Original Article

Low Correspondence of EGFR Mutations in Tumor Tissue And Paired Serum of Non-Small-Cell Lung Cancer Patients

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ABSTRACT

Objective: Epidermal growth factor receptor (EGFR) mutations are strong determinants of tumor response to EGFR tyrosine kinase inhibitors in non-small-cell lung cancer (NSCLC) patients. The aim of this study was to evaluate the correspondence between EGFR mutations in non-small-cell lung cancer tissues and in circulating DNA.

Methods: The research was conducted in 50 non-small-cell lung cancer patients who had undergone curative surgery, and in whom both serum and neoplastic tissues were available. Meanwhile sera of 33 cases of advanced NSCLC patients were also analyzed. DNA were extracted from each sample. Mutations of EGFR in exon18-21 were examined by PCR amplification method and direct sequencing.

Results: EGFR mutations were detected in 15 (30%) of 50 neoplastic tissue samples, 6 cases were in-frame deletion del E746-A750 in exon19, 9 cases were substitution in exon 21 (all were L858R except one was L861Q), but no mutated DNA resulted in paired serum circulating DNA samples of 50 resectable patients. As the 33 advanced NSCLC patients, EGFR mutations were detected in only 2 serum circulating DNA samples, all were L858R mutation in exon 21.

Conclusion: These data indicated that it was difficult to identify EGFR mutations in circulating DNA of NSCLC patients. The use of EGFR mutation in serum as a clinical method for decision making of TKI therapy is unsatisfactory.

Key words: Circulating DNA; Epidermal growth factor receptor (EGFR); Mutation; Non-small-cell lung cancer (NSCLC)

INTRODUCTION

Lung cancer is a major cause of cancer-related mortality worldwide and is expected to remain a major health problem in the future[1]. Targeting the epidermal growth factor receptor (EGFR) is an encouraging strategy for the treatment of non-small-cell lung cancer (NSCLC) as EGFR has been found to be expressed, sometimes strongly, in NSCLC tumors[2]. Studies have reported that EGFR mutations are strong determinants of tumor response to EGFR tyrosine kinase inhibitors (TKI) in NSCLC patients[3–6]. Most EGFR mutations have been identified retrospectively from operative resected tumor samples. However, it is sometimes difficult to obtain tumor samples from patients with inoperable NSCLC in prospective studies; thus, it is necessary to establish a method to detect mutant EGFR from other more readily accessible patient samples.

The finding that tumors are capable of shedding nucleic acids into the bloodstream has opened new areas in cancer research[7, 8]. Large amounts of tumor-derived DNA may be released from a tumor mass in which cell necrosis or lysis of tumor cells occurs, resulting in a very elevated serum DNA

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concentration[9–12]. Although considerably degraded, DNA can be recovered from a patient's serum or plasma and used as a surrogate source of tumor DNA. Accordingly, the number of studies evaluating the potential use of serum or plasma DNA in cancer diagnosis and prognosis has increased steadily in the past decade. In the case of non-small-cell lung cancer (NSCLC), some reports showed that K-ras point mutations and P53 mutation can be found in circulating DNA and were identical to the mutations found in the tumors[13, 14], but the results were not the same[15]. As to EGFR mutation in circulating DNA, there is only two reports showing the EGFR mutation in the serum consistent with those in the tumor samples of advanced NSCLC patients[16, 17]. The detection of EGFR mutations in serum DNA may provide a noninvasive and repeatable source of genotypic information that might facilitate clinical decision making at the time of diagnosis and in the later course of the disease, especially in patients with NSCLC treated with TKIs.

The purpose of the present study was to evaluate the correspondence of EGFR mutation of NSCLC patients in whom it was possible to analyze both tissue samples and serum.

MATERIALS AND METHODS

Patients

Clinical samples comprised 50 tumor samples that had been resected from non-small cell lung cancer patients (27 men and 23 women) at the time of operation From March 2004 to July 2006 and immediately frozen at -80°C. Peripheral Blood samples were obtained from each patient before surgery. Meanwhile blood samples of 33 cases of advanced NSCLC patients were also available. Separated serum was stored at -80°C until use. Written informed consent was obtained before collecting samples from each individual. Histopathologic diagnosis for these 50 resectable tumors included adenocarcinoma (n=24), squamous cell carcinoma (n=20), bronchioloalveolar carcinoma (n=2), large-cell carcinoma (n=2), and adenosquamous carcinoma (n=2). No patients received preoperative antitumor therapy except two that had received two cycles of neoadjuvant chemotherapy. Staging for these patients resulted as follows: stage I (n=16), stage II (n=18) and stage IIIa (n=16), according to TNM staging system. Histopathologic diagnosis for the 33 advanced patients included adenocarcinoma (n=19), squamous cell carcinoma (n=11), bronchiolo-alveolar carcinoma (n=2), and adenosquamous carcinoma (n=1). We analyzed EGFR mutations in the tissue samples and investigated EGFR mutations in the serum of all the patients.

DNA Extraction

DNA was extracted from tumor samples using a DNAeasy kit (U-gene biotechnology CO., LTD, China) according to the instructions of the manufacturer. The concentration and purity of the extracted DNA were determined by Beckman Coulter DU800 spectrophotometer.

DNA from serum samples of these patients was extracted using QIAamp (Qiagen, Basel, Switzerland) DNA blood kit. The extraction was performed according to the supplier's recommendations. The resulting DNA was eluted in 50 μl buffer (provided by the kit). The serum DNA can not be quantified by spectrophotometer because of the very low amount of DNA.

PCR Amplification and Sequence Analysis

Amplification of exons 18 to 21 were done in duplicate for each sample obtained from serum and tissue specimens. Primer sequences for EGFR exons 18 to 21 were obtained from Shigematsu H, et al.[6]. All PCR assays were carried out in a 50 μl volume that contained 200 ng of tissue genomic DNA or 6 μl of serum DNA and 2.5 units of Taq DNA polymerase (Takara). DNA was amplified for 35 cycles at 95°C for 45 s, 65°C for 30 s, and 72°C for 45 s, followed by a 10 min extension at 72°C. PCR products were electrophoresed in 1% agarose gel .Only those PCR products producing a positive band were send to Beijing AuGCT Biotechnology Co., Ltd, China for purification and sequence analysis. Sequencing was carried out in an automated DNA analyzer (ABI Prism 3730; PE Biosystems). The sequences were compared with the GenBank-archived human sequence for EGFR (accession no.AY588246). All sequence variants were confirmed by sequencing the products of independent PCR amplifications.

RESULTS

PCR Analysis

Exon 18 to 21 could be amplified in all tissue samples, 42 of 50 resectable patients serum and 24 of 33 advanced patients serum. Each PCR product

produced a positive band. Exon18 was a 262 bp band, exon19 was a 265 bp band, exon20 was a 362 bp band and exon21 was a 297 bp band (Figure 1).

Detection of EGFR Mutations in Serum and Tissue by Direct Sequencing

In 50 tumor tissue samples, we detected 15 EGFR mutations (30%). Sequence analysis revealed deletions within exon 19 in 6 of the 50 NSCLC samples. The types of deletion all were the same kind- del E746-A750 (2235–2249 del GGAA-TTAAGAGAAGC). Sequence analysis of exon 21 showed that 8 of the 50 NSCLC samples contained the L858R mutation (2573 T>G), and only one contained the L861Q (2582 T>A) mutation (Figure 2). No mutations were detected in exon 18 and exon 20 in the series of patients. All samples with sequence mutations were heterozygous mutation. Most patients with mutation were adenocarcinoma (13 of 15 mutant samples) and female (12/15).

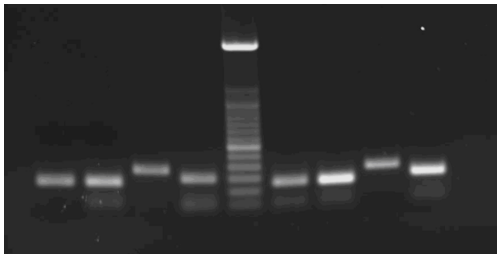

Figure 1. PCR amplification of EGFR gene in exon 18–21. The four band on the left is from serum DNA, the four band on the right is from tissue DNA.

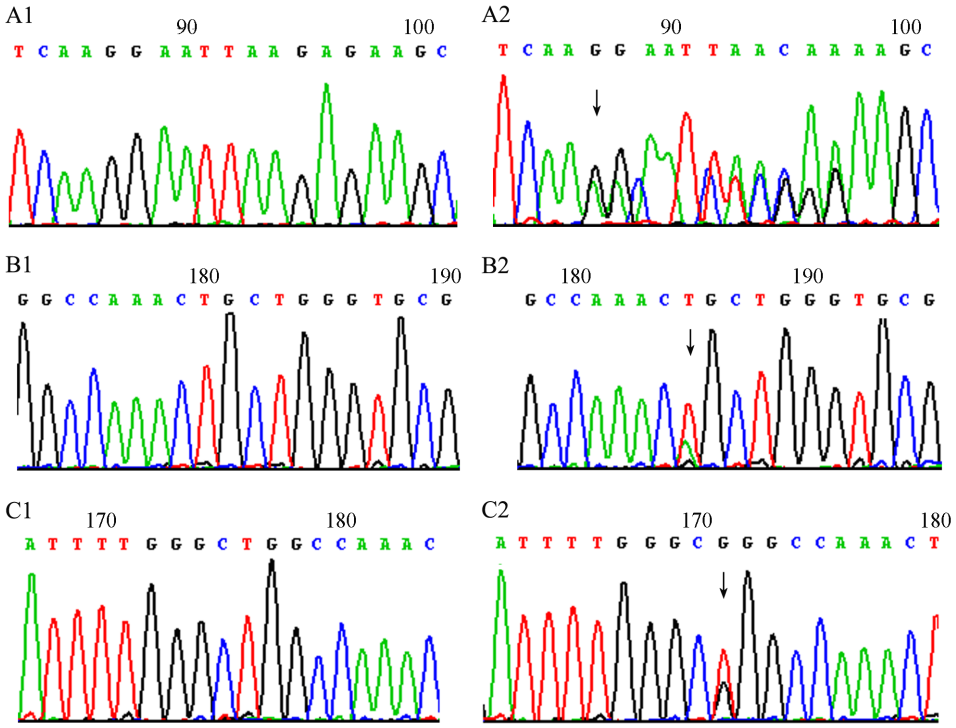

Figure 2. Sequence electropherograms of mutations in the EGFR gene of tumor tissue sample. Sequence of nucleotides and amino acids according to Genbank Accession No. AF288738. A1: exon19 wild type; A2: exon19 del E746–A750 (2235–2249 del GGAATTAAGAGAAGC); B1: exon21 wild type; B2: exon21 L861Q (2582 T>A); C1: exon21 wild type; C2: exon21 L858R (2573 T>G).

No mutation of EGFR was detected in the serum DNA of the 15 patients with tissue EGFR mutations as well as of the 35 patients without tissue EGFR mutations. No correspondence of EGFR mutation was observed between cancer tissue and paired circulating DNA in resectable NSCLC.

However, in the 33 advanced NSCLC patients serum samples, EGFR mutations were detected in only 2 serum circulating DNA samples, all were L858R mutation in exon21 (Figure 3).

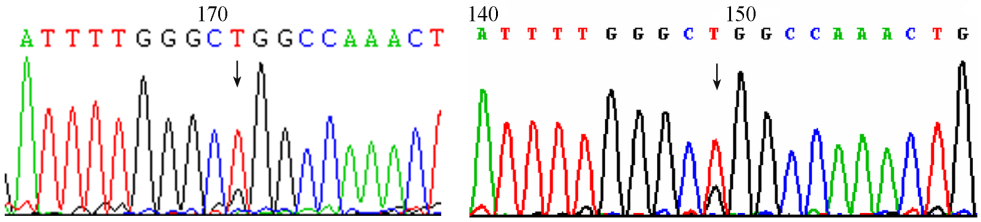

Figure 3. Exon21 mutation sequence L858R (2573 T>G) of 2 advanced patients serum samples.

DISCUSSION

The targeting of the ATP binding pocket of the epidermal growth factor receptor (EGFR) tyrosine kinase, by the small molecule drugs gefitinib and erlotinib, represents a promising new therapeutic strategy in non-small cell lung cancer. However, it is now apparent that only a subset of patients respond to such treatment. Two publications in early 2004 reported the presence of activating mutations in the EGFR tyrosine kinase gene conferring exquisite sensitivity to these drugs[3, 4]. Several publications have since reported prospective data consistent with this finding. Mutation rates are higher in non-smokers, women, East Asians and adenocarcinoma or bronchioloalveolar carcinoma patients[5, 6, 18–20].

In this study we detected 15 EGFR mutations in 50 early stage NSCLC patients. The mutation rate is 30%, just like other data about EGFR mutation in Asian. The types of mutation are also the major types in exon19 and exon 21. Most patients with mutation were adenocarcinoma (13 of 15 mutant samples), female (12/15) and non-smokers (10/15), as same as the other reports[21]. On the contrast, there is no mutation detected in paired serum of the patients, even in the tumor positive cases.

The aim of our study was to assess the clinical reliability of EGFR mutation detection in serum. Despite what was mentioned in some articles, we failed to find any mutation in all early stage resectable patients we analyzed, in contrast with the positive findings of other reports[16, 17]. The lack of assess ability of this alteration in our serum series may be ascribed to several reasons.

EGFR mutation in circulating DNA has been previously reported only in two studies for NSCLCs. In the two reports, all samples were stage IIIB and IV, but for our study the patients were stage I-IIIA resected patients. Some studies have shown that significantly higher DNA levels were found in the serum of patients with metastatic disease[9], and circulating DNA concentrations correlated to advanced tumour stages[22]. So maybe the small amount of circulating DNA of our samples is not enough for the detection of EGFR mutation.

On the other hand, all samples with sequence mutations were heterozygous mutation. That means the wild type and mutant type sequence coexist in the samples. The percentage of mutant sequence may be different in early stage and advanced NSCLC.

However in the 33 advanced NSCLC patients serum samples, EGFR mutations were detected only in 2 serum circulating DNA samples, all were L858R mutation in exon21. The finding of the mutation in the advanced patients was far less than the mutation rate previous reported.

Thirdly we think that the routine methods are not sensitive enough for detecting EGFR mutation in the serum of NSCLC patients. Sequence analysis of tumor DNA is the most widely applied method for detecting EGFR mutations, and is considered the most direct and reliable approach. However, the method has several disadvantages, the most notable being difficulties in detecting mutations in the presence of contaminated normal tissues in tumor samples. Specimens usually contain substantial proportions of normal cells, such as those from fibrous tissue and peripheral blood. Previous reports have noted that a positive result from direct sequencing requires ≥30% of mutant DNA in the sample[23]. The DNA freely circulates in blood plasma not only from tumor cells' necrosis or lysis[9–12], but also from normal cells' renewal[24]. Furthermore the DNA from tumor cells includes both wild type and mutant type. The interfusion of normal or wild type DNA with mutant type DNA prevents the detection of mutations in the serum or plasma. Therefore, it is necessary to enhance the sensitivity and specificity of the detection of EGFR mutations from tumor-derived DNA.

The results of the present investigation indicate that it is difficult to identify EGFR mutations in DNA extracted from the serum of NSCLC patients.

This lack of detection would preclude, at present, the use of EGFR mutation in serum as a clinical method for decision making of TKI therapy.

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