EGFR mutations predict a favorable outcome for malignant pleural effusion of lung adenocarcinoma with Tarceva therapy

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Abstract. The aim of the present study was to evaluate the therapeutic effects and adverse reactions of Tarceva treatment for malignant pleural effusion (MPE) caused by metastatic lung adenocarcinomas. One hundred and twenty-eight patients who failed first-line chemotherapy drug treatment were divided into a mutation and a non-mutation group according to the presence or absence of epidermal growth factor receptor (EGFR) mutations. Each patient received closed drainage combined with simple negative pressure suction after thoracoscopic talc poudrage pleurodesis and oral Tarceva treatment. Short-term and long-term clinical therapeutic effects of Tarceva were evaluated. The EGFR mutation rate in pleural metastatic tissues of lung adenocarcinoma acquired through video-assisted thoracoscopic surgery was higher compared to that in surgical resection specimens, plasma specimens and pleural effusion specimens compared to previously reported results. There were significant statistical differences in the average extubation time ($p<0.01$), drainage volume of pleural effusion ($p<0.05$), Karnofsky score and formation of encapsulated pleural effusion 4 weeks after surgery ($p<0.05$) between these two groups. The number of patients with mild pleural hypertrophy in the mutation group was significantly higher compared to the non-mutation group ($p<0.01$), while the number of patients with severe pleural hypertrophy was significantly reduced ($p<0.05$). There was significant statistical discrepancy between these two groups in terms of improvement of peripheral blood carcinoembryonic antigen and tissue polypeptide antigen after 4 weeks of therapy. The complete remission rate and the efficacy rate were higher in the mutation group compared to that in the non-mutation group ($p<0.05$). There was a longer overall survival time after Tarceva treatment in patients with EGFR mutations than those without EGFR mutation. EGFR mutations predict a favorable outcome for malignant pleural effusion of lung adenocarcinoma with Tarceva therapy. Detection of EGFR mutations may determine the responsiveness of malignant pleural effusion to Tarceva treatment.

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Introduction

Lung cancer ranks as the leading cause of cancer-related death among all malignant tumors (1). At a late stage of the disease, pleural metastasis and malignant pleural effusions (MPEs) are common. Although all cell types of lung cancer may cause MPEs, adenocarcinoma is the most common cell type (5). MPE is also a common complication of other advanced tumors such as breast cancer, lymphoma and mesothelioma. MPEs caused by breast and lung cancer account for approximately 75% of all cases (2-4).

At present, the treatments for MPEs include perfusion, surgery, radiotherapy and hyperthermia. Chest tube drainage and chemical pleurodesis are the standard care for malignant pleural effusions (6). Closed drainage with negative pressure suction helps to expel the residual gas and pleural effusion, adhere visceral and parietal pleural, and thus reduce the incidence of pleural hypertrophy and the formation of encapsulated pleural effusion (7).

Pleurodesis is an effective manner by which to treat malignant pleural effusion (8-10). In recent years, thoracoscopic talc poudrage pleurodesis (TTP) has been considered as an effective approach (11,12). Closed drainage after thoracoscopic operation combined with negative pressure suction can dramatically shorten the drainage time and increase complete remission. Usually, application of the above method alone for MPEs is not sufficient, and chemotherapy drugs are needed. The optimal treatment for lung cancer patients who fail first-line chemotherapy is thus an important issue.

In recent years, targeting of the epidermal growth factor receptor (EGFR) signaling pathway has become a focus of individualized therapy within the international oncology community. The clinical application of EGFR-targeted therapy for cancer patients has provided beneficial effects, and tyrosine kinase inhibitors (TKIs) have become important cancer treatment drugs.

Previous studies (13,14) have shown that EGFR plays a critical role in cell proliferation, differentiation, metastasis and survival. Targeting of EGFR has had an effect on the prognosis of cancer patients.

The EGFR family includes the most representative molecules of the transmembrane-receptor-type tyrosine kinases with a wide range of biological functions. It plays important roles in growth, proliferation and differentiation of cells (16). The human EGFR family members including c-erbB-1 (HER-1), c-erbB-2 (HER-2), c-erbB-3 (HER-3) and c-erbB-4 (HER-4) are located on the cell membrane. EGFR is a transmembrane glycoprotein (170 kDa) encoded by oncogene c-erbB-1. It is composed of three parts, an extracellular domain, a transmembrane domain and an intracellular region which is composed of 621,23,542 amino acid residues, respectively. The N-terminal extracellular region is a ligand binding domain of EGFR with high affinity (15).

EGFR and its natural ligands including epidermal growth factor (EGF), transforming growth factor (TGF), two-way adjustment factor, B cell growth factor and heparin-binding EGF are overexpressed in most non-small cell lung cancer (NSCLC) patients (5). Abnormal activation, amplification and overexpression of the EGFR gene were also found in lung adenocarcinoma. Pleural metastasis of lung adenocarcinoma was found to be closely related to EGFR (5,13).

Ligands bind to the extracellular region of the receptor and activated receptor by inducing two neighboring receptor to form a dimer and further activate tyrosine kinase by autophosphorylation of tyrosine residues.

Activated HER-1/EGFR which combine with a multitude of different molecules in cells trigger various downstream signaling pathways, such as ras-raf-MEK-MAPK and PI3K-PKC-κB. Activated signaling pathways further activate transcription factors at the levels of transcription and translation. These transcription factors mediate a series of processes including cell differentiation, survival, migration, invasion, adhesion and cell damage repair (17).

Treatment techniques for malignant pleural effusion have greatly improved, yet no significant improvement in the survival rate and the quality of life of patients has been noted (18).

Iressa (gefitinib) and Tarceva (erlotinib) are the two main EGFR tyrosine kinase inhibitors (TKIs) used in the clinic (19,20). Tarceva is the only one that possesses a survival advantage in treating lung cancer (21,22). It is a new targeted therapy drug which can be selected after failed first- or second-line chemotherapy in patients with progressive and metastatic NSCLC (Tarceva received a certification license in the US in 2004 and in the European Union in 2005, respectively). Through specific inhibition of human EGFR tyrosine kinase activity and further inhibition of the EGFR downstream signaling pathway, Tarceva inhibits the growth of tumor cells by enhancing sensitivity to chemotherapy, promoting apoptosis, inhibiting proliferation, invasion, metastasis, angiogenesis and reducing adhesive ability (23).

However, not all patients with lung cancer have a favorable response to EGFR TKIs. In 2004, Lynch *et al* (24) and Paez *et al* (25) reported that EGFR mutations in lung cancer cells were a prerequisite for targeted drugs. That EGFR mutations are closely related to targeted therapy in NSCLC is widely recognized (13,18,16-28).

Tarceva was found to be effective in >80% of patients with EGFR mutations while it was largely ineffective in a non-mutation group (29-31). Thus, investigation of EGFR gene mutations in patients is necessary prior to using Tarceva. Currently, the best method for detecting EGFR mutations is through direct sequencing, Lynch *et al* (24) and Paez *et al* (25) were the first to report this method. This method accurately determines the scope and types of EGFR mutations. In this study, we applied this method to detect EGFR gene mutations.

Previous studies (30-33) have shown that lung cancer patients with an EGFR mutation have a favorable response to EGFR TKIs. However, the correlation between EGFR mutations in pleural metastatic tissues of lung adenocarcinoma and the therapeutic effect of Tarceva on MPE caused by metastatic lung adenocarcinoma remains uncertain. Questions concerning the association between the EGFR gene mutation and pleural metastasis tissues of lung adenocarcinoma; the therapeutic effects of Tarceva on malignant pleural effusion caused by metastatic lung adenocarcinoma; the relationship between EGFR mutations in pleural metastatic tissues of lung adenocarcinoma and the therapeutic effect of Tarceva on malignant pleural effusion caused by metastatic lung adenocarcinoma are still as yet unanswered. In this study, our aim was to systematically evaluate the relationship between the EGFR mutation in pleural metastasis tissues of lung adenocarcinoma and the therapeutic effect of Tarceva on malignant pleural effusion caused by metastatic lung adenocarcinoma.

Materials and methods

Patients and groups. The present study was approved by the Institutional Review Board of Shandong University, and requirement of informed consent was waived. Patients were enrolled at the Department of Respiratory Medicine and the Center of Cancer Treatment of Shandong Provincial Hospital, Chest Hospital, Shandong, China and the Central Hospital of Shengli Oil Field, Dongying, China from July, 2007 to August, 2009. A total number of 128 cases with malignant pleural effusion caused by metastatic lung adenocarcinoma diagnosed by thoracoscopic lung biopsy were recruited. The expected survival of these patients was longer than 3 months. All patients were divided into a mutation group and a non-mutation group according to the presence or absence of an EGFR mutation in pleural metastatic tissues of lung adenocarcinoma. The mutation group consisted of 90 cases including 48 males and 42 females with a mean age of 64.5±9.8 years. The non-mutation group consisted of 38 cases including 20 males and 18 females with a mean age of 60.4±11.2 years. Differences between the two groups were not significant, as shown in Table I.

All subjects were diagnosed by thoracoscopic lung biopsy, pathology or cytology. The patients were poorly responsive

Table I. Clinical characteristics of the patients before treatment.

Parameters	Mutation group n (%)	Non-mutation group n (%)	P-value
Age (years)			0.9681
<50	18 (20.00)	7 (18.42)	
50-60	29 (32.22)	12 (31.58)	
>60	43 (47.78)	19 (50.00)	
Gender			0.9036
Male	48 (53.33)	20 (52.63)	
Female	42 (46.67)	18 (47.37)	
Smoking habit			0.5372
Current	16 (17.78)	7 (18.42)	
Former	30 (33.33)	9 (23.68)	
Never	44 (48.89)	22 (57.89)	
Grade			0.9147
Well differentiated	23 (25.56)	9 (23.68)	
Moderately differentiated	32 (35.56)	15 (39.47)	
Poorly differentiated	35 (38.89)	14 (36.84)	
Karnofsky score			0.9790
<40	18 (20.00)	7 (18.42)	
40-70	51 (56.67)	22 (57.89)	
>70	21 (23.33)	9 (23.68)	

Based on statistical analysis, no significant statistical discrepancy in the clinical characteristics of the patients between the two groups prior to treatment was noted (p>0.05).

to radiotherapy and chemotherapy or exhibited toxicity for the chemotherapy drugs, and thus did not continue to receive first-line drugs such as docetaxel, gemcitabine, carboplatin or cisplatin.

Methods for specimen sampling and patient therapy. Preoperative examination for each patient included clotting time, ECG, as well as a chest X-ray for inspection of pleural adhesion. Artificial pneumothorax was created 1-2 days before surgery. Diazepam (10 mg) was injected intramuscularly one half hour before surgery.

Under conventional thoracoscopy, 4-5 spots in the pleural lesion tissues were taken for biopsy. These lesions were stored at -80°C for pathological examination. Following biopsy, TTP was given to each patient. Medical sterilized talc powder was chosen as the sclerosing agent. We poudraged 2-4 g of talc powder to the pleural cavity uniformly under direct thoracoscope assistance after the injection of 50 mg of pethidine (meperidine) intramuscularly. After TTP, each patient received closed drainage combined with simple negative pressure suction device to help expel the pleural effusion and gas in the thoracic cavity. Each patient was administered Tarceva chemotherapy in accordance with the scheduled treatment. Tarceva was taken orally at a dose of 150 mg daily at 1 h before eating food or 2 h after eating food. After 4 weeks of treatment, we evaluated the short-term clinical therapeutic

effects through imaging method such as CT and MRI. In order to observe the long-term therapeutic effects of Tarceva, each patient was followed up for one year. Peripheral blood (3 ml) of each patient was collected to detect CEA and TPA prior to and after 4 weeks of treatment.

The tubes were removed when the drainage volume of the pleural effusion was <50 ml/days. We reviewed the chest X-ray examination and chest B-ultrasonic examination, and carried out Karnofsky scoring immediately after extubation and at 4 weeks after surgery. The patients without pleural effusion via B-ultrasonic examination were requested to receive anteroposterior chest X-ray to examine the extent of pleural hypertrophy. Complete remission (CR), partial remission (PR), no change (NC) and disease progression (PD) were determined according to the WHO criteria 4 weeks after surgery.

DNA extraction. DNA was extracted using the EZNA™ Tissue DNA kit (Shanghai Kenqiang Instrument Co., Ltd., Shanghai, China) from frozen tissues acquired from thoracoscopic biopsy.

DNA preparation. DNA extracted from frozen tissues was diluted 100 times with TE. A Nano Drop® (ND-1000) ultraviolet spectrophotometer was used to determine DNA concentration (Based on 1 OD=50 μg/ml double-stranded nucleic acid the concentration of DNA samples was calculated)

Table II. Primers of EGFR exons 18-21.

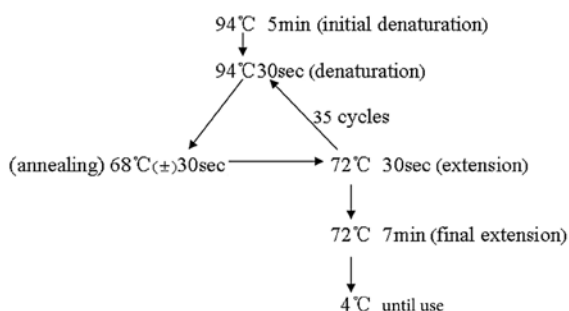| Exons	Primers
18	F: 5'-GCTGAGGTGACCCTTGTCTC-3' R: 5'-ACAGCTTGCAAGGACTCTGG-3'
19	F: 5'-GCAATATCAGCCTTAGGTGCGGTC-3' R: 5'-CATAGAAAGTGAACATTTAGGATGTG-3'
20	F: 5'-CATTCATGCGTCTTCACCTG-3' R: 5'-CCGTATCTCCCTTCCCTGAT-3'
21	F: 5'-CTAACGTTCGCCAGCCATAAGTCC-3' R: 5'-GCTGCGAGCTCACCCAGAATGTCTGG-3'

Figure 1. The PCR reaction conditions consisted of the following steps: initial denaturation at 94°C for 5 min, 35 cycles of denaturation at 94°C for 30 sec, annealing at 68°C for 30 sec for exons 18, 19, 20 and 2l of the EGFR gene, and extension at 72°C for 30 sec, and a final extension step at 72°C for 7 min, and stored at 4°C until used.
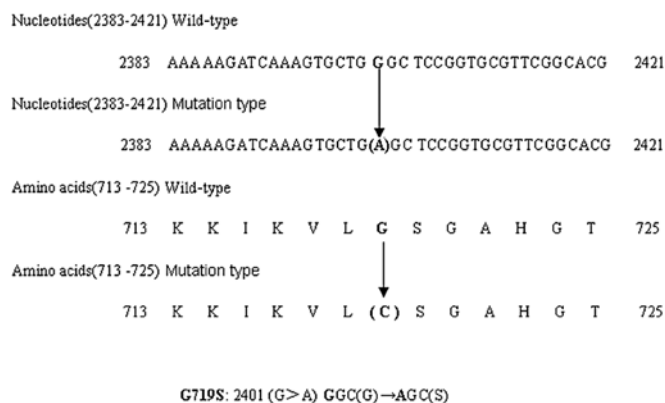

Figure 2. Site of the mutation in exon 18 of EGFR (G719S). The mutation was in codon 719 and appeared as a G→A transition, which resulted in glycine (Gly, G) to serine (Ser, S) amino acid mutation. [The nucleic acid and protein used to name the mutations were based on NG_007726.1 GI:188219609 (nucleotide) and NP_005219.2 GI:29725609 (amino acid) according to GenBank Accession, respectively].

and purity (The ratio of OD260/OD280 was used to measure the purity of the extracted DNA). Test results: DNA purity, OD260/OD280=1.60-1.95; DNA concentration, 40-75 ng/μl. The extracted DNA was stored at -20°C until its use.

PCR amplification and direct sequencing. According to a previously reported method (34), exons 18, 19, 20 and 2l of the EGFR gene were amplified by polymerase chain reaction (PCR). Primers used for amplification of exons 18-21 are showed in Table II Primers were synthesized by Shanghai Generay Biotech Co., Ltd., Shanghai. PCR was performed for 35 cycles in a programmable thermal cycler (Gene Amp PCR System 9600, Applied Biosystems, Foster City, CA, USA) with a volume of 50 μl which contained 2 μl of template DNA, 2 μl of each primer (10 μM), 25 μl of HotStarTaq Master Mix (Qiagen, product type: 203443; Merchant, Shanghai Pu Sheng Biotech Co., Ltd.) and 19 μl of ddH$_2$O. The PCR reaction conditions are shown in Fig. 1.

PCR products were directly sequenced in both sense and antisense directions in the ABI Prism 3100 Avant Genetic Analyzer. Only specimens in which a mutation was identified in both rounds were considered as mutation-positive. The nucleic acid and protein used to name the mutations were based on NG_007726.1 GI:188219609 (nucleotide) and NP_005219.2 GI:29725609 (amino acid) according to GenBank Accession, respectively.

Evaluation of the therapeutic effects. Therapeutic efficacy was evaluated for pleural effusion according to the WHO criteria as determined by chest X-ray (35), Karnofsky score (36) and the extent of pleura hypertrophy through chest X-ray (37).

Statistical analysis. Data are shown as the mean ± SD, using the SPSS16.0 software program. T-test, χ^2 test and Fisher's exact test probability tests were used for statistics. $p<0.05$ was considered to denote a significant difference. The log-rank test was used to compare progression-free survival and overall survival time. We calculated the total efficacy rate using CR+PR.

Results

Sequencing of EGFR exons 18-21. EGFR gene mutations were found in 90 of the 128 cases. The mutation rate was 70.31%. The mutation types were missense mutation G719S in exon 18, L858R in exon 21 and in-frame mutation in exon 19.

G719S of exon 18 was detected in 5 patients (3.90%). The mutation was in codon 719 and appeared to be a G→A transition, which results in a glycine (Gly, G) to serine (Ser, S) amino acid mutation (Fig. 2). Exon 19 mutations were detected in 56 cases, which accounted for 43.75% of the total number of patients.

The major mutations in exon 19 were the loss of codons from 746 to 750 (del E746-A750). There were 7 different mutations. Type I was a loss of 15 bases which resulted in a loss of EGFR protein 746-750 amino acids (ELREA). It is referred to here as del E746-A750 (I). Type II was characterized by the position of a base deletion which was shifted backward one base compared to type I. A loss of 15 bases was noted, but the lack of amino acids was the same as type I. Therefore, this type of mutation was termed del E746-A750 (II). Del E746-A750 (I) and del E746-A750 (II) were the main types of EGFR mutations in exon 19 and were noted in a total of 35 cases, accounting for 27.34% of the total number of patients. Type III was characterized by a loss of nine bases, with a subsequent guanine (G) conversion to cytosine (C), which led to a

Figure 3. Sites of mutations in exon 19 of EGFR. Seven types of in-frame mutation in exon 19 were detected, from top to bottom as follows: del E746-A750 (I), del E746-A750 (II), del L747-E749insP, del L747-A750insP, del L747-T751insA, del L747-S752insV and del L747-S752. [The nucleic acid and protein used to name the mutations were based on NG_007726.1 GI:188219609 (nucleotide) and NP_005219.2 GI:29725609 (amino acid) according to GenBank Accession, respectively].

Figure 4. Site of the mutation in exon 21 of EGFR (L858R). The mutation T→G transition was in codon 858, and caused a conversion from leucine (Leu, L) to arginine (Arg, R). [The nucleic acid and protein used to name the mutations were based on NG_007726.1 GI:188219609 (nucleotide) and NP_005219.2 GI:29725609 (amino acid) according to GenBank Accession respectively].

loss of EGFR protein 747-749 amino acids (LRE), and at the same time introduced a new mutation, from alanine (A) to a proline (P). This type of mutation was termed del L747-E-749insP. This mutation was detected in 6 patients, accounting for 4.69% of the total number of patients. Type IV included a loss of 13 bases, and the subsequent adenine (A) was converted to cytosine (C). This led to a loss of EGFR protein 747-750 amino acids (LREA), while introducing a new mutation from threonine (T) into a proline (P). This type of mutation was termed del L747-A750insP. This mutation occurred in 3 cases, accounting for 2.34% of the total number of patients. Type V was a 2482-2497 nucleotide deletion, resulting in an amino acid deletion (747 leucine to 751 threonine) and inserted alanine. This type of mutation was termed del L747-T751ins A. This mutation was found in 4 cases, accounting for 3.13% of the total number of patients. Type VI mutation was characterized by 2 base deletions (AA and AAGAGAAGCAACATCT) composition, resulting in the loss of EGFR protein 746-752 amino acids (LREATS), while introducing a new mutation with a change from glutamine (E) to valine (V). This type of mutation was termed del E747-S752insV. This mutation was found in 5 patients, accounting for 3.91% of the total number of patients. Type VII included a loss in 18 bases, resulting in a loss of EGFR protein 747-752 amino acids (LREATS). This mutation was termed del L747-S752. This mutation was present in 3 cases, accounting for 2.34% of the total number of patients (Fig. 3).

Table III. Correlation between EGFR mutations and clinical features.

Parameters	Mutation in exon			Mutation rate (%)	p-value
	18	19	21		
Age (years)					0.6483
<50	1	20	10	34.44	
50-60	1	17	8	28.89	
>60	3	19	11	36.67	
Gender (n)					0.5271
Male	2	31	15	53.33	
Female	3	25	14	46.67	
Smoking (n)					0.0033
Current	0	16	5	23.33	
Former	1	14	9	26.66	
Never	4	26	15	50.00	

EGFR mutations were detected more frequently in samples from non-smokers than in current smokers and former smokers (p=0.0033). Age (p=0.6483) and gender (p=0.5271) were not associated with EGFR mutations.

L858R of exon 21 was detected in 29 patients, which accounted for 22.66% of all patients. This type of mutation was in codon 858 and appeared to be a T→G transition, resulting in conversion of leucine (Leu, L) to arginine (Arg, R) (Fig. 4).

Four patients carried both the del E746-A750 and L858R mutations. This type of mutation accounted for 3.13% of all patients. No mutations were detected in exon 20. In order to further verify the above results, these mutations were cloned and sequenced twice.

EGFR mutations were correlated with clinical features (Table III). EGFR mutations were detected more frequently in the samples from non-smokers than in current smokers and former smokers (p=0.0033). Age (P=0.6483) and gender (p=0.5271) were not associated with EGFR mutations.

Table IV. Results of the clinical study.

Parameters	Mutation group	Non-mutation group	p-value
Average extubation time (h)	64.4±12.5	144.2±15.4	8.119×10^{-36}
Drainage volume (ml)	840.0±140.0	1780.0±160.0	7.011×10^{-36}
Formation of encapsulated pleural effusion (cases, %)	8 (8.51)	16 (17.02)	1.088×10^{-5}
Pleural hypertrophy (cases, %)			
Mild	29 (30.85)	11 (28.95)	0.715
Moderate	18 (19.15)	16 (42.11)	0.00968
Severe	4 (4.26)	16 (42.11)	8.256×10^{-8}

In the above table parameters, apart from the moderate pleural hypertrophy, there were significant statistical differences between the mutation group and non-mutation group ($p<0.01$).

Table V. Changes in Karnofsky score.

Groups	Total no. of patients	Increase			No change	Decrease
		30	20	10		
Mutation group (cases, %)	90	13 (14.44)	34 (37.78)	20 (22.22)	13 (14.44)	10 (11.11)
Non-mutation group (cases, %)	38	1 (2.63)	11 (28.95)	10 (26.32)	5 (13.16)	11 (28.95)
p-value		0.0304	0.4512	0.7863	0.9307	0.0259

The increase in the ratio of 30 points in the mutation group was significantly higher than that in the non-mutation group. The decrease in the ratio of the Karnofsky score in the mutation group was significantly lower than that in the non-mutation group. There was a significant statistical difference in the above parameters between the two groups ($p<0.05$); there was no statistical difference in other parameters between the two groups ($p>0.05$).

Table VI. Comparison of the clinical efficacy.

Group	Total no. of patients	Efficacy (no. of patients)			CR rate (%) (CR/total no. of cases)	Efficacy rate (%) (CR+PR/total no. of patients)
		CR	PR	NC		
Mutation group	90	55	20	15	61.11	83.33
Non-mutation group	38	17	9	12	44.74	68.42
p-value					0.02945	0.02151

CR, complete remission; PR, partial remission; NC, no change.

Clinical study results. The average extubation time in the mutation group (64.4±12.5 h) was shorter than that of the non-mutation group (144.2±15.4 h) ($p<0.01$) (Table IV). The drainage volume of pleural effusion in the mutation group was 840±140 ml and in the non-mutation group was 1780±160 ml ($p<0.01$). Four weeks after surgery, 8 patients in the mutation group and 16 patients in the non-mutation group were found with formation of encapsulated pleural effusion ($p<0.01$). The number of the patients with mild, moderate and severe pleural hypertrophy were 29, 18 and 4 in the mutation group and 11, 16 and 16 in the non-mutation group, respectively. The number of patients with mild pleural hypertrophy in the mutation group was significantly higher than that in the non-mutation group ($p<0.01$), while the number of patients with severe pleural hypertrophy was significantly reduced ($p<0.01$) (Table IV).

Changes in Karnofsky score. Four weeks after surgery, the Karnofsky score was increased by 30 in 13 patients in the mutation group (14.44%), while this increase was noted in only 1 patient in the non-mutation group (2.63%) (Table V). The Karnofsky score was increased by 20 in 34 patients in the mutation group (37.78%), while this increase was noted in only 11 patients in the non-mutation group (28.95%). The

Table VII. Changes in CEA and TPA in peripheral blood after one month of treatment.

Parameters	Groups	Before treatment	After one month of treatment	p-value
CEA (μg/l)	Mutation group	101.2±42.5	14.6±6.5	
	Non-mutation group	98.8±50.4	84.5±7.8	0.009158
TPA (μg/l)	Mutation group	7.6±1.4	1.4±0.3	
	Non-mutation group	8.2±1.2	7.8±1.5	0.049836

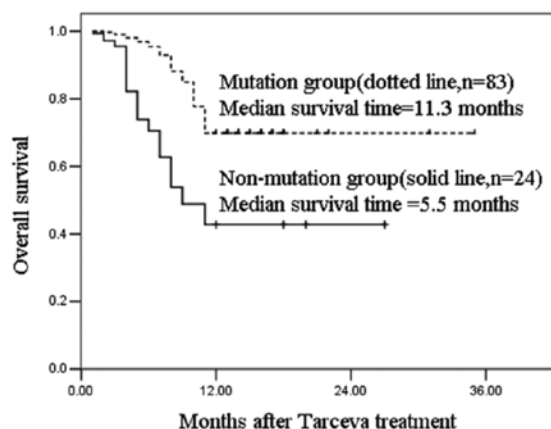CEA, carcinoembryonic antigen; TPA, tissue polypeptide antigen.

Figure 5. Comparison of overall survival time between the mutation group (dotted line) and the non-mutation group (solid line). Kaplan-Meier survival curve showed that the overall survival was significantly improved in the EGFR mutation group (p<0.001 by log-rank test).
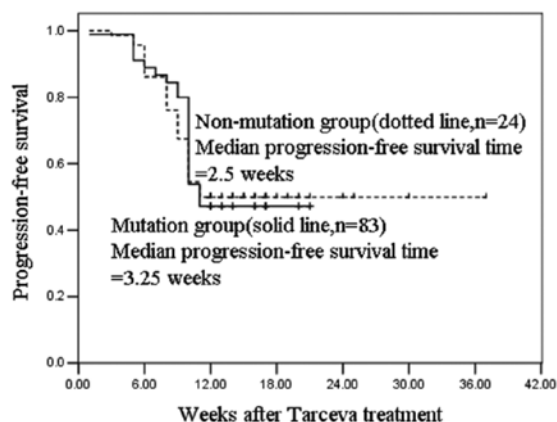

Figure 6. Comparison of progression-free survival time between the mutation group (solid line) and the non-mutation group (dotted line), Kaplan-Meier survival curve showed that there was no significant difference in progression-free survival time between these two groups (p=0.93 by log-rank test).

Karnofsky score was decreased in 11 patients in the non-mutation group (28.95%), while this decrease was noted in only 10 patients in the mutation group (11.11%). These differences were statistically significant (p<0.05). However, there was no significant statistical difference in patients whose Karnofsky score increased by 10 and no change between these two groups

was noted (p>0.05). Taken together, these data suggest that the quality of life of the patients in the mutation group was significantly improved than that in the non-mutation group with the combined therapy (Table V).

Comparison of clinical efficacy. Comparing the two groups in clinical efficacy, the CR rate and efficacy rate were 61.11 and 83.33% respectively in the mutation group, which were significantly higher than these values (CR rate, 44.74%; efficacy rate, 68.42%) in the non-mutation group, which showed a significant statistical discrepancy (p<0.05) (Table VI).

Changes in CEA and TPA in peripheral blood extracted from each patient before treatment and after one month of treatment. Electrochemical luminescence was applied to test serum CEA and TPA of all patients before receiving Tarceva and one month after treatment, respectively. CEA and TPA in peripheral blood decreased gradually, but the degree of decrease was higher in the mutation group than that in the non-mutation group. Based on the statistical analysis, a significant statistical difference was achieved between the two groups (p-value <0.05) (Table VII).

Results of the patient follow-up. Patients enrolled in this study were followed up for one year. During this period, 9 patients were lost in the follow-up (2 cases in the mutation group; 7 cases in the non-mutation group). Twelve patients (5 cases in the mutation group; 7 cases in the non-mutation group) discontined treatment due to moderate or severe liver and renal dysfunction.

Fifty-six patients died in the mutation group and 18 patients died in the non-mutation group. One-year survival rate was 32.53% in the mutation group and 25.00% in the non-mutation group.

The median progression-free survival time and the median overall survival time were 3.25 and 11.30 months in the mutation group and 2.50 and 5.50 months in the non-mutation group, respectively. The overall survival was significantly improved in the mutation group (p<0.001 by log-rank test) (Fig. 5). However, there was no significant difference in progression-free survival time between these two groups (p=0.93 by log-rank test) (Fig. 6).

Adverse reactions during Tarceva treatment. In the course of treatment, different types and different levels of side effects occurred. The main adverse responses are shown in Table VIII.

Table VIII. Main adverse reactions of all patients.

Parameters	Mutation group	Non-mutation group	p-value
Fever (days)	3.2±1.2	2.8±1.6	0.1715
Chest pain (cases, %)	46 (51.11)	23 (60.53)	0.3289
Rash (cases, %)	62 (68.89)	9 (23.68)	2.583×10^{-6}
Diarrhea (cases, %)	24 (26.67)	14 (36.84)	0.2496
Anorexia (cases, %)	22 (24.44)	16 (42.11)	0.4152
Moderate and severe liver dysfunction (cases,%)	6 (6.67)	2 (5.26)	0.7640
Moderate and severe renal dysfunction (cases, %)	3 (3.33)	1 (2.63)	0.8350

For the above table parameters, apart from rash (p<0.01), no significant statistical differences were achieved between the mutation and non-mutation group (p>0.01).

Twenty-four patients had fever for 2-5 days in the mutation group, of whom the temperature of 4 patients was >39˚C, and the average duration of fever was 3.2±1.2 days. In contrast, 27 patients had fever for 2-5 days in the non-mutation group, of whom the temperature of 3 patients was >39˚C, and the average duration of fever was 2.8±1.6 days. Forty-six patients presented with chest pain in the mutation group, while 23 patients presented with chest pain in the non-mutation group. The differences were not significant between these two groups (p>0.05).

After 7-10 days of treatment, rash occured in 62 cases (68.89%) with EGFR mutations and 9 cases (23.68%) without EGFR mutations. The difference was statistically significant (p<0.05). These rashs disappeared a few days after clindamycin treatment.

Mild diarrhea and anorexia were also observed in the two groups (p>0.05). No special treatments were required for these symptoms. Several patients experienced moderate to severe liver and renal dysfunction. After stopping Tarceva treatment, liver and renal functions gradually returned to normal. There were no other significant adverse reactions.

Discussion

In this study, EGFR mutations were examined in pleural metastatic tissues of lung adenocarcinoma biopsied through video-assisted thoracoscopic surgery (**VATS) by PCR ampli-**fication and direct sequencing. The therapeutic effects of Tarceva on MPEs were compared between the EGFR mutation and non-mutation group.

DNA sequencing is the gold-standard method for detection of mutations. In this study, we applied this gold-standard method to detect EGFR mutations in pleural metastatic tissues of lung adenocarcinoma acquired through VATS. This procedure included four steps: firstly, we acquired lesion tissues through VATS; secondly, the histopathological type of the acquired lesion tissue was determined; thirdly, DNA was extracted and amplified by PCR; and fourthly EGFR mutations were detected by direct sequencing.

It was well known that EGFR mutations are frequently observed in lung adenocarcinomas. Soh *et al* (38) and Gow *et al* (39) reported an EGFR mutation rate of 24.5% (13 out of 53) in MPEs of lung adenocarcinoma. Kimura *et al* (40) found a 13% (3 out of 23) EGFR mutation rate by direct sequencing of EGFR mutations in MPEs of lung adenocarcinoma, of which 9.1% (1 out of 11) were females and 10% (1 out of 10) were never smokers. Overall, the EGFR mutation rate of MPEs ranged from 9.1 to 68.4%. This variability probably reflects the methodology, selection of patients, and geographic differences. The incidence of EGFR mutations in NSCLC ranged from 10 to 50% (34,41-45).

In the present study, EGFR mutations were detected in 90 of the 128 patients with pleural metastasis of lung adenocarcinoma. The EGFR mutation rate was 70.31%. There were 5 missense mutations in exon 18, 56 in frame deletion mutations in exon 19 and 29 missense mutations in exon 21, which accounted for 3.90, 43.75 and 22.66% of the total number of patients, respectively. Four patients carried both del E746-A750 and L858R mutations. This type of mutation accounted for 3.13% of the total number of patients. No mutations were detected in exon 20. The EGFR mutations found in this study (43.75% in exon 19, 22.66% in exon 21) were consistent with previous findings (20,24,25,28,30,46).

Jian *et al* (47) used free DNA from plasma and pleural effusion to detect EGFR mutations by PCR using Taqman-MGB probes. The EGFR mutation rate was 23.2% in 56 plasma samples and 28.1% in 32 pleural effusion samples. The EGFR mutation rate was 31.9% in 69 patients with lung adenocarcinoma. In our study, the EGFR mutation rate was 70.31%, which was much higher than the above results. Wu *et al* (48) detected EGFR mutations in surgical resected specimens of lung adenocarcinoma. The results showed that 93 out of 136 patients with MPEs caused by lung adenocarcinoma had positive EGFR mutations. The mutation rate was 68.4%, which was close to our findings (70.31%). These findings showed that the EGFR mutation rate detected in lesion tissues was much higher than that in plasma and pleural effusion samples indicating that there were more false-negative results in plasma and pleural effusion samples.

It has been reported that EGFR mutations occured more frequently in female patients and never smokers (49,50). Yang (32) reported that the EGFR mutation rate was significantly higher in adenocarcinoma than in other types of NSCLC (21

vs. 2%), higher in females than in males (20 vs. 9%), and higher in Asian patients than in European and American patients (26 vs. 2%). In another report, Huang *et al* (45) showed that the EGFR mutation rate in resected lung adenocarcinoma was not associated with gender (females vs. males, 54.5 vs. 55.5%) and smoking history in Taiwan. In our study, EGFR mutations were detected more frequently in non-smoking patients (50.00% in non-smokers, 23.33% in current smokers and 26.66% in former smokers respectively, p=0.0033). However, EGFR mutations were not closely related to age (p=0.6483) and gender (p=0.5271). The reasons for the differences between these studies are unclear.

In recent years, many studies (24,25,51-53) have reported that the clinical therapeutic effects of TKIs are closely associated with EGFR mutations in NSCLC patients. In a clinical study by Shepherd *et al* (54), 731 patients with NSCLC were randomly treated with oral Tarceva (150 mg/day) and a placebo at the ratio of 2:1 until the ocurrence of disease progression or unacceptable toxicity. The results showed that Tarceva significantly prolonged the survival time of patients with advanced NSCLC. The median survival time increased by 42.5% when compared to the control group (6.7 vs 4.7 months). The 1-year survival rate increased by 45% compared with the control group (31.2 vs. 21.5%). The median progression-free survival (PFS) was 9.7 weeks in the treatment group and 8.0 weeks in the placebo group (HR=0.61, p<0.001). Tarceva was the only EGFR TKI confirmed by the test that may improve the progression-free survival (PFS) of patients.

TTP combined with closed drainage and simple negative pressure suction is an effective therapy for malignant and refractory benign pleural effusions (55-57). In our study, TTP was applied through VATS and then closed thoracic drainage combined with simple negative pressure suction was administered to each patient. TTP applied through VATS had several advantages: i) pleural effusions could be fully suctioned under direct vision assisted by thoracoscope; ii) pleura was better exposed via releasing the pleural adhesion which ensured the accuracy and reliability of specimen sampling; iii) talc powder could be distributed uniformly on both visceral and parietal pleura; iv) closed drainage after thoracoscopic operation with the addition of negative pressure suction could help expel the residual gas and pleural effusion caused by tumor infiltration or stimulation of pleurodesis agent. Thus, the visceral and parietal pleural could adhere closely which would reduce the incidence of pleural hypertrophy and the formation of encapsulated pleural effusion. This may result in a synergistic effect with Tarceva. After one month of treatment, the clinical therapeutic results showed that average extubation time was significantly shorter and drainage volume was significantly less in the mutation group compared with that in the non-mutation group. Formation of encapsulated pleural effusion in the non-mutation group was significantly more than that in the mutation group. The number of cases forming severe pleural hypertrophy in the non-mutation group was significantly higher than that in the mutation group.

The Karnofsky score in patients varied significantly. From the changes in Karnofsky score, we determined that the improvement in the quality of life of the patients with an EGFR mutation was obviously better than that of the patients without mutations.

CEA is a broad spectrum tumor marker that reflects the existence of a variety of tumors, such as colorectal, breast and lung cancer. It is also a good tumor marker to determine efficacy of treatment, disease progression and prognosis. Serum TPA is a non-specific tumor marker which is closely related to cell proliferation. Serum TPA increases during the division and proliferation of tumor cells and thus it is commonly used in the diagnosis of malignant tumors. TPA in blood decreases to normal levels indicating that the treatment for cancer was effective. In our study, peripheral blood CEA and TPA in the mutation group gradually returned to normal one month after treatment. These parameters also slightly decreased in the non-mutation group. These results suggest that the clinical efficacy of Tarceva for MPEs in the mutation group was significantly better than that in the non-mutation group.

According to the WHO criteria of treatment for pleural effusion, 4 weeks after surgery, the CR and efficacy rates (CR+PR/total no. of patients) were 61.11 and 83.33%, respectively, in the mutation group, which were significantly higher than that (CR rate, 44.74%; efficacy rate, 68.42%) in the non-mutation group (p<0.05).

After the one-year follow-up, we found that the EGFR mutation is a prognostic factor for progression-free survival time and overall survival time. Patients with an EGFR mutation have a longer median overall survival time (11.30 vs. 5.50 months) than patients without an EGFR mutation. Overall survival was significantly improved in the mutation group (p<0.001 by log-rank test) (Fig. 5). Patients with an EGFR mutation also had a longer median progression-free survival time (3.25 vs. 2.50 months). However, the difference in progression-free survival time between the two groups was not significant (p=0.93 by log-rank test) (Fig. 6).

The 1-year survival rate was 32.53% in the mutation group vs. 25.00% in the non-mutation group. The above rates were equivalent to the reported literature (24,25,58-60).

In the course of treatment, side effects observed included rash, diarrhea, liver dysfunction and renal dysfunction. The incidence of rash was 34.04% in the mutation group and 5.32% in the non-mutation group. There was a significant statistical discrepancy between the two groups. The severity of rash was correlated with better clinical efficacy.

There were several limitations in this study. For example, geographic (ethnic) differences in detection of the EGFR mutation may result in variability. The results of previous studies (30,34,42,61-66) showed that there were significant differences in the sites and main types of EGFR mutations in different races and regions. Most of the specimens in this study came from Shandong, China. Thus, conclusions from our study might not be representative of other regions. On the other hand, methods for detecting EGFR mutations and pathological types of lung cancer may account for the differences found in EGFR gene mutations. An extensive nation-wide clinical trial may be able to solve some of these issues.

In conclusion, our results suggest that there was a higher EGFR mutation rate in pleural metastatic tissues of lung adenocarcinoma obtained by VATS than that in the surgically resected specimens, plasma specimens and pleural effusion specimens. The therapeutic effects of Tarceva for malignant pleural effusion caused by metastatic lung adenocarcinoma was closely related to EGFR mutations of pleural metastatic tissues.

There was a longer overall survival time after Tarceva treatment for those patients with EGFR mutations than those without EGFR mutations. Although there was a longer progression-free survival time, after analysis of medical statistics, there were no significant differences between the two groups.

Acknowledgements

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