Sir,

Both single-arm studies (Di Fiore et al, 2007; Lièvre et al, 2008) and randomised clinical trials (Amado et al, 2008; Karapetis et al, 2008) for the treatment of metastatic colorectal cancer (mCRC) with anti-EGFR monoclonal antibodies (mAbs) have unambiguously demonstrated that the occurrence of somatic KRAS mutations is a highly predictive marker of resistance to such therapies. Based on these data, analysis of KRAS mutation status in mCRC patients is now routinely carried out to select candidates for anti-EGFR treatment. However, it is still a matter of debate which KRAS codons should be investigated and which genotyping methods should be used.

To date, all of the reported studies focused on the analysis of missense KRAS mutations in codon 12 and 13. Indeed, substitutions at these codons (i) represent up to 90% of KRAS alterations found in colorectal cancer (Forbes et al, 2009), (ii) result in the accumulation of the protein in the active GTP-bound conformation (Malumbres and Barbacid, 2003) and (iii) impair the therapeutic effect of anti-EGFR mAb. Nevertheless, the recent data reported by Loupakis et al (2009) showed that rare mutations occurring at codon 61 or 146 are clinically relevant and predict resistance to cetuximab and irinotecan treatment. Furthermore, according to our personal experience, in a series of 845 surgical samples from mCRC patients, who were referred to our institution for diagnosis of KRAS mutations, the analysis by high-resolution melting (HRM) followed by bi-directional direct sequencing of exon 2 identified 307 (36.33%) mutated samples, among which 12 showed rare variants. Six tumours carried rare codon 13 substitutions (five c.37G > T, p.G13C and one c.37C > G, p.G13R). Four tumours presented double-point mutations, one patient had the c.38_39GC > AA, p.G13E modification, one patient the c.34_35GG > CT, p.G12A modification and one patient the c.34_35GG > TT, p.G12F modification) and two patients showed two uncommon KRAS mutations. The first one was an in-frame c.30_31 insGGA insertion resulting in a glycine insertion (p.G10_A11insG) in a rectal lesion located 7 cm from the anal verge of a 74-year-old man. Histological examination classified this tumour as well differentiated with a T3NxM1 stage. This uncommon mutation has only been previously reported in one CRC (Simi et al, 2008) patient and in a case of childhood myeloid leukaemia (Bollag et al, 1996). This duplication has been shown to induce cellular transformation in vitro and activation of the RAS–MAPK signalling pathway linked to impaired intrinsic GTP hydrolysis and resistance to GAPs (Bollag et al, 1996). Based on these data, the presence of this KRAS variant could be supposed to predict resistance to anti-EGFR therapies. The second uncommon KRAS mutation was a heterozygous 6-bp in-frame c.36_37insGCTGGT insertion that was responsible for the insertion of both one extra alanine and one extra glycine (p.G12_G13insAG). This mutation was found in a hepatic metastasis lesion from a 65-year-old man with a sigmoid colon cancer. To the best of our knowledge, this tandem duplication of both codon 12 and 13 has not been described before in any cancer type. Although the functional characterisation of this insertional variant is not available, previous in vitro data (Klockow et al, 2000) have reported that synthetic mutants showing insertions of extra amino acids in the KRAS codon 10–17 region (phosphate-binding loop) can promote cellular outgrowth and stimulate the MAP-kinase pathway more efficiently than the common oncogenic p.G12V variant. Thus, also the specific alteration found in this patient could possibly be associated with resistance to anti-EGFR therapy.

Together, these data showed the clinical necessity to investigate the presence of rare or complex KRAS variants in mCRC patients before anti-EGFR therapy decision. Moreover, the occurrence of these allelic variants has certainly been underestimated, because many laboratories or commercial KRAS detection solutions focus only on the analysis of seven hotspots corresponding to amino-acid changes G12D, G12V, G12C, G12S, G12A, G12R and G13D. Particularly, these additional mutations have an impact on both therapeutic decision-making and molecular testing. We believe that in the absence of (i) KRAS standardised testing procedures and (ii) sufficient data on the functional role of rare variants, a comprehensive analysis to identify all KRAS mutations in tumour samples is required.

At the present time, no ideal mutation testing method is in use universally for KRAS status determination (Jimeno et al, 2009). To circumvent KRAS mutation targeting, we propose a two-step diagnostic approach. First, an exhaustive analysis through genetic screening of exon 2 and 3 could be carried out by HRM (Wittwer et al, 2003). Then, for exons with an altered melting profile, characterisation of the KRAS variants by sequencing could be performed. In addition, the use of traditional PCR could be replaced by coamplification at lower denaturation temperatures. As a result, the KRAS genetic screening can be extended to include all tumour samples. Furthermore, the implementation of this strategy could reduce the costs of KRAS genetic screening and could be replaced by coamplification at lower denaturation temperatures. As a result, the KRAS genetic screening can be extended to include all tumour samples. Furthermore, the implementation of this strategy could reduce the costs of KRAS genetic screening and could be extended to all tumours.
temperature-PCR (Li et al., 2008) to reach the detection of low-level somatic mutations that characterise heterogeneous or stromal contaminated samples.

We therefore suggest taking into account the occurrence of rare KRAS gene substitutions or more complex alterations for a more accurate patient selection for anti-EGFR therapies. A comprehensive detection of these alterations is not an inaccessible summit. The rapid, reliable, comprehensive and cost-limited two-step approach we propose is relatively easy to implement without any waste of time or money.

REFERENCES

Amado RG, Wolf M, Peeters M, Van Cutsem E, Siena S, Freeman DJ, Juan T, Sikorski R, Suggs S, Radinsky R, Patterson SD, Chang DD (2008) Wildtype KRAS is required for panitumumab efficacy in patients with metastatic colorectal cancer. *J Clin Oncol* 26: 1626 – 1634

Bollag G, Adler F, elMasry N, McCabe PC, Conner Jr E, Thompson P, McCormick F, Shannon K (1996) Biochemical characterization of a novel KRAS insertion mutation from a human leukemia. *J Biol Chem* 271: 32491 – 32494

Di Fiore F, Blanchard F, Charbonnier F, Le Pessot F, Lamy A, Galais MP, Bastit L, Killian A, Sesboüé R, Tuche JJ, Queuniet AM, Paillot B, Sabourin JC, Michot F, Michel P, Frebourg T (2007) Clinical relevance of KRAS mutation detection in metastatic colorectal cancer treated by Cetuximab plus chemotherapy. *Br J Cancer* 96: 1166 – 1169

Forbes SA, Tang G, Bindal N, Bamford S, Dawson E, Cole C, Kok CY, Jia M, Ewing R, Menies A, Teague JW, Stratton MR, Futreal PA (2009) COSMIC (the Catalogue of Somatic Mutations in Cancer): a resource to investigate acquired mutations in human cancer. *Nucleic Acids Res* 38(database issue): D652 – D657

Jimeno A, Messersmith WA, Hirsch FR, Franklin WA, Eckhardt SG (2009) KRAS mutations and sensitivity to epidermal growth factor receptor inhibitors in colorectal cancer: practical application of patient selection. *J Clin Oncol* 27: 1130 – 1136

Karapetis CS, Khambata-Ford S, Jonker DJ, O’Callaghan CJ, Tu D, Tebbutt NC, Simes RJ, Chalchal H, Shapiro JD, Robitaille S, Price TJ, Shepherd L, Au HJ, Langer C, Moore MJ, Zalcberg JR (2008) K-ras mutations and benefit from cetuximab in advanced colorectal cancer. *N Engl J Med* 359: 1757 – 1765

Klockow B, Ahmadian MR, Block C, Wittinghofer A (2000) Oncogenic insertional mutations in the P-loop of Ras are overactive in MAP kinase signaling. *Oncogene* 19: 5367 – 5376

Li J, Wang L, Mamou H, Kulke MH, Berbeco R, Makrigiorgos GM (2008) Replacing PCR with COLD-PCR enriches variant DNA sequences and redefines the sensitivity of genetic testing. *Nat Med* 14: 579 – 584

Lièvre A, Bachet JB, Boige V, Cayre A, Le Corre D, Buc E, Ychou M, Bouché O, Landi B, Louvet C, André T, Bibeau F, Diebold MD, Rougier P, Ducrex M, Tomasic G, Emile JF, Penault-Llorca F, Laurent-Puig P (2008) KRAS mutations as an independent prognostic factor in patients with advanced colorectal cancer treated with cetuximab. *J Clin Oncol* 26: 374 – 379

Loupakis F, Ruzzo A, Cremolini C, Vincenzi B, Salvatore L, Santini D, Masi G, Stasi L, Canestri E, Rulli E, Floriani I, Bencardino K, Galluccio N, Catalano V, Tonini G, Magnani M, Fontanini G, Basolo F, Falcone A, Graziano F (2009) KRAS codon 61, 146 and BRAF mutations predict resistance to cetuximab plus irinotecan in KRAS codon 12 and 13 wild-type metastatic colorectal cancer. *Br J Cancer* 101: 715 – 721

Malumbres M, Barbacid M (2003) RAS oncopgenes: the first 30 years. *Nat Rev Cancer* 3: 459 – 465

Simi L, Pratesi N, Vignoli M, Sestini R, Gianchi F, Valanzano R, Nobili S, Mini E, Pazzagli M, Orlando C (2008) High-resolution melting analysis for rapid detection of KRAS, BRAF, and PIK3CA gene mutations in colorectal cancer. *Am J Clin Pathol* 130: 247 – 253

Wittwer CT, Reed GH, Gundry CN, Vandersteen JG, Pyyor RJ (2003) High-resolution genotyping by amplicon melting analysis using LCGreen. *Clin Chem* 49: 853 – 860