Japanese Society of Medical Oncology Clinical Guidelines: Molecular Testing for Colorectal Cancer Treatment Fifth Edition

Japanese Society of Medical Oncology

Japanese Society of Medical Oncology Clinical Guidelines: Molecular Testing for Colorectal Cancer Treatment, Fifth Edition
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Abbreviations list

ASCO	American Society of Clinical Oncology
APC	Adenomatous polyposis coli
BSC	best supportive care
CAPOX	Capecitabine + oxaliplatin
CDx	companion diagnostics
cfDNA	cell-free DNA
CGP	comprehensive genomic profiling
CHIP	clonal hematopoiesis of indeterminate potential
CI	confidence interval
CIMP	CpG island methylator phenotype
CIN	chromosomal instability
CMS	consensus molecular subtypes
CNAs	copy number alteration
COSMIC	Catalog of Somatic Mutations in Cancer
CRC	Colorectal cancer
ctDNA	circulating tumor DNA
DFS	disease-free survival
dMMR	mismatch repair-deficient
EGF	epidermal growth factors
EGFR	epidermal growth factor receptor
ESMO	European Society for Medical Oncology
FDA	Food and Drug Administration
FFPE	formalin-fixed paraffin-embedded
FGF	fibroblast growth factor
FISH	fluorescence in situ hybridization
FOLFIRI	5-FU + leucovorin + irinotecan
FOLFOX	5-FU + leucovorin + oxaliplatin
FOLFOXIRI	5-FU + leucovorin + oxaliplatin + irinotecan
GCHP	goblet cell-rich type hyperplastic polyp

HMCC	high-methylated colorectal cancer
HNPCC	hereditary non-polyposis colorectal cancer
HP	hyperplastic polyp
HR	hazard ratio
IHC	Immunohistochemistry
ISH	in situ hybridization
IUO	investigational use only
IVDs	in-vitro diagnostics
LDT	laboratory developed test
LMCC	low-methylated colorectal cancer
MAF	mutant allele frequency
MMR	mismatch repair
MRD	minimal residual disease
mRNA	messenger RNA
MSI	microsatellite instability
MSI-H	microsatellite instability-high
MSI-L	microsatellite instability-low
MSS	microsatellite stable
MVHP	microvascular type hyperplastic polyp
NGS	next generation sequencing
NTRK	neurotrophin receptor tyrosine kinase
OR	odds ratio
PCR	polymerase chain reaction
PDGF	platelet-derived growth factors
PFS	progression-free survival
PlGF	placental growth factors
pMMR	mismatch repair-proficient
RFS	relapse-free survival
RR	relative risk
RT-PCR	reverse transcription-polymerase chain reaction
RUO	research use only

SNVs	single nucleotide variation
SSA	sessile serrated adenoma
SSL	sessile serrated lesion
TAT	turnaround time
TMB	tumor mutational burden
TSA	traditional serrated adenoma
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
VUS	variant of unknown significance
WES	whole exome sequencing

Introduction

This guidance originates from the 2008 issue of the "Guidance on measuring *KRAS* gene mutations in patients with colorectal cancer." In 2008, the anti-EGFR antibody cetuximab was approved in Japan. Around this time, reports emerged regarding the involvement of *KRAS* gene mutations in early resistance to anti-EGFR antibodies. There was a need to determine how to incorporate *KRAS* genetic testing, which was not covered by insurance at that time. After the publication of the 1st edition, *KRAS* genetic testing became covered by insurance. Since then, this guidance has been revised whenever new testing methods and evidence emerge, contributing to the range and approval of testing methods.

The purpose of this guidance is to provide clinicians and testing physicians with information on the basic requirements of gene-related testing for colorectal cancer treatment, how to perform the tests currently covered by insurance, and their application for treatment. As the purpose of this report is to provide information on the status and future prospects of new testing techniques, we have also included recommendations for tests that are not currently covered by insurance but have been scientifically validated, and we have also explained their significance. The 5th edition incorporates new evidence that has emerged since the publication of the 4th edition, including the HER2 test, which became covered by insurance in March 2022.

In this revised version of the guidance, basic requirements were established when consistent reports were confirmed from multiple research groups regarding testing for genetic abnormalities related to treatment selection or prognosis prediction in colorectal cancer treatment (Table 1, Figure 1). Each requirement is voted on by committee members, to determine the degree of recommendation (Table 2). The degree of recommendation for each requirement is determined based on the evidence for each test and the balance between the expected benefits and losses that the patient will receive if the test is performed; crucially, these recommendations do not take into consideration the health insurance coverage of each test in Japan. If the voting resulted in a consensus of 70% or more, it was taken as the overall opinion. If a consensus of 70% or more cannot be obtained for all recommendations, the results were announced and voting was conducted again. If the percentage of votes indicating "Strongly recommended" were not 70% or more and if the sum total of votes indicating "Strongly recommended" and "Recommended" was greater than 70%, the degree of recommendation was set to "Recommended." In addition to the main text, information directly related to the basic requirements is described in "comments," and information that is not directly related to the basic requirements but is considered necessary as peripheral information of the basic requirements is described in "side notes." The guidance also describes the status and prospects of the test technology currently under development. Please refer to the remarks column for the definitions of the guidance provided by the Japanese Society of Clinical Oncology and insurance coverage for each test.

Table 1: Basic requirements indicated in this guidance

Basic requirements	Degree of recommendation	Breakdown
RAS mutation testing		
3.2: *RAS* mutation testing is strongly recommended prior to first-line therapy to assess the indications for anti-EGFR antibody in patients with unresectable CRC. (p.26)	Strongly recommended	9 SRs
3.3: *RAS* mutation testing is recommended prior to adjuvant chemotherapy to access the optimal chemotherapy based on the risk of recurrence in patients with resectable CRC. (p.31)	Recommended	2 SRs R 7 people
3.4: Circulating tumor DNA (ctDNA)-based *RAS* mutation testing is strongly recommended to assess the indication for re-administration of anti-EGFR antibody in patients with unresectable CRC. (p.32)	Strongly recommended	8 SRs R 1 person
BRAF mutation testing		
4.2: *BRAF* V600E mutation testing is strongly recommended prior to first-line therapy to predict the prognosis and assess the indication for the combination of BRAF inhibitor and anti-EGFR antibody, with or without MEK inhibitor in patients with unresectable CRC. (p.42)	Strongly recommended	9 SRs
4.3: *BRAF* V600E mutation testing is recommended prior to adjuvant chemotherapy to access the optimal chemotherapy based on the risk of recurrence in patients with resectable CRC. (p.43)	Recommended[1]	6 SRs 3 R
4.4: *BRAF* V600E mutation testing is strongly recommended to help diagnose Lynch syndrome. (p.45)	Strongly recommended	9 SRs
HER2 testing		
5.2: HER2 testing is strongly recommended prior to anti-HER2 therapy to assess the indication of anti-HER2 therapy in patients with unresectable CRC. (p.51)	Strongly recommended[2]	9 SRs
5.3: In HER2 testing for unresectable advanced CRC, IHC testing is strongly recommended first. ISH testing is added in case of IHC 2+. (p.54)	Strongly recommended[3]	9 SRs
Testing for mismatch repair deficiency		
6.2: Mismatch repair (MMR) deficiency testing is strongly recommended	Strongly	9 SRs

prior to first-line therapy to assess the indications for immune checkpoint inhibitors in patients with unresectable CRC. (p.60)	recommended	
6.3: MMR deficiency testing is strongly recommended to assess the optimal chemotherapy based on the risk of recurrence in patients with resectable CRC. (p.63)	Strongly recommended	7 SRs R 2 people
6.4: MMR deficiency testing is strongly recommended to screen for Lynch syndrome (p.66)	Strongly recommended	9 SRs
6.5: The following methods are strongly recommended when assessing for MMR deficiency:		
➢ Microsatellite instability (MSI) testing	Strongly recommended	9 SRs
➢ Immunohistochemistry (IHC) testing	Strongly recommended	9 SRs
➢ Next-generation sequencing (NGS)-based testing (p.69)	Strongly recommended	SR 7 / R 2
Tissue-based comprehensive genomic profiling tests		
7.2: Tissue-based comprehensive genomic profiling (CGP) testing is strongly recommended to assess the indications for molecular targeted drugs in patients with unresectable CRC. (p.82)	Strongly recommended[4]	9 SRs
Liquid biopsy		
8.2: ctDNA-based CGP testing is strongly recommended to assess the indications for molecular targeted drugs in patients with unresectable CRC. (p.95)	Strongly recommended[4]	9 SRs
8.3: Gene panel test detecting minimal residual disease is strongly recommended to assess the optimal adjuvant chemotherapy in patients with CRC having received curative resection. (p.99)	Strongly recommended[5]	8 SRs R 1 person
Specimen handling for molecular testing		
9.1: Formalin-fixed paraffin-embedded (FFPE) tissue is suitable for genetic testing of somatic mutations in cancers. It is able to assess whether samples have sufficient amount of tumor cells by examining histologic findings using matched hematoxylin and eosin-stained slides. Selection of FFPE samples, decision on the	Strongly recommended	9 SRs

need for macrodissection, and assessment of tumor cellularity should be performed by a pathologist. (p.106)		
9.2: In performing circulating tumor DNA testing, the manufacturer's instructions concerning the use of a collection tube and plasma preparation procedure should be followed. (p.109)	Strongly recommended	9 SRs
Quality assurance requirements for testing		
10: Genetic testing for CRC treatment should be carried out under a quality assurance system. (p.115)	Strongly recommended	9 SRs

CRC, colorectal cancer; EGFR, epidermal growth factor receptor; ctDNA, circulating tumor DNA; IHC, immunohistochemistry; ISH, in-situ hybridization; MMR, mismatch repair; MSI, Microsatellite instability; NGS, next-generation sequencing; CGP, comprehensive genomic profiling; FFPE, Formalin-fixed paraffin-embedded

SR: Strongly recommended, R: Recommended

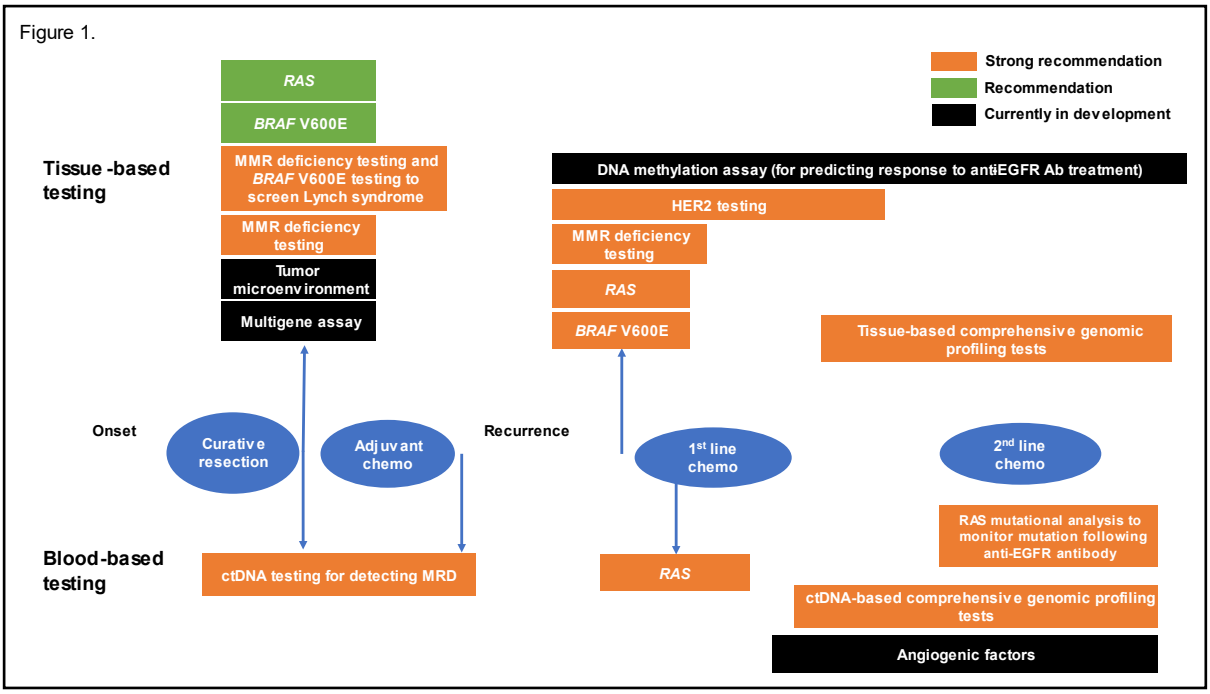
[1]Designated as "Recommended" because the total of "Strongly recommended" and "Recommended" votes comprised 70% or more of voters. "Strongly recommended" votes alone did not surpass 70%.

[2]As of January 1, 2023, trastuzumab + pertuzumab therapy, which is approved for patients with unresectable, advanced, recurrent HER2-positive colorectal cancer, has shown efficacy only in *RAS* wild-type cases.

[3]Trastuzumab + pertuzumab therapy is "IHC 3+ or ISH positive." However, from the perspectives of HER2-positive frequency, international uniform criteria for HER2 diagnosis, the view of the Japanese Society of Pathology "Working Group for Solid Tumor HER2 Testing Guidance," and the efficacy of trastuzumab + pertuzumab therapy, it was recommended that the IHC test should be performed first, and that the ISH test be performed in cases judged to be 2+.

[4]The current comprehensive genome profiling test is defined as "patients with solid cancer for whom there is no standard treatment, or patients with solid cancer for whom standard treatment has been completed due to local progression or metastasis (including those who are expected to complete)."

[5]As of January 1, 2023, in patients with resectable, advanced, recurrent colorectal cancer, no panel test for detecting minimal residual tumor is approved and covered by insurance, for the purpose of selecting treatment according to the risk of recurrence. However, since clinical usefulness has already been demonstrated in prospective phase II studies, etc., it was "strongly recommended."



MMR: mismatch repair, ctDNA: circulating tumor DNA, MRD: minimal residual disease

Figure 1 Test timing

Table 2 Recommendation level and judgment criteria

Degree of recommendation	Criteria for recommendation
Strongly recommended (SR)	Sufficient evidence and benefits outweigh losses, strongly recommended
Recommended (R)	There is a certain amount of evidence, and the course of action is recommended considering the balance between benefits and losses
Expert consensus (ECO)	Evidence and usefulness information are not sufficient, but a certain consensus has been obtained
Not recommended (NR)	No evidence, not recommended

Basic requirements

&

Commentary

1 Recommendations in this guidance and relevance of insurance approval conditions

Table 1 shows the history of approval of gene-related tests for colorectal cancer. As described in the "Introduction," this revised version of the guidance is based on confirmation of consistent reports from multiple research groups regarding testing for genetic abnormalities related to treatment selection or prognosis prediction in colorectal cancer treatment. As a result, it was decided to include all examinations that are covered by insurance as basic requirements. In addition, as of January 1, 2023, ctDNA testing for the detection of minimal residual tumor and recurrence monitoring has not been reimbursed by insurance, but sufficient evidence has already been accumulated to warrant a recommendation and add it to the basic requirements. In addition, the current insurance reimbursement conditions and the best timing of comprehensive genomic profiling testing considered by this guidance do not necessarily match.

In this way, this guidance sets out basic requirements based on scientific evidence, but in actual clinical practice, it is necessary to comply with the insurance reimbursement situation in Japan. To avoid confusion for readers, Table 2 lists the insurance reimbursement status as of January 1, 2023. Please refer to the listed insurance reimbursement conditions when ordering tests.

Table 1 History of approval of gene-related tests for colorectal cancer

Insurance application date	Testing for genetic abnormalities, etc.	Approved content	Description in this guidance
June 2007	MSI test	Screening tests for Lynch syndrome	Chapter 6
April 2010	K-*RAS* genetic testing	Determining the suitability of anti-EGFR antibody drugs	Chapter 3
April 2015	*RAS* (*KRAS*/*NRAS)* genetic testing	Determining the suitability of anti-EGFR antibody drugs	Chapter 3
August 2018	*BRAF* V600E mutation test	Assistance in selection of treatment for unresectable, advanced, recurrent colorectal cancer and diagnosis of Lynch syndrome in colorectal cancer	Chapter 4
December 2018	MSI test	PD-1 antibody drug pembrolizumab (Keytruda®) to patients with locally advanced or metastatic cancer	Chapter 6
June 2019	Comprehensive cancer gene profiling test using tissue samples	Patients with solid tumors for whom there is no standard therapy, or for whom standard therapy has been completed due to local progression or metastasis (including those who are expected to complete), based on the general condition, organ function, etc., based on the guidelines for chemotherapy of related societies. Collectively detect the presence or absence of multiple gene mutations for those who are judged by the attending physician to be highly likely to be eligible for chemotherapy	Chapter 7
August 2020	OncoBEAM ™ *RAS* CRC Kit	Aiming to select a treatment method using antineoplastic agents	Chapter 3
August 2021	MSI test	Selection of PD-1 antibody drug pembrolizumab (Keytruda®) to treat unresectable, advanced, or recurrent microsatellite instability-high (MSI-H) colorectal cancer	Chapter 6
August 2021	Comprehensive genomic profiling test using blood samples	Patients with solid tumors for whom there is no standard therapy, or for whom standard therapy has been completed due to local progression or metastasis (including those who are expected to complete), based on the general condition, organ function, etc., based on the guidelines for chemotherapy of related societies. Collectively detect the presence or absence of multiple gene mutations for those who are judged by the attending physician to be highly likely to be eligible for chemotherapy	Chapter 8
March 2022	Colon cancer HER2 protein (IHC)	Purpose of determining the application of anti-HER2 antibody to patients with locally advanced or metastatic cancer	Chapter 5
	Colon cancer *HER2* gene (FISH)		
October 2022	MMR protein (IHC)	Assistance in determining the indication of pembrolizumab (genetical recombination) in patients with solid tumors Aid in the diagnosis of Lynch syndrome in colorectal cancer Chemotherapy selection aids in colorectal cancer	Chapter 6
January 2023	BRAF V600E mutant	Detection of BRAF V600E mutant protein in cancer	Chapter 4

	protein (IHC)	tissue Aid in the diagnosis of Lynch syndrome in colorectal cancer Chemotherapy selection aids in colorectal cancer	

Table 2 Status of pharmaceutical approval and insurance coverage for various tests as of January 1, 2023

RAS mutation test	・The *RAS* mutation test is covered by insurance for the purpose of assisting treatment selection for colorectal cancer as "*RAS* gene test (2,500 points)" in addition to "D004-2 malignant tumor tissue test." ・As a *RAS* mutation test using blood samples, the OncoBEAM™ *RAS* CRC kit became covered by insurance in August 2020 for the purpose of selecting chemotherapy for colorectal cancer patients. "D006-22 *RAS* gene test (plasma)" is calculated as 7,500 points, which can be calculated only once per patient, but can also be calculated when it is necessary to select a treatment method again. This test is to be performed only when it is difficult to perform the *RAS* gene test or K-*RAS* gene test of "D004-2 Malignant tumor tissue test" using tissue specimens of colorectal cancer.
BRAF V600E mutation test	・The *BRAF* mutation test is applied as a "*BRAF* gene test (2,100 points)" to "D004-2 malignant tumor tissue test" to assist in the selection of chemotherapy (including postoperative adjuvant chemotherapy) for colorectal cancer. In addition, it is covered by insurance to aid in diagnosing Lynch syndrome in colorectal cancer. When used as CDx for encorafenib and binimetinib, 2,500 points will be calculated as "(1) Used to assist drug indication determination" in "D004-2 Malignant tumor tissue examination." ・If *RAS*/*BRAF* mutation tests are performed at the same time, it will be a comprehensive provision of "2 items 4,000 points" of "D004-2 malignant tumor tissue examination." ・As an immunostaining reagent, Ventana OptiView BRAF V600E (VE1) was approved as an in-vitro diagnostic agent and was covered by health insurance in January 2023. 1,600 points can be scored only once per patient.
HER2 test	- HER2 testing by immunohistochemical staining (IHC) and fluorescence in situ hybridization (FISH) is covered by health insurance as an aid in selecting anti-HER2 antibody combination therapy for colorectal cancer. The IHC test can calculate 690 points in "N002 immunostaining (immunoantibody method) histopathological specimen preparation." The FISH method can calculate 2,700 points in "N005 *HER2* gene sample preparation," and if "N002 immunostaining (immunoantibody method) histopathological sample preparation" for IHC test is performed for the same purpose, it totals 3,050 points.
Tests to determine mismatch repair deficiency	・As a test to determine mismatch repair function deficiency, if the microsatellite instability test is performed as "(1) Used to assist drug indication determination" in "D004-2 Malignant tumor tissue test," 2,500 points will be calculated. When performed as a genetic test to assist in the diagnosis of Lynch syndrome, 2,100 points will be calculated as "(2) Others" of the category number "D004-2." The microsatellite instability test, calculated at 2,500 points, only once per patient, is used for the purpose of assisting the diagnosis of Lynch syndrome or for the purpose of selecting a treatment method with antineoplastic agents for solid tumors. Even if the test is carried out for another purpose after the test has been carried out, it can be calculated separately only once. ・Among tests using next-generation sequencing (NGS), comprehensive genomic profiling tests "FoundationOne® CDx Cancer Genomic Profile" and "Guardant360® CDx Cancer Gene Panel" can obtain results of microsatellite instability, and have been approved as a companion diagnostic for determining drug indications. The "OncoGuide™ NCC Oncopanel System" can also obtain microsatellite instability results, but it is not approved under the Pharmaceutical Affairs Law or covered by insurance. However, according to the "Points to note regarding insurance coverage of gene panel testing" issued by the Ministry of Health, Labor, and Welfare, if an expert panel held after a CGP test is deemed appropriate, the drug will be administered

	without another companion test.

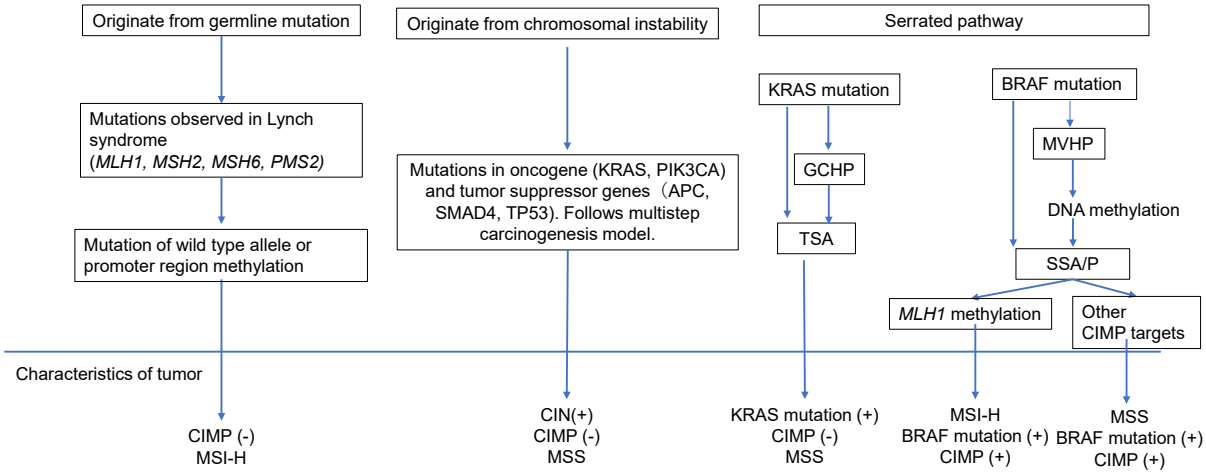
Table 2 continued

	・Ventana OptiView MLH1 (M1), PMS2 (A16-4), MSH2 (G219-1129), and MSH6 (SP93) became available as companion diagnostics in December 2021 as tests to determine mismatch repair function deficiency. They were covered by health insurance in September 2022. According to "N005-3," 2,700 points can be calculated only once per patient as "assistance in determining whether pembrolizumab (genetical recombination) is suitable for solid cancer patients." Even if the test is performed for other purposes, such as "supporting the diagnosis of Lynch syndrome in colorectal cancer" or "supporting the selection of chemotherapy for colorectal cancer," it can be calculated separately only once.
Comprehensive genomic profiling test using tissue samples	・ Comprehensive genomic profiling tests, "OncoGuide™ NCC Oncopanel System" and "FoundationOne® CDx Cancer Genomic Profile" were approved by the Pharmaceutical Affairs in December 2018 and covered by insurance in June 2019. In addition, the "GenMineTOP cancer genome profiling system" received pharmaceutical approval in July 2022. As of January 2023, the GenMineTOP cancer genome profiling system is not covered by health insurance. Initially, it was possible to calculate 8,000 points at the time of test submission, and 48,000 points at the time of explanation to the patient after the results were judged by the expert panel. According to "D006-19 cancer genome profiling test," 44,000 points can be calculated. At the time of explanation to the patient, 12,000 points will be calculated as "B011-5 cancer genome profiling evaluation fee." It can be calculated only once per patient. ・ If "FoundationOne® CDx Cancer Genomic Profile" is used as companion diagnostic for cetuximab, panitumumab, encorafenib, and binimetinib for colorectal cancer, it is possible to calculate 2,500 points for "D004-2 Malignant Tumor Tissue Examination;" in that case, 44,000 points for cancer genome profiling test cannot be calculated. After that, when the results are used as a comprehensive genomic profiling test, it is possible to calculate the score obtained by subtracting the points already requested for the companion test and 12,000 points for the panel test judgment and explanation fee.
Comprehensive genomic profiling test using plasma samples	- The FoundationOne® Liquid CDx cancer genomic profile and Guardant360® CDx cancer gene panel have been approved as comprehensive genomic profiling tests using ctDNA. The Guardant360® CDx Oncogene Panel is not covered by health insurance as of September 2022. Similar to the comprehensive genomic profiling test using tissues, 44,000 points are indicated as "D006-19 Cancer genomic profiling test" at the time of test submission, and "B011-5 Cancer genomic profiling evaluation provided" when explaining the results to the patient after the expert panel has judged the results. A total of 12,000 points can be calculated as a "fee," and can be calculated only once per patient.

2 General remarks

2.1 Molecular background of colorectal cancer

Most colorectal cancers develop in stages due to the accumulation of various abnormalities in multiple genes, and progress to malignant transformation. Genetic abnormalities include genetic changes such as mutations and deletions due to genetic background and environmental factors, as well as epigenetic changes such as expression dysregulation at the transcription level. At present, colorectal cancer is broadly classified into those caused by germline mutations, those caused by chromosomal instability, and those caused by serrated lesions (**Figure 1**)[1].



APC: adenomatous polyposis coli, CIMP: CpG island methylator phenotype, CIN: chromosomal instability, GCHP: goblet cell-rich type hyperplastic polyp, MSI: microsatellite instability, MSS: microsatellite stable, MVHP: microvascular type hyperplastic polyp, TSA: traditional serrated adenoma, SSL: sessile serrated lesion

Figure 1 Presumed pathogenesis of colorectal cancer

Colorectal cancer due to germline mutations includes Lynch syndrome, one of the hereditary colorectal cancers, and microsatellite instability (MSI), in which genetic abnormalities accumulate due to the lack of DNA mismatch repair (MMR) function. MSI is involved in tumor development and progression. Tumors derived from chromosomal instability are carcinogenic through a multistage carcinogenesis model, in which APC mutation occurs when normal mucosa develops into low-grade adenoma, and *KRAS* mutation develops when it becomes high-grade adenoma. It is believed that mutations are associated with the accumulation of mutations in tumor suppressor genes such as TP53. Colorectal serrated lesions are classified into hyperplastic polyp (HP), traditional serrated adenoma (TSA), and sessile serrated lesion (SSL). Serrated lesions have been proposed to cause HP to develop microsatellite instability-high (MSI-H) colorectal cancer via SSL, and HP to develop MSS via TSA. SSL occurs most frequently in the right colon, is associated with high frequency of *BRAF* mutations and CpG island methylator phenotype (CIMP), presents with MSI, and has attracted

particular attention as a precursor lesion of right colon cancer (**Figure 1**). The frequency of colorectal cancer associated with serrated lesions is estimated to be about 5%–10% of all colorectal cancers.

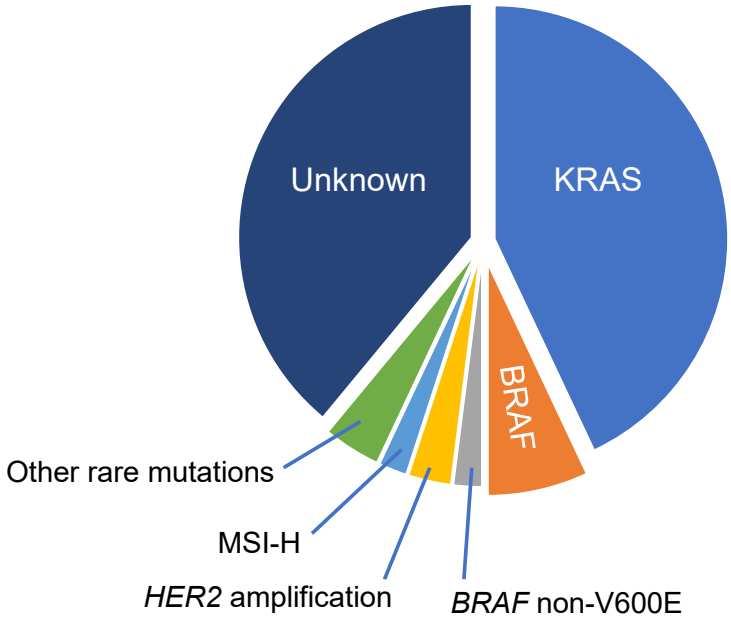
In addition, colorectal cancer is classified into four types based on gene expression profiles (**Table 1**)[2]. Consensus molecular subtypes (CMS) 1 are predominant in females with a right-sided primary colon and have a high proportion of MSI-H and *BRAF* mutations. Although the genetic mutation rate is high, the rate of genomic copy number alteration (CNA) is considered low. CMS2 has a high proportion of CNAs and is characterized by activation of the WNT pathway. CMS3 has a high *KRAS* mutation rate and is characterized by high expression of IGFBP2. CMS4 is characterized by a high CNA and a high percentage of advanced stage cases. In this way, the origin and mechanism of colorectal cancer are correlated with the expression profile and molecular biological characteristics of the formed tumor.

Table 1 Colorectal cancer subtype classification based on expression analysis

	CMS1	CMS2	CMS3	CMS4
	MSI immune	Canonical	Metabolic	Mesenchymal
MSI	MSI-H	MSS	Mixed	MSS
CIMP	High		Low	
Chromosomal abnormality		High	Low	High
Genetic mutation	Many			
Genetic abnormality	*BRAF* mutation		*KRAS* mutation	
Other features	Infiltration of immune cells	Activation of WNT and MYC		Angiogenesis and infiltration into the stroma
Prognosis	Poor prognosis after recurrence			Recurrence-free survival and poor overall survival

MSI: microsatellite instability, CIMP: CpG island methylator phenotype,
CMS: consensus molecular subtypes, MSI-H: microsatellite instability-high, MSS: microsatellite stable

Many colorectal cancers are thought to develop in a multistage carcinogenesis model, and among these, *KRAS* and *BRAF* are thought to be driver gene mutations that play an important role in the development and progression of colorectal cancer. Driver gene abnormalities are mutually exclusive and are rarely detected simultaneously. With the recent introduction of comprehensive genomic profiling tests, other driver genetic abnormalities, such as *HER2* amplification, have been identified, albeit at low frequency (**Figure 2**).



Created by the Guidance Committee based on the frequency of genetic alterations reported in the TCGA pan cancer atlas. Infrequent driver gene abnormalities include *ALK* and *NTRK*.

Figure 2 Driver gene mutations and their frequency in colorectal cancer

2.2 Clinical significance of genetic abnormalities observed in colorectal cancer and advances in genetic testing methods

Genetic abnormalities involved in the development and progression of colorectal cancer can affect the therapeutic effects of drugs used for colorectal cancer diagnosis. For example, by analyses of numerous prospective studies, antibody drugs against epidermal growth factor receptor (EGFR) have been shown to be ineffective when *RAS* (*KRAS/NRAS*) mutations are present (see Chapter 3, *RAS* Mutation Testing). To determine the appropriateness of anti-EGFR antibody drugs, *KRAS* genetic testing was covered by insurance in April 2010 and *RAS* (*KRAS/NRAS*) genetic testing was covered by insurance in April 2015, and both these tests are widely used in clinical practice. Furthermore, in recent years, drugs that target genetic abnormalities have been developed. Certain treatment options are now determined by the presence of genetic abnormalities. In the 2000s, the direct sequencing method was used to directly evaluate gene mutations. In addition, the advent of next-generation sequencing (NGS) has made it possible to simultaneously assess a large number of genes with high sensitivity, and previously approved tests have been replaced with those using next-generation sequencing.

2.3 Methods used for gene-associated testing for colorectal cancer and their

positioning

Gene-related tests used for diagnosing diseases such as colorectal cancer are mainly classified into two types: in-vitro diagnostics (IVD) and laboratory developed tests (LDT), which are classified as reagents. IVDs are marketed after being approved by the Pharmaceutical Affairs Law, based on the Pharmaceuticals and Medical Devices Act. When the *KRAS* mutation test was first developed, unapproved tests were conducted under medical insurance, but currently, with some exceptions, gene-related tests in the field of cancer are approved as IVDs. In addition to IVDs and unapproved tests under the Pharmaceutical Affairs Law, RUO (research use only) tests can be used for research purposes, without approval as IVDs. In the United States and other countries, there is also a classification called IUO (investigational use only), which is used in clinical trials after its analytical performance has been confirmed.

Among IVDs and some medical devices used for diagnosing diseases and pathological conditions, companion diagnostics (CDx) are products that are used for the purpose of improving the efficacy or safety of specific drugs and are indispensable for the use of such drugs. Therefore, CDxs must be designed and verified to have the ability to correctly analyze the substance to be measured, and their clinical performance must support their clinical usefulness. In other words, it is important that the cut-off is set based on the clinical performance and assumed clinical significance and that it contributes to the clinical performance of the target drug. Tests recommended in this guidance should be performed using analytically validated tests, and this applies to IVDs and some medical devices, including CDxs.

Testing methods using NGS and comprehensive genomic profiling (CGP) have been pharmaceutically approved. These methods simultaneously evaluate various types of abnormalities for many genes including single nucleotide variation (SNV), insertion/deletion (In/Del), copy number alteration (CNA) and chromosomal translocation. **Figure 3** shows the relationship between gene-related tests and representative gene panel tests used in Japan and overseas. Whole-genome sequencing of each individual and whole-exome sequencing, which concentrates and analyses the exons on the genome, are also performed in some cases. **Figure 4** shows the relationship between companion diagnostics and whole-genome sequencing.

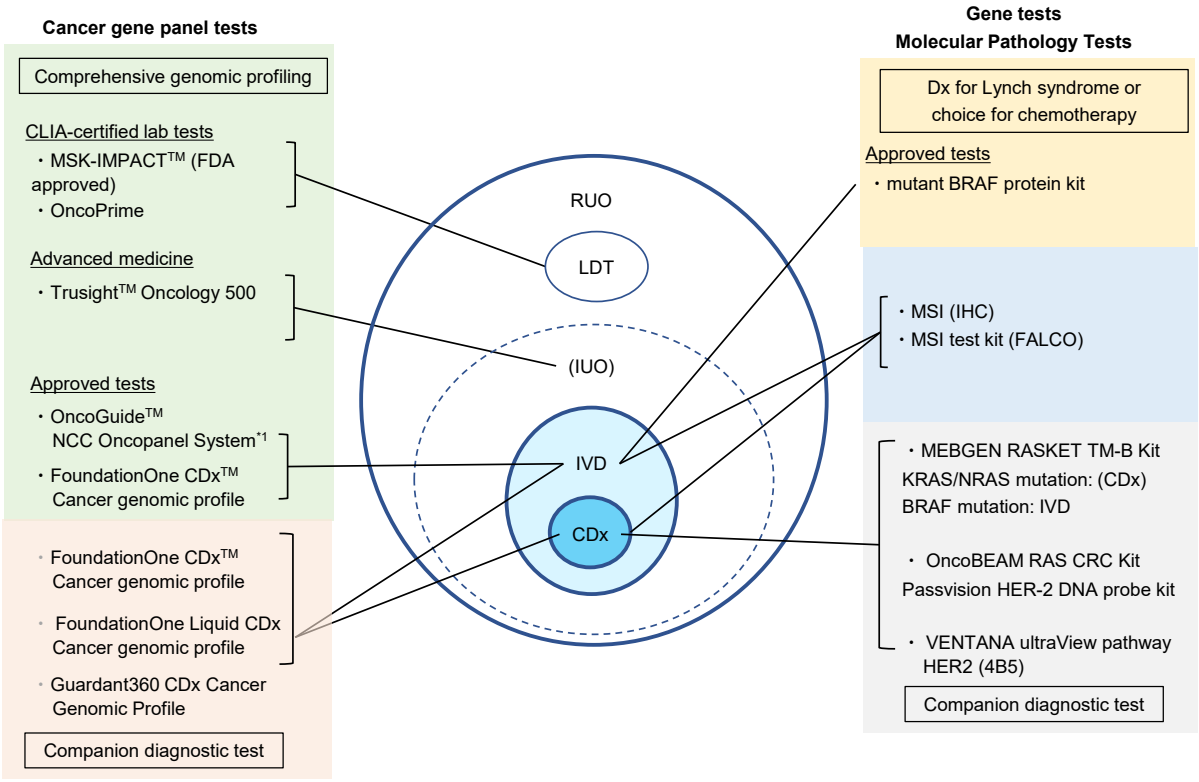


Figure 3 Schematic diagram of gene-related tests and corresponding tests

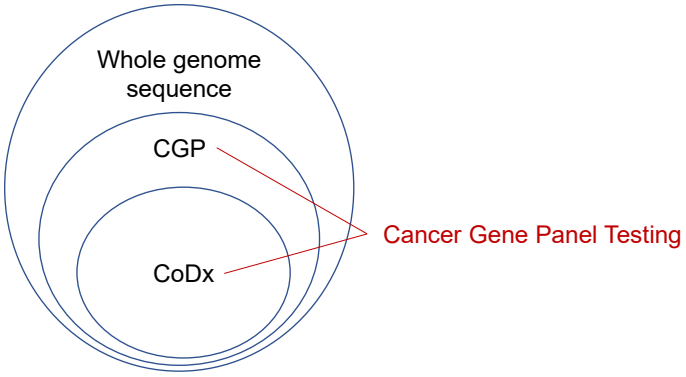


Figure 4 Correlation diagram of tests using NGS

When selecting therapeutic agents based on CGP results, analytical performance is important for clinical usefulness (**Table 2)**. To demonstrate analytical performance, the positive concordance rate and positive predictive value should be calculated for each representative mutation type (SNV, Ins/Dels, CNAs, chromosomal translocations) using control test methods and samples. Accuracy should then be assessed and presented. At present, comparative tests are often conducted with already approved companion diagnostics as controls, but it is quite laborious to conduct performance comparison tests with approved companion diagnostics for each abnormality detected by CGP. Therefore, how to select the appropriate control method

in Japan may become an issue in future. In recent years, there has been a rapid increase in the number of drugs based on genetic driver mutations and the biological background of cancer, making it necessary to evaluate many genes before deciding on treatment. Performing CGP for the purpose of treatment selection before starting first-line therapy is more time- and cost-effective than evaluating individual companion diagnostics at each stage of treatment selection. If the gene profile of a tumor is identified by CGP testing in the early stage of treatment, there is concern about changes in the profile due to subsequent treatment modifications. However, it is possible to administer efficient treatment while considering prognosis and candidate drugs that can be used in the future.

Table 2 Differences between companion diagnostics and comprehensive gene profiling tests

	CDx	Comprehensive genetic profiling test
Possible treatment	Treatment methods established by evidence	In principle, there is no standard treatment, and treatment with a low evidence level is assumed
Positioning of output test results	Direct indication of approved drug indications	Physicians interpret results based on output results and formulate treatment policies
Assumed use facility	Each medical institution	Cancer genome medicine core bases/center hospitals with expert panels and cancer genome medicine collaborative hospitals
Parameters to demonstrate clinical usefulness	Diagnostic accuracy	Analytical performance (accuracy, reproducibility, etc.) as a measuring instrument assuming comprehensive profile test

[References]

1) Leggett B, Whitehall V: Role of the serrated pathway in colorectal cancer pathogenesis. Gastroenterology 138: 2088-100, 2010

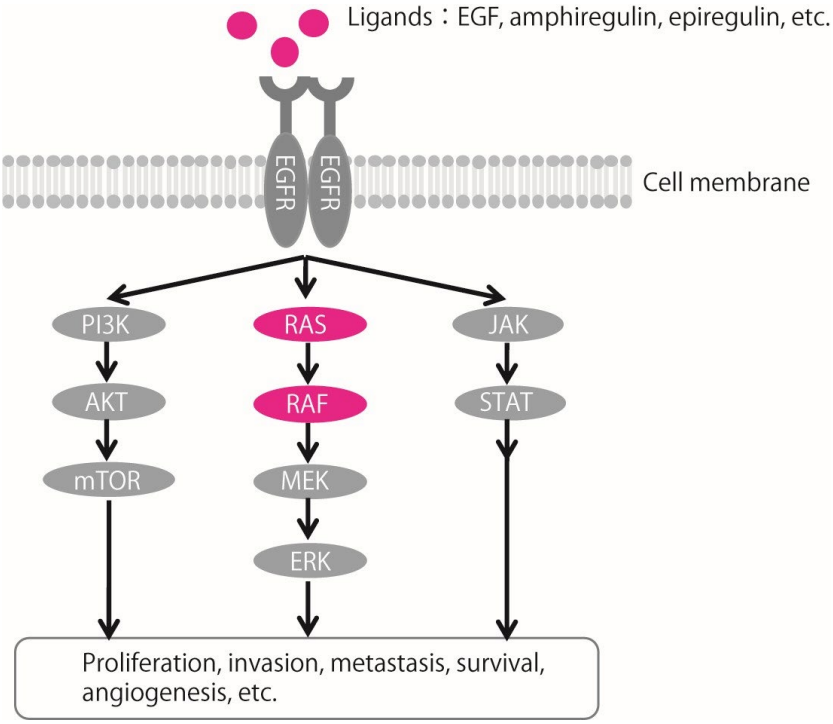
2) Guinney J, Dienstmann R, Wang X, et al: The consensus molecular subtypes of colorectal cancer. Nat Med 21: 1350-6, 2015

3 *RAS* mutation test

3.1 Background

Colon cancer and the EGFR pathway

EGFR, also called HER1, erbB1, is a 170 kDa transmembrane glycoprotein receptor tyrosine kinase, highly expressed in about 80% of colorectal cancers. When a ligand such as epidermal growth factor (EGF), amphiregulin, or epiregulin binds to EGFR from outside the cell, it forms a dimer with EGFR or other HER family molecules, and autophosphorylates the intracellular tyrosine kinase domain. It is activated through oxidation and transmits signals downstream. Downstream signaling pathways include the RAS/RAF (MAPK) pathway, PI3K/AKT/mTOR pathway, and JAK/STAT pathway. While these EGFR pathways play important roles in cell differentiation, proliferation, and maintenance in normal tissues, they are involved in cancer growth, invasion, metastasis, survival, angiogenesis, etc. due to their hyperfunction in colon cancer tissues (**Figure 1**).



EGFR activates the downstream PI3K/AKT/mTOR, RAS/RAF, and JAK/STAT pathways upon ligand stimulation, and is involved in the survival and proliferation of cancer cells. The anti-EGFR antibody drugs cetuximab and panitumumab are mouse/human chimeric IgG1 subclass monoclonal antibody drugs and fully human IgG2 subclass monoclonal antibody drugs against EGFR, respectively. Inhibition of binding results in cell growth inhibition. In Japan, cetuximab was covered by health insurance in 2008 and panitumumab in 2010. However, in cancer cells with gain-of-function mutations in *RAS and RAF,* which are downstream of the EGFR signaling pathway, each mutant protein constitutively activates the MEK-ERK pathway regardless of the presence or absence of stimulation from EGFR. To

maintain cell survival and proliferation, it becomes resistant to anti-EGFR antibody drugs.

Figure 1 Colorectal cancer and EGFR signaling pathway

RAS mutations in colorectal cancer

RAS point mutations are reported to occur early in the development of colorectal cancer and are detected at a constant frequency in all stages of colorectal cancer (**Table 1**). The frequency of *KRAS* exon 2 (codons 12, 13) mutations is approximately 35%–40% of colorectal cancers, and there is no difference between Western and Japanese reports. In *KRAS* exon 2 wild-type, the frequency of *KRAS* exon 3, 4 and *NRAS* exon 2, 3 mutations is approximately 3% and 6% respectively, and the frequency of *NRAS* exon 4 mutations is less than 1%, for a total of approximately 20% (10% of all colorectal cancers) (**Appendix Table 1)**.

Table 1 Frequency of *KRAS* exon 2 mutations by stage

	Dukes' stage	Frequency (%)		Stage	Frequency (%)
Andreyev HJ, et al. (RASCAL) [1] n = 2,721	Dukes' A	33.9	Watanabe T, et al.[2] n = 5,887	Stage I	33.1
	Dukes' B	39.8		Stage II	37.3
	Dukes' C	38.3		Stage III	38.1
	Dukes' D	35.8		Stage IV	37.5

3.2

Basic requirements

RAS mutation testing is strongly recommended prior to first-line therapy to assess the indications for anti-EGFR antibody in patients with unresectable colorectal cancer.

Degree of recommendation

Strongly recommended [SR 9]

Treatment outcomes of anti-EGFR antibody drugs for *RAS* mutation-positive cases

Multiple phase III trials comparing anti-EGFR antibody monotherapy and chemotherapy in combination with standard therapy in patients with unresectable, advanced, recurrent colorectal cancer reported that anti-EGFR antibody drugs did not increase response rate or prolong progression-free survival or overall survival in patients with *KRAS* exon 2 mutations.

After that, in several phase III trials of panitumumab, additional analyses were conducted on the presence or absence of mutations in *KRAS* exons 3 and 4, and *NRAS* exons 2, 3, and 4 and the effect of panitumumab. While panitumumab is expected to be effective in wild-type *RAS*, it is unlikely to significantly benefit cases with mutations in *KRAS* exons 3, or 4 or *NRAS* exons 2, 3, or 4 (**Appendix Tables 2 and 3**). Furthermore, analyses that divided *KRAS* exon 2 mutation-positive cases and other *KRAS*/*NRAS* mutation-positive cases also showed that the additional effect of panitumumab could not be expected. A randomized controlled trial

of cetuximab also showed a tendency for cetuximab to be effective only in wild-type *RAS*.

Thus, patients with mutations in *KRAS* exons 2, 3, 4 and *NRAS* exons 2, 3, 4 are unlikely to benefit from anti-EGFR antibody drugs. This trend was reproducible regardless of the type of anti-EGFR antibody drug, treatment line, and the presence or type of concomitant chemotherapy and was also confirmed by a meta-analysis[3]. As of January 1, 2023, the package inserts for cetuximab and panitumumab include the following precautions regarding efficacy: "Eligible patients should be selected based on a thorough understanding of efficacy and safety."

Colorectal cancer treatment algorithm and *RAS* mutation test

In colorectal cancer, *BRAF* V600E mutation, *PIK3CA* mutations, CpG island methylator phenotype-high (CIMP-high), and microsatellite instability-high (MSI-H) are common on the right colon cancer (cecum, ascending colon, and transverse colon)[4]. However, there is a high frequency of *TP53* mutations on the left colon cancer (descending colon, sigmoid colon, and rectum) [4]. Thus, it has been suggested that the frequency of each gene mutation and the pattern of gene expression differ depending on the site of the primary tumor. Furthermore, in recent years, it has been reported that in *RAS* wild-type colorectal cancer, the prognosis and efficacy of anti-EGFR antibody drugs differ between left-sided and right-sided primary tumors. In an analysis combining data from six large-scale clinical trials (CRYSTAL study, FIRE-3 study, CALGB80405 study, PRIME study, PEAK study, and 20050181 study) investigating the efficacy of anti-EGFR antibody drugs, *RAS* wild-type right colon cancer was worse than left colon cancer in terms of overall survival, progression-free survival, and response rate. Furthermore, while anti-EGFR antibody drugs have a significant additional effect on overall survival and progression-free survival in *RAS* wild-type left colon cancer, anti-EGFR antibody drugs does not show an additional effect in right colon cancer[5]. In a phase III trial (PARADIGM study) conducted in Japan, the prolongation of overall survival was prospectively confirmed in the panitumumab combination group compared with the bevacizumab combination group in the first-line treatment of *RAS* wild-type left colon cancer, while no difference was observed in right colon cancer[6].

According to the "Japanese Society for Cancer of the Colon and Rectum (JSCCR) Guidelines 2022 for the Treatment of Colorectal Cancer" published in January 2022[7] and Pan-Asian adapted ESMO consensus guidelines[8], MSI, *RAS,* and *BRAF* V600E tests are to be performed prior to first-line therapy in patients with unresectable colorectal cancer. If MSI-H is not detected and *RAS/BRAF* are wild-type, standard chemotherapy FOLFOX or FOLFIRI combined with an anti-EGFR antibody drug is recommended for left colon cancer, based on the site of primary tumor occupancy. For right colon cancer, the combination of standard chemotherapy such as FOLFOX or FOLFIRI and FOLFOXIRI with bevacizumab is recommended as the first-line therapy. Therefore, since the first-line treatment regimen depends on the results of *RAS* mutation testing, it is strongly recommended that *RAS* mutation testing should be performed prior to initiation of first-line therapy for patients with unresectable, advanced, recurrent colorectal cancer, to determine the indications for anti-EGFR antibody drugs.

RAS mutation test (**Table 2**)

For the detection of *RAS* mutations, CDxs based on various measurement principles have already been covered by health insurance and are widely used in Japan.

Table 2 Representative companion diagnostics for *RAS mutation testing*

Companion diagnostic	Specimen	Detection limit (%)	Measuring principle
MEBGEN™ RASKET-B Kit[1]	Tumor tissue	1–5	PCR-rSSO method
FoundationOne® CDx[2]	Tumor tissue	2.3	Hybrid capture method
OncoBEAM™ RAS CRC Kit[3]	Plasma	0.03	BEAMing method

[1]*KRAS*/*NRAS* codon 12 (G12S, G12C, G12R, G12D, G12V, and G12A), codon 13 (G13S, G13C, G13R, G13D, G13V, and G13A), codon 59 (A59T and A59G), codon 61 (Q61K, Q61E, Q61L, Q61P, Q61R, and Q61H), codon 117 (K117N), codon 146 (A146T, A146P, and A146V) mutations

[2]In FoundationOne® CDx, the above mutations are returned as companion diagnostics, and other *RAS* mutations are returned as new mutations whose significance has not been established.

[3]*KRAS* codon 12 (G12S, G12C, G12R, G12D, G12V, and G12A), codon 13 (G13D), codon 59 (A59T), codon 61 (Q61L, Q61R, and Q61H), codon 117 (K117N), codon 146 (A146T and A146V) mutation. *NRAS* codon 12 (G12S, G12C, G12R, G12D, G12V, and G12A), codon 13 (G13R, G13D, and G13V), codon 59 (A59T), codon 61 (Q61K, Q61L, Q61R, and Q61H), codon 117 (K117N), codon 146 (A146T) mutation.

PCR-rSSO: PCR-reverse sequence specific oligonucleotide

RAS mutation testing using blood specimens (**Table 3**)

Currently, most genetic mutation diagnoses for cancer are performed using DNA derived from tumor tissue. However, tissue cannot be collected from all cases, and repeated examinations are difficult given the invasive nature of tissue collection. To solve these problems, various techniques have been developed to analyze plasma-derived DNA (circulating tumor DNA; ctDNA) using blood samples. The digital PCR method has been developed as a technique suitable for detecting minute amounts of DNA derived from blood samples, and one of them, the BEAMing method, has shown excellent detection sensitivity of 0.03%. The *RAS* mutation test kit (OncoBEAM™ RAS CRC kit) using the BEAMing method acquired the CE mark in Europe in 2016 (a mark indicating that it meets the safety standards of the member countries of the European Union; EU). Retrospective and prospective clinical performance studies conducted in Europe have shown high concordance with *RAS* mutation testing using tumor tissue[9-13]. A clinical performance test conducted in Japan also showed high concordance between the OncoBEAM™ RAS CRC kit and the BEAMing method for *RAS* mutation testing using tumor tissue[14]. The OncoBEAM™ RAS CRC kit was approved in July 2019 and was covered by health insurance in August 2020 for "Detection of *RAS* (*KRAS* and *NRAS)* gene mutations in genomic DNA extracted from plasma (used to assist in determining the indications of cetuximab (genetical recombination) or panitumumab (genetical recombination) in patients with colorectal cancer)". This test is limited to cases where it is difficult to perform *RAS* genetic testing using colorectal cancer tissue specimens,

for example when tissue specimen is not stored correctly, or it is not suitable for testing due to long-term storage or formalin fixation conditions.

Table 3 Concordance of blood- and tissue-based *RAS* mutation testing

	n	Sensitivity (%)	Specificity (%)	Concordance (%)
Grasselli J, et al.[9]	146	88.9	90.2	89.7
Vidal J, et al.[10]	115	96.4	90.0	93.0
Garcia-Foncillas J, et al.[11]	238	92.6	94.0	93.3
Schmiegel W, et al.[12]	98	90.4	93.5	91.8
Garcia-Foncillas J, et al.[13]	236	86.3	92.4	89.0
Bando H, et al.[14]	280	82.1	90.4	86.4

Comment 1 Limitation of *RAS* Mutation Testing Using Blood Specimens

The amount of ctDNA released into the plasma varies depending on the organ to which the tumor metastasized. In particular, the mutation allele frequency (MAF, ratio of mutant alleles among the total DNA alleles in plasma) detected in cases of lung metastasis alone has been reported to be lower than that in cases of metastasis to other organs[13-15]. An analysis using data from studies conducted in Japan and Europe investigated the concordance of the *RAS* status between the OncoBEAM ™ RAS CRC kit and tissue-based *RAS* mutation testing. In patients with lung metastasis alone, tumors less than 20 mm in longest diameter, and fewer than 10 lesions, the overall concordance rate was 46%, the positive concordance rate was 30%, and the negative concordance rate was 88%[15]. These results suggest that more patients were identified as *RAS* mutation-positive in tissue-based but *RAS* wild-type in plasma-based testing. In patients with peritoneal metastasis alone with tumors less than 20 mm in longest diameter, the overall concordance and positive concordance rate was low[15]. In ctDNA analysis of *RAS*/*BRAF* mutations using the Guardant 360 test, the concordance rate was low in patients with lung metastasis alone or peritoneal metastasis alone, and there were many cases in which the max MAF was below the detection limit[16]. Based on the above findings, in patients with lung or peritoneal metastasis alone, even if *RAS* mutations are detected in the tissue-based testing, blood-based testing may determine to be wild-type (false-negative results). Care must be taken in interpretation of these results.

Side note 1 *KRAS* G12C mutation

The *KRAS* G12C mutation is found in approximately 3% of unresectable, advanced, recurrent colorectal cancers[17] and is a relatively rare mutation among the *RAS* mutations detected in colorectal cancer. *RAS* mutation-positive colorectal cancer has a shorter overall survival period than *RAS* wild-type[18], and *KRAS* G12C mutation cases have been reported to have poor prognosis among *RAS* mutation-positive cases[17]. The *KRAS* G12C selective inhibitor sotorasib was reported to have a response rate of 37.1% in a phase I/II study (CodeBreaK100 study) for unresectable, advanced, recurrent non-small cell lung cancer (NSCLC)[19].

Sotorasib was approved by insurance for *KRAS* G12C mutation-positive NSCLC in April 2022. On the other hand, in the cohort of colorectal cancer in the CodeBreaK100 trial, the response rate was 9.7% (6 of 62 patients), which was lower than the preset expected response rate of 20%[20]. In colorectal cancer, the enhancement of the EGFR signaling pathway is one of the causes of primary resistance to *KRAS* G12C inhibitors[21], and combination therapy with anti-EGFR antibody drugs is being developed. A phase Ib study (CodeBreaK101 subprotocol H) of sotorasib and panitumumab combination therapy for unresectable, advanced, recurrent colorectal cancer with *KRAS* G12C mutation reported a confirmed response rate of 15.4% (4 of 26 patients)[22]. In a phase I/II study (KRYSTAL-1 study) of the *KRAS* G12C selective inhibitors adagrasib and cetuximab, the response rate, including unconfirmed partial responses, was 43% (12/28) with combination therapy, and was 22% (10/45) with adagrasib monotherapy[23]. Randomized controlled trials (CodeBreaK300 study, KRYSTAL-10 study) are currently underway to evaluate the efficacy of combination therapy with a *KRAS* G12C inhibitor and an anti-EGFR antibody for *KRAS* G12C mutation-positive colorectal cancer.

Side note 2 Handling of *RAS* mutations other than *KRAS*/*NRAS* codons 12, 13, 59, 61, 117, and 146

Among *RAS* mutations, mutations in codons 12, 13, 59, 61, 117, and 146, which are hotspots of *KRAS*/*NRAS* mutations, have been shown to be negatively correlated with the therapeutic efficacy of anti-EGFR antibody drugs. On the other hand, comprehensive genomic profiling by next-generation sequencing (NGS) can detect rare *RAS* mutations other than the codons listed above. In two large-scale retrospective studies using tissue specimens from unresectable, advanced, recurrent colorectal cancer (n = 18,270 and n = 9,485, respectively), the frequency of non-hotspot *RAS* mutations ranged from 0.9% to 1.2%. The overall survival in patients with non-hotspot *RAS* mutations was shorter than that in patients with *RAS* wild-type, but was similar to *RAS* hotspot mutations[24,25]. Of the 6 cases with non-hotspot *RAS* mutations for which treatment information was available, 4 cases with mutations that were preclinically known to be activating mutations in the MAPK pathway did not respond to anti-EGFR antibody drugs. Only one patient with a mutation of uncertain significance had a response[24]. Clinical data on the efficacy of anti-EGFR antibody drugs in patients with non-hotspot *RAS* mutations are scarce, and the indication for administration of anti-EGFR antibody drugs cannot be uniformly denied. Therefore, it is appropriate to make a comprehensive judgment by taking into consideration the following: (1) whether the detected mutation is an activating mutation, (2) existing patient reports in which anti-EGFR antibody drugs were administered for the detected *RAS* mutation, (3) side effects of anti-EGFR antibody drugs, and (4) whether there are treatment options other than anti-EGFR antibody drugs.

Side Note 3 Other *RAS* mutation testing methods

In addition to the companion diagnostics listed in **Table 2,** *RAS* mutation tests are being developed. The Idylla™ KRAS Gene Mutation Detection Kit and the Idylla™ BRAF/NRAS Gene Mutation Detection Kit,

which involve real-time PCR using tumor tissue samples, was reported an overall concordance rate of 95.3% with the MEBGEN ™ RASKET-B kit in clinical performance tests[26]. As of January 1, 2023, an application for manufacturing and marketing approval has been filed. In addition, Idylla™ MSI Test, which detects MSI-High by real-time PCR and High-Resolution Melting methods, was approved in August 2022. These tests use a dedicated cartridge and measuring instrument to fully automate the entire process from pretreatment of formalin-fixed paraffin-embedded (FFPE) sections to obtaining results. For this reason, it is possible to perform measurements in the laboratory of one's own facility, which has the advantage of shortening the time required to report test results.

3.3

Basic requirements

RAS mutation testing is recommended prior to adjuvant chemotherapy to access the optimal chemotherapy based on the risk of recurrence in patients with resectable colorectal cancer.

Degree of recommendation

Recommended [SR 2 and R 7]

Clinical significance of *RAS* mutations in patients with resectable, advanced, recurrent colorectal cancer

Two phase III trials were conducted comparing FOLFOX therapy and FOLFOX plus cetuximab therapy as adjuvant setting for stage III colon cancer. However, even in *KRAS* exon 2 wild-type patients, concomitant use of cetuximab did not improve recurrence-free survival or overall survival[27,28]. In addition, a phase III study that examined the effect of adding cetuximab to preoperative and postoperative chemotherapy for patients with resectable synchronous or metachronous liver metastases found no efficacy with cetuximab combination therapy, and actually, progression-free survival tended to be worse in the cetuximab combination group[29]. Based on the above, the efficacy of cetuximab for resectable advanced or recurrent colorectal cancer has not been demonstrated.

Regarding whether *RAS* mutation is a prognostic factor in resectable, advanced, recurrent colorectal cancer, additional analyses of phase III studies on adjuvant therapy for stage II/III colon cancer found no difference in recurrence-free survival or overall survival between patients with and without *KRAS* mutations[30,31]. On the other hand, there have also been reports that the prognosis of *KRAS* mutation-positive cases was significantly worse[32-34] **(Table 4)**. In a meta-analysis of additional analyses of phase III trials on adjuvant therapy for stage II/III colorectal cancer, it was shown that although the results varied from trial to trial, overall, recurrence-free survival and overall survival were significantly shorter in *KRAS* mutation-positive patients (recurrence-free survival; pooled HR 1.36, 95% CI 1.15-1.61, $p < 0.001$, overall survival; pooled HR 1.27, 95% CI 1.03–1.55, $p = 0.03$)[35]. It has also been reported that *KRAS* mutations are associated with recurrence of lung metastasis after resection of stage II/III colon cancer[36]. In addition, it has been reported that *RAS* mutation-positive cases have shorter recurrence-free and overall survival times than *RAS* wild-type cases in cases of

resection of metastatic lesions such as liver metastasis[37]. Thus, there are many reports that *RAS* mutations are a poor prognostic factor in resectable, advanced, recurrent colorectal cancer. Although it does not directly affect the selection of therapeutic drugs at present, it is recommended that *RAS* mutation testing be performed in patients with resectable, advanced, recurrent colorectal cancer as it will serve as a reference for subsequent treatment decisions.

Table 4 Proportion of recurrence in resectable colorectal cancer patients with or without *RAS* mutation

	Stage	*RAS*	N	5-year RFS (%)	HR	5-year OS (%)	HR
CALGB8980 3[30]	III	*KRAS* WT	330	64	0.97 (p = 0.84)	75	0.90 (p = 0.56)
		KRAS MT	178	66		73	
PETACC-3, EORTC40993, SAKK 60-00[31]	II/III	*KRAS*	818	—	1.05 (p = 0.66)	—	1.09 (p = 0.48)
		KRAS MT	481	—		—	
N0147[32]	III	*KRAS*	1,479	77[1]	1.50 (p < 0.0001) 1.46 (p = 0.0035)	—	—
		KRAS MT	779 220	68 (codon 12)[1] 67 (codon 13)[1]		—	
PETACC-8[33]	III	*KRAS* WT	1,019	—	1.56[2] (p <0.001)	—	—
		KRAS MT	638	—		—	

WT: wild-type, MT: mutation-positive, RFS: recurrence-free survival, HR: hazard ratio, OS: overall survival
[1]3-year RFS, [2]HR for time to recurrence

3.4

Basic requirements

Circulating tumor DNA -based *RAS* mutation testing is strongly recommended to assess the indication for re-administration of anti-EGFR antibody in patients with unresectable colorectal cancer.

Degree of recommendation

Strongly recommended [SR 8 and R 1]

Acquired mutation of *RAS* by anti-EGFR antibody drug

After chemotherapy containing anti-EGFR antibody drugs, *RAS* mutations, pathogenic mutations in the extracellular domain of *EGFR*, and activating mutations in the MAPK pathway, which were not observed before treatment, may be detected. These possibly contribute to acquired resistance to anti-EGFR antibody

drugs[38]. It is extremely rare for tumors to change *RAS* mutational status after anti-EGFR-free chemotherapy. On the other hand, *RAS* mutations may be detected after administration of anti-EGFR antibody drugs, and the newly detected *RAS* mutations reflect the result that *RAS* mutant clones became dominant in tumor clone selection by anti-EGFR antibody drugs[40,41]. *RAS* mutations that emerged as acquired resistance have been reported to decay over time as long as anti-EGFR antibody drugs were not administered[42].

RAS mutation testing in determining eligibility for re-administration of anti-EGFR antibody drugs

A treatment strategy in which anti-EGFR antibody drugs are administered again after a certain period of treatment without anti-EGFR antibody drugs for unresectable, advanced, recurrent colorectal cancer that is refractory to anti-EGFR antibody drugs (rechallenge therapy) is being developed. It has been reported that the presence or absence of *RAS* mutations in the ctDNA collected immediately before rechallenge therapy may be a predictor of the efficacy of rechallenge therapy. In patients with no detectable *RAS* mutations in ctDNA before initiation of rechallenge therapy, the response rate to treatment including anti-EGFR antibody drugs in the third or later line setting was ~30.8%(**Table 5**). On the other hand, only 1 patient (5.3%) in the CAVE study[46] who had a *RAS* mutation experienced a response, and no response was reported in other studies (**Table 5**). Similar trends were observed in several retrospective and prospective studies, albeit small, indicating that *RAS* mutations in ctDNA collected before initiation of rechallenge therapy may be a predictor of the efficacy of rechallenge therapy.

Table 5 Therapeutic effect of anti-EGFR antibody rechallenge therapy

	Treatment line	Regimen	Mutation at readministration		n	RR (%)	PFS (M)	HR	OS (M)	HR
CRICKET[43]	3	Cetuximab + Irinotecan	*RAS*/ *BRAF*	WT	13	30.8	4.0	0.44 (p = 0.03)	12.5	0.58 (p = 0.24)
				MT	12	0	1.9		5.2	
E-Rechallenge[44]	≥3	Cetuximab + Irinotecan	*RAS*/ *BRAF*/ *EGFR*	WT	12	25	111 days	—	—	—
				MT	12	0	84 days		—	
JACCRO CC-08/09AR[45]	3/4	Cetuximab / Panitumumab	*RAS*	WT	10	0	4.7	0.16 (p = 0.01)	16.0	0.08 (p = 0.003)
				MT	6	0	2.3		3.8	
CAVE[46]	≥3	Cetuximab + Avelumab	*RAS*/ *BRAF*/ *EGFR*	WT	48	8.3	4.1	0.42 (p = 0.004)	17.3	0.49 (p = 0.02)
				MT	19	5.3	3.0		10.4	
CHRONOS[47]	≥3	Panitumumab	*RAS*/ *BRAF*/	WT	27	30[1]	16 wee	—	55 weeks	—

			EGFR				ks			
PURSUIT[48]	≥3	Panitumumab + Irinotecan	*RAS*	WT	50	14	3.6	—	—	—
NCT03087071[49]	≥3	Panitumumab	*RAS* / *BRAF* / *EGFR* / *MAP2K1*	WT	33	18	4.1	—	10.9	—
		Panitumumab + trametinib		MT	20	0	2.1		5.9	

WT: wild-type, MT: mutation-positive, RR: response rate, PFS: progression-free survival, M: month, HR: hazard ratio, OS: overall survival.
[1]Includes 2 unconfirmed partial responses

These results suggest that *RAS* mutation testing using blood samples is useful for determining the suitability of anti-EGFR antibody rechallenge therapy.

However, the appropriate timing of evaluation of *RAS* mutational status for predicting treatment efficacy to rechallenge therapy have not been clarified yet. Many of the previous reports evaluated *RAS* in specimens collected immediately before the start of rechallenge therapy, while one study has reported that the presence or absence of *RAS* mutations at the time of resistance to the initial anti-EGFR antibody drug may predict the therapeutic effect of subsequent rechallenge therapy[48]. Furthermore, in ctDNA-based *RAS* mutation analysis, the appropriate cut-off value (MAF 0.1%, etc.) for predicting therapeutic efficacy is currently unknown, and it is necessary to wait for the accumulation of data in the future to determine this accurately.

Thus, although there is still room for debate regarding the appropriate timing for evaluating *RAS* mutations and the appropriate cutoff value for MAF, monitoring with *RAS* mutation testing using blood samples is strongly recommended because it may be useful in determining indications for treatment. Multiple trials have reported the clinical usefulness of rechallenge therapy with anti-EGFR antibody drugs, and verification by randomized controlled trials (AIO-KRK-0114 trial and PARERE trial) is currently underway.

Side note 1 Significance of *RAS* mutation testing using blood specimens in *RAS* mutation-positive cases

A phenomenon (NeoRAS) has been reported in which colorectal cancer determined to be *RAS* mutation-positive by examination using tumor tissue or blood specimens transforms to *RAS* wild-type during the course of treatment. A prospective observational study was conducted in which blood samples were collected periodically for unresectable, advanced, recurrent colorectal cancer and analyzed for ctDNA. The study defined *RAS* wild-type (NeoRAS) as cases in which *RAS* mutations disappeared with the course of treatment, and *APC* and *TP53* mutations etc., which had been detected before treatment, were still detected. NeoRAS, defined as reversion from *RAS* mutation to *RA*S wild-type was observed in 2%–8% of *RAS*-mutant colon cancer cases. In this study, baseline MAF values of *RAS* mutations detected in NeoRAS cases tended to be low, suggesting to be subclones[50]. In addition, for patients who were *RAS* mutation-positive in the tissue sample of the primary tumor but *RAS* wild-type in blood samples collected after chemotherapy containing

fluoropyrimidines, cetuximab plus FOLFIRI therapy showed complete response/partial response in 5 out of 9 patients, and the median progression-free survival was 9.0 months (95% CI 4.7–13.3)[51]. Thus, even if the tumor tissue is *RAS* mutation-positive before treatment, monitoring with *RAS* testing using a blood sample may be useful for changing treatment. A clinical trial is currently being conducted on the usefulness of anti-EGFR antibody drugs for NeoRAS cases.

[References]

1) Andreyev HJ, Norman AR, Cunningham D, et al: Kirsten *RAS* mutations in patients with colorectal cancer : the multicenter "*RAS*CAL" study. J Natl Cancer Inst 90:675-84, 1998

2) Watanabe T, Yoshino T, Uetake H, et al: *KRAS* mutational status in Japanese patients with colorectal cancer: results from a nationwide, multicenter, cross-sectional study. Jpn J Clin Oncol 43: 706-12, 2013

3) Sorich MJ, Wiese MD, Rowland A, et al: Extended *RAS* mutations and anti-EGFR monoclonal antibody survival benefit in metastatic colorectal cancer: a meta-analysis of randomized, controlled trials. Ann Oncol 26: 13-21, 2015

4) Loree JM, Pereira AAL, Lam M, et al: Classifying Colorectal Cancer by Tumor Location Rather than Sidedness Highlights a Continuum in Mutation Profiles and Consensus Molecular Subtypes. Clin Cancer Res 24: 1062-72, 2018

5) Arnold D, Lueza B, Douillard JY, et al: Prognostic and predictive value of primary tumor side in patients with *RAS* wild-type metastatic colorectal cancer treated with chemotherapy and EGFR directed antibodies in six randomized trials. Ann Oncol 28:1713-29, 2017

6) Yoshino T, Uetake H, Tsuchihara K, et al: PRADIGM study: A multicenter, randomized, phase III study of mFOLFOX6 plus panitumumab or bevacizumab as first-line treatment in patients with *RAS* (*KRAS*/N*RAS*) wild-type metastatic colorectal cancer. Journal of Clinical Oncology 39 (3_suppl): 85-85, 2021

7) Colorectal Cancer Study Group: Colorectal Cancer Treatment Guidelines 2022 Edition for Doctors. Kanehara Publishing, 2022

8) Yoshino T, Arnold D, Taniguchi H, et al: Pan-Asian adapted ESMO consensus guidelines for the management of patients with metastatic colorectal cancer: a JSMO-ESMO initiative endorsed by CSCO, KACO, MOS, SSO and TOS. Ann Oncol 29: 44-70, 2018

9) Grasselli J, Elez E, Carat- G, et al: Concordance of blood- and tumor-based detection of *RAS* mutations to guide anti-EGFR therapy in metastatic colorectal cancer. Ann Oncol 28: 1294-301, 2017

10) Vidal J, Muinelo L, Dalmases A, et al: Plasma ctDNA *RAS* mutation analysis for the diagnosis and treatment monitoring of metastatic colorectal cancer patients. Ann Oncol 28:1325-32, 2017

11) Garc-a-Foncillas J, Alba E, Aranda E, et al: Incorporating BEAMing technology as a liquid biopsy into clinical practice for the management of colorectal cancer patients: an expert taskforce review. Ann Oncol 28: 2943-9, 2017

12) Schmiegel W, Scott RJ, Dooley S, et al : Blood-based detection of *RAS* mutations to guide anti-EGFR therapy in colorectal cancer patients: concordance of results from circulating tumor DNA and tissue-based *RAS* testing. Mol Oncol 11: 208- 19, 2017

13) Garc-a-Foncillas J, Tabernero J, -lez E, et al: Prospective multicenter real-world *RAS* mutation comparison between OncoBEAM-based liquid biopsy and tissue analysis in metastatic colorectal cancer. Br J Cancer 119: 1464-70, 2018

14) Bando H, Kagawa Y, Kato T, et al: A multicentre, prospective study of plasma circulating tumor DNA test for detecting *RAS* mutation in patients with metastatic colorectal cancer. Br J Cancer 120: 982-6, 2019

15) Kagawa Y, Elez E, Garc-a-Foncillas J, et al: Combined Analysis of Concordance between Liquid and Tumor Tissue Biopsies for *RAS* Mutations in Colorectal Cancer with a Single Metastasis Site: The METABEAM Study. Clin Cancer Res 27: 2515-22, 2021

16) Bando H, Nakamura Y, Taniguchi H, et al: Effects of Metastatic Sites on Circulating Tumor DNA in Patients With Metastatic Colorectal Cancer. JCO Precis Oncol 6: e2100535, 2022

17) Henry JT, Coker O, Chowdhury S, et al: Comprehensive Clinical and Molecular Characterization of *KRAS*G12C-Mutant Colorectal Cancer. JCO Precis Oncol 5: PO.20.00256, 2021

18) Modest DP, Ricard I, Heinemann V, et al: Outcome according to *KRAS*-, N*RAS*- and BRAF-mutation as well as *KRAS* mutation variants: pooled analysis of five randomized trials in metastatic colorectal cancer by the AIO colorectal cancer study group. Ann Oncol 27: 1746-53, 2016

19) Skoulidis F, Li BT, Dy GK, et al: Sotorasib for Lung Cancers with *KRAS* p.G12C Mutation. N Engl J Med 384:2371-81, 2021

20) Fakih MG, Kopetz S, Kuboki Y, et al: Sotorasib for previously treated colorectal cancers with *KRAS*G12C mutation (CodeBreaK100): a prespecified analysis of a single-arm, phase 2 trial. Lancet Oncol 23: 115-24, 2022

21) Amodio V, Yaeger R, Arcella P, et al: EGFR Blockade Reverts Resistance to *KRAS*G12C Inhibition in

Colorectal Cancer. Cancer Discov 10: 1129-39, 2020

22) Fakih M, Falchook GS, Hong DS, et al: 434P-CodeBreaK 101 subprotocol H: Phase Ib study evaluating combination of sotorasib (Soto), a *KRAS*G12C inhibitor, and panitumumab (PMab), an EGFR inhibitor, in advanced *KRAS* p.G12C -mutated colorectal cancer (CRC). Annals of Oncology 32 (suppl_5): S530-S582, 2021

23) Weiss J, Yaeger RD, Johnson ML, et al: LBA6-KRYSTAL-1: Adagrasib (MRTX849) as monotherapy or combined with cetuximab (Cetux) in patients (Pts) with colorectal cancer (CRC) harboring a *KRAS*G12C mutation. Annals of Oncology 32 (suppl_5): S1283-S1346, 2021

24) Pietrantonio F, Yaeger R, Schrock AB, et al: Atypical *RAS* Mutations in Metastatic Colorectal Cancer. JCO Precis Oncol 3:1-11, 2019

25) Loree JM, Wang Y, Syed MA, et al: Clinical and Functional Characterization of Atypical *KRAS*/N*RAS* Mutations in Metastatic Colorectal Cancer. Clin Cancer Res 27:4587-98, 2021

26) Makutani Y, Sakai K, Yamada M, et al: Performance of IdyllaTM *RAS*-BRAF mutation test for formalin-fixed paraffin-embedded tissues of colorectal cancer. Int J Clin Oncol 27:1180-7, 2022

27) Alberts SR, Sargent DJ, Nair S, et al: Effect of oxaliplatin, fluorouracil, and leucovorin with or without cetuximab on survival among patients with resected stage III colon cancer: a randomized trial. JAMA 307: 1383-93, 2012

28) Taieb J, Tabernero J, Mini E, et al; PETACC-8 Study Investigators: Oxaliplatin, fluorouracil, and leucovorin with or without cetuximab in patients with resected stage III colon cancer (PETACC-8): an open-label, randomized phase 3 trial. Lancet Oncol 15: 862-73, 2014

29) Primrose J, Falk S, Finch-Jones M, et al: Systemic chemotherapy with or without cetuximab in patients with resectable colorectal liver metastasis: the New EPOC randomized controlled trial. Lancet Oncol 15: 601-11, 2014

30) Ogino S, Meyerhardt JA, Irahara N, et al; Cancer and Leukemia Group B; North Central Cancer Treatment Group; Canadian Cancer Society Research Institute; Southwest Oncology Group: *KRAS* mutation in stage III colon cancer and clinical outcome following intergroup trial CALGB 89803. Clin Cancer Res 15:7322-9, 2009

31) Roth AD, Tejpar S, Delorenzi M, et al: Prognostic role of *KRAS* and BRAF in stage II and III resected colon cancer: results of the translational study on the PETACC-3, EORTC 40993, SAKK 60-00 trial. J Clin Oncol 28 :466-74, 2010

32) Yoon HH, Tougeron D, Shi Q, et al; Alliance for Clinical Trials in Oncology: *KRAS* codon 12 and 13 mutations in relation to disease-free survival in BRAF-wild-type stage III colon cancers from an adjuvant chemotherapy trial (N0147 alliance). Clin Cancer Res 20:3033-43, 2014

33) Blons H, Emile JF, Le Malicot K, et al: Prognostic value of *KRAS* mutations in stage III colon cancer: post hoc analysis of the PETACC8 phase III trial dataset. Ann Oncol 25: 2378-85, 2014

34) Shida D, Kuchiba A, Shibata T, et al: The genomic landscape and its prognostic significance for stage III colorectal cancer in Japan: An ancillary study of JCOG0910 trial—JCOG1506A1. Journal of Clinical Oncology 37 (15_suppl): 3607-3607, 2019

35) Formica V, Sera F, Cremolini C, et al: *KRAS* and BRAF Mutations in Stage II and III Colon Cancer: A Systematic Review and Meta-Analysis. J Natl Cancer Inst 114: 517-27, 2022

36) Tie J, Lipton L, Desai J, et al: *KRAS* mutation is associated with lung metastasis in patients with curatively resected colorectal cancer. Clin Cancer Res 17:1122-30, 2011

37) Schirripa M, Bergamo F, Cremolini C, et al: BRAF and *RAS* mutations as prognostic factors in metastatic colorectal cancer patients undergoing liver resection. Br J Cancer 112:1921-8, 2015

38) Strickler JH, Loree JM, Ahronian LG, et al: Genomic Landscape of Cell-Free DNA in Patients with Colorectal Cancer. Cancer Discov 8: 164-73, 2018

39) Kawamoto Y, Tsuchihara K, Yoshino T, et al: *KRAS* mutations in primary tumors and post-FOLFOX metastatic lesions in cases of colorectal cancer. Br J Cancer 107: 340-4, 2012

40) Goldberg RM, Montagut C, Wainberg ZA, et al: Optimizing the use of cetuximab in the continuum of care for patients with metastatic colorectal cancer. ESMO Open 3: e000353, 2018

41) Van Emburgh BO, Arena S, Siravegna G, et al: Acquired *RAS* or EGFR mutations and duration of response to EGFR blockade in colorectal cancer. Nat Commun 7: 13665, 2016

42) Parseghian CM, Loree JM, Morris VK, et al: Anti-EGFR-resistant clones decay exponentially after progression: implications for anti-EGFR re-challenge. Ann Oncol 30: 243-9, 2019

43) Cremolini C, Rossini D, Dell'Aquila E, et al: Rechallenge for Patients With *RAS* and BRAF Wild-Type Metastatic Colorectal Cancer With Acquired Resistance to First-line Cetuximab and Irinotecan: A Phase 2 Single-Arm Clinical Trial. JAMA Oncol 5 : 343-50, 2019

44) Osawa H, Shinozaki E, Nakamura M, et al: 5820-Phase II Study of Cetuximab Rechallenge in Patients with *RAS* Wild-Type metastatic Colorectal Cancer: E-Rechallenge Trial. Annals of Oncology 29

(suppl_8): viii150-viii204, 2018

45) Sunakawa Y, Nakamura M, Ishizaki M, et al：*RAS* Mutations in Circulating Tumor DNA and Clinical Outcomes of Rechallenge Treatment With Anti-EGFR Antibodies in Patients With Metastatic Colorectal Cancer. JCO Precis Oncol 4：898-911, 2020

46) Martinelli E, Martini G, Famiglietti V, et al: Cetuximab Rechallenge Plus Avelumab in Pretreated Patients With *RAS* Wild-type Metastatic Colorectal Cancer: The Phase 2 Single-Arm Clinical CAVE Trial. JAMA Oncol 7: 1529-35, 2021

47) Sartore-Bianchi A, Pietrantonio F, Lonardi S, et al: Circulating tumor DNA to guide rechallenge with panitumumab in metastatic colorectal cancer: the phase 2 CHRONOS trial. Nat Med 28: 1612-8, 2022

48) Kagawa Y, Kotani D, Bando H, et al: Plasma *RAS* dynamics and anti-EGFR rechallenge efficacy in patients with *RAS*/BRAF wild-type metastatic colorectal cancer: REMARRY and PURSUIT trials. Journal of Clinical Oncology 40 (16_suppl): 3518- 3518, 2022

49) Megerdichian-Parseghian C, Vilar-Sanchez E, Sun R, et al: Phase 2 study of anti-EGFR rechallenge therapy with panitumumab with or without trametinib in advanced colorectal cancer. Journal of Clinical Oncology 40 (16_suppl): 3520-3520, 2022

50) Henry J, Willis J, Megerdichian-Parseghian C, et al: Neo*RAS*: Incidence of *RAS* reversion from *RAS* mutated to *RAS* wild type. Journal of Clinical Oncology 38 (4_suppl): 180-180, 2020

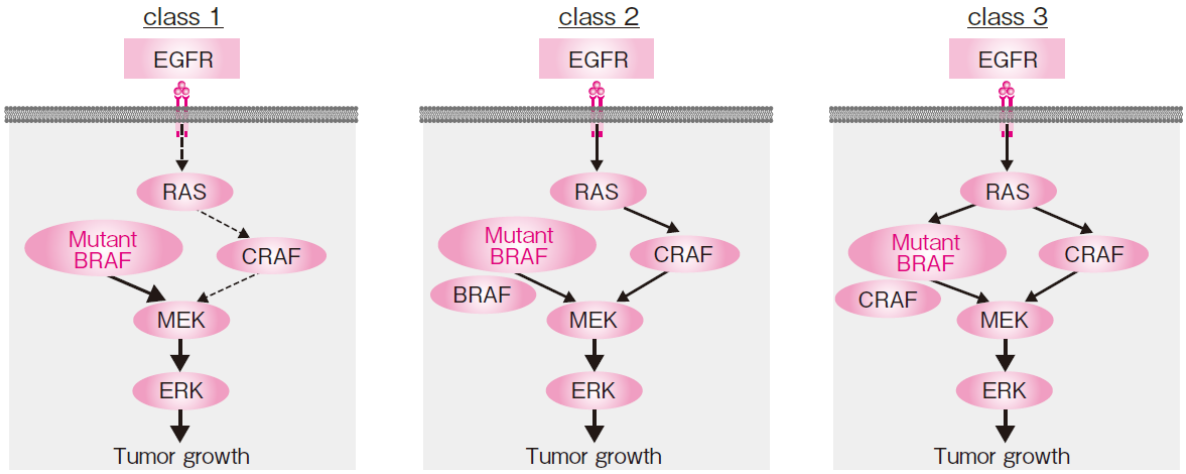
51) Bouchahda M, Saffroy R, Karabou- A, et al: Undetectable *RAS*-Mutant Clones in Plasma: Possible Implication for Anti-EGFR Therapy and Prognosis in Patients With *RAS*-Mutant Metastatic Colorectal Cancer. JCO Precision Oncology 4: 1070-9, 2020

4 *BRAF* mutation tests

4.1 Background

BRAF function and *BRAF* mutations

RAF consists of three isoforms: ARAF, BRAF, and CRAF[1]. The BRAF protein is a serine-threonine kinase of approximately 74 kDa consisting of 766 amino acids. Its signal transduction is carried out from *RAS* activated by receptor tyrosine kinases such as EGFR, and by activating the downstream MEK-ERK pathway, it is involved in cell proliferation and survival (See Chapter 3 *RAS* Mutation Testing/Figure 1)[2]. *BRAF* is located on chromosome 7 and consists of 18 exons. In 2002, it was reported for the first time that *BRAF* mutations were found in human cancers, and they are known to occur frequently in malignant melanoma (43%), thyroid cancer (27%), and biliary tract cancer (14%)[3]. The frequency of *BRAF* mutations in colorectal cancer is 12.4%. The most common *BRAF* mutation involves a thymine to adenine substitution at position 1799 of the *BRAF* gene (c.1799T>A), resulting in a valine to glutamate substitution at amino acid codon 600 (p.Val600GLU;V600E). In recent years, with the spread of next-generation sequencing, mutations other than the V600E mutation (*BRAF* non-V600E mutations) have been reported. Although it has been proposed to classify these mutations into three subtypes (**Figure 1**)[4], in this chapter, only the V600E mutation (class 1) has been described.



In class 1, the kinase activity of the BRAF mutant protein is extremely high, and the monomeric mutant BRAF directly activates downstream signals. However, in class 2, kinase activity is mildly elevated, and downstream signals are activated along with CRAF-mediated signaling pathways. In class 3, the kinase activity is rather reduced, so that it forms a dimer with wild-type BRAF or CRAF, and the dimer is activated by upstream signals to perform signal transduction.

Figure 1 Classification of *BRAF* mutations

Frequency and clinicopathologic features of *BRAF V600E* mutations in colorectal cancer

The *BRAF* V600E mutation reportedly occur in the initial stage of carcinogenesis of colorectal cancer, and its frequency is reported to be slightly higher at 6.9% in Stage IV compared to that in Stages 0–III (around 4%)[5]. This high frequency at advanced stages can be explained by aggressive features of *BRAF* V600E-mutant colorectal cancer. The frequency of *BRAF* V600E mutations has been reported as 4.5%–6.7% in Japanese colorectal cancer patients [6,7], which is slightly lower than that in Western patients (5%–12%). *RAS* mutations and *BRAF* V600E mutations in colorectal cancer are thought to be mutually exclusive.

In addition, *BRAF* V600E mutation cases have different clinicopathological features from those of the wild type. A meta-analysis including 11,955 colorectal cancers from 25 studies reported a high frequency of *BRAF* V600E mutations in those with the following characteristics: female, ≥60 years old, right-sided primary tumor location, poorly differentiated adenocarcinoma, the presence of mucinous component, microsatellite instability-high (MSI-H) tumor (**Table 1**)[8].

Table 1 Frequency of *BRAF* V600E by patient background

Patient background		n	Frequency (%)	Odds ratio
Sex	Male	6,186	8.0	1.71 (1.42-2.07)
	Female	5,489	13.7	
Age	Under 60 years	1,351	6.7	2.29 (1.13-4.61)
	Over 60 years	1,631	18.6	
Primary tumor location	Left colon to rectum	5,806	4.8	4.85 (3.59-6.56)
	Right colon	4,007	21.6	
Stage at diagnosis	I/II	1,806	8.0	1.59 (1.16-2.17)
	III/IV	2,630	11.6	
Tumor differentiation	High to medium differentiation	4,257	8.0	3.89 (2.94-5.17)
	Poorly differentiated	766	25.6	
Mucinous components	Absent	2,134	8.1	2.99 (2.20-4.07)
	Present	392	19.4	
Microsatellite instability	Absent	1,371	9.3	8.18 (5.08-13.17)
	Present	352	38.9	

4.2

Basic requirements

BRAF V600E mutation testing is strongly recommended prior to first-line therapy to predict the prognosis and assess the indication for the combination of BRAF inhibitor and anti-EGFR antibody, with or without MEK inhibitor in patients with unresectable colorectal cancer.

Degree of recommendation

Strongly recommended [SR 9]

Clinical significance of *BRAF* V600E mutation in patients with unresectable, advanced, recurrent colorectal cancer

Patients with *BRAF* V600E mutations have a poorer prognosis than wild-type patients, and a meta-analysis of 26 studies reported an overall survival HR of 2.25 (95% CI 1.82–2.83). A pooled analysis of randomized controlled trials in first-line chemotherapy patients with unresectable, advanced, recurrent colorectal cancer also showed that the survival period of *BRAF* V600E mutation patients was significantly inferior to that of wild-type patients (**Table 2**)[9,10]. The association of worse survival with *BRAF* V600E mutation has also been shown in Japanese patients[6,11].

Table 2 Treatment results of *BRAF* V600E mutation-positive cases (pooled analysis)

		n	PFS (M)	HR	OS (M)	HR
Venderbosch S, et al[9]	*BRAF* WT	2,813	7.7	1.34 (p = 0.001)	17.2	1.91 (p = 0.001)
	BRAF MT	250	6.2		11.4	
Modest DP, et al[10]	*RAS*/*BRAF* WT	664	10.3	2.19 (p <0.001)	26.9	2.99 (p <0.001)
	BRAF MT	74	7.4		11.7	

WT: wild-type, MT: mutation-positive, PFS: progression-free survival, HR: hazard ratio, OS: overall survival, M: months

A subgroup analysis of a phase III study (TRIBE study) comparing FOLFOXIRI + bevacizumab therapy and FOLFIRI + bevacizumab therapy as first-line treatment for colorectal cancer showed that FOLFOXIRI + bevacizumab therapy tended to have a greater survival benefit, especially in patients with *BRAF* V600E mutations[12]. However, a subsequent meta-analysis including the TRIBE trial did not reproduce the superiority of triplet regimen, and no significant difference in survival was observed between FOLFOXIRI + bevacizumab therapy and FOLFOX/FOLFIRI + bevacizumab therapy[13]. Based on the results, the "Japanese Society for Cancer of the Colon and Rectum (JSCCR) guidelines 2022 for the treatment of colorectal cancer" recommended both the triplet regimen such as FOLFOXIRI and the doublet regimens with the same recommendation level[14]. Whereas the benefit of adding anti-EGFR antibody alone to patients with *BRAF* V600E mutations is limited. In fact, in the FIRE-4.5 study, a phase II study comparing FOLFOXIRI + bevacizumab therapy and FOLFOXIRI + cetuximab therapy in patients with *BRAF* V600E mutations, the

bevacizumab arm had a better tendency in both objective response rate and survival than in the cetuximab arm[15]. This finding supported the recommendation of combination of bevacizumab and cytotoxic agents in 1st line setting.

BRAF inhibitors for unresectable, advanced, recurrent colorectal cancer were tested in the BEACON CRC trial. Encorafenib (BRAF inhibitor) + binimetinib (MEK inhibitor) + cetuximab (BEACON triplet) or encorafenib + cetuximab (BEACON doublet) were administered to *BRAF* V600E mutation-positive patients whose disease had progressed after 1 or 2 prior treatments. Compared with a control group, FOLFIRI (or irinotecan) + cetuximab, both the BEACON triplet and BEACON doublet groups showed superiority in the primary endpoint of overall survival. Improvement compared with the control arm was also seen in secondary endpoints such as progression-free survival, and objective response rate (**Table 3**)[16]. Based on these results, the BEACON triplet and the BEACON doublet were approved in Japan in November 2020. Since an exploratory analysis showed no significant difference in survival between the BEACON triplet and the BEACON doublet, Japanese Guidelines recommends the selection of these two regimens depending on patients' condition[14].

Table 3 BEACON CRC trial outcomes[16]

	n	ORR (%)	PFS (M)	HR	OS (M)	HR
FOLFIRI (or irinotecan) + cetuximab	221	2	1.5		5.4	
Encorafenib + cetuximab	220	20	4.2	0.40 (p <0.001)	8.4	0.60 (p <0.001)
Encorafenib + binimetinib + cetuximab	224	26	4.3	0.38 (p <0.001)	9.0	0.52 (p <0.001)

ORR: response rate, PFS: progression-free survival, HR: hazard ratio, OS: overall survival, M: months

Based on the above reported results, confirming the presence or absence of BRAF V600E mutation is useful for prognosis prediction, selection of first-line therapy, and eligibility for cetuximab + encorafenib (+ binimetinib) after second-line therapy. Thus, *BRAF* V600E mutation testing is strongly recommended.

4.3

Basic requirements

BRAF V600E mutation testing is recommended prior to adjuvant chemotherapy to access the optimal chemotherapy based on the risk of recurrence in patients with resectable colorectal cancer.

Degree of recommendation

Recommended [SR 6 and R 3]

Clinical significance of *BRAF* V600E mutation in patients with resectable advanced recurrent colorectal

cancer

In recent years, studies have reported that *BRAF* V600E mutation is a strong prognostic factor even in patients with resectable cases. A meta-analysis of phase III trials of postoperative adjuvant chemotherapy for stage II/III colon cancer showed that the presence of *BRAF* V600E mutation is a risk factor of recurrence with HR of 1.49 (1.31–1.70) and 1.33 (1.00–1.78) for overall survival and disease-free survival and that the HR adjusted for MSI status was 1.67 (1.37–2.04) and 1.59 (1.22–2.07) for overall survival and disease-free survival[17]. In addition, a subgroup analysis from the MOSAIC trial, a phase III study comparing 5-FU/LV (5-FU + leucovorin) therapy and FOLFOX therapy as adjuvant chemotherapy, suggested that the benefit of adding oxaliplatin may differ depending on *BRAF* mutation status (HR for overall survival 0.93 (0.25–1.00) versus 0.66 (0.31–1.42), although, its difference was no statistically significant[18].

In addition, in a prospective observational study, metastatic colorectal cancer patients with *BRAF* V600E mutation who underwent resection and/or local ablative therapy had a significantly worse overall survival with HR of 3.11 (1.49-6.49) than *RAS*/*BRAF* wild-type patients[19]. As for liver metastasectomy, it has been reported that recurrence within 1 year after surgery is extremely high in patients with *BRAF* V600E mutations, and a meta-analysis including reports from Japan have shown significantly worse treatment outcomes[20,21].

A meta-analysis including 24,067 cases with colorectal cancer compared the prognosis difference with four subtypes according to MSI and *BRAF* V600 mutation. In resectable cases, HRs of MSS/*BRAF*-mutated, MSI-H/*BRAF* wild-type, and MSI-H/*BRAF*-mutated were 1.54 (95%CI = 1.16–2.05), 0.51 (95%CI = 0.31–0.83), and 0.54 (95%CI = 0.30–0.94). The significance of the *BRAF* V600E mutation as a prognostic factor differed between patients with MSI-H and those with MSS, and was powerful in those with MSS[22].

Thus, even in resectable cases, the presence of the *BRAF* V600E mutation serve as a very strong prognostic factor, especially in MSS cases. The Japanese "JSCCR guidelines 2022 for the treatment of colorectal cancer" recommends taking recurrence risk into account and selecting either fluoropyrimidine monotherapy or combination therapy with oxaliplatin for adjuvant chemotherapy after curative resection[14]. In addition, *BRAF* mutation is a poor prognostic factor even in patients who underwent metastasectomy. Therefore, it may affect treatment selections such as indications for resection of metastatic lesions and the implementation of adjuvant chemotherapy. Therefore, *BRAF* V600E mutation testing is considered useful and is recommended in patients with resectable colorectal cancer. Also, since the value of prognostic factors for *BRAF* V600E mutations can differ depending on MSI status, simultaneous testing for mismatch-repair deficiency is desirable.

In Japan, the *BRAF* V600E mutation test using the MEBGEN RASKET™ -B kit was launched in April 2020 for the purpose of helping selecting the optimal perioperative chemotherapy for colorectal cancer, and the *BRAF* V600E mutation test using the Ventana OptiView BRAF V600E (VE1) was launched in January 2023. Insurance coverage for resectable colorectal cancer has been expanded for the V600E mutant protein test.

4.4

Basic requirements

BRAF V600E mutation testing is strongly recommended to help diagnose Lynch syndrome.

Degree of recommendation

Strongly recommended [SR 9]

BRAF V600E mutation testing for the exclusion diagnosis of Lynch syndrome

(See Chapter 6, Tests to determine mismatch repair deficiencies for more information on Lynch syndrome and MMR testing.)

The frequency of *BRAF* V600E mutations in colorectal cancer differs greatly between mismatch repair-deficient (dMMR: MSI-H or loss of MMR protein expression on immunohistochemistry) and mismatch repair-proficient (pMMR: MSS, or MMR expression on immunohistochemistry). It was found that *BRAF* V600E mutations were more frequent in dMMR than MSS tumors (38.9% vs. 9.3%, odds ratio 8.18 (5.08–13.2))[8]. While dMMR colorectal cancer in Lynch syndrome is caused by germline mutation, most sporadic dMMR colorectal cancers are considered to be caused by promoter region methylation. For example, acquired aberrant methylation of the promoter region of the *MLH1* gene causes MLH1 loss of expression. *BRAF* V600E mutations were frequently found in sporadic dMMR colorectal cancer. In a review of 35 studies comprising 4,562 cases, the frequency of *BRAF* V600E mutations in colorectal cancers with possible Lynch syndrome was 1.4%, and the frequency of mutations was 63.5% in colorectal cancers with MLH1 loss, which was considered sporadic[23]. Thus, in cases of MSI-H or dMMR tumors, if *BRAF* V600E mutation is observed, Lynch syndrome can be ruled out with high probability. In fact, Western guidelines for Lynch syndrome recommended *BRAF* V600E mutation testing before genomic testing in patients with MSI-H or deficient MMR protein like MLH1. It is considered a cost-effective method of screening for Lynch syndrome because it reduces the number of patients who require genomic testing for a definitive diagnosis of Lynch syndrome[24,25]. The Japanese Guidelines for the Treatment of Hereditary Colorectal Cancer also recommend *BRAF* V600E mutation testing as an option that can be implemented before genomic testing in the case with MSI-H or deficient MLH1 protein expression[26].

Thus, it is strongly recommended to perform *BRAF* V600E mutation testing as an aid in the diagnosis of Lynch syndrome. In Japan, *BRAF* V600E mutation testing has been covered by health insurance since August 2018 as an aid in diagnosing Lynch syndrome. It should be noted that the significance of *BRAF* V600E mutation testing for the exclusion of Lynch syndrome is applicable to only colorectal cancer.

4.5 BRAF mutation assay (**Table 4**)

In Japan, the MEBGEN RASKET™-B kit was approved in December 2017 as an in vitro diagnostics (IVD) that simultaneously detects *RAS* and *BRAF* V600E mutations, and the *BRAF* V600E mutation test for

colorectal cancer was covered by insurance in August 2018. In addition, the FoundationOne® CDx Cancer Genome Profile and OncoGuide™ NCC OncoPanel System, which have been approved as genomic profiling tests, include *BRAF* gene in the analysis targets, and test results can be used. In addition, the FoundationOne® Liquid CDx cancer genome profile and Guardant360® CDx cancer gene panel, which are approved as genomic profiling tests using blood samples, are also capable of detecting *BRAF* mutations. However, a previous study revealed that the sensitivity is inferior to tests using tumor tissue, with a sensitivity of 0.71 (0.62–0.78) and a specificity of 0.99 (0.98–0.99), although older studies are included and the analysis methods are different, [27] (see Chapter 8, Liquid biopsy for details). Furthermore, along with the approval of BRAF inhibitors, the therascreen® *BRAF* V600E RGQ PCR kit and the MEBGEN RASKET™-B kit have been newly approved as a CDxs for BRAF inhibitors in Japan.

In addition, another *BRAF* V600E test is immunohistochemistry (IHC), which uses the VE1 monoclonal antibody against the BRAFV600E mutant protein. A meta-analysis of 1,021 colorectal cancer patients from eight studies reported a concordance rate of 0.94 (95% CI 0.87–0.98) between IHC and mutation testings[28]. Observational studies that compared the prognosis of *BRAF* V600E mutant protein-positive patients and wild-type patients using the IHC method also confirmed that *BRAF* V600 mutations are extremely strong prognostic factors[29,30]. Strictly standardized staining techniques, reagents, and determination methods are essential because the degree of staining in the IHC method can vary depending on the antibody clones, staining conditions, and automated immunostaining equipment used. Ventana OptiView BRAF V600E (VE1), an immunostaining reagent for BRAF VE1 clones, received regulatory approval in December 2021 and insurance coverage in January 2023, and the IHC method is also recommended as a *BRAF* V600E mutation detection method.

Table 4 *BRAF* mutation tests approved in Japan

Test method name	Sample used	Test system	Target gene	Approval as companion diagnostics for BRAF inhibitors
MEBGEN RASKET™-B Kit	Tumor tissue	PCR-rSSO method	*RAS* (exons 2, 3, and 4) *BRAF* V600E	Yes
FoundationOne® CDx Cancer Genomic Profile	Tumor tissue	Next generation sequencing	324 genes	No
OncoGuide™ NCC Oncopanel System	Tumor tissue	Next generation sequencing	124 genes	No
FoundationOne® Liquid CDx Cancer Genomic Profile	Blood sample	Next generation sequencing	324 genes	No
Guardant360® CDx Oncogene Panel	Blood sample	Next generation sequencing	74 genes	No
therascreen® BRAF V600E Mutation Detection Kit	Tumor tissue	Real-time PCR method	*BRAF* V600E	Yes

Ventana OptiView BRAF V600E (VE1)	Tumor tissue	Immunohistochemical staining method	*BRAF* V600E	No

[References]

1) Rapp UR, Goldsborough MD, Mark GE, et al: Structure and biological activity of v-raf, a unique oncogene transduced by a retrovirus. Proc Natl Acad Sci USA 80: 4218-22, 1983

2) Chong H, Vikis HG, Guan KL: Mechanisms of regulating the Raf kinase family. Cell Signal 15: 463-9, 2003

3) Schubert S, Shannon K, Bollag G: Hyperactive *RAS* in developmental disorders and cancer. Nat Rev Cancer 7: 295-308, 2007.

4) Yaeger R, Kotani D, Mondaca S, et al: Response to Anti-EGFR Therapy in Patients with BRAF non-V600-Mutant Metastatic Colorectal Cancer. Clin Cancer Res 25: 7089-97, 2019

5) Ogura T, Kakuta M, Yatsuoka T, et al: Clinical pathological characteristics and prognostic impact of colorectal cancers with N*RAS* mutations. Oncol Rep 32: 50-6, 2014

6) Yokota T, Ura T, Shibata N, et al: BRAF mutation is a powerful prognostic factor in advanced and recurrent colorectal cancer. Br J Cancer 104: 856-62, 2011

7) Nakanishi R, Harada J, Tuul M, et al: Prognostic relevance of *KRAS* and BRAF mutations in Japanese patients with colorectal cancer. Int J Clin Oncol 18: 1042-8, 2013

8) Chen D, Huang JF, Liu K, et al: BRAFV600E mutation and its association with clinicopathological features of colorectal cancer: a systematic review and meta-analysis. PLoS One 9: e90607, 2014

9) Venderbosch S, Nagtegaal ID, Maughan TS, et al: Mismatch repair status and BRAF mutation status in metastatic colorectal cancer patients: a pooled analysis of the CAIRO, CAIRO2, COIN, and FOCUS studies. Clin Cancer Res 20: 5322-30, 2014

10) Modest DP, Ricard I, Heinemann V, et al: Outcome according to *KRAS*-, N*RAS*- and BRAF-mutation as well as *KRAS* mutation variants: pooled analysis of five randomized trials in metastatic colorectal cancer by the AIO colorectal cancer study group Ann Oncol 27:1746-53, 2016

11) Mitani S, Taniguchi H, Sugiyama K, et al: The impact of the Glasgow Prognostic Score on survival in second-line chemotherapy for metastatic colorectal cancer patients with BRAF V600E mutation. Ther Adv Med Oncol 11: 1758835918820298, 2019

12) Cremolini C, Loupakis F, Antoniotti C, et al: FOLFOXIRI plus bevacizumab versus FOLFIRI plus bevacizumab as first-line treatment of patients with metastatic colorectal cancer: updated overall survival and molecular subgroup analyses of the open-label, phase 3 TRIBE study Lancet Oncol 16:1306-15, 2015

13) Cremolini C, Antoniotti C, Stein A, et al: Individual Patient Data Meta-Analysis of FOLFOXIRI Plus Bevacizumab Versus Doublets Plus Bevacizumab as Initial Therapy of Unresectable Metastatic Colorectal Cancer. J Clin Oncol JCO2001225, 2020

14) Colorectal Cancer Study Group: Colorectal Cancer Treatment Guidelines 2022 Edition for Doctors. Kanehara Publishing, 2022

15) Stintzing S, Heinrich K, Tougeron D, et al: Randomized study to investigate FOLFOXIRI plus either bevacizumab or cetuximab as first-line treatment of BRAF V600E-mutant mCRC: The phase-II FIRE-4.5 study (AIO KRK-0116) J Clin Oncol: 39 (15_suppl): 3502-3502, 2021

16) Kopetz S, Grothey A, Yaeger R, et al: Encorafenib, Binimetinib, and Cetuximab in *BRAF* V600E-Mutated Colorectal Cancer. N Engl J Med 381:1632-43, 2019

17) Formica V, Sera F, Cremolini C, et al: *KRAS* and BRAF Mutations in Stage II and III Colon Cancer: A Systematic Review and Meta-Analysis. J Natl Cancer Inst 114: 517-27, 2022

18) André T, de Gramont A, Vernerey D, et al: Adjuvant Fluorouracil, Leucovorin, and Oxaliplatin in Stage II to III Colon Cancer: Updated 10-Year Survival and Outcomes According to BRAF Mutation and Mismatch Repair Status of the MOSAIC Study. Clin Oncol 33:4176-87, 2015

19) Uutela A, Osterlund E, Halonen P, et al: Resectability, conversion, metastasectomy and outcome according to *RAS* and BRAF status for metastatic colorectal cancer in the prospective RAXO study. Br J Cancer 127: 686-94, 2022

20) Margonis GA, Buettner S, Andreatos N, et al: Association of BRAF Mutations With Survival and Recurrence in Surgically Treated Patients With Metastatic Colorectal Liver Cancer. JAMA Surg 153: e180996, 2018

21) Gau L, Ribeiro M, Pereira B, et al: Impact of BRAF mutations on clinical outcomes following liver surgery for colorectal liver metastases: An updated meta-analysis. Eur J Surg Oncol 47: 2722-33, 2021

22) Yang Y, Wang D, Jin L, et al: Prognostic value of the combination of microsatellite instability and *BRAF* mutation in colorectal cancer. Cancer Manag Res 10: 3911-29, 2018

23) Parsons MT, Buchanan DD, Thompson B, et al: Correlation of tumor BRAF mutations and MLH1 methylation with germline mismatch repair (MMR) gene mutation status: a literature review assessing utility of tumor features for MMR variant classification. J Med Genet 49 : 151-7, 2012

24) Rubenstein JH, Enns R, Heidelbaugh J, et al; Clinical Guidelines Committee: American Gastroenterological Association Institute Guideline on the Diagnosis and Management of Lynch Syndrome. Gastroenterology 149: 777-82; quiz e16-7, 2015

25) Stoffel EM, Mangu PB, Gruber SB, et al; American Society of Clinical Oncology; European Society of Clinical Oncology: Hereditary colorectal cancer syndromes: American Society of Clinical Oncology Clinical Practice Guideline endorsement of the familial risk-colorectal cancer: European Society for Medical Oncology Clinical Practice Guidelines. J Clin Oncol 33: 209-17, 2015

26) Colorectal cancer research group Ed: Hereditary colorectal cancer clinical practice guideline 2020 edition. Kanehara Publishing, 2020

27) Ye P, Cai P, Xie J, et al: Reliability of BRAF mutation detection using plasma sample: A systematic review and meta-analysis. Medicine (Baltimore) 100: e28382, 2021

28) Pyo JS, Sohn JH, Kang G: Diagnostic Accuracy of BRAF Immunohistochemistry in Colorectal Cancer: a Meta-Analysis and Diagnostic Test Accuracy Review. Pathol Oncol Res 22: 831-7, 2016

29) Kwon JH, Jeong BK, Yoon YS, et al: Utility of BRAF VE1 Immunohistochemistry as a Screening Tool for Colorectal Cancer Harboring *BRAF* V600E Mutation. J Pathol Transl Med 52: 157-63, 2018

30) Nakaji Y, Oki E, Nakanishi R, et al: Prognostic value of BRAF V600E mutation and microsatellite instability in Japanese patients with sporadic colorectal cancer. J Cancer Res Clin Oncol 143: 151-60, 2017

5 HER2 test

5.1 Background

Colorectal cancer and the HER2 pathway

HER2 belongs to the EGFR tyrosine kinase family. It is a 185 kDa transmembrane glycoprotein receptor tyrosine kinase, and the *ERBB2*/*HER2* gene is located on the long arm of chromosome 17. Although HER2 has no endogenous ligand, it is activated through autophosphorylation of the intracellular tyrosine kinase domain by forming heterodimers with other HER family molecules that have ligands bound to their extracellular domains, leading to downstream activation. They transmit a signal similar to EGFR, and downstream signaling pathways include the *RAS*/RAF (MAPK) and PI3K/AKT/mTOR pathways. While these HER2 pathways play important roles in cell differentiation, proliferation, and maintenance in normal tissues, their hyperfunctions in colorectal cancer tissues are involved in cancer proliferation, suppression of apoptosis, differentiation, and metastasis (**Figure 1**)[1-3].

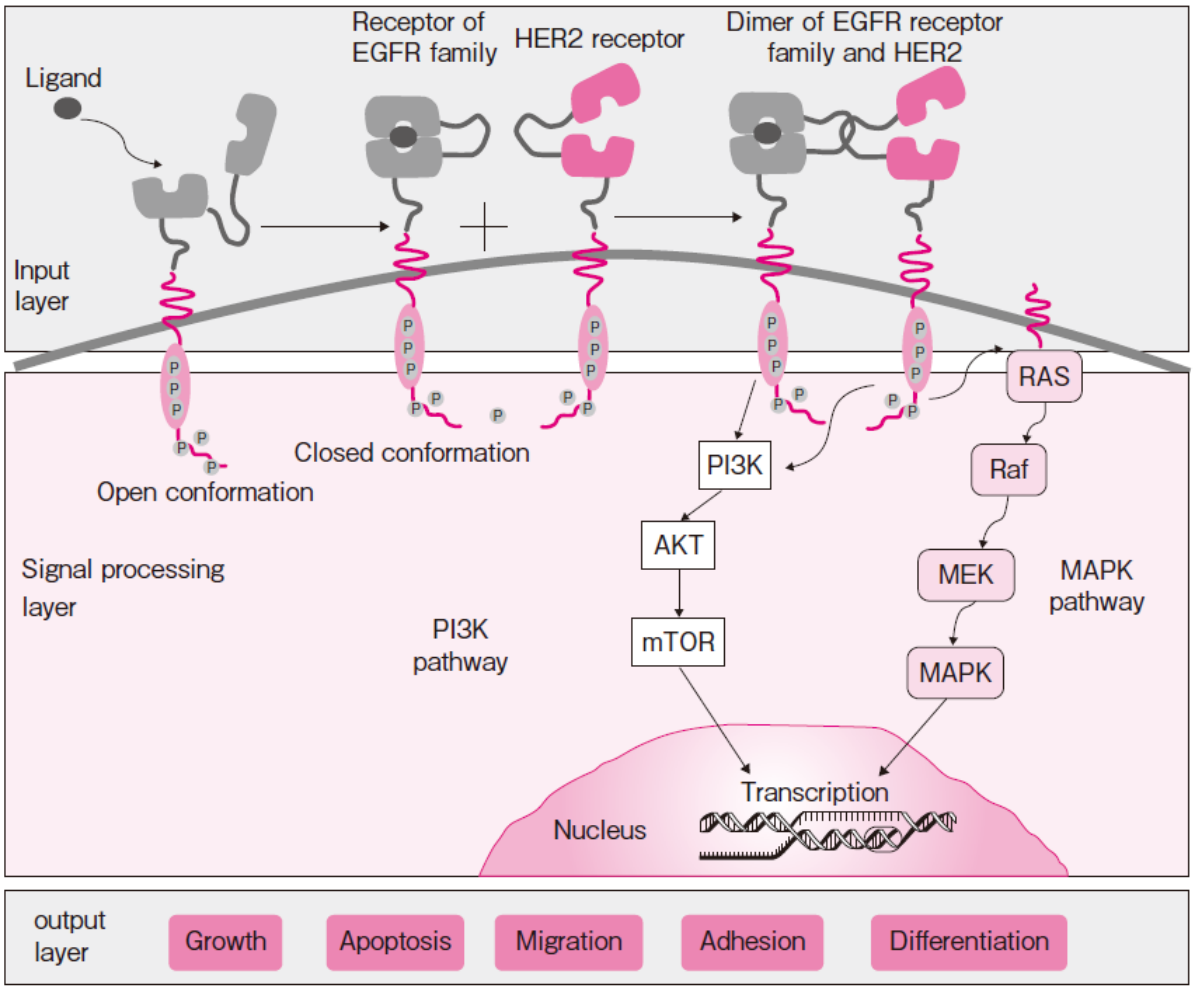


Figure 1 Colorectal cancer and HER2 signaling pathway[3]

Frequency and clinical features of HER2-positive colorectal cancer

The frequency of HER2 overexpression and *HER2* amplification in colorectal cancer is 2%–4%, and it differs among detection methods such as immunohistochemical staining (IHC), in situ hybridization (ISH) and next-generation sequencing (NGS) (**Table 1)**[4-8]. It is more common in left-sided colon and rectal primary tumors and is even more common in *RAS/BRAF* wild-type tumors (2.1%–5.4% in *RAS/BRAF* wild-type and 0.2%–1.4% in *RAS/BRAF* mutants). However, there is no mutual exclusivity with *RAS/BRAF* mutations[9,10]. A retrospective study of 370 cases using IHC/fluorescence in situ hybridization (FISH) in Japan reported that HER2-positive cases accounted for 4.1% of all colorectal cancers and 7.7% of *RAS*/*BRAF* wild-type cases[8].

Table 1 Percentage of HER2 overexpression and *HER2* amplification

	Test method	Stage	N	HER2 positive rate (%)
Marx et al[4]	IHC, FISH	I～IV	1,851	2.5
Heppner et al[5]	IHC, CISH	I to IV	1,64 5	1.6
Richman et al[6]	IHC, FISH	IV	1,342	2.2 (5.2 in *KRAS* wt)
Valtorta et al[7]	IHC, SISH	IV	304	5.6 in *KRAS* wt
Sawada et al[8]	IHC. FISH	IV	370	4.1 (7.7 in *RAS* / *BRAF* wt)

IHC: immunohistochemical staining, FISH: fluorescence in situ hybridization,
CISH: chromogenic in situ hybridization, SISH: silver in situ hybridization,
wt: wild type

It has been reported that HER2-positive breast and gastric cancers often metastasize to the central nervous system[11,12], and a similar tendency has been reported for colorectal cancer[13]. In addition, in colorectal cancer, it was reported that ovarian metastases were observed frequently in HER2 positive tumors [14].

5.2

Basic requirements

HER2 testing is strongly recommended prior to anti-HER2 therapy to assess the indication of anti-HER2 therapy in patients with unresectable colorectal cancer.

Degree of recommendation

Strongly recommended [SR 9]

*As of January 1, 2023, trastuzumab + pertuzumab therapy, which is approved for patients with unresectable, advanced, recurrent HER2-positive colorectal cancer, has shown efficacy only in *RAS* wild-type cases.

Anti-HER2 therapy for colorectal cancer

The results of clinical trials of multiple anti-HER2 therapies for HER2-positive colorectal cancer are presented in **Table 2**. The HERACLES-A trial reported the efficacy of trastuzumab + lapatinib therapy in patients with HER2-positive colorectal cancer who had failed to respond to treatments containing 5-FU, irinotecan, and oxaliplatin. Progression-free survival was 4.7 months, which was favorable for a trial targeting post-standard therapy[15,16].

Table 2 Therapeutic effect of anti-HER2 therapy for HER2-positive colorectal cancer

	Regimen	Test method	n	Line number for pretreatment	Response rate (%)	PFS (m)
HERACLES[15,16]	Trastuzumab + Lapatinib	IHC and FISH	32	≥2	28	4.7
My Pathway[17]	Trastuzumab + Pertuzumab	IHC, ISH, and NGS	57	≥1	32	2.9
TRIUMPH[18]	Trastuzumab + Pertuzumab	IHC, FISH, and ctDNA	27	≥1	30	4.0
HERACLES-B[3]	Trastuzumab + T-DM1	IHC and FISH	31	≥2	10	4.1
MOUNTAINEER[4]	Trastuzumab + Tucatinib	IHC, ISH, and ctDNA	23	≥2	52	8.1
DESTINY-CRC 01[21]	Trastuzumab + Deruxtecan	IHC and FISH	53	≥2	45	6.9

T-DM1: trastuzumab emtansine, IHC: immunohistochemistry, FISH: fluorescence in situ hybridization, ISH: in situ hybridization NGS: next generation sequencing, ctDNA: circulating tumor DNA, PFS: progression-free survival, m: month

In the cross-organ MyPathway study, solid tumors with *HER2* copy number ≥6, *HER2/CEP17* ratio ≥2.0 by ISH method, or strongly positive (3+) by IHC method were targeted. The efficacy and safety of trastuzumab + pertuzumab therapy were investigated, and the response rate was 32% and the progression-free survival was 2.9 months in colorectal cancer cohort (n = 57). Enrollment was performed regardless of *RAS* status in this study, but the response rate for wild-type *RAS* was 40%, while the response rate for *RAS* mutant-type was 8%. The results suggested it is difficult to obtain a therapeutic effect of trastuzumab + pertuzumab therapy in tumors with *RAS* mutants[17].

The TRIUMPH trial, which was a single-arm phase II trial in Japan investigating the efficacy of trastuzumab + pertuzumab therapy for *RAS* wild-type HER2-positive colorectal cancer refractory to treatments including 5-FU, irinotecan, oxaliplatin, and anti-EGFR antibody drugs. In addition to cases diagnosed as HER2-positive by HER2 testing using IHC/FISH in tissue specimens (IHC 3+ or FISH-positive), patients with *HER2* amplification confirmed by liquid biopsy (Guardant360® CDx) were also enrolled in this study.

The significance of performing HER2 testing in colorectal cancer patients is to determine the indications for anti-HER2 therapy. HER2 testing is recommended prior to anti-HER2 therapy. In addition, the eligibility

criteria for the TRIUMPH trial were limited to those with *RAS* wild type. The clinical practice as of January 1, 2023, is meaningful only for those with wild-type *RAS*. However, HER2 amplification is not mutually exclusive with *RAS/BRAF* status and some other clinical trials of anti-HER2 therapy in colorectal cancer include HER2 positive tumors regardless of *RAS* status. Therefore, it is considered appropriate to conduct HER2 testing in patients with unresectable, advanced, recurrent colorectal cancer regardless of *RAS/BRAF* status.

Comment 1 Prognosis of HER2-positive colorectal cancer and therapeutic effect of anti-EGFR antibody drug

There are several reports on the association between HER2 positivity and prognosis in colorectal cancer. A retrospective study of 1,645 patients by Heppner et al. reported that HER2-positive colorectal cancer had a poorer prognosis than HER2-negative patients. However, an analysis using samples from the FOCUS/PICCORO trial by Richman et al. did not show any difference in prognosis, and there is currently no consensus regarding *HER2* amplification and prognosis[5,6,8].

In addition, the signaling pathway suggests that *HER2* amplification, like *RAS* and *BRAF* mutations, is a negative predictor of therapeutic efficacy of anti-EGFR antibody drugs. Although no prospective studies have been reported, multiple retrospective studies have reproducibly shown that anti-EGFR antibody drugs are poorly effective in HER2-positive colorectal cancer (**Table 3**)[8,9,22-24].

Regarding the timing of HER2 testing in colorectal cancer patients, it is mentioned above that it should be performed before anti-HER2 therapy is started.

Table 3 Therapeutic effect of anti-EGFR antibody drugs on HER2-positive colorectal cancer

	treatment	Group	n	Response rate (%)	PFS (m)	*p* -value
Sartore-Bianchi A et al [22]	Anti- EGFR antibody drug + Chemotherapy	HER2 positive	79	31	5.7	0.031
		HER2 negative and *RAS* wild type	113	47	7.0	
Martin et al[23]	Anti-EGFR antibody drug	HER2 IHC positive and FISH-positive	6	NA	2.5	<0.0001
		other than that	156	NA	6.7	
Raghav et al [9]	Anti-EGFR antibody drug	*HER2* DISH positive	14	NA	2.9	<0.0001
		HER2 DISH negative	83	NA	8.1	
Sawada et al[8]	Anti-EGFR antibody drug	HER2 positive	11	20	2.6	0.006
		HER2 negative and *RAS* / *BRAF* wild type	132	45	6.0	
Jeong et al[24]	Anti-EGFR antibody drug	HER2 positive	7	NA	3.1	0.019
		HER2 negative and *RAS* / *BRAF* wild type	135	NA	5.6	

FISH: fluorescence in situ hybridization, DISH: dual color in situ hybridization, NA: not available, PFS: progression-free survival, m: month

5.3

Basic requirements

In HER2 testing for unresectable advanced colorectal cancer, IHC testing is strongly recommended first. ISH testing is added in case of IHC 2+*.

Degree of recommendation

Strongly recommended [SR 9]

* Trastuzumab + pertuzumab therapy is reimbursed for "IHC 3 + or ISH positive" colorectal cancer. However, according to the international unified criteria for HER2 diagnosis and the view of the Japanese Society of Pathology "Solid Tumor HER2 Test Guidance Development Working Group," it was recommended that "IHC tests should be performed first, and ISH tests should be performed in patients judged to be 2+" from the perspective of the efficacy of pertuzumab + trastuzumab therapy,

Testing methods and diagnostic criteria for HER2-positive colorectal cancer

IHC, which measures the expression of HER2 protein on the cell membrane, and ISH, which measures the presence of *HER2* amplification, are mainly used for HER2-positive diagnosis. In addition, in recent years, a comprehensive genome profiling test that can detect multiple genetic abnormalities with a small amount of sample (tissue sample or blood sample) using NGS has been performed [Comment 2].

IHC is the simplest method, and the Ventana ultraView Pathway HER2 (4B5) (Roche Diagnostics Co., Ltd.) is approved as a companion diagnostic agent for trastuzumab + pertuzumab therapy for colorectal cancer in Japan. HER2 IHC is determined using formalin-fixed paraffin-embedded (FFPE) colorectal cancer tissue specimens, based on the criteria shown in **Table 4**[29].

Table 4 HER2 IHC scoring algorithms used for colorectal cancer

IHC score	Surgical sample	Biopsy sample
3+	>10% of tumor cells have positive staining with strong staining intensity in the lateral intact or circumferential plasma membranes.	Regardless of the percentage of staining-positive tumor cells, staining-positive images are seen with strong staining intensity in the lateral complete plasma membrane or in the circumferential plasma membrane.
2+	>10% of tumor cells have weak to moderate staining intensity and positive staining in lateral incomplete or circumferential plasma membranes. Alternatively, ≤10% of tumor cells have positive staining with strong staining intensity in the lateral intact or circumferential plasma membranes.	Regardless of the percentage of positive tumor cells, weak to moderate staining intensity and positive staining are seen in the lateral incomplete or circumferential plasma membranes.
1+	>10% of tumor cells have a faint/barely perceptible staining positive staining in defective lateral or circumferential	A faint/barely perceptible staining intensity is present in the plasma membrane regardless of

	membranes.	the percentage of positive tumor cells.
0	No staining-positive image is observed, OR ≤10% of tumor cells have a faint/barely perceptible staining positive staining in defective lateral or circumferential plasma membranes.	No cells showing a positive image in the cell membrane are observed.

The ISH method detects the distribution and amount of specific DNA and mRNA in cells by utilizing the specific binding between single-stranded nucleic acid molecules with complementary nucleotide sequences and is widely used to determine the presence or absence of *HER2* amplification. The ISH method can be classified into FISH, chromogenic in situ hybridization (CISH), dual color in situ hybridization (DISH), silver in situ hybridization (SISH) and other methods. FISH is one of the most widely used methods for HER2 diagnosis, and in Japan, the Pathvision HER-2 DNA Probe Kit (Abbott Japan G.K.) has been approved as a companion diagnostic agent for trastuzumab + pertuzumab therapy for colorectal cancer.

Although diagnostic criteria for determining HER2-positive colorectal cancer differed depending on the test, a Japanese researcher took the lead in creating an international standard for HER2 diagnosis[26]. As a result, the definition of HER2-positive in colorectal cancer was defined as (1) more than 10% of the tumor cells are IHC 3+, or (2) more than 10% of the tumor cells are IHC 2+ and ISH-positive. Biopsy specimens were defined as (1) presence of IHC 3+ tumor cells regardless of the percentage of positive cells, or (2) presence of IHC 2+ and ISH-positive cells regardless of the percentage of positive cells.

In Japan, the administration standard for trastuzumab + pertuzumab therapy for colorectal cancer is "IHC 3+ or ISH positive," and either IHC or FISH companion diagnostic reagents can be used first for diagnosis. Furthermore, since the *HER2* amplification rate in colorectal cancer is as low as 2%–4%, the IHC method is recommended as an initial test because it is inexpensive and simple (preparation of HER2 protein histopathological samples = 690 points, *HER2* gene sample production = 2,700 points). In the case with IHC 2+, it is desirable to confirm *HER2* amplification by ISH because the TRIUMPH study contained four IHC2+/FISH-positive cases among all cases were confirmed to be HER2-positive by tissue (n = 27). A similar diagnostic algorithm is also proposed in the opinion of the Japanese Society of Pathology "Working Group for HER2 Testing Guidelines for Solid Tumors" issued on September 22, 2022.

Comment 2 Detection of HER2 amplification using comprehensive genomic profiling test

In the SCRUM-Japan GI-SCREEN study, NGS using tissue samples was performed using Thermo's Oncomine Comprehensive Assay (OCA), and *HER2* amplification was reported in 2.8% of cancers[25]. Regarding the concordance rate between NGS and IHC/ISH, it has been reported that the concordance rate between NGS and IHC was 92% in 102 cases of *HER2*-amplified colorectal cancer, and 99% if borderline type is included[26]. In addition, in the MyPathway trial, the diagnostic concordance rate for *HER2* amplification in patients who underwent FISH/CISH in addition to NGS was 81%[17].

Regarding comprehensive genomic profiling tests using plasma specimens, HER2-positive tissue samples by ctDNA testing using Guardant360 was included in the eligibility criteria for the TRIUMPH trial, and the

response rate in cases with *HER2* amplification in ctDNA was 28%. The positive percent agreement (PPA) between OCA and Guardant360 was reported to be 82.1%, negative percent agreement (NPA) 83.3%, and overall agreement 82.6%[18].

In this way, the comprehensive genomic profiling test using tissue/blood specimens and the HER2 diagnosis by IHC/ISH show a high concordance rate. Since there are no reports directly examining the correlation between *HER2* amplification using a comprehensive genomic profile (FoundationOne® CDx, OncoGuide™ NCC Oncopanel System, FoundationOne® Liquid CDx, Guardant360® CDx) and *HER2* amplification by IHC/FISH, HER2 testing using a companion diagnostic is recommended.

In a study of 40 colorectal cancer specimens using NGS in Japan, in cases with *HER2* copy number ≥7 (n = 14), IHC 3+ 78.6%, IHC 2+/FISH positive 21.4%, all cases were HER2 positive (definition: IHC 3+ or IHC 2+/FISH positive)[27]. Furthermore, a cross-validation study in which the same sample was analyzed on different NGS panels also showed a very high correlation (r = 0.98) in *HER2* copy number between NGS panels[27]. Therefore, if a comprehensive genomic profile test shows a high copy number of *HER2*, and the patient's performance status, and the initiation of treatment is urgent, omitting the HER2 test using a companion diagnostic may be considered.

Side note 1 Specimens used for HER2 testing

HER2-positive colorectal cancer has been reported to exhibit intratumoral heterogeneity. In a study using 19 HER2 positive surgical specimens, 37% (7/19) of cases showed HER2-positivity only in less than 50% of tumor cells[27]. Therefore, when biopsy specimens are used for HER2 testing, it is considered desirable to obtain biopsies from multiple sites. However, a discrepancy in HER2 overexpression between the primary and metastatic lesions has been reported in approximately 14% of cases[28] reported when the primary lesion was HER2-positive but the metastatic lesion was negative or vice versa. Therefore, there is no consensus on whether primary or metastatic lesions should be used for HER2 testing.

[References]

1) Akiyama T, Sudo C, Ogawara H, et al: The product of the human c-erbB-2 gene: a 185-kilodalton glycoprotein with tyrosine kinase activity. Science 232: 1644-6, 1986
2) Coussens L, Yang-Feng TL, Liao YC, et al: Tyrosine kinase receptor with extensive homology to EGF receptor shares chromosomal location with neu oncogene. Science 230: 1132-9, 1985
3) Lv Q, Meng Z, Yu Y, et al: Molecular Mechanisms and Translational Therapies for Human Epidermal Receptor 2 Positive Breast Cancer. Int J Mol Sci 17: 2095, 2016
4) Marx AH, Burandt EC, Choschzick M, et al: Heterogeneous high-level HER-2 amplification in a small subset of colorectal cancers. Hum Pathol 41: 1577-85, 2010
5) Ingold Heppner B, Behrens HM, Balschun K, et al: HER2/neu testing in primary colorectal carcinoma. Br

J Cancer 111: 1977-84, 2014
6) Richman SD, Southward K, Chambers P, et al: HER2 overexpression and amplification as a potential therapeutic target in colorectal cancer: analysis of 3256 patients enrolled in the QUASAR, FOCUS and PICCOLO colorectal cancer trials. J Pathol 238 : 562-70, 2016
7) Valtorta E, Martino C, Sartore-Bianchi A, et al: Assessment of a HER2 scoring system for colorectal cancer: results from a validation study. Mod Pathol 28: 1481-91, 2015
8) Sawada K, Nakamura Y, Yamanaka T, et al: Prognostic and Predictive Value of HER2 Amplification in Patients With Metastatic Colorectal Cancer. Clin Colorectal Cancer 17: 198-205, 2018
9) Raghav K, Loree JM, Morris JS, et al: Validation of HER2 Amplification as a Predictive Biomarker for Anti-Epidermal Growth Factor Receptor Antibody Therapy in Metastatic Colorectal Cancer. JCO Precis Oncol 3: 1-13, 2019
10) Salem ME, Weinberg BA, Xiu J, et al: Comparative molecular analyses of left-sided colon, right-sided colon, and rectal cancers. Oncotarget 8: 86356-68, 2017
11) Hosonaga M, Saya H, Arima Y: Molecular and cellular mechanisms underlying brain metastasis of breast cancer. Cancer Metastasis Rev 39: 711-20, 2020
12) Cavanna L, Seghini P, Di Nunzio C, et al: Gastric cancer with brain metastasis and the role of human epidermal growth factor 2 status. Oncol Lett 15: 5787-91, 2018
13) Sartore-Bianchi A, Lonardi S, Aglietta M, et al: Central Nervous System as Possible Site of Relapse in ERBB2-Positive Metastatic Colorectal Cancer: Long-term Results of Treatment With Trastuzumab and Lapatinib. JAMA Oncol 6: 927-9, 2020
14) Li JL, Lin SH, Chen HQ, et al: Clinical significance of HER2 and EGFR expression in colorectal cancer patients with ovarian metastasis. BMC Clin Pathol 19: 3, 2019
15) Sartore-Bianchi A, Trusolino L, Martino C, et al: Dual-targeted therapy with trastuzumab and lapatinib in treatment-refractory, *KRAS* codon 12/13 wild-type, HER2-positive metastatic colorectal cancer (HERACLES): a proof -of-concept, multicentre, open-label, phase 2 trial. Lancet Oncol 17: 738-46, 2016
16) Tosi F, Sartore-Bianchi A, Lonardi S, et al: Long-term Clinical Outcome of Trastuzumab and Lapatinib for HER2-positive Metastatic Colorectal Cancer. Clin Colorectal Cancer 19: 256-62.e2, 2020
17) Meric-Bernstam F, Hurwitz H, Raghav KPS, et al: Pertuzumab plus trastuzumab for HER2-amplified metastatic colorectal cancer (MyPathway): an updated report from a multicentre, open-label, phase 2a, multiple basket study. Lancet Oncol 20:518-30, 2019
18) Nakamura Y, Okamoto W, Kato T, et al: Circulating tumor DNA-guided treatment with pertuzumab plus trastuzumab for HER2-amplified metastatic colorectal cancer: a phase 2 trial. Nat Med 27: 1899-903, 2021
19) Sartore-Bianchi A, Lonardi S, Martino C, et al: Pertuzumab and trastuzumab emtansine in patients with HER2-amplified metastatic colorectal cancer: the phase II HERACLES-B trial. ESMO Open 5: e000911, 2020
20) Strickler JH, Zelma T, Ou F, et al: Trastuzumab and tucatinib for the treatment of HER2 amplified

metastatic colorectal cancer (mCRC): Initial results from the MOUNTAINEER trial. Ann Oncol 30 (suppl_5): v200, 2019

21) Yoshino T, Bartolomeo MD, Raghav KPS, et al: Trastuzumab deruxtecan (T-DXd; DS-8201) in patients (pts) with HER2-expressing metastatic colorectal cancer (mCRC): Final results from a phase 2, multicenter, open-label study (DESTINY-CRC01). J Clin Oncol 39 (15_suppl), 2021

22) Sartore-Bianchi A, Amatu A, Porcu L, et al: HER2 Positivity Predicts Unresponsiveness to EGFR-Targeted Treatment in Metastatic Colorectal Cancer. Oncologist 24: 1395-402, 2019

23) Martin V, Landi L, Molinari F, et al: HER2 gene copy number status may influence clinical efficacy to anti-EGFR monoclonal antibodies in metastatic colorectal cancer patients. Br J Cancer 108: 668-75, 2013

24) Jeong JH, Kim J, Hong YS, et al: HER2 Amplification and Cetuximab Efficacy in Patients With Metastatic Colorectal Cancer Harboring Wild-type *RAS* and BRAF. Clin Colorectal Cancer 16: e147-52, 2017

25) Yuki S, Kato T, Taniguchi H, et al: The Nationwide Cancer Genome Screening Project in Japan SCRUM-Japan GI-SCREEN: Efficient Identification of Cancer Genome Alterations in Advanced Colorectal Cancer. Ann Oncol 28 (suppl_5): v192, 2017

26) Cenaj O, Ligon AH, Hornick JL, et al: Detection of ERBB2 Amplification by Next-Generation Sequencing Predicts HER2 Expression in Colorectal Carcinoma. Am J Clin Pathol 152: 97-108, 2019

27) Fujii S, Magliocco AM, Kim J, et al: International Harmonization of Provisional Diagnostic Criteria for ERBB2-Amplified Metastatic Colorectal Cancer Allowing for Screening by Next-Generation Sequencing Panel. JCO Precis Oncol 4: 6-19, 2020

28) Lee WS, Park YH, Lee JN, et al: Comparison of HER2 expression between primary colorectal cancer and their corresponding metastases. Cancer Med 3: 674-80, 2014

29) Ventana ultraView pathway HER2 (4B5) package insert

6 Tests for mismatch repair deficiencies

6.1 Background

Molecular mechanism of mismatch repair (MMR) deficiency

DNA accumulates replication errors at a certain frequency each time replication is repeated. The main mechanisms for repairing DNA replication errors include direct repair, excision repair, post-replication repair, and mismatch repair. Abnormalities in the repair mechanism of non-complementary base combinations (DNA mismatches) play an important role in colorectal cancer development. At least six genes, *MLH1*, *MSH2*, *MSH6*, *PMS2*, *MLH3*, and *MSH3*, are known to be involved in mismatch repair. The tetramer consisting of MSH2, MSH3, MLH1, and PMS2 (or MLH3) repairs insertion/deletion mismatches with 2- to 4-base loops. DNA replication errors are likely to occur in the repeated sequences of one to several bases of DNA, called microsatellites, and defects in the mismatch repair function cause abnormalities in the number of microsatellite repeats, leading to microsatellite instability (MSI). MSI-induced frameshifting of the genes involved in tumor suppression, cell proliferation, DNA repair, and apoptosis can lead to carcinogenesis.[1]

Definition of dMMR and MSI

When a pathogenic variant or epigenetic change occurs in both alleles of the *MLH1, MSH2, PMS2,* or *MSH6* genes involved in mismatch repair, proteins with normal function are not synthesized, resulting in a deficient mismatch repair function. This state is called deficient mismatch repair (dMMR). Consequently, DNA replication errors cannot be repaired and are fixed in the genome as variants. MSI tests detect changes in the number of repeated sequences caused by DNA replication errors that cannot be repaired, particularly in multiple microsatellite regions. Generally, dMMR and proficient mismatch repair (pMMR) are terms that describe the state of mismatch repair function. A dMMR status is expressed by either loss of MMR expression by immunohistochemistry (IHC) or a high-frequency of MSI (MSI-H) by the MSI test. A pMMR status is expressed as either positive MMR expression by IHC test, or microsatellite stable (MSS) or a low frequency of MSI (MSI-L) by the MSI test.

Immunological mechanisms of the tumor microenvironment in MMR-deficient colorectal cancer

In dMMR colorectal cancer, hypermutations develop due to DNA replication errors. This increases the probability that highly immunogenic variants are presented as antigens on the cell surface, subsequently leading to the activation of T lymphocytes. A resultant significant increase in the number of $CD8^+$ T cells infiltrating the tumor and microenvironment has been reported, which may be one of the reasons for dMMR colorectal cancer having a better prognosis than MSS and pMMR colorectal cancer.[2,3] However, dMMR colorectal cancer has also been reported to increase tumor cell PD-L1 expression and evade tumor immunity.[4] Although dMMR colorectal cancer is highly immunogenic due to disruption of the mismatch repair

mechanism, making it easier for tumor cells to be recognized, the increase in PD-L1 expression suppresses the immune response. Therefore, blocking the PD-1/PD-L1 pathway with checkpoint inhibitors may be an effective treatment in dMMR colorectal cancer.

6.2

Basic requirements

Mismatch repair deficiency testing is strongly recommended prior to first-line therapy to assess the indications for immune checkpoint inhibitors in patients with unresectable colorectal cancer.

Degree of recommendation

Strongly recommended [SR 9]

Clinical significance of testing for MMR deficiency in unresectable metastatic colorectal cancer (**Table 1)**

The anti-PD-1 antibody drug pembrolizumab was used in previously treated unresectable metastatic colorectal cancer in a phase II study (KEYNOTE-016 study). No response was observed in those with MSS, whereas a 40% response rate was observed in those with MSI-H[5]. Additionally, a phase II study of pembrolizumab for previously treated unresectable metastatic MSI-H/dMMR colorectal cancer was conducted (KEYNOTE-164 study). For third-line treatment and beyond, pembrolizumab achieved a response rate of 27.9% in 61 patients (95% CI: 17.1-40.8%), a 12-month progression-free survival rate of 34.3%, and a 12-month overall survival rate of 71.7%. Based on these results, pembrolizumab was approved in Japan in December 2018 for MSI-H solid tumors including colorectal cancer, using the MSI test kit (FALCO) as a companion diagnostic agent.

Subsequently, the Phase III KEYNOTE-177 study was conducted to verify the efficacy of standard therapy and pembrolizumab monotherapy for unresectable metastatic colorectal cancer. The median survival time was 16.5 months in the pembrolizumab group and 8.2 months in the standard treatment group, showing a significant improvement in progression-free survival in the pembrolizumab group. The response rate was higher in the pembrolizumab group (43.8%) than in the standard therapy group (33.1%).[9] Median overall survival was not reached with pembrolizumab alone and was 36.7 months with standard chemotherapy (HR 0.74, $p = 0.036$). Based on the results of this study, pembrolizumab was approved by the U.S. Food and Drug Administration (FDA) as a first-line treatment for unresectable metastatic dMMR colorectal cancer in June 2020.[9,10] The “Colorectal Cancer Treatment Guidelines for Physicians 2022” also strongly recommends the use of pembrolizumab as first-line treatment for unresectable metastatic dMMR colorectal cancer. It is strongly recommended that testing to determine MMR dysfunction for the purpose of determining suitability be performed before starting first-line therapy.

Another anti-PD-1 antibody, nivolumab, was evaluated in a phase II study (CheckMate142 study) investigating the efficacy and safety of unresectable metastatic MSI-H/dMMR colorectal cancer. In the nivolumab monotherapy group, efficacy was demonstrated with a response rate of 31.1% and a median

progression-free survival of 14.3 months.[11] Furthermore, in the treatment-experienced nivolumab + ipilimumab combination therapy group (nivolumab 3 mg/kg + ipilimumab 1 mg/kg every 3 weeks), the response rate, 12-month progression-free survival rate, and the overall survival rate were 55%, 71% and 85%, respectively. The response of the nivolumab + ipilimumab combination therapy group was favorable compared to that of nivolumab therapy alone. The incidence of grade 3 or higher immune-related adverse events was 32%, but it was judged to be well tolerated. As a result of this trial, in July 2018, the FDA granted accelerated approval of nivolumab plus ipilimumab for MSI-H/dMMR unresectable metastatic colorectal cancer that progressed after chemotherapy with fluoropyrimidine, oxaliplatin, and irinotecan. In Japan, the indications of nivolumab monotherapy and nivolumab + ipilimumab combination therapy were expanded in February 2020 and September 2020 to include unresectable metastatic colorectal cancer with MSI-H that has progressed after cancer chemotherapy. Further, the CheckMate 142 study reported high efficacy of nivolumab/low-dose ipilimumab combination therapy consisting of ipilimumab 1 mg/kg every 6 weeks and nivolumab 3 mg/kg every 2 weeks as first-line therapy[12]. Additionally, there is a phase III trial underway (CheckMate-8HW), in which the efficacy of nivolumab + ipilimumab combination for first line therapy is being investigated.

Based on the results of these trials, it is strongly recommended to perform tests to determine MMR dysfunction at an early stage, to maximize the possibility of effective treatment for unresectable metastatic colorectal cancer. As MMR deficiency is not mutually exclusive of the presence of *RAS/BRAF* mutations, testing to determine MMR deficiency is recommended regardless of *RAS/BRAF* mutation status. Changes in MMR status over time have not been reported in colorectal cancer. Therefore, for efficient use of histopathological materials and cost effectiveness, tests to determine MMR function deficiency should be performed while determining *RAS/BRAF* mutation, before starting first-line treatment.

Table 1 Effects of immune checkpoint inhibitors for unresectable metastatic MSI-H/dMMR colorectal cancer that are approved in Japan.

author	exam name	Phase	immune check point inhibitor	treatment line	subject	n	RR (%)	PFS (M)	OS (M)
Le DT, et al [5]	KEYNOTE-016	II	pembrolizumab	Tertiary and beyond	MSI-H	11	40	Unachieved	Unachieved
					MSS	twenty one	0	2.2	Five
Le DT, et al [13]	KEYNOTE-	II	pembrolizumab	Tertiary	MSI-H	40	52	Unac	Unachie

	016			and beyond				hieved	ved
Le DT, et al [14]	KEYNOTE-164	II	pembrolizumab (Cohort A)	Tertiary and beyond	MSI-H/ dMMR	61	33	2.3	31.4
			pembrolizumab (Cohort B)	Secondary and beyond		63	33	4.1	Unachieved
Overman MJ, et al [11]	CheckMate-142	II	Nivolumab	Secondary and beyond	MSI-H/ dMMR	74	31.1	14.3	Unachieved
Overman MJ, et al [15]	CheckMate-142	II	Nivolumab + ipilimumab	Secondary and beyond	MSI-H/ dMMR	119	55	Unachieved	Unachieved
Lenz HJ, et al [12]	CheckMate-142	II	Nivolumab + ipilimumab	once	MSI-H/ dMMR	45	69	Unachieved	Unachieved
André T, et al [9]	KEYNOTE-177	III	pembrolizumab	once	MSI-H/ dMMR	153	45	16.5	Unachieved
			chemical treatment			154	51	8.2	36.7

RR: response rate, PFS: progression-free survival, OS: overall survival, M: months, MSI-H: high frequency microsatellite instability, MSS: microsatellite stable, dMMR: mismatch repair-deficient.

6.3

Basic requirements

Mismatch repair deficiency testing is strongly recommended to assess the optimal chemotherapy based on the risk of recurrence in patients with resectable colorectal cancer.

Degree of recommendation

Strongly recommended [SR 7, R 2]

Clinical significance of testing for MMR deficiency in resectable colorectal cancer

The prevalence of dMMR in stage II and stage III colon cancer is reported to be 15-22% and 12-14%[16-18] respectively, while in Japan it is 6-10% and 5% respectively.[19,20] As cell line studies have reported a relationship between dMMR and 5-FU resistance,[21] the efficacy of 5-FU in dMMR cases has been extensively investigated.

In stage II/III colorectal cancer, cases with dMMR have a significantly lower risk of recurrence compared with pMMR (11% vs 26%, HR 0.53, 95% CI 0.40-0.70), with a stronger tendency for stage II colon cancer (8% vs 21%, HR 0.44, 95% CI 0.29-0.67).[16] Postoperative 5-FU combination therapy was also compared against surgery alone in Stage II/III colon cancer. In low-frequency microsatellite instability (MSI-L)/MSS cases, there was a significant additive effect on overall survival in the adjuvant chemotherapy group. However, no additional effect was observed in MSI-H cases, and the surgery alone group was significantly superior in overall survival. Thus, adjuvant chemotherapy containing 5-FU is effective in MSI-L/MSS colon cancer but may have adverse effects in MSI-H colon cancer (Tables 2, 3).[2,22]

Table 2 Meta-analysis of a phase III trial comparing postoperative 5-FU therapy with surgery alone in stage II/III colon cancer (reference 2)

	overall survival			
	Surgery alone MSI-H vs MSI-L/MSS	Postoperative 5-FU therapy MSI-H vs MSI-L/MSS	MSI-H Postoperative 5-FU therapy vs surgery alone	MSI-L/MSS Postoperative 5-FU therapy vs surgery alone
HR	0.31	1.07	2.17	0.69
95%CI	0.14-0.72	0.62-1.86	0.84-5.55	0.50-0.94
p	0.004	0.80	0.10	0.02

HR: hazard ratio, CI: confidence interval, MSI-L/MSS: low frequency microsatellite instability or microsatellite stability, MSI-H: high frequency microsatellite instability

Table 3 Meta-analysis of a phase III trial comparing disease-free survival between postoperative 5-FU therapy and surgery alone in stage II/III colon cancer (reference 22)

dMMR vs pMMR			
	HR	95%CI	*p* -value
Surgery alone	0.51	0.29-0.89	0.009
Postoperative 5-FU therapy	0.79	0.49-1.25	0.3
Postoperative 5-FU therapy vs surgery alone			
	HR	95%CI	*p* -value
dMMR			
StageII	2.3	0.84-6.24	0.09
Stage III	1.01	0.41-2.51	0.98
pMMRs			
StageII	0.84	0.57-1.24	0.38
Stage III	0.64	0.48-0.84	0.001

HR: hazard ratio, CI: confidence interval, dMMR: mismatch repair-deficient, pMMR: mismatch repair proficient

BRAF V600E mutations are more common in dMMR (35.3%) than in pMMR (11.5%) in stage II/III colon cancer.[23] A pooled analysis of the N0147 and PETACC8 trials in stage III colon cancer showed that dMMR is a favorable prognostic factor, whereas pMMR with *BRAF* V600E and *KRAS* exon 2 mutations has a significantly higher risk of recurrence and a poorer prognosis.[24,25] Based on these findings, recurrence-free survival in stage III colon cancer can be stratified by risk through simultaneous evaluation of *BRAF* V600E mutation and dMMR.[24,26]

However, the frequency of dMMR in rectal cancer is low. In a retrospective study of stage I to IV colorectal cancer in Japan, the frequency of MSI-H was 13% (36/275) in the right colon, 4% (12/271) in the left colon, and 2% (7/394) in the rectum.[20] In addition, the 5-year survival rate of dMMR rectal cancer was good in all stages, and the pathological complete response (pCR) rate in the stage II/III group who underwent preoperative chemoradiotherapy was 27.6%. MSI-H showed a favorable effect compared to the pCR rate of 18.1% for all types of rectal cancer (including MSS/pMMR),[27] indicating the possibility of MSI-H being a predictor of therapeutic effect.[28] Similarly, an analysis of 5,086 patients with locally advanced rectal cancer from the US National Cancer Database reported that MSI status was an independent predictor of pCR therapeutic response.[29]

Thus, dMMR is recognized as a recurrence and prognostic factor for stage II/III colorectal cancer, and fluoropyrimidine monotherapy, especially for stage II colorectal cancer, may increase the recurrence rate. In stage III colon cancer, the risk of recurrence can be stratified according to the presence or absence of *BRAF*

mutations, and the 2022 Physician Treatment Guidelines for Colorectal Cancer clearly states that fluoropyrimidine therapy alone is not recommended for MSI-H. As postoperative adjuvant therapy for stage III colon cancer, it is recommended to select a treatment regimen and treatment period according to the risk of recurrence. Based on these studies, it is strongly recommended that patients with resectable colorectal cancer undergo tests to determine MMR dysfunction before starting adjuvant chemotherapy, with the aim of selecting treatment according to the risk of recurrence.

Side note 1　Significance of preoperative testing to determine MMR deficiency

In recent years, there has been a series of reports on the significance of testing for MMR dysfunction in determining the applicability of immune checkpoint inhibitors to perioperative chemotherapy. In the NICHE trial, which evaluated the utility of nivolumab plus ipilimumab as preoperative chemotherapy in resectable colon cancer, dMMR colon cancer had a response rate of 100% (32/32) and a pCR rate of 69% (22).[30] In the VOLTAGE study, which evaluated the efficacy and safety of nivolumab administration after preoperative chemoradiotherapy for resectable, locally advanced rectal cancer followed by radical resection, MSI-H rectal cancer had a pCR rate of 60% (3/5 patients).[31] In addition, a phase II trial evaluated the efficacy of the anti-PD-1 antibody, dostarlimab, as preoperative treatment for dMMR Stage II/III locally advanced rectal cancer, 6 months after treatment.[12] A clinical complete response (cCR) was obtained in all patients, and ASCO2022 additionally reported that cCR was observed in all 14 patients. These studies demonstrate the usefulness of immune checkpoint inhibitors as a preoperative treatment for resectable colorectal cancer. To determine whether these should be used in treatment, it is important to perform preoperative tests to determine MMR dysfunction.[32,33]

6.4

Basic requirements

Mismatch repair deficiency testing is strongly recommended to screen for Lynch syndrome.

Degree of recommendation

Strongly recommended [SR 9]

Screening for Lynch syndrome

Lynch syndrome is an autosomal dominant disorder caused primarily by germline pathogenic variants in the MMR genes *MLH1, MSH2, MSH6,* and *PMS2*. Although it is a rare disease, Lynch syndrome accounts for 2-4% of all colorectal cancers in Europe and the United States,[34,35] and approximately 0.7-1% of all colorectal cancers in Japan.[36,37] Its diagnosis is clinically important because it causes a variety of malignant tumors, including colorectal cancer and endometrial cancer. In Lynch syndrome, one MMR gene has a germline pathogenic variant, and an acquired variant (or promoter region methylation) in the other wild-type allele impairs MMR function. This thought to lead to carcinogenesis.

The frequencies of MSI-H in all types of colorectal cancer are 12-16%[38-40] in Western reports and 6-7%[20,41] in Japanese reports. Most MSI-H cases have MMR gene function defects, acquired through methylation of the MLH1 promoter region, but approximately 10-20% of MSI-H colorectal cancer cases are thought to have Lynch syndrome. Therefore, although it is not appropriate to uniformly perform genetic testing for MSI-H colorectal cancer, it increases the likelihood of detecting latent Lynch syndrome. Understanding the screening process to determine Lynch syndrome is strongly recommended. Internationally, universal screening for Lynch syndrome using MSI and immunohistochemical tests for all patients with colorectal and endometrial cancers (or those aged 70 years or younger) has been proposed [Side note 2]. In Japan, as described in the "Hereditary Colorectal Cancer Clinical Practice Guideline 2020," testing for Lynch syndrome is recommended as a secondary screening when the clinical information satisfies the Amsterdam criteria II (**Table 4)** or the revised Bethesda guidelines (**Table 5);** these tests are also specified as universal screening (**Figure 1)**. For details on the diagnosis procedure, surveillance, and treatment policy for Lynch syndrome, please refer to the "Hereditary Colorectal Cancer Clinical Practice Guideline 2020."

Table 4 Amsterdam Criteria II (1999)

At least 3 relatives with HNPCC (Lynch syndrome)-related cancers (colorectal cancer, endometrial cancer, renal pelvic/ureteral cancer, small bowel cancer) have all of the following:
1. One affected person is a first-degree relative to the other two.
2. Affected in at least 2 consecutive generations.
3. At least one cancer was diagnosed before age 50 years.
4. The tumor is pathologically confirmed to be cancerous.

5. FAP is excluded.

HNPCC: familial nonpolyposis colorectal cancer (Lynch syndrome), FAP: familial adenomatous polyposis

Table 5 Revised Bethesda Guidelines (2004)

Tumor MSI testing is recommended for patients with colorectal cancer who have any of the following:
1. Colorectal cancer diagnosed before age 50 years.
2. Synchronous or metachronous colorectal cancer or other Lynch syndrome-related tumors*, regardless of age.
3. Colorectal cancer with MSI-H histologic findings ** diagnosed <60 years of age.
4. One or more 1st-degree relatives with a Lynch syndrome-related tumor, one of whom had colorectal cancer diagnosed before age 50 years.
5. Colorectal cancer in patients of any age who have two or more first- or second-degree relatives diagnosed with Lynch syndrome-associated tumors.

* Colorectal cancer, endometrial cancer, gastric cancer, ovarian cancer, pancreatic cancer, biliary tract cancer, small bowel cancer, renal pelvic/ureteral cancer, brain tumor (glioblastoma usually seen in Turcotte syndrome), Muir Sebaceous adenoma and keratoacanthocytoma in Tre syndrome.

** Intratumoral lymphocyte infiltration, clonal lymphocyte reaction, mucinous carcinoma/signet ring cell carcinoma-like differentiation, medullary proliferation

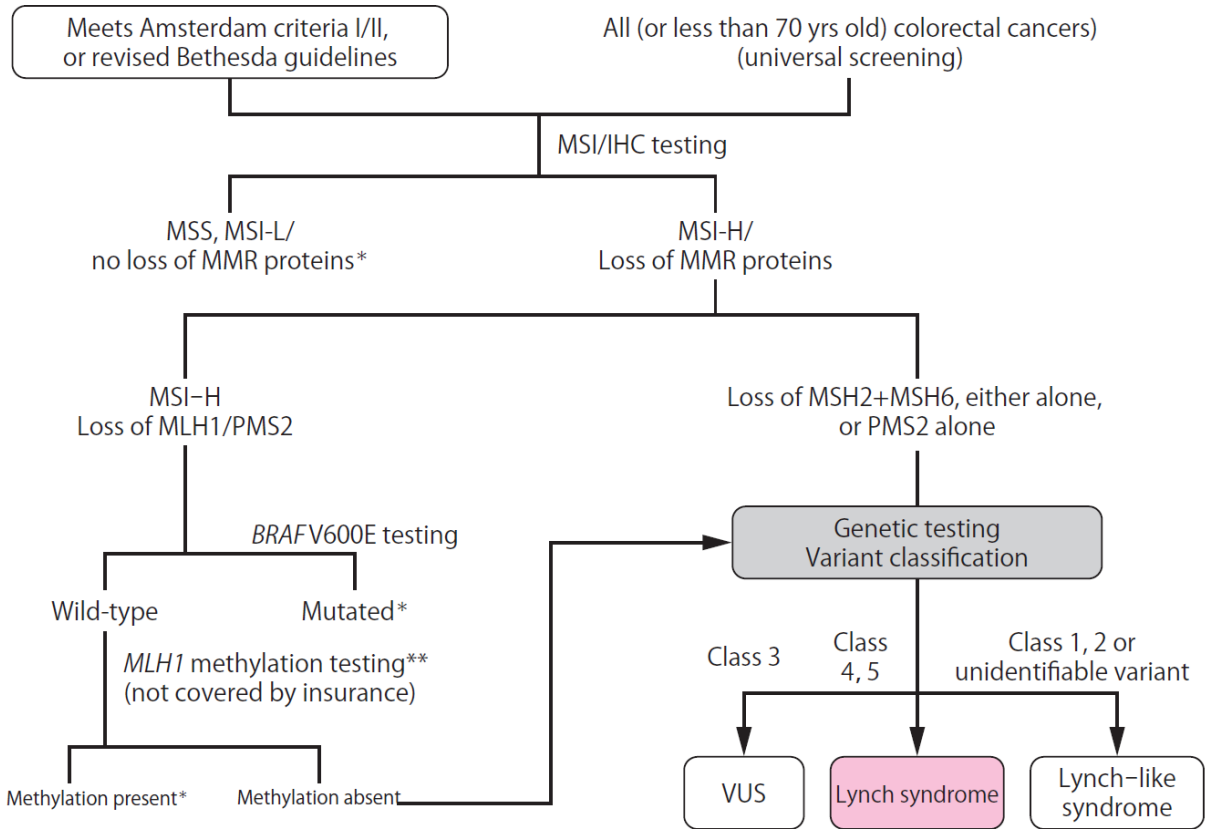


Figure 1 Lynch syndrome diagnosis procedure ("Hereditary Colorectal Cancer Clinical Practice Guideline 2020 ")

MSI: microsatellite instability, IHC: immunohistochemistry, MSI-H: high-frequency MSI, MSI-L: low-frequency MSI, MSS: microsatellite stable, MMR: mismatch repair, VUS: variant of unknown significance.

*Do not proceed to genetic test, ***MLH1* methylation test alone may be performed without *BRAF V600E* test. (Reprinted from Colorectal Cancer Research Group: Hereditary Colorectal Cancer Treatment Guidelines 2020 Edition. Kanehara Publishing, 2020)

Side Note 2 Universal Screening for Lynch Syndrome

In Europe and the United States, universal screening with an MSI test and an IHC test of MMR protein is cost-effective for diagnosing Lynch syndrome for all patients with colorectal cancers (or those under 70 years old), regardless of stage. It is recommended as a high-performance method, and the incidence of Lynch syndrome obtained from universal screening has been reported to be 2.4-3.7%.[42,43] Analysis of MSI testing and germline variants of *MLH1*, *MSH2*, *MSH6*, *PMS2*, and *EPCAM* in 15,045 patients with more than 50 types of cancer revealed that Lynch syndrome accounted for 16.3% of MSI-H cases, 1.9% of MSI-Intermediate (I) (defined as 3 ≤ MSI score < 10 on the MSI sensor in MSK-IMPACT) cases, and 0.3% of MSS cases. In addition, 50% of Lynch syndrome patients with MSI-H/MSI-I developed malignant tumors other than colorectal cancer and ovarian cancer. Of these, 45% did not meet the revised Bethesda criteria, suggesting the need for universal screening for all cancer types using tests to determine MMR dysfunction.[44] Colonoscopy surveillance in Lynch syndrome has long been reported for the early detection of colorectal adenoma/colorectal cancer and the reduction of colorectal cancer mortality,[45] but the usefulness of universal screening in Japan has not been verified. As it is also necessary to consider tumors in the reproductive organs, considerations specific to hereditary diseases are necessary when performing this procedure.

Side Note 3 *EPCAM* and Lynch Syndrome

EPCAM is an adjacent gene located upstream to the *MSH2* gene, and deletion of this 3' side causes aberrant methylation of the *MSH2* gene promoter region, resulting in loss of MSH2 protein expression, which is the etiology of Lynch syndrome. However, germline *EPCAM* gene deletion cases are extremely rare, and the risk of colorectal cancer is almost the same as that of germline *MSH2* loss-of-function variant carriers. The risk of endometrial cancer development is low, yet caution is required.[46]

6.5 Types of Tests for Determining Mismatch Repair Deficiency

As a representative method for determining MMR function deficiency, fragment analysis is performed using PCR products targeting microsatellite markers, and MSI testing is performed to evaluate the deviation of the marker waveform. Other methods for determining MMR deficiency include IHC testing to examine the

expression of proteins (MLH1, MSH2, MSH6, and PMS2) in tumor tissue, and methods to assess mismatch repair function by next-generation sequencing (NGS).

6.5

Basic requirements

The following methods are strongly recommended when assessing for Mismatch repair deficiency:

- Microsatellite instability (MSI) testing (strongly recommended [SR 9])
- Immunohistochemistry (IHC) testing (strongly recommended [SR 9])
- Next-generation sequencing (NGS)-based testing (strongly recommended [SR 7, R 2])

MSI test as a test to determine MMR dysfunction

The Bethesda panel **(Table 6)** is used to screen for Lynch syndrome and consists of 2 mono-nucleotide markers (BAT25, BAT26) and 3 di-nucleotide markers (D2S123, D5S346, D17S250).[47-49] Dinucleotide markers are generally more effective in diagnosing MSI-L, but Lynch syndrome with a germline pathogenic variant in *MSH6* and *PMS2* may not show MSI-H.[50,51] In contrast, mono-nucleotide markers can diagnose MSI-H with high sensitivity and specificity and are less susceptible to genetic polymorphisms, so they can be evaluated only in tumor tissue. In addition, the mono-marker panel can identify *MSH6*- deficient cases at a relatively high rate (62.5%), where MSI-H is unlikely to be detected by the Bethesda panel.[52]

For this reason, a panel consisting of single base repeat markers was developed. The MSI test kit (FALCO) detects five single-nucleotide repeat markers, and is also used to determine whether pembrolizumab can be indicated **(Table 6)**.[53] In this test, formalin-fixed, paraffin-embedded specimens are used, DNA is extracted from tumor tissue, and 5 microsatellite regions are amplified. The amplified base sequences are then separated according to their length by capillary electrophoresis. The migration patterns are then evaluated. Waveforms derived from normal cells are recognized within a certain range. If an abnormal waveform is detected outside the normal range, it is reported as MSI positive **(Figure 2)**. When some markers are unavailable, MSI-H is reported if ≥2 other markers are positive for MSI **(Table 7)**. If a diagnosis cannot be made based on the tumor site alone, comparison with normal tissue (a blood sample can be used as a substitute) is required.

Table 6 Overview of various panels

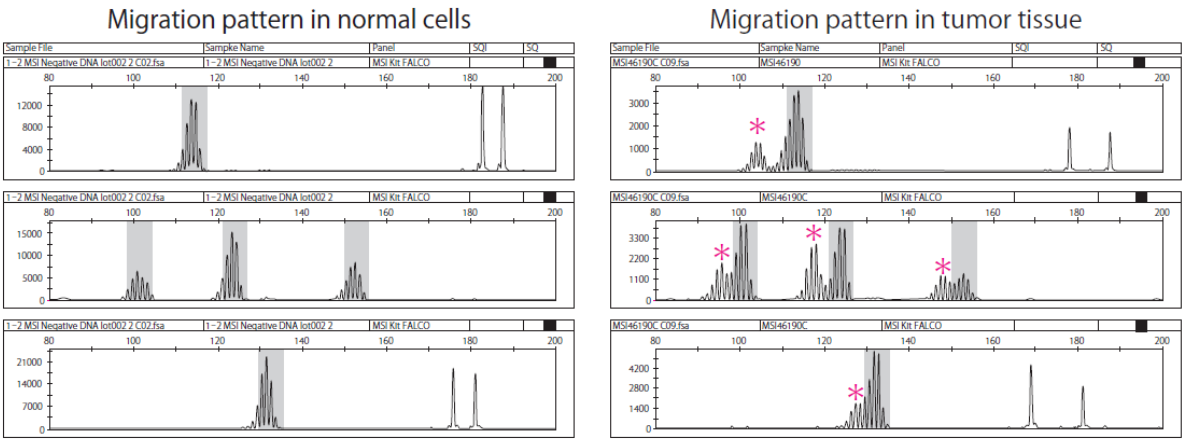
Bethesda panel

Marker name	Array structure
BAT25	1 base repeat
BAT26	1 base repeat

MSI test kit (FALCO)

Marker name	Array structure
BAT25	1 base repeat
BAT26	1 base repeat

D2S123	2-base repeat		NR21	1 base repeat
D5S346	2-base repeat		NR24	1 base repeat
D17S250	2-base repeat		MONO27	1 base repeat



After PCR amplification of the microsatellite marker region, fragment analysis is performed by capillary electrophoresis. A decrease in the number of repeats (*) is observed in tumor cell-derived DNA compared to normal cells. It is known that waveforms derived from normal cells are recognized within a certain range. The MSI test kit (FALCO) determines MSI positive status in tumor tissue by detecting waveforms outside the normal range.

Figure 2. MSI-H patients measured with MSI test kit (FALCO) (MSI positive for all 5 markers)

[Modified package insert of MSI test kit (FALCO)]

Table 7 Results of Cases A-E, in which some markers are not available and could not be inspected

Case	Marker A	Marker B	Marker C	Marker D	Marker E	Result
A.	(-)	(-)	(-)	(-)	unable to inspect	MSI-L or MSS
B.	(+)	(-)	(-)	(-)	unable to inspect	undecidable
C.	(+)	(+)	(-)	(-)	unable to inspect	MSI-H
D.	(+)	(+)	(-)	unable to inspect	unable to inspect	MSI-H

E.	(+)	(+)	unable to inspect	unable to inspect	unable to inspect	MSI-H

(+): MSI positive, (-): MSI not positive

Subject to MSI test

The MSI test (FALCO) is used for diagnosing Lynch syndrome, in patients with resectable colorectal cancer for the purpose of treatment selection according to the risk of recurrence, or in patients with unresectable metastatic colorectal cancer for the purpose of determining whether immune checkpoint inhibitors. This test is covered by insurance only once for one of these purposes. If this test is performed again for other purpose, the cost can be calculated separately only once in Japan.

IHC test as a test to determine MMR dysfunction

IHC evaluation of the expression of MMR proteins (MLH1, MSH2, MSH6, and PMS2) in tumors is another a common method for determining MMR dysfunction. During staining evaluation, internal positive controls (glandular bases of colonic mucosa and germinal centers of lymphoid follicles in non-tumor tissue) are used to confirm the adequacy of staining. All four proteins are expressed in tumors without MMR dysfunction, but in Lynch syndrome-associated tumors with MMR dysfunction, the expression of the protein corresponding to the inactivated MMR gene is lost.

There is no one-to-one correspondence between individual MMR gene abnormalities and loss of protein expression. MLH1 mutation is accompanied by loss of expression of MLH1 and PMS2, and MSH2 mutation is accompanied by loss of expression of MSH2 and MSH6. Most of these scenarios show a staining pattern as shown in **Table 8**. If staining results are obtained that do not fit the scenarios listed in **Table 8**, the validity of the staining should be confirmed before considering the patient as a possible exception, and MSI testing should be performed if necessary. For more details, please refer to the 2020 Guidelines for the Treatment of Hereditary Colorectal Cancer.

It has been reported that the IHC test shows a high concordance rate with the MSI test results in colorectal cancer. Four in-vitro diagnostic agents for examining the expression of MSH2 and MSH6 have been approved for manufacturing and marketing as companion diagnostics for pembrolizumab and are covered by health insurance since October 2022 (**Table 9**)**.** These tests are also covered by health insurance as screening tests for Lynch syndrome in colorectal cancer and as aids in the selection of chemotherapy for colorectal cancer.

Table 8 Relationship between MMR protein staining and each gene mutation by IHC method

		Expression in IHC staining			
		MLH1	MSH2	PMS2	MSH6
mutated gene	*MLH1*	-	+	-	+

	MSH2	+	-	+	-
	PMS2	+	+	-	+
	MSH6	+	+	+	-

Table 9 In-vitro diagnostics approved for IHC testing of MMR proteins

MMR protein	Roche/Ventana
MLH1	Ventana OptiView MLH1 (M1)
MSH2	Ventana OptiView MSH2 (G219-1129)
PMS2	Ventana OptiView PMS2 (A16-4)
MSH6	Ventana OptiView MSH6 (SP93)

Five KEYNOTE pooled analyses [KEYNOTE-012, -016, -028, -158, -164 (cohort A)] and the CheckMate-142 study found that anti-PD-1 antibody therapy was effective in patients diagnosed with dMMR by IHC testing. Additionally, in patients who were MSI-H negative by central review and in whom dMMR was detected by IHC testing, anti-PD-1 antibody therapy was shown to be effective.[15] In future clinical practice, even if the MSI test is determined to be MSS, it is assumed that re-evaluation by IHC would be useful in case false negative result was obtained for the reasons described later.

It has been reported that surgical specimens and biopsied tissues are comparable or superior to biopsied tissues in tests for determining MMR functional defects by IHC, and the uniformity of formalin fixation has suggested as the reason for this.[54-56] MSI testing using biopsy tissue requires attention to tumor cell content and DNA yield, but IHC testing has the advantage of confirming tumor cell content using hematoxylin and eosin (H&E) specimens.

However, there are a small number of mismatched cases between MSI and IHC tests. Even in MSI-H tumors, loss-of-function missense mutations may result in staining positive (pMMR) by IHC test, and cases may be MSS by MSI test due to low tumor cell ratio or MSH6 mutation. A thorough understanding of the characteristics of both tests is required. In addition, loss of expression of MSH6 and MLH1 proteins has been reported in specimens after preoperative chemoradiation therapy or cisplatin-containing regimens.[51,57-59] Particular attention should be paid to lower rectal cancer, for which chemoradiotherapy is one of the standard treatments. In addition, only pembrolizumab has been approved as a companion diagnostic test for determining MMR dysfunction using immunohistochemical staining, which was recently approved by the Pharmaceutical Affairs Law. Nivolumab is not approved (as of January 1, 2023). In clinical practice, there are quite a few cases in which it is impossible to collect a sufficient amount of tissue or cases in which the ratio of tumor cells is low. Therefore, any immune checkpoint inhibitor can be administered if it is performed using a test with already confirmed analytical validity.

NGS as a test to determine MMR functional defects

Testing to determine MMR dysfunction using NGS is also clinically useful. FoundationOne ® CDx Cancer Genomic Profile and OncoGuide ™ NCC Oncopanel System have been approved in Japan as comprehensive cancer genomic profiling tests using tissues (see Chapter 7, Comprehensive Genomic Profiling Tests Using Tissue Specimens). The FoundationOne ® CDx Cancer Genomic Profile can assess microsatellite markers in 95 intronic regions to determine MSI. The concordance rate with MSI and IHC tests is as high as 97%,[60] and it has been shown that the NGS method may be able to diagnose cases that are shown as MSS in the MSI test but yield dMMR results in the IHC test.[61] In June 2021, FoundationOne ® CDx was approved in Japan as a companion diagnostic for nivolumab and pembrolizumab for MSI-H cancer. The OncoGuide™ NCC Oncopanel covers 576 monorepeats to microsatellites of up to 5 bases, and the MSI score is calculated by comparing the tumor tissue and blood cells (normal). If the MSI score is 30 or higher, it is judged as MSI-H. (Not approved as a companion diagnostic as of January 1, 2023). In addition, algorithms such as the MSIsensor[62] (used in the MSK-IMPACT test), MOSAIC and MANTIS which use whole exome nucleotide sequence analysis[63,64] have been reported. However, the MSI determination method differs depending on the included microsatellite markers and algorithms, and this must be considered. In addition, NGS tests are more likely than other tests to be unmeasurable due to the influence of the amount and quality of submitted samples, and the turnaround time (TAT) required from sample submission to arrival of results takes several weeks. Therefore, it is necessary to carefully judge its use in clinical practice. As NGS results may not be completely consistent with the conventional companion test results, interpretation requires comprehensive judgment by an expert panel.

A high concordance rate has been reported in NGS using blood, compared with the conventional method using tissues for determination of MMR dysfunction.[65] In Japan, the Guardant360® CDx cancer gene panel obtained manufacturing and marketing approval on March 10, 2022 as a companion diagnostic for identifying patients with MSI-H solid tumors who are likely to respond to pembrolizumab and patients with MSI-H colorectal cancer who are likely to respond to nivolumab.

Comment 1　Regarding informed consent for testing to determine MMR functional deficit

Tests to determine MMR dysfunction have been performed as an adjunctive diagnosis and secondary screening for colorectal cancer patients suspected of having Lynch syndrome. Since the MSI test (FALCO) has been approved as a companion diagnostic for determining the indications of immune checkpoint inhibitors and as a treatment option for postoperative adjuvant therapy, the scope of testing has expanded to include all colorectal cancers, and the demand for MSI testing has surged. A dMMR test that uses immunostaining to evaluate protein expression has already been approved in Japan. If patients are undergoing testing to select treatment, they should be informed about a possible diagnosis of Lynch syndrome and informed consent should be obtained before the test is performed. The Japanese Society of Genetic Oncology

recommends that patients who do not actively suspect the possibility of Lynch syndrome should have a detailed understanding of Lynch syndrome before the test and consent based on the diagnosis of a hereditary tumor. In January 2022, an opinion was also issued stating that there is no need to obtain a medical certificate, necessary explanations should be given as part of normal medical practice, consent should be obtained, and this should be recorded in the medical record. In addition to providing information on Lynch syndrome according to test results and clinical necessity, medical professionals with experience in genetic medicine (clinical geneticists, certified genetic counselors, hereditary oncologists, familial tumor specialists, counselors, hereditary tumor coordinators, etc.) are recommended. If necessary, a system for providing genetic counseling by collaborating with medical professionals involved in genetic medicine should be established. For more information, please refer to the "Hereditary Colorectal Cancer Clinical Practice Guideline 2020."

[References]

1) Boland CR, Goel A: Microsatellite instability in colorectal cancer. Gastroenterology. 2010;138:2073-87.e3. https://doi.org/10.1053/j.gastro.2009.12.064

2) Ribic CM, Sargent DJ, Moore MJ, et al. Tumor microsatellite-instability status as a predictor of benefit from fluorouracil-based adjuvant chemotherapy for colon cancer. N Engl J Med. 2003;349:247-257. https://doi.org/10.1056/nejmoa022289

3) Chen DS, Mellman I. Elements of cancer immunity and the cancer-immune set point. Nature. 2017;541:321-330. https://doi.org/10.1038/nature21349

4) Llosa NJ, Cruise M, Tam A, et al. The vigorous immune microenvironment of microsatellite instable colon cancer is balanced by multiple counter-inhibitory checkpoints. Cancer Discov. 2015;5:43-51. https://doi.org/10.1158/2159-8290.cd-14-0863

5) Le DT, Uram JN, Wang H, et al. PD-1 Blockade in Tumors with Mismatch-Repair Deficiency. N Engl J Med. 2015;372:2509-2520.

6) Dung L, Thierry A, Won K, et al. P-274Pembrolizumab for patients with previously treated, mismatch repair-deficient microsatellite instability-high advanced colorectal carcinoma: phase 2 KEYNOTE-164 study. Annals of Oncology. 2016; 27 (Suppl 2): ii79-ii79. https://doi.org/10.1093/annonc/mdw199.264

7) Diaz L, Marabelle A, Kim TW, et al. 386PEfficacy of pembrolizumab in phase 2 KEYNOTE-164 and KEYNOTE-158 studies of microsatellite instability high cancers. Annals of Oncology. 2017;28(suppl_5). https://doi.org/10.1093/annonc/mdx367.020

8) Hara H, Yoshino T, Taniguchi H, et al. 1062-Phase 2 KEYNOTE-164 Study of Pembrolizumab (pembro) Monotherapy for Patients (pts) With Previously Treated, Mismatch Repair-Deficient (dMMR) Advanced

Colorectal Cancer (CRC): Primary and Japan Subgroup Analyses. Annals of Oncology. 2018;29(suppl_9):ix28-ix45.
https://doi.org/10.1093/annonc/mdy431.014
9) André T, Shiu KK, Kim TW, et al. KEYNOTE-177 Investigators: Pembrolizumab in Microsatellite-Instability-High Advanced Colorectal Cancer. N Engl J Med. 2020;383:2207-2218.
10) Diaz LA Jr, Shiu KK, Kim TW, et al. KEYNOTE-177 Investigators: Pembrolizumab versus chemotherapy for microsatellite instability-high or mismatch repair-deficient metastatic colorectal cancer (KEYNOTE-177): final analysis of a randomized, open-label, Phase 3 study. Lancet Oncol. 2022;23:659-670.
https://doi.org/10.1016/s1470-2045(22)00197-8
11) Overman MJ, McDermott R, Leach JL, et al. Nivolumab in patients with metastatic DNA mismatch repair-deficient or microsatellite instability-high colorectal cancer (CheckMate 142): an open-label, multicentre, phase 2 study. Lancet Oncol. 2017;18:1182-1191.
https://doi.org/10.1016/s1470-2045(17)30422-9
12) Lenz HJ, Van Cutsem E, Luisa Limon M, et al. First-Line Nivolumab Plus Low-Dose Ipilimumab for Microsatellite Instability-High/Mismatch Repair-Deficient Metastatic Colorectal Cancer: The Phase II CheckMate 142 Study. J Clin Oncol. 2022;40:161-170.
https://doi.org/10.1200/jco.21.01015
13) Le DT, Durham JN, Smith KN, et al. Mismatch repair deficiency predicts response of solid tumors to PD-1 blockade. Science. 2017;357:409-413.
14) Le DT, Kim TW, Van Cutsem E, et al. Phase II Open-Label Study of Pembrolizumab in Treatment-Refractory, Microsatellite Instability-High/Mismatch Repair-Deficient Metastatic Colorectal Cancer : KEYNOTE-164. J Clin Oncol. 2020;38:11-19.
https://doi.org/10.1200/jco.19.02107
15) Overman MJ, Lonardi S, Wong KYM, et al. Durable Clinical Benefit With Nivolumab Plus Ipilimumab in DNA Mismatch Repair-Deficient/Microsatellite Instability-High Metastatic Colorectal Cancer. J Clin Oncol. 2018;36:773-779.
https://doi.org/10.3410/f.732538488.793550886
16) Hutchins G, Southward K, Handley K, et al. Value of mismatch repair, KRAS, and BRAF mutations in predicting recurrence and benefits from chemotherapy in colorectal cancer. J Clin Oncol. 2011;29:1261-1270.
https://doi.org/10.1200/jco.2010.30.1366
17) Bertagnolli MM, Redston M, Compton CC, et al. Microsatellite instability and loss of heterozygosity at chromosomal location 18q: prospective evaluation of biomarkers for stages II and III colon cancer--a study of CALGB 9581 and 89803. J Clin Oncol. 2011;29:3153-162.
https://doi.org/10.1200/jco.2010.33.0092
18) Roth AD, Tejpar S, Delorenzi M, et al. Prognostic role of KRAS and BRAF in stageII and III resected

colon cancer：results of the translational study on the PETACC-3, EORTC 40993, SAKK 60-00 trial. J Clin Oncol. 2010;28:466-474.
https://doi.org/10.1200/jco.2009.23.3452
19) Yamanaka T, Oki E, Yamazaki K, et al. 12-Gene Recurrence Score Assay Stratifies the Recurrence Risk in StageII/III Colon Cancer With Surgery Alone：The SUNRISE Study. J Clin Oncol. 2016; 34:2906-2913.
https://doi.org/10.1200/jco.2016.67.0414
20) Asaka S, Arai Y, Nishimura Y, et al. Microsatellite instability-low colorectal cancer acquires a KRAS mutation during the progression from Dukes'A to Dukes'B. Carcinogenesis, 2009;30:494-499.
https://doi.org/10.1093/carcin/bgp017
21) Carethers JM, Chauhan DP, Fink D, et al. Mismatch repair proficiency and in vitro response to 5-fluorouracil. Gastroenterology. 1999;117:123-131.
https://doi.org/10.1016/s0016-5085(99)70558-5
22) Sargent DJ, Marsoni S, Monges G, et al. Defective mismatch repair as a predictive marker for lack of efficacy of fluorouracil-based adjuvant therapy in colon cancer. J Clin Oncol. 2010;28:3219-3226.
https://doi.org/10.1200/jco.2009.27.1825
23) Gavin PG, Colangelo LH, Fumagalli D, et al. Mutation profiling and microsatellite instability in stage II and III colon cancer: an assessment of their prognostic and oxaliplatin predictive value. Clin Cancer Res. 2012;18:6531-6541.
https://doi.org/10.1158/1078-0432.ccr-12-0605

24) Taieb J, Le Malicot K, Shi Q, et al. Prognostic Value of BRAF and KRAS Mutations in MSI and MSS StageIII Colon Cancer. J Natl Cancer Inst. 2016;109:djw272.
https://doi.org/10.1093/jnci/djw272
25) Zaanan A, Shi Q, Taieb J, et al. Role of Deficient DNA Mismatch Repair Status in Patients With StageIII Colon Cancer Treated With FOLFOX Adjuvant Chemotherapy：A Pooled Analysis From 2 Randomized Clinical Trials. JAMA Oncol. 2018;4:379-383.
https://doi.org/10.1001/jamaoncol.2017.2899
26) Sinicrope FA, Shi Q, Allegra CJ, et al. Association of DNA Mismatch Repair and Mutations in BRAF and KRAS With Survival After Recurrence in StageIII Colon Cancers: A Secondary Analysis of 2 Randomized Clinical Trials. JAMA Oncol. 2017;3:472-480.
https://doi.org/10.1001/jamaoncol.2016.5469
27) Park IJ, You YN, Agarwal A, et al. Neoadjuvant treatment response as an early response indicator for patients with rectal cancer. J Clin Oncol. 2012;30:1770-1776.
https://doi.org/10.1200/jco.2011.39.7901
28) de Rosa N, Rodriguez-Bigas MA, Chang GJ, et al. DNA Mismatch Repair Deficiency in Rectal Cancer: Benchmarking Its Impact on Prognosis, Neoadjuvant Response Prediction, and Clinical Cancer Genetics. J

Clin Oncol. 2016;34:3039-3046.
https://doi.org/10.1200/jco.2016.66.6826
29) Hasan S, Renz P, Wegner RE, et al. Microsatellite Instability (MSI) as an Independent Predictor of Pathologic Complete Response (PCR) in Locally Advanced Rectal Cancer: A National Cancer Database (NCDB) Analysis. Ann Surg. 2020;271:716-723.
https://doi.org/10.1097/sla.0000000000003051
30) Verschoor YL, Berg Jvd, Beets G, et al. Neoadjuvant nivolumab, ipilimumab, and celecoxib in MMR-proficient and MMR-deficient colon cancers: Final clinical analysis of the NICHE study. Journal of Clinical Oncology. 2022;40:3511-3511.
https://doi.org/10.1200/jco.2022.40.16_suppl.3511
31) Bando H, Tsukada Y, Inamori K, et al. Preoperative Chemoradiotherapy plus Nivolumab before Surgery in Patients with Microsatellite Stable and Microsatellite Instability-High Locally Advanced Rectal Cancer. Clin Cancer Res. 2022;28:1136-1146.
https://doi.org/10.1158/1078-0432.ccr-21-3213
32) Cercek A, Lumish M, Sinopoli J, et al. PD-1 Blockade in Mismatch Repair-Deficient, Locally Advanced Rectal Cancer. N Engl J Med. 2022;386:2363-2376.
33) Cercek A, Lumish MA, Sinopoli JC, et al. Single agent PD-1 blockade as curative-intent treatment in mismatch repair deficient locally advanced rectal cancer. Journal of Clinical Oncology. 2022;40: LBA5-LBA5.
https://doi.org/10.1200/jco.2022.40.17_suppl.lba5
34) Hampel H, Frankel WL, Martin E, et al. Screening for the Lynch syndrome (hereditary nonpolyposis colorectal cancer). N Engl J Med. 2005;352:1851-1860.
https://doi.org/10.1056/nejmoa043146
35) Barrow E, Hill J, Evans DG. Cancer risk in Lynch Syndrome. Fam Cancer. 2013;12:229-40.
36) Chika N, Eguchi H, Kumamoto K, et al. Prevalence of Lynch syndrome and Lynch-like syndrome among patients with colorectal cancer in a Japanese hospital-based population. Jpn J Clin Oncol. 2017;47: 108-117.
https://doi.org/10.1093/jjco/hyw178
37) Fujita M, Liu X, Iwasaki Y, et al. Population-based Screening for Hereditary Colorectal Cancer Variants in Japan. Clin Gastroenterol Hepatol. 2022;20:2132-41.
38) Aaltonen LA, Peltomäki P, Mecklin JP, et al. Replication errors in benign and malignant tumors from hereditary nonpolyposis colorectal cancer patients. Cancer Res. 1994;54:1645-1648.
39) Aaltonen LA, Salovaara R, Kristo P, et al. Incidence of hereditary nonpolyposis colorectal cancer and the feasibility of molecular screening for the disease. N Engl J Med. 1998;338:1481-1487.
https://doi.org/10.1056/nejm199805213382101
40) Peltomäki P. Role of DNA mismatch repair defects in the pathogenesis of human cancer. J Clin Oncol. 2003;21:1174-1179.

https://doi.org/10.1200/jco.2003.04.060
41) Ishikubo T, Nishimura Y, Yamaguchi K, et al. The clinical features of rectal cancers with high-frequency microsatellite instability (MSI-H) in Japanese males. Cancer Lett. 2004;216:55-62.
https://doi.org/10.1016/j.canlet.2004.07.017
42) van Lier MG, Leenen CH, Wagner A, et al. LIMO Study Group: Yield of routine molecular analyses in colorectal cancer patients ≤70 years to detect underlying Lynch syndrome. J Pathol. 2012;226:764-774.
https://doi.org/10.1002/path.3963
43) Julié C, Trésallet C, Brouquet A, et al. Identification in daily practice of patients with Lynch syndrome (hereditary nonpolyposis colorectal cancer): revised Bethesda guidelines-based approach versus molecular screening. Am J Gastroenterol. 2008;103:2825-35;quiz 2836.
https://doi.org/10.1111/j.1572-0241.2008.02084.x
44) Latham A, Srinivasan P, Kemel Y, et al. Microsatellite Instability Is Associated With the Presence of Lynch Syndrome Pan-Cancer. J Clin Oncol. 2019;37:286-295.
45) Järvinen HJ, Aarnio M, Mustonen H, et al. Controlled 15-year trial on screening for colorectal cancer in families with hereditary nonpolyposis colorectal cancer. Gastroenterology. 200;118:829-834.
https://doi.org/10.1016/s0016-5085(00)70168-5
46) Kempers MJ, Kuiper RP, Ockeloen CW, et al. Risk of colorectal and endometrial cancers in EPCAM deletion-positive Lynch syndrome: a cohort study. Lancet Oncol. 2011;12:49-55.
47) Dietmaier W, Wallinger S, Bocker T, et al. Diagnostic microsatellite instability: definition and correlation with repair mismatch protein expression. Cancer Res. 1997;57:4749-56.
48) Bocker T, Diermann J, Friedl W, et al. Microsatellite instability analysis: a multicenter study for reliability and quality control. Cancer Res. 1997;57:4739-4743.
49) Boland CR, Thibodeau SN, Hamilton SR, et al. A National Cancer Institute Workshop on Microsatellite Instability for cancer detection and familial predisposition: development of international criteria for the determination of microsatellite instability in colorectal cancer. Cancer Res. 1998;58:5248-5257.
https://doi.org/10.32388/zgr9m1
50) Clendenning M, Senter L, Hampel H, et al. A frame-shift mutation of PMS2 is a widespread cause of Lynch syndrome. J Med Genet. 2008;45:340-345.
https://doi.org/10.1136/jmg.2007.056150
51) Shia J, Zhang L, Shike M, et al. Secondary mutation in a coding mononucleotide tract in MSH6 causes loss of immunoexpression of MSH6 in colorectal carcinomas with MLH1/PMS2 deficiency. Mod Pathol. 2013;26:131-138.
https://doi.org/10.1038/modpathol.2012.138
52) Goel A, Nagasaka T, Hamelin R, et al. An optimized pentaplex PCR for detecting DNA mismatch repair-deficient colorectal cancers. PLoS One. 2010;5:e9393.
https://doi.org/10.1371/journal.pone.0009393

53) Bando H, Okamoto W, Fukui T, et al. Utility of the quasi-monomorphic variation range in unresectable metastatic colorectal cancer patients. Cancer Sci. 2018;109:3411-3415.
https://doi.org/10.1111/cas.1377
54) Shia J, Stadler Z, Weiser MR, et al. Immunohistochemical staining for DNA mismatch repair proteins in intestinal tract carcinoma: how reliable are biopsy samples- Am J Surg Pathol. 2011;35:447-454.
https://doi.org/10.1097/pas.0b013e31820a091d
55) Kumarasinghe AP, de Boer B, Bateman AC, et al. DNA mismatch repair enzyme immunohistochemistry in colorectal cancer: a comparison of biopsy and resection material. Pathology. 2010;42:414-420.
https://doi.org/10.3109/00313025.2010.493862
56) O'Brien O, Ryan É, Creavin B, et al. Correlation of immunohistochemical mismatch repair protein status between colorectal carcinoma endoscopic biopsy and resection specimens. J Clin Pathol. 2018;71:631-636.
https://doi.org/10.1136/jclinpath-2017-204946
57) Bao F, Panarelli NC, Rennert H, et al. Neoadjuvant therapy induces loss of MSH6 expression in colorectal carcinoma. Am J Surg Pathol. 2010;34:1798-1804.
https://doi.org/10.1097/pas.0b013e3181f906cc
58) Watanabe Y, Koi M, Hemmi H, et al. A change in microsatellite instability caused by cisplatin-based chemotherapy of ovarian cancer. Br J Cancer. 2001;85:1064-1069.
https://doi.org/10.1038/sj.bjc.6692037
59) Vilkin A, Halpern M, Morgenstern S, et al. How reliable is immunohistochemical staining for DNA mismatch repair proteins performed after neoadjuvant chemoradiation- Hum Pathol. 2014;45:2029-2036
https://doi.org/10.1016/j.humpath.2014.07.005
60) FoundationOne: SUMMARY OF SAFETY AND EFFECTIVENESS DATA (SSED).
61) Vanderwalde A, Spetzler D, Xiao N, et al. Microsatellite instability status determined by next-generation sequencing and compared with PD-L1 and tumor mutational burden in 11,348 patients. Cancer Med. 2018;7:746-756.
https://doi.org/10.1002/cam4.1372
62) Middha S, Zhang L, Nafa K, et al. Reliable Pan-Cancer Microsatellite Instability Assessment by Using Targeted Next-Generation Sequencing Data. JCO Precis Oncol 2017;PO.17.00084.
https://doi.org/10.1200/po.17.00084
63) Hause RJ, Pritchard CC, Shendure J, et al. Classification and characterization of microsatellite instability across 18 cancer types. Nat Med. 2016;22:1342-1350.
https://doi.org/10.1038/nm.4191
64) Bonneville R, Krook MA, Kautto EA, et al. Landscape of Microsatellite Instability Across 39 Cancer Types. JCO Precis Oncol 2017;PO.17.00073.
https://doi.org/10.1200/po.17.00073
65) Willis J, Lefterova MI, Artyomenko A, et al. Validation of Microsatellite Instability Detection Using a

Comprehensive Plasma-Based Genotyping Panel. Clin Cancer Res. 2019;25:7035-7045.

7 Comprehensive genomic profiling test using tissue samples

7.1 Background

Outline of comprehensive genomic profiling test (CGP) using next-generation sequencing

Next-generation sequencing (NGS) is a nucleotide sequence analysis method based on the principle of massively parallel sequencing. Compared to the conventional Sanger method, the ability to decode base sequences has dramatically improved, and it has become possible to decode a large amount of genomes at ultra-high speeds.[1,2] In addition to identification of differences (mutations) in genome sequences, genome copy number analysis (amplification/deletion), identification of modified genome sites and quantification of their frequency can be done. It is also possible to determine the amount (expression) of RNA by transcriptome analysis and search for fusion genes. Conventional cancer-related genomic testing has focused on single genes or a small number of genes, but NGS has made it possible to evaluate multiple genomic abnormalities at once. Consequently, driver gene abnormalities with low frequency have been identified, and molecular-targeted treatment for these driver genes has been developed.

Analytical Validity of CGP Testing

The purpose of comprehensive genomic profiling (CGP) testing is to obtain information on genetic abnormalities that will assist in the formulation of treatment strategies and the determination of therapeutic drug indications. It is essential that CGP has adequate clinical performance and that relevant results are reported.

All genes associated with molecularly targeted drugs, companion diagnostics and biomarkers in patients with solid tumors as well as genes associated with cancer initiation, proliferation, or suppression should be comprehensively included in the analysis.

The adequacy of the detection performance of a gene mutation is judged by its accuracy, precision, specificity and minimum detection sensitivity for representative base substitutions, insertions/deletions, copy number abnormalities, and fusion genes. In addition, clinical performance of its use as a companion diagnostic should be demonstrated through analytical equivalence with other companion diagnostics currently approved in Japan. In a study comparing *RAS* mutation testing or *BRAF* mutation testing by the NGS method with standard methods such as the direct sequencing method (Sanger method) using colorectal cancer tumor tissue samples, the concordance rate between the two results was reported to be as high as 92-100%.[3-6] In addition, panel testing of *KRAS/NRAS* exons 2, 3, 4 and *BRAF* exon 15 is similar in cost and time to results and requires less amount of DNA compared to testing all regions by the Sanger method .[7] FoundationOne® CDx Cancer Genomic Profile includes multiple companion diagnostics, and non-inferiority of concordance has been

confirmed for analytical equivalence with approved companion diagnostics. The result reporting process and content of the report must be appropriately managed based on the genetic abnormality detection criteria, data quality evaluation criteria, and report output criteria.

7.2

Basic requirements

Tissue-based comprehensive genomic profiling testing is strongly recommended to assess the indications for molecular targeted drugs in patients with unresectable colorectal cancer*.

Degree of recommendation

Strongly recommended [SR 9]

*The current comprehensive genomic profiling test targets “patients with solid cancer for whom there is no standard treatment, or patients with solid cancer for whom standard treatment has been completed due to local progression or metastasis (including those who are expected to complete).”

CGP testing encompasses genes reported to have abnormalities associated with the development, proliferation, or suppression of cancer, as well as genomic abnormalities associated with molecular-targeted drugs approved or under development as companion diagnostics and biomarkers. Therefore, it is possible to obtain information related to prognosis and the selection of drugs that are expected to have therapeutic effects. Treatment with targeted drugs has been shown to prolong prognosis of patients who have tumors with corresponding genomic alterations, compared with patients who cannot be treated with targeted drugs. Several integrated analysis of phase I trials of targeted therapy including colorectal cancer patients showed that the response rate, progression-free survival, and overall survival were favorable.[8,9,10] In addition, a meta-analysis verified the usefulness of CGP testing, where treatment selection was based on the results of gene panel testing in a total of 570 trials of 32,148 patients. These phase II trials targeted various cancer types, including gastrointestinal cancer and have shown favorable response rates, progression-free survival, and overall survival.[11] Therefore, CGP testing is strongly recommended for patients with unresectable advanced recurrent colorectal cancer, as it may lead to the selection of effective cancer drug therapy. However, the percentage of patients who receive treatment based on CGP testing remains at less than 10%.[12] The disadvantages of this method must be considered, such as the time taken to report results, and the possibility of detecting a hereditary tumor as a secondary finding, which may impose a psychological burden on the patient and his/her family.

The profile of detected genomic alterations is different for each type of cancer, as well as the treatment success rate. In a prospective observational study, the detection rate of genomic abnormalities linked to some form of treatment in colorectal cancer was 48 out of 60 (80%), and 124 out of 197 (62.9%) in the total analyzed cases, suggesting that CGP testing is a useful test for colorectal cancer.[13] In addition, in determining the indications for molecular-targeted treatment, it is more time and cost effective to determine the indications

for multiple targeted drugs through a single panel test, rather than conducting multiple companion diagnostics individually. It has been shown that CGP testing is more cost-effective than repeated conventional single-gene testing in non-small cell lung cancer, which is often treated with companion diagnostics[14]. Based on an analysis of 5 million people in the US insurance system, if 20% of tests normally performed are changed to CGP tests before first-line treatment, it is estimated that an additional 15.5 people with colorectal cancer will be able to receive treatment based on genomic information for a mere $0.003/month increase in testing costs per person.[15] Currently, CGP testing is intended for "patients with solid tumors for whom there is no standard treatment, or patients with solid tumors for whom standard treatment has been completed due to local progression or metastasis (including those whose completion is expected)." Despite the disadvantages discussed previously, which must be dealt with, it is desirable that CGP testing be performed before the start of first-line treatment in the future.

Pharmaceutically approved cancer gene panel tests **(Table 1)**

① OncoGuide ™ NCC Oncopanel System

DNA extracted from the tumor tissue and DNA derived from the patient's leukocytes are sequenced and the results are compared to accurately identify tumor-specific genomic abnormalities. Abnormalities in 124 cancer-related genes can be determined as well as the identification of 13 fusion genes, MSI, the tumor mutational burden (TMB), and features that define the germline variant.

② FoundationOne ® CDx Cancer Genomic Profile

Abnormalities in 324 cancer-related genes, 36 fusion genes, MSI, and TMB in DNA extracted from tumor tissue can be analyzed. In colorectal cancer, *KRAS/NRAS* gene mutation and MSI-H are established as companion diagnostics. In addition, MSI-H, TMB-H, and *NTRK1/2/3* fusion genes are established as companion diagnostics for solid tumors.

③ GenMineTOP cancer genome profiling system

By performing pair analysis of DNA base sequences derived from tumor tissue and non-tumor cells, base substitutions, insertions/deletions, and copy number abnormalities are detected for 737 cancer-related genes. At the same time, RNA analysis detects fusion genes and exon skipping and acquires expression information.

Table 1 Details of pharmaceutical approved cancer gene panel tests

Gene panel name	OncoGuide ™ NCC Oncopanel System	FoundationOne® CDx – cancer genomic profile
Number of targeted genes Nucleotide substitution,	124 13 (fusion gene)	324 36 (fusion gene)

insertion/deletion, copy number aberration, fusion, etc.		
Required sample	tissue DNA, blood DNA	tissue DNA
MSI test	Possible	Possible
TMB evaluation	Possible	Possible
Germline variant detection	Possible	Putative
Detection Criteria for Genetic Abnormalities	Base substitution Allele frequency 5% or more Insertion/Deletion Allele frequency 5% or more Abnormal copy number Median Depth ≥200, copy number ≥8 (Depth) ratio ≥4, log (Depth ratio) ≥2 in the region showing gene amplification Fusion gene Allele frequency 3% or more 2.0×10^{-6} or more as a percentage of the total number of reads	Base substitution Allele frequency 5% or more Insertion/Deletion Allele frequency 5% or more Abnormal copy number Tumor rate 20% or more Gene amplification: diploid: 6 or more copies (however, in the case of *ERBB2,* diploid: 5 or more copies), triploid: 7 or more copies, tetraploid: 8 or more copies Homozygous deletion: 0 copies Fusion gene ≥5 read pairs on different chromosomes or ≥10 Mbp apart (≥3 for known fusions)
Pharmaceutical approval date	December 25, 2018	December 27, 2018

MSI: microsatellite instability, TMB: tumor mutational burden

Treatment Based on Comprehensive Genomic Profiling Testing

If abnormalities are detected by the companion diagnostics that are approved by the Pharmaceutical Affairs Law, use of approved drugs is recommended. In other cases, based on the level of evidence and evaluation of clinical trials and advanced medical care, off-label use of drugs listed in the National Health Insurance drug price list can be used for treatment. Practical use of the patient-proposed healthcare services is also considered. At designated core hospitals, the percentage of patients who were treated based on CGP testing was 3.7% (28/754) from June 2019 to January 2020, and 7.7% (176/2,295) in February 2020 to January 2021, showing an increase over time ($p < 0.001$). In these time periods, the percentage of patients enrolled in clinical trials was 2.1% and 4.7% ($p = 0.002$), respectively, and the percentage of patients receiving approved drugs was 1.1% and 2.3% ($p = 0.048$), respectively.[12]

The patient-proposed healthcare services permits the use of off-label drugs at the request of the patient, but

the costs associated with implementation, including drug costs, are borne by the patient, and the actual procedure takes several months. As of January 1, 2023, the BELIEVE study is being conducted, which collects data on treatment results and safety of off-label patient-requested drugs. By preparing a master protocol for this trial and establishing the procedure up to drug delivery, it is possible to efficiently treat multiple genomic abnormalities. In this trial, the patient bears the costs related to conducting the trial, but the drug is provided free of charge.

Actionable Rare Fraction Recognized by CGP Testing

Among the actionable rare fractions detected in colorectal cancer, the main genomic abnormalities expected to be therapeutically effective are shown in **Table 2** (For MSI-H, see Chapter 6, Tests for Defective Mismatch Repair Function). Of these, the *NTRK1/2/3* fusion gene and TMB-H (described in the following sections) have been approved as companion diagnostics. If a CGP test confirms a genomic abnormality for which there is a companion diagnostic, and the expert panel recommends that drug administration for the genomic abnormality is appropriate based on package inserts, guidelines, literature, etc., the drug can be administered without having to perform a companion test again (Notification of the Health Insurance Bureau, Ministry of Health, Labour and Welfare on June 4, 2019). Therefore, entrectinib and larotrectinib can be administered as covered by health insurance even if the *NTRK1/2/3* fusion gene is detected in the OncoGuide ™ NCC oncopanel system. Similarly, pembrolizumab can be administered if TMB-H is observed. On the other hand, if other genomic abnormalities are detected by CGP testing for colorectal cancer, active participation in clinical trials is recommended because currently, no drugs are approved in Japan which target these abnormalities.

Table 2 Actionable rare fractions and testing methods

	Frequency in colorectal cancer [19,20] (%)	OncoGuide ™ NCC Oncopanel System	FoundationOne ® CDx Cancer Genomic Profile
NTRK1/2/3 fusion gene	~1	○	◎
TMB-H	5	○	◎
ERBB2 amplification	2 to 4	○	○
KRAS G12C	2	○	○
BRAF non V600E	2	○	○
MET amplification	2	○	○
ALK fusion gene	~1	○	○

ROS1 fusion gene	~1	○	○
RET fusion gene	~1	○	○

TMB: tumor mutational burden, ◎ : Approved as a companion diagnostic, ○: Approved as an in-vitro diagnostic

NTRK fusion gene

The neurotrophin receptor tyrosine kinase (NTRK) gene has subtypes *NTRK1, NTRK2,* and *NTRK3,* which encode the tropomyosin receptor kinase (TRK) proteins TRKA, TRKB, and TRKC, respectively. Activation of *NTRK* as an oncogene most commonly occurs as a fusion gene, and fusion genes are observed across organs. The *NTRK* fusion gene is found in more than 90% of rare cancer types, such as mammary gland carcinoma and salivary gland carcinoma, which develops in the head and neck region, and is found in less than 1% of colorectal cancer.[16] Entrectinib, a ROS1/TRK inhibitor, was approved in June 2019, and larotrectinib, a selective TRK inhibitor, approved in March 2021 both target NTRK fusion-positive solid tumors.

Since the *NTRK* fusion gene spans *NTRK1-3* and has a wide variety of fusion partners, an NGS test that can detect any fusion gene of *NTRK1-3* is recommended. A study in which MSK-IMPACT analyzed 38,095 specimens from 33,997 patients, including 2,929 colorectal cancer patients, reported that the sensitivity and specificity of the detection of *NTRK* fusion gene was 81.1% and 99.9% respectively.[17] The fusion genes covered by each test are different, so it is necessary to be familiar with the test details. FoundationOne ® CDx Cancer Genomic Profile and FoundationOne ® Liquid CDx Cancer Genomic Profile do not cover the intron region of *NTRK3*. These tests target *ETV6,* which is a frequent translocation partner.

TMB-H

Tumor mutational burden (TMB) is expressed in units of gene mutations per million bases (mut/Mb). TMB has traditionally been assessed by whole-genome testing and whole-exon testing. However, panel testing with TMB analysis regions greater than 1.1 mut/Mb correlated with results from whole-exon testing. As of January 1, 2023, it has been reported that both FoundationOne® CDx Cancer Genomic Profile and OncoGuide™ NCC Oncopanel System, which are approved for insurance in Japan, show high correlation.[18,19] The efficacy of pembrolizumab for treatment-refractory/intolerant, unresectable, advanced, recurrent solid tumors showing TMB-H was demonstrated in the KEYNOTE-158 phase II study, and was approved in Japan in February 2022.[20] In this study, TMB-H is defined as a TMB of 10 mut/Mb or higher, as determined by the FoundationOne ® CDx, which is also a companion diagnostic in Japan. The cutoff value of 10 mut/Mb for TMB was reached by consensus in a consortium of industry, government, and academia aimed at applying cutoff values across cancer types.[21,22]

On the other hand, it has been suggested that the TMB cut-off value related to the efficacy of immune checkpoint inhibitors may differ for each cancer type. A retrospective analysis of 1,678 patients with 16 types

of cancer treated with immune checkpoint inhibitors showed that among 50 patients with colorectal cancer, the response rate in those with a TMB < 10 mut/Mb (43 patients) was 5%, and 14% in those with a TMB ≥ 10 (7 patients).[23] In a retrospective analysis of 137 colorectal cancer patients treated with immune checkpoint inhibitors, excluding dMMR patients and patients with POLD1 or POLE mutations from the group with TMB ≥ 10 mut/Mb, no significant difference was observed in overall survival in those with a TMB < 10 mut/Mb compared to those with a TMB ≥ 10 mut/Mb.[24] The TMB value calculated by each gene panel test may differ even in the same case. It should be noted that the TMB threshold in other panels may differ from the FoundationOne ® CDx Cancer Genomic Profile.[25]

Side Note 1 Variant of unknown significance (VUS)

Genomic analysis of tumor cells using NGS reveals many mutations, many of which are VUS. In many cases, the significance of low-frequency mutations in oncogenes is not clear, such as whether they are driver mutations that cause carcinogenesis, or whether they are accidental passenger mutations. Interpretation of such VUS is often difficult because it is unclear whether the mutations are carcinogenic or whether the patient has drug susceptibility. Regarding the response to VUS, it is considered appropriate for an expert panel to decide on a policy based on reference to public databases such as ClinVar and COSMIC, and gene-related genome information databases that have already been sufficiently annotated.

Side Note 2 Secondary Findings

Finding a conclusive germline pathogenic mutation on CGP testing is termed a secondary finding. Of the 1,040 individuals who underwent the MSK-IMPACT test, 101 (56%) of the 182 individuals with blood-confirmed germline variants had hereditary tumors not suspected based on family history or phenotype,[26] thus CGP testing should be performed on the assumption that secondary findings are present. CGP testing in colorectal cancer patients reportedly found 15 of 151 (9.9%) to have a germline variant associated with colorectal cancer.[27] As for the response to the secondary findings, we will refer to the "Guidelines for Communication Processes in Genomic Medicine" of “Extraction of ethical and social issues and improvement of social environment toward the realization of a society where people can benefit from genomic medicine without anxiety,” which was conducted as part of the research project on ethical, legal, and social issues supported by the Health, Labour and Welfare sciences research grants.

[References]

1) Meyerson M, Gabriel S, Getz G: Advances in understanding cancer genomes through second-generation sequencing. Nat Rev Genet. 2010;11:685-696.

https://doi.org/10.1038/nrg2841

2) Mardis ER. A decade's perspective on DNA sequencing technology. Nature. 2011;470:198-203.

3) Gao J, Wu H, Wang L, et al. Validation of targeted next-generation sequencing for RAS mutation detection in FFPE colorectal cancer tissues: comparison with Sanger sequencing and ARMS-Scorpion real-time PCR. BMJ Open. 2016;6:e00953.2

https://doi.org/10.1136/bmjopen-2015-009532

4) Sakai K, Tsurutani J, Yamanaka T, et al. Extended RAS and BRAF Mutation Analysis Using Next-Generation Sequencing. PLoS One. 2015;10:e0121891.

https://doi.org/10.1371/journal.pone.0121891

5) Kothari N, Schell MJ, Teer JK, et al. Comparison of KRAS mutation analysis of colorectal cancer samples by standard testing and next-generation sequencing. J Clin Pathol. 2014;67:764-767.

https://doi.org/10.1136/jclinpath-2014-202405

6) Malapelle U, Vigliar E, Sgariglia R, et al. Ion Torrent next-generation sequencing for routine identification of clinically relevant mutations in colorectal cancer patients. J Clin Pathol. 2015;68:64-68.

https://doi.org/10.1136/jclinpath-2014-202691

7) Belardinilli F, Capalbo C, Buffone A, et al. Validation of the Ion Torrent PGM sequencing for the prospective routine molecular diagnostic of colorectal cancer. Clin Biochem. 2015;48:908-910.

https://doi.org/10.1016/j.clinbiochem.2015.04.003

8) Tsimberidou AM, Wen S, Hong DS, et al. Personalized medicine for patients with advanced cancer in the phase I program at MD Anderson: validation and landmark analyses. Clin Cancer Res. 2014;20: 4827-4836.

https://doi.org/10.1158/1078-0432.ccr-14-0603

9) Tsimberidou AM, Hong DS, Ye Y, et al. Initiative for Molecular Profiling and Advanced Cancer Therapy (IMPACT): An MD Anderson Precision Medicine Study. JCO Precis Oncol 2017;PO.17.00002.

https://doi.org/10.1200/po.17.00002

10) Schwaederle M, Zhao M, Lee JJ, et al. Association of Biomarker-Based Treatment Strategies With Response Rates and Progression-Free Survival in Refractory Malignant Neoplasms: A Meta-analysis. JAMA Oncol. 2016;2:1452-1459.

https://doi.org/10.1001/jamaoncol.2016.2129

11) Schwaederle M, Zhao M, Lee JJ, et al. Impact of Precision Medicine in Diverse Cancers: A Meta-Analysis of Phase II Clinical Trials. J Clin Oncol. 2015;33:3817-3825.

https://doi.org/10.1200/jco.2015.61.5997

12) Sunami K, Naito Y, Komine K, et al. Chronological improvement in precision oncology implementation in Japan. Cancer Sci. 2022;113:3995-4000.

https://doi.org/10.1111/cas.15517

13) Kondo T, Matsubara J, Quy PN, et al. Comprehensive genomic profiling for patients with chemotherapy-naïve advanced cancer. Cancer Sci. 2021;112:296-304.

https://doi.org/10.1111/cas.14674

14) Pennell NA, Mutebi A, Zhou ZY, et al. Economic Impact of Next-Generation Sequencing Versus Single-Gene Testing to Detect Genomic Alterations in Metastatic Non-Small-Cell Lung Cancer Using a Decision Analytic Model. JCO Precis Oncol. 2019;3:1-9.

https://doi.org/10.1200/po.18.00356

15) Proudman D, DeVito NC, Belinson S, et al. Comprehensive genomic profiling in advanced/metastatic colorectal cancer: number needed to test and budget impact of expanded first line use. J Med Econ. 2022;25:817-825.

https://doi.org/10.1080/13696998.2022.2080463

16) Cocco E, Scaltriti M, Drilon A: NTRK fusion-positive cancers and TRK inhibitor therapy. Nat Rev Clin Oncol. 2018;15:731-747.

https://doi.org/10.1038/s41571-018-0113-

17) Solomon JP, Linkov I, Rosado A, et al. NTRK fusion detection across multiple assays and 33,997 cases: diagnostic implications and pitfalls. Mod Pathol. 2020;33:38-46.

https://doi.org/10.1038/s41379-019-0324-7

18) Chalmers ZR, Connelly CF, Fabrizio D, et al. Analysis of 100,000 human cancer genomes reveals the landscape of tumor mutational burden. Genome Med. 2017;9:34.

19) Sunami K, Ichikawa H, Kubo T, et al. Feasibility and utility of a panel testing for 114 cancer-associated genes in a clinical setting: A hospital-based study. Cancer Sci. 2019;110:1480-1490.

20) Marabelle A, Fakih M, Lopez J, et al. Association of tumor mutational burden with outcomes in patients with advanced solid tumors treated with pembrolizumab: prospective biomarker analysis of the multicohort, open-label, phase 2 KEYNOTE-158 study. Lancet Oncol. 2020;21:1353-1365.

https://doi.org/10.1016/s1470-2045(20)30445-9

21) Japanese Society of Clinical Oncology/Japanese Society of Oncology/Japanese Society of Pediatric Hematology/Oncology: Guideline for cross-organ genomic diagnosis in advanced solid tumors in adults and children, 3rd edition. Kanehara Publishing, 2022.

22) Merino DM, McShane LM, Fabrizio D, et al. Establishing guidelines to harmonize tumor mutational burden (TMB): in silico assessment of variation in TMB quantification across diagnostic platforms: phase I of the Friends of Cancer Research TMB Harmonization Project. J Immunother. 2020;e000147.

https://doi.org/10.1136/jitc-2019-000147

23) Valero C, Lee M, Hoen D, et al. Response Rates to Anti-PD-1 Immunotherapy in Microsatellite-Stable Solid Tumors With 10 or More Mutations per Megabase. JAMA Oncol. 2021;7:739-743.

https://doi.org/10.1001/jamaoncol.2020.7684

24) Rousseau B, Foote MB, Maron SB, et al. The Spectrum of Benefit from Checkpoint Blockade in Hypermutated Tumors. N Engl J Med. 2021;384:1168-1170.

https://doi.org/10.1056/nejmc2031965

25) AMED Deregulation/Evaluation Research Project for Pharmaceuticals, etc. “Considerations for Standardization of Companion Diagnostic Systems Using Gene Panel Testing” (Nagai Group) Proposal “Points to Note Regarding Analytical Equivalence Evaluation of Gene Panel Testing”.

26) Mandelker D, Zhang L, Kemel Y, et al. Mutation Detection in Patients With Advanced Cancer by Universal Sequencing of Cancer-Related Genes in Tumor and Normal DNA vs Guideline-Based Germline Testing. JAMA. 2017;318:825-835.

27) You YN, BorRAS E, Chang K, et al. Detection of Pathogenic Germline Variants Among Patients With Advanced Colorectal Cancer Undergoing Tumor Genomic Profiling for Precision Medicine. Dis Colon Rectum. 2019;62:429-437.
https://doi.org/10.1097/dcr.0000000000001322

8 Liquid biopsy

8.1 Background

Liquid biopsy for colorectal cancer

Liquid biopsy for colorectal cancer

Liquid biopsy is a test method for diagnosing tumor status using body fluid samples such as blood and urine, without directly collecting tumor tissue. Human blood normally contains a certain amount of free DNA, but cancer patients are known to have an increased amount. DNA present in plasma, including those derived from normal cells and tumors, is called cell free DNA (cfDNA), and tumor-derived cfDNA in cancer patients is called circulating tumor DNA (ctDNA). Somatic cell genetic testing using ctDNA is expected to be used in various aspects of colorectal cancer treatment as a minimally invasive, real-time testing method for detecting genetic abnormalities in tumors[1,2] (Figure 1).

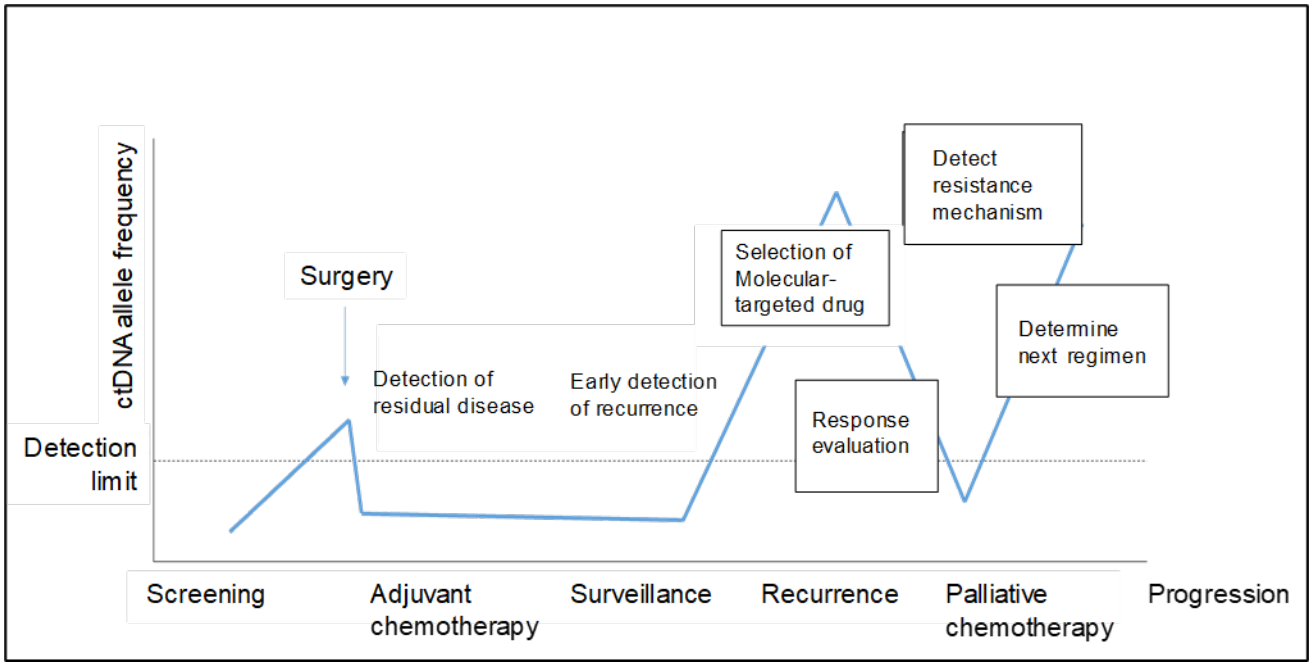


Figure 1 Assumed clinical usefulness of ctDNA testing

Concordance with genetic aberrations and ctDNA abundance in tumor tissue

The advent of ultra-sensitive mutation detection methods such as the BEAMing method and droplet digital PCR method has dramatically improved the sensitivity of gene mutation detection using ctDNA. The OncoBEAM™ *RAS* CRC kit, which uses ctDNA to detect *KRAS/NRAS* mutations by the BEAMing method, has been reported to have a high concordance rate with tumor tissue samples and has been approved in Japan (see Chapter 3, *RAS Mutation* Test). In addition, it has been reported that the amount of ctDNA before starting

chemotherapy is a poor prognostic factor in unresectable cases.[3] A meta-analysis of 1,076 colorectal cancer patients from 10 studies found that the group with high pretreatment levels of ctDNA above the median had a significantly worse prognosis (HR 2.39, 95% CI 2.03-2.82, $p < 0.001$).[4] In addition, there are many reports that the reduction of ctDNA in the early posttreatment period is useful as a predictor of early treatment efficacy. Tie et al. reported that ctDNA can be detected in 48 cases (92.3%) of 52 cases of colorectal cancer in which mutated alleles were detected in the tumor tissue before starting treatment. In addition, they showed that reduction in ctDNA early in treatment (before the start of the second course) correlated with treatment efficacy[5]. It may be possible to predict tumor burden and chemotherapy efficacy more sensitively than existing tumor markers.

Comprehensive genomic profiling test using plasma samples

Patients with recurrent colorectal cancer may have tumors in internal organs such as the liver and lungs, and collecting tumor tissue to perform genetic testing is invasive. If genetic testing can be performed using ctDNA, tumor tissue collection can be avoided. Furthermore, genetic testing using ctDNA is not affected by tumor heterogeneity, and allows evaluation of genetic abnormalities in the patient's tumor as a whole. From this, it is possible to estimate changes over time in clonal evolution, the degree of tumor progression, and the state of response to and resistance to therapeutic drugs. Since there is no need to process histopathological specimens, the turnaround time (TAT) can be shortened, and it is particularly useful when a treatment regimen is quickly determined and drug therapy can commence.

On the other hand, the accuracy of ctDNA testing is affected by the amount of ctDNA exuded into the blood. Care must be taken because the ctDNA exudation rate is affected by metastatic organs and tumor burden. In addition, ctDNA has false positives due to clonal hematopoiesis of indeterminate potential (CHIP). CHIP is a phenomenon in which somatic mutations accumulate in hematopoietic stem cells with aging, resulting in clonal proliferation of blood cells with specific mutations, and is reported to be associated with blood cancer and coronary heart disease. CHIP-derived mutations can be reported as pathogenic variants in plasma CGP testing. CHIP-related mutation allele frequencies are generally considered to be lower than those for advanced cancer, but there is no method to clearly distinguish between the two, so caution is required in interpreting the results. **Table 1** summarizes the advantages and precautions of CGP testing with ctDNA and tumor tissue.

Table 1 Advantages and precautions of CGP testing using plasma and tissue specimens (partially modified from “Policy Recommendations for Appropriate Use of Cancer Genome Profiling Tests Using Circulating Tumor DNA”)

	Advantage	Precautions
Plasma	• Less invasive, allowing repeated	• May not be detected if the tumor burden is not sufficient

	sample collection • Current genome profile can be obtained • Short time to obtain results • Evaluation of heterogeneity	• Amount of false negatives may be higher than tissue samples • Frequency of false positives by CHIP increases with age • There is a possibility of false negatives in cancer types and pathological conditions where the detection rate of genetic abnormalities in plasma samples is low * • Evaluation of copy number alterations and gene fusions can be difficult
Organization	• Direct evaluation of genetic abnormalities in tumor cells	• Invasive sample collection • It takes time to obtain the result • False negative results when the tumor cell ratio is low • Historical specimens may not reflect current genetic abnormalities in tumor cells • If a long time has passed since sample collection, sample deterioration may occur.

CHIP: clonal hematopoiesis of indeterminate potential

* Brain, bladder, and pancreatic cancers are reported to have low detection rates of genetic alterations in plasma specimens.[6,7] It has also been reported that the detection rate is low for colorectal cancer with only lung metastasis or only peritoneal metastasis.[8,9]

ctDNA testing for minimal residual tumor (MRD) detection

ctDNA is known to have an extremely short half-life in plasma compared to tumor markers such as CEA, CA19-9, etc.[10] Therefore, after curative resection, ctDNA rapidly disappears from the blood if there is no residual cancer. Taking advantage of this characteristic of ctDNA, many diagnostic systems using next-generation sequencing technology for detecting minimal residual disease (MRD) have been developed.

Diagnostic systems can be divided into two types: the tumor-informed approach and the tumor-uninformed/tumor-naive approach (**Table 2**). Tumor-informed approach is a method of performing genetic analysis of tumor tissue, creating a custom gene panel based on the results, and evaluating ctDNA. Signatera™ is an example of a tumor-informed approach. It performs whole-exome analysis of tumor tissue, selects 16 tumor-specific and clonal single-nucleotide variations (SNVs) present in the patient, and creates a custom gene panel. The tumor-uninformed/tumor-naive approach is a method of evaluating pre-established genetic alterations with ctDNA. Guardant Reveal™ is an example of a tumor-uninformed/tumor-naive approach. Compared to tumor-uninformed/tumor-naive approaches, the tumor-informed approach reduces background signaling due to non-tumor-derived mutations, so it is possible to lower the detection limit by increasing the sequencing depth.[11] On the other hand, the tumor-informed approach requires tumor tissue and

a long TAT to create a custom gene panel. It has been reported that the tumor-uninformed/tumor-naive approach can be more sensitive by comprehensively analyzing changes in the epigenome.[12]

Table 2 MRD-detecting ctDNA gene panel test

	Tumor-informed approach	Tumor-uninformed/tumor-naive approach
Baseline sample	Requires tumor tissue or plasma	Not required
Analysis target gene	Determined based on results of genetic analysis of baseline samples	Predefined genes are targeted for analysis
Advantage	Low detection limit due to selection of specific genes	Shorter TAT compared to tumor-informed approaches

MRD: minimal residual tumor, ctDNA: circulating tumor DNA, TAT: Turnaround time

8.2 Comprehensive Genomic Profiling Test Using Plasma Specimens

Basic requirements

Circulating tumor DNA-based comprehensive genomic profiling testing is strongly recommended to assess the indications for molecular targeted drugs in patients with unresectable colorectal cancer.

Degree of recommendation

Strongly recommended [SR 9]

*The current comprehensive genomic profiling test includes “patients with solid cancer for whom there is no standard treatment, or patients with solid cancer for whom standard treatment has been completed due to local progression or metastasis (including those who are expected to complete).” Targeted.

Significance of Comprehensive Genomic Profiling Test Using Plasma Specimens

According to a report by SCRUM-Japan, a large-scale genomic screening project in Japan, CGP tests using plasma samples have a significantly shorter TAT than tests using tissue samples. The median TAT for plasma samples was 11 days, whereas the median TAT for tissue samples was 33 days ($p < 0.0001$), and the rate of participation in clinical trials based on test results was 9.5% for blood samples and 4.1% for tissue samples ($p < 0.0001$).[13] CGP tests using plasma specimens are difficult to detect in cases where there is insufficient ctDNA in the blood, such as cases with lung metastasis only or peritoneal metastasis only, and evaluation of copy number alterations and gene fusions is difficult. As detailed in Table 1, there are precautions to note regarding CGP testing using plasma such as the possibility of false positives due to CHIP, whether sufficient tissue specimens suitable for testing can be obtained, or results need to be returned in a short timeframe. It is strongly recommended to perform this testing on patients who can adequately benefit from it.

Under current insurance coverage, it can be calculated only once per patient in cases where standard treatment

has been completed (including those that are expected to be completed), as an aid in assessing therapeutic drug eligibility.

Discovery and monitoring of therapeutic targets using ctDNA

In colorectal cancer, development of molecular-targeted drugs for new therapeutic targets is progressing. A phase Ib trial of vemurafenib (BRAF inhibitor) + irinotecan + cetuximab therapy for *BRAF* V600E mutation-positive, unresectable, recurrent, colorectal cancer reported a response rate of 35%, and pretreatment ctDNA testing detected the *BRAF* V600E in all 12 cases. In addition, it has been reported that the degree of ctDNA reduction after commencing treatment was strongly correlated with the degree of response.[14] Similarly, in a phase II trial of HER2 antibody drug trastuzumab therapy targeting HER2-positive colorectal cancer in tumor tissue, HER2 amplification was detectable in 96.6% (28/29) of ctDNA before treatment. A correlation between the therapeutic effect and the copy number in ctDNA has been reported.[15] In the TRIUMPH study (see Chapter 5, HER2 testing for details), the positive concordance rate, negative concordance rate, and overall concordance rate for *HER2* amplification in tissue and ctDNA analysis were 82%, 83%, and 83%, respectively. Furthermore, it has been reported that the response rate to pertuzumab and trastuzumab combination therapy in HER2-positive patients by liquid biopsy was 28%, exceeding the preset efficacy evaluation criteria. The study also included ctDNA testing over time and reported the following[16]: (1) efficacy was higher in patients with high HER2 gene copy number and in the absence of other cancer genomic abnormalities compared to other patients; (2) effectiveness was high in cases where ctDNA decreased after 3 weeks of treatment compared to the pretreatment level; (3) various cancer genome abnormalities newly appeared in ctDNA after treatment stopped working.

In this way, considering the development of new molecular-targeted treatments for unresectable colorectal cancer, CGP testing using ctDNA, similar to CGP testing using tumor tissue, is clinically useful in terms of searching for therapeutic targets. However, to increase sensitivity to a level comparable to tissue CGP using tumor tissue, it is necessary to limit the number of genes analyzed to a few dozen. There are short-term benefits to GP testing using ctDNA.

Detection of resistance mechanisms and selection of new therapeutic agents

After administration of anti-EGFR antibody drugs to *RAS* wild-type unresectable advanced colorectal cancer, multiple genetic abnormalities that are assumed to be resistance factors such as *EGFR, KRAS, NRAS, BRAF* mutations, *HER2* amplification, and *MET* amplification are detected in ctDNA.[6,17] In addition, it has been reported that the treatment effect is high in patients in whom multiple resistance gene abnormalities were not detected in the ctDNA test before readministration of anti-EGFR antibody drugs.[18]

In this way, ctDNA somatic genetic testing using blood samples for unresectable, advanced, recurrent colorectal cancer is not only an alternative to tumor tissue testing, but also a tumor-wide analysis that considers heterogeneity within the tumor. It has been established as a minimally invasive test that can

determine genetic status over time. It is possible to obtain useful information not only for finding therapeutic targets but also for predicting therapeutic effects and acquisition of resistance. For example, when re-challenging with anti-EGFR antibody drugs, a correlation is observed between the genetic status of ctDNA immediately before treatment and the therapeutic effect.

Based on the above, ctDNA gene panel testing for unresectable colorectal cancer is strongly recommended as a repeatable, minimally invasive test for identifying therapeutic targets and monitoring therapeutic efficacy. As chronological monitoring is useful in treatment of unresectable colorectal cancer, the fact that ctDNA testing can be performed multiple times over the clinical course will be advantageous on introducing it in clinical practice.

Pharmaceutically Approved Comprehensive Genomic Profiling Test Using Plasma Specimens

In Japan, the FoundationOne ® Liquid CDx Cancer Genome Profile and Guardant360 ® CDx Cancer Gene Panel have received regulatory approval (**Table 3)**.

① FoundationOne ® Liquid CDx Cancer Genomic Profile

This test targets all coding exons of 309 genes, including introns or noncoding regions in 21 genes. In another 15 genes, introns or non-coding regions are targeted, and it is possible to test mutations (SNVs, insertion/deletions or fusion genes) in a total of 324 genes. In a study comparing the results of tests using blood samples and tissue samples collected from the same patient, the concordance rate of gene mutations detected in both tests was 75%.[19] In addition, although this test can measure copy number alterations (not subject to pharmaceutical approval), it is difficult to evaluate copy number alterations when the ratio of ctDNA to cfDNA (tumor fraction: TF) is low. In the FoundationOne® Liquid CDx Cancer Genomic Profile, the detection limit for copy number changes in the TF is 20%, so caution is required for the potential of false negatives for copy number changes.[20,21]

② Guardant360® CDx Oncogene Panel

This panel detects 74 cancer-related gene abnormalities and MSI status by digital sequencing technology which combines NGS and Guardant Health's proprietary bioinformatics technology. Concordance with the comparator NGS assay was 82.5%. The positive concordance rate for detection of insertion/deletions and SNVs was 91.4% and the negative concordance rate was a ≥99%.[22]

Table 3 Biomarkers and test methods

	FoundationOne® Liquid CDx cancer genomic profile	Guardant360® CDx cancer gene panel
Number of target genes	324	74

TMB	△	—
MSI	△	◎
NTRK1/2/3 fusion gene	◎	○
KRAS/NRAS	○	○
BRAF V600E	○	○
HER2 amplification	△	○

◎ Approved as a companion diagnostic, ○Approved as an in-vitro diagnostic, △ Measurable but not approved under the Pharmaceutical Affairs Law

TMB in a comprehensive genomic profiling assay using plasma samples

In 69 patients with solid tumors who were treated with immune checkpoint inhibitors, those with a variant of unknown significance (VUS) exceeding 3 were analyzed using Guardant360® CDx Oncogene Panel, and these patients were reported to have significantly longer progression-free survival.[23] The OAK and POPLAR trials validated the superiority of atezolizumab over docetaxel in non-small cell lung cancer. These studies were retrospectively analysed with Foundation Medicine (may not be identical to FoundationOne ® Liquid CDx cancer genomic profile covered by insurance) to perform ctDNA analysis of blood TMB (bTMB). It found that atezolizumab is most effective in patients with a TMB of 16 mut/Mb or higher.[24] On the other hand, in the B-F1RST study, a prospective study which evaluated the efficacy of atezolizumab in patients with elevated bTMB, there was no statistically significant difference in progression-free survival between the bTMB ≥ 16 group and the bTMB < 16 group (HR 0.80, p =0.35).[25] However, the response rate improved with increasing bTMB score, and the bTMB ≥ 16 group had a significantly longer overall survival than the bTMB < 16 group (HR 0.54, p = 0.032). Similarly, a prospective phase II study called CheckMate-848 evaluated nivolumab monotherapy and combination therapy with nivolumab and ipilimumab in immunotherapy-naïve patients with advanced solid tumors who were refractory to standard therapy. It found that the tissue TMB (tTMB) ≥ 10 group had a better response rate than the bTMB ≥ 10 group for both nivolumab monotherapy and nivolumab and ipilimumab combination therapy.[26]

8.3 ctDNA Testing for Minimal Residual Tumor Detection and Recurrence Monitoring

Basic requirements

Gene panel test detecting minimal residual disease is strongly recommended to assess the optimal adjuvant chemotherapy in patients with CRC having received curative resection.

Degree of recommendation

Strongly recommended [SR 8, R 1]

*As of January 1, 2023, there is no panel test for detecting minimal residual tumor that is approved by the pharmaceutical affairs and covered by insurance for the purpose of selecting treatment according to the risk of recurrence in patients with resectable, advanced, recurrent colorectal cancer, and clinical efficacy has already been demonstrated in prospective phase II studies. Therefore, it was "strongly recommended."

By tagging the genes to be amplified, Tie et al. made it possible to easily distinguish between genetic mutations and read errors[27]. Using Plasma-Safe-SeqS, which performs next-generation sequencing with increased sensitivity, they have developed a diagnostic panel for over 10 genes which are frequently mutated in colorectal cancer, including *APC, TP53*, and *SMAD4*. Of the 231 patients with Stage II colon cancer who underwent curative resection, tumor tissue was examined, and at least one mutations was detectable in 230 (99.6%). Among 178 patients who did not receive adjuvant chemotherapy, ctDNA was detected in 14 patients (7.9%), and recurrence occurred in 11 (79%) of these. Recurrence occurred in 16 (9.8%) of 164 patients who did not have ctDNA (HR 18, 95% CI 7.9-40, $p < 0.001$). ctDNA detection after curative resection identifies patients at a high risk of recurrence.[27] Similarly, in the 37 cases of curative resection of liver metastases, the 3-year recurrence-free survival rate was 0% in the mutation allele-positive group and 84% in the mutation-negative group, showing a large difference (HR 13, 95% CI 19-325, $p < 0.001$). A prospective observational study including stage III colon cancer (58 cases, HR 17) and locally advanced rectal cancer (159 cases, HR 13) reported similar results.[28,29] In addition, Reinert et al. evaluated recurrence using the Signatera method, in which 16 genes were extracted from all exons of resected tumor tissue, primers were prepared, and MRD was monitored from postoperative blood samples. They tested 130 patients with Stage I to III colorectal cancer after curative resection and found that the recurrence rate was significantly higher in patients who were ctDNA-positive 30 days after surgery (HR 7.2, $p < 0.001$).[30] In Japan, the COSMOS-CRC-01 study is evaluating the chronological changes in ctDNA using Guardant Reveal for resectable Stage 0-III colorectal cancer. An interim analysis for Stage II to III cases reported one year disease free survival in patients with positive MRD as 81.2%. The average time from when postoperative ctDNA was detected to the confirmation of recurrence by radiological imaging was 6.6 months.[31] In addition, the GALAXY trial evaluating Signatera in radically resectable colorectal cancer is underway. The study reported that ctDNA-positive cases at 4 weeks had a very high risk of recurrence (HR 10.9, $p < 0.001$). Multivariate analysis of factors related to recurrence

in Stage II/III cases showed that ctDNA positivity at 4 weeks was the most powerful predictor of recurrence, more than T factor, N factor, *RAS* mutation, and *BRAF* mutation. In addition, in the ctDNA-positive patients at 4 weeks, the negative conversion rate at 6 months after surgery was significantly higher in the group receiving adjuvant chemotherapy, regardless of pathological stage (HR 9.3, $p < 0.001$). If ctDNA was detected 4 weeks post-surgery, but was not detected at 12 weeks, the disease-free survival (DFS) was similar to that of the negative group at 4 and 12 weeks (HR 0.8, $p = 0.6$). On the other hand, in the ctDNA-negative group at 4 weeks, there was no significant difference in DFS, regardless of the presence or absence of adjuvant chemotherapy (HR 1.3, $p = 0.63$).[32]

In addition, a phase II study (DYNAMIC study) was conducted to compare recurrence-free survival (RFS) in stage II colorectal cancer. Two groups were compared; 1) a ctDNA-guided group in which positive cases were treated with oxaliplatin or fluoropyrimidine monotherapy as adjuvant chemotherapy, according to postoperative ctDNA status, and negative cases were followed up; and 2) a standard group with standard postoperative follow-up. A lower proportion of patients in the ctDNA-guided group received adjuvant chemotherapy than in the standard follow group (15% vs. 28%, RR: 1.82). For ctDNA-guided management, the 2-year RFS was noninferior to standard follow-up (93.5% vs. 92.4%)[33]. Many trials are currently underway to select adjuvant chemotherapy according to postoperative ctDNA status.

Thus, the ctDNA gene panel test for MRD detection in patients with curative resection of colorectal cancer is considered useful for identifying patients with a very high risk of recurrence. The 2022 edition of the Japanese “Colorectal Cancer Treatment Guidelines” recommends selection of a treatment regimen according to the risk of recurrence in postoperative adjuvant therapy.[34] In addition, if the high-risk recurrence group can be excluded, it will be possible to extract a group with a favorable prognosis, and it will be possible to omit postoperative adjuvant chemotherapy in consideration of other clinical prognostic factors. Since regular evaluations such as CT scans and blood sampling are effective as surveillance for postoperative recurrence, repeated MRD monitoring using liquid biopsy is expected to lead to early detection of recurrence.

Based on the above, ctDNA gene panel testing for detecting MRD in patients with resectable, advanced, recurrent colorectal cancer is strongly recommended as a repeatable test for identifying high-risk recurrence groups.

[References]

1) Schwarzenbach H, Hoon DS, Pantel K: Cell-free nucleic acids as biomarkers in cancer patients. Nat Rev Cancer. 2011;11:426-437.

https://doi.org/10.1038/nrc3066

2) Diaz LA Jr, Bardelli A. Liquid biopsies: genotyping circulating tumor DNA. J Clin Oncol. 2014;32:579-586.

https://doi.org/10.1200/jco.2012.45.2011

3) El Messaoudi S, Mouliere F, Du Manoir S, et al. Circulating DNA as a Strong Multimarker Prognostic Tool for Metastatic Colorectal Cancer Patient Management Care. Clin Cancer Res. 2016;22:3067-3077.

https://doi.org/10.1158/1078-0432.ccr-15-0297

4) Spindler KG, Boysen AK, Pallisgård N, et al. Cell-Free DNA in Metastatic Colorectal Cancer: A Systematic Review and Meta-Analysis. Oncologist. 2017;22:1049-1055.

https://doi.org/10.1634/theoncologist.2016-0178

5) Tie J, Kinde I, Wang Y, et al. Circulating tumor DNA as an early marker of therapeutic response in patients with metastatic colorectal cancer. Ann Oncol. 2015;26:1715-1722.

https://doi.org/10.1093/annonc/mdv177

6) Bettegowda C, Sausen M, Leary RJ, et al. Detection of circulating tumor DNA in early- and late-stage human malignancies. Sci Transl Med. 2014;6:224ra24.

https://doi.org/10.1093/neuonc/nou206.24

7) Zhang Q, Luo J, Wu S, et al. Prognostic and Predictive Impact of Circulating Tumor DNA in Patients with Advanced Cancers Treated with Immune Checkpoint Blockade. Cancer Discov. 2020;10:1842-1853.

https://doi.org/10.1158/2159-8290.cd-20-0047

8) Vidal J, Muinelo L, Dalmases A, et al. Plasma ctDNA RAS mutation analysis for the diagnosis and treatment monitoring of metastatic colorectal cancer patients. Ann Oncol. 2017;28:1325-1332.

https://doi.org/10.1093/annonc/mdx125

9) Bando H, Kagawa Y, Kato T, et al. A multicentre, prospective study of plasma circulating tumor DNA test for detecting RAS mutation in patients with metastatic colorectal cancer. Br J Cancer. 2019;120: 982-986.

https://doi.org/10.1038/s41416-019-0457-y

10) Diehl F, Schmidt K, Choti MA, et al. Circulating mutant DNA to assess tumor dynamics. Nat Med. 2008;14:985-990.

https://doi.org/10.1038/nm.1789

11) Dudley JC, Diehn M: Detection and Diagnostic Utilization of Cellular and Cell-Free Tumor DNA. Annu Rev Pathol. 2021;16:199-222.

https://doi.org/10.1146/annurev-pathmechdis-012419-032604

12) Parikh AR, Van Seventer EE, Siravegna G, et al: Minimal Residual Disease Detection using a Plasma-only Circulating Tumor DNA Assay in Patients with Colorectal Cancer. Clin Cancer Res. 2021;27:5586-5594.

https://doi.org/10.1016/j.annonc.2020.08.530

13) Nakamura Y, Taniguchi H, Ikeda M, et al. Clinical utility of circulating tumor DNA sequencing in advanced gastrointestinal cancer: SCRUM-Japan GI-SCREEN and GOZILA studies. Nat Med. 2020;26:1859-1864.

https://doi.org/10.1038/s41591-020-1063-5

14) Hong DS, Morris VK, El Osta B, et al. Phase IB Study of Vemurafenib in Combination with Irinotecan and Cetuximab in Patients with Metastatic Colorectal Cancer with BRAFV600E Mutation. Cancer Discov. 2016;6:1352-1365.

https://doi.org/10.1158/2159-8290.cd-16-0050

15) Siravegna G, Sartore-Bianchi A, Nagy RJ, et al. Plasma HER2 (ERBB2) Copy Number Predicts Response to HER2-targeted Therapy in Metastatic Colorectal Cancer. Clin Cancer Res. 2019;25:3046-3053.

https://doi.org/10.1158/1078-0432.ccr-18-3389

16) Nakamura Y, Okamoto W, Kato T, et al. Circulating tumor DNA-guided treatment with pertuzumab plus trastuzumab for HER2-amplified metastatic colorectal cancer: a phase 2 trial. Nat Med. 2021;27:1899-1903.

https://doi.org/10.1038/s41591-021-01553-

17) Diaz LA Jr, Williams RT, Wu J, et al. The molecular evolution of acquired resistance to targeted EGFR blockade in colorectal cancers. Nature. 2012;486:537-540.

https://doi.org/10.1038/nature11219

18) Montagut C, Argilés G, Ciardiello F, et al. Efficacy of Sym004 in Patients With Metastatic Colorectal Cancer With Acquired Resistance to Anti-EGFR Therapy and Molecularly Selected by Circulating Tumor DNA Analyses : A Phase 2 Randomized Clinical Trial. JAMA Oncol. 2018;4:e175245.

https://doi.org/10.1001/jamaoncol.2017.5245

19) Shu Y, Wu X, Tong X, et al: Circulating Tumor DNA Mutation Profiling by Targeted Next Generation Sequencing Provides Guidance for Personalized Treatments in Multiple Cancer Types. Sci Rep. 2017;7:583.

https://doi.org/10.1038/s41598-017-00520-1

20) Woodhouse R, Li M, Hughes J, et al. Clinical and analytical validation of FoundationOne Liquid CDx, a novel 324-Gene cfDNA-based comprehensive genomic profiling assay for cancers of solid tumor origin. PLoS One. 2020;15:e0237802.

https://doi.org/10.1371/journal.pone.0237802

21) Clark TA, Chung JH, Kennedy M, et al. Analytical Validation of a Hybrid Capture-Based Next-Generation Sequencing Clinical Assay for Genomic Profiling of Cell-Free Circulating Tumor DNA. J Mol Diagn. 2018;20:686-702.

22) Guardant360 Technical information. https://guardant360cdx.com/wp-content/uploads/guardant360-cdx-technical-information.pdf.

23) Khagi Y, Goodman AM, Daniels GA, et al. Hypermutated Circulating Tumor DNA: Correlation with Response to Checkpoint Inhibitor-Based Immunotherapy. Clin Cancer Res. 2017;23:5729-5736.

https://doi.org/10.1158/1078-0432.ccr-17-1439

24) Gandara DR, Paul SM, Kowanetz M, et al. Blood-based tumor mutational burden as a predictor of clinical benefit in non-small-cell lung cancer patients treated with atezolizumab. Nat Med. 2018;24:1441-1448.

https://doi.org/10.1038/s41591-018-0134-3

25) Kim ES, Velcheti V, Mekhail T, et al. Blood-based tumor mutational burden as a biomarker for atezolizumab in non-small cell lung cancer: the phase 2 B-F1RST trial. Nat Med. 2022;28:939-945.

https://doi.org/10.1038/s41591-022-01754-x

26) Schenker M, Burotto M, Richardet M, et al. Abstract CT022 CheckMate 848: A randomized, open-label, phase 2 study of nivolumab in combination with ipilimumab or nivolumab monotherapy in patients with advanced or metastatic tumors of high tumor mutational solid burden. Cancer Res. 2022;82(12_Suppl): abstr CT022.

https://doi.org/10.1158/1538-7445.am2022-ct022

27) Tie J, Wang Y, Tomasetti C, et al. Circulating tumor DNA analysis detects minimal residual disease and predicts recurrence in patients with stage II colon cancer. Sci Transl Med. 2016;8:346ra92.

https://doi.org/10.1007/s11725-017-0702-6

28) Reinert T, Henriksen TV, Rasmussen MH, et al. Serial circulating tumor DNA analysis for detection of residual disease, assessment of adjuvant therapy efficacy and for early recurrence detection. Annals of Oncology. 2018;29(suppl_8):viii151.

https://doi.org/10.1093/annonc/mdy281.004

29) Tie J, Cohen JD, Wang Y, et al. Serial circulating tumor DNA analysis during multimodality treatment of locally advanced rectal cancer: a prospective biomarker study. Gut. 2019;68:663-671.

https://doi.org/10.1136/gutjnl-2017-315852

30) Reinert T, Henriksen TV, Christensen E, et al. Analysis of Plasma Cell-Free DNA by Ultradeep Sequencing in Patients With Stages I to III Colorectal Cancer. JAMA Oncol. 2019;5:1124-1131.

31) Tsukada Y, Matsuhashi N, Murano T, et al. Impact of postoperative integrated genomic and epigenomic signatures of circulating tumor DNA (ctDNA) on recurrence in resected colorectal cancer: Initial report of a prospective ctDNA monitoring study COSMOS-CRC-01. Journal of Clinical Oncology. 2022;40 (4_suppl):168.

https://doi.org/10.1200/jco.2022.40.4_suppl.168

32) Kotaka M, Shirasu H, Watanabe J, et al. Association of circulating tumor DNA dynamics with clinical outcomes in the adjuvant setting for patients with colorectal cancer from an observational GALAXY study in CIRCULATE-Japan. Journal of Clinical Oncology. 2022;40(4_suppl):9.

https://doi.org/10.1200/jco.2022.40.4_suppl.009

33) Tie J, Cohen JD, Lahouel K, et al. DYNAMIC Investigators：Circulating Tumor DNA Analysis Guiding Adjuvant Therapy in StageII Colon Cancer. N Engl J Med. 2022;386:2261-2272.

34) Colorectal Cancer Study Group: Colorectal Cancer Treatment Guidelines for Physicians 2022 Edition. Kanehara Publishing, 2022

9 Samples for molecular testing

9.1 Tissue samples

Basic requirements

Formalin-fixed paraffin-embedded (FFPE) tissue is suitable for genetic testing of somatic mutations in cancers. It is able to assess whether samples have sufficient amount of tumor cells by examining histologic findings using matched hematoxylin and eosin-stained slides. Selection of FFPE samples, decision on the need for macrodissection, and assessment of tumor cellularity should be performed by a pathologist.

Degree of recommendation

Strongly recommended [SR 9]

Recommended test material

Formalin-fixed, paraffin-embedded tissue is the most widely used for fixation and preservation of tumor samples and is suitable for use in most genetic tests. Fresh-frozen tissue can also be used for genetic testing material, if sufficient amount of tumor cells is histologically confirmed.

Although a surgically resected specimen is ideal to genetic testing, examinations for *RAS* mutations, *BRAF* mutations, and MSI is possible using several small pieces of biopsied tissue (endoscopic biopsy or needle biopsy) if it contains a high proportion of tumor cells. The FoundationOne ® CDx (F1CDx) and OncoGuide ™ NCC Oncopanel System (NCC Oncopanel), now approved by the Ministry of Health, Labor and Welfare (MHLW) and covered by national health insurance in Japan, are used as comprehensive genomic profiling tests (CGP tests) for patients with advanced colorectal cancer. F1CDx has a larger gene panel and requires more tissue volume (25 mm^2 or larger) than the NCC Oncopanel (16 mm^2 or larger). Although CGP test can be successfully analyzed using endoscopic biopsy tissues or needle biopsy tissues, one or two biopsied specimens may not be allowed for CGP tests with larger gene panel such as F1CDx due to insufficient yield of input DNA.

Selection of organs

RAS mutations, *BRAF* mutations, and mismatch repair (MMR) deficiency play a crucial role in the formation of the colorectal cancer and are thought to be acquired early in tumor development. Therefore, genetic alterations of these genes or MMR deficiency are consistently seen in both primary and metastatic lesions [Comment 1]. Similarly, it is thought that no differences in alterations of these genes or MMR deficiency are observed between biopsy specimens and surgical specimens of the same tumor[1]. Regarding *HER2* amplification, it has been reported that HER2 protein expression is uniformly observed within lesions, and that intratumoral heterogeneity, commonly observed in gastric and esophageal adenocarcinoma, occur less

frequently in colorectal cancer[2]. However, at present, studies are limited that investigated intratumoral heterogeneity of HER2 protein expression in colorectal cancer, and it is recommended that surgically resected materials should be used if available. When using biopsy specimens, it is desirable to obtain multiple sites of the lesion. Care should be taken that a lymph node with metastatic carcinoma may not be an optimal sample for CGP tests because of high proportion of background lymphocytes, which can reduce the sensitivity of genetic tests.

Formalin fixation

Appropriate formalin fixation of tumor samples is the most important factor that determines successful molecular testing including immunohistochemistry, fluorescence in situ hybridization (FISH), and genetic analysis. Clinicians and pathologists need to work together to ensure that optimal tissue fixation is conducted at their institution according to the guidelines for appropriate formalin fixation procedures ("Recommendations for Handling Pathological Tissue Specimens for Genomic Medicine" issued by the Japanese Society of Pathology).

Key procedures for tissue handling and formalin fixation are listed below[3].

- Promptly start formalin fixation after surgical excision. If there is a delay, the specimen should be refrigerated and start formalin fixation within 1 hour after excision.
- Promote formalin fixation: open the intestine and fix it on a board with pins. For organs with metastatic tumors, divide the specimen in order to maximize surface exposure to fixative reagents.
- Fixative: 10% neutral buffered formalin is recommended.
- Amount of fixative: there should be at least 10:1 ratio of fixative to tissue specimen.
- Fixation time: it can be completed within 6 to 48 hours, depending on the volume of the tissue specimen.

Selection of FFPE blocks

Tumor cellularity is an index that expresses the degree of contaminated non-tumor cells in a tumor and is defined as the ratio of the number of tumor cells to the total number of cells [Comment 2]. When a section contains a considerable amount of non-neoplastic cells, macrodissection is necessary for obtaining optimal test results with sufficient analytical quality. Selection of an ideal tissue section and determination of the position of macrodissection should be performed by a pathologist. Macrodissection of biopsy materials with low tumor cellularity may be difficult, and as such the entire tissue piece may be submitted for genetic testing. However, it should be noted that biopsy specimens with a low tumor cellularity can cause a false negative result, and thus the indication of rebiopsy or liquid biopsy should be considered. When there are multiple specimens available, ideal specimen for genetic testing is selected based on the conditions including shorter storage period, larger amount of tumor cells in a section, residual tumor volume in a specimen after preoperative treatment, and so on. Generally, resected materials after preoperative treatment may be a challenge for genetic testing as these samples tend to have an abundance of stromal fibrosis and inflammatory

cell infiltrates, causing decreased tumor cell ratio.

Comment 1 Correlation between genetic abnormalities in primary and metastatic lesions

The correlation of *KRAS* mutations between the primary and metastatic lesions is consistently high, with most reports showing 90% or higher concordance[4,5]. However, the concordance rates differ depending on the metastatic organ being tested. For example, care must be taken when examining lymph node metastasis, as concordance between the lymph node metastasis and its primary tumor tends to be lower than that between liver metastasis and its primary tumor. A high concordance rate for MSI between the primary tumor and liver metastasis has also been confirmed, including synchronous and metachronous cancers[6]. Reports also show that the concordance rate of *KRAS* mutations, *BRAF* mutations and MSI status between primary and metastatic lesions is as high as 90% or more. However, as mentioned above, it was reported that the concordance rate for *KRAS* mutation and MSI-H was lower in lymph node metastasis than that in liver metastasis[7]. Data on the correlation of HER2 protein expression is currently limited. It has been reported that the concordance rate between the primary tumor and lymph node metastasis is 90%, and between the primary tumor and liver metastasis is 73%. Discrepancy in HER2 overexpression/*HER2* amplification between primary and metastatic lesions was reported in 15% of cases[8,9]. The Colorectal Cancer Biomarker Guidelines jointly created by four American societies (ASCP/CAP/AMP/ASCO [Note]) recommends that molecular tests should use tissue of the metastatic lesion rather than primary when available[10].

Note: American Society for Clinical Pathology (ASCP), College of American Pathologists (CAP), Association for Molecular Pathology (AMP), American Society of Clinical Oncology (ASCO)

Comment 2 Tumor cellularity and detection limit of genetic testing

Accurate estimation of tumor cellularity is a crucial factor in pre-analytical process in genetic testing. Note that this ratio means the number of cell nuclei, not the area occupied by tumor cells. The percentage of tumor cells required for genetic testing differs depending on the tests applied. A tumor cell ratio of 30% or more (at least 20%) is required for detection of gene mutation and high copy number amplification in CGP tests. The limit of detection of the MSI test is a subpopulation of 2-10% tumor alleles, and thus the tumor cellularity needs to be 20% if the tumor cells are diploid[11,12]. In addition, interobserver variability in estimating tumor cellularity among pathologists should be considered. The actual percentage of tumor cells may occasionally be much lower than the estimate by eyeballing method, which can be a source of false-negative results[13-16]. Thus, when analyzing all sets of genetic tests at a time from one specimen, it is desirable that the ratio of tumor cell content be twice or higher than the detection limit of all tests applied (ideally 40-50% or higher)[10].

Side note 1 Handling tissue specimens containing bone tissue

Specimens of bone metastases need to be decalcified because they contain bone tissue, but as most decalcification procedures severely fragment nucleic acid, post-fixation procedures must also be considered.

When EDTA demineralized solution is used, the effect of denaturation is small, and similar results can be obtained for both genetic testing and IHC. Acid decalcification should be avoided and EDTA decalcification should be performed if tissue-containing specimens are likely to be subjected to genomic diagnosis.

9.2 Blood samples

Basic requirements

In performing circulating tumor DNA testing, the manufacturer's instructions concerning the use of a collection tube and plasma preparation procedure should be followed.

Degree of recommendation

Strongly recommended [SR 9]

Recommended blood sample

Genetic testing using circulating tumor DNA (ctDNA) in blood (liquid biopsy) for detecting somatic mutations of cancers is generally performed using plasma rather than serum (see Chapter 8, Liquid Biopsy). An EDTA tube, commonly used for whole blood collection, can be used in isolating cell free DNA (cfDNA), but a verified and specific blood collection tube should be used. These specific blood collection tubes can be stored at room temperature after blood sampling. It is desirable that they are submitted to the laboratory on the same day of blood sampling.

Blood sampling and preparation of plasma samples

Blood contains cfDNA released from normal and tumor cells via the process of cell death (apoptosis and necrosis), and the amount of ctDNA derived from tumor cells is in most cases very low. Improper handling of the specimens after blood sampling (e.g., long interval period after blood sampling or storing at a non-recommended temperature) can promote damage of nucleated cells including non-neoplastic white blood cells, resulting in increase in normal genomic DNA and diluting ctDNA. When nucleated cells such as leukocytes are contaminated during the process of plasma separation, ctDNA is similarly diluted by normal genomic DNA. To minimize the effect of dilution by contaminated normal DNA, it is essential to handle specimens according to the instructions of the test kit package or the standard operating procedures. **Table 1** summarizes the three ctDNA tests approved by the Ministry of Health, Labor and Welfare (MHLW) in Japan for colorectal cancer as of January 1, 2023.

Table 1 Gene panel tests for colorectal cancer using plasma ctDNA as a sample

	OncoBEAM™ *RAS* CRC Kit [17-19] (Sysmex)	Guardant360® CDx Oncogene Panel[20,21] (Guardant Health)	FoundationOne® Liquid Cancer Genomic Profile[22] (Chugai

			Pharmaceutical/Foundation Medicine)
Types of Somatic Genetic Testing	Companion diagnostics	Comprehensive genomic profiling test (some companion diagnostics)	Comprehensive genomic profiling test (some companion diagnostics)
Pharmaceutical approval	Approved July 2019	Approved March 2022	Approved March 2021
Number of target genes (CDx items)	2 genes (*KRAS, NRAS)*	74 genes (MSI[*)]	324 genes (*NTRK*[**)]
Blood collection tube used	Streck blood collection tube (cell-free DNA BCT), blood collection tube for cell-free DNA extraction (Roche)	Streck blood collection tube (cell-free DNA BCT)	Blood collection tube for cell-free DNA extraction (Roche)
Storage conditions from blood collection to plasma separation	Storage temperature: room temperature (15 to 25°C)	Storage temperature: room temperature (6 to 37°C)	Storage temperature: room temperature (4 to 35°C)
Location of plasma separation	Medical institutions and domestic laboratories	Specific overseas laboratory (Redwood City, USA)	Specific overseas laboratory (Cambridge, USA)

[*] Pembrolizumab (solid tumors) and nivolumab (colorectal cancer), [**] Entrectinib (solid tumors), CDx: companion diagnostics, BCT: blood collection tube.

[References]

1） Fadhil W, Ibrahem S, Seth R, et al. The utility of diagnostic biopsy specimens for predictive molecular testing in colorectal cancer. Histopathology. 2012;61:1117-1124.

https://doi.org/10.1111/j.1365-2559.2012.04321.x

2） Valtorta E, Martino C, Sartore-Bianchi A, et al. Assessment of a HER2 scoring system for colorectal cancer: results from a validation study. Mod Pathol. 2015;28:1481-1491.

3 ） Rules for handling pathological tissue specimens for genomic medicine. https://pathology.or.jp/genome_med/pdf/textbook.pdf.

4） Baas JM, Krens LL, Guchelaar HJ, et al. Concordance of predictive markers for EGFR inhibitors in primary tumors and metastases in colorectal cancer: a review. Oncologist. 2011;16:1239-1249.

https://doi.org/10.1634/theoncologist.2011-0024

5） Knijn N, Mekenkamp LJ, Klomp M, et al. KRAS mutation analysis: a comparison between primary tumors and matched liver metastases in 305 colorectal cancer patients. Br J Cancer. 2011;104:1020-1026.

https://doi.org/10.1038/bjc.2011.26

6） Koi M, Garcia M, Choi C, et al. Microsatellite Alterations With Allelic Loss at 9p24.2 Signify Less-Aggressive Colorectal Cancer Metastasis. Gastroenterology. 2016;150:944-955.

https://doi.org/10.1053/j.gastro.2015.12.032

7） Fujiyoshi K, Yamamoto G, Takahashi A, et al. High concordance rate of KRAS/BRAF mutations and MSI-H between primary colorectal cancer and corresponding metastases. Oncol Rep. 2017;37:785-792.

https://doi.org/10.3892/or.2016.5323

8） Shan L, Lv Y, Bai B, et al. Variability in HER2 expression between primary colorectal cancer and corresponding metastases. J Cancer Res Clin Oncol. 2018;144:2275-2281.

https://doi.org/10.1007/s00432-018-2744-z

9） Lee WS, Park YH, Lee JN, et al. Comparison of HER2 expression between primary colorectal cancer and their corresponding metastases. Cancer Med. 2014;3:674-680.

https://doi.org/10.1002/cam4.228

10） Sepulveda AR, Hamilton SR, Allegra CJ, et al. Molecular Biomarkers for the Evaluation of Colorectal Cancer: Guideline From The American Society for Clinical Pathology, College of American Pathologists, Association for Molecular Pathology, and the American Society of Clinical Oncology. Journal of Clinical Oncology. 2017;35:1453-1486.

https://doi.org/10.1200/jco.2016.71.9807

11） Berg KD, Glaser CL, Thompson RE, et al. Detection of microsatellite instability by fluorescence multiplex polymerase chain reaction. J Mol Diagn. 2000;2:20-28.

https://doi.org/10.1016/s1525-1578(10)60611-3

12） Trusky CL, Sepulveda AR, Hunt JL. Assessment of microsatellite instability in very small microdissected samples and in tumor samples that are contaminated with normal DNA. Diagn Mol Pathol 2006;15:63-69.

https://doi.org/10.1097/00019606-200606000-00001

13） Smits AJ, Kummer JA, de Bruin PC, et al. The estimation of tumor cell percentage for molecular testing by pathologists is not accurate. Mod Pathol. 2014;27:168-174.

https://doi.org/10.1038/modpathol.2013.134

14） Viray H, Li K, Long TA, et al. A prospective, multi-institutional diagnostic trial to determine pathologist accuracy in estimation of percentage of malignant cells. Arch Pathol Lab Med. 2013;137:1545-1549.

https://doi.org/10.5858/arpa.2012-0561-cp

15） Boyle TA, Bridge JA, Sabatini LM, et al. College of American Pathologists Molecular Oncology Committee: Summary of microsatellite instability test results from laboratories participating in proficiency surveys: proficiency survey results from 2005 to 2012. Arch Pathol Lab Med. 2014;138:363-370.

https://doi.org/10.5858/arpa.2013-0159-cp

16） Chen G, Yang Z, Eshleman JR, et al. Molecular Diagnostics for Precision Medicine in Colorectal Cancer: Current Status and Future Perspective. BioMed Research International 2016:9850690.

https://doi.org/10.1155/2016/9850690

17） Medina Diaz I, Nocon A, Mehnert DH, et al. Performance of Streck cfDNA Blood Collection Tubes for Liquid Biopsy Testing. PLoS One. 2016;11:e0166354.

https://doi.org/10.1371/journal.pone.0166354

18） García-Foncillas J, Tabernero J, Élez E, et al. Prospective multicenter real-world RAS mutation comparison between OncoBEAM-based liquid biopsy and tissue analysis in metastatic colorectal cancer. Br J Cancer. 2018;119:1464-1470.

https://doi.org/10.1038/s41416-018-0293-5

19） Kagawa Y, Kato T, Bando H, et al. A multicentre, prospective clinical evaluation study for analyzing RAS mutational status utilizing plasma circulating tumor DNA in patients with metastatic colorectal cancer. Annals of Oncology. 2018;29(Suppl 5):v101-v102.

https://doi.org/10.1093/annonc/mdy149.004

20） Odegaard JI, Vincent JJ, Mortimer S, et al. Validation of a Plasma-Based Comprehensive Cancer Genotyping Assay Utilizing Orthogonal Tissue- and Plasma-Based Methodologies. Clin Cancer Res. 2018;24:3539-3549.

https://doi.org/10.1158/1078-0432.ccr-17-3831

21） Lanman RB, Mortimer SA, Zill OA, et al. Analytical and Clinical Validation of a Digital Sequencing Panel for Quantitative, Highly Accurate Evaluation of Cell-Free Circulating Tumor DNA. PLoS One. 2015;10: e0140712.

https://doi.org/10.1371/journal.pone.0140712

22) Clark TA, Chung JH, Kennedy M, et al. Analytical Validation of a Hybrid Capture-Based Next-Generation Sequencing Clinical Assay for Genomic Profiling of Cell-Free Circulating Tumor DNA. J Mol Diagn. 2018;20:686-702.

https://doi.org/10.1016/j.jmoldx.2018.05.004

10 Ensuring test accuracy

Basic requirements

Genetic testing for colorectal cancer treatment should be carried out under a quality assurance system.

Degree of recommendation

Strongly recommended [SR 9]

Requirements for ensuring test accuracy in laboratories

As tests for cancer treatment become more sophisticated, to ensure accurate results, quality control at clinical testing laboratories is important. This includes the handling of specimens from when they are collected from patients to the subsequent handling, so quality control is also important for tests not performed at our own facilities. In cancer genome testing, the FFPE specimen management process (specimen collection-storage-transport) also contributes to quality control. For the handling of pathological specimens, please refer to Chapter 9, Samples Used as Specimens. In addition, part of the provisions of the Act for Partial Revision of the Medical Care Act, etc., came into effect on December 1, 2018. Along with this, revisions to the ministerial ordinance were implemented based on the summary of the study group on quality control of laboratory tests[1]. The following were stipulated: (1) Placement of person in charge of gene-related testing and chromosome testing, (2) internal quality control and implementation of appropriate training, and (3) participation in external quality control surveys. To ensure the quality of testing itself and laboratory personnel, testing should be performed in accordance with the technical requirements of ISO15189:2012 Chapter 5[2], which is the international standard for the quality and competence of clinical laboratories, and the required standards of the Japanese version of the Best Practice Guideline for Genetic Testing[3]. In addition, for the time being, it was decided to recommend the acquisition of third-party accreditation for testing facilities.

The main points of the revised ministerial ordinance concerning the “Standards To Be Established To Ensure The Accuracy Of Gene-Related Tests And Chromosomal Tests” are as follows.

1. In addition to the person responsible for overall quality control of specimen testing, it was made mandatory to appoint a person responsible for gene-related testing and chromosome testing. In principle, the person should be a doctor or a clinical laboratory technologist with work experience.
2. Implementation of internal quality control (management of accuracy, reproducibility, etc. of testing within the facility) and preparation of a statistical quality control ledger, a standard work manual, a work diary, etc. were made obligatory. Appropriate training was also mandated to ensure the quality of test personnel.
3. Participation in external quality control surveys. If there is no external quality control survey system,

facilities such as medical institutions and health laboratories may work together and mutually confirm the accuracy of tests using the specimens they store and handle. Efforts should be made to utilize alternative methods.

4. Testing facilities acquire and maintain third-party accreditation such as the international standard ISO15189 and the Laboratory Accreditation Program (LAP) of the College of American Pathologists (CAP).[4] Ensuring reliability of test accuracy is recommended. In June 2019, the Ministry of Health, Labour and Welfare responded to a questionable interpretation, stating that "third-party accreditation with accreditation related to accuracy control of testing using a sequencer system corresponds to CAP." The next-generation sequencing method was added for ISO15189 in December 2019, and its certification is gradually progressing.

At present, the Japanese Society of Pathology and the Japanese Society of Clinical Laboratory Medicine are formulating a "testing guideline for cancer genome testing in general". In the draft guidelines, external accuracy evaluation is raised as an urgent domestic issue. In the draft guidelines, external accuracy evaluation is raised as an urgent domestic issue. Currently, the only external system evaluations related to cancer genome testing by domestic organizations are the evaluation of leukemia-related genes sponsored by the Gene and Proteomics Committee of the Japanese Society of Medical Laboratory Sciences and the evaluation of nucleic acid quality, etc. of pathological specimens conducted by the Japan Institute for Quality Assurance of Pathology, a domestic third-party external system evaluation organization. Regarding cancer genome testing, a pilot study by Maekawa et al. using next-generation sequencing also reported some disparities between facilities5. The Genetic and Proteomics Committee of the Japanese Society of Medical Laboratory Sciences, the Genetic Committee of the Japanese Society of Clinical Laboratory Medicine, and the Japanese Pathological Accuracy Assurance Organization jointly examined how to proceed with external accuracy evaluation for cancer gene panel tests covered by insurance.

[References]

1) Summary of Review Committee on Accuracy Control of Laboratory Tests, June 6, 2018. The 62nd meeting of the Social Security Council Medical Subcommittee
2) Japan Accreditation Board: Guidance on "Accreditation Criteria" - Clinical Laboratories - JAB RM300: 2014 https://www.jab.or.jp/files/items/4908/File/RM3002014V3.pdf
3) Japan Association for Clinical Laboratory Standards: Japanese Best Practice Guidelines for Genetic Testing　Explanation version. Academic advertising company, 2016
4) College of American Pathologists https://www.cap.org
5) Maekawa M, Taniguchi T, Nishio K, et al. Precision cancer genome testing needs proficiency testing involving all stakeholders. Sci Rep. 2022;12:1494. https://doi.org/10.1038/s41598-022-05589-x

11 Tests currently in development

11.1 Assays using angiogenic factors as indicators

History of assay development using angiogenesis factors as indicators

Tumor growth and progression requires tumor angiogenesis, which involves vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), angiopoietin, and various other factors. VEGF is a dimer-forming glycoprotein that binds to transmembrane receptors (VEGF receptor: VEGFR) expressed on vascular endothelial cells to activate signal transduction pathways. Seven VEGFs, VEGF-A, -B, -C, -D, -E, and placental growth factor (PlGF)-1, 2, and three VEGFRs, VEGFR-1, -2, and -3, have been identified. Among them, binding of VEGF-A to VEGFR-2 is thought to be central to the signal transduction pathway. Angiogenesis inhibitors include bevacizumab, a humanized monoclonal antibody against VEGF-A, and ramucirumab, a fully humanized anti-VEGFR-2 monoclonal antibody that inhibits the binding of VEGF-A, -C, and -D to VEGFR-2. Aflibercept, a recombinant protein that traps VEGF-A, -B, and PlGF in the blood by fusing the Fc domain of an IgG1 antibody with aflibercept, has been reported to be effective in combination with cytotoxic anticancer drugs. It is important to develop a test to assist treatment selection, especially in second-line treatment, where the combination effect of ramucirumab and aflibercept has been proven.

Significance of plasma VEGF-D levels in ramucirumab treatment

The RAISE study validated the efficacy of FOLFIRI plus ramucirumab as second-line treatment for patients with unresectable, advanced, recurrent colorectal cancer. Biomarker analysis was performed, with 1,050 patients enrolled and divided into exploratory and validation cohorts[1]. Patients were randomized at a ratio of 2:2, and the expression of VEGF-C, -D, sVEGFR-1, -2, -3, and VEGFR-2 in tumor tissue before treatment and the therapeutic effect were investigated[1]. It was found that VEGF-D level was strongly correlated with the effect of ramucirumab combination on overall survival and progression-free survival in the exploratory cohort, and a significant interaction was also observed in the validation cohort (overall survival: $p = 0.01$, progression-free survival: $p = 0.001$). Analysis of all subjects (the exploratory and validation cohort) also showed a significant interaction between high/low VEGF-D level and ramucirumab treatment effect (overall survival: p = 0.0005, progression-free survival: $p < 0.0001$). In the high VEGF-D level group (n = 536), both overall survival and progression-free survival were significantly better in those who received ramucirumab combination therapy. In the low VEGF-D group, overall survival was significantly worse in those who received placebo (**Table 1)**. In addition, since the measurement method used in this study was designated as research use only (RUO), a clinically relevant VEGF-D measurement method, the Corgenix assay, was developed at the investigational use only (IUO) status. A similar analysis using the 878 available plasma samples that were used in the biomarker analysis of the RAISE study showed that

although the cut-off values differed due to differences in measurement methods, the VEGF-D high group (n = 313) showed an effect with ramucirumab combination compared to the VEGF-D low group (n = 565) [progression-free survival (high-value group HR 0.59, low-value group HR 0.96)], and a similar trend was observed in overall survival. Yes (high group HR 0.78, low group HR 1.00) [2]. The above results suggest that the pretreatment plasma VEGF-D level may be a predictor of therapeutic efficacy of ramucirumab, although this finding was reported in the RAISE study only.

Table 1a Relationship between overall survival and VEGF-D

	High VEGF-D				Low VEGF-D			
Treatment Arm	n	OS (m)	HR	*p*	n	OS (m)	HR	*p*
RAM	270	13.9	0.73	0.0022	176	12.6	1.32	0.0344
Placebo	266	11.5			172	13.1		

RAM: ramucirumab, OS: overall survival, HR: hazard ratio, PFS: progression-free survival, m: month

Table 1b Relationship between progression-free survival and VEGF-D

	High VEGF-D				Low VEGF-D			
Treatment Arm	n	PFS (m)	HR	p	n	PFS (m)	HR	p
RAM	270	6.0	0.62	<0.0001	176	5.4	1.16	0.1930
Placebo	266	4.2			172	5.6		

RAM: ramucirumab, OS: overall survival, HR: hazard ratio, PFS: progression-free survival, m: month

Treatment results of other angiogenic factor inhibitors and the significance of measuring angiogenic factors using blood

Since the development of bevacizumab, various efficacy predictors have been investigated, but no established factors have been identified at present. In a meta-analysis of 11 studies using bevacizumab combination therapy, the overall survival (HR 1.30, $p < 0.0001$) and progression-free survival (HR 1.26, $p = 0.0001$) were reported to be significantly worse [3]. Biomarker analysis was conducted in the AGITG-MAX trial, which verified the efficacy of bevacizumab in combination with capecitabine (± mitomycin). The expression of VEGF-A, -B, -C, -D, and VEGFR-1, and -2 proteins in tumor tissue and the effect of bevacizumab combination was investigated. Only the degree of VEGF-D protein expression was found to be significantly associated with bevacizumab effect in multivariate analysis[4]. However, a subsequent analysis

using tumor tissue from the control group [CAPOX (capecitabine + oxaliplatin) + bevacizumab group] of the CAIRO-2 study showed no association between VEGF-D protein expression and progression-free survival and overall survival. In the CALGB80405 study, which compared the combination of bevacizumab and cetuximab for FOLFOX or FOLFIRI therapy, a biomarker analysis using pretreatment plasma specimens showed that in the VEGF-D low group (low level 1/4 group), overall survival and progression-free survival were both favorable (HR 0.62 and 0.59 respectively), but the same trend was not observed when combined with FOLFIRI[5].

In addition, the VELOUR study validated the efficacy of FOLFIRI plus aflibercept as second-line therapy for patients with unresectable, advanced, recurrent colorectal cancer. In this study, a retrospective biomarker analysis of 98 angiogenic factors and inflammatory cytokines in pretreatment plasma was performed and it was found that VEGF-A, PlGF, serum amyloid component, and C-reactive protein are highly expressed in patients with a history of bevacizumab treatment[6]. Furthermore, in patients with plasma VEGF-A and PlGF levels higher than the median, the overall survival was better in the aflibercept combination group, regardless of prior bevacizumab treatment. However, no biomarkers useful for predicting efficacy have been established thus far.

Factors that predict the efficacy of angiogenesis factor inhibitors are still being investigated. The PERMAD trial consisted of 647 patients with unresectable, advanced, recurrent colorectal cancer and investigated bevacizumab combination therapy as first-line therapy. It measured five plasma cytokines and angiogenic factors, including PlGF and VEGF-B, and found that resistance to bevacizumab combination therapy could be indicated 100 days before imaging progression with an accuracy of 83%, sensitivity of 76%, and specificity of 88%. Interim results of a prospective multicenter study (UMIN000028616) in Japan investigating the Angiogenesis Panel in unresectable, advanced, recurrent colorectal cancer have been reported. Plasma PlGF levels increased significantly before and after first-line bevacizumab administration[9]. Before and after administration of various angiogenesis factor inhibitors in second-line therapy, VEGF-A levels significantly decreased after administration in the bevacizumab group, but increased after administration of other angiogenesis factor inhibitors. VEGF-D levels rose only after ramucirumab administration. PIGF levels are elevated after administration of all angiogenesis inhibitors. These findings demostrate that VEGF-D, VEGF-A, and PlGF fluctuate independently. In addition, it was reported that low VEGF-D levels, high VEGF-A levels, and low PlGF levels before treatment may be favorable factors for progression-free survival when bevacizumab is administered as second-line therapy. As described above, it has been reported that various angiogenic factors fluctuate before and after treatment with angiogenesis inhibitors, and monitoring these fluctuations is expected to lead to the selection of the optimal angiogenesis inhibitor.

11.2 DNA methylation assay for anti-EGFR antibody therapeutic effect prediction

Aberrant DNA methylation in colorectal cancer

The CpG island methylator phenotype (CIMP) is an important oncogenic mechanism of colorectal cancer associated with DNA methylation, and is involved in approximately 20% of all colorectal cancers [11]. CIMP-positive colorectal cancers have a higher proportion of right-sided colons, [12] and a higher proportion of cases with *BRAF* gene mutations and microsatellite instability (MSI)[13]. In addition, as a histopathological feature, many hyperplastic polyps and sessile serrated polyps are observed as precursor lesions[14] and it is thought to have a different carcinogenic mechanism from CIMP-negative colorectal cancer arising from tubular adenoma.

CIMP is generally determined to be CIMP-positive when a certain percentage of the gene set extracted as a CIMP marker is methylated. However, there is no established marker set for classifying CIMP-positive and CIMP-negative CRC[11-13,15,16].

DNA methylation status as a predictor of anti-EGFR antibody therapeutic efficacy

In a retrospective study, bead array (Infinium450K, Illumina) was used to investigate the association between genome-wide DNA methylation status (GWMS) and response to anti-EGFR treatment in 97 patients who received anti-EGFR treatment for *KRAS* wild-type metastatic colorectal cancer. Patients were classified into high-methylated colorectal cancer (HMCC) and low-methylated colorectal cancer (LMCC) subgroups based on GWMS. It was found that clinical outcomes were significantly better in the LMCC subgroup than in the HMCC subgroup[17]. When only *RAS* wild-type cases were analyzed, the HMCC group (n = 28) had a significantly lower response rate (3.7% vs. 37.9%, $p < 0.001$) and shorter progression-free survival (median: 2.3 months vs. 6.6 months, HR 0.22, 95%CI 0.13-0.37, $p < 0.001$) and overall survival (median: 8.5 months vs. 20.9 months, HR 0.24, 95% CI 0.11-0.53, $p < 0.001$) than the LMCC group. Furthermore, it was suggested that *RAS* wild-type HMCC is as resistant to anti-EGFR antibody drugs as *RAS*-mutant colorectal cancer[17]. In addition, in a retrospective study of 103 metastatic *RAS* wild-type colorectal cancer patients, a multivariate analysis which included the consensus molecular subtypes (CMS) classification, primary tumor site and GWMS revealed GWMS as only a significant independent factor for both progression-free survival (HR 0.21, $p < 0.01$) and overall survival (HR 0.30, $p = 0.04$) associated with anti-EGFR antibody treatment[18]. Although it is a retrospective study, overseas research groups have also reported CIMP positivity (CIMP-High) as a poor prognostic factor in those treated with anti-EGFR antibody[12]. These results suggest that GWMS is a novel therapeutic effect predictor of anti-EGFR antibody drugs in *RAS* wild-type colorectal cancer.

Development and Usefulness of a DNA Methylation Assay

Development of *in vitro* diagnostics that can easily diagnose DNA methylation status is currently underway. The 16 CpG regions that reflect the genome-wide DNA methylation status are analyzed using a measurement system based on the MethyLight assay[19] (MeC-mML assay, DNA methylation status assay of mCRC by modified MethyLight). If 8 or more sites are positive for methylation, the sample is determined as HMCC,

and 7 sites or less are positive for methylation, it is determined as LMCC[20].

The ability of the MeC-mML assay to predict the therapeutic effect of anti-EGFR antibody drugs was verified in a retrospective analysis of 101 previously treated patients with *RAS* wild-type metastatic colorectal cancer (mCRC) who received anti-EGFR antibody drugs. The therapeutic effect of anti-EGFR antibody drugs in the HMCC group (n=24) was shown to be significantly worse than that in the LMCC group (n=77) (response rate: 4.2% vs 33.3%, p = 0.001, progression-free survival: median 2.5 months vs 6.6 months, HR 0.22, p < 0.001, overall survival: median 5.6 months vs 15.5 months, HR 0.23, p <0.001)[20]. In this study, the GWMS measured by the MeC-mML assay showed a strong relationship with the therapeutic effect of anti-EGFR antibody drugs regardless of the primary tumor site. It was also suggested that the therapeutic effect of anti-EGFR antibody drugs can be similarly predicted even when the analysis object is narrowed down to the patients with *RAS*/*BRAF* wild-type mCRC. In addition, the significance of the GWMS measured by the MeC-mML assay as a predictor of anti-EGFR antibody treatment efficacy in first-line treatment was also investigated retrospectively. It was suggested that the therapeutic effect of anti-EGFR antibody drug combination regimen in first-line treatment was worse in the HMCC group (n=15) than in the LMCC group (n=154) (response rate: 53.3% vs 81.8%, p = 0.017, median progression-free survival: 5.7 months vs. 13.1 months, HR 3.13, p = 0.004, overall survival: median 31.1 months vs. 51.4 months, HR 2.35, p = 0.019) [21].

Future clinical application

DNA methylation assays are considered useful to assist the selection of anti-EGFR treatment in the first-line and previously treated patients with metastatic colorectal cancer. Due to treatment selection in first-line treatment and tissue sparing, it is more appropriate to perform DNA methylation assays before starting first-line treatment. A retrospective analysis using clinical specimens collected in prospective studies is currently underway, and approval as an *in vitro* diagnostics is expected.

11.3 Multigene Assays in Predicting Postoperative Recurrence of Colon Cancer

History of the development of multigene assays in cases of curative resection of colon cancer

Postoperative adjuvant chemotherapy is generally recommended for Stage III colon cancer patients undergoing curative R0 resection to reduce recurrence risk, but it is reported that the recurrence risk of T1-2N1M0 cases was comparable to that in high risk Stage II cases [22]. In addition, the benefit of postoperative adjuvant chemotherapy for Stage II colon cancer has not yet been established in prospective studies, and the guidelines from both the American Society of Clinical Oncology and European Society for Medical Oncology stipulate Stage II at a high risk of recurrences using clinicopathological factors. However, no robust evidence exists to support these recommendations [23,24]. Therefore, attempts have been made to develop multigene assays to identify high-risk groups for postoperative recurrence in Stage II/III colon cancer.

Oncotype DX ® Colon Cancer Assay

The Oncotype DX ® Colon Cancer Assay detects 7 cancer-related genes (BGN, *FAP, INHBA, GADD45B, Ki - 67, C - MYC, MYBL2)* and 5 reference genes (*ATP5E, GPX1, PGK1, UBB, VDAC2)*. A total of 12 constituent genes are extracted, and the recurrence score (RS) is calculated from the expression level of the 12 genes. An RS of 0-29 are classified as low risk, 30-40 as intermediate risk, and 41-100 as high risk[25].

In the CALGB9581 study[26], which compared surgery alone and postoperative adjuvant chemotherapy with an anti-EpCAM antibody for stage II colon cancer, in T3 and microsatellite stable (MSS) cases, the RS calculated by the Oncotype DX® Colon Cancer Assay was shown to enable stratification of risk recurrence, showing the utility of RS in stage II colon cancer (**Table 2**). Furthermore, in the NSABP-07 study[27] comparing 5-FU/LV and FLOX (5-FU + leucovorin + oxaliplatin) for stage II /III colon cancer, HR for recurrence risk was 1.96 (95% CI 1.50-2.55, $p < 0.001$), indicating that RS can predict recurrence risk regardless of the therapy type, not only in Stage II, but also Stage.

Table 2 Oncotype DX ® Colon Cancer Assay 5-year recurrence rate by recurrence risk group

	Stage	Low risk group (%)	Intermediate risk group (%)	High risk group (%)
CALGB9581 Study [26]	StageII (T3 and MSS)	13	16	21
	StageII	9	14	19
SUNRISE Study [28]	StageIIIA/IIIB	20	29	38
	Stage IIIC	38	51	62

In the SUNRISE study[28] conducted, recurrent and non-recurrent cases were retrospectively extracted at a ratio of 1:2 from 1,568 pathological stage II/III Japanese colon cancer patients, who had not undergone postoperative adjuvant chemotherapy. Of the 630 patients, RT-PCR analysis was performed on 597of these patients. The primary endpoint, recurrence-free interval, was significantly correlated with RS, with an HR per 25 RS points of 2.05 ($p < 0.001$). RS was also significantly correlated with the secondary endpoints of recurrence-free survival, disease-free survival, and overall survival with HRs per 25 RS points of 1.77, 1.90, and 2.02 (all $p < 0.001$), respectively. In addition, the 5-year recurrence rates were similar between the stage II high risk group and stage IIIA/IIIB low risk group (**Table 2**). It was suggested that the risk of recurrence can be predicted more accurately by adding RS to pathological staging. In addition, in a meta-analyses which included the above studies, factors such as stage, T factor, number of lymph nodes dissected, MMR status, and RS were significant risk factors for recurrence in all cases[29].

In postoperative adjuvant chemotherapy for colorectal cancer, the results of the IDEA collaboration, which showed the non-inferiority of 3 months to 6 months of oxaliplatin combination chemotherapy

(FOLFOX/CAPOX), suggest that the treatment period may be shortened depending recurrence risk. Japanese guidelines also recommend taking account of recurrence risk in decision to implement adjuvant therapy[30]. Against this background, a prospective study (SUNRISE-DI) was conducted in Japan and investigated whether the Oncotype DX® ColonCancer Assay change treatment recommendations from before to after the assay in Stage II/III colon cancer. A total of 275 cases were enrolled, and the treatment recommendations changed in 40% of cases after obtaining 12-RS assay results. Furthermore, when the stage III cases were classified by IDEA collaboration into low-risk (T1 to T3 and N1) and high-risk (T4 and/or N2) cases, the recommended treatment was changed in 48% and 38% of the cases, respectively. Even in the era of the IDEA collaboration, the Oncotype DX ® Colon Cancer Assay can be useful[31].

Future perspective of Multigene Assays in Colon Cancer

The Oncotype DX ® Colon Cancer Assay has been consistently reported to be useful in predicting the recurrence and prognosis of stage II/III colon cancer, regardless of the presence or absence of postoperative adjuvant chemotherapy, types of regimen, and race. In addition, it has been reported from Japan that the recurrence risk of stage II/III colon cancer can be classified into high-risk and low-risk by combining 55 gene expressions and *RAS* mutations[32]. The value of gene expression has also been validated by other studies [33,34]. In addition, the usefulness of ColoPrint®, which can predict recurrence of stage II colon cancer from the expression of 18 genes, has also been reported in overseas studies[35]. Postoperative adjuvant chemotherapy may result in overtreatment or undertreatment in some cases when patient selection using only clinicopathological factors is carried out. Development of a new testing based on molecular biological features is sorely needed to predict recurrence risk more accurately and optimize strategies of adjuvant treatments.

11.4 Tumor microenvironment

Assessment of tumor microenvironment and prognostic factors

In tumor tissue, tumor infiltrating lymphocytes (TIL), dendritic cells, tumor-associated macrophages, tumor-associated fibroblasts, etc. form the tumor microenvironment (TME). The TME is known to contribute to development or inhibition of cancer. It has been reported that the tumor microenvironment correlates with prognosis in multiple cancer types including colorectal cancer[36,] and in recent years it has also been researched and developed as a therapeutic target. The number of CD3-positive T cells, CD8-positive T cells, helper T1 cells in tumor tissue, the expression of programmed death-1 (PD-1) molecules in TILs, and local formation of lymphoid structures have been reported as a good prognostic factors in colorectal cancer. The number of helper T17 cells and tumor-associated macrophages have been reported as a poor prognostic factor [36-39].

The TME has been evaluated by immunohistochemistry (IHC) using formalin-fixed paraffin-embedded

(FFPE) sections, real-time PCR using unfixed specimens, and microarray. In recent years, next-generation sequencers have been used to perform comprehensive analysis, not only on organizational units but also on single-cell units. In addition, analyses using multiplex immunostaining and imaging mass cytometry are also being conducted, and elucidation of the TME of colorectal cancer is underway.

Evaluation method and significance of TME in resectable colorectal cancer

Immunoscore ® is a standardized TME evaluation method to quantify CD3- and CD8-positive cells. It involves performing IHC of FFPE sections of surgical specimens from colorectal cancer patients. Quantitative analysis is then carried out with automatic measurement software (Immunoscore ® Analyzer) on image data acquired with a digital microscope[40]. The software automatically distinguishes between tumor tissue and normal tissue and recognizes the area within a certain distance from the margin of tumor invasion as the invasive margin (IM). The cell density of CD3-positive cells and CD8-positive cells in the tumor core (TC) and IM are measured. The immunoscore (IS) is the average value of the four cell densities, with 0-25% classed as low score, 25-70% as intermediate score, and 70%-100% as high score[40]. These measurements are objective and reproducible, as the results are not affected by the number of years since specimen collection, the position of the selected FFPE in the tumor tissue, or the software operator (pathologist)[40,41]. Immunoscore ® has obtained the CE-IVD mark as an in-vitro diagnostic device in Europe but is not yet approved in Japan. An international multicenter study retrospectively examined the standardization of IS and its significance as a prognostic predictor, using surgical specimens from 2,681 patients with stage I-III colorectal cancer. In the training set, the 5-year recurrence rate in those with a low, intermediate and high IS was 32%, 19% and 8% respectively, and the HR for risk of recurrence in the high-score group versus the low-score group was 0.20 ($p < 0.0001$). Findings were confirmed in two validation sets[40]. Multivariate analysis showed that IS was independent of histopathological prognostic factors such as age, sex, T stage, N stage, microsatellite instability, vascular invasion, and peritoneal invasion ($p < 0.0001$). IS had the highest relative contribution to recurrence risk compared to known prognostic factors, including the TNM classification system. The authors suggested that a new staging system that combines the TNM classification with IS (TNM-Immune) is prognostically useful.[40] This new staging system has been investigated elsewhere.[42] A post-hoc analysis using resected specimens from cases enrolled in two randomized controlled phase III studies (IDEA France study[43], NCCTG N0147 study[44]) for Stage III colorectal cancer also showed IS to be an independent prognostic factor in predicting recurrence after adjuvant chemotherapy (low IS score vs. high score HR 2.28, $p = 0.00145$, IS HR per 10% increase 0.90, $p = 0.00446$). These results suggest that IS is a prognostic predictor for resectable advanced colorectal cancer, and the addition of IS to the TNM classification is expected to further improve prognostic accuracy.

In the aforementioned IDEA France study, 481 patients who received FOLFOX therapy and who were evaluable for IS showed that the 6-month treatment group had a longer recurrence-free survival than the 3-month group in the IS high-score or intermediate-score group (HR 0.53, $p = 0.0004$). The results were

similarly significant in both the clinically high-risk group (T4 and/or N2) and the clinically low-risk group (T1-T3 and N1) (clinically high-risk group: HR 0.54, $p = 0.007$, clinically low-risk group: HR 0.47, $p = 0.01$).[45] On the other hand, in the IS low score group, there was no significant difference in recurrence-free survival between the 6-month group and the 3-month group (HR 0.84, $p = 0.27$), and no difference was observed in the analysis by clinical risk of recurrence.[45] In addition, among the cases registered in the above-mentioned international study[40] to examine the classification of IS, an analysis focused on Stage III colorectal cancer also showed that only the IS high-score or intermediate-score group had no recurrence in both the clinically high-risk and clinically low-risk groups. A significant prolongation of recurrence-free survival was observed in the adjuvant therapy group compared with the treatment follow-up group [IS high or intermediate score group: HR 0.5, $p = 0.0015$ (clinically high-risk group), HR 0.42, $p = 0.0011$ (clinically low-risk group), IS low score group: $p = 0.12$ (clinically high-risk group), $p = 0.18$ (clinically low-risk group)].[47] Thus, IS has been shown to be a potential predictor of postoperative adjuvant chemotherapy in stage III colorectal cancer.

Future prospects

In recent years, advances have been made in the development of biopsy-adapted IS (IS_B). IS_B follows the same procedure as for surgical specimens, but IM regions are not included in biopsy specimens, therefore the average IS score for CD3 and CD8 positive cells in TC alone is calculated for IS_B.[48] However, there are no reports of equivalence for prognostic prediction between resected specimens and biopsy specimens. In a retrospective study, IS_B was measured using biopsy specimens obtained at the time of diagnosis of rectal cancer for patients who underwent preoperative chemoradiation therapy or radiotherapy followed by radical surgery.[48] The study found that a high IS_B was an independent favorable prognostic factor in two independent cohorts (n = 124, n = 114)[48]. The usefulness of IS_B in the watch-and-wait strategy in rectal cancer patients with excellent response to preoperative chemoradiotherapy has been reported mainly in Europe and the United States.[48-50]

In addition, a variant of Immunoscore IC for measuring and scoring the density of CD8-positive cells and programmed death-ligand 1 (PD-L1)-positive cells by IHC of tumor tissue (and the proximity of both), and to predict the effect of immune checkpoint inhibitors, is also under development. A subgroup analysis of a phase II study (AtezoTRIBE study) investigating the additional effect of anti-PD-L1 antibody drug atezolizumab on FOLFOXIRI + bevacizumab combination therapy for first-line treatment of unresectable advanced colorectal cancer showed that in the Immunoscore IC high-score group, concomitant use of atezolizumab extended progression-free survival, but in the low-score group, concomitant use of atezolizumab did not prolong progression-free survival[51]. The system is also expected to be developed in the future as a predictor of the therapeutic effect of immune checkpoint inhibitors.

[References]

1) Tabernero J, Hozak RR, Yoshino T, et al. Analysis of angiogenesis biomarkers for ramucirumab efficacy in patients with metastatic colorectal cancer from RAISE, a global, randomized, double-blind, phase III study. Ann Oncol 29. 2018;602-609.

https://doi.org/10.1093/annonc/mdx767

2) Taniguchi H, Yoshino T, Yamaguchi K, et al. Clinical development and evaluation of a VEGF-D assay in plasma from patients with metastatic colorectal cancer in the RAISE study. Curr Med Res Opin. 2021;37:1769-1778.

3) Zhao L, Zhang D, Ma H, et al. High VEGF-A level at baseline predicts poor treatment effect of bevacizumab-based chemotherapy in metastatic colorectal cancer: a meta-analysis. Panminerva Med. 2016;58:48-58.

4) Weickhardt AJ, Williams DS, Lee CK, et al. Vascular endothelial growth factor D expression is a potential biomarker of bevacizumab benefit in colorectal cancer. Br J Cancer. 2015;113:37-45.

https://doi.org/10.1038/bjc.2015.209

5) Nixon AB, Sibley A, Hatch AJ, et al. Blood-based biomarkers in patients (pts) with metastatic colorectal cancer (mCRC) treated with FOLFOX or FOLFIRI plus bevacizumab (Bev), cetuximab (Cetux), or Bev plus Cetux: Results from CALGB 80405 (Alliance). Journal of Clinical Oncology. 2016;34(15_suppl): 3597-3597.

https://doi.org/10.1200/jco.2016.34.15_suppl.3597

6) Tabernero J, Paccard C, Chiron M, et al. Placental growth factor and the angiogenic environment based on analysis of baseline plasma biomarkers from the VELOUR trial. Journal of Clinical Oncology. 2017;35 (4_suppl):592.

https://doi.org/10.1200/jco.2017.35.4_suppl.592

7) Dochy EC PM, Tabernero C. Impact of prior bevacizumab treatment of VEGFA and PIGF levels and patient outcomes: A retrospective analysis of baseline plasma samples from the VELOUR trial. WCGI. 2017;O-012.

https://doi.org/10.1093/annonc/mdx262.011

8) Seufferlein T, Ettrich TJ, Stein A, et al. Predicting resistance to first-line FOLFOX plus bevacizumab in metastatic colorectal cancer: Final results of the multicenter, international PERMAD trial. Journal of Clinical Oncology. 2021;39(3_suppl):115-115.

https://doi.org/10.1200/jco.2021.39.3_suppl.115

9) Taniguchi H, Yuki S, Shiozawa M, et al. Plasma VEGF-D and PlGF levels according to prior use of biologics among metastatic colorectal cancer: Preliminary results from GI-SCREEN CRC-Ukit study. Journal of Clinical Oncology. 2020;38(4_suppl):178-178.

https://doi.org/10.1200/jco.2020.38.4_suppl.178

10) Yuki S, Taniguchi H, Masuishi T, et al. 463P Impact of plasma angiogenesis factors on the efficacy of 2nd-line chemotherapy combined with biologics in metastatic colorectal cancer (mCRC): Early efficacy results from GI-SCREEN CRC Ukit study. Oncology. 2021;32:(Suppl 5):S563-S564.

https://doi.org/10.1016/j.annonc.2021.08.984

11) Ogino S, Cantor M, Kawasaki T, et al. CpG island methylator phenotype (CIMP) of colorectal cancer is best characterized by quantitative DNA methylation analysis and prospective cohort studies. Gut. 2006;55: 1000-1006.

https://doi.org/10.1136/gut.2005.082933

12) Lee MS, McGuffey EJ, Morris JS, et al. Association of CpG island methylator phenotype and EREG/AREG methylation and expression in colorectal cancer. Br J Cancer. 2016;114:1352-1361.

https://doi.org/10.1038/bjc.2016.87

13) Weisenberger DJ, Siegmund KD, Campan M, et al. CpG island methylator phenotype underlies sporadic microsatellite instability and is tightly associated with BRAF mutation in colorectal cancer. Nat Genet. 2006;38:787-793.

https://doi.org/10.1038/ng1834

14) Torlakovic EE, Gomez JD, Driman DK, et al. Sessile serrated adenoma (SSA) vs. traditional serrated adenoma (TSA). Am J Surg Pathol. 2008;32:21-29.

https://doi.org/10.1097/pas.0b013e318157f002

15) Toyota M, Ahuja N, Ohe-Toyota M, et al. CpG island methylator phenotype in colorectal cancer. Proc Natl Acad Sci USA. 1999;96:8681-866.

https://doi.org/10.1073/pnas.96.15.8681

16) Kaneda A, Yagi K: Two groups of DNA methylation markers to classify colorectal cancer into three epigenotypes. Cancer Sci. 2011;102:18-24.

https://doi.org/10.1111/j.1349-7006.2010.01712.x

17) Ouchi K, Takahashi S, Yamada Y, et al. DNA methylation status as a biomarker of anti-epidermal growth factor receptor treatment for metastatic colorectal cancer. Cancer Sci. 2015;106:1722-1729.

https://doi.org/10.1111/cas.12827

18) Okita A, Takahashi S, Ouchi K, et al. Consensus molecular subtypes classification of colorectal cancer as a predictive factor for chemotherapeutic efficacy against metastatic colorectal cancer. Oncotarget. 2018;9: 18698-18711.

https://doi.org/10.18632/oncotarget.24617

19) Eads CA, Danenberg KD, Kawakami K, et al. MethyLight: a high-throughput assay to measure DNA methylation. Nucleic Acids Res. 2000;28:E32.

https://doi.org/10.1093/nar/28.8.e32

20) Ouchi K, Takahashi S, Okita A, et al: A modified MethyLight assay predicts the clinical outcomes of anti-epidermal growth factor receptor treatment in metastatic colorectal cancer. Cancer Sci. 2022;113:1057-1068.

https://doi.org/10.1111/cas.15252

21) Osumi H, Ouchi K, Shinozaki E, et al. Effect of DNA methylation status on first-line anti-epidermal growth factor receptor treatment in patients with metastatic colorectal cancer. Int J Colorectal Dis. 2022;37:1439-1447.

https://doi.org/10.1007/s00384-022-04177-9

22) O'Connell JB, Maggard MA, Ko CY: Colon cancer survival rates with the new American Joint Committee on Cancer sixth edition staging. J Natl Cancer Inst. 2004;96:1420-1425.

https://doi.org/10.1093/jnci/djh275

23) Benson AB 3rd, Schrag D, Somerfield MR, et al. American Society of Clinical Oncology recommendations on adjuvant chemotherapy for stage II colon cancer. J Clin Oncol. 2004;22:3408-3419.

https://doi.org/10.1200/jco.2004.05.063

24) Labianca R, Nordlinger B, Beretta GD, et al. ESMO Guidelines Working Group: Early colon cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. Ann Oncol. 2013;24 Suppl 6:vi64-vi72.

https://doi.org/10.1093/annonc/mdt354

25) O'Connell MJ, Lavery I, Yothers G, et al. Relationship between tumor gene expression and recurrence in four independent studies of patients with stage II/III colon cancer treated with surgery alone or surgery plus adjuvant fluorouracil plus leucovorin. J Clin Oncol. 2010;28:3937-3944.

https://doi.org/10.1200/jco.2010.28.9538

26) Venook AP, Niedzwiecki D, Lopatin M, et al. Biologic determinants of tumor recurrence in stage II colon cancer: validation study of the 12-gene recurrence score in cancer and leukemia group B (CALGB) 9581. J Clin Oncol. 2013;31:1775-1781.

https://doi.org/10.1200/jco.2012.45.1096

27) Yothers G, O'Connell MJ, Lee M, et al. Validation of the 12-gene colon cancer recurrence score in NSABP C-07 as a predictor of recurrence in patients with stage II and III colon cancer treated with fluorouracil and leucovorin (FU/ LV) and FU/LV plus oxaliplatin. J Clin Oncol. 2013;31:4512-4519.

https://doi.org/10.1200/jco.2012.47.3116

28) Yamanaka T, Oki E, Yamazaki K, et al. 12-Gene Recurrence Score Assay Stratifies the Recurrence Risk in StageII/III Colon Cancer With Surgery Alone: The SUNRISE Study. J Clin Oncol. 2016;34:2906-13.

https://doi.org/10.1200/jco.2016.67.0414

29) Yothers G, Venook AP, Oki E, et al. Patient-specific meta-analysis of 12-gene colon cancer recurrence score validation studies for recurrence risk assessment after surgery with or without 5FU and oxaliplatin. J Gastrointest Oncol. 2022;13:126-136.

https://doi.org/10.21037/jgo-21-620

30) Colorectal cancer research group Ed. Colorectal Cancer Treatment Guidelines 2022 Edition for Doctors. Kanehara Publishing, 2022

31) Oki E, Watanabe J, Sato T, et al. Impact of the 12-gene recurrence score assay on deciding adjuvant chemotherapy for stageII and IIIA/B colon cancer：the SUNRISE-DI study. ESMO Open. 2021;6:100146.

https://doi.org/10.1016/j.esmoop.2021.100146

32) Gotoh K, Shinto E, Yoshida Y, et al. Prognostic Model of StageII/III Colon Cancer Constructed using Gene Expression Subtypes and KRAS Mutation Status. J Clin Exp Oncol. 2018;7:2.

https://doi.org/10.4172/2324-9110.1000214

33) Shinto E, Oki E, Shimokawa M, et al. A Validation Study for Recurrence Risk Stratification of Stage II Colon Cancer Using the 55-Gene Classifier. Oncology. 2020:98:534-541.

https://doi.org/10.1159/000506369

34) Oki E, Shinto E, Shimokawa M, et al. Evaluation of a 55-gene classifier as a prognostic biomarker for adjuvant chemotherapy in stage III colon cancer patients. BMC Cancer. 2021:21:1332.

https://doi.org/10.1186/s12885-021-09088-6

35) Salazar R, Roepman P, Capella G, et al. Gene expression signature to improve prognosis prediction of stage II and III colorectal cancer. J Clin Oncol. 2011;29:17-24.

https://doi.org/10.1200/jco.2010.30.1077

36) Fridman WH, Pagès F, Sautès-Fridman C, et al. The immune context in human tumors: impact on clinical outcome. Nat Rev Cancer. 2012;12:298-306.

https://doi.org/10.1038/nrc3245

37) Wilkinson K, Ng W, Roberts TL, et al. Tumor immune microenvironment biomarkers predicting cytotoxic chemotherapy efficacy in colorectal cancer. J Clin Pathol. 2021;74:625-634.

https://doi.org/10.1136/jclinpath-2020-207309

38) Di Caro G, Bergomas F, Grizzi F, et al. Occurrence of tertiary lymphoid tissue is associated with T-cell infiltration and predicts better prognosis in early-stage colorectal cancers. Clin Cancer Res. 2014;20:2147-2158.

https://doi.org/10.1158/1078-0432.ccr-13-2590

39) Li Y, Liang L, Dai W, et al. Prognostic impact of programmed cell death-1 (PD-1) and PD-ligand 1 (PD-L1) expression in cancer cells and tumor infiltrating lymphocytes in colorectal cancer. Mol Cancer. 2016;15:55.

https://doi.org/10.1186/s12943-016-0539-x

40) Pagès F, Mlecnik B, Marliot F, et al. International validation of the consensus Immunoscore for the classification of colon cancer: a prognostic and accuracy study. Lancet. 2018;391:2128-2139.

41) Marliot F, Chen X, Kirilovsky A, et al. Analytical validation of the Immunoscore and its associated prognostic value in patients with colon cancer. J Immunother Cancer. 2020;8:e000272.

https://doi.org/10.1136/jitc-2019-000272

42) Mlecnik B, Bifulco C, Marliot F, et al. A TNM-Immune (TNM-I) classification staging system for predicting survival in colon cancer in a multicenter international SITC study. Annals of Oncology. 2020;31 suppl_3:238.

https://doi.org/10.1016/j.annonc.2020.04.070

43) André T, Vernerey D, Mineur L, et al ; for PRODIGE investigators, GERCOR, Fédération Française de Cancérologie Digestive, and UNICANCER : Three Versus 6 Months of Oxaliplatin-Based Adjuvant Chemotherapy for Patients With StageIII Colon Cancer : Disease-Free Survival Results From a Randomized, Open-Label, International Duration Evaluation of Adjuvant (IDEA) France, Phase III Trial. J Clin Oncol. 2018;36:1469-1477.

https://doi.org/10.1200/jco.2017.76.0355

44) Alberts SR, Sargent DJ, Nair S, et al. Effect of oxaliplatin, fluorouracil, and leucovorin with or without cetuximab on survival among patients with resected stage III colon cancer: a randomized trial. JAMA. 2012;307:1383-1393.

https://doi.org/10.1001/jama.2012.385

45) Pagès F, André T, Taieb J, et al. Prognostic and predictive value of the Immunoscore in stage III colon cancer patients treated with oxaliplatin in the prospective IDEA France PRODIGE-GERCOR cohort study. Ann Oncol. 2020;31:921-929.

https://doi.org/10.1016/j.annonc.2020.03.310

46) Sinicrope FA, Shi Q, Hermitte F, et al. Contribution of Immunoscore and Molecular Features to Survival Prediction in StageIII Colon Cancer. JNCI Cancer Spectr. 2020;4:pkaa023.

https://doi.org/10.1093/jncics/pkaa023

47) Mlecnik B, Bifulco C, Bindea G, et al. Multicenter International Society for Immunotherapy of Cancer Study of the Consensus Immunoscore for the Prediction of Survival and Response to Chemotherapy in StageIII Colon Cancer. J Clin Oncol. 2020;38:3638-3651.

48) El Sissy C, Kirilovsky A, Van den Eynde M, et al. A Diagnostic Biopsy-Adapted Immunoscore Predicts Response to Neoadjuvant Treatment and Selects Patients with Rectal Cancer Eligible for a Watch-and-Wait Strategy. Clin Cancer Res. 2020;26:5198-5207.

https://doi.org/10.1158/1078-0432.ccr-20-0337

49) Pagès F, El Sissy C, Kirilovsky A, et al. International validation of the Immunoscore-biopsy (ISB) to guide selection and monitoring of patients treated with watch-and-wait (WW) strategy for rectal cancer. Journal of Clinical Oncology. 2022;40(16_suppl): 3517-3517.

https://doi.org/10.1200/jco.2022.40.16_suppl.3517

50) Shamseddine A, Khalifeh IM, Elias C, et al. High immunoscore as a predictor of outcome in patients who underwent chemoimmuno-therapy in locally advanced rectal cancer: A post-hoc analysis of the correlation between immunoscore and pCR in the Averectal study. Clinical Oncology. 2022;40(4_suppl):184-184.

https://doi.org/10.1200/jco.2022.40.4_suppl.184

51) Antoniotti C, Rossini D, Pietrantonio F, et al. GONO Foundation Investigators: Upfront FOLFOXIRI plus bevacizumab with or without atezolizumab in the treatment of patients with metastatic colorectal cancer (AtezoTRIBE): a multicentre, open-label, randomized, controlled, phase 2 trial. Lancet Oncol. 2022;23:876-887.

https://doi.org/10.1016/s1470-2045(22)00274-1

12 Remarks

Definitions of guideline, guidance, etc. by the Japanese Society of Medical Oncology

1. Guideline

Used when the target disease/treatment area is wide, when a lot of evidence has been accumulated, or when multi-disciplinary involvement is required for preparation, or when the target is multi-disciplinary in nature.

2. Guidance

When the target disease or treatment area is narrow ranging, the evidence is limited, and the authors and subjects are limited to a narrow area.

3. Consensus report

Greatest common denominator opinions and clinical guidelines determined by voting by a group of experts.

4. Expert opinion

Opinions of experts or groups of experts, clinical guidelines.

5. Provisional view

Temporary, provisional opinion, statement. It is used when it is undecided but necessary and should be stated as a provisional opinion at this time. Synonymous with provisional statement/opinion.

Appendix

Table 1 Frequency of *RAS* mutations

	KRAS exon 2	*KRAS* exon 3	*KRAS* exon 4	*NRAS* exon 2	*NRAS* exon 3	*NRAS* exon 4	Total *
PRIME [1]	40% (440/1,096)	4% (24/638)	6% (36/620)	3% (22/637)	4% (26/636)	0% (0/629)	17%
20050181 [2]	45% (486/1,083)	4.4% (24/548)	7.7% (41/534)	2.2% (12/536)	5.6% (30/540)	0% (0/532)	20%
20020408 [3]	43% (184/427)	4.8% (8/166)	5.0% (9/180)	4.2% (7/166)	3.0% (5/168)	1.1% (2/180)	18%
CRYSTAL [4]	N/A	3.3%	5.6%	3.5%	2.8%	0.9%	15%
FIRE-3 [5]	N/A	4.3% (21/431)	4.9% (24/458)	3.8% (18/464)	2% (10/468)	0% (0/458)	16%
CALGB 80405 [6]	N/A	1.8%	5.9%	2.3%	4.2%	0%	14%
RASKET [7]	37.8% (116/307)	3.1% (6/191)	5.2% (10/191)	3.1% (6/191)	4.2% (8/191)	0% (0/191)	16%

* Frequency of *KRAS/NRAS* mutations in *KRAS* exon 2 wild type

N/A: not available

Table 2 Therapeutic effects of anti-EGFR antibody drugs against *RAS* wild-type

	RAS Evaluable *	regimen	n	RR (%)	PFS (m)	HR	OS (m)	HR
PRIME [1] (first-line treatment)	90% (1,060/1,183)	FOLFOX4	253	—	7.9	0.72 (p =0.004)	20.2	0.78 (p = 0.04)
		FOLFOX4 + Pmab	259	—	10.1		26.0	
20050181 [2] (second-line treatment)	85% (1,008/1,186)	FOLFIRI	213	10	4.4	0.70 (p = 0.007)	13.9	0.81 (p =0.08)
		FOLFIRI + Pmab	208	41	6.4		16.2	

20020408 [3] (third-line treatment)	82% (378/463)	BSC	63	0	7 weeks	0.36 ($p < 0.0001$)	—	—
		BSC + Pmab	73	16	14.1 weeks		—	
20100007 [4] (third-line treatment)	87% (328/377)	BSC	128	2.3	1.7	0.46 ($p < 0.0001$)	6.9	0.70 ($p = 0.0135$)
		BSC + Pmab	142	31.0	5.2		10.0	
OPUS [5] (first-line treatment)	75% (254/337)	FOLFOX4	49	29	5.8	0.53 ($p = 0.0615$)	17.8	0.94 ($p = 0.80$)
		FOLFOX4 + Cmab	38	58	12.0		19.8	
Crystal [6] (first-line treatment)	69% (827/1,198)	FOLFIRI	189	38.6	8.4	0.56 ($p = 0.0002$)	20.2	0.69 ($p = 0.0024$)
		FOLFIRI + Cmab	178	66.3	11.4		28.4	
FIRE-3 [7] (first-line treatment)	78 % (588/752)	FOLFIRI + Bmab	201	58.7	10.2	0.97 ($p = 0.77$)	25.0	0.70 ($p = 0.0059$)
		FOLFIRI + Cmab	199	65.3	10.3		33.1	
PEAK [8] (first-line treatment)	82% (233/285)	mFOLFOX6 + Bmab	82	54	10.1	0.66 ($p = 0.03$)	28.9	0.63 ($p = 0.06$)
		mFOLFOX6 + Pmab	88	58	13.0		41.3	
CALGB80405 [9] (first-line treatment)	59% (670/1,137)	FOLFOX/IRI＋Bmab	256	53.8	11.0	1.03 ($p = 0.71$)	31.2	0.9 ($p = 0.40$)
		FOLFOX/IRI + Cmab	270	68.6	11.2		32.0	

Table 3 Therapeutic effect of anti-EGFR antibody drugs on *RAS* mutation-positive cases

	regimen	n	RR (%)	PFS (m)	HR	OS (m)	HR
PRIME [1] (first-line treatment)	FOLFOX4	276	—	8.7	1.31 (p =0.008)	19.2	1.25 ($p = 0.034$)
	FOLFOX4 + Pmab	272	—	7.3		15.6	

20050181 [2] (second-line treatment)	FOLFIRI	294	13	4.0	0.86 (p = 0.14)	11.1	0.91 (p = 0.34)
	FOLFIRI + Pmab	299	15	4.8		11.8	
20020408 [3] (third-line treatment)	BSC	114	0	7.3 weeks	0.97 (p = 0.729)	—	—
	BSC + Pmab	99	1	7.4 weeks		—	
20100007 [4] (third-line treatment)	BSC	28	—	1.6	1.03 (p = 0.9429)	7.5	0.99 (p = 0.9625)
	BSC + Pmab	26	—	1.6		7.6	
OPUS [5] (first-line treatment)	FOLFOX4	75	50.7	7.8	1.54 (p = 0.0309)	17.8	1.29 (p = 0.1573)
	FOLFOX4 + Cmab	92	37.0	5.6		13.5	
Crystal [6] (first-line treatment)	FOLFIRI	214	36.0	7.5	1.10 (p = 0.47)	17.7	1.05 (p = 0.64)
	FOLFIRI + Cmab	246	31.7	7.4		16.4	
FIRE-3 [7] (first-line treatment)	FOLFIRI＋Bmab	91	50.5	9.6	1.25 (p = 0.14)	20.6	1.05 (p =0.75)
	FOLFIRI + Cmab	97	38.1	7.5		20.2	
PEAK [8] (first-line treatment)	mFOLFOX6 + Bmab	27	56	8.9	1.39 (p = 0.318)	16.6	0.41 (p = 0.020)
	mFOLFOX6 + Pmab	24	60	7.8		27.0	
CALGB80405 [9] (first-line treatment)	FOLFOX/IRI＋ Bmab	42	—	—	—	22.3	0.74 (p = 0.21)
	FOLFOX/IRI + Cmab	53	—	—		28.7	

Pmab: panitumumab, Cmab: cetuximab, Bmab: bevacizumab, IRI: irinotecan, RR: response rate, PFS: progression-free survival, HR: hazard ratio, OS: overall survival, m: month, * *RAS* evaluable: percentage of cases evaluated for *RAS* mutations among randomized cases, BSC: best supportive care, FOLFOX: 5-FU + leucovorin + oxaliplatin, FOLFIRI: 5-FU + leucovorin + irinotecan

[References]

1) Douillard JY, Oliner KS, Siena S, et al. Panitumumab-FOLFOX4 treatment and RAS mutations in

colorectal cancer. N Engl J Med. 2013;369:1023-1034.
https://doi.org/10.1056/nejmoa1305275
2) Peeters M, Oliner KS, Price TJ, et al. Analysis of KRAS/NRAS Mutations in a Phase III Study of Panitumumab with FOLFIRI Compared with FOLFIRI Alone as Second-line Treatment for Metastatic Colorectal Cancer. Clin Cancer Res. 2015;21:5469-5479.
https://doi.org/10.1158/1078-0432.ccr-15-0526
3) Peeters M, Oliner KS, Parker A, et al. Massively parallel tumor multigene sequencing to evaluate response to panitumumab in a randomized phase III study of metastatic colorectal cancer. Clin Cancer Res. 2013;19:1902-1912.
https://doi.org/10.1158/1078-0432.ccr-12-1913
4) Kim TW, Elme A, Kusic Z, et al. A phase 3 trial evaluating panitumumab plus best supportive care vs best supportive care in chemrefractory wild-type KRAS or RAS metastatic colorectal cancer. Br J Cancer. 2016;115:1206-1214.
https://doi.org/10.1038/bjc.2016.309
5) Bokemeyer C, Köhne CH, Ciardiello F, et al. FOLFOX4 plus cetuximab treatment and RAS mutations in colorectal cancer. Eur J Cancer. 2015;51:1243-1252.
https://doi.org/10.1016/j.ejca.2015.04.007
6) Van Cutsem E, Lenz HJ, Köhne CH, et al. Fluorouracil, leucovorin, and irinotecan plus cetuximab treatment and RAS mutations in colorectal cancer. J Clin Oncol. 2015;33:692-700.
https://doi.org/10.1200/jco.2014.59.4812
7) Stintzing S, Modest DP, Rossius L, et al. FIRE-3 investigators: FOLFIRI plus cetuximab versus FOLFIRI plus bevacizumab for metastatic colorectal cancer (FIRE-3): a post-hoc analysis of tumor dynamics in the final RAS wild- type subgroup of this randomized open-label phase 3 trial. Lancet Oncol. 2016;17: 1426-1434.
https://doi.org/10.1016/s1470-2045(16)30269-8
8) Schwartzberg LS, Rivera F, Karthaus M, et al. PEAK: a randomized, multicenter phase II study of panitumumab plus modified fluorouracil, leucovorin, and oxaliplatin (mFOLFOX6) or bevacizumab plus mFOLFOX6 in patients with previously untreated, unresectable, wild- type KRAS exon 2 metastatic colorectal cancer. J Clin Oncol. 2014;32:2240-2247.
https://doi.org/10.1200/jco.2013.53.2473
9) Venook AP, Niedzwiecki D, Lenz HJ, et al. Effect of First-Line Chemotherapy Combined With Cetuximab or Bevacizumab on Overall Survival in Patients With KRAS Wild-Type Advanced or Metastatic Colorectal Cancer: A Randomized Clinical Trial. JAMA. 2017;317:2392-2401.
https://doi.org/10.3410/f.727734555.793550882