Received: 26 November 2021 | Accepted: 4 April 2022

DOI: 10.1111/1759-7714.14432

ORIGINAL ARTICLE

WILEY

Clinical features and prognosis according to genomic mutations in primary and metastatic lesions of non-small-cell lung cancer

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Funding information
None.

Abstract

Non-small-cell lung cancer (NSCLC) is an important cause of cancer-related death worldwide. The distant metastasis heterogeneity of gene tumor mutations in tumors of NSCLC patients brings critical challenges for treatment. We sequenced the primary tumors and metastatic tissues of 48 NSCLC patients through 363 tumor-related gene panels to examine gene mutations in primary tumors and metastatic tissues, and screen candidate carcinogenic and metastatic-related driver mutations. The patient group included 21 patients in the metastatic group and 27 patients in the non-metastatic group. The patient's median age was 62 years and 54% (26/48) of patients were women. Approximately 75% (36/48) of patients were non-smokers. The mutation spectrum results showed that epidermal growth factor receptor (EGFR) gene mutation was the most frequent mutation (68.75%), followed by TP53 mutation (45.83%); 19del accounted for the largest proportion of EGFR mutations. Copy number variation (CNV) mutation spectrum results showed that EGFR amplification was more common in the metastatic group than the non-metastatic group. The mutant-allele tumor heterogeneity value of the metastatic group was higher than that of the non-metastatic group ($p = 0.013$). The progression-free survival of the metastatic group was significantly shorter than that in the non-metastatic group ($p = 0.041$). Single nucleotide variant difference analysis showed that the frequency of TP53 mutations was higher in the metastasis group. The number of subclonal mutations in the primary and metastatic lesions in the metastasis group was significantly different; the number of subclonal sites in metastatic lesions was higher than that in primary lesions. Our results suggested that the gene mutations of NSCLC in primary and metastatic lesions and identified specific mutations related to metastasis of NSCLC. Our research will help to clarify key differences between gene mutations between primary and metastatic NSCLC. These findings will help to provide new theoretical support for the future targeted therapy of metastatic NSCLC.

KEYWORDS
metastasis, mutation, non-small-cell lung cancer, tumor heterogeneity

INTRODUCTION

Lung cancer (LC) is the leading cause of cancer-related mortality and a serious threat to human health worldwide.[1] Non-small-cell lung cancer (NSCLC) accounts for approximately 80% of LCs and the 5-year survival rate of NSCLC is less than 15%.[2] Although surgical resection of NSCLC is the most effective treatment at the early stage, many patients have already developed distant metastases at the time of diagnosis and are not eligible for surgical treatment.[3] In

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addition, approximately 65% of patients with NSCLC experience recurrence and metastasis after surgery, and show a poor prognosis.[4] In the past few decades, chemotherapy and radiotherapy have become the main treatment strategy for perioperative or late palliative care of patients with NSCLC.[5,6] However, the efficacy of these treatments for patients with advanced NSCLC is unsatisfactory. Therefore, improving the early detection and treatment of NSCLC for patients with advanced NSCLC in the curable stage has important clinical significance.

With the development and progress of large-scale whole-genome sequencing, research and development of molecular targeted therapies, especially therapies targeting cancer driver mutations, have furthered the realization of personalized treatment of NSCLC.[7] Targeted therapy of mutated genes has become an important treatment strategy for NSCLC patients. Somatic mutation and genome rearrangement rates are both high in the development of NSCLC carcinogenesis. Previous studies have identified some NSCLC associated genes, including the genes encoding tumor protein p53 (TP53), epidermal growth factor receptor (EGFR), Kirsten rat sarcoma viral oncogene homolog (KRAS), and anaplastic lymphoma kinase (ALK). Drugs that target EGFR and ALK mutations have been shown to significantly improve the survival time of patients with NSCLC.[8–10] However, because of the heterogeneity of tumor biological characteristics and the mutual influence between genes, these targeted therapies have limitations. The metastasis and aggressiveness of tumors are the main reasons. Approximately 90% of cancer deaths are related to metastasis.[11] In metastatic NSCLC, the mutated gene of the metastatic tumor usually has new mutations.[12] The complexity and heterogeneity of gene mutations increase the difficulty of clinical treatment. Therefore, study of the gene expression of metastatic tumors has important clinical significance to improve the survival status of patients with NSCLC.

To further understand the molecular pathogenesis of metastatic NSCLC, we performed high-throughput sequencing on primary tumor and metastatic tumor tissue samples of patients with NSCLC. We explored the differences in gene mutations in the primary foci and the differences in genetic composition between the primary lesion and metastatic lesions in the metastatic group. Clarifying the relationship between genetic differences and clinical metastasis may lead to the identification of target genes and mechanisms related to metastasis, and provide support for the development of novel clinical treatments.

METHODS

Study population and collection of tumor specimens

This study included clinical specimens from 49 patients undergoing LC resection in hospital. All specimens were pathologically diagnosed as NSCLC after surgery. The patient inclusion criteria were as follows: (1) age 18–75 years; (2) histological or pathological diagnosis of NSCLC stages I to IIIB; (3) Eastern Cooperative Oncology Group (ECOG) performance status of 0–1; (4) compliance with Response Evaluation Criteria in Solid Tumors, version 1.1 (RECIST 1.1)[13]; and (5) no treatment before enrolment and agreement to the procedure. Exclusion criteria were as follows: (1) postoperative histopathological diagnosis was not NSCLC; (2) the patient had other active malignant neoplastic diseases; (3) the patient had evidence of serious or uncontrolled systemic disease, including uncontrolled hypertension and active hemorrhagic factors (these factors were assessed by the investigator as to whether they were causing the patient's reluctance to participate in the trial or the patient's compliance with the study's treatment regimen decreased) or active infectious diseases such as hepatitis B, hepatitis C, and human immunodeficiency virus (HIV) infection; and (4) history of previous interstitial lung disease (ILD), drug-induced ILD, radiation pneumonitis requiring hormone therapy, or evidence of any clinically active ILD.

The fresh specimens were placed in liquid nitrogen and transferred to the refrigerator for storage. We obtained 48 primary tumor samples, including 21 samples with metastases and 27 samples without metastases; one patient's primary lesion sample information was excluded if the experiment was failed. No patient had received any treatment that may impact the experiment results, such as chemotherapy or radiotherapy before surgery. Clinical characteristics such as age, sex, smoking status, type of surgery, recurrence, and outcome were collected from the clinical records. The study was approved by the ethics committee of the research institution. The personal information of all patients was confidential and all patients signed an informed consent form.

Targeted DNA sequencing and analysis

We conducted targeted next-generation sequencing (NGS) on 80 FFPE samples. Library preparations were performed using the Paragon Genomics manufacturer's protocol. In brief, 40 ng of human genomic DNA from FFPE tissue was used for each multiplex PCR reaction, and the library concentrations were measured. To compare the gene mutations of the primary and metastatic lesions from the same patients, exome sequencing was conducted for 363 tumor-associated genes by the NextSeq CN500 NGS platform. The 363 cancer driver gene profiles were selected from Catalogue of Somatic Mutations in Cancer,[14] The Cancer Genome Atlas,[15,16] and Oncomine.[17] The tumor-associated genes included oncogenes, tumor suppressor genes, and protein kinase family genes. The major mutations examined in this study included single nucleotide variants (SNVs), insertions or deletions (Indels), and copy number variations (CNVs). Paired-end reads (2 × 150 base pairs) were derived from the Amplicon libraries using NextSeq CN500. We used ANNOVAR and TransVar for annotation with the public variant databases.[18–20] Variants were filtered if the baseline population frequency ≥5%.

Statistical analysis

Statistical analysis was performed using SAS 9.4 statistical analysis software. The normality test was conducted by Shapiro–Wilk test. Variables conforming to the normal distribution are represented by mean ± SD, and categorical variables are represented by *n* (%). The comparison of categorical data between groups was performed by chi-square test or the Fisher exact probability method according to the conditions of use. The Kaplan–Meier method was used to map disease-free survival. $p < 0.05$ indicated statistical significance.

RESULTS

Analysis of baseline characteristics of primary tumors

Surgical specimens of primary lung adenocarcinoma were collected from 48 patients who underwent surgical resection (Table 1). Among the 48 patients, 21 patients were in the metastatic group and 27 patients were in the non-metastatic group. The median patient age was 62 years and 54% (26/48) of the patients were women. Approximately 75% (36/48) of the patients were non-smokers. All patients had no complications. In the metastatic group, 15 patients' tumor stage was III and six patients' tumor stage was II. There were 25 patients with tumor size ≤2 cm and 14 patients had pleural invasion. Carcinoembryonic antigen (CEA) positivity and intravascular cancer emboli were significantly correlated with metastases ($p = 0.0003$ and $p = 0.0044$, respectively) (Table 2).

The mutation spectrum (top 40 mutated genes) of the primary tumor of all samples is shown in Figure 1a. EGFR mutation was the most frequent mutation (68.75%), with 19del accounting for the largest proportion of EGFR mutations, and TP53 mutation was the second most frequent mutation (45.83%). Analysis of the CNV mutation spectrum (report gene) showed that EGFR amplification was more common in the CNVs of the metastatic group than the CNVs of the non-metastatic group. There was no statistically significant difference in CNVs between the two groups (Figure 1b). Analysis of the primary lesions between the two groups showed that only CNV was different between the two groups ($p = 0.049$); there was no statistically significant difference distribution in SNV/indel and fusion between the two groups (Figure 1c).

TABLE 1 Baseline characteristics of primary tumors

Adenocarcinoma sample information	The metastatic group ($n = 21$)	The non-metastatic group ($n = 27$)	*p* value
Age			
≤65	16	17	0.3660
>65	5	10	
Gender			
Female	11	15	1.000
Male	10	12	
Smoking			
Yes	6	6	0.7406
No	15	21	
Complication			
Yes	0	0	1.0000
No	21	27	
Pathological stage			
Stage I	0	27	<0.001
Stage II	6	0	
Stage III	15	0	
Stage IV	0	0	
Pathologic M			
M0	21	27	1.0000
M1	0	0	
Regional lymph nodes			
N0	0	27	<0.001
N1	6	0	
N2	15	0	
Primary tumor			
T0	0	0	0.1082
T1	13	21	
T2	8	6	
T3	0	0	
T4	0	0	

Comparisons of the metastatic group and the non-metastatic group

In the analysis of the primary tumor samples, the tumor mutation burden (TMB) of the metastatic group and the non-metastatic group was not statistically different (Figure 2a). The primary tumors in the metastasis group were more prone to T > G mutation and C > T mutation, while the primary tumors in the non-metastasis group were more likely to have C > G and C > A mutations (Figure 2b). Analysis of tumor heterogeneity showed that there were no significant differences in the clonal and subclonal mutations of the primary tumor in the metastatic group and the non-metastatic group, and the difference in the cancer cell fraction (CCF) value of the clonal/subclonal mutations was not significant (Figure 2c,d). The mutant-allele tumor heterogeneity (MATH) value of the metastatic group was higher than that of the non-metastatic group ($p = 0.013$, Figure 2e). This indicates that the tumor heterogeneity in the metastatic group is higher.

The prognostic analysis of the primary tumor in the metastatic group and the non-metastatic group is summarized in Figure 2f and the results revealed a significant difference in prognosis between the two groups. The progression-free survival (PFS) of patients in the metastatic

group was significantly shorter than that in the non-metastatic group ($p = 0.0041$). Analysis of SNVs between the two groups revealed that the frequency of TP53 mutations was higher in patients of the metastasis group. The frequency of TP53 mutations in patients ≤62 years of age was higher than that of patients older than 62 years of age. No differences in genes were found in smoking, gender, and other groups. The prognosis of patients with TP53 mutations was worse (Figure 2g).

TABLE 2 Analysis of clinical information of all samples of primary tumor

	The metastatic group ($n = 21$)	The non-metastatic group ($n = 27$)	*p* value
Tumor size			
0–2 cm (contains two)	11	14	1
2–3 cm	10	13	
CEA (carcinoembryonic antigen)			
Negative	5	21	0.00035
Positive	16	6	
Thoracic			
Yes	7	7	0.7502
No	14	20	
Vascular			
Yes	6	0	0.0044
No	15	27	

Analysis of the difference between primary and metastatic lesions in the metastatic group

The top 20 genes in the SNV mutation spectrum of the primary and metastatic lesions from the 21 patients in the metastasis group are shown in Figure 3a. The results showed that EGFR, TP53, and ALK genes were all frequently mutated. The CNV detection results of primary and metastatic lesions in the metastasis group revealed that EGFR was highly expressed in the primary lesions, and VEGFA, ERBB2, and MDM2 were only expressed in the primary lesions (Figure 3b). The overall TMB of the primary lesions was higher than that of the metastasis lesions, and there was no significant difference between the primary and metastatic lesions (Figure 3c). The primary lesions had a larger

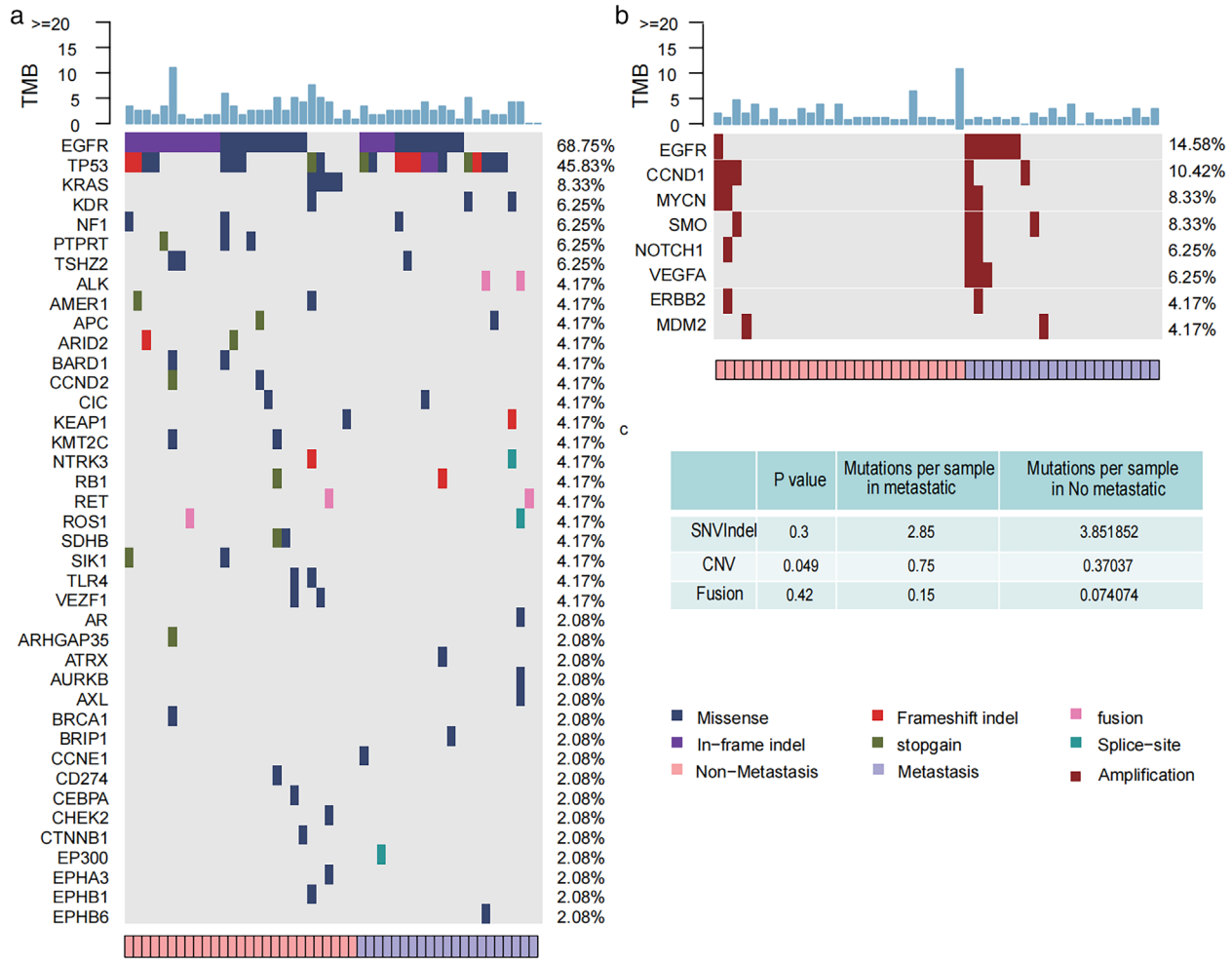

FIGURE 1 (a) Mutation spectrum of all samples from the primary lesion. (b) CNV mutation spectrum of all samples from the primary lesion. (c) Overall comparison results of CNV/SNV/fusion

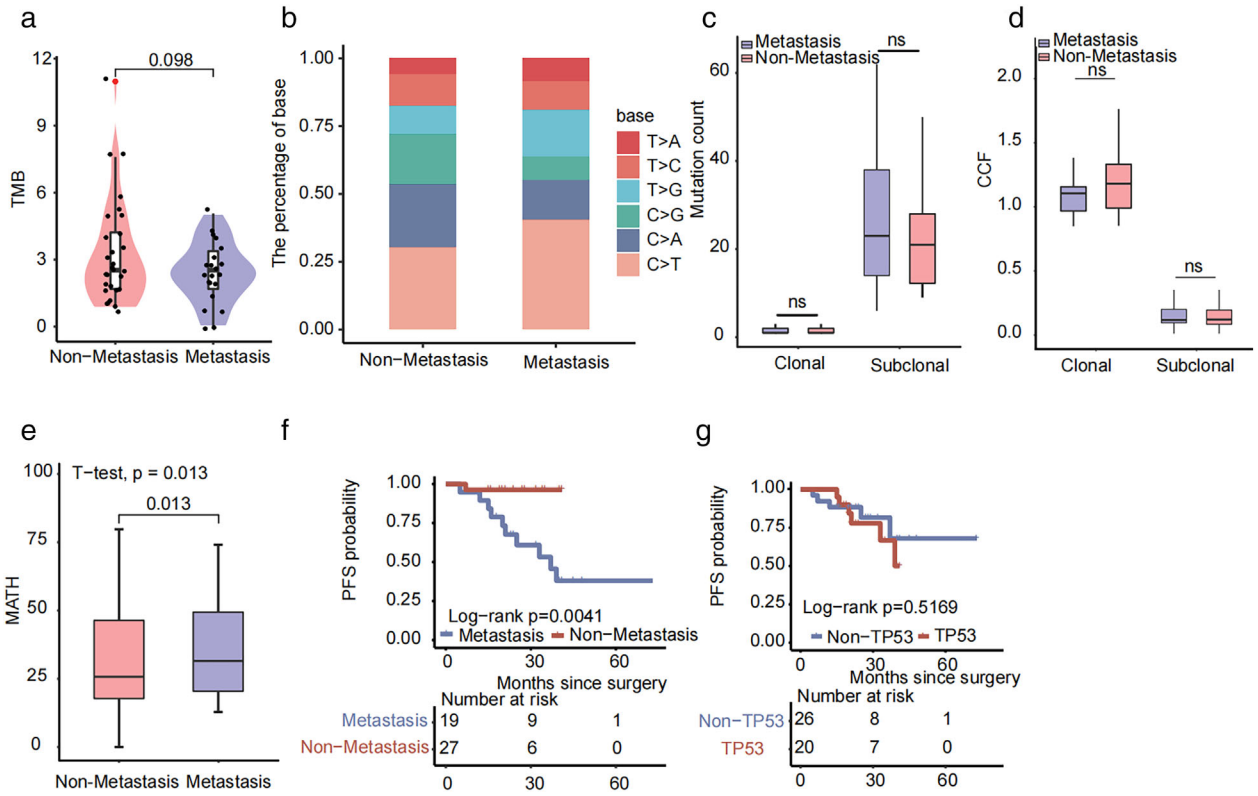

FIGURE 2 (a) Analysis of the difference of TMB of primary tumor in the metastatic and non-metastatic groups. (b) Analysis of the difference in base composition of primary lesions between the metastatic and non-metastatic groups. (c) Analysis of the difference in the number of clonal and subclonal mutation counts between the two groups. (d) Analysis of CCF difference between two groups of clonal and subclonal. (e) Analysis of the difference between the MATH value of the primary tumor in the metastatic group and the non-metastatic group. (f) Analysis of the difference in prognosis of primary tumors between the metastatic group and the non-metastatic group. (g) The prognostic correlation analysis of the differential gene TP53 between the metastatic group and the non-metastatic group

proportion of T > C mutations compared with the metastatic lesions, and the metastatic lesions had more C > T mutations than the primary lesions (Figure 3d). Comparing the difference in CCF values between the clonal/subclonal sites of the primary and metastatic lesions showed that in the subclonal sites, the CCF of the primary and metastatic sites were significantly different (Figure 3e). The number of subclonal mutations in the primary and metastatic lesions in the metastasis group was significantly different, and the number of subclonal sites in the metastatic lesions was higher than that in the primary lesions (Figure 3f). Analysis of tumor heterogeneity showed that the primary lesions in the metastasis group had a higher MATH value ($p < 0.01$) and the tumor heterogeneity in the primary lesions was greater (Figure 3g). As shown in Figure 3h, the less clonal mutations in the primary lesions was, the longer the PFS patients would get. The number of clonal and subclonal metastases had little effect on PFS.

DISCUSSION

NSCLC is an important cause of cancer-related death worldwide. According to China's 2015 Cancer Statistics, there were 4.29 million new cases of LC and 2.81 million cases of deaths from LC in 2015.[21] Approximately 80% of LC patients have NSCLC, and 60–70% of patients with NSCLC are in the middle and advanced stages at diagnosis and do not have the opportunity for surgery.[1] With the advancement of gene-targeted therapy, more cancer mutant genes have been discovered. Drugs targeting EGFR mutations and ALK translocations can improve patient treatment response and survival.[22–24] However, the heterogeneity of the mutant gene has brought great challenges for the treatment of NSCLC patients with distant metastasis.[25] In this study, we not only analyzed the mutated genes of primary NSCLC tumors but also conducted a comprehensive analysis of the tissues from metastatic tumors from small primary tumors. We identified several mutated genes and performed tumor heterogeneity analysis in the metastases. We revealed the relationship between the clinical prognosis of primary and metastatic lesions. We also conducted a comprehensive analysis of the mutated genes of metastases, an advantage of this study, which may have an impact on individualized gene therapy for patients with metastatic NSCLC in the future.

With the clinical application and promotion of NGS technology, TMB has gradually become well known.[26] TMB

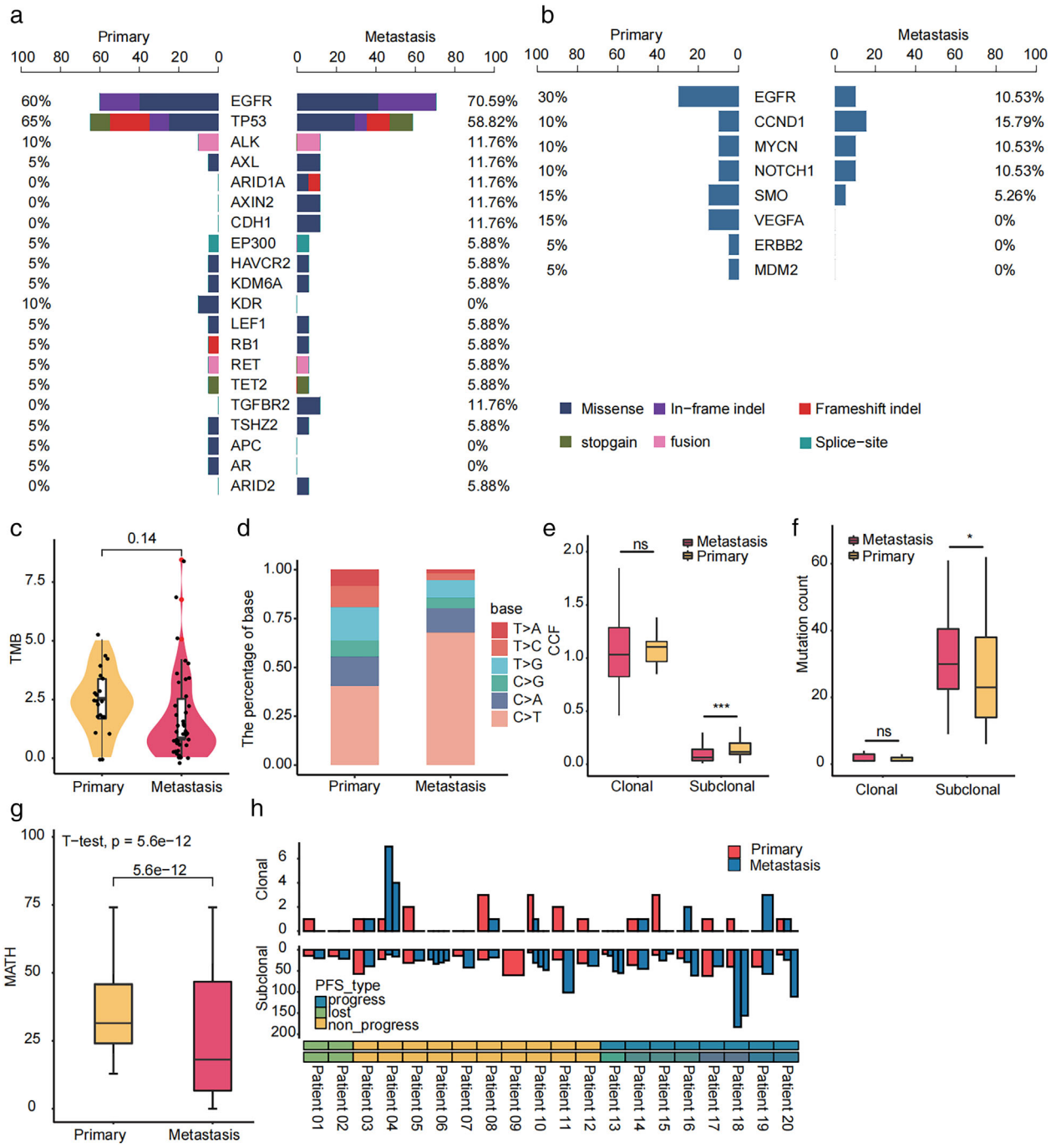

FIGURE 3 (a) SNV mutation bar chart of primary and metastatic lesions in the metastasis group (top 20). (b) CNV detection results of primary and metastatic lesions in the metastasis group. (c) Analysis of the difference of TMB between primary and metastatic lesions in the metastasis group. (d) Analysis of the difference in base composition between primary and metastatic lesions in the metastasis group. (e) Analysis of the difference in CCF value of the primary and metastatic lesions in the metastasis group. (f) Analysis of the difference in the number of clonal/subclonal between primary and metastatic lesions in the metastasis group. (g) Analysis of the difference in MATH value between primary and metastatic lesions in the metastasis group. (h) Analysis of the relationship between the cloning status and clinical PFS of the primary tumor and metastatic tumor samples of patients in the metastasis group

is an independent molecular marker for screening potential beneficiaries of immunotherapy. However, large-scale studies on the distribution of TMB in NSCLC and its associated factors has been lacking. In this study, we found that the overall TMB of the primary lesions was higher than that of the metastatic lesions. This suggests that NSCLC patients without metastasis can benefit from immunotherapy. Notably, the small number of patients included in this study and the different proportions of patients in various stages may influence the difference in TMB values between the groups. Therefore, the distribution trend of TMB values in patients should be studied on a larger population scale. Studies have

shown that the use of expression of the molecular marker Programmed death 1 ligand 1 (PD-L1) in combination with TMB can improve the predictive power of immunotherapy efficacy.[27,28] The KEYNOTE-158 is a landmark study with 1032 patients with refractory solid tumors from 10 cancer species that showed that pembrolizumab had an overall objective response rate (ORR) of 29% in patients with high TMB and only 6% in patients with low TMB.[29] On the basis of this result, pembrolizumab was approved by the FDA for use in patients with non-surgical or metastatic solid tumors with high TMB and disease progression after previous treatment. A retrospective study in 2018 analyzed the correlation between TMB and the efficacy of atezolizumab.[27] The study found significant improvements in ORR and PFS after treatment with atezolizumab in patients with advanced NSCLC with high TMB. Some studies confirmed that patients with first-line EGFR-TKI (Tyrosine Kinase Inhibitors) resistance can still benefit from immune checkpoint inhibitor (ICI) therapy, particularly those with T790M negative acquired mutations and L858R mutations.[30,31] Both mutation subtypes were relatively high TMB in EGFR mutations, so high TMB can guide the application of ICI in the EGFR mutant NSCLC. The combination of TMB and other biomarkers to guide clinical decision-making and personalized drug regimens will be critical in future research and clinical practice to maximize the clinical benefits of patients.

In this study, most of the LC patients were non-smokers and more than half of the patients were female. Treatment-affected factors include environment, living habits, and mutations. In a previous study, NGS was used for genomic testing of non-smokers with NSCLC and the results showed that non-smokers with NSCLC had a lower burden of somatic mutations.[32] A low number of non-activating mutations in KRAS was observed, which was associated with TKI resistance.[33] In addition, EGFR activating mutations and EML4-ALK fusions were relatively more frequent in non-smokers.[34] These mutations all enabled non-smokers to achieve longer survival after targeted therapy. Our results did not show an association between gene mutations and smoking. However, the mutation rates of EGFR and TP53 in primary tumors were higher. These results are similar to those of previous studies. A meta-analysis showed that non-smokers receiving first-line EGFR-TKI treatment achieved greater clinical benefits.[35] In addition, some studies have shown that smoking patients are relatively less exposed to drugs.[36,37] In this way, the efficacy of EGFR-TKIs is reduced. Therefore, based on the results of this study, we found and further confirmed that non-smokers have a higher risk of NSCLC, and the mutation rate of EGFR and TP53 is higher than the mutation rate of other genes. This provides a further clinical basis for patient-targeted therapy.

In this study, we identified the genetic mutations in primary and metastatic lesions. In the primary tumors, EGFR and TP53 mutations were the most common, and the 19del site accounted for the largest proportion of EGFR mutations. In the primary tumor samples, we did not find a difference in TMB between the metastatic group and the non-metastatic group. Tumor heterogeneity analysis revealed that the heterogeneity of the metastatic group was higher than that of the non-metastatic group. The PFS of patients in the metastatic group was significantly shorter than that in the non-metastatic group. SNV difference analysis showed that the frequency of TP53 mutation was higher in the metastatic group. Patients with metastatic tumors had high tumor heterogeneity and poor prognosis. We speculate that these mutations might have critical roles in tumor metastasis. EGFR and TP53 have been demonstrated to play critical roles in NSCLC metastasis in other studies. EGFR bound epidermal growth factor to produce a molecular effect that initiated the signaling pathway after structural changes.[38] Some studies have shown that EGFR and mitogen-activated protein kinase pathway (MAPK), phosphatidylinositol 3 kinase (PI3K), signal transducer and activator of transcription 3 pathway (STAT3), and signal transducer and activator of transcription 5 (STAT5) have critical roles in tumor metastasis.[39,40] The 19del site in the EGFR mutation in this study was also extensively detected in other studies. EGFR inhibitor drugs exhibit better treatment effects for patients with 19del site mutation.[41,42]

In the metastatic group, EGFR mutation was the most common mutation. Moreover, we found that EGFR is expressed at higher levels in primary lesions than metastatic lesions, and VEGFA, ERBB2, and MDM2 are only expressed in primary lesions. The number of TMB, heterogeneity, and subclonal mutations in primary lesions were higher than those in metastatic lesions. These results further indicate that EGFR mutations play an important role in tumor metastasis. Moreover, the PFS was related to the clonality in the primary lesions. The counts of clonal or subclonal amount in metastases might not be effective on PFS. The genetic mutations in the metastases were significantly different from those in the primary tumors. This may explain the poor effect of targeted drugs in the treatment of metastatic patients, and this finding has important clinical significance for clinical targeted therapy. This might also be related to the synergistic effect of assisting other drugs while metastatic patients were receiving targeted therapy. The effective treatment management of primary and metastatic lesions of patients with NSCLC requires further research.

Our research has some limitations. First, this study included a small sample size with only NSCLC patients in Asia, therefore selection bias is a possibility. Second, we did not perform genetic testing on matched normal tissues during testing and only tumor tissue and metastatic tumor tissue were evaluated. This reduces the detection accuracy. Third, we did not differentiate NSCLC patients by pathological classification.

CONCLUSIONS

Our results suggest that the mutations of NSCLC in primary and metastatic lesions, and identified specific mutations relate to the spread and metastasis of NSCLC. Our research

provides new insights into gene mutations in NSCLC and will help to clarify the key difference between gene mutations between primary and metastatic NSCLC. These findings will help to provide new theoretical support for the future targeted therapy of metastatic NSCLC.

CONFLICTS OF INTEREST

None.

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How to cite this article: Zhang W, Han W, Yu B, Zhao X, Lu G, Wu W, et al. Clinical features and prognosis according to genomic mutations in primary and metastatic lesions of non-small-cell lung cancer. Thorac Cancer. 2022;13(11):1642–50. https://doi.org/10.1111/1759-7714.14432