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Report

Japanese Society of Medical Oncology Clinical Guidelines: *RAS* (*KRAS/NRAS*) mutation testing in colorectal cancer patients[11]

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[11]This report is an English translation of "Japanese Society of Medical Oncology Clinical Guidelines: *RAS* (*KRAS/NRAS*) mutation testing in colorectal cancer patients". The original version written in Japanese is available at the Japanese Society of Medical Oncology website.[1]

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The Japanese guidelines for the testing of *KRAS* mutations in colorectal cancer have been used for the past 5 years. However, new findings of *RAS* (*KRAS/NRAS*) mutations that can further predict the therapeutic effects of anti-epidermal growth factor receptor (EGFR) antibody therapy necessitated a revision of the guidelines. The revised guidelines included the following five basic requirements for *RAS* mutation testing to highlight a patient group in which anti-EGFR antibody therapy may be ineffective: First, anti-EGFR antibody therapy may not offer survival benefit and/or tumor shrinkage to patients with expanded *RAS* mutations. Thus, current methods to detect *KRAS* exon 2 (codons 12 and 13) mutations are insufficient for selecting appropriate candidates for this therapy. Additional testing of extended *KRAS/NRAS* mutations is recommended. Second, repeated tests are not required for the detection; tissue materials of either primary or metastatic lesions are applicable for *RAS* mutation testing. Evaluating *RAS* mutations prior to anti-EGFR antibody therapy is recommended. Third, direct sequencing with manual dissection or allele-specific PCR-based methods is currently applicable for *RAS* mutation testing. Fourth, thinly sliced sections of formalin-fixed, paraffin-embedded tissue blocks are applicable for *RAS* mutation testing. One section stained with H&E should be provided to histologically determine whether the tissue contains sufficient amount of tumor cells for testing. Finally, *RAS* mutation testing must be performed in laboratories with appropriate testing procedures and specimen management practices.

Cetuximab and panitumumab are monoclonal antibodies targeting the epidermal growth factor receptor (EGFR) and have demonstrated survival benefits in randomized control trials (RCT) of metastatic colorectal cancer (mCRC). Since 2008, retrospective analyses of previous RCT have shown that cetuximab and panitumumab are contraindicated in patients with *KRAS* exon 2 (codons 12 and 13) mutations. Moreover, patients with *KRAS* mutations exhibited detrimental effects on receiving oxaliplatin, folic acid, and infusional 5-FU (FOLFOX4) plus cetuximab or panitumumab compared with FOLFOX4 alone. Since the Japanese Society of Medical Oncology (JSMO) published "Japanese guidelines for testing of *KRAS* gene mutation in colorectal cancer" in 2008, testing for *KRAS* mutation prior to anti-EGFR antibody therapy has been widely accepted in clinical practice and three types of quality-assured diagnostic kits have been approved in Japan (Table 1).

A therapeutic strategy for mCRC has been continuously improved since the publication of the Japanese guidelines. In

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Table 1. Summary of the commonly used assays in Japan for KRAS testing of colorectal cancer

Assay	Sanger sequencing	PCR-rSSO	Scorpion-ARMS	F-PHFA
Commercial diagnostic kit	—	MEBGEN KRAS	TheraScreen: K-RAS Mutation Kit DxS-QIAGEN, Manchester, UK	OncoGuide KRAS
Limit of detection (%)	10–25	5	1–5	5–10
Detectable types of mutations	All types of mutations	G12S, G12C, G12R G12D, G12V, G12A G13S, G13C, G13R G13D, G13V, G13A	G12S, G12C, G12R G12D, G12V, G12A G13D	G12S, G12C, G12R G12D, G12V, G12A G13D

addition, patients with *KRAS* or *NRAS* mutations except those with *KRAS* exon 2 mutations are reported to be primarily resistant to anti-EGFR antibody therapies.[2,3] Because these patients account for approximately 20% of *KRAS* exon 2 wild-type patients, "minor" *RAS* mutations are not negligible in daily clinical practice.

The Japanese Society of Medical Oncology established a working group to revise *KRAS* guidelines in December 2013, and published a revised version of the guidelines in April 2014 after independent review and public comments. Here, we summarize the new clinical guidelines. Additional references related to each section are listed as supplemental information.

Basic Requirements for Testing *RAS* Mutations

Anti-epidermal growth factor receptor antibody therapy may be ineffective in terms of survival benefit and/or tumor shrinkage in patients with expanded *RAS (KRAS/NRAS)* mutations. Randomized control trials (RCT) of chemotherapy with or without anti-EGFR antibody in mCRC revealed that anti-EGFR antibody had no benefit on the response rate, progression-free survival and overall survival in patients with *KRAS* exon 2 (codons 12 and 13) mutations.[4] This finding is consistent with other anti-EGFR therapies, including cetuximab or panitumumab, therapeutic lines and combined chemotherapies. Although increased survival with cetuximab of the patients with *KRAS* codon 13 (G13D) mutation was reported,[5] patients with any *KRAS* exon 2 mutations are unlikely to benefit from cetuximab or panitumumab.[6] Therefore, anti-EGFR antibody therapy is not recommended for patients with *KRAS* exon 2 mutations.

Since 2013, prospective-retrospective analyses of phase III studies have revealed that patients with wild-type *RAS* were expected to benefit from panitumumab, although benefits were not obtained in patients with mutations including *KRAS* exons 3 and 4, and *NRAS* exons 2, 3 and 4, similar to patients with *KRAS* exon 2 mutations (Tables 2 and 3).[2]

Retrospective analyses of RCT suggested that cetuximab also has a favorable survival impact only in patients with wild-type *RAS*. Furthermore, two RCT that compared anti-EGFR antibody therapy to bevacizumab revealed that a subgroup of patients with *RAS* mutations, except those with *KRAS* exon 2 mutations, did not show benefits.[3] Based on these results, anti-EGFR antibody therapy is ineffective in patients with previously known *KRAS* exon 2 mutations or those with mutations in *KRAS* exons 3 and 4 and *NRAS* exons 2, 3 and 4. *In vitro* studies revealed that the overexpression of *KRAS* transgenes with mutations in codons 12, 13, 59, 61, 117 and 146 induced constitutive RAS protein activation; however, the impact of individual mutations on the therapeutic efficacy remains unclear. While several patients with *KRAS* codon 146 mutation respond to anti-EGFR antibody therapy,[7] we assume that further subgroup analyses of RCT may provide information to conclude these issues. Thus, current procedures to detect only *KRAS* exon 2 mutations are insufficient for selecting appropriate patients. Additional testing of expanded *KRAS/NRAS* mutations is recommended.

Clinicians should properly interpret the immeasurable or unmeasured mutation status. When one or some exons/codons have undetermined mutational statuses while all the other evaluable exons are determined as *RAS* wild-type, these patients should be diagnosed as *RAS* unknown (Table S1). Potential causes of the failures are sample and/or technical issues of testing. If the test failure is due to the sample, re-examination using the remnant or newly obtained tumor samples should be considered. If the test failure is due to technical

Table 2. Therapeutic effects on wild type *RAS*

	RAS ascertainment†	Regimen	*n*	RR (%)	PFS (M)	HR	OS (M)	HR
PRIME	90% (1060/1183)	FOLFOX4	253	—	7.9	HR 0.72 ($P = 0.004$)	20.2	HR 0.78 ($P = 0.04$)
		FOLFOX4 + Pmab	259	—	10.1		26.0	
20050181	85% (1008/1186)	FOLFIRI	211	10	4.4	HR 0.695 ($P = 0.006$)	13.9	HR 0.803 ($P = 0.08$)
		FOLFIRI + Pmab	204	41	6.4		16.2	
20020408	82% (378/463)	BSC	63	0	7 weeks	HR 0.36 ($P < 0.0001$)	—	—
		BSC + Pmab	73	16	14.1 weeks		—	
OPUS	75% (254/337)	FOLFOX4	49	28.6	5.8	HR 0.53 ($P = 0.0615$)	17.8	HR 0.94 ($P = 0.80$)
		FOLFOX4 + Cmab	38	57.9	12.0		19.8	
CRYSTAL	69% (827/1198)	FOLFIRI	189	38.6	8.4	HR 0.56 ($P = 0.0002$)	20.2	HR 0.69 ($P = 0.0024$)
		FOLFIRI + Cmab	178	66.3	11.4		28.4	
PEAK	82% (233/285)	mFOLFOX6 + Bev	82	54	10.1	HR 0.66 ($P = 0.03$)	28.9	HR 0.63 ($P = 0.06$)
		mFOLFOX6 + Pmab	88	58	13.0		41.3	
FIRE-3	69% (520/752)	FOLFIRI + Bev	171	59.6	10.2	HR 0.93 ($P = 0.54$)	25.6	HR 0.70 ($P = 0.011$)
		FOLFIRI + Cmab	171	65.5	10.4		33.1	

†*RAS* ascertainment: ratio of randomized patients whom *RAS* mutations were evaluated. Bev, bevacizumab; Cmab, cetuximab; HR, hazard ratio; OS, overall survival; PFS, progression free survival; Pmab, panitumumab; RR, response rate.

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Table 3. Therapeutic effects on mutant *RAS*

	Regimen	*n*	RR (%)	PFS (M)	HR	OS (M)	HR
PRIME	FOLFOX4	276	—	8.7	HR 1.31 (P = 0.008)	19.2	HR 1.25 (P = 0.034)
	FOLFOX4 + Pmab	272	—	7.3		15.6	
20050181	FOLFIRI	294	13	4.0	HR 0.861 (P = 0.14)	11.1	HR 0.914 (P = 0.34)
	FOLFIRI + Pmab	299	15	4.8		11.8	
20020408	BSC	114	0	7.3 weeks	HR 0.97 (P = 0.729)	—	—
	BSC+Pmab	99	1	7.4 weeks		—	
OPUS	FOLFOX4	75	50.7	7.8	HR 1.54 (P = 0.0309)	17.8	HR 1.29 (P = 0.1573)
	FOLFOX4+Cmab	92	37.0	5.6		13.5	
CRYSTAL	FOLFIRI	214	36.0	7.5	HR 1.10 (P = 0.47)	17.7	HR 1.05 (P = 0.64)
	FOLFIRI+Cmab	246	31.7	7.4		16.4	
FIRE-3	FOLFIRI+Bev	86	51.2	10.1	HR 1.31 (P = 0.085)	20.6	HR 1.09 (P = 0.60)
	FOLFIRI+Cmab	171	65.5	10.4		33.1	

Bev, bevacizumab; Cmab, cetuximab; HR, hazard ratio; OS, overall survival; PFS, progression free survival; Pmab, panitumumab; RR, response rate.

problems, re-examination should be performed with other methods. Indications for anti-EGFR antibody therapy for patients with *RAS* unknown status should be determined according to: (i) the reported frequency of mutations of immeasurable or unmeasured codons; (ii) evidence of no expected effects on patients if they have *RAS* mutations in immeasurable or unmeasured codons; (iii) side effects of anti-EGFR antibody therapy; and (iv) alternative therapeutic options except anti-EGFR antibody therapy.

Repeated tests are not required for the detection; tissue materials of either primary or metastatic lesions are applicable for *RAS* mutation testing. Evaluating *RAS* mutations prior to anti-epidermal growth factor receptor antibody therapy is recommended. *RAS* mutation is an early event in the tumorigenesis, and the frequency of *RAS* mutations might not be altered in any clinical stage (Table S2). The frequency of *KRAS* exon 2 mutations is approximately 35–40% in colorectal cancer patients, and the frequency of other *RAS* mutations is 10–15%; the same trend exists in Europe and the USA, and Japan (Table 4).[8]

The concordance rate of the mutation status between primary tumors and metastatic sites reached 93% by meta-analysis.[9] *RAS* mutational status of tumor tissue from endoscopic biopsies and matched resected specimens is highly concordant and the concordant rate is ≥97%. The mutational status of *RAS* was not altered by chemotherapy without cetuximab or panitumumab, whereas chemotherapies including cetuximab or panitumumab reportedly induced secondary *RAS* mutation and amplification. The clinical implications of the secondary *RAS* mutation, including the potential efficacy of anti-EGFR antibody therapy, remain unknown. Based on these findings, repeated testing of *RAS* mutations is currently not recommended.

Direct sequencing with manual dissection or allele-specific PCR-based methods is currently applicable for *RAS* mutation testing. Direct sequencing is able to detect both known and unknown gene mutations, whereas the detection sensitivity of the assay is limited to 10–25%, which is less sensitive than that of allele-specific PCR-based methods. Therefore, direct sequencing requires the condensation of tumor cells by manual dissection of the tissue sections in which tumor cells are densely contained (manual microdissection).[10] A multiplex mutation detecting kit using Luminex technology (Mebgen Rasket Kit; Medical and Biological Laboratories, Nagoya, Japan) has been approved for the simultaneous detection of 48 types of *RAS* mutations.[8]

In previous clinical studies, *RAS* testing was performed using various assays (Table 4). The detection limit of these methods was within 10–25% (direct sequencing) to <1% (BEAMing method) and that of the other methods was within 1–10%. Regardless of the difference in the detection limit between each method, the subgroup analyses of these RCT consistently demonstrated that *RAS* status is a predictive factor for anti-EGFR antibody therapy. Therefore, while the most suitable detection sensitivity remains to be determined, the detection limit within 1–10% should be practically considered for *RAS* mutation testing.

Thinly sliced sections of formalin-fixed, paraffin-embedded tissue blocks are applicable for *RAS* mutation testing. One section should be stained with H&E and provided for histological

Table 4. Frequencies of exon mutations

	KRAS exon 2 (%)	*KRAS* exon 3 (%)	*KRAS* exon 4 (%)	*NRAS* exon 2 (%)	*NRAS* exon 3 (%)	*NRAS* exon 4 (%)	Total†	Method
PRIME	40 (440/1096)	4 (24/638)	6 (36/620)	3 (22/637)	4 (26/636)	0 (0/629)	17	Sanger SURVEYOR
20050181	45 (486/1083)	4.4 (24/548)	7.7 (41/534)	2.2 (12/536)	5.6 (30/540)	0 (0/532)	20	Sanger SURVEYOR
20020408	43 (184/427)	4.8 (8/166)	5.0 (9/180)	4.2 (7/166)	3.0 (5/168)	1.1 (2/180)	18	Sanger‡ SURVEYOR
OPUS	43 (136/315)	6.8	9.3	6.8	5.1	0.8	26	BEAMing
CRYSTAL	37 (136/315)	3.3	5.6	3.5	2.8	0.9	15	BEAMing
PEAK	N/A	4 (9/225)	7 (17/223)	5 (12/224)	6 (13/225)	0 (0/223)	22	Sanger SURVEYOR
FIRE-3	N/A	4.3 (21/431)	4.9 (24/458)	3.8 (18/464)	2 (10/468)	0 (0/458)	16	Pyrosequencing

†*KRAS/NRAS* mutation ratio in wild type *KRAS* exon 2. ‡Next generation sequencers were used to confirm some of codon mutations.

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examination to confirm whether tissue contains a sufficient amount of tumor cells for testing. The paraffin-embedded (FFPE) tissue sample is widely used as a sample for *RAS* mutation testing. If sufficient tumor cells are confirmed histologically, the use of fresh frozen tissue samples will also be considered.

It is recommended to select tissue sections containing ≥50% tumor cells estimated by the area of tumor cells. When performing *RAS* mutation testing using sections with fewer tumor cells coupled with low sensitivity methods, manual microdissection should be performed to increase tumor cell/non-tumor cell ratio. Samples with apoptosis and necrosis are unsuitable due to the degradation of genomic DNA. If multiple samples are obtained from the same patient, select the sample that was archived for a shorter period, has a higher tumor cell ratio, and has fewer effects of prior chemotherapy or radiotherapy. These parameters should be discussed with the pathologists and laboratory staff prior to *RAS* mutation testing.

Formalin fixation leads to DNA fragmentation in FFPE tissue block samples. Thus, sample fixation (e.g. formaldehyde concentration, buffered or non-buffered formalin, duration of fixation, tissue size and sample segmentation) should be carefully considered. Using a 10% buffered formaldehyde solution is recommended. The duration of fixation is dependent on the sample size. In general, 6–48 h of fixation is recommended.

RAS mutation testing must be performed in laboratories well-qualified to perform both the testing procedures and specimen management. The clinical laboratories should verify the quality of testing procedures. Clinical laboratories are recommended to obtain a certificate of International Standard (e.g. ISO/IEC 17025, ISO 15189) from the International Organization for Standardization (ISO). The laboratories should undergo regular evaluations by authorized inspectors to maintain laboratory quality. Quality assurance (QA) should adhere to both international OECD and Japanese guidelines.

Testing must be performed according to standard operation procedures. The items suggested in the European QA program (Table S3) are used for the validation of testing procedures. Finally, the items shown in Table S4 should be included in the report of *RAS* mutation testing.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Data S1. Supplemental references.

Table S1. Defined RAS status with unevaluable exons.

Table S2. Ratio of KRAS exon 2 mutation in each stage.

Table S3. Validation of testing procedures.

Table S4. The report of RAS mutation testing.

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