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PERSPECTIVE

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Improving the value of molecular testing: current status and opportunities in colorectal cancer precision medicine

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Colorectal cancer (CRC) is the second leading cause of cancer-related deaths worldwide[1]. Surgical radical resection with adjuvant chemotherapy remains the primary treatment choice for CRC, but the 5-year postoperative survival rate is only approximately 60%, and approximately one-third of patients with CRC experience recurrence within 2 years of surgery[2]. Fortunately, the transformation of high-throughput sequencing has accelerated the development of precision medicine. For example, *KRAS* mutations indicate resistance to anti-epidermal growth factor receptor (*EGFR*)-targeted therapies in CRC[3]. Furthermore, molecular-guided individualized therapy has brought new promise in major clinical areas and challenges, such as novel biomarkers predicting sensitivity and resistance to immunotherapy for microsatellite stable (MSS) CRC. Consequently, identifying more potential targets is imperative to improve the stratification of patients with CRC through molecular testing and to achieve precision treatment of CRC.

In this perspective, on the basis of our previous research and experience, we discuss the current status and future directions of molecular testing-guided targeted and immunological therapies for CRC. We also briefly outline the essential aspects of conducting molecular testing in large cancer centers (**Figure 1**).

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Gene-targeted precision medicine for CRC

Single-gene variation-guided clinical management of CRC

In recent years, with the refinement of precise therapeutic targets, targeted drugs for specific gene variations have substantially advanced from preclinical research to clinical trials. Beyond approved targeted drugs, such as cetuximab and bevacizumab, some emerging targeted drugs have shown promising outcomes in CRC populations with targeted single-gene variations, including *RAS*, *BRAF* mutations, *HER2* amplification, and *RET* and *NTRK* fusion. Molecular testing of these variations has been recommended as standard testing for metastatic CRC (mCRC) in prominent guidelines such as those from the National Comprehensive Cancer Network (NCCN), the European Society for Medical Oncology (ESMO), and the Chinese Society of Clinical Oncology (CSCO)[4-6]. Furthermore, some potential targeted drugs, such as the *KRAS G12C* inhibitor sotorasib (AMG-510), the *KRAS G12D* inhibitor MRTX1133, a pan-*KRAS* inhibitor, and the NTRK inhibitor entrectinib (RXDX-101), may be considered as later-line treatments. Relevant clinical trials are presented in **Table 1**.

Nonetheless, a sizeable patient population does not respond to existing targeted drugs or develops acquired resistance, thus necessitating the investigation of novel viable targets. In our recent study, we have found that *RBM10* mutation is an independent prognostic factor for mCRC and is associated with elevated risk of early recurrence or secondary metastasis; these findings must be confirmed in an extensive cohort[7]. Notably, blockade of upstream single-gene targets affects the activation

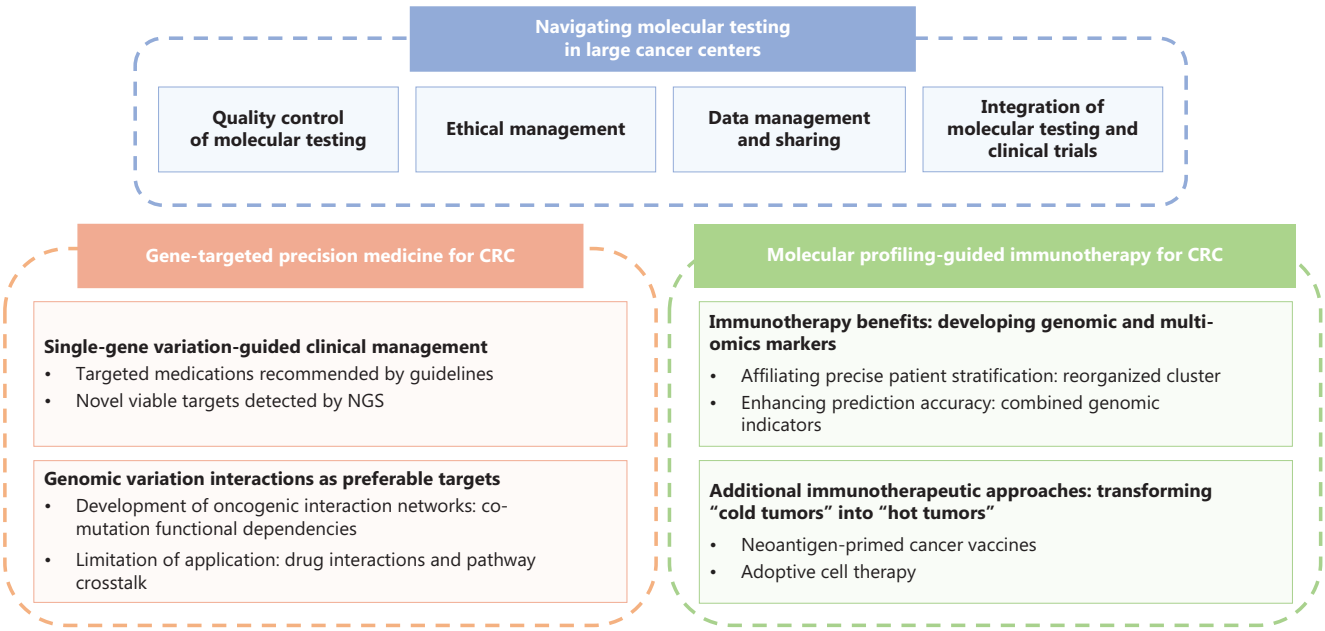

Figure 1 Schematic diagram of the overall structure of this perspective.

of the entire pathway, whereas inhibition of a single gene downstream is associated with potential secondary resistance *via* bypass activation. Thus, the discovery of genomic variants that interact with existing targets may yield new insights into molecular-guided targeted therapies.

Future insights: genomic variation interactions as preferable targets

As previously illustrated, potential effects of genetic variation interactions, co-occurrence (CO) or mutual exclusion (ME), have been overlooked. Several CO gene pairs have been identified as predictors of poor prognosis in CRC. Genomic analysis has revealed that *RAS* or *BRAF* and *TP53*, as well as the tumor suppressor gene *APC* and the oncogene *KRAS*, are typical co-mutations that promote the development of CRC[8,9]. ME is caused primarily by functional redundancy, as exemplified by *KRAS*-*BRAF* in the MAPK pathway and β-catenin regulatory domain (*CTNNB1*)-*APC* in the WNT signaling pathway[10,11]. Despite the discovery of multiple oncogenic dependencies in CRC, their clinical applications remain constrained, because of the lack of inhibitors that target these co-mutated signaling pathways synergistically. Furthermore, Fisher's exact test has been used to identify potential interactions by incorporating all mutations in the gene of interest; however, some of these gene pairings are only statistically significant, but have no pathogenicity or clinical significance associated with genetic interactions.

To identify more clinically significant co-occurring mutations and potential effective targeted treatment regimens, an interaction network model must be developed. In our study, we constructed the first oncogenic-dependent network of CRC by using the innovative SELECT algorithm. This algorithm incorporates all known functional mutations and copy number variation in oncogenes and tumor suppressor genes into the calculation of SELECT scores, thereby accurately quantifying the functional dependency strength of CO or ME gene pairs. Through the network, we discovered that the co-occurrence of oncogenic *KRAS* and the loss of *APC* or *AMER1* resulted in specific aggressive biological behavior and predicted the onset of metastasis. The combination of bevacizumab with first-line chemotherapy did not improve the prognosis of patients with CRC with co-mutations of *KRAS*/*AMER1* and *KRAS*/*APC*. The minimal clinical benefit of chemotherapy-based treatments was found to be due to co-mutations that accelerated the phase I/II metabolism of medications[7]. Hence, therapeutic targeting of co-mutations involving *KRAS* and the WNT pathway, as well as co-mutations involving other pathways, requires further investigation. To address the problem of insufficient effective inhibitors, multiple factors must be considered during regimen development research. These factors include assessing the synergistic or antagonistic effects

Table 1 Main clinical trials evaluating key site-specific targeted drugs in CRC treatment

Trial	Targeted gene variations	Treatment regimen	Target population	Outcomes
Recommended by guidelines				
BEACON	BRAF p.V600E	Encorafenib + cetuximab	mCRC with BRAF p.V600E mutation	mOS 9.3 months, ORR 19.5%
		Encorafenib + cetuximab + binimetinib		mOS 9.3 months, ORR 26.8%
SWOG S1406	BRAF p.V600E	Vemurafenib + cetuximab + irinotecan	mCRC with BRAF p.V600E mutation	ORR 17%, DCR 65%
MyPathway	HER2 amplification	Trastuzumab + pertuzumab	HER2-amplified mCRC	ORR 32%
DESTINY-CRC01	HER2 amplification	Trastuzumab deruxtecan (DS8201)	HER2-positive mCRC, immunohistochemistry (IHC) 3+ or IHC2+ and in-situ hybridization (ISH)-positive	ORR 45.3%
NAVIGATE	NTRK fusion	Larotrectinib	CRC with NTRK gene fusion	mPFS 5.3 months, mOS 33.4 months
CONCUR	Multiple kinases (including VEGF receptors, fibroblast growth factor receptors, platelet-derived growth factor receptors, BRAF, KIT, and RET)	Regorafenib	Refractory progressive mCRC	mOS 8.8 months *vs.* 6.3 months
Potential and ongoing				
Hong 2020	KRAS p.G12C	Sotorasib (AMG-510)	CRC with KRAS p.G12C mutation	ORR 7.1%, DCR 73.8%, mPFS 4.0 months
KRYSTAL-1	KRAS p.G12C	Adagrasib	CRC with KRAS p.G12C mutation	ORR 22%, DCR 87%, mPFS 5.6 months
		Adagrasib + cetuximab		ORR 43%, DCR 100%
NCT05737706	KRAS p.G12D	MRTX1133	CRC with KRAS p.G12D mutation	Status: recruiting
AMPLIFY-201	KRAS G12D, KRAS G12R	ELI-002 2P	CRC with KRAS/NRAS p.G12D or p.G12R mutation	Status: active, not recruiting
NCT04627142	pan-KRAS	BI 1701963	mCRC with confirmed KRAS mutations	Status: terminated
STARTRK-2	NTRK1/2/3	Entrectinib (RXDX-101)	mCRC with NTRK1/2/3-rearrangement (fusion)	Status: active, not recruiting

DCR, disease control rate; mPFS, median progression-free survival; mOS, median overall survival; ORR, objective response rate.

of novel combination regimens, evaluating potential adverse effects after administration of drug combinations, and analyzing the possibility of aberrant activation or inhibition of relevant pathways due to crosstalk.

Molecular profiling-guided immunotherapy for CRC

Immunotherapy benefits: developing genomic markers and omics-based patient stratification

Currently, genomic indicators used for guiding the use of immunotherapeutic agents in patients with CRC focus on microsatellite instability high (MSI-H)/mismatch repair deficiency (dMMR) and MSS/proficient mismatch repair (pMMR). On the basis of promising results from multiple clinical trials, immune checkpoint inhibitors (ICIs) have been approved as first-line or neoadjuvant therapies for unresectable or metastatic CRC with MSI-H/dMMR characteristics. The effective use of ICIs is primarily limited to MSI-H/dMMR CRCs with moderate proportions, which are regarded as "immunologically hot". However, not all MSI-H/dMMR CRCs are suitable for immunotherapy, because the ORRs of immunotherapy guided by MSI-H/dMMR range from 33% to 60%. In contrast, not all MSS/pMMR CRCs, which represent 95% of all CRCs, are "cold tumors" with poor immunotherapy efficacy. Recent clinical trials have demonstrated initially favorable results of the combination of bevacizumab with chemotherapy and immunotherapy in some MSS CRCs (**Table 2**).

Future development of molecular testing-guided immunotherapy will prioritize more precise identification of patients with CRC who stand to truly benefit from immunotherapy. A principal direction involves the integration of transcriptomic and genomic data to accomplish more precise stratification, such as the use of DNA or RNA signatures to score MSI-H or MSS CRC. For example, the most recent AtezoTRIBE trial has identified a novel 27-gene expression signature, DetermaIO, with the potential to predict the effectiveness of atezolizumab plus chemotherapy and bevacizumab in MSS mCRCs. Higher DetermaIO scores correlate with a greater progression-free survival (PFS) benefit from the addition of atezolizumab[12]. Similarly, in cohorts of MSI-H mCRC, analysis of the mutational status of DNA microsatellite-containing genes in epithelial cells and non-epithelial transforming growth factor beta (TGFB)-related desmoplastic RNA markers has been found to predict the PFS associated with ICI-based immunotherapy[13]. Additional transcriptomic information, such as PD-L1 expression, may assist in screening patients with MSS CRC who are suitable candidates for immunotherapy. We anticipate that future clinical studies will validate the prognostic value of these markers.

In contrast, the use of novel predictive biomarkers and the combination of multiple genomic indicators are promising avenues for refining CRC immunotherapy prediction. For example, the burden of insertion-or-deletion alteration (INDEL)-derived neoantigens may serve as a potential biomarker of ICI response. Pan-cancer analysis of The Cancer Genome Atlas (TCGA) data has revealed that INDEL-derived neoantigens are more immunogenic than single-nucleotide variant-derived neoantigens. The presence of INDEL-derived neoantigens is associated with prolonged PFS in patients treated with ICIs in certain cancer cohorts, including melanoma, clear cell renal cell carcinoma, and non-small cell lung cancer[14]. In the context of CRC, our study has revealed that MSI-H CRCs with high tumor INDEL burden (TIB-H) are positively associated with CD8+ T-cell infiltration[7], in agreement with previous findings[15]. Furthermore, we discovered a strong correlation between MSI score and TIB, thus suggesting that the use of MSI-H and TIB-H in combination is a superior biomarker. MSI-H/TIB-H has been confirmed to be a positive marker of increased immune cell infiltration, up-regulated expression of immune checkpoints, and PD-1/PD-L1 co-localization[7]. In addition to MSI-H/TIB-H, MSI-H in combination with *B2M* mutations or tumor-infiltrating lymphocytes may also serve as optimal markers of ICI therapy in CRCs[16,17]. The predictive value of MSI-H in conjunction with *RAS*/*RAF* mutations is expected to be examined in future research.

Additional immunotherapeutic approaches: transforming "cold tumors" into "hot tumors"

Except for conventional immune medications such as PD-1/PD-L1 and CTLA-4 antibodies, emerging immunotherapeutic approaches aimed at transforming "cold tumors" into "hot tumors" may increase the number of patients with CRC who can benefit from immunotherapy, thus providing potential avenues for the future development of CRC immunotherapy.

Neoantigen-primed cancer vaccines are effective strategies for expanding CRC immunotherapy. Research on the Fudan University Shanghai Cancer Center (FUSCC)-CRC cohort has

Table 2 Key clinical trials of ICI-based immunotherapy in MSI-H/dMMR and/or MSS/pMMR CRC cohorts

Trial	Treatment regimen	Target population	Number of patients	Sponsor	Outcomes
Targeting MSI-H/dMMR CRCs					
KEYNOTE-016	Pembrolizumab in chemorefractory patients	MSI-H/dMMR CRC; MSS/pMMR CRC	9 (MSI-H)/41	Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins	ORR of MSI-H CRC 40.0%
KEYNOTE-164	Pembrolizumab in chemorefractory patients	MSI-H/dMMR mCRC with ≥ 1 prior line of therapy	63	Merck Sharp & Dohme LLC	ORR 33%, mPFS 4.1 months
CheckMate-142	Nivolumab plus ipilimumab	MSI-H/dMMR mCRC with no prior treatment	45	Bristol-Myers Squibb	ORR 69%, DCR 84%
KEYNOTE-177	Pembrolizumab as first- line therapy	MSI-H/dMMR CRC	307	Merck Sharp & Dohme LLC	ORR 45.1%, mPFS 16.5 months
NICHE	Ipilimumab plus nivolumab; pMMR group with or without celecoxib	MSI-H/dMMR or MSS/pMMR CRC	40	The Netherlands Cancer Institute	ORR 100% in dMMR CRC, 27% in pMMR CRC
Targeting MSS/pMMR CRCs					
REGONIVO	Regorafenib plus nivolumab	MSS/pMMR CRC with ≥ 2 previous lines of chemotherapy	25	Kohei Shitara, National Cancer Center Hospital East	ORR 36%, mPFS 7.9 months
REGOTORI	Regorafenib plus toripalimab	MSS/pMMR CRC with ≥ 2 previous lines of chemotherapy	42	Second Affiliated Hospital, School of Medicine, Zhejiang University	ORR 15.2%, mPFS 2.1 months, mOS 15.5 months
Fakih 2023	Regorafenib, ipilimumab and nivolumab	MSS/pMMR mCRC	39	City of Hope Medical Center	ORR 27.6%, mPFS 4 months, mOS 20 months
KEYNOTE-651	mFOLFOX7 plus pembrolizumab	MSS/pMMR mCRC with no prior treatment	31	Merck Sharp & Dohme LLC	mPFS 9 months, mOS 29 months
METIMMOX-2	Oxaliplatin plus nivolumab	MSS/pMMR mCRC with no prior treatment	28	University Hospital, Akershus	ORR 32%
BBCAPX	Sintilimab plus bevacizumab and oxaliplatin and capecitabine	RAS-mutant and MSS/pMMR mCRC with no prior treatment	25	Second Affiliated Hospital, School of Medicine, Zhejiang University	ORR 84.0%, DCR 100%, mPFS 18.2 months
NIVACOR	FOLFOXIRI plus bevacizumab and nivolumab	RAS/BRAF-mutant mCRC	52 (MSS)/73	Gruppo Oncologico Italiano di Ricerca Clinica	MSS CRC: ORR 78.9%, DCR 96.2%, mPFS 9.8 months

DCR, disease control rate; mPFS, median progression-free survival; mOS, median overall survival; ORR, objective response rate.

confirmed that higher tumor INDEL burden and less neoantigen depletion promote an immune-active phenotype, and that the potency of the immune response does not depend solely on the quantity of neoantigens but instead depends on their qualities. Therefore, developing vaccines against potential tumor neoantigens that are then infused back into patients to stimulate T cell responses should be beneficial. Several ongoing clinical trials, including national clinical trial (NCT) 03639714, NCT03953235, and NCT04117087, are aimed at validating neoantigen-primed vaccines to boost the immune response in MSS/pMMR CRCs.

Moreover, certain drugs and molecular inhibitors might have an effect on CRC immunotherapy expansion. Temozolomide pharmacologically induces *MSH6* mutations and increases the tumor mutation burden, thereby promoting the immunogenicity of tumor cells and rendering certain MSS/pMMR CRCs susceptible to ICIs[18]. Inhibitors of particular cytokines and chemokines, such as interleukin-2 (IL-2) and chemokine receptor 4 (CXCR4), may also facilitate the transition from "cold" to "hot" by enhancing cytotoxic activity and antigen presentation[19].

Furthermore, adoptive cell therapy, such as chimeric antigen receptor-modified T (CAR-T) cell therapy or tumor-infiltrating lymphocyte therapy, can be effective approaches. Magee et al.[20] have demonstrated that CAR-T cells targeting guanylyl cyclase C (GUCY2C) recognize and destroy GUCY2C-expressing CRC cells and resist lung metastasis.

Navigating molecular testing in large cancer centers

Quality control of molecular testing

Because standard diagnostic markers are adapted to the genetic composition of primarily Western populations, customized tumor diagnostic markers must be developed on the basis of the genetic background of Chinese populations. Moreover, a standard operating procedure must be established to ensure the reproducibility and reliability of molecular testing across laboratories. Sample qualities, reagent qualities, personnel skills, environmental factors, and analysis procedures should be strictly controlled. Laboratories must obtain external quality assessment certifications, such as ISO 15189:2022. Furthermore, continual quality assurance is required throughout the data analysis process, and a cycle of optimization should be established on the basis of clinical feedback. For example, the MSI results obtained *via* next-generation sequencing (NGS) testing should be compared with those detected by polymerase chain reaction, or dMMR results detected by immunohistochemistry. Inconsistencies can be addressed by modifying analysis algorithms and data annotation criteria, thus increasing the reliability of the molecular testing platform. Finally, the entire laboratory testing procedure should undergo risk analysis, management, and control in accordance with ISO 14971: 2019.

Ethical management

Acquiring and managing the genetic information of patients requires strict adherence to ethical principles. All samples must be approved and authorized by institutional ethics committees, and all participants must sign informed consent. Clinical centers must protect genetic information with the utmost discretion. Sharing of data should be limited to basic details useful to scientists, such as the type of cancer as well as patients' age, gender, racial and ethnic background, and when the sample was collected during the course of treatment.

Data management and sharing

The large number of cancer patients in China provides a favorable foundation for the establishment of reliable genomic reference databases. Our team is currently constructing a standardized omics database, FD-Dataportal, by using paired genomic DNA and transcriptomic RNA data derived from clinical tumor samples as standard candidates. We seek to improve the accuracy of clinical annotation matching and drug knowledgebase matching by integrating sequencing data with clinical pathological characteristics and outcomes. We hope that sharing these data will facilitate the future development of innovative diagnostic and therapeutic approaches for precision oncology.

Integration of molecular testing and clinical trials

On the basis of the aforementioned factors, the incorporation of molecular testing and clinical trials has the potential to substantially advance clinical research. Results of molecular testing can identify patients who are candidates for targeted therapy clinical trials. These patients can be promptly enrolled

in the trials, thereby maximizing their likelihood of benefiting from novel interventions. With the accumulation of sample data, molecular characteristics with previously unknown clinical significance may be uncovered and become novel molecular biomarkers or target sites, thereby facilitating future clinical trials.

Conclusions

Overall, molecular testing is critical for the clinical management of CRC: it has enabled personalized treatment to increase efficacy, promoted the development of new drugs, and encouraged the conduct of novel clinical trials. Future efforts should concentrate on the establishment of more precise biomarkers and more comprehensive classification of CRCs according to combinations of multi-omics biomarkers. These efforts may enable a larger population of patients with CRC to derive clinical benefits from precision medicine, and support the realization of precision medicine for CRC.

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Conflict of interest statement

No potential conflicts of interest are disclosed.

Author contributions

Wrote the paper: Haiyun Li, Linwei Guo.
Designed the paper and provided supervision: Chenchen Wang, Ye Xu, Xin Hu.

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