12–13.

All mutations and polymorphisms in coding and not coding regions of the K-ras gene are available on the websites: www.sanger.ac.uk/genetics/CGP/cosmic/ and dbSNP: www.ncbi.nlm.nih.gov/SNP/. While mutations in codon 12 are well documented, a few authors have also reported mutations in codons 11 and 57, such as the cases of double mutations in the K-ras gene.

In the present study, we report for the first time a colon cancer patient presenting a polymorphism at codon 11 and point mutations at codons 12 (exon1) and 57 (exon 2), concurrently.

Material and methods

A colon cancer paraffin-embedded tissue of a 79 year-old male diagnosed with metastatic colorectal cancer and who had undergone surgical resection, was referred to our Institutions following a request from the oncologist. Histological diagnosis was a moderately differentiated adenocarcinoma of the colon-rectum (G2) infiltrating the subserosal adipose tissue (T3) and showing glandular structures with atypical epithelium and some mitotic figures.

Molecular analysis of K-ras was carried out from DNA extracted by the paraffin-embedded tumor sections. Tumor and tumor-free areas were identified within 15 μ m-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. DNA extraction from the microdissected area was performed as previously reported [6]. Exons 1 and 2 of K-ras were individually amplified using a 'seminested' amplification protocol [7]. DNA extractions and set-up of PCR reactions were performed in a different laboratory. Direct sequencing reactions were performed using a Big Dye Terminator (Applied Biosystems, Foster City, CA, USA), and run on an ABI 3130 Genetic Analyzer (Applied Biosystems). In order to exclude pre-analytical and analytical errors, all sequencing analyses were carried out on both strands and were repeated on PCR products obtained from new nucleic acid

extractions. In order to separate alleles and assess the location in cis or trans of the variations on codons 11 and 12, the amplified product was cloned into Pcr4-TOPO Vector using TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA) and the haplotypes of each heterozygote were purified and sequenced.

All analysis was confirmed in duplicate experiments also in this case, 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 and discussion

Direct sequencing of tumor-derived PCR products identified two heterozygous point mutations on exon 1 which led to a substitution of Cysteine for Glycine (TGT to GGT) at codon 12 and a silent variant (GCC to GCT) involving Alanine at codon 11 (Figure 1). In addition, in exon 2 a sequence variant in codon 57 was identified, determining a substitution of Asparagine for Aspartic Acid (AAC to GAC). The results of PCR analysis of exon 1 were cloned and the haplotypes of each heterozygote were sequenced in order to confirm whether the exon 1 sequence variants occurred in the same or different alleles. By cloning the PCR products into TOPO TA Cloning Kit, we demonstrated the presence of sequence variants in codons 11 and 12 on the same allele (Figure 1). It was not possible to extend the analysis to Gly12Ala and Asp57Asn variants, due to the excessive intronic region length (17,861 bp Ensemble # ENSE00001428812) and to the nature of the source paraffin tissue.

Concurrent mutations detected during mutational analysis of K-Ras gene have until now been considered to be relatively rare. The most widely described case in literature concerns a patient with colon cancer who presented concurrent point mutations of the K-ras oncogene at codons 12 (Gly12Ser) and 22 (Glu22Arg). This study, published in 2002, focused attention for the first time on determining the presence of the double mutation in heterozygosity or on the same allele [8]. In a recent study performed on 186 adenocarcinomas and 16 adenomas from the EPIC-Norfolk study, one sample, harboring a double mutation at codons 19 (Leu to Phe) and 20 (Thr to Ala), showed these two changes based on the same allele [9].

In fact, a few previous studies had exclusively described the double mutations at codon 12, consequently assuming that these concurrent mutations inevitably affected both alleles [10, 11]. Sato et al., using dot blot hybridization, reported a double codon 12 mutation from wild type GGT (Gly) to GAT (Asp) and GCT (Ala), observed in a case of human endometri-

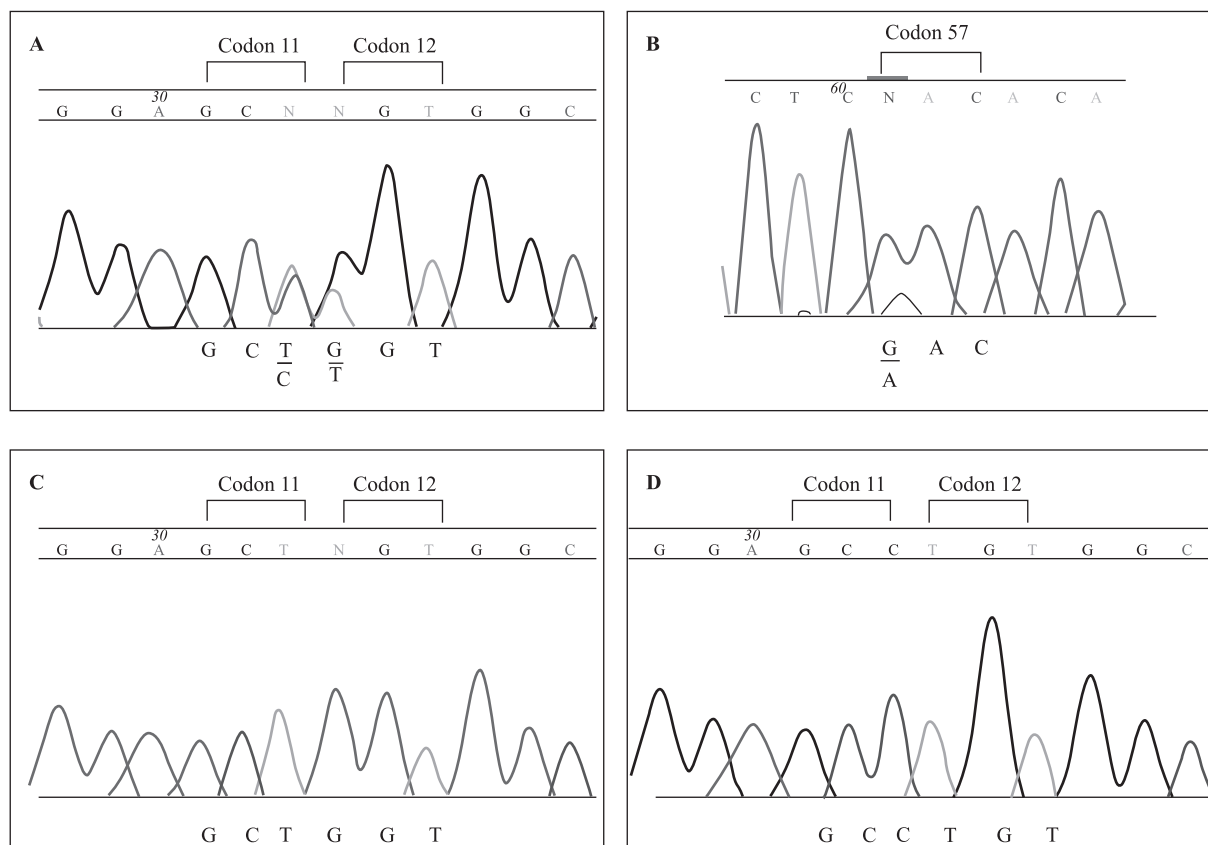


Figure 1. Direct sequencing of the PCR products showing substitutions of GCT (Ala) to GCC (Ala) at codon 11, GGT (Gly) to TGT (Cys) at codon 12 (A) and GAC (Asp) to AAC (Asn) at codon 57 (B). TOPO TA cloning sequencing of exon 1 of K-ras PCR product showing a normal allele (C) and the concurrent presence of the sequence variants in codons 11 and 12 on the same allele (D)

al carcinoma [10]. Using a novel multiplex PCR/ligase detection to analyze 144 paraffin-embedded archival colon carcinomas, Khanna et al. identified a novel double mutation in codon 12 consisting of a concomitant presence of Asp and Cys, instead of Gly [11]. However, the screening methods used in these two studies, such as dot blot hybridization or multiplex PCR/ligase, were not able to proceed with a consequent allelic separation [10, 11]. Most studies in which double mutations have been identified on different codons have not assessed the allelic distribution of the K-ras variants.

In a screening of 136 colorectal cancers, Jönsson et al. identified 53 K-ras mutations, of which two had double variants in codon 12 and 13 and one had multiple concurrent variants in codon 12 [12].

In order to investigate the incidence of point mutations at K-ras codons 12, 13 and 61, a study conducted on 101 colorectal tumor specimens from a Mexican population reported two patients with a double mutation of codon 12 and one with a concurrent point mutation at codon 12 and 13 [3]. Two recent experimental studies performed on tumors derived

from animal models, despite showing the presence of double mutations, did not focus on evaluating the allelic distribution of concurrent K-ras mutations [13, 14]. Moreover, in two mice ethylene oxide induced lung cancers, Hong et al. detected K-ras double mutations concurrently in codons 12 and 13, and 12 and 61, respectively [13].

In a recent study conducted in pancreas tumor biopsies obtained from 222 patients, Guo et al. identified a new K-ras mutation at codon 76 (Glu76Gly), simultaneously with a codon 12 (Gly12Val) Ras mutation [14]. An accurate analysis of the allelic distribution of multiple K-ras mutations was described in a recent paper performing an extensive mutational screening on 236 gastrointestinal human tumors [15]. In this work, Kimura et al. reported four colorectal cancer and one gastric cancer cases with double K-ras codon 12 and 13 mutations. Allelic separation confirmed the presence of point mutations on different alleles, suggesting the simultaneous presence of different cell clones in the same tumor, with only one mutation *per clone* [15].

These considerations may be in agreement with the theory that even a single mutation of K-ras is sufficient to sustain the cancer cell and to explain the mechanisms of neoplastic progression [15]. Another paper reports the case of a patient with metachronous double lung cancers, which occurred over a period of four years, presenting two different mutations in codon 12 of K-ras gene. In the first tumor, a variant Gly12Val was described, while the second episode of lung cancer presented a Gly12Cys, proving the metachronous nature of these double cancers [16]. In our study, in addition to the canonical sequence variant at codon 12 (Gly12Cys), we also detected an unusual silent polymorphism on codon 11 (Ala11Ala) and a novel GAC to AAC substitution at codon 57 (Asp57Asn). In a study performed on 161 patients with various forms of hematologic malignancies, Ahuja et al. reported, in one case, a codon 11 mutation with a threonine for alanine substitution [17]. Two novel codon 11 missense mutations (Gly11Pro and Gly11Val) were described in an Italian study performed on 34 cases of gastric cancer. These authors suggested that these point mutations in codon 11, not previously reported, might result in transformation, since codon 10 of similar gene H-ras has been shown to be transforming when altered by site-directed mutagenesis [6]. In a study performed on DNA from peripheral blood of lung cancer patients, Gu et al. argued that codon 11 CCT (Pro) instead of GCT (Ala) was a frequent polymorphism in a Chinese population [18]. More recently, Zaravinos et al. reported, in 23 cases of human nasal polyps, in addition to classical mutations at codon 12 of K-ras gene, evidence for an unusually high frequency of mutations in codon 11, without sequence variants in codon 13. All mutations in codon 11 were homozygous and in six cases were concurrent with mutations in codon 12 [19]. Unlike the codon 11, to our knowledge, there are no reports that describe human sequence variants on codon 57 of K-ras gene. *In vitro* studies have demonstrated that the Ras mutant, in which Asp57 is replaced with tyrosine (Asp57Tyr), abolished the insulin-induced increase in mitogen-activated protein kinase activity [20], while a previous study showed that Asp57 in p21ras plays a considerable biological role, since it is a part of the switch region II of G proteins [21]. In our findings, it was not identified whether this Asp57 mutation gives rise to tumorigenesis. The currently available biotechnologies, applicable to paraffin extracted nucleic acids, in most cases degraded, have not allowed the allelic separation involving both exons, so that it is not possible to determine if a double mutation is present in one or two distinct cellular clones. Heterogeneity in the tumor tissue with multi-

ple K-ras gene alterations in the same tumor has been documented, suggesting that different cellular clones arising from the same original transformed cell may harbor different genetic disruptions of the same gene [8, 15]. Recent evidence suggests the reason for heterogeneity of colon cancer, providing a plausible model to explain the sequence of events and molecular somatic alterations for the major pathways involved in colorectal cancer progression [22]. How this mutational 'clonality' of K-ras may overall influence cancer growth is still unknown, even though these mutations must be late changes in tumor progression. It is imperative that there should be further studies to clarify the biological activity of combination in point mutations at exon 1 and 2.

Acknowledgements

We wish to thank Barbara Leone for her excellent technical assistance. The authors declare that there is no conflict of interest in regard to this paper. This study was partially supported by an Italian Ministry of Health Research Grant RFPS-2006-7-342220 and ACC-WP 3/1b.

References

1. van Krieken JH, Jung A, Kirchner T et al. K-RAS mutation testing for predicting response to anti-EGFR therapy for colorectal carcinoma: proposal for an European quality assurance program. *Virchows Arch.* 2008;453:417–423.
2. Andreyev HJ, Norman AR, Cunningham D, Oates JR, Clarke PA. Kirsten ras mutations in patients with colorectal cancer: the multicenter 'RASCAL' study. *J Natl Cancer Inst.* 1998;90:675–684.
3. Martinez-Garza SG, Nunez-Salazar A, Calderon-Garciduenas AL, Bosques-Padilla FJ, Niderhauser-Garcia A, Barrera-Saldana HA. Frequency and clinicopathology associations of K-ras mutations in colorectal cancer in a northeast Mexican population. *Dig Dis.* 1999;17:225–229.
4. Urošević N, Krtolica K, Skaro-Milic A, Knezevic-Usaj S, Dujic A. Prevalence of G-to-T transversions among K-ras oncogene mutations in human colorectal tumors in Yugoslavia. *Int J Cancer.* 1993;54:249–254.
5. Breivik J, Meling GI, Spurkland A, Rognum TO, Gaudernack G. K-ras mutation in colorectal cancer: relations to patient age, sex and tumor location. *Br J Cancer.* 1994;69:367–371.
6. Hongyo T, Buzard GS, Palli D et al. Mutations of the K-ras and p53 genes in gastric adenocarcinomas from a high-incidence region around Florence, Italy. *Cancer Res.* 1995;55:2665–2672.
7. Palmirotta R, Savonarola A, Formica V et al. A novel K-ras mutation in colorectal cancer. A case report and literature review. *Anticancer Res.* 2009;29:3369–3374.
8. Miyakura Y, Sugano K, Fukayama N, Konishi F, Nagai H. Concurrent mutations of K-ras oncogene at codons 12 and 22 in colon cancer. *Jpn J Clin Oncol.* 2002;32:219–221.
9. Naguib A, Mitrou PN, Gay LJ et al. Dietary, lifestyle and clinicopathological factors associated with BRAF and K-ras mutations arising in distinct subsets of colorectal cancers in the EPIC Norfolk study. *BMC Cancer.* 2010;10:99.

10. Sato S, Ito K, Ozawa N, Yajima A, Sasano H. Analysis of Point Mutations at Codon 12 of K-ras in Human Endometrial Carcinoma and Cervical Adenocarcinoma by Dot Blot Hybridization and Polymerase Chain Reaction. *Tohoku J Exp Med.* 1991;165:131–136.
11. Khanna M, Park P, Zirvi M et al. Multiplex PCR/LDR for detection of K-ras mutations in primary colon tumors. *Oncogene.* 1999;18:27–38.
12. Jönsson M, Ekstrand A, Edekling T et al. Experiences from treatment-predictive KRAS testing; high mutation frequency in rectal cancers from females and concurrent mutations in the same tumor. *BMC Clin Pathol.* 2009;9:8.
13. Hong HH, Houle CD, Ton TV, Sills RC. K-ras Mutations in lung tumors and tumors from other organs are consistent with a common mechanism of ethylene oxide tumorigenesis in the B6C3F1 mouse. *Toxicol Pathol.* 2007;35:81–85.
14. Guo Z, Lu Y, Oakes S et al. Transnational patterns of pancreas cancer Ras mutations and discovery of a new Ras mutation with oncogenic synergy when found with Ras codon 12 mutations. *AACR-NCI-EORTC Poster presentation.# 22;* 2008.
15. Kimura K, Nagasaka T, Hoshizima N et al. 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.* 2007;35:450–457.
16. Sugio K, Inoue T, Inoue K et al. Different site mutation of the K-ras gene in a patient with metachronous double lung cancers. *Anticancer Res.* 1993;13:2469–2471.
17. Ahuja HG, Foti A, Bar-Eli M, Cline MJ. The pattern of mutational involvement of RAS genes in human hematologic malignancies determined by DNA amplification and direct sequencing. *Blood.* 1990;75:1684–1690.
18. Gu Q, Li L, Zhang Y, Li X, Xiao Y, Wu X. Sequencing analysis of human K-ras oncogene exon 1 in Chinese people. *Zhonghua Yi Xue Yi Chuan Xue Za Zhi.* 1998;15:98–100.
19. Zaravinos A, Bizakis J, Soufla G, Sourvinos G, Spandidos DA. Mutations and differential expression of the ras family genes in human nasal polyposis. *Int J Oncol.* 2007;31:1051–1059.
20. Sakaue H, Ogawa W, Takata M et al. Phosphoinositide 3-kinase is required for insulin-induced but not for growth hormone- or hyperosmolarity-induced glucose uptake in 3T3-L1 adipocytes. *Mol Endocrinol.* 1997;11:1552–1562.
21. Sun X, Bognar AL, Baker EN, Smith CA. Structural homologies with ATP- and folate-binding enzymes in the crystal structure of folylpolyglutamate synthetase. *Proc Natl Acad Sci USA.* 1998;95:6647–6652.
22. Sweeney C, Boucher KM, Samowitz WS et al. Oncogenetic tree model of somatic mutations and DNA methylation in colon tumors. *Genes, Chromosomes & Cancer.* 2009;48:1–9.

Submitted: 6 August, 2010

Accepted after reviews: 7 June, 2011