**KRAS mutational test for metastatic colorectal cancer patients: not just a technical problem**


The identification of KRAS and BRAF mutations as predictive molecular alterations of resistance to EGF receptor monoclonal antibody therapy in metastatic colorectal cancer have significantly improved the selection of patients more likely to be eligible for the treatment with these targeted agents. Several methods are available for KRAS and BRAF mutation detection but few studies have compared different techniques, especially in the clinical setting. In this article, we contextualize the wobble-enhanced amplification refractory mutation sequencing method for the identification of KRAS and BRAF mutations with the other methodologies frequently used for the assessment of these alterations in colorectal cancer, discussing advantages and limitations over other frequently used diagnostic methods.

**Keywords:** BRAF mutation • colorectal cancer • direct sequencing • KRAS mutation • real-time PCR • sensitivity • specificity • targeted therapies • wobble-enhanced amplification refractory mutation sequencing

The analysis of KRAS mutational status is mandatory, according to the US FDA and the EMA guidelines, for the selection of patients who must be excluded from treatment with monoclonal antibodies, cetuximab and panitumumab (targeting EGF receptor [EGFR]) in chemorefractory metastatic colorectal cancer (mCRC) [1,2]. The same role played by KRAS has been proposed for BRAF mutations, although BRAF testing has not yet entered in clinical practice because no precise statements by regulatory agencies have been established [3,4].

Several methods are available for the analysis of KRAS and BRAF mutations, all based on the PCR technique. The most widely applied method for mutational testing is direct sequencing, characterized by a sensitivity of 10–20% [5]. In recent years, a number of more sensitive techniques have been developed, including both commercial kits, such as MALDI-TOF mass spectrometry technology (Sequenom GmbH, CA, USA), TheraScreen® (DxS, Manchester, UK), pyrosequencing or laboratory-made mutational methods, such as mutant-enriched PCR (ME-PCR) or high-resolution melting analysis [6,7]. Among tests with higher sensitivity and specificity than direct sequencing, the most used is TheraScreen, based on an amplification refractory mutation sequencing (ARMS) method followed by real-time PCR.

A novel wobble-enhanced ARMS (WE-ARMS) method, combined with the real-time methodology, has recently been developed by Hamfjord et al. for the detection of both KRAS and BRAF mutations [8]. In this article, we will discuss the advantages and limitations of such a methodology by focusing on the importance of the use of sensitive techniques for the evaluation of resistance-related mutations in the clinical setting.

**Summary of methods & results**

Hamfjord et al. developed a novel sensitive mutation assay, WE-ARMS, for detecting KRAS and BRAF mutations [8]. This test can identify the eight most commonly reported mutations in the KRAS (G12R, G12S, G12C, G12D, G12A, G12V, G13D) and BRAF (V600E) genes, by combining ARMS with the real-time
PCR technique (based on TaqMan® technology). To enhance the sensitivity and specificity of ARMS, the authors designed and tested sequence-specific primers with induced mismatches (wobbles) and sequence-specific probes. To preliminarily validate the assay, the authors tested primers and probes on DNA from mutated formalin-fixed paraffin-embedded (FFPE) tumor cells with less than 10% and less than 1% of tumor cells by evaluating an internal control in each reaction. The results indicated that the method is optimized for a template DNA from 1 to 20 ng from FFPE tissues for each reaction, and that the detection limit is approximately 1%. The cut-off values to discriminate between mutated and wild-type samples were experimentally established by the analysis of DNA from less than 10% FFPE tumor cells mixed with a background of wild-type cell line DNA. The authors state that each laboratory needs to establish the optimal cut-off, because it can depend on the type of sample, as well as on the method of DNA extraction. Finally, the accuracy of the test was evaluated by comparing WE-ARMS and TheraScreen in 49 FFPE mCRC cases. The sensitivity of WE-ARMS and TheraScreen were superimposable, whereas the cross-bindings were quite different between the two methods: they were observed in several set ups for the TheraScreen kit and only once in the WE-ARMS method.

Discussion

**KRAS** mutations identify mCRC patients resistant to EGFR-targeted therapies [1,2]. The same has been proposed for **BRAF**, **HRK3CA** and **NRAS** mutations [3,4]; however, these analyses are not mandatory for the selection of patients because, in contrast to **KRAS** mutations, no regulatory guidelines have been established yet.

Several methodologies are available for the detection of somatic mutations. The gold-standard method for investigating KRAS is direct sequencing, characterized by low sensitivity (10–20%) [9,10]. Therefore, mutations in cases showing tumor heterogeneity or few tumoral cells (e.g., small biopsies) can be missed.

A more sensitive method has been recently developed by Hamfjord et al. [8]. The authors presented a novel, quick and cost-effective mutation assay based on real-time PCR analysis (WE-ARMS) for the evaluation of the eight more frequent types of mutations in **KRAS** and **BRAF** oncoproteins, with a sensitivity of 1%. Several other methods have been developed accordingly to the same ARMS-based real-time technology, among which the most used is the TheraScreen, used by the authors for validating WE-ARMS accuracy. Both these methodologies showed the same sensitivity. However, WE-ARMS seems to have some advantages with respect to TheraScreen, because it is more specific, cheaper and the normalization is performed using an internal control, minimizing pipetting errors and heat-block irregularities. An enormous advantage of WE-ARMS is the possibility of adequately performing the assay starting from a very low DNA concentration (1–20 ng) as opposed to the other methods for **KRAS** evaluation, which need approximately 60–100 ng of DNA for each analysis. This is a very important point, particularly when the sample is very small (e.g., tumor biopsy).

As it is a real-time based methodology, WE-ARMS could be easily performed in all laboratories that have a real-time instrument capable of handling TaqMan technology. An automated sequencer, which is very expensive in terms of buying, maintenance and control, is therefore not required. However, the cut-off values for discriminating mutated from wild-type alleles are not standardized, but have to be established and optimized in each laboratory, as they may change according to the sample analyzed and to the DNA extraction method. This last evidence could create some problems for the analysis of samples processed in different laboratories, especially because pathology laboratories sometimes analyze cases that have been resected in another institute (and are, therefore, processed in a different manner).

Another limitation of WE-ARMS, and in general for those techniques based on real-time PCR, is that seven reactions for each sample (double for confirmation) are required for **KRAS** gene status assessment. Furthermore, only known and commonly reported mutations are detected. For example, WE-ARMS is designed to specifically recognize only **KRAS** G13D change for codon 13 alterations; therefore, missing other point mutations involving codon 13 (e.g., G13C), which cumulatively represent approximately 10% of alterations occurring in this codon. The same is true for **BRAF** mutations: indeed, the **V600K** change or those occurring in other codons (not so frequent in CRC but that can occur at higher rate in melanoma patients, where **BRAF** testing is now entering into clinical practice) are not detected. By contrast, both direct sequencing and a more sensitive technique such as ME-PCR (characterized by a sensitivity of ~0.1%) are able to characterize all the mutations in codons 12 and 13 of **KRAS** gene and in exon 15 of **BRAF** gene after a single or double PCR reaction (for direct sequencing and ME-PCR, respectively) [11]. However, direct sequencing suffers from low sensitivity, whereas ME-PCR, although extremely sensitive, is time-consuming and requires considerable manual input to avoid contamination.

Although WE-ARMS has only been validated in mCRC patients, it should also be tested in patients treated with anti-EGFR monoclonal antibodies to verify the clinical impact of such a methodology. A similar assay to WE-ARMS has been successfully used in a Phase II trial for mCRC patients treated with panitumumab [1] and in a very recent study, in which the new assay was compared with direct sequencing [12]. These papers demonstrated that the overall mutation rate found by applying ARMS was higher than that found by direct sequencing and that all the mutations found by ARMS occurred in nonresponder patients. These studies confirm the results reported by Molinari et al., in which ME-PCR was compared with direct sequencing in a clinical setting, and in which the mutational rate found by ME-PCR reached 50% and significantly improved the selection of patients [11]. Overall, these studies therefore demonstrate the importance of the use of sensitive KRAS tests for tailoring the best treatment in mCRC patients.

ARMS real-time based technology (such as WE-ARMS) is therefore very promising for the identification of specific oncogene mutations because it is extremely sensitive, rapid and does not need confirmation by direct sequencing. Its application for
diagnostic purposes could be further improved by also including the less frequent alterations occurring both in KRAS and BRAF genes, and by multiplexing the analysis of different mutations in a single or few reactions.

**Expert commentary & five-year view**
The investigation of tumor genetic profile plays a fundamental role in driving the most appropriate treatment for targeted drugs. Examples of oncogene mutations significantly correlated with targeted therapy benefit include eKit mutations in gastrointestinal stromal tumors, EGFR mutations in lung cancer and BRAF mutations in melanoma. By contrast, other genetic alterations are responsible for resistance to targeted drugs. In this field, the most important drug-resistant biomarker is KRAS, whose mutation evaluation has been introduced into the clinical setting following FDA and EMA guidelines.

The development of new methodologies for the identification of oncogene mutations is therefore clearly a priority owing to the clinical impact in the decisional approach to targeted therapy treatments. Researchers and companies will invest a great deal of effort into the identification of new methods, which should be sensitive, specific, rapid, cheap, easy to set up in pathology laboratories and require as few ng of DNA as possible. Another challenge will be to multiplex the analyses comprising all the molecular tests necessary for a specific tumor type (e.g., BRAF–NRAS for melanoma and KRAS–BRAF–PIK3CA for colon and ovarian cancer). All the methods developed have to be tested in large series of patients treated with targeted drugs to verify the clinical impact of these techniques and to establish the cut-off values of sensitivity that a clinical test should have for a better selection of patients in the diagnostic setting. Because a plethora of new mutational analyses will be conducted in the decisional approach to targeted therapy treatments in the near future, a tremendous challenge lies not only in the development of new methodologies but also, and more importantly, in the establishment of accepted guidelines for samples management, analysis and evaluation criteria.

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**Key issues**

- The investigation of KRAS and BRAF mutations is mandatory to identify metastatic colorectal cancer patients resistant to EGF receptor-targeted therapies.
- The most used methodology for assessing KRAS and BRAF mutations is direct sequencing; however, this technique has a low sensitivity.
- Hamford *et al.* developed a novel sensitive mutation assay, wobble-enhanced amplification refractory mutation sequencing (WE-ARMS), for detecting the eight most commonly reported mutations in the KRAS and BRAF genes, by combining ARMS with the real-time PCR technique (based on TaqMan® technology).
- The WE-ARMS methodology is sensitive, specific, quick and cost effective, only needs a low DNA concentration to perform the analysis and was validated in formalin-fixed, paraffin-embedded tissues.
- With respect to a method based on a similar technology and used in many diagnostic laboratories (the TheraScreen® kit), WE-ARMS is characterized by lower cross-bindings and by superimposable sensitivity.
- WE-ARMS represents a valid improvement in the field of KRAS testing.
- WE-ARMS can be further implemented by including less common KRAS and BRAF mutations and by multiplexing the analysis of different mutations in a single or few reactions.
- WE-ARMS should be tested on a series of metastatic colorectal cancer patients treated with anti-EGF receptor monoclonal antibodies to verify the clinical impact of such a methodology.

**References**

Papers of special note have been highlighted as:
- of interest
- of considerable interest


**First study investigating several methods for detecting KRAS mutations, characterized by different sensitivities, in mCRC treated with monoclonal antibodies against EGF receptor.**