

Mutational Characterization and Potential Prognostic Biomarkers of Chinese Patients with Esophageal Squamous Cell Carcinoma

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Purpose: Esophageal squamous cell carcinoma (ESCC) is the most common type of esophageal cancer in China and the 5-year mortality rate is up to 70%. Studies on the ESCC genetic landscape are needed to further explore clinical therapeutic strategies. In this study, we evaluated the genetic landscape of ESCC to aid the search for clinical therapeutic strategies.

Patients and Methods: A total of 225 ESCC patients were enrolled in this study. Deep sequencing of 450 cancer genes was performed on formalin-fixed paraffin-embedded tumor biopsies and matched blood samples from patients. Tumor mutational burden (TMB) was calculated using an algorithm developed in-house.

Results: Our results showed that the most commonly mutated genes in ESCC were *TP53* (96%), *CCND1* (46%), *FGF4* (44%), *FGF19* (44%), *FGF3* (44%), *CDKN2A* (31%), *PIK3CA* (26%), *NOTCH1* (24%), *KMT2D* (18%), *FAT1* (16%), and *LRP1B* (16%). We found that TMB correlated with patient drinking status. We identified mutations associated with sex, early ESCC, high TMB, and metastasis lymph nodes. *KMT2D* mutations associated with sex ($P = 0.035$), tumor stage ($P = 0.016$), high TMB ($P = 0.0072$), and overall survival of patients ($P = 0.0026$). *SPEN* mutations associated with high TMB ($P = 0.0016$) and metastasis-positive lymph nodes ($P = 0.027$). These results suggested that *SPEN* and *KMT2D* could be potential prognosis biomarkers for Chinese patients with ESCC. We also found that the number of positive lymph nodes was associated with disease-free survival. Clinical target gene analysis indicated that nearly half of Chinese ESCC patients might benefit from treatment with gene-specific target drugs.

Conclusion: Our study revealed the ESCC mutational landscape in 225 Chinese patients and uncovered the potential prognosis biomarker for Chinese patients with ESCC.

Keywords: genomic variation, deep sequencing, esophageal cancer, tumor mutational burden, biomarker

Introduction

Esophageal cancer, a malignant lesion formed by abnormal hyperplasia of the esophageal epithelium or squamous epithelium, is one of the most aggressive tumors worldwide.¹ Esophageal squamous cell carcinoma (ESCC) is the most common type of esophageal cancer in China.² Surgery is the primary method to treat ESCC, but the 5-year mortality rate remains as high as 70%.³ Progress in gene-targeted therapies and our increasing understanding of the molecular mechanisms driving esophageal cancer provide hope for developing treatments to improve

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survival.⁴⁻⁷ Characterizing the genetic landscape of ESCC will facilitate the development of targeted therapies.

With the emergence of next-generation sequencing technology, tremendous progress has been made in oncogenomics. Compared to other solid tumors, ESCC has a higher somatic mutation rate.^{8,9} The most frequently mutated genes in ESCC include *TP53*, *NOTCH1*, *PIK3CA*, *CDKN2A*, *CCND1*, and *FAT1*.¹⁰ The mutation of *TP53* is considered an early event in ESCC carcinogenesis, and some studies have shown that patients with *TP53* alterations respond better to angiogenesis inhibitors than those without the alterations.¹¹ As both an oncogene and a tumor suppressor, *NOTCH1* is closely related to human carcinogenesis, and *NOTCH1* mutations have been observed in many cancers.¹²⁻¹⁵ NOTCH signaling may inhibit tumorigenesis in ESCC.¹⁶ Curcumin can affect the interaction between NOTCH signal and other signals, which is of great significance in chemoprevention and clinical treatment of ESCC.¹⁷ *PIK3CA* participates in the receptor tyrosine kinase/mitogen-activated kinase/phosphatidylinositol-3 kinase (PI3K) pathway, which is downstream of *RTKS*, *EGFR*, *ERBB2*, *ERBB4*, and *MET*. Many biomarkers associated with the prognosis of ESCC have been reported, including circulating microRNAs, circular RNAs, and specific prognostic associated oncogenes.¹⁸⁻²⁰ Autophagy was also suggested to be a suppressor in cancer progression and could be regulated by miRNA level in different stages of cancer.²¹ Despite efforts in recent years to develop a targeted molecular therapy for ESCC, an effective drug has still not been found.

Smoking and drinking are high-risk factors for ESCC. Cigarette smoke contains thousands of components, including cancer initiators, promoters, and carcinogens.²² Long-term exposure to smoke and acetaldehyde can lead to DNA damage and a series of genetic changes.²³ Many studies have focused on the molecular mechanism of ESCC incidence and found that tobacco and alcohol use contribute to genome variations, such as *TP53* gene mutations, in ESCC.^{24,25} However, a definitive molecular mechanism remains to be determined.

In this study, we comprehensively analyzed the genomic features of 225 Chinese patients with ESCC, performed drug-target analysis, and examined correlations between prognosis and metastasis-positive lymph nodes. We aimed to identify specific biomarkers for the early diagnosis and prognosis of ESCC, as well as potential therapeutic targets.

Patients and Methods

Patient Enrollment and Sample Collection

From 2014 to 2019, 225 patients with ESCC were enrolled in the Fourth Hospital of Hebei Medical University. The inclusion criteria were as follows: the patients did not suffer from other tumors before surgery; the patients did not receive any other anti-tumor treatment before surgery; the postoperative pathology diagnosis was squamous cell carcinoma and at least 12 lymph nodes were removed. Formalin-fixed paraffin-embedded (FFPE) tumor tissues and matched blood samples were transferred to the laboratory of OrigiMed (Shanghai, China), which is accredited by the College of American Pathologists and certified by Clinical Laboratory Improvement Amendments for genetic variation detection. Genomic DNA was prepared using a QIAamp DNA FFPE Tissue Kit and QIAamp DNA Blood Midi Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. This study was approved by the Ethics Committee of the Fourth Hospital, Hebei Medical University (2018MEC161). This study was conducted in accordance with the declaration of Helsinki. Written informed consent was obtained from all participants.

Identification of Genomic Alterations

The ESCC genomic profile was produced using the next-generation sequencing-based YuanSu 450 gene panel (OrigiMed, Shanghai, China). The genes were sequenced to a mean depth of 1000× with an Illumina NextSeq 500. Genomic alterations were identified according to a previous study.²⁶ Briefly, single nucleotide variants were identified with MuTect (v1.17) and insertion-deletion polymorphisms were identified using PINDEL (v0.2.4); the functional impact of these mutations was annotated with SnpEff3.0. Copy number variation regions were identified with Control-FREEC (v9.4) using the following parameters: window = 50,000 and step = 10,000. Gene fusions were detected using an in-house pipeline. Gene rearrangements were assessed with Integrative Genomics Viewer. We estimated the Tumor mutational burden (TMB) in each patient by counting the somatic mutations from the coding region, including single nucleotide variants and insertion-deletion polymorphisms, per megabase of sequence examined. We did not count driver mutations and known germline alterations.

Statistical Analysis

Statistical analyses were performed using SPSS version 22.0 (SPSS Inc., Chicago, IL, USA). We applied multivariate Cox and logistic regression models to analyze associations between mutational features and clinical parameters. Differences with a *P*-value <0.05 were considered statistically significant.

Results

Characterization of Chinese Patients with ESCC

A total of 225 ESCC patients (186 male and 39 female patients) confirmed by pathology after esophagectomy for cancer were enrolled in this study. The median age of the patients was 63 years, ranged from 37 to 90 years. Except for 39 patients with unknown smoking and drinking histories, the percentages of patients who had a smoking or drinking history were 61.8% (115/186) and 54.8% (102/186), respectively. Based on the classifications for esophageal cancer defined by the 8th edition American Joint Committee on Cancer (AJCC) Cancer Staging Manual²⁷ there were 16 stage I, 66 stage II, 86 stage III, and 39 stage IV patients (Table 1). 48.44% of patients were identified as lymph node metastasis positive. Based on tumor differentiation, 25.33%, 42.67%, and 17.78% of patients were poorly, moderately differentiated, and well differentiated, respectively. The clinicopathological characteristics of the patients are shown in Table 1.

Genome Alterations in Chinese Patients with ESCC

We detected 2,859 alterations in the 225 samples of Chinese patients with ESCC, including 1,109 substitution/insertion-deletion polymorphisms, 1,172 gene amplifications, 420 truncations, 90 homozygous gene deletions, and 66 fusions/rearrangements. The most commonly mutated genes were *TP53* (96%), *CCND1* (46%), *FGF4* (44%), *FGF19* (44%), *FGF3* (44%), *CDKN2A* (31%), *PIK3CA* (26%), *NOTCH1* (24%), *KMT2D* (18%), *FAT1* (16%), and *LRP1B* (16%) (Figure 1A). The most frequent gene amplifications were of *CCND1*, *FGF19*, *FGF3*, and *FGF4*. The amplification of *FGF19*, *FGF3* and *FGF4* were all located on chromosome 11q13 and occurred together in patients. The most common homozygous gene deletions were in *CDKN2A* and *CDKN2B*. The most common fusion/rearrangement mutations occurred in *CDKN2A*.

We also analyzed mutations in common pathways in this cohort (Figure 1B). We observed more mutations in

Table 1 The Characterization of 225 ESCC Chinese Patients

	N=225
Gender	
Male	186(82.67%)
Female	39(17.33%)
Age	
< 60 years old	73(32.44%)
≥ 60 years old	152(67.56%)
Smoking history	
Yes	115(51.11%)
No	71(31.56%)
Unavailable	39(17.33%)
Drinking status	
Yes	102(45.33%)
No	84(37.33%)
Unavailable	39(17.33%)
Tumor differentiation degree	
Poor differentiation	57(25.33%)
Middle differentiation	96(42.67%)
High differentiation	40(17.78%)
Unclear	32(14.22%)
Metastatic lymph nodes	
Yes	109(48.44%)
No	78(34.67%)
Unclear	38(16.89%)
Tumor stage	
I	16(7.11%)
II	66(29.33%)
III	86(38.22%)
IV	39(17.33%)
Unclear	18(8.00%)

the cell cycle, fibroblast growth factor, PI3K/MTOR, and histone modification pathways in ESCC than in the homologous recombination deficiency, DNA mismatch repair, and WNT pathways. Mutations in genes related to the cell cycle pathway, especially gene copy variations, occurred with high frequency in ESCC, suggesting that targeting this pathway may be a promising therapeutic strategy.

Tumor Mutational Burden Significantly Correlated with Drinking Status in Chinese Patients with ESCC

The median TMB of the 225 ESCC patients was 7.7 mutations/Mb (range: 0–43.3 mutations/Mb). We identified 74 cases (32.9%, 74/225) with a high TMB (≥10 mutations/Mb) and 151 cases (67.1%, 151/225) with a low



Figure 1 The mutational landscape of 225 Chinese patients with ESCC. **(A)** The top 40 mutated genes. **(B)** Commonly mutated pathways. The X-axis displays each case sample and the Y-axis displays each mutated gene. The bar graph above shows the tumor mutational burden for each sample. The mutation frequency of each mutated gene is shown on the right. Green represents substitution or insertion-deletion mutations, red represents gene amplification mutations, blue represents homozygous gene deletion mutations, yellow represents fusion/rearrangement mutations, and purple represents truncation mutations.

mutational burden (<10 mutations/Mb). The patient with the highest TMB had a positive excision margin, >2 metastases, and grade III tumor with moderate differentiation. We evaluated the correlation between TMB and clinical characteristics, such as tumor stage, tumor differentiation, smoking status, and drinking status. Our results showed that TMB was significantly associated with patient drinking status ($P=7.5\times 10^{-5}$). We also detected a trend that did not reach statistical significance between smoking and higher TMB ($P=0.19$). However, no correlation was observed between TMB and tumor stage or tumor differentiation. Upon analysis of lymph node metastasis information from 187 patients, we observed no statistical correlation between the number of lymph node metastases and TMB (Figure 2).

Correlations Between Mutated Genes and the Clinical Characteristics of Chinese Patients with ESCC

To compare the different molecular features of male and female patients, we performed association analyses between mutated genes and sex. Interestingly, we found that mutations

of *LRP1*, *AXIN2*, *CFTR*, *CREB3L1*, and *TAF1* occurred exclusively in female patients. The mutational frequencies of *LRP1* ($P=0.0049$), *JAK2* ($P=0.0091$), *CDK12* ($P=0.018$), *AXIN2* ($P=0.029$), *CFTR* ($P=0.029$), *CREB3L1* ($P=0.029$), *TAF1* ($P=0.029$), and *KMT2D* ($P=0.035$) were significantly higher in female than in male patients (Figure 3A).

In this cohort, 48.4% (109/225) of patients were positive for lymph node metastases and 34.7% (78/225) of patients were negative. We analyzed the differences in gene mutation frequencies between positive and negative patients. We found that the mutational frequencies of *NOTCH2* (11.93% vs 2.56%, $P=0.027$) and *SPEN* (9.17% vs 1.28%, $P=0.027$) were significantly higher in lymph node metastasis-positive patients than in negative patients, whereas the mutational frequencies of *FOS* (0.92% vs 8.97%, $P=0.01$), *DOTIL* (0.00% vs 6.41%, $P=0.012$), and *VEGFA* (0.00% vs 5.13%, $P=0.029$) were significantly lower in lymph node metastasis-positive patients than in negative patients (Figure 3B).

We also analyzed the associations of gene alterations with tumors stage. We classified 82 stage I and II tumors as early-stage and 125 stage III and IV tumors as advanced stage. The mutational frequencies of *DOTIL* ($P=0.009$), *BMPRIA*

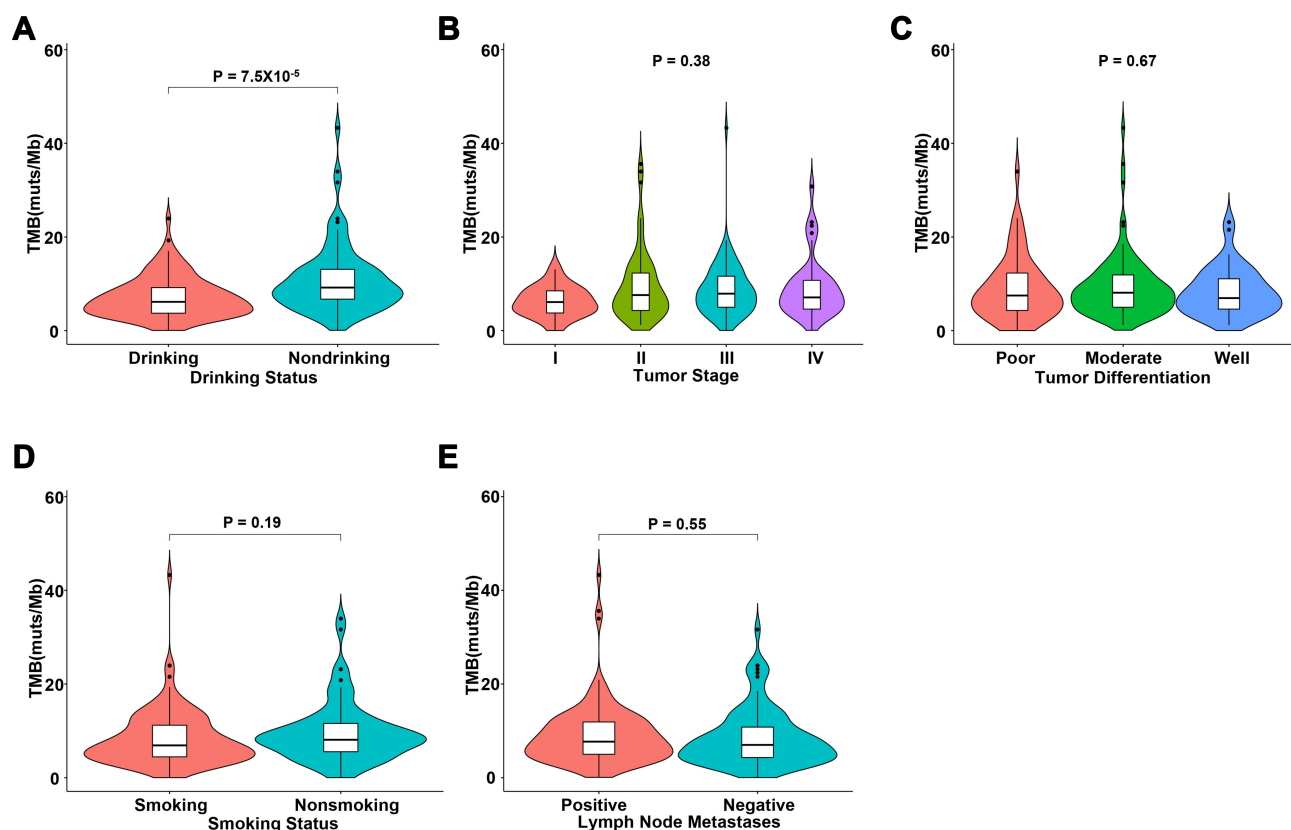


Figure 2 Correlation analyses between tumor mutational burden and clinical characteristics. (A) Drinking status, (B) tumor stage, (C) tumor differentiation, (D) smoking status, and (E) the number of metastatic lymph nodes.

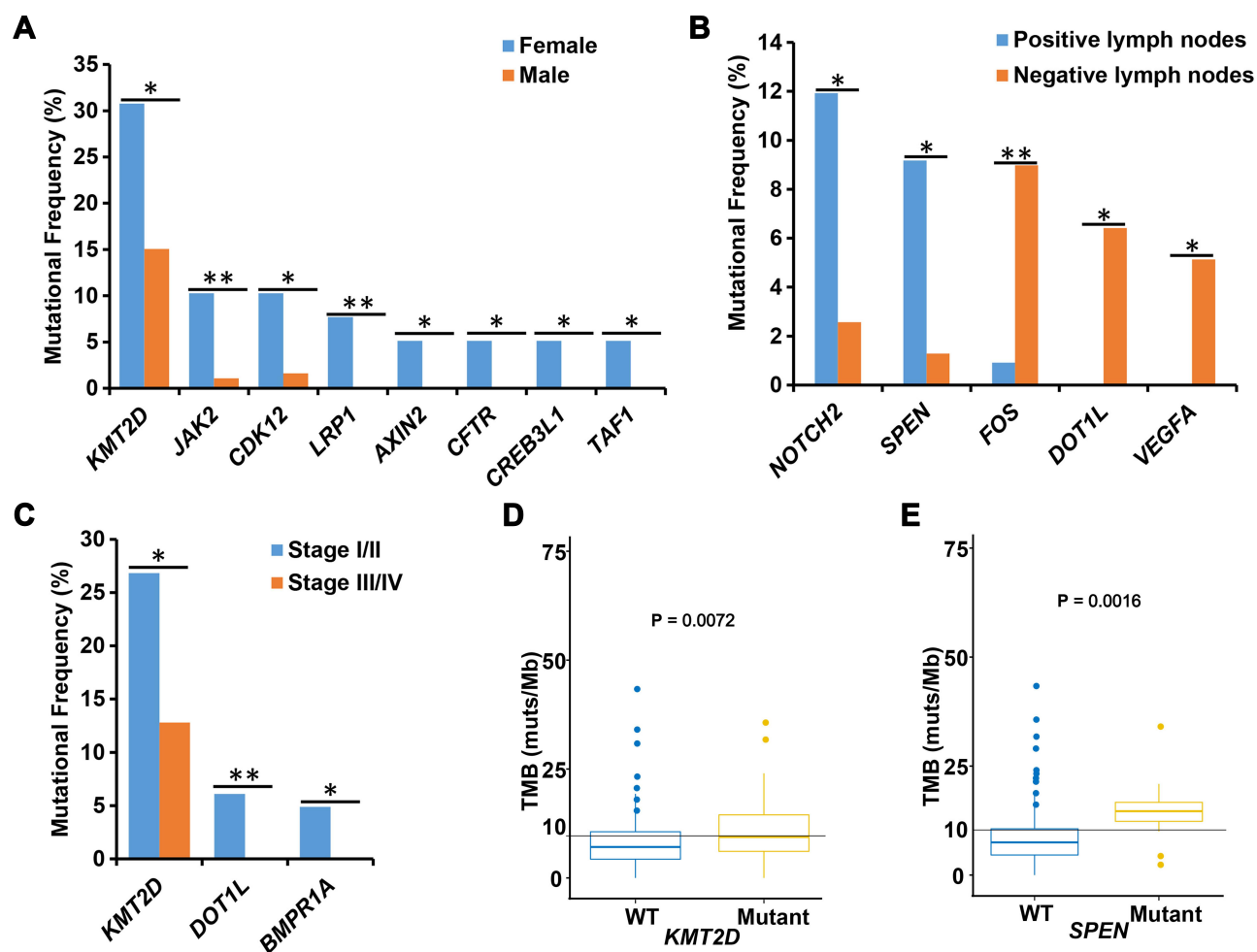


Figure 3 Correlation analyses between mutated genes and clinical characteristics. **(A)** The association between mutated genes and sex. The X-axis displays each detected gene and the Y-axis displays the mutation frequency of these genes. Blue represents female patients and red represents male patients. **(B)** The correlation analysis between lymph node status and mutated genes. The X-axis displays each mutated gene and the Y-axis displays the mutation frequency of those genes. **(C)** The association between mutated genes and tumor differentiation. The X-axis displays each detected gene and the Y-axis displays the mutation frequency of these genes. Blue represents tumor stage I/II (early) and red represents tumor stage III/IV (advanced). **(D and E)** The association between mutated genes and tumor mutational burden. The X-axis displays mutation status of each target gene, and Y-axis displays the tumor mutational burden value. WT, wild type target gene; Mutant, mutated target gene. *, $P < 0.05$ and **, $P < 0.01$.

($P=0.024$) and *KMT2D* ($P=0.016$) were significantly higher in early-stage tumors than in advanced-stage tumors (Figure 3C).

To explore TMB-related mutations, we divided patients into mutant and wild type groups for each mutated gene; genes mutated in fewer than five cases were excluded from the association analysis. Statistical analysis revealed that mutations of *KMT2D* ($P=0.0072$) and *SPEN* ($P=0.0016$) were significantly associated with high TMB (Figure 3D and E).

The Correlation Between *KMT2D* Mutation and Disease-Free Survival of Chinese Patients with ESCC

In this study, we selected 46 squamous cell carcinoma patients who had not received anti-tumor treatment prior to surgery for correlation analysis between gene mutations

and clinical follow-up data. We analyzed the correlations between the 10 most frequently mutated genes and disease-free survival and overall survival of patients. We found that *KMT2D* mutation was significantly associated with overall survival ($P=0.026$), but did not correlate with disease-free survival ($P=0.08$) (Figure 4). We also analyzed the relationship between mutations in genes in related pathways and the prognosis of ESCC patients but did not detect a correlation.

Analysis of Genes Targeted by Available Drugs

Based on the alterations detected in the 225 Chinese patients with ESCC, 82% (184/225) of patients in this cohort were candidates for potential molecularly targeted

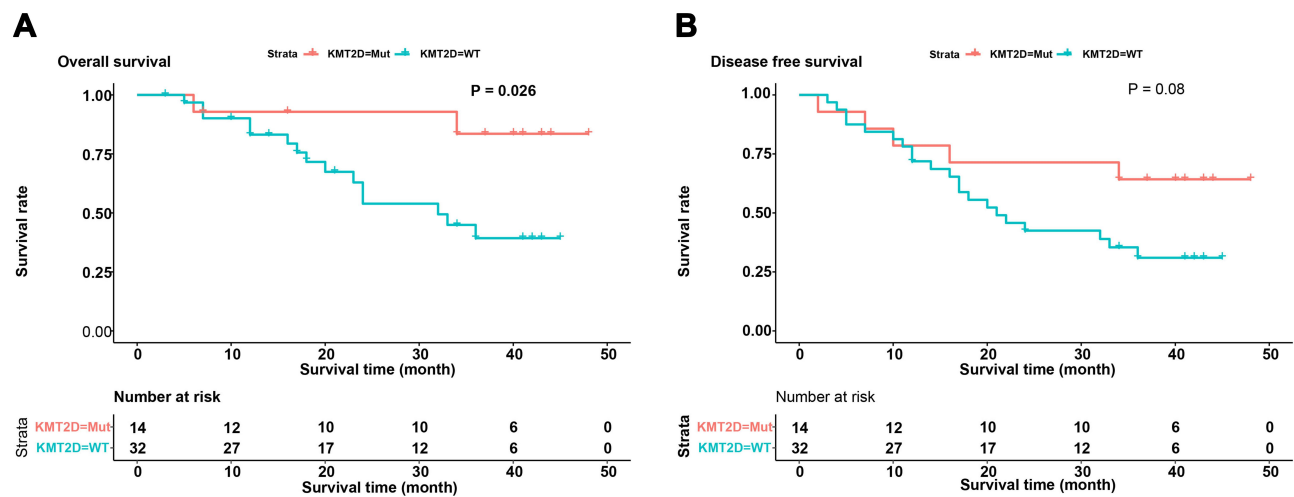


Figure 4 Analysis of disease-free survival and overall survival of patients with or without *KMT2D* mutations. **(A)** The correlation analysis of disease-free survival and mutation of *KMT2D*. **(B)** The correlation analysis of overall survival and mutation of *KMT2D*. The X-axis displays survival time and the Y-axis displays survival rate. Blue represents the patients without *KMT2D* mutations and red represents the patients with *KMT2D* mutations.

treatments. We detected mutations in 24 genes—*CCND1*, *CDKN2A*, *PIK3CA*, *CDKN2B*, *FBXW7*, *EGFR*, *FGFR1*, *PTEN*, *BRCA1*, *ERBB2*, *BRCA2*, *MET*, *STK11*, *CDK4*, *CDK6*, *ERBB3*, *KDR*, *VEGFRA*, *ARAF*, *CD274*, *FGFR3*, *PDCD1LG2*, *PDGFB*, and *TSC1*—that are targeted by available drugs. In this cohort, nearly 61% (137/225) of patients harbored at least 1 mutation of *CCND1*, *CDKN2A*, *CDKN2B*, *CDK4*, or *CDK6*, and nearly 37% (84/225) of patients harbored at least 1 mutation of *PIK3CA*, *FBXW7*, *PTEN*, *STK11*, or *TSC1*. Cancers with mutations in *CCND1*, *CDKN2A*, *CDKN2B*, *CDK4*, and *CDK6* respond to drugs such as palbociclib, ribociclib, and abemaciclib. Cancers with mutations in *PIK3CA*, *FBXW7*, *PTEN*, *STK11*, and *TSC1* respond to drugs such as everolimus and temsirolimus (Figure 5).

Metastasis-Positive Lymph Node Status Affects the Prognosis of Chinese Patients with ESCC

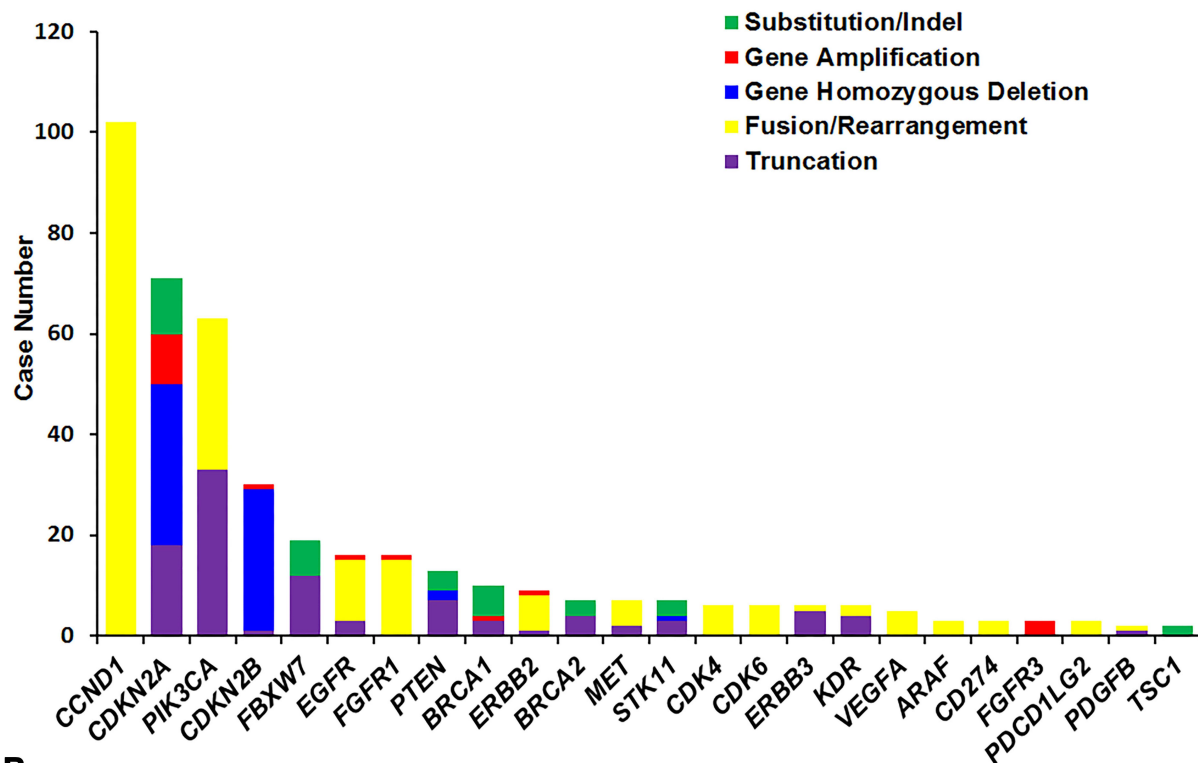
A follow-up for 46 patients was completed with a median follow-up time of 24 months (range: 3–48 months). Survival analysis showed that disease-free survival of lymph node metastasis-positive patients was significantly higher than that of lymph node metastasis-negative patients ($P=0.0094$) (Figure 6A). Although overall survival did not negatively correlate with the presence of lymph node metastases ($P=0.23$), we observed a worse trend for the overall survival of lymph node metastasis-positive patients (Figure 6B). We further divided the patients into groups based on the number of positive lymph nodes.

When the cut-off value was set to 3, we observed the greatest difference in the overall survival of patients with ≥ 3 positive lymph nodes and that of patients with < 3 positive lymph nodes ($P=0.079$) (Figure 6C), suggesting that the number of positive lymph nodes influences overall survival.

Discussion

ESCC is one of the most prevalent cancers in China, and Chinese cases account for half of the global incidence.²⁸ Recently, many studies have characterized ESCC genomes as having hundreds of somatic mutations.^{8,29–31} Many important mutated genes, including *TP53*, *NOTCH1*, *NOTCH2*, *NOTCH3*, *FBXW7*, *KIF16B*, *KIF21B*, and *MYCBP2* have been identified in Western populations, and *TP53*, *PIK3CA*, *NOTCH1*, *FAT1*, *FAT2*, *ZNF750*, and *KMT2D* have been identified in Chinese populations.^{29,32} Based on 113 pairs of matched tumor–normal samples, Gao et al also reported the most common alterations in Chinese patients with ESCC as *TP53*, *CCND1*, *CDKN2A*, *NFE2L2*, and *RBI*.³⁰ In order to study ESCC-associated mutations in local Chinese patients, we enrolled 225 patients from China and performed next-generation sequencing on their tumor and blood samples. We detected frequent mutations in *TP53*, *CCND1*, *FAT1*, and *CDKN2A*, as in other studies, and further identified frequent mutations in *FGF19*, *FGF3*, *FGF4*, and *KMT2D*. *FGF19*, *FGF4*, *FGF3*, and *CCND1* are located on human chromosome 11q13.³³ Previous studies have proposed important roles for *FGF19*, *FGF4*, *FGF3*, and *CCND1* in various

A



B

Drug Target Genes	Case Number	Drugs
CCND1	102	Palbociclib,Ribociclib,Abemaciclib
CDKN2A	71	
CDKN2B	30	
CDK4	6	
CDK6	6	
PIK3CA	63	Everolimus,Temsirolimus,Alpelisib,Copanlisib
PTEN	13	Everolimus,Temsirolimus,Copanlisib
FBXW7	19	Everolimus,Temsirolimus
STK11	7	
TSC1	2	
EGFR	16	Osimertinib,Gefitinib,Erlotinib,Icotinib,Afatinib,Dacomitinib
FGFR1	16	Pazopanib,Ponatinib
FGFR3	3	Pazopanib,Erdafitinib
BRCA1	10	Olaparib,Niraparib,Rucaparib,Talazoparib
BRCA2	7	
ERBB2	9	Trastuzumab,Pertuzumab,T-DM1,Lapatinib,Neratinib,Pyrotinib
ERBB3	6	
MET	7	Crizotinib,Cabozantinib
KDR	6	Regorafenib,Pazopanib
VEGFA	5	Sorafenib
ARAF	3	
CD274	3	Pembrolizumab,Nivolumab,Atezolizumab,Avelumab,Durvalumab,Cemiplimab,Toripalimab,sintilimab,Camrelizumab
PDCD1LG2	3	Pembrolizumab,Nivolumab,Cemiplimab,Toripalimab,sintilimab , Camrelizumab
PDGFB	2	Imatinib,Sunitinib , Pazopanib , Sorafenib

Figure 5 Analysis of mutations in target genes for available drugs. (A) The mutation of actionable genes in 225 ESCC patients. The X-axis displays each detected actionable gene and the Y-axis displays the mutation frequency of these genes. Green represents substitution/insertion-deletion mutations, red represents gene amplification mutations, blue represents homozygous gene deletion mutations, yellow represents fusion/rearrangement mutations, purple represents truncation mutations, and light blue represents splice site mutations. (B) Drugs corresponding to the actionable genes are shown above the bar for each gene.

cancers.^{34–37} Similar to a previous study,³⁷ we also observed frequent amplification of *CCND1*, *FGF19*, *FGF3*, and *FGF4*, which supports the occurrence of chromosome 11q13 instability in ESCC patients. Together, our

results confirmed previous studies and supported the existence of geographically distinct molecular ESCC profiles.

KMT2D, also known as *MLL2/ALR/MLL4*, encodes a conserved protein of the SET1 family of histone lysine

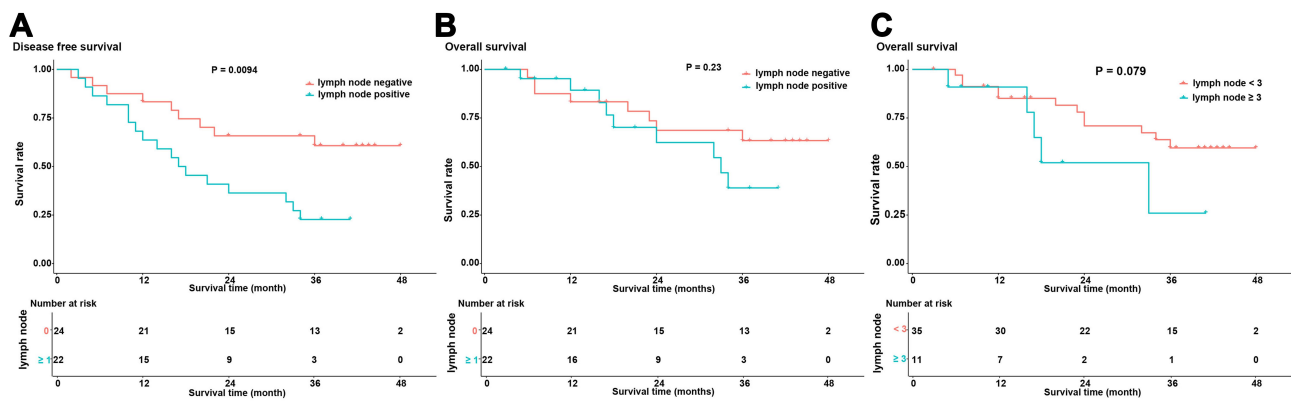


Figure 6 The correlation between metastatic lymph node status and the prognosis of ESCC patients. **(A)** The differential analysis of disease-free survival of patients with and without metastatic lymph nodes. The X-axis displays survival time and the Y-axis displays survival rate. Blue represents the patients without metastatic lymph nodes and red represents the patients with metastatic lymph nodes. **(B)** The differential analysis of overall survival of patients with and without metastatic lymph nodes. The X-axis displays survival time and the Y-axis displays survival rate. Blue represents the patients without metastatic lymph nodes and red represents the patients with metastatic lymph nodes. **(C)** Analysis of the overall survival of patients with <3 metastatic lymph nodes and greater ≥ 3 metastatic lymph nodes. The X-axis displays survival time and the Y-axis displays survival rate. Blue represents the patients with <3 metastatic lymph nodes and red represents the patients with ≥ 3 metastatic lymph nodes.

methyltransferases; it functions as a major histone H3 lysine 4 mono- and di-methyltransferase at enhancer regions in mammalian cells.³⁰ Mutation of *KMT2D* frequently occurs in follicular lymphoma and diffuse large B-cell lymphoma.^{38,39} Mutation of *KMT2D* has also been reported as a tumor inhibitor, but few studies have reported a high frequency of *KMT2D* mutations in ESCC.⁴⁰ Mutation of *KMT2D* is reportedly associated with poor prognosis in many cancers, such as non-small-cell lung carcinoma, breast cancer, and ovarian metastases of colorectal cancer.^{41–43} On the contrary, mutation of *KMT2D* correlates with longer survival of small cell lung cancer patients.⁴⁴ These findings indicate that the molecular mechanism of *KMT2D* in tumors is complex and incompletely understood. In this study, we identified frequent mutation of *KMT2D* and found that these mutations were associated with sex, early tumor stage, and high TMB. High TMB is considered to associate with a greater opportunity to benefit from immunotherapy.⁴⁵ In addition, we found that patients with a *KMT2D* mutation had better disease-free and overall survival than those without a *KMT2D* mutation, suggesting that *KMT2D* mutation is associated with a good ESCC prognosis. Together, our results suggest that *KMT2D* could be used as a prognostic biomarker for Chinese patients with ESCC.

NOTCH2 has been reported as an independent prognostic biomarker for ESCC.⁴⁶ *SPEN* is a regulator of NOTCH signaling.⁴⁷ The high expression of *SPEN* was associated with metastasis in breast cancer.⁴⁸ In nasopharyngeal carcinoma, *SPEN* expression is also associated with lymph node metastasis and tumor stage.⁴⁹ The increased

expression of *SPEN* was found to lead to poor prognosis, while reduced expression of *SPEN* suppressed migration and invasion of nasopharyngeal carcinoma.⁴⁹ These studies suggested that *SPEN* played an important role in tumor pathogenesis. Interestingly, both *NOTCH2* and *SPEN* mutations were associated with lymph node metastases in our cohort. A metastasis-positive lymph node is considered an important prognostic indicator for ESCC.⁵⁰ These results suggested that *SPEN* mutations may predict the poor prognosis of ESCC patients. Meanwhile, our results also showed the association between *SPEN* mutation and high TMB. Many studies have shown that with the increase of TMB-based immunotherapy, progression-free and overall survival are prolonged, and higher TMB is associated with a better prognosis.^{51–53} These results also suggested the potential association between *SPEN* mutations and a good prognosis. However, we did not find any correlation. Although the detection of *SPEN* expression was not performed, our results suggest that the role of *SPEN* in ESCC is also complex.

Lymph node metastasis often occurs in the development of many types of cancer, and its existence often implies a poor prognosis.⁵⁴ Interestingly, we identified associations between mutation of *FOS*, *DOT1L*, and *VEGFA* and metastasis-negative lymph nodes in ESCC. The mutation of *FOS* is reportedly associated with poor prognosis in colorectal cancer,⁵⁵ and high expression of *DOT1L* and *VEGFA* indicates poor prognosis in many cancers, such as lung cancer, gastric cancer, and clear cell renal cell carcinoma.^{56–59} These reports imply that mutation of these genes is potentially associated with the

prognosis of ESCC as well. However, we failed to detect a correlation between these mutations and the disease-free survival of ESCC. This may be due to the small number of patients with clinical follow-up. Meanwhile, based on the follow-up information of 46 patients, we observed that 3 positive lymph nodes may be a potential threshold for Chinese patients with ESCC to predict the prognosis. The optimal number of positive lymph nodes needed to predict prognosis remains controversial. This may be due to the different clinical and pathological manifestations of ESCC patients from different regions, including variations in histology, tumor location, and patient age.⁶⁰ Since China accounts for half of the global ESCC incidence,²⁸ it is important to determine the optimal threshold using more data from Chinese populations.

TMB is an emerging biomarker that correlates with the clinical outcome of cancer patients.^{61,62} Alexandrov et al showed lower TMB in esophageal cancer than that in lung cancer and melanoma.⁶³ In ESCC, it is reported that the optimal critical value of TMB was 7.3 mutations/Mb.⁶⁴ Although the median TMB was 7.7 mutations/Mb in this study, only TMB values higher than 10 mutations/Mb were considered high. This supported the reliability of correlations between high TMB-related gene mutations such as those in *KMT2B* and *SPEN*, and between high TMB and clinical characteristics such as drinking status. In addition, TMB is one of the indexes related to the effect of immunotherapy.⁶⁵ High TMB was reported to associate with prolonged progression-free and overall survival after immunotherapy.^{51–53} Together, our results support that *KMT2B* and *SPEN* may be used as potential prognostic biomarkers, as well as to provide more opportunities for non-drinking patients to benefit from immunotherapy.

Molecularly targeted therapy is a relatively new treatment for tumors. Drugs that target specific molecular targets, such as *EGFR*, *MTOR*, *PDCDI*, and *ALK*, have been developed and thoroughly investigated in the past decades.^{66–69} Targeting these genes influences the occurrence and development of tumors by inhibiting cell proliferation, angiogenesis, invasion, and metastasis; promoting cell apoptosis; and regulating key molecules or signal transduction pathways of inflammation.⁷⁰ Recently, studies reported that targeted therapies for *EGFR* mutations showed only limited success in improving the overall survival of ESCC patients in clinical trials.^{71,72} Although it is not an ideal guide for drug treatment, the assessment of genomic alterations can help us to better understand the molecular mechanisms of tumor development and identify targets for treatment. In this

cohort, for example, genetic profiling revealed that many of the patients were candidates for treatment with palbociclib, ribociclib, abemaciclib, everolimus, temsirolimus, olaparib, rucaparib, niraparib, talazoparib, trastuzumab, and pertuzumab based on their mutations.

In conclusion, we studied the mutational landscape of 225 Chinese patients with ESCC and found a correlation between TMB and tumor stage. We also analyzed the relationship between the number of metastatic lymph nodes and prognosis, and the potential prognostic role of *SPEN* and *KMT2D* mutations. However, a shortcoming of this study is the small number of samples with clinical follow-up. Future studies with larger cohorts and more clinical follow-up are necessary to confirm and expand on our results.

Abbreviations

AJCC, American Joint Committee on Cancer; ESCC, esophageal squamous cell carcinoma; FFPE, formalin-fixed paraffin-embedded; PI3K, phosphatidylinositol-3 kinase; TMB, tumor mutational burden.

Data Sharing Statement

The datasets used and analyzed in this study are available from the corresponding author upon reasonable request.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

Junping Shi, Xiaoliang Shi, and Wenting Chen are employees of Origimed Co. Ltd. The authors report no other potential conflicts of interest for this work.

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