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Review

The ideal reporting of *RAS* testing in colorectal adenocarcinoma: a pathologists' perspective

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Summary

RAS gene mutational status represents an imperative predictive biomarker to be tested in the clinical management of metastatic colorectal adenocarcinoma. Even if it is one of the most studied biomarkers in the era of precision medicine, several pre-analytical and analytical factors may still impasse an adequate reporting of *RAS* status in clinical practice, with significant therapeutic consequences. Thus, pathologists should be aware on the main topics related to this molecular evaluation: (i) adopt diagnostic limit of detections adequate to avoid the interference of sub-clonal cancer cell populations; (ii) choose the most adequate diagnostic strategy according to the available sample and its qualification for molecular testing; (iii) provide all the information regarding the mutation detected, since many *RAS* mutation-specific targeted therapeutic approaches are in development and will enter into routine clinical practice. In this review, we give a comprehensive description of the current scenario about *RAS* gene mutational testing in the clinic focusing on the pathologist's role in patient selection for targeted therapies.

Key words: colorectal cancer, precision medicine, *RAS*, biomarkers

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Introduction

In the era of precision medicine, the implementation of testing for established predictive biomarkers has become a crucial step of therapeutic management of patients with advanced colorectal cancer (CRC) [1].
At present, international guidelines recommend testing for DNA mismatch repair (MMR)/ microsatellite instability (MSI) status and *KRAS* (exons 2, 3 and 4), *NRAS* (exons 2, 3 and 4) and *BRAF* (exon 15) mutations in all metastatic CRC (mCRC) patients [2]. *RAS* testing is mandatory before treatment with anti-EGFR therapies. *BRAF* mutational status should be assessed for prognostic stratification and eligibility to be treated with ce-

tuximab-encorafenib [3], in addition to its negative predictive value for response to anti-EGFRs. The evaluation of MMR status by immunohistochemistry (IHC) is recommended in all CRC patients (i.e., universal screening for Lynch syndrome) and MMR/MSI status assessment is also mandatory for treating advanced CRC with immune-checkpoint inhibitors. According to recent literature data, the identification of *HER2* amplification by IHC or fluorescent *in-situ* hybridization (FISH) is recommended in *RAS*-wild-type (wt) patients to detect those who may benefit from anti-HER2 targeted therapy [4].

The *RAS* genes family

The *RAS* genes family encodes four small, cytoplasmic proteins with GTPase activity: H-Ras, K-Ras4a, K-Ras4b, and N-Ras [5]. The RAS-RAF-MEK-ERK mitogen-activated kinase signaling (MAPK) pathway is activated by growth factors, cytokines, immunological receptors, integrins, and chemokine receptors and promotes cell growth, differentiation, proliferation and survival [6]. Thus, mutations that affect RAS-RAF-MEK-ERK pathway components can result in aberrant and constitutive activation of tumorigenesis.

RAS mutations or amplifications are among the most common genetic alterations in cancer and single nucleotide activating point mutations in members of *RAS* family have been found in 20-25% of human cancers [7]. *KRAS* mutations occur in a wide range of solid tumors; *NRAS* mutations are found in melanoma and many hematological malignancies; *HRAS* mutations are present in head and neck cancers and genitourinary tract malignancies [8].

Approximately 40% of CRC harbor *KRAS* mutations mostly in exon 2 codons 12 (70-80%) and 13 (15-20%). Additional *KRAS* mutations are mainly located in exon 3 codons 59-61 and in exon 4 codons 117 and 146. Mutations in *NRAS* are present in approximately 3-5% of CRC and are located in exon 3 codon 61 in the majority of cases and in exon 2 codons 12, 13. *NRAS* and *KRAS* mutations are typically mutually exclusive [9].

Due to the high concordance rates between primary and metastatic CRC samples, *KRAS* mutations are believed to be early events in CRC carcinogenesis, which are maintained during CRC development and progression [10].

The clinical perspective

More than a decade ago, retrospective analyses of seminal clinical trials for the anti-EGFR monoclonal antibodies cetuximab and panitumumab reported that patients with mCRC harboring activating point mutations in *KRAS* exon 2 (codons 12/13) do not derive clinical benefit from anti-EGFR therapy [11-13]. Evidence from the PRIME study [14] and CRYSTAL study [15] showed that mutations in exons 3 and 4 of *KRAS* and exons 2, 3 and 4 of *NRAS* also predict a lack of response to EGFR-targeting monoclonal antibodies. Since then, several studies have investigated the role of *RAS* mutations in predicting response to anti-EGFR therapy [16,14,17,18]. Cumulative data strongly indicate that the presence of any mutation is a negative predictive marker of response to anti-EGFR monoclonal antibodies, alone or in combination. Thus, expanded *RAS* analyses should be conducted on all patients eligible/being considered for EGFR antibody therapy.

Despite numerous efforts, *KRAS* mutation has long been deemed "undruggable". However, preclinical studies and early-phase trials have recently demonstrated the safety and promising efficacy of the $KRAS^{G12C}$ inhibitors sotorasib and adagrasib [19,20]. This has an important impact on *RAS* testing, because the definition of the exact alteration observed in the analysis is mandatory in the molecular report. The $KRAS^{G12C}$ mutation is found in 14% of non-small cell lung cancers (NSCLC), 3% of CRC [21], and 1-3% of other solid tumors [22]. CRC patients derive fewer benefits from $KRAS^{G12C}$ inhibitors, in comparison with NSCLC patients, due to the rapid onset of resistance by cellular, molecular, and genetic mechanisms. As a result, combination approaches with EGFR inhibitors are currently being investigated [23].

Pre-analytical variables of the formalin-fixed paraffin-embedded (FFPE) specimen

Data from retrospective studies recommend resection of the primary tumor also in metastatic disease because of obstructive symptoms and/or bleeding, and thus in the CRC setting most of the molecular testing is performed on surgical specimens [24-26].

Several sequential steps are involved in the preanalytical handling of surgical specimens from resection in the surgical theater to paraffin-embedding and storage. Each step is highly critical and can significantly affect the preservation of morphology, antigens, and nucleic acids. Some key points in this process are still undefined and are subject to high variability among hospitals.

Fixation

Specimens are ideally received as quickly as possible, fresh, and unopened. Fresh specimens allow opti-

mal macroscopic assessment of the resection, which is crucial in rectal cancers, which may otherwise be affected by distortion resulting from formalin fixation. It has been recommended that the "cold ischemia time" remain below 1 h to allow proper processing of cancer specimens and permit correct evaluation of both morphological and therapeutic-prognostic parameters [27,28]. Moreover, tissue handling procedures should be optimized to allow biomarker testing. The specimen should undergo fixation with 10% neutral buffered formalin (4% formaldehyde) for no less than 6 h and no more than 48 h. If prompt transport to the laboratory is not possible, the specimen should be placed unopened in an adequate quantity of formalin fixative. Refrigeration should also be considered if a delay longer than a day or more is anticipated [29].

Evaluation of the biospecimen and its qualification for molecular testing

Surgical pathologists play an important role in the molecular diagnosis of solid tumors. They make the histologic diagnosis of CRCs, request mandatory molecular tests, and submit appropriate neoplastic tissues for molecular testing [1,30,31]. Most importantly, they designate areas for microdissection or macrodissection and estimate tumor cellularity of those areas to ensure it is adequate for the limit of detection (LoD) of the requested molecular assays [32]. In the qualification process of the specimen for molecular testing, the pathologist should review all available tumor specimens and enrich samples by macrodissection to maximize tumor cell content (> 20%) before DNA extraction. Specimens resected after chemotherapy/radiation therapy (at present limited to rectal adenocarcinomas in the CRC setting) are not ideal for molecular analysis and should be avoided; moreover, pathologists should avoid areas with potential PCR inhibitors, such as mucin and necrotic debris, and areas with reduced overall tumor cell percentage, such as infiltrative or subcapsular metastasis of lymph nodes with no nodular formation, tumors with a prominent desmoplastic reaction or lymphocytic infiltration, and the superficial portion of ulcerated tumors with prominent neutrophil infiltration [1,30,33].

A crucial step in the diagnostic process is the integration of the molecular test results into pathology reports. In cases with a tumor cell percentage potentially lower than the LoD of the assay, a note indicating potential false-negative results should be noted in the report. Neoadjuvant therapy poses challenges to molecular diagnostics insofar as it depletes the tumor cellularity of specimens. For patients undergoing neoadjuvant therapy, pathologists should ideally obtain alternative specimens, such as pretreatment biopsy samples. In fact, the depletion of tumor cellularity after neoadjuvant therapy may lead to false-negative results. Boissiere-Michot et al. [34] examined pre-neoadjuvant and post-neoadjuvant treatment rectal cancers from 31 patients. *KRAS* mutations were identified in 12 pretreatment specimens, but only 9 post-treatment specimens, despite using laser capture microdissection to increase the proportion of tumor tissues and allele-specific PCR to improve the sensitivity of assays in the latter samples. These results demonstrate that pretreatment biopsy specimens represent the best option for *KRAS* mutation detection. At a referral center for neoadjuvant chemotherapy/radiation therapy, biopsy specimens are usually taken before the patient arrives. In these cases, it is recommended to request the tissue blocks or unstained slides for potential molecular tests when the H&E-stained slides are sent for confirmation of diagnosis.

Tumor cellularity and (macro/micro)dissection

In the clinical molecular diagnostics setting, specimens from unselected patients often have a low tumor cellularity and therefore require assays with higher analytic sensitivity, especially in the metastatic setting [35]. Improvement of sensitivity can also be achieved at the preanalytical step by macrodissection or microdissection. Manual macrodissection from the area designated by pathologists has been a routine preanalytical approach to enrich tumor cellularity [32]. Selection and designation of adequate specimens by pathologists is still the most cost-effective option for accurate molecular diagnosis.

The estimation of tumor cell percentage by pathologists is important both to ensure that submitted specimens meet the LoD of requested molecular assays and for quality control purposes. Ideally, pathologists who interpret molecular test results should be responsible for the estimation of tumor cellularity since they are aware of the analytic sensitivity of the requested assay. In addition, molecular pathologists can improve their precision and accuracy in estimating tumor cellularity through prospective and retrospective quality management processes correlating the estimated tumor cell percentage with the observed mutant allele percentage. In general, the tumor cell percentage, if accurately estimated, should correlate with the mutant allele percentage as determined by the molecular assays. For heterozygous mutations, the tumor cell percentage should be roughly twice the percentage of the mutant allele in a mixture of diploid DNA. Mutant allele frequencies of more than 50% may indicate mutant allele amplification bias of the assay, genomic amplification of the mutant allele, or loss of the wild-type allele.

DNA integrity and qualification

Other critical preanalytical factors should be considered in DNA evaluation from FFPE tissue such as storage conditions, deparaffinization, and DNA extraction. Storage of FFPE blocks at room temperature for more than 3 years detrimentally affects their fitness for the purpose of NGS [36]. However, even DNA extracted from blocks stored for 32 years can successfully be used in whole exome sequencing, albeit with lower coverage and read depth [37].

Slide deparaffinization has been shown to be more efficient than in tubes in terms of DNA yield and integrity [38]. However, waxing of the slides did not improve the quality of the DNA extracted after several months of storage at 4 °C [39]. Xylene alternatives exist for deparaffinization of FFPE tissue, namely mineral oil, hexadecane, pentadecane or tetradecane, and may give superior DNA yields [40].

The extraction method and heat treatment conditions applied for reversal of crosslinks is another critical factor. Comparison of two silica membrane-based kits, the QIAamp DNA FFPE tissue kit (Qiagen) and the Nucleospin FFPE DNA kit (Machery-Nagel) for whole exome sequencing found higher yields of dsDNA with QIAamp [36]. In another comparative study, 7 silica column based methods (Syngen Tissue DNA, ZR FFPE DNA, QIAamp DNA FFPE Tissue, GeneMATRIX Tissue DNA, ReliaPrep FFPE gDNA, E.Z.N.A. FFPE DNA, and Invisorb Spin Tissue) and one magnetic bead-based method (MagMAX FFPE DNA) were compared, and the best results in terms of DNA yield and integrity were obtained with the QIAamp DNA FFPE and the ReliaPrep FFPE gDNA kit [41]. Dual DNA/RNA extraction kits generally give lower yields [41]. DNA extraction from FFPE for downstream NGS can also be automated [42]. Five different automated FFPE DNA extraction methods were compared, 4 magnetic bead-based methods (MagAttract DNA, QIA- symphony DNA, InnuPREP FFPE DNA, Maxwell 16 FFPE) and one silica membrane-based kit (QIAamp DNA FFPE) in terms of DNA yield and amplifiability, with the Maxwell method showing the best results [43].

The current *RAS* diagnostic scenario

Several testing strategies are currently available (Tab. I). Any validated test strategy is deemed acceptable provided that it satisfies the minimal requirements of a mutation-detection sensitivity between 95% and 99%, and a specificity of 100%. *KRAS* testing must be reproducible and should be performed by an accredited laboratory that routinely participates in proficiency testing with external validation. The implementation of most adequate testing strategy takes into account several challenging points including pre-analytical (as stated before) and analytical aspects [1].

An important aspect of clinical sequencing tests to consider is the lower LoD, which refers to the lowest levels of genomic variants that the platform can detect consistently in the background of wild-type sequences. Any sequencing method is characterized by a specific LoD and can produce both false positive (results where the analyte is declared to be present although actually it is below LoD) and false negative (results where analyte is declared to be below LoD although it is not) errors at the same time [44]. The most important point in the selection of the most adequate test to be implemented for *RAS* mutational analysis is to avoid

Table I. Technical features of analytical platforms available for *RAS* molecular testing in CRC patients.

Technique	Sample type	Biomarkers	Limit of Detection (LoD) (%)	Reference range	Turnaround time (TAT)	Reference
Sanger Sequencing	Frozen tissue, FFPE, direct smears, Thin Prep	*KRAS*, *NRAS*, *BRAF*	15-20	All mutations present in the analyzed gene regions	3 days	Zaidi et al. [45]
RT-PCR	Frozen tissue, FFPE, direct smears, Thin Prep, cell-block, LBC	*KRAS*, *NRAS*, *BRAF*, MSI	1-10	Only «hot spot» Mutations (probe based)	1 day	Zhang et al. [46]
digital-PCR	Liquid biopsy	*KRAS*, *NRAS*, *BRAF*	0.0001-0.01	Only «hot spot» Mutations (probe-based)	1 day	Mauri et al. [47]
NGS	Frozen tissue, FFPE, direct smears, Thin Prep, cell-block, LBC, liquid biopsy	*KRAS*, *NRAS*, *BRAF*, MSI	0.001-5	All mutations present in the analyzed gene regions	1-3 days	Jan et al. [48]

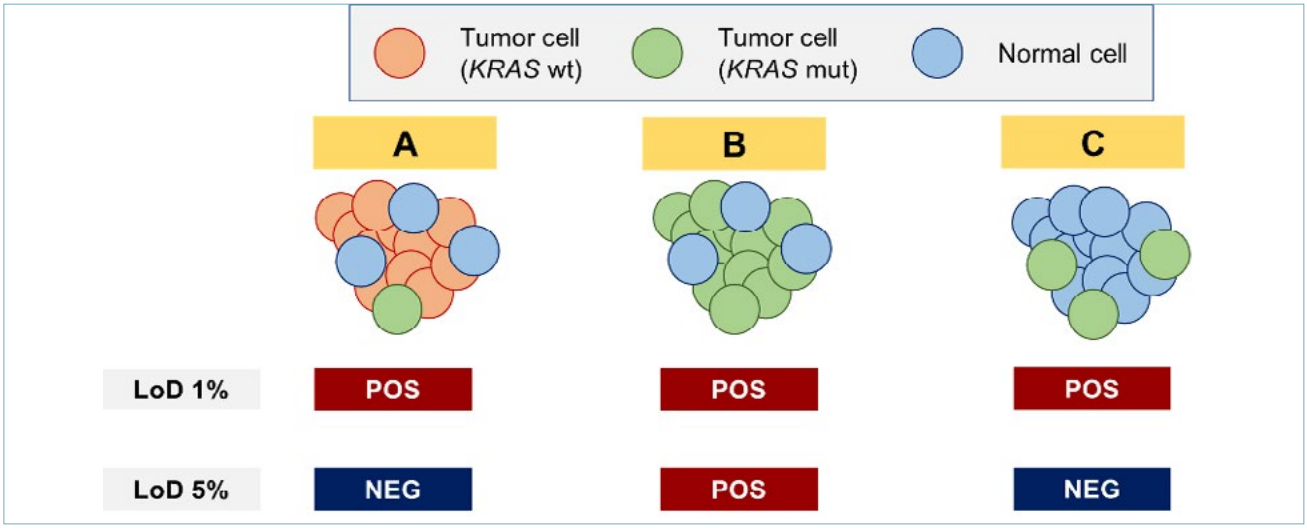

Figure 1. The adoption of a low Limit of Detection (LoD) is detrimental in the case of (sub)clonal mutation, negatively affecting the adoption of anti-EGFR targeting therapeutic approaches (A). This is a relatively frequent situation in the clinical practice, and, thus, most samples (A and B) should be tested with technologies applying at least a 5% LoD cut-off. The only situation in which a low LoD should be applied to *RAS* mutational testing are cases characterized by low tumor cellularity, avoiding false negative results (C).

low LoD in cases of good samples characterized by an adequate tumor cellularity, limiting the influence of (sub)clonal mutations, which are not significant in the therapeutic setting (Fig. 1). On the other hand, low LoD strategies should be considered only in specific cases, such as in the presence of a significant inflammatory intra-tumor infiltration, or when looking for minimal residual disease, applying liquid biopsy methodologies. These data confirm the central role of histopathological qualification of the biospecimen in molecular diagnostics and in the selection of the most adequate diagnostic approach according to the characteristics of the biospecimen to be analyzed.

To date, a heterogeneous landscape of testing strategies may be observed in the diagnostic setting. As mentioned, each technical approach shows analytical variability in terms of reference range, technical sensitivity/specificity, and turnaround time (TAT) [1,49].

Sanger sequencing

Sanger sequencing, also known as the "chain termination method", is a method to determine the nucleotide sequence of DNA. Sanger sequencing platforms have represented the "gold standard" testing approach for the molecular analysis of clinically approved biomarkers in clinical practice, due to low false-positive rates and high specificity. Sanger sequencing is a robust and affordable technique that enables the detection of point mutations and small insertions and deletions. Sanger sequencing can be used to detect rare mutations not covered by commercial panels. However, this method is burdened by a medium-high turnaround time (TAT), due to the need to manually analyze sequencing chromatograms to search for mutations.

This approach has a LoD of approximately 15-20% mutant allele frequency, which entails a not-negligible risk to yield false negative results. No difference in the detection of *RAS* mutations has been reported in comparison with real time-PCR for samples with at least 30% of tumor cells. However, a tumor cell content of at least 50% is recommended to avoid false negative results [50]. Moreover, data interpretation from Sanger sequencing approach requires highly-trained personnel due to technical artifacts when the starting sample is a "scant" diagnostic material [51].

In summary, Sanger sequencing should be used only in accredited laboratories that incorporate internal quality control systems in their routine works and that have access to a high-sensitivity technique to test doubtful cases.

Mass spectrometry

MALDI-TOF (matrix-assisted laser desorption/ionization time-of-flight) mass spectrometry is an analytical technique in which samples are ionized into charged molecules and the ratio of their mass-to-charge (m/z) can be measured. Primer-extension-based MALDI-TOF technology enables genotyping through the

detection of single-base extension products of a primer immediately adjacent to the single nucleotide polymorphism (SNP) site. The LoD of this method may vary from 2.5% to 10% depending on the mutation type. Several commercial kits using MALDI-TOF mass spectrometry technology associated with the single base extension technology are currently available for diagnostic purposes. These kits use multiplex PCR to detect the most relevant *KRAS* and *NRAS* mutations in codons 12, 13, 59, 61, 117, and 146. Mass spectrometry has proved to be a reliable, cost-effective technology, with a low TAT, and requires a lower input of DNA in comparison with the other technologies [52]. Conversely, a not-negligible percentage of false negative results and a low reference range in comparison with sequencing-based approaches affect these platforms [53].

Real-time PCR (RT-PCR)

Numerous RT-PCR-derived systems have also been implemented for the molecular analysis of diagnostic routine samples in Italian laboratories. Although the vast majority of small/medium laboratories adopt high-sensitive RT-PCR systems in routine practice, low multiplexing capability and challenging visual data interpretation discourage the widespread diffusion of RT-PCR-based assays as unique testing platforms for molecular analysis of routine samples. RT-PCR systems are conventionally grouped in semi and fully automated systems in accordance with the manual series of technical procedures covered by each approach [54,55].

RT-PCR is a sensitive technique with a LoD of 1-10% and a recommended threshold of neoplastic cells of 10% [56,46]. RT-PCR methods employ primers and probes that look for a pre-defined set of mutations. This makes them less comprehensive, but makes interpretation easier thanks to proprietary software able to easily carry out molecular data. Moreover, certain RT-PCR assays do not allow to discriminate between different variants, thus failing to identify the $KRAS^{G12C}$, which is now mandatory to be included in the molecular pathology report for its therapeutic implications.

The TheraScreen *KRAS* Mutation Detection Kit (DxS-Qiagen), COBAS *KRAS* Mutation Test (Roche Molecular Systems), Idylla™ *KRAS* mutation test and the EasyPGX® ready *KRAS* are widespread commercial RT-PCR panels in the Italian setting.

The TheraScreen *KRAS* Mutation Detection Kit can identify 7 mutations in codons 12 and 13 of *KRAS* gene, and additional techniques are needed to analyze other *loci* of interest [57]. The COBAS test offers the possibility, by using the combination of two kits, to identify the most relevant mutations in *KRAS* (codons 12, 13, 61) and *NRAS* [58].

The Idylla™ *KRAS* mutation test covers 21 *KRAS* mutations in exons 2, 3 and 4 (codons 12, 13, 59, 61, 117 and 146) providing a broader mutation screening platform compared to other commercially established systems. Although the Idylla *KRAS* mutation platform tests for all the common and some of the rarer *KRAS* mutations, some rare mutations such as those as the p.G13C are not covered [59].

The EasyPGX® ready *KRAS* detects the main mutations of exon 2 (codons 12, 13) of exon 3 (codons 59, 61) and of exon 4 (codons 117, 146) of the gene *KRAS* gene. The kit allows the detection of low percentages of mutated allele in the presence of high amounts of wild-type genomic DNA by real-time amplification with sequence-specific probes with a LoD down to 0.5% [60].

The main issue regarding the application of RT-PCR based systems is represented by the partial reference range discouraging this approach when a scant diagnostic sample is only available for molecular tests [61].

Next-Generation Sequencing

In recent years, Next-Generation Sequencing (NGS) has transitioned from research to clinical practice, due to the convergence of advances in molecular technology and the rapid expansion of targeted therapeutics [1,51]. According to ESMO guidelines, NGS testing can be implemented in the CRC setting if its cost is similar/lower to that of orthogonal technologies [62].

NGS methods can detect any variant from single-nucleotide changes to insertions or deletions, and translocations, in a single multiplex PCR reaction. NGS has a high sensitivity, with a LoD of 5-10%, enabling to successfully analyze scant diagnostic samples (i.e., small biopsies). The LoD is an important tool to determine whether a variant with a low number of reads and low frequency should be reported as a mutation or should be regarded as a false-positive or (sub)clonal result [63].

NGS assays can be used to investigate the mutational status of a specific set of genes (targeted panels), to sequence the coding regions of the genome (whole-exome sequencing [WES]) or to sequence the entire genome, including the intronic regions (whole-genome sequencing [WGS]). Targeted panels are being used in clinical settings because they allow the detection of clinically relevant variants in multiple genes of interest, overcoming single-plex issues related to RT-PCR systems; furthermore, they have a decreased data burden with simplified data analysis [64]. At present, numerous commercial NGS panels are available for CRC diagnostics, covering a variable number of genes ranging from five to hundreds. NGS panels allow combining extended *RAS* and *BRAF*

analysis, which is currently recommended in routine practice, with testing for additional emerging biomarkers (i.e., *PIK3CA*, *EGFR, CTNNB1, ERBB2, PTEN,* and *SMAD4*) [65]. Similarly, customized NGS panels also represent technically available solutions for molecular analysis of predictive biomarkers. Customized panel consists of a "tailored" approach in accordance with clinical requests received by a specific institution. After preliminary validation procedures, these panels allow to maintain high quality in terms of molecular analysis of predictive molecular biomarkers from diagnostic routine specimens [66,67].

Another pillar of the tailored therapeutic approach in CRC patients is MSI status evaluation. Conventionally, MSI status was detected on Sanger sequencing platforms, but, because of the aforementioned technical limitations, novel promising approaches have been developed. High-sensitive microfluidic platforms and automated RT-PCR systems demonstrated technical feasibility to accurately detect MSI status on diagnostic specimens. Several NGS assays are currently in use to provide comprehensive data regarding hot spot mutations and MSI status combining ultra-sensitive systems with an optimized bioinformatic algorithm aimed to promote a single-step multi-testing analysis system for the clinical management of CRC patients [68,69].

Liquid biopsy

Liquid biopsy methods are based on a heterogeneous group of biological fluids from which several analytes may be isolated and evaluated. Benefiting from a dynamic, comprehensive, and less invasive sampling approach, liquid biopsy from peripheral blood has been approved by international societies to select NSCLC patients who could benefit from TKI-based therapeutic regimens [70]. To date, only circulating free DNA (cfDNA), including circulating tumor DNA (ctDNA), represents a clinically available source of nucleic acids for predictive molecular testing in routine practice. Moreover, the application of liquid biopsy specimens in clinical practice has to overcome several challenges including clonal hematopoiesis effect and low amount of nucleic acids available for molecular tests [70]. Liquid biopsies are gaining momentum in clinical management of CRC patients as a minimally invasive approach for treatment monitoring [71]. The implementation of liquid biopsy samples for "re-challenge" strategy with EGFR inhibitors in *RAS* wild-type cases may be considered the most promising area for CRC patients. Other important applications of liquid biopsy regarding detection of ctDNA in the CRC setting are: (i) analysis of minimal residual disease (MRD) as a prognostic factor and for adjuvant therapy selection [68,72], (ii) identification of *RAS* and *BRAF* mutations in the absence of an adequate tissue biopsy in the metastatic setting, and (iii) evaluation of the prognostic significance of cfDNA in early-stage disease [71,73]. Technical administration of liquid biopsy samples requires comprehensive, ultra-deep technologies, like NGS platforms or digital PCR (dPCR) systems [74] enabling to detect cancer-related molecular alterations from scant samples. In particular, NGS provides a fast high-throughput, and cost-effective alternative to traditional Sanger sequencing to accurately identify alterations in multiple genes of interest and to provide clinically-useful information.

RAS testing in the real-world setting

The widespread diffusion of NGS platforms has increased the rate of *RAS* biomarker testing in diagnostic routine specimens. Accordingly, the identification of "uncommon" *RAS* molecular alterations has had significant impact on the clinical management of CRC patients [75]. Public databases that are routinely queried by personnel involved in molecular tests annotate a plethora of *RAS* mutations derived from pre-clinical and clinical trials with various clinical roles in the selection of CRC patients [76]. On the other hand, *RAS* molecular alterations from real-world diagnostic practice may significantly impact the reporting of clinically relevant molecular alterations identified on routine diagnostic specimens [76]. This issue encouraged seven referral Italian institutions in molecular testing to retrospectively collect data from routine diagnostic practice about *KRAS* testing in CRC patients in order to develop a "knowledge-based" database with real-world data [20]. Briefly, a series of 1523 archival data from two previous years of diagnostic activity were retrieved. Overall, 728 of 1523 (47.8%) of cases harbored *K-* and/or *NRAS* hotspot mutations. The data also showed that NGS, alone or in combination with orthogonal technologies, was selected for *RAS* molecular testing in routine diagnostic practice by participating institutions. These data, periodically updated and supervised by a dedicated group of data managers with proven expertise in this field, improve knowledge about the real-world data from *RAS* testing in Italian institutions and support healthcare personnel involved in *RAS* mutation testing.

RAS testing: the molecular pathology report

Molecular pathology reports should present results in a clear and concise manner to guide clinicians in the selection of the best treatment options [77]. The molecular pathology should contain the following information:

- Identification of laboratory, patient (name, surname, date of birth, sex), ordering physician, sample (ID, date specimen collected).
- Specimen used for analysis (FFPE, frozen tissue, liquid biopsy), and if tumor tissue also to be included: microscopic diagnosis, tumor cell content, whether microdissection was performed, and the identification of the physician who performed it.
- Methodology: assay, LoD, target genes with exons/codons analyzed (if applicable).
- Test results: list of genetic alterations detected using standard nomenclature (for variant also include variant allele frequency if applicable), additional analytic and clinical interpretative comments.

In accordance with ESMO guidelines [4], *RAS* testing should be completed and reported within a TAT of ≤ 7 working days for at least 90% of the test requests.

Conclusions

RAS gene mutational status is a central dogma in the therapeutic management of metastatic colorectal adenocarcinoma patients. Despite being one of the most studied biomarkers in the era of precision medicine, several pre-analytical and analytical factors may still impasse adequate reporting in clinical practice, with significant therapeutic consequences. Thus, pathologists should be aware of the challenges related to the molecular evaluation of this biomarker: (i) adopt a method with an adequate LoD in order to avoid the interference of sub-clonal cancer cell populations; (ii) choose the most adequate diagnostic strategy according to the available sample and its characteristics in relation to molecular testing; (iii) provide all the information regarding the mutation detected, since many *RAS* mutation-specific targeted therapeutic approaches are in development and will enter into routine clinical practice. Literature data has recognized the pathologist as a key player in the *RAS* diagnostic algorithm and the person in charge of personalized diagnostics, which is the basis of current precision oncology.

Conflicts of interest

U.M. has received personal fees (as consultant and/or speaker bureau) from Boehringer Ingelheim, Roche, MSD, Amgen, Thermo Fisher Scientific, Eli Lilly, Diaceutics, GSK, Merck, AstraZeneca, Janssen, Diatech, Novartis, and Hedera unrelated to the current work. M.F. reports research funding (to Institution) from QED, Macrophage pharma, Astellas, Diaceutics; personal honoraria as invited speaker from Roche, Astellas, AstraZeneca, Incyte, Bristol Myers Squibb, Merck Serono, Pierre Fabre, GlaxoSmithKline, Novartis, Amgen; participation in advisory board for Amgen, Astellas, Roche, Merck Serono, GlaxoSmithKline, Novartis, Janssen. S.L. reports research funding (to Institution) from Amgen, Astellas, Astra-Zeneca, Bayer, Bristol-Myers Squibb, Daichii Sankyo, Hutchinson, Incyte, Merck Serono, Mirati, MSD, Pfizer, Roche, Servier; personal honoraria as invited speaker from Amgen, Bristol-Myers Squibb, Incyte, GSK, Lilly, Merck Serono, MSD, Pierre-Fabre, Roche, Servier; participation in advisory board for Amgen, Astra Zeneca, Bristol-Myers Squibb, Daiichi-Sankyo, Incyte, Lilly, Merck Serono, MSD, Servier, GSK. G.P. has received personal fees (as consultant and/or speaker bureau) from Roche, Eli Lilly, Diaceutics, AstraZeneca, Novartis, Exact Sciences and ADS Biotec.

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Authors' contributions

Conceptualization, MF, GP and UM; methodology GF, SL and MS; data curation FP, AV; writing-original draft preparation, GF, SL, AV, FP, MS, LM, GP, AM; writing-review and editing, MF, GP and UM. All authors have read and agreed to the published version of the manuscript.

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