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KRAS mutations in primary tumours and post-FOLFOX metastatic lesions in cases of colorectal cancer

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BACKGROUND: *KRAS* mutations are predictive markers for the efficacy of anti-EGFR antibody therapies in patients with metastatic colorectal cancer. Although the mutational status of *KRAS* is reportedly highly concordant between primary and metastatic lesions, it is not yet clear whether genotoxic chemotherapies might induce additional mutations.

METHODS: A total of 63 lesions (23 baseline primary, 18 metastatic and 24 post-treatment metastatic) from 21 patients who were treated with FOLFOX as adjuvant therapy for stage III/IV colorectal cancer following curative resection were examined. The DNA samples were obtained from formalin-fixed paraffin-embedded specimens, and *KRAS, NRAS, BRAF* and *PIK3CA* mutations were evaluated.

RESULTS: The numbers of primary lesions with wild-type and mutant *KRAS* codons 12 and 13 were 8 and 13, respectively. The mutational status of *KRAS* remained concordant between the primary tumours and the post-FOLFOX metastatic lesions, irrespective of patient background, treatment duration and disease-free survival. Furthermore, the mutational statuses of the other genes evaluated were also concordant between the primary and metastatic lesions.

CONCLUSION: Because the mutational statuses of predictive biomarker genes were not altered by FOLFOX therapy, specimens from both primary tumours and post-FOLFOX tumour metastases might serve as valid sources of DNA for known genomic biomarker testing.

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KRAS mutations are predictive markers for the poor efficacy of anti-EGFR antibody therapies in patients with metastatic colorectal cancer (Lievre *et al*, 2006; Benvenuti *et al*, 2007; Di Fiore *et al*, 2007; Frattini *et al*, 2007; Khambata-Ford *et al*, 2007; Amado *et al*, 2008; De Roock *et al*, 2008; Freeman *et al*, 2008; Karapetis *et al*, 2008; Lievre *et al*, 2008). Point mutations in the *KRAS* gene occur early in the progression from colorectal adenoma to carcinoma and are detected in 35–40% of patients, regardless of their Dukes stage (Andreyev *et al*, 1998). More than 90% of the *KRAS* mutations in these patients have been detected in codons 12 (GGT) and 13 (GGC) (Oliveira *et al*, 2004). Activating mutations at codons 61 and 146 have also been reported in a small number of these tumours. In addition, mutations in the molecules involved in signalling pathways downstream of EGFR, such as *NRAS*, *BRAF* and *PIK3CA*, have also been reported in colorectal cancers. These mutations have been suggested to modify the efficacy of anti-EGFR antibody therapies, although their predictive value has not yet been established (De Roock *et al*, 2010).

Oxaliplatin [*trans*-R,R-1,2-diaminocyclohexaneoxalatoplatinum (II), L-OHP] is a third-generation platinum (Pt)-containing anti-tumour compound. It is frequently administered as a component of FOLFOX therapy in combination with 5-FU for patients with metastatic colorectal cancer. Oxaliplatin induces DNA damage associated with intra- and inter-strand cross-links (Pt-GG adducts) and can induce gene mutations (Woynarowski *et al*, 2000; Hah *et al*, 2007; Sharma *et al*, 2007). The mutagenic activity of oxaliplatin has been demonstrated in cultured cells (Silva *et al*, 2005).

The *KRAS* mutation status of primary and metastatic lesions is reportedly highly concordant (Oudejans *et al*, 1991; Losi *et al*, 1992; Suchy *et al*, 1992; Zauber *et al*, 2003; Weber *et al*, 2007; Etienne-Grimaldi *et al*, 2008; Santini *et al*, 2008; Garm Spindler *et al*, 2009; Loupakis *et al*, 2009; Perrone *et al*, 2009; Baldus *et al*, 2010; Italiano *et al*, 2010; Knijn *et al*, 2011). However, whether long-term treatment with genotoxic chemotherapies, such as oxaliplatin, can induce additional mutations in metachronous metastatic lesions has not yet been well examined.

Assuming that FOLFOX therapy has the potential to alter the biomarker mutation profile, it is important to determine whether

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the primary or relapsed tumour represents the more appropriate source of DNA for testing. We examined the mutation status of *KRAS* and other biomarker genes in primary and synchronous/metachronous metastatic lesions in patients with stage III/IV colorectal cancer treated with adjuvant FOLFOX therapy following curative resection.

PATIENTS AND METHODS

Patient selection

A total of 63 lesions from 21 patients who had received adjuvant FOLFOX therapy for stage III/IV colorectal cancer following curative resection at the National Cancer Center Hospital East, Japan, between January 2006 and December 2009 were examined.

All patients were treated with a modified FOLFOX6 regimen, with a reduced oxaliplatin dose of 85 mg m^{-2} administered every 14 days, and 12 cycles were planned as the full therapy course (Andre *et al*, 2004; Allegra *et al*, 2009). FOLFOX therapy was discontinued when tumour relapse was demonstrated by imaging or when intolerable adverse events occurred.

DNA samples and mutational analyses

The DNA samples were obtained from macroscopically dissected formalin-fixed paraffin-embedded specimens cut into 10-μm-thick sections. Genomic DNA was extracted using the EZ1 Advanced XL and EZ1 DNA Tissue Kits (Qiagen, Hilden, Germany) according to the manufacturer's instructions (Bando *et al*, 2011). Mutations in *KRAS* codons 12 and 13 were detected using the ARMS/Scorpions technology-based *KRAS* PCR Kit (Qiagen) according to the manufacturer's instructions. Mutations in *KRAS* codons 61 and 146, *NRAS* codons 12, 13 and 61, *BRAF* codon 600 and *PIK3CA* codons 542, 545, 546 and 1047 were detected using the multiplex PCR-Luminex method-based MEBGEN Mutation Kit (Medical & Biological Laboratories, Nagoya, Japan). Mutations detected with the MEBGEN Mutation Kit were confirmed by direct sequencing. Mutations in *PIK3CA* codons 542, 545 and 546 were further confirmed using the ARMS/Scorpions technology-based PI3K Mutation Test Kit (Qiagen). The study was approved by the Institutional Review Board of the National Cancer Center.

RESULTS

Patient and tumour site characteristics

We reviewed 151 consecutive cases of stage III/IV colorectal cancer treated with an adjuvant FOLFOX therapy after curative resection. Among these cases, 21 patients developed metastatic tumours that were diagnosed during or after the FOLFOX therapy and surgically resected. The patient and tumour site characteristics are shown in Table 1. The primary tumour sites were the colon and rectum in 8 and 13 patients, respectively. The most abundant primary tumour histopathological type was differentiated adenocarcinoma. Well- and moderately differentiated adenocarcinomas and mucinous adenocarcinomas were observed in 5, 14 and 2 patients, respectively. All metastatic tumours exhibited histology concordant with that of the associated primary colorectal adenocarcinoma.

In all, 12 patients had stage III disease, whereas the remaining 9 patients had synchronous metastatic lesions and were diagnosed as stage IV at the initial operation. There were 12 synchronous metastatic lesions in the patients with stage IV disease. In addition, six metastatic lesions were detected in five patients with stage III disease at operation that were resected prior to the start of FOLFOX therapy. These 18 lesions were regarded as 'pre-FOLFOX' metastatic lesions. The pre-FOLFOX metastases were found in the liver (11 lesions), lung (5 lesions), as a local recurrence (1 lesion) and as a subcutaneous recurrence (1 lesion). Meanwhile, 24 metastatic lesions in the 21 patients were detected during or after FOLFOX therapy. These lesions were regarded as 'post-FOLFOX' metastatic lesions. The post-FOLFOX metastases were found in the liver, lung, as a local recurrence and lymph node in 6, 14, 3 and 1 patients, respectively.

Table 1 Characteristics

Patient characteristics	Number
Sex (female/male)	8/13
Median age (range)	64 (36–75) years
Primary tumour site	
Colon	8
Rectum	13
Histopathological type of primary site	
Well-differentiated adenocarcinoma	5
Moderately differentiated adenocarcinoma	14
Mucinous adenocarcinoma	2
Stage before initial operation	
III	12
IV (synchronous metastases)	9
Tumour site characteristics	
Metastases	
Pre-FOLFOX	18
Synchronous	12
Metachronous	6
Post-FOLFOX	24
Sites of metastases	
Pre-FOLFOX	
Liver	11
Lung	5
Local recurrence	1
Subcutaneous	1
Post-FOLFOX	
Liver	6
Lung	14
Local recurrence	3
Lymph node	1

The median number of FOLFOX therapy cycles administered was 9 (3–12 cycles). Five patients experienced relapse during FOLFOX therapy (case 1, 2, 3, 7 and 12), whereas the remaining 16 patients experienced relapse after the end of FOLFOX therapy. The median disease-free survival, calculated from the time of the last operation until post-FOLFOX recurrence, was 409 days (97–1077). The median period from the start of FOLFOX therapy until recurrence was 373 days (35–1029). Relapses developed within 180 days after the end of FOLFOX therapy in 10 of the 21 patients (Table 2).

Mutational status of *KRAS* and other genes

The mutational statuses of *KRAS* and other genes in primary and metastatic lesions are shown in Table 3. Mutations in *KRAS* codons 12 and 13 were detected in 13 of the 21 primary colorectal tumours. Among the remaining eight tumours with wild-type *KRAS* codons 12 and 13, two tumours exhibited *KRAS* codon 146 mutations (A146V and A146T) and one tumour exhibited *NRAS* codon 61 mutation (Q61H). Two tumours exhibited mutations in *PIK3CA* codon 542 (E542K), one tumour exhibited a *KRAS* G12S mutation and one tumour had no mutations in any of the genes examined. No apparent mutations of *KRAS* codon 61, *NRAS* codon

Table 2 FOLFOX treatment, metastasis status and tumour recurrence sites

Case	Primary site	Histopathological type	Pre-FOLFOX metastatic site	Synchronous/ metachronous	FOLFOX cycles	DFS (days)	Days from end of FOFLOX until recurrence	Post-FOLFOX recurrence site
1	Rectum	Mode	—	—	3	124	6	Liver
2	Colon	Mode	Liver	Synchronous	4	97	−16[a]	Liver
3	Colon	Mode	Liver	Synchronous	4	116	26	Liver
4	Rectum	Well	Local recurrence	Metachronous	4	469	363	Local recurrence
5	Rectum	Mode	—	—	5	827	603	Lung
6	Colon	Mode	—	—	5	350	244	Lymph node
7	Rectum	Mode	Liver Lung	Synchronous Synchronous	8	214	1	Lung
8	Rectum	Muc	—	—	8	538	318	Lung
9	Colon	Well	—	—	8	1077	903	Liver
10	Colon	Mode	Liver Liver Lung	Synchronous Synchronous Synchronous	8	344	120	Lung Lung
11	Colon	Muc	Lung	Synchronous	9	721	401	Lung
12	Rectum	Well	Liver	Synchronous	9	109	−88[a]	Liver
13	Rectum	Mode	Liver Lung	Metachronous Metachronous	11	328	120	Liver
14	Rectum	Mode	Subcutaneous	Metachronous	12	519	156	Lung
15	Colon	Mode	—	—	12	388	176	Local recurrence
16	Rectum	Mode	Liver	Synchronous	12	466	210	Lung
17	Rectum	Well	Lung	Synchronous	12	556	264	Lung
18	Colon	Mode	Liver	Metachronous	12	531	231	Lung Lung
19	Rectum	Mode	Liver	Synchronous	12	409	217	Lung
20	Rectum	Mode	—	—	12	455	243	Local recurrence
21	Rectum	Well	Liver	Metachronous	12	346	71	Lung Lung

Abbreviations: DFS = disease-free survival; mode = moderately differentiated adenocarcinoma; muc = mucinous adenocarcinoma; well = well-differentiated adenocarcinoma.
[a]The cases that FOLFOX therapies were administered after recurrence.

Table 3 Mutational status of *KRAS* and other genes

Case	Primary site	Mutation status	Pre-FOLFOX metastatic site	Mutation status	Post-FOLFOX recurrence site	Mutation status
1	Rectum	KRAS G12D	—	—	Liver	KRAS G12D
2	Colon	KRAS G12D	Liver	KRAS G12D	Liver	KRAS G12D
3	Colon	KRAS G12D	Liver	KRAS G12D	Liver	KRAS G12D
4	Rectum	KRAS G12R	Local recurrence	KRAS G12R	Local recurrence	KRAS G12R
5	Rectum	KRAS G12D	—	—	Lung	KRAS G12D
6	Colon	WT	—	—	LN	WT
7	Rectum	KRAS G12S	Liver Lung	KRAS G12S KRAS G12S	Lung	KRAS G12S
8	Rectum	WT	—	—	Lung	WT
9	Colon	WT	—	—	Liver	WT
10	Colon	KRAS G12A	Liver Liver Lung	KRAS G12A KRAS G12A WT	Lung Lung	KRAS G12A KRAS G12A
11	Colon	KRAS G13D	Lung	KRAS G13D	Lung	KRAS G13D
12	Rectum	KRAS A146V	Liver	KRAS A146V	Liver	KRAS A146V
13	Rectum	KRAS G12V	Liver Lung	KRAS G12V KRAS G12V	Liver	KRAS G12V
14	Rectum	KRAS G12D	Subcutaneous	KRAS G12D	Lung	KRAS G12D
15	Colon	WT	—	—	Local recurrence	WT
16	Rectum	KRAS G12S, PIK3CA E542K	Liver	KRAS G12S, PIK3CA E542K	Lung	KRAS G12S, PIK3CA E542K
17	Rectum	KRAS G12D	Lung	KRAS G12D	Lung	KRAS G12D
18	Colon	KRAS G12D	Liver	KRAS G12D	Lung Lung	KRAS G12D KRAS G12D
19	Rectum	NRAS Q61H	Liver	NRAS Q61H	Lung	NRAS Q61H
20	Rectum	PIK3CA E542K	—	—	Local recurrence	PIK3CA E542K
21	Rectum	KRAS A146V	Liver	KRAS A146V	Lung Lung	KRAS A146V KRAS A146V

Abbreviations: LN = lymph node; WT = wild-type.

12 or 13, *BRAF* codon 600, or *PIK3CA* codon 1047 were detected in any sample in this study.

The degree of concordance of the gene mutations in primary and pre-FOLFOX metastatic lesions was examined. In case 10, a *KRAS* G12A mutation was detected in the primary lesion, whereas the metastatic lesion in the lung had wild-type *KRAS*. Although the histological features of the lung lesion were consistent with metastatic adenocarcinoma of the colon, no mutations in the metastatic lesion were detected, even after repeated high-sensitivity examinations. The remaining 17 metastatic lesions in 14 patients, including 2 liver metastatic lesions in case 10, showed the same mutational statuses as the primary tumours for all of the genes examined.

Then, the mutational statuses of the post-FOLFOX metastatic lesions were examined. The mutational statuses of all genes examined were identical in the 21 primary tumours and the corresponding 24 post-FOLFOX metastatic lesions, regardless of the sites involved, duration of FOLFOX treatment or disease-free survival period.

DISCUSSION

Previous studies have reported a high concordance rate of the *KRAS* mutations in primary and metastatic tumours (Oudejans *et al*, 1991; Losi *et al*, 1992; Suchy *et al*, 1992; Zauber *et al*, 2003; Weber *et al*, 2007; Etienne-Grimaldi *et al*, 2008; Santini *et al*, 2008; Garm Spindler *et al*, 2009; Loupakis *et al*, 2009; Perrone *et al*, 2009; Baldus *et al*, 2010; Italiano *et al*, 2010; Knijn *et al*, 2011). However, in patients receiving long-term chemotherapy, the effects of genotoxic chemotherapies, such as oxaliplatin, have not been well investigated.

In this study, we examined 21 patients with metastatic colorectal cancer who received adjuvant FOLFOX therapy. The recurrent tumours in three patients who showed relapse within 4 months after the primary surgery or during the first 3 or 4 cycles of adjuvant FOLFOX therapy (cases 1–3) were regarded as synchronous metastases arising from micrometastases that likely existed prior to the start of the adjuvant chemotherapy. The remaining 18 patients who developed relapses more than 8 months from the end of adjuvant FOLFOX therapy or after more than 6 cycles of adjuvant FOLFOX therapy were regarded as having metachronous metastatic tumours that had developed after exposure to oxaliplatin. Among these cases, tumour relapse occurred within 180 days after FOLFOX therapy in 7 patients and more than 180 days after FOLFOX therapy in the remaining 11 patients. Regardless of the treatment duration, 8 of the primary tumours with wild-type *KRAS* codons 12 and 13 did not acquire *KRAS* mutations. The remaining tumours with *KRAS* mutations also did not show additional mutations after FOLFOX therapy. Furthermore, none of the other genes that might potentially affect the efficacy of anti-EGFR antibody therapy were altered.

KRAS, *NRAS* and *BRAF* mutations are all regarded as strong driver mutations that induce cell proliferation. These mutations might be acquired in the early stages of carcinogenesis and have generally been reported as mutually exclusive (Andreyev *et al*, 1998). Consistent with this observation, the *KRAS* and *NRAS* mutations in this study were found to be mutually exclusive. In the rest of the tumours, other unidentified driver mutations or amplifications may have activated the signalling pathways promoting cell proliferation. Considering the exclusive nature of the tested mutations, the acquisition of additional driver mutations may not be advantageous to these tumour cells for clonal selection. This could be one explanation for why the mutational statuses of *KRAS* and other genes were not altered during the development of metastatic tumours.

Our findings suggest that both the primary tumours and metastatic tumours arising during or after FOLFOX therapy could be valid sources of DNA for *KRAS* testing prior to treatment with anti-EGFR antibodies, although the number of cases in this study was limited. This finding should be further confirmed in a larger number of cases. Though collecting surgically resected metastatic tumour tissues is often difficult, circulating tumour cells may be a useful alternative DNA source for highly reliable and sensitive mutation detection systems such as the ARMS/Scorpion method for further analyses.

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