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A Comprehensive Procedural Approach to Genotyping *KRAS* and *BRAF* from Paraffin Embedded Tissues for Diagnostic Purposes

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Abstract. *Background: Mutations in the Kirsten Ras 1 (KRAS) and V-Raf Murine Sarcoma Viral Oncogene Homolog B1 (BRAF) genes may be predictive of response to drugs directly linked to the Epidermal Growth Factor Receptor (EGFR) signaling pathway. Materials and Methods: A total of 230 samples from patients with metastatic colorectal cancer were analyzed for KRAS exon 1 and 2 and for BRAF exon 15 mutations. DNA from paraffin-embedded tumor sections was analyzed using microdissection, direct sequencing analysis and allelic separation by cloning. Results: KRAS mutations were present in 44.3% of the tumor samples. The mutation frequency at hot-spot codons of exon 1 was 84.2%, whereas non-canonical variants had a frequency of 11.8%. Approximately 4% of the cases exhibited concomitant variations. BRAF mutations were present in 3.9% of the tumor samples. Conclusion: Our experience suggests that sequential microdissection, direct sequencing and allelic separation by cloning may improve the approach to mutational analysis of KRAS and BRAF in patients with colorectal cancer.*

Cancer development and progression is strongly associated with cell membrane receptors' activity and the intracellular signal transduction pathways regulating several cell functions, including proliferation, apoptosis, motility, adhesion and angiogenesis (1).

In this context, based on the major role of *EGFR* in the pathogenesis of a number of solid tumor types (2), the transcriptional factors of EGFR pathway are regarded as putative targets of anticancer treatments (1). Ligation of EGFR is followed by auto-phosphorylation of SRC homology regions 2 (SH2) binding domains that recruit small adaptor proteins, GTPases, Son of Sevenless (SOS) and Growth Factor Receptor-Bound Protein 2 (GRB2). The GRB2/SOS complex, in turn, stimulates the activation of KRAS by Guanosine-5'-triphosphate (GTP) binding and signal transduction into the nucleus (3). RAS signaling is terminated when GTPase stimulates RAS to hydrolize GTP to guanosine diphosphate (GDP). In particular, GTP-bound RAS activates the RAF/Mitogen-Activated protein Kinase (MEK)/extracellular signal regulated kinase (ERK) pathway, which regulates cell proliferation and motility, and the phosphatidyl inositol 3-kinase (PIK3)/phosphatase and tensin homolog (PTEN)/Protein Kinase B-alpha (AKT)/ Mammalian Target Of Rapamycin (mTOR) cascade, which is involved in anti-apoptotic responses (4, 5).

KRAS proto-oncogene is frequently mutated in colorectal cancer, where it has been associated with both tumor initiation and progression (6, 7). Activating mutations of *KRAS* are observed in approximately 40% of sporadic colorectal cancer cases (8), and up to 90% include G-to-A transitions (9) and G-to-T transversions (10) in codons 12 and 13. Less frequently, mutations occur in codons 61, 63 and 146, and rarely, in other codons, or as concomitant mutations (11-13).

Given that KRAS is a downstream effector of EGFR, mutational changes of *KRAS* lead to a permanently activated

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status and to the induction of the signal transduction pathway regardless of upstream EGFR status. These considerations are of particular interest considering that monoclonal antibodies directed against EGFR (anti-EGFR MoAbs), namely Cetuximab® and Panitumumab®, have recently been introduced into clinical practice for the treatment of EGFR-expressing metastatic colorectal cancer (mCRC) with disease progression, in which mutational changes leading to RAS activated status is responsible for a lack of activity of the drug itself (5). Indeed, after their initial approvals in 2007 by the U.S. Food and Drug Administration (FDA) and by the European Medicines Agency (EMEA), in July 2009, the FDA updated the labels of both anti-EGFR MoAbs to include information about *KRAS* mutations (14). Therefore, a correct assessment of *KRAS* mutational status is required for the selection of patients who can benefit from anti-EGFR MoAb-based treatment, as evidenced by a number of clinical trials (15-20).

In this context, however, we should also consider that a minor subset of patients with wild-type (wt) *KRAS* do not benefit from EGFR MoAb treatment, due either to alteration of other genes enrolled in the EGFR signaling pathway, such as *BRAF* (20-22), or, to a lesser extent, to *PIK3CA* mutations (23), or overexpression of PTEN protein (24) and EGFR ligands (1, 25). Accordingly, the exclusion of EGFR MoAb-based treatment in the presence of mutated *KRAS*/*BRAF* is becoming a standard in clinical practice (20, 26).

Of interest, the most important multicenter studies carried out to date, such as the Kirsten ras in-colorectal-cancer collaborative group (RASCAL) I and II, the Panitumumab Randomized Trial in Combination With Chemotherapy for Metastatic Colorectal Cancer to Determine Efficacy (PRIME) and the Oxaliplatin and Cetuximab in First-Line Treatment of Metastatic Colorectal Cancer (OPUS) (15, 16, 18, 19), reported mutational results obtained from various laboratories using methods that significantly differed from each other, such as single strand conformation polymorphism (SSCP), melting curve technique, direct sequencing analysis and allele-specific-hybridization, the latter being the procedure most commonly used in commercial kits (15, 16, 18, 19, 27). Therefore, despite the emerging need to tailor CRC treatment on the basis of mutational analysis, there are still considerable differences in tissue processing, storage conditions and, most importantly, in molecular screening techniques among different laboratories, which have prompted the European Society of Pathology and the College of American Pathologists to issue technical guidelines for *KRAS* mutational analyses (22, 26, 28).

Here, we present our experience using 230 formalin-fixed paraffin-embedded colorectal cancer samples for *KRAS* exon 1 and 2 and *BRAF* exon 15 mutation screening analysis by microdissection, polymerase chain reaction (PCR), direct sequencing and allelic cloning. Moreover, the applicability of these methods for routine testing is briefly reviewed in the context of the available international literature.

Materials and Methods

Between July 2008 and September 2011, paraffin-embedded colon cancer sections obtained from 230 consecutive patients with mCRC (93 women, 137 men; mean age 66.2 years, ranging from 31 to 86 years) were delivered to our Laboratory for molecular analysis of *KRAS* and *BRAF* genes.

All patients were referred to our Institution following an oncologist's request. There were no statistical differences between sexes. Written informed consent was obtained from each participating subject and the study was performed under the appropriate institutional ethics approvals and in accordance with the principles embodied in the Declaration of Helsinki. All biological data were treated for purposes of scientific research and dissemination of the results occurred only anonymously in an aggregated or summarized form.

Each paraffin-embedded section was collected on microscope slides and first examined under the microscope to ensure that it contained sufficient tumor material. 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. DNA extraction from the microdissected area was performed as previously reported (11, 12).

Briefly, formalin-fixed paraffin-embedded microdissected areas were dipped into xylene to remove paraffin, rehydrated in a series of ethanol and incubated in 100 ml of digestion buffer, containing 1 M Tris-HCl (pH 8.0), 0.5 M EDTA, 0.02% Tween 20, and 100 mg/ml proteinase K. After an incubation of 3 h at 55˚C, proteinase K was inactivated at 95˚C for 10 min, and samples were centrifuged at 16000 ×g. The recovered supernatant was first purified by adding a saturated sodium chloride solution and centrifuged for 30 min at 16000 ×g and after precipitated by adding two volumes of 100% ethanol. The DNA pellet was finally dried and the pellet was re-dissolved in 50 ml of DNase-free water.

KRAS and *BRAF* PCR conditions are described in Table I. Exons 1 and 2 of *KRAS* were individually amplified using a nested amplification protocol.

DNA extraction was performed in a dedicated area different from that used for the set-up of PCR reactions. 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.

To separate alleles and assess the putative location in *cis* or *trans* of the concomitant variations, the amplified product was cloned into Pcr4-TOPO Vector using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA) and the haplotypes of each heterozygote were purified and sequenced (12). All analyses were confirmed in duplicate experiments as above, using independently extracted DNA samples.

Results

Mutational analysis of the KRAS gene: KRAS gene sequence variants were detected in 102 (44.3%) out of the 230 biopsies analyzed. In particular, mutations in exon 1 and 2 were detected in 88 (86.3%) and 10 (9.8%) of samples from 102

Table I. *PCR primers, product size and reaction conditions for amplification and direct sequencing of KRAS and BRAF genes.*

Gene		Primers	PCR product size (bp)	PCR annealing temp (˚C)
KRAS	1st PCR	F5'-GTACTGGTGGAGTATTTGATAGTG-3'		
(exon 1)		R5'-GGTCAGAGAAACCTTTATCTGTATC-3'	278	55
	Nested PCR	F5'-TTTTTATTATAAGGCCTGCT-3'		
		R5'-GTCCTGCACCAGTAATATGC-3'	174	54
KRAS	1st PCR	F5'-AGGTGCACTGTAATAATCCAG-3'		
(exon 2)		R5'-ATTATATGCATGGCATTAGC-3'	309	52
	Nested PCR	F5'-ATCCAGACTGTGTTTCTCCC-3'		
		R5'-AACTATAATTACTCCTTAATGTCAGC-3'	256	55
BRAF		F5'-TCATAATGCTTGTTGCTCTGATAGGA-3'		
(exon 15)		R5'-GGCCAAAAATTTAATCAGTGGA-3'	193	55

patients, respectively. The remaining 4 (3.9%) patients exhibited the concomitant presence of different *KRAS* gene variants in the same biological sample.

Pathogenic analysis of the observed mutations revealed the presence of three silent mutations, one in exon 1 (Gln22Gln) and two in exon 2 (Gly60Gly and Glu63Glu). Accordingly, the number of sequence variants was recalculated taking into consideration only those with pathogenic significance, with a resulting frequency of somatic mutation of 43% (99 out of 230 biological samples examined). All variants of the *KRAS* gene are summarized in Table II. All known mutations and polymorphisms identified are reported in the WEB-database http://www.sanger.ac.uk/genetics/CGP/cosmic/. Mutations were named according to the recommendations of the Nomenclature System for Human Gene Mutations. The GenBank mRNA sequence (M54968) of *KRAS* was used as a reference.

KRAS exon 1 mutations: As for single-mutations in exon 1, 63 variants (61.8%) were found at codon 12. In particular, the Gly12Asp variant was identified in 31 patients (30.4%), the Gly12Val variant in 17 patients (16.7%), the Gly12Ala variant in 8 patients (7.8%), the Gly12Ser and the Gly12Cys in three patients each (2.9%) and the Gly12Arg variant in only one patient (0.9%).

Twenty-three patients showed single variants of codon 13, the most represented being the Gly13Asp variant, which was identified in 21 patients (20.6%). The Gly13Arg (0.9%) and the Gly13Val (0.9%) variants were observed in one case each. Finally, mutations at codon 22 were identified in two patients. The first, recently described by our group and to our knowledge never reported previously in the literature, was responsible for the substitution of a glycine with a premature stop codon (Gln22Stop) (11). The second was represented by a silent Gln22Gln variant sequence (Table II).

KRAS exon 2 mutations: KRAS exon 2 pathogenic single-mutations were identified in 10 cases, including three patients (2.9%) with a Gln61His variant, two patients (1.9%) with a Gln61Lys variant and three patients with a Ala59Thr, a Gln61Leu and a Glu63Lys variant, respectively. In addition,

Table II. *Mutations of KRAS gene detected by direct sequencing assay.* [a]*The frequency of mutation tumour from our patients was compared with data extracted from (4), the RASCAL II study (16) and from The Catalogue of Somatic Mutation in Cancer (COsMiC; Sanger Institute, http://www.sanger.ac.uk/genetics/CGP/cosmic/).* [b]*Taken from reference 11,* [c]*reference 12.*

KRAS mutations	Nucleotide variants	No of patients	Mutation frequency (%)	
			Present study	Other studies[a]
Codon 12				
Gly12Asp (G12D)	G35A	31/102	30.4%	32.5
Gly12Val (G12V)	G35T	17/102	16.6%	22.5
Gly12Cys (G12C)	G34T	3/102	2.9%	8.8
Gly12Ser (G12S)	G34A	3/102	2.9%	7.6
Gly12Ala (G12A)	G35C	8/102	7.8%	6.4
Gly12Arg (G12R)	G34C	1/102	0.9%	0.9
Codon 13				
Gly13Asp (G13D)	G38A	21/102	20.6%	19.5
Gly13Arg (G13R)	G38C	1/102	0.9%	n.d.
Gly13Val (G13V)	G38T	1/102	0.9%	n.d.
Other				
Gln22Gln (Q22Q) (exon 1)	G66A	1/102	0.9%	1.8
Gln22STOP[b] (exon 1)	C64T	1/102	0.9%	
Ala59Thr (A59T) (exon 2)	G175A	1/102	0.9%	
Gly60Gly (G60G) (exon 2)	T180C	1/102	0.9%	
Gln61His (Q61H) (exon 2)	A183C	3/102	2.9%	
Gln61Leu (Q61L) (exon 2)	A182T	1/102	0.9%	
Gln61Lys (Q61K) (exon 2)	C181A	2/102	1.9%	
Glu63Glu (E63E) (exon 2)	G189A	1/102	0.9%	
Glu63Lys (E63K) (exon 2)	G187A	1/102	0.9%	
Concomitant mutations				
Gly12Asp (G12D) (exon1)	G35A	1/102	0.9%	n.d.
Asp30Glu (D30E) (exon 1)	C90A			
Gly13Asp (G13D) (exon1)	G38A	1/102	0.9%	n.d.
Gln61Hys (Q61H) (exon 2)	A182C			
Gly12Cys (G12C)[c] (exon1)	G34T			
Ala11Ala (A11A) (exon1)	T33C	1/102	0.9%	n.d.
Asp57Asn (D57N) (exon2)	G169A			
Gln61Arg (Q61R) (exon 2)	A182G A183T	1/102	0.9%	n.d.

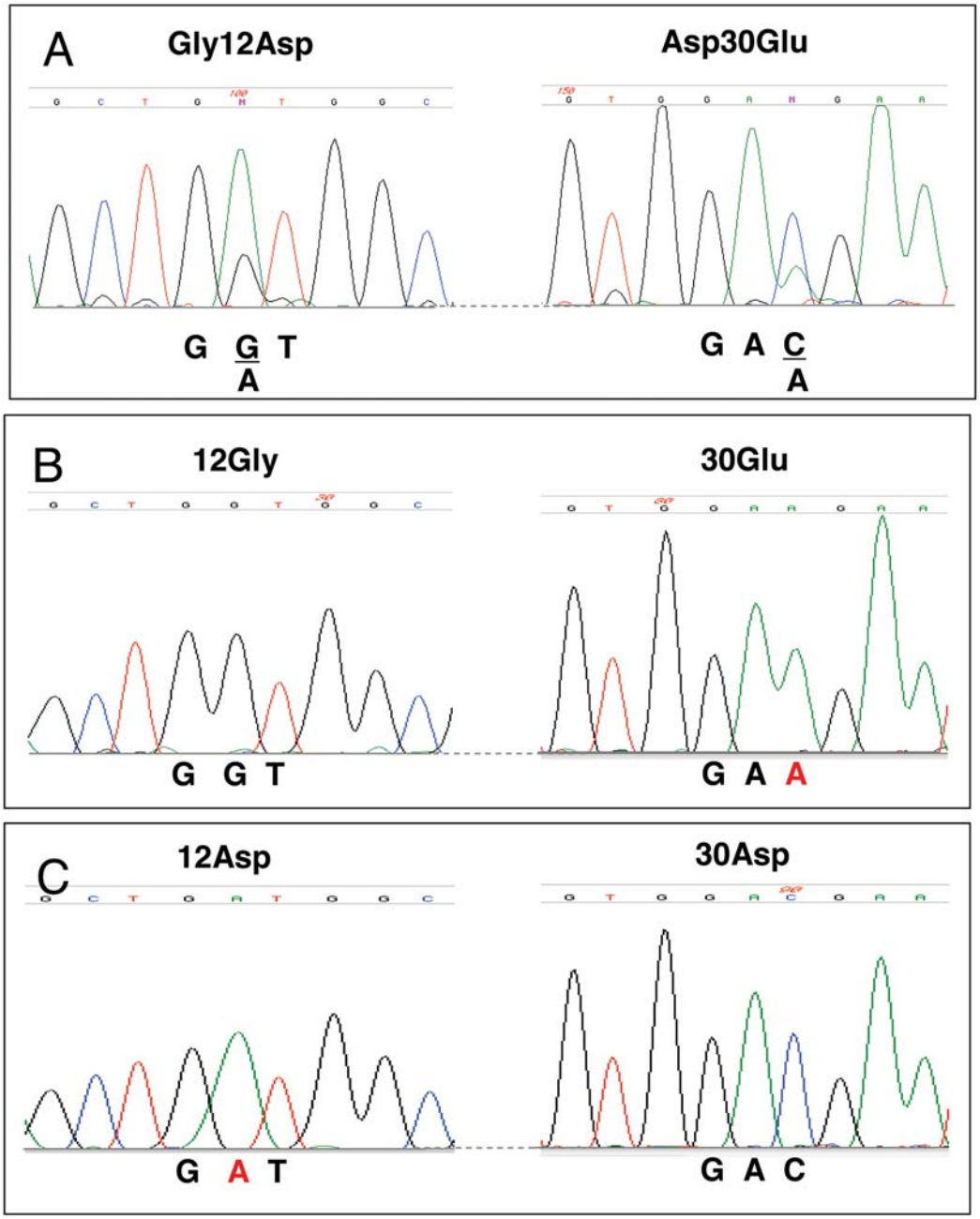

Figure 1. *Direct sequencing of the KRAS gene exon 1 showing concurrent substitutions of GGT (Gly) to GAT (Asp) at codon 12 and GAC (Asp) to GAA (Glu) to codon 30 (A). The cloning sequencing of PCR product shows an allele with a wild-type codon 12 and a mutated codon 30 (B) and an allele with a mutated codon 12 and a wild-type codon 30 (C).*

silent variants at codon 60 (Gly60Gly) and codon 63 (Glu63Glu) were identified in two additional patients (Table II).

Concurrent mutations: KRAS mutational analysis of the biological samples under examination showed the occurrence of different somatic variants in four cases. The simultaneous presence of variants on both exons was detected in two out of four cases.

One patient showed the presence in exon 1 of a sequence variant due to the substitution of a glycine with an aspartic acid (GGT to GAT) at codon 12, and the concomitant replacement of an aspartic acid with a glutamic acid (GAC to GAA) at codon 30 (Figure 1A). By cloning the PCR products with the TOPO TA Cloning kit, we demonstrated the presence of the two variants *in-trans* on different alleles (Figure 1B and C).

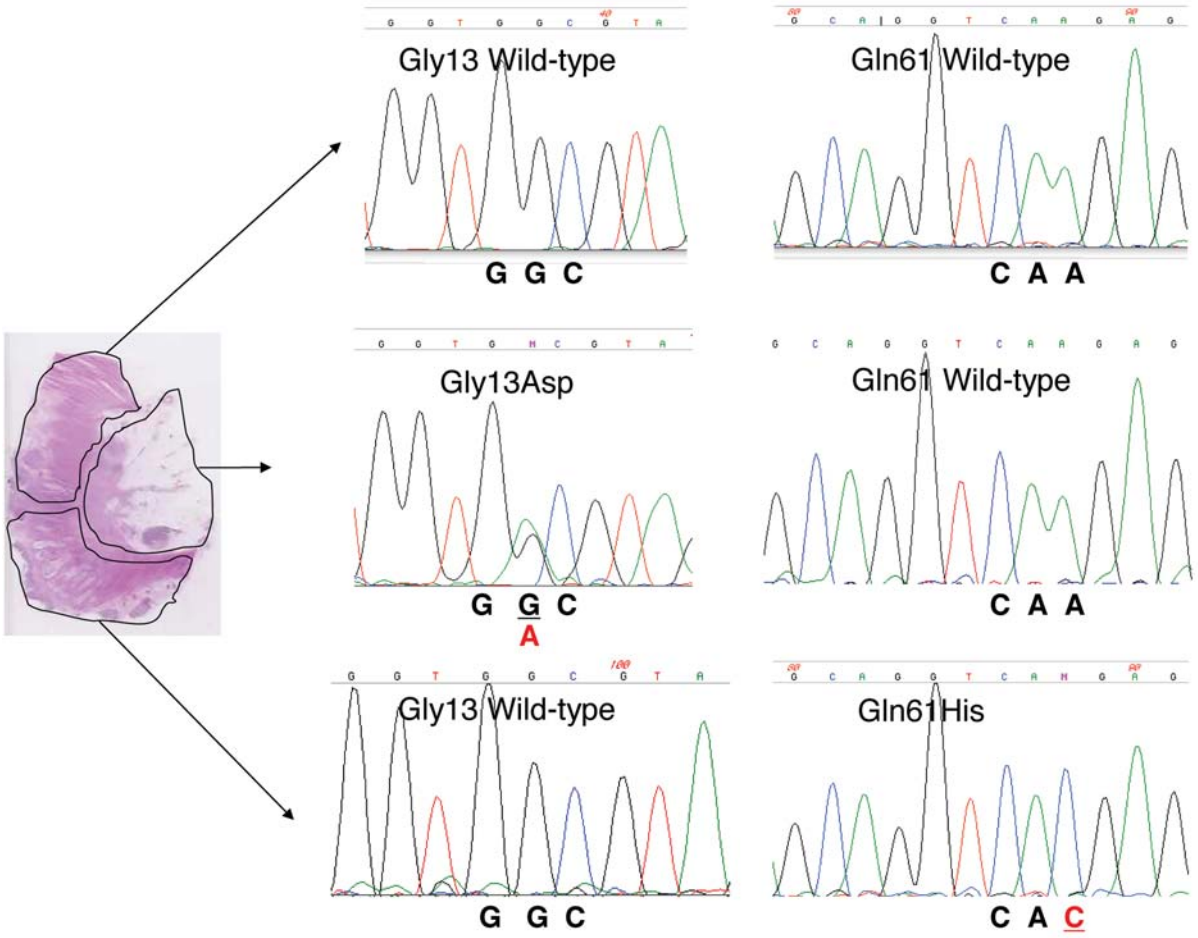

Figure 2. *Direct sequencing of the KRAS gene exons 1 and 2 from DNA extracted from different microdissected areas, showing concurrent substitutions of GGC (Gly) to GAC (Asp) at codon 13 and a homozygous substitution CAA (Gln) to CAC (His) at codon 61.*

In a second patient, we found the substitution of a glycine with an aspartic acid (GGC to GAC) at codon 13 of exon 1 and the replacement of a glutamine with a homozygous histidine at codon 61 (CAA to CAC) of exon 2. Both mutations were present in different areas of microdissection (Figure 2).

In the third case, already described in a previous report (12), two heterozygous point mutations in exon 1 were identified by direct sequencing of tumor-derived PCR products. These were represented by the substitution of a cysteine with a glycine (GGT to TGT) at codon 12 (Gly12Cys) and by a silent variant (GCC to GCT) without alanine substitution at codon 11 (Ala11Ala). In addition, a sequence variant was identified (AAC to GAC) at codon 57 of exon 2, which determined a replacement of one asparagine with an aspartic acid (Asp57Asn). PCR products of exon 1 analysis were cloned and the haplotypes of each heterozygous have been sequenced, in order to confirm whether the sequence variants of exon 1 occurred in the same allele or in different alleles. By cloning the PCR products, we demonstrated the presence of sequence variants of codons 11 and 12 *in-cis* on the same allele, while it was not possible to extend the analysis to the variant Asp57Asn, due to the excessive length of the intronic region that separates the two exons (17,861 bp, Ensamble # ENSE00001428812) and the nature of the degraded paraffin-embedded tissue.

Finally, in a fourth case we observed the simultaneous presence of two nucleotide substitutions at codon 61, with the identification, by direct sequencing analysis, of a CGT in place of CAA resulting in an amino acid change from a glutamine to an arginine (Gln61Arg) (Figure 3A). Allelic separation analysis of the PCR product, allowed also in this case to assert that both nucleotidic variants were *in-cis* on the same allele (Figure 3 B and C).

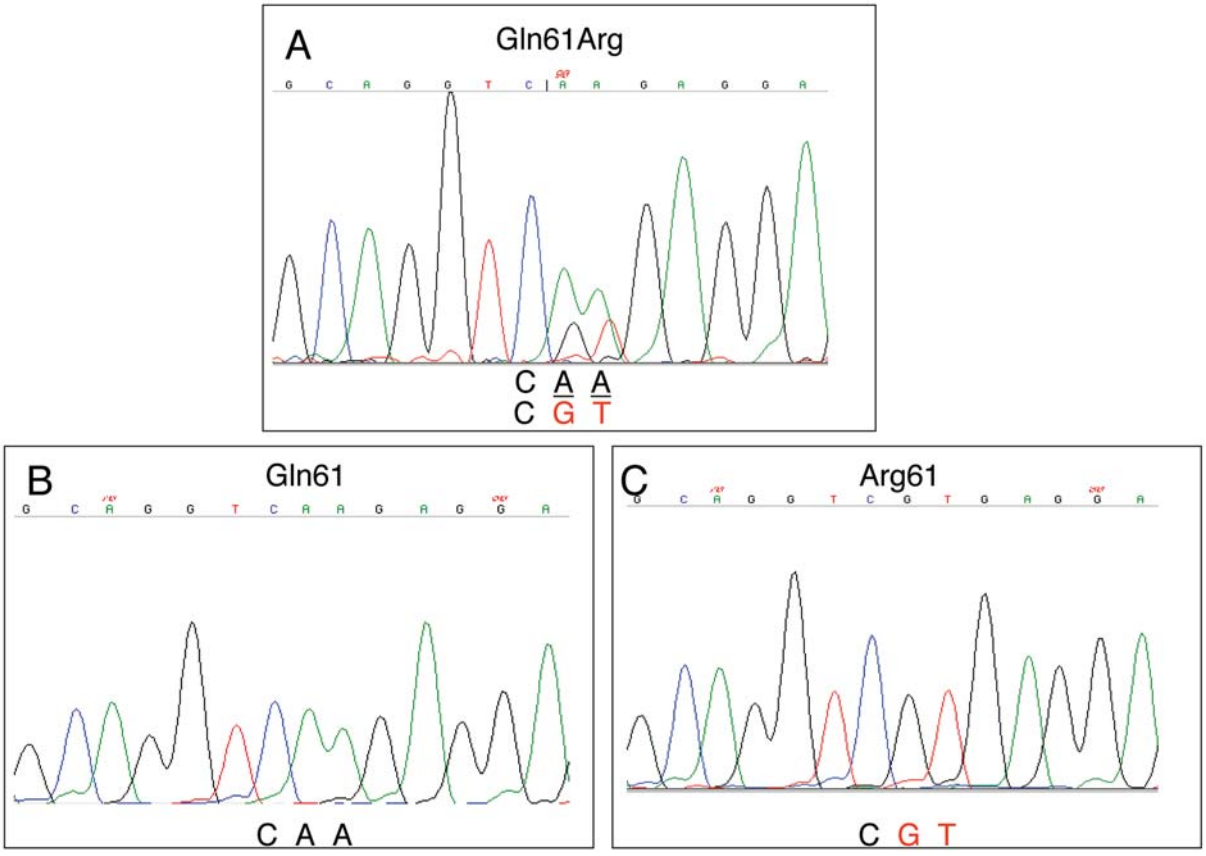

Figure 3. *Direct sequencing of the KRAS gene exon 2 showing concurrent substitutions of two nucleotides at codon 61 (CAA to CGT) determining the substitution of a glutamine to arginine (A). The cloning sequencing of PCR product reveals a wild-type allele (A) and an allele mutated with the concurrent presence of the two nucleotide variants (B).*

Mutational analysis of BRAF. Mutational analysis of the exon 15 of the *BRAF* gene was also performed on all samples. This region is characterized by a hot-spot mutation which determines the substitution of a valine with a glutamic acid at codon 600 (V600E, GTG to GAG). Pathogenic sequence variants were detected in samples from 9 (3.9%) out of 230 patients.

The canonical variant V600E was observed in eight cases, whereas a frameshift deletion of two nucleotides at codon 600 (1799 TG deletion) was found in a single case (Figure 4 A), which determines the substitution at codon 600 of a valine with a glutamic acid and the substitution of a phenylalanine with a stop signal 10 codons downstream (Phe610Stop) (Figure 4B). To our knowledge this frameshift mutation has never been reported.

Microdissection and preferential allele amplification. From our experience, we have been able to assess the importance of microdissection for a better visualization and interpretation of the mutational results. We noticed that when we proceeded to nucleic acid extraction without prior microdissection, the analysis of electropherograms was in some cases very difficult. In fact, even in the presence of canonical and relatively frequent sequence variants, the intensity of the peak corresponding to the nucleotidic variant was much less pronounced than the peak of the wild-type nucleotide. In some cases, there was complexity in distinguishing the presence of a single variant from the simple 'background noise' which is normally found in an electropherogram (Figure 5A). For this reason, whenever possible, we proceeded to a preliminary morphological evaluation of the biopsy sample before the step of nucleic acid extraction, outlining under microscopy (from ×4 to ×20) the boundaries between frankly neoplastic tissue and normal tissue. Then for each sample, we proceeded to extraction, amplification and sequence analysis of different portions of the same microdissected biological sample. Exemplifications this are shown in Figures 5 and 6.

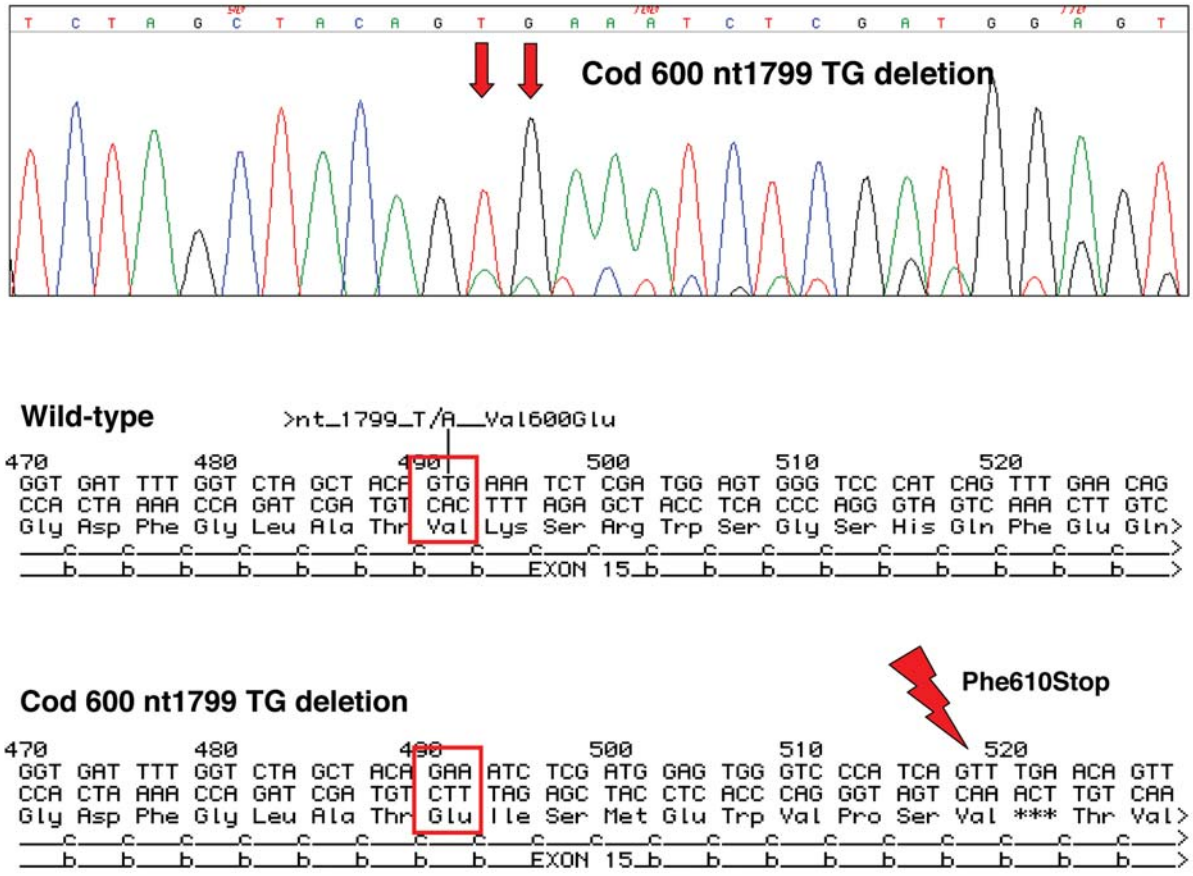

Figure 4. *Codon 600 deletion (1799 TGdel) in the BRAF gene exon 15 determining a stop signal at codon 610 (Phe610Stop).*

Discussion

In recent years, new therapeutic approaches have been developed to treat mCRC. Among these, the most helpful in clinical practice are the MoAbs to EGFR (1). EGFR is highly expressed (80%) in CRC (14). However, despite high levels of EGFR expression being predictive of a positive response to treatment, experimental studies recently clearly demonstrated that other factors, such as genetics, may identify the 'non-responder' patients (15-19, 29). In particular, mutations of *KRAS* and *BRAF* genes have been associated with treatment failure in patients treated with EGFR to MoAbs (20, 21).

Based on this evidence, in the present study, we aimed to determine the somatic genetic variations of *KRAS* and *BRAF* in patients with mCRC. The results obtained showed that approximately 48% of mCRC tissue samples exhibit genetic variants of *KRAS* (44%) or *BRAF* (4%) genes. These findings are in agreement with the frequencies reported in other genetic studies, with *KRAS* mutation rates ranging from 30 to 54% (2, 15, 16). Most mutations were represented by single, mutations in exon 1 (86%) and exon 2 (10%) of *KRAS* gene, however, concomitant variations of *KRAS* were found in 4% of the mutated cases.

A high frequency of mutations was found in this study in codons 12 and 13. In particular, approximately 62% and 23% of mutations were located on codons 12 and 13, respectively, which is consistent with previously published studies reporting a variability in mutation frequency ranging from 78% to 82% (26, 29, 30) and from 17% to 20% (2, 26, 29, 30, 31), respectively.

On the other hand, we found a frequency of non-canonical variants, elsewhere, of approximately 12% (7% on codon 61 and lower frequencies on codons 22, 30, 57, 59, 60, and 63). This is in contrast with the scientific literature and specific databases (COSMIC) reporting a frequency of mutations which are not hot-spots, ranging between 1 and 4% (2, 15, 16).

The discrepancies found in our study compared to others may be at least partially explained by the different

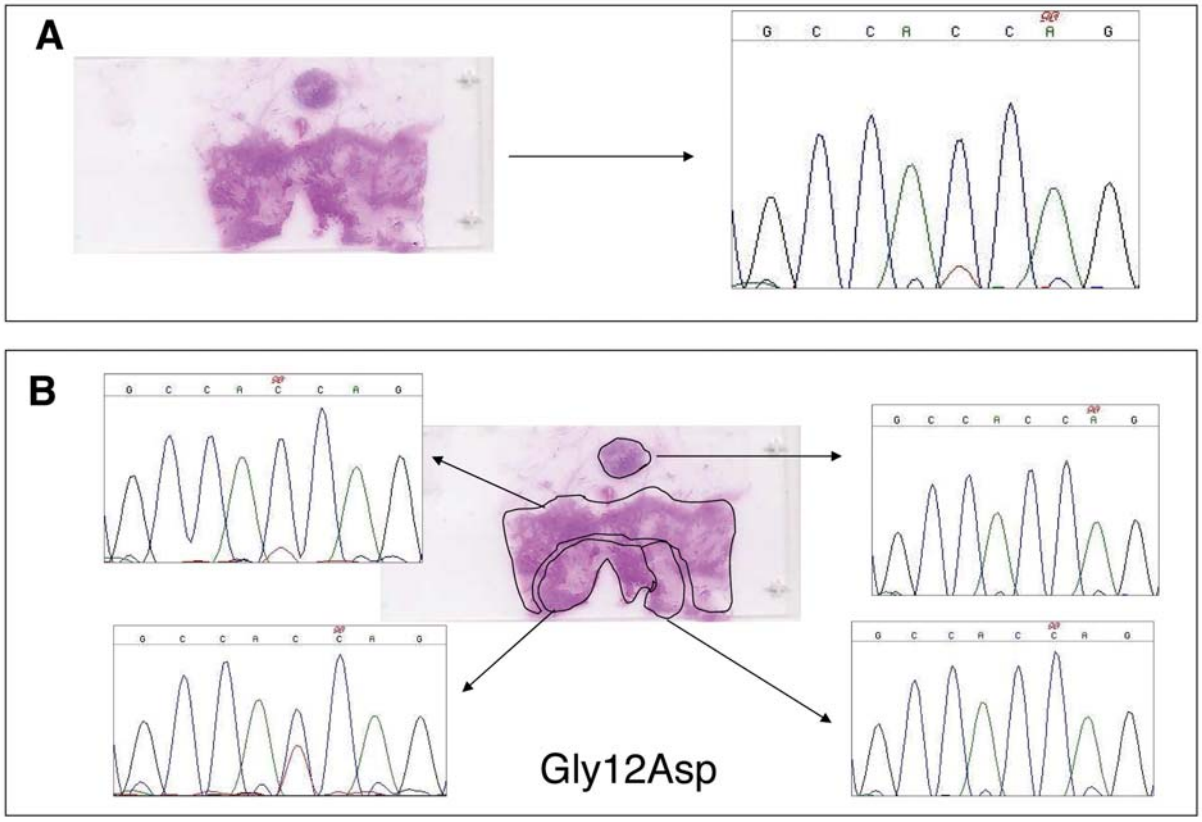

Figure 5. *Sequence analysis of KRAS exon 1 performed on DNA extracted from an entire slide of paraffin-embedded tumour section not microdissected (A) and from different microdissected areas of the same sample area (B).*

methodologies used for the molecular screening of *KRAS* gene. As stated in the introductory section, important multicentric studies such as RASCAL (15, 16) PRIME (18), and OPUS (19) reported data obtained from different laboratories using different techniques (18, 19, 27). Among these, allele-specific hybridization-based commercial kits were the most widely used; accordingly, the majority of the studies focused on canonic hot-spot *KRAS* mutations of codons 12 and 13. However, as shown in the present (by direct sequence methodology) and in previous (by denaturing high performance liquid chromatography or pyrosequencing) (31, 32) studies, mutations outside hot-spot codons can be identified by means of different analytical tools.

The use of commercial kits may be also responsible for a lower identification of concomitant mutations in *KRAS*. To date the presence of simultaneous mutations on *KRAS* are considered rare (12, 31, 33, 34, 35) and their implication in mCRC is not still fully understood. In this study, allelic separation confirmed the presence of point mutations in different alleles, in one case of double mutations, suggesting the simultaneous presence of different cellular clones in the same tumour, with a singular mutation for each clone, as previously reported (36). These findings are in agreement with the theory that a single mutation in *KRAS* may trigger the tumourigenesis (37). Intratumoral heterogeneity for *KRAS* mutations, with a high frequency in the earliest stages of colorectal cancer, has already been described (22, 38). The heterogeneity of tumour tissue, with multiple *KRAS* mutations, as found in the present study, most likely confirms that different cellular clones derived from the same cellular line may include different alterations in the same gene (35, 36). The pathogenic mechanisms by which the clonal mutation in *KRAS* may impact on tumour growth has not been fully explained yet, but interesting experimental studies provided a reasonable model for the molecular pathways underlying tumour progression of mCRC (39) and leading to a variability in drug response (39). Further studies are imperative to clarify the biological activity of simultaneous mutations in oncology.

In our opinion, a careful evaluation of paraffin-embedded sections and their microdissection represent fundamental steps in the definition of *KRAS* genetic mutations, as currently used mutational tests allow just an on/off result

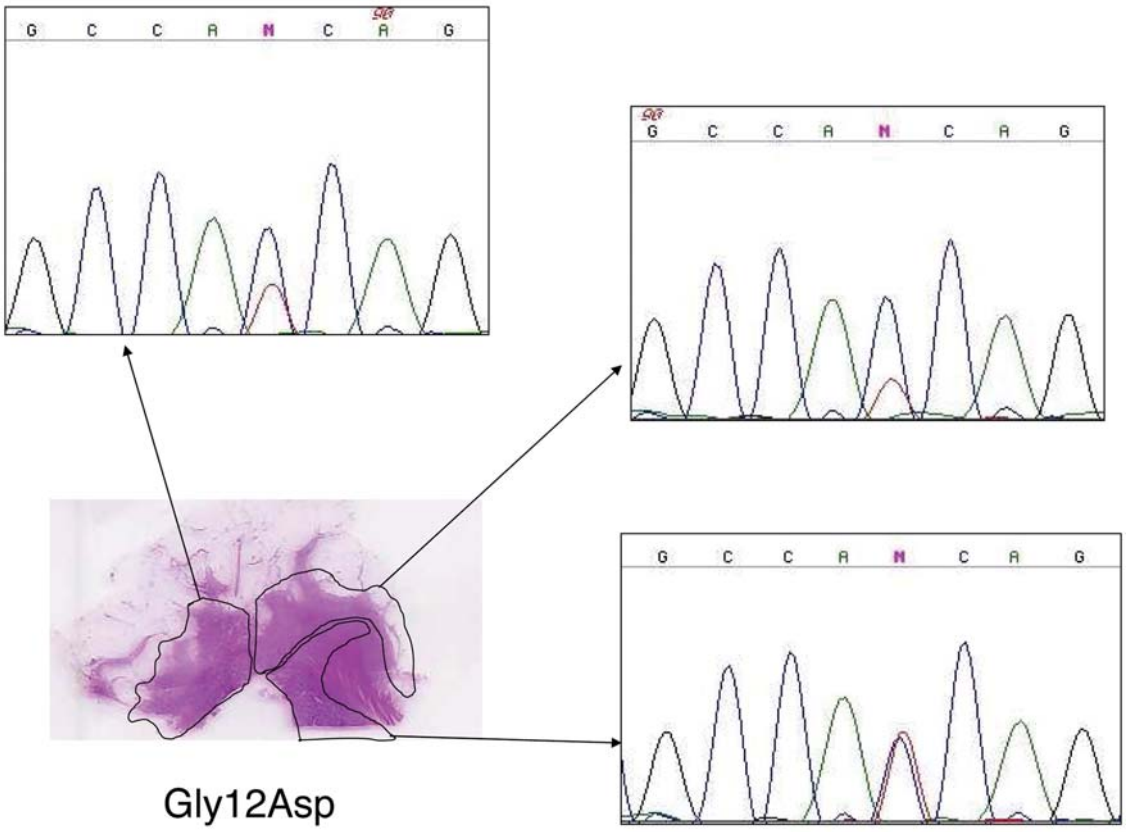

Figure 6. *Example of electropherograms originated from the extraction of nucleic acids from different microdissected areas of a section of paraffin-embedded tumour sample. The intensity of the peaks corresponding to the mutated allele is markedly dissimilar in each microdissected area.*

(wild-type *vs*. mutation) without taking into consideration the relative frequency of neoplastic cells in the tissue.

The heterogeneity of the tumour tissue arises from the concomitant presence of normal and transformed cells which may also include, as previously described, different clones with multiple genetic mutations in *KRAS*. Under these circumstances, the PCR reaction might result in the enhancement of some alleles at the expense of others, a phenomenon which is called allelic preferential amplification (40). The greater amplification of an allele at the expense of another in a heterozygous DNA sample may, thus, result in an incorrect or ambiguous genotyping of a heterozygous individual during genetic analysis.

Additional cases in which the PCR reaction can generate artifacts are those in which samples contain a low amount or poor quality of target heterozygous DNA, due to damage occurring during fixation or storage processes of paraffin-embedded tissues (41). This may cause a stochastic fluctuation in the number of copies of each amplified allele (40), leading to an allele drop-out phenomenon, as we suspect has occurred in the case shown in Figure 2.

In light of these considerations, we believe that microdissection of the analyzed tissue represents an essential step in the molecular analysis that prevents an erroneous mixture of healthy and transformed tissues being analysed.

In conclusion, standardization of the collection and handling of biological samples for genetic analyses is necessary to improve pharmacogenetic studies (28, 42), and to improve the ability to identify new genetic mutations in small sub-clones that may result in cellular resistance to conventional therapy. Thus, we suggest that some analytical phases, essential in making a diagnosis, should be better standardized in scientific studies. In this respect, tissue microdissection, direct gene sequencing, and allelic separation by cloning are steps that may increase the skill of pharmacogenetic studies in recognizing particular subpopulations of patients. The ultimate goal is the 'deep sequencing' of the neoplastic genome, which allows the detection of a small number of resistant cells, thus influencing the choice of primary therapy or the development of combined treatment strategies.

Continuous development of new pharmacogenetic technologies may allow us to quickly gain a valuable advantage in terms of diagnosis, prognosis and therapy of

patients with neoplasms. Further studies are eagerly awaited to establish the economic impact and the clinical utility of pharmacogenomics in the context of personalized medicine.

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