Introduction
Esophageal squamous cell carcinoma (ESCC) is one of the most common and invasive cancers worldwide, with nearly 79% of ESCC occurring in Asian countries.[1] The incidence of ESCC had obvious regional characteristics, especially in Hebei province. Although great progress has been made in the traditional treatment of ESCC, the survival rate of patients has not significantly improved. Chinese and Japanese researchers in related fields have revealed the genetic landscape of ESCC in different regions by using next-generation sequencing (NGS), which provides an important basis for further exploration of the pathogenesis of ESCC and the search for therapeutic targets.[2-4] In this study, we collected 29 ESCC samples and detected 520 tumor-related genes. The preliminary construction of mutation gene profile related to surgically resected ESCC in high-incidence areas can further complement the genomic research of ESCC in China.

Endogenous (such as spontaneous deamination of 5-methylcytosines) and exogenous (such as ultraviolet radiation) factors cause DNA damage and induce DNA damage repair, leaving genomic imprints that can be detected by sequence analyses. Mutation spectra
mathematically extracted from these genomic imprints are called mutation signatures. These signatures reflect different genetic perturbations that occur before and during malignant transformation and progression.\(^5\) Currently, 30 different mutational signatures are published in the Catalogue of Somatic Mutations in Cancer (COSMIC). Previous studies have verified that mutational signature 1 and apolioprotein B messenger RNA-editing enzyme catalytic polypeptide (APOBEC)-mediated mutational signatures are common mutational signatures in ESCC.\(^5,6\) However, patients with ESCC have high heterogeneity and complex genomic mutational landscape. In order to further understand the details of complex mutation mechanisms, we quantified the contribution of the mutation spectrum of COSMIC to the sample mutation spectrum.

With the in-depth development of molecular biology, immunotherapy has shown its unique curative effect in advanced ESCC. A multi-center phase Ib/II trial (NCT02915432) showed that 59 patients with advanced refractory ESCC were treated with toripalimab (programmed death-1 [PD-1] inhibitors). Patients without CCND1/FGF3/FGF4/FGF19 amplification had significantly better objective response rates and progression-free survival than individuals with CCND1/FGF3/FGF4/FGF19 amplification.\(^7\) The genomic amplification of CCND1/FGF3/FGF4/FGF19 may be related to the poor prognosis of immunotherapy. The results of this study provide important ideas for further exploration of the treatment of ESCC, such as improving the accuracy of immunotherapy screening to improve efficacy. However, there are few biomarkers that can accurately predict the effects of immunotherapy. Previous analyses have shown that programmed death-ligand 1 (PD-L1) expression, tumor mutational burden (TMB), and microsatellite instability (MSI) may be biomarkers for the immunotherapy response.\(^8\) Although nivolumab and pembrolizumab (PD-1 inhibitors) are currently used for patients with advanced ESCC, the expression of PD-L1 in patients with resectable ESCC has not been explored, and few researches have been conducted on TMB and the MSI status in ESCC. We used immunohistochemical (IHC) 22C3 technology to detect PD-L1 expression, and further explored the correlation between PD-L1 expression and the detected gene mutation profile which can help to identify the best candidate for immunotherapy. Next-generation sequencing technology to detect TMB and MSI provides a theoretical basis for immunotherapy for patients with resectable ESCC.

**Methods**

**Ethical approval**

The study protocol was reviewed and approved by the Ethics Committee of the Fourth Hospital of Hebei Medical University (No. 2017009) and the requirement for written informed consent was waived.

**Patients and sample collection**

A total of 29 pairs of matched tissues (cancer tissues and adjacent tissues) were obtained from 29 patients with ESCC who underwent resection between 2015 and 2019 at The Fourth Hospital of Hebei Medical University. Among all patients, there were 16 males and 13 females, the mean age was 62 years (ranging from 51 to 73 years), and the patients with ESCC were at an early clinical stage (tumor, node, and metastasis [TNM] stage: IA-IIIB). There were nine patients with lesions in the upper segment, 13 patients with lesions in the middle segment, and seven patients with lesions in the lower segment [Supplementary Table 1, http://links.lww.com/CM9/A476].

**DNA extraction and sequencing**

DNA was extracted using QIAamp DNA formalin-fixed paraffin-embedded (FFPE) Tissue Kits (Qiagen, Valencia, CA, USA) according to the manufacturer’s instructions. The quality of DNA was evaluated using a NanoDrop 8000 (Nanodrop Technologies, Wilmington, DE, USA) and quantified using Qubit double-stranded DNA HS Assay Kits (Invitrogen, Carlsbad, CA, USA) on a Qubit 3.0 Fluorometer (Invitrogen). Shearing, end repair, and ligation were performed on DNA. The DNA fragments with 200 to 400 bp in size were selected with beads (Agencourt AMPure XP Kit; Beckman Coulter, Brea, CA, USA), followed by hybridization with probe baits, selection with magnetic beads, and polymerase chain reaction amplification. The indexed samples were sequenced on a NextSeq 500 sequencer (Illumina, San Diego, CA, USA).

**Sequencing data analysis**

Burrows-Wheeler aligner 0.7.10 (Dice Holdings, New York, NY, USA) was used to map reads to the human genome (hg19). Genome Analysis Toolkit 3.2 (Broad Institute, Cambridge, MA, USA), Picards (Broad Institute), and VarScan (Sourceforge, Mountain View, CA, USA) were optimized for local comparison, tag repetition, and mutation calling. The VarScan filter pipeline was used to filter the variants and loci with depths less than 100. Insertions or deletions required at least five supported reads, and single nucleotide variants (SNVs) required eight supported reads. A panel of 520 genes which are closely related to cancer mechanisms and targeted therapies were analyzed using probe hybridization to detect the entire exon region of 312 genes and hot spot mutations (exons, introns, and promoter regions) of 208 genes. Aberrations, such as gene mutations, amplifications, and fusions, which had a clear clinical correlation with cancer, were detected in a comprehensive and accurate manner [Supplementary Table 2, http://links.lww.com/CM9/A476]. TMB was calculated by adding all of the detected somatic variants and dividing this number by the size of the target region. Gene detection can identify the microsatellite status. The presence of 13 or more microsatellite site errors indicated the MSI-H status. In most MSI-H tumors, but not in MSI-low or microsatellite stable (MSS) tumors, the expression of mismatch repair (MMR) proteins is absent or significantly decreased.

**Evaluation of PD-L1 expression**

The expression levels of PD-L1 were evaluated by the PD-L1 IHC 22C3 pharmDx assay (Merk, Kenilworth, NJ, USA). The tumor proportion score refers to the percentage...
of tumor cells with partial or intact membrane staining in all live tumor cells in the sample.[11]

Statistical analysis

The R Package Mutational Patterns (https://doi.org/10.1101/071761) were used to evaluate and visualize a multitude of mutational patterns in base substitution catalogs. Mutational signatures can be extracted from the mutation count matrix by using non-negative matrix factorization.[12]

The IBM SPSS Statistics software application (version 24.0; IBM, Armonk, NY, USA) was used for the data analysis. Spearman rank correlation was used to analyze correlations between detected genomic alterations and clinicopathological characteristics and PD-L1 expression. A two-sided P value less than 0.05 was considered statistically significant.

Results

High-frequency mutation gene profile of 29 patients with ESCC

Targeted NGS was performed on 29 surgically resected ESCC tissues and adjacent normal tissues using a 520-cancer-gene panel. Among 520 genes closely related to cancer mechanism and targeted therapy, 421 genomic alterations were identified, of which there were 230 SNVs, 35 insertions and deletions (INDELs), 147 copy number amplifications, and nine copy number deletions [Figure 1, Supplementary Table 3, http://links.lww.com/CM9/A476]. The most frequently mutated gene was TP53 (17.2%, 5/29), followed by NOTCH1 (96.6%, 28/29), and EP300 (27.6%, 8/29), PRKDC (17.2%, 5/29), and KMT2C (17.2%, 5/29).

Comparing the detected mutation sites with those in the COSMIC database, 192 novel mutation sites were discovered, with the TP53 hotspot mutations, p.R248Q, and p.R175H, in four samples and the PIK3CA hotspot mutation, p.H1047L, in one sample.

Copy number variation occurred in 69.0% (20/29) of patients with ESCC. CCND1/FGF3/FGF4/FGF19, colocalized on 11q13.3, were co-amplified in The Cancer Genome Atlas database. Among the copy number amplified genes, the CCND1/FGF3/FGF4/FGF19 gene cluster had the highest frequency (41.4%, 12/29), followed by NKK2-1 (17.2%, 5/29), NFKBIA (13.8%, 4/29), TP63 (10.3%, 3/29), IL7R (10.3%, 3/29), PGR (10.3%, 3/29), and ERBB2 (10.3%, 3/29). The copy number deleted genes were CDKN2A/2B (10.3%, 3/29) and MET (6.9%, 2/29).

Notably, NOTCH1 (31.0%, 9/29), NFKBIA (20.7%, 6/29), CDKN2A (20.7%, 6/29), TRRAP (13.8%, 4/29), PIK3CA (13.8%, 4/29), CHD4 (13.8%, 4/29), DOTIL (10.3%, 3/29), FAT3 (10.3%, 3/29), KDM5A (10.3%, 3/29), and KEAP1 (10.3%, 3/29) harbored alterations in at least 10% of the primary tumors.

Mutational signatures of ESCC

The total number of point mutations was 183, and the most common type of mutation was the C>T transition, followed by C>G and C>A [Figure 2A, Supplementary Table 4, http://links.lww.com/CM9/A476]. We extracted four mutational signatures from ESCC with varying mutational activities [Figure 2B, Supplementary Table 5, http://links.lww.com/CM9/A476]. The similarities between signatures A–D and each COSMIC signature were calculated [Figure 2C, Supplementary Table 6, http://links.lww.com/CM9/A476]. Signatures A–D showed low similarity to any COSMIC signature (the highest cosine similarity was 0.67), which may be due to the limited clinical sample size or novel mutational signatures in ESCC.

In order to further understand the details of the complex mutation mechanism, the contribution of any signature set to the sample mutation spectrum was also quantified. The contribution of signature 1, APOBEC-mediated mutational signatures (signature 2 and signature 13), signature 10, signature 12, and signature 17 was relatively high in all samples (58.6%, 51.7%, 41.4%, 31.0%, and 31.0%, respectively) [Supplementary Table 7, http://links.lww.com/CM9/A476]. We noted that two samples showed high similarity to COSMIC signature 1 (cosine similarity 0.76 and 0.71, respectively). Signature 1 was the result of an endogenous mutational process initiated by spontaneous deamination of 5-methylcytosine [Figure 2D, Supplementary Table 8, http://links.lww.com/CM9/A476].[13] Signature 2 was characterized by C>T mutation. One sample was highly similar to COSMIC signature 13 (cosine similarity 0.93). Signature 13, which primarily resulted in the C>G mutation, was related to the increase of the activity of the APOBEC family-mediated mutagenesis.[14] Signature 10 exhibited strong transcriptional strand bias for T>C substitutions. The etiology of signature 17 remains unknown. It showed that signature 6 and signature 16 highly contributed to sample 16 and sample 24, respectively [Figure 2E]. Signature 6 was associated with small INDELs (mostly 1 bp) with a large number of single/poly nucleotide repeats. Signature 16 was characterized by T>C mutations in the ApTpN context.

Functionally aberrant pathways in ESCC

Cell cycle regulators constituted the most frequently disrupted category, including mutations in TP53 (96.6%, 28/29), ER300 (17.2%, 5/29), CREBBP (10.3%, 3/29), STAG2 (10.3%, 3/29), RB1 (6.9%, 2/29), PRKDC (6.9%, 2/29), ATM (6.9%, 2/29), ATR (6.9%, 2/29), MYC (3.4%, 1/29) and amplifications or deletions of CCND1 (41.4%, 12/29), CDKN2A/B (10.3%, 3/29), CDK6 (6.9%, 2/29), and CCNE1 (6.9%, 2/29).

Genetic alterations associated with chromatin modification occurred in 79.3% (23/29) of ESCC samples. Components of the SWI/SNF (SWItch/Sucrose Non-Fermentable) complex, including AT-rich interaction domain 1A (ARID1A) (4/29, 13.8%), ATRX (3/29, 10.3%), ARID2 (1/29, 3.4%), and SMARCA4 (1/29, 3.4%), were mutated in ESCC. Four mutations and two amplifications in CHD4 were identified. TP63 was amplified in 10.3% (3/29) of ESCC tumors.
Genes involved in the Janus kinase-signal transducer and activator of transcription (JAK-STAT) signaling pathway were altered in 75.9% (22/29) of tumors, and included mutations in JAK1, JAK2, JAK3, STAT3, and PIK3CA. The amplification of IL7R was found in 10.3% (3/29) of cases. Altered genes in the Notch signaling pathway, playing an important role in regulating normal cell differentiation, were mutated in 48.3% (14/29) of cases. NOTCH1 showed mutations in eight cases. In addition, the mutations in CREBBP and EP300 were detected in three and five samples, respectively. NOTCH1 and NOTCH3 were amplified in 6.9% (2/29) of cases.

**Biomarkers to predict the efficacy of immunotherapy**

Among 29 samples evaluated for PD-L1 expression, 13.8% (4/29) of samples had TPS ≥1% [Figure 3A–C]. By performing targeted NGS in 29 ESCC samples, we found the median value of TMB was 5.6 mut/Mb, ranging from 0.8 to 42.9 mut/Mb [Figure 3D]. There were 17.2%
Figure 2: Mutational signatures of ESCC. (A) Point mutation type. Single nucleotide substitutions are divided into six categories. (B) Signature A–D. (C) Pairwise cosine similarity between signature A–D and COSMIC signatures. (D) Cosine similarity between mutational profiles and COSMIC signatures. (E) The contribution of any set of signatures to the mutational profile of a sample can be quantified. COSMIC: Catalogue of somatic mutations in cancer; ESCC: Esophageal squamous cell carcinoma.
which were higher than 10 mut/Mb. All samples were MSS.

**Relationship between gene mutation and clinicopathological characteristics and PD-L1 expression**

KMT2D mutations were associated with lymph node metastasis \((r = 0.407, \ P = 0.028)\), CCND1/FGF3/FGF4/FGF19 amplification \((r = -0.473, \ P = 0.009)\) and CDKN2A deletion \((r = -0.654, \ P < 0.001)\) were associated with the depth of infiltration. TMB was associated with lymph node metastasis \((r = 0.468, \ P = 0.010)\), and it was not significantly associated with PD-L1 expression \((r = 0.246, \ P = 0.198)\). PD-L1 expression was not significantly associated with the detected genetic variations (all \(P > 0.05\)) [Table 1].

**Table 1: Correlation analysis results between gene mutation and clinicopathological characteristics and PD-L1 expression in 29 esophageal squamous cell carcinoma samples.**

| Items                  | Age     | Smoking | Drinking | Site     | Differentiation | T-stage | N-stage | PD-L1 |
|------------------------|---------|---------|----------|----------|-----------------|---------|---------|-------|
| TMB                    | 0.068   | -0.236  | -0.256   | -0.189   | 0.230           | -0.320  | 0.468†  | 0.246 |
| CCND1/FGF3/FGF4/FGF19  | 0.164   | -0.080  | -0.362   | -0.198   | 0.165           | -0.473* | -0.023  | 0.042 |
| NOTCH1                 | 0.175   | -0.064  | -0.081   | 0.153    | 0.181           | 0.074   | -0.144  | -0.267 |
| CDKN2A                 | 0.010   | -0.048  | -0.315   | 0.022    | 0.024           | -0.654† | -0.301  | 0.025 |
| KMT2D                  | 0.018   | 0.099   | 0.201    | 0.051    | 0.285           | 0.043   | 0.407†  | -0.160 |
| NFKBIA                 | 0.363   | -0.048  | -0.315   | -0.175   | 0.024           | -0.339  | -0.227  | -0.204 |
| ARID1A                 | -0.222  | -0.107  | 0.201    | -0.282   | 0.139           | 0.043   | 0.240   | 0.110 |
| EP300                  | 0.099   | 0.019   | 0.127    | 0.141    | -0.038          | 0.260   | 0.067   | -0.182 |

Data are presented as correlation coefficients \((r)\). †\(P < 0.05\), *\(P < 0.01\). ARID1A: AT-rich interaction domain 1A; PD-L1: Programmed death-ligand 1; TMB: Tumor mutation burden.

(5/29) which were higher than 10 mut/Mb. All samples were MSS.

**Discussion**

Although the traditional treatment methods have made great progress in ESCC, the survival rate of patients has not significantly improved, so exploring the pathogenesis and biological characteristics has become the primary task. We used next-generation sequencing to preliminarily construct mutation gene profile related to surgically resected ESCC in high-incidence areas, and further explored the details of the complex mutation mechanism and biomarkers to...
predict the efficacy of immunotherapy, which provides the possibility for developing more precise treatment options.

This study found that the most frequently mutated genes were TP53, followed by NOTCH1, EP300, KMT2C, and so on. The most frequently amplified and deleted genes were CCND1/FGF3/FGF4/FGF19 and CDKN2A/2B. This is consistent with the results of a previous study. Although the sample size of this study is small, this study preliminarily constructed mutation gene profile related to surgically resected ESCC in a high-incidence area of Hebei Province, which can further complement the genomic research of ESCC in China.

The ESCC has high heterogeneity and complex molecular mechanism, and the details of these mechanisms require further exploration. An analysis of mutational signatures could be a promising new tool for molecular tumor diagnosis and classification. In this study, we identified four mutational signatures (signatures A–D) in ESCC. The similarity between mutation signatures A–D and COSMIC mutation signatures was low, and their clinical significance was not clear.

We quantified the contribution of any set of COSMIC signatures to the sample mutation spectrum. Signature 1 and APOBEC-mediated mutational signatures (signature 2 and signature 13) were the most commonly observed signatures in ESCC samples, which is consistent with the previous study. Lin et al. found that patients with APOBEC-mediated mutational signature had more targeted driver genes including ZNF750, PIK3CA, MLL2, MLL3, and RB1. We also found that signature 10, signature 12, and signature 17 had relatively high contributions. Signature 10, which was previously associated with altered activity of the error-prone POLE, generates a massive number of mutations in uterine cancer and colorectal subsets. However, POLE was not altered in our samples, which may be related to the low mutational activity of signature 10. Signature 17 is associated with lung adenocarcinoma, breast cancer, B-cell lymphoma, liver cancer, gastric cancer, and melanoma. Signature 17 has been shown to be associated with high neoantigen load, which means that these patients may require immunotherapy. Signature 6 and signature 16 had relatively high contributions in two samples. The signature 6, present in microsatellite unstable tumors, is closely related to the inactivation of the DNA MMR gene. However, the samples with the contribution of signature 6 in our cohort were all MSS. Li et al. found that signature 16 was significantly associated with alcohol consumption. The patient linked to sample 24 had a long history of drinking, indicating a possible association between signature 16 and alcohol consumption in ESCC.

Further analysis revealed genetic variants were associated with cell cycle, chromatin modification, Notch and JAKSTAT signaling pathways, which may be key pathways in the development and progression of ESCC.

Genetic alterations in the SWI/SNF complex were induced at an early stage of esophageal squamous cell carcinoma, which were detected in 13.8% (4/29) of ESCC samples in the study, is a non-catalytic subunit of the SWI/SNF chromatin-remodeling complex that regulates gene transcription. TP63 (10.3%, 3/29) encodes TAp63, which is functionally similar to TP53 and ΔNp63, which lacks the transcription-activating domain of TAp63, and seems to have strong carcinogenicity. The CDKN2A expression encoding the E1A-binding protein p300 was mutated in 5 samples (5/29, 17.2%), and the incidence was higher than a Japanese study (8.3%). Cancer-associated histone acetyl transferases (HAT) domain-altering mutations and deletions impair the HAT activity of p300, leading to the hypothesis that p300 and CREBBP acetyltransferase activities might be tumor-suppressive.

Effective molecular targeted drugs for ESCC with improved therapeutic efficacy and few adverse reactions are highly anticipated. CDKN2A was amplified or mutated in 20.6% of samples in the study. CDKN2A is a multifunctional gene that produces p16 and p19 to arrest the cell cycle at the G1/S checkpoint through cyclin-dependent kinases 4/6 (CDK4/6)-regulated mechanism, and the proteins bind to murine double minute 2 to block the reduction in p53 levels. CDK4/6 is a potential target in CDKN2A-deficient tumors. Palbociclib has already shown efficacy and safety in metastatic liposarcoma. CDKN2A could also be a target for anti-cancer therapy in ESCC.

Traditional anti-tumor strategies have not shown significant survival benefits, which prompted the development of new treatments for patients with ESCC. Immunotherapy showed good efficacy for esophageal cancer, and the commonly used efficacy predictors included PD-L1 and TMB. This study found that TMB is associated with lymph node metastasis and has no significant association with PD-L1 expression, consistent with prior reports. Singal et al. explored the correlation between the genome and clinicopathological characteristics of 4064 patients with non-small cell lung cancer, and found that there was no significant correlation between TMB level and PD-L1 expression. Therefore, PD-L1 and TMB may be two independent biomarkers. However, there are not many studies on TMB in ESCC. The relationship between TMB and PD-L1 still needs to be further explored in large samples.

Exploring the correlation between PD-L1 expression and detected gene mutations can help further understand the carcinogenesis mechanism regulated by PD-L1. Kim et al. found that the loss of ARID1A was closely related to the high expression of PD-L1 in gastric cancer. In this study, due to the limited sample size, the positive rate of PD-L1 expression was low, and there was no significant correlation between PD-L1 expression and the detected genetic variation. Further expanding the sample size in the future and exploring the correlation between PD-L1 expression and the ESCC will help find the best candidate for immunotherapy.

In this study, the clinical significance of KMT2D mutation, CCND1/FGF3/FGF4/FGF19 amplification, and CDKN2A deletion in ESCC progression and metastasis was identified by analyzing the relationship between gene mutation and clinicopathological characteristics. KMT2D mutation, CCND1/FGF3/FGF4/FGF19 amplification, and CDKN2A...
deletion may be new prognostic factors and therapeutic targets for ESCC.

This study also had some limitations. The sample size was limited. Furthermore, this study was based on selected cancer-related genomes, and we may have missed some important genes or signaling pathways.

In conclusion, our research initially constructed mutation gene profile related to surgically resected ESCC in high-incidence areas, and provided new ideas for precise targeted therapy and precise immunotherapy of ESCC.

Conflicts of interest

None.

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