

# Comprehensive Analysis of Aneuploidy Status and Its Effect on Efficacy of EGFR-TKIs in Lung Cancer

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## Research Article

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# Abstract

**Background:** The evaluation of the clinical efficacy of molecularly targeted cancer therapies remains a great challenge.

**Methods:** Next-generation sequencing (NGS) analysis and fluorescence in situ hybridization (FISH) were used to evaluate the clinical efficacy in terms of EGFR mutation abundance and aneuploidy status.

**Results:** The PFS of patients diagnosed as euploidy was actually higher than that of patients diagnosed with aneuploidy, and was related to both ORR and DCR. Patients with EGFR mutation abundance  $\geq 28.86\%$  had slightly higher ORR and similar DCR. Two-way analysis of variance was used to assess the effects of EGFR mutation abundance and tumor aneuploidy status on patients' PFS, supporting a strong correlation between aneuploidy status and clinical efficacy, euploid patients have higher ORR and DCR.

**Conclusions:** Aneuploidy status could effectively evaluate clinical efficacy of patients with lung cancer, whereas EGFR mutations abundance couldn't predict the extent of benefit from EGFR-TKI treatment.

## Introduction

Lung cancer is the leading global cause of cancer death, which arise because of the acquisition of somatic alterations in their genomes that alter the function of key cancer genes. A number of these alterations are implicated as determinants of treatment response in clinical practice[1]. Currently, Lung Cancer driver mutations and mutational signatures have been described in detail[2, 3], and numerous clinical trials have demonstrated that targeted therapies can delay the progression of the disease in patients. Targeted drugs have achieved great clinical success and significantly extended progression-free survival and overall survival in lung cancer patients with driver gene mutations. However, accurate and effective evaluation of patient response to targeted drugs has always been a difficult problem for clinicians due to tumor heterogeneity and individual differences among patients.

In order to select the best therapy, there is a great need for biomarkers, that is, an identifiable, tumor-specific feature that can predict drug response. Aneuploidy-driven phenotypes are caused by copy number changes of genes. Recent studies suggest that aneuploidy is a context-dependent, cancer-type-specific oncogenic event that may have clinical relevance as a prognostic marker and as a potential therapeutic target[4, 5]. Aneuploidy is a hallmark of most cancers and is associated with increased malignancy, tumor recurrence and drug resistance[6–8]. Indeed, roughly two out of three human tumors display aneuploidy[9–11], and increasing aneuploidy levels are generally correlated with later tumor stages[12]. Emerging evidence indicates that aneuploidy is a new driving force in tumorigenesis and is associated with prognosis beyond strong predictors[13]. The prognostic value of aneuploidy has long been demonstrated for several indications[14, 15], with high levels of aneuploidy being associated with poorer prognosis in the vast majority of cases. Previous studies confirms that aneuploidy deserves as a predictive biomarker for benefit from adjuvant docetaxel[16]. Tumor aneuploidy may also be a useful biomarker for predicting which patients are most likely to benefit from immunotherapy[17]. However, an

important question is whether aneuploidy can also inform treatment decisions for targeted therapy. However, how aneuploidy influences cancer progression and whether degree of aneuploidy can be implemented clinically to inform the care of patients with cancer are still unclear.

In our study, aneuploidy status and EGFR mutation abundance were used to evaluate clinical efficacy of patients with lung cancer. The results support a strong correlation between aneuploidy status and patient efficacy, euploid patients have better clinical efficacy.

## Methods

### Patient specimen acquisition

We retrospectively investigated the medical records of Lung Cancer patients who underwent EGFR mutation and mutation abundance examinations in tumor tissues by NGS of 8 gene panel and FISH from July 7,2018, and June 27,2020. Clinical data of all patients were from the electronic medical record database of Henan Cancer Hospital. The present study included 63 lung cancer patients received NGS assessment and FISH for molecularly matched therapy and follow up. Study protocols were approved by the Ethical Review Community of Henan Cancer Hospital.

### DNA extraction and qualification and target gene sequencing

Genomic DNA was extracted from tumor FFPE tissues using a DNeasy Blood & Tissue Kit (Qiagen Inc., USA). The KAPA Hyper Prep Kit (Kapa Biosystems, USA) was utilized for DNA library preparation as a versatile reagent kit adapted to the Illumina platform. For hybridization enrichment, customized xGen lockdown probes (Integrated DNA Technologies, USA) were applied. The probes panel were designed to target 8 tumor-specific genes. All procedures were conducted according to the manufacturers' protocols.

### FISH experiments and the detection result of the determination criteria

All FISH tests were conducted in the department of pathology in Henan Cancer Center. FISH was carried out on formalin-fixed paraffin-embedded (FFPE) sections using the ALK Break Apart Detection kit (Guangzhou LBP Medicine Science & Technology Co., Ltd) according to manufacturer's instructions. The GSP ALK 5' probe (Spectrum Green) and the GSP LAF4 3' probe (Spectrum RED) were labeled, hybridized and evaluated along with standard controls. FISH signals were evaluated independently by two technicians who were blinded to the patient's history and histologic findings. All samples were examined by pathologists to identify tumor cell enriched areas which were marked on the underside of the slides with a diamond-tipped scribe. The percentage of tumor cells in each case was over 60%. The presence of separated green-red signals (> 2 signal diameters) or individual red signals in tumor cells was considered FISH-positive, while FISH-negative was defined as overlapping red-green signals (slightly yellow).

The criteria for detecting ALK status in the sample was that at least 50 tumor cells were observed. If > 25 cells out of 50 (> 25/50 or > 50%) were positive, the sample was considered positive. If 5 to 25 cells out of 50 (10–50%) are positive, the sample was considered equivocal, and the slide should be evaluated by the second reader who selected additional 50 nuclei. In this case, the sample was considered positive if more than 15 of the accumulated 100 cells have a separation signal ( $\geq 15\%$ ).

## Study endpoints

The primary endpoint of this study was to determine the association between PFS and mutation abundance of eligible patients after EGFR-TKI therapy. PFS was the time from starting administration of oral EGFR-TKI to disease progression. The secondary endpoints included the objective response rate, the association between PFS and age, sex, and whether EGFR-TKI was administered as the first-line treatment. The therapeutic effect of EGFR-TKI was evaluated according to the Response Evaluation Criteria In Solid Tumors with Complete Response(CR), disappearance of all target lesions; Partial Response (PR): with at least a 30% decrease in the sum of the longest diameter of target lesions with the baseline sum of the longest diameter as the reference; Progressive Disease(PD) as the ratio between the longest diameter sum of the target lesion and the longest diameter of the smallest target lesion was recorded after starting administration of oral EGFR-TKI, and increased by at least 20% or the presence of one or more new lesions. Objective Response Rate (ORR) was defined as achieving CR or PR.

## Statistical methods

All statistical analyzes were performed using GraphPad Prism version 7.0. Kaplan-Meier and Cox survival regression models were used to analyze the influence of age, gender and aneuploidy status as well as EGFR mutation abundance on survival and prognosis.  $P$  value < 0.05 was regarded as statistically significant.

## Result

### *Patient characteristics*

A total of 291 patients diagnosed with lung cancer and were analyzed by both NGS and FISH methods. Among them, 63 (21.6%) patients were treated according to the molecular testing and followed up all the time. The clinical and pathological features of patients with Lung Cancer were summarized in Table 1. There were 23 males (36.5%) and 40 females (63.5%), 20.6% (13/63) had a smoking history, and 22.2% (14/63) were over 65 years old. The median age was 57 years (ranged from 31 to 76 years). There were 60 patients with Adenocarcinoma (94%), 1 with Squamous (1%), and 2 patients with NSCLC (5%).

### *PFS correlated with EGFR mutation abundance after EGFR-TKI therapy*

The median PFS was 10.0 months (range, 1.0-33.0 months), with an overall disease control rate of 42.4%. In order to establish the accurate cutoff value for distinguishing EGFR mutation abundance and thus

providing useful evidence for clinical practice, combining the median PFS data of patients given EGFR-TKI orally were analyzed by stratification.

stratified analyses were conducted by combining the median PFS data of patients given EGFR-TKI orally. We respectively tried to use 10%, 20%, 30%, 40%, or 50% as the cutoff values to analyze the medium PFS of the high and low abundance groups. The results revealed that when 10%, 20%, or 30% were used as the cutoff values, the medium PFS achieved a statistically significant therapeutic effect in > 10%, 20%, 30% compared to < 10%, 20% and 30% mutation abundances, respectively (Supplementary Table 1). The medium PFS was most significant (11.0 months vs 8.0 months) when EGFR mutation abundance value of 25% was used as the cutoff value. Although the median PFS was increased initially with an increase in the abundance of EGFR mutation, the median PFS did not continue to increase when the mutation abundance increased more than 30%. Further, we estimated the accurate cut-off value of EGFR mutation abundance by ROC analysis in 63 patients. We found that the cut-off value for it was 28.86% and the area under the curve (AUC) were 0.571 (0.426-0.716) for EGFR mutation abundance (Figure 1A). When the cut-off value of the abundance of EGFR mutations was  $\geq 28.86\%$ , the median of mutation abundance was about 40% (Figure 1B), and Kaplan-Meier analysis showed that cut-off value of 28.86% was no significant difference between PFS duration and EGFR mutation abundance (Figure 1C). Simultaneously, we investigated the difference in clinical efficacy between patients with EGFR mutation abundance < 28.86% and  $\geq 28.86\%$ , and found that patients with EGFR mutation abundance  $\geq 28.86\%$  had slightly higher ORR and similar DCR (Figure 1D-E).

### ***PFS correlated with aneuploidy status after EGFR-TKI therapy***

Simultaneously, all 63 cases also received FISH assessment for Aneuploidy status. The mPFS of euploid patients was 9.0 months (40 cases), and that of patients with aneuploidy was 10.0 months (23 cases) (Figure 2A). To further investigate whether euploidy and aneuploidy affected patient's PFS, Kaplan-Meier analysis showed no significant difference between euploid and aneuploid patients (Fig. 2B). Furthermore, we explored the dissimilitude of clinical efficacy between euploid and aneuploid patients, and found a higher ORR and DCR displayed in euploid patients. The ORR of euploid and aneuploid patients were 7.7% and 0%, and the DCR were respectively 51.3%, 25.0% (Figure 2C-D). The results showed that the PFS of patients diagnosed as euploidy was actually higher than that of patients diagnosed with aneuploidy, and was related to both ORR and DCR, giving a clue that patients diagnosed with euploidy had a better clinical efficacy.

### ***Association among aneuploidy status, EGFR mutation abundance and clinical efficacy***

To further elucidate whether tumor aneuploidy status and the abundance of EGFR mutations together affect PFS, there are significant differences both aneuploidy status and EGFR mutation abundance in 63 patients. The median of the abundance of EGFR mutations in patients with euploidy was 25.51%, while that in aneuploid patients was 28.59% (Figure 3A). Two-way analysis of variance was also used to assess the effects of EGFR mutation abundance and tumor aneuploidy status on patients' PFS. In patients with EGFR mutation abundance less than 28.86%, euploid patients was slightly more significant

than aneuploid patients. However, in patients with EGFR mutation abundance more than 28.86%, there was not significant difference between euploid and aneuploid patients (Figure 3B). When the EGFR mutation abundance was less than 28.86%, the mPFS of patients with euploidy and aneuploidy were 9 and 11 months, respectively. When patients with the EGFR mutation abundance was more than 28.86%, the mPFS of patients were 11 and 10 months. We also assessed the dissimilitude of clinical efficacy between both EGFR mutation abundance and aneuploidy status. Euploid patients had higher ORR and DCR than aneuploid patients, regardless of EGFR mutation abundance (Figure 3C-D). Next, we also conducted univariate analysis for the general characteristic of age, gender, smoking history by Kaplan-Meier survival analyses. The results showed that these factors were not associated with the median PFS after EGFR-TKI (Supplementary Table 2).

## Discussion

Cancer is driven by multiple types of genetic alterations, ranging in size from point mutations to whole-chromosome gains and losses, known as aneuploidy. Chromosome instability, the process that gives rise to aneuploidy and can promote tumorigenesis by increasing genetic heterogeneity and promoting tumor evolution. The classical definition of aneuploidy is the numerical aberrations of whole chromosomes. In the recent cancer genome literature, this definition has been extended to include gains or losses of chromosome arms [18, 19]. Unlike 'aneuploidy', the term 'focal copy number alterations (focal CNAs)' is usually used to describe smaller copy number changes that encompass fewer genes. Although this qualitative definition of aneuploidy is operationally convenient, it is ambiguous [4]. The consequence of aneuploidy for the genome is mostly limited to alterations in gene copy numbers. It is generally believed that changes in the copy number of specific genes are responsible for the increased fitness of cells harboring specific aneuploidies [20–25]. Recent studies have further suggested that aneuploidies are largely driven by the cumulative effects of oncogenes and tumor suppressor genes that reside within the aberrant chromosome [26, 27]. A key feature of aneuploid cells is that they often provoke genomic instability [28–30]. A single episode of genomic instability generates multiple subclones that attain clonal dominance at different rates and under various selective pressures [31], and clonal suppression and recrudescence appear to correspond to drug sensitivity and resistance. Since therapeutic agents can be regarded as new selection pressures, aneuploidy might differentially influence therapy response and tumor relapse in ways similar to their impact on tumor evolution [32]. Aneuploidy, can have profound impact on therapy by accelerating tumorigenesis and the outgrowth of resistant clones followed by tumor release. Targeting the aneuploid state, specific aneuploidy drivers or specific aneuploidy passengers has been demonstrated to be useful in selectively killing aneuploid cells. Therapeutically exploiting aneuploidy will likely depend on the mechanism and level of aneuploidy tolerance in aneuploid cells. Since aneuploidy tolerance might be a bottleneck for increasing karyotypic divergence, low divergence might predict higher sensitivity toward such therapeutic strategies.

Aneuploidy is associated with tumor progression and poor prognosis [16]. Aneuploidy and SCNA levels in cancers were shown to positively correlate with mutation load and cell proliferation [11, 24]. By determining the copy number of specific chromosomes in single cells, it was shown that the

chromosomal content within certain cell populations varied over time. Aneuploidy is usually quantified by measuring intracellular DNA content or chromosome structure and number [16]. Therefore, aneuploidy can be considered as a biomarker for evaluating clinical efficacy.

By contrast, single-cell methods, such as FISH, provide a more accurate measure of focal SCNAs. In order to examine the correlation between focal SCNAs and clinical outcomes, we simultaneously used NGS and FISH to obtain clinical information of patients. Our results demonstrated that patients diagnosed with euploidy had a better clinical efficacy, and a higher ORR and DCR displayed in euploid patients (Fig. 2C-D). Two-way analysis of variance also shown that the mPFS of euploid patients was higher than that of aneuploid patients, and ORR and DCR of euploid patients were better than those of aneuploid patients (Fig. 3C-D). Our results support the strong correlation between clinical efficacy and cancer aneuploidy status.

The success of precision medicine depends on our ability to effectively translate genomic data into actionable, customized prognosis and treatment regimens for individual patients. Valid predictive factors of the efficacy of EGFR-TKIs are important for selecting patients who may benefit more from EGFR-TKI treatment. Several previous studies suggested that the relative abundance of EGFR mutations could predict the extent of benefit from EGFR-TKI treatment [33, 34]. However, our study confirmed that the PFS of patients was not closely related with the mutation abundance of the EGFR gene after treatment with EGFR-TKI (Fig. 3B-D). In comparison to aneuploidy status, treatment with EGFR-TKI in patients with high EGFR mutation abundance didn't achieve more benefits in terms of ORR and DCR, while 28.86% was the best cutoff value to separate the low and high EGFR mutant abundance (Fig. 1D-E). Our results support that clinical efficacy is no correlation with EGFR mutation abundance.

The clinical development of molecularly targeted cancer therapies remains a huge challenge. The FISH technique that plays a leading role in diagnostic pathology for its single-cell analysis has provided crucial information regarding genomic variations in malignant cells. Although aneuploidy status can relatively quickly be determined by FISH, such technique has several limitations. The main drawback of this technique is the fact that most tumor cannot be scored in an automated fashion, which renders FISH extremely labor intensive, limiting its potential use in the clinical setting. Secondly, FISH can be performed on interphase nuclei but can only analyze a limited number of loci. It seems probable that such single-cell, next-generation sequencing approaches to define tumor aneuploidy status will become more prevalent as costs decrease and technology improves. In recent years, aneuploidy can readily be detected using multiple technologies, including various conventional and molecular cytogenetic methods, FISH [35], single-nucleotide polymorphism arrays (SNP array), and genome-wide DNA and RNA sequencing[4]. Furthermore, other molecular cytogenetic methodologies such as chromosome specific FISH karyotyping and comparative genomic hybridization have also helped in the detection of cryptic genetic changes in cancer.

In our study, aneuploidy status and EGFR mutation abundance were used to evaluate clinical efficacy of patients with lung cancer. The results support a strong correlation between aneuploidy status and clinical

efficacy, euploid patients had higher ORR and DCR.

## **Conclusions**

In summary, our data suggest that assays of tumor aneuploidy status might be useful for determining which patients are most likely to respond to therapies based on EGFR-TKIs. Information on aneuploidy status can be derived from NGS analysis and FISH performed on patient tumors. Our results suggest that important clues to the progression of lung cancer lie in the aneuploidy status.

## **Declarations**

### **Ethical Statement**

The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the ethics committee of Henan Cancer Hospital. Written informed consent was obtained from all individuals included in the study.

### **Consent for publication**

All authors confirmed they have contributed to the intellectual content of this paper and have met the following 4 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; (c) final approval of the published article; and (d) agreement to be accountable for all aspects of the article thus ensuring that questions related to the accuracy or integrity of any part of the article are appropriately investigated and resolved.

### **Data Availability Statement**

All the related software and scripts are available from the corresponding author on reasonable request.

### **Competing interests**

Upon manuscript submission, all authors completed the author disclosure form. The authors declare that they have no competing interests.

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### **Authors' contributions**

C Zhao., K Yang., and C Yan. performed the experimented and analyzed the data. Y Chang., J Ma., and Y Guo. performed the experiments, analyzed the data, and wrote the manuscript. H Gao. supervised aspects of the project. B Wei. conceived and managed the project.

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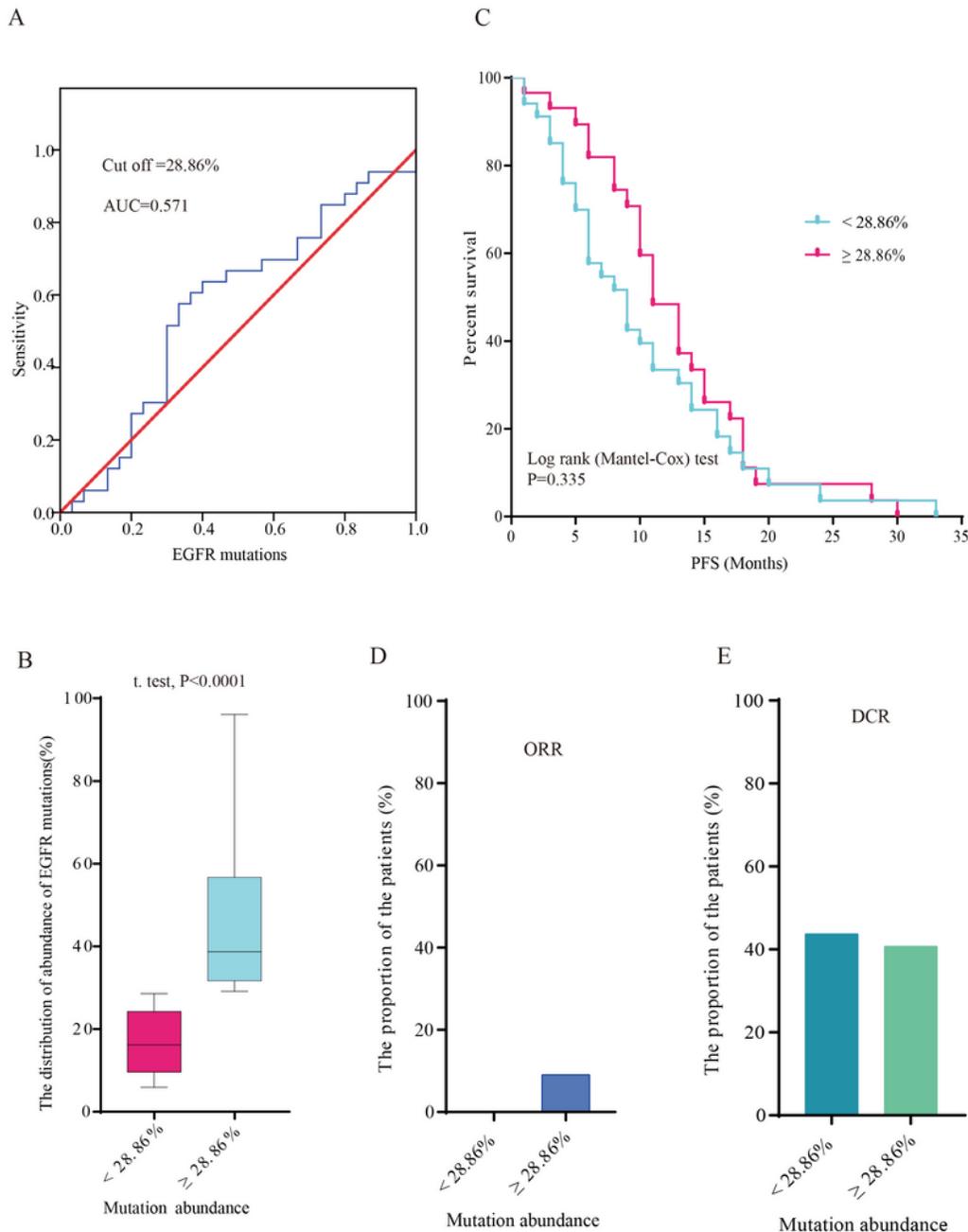
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## Tables

**Table 1.** Clinical characteristics of patients at baseline.

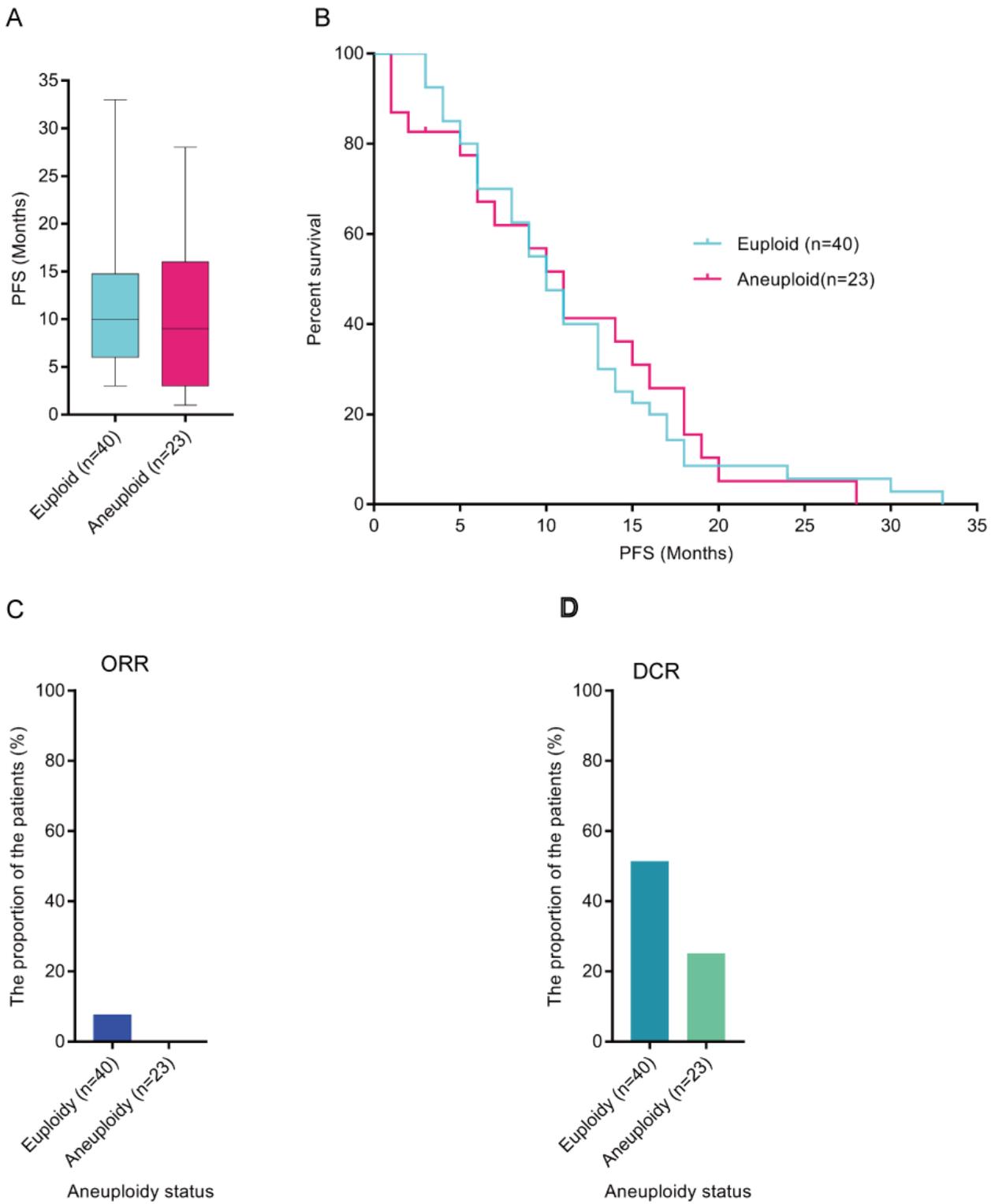
Characteristic	All patients(n=63)	
	Number	Proportion (%)
<b>Age</b>		
Median	57	
range	31-76	
<65	49	77.8
≥65	14	22.2
<b>Sex</b>		
Male	23	36.5
Female	40	63.5
<b>Smoking History</b>		
Ever smoker	13	20.6
Never smoker	50	79.4
Pathology Type		
Adenocarcinoma	60	95.2
Squamous	1	1.6
NSCLC	2	3.2
<b>Stage</b>		
I-III	15	23.8
IV	48	76.2

## Figures



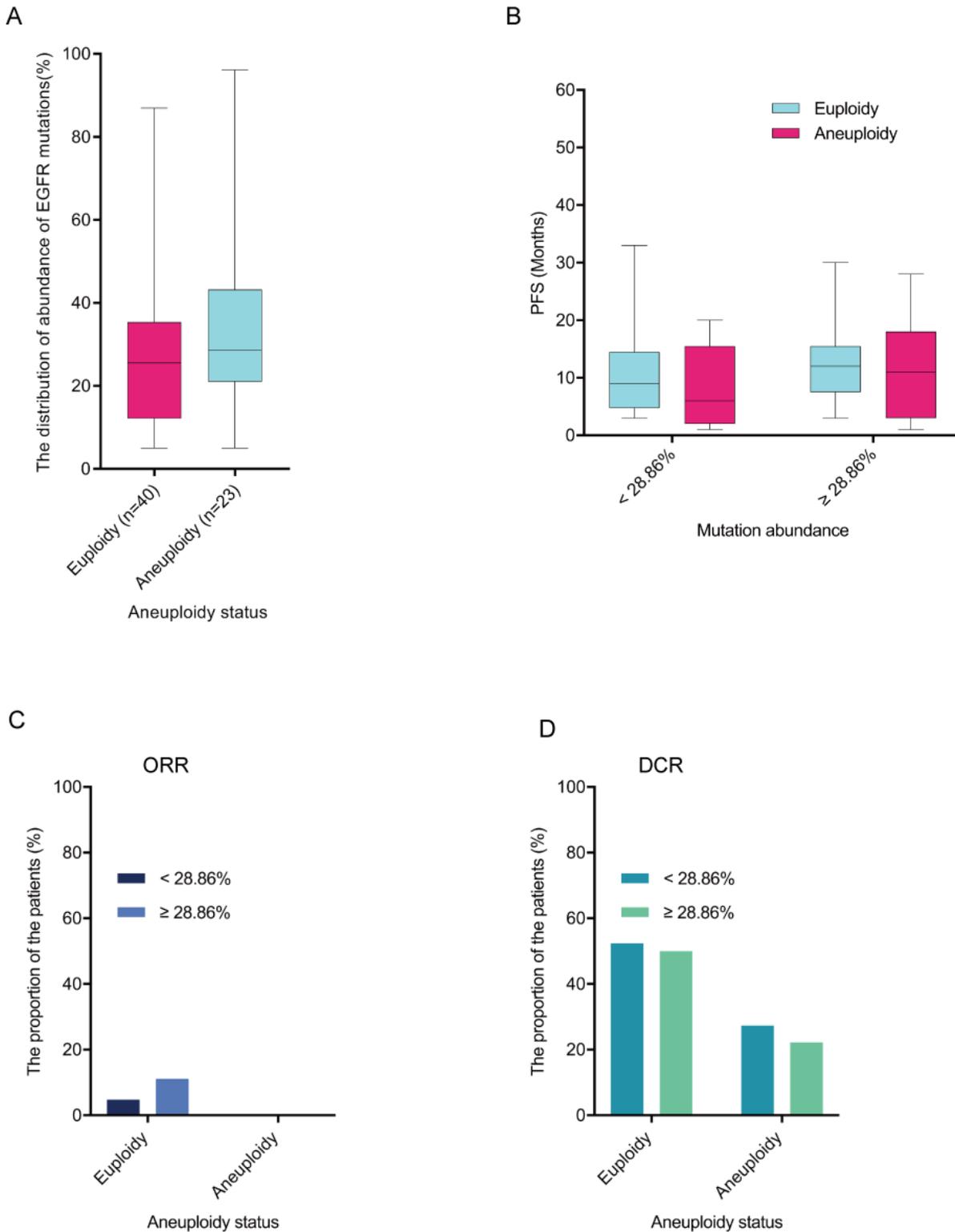
**Figure 1**

EGFR mutation abundance affect the clinical efficacy of patients with lung cancer. A. The district of PFS in 25% cutoff value mutation abundance; B. Kaplan-Meier curves for median PFS in high and low mutation abundance; C. The comparison of ORR between high and low mutation abundance; D. The comparison of DCR between high and low mutation abundance.



**Figure 2**

Aneuploidy status affect the clinical efficacy of patients with lung cancer. A. The district of PFS in aneuploidy status; B. Kaplan-Meier curves for median PFS in euploid and aneuploid patients; C. The comparison of ORR between euploid and aneuploid patients; D. The comparison of DCR between euploid and aneuploid patients.



**Figure 3**

The patient clinical efficacy in both EGFR mutation abundance and aneuploidy status. A. The proportion of aneuploidy status in the abundance of EGFR mutations; B. Two-factor analysis of the effects of EGFR mutation abundance and aneuploidy status for PFS; C. The comparison of ORR between EGFR mutation abundance and aneuploidy status; D. The comparison of DCR between EGFR mutation abundance and

aneuploidy status; E. Kaplan-Meier curves for median PFS in EGFR mutation abundance and aneuploidy status.

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementalTable1.docx](#)
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