CANCER BIOLOGY & THERAPY
2019, VOL. 20, NO. 8, 1097–1104
https://doi.org/10.1080/15384047.2019.1595280

RESEARCH PAPER

The *in cis* compound *EGFR* mutations in Chinese advanced non-small cell lung cancer patients

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ABSTRACT

Literatures regarding the prevalence and clinical significance of compound EGFR mutations are limited. Until now, none of retrospective or prospective research has focused on *in cis* compound *EGFR* mutations except case reports. In this study, we screened a cohort of 3,000 treatment-naïve Chinese advanced NSCLC patients using capture-based ultra-deep targeted sequencing to evaluate the prevalence of *EGFR in cis* compound mutations and the efficacy of EGFR-TKI in this population. Of the 3,000 patients screened, 1,266 (42.2%) had *EGFR* mutation; among them, 15 patients (1.2%) harboring *in cis* compound *EGFR* mutations, with 10 patients carrying *EGFR* L858R in combination with a rare mutation and five patients carrying two rare *EGFR* mutations. No patient with *EGFR* 19del was observed. Interestingly, no *in trans* configuration was identified in this cohort. All of the patients harboring *in cis* compound *EGFR* mutations were non-smokers, histologically diagnosed with adenocarcinoma and received first-generation EGFR-TKI. Furthermore, our data also revealed that patients with *in cis* compound *EGFR* mutations exhibit comparable PFS to first generation EGFR-TKI comparing to patients with single activating *EGFR* mutation. This observation was further supported by *in silico* molecular modeling analyses which demonstrated *in cis* compound mutations do not alter the ATP-binding pocket of *EGFR*, thus having no effect on the interaction between gefitinib and *EGFR*.

ARTICLE HISTORY
Received 31 October 2018
Accepted 25 December 2018

KEYWORDS
In cis; compound mutations; EGFR; non-small cell lung cancer; Chinese

Introduction

The clinical application of molecular targeted therapy such as epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) has greatly improved the prognosis of *EGFR*–mutant non-small cell lung cancer (NSCLC) patients.[1,2] Three generations of EGFR-TKIs have been developed. Gefitinib, erlotinib, and icotinib are first-generation TKIs that bind to the tyrosine kinase domain of *EGFR* in a competitive and reversible manner. Afatinib, currently the only clinically available second-generation inhibitor, binds covalently and irreversibly to tyrosine kinase receptors.[3,4] The third-generation inhibitor, osimertinib (AZD-9291), irreversibly and preferentially binds to mutated receptors and is the treatment of choice for patients who have acquired T790M mutation as the resistance mechanism to first-generation inhibitors.[5] These inhibitors bind to the tyrosine kinase domain of *EGFR* which spans from exon 18 to 24. The majority of the mutations identified in the *EGFR* gene are in exon 18, 19 and 21.[6,7]

Two of the most common mutations associated with the efficacy of EGFR-TKIs are the small in-frame deletions between E746_A750 in exon 19 (19del) and a substitution mutation, L858R, in exon 21.[8] These two mutations together comprise about 80–90% of all *EGFR* mutations.[1] The remaining 10–20% are composed of rare mutations including E709X (where X indicates the substitution of Glu residue for either Ala, Gly, His, Lys or Val), G719X (where X indicates a substitution of the Gly residue for either Ala, Arg, Cys, Ser or Val), S768I, T790M, insertions in exon 20, L861X (where X indicates a substitution of the Leu residue for either Arg, or Glu) and others.[9] *EGFR* mutations are more frequently seen in Asians than Caucasians,[10] where common mutations are largely found in females and never-smokers while rare mutations are observed to be more frequent in males and smokers.[9] Mutations are also more predominantly found in high grade adenocarcinoma than in low grade tumor.[11]

A majority of the rare *EGFR* mutations occurs as single mutations while some occur as compound mutations. Compound mutations are combinations of two different mutations; wherein often one is rare, and the other mutation is a common mutation. Under rare circumstances, both mutations can be rare mutations.[9,11] Surprisingly, compound mutations consisting of 19del and L858R have also been reported.[11–13] The most frequently reported compound

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mutations are a combination of any two of the following five mutations, L858R, E709X, G719X, L861X, and S768I.[9,14–16] Depending on the population, compound mutations could account for 2–14% of the total *EGFR* mutations.[8,9,12,13,15,17,18]

Some studies have investigated the efficacy of EGFR-TKIs on patients harboring compound mutations but yielded conflicting results. Some studies reported an unfavorable progression-free (PFS) and overall survival (OS) of patients harboring compound mutation compared to patients harboring a single *EGFR* sensitizing mutation.[9,17] In contrast, other studies, both *in vitro* and clinical studies, have shown moderate responses.[9–11,13,18–20] In addition, studies on compound mutation consisting of only rare mutations reported an unfavorable response.[12,13]

Increasing attention has been given to compound *EGFR* mutations to understand the efficacy of EGFR-TKI on patients with such mutations. However, less attention has been invested in distinguishing the molecular configuration of these compound *EGFR* mutations. No retrospective or prospective research has focused on the configuration of compound *EGFR* mutations except a few case reports. Furthermore, whether the configuration of *EGFR* compound mutation affects the efficacy of EGFR-TKI remains elusive. In this study, we screened 3,000 NSCLC patients and investigated the configuration of those with compound *EGFR* mutation. We also examined the association between the configuration of *EGFR* compound mutation and responses to EGFR-TKIs.

Results

Patient characteristics

We screened 3,000 treatment-naïve advanced NSCLC patients for *EGFR* status using capture-based targeted sequencing. Among them, 1,266 (42.2%) were found to carry *EGFR* mutation, including 501 and 489 patients with exon 19 deletion and L858R, respectively. The remaining 276 patients harbored rare mutations. Among them, 95 patients (7.5%) harbored compound *EGFR* mutations. Fifteen patients had an evaluable configuration, both mutations located on the same read. All of them harbored *in cis* compound *EGFR* mutation. No patient carrying *in trans* compound *EGFR* mutation was identified. The configuration of the remaining 80 patients cannot be evaluated due to the distance between two mutations is longer than 170 bp; therefore, the two mutations were distributed on different reads. The median age of patients with *in cis* compound *EGFR* mutations was 67 years (ranged from 45 to 77 years). Nine were females; six were males. All of the patients were non-smokers, histologically diagnosed with adenocarcinoma and received first-generation EGFR-TKI. The detailed patient characteristics were summarized in Table 1.

In cis compound *EGFR* mutations

Of the 15 patients with *in cis* compound *EGFR* mutations, 10 (67%) of them harbored L858R coupled with a rare mutation. The most frequent *in cis* compound mutations consisted of L858R and V834L (2/15) or G873E (2/15). The *in cis* compound mutation of the remaining 5 patients consisted of two rare mutations, which co-located on either exon 18 (n = 2) or exon 21 (n = 3). Interestingly, both patients with *in cis* compound mutations in exon 18 were a combination of G719X and E709X. One carried G719A + E709A and the other carried G719S + E709K. Collectively, we revealed that EGFR L858R is significantly more likely to couple with a rare mutation forming an *in cis* compound mutation ($P < 0.001$). It is interesting to note that none of the *in cis* compound mutation found in our cohort involved 19del, suggesting EGFR 19del is a stronger oncogenic driver than EGFR L858R ($P = 0.000197$, Fisher's exact test). The allelic fractions (AF) of both mutations were similar. The AF of either *EGFR* mutations was the maximum AF in all patients, demonstrating the clones harboring *EGFR* mutations were major clones. Table 2 summarizes the details of compound *EGFR* mutations found in this cohort. The representative configuration of an *in cis* EGFR *V843I* and *L858R* is depicted in Supplementary Figure 1.

Mutation profile of patients with *in cis* compound *EGFR* mutation

Next, we investigated concurrent mutations occurring in patients with *EGFR in cis* compound mutation. Five patients

Table 1. Patient clinical characteristics.

Patient number	Gender	Age	Smoking Status	Pathology	Stage	EGFR mutations	TKI Administered	PD Status	TKI PFS (Days)
1	Female	72	No	Adenocarcinoma	IV	G719S+E709K	Erlotinib	PD	70
2	Male	67	No	Adenocarcinoma	IV	G719A+E709A	Erlotinib	PD	68
3	Male	67	No	Adenocarcinoma	IV	L858R+V834L	Gefitinib	PD	360
4	Female	70	No	Adenocarcinoma	IV	L858R+G873E	Erlotinib	PD	146
5	Female	53	No	Adenocarcinoma	IV	L858R+A871E	Icotinib	PD	240
6	Male	53	No	Adenocarcinoma	IV	L858R+G873E	Gefitinib	PD	120
7	Female	50	No	Adenocarcinoma	IV	L858R+H870R	Gefitinib	PD	219
8	Male	67	No	Adenocarcinoma	IV	L858R+V834L	Erlotinib	PD	420
9	Male	70	No	Adenocarcinoma	IV	L858R+A859S	Erlotinib	- *	285
10	Female	53	No	Adenocarcinoma	IV	L858R+V843I	Gefitinib	PD	225
11	Female	55	No	Adenocarcinoma	IV	L858R+K860I	Gefitinib	PD	260
12	Female	67	No	Adenocarcinoma	IV	L858R+L833F	Icotinib	PD	464
13	Male	45	No	Adenocarcinoma	IV	L861R+L833F	Gefitinib	PD	193
14	Female	67	No	Adenocarcinoma	IV	L861Q+V834L	Gefitinib	PD	543
15	Female	77	No	Adenocarcinoma	IV	H835L+L833V	Gefitinib	-*	450

Abbreviations: TKI, EGFR tyrosine kinase inhibitor administered to the patient; PD, disease progression; TKI PFS, progression-free survival after starting the EGFR-TKI treatment;

* Patients number 9 and 15 have not experienced disease progression as of the last follow up on August 30, 2018.

Table 2. Distribution of EGFR compound mutations.

Compound *EGFR* mutations	Mutation 1 Location	Mutation 2 Location	Number of patients with this compound mutation
***EGFR* L858R in combination with a rare mutation**			
L858R + L833F	Exon 21	Exon 21	1
L858R + V834L	Exon 21	Exon 21	2
L858R + V843I	Exon 21	Exon 21	1
L858R + A859S	Exon 21	Exon 21	1
L858R + K860I	Exon 21	Exon 21	1
L858R + H870R	Exon 21	Exon 21	1
L858R + A871E	Exon 21	Exon 21	1
L858R + G873E	Exon 21	Exon 21	2
		Total:	10
Combination of 2 rare mutations			
G719A + E709A	Exon 18	Exon 18	1
G719S + E709K	Exon 18	Exon 18	1
H835L + L833V	Exon 21	Exon 21	1
L861R + L833F	Exon 21	Exon 21	1
L861Q + V834L	Exon 21	Exon 21	1
		Total:	5

harbored *EGFR* amplifications (Figure 1). Interestingly, 4 out of 5 patients who had the *EGFR* amplifications harbored *in cis* compound mutations with L858R (Figure 1, red-colored boxes) and the remaining patient had EGFR L861R + L833F. In addition to *EGFR in cis* compound mutations, 3 patients had concurrent driver mutations, 2 with *MET* amplification and 1 with *KRAS* mutation. Moreover, we have found 7 (47%) patients harbored concurrent *TP53* mutation. Of these 7 patients with *TP53* mutations, 6 of them harbored *in cis* compound mutation with L858R (Figure 1). Interestingly, the patient with H835L + L833V was also the only patient in the cohort found to co-harbor numerous gene amplifications including *STK11, CCND1, FGFR3* and *HRAS*.

EGFR-TKI efficacy in patients with *in cis* compound *EGFR* mutations

The efficacies of EGFR-TKI in patients with *EGFR* compound mutation have been controversial. In our cohort, patients harboring *EGFR in cis* compound mutation, both occurring in exon 18, had the shortest progression-free survival (PFS) (P = 0.0021, Figure 2). Since the majority of the *in cis* compound mutations involved L858R, we then compared the PFS in patients with *in cis* compound *EGFR* mutation and single EGFR L858R. We randomly selected 23 patients from our screened cohort harboring single EGFR L858R. The clinical characteristics of this cohort were comparable to the cohort with *in cis* compound *EGFR* mutation. The median PFS (mPFS) of the patients with *in cis* compound mutations was 9 months, which is comparable to the mPFS of patients with single EGFR L858R, 7 months (P = 0.56, Figure 3(a)). Interestingly, we observed that patients with concurrent *EGFR* amplification had a shorter PFS than patients without (P = 0.027, Figure 3(b)). *TP53*, another frequently co-occurring mutation, was mutated in seven patients, who showed a comparable PFS with patients harboring WT *TP53* (Figure 3(c)). Collectively, these data shows that the patients with *in cis* compound mutations and single L858R mutation had a comparable PFS to first-generation TKIs. In addition,

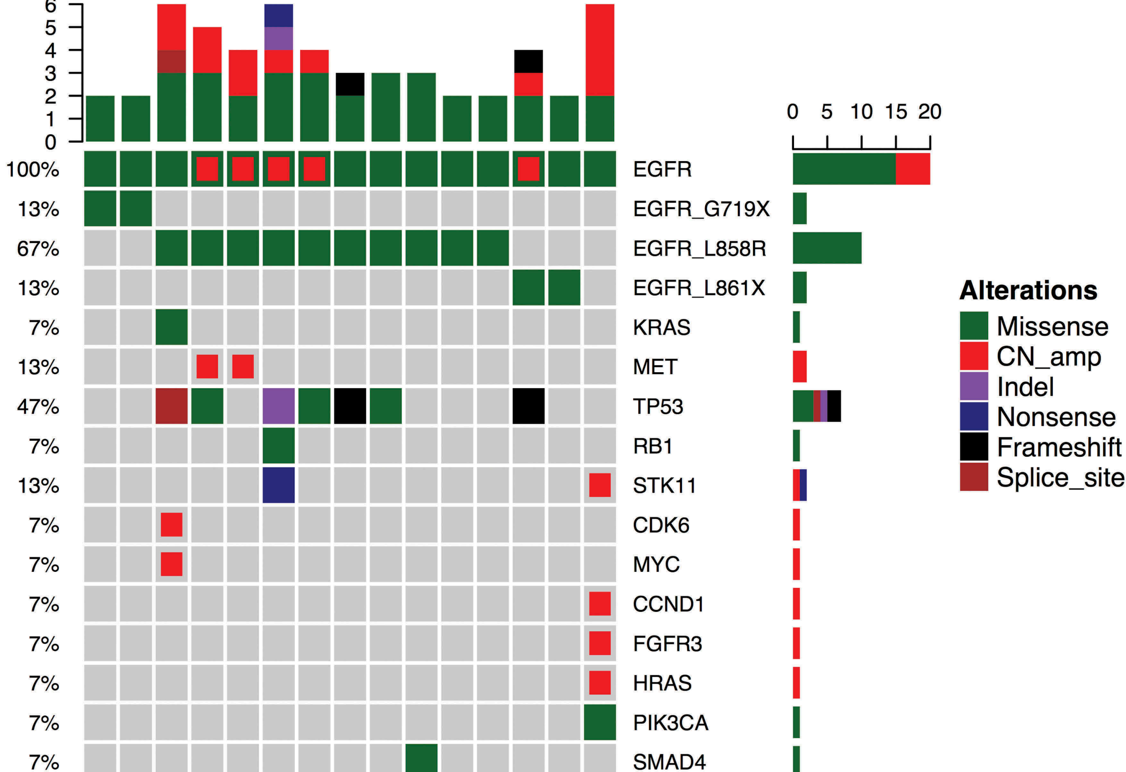

Figure 1. Mutational Profile of the 15 patients with compound *EGFR* mutations.
Each column represents a patient and each row represents a gene. Top bars represent the number of mutations a patient carried and sidebars represents the percentage of patients with a certain mutation. Different colors denote different types of mutation.

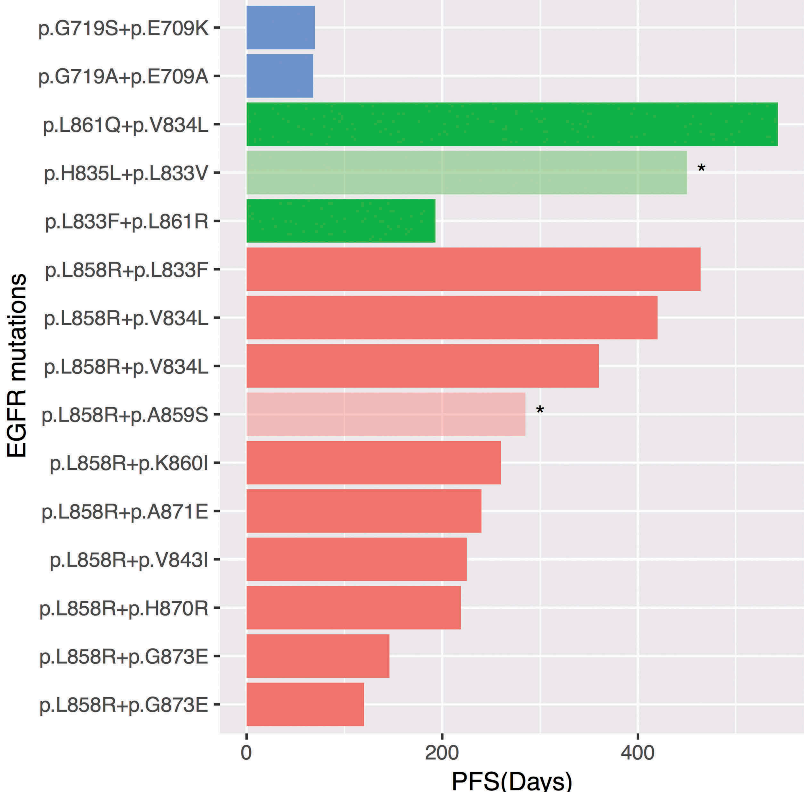

Figure 2. Compound *EGFR* mutations and treatment response of each patient.

X-axis indicates the progression-free survival (PFS) of each patient. Y-axis denotes the compound *EGFR* mutations of each patient. Orange bars denote compound mutations with L858R. Green bars denote compound mutations in exon 21, and blue bars denote compound mutations in exon 18. Asterisks denote patients who have not experienced progression at the time of the last follow-up on August 30, 2018.

patients with concurrent *EGFR* amplification in addition to *in cis* compound *EGFR* mutation showed an inferior PFS than patients without.

EGFR conformation transitions in patients with *in cis* compound *EGFR* mutations

We also performed molecular modeling to simulate the interaction between various EGFR mutations and gefitinib and to predict the efficacy. EGFR-TKI, such as gefitinib, binds to the ATP-binding site of EGFR by forming a hydrogen bond in the pocket of Met793, Asp800, which reversibly and competitively inhibits ATP binding, thereby preventing the activation of downstream signaling pathways. Aromatic groups need to be embedded in the hydrophobic pockets with T790 gatekeeper. Consequently, a mutation in any of the three amino acids, Met793, Asp800, and T790, may affect the binding of gefitinib to EGFR. However, the EGFR conformation transitions showed that amino acids E709, L833, V834, H835, V843, L858, A859, K860, L861, H870, A871, G873 are located outside the EGFR ATP-binding pocket (Figure 4). Hence, compound mutations involving L858 and another amino acid, such as L833, V834, V843, A859, K860, H870, A871, or G873, do not affect the binding of gefitinib to EGFR (Figure 4(a-h)). On the other hand, G719 amino acid is located near the ATP-binding pocket. However, mutations involving substitution of G719 glycine with small amino acids such as serine with hydroxymethyl (CH_2OH) side chain or alanine with a methyl side chain, resulting in G719S or G719A, do not affect the interaction between EGFR and gefitinib. Moreover, compound mutations with G719X and E709X also did not affect the binding of gefitinib to EGFR (Figure 4(i, j)).

Discussion

The *EGFR* mutation has been well-elucidated in different ethnicities.[21–25] However, literatures regarding the prevalence and clinical significance of compound EGFR mutations are limited and deserve further investigation. To date, this is the first study that focused on the configuration of compound *EGFR* mutations and investigated the efficacy of EGFR-TKI in patients with *in cis* compound *EGFR* mutation. This collaborative effort involving multiple cancer centers represents a more accurate estimation of the prevalence of *in cis* compound mutation and the efficacy of EGFR-TKI in this population. We derived a 1.2% prevalence of *in cis* compound mutation in *EGFR* mutant Chinese advanced NSCLC patients. We also correlated clinical parameters with *in cis* compound *EGFR* mutations, which were more likely to occur in non-smokers and adenocarcinomas. EGFR L858R was significantly more likely to couple with a rare *in cis* compound mutation than 19 del. This phenomenon can be potentially explained by the notion that EGFR 19del might be a stronger oncogenic driver than L858R.

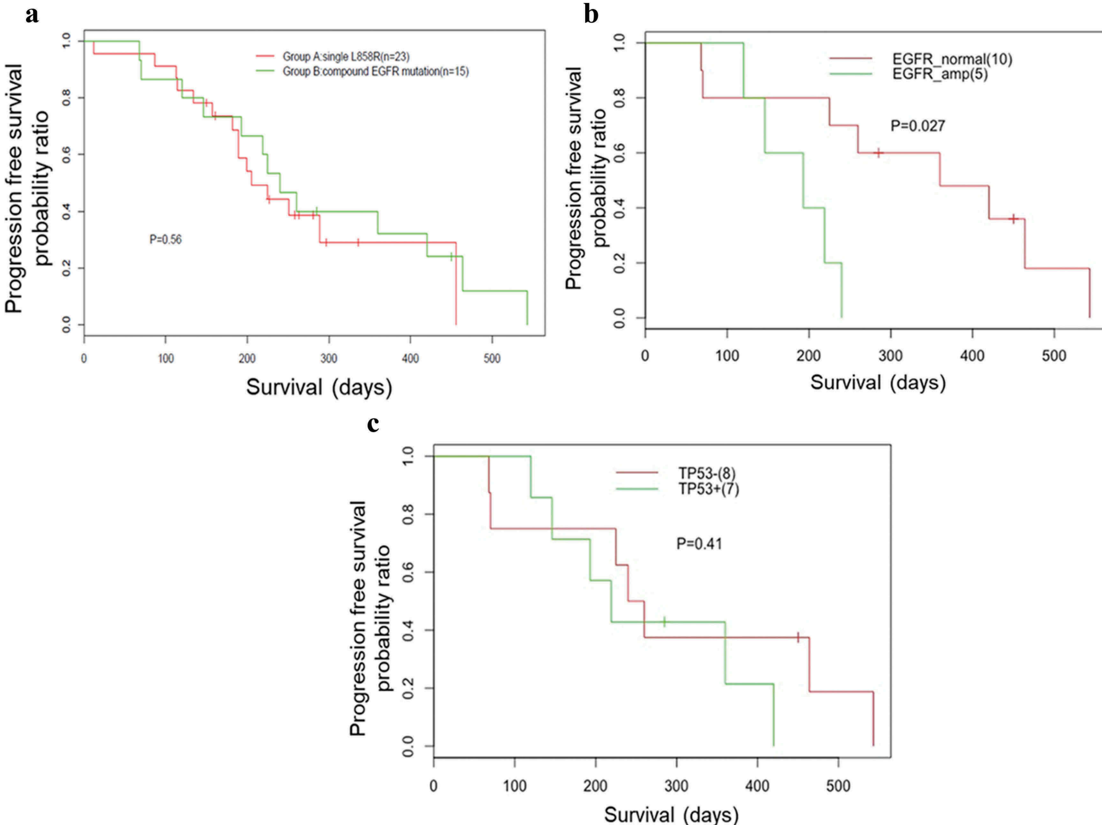

Figure 3. Kaplan-Meier analysis of patients with compound mutations.

(a). Comparison between compound *EGFR* and single *EGFR* L858R mutations. Group A includes patients with a single L858R mutation (n = 23); Group B includes all 15 patients with compound *EGFR* mutations. (**b-c). Comparison between compound *EGFR* mutation patients with and without concurrent variations. B. *EGFR* amplification**. EGFR_ normal denotes patients without concurrent *EGFR* amplification (n = 10). EGFR_amp denotes patients with concurrent *EGFR* amplification (n = 5). (**c). *TP53* mutation**. TP53- denotes patients without concurrent *TP53* mutation (n = 9). TP53+ denotes patients with concurrent *TP53* mutation (n = 6). X-axis represents progression-free survival (PFS) expressed in days. Y-axis denotes the PFS ratio.

Furthermore, we investigated the efficacy of first-generation EGFR-TKI on patients with *in cis* compound *EGFR* mutation. In our present study, albeit having a limited number of patients, we have found that different combinations of mutations have distinct treatment responses. From our cohort, the most common *in cis* compound mutation was the combination of L858R and another rare mutation. Similar to the observations of Keam *et al.*,[13] our data also showed that patients with *in cis* compound mutations involving L858R and patients with single L858R have comparable responses to first-generation EGFR-TKIs. This observation was further supported by the *in silico* analysis which demonstrated the additional mutations do not alter the ATP-binding pocket of *EGFR*, thus allowing the interaction between *EGFR* and gefitinib.

Further analysis revealed that patients with *in cis* compound mutations in exon 21, including L858R, with one of the mutations being either V834L or L833X had a significantly better response to first-generation EGFR-TKIs (P = 0.0039). The PFS of these patients ranged from 12 to 18.1 months (Table 1, patients 3, 8, 12, 14 and 15). On the other hand, we also revealed a trend of having an unfavorable response in patients harboring *EGFR* L858R in combination with a second mutation located between amino acid positions 870 to 873, i.e. H870R, A871E or G873E (P = 0.08). It is interesting to note that patients with concurrent *EGFR* amplifications tend to have shorter PFS, ranged from 4.8 to 8 months (P = 0.0027, Table 1 and Figure 1, patients 4, 5, 6, 7 and 13). Among all the patients in our cohort, both patients with G719X + E709X had the worse response to first-generation EGFR-TKIs. However, reports on G719X and E709X, as single or compound mutations, have shown sensitivity towards EGFR-TKIs.[8,9,20]

In our study, we have found that *in cis* compound mutations occurring in exon 21, including L858R, responded similarly as single L858R to first-generation EGFR-TKIs. In contrast, *in cis* compound mutations in exon 18 had an unfavorable response. In addition, we revealed certain concurrent mutations, such as *EGFR* amplification, can have an effect on treatment responses, highlighting the importance of elucidating concurrent mutations. Due to the limited number of patients in our cohort, further investigations regarding the efficacy of EGFR-TKIs in patients with *in cis* compound *EGFR* mutations are needed to validate our results.

Materials and methods

Patient selection

We screened 3,000 treatment-naïve advanced adenocarcinomas NSCLC patients from nine participating hospitals. Either tumor

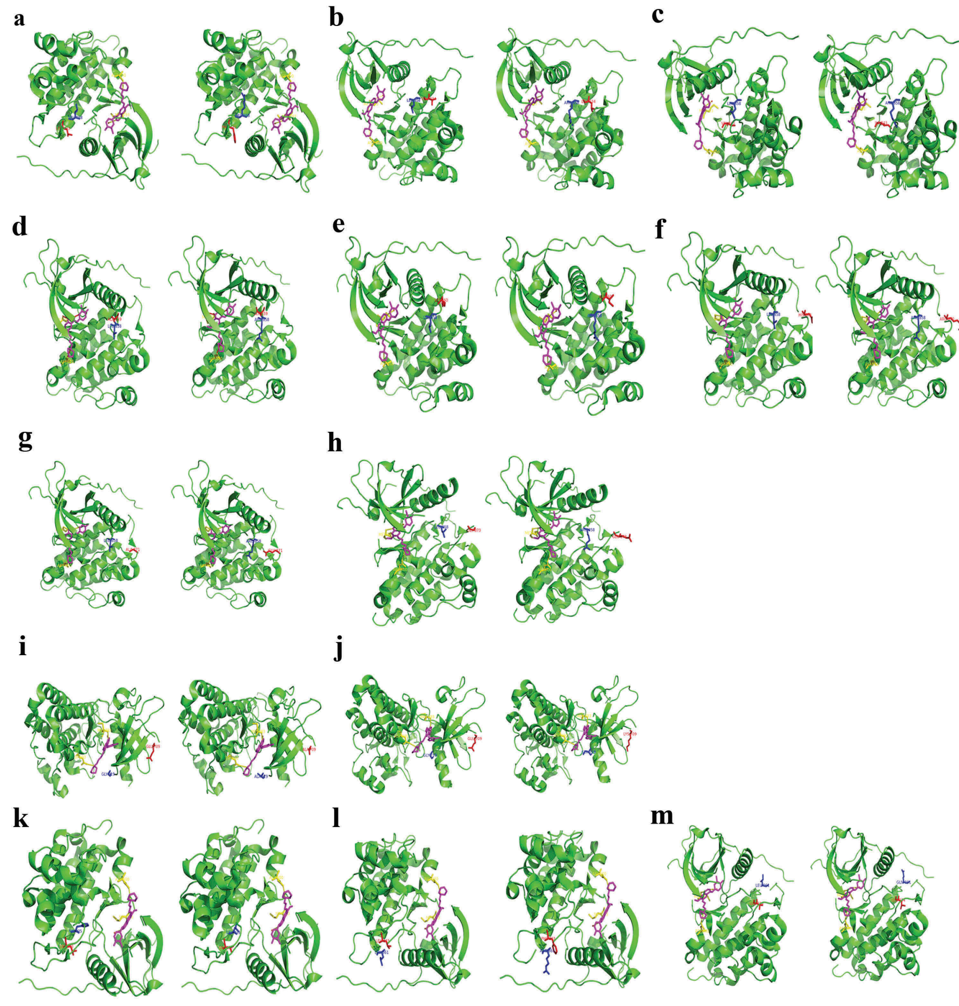

Figure 4. EGFR conformation transitions in patients with *in cis* compound *EGFR* mutations.

(a). EGFR L858R+L833F; (**b**). EGFR L858R+V834L; **c**. EGFR L858R+V843I; **d**. EGFR L858R+A859S; €. EGFR L858R+K860I; (**f**). EGFR L858R+H870R; (**g**). EGFR L858R+A871E; (**h**). EGFR L858R+G873E; **i**. EGFR G719A+E709A; **j**. EGFR G719S+E709K; (**k**). EGFR H835L+L833V; (**l**). EGFR L861R+L833F; (**m**). EGFR L861Q+V834L. EGFR protein is represented by green alpha helix, beta folding, loop. Gefitinib is represented by a magenta stick. The *in cis* compound mutations of EGFR protein are represented by blue and red sticks. Met793 and Asp800 of EGFR protein are represented by yellow sticks. The hydrogen bond is represented by a yellow dashed line.

tissue or plasma sample was obtained from each patient. This study was performed in concordance with the guideline of the ethics committee of each of the participating hospital. Written informed content was obtained from each patient.

Preparation of tissue and plasma cell-free DNA

Tissue and circulating cell-free DNA was extracted using QIAamp DNA FFPE tissue kit (Qiagen) and QIAamp Circulating Nucleic Acid kit (Qiagen), respectively, according to manufacturer's instructions.

Capture-based targeted DNA sequencing

DNA concentration and genomic DNA quality were measured by Qubit dsDNA assay kit (Life Technologies, Carlsbad, CA) and 260 nm/280 nm absorption ratio, respectively. A minimum of 50 ng of cfDNA is required for NGS library construction. DNA shearing was performed on tissue DNA using Covaris M220, followed by end repair, phosphorylation, and adaptor ligation. Fragments of size 200–400 bp from sheared tissue DNA and plasma cell-free DNA were selected by a bead (Agencourt AMPure XP Kit), followed by hybridization with capture probes baits, hybrid selection with magnetic beads and PCR amplification. A bioanalyzer high-sensitivity DNA assay was then performed to assess the quality and size of the fragments and indexed samples were sequenced on Nextseq500 sequencer (Illumina, Inc., USA) with paired-end reads.

Sequence data analysis

Sequence data were mapped to the reference human genome (hg19) using Burrows-Wheeler Aligner 0.7.10. Local alignment optimization, variant calling, and annotation were performed using GATK 3.2, MuTect, and VarScan. Plasma sample was compared against its own white blood cell control to identify somatic variants. Variants were filtered using the VarScan FP filter pipeline, with loci depth less than 100 filtered out. Base calling in plasma and tissue samples required at least eight supporting reads for single nucleotide variations (SNV) and 2 and 5 supporting reads for insertion-

deletion variations (INDEL), respectively. Variants with population frequency over 0.1% in the ExAC, 1000 Genomes, dbSNP or ESP6500SI-V2 databases were grouped as single nucleotide polymorphisms (SNP) and excluded from further analysis. Remaining variants were annotated with ANNOVAR and SnpEff v3.6. Analysis of DNA translocation was performed using both Tophat2 and Factera 1.4.3.

Statistical analysis

All the data were analyzed using R software. Survival data were analyzed by Kaplan–Meier and log-rank test were used to compare the difference between survival groups. Difference in *EGFR* frequency was calculated and presented using paired, two-tailed Student's t-test in p-value. For all statistical tests, $P < 0.05$ was considered statistically significant.

Funding

This investigation was supported by National Key R&D Program of China (2016YFC1303300) and Xiangya clinical big data system construction project of Central South University (Clinical big data construction project of lung cancer).

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