RAS-testing in colorectal cancer: Belgian guidelines

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There is an urgent need for predictive biomarkers in several cancers. In colorectal cancers, KRAS exon 2 mutation analyses were mandatory when considering anti-epidermal growth factor antibody therapy with agents such as cetuximab or panitumumab. However, since the introduction of this testing, a cohort of patients still did not appear to benefit from this therapy. Recently, additional testing for KRAS exon 3 and 4, and NRAS considerably improved the predictive power for therapy success. Therefore, an update of the Belgian guidelines for RAS testing was urgently needed.

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Introduction

RAS molecules participate in the activation of important oncogenic signalling pathways. They are involved in processes such as cell proliferation, anti-apoptosis, invasion and migration.[1,2] An activating mutation may induce an oncogenic transformation. Initial single-agent studies of anti-epidermal growth factor receptor (anti-EGFR) antibodies in the treatment of metastatic colorectal cancer (mCRC) showed that these agents were marginally effective.[3,4] Objective response rates were approximately 10% when used as monotherapy for irinotecan-refractory and/or oxaliplatin-refractory mCRC. RAS mutation status has emerged as an important predictive marker for anti-EGFR therapy in patients with mCRC because patients with mutant RAS are unlikely to benefit from treatment with the anti-EGFR antibodies. Moreover such a treatment may be noxious: patients with KRAS mutated CRC may have inferior outcomes if this therapy is combined with an oxaliplatin-containing chemotherapy regimen compared to patients which are treated with oxaliplatin alone.[5]

Mutations in KRAS exon 2 became the first predictive biomarker for colorectal cancer.[6] Even with testing of KRAS exon 2, however, a cohort of patients still did not benefit from anti-EGFR antibody therapy. Numerous publications showed that looking at more infrequent RAS mutations could predict response to anti-EGFR therapy more reliably. In particular, the pivotal publication of Douillard et al showed the importance of these mutations. In an analysis of the phase III panitumumab randomised trial in combination with chemotherapy for mCRC to determine efficacy (PRIME), the authors clearly demonstrated that additional RAS testing further delineated the population most likely to benefit from the addition of anti-EGFR antibody therapy to standard chemotherapy.[7]

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Accurate RAS mutation testing is important as the toxicity of anti-EGFR antibody therapy for mCRC is not negligible. In addition, false negative results represent significant health care costs.[8]

These molecular studies need competence. The procedure needs to be standardised and performed in reference labs which are accredited following the International Organisation for Standardisation ISO 15189 (2012).[9] Pathologists are part of the multidisciplinary oncology treatment team. As such, pathologists should understand the clinical significance of RAS mutation status and its role in the evaluation of treatment options for targeted therapy with anti-EGFR antibodies.[10,11]

EGFR, RAS, cetuximab and panitumumab

EGFR is a transmembrane glycoprotein tyrosine kinase receptor that binds soluble ligands such as epidermal growth factor and transforming growth factor-alpha. EGFR forms dimers when ligand-bound and stimulates an intracellular phosphorylation cascade that transmits the original ligand-generated signal from the cell surface to the nucleus. Activation of the EGFR stimulates angiogenesis, migration, proliferation and survival of cancer cells. RAS is downstream from the EGFR in the RAS-BRAF-MEK-ERK pathway and therefore anti-EGFR antibodies are unable to block receptor signalling in tumours with activating RAS mutations.[11]

RAS proteins are GTPases, which function as molecular switches: 'on' when bound to GTP, and 'off' when bound to GDP. RAS-GTP can bind to numerous partner proteins, termed 'effectors' and these RAS-effector interactions lead to a cascade of downstream signal events.[12]

The hydrolysis of GTP to GDP by RAS is a slow process, and therefore RAS cycles between these states with the aid of regulatory proteins.

GTPase activating proteins (GAP) catalyse the hydrolysis of GTP to GDP ('on' to 'off'), whereas guanine nucleotide exchange factors (GEF) catalyse the dissociation of GDP, with GTP binding afterwards due to its high concentration in cells. However, this pathway is co-opted by oncogenic mutations in RAS. For example, Q61 mutants prevent co-ordination of a water molecule necessary for GTP hydrolysis, whereas G12 and G13 mutants prevent binding of RAS to its GAP. It seems that all activating RAS mutations result in RAS-GTP in an 'on' state, driving oncogenesis.[2]

Cetuximab, a human-mouse chimeric monoclonal antibody, and panitumumab, a fully human monoclonal antibody, are directed against the extracellular domain of EGFR and can be used as monotherapy or combined with chemotherapy.[3-5]

Both drugs have very similar efficacy. Blocking EGFR while RAS remains in the 'on' mode by an activating mutation is pointless. It was, however, not anticipated that anti-EGFR antibodies might harm patients who have mutated RAS. Preclinical data suggested that non-mutated RAS isoforms can suppress the activity of mutated RAS isoforms. Suppression of non-mutated RAS with an EGFR inhibitor would theoretically activate mutated RAS isoforms by releasing the suppressive effects of the non-mutated RAS isoforms.[1]

Which RAS mutation testing ?

Until recently, only KRAS exon 2 mutation testing was routinely performed in patients with mCRC who were candidates for anti-EGFR therapy. As already explained, significant work has been done to further delineate the group of patients likely to respond to anti-EGFR therapy. Newly presented data clearly show that genetic profiling should be expanded to include other RAS mutations.[13]

Based on this new evidence, the most recent National Comprehensive Cancer Network (NCCN) guidelines for colon cancer strongly recommend KRAS exons 2, 3, 4 and NRAS genotyping for all patients with mCRC (http://www.nccn.org/professionals/physician_gls/recently_updated.asp). Recently, the European Society for Medical Oncology (ESMO) issued similar guidelines.[14] Also, the European Medicines Agency (EMA) indications for cetuximab and panitumumab have been updated to include only patients with RAS wild type (KRAS/NRAS) mutations.

The most common RAS mutations, seen in 35-40% of patients with mCRC, are activating mutations of KRAS exon 2 at codons 12 or 13. These mutations represent more than 90% of all the KRAS mutations. Different studies have demonstrated that other mutations can be found and need to be looked for before starting or not starting an anti-EGFR therapy. KRAS exon 3 mutations (in particular codon 61) account for 4,3% of the RAS mutations, while KRAS exon 4 mutations (codon 117 and 146) account for a further 6,7% of mutations.[15]

NRAS mutations are seen less frequently in colorectal cancer, accounting for 10%. NRAS exon 3 (codon 61) mutations are most frequently detected (+/- 4,8%), while NRAS exon 2 (codon 12 and 13) and NRAS exon 4 (codon 117 and 146) are less frequently detected, in

3,8 and 0,5% of cases, respectively. Hence testing for RAS mutations besides KRAS exon 2 mutations adds 20% more cases in which anti-EGFR therapy should not be given. Generally speaking, 53% of mCRC tumours are resistant to anti-EGFR mAbs.[15]
This is why routine RAS screening is required before initiating anti-EGFR therapy in patients with mCRC to predict non responsiveness to anti-EGFR therapy.

Procedure

These molecular studies need competence, standardised equipment and standardised procedures. In practice, RAS mutation detection involves two different, but complimentary, fields, i.e. pathology and molecular medicine. The procedure needs to be standardised and performed in reference labs which are accredited as ISO 15189 and participate in internal and external quality controls as detailed in law article 33bis (published in July 2009).
Pathologists have a crucial and responsible role in coordinating RAS testing as mutation analysis is performed on paraffin-embedded tissue selected by the pathologist. Their role includes the tumour diagnosis, the careful attention to fixation procedures to preserve tissue quality, the selection of the most appropriate tumour block with evaluation of the percentage of tumour cells and their composition (necrosis, mucinous changes).[10]

Sample quality in relation to test sensitivity

There are several important issues that need to be considered by the pathologist to ensure high quality tissue collection.[16,17]

A. Fixation

Pathologists should be aware of the fact that adequate fixation is important. To avoid degradation of the tissue, fixation should start as soon as possible after biopsy or surgical removal. Delayed or suboptimal fixation results in DNA degradation due to apoptosis and/or necrosis. Fixatives such as Bouin or B5 fixative are not compatible with molecular testing. Ten percent neutral buffered formalin is the gold standard fixative.
Fixation time is also a critical issue. It has been shown that tissues fixed for more than 24 hours already have a lower yield and poor quality of DNA. Formalin over fixation can damage DNA and introduce artificial mutations through excessive cross-linking. This is a general problem in molecular testing.[18,19]
Every specimen should be rapidly fixed (within 1 hour of being obtained), preferentially in 10% neutral buffered formalin for <72 hours: six to twelve hours for endoscopic biopsy specimens and eight to maximum 72 hours for surgical specimens.

B. Paraffin embedding

Paraffin embedded tissues are preferred over frozen tissue or fresh material because it is important to know the percentage of tumour cells in the analysed tissue sample, which is easier to assess on a HE-stained section. Paraffin-embedded tissues are also easier to transport than frozen tissue in case the test needs to be sent to a reference lab. Finally, paraffin embedding allows the use of archived samples.

C. Appropriate block

The pathologist must decide what tumour block is best suited for molecular testing. Whatever the material used, the fundamental question is: is this material sufficiently representative of the tumour to be used to make treatment decisions?
- resection specimens or endoscopic biopsies?[17,20]

When looking at the RAS mutation status in the biopsy and in the corresponding resection specimens, literature suggests that the concordance status is very high regardless of the method used. However, endoscopic biopsies represent only a limited, superficial sampling, so the possibility exists that mutant clones are not sampled if they lie within the deeper parts of the carcinoma. Also, data regarding intra tumour heterogeneity are likely to evolve especially as more sensitive assays are developed. Practically speaking, analysis of blocks from resected specimens is preferable to that of endoscopic biopsies or fine needle aspiration biopsies.
A particular problem is rectal cancer. Neoadjuvant chemoradiation therapy leads to complete tumour regression in approximately 10-20% of patients and to an almost complete tumour regression in a further 20-30% of cases. Although chemotherapy or radiotherapy does not significantly alter the genetic status of cancer cells, RAS genotyping on post-treatment samples can be challenging because of the paucity of neoplastic cells. Therefore, the quantity and quality of the pre-treatment biopsies are of utmost importance, and gastroenterologists should be aware of this. Patients operated after radio-chemotherapy and without tumour, pre-treatment biopsies can represent the most cost-effective option for reliable RAS genotyping. When small tissue samples are available, the molecular pathology laboratory has to report it because size and amount of tissue affect the specificity and sensitivity of the test.

As has been stated in earlier guidelines, in case the rectal surgical specimens after radio-chemotherapy contain very few tumour cells, it should be precised in the final report. Larger tumour sampling before therapy by rigid rectoscopy should be considered.[17]

In this regard, endoscopic biopsies sometimes show only adenoma, while there is clear-cut clinical and radiologic evidence for a (metastatic) colorectal cancer. Generally speaking, RAS mutations occur early on in the adenoma-carcinoma pathway and are key driver mutations.

So, a RAS mutation demonstrated in an adenoma is likely to be harboured by the CRC arising from the adenoma. However, there are exceptions and RAS mutations can occur as a late event in colorectal cancer. So, repeat biopsy of either the primary tumour or a metastasis should be requested in case of a negative test result on a sample containing only adenomatous tissue.

- primary or metastatic site?

Currently, biopsy of the metastatic site is not necessary because the test can be reliably performed on the archival tissue blocks containing primary tumour. It is well established that RAS mutations are highly stable during the course of the disease. However, a slight difference in concordance has been reported depending on the site of metastasis. This is a matter of debate and continuous research. Concordance between primary CRC and liver metastasis regarding KRAS status seems excellent.[21]

Practically speaking, there is no need for biopsy of metastatic site, except perhaps in the case of lung metastasis, where a greater discrepancy is seen.[22] When metastatic tissue is available, the choice between primary tumour and metastatic tumour tissue should be dictated by the amount of tumour cells, the ratio of tumour cell content, the absence of significant mucin and/or necrosis and fixation conditions.

D. Slide preparation

Preparing the slides for macrodissection is important. The technician has to orientate the samples so that the same areas are at the same place on the slides. It is also important that a contemporaneous HE recut is obtained when sections are made for DNA extraction, because the appearance of tissue sometimes changes very dramatically in recut slides.

E. Tumour cell quantity - LOD

The tumour content (based on a haematoxylin and eosin staining) should be estimated by a pathologist. Unfortunately, even among experienced pathologists, high variability has been observed in the estimation of tumour cells. It must be stressed that the ratio between tumour cells and all other cells (fibroblasts, normal epithelial cells, endothelial cells, and particularly lymphocytes and other inflammatory cells) is of utmost importance, and not the ratio between the tumour surface area and the non-tumoral surface area. Also, areas of necrosis and acellular mucin should not be included in calculations of neoplastic cell content and blocks containing a lot of mucin and/or necrosis should be avoided when possible. The percentage of viable tumour cells is very important. Each molecular pathology laboratory has to know the sample requirements to produce reliable results with the test.

All methods show a decreasing correct mutation call rate proportionally with decreasing percentage of tumour cells. Neoplastic cell frequency cut-off levels depend on the molecular technique used.[23] For example, Sanger sequencing is less sensitive in detecting mutations than PCR-based techniques. The meaning of limit of detection (LOD) should be fully understood by the pathologist. LOD in RAS testing is on the DNA level. LOD means: how sensitive is the platform in detecting mutated DNA in a pool of non-mutated DNA? A LOD of 5% means that 10% of the cells present should be cancer cells, because assuming that cancer cells are diploid, only one of the two alleles is (theoretically) mutated. It is acknowledged that aneuploidy is common and can potentially result in increased (if mutant alleles are amplified or wild-type alleles are deleted) or decreased (if the wild-type allele is amplified) sensitivity of detection. Particular attention should be given to macrodissection in order to select areas in which the ratio between neoplastic and non-neoplastic cells is highest and in order to eliminate necrotic and/or mucin-rich areas.

With the increasing sensitivity of RAS testing methods, the detection of mutations present at low relative level in CRC is becoming optimised. But how clinically meaningful is higher sensitivity? A LOD of 5% has already proven to be significant, but at the ASCO 2014 meeting, a LOD of 1% was also significant (oral presentation). Further studies are awaited, but from a practical point of view, a LOD of at least 5% is mandatory. The minimum neoplastic cell content tested should be at least two times the assay's LOD.

F. Methods of detection[23]

Despite the wide acceptance of RAS mutation testing as a diagnostic tool for mCRC, a uniform consensus on optimal test methods is lacking. The most commonly

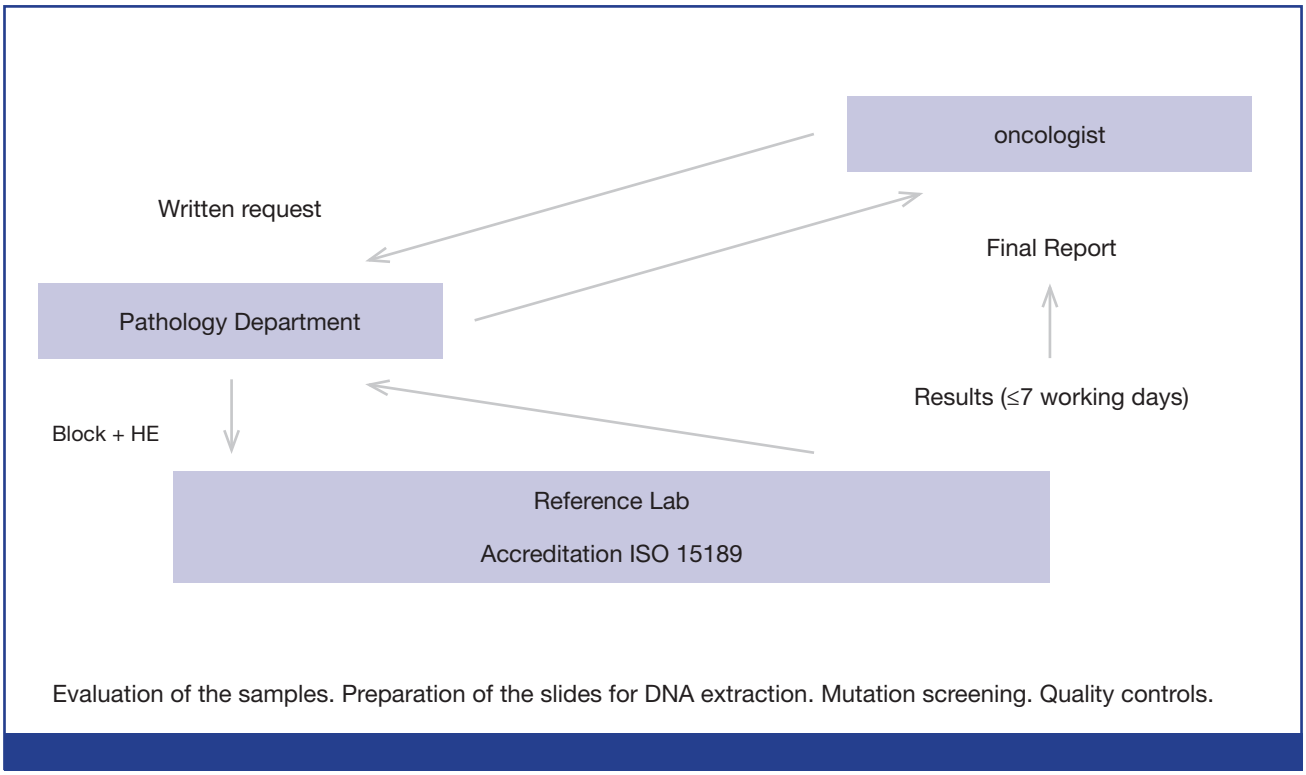

Figure 1. Standardisation of the procedure.

used methods for analysing RAS mutations can be categorised as direct sequencing and PCR-based detection. The current gold standard for detection of RAS mutations remains direct sequencing of PCR amplification products. This technique identifies all possible mutations in amplified DNA sequences. The major pitfall in direct sequencing is that it is not very sensitive. For clinical routine we have to consider the limits of detection, the time to give the result, the cost of the technique and the possibility to detect most of the mutations in the shortest time.

Next generation sequencing (NGS) technologies, by simultaneous sequencing of thousands of short sequences in a massively parallel way, may offer a cost effective approach for detecting multiple mutations with a minimum amount of DNA. Several studies have already validated the clinical use of NGS in terms of sensitivity, speed and cost.[24-26] Moreover, this technology allows the screening of hundreds of potentially clinically actionable alterations in cancer-related genes including all RAS mutations. The challenge is to identify clinically relevant gene alterations that could help drive optimal therapeutic decisions for mCRC patients. This equipment is expensive and needs Belgian rationalisation and optimisation so that the number of tests per machine increases, thereby lowering the total cost.

When doing the test: reflex testing or 'on-demand' testing?

One model of reflex testing requires all surgically excised CRCs to be RAS genotyped and these data integrated into the resection specimen pathology report. However, according to Belgian law, at least for KRAS testing, this test can only be done in the metastatic setting of CRC.

Since pathologists are not always aware of the clinical stage of the patient, it is reasonable that the physician in charge of the patient orders the testing led by the decision of the multidisciplinary oncology team. Even when distant metastases are not present, those patients with lymph node metastases should be considered for testing. Everyone in the multidisciplinary oncology team must be aware of the importance of RAS testing in CRC (together with MSI testing).

Turnaround time

In the scope of 'on-demand' testing, a crucial item is the turnaround time (TAT). Several definitions of TAT can be used: the time to issuing a final report from:

- The clinical request for RAS testing,
- The request of histological tissue from its source laboratory, or
- The receipt of the tissue block at the testing laboratory.

The last definition is most commonly used.
In a recent paper about EGFR testing in Flanders, the median time for the local pathology labs to prepare and ship the tumour samples was, in 37,5% of cases, between five and sixteen days.[27] Local pathologists should be aware of this problem and instruct their secretary accordingly. The sending of the appropriate blocks should be done as soon as possible, within 72 hours. Also, when test results arrive, they must be urgently communicated to the physician in charge of the patient.[27]
Different laboratories may adapt different approaches to RAS multiplex testing with some implementing a sequential approach; for example, KRAS codon 12 and 13 (representing up to 80% of RAS mutations in CRC) are analysed first and if these are wild-type, the remaining codons are studied. An alternative approach is blanket testing of all RAS codons. Even in this case, there are variations on how this is done. Some laboratories choose a screening assay (e.g., single-strand conformation analysis or HRM) and then a sequencing assay for the codon found to bear mutation.
From the patient perspective, it is not acceptable that an increased range of biomarker testing leads to even increasing TAT and potential treatment delays. Testing for some RAS mutations in one lab and sending tissue out to another lab for further RAS testing is inacceptable.

Standardisation of the procedure (*Figure 1*)

To standardise the procedure, a written request should be sent by the oncologist to the pathology lab, which chooses the best block as already described. The pathologist sends the block to the reference ISO 15189 lab, which evaluates the block, prepares the slides for DNA extraction and mutation analysis.
So, RAS testing (using the above recommended panel) should be completed and reported in >90% of specimens with a TAT of ≤7 working days from the receipt of the specimen in the testing laboratory (in accordance with the recently published UK guidelines).[16]

Reporting and interpretation

Guidelines for reporting results of molecular tests are based on ISO 15189 (2012) requirements for medical laboratories. Some of the key items that should be included in a test report are sample collection details, percentage of tumour cells, genotype, correct nomenclature, list of the RAS mutations tested, the testing methodology used (including version number of the kit, if used), and the LOD of the assay (*Table 1*). Clinicians unfamiliar with the various mutations and the clinical data related to them, may find the reporting details on mutations somewhat confusing if provided only with a list without context. A clear note should be made of the clinically actionable items: simply, whether the tumour sample has a wild-type or mutated RAS that warrants specific treatment choices.

Table 1. Reporting of RAS testing results.

Sample collection details (fixative, primary or metastatic samples, pre-radio-chemotherapy, etc.)
Percentage of tumour cells
Specific reason if possibility of false negative (mucus, necrosis, low percentage of tumour cells, etc.)
List of the RAS mutations tested and results
Specific mutation sites
Testing method with sensibility (LOD) and specificity
Correct nomenclature describing the mutation
Clinical implication

Assay validation

The assay's precision and accuracy need to be analysed and recorded. Precision refers to how reproducible the assay can detect the same mutation, whereas accuracy refers to whether or not the assay can detect reference genotypes, whether mutant or wild-type. Precision can therefore be assessed through repeat analysis of the same DNA sample within the same run, between runs and between operators at different times and in different conditions. Accuracy encompasses key aspects of a qualitative test (including its sensitivity and specificity) and is best assessed using clinical samples which have been genotyped either with a different, previously validated assay in the same laboratory or by the same assay in a different laboratory. The number of clinical samples required for validation depends on the statistical power required in each laboratory and for each test. A recent publication from the College of American Pathologists has suggested validation with at least 40 specimens, though it is noted that statistically speaking, a perfect correlation with 40 specimens predicts for a test sensitivity of 92,5%.[28,29]
When a CE-marked IVD-compliant test is being used, a process of verification, rather than formal validation, is required to ensure that the test manufacturer's specifications are met in the laboratory which is starting to use the test.
A particular problem is validating the test on low percentages of tumour cells. A recent study showed that the

Key messages for clinical practice

1. Either primary or metastatic CRC can be used in most cases.
2. Either biopsy or resection specimen tissue can be used, though if both are equally available, use of resection tissue is preferable.
3. RAS analysis should include KRAS codons 12, 13, 61, 117 and 146, and NRAS codons 12, 13, 61 and 146.
4. Turnaround time for RAS testing should be ≤7 working days from receipt of the specimen in the reference laboratory to issuing of the final report, for >90% of specimens.
5. Laboratories should audit their results to ensure that the proportion of mutant cases for each gene and codon is in line with published data.
6. Laboratories providing RAS testing for CRC should demonstrate successful participation in a relevant EQA scheme and be accredited (BELAC ISO 15189).

percentage of correct mutation analysis rates decreases proportionally with lower percentage of tumour cells, especially when the percentage of tumour cells is lower than 10%.[28,29] One of the likely explanations is that DNA extraction methods are a crucial factor in the process.

Quality control and assurance[30]

A record should be kept of the test results. These results should be compared with results from scientific literature in order to see if the results obtained match with the literature.

Also, the proportion of test failures should be documented and in each case, a likely reason for such failure should be mentioned. For every batch of samples analysed, a minimum of one positive control and one negative control (including a non-template control) per analysed target is recommended.

For assays aiming to report low-level mutations, it is recommended that the LOD is analysed and recorded regularly by including known DNA samples with the required low level of mutant allele burden. Any lab offering a RAS testing service should be BELAC accredited (implementation of the ISO 15189 standard). By definition, this lab must be involved in a RAS external quality assurance scheme, for example, those run by the European Society of Pathology or the UK NEQAS Molecular Pathology. It is also encouraged that laboratories participate in a sample exchange program with other laboratories to allow for cross-analysis of, in particular, samples yielding failed or equivocal results.

Conclusion

RAS genotyping of mCRC to guide anti-EGFR therapy is still evolving and rapidly being updated. Currently, it is necessary to check for KRAS and NRAS mutations. A summary of the main recommendations is given in the 'key messages'.

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