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ORIGINAL RESEARCH

High Tumor Mutation Burden and DNA Repair Gene Mutations are Associated with Primary Resistance to Crizotinib in *ALK*-Rearranged Lung Cancer

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Background: About 20% of patients with *ALK*-rearranged non-small cell lung cancer (NSCLC) develop acquired resistance to tyrosine kinase inhibitor (TKI) during the first 6 months. This study aimed to examine the molecular mechanisms of early TKI resistance and prognosis in *ALK*-rearranged NSCLC.

Methods: Ten patients with *ALK*-rearranged NSCLC were included: five who developed rapid resistance to crizotinib (progression-free survival (PFS) ≤3 months) and five who exhibited a good response to crizotinib (PFS ≥36 months). The tumor specimens were subjected to whole-exome sequencing (WES). The validation cohort included 19 patients with *ALK*-rearranged NSCLC who received crizotinib; targeted sequencing of 43 selected genes was performed. The effect of the *TP53* G245S mutation on crizotinib sensitivity was tested in H3122 cells.

Results: Mutations in DNA repair-associated genes were identified in primary resistance to crizotinib. Patients with a poor response to crizotinib harbored a greater burden of somatic mutations than those with a good response [median somatic mutations, 136 (range, 72–180) vs 31 (range, 10–48)]. Compared with the patients carrying wild-type *TP53* or *TP53* exon 3 deletion, 29 patients with *TP53* G245S mutation showed a shorter survival time ($P < 0.05$), with a median PFS of 3 (95% CI: 1.9–4.1) months and a median overall survival of 7 (95% CI: 3.4–10.5) months. *TP53* mutation promoted the proliferation of *EML4-ALK*-rearranged H3122 cells by approximately 3 folds ($P < 0.001$). H3122 cells with *TP53* mutant were more sensitive to crizotinib compared with control cells.

Conclusion: A higher mutation burden and mutations in DNA repair gene, including *TP53*, were potentially associated with primary resistance to crizotinib in *ALK*-rearranged NSCLC. An immune-checkpoint inhibition strategy could be examined, which might overcome primary resistance to crizotinib in *ALK*-rearranged NSCLC.

Keywords: *ALK*, non-small cell lung cancer, TKI, resistance, prognosis

Introduction

Anaplastic lymphoma kinase (*ALK*)-fusion genes represent a small but important part of oncogenic driver mutations in NSCLC, accounting for approximately 3%-7% of all cases worldwide.[1,2] Small molecule tyrosine kinase inhibitors (TKIs) are the standard therapy for *ALK*-rearranged NSCLC. Crizotinib, a first-generation TKI, is the most widely used targeted drug to treat *ALK*-rearranged NSCLC. Patients receiving first-line crizotinib can survive for more than 4 years,[3]

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even 5–7 years after sequential treatment with next-generation TKIs.[4,5] Nevertheless, based on the ALEX study,[6] about 20% of patients with *ALK*-rearranged NSCLC developed acquired resistance to first-generation TKI (crizotinib) or second-generation TKI (alectinib) during the first 6 months of treatment and survived no more than 12 months. The ALEX study[6] suggested that patients with early resistance to TKIs had a poor prognosis.

The resistance to first- and second-generation TKIs is mediated by a variety of mechanisms, including secondary *ALK* mutations in 20–30% of cases, *ALK*-rearranged gene amplification in 10% of cases, and activation of alternative signaling pathways and wild-type resistance in other cases.[7–9] The three main *ALK* resistance mutations are L1196M, C1156Y, and G1202R, and not all *ALK* TKIs are active against them. In addition, other mutations may also lead to resistance to specific TKIs.[10,11] Crizotinib resistance can be overcome in many cases by using next-generation TKIs, and the overall response rate (ORR) is as high as 70%–80%.[12,13] Nevertheless, patients with early acquired resistance to crizotinib often fail to respond to next-generation TKIs and display a short survival. *EML4-ALK* variant 3a/b and high levels of p-c-Kit might be associated with TKI resistance and short survival,[14,15] but little is known about the molecular mechanism of early TKI resistance or poor prognosis in patients with *ALK* fusion NSCLC.

Whole-exome sequencing (WES) is a powerful tool for identifying genetic variants related to cancer and treatment outcomes. It can provide biologically relevant information about genetic predisposition to cancer occurrence,[16,17] development of metastasis,[18] resistance to treatments,[19] and the best treatment options.[20] On the other hand, WES has the disadvantage of revealing many genetic variants with unknown biological meanings. It is necessary to process a large amount of data to determine the pathogenicity of specific genetic variants, and a lot of effort has been made to achieve this goal.[20,21] One study used WES to examine genetic variants associated with the inflammatory microenvironment of NSCLC.[22]

Therefore, this study aimed to gain a comprehensive understanding of the possible molecular mechanisms of early TKI resistance and prognosis in *ALK*-rearranged NSCLC by using WES in lung adenocarcinoma specimens with early failure (≤3 months) to crizotinib vs very good response (≥36 months) to crizotinib. In addition, the identified genetic variants were validated by targeted sequencing in specimens from other NSCLC patients who received crizotinib.

Materials and Methods

Patients and Specimens

Patients with *ALK*-rearranged NSCLC who received crizotinib as first-line treatment were included. Nineteen validation patients came from a cohort of 87 *ALK*-positive NSCLC patients who received crizotinib. All patients were admitted to the First Affiliated Hospital of Guangzhou Medical University from August 2014 to February 2018 and were pathologically confirmed with stage IV NSCLC. The study was approved by the ethics committee of the First Affiliated Hospital of Guangzhou Medical University and conducted in accordance with the Declaration of Helsinki.

HE-stained sections from each patient specimen were subjected to an independent pathology review to confirm that the tumor specimen was histologically consistent with NSCLC (>70% tumor cells). *ALK* rearrangement was determined using VENTANA immunohistochemical system (Roche Diagnostics, Basel, Switzerland) or PCR (Amoy Diagnostics Company, Haicang, China). WES was performed on specimens from five patients with very early failure (≤3 months) to crizotinib and five patients with a very good response (≥36 months) to crizotinib. Probe capture sequencing was conducted on specimens from 19 validation patients who received crizotinib.

The progression-free time (PFS) was measured from the first treatment until progression or death. The overall survival (OS) was measured from the first treatment until death.

DNA Extraction and Next-Generation Sequencing

DNA was extracted from paraffin-embedded lung tissues using the QIAamp DNA Kit (Qiagen, Venlo, The Netherlands). The qualified genomic DNA samples were randomly fragmented using the Covaris technology, and the size of the library fragments was mainly distributed between 200 and 300 bp. The extracted DNA was amplified and purified. Genomic DNA was captured on the IDT xGen Lockdown probe for WES (Integrated DNA Technologies, Inc., Coralville, IA, USA) or IDT Individually Synthesized Panel for target region sequencing, according to the manufacturer's instructions. Then, high-throughput sequencing of the library was performed

using the Illumina Novaseq 6000 platform (Illumina, Inc., San Diego, CA, USA).

BWA (version 0.7.17-r1188) was used to align the short sequence reads to the hg19 human reference genome (NCBI build 37). The generated .sam file was converted to the .bam format using the SAMtools software (version 1.7). The raw .bam file was deduped using fgbio (version 0.12) and a unique molecular barcode sequence. After obtaining the deduped .bam file, fgbio was used to call the genetic variants from the .bam file with the recommended parameters.

The tumor mutational burden (TMB) was calculated as previously reported.[23] The signaling pathways of the variants were analyzed using the KEGG database. The predicted protein functions were subjected to the online STRING database analysis (https://string-db.org/).

Cell Culture and P53 G245S Transfection

The lung cancer cell line H3122 (*EML4-ALK* fusion) was obtained from Dana–Farber Cancer Institute (Boston, MA), which was cultured in RPMI-1640 medium supplemented with 10% FBS and kanamycin/penicillin. Crizotinib was purchased from Selleck (Houston, TX, USA) and dissolved in DMSO. The wild-type *TP53* fragment was amplified from normal lung tissue using the forward primer 5′-TCT AGA GCC ACC ATG GAG GAG CCG CAG TCA GAT CC-3′ and reverse primer 5′-GGA TCC TCA CTT ATC GTC GTC ATC CTT GTA ATC GTC TGA GTC AGG CCC TTC TGT C-3′. The fragment was ligated into pGEM-T easy (Promega, Madison, WI, USA) to generate pGEM-T-*TP53*. The *TP53* mutant p53 G245S was generated by site-directed mutagenesis (Agilent Technologies, Santa Clara, CA, USA). PCR was performed with pGEM-T-*TP53* as a template and by using the mutagenic primers: 5′-Cct ccg gtt cat gct gcc cat gca gga ac-3′ and 5′-gtt cct gca tgg gca gca tga acc gga gg-3′. The purified fragments of WT and mutant *TP53* were inserted into the lentiviral expression vector pCDH-CMV-MCS-EF1-GFP-Puro (System Biosciences, Palo Alto, CA, USA). The lentiviral-based expression vector and the packaging vectors psPAX2 and pMD2.G were transiently transfected into 293T human embryonic kidney cells using Lipofectamine 2000 (Life Technologies Co., Grand Island, NY, USA) (Supplemental Methods). The lentiviruses were transduced into H3122 cells in the presence of polybrene (8 μg/mL).

MTT Assay

Cell proliferation was determined using the MTT assay. Cells seeded in 96-well plates (3×10^3 cells/well) were treated with various concentrations of crizotinib. After 48 h, the cells were incubated with MTT (1 mg/mL) for 3 h. The absorbance was determined at 570 nm with the cell imaging multi-mode microplate reader (Cytation 3, Bio-Tek, Winooski, VT, USA).

Statistical Analysis

Continuous data were analyzed using the Mann–Whitney *U*-test. Fisher's exact test or chi-square test was used to compare the categorical data between the two groups. The Kaplan–Meier method was used to analyze the PFS and OS. Statistical analyses were carried out using SPSS 22.0 (IBM Corp., Armonk, NY, USA). Statistical significance was defined as a two-tailed P-value <0.05.

Results

Characteristics of the Patients

Eighty-seven NSCLC patients with *ALK* rearrangement who received crizotinib were included. In order to investigate the molecular mechanism underlying the primary or rapid resistance to crizotinib, ten *ALK*-rearranged NSCLC patients were selected based on their response to crizotinib. Five patients showed an extremely poor response, while the other five patients exhibited a good response to crizotinib. As shown in Table S1, the five poor responders (median PFS 2±1 months) had a dramatically shorter PFS than those with a good response (median PFS 36±12.8 months). There were no significant differences in age, sex, and other clinical characteristics between the two groups. Only 19 NSCLC patients had complete follow-up data and qualified samples for the validation study. For the validation cohort of 19 NSCLC patients with *ALK* rearrangement, the median PFS and OS were 15 (range, 1–44) and 19 (range, 2–44) months, respectively (Table 1).

Identification of Somatic Genetic Alterations Involved in Primary Resistance to Crizotinib by WES

WES was performed on the tumor specimens from 10 NSCLC patients with a good or poor response to crizotinib. The whole-exome was sequenced to a mean depth of 828×, covering approximately 0.6–0.8 million small indels and 3.9–4.1 million single nucleotide variants (SNVs). The specimens harbored an average of 264 somatic mutations,

Table 1 Clinical Characteristics of Patients in the Validation Cohort (n=19)

Characteristics	
Age (m±SD), years	56±14
Sex, n Female Male	n=19 8 11
Pathology, n Adenocarcinoma Adenosquamous carcinoma	n=19 18 1
PFS (m±SD), n PFS≤ 6 (months) 6<PFS≤18 (months) PFS>18 (months)	 4±1.9, n=4 14±4.7, n=8 26±4.8, n=7

which were differently distributed between the good and poor response groups. The number of somatic coding mutations was higher in the poor response group ($P < 0.001$). The median number of co-mutations was 31 (range, 10 to 48) in the good response group and 136 (range, 72 to 180) in the poor response group (Figure 1). The poor response group had a higher TMB than the good response group (median TMB, 18 vs 8, P=0.002) (Table S1). Hence, NSCLC that responded poorly to crizotinib harbored more mutations than NSCLC with a good response to the drug.

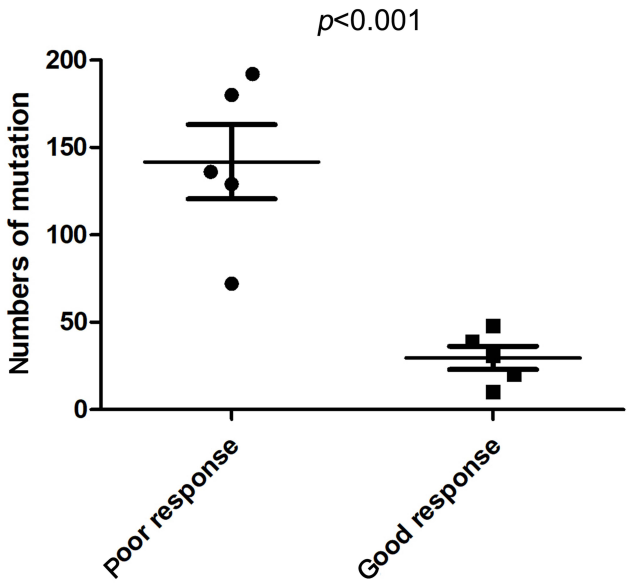

Figure 1 The number of co-mutations in groups with a poor or good response to crizotinib.

Some genes that were frequently mutated were identified. According to the KEGG database, the affected pathways included mitochondrial apoptosis, vascular endothelial growth factor (VEGF) angiogenesis, DNA repair, and platinum resistance (Figure S1). DNA mismatch repair-related gene variations such as *TP53*, MLH1, MSH2, and XPA were associated with survival or drug responsiveness. Interestingly, the results of the pathway analysis were in accordance with the functional protein analysis in the STRING database (Figure S2). It suggested that these genetic variations in patients with *ALK*-positive NSCLC were involved in different responses to crizotinib treatment or survival, and *TP53* was the key player in these variations.

Next-Generation Sequencing for Validation

In order to validate the gene mutations identified by WES, 43 frequently mutated genes associated with tumor growth, metastasis, and drug therapy (Table S2) were selected for hybrid-recapture-based targeted sequencing in a cohort of 19 NSCLC patients with *ALK*-rearrangement. A total of 774 genomic variations could be matched to the crizotinib responses and were located within regions of DNA repair, mitochondrial apoptosis, and tumor angiogenesis-target genes. Among these variations, 20 were located in the coding regions of 18 genes, including SNV, frameshift deletion, and stopgain, and were identified in 19 patients treated with crizotinib (Figure 2). The *TP53* exon 3 G245S mutation and frameshift deletion were among them.

TP53 Mutation and Survival

Compared with patients with wild type *TP53* or exon 3 deletion, 29 patients carrying the *TP53* G245S mutation showed a shorter survival time after crizotinib treatment ($P < 0.05$), with a median PFS of 3 (95% CI: 1.9–4.1) months (Figure 3A), and a median OS of 7 (95% CI:3.4–10.5) months (Figure 3B). None of these patients survived more than 12 months. There was no significant difference in PFS or OS between the *TP53* exon 3 deletion and wild-type group.

Functional Analysis of *TP53* G245S Mutation in Lung Cancer Cell

In order to determine the effect of *TP53* mutation on the response of *EML4-ALK* rearranged H3122 cells to crizotinib, cells stably expressing wild type or mutant *TP53* (Figure 4A) were established. The *TP53* mutation promoted

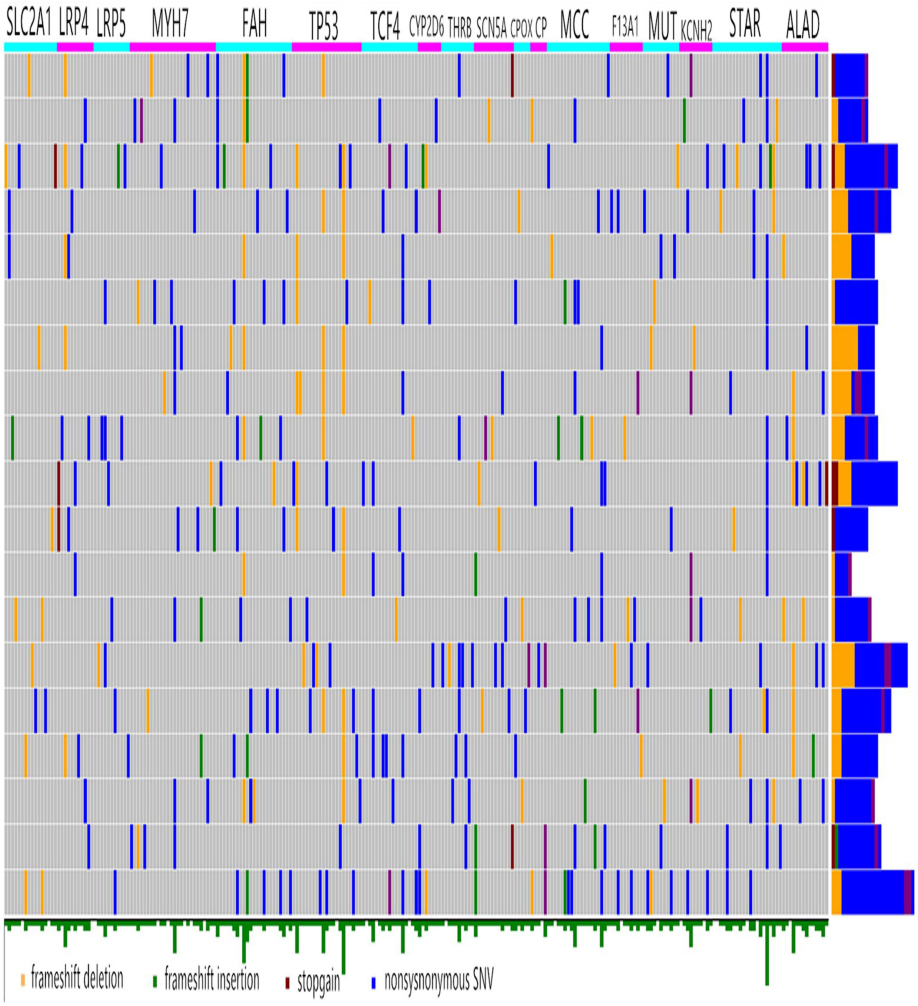

Figure 2 Next-generation sequencing (NGS) was performed on an individually synthesized panel containing 43 target gene regions in 19 validation patients. A total of 774 genomic variations could be matched to the crizotinib responses and were located within regions of DNA repair, mitochondrial apoptosis, and tumor angiogenesis target genes.

the proliferation of H3122 cells by approximately 3 folds (Figure 4B) ($P < 0.001$). When the cells were treated with crizotinib, the *TP53* mutant cells were more sensitive to crizotinib compared with the control cells (Figure 4C).

Discussion

About 20% of patients with *ALK*-rearranged NSCLC develop rapid TKI primary resistance during the first 6 months of treatment.[6] This study aimed to examine the molecular mechanisms of early TKI resistance and prognosis in *ALK*-rearranged NSCLC. The results suggested that high TMB and mutations in DNA repair genes (including *TP53* G245S) in *ALK*-positive NSCLC conferred rapid resistance to crizotinib.

To date, the mechanisms of primary resistance to *ALK*-TKIs have mainly focused on secondary mutations in the *ALK* gene. In this study, we observed that a subset of patients had an extremely poor response to crizotinib (PFS <3 months) with poor survival (no longer than 6 months) compared with the literature.[2–6] Although they did not receive subsequent next-generation TKIs, their survival rate was worse than patients who only received crizotinib followed by chemotherapy, with a median survival of 20 months.[3] In the ALEX study, among patients who received crizotinib and alectinib, patients with early resistance to TKI had shorter survival (no longer than 12 months).[6] Therefore, we hypothesized that patients with early resistance to ALK-TKI had a poor prognosis, and some cases might be caused by mutations in genes other than *ALK*.

In the WES analysis, 264 somatic coding mutations were identified. The results showed that tumors that

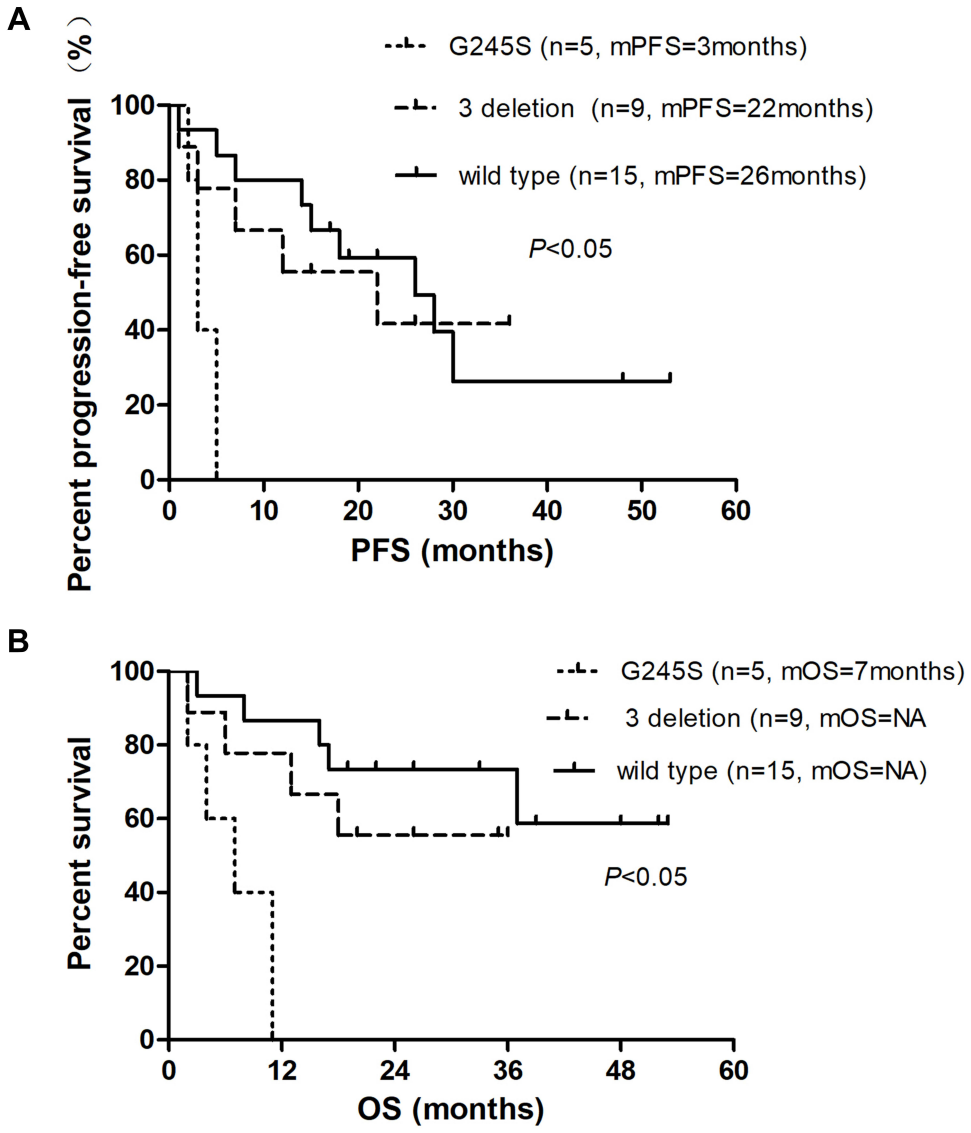

Figure 3 Survival analysis. The Kaplan–Meier curve showed the survival of three subgroups of NSCLC patients: patients with wild-type *TP53*, *TP53* G245S mutation, and *TP53* exon 3 deletion. (**A**) Progression-free survival (PFS) curve. (**B**) Overall survival (OS) curve. The statistical difference was shown on the graph.

responded poorly to crizotinib exhibited a higher number of somatic mutations and higher TMB compared with tumors that responded well. In many cancer types, higher TMB was associated with poorer survival, in contrast to ICI-treated patients in whom higher TMB was associated with longer survival, reported by Valero et al.[24] Similar findings have previously been shown in EGFR-mutant lung cancer. For instance, in TKI treatment, a higher number of concurrent driver gene mutations in patients with EGFR-mutant NSCLC was reported to be associated with poor PFS.[25] As a novel biomarker for immune checkpoint inhibitors, TMB is lower in EGFR-mutant or *ALK*-rearranged NSCLC. Using the Cancer Genome Atlas (TCGA) database, TMB has also been identified as a negative prognostic biomarker for OS in EGFR-mutated patients, especially those with P53 mutations.[26]

We selected 43 genes covering 774 genomic variations for validation. Those genes were selected because of their known involvement in drug resistance, tumor prognosis, mitochondrial apoptosis, VEGF angiogenesis, DNA repair, and platinum-resistance pathway.[27–31] It is worthwhile to note that *TP53* mutations are often associated with high TMB.[26] DNA repair-related gene variations, including MLH1, MSH2, and XPA, are associated with chemotherapy resistance. As previously reported, variations in these genes are important predictors of superior response to

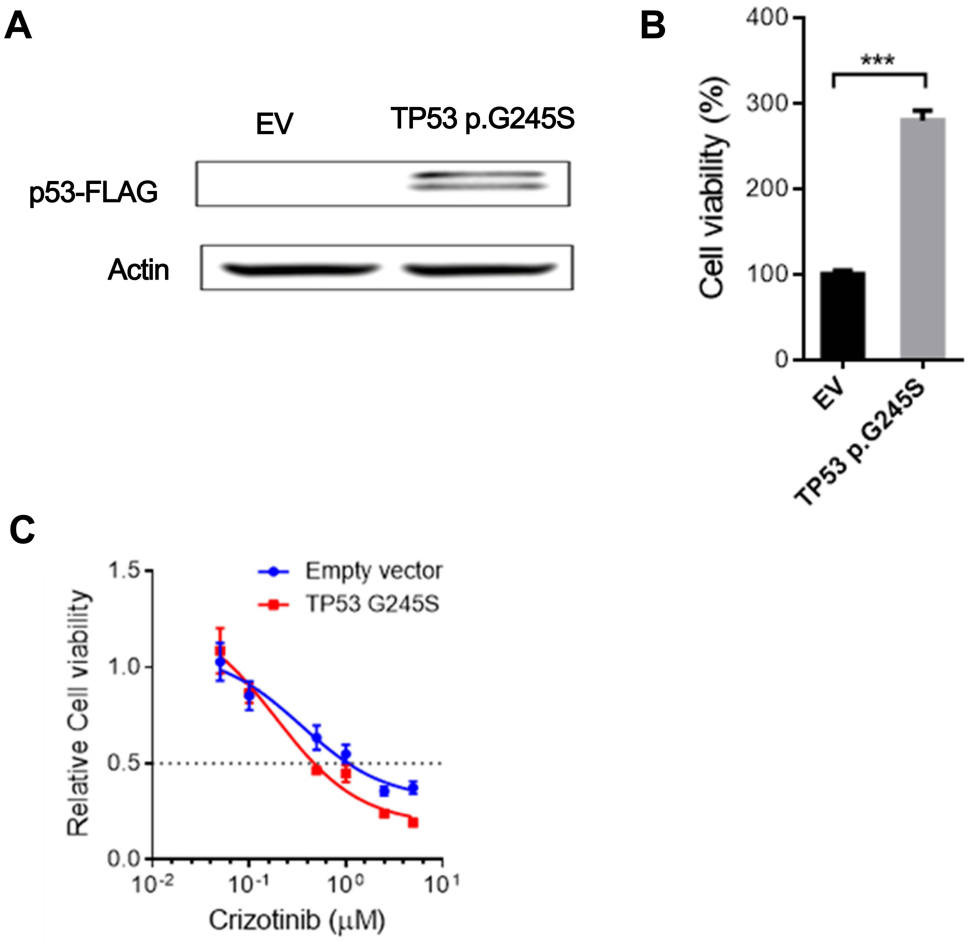

Figure 4 The effect of *TP53* G245S mutation on the response of H3122 cells to crizotinib. Statistical significance was determined by unpaired t-tests, ***$P<0.001$. (**A**) Western blot of p53 expression in H3122 cells stably expressing wild type TP53 (control, EV) or TP53 G245S mutant. (**B**) Cell viability assay in control and mutant H3122 cells. (**C**) Sensitivity of control and mutant H3122 cells to crizotinib.

immune checkpoint inhibitors.[30–33] It is plausible that *TP53* or DNA repair-related mutations and high TMB are associated with the early resistance to crizotinib or poor prognosis. The results suggested that the subset of those patients might benefit from immune checkpoint inhibitors combined with TKI, which warranted further investigation.

In the validation analysis, the *TP53* G245S mutation indicated extremely poor PFS and OS after crizotinib treatment. It was still not clear whether the *TP53* G245S mutation affected TKI sensitivity or prognosis, or both. In this context, we sought to investigate the function of the *TP53* G245S mutation in *EML4-ALK* fusion lung cancer cells. The results demonstrated that the *TP53* G245S mutation promoted the proliferation of H3122 cells but did not show resistance to crizotinib compared with controls. Those results indicated that early TKI resistance is probably a complex event involving intrinsic and extrinsic factors. Only *TP53* G245S is not enough to cause TKI resistance. We need to perform more research on DNA repair deficiency and *TP53* mutations in TKI-resistant tumors. The *TP53* G245S mutant has been associated with a poor prognosis in colon cancer.[34] This mutation can cause decreased levels of the 53BP1 protein and destabilize several structural regions of the protein that are crucial for DNA binding.[35,36]

Conclusions

Although the sample size was small and the patients came from a single hospital, this study suggests that a high mutation burden and mutations in DNA repair genes, including TP53, might be associated with primary resistance to crizotinib in ALK-rearranged NSCLC, leading to poor survival outcomes. Further prospective clinical studies are needed to confirm the role of TP53 or DNR repair-related gene variations in responses to ALK-TKI treatment or an immune-checkpoint inhibition strategy for ALK-rearranged NSCLC.

Data Sharing Statement

The raw data supporting the conclusions of this article will be made available by the authors without undue reservation.

Ethics and Consent Statement

The studies involving human participants were reviewed and approved by the Medical Ethics Committee in the First Affiliated Hospital of Guangzhou Medical University and carried out in accordance with the Declaration of Helsinki. The patients/participants provided their written informed consent to participate in this study.

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Author Contributions

All authors contributed to data analysis, drafting or revising the article, have agreed on the journal to which the article will be submitted, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

Disclosure

The authors declare no conflict of interest.

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