The clinicopathological and molecular characteristics of resected EGFR-mutant lung adenocarcinoma

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Abstract
Background: Epidermal growth factor receptor (EGFR) mutations were frequently found with concomitant genetic alterations in lung adenocarcinoma (LUAD). This study aimed to investigate the profile of concomitant alterations of EGFR-mutant LUAD ≤3 cm in size and its prognostic effect on recurrence.

Methods: From January 2018 to December 2018, patients with resected LUAD ≤3 cm in size in Shanghai Chest Hospital were identified. All patients underwent capture-based targeted next-generation sequencing (NGS) with a panel of 68 lung cancer-related genes and were found with EGFR mutation. Clinicopathological and molecular characteristics and recurrence-free survival (RFS) were analyzed.

Results: A total of 637 patients were enrolled in this study. The top three frequent co-mutational genes were TP53 (179 of 637, 28.1%), PIK3CA (27 of 637, 4.2%), and ATM (22 of 637, 3.5%). The most common amplified genes were EGFR (37 of 637, 5.8%), followed by CDK4 (37 of 637, 5.8%) and MYC (12 of 637, 2.0%). Only TP53 mutation and EGFR amplification were adverse prognostic factors for RFS (all p < 0.001) in univariate analysis. Multivariable analysis further demonstrated that TP53 mutation and EGFR amplification were independent risk factors for RFS [(hazard ratio (HR) 2.07, 95% confidence interval (CI) 1.07–4.00, p = 0.030; HR 3.09, 95% CI 1.49–6.40, p = 0.002, respectively].

Conclusions: Concomitant TP53 mutation and EGFR amplification were poor prognostic factors for RFS in patients with EGFR-mutant resected LUAD. Our findings provide valuable understanding of the impact of concurrent alterations and implication for better implementation of precision therapy for patients.

KEYWORDS
concomitant mutation, EGFR, lung adenocarcinoma, recurrence-free survival
1 | INTRODUCTION

Lung adenocarcinoma (LUAD) is the most common pathological subtype of lung cancer, which is the leading cause of cancer-related mortality worldwide. For patients with early-stage LUAD, surgery is the standard treatment. But even underwent radical resection, recurrence still takes place.

Previous studies explored the relationship between oncogene alteration and clinical outcome of early-stage non-small cell lung cancer (NSCLC). Epidermal growth factor receptor (EGFR) mutation represents the most common druggable driver mutation in East Asian LUAD patients, found as favorable prognostic factor. However, some studies showed that EGFR mutation is a negative prognostic indicator of recurrence-free survival (RFS). Intra-tumor heterogeneity of LUAD lead to different biological behaviors, which may explain the inconsistent conclusions. Concomitant alterations reflected genetic characteristics of different clones in tumor, which were related to intra-tumor heterogeneity. In advanced EGFR-mutant LUAD, several studies revealed that concomitant alterations were associated with the efficacy of tyrosine-kinase inhibitors (TKIs).

Only few studies have illustrated the concomitant mutations in EGFR-mutant resected LUAD. Nowadays, next-generation sequencing (NGS) was applied widely in clinical practice. Further research is thus needed to explore the association between gene alteration and RFS, to develop a more precise management after surgical resection.

In this study, we hypothesized that concurrent gene alterations have critical impact on RFS. In order to understand the clinicopathological and molecular characteristics in EGFR-mutated patients, we performed this study to reveal the prevalence of EGFR concomitant alterations and their effect on RFS.

2 | MATERIALS AND METHODS

2.1 | Patients and sample collection

The study cohort consisted of 637 patients who underwent completely surgical resection and histologically confirmed with pathological size ≤3 cm LUAD at Shanghai Chest Hospital from January 2018 to December 2018 and was defined as Shanghai Chest cohort. All these patients have available NGS reports and confirmed with EGFR-mutant status. The patients were staged based on the eighth edition of the International Association for the Study of Lung Cancer TNM classification for lung cancer.

Inclusion criterion were: (1) primary LUAD; (2) underwent completely surgical resection; (3) confirmed pathological size ≤3 cm; (4) all resected-tissues were performed genetic analysis using 68-gene NGS panel in Shanghai Chest Hospital; (5) NGS tests reported EGFR mutation positive (including exon 19 deletion, exon 21 L858R mutation, exon 20 insertion and exon 18 G719A mutation et al). Patients were excluded for: (1) non-invasive LUAD (e.g., adenocarcinoma in situ, minimally invasive adenocarcinoma) or non-adenocarcinoma; (2) preoperative neoadjuvant therapy. The study flowchart is shown in Figure 1.

The final follow-up date was March 2021. Postoperative follow-up was started the day the patient received surgery and performed every 3 months for the first 2 years, every 6 months for the next 2 years. The follow-up data were obtained from hospital records or collected by telephone. RFS was calculated from the surgery date to recurrence or last follow-up. All clinical data were collected from electronic records. The research was approved by the Research Ethics Board of Shanghai Chest Hospital and conducted in accordance with the Declaration of Helsinki as well, and it was deemed exempt from the requirement to gather participant consent by the Institutional Review Board (KS2039).

2.2 | Targeted NGS

The DNA extraction was performed using the QIA amp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany). Targeted NGS was performed to detect somatic mutations within each sample using a 68-gene panel on the Nextseq500 sequencer (Illumina, Inc, Madison, WI, USA). The genomic profiles were assessed using Lung Core panel from Burning Rock Biotech (Guangzhou, China) (list of genes was provided in Table S1).

2.3 | Sequence data analysis

Sequence data were mapped to the reference human genome (hg19) using Burrows-Wheeler aligner v.0.7.10. Local alignment optimization, variant calling, and annotation were performed using Genome Analysis Tool Kit v.3.2 and VarScan. Variants were filtered using the VarScan. Loci with depth less than 100 were filtered out. Minimal of five supporting reads were needed for INDELs and eight supporting reads were needed for SNV calling. According to the ExAC, 1000 Genomes, dbSNP, ESP6500SI-V2 database, variants with population frequency over 0.1% were grouped as SNP and excluded from further analysis. Remaining variants were annotated with ANNOVAR.
2.4 | External cohort from cBioPortal database

The cBioPortal for Cancer Genomics (http://cBioPortal.org/) is an open source for interactive exploration of multidimensional cancer genomic data that aims to translate data sets into biologic insights and clinical applications.\(^\text{20,21}\) Patients with LUAD and available NGS as well as RFS data were identified in MSK-IMPACT Clinical Sequencing Cohort (MSKCC, 2020; dataset ID: luad_mskcc_2020) as an external cohort for validation. Finally, 184 EGFR-mutated patients were included in further analysis and defined as MSKCC cohort.

2.5 | Statistical analysis

Fisher’s exact test or Chi-square test was used to compare the categorical data between two groups. RFS analysis was performed using the Kaplan-Meier method and log-rank test. Multivariable Cox proportional hazards model was applied to analyze factors correlating to RFS. SPSS (version 24.0, SPSS Inc, Chicago, IL, USA) and Prism software (version 8.0, GraphPad Software, San Diego, CA, USA) and R software (version 4.0.5, the R Foundation for Statistical Computing, Vienna, Austria) served for statistical analysis. Genes altered in 10 patients at least were considered. Gene amplification defined as copy number gains more than 2 times. Significant factors in univariable Cox proportional hazards model. A \( p \) value <0.05 was considered to be statistically significant. Multiple testing was corrected by the false discovery rate (FDR) method on molecular variables’ univariable Cox analysis. The FDR was calculated by the `p.adjust` function derived from ‘Stats’ package in R software. The molecular variables with 2-tailed \( p \) value <0.05 and FDR <0.05 were considered statistically significant with an acceptable FDR.

3 | RESULTS

3.1 | Baseline demographics

A total of 637 patients met the inclusion criteria and were enrolled for analysis. The clinicopathological and molecular characteristics of study cohort were shown in Table 1. In general, 424 (66.6%) patients were female, 385 (60.4%) patients were older than 60 years and most of patients (92.3%) were never smokers. Two hundred and forty-six (38.6%) patients were found with EGFR exon 19 deletion (19Del), while EGFR exon 21 L858R mutation (21L858R) was identified in 338 (53.1%) patients. The clinicopathological and molecular characteristics of MSKCC cohort can be found in Table S2.

3.2 | Somatic mutation and amplification of major genes

The most frequent co-mutational genes were TP53 (179 of 637, 28.1%), followed by PIK3CA (27 of 637, 4.2%),
The most common amplification genes were EGFR (37 of 637, 5.8%), followed by CDK4 (37 of 637, 5.8%), MYC (12 of 637, 2.0%). The overview of top 12 concurrent alterations and top 3 amplified genes are shown in Figure 2.

### 3.3 Comparison of characteristics between patients with or without TP53 mutation and EGFR amplification

To evaluate the clinical and pathological characteristics of co-occurred TP53 mutation or EGFR amplification in EGFR-mutant patients, comparisons were summarized. TP53 mutation was associated with cigarette exposure, larger tumor size, higher TNM stage, high-grade-component predominance, and visceral pleural invasion (VPI). And the presence of EGFR amplification was associated with larger tumor size, higher TNM stage, high-grade-component predominance and VPI (Table S3).

The association of TP53 mutation and EGFR amplification in EGFR-mutated patients was also explored, showing that the presence of EGFR amplification was significantly associated with mutations in TP53 in both cohorts (Figure S1). And the prevalence of EGFR amplification showed no difference between study cohort and external cohort [5.81% (37/637) vs. 4.89% (9/184), p = 0.719].

According to concomitant status of gene alterations, four groups of patients can be identified: (1) EGFR...
mutation only, (2) concomitant TP53 mutation only, (3) concomitant EGFR amplification only, and (4) concomitant TP53 and EGFR amplification. The analysis of tumor mutation burden and EGFR copy number gains in patients with EGFR amplification was showed in Figures S2 and S3, respectively.

3.4 Prognostic value of concurrent TP53 mutations or EGFR amplification in EGFR-mutant lung adenocarcinoma patients

The median follow-up duration was 30.57 months (interquartile range, 27.87–32.40). A total of 49 patients (7.7%) experienced recurrences. Survival analyses were performed and demonstrated the prognostic value of concomitant TP53 mutation or EGFR amplification in EGFR-mutant patients (Table S4).

Compared with patients harboring TP53 WT (wild type), patients harboring TP53 mutation had a significant worse RFS in Shanghai Chest cohort (p < 0.001; Figure 3A). Compared with patients with EGFR mutation only, patients with both EGFR mutation and amplification had a significant worse RFS (p < 0.001; Figure 3B). Similar results were observed in the external MSKCC cohort (p = 0.002, p < 0.001, respectively) in Figure 3C,D.

Furthermore, in different mutation subtypes of EGFR (19Del and L858R), similar findings were also observed (p = 0.005, p < 0.001, p < 0.001, p < 0.001, respectively; Figure 3E–H).

3.5 Concomitant TP53 mutations or EGFR amplification is an independent prognostic factor of patients with EGFR-mutant lung adenocarcinoma

Survival was analyzed using univariable and multivariable Cox proportional hazard regress model for RFS (Table 2). Univariable analysis revealed that TNM stage, high-grade component predominant, TP53 mutation and EGFR amplification were significant prognostic factors for RFS. Multivariable analysis further demonstrated that concurrent TP53 mutation (HR 2.07, 95% CI 1.07–4.00, p = 0.030) and EGFR amplification (HR 3.09, 95% CI 1.49–6.40, p = 0.002) were independent adverse factors for RFS.

Compared with others, the patients harboring EGFR amplification had a significant worse RFS, regardless of TP53 status (Figure 4A). Similarly, we found that patients with concomitant TP53 mutation and EGFR amplification in the external MSKCC cohort had poorer RFS (Figure 4B).
Figure 3 Kaplan–Meier survival curves of RFS for patients with and without EGFR amplification in Shanghai Chest cohort (A, B), in MSKCC cohort (C, D), EGFR 19 Del subgroup (E, F) and EGFR 21 L858R subgroup (G, H) in Shanghai Chest cohort. Abbreviations: RFS, recurrence-free survival; 19 Del, exon 19 deletion; 21 L858R, exon 21 L858R mutation.

The prognosis values of TP53 mutation and EGFR amplification in RFS were also estimated by nomogram (Figure S4) and validated by calibration curves (Figure S5). Subgroup analyses of stage IA and stage IB-IIIA were performed (Figure S6).

4 DISCUSSION

Recurrence is a critical problem in postoperative management of LUAD. Therefore, recurrence risk stratification is vital for identifying those who might benefit from more intensive adjuvant treatment for resected LUAD. EGFR is reported as the most frequent altered driver gene in Asian patients. Concomitant alterations are frequently noticed in LUAD. However, their prognostic role remains unclear in early-stage LUAD. In the current study, we analyzed the data of 637 EGFR-mutated patients and explored the prognostic value of concomitant alterations on recurrence. In order to minimize the impact of surgical approach and dissection extent and the effect of tumor’s burden in primary site on prognosis, only small-size (≤3 cm) cases were included in our study. Our study demonstrated that concomitant TP53 mutations and EGFR amplification were poor prognostic factors for RFS in patients with EGFR-mutant resected LUAD, indicating that early interventions may be considered in these patients. To our knowledge, this is the largest study that comprehensively focused on both TP53 mutation and EGFR amplification in resected EGFR-mutant patients.

TP53, functioning critically in cell cycle, DNA repair and metabolism, is the most common tumor suppressor gene in EGFR-mutated lung adenocarcinoma. Mutation in TP53 gene was reported as a poor prognostic predictor of EGFR-TKIs treatment in advanced LUAD. Zhao et al analyzed 409 EGFR-mutated patients and confirmed that patients with concurrent TP53 mutation have worse (disease-free survival) DFS, and Long et al as well as Lee et al showed the similar observations. In the current study, we found that 28.1% of patients carrying TP53 mutation, which is similar to previous researches focusing on surgical EGFR-mutated patients (15.83–53.54%). Moreover, TP53 mutation was associated with aggressive clinicopathological features as previously reported. The survival analysis of both two cohorts further demonstrated that patients with concurrent TP53 mutation have poorer DFS. In addition, mutations in TP53 occur as early truncal events in tumor evolution and allow tolerance of a greater degree of genomic instability, resulting subclonal diversification and intra-tumor, which correlated with aggressively biological behaviors and higher tumor mutation burden (TMB). These findings indicated that TP53-mutant status was an independent prognostic factor in EGFR-mutant
LUAD. As such, the postoperative management of LUAD should consider the mutation not only in *EGFR* but also in *TP53*, given its negative impact.

*EGFR* amplification occurred usually in *EGFR*-mutant patients. Some studies showed that *EGFR* amplification is one of the resistance mechanisms of the third generation inhibitors.
EGFR-TKIs treatment, which lead to worse survival benefits in advanced patients.\textsuperscript{27–29} But in gefitinib-treated studies, patients with \textit{EGFR} amplification had better progression-free survival than those without \textit{EGFR} amplification.\textsuperscript{30,31} However, there remains no study focusing on the impact of \textit{EGFR} amplification in \textit{EGFR}-mutated surgical resected LUAD. In current research, 5.8\% of patients have concurrent \textit{EGFR} amplification. We investigated the characteristics of \textit{EGFR} amplification in postoperative patients with \textit{EGFR} mutation and explored the impact to RFS. Patients harboring \textit{EGFR} amplification had worse RFS, regardless of \textit{TP53} mutation subtype. Possible reason may be that \textit{EGFR} amplification was associated with increased mutant allele transcription and gene activity on the basis that mutation of \textit{EGFR} activated receptor tyrosine kinase (RTK) pathway, cooperating with tumorigenesis and resulting in aggressive characteristics.\textsuperscript{32}

Cancer develops through a process of somatic evolution.\textsuperscript{33} The association between \textit{TP53} mutation and \textit{EGFR} amplification may be complex. Previous research revealed that mutations in \textit{TP53} lead to genetic instability and result in focal high-amplitude amplifications that occur late during the evolution of lung cancer.\textsuperscript{34} Zhang et al.\textsuperscript{35} also reported that \textit{EGFR} copy number gains occurred relatively late compared with \textit{EGFR} mutation and \textit{TP53} mutation in molecular time scale. In our search, the presence of \textit{EGFR} amplification was correlated with \textit{TP53} mutation in both two cohorts, which indicated \textit{EGFR} amplification arise relatively late and toward the end of the evolution of \textit{EGFR}-mutated adenocarcinoma, resulting in aggressive pathological characteristics (e.g., high-grade-component predominance and lymphatic metastases). Therefore, patients with \textit{EGFR} amplification should be regarded as high recurrence-risk population in \textit{EGFR}-mutated patients.

According to previous researches, mutation in \textit{TP53} correlated with higher TMB.\textsuperscript{36,37} TMB significantly distinguished the patients with inferior RFS from entire MSKCC cohort, but there was no difference in TMB between tumors with \textit{TP53} mutation and those with \textit{TP53} mutation and \textit{EGFR} amplification concurrently, indicating that the reasons were likely to be that TMB is not the major prognostic factor in patients with \textit{TP53} mutation. The inner mechanism of the inferior RFS of patients with concomitant \textit{TP53} and \textit{EGFR} amplification is complex and needed to be explored in further studies.

The analysis of \textit{EGFR} copy number displayed that \textit{EGFR} amplification was significantly associated with recurrence, while \textit{EGFR} copy number showed no different between recurrent patients and non-recurrent patients. In addition, patients with lower copy number has no better survival than those with higher copy number. It suggested that the status of \textit{EGFR} amplification happening may weight more important than its frequency (copy number); therefore, further mechanism account for this result still needs future exploration.

With the improvement of early lung cancer scanning, the proportion of surgical lung cancer patients is increasing.\textsuperscript{38} and \textit{EGFR}-mutate patients are the major part of them especially in Asia.\textsuperscript{2} For surgical resected \textit{EGFR}-mutated patients, monitoring the recurrence of tumor is performed currently according to clinicopathologic risk stratification, which could be improved in some way. In recent years, the development of adjuvant therapy was promoted due to the clinical trials focusing on the use of EGFR-TKIs in post-operative management.\textsuperscript{39–41} ADUARA trial on adjuvant EGFR-TKIs revealed that even the stage IB patients can benefit from adjuvant EGFR-TKIs, which indicated that the better postoperative management is needed for \textit{EGFR}-mutated patients.\textsuperscript{40} Providing predictive and prognostic values in advanced cancer treatment, NGS was used to guide clinical practice. Genomic alterations stratified the treatment-benefit cohort in targeted therapy and immunotherapy,\textsuperscript{42} which confirms the clinical value of genetic alterations. Risk stratification by molecular features can help to differentiate the high-risk \textit{EGFR}-mutant patients from low-risk \textit{EGFR}-mutated patients. This study contributed to uncovering the risk cohorts according to molecular risk stratification, which may benefit more

\begin{figure}[h]
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\caption{Kaplan–Meier survival curves of RFS for patients with no mutation in \textit{TP53} or no amplification in \textit{EGFR}, mutations in \textit{TP53} alone, amplification in \textit{EGFR} alone and both mutations in \textit{TP53} and amplification in \textit{EGFR} in Shanghai Chest cohort (A) and in MSKCC cohort (B). Abbreviations: RFS, recurrence-free survival.}
\end{figure}
from earlier and more intensive adjuvant therapy. There is no research focusing on the postoperative management of these specific population, but researches focusing on advanced NSCLC patients have showed that EGFR amplification was associated with treatment guiding benefits. The subgroup analyses of IPASS trial were reported that PFS favored gefitinib over carboplatin/paclitaxel in EGFR-mutated patients with high EGFR copy number. A. Ruiz-Patiño et al. and Cui J et al. had reported that EGFR amplification was associated with better survival when treated with EGFR-TKIs. Additionally, among patients with EGFR amplification, high or low copy number did not affect the treatment outcomes. However, EGFR amplification was also reported as one of the resistance mechanisms of 3rd generation EGFR-TKIs, which may indicated that patients with EGFR amplification benefit less from 3rd generation EGFR-TKIs therapy, compared to patients without EGFR amplification. But there is no research comparing the clinical outcomes of being treated with different generation EGFR-TKIs in patients with EGFR amplification, which should be explored in further research. Moreover, 1st plus 3rd generation EGFR-TKIs was designed following biomarkers strategy to overcome the resistance of 3rd generation TKIs (EGFR amplification) in the phase II ORCHARD trial, which will provide valuable guidance for these specific population.

Our study has several limitations. Selection bias is inevitable for single-center retrospective study. The frequency of different mutation subtypes in EGFR was similar with previous researches, which may indicate the minor selection bias. The number of EGFR amplification events was small, but there was no statistically significant difference in frequency of EGFR amplification between Shanghai Chest cohort and MSKCC cohort. Postoperative NGS detection was performed widely since 2018 in our institution. Long-term follow-up is needed for more conclusive statements. What’s more, the mechanism should be explored in experiment research.

5 | CONCLUSIONS

Concomitant TP53 mutation and EGFR amplification were independent adverse factors for RFS in patients with EGFR-mutant resected LUAD. Our findings provide valuable understanding of the impact of concurrent alterations and implication for better implementation of precision therapy for these patients.

ETHICAL APPROVAL STATEMENT

Data are collected from Shanghai Chest Hospital and are devoid of any personal identifiable information (KS2039).

CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

AUTHOR CONTRIBUTIONS

Wensheng Zhou: Conceptualization, Data curation, Formal analysis, Project administration, Software, Writing - original draft, Writing - review & editing. Zhichao Liu: Data curation, Formal analysis, Investigation, Methodology, Software. Yanan Wang: Investigation, Software, Writing - review & editing. Yanwei Zhang: Project administration, Methodology. Fangfei Qian: Software, Formal analysis. Jun Lu: Software, Formal analysis, Data curation. Huimin Wang: Data curation, Investigation, Project administration. Ping Gu: Software, Project administration. Minjuan Hu: Data curation, Software. Ya Chen: Data curation, Investigation. Zhenyu Yang: Data curation, Investigation. Ruiying Zhao: Software, Formal analysis. Yuqing Lou: Supervision, Validation, Visualization, Writing – review. Baohui Han: Conceptualization, Supervision, Writing - review & editing. Wei Zhang: Conceptualization, Project administration, Supervision, Validation, Visualization, Writing - review & editing. All authors reviewed and approved the final version of the manuscript.

DATA AVAILABILITY STATEMENT

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

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SUPPORTING INFORMATION
Additional supporting information may be found in the online version of the article at the publisher’s website.

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