

Mutations of the epidermal growth factor receptor gene in gastrointestinal tract tumor cell lines

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Received November 29, 2005; Accepted January 16, 2006

Abstract. The epidermal growth factor receptor (EGFR) is commonly overexpressed in many human tumors including gastrointestinal tract tumors. Gefitinib is a selective inhibitor of EGFR tyrosine kinase, and blocks several signal transduction pathways including those involved in tumor cell proliferation, angiogenesis and metastasis. Recent mutational and biological studies have suggested that mutations in the tyrosine kinase domain of the EGFR gene are well correlated with the response to gefitinib, and that these mutations are frequently observed in non-small cell lung cancers affecting women, East Asians and nonsmokers. This led us to speculate that EGFR gene mutations may occur frequently in gastrointestinal tract carcinomas (GITCs) because over-expression is observed in these tumor types. To investigate EGFR mutations in GITCs, we studied 11 esophageal, 6 gastric, and 12 colorectal cancer cell lines. We found a missense mutation in a gastric cancer cell line, and 10 single nucleotide polymorphisms. The occurrence of rare mutations in the tyrosine kinase domain of the EGFR gene suggests that gefitinib is unlikely to be reliable as single-drug therapy for GITCs.

Introduction

Advances in cancer research have directly contributed to the development of so-called molecular target medicine, in which numerous agents can be used to target cancer-specific molecules. Some of these agents (such as imatinib for chronic myeloid leukemia and gastrointestinal stromal tumors) have already been evaluated in clinical trials, and their efficacy

has been widely accepted (1-3). Small-molecule kinase inhibitors have been developed against growth factor receptors that are frequently expressed in epithelial cancers. The first of these was gefitinib (Iressa; AstraZeneca, Wilmington, DE), which targets the epidermal growth factor receptor (EGFR) (4). Gefitinib has been tested in patients with chemotherapy-refractory non-small cell lung cancer (NSCLC), as this cancer shows frequent expression of EGFR and responds poorly to standard therapies (5-8).

Initial trials of gefitinib failed to demonstrate activity in the majority of NSCLC cases, although a small subset of cases did respond and showed rapid and dramatic tumor shrinkage (5-8). The level of EGFR expression was found not to correlate with the response to gefitinib. Some investigators have recently demonstrated that the majority of tumors showing marked responses harbor mutations in the EGFR kinase domain that are not present in non-responsive cases (9-11). These mutations have been detected in approximately 10% of NSCLC cases in North America and 30% in Asia (10-23). EGFR mutations associated with gefitinib response include amino acid substitutions and in-frame deletions clustered around the ATP binding pocket, which also serves as the drug binding site. A small number of different mutations account for most cases, suggesting that they confer specific enzymatic properties. Indeed, reconstitution of these mutations *in vitro* reveals that they result in a dramatic increase of anti-apoptotic signals following binding of the EGF ligand to the receptor, compared with wild-type EGFR (24-26). Suppression of these survival signals, either by gefitinib or by direct targeting of the mutant EGFR transcript using small interfering RNA, leads to rapid apoptosis, consistent with the oncogene addiction model (24).

Two types of EGFR mutation have been reported previously. One occurs naturally, due possibly to alternative splicing, and is best characterized by the oncogenic mutant designated as del2-7 EGFR or EGFRvIII (9,27-34). This type of mutation has been observed in glioblastoma, and ovarian and breast cancers (9,27-34). Other EGFR mutations around the ATP binding pocket associated with the gefitinib response have been frequently found in women, East Asians, and nonsmokers (10-23). The tumors harboring these mutations are primarily adenocarcinomas, often with areas of bronchoalveolar histology. An especially high frequency of EGFR mutations associated with the gefitinib response has been

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Key words: EGFR, mutation, gefitinib, gastrointestinal tract tumors, splice variant

Table I. PCR primers used for genomic DNA amplification.

Exon	Forward (5'-3')	Reverse (5'-3')
Exon 1	CCTCTCGGAAATTAACCTCTCA	GTTTCCTTGAGATCAGCTGC
Exon 2	CAGGAATGGGTGAGTCTCTG	TCGTTTGTAGCTCTGTAAGACTTG
Exon 3	CCATGACTGCAATCGTCTACC	ATCTTACACACAGCCGGCAC
Exon 4	GTTCACTGGGCTAATTGCG	ATGTCCGTGGTAAATACATGC
Exon 5	ATTCTACAAACCAGCCAGCC	ACTGCATGCGGTGAGATTTG
Exon 6	TCGTTGGAAGCAAATGTGTC	GCAGAGGGCAATATCCTGTC
Exon 7	TGGAACACTAGGCTGCAAAG	CTCCAAGCAAGGCAAACAC
Exon 8	TCCTTCTCTATTTGCAACCTTC	TGCCATTGCCTAACCTAGC
Exon 9	TGAAGGATGATGTGGCAGTG	GGATCCAGAGGAGGAGTATGTG
Exon 10	TTCAAAAACCTGCACCTCCATC	TTGCCTGCAGGAGAAACTG
Exon 11	CTGTTTCATATAATACAGAGTCCCTGAG	CTCTCCTGTTAAGCCTAATTTCCAAC
Exon 12	CTCCCACAGCATGACCTACC	GGAATTCACATGGTAATTTACAG
Exon 13	GGTAGCCAGCATGTCTGTGTC	CAAAACCTCCAAAAGCCAAG
Exon 14	TCAGAGAGAAGATGACCCAGG	TGTGTGTGCTAATGTCACCG
Exon 15	TTTAAGAATTTTTCTATCATTTGGC	TGTGGGGACCAAAAACACC
Exon 16	CCAATCCAACATCCAGACAC	GGCCCAGAGCCATAGAAAC
Exon 17	GGAAACGTTGCCTTAGAAGC	CCTCGGATGGATGTACCAAC
Exon 18	GCAAGTGCCGTGTCCTG	CCCAAACACTCAGTGAAACAAAG
Exon 19	CTCCACAGCCCCAGTGTC	TGGAGTTTCCCAAACACTCAG
Exon 20	CCATGCGAAGCCACACTGAC	ACATATCCCCATGGCAAACCTTG
Exon 21	TAACGTTTCGCCAGCCATAAG	AAAGGAATGTGTGTGTGCTG
Exon 22	AGACTGAAATCCCCTGTTGC	CGAGCTCACCCAGAATGTC
Exon 23	TCATGATCCCCTGCCTTCT	AGGGGTATTTACAGATGTTTCTGG
Exon 24	GTACAGTGCTGGCATGGTCTT	AATAATGCGATCTGGGACACA
Exon 25	AGAGAACCAAGGGGATTTTC	GGACCTAAAAGGCTTACAATCAAG
Exon 26	AACGATTAAGACAAAAATTAACACC	GGAAAAACCCACACAGGAAG
Exon 27	ACCAGGGTGCAGACTCTCAG	TGCAAATCTGCCACTGTTTC
Exon 28-1	AGGCTCCTGCTCCCTGTC	TTGGGAAAGAAGTCCTGCTG
Exon 28-2	CTGTGTCAACAGCACATTTCG	GTCTAAGAGCTAATGCGGGC
Exon 28-3	CACGGAGGATAGTATGAGCCC	TCCCCAATCAATAAAAATCCTC
Exon 28-4	TTTACAGAAACGCATCCAGC	AGTGTTTTGCAGTGGAAGCC
Exon 28-5	CCAACTGTGAGCAAGGAGC	TGGAATGGAAGACAAACAAGTC
Exon 28-6	AAGAAGAAACGGAGGGGATG	TTTTGCGAGAACAAAAGCG
Exon 28-7	AATAACTCGGATTCCAGCCC	CAGCTGACCTGGAGGGAAC
Exon 28-8	TCTCCTTTAGCCATCACCCC	AGCTGAATCTTCCAATCTTCC
Exon 28-9	ACAGGGCATTTTACAGGTGC	TGTGTGTGTGACTGAACATAACTG
Exon 28-10	TGTGCCCTGTAACCTGACTG	GTCTGGCATTGTGTCACGTTG

reported in primary tumors and cell lines from Japanese NSCLC patients. The subset of EGFR mutations associated with the gefitinib response might therefore be characteristic to Japanese cancer patients.

Overexpression of EGFR protein has been documented in gastrointestinal tract carcinoma (GITC) (34-36), but both types of EGFR mutation have not been fully examined in Japanese GITCs. In this study, therefore, we screened two

types of EGFR mutation in GITCs established mainly from Japanese patients.

Materials and methods

Cell lines and blood samples. We examined 29 human GITC cell lines comprising 11 esophageal (TE1, -2, -3, -4, -5, -6, -7, -8, -9, -10, -14, and -15; Cell Resource Center for Biomedical

Segment	Forward (5'-3')	Reverse (5'-3')
S1	CCCCTGACTCCGTCCAGTATT	GCTCATACTATCCTCCGTGGTCATG
S2	CTCGCCGCCAACGCCACAACC	GGCAGTTCTCCTCTCCTGC
S3	CTGACTCCGTCCAGTATTGA	CTGTGGATCCAGAGGAGGAGTAT
S4	CAACATGTTCGATGGACTTCCA	CTGTGGATCCAGAGGAGGAGTAT
S5	GCAAAGTGTGTAACGGAATAGG	AGAGTTCTCCACAAACTCCC
S6	GTGAAAACAGCTGCAAGGCC	TTCGCATGAAGAGGCCGATC
S7	GCCTAAGATCCCGTCCATCG	CCAGTTGAGCAGGTACTGGG
S8	AATCCTCGATGAAGCCTACG	GGCGCGGGGTTTCAGAGGCTGATTGTGAT
S9	AGAGTGATGTCTGGAGCTACGGGGTGAC	CTATCCTCCGTGGTCATGCT

Research, Tohoku University, Sendai, Japan), 6 gastric (MKN7, MKN28, MKN45, MKN74, GCIY, and GT3TKB; RCB, Tsukuba, Japan), and 12 colon (SW403, SW480, SW620, SW837, SW1463, CaCO₂, DLD1, HCT8, HT29, LS513, LS1034, and T84; American Type Culture Collection, Rockville, MD, USA) carcinomas. All of the esophageal and gastric cancer cell lines were established from Japanese patients, while the colon cancer cell lines were from non-Japanese patients. The cell lines were maintained under the recommended conditions.

DNA and RNA isolation. Genomic DNA was isolated with a Puregene DNA isolation kit (Gentra Systems, Inc., Minneapolis, MN, USA), and total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). mRNA was reverse-transcribed with a ThermoScript™ RT-PCR system and oligo (dT) (Invitrogen) to produce cDNA. DNA samples were extracted from blood of 30 healthy Japanese volunteers using an EZ1 DNA Blood kit (Qiagen Sciences, Valencia, CA, USA).

Polymerase chain reaction. Thirty-seven sets of primers corresponding to exons 1-28 of EGFR were synthesized Table I (primers are published as supporting information on the PNAS website: <http://www.pnas.org/cgi/content/full/0405220101/DC1>) (11). Primers of exons 1, 11, 20 and 23 were originally designed. DNAs isolated from cell lines were amplified by PCR. Briefly, 100 ng of DNA was amplified using a protocol consisting of 95°C for 2 min for initial denaturing, 35 cycles at 95°C for 1 min, 58°C for 30 sec, and 72°C for 2 min, with a final extension at 72°C for 5 min. The primers used for RT-PCR were synthesized according to a previous report (Table II) (29). Fifty ng of cDNA was amplified at 95°C for 9 min for initial denaturing, followed by 40 cycles at 95°C for 30 min, 58°C for 30 sec, and 72°C for 1 min, and a final extension at 72°C for 10 min. The final reaction volume for all PCRs was 50 µl, and the mixture contained 0.2 µl of each primer, 100 ng of DNA or 50 ng cDNA, 3 mM MgCl₂, 50 mM KCl, 2 mM each dNTP, and 1.25 U of AmpliTaq Gold (Applied Biosystems, Foster City, CA, USA).

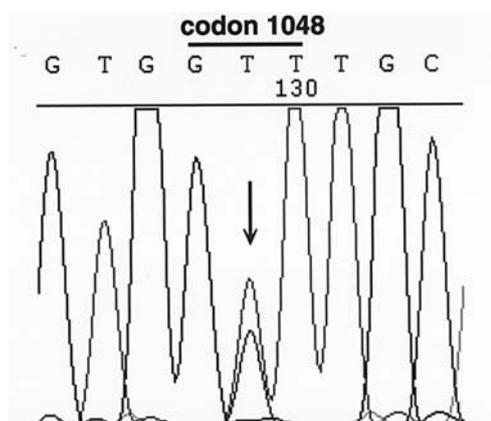


Figure 1. Sequence histogram of PCR-direct sequencing of exon 26 of the EGFR gene in the gastric cancer cell line MKN45. A point mutation at codon 1048 (GCT/GTT, Ala/Val) was observed. The remaining allele revealed the wild-type sequence.

Direct sequencing. The PCR products were electrophoresed on a 2% agarose gel and stained with ethidium bromide. A clonal band of the appropriate size was excised from the gel electrophoresis material and purified with a QIA quick Gel Extraction Kit (Qiagen Sciences). Cycle sequencing was performed using the same primer as that used for amplification of each segment, a BigDye Terminator Cycle Sequencing FS Ready Reaction Kit ver. 3.0 (Applied Biosystems), and an ABI PRISM 3100 DNA sequencer (Applied Biosystems).

Results

In the gastric MKN45 carcinoma cell line, we found a missense mutation at codon 1048 (GCT/GTT, Ala/Val) around the autophosphorylation site of the EGFR gene (Figs. 1 and 2). The remaining allele of the segment revealed the wild-type sequence (Fig. 1). This base substitution was not observed in any of the cell lines or DNA samples extracted from the healthy volunteers. Otherwise, 10 single nucleotide polymorphisms (SNPs) were detected; one with an amino acid substitution (codon 521, AGG/AAG, Arg/Lys) and the remaining 9 without

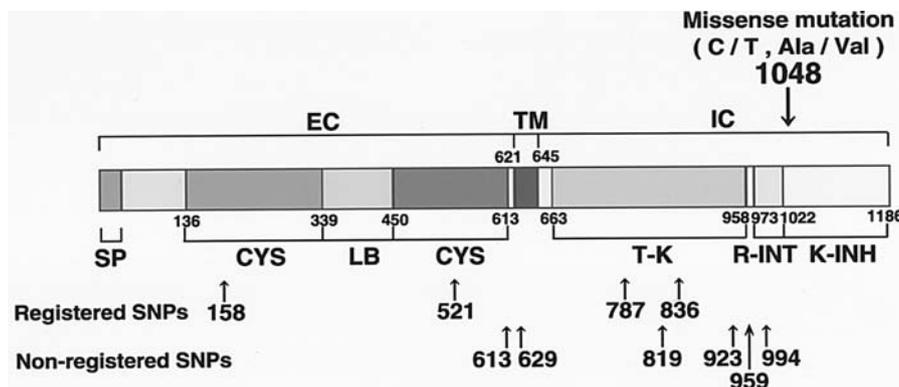


Figure 2. Diagram of structural and functional domains of wild-type EGFR, and summary of a point mutation and registered and non-registered SNPs detected in gastrointestinal tract carcinoma cell lines. EC, extracellular; TM, transmembrane; IC, intracellular; SP, signal peptide; CYS, cysteine-rich; LB, ligand binding; T-K, tyrosine kinase; R-INT, receptor internalization; K-INH, kinase-inhibitory. Numbers show location of the codon numbers.

it. Four SNPs (codon 158, GCT/GTT, Asn; codon 521, AGG/AAG, Arg/Lys; codon 787, GTG/GTA, Val; codon 836, CGC/CGT, Arg) have already been nominated in the Entrez SNP database (<http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Snpcmd=Limits>), but the remaining 6 have not (codon 613, GCC/GCT, Ala; codon 629, ACT/ACA, Thr; codon 819, GTG/GTA, Val; codon 923, ATC/ATT, Ile; codon 959, CCA/CCT, Pro; codon 994, GAC/GAT, Asp) (Fig. 2). No somatic mutations were found in any other PCR products. We further searched for splicing variants of EGFR mRNA by RT-PCR analysis, but none were found in any of the GITC cell lines.

Discussion

EGFR is widely distributed in human tissues, including endocrine glands, epithelia, liver, brain, placenta, and skin. Many studies have reported increased expression of EGFR in several human malignancies. EGFR, a 170-kDa glycoprotein, is composed of three domains; an internal domain with tyrosine kinase activity, an external ligand-binding domain, and a transmembrane domain (37). Because in Japanese NSCLCs somatic mutations in the tyrosine kinase domain of EGFR are more common in adenocarcinomas and non-smokers (10-23), we expected that a high proportion of Japanese GITCs would harbor EGFR mutations. However, we failed to find any somatic mutations in the kinase domain or EGFRvIII, which displays constitutive activity and is transformed in the absence of the ligand (24-26). The biological significance of the missense mutation detected in one of the gastric cancer cell lines should be further studied.

Reports from Western countries suggest an extremely rare incidence of EGFR mutation associated with the gefitinib response. Lynch *et al* (9) searched for EGFR mutations (exons 19-21) in a panel of 108 cancer-derived cell lines from various origins, including 7 head and neck and 5 colonic cancer cell lines (not including gastric cancer), but failed to detect any except in lung cancer cell lines. Moreover, Barber *et al* (38) demonstrated only one (0.3%) mutation in 293 colorectal cancers and 59 glioblastomas (exons 17-24). In an Asian study, Su *et al* (39) examined exons 18-21 in 89 hepatocellular carcinomas, but detected no EGFR mutation. Lee *et al* (40)

demonstrated the same mutation (an in-frame deletion mutation in exon 19, E746_A750del) in 3 (7.3%) of 41 head and neck carcinomas. Despite the large series of non-lung cancers examined in these Asian studies, EGFR mutations in NSCLC may show a degree of geographic restriction. Similar to the situation in Western countries, mutations of the EGFR gene associated with the gefitinib response may be infrequent in non-lung cancers.

A phase II trial of two doses of gefitinib monotherapy for refractory NSCLC in the United States reported an overall partial response (PR) rate (IDEAL-2) of 10% (5,6), and a 19% PR was observed in a companion European/Japanese study (IDEAL-1). Two subsequent phase III trials randomized previously untreated patients with advanced NSCLC to standard platinum-based chemotherapy, with or without addition of gefitinib at two doses (INTACT-1, cisplatin and gemcitabine ± gefitinib; INTACT-2, carboplatin and paclitaxel ± gefitinib) (7,8). These trials reported no difference in response rate, time to progression (TTP), or 1-year or overall survival (OS) with the addition of gefitinib to standard chemotherapy. These findings were nearly identical to the results of the TALENT and TRIBUTE studies, which had a similar design to INTACT but used the EGFR-tyrosine kinase inhibitor (TKI), erlotinib (41). Thus, despite randomized clinical studies involving nearly 4000 patients with advanced disease, there was no discernible improvement in outcome following the addition of EGFR-TKI to standard cytotoxic chemotherapy. Bell *et al* (42) also examined whether molecular alterations to EGFR affected response and survival within the above phase II (IDEAL) and phase III (INTACT) trials of gefitinib. Their data suggested that EGFR mutations and, to a lesser extent, amplification appear to identify distinct subsets of NSCLC that have an increased response to gefitinib. Combination of gefitinib with chemotherapy does not improve survival in patients with these molecular markers. Since almost all GITCs have no EGFR mutations, gefitinib is unlikely to be of value as a single drug or in combination with conventional chemotherapeutic agents.

However, for many kinds of solid tumors both *in vitro* and *in vivo*, various additive/cooperative effects of gefitinib combined with other types of chemotherapeutic agents, radiotherapy, and other forms of targeted molecular medicine



SPANDIDOS[®] tested (43-45). Teraishi *et al* (46) demonstrated gefitinib enhanced TNF-related apoptotic cell death, including ligand (TRAIL)-induced apoptosis, via activation of caspase-3 and -9, and inactivation of Bcl-xL in an esophageal cancer cell line (TE-8, which is a TRAIL resistant cell line). Thus, gefitinib may enhance other known and unknown molecular mechanisms that can be exploited for their anti-cancer effects. Even if gefitinib has no marked efficacy when used as a single drug or in combination with conventional chemotherapeutic agents in clinical trials of GITCs, there is no doubt that it does exert inhibitory effects on EGFR signaling pathways. Further studies aimed at strategies for targeting EGFR in GITCs are warranted, and gefitinib may still be a candidate agent for such strategies.

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