·Clinical Research·

Epidermal growth factor receptor mutations, HER2/3 protein expressions and clinical outcome in the Chinese patients with advanced non-small cell lung cancer treated with gefitinib

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[Abstract] Background and Objective: The effect of gefitinib varied on advanced non-small cell lung cancer (NSCLC). It is of clinical significance to choose the sensitive patients and improve the effect of treatment. The objective of this research was to assess the role of epidermal growth factor receptor (EGFR) mutations and HER2/3 protein expression as predictive markers of responsiveness to gefitinib in Chinese patients with NSCLC. **Methods:** From May 2002 to February 2005, a total of 106 Chinese NSCLC patients who had failed at least one chemotherapy regimen received gefitinib 250 mg once a day. All the tumors from these patients were correlated with clinical treatment outcome and screened for mutations in the exons 18–24 of EGFR gene, and 84 tumors were studied by immunohistochemistry for HER2/3 expression. **Results:** EGFR mutations were identified in 32 patients (30.2%). The objective response rate (ORR) was significantly higher in the patients with HER2 high expression than in those with HER2 low expression (36.8% vs. 17.4%, P=0.044). patients. HER2 and HER3 expression levels were not associated with time-to-progression (TTP) and overall survival (OS). The OS was slightly longer in the patients with HER2 high expression than in those with HER2 low expression (6.1 months vs. 9.1 months, P=0.725) and longer in the patients with HER3 high expression than in those with HER3 low expression (6.1 months vs. 9.0 months, P=0.862). The difference was even obvious for those with concomitant HER2/3 high expression. EGFR-mutated patients with HER2 expression or high HER2 and HER3 expressions were more sensitive to gefitinib. **Conclusion:** EGFR mutations and HER2/3 expressions are effective predictors for gefitinib efficacy in Chinese patients with advanced NSCLC.

Key words: Gefitinib, epidermal growth factor receptor, HER2, HER3, mutation, non-small cell lung cancer, efficacy, prognosis

Gefitinib (Iressa, ZD1893) is an inhibitor of epidermal growth factor receptor tyrosine kinase (EGFR-TK). Due to the variability of efficacy of gefitinib in non-small cell lung cancer (NSCLC),[1] how to identify target patients for gefitinib treatment to enhance efficacy is a hot spot in research. This study aimed to investigate the relationship between EGFR-TK domain mutation plus HER2/3 protein expression in tumor tissues and efficacy of gefitinib for NSCLC treatment.

Materials and Methods

Clinical materials

From May 2002 to February 2005, the Cancer Center of the No. 307 Hospital, Chinese Academy of Military Medical Sciences and the Cancer Center of the Second Affiliated Hospital, Dalian Medical University enrolled patients in accordance to the following criteria. The patient was confirmed as having NSCLC by pathology. Local lesions or metastatic lesions were measurable. Tumor tissues were available for gene analysis.The patient was older than 18 years. The Eastern Cooperative Oncology Group (ECOG) performance status ranged between 0–3. The expected survival was over 3 months. The leucocyte count was $\geqslant 3.0 \times 10^9$/L; the platelet count $\geqslant 100 \times 10^9$/L; the bilirubin level was below 1.5 times of the upper limit of normal range (ULN); the ALT and

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AST levels were below 3 times of the ULN (or below 5 times of ULN if liver metastases existed); the creatinine clearance rate (CCR) was greater than 45 mL/min; and no history of other tumors. The number of chemotherapy schemes was not considered. The informed consent was obtained from all patients prior to the research.

Gefitinib was provided by the Expanded Access Program from AstroZeneca International (Wilmington, Delaware, US). Gefitinib 250 mg was administered to the patients at a fixed time in the morning or afternoon each day. The medication was discontinued whenever progressive disease (PD) or intolerable adverse events occurred. A total of 106 patients with a median age of 62 years (range, 31–78 years) were enrolled in the research . The patient population consisted of 55 (51.9%) men and 51 (48.1%) women. Eighty (78.3%) patients were diagnosed with Stage IV NSCLC. Forty-four (41.5%) patients got an ECOG performance status of 2–3. Seventy-eight patients had adenocarcinoma, accounting for 73.6%. The patients had been treated with platinum-based chemotherapy prior to this research, and 94 (88.7%) received over two courses of chemotherapy. Seventy-one (67%) patients had no history of smoking.

DNA extraction, PCR and sequencing

Prior to medication, tumor tissues were verified to have over 80% tumor components using HE staining. The tumor tissues embedded in paraffin were sectioned into thin slices at 5 μm. Five to 8 slices were dewaxed in xylene and cleared with absolute ethanol. Thirty mg additional fresh tissues were selected for the procedure. The genomic DNA was extracted using the DNA extraction kit (Omega Bio-Tek, Doraville, Georgia, US). The extrons 18–24 of the EGFR-TK domain from genomic DNA were amplified using the nested PCR. The PCR products were sequenced in two directions via the ABI PRISM 3100 Sequence Detection System (PE Applied Biosystems, Foster City, California, US). The sequencing results were compared with the EGFR-TK domain from the gene sequence database (registration No. NM00528.3) using the Blast2 program. Each sample underwent the procedure at least twice.

Selection of immunohistochemical reagents, methods and determination of results

The immunohistochemical (IHC) kit and monoclonal antibodies (produced by Zymed Laboratories, San Francisco, California, US; BD, Franklin Lakes, New Jersey, US; and Thermo Scientific, Waltham, Massachusetts, US) were purchased from Maxin.Bio (Fuzhou, Fujian, China) and Zhongshan Goldenbridge Biotechnology. Co., Ltd (Beijing, China). The tissue samples were fixed in formaldehyde and embedded in paraffin. The streptavidin-proxidase (SP) method was adopted for routine IHC procedure.

Twenty slides of normal lung tissues were regarded as the standard negative control in staining with PBS in replacement of the primary antibody. The staining effects were observed by two technicians in a blind manner. The IHC staining results were rated in both the staining intensity and the percentage of stained cells: (-) and (+) as negative, and (++) and (+++) as positive.

Efficacy evaluation and survival of patients

In reference to the baseline data of the patients, the efficacy was evaluated as complete remission (CR), partial response (PR), stable disease (SD) and progressive disease (PD) according to the RECIST guidelines. CR and PR were verified again 4 weeks later. The time to progression (TTP) was the duration from medication of gefitinib to occurrence of PD. The last efficacy evaluation was regarded as the end of the TTP for patients who did not achieve PD in the last efficacy analysis or who were lost to follow-up. The overall survival (OS) was considered to be started from medication of gefitinib to death. The last efficacy evaluation was regarded as the end of the OS for patients who survived in the last efficacy analysis or who were lost to follow-up. Each course of chemotherapy lasted 4 weeks and the efficacy was evaluated at the end of each course.

Statistical analysis

The Chi-square test, Fisher's exact test, and Wilcoxon rank-sum test were used for univariate analysis. The logistic regression model was applied for multivariate analysis. The survival analysis was done by the Kaplan-Meier method. Between-group comparison was conducted via the Log-rank test. The Cox regression model was used for multivariate analysis of time variables. A statistically significant difference was considered if P was < 0.05 (two sided). All analyses were conducted using the SPSS16.0 software.

Result

EGFR-TK domain mutation in tumor tissues

Included for gene analysis were 25 frozen fresh tumor tissue samples and 81 embedded tissue samples. DNA sequencing found EGFR-TK domain mutation in 32 samples (30.2%), mainly at Exon 19 (17/32, 53.1%), Exon 18 (6/32, 18.8%) and Exon 21 (6/32, 18.8%) (Figure 1). The mutation rate of EGFR-TK domain was significantly higher for adenocarcinoma (35.9%) than for squamous cell carcinoma (14.3%) (P = 0.033) among the 106 patients. The mutation rate was not obviously affected by the gender, smoking history, age, general condition and tumor staging (Table 1). The median duration of gefitinib medication was 6.4 months (range, 1.0–26.0 months). The effectiveness rate for the patients with EGFR-TK domain mutation was significantly higher than the patients without mutation (71.9% vs. 13.5%, P < 0.001). The Kaplan-Meier method implied that the median TTP for the patients with mutation was 15.0 months (95% CI, 6.0–23.9 months), while it was 3.0 months (95% CI, 2.6–3.3 months) for the patients without mutation. There was a significant difference between these two groups (P < 0.001). The median OS for the patients with mutation was 18.5 months (95% CI, 8.3–28.6 months), which was significantly higher than 6.0 months for the patients without mutation (95% CI 5.0–6.90 months) (P < 0.001) (Figure 2).

HER2/HER3 protein expression

HER2/HER3 protein expression was detected in 84 of 106 patients. HER2/HER3 protein expression was not associated with the age, gender, ECOG performance status, pathologic type,

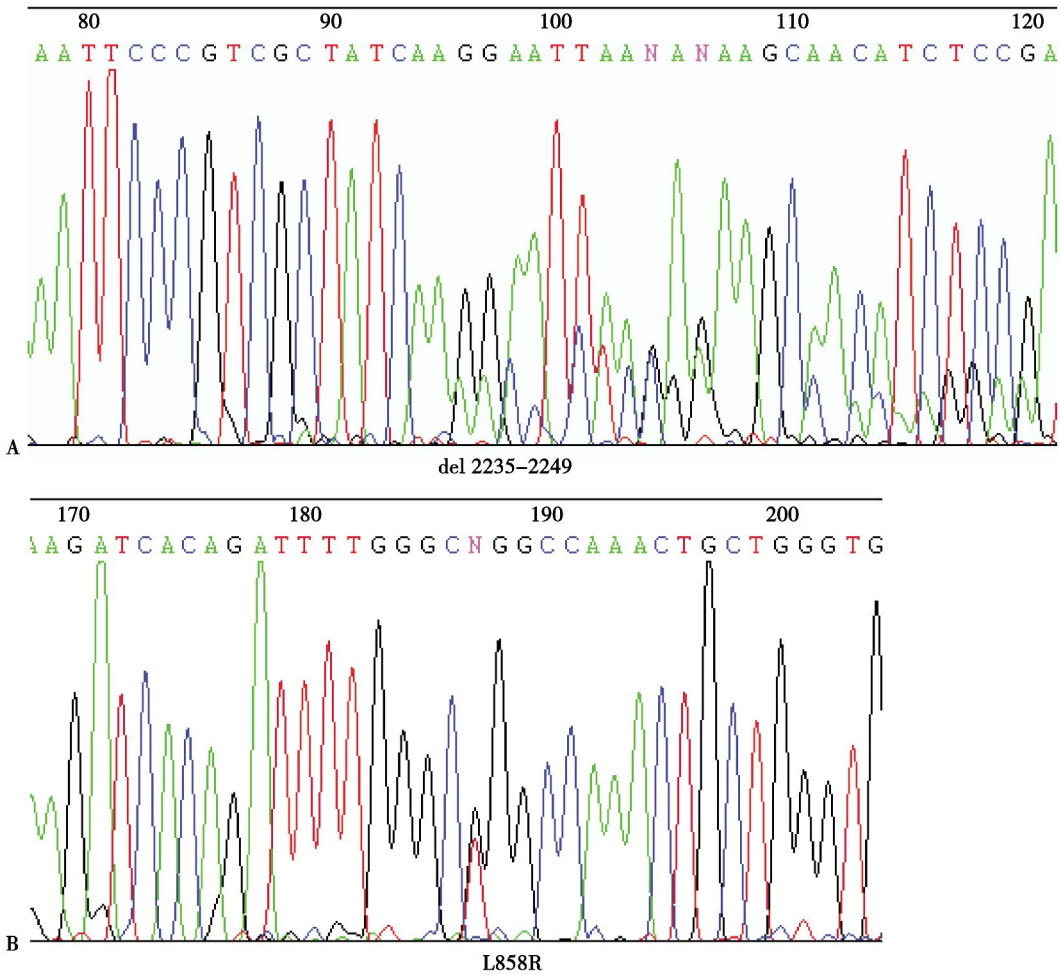

Figure 1 EGFR gene mutation sequencing results

A, the deletion mutation in exon 18; B, the point mutation in exon 21. All are somatic mutations.

prior chemotherapy, and tumor stage. High HER3 protein expression was only related to non-smoking behavior (P=0.016) (Table 2).

In the 84 patients with HER2/HER3 protein expression, 38 (45.2%) and 17 (20.2%), respectively, had HER2 (2+/3+) and HER3 (2+/3+) protein expression. Forty-one (48.8%) patients had both low HER2 and HER3 protein expressions, whereas 12 (14.2%) patients had high protein expressions (Figure 3).

The effectiveness rate was 36.8% for the patients with high HER2 protein expression, which was significantly higher than 22.2% for the patients with low expression (P = 0.044). The effectiveness rate for the patients with high HER3 protein expression was 41.2%, while it was 22.4% for the patients with low expression. No significant difference was noted between the two groups (P > 0.05). Expression of HER2 or HER3 was not associated with the median TTP or OS. The patients with high HER2 or HER3 protein expression had a higher effectiveness rate than those with low protein expression (50.0% vs. 17.1%, P = 0.05).

Effects of EGFR-TK domain mutation and HER2/HER3 protein expression on efficacy and prognosis

It was found that, among the patients with EGFR-TK domain mutation, those with high HER2 protein expression had an apparently higher effectiveness rate than those with low expression (84.6% vs. 37.3%, P = 0.025), as well as a longer median TTP and OS. However, there was no significant difference between the two groups (P > 0.05). Compared with the patients without EGFR-TK domain mutation and/or with low HER2 protein expression, those with both EGFR-TK mutation and high HER2 protein expression had a significantly higher effectiveness rate, longer 1-year survival, and longer median TTP and OS (P < 0.001). Among the patients with EGFR-TK domain mutation, the RR, and median TTP and OS were not significantly higher in the patients with high HER3 protein expression than those with low expression, possibly due to limited cases of HER3 high expression. Five of 7 patients with both EGFR-TK domain mutation and high HER3 protein expression showed responses to

Table 1 Patient characteristics, EGFR mutation and /HER2/HER3 status

Characteristic	EGFR [patient No.(%)]		HER2 [patient No.(%)]		HER3 [patient No.(%)]	
	Mutation	Wildtype	Positive	Negative	Positive	Negative
Total	32(30.2)	74(69.8)	38(45.2)	46(54.8)	17(20.2)	67(79.8)
Gender						
Male	13(23.6)	42(76.4)	17(38.6)	27(61.4)	8(18.2)	36(81.8)
Female	19(37.3)	32(62.7)	21(52.5)	19(47.5)	9(22.5)	31(77.5)
P	0.127		0.202		0.707	
Age(years)						
<65	18(30)	42(70)	20(44.4)	25(55.6)	12(26.7)	33(73.3)
⩾65	14(30.4)	32(69.6)	18(46.2)	21(53.8)	5(12.8)	34(87.2)
P	0.961		0.875		0.115	
ECOG						
0–1	19(30.6)	43(69.4)	25(51.0)	24(49.0)	14(28.6)	35(71.4)
2–3	13(29.5)	31(70.5)	13(37.1)	22(62.9)	3 (8.6)	32(91.4)
P	0.903		0.208		0.024	
Histologic type						
Adenocarcinoma	28(35.9)	50(64.1)	28(45.9)	33(54.1)	12(19.7)	49(80.3)
Non-adenocarcinoma	4(14.3)	24(85.7)	10(43.5)	13(56.5)	5(21.7)	18(78.3)
P	0.033		0.842		1.000	
Smoking						
No	25(35.2)	46(64.8)	26(45.6)	31(54.4)	7(25.9)	20(74.1)
Yes	7(20)	28(80)	12(44.4)	15(55.6)	10(17.5)	47(82.5)
P	0.109		0.920		0.372	
Failure regimens						
1	5(41.7)	7(58.3)	5(41.7)	7(58.3)	4(33.3)	8(67.7)
⩾2	27(28.7)	67(71.3)	33(45.8)	39(54.2)	13(18.1)	59(81.9)
P	0.358		0.788		0.251	
Stage						
Ⅲb	5(21.7)	18(78.3)	7(35.0)	13(65.0)	3(15.0)	17(85.0)
Ⅳ	27(32.5)	56(67.5)	31(48.4)	33(51.6)	14(21.9)	50(78.1)
P	0.319		0.292		0.751	

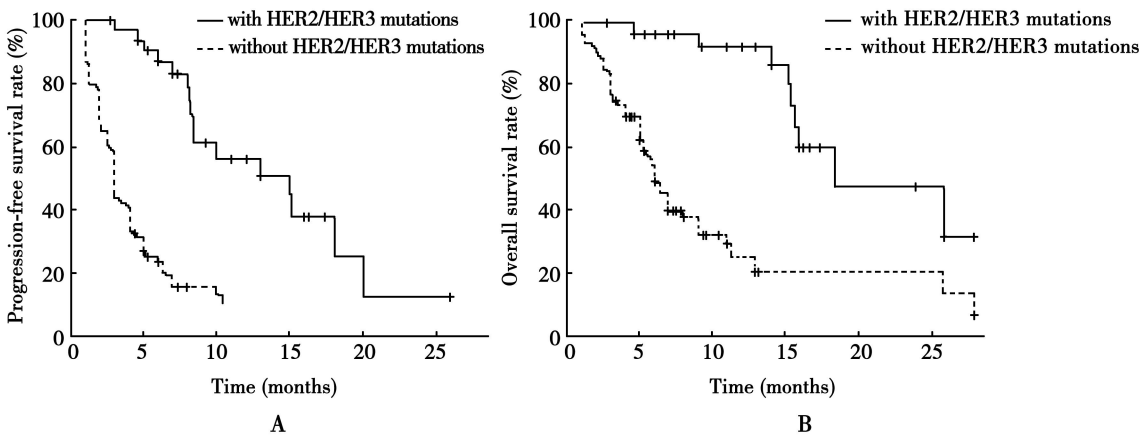

Figure 2 Kaplan-Meier survival curves for time to disease progression (A) and overall survival (B) between the patients mutant EGFR and those with wild-type EGFR

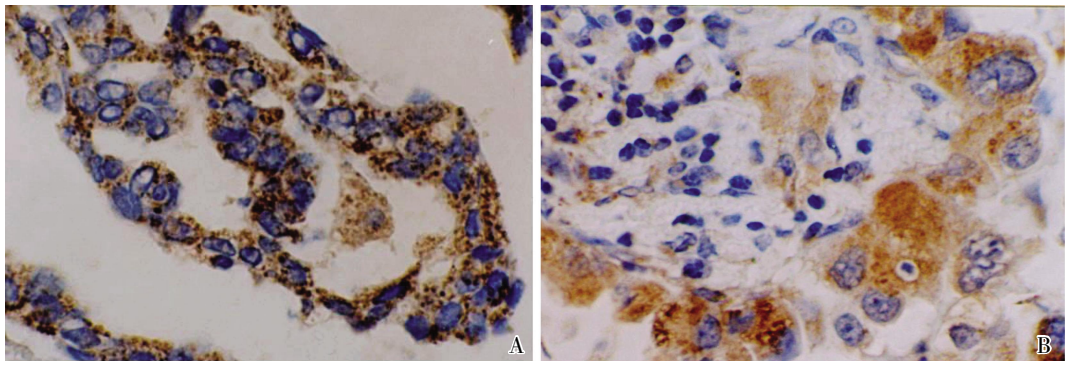

Figure 3 Expression of HER2 and HER3 proteins in non-small cell lung cancer (NSCLC) tissues (IHC ×500)

A, HER2 protein expression (+++); B, HER3 protein expression (+++).

Table 2 Association between tumor response and prognosis according to EGFR mutation/HER2/HER3 status

Markers	Number (%)	Overall efficacy rate (%)	Time to progression (months, mean)	Overall survival time (months, mean)	1-year survival rate (%)
EGFR mutation/HER2/HER3	84(100)	26.2	4.0	11.4	47.6
HER2+/HER3+	12 (14.2)	50.0	4.0	13.0	53.1
HER2−/HER3+	5 (6.0)	20.0	7.0	9.3	60.3
HER2+/HER3−	26 (31.0)	30.8	5.0	13.5	54.5
HER2−/HER3−	41 (48.8)	17.1	3.0	7.0	39.2
Any negative	72 (85.7)	22.2	4.0	8.0	42.3
P (any negative vs. HER2+/HER3+)		0.071	0.960	0.636	0.119
EGFR mutation/HER2/HER3	84(100)	26.2	4.0	11.4	47.5
EGFR mutation+/HER2+	13 (15.5)	84.6	18.1	26.0	75.8
EGFR mutation+/HER2−	8 (9.5)	37.3	15.1	21.2	58.2
EGFR mutation−/HER2+	25 (29.8)	12.0	3.0	6.4	23.1
EGFR mutation−/HER2−	38 (45.2)	13.2	3.4	6.0	29.1
Any negative	71 (84.5)	15.5	3.0	7.0	30.3
P (any negative vs. HER2+/HER3+)		<0.001	<0.001	<0.001	<0.001
EGFR mutation+/HER3+	7 (8.3)	71.4	8.5	18.3	85.2
EGFR mutation+/HER3−	14 (16.7)	64.3	18.1	26.0	79.9
EGFR mutation−/HER3+	10 (11.9)	20	3.0	5.4	18.2
EGFR mutation−/HER3−	53 (63.1)	11.3	3.0	6.4	29.5
Any negative	77 (91.7)	22.1	3.4	15.2	42.5
P (any negative vs. HER2+/HER3+)		0.012	0.027	0.013	<0.001
EGFR mutation+/HER2+/HER3+	4 (4.8)	100	8.0	13.0	75.2
EGFR mutation+/HER2−/HER3−	5 (6.0)	40.0	6.4	15.2	80.2
EGFR mutation−/HER2+/HER3+	8 (9.5)	25.0	3.0	9.0	23.6
EGFR mutation−/HER2−/HER3−	36 (42.9)	13.9	3.0	6.4	31.7
Any negative	80 (95.2)	22.5	4.0	9.0	44.5
P (any negative vs. HER2+/HER3+)		0.004	0.325	0.722	<0.001

gefitinib, while 9 of 14 patients with EGFR-TK mutation but low HER3 expression showed responses to gefitinib. Compared with the patients without EGFR-TK mutation and/or with low HER3 protein expression, the patients with GFR-TK domain mutation and high HER3 protein expression achieved a significantly higher effectiveness rate and one-year survival ($P < 0.05$), as well as a significantly longer median TTP (8.5 months vs. 3.4 months, $P = 0.027$) and OS (18.3 months vs. 15.2 months, $P = 0.013$) (Table 2).

Compared with other patients, those with high protein expression of both HER2 and HER3 in addition to EGFR-TK domain mutation had a significantly higher effectiveness rate

(P = 0.009). This was true especially when the effectiveness rate was weighted as against the patients without EGFR-TK domain mutation and/or low HER2/HER3 protein expression (P = 0.004); they also had a significantly longer 1-year survival (P < 0.001), and a longer median TTP (8.0 months vs. 4.0 months, P = 0.325) and longer OS (13.0 months vs. 9.0 months, P = 0.722) (Table 2).

The Cox regression model added that adenocarcinoma (RR = 2.1 and P = 0.005) and EGFR-TK domain mutation (RR = 4.7 and P < 0.001) were independent factors significantly affecting the median TTP while the EGFR-TK mutation (RR = 5.2 and P < 0.001) and non-smoking history (RR = 2.0 and P = 0.016) were independent factors significantly affecting the median OS, but the protein expression of HER2 or HER3 alone did not affect the median TTP or OS.

Discussion

EGFR is a member of the erbB receptor family including EGFR (erbB1/HER1), erbB2 (HER2/neu), erbB3 (HER3) and erbB4 (HER4). All the members are essential factors in the signal transduction pathway, affecting cell differentiation, proliferation, motility, invasion, adhesion, recovery and vessel regeneration.[2] Due to fixed extracellular region, HER2 does not bind to the ligand and passes the signal through formation of heterodimers. HER3 lacks the tyrosine kinase activity and has to rely on formation of heterodimer for passage of signal. Researches on functions and characteristics of HER4 are being conducted.[3] After gefitinib enters the cell, it competes with ATP for specific EGFR binding sites and suppresses activation of EGFR. Theoretically, it inhibits signal transduction by EGFR homodimers, EGFR/HER2 heterodimers and EGFR/HER3 heterodimers, but not by HER2/HER3 heterodimers, which renders limited efficacy of gefitinib.[4]

Some researches indicated that EGFR and HER3 formed heterodimers inside cells sensitive to gefitinib to trigger the P13K/Akt signal pathway,[5] while HER2/HER3 heterodimers ignited the Akt pathway. However, gefitinib predominantly suppresses the P13K/Akt pathway among the downstream signal transduction pathways by EGFR.[6] It was also reported that EGFR-TK1 inhibited erbB homodimers and heterodimers, and that gefitinib interfered with formation of HER2/HER3 heterodimers, especially in cells with high HER2 protein expression.[7] It is thus concluded that gefitinib inhibits the signal pathway not only through suppression of EGFR activation, but also via affecting interaction within erbB members, especially EGFR, HER2 and HER3.

It is commonly regarded that EGFR-TK domain mutation is closely correlated with chemotherapeutic efficacy. The mutation of sites within or adjacent to the EGFR-TK domain tends to result in structural change of EGFR, essential for self-adaptation of various protein kinases. Mutated EGFR strengthens the activity of tyrosine kinases for epidermal growth factors and the sensitivity to gefitinib as well. It was also demonstrated that patients with EGFR-TK mutation had a significantly longer TTP and OS than patients without mutation.[8,9] However, EGFR-TK domain mutation cannot be used to predict the efficacy of gefinitib. There are complex interactions between EGFR and other erbB family members; heterodimers from synthesis with other members or activation of other members exhibit higher receptor activity and signal transduction capacity than EGFR homodimers, indicating that the activity of a member alone does not represent the overall status of the signal transduction pathway.[10,11]

The current research further analyzed the association between EGFR-TK domain mutation plus HER2/HER3 protein expression and efficacy plus prognosis in erbB family. The EGFR-TK domain mutation rate and the positive protein expression rates of HER2 and HER3 were consistent with the findings in literature.[7,12] The effectiveness rate for the patients with EGFR-TK domain mutation was significantly higher than that for the patients without mutation, and the patients with mutation also had a longer median TTP and OS. Regardless of EGFR-TK domain mutation, the HER2 and HER3 protein expressions were not associated with efficacy or prognosis of patients.

In this study, among the patients with EGFR-TK domain mutation, the effectiveness rate for the patients with high HER2 protein expression was significantly higher than that for the patients with low expression, as well as a longer median TTP and OS. It is also true for HER3. Compared with the patients without EGFR-TK domain mutation and/or with low HER3 protein expression, the patients with both EGFR-TK domain mutation and high expression of HER2/HER3 had a longer median TTP and OS, demonstrating that the tumors with high HER2 and HER3 protein expression are sensitive to gefitinib. Because of limited samples, the stratified analysis was inadequate and new findings might emerge had more samples been collected in the current research. In the patients without EGFR-TK domain mutation, the patients with high expression of both HER2 and HER3 had a significantly higher effectiveness rate than the patients with low expression, as well as a longer survival. Consequently, it is proposed that the patients with high HER2 protein expression are comparatively more sensitive to gefitinib and those with high expression of both HER2 and HER3 especially can achieve better efficacy and prognosis among the NSCLC patients with EGFR-TK domain mutation. For the NSCLC patients without EGFR-TK domain mutation, though it is difficult to estimate the efficacy of gifitinib through high protein expression of HER2 or HER3 alone, high protein expression of both HER2 and HER3 may predict favorable benefits from gefitinib treatment.

Combined detection of EGFR-TK domain mutation and HER2/HER3 protein expression turns out to be a simple, feasible and functional approach to predict the sensitivity of NSCLC patients to gefitinib in expanded clinical practice.

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