Harmonization of Molecular Oncology testing in Belgium: introduction of KRAS testing for colorectal cancer

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Summary

Recent re-analysis of phase II and III trials with the Epidermal Growth Factor (EGF) targeting monoclonal antibodies cetuximab and panitumumab have shown that patients with metastatic colorectal cancer who have KRAS mutations detected in codon 12 or 13 do not benefit from these therapies.

In Europe, a recent EMEA directive has limited the use of these drugs to patients with KRAS wild type colorectal cancer and a similar directive is to be expected shortly in the USA. KRAS testing thus needs to be implemented in all countries at a relatively short notice. In Europe, different testing methodologies are currently accepted and laboratories have chosen to either develop their own test or utilize CE marked kits. In both cases, standard operating procedures need to be developed to handle the workflow of obtaining an archival colorectal tumor specimen, verifying if it is suitable for molecular diagnostics, and performing the test itself. Validation of the molecular test and proficiency testing of the laboratories is paramount. This document contains some guidelines formulated by the working group, to help realize these important steps in Belgium.

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Introduction

In the last 5 years, cancer therapy has undergone a major revolution characterized by the introduction of targeted drugs that inhibit specific proteins. Among those, the monoclonal antibodies (mAbs) (cetuximab and panitumumab) targeting the Epidermal Growth Factor Receptor (EGFR) have shown remarkable efficacy in the treatment of metastatic colorectal cancer (mCRC). Similar to other targeted therapies, anti-EGFR drugs are only active in a fraction of patients. Accordingly, a major challenge is to identify the genetic alterations associated with the clinical response to anti-EGFR mAbs. It has been shown that most patients with CRC carrying mutated KRAS or BRAF are not likely to experience significant benefit of either cetuximab or panitumumab treatment.[1] As more drugs that target specific components of signal-transduction pathways become available and as we increase our knowledge on the complexity of these signaling networks, the burden of selecting the correct drug combinations for each individual cancer patient will ultimately shift to the pathologist, who must identify the underlying defect in each tumor. This will require new diagnostic technologies and will be a major challenge over the next decade.

Harmonization of testing and Quality Assurance are key elements to improve testing specificity. As was concluded in the recent ASCO/CAP HER2 testing guidelines: "The panel strongly recommends validation of laboratory assay or modifications, use of standardized operating procedures, and com-

pliance with new testing criteria to be monitored with the use of stringent laboratory accreditation standards, proficiency testing, and competency assessment".[2]

Working Group on KRAS testing in Colorectal Cancer

The Working Group on KRAS testing in Colorectal Cancer was initiated in February 2008 by the Belgian National Working Group for Molecular Pathology. It consists of pathologists, oncologists and molecular biologists with special expertise in the clinical and molecular pathology aspects of colorectal cancer biology and KRAS testing and represents all Belgian Centers currently performing KRAS testing. Through expert discussion, based on own experience and published literature, it aims to provide recommendations for standardized application and interpretation of KRAS mutation detection testing in clinical practice. It is also a key player in the recent European initiative, taken by the European Society of Pathology (ESP), on harmonization of KRAS testing throughout Europe. The KRAS Working Group will coordinate proficiency testing for KRAS mutation detection at a national level and in collaboration with a European network to be organized by the ESP.

Many different tests are available to detect KRAS mutations, and many labs will undoubtedly want to use their own preferred test. This is not a problem, as long as the sensitivity and specificity of each test is defined and reaches standard norms defined by the KRAS Working Group. It is suggested that all laboratories performing KRAS testing seek accreditation for this test under a formal quality system like ISO15189 and that they perform continuous quality control and proficiency testing to ensure optimal test performance.

Some of the challenges in the introduction of a new pharmaco-diagnostic molecular test can be derived from the recent experience with HER2 Neu testing for response prediction to Herceptin in breast cancer. In April 2002, the drug Herceptin, a monoclonal antibody directed against the epidermal growth factor receptor type 2, used for the treatment of breast carcinoma and shown to have efficacy only for patients with HER2 overexpression in tumoral cells was approved for the Belgian market. As a condition for reimbursement national authorities require that HER2 gene amplification status is determined by a molecular fluorescence in situ hybridisation test (FISH) prior to treatment. Similar requirements apply in other countries, where FISH testing is often combined with immunohistochemical (IHC) testing for HER2 to establish overexpression. Only recently, more than five years after the introduction of HER2 testing, evidence based guidelines on the ideal testing algorithm and test requirements have become available.[2] One of the primary aims of the KRAS Working Group is to avoid such a delay and provide timely and evidence based guidelines for KRAS testing. In view of the high cost and potential side-effects of the new anticancer treatments, it is important that false positive tests (in the case of HER2) or false negative tests (in the case of KRAS) are avoided. In this respect, harmonization of testing and quality assurance are key elements to improve testing specificity. The role of National and European KRAS QA programs would be (a) to stimulate collaboration and facilitate administrative and logistic support for QA testing, (b) to provide validated SOP's, proficiency testing and competency assessments , (c) encouraging and assisting participating institutions at the National and European level in attaining and maintaining ISO15189 accreditation.

The introduction of HER2 testing also highlighted the lack of coordination between medical and technical national competent authorities. In some countries, the introduction of Herceptin was not accompanied by adequate provision for diagnostic testing. In Belgium, formal inclusion of HER2 testing in the reimbursed nomenclature was only achieved in August 2007, five years after the introduction of the drug on the market. National and European KRAS QA programs should therefore also aim to provide local regulatory and health care authorities with the necessary technical information to facilitate reimbursement and help to implement regulatory oversight.

Recommendations of the KRAS Working Group

The KRAS testing process comprises three parts: (1) the selection of the block and tumor area from which DNA will be extracted, (2) the DNA extraction process, and (3) the KRAS mutation analysis. Different strategies are possible for all three parts; the KRAS Working Group will provide general recommendations in this text, and in addition will provide detailed SOPs as help for laboratories to harmonize their procedures.

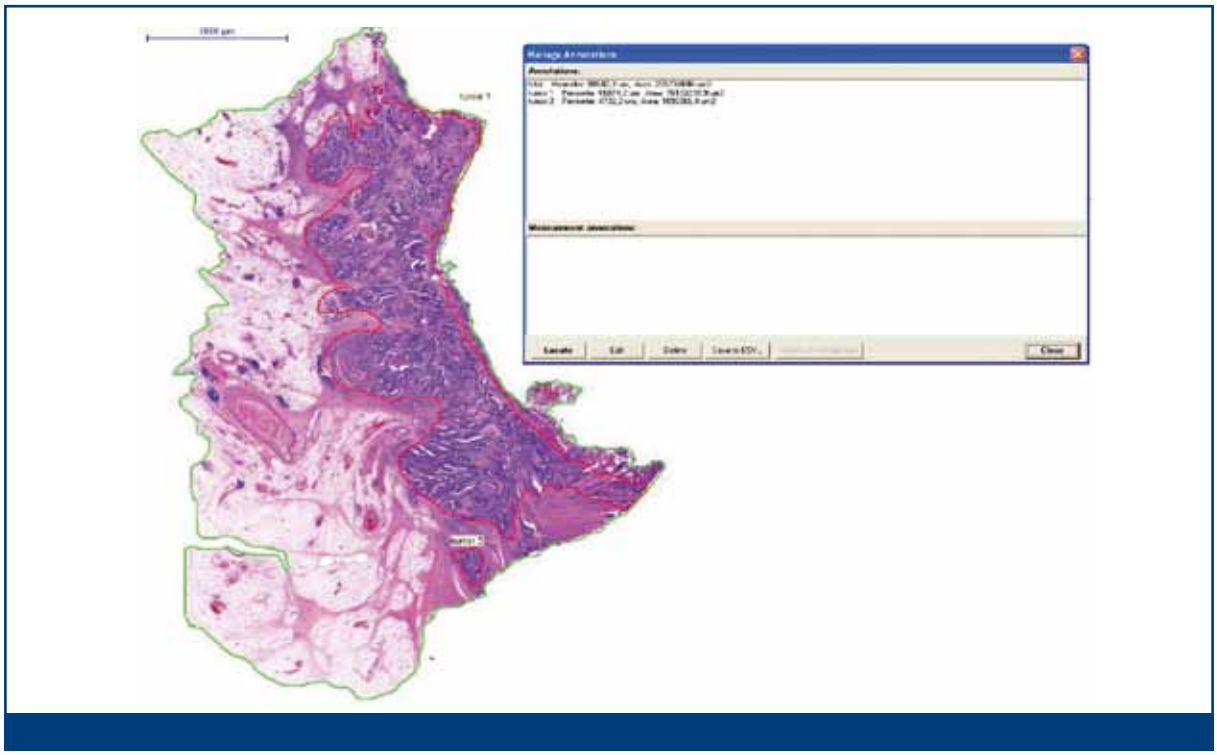

Figure 1. The percentage tumor area versus non tumor tissue is calculated. The example shows an example of a tumor area calculation done at a reference laboratory (HistoGeneX).

General procedure "on demand testing"

The clinician will contact the pathologist having stored the archival primary tumor or metastatic tumor. Either the pathologist's laboratory is accredited for KRAS testing and will perform the test itself, or the pathologist will send the block to a referral center accredited for KRAS testing. In both situations the local pathologist will be a key player in the process of testing, will receive the test result and will integrate it into the original pathology report or in a complementary pathology report. The KRAS Working Group suggests that the local pathologist should receive reimbursement for his coordinating role in the process.

1. Clinician → primary pathologist → perform KRAS test → report to clinician.
2. Clinician → primary pathologist →central KRAS testing → report to primary pathologist and clinician.

Material used for KRAS testing

Archival material of the tumor is available

The role of the primary pathologist is to choose the most appropriate block for KRAS testing and evaluate the tumor content.

Criteria: The most appropriate block of a tumor is the block containing a large amount of tumor. For an endoscopic biopsy of the primary tumor, invasive adenocarcinoma needs to be present. Tissue blocks containing only adenomatous tissue, including high-grade intraepithelial neoplasia or intramucosal neoplasia, should not be used for testing. For a biopsy of a metastatic site, eg liver metastasis, or lymph node metastasis, carcinoma cells with morphology and/or immunohistochemical profile compatible with colorectal origin need to be present. The minimum percentage of tumor area versus non tumor area required will depend on the method used for KRAS testing. In any case the percentage tumor versus non tumor tissue needs to be assessed and reported by the primary pathologist or the pathologist of the reference laboratory for that specific block. Evaluation of tumor content will by done by review of the existing H&E slide corresponding to the tissue block (*Figure 1*). If not available, a new H&E slides needs to be made. H&E slides should be reviewed by an experienced pathologist.

Archival material of the tumor is not available

About 20% of the target patients will present with upfront metastatic disease and will not have had the

primary tumor resected. Material will need to be acquired for the KRAS testing to be performed. If endoscopic biopsies of the primary tumor or biopsies of a metastatic site will be obtained for KRAS testing, the role of the primary pathologist will be to ensure that invasive adenocarcinoma is present in the material, and to indicate which blocks can be used for testing.

Choice of material to be used

In case that different types of material are available (primary and resected metastasis) or in case that only non primary sites are available, the question arises what can and should be used? KRAS mutations are an early event in the colorectal carcinogenesis and are expected to be clonally present throughout the primary tumor and derived metastatic lesions. In this case, primary as well as metastatic sites will be representative of the KRAS status of the lesion being treated by EGFR inhibitors.

There is however not yet much experimental data addressing the question of concordance for KRAS status between primary and metastatic or different metastatic sites. The Working Group will follow up on concordance/discordance rates from the literature as they become available and adapt guidelines according to these data.

Processing by primary pathologist in case of central lab testing

A: If sending an uncut FFPE block

Select a formalin-fixed, paraffin-embedded block according to the criteria described above. Evaluate area and percent tumor content on H&E section; select block with sufficient tumor tissue according to requirements of central lab. Include name and address for returning the block and original pathology report as well as coordinates of requesting clinician. A specific request form and procedure will be developped by every testing laboratory, to optimize transfer of information from clinician and primary pathologist. Additional requested data may include pre-analytical variables such as location of the tumor, pretreatment with chemo and/or radiotherapy, type and duration of fixation. Results will be reported to the primary pathologist and the clinician to minimize delays.

B: If sending slides

Select a formalin-fixed, paraffin-embedded block according to criteria described above. Evaluate area and percent tumor content on H&E section; select block with sufficient tumor tissue according to requirements of central lab. Cut consecutive sections (amount according to central lab requirements) of 4-5µm thickness and number the consecutive slides. When cutting sections care should be taken to avoid interpatient contamination (clean knife between patients, avoid floaters,..). Provide the central lab with: clearly labeled slides with a specimen ID and the corresponding H&E slide.

The option of sending slides instead of the tissue block for testing will only work if there are specific agreements with the testing lab on the quantity and quality of the slides to be sent. This in turn will depend on the methodology used for KRAS mutation detection in that specific lab. In general, it is therefore preferable to send tissue blocks for testing as outlined in section A.

General procedure "upfront testing"

Several reasons argue for "upfront" KRAS testing on all colorectal cancer samples at diagnosis.: (1) Increased efficiency of the process: identification of invasive tumor is part of the routine pathology assessement; (2) the additional slides necessary for KRAS testing can be made at the first routine examination; this will use less material than going back to an archival block and avoids reorientation and re-cutting of the block, with inevitable loss of tissue; (3) there is less risk of untraceable specimens and thus the need for additional biopsies of the patient; (4) there is less risk of loss of blocks during shipment; (5) the turn around time will be lower: KRAS test results will be immediately available to the clinician upon his decision to treat a metastatic patient. Retrospective analysis will involve a variable lag time before test results on archival material will be available. This causes stress for the patient and the clinician and is inefficient for therapy planning. At this point, the KRAS Working Group recommendations are tailored to "on-demand" testing. Depending on a reassessment of this process after implementation, and depending on the processes followed in other European countries, the need and modalities for upfront KRAS testing for all newly diagnosed colorectal tumors of stage III or higher can be reconsidered.

Performance indices required for KRAS testing

General comments

KRAS testing should preferentially be performed in laboratories accredited (ISO 15189) for the mu-

Figure 2. Macrodissection of a colorectal cancer. The dissection is guided by a print out of a serial HE stained slide of the tumor. (HistoGeneX)

tation analysis as well as for DNA extraction from FFPE material. All laboratories should participate in national and international Quality Control rounds. Reimbursement of the test could be linked to these requirements. Testing laboratories should validate the test according to internationally accepted criteria. Validation criteria should include the following elements:

Sensitivity and specificity of the chosen test

Sensitivity: Lower detection limit of mutant signal. The working group recommends to use a test that has a sensitivity threshold of at least 20% (ie that can detect 20% tumor cells against a background of 80% normal tissue). For tests that have a sensitivity threshold between 1 and 10%, no macrodissection for tumor enrichment prior to DNA extraction is required. For tests that have a sensitivity threshold of more than 10%, macrodissection should be performed (*Figure* 2).

Specificity: Tests should be able to detect the seven most common somatic mutations located in codons 12 and 13 (Gly12Asp, Gly12Ala, Gly12Val, Gly12Ser, Gly12Arg, Gly12Cys, and Gly13Asp). The test result should specify which mutations have been tested and which mutations might be present but were not tested for: ie codon 61 mutations or other codon 13 mutations. Depending on the test specifications, false negative results can be expected for specific infrequent mutations at codon 12 and 13, codon 61 and 146.

Method validation; Analytical validation

More detailed guidelines on how to validate a test are being developed by the ESP.[3] The European KRAS mutation detection program of ESP will also provide laboratories with the opportunity to compare their methodology to tests used in ESP reference labs.

In general, validation of a new test should address the following questions:

- Determine cut-off values for discerning mutant from wt.
- Determine sensitivity of the test (amount of tumor DNA or mm2 tumor area).
- Accuracy
- Reproducibility : ie different machines etc
- Robustness:
  - influence of varying DNA concentrations
  - Manual vs robotic

Analysis success rate

Determine the fraction of samples with successful DNA extraction and/or determine the fraction of samples with a valid test result. The working group recommends that both these indices are above 95% for samples that fulfill the acceptance criteria. Laboratories may opt to test only those samples for which their test system is suitable. If test systems are used that have a relatively low sensitivity but a high specificity, they may opt to limit their analysis to samples that have sufficient DNA content (cq tumor area) or have a sufficiently high fraction of tumoral cells. Laboratories should have clear acceptance criteria that outline the prerequisites for the test with regard to these parameters and maximize their control over the pre-analytical phase. In case the material does not meet the acceptance criteria, the laboratory will take all necessary action to ensure that a timely result is obtained by a laboratory with a more suitable test system (i.e. high sensitivity but possibly lower specificity).

Turn around time

Lab turn around time of the laboratory performing the KRAS test, is defined as the time between receipt of a FFPE block and sending out the report to the pathologist/clinician. The total turn around time will be longer as this will also include additional elements as the time to trace the block, time to send the block to the laboratory for testing, time before the requesting clinician receives the result. The working group recommends that the Lab turn around time should not exceed 10 working days. Strategies to minimize the time before result for the clinician will need further discussion.

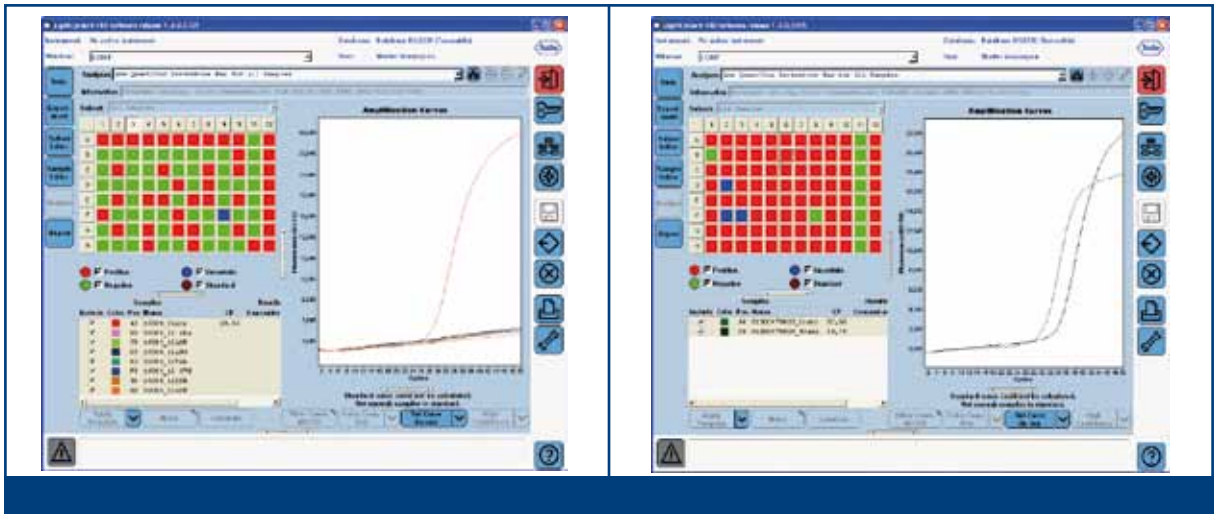

Figure 3. An example of ARMS/ Scorpion analysis (DxS kit) done on a Lightcycler LC480. The method was validated to test with a limit of detection of 1%. (HistoGeneX)

Reporting of the mutation analysis result

A standard way of reporting the KRAS status of a tumor block will be developed. This report will specify which mutations have been tested and which mutations might be present but were not tested for or could have been missed by the test, based on it's specifications. A disclaimer should be added.
The accredited laboratory should report to the primary pathologist, who will include the result in a standardized fashion into the original or complementary pathology report and to the requesting clinician.

Quality assurance project

A quality assurance project is under development for Belgium and at a European level, and will be detailed in future publications.

Conclusion

The detection of KRAS mutations in colorectal cancer has become a predictive test, able to identify patients which may be resistant to EGFR inhibitor therapy. This allows the differentiation between patients likely to benefit from and those unlikely to respond to expensive new EGFR inhibitor drugs. Because of the impact of the test result on patient care, it is essential that a KRAS mutation assay is done with a validated method based on ISO 15189 criteria. The analytical validation of the KRAS assay includes defining the performance characteristics (i.e. sensitivity, specificity, precision and robustness). In addition, also the pre-analytical conditions are critical for this molecular assay since the tumor content present in the starting material can influence the test result. The anatomic pathologist is the key player in the pre-analytical phase of this assay since the selection of the tumor tissue is essential for the performance of the test. Therefore a close communication between the anatomic pathologist and the KRAS reference laboratories should prevail. The Belgian working group of molecular pathology has written guidelines for the submission of samples and the performance of the assay.

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Key messages for clinical practice

1. The oncologist, who needs to know the KRAS status of the patient's tumor, needs to send a request to the anatomic pathologist of his hospital.
2. The anatomic pathologist selects a tumor block containing sufficient invasive colorectal cancer tissue.
3. The block is submitted to a laboratory accredited for KRAS testing.
4. The KRAS testing laboratory reports to the anatomic pathologist, who integrates the mutation status in his histopathological report.

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