siRNA Targeting against *EGFR*, a Promising Candidate for a Novel Therapeutic Application to Lung Adenocarcinoma

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Key Words

Epidermal growth factor receptor • Gefitinib • Lung cancer • siRNA

ated knockdown is effective in lung adenocarcinomas with *EGFR* mutation, probably in those with resistance to gefitinib by acquired mutation in *EGFR*. Copyright © 2008 S. Karger AG, Basel

Abstract

Objective: To understand the molecular pathogenesis of lung cancer and to establish a novel therapeutic application, we examined the genetic alterations in lung cancer, and studied the effects of gefitinib and siRNA-mediated knockdown of *EGFR* on lung cancer. **Methods:** We analyzed mutations in *EGFR*, *KRAS*, *TP53*, and *ERBB2* in 198 surgically resected lung cancer specimens. We then analyzed the effects of gefitinib and siRNA treatment on lung adenocarcinoma cell lines. **Results:** Mutations in *EGFR* were found only in adenocarcinoma (35 of 106 adenocarcinoma), mainly in females (73%). Mutually exclusive mutations of *EGFR* and *KRAS* genes were observed. Mutations of *EGFR* were well associated with a positive response to gefitinib. Cells with *EGFR* mutations were very sensitive to gefitinib as well as siRNA-mediated knockdown of *EGFR*, those with *KRAS* mutations responded poorly, and those without mutations of *KRAS* and *EGFR* showed moderate responses to both treatments. **Conclusions:** Our present results imply that (1) mutation analyses of *EGFR* and *KRAS* provide valuable information about whether or not to apply treatments targeting against *EGFR* and the selection of dosage for such treatments, and (2) siRNA-medi-

Introduction

Recently, lung cancer has become the most frequent cause of cancer death worldwide [1]. In Japan, the number of cancer deaths from this disease accounts for 19.1% of the total, and it is the leading cause of cancer-related death in 2005 (<http://ganjoho.ncc.go.jp/professional/statistics/statistics.html>). The mean 5-year survival rate of this disease in Japan is only 14.9% (http://www.mc.pref.osaka.jp/ocr_e/ocr/survival.xls). Hence, the establishment of more effective methods for the treatment of patients with this disease is an urgent task with the highest priority. Although various refinements of the conventional therapies for lung cancer such as surgery, radiotherapy, chemotherapy, and their multidisciplinary applications have been tried, the improvement in the survival rate remains far from satisfactory. A newly developed, molecular-targeted antitumor drug, gefitinib (Iressa, AstraZeneca Pharmaceuticals, Macclesfield, UK), has recently become one of the main foci of the researchers' attention. Gefitinib interacts with the ATP cleft within

Table 1. Nucleotide sequences of the primer sets and PCR conditions

Name	Forward primer 5' to 3'	Reverse primer 5' to 3'	Size of product bp	Annealing temperature °C
<i>EGFR</i> exon 18	AGCTTGTGGAGCCTCTTACACC	CTGTGCCAGGGACCTTACCT	143	60
<i>EGFR</i> exon 19	CCATCTCACAATTGCCAGTT	ATCGAGGATTTCTTGTG	146	60
<i>EGFR</i> exon 21	GTGAAAACACCGCAGCATGT	TGTCAGGAAAATGCTGGCTG	133	55
<i>ERBB2</i> exon 20	GTGTGTGGTCTCCCATACCCCT	CCGTGGATGTCAGGCAGAT	116	60
<i>KRAS</i> exon 2	AGGCCTGCTGAAAATGACTG	CCTCTATTGTTGGATCATATTC	125	55
<i>TP53</i> exon 5	CGTGTTCAGTTGCTTTATC	CAATCAGTGAGGAATCAGAG	328	60
<i>TP53</i> exon 6	AGAGACGACAGGGCTGGTT	CACTGACAACCACCTTAAC	230	60
<i>TP53</i> exon 7	CTGCTTGCCACAGGTCTCC	ATGGAAGAAAATCGGTAAG	268	60
<i>TP53</i> exon 8	GACAGGTAGGACCTGATTTTC	TGAATCTGAGGCATAACTGCA	256	60

the tyrosine kinase domain of the epidermal growth factor receptor (EGFR) and inactivates its tyrosine kinase activity, resulting in downregulation of downstream effectors [2]. Several clinical trials have indicated that the patients who enjoy benefits from gefitinib were non-smoking Japanese female adenocarcinoma patients [2, 3]. Recently, other studies have found that such gefitinib-responsive cases harbor highly frequent somatic mutations within the EGFR kinase domain [4, 5]. As a contraindication, gefitinib possibly increases an adverse side effect affecting the lung, interstitial pneumonia, that can result in a fatal outcome within a short period of time [6]. Therefore, we need to establish a more accurate compass for the usage of gefitinib. Alternative therapeutic methods without adverse side effects also need to be developed. In this study, we first explored the mutations in *EGFR* along with lung cancer-associated genes such as *KRAS*, *TP53*, and *ERBB2*; the last encodes EGFR homologous protein [7]. To shed new light on the understanding of the molecular pathogenesis of lung cancer and to establish a novel therapeutic application, we examined the effects of siRNA-mediated knockdown of *EGFR* and compared them with effects of gefitinib on lung cancer cells.

Materials and Methods

Lung Cancer Tissues and Cell Lines and Their Mutation Analyses

A total of 198 lung cancer specimens (all from Japanese patients; 139 males and 59 females; 106 adenocarcinomas, 52 squamous cell carcinomas, 20 large cell carcinomas, 12 small cell carcinomas, and 8 others) were analyzed for mutations at hot spots in the *EGFR* (exons 18, 19 and 21), *ERBB2* (exon 20), *TP53* (exons 5 through 8), and *KRAS* (exon 2) genes. In our series of 198 pa-

tients, 18 cases (17 adenocarcinomas and 1 large cell carcinoma; 7 males and 11 females) were treated with gefitinib. Fresh-frozen primary tumor tissues were collected at the time of surgery and cut into pieces. Six-micrometer-thick sections were stained with hematoxylin and eosin and evaluated by microscopy to assess the percentage of neoplastic cells. Tumors with more than 80% neoplastic cellularity were microdissected. Genomic DNAs were isolated with a Qiagen DNAamp microkit (Qiagen, Valencia, Calif., USA) according to the manufacturer's instructions. Nucleotide sequences of the primer sets and the PCR conditions are summarized in table 1, and the experiments were performed with the methods described by Sakurada et al. [8].

Six human lung adenocarcinoma cell lines were used for in vitro studies. A549, LK87, 11-18, and HT-1 were obtained from the Cell Resource Center for Biomedical Research (IDAC, Tohoku University, Sendai, Japan), PC-9 was provided by Dr. K. Nishio (National Cancer Center Research Institute, Tokyo, Japan), and H3255 was provided by Dr. P.A. Jänne (Dana Farber Cancer Institute, Boston, Mass., USA). All these cell lines were derived from lung adenocarcinoma cases. Cells were maintained by methods recommended by the suppliers. PC-9 and H3255 harbor mutations in *EGFR* (15-bp deletion and L858R, respectively), A549 and LK87 harbor mutations in *KRAS* (G12S and G12D, respectively), and 11-18 and HT-1 harbor no mutations in either of these two genes.

siRNA Design and Transfection to Lung Cancer Cell Lines

We designed oligonucleotides for siRNA targeting against *EGFR* using the siDirect website (<http://design.RNAi.jp>) [9]; two pairs of siRNAs, termed 1565 and 1252, were purchased from Japan Bioservices (Asaka, Japan). These correspond to nucleotides 1565–1583 and 1252–1270 of *EGFR*, respectively, and the nucleotide sequences were 5'-GUCAGCCUGAACAUACAUC-3' and 5'-GUGUACGGAAUAGGUAAU-3' in the sense strand. Both of the sense and corresponding antisense strands harbor two deoxyribonucleotides dTdT overhangs, and were annealed as previously described [10]. Then the siRNAs were transfected into the cell lines using the oligofectamine reagent (Invitrogen, Carlsbad, Calif., USA) according to the manufacturer's instructions. *GL2* (the luciferase gene) siRNA was used as the experimental control as described previously [10].

a			
Type of Mutation	No. of Cases	Sequence	
L858R	9	codon 858 CTG to CGG	
G719S	1	codon 719 GGC to AGC	
Del-1 (15-bp del)	12	AA- --- --- --- --- --A ACA TCT CCG	
Del-2 (15-bp del)	7	AAG --- --- --- --- --A ACA TCT CCG	
Del-3 (15-bp del) AB221348	1	AAG --A T-- --- --- --- --A TCT CCG	
Del-4 (15-bp del)	1	AAG GA- --- --- --- --- --A TCT CCG	
Del-5 (10-bp del/1-bp ins)	1	AAG GAA --- -C- --- -CA ACA TCT CCG	
Del-6 (18-bp del)	3	AAG GAA T-- --- --- --- --- -CG	
EGFR	codon 745	K	E L R E A T S P
EGFR (NM_005228)	nt 2233	AAG GAA TTA AGA GAA GCA ACA TCT CCG	

b			
Type of Mutation	No. of Cases	Sequence	
Ins-1 (12-bp ins)	2	GAA <u>GCA TAC GTG ATG</u> GCT GGT GTG GGC TCC CCA TAT <div style="text-align: right;"><u>GGTCCCCA</u></div>	
Ins-2 (9-bp ins) AB221350	1	GAA GCA TAC GTG ATG GCT GGT GTG <u>GGC TCC CCA</u> TAT <div style="text-align: right;"><u>GGG</u></div>	
Ins-3 (3-bp ins) AB221349	2	GAA GCA TAC GTG ATG GCT GGT GTG <u>GGC</u> TCC CCA TAT <div style="text-align: right;"><u>GGG</u></div>	
ERBB2	codon 770	E	A Y V M A G V G S P Y
ERBB2 (NM_004448)	nt 2308	GAA GCA TAC GTG ATG GCT GGT GTG GGC TCC CCA TAT	

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Fig. 2. a The human lung adenocarcinoma cell lines PC-9, H3255, A549, LK87, 11-18, and HT-1 were used. PC-9 and H3255 harbor mutations (Del-1 type 15-bp deletion and L858R, respectively) in *EGFR*. A549 and LK87 harbor mutations in *KRAS* (G12S and G12D, respectively) but not in *EGFR*, and 11-18 and HT-1 harbor no mutations in either of these two genes. MTT assay measured after 72 h culture in media with gefitinib. A total of 5×10^3 cells were seeded in 96-well plates, and 72 h after siRNA transfection or addition of appropriate concentration of gefitinib, the medium was replaced with 100 μ l of 0.05% MTT/PBS(-). After 1 h of incubation, the MTT solution was replaced with 100 μ l of 100% ethanol. The absorption of the cells that were completely suspended in ethanol was measured with a Versamax microplate reader (Amersham Bioscience). Results are plotted in a logarithmic manner. Concentration of gefitinib in culture medium is indicated below the horizontal axis. Viability indicated on the vertical axis represents the absorption ratio to cells without gefitinib. **b** Cells after 72 h culture in media with gefitinib are shown. LK87, HT-1, and PC-9 are representatives from the three groups: mutation with *KRAS* but not in *EGFR*, no mutations in either of these two

genes, and mutation with *EGFR*. Surviving cells dramatically decrease in accordance with the increased gefitinib concentration in PC-9. HT-1 was moderately decreased, and LK87 was slightly decreased. In contrast, dead cells that are floating in media dramatically increased in accordance with the increased concentration of gefitinib in PC-9, and moderately increased in HT-1. A small number of floating dead cells were observed in LK87. **c** MTT assay measured after 72 h culture in medium with siRNA against *EGFR*. siRNA 1565 was used, and results are plotted in a logarithmic manner. Concentration of siRNA used for transfection is indicated below the horizontal axis. **d** Cells after 72 h culture in media with siRNA against *EGFR* are shown. The proportion of the surviving and dead cells showed in general the same tendency as the results after gefitinib treatment. **e** Western blot analyses after siRNA treatment targeting against *EGFR* are shown. The antibodies used were rabbit antihuman *EGFR* (Cell Signaling Technology, Beverly, Mass., USA) and mouse anti-human β -actin antibody (Sigma, St. Louis, Mo., USA); the latter was used as an internal control. Concentrations of siRNA are indicated above the lanes.

tions had good responses to gefitinib; 6 of them were partial response and 2 were stable disease. One case initially responded to gefitinib, but then developed progressive disease and died. In 2 cases that we judged as stable disease, the apparent lung lesions were stable, but the abdominal lesions such as those in the para-aortic lymph node regressed in size. Hence gefitinib was more or less effective in lung adenocarcinoma patients with *EGFR* mutation. On the other hand, none of the cases without *EGFR* mutations responded markedly to gefitinib treatment. The efficacy of gefitinib was significantly different between cases with and without *EGFR* mutations ($p = 0.0008$). One patient with a mutation in *ERBB2* did not respond to gefitinib treatment.

In vitro Studies Looking for Ideal Usage of Gefitinib

To establish personalized medicine for lung cancer patients, we need to obtain more information about the appropriate use of gefitinib. To establish the ideal dosage of gefitinib, with minimum frequency of adverse side effects without decreasing the responsiveness of this drug, is one of the most important demands with the highest priority. For this purpose, we performed in vitro studies using human cancer cell lines with *KRAS* mutations (A549 and LK87), *EGFR* mutations (PC-9 and H3255), and without mutations in either *KRAS* or *EGFR* (11-87 and HT-1). An MTT assay measured after 72 h of culture in medium with gefitinib showed three clearly different patterns of growth curves (see fig. 2a). Cells with *KRAS* mutations did not show any apparent responsiveness to a high concentration of gefitinib ($IC_{50} > 2 \mu M$), but cells with *EGFR* mutations were responsive even to lower concentrations (IC_{50} is about 5 nM). Gefitinib was also effective against cells without mutations in either *KRAS* or *EGFR* when the concentration was increased (IC_{50} is about 2 μM). Typical examples of the three different types of cells after gefitinib treatment are shown in figure 2b; the results are very consistent with those with the MTT assay.

Effects of siRNA-Mediated Knockdown against EGFR

To study whether or not the knockdown of *EGFR* has the same effects on cancer cells as gefitinib, we performed siRNA experiments to observe the effects on the in vitro growth of several human lung cancer cell lines. We designed two siRNAs: because the efficiency of siRNA 1565 was slightly better than siRNA 1252 although the results were basically the same, we used siRNA 1565 for further experiments. The growth curve we obtained showed the expected results; as shown in figure 2c, cells with *EGFR* mutations were very sensitive to the *EGFR* knockdown

(IC_{50} is about 10 nM), those without mutations in either *EGFR* or *KRAS* were moderately sensitive (IC_{50} is about 200 nM), and those with *KRAS* mutations were poorly sensitive ($IC_{50} > 200$ nM). Typical examples of the three different types of cells after siRNA treatment are shown in figure 2d; the results were very consistent with those of the MTT assay. We observed no growth suppression by *GL2* siRNA treatment in the cells used (data not shown). When we monitored the efficiency of siRNA intake, we analyzed surviving cells by Western blot analysis; the expression of *EGFR* protein was markedly suppressed in cells without *EGFR* mutations (see fig. 2e). In the cells with *EGFR* mutations, it was not possible to monitor the *EGFR* protein level after siRNA treatment because of the immediate death of most of the transfected cells.

Discussion

Gefitinib interacts with the *EGFR* protein and inhibits the tyrosine kinase activity by binding to the ATP binding cleft of the tyrosine kinase domain [3]. Several reports have suggested that gefitinib harbors a valuable antitumor activity, and there is the promising possibility that it is a novel treatment option for patients with advanced non-small cell lung cancer, especially adenocarcinoma [2, 3, 17–19]. However, the use of this drug is controversial; some reports support and others deny a benefit for lung cancer patients. In our series of patients, 18 were treated with gefitinib; all the cases with *EGFR* mutations showed tumor regression after gefitinib administration. On the other hand, none of the cases without *EGFR* mutations responded markedly to gefitinib treatment. These results suggest the importance of checking *EGFR* mutation status before administration of gefitinib.

ERBB2 is one of the *EGFR* kinase subfamilies, and the *ERBB2* mutation was reported at a frequency of 9.8% (5/51) in lung adenocarcinoma [7]. Although this frequency is lower than that of *EGFR*, it is likely that these mutations also play important roles in the pathogenesis of lung adenocarcinomas. However, the effectiveness of gefitinib against tumors with *ERBB2* mutations is not clear in this study; only one case was treated with gefitinib and did not respond to the drug. We did not have any lung cancer cell lines with mutations in *ERBB2*, so it was not possible to further analyze the sensitivity of such mutations to gefitinib and siRNA-mediated knockdown.

Mutations of *EGFR* and *KRAS* were mutually exclusive, consistent with previous reports [12, 16]. *TP53*, how-

ever, did not show any association with *EGFR* mutations. These results may reflect one of the aspects of the differing molecular pathogenesis of lung adenocarcinomas. This evidence is reasonable; *KRAS* is on the downstream of *EGFR* in the MAPK cascade, and activation of one of these molecules should be enough for upregulation of cell growth mediated by MAPK cascade. Hence tumors with activated *KRAS* do not require activated *EGFR* in their tumorigenesis, and gefitinib will not be effective against tumors with *KRAS* mutations. Indeed, such cells were very resistant to gefitinib treatments.

A previous in vitro study showed that there were more than 10-fold differences in the effective dose of gefitinib in resistant and sensitive cell lines [20]. In our study however, the IC_{50} for cells with *EGFR* mutations was less than that for other cells by 400-fold or more. Our results may have implied a possibility that a lower concentration of gefitinib is enough to suppress the growth in cells with *EGFR* mutations. The present clinical dose of 250 mg/day, resulting in mean steady-state plasma concentrations of 400–1,400 nM [21], could be excessive for cases with *EGFR* mutations (see fig. 2a, b); 200 nM is enough in these two cells with *EGFR* mutation. To avoid the various adverse side effects of gefitinib, our data imply that the ideal dose of gefitinib can be settled depending on the mutation status. Second, it is possible that an increased dose of gefitinib lowers the viability of cells without *EGFR* mutations, particularly those also without a *KRAS* mutation. Third, cells with *KRAS* mutations probably do not have a beneficial response to gefitinib intake. Decreasing the amount of gefitinib will probably reduce the fatal adverse effects of the medication such as interstitial pneumonia. Because the risk factors for such adverse side effects are not clearly understood at present, decreasing the dose of gefitinib will surely help reduce the incidence of lethal side effects.

In the present study, we considered the possibility of better alternative therapies other than gefitinib and tested siRNA-mediated *EGFR* knockdown. Our MTT assay demonstrated promising possibilities for siRNA treatment; cells with the *EGFR* mutation responded well, similarly to their response to gefitinib treatment. Furthermore, cells without mutations in *EGFR* and *KRAS* showed moderate sensitivities to siRNA-mediated *EGFR* knockdown. It is notable that cells with *KRAS* mutation were also slightly sensitive to *EGFR* knockdown. The possibility that siRNA-mediated knockdown may provide a novel method for the treatment of lung adenocarcinoma includes the hope that it may prevent adverse side effects such as interstitial pneumonia. There is also a possibility

that siRNA-mediated knockdown might show synergistic enhancement with other anticancer drugs as was observed in other human cancer [10]. However, we need further investigation to overcome the hurdles for clinical application including the delivery system or effects on tissues other than tumor.

Recently, the second point mutation of T790M in *EGFR*, and also D761Y, led to losing the gefitinib sensitivity [22–24]. The T790M mutation leads to the steric hindrance of *EGFR* inhibitor binding, owing to the presence of the bulkier methionine side chain in the ATP-kinase binding pocket [22]. The siRNA-mediated knockdown of *EGFR* has the possibility of being effective even for cases with acquisition of additional *EGFR* mutations that cause gefitinib resistance, as long as without *KRAS* mutation, because the RNAi interaction is not affected by the structural change of protein.

Cells without *EGFR* mutation also showed some sensitivity to siRNA, although the sensitivity was lower than that with *EGFR* mutations. A previous report of siRNA targeting against *EGFR* failed to suppress the growth of cancer cells with a wild-type *EGFR* gene [20], but our present siRNA results may provide the option of a novel and effective treatment for such cancer patients. The reasons for these differences are not clear, but some factors such as differences in target sequences or the mutation status of *KRAS* in the studied cells may have been involved.

In this report, we have suggested that the aspects of *EGFR* mutations are significant characteristics of Japanese lung adenocarcinoma patients and indicated the importance of considering the mutation status of tumor when calculating the administration dose of gefitinib. Additionally, we discussed the possibility of the RNAi technique for in vivo abrogation of the progression in lung cancer.

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