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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OXaliplatin [trans-R, R-1,2-diaminocyclohexanooxalato(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; Etiennie-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 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
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 PISK 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.

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 (ES42K), 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 600 and BRAF codon 600 were detected in any of the lesions.

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   |

Table 2 Sites of metastases

| Sites of metastases | Number |
|---------------------|--------|
| Pre-FOLFOX          |        |
| Liver               | 11     |
| Lung                | 5      |
| Local recurrence    | 1      |
| Subcutaneous        | 1      |
| Post-FOLFOX         |        |
| Liver               | 6      |
| Lung                | 14     |
| Local recurrence    | 3      |
| Lymph node          | 1      |

Table 3 Mutations in KRAS codons

| KRAS Codon | Mutation |
|------------|----------|
| 12         | G12S     |
| 13         | A146V, A146T |
| 61         | Q61H     |

Conclusion

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. Our results suggest that KRAS and other biomarker genes may be useful as predictive markers for adjuvant FOLFOX therapy in patients with colorectal cancer.
### 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 FOLFOX until recurrence | Post-FOLFOX recurrence site |
|------|--------------|------------------------|----------------------------|---------------------------|---------------|-----------|------------------------------------------|-----------------------------|
| 1    | Rectum       | Mode                   | —                          | —                         | 3             | 124       | 6                                       | Liver                       |
| 2    | Colon        | Mode                   | Liver                      | Synchronous               | 4             | 97        | — 16<sup>a</sup>                       | 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                   | Liver                      | Synchronous               | 5             | 350       | 244                                     | Lymph node                  |
| 7    | Rectum       | Mode                   | Liver                      | Synchronous               | 8             | 214       | 1                                       | Lung                        |
| 8    | Rectum       | Muc                    | —                          | —                         | 8             | 538       | 318                                     | Lung                        |
| 9    | Colon        | Well                   | —                          | —                         | 8             | 1077      | 903                                     | Liver                       |
| 10   | Colon        | Mode                   | Liver                      | Synchronous               | 8             | 344       | 120                                     | Lung                        |
|      |              |                        |                            |                           |               |           |                                         |                             |
| 11   | Colon        | Muc                    | Lung                       | Synchronous               | 9             | 721       | 401                                     | Lung                        |
| 12   | Rectum       | Well                   | Liver                      | Synchronous               | 9             | 109       | — 88<sup>a</sup>                       | Liver                       |
| 13   | Rectum       | Mode                   | Liver                      | 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                        |
|      |              |                        |                            |                           |               |           |                                         |                             |
| 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                        |

<sup>a</sup>The cases that FOLFOX therapies were administered after recurrence.

Abbreviations: DFS = disease-free survival; mode = moderately differentiated adenocarcinoma; muc = mucinous adenocarcinoma; well = well-differentiated adenocarcinoma.

### 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       | Liver                      | KRAS G12D       | Lung                       | KRAS G12D       |
| 6    | Colon        | WT               | —                          | —               | LN                         | WT              |
| 7    | Rectum       | KRAS G12S       | Liver                      | KRAS G12S       | Liver                      | KRAS G12S       |
| 8    | Rectum       | WT               | —                          | —               | Lung                       | WT              |
| 9    | Colon        | WT               | —                          | —               | Liver                      | WT              |
| 10   | Colon        | KRAS G12A       | Liver                      | KRAS G12A       | Lung                       | 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                      | 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       | Lung                       | KRAS G12D       | Lung                       | 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                       | KRAS A146V      |

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