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Clinical significance of tumor mutation burden and DNA damage repair in advanced stage non-small cell lung cancer patients

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Abstract. – OBJECTIVE: This study aimed to investigate the impact of tumor mutational burden (TMB) and DNA damage repair (DDR) gene alteration on overall survival (OS) in advanced non-small cell lung cancer (NSCLC) patients.

PATIENTS AND METHODS: A DNA library of cancer cells from 67 NSCLC patients in stages III-IV was constructed for next-generation sequencing (NGS). Geneseeq422 probes were used for hybridization enrichment. The target-enriched library was sequenced on HiSeqNGS platforms, and we analyzed the relevant signaling pathways. Then, we correlated the OS of the patients with TMB and DDR mutations.

RESULTS: Many significant alterations were found, including in the *EGFR, p53, KRAS, RB1, ERBB2, NF1, DNMT3A, ALK, MYC, PIK-3CA, ROS1, BRAF, ARID1A, PTEN, CDKN2A,* and *FGF19* genes. We also identified many mutations in the genes relevant to the DDR pathway. Interestingly, we found that the TMB of patients with DDR gene mutations was dramatically higher than that in the DDR wild-type (WT). Univariable analysis showed that *DNMT3A*, *RB1*, DDR pathway-related gene mutations, and TMB were critical factors for the effects on OS. Multivariable analysis confirmed that *DNMT3A* and mutations in the DDR pathway-related genes were important for predicting OS.

CONCLUSIONS: Multiple mutations in the genes of the DDR pathway caused higher TMB levels, which resulted in longer OS. By contrast, OS was significantly longer in patients with non-DNMT3A mutations than in those with *DNMT3A* variants. *DNMT3A* alteration in NSCLC patients led to poor outcomes.

Key Words:
DNA damage and repair, DNMT3A, Non-small cell lung cancer, Tumor mutation burden, Overall survival.

Introduction

Worldwide, lung cancer is the leading cause of cancer-related deaths in men and the second highest cause of cancer-related mortality in women. In China, the incidence and the fatality rate of lung cancer are ranked first among all types of cancer[1]. Lung cancer types include small-cell lung carcinoma (SCLC) and non-small cell lung carcinoma (NSCLC). NSCLC accounts for 85% of all lung cancer cases. Lung cancer treatment includes surgery, chemotherapy, and radiotherapy. Recently, therapies targeting the epidermal growth factor receptor (*EGFR*)[2] or the anaplastic lymphoma kinase (*ALK*)[3,4], and immunotherapy have greatly improved patients' outcome. Risk factors for lung cancer development include smoking, air pollution, genetics, and asbestos inhalation[5,6]. Similar to other cancers, lung cancer is associated with many gene mutations, including those in *K-ras, EGFR, LKB1, PIK3CA*, and *BRAF*[7]. These gene alterations are both causes of carcinogenesis and therapeutic targets. Recently, in addition to the development of many drugs targeting the *EGFR* and *ALK* genes[8], immunotherapy for lung cancer has also shown great progress[9,10]. However, the individual responses to immunotherapy are diverse.

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Next-generation sequencing (NGS) has high sensitivity and accuracy for detecting gene mutations. It can detect a large number of gene alterations in a short time[11]. In the past decades, NGS has been widely applied for the diagnosis, treatment, drug-resistance exploration, and outcome decision of various cancers. Bordi et al[12] reported that NGS was used for monitoring resistance mechanisms of advanced T790M-positive NSCLC. Pepe et al[13] reported that *EGFR*, *KRAS*, *NRAS*, *BRAF*, *c-KIT,* and *PDGFRα* mutations detected using NGS were excellent biomarkers for the assessment of the treatment.

Mutations in DNA damage repair (DDR) genes occur frequently in both germline and somatic cells[14,15]. If DNA replication defects are not repaired promptly, then, they can lead to elevated rates of somatic mutations. One study indicated that alterations in DDR-relevant genes in cancer patients were closely associated with the outcome of the disease[16]. Tumor mutational burden (TMB) is the total number of somatic mutations in the whole genome. Cancer patients with a higher TMB have higher neoantigen loads, useful for immunotherapy[17]. Mei et al[18] indicated that high TMBs were associated with markedly higher lymphocytic infiltrates. Therefore, higher TMB rates in cancer patients translate into a good response to immunotherapy[19,20]. TMB rates also correlated with the outcome of cancer patients. Alterations in DDR pathway genes are closely associated with the response to immunotherapy and the long-term outcomes in cancer patients.

In this study, we performed a NGS study of tumor samples collected from 67 advanced stage (stages III-IV) NSCLC patients and we analyzed the patterns of somatic gene mutations and alterations in DDR pathway genes.

Patients and Methods

Patients and Samples

We collected 67 tumor samples from stages III-IV NSCLC patients treated in our department between January 2016 and February 2019. Cancer tissue samples were fixed in 10% formalin and embedded in paraffin (FFPE) after biopsy. Genomic DNA from patients' peripheral blood was used as a control for detecting somatic mutations. The clinical characteristics of the 67 NSCLC patients are shown in Table I. All sequencing data were obtained from the initial biopsy samples. This study protocol was approved by our Hospital Ethical Committee. All participants had given informed consent.

Table I. Clinical profile of the 67 patients with non-small cell lung cancer (NSCLC).

Characteristics	Category	Number
Age, median, (range)		65 (38-80)
Sex, N, (%)	M	45 (0.67)
	F	22 (0.33)
Smoking history, N, (%)	Yes	25 (0.37)
	No	42 (0.63)
Histology type at initial diagnosis, N, (%)	Adenocarcinoma	60 (0.90)
	Squamous	6 (0.09)
	Adenosquamous	1 (0.01)
Surgical history, N, (%)	Yes	18 (0.27)
	No	49 (0.73)
Clinical stage at initial diagnosis, N, (%)	IIIa	7 (0.10)
	IIIb	6 (0.09)
	IV	53 (0.79)
	Unknown	1 (0.02)
History of treatment, N, (%)	Treatment-naive	22 (0.33)
	First-line	10 (0.15)
	Second-line	4 (0.06)
	Third-line and above	7 (0.10)
	Unknown	24 (0.36)
History of treatment with TKI, N, (%)	Yes	11 (0.16)
	No	30 (0.45)
	Unknown	26 (0.39)

M, male; F, female; N, patient's number; TKI: Tyrosine kinase inhibitors.

DNA Preparation and NGS

The cell pellets obtained from the samples were thawed to room temperature, and as much media or phosphate-buffered saline (PBS) as possible was removed from the thawed pellets. DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen, Venlo, Hilden, Germany). The purified DNA was analyzed qualitatively using Nanodrop One (Thermo Fisher Scientific, Waltham, MA, USA) and quantitatively with Qubit 3.0 (Life Technologies, Singapore, Singapore) using the ds DNA HS Assay Kit (Life Technologies, Eugene, OR, USA) according to the manufacturer's recommendations.

Library Preparation

Sequencing libraries were prepared using the KAPA Hyper PreP Kit (KAPA, Biosystem, Cape Town, South Africa) with an optimized manufacturer's protocol. In brief, 50 ng-1 μg of genomic DNA was sheared into 350-bp fragments using Bioruptor Pico (Denville, NJ, USA). The fragments were subjected to end-repairing, A-tailing, and ligation process that had indexed adapters sequentially, followed by size selection using Agencourt AMPure XP beads (Beckman Coulter, Inc., Indianapolis, IN, USA). Finally, the libraries were amplified by Polymerase Chain Reaction (PCR) and purified for target enrichment.

Hybridization Capture and Sequencing

Different libraries with unique indices were pooled together in desirable ratios for up to 2 μg of total library input. Human cot-1 DNA (Life Technologies, Waltham, MA, USA) and xGen Universal blocking oligos (Integrated DNA Technologies, Coralville, IA, USA) were added as blocking reagents. Geneseeq 422 probes (Geneseeq ONE, Nanjing, Jiangsu, China) were used for hybridization enrichment. The capture reaction was performed with the NimbleGen SeqCap EZ Hybridization and Wash Kit (Roche, Madison, WI, USA) and Dynabeads M-270 (Life Technologies,Vilnius, Lithuania) with optimized manufacturers' protocols. Captured libraries were on-beads amplified with Illumina p5 (5'-AAT GAT ACGGCG ACC ACC GA-3') and p7 primers (5'-CAA GCAGAAGACGGC ATA CGA GAT-3') in KAPA HiFi HotStartReadyMix (KAPA Biosystems,Cape Town, South Africa). The postcapture amplified library was purified using AgencourtAMPure XP beads and quantified by qPCR using the KAPA Library Quantification kit (KAPA Biosystems, Cape Town, South Africa). Library fragment size was determined by using the Agilent Technologies 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA). The target-enriched library was then sequenced on HiSeqNGS platforms (Illumina, San Diego, CA, USA) according to the manufacturer's instructions. The mean coverage depth was over 300× for lung cancer tissues.

Sequence Data Processing

Trimmomatic software was used for FASTQ file quality control. Readings from each sample were mapped to the reference sequence hg19 (Human Genome version 19, NCBI, NIH, USA) using Burrows-Wheeler Aligner (BWA-mem, v0.7.12, Broad institute, Cambridge, MA, USA). VarScan2 (Washington University, St. Louis, MI, USA) was employed for the detection of somatic mutations. Somatic variants were selected with at least 0.2% mutant allele frequency (MAF) and three supporting-reads from both directions. Common SNPs were screened with dbSNP (v137) and the 1000 Genomes database. Annotation was performed using ANNOVAR software on the hg19 reference genome and 2014 versions of standard databases and functional prediction programs.

Genomic fusions were identified by FACTERA with default parameters. Copy number variations (CNVs) were detected using ADTEx (http://adtex.sourceforge.net) with default parameters. TMB was defined as the number of somatic, coding base substitutions, short insertions and deletions (indels) per megabase (MB) of the genome examined using Geneseeq ONE (Nanjing, Jiangsu, China). The frequently mutated genes are identified by MutSigCV and Lauren classification. A total of 24 DDR gene panels were used for determining genetic alterations using in Geneseeq ONE.

Statistical Analysis

For OS analysis, Kaplan-Meier curves were constructed using a log-rank test. Statistical analysis was performed with GraphPad Prism software, version 5.0 (GraphPad Software Inc., San Diego, CA, USA) and R software, version 3.5.0 (R Foundation for Statistical Computing, Boston, MA, USA). p-value of less than 0.05 was considered to indicate statistical significance.

Results

Clinical Characteristics

The clinical features of the 67 advanced stage (III-IV) NSCLC patients are shown in Table I. Their age range was 38-80 years old. Thirty-sev-

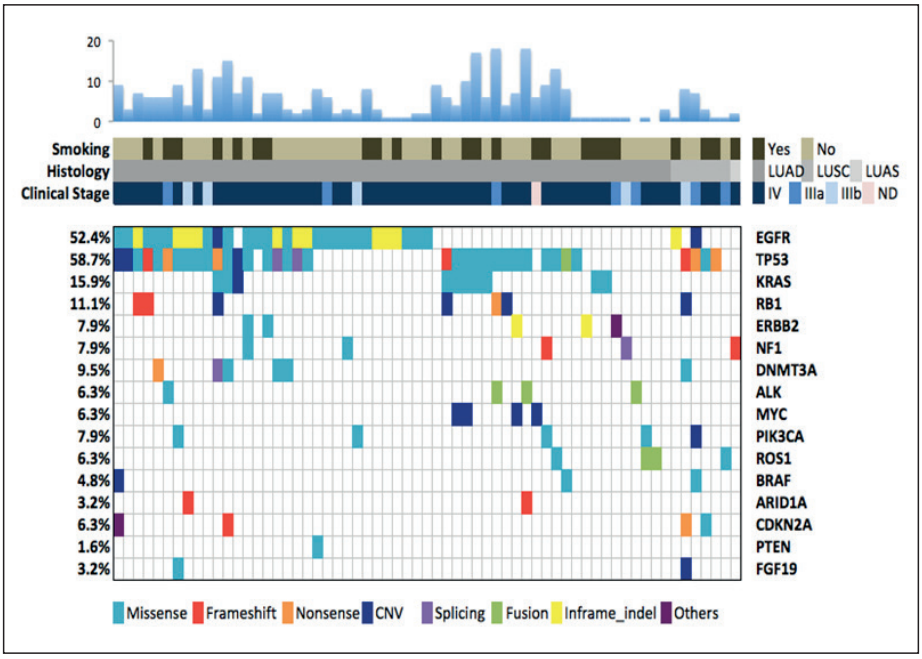

Figure 1. Landscape of mutations from patients with stages III and IV lung cancer. The frequently mutated genes are identified by MutSigCV and Lauren classification. The top panel presents tumor mutation burden (TMB), mutations per MB. The middle panel indicates smoking history, histological types, and clinical stages for 67 patients. The lower panel shows the percentages of the frequently mutated genes. LUAD: lung adenocarcinoma; LUSC: lung squamous cell carcinoma; LUAS: lung adenosquamous carcinoma; ND: no disease; CNV: copy number variation.

en percent of the patients had a history of smoking. Adenocarcinoma, squamous carcinoma, and adenosquamous carcinoma at the initial histology diagnosis were 90% (n=60), 9% (n=6), and 1% (n=1), respectively. Patients with stages IIIa and IIIb accounted for 19% of all patients. By contrast, 79% of the patients had stage IV. Twenty-seven percent of the patients had a surgical history. Treatment-naïve, first-line, second-line, and third-line and beyond targeted drug therapy was given in 33%, 15%, 6%, and 10%, respectively. Sixteen percent of patients experienced treatment with a tyrosine kinase inhibitor (TKI).

Mutation Landscape

The profile of mutated genes from the 67 NSCLC patients before treatment is shown in Figure 1. The most frequently mutated genes were *EGFR* (52.4%), *TP53* (58.7%), *KRAS* (15.9%), *RB1* (11.1%), *ERBB2*(7.9%), *NF1* (7.9%), *DNMT3A* (9.5%), and *PIK3CA* (7.9%) (Figure 1). In addition to these frequently mutated genes, in most cancers, we also found less frequently mutated genes including *ALK* (6.3%), *MYC* (6.3%), *ROS1* (6.3%), *CDKN2A* (6.3%), *BRAF*(4.8%), *ARID1A* (3.2%), *FGF19* (3.2%), and *PTEN* (1.6%). Different gene alterations had distinct mutation styles. For example, *EGFR* mainly had missense, frameshift, indel, and copy number variation (CNV) mutations. By contrast, *TP53* had other mutations including frameshift, nonsense, and fusion except missense and CNV. These mutations also occurred in other cancers (gastric cancer, pancreatic cancer[21], colon cancer[22], etc.).

Tumor Mutation Burden and DDR of NSCLC

Recent reports revealed that TMB is a critical biomarker for assessing the response of cancer patients to immune checkpoint inhibitors, such asanti-PD-1 or anti-PD-L1 antibody therapy[14,23]. NGS is a sensitive and reliable tool to detect TMB, and it has been used in many investigations[24,25]. Alterations in DDR pathway genes, including genes related to mismatch repair (MMR), base excision repair (BER), homology-dependent recombination (HDR)[26], and nucleotide excision repair (NER), were frequently identified in both germline and somatic cells. Previous studies identified 27 genes relevant to DDR. In the current study, we found that most DDR-related gene mutations had missense-type alteration and, in addition to *MLH1*, frameshift of *MLH1*, fusion of *RECQL4*, and nonsense of *ATM* (Figure 2A). We also compared the TMB of wild-type (n=43) and mutat-

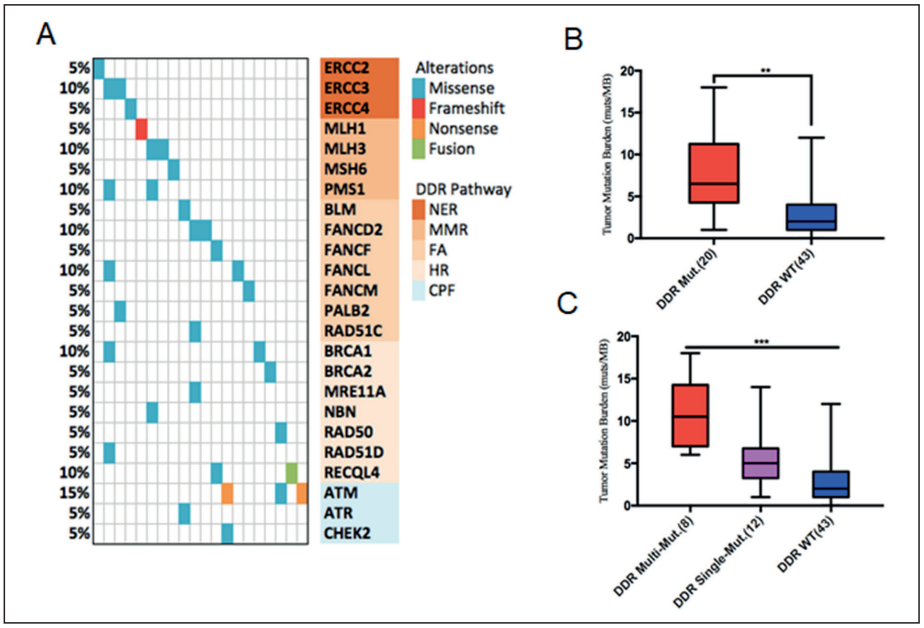

Figure 2. DDR mutations are associated with TMB in lung cancers. **A**, Landscape of the mutated genes in the DDR pathway in NSCLC. **B**, Comparison of tumor mutational burden in samples of DDR wild-type (WT, n=43) and DDR mutations (DDR mut, n=20). **C**, Comparison of DDR wt, DDR-single mut and multi-mut.NER: nucleotide excision repair; MMR: mismatch repair; FA: Fanconi anemia; HR: homology-dependent recombination; CPF: cleavage and polyadenylation factor; DDR mut: DNA damage and repair mutations; DDR WT: DNA damage and repair mutations wild type.**p<0.01; ***p<0.001.

ed (n=20) DDR-relevant genes, which revealed significant differences between the wild-type and the mutated genes (p<0.01, Figure 2B). We further classified TMB as caused by a single mutation, multiple mutations, or wild type (WT). We found that the TMB of multiple mutated genes was dramatically higher than that of single mutated and wild-type genes, respectively (p<0.001, Figure 2C). These results imply that patients with multiple DDR mutations may show a good response to therapy with immune checkpoint inhibitors.

Outcome Evaluation of TMB and DDR Mutations in NSCLC Patients for Targeted Therapy

To assess the impact of the gene mutation profile on treatment decisions, we performed univariable and multivariable analyses of OS in a few gene mutations, taking into account the TKI therapy history, TMB levels, *DNMT3A*, *FLT4, RB1,* and the DDR pathway (Table II). Univariable analyses revealed that TMB high *vs.* low levels (p=0.0375), *DNMT3A* mutation *vs.* WT (p=0.0151), *RB1* gene mutation *vs.* WT (p=0.0217) and DDR pathway gene alteration *vs.* WT (p=0.0036) had significant effects on OS. By contrast, the multivariable analysis revealed that mutations in DDR pathway genes (Table II and Figure 3A) and *DNMT3A* (Figure 3B) were critical factors for OS as assessed by log-rank analysis. However, DDR pathway gene alterations had more benefits than the *DNMT3A* mutation (Figure 3A, B).

Discussion

In this study, we performed an NGS study for the detection of individual gene mutations, DDR pathway mutated genes, and TMB evaluation of tumors in 67 NSCLC patients. Our results showed that the most frequently observed mutations were *TP53* (58.7%), *EGFR* (52.4%), and *KRAS* (15.9%). The major genes in the DDR pathway had 5-10% alterations. Interestingly, TMB levels in patients with DDR pathway mutated genes were significantly higher than those in patients with non-DDR gene alterations. Finally, we found that alterations in the *DNMT3A* and DDR genes were relevant to an NSCLC patient's outcomes as determined on univariable and multivariable analyses.

In this study, we identified 16 significantly mutated genes (Figure 1). Similar to other can-

Table II. Univariable and multivariable analysis of overall survival.

	Univariable Analysis			Multivariable Analysis		
Parameter	**HR**	**95% CI**	***p*-value**	**HR**	**95% CI**	***p*-value**
History of treatment with TKI						
Yes vs. No	2.28	0.968 to 5.35	0.0528			
TMB						
High *vs.* Low	2.99	1.01 to 8.84	0.0375			
DNMT3A gene						
Alterations vs. WT	3.72	1.19 to 11.6	0.0151	4.455	1.357 to 14.628	0.014
FLT4 gene						
Alterations vs. WT	1.23E-08	0 to Inf	0.0927			
RB1 gene						
Alterations vs. WT	0.134	0.018 to 1	0.0217			
DDR pathway gene						
Alterations vs. WT	0.196	0.058 to 0.663	0.0036	0.181	0.053 to 0.620	0.006

HR, hazard ratio; OR, odds ratio; CI, confidence interval; TKI, tyrosine kinase inhibitors; WT, wild type; DDR, DNA damage repair; TMB, tumor mutation burden.

cers, the tumor suppressor gene *TP53* and the oncogene *KRAS* were the most frequently mutated genes[21,27,28]. *TP53* mutation was associated with not only clinical characteristics such as gender and tumor location[29], but also TMB rate. Interestingly, we found *EGFR* and *ALK* gene alterations in 52.4% and 6.3% of the cases, respectively. This is a critical finding for clinical therapy because many drugs targeting *EGFR* and *ALK* mutations are widely used in clinical therapy[30-32].

We observed extensive mutations in the genes of the DDR pathway, as shown in Figure 2. There are evident differences concerning DDR gene alterations between different kinds of cancers. Gee et al[33] reported 63-67% *BER* and 50% *HR* alterations in ovarian cancer patients. Mouw[34] indicated that the major DDR mutation types were double-strand break (DSB) and NER in bladder tumors. Here, our data showed that the alteration rate in the genes of the DDR pathway was 5-15% in NSCLC patients. This alteration rate was lower than that observed in ovarian cancer and bladder cancer. However, TMB levels of patients with DDR gene alteration were higher than those of patients without DDR

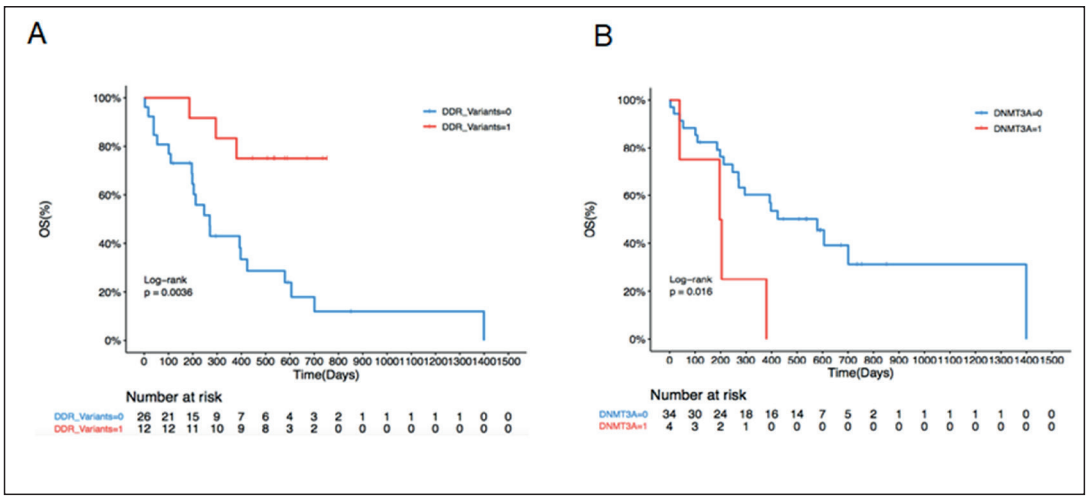

Figure 3. Overall survival (OS) of lung cancer patients with DDR variants and DNMT3A mutation. **A**, OS of with and without DDR variants; p=0.0036 calculated using log-rank test. **B**, Comparison of OS with and without DNMT3A mutation

gene mutations. Our results also demonstrated that more DDR gene alterations caused higher TMBs, which indicates that these patients may show a good response to immune checkpoint inhibitors[25,35] and platinum-based therapy.

DNMT3A alteration had an important impact on the outcome of cancer[36,37]. *DNMT3A* encodes a DNA methyltransferase that is involved in gene transcription and maintain de novo DNA methylation. Husni et al[38] showed that high *DNMT3A* alteration had poor outcome in lung adenocarcinoma patients. Chen et al[39] reported that high *DNMT3A* alteration was relevant to the severity of leukemia. Our data showed that *DNMT3A* alteration led to a short OS. This result indicates that *DNMT3A* alteration plays a critical role in NSCLC outcome.

Higher TMB levels in cancer patients had greater benefits for the treatment of disease[20]. Wang et al[25] reported that TMB in melanoma patients had a direct impact on the outcome of cancer. Our current data show that higher TMB in NSCLC patients led to better OS as observed on univariable analysis, although there were no significant correlations with OS on multivariable analysis. This finding implies that NSCLC patients with high TMB may show good responses to immunotherapy.

Our study indicated that DDR gene and *DNMT3A* alterations had tremendous the effects on the outcomes of NSCLC patients. To the best of our knowledge, our results are the first to uncover a relationship between *DNMT3A* and the outcomes of NSCLC.

Conclusions

We performed a comprehensive mutational landscape in 67 NSCLC patients. We showed that alterations in DDR genes and *DNMT3A* significantly contributed to OS in lung cancer, which was mediated by increasing TMB in patients. This provides a theoretical foundation for therapy targeting DDR genes and *DNMT3A*.

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Conflict of Interests

The authors have no conflicts of interest to declare.

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