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REVIEW

Molecular testing for colorectal cancer: Clinical applications

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Abstract

Molecular genetic analysis is an integral part of colorectal cancer (CRC) management. The choice of systemic therapy for CRC is largely based on the results of tumor molecular testing. Evaluation of the *KRAS* and *NRAS* gene status is mandatory for consideration of anti-epidermal growth factor receptor (EGFR) therapy. Tumors with the *BRAF* V600E substitution are characterized by aggressive behaviour, may require intensified cytotoxic regimens and benefit from combined BRAF and EGFR inhibition. The inactivation of DNA mismatch repair (MMR), or *MUTYH* gene, or DNA polymerase epsilon results in excessive tumor mutational burden; these CRCs are highly antigenic and therefore sensitive to immune checkpoint inhibitors. Some CRCs are characterized by overexpression of the *HER2* oncogene and respond to the appropriate targeted therapy. There are CRCs with clinical signs of hereditary predisposition to this disease, which require germline genetic testing. Liquid biopsy is an emerging technology that has the potential to assist CRC screening, control the efficacy of surgical intervention and guide disease monitoring. The landscape of CRC molecular diagnosis is currently undergoing profound changes due to the increasing use of next generation sequencing.

Key Words: Colorectal cancer; Anti-epidermal growth factor receptor therapy; *KRAS*; *NRAS*; *BRAF*; *HER2*; Microsatellite instability; *MUTYH*; Hereditary cancer

Core Tip: Molecular genetic analysis is an integral component of colorectal cancer (CRC) management. Comprehensive *KRAS* and *NRAS* testing is mandatory for selection of patients for anti-epidermal growth factor receptor (EGFR) therapy. *BRAF* V600E mutated cancers are responsive to combination of BRAF and EGFR inhibitors. CRCs with *HER2* amplification and overexpression can be controlled by the down-regulation of this receptor. Immune therapy is highly effective in CRCs with exceptionally high tumor mutation burden, *e.g.*, in cancers with microsatellite instability, *MUTYH* gene inactivation or mutations in the *POLE* gene. CRC patients with early disease onset, specific tumor features or family history of the disease require germ-line DNA testing.

INTRODUCTION

Colorectal cancer (CRC) holds the third position for cancer morbidity and accounts for approximately 1.8 million new cases per year worldwide. Approximately half of the patients with CRC diagnosis die from this disease[1]. CRC is commonly classified as right-sided (proximal) cancers affecting cecum, ascending colon, hepatic flexure and transverse colon, and left-sided (distal) tumors, which include CRCs located at the splenic flexure, descending colon, sigmoid colon and rectum. Right-sided and left-sided CRCs have a distinct embryological origin, being developed from midgut and hindgut, respectively. Right-sided CRCs are more characteristic for females and often demonstrate peritoneal metastatic spread. Left-sided tumors are more frequent among men and tend to metastasize to the liver and lungs. The distinct biologic behavior of left- and right-sided tumors is at least in part attributed to the difference in the spectrum of cancer-associated mutations[2,3].

CRCs can be broadly divided into microsatellite stable tumors, which account for the vast majority of CRC cases, and carcinomas with so-called high-level microsatellite instability (MSI-H). A subset of CRCs is characterized by wide-spread methylation of cytosine residues, defined as CpG island methylator phenotype (CIMP). There are also classifications of CRCs based on tumor transcriptional profiles. The most characteristic molecular features of CRCs include dysregulation of MAPK and WNT signalling pathways, chromosomal imbalances at chromosomal loci 1p, 5q, 17p, 18p, 18q, 20p and 22q, mutations in *KRAS*, *NRAS* or *BRAF* oncogenes, activation of PI3 kinase, and *TP53* gene inactivation[4-7]. Recent data suggest that the gut microbiome at least partially contributes to molecular pathogenesis of CRC[8,9]. While many deaths from CRC may be prevented by early tumor detection[3], some CRCs generate metastases in the very early phases of their development when the primary tumor is invisible by available diagnostic tools[10].

MARKERS FOR THE CHOICE OF SYSTEMIC THERAPY

Analysis of mutations in RAS genes for the administration of anti-EGFR therapy

The majority of CRCs are characterized by the activation of MAPK signalling cascade. Some CRCs demonstrate activation of receptor tyrosine kinases (RTKs), mainly the epidermal growth factor receptor (EGFR). Up-regulation of the MAPK pathway can also be achieved by mutations in the downstream targets of RTKs, *i.e.*, *RAS* or *RAF* oncogenes. Consequently, CRCs can be broadly divided into two categories: approximately a third of colorectal tumors appear to be EGFR-dependent, while the remaining cases are triggered either by the activation of collateral cascades (*e.g.*, *HER2*-amplified cancers; approximately 2%-5% of CRCs) or genetic events affecting *RAS* or *RAF* genes (> 60% of CRC cases). EGFR-driven CRCs are likely to be responsive to anti-EGFR antibodies, panitumumab or cetuximab, while patients with EGFR-independent tumors usually do not benefit from EGFR-targeted therapy (Figure 1).
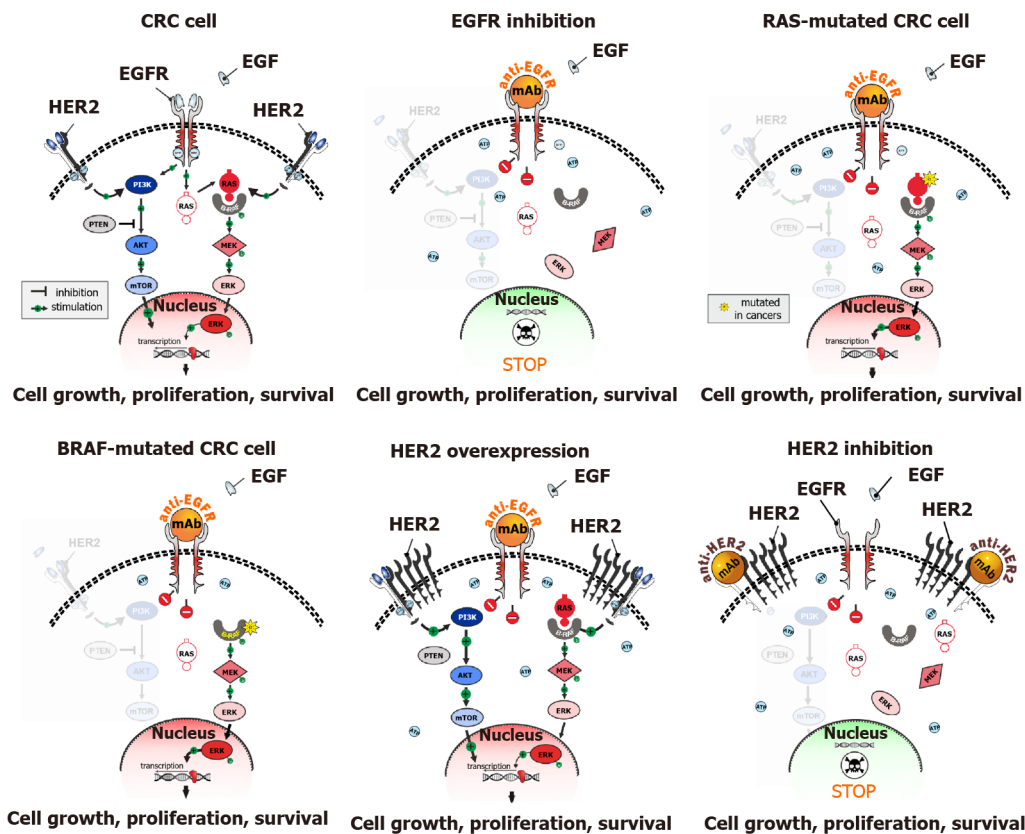
Figure 1 Molecular cascades underlying colorectal cancer responsiveness and resistance to targeted therapy. Virtually all colorectal cancers (CRCs) are characterized by upregulation of mitogen-activated protein kinases (MAPK) signaling pathway. If MAPK cascade is activated by epidermal growth factor receptor (EGFR) receptor, the administration of anti-EGFR therapeutic antibodies results in the cessation of tumor growth. There are instances of EGFR-independent activation of MAPK pathway caused by mutations in the molecules located downstream to EGFR (KRAS, NRAS or BRAF) or by collateral signaling (*e.g.*, HER2 overexpression); these CRCs are resistant to EGFR blockade, however they may be sensitive to appropriate targeted drugs. CRC: Colorectal cancer; EGFR: Epidermal growth factor receptor.

Although this statement is an oversimplification, with many nuances requiring detailed clarification, it generally reflects the framework for the administration of anti-EGFR therapy[3,11-13].

Cetuximab and panitumumab may render a significant improvement in the disease outcomes when applied to *RAS/RAF* wild-type patients in combination with the standard cytotoxic regimens. This is particularly true for left-sided tumors, while right-sided CRCs appear to derive less benefit from EGFR down-regulation[14,15]. Panitumumab and cetuximab may also be utilized as single agents after the failure of chemotherapy. There were attempts to use monotherapy by anti-EGFR antibodies as an upfront treatment to delay the administration of cytotoxic drugs, however, these trials failed to generate promising clinical data[16]. Consequently, the combined use of anti-EGFR and chemotherapy in *RAS/RAF* wild-type patients at the very beginning of systemic intervention is a preferable option. This approach also leaves room for the re-challenge of cetuximab or panitumumab for CRC patients in the third-line treatment, *i.e.,* after the failure of the first-line therapy and subsequent cessation of EGFR down-regulation during the second-line treatment[14,15].

Technical nuances concerning testing for mutations in *KRAS* and *NRAS* oncogenes deserve some discussion. Proper analysis of *RAS* gene status is critical for the fate of the patients: some data indicate that erroneous administration of anti-EGFR antibodies to CRC patients with *RAS* mutations, which are present in tumor tissue but were missed due to deficiency in the laboratory procedures, may lead to the acceleration of tumor growth[17]. Both *KRAS* and *NRAS* need to be screened at least for the presence of mutations in codons 12, 13, 59, 61, 117 and 146[18]. Given that each mentioned codon can be affected by a multitude of various mutations, there is almost an indefinite diversity of substitutions, with some rare variants not detectable by allele-specific PCR kits[19]. Some early clinical trials utilized conventional DNA sequencing for the analysis of exons 2, 3 and 4; however, this method cannot reliably detect mutations when the tumor specimen has a high admixture of normal cells. These drawbacks can be easily overcome by next generation sequencing (NGS), which

applies multiple reads to each analyzed genomic fragment and, therefore, can detect mutated gene copies even in the presence of a high amount of normal DNA[20]. NGS remains expensive for the time being and this method is still not easily accessible in all hospitals around the world. NGS usually requires an accumulation of several samples for a single run that may affect turn-around-time for a given assay[21]. Anti-EGFR therapy provides remarkable improvement of the overall survival when given to left-sided *KRAS/NRAS* wild-type cancers as first-line therapy[14,15] therefore it is important to know the *RAS* status of CRC patients already at the time of initial treatment planning.

The "classical" model of CRC development, which was described by Fearon and Vogelstein, suggested that the emergence of *RAS* mutations provides an advantage to the evolving tumor clone, so the newly appeared *RAS*-mutated tumor subclones, in theory, should rapidly outperform their *RAS*-wild-type predecessors and entirely repopulate the tumor mass[22]. The molecular genetic portrait of the majority of CRCs appears to support this concept. However, there are notable exceptions. Some CRCs have *RAS* wild-type status when analyzed as a gross tumor mass, however, the use of ultrasensitive methods often allows to reveal a minor fraction of *RAS*-mutated cells in these carcinomas. These tumors appear to benefit from anti-EGFR therapy, as the majority of malignant cells forming the cancer lump remain vulnerable to EGFR inhibition[23,24]. Consequently, the mere detection of mutated *RAS* clones, as achieved by allele-specific PCR, may not be sufficient to guide the choice of CRC therapy; indeed, the quantitation of the proportion of mutated *RAS* clones is likely to be essential for proper therapeutic decisions[25,26]. Furthermore, when presumably *RAS* wild-type tumors undergo anti-EGFR therapy, the acquired resistance to EGFR down-regulation is often attributed to the emergence of *RAS* mutations. Interestingly, tumors exposed to anti-EGFR therapy demonstrate inhibition of DNA repair pathways, which results in the accelerated accumulation of mutations, and, consequently, increased probability of acquiring activating substitutions in *RAS* oncogenes[27]. The proportion of newly developed *RAS*-mutated clones often decreases after cessation of the anti-EGFR therapy, thus explaining the efficacy of cetuximab or panitumumab re-challenge[14,15]. In aggregate, these data suggest that the concept of the "evolutionary" advantage of *RAS* mutations is an oversimplification of the natural history of CRC development. Instead, some tumors demonstrate an example of an "ecosystem", where *RAS*-mutated clones co-exist with *RAS*-wild-type malignant cells. The co-existence of cells with different status of driver mutations has been demonstrated for other tumor types; there are sound arguments suggesting that this intratumoral heterogeneity is not a result of the random expansion of distinct malignant clones, but a built-in biological property warranting tumor plasticity and adaptation to external hazards[28,29].

BRAF mutations

Approximately 5%-10% of CRCs contain activating V600E mutation in the *BRAF* oncogene. BRAF kinase is located within the MAPK signalling cascade; therefore, its mutation-driven up-regulation is very likely to render tumor independence from EGFR inhibition, similar to the role of *RAS* mutations. The combined analysis of available clinical trials generally supports this concept[30]. However, there are some clinical observations, which suggest that a subset of *BRAF*-mutated patients may still derive benefit from the addition of EGFR antibodies to some chemotherapy regimens [31]. The mechanistic basis for these observations is unclear. It has to be commented that clinical trials involving CRCs with *BRAF* mutations are prone to biases due to the rarity of this genetic event, and, consequently, a small number of recruited patients[12, 30].

BRAF V600E-mutated tumors have aggressive behavior and poor prognosis. It is advised to start the therapy of these CRCs with intensive chemotherapy in combination with bevacizumab[12,30]. Second-line treatment of *BRAF* V600E-driven CRCs by single-agent BRAF inhibitors turned out to be non-successful, as CRCs adapt to this therapy by activation of EGFR-MAPK bypass pathways[32]. Several studies confirmed the efficacy of the combined use of EGFR and BRAF V600E inhibitors. The addition of a MEK-targeted drug to the above combination did not result in medically relevant improvement of treatment outcomes[33,34]. The doublet therapy composed of cetuximab and encorafenib has recently received FDA approval[30].

Some CRCs carry *BRAF* mutations in codons 594, 596, 597 or 601[35]. Mutations in positions 594 and 596 are not kinase-activating. Substitutions affecting codons 597 or 601 result in BRAF kinase activation[36]. There are instances of clinical responses to vemurafenib in tumors carrying substitutions in the codon 597[37,38]. Cancers with mutations in position 601 cannot be targeted by BRAF V600E-specific inhibitors[39].

The analysis of *BRAF* actionable mutations is not complicated. It can be done by various commercial allele-specific PCR kits or by DNA sequencing.

Microsatellite instability

Some CRCs accumulate an excessive number of small mutations due to deficiency in DNA mismatch repair (MMR). These tumors contain multiple genetic alterations in the repetitive nucleotide sequences (for example, …AAAAAAAAAAAAAA…, or …CACACACACACACACACA…, *etc.*), which are called microsatellites. Consequently, MMR deficiency results in so-called high-level microsatellite instability (MSI-H).

Historically, microsatellite instability was simultaneously reported by several research groups, which applied different criteria for describing this phenotype. Dr. Manuel Perucho, whose group was apparently the first to submit for publication an article describing MSI phenotype, proposed to focus on length alterations in mononucleotide markers, which provided clear-cut discrimination between microsatellite "stable" and "unstable" tumors. Some other reports suggested to include dinucleotide repeats in PCR panels, and these efforts resulted in the development of the so-called consensus "Bethesda panel" consisting of two mono- and three dinucleotide markers (BAT-25, BAT-26, D2S123, D5S346, D17S250)[40-42]. The use of combinations of mononucleotide and dinucleotide markers led to 3-tier classification of CRCs, *i.e.*, microsatellite-stable (MSS), MSI-H (*i.e.*, CRCs containing alterations in the majority of markers analyzed), and MSI-L (MSI-low; *i.e.*, the condition, when only a few analyzed markers are altered)[41]. Some contemporary reports still refer to the "Bethesda panel" [43], although subsequent research revealed, that only the MSI-H but not MSI-L condition has an established clinical significance and that the use of mononucleotide but not dinucleotide markers is strongly preferable for reliable discrimination between MSI-H and MSS tumors. Furthermore, while dinucleotide markers are highly polymorphic and thus require the analysis of normal DNA obtained from the same patient, some quasi-monomorphic mononucleotide microsatellites can be analyzed without comparison to the control DNA sample[44-46].

PCR analysis for MSI-H includes electrophoretic evaluation of the length of analyzed markers, *i.e.*, an additional relatively sophisticated manipulation carried out after PCR amplification. While many other PCR mutation tests only require a PCR machine and commercially available reaction kits, MSI-H testing is not fully compatible with the establishment of a conventional morphological laboratory. Fortunately, this phenotype can also be detected by immunohistochemical (IHC) staining for key proteins involved in mismatch repair. MMR-D phenotype is manifested by the lack of expression of MMR proteins (MLH1, MSH2, MSH6, PMS2). MLH1 forms heterodimers with PMS2, while MSH2 heterodimerizes with MSH6. Inactivation of the *MLH1* gene usually results in concomitant loss of expression of both MLH1 and PMS2; similarly, inactivation of the *MSH2* is accompanied by the lack of staining for both MSH2 and MSH6 proteins. Isolated loss of expression of either MSH6 or PMS2 indicates alterations in the *MSH6* or *PMS2* genes, respectively[47]. The terms "MSI-H" and "MMR-D" are often used interchangeably, although it is more appropriate to utilize "MSI-H" for the analysis of the length of mononucleotide repeats, and "MMR-D" for IHC. It is frequently stated that PCR-driven and IHC analyses for MSI-H/MMR-D usually produce highly concordant results, although it is necessary to keep in mind that both procedures are relatively error-prone and, therefore, require rigorous quality assessment[48,49]. In addition to PCR or IHC, an MSI-H/MMR-D phenotype can be reliably detected by NGS[43].

The discovery of MSI-H was initially viewed mainly as an advance in the fundamental understanding of cancer pathogenesis and clinical application of MSI-H testing was limited to the identification of patients requiring genetic testing for the Lynch syndrome. It was subsequently revealed that MSI-H/MMR-D phenotype is associated with decreased risk of CRC relapse after surgery, the sensitivity of the tumor to the therapy by immune checkpoint inhibitors (ICIs), and, perhaps, a distinct pattern of tumor sensitivity to various cytotoxic drugs. Consequently, MSI-H/MMR-D has nowadays an utmost medical value, therefore, proper utilization of this test is a critical component of CRC management[43,47,50].

MSI-H/MMR-D status is characteristic of two clinically distinct categories of CRC patients. Wide-spread microsatellite instability is an obligatory feature of hereditary CRCs arising in subjects affected by Lynch syndrome. These patients tend to have an extensive family history of colorectal, endometrial and some other cancers and often present with the malignant disease at a relatively young age (< 50 years). MSI-H/MMR-D may also arise due to hypermethylation of the *MLH1* gene promoter; these patients with sporadic (*i.e.*, non-hereditary) CRCs dislaying MSI-H/MMR-D are

usually of elderly age[43,47,50]. Hence, the frequency of MSI-H depends on two factors, *i.e.,* the population-specific contribution of Lynch syndrome-related germ-line mutations in CRC incidence and the average age of CRC patients; the latter parameter is highly influenced by life expectancy observed in a given country. Studies performed in the countries of the Western world usually claim that up to 10%-15% of CRCs are characterized by MSI-H/MMR-D phenotype; these estimates may be substantially lower in some other patient series[51]. Mode of the recruitment of the CRC cases also plays an essential role, as MSI-H/MMR-D is observed at a higher frequency in patients with localized vs. metastatic cancers[43,47,50].

MSI-H/MMR-D phenotype is generally associated with a lower risk of relapse in surgically treated patients. Systematic analysis of multiple studies suggested that stage II MSI-H/MMR-D CRCs do not benefit from adjuvant chemotherapy, therefore, abstinence from postoperative systemic treatment in this category of patients is suggested by CRC clinical guidelines[43,47,50].

MMR-D results in an extensive accumulation of various mutations: tumor mutational burden (TMB) in these CRCs may exceed the number of mutations observed in MSS tumors by two orders of magnitude. High TMB is associated with an increased amount of tumor antigens thus rendering the responsiveness of MSI-H/MMR-D CRCs to immune therapy. There are several clinical trials, which demonstrated the remarkable efficacy of immune therapy for microsatellite unstable CRCs. In particular, pembrolizumab has been approved for the treatment of MSI-H/MMR-D CRC both in a first-line setting and for tumors with prior exposure to cytotoxic agents[52,53]. In addition, treatment of mismatch repair-deficient CRCs after the failure of chemotherapy may rely on nivolumab given alone or in combination with ipilimumab[54].

Combination of BRAF V600E mutation and microsatellite instability

Almost half of *BRAF* V600E-mutated tumors observed in elderly patients have a microsatellite unstable phenotype. Similarly, up to half of sporadic late-onset CRCs, which render the MSI-H phenotype, carry the *BRAF* V600E mutation[55,56]. Consequently, if we consider CRC patients, who were diagnosed with this disease in the second half of their life, especially in their seventies or eighties, there will be a significant enrichment for tumors carrying both the above targets. These tumors are potentially sensitive both to BRAF V600E inhibition and to the immune checkpoint blockade. For the time being, the experience of treating these tumors is limited to applying either of these options[34,52]. There are several lines of evidence suggesting a cross-talk between EGFR-BRAF-MAPK pathways and immune signalling cascades [57]. Consequently, it is tempting to expect that the combination of EGFR/BRAF and immune checkpoint inhibition will result in a dramatic improvement of the disease outcomes. A trial combining cetuximab, encorafenib and nivolumab in CRCs carrying a combination of *BRAF* V600E and MSI-H is currently underway (NCT04017650).

HER2 overexpression

Up to 5% of *RAS/RAF* mutation-negative CRCs are driven by the activation of *HER2*. It is essential to emphasize that the definition of HER2 up-regulation deserves particular attention. *HER2* was initially studied as a breast cancer gene. In breast cancer, *HER2* amplification is almost always accompanied by gene overexpression; consequently, it was suggested that FISH and IHC tests can be used interchangeably for the management of breast cancer patients[58]. The biology of CRC has some differences as compared to breast cancer. It appears that a subset of CRCs carries *HER2* extra copies, which do not result in the increased production of the HER2 protein. This "non-productive" *HER2* amplification is not mutually exclusive with other activating genetic events in the MAPK pathway and is not associated with clinical benefit from HER2 down-regulation[59].

"Genuine" *HER2* activation, which is usually manifested by a combination of *HER2* amplification and overexpression, is associated with the lack of tumor responsiveness to anti-EGFR therapy. There are several successful clinical studies utilizing rigorous HER2 inhibition achieved by the combination of two HER2-targeted agents. For example, Sartore-Bianchi *et al*[60] demonstrated high efficacy of the combination of trastuzumab and lapatinib in *HER2*-driven cancers. Similarly, Meric-Bernstam *et al*[59] showed the utility of pertuzumab plus trastuzumab combination. There are a number of ongoing clinical trials involving various HER2-targeted therapeutic regimens[61,62].

Emerging predictive markers and therapeutic approaches

More than half of CRCs carry mutations in *KRAS* or *NRAS* oncogenes. These patients

are excluded from the anti-EGFR treatment and therefore have limited treatment options. The development of specific inhibitors of mutated RAS proteins is highly complicated due to their small size, limited conformational change upon mutational activation and high affinity to their enzymatic substrate, *i.e.,* GTP. For the time being, only the inhibitors of *KRAS* carrying glycine-to-cysteine substitution in codon 12 (*KRAS* G12C) succeeded to enter late phases of clinical development. A *KRAS* G12C mutation occurs in approximately 15% of non-small cell lung cancers, mainly in smokers[63], but is relatively rare in CRCs: it accounts for approximately 1 out of 12 *KRAS* mutations, and its frequency in unselected metastatic CRCs is about 4%[64]. A recent phase I trial involving *KRAS* G12C inhibitor sotorasib (AMG 510) included 42 CRC patients; 3 (7%) subjects experienced partial response and 28 (67%) cases demonstrated the disease stabilization[65]. Preclinical studies suggest that combined inactivation of *KRAS* G12C and EGFR may prevent CRC escape from a single-agent KRAS inhibition[66].

Mutation-driven activation of RAS results in the upregulation of MEK kinase. However, MEK kinase inhibitors do not show significant clinical efficacy in RAS-driven tumors. Some data indicate that tumor escape for MEK down-regulation may be attributed to autophagy. Preclinical experiments and some case reports involving KRAS-mutated pancreatic cancer patients suggest that the addition of an autophagy inhibitor, hydroxychloroquine (Plaquenil), may augment the efficacy of MEK antagonists[67,68]. There is a report describing the response of CRC carrying the *KRAS* G12D mutation to the combination of binimetinib, hydroxychloroquine, and bevacizumab[69]. This approach may have significant implications, given that almost a million people worldwide develop RAS-mutated CRC every year.

Microsatellite instability caused by the inactivation of mismatch repair genes is the most frequent cause of excessive tumor mutation burden, and, consequently, sensitivity to immune therapy. There are other varieties of CRC driven by the inactivation of DNA repair. For example, *MUTYH*-associated CRC is a rare example of a recessive hereditary cancer syndrome, which is characterized by a deficiency in base excision repair. CRCs with biallelic inactivation of the *MUTYH* gene accumulate a huge amount of G:C > T:A transversions. These tumors are responsive to immune checkpoint blockade[64]. Biallelic carriers of pathogenic *MUTYH* alleles often develop CRC *via* the acquisition of the KRAS G12C substitution. Given the existence of recurrent *MUTYH* variants, it is advisable to screen individuals with *KRAS* G12C mutated CRC for the presence of *MUTYH* germ-line mutations[64,70].

POLE-associated CRC is another example of rare CRC variety with ultra-high mutational load. POLE (polymerase epsilon) is a DNA polymerase with proofreading activity. Mutations in the exonuclease domain of the *POLE* gene result in an excessive number of errors occurring during DNA replication. There are cancers arising due to inheritance of a *POLE* pathogenic allele as well as instances of somatic inactivation of the *POLE* gene in sporadic CRCs and other tumor types. POLE deficiency is associated with tumor response to inhibitors of immune checkpoints[71,72].

Actionable rearrangements involving receptor tyrosine kinases are characteristic mainly for non-small cell lung carcinomas and some pediatric tumors[73,74]. Somewhat unexpectedly, instances of gene fusions resulting in the activation of involved kinases have been repeatedly demonstrated in microsatellite-unstable cancers arising due to somatic methylation of the *MLH1* gene promoter[75-77]. There are also rare instances of druggable gene rearrangements in microsatellite stable cancers[75,76]. CRCs carrying activating gene fusions are responsive to the administration of appropriate tyrosine kinase inhibitors[78,79].

HEREDITARY PREDISPOSITION TO CRC

Approximately 3% of CRCs develop due to inherited mutations in *MMR* genes. This condition is called Lynch syndrome and remains the only well-established cause of hereditary non-polyposis CRC (HNPCC). Lynch syndrome is associated with germ-line pathogenic variants affecting *MLH1*, *MSH2*, *MSH6* or *PMS2* genes. The fifth HNPCC gene is *EPCAM*, which is involved in the disease pathogenesis *via* inactivation of the *MSH2* gene. The penetrance of the above-mentioned genes tended to be overestimated in the past because studies of HNPCC were focused on the analysis of large cancer pedigrees. The invention of NGS led to significantly increased accessibility of the mutational testing in the *HNPCC* genes, so the appropriate DNA analysis was applied to unselected CRC cases and healthy controls. It is currently estimated that the individual CRC risk for carriers of germline variants in the *MLH1* and *MSH2* genes

falls within the range of 40%-80%. Inheritance of mutations in the *MSH6* and *PMS2* genes is associated with a 10%-20% probability of developing CRC during the lifetime, which is only 2-4 times higher than among unselected subjects. The contribution of Lynch syndrome in CRC incidence significantly varies between different populations[80-84].

Tumors arising in patients affected by Lynch syndrome always demonstrate MSI if they are causally linked to an inherited mutation in an *MMR* gene. MSI-H/MMR-D testing is now applied to all CRC patients; therefore, this assay serves as a screening test for HNPCC. The analysis of germ-line variants in *MLH1*, *MSH2*, *MSH6*, *PMS2* and *EPCAM* genes requires consideration of both small mutations (protein-truncating variants or pathogenic missense mutations) and so-called large gene rearrangements (LGRs). IHC analysis of selective loss of MMR protein expression was used in the past to guide the identification of the involved gene. Nowadays, testing for Lynch syndrome genes is almost always done by NGS, except for communities with a pronounced founder effect[85,86].

There are multiple instances of familial aggregation of colorectal tumors not displaying a MSI-H/MMR-D phenotype and that are not related to pathogenic variants in *MLH1, MSH2, MSH6, PMS2* or *EPCAM*. Despite intensive research efforts, RPS20 is currently the only gene showing reliable association with HNPCC. Germ-line mutations in the RPS20 are exceptionally rare and their spread is limited to particular ethnic groups[81,84].

There is a number of genes associated with colon polyposis and subsequent development of CRC. Dominant mutations in the *APC* gene are the most recognized cause of this condition. Other genetic causes of polyposis and CRC are relatively rare and include alterations in *MUTYH*, *POLE*, *POLD1*, *NTHL1*, *MSH3*, *STK11*, *SMAD4*, *PTEN*, *GERM1* and some other genes[81-83].

LIQUID BIOPSY FOR CRC

Many cancer patients have detectable tumor-derived DNA in their plasma, probably due to decay of malignant cells and consequent DNA shedding in the bloodstream. The detection of circulating tumor DNA (ctDNA) has multiple potential applications for CRC patients. Some studies suggest that ultrasensitive detection of mutations in genes, which are frequently somatically altered in CRC, may facilitate CRC screening, especially when coupled with the use of other markers[87,88]. ctDNA testing may be used to control the success of surgical tumor resection. It is anticipated that patients who achieved ctDNA clearance after surgery have a good long-term prognosis and do not need adjuvant therapy, while subjects with residual ctDNA in the bloodstream are likely to relapse and require systemic drug exposure to reduce the risk of the disease recurrence[89-91]. Postsurgical monitoring for CRC-specific mutations may support early diagnosis of the tumor relapse[92]. Successful systemic therapy is accompanied by a rapid decline of the ctDNA concentration, while the maintenance of a stable ctDNA level indicates the lack of treatment efficacy[93]. A liquid biopsy allows the detection of secondary mutations, which are associated with acquired tumor resistance to therapy[94]. Clearance of these mutations from blood provides a rationale for re-challenge with the same targeted drug[95]. The various clinical applications of ctDNA analysis have been evaluated in many ongoing trials[96].

CONCLUSION

CRC was the first common cancer type, whose molecular testing became a mandatory component of the therapeutic decisions: indeed, *KRAS* status assessment was incorporated in the drug labels for panitumumab and cetuximab already in the year 2009, *i.e.*, sometime before the integration of *EGFR* mutation analysis into the non-small cell lung cancer management. Nowadays, all CRCs undergo comprehensive genetic analysis for somatic mutations and selected patients are subjected to germ-line DNA testing. Rapid spread of NGS technology is likely to affect attitudes towards CRC screening, diagnosis, treatment and monitoring in the near future.

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