KRAS Genotyping as Biomarker in Colorectal Cancer: A Comparison of Three Commercial Kits on Histologic Material

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Abstract. *Background/Aim: The crucial role of KRAS status in new colorectal cancer target therapy raises the issue regarding which testing method to use. This study analysed 112 formalin fixed, paraffin-embedded (FFPE) metastatic tissue samples using three different commercially available kits. Patients and Methods: A group of 40 KRAS wild-type (wt), 40 codon 12-mutated and 32 codon-13 mutated samples, previously evaluated by real-time PCR (TheraScreen kit), used as reference method, were analysed by Ampli-set-K-RAS and K-RAS StripAssay kit (herein called kit A and B, respectively) based on two different technologies. Results: The sensitivity of both kits was 92.5% for wt samples, 100% and 95.0% for kit A and B, respectively for samples mutated in codon 12. The specificity was 100% for both kits for all groups of samples. After a minor modification of the kit A method, its specificity reached 100%. Conclusion: of low cost and easy to use, kit A may be suitable for use in a routine diagnostic setting.*

Inhibition of the epidermal growth factor receptor (EGFR) signalling pathway represents a therapeutic option in metastatic colorectal cancer (mCRC). Improved response rates and prolonged time to metastasis and/or survival have been demonstrated with the EGFR-blocking antibodies cetuximab and panitumumab (1). However, this therapy is effective only in a subset of patients since it is limited to patients with wild-type (wt) *KRAS* gene (2).

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KRAS mutations, which typically occur in the precursor lesions and at the late adenoma stage, are found in about 40% of all colorectal tumours, with predominant locations in codons 12 (82%) and 13 (17%) of the gene sequence (3). Since the mutations lead to the activation of the KRAS protein in the absence of upstream EGFR stimulation, the presence of *KRAS* mutation represents a negative predictor of response to anti-EGFR therapy. Quality assurance programmes for *KRAS* mutation testing and practice guidelines related to the optimal testing material, methodological considerations and recommendations for result reporting are currently being developed (4).

In general, *KRAS* diagnostics in the clinical setting are limited by two factors, namely the type of tissue samples used for the assay and the choice of method to analyse the *KRAS* mutations. Although it is preferable to use snap-frozen tumour samples, such samples are rarely available for this purpose. Therefore, formalin-fixed paraffin-embedded (FFPE) tissue is usually used, considering also that the therapy is often performed at a metastatic stage after surgical treatment.

A major problem with FFPE specimens is DNA fragmentation due to the duration of fixation, pH, salt concentration and temperature (5, 6). However, this problem may be circumvented by the amplification of a small segment of DNA and since the majority (>98%) of *KRAS* mutations in mCRC are point mutations, they may be easily evaluated by PCR-based methods instead of sequencing methods (7-9).

The optimal method for detecting the mutations in the *KRAS* gene is still a matter of debate. Allele-specific PCR, standard Sanger sequencing, and pyrosequencing all have advantages and disadvantages. Recently, some studies have raised this issue comparing different commercial kits for results, costs and working times (10-13).

There are no tests for *KRAS* analysis currently approved by the U.S. Food and Drug Administration (FDA), while the European guidelines have divided the diagnostic tests into two categories: those able to detect *KRAS* mutations by

sequencing and those by real-time PCR. Among the latter, the first kit approved for diagnostic purposes was the TheraScreen kit (DxS, Manchester, UK). The TheraScreen kit was quoted by European directives because it is able to identify six mutations in codon 12 and one mutation in codon 13 using the principle of the ARMS/Scorpion method (14-16).

Since the detection of the mutations affecting codons 12 and 13 of the *KRAS* gene is almost exclusively performed by real-time PCR or sequencing techniques, the present study evaluated the performance of two commercial kits: Ampli-set-K-RAS kit (Bird, Siena, Italy) and K-RAS StripAssay (ViennaLab Diagnostics, Vienna, Austria), which are based on methods different from that of real-time PCR. The first kit is based on the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method, while the second kit is based on the PCR-hybridisation strip method (17, 18). The results obtained from these two kits were then compared to those obtained from the TheraScreen kit, which was used as a reference kit, since it is the first kit approved in Europe and of which the authors had more laboratory experience. Finally, the study also estimated the ease of assay implementation, total time, costs per sample and the potential equipment costs of the kits; all important factors for routine diagnostic testing.

Patients and Methods

Tissue specimens and processing. From October 2008 to May 2010, 342 FFPE samples were investigated for *KRAS* mutational status in order to predict the eligibility of patients with mCRC for anti-EGFR antibody treatment. A total of 170 colorectal samples, obtained from the Department of Clinical Surgery of the host institute (Scientific Institute for Digestive Diseases, I.R.C.C.S. Saverio de Bellis, Italy) were processed and included in paraffin blocks for histological diagnosis in the Department of Pathology, and 172 FFPE samples included in paraffin were obtained from the Departments of Pathology of other Institutes.

For this study, 112 of 170 patients followed-up in the host institute were selected. Informed consent for use of biological material for research purposes was obtained from the patients and the study was design was approved by the local Ethics Committee.

The *KRAS* mutational status of 112 FFPE tissue samples was identified previously by the TheraScreen kit following the manufacturer's instructions. The TheraScreen kit was able to identify six mutations of codon 12 (*p.G12A, p.G12D, p.G12R, p.G12C, p.G12S* and *p.G12V*) and one mutation of codon 13 (*p.G12D*) of the *KRAS* gene. The distribution of the *KRAS* mutational status in the 112 FFPE samples selected for this study was as follows: 40 samples with mutation at codon 12 (55% with *p.G12D* mutation; 32.5% with *p.G12V* and 12.5% with *p.G12A*), 32 samples with mutation at codon 13 (*p.G13D*) and 40 with *KRAS* wt.

The same procedures for FFPE sample processing and for DNA extraction used in the TheraScreen analysis were also followed for the two commercial kits. In brief, the area for DNA extraction was identified on tissue sections stained with haematoxylin-eosin by a pathologist, avoiding necrotic and stromal tissues with a percentage of tumour cells ≥70%. This area was marked on parallel serial unstained tissue sections (10-μm thick) of the same paraffin block, and then dissected with a scalpel. After deparaffinization with d-limonene solution (K-Clear; Kaltek, Padua, Italy), the DNA was extracted by Qiamp DNA FFPE tissue Kit (Qiagen, Milan, Italy) according to the manufacturer's instructions. DNA quality and concentration (μg/ml) were assessed by A260/280 absorbance ratio in an ultraviolet spectrophotometer (BioPhotometer; Eppendorf, Milan, Italy).

Since DNA integrity is a limiting factor in routine *KRAS* analysis, the PCR products obtained from two commercial kits were also evaluated by the electrophoresis on 3% agarose gel before the *KRAS* analysis.

Two commercial kits and their methodologies.
Ampli-set-K-RAS kit: The Ampli-set-K-RAS kit was based on the PCR-RFLP method and consisted of two separate PCR reactions to highlight the codon 12 and 13 mutations. The PCR products of 107 bp and 171 bp, obtained to evaluate the mutations of codons 12 and 13, respectively, were submitted to restriction enzyme action: *MvaI* for PCR products of 107 bp and *HaeIII* for PCR products of 171 bp, as recommended in the manufacturer's instructions (at 37°C for at least 4 h). DNA input for each PCR reaction was approximately 60 ng and each sample was analysed in duplicate. Prior to and following digestion with restriction enzymes, the PCR products were resolved in 3% agarose gel stained with ethidium bromide. To assess the DNA fragmentation, aliquots of PCR products were subjected to electrophoresis prior to enzymatic digest, whereas, after enzymatic digest, electrophoresis was performed to analyse the *KRAS* gene mutational status. The samples with codon 12 mutations showed three electrophoretic bands of 107, 77 and 30 bp in heterozygosis status and one band of 107 bp in homozygosis. Fragment lengths of 77 and 30 bp were seen in cases of no codon 12 mutation. The specimens with codon 13 mutations showed four electrophoretic bands of 102, 69, 48 and 21 bp in heterozygosis, two bands of 102 and 69 bp in homozygosis and three bands of 102, 48 and 21 bp for wt samples. The kit was unable to highlight the type of mutation in codon 12 or 13.

K-RAS StripAssay kit: The protocol included three steps: (i) DNA isolation, (ii) PCR amplification using biotinylated primers and (iii) hybridization of PCR products to a test strip containing allele-specific oligonucleotide probes immobilised as an array of parallel lines. After PCR reaction, the products of 151 and 204 bp in each tube were separated into two strands by raising the temperature. The biotinylated strand was hybridised with a probe immobilised on the strip and this binding was visualised by streptavidin-alkaline phosphatase and colour substrate in a shaking water bath (18). The assay was performed in duplicate for each sample examined. The kit covered seven mutations of codon 12 (*p.G12A, p.G12D, p.G12R, p.G12C, p.G12I, p.G12S* and *p.G12V*) and two mutations of codon 13 (*p.G12D* and *p.G13C*). The assay had an internal positive control in order to ascertain PCR amplification. The DNA input for each tube reaction ranged from 5 to 50 ng. To assess the DNA fragmentation in each sample, the PCR products of 151 and 204 bp were visualised on 3% agarose gel before the hybridisation step. The kit was unable to highlight the mutations of the *KRAS* gene in heterozygosis or homozygosis.
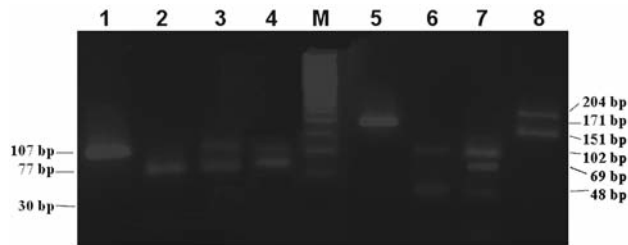
Figure 1. *Rappresentative electrophoresis for KRAS mutation analysis by Ampli-set-K-RAS kit. Lane 1: PCR product of 107 bp used to identify the codon 12 mutation before enzymatic cut. Lane 2-4: the same PCR product after the enzymatic cut. Lane 2: Wild type sample (bands of 77 and 30 bp). Lane 3: Sample mutated in codon 12 (bands of 107, 77 and 30 bp). Lane 4: Wt sample by TheraScreen kit was branded as positive by Ampli-set-K-RAS kit (false-positive). Lane 5: PCR product of 171 bp used to identify the codon 13 mutation before enzymatic cut. Lane 6: Wt sample (bands of 102, 48 and 21 bp). Lane 7: Sample with mutation in codon 13 (bands of 102, 69, 48 and 21 bp). Lane 8: Example of PCR product (bands of 204 and 151 bp) used with K-RAS StripAssay kit before hybridization step. Lane M is a DNA ladder of 50 bp.*

Figure 2. *Example of results obtained by the K-RAS StripAssay kit. Strip 1: Wt sample without bands except for marker and control band. Strips 2 and 3: Presence of mutations in codons 12 (p.G12D) and 13 (p.G13D), respectively. Strip 4: Example of a sample with double mutation. The black arrow shows the band corresponding to the mutation of codon 12, whereas the white arrow shows the non-specific additional band corresponding to the mutation of codon 13 (false-positive). The decoder table (shown on the right) was included in the operator's manual and was used to identify the type of mutation.*

Laboratory working time and costs. The laboratory working time and the costs of the assay and the instruments are also important factors for routine clinical testing. Therefore, the study also considered the total testing times and the approximate cost for a single assays because the number of assays may vary for each run. The cost of the DNA extraction kit was excluded from the economical evaluation. The potential equipment costs for each method used were also estimated taking into account the manufacturer's information and personal communications with specialists of the local distributors of the kits evaluated.

Statistcal analysis. For the evaluation of the validity of each kit, in comparison to the reference (TheraScreen kit), their sensitivity and specificity were determined. A technique was considered to be in agreement with TheraScreen, if both of them identified a wt or a mutant, regardless of the mutation in homozygosis or heterozygosis.

Results

DNA fragmentation analysis. DNA quality is a potentially important factor affecting the performance of *KRAS* mutation assays and the DNA extraction procedure used in this study for FFPE tissues worked particularly well for the PCR-RFLP and PCR-hybridization strip assay methods. DNA purity did not differ substantially among tissue samples (A260/280 mean ratio was 1.7±2.0).

Before the analysis of the *KRAS* mutational status, all PCR products were visualised on 3% agarose gel stained with ethidium bromide (Figure 1). This also suggested that the DNA extracted from FFPE tissues may be successfully used in PCR with short amplification products up to approximately 200 bp in length.
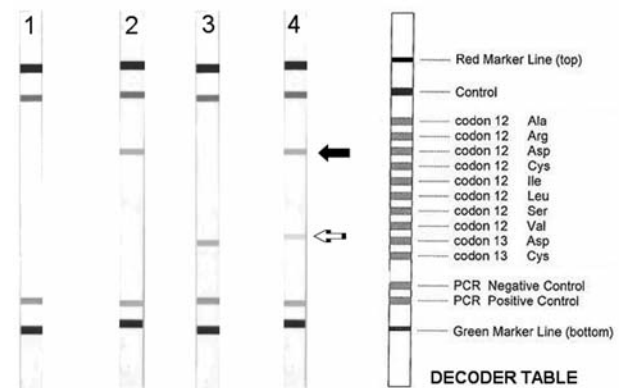
Specificity and sensitivity of the two commercial kits. The results of the Ampli-set-K-RAS kit were in full concordance with those of the reference kit (TheraScreen) for the mutations of codons 12 and 13 (Figure 1). Among the 40 tissue samples evaluated as wt by TheraScreen, three showed a mutation in codon 12 (false-positives) with the Ampli-set-K-RAS kit (Figure 1, lane 4).

Total agreement was observed between K-RAS StripAssay kit and TheraScreen kits for the samples mutated in codon 13 (Figure 2). However, with the K-RAS StripAssay kit, 3 of 40 wt samples showed a mutation in codon 12 (false positives). In addition, using the same kit, 2 of 40 cases with mutation in codon 12 showed a second band (double mutation) with less colour intensity corresponding to the mutation of codon 13 (double mutation) (Figure 2, strip 4). Generally, the presence of a double mutation in the *KRAS* gene is a rare possibility in colorectal tumours. In the present study, this event was excluded by the TheraScreen kit. For this reason, in statistical evaluation, the double mutation of two samples was considered as a discordant results compared to that obtained by the reference kit.

In terms of specificity and sensitivity, both commercial kits showed the same values for wt and codon 13-mutated samples, while the Ampli-set-K-RAS kit, when compared to the K-RAS StripAssay kit, showed higher sensitivity in detecting mutations in codon 12 of the *KRAS* gene. The diagnostic specificity and sensibility of two kits are shown in Table I.

Table I. *Sensitivity and specificity of two commercial kits (Ampli-set-K-RAS kit and K-RAS StripAssay kit) compared to the TheraScreen kit, used as a reference.*

KRAS mutational status	Ampli-set-K-RAS kit		K-RAS StripAssay kit	
	Sensitivity (%)	Specificity (%)	Sensitivity (%)	Specificity (%)
Wild-type (n=40)	92.5	100	92.5	100
Codon 12 mutated (n=40)	100	100	95.0	100
Codon 13 mutated (n=32)	100	100	100	100

Laboratory working time and costs. The working time and cost per assay performed with the two kits were investigated considering the TheraScreen kit as reference. The reference kit offered a rapid, standardised and high-throughput system, but the high cost of the assay, partly justified by the Scorpion/Arms technique used, may be prohibitive for many clinical tests. On the contrary, the assay of the K-RAS StripAssay kit was less expensive, but also less rapid compared to the reference kit because it provided several reaction steps, namely amplification, hybridisation, detection and washing. The hybridisation and detection steps may become automated to enable analysis of more samples in the same batch assay, but the apparatus required for this purpose is dedicated to the hybridisation analysis with strips. The PCR-FRLP method of the Ampli-set-K-RAS kit had similar working time with the PCR-hybridisation method. Compared to the reference kit, the assay of the Ampli-set-K-RAS kit was less expensive, easily applicable and did not require high-laboratory expertise, also taking into account the use of the commercial pre-cast agarose gels. In addition, the equipment cost was lower than that of a real-time PCR. The comparison of the working time and costs of the kits are given in Table II.

Discussion

A total of 112 of FFPE tissue sections were tested by two commercial kits based on methods different from that of the real-time PCR. The results obtained were then compared to those obtained with TheraScreen, used here as reference kit.

The first stage of a diagnostic test is the preparation and storage conditions of the tissue samples to ensure the integrity of target genes for the downstream analysis. In the present study, tissue samples of patients surgically treated in the host institute were used, as distinct from the samples obtained in other institutions, because the samples used were fairly homogeneous and standardised in processing and storage.

Although the two kits were declared usable on FFPE tissue samples, it was important to assess the quality of the extracted DNA for an accurate interpretation of the results.

Table II. *Estimates of assay cost and working time among the three kits (TheraScreen, Ampli-set-K-RAS and K-RAS StripAssay). The + signs are only an indication of magnitude. Working time excluded the time required for DNA extraction.*

Kit	Reagent costs	Working time
TheraScreen	+ + + + +	+ +
Ampli-set-K-RAS	+ + +	+ + + +
K-RAS StripAssay	+ + + +	+ + + +

In fact, a potential problem that may affect test results was the DNA integrity after the steps of formaldehyde fixation and paraffin embedding. Formaldehyde, the effective component of formalin, leads to the generation of cross-linkage between nucleic acids and proteins, while paraffin embedding may cause DNA fragmentation (19).

To overcome this pre-analytical confound, tamponated formalin and d-limonene solution (K-Clear), were used instead of xylene solution, for the deparaffinisation step. The use of these two solutions led to improved DNA quality as previously described for RNA by Roberts *et al*. (20). In addition, before the analysis of the *KRAS* mutational status, the PCR products were always subjected to electrophoresis on 3% agarose gel to monitor and control the amplification process. Overcoming the problems related to the DNA quality and integrity enable an unbiased and systematic comparison between the two kits.

In the PCR-FRLP method (Ampli-set-K-RAS kit), the 107 bp band was the principal electrophoretic band for identifying the mutations in codon 12. When this PCR product was cleaved into two bands of 77 and 30 bp by a restriction enzyme, the sample was scored as wt, otherwise the mutant codon 12 fragment was cut into three bands (107, 77 and 30 bp). In 3 out of 40 wt samples, the 107 bp band was partially cut and, in this case, the presence of three bands was interpreted as mutation of codon 12 (false-positive). It is likely the incomplete enzymatic cut was due to an insufficient amount of enzyme or to a short time of

the digestion process (21). Comparing the assay procedures step by step and in order to rule out the incomplete digestion-generated artifacts, the restriction enzyme units for single reaction and the enzymatic digestion time were increased (10 U/μl *vs*. 5 U/μl and 12 h *vs*. approximately 4 h, respectively). Improving the method slightly, the 107 bp bands of three cases were fully cut, identifying correctly the samples as wt.

The analysis of the other kit, K-RAS StripAssay, showed 3 of 40 wt samples mutated at codon 12 (false-positive) and 2 of 40 samples mutated at codon 12 showed an additional mutation at codon 13 (double mutation).

To overcome the ambiguous identification of the *KRAS* status, several modifications were introduced to the method, similar to those performed for the Ampli-set-K-RAS kit. An automatic shaking water bath (ProfiBlot T48, Tecan, Milan, Italy) was used to improve the reproducibility of the assay and the ramp rate of the PCR instrument was decreased to improve the amplification product. However, the final result was not improved.

Cost and turnaround time of assays are also important factors for routine clinical testing of *KRAS* mutational status. The assay of the Ampli-set-K-RAS kit was less expensive when compared to that of the K-RAS StripAssay kit and the DxS-Kras (TheraScreen) kit. In particular, even though the real-time PCR (TheraScreen) was markedly fast, it had a higher cost per assay. In addition, the cost of the real-time PCR instrument was high if compared to that of the other kits. The cost per assay and equipments to apply the PCR-RFLP method (Ampli-set-K-Ras) was less expensive. However, despite its low cost, the Ampli-set-K-RAS kit was unable to identify the type of mutations present in codon 12 and 13 of the *KRAS* gene.

There is a general consensus that seven mutations in codons 12 and 13 of the *KRAS* gene should be covered. However, the type of mutation in codon 12 or 13 is significantly linked to the biologically aggressive potential of the tumour and it is most important for the clinical outcome (prognosis) of patients with mCRC rather than for a diagnostic role in target therapy (22, 23). Other mutations also need to be analysed, such as the *BRAF* gene mutation that is present in about 60% of non-responder patients with *KRAS* wt (24).

In conclusion, PCR-RFLP, a non-real-time PCR-based technology, was comparable in analytical specificity and sensitivity with the Scorpion/Arms method and may be used, except for the laboratory working time, as an accurate, easy and inexpensive test for FFPE colorectal cancer samples.

Acknowledgements

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