Clinical implications of PNA-sequencing as a complementary test for EGFR mutation analysis in human lung cancer

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Abstract. Epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs) are the first-line regimen for the treatment of non-small cell lung cancer (NSCLC) patients with EGFR mutations. However, false-negative results are occasionally observed, even with FDA-approved molecular tests. Such examples in have been reported in our pilot study showing a slightly upward-shifted amplification curve using commercial reverse transcription-quantitative (RT-q)PCR. Verification using peptide nucleic acid (PNA) clamping-sequencing, which has a sensitivity of ~0.1%, may allow better prediction of which patients will benefit from EGFR-TKI therapy. To confirm this hypothesis, samples were prospectively collected from 1,783 lung cancer cases diagnosed in National Cheng Kung University Hospital between 2012-2018. An independent lung cancer cohort of 1,944 cases was also recruited from other hospitals. The clinical significance of mutant-enriched PCR with PNA-sequencing was analyzed and patient outcomes were followed. A total of 17 of 34 cases (50%) were found to harbor EGFR mutations by PNA-sequencing. A total of 22 cases were discovered in the independent lung cancer cohort, and 14 of these (63.6%) cases had EGFR mutations. TKIs were administered to 14 of the 17 mutation-positive patients, and a partial response was observed in 4 cases and stable disease in 10 cases. Patients with EGFR mutations receiving a TKI regimen had a longer overall survival (OS) (median: 40.0 vs. 10.0 months) compared with those without treatment. The difference in OS was not significant. Based on the results of the present study, combining RT-qPCR with PNA-sequencing may be a practical supplementary technology in a clinical molecular laboratory for a subset of lung cancer patients in selection of EGFR TKI therapy.

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Introduction

Accumulating evidence indicates that activating mutations of the epidermal growth factor receptor (EGFR) are present in ~50% of patients with advanced non-small cell lung cancer (NSCLC) of Eastern Asian ethnicity compared with 12-15% in the Caucasian population (1,2). These mutations predict sensitivity to first- and second-generation tyrosine kinase inhibitors (TKIs), such as gefitinib, erlotinib, or afatinib, and are pivotal molecular biomarkers in the selection of targeted therapy (3-5). Thus, a sensitive and reliable laboratory test is critical in the design of personalized therapy for this subset of patients.

Recent advances in molecular pathology have introduced highly sensitive technology platforms for detecting EGFR mutations by reverse transcription-quantitative (RT-q)PCR using specific probes or amplified refractory mutation system (ARMS), such as the EGFR PCR Kit or therascreen EGFR RGQ PCR kit provided by Qiagen GmbH (6). Both kits characteristically produce flat or linear lines for negative results, making it difficult to ignore the occasional late upward-shifted PCR curves beyond the limits of detection (LOD). The peptide nucleic acid (PNA)-sequencing was designed to construct the PCR clamp reactions, in which the PNA clamp suppresses the amplification of wild-type DNA and thus enriches the amplification of mutant sequences. PNA-mediated PCR clamping is an interesting technology in the detection of gene mutations. Both simplicity and versatility make it especially advantageous for large-scale screening programs. Thiede *et al* (7) proposed the simple and sensitive method of PNA-mediated PCR clamping in the detection of mutations in the ras proto-oncogenes. Sano *et al* (8) reported that PNA-clamped probe assay is more sensitive than direct sequencing in the detection of mutations in samples containing <1% mutant alleles. Our previous study confirmed its comparable performance in terms of EGFR mutation detection compared with RNA sequencing but was

more sensitive in malignant pleural effusion than by direct sequencing (9).

In a pilot study performed on a lung cancer cohort (n=346), 9 equivocal cases with late upward PCR curves were further investigated using mutant-enriched PCR with PNA-sequencing, of which the analytical sensitivities were conservatively estimated at 1% or better for both E746_A750del and L858R mutations using cloned DNA fragments mixed with a wild-type EGFR background. All 20 cases revealed unequivocally negative results in exons 19 and 21 by PNA-sequencing, supporting the notion that a flat baseline or linearly increasing straight line observed in reverse transcription-quantitative PCR is genuinely negative. The data verified no false positive and false negative results for PNA-sequencing (10). PNA-sequencing showed that 6 of the 9 equivocal cases harbored EGFR mutations. The results supported the usage of PNA sequencing as a supplementary method in a clinical diagnostic setting.

Herein, the validation of the clinical value of the supplementary PNA-sequencing technology in a larger cohort and an independent referral cohort from other institutes is reported. Clinical responses to first- or second-generation EGFR-TKIs were assessed in 17 PNA-positive cases in the recruited cohort (n=1,783) and 3 PNA-positive cases from the referral cohort (n=1,944).

Materials and methods

Patients and samples. For the retrospective cohort study, the need for informed consent from the patients was waived by the Institutional Review Board of National Cheng Kung University Hospital (Tainan, Taiwan). Informed consent was obtained for the use of patient tissues but was waived for the use of patient data. A total of 1,783 cases of formalin-fixed, paraffin-embedded (FFPE) NSCLC, diagnosed between September 2012 and February 2018 in our institute were included in the present study. Out of 1,783 patients, 847 (47.5%) were males. The median age was 66 years (range, 26-99 years). All cases were diagnosed as stage III or IV adenocarcinoma and reviewed independently by two pathologists. The clinical information of the patient cohort was independently reviewed by two clinicians. Any disagreement in the reviewing of samples was resolved by a third author. Basic demographic information, smoking status, stage at presentation, EGFR mutation type, and treatment details were obtained from the medical records. The response of the tumor to EGFR TKI was evaluated by chest radiography and computed tomography (CT) every 2-4 and 8-12 weeks after treatment, respectively. Treatment response was assessed according to the Response Evaluation Criteria in Solid Tumors (RECIST) v1.1 (11). Progression of disease was evaluated on scan imaging (11). In addition, clinical deterioration or death from any cause without evidence of disease progression was also defined as progression in the calculation of progression-free survival (PFS). Objective response rate (ORR) was defined as the achievement of either a complete or partial response.

An independent lung cancer cohort of 1,944 cases was also collected from other referral hospitals between September 2012 and February 2018. Out of 1,944 patients, 953 (49.0%) were male. The median age was 67 years (range, 24-98 years). Referral hospitals included: The Department of Surgery, E-DA hospital; Department of Oncology, Tainan Hospital, Ministry of Health and Welfare; Department of Oncology, Tainan Municipal Hospital; and Department of Oncology, Dalim, Tzu Chi Hospital.

The independent lung cancer cohort from other hospitals was recruited to reduce potential selection bias. All cases were diagnosed as stage III or IV adenocarcinoma. Only 3 cases with EGFR mutations from E-DA hospital had follow-up data.

FFPE DNA extraction. FFPE tissues were used for molecular diagnosis. Hematoxylin and Eosin-stained histological sections were reviewed by a pathologist who marked the tumor area (>20%) for macrodissection. DNA was then extracted using a QIAcube automated extractor (Qiagen GmbH) using the QIAamp DNA FFPE tissue kit (Qiagen GmbH) according to the manufacturer's instructions.

EGFR mutation analysis. The EGFR mutations were analyzed using an EGFR PCR Kit (EGFR RUO Kit; Qiagen GmbH) (n=256) and therascreen EGFR RGQ PCR Kit (EGFR IVD Kit; Qiagen GmbH) (n=3,471). Both diagnostic kits combine Scorpion probe/primers and ARMS technologies to detect the mutations by RT-qPCR. The assay was performed according to the manufacturer's instructions. This assay system was developed for the detection of the most commonly reported EGFR mutations, including 19 deletions in exon 19, 3 insertions in exon 20, and point mutations of G719X (in exon 18), S768I (in exon 20), L858R and L861Q (in exon 21).

EGFR IVD Kit assessing a T790M (exon 20) mutation, an important TKI-resistant mutation (12,13), was used for clinical analysis. Analysis was performed using the Rotor-Gene Q series built-in software version 2.0.3 (Build 2) for the EGFR RUO Kit and EGFR IVD Kit (Qiagen GmbH). The EGFR PCR Kit (EGFR RUO Kit) was first introduced to our laboratory for EGFR mutations, and subsequently replaced by the therascreen EGFR RGQ PCR Kit (EGFR IVD Kit) to improve the detection of T790M (exon 20). The EGFR mutation rate was 54.4 and 54.9% for the EGFR RUO Kit and IVD Kit, respectively based on the results. No significant differences between the methods were observed. Real-time curves were generated using FAM-labeled probes for exon 2 control and each mutation in separate tubes. To calculate a ΔCq value for each mutation reaction, the following equation was used: [Mutation Cq]-[Control Cq]=ΔCq. Manufacturer-supplied ΔCq thresholds were used as the limit of detection (LOD) to call a mutation (≤ΔCT threshold is positive for mutation).

During the study period, a total of 34 and 22 cases showing upward-shifted amplification curves in the late cycles beyond the LOD were discovered in our hospital and other institutes, respectively. They were submitted for further investigation using direct sequencing and PNA-sequencing.

PNA-sequencing. For PNA-sequencing of *EGFR* exons 19 and 21, PNA was used to construct the PCR clamp reactions, in which the PNA clamp suppresses the amplification of wild-type DNA, and thus increases the preferential amplification of mutant sequences (10). PNA oligos were synthesized by PANAGENE Inc. The PNA clamp primer for exons 19 and 21 was designed to be homologous to the wild-type

allele at codons 746-751 and 855-860, respectively (RefSeq accession no. NM_005228.3).

The analytical sensitivity of PNA-sequencing was estimated at ~1% for both E746_A750del and L858R mutations using cloned DNA fragments serially diluted with A549 cells containing wild-type EGFR. They were mixed with ratios from 1:1 to 1:1,000 in the prior pilot study (10).

PNA-sequencing was performed in a mixture of PCR primers (10 mmol/l each), 100 mmol/l PNA oligos, 25 ng genomic DNA (gDNA), and polymerase mix (Super Therm Gold MasterMix; Bionovas Biotechnology). The PCR amplicons were purified and then subjected to bidirectional sequencing.

Clinical response to EGFR-TKIs. All of the patients received a CT scan of the chest before initiation of the treatment and every 12 weeks thereafter to evaluate the tumor response. Brain imaging and bone scans were performed if associated symptoms occurred in patients. The primary endpoint was progression-free survival (PFS). The secondary endpoints included disease control rate, overall response rate, and overall survival (OS). The EGFR mutation status was analyzed by PNA-sequencing.

PFS was calculated from the date of TKI treatment until the date of radiological progression according to the RECIST v1.1 guidelines or death, with censoring at the date of last follow-up in the event that the patient did not progress. The overall response rate was calculated as the percentage of patients who exhibited a partial response or complete response in the first image study after the introduction of the TKI treatment, while the disease control rate was defined as the percentage of patients who exhibited a partial response, complete response, or stable disease. Furthermore, the duration of OS was defined as the period from the beginning date of TKI treatment until the date of death. If follow-up was incomplete or interrupted at the end of the study, the nurse practitioner was asked to call their families to clarify the status of the clinical response.

Major changes in the status according to the RECIST v1.1 guidelines related to imaging include the following: i) Number of target lesions; ii) assessment of pathologic lymph nodes; iii) status of disease progression; iv) clarification of unequivocal progression of non-target lesions; and v) hybrid imaging with F-18 fludeoxyglucose positron emission tomography/magnetic resonance imaging (FDG PET/MRI) that correlated with the results of a CT scan in the detection of new lesions. The progression of the disease was evaluated using scan imaging. Primary outcomes were OS and PFS; secondary outcomes were overall ORR based on the RECIST v 1.1 guidelines.

Chemotherapy. Of 17 NSCLC patients with low levels of EGFR mutation discovered in our hospital, three patients did not receive TKIs due to presence of a brain metastasis (n=2) or as a conservative strategy following after lobectomy. The remaining patients with activating EGFR mutations (exon 19 deletions and exon 21 L858R received TKIs. A first-generation EGFR-TKI (gefitinib and erlotinib) is the standard treatment for patients with locally advanced or metastatic NSCLC, in particular for those patients harboring EGFR mutations. Afatinib is an oral irreversible second-generation EGFR TKI. This drug was developed for patients showing resistance to the first-generation EGFR TKIs. If resistance is still observed, Osimertinib is a potent irreversible third generation EGFR-TKI targeting EGFR mutations with very little effect on wild-type EGFR (14,15).

If chemotherapy is considered (for example, in case of EGFR exon 20 insertion), patients should receive pemetrexed (Alimta®) 500 mg/m^2 combined with carboplatin (area under the concentration-time curve of 5 mg/ml/min,) both administered on day 1 of a 21-day cycle. The treatment should be continued for 4 cycles or until unacceptable toxicity or disease progression appears, in which case bevacizumab may be administered as a salvage chemotherapy (16,17).

Statistical analysis. Patient characteristics (age, sex, smoking history, stage and histology) were tabulated in relation to mutation status. A Fisher's exact test was used to analyze the relationship between patient characteristics and EGFR mutations. $P<0.05$ was considered to indicate a statistically significant difference.

The PFS after TKI therapy was calculated from the first day of TKI treatment until the earliest sign of disease progression or death from any cause. OS for the entire cohort was calculated from the date of diagnosis to the time of death or last follow-up. The follow-up data was censored on October 30, 2022. Kaplan-Meier curves were used to estimate the probabilities at each time point when an event occurred and the probability of survival at each point in time. A log-rank test was used to identify factors affecting the OS of the entire cohort, and multivariate analysis was performed using Cox regression analyses. SPSS version 17.0 (SPSS, Inc.) was used for statistical analysis.

Results

Patient characteristics. A total of 3,727 patients (NCKUH, 1,783; other institutes, 1,944) were enrolled in the present study. The patient characteristics (age and sex) and histological profile did not differ significantly between our institute and the other institutes (Table SI). For the 3,727 patients, the median age was 66.5 years (range, 20-99 years). EGFR mutation status was not associated with patient age ($P>0.999$) but was more frequent in females than in males (60.1 vs. 39.9%; $P<0.05$). The histology of the tumors included adenocarcinoma (89.3%), adenosquamous carcinoma (1.4%), squamous cell carcinoma (4.0%), and NSCLC-NOS (5.3%). The rate of EGFR mutation was significantly higher than that of wild-type EGFR in patients with adenocarcinoma (60 vs. 40%; $P<0.05$) but did not differ in terms with regard to smoking habits and clinical stage. Around one-fifth (22.5%) of the specimens came from surgical resection and the others (77.5%) were small biopsy or cell block specimens. Among these patients, 2,075 cases (55.7%) had EGFR mutations, with exon 19 deletions in 831 cases (40%), L858R mutation in 1,001 cases (48.2%), and other mutations in 243 cases (11.7%) (Table I). Interestingly, 68 cases (3.3%) had T790M mutation in combination with other mutations analyzed by the EGFR IVD Kit.

EGFR mutation analysis. Between September 2012 and February 2018, 34 cases were categorized as equivocal from

Table I. Summary of the characteristics of the patients based on the EGFR mutation status.

Characteristic	EGFR mutant	EGFR wild-type	P-value
Age, years			
Mean ± SD	66.5±11.6	66.0±12.7	>0.05
Median	67	66	
Range	26-99	20-98	
Sex, n (%)			
Male	827 (43.4)	1,078 (56.6)	<0.05
Female	1248 (68.5)	574 (31.5)	
Histological type, n (%)			
Adenocarcinoma	1,999 (60.0)	1,331 (40.0)	<0.05
Adenosquamous carcinoma	4 (47.1)	27 (52.9)	
Squamous cell carcinomas	13 (8.8)	135 (91.2)	
NSCLC-NOS	39 (19.7)	159 (80.3)	
Smoking history, n (%)			
Never smoker	1,452 (56.1)	1,135 (43.9)	>0.05
Smoker	623 (54.6)	517 (45.4)	
Stage, n (%)			
III	15 (37.5)	25 (62.5)	>0.05
IV	2,060 (55.9)	1,627 (44.1)	
Tumor sample, n (%)			
Surgical specimen	481 (57.4)	357 (42.6)	>0.05
Small biopsy or cell block specimen	1,594 (55.2)	1,295 (44.8)	
Mutation Type			
Exon 19 deletion	831 (40.0)		
Exon 21 L858R	1,002 (48.3)		
Other (including double mutation)	172 (8.3)		
Exon 20 T790M	2 (0.1)		
Exon 20 T790M combined with other mutations	68 (3.3)		

EGFR, epidermal growth factor receptor; NSCLC-NOS, non-small cell lung cancer-not otherwise specified.

among the 1,783 lung cancer cases analyzed in our institute (Table II). Of these, 6 cases were reported in our prior pilot study (2). To validate the performance of PNA sequencing technology, an independent lung cancer cohort of 1,944 cases was collected from other institutes between September 2012 and February 2018.

In total, EGFR mutations were detected in 2,044 of 3,727 cases of NSCLC (54.8%) using the EGFR RUO Kit or the EGFR IVD Kit. The control Cq fell within the recommended range of the kits. The Cq values were calculated and the threshold was set at 0.075. Then, ΔCq (mutation Cq-control Cq) was calculated. In the Qiagen EGFR assay, a negative result (wild-type) usually produced an undoubtedly flat baseline. Additionally, a linearly increasing straight line may have been observed in certain negative cases. In certain cases, however, an upward curve appeared in the later cycles that did not reach the threshold. The result was designated as negative according to the manufacturer's instructions. In our laboratory, these cases were reported as equivocal with concerns of potential false negativity.

Confirmation of the EGFR mutation status using PNA-sequencing. Of 34 equivocal cases discovered in our hospital, 4 cases were observed in the exon 19 assays and 13 cases in the exon 21 assays. They were submitted for PNA-sequencing and found to harbor EGFR mutations in 17 cases (50%). These mutations included 13 cases of L858R mutation and 4 cases of exon 19 mutations (two L746_A750del, one L747_A750delinsP and one E746_P753delinsVP).

Of 22 equivocal cases collected from referral institutes, 4 cases were found in the exon 19 assays and 10 cases in the exon 21 assays. PNA sequencing revealed 14 cases (64%) containing EGFR mutations, including 7 cases of L858R mutation, 1 case of L858P mutation, 2 cases of L861Q mutation, and 4 cases of exon-19 mutations (L746_A750del, L747_A751del, E746_P752delinsV and E746_P751delinsVA).

TKI response rate. Of 17 NSCLC patients with low levels of EGFR mutations (where the amount of mutant DNA was lower than the LOD), discovered in our hospital, the median age was 69 years and 15 patients (88%) were female (Table III).

Table II. Summary of the equivocal samples and EGFR mutation rates.

Cohort	Cases	Equivocal, n (%)	Detected by PNA-sequencing, n (%)	Exon 19 deletion, n	Exon 21 L858R/P[b], n	Exon 21 L861Q, n
NCKUH	1,783	34 (1.9)	17 (50.0)	4	13	0
Other hospitals	1,944	22 (1.1)	14 (63.6)	4	8	2
Total cases[a]	3,727	56 (1.5)	31 (55.4)	8	21	2

[a]Includes NCKUH and other hospitals; [b]L858R and L858P. EGFR, epidermal growth factor receptor; NCKUH, National Cheng Kung University Hospital; PNA, peptide nucleic acid.

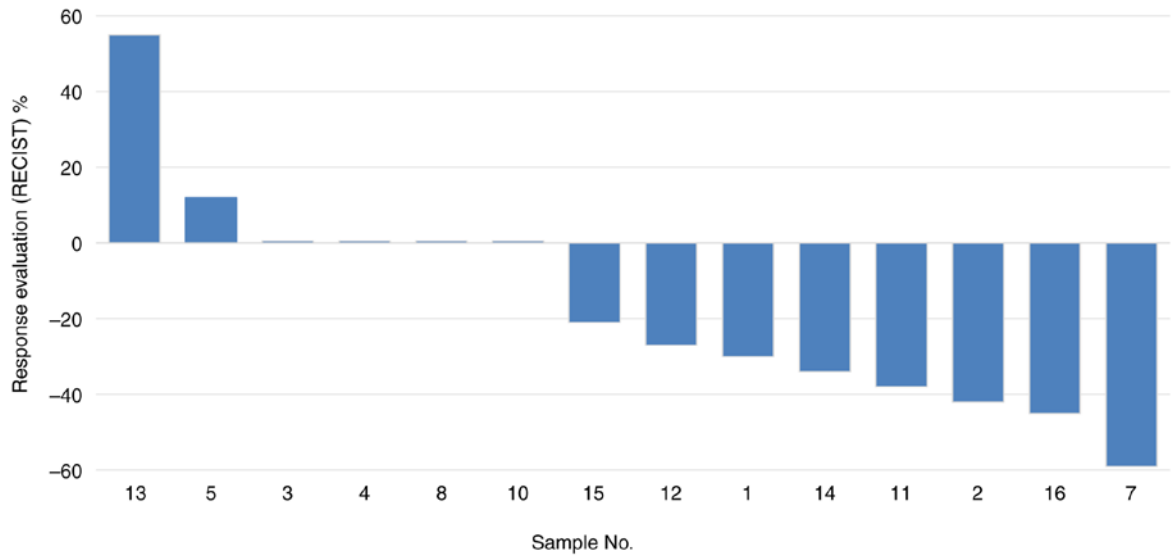

Figure 1. Percentage change in target-lesion size. Waterfall plot of EGFR-positive NSLC patients treated with TKIs. Response assessments of target lesions were assessed based on the RECIST 1.1 guidelines, as indicated by the percentage changes in the sum of the diameters of the target lesions as the optimum response during therapy. Of 17 NSCLC patients with low levels of EGFR mutation discovered in our hospital, three patients (No. 6, 9, and 17) did not receive TKIs due to the presence of a brain metastasis (n=2) or as a conservative strategy chosen after lobectomy (n=1). The remaining 14 patients received TKIs and were evaluated for clinical response, the distribution of reduction in target-lesion size (waterfall plot) after TKI treatment is shown. EGFR, epidermal growth factor receptor; NSCLC, non-small cell lung cancer; TKI, tyrosine kinase inhibitor.

Of these, 3 patients (patients no. 6, 9, and 17) did not receive TKIs due to a brain metastasis (n=2) or a conservative strategy chosen after lobectomy (n=1). The remaining 14 patients received TKIs and were evaluated for clinical response, in which 10 cases had an L858R mutation and two had an exon 19 deletion. Of the 14 patients, 7 patients (50%) showed partial response, of which, 6 patients (42.8%) had stable disease and 1 patient exhibited disease progression. The distribution of reduction in target-lesion size (waterfall plot) after TKI treatment is shown in Fig. 1.

Correlation between EGFR mutation status and clinical response to EGFR TKIs. Case 10 was a 78 year female who had stage IIA squamous cell carcinoma (T2aN1M0) and received video-assisted thoracic surgery for the right lower lobe of lung and mediastinal lymph node dissection in 2013. The EGFR RUO kit initially yielded a negative result, with a late upward shifted curve, but was shown to have an EGFR L858R mutation by PNA-sequencing in October 2014. The recurrence of lung cancer with left pleural seeding occurred in November 2014. The patient first received Gefitinib (Iressa; 250 mg OD) treatment for 25 months, which showed mild regression of the left lingual lung. Later, Gefitinib was replaced with Erlotinib (Tarceva; 150 mg OD) for severe xerosis.

The follow-up CT at 31 months after TKI treatment showed a reduction of the right lung tumor of ~53% (from 2.8 to 1.3 cm in diameter) with slight regression of left side pleural seeding (Fig. 2). The patient was lost to follow-up in March 2018.

Overall survival of equivocal cases. A total of 14 patients with EGFR mutations had a longer 5-year OS (median: 40.0 vs. 10.0 months) after TKI treatment than those with wild-type EGFR (n=17; P=0.085, log-rank test) using a univariate analysis model (Fig. 3). The OS of patients with or without EGFR gene mutations was 40.0 months (95% CI, 24.2-33.8) and 10.0 months (95% CI, 14.7-27.3), respectively. The difference in OS was not significant (P>0.05).

Discussion

The Qiagen kits are trustworthy methods that have been used daily for diagnosis for years in our practice. The infrequent

Table III. Summary of equivocal samples and treatment response.

Case no.	Sex	Age	Tumor, %	Pathology	Stage	Smoking	TTF-1	EGFR result	Systemic therapy	Response evaluation	RECIST, %	Time since last TKI use, months	Survival
1	F	90	20	AD	IV	Y	+	L858R	TT	PR	-30	50	No
2	F	71	20	AD	IV	N	+	L858R	TT	PR	-42	40	No
3	F	70	20	AD	IV	N	+	L858R	TT	SD	0	5	No
4	F	73	20	AD	IV	N	NA	L858R	TT	SD	0	10	No
5	F	84	35	AD	IV	Y	NA	L858R	TT	SD	+12	40	No
6	F	52	40	AD	IV	N	+	L858R	Lobectomy	None[a]	None	NA	Yes
7	F	40	30	AD	IV	N	+	L858R	TT	PR	-59	19	No
8	F	54	20	AD	IV	N	NA	E746_A750del	TT	SD	0	80	Yes
9	M	56	70	NSCLC-NOS	IV	N	+	E746_S752delinsV	None	None[a]	None	NA	No
10	F	52	40	AD	IV	Y	+	E746_T751delinsVA	TT	SD	0	40	No
11	F	78	70	SCC	IV	N	NA	L858R	TT	PR	-38	31	No
12	F	69	2	AD	IV	N	+	L858R	TT	SD	-27	51	No
13	F	86	10	AD	IV	N	+	L858R	TT	PD	+55	2	No
14	F	81	30	AD	IV	N	NA	L858R	TT	PR	-34	30	No
15	M	55	20	AD	IV	N	NA	L858R	TT	SD	-21	60	Yes
16	F	60	30	AD	IV	N	+	L858R	TT	PR	-45	20	No
17	F	58	40	AD	IV	N	+	L747_T751del, Exon 20 Insertion	None	None[a]	None	NA	No

[a]Brain metastasis. AD, adenocarcinoma; NSCLC-NOS, non-small cell lung cancer-not otherwise specified; SCC, squamous cell carcinoma; TTF1, thyroid transcription factor-1; NA, not available; TT, targeted therapy; None, the patient did not receive systemic therapy; RECIST, Response Evaluation Criteria in Solid Tumors; PR, partial response; SD, stable disease; PD, progressive disease; M, male; F, female; EGFR, epidermal growth factor receptor; TKI, tyrosine kinase inhibitor.

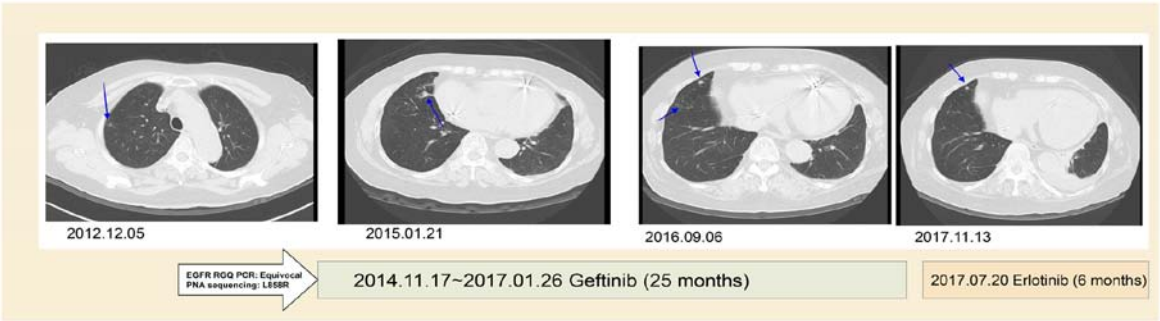

Figure 2. Example of a partial response to TKI treatment. The patient was a 78-year-old woman with squamous cell carcinoma. The computed tomography images pretreatment (left) and post-treatment (right). The tumor size decreased from 2.8 to 1.3 cm (53%) after 31 months of TKI treatment. The patient was lost to follow-up in March 2018. Blue arrows designate the tumor lesion. TKI, tyrosine kinase inhibitor.

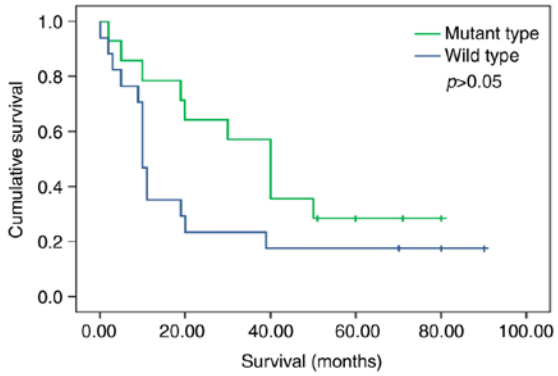

Figure 3. Kaplan-Meier estimations of OS in patients with different *EGFR* mutations using PNA-sequencing. *EGFR* mutation vs. wild-type cases for the 5-year OS. The median survival was 40.0 vs.10.0 months (P=0.085 using a Log-rank test) in the univariate analysis model. The OS of the patients with or without EGFR gene mutations were 40.0 months (95% CI, 24.2-33.8) and 10.0 months (95% CI, 14.7-27.3) respectively (P>0.05). The difference in OS was not significant. OS, overall survival; EGFR, epidermal growth factor receptor; CI, confidence interval.

occurrence of a late upward shift in the PCR curve renders them useful only in specific cases. In our experience, homemade RT-qPCR tests very often produce late upward PCR curves, but these negative results should be neglected. In contrast, the Scorpion ARMS system characteristically almost always produces flat or linear lines for these same negative results. At least three possible explanations can account for the upward curves in the later cycles of amplification. First, the amount of mutant DNA may be just lower than the detection limit of the Qiagen kits. This is because most of the positive cases are taken from small biopsy specimens with a low percentage of tumor cells. Second, the heterogeneous distribution of the low levels of mutant tumor cells in the specimen. Third, low levels of mutant DNA in these samples result in sampling bias, resulting in divergent results when analyzed using different techniques. The primary purpose of this study was to share our experience on how to pick up the rare drop-off of a robust system by rechecking cases with late upward shifts in the PCR curve, which occur infrequently.

In the prior pilot study, it was demonstrated that a small subset (6/346) of NSCLC lung cancer patients with low levels of EGFR mutations may have been misclassified by a commercial RT-qPCR assay system. The apparent reduction of lesion size or partial response of a tumor to TKI treatment supports the application of PNA-sequencing as an important supplementary technology for patients with a low tumor burden (18-21).

The present study validated the performance and clinical value of the PNA-sequencing platform. Between September 2012 and February 2018, 34 equivocal cases were encountered in 1,783 patients with lung cancer patients in our institute and 17 of these (50%) were proven to harbor EGFR mutations by PNA-sequencing. These patients achieved either partial response or stable disease (92.8% disease control rate) to TKI treatment, which indicated a subset of patients may benefit from a more sensitive diagnostic method.

PNA oligos can be used as a sequence-specific PCR blocker given its higher binding affinity and specificity to the target DNA without recognition by DNA polymerase. PNA clamping is inexpensive and convenient to set up in a clinical diagnostic laboratory (22,23). In the present cohort study, several uncommon subtypes of EGFR exon-19 deletions were detected, including L747_A750delinsP, E746_P753delinsVP, L747_A751del, E746_P752delinsV, and E746_P751delinsVA. The results confirm that PNA clamping is useful for the detection of rare alleles at hotspots of sequence variations.

The Qiagen kits were designed to detect 29 somatic mutations of the EGFR gene, which covers the majority of the mutational spectra. Approximately 90% of EGFR mutations are either exon 19 deletions or exon 21 L858R point mutations (24). Both RUO and IVD kits utilize the Scorpion ARMS technology and typically produce flat or linear lines in the cases of negative results. The aim of this investigation was to share our experience with the 'drop-off' of an optimized system. Evidence that cases with a late upward shift in the PCR curve may benefit from TKI treatment and thus confer a survival advantage to these patients was presented here. However, PNA-sequencing technology has its own inherent weaknesses: i) The method was designed to detect exon 19 deletions and L858R mutations only, excluding exons 18 and 20; ii) DNA-based PNA-sequencing depends solely upon the proportion of non-tumor cells. The non-tumor cells contain considerably lower EGFR expression levels compared with EGFR overexpression and mutant DNA in the NSCLC cells. As the wild-type background signal cannot be completely blocked, the genomic DNA of non-tumor cells inevitably affects the performance of PNA sequencing (3). The primary

drawback of PNA sequencing is it is extremely sensitive (<1%) with a higher risk of contamination when handling the post-PCR material.

In daily practice, ~75% of EGFR mutation analysis is performed on small biopsies or cell block specimens. Whether PNA-sequencing technology should be included in the routine diagnostic algorithm requires further investigation. However, clinicians should be cognizant of the possibility of low-level EGFR mutations in the interpretation of companion diagnostic molecular tests, since testing accuracy is critical in the selection of patients for optimal therapy and reducing treatment-related toxicity.

In conclusion, a small subset of NSCLC patients with a low tumor burden may be misclassified by currently approved tests and thus delay starting the correct therapy. The evidence highlights the benefit of a complementary technique to maximize the potential of precision medicine. The novelty and significance of this manuscript is to draw attention to the cases with a late upward shift in the PCR curve during EGFR mutation analysis. The complementary technique may be of assistance in the identification of a small subset of NSCLC patients with a low tumor burden misclassified by currently approved tests and thus delay starting the correct therapy. The evidence highlights the benefit of this strategy to maximize the potential of precision medicine.

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Availability of data and materials

The datasets for this manuscript are not publicly available due to legal restrictions imposed by the government of Taiwan in relation to the Personal Information Protection Act. Requests for access to the datasets should be directed to the corresponding author.

Authors' contributions

CLH and YLC conceived the study. CCL, SCY, and WCS collected the samples and designed the study. YTY and WLC performed the experiments. YLC and WLC confirm the authenticity of all the raw data. NHC and WH contributed to the data interpretation. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Institutional Review Board of National Cheng Kung University Hospital, Tainan, Taiwan. In the study design, NSCLC patients diagnosed at the National Cheng Kung University Hospital between 2012 and 2018 were retrospectively collected with concurrent analysis of EGFR status by Qiagen EGFR kits and PNA-sequencing according to an approved protocol (approval no. A-ER-108-311).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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