Accurate KRAS mutation testing for EGFR-targeted therapy in colorectal cancer : emphasis on the key role and responsibility of pathologists

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Abstract

Patients with metastatic colorectal cancer and KRAS mutation are unlikely to benefit from treatment with anti-EGFR antibodies, and testing for KRAS mutation in this setting is recommended. Pathologists have a crucial role in accurate testing for KRAS mutations, whether or not testing is performed in their own laboratory, as mutation analysis is performed on paraffin embedded tissue selected by the pathologists. The type of fixative used is a very important issue, as some fixatives do not allow molecular testing. Pathologists must select the most appropriate tumoral tissue block for KRAS mutation analysis and hence, must know the sensitivity of the KRAS mutation detection methodology utilized in their reference laboratory. It is essential that they select a tissue block that contains enough percentage of viable tumour cells, as false negative results will occur when the sample is contaminated with high levels of nontumour elements. Pathologists not only have to recognize the area of invasive carcinoma and distinguish it from non-invasive neoplastic components, but also have to estimate the percentage of necrotic debris and nontumoural elements. For tests that require a high percentage of tumour cells, macrodissection before extraction of nucleic acids is often indicated. The primary pathologists in addition are responsible for preparation of the pathology report for the tissue block on which the KRAS mutation analysis was performed and should transmit the results to the requesting clinician. Pathologists should participate in a multidisciplinary oncologic consult to achieve correct interpretation of the results e.g. in case of potential false negative results. (Acta gastroenterol. belg., **2010**, 73, **497-503**).

Key words : KRAS mutation, EGFR, colorectal cancer, cetuximab, panitumumab.

Introduction

The epidermal growth factor receptor (EGFR) constitutes the link between the extracellular space and intracellular signal transduction pathways which regulate nuclear processes involved in cell growth, cell differentiation, inhibition of apoptosis and vascular proliferation. EGFR (ErbB1) is a member of the erbB family of receptor tyrosine kinases, which also includes ErbB2 (HER2/neu), ErbB3 (HER3), and ErbB4 (HER4). EGFR is a transmembrane protein composed of three components : an extracellular ligand binding domain, a lipophilic transmembrane domain, and an intracellular tyrosine kinase domain. The main ligands responsible for activation of EGFR are epidermal growth factor (EGF) and transforming growth factor-α (TGF-α). Extracellular ligand binding induces homo- or heterodimerization between the different ErbB receptors and intracellular autophosphorylation at the tyrosine kinase domain which, in turn, activates downstream signalling pathways. Several pathways of signal transduction have been identified, including activation of the RAS/RAF/MAPK pathway and the PI3K/AKT pathway (1-4).

The EGFR pathway plays an important role in tumourigenesis and tumour progression of colorectal cancer and hence, EGFR has evolved as a relevant target in the treatment of colorectal cancer (5). Two monoclonal antibodies targeted against the extracellular domain of the EGFR that are effective inhibitors of EGFR, cetuximab and panitumumab, were introduced in the treatment of metastatic colorectal carcinoma (6,7). Cetuximab is a chimeric mouse/human IgG1 anti-EGFR antibody, while panitumumab is a fully human IgG2 anti-EGFR antibody (6-8).

KRAS is an oncogene that has been long known to be involved in the development and progression of colorectal cancer and is mutated in about 40% of colorectal cancers (9). In the course of the adenoma-carcinoma progression sequence a relatively early occurrence of KRAS mutations is observed (10-12). Retrospective analyses of several clinical trials have consistently demonstrated that patients with metastatic colorectal cancer and mutant KRAS are unlikely to benefit from treatment with the anti-EGFR antibodies cetuximab and panitumumab (13-15). It has been shown that these therapies have no effect in tumours with mutations targeting codon 12 and 13 (of exon 2) of the KRAS gene (9,13). These KRAS mutations are the most commonly found KRAS mutations in colorectal cancer. It are activating missense mutations that prevent dephosphorylation and inactivation of the protein, causing it to be permanently switched on, even in the absence of EGFR-mediated signalling/stimulation (16). Since KRAS acts downstream of EGFR in the signal transduction pathways, activating mutations of KRAS renders the tumour cells independent from EGFR

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Submission date : 12/10/2010
Acceptance date : 26/10/2010

activity. This explains why activated mutant KRAS protein cannot be modulated (affected) by inhibition of EGFR. If a codon 12 or 13 KRAS mutation is detected in patients with metastatic colorectal carcinoma, they should not receive anti-EGFR antibody therapy as part of their treatment. As a consequence, testing of the tumour for KRAS mutation in all patients with metastatic colorectal carcinoma who are candidates for anti-EGFR antibody therapy is recommended (13). Pathologists have a crucial and responsible role in coordinating the testing for KRAS mutations, whether or not testing is performed in their own laboratory, as mutation analysis is performed on paraffin embedded tissue selected by the pathologist (13,15,17-19).

Selecting the most appropriate tumoural tissue block for KRAS mutation analysis

Fixation and processing of tissues

KRAS mutation status is a predictive biomarker recently reported for colorectal cancer and consequently, it has been introduced clinically over a very short period of time (13). The starting material for mutation analysis, however, is paraffin embedded tissue, often archival material that sometimes dates back to several years ago. Paraffin embedded tissue is preferred over frozen tissue or fresh material, as it is important to know the percentage of tumour cells in the analysed tissue sample, which is most easy to assess on a haematoxylin-eosin (HE) stained section from the paraffin embedded tumour tissue block that is used for DNA extraction. Moreover, paraffin embedded tissue is more easily to transport than frozen tissue in case the test is not performed in-house. In case freshly extracted tissue is used, either stored in RNA preservation solution or rapidly frozen and stored frozen, the percentage of tumour cells must be assessed on frozen tissue sections (13).

To avoid degradation, it is evident that fixation should start as soon as possible after surgical removal of tissues. The type of fixative used is a very important issue, as some fixatives do not allow molecular testing, e.g. fixatives that have a low pH such as picric acid containing Bouin's fixative and acid decalcification solutions or fixatives that contain heavy metals such as B5 fixative with mercury (20,21). Most standard formalin-based fixatives can be used. The fixation time, however, is also a critical issue. It has been shown that tissues fixed for more than 24 h have a much lower yield and poorer quality of DNA (21). With archival tissue we cannot modulate these factors any more. Today, it is however the responsibility of the pathologist to pay careful attention to the type of fixative and fixation time in order to anticipate the requests for possible ulterior molecular analyses.

Sample size and tumour cell percentage

In case of biopsy specimens, there is usually only one tissue block available. With resection specimens, however, there are almost always several tissue blocks containing tumour prelevated, as this is required for accurate grading and staging of the tumour. Pathologists should carefully select the tissue block that should be used for testing. They must be aware of which KRAS mutation detection methodology is utilized in their reference laboratory and they have to know the sensitivity of the method used, as it is essential that they select a tissue block for KRAS mutation analysis that contains enough tumour cells, as false negative results will occur when the sample is contaminated with high levels of nontumour elements. For instance, some KRAS mutation tests require tissue samples containing at least 70 % adenocarcinoma cells for DNA extraction (9). In general, a paraffin block containing only tissue from adenoma or non-invasive carcinoma should not be used for KRAS mutation analysis (22-25). It is clear that selecting the appropriate tissue block requires a trained pathologist. He not only has to recognize the area of invasive carcinoma and distinguish it from non-invasive neoplastic components, but he also has to estimate the percentage of necrotic debris and nontumoural compounds such as supporting stroma and infiltrating inflammatory cells. For tests that require a high percentage of tumour cells, macrodissection before extraction of nucleic acids is often indicated (14). Macrodissection requires familiarity with the morphology of the tissues being macrodissected, hence pathologists should be involved in this process. Standard manual macrodissection can be performed (Fig. 1). It is easy, inexpensive to perform and tumour enrichment can be achieved to very high levels (20). It will reduce false negatives. It is not indicated to measure the tumour surface over total tissue surface with image analysis, as tissue surface is not necessarily correlated to the amount of DNA present in that region. Fat cells, for example, are large cells, but they do not contain more DNA than a small fibroblast with very little cytoplasm and a high nucleo-cytoplasmic ratio. In mucinous adenocarcinomas (per definition more than 50% of the lesion composed of pools of extracellular mucin containing malignant epithelium as acinar structures, strips of cells or single cells) a high tumour purity will be obtained if only these areas consisting of pools of extracellular mucin with dispersed tumour cells are selected for DNA extraction, as the extracellular mucin does not contain DNA. Here the problem will however be the low yield of extracted DNA (Fig. 2).

It is also important that a contemporaneous HE recut is made when new sections are prepared for DNA extraction, as the appearance of tissue sometimes changes very dramatically in recut slides because they provide a view of tissue deeper in the paraffin block (26). HE recuts should be evaluated by a pathologist, and preferentially two HE recuts should be obtained for each analyzed sample, one serial section before and one immediately after the sections used for DNA extraction.

Each molecular laboratory performing the mutation analysis has to inform the pathologist about the minimal

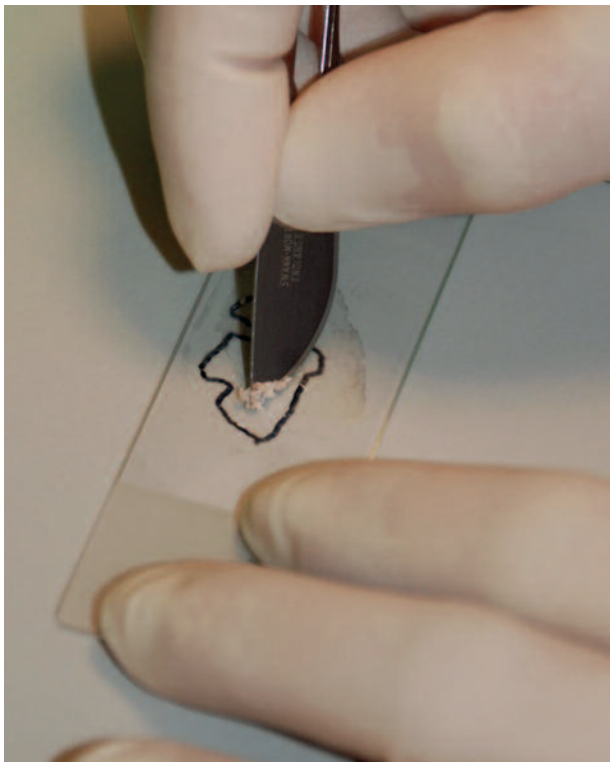

Fig. 1. — For tests that require a high percentage of tumour cells, standard manual macrodissection before extraction of nucleic acids can be performed.

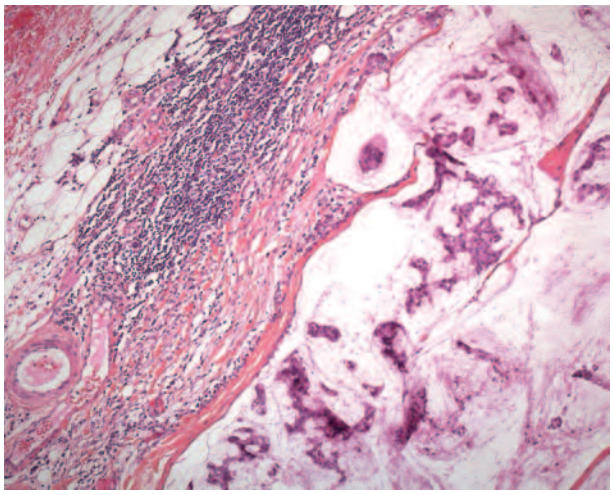

Fig. 2. — In mucinous adenocarcinomas a high tumour purity will be obtained if only these areas consisting of pools of extracellular mucin with dispersed tumour cells are selected for DNA extraction, as the extracellular mucin does not contain DNA. Here the problem will, however, be the low yield of extracted DNA.

sample size (equivalent amount of cells) that is required to produce reliable results with the test that he is using. The pathologist should provide a tissue block with an as high amount of invasive tumour as possible. Surgical resection usually yields much more tissue material for testing than more limited diagnostic procedures such as snare or fine needle aspiration biopsies. For this reason testing of resection specimens is favoured whenever these are available (27).

Tumour cell quantity may be insufficient and be a source of false "KRAS wild type" results in particular after neoadjuvant therapy in rectal cancer. Sometimes only very sparse tumour cells are left embedded in dense fibrotic tissue. In this case it is best to do the test on pretherapy biopsy material. It needs to be emphasized that it is important to make gastroenterologist aware that they should try to obtain sufficient material in diagnostic biopsies. This holds in particular for rectal carcinoma, where often neoadjuvant radiotherapy/chemoradiotherapy is administered prior to surgery. Moreover, rectum resection specimens require a special macroscopic handling and pathological workup with fixation of the tissue for 48 h prior to cutting in order to allow adequate assessment of the circumferential resection margin (28). If possible it is advisable to take a tumour sample from the luminal surface and apply a shorter fixation time for this tumour sample. Here also, pre-treatment samples often prove invaluable (19). In case the diagnostic biopsy contains very few tumour cells, taking a larger tumour sample during preradiotherapy rigid rectoscopy may be considered.

Careful microdissection under direct visual guidance with a microdissection microscope has been reported, however, not always successful (14). Eventually, after discussing with the oncologist about the high possibility of false negative results because of insufficient tumoral tissue for reliable testing, it can be considered to take a new biopsy from a metastatic site. If it is not possible to obtain a larger tissue sample, the test can be performed in case of borderline amount of tissue and/or tumour cell percentage, but the molecular pathology laboratory has to report that because of the small size and small amount of tissue the specificity and sensitivity of the test may be affected (19).

In cases when only very small amounts of DNA are available as starting material, caution must be taken for the occurrence of false positive results eventually generated by mutations that were artificially introduced by the formalin fixation step (14,27), or mutations introduced by the DNA polymerase during the initial amplification steps.

Primary tumour versus metastasis

It is often stated that KRAS mutation analysis preferentially should be performed on primary tumour tissue (25). However, 20% of colorectal carcinomas present with metastatic disease at initial diagnosis and an additional 30-40% will develop metastasis during the disease course and testing the primary tumour is sometimes simply not possible. Sometimes only a biopsy is taken from the metastasis and not from the primary tumour or the primary tumour is e.g. fixed in Bouin's

solution, which does not allow molecular testing, or very little tissue with a low tumour cell percentage is available from the primary tumour, while a large tissue sample with a high tumour purity is available from a metastatic site. Moreover, some authors, in contrast, have recommended testing the metastasis rather than the primary tumour, primarily because metastatic disease is the target of anti-EGFR therapy (29). In addition, KRAS mutation is known to be an early event in colorectal carcinoma carcinogenesis and is unlikely to change during disease progression (22). Concordance rates of KRAS mutations between the primary tumour and metastasis are higher than 90% in several studies. Some series observed complete concordance between the primary tumour and lymph node metastases (24,29). One series reported a concordance rate of only 68%, but this study also reported 36% cases with concomitant BRAF and KRAS mutations, that are considered mutually exclusive (30,31). As anti-EGFR efficacy data are often based on KRAS mutation testing from the primary tumour, current practice is not to re-biopsy a tumour recurrence for KRAS testing if there is sufficient material available from the previous biopsy or resection, although not confirmed by prospective studies.

Before versus after chemoradiotherapy

It has not yet been investigated in large studies whether chemotherapy or radiation alters KRAS mutation status. Preliminary data, however, indicate that previous chemotherapy does not seem to impact the KRAS mutation status. Moreover, most published studies that showed the effect of KRAS mutation were performed after failing of at least one chemotherapy regimen (19).

Upfront testing or only if therapy is requested

There are no scientific arguments to defend upfront (reflex) testing of colorectal cancer for KRAS mutation analysis, and there are of course logistic and financial aspects linked to it. However, in case testing is not performed in-house, it often takes several weeks before the tissue block gets in the reference laboratory, which implies a long waiting period before therapy can be started in these patients with often advanced and rapidly progressive metastatic disease. This could be avoided by upfront testing. Moreover, costs of KRAS mutation analysis are low compared to costs of therapy. Hence, it is not unlikely that in the near future, KRAS mutational analysis will become the standard of care for all colorectal carcinomas.

Assays for KRAS mutation testing

There are multiple assay types available for determining the KRAS mutation status of a tumour, and each shows advantages and disadvantages (14,19). Several methods were compared and yielded comparable results (18). In all cases, DNA is first extracted by laboratory specific and standardized protocols that incorporate standards to assure adequate and specific extraction. All methods of KRAS mutation detection are based on the polymerase chain reaction (PCR).

The current gold standard for detection of KRAS mutations remains direct sequencing (Sanger sequencing) of PCR amplification products (27). This technique identifies all possible mutations in amplified DNA sequences. The major pitfall in direct sequencing is that it is not very sensitive. Mutant copies must have a relative abundance that is at least 20% in a background of wild type alleles, a sensitivity that may not be optimal for clinical testing (14). Pyrosequencing is an alternative methodology for DNA sequencing that has a somewhat higher sensitivity than Sanger sequencing.

Real-time PCR-based methods are easy to use, allow fast turnaround time, and have in some cases a sensitivity down to 1% of mutant alleles. These methods employ e.g. allele-specific probes or amplification refractory mutation system (ARMS). They have however a higher cost and false-negative results can be obtained if mutations are present in the sample for which the assays were not designed (18).

Other assays for detecting point mutations in tumour samples use a 2 steps strategy where a pre-screening method generates results that are eventually confirmed by sequence analysis. The pre-screening methodologies often use DNA conformation based separation techniques, including single strand conformation polymorphism (SSCP), denaturing gradient gel electrophoresis (DGGE) or high resolution melting curve analysis (HRMA), but also other techniques like restriction fragment length polymorphism (RFLP). These methods are often inexpensive, sensitive and specific, but they cannot provide the identity of the detected mutation with accuracy. Therefore, pre-sceening results need to be confirmed by sequence analysis, if needed after enrichment for the mutated variant, which increases the turnaround time and expenses (14).

The specificity and sensitivity of the tests should be known and high-quality validation procedures are needed to ensure reliability of results (21). Evaluation of the method proficiency at each individual laboratory by subscribing to proficiency-testing programs or performing sample exchanges with other laboratories is important. Proficiency testing for KRAS is available through the College of American Pathologists (CAP) and through an European quality-assurance program developed by the European Society of Pathology. Very important is also that turnaround time requirements of current targeted treatment protocols are met (32).

Reporting the KRAS mutation analysis

It is generally accepted that a molecular pathology report should include at least the results of the molecular

analysis, as well as the interpretation of these results and the methodology that was used (33). The KRAS mutation analysis report must specify which mutations have been tested and if mutations have been found. It is advised to report the exact KRAS mutation that is found and not just the fact that a mutation is present (18). There are not yet data published that demonstrate that different KRAS mutations have a different response to therapy or a different prognosis (34). It is, however, not excluded that further studies and follow-up will show differences in behaviour for different mutations (19).

The CAP proposed to provide the following interpretive comment related to therapy : "Accumulating evidence indicates that the presence of a KRAS mutation in metastatic colorectal cancer predicts resistance to anti-EGFR targeted therapy using cetuximab and panitumumab" (13,14,17,18).

The method that was used should be reported. It is advisable to include the minimum required cellularity (the minimum tumour cell percentage) (18). This will be different for different laboratories as it depends on the analytical sensitivity of the mutation detection technique used in the laboratory. Potential errors should be included e.g. potentially false negative results due to insufficient/inadequate template material because of a too low percentage of tumour cells or because of too little tissue with subsequently a too small recovered DNA amount (33). Also the specificity of the method should be included (18).

The molecular pathology laboratory should report to the primary pathologist, who has initially analysed the biopsy or resection specimen and who is responsible for preparation of the pathology report for the tissue block on which the KRAS mutation analysis was performed. The primary pathologist will include the result into the original or a complementary pathology report and transmit the results to the requesting clinician. In the report must be included in which molecular laboratory the test was performed, which tissue (primary tumour or metastasis) was tested and on which tissue block the analysis was performed. It seems also appropriate to report the percentage of tumour cells in the sample and to specify if macro- or microdissection was applied prior to DNA extraction, in which case the percentage of tumour cells after tumour cell enrichment needs to be reported. Finally, pathologists should participate in a multidisciplinary oncologic consult to achieve correct communication of the results e.g. in case of potential false negative results (25).

Summary

Tailored therapy for each patient should maximise efficacy and minimise toxicity of chemotherapeutic agents and improve the outcome of cancer patients. The discovery of KRAS mutation as a marker of resistance to EGFR-targeted therapy with monoclonal antibody inhibitors is a first step in the tailoring of treatment to the individual colorectal cancer patient (31). KRAS mutation testing is important as the toxicity of anti-EGFR antibody therapy for metastatic colorectal cancer is not negligible and administering an ineffective therapy delays the use of a potentially more effective therapy. Moreover, some studies suggest that patients with KRAS mutations treated with chemotherapy and an EGFR inhibitor had a worse outcome than those treated with chemotherapy alone in an advanced setting (14). In addition, EGFR inhibitor treatment of KRAS false negative patients represent significant and unnecessary health care costs. Not treating KRAS false positive patients would even represent a greater catastrophe, as from the clinical point of view we can risk treating a non-responsive patient, but we cannot risk not treating a potentially responsive one.

Pathologists play an essential role in KRAS mutation testing, whether or not testing is performed in their own laboratory (13,15,17-19). First of all, it is the responsibility today of the pathologist to pay careful attention to the type of fixative and fixation time in order to anticipate the requests for possible ulterior molecular analyses. The next important challenge for the pathologist is the selection of appropriate material for KRAS mutation testing. Paraffin blocks are highly heterogeneous with respect to the quantity and distribution of tumour within the blocks and microscopic verification that sufficient tumour cells are present in the tissue block for analysis is critical for accurate testing. Pathologists must select a tissue block that contains sufficient quantity and quality of tumour material for KRAS testing. The samples should be specifically chosen to include predominantly tumour cells without significant necrosis or inflammation. Pathologists also determine whether the percentage of tumour tissue and absolute amount of material present in the selected tissue block fulfil the minimum requirements for the KRAS test used in the laboratory. In some cases blocks may be directly sectioned to obtain tumour material for DNA extraction. In other cases with a low tumour cell percentage an attempt should be made to enrich to a tumour cell DNA level suitable for the assay being used, e.g. by macrodissection of the histological sections (18). In addition, pathologists have to evaluate and select a molecular diagnostic laboratory for KRAS testing. Pathologists who use reference laboratories for this testing should be able to carefully evaluate the KRAS testing technique used and quality processes employed to ensure confidence in the results. Finally, pathologists should assist oncologists in the appropriate interpretation of the test and guide the use of the results (15,17,18,25).

Reliable results require sensitive and accurate mutation detection methodologies due to the frequent predominance of non-neoplastic cells in colorectal cancer specimens. It is, however, important to note that it is currently not known what level of sensitivity is required to provide useful information to clinicians. Indeed, pathologists must be aware that assays with

exceptionally low detection limits may identify patients with mutant KRAS expression in only a limited proportion of tumour tissue (15). Tests that have a detection sensitivity of 1% might detect subclones in a tumour that have acquired a mutation. In fact, at present, not much is known about intratumoural heterogeneity for KRAS mutation. Baisse B. *et al*. reported intratumour heterogeneity for KRAS mutation in 20% of the primary tumour colorectal carcinomas studied, either a clone with and a clone without mutation, or 2 different mutations were observed (35). This needs to be further investigated as this finding might have consequences for treatment response.

The most common KRAS mutations occur in codons 12 and 13 of exon 2, with approximately 80% occurring in codon 12 and 20% in codon 13. Activating mutations in codon 61 (exon 3) and codon 146 have also been reported, but these make up < 1% of mutations (19,27,34). Some methods cover only the common codon 12 and 13 mutations but miss uncommon mutations in codon 13, codon 61 or codon 146. Preliminary data suggest that mutations in codons other than 12 and 13 also predict response to anti-EGFR therapy. Consequently, the ability to detect mutations in codons other than 12 and 13 may be necessary to avoid unnecessary use of anti-EGFR therapies in some patients (36)

Finally, it needs to be emphasized that e.g. in Belgium there is reimbursement for the KRAS-test itself, but not for the inspection of the sections, selection of the most appropriate tissue block out of the different archived tumour samples and preparation of the material for KRAS testing. As it is likely that there will follow an explosive increase in the number of markers associated with response to therapy in colorectal cancer and other cancers, this will represent a considerable burden for pathologists and adequate regulation is mandatory.

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