

Influence of Chemotherapy on *EGFR* Mutation Status Among Patients With Non–Small-Cell Lung Cancer

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ABSTRACT

Purpose

EGFR mutation is a predictor of epidermal growth factor receptor–tyrosine kinase inhibitor treatment response in patients with non–small-cell lung cancer (NSCLC). However, it remains unclear whether chemotherapy affects *EGFR* mutation status in NSCLC. We investigated the influence of chemotherapy on *EGFR* mutations in plasma and tumor tissues from patients with NSCLC.

Patients and Methods

Samples were derived from three cohorts: one, 264 patients with advanced NSCLC who received first-line chemotherapy with matched pre- and postchemotherapy blood samples; two, 63 patients with stages IIb to IIIb disease with pre- and post-neoadjuvant chemotherapy tumor tissues; and three, 79 patients with advanced NSCLC who underwent palliative surgery. *EGFR* mutation status was determined and analyzed to reveal potential impact of chemotherapy.

Results

In the first cohort, *EGFR* mutations were detected in 34.5% of the prechemotherapy plasma samples (91 of 264) but in only 23.1% of the postchemotherapy plasma samples (61 of 264). The decrease in *EGFR* mutation rate was statistically significant ($P < .001$). Patients whose *EGFR* mutations switched from positive to negative after chemotherapy had a better partial response (PR) than patients with a reverse change ($P = .037$). A similar decrease in *EGFR* mutation rate was observed in tissues after neoadjuvant chemotherapy in the second cohort (34.9% [22 of 63] v 19.0% [12 of 63]; $P = .013$). In the third cohort, 38.0% of the tumors (30 of 79) showed an intratumor heterogeneity of *EGFR* mutation, whereas 62.0% (49 of 79) were homogeneous, either with *EGFR* mutation or no mutation.

Conclusion

Our results suggest that chemotherapy may reduce *EGFR* mutation frequency in patients with NSCLC, likely the result of a preferential response of subclones with *EGFR* mutations in tumors with heterogeneous tumor cell populations.

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INTRODUCTION

Oral tyrosine kinase inhibitors (TKIs) targeting epidermal growth factor receptor (EGFR) have become an indispensable and important modality for treating advanced non–small-cell lung cancer (NSCLC). Because only a limited number of patients will likely benefit from these agents,^{1,2} the identification of such patients is urgently needed.

Somatic mutations in the *EGFR* tyrosine kinase domain have been linked to EGFR-TKI treatment response in patients with advanced NSCLC.³⁻¹¹ Recent phase III clinical studies of advanced NSCLC have demonstrated that *EGFR* mutations are the most effective predictor of clinical outcome in re-

sponse to first-line TKIs.¹²⁻¹⁶ These mutations have become important biomarkers in determining optimal first-line therapy (chemotherapy or TKI therapy), and the use of these biomarkers is accepted as a paradigm of genotype-based individualized target therapy for patients with NSCLC.

However, the significant predictive value of *EGFR* mutations observed in first-line TKI treatment has not been maintained in second-line TKI treatment.^{2,17} Biomarker analysis indicated that *EGFR* mutations were not associated with the outcomes of TKI treatment in the BR.21 trial² or in the ISEL (IRESSA Survival Evaluation in Lung Cancer) study, which compared erlotinib or gefitinib with placebo in patients for whom

platinum-based chemotherapy had failed.¹⁷ Moreover, the rate of tumor response to second-line TKI therapy was lower than that for first-line therapy in patients with *EGFR* mutations. The reason for the inconsistency in the predictive value of *EGFR* mutations between first- and second-line treatments is unknown. We postulated that first-line chemotherapy may influence the status of *EGFR* mutations, and thus, assessment of *EGFR* mutations using specimens collected at the initial diagnosis might be inadequate for predicting response to *EGFR*-TKI treatment after chemotherapy.

However, it is difficult to obtain tumor biopsies from patients for whom chemotherapy has failed. Plasma DNA may provide a noninvasive and repeatable source of genotypic information. We and others have previously shown that plasma DNA is a reliable source for *EGFR* mutation analysis in patients with advanced NSCLC.¹⁸⁻²⁰ The aims of the current study were threefold: first, to compare *EGFR* mutation status before and after first-line chemotherapy in plasma DNA from patients with advanced NSCLC; second, to identify neoadjuvant chemotherapy-related variation in *EGFR* mutation in tissue samples from patients with stages IIB to IIIB NSCLC; and third, to explore the potential mechanism of *EGFR* mutation variation by analyzing the heterogeneity of intratumoral *EGFR* mutations in tissue samples obtained during palliative surgical resection.

PATIENTS AND METHODS

Study Cohorts

Three cohorts of patients with NSCLC were enrolled onto this study. All patients were treated at the Peking University Cancer Hospital (Beijing, China) between April 1, 2006, and December 31, 2009.

The first cohort consisted of 264 consecutive patients with histologically confirmed stages IIIb to IV NSCLC who had received two cycles of platinum-based first-line chemotherapy (cisplatin and carboplatin plus gemcitabine, vinorelbine and taxanes). Pre- and postchemotherapy peripheral blood were collected from each patient.

The second cohort included patients with locally advanced NSCLC ($n = 63$) who had received two to four cycles of neoadjuvant chemotherapy (gemcitabine plus cisplatin, vinorelbine plus cisplatin) to confirm chemotherapy-related *EGFR* mutation status changes observed in the first cohort. Matched biopsy and surgical resection samples were collected before and after neoadjuvant treatment.

The third cohort consisted of 79 patients with stages IIIa to IV NSCLC who had received palliative surgical resection without prior treatment. Tumor cells were microdissected at multiple small regions of the formalin-fixed paraffin-embedded specimens individually and subjected to *EGFR* mutation analysis.

Patients' clinical information was derived from the clinical database established in 1999. Smoking status was defined as those who had smoked more than 100 lifetime cigarettes and was based on records at patients' first clinic visit. Histologic subtypes were based on WHO criteria.²¹ Staging was based on the 2009 International Union Against Cancer–American Joint Committee on Cancer–TNM system (version 7).²²

The study was reviewed and approved by the Institutional Ethic Committee at Peking University Cancer Hospital. All patients provided written informed consent before samples were collected.

Sample Collection and Processing

Blood was collected in anticoagulated tubes from patients in the first cohort before and after two cycles of first-line chemotherapy. Plasma DNA was extracted according to a method reported previously.¹⁸

For the second cohort, tumor tissues were macrodissected to avoid influence of necrotic tissues resulting from neoadjuvant chemotherapy. For the third cohort, one section 15 μm in thickness for each patient case was

stained with hematoxylin and eosin and microdissected using the AS Laser Microdissection system (Leica Microsystems, Wetzlar, Germany). Thirty to 40 tumor foci containing approximately 100 cancer cells in each were obtained. E.Z.N.A formalin-fixed paraffin-embedded DNA kits (Omega Bio-Tek, Norcross, GA) were used to extract DNA from the tissues. The quality and concentration of extracted DNA were determined using NanoDrop 2000 (Thermo Fisher Scientific, Wilmington, DE). The extracted DNA was then used for qualitative or semiquantitative *EGFR* mutation analysis by denaturing high-performance liquid chromatography (DHPLC).

Mutation Analysis

We analyzed all matched samples in the same condition to equalize the detection conditions. The *EGFR* exon 19 deletion or exon 21 substituted mutations were detected according to the method reported by us previously.¹⁸ The amplification refractory mutation system (ARMS), a more sensitive method, was used to re-evaluate the patient cases with *EGFR* mutation discrepancies before and after chemotherapy or neoadjuvant chemotherapy.

A semiquantitative analysis of mutation abundance was performed by calculating a ratio (M/W) between the peak heights for mutant and wild-type product. M represents mutant peak height, and W represents normal peak height. The analysis was only used in exon 19 mutation examination, not in exon 21 analysis, because mutant peak and wild peak were separate in exon 19 but overlapped in exon 21.

Statistical Analysis

Frequency tabulation and summary statistics were provided to characterize the data distribution. The McNemar test was applied to compare the change of mutation status before and after treatment. The Cochran-Armitage trend test was used to test whether change in mutation status was associated with clinical outcome in terms of partial response (PR), stable disease (SD), or disease progression (PD). The associations of unpaired categorical variables were analyzed using the χ^2 test; however, Fisher's exact test was used for small sample sizes (expected value < 5 in any cell of the contingency table). The Wilcoxon rank sum test was applied to compare the mutant abundance between the different mutation groups. Statistical significance was set at a level of .05. Two-sided tests were performed in all settings except for the trend test, in which a one-sided test was applied. All calculations were performed using SAS version 10.0 (SAS Institute, Cary, NC).

RESULTS

Patient Characteristics

Patients' sociodemographic and clinical characteristics are listed in Table 1.

EGFR Mutation Shift in Plasma DNA Before and After First-Line Chemotherapy

Change in *EGFR* mutation status in plasma DNA was assessed for the 264 patients who had undergone first-line chemotherapy (cohort one). *EGFR* mutations were detected by DHPLC and confirmed by ARMS for 91 patients (34.5%) before first-line chemotherapy and for 61 (23.1%) after chemotherapy. There was only one patient case in which different mutation status was determined by the two methods (the patient's *EGFR* 21 exon mutation switched from positive to negative after chemotherapy based on the DHPLC result, but no switch was observed based on ARMS). The decrease in *EGFR* mutation rate after chemotherapy was statistically significant ($P < .001$ [McNemar test]; Table 2). No shift in subtype of mutation was observed after chemotherapy.

Influence of Tumor Response to First-Line Chemotherapy on *EGFR* Mutation Shift

In cohort one, the objective response rate to first-line chemotherapy was 31.1% (82 of 264), without significant statistical association

Table 1. Sociodemographic and Clinical Characteristics by Study Cohort

Characteristic	Cohort One: First-Line Chemotherapy (n = 264)		Cohort Two: Neoadjuvant Chemotherapy (n = 63)		Cohort Three: Palliative Surgery (n = 79)	
	No.	%	No.	%	No.	%
Age, years						
≤ 60	160	60.6	42	66.7	38	48.1
> 60	104	39.4	21	33.3	41	51.9
Sex						
Male	144	54.5	47	74.6	44	55.7
Female	120	45.5	16	25.4	35	44.3
Histologic subtype						
Adenocarcinoma	205	77.7	25	39.7	58	73.4
Nonadenocarcinoma	59	22.3	38	60.3	21	26.6
Smoking status						
Former smoker	136	51.5	33	52.4	36	45.6
Never smoker	128	48.5	30	47.6	43	54.4
Stage						
IIb to IIIa	0	0.0	58	92.1	25	31.6
IIIb to IV	264	100.0	5	7.9	54	68.4

with the pretreatment *EGFR* mutation status (prechemotherapy: mutant, 31.9% v wild type, 30.6%; $P = .84$). Among the 264 patients, 54 (20.5%) had mutation before treatment, but this changed to wild type after chemotherapy; 186 (70.4%) remained at mutation or wild type before and after treatment; and 24 (9.1%) had wild type, but this changed to mutation after treatment (Table 2). The PR rates in these three groups were 38.9%, 30.1%, and 20.8%, respectively. Conversely, the PD rates were 13.0%, 23.7%, and 25.0%, respectively. Among patients with discordant *EGFR* mutation status before and after chemotherapy, decrease in mutation was significantly associated with better clinical response ($P = .037$ [Cochran-Armitage trend test]; Fig 1; Table 3).

EGFR Mutation Shift in Tumor Tissues Before and After Neoadjuvant Chemotherapy

We also assessed change in *EGFR* mutation status in cohort two (n = 63). Twenty-two patients (34.9%) had *EGFR* mutations before neoadjuvant chemotherapy; this dropped to 12 (19.0%) after treatment (Table 4). A total of 49 patients (77.8%) retained their initial *EGFR* status (39 wild type, 10 *EGFR* mutant); in two patient cases (3.2%), wild type switched to mutation; and in 12 patient cases (19.0%), mutation switched to wild type. The discordant rate of *EGFR* mutation between pre- and post-neoadjuvant chemotherapy was

Table 2. Effect of First-Line Chemotherapy on *EGFR* Mutation Status Before and After Treatment in Plasma Samples From Patients With Stages IIIb to IV NSCLC (n = 264)

Prechemotherapy	Postchemotherapy				Total	
	Wild Type		Mutated		No.	%
Wild type	149	56.4	24	9.1	173	65.5
Mutated	54	20.5	37	14.0	91	34.5
Total	203	76.9	61	23.1	264	100.0

NOTE. $P < .001$ (McNemar test).
Abbreviation: NSCLC, non-small-cell lung cancer.

22.2%. The decrease in *EGFR* mutation rate after chemotherapy was statistically significant ($P = .013$ [McNemar test]; Table 4).

Evaluation of tumor response to neoadjuvant chemotherapy revealed that 38 patients had achieved PR, and 24 had achieved SD. One patient case was not evaluable because of no response data. There was no patient with PD in this cohort. In 14 patients, *EGFR* mutation status changed before and after treatment, with 12 patients changing from mutation to wild type and two from wild type to mutation. The PR rates for these two groups were 63.6% and 50%, respectively. The difference was not statistically significant ($P = .64$ [Cochran-Armitage trend test]). Sociodemographic and clinical factors, including chemotherapy regimen, number of cycles, age, sex, smoking status, and histologic subtype, were not associated with *EGFR* shift ($P > .05$).

Heterogeneity Analysis of EGFR Mutation in Intratumor Tissue Samples

We performed genetic heterogeneity analysis for samples from 79 patients with stages IIIa to IV NSCLC who had received palliative

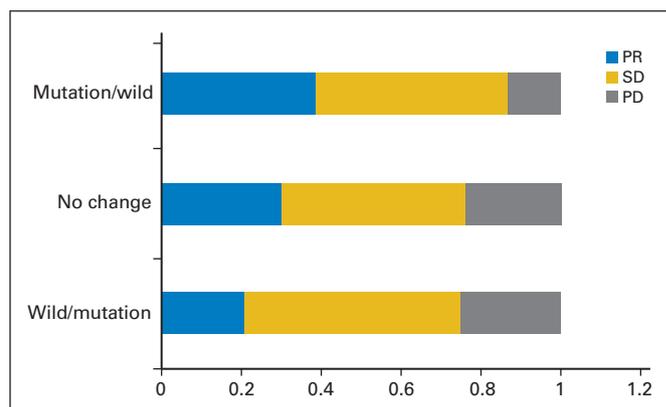


Fig 1. Response outcome between different subgroups of mutation change in cohort one. The x-axis indicates percentage of different response group; the y-axis indicates three groups according to *EGFR* mutation variation. PD, progressive disease; PR, partial response; SD, stable disease.

Table 3. Effect of First-Line Chemotherapy on Change in *EGFR* Mutation in Plasma Samples Before and After Treatment and Association With Tumor Response (n = 264)

<i>EGFR</i> Mutation Change From Pre- to Postchemotherapy	Tumor Response						Total (n = 264)	
	PR		SD		PD		No.	%
	No.	%	No.	%	No.	%		
Mutation → wild type*	21	38.9	26	48.1	7	13.0	54	100.0
No change	56	30.1	86	46.2	44	23.7	186	100.0
Wild → mutation*	5	20.8	13	54.2	6	25.0	24	100.0

Abbreviations: PD, progressive disease; PR, partial response; SD, stable disease.
**P* = .037 (Cochran-Armitage trend test).

surgery. Forty-two patient cases were *EGFR* mutation positive, including 23 with exon 19 mutation (group E19) and 19 with exon 21 mutation (group E21). Of these 42 patient cases with *EGFR* mutation, a total of 1,331 tumor foci were fractionated, with a median mutation rate of 74.75%. Sixteen of 42 samples consisted of only *EGFR*-mutated cells, whereas the other 26 samples consisted of cells with both wild-type and mutated *EGFR*, with the proportion of *EGFR*-mutant cells ranging from 30% to 90%. Among the 37 patient cases (group W) previously determined in routine detection to be *EGFR* wild type, 1,175 tumor foci were fractionated for analysis of *EGFR* mutation, and four (10.8%) showed low mutant frequency (three with *EGFR* exon 19 mutation, one with *EGFR* exon 21 mutation) ranging from 7.69% to 20.83%. After combining the 26 mutated patient cases with both wild-type and mutant *EGFR* cells, and the for nonmutated patient cases with low abundant mutated cells, 38% of the samples (30 of 79) had intratumoral heterogeneity regarding *EGFR* mutation. The remaining 62% of the samples (49 of 79) contained only *EGFR* mutation-positive cells or only wild-type cells. In semiquantitative analysis, the mutant abundance values for the three patient cases in group W with low mutation rates were 0.06, 0.08, and 0.22, respectively. On the other hand, the mutant abundance values for the 23 patient cases with exon 19 mutation ranged from 0.4 to 9.5. Not surprisingly, the difference in mutant abundance values was statistically significant (*P* = .011 [Wilcoxon rank sum test]).

DISCUSSION

Heterogeneity in *EGFR* gene expression, mutation, or amplification between primary and metastatic tumors has been observed in several

small-sample studies, with discordance seen in 25% to 35% of patients.²³⁻²⁵ However, to our knowledge, none of these studies evaluated the influence of chemotherapy on *EGFR* mutation. Our study provides evidence that first-line chemotherapy may influence *EGFR* mutation status in both tissue and peripheral blood samples. The decrease in mutation rate was significantly associated with better clinical response (PR v PD).

Recently, several studies have focused on exploring the potential possibility that *EGFR* mutation detection in circulating tumor DNA from plasma or serum samples had been used as a surrogate of primary tumor tissue samples.^{18,26-28} The concordance of *EGFR* mutation status between peripheral blood samples and matched tumor tissue had been reported as varying from 59.1% to 92%, with minimal false-positive rates and variable false-negative rates.^{18,26-28} In our previous study, 230 plasma samples and matched tumor tissue samples were used for *EGFR* mutation detection, and concordance was 78%. Recently, we extended the study to include an additional 822 matched plasma and tissue samples, in which 744 patients had advanced NSCLC.²⁹ The mutation concordances of the two kinds of samples were 70% and 78% for total population and patients with advanced disease, respectively, similar to results in our previous report.¹⁸ Although the DHPLC method has not been widely used for *EGFR* mutation analysis, its high sensitivity and specificity have been shown in our and other studies (detection limit approximately 3%-5%).^{18,30} To minimize the possibility of false results by the DHPLC method, we verified *EGFR* mutation status using the ARMS method, which is considered more sensitive in mutation analysis; the results were identical, except for one mutation sample deemed negative after chemotherapy by DHPLC but not ARMS. Overall, the DHPLC method for *EGFR* mutation analysis in plasma is feasible and reproducible.

We showed that the overall incidence of *EGFR* mutation was lower in plasma DNA after first-line chemotherapy and in tumor tissues after neoadjuvant chemotherapy (Tables 2 and 4). This chemotherapy-related change may partially explain why chemotherapy-resistant tumors are less sensitive to *EGFR*-TKI treatment than chemotherapy-naïve tumors.³¹ It may also explain why almost all clinical trials involving second-line TKI therapy have failed to show a positive correlation between *EGFR* mutation and progression-free or overall survival.^{2,17} *EGFR* mutation detection at the time of initial diagnosis has failed to reflect exactly mutation status at the initiation of *EGFR*-TKIs as second- or third-line treatment. Therefore, future prospective trials should consider analyzing biopsies taken immediately before second- or third-line *EGFR*-TKI therapy. Our study provides an alternative blood-based strategy to assess *EGFR* mutation status.

Table 4. Effect of Neoadjuvant Chemotherapy on *EGFR* Mutation Status Before and After Treatment in Tissue Samples From Patients With Stages IIb to IIIb NSCLC (n = 63)

Prechemotherapy	Postchemotherapy				Total	
	Wild Type		Mutated		No.	%
No.	%	No.	%			
Wild type	39	61.9	2	3.2	41	65.1
Mutated	12	19.0	10	15.9	22	34.9
Total	51	81.0	12	19.0	63	100.0

NOTE. *P* = .013 (McNemar test).
Abbreviation: NSCLC, non-small-cell lung cancer.

Whether EGFR-TKI treatment should precede or be followed by chemotherapy remains an unresolved clinical issue. In a recent multicenter randomized phase III study comparing the order of erlotinib and cisplatin plus gemcitabine in unselective advanced NSCLC, chemotherapy followed by erlotinib resulted in better progression-free and overall survival compared with erlotinib followed by chemotherapy.³² Unfortunately, this study did not address the question of the optimal order of EGFR-TKIs and chemotherapy for patients harboring *EGFR* mutations. Chin et al³³ used an erlotinib-sensitive *EGFR*-mutant NSCLC cell line to explore whether its prior exposure to platinum agents would affect subsequent response to erlotinib. Their results suggest that first-line chemotherapy in *EGFR*-mutant NSCLC may reduce the benefit of subsequent EGFR-TKI treatment. Our results are consistent with the observation of Chin et al; decreased *EGFR* muta-

tion frequency after chemotherapy may reduce the overall clinical benefit of subsequent EGFR-TKI treatment. If these results are confirmed in future prospective and multicenter studies, the therapeutic strategy of using EGFR-TKIs followed by chemotherapy should be considered as an optimal option for patients who harbor *EGFR* mutations.

The shift in tumors from *EGFR* mutation status to wild-type status observed in this study suggests that both mutant and nonmutant cancer cells coexist in the same tumor. To identify intratumor heterogeneity, we microdissected and analyzed *EGFR* mutation status in more than 2,506 tumor foci of 79 tumors from patients with NSCLC who underwent palliative surgery (Fig 2). Approximately 38% of tumors contained both *EGFR*-mutant and wild-type foci, similar to the rate reported by Taniguchi et al.³⁴ Recently, the study by

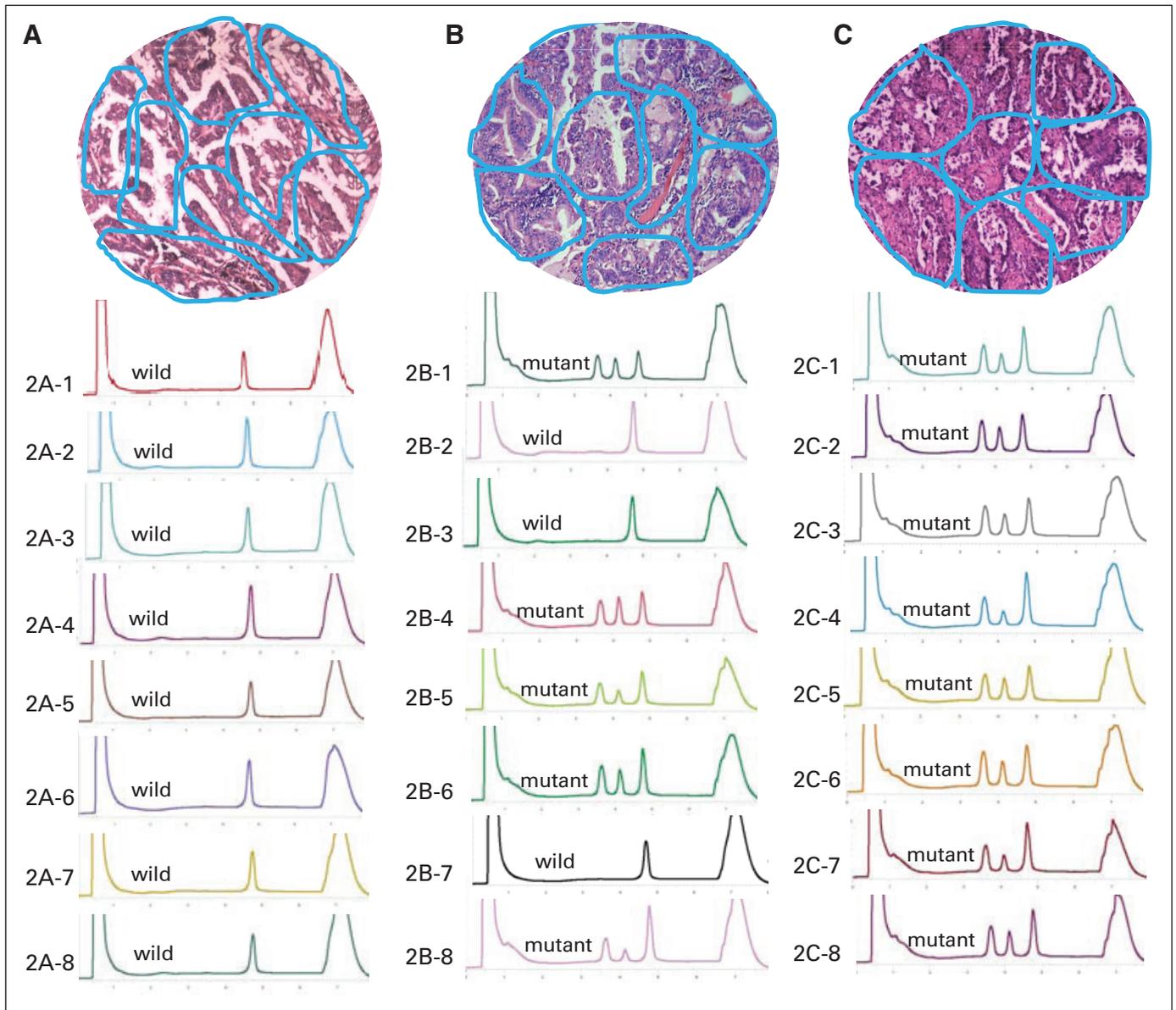


Fig 2. Multiple foci microdissection and analysis of intratumor genetic heterogeneity on *EGFR* mutation. Blue circles in hematoxylin and eosin staining tissue ($10 \times$) represent microdissected foci; DHPLC graph represents corresponding tumor foci mutation status. (A) Tissue with homogeneity of wild-type *EGFR*; (B) tissue with heterogeneity of *EGFR* mutation; (C) tissue with homogeneity of mutant *EGFR*.

Gerlinger et al³⁵ revealed high intratumoral heterogeneity of multiple tumor-suppressor genes converging on loss of function. Conversely, Yatabe et al³⁶ reported a rare occurrence of the heterogeneous distribution of *EGFR* mutation. However, it should be noted that only 450 tumor micropoints were analyzed in the Yatabe et al study.

It is interesting to note that a majority of *EGFR* mutation changes after chemotherapy were from mutant state to wild type, suggesting that cancer cells harboring *EGFR* mutations might be more sensitive to chemotherapy than those without mutation. We further analyzed the relationships between chemotherapy responses and the shift of *EGFR* mutation status and found patients who achieved PR were more likely to have had *EGFR* mutation shift than those achieving SD or PD after chemotherapy. This is consistent with the results from the IPASS trial, where the objective response rate was 47.3% in *EGFR*-mutant patients treated with chemotherapy, significantly higher than the rate in *EGFR* mutation–negative patients (23.5%).¹² Together with previous studies, this study suggests that *EGFR* mutation shift may be related to the heterogeneity of intratumoral *EGFR* mutation and to different chemosensitivity levels of mutant and wild-type cells. Interestingly, in our study, four patients (10.8%) had low frequency and abundance of *EGFR* mutations, which were detected only through microsamples, thus explaining in part why some patients' *EGFR* mutation status shifted from negative to positive after chemotherapy. To further confirm these results, a study in vivo exploring molecular mechanisms resulting in chemotherapy-related shift of *EGFR* mutation status is ongoing in our group.

It has been shown that chemotherapy may alter DNA concentration in serum or plasma.^{37,38} To exclude the influence of DNA concentration change after chemotherapy on the shift of *EGFR* mutation status, the relationship between them was analyzed. The results revealed that there was no association between change in plasma DNA levels and shift in *EGFR* gene mutation after chemotherapy, suggesting that the influence of chemotherapy on mutation of the *EGFR* gene is independent of the change in the level of DNA in the plasma (Appendix; online only).

One of the limitations of the current study is the lack of blood and tissue samples from the same patients. The first cohort provided plasma only, whereas the other two cohorts provided tissue samples only. Nevertheless, we used the plasma samples to show that frequency of *EGFR* mutation was significantly decreased after two cycles of chemotherapy; we used paired tumor tissues to show that mutation frequency was reduced in the tumors as well; and we used large advanced tumor tissues to show the heterogeneity of *EGFR* mutations within some of the tumors. Results from the three interconnecting cohorts provided a solid foundation to support our conclusions.

In conclusion, chemotherapy may affect *EGFR* mutation status in patients with NSCLC, lowering the number of mutations. This observation may be attributable to the heterogeneity of intratumoral *EGFR* mutations and the different sensitivities of *EGFR*-mutated and wild-type tumor cells to chemotherapy. These findings should be considered in future studies designed to elucidate the predictive role of *EGFR* mutation in second-line TKI therapy for patients with NSCLC.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

The author(s) indicated no potential conflicts of interest.

AUTHOR CONTRIBUTIONS

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Administrative support: Jie Wang

Provision of study materials or patients: Zhijie Wang, Keneng Chen, Jun Zhao, Qinghua Zhou

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