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Concomitant Genetic Alterations are Associated with Worse Clinical Outcome in *EGFR* Mutant NSCLC Patients Treated with Tyrosine Kinase Inhibitors

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Abstract

Epidermal growth factor receptor- tyrosine kinase inhibitors (*EGFR*-TKI) are recommended first-line therapy for advanced non-small cell lung cancer (NSCLC) with sensitizing *EGFR* mutations. It is of clinical interest to identify concurrent genetic mutations in NSCLC patients with *EGFR* mutations in the hopes of discovering predictive biomarkers towards EGFR-TKI treatment. We retrospectively analyzed a cohort of patients with advanced *EGFR* mutant NSCLC who underwent treatment with first generation TKIs at our hospital by a multi-gene panel via next generation sequencing. A total of 33 patients with mutant EGFR were enrolled. Up to 26 (78.8%) patients had at least one concomitant genetic alteration coexisting with mutant *EGFR*. Among the concomitant genetic alterations discovered, *TP53* mutation was most common (n = 10,30.3%), followed by CDK4 (n = 8, 24.2%) and CDKN2A (n = 7, 21.2%)copy number variation (CNV). Progression-free survival was shorter in patients with concomitant *FGFR3* mutation (1.6 vs. 12.6 months, $P = .003$) and *CDKN2A* CNV loss (6.5 vs. 13.4months, $P = .019$). Patients with any concomitant genetic alterations also had significant worse overall survival (24.1 vs. 40.8 months, $P = .029$). In summary, our study revealed an unfavorable association between concomitant genetic mutations and treatment response towards EGFR-TKI. FGFR3 mutation and CDKN2A CNV loss may be potential predictive markers for treatment outcome and warrant further investigation.

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Introduction

Lung cancer is one of the most prevalent human cancers in the world. According to the US CDC (Centers for Disease Control and Prevention), lung cancer is responsible for the most deaths from cancer compared to other cancer types [1]. Lung cancers can be classified into small cell lung cancer and non-small cell lung cancer (NSCLC) [2]. The success of tyrosine kinase inhibitors (TKIs) has significantly improved outcomes in *EGFR* mutant NSCLC [3]. "First generation" TKIs including gefitinib and erlotinib are now standard treatment for NSCLC [4], and "next generation" TKIs (such as osimertinib [5]) that are effective even in gefitinib/erlotinib resistant

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tumors are now approved and established in guidelines for NSCLC treatment. The recent success of immunotherapy in NSCLC has now widened the therapeutic armory for oncologists [6]. Indeed, recent studies combining immunotherapy with chemotherapy in NSCLC patients with non-mutant *EGFR* have demonstrated significant improvement in survival [7]. However, a meta-analysis of several large scale clinical trials concluded no benefit of immunotherapy in *EGFR* mutant NSCLC [8]. Therefore, in NSCLC patients with mutant *EGFR*, *EGFR*-TKIs remain the current mainstay of treatment.

Despite high response rates of TKIs from first-line use in advanced, EGFR mutant NSCLC (up to 70% with gefitinib as first line use [9]), this response is not extremely durable and usually lasts less than 12 months [9,10]. It is generally thought that approximately half of the cases that are resistant to TKIs acquire a secondary mutation in *EGFR* (T790M) [11,12], while other cases mostly acquire mutations in other oncogenes such as *MET* [13]. Novel TKIs (such as osimertinib) have been developed that can overcome T790M mutations [14]. However, the high cost of osimertinib have precluded its widespread use, despite studies that demonstrate significant superiority of osimertinib compared to first generation TKIs in the first line setting[15]. Indeed, recent studies have proposed that osimertinib is not cost effective in countries such as the United States and China [16]. Before the affordability of novel TKIs improve for patients, first generation TKIs still play an invaluable role in treatment for EGFR mutant NSCLC. In the era of precision medicine[17], real world information regarding molecular analysis of patients treated with first generation TKI is of immense value to clinicians.

With this perspective, we retrospectively analyzed a cohort of patients with advanced *EGFR* mutant NSCLC who underwent treatment with first generation TKIs at our hospital. Patient samples were assayed for a multi-gene panel (ACTonco®+ , ACT Genomics, Taiwan) using next-generation sequencing (NGS). Our goal was to identify genetic alterations that co-existed with EGFR mutation in our cohort. The identified mutations could then be correlated with patient outcome and treatment response to *EGFR*-TKI. Our goal is to discover novel molecular interactions and possible predictive factors for *EGFR*-TKI treatment, and uncover implications for novel pathway interactions with mutant *EGFR*. We believe that this study will expand the knowledge base for oncologists treating NSCLC.

Methods

Study Design

This study was conducted at single cancer center (National Yang-Ming University hospital, Taiwan). NSCLC patients with metastatic disease on diagnosis or tumor recurrence after complete surgical resection (stage IV by AJCC 7th edition staging) [18] were enrolled.

Inclusion criteria included detection of sensitizing *EGFR* mutations in exon 18-21 by methods of cobas RT-PCR test (Roche Molecular Systems Inc., Branchburg, NJ, USA) from initial tumor tissue, first line treatment with either gefitinib, erlotinib, or afatinib; and adequate tumor tissue for further NGS testing.

Exclusion criteria included life expectancy of less than three months, Eastern Cooperative Oncology Group (ECOG) performance status >3, and impaired major organ function functions.

All patients received *EGFR*-TKI monotherapy till disease progression. Tumor treatment response for *EGFR*-TKI was evaluated according to RECIST criteria version 1.1. [19] Subsequent treatment after disease progression was determined by individual clinician judgment. The study was approved by the Institutional Review Board of National Yang-Ming University Hospital (IRB No. 2016B007).

Table 1. A list of the sequenced genes

ABL1	*AKT1*	*ALK*	*BRAF*	*CCND1*	*CDK4*	*CDK6*
CDKN2A	*CTNNB1*	*EGFR*	*ERBB2*	*ERBB4*	*ESR1*	*FGFR1*
FGFR2	*FGFR3*	*FLT3*	*HRAS*	*IDH1*	*IDH2*	*JAK2*
JAK3	*KDR*	*KIT*	*KRAS*	*MAP2K2*	*MAP2K1*	*MET*
NRAS	*PDGFRA*	*PIK3CA*	*PTEN*	*RET*	*TP53*	*UGT1A1*

Copy number variation analysis is performed only on 14 genes.

Sample Processing and Sequencing

Genomic DNA was extracted from FFPE tumor samples using the QIAamp DNA FFPE Tissue Kit (Qiagen) and quantified using the Quant-iT dsDNA HS Assay (Invitrogen). The integrity of genomic DNA was evaluated by Fragment AnalyzerTM (Advanced Analytical Technologies, Inc.).

Twenty nanograms of extracted genomic DNA was amplified using one pool primer pairs (Life Technologies) to target important and hotspot regions of analyzed genes. The analyzed genes are listed in Table 1. Amplicons were ligated with barcoded adaptors using the Ion Amplicon Library Kit (Life Technologies). Barcoded libraries were subsequently conjugated with sequencing beads by emulsion PCR and enriched using IonChef (Life Technologies) according to the Ion Torrent protocol (Life Technologies). Quality and quantity of amplified library were determined using the fragment analyzer (AATI) and Qubit (Invitrogen). Sequencing was performed on the Ion Proton sequencer using the Ion PI chip (Life Technologies) according to the manufacturer's protocol.

Raw reads generated by the sequencer were mapped to the hg19 human reference genome using the Ion Torrent Suite (version 5.2).

Table 2. Characteristics of the *EGFR* mutant NSCLC cohort

Patients (N = 33)		
Average age	69.9 (range:45-92)	
Number		(%)
Gender		
Male	13	39.4
Female	20	60.6
Smoking history		
never smoker	24	72.7
current & ex-smoker	9	27.3
weight loss		
no	25	75.8
yes	8	24.2
PS (ECOG)		
0-1	19	57.6
2-3	14	42.4
Brain metastasis at diagnosis		
no	24	72.7
yes	9	27.3
Liver metastasis at diagnosis		
no	28	84.8
yes	5	15.2
Initial treatment		
Afatinib	2	6.1
Gefitinib	18	54.5
Erlotinib	13	39.4
EGFR mutation		
Exon 18	1	3
Exon 19	17	51.5
Exon 21	15	45.5

Coverage depth was calculated by Torrent Coverage Analysis plug-in. Variants, including Single nucleotide variants (SNVs) and short insertions/deletions (INDELs), were identified using the Torrent Variant Caller plug-in (version 5.2). Variants were annotated using VEP (Variant Effect Predictor) (version 88) and the databases from COSMIC v.83 and 1000 Genomes Project Phase 3. Non-synonymous mutations with coverage ≥25, allele frequency ≥5% and actionable variants with allele frequency ≥2% were retained.

Amplicons with read counts in the lowest 5th percentile of all detectable amplicons and amplicons with a coefficient of variation ≥0.3 were removed. ONCOCNV (an established method for calculating copy number aberrations in amplicon sequencing data by Boeva et al., 2014 [20]) was applied for the normalization of total amplicon number, amplicon GC content, amplicon length, and technology-related biases, followed by segmenting the sample with a gene-aware model. The method was used as well for establishing the baseline of copy number variations from samples in ACT Genomics in-house FFPE database.

Statistical Analysis

The statistical analyses of clinical and genetic data were performed using SPSS software for Windows (version 22; IBM Corporation, Armonk, NY). Data was presented as frequencies for categorical variables and by mean ± SD for numerical variables. Categorical variables were compared using a chi-square test or Fisher's exact test, and continuous variables were compared using an independent unpaired t-test. Progression-free survival and overall survival were represented by Kaplan−Meier survival curves and calculated by a log-rank test. *P*-values of less than .05 were considered to be statistically significant.

Results

Clinical and Molecular Characteristics of EGFR Mutant NSCLC Patients Receiving EGFR-TKI as First-Line Treatment

We present the characteristics of the 33 cases in Table 2. The mean age was 69.9 years old (range 45-92 years old), and 20 out of 33 were

Table 3. Multivariate analysis of genetic mutations and clinical outcomes. Significant concurrent mutations with impact on survivals are highlighted.

		Objective response		PFS			OS		
	No. (%)	No. (%)	*P* value	*PFS* (month)	*HR (95% CI)*	*P* value	*OS* (month)	*HR (95% CI)*	*P* value
***TP53* mutant**			1			0.469			0.134
Yes	10 (30.3)	7 (70.0%)		9.10±1.46	1.37 (0.58-3.23)		20.43±3.08	2.00 (0.81-4.93)	
No	23 (69.7)	17 (73.9%)		12.59±1.83	1		29.84±3.50	1	
***CTNNB1* mutant**			0.477			0.435			0.867
Yes	2 (6.1)	1 (50.0%)		8.03±2.34	1.79 (0.41-7.75)		28.50±3.40	1.13 (0.26-4.88)	
No	31 (93.9)	23 (74.2%)		12.15±1.55	1		27.74±3.03	1	
***FGFR1* mutant**			0.477			0.029			0.588
Yes	2 (6.1)	1 (50.0%)		3.52±2.90	5.52 (1.19-25.65)		23.85±4.15	1.50 (0.35-6.51)	
No	31 (93.9)	23 (74.2%)		12.43±1.51	1		28.01±3.02	1	
***FGFR3* mutant**			0.068			0.003			0.002
Yes	2 (6.1)	0 (0.0%)		1.60±0.12	38.82 (3.42-440.69)		5.40±2.70	49.57 (4.38-560.43)	
No	31 (93.9)	24 (77.4%)		12.56±1.49	1		29.17±2.83	1	
***ALK* mutant**			1			0.876			0.595
Yes	1 (3.0)	1 (100.0%)		9.93±0.00	1.17 (0.16-8.83)		20.60±0.00	1.74 (0.23-13.22)	
No	32 (97.0)	23 (71.9%)		11.96±1.51	1		27.95±2.92	1	
***CCND1* mutant**			1			0.669			0.146
Yes	1 (3.0)	1 (100.0%)		8.58±0.00	1.56 (0.21-11.81)		11.20±0.00	4.81 (0.58-40.01)	
No	32 (97.0)	23 (71.9%)		12.01±1.51	1		28.24±2.88	1	
***CDK4* mutant**			1			NA			0.532
Yes	1 (3.0)	1 (100.0%)		0.62±0.00	NA		19.70±0.00	1.92 (0.25-14.67)	
No	32 (97.0)	23 (71.9%)		12.24±1.47	NA		27.98±2.92	1	
***FLT3* mutant**			1			0.946			0.987
Yes	1 (3.0)	1 (100.0%)		10.36±0.00	1.07 (0.14-8.05)		31.90±0.00	0.98 (0.13-7.37)	
No	32 (97.0)	23 (71.9%)		11.95±1.51	1		27.66±2.93	1	
***JAK2* mutant**			1			0.294			0.072
Yes	1 (3.0)	1 (100.0%)		6.21±0.00	3.025 (0.38-23.90)		9.80±0.00	7.49 (0.84-67.01)	
No	32 (97.0)	23 (71.9%)		12.07±1.50	1		28.29±2.87	1	
***PIK3CA* mutant**			1			0.55			0.302
Yes	1 (3.0)	1 (100.0%)		24.20±0.00	0.54 (0.07-4.08)		57.60±0.00	0.038 (0.00-18.76)	
No	32 (97.0)	23 (71.9%)		11.52±1.47	1		26.56±2.63	1	
***EGFR* CNV gain**			1			0.331			0.449
(+)	6 (18.2)	5 (83.3%)		9.29±2.05	1.58 (0.63-3.97)		26.05±6.36	1.43 (0.57-3.64)	
(-)	27 (81.8)	19 (70.4%)		12.57±1.74	1		28.46±3.32	1	
***CDK4* CNV loss**			0.651			0.813			0.411
(+)	8 (24.2)	5 (62.5%)		11.14±2.88	1.11 (0.48-2.54)		25.46±4.33	1.42 (0.61-3.31)	
(-)	25 (75.8)	19 (76.0%)		12.19±1.74	1		28.73±3.61	1	
***CDKN2A* CNV loss**			0.358			0.019			0.081
(+)	7 (21.2)	4 (57.1%)		6.54±1.22	3.03 (1.20-7.64)		19.46±4.92	2.20 (0.91-5.34)	
(-)	26 (78.8)	20 (76.9%)		13.38±1.74	1		30.09±3.29	1	
Any genetic alteration			1			0.075			0.029
Yes	26 (78.8)	19 (73.1%)		10.30±1.40	2.68 (0.91-7.93)		24.12±2.84	3.42 (1.14-10.29)	
no	7 (21.2)	5 (71.4%)		17.86±3.88	1		40.76±5.68	1	
***EGFR* mutant**			0.044						NA
Exon19 Del	17 (51.5)	15 (88.2%)		12.46±2.02	1		29.17±4.14	NA	
Exon21 L858R	15 (45.5)	9 (60.0%)		12.02±2.33	1.07 (0.49-2.31)	0.869	27.37±3.59	NA	
Exon18	1 (3.0)	0 (0.0%)		1.71±0.00	15.97 (1.41-181.21)	0.025	2.70±0.00	NA	

females (60.6%). Twenty-four patients (72.7%) were non-smokers, and 19 patients (57.6 %) had an ECOG 0-1. 9 patients had an initial diagnosis with brain metastasis (27.3%) and 5 patients (15.2%) had liver metastasis. Choices of the first line EGFR TKI use included 2 cases of afatinib (6.1 %), 18 cases of gefitinib (54.5 %) and 13 cases of erlotinib (39.4%). Regarding the EGFR mutation profile, 17 cases (51.5%) had exon 19 deletion, 15 (45.5%) had exon 21 mutation and 1 (3%) had exon 18 G719X mutation.

Genomic Profiling Reveals Commonly Mutated Oncogenes in our Cohort of EGFR Mutated NSCLC

Genomic DNA from each patient sample was analyzed for genetic alterations using the ACTonco®+ panel. All the patients had confirmed *EGFR* mutations as listed in Table 2**.** No T790M mutations were present in this cohort. The most common gene alteration occurred in *TP53* (10/31), followed by *CDK4* (8/31), *CDKN2A* (7/31). Interestingly, gene alterations in *TP53* were mutations in all 10 patients, while almost all alterations in *CDK4* were CNV gains, and all genetic alterations in CDKN2A were CNV loss. A complete list of identified mutations is shown in Table 3.

Multivariate Analysis of Genetic Mutations and Clinical Outcomes

We performed a multivariate analysis in Table 3 to examine association of genetic alterations with clinical outcomes. Our analysis revealed that mutations in *FGFR3* (1.6 vs. 12.6 months, $P = .003$), loss of CNV in *CDKN2A* (6.5 vs. 13.4 months, $P = .019$) influenced PFS (Figure 1), and the presence of any concomitant genetic alteration significantly influenced OS (24.1 vs. 40.8 months, $P = .029$) (Figure 2). As shown in Figure 2, patients with *FGFR3* mutant had shorter OS (5.4 months V.S 29.2 months, $P = .002$).

In summary, our findings reveal that *EGFR* mutant patients with concomitant FGFR3 mutations or *CDKN2A* CNV loss presented with differential outcomes when treated with *EGFR*-TKI.

Discussion

Our study demonstrated the efficacy of *EGFR*-TKIs in first-line treatment for patients of stage IV adenocarcinoma with mutant *EGFR* (ORR:72.3%, PFS:10.9 months). This data is consistent with the multiple phase 3 clinical trials that are published regarding TKIs in *EGFR* mutant stage IV NSCLC [9,21−24]. Standard TKIs such as gefitinib, erlotinib are already well established as first-line treatment in this population, while osimertinib, a third generation EGFR-TKI that is selective for both EGFR sensitizing and T790M resistance mutations, may offer further advantage than standard TKIs as frontline therapy [15]. The optimal first-line TKI (selection of gefitinib/erlotinib or osimertinib as first-line) is therefore currently being actively investigated [25,26]. With the increasing availability and popularity of multi-gene testing panels, genetic data from real world patients can provide important insights for predictors of response. It is of clinical interest to identify concurrent genetic mutations in patients with *EGFR* mutations in the hopes of discovering a predictive biomarker. Another recent retrospective study [27] analyzed 49 cancer-related genes by NGS in 58 *EGFR*-mutant metastatic NSCLC patients and discovered that 32 of 58 (55%) harbored concomitant genetic alterations. These patients with concomitant genetic alterations were associated with poor outcomes including worse ORR, shorter PFS and OS with first-line treatment *EGFR*-TKI. Our study incorporated a similar approach by using NGS to detect mutations in 35 genes (with CNV analysis in 14 genes) in tumor tissues of *EGFR*-mutant NSCLC before treatment. In our study, up to 78.8% of patients had concomitant genetic mutations and were associated with poor clinical outcomes. The mutation frequency of *TP53* was the most common alteration in our study (30.3%), which is in line with previous studies [27−29]. TP53 co-mutation in patients *EGFR*-mutated advanced NSCLC have been reported with no difference in ORR with *EGFR*-TKIs (TP53-mutant 54% vs. wild type 66%, $P = .42$) but a non-significant shorter PFS (HR 1.74, CI 0.98-3.10, $P = .06$) [30]. In our study, similar

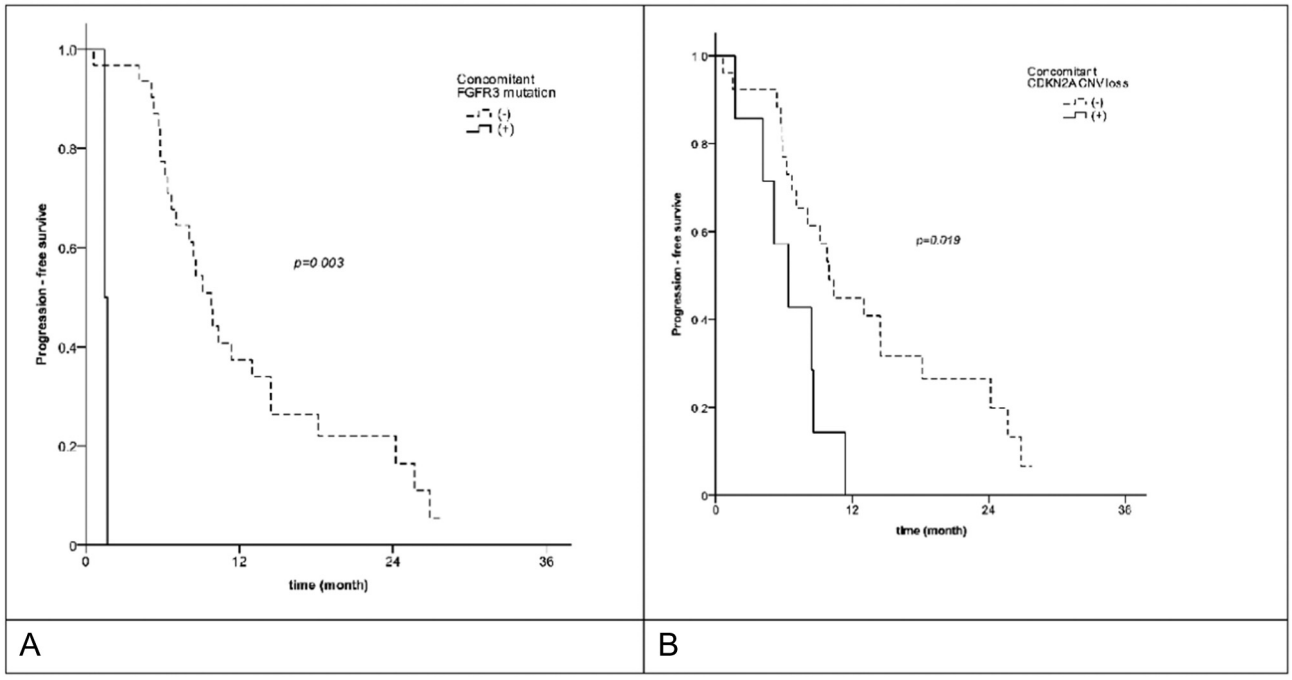

Figure 1. Progression-free survival curves of *EGFR*-mutated patients with and without *FGFR3* mutation, *CDKN2A* copy number loss (B).

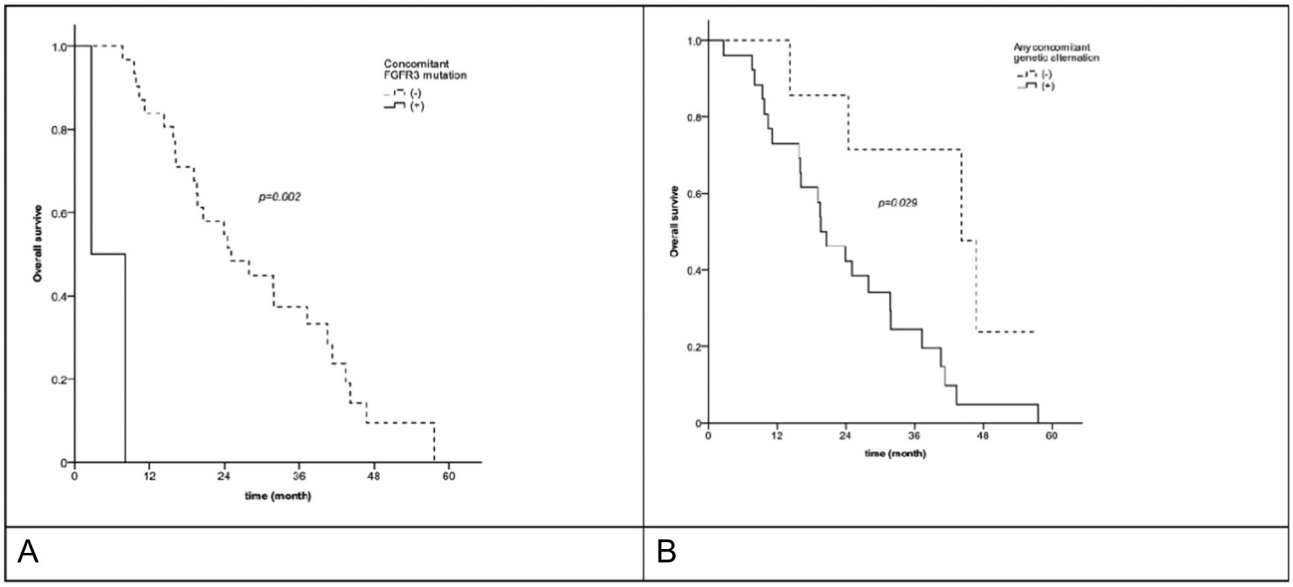

Figure 2. Overall survival curves of *EGFR*-mutated patients with and without *FGFR3* mutation (A), and any concomitant genetic alteration (B).

objective response rates but non-significant shorter PFS were observed in patients with *TP53/EGFR* co-mutations in first-line treatment of EGFR-TKIs. A very recent study retrospectively examined genetic alterations by comprehensive gene panel with a similar setup to our study [31]. Interestingly, the authors reported that mutations in TP53, PTEN, RB1, MDM2 were associated with worse PFS in NSCLC. In our study, mutations in TP53 was associated with a trend towards worse PFS and OS (See Table 3: Hazard ratio: 1.37, 2.0 respectively; *P* values:0.469, 0.134 respectively). *PTEN, RB1, MDM2* were not examined in the panel used for our study.

Although significance of this association was not achieved (presumably due to relatively small samples size and thus lack of statistical power), our finding is in line with the aforementioned study [31]. Taken whole these two aforementioned studies and our cohort, similarities in prevalent genetic mutations (such as *TP53*) are present, although a more definite conclusion regarding gene alterations and survival would be more appropriately addressed with a larger study size. We identified other concurrent genetic mutations that were associated with response towards *EGFR*-TKI. Fibroblast growth factor receptor 3 (*FGFR3*) mutations was found in 2 patients (6.1%) in our study and were associated with a worse response to EGFR-TKIs by multivariate analysis. In a recent retrospective study, a cohort of 23 *EGFR* mutant NSCLC with concomitant genetic alterations was analyzed and demonstrated 1 patient with *FGFR3* mutation [32]. This patient was also associated with unfavorable response towards first-line TKIs, which is in line with our current findings. Another case report described a similar association of FGFR3 mutation with poor response to *EGFR*-TKI, and suggested *FGFR3* mutation as an alternate pathway for *EGFR* signaling, thus providing resistance to TKIs [33]. Our study, in addition to the currently reported cases in the literature, propose *FGFR3* mutation as a clinical meaningful mutation for pre-TKI treatment in this patient population.

In our study, loss of CNV in *CDKN2A* was also significantly associated with shorter PFS in *EGFR* mutant NSCLC. Interestingly, CNV loss in *CDKN2A* occurred in a significant proportion of our cohort (7/31, 22.6%). In the 7 patients with this genetic alteration, 4 patients had an intermediate response (PFS 6-12 months on first-line TKI) and 3 patients had an unfavorable response (PFS <6 months). *CDKN2A* encodes for p16, an important tumor suppressor gene of which its loss has long been known to be associated with poor outcome in NSCLC [34,35]. In a retrospective study analyzing 127 patients with *EGFR* mutant NSCLC, homologous deletion of p16/*CDKN2A* was present in 24.4% of the cohort and was associated with significant worse response and survival [36]. This reported study is consistent with findings in our cohort. A recent study proposed that the high occurrence of gene alterations in *TP53*, *CDKN2A* and *RB1* suggest a propensity of *EGFR* mutant NSCLC cells to acquire early genetic mutations in cell cycle related genes [37]. The success of CDK4/6 inhibitors in breast cancer [38] has sparked much hope to probe for similar activity in other cancer types, and the aforementioned studies may serve as a rationale for drug development in NSCLC. Indeed, preclinical studies have demonstrated promising results [39], and clinical trials are currently ongoing to probe this issue (https://clinicaltrials.gov/ct2/show/NCT03455829).

Our study is not without limits. Our analysis is limited to the genes analyzed by the multigene panel, which are arbitrarily selected due to possible druggability and high clinical genetic relevance. It is very likely that other concomitant genetic alterations could also impact treatment response towards EGFR-TKI that is not discovered in this study. However, it must be noted that the high cost of comprehensive whole genome sequencing prevents its wide availability, thus it may be difficult to identify other concomitant genetic alterations in most cases. Another limitation is our study is completely retrospective clinical analysis in nature, lacking mechanistic studies. Further animal studies will be beneficial in elucidating the mechanisms of interactions between possible *FGFR3* mutation and or *CDKN2A* CNV alteration with mutant *EGFR* signaling pathway. Nevertheless, we believe that our study provides valuable clinical observation that can guide future work to ultimately identify predictive biomarkers for treatment response by *EGFR*-TKI.

In conclusion, in patients of advanced NSCLC with *EGFR* mutation, concomitant genetic alterations are not uncommon and may be associated with poor outcomes under standard *EGFR*-TKI treatment. Our study identifies *FGFR3* mutations and *CDKN2A* CNV abnormality as meaningful concomitant genetic alterations with mutant *EGFR* that could be predictive of treatment response by TKIs. Further data is needed to validate the clinical usefulness of the findings in this study.

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Author Contributions

Shih-Chieh Chang: conceptualization, validation, formal analysis, writing, data curation, original drift preparation, review and editing

Yi-Chun Lai: writing, data curation, original drift preparation

Cheng-Yu Chang: original drift preparation

Li-Kuo Huang: original drift preparation

Shu-Jen Chen; Kien Thiam Tan, Pei-Ning Yu: methodology, validation, formal

Jiun-I Lai: validation, writing, data curation, original drift preparation, review and editing.

Conflicts of Interest

The authors declare no conflict of interest.

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