Detection of KRAS Oncogene in Peripheral Blood as a Predictor of the Response to Cetuximab Plus Chemotherapy in Patients with Metastatic Colorectal Cancer

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Abstract

Purpose: Previously we developed membrane-arrays as a promising tool to detect circulating tumor cells (CTC) with KRAS oncogene in patients with malignancies. This study was conducted to determine the predictive values of CTCs with KRAS mutation by membrane-arrays for metastatic colorectal cancer patients treated with cetuximab plus chemotherapy.

Experimental Design: Seventy-six metastatic colorectal cancer patients receiving cetuximab plus FOLFIRI or FOLFOX-4 chemotherapy were enrolled. KRAS mutation status in the peripheral blood of these patients was analyzed using membrane-arrays, and KRAS mutation status in tumors was analyzed by DNA sequencing.

Results: Among 76 metastatic colorectal cancer patients, KRAS mutations in tumors and in peripheral blood were identified in 33 (43.4%) and 30 (39.5%) patients, respectively. The detection sensitivity, specificity, and accuracy of membrane-arrays for CTCs with KRAS oncogene were 84.4%, 95.3%, and 90.8%, respectively, and indeed a highly significant correlation to KRAS mutations in tumors (P < 0.0001) was observed. Forty-five (59.2%) patients responded to cetuximab plus chemotherapy, and 41 and 40 were wild-type KRAS in tumors and peripheral blood, respectively (both P < 0.0001). Patients with tumors that harbor wild-type KRAS are more likely to have a better progression-free survival and overall survival when treated with cetuximab plus chemotherapy (P < 0.0001). Likewise, patients with CTCs of wild-type KRAS in peripheral blood express a better progression-free survival and overall survival when treated with cetuximab plus chemotherapy (P < 0.0001).

Conclusions: These findings provide evidence that detection of KRAS mutational status in CTCs, by gene expression array, has potential for clinical application in selecting metastatic colorectal cancer patients most likely to benefit from cetuximab therapy.

Colorectal cancer is the second leading cause of cancer-related death in western countries and is the third major cause of cancer-related death in Taiwan, with >9,000 new cases and 4,000 deaths per year.12 In the past decade, significant improvements have been made in response rates, progression-free survival (PFS), and overall survival (OS) of metastatic colorectal cancer patients (1–4). This prominent improvement is mainly due to the recent introduction of new combinations of standard chemotherapy, including 5-fluorouracil/folinic acid, irinotecan, and oxaliplatin, and to the new therapeutic agents targeting molecular events involved in colorectal carcinogenesis such as monoclonal antibodies (mAb) against epidermal growth factor receptor (EGFR) or mAbs against vascular endothelial growth factor. Previous studies (5–11) showed that the benefits of the anti-EGFR mAb cetuximab among patients with metastatic colorectal cancer are limited to those who have colorectal tumor tissues with wild-type KRAS genes, and KRAS genes with mutations are...
essentially insensitive to EGFR inhibitors. Therefore, the identification of metastatic colorectal cancer patients who harbor KRAS mutants is an important work prior to the addition of such expensive targeted therapies to standard chemotherapy.

Nowadays, KRAS genotyping of tumor tissues or metastatic lesions is strongly recommended by the National Comprehensive Cancer Network (NCCN) Clinical Practice Guidelines in Oncology Version 3, 2008, in patients with metastatic colorectal cancer before treatment with EGFR inhibitors.13 Consequently, KRAS genotyping highlights the value of banking tumor specimens obtained from primary tumor or a metastasis. In most of these studies, however, KRAS genotyping has been done on primary colorectal cancers, whereas anti-EGFR antibodies are used to treat the metastatic disease. This strategy might, at least in certain circumstances, present two limitations (12). First, systematic KRAS genotyping in metastatic colorectal cancer patients might be hampered in the future, at least for some patients, by the difficulty of obtaining tumor samples suitable for molecular analyses. Second, considering the genetic heterogeneity of colorectal cancers, the absence of detectable KRAS mutations in the primary tumor cannot formally exclude the presence of a KRAS mutation in metastases. Hence, an alternative method to detect KRAS gene mutation in these metastatic colorectal cancer patients treated with anti-EGFR is an urgent and relevant event.

Our recently developed membrane-array–based multimarker assay can detect circulating tumor cells (CTC) with KRAS oncogene in the peripheral blood of patients with various malignancies, including colorectal cancer, achieving a considerable sensitivity, specificity, and accuracy when compared with direct sequencing of tumor tissues (13). The aim of this study was conducted to detect CTCs with KRAS mutation status in the peripheral blood of metastatic colorectal cancer patients by a panel of molecular markers using a constructed membrane-array method, and to evaluate whether the mutation status of KRAS gene in peripheral blood is related to the clinical outcome of cetuximab therapy.

### Materials and Methods

**Patients and samples.** From February 2006 to February 2008, we retrospectively analyzed 76 histologically confirmed metastatic colorectal cancer patients treated with cetuximab plus either FOLFIRI or FOLFOX-4 chemotherapy and for whom tumor and peripheral blood were available. FOLFOX was conducted comprising irinotecan 180 mg/m² as a two-hour infusion on day 1, 5-FU 400 mg/m² as a two-hour infusion concurrently with irinotecan on day 1, 5-FU 400 mg/m² as an i.v. bolus infusion followed by 2400 mg/m² infusion IV over a 46-hour period, and repeated every 2 weeks. FOLFOX-4 was conducted comprising oxaliplatin 85 mg/m² as a two-hour infusion on day 1, i.v bolus 200 mg/m² as a two-hour infusion concurrently with oxaliplatin on day 1, followed by a bolus of 5-FU 400 mg/m² then and continuous infusion of 5-FU 600 mg/m² over 22 hours, and repeated every 2 weeks. To be eligible for the study, metastatic colorectal cancer patients with measurable lesions by computed tomographic scan were enrolled. Patients were required to be at least 18 y of age with a life expectancy of 3 mo, and have an Eastern Cooperative Oncology Group (ECOG) performance status of 0 to 2. The patients’ clinical and pathologic characteristics are listed in Table 1. Tumor response was evaluated according to the Response Evaluation Criteria for Solid Tumors (RECIST, ref. 14). A combination of complete response and partial response was subsequently defined as responder, whereas stable disease and progressive disease were classified as nonresponders. The median follow-up period was 20 mo (range, 4-34 mo). Primary end point was the clinical response to cetuximab plus chemotherapy, and second end point was PFS and OS of these patients. This study was approved by the Institutional Review Board of Kaohsiung Medical University Hospital, and was not supported by any commercial company.

| Table 1. Characteristics of the patients
|---|---|
| No. (%) | Total no. of patients 76
| | 64 (39-83)
| Sex | Male 44 (57.9)
| | Female 32 (42.1)
| Performance status | 0 53 (69.7)
| | 1 21 (27.6)
| | 2 2 (2.6)
| Primary tumor | Colon 55 (72.4)
| | Rectum 21 (27.6)
| Metastatic site | Liver only 35 (46.1)
| | Lung only 9 (11.8)
| | Local recurrence 8 (10.5)
| | Peritoneum only 7 (9.2)
| | >2 sites 17 (22.4)
| Differentiation | Well 9 (11.8)
| | Moderately 54 (71.1)
| | Poorly 13 (17.1)
| Chemotherapy regimen | Cetuximab + FOLFIRI 54 (71.1)
| | Cetuximab + FOLFOX 22 (28.9)
| Objective response | Responders 45 (59.2)
| | Complete response 2 (2.6)
| | Partial response 43 (56.6)
| | Nonresponders 31 (40.8)
| | Stable disease 16 (21.1)
| | Progressive disease 15 (19.7)

RNAs from blood specimens was done with ISOGEN (Nippon Gene Co., Ltd) following the modified acid guanidine thiocyanate and phenol/chloroform extraction method (15). Poly (A)^+ -enriched RNAs were then purified from total RNAs with a Dynabead mRNA DIRECT kit (Dynal A.S.). Two micrograms of poly (A)^+ RNAs of each blood specimen were used for double-stranded cDNA synthesis with a SMART PCR cDNA synthesis kit (Clontech), according to the manufacturer’s protocol.

mRNA isolation and first-strand cDNA synthesis. The isolation of total RNAs from blood specimens was done with ISOGEN (Nippon Gene Co., Ltd) following the modified acid guanidine thiocyanate and phenol/chloroform extraction method (15). Poly (A)^+ -enriched RNAs were then purified from total RNAs with a Dynabead mRNA DIRECT kit (Dynal A.S.). Two micrograms of poly (A)^+ RNAs of each blood specimen were used for double-stranded cDNA synthesis with a SMART PCR cDNA synthesis kit (Clontech), according to the manufacturer’s protocol.

Membrane-arrays. The procedure of the membrane-array method for the detection of CTCs with KRAS oncogene was done according to our recent study (13). The primers of oligonucleotide probes were designed by using primer 3 free online software,14 and then synthesized, purified, and controlled before being grafted onto the membranes. The newly synthesized oligonucleotide fragments were then dissolved in di-water to a concentration of 20 mmol/L applied to a BioJet Plus 3000 nanoliter dispensing device (BioDot), which blotted sequentially the 22 target genes, 1 tuberculosis gene, and 1 housekeeping gene (β-actin) on a Nytran SuperCharge nylon membrane (Schleicher and Schuell) in triplicate, and then cross-linked to the membrane using a UV Stratalinker 1800 (Stratagene). The lifts were covered with the ExpressHyb hybridization solution (BD biosciences,) containing DIG-11-UTP–labeled cDNA probes, and then incubated with an alkaline phosphatase–conjugated antidigoxigenin antibody (Roche Diagnostics GmbH). The hybridized membrane-arrays were then cross-linked to the membrane using a UV Stratalinker 1800 (Stratagene). The hybrids were washed and detected with the chemiluminescent reagent. The hybridized membrane-arrays were then cross-linked to the membrane using a UV Stratalinker 1800 (Stratagene).

Preparation of digoxigenin-labeled cDNA targets and hybridization. First-strand cDNA targets for hybridization were produced by reverse transcription of mRNAs from the peripheral blood of metastatic colorectal cancer patients by using SuperScript II reverse transcriptase (Gibco-BRL) in the presence of digoxigenin (DIG)-labeled UTP (Roche Diagnostics GmbH). The lifts were covered with the ExpressHyb hybridization solution (BD biosciences,) containing DIG-11-UTP–labeled cDNA probes, and then incubated with an alkaline phosphatase–conjugated antidigoxigenin antibody (Roche Diagnostics). For hybridization, the arrays were incubated at 42°C for 12 h in a humid chamber. In signal detection, the membranes were incubated for 15 min in a chromogen solution containing nitroblue-tetrazolium and 5-bromo-4-chloro3-indoly-phosphate. The hybridized membrane-arrays were then scanned using an Epson Perfection 1670 flat bed scanner (SEIKO EPSON Corp.). Subsequent quantification analysis of each spot’s intensity was carried out using AlphaEase FC software (Alpha Innotech Corp.). The density ratio of each gene was divided with β-actin as an internal control. Mean spot densities consistently varying by a factor of 2 or more were taken as overexpressed.

DNA extraction and direct sequencing of KRAS gene. Genomic DNA was isolated from frozen primary colorectal cancer tissues using proteinase-K (Stratagene) digestion and phenol/chloroform extraction procedure according to the method by Sambrook (16). The designed sequences of oligonucleotide primers for exons 1 and 2 of the KRAS and the operation procedure of direct sequencing were according to our previous study (17). An automated DNA electrophoresis system (Model 4200; LI-COR) with a laser diode emission at 785 nm and fluorescence detection between 815 and 835 nm was used to detect and analyze the sequencing ladders.

Receiver-operating characteristic curves. Receiver-operating characteristic curves were constructed by plotting all possible sensitivity/specificity pairs for the membrane arrays analysis, resulting from continuously varying the cutoff values over the entire range of results obtained. According to the analysis of receiver-operating characteristic curves, the optimal cutoff point for the number of differentially expressed genes was 11. That is, a diagnostic membrane on which 11 (or >11) of 22 genes were expressed 2-fold higher than normal levels was considered to be positive and vice versa (Fig. 1). At this cutoff point, the sensitivity and specificity of membrane arrays would also achieve optimal levels. In our previous investigations, the sensitivity limit of this technique was established at approximately 1 tumor cell per 10^6 WBC (5 cells per 1 ml blood).

Statistical analysis. All data were analyzed by using the Statistical Package for the Social Sciences Version 12.0 software (SPSS Inc.). The correlation between the membrane-arrays and direct sequencing for the detection of KRAS oncogene in peripheral blood and tumor tissues was compared using the χ^2 test. Also, the correlation between the clinical response to cetuximab plus chemotherapy and the clinicopathological features/regimen/KRAS mutations in metastatic colorectal cancer patients was compared using the χ^2 test. A multivariate Cox proportional hazard model was used to determine the effect of KRAS gene mutations in tumor tissues and peripheral blood on PFS/OS. P < 0.05 was considered statistically significant.

Results

Forty-four men (57.9%) and 32 women (42.1%) were recorded, with a median age of 64 years (range, 39-83 years). Fifty-five tumors (72.4%) were in the colon and 21 (27.6%) in the rectum. Seventy-four patients were ECOG performance status 0 or 1, and only two patients were ECOG performance status 2. With regard to the histologic type of these tumors, 9 (11.8%) were well-differentiated, 54 (71.1%) were moderately...

14 http://web.umassmed.edu/bioapps/primer3_www.cgi

Fig. 1. Comparing CTCs with detectable KRAS oncogene in peripheral blood of metastatic colorectal cancer patients. A triplicated set of 22 mRNA markers for KRAS gene mutation was blotted on nylon membrane. In addition, a housekeeping gene (β-actin) and a tuberculosis gene serving as positive and negative controls were also blotted on the membrane. The spots within the red rectangle of each image represent β-actin (positive control), and the spots within the red circle of each image represent tuberculosis gene (negative control). Eleven or >11 of 22 genes that expressed 2-fold higher than normal levels were considered to be positive and vice versa. A, CTCs with detectable KRAS oncogene. B, CTCs with undetectable KRAS oncogene.

Specimen collection. All tissue samples were collected and separated into various tubes immediately after surgical resection. They were frozen instantly in liquid nitrogen, and then stored in the freezer at -70°C until analyzed. RNA were extracted from all blood samples and reverse-transcribed to cDNA, and then stored in various tubes and placed in the freezer at -70°C until analyzed. Each tube of biological sample was used individually while experiments were done to ensure the quality.

A triplicated set of 22 mRNA markers for KRAS gene mutation was blotted on nylon membrane. In addition, a housekeeping gene (β-actin) and a tuberculosis gene serving as positive and negative controls were also blotted on the membrane. The spots within the red rectangle of each image represent β-actin (positive control), and the spots within the red circle of each image represent tuberculosis gene (negative control). Eleven or >11 of 22 genes that expressed 2-fold higher than normal levels were considered to be positive and vice versa. A, CTCs with detectable KRAS oncogene. B, CTCs with undetectable KRAS oncogene.

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Table 3. The correlation between the membrane-arrays and direct sequencing for the detection of activated KRAS oncogene in peripheral blood and tumor tissues of metastatic colorectal cancer patients, respectively

<table>
<thead>
<tr>
<th>KRAS in peripheral blood</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>28</td>
</tr>
<tr>
<td>Negative</td>
<td>5</td>
</tr>
</tbody>
</table>

Table 2. KRAS mutation detection in tumor tissues by direct sequencing and in peripheral blood by membrane-array method

<table>
<thead>
<tr>
<th>No. (%)</th>
<th>Tumor tissues</th>
<th>KRAS mutations</th>
<th>Mutations</th>
<th>Wild type</th>
<th>KRAS mutation sites (n = 33)</th>
<th>Codon 12</th>
<th>Codon 13</th>
<th>Codon 15</th>
<th>Codon 18</th>
<th>Codon 20</th>
<th>Codon 30</th>
<th>Codon 31</th>
<th>Activating KRAS mutant (n = 33)</th>
<th>Positive (codons 12, 13, 15, 18)</th>
<th>Negative (codons 20, 30, 31)</th>
<th>Peripheral blood</th>
<th>KRAS mutations</th>
<th>Activating mutations (codons 12, 13, 15, 18)</th>
<th>Active</th>
<th>Wild type</th>
<th>46 (60.5)</th>
</tr>
</thead>
</table>

well-differentiated, and 13 (17.1%) were poorly differentiated carcinomas. The main site of metastases (one lesion only) was liver (35; 46.1%), followed by lung (9; 11.8%), local recurrence (8; 10.5%), and peritoneum (7; 9.2%), and 17 (22.4%) patients developed at least 2 metastatic sites. Among 76 metastatic colorectal cancer cases, 54 (71.1%) cases received cetuximab plus FOLFIRI regimen and gained a response rate of 59.2% (45/76), including 2 (2.6%) patients with complete response, 43 (56.6%) patients with partial response, 16 (21.1%) patients with stable disease, and 15 (19.7%) patients with progressive disease (Table 1). KRAS mutations were identified in 33 (43.4%) tumor tissues, and detected in 30 (39.5%) peripheral blood samples of metastatic colorectal cancer patients (Table 2). The detection sensitivity, specificity, and accuracy of membrane-arrays for CTCs with KRAS mutations were 84.4%, 95.3%, and 90.8%, respectively. In fact, a statistically significant correlation between KRAS mutations in tumors and peripheral blood was observed (Table 3;  \( P < 0.0001 \)).

Table 4 shows the correlation between the clinical response to cetuximab plus chemotherapy and the clinicopathologic features/KRAS mutation status of 76 metastatic colorectal cancer patients. We found that both KRAS mutation status in tumors and peripheral blood were significantly different between responders and nonresponders (Table 4;  \( P < 0.0001 \)), but gender, depth of tumor invasion, lymph node metastases, cell differentiation, or chemotherapy regimen were not (all  \( P > 0.05 \)). Of 76 metastatic colorectal cancer patients, 41 of 48 (85.4%) patients with tumors that either expressed wild-type KRAS (43 tumors) or nonactivating KRAS mutations (5 tumors) responded to cetuximab plus chemotherapy, but 4 of 28 (14.3%) patients with KRAS mutations responded to cetuximab plus chemotherapy. Likewise, 40 of 46 (87%) patients’ CTCs without detectable KRAS mutations responded to cetuximab plus chemotherapy, but 5 of 30 (16.7%) patients’ CTCs with detectable KRAS oncogene responded to cetuximab plus chemotherapy. Using a Cox proportional hazard regression analysis, tumors with wild-type KRAS ( \( P < 0.0001 \); hazard ratio, 0.253; 95% confidence interval, 0.149-0.478) and CTCs of peripheral blood without detectable KRAS mutation ( \( P < 0.0001 \); hazard ratio, 0.189; 95% confidence interval, 0.113-0.305) were shown to be independent predictive factors for PFS (Table 5). Meanwhile, tumors with wild-type KRAS ( \( P < 0.0001 \); hazard ratio, 0.151; 95% confidence interval, 0.077-0.365) and CTCs without detectable KRAS mutation ( \( P < 0.0001 \); hazard ratio, 0.112; 95% confidence interval, 0.053-0.274) were shown to be independent predictive factors for OS (Table 5).

Discussion

Cetuximab, a mAb against the EGFR, was the second targeted agent that was approved for metastatic colorectal cancer based on improved response rates, time to progression, PFS/OS, and quality of life when administered with irinotecan or oxaliplatin (3, 9–11, 18, 19). Previously, the level of EGFR immunohistochemical expression has been considered adequate for predicting sensitivity to anti-EGFR antibodies therapy. Meanwhile, Food and Drug Administration approval of cetuximab for the treatment of metastatic colorectal cancer included a requirement that there be positive immunohistochemical staining for the presence of EGFR in the tumor. However, accumulated evidence of the lack of usefulness of EGFR detection by immunohistochemistry for cetuximab therapy in metastatic colorectal cancer patients was addressed, irrespective of both the percentage of EGFR-positive tumor cells and the intensity of the staining per cell (18, 19), and clinical response could be obtained in colorectal cancers that do not express EGFR by immunohistochemistry in 25% of patients (20, 21).

The predictive value of KRAS mutation in metastatic colorectal cancer patients treated with cetuximab plus chemotherapy has recently been shown in that patients with tumor KRAS mutation were resistant to cetuximab and had shorter PFS and OS times compared with patients without mutation (5–11). Additionally, NCCN Clinical Practice Guidelines in Oncology Version 3, 2008, strongly recommends KRAS genotyping of tumor tissue (either primary tumor or metastasis) in all patients with metastatic colorectal cancer before treatment with EGFR inhibitors. KRAS mutational analysis has advantages over attempts to predict responsiveness to anti-EGFR antibodies with the use of...
immunohistochemical analysis. DNA is stable in fixed tissue samples, and mutational analysis is therefore less likely than immunohistochemical analysis to be affected by fixation and storage. Moreover, mutational analysis is a yes-or-no result: either the mutation is present or it is not, whereas immunohistochemical analysis entails subjective grading of the intensity of the staining (22). It is sometimes difficult, however, to obtain tumor samples from all metastatic colorectal cancer patients suitable for mutational analysis; thus, it is mandatory to establish an alternative method to detect mutant KRAS from other more readily accessible patient samples.

The recent advent of PCR technology for the amplification of small amounts of DNA using serum samples has made it possible to identify the same gene alterations from patients with metastatic colorectal cancer. However, despite our limited analyzed numbers of colorectal cancer patients, the relatively low detection sensitivity (<50% in International Union against Cancer stage IV patients) of KRAS gene by conventional sequencing techniques would make this assay unreliable in clinical implication (23, 24). In contrast, the current study has shown that our constructed membrane-arrays not only detected KRAS mutations in peripheral blood samples obtained from metastatic colorectal cancer patients, but also achieved a considerable high sensitivity of 84.4% and specificity of 95.3%. Again, a significant correlation of KRAS mutation status between the tumor tissues and peripheral blood is further shown. The high accuracy (90.8%) of constructed membrane-arrays may have resulted from our having screened a set of genes related to KRAS oncogene instead of a single gene (13). Moreover, this membrane-array method is a high-throughput assay with the advantages of time-saving and cost-effectiveness when compared with PCR and sequencing techniques in future clinical practice (13, 25).

Table 4. Correlation between clinical response to cetuximab plus chemotherapy and clinicopathologic features of 76 metastatic colorectal cancer patients

<table>
<thead>
<tr>
<th>Variables</th>
<th>Responders (n = 45), no. (%)</th>
<th>Nonresponders (n = 31), no. (%)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>25 (55.6)</td>
<td>19 (61.3)</td>
<td>0.619</td>
</tr>
<tr>
<td>Female</td>
<td>20 (45.4)</td>
<td>12 (38.7)</td>
<td></td>
</tr>
<tr>
<td>Tumor location</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Colon</td>
<td>30 (66.7)</td>
<td>25 (80.6)</td>
<td>0.180</td>
</tr>
<tr>
<td>Rectum</td>
<td>15 (33.3)</td>
<td>6 (19.4)</td>
<td></td>
</tr>
<tr>
<td>Depth of tumor invasion</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T2</td>
<td>3 (6.7)</td>
<td>0 (0)</td>
<td>0.139</td>
</tr>
<tr>
<td>T3</td>
<td>37 (82.2)</td>
<td>30 (96.8)</td>
<td></td>
</tr>
<tr>
<td>T4</td>
<td>5 (11.1)</td>
<td>1 (3.2)</td>
<td></td>
</tr>
<tr>
<td>Lymph node metastases</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N0</td>
<td>3 (6.7)</td>
<td>0 (0)</td>
<td>0.334</td>
</tr>
<tr>
<td>N1</td>
<td>20 (44.4)</td>
<td>14 (45.2)</td>
<td></td>
</tr>
<tr>
<td>N2</td>
<td>22 (48.9)</td>
<td>17 (54.8)</td>
<td></td>
</tr>
<tr>
<td>Differentiation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Well</td>
<td>5 (11.1)</td>
<td>4 (12.9)</td>
<td>0.867</td>
</tr>
<tr>
<td>Moderately</td>
<td>33 (73.3)</td>
<td>21 (67.7)</td>
<td></td>
</tr>
<tr>
<td>Poorly</td>
<td>7 (15.6)</td>
<td>6 (19.4)</td>
<td></td>
</tr>
<tr>
<td>Chemotherapy regimen</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cetuximab + FOLFOX</td>
<td>34 (75.6)</td>
<td>20 (64.5)</td>
<td>0.297</td>
</tr>
<tr>
<td>FOLFIRI</td>
<td>11 (24.4)</td>
<td>11 (35.5)</td>
<td></td>
</tr>
<tr>
<td>Activating KRAS mutant in tumor tissues</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mutant</td>
<td>4 (8.9)</td>
<td>24 (77.4)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Wild type</td>
<td>41 (91.1)</td>
<td>7 (22.6)</td>
<td></td>
</tr>
<tr>
<td>KRAS mutant in peripheral blood</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mutant</td>
<td>5 (11.1)</td>
<td>25 (80.6)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Wild type</td>
<td>40 (88.9)</td>
<td>6 (19.4)</td>
<td></td>
</tr>
</tbody>
</table>

Table 5. Correlation between progression-free and overall survival in metastatic colorectal cancer patients using multivariate Cox proportional hazard regression analysis according to KRAS mutations in tumor or peripheral blood

<table>
<thead>
<tr>
<th>Variables</th>
<th>Progression-free survival</th>
<th>Overall survival</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hazard ratio (95% CI)</td>
<td>P</td>
</tr>
<tr>
<td>Activating KRAS mutations in tumor</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>0.253 (0.149-0.478)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Yes</td>
<td>1.000 (reference)</td>
<td></td>
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<tr>
<td>KRAS mutations in peripheral blood</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>0.189 (0.113-0.305)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Yes</td>
<td>1.000 (reference)</td>
<td></td>
</tr>
</tbody>
</table>
Nevertheless, among patients with wild-type KRAS colorectal cancer, the objective response rate is 59% and 61% (versus 43% and 33% in unselected patients) with cetuximab plus either irinotecan- or oxaliplatin-based chemotherapy, respectively (10, 11). These data indicate that other mechanisms of anti-EGFR resistance play an important role. Remarkably, evaluation of metastatic rather than primary sites could be of clinical relevance because the occurrence of a mutation in the metastasis could, at least theoretically, explain resistance despite a wild-type primary tumor. Suchy et al. have shown the concordance of KRAS mutations in primary tumors and respective metastases in 15 patients, and the type of mutation was also identical in the instance of different metastases from the same primary tumor localized in different organs, indicating a stability of these mutations during metastatic progression (26). Recently, Artal et al. indicated a concordance of KRAS mutational status between primary tumor and metastasis in 10 of 13 (77%) metastatic colorectal cancer patients (27). Also, they suggested that evaluation of the KRAS mutations can be done in either primary tumor or metastatic site(s) and that absence of such mutations could be enough to drive the selection of metastatic colorectal cancer patients who are candidates for anti-EGFR mAb therapy. Therefore, the detectable KRAS mutations in peripheral blood might probably reflect the actual metastatic status and prediction of clinical response to cetuximab therapy in these patients, because anti-EGFR antibodies are used to treat the metastatic disease. Recently, Fiore and his colleagues detected KRAS mutations in blood using real-time PCR for anti-EGFR therapies in metastatic colorectal cancer patients, and suggested that it is probably useful to collect blood samples before treatment and that the clinical interest of such blood tests is to detect KRAS mutations (12), of which findings are consistent with our results.

In conclusion, the diagnostic membrane-array is a great potential tool in the clinical detection of CTCs with KRAS mutations of metastatic colorectal cancer patients before the introduction of cetuximab therapy, in addition to KRAS mutation status from primary tumor or metastatic site. These results also highlight the need to prompt further prospective studies on larger metastatic colorectal cancer cases to definitely establish the clinical relevance of KRAS mutation detection from peripheral blood in anti-EGFR–based chemotherapy.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Acknowledgments**

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**References**

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