Molecular Testing for Colorectal Cancer Treatment

4th Edition

Compiled by the Japanese Society of Medical Oncology

4.1 ed. Draft Version 0.01	January 5, 2019
4.1 ed. Draft Version 0.02	March 4, 2019
4.1 ed. Draft Version 0.03	March 18, 2019
4.1 ed. Draft Version 0.04	March 23, 2019
4.1 ed. Draft Version 0.05	March 26, 2019
4.1 ed. Draft Version 0.06	April 19, 2019
4.1 ed. Version 1.	May 15, 2019
4.2 ed. Version 1.	September 25, 2019
4.2 ed. Version 2.	October 25, 2019

Japanese Society of Medical Oncology Clinical Guidelines: Molecular Testing for Colorectal Cancer Treatment, 4th Edition Working Group

Committee chair

Hiromichi Ebi	Division of Molecular Therapeutics, Aichi Cancer Center Research Institute

Committee members

Hideaki Bando	Department of Clinical Oncology, Aichi Cancer Center
Yutaka Hatanaka	Hokkaido University Hospital Research Division of Genome Companion Diagnostics
Waki Hosoda	Department of Molecular Diagnostics, Aichi Cancer Center
Kensuke Kumamoto	Department of Gastroenterological Surgery, Faculty of Medicine, Kagawa University
Kaname Nakatani	Mie University Hospital, Clinical Laboratory
Yoshinaga Okugawa	Department of Genomic medicine, Mie University Faculty of Medicine
Yu Sunakawa	St. Marianna University School of Medicine, Department of Clinical Oncology
Hiroya Taniguchi	Department of Gastrointestinal Oncology, National Cancer Center Hospital East
Kentaro Yamazaki	Shizuoka Cancer Center, Gastroenterology

（Alphabetical order）

Contributor

Saori Mishima	Department of Gastrointestinal Oncology, National Cancer Center Hospital East

External Evaluation Committee

Eishi Baba	Department of Oncology and Social Medicine, Kyushu University Graduate School of Medical Sciences
Hideyuki Ishida	Department of Digestive Tract and General Surgery, Saitama Medical Center, Saitama Medical University
Kazuto Nishio	Department of Genome Biology, Kindai University Faculty of Medicine
Katsuya Tsuchihara	Division of Translational Informatics, Exploratory Oncology Research and Clinical Trial Center, National Cancer Center

（Alphabetical order）

Table of Contents

5 Next-generation sequencing-based comprehensive genomic profiling tests

5.1 Background

5.2 Comprehensive genomic profiling tests are recommended to assess the benefits of molecular targeted drugs in patients with unresectable CRC.

5.3 Use test systems with verified analytical validity to perform comprehensive genomic profiling tests.

6 Liquid biopsy

6.1 Background

6.2 ctDNA testing is recommended to determine the optimal perioperative chemotherapy based on presumed recurrence risk of patients with resectable CRC.

6.3 ctDNA testing is recommended to evaluate the suitability of and monitor the therapeutic effects of anti-EGFR antibody therapy in patients with unresectable CRC.

6.4 ctDNA-based comprehensive genomic profiling tests are recommended to assess the benefits of molecular targeted drugs for patients with unresectable CRC.

7 Angiogenic factors

7.1 Background

7.2 Measurement of VEGF-D level is performed to identify the appropriate angiogenesis inhibitors for patients with unresectable CRC.

8 Other tests

8.1 Multi-gene assays to predict colon cancer recurrence after surgery

8.2 CDX2

8.3 CIMP

9 Samples for molecular testing

9.1 FFPE tissue is suitable for genomic testing of somatic mutations. It is recommended to confirm that the samples have an adequate amount of tumor cells and expect sufficient quality of nucleic acids by assessing the matched reference hematoxylin and eosin stained slides. Selection of FFPE samples, decision on the need for macrodissection, and assessment of tumor cell content should be performed by a pathologist.

9.2 In ctDNA testing, use of collection tubes and the preservation and adjustment of plasma after blood collection should be performed in accordance with the manufacturer’s instructions.

10 Quality assurance requirements for testing

Genomic testing for CRC treatment should be carried out under a quality assurance system.

11 Notes

11.1 Definitions of “guidelines,” “guidance,” etc. by the Japanese Society of Medical Oncology

11.2 Pharmaceutical approval and insurance coverage status for each test as of September 2019

11.3 On participation in voting

Abbreviations

ASCO	American Society of Clinical Oncology
APC	adenomatous polyposis coli
BSC	best supportive care
CAPOX	Capecitabine + Oxaliplatin
CDx	companion diagnostics
CDX2	caudal-related homeobox transcription factor 2
cfDNA	cell free DNA
CGP	comprehensive genomic profiling
CI	confidence interval
CIMP	CpG island methylation phenotype
CIN	Chromosomal instability
CMS	Consensus Molecular Subtypes
CNA	copy number alterations
COSMIC	Catalogue of Somatic Mutations in Cancer
CRC	Clinical Research Coordinator
ctDNA	circulating tumor DNA
DFS	Disease-free survival
dMMR	mismatch repair–deficient
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ESMO	European Society for Medical Oncology
FGF	fibroblast growth factor
FOLFIRI	5-FU＋leucovorin＋irinotecan
FOLFOX	5-FU＋leucovorin＋oxaliplatin
FOLFOXIRI	5−FU＋leucovorin＋oxaliplatin＋irinotecan
GCHP	Goblet cell−rich type hyperplastic polyp
HGF	hepatocyte growth factor
HNPCC	Hereditary Non−polyposis colorectal cancer
HP	hyperplastic polyp
HR	Hazard Ratio
IHC	Immunohistochemistry
IUO	investigational use only
IVD	*in-vitro* diagnostics

LDT	laboratory developed test
MCP-3	macrophage chemoattractant protein-3
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
MVD	microvessel density
MVHP	Microvesicular type hyperplastic polyp
NCA	copy number alterations
NCCN	The National Comprehensive Cancer Network
NGS	next generation sequencing
NTRK	neurotrophin receptor tyrosine kinase
OR	Odds ratio
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PFS	progression-free survival
PlGF	placental growth factor
pMMR	mismatch repair−proficient
RFS	relapse-free survival
RR	Risk Ratio
RT-PCR	reverse transcription-polymerase chain reaction
RUO	research use only
SSA	sessile serrated adenoma
SSA/P	sessile serrated adenoma/polyp
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 uncertain significance
WES	whole exome sequencing

Summary

Earlier editions of these guidelines, including the first edition, “Japanese Guidelines for Testing of the *KRAS* Gene Mutation in Colorectal Cancer, First Edition,” and the subsequently published “Japanese Society of Medical Oncology Clinical Guidelines: *RAS (KRAS/NRAS*) Mutation Testing in Colorectal Cancer Patients, Second Edition,” have contributed to the proper use of *KRAS* and *RAS* mutation testing in clinical practice. Thereafter, in accordance with recognizing the importance of abnormalities other than the *RAS* mutation in the treatment of colorectal cancer, guidelines on properly testing for the *BRAF* V600E mutation and MMR deficiency were presented in “Japanese Society of Medical Oncology Clinical Guidelines: Molecular Testing for Colorectal Cancer Treatment, Third Edition.”

It has been approximately two years since the third edition was released. During this time, not only have tests for the *BRAF* V600E mutation and MMR deficiency received insurance coverage, but also there has been a rapid introduction of precision medicine based on cancer gene panel testing, which led to the approval of the second cancer gene profiling test in December 2018 at a medical device and *in vitro* diagnostics conference. With the expectation of targeted treatments for low-frequency driver gene mutations based on panel testing, efforts behind liquid biopsy-based profiling tests are also rapidly progressing. Further, the use of tumor tissue or blood samples suitable for these tests is indispensable for obtaining accurate results. Therefore, in November 2018, the working group behind this guidance began revision; as such, evaluation was undertaken by an independent review committee from March to April 2019, and a provisional version was prepared. Further, because next-generation sequencing-based comprehensive genomic profiling tests received insurance coverage in June 2019, the content of the guidance was updated and published as a fourth edition.

The objectives of this revision are to provide clinicians and pathologists involved in genomic testing for colorectal cancer treatment with basic requirements regarding the proper performance and use in the treatment of tests currently covered by insurance, and to present information about both the status and prospects of new test technologies. Therefore, the revised guidance describes the basic requirements indicated in **Table 1** for testing genomic abnormalities involved in treatment selection and outcome prediction in colorectal cancer treatment (indications and timing of each genomic test are shown in **Figure 1**). The strength of recommendation for each requirement was determined by a vote among working group members (**Table 2**). The degree of recommendation for each requirement was determined based on evidence for each test and the expected balance of benefits and disadvantages for patients when testing is performed; the status of insurance coverage in Japan for each test was not considered. When an agreement of at least 70% was reached through voting, the majority stance was set as the collective opinion. If, for all degrees of recommendation, an agreement of at least 70% could not be reached, voting was repeated after a reveal of the prior results. If, after repeating this process three times, the degree of recommendation could not be determined, the judgment for that requirement was “No degree of

recommendation." Outside the main text, information directly related to the basic requirements is presented in the "Comments" section, while information not directly related but considered necessary as peripheral information to the basic requirements is presented in the "Memos" section. We have also noted the status and prospects of testing technologies that are currently being developed. Further, please refer to the "Notes" section for the definition of "guidance" used by the Japanese Society of Medical Oncology, the insurance coverage status of each test, and the status of each working group member's participation in voting.

Table 1. Basic requirements presented in this guidance

Basic requirement	Strength of recommendation
***RAS* mutation testing**	
● *RAS* mutation testing is recommended prior to first-line chemotherapy to assess the benefit of anti-EGFR antibody therapy in patients with unresectable CRC. Use a companion diagnostic to perform *RAS* mutation testing.	Strong recommendation
● *RAS* mutation testing can determine the optimal perioperative chemotherapy based on the presumed recurrence risk in patients with resectable CRC. Use a test with verified analytical validity to perform *RAS* mutation testing.	Expert Consensus Opinion
***BRAF* mutation testing**	
● *BRAF* V600E mutation testing is recommended prior to first-line chemotherapy to determine the optimal treatment based on the prognosis of patients with unresectable CRC. Use a test with verified analytical validity to perform *BRAF* V600E mutation testing.	Strong recommendation
● *BRAF* V600E mutation testing is recommended to determine the optimal perioperative chemotherapy based on the presumed recurrence risk in patients with resectable CRC. Use a test with verified analytical validity to perform *BRAF* V600E mutation testing.	Recommendation
● *BRAF* V600E mutation testing is recommended to help diagnose Lynch syndrome.	Recommendation

Use a test with verified analytical validity to perform *BRAF* V600E mutation testing.	

Testing for mismatch-repair deficiency

● Mismatch repair (MMR) deficiency testing is recommended to evaluate the benefit of immune checkpoint inhibitors in patients with unresectable CRC. Use a companion diagnostic to perform MMR deficiency testing to evaluate the suitability of immune checkpoint inhibitor therapy.	Strong recommendation
● MMR deficiency testing is recommended to assess the risk of recurrence and stratify optimal perioperative chemotherapy in patients with resectable CRC. Use a test with verified analytical validity to perform MMR deficiency testing with the aim of treatment selection appropriate to recurrence risk.	Recommendation
● MMR deficiency testing is recommended to screen for Lynch syndrome.	Strong recommendation
● The following methods are recommended when assessing for MMR deficiency:	
➢ MSI testing.	Strong recommendation
➢ IHC testing	Strong recommendation
➢ NGS-based testing.	Recommendation

Next-generation sequencing-based comprehensive genomic profiling tests

● Comprehensive genomic profiling tests are recommended to assess the benefits of molecular targeted drugs in patients with unresectable CRC.	Strong recommendation
Use test systems with verified analytical validity for the performance of comprehensive genomic profiling tests.	Strong recommendation

Liquid biopsy

● ctDNA testing is recommended to determine the optimal perioperative chemotherapy based on presumed recurrence risk of patients with resectable CRC. Use a quality-assured panel test for the detection of minimal residual disease to perform testing.	Recommendation

● ctDNA testing is recommended to evaluate the suitability of and monitor the therapeutic effects of anti-EGFR antibody therapy in patients with unresectable CRC. Use a test with verified analytical validity to perform testing.	Recommendation
● ctDNA-based comprehensive genomic profiling tests are recommended to assess the benefits of molecular targeted drugs for patients with unresectable CRC. Use a test with verified analytical validity to perform testing.	Recommendation

Angiogenic factors

● Measurement of VEGF-D level is performed to identify the appropriate angiogenesis inhibitors for patients with unresectable CRC. Use a test with verified analytical validity to perform testing.	Expert Consensus Opinion

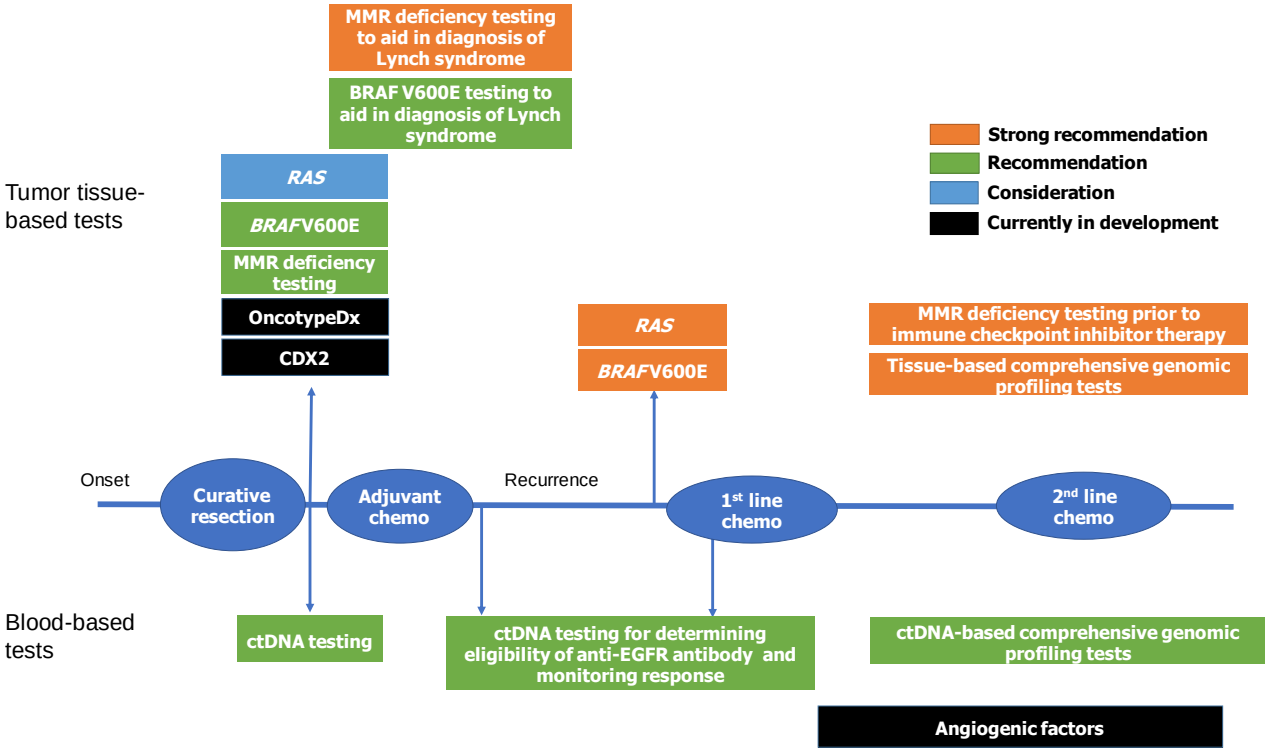
Specimen processing

● FFPE tissue is suitable for genomic testing of somatic mutations. It is recommended to confirm that the samples have an adequate amount of tumor cells and expect sufficient quality of nucleic acids by assessing the matched reference hematoxylin and eosin stained slides. Selection of FFPE samples, decision on the need for macrodissection, and assessment of tumor cell content should be performed by a pathologist.	Strong recommendation
● In ctDNA testing, use of collection tubes and the preservation and adjustment of plasma after blood collection should be performed in accordance with the manufacturer's instructions.	Strong recommendation

Quality assurance for testing

● Genomic testing for CRC treatment should be carried out under a quality assurance system.	Strong recommendation

Figure 1. Timing of tests



MMR：mismatch repair, ctDNA：circulating tumor DNA

Table 2. Strength of recommendation and decision criteria

Degree of recommendation	Decision criteria
Strong recommendation（SR）	Sufficient evidence and the benefits of testing outweigh the losses
Recommendation（R）	Evidence considering the balance between benefits and losses
Expert consensus opinion（ECO）	Consensus obtained although not enough evidence and information
No recommendation（NR）	Not recommended owing to the lack of evidence

Sufficient evidence, consistent evidence from randomized control trials (RCT) without important limitations or exceptionally strong evidence from observational studies; evidence, evidence from RCT with important limitations or strong evidence from observational studies; consensus, evidence for at least 1 critical outcome from observational studies, case series, or RCTs with serious flaws or indirect evidence

1 Introduction

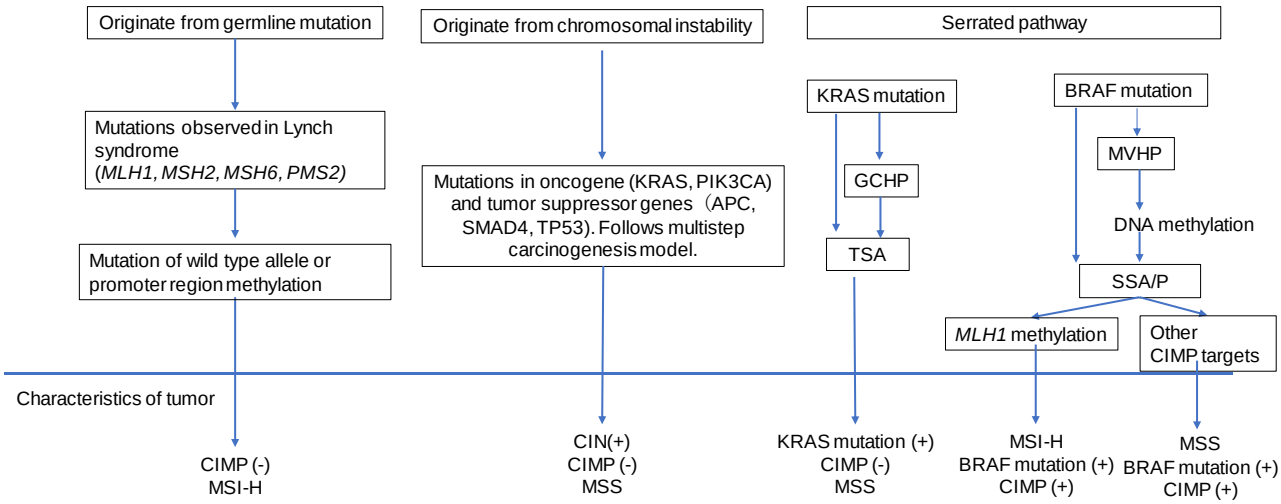
1.1 Molecular biological background of colorectal cancer

Most colorectal cancers develop in stages and undergo malignant progression due to the accumulation of various abnormalities on multiple genes. Genomic abnormalities include gene mutations due to genomic or environmental factors, such as spontaneous mutations and deletions, as well as epigenomic changes, such as dysregulation of genomic expressions at the transcriptional level. At present, colorectal cancer is broadly divided into cases originating from a germline mutation, chromosomal instability, or serrated lesions (**Figure 1**) [1]. In colorectal cancer caused by germline mutations, including Lynch syndrome and hereditary colorectal cancer, microsatellite instability (MSI), which causes genomic abnormalities to accumulate due to a DNA mismatch-repair (MMR) deficiency, plays a role in tumor initiation and development. In colorectal cancer that involves chromosomal instability, the mechanism of carcinogenesis is believed to follow a multi-step model in which abnormalities accumulate in tumor suppressor genes, such as *APC* and *TP53,* and oncogenes, such as *KRAS and PIK3CA*. Colorectal serrated lesions are classified into hyperplastic polyps (HP), traditional serrated adenomas (TSA), and sessile serrated adenomas/polyps (SSA/P). Further, SSA/P, which develop preferentially in the right colon and which frequently show MSI, have *BRAF* mutations, or present the CpG island methylation phenotype (CIMP), have drawn attention as a potential precursor lesion, especially for right colon cancer.

*KRAS and BRAF a*bnormalities are considered driver gene mutations that play important roles in the development and progression of colorectal cancer. Considered mutually exclusive, these mutations are rarely detected at the same time. In addition, with the introduction of comprehensive genomic profiling tests in recent years, other driver gene mutations, though infrequent, have been identified, such as *HER2* amplification.

On the other hand, when classified based on the gene expression profile, colorectal cancers are grouped into four subtypes（**Table 1**）[2]. Consensus Molecular Subtype (CMS) 1 is frequent among females with right colon primary tumors, and the rates of microsatellite instability (MSI)-high (MSI-H) and *BRAF* mutation are high. Although the rate of genomic mutation is high, the rate of copy number alterations (CNAs) is low. CMS2 has a high rate of CNAs and is characterized by activation of the WNT pathway; CMS3 has a high rate of *KRAS* mutation and is characterized by overexpression of IGFBP2; and CMS4 is high in CNAs and is characterized by a high proportion of cases in advanced stages. In this way, *KRAS* and *BRAF* abnormalities and MSI are also believed to influence the gene expression profile of colorectal cancers that have been developed.

Figure 1. Presumptive pathways for pathogenesis of colorectal cancer



APC : adenomatous polyposis coli, CIMP : CpG island methylation phenotype, CIN : Chromosomal instability, GCHP : Goblet cell-rich type hyperplastic polyp, MSI : microsatellite instability, MSS : microsatellite stable, MVHP : microvesicular type hyperplastic polyp, SSA : sessile serrated adenoma, TSA : traditional serrated adenoma, SSA/P : sessile serrated adenoma/polyp

Table 1. Classification of colorectal cancer subtypes by gene expression

	CMS1	CMS2	CMS3	CMS4
	MSI immune	Canonical	Metabolic	Mesenchymal
MSI	MSI-H	MSS	Mixed	MSS
CIMP	High		Low	
Chromosomal abnormalities		High	Low	High
Gene mutations	Many			
Genomic abnormalities	*BRAF* mutation		*KRAS* mutation	
Other features	Immune cell infiltration	WNT, MYC activation		Angiogenesis, stromal invasion
Prognosis	Poor outcome after recurrence			Poor recurrence-free survival and overall survival

MSI : microsatellite instability, CIMP : CpG island methylation phenotype, CMS : consensus molecular

subtype, MSI-H：MSI-high, MSS：microsatellite stable

1.2 Clinical significance of genomic abnormalities in colorectal cancer

There have also been advancements in the investigation of genomic abnormalities and their clinical significance in the development of colorectal cancer. Analysis based on numerous prospective studies has shown that anti- epidermal growth factor receptor (EGFR) antibody therapy is ineffective for patients with *RAS (KRAS/NRAS)* mutation (see Section 2: *RAS* mutation testing). Therefore, *KRAS* (K-ras) and *RAS* (*KRAS/NRAS)* gene tests to evaluate the adequacy of anti-EGFR antibody therapy have become covered by health insurance since April 2010 and April 2015, respectively, and the tests are being widely implemented in clinical practice.

The *BRAF* V600E mutation has demonstrated a strong independent predictive value for poor prognosis in patients with unresectable advanced or recurrent colorectal cancer. However, it has been recently reported that treatment with FOLFOXIRI (5-FU + leucovorin + oxaliplatin + irinotecan) and bevacizumab may be more effective for *BRAF* V600E-mutated cases compared to other existing chemotherapies (see Section 3: *BRAF* mutation testing). In addition, development of treatments using BRAF inhibitors is advancing[3]. Therefore, *BRAF* V600E mutation testing, to select treatment for unresectable advanced or recurrent colorectal cancer, is covered under health insurance since August 2018. Moreover, to diagnose Lynch syndrome, Lynch syndrome can be essentially ruled out in *BRAF* V600E- mutant cases, in which immunostaining shows loss of MLH1 expression; therefore, *BRAF* mutation testing is simultaneously covered by health insurance as a diagnostic tool for diagnosing Lynch syndrome in patients with colorectal cancer.

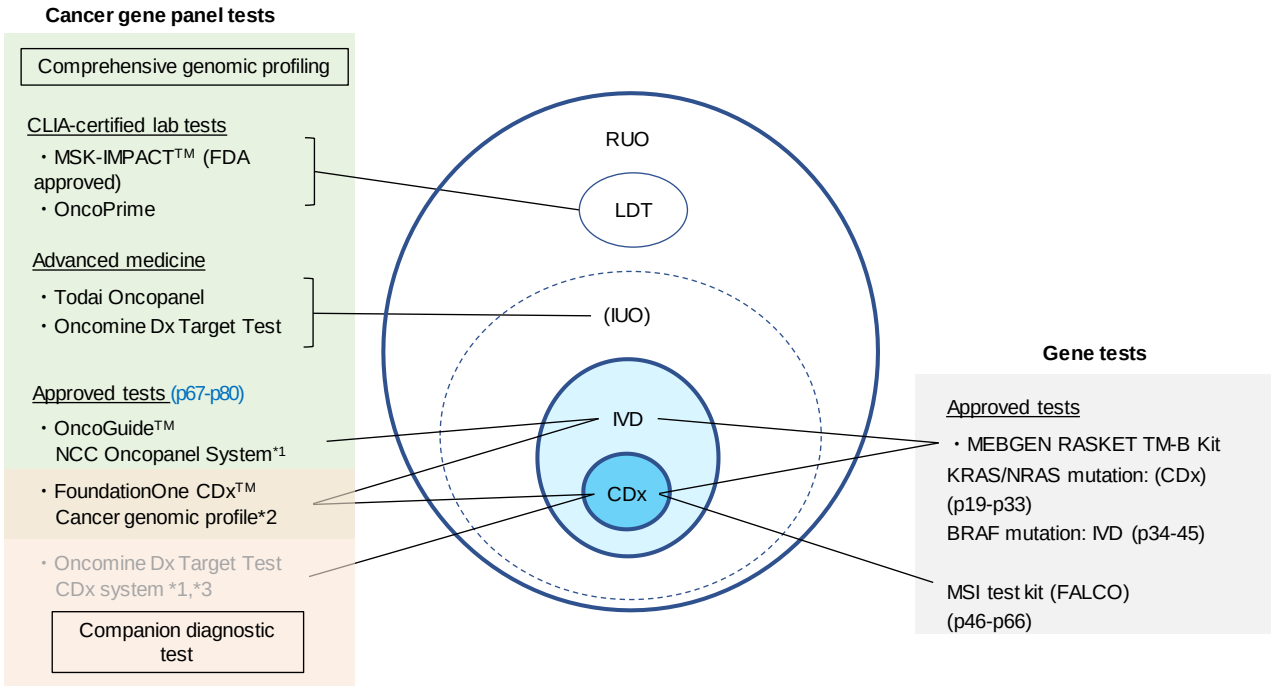
Meanwhile, MSI testing, which evaluates MMR deficiency, has been covered by insurance as a screening test for Lynch syndrome since June 2007. In patients with stage II colon cancer who have undergone curative resection, MMR deficiency predicts low recurrence risk, favorable outcomes, and inefficacy of single-drug postoperative adjuvant chemotherapy with 5-FU; meanwhile, patients with MMR-deficient *BRAF* V600E wild-type unresectable advanced or recurrent colorectal cancer tend to present poor outcomes. Since anti-PD-1 antibody therapy has recently been found effective in patients with MMR-deficient unresectable advanced or recurrent colorectal cancer, health insurance has, since December 2018, covered this test for patients with locally advanced or metastatic cancer, in order to evaluate the adequacy of the anti-PD-1 drug pembrolizumab (Keytruda®) (see Section 4: Testing for mismatch-repair deficiency).

1.3 Methods of genomic testing for colorectal cancer

Genomic tests to evaluate diseases, such as colorectal cancer, can be divided into two main categories: *in vitro* diagnostics (IVDs) and laboratory developed tests (LDTs), which are classified as

reagents. In vitro diagnostics are market-distributed pharmaceuticals approved by pharmaceutical and medical device regulations. When *KRAS* mutation testing was first introduced, it was performed under health insurance as a laboratory developed test; at present, however, genomic tests in the oncology field are, with some exceptions, moving toward IVD status. IVDs are used to diagnose diseases and conditions, but among these, companion diagnostics (CDx) are used to improve the efficacy and safety of specific pharmaceutical agents, and are used as in vitro diagnostics that are indispensable when using the corresponding drugs. Therefore, in the case of a companion diagnostic, not only are sensitivity and specificity to the test object important, but so is the setting of a clinical cut-off to contribute to the clinical data of the corresponding drug. Besides IVDs and LDTs, there are also the categories research use only (RUO), which includes products used in research without obtained approval as IVDs, and investigational use only (IUO), which applies to products and clinical tests with analytical validity that has been verified in countries such as the United States; as such, gene panel tests that belong to this category have been implemented in clinical practice. In **Figure 2,** these genomic tests, including gene panel tests, are organized and shown in relation to the representative gene panel tests used in Japan and internationally. Further, due to recent innovations in next-generation sequencers, there have also been individual whole genome sequencing and exome sequencing practices, in which the exomes of the genome are concentrated and analyzed. **Figure 3** shows the relationship between companion diagnostic tests and whole genome sequencing in the comprehensive genomic profiling (CGP) tests that are currently approved in Japan.

Figure 2. Conceptual diagram of genomic tests and corresponding test products

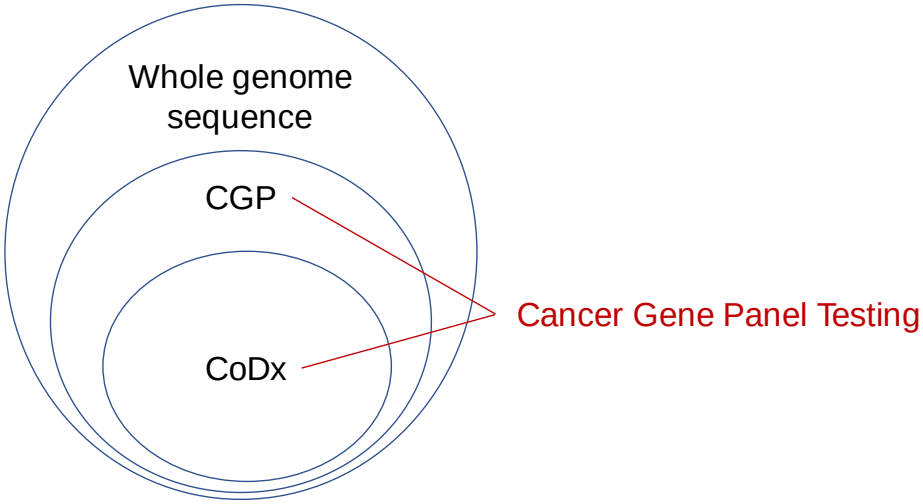


*1 Approved as a medical device and IVD system.

*2 Disease diagnostic program (medical devices) approval only; IVD not included.

*3 Not approved or covered by health insurance for colorectal cancer. Handled as RUO except for CDx genes.

Figure 3. Correlation diagram of NGS-based testing



【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. 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

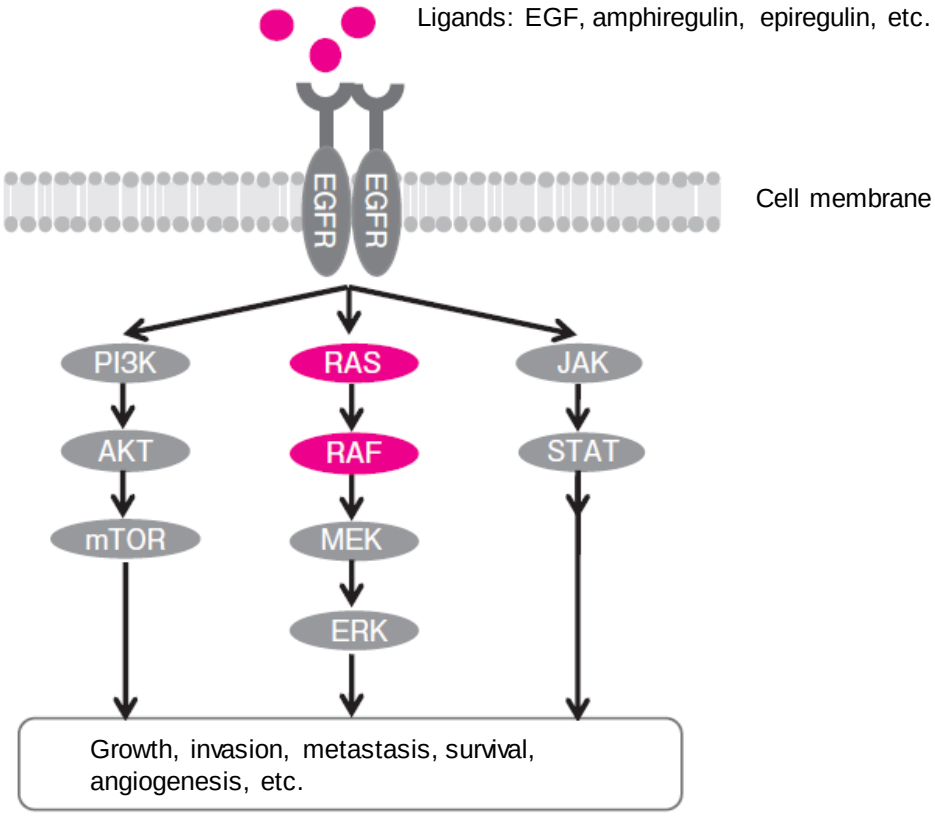
2 *RAS* Mutation Testing

2.1 Background

Colorectal cancer and the EGFR pathway

EGFR, also known as HER1 or ErbB-1, is a 170-kDa transmembrane glycoprotein receptor tyrosine kinase, and is overexpressed in approximately 80% of colorectal cancers. Upon extracellular binding of a ligand such as epidermal growth factor (EGF), amphiregulin, or epiregulin, EGFR dimerizes with another EGFR or another HER family molecule and is activated through autophosphorylation of its intracellular tyrosine kinase domain, which induces downstream signalling. Downstream signalling pathways include the RAS/RAF (MAPK), PI3K/AKT/mTOR, and JAK/STAT pathways. In normal tissues, these EGFR pathways perform important roles in cell differentiation, proliferation, and maintenance, but in colorectal cancer tissues, they are hyperactive and contribute to functions such as growth, invasion, metastasis, survival, and angiogenesis (**Figure 1).**

Figure 1. Colorectal cancer and the EGFR signal transduction pathway



When stimulated by ligands, EGFR activates downstream PI3K/AKT/mTOR, RAS/RAF, and JAK/STAT pathways, which affect cancer cell survival, proliferation, etc. In cancer cells with *RAS* and *RAF* mutations, each mutant protein supports cell survival and proliferation by activating the MEK-ERK pathway regardless of the presence or absence of stimulation by EGFR.

Anti-EGFR antibody therapy for colorectal cancer

Anti-EGFR antibody products include cetuximab and panitumumab. Cetuximab is a chimeric human mouse IgG1 monoclonal antibody that targets EGFR, and panitumumab is a fully human IgG2 monoclonal antibody. Both drugs bind to the antigen epitope of EGFR on the cell membrane surface and inhibit ligand binding, which suppresses cell proliferation. The effectiveness of both cetuximab and panitumumab has been recognized in clinical trials on metastatic colorectal cancer, and both drugs have been approved for pharmaceutical use in Japan since July 2008 and April 2010, respectively.

Ras protein function and RAS mutations

Ras proteins are low molecular weight GTPases that consist of 188-189 amino acids and measure approximately 21 kDa, of which three isoforms exist: KRAS, NRAS, and HRAS. Ras is activated when upstream stimulation from EGFR, etc., causes the release of guanosine diphosphate (GDP) from Ras, followed by replacement with intracellular GTP. The activated form of Ras binds to a maximum of 20 types of effector proteins, including RAF, PI3K, and RALGDS, which then activate downstream signal cascades. On the other hand, activated Ras is inactivated via hydrolyzing GTP, when binding to a GTPase activating protein (GAP). The *RAS* gene family includes *KRAS* on chromosome 12, *NRAS* on chromosome 1, and *HRAS* on chromosome 11, and each gene is composed of four exons and three introns. When amino acid substitution occurs due to a *RAS* mutation, the mutated Ras protein cannot hydrolyze GTP once it is bound to it, which results in a constitutively active state with continuous downstream signaling. This excess signaling is involved in carcinogenesis and cancer growth.

Frequency of *RAS* mutation in colorectal cancer

According to the Catalogue of Somatic Mutations in Cancer (COSMIC) database (v87), the reported frequency of *RAS* mutations in colorectal cancer is 33.03% for *KRAS*, 3.68% for *NRAS*, and 0.62% for *HRAS*, with a large proportion of *KRAS* exon 2 (codons 12 and 13) mutations. *RAS* gene point mutations reportedly occur early in the development of colorectal cancer and are found at a set frequency irrespective of disease stage (**Table 1**)[1,2]. The frequency of *KRAS* exon 2 (codons 12,13) mutations in colorectal cancer is approximately 35-40%, with no difference between reports from Western countries and those from Japan. According to clinical trials conducted primarily in the West, the combined frequency of *KRAS* exon 3 and 4 mutations and *NRAS* exon 2, 3, and 4 mutations is 10-15% (approximately 20% of wild type *KRAS* exon 2) (**Table 2**).

Table 1. Frequency of *KRAS* exon 2 mutation by stage

	Dukes' stage	Frequency (%)		Stage	Frequency (%)
Andreyev HJ, et al. (RASCAL)[1] N=2721	Dukes'A	33.9	Watanabe T, et al.[2] N=5887	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

Table 2. Frequency of *RAS* mutations

	KRAS Exon 2	*KRAS* Exon 3	*KRAS* Exon 4	*NRAS* Exon 2	*NRAS* Exon 3	*NRAS* Exon 4	Total*
PRIME[3]	40% （440／1096）	4% （24／638）	6% （36／620）	3% （22／637）	4% （26／636）	0% （0／629）	17%
20050181[4]	45% （486／1083）	4.4% （24／548）	7.7% （41／534）	2.2% （12／536）	5.6% （30／540）	0% （0／532）	20%
20020408[5]	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[6]	N／A	3.3%	5.6%	3.5%	2.8%	0.9%	15%
FIRE–3[7]	N／A	4.3% （21／431）	4.9% （24／458）	3.8% （18／464）	2% （10／468）	0% （0／458）	16%
CALGB 80405[8]	N／A	1.8%	5.9%	2.3%	4.2%	0%	14%
RASKET[9]	37.8% （116／307）	3.1% （6／191）	5.2% （10／191）	3.1% （6／191）	4.2% （8／191）	0% （0／191）	16%

*Rate of *KRAS/NRAS* mutation in wild type *KRAS* exon 2

N／A：not available

2.2

Basic requirements

To evaluate the suitability of anti-EGFR antibody therapy, implement *RAS* mutation testing prior to initiation of first-line therapy for patients with unresectable metastatic colorectal cancer.

Use a companion diagnostic to perform *RAS* mutation testing.

Strength of recommendation

Strong recommendation ［SR: 10 members］

Treatment outcomes of anti-EGFR antibody therapy for patients with *RAS* mutation

In terms of response rate, progression-free survival, and overall survival, anti-EGFR antibody has not been observed to serve advantages for patients with *KRAS* exon 2 (codons 12, 13) mutations in analyses based on the following: randomized controlled studies of standard chemotherapy with or without anti-EGFR antibody as a first-line therapeutic treatment for patients with metastatic colorectal cancer; phase III clinical studies of chemotherapy with or without anti-EGFR antibody for patients previously treated with chemotherapy; and phase III clinical studies comparing anti-EGFR monotherapy versus best supportive care (BSC) for patients who are resistant to standard chemotherapy.

Later, in phase III trials of panitumumab, the efficacy of the drug was further analyzed in the presence of mutations other than *KRAS* exon 2 (codons 12 and 13), namely *KRAS* exon 3 (codons 59 and 61) and exon 4 (codons 117 and 146), along with *NRAS* exon 2 (codons 12 and 13), exon 3 (codons 59 and 61), and exon 4 (codons 117 and 146) [3-5,10]. As a result, it was revealed that, whereas panitumumab is reliably effective in patients with wild type *RAS* and who do not have any *KRAS/NRAS* mutations, panitumumab is not expected to be effective in patients with any mutations on *KRAS* exons 3 or 4 or *NRAS* exons 2, 3, or 4 (**Table 3, Table 4**). In addition, in subgroup analyses in which patients were divided into those with *KRAS* exon 2 mutations and those with other *KRAS/NRAS* mutations, the addition of panitumumab was found to be similarly ineffective for both groups. Further, randomized controlled studies of cetuximab showed that cetuximab also provides reliable benefit only in patients with wild type *RAS* and who do not have any *KRAS/NRAS* mutations[6,11,12]. It has also been reported in Japan, by retrospective analyses of patients who were administered anti-EGFR therapy, that patients with *KRAS/NRAS* mutations other than *KRAS* exon 2 did not benefit from therapy.

From the above results, it has been demonstrated that patients with mutated *KRAS* exon 2, 3, or 4, or *NRAS* exon 2, 3, or 4, are unlikely to benefit from anti-EGFR antibody therapy. This tendency has been confirmed in a meta-analysis, and reproducibility has been observed regardless of the anti-EGFR antibody (cetuximab or panitumumab) type, treatment line, or type of combining chemotherapy[13]. Currently, package inserts for cetuximab and panitumumab include a precaution related to effects and indications, which states, “Select appropriate patients when you use this drug based on *RAS* (*KRAS* or *NRAS* gene) mutational status.”

Colorectal cancer treatment algorithms and *RAS* mutation testing

Three randomized controlled studies in Western countries had previously compared anti-EGFR antibody combination versus bevacizumab combination in first-line treatment for patients with metastatic

colorectal cancer. In the FIRE-3[7] and PEAK[14] trials, among patients with wild type *RAS*, improved overall survival was observed in patients treated with anti-EGFR antibody combination compared to those treated with bevacizumab combination. On the other hand, for the CALGB80405 trial, no significant differences were found between the two groups in the primary endpoint of the overall survival[8].

It has also been noted that among colorectal cancers, patterns of gene mutation frequencies and gene expression vary depending on the primary location; for instance, the *BRAF* V600E mutation, *PIK3CA* mutation, CpG island methylator phenotype–high（CIMP–high）, and microsatellite instability are frequent in right colon cancers, while the *TP53* mutation is frequent[15] in left colon cancers. Additionally, it has been recently reported that, among patients with *RAS* wild-type colorectal cancers, differences regarding efficacy of anti-EGFR antibody exist between patients with right- versus left-side colon. From data analyses pooled from six large-scale clinical trials (CRYSTAL, FIRE-3, CALGB80405, RRIME, PEAK, and 20050181) that investigated the efficacy of anti-EGFR antibody, *RAS* wild-type right-side colon cancers showed poorer overall survival, progression-free survival, and response rates compared to left-side colon cancers. It was also reported that, although the addition of anti-EGFR antibody significantly improved overall survival and progression-free survival in patients with *RAS* wild-type left-side colon cancers, anti-EGFR antibody therapy was not found to be beneficial for patients with right colon cancers[16]. Moreover, retrospective analysis of past clinical trials found that patients with *RAS* mutations tend to have shorter overall survival than do patients with wild-type *RAS*[17]; the *NRAS* mutant cases, in particular, may have worse outcomes than cases with either *KRAS* mutation or wild-type *RAS*[18,19].

Publications, such as 2019 Japanese G*uidelines for the Treatment of Colorectal Cancer* and ESMO's Pan-Asian adapted ESMO consensus guidelines[21], describe the latest treatment algorithms. *RAS* and *BRAF* testing is conducted prior to the initiation of first-line therapy, and if the cancer is *RAS/BRAF* wild type, first-choice drugs are indicated on the additional basis of primary location: for left colon cancer, these are standard chemotherapies, such as FOLFOX (5-FU, l-leucovorin, and oxaliplatin) and FOLFIRI (5-FU, l-leucovorin, and irinotecan) in combination with anti-EGFR antibodies; for right colon cancer, standard chemotherapies, such as FOLFOX, FOLFIRI, and FOLFOXIRI (5-FU, l-leucovorin, oxaliplatin, and irinotecan) combined with bevacizumab are indicated as first-choice treatment. From the above results, since the choice of first-line regimen differs based on *RAS* mutation status, *RAS* mutation testing prior to the initiation of first-line therapy is strongly recommended to evaluate the adequacy of anti-EGFR therapy for patients with metastatic colorectal cancer. Regarding methods of *RAS* mutation testing, refer to the "Comments" section of the following section.

Table 3. Therapeutic effects of anti-EGFR antibody therapy in patients with wild type *RAS*

	RAS evaluable *	Regimen	N	RR (%)	PFS (M)	HR	OS (M)	HR
PRIME[3] (First-line)	90% (1060／1183)	FOLFOX4	253	–	7.9	0.72 (p=0.004)	20.2	0.78 (p=0.04)
		FOLFOX4＋Pmab	259	–	10.1		26.0	
20050181[4] (Second-line)	85% (1008／1186)	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[5] (Third-line)	82% (378／463)	BSC	63	0	7 weeks	0.36 (p<0.0001)	–	–
		BSC＋Pmab	73	16	14.1 weeks		–	
20100007[10] (Third-line)	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[11] (First-line)	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)	69% (827／1198)	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)	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[14] (First-line)	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[8] (First-line)	59% (670／1137)	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	

**RAS* evaluable: ratio of randomized patients in whom *RAS* mutations were evaluable

FOLFOX：5-FU＋leucovorin＋oxaliplatin, Pmab：panitumumab, FOLFIRI：5-FU＋leucovorin＋irinotecan, BSC：best supportive care, Cmab：cetuximab, Bmab：bevacizumab, IRI：irinotecan, RR：response rate, PFS：progression-free survival, M：month, HR：hazard ratio, OS：overall survival

Table 4 Therapeutic effects of anti-EGFR antibody therapy in patients with *RAS* mutation

	Regimen	N	RR (%)	PFS (M)	HR	OS (M)	HR
PRIME[3] (First-line)	FOLFOX4	276	–	8.7	1.31 (p=0.008)	19.2	1.25 (p=0.034)
	FOLFOX4＋Pmab	272	–	7.3		15.6	
20050181[4] (Second-line)	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[5] (Third-line)	BSC	114	0	7.3 weeks	0.97 (p=0.729)	–	–
	BSC＋Pmab	99	1	7.4 weeks		–	
20100007[10] (Third-line)	BSC	28	–	1.6	1.03 (p=0.9429)	7.5	0.99 (p=0.9625)
	BSC＋Pmab	26	–	1.6		7.6	
OPUS[11] (First-line)	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)	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)	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[14] (First-line)	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[8] (First-line)	FOLFOX／IRI＋Bmab	42	–	–	–	22.3	0.74 (p=0.21)
	FOLFOX／IRI＋Cmab	53	–	–		28.7	

FOLFOX：5-FU＋leukovorin＋oxaliplatin, Pmab：panitumumab, FOLFIRI：5-FU＋leucovorin＋irinotecan, BSC：best supportive care, Cmab：cetuximab, bmab：bevacizumab, IRI：irinotecan, RR：response rate, PFS：progression-free survival, M：month, HR：hazard ratio, OS：overall survival

Comment 1 Management by *RAS* mutation subtype

Studying cell lines has shown that *RAS* codon 12, 13, 59, 61, 117, and 146 mutations induce constitutive activation of Ras proteins, increases in tumor cell growth rates, and activation of downstream proliferative signals. However, it is not currently clear whether these effects of mutant Ras protein on tumor cells vary depending on which codon is mutated.

Post-hoc analysis of randomized controlled studies on cetuximab has suggested the possibility that cetuximab may be equally effective in patients with the wild-type *KRAS* exon 2 and in patients with the mutant *KRAS* exon 2 codon 13D (G13D) [22]. However, post-hoc analyses of other phase III trials of cetuximab and panitumumab as well as a meta-analysis[23] of eight trials that include the above-mentioned findings have found that anti-EGFR antibody is ineffective in patients with the *KRAS* G13D mutation as well as in patients with other *KRAS* exon 2 mutations. Results of phase II trials of combination therapy with anti-EGFR antibodies exclusively in patients with the *KRAS* G13D mutation have also yielded reports of limited efficacy for this treatment[24,25].

Since it has also been reported that patients with *KRAS* codon 146 mutation responded to combination therapy with anti-EGFR antibody (+irinotecan) [26] as a later-line treatment, the accumulated evidence is not sufficient to judge whether anti-EGFR antibody is equally effective for all codon mutations. However, although there are some codon differences studied in each clinical trial, the lack of additional benefits from anti-EGFR antibody in patients with *RAS* mutations is a reproducible point. Therefore, in evaluating the appropriateness of administering anti-EGFR antibody, it is desirable to perform *RAS* mutation testing to investigate whether mutations are present on the *KRAS/NRAS* codons 12, 13, 59, 61, 117, or 146.

Comment 2 Changes in *RAS* mutations due to chemotherapy

Since *RAS* mutations occur at an early stage in colorectal cancer development, it is considered extremely rare for tumoral *RAS* mutational status to change after chemotherapy, other than anti-EGFR antibody therapy[27]. Therefore, it is believed that *RAS* mutation testing performed on existing cancer tissue from any point in time generally yields coinciding results. In contrast, after chemotherapy that includes anti-EGFR antibodies, *RAS* mutations that have not been seen before treatment emerge sometimes. Moreover, such new *RAS* mutations are a factor in acquired resistance to anti-EGFR antibodies[28]. When *RAS* mutation testing is performed on cancer tissues collected by re-biopsy or on blood samples, as described later, it is necessary to interpret results with adequate consideration of sample collection time and history of anti-EGFR antibody administration.

2.3

Basic requirements

To select treatment appropriate to recurrence risk for patients with unresectable metastatic colorectal cancer, implement *RAS* mutation testing prior to initiation of adjuvant chemotherapy.

Use a test with proven analytical validity to perform *RAS* mutation testing.

Strength of recommendation

Expert Consensus Opinion [ECO: 9 members, R: 1 member]

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

Although two phase III trials have compared FOLFOX with and without cetuximab as adjuvant therapies for resected stage III colon cancer, the addition of cetuximab did not improve recurrence-free survival or overall survival even in patients with wild-type *KRAS* exon 2 [29,30]. Additionally, a phase III trial that investigated the effects of adding cetuximab to pre- and post-operative chemotherapy in patients with resectable synchronous/metachronous liver metastases demonstrated no benefits from the addition of cetuximab in progression-free survival [31]. Based on the above results, it has not been demonstrated that cetuximab is effective against resectable advanced or recurrent colorectal cancer.

On the other hand, it remains debatable whether *RAS* mutation is a prognostic factor in resectable advanced or recurrent colorectal cancer. Additional analyses of phase III trials of adjuvant therapies for stage II/III colon cancer found no difference in recurrence-free survival or overall survival between patients with and without *KRAS* mutations [32,33]. On the other hand, there have also been reports that patients with *KRAS* mutations have significantly worse outcomes[34,35] (**Table 5).** Additionally, among patients with resected metastasic lesions such as liver metastases, patients with *RAS* mutation had shorter recurrence-free survival and overall survival than those of patients with *RAS* wild type [36]. Furthermore, an association has been reported between the *KRAS* mutation and lung metastasis after curative resection in patients with stage II/III colon cancers[37]. Therefore, although no current consensus has been reached on whether *RAS* mutation is a prognostic factor in patients with resectable advanced or recurrent colorectal cancer, testing can serve as a reference for future therapeutic strategy and should be considered depending on each patient's situations.

Table 5 Recurrence rate by *RAS* mutation status in patients with resectable colorectal cancer

	Stage	*RAS*	N	5 年 RFS (%)	HR	5 年 OS (%)	HR
CALGB 89803[32]	Ⅲ	*KRAS* WT	330	6	0.97 (*p*=0.84)	75	0.90 (*p*=0.56)
		KRAS MT	178	66		73	
PETACC–3, EORTC 40993, SAKK 60–00[33]	Ⅱ/Ⅲ	*KRAS* WT	818	–	1.05 (*p*=0.66)	–	1.09 (*p*=0.48)
		KRAS MT	481	–		–	
N0147[34]	Ⅲ	*KRAS* WT	1479	77*	1.50	–	–

		KRAS MT	779 220	68 (codon 12)* 67 (codon 13)*	(*p*<0.0001) 1.46 (*p*=0.0035)	–	
PETACC–8[35]	Ⅲ	*KRAS* WT	1019	–	1.56** (*p*<0.001)	–	–
		KRAS MT	638	–		–	

*3-year RFS **HR of time to recurrence

WT：wild type，MT：mutant，RFS：relapse-free survival，HR：hazard ratio，OS：overall survival

Comment *RAS* mutation test methods

Tests with regulatory approval for use as *in vitro* diagnostic products

To detect *RAS* mutations, *in vitro* diagnostic products based on various measurement principles have already been approved and are widely distributed in Japan; thus, it is recommended to use the following products to perform testing (**Table 6).**

Table 6 Representative *in vitro* diagnostic products for *RAS*（*KRAS*） mutation testing

In vitro diagnostic product	*KRAS* Exon 2 mutation	*KRAS* exon 3 、4 mutation And *NRAS* mutation	Detection limit	Measurement principle
MEBGEN RASKET™-B Kit	Detectable*	Detectable*	1–5%	PCR–rSSO
FoundationOne® CDx	Detectable**	Detectable**	2.3%	Hybrid capture
OncoBEAM™ RAS CRC Kit	Detectable***	Detectable****	0.03%	BEAMing

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

**From the FoundationOne® CDx, results for the above mutations are returned as companion diagnostics, and results for other *RAS* mutations are returned as novel mutations of unverified significance.

*** *KRAS* codon 12（G12S, G12C, G12R, G12D, G12V, G12A），codon 13（G13D），codon 59（A59T），codon 61（Q61L, Q61R, Q61H），codon 117（K117N），codon 146（A146T, A146V） mutations

****NRAS* codon 12（G12S，G12C，G12R，G12D，G12V，G12A），codon 13（G13R，G13D，G13V），codon 59（A59T），codon 61（Q61K，Q61L，Q61R，Q61H），codon 117（K117N），codon 146（A146T）mutations

PCR–rSSO：PCR–reverse sequence specific oligonucleotide

RAS mutation testing using blood samples (**Table 7**)

Currently, most cancer gene mutation tests are performed using DNA from tumor tissues. However, these tissues cannot be collected in all cases, and moreover, given the invasive nature of tissue collection, it is difficult to perform repeated testing. To resolve such issues, a variety of technologies have been developed to use blood samples to analyze DNA extracted from plasma (circulating tumor DNA [ctDNA]). Digital PCR techniques have been developed to detect very a small amount of DNA in blood samples. One

such technique, BEAMing, excels with its high sensitivity, demonstrating a detection threshold of 0.03%. In 2016, a *RAS* mutation test kit (OncoBEAM™ RAS CRC kit) based on BEAMing technology obtained the European CE mark (a mark indicating conformity with EU member states' safety standards) in 2016, and retrospective and prospective clinical performance tests conducted in Europe have shown high concordance with tumor tissue-based *RAS* mutation tests[38-42]. Clinical evaluation study in Japan has also confirmed high concordance between *RAS* mutation testing by the OncoBEAM™ RAS CRC kit and BEAMing of tumor tissues[43], and in July 2019, the kit obtained regulatory approval for use in "detecting *RAS (KRAS* and *NRAS*) mutations in genomic DNA extracted from plasma (to aid in evaluating the application of cetuximab and panitumumab for patients with colorectal cancer)."

Table 7 Concordance of blood- and tissue-based *RAS* mutation testing

	N	Sensitivity (%)	Specificity (%)	Concordance (%)
Grasselli, J., et al.[38]	146	88.9	90.2	89.7
Vidal J, et al.[39]	115	96.4	90.0	93.0
Garcia-Foncillas, J., et al.[40]	238	92.6	94.0	93.3
Schmiegel W, et al.[41]	98	90.4	93.5	91.8
Garcia-Foncillas, J., et al.[42]	236	86.3	92.4	89.0
Bando H, et al.[43]	280	82.1	90.4	86.4

Memo 1 Management of cases in which different tests on the same sample yield inconsistent *RAS* mutation test results

There is possible disagreement between results of *RAS* mutation testing that use in vitro diagnostic products and results from research use testing or next-generation sequencing (NGS)-based comprehensive genomic profiling. In case of disagreement between results from two different test performed on the same sample, it is necessary to confirm each test method. Further, as a general principle, test results from the in vitro diagnostic product should be prioritized in regard to evaluating the application of anti-EGFR antibody therapy.

Memo 2 Management of cases in which *RAS* mutations are detected outside of *KRAS/NRAS* codons 12, 13, 59, 61, 117, and 146

According to randomized controlled trials of anti-EGFR antibody on patients with metastatic colorectal cancer, *RAS* mutations that predict a lack of therapeutic benefit from anti-EGFR antibody therapy are *KRAS/NRAS* codons 12, 13, 59, 60, 117, and 146. However, with the expanded use of NGS-based comprehensive genomic profiling tests, detection of *RAS* mutations on other codons can be expected in rare cases. For example, there are reports of missense mutations detected on *KRAS* codon 22 and *HRAS* codon 22 in colorectal cancer[44,45]. Clinical data are scarce regarding the efficacy of anti-EGFR antibody for patients in whom *RAS* mutations outside of *KRAS/NRAS* codons 12, 13, 59, 61, 117, and 146 are detected, and anti-EGFR antibody cannot be uniformly dismissed as inappropriate for such

patients. When these gene mutations are detected, the application of anti-EGFR antibody shall be evaluated holistically upon considering 1) whether Ras proteins that result from the mutation show enhanced activity, 2) any existing reports on patients with the *RAS* mutation who were administered anti-EGFR therapy, 3) side effects of anti-EGFR antibody therapy, 4) existence of treatment options other than anti-EGFR antibody, etc.

Memo 3 Assessment of EGFR expression by immunohistochemistry (IHC) testing

Cetuximab was originally developed exclusively to treat patients with tumor tissue EGFR expression using IHC (positive EGFR expression). Therefore, the current package insert for cetuximab describes its indication as “unresectable EGFR-positive advanced or recurrent colorectal cancer.” IHC for EGFR expression is covered by health insurance under the category “N002 Immunostaining (antibody-based), preparation of histopathological specimen for EGFR protein (690 points),” and approved in vitro diagnostic products include Dako EGFR pharmDx（2–18C9） and Histofine® Simple Stain MAXPO (MULTI) anti-EGFR monoclonal antibody (31G7). However, subsequent investigation has revealed that some patients with EGFR-positive colorectal cancer also respond to cetuximab, and that the therapeutic effect of cetuximab does not correspond to the level of EGFR expression. Based on the above results, there is no need to perform IHC to evaluate the application of anti-EGFR antibody therapy. Likewise, European and U.S. guidelines do not recommend assessing EGFR expression by IHC to evaluate the application of anti-EGFR antibody.

【References】

1. Andreyev HJ, Norman AR, Cunningham D, et al: Kirsten ras mutations in patients with colorectal cancer: the multicenter "RASCAL" 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. Douillard JY, Oliner KS, Siena S, et al: Panitumumab-FOLFOX4 treatment and RAS mutations in colorectal cancer. N Engl J Med 369:1023-34, 2013

4. 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 21:5469-79, 2015

5. 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 19:1902-12, 2013

6. Van Cutsem E, Lenz HJ, Kohne CH, et al: Fluorouracil, leucovorin, and irinotecan plus cetuximab treatment and RAS mutations in colorectal cancer. J Clin Oncol 33:692-700, 2015

7. Stintzing S, Modest DP, Rossius L, et al: FOLFIRI plus cetuximab versus FOLFIRI plus bevacizumab for metastatic colorectal cancer (FIRE-3): a post-hoc analysis of tumour dynamics in the final RAS wild-type subgroup of this randomised open-label phase 3 trial. Lancet Oncol, 2016

8. 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 317:2392-2401, 2017

9. Yoshino T, Muro K, Yamaguchi K, et al: Clinical Validation of a Multiplex Kit for RAS Mutations in Colorectal Cancer: Results of the RASKET (RAS KEy Testing) Prospective, Multicenter Study. EBioMedicine 2:317-23, 2015

10. Kim TW, Elme A, Kusic Z, et al: A phase 3 trial evaluating panitumumab plus best supportive care vs best supportive care in chemorefractory wild-type KRAS or RAS metastatic colorectal cancer. Br J Cancer 115:1206-1214, 2016

11. Bokemeyer C, Kohne CH, Ciardiello F, et al: FOLFOX4 plus cetuximab treatment and RAS mutations in colorectal cancer. Eur J Cancer 51:1243-52, 2015

12. Sobrero A, Lenz H-J, Eng C, et al: Retrospective RAS analysis of the EPIC trial: Cetuximab plus irinotecan vs irinotecan in patients with second-line metastatic colorectal cancer. ESMO Congress 2018 484P, 2018

13. 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

14. 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 32:2240-7, 2014

15. 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 24:1062-1072, 2018

16. Arnold D, Lueza B, Douillard JY, et al: Prognostic and predictive value of primary tumour side in patients with RAS wild-type metastatic colorectal cancer treated with chemotherapy and EGFR directed antibodies in six randomised trials. Ann Oncol, 2017

17. Modest DP, Ricard I, Heinemann V, et al: Outcome according to KRAS-, NRAS- 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

18. Schirripa M, Cremolini C, Loupakis F, et al: Role of NRAS mutations as prognostic and predictive markers in metastatic colorectal cancer. Int J Cancer 136:83-90, 2015

19. Cercek A, Braghiroli MI, Chou JF, et al: Clinical Features and Outcomes of Patients with Colorectal Cancers Harboring NRAS Mutations. Clin Cancer Res 23:4753-4760, 2017

20. 大腸癌研究会編：大腸癌治療ガイドライン―医師用 2019 年版. 金原出版, 東京, 2019

21. 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

22. De Roock W, Jonker DJ, Di Nicolantonio F, et al: Association of KRAS p.G13D mutation with outcome in patients with chemotherapy-refractory metastatic colorectal cancer treated with cetuximab. Jama 304:1812-20, 2010

23. Rowland A, Dias MM, Wiese MD, et al: Meta-analysis comparing the efficacy of anti-EGFR monoclonal antibody therapy between KRAS G13D and other KRAS mutant metastatic colorectal cancer tumours. Eur J Cancer 55:122-30, 2016

24. Schirripa M, Loupakis F, Lonardi S, et al: Phase II study of single-agent cetuximab in KRAS G13D mutant metastatic colorectal cancer. Ann Oncol 26:2503, 2015

25. Segelov E, Thavaneswaran S, Waring PM, et al: Response to Cetuximab With or Without Irinotecan in Patients With Refractory Metastatic Colorectal Cancer Harboring the KRAS G13D Mutation: Australasian Gastro-Intestinal Trials Group ICECREAM Study. J Clin Oncol 34:2258-64, 2016

26. De Roock W, Claes B, Bernasconi D, et al: Effects of KRAS, BRAF, NRAS, and PIK3CA mutations on the efficacy of cetuximab plus chemotherapy in chemotherapy-refractory metastatic colorectal cancer: a retrospective consortium analysis. Lancet Oncol 11:753-62, 2010

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

28. Misale S, Yaeger R, Hobor S, et al: Emergence of KRAS mutations and acquired resistance to anti-EGFR therapy in colorectal cancer. Nature 486:532-6, 2012

29. 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

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

31. 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 randomised controlled trial. Lancet Oncol

15:601-11, 2014

32. Ogino S, Meyerhardt JA, Irahara N, et al: KRAS mutation in stage III colon cancer and clinical outcome following intergroup trial CALGB 89803. Clin Cancer Res 15:7322-9, 2009

33. 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

34. Yoon HH, Tougeron D, Shi Q, et al: 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

35. 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

36. 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

37. 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

38. Grasselli J, Elez E, Caratu G, et al: Concordance of blood- and tumor-based detection of RAS mutations to guide anti-EGFR therapy in metastatic colorectal cancer. Ann Oncol 28:1294-1301, 2017

39. 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 28:1325-1332, 2017

40. Garcia-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, 2017

41. 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 11:208-219, 2017

42. Garcia-Foncillas J, Tabernero J, Elez 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

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

44. Palmirotta R, Savonarola A, Formica V, et al: A novel K-ras mutation in colorectal cancer. A case report and literature review. Anticancer Res 29:3369-74, 2009

45. Boidot R, Chevrier S, Julie V, et al: HRAS G13D, a new mutation implicated in the resistance to anti-EGFR therapies in colorectal cancer, a case report. Int J Colorectal Dis 31:1245-6, 2016

3 *BRAF* mutation testing

3.1 Background

BRAF protein function and *BRAF* mutation

The RAF protein family includes three isoforms: ARAF, BRAF, and CRAF[1]. BRAF is an approximately 74 kDa serine/threonine kinase composed of 766 amino acids. It binds directly to an RAS protein activated by a receptor tyrosine kinase, such as EGFR, and becomes activated by forming a dimer with another BRAF or CRAF, which activates a downstream MEK-ERK pathway, contributing to cell proliferation and survival (see Chapter 2, **Figure 1**)[2]. The *BRAF* gene is located on chromosome 7 and contains 18 exons. In 2002, it was first reported that *BRAF* mutations are frequently found in human cancers, and since then have been known to occur with high frequencies in malignant melanoma (43%), thyroid cancer (27%), and biliary tract cancer (14%), among others[3]. When an amino acid substitution occurs due to the *BRAF* mutation, downstream proteins become constitutively activated, regardless of whether there is stimulation from upstream RAS proteins.

Frequency and clinicopathological features of the *BRAF* V600E mutation in colorectal cancer

According to the COSMIC database (v87), the frequency of the *BRAF* mutation in colorectal cancer is 10.3% (colon cancer: 13.4%, rectal cancer: 3.2%), with a high number of V600E mutations (p.V600E), in which thymine is substituted by adenine at position 1799 of exon 15, and valine is substituted by glutamic acid at codon 600. Although the *BRAF* V600E mutation reportedly arises at an early stage in colorectal cancer development, it is slightly more prevalent in Stage III/IV compared to Stage I/II of the disease (**Table 1)** [4,5]. It has been inferred that this is related to the fact, as discussed below, that *BRAF* V600E-mutated colorectal cancer has a poor prognosis, and is thus frequently discovered at an advanced stage. The reported frequency of the *BRAF* V600E mutation in colorectal cancer in Japan is 4.5%-6.7%[6,7], which is slightly lower than that of Western countries (5-12%). The *RAS* mutation and *BRAF* V600E mutation are considered mutually exclusive in colorectal cancer.

Patients with the *BRAF* V600E mutation show different clinicopathological features than wild-type patients. Results of a meta-analysis of 25 studies that feature a total of 11,955 patients with colorectal cancer showed that the *BRAF* V600E mutation is more frequent in females, patients aged ≥60 years, and patients with right colon primary tumors and tumors with low differentiation, mucinous histology, and microsatellite instability (**Table 2)**[8]**.**

Table 1. Frequency of *BRAF* V600E mutation by stage

	Stage	N	Frequency (%)		Stage	N	Frequency (%)
Phipps AI, et al.[4] N＝1980	Localized	785	12.1	Ogura T, et al.[5] N＝1304	Stage 0/Ⅰ	296	3.4
	Regional	937	14.2		StageⅡ	407	4.2
	Distant	226	6.6		StageⅢ	384	4.4
	Unknown	32	–		StageⅣ	217	6.9

Table 2. Frequency of *BRAF* V600E mutation by patient background[6]

Patient Background		N	Frequency (%)	Odds ratio
Sex	Male	6186	8.0	1.71 (1.42–2.07)
	Female	5489	13.7	
Age	<60	1351	6.7	2.29 (1.13–4.61)
	≥60	1631	18.6	
Primary lesion location	Left-sided colon and rectum	5806	4.8	4.85 (3.59–6.56)
	Right-sided colon	4007	21.6	
Stage at diagnosis (TNM Classification)	Ⅰ/Ⅱ	1806	8.0	1.59 (1.16–2.17)
	Ⅲ/Ⅳ	2630	11.6	
Differentiation	Well- to moderately differentiated	4257	8.0	3.89 (2.94–5.17)
	Poorly differentiated	766	25.6	
Mucinous adenocarcinoma	Non-mucinous	2134	8.1	2.99 (2.20–4.07)
	Mucinous	392	19.4	
Microsatellite instability	MSS	1371	9.3	8.18 (5.08–13.17)
	MSI	352	38.9	

3.2

Basic requirements

BRAF V600E mutation testing is recommended prior to first-line chemotherapy to determine the optimal treatment based on the prognosis of patients with unresectable CRC.

Use a test with verified analytical validity to perform *BRAF* V600E mutation testing.

Strength of recommendation

Strong recommendation ［SR: 9 members, R: 1 member］

Clinical significance of *BRAF* V600E testing for patients with metastatic colorectal cancer

Patients with the *BRAF* V600E mutation have a poor prognosis than those with wild-type *BRAF*, with a hazard ratio of 2.25 for overall survival (95% confidence interval [CI], 1.82 to 2.83), as reported from a meta-analysis of 26 studies. Also, a pooled analysis of randomized controlled trials on patients receiving first-line chemotherapy for metastatic colorectal cancer demonstrated a considerably worse overall survival among patients with the *BRAF* V600E mutation than that among patients with wild-type *BRAF* （**Table 3**）[9,10]. Likewise, in Japan, from an analysis on patients with metastatic colorectal cancer, poor prognosis has been reported in patients with the *BRAF* V600E mutation.

From a phase III trial (TRIBE study) that compared FOLFOXIRI combined with bevacizumab versus FOLFIRI combined with bevacizumab as first-line therapies for colorectal cancer, FOLFOXIRI with bevacizumab tended to better prolong survival, especially for patients with the *BRAF* V600E mutation （Table **4**）[11]. In addition, a phase II trial of FOLFOXIRI in combination with bevacizumab that included only patients with the *BRAF* V600E mutation also demonstrated favorable therapeutic effects[12]. Based on these results, the 2019 Japanese G*uidelines for the Treatment of Colorectal Cancer* and ESMO's Pan-Asian adapted ESMO consensus guidelines indicate therapy with FOLFOXIRI and bevacizumab as the first-choice regimen for first-line treatment in patients with the *BRAF* V600E mutation[13].

Additionally, the results of the BEACON CRC trial have been reported. This phase 3 study compared three groups of patients with metastatic colorectal cancer with the *BRAF* V600E mutation who had previously undergone one or two treatment regimens: a triplet-therapy group, which received encorafenib (BRAF inhibitor), binimetinib (MEK inhibitor), and cetuximab (anti-EGFR antibody); a double-therapy group, which received encorafenib and cetuximab; and a control group, which received FOLFIRI (or irinotecan) and cetuximab. Results were revealed after the first interim analysis as the primary endpoints, whereby response rates and overall survival were clearly higher in the triplet-therapy group than in the control group; further, compared to the control group, the triplet-therapy and double-therapy groups had significantly better overall survival (median: triplet-therapy, 9.0 months [HR 0.52, p<0.001]; double-therapy, 8.4 months [HR 0.60, p<0.001]; control, 5.4 months), progression-free survival (median: triplet-therapy, 4.3 months [HR 0.38, p<0.001]; double-therapy, 4.2 months [HR 0.40, p<0.001]; control, 1.5 months), and response rates (triplet-therapy: 26% (p<0.001), double-therapy: 20% [p<0.001], control 2%[14]).

From the above results, confirmation of the *BRAF* V600E mutation status is useful for treatment selection as it provides more accurate information about the therapeutic effects and outcomes of chemotherapy. As such, it is strongly recommended to perform *BRAF* V600E mutation testing prior to initiating first-line therapy in patients with metastatic colorectal cancer.

Table 3. Treatment outcomes for patients with *BRAF* V600E mutation（pooled analysis）

		N	PFS（M）	HR	OS（M）	HR
Venderbosch S, et al. [9]	*BRAF* WT	2813	7.7	1.34	17.2	1.91
	BRAF MT	250	6.2	（p＝0.001）	11.4	（p＝0.001）
Modest DP, et al. [10]	*RAS / BRAF* WT	664	10.3	2.19	26.9	2.99
	BRAF MT	74	7.4	（p＜0.001）	11.7	（p＜0.001）

WT；wild type, MT；mutant, PFS；progression-free survival, HR；hazard ratio, OS；overall survival, M; months

Table 4. Treatment outcomes of first-line therapy with FOLFOXIRI and bevacizumab

		Regimen	N	RR（%）	PFS（M）	HR	OS（M）	HR
TRIBE[11]	*RAS / BRAF* WT	FOLFIRI＋Bmab	45	60	12.2	0.85	33.5	0.77
		FOLFOXIRI＋Bmab	48	65	13.7		41.7	
	RAS MT	FOLFIRI＋Bmab	119	55	9.5	0.78	23.9	0.88
		FOLFOXIRI＋Bmab	117	66	12.0		27.3	
	BRAF MT	FOLFIRI＋Bmab	12	42	5.5	0.57	10.7	0.54
		FOLFOXIRI＋Bmab	16	56	7.5		19.0	
Loupakis F, et al. [12]	*BRAF* MT	FOLFOXIRI＋Bmab	25	72	11.8	–	24.1	–

Bmab：bevacizumab, WT：wild type, MT：mutant, RR：response rate, PFS：progression-free survival, HR：hazard ratio, OS：overall survival, M：months

Comment 1 Efficacy of anti-EGFR antibody therapy in patients with the *BRAF* V600E mutation

The therapeutic efficacy of anti-EGFR antibody therapy in patients with the *BRAF* V600E mutated-metastatic colorectal cancer remains disputed. Initially, based on retrospective analysis of patients who received anti-EGFR antibody therapy, anti-EGFR antibody therapy was reported to be ineffective in patients with the *BRAF* V600E mutation, as it is in patients with the *KRAS* mutation. Later, however, multiple subgroup analyses of phase III trials of chemotherapy with and without the addition of anti-EGFR antibody drugs yielded conflicting reports on the efficacy of anti-EGFR antibody therapy. Furthermore, a meta-analysis on these randomized controlled trials, which compared chemotherapy with and without the addition of anti-EGFR antibodies, reported that there is no increase in survival from the addition of anti-EGFR antibody therapy in patients with the *BRAF* V600E mutation[15], while another has reported that patients with the *BRAF* V600E mutation, as with those with wild-type *KRAS/BRAF,* can therapeutically benefit from anti-EGFR antibody therapy[16].

Based on these results, with the possibility that the risk-benefit balance of anti-EGFR antibody therapy may differ between patients with wild-type *RAS/BRAF* and those with the *BRAF* V600E mutation, it is considered beneficial for treatment regimen selection to clarify *BRAF* V600E mutation status prior to

administering anti-EGFR antibody therapy. In fact, based on the expectation that anti-EGFR antibody drugs are highly unlikely to have therapeutic efficacy in patients with the *BRAF* V600E mutation, the United States National Comprehensive Cancer Network (NCCN) Guidelines (Version 1.2019) recommend testing for the *BRAF* V600E mutation at the time of Stage IV colorectal cancer diagnosis[17]. However, it cannot be concluded from the previously mentioned evidence that anti-EGFR antibody therapy is definitively ineffective in patients with the *BRAF* V600E mutation; therefore, in clinical practice, therapies, including anti-EGFR antibody drugs, are expected to be selected as a line of treatment.

3.3

Basic requirements

BRAF V600E mutation testing is recommended to determine the optimal perioperative chemotherapy based on the presumed recurrence risk in patients with resectable CRC.

Use a test with verified analytical validity to perform *BRAF* V600E mutation testing.

Strength of recommendation

Recommendation ［R: 7 members, ECO: 3 members］

Clinical significance of *BRAF* V600E mutation testing for resectable colorectal cancer

In recent years, reports have accumulated that the *BRAF* V600E mutation is a strong predictor of poor outcomes even in resectable cases. A meta-analysis on phase 3 trials of postoperative adjuvant chemotherapy in patients with stage II/III colon cancer has reported that the *BRAF* V600E mutation is a risk factor for recurrence, with a hazard ratio of 1.42 (1.25-160) for overall survival and 1.26 (1.07-1.48) for disease-free survival for patients with the *BRAF* V600E mutation compared to those with wild-type *BRAF* [18]. In subgroup analyses from the MOSAIC study, a phase 3 trial that compares postoperative adjuvant chemotherapy with 5–FU/LV（5–FU and leucovorin）therapy versus FOLFOX, which suggests a possibility that the benefits of adding oxaliplatin may differ between patients with wild-type *BRAF* and those with the *BRAF* V600E mutation, although there were no significant differences (hazard ratio 0.93 vs. 0.66 for overall survival). In addition, an observational study on patients with liver metastases who undergo curative resection has also reported that patients with the *BRAF* V600E mutation have significantly worse outcomes than those with wild-type *BRAF*, with hazard ratios of 1.62 (1.07 to 2.47) for recurrence-free survival and 2.39 (1.53 to 3.72) for overall survival, and there was considerably more recurrence among patients with the *BRAF* V600E mutation within one year of resection[19]. Furthermore, a meta-analysis that includes reports from Japan has confirmed similar trends[20].

However, a meta-analysis on 27 studies comprising 24,067 patients with unresectable and resectable colorectal cancer has reported that, compared to the microsatellite stable (MSS)/*BRAF* wild type, the hazard ratio for progression-free survival in patients with resectable tumors was 1.54（1.16 to 2.05）for the MSS/*BRAF* V600E mutant subtype and 0.51（0.31–0.83）for the MSI-H/*BRAF* V600E mutant subtype. The hazard ratio compared to MSS/*BRAF* wild type for overall survival in all patients, including those with unresectable cancer, was 2.02（1.71–2.39） for the MSS/*BRAF* V600E mutant subtype and 1.32（0.94–1.87） for the MSI-H/*BRAF* V600E mutant subtype. Thus, the degree to which the *BRAF* V600E mutation influences outcomes reportedly differs greatly between patients with MSI-H and those with MSS tumors[21].

In this way, even in resectable cases, the presence of the *BRAF* V600E mutation is a very strong negative prognostic factor, especially in patients with MSS. *Japanese Guidelines for the Treatment of Colorectal Cancer* recommend taking recurrence risk into account and selecting either fluoropyrimidine monotherapy or combination therapy with oxaliplatin for adjuvant chemotherapy after curative resection[22]. It is also possible that, depending on recurrence risk, the adequacy of metastatic lesion resection and treatment selection, such as adjuvant chemotherapy, could also differ in cases of distant metastasis resection. Therefore, *BRAF* V600E mutation testing is considered useful for treatment selection and is recommended

in patients with resectable colorectal cancer. Also, since the prognostic value of the *BRAF* V600E mutation differs greatly between patients with MSI-H and those with MSS, simultaneous testing for mismatch-repair deficiency is desirable. Treatment guidelines in both the United States and Europe also recommend *BRAF* V600E mutation testing in the work-up for determining therapeutic strategy in patients with distant metastasis, including those with tumors that are resectable[17, 23]. However, since September 2019, *BRAF* V600E mutation testing is not yet covered by health insurance in Japan for treatment selection in resectable colorectal cancer.

3.4

Basic requirements

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

Use a test with verified analytical validity to perform *BRAF* V600E mutation testing.

Recommendation

Recommendation ［SR: 1 members, R: 9 member］

BRAF V600E mutation testing to diagnose exclusion for Lynch syndrome

(For details regarding Lynch syndrome, dMMR, and pMMR, see Chapter 4)

The frequency of the *BRAF* V600E mutation in colorectal cancer differs greatly between mismatch-repair deficient (dMMR:MSI-H or negative MMR protein expression as determined by immunohistochemical testing) and mismatch-repair proficient (pMMR:MSS or positive MMR protein expression as determined by immunohistochemical testing) patients, and the frequency is higher in dMMR patients (9.3% vs 38.9%, odds ratio 8.18 [5.08-13.2]). While most sporadic dMMR colorectal cancers are considered to be caused by promoter region methylation, dMMR colorectal cancer in Lynch syndrome is caused by germline mutation. For example, abnormal-acquired methylation of the promoter region of *MLH1* results in deficient MLH1 protein expression. A high frequency of the *BRAF* V600E mutation is observed in sporadic dMMR colorectal cancers; in a review of 35 studies comprising 4562 patients, the *BRAF* V600E mutation occurred in 1.4% of patients with colorectal cancer who were considered to have Lynch syndrome and in 63.5% of patients with apparently sporadic MLH1 deficient colorectal cancer[24]. Therefore, in patients with MSI-H or deficient expression of MMR proteins, specifically MLH1, Lynch syndrome can be excluded with high probability if the *BRAF* V600E mutation is present. In fact, Western guidelines for Lynch syndrome recommend testing for the *BRAF* V600E mutation before genomic testing in patients with MSI-H or deficient MMR protein expression, and because this reduces the number of patients who require genomic testing for a definitive diagnosis of Lynch syndrome, it is a cost effective screening method for Lynch syndrome[25,26]. 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 cases with MSI-H or deficient MLH1 protein expression.

Based on the above results, it is recommended to perform *BRAF* V600E mutation testing as an aid in diagnosing Lynch syndrome in patients with colorectal cancer. In Japan, *BRAF* V600E mutation testing to diagnose Lynch syndrome has been covered by health insurance since August 2018. However, it must be noted that for cancers other than colorectal cancer, the use of *BRAF* V600E mutation testing to exclude Lynch syndrome diagnosis has no known clinical significance and is unnecessary.

Comment 1 *BRAF* mutation testing methods

Clinical studies that found the *BRAF* V600E mutation to be a negative prognostic factor used a variety of major testing methods, including direct sequencing, SURVEYOR, next generation sequencing, pyrosequencing, MALDI-MS, and real-time PCR clamping（**Table 5**）. With the exception of direct sequencing, these techniques have detection limits of 1-10%. Further, since results obtained through these various testing methods have consistently shown that patients with *BRAF* V600E mutations have

poor outcomes, it is recommended to select a method with a detection limit of 1-10% to test for the *BRAF* mutation.

In Japan, the MEBGEN RASKET™-B kit was approved for manufacture and sale in December 2017 as an in vitro diagnostics (IVD) that simultaneously detects *RAS* and *BRAF* V600E mutations, and since August 2018, *BRAF* V600E mutation testing has been covered by health insurance. In addition, the FoundationOne® CDx and the OncoGuide™ NCC Oncopanel System include *BRAF* V600E in their analyses, and the test results may be utilized.

On the other hand, a distinct BRAF V600E test exists in the form of immunohistochemical (IHC) testing for mutant BRAF protein (VE1). A meta-analysis that includes 8 cohort studies comprising 1021 patients found that IHC testing had a concordance rate of 0.94（95%CI 0.87–0.98）with *BRAF* V600E mutation testing, a sensitivity rate of 0.94 (0.91-0.96), and a specificity rate of 0.96 (0.95-0.98) [27]. Further, IHC-based observational studies that compared outcomes in patients with mutant and wild type *BRAF* V600E have reproduced the extremely strong negative prognostic value of *BRAF* V600E [28,29]. The use of rigorously standardized staining techniques and reagents is essential, since the intensity of staining in IHC can vary depending on the antibody clone, staining conditions, and automated immunostaining system. In the future, if reagents for immunostaining BRAF clone VE1 are approved as IVDs, IHC testing may also be recommended as a method for detecting the *BRAF* V600E mutation.

Table 5. *BRAF* V600E mutation test method used in each clinical trial

Trial name	Test method	N	Rate of *BRAF* V600E mutation
CAIRO–2	Direct sequencing	519	8.7%
FOCUS	Pyrosequencing	711	7.9%
CRYSTAL	Real-time PCR clamping	999	6%
COIN	Pyrosequencing, MALDI-MS	1316	8%
MAX	HRM analysis	315	10.6%
NORDIC–Ⅶ	Wobble–enhanced ARMS, Real-time PCR clamping	457	12%
PRIME	Direct sequencing Wave–based Surveyor	641*	8%
TRIBE	Pyrosequencing, MALDI-MS	508	5.5%

*Performed for patients with wild-type *KRAS* exon 2

Memo1 *BRAF* mutation testing as companion diagnostics

In malignant melanoma, the *BRAF* V600 mutation (V600E mutation: 90%, V600K mutation: 10%) is found in approximately 50-60% of Western and 20-30% of Japanese patients. The efficacy of BRAF inhibitors in *BRAF* V600 mutant malignant melanoma has been proven, and in Japan, the BRAF inhibiting drugs vemurafenib and dabrafenib are approved for pharmaceutical use. To predict the therapeutic effects of each drug prior to administration, the real-time PCR-based COBAS® BRAF V600 mutation detection kit and THxID® BRAF kit, respectively, are approved as companion diagnostics. The *BRAF* V600E mutation is seen in approximately 3% of patients with non-small-cell

lung carcinoma, and combination therapy with dabrafenib and the MEK inhibitor trametinib is also approved in Japan. As companion diagnostics, the next-generation sequencing-based Oncomine ™ Dx Target Test Multi-CDx System is approved.

With regard to metastatic colorectal cancer, combination therapy with a BRAF inhibitor, MEK inhibitor, and anti-EGFR antibody has been developed for patients with *BRAF* V600E mutation, and in July 2019, its efficacy was verified in phase 3 of the BEACON CRC trial. As such, it seems that *BRAF* mutation testing will receive regulatory approval in Japan for use as companion diagnostics. Because Since patient eligibility for this trial was based on *BRAF* V600E mutation testing at each facility, the trial enrolled patients through a variety of test methods to include the *BRAF* V600E mutation. If a specific companion diagnostic is designated for this therapy, appropriate expert guidance will likely be needed to determine whether this therapy is appropriate and whether a retest using the companion diagnostic is necessary in patients with detected *BRAF* V600E mutations via a test method of verified analytical validity, such as a different IVD.

【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 U S A 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. Schubbert S, Shannon K, Bollag G: Hyperactive Ras in developmental disorders and cancer. Nat Rev Cancer 7:295-308, 2007

4. Phipps AI, Buchanan DD, Makar KW, et al: BRAF mutation status and survival after colorectal cancer diagnosis according to patient and tumor characteristics. Cancer Epidemiol Biomarkers Prev 21:1792-8, 2012

5. Ogura T, Kakuta M, Yatsuoka T, et al: Clinicopathological characteristics and prognostic impact of colorectal cancers with NRAS 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-, NRAS- 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. 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

12. Loupakis F, Cremolini C, Salvatore L, et al: FOLFOXIRI plus bevacizumab as first-line treatment in BRAF mutant metastatic colorectal cancer. Eur J Cancer 50:57-63, 2014

13. 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

14. Kopetz S, Grothey A, Yaeger R, et al: Encorafenib, Binimetinib, and Cetuximab in BRAF V600E-Mutated Colorectal Cancer. N Engl J Med, 2019

15. Pietrantonio F, Petrelli F, Coinu A, et al: Predictive role of BRAF mutations in patients with advanced colorectal cancer receiving cetuximab and panitumumab: a meta-analysis. Eur J Cancer 51:587-94, 2015

16. Rowland A, Dias MM, Wiese MD, et al: Meta-analysis of BRAF mutation as a predictive biomarker of benefit from anti-EGFR monoclonal antibody therapy for RAS wild-type metastatic colorectal cancer. Br J Cancer 112:1888-94, 2015

17. NCCN Clinical Practice Guidelines in Oncology Colon/Rectal Cancer 2019. Version 1. 2019.

18. Zhu L, Dong C, Cao Y, et al: Prognostic Role of BRAF Mutation in Stage II/III Colorectal Cancer Receiving Curative Resection and Adjuvant Chemotherapy: A Meta-Analysis Based on Randomized Clinical Trials. PLoS One 11:e0154795, 2016

19. 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

20. Tosi F, Magni E, Amatu A, et al: Effect of KRAS and BRAF Mutations on Survival of Metastatic Colorectal Cancer After Liver Resection: A Systematic Review and Meta-Analysis. Clin Colorectal Cancer 16:e153-e163, 2017

21. 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 10:3911-3929, 2018

22. 大腸癌治療ガイドライン　医師用　2019 年版　大腸癌研究会　編.

23. Glynne-Jones R, Wyrwicz L, Tiret E, et al: Rectal cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. Ann Oncol 28:iv22-iv40, 2017

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

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

26. Stoffel EM, Mangu PB, Gruber SB, et al: 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

27. 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

28. 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 52:157-163, 2018

29. 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 143:151-160, 2017

4 Testing for mismatch-repair deficiency

4.1 Background

Molecular mechanisms of mismatch repair (MMR) deficiency

With each DNA replication cycle, replication errors occur at a certain rate. The predominant mechanisms for repairing of DNA replication errors include direct repair, excision repair, post-replication repair, and mismatch repair. Moreover, abnormalities in the repair of DNA mismatches, which are errors in which incompatible bases are combined, play a pivotal role in gastrointestinal carcinogenesis. To date, six genes, including *MLH1, MSH2, MSH6, PMS2, MLH3*, and *MSH3*, are well elucidated to be involved in mismatch repair. The tetramer composed of MSH2, MSH6, MLH1, and PMS2 primarily repairs base:base mismatches and +1/-1 insertion/deletion loops, and the tetramer formed by MSH2, MSH3, MLH1, and PMS2 (or MLH3) primarily repairs 2-4 base insertion/deletion loops. DNA replication errors tend to occur in microsatelliteregions, wherein sequences of one to several bases are repeated, and due to mismatch repair deficiency, an abnormal number of microsatellite repetitions results in microsatellite instability (MSI). Frameshift mutations due to MSI in genes associated with cellular functions, such as tumor suppression, cell growth, DNA repair, and apoptosis, can cause malignant transformation[1].

Definition of dMMR and MSI in MMR deficient colorectal cancer

If pathological variations or epigenetic changes occur in any of the mismatch repair genes— *MLH1*, *MSH2*, *PMS2,* or *MSH6*—proteins that carry out the typical functions cannot be synthesized, which results in impaired mismatch repair function. This condition is known as deficient mismatch repair (dMMR) and gives rise to DNA replication errors in microsatellite regions. MSI testing assesses the number of sequence repetitions at multiple microsatellite regions. Based on this background, the term dMMR is used to refer to both the deficient expression of MMR proteins as assessed by IHC and the microsatellite instability-high (MSI-H) as assessed by MSI testing. Conversely, tumors that do not show abnormalities in MSI testing are referred to as microsatellite stable (MSS) colorectal cancer, while proficient mismatch repair (pMMR) colorectal cancer refers to IHC testing that shows positive MMR protein expression. Therefore, MSS and pMMR typically refer to tumors in which mismatch repair function is retained.

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

Due to DNA replication errors, dMMR colorectal cancer is of a hyper-mutated type in which T-lymphocyte activation is observed, along with the increased probability that highly immunogenic mutations are expressed as neoantigens on the cell surface. As a result, there is a significant increase in invasive CD8+ T-cells in the tumor and its microenvironment, which is considered a factor in favorable outcomes compared to MSS and pMMR colorectal cancers[2,3]. However, it has been reported that dMMR colorectal cancer evades tumor immunity through upregulation of PD-L1 expression in cancer cells. Further, upregulated PD-L1 expression of cancer cells in dMMR colorectal cancer facilitates evasion of tumor

immunity[4]. In this way, the disruption of mismatch repair in dMMR colorectal cancer leads not only to high immunogenicity, but also to immunotolerance; for this reason, immune checkpoint inhibitors are believed to be effective in patients with dMMR colorectal cancer.

4.2

Basic requirements

MMR deficiency testing is recommended to evaluate the benefit of immune checkpoint inhibitors in patients with unresectable CRC.

Use a companion diagnostic to perform MMR deficiency testing to evaluate the suitability of immune checkpoint inhibitor therapy.

Strength of recommendation

Strong recommendation［SR, 10 members］

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

In the KEYNOTE-016 study, a phase 2 trial of the anti-PD-1 antibody drug pembrolizumab in patients with previously treated unresectable metastatic colorectal cancer, no MSS patients responded to the drug, while 40% of MSI-H patients responded[5]. Further, in the phase 2 KEYNOTE-164 trial of pembrolizumab in patients with previously treated MSI-H/dMMR unresectable metastatic colorectal cancer, among the 61 enrolled patients who received their third or later treatment line (cohort A), the response rate was 27.9% (95%CI 17.1–40.8%), the 12-month progression-free survival rate was 34.3%, and the 12-month overall survival rate was 71.7%; subgroup analysis of Japanese patients found that 2 out of 7 patients responded to therapy, while long-term survival of at least 10 months was confirmed for both of these patients[6,7]. Based on these results, pembrolizumab was approved in Japan in December 2018 for treatment of MSI-H solid tumors, which includes colorectal cancer, with an MSI test kit (FALCO) as its companion diagnostic. Regarding treatment line, due to the approval application that was based on data from cohort A, the package insert includes the effects and indications statement as follows: “The safety and efficacy of this drug has not been established in patients who have not previously been treated with an anti-cancer drug of the fluoropyrimidine class, oxaliplatin, and irinotecan hydrochloride hydrate”. However, the use in second-line or later therapy can nevertheless be considered, as efficacy (RR 32%) was also demonstrated in cohort B of the KEYNOTE-164 trial, which consisted of patients who received their second or later line of treatment. Likewise, in the phase 2 of trial CheckMate142, the anti-PD-1 antibody nivolumab also achieved a response rate of 31.1%（95%CI 20.8–42.9%）with a median progression-free survival of 14.3 months in patients with unresectable or recurrent MSI-H/dMMR colorectal cancer[8].

Therefore, the efficacy of pembrolizumab for unresectable or recurrent MSI-H colorectal cancer has been established, and MMR deficiency testing is strongly recommended to evaluate indication for treatment of pembrolizumab. As will be discussed later, poor outcomes have been reported in patients with unresectable MSI-H colorectal cancer, regardless of whether the *BRAF* V600E mutation is present. Testing for MMR deficiency at an early line in the treatment of unresectable metastatic colorectal cancer is also recommended to avoid missing opportunities to administer potent therapies. In addition, because

MMR deficiency and *RAS/BRAF* mutation are not mutually exclusive, implementation of MMR testing is recommended regardless of genotype. Furthermore, because changes in MMR status over time have not been reported in colorectal cancer, from the viewpoint of cost and effective use of histopathological materials, it is efficient to test for MMR deficiency at the same time as *RAS/BRAF* mutation testing.

Recently, the efficacy of immune checkpoint inhibitors in first-line therapy for unresectable metastatic colorectal cancer has also been reported[9], and phase 3 of the KEYNOTE-177 trial is currently in progress. Depending on the results, the use of immune checkpoint inhibitors could potentially be expanded to first-line therapy, and simultaneously, it may become necessary to test for MMR deficiency prior to initiating first-line therapy for appropriate treatment strategy.

Memo 1 Stage IV colorectal cancer and MMR status

In stage IV colorectal cancer, dMMR is observed in 5-11%[10,11] of patients in Western countries and approximately 2%[12,13] of patients in Japan, and it is a negative prognostic factor. For example, in pooled analyses of phase 3 trials assessing treatment of unresectable metastatic colorectal cancer, prognosis was poor among patients with dMMR compared to those with pMMR (13.6 months vs. 16.8 months, HR 1.35, 95%CI 1.13–1.61) [10,11]. In combined analysis with *BRAF* V600E mutation status, the *BRAF* V600E mutation was a strong negative prognostic factor regardless of MMR status, but among patients with wild-type *BRAF*, outcomes were poorer for dMMR than for pMMR, and progression-free survival also tended to be shorter (**Table 2).**

On the other hand, regarding the efficacy of chemotherapy for unresectable metastatic dMMR colorectal cancer, it has been reported that the response rate tends to be better than in pMMR (Odds ratio: 0.81、95%CI 0.65–1.03) [14] and that the response rate to second-line treatment with irinotecan after resistance to 5-FU is higher; however, a unified view has not been reached[15]. Accordingly, although any of the typically selected chemotherapies could be appropriate for unresectable metastatic colorectal cancer regardless of MMR status, MMR deficiency testing in conjunction with *BRAF* V600E testing can be considered for predictive factors for prognosis.

Table 1. Efficacy of immune checkpoint inhibitors in unresectable metastatic dMMR colorectal cancer

Authors	Trial name	Phase	Immune checkpoint inhibitor	Target	N	RR (%)	PFS (M)	OS (M)
Le DT, et al.[5]	KEYNOTE –016	Ⅱ	Pembrolizumab	MSI–H	11	40	Not reported	Not reported
				MSS	21	0	2.2	5
Le DT, et al.[16]		Ⅱ	Pembrolizumab	MSI–H	40	52		

	KEYNOTE–016						Not reported	Not reported
Dung L, et al[6]	KEYNOTE–164 (Cohort A)	Ⅱ	Pembrolizumab	MSI–H/dMMR	61	27.9	Not reported	Not reported
Overman MJ, et al[8]	CheckMate–142	Ⅱ	Nivolumab	MSI–H/dMMR	74	31.1	14.3	Not reported
Overman MJ, et al[8]	CheckMate–142	Ⅱ	Nivolumab + Ipilimumab	MSI–H/dMMR	119	54.6	Not reported	Not reported

RR：Response rate, PFS：Progression-free survival, OS：Overall survival, M：months

Table 2. Overall survival and progression-free survival of patients with unresectable metastatic colorectal cancer by MSI/MMR and *BRAF* V600E mutation status

		BRAF WT		*BRAF* MT		MSS/pMMR		MSI−H/dMMR	
		MSI–H /dMMR	MSS /pMMR	MSI–H /dMMR	MSS /pMMR	*BRAF* MT	*BRAF* WT	*BRAF* MT	*BRAF* WT
Tran B[10]	OS	17.2	26.6	8.0	8.2	8.2	25.5	8.0	17.2
	HR	–		–		–		–	
	p	0.021		0.363		＜0.001		0.011	
Venderbosch S[11]	OS	15.0	17.3	11.7	11.3	11.3	17.3	11.7	15.0
	HR	1.22（0.91–1.65）		1.05（0.68–1.63）		1.94（1.57–2.40）		1.51（0.93–2.46）	
	p	–		–		–		–	
	PFS	6.3	7.8	6.1	6.2	6.2	7.8	6.1	6.3
	HR	1.32（1.00–1.75）		0.95（0.62–1.46）		1.34（1.10–1.64）		1.07（0.67–1.70）	
	p	–		–		–		–	

WT：wild type, MT：mutant, OS: Overall survival, HR：hazard ratio, PFS：progression-free survival

4.3

Basic requirements

MMR deficiency testing is recommended to assess the risk of recurrence and stratify optimal perioperative chemotherapy in patients with resectable CRC.

Use a test with verified analytical validity to perform MMR deficiency testing and to select treatments that are appropriate to recurrence risk.

Strength of recommendation

Recommendation［SR: 2 members, R: 8 members］

Clinical significance of MMR deficiency testing in metastatic colorectal cancer

The rates of dMMR in stage II/ III colon cancer have been reported as 15-22% and 12-14%[17-19], respectively, and in Japan as 6-10% and 5%[13,20], respectively. Since an association between dMMR and 5-FU resistance was reported based on the experiment using cancer cell lines [21], many trials on the efficacy of 5-FU on patients with dMMR have been conducted.

In stage II/III colorectal cancer, patients with dMMR have a significantly lower recurrence risk than patients with pMMR（11% vs 26%； HR 0.53、95%CI 0.40–0.70）, and this trend was marked in stage II colon cancer（8% vs 21%、 HR 0.44、95%CI 0.29–0.67）[17]. Also, when postoperative adjuvant 5-FU therapy was compared with surgery alone in stage II/III colon cancer, patients with MSI-H had significantly better overall survival than those with MSI-L or MSS in a group of surgery alone; however, among patients who received adjuvant chemotherapy, there were no significant differences in overall survival. Thus, adjuvant chemotherapy that includes 5-FU shows efficacy in MSI-L and MSS colon cancer, but in MSI-H colon cancer, it could instead potentially cause harm (**Table 3, 4**) [2, 22].

In stage II/III colon cancer, the *BRAF* V600E mutation is found at a higher rate in patients with dMMR (35.3%) compared to those with pMMR (11.5%)[23]. Pooled analyses of the N0147 and PETACC8 trials of stage III colon cancer demonstrated that, whereas dMMR is a favorable prognostic factor, when *BRAF* V600E or *KRAS* exon 2 mutation is present with pMMR, recurrence risk is significantly higher and prognosis is poor[24,25]. Based on this, it has been reported that recurrence-free survival for stage III colon cancer can be stratified by risk through a simultaneous assessment of the *BRAF* V600E mutation and dMMR[24,26].

On the other hand, the frequency of dMMR in rectal cancer is low; in a retrospective study on stage I-IV colorectal cancer in Japan, frequency of MSI-H was 4% (12/271) for left colon cancer and 2% (7/394) for rectal cancer versus 13% (36/275) for right colon cancer [13]. A recent observational study found not only a favorable five-year survival rate for dMMR rectal cancer, but also a pathological complete response（pCR）rate of 27.6% among stage II/III patients who received neoadjuvant chemoradiotherapy, which is higher than the overall rate (pCR=18.1%) reported for all rectal cancers, including MSS/pMMR [27], which

demonstrates that dMMR could potentially be a favorable predictive factor for therapeutic responses [28]. In addition, an analysis of 5086 patients with locally advanced rectal cancer in the United States National Cancer Database has similarly reported that the MSI status serves as an independent predictive factor of the therapeutic response in terms of pCR[29].

Based on the above, dMMR is a recognized as a favorable predictor of recurrence and outcomes for stage II/III colorectal cancer, and it is possible that independent therapy with fluoropyrimidine could increase the recurrence rate for dMMR stage II colon cancer. In stage III colon cancer, it can be used with *BRAF* mutational status to stratify recurrence risk, and because the 2019 Japanese G*uidelines for the Treatment of Colorectal Cancer* recommend selecting a postoperative adjuvant therapy regimen and duration for stage III colon cancer that is in accordance with recurrence risk, it is useful to test for MMR deficiency prior to the initiation of adjuvant chemotherapy and prior to treatment selection. Based on these results, MMR deficiency testing prior to the initiation of adjuvant chemotherapy is recommended to determine appropriate treatments for recurrence risk in patients with metastatic colorectal cancer.

Table 3. Meta-analysis of phase 3 trials that compare surgery alone to adjuvant 5-FU therapy for stage II/III colon cancer (Reference 2)

	Overall survival			
	Surgery alone MSI–H vs MSI–L/MSS	Adjuvant 5–FU therapy MSI–H vs MSI–L/MSS	MSI–H Adjuvant 5–FU therapy vs surgery alone	MSI–L/MSS Adjuvant 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：microsatellite instability-low and microsatellite-stable

Table 4. Meta-analysis of phase 3 trials that compare surgery alone to adjuvant 5-FU therapy for stage II/III colon cancer (Reference 22)

	Disease-free survival					
	Surgery alone dMMR vs pMMR	Adjuvant 5-FU therapy dMMR vs pMMR	dMMR Stage Ⅱ Adjuvant 5-FU therapy vs surgery alone	dMMR Stage Ⅲ Adjuvant 5-FU therapy vs surgery alone	pMMR Stage Ⅱ Adjuvant 5-FU therapy vs surgery alone	pMMR Stage Ⅲ Adjuvant 5-FU therapy vs surgery alone
HR	0.51	0.79	2.30	1.01	0.84	0.64
95 %CI	0.29–0.89	0.49–1.25	0.084–6.24	0.41–2.51	0.57–1.24	0.48–0.84
p	0.009	0.30	0.09	0.98	0.38	0.001

HR：hazard ratio, CI：confidence interval

Memo 1 Clinical significance of MSI-L in resectable colorectal cancer

In 1997, the United States National Cancer Institute established international standards based on a total of 5 markers (the Bethesda panel), which include 2 mononucleotide markers (BAT25, BAT26) and 3 dinucleotide markers (D2S123, D5S346, D17S250) [30,31], and these standards define tumors with instability in 2 or more markers as MSI-H, in 1 marker as MSI-L, and in no markers as MSS[32]. However, because determination of MSI-L and MSS depends on the numbers and types of markers measured, there is no unified view of either, and they are often even considered virtually void of clinical significance[32,33]. Among recent reports, there are some findings that MSI-L is a recurrence risk factor and negative prognostic factor compared to MSS[34-37], but these reports are based on the use of different definitions and types and numbers of microsatellite markers for determining MSI-L. There is a need to establish a method of identification, as it has been reported that, when determining MSI using markers in 377 locations, approximately 80% of colorectal cancer patients who were not considered MSI-H were found to have MSI in at least one location[33, 38].

4.4

Basic requirements

MMR deficiency testing is recommended to screen for Lynch syndrome.

Use a test with verified analytical validity to perform MMR deficiency testing for Lynch syndrome screening.

Strength of recommendation

Strong recommendation［SR: 10 members］

Lynch syndrome screening

Lynch syndrome is an autosomal dominant inherited disease primarily caused by the germline mutations of any of the MMR genes: *MLH1, MSH2, MSH6,* or *PMS2*. Although it is a rare disease, it reportedly affects 2-4% of all patients with colorectal cancer in the Western countries[39,40]. Further, it is a clinically significant diagnosis, as the patient and family members are prone to various malignancies, including colorectal cancer and endometrial cancer. In Lynch syndrome, one of the MMR gene alleles carries a pathogenic germline mutation. It is believed that if the other wild-type allele also acquires a mutation or undergoes promoter region methylation, MMR function is lost, which leads to cancer.

The overall frequency of MSI-H in all colorectal cancers is reportedly 12-16% in the Western countries[41-43] and 6-7% in Japan[13,44]. In most cases, MMR deficiency is acquired through methylation of the promoter region of *MLH1*, but it is considered that 10-20% of patients with MSI-H colorectal cancer are affected by Lynch syndrome. Thus, although it is inappropriate to perform universal genetic testing for patients with MSI-H colorectal cancer, it is essential to understand the screening process for Lynch syndrome, as it can increase the chance of detecting potential Lynch syndrome.

Outside Japan, universal screening for Lynch syndrome is advocated for all patients with colorectal cancer using MSI or immunohistochemical testing【Memo 1】. However, as stated in Japanese Society for Cancer of the Colon and Rectum (JSCCR) Guidelines 2016 for the Clinical Practice of Hereditary Colorectal Cancer, such testing is currently recommended in Japan for secondary screenings in the case that clinical information fulfills either Amsterdam criteria II (**Table 5)** or the revised Bethesda guidelines (**Table 6**) (**Figure 1**). Please refer to the JSCCR Guidelines 2016 for the Clinical Practice of Hereditary Colorectal Cancer for details on diagnostic procedures, surveillance, and treatment strategies for Lynch syndrome.

Table 5. Amsterdam criteria Ⅱ（1999）

Three or more relatives have been affected by HNPCC (Lynch syndrome)-associated cancers (colorectal cancer, endometrial cancer, carcinoma of the renal pelvis or ureter, small bowel cancer), and all criteria below are met:

1. One of the affected relatives is a first-degree relative of the other two.
2. At least two consecutive generations are affected.
3. At least one cancer diagnosed before 50 years of age.
4. Tumors have been histologically confirmed as cancerous.
5. FAP has been excluded.

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

Table 6. Revised Bethesda guidelines（2004）

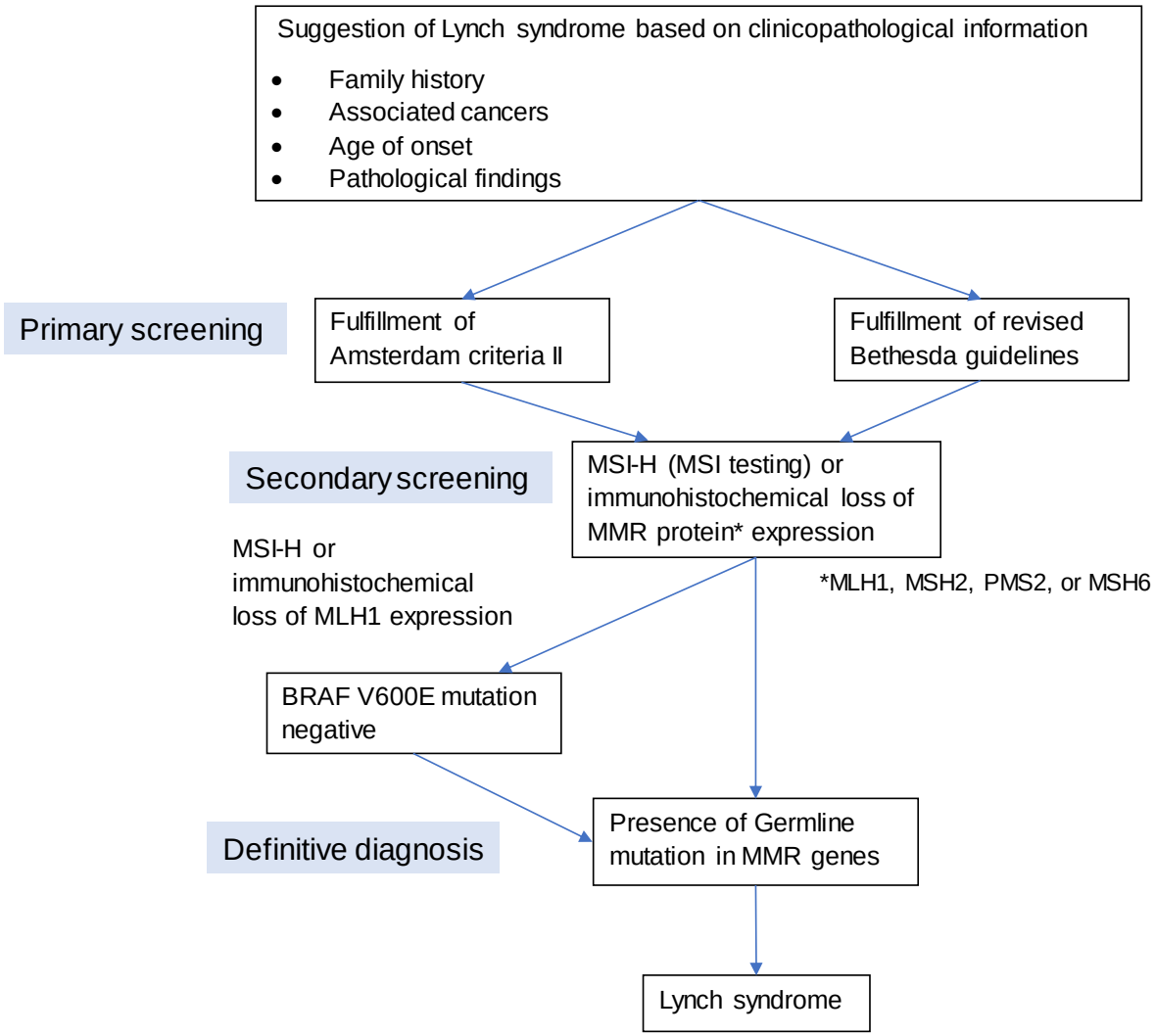
Tumor MSI testing is recommended for patients with colorectal cancer who meet any of the below criteria:

1. Colorectal cancer diagnosed before age 50.
2. Regardless of age, synchronous or metachronous colorectal or other Lynch syndrome-associated* tumors present
3. Colorectal cancer with MSI-H histology** diagnosed before age 60.
4. One or more first-degree relatives diagnosed with a Lynch syndrome-associated tumor, including one diagnosis of colorectal cancer before age 50.
5. Colorectal cancer diagnosed in two or more first- or second-degree relatives with Lynch syndrome-associated tumors, regardless of age.

*colorectal cancer, endometrial cancer, gastric cancer, ovarian cancer, pancreatic cancer, biliary tract cancer, small bowel cancer, cancer of the ureter and or renal pelvis, brain tumor (usually glioblastoma as seen in Turcot syndrome), sebaceous gland adenomas and keratoacanthomas in Muir-Torre syndrome

** tumor infiltration by lymphocytes, Crohn's-like lymphocytic reaction, mucinous/signet-ring differentiation, medullary growth pattern

Figure 1. Diagnostic process for Lynch syndrome (revised from the 2016 JSCCR Guidelines for the Clinical Practice of Hereditary Colorectal Cancer)



Memo 1 Universal screening for Lynch syndrome

In the Western countries, universal screening with MSI testing or IHC examination of MMR proteins is recommended for all patients (or all patients age 70 or under) with colorectal or endometrial cancer, regardless of stage, as a cost-effective method for Lynch syndrome diagnosis, and Lynch syndrome is reportedly found in 2.4-3.7% of patients who undergo universal screening[45,46]. Results of MSI testing and *MLH1, MSH2, MSH6, PMS2,* and *EPCAM* germline mutation analysis for a total of 15045 patients who were diagnosed with over 50 types of cancer indicated that Lynch syndrome is in 16.3% of patients with MSI-H, 1.9% of patients with MSI-Intermediate (I), and 0.3% of patients with MSS; in addition, 50% of Lynch syndrome patients with MSI-H/I developed malignancies other than colorectal and ovarian cancers. Of these patients, 45% did not meet the revised Bethesda criteria, which suggests that it may be necessary to implement universal screening using MMR deficiency testing for all cancer types[47]. The

importance of testing all cancer types for MMR deficiency is highlighted by the fact that MSI-H/dMMR is also a predictive factor for the therapeutic response to immune checkpoint inhibitors in solid tumors other than colorectal cancer. However, the value of universal screening in Japan has not been verified yet; thus, it will be necessary to consider relevant points specific to hereditary disease when implementing universal screenings, as hereditary tumor management will also be needed.

Memo 2 Lynch syndrome and hereditary non-polyposis colorectal cancer

Lynch syndrome is named after Henry T. Lynch, who in 1971 with his team discovered the autosomal dominant inheritance pattern in the genealogy of “Cancer Family G”— a family of which 95 of 650 members developed cancer. Later, in 1985, Lynch himself named the syndrome hereditary non–polyposis colorectal cancer (HNPCC) to differentiate it from familial adenomatous polyposis. However, as the causes and clinical picture of Lynch syndrome were gradually elucidated, and given the high risk of cancer development other than colorectal cancer, “Lynch syndrome” is considered a more appropriate name for the disease, rather than “hereditary non-polyposis colorectal cancer.” Currently, health insurance coverage for MSI testing is designated under “hereditary non-polyposis colorectal cancer or…,” but at present, the term “Lynch syndrome” is considered more suitable.

4.5 Types of tests to evaluate mismatch repair deficiency

Typical methods for evaluating MMR deficiency include: MSI testing, in which microsatellite instability is assessed as marker peaks shift in the fragment analysis performed using PCR products that correspond to microsatellite markers; IHC testing, which assesses the expression of MMR proteins (MLH1, MSH2, MSH6, and PMS2) in cancer tissues; and NGS-based evaluation of mismatch repair function.

Basic requirements

The following methods are recommended when assessing for MMR deficiency:

- MSI testing (Strong recommendation ［SR: 10 members］)
- IHC testing (Strong recommendation ［SR: 7 members, R: 3 members］)
- NGS-based testing (Recommendation ［R: 9 members, ECO: 1 member］)

MSI testing to evaluate MMR deficiency

The Bethesda panel (**Table 7),** which has been used for Lynch syndrome screening, is composed of 5 markers total, including 2 mononucleotide markers (BAT25, BAT26) and 3 dinucleotide markers (D2S123, D5S346, and D17S250) [30-32]. Although dinucleotide markers are typically more valid for diagnosing MSI-L, these markers can fail to indicate MSI-H in Lynch syndrome, in which germline mutations exist on *MSH6* or *PMS2* [48,49]. On the other hand, mononuclear markers detect MSI-H with high sensitivity and specificity and, as they are less influenced by polymorphisms, can be evaluated in cancer tissues alone. Moreover, a mono-marker panel can identify patients with MSH6 deficiency and in whom MSI-H is difficult to detect at a comparatively high rate (62.5%)[50].

Based on these backgrounds, a panel composed exclusively of mononucleotide markers was developed. The MSI test kit (FALCO), which is used to assess the indication of pembrolizumab, is another diagnostic product based on 5 mononuclear markers (**Table 7**)[51]. In this test, DNA is extracted from cancer tissues using a formalin-fixed paraffin-embedded specimen, and 5 microsatellite regions are amplified and evaluated. Upon sorting, the amplified base sequences are sorted by the length of their repetitive base sequences, and a judgment of MSI-positive is established if abnormal peaks are observed outside the normal peak ranges; further, if two or more markers are MSI-positive, a judgment of MSI-H is made (**Figure 2**). If a judgment cannot be made solely based on a tumor sample, a normal tissue sample (can be substituted by a blood sample) will be needed for comparison. In MSI testing, if some markers are untestable, but at least two others are confirmed as MSI-positive, the tumor will be diagnosed as MSI-H (**Table 8**).

Candidates for MSI testing

In December 2018, the MSI test (FALCO) became covered by health insurance in Japan as the companion diagnostic for use to evaluate the indication of pembrolizumab for MSI-H solid tumors. Insurance covers MSI testing on only one occasion— if performed for diagnosis of Lynch syndrome or

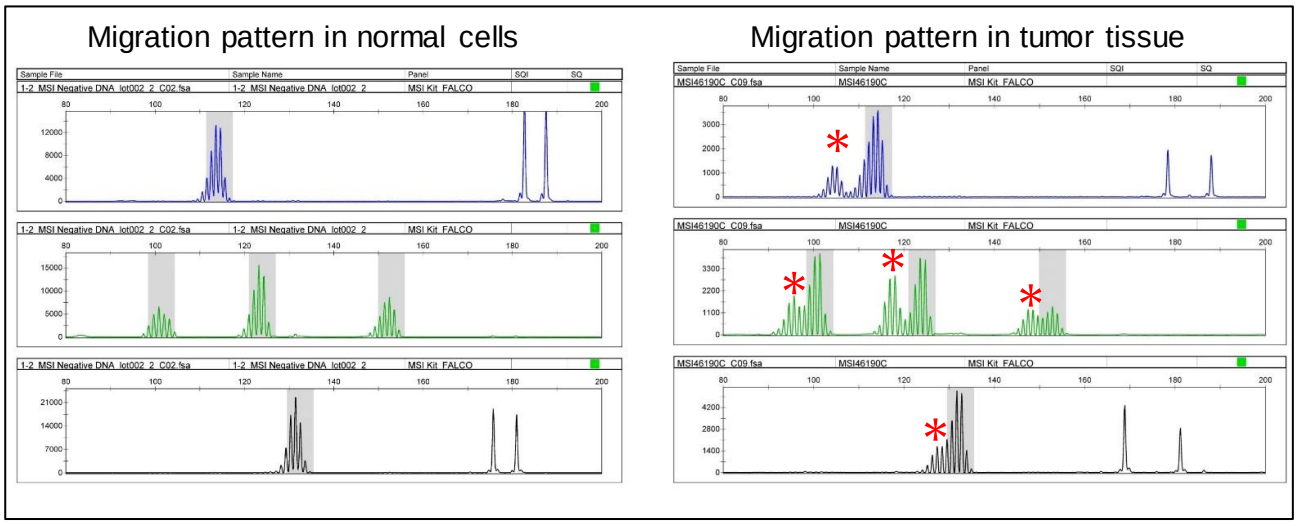
for selection of drug therapy strategies for locally advanced or metastatic solid cancer that are difficult to treat with standard therapies. However, if the test is repeated for the other purpose, it can be calculated separately for only one occasion.

Table 7. Panel overviews

Bethesda panel		MSI test kit（FALCO）	
Marker	Microsatellite type	Marker	Microsatellite type
BAT25	Mononucleotide	BAT25	Mononucleotide
BAT26	Mononucleotide	BAT26	Mononucleotide
D2S123	Dinucleotide	NR21	Mononucleotide
D5S346	Dinucleotide	NR24	Mononucleotide
D17S250	Dinucleotide	MONO27	Mononucleotide

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

（Revised from the MSI test kit [FALCO] package insert）



After PCR amplification of microsatellite marker regions, fragments are analyzed using capillary electrophoresis. A reduced number of repetitions (＊) is observed in DNA from cancer cells compared to DNA from normal cells. As peaks are known to occur in certain ranges in normal-cell DNA, the MSI test kit (FALCO) determines MSI status using cancer tissues alone, by judging peaks outside these ranges as MSI-positive.

Table 8. Examples of judgments with untestable markers

Patient	Marker A	Marker B	Marker C	Marker D	Marker E	Judgment
A	(−)	(−)	(−)	(−)	Untestable	MSI–L or MSS
B	(+)	(−)	(−)	(−)	Untestable	Indeterminate
C	(+)	(+)	(−)	(−)	Untestable	MSI–H
D	(+)	(+)	(−)	Untestable	Untestable	MSI–H
E	(+)	(+)	Untestable	Untestable	Untestable	MSI–H

(+): MSI-positive, (−): MSI-negative

IHC testing to test for MMR deficiency

Another typical method to test for MMR deficiency is the IHC-based assessment of MMR protein (MLH1, MSH2, MSH6, and PMS2) expression in cancer tissues. When evaluating stains, stain adequacy is confirmed using an internal positive control (noncancerous tissue, such as the glandular base of the large intestine mucosa or the germinal center of the lymphoid follicle). All four proteins are expressed in tumors without MMR deficiency, but in Lynch syndrome-associated tumors, which present abnormal MMR function, expression of proteins that correspond to the inactivated MMR genes is lost.

Individual MMR gene abnormalities and proteins with lost expression do not correspond one to one. For example, *MLH1* mutation results in the loss of both MLH1 and PMS2 expression, while *MSH2* mutation results in the loss of both MSH2 and MSH6 expression, and many show stain patterns, such as those in **Table 9.** If a stain pattern that does not fit **Table 9** is obtained, verify stain validity and perform MSI testing if necessary, before considering that the patient may be exceptional. For details, please refer to the JSCCR Guidelines 2016 for the Clinical Practice of Hereditary Colorectal Cancer.

Table 9. MMR protein IHC stain results associated with each gene mutation

		Expression in IHC staining			
		MLH1	MSH2	PMS2	MSH6
Mutant gene	*MLH1*	−	+	−	+
	MSH2	+	−	+	−
	PMS2	+	+	−	+
	MSH6	+	+	+	−

It has been reported that the result of IHC has a high concordance rate with that of MSI test in colorectal cancer. Beyond its use as a screening test for Lynch syndrome, it is expected to become an *in vitro* diagnostics (IVD) in the future for use as a companion diagnostic in immune checkpoint inhibitor therapy. In fact, in a pooled analysis of the 5 KEYNOTE studies (KEYNOTE-012, -016, -028, -158, and -164 [cohort A]) and the CheckMate 142 trial, the efficacy of anti-PD-1 antibody therapy was demonstrated in patients with dMMR, as determined by IHC; further, efficacy was also observed in patients of intermediate status, who were identified as negative for MSI-H, though dMMR by IHC[52]. In clinical practice, re-

evaluation using IHC may be useful when a false negative result is suspected for reasons such as those described below, despite a MSI test judgement of MSS.

Due to their uniform formalin fixation, biopsy tissues are reportedly equal or superior to surgical specimens for IHC examination to evaluate MMR deficiency[53-55]. In MSI testing, biopsy tissues must be used with caution in terms of tumor cell content and DNA yield, whereas IHC offers the advantage of verifying tumor cell percentage using H&E-stained slides. On the other hand, since each antibody clone recognizes an epitope with different antigenic molecules, IHC requires the use of individually optimized detection methods for each antibody clone. Accordingly, IHC must be performed using standardized test methods and highly specific and reactive antibodies. **Table 10** shows typical antibody clones for MMR protein IHC that have been highly rated by external quality assessment organizations in other countries or that have been already approved as IVD in other countries— all of which are expected to further develop in Japan.

However, there are limited cases of discrepancy between IHC and MSI testing. For example, given a dMMR tumor, IHC could yield a positive (pMMR) stain due to a loss-of-function missense mutation, or MSI testing could yield a result of MSS due to low tumor cell percentage or the *MSH6* mutation. Therefore, it is important to adequately comprehend the features of both tests. Additionally, the loss of MSH6 and MLH1 expression has been reported in specimens collected after neoadjuvant chemoradiotherapy and regimens that include cisplatin[49, 56-58]. Special caution must be exercised with low rectal cancer, for which chemoradiotherapy is a standard treatment.

Table 10. Typical antibody clones used in IHC for MMR proteins

MMR Protein	Agilent/Dako Co.	Roche/Ventana Co.
MLH1	ES05	M1
MSH2	FE11	G219–1129
PMS2	EP51	A16–4
MSH6	EP49	SP93

※Reference：http://www.nordiqc.org/about.php

Next-generation sequencing (NGS) as MMR deficiency testing

NGS-based tests to evaluate MMR deficiency are also clinically useful. The FoundationOne® CDx (refer to Section 5) can determine MSI by evaluating 95 intronic microsatellite markers. Concordance with MSI testing and IHC is high at 97%[59], and it has been shown that patients whose MSI testing indicates MSS while IHC indicates dMMR could also potentially be diagnosed using NGS-based testing[60]. Other algorithms have been reported, such as the MSIsensor algorithm, which is based on the MSK-IMPACT assay[61], and the MOSAIC and MANTIS algorithms, which are based on whole exome sequencing[62,63]; however, caution is necessary, as the method of determining MSI varies according to which microsatellite markers and algorithms are used. Furthermore, the use of NGS tests in clinical practice must be approached with care, due to some the following issues: a higher risk that sample quantity and quality

are impeded, compared to other test methods; turnaround time (TAT) of several weeks; and existing clinical trials on immune checkpoint therapy have not yet included NGS-based determination of MSI among eligibility criteria.

Comment 1 Informed consent for MMR deficiency testing

MMR deficiency testing is performed as an auxiliary diagnostic and secondary screening test for patients with colorectal cancer in whom Lynch syndrome, which is a hereditary colorectal cancer, is suspected. Since the MSI test (FALCO) was approved as a companion diagnostic to evaluate the indication of pembrolizumab, the MSI test has been performed to all solid cancers, and the demand for MSI testing is rapidly increasing. If testing is not intended to screen for Lynch syndrome, the following explanations should be provided: (1) the objectives of the test are to evaluate the indication of immune checkpoint inhibitor therapy and to select postoperative adjuvant chemotherapy, and (2) the test is used as a screening tool for Lynch syndrome, given that testing is performed with the obtained consent of the patient. It must be clearly noted in the records of the patient that the contents of (1) and (2) were explained, and that the consent of the patient was obtained. In cases where Lynch syndrome is strongly suspected based on the test results, if the patient desires a definitive diagnosis, an explanation on genetic testing should be provided, along with an explanation that genetic testing, which is not covered by health insurance, will be necessary. Further, since coordinating with experienced healthcare professionals in genetic medicine (Japanese board of medical genetics and genomics, clinical genetics, certified genetic counselors, hereditary tumor specialists, hereditary tumor counselors, familial tumor coordinators, etc.) when performing genetic testing for definitive diagnoses is recommended, you must coordinate in advance with healthcare professionals involved in genetic medicine and arrange a system whereby patients can receive genetic counseling on test objectives, results interpretations, etc., specifically if genetic counseling is unavailable at your facility. For details, please refer to the JSCCR Guidelines 2016 for the Clinical Practice of Hereditary Colorectal Cancer.

【References】

1. Boland CR, Goel A: Microsatellite instability in colorectal cancer. Gastroenterology 138:2073-2087.e3, 2010

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 349:247-57, 2003

3. Chen DS, Mellman I: Elements of cancer immunity and the cancer-immune set point. Nature 541:321-330, 2017

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 5:43-51, 2015

5. Le DT, Uram JN, Wang H, et al: PD-1 Blockade in Tumors with Mismatch-Repair Deficiency. N Engl J Med 372:2509-20, 2015

6. Dung L, Thierry A, Won KT, 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 27:ii79-ii79, 2016

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 28:mdx367.020-mdx367.020, 2017

8. 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 18:1182-1191, 2017

9. Lenz HJJ, Van Cutsem E, Limon ML, et al: LBA18_PRDurable clinical benefit with nivolumab (NIVO) plus low-dose ipilimumab (IPI) as first-line therapy in microsatellite instability-high/mismatch repair deficient (MSI-H/dMMR) metastatic colorectal cancer (mCRC). Annals of Oncology 29:mdy424.019-mdy424.019, 2018

10. Tran B, Kopetz S, Tie J, et al: Impact of BRAF mutation and microsatellite instability on the pattern of metastatic spread and prognosis in metastatic colorectal cancer. Cancer 117:4623-32, 2011

11. 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

12. Kajiwara T SK, Denda T, et al.: The Nationwide Cancer Genome Screening Project for Gastrointestinal Cancer in Japan（GI—SCREEN）：MSI—status and cancer—related genome alterations in advanced colorectal cancer（CRC）—GI—SCREEN 2013—01—CRC sub—study. . J Clin Oncol. 34:suppl；abstr 3573, 2016

13. 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 30:494-9, 2009

14. Des Guetz G, Uzzan B, Nicolas P, et al: Microsatellite instability does not predict the efficacy of chemotherapy in metastatic colorectal cancer. A systematic review and meta-analysis. Anticancer Res 29:1615-20, 2009

15. Fallik D, Borrini F, Boige V, et al: Microsatellite instability is a predictive factor of the tumor response to irinotecan in patients with advanced colorectal cancer. Cancer Res 63:5738-44, 2003

16. Le DT, Durham JN, Smith KN, et al: Mismatch repair deficiency predicts response of solid tumors to PD-1 blockade. Science 357:409-413, 2017

17. 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 29:1261-70, 2011

18. 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 29:3153-62, 2011

19. 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

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

21. Carethers JM, Chauhan DP, Fink D, et al: Mismatch repair proficiency and in vitro response to 5-fluorouracil. Gastroenterology 117:123-31, 1999

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 28（20）：3219—26, 2010

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 18:6531-41, 2012

24. Taieb J, Le Malicot K, Shi Q, et al: Prognostic Value of BRAF and KRAS Mutations in MSI and MSS Stage III Colon Cancer. J Natl Cancer Inst 109, 2017

25. Zaanan A, Shi Q, Taieb J, et al: Role of Deficient DNA Mismatch Repair Status in Patients With Stage III Colon Cancer Treated With FOLFOX Adjuvant Chemotherapy: A Pooled Analysis From 2 Randomized Clinical Trials. JAMA Oncol 4:379-383, 2018

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 Stage III Colon Cancers : A Secondary Analysis of 2 Randomized Clinical Trials. JAMA Oncol 3:472-480, 2017

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 30:1770-6, 2012

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 34:3039-46, 2016

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, 2018

30. Dietmaier W, Wallinger S, Bocker T, et al: Diagnostic microsatellite instability: definition and correlation with mismatch repair protein expression. Cancer Res 57:4749-56, 1997

31. Bocker T, Diermann J, Friedl W, et al: Microsatellite instability analysis: a multicenter study for reliability and quality control. Cancer Res 57:4739-43, 1997

32. 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 58:5248-57, 1998

33. Laiho P, Launonen V, Lahermo P, et al: Low-level microsatellite instability in most colorectal carcinomas. Cancer Res 62:1166-70, 2002

34. Lee SY, Kim DW, Lee HS, et al: Low-Level Microsatellite Instability as a Potential Prognostic Factor in Sporadic Colorectal Cancer. Medicine (Baltimore) 94:e2260, 2015

35. Nazemalhosseini Mojarad E, Kashfi SM, Mirtalebi H, et al: Low Level of Microsatellite Instability Correlates with Poor Clinical Prognosis in Stage II Colorectal Cancer Patients. J Oncol 2016:2196703, 2016

36. Garcia M, Choi C, Kim HR, et al: Association between recurrent metastasis from stage II and III primary colorectal tumors and moderate microsatellite instability. Gastroenterology 143:48-50.e1, 2012

37. Torshizi Esfahani A, Seyedna SY, Nazemalhosseini Mojarad E, et al: MSI-L/EMAST is a predictive biomarker for metastasis in colorectal cancer patients. J Cell Physiol 234: 13128-36, 2019

38. Halford S, Sasieni P, Rowan A, et al: Low-level microsatellite instability occurs in most colorectal cancers and is a nonrandomly distributed quantitative trait. Cancer Res 62:53-7, 2002

39. Hampel H, Frankel WL, Martin E, et al: Screening for the Lynch syndrome (hereditary nonpolyposis colorectal cancer). N Engl J Med 352:1851-60, 2005

40. Barrow E, Hill J, Evans DG: Cancer risk in Lynch Syndrome. Fam Cancer 12:229-40, 2013

41. Aaltonen LA, Peltomaki P, Mecklin JP, et al: Replication errors in benign and malignant tumors from hereditary nonpolyposis colorectal cancer patients. Cancer Res 54:1645-8, 1994

42. 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 338:1481-7, 1998

43. Peltomaki P: Role of DNA mismatch repair defects in the pathogenesis of human cancer. J Clin Oncol 21:1174-9, 2003

44. 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 216:55-62, 2004

45. van Lier MG, Leenen CH, Wagner A, et al: Yield of routine molecular analyses in colorectal cancer patients </=70 years to detect underlying Lynch syndrome. J Pathol 226:764-74, 2012

46. Julie C, Tresallet 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 103:2825-35; quiz 2836, 2008

47. Latham A, Srinivasan P, Kemel Y, et al: Microsatellite Instability Is Associated With the Presence of Lynch Syndrome Pan-Cancer. J Clin Oncol:Jco1800283, 2018

48. Clendenning M, Senter L, Hampel H, et al: A frame-shift mutation of PMS2 is a widespread cause of Lynch syndrome. J Med Genet 45:340-5, 2008

49. 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 26:131-8, 2013

50. Goel A, Nagasaka T, Hamelin R, et al: An optimized pentaplex PCR for detecting DNA mismatch repair-deficient colorectal cancers. PLoS One 5:e9393, 2010

51. Bando H, Okamoto W, Fukui T, et al: Utility of the quasi-monomorphic variation range in unresectable metastatic colorectal cancer patients. Cancer Sci 109:3411-3415, 2018

52. 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 36:773-779, 2018

53. 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 35:447-54, 2011

54. 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 42:414-20, 2010

55. O'Brien O, Ryan E, Creavin B, et al: Correlation of immunohistochemical mismatch repair protein status between colorectal carcinoma endoscopic biopsy and resection specimens. J Clin Pathol 71:631-636, 2018

56. Bao F, Panarelli NC, Rennert H, et al: Neoadjuvant therapy induces loss of MSH6 expression in colorectal carcinoma. Am J Surg Pathol 34:1798-804, 2010

57. Watanabe Y, Koi M, Hemmi H, et al: A change in microsatellite instability caused by cisplatin-based chemotherapy of ovarian cancer. Br J Cancer 85:1064-9, 2001

58. Vilkin A, Halpern M, Morgenstern S, et al: How reliable is immunohistochemical staining for DNA mismatch repair proteins performed after neoadjuvant chemoradiation? Hum Pathol 45:2029-36, 2014

59. FoundationOne SUMMARY OF SAFETY AND EFFECTIVENESS DATA (SSED).

60. 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 7:746-756, 2018

61. 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, 2017

62. Hause RJ, Pritchard CC, Shendure J, et al: Classification and characterization of microsatellite instability across 18 cancer types. Nat Med 22:1342-1350, 2016

63. Bonneville R, Krook MA, Kautto EA, et al: Landscape of Microsatellite Instability Across 39 Cancer Types. JCO Precis Oncol 2017, 2017

5 Next-generation sequencing-based comprehensive genomic profiling tests

5.1 Background

Overview of next-generation sequencing-based comprehensive genomic profiling tests

Next-generation sequencing (NGS) is a base sequence analysis technique based on the principles of massively parallel sequencing. Compared to the earlier Sanger method, NGS has a tremendously improved ability to decode base sequences, which has enabled extremely rapid and high-volume decoding of genomes[1,2]. With NGS, it is possible not only to identify the genomic variations (mutations), but also to analyze the genomic copy number for elevation and loss, identify and determine the frequency of modified regions of the genome, quantify RNA (expression) by means of transcriptome analysis, search for fusion genes, and more.

Clinical sequencing refers to the genomic analysis of patient samples, of which the analytical results are intended for use in treatment selection. Although past cancer-related genomic testing has tested a single gene or a small number of genes, the development of molecular targeted therapies for rare genomic abnormalities has inspired the pursuit of tests, which can simultaneously identify many genomic abnormalities. Comprehensive genomic profiling (CGP) is one NGS-based clinical sequencing form that has been developed for clinical implementation in response to this setting. The clinical value of using CGP testing to comprehensively analyze many genes and select optimal treatment for individual cancer patients has been indicated on the Clinical Practice Guidance for Next-generation Sequencing in Cancer Diagnosis and Treatment (Edition 1.0), which is a collaborative work of the Japan Society of Clinical Oncology, Japanese Society of Medical Oncology, and Japanese Cancer Association, and which was published on October 1, 2017 (henceforth the “3-Society Guidance”)

Comprehensive genomic profiling test workflow

The clinical sequencing process using CGP is composed of several steps. After obtaining the consent of the patient to pursue the test, a sample appropriate for the test is selected (or newly collected), and then checked for quality. The content ratio of tumor cells in the sample is estimated, and if the ratio is low, microdissection is recommended. After the extracted DNA quality and concentration are measured, the NGS library is prepared and sequenced. In data analysis, the obtained base sequence data is first fitted to a human reference sequence, and portions with bases that differ from those of the reference allele are extracted. Information is added in regard to the gene and amino acid mutations coded by the extracted base sequences and whether these mutations are single nucleotide polymorphisms. Information on the gene function changes associated with obtained variants is retrieved— in the case of known variants— from public databases, such as ClinVAr and COSMIC (known as clinical variant annotation), whereas variants of uncertain significance are reported as variants of uncertain significance (VUS) (**Memo 1**). Further, whether the detected genomic alterations correspond to a secondary finding is determined (**Memo 2**). For the detected genomic alterations, a level of evidence is determined by comprehensive evaluation of the availability of anti-cancer targeted drugs, the types of cancer applicable, and the stage of drug development（**Table 1, next section**）[3].

A multidisciplinary molecular tumor board (MTB) assembles to discuss the medical interpretation of the analytical results obtained from annotations. In addition to the attending physician, specialists in disciplines such as cancer drug therapy, medical genetics, genetic counseling, pathology, molecular genomics, cancer

genomic medicine, and bioinformatics participate in the MTB; further, after discussion, a report is prepared and returned to the attending physician. Based on the interpretation of the genomic profile and its alterations, therapeutic options are investigated, and the final report includes descriptions of sample quality, reliability of analytical results, genomic alterations detected and their physiological significance, and possibility of treatment with molecular targeted drugs. For example, approval status and indications of drugs, information about clinical trials in progress, management of VUS and secondary findings, necessity of genetic counseling, etc. are provided in this final report. Genomic information obtained by sequencing is collected in the Cancer Genome Knowledge Database of the Center for Cancer Genomics and Advanced Therapeutics (C-CAT), which not only enables treatment selection that is suited to the individual's condition based on the genomic information of the Japanese population, but is also expected to be used in new developments.

Memo 1 Variants of uncertain significance (VUS)

Many variants are found when cancer cell genes are analyzed using NGS, many of which are VUS in reality. Further, in many cases, the significance of rare mutations in cancer genes is uncertain; for example, it may be unclear whether they are driver mutations that strongly induce malignant transformation, or incidentally occurring passenger mutations. Since their drug sensitivities, such as their potential for malignant transformation, are unclear, interpretation is often difficult if such VUS are found in sequencing. Currently, in regard to VUS, it is considered adequate for a MTB to decide on a strategy by referring to public databases, such as ClinVar and COSMIC or sufficiently annotated genome databases.

Memo 2 Secondary findings

The discovery of a conformably pathological germline gene mutation in CGP testing is known as a secondary finding. In regard to management of secondary findings, please refer to "Recommendations regarding the signal transduction process in genomic medicine: regarding cancer gene panel testing and germline whole genome/whole exome analysis" in "Research regarding the establishment of systems for appropriate disclosure of genomic information in medical settings," conducted at the "Program for Promoting Platform of Genomics Based Drug Discovery" of the Japan Agency for Medical Research and Development (AMED).

5.2

Basic requirement

Comprehensive genomic profiling tests are recommended to assess the benefits of molecular targeted drugs in patients with unresectable CRC.

Strength of recommendation

Strong recommendation［SR: 7 members, R: 3 members］

CGP includes genes in which the alterations involved in the development, growth, or inhibition of cancer have been reported, as well as the genomic alterations approved as companion diagnostics or biomarkers, or those that are related to molecular targeted drugs currently in development; therefore, CGP can be used to obtain information relevant to prognosis prediction and to drug selection in which therapeutic effectiveness is expected based on information on these alterations. Greater extension of prognosis with molecular targeted drug therapy has been demonstrated in patients with tumors that feature genomic alterations targeted by therapeutic drugs compared to tumors without a target. In retrospective analyses of patients that include those with colorectal cancer and who had participated in phase I trials, significant benefit in the response rate, progression-free survival, and overall survival were reported among patients in whom targetable molecular abnormalities were observed[4,5]. In a meta-analysis that verified the usefulness of CGP, it was reported that treatment selection based on gene panel test results correlates with treatment outcome. From a total of 570 studies, 32,148 patients with various types of cancers, including gastrointestinal cancers, were grouped by whether treatment was selected based on biomarkers that use gene panel testing; comparative analysis of these two groups found significantly more favorable response rates, progression-free survival, and overall survival in the group that received gene panel testing[6]. In addition, of the 13,203 patients from the 346 phase I trials that include colorectal cancer, it was reported that patients who received gene panel testing had significant benefit in response rates and progression-free survival, and that the response rate was even more favorable among patients whose genomic alterations were used as biomarkers[7].

CGP has been optimized by the 3-Society Guidance, in addition to proposals from Japan's "Roundtable Consortium on the Promotion of Cancer Genomic Medicine" with the establishment of an upgraded medical system centered on Core Hospitals for Cancer Genomic Medicine, and upgraded systems for the collection and provision of information by the establishment of the Center for Cancer Genomics and Advanced Therapeutics Cancer Genome Information Repository. This prescribed use of CGP contributes to the selection of optimal cancer drug therapies for individual patients. Therefore, given that genomic profiling potentially provides information on genomic alterations, which aids in determining treatment strategy and which leads to the optimal choice of cancer drug therapy, it is strongly recommended to perform CGP testing for patients with metastatic colorectal cancer.

Treatment based on comprehensive genome profiling tests

In the report for the attending physician, several therapies are recommended according to the level of evidence (**Table 1**) (see the 3-Society Guidance regarding levels of evidence). If an alteration approved as a companion diagnostic is detected, approved therapeutic agents are considered for use based on the results of the companion diagnostic (evidence level 1A). However, for colorectal cancer, this currently

applies only to *KRAS/NRAS*. Clinical trials, advanced healthcare, healthcare under assessments such as off-label use of items that are listed on the National Health Insurance drug price list, and use of Special or Specified Medical Care Coverage, such as Patient-Proposed Health Services, are considered if their usefulness is demonstrated by prospective studies or meta-analyses (evidence level 1B-2B), However, study results from outside Japan have shown that approximately 20% of patients are able to access therapeutic drugs based on CGP testing[8], while no recommended treatment options were indicated for most patients.

Since clinical trials, expanded access, and advanced medical care are limited, not all patients receive the opportunity to undergo treatment. However, cost and safety are concerns in private treatment with the off-label use of drugs on the NHI price list. Expanded access allows the use of drugs that have not been approved by the PMDA, such as drugs in the pre-approval stage that have already undergone phase 3 trials. Off-label use is also permitted under advanced medical care, which is implemented by physicians with the aim of future approval, but the cost of the drug is essentially the patient's responsibility. Off-label drug use is also permitted at the patient's request under the Patient-Proposed Health Services program, but as in advanced medical care, the patient is responsible in some cases for the cost of the drug, and several months are required for the actual process.

Table 1. Results and treatment options in the report for the attending physician

Content of genomic alterations		Status of genomic alterations and therapeutic drugs <3-Society Guidance Evidence Level>	Treatment options
Detection of known genomic alteration	Present	Approved as a companion diagnostic product for the type of cancer in question <Evidence level：1A>	Use of approved therapeutic drugs
		For the type of cancer concerned ・FDA-approved as a companion diagnostic ＜Evidence level：1B＞ ・Utility demonstrated in a meta-analysis or a prospective study <Evidence level：1B> ・Utility demonstrated in a subgroup analysis of a prospective study <Evidence level：2A>	Consider clinical trials, advanced medical care, healthcare under assessment, e.g. off-label use of items listed on the National Health Insurance drug price list, Special or Specified Medical Care Coverage, e.g. Patient Proposed Health Services
		Approved for a different type of cancer ＜Evidence level：2B＞	Clinical trials, advanced medical care, health care under assessment, e.g. off-label use of items listed on the National Health Insurance drug price list, and use of Special or

			Specified Medical Care Coverage, e.g., Patient Proposed Health Services
	Absent	Drug recommendation by molecular tumor board ＜Evidence level：3A＞	May consider clinical trials or advanced medical care
		No drug recommendation by molecular tumor board	No treatment proposal
MSI-H*	Present	Immune checkpoint inhibitor	Use of approved drugs
TMB-H**	Present	Immune checkpoint inhibitor	May consider clinical trials or advanced medical care

*MSI-H：microsatellite instability-high, **TMB-H：tumor mutational burden-high

Timing for implementation of comprehensive genomic profiling testing

Clinical implementation of CGP testing in Japan earnestly began in June 2019, when two cancer gene panel tests had become covered by health insurance coverage. Indicated timings for CGP testing were determined as at the conclusion of standard therapies (or when exhaustion of standard therapies is predicted) and prior to initiation of treatment for cancer types that lack standard treatment. However, the FoundationOne® CDx cancer genome profile (discussed below) includes genomic alterations that can be used as companion diagnostics, and it can be used not only to grasp the genomic profile of the tumor, but also as a companion diagnostic to predict the therapeutic effect of corresponding drugs. *RAS/BRAF* mutation testing prior to the initiation of first-line treatment is strongly recommended for metastatic colorectal cancer, and given that *RAS* mutation testing is included in the FoundationOne® CDx cancer genome profile, it is possible to perform testing prior to the initiation of first-line treatment; however, it must be cautioned that testing under these circumstances cannot be claimed as CGP testing for insurance purposes, as the purpose is considered companion diagnostic testing, rather than CGP testing.

In practice, when evaluating the suitability of molecular targeted drugs, it is more reasonable for efficient time and resources to evaluate the suitability of multiple targeted therapies using a single panel test rather than multiple companion diagnostic tests. Despite the concern that a tumor's genomic profile could subsequently change due to therapeutic modification, obtaining the profile at an early stage in treatment by means of CGP is believed to contribute to effective treatment in which considerations, such as prognosis and candidate drugs for future use, are considered. In terms of global considerations, it would be ideal for CGP testing to be performed prior to initiation of first-line therapy in the future.

Memo 1 Tumor Mutational Burden（TMB）

TMB is an index of the number of genomic mutations in the DNA of tumor tissues. Although there is no consensus on a standard method for TMB quantification, Chalmers et al. have used the quantity of somatic gene mutations (base substitutions and insertions/deletions, including synonymous alternations) in 1 Mb of coding sequence as the TMB[9]. Although determination of TMB normally requires whole exome sequencing (WES), Chalmers et al. have reported that an adequate estimation of TMB is possible with

gene panel testing; in samples from 29 patients, TMB estimates based on a gene panel test that included 315 cancer genes and covered 1.1 Mb mostly corresponded with analytical results from whole exome sequencing[9]. The benefit of immune checkpoint inhibitor therapy in patients with high TMB has been reported in various cancer types[10-14], and in colorectal cancer, the benefit of immune checkpoint inhibitor therapy is reported to increase with TMB even among MSI-H colorectal cancer patients[15].

Memo 2 Basket and umbrella trials based on clinical sequencing results

In basket trials, a single-therapy treatment is tested in patients with various cancer types who have been screened for a therapeutically targeted subtype that was identified by a comprehensive genomic analysis. Umbrella trials, on the other hand, simultaneously test multiple treatments in multiple subtypes of a specific cancer identified by comprehensive genomic analyses. The performance of basket and umbrella trials based on genomic alterations identified by CGP testing is effective in the data collection for therapeutic drug development, drug efficacy, and pharmaceutical approval[16]. Currently, many clinical trials that are based on genomic alterations, which are identified by CGP testing, are in progress[17,18].

Memo 3 Development of treatments for rare colorectal cancers

With CGP testing, it is becoming clear that driver mutations previously known to be involved in the survival and growth of other cancer types also rarely exist in colorectal cancer. The following are descriptions of genomic alterations that currently show promise as therapeutic targets:

① *NTRK* gene fusion

The NTRK (neurotrophin receptor tyrosine kinase) genes include the subtypes *NTRK1*, *NTRK2,* and *NTRK3*, which encode the tropomyosin receptor kinase (TRK) proteins TRKA, TRKB, and TRKC, respectively. Activation of *NTRK* as a cancer gene is most often seen in gene fusions, and the fusion genes are observed in multiple organs. Although *NTRK* gene fusion is found in over 90% of rare cancers, such as secretory carcinoma of the breast and the mammary analog secretory cancer of the head and neck, it has a low frequency of less than 1% in colorectal cancer[19]. The selective TRK inhibitor larotrectinib and the ROS1/TRK inhibitor entrectinib have been reported as highly effective in various cancer types with *NTRK* gene fusions [20,21], and entrectinib was approved in Japan in June 2019 and has been covered by health insurance since September 2019 for solid cancers with *NTRK* gene fusions. Additionally, expanded use of the FoundationOne® CDx cancer genome profile as a companion diagnostic for entrectinib was approved in June 2019. However, if used as a companion diagnostic prior to the initiation of first-line therapy, it cannot be claimed as CGP testing; thus, in clinical practice, entrectinib may potentially be used for patients in which *NTRK* gene fusion is found through CGP testing.

② *HER2* amplification

Overexpression of HER2 protein and elevation of the *HER2* copy number reportedly occurs in 1.6-5.2% of colorectal cancers[22-26]. Promising results have been reported from trials such as the HERACLES, which tested the efficacy of trastuzumab with lapatinib in treatment-refractive metastatic *KRAS* wild type, HER2-positive colorectal cancer,[27] as well as a study on the efficacy of trastuzumab

and pertuzumab against HER2-positive metastatic colorectal cancer[28,29], which is performed as part of the MyPathway trial— a basket trial that tested the efficacy of molecular targeted therapies against a variety of genomic alterations. The MyPathway study also reported that the objective response rate was high among patients with wild-type *KRAS,* whereas no patient with mutant *KRAS* had demonstrated an objective response[28]. Currently, studies are in progress, such as a phase 2 physician-led trial (UMIN000027887) on combination therapy with trastuzumab and pertuzumab, and an international phase 3 trial (NCT03384940) on HER2-targeted antibody-drug conjugate DS-8201a.

③ *BRAF* Non-V600E mutations

Certain *BRAF* mutations are non-V600E *BRAF* mutations, which are found outside the V600E hotspot and occur in 2.2-5.5% of patients with metastatic colorectal cancer[30-33]. The BREAC trial conducted in Japan reported that not only the *BRAF* V600E mutation, but potentially also non-V600E *BRAF* mutations may predict a lack of therapeutic benefits from anti-EGFR antibody therapy and serve as negative prognostic factors. On the other hand, a large-scale analysis in the United States reported that, unlike colorectal cancer with the *BRAF* V600E mutation, non-V600E *BRAF*-mutated colorectal cancer is characterized by younger patient ages, less differences between male and female patients, fewer right-sided primary tumors, less peritoneal metastasis, and potentially more favorable prognosis[33].

A basic study has reported that the inhibition of BRAF, MEK, and EGFR in non-V600E *BRAF*-mutated cell lines results in a synergistic effect[34], and a phase 2 physician-led trial (UMIN000031857) in Japan is currently underway to investigate the efficacy of combination therapy with encorafenib (BRAF inhibitor), binimetinib (MEK inhibitor), and cetuximab (anti-EGFR antibody) in patients with non-V600E *BRAF* mutation.

④ Significance of other genomic mutations and amplifications

Besides *NTRK, HER2*, and *BRAF* genes that include *MET*, *FGFR*, *ROS1*, and *ALK* are also considered therapeutic targets, which are expected to be effective in multiple organs, and the future development of corresponding treatments is also expected.

5.3

Basic requirement

Use test systems with verified analytical validity to perform comprehensive genomic profiling tests.

Strength of recommendation

Strong recommendation［SR: 10 members］

CGP testing is conducted to obtain information on genomic alterations, which can aid in selecting therapeutic strategies and in determining the suitability of therapeutic drugs. Therefore, it is essential that the clinical performance of test systems be based on valid selection of analyzed genes, valid detection of analyzed abnormalities, valid reported generation processes, and valid reported content.

Valid selection of genes for analysis should be comprehensive; for patients with solid cancers, it is important to include alterations that are related to molecular targeted drugs, for which companion diagnostics or biomarkers have been approved or developed, as well as genes in which alterations have been reported, related to tumor development, growth, or suppression. Given that the test will be used to select appropriate patients for drug therapy, analysis should ideally include a sufficient number of genes that correspond to the evidence level of 3A or higher in the 3-Society Guidance report. Valid detection of analyzed variants must be demonstrated by accuracy, precision, specificity, and minimum detection sensitivity, with respect to typical base substitutions, indels, abnormal copy numbers, and fusion genes. In addition, for clinical performance as a companion diagnostic, it is important that the CGP test systems demonstrate analytical equivalence with companion diagnostics that are already approved in Japan. Expectations regarding the validity of reported generation processes and content are needed for appropriate management based on data quality and standards for mutation detection, data quality evaluation, and output to be reported. In Japan, the assessment of genomic panel tests for manufacturing and sales approval, which includes evaluation of each of the clinical performance criteria, was developed, and in December 2018, two cancer gene panel tests that include a CGP test were also approved for manufacture and sale.

Based on these results, test systems with verified analytical validity are strongly recommended for comprehensive genomic profiling to select treatment strategies and assess the suitability of therapeutic drugs in patients with metastatic colorectal cancer.

Approved cancer gene panel tests (**Table 2**)

① OncoGuide™ NCC Oncopanel System

DNA, which had been extracted from tumor tissues and white blood cells of patients, are sequenced; further, the results from each source type are compared, and tumor-specific genomic alterations are accurately identified. DNA extracted from the tumor tissues can be analyzed for any mutations in 114 cancer-related genes, 12 gene fusions, and TMB.

② FoundationOne® CDx Cancer Genomic Profile

In addition to obtaining a mutation profile of 324 cancer-related genes in DNA extracted from tumor tissues, this system also includes *KRAS/NRAS* gene mutations for colorectal cancer as a companion diagnostic for cetuximab and panitumumab. Along with detecting the above genomic

alterations as genomic findings, this test can also analyze microsatellite instability (MSI) and TMB as biomarker findings.

Table 2. Details of approved cancer gene panel tests

Name of gene panel	OncoGuide™ NCC Oncopanel System	FoundationOne® CDx Cancer Genomic Profile
Genes targeted Base substitutions, insertions/deletions, abnormal copy numbers, fusions, etc.	114 12	324 36
MSI	–	Analyzable
TMB	Analyzable	Analyzable
Evidence level 3A or above	Over 50	Over 120
Sample(s) needed	Tissue DNA、Blood DNA	Tissue DNA
Germline mutations	Included	Not included
Judgment criteria for detection of mutations	Base substitutions Allele frequency ≥5% Insertions/deletions Allele frequency ≥5% Copy number variations Median read depth of amplified region ≥200 and copy number ≥8 (Depth) ratio ≥4, log (Depth ratio) ≥2 Fusion genes Allele frequency ≥3% at least 2.0e-6 percent of total number of reads	Base substitutions Allele frequency ≥5% Insertions/deletions Allele frequency ≥5% Copy number variations Tumor percentage: ≥20% Gene amplification : Diploid : ≥6 copies（≥5 copies for *ERBB2*） Triploid：≥7 copies tetraploid ：≥8 copies homozygous deletion：0 copies Fusion genes at least 5 read pairs on different chromosomes or separated by at least 10 Mb (for known fusion genes, at least 3)
Test provider	RIKEN Genesis	Foundation Medicine（USA）
Date of approval in Japan	December 25, 2018	December 27, 2018

MSI：microsatellite instability, TMB：tumor mutational burden

Comment1 Companion diagnostic testing using gene panel tests

Studies have reported concordance rates of 92-100% between the tumor tissue-based *RAS* and *BRAF* mutation test results in colorectal cancer, which is based on both NGS and testing with standard techniques, such as direct sequencing (Sanger) [35-38]. Furthermore, use of a panel test to assess *KRAS/NRAS* exons 2, 3, and 4 and *BRAF* exon 15 requires a smaller quantity of DNA, but is similar in cost and time requirements for analysis of all regions by Sanger sequencing[39]. The FoundationOne® CDx Cancer Genomic Profile includes multiple companion diagnostics, and is a good judgment match rate that has been verified in terms of analytical equivalence as an approved companion diagnostic.

In the past, the development of a molecular targeted drug has frequently been accompanied by simultaneous development and approval of a specific companion diagnostic test, and the need for new testing with a different companion diagnostic test arose when a different drug for the same treatment target was used. On June 4, 2019, the Japanese Ministry for Health, Labour and Welfare announced— in regard to the genomic mutations that were identified by genomic panel testing using the FoundationOne® CDx Cancer Genomic Profile or the OncoGuide™ NCC Oncopanel System— that if the administration of a drug that is relevant to the genomic mutation of interest is recommended by the MTB held after the test, based on the package insert, practice guidelines, literature, etc., it would be acceptable to administer that drug without performing a new companion test. It is hoped that, in the future, indications of quality-assured test systems, such as those approved as IVDs, can be expanded across therapeutic agents, e.g. by performing equivalence testing.

Memo 1 Facility requirements for comprehensive genomic profiling tests

On April 1, 2018, 11 facilities in Japan were designated as “Core Hospitals for Cancer Genomic Medicine;” further, “Liaison Hospitals for Cancer Genomic Medicine” were designated in each region to develop cancer genomic medicine in cooperation with the core hospitals. In addition, 34 facilities that can provide complete cancer genomic medical care were designated as “Hub Hospitals for Cancer Genomic Medicine.” After the patient is provided with relevant information, core, hub, and liaison hospitals prepare and submit samples for panel testing in accordance with procedures for specimen handling. At core and hub hospitals, annotation of analyzed data, along with curation, i.e. functional annotation of results, is performed, and a MTB is held for the medical interpretation of genomic data obtained using CGP.

Table 3. Facility functions by designation, e.g. Core Hospital for Cancer Genomic Medicine

	Patient explanation, specimen preparation	**Sequencing**	**Molecular tumor board**	**Report preparation**	**Patient explanation**	**Treatment**	**Research and development**	**Human resource development**
Core Hospitals	Required	May outsource	Required		Required	Required	Required	Required
Hub Hospitals	Required	May outsource	Required		Required	Required	Cooperate	Cooperate
Liaison Hospitals	Required	May outsource	Participation in meetings, etc. at Core and Base Hospitals or		Required	Required	Cooperate	Cooperate

【References】

1. Meyerson M, Gabriel S, Getz G: Advances in understanding cancer genomes through second-generation sequencing. Nat Rev Genet 11:685-96, 2010

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

3. Carr TH, McEwen R, Dougherty B, et al: Defining actionable mutations for oncology therapeutic development. Nat Rev Cancer 16:319-29, 2016

4. 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 20:4827-36, 2014

5. 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, 2017

6. 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 33:3817-25, 2015

7. 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 2:1452-1459, 2016

8. Johnson A, Khotskaya YB, Brusco L, et al: Clinical Use of Precision Oncology Decision Support. JCO Precis Oncol 2017, 2017

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

10. Wang Z, Duan J, Cai S, et al: Assessment of Blood Tumor Mutational Burden as a Potential Biomarker for Immunotherapy in Patients With Non-Small Cell Lung Cancer With Use of a Next-Generation Sequencing Cancer Gene Panel. JAMA Oncol, 2019

11. Ready N, Hellmann MD, Awad MM, et al: First-Line Nivolumab Plus Ipilimumab in Advanced Non-Small-Cell Lung Cancer (CheckMate 568): Outcomes by Programmed Death Ligand 1 and Tumor Mutational Burden as Biomarkers. J Clin Oncol:JCO1801042, 2019

12. Hellmann MD, Callahan MK, Awad MM, et al: Tumor Mutational Burden and Efficacy of Nivolumab Monotherapy and in Combination with Ipilimumab in Small-Cell Lung Cancer. Cancer Cell 35:329, 2019

13. Samstein RM, Lee CH, Shoushtari AN, et al: Tumor mutational load predicts survival after immunotherapy across multiple cancer types. Nat Genet 51:202-206, 2019

14. Ott PA, Bang YJ, Piha-Paul SA, et al: T-Cell-Inflamed Gene-Expression Profile, Programmed Death Ligand 1 Expression, and Tumor Mutational Burden Predict Efficacy in Patients Treated With Pembrolizumab Across 20 Cancers: KEYNOTE-028. J Clin Oncol 37:318-327, 2019

15. Schrock AB, Ouyang C, Sandhu J, et al: Tumor mutational burden is predictive of response to immune checkpoint inhibitors in MSI-high metastatic colorectal cancer. Ann Oncol, 2019

16. Billingham L, Malottki K, Steven N: Research methods to change clinical practice for patients with rare cancers. Lancet Oncol 17:e70-e80, 2016

17. Hurwitz H, Hainsworth JD, Swanton C, et al: Targeted therapy for gastrointestinal (GI) tumors based on molecular profiles: Early results from MyPathway, an open-label phase IIa basket study in patients with advanced solid tumors. Journal of Clinical Oncology 34:653-653, 2016

18. Conley BA, Chen AP, O'Dwyer PJ, et al: NCI-MATCH (Molecular Analysis for Therapy Choice) – a national signal finding trial. Journal of Clinical Oncology 34:TPS2606-TPS2606, 2016

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

20. Drilon A, Laetsch TW, Kummar S, et al: Efficacy of Larotrectinib in TRK Fusion-Positive Cancers in Adults and Children. N Engl J Med 378:731-739, 2018

21. Drilon A, Siena S, Ou SI, et al: Safety and Antitumor Activity of the Multitargeted Pan-TRK, ROS1, and ALK Inhibitor Entrectinib: Combined Results from Two Phase I Trials (ALKA-372-001 and STARTRK-1). Cancer Discov 7:400-409, 2017

22. Comprehensive molecular characterization of human colon and rectal cancer. Nature 487:330-7, 2012

23. Ingold Heppner B, Behrens HM, Balschun K, et al: HER2/neu testing in primary colorectal carcinoma. Br J Cancer 111:1977-84, 2014

24. 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

25. 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

26. Kato T, Hamaguchi T, Hara 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. J Clin Oncol. abstr 3591, 2016

27. 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. The Lancet Oncology 17:738-746, 2016

28. Hurwitz H RK, Burris HA, Kurzrock R, Sweeney C, Meric-Bernstam F, Vanderwalde AM, Spigel DR, Bose R, Fakih Marwan, Swanton C, Guo S, Bernaards C, Beattie MS, Sommer N, Hainsworth JD: Pertuzumab + trastuzumab for HER2-amplified/overexpressed metastatic colorectal cancer (mCRC): Interim data from MyPathway. J Clin Oncol 35:abstr 676, 2017

29. Hainsworth JD, Meric-Bernstam F, Swanton C, et al: Targeted Therapy for Advanced Solid Tumors on the Basis of Molecular Profiles: Results From MyPathway, an Open-Label, Phase IIa Multiple Basket Study. J Clin Oncol 36:536-542, 2018

30. Ciardiello F, Normanno N, Maiello E, et al: Clinical activity of FOLFIRI plus cetuximab according to extended gene mutation status by next-generation sequencing: findings from the CAPRI-GOIM trial. Ann Oncol 25:1756-61, 2014

31. Haley L, Tseng LH, Zheng G, et al: Performance characteristics of next-generation sequencing in clinical mutation detection of colorectal cancers. Mod Pathol 28:1390-9, 2015

32. Shen Y, Wang J, Han X, et al: Effectors of epidermal growth factor receptor pathway: the genetic profiling ofKRAS, BRAF, PIK3CA, NRAS mutations in colorectal cancer characteristics and personalized medicine. PLoS One 8:e81628, 2013

33. Jones JC, Renfro LA, Al-Shamsi HO, et al: Non-V600 BRAF Mutations Define a Clinically Distinct Molecular Subtype of Metastatic Colorectal Cancer. J Clin Oncol 35:2624-2630, 2017

34. Kotani H, Adachi Y, Kitai H, et al: Distinct dependencies on receptor tyrosine kinases in the regulation of MAPK signaling between BRAF V600E and non-V600E mutant lung cancers. Oncogene 37:1775-1787, 2018

35. 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 6:e009532, 2016

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

37. 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 67:764-7, 2014

38. 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 68:64-8, 2015

39. 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 48:908-10, 2015

6 Liquid Biopsy

6.1 Background

Liquid biopsy for colorectal cancer

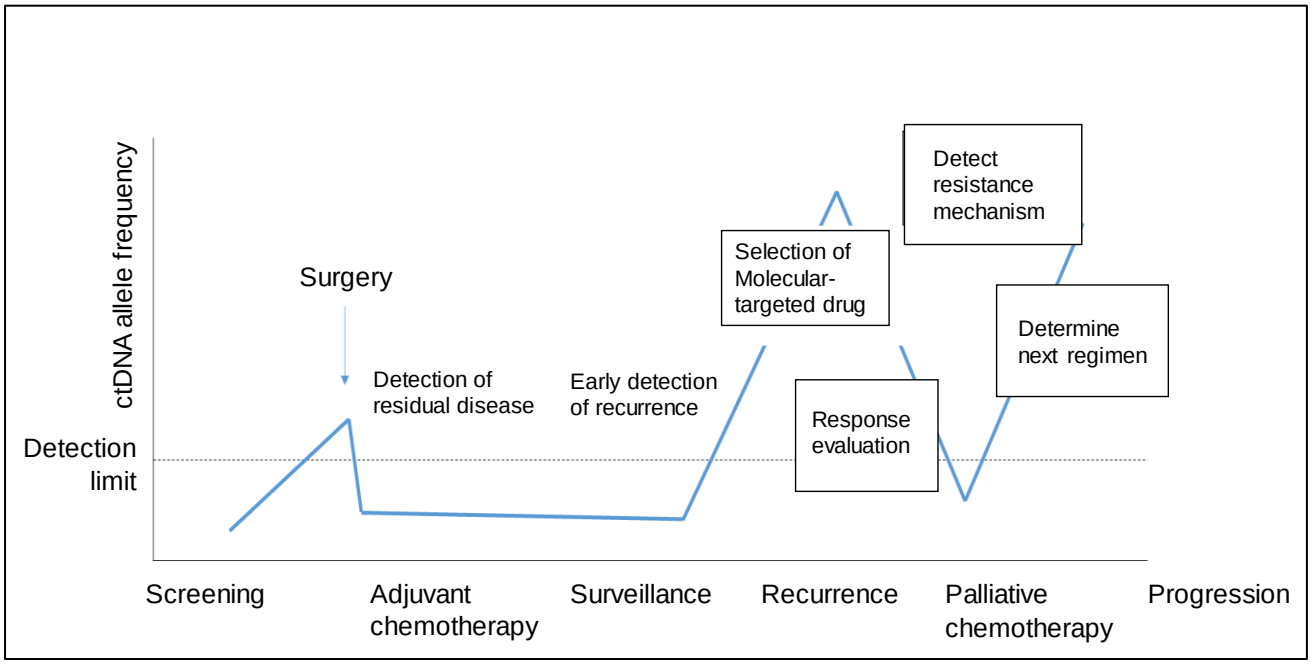
Liquid biopsy is a test technique, in which tumor status is evaluated without direct tumor tissue collection, using samples of bodily fluids such as blood or urine. A quantity of free DNA is present in human blood even in normal circumstances, but this quantity is known to increase in patients with cancer. DNA present in plasma, including DNA of normal cell and tumor origins, is referred to as cell-free DNA（cfDNA）. Because cfDNA in patients with cancer includes tumor-derived DNA, it is also often known as circulating tumor DNA（ctDNA）. Somatic genomic testing, which is performed using ctDNA samples instead of tumor tissue samples, is an anticipated low-invasive test technique for the real-time detection of tumor genomic abnormalities (**Figure 1**) [1,2]. In patients with recurrent colorectal cancer, tumor tissue may be present only in deep regions of the body, such as the liver or lungs, and genomic testing that requires tumor tissue collection is invasive. Therefore, it is possible to avoid tumor tissue collection if genomic testing can be performed on ctDNA. In addition, since ctDNA-based genomic testing does not require histopathological specimen processing, TAT can be shortened, allowing this to be a particularly useful option; for example, when expedited treatment selection and initiation of drug therapy are desired.

Concordance with genomic abnormalities in tumor tissue and ctDNA quantity

The sensitivity of ctDNA-based detection of genomic mutations is rapidly increasing due to the introduction of highly sensitive mutation-detecting methods, such as BEAMing and droplet digital PCR. In patients with metastatic colorectal cancer who have never received anti-EGFR antibody therapy, *KRAS* mutation tests performed on tumor tissue specimens and on blood specimens have a high concordance rate of 78-96%[3-6]. When *KRAS/NRAS* mutation results from ctDNA testing with BEAMing were compared to those from tumor tissues in 205 patients with metastatic colorectal cancer prior to anti-EGFR antibody administration, a concordance rate of 92.2% was reported, with 90.4% for the positive percent agreement, and 92.2% for the negative percent agreement[7]. The OncoBEAM™ RAS CRC kit, which uses BEAMing to detect *KRAS/NRAS* mutation in ctDNA, already bears the CE mark as an *in vitro* diagnostic product in Europe, and as discussed below, the kit has also obtained pharmaceutical approval in Japan.

In patients with metastatic colorectal cancer, the amount of ctDNA prior to initiation of chemotherapy has been reported as a negative prognostic factor[8]. In a meta-analysis of ten studies comprising 1076 patients with colorectal cancer, prognosis was significantly poorer in patients with a ctDNA value greater than the median pre-treatment（HR 2.39, 95% CI 2.03–2.82, $p < 0.001$）[9]. In addition, many reports have suggested that early reduction in ctDNA after treatment is a useful predictor of early treatment effect. Tie et al. have reported that ctDNA was detectable prior to initiation of treatment in 48 of 52 patients (92.3%) with colorectal cancer, in which mutant alleles were detected in tumor tissues; further, the results showed that a reduced ctDNA early in treatment (prior to initiation of the second course) correlates with a therapeutic response[10]. ctDNA could potentially predict tumor load and effectiveness of chemotherapy with more sensitivity than previously used tumor markers did.

Figure 1. Expected clinical utility of ctDNA testing



6.2 ctDNA testing for detection of minimal residual disease and recurrence monitoring

Basic requirements

ctDNA testing is recommended to determine the optimal perioperative chemotherapy based on presumed recurrence risk of patients with resectable CRC.

Use a quality-assured panel test for the detection of minimal residual disease to perform testing.

Strength of recommendation

Recommendation ［R: 10 members］

ctDNA testing to detect minimal residual disease (MRD)

ctDNA (alleles with mutant genes) is known to have an extremely short plasma half-life compared to tumor markers such as CEA and CA 19-9[11]. Therefore, ctDNA rapidly disappears from the blood after curative resection if there is no residual cancer. Taking advantage of this characteristic of ctDNA, test systems using next-generation sequencing technology are being developed to detect minimal residual disease (MRD).

Tie et al. have used Plasma Safe-SeqS, which performs high-sensitivity next-generation sequencing by tagging and amplifying genes and which facilitates the distinction of gene mutations from read errors, to develop a diagnostic panel that targets 10+ genes that are frequently mutated in colorectal cancer, such as *APC, TP53,* and *SMAD4*. A genomic mutation was detectable in 230 (99.6%) of 231 patients with curatively resected stage 2 colon cancer who were tested using tumor tissue. Of 178 patients who did not undergo adjuvant chemotherapy, the recurrence rate was significantly higher（HR 18, 95%CI 7.9–40, p<0.001）in 14 patients whose ctDNA mutated alleles were detected at 4-7 weeks after curative resection compared to the 164 patients with undetected mutations; in 85% of patients with mutated ctDNA, the mutated alleles were reportedly found in the ctDNA before imaging tests had confirmed recurrence[12]. Similarly, in 37 patients with curatively resected liver metastases, those with mutated alleles had a 3-year recurrence-free survival rate of 0%, which represents a large difference from the 84% of patients without a mutation (HR 13, 95%CI 19–325, p<0.001). In addition, similar results have been reported from an analysis that included stage III colon cancer (58 patients, HR: 17) and a prospective observational study of locally advanced rectal cancer (159 patients, HR 13) [13,14]. Further, Reinert et al. have reported an MRD-monitoring method (Signatera) that uses postoperative blood samples by the extraction of 16 genes from whole exome analysis of resected tumor tissues to generate primers. 130 patients with curatively resected stage I-III colorectal cancer were tested, and the recurrence rate was significantly higher (HR 7.2, 95%CI 2.7–19.0, p<0.001) among patients who were positive for ctDNA on postoperative day 30 [15].

Based on this, ctDNA gene panel tests for detecting MRD in patients with curatively resected colorectal cancer appears to be useful for identifying patients at high risk of recurrence. In the *2019 Japanese Guidelines for the Treatment of Colorectal Cancer*, selecting an adjuvant chemotherapy treatment regimen in accordance with recurrence risk is recommended[16]. Furthermore, by excluding patients with high recurrence risk, it also becomes possible to select patients with favorable prognosis and potentially omit adjuvant chemotherapy in view of other clinical prognostic factors. Moreover, since assessment of CT scans, blood samples, etc. over time is effective for surveillance of recurrence after surgery, it is predicted

that liquid biopsy-based MRD monitoring on a repetitive basis will allow early detection of recurrences. Based on the above, for patients with resectable advanced or recurrent colorectal cancer, ctDNA gene panel testing for detecting MRD is recommended as a test that can be implemented repeatedly for the identification of patients at high risk of recurrence.

6.3 ctDNA testing to evaluate appropriateness of anti-EGFR antibody drugs

Basic requirements

ctDNA testing is recommended to evaluate the suitability of and monitor the therapeutic effects of anti-EGFR antibody therapy in patients with unresectable CRC.

Use a test with verified analytical validity to perform testing.

Strength of recommendation

Recommendation ［SR: 1 member, R: 9 members］

ctDNA-based *RAS* mutation testing to evaluate the suitability of anti-EGFR antibody therapy

RAS (KRAS/NRAS) testing is recommended for patients with metastatic colorectal cancer as a test to select a first-line therapeutic regimen. A *RAS* mutation test that uses ctDNA has the considerable advantages of short TAT and not requiring tumor tissue collection. A Japanese clinical performance test has reported that the OncoBEAM™ RAS CRC kit, which uses BEAMing to detect *RAS* mutations in ctDNA, shows a satisfactory rate of concordance (86.4%) with *RAS* testing that was performed using tumor tissues collected prior to drug therapy[17], and the kit has been approved for testing since July 2019. However, caution is necessary, as low concordance between tumor tissue and ctDNA genotypes has been reported in patients with lung metastasis alone.

Additionally, reports have also accumulated on mutant allele detection as a predictor in response to anti-EGFR antibody therapy. In studies based on blood that was sampled prior to administration of anti-EGFR antibody therapy, the presence of *RAS* mutant alleles in ctDNA prior to treatment initiation has been reported as a negative prognostic factor[18,19]. Further, the efficacy of rechallenge with anti-EGFR antibody therapy has been reported in patients with colorectal cancer who do not have these genomic abnormalities. In the CRICKET trial, which investigated the efficacy of combination therapy with cetuximab as a third-line therapy after anti-EGFR antibody drug resistance, the overall response rate for all patients was 21%; however, none of the patients with a detected *RAS* mutation in the collected blood ctDNA before the rechallenge responded to the therapy[20]. The detection of the *RAS* mutation is believed to reflect proliferation during anti-EGFR antibody administration of resistant clones within tumor tissues.

Based on the above results, ctDNA-based *RAS* testing prior to the administration of anti-EGFR antibody therapy (including rechallenge anti-EGFR therapy) appears to be useful for evaluating the appropriateness of anti-EGFR antibody therapy. *RAS* mutations detected in ctDNA, which appear as acquired tolerance, have been reported to decay over time as long as anti-EGFR is not administered[21]; in an anticipated therapeutic strategy, the timing for rechallenge with anti-EGFR antibody therapy will be selected while monitoring the presence of the *RAS* mutations in ctDNA through repeated measurements over time. Although more knowledge must be accumulated in the future before a clear cutoff can be determined, many reports indicate that anti-EGFR antibody therapy is ineffective in the presence of at least 1% *KRAS*-mutated alleles. Based on these results, ctDNA testing can be used to evaluate the suitability of and to monitor the therapeutic effects of anti-EGFR antibody therapy in patients with metastatic colorectal cancer.

6.4 ctDNA testing to monitor cancer gene abnormalities

Basic requirements

ctDNA-based comprehensive genomic profiling tests are recommended to assess the benefits of molecular targeted drugs for patients with unresectable CRC.

Use a test with verified analytical validity to perform testing.

Strength of recommendation

Recommendation ［R: 8 members, ECO: 2 members］

Selecting and monitoring treatment targets using ctDNA

Molecular-targeted drugs that correspond to new therapeutic targets are also being developed for therapeutic use in colorectal cancer. A response rate of 35% was reported in phase 1b of a trial that included vemurafenib (BRAF inhibitor) in combination with irinotecan and cetuximab in patients with metastatic colorectal cancer with tumor tissues that were positive for *BRAF* V600E mutation; in all 12 patients whose ctDNAs were tested prior to treatment, the *BRAF* V600E mutation was detectable. In addition, the degree of ctDNA reduction after initiating treatment showed a strong correlation with the degree of the therapeutic response[22]. Similarly, in phase 2 of the trial of the HER2-inhibiting drug trastumumab in colorectal cancer patients with HER2-positive tumor tissues, *HER2* amplification was detectable by pre-therapy ctDNA in 96.6% (28/29) of patients, and a correlation was observed between the copy number in ctDNA and the therapeutic response[23]. Therefore, with the development of new molecular targeted therapies for unresectable colorectal cancer, CGP that is based on ctDNA, as with CGP based on tumor tissues, is considered clinically useful for determining treatment targets. Despite the disadvantage that high sensitivity in ctDNA-based CGP testing depends on limiting the number of genes analyzed to several dozens, ctDNA has the advantage of low invasiveness and low TAT compared to using tumor tissues. Further, testing is easy to repeat and is also reported to be useful for early evaluation of treatment response. In the United States, Guardant360®, FoundationOne® Liquid, and PGDx elio™ Plasma have already been approved since 2018 as ctDNA-based CGP tests.

Detecting resistance mechanisms and selecting new therapeutic agents

Multiple genomic abnormalities that are believed to be resistance factors, including *EGFR, KRAS, NRAS,* and *BRAF* mutations, and *HER2* and *MET* amplifications, are known to be detectable in ctDNA after anti-EGFR antibody therapy administration in *RAS* wild type metastatic colorectal cancer[24, 25]. In addition, a greater therapeutic response has been reported in patients who did not have such resistance alterations detected in ctDNA testing prior to rechallenge with anti-EGFR antibody therapy[26].

Therefore, in patients with unresectable colorectal cancer, blood-based ctDNA somatic genomic testing has been established not only as an alternative to tumor tissue testing, but also as a less invasive test that can be used over time to capture the predominant genomic status of a tumor, complemented by the internal heterogeneity of the tumor. It can be used not only to find treatment targets, but also to obtain useful information related to treatment response prediction and resistance acquisition. In particular, for

rechallenge therapy with (re-administration of) anti-EGFR antibody therapy, ctDNA testing prior to treatment has great impact on treatment selection given the recognized correlation between treatment response and the genomic status of the ctDNA immediately prior to treatment.

Based on these results, for patients with unresectable colorectal cancer, ctDNA genomic panel testing is recommended as a low-invasive test that can be repeatedly performed to identify treatment targets and monitor treatment responses. Given the value of chronological monitoring, it is desired that once clinically introduced, testing on multiple occasions will be viable according to the clinical course of the tumor.

【References】

1. Schwarzenbach H, Hoon DS, Pantel K: Cell-free nucleic acids as biomarkers in cancer patients. Nat Rev Cancer 11:426-37, 2011

2. Diaz LA, Jr., Bardelli A: Liquid biopsies: genotyping circulating tumor DNA. J Clin Oncol 32:579-86, 2014

3. Taly V, Pekin D, Benhaim L, et al: Multiplex picodroplet digital PCR to detect KRAS mutations in circulating DNA from the plasma of colorectal cancer patients. Clin Chem 59:1722-31, 2013

4. Danese E, Minicozzi AM, Benati M, et al: Comparison of genetic and epigenetic alterations of primary tumors and matched plasma samples in patients with colorectal cancer. PLoS One 10:e0126417, 2015

5. Spindler KL, Pallisgaard N, Vogelius I, et al: Quantitative cell-free DNA, KRAS, and BRAF mutations in plasma from patients with metastatic colorectal cancer during treatment with cetuximab and irinotecan. Clin Cancer Res 18:1177-85, 2012

6. Thierry AR, Mouliere F, El Messaoudi S, et al: Clinical validation of the detection of KRAS and BRAF mutations from circulating tumor DNA. Nat Med 20:430-5, 2014

7. Jones FS, Edelstein D, Wichner K, et al: Performance of standardized BEAMing platform for detecting RAS mutations in the blood of metastatic colorectal cancer (mCRC) patients. Journal of Clinical Oncology 34:11538-11538, 2016

8. 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 22:3067-77, 2016

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

10. 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 26:1715-22, 2015

11. Diehl F, Schmidt K, Choti MA, et al: Circulating mutant DNA to assess tumor dynamics. Nat Med 14:985-90, 2008

12. Tie J, Wang Y, Tomasetti C, et al: The potential of circulating tumor DNA (ctDNA) to reshape the design of clinical trials testing adjuvant therapy in patients with early stage cancers. Journal of Clinical Oncology 34:3511-3511, 2016

13. Reinert T HT, Rasmussen MH, Sethi H, Salari R, Shchegrova S, Swenerton R, Wu H, Sharma S, Christensen E, Natarajan P, Dashner S, Tin T, Olson A, Pawar H, Zimmermann B, Iversen LH, Madsen MR, Lin J, Andersen CL. : 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): viii150-viii204. 10.1093/annonc/mdy281, 2018

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

15. 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 May 9. doi: 10.1001/jamaoncol.2019.0528. [Epub ahead of print]

16. 大腸癌治療ガイドライン　医師用　2019 年版　大腸癌研究会　編.

17. Bando H, Kagawa Y, Kato T, et al: A multicentre, prospective study of plasma circulating tumour DNA test for detecting RAS mutation in patients with metastatic colorectal cancer. Br J Cancer 120:982-986, 2019
18. Azuara D, Santos C, Lopez-Doriga A, et al: Nanofluidic Digital PCR and Extended Genotyping of RAS and BRAF for Improved Selection of Metastatic Colorectal Cancer Patients for Anti-EGFR Therapies. Mol Cancer Ther 15:1106-12, 2016
19. Laurent-Puig P, Pekin D, Normand C, et al: Clinical relevance of KRAS-mutated subclones detected with picodroplet digital PCR in advanced colorectal cancer treated with anti-EGFR therapy. Clin Cancer Res 21:1087-97, 2015
20. 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, 2018
21. Parseghian CM, Loree JM, Morris VK, et al: Anti-EGFR-resistant clones decay exponentially after progression: implications for anti-EGFR re-challenge. Ann Oncol 30:243-249, 2019
22 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 6:1352-1365, 2016
23 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
24 Diaz LA, Jr., Williams RT, Wu J, et al: The molecular evolution of acquired resistance to targeted EGFR blockade in colorectal cancers. Nature 486:537-40, 2012
25 Bettegowda C, Sausen M, Leary RJ, et al: Detection of circulating tumor DNA in early- and late-stage human malignancies. Sci Transl Med 6:224ra24, 2014
26 Montagut C, Argiles 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 4:e175245, 2018

7 Angiogenic factors

7.1 Background

Colorectal cancer and angiogenesis

In colorectal cancer as well as other cancers, tumor angiogenesis plays an important role in tumor proliferation and progression, and numerous factors, such as vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), and angiopoietin, are involved in angiogenesis. VEGF is a dimerizing glycoprotein that activates a signal transduction pathway by binding to VEGF receptors (VEGFR) on vascular endothelial cell surfaces. Seven types of VEGF (VEGF-A, B, C, D, and E; placental growth factor [PlGF]-1 and -2) and three types of VEGFR (VEGFR-1, 2, and 3) have been identified. Of these, the binding of VEGF-A to VEGFR-2 is believed to be essential to the signal transduction pathway.

7.2

Basic requirements

To select angiogenesis inhibitors for patients with unresectable metastatic colorectal cancer, perform VEGF-D measurement.

Use a test with verified analytical validity to perform testing.

Strength of recommendation

Expert Consensus Opinion ［ECO: 6 members, R: 3 members］

Plasma VEGF-D levels and therapeutic outcomes of ramucirumab

Ramucirumab, a fully human monoclonal anti-VEGFR-2 antibody, inhibits angiogenesis by blocking VEGF-A, C, and D from binding to VEGFR-2. In the RAISE trial, which investigated the efficacy of a second-line therapy with FOLFIRI in combination with ramucirumab for patients with unresectable colorectal cancer, a pre-planned biomarker analysis was performed. The 1050 patients enrolled in the trial were randomized into marker exploratory and marker confirmatory groups at a ratio of 1:2, and a relationship between treatment outcomes and biomarkers such as baseline plasma VEGF-C and D; sVEGFR-1, 2, and 3; tumor tissue VEGFR-2 protein expression were analyzed[1]. Among the results, in the marker exploratory group, a strong association was observed between higher VEGF-D levels and increased benefits in terms of overall survival and progression-free survival due to the addition of ramucirumab. Using a high/low cutoff for VEGF-D levels based on analyses of the marker exploratory group, a significant relationship between VEGF-D levels and efficacy of ramucirumab was likewise recognized in the marker confirmatory group (overall survival: p=0.01, progression-free survival: p=0.001).

A significant relationship between high/low VEGF-D levels and efficacy of ramucirumab (Overall survival: p=0.0005, progression-free survival: p=<0.0001) was also observed in an overall analysis that included both the exploratory and confirmatory groups. In the high VEGF-D group (n=536), both overall survival and progression-free survival were significantly improved in patients who had received ramucirumab; however, in the low VEGF-D group (n=348), the overall survival was significantly shorter, with no indication of any benefits due to ramucirumab (**Table 1**). Although the previously mentioned results are solely from the RAISE trial, these results suggest that the baseline plasma VEGF-D level may be a predictive marker for the therapeutic benefits of ramucirumab. Currently, its significance is also being evaluated in Japan in a prospective multicenter study (UMIN000028616). Fluctuations in a variety of angiogenic factors have been reported before and after treatment with angiogenesis inhibitors, and monitoring these fluctuations is expected to contribute to the selection of an optimal angiogenesis inhibitor[2-4].

Table 1a. Association between overall survival and VEGF-D

	High VEGF-D				Low VEGF-D			
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		

Table 1b. Association between progression-free survival and VEGF-D

	High VEGF-D				Low VEGF-D			
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		

OS：Overall survival，HR：Hazard ratio，PFS：Progression-free survival，M：month

Comment 1　Angiogenic factors and therapeutic outcomes of bevacizumab

Bevacizumab, which is a human monoclonal antibody against VEGF-A, has demonstrated efficacy when used in combination with various cytotoxic agents. A meta-analysis of 11 trials on the use of combination therapy with bevacizumab has reported that patients with high levels of VEGF-A (in the plasma or tumor tissue) prior to treatment had significantly shorter overall survival (HR 1.30, p<0.0001) and progression-free survival（HR 1.26, p=0.0001）[5]. The relationship between the efficacy of adding bevacizumab and VEGF-A, B, C, and D, and VEGFR-1 and 2 protein expression in tumor tissue was analyzed in the AGITG-MAX trial, which had investigated the efficacy of bevacizumab in combination with capecitabine (± mitomycin). A multivariate analysis showed that only the protein expression level of VEGF-D was significantly associated with benefits of adding bevacizumab[6]. However, when analyzing tumor tissues from the control group (CAPOX [capecitabine and oxaliplatin] + bevacizumab) of the CAIRO-2 trial, no association was observed between VEGF-D protein expression and both overall and progression-free survival. Moreover, in the biomarker analysis on the baseline plasma samples from the CALGB 80405 trial, which compered FOLFOX/FOLFIRI plus bevacizumab with FOLFOX/FOLFIRI plus cetuximab, patients in the bottom quartile for the VEGF-D value (low VEGF-D) and who received FOLFOX plus bevacizumab had longer overall survival（HR 0.62, 95% CI 0.41–0.92）and progression-free survival（HR 0.59, 95%CI 0.41–0.85）compared to those who received FOLFOX plus cetuximab. However, the similar trend was not observed in treatments that involved combinations with FOLFIRI[7]. Based on the above results, although various angiogenic factors are being evaluated as predictive marker regarding the effectiveness of bevacizumab, currently, no biomarker has been established.

Comment 2　Angiogenic factors and therapeutic outcomes of aflibercept

Aflibercept is a recombinant protein that consists of VEGFR-1 and 2 domains fused with the Fc domain of the IgG1 antibody, and it inhibits angiogenesis by blocking VEGF-A, VEGF-B, and PlGF. Further, in the VELOUR trial, which investigated the efficacy of FOLFIRI in combination with aflibercept as a second-line

therapy for patients with unresectable colorectal cancer, a retrospective biomarker analysis was performed on 98 angiogenic factors and inflammatory cytokines in pre-treated plasma. High expression of VEGF-A, PlGF, serum amyloid component, and C-reactive protein were reported in patients with a history of bevacizumab therapy[3]. Furthermore, in patients with plasma VEGF-A and PlGF levels above the median, overall survival was longer for those in the aflibercept combination group, regardless of whether they had previously received bevacizumab therapy[8]. Although results of this analysis suggest that the expression of angiogenic factors and inflammatory cytokines may differ based on whether a patient has prior history of bevacizumab therapy, no useful biomarker is currently available for predicting the effectiveness of aflibercept. Currently, the PERMAD trial (NCT02331927) is taking place to determine biomarkers for the early prediction of an ineffective response to combination therapy with bevacizumab using fluctuations in cytokines and angiogenic factors (part 1), and to investigate the clinical significance of an early biomarker-based switch from bevacizumab to aflibercept prior to radiological progression (part 2)[4].

Memo Angiogenic factors and outcomes of colorectal cancer

Along with tumor invasion and disease stage, the degree of angiogenesis has been reported as a negative prognostic factor. Significantly higher expression of VEGF mRNA has been reported in patients with depth of invasion and metastases to the lymph nodes or liver, and significantly less favorable prognosis has been reported in patients with higher expression of VEGF mRNA compared to those with lower expression (Risk Ratio [RR]=2.49, p<0.0001)[9]. A meta-analysis of VEGF expression (mostly of the VEGF protein), tumor microvessel density (MVD), and outcome has reported that both high VEGF expression and high MVD were associated with significantly worse outcomes in terms of both progression-free survival（VEGF：RR 2.84, p<0.001, MVD：RR 2.32, p<0.001）and overall survival（VEGF：RR 1.65, p<0.001, MVD： RR 1.44, p<0.001）. In preoperative patients, significant correlations were also reported between preoperative plasma VEGF and age and stage, as patients with low preoperative plasma VEGF levels had a higher rate of curative resection and faster postoperative drop in plasma VEGF levels compared to patients with high preoperative plasma VEGF levels[11]. Based on the above findings, prognosis is considered poor in colorectal cancer with high tumor VEGF expression or plasma VEGF levels.

【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 29:602-609, 2018

2. Kopetz S, Hoff PM, Morris JS, et al: Phase II trial of infusional fluorouracil, irinotecan, and bevacizumab for metastatic colorectal cancer: efficacy and circulating angiogenic biomarkers associated with therapeutic resistance. J Clin Oncol 28:453-9, 2010

3. 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 35 no. 4_suppl:592, 2017

4. Seufferlein T, Lausser L, Stein A, et al: A novel biomarker combination and its association with resistance to chemotherapy combinations with bevacizumab: First results of the PERMAD trial. Annals of Oncology 29 suppl_8, 2018

5. 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 58:48-58, 2016

6. 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 113:37-45, 2015

7. 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 34:3597-3597, 2016

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

9. Ishigami SI, Arii S, Furutani M, et al: Predictive value of vascular endothelial growth factor (VEGF) in metastasis and prognosis of human colorectal cancer. Br J Cancer 78:1379-84, 1998

10. Liang JF, Wang HK, Xiao H, et al: Relationship and prognostic significance of SPARC and VEGF protein expression in colon cancer. J Exp Clin Cancer Res 29:71, 2010

11. De Vita F, Orditura M, Lieto E, et al: Elevated perioperative serum vascular endothelial growth factor levels in patients with colon carcinoma. Cancer 100:270-8, 2004

8 Other tests

8.1 Multi-gene assays to predict colon cancer recurrence after surgery

Developmental history of multi-gene assays for patients with curatively resected colon cancer

Although adjuvant chemotherapy is uniformly implemented to prevent recurrence in patients with curatively resected (R0) stage III colon cancer, it is has been reported that prognosis in patients with T1-2N1M0 disease is more favorable than that of patients with stage II disease[1]. Further, the benefits of adjuvant chemotherapy for patients with stage II colon cancer have not yet been established, and although guidelines from both the American Society of Clinical Oncology and European Society for Medical Oncology use clinicopathological factors to define populations with high recurrence risk, the evidence level is not high[2,3]. Therefore, multi-gene assays were developed to achieve more accurate predictions of recurrence and outcomes for patients with curatively resected stage II/III colon cancer. One of these is the Oncotype DX® Colon Cancer Assay, in which gene expression is analyzed in archived specimens from participants in previous large-scale clinical trials of adjuvant chemotherapy. For this analysis, a total of 12 genes, including 7 cancer-related genes（*BGN*, *FAP*, *INHBA*, *GADD45B*, *Ki-67*, *C-MYC*, and *MYBL2*）, and 5 reference genes（*ATP5E*, *GPX1*, *PGK1*, *UBB*, *VDAC2*） were selected based on their correlation with recurrence time, consistency between stage II/III, and clustering results. A recurrence score (RS) is calculated based on the degree of expression for these 12 genes, and classified as either low risk (0-29), medium risk (30-40), or high risk (41-100) [4].

Trials that have verified the utility of the Oncotype DX® Colon Cancer Assay

RS was calculated for patients (n=1436) with stage II colon cancer and whose formalin-fixed specimens were available for use as they had participated in the QUASAR trial[5], which had compared adjuvant chemotherapy with 5-FU/LV, either with or without levamisole, against surgery alone in patients with curatively resected colorectal cancer. The 3-year recurrence rates for the low-, medium-, and high-risk groups were estimated at 12%, 18%, and 22%, respectively, and the recurrence risk hazard ratio per 25 RS points was 1.38（95%CI 1.11–1.74, p=0.004). In addition, it was found that patients with T4 pMMR tumors had a high recurrence rate even with low RS, and conversely, patients with T3 dMMR tumors had a low recurrence rate even with high RS; thus, it was concluded that RS may be especially useful for evaluating recurrence risk for the 74% of patients who have T3 pMMR tumors.

In the CALGB 9581 trial[6] to assess postoperative EpCAM antibody therapy for stage II colon cancer, compared to only surgery, patients with T3 microsatellite-stable cancer with low, medium, and high recurrence risk based on RS had 5-year recurrence rates of 13%, 16%, and 21%, respectively. The hazard ratio for recurrence risk was 1.68 （95%CI 1.18–2.38, p=0.004） per 25 RS points, which again demonstrates the utility of RS in stage II colon cancer. In addition, in the NSABP-07 trial[7], which compared 5-FU/LV to FLOX (5-FU, leucovorin, and oxaliplatin) in stage II/III colon cancer, the recurrence risk hazard ratio per 25 RS points, when adjusted for stage and therapy, was 1.96（95%CI 1.50–2.55, p<0.001）, which

indicates that RS can significantly predict recurrence irrespective of therapy type, not only in stage II, but also in stage III colon cancer. There were also significant differences in disease-free survival and overall survival, with hazard ratios per 25 RS points of 1.60 and 1.89, respectively.

Out of 1568 patients in Japan with stage II/III colon cancer who underwent curative resection but did not receive adjuvant chemotherapy, 630 patients were enrolled in the SUNRISE trial at a 1:2 ratio of recurrence to non-recurrence, and analysis was performed on 597 of these patients by RT-PCR. For stage II disease, the 3-year recurrence rate was 9%, the 5-year recurrence rate was 11%, and the median RS was 27 (interquartile range [IQR] 20-36); for stage III disease, they were 26%, 31%, and 32 (IQ 22-40), respectively. The primary endpoint for the recurrence-free interval was found to correlate significantly to RS, with a hazard ratio per 25 RS points of 2.05（95%CI 1.47–2.86, p<0.001）. Significant correlations (all p<0.001) were also recognized between RS and the secondary endpoints of recurrence-free survival, disease-free survival, and overall survival; the hazard ratio per 25 RS points was 1.77, 1.90, and 2.02, respectively. Further, 5-year recurrence rates for the stage II high-risk and stage IIIA/B low-risk groups were similar at 19% and 20%, respectively, and the 5-year recurrence rate was 38% for both the stage IIIA/B high-risk group and the stage IIIC low-risk group.

Based on results from the IDEA collaboration, which tested whether a 3-month course of oxaliplatin-based (FOLFOX/XELOX) adjuvant chemotherapy was as beneficial for patients with stage III colon cancer as the 6-month course, treatment duration was appropriately shortened for patients; for example, a recommendation for 3 months of postoperative adjuvant chemotherapy with CAPOX was established for low-risk (T1-T3 and N1) patients with stage III colon cancer. With this background information, in Japan, the prospective SUNRISE-DI study investigated the decision impact of the Oncotype DX® Colon Cancer Assay on the therapeutic strategy in stage II/III colon cancer. Overall, treatment recommendations were altered for 40% of the 275 analyzed patients; in terms of staging, they were altered in 30% of stage II patients and 45% of stage III patients, which indicates a significant change in treatment recommendations for stage III patients. In addition, when stage III patients were classified according to the risk levels used in the IDEA collaboration, 48% of low-risk (T1-T3 and N1) and 38% of high-risk (T4 and/or N2) patients experienced changes to their treatment recommendations, suggesting the utility of this test even in the post-IDEA era[9].

Future prospects for multi-gene assays in patients with resected colon cancer

The utility of the Oncotype DX® Colon Cancer Assay for predicting recurrence and outcomes of patients with stage II/III colon cancer has been suggested, regardless of whether postoperative adjuvant chemotherapy is administered or the differences in regimens. Since the selection of patients for postoperative adjuvant chemotherapy based solely on clinicopathological factors can result in overtreatment or undertreatment of some patients, the development of highly accurate tests for predicting recurrence based on molecular biological factors is anticipated.

8.2 CDX2

Caudal-related homeobox transcription factor 2（CDX2）

CDX2 is a member of the homeobox transcription factor family involved in the development and differentiation of intestinal epithelial cells [10]. Expressed in the nuclei of intestinal mucosal epithelial cells, it plays an important role in intestinal metaplasia[11]. It is also involved in controlling the expression of genes related to cellular processes, such as cell adhesion, differentiation, cell cycle regulation, and apoptosis[12], and functions as a tumor suppressor in colorectal cancer[13-16]. Further, CDX2 is strongly expressed in at least 90-95% of patients with colorectal cancer[17-18], and loss of expression is correlated to low differentiation, advanced stages, right side primary tumors, and microsatellite instability[10]. This transcription factor is also useful as a tissue marker for gastrointestinal cell differentiation[19].

Evaluation of prognosis after curative resection

CDX2 was identified when searching for prognostic biomarkers for high-risk colon cancer[20]. In the discovery data set, which included 466 patients, the 5-year disease-free survival rate among CDX2-negative patients, which was 6.9%, was significantly lower（HR 3.44, 95%CI 1.60–7.38, *p*=0.002）than that of CDX2-positive patients (93.1%), and in a multivariate analysis, CDX2 was found to be a prognostic factor independent of stage, age, and sex. The significant correlation of CDX2 expression and 5-year disease-free survival rate was also confirmed in the validated data set of 314 patients（HR 2.42, 95%CI 1.36–4.29, *p*=0.003）. In particular, it was shown that the 5-year disease-free survival rate for patients with stage II disease was significantly lower in CDX2-negative patients（discovery set：positive[n=191] 87%, negative [n=15] 49%, *p*=0.003, validation set： positive[n=106] 80%, negative [n=15] 51%, *p*=0.004）. Additionally, postoperative adjuvant chemotherapy improved disease-free survival rates for stage III patients regardless of CDX2 expression; however, among stage II patients, improvement was only seen among CDX2-negative patients (CDX2-negative *p*=0.006 vs CDX2-positive *p*=0.4. Furthermore, in a retrospective study that included 1045 patients with stage I-III colorectal cancer, CDX2 was a negative prognostic factor independent of MSI and Consensus Molecular Subtype (CMS) in patients with *BRAF* mutations. In this study, the 5-year recurrence-free survival rate in patients with CDX2-negative stage III cancer improved significantly with postoperative adjuvant chemotherapy[21]. In a systematic review and meta-analysis that included 14 studies, patients with CDX2-positive colorectal cancer had a significantly lower death rate（HR 0.5, 95%CI 0.38–0.66, *p*<0.001), and an analysis of 6 other studies also indicated a significant correlation between CDX2 expression and lower rates of recurrence or death（HR 0.48, 95%CI 0.39–0.59, *p*<0.001）[22]. In cell lines, it has been demonstrated that CDX2-negative cell lines are more sensitive to chemotherapeutics than CDX-positive lines[21]. Based on the above results, CDX2 is a prognostic factor in resectable colorectal cancer and potentially a predictive factor for the effectiveness of postoperative adjuvant chemotherapy, especially in patients with stage II disease.

Prognostic factors in metastatic colorectal cancer

A retrospective study on patients with metastatic colorectal cancer reported that low expression of CDX2 is a negative prognostic factor that is independent of patient background[21]. A Japanese study on patients with metastatic colorectal cancer also found that out of the 2560 genes examined, CDX2 expression showed the strongest correlation with prognosis[23]. In addition, an association between low CDX2 expression and resistance to chemotherapy has also been demonstrated[24]. However, correlations have been reported between the loss of CDX2 expression and *BRAF* mutation, MSI, and CMS (especially CMS4), in addition to the stage and grade of the colorectal cancer [10,25-34]. The association between CDX2 expression and prognosis was also dependent on MSI and the *BRAF* mutation in an additional study on the FIRE-3 trial that was conducted overseas[35].

Future clinical application

CDX2 expression has demonstrated potential as a biomarker for selecting patients with stage II colorectal cancer who can benefit from postoperative adjuvant chemotherapy. However, there are also reports that such expression correlates to known prognostic factors, such as MSI and the *BRAF* mutation, and thus further analysis is necessary, due to the lack of studies that have prospectively evaluated CDX2 and outcomes in colorectal cancer.

8.3 CIMP

CpG island methylator phenotype（CIMP）

CpG islands are found in 50-70% of promoter regions in human genes, and global genome aberrant methylation occurs in 15-20% of colorectal cancers, which is referred to as the CpG island methylator phenotype（CIMP）. Such a phenotype is considered to occur at a high rate among colorectal cancers that originate from sessile serrated adenomas/polyps (SSA/P), which is a type of colorectal serrated lesion. Features of CIMP-positive colorectal cancer include MSI-High, *BRAF* mutation-positive, *TP53* mutation-negative, poorly differentiated adenocarcinoma, right-sided primary tumor, and development in females of advanced age[36].

CIMP as a prognostic factor

In MSI-High colorectal cancer, CIMP is considered unrelated to prognosis, but in MSS colorectal cancer, multiple analyses have found it to be a negative prognostic factor, regardless of disease stage. In addition, it has been reported that CIMP-positive patients also have poor prognosis among patients with MSS and *BRAF*-mutated colorectal cancer[37,38].

CIMP as a predictive factor for the therapeutic benefits of anti-EGFR antibody therapy

In a Japanese retrospective analysis that includes patients with *KRAS*-mutated metastatic colorectal cancer who were administered anti-EGFR antibody therapy, 97 patients (first cohort: 45 patients, second cohort: 52 patients) were classified to have either high or low methylated colorectal cancer using DNA chips that cover 99% of CpG islands. The results, reproduced in both cohorts, indicated significantly better

response rates, disease control rates, progression-free survival, and overall survival in low-methylated colorectal cancer than in high-methylated colorectal cancer, which suggests that genome-wide methylation is an independent predictor of therapeutic response to anti-EGFR antibody therapy[39].

Currently, an in vitro diagnostic product is being developed to assess the methylation of 16 CpG islands using methylation-specific PCR. In a retrospective analysis on 217 patients with metastatic colorectal cancer who were administered anti-EGFR antibody therapy, this product was effective in predicting the therapeutic benefit of anti-EGFR antibody therapy[40]. Further development and approval of the product as an IVD are anticipated.

【References】

1. 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 96:1420-5, 2004

2. 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 22:3408-19, 2004

3. Labianca R, Nordlinger B, Beretta GD, et al: Early colon cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. Ann Oncol 24 Suppl 6:vi64-72, 2013

4. 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 28:3937-44, 2010

5. Gray RG, Quirke P, Handley K, et al: Validation study of a quantitative multigene reverse transcriptase-polymerase chain reaction assay for assessment of recurrence risk in patients with stage II colon cancer. J Clin Oncol 29:4611-9, 2011

6. 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 31:1775-81, 2013

7. 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 31:4512-9, 2013

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

9. Watanabe J, Sato T, Kagawa Y, et al: SUNRISE-DI Study: Decision Impact of the 12-Gene Recurrence Score (12-RS) Assay on Adjuvant Chemotherapy Recommendation for Stage II and IIIA/B Colon Cance. ESMO World Congress on Gastrointestinal Cancer O-017, 2019

10. Olsen J, Espersen ML, Jess P, et al: The clinical perspectives of CDX2 expression in colorectal cancer: a qualitative systematic review. Surg Oncol 23:167-76, 2014

11. Verzi MP, Shin H, Ho LL, et al: Essential and redundant functions of caudal family proteins in activating adult intestinal genes. Mol Cell Biol 31:2026-39, 2011

12. Witek ME, Nielsen K, Walters R, et al: The putative tumor suppressor Cdx2 is overexpressed by human colorectal adenocarcinomas. Clin Cancer Res 11:8549-56, 2005

13. Abate-Shen C: Deregulated homeobox gene expression in cancer: cause or consequence? Nat Rev Cancer 2:777-85, 2002

14. Bonhomme C, Duluc I, Martin E, et al: The Cdx2 homeobox gene has a tumour suppressor function in the distal colon in addition to a homeotic role during gut development. Gut 52:1465-71, 2003

15. Gross I, Duluc I, Benameur T, et al: The intestine-specific homeobox gene Cdx2 decreases mobility and antagonizes dissemination of colon cancer cells. Oncogene 27:107-15, 2008

16. Hryniuk A, Grainger S, Savory JG, et al: Cdx1 and Cdx2 function as tumor suppressors. J

Biol Chem 289:33343-54, 2014

17. Wicking C, Simms LA, Evans T, et al: CDX2, a human homologue of Drosophila caudal, is mutated in both alleles in a replication error positive colorectal cancer. Oncogene 17:657-9, 1998

18. Saad RS, Ghorab Z, Khalifa MA, et al: CDX2 as a marker for intestinal differentiation: Its utility and limitations. World J Gastrointest Surg 3:159-66, 2011

19. Liu Q, Teh M, Ito K, et al: CDX2 expression is progressively decreased in human gastric intestinal metaplasia, dysplasia and cancer. Mod Pathol 20:1286-97, 2007

20. Dalerba P, Sahoo D, Paik S, et al: CDX2 as a Prognostic Biomarker in Stage II and Stage III Colon Cancer. N Engl J Med 374:211-22, 2016

21. Bruun J, Sveen A, Barros R, et al: Prognostic, predictive, and pharmacogenomic assessments of CDX2 refine stratification of colorectal cancer. Mol Oncol 12:1639-1655, 2018

22. Tomasello G, Barni S, Turati L, et al: Association of CDX2 Expression With Survival in Early Colorectal Cancer: A Systematic Review and Meta-analysis. Clin Colorectal Cancer 17:97-103, 2018

23. Sunakawa Y, Stintzing S, Cao S, et al: 483PA biomarker study to validate predictors for clinical outcome of cetuximab based chemotherapy in first-line metastatic colorectal cancer (mCRC) patients: JACCRO CC-05/06AR and FIRE-3. Annals of Oncology 29:mdy281.031-mdy281.031, 2018

24. Zhang BY, Jones JC, Briggler AM, et al: Lack of Caudal-Type Homeobox Transcription Factor 2 Expression as a Prognostic Biomarker in Metastatic Colorectal Cancer. Clin Colorectal Cancer 16:124-128, 2017

25. Pilati C, Taieb J, Balogoun R, et al: CDX2 prognostic value in stage II/III resected colon cancer is related to CMS classification. Ann Oncol 28:1032-1035, 2017

26. Trinh A, Trumpi K, De Sousa EMF, et al: Practical and Robust Identification of Molecular Subtypes in Colorectal Cancer by Immunohistochemistry. Clin Cancer Res 23:387-398, 2017

27. Baba Y, Nosho K, Shima K, et al: Relationship of CDX2 loss with molecular features and prognosis in colorectal cancer. Clin Cancer Res 15:4665-73, 2009

28. Dawson H, Galvan JA, Helbling M, et al: Possible role of Cdx2 in the serrated pathway of colorectal cancer characterized by BRAF mutation, high-level CpG Island methylator phenotype and mismatch repair-deficiency. Int J Cancer 134:2342-51, 2014

29. Dawson H, Koelzer VH, Lukesch AC, et al: Loss of Cdx2 Expression in Primary Tumors and Lymph Node Metastases is Specific for Mismatch Repair-Deficiency in Colorectal Cancer. Front Oncol 3:265, 2013

30. Hinoi T, Tani M, Lucas PC, et al: Loss of CDX2 expression and microsatellite instability are prominent features of large cell minimally differentiated carcinomas of the colon. Am J Pathol 159:2239-48, 2001

31. Lugli A, Tzankov A, Zlobec I, et al: Differential diagnostic and functional role of the multi-marker phenotype CDX2/CK20/CK7 in colorectal cancer stratified by mismatch repair status. Mod Pathol 21:1403-12, 2008

32. Olsen J, Eiholm S, Kirkeby LT, et al: CDX2 downregulation is associated with poor differentiation and MMR deficiency in colon cancer. Exp Mol Pathol 100:59-66, 2016

33. Herr R, Brummer T: BRAF inhibitors in colorectal cancer: Toward a differentiation therapy? Mol Cell Oncol 2:e1002709, 2015

34. Landau MS, Kuan SF, Chiosea S, et al: BRAF-mutated microsatellite stable colorectal carcinoma: an aggressive adenocarcinoma with reduced CDX2 and increased cytokeratin 7 immunohistochemical expression. Hum Pathol 45:1704-12, 2014

35. Neumann J, Heinemann V, Engel J, et al: The prognostic impact of CDX2 correlates with the underlying mismatch repair status and BRAF mutational status but not with distant metastasis in colorectal cancer. Virchows Arch 473:199-207, 2018

36. Samowitz WS, Albertsen H, Herrick J, et al: Evaluation of a large, population-based sample supports a CpG island methylator phenotype in colon cancer. Gastroenterology 129:837-45, 2005

37. Dahlin AM, Palmqvist R, Henriksson ML, et al: The role of the CpG island methylator phenotype in colorectal cancer prognosis depends on microsatellite instability screening status. Clin Cancer Res 16:1845-55, 2010

38. Vedeld HM, Merok M, Jeanmougin M, et al: CpG island methylator phenotype identifies high risk patients among microsatellite stable BRAF mutated colorectal cancers. Int J Cancer 141:967-976, 2017

39. 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 106:1722-9, 2015

40. Ouchi K, Takahashi S, Okita A, et al: Development of novel in vitro diagnostics to predict the efficacy of anti-EGFR treatment based on DNA methylation status. Annals of Oncology 30, 2019

9 Samples for molecular testing

9.1 Tissue specimens

Basic requirements

FFPE tissue is suitable for genomic testing of somatic mutations. It is recommended to confirm that the samples have an adequate amount of tumor cells and expect sufficient quality of nucleic acids by assessing the matched reference hematoxylin and eosin stained slides. Selection of FFPE samples, decision on the need for macrodissection, and assessment of tumor cell content should be performed by a pathologist.

Strength of recommendation

Strong recommendation ［SR: 10 members］

Specimen processing

Formalin-fixed paraffin-embedded (FFPE) tissue is a well-suited specimen preparation method for clinical genomic testing of somatic mutations of cancer because it is widely used for tissue preservation in most clinical laboratories and allows for histologic assessment with H&E staining in parallel with molecular testing. Surgically resected specimens are most suitable materials for molecular tests, but biopsy specimens (such as endoscopic and needle biopsies), if containing a high amount of tumor cells, can also be used for mutation analyses of *RAS* and *BRAF* or mismatch-repair deficiency testing. DNA extraction should ideally be performed soon after the cut of the paraffin blocks; unstained slides that have been stored at room temperature for a prolonged period of time should be avoided for genomic testing. This is because nucleic acids preserved in unstained slides degrade faster over time. We should consider this when we perform genomic tests using unstained slides that are provided by other facilities.

Selection of sites

The *RAS* and *BRAF* mutations and mismatch-repair deficiency play important roles in the formation of cancer. Cancer is considered to acquire these alterations at an early stage of development. Indeed, many studies showed that these alterations did not differ significantly when compared between primary and metastatic lesions【Comment 1】. Further, it can be reasonably considered that there is no difference in genomic mutations/ abnormalities detected between biopsy and surgical specimens, as long as both are collected from the same tumor[1].

Formalin fixation

Formalin causes a wide variety of DNA degradation including DNA fragmentation. Thus, it is important to note that the condition of formalin fixation greatly affects the quality of DNA and the result of molecular

tests. Clinicians and pathologists should develop an ideal system in their own hospital to fulfill optimal fixation conditions of resected specimens for genomic testing. Some of the crucial procedures are listed below[2].

- Fix surgically resected samples in formalin within one hour after resection: if it is not possible, keep the specimen at 4℃ (in a refrigerator, etc.) until fixation.
- Fixative solution: 10% neutral buffered formalin is recommended.
- Fixation time: Fix samples for 6 to 48 hours, depending on tissue size.
- Volume of fixative solution: Use a fixative volume of 10 times the tissue volume.
- Promoting penetration of formalin solution: Open the bowel and extend it on a board using pins before fixation. For a metastatic lesion, make one or two sections in the tumor to promote penetration of the formalin.

Tumor area for dissection, and tumor cell percentage

Tumor sections with high tumor cell percentage as well as sufficient tumor volume need to be submitted for genomic testing. Then tumor cell percentage (ratio of the number of tumor nuclei per that of the entire cells in an area) is estimated from the matched H&E section. A thin-sliced tissue is placed on a glass slide, and tissue is scraped from the tumor and collected into sample tubes. If a tumor comprises the entire tissue block, the whole tissue on the slide can be submitted. If the block also contains normal tissue and the dissection of the entire tissue may result in low tumor cell percentage, macrodissection needs to be performed by marking areas with a high cellularity of tumor cells on the reference H&E slide (enrichment). For MSI testing, if dissection of the entire section results in a tumor cell percentage of less than 50%, macrodissection is needed to avoid false negative results[3]. Areas with a prominent coagulative necrosis or mucin deposition should be avoided. Also, areas with histologic findings of marked tumor cell degradation, commonly seen in the center of a large mass, are not suitable for molecular analysis. Preferably, the selection of the FFPE block as well as markings of the area for macrodissection should be conducted by the pathologist.

In using biopsy specimens, the entire tissue would be dissected due to the difficulty of selective macrodissection. It should be noted that the biopsy specimens with low tumor volume or with low tumor cell percentage should not be submitted for molecular genomic testing as such samples may result in false-negative results.

Accurate estimation of tumor cell percentage is one of the most important pre-analytical variables in genomic testing. Note that the proportionate number of tumor cell nuclei to normal cell nuclei is estimated, not the ratio of the area occupied by tumor cells. The tumor cell percentage required for genomic testing varies depending on the sensitivity each molecular test has. For comprehensive genomic panel tests, tumor cell percentage of 30% or higher is desirable; 20% is the minimum. For MSI testing, the limit of detection is 2-10% for allele frequency, with a reported detection limit of 20% tumor cells for diploid tumor cells[4,5].

Moreover, the pathological estimation of tumor cell percentage has significant intra-observer variability. The actual tumor cell percentage is sometimes much lower than the estimated value by pathologists, which can cause false-negative results[6-9]. Thus, it is recommended that a tumor cell percentage of at least 2 times the limit of detection of a molecular genomic test is necessary in clinical samples. When one tissue block is submitted for the analysis of multiple genomic tests with relatively low sensitivity (including genomic panel testing and MSI testing)[10], the tumor cell content of the block needs to be at least 40-50%.

When a patient has multiple specimens (resected or biopsied) during its course of treatment, it is recommended that the samples be selected for genomic testing with shorter storage period, higher amount of tumor cells, and those that have not been affected by previous treatments such as chemotherapy or radiation. Resected specimens after neoadjuvant chemotherapy tend to contain more stromal fibrosis and inflammatory cell infiltrates, resulting in lower tumor cell percentage. In addition, tumor cell percentage also tends to be low in samples of lymph node metastases.

Comment 1 Correlation of genomic abnormalities in primary and metastatic lesions

In colorectal cancer, the concordance rate for the *KRAS* mutations between primary and metastatic lesions is typically high, with most studies reporting a rate of 90% or higher (**Table 1**)[11,12]. However, caution must be exercised in using samples of lymph node metastasis. It has been reported that the concordance between a primary lesion and a lymph node metastasis tends to be lower than that between a primary lesion and a metastatic lesion to the liver. A high concordance rate has also been confirmed for MSI in primary lesions and in synchronous/metachronous liver metastases[13]. Similarly, a study from Japan reports that, although the concordance rate between primary and metastatic lesions (mostly to the liver) for mutations of *KRAS* and *BRAF* and MSI status were high at 90% and above, the concordance rates for the *KRAS* mutation and MSI status were lower between primary and lymph-node metastatic lesions[14].

Synchronous or metachronous multiple primary colorectal cancer is common. This poses a question as to which lesion should be used for molecular testing. The joint ASCP/CAP/AMP/ASCO Guideline on Molecular Biomarkers in Colorectal Cancer states that metastatic or recurrent colorectal carcinoma tissues are the preferred specimens for treatment predictive biomarker testing and should be used if such specimens are available and adequate[10].

Table 1. Summary of the concordance rates of *KRAS* status in primary tumors and matched

metastases in colorectal cancer（revised from Baas et al. [11]）

	N	Frequency of *KRAS* mutation in primary tumors（%）	Site of metastasis（%）	Overall concordance（%）	*KRAS* mutation detection method
Vakiani et al. (2012) [15]	613	36	Liver 78、Lung 12 Other 10	93*	MassARRAY and sequencing
Knijn et al.（2011） [12]	305	35	Liver 100	96	Sequencing
Italiano et al.（2010）[16]	59	39	Not specified	95	Sequencing
Baldus et al.（2010）[17]	75	41	Lymph node 73 Other 27	76	Sequencing and pyrosequencing
Cejas et al.（2009）[18]	110	34	Liver 83、Lung 17	94	Sequencing
Molinari et al.（2009）[19]	37	43	Liver 74、Lung 8 Other 18	92	Sequencing
Loupakis et al.（2009）[20]	43	40	Not specified	95	Sequencing
Garm Spindler et al.（2009）[21]	31	29	Not specified	94	Sequencing
Santini et al.（2008）[22]	99	38	Liver 81、Lung 7、Other 12	96	Sequencing
Artale et al.（2008）[23]	48	27	Liver 81 Other 19	94	Sequencing
Etienne–Grimaldi et al.（2008）[24]	48	33	Liver 100	100	PCR–RFLP
Perrone et al.（2009）[25]	10	20	Not specified	80	Sequencing
Albanese et al.（2004）[26]	30	47	Liver 100	70	SSCP
Zauber et al.（2003）[27]	42	52	Lymph node 93、Liver 5、mesentery 2	100	SSCP
Thebo at al.（2000）[28]	20	100	Lymph node 100	80	AS–PCR
Schimanski et al.（1999） [29]	22	95	Liver 100	95	PCR–RFLP
Al–Mulla et al.（1998）[30]	47	34	Lymph node、Liver	83	ASO

Finkelstein et al.（1993）[31]	NR	35	Not specified	100	Sequencing
Losi et al.（1992）[32]	18	83	Liver 33 Other 67	100	AS–PCR
Suchy et al.（1992）[33]	66	21	Not specified	100	ASO
Oudejans et al.（1991）[34]	31	42	Lung、Liver	87	ASO

* Concordance for *KRAS*, *NRAS*, and *BRAF* mutations between primary and metastatic lesions

SSCP：single strand conformation polymorphism，AS-PCR：allele specific PCR，ASO：allele-specific oligonucleotide

Comment 2　Assessment of quality of nucleic acids

It is reported that underfixation lowers the reactivity of IHC stains but has a negative effect on nucleic acids to a lesser degree. Overfixation, on the contrary, is known to lower the reactivity of IHC stains and cause prominent fragmentation of nucleic acids[35]. Although underfixation can easily be judged morphologically, overfixation is more difficult to acknowledge because the morphological changes associated with overfixation are generally subtle. Multiple tools for assessing the quality of nucleic acid are widely used such as real-time PCR-based assay kits. Normal tissue that is attached to the tumor sample may be useful for the quality check of the specimen.

Memo 1 Handling tissue specimens that contain bone

Samples that contain bone or calcified tissue and thus processed with decalcifying agents are not suitable for genomic testing because decalcifying agents (particularly strong acids) cause marked fragmentation of nucleic acid. Use chelating agents (EDTA-decalcifying agents), which is appropriate for genomic testing or IHC.

Memo 2 Cell block

Cell block is a method of processing cytologic material into a FFPE block after cells are fixed and the cell pellet was solidified by agar. This technique is also applied to aspiration samples obtained by endoscopic ultrasound-guided fine needle aspiration (EUS-FNA). Various techniques for preparing cell block have been devised by multiple institutions[36] and its utility in IHC, in situ hybridization, and molecular genomic analysis is widely acknowledged. Although nucleic acids with high quality can be obtained from cell block samples if handled properly and in good condition, there is no standardized procedure in the preparation of cell block for genomic testing.

9.2 Blood specimens

Basic requirement

In ctDNA testing, use of collection tubes and the preservation and adjustment of plasma after blood collection should be performed in accordance with the manufacturer's instructions.

Strength of recommendation

Strong recommendation ［SR: 10 members］

Recommended blood specimens

Plasma, instead of serum, is generally used for performing somatic genomic testing using ctDNA in blood, as in a liquid biopsy (refer to Section 6 for more on liquid biopsy). Single purpose collection tubes approved and designated for each test method should be used to collect blood that will be used for isolation of cell-free DNA (cfDNA) . However, typical EDTA blood collection tubes may also be used【Comment 1】.

Blood collection and plasma sample adjustment

Blood contains cfDNA released from normal and tumor tissues due to cell death events, such as apoptosis and necrosis. The quantity of tumor-derived ctDNA contained in the total ctDNA is very little in many cases. Therefore, if blood collection tubes are handled inappropriately after collection, such as by being allowed to stand at temperatures outside the recommended range or for prolonged periods, nucleated cells, such as leukocytes, which are found in large numbers in blood, might get damaged, and genomic DNA from these cells might leak into the plasma, diluting the ctDNA. Furthermore, contamination by nucleated cells, such as leukocytes, during plasma separation can similarly dilute the ctDNA, resulting in inaccurate test results. To avoid such situations, it is essential to handle specimens according to the test kit package insert or standard operation procedures for each test method.

Comment 1　Use and handling of blood collection tubes after blood collection

For blood-based somatic genomic testing, the EGFR mutation test kit (Cobas® EGFR Mutation Test v2; Roche Diagnostics) approved as a companion diagnostics in Japan and US has been clinically introduced for non-small cell lung cancer. For *RAS* mutation testing in colorectal cancer, the OncoBEAM™ RAS CRC kit (Sysmex Corporation) was approved as a companion diagnostics in 2019. On the contrary, cancer gene panel tests are being developed for approval in Japan, although no product has yet been approved. The major tests along with their respective recommended blood collection tubes and post-collection handling procedures are summarized in **Table 2.**

Table 2. Plasma ctDNA-based cancer genomic panel tests

	OncoBEAM™ RAS CRC Kit[1-3] (Sysmex)	Guardant 360® [40,41] (Guardant Health)	FoundationOne® Liquid[42] (Chugai Pharmaceutical Co. ／ Foundation Medicine)
Type of somatic genomic test	Companion diagnostic test	Comprehensive genomic profiling test	Comprehensive genomic profiling test
Pharmaceutical approval Insurance coverage	In vitro diagnostic product (Companion diagnostic)	Not approved in Japan	Not approved in Japan
Number of target genes	2 genes (*KRAS*, *NRAS*)	74 genes	70 genes
Blood collection tube used	Streck cell–free DNA BCT (Recommended)	Streck cell–free DNA BCT (Specified)	Streck cell–free DNA BCT (Specified)
Storage requirements from blood collection to plasma separation	Storage temperature: 15–25°C Storage time: Up to 3 days after collection	Storage temperature: 15–30°C Storage time: Up to 7 days after collection	Store at 18–25°C and, as a rule, submit on the day of collection
Location of plasma separation	Medical institution or domestic laboratory	Specific foreign laboratory (Redwood City, CA)	Specific foreign laboratory (Cambridge, MA)
Storage of separated plasma	Stable for 6 months if stored at -30～-15°C or -70℃ or below	-	-
Storage of extracted cfDNA	Stable for 24 hours at 2–8°C, 30 days at -30～-15°C	-	-

【References】

1. Fadhil W, Ibrahem S, Seth R, et al: The utility of diagnostic biopsy specimens for predictive molecular testing in colorectal cancer. Histopathology 61:1117-24, 2012

2. The Japanese Society of Pathology Guidelines on the handling of pathological tissue samples for genomic medicine (Japanese). http://pathology.or.jp/genome_med/pdf/textbook.pdf.

3. http://www.info.pmda.go.jp/downfiles/ivd/PDF/201680_23000EZX00047000_A_01_01.pdf.

4. Berg KD, Glaser CL, Thompson RE, et al: Detection of microsatellite instability by fluorescence multiplex polymerase chain reaction. J Mol Diagn 2:20-8, 2000

5. 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 15:63-9, 2006

6. 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 27:168-74, 2014

7. 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 137:1545-9, 2013

8. Boyle TA, Bridge JA, Sabatini LM, et al: Summary of microsatellite instability test results from laboratories participating in proficiency surveys: proficiency survey results from 2005 to 2012. Arch Pathol Lab Med 138:363-70, 2014

9. Chen G, Yang Z, Eshleman JR, et al: Molecular Diagnostics for Precision Medicine in Colorectal Cancer: Current Status and Future Perspective. Biomed Res Int 2016:9850690, 2016

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. J Clin Oncol 35:1453-1486, 2017

11. 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 16:1239-49, 2011

12. Knijn N, Mekenkamp LJ, Klomp M, et al: KRAS mutation analysis: a comparison between primary tumours and matched liver metastases in 305 colorectal cancer patients. Br J Cancer 104:1020-6, 2011

13. Koi M, Garcia M, Choi C, et al: Microsatellite Alterations With Allelic Loss at 9p24.2 Signify Less-Aggressive Colorectal Cancer Metastasis. Gastroenterology 150:944-55, 2016

14. 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 37:785-792, 2017

15. Vakiani E, Janakiraman M, Shen R, et al: Comparative genomic analysis of primary versus metastatic colorectal carcinomas. J Clin Oncol 30:2956-62, 2012

16. Italiano A, Hostein I, Soubeyran I, et al: KRAS and BRAF mutational status in primary

colorectal tumors and related metastatic sites: biological and clinical implications. Ann Surg Oncol 17:1429-34, 2010

17. Baldus SE, Schaefer KL, Engers R, et al: Prevalence and heterogeneity of KRAS, BRAF, and PIK3CA mutations in primary colorectal adenocarcinomas and their corresponding metastases. Clin Cancer Res 16:790-9, 2010

18. Cejas P, Lopez-Gomez M, Aguayo C, et al: KRAS mutations in primary colorectal cancer tumors and related metastases: a potential role in prediction of lung metastasis. PLoS One 4:e8199, 2009

19. Molinari F, Martin V, Saletti P, et al: Differing deregulation of EGFR and downstream proteins in primary colorectal cancer and related metastatic sites may be clinically relevant. Br J Cancer 100:1087-94, 2009

20. Loupakis F, Pollina L, Stasi I, et al: PTEN expression and KRAS mutations on primary tumors and metastases in the prediction of benefit from cetuximab plus irinotecan for patients with metastatic colorectal cancer. J Clin Oncol 27:2622-9, 2009

21. Garm Spindler KL, Pallisgaard N, Rasmussen AA, et al: The importance of KRAS mutations and EGF61A>G polymorphism to the effect of cetuximab and irinotecan in metastatic colorectal cancer. Ann Oncol 20:879-84, 2009

22. Santini D, Loupakis F, Vincenzi B, et al: High concordance of KRAS status between primary colorectal tumors and related metastatic sites: implications for clinical practice. Oncologist 13:1270-5, 2008

23. Artale S, Sartore-Bianchi A, Veronese SM, et al: Mutations of KRAS and BRAF in primary and matched metastatic sites of colorectal cancer. J Clin Oncol 26:4217-9, 2008

24. Etienne-Grimaldi MC, Formento JL, Francoual M, et al: K-Ras mutations and treatment outcome in colorectal cancer patients receiving exclusive fluoropyrimidine therapy. Clin Cancer Res 14:4830-5, 2008

25. Perrone F, Lampis A, Orsenigo M, et al: PI3KCA/PTEN deregulation contributes to impaired responses to cetuximab in metastatic colorectal cancer patients. Ann Oncol 20:84-90, 2009

26. Albanese I, Scibetta AG, Migliavacca M, et al: Heterogeneity within and between primary colorectal carcinomas and matched metastases as revealed by analysis of Ki-ras and p53 mutations. Biochem Biophys Res Commun 325:784-91, 2004

27. Zauber P, Sabbath-Solitare M, Marotta SP, et al: Molecular changes in the Ki-ras and APC genes in primary colorectal carcinoma and synchronous metastases compared with the findings in accompanying adenomas. Mol Pathol 56:137-40, 2003

28. Thebo JS, Senagore AJ, Reinhold DS, et al: Molecular staging of colorectal cancer: K-ras mutation analysis of lymph nodes upstages Dukes B patients. Dis Colon Rectum 43:155-9; discussion 159-62, 2000

29. Schimanski CC, Linnemann U, Berger MR: Sensitive detection of K-ras mutations augments diagnosis of colorectal cancer metastases in the liver. Cancer Res 59:5169-75, 1999

30. Al-Mulla F, Going JJ, Sowden ET, et al: Heterogeneity of mutant versus wild-type Ki-ras in primary and metastatic colorectal carcinomas, and association of codon-12 valine with early mortality. J Pathol 185:130-8, 1998

31. Finkelstein SD, Sayegh R, Christensen S, et al: Genotypic classification of colorectal adenocarcinoma. Biologic behavior correlates with K-ras-2 mutation type. Cancer 71:3827-38, 1993

32. Losi L, Benhattar J, Costa J: Stability of K-ras mutations throughout the natural history of human colorectal cancer. Eur J Cancer 28A:1115-20, 1992

33. Suchy B, Zietz C, Rabes HM: K-ras point mutations in human colorectal carcinomas: relation to aneuploidy and metastasis. Int J Cancer 52:30-3, 1992

34. Oudejans JJ, Slebos RJ, Zoetmulder FA, et al: Differential activation of ras genes by point mutation in human colon cancer with metastases to either lung or liver. Int J Cancer 49:875-9, 1991

35. Werner M, Chott A, Fabiano A, et al: Effect of formalin tissue fixation and processing on immunohistochemistry. Am J Surg Pathol 24:1016-9, 2000

36. Matsunaga T, Hana R: Byori-to-Rinsho 32:45-53, 2014 (Japanese)

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

38. Garcia-Foncillas J, Tabernero J, Elez 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 119:1464-1470, 2018

39. 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 29:Suppl 5: v101-v102, 2018

40. 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 24:3539-3549, 2018

41. 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 10:e0140712, 2015

42. 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 20:686-702, 2018

10 Quality assurance requirements for testing

Basic requirement

Genomic testing for CRC treatment should be carried out under a quality assurance system.

Strength of recommendation

Strong recommendation［SR: 10 members］

Laboratory requirements for quality assurance testing

Based on legal provisions effective December 1, 2018 for the revision of Medical Care Act, quality assurance of genomic and chromosomal testing in medical institutions, clinical laboratories, etc. was controlled under the law. Accordingly, ministerial ordinances were revised based on a summary of the panel on quality control in laboratory testing[1], which results in prescriptions for #1 allocation of a responsible person for accuracy control of genomic and chromosomal testing, #2 internal accuracy control, implementation of adequate training, and #3 external investigation of accuracy control. Quality assurance of laboratory tests and personnel should be performed in conformity with the standards required in the “Technical Requirements” of Part 5 of ISO 15189:JAB RM300:2019[2] and “OECD Guidelines for Quality Assurance in Molecular Genetic Testing, Japanese edition, Commentary Version[3].” In addition, at the present time, it was recommended that test facilities obtain third-party certification.

Key points in the revised ministerial ordinance on “standards to be established for the quality assurance of genomic and chromosomal testing” are as follows:

1. The allocation of genomic and chromosomal testing to a responsible person of accuracy control is mandated, in addition to mandating responsible persons involved for the general quality control of all laboratory testing. As a rule, this responsible person should be a physician or clinical laboratory technician who has considerable experience in examination services; however, taking expertise and experience into account, persons of other occupations shall not be impeded from serving as responsible parties.
2. Implementation of internal investigation of accuracy control (control of test accuracy, reproducibility, etc.) within a facility, and preparation of statistical accuracy control ledger, standard operation manuals, operation diaries, etc. were also mandated. Implementation of adequate training was further mandated to ensure quality of the laboratory personnel.
3. Receiving external investigations of accuracy control. If there is no system for external investigation of accuracy control, it is mandatory to attempt alternative methods; for example, individual medical

institutions, clinical laboratories, etc. should be cooperated among themselves and mutually confirm laboratory test accuracy using samples in their archives/possession.

4. In addition, testing facilities are encouraged to ensure reliability of test accuracy by obtaining and maintaining third-party certification, such as through the international standard of ISO 15189 or the Laboratory Accreditation Program (LAP) of the College of American Pathologists (CAP)[4].

【References】

1. A summary in the panel for quality assurance of laboratory testing, https://www.mhlw.go.jp/file/05-Shingikai-12601000-Seisakutoukatsukan-Sanjikanshitsu_Shakaihoshoutantou/0000210421.pdf
2. Japanese Accreditation Board. Guideline for accreditation -Medical Laboratories-JAB RM300:2019 https://www.jab.or.jp/files/items/common/File/RM3002019V5.pdf
3. OECD Guidelines for Quality Assurance in Molecular Genetic Testing, Japanese edition, Commentary Version, JCCLS.2016
4. https://www.cap.org

11 Notes

11.1 Definitions of “guidelines,” “guidance,” etc. used by the Japanese Society of Medical Oncology

1. Guidelines

Used when the targeted disease or clinical practice is broad in scope, accumulated evidence is abundant, development requires a wide range of professionals, or a wide range of professionals are targeted.

2. Guidance

Used when the targeted disease or clinical practice is narrow in scope, evidence is limited, or authors or audience are limited in range.

3. Consensus report

Overall opinions or practice policies determined through voting by a group of experts.

4. Expert opinion

Opinions or practice policies of an expert or group of experts.

5. Provisional opinion (Provisional statement)

A temporary and provisional opinion or statement. Used when a current tentative view, albeit unconfirmed, should be stated by necessity.

11.2 Pharmaceutical approval and health insurance coverage status as of September 2019

1. *RAS* mutation testing

- *RAS* mutation testing is covered by insurance as “D004-2: Histological examination for malignant tumor, RAS gene testing (2500 points)” to assist in selecting treatment for patients with unresectable advanced or recurrent colorectal cancer.
- The OncoBEAM™ RAS CRC kit was approved as a blood-based *RAS* mutation test in July 2019, but is currently not covered by health insurance (as of September 2019).
- If both *RAS* and *BRAF* mutation tests are performed at the same time, they are covered together by insurance under the category “D004-2: Histological examination for malignant tumor, 2 items (4000 points).”

2. *BRAF* V600E mutation testing

- *BRAF* mutation testing is covered by insurance under the category “D004-2: Histological examination for malignant tumor, BRAF gene testing (2100 points)” to assist in selecting treatment for patients with unresectable advanced or recurrent colorectal cancer and to aid diagnosing Lynch syndrome in patients with colorectal cancer.

3. Testing for mismatch-repair deficiency

- Microsatellite instability testing to test for mismatch-repair deficiency is covered by insurance under the category “D004-2: Histological examination for malignant tumor, Microsatellite instability testing (2100 points)” for “hereditary non-polyposis colorectal cancer or locally advanced or metastatic solid cancers in which standard treatments are difficult.”
- Next-generation sequencing (NGS)-based tests for mismatch-repair deficiency are not approved or covered by insurance. However, if using the FoundationOne® CDx Cancer Genomic Profile as a comprehensive genomic profiling test, results for microsatellite instability can be obtained, and

the Japanese Ministry of Health, Labour and Welfare's statement "Considerations Related to Health Insurance Coverage for Genetic Panel Testing" expresses that it is acceptable to administer a given drug without performing a companion diagnostic test, if deemed appropriate by an expert panel held after CGP testing.

- IHC as a test for mismatch-repair deficiency is not approved or covered by health insurance. However, IHC fee can be calculated as "N002 8: Preparation of immunostained histopathological sample (antibody-based methods), Other (per organ) 400 points."

4. Comprehensive genomic profiling tests

- The OncoGuide™ NCC Oncopanel System and the FoundationOne® CDx Cancer Genomic Profile were both approved in Dec 2018 for comprehensive genomic profile testing and received health insurance coverage in June 2019. The fee for performing the panel test can be calculated at the time of test submission as "D006-4: Genetic testing (3) Test requiring highly complex processing, 8000 points." At the time of result evaluation by an expert panel and explanation to the patient, the panel test evaluation and patient explanation fees can be calculated only once per patient at a total of 48000 points, which represents the sum of "D006-4: Genetic testing (3) Test requiring highly complex processing, 4 times, 32,000 points," "D004-2: Histological examination for malignant tumor, 3 or more items, 6000 points," and "M001-4: Particle beam therapy (per series), Particle beam therapy medical management supplement, 10,000 points."
- If the FoundationOne® CDx Cancer Genomic Profile is used as a companion diagnostic for cetuximab or panitumumab in colorectal cancer, it can be counted as "D004-2: Histological examination for malignant tumor, K-ras gene test, 2,100 points;" however, the 8000 point fee for performing the panel test cannot be calculated. If the results are later used as a comprehensive genomic profiling test, the panel test evaluation and patient explanation fee can be counted as 48,000 points.
- The FoundationOne® CDx Cancer Genomic Profile was approved as a companion diagnostic test for entrectinib in June 2019, and it became covered by health insurance in September 2019. Further, it can be inferred that results from the insurance-covered CGP tests other than the FoundationOne® CDx Cancer Genomic Profile can also be used to determine whether to administer entrectinib, as it is stated in the Japanese Ministry of Health, Labour and Welfare's statement "Considerations Related to Insurance Coverage for Genetic Panel Testing" that it is acceptable to administer a given drug without performing a companion diagnostic test, if deemed appropriate by an expert panel held after CGP testing.

5. Liquid biopsy（ctDNA testing）

- ctDNA tests other than the OncoBEAM™ RAS CRC kit are not approved or covered by insurance to select treatments for unresectable advanced or recurrent colorectal cancer, nor for resectable colorectal cancer.
- ctDNA-based comprehensive genomic profiling tests are not approved or covered by insurance to select treatment for unresectable advanced or recurrent colorectal cancer.

11.3 Participation in voting

Guidance was developed based on a discussion among all committee members. Committee members actively involved in clinical evaluation studies of test reagents that are currently underway or being planned were excluded from participation in voting for the “Basic Requirements” in the relevant section. Thus, Dr. Hiroya Taniguchi did not participate in voting on the recommendations included in the “Basic Requirements” of the section titled “7. Angiogenic Factors.”