Somatic Mutations of Epidermal Growth Factor Receptor in Colorectal Carcinoma

Hisashi Nagahara,1,2 Koshi Mimori,1 Mitsuhiko Ohta,1 Tohru Utsunomiya,1 Hiroshi Inoue,1 Graham F. Barnard,3 Masachi Ohira,2 Kosei Hirakawa,2 and Masaki Mori1

1Department of Surgery, Medical Institute of Bioregulation, Kyushu University, Beppu, Japan; 2Department of Surgical Oncology, Osaka City University Graduate School of Medicine, Osaka, Japan; and 3Department of Medicine, University of Massachusetts, Worcester, Massachusetts

ABSTRACT

Purpose: Somatic mutations of the epidermal growth factor receptor (EGFR) gene may predict the sensitivity of non–small cell lung carcinoma to gefitinib. However, no mutations have been reported for colorectal carcinoma. We therefore analyzed EGFR mutations in colorectal adenocarcinomas by the combined use of laser microdissection and sequencing of genomic DNA.

Experimental Design: We examined 11 representative colorectal adenocarcinoma cell lines and 33 clinical samples of colorectal carcinoma. In the clinical cases, we carefully dissected only carcinoma cells from frozen sections by laser microdissection. After DNA extraction and PCR, we examined EGFR mutations by sequencing genomic DNA.

Results: None of 11 colorectal carcinoma cell lines exhibited somatic mutations, but 4 of 33 clinical tumors (12%) exhibited mutations in the EGFR kinase domain. This may be the first report of somatic mutations in colorectal adenocarcinoma.

Conclusions: Our findings suggest that a distinct minority of colorectal adenocarcinomas exhibit somatic mutations of EGFR, and these tumors may be susceptible to gefitinib treatment.

INTRODUCTION

Epidermal growth factor receptor (EGFR) is more abundantly expressed in lung carcinoma tissue than in adjacent normal lung tissue (1). Recently, the EGFR tyrosine kinase inhibitor gefitinib (Iressa) was approved for use in patients with non–small cell lung carcinomas (NSCLC) in Japan and the United States. EGFR expression as detected by immunohistochemistry has not been found an effective predictor of response to gefitinib (2). Moreover, in clinical studies, the effectiveness of gefitinib is variable. In patients exhibiting a dramatic response, both primary and metastatic lesions disappeared completely; however, only 27.5% of Japanese patients exhibited a response to gefitinib. In Europe, this drug was even less effective (10.4% efficacy, both studies in a multi-institutional phase II trial; ref. 3). In the United States, partial clinical response to gefitinib has been observed most frequently in women, in nonsmokers, and in patients with adenocarcinoma (4–6). Lynch et al. and Paez et al. each reported that EGFR mutations in NSCLC were related to a clinical response to gefitinib (7, 8). These mutations centered on exons 18 to 21 of the EGFR kinase domain. These authors found that phosphorylation of EGFR was much stronger in NSCLC cases with any mutation in these exons, compared with cells without such mutations, and that cases with a high degree of EGFR phosphorylation were appropriate for treatment with gefitinib. In cancers of other organs, such as colorectal carcinoma, however, such mutations have never been reported in either cell lines or primary tumors (7). Colorectal carcinoma is one of the most common malignancies throughout the world. We examined both carcinoma cell lines and primary tumors of the colorectum. For primary tumors, it is desirable to study DNA extracted specifically from carcinoma cells within the sample. To determine with precision the presence or absence of DNA mutations in EGFR, we therefore carefully harvested carcinoma cells from frozen sections of Japanese cases of colorectal adenocarcinoma by laser microdissection and extracted this DNA for sequence analysis.

MATERIALS AND METHODS

Primer Design and Samples. Primers for amplification of the selected EGFR exons, including exons 18, 19, 20, and 21, that contained intronic sequences were designed according to the previous study by Paez et al. (8). We examined 11 representative colorectal adenocarcinoma cell lines (i.e., colo201, colo205, WiDr, DLD-1, Lovo, CaR-1, RCMI-1, VMRC-MERG, CCK81, HT29, and LS174T) and 33 clinical samples of colorectal carcinoma. The cell lines were maintained in RPMI 1640 containing 10% fetal bovine serum and antibiotics at 37°C in a 5% humidified CO2 atmosphere. Clinical samples were surgically removed at the Department of Surgical Oncology, Medical Institute of Bioregulation Hospital, Kyushu University, Beppu, Japan. Specimens of 5 × 5 mm size were removed, embedded in tissue fixative medium (Tissue-Tek), immediately frozen and stored at −90°C until use. Frozen samples were thinly sliced (8–10 μm), and we carefully dissected out only carcinoma cells from whole specimens by laser.
microdissection (Leica, Tokyo Japan) to avoid contamination by stromal cells and/or normal epithelial cells (9). In addition, to confirm the results from laser microdissection with its small amounts of DNA, we reexamined the altered nucleotide by macrodissected samples adjacent to the microdissected specimens. Genomic DNA was then extracted with a DNA tissue kit (Qiagen, Chatsworth, CA), following the manufacturer's protocol.

**PCR and Sequencing Methods for Genomic DNA.** EGFR exons and flanking intronic sequences were amplified using specific primers in a nested PCR setup. Each PCR reaction contained 20 ng of DNA in 30-μL reaction mixture containing 10 mmol/L Tris-HCl, 1.5 mmol/L MgCl₂, 50 mmol/L KCl (pH 8.3, 20°C), 200 μmol/L of each deoxynucleotide triphosphate, 12.5 μmol/L of each primer, and 1 unit of Taq DNA polymerase (Boehringer, Mannheim, Germany). PCR cycling variables were one cycle at 95°C for 10 minutes, 35 cycles at 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 1 minute followed by one cycle at 72°C for 7 minutes. The resulting PCR products were sequenced using the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA) following the manufacturer's protocol. The EGFR sequences were aligned and analyzed with the Sequencher 4.1.2 (Gene Codes Co., Ann Arbor, MI).

**RESULTS**

The quality of the genomic DNA was validated using PCR amplification of GAPDH as control gene, and all DNA samples extracted from the 11 cell lines and 33 clinical specimens were confirmed to be of good quality (data not shown). In addition, at least 200 ng of DNA were obtained with these procedures. Sequencing revealed no mutations in EGFR in the 11 colorectal carcinoma cell lines nor in the 33 normal colorectal tissues. On the other hand, somatic EGFR mutations were identified in 4 of 33 patients (12%), as shown in Fig. 1 and Table 1. Patients 1 and 2 had mutations at the same site. All these mutations were substitutions.

![Fig. 1 A, laser microdissection (LMD) in colorectal carcinoma. Only carcinoma cells were specifically dissected. B, three types of mutations in cases of colorectal carcinoma. Amplified genomic DNA from carcinoma cells obtained using LMD (T). Amplified genomic DNA from adjacent normal tissue (N). All sequencing was performed in the forward and reverse directions. Arrowhead, ambiguous nucleotide change of two overlapping peaks (A and G) in the forward sequence at exon 19. We confirmed two overlapping peaks (T and C) at the identical nucleotide position on sequencing in the reverse direction.](image)

**Table 1**

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Exon</th>
<th>Mutation type</th>
<th>Nucleotide</th>
<th>Amino acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>19</td>
<td>Substitution</td>
<td>2245 G &gt; A</td>
<td>E749K</td>
</tr>
<tr>
<td>2</td>
<td>19</td>
<td>Substitution</td>
<td>2245 G &gt; A</td>
<td>E749K</td>
</tr>
<tr>
<td>3</td>
<td>20</td>
<td>Substitution</td>
<td>2285 A &gt; G</td>
<td>E762G</td>
</tr>
<tr>
<td>4</td>
<td>20</td>
<td>Substitution</td>
<td>2299 G &gt; A</td>
<td>A767T</td>
</tr>
</tbody>
</table>

NOTE. Patients 1 and 2 had mutations at the same site. All mutations were substitutions.

Abbreviations: G, guanine; A, adenine Amino Acid; E, glutamic acid; K, lysine; G, glycine; A, alanine; T, threonine.
substitutions, and were nonsynonymous, missense mutations. There were no frame-shift type deletion mutations identified in our study, as have been frequently observed in lung carcinomas. There were no frame-shift type deletion mutations identified in another way. We dissected carcinoma cells whereas viewing prepared from laser microdissection, we extracted genomic DNA.

In a previous study, EGFR was found to be highly overexpressed in 45% of NSCLC cases examined (1). Lynch et al. reported identifying EGFR mutations in the tyrosine kinase domain of the EGFR gene in eight of nine patients with gefitinib-responsive lung carcinoma, compared with none of seven patients with no response to gefitinib (7). Paz et al. have also reported identifying EGFR kinase domain mutations in all five of five patients with gefitinib-responsive lung carcinoma, compared with none of four patients with no response to gefitinib (8). They found that, in vitro, EGFR mutants were enhanced for tyrosine kinase activity in response to epidermal growth factor and exhibited increased sensitivity to inhibition by gefitinib. Thus, screening for such mutations in lung carcinomas may identify patients who will exhibit a response to gefitinib.

In colorectal carcinoma, expression of EGFR was found in 60% to 75% of cases (10). Based on this finding, inhibition of EGFR is evolving as another modality of treatment for colorectal carcinoma. For example, cetuximab, an anti-EGFR antibody, has been studied in combination with irinotecan for metastatic colorectal carcinoma (11). Cunningham et al. reported identifying EGFR mutations in the tyrosine kinase domain in eight of nine patients with gefitinib (7), and none of seven patients with no response to gefitinib (8).

In a previous study, EGFR was found to be highly overexpressed in 45% of NSCLC cases examined (1). Lynch et al. reported identifying EGFR mutations in the tyrosine kinase domain of the EGFR gene in eight of nine patients with gefitinib-responsive lung carcinoma, compared with none of seven patients with no response to gefitinib (7). Paz et al. have also reported identifying EGFR kinase domain mutations in all five of five patients with gefitinib-responsive lung carcinoma, compared with none of four patients with no response to gefitinib (8). They found that, in vitro, EGFR mutants were enhanced for tyrosine kinase activity in response to epidermal growth factor and exhibited increased sensitivity to inhibition by gefitinib. Thus, screening for such mutations in lung carcinomas may identify patients who will exhibit a response to gefitinib.

In colorectal carcinoma, expression of EGFR was found in 60% to 75% of cases (10). Based on this finding, inhibition of EGFR is evolving as another modality of treatment for colorectal carcinoma. For example, cetuximab, an anti-EGFR antibody, has been studied in combination with irinotecan for metastatic colorectal carcinoma (11). Cunningham et al. reported that the response rate to this combination was 22.9% (11). Other studies with various monoclonal antibodies have recently been reported, and are summarized in Table 2. As with lung carcinomas, mutations in the tyrosine kinase domain of EGFR may be associated with clinical efficacy of EGFR inhibitors in the treatment of colorectal carcinomas. It was therefore important to determine how frequently EGFR mutations actually occur in colorectal carcinomas.

<table>
<thead>
<tr>
<th>Antibody name</th>
<th>Target</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cetuximab (Imclone/Bristol Myers Squibb)</td>
<td>EGFR</td>
<td>(11)</td>
</tr>
<tr>
<td>ABX-EGF (Abgenix/Amen)</td>
<td>EGFR</td>
<td>(12)</td>
</tr>
<tr>
<td>EMD 72000 (Merck)</td>
<td>EGFR</td>
<td>(13)</td>
</tr>
<tr>
<td>Edrecolomab (Centocor/GlaxoSmithKline)</td>
<td>Ep-CAM</td>
<td>(14)</td>
</tr>
<tr>
<td>Bevacizumab (Genetech)</td>
<td>VEGF</td>
<td>(15)</td>
</tr>
</tbody>
</table>

Abbreviations: Ep-CAM, 17-1A antigen; VEGF, vascular endothelial growth factor.
Somatic Mutations of Epidermal Growth Factor Receptor in Colorectal Carcinoma

Hisashi Nagahara, Koshi Mimori, Mitsuhiko Ohta, et al.


Updated version
Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/11/4/1368

Cited articles
This article cites 15 articles, 5 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/11/4/1368.full.html#ref-list-1

Citing articles
This article has been cited by 21 HighWire-hosted articles. Access the articles at:
/content/11/4/1368.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.