Epidermal Growth Factor Receptor (EGFR): 
Mutational and Protein Expression Analysis in Gastric Cancer

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Abstract. Background: Since specific epidermal growth factor receptor (EGFR) somatic mutations have been demonstrated to influence the response to anti-EGFR therapy in non-small cell lung cancer, EGFR gene mutational analysis of different types of neoplasm is under investigation. Patients and Methods: EGFR protein expression and gene mutations in exons 18, 19 and 21 were investigated in 49 gastric adenocarcinomas. Results: no specific EGFR gene mutations were detected, while EGFR positive staining was detected in 6% of the cases. Conclusion: Our findings indicate that in gastric adenocarcinoma, specific EGFR gene mutations are very rare or absent and the rate of EGFR protein expression is low.

Gastric cancer is the second leading cause of death-related cancer worldwide. The incidence varies greatly, ranging from 8.4 per 100,000 in USA to 77.8 per 100,000 in Japan, while in Europe, it is low in Northern and high in Eastern countries (1).

More than two-thirds of patients diagnosed with gastric cancer have unresectable disease and a median survival of 6 to 9 months. Moreover, in patients with resectable tumors, the local and distant recurrence rates are high, and the 5-year survival rate is less than 30% (2). Therefore, more effective agents are needed for the treatment of patients with advanced gastric cancer.

Protein kinases, by phosphorylating substrate proteins, direct the activity, localization and overall function of numerous proteins, and co-ordinate complex functions, such as growth and differentiation, cell cycle control and apoptosis, which are often disregulated in cancer development and progression (3, 4). Protein kinases are, therefore, important targets for molecular therapy and the inhibition of protein kinase by monoclonal antibodies or small-molecule drugs has been shown to be effective against cancer cells (5, 6).

The epidermal growth factor receptor (EGFR) is a 170,000 kDa transmembrane glycoprotein with a cytoplasmic tyrosine kinase domain (7). EGFR contributes to several essential tumorigenic mechanisms, such as tumor survival, invasion, angiogenesis and metastatic spread (8-10). EGFR expression has been described in numerous human tumors, and several authors have demonstrated that it is correlated with outcome (11).

Gefitinib (IRESSA, ZD1839), a low molecular weight, quinazoline derivative, inhibits EGFR tyrosine kinase (EGFR-TKI), which in patients with non-small cell lung cancer (NSCLC) has been shown to achieve a response rate of about 10% (12).

Two recent studies using NSCLC tissue samples from USA and Japan demonstrated a close association between the presence of somatic mutations on the EGFR gene and the response to gefitinib treatment (13, 14). More specifically, these mutations were clustered within exons 18, 19 and 21, and were detected in 2% of the cases from USA and 26% of the cases from Japan (13). In a retrospective analysis of 291 NSCLC patients, EGFR mutations in exons 19 and 21 were correlated with different tumor stages at the time of presentation; moreover, mutations in exon 19 were correlated with better outcome compared to mutations in exon 21 (15).

Recent experimental data indicate that the functional significance of these mutations is related to apoptosis inhibition via the down-stream targets of EGFR, such as extracellular signal-regulated kinase 1/2 (ERK1/2) and the v-akt murine thymoma viral oncogene homolog (AKT) (16).

EGFR gene mutational analysis of different types of neoplasm has been advocated since it may lead to the identification of tumor sub-sets more likely to respond to gefitinib. Moreover, an important synergistic effect of Gefitinib with Paxitaceel has been demonstrated in gastric cancer in vitro (17).
In addition, there is an increasing interest in immunohistochemistry-based EGFR screening methods to select cancer patients eligible for treatment with IMC-C225 (Cetuximab), a monoclonal antibody (MAb) directed against the EGFR protein, which has been shown to be effective in advanced colorectal cancer (18).

Therefore, the aim of this study was to evaluate the frequency of specific EGFR gene mutations and protein expression in a group of previously untreated gastric adenocarcinomas.

Patients and Methods

Patients. The patient population consisted of 49 fully informed consent patients (29 men and 20 women; mean age 69 years; age range 28-87 years), who underwent surgical resection for gastric adenocarcinoma between January 2002 and June 2004 at the Department of Oncological and Surgical Science, University of Padova, Padova, Italy. None of the patients had undergone preoperative chemo-radiotherapy. The gastric tumors were located in the upper third in 7 cases (14.3%), in the middle third in 13 cases (26.5%) and in the lower third in 27 cases (55.1%); two (4.1%) patients had diffuse disease. Tumors were staged according to the International tumor node metastasis (TNM) staging system, and the histologic grade was assessed according to World Health Organization (WHO) criteria: one of the tumors (2%) was stage IA, 12 (24%) were stage IB, 13 (27%) were stage II, 4 (8%) were stage IIIA, 4 (8%) were stage IIIB and 15 (31%) were stage IV.

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Two of the tumors (4.1%) were well differentiated, 14 (28.6%) were moderately differentiated, 27 (55.1%) were poorly differentiated and 6 (12.2%) were undifferentiated.

EGFR sequence analysis. Tumor DNA for the mutational analyses was isolated from paraffin-embedded tissue (QIAamp® DNA Mini Kit, Qiagen Inc., CA, USA) samples of each proband. Samples of tumor DNA were used to amplify sequences from encoding nucleotide region exons 18, 19 and 21 of the EGFR gene using polymerase chain reaction (PCR).

Mutation analyses were carried out using bi-directional sequencing on an automatic ABI3100 DNA analyser (Applera Corp., CT, USA). Point mutations of exons 18, 19 and 21 of the EGFR gene were sought using PCRs in tumor DNA with exon-specific primer pairs and bidirectional sequencing. We followed the method described by Lynch et al. (14), after making modifications to the protocol in order to achieve the specific amplification of specific exons. In particular, we used the new internal primer for subsequent exon-specific amplification from the template obtained. The results of sequence analysis were repeatedly confirmed on multiple aliquots of DNA.

Immunohistochemistry. Immunostaining for EGFR was performed on haematoxylin-/eosin-stained sections using the Dako EGFrpharmDx™ assay detection system (Dako Corporation, Carpinteria, CA, USA). After dewaxing in fresh xylene, 100% ethanol, 95% ethanol and 70% ethanol (four baths each), the slides were placed in a humid chamber for proteolytic digestion with proteinase K solution (100 μl for 5 min), followed by quenching of endogenous peroxidase for 5 min. After incubation for 30 min, the primary mouse anti-EGFR Mab was incubated for another 30 min with a labelled polymer, after which DAB localization of the positive cells was performed. Counterstaining was achieved with hematoxylin, followed by 10 slide dips in a bath containing 37 mmol/l ammonia water. The control slides, included in every run, contained sections of pelleted, formalin-fixed, paraffin-embedded cell line HT-29 with a moderate level of EGFR protein expression (positive control, IHC staining score of cell pellet is 2.5±0.5) and of the EGFR negative CAMA-1 cell line (negative control, score 0).

Membrane staining evaluated in the neoplastic cells was quantified and graded as recommended in the detection kit: 0 score: no staining observed, or membrane staining in >10% neoplastic cells, negative; 1+ score: weak complete and/or incomplete membrane staining in >10% neoplastic cells, positive; 2+ score: moderate complete and/or incomplete membrane staining in >10% neoplastic cells, positive; 3+ score: strong complete and/or incomplete membrane staining in >10% neoplastic cells, positive.

Results

Mutational analysis was achieved in all 49 gastric adenocarcinomas examined. Genomic DNA sequencing of exons 18, 19 and 21 failed to demonstrate any mutations.

Immunohistochemical analysis for the evaluation of EGFR membrane staining showed no staining in 46 cases (94%, score 0) and weak staining (1+) in two (4%) cases (the tumors were located in the antrum; one was T2bN2M0, poorly differentiated, the other T2aN0M0, moderately differentiated), while strong (3+) staining was observed in one (2%) case (the tumor was located in the antrum, was T3N2M1 and moderately differentiated) (Figure 1).

Discussion

The findings made in the present study indicate that EGFR gene mutations on exons 18, 19 and 21, known to be correlated with response to gefitinib in patients with NSCLC, are absent or very rare in gastric adenocarcinomas. This observation is in line with the recent findings made by Lee et al., who found no EGFR gene mutations in 185 gastric adenocarcinomas in a series of Korean patients (19), and by Mimori et al., who found a silent mutation in exon 20 in only 5 of 39 (5.1%) Japanese patients, but without any predicted amino-acid changes (20). It confirms that specific EGFR mutations are absent in gastric adenocarcinomas in western countries, too. This is relevant since the incidence of specific EGFR mutations can vary substantially with ethnicity, as demonstrated in the study by Paez et al., who found a significant difference between the mutation rate in NSCLC from USA (2%) and from Japan (26%) (13).

Since there is an increasing interest in immuno-histochemistry-based EGFR-screening methods using paraffin-embedded tumor specimens to select cancer patients eligible for treatment with IMC-C225 (Cetuximab), a promising monoclonal antibody (MAb) directed against...
Figure 1. EGFR protein expression in a case of moderately differentiated T3N2M1 human gastric adenocarcinoma. Formalin-fixed paraffin-embedded sections were immunostained using the primary mouse anti-EGFR monoclonal antibody. Immunohistochemistry showed strong (3+) membrane staining (A: 20x; B: 60x).
the EGFR protein (18), in this study we also assessed the status of the EGFR protein. Contrary to other epithelial malignances, including esophageal and colorectal cancer in which EGFR protein expression has been reported to be high (11), immunohistochemistry detected EGFR protein expression in only 6% of the cases. This is in agreement with the data reported by Takehana et al., who, in their series of 413 gastric carcinomas, found negative EGFR protein expression in 89.6%, low level in 8.2% and high level in 2.2% of cases (21). In addition, our mutation and immunohistochemistry data indicate that EGFR protein expression does not represent underlying exon 18, 19 and 21 alterations.

The results of the present study on EGFR mutational and protein expression indicate that in gastric adenocarcinomas, EGFR gene mutations on exons 18, 19 and 21 are very rare or absent and the EGFR protein expression rate is low.

References


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