

# Somatic Mutations of Epidermal Growth Factor Receptor in Colorectal Carcinoma

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## 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, RCM-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 CO<sub>2</sub> 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

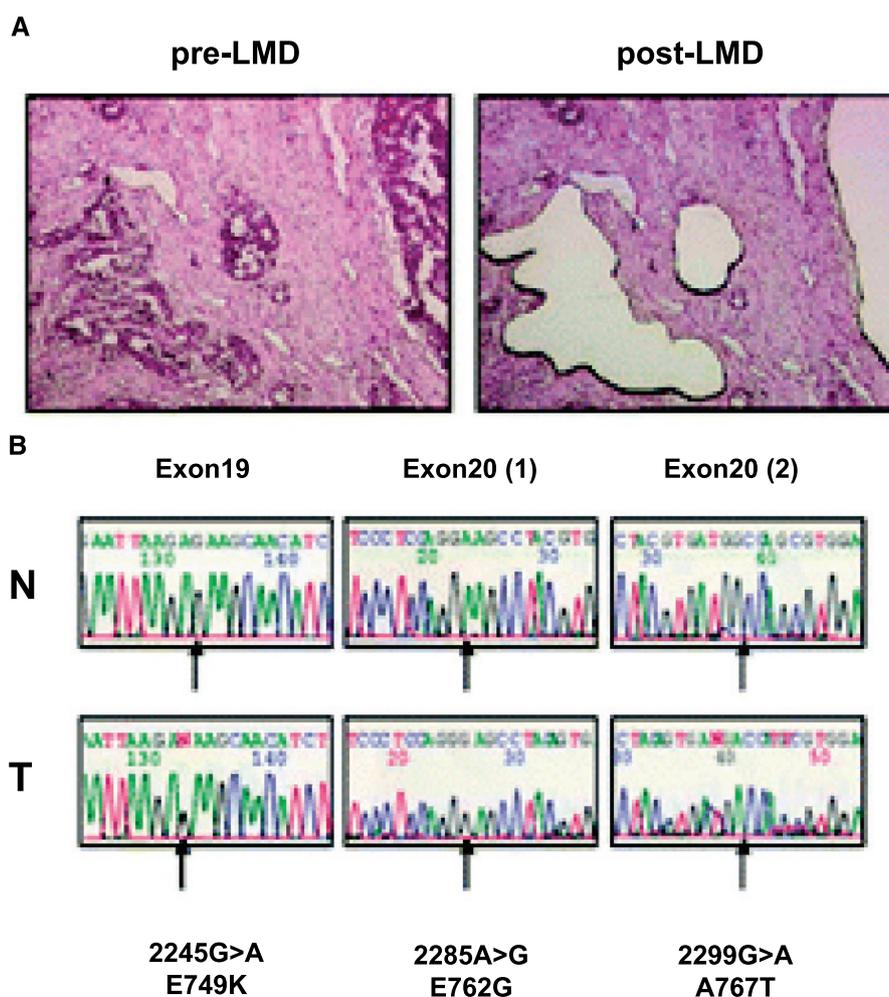
Received 9/16/04; revised 10/28/04; accepted 11/5/04.

**Grant support:** Japan Society for the Promotion of Science Scientific Research (B) grants-in-aid 15390398, 15390379, and 16390381 and Scientific Research (C) grants-in-aid 15591412 and 15591411; Ministry of Education, Culture, Sports, Science and Technology (Japan) for Exploratory Research grant-in-aid 16659337; Public Trust Haraguchi Memorial Cancer Research Fund; and Japan Chemical Industry Association, Long-range Research Initiative project.

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*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.

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- $\mu$ L reaction mixture containing 10 mmol/L Tris-HCl, 1.5 mmol/L MgCl<sub>2</sub>, 50 mmol/L KCl (pH 8.3, 20°C), 200  $\mu$ mol/L of each deoxynucleotide triphosphate, 12.5  $\mu$ mol/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 Sequence 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

Table 1 EGFR mutation status in colorectal cancer

Patient no.	Exon	Mutation type	Nucleotide	Amino acid
1	19	Substitution	2245 G > A	E749K
2	19	Substitution	2245 G > A	E749K
3	20	Substitution	2285 A > G	E762G
4	20	Substitution	2299 G > A	A767T

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.

Table 2 Representative monoclonal antibodies used for targeted therapy for colorectal carcinoma

Antibody name	Target	Reference
Cetuximab (Imclone/Bristol Myers Squibb)	EGFR	(11)
ABX-EGF (Abgenix/Amgen)	EGFR	(12)
EMD 72000 (Merck)	EGFR	(13)
Edrecolomab (Centocor/GlaxoSmithKline)	Ep-CAM	(14)
Bevacizumab (Genentech)	VEGF	(15)

Abbreviations: Ep-CAM, 17-1A antigen; VEGF, vascular endothelial growth factor.

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.

Silent mutations (or variants) were found in 9 of 11 cell lines and in a total of 6 of the 33 patients: two patients at codon 787 (Gln; CAG to CAA) in exon 20 of the EGFR gene; one patient at codon 802 (Val; GTC to GTT) in exon 20; one patient at codon 832 (Arg; CGC to CGA) in exon 21; two patients at codon 858 (Leu; CTG to CTA) in exon 21.

To validate the results from the small amounts of DNA prepared from laser microdissection, we extracted genomic DNA in another way. We dissected carcinoma cells whereas viewing under an optical microscope with a surgical knife for “macrodissection” samples, and analyzed *EGFR* gene mutations in the same way. This specimen consisted of ~70% tumor cells and ~30% stromal cells. Both methods lead to the same results, therefore this confirmed the original findings.

## DISCUSSION

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). Paez 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.

There have been no previous reports demonstrating EGFR mutations in colorectal carcinoma. Lynch et al. studied five colorectal carcinoma-derived cell lines, and reported finding no mutations in the tyrosine kinase domain of EGFR. To determine DNA mutations in clinical samples with greater precision, it was desirable to study carcinoma cells alone without their surrounding stromal cells. We therefore carefully dissected carcinoma cells from frozen sections by laser microdissection to avoid contamination by stromal cells and/or normal epithelial cells. We found mutations (amino acid substitutions) in exons 19 and 20 of the EGFR gene in 4 of 33 clinical colorectal carcinomas. In previous reports (7, 8), two types of mutations, either small, in-frame deletions, or amino acid substitutions clustered around the ATP-binding pocket of the tyrosine kinase domain were detected in NSCLCs. In this study, we found three patterns of amino acid substitutions (E749K within exon 19 and E762G and A767T within exon 20), which are also clustered within the tyrosine kinase domain of EGFR, although no in-frame deletions were observed. Because both types of mutations have correlated with clinical responsiveness to gefitinib (7, 8), it is quite likely that the amino acid substitutions, that we here newly detect in colorectal carcinomas, may also be associated with clinical responsiveness to EGFR inhibitors.

In conclusion, we have discovered somatic mutations in critical EGFR domains in a distinct minority of colorectal carcinomas. Our findings may well be helpful in selecting appropriate candidates for administration of EGFR inhibitors. The large number of clinical trials of diverse agents targeting EGFR will make this an important result.

## ACKNOWLEDGMENTS

We thank Drs. T. Sudo and T. Ohmachi for critical advice, K. Ogata and M. Kayashima for technical assistance, and Dr. T. Mitsudomi (Aichi Cancer Center Research Institute, Nagoya, Japan) for helpful discussions.

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*Clin Cancer Res* 2005;11:1368-1371.

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