

# Genetic Heterogeneity of Esophageal Squamous Cell Carcinoma with Inherited Family History

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**Background:** Esophageal squamous cell carcinoma (ESCC) is a common malignant tumor with significant geographical variation and familial aggregation. However, the potentially different mechanisms underlying tumorigenesis in patients with ESCC with and without a family history of the disease remain unclear. In this study, the genes mutated in familial and nonfamilial ESCC were analyzed. Further, we aimed to explore the genes related to ESCC and attempt to identify potential patients in families with a history of ESCC.

**Methods:** Next-generation sequencing technology was used to examine germline mutations and mutation profiles in 36 matched tumor-normal ESCC specimens. Additionally, tumor mutational burden (TMB) values were measured in two cohorts.

**Results:** We identified four novel germline mutations in patients with familial ESCC, in *BAX* (c.121dupG: p.E41G), *CDKN2A* (c.374dupA: p.D125E), *TP53* (c.856G>A: p.E286K), and *CHEK1* (c.923+1G>A). Mutation profiles revealed that patients with and without a family history of ESCC had similar high-frequency gene mutation profiles, among which *TP53* was the most commonly mutated gene. Additionally, tumor-specific mutated genes in patients with a positive family history of ESCC were *APC*, *AKT3*, *DPYD*, *EP300*, *NFE2L2*, *PPP2R1A*, *RUNX1*, and *VEGFA*, while those in patients without a family history of ESCC were *CXCR4*, *PIK3R2*, *SMARCA4*, and *TTF1*. Moreover, patients with positive family history had significantly higher TMB values ( $7.8 \pm 4.1$  vs  $5.0 \pm 2.4$ , for patients with and without a family history, respectively;  $P = 0.038$ ).

**Conclusion:** Our results identified mutation profiles in patients with familial and nonfamilial ESCC, and identified germline mutations in patients with positive history. TMB values may be informative for immunotherapy approaches in familial ESCC.

**Keywords:** esophageal squamous cell carcinoma, genetic heterogeneity, family heredity

## Introduction

Esophageal cancer, of which esophageal squamous cell carcinoma (ESCC) is the major histological subtype, is the eighth leading cause of cancer-related death worldwide.<sup>1,2</sup> The burden of ESCC incidence is closely related to geographical distribution and more than half of all ESCC cases worldwide are diagnosed in China.<sup>3,4</sup> The significant geographical variation implies that environmental factors may play important roles in the development of ESCC. However, genetic factors may also contribute to the susceptibility to ESCC.<sup>5</sup> Data from our hospital showed that individuals whose parents or siblings had ESCC account for 11.06% of all patients with ESCC. Despite improved treatments, including curative surgery, chemoradiotherapy, and other treatments, the long-term outcomes of ESCC have remained unsatisfactory.<sup>6,7</sup>

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The development of new biochemical technologies, especially next-generation sequencing (NGS), has greatly advanced our understanding of the genomic features of ESCC. Previous genome-wide association or whole exon sequencing analyses have mainly compared patients with ESCC to those without ESCC to screen out mutated genes, including *TP53*, *CDKN2A*, *NOTCH1*, and *PIK3CA*.<sup>8,9</sup> However, few studies have reported genetic mutations associated with familial ESCC and none have not concentrated on the different tumorigenesis mechanisms in familial and non-familial ESCC.<sup>10</sup>

In this study, we used NGS technology to explore the inheritance of genes related to ESCC and attempted to identify potential patients in families with a history of ESCC.

## Methods

### Study Subjects

A total of 40 patients with ESCC were recruited from Sichuan Cancer Hospital from February 2013 to February 2017. All diagnoses were confirmed by independent experienced pathologists. At the time of recruitment, study subjects were interviewed in detail about whether their parents, siblings, or children had ever been diagnosed with ESCC. For patients whose relatives had a history of cancer, we gathered information about their age at cancer diagnosis, physical condition, or age at death. We collected the vital status of patients with relatives with no history of cancer. The inclusion criteria were: 1) histological diagnosis of resectable ESCC in patients over the age of 18; 2) availability of tumor tissue samples by operation or puncture and corresponding adjacent non-tumor tissues (located more than 3 cm from the tumors); and 3) availability of blood samples. Patients with other severe systemic disease, metastasis, or any preoperative neoadjuvant therapy were excluded from the study. This study was approved by the Ethics Committee of Sichuan Cancer Hospital (SCCHEC-02-2017-043). All participants signed informed consent, in accordance with the Declaration of Helsinki, for specimen collection and genetic testing following a detailed description of the purpose of the study.

### DNA Extraction and Quantification

Formalin-fixed and paraffin-embedded (FFPE) blocks were de-paraffinized twice with xylene and DNA was extracted using a QIAamp DNA FFPE Tissue Kit (Qiagen) following the manufacturer's instructions.

Unstained FFPE sections (n = 5–8) with an area greater than 0.5 × 0.5 cm and thickness of 6–10 µm were used for sequencing. More sections were used if the tissue area was less than 0.5 cm. In this study, the total amount of DNA extracted from samples ranged from 88 to 17,300 ng. All extracted samples met the required DNA yield for quality control, which is 50 ng. Extracted DNA was purified, qualified using a Nanodrop2000 (Thermo), and quantified by Qubit3.0 (Life Technology) using a dsDNA HS Assay Kit (Life Technology) following the manufacturer's protocols.

### Library Preparation and Sequencing

DNA libraries were subjected to polymerase chain reaction (PCR) amplification and purification before targeted enrichment. Libraries from different samples were marked with unique indices during library preparation and up to 2 µg of different libraries were pooled together for targeted enrichment. Human cot-1 DNA (Life Technologies) and xGen Universal Blocking Oligos (Integrated DNA Technologies) were added to block nonspecific binding of library DNA to targeted probes. Customized xGen lock-down probe panels (Integrated DNA Technologies) were used for targeted enrichment of 425 predefined genes. The hybridization reaction was performed using NimbleGen SeqCap EZ Hybridization and Wash Kit (Roche). Dynabeads M-270 (Life Technologies) were used to capture probe-bound fragments. Then, the library was amplified with Illumina p5 and p7 primers in KAPA HiFi HotStart ReadyMix (KAPA Biosystems) and purified using Agencourt AMPure XP beads. Library quantification was achieved using the KAPA Library Quantification kit (KAPA Biosystems). The size distribution was measured using Agilent Technologies 2100 Bioanalyzer (Agilent Technologies). Enriched libraries were sequenced on HiSeq 4000 NGS platforms (Illumina) to coverage depths of at least 100 x and 300 x for normal tissue and tumor, respectively, after removing PCR duplicates.

### Bioinformatics Analysis

Original HiSeq 4000 sequencing platform data were transferred by base calling analysis into raw sequence data containing both sequence and sequencing quality information. Single-nucleotide variants (SNVs) and short insertions/deletions (indels) were identified using VarScan2 with the minimum variant allele frequency threshold set at 0.01, and the p-value threshold for calling variants set at 0.05 to generate Variant Call Format (VCF) files. All

**Table 1** Clinical Characteristics of Esophageal Squamous Cell Carcinoma Patients

Characteristics	Positive Family History (N=18)	Negative Family History (N=18)	P-value
Age at initial diagnosis (years) Median (range)	62.5 (45–77)	61.5 (44–76)	0.969
Gender			>0.999
Male	14 (77.8%)	14 (77.8%)	
Female	4 (22.2%)	4 (22.2%)	
Stage at initial diagnosis			0.764
IIA	5 (27.8%)	4 (22.2%)	
IIB	5 (27.8%)	7 (38.9%)	
IIIA	5 (27.8%)	3 (16.7%)	
IIIB	3 (16.6%)	3 (16.7%)	
IIIC	0 (0)	1 (5.5%)	
Tumor location			0.776
Upper	4 (22.2%)	5 (27.8%)	
Middle	12 (66.7%)	11 (61.1%)	
Lower	2 (11.1%)	2 (11.1%)	
Smoking history			0.760
Yes	9 (50.0%)	7 (38.9%)	
No	8 (44.4%)	11 (61.1%)	
Unknown	1 (5.6%)	0 (0)	

**Table 2** Summary of Pathogenic Germline Mutations Detected in Esophageal Squamous Cell Carcinoma Patients

Patient ID	Gene	cDNA Change (Protein Change)	NM_ID	Hom/Het
M09-F	BAX	c.121dupG (p.E41Gfs*33)	NM_004324.3	Het
M10-F	CHEK1	c.923+1G>A	NM_001114121.2	Het
M14-F	TP53	c.856G>A (p.E286K)	NM_001126112.2	Het
F02-F	CDKN2A	c.374dupA (p.D125Efs*17)	NM_000077.4	Het

SNVs/indels were annotated with ANNOVAR, and each SNV/indel was manually checked on the Integrative Genomics Viewer (IGV). Copy number variations (CNVs) were detected using software developed in-house.

## Results

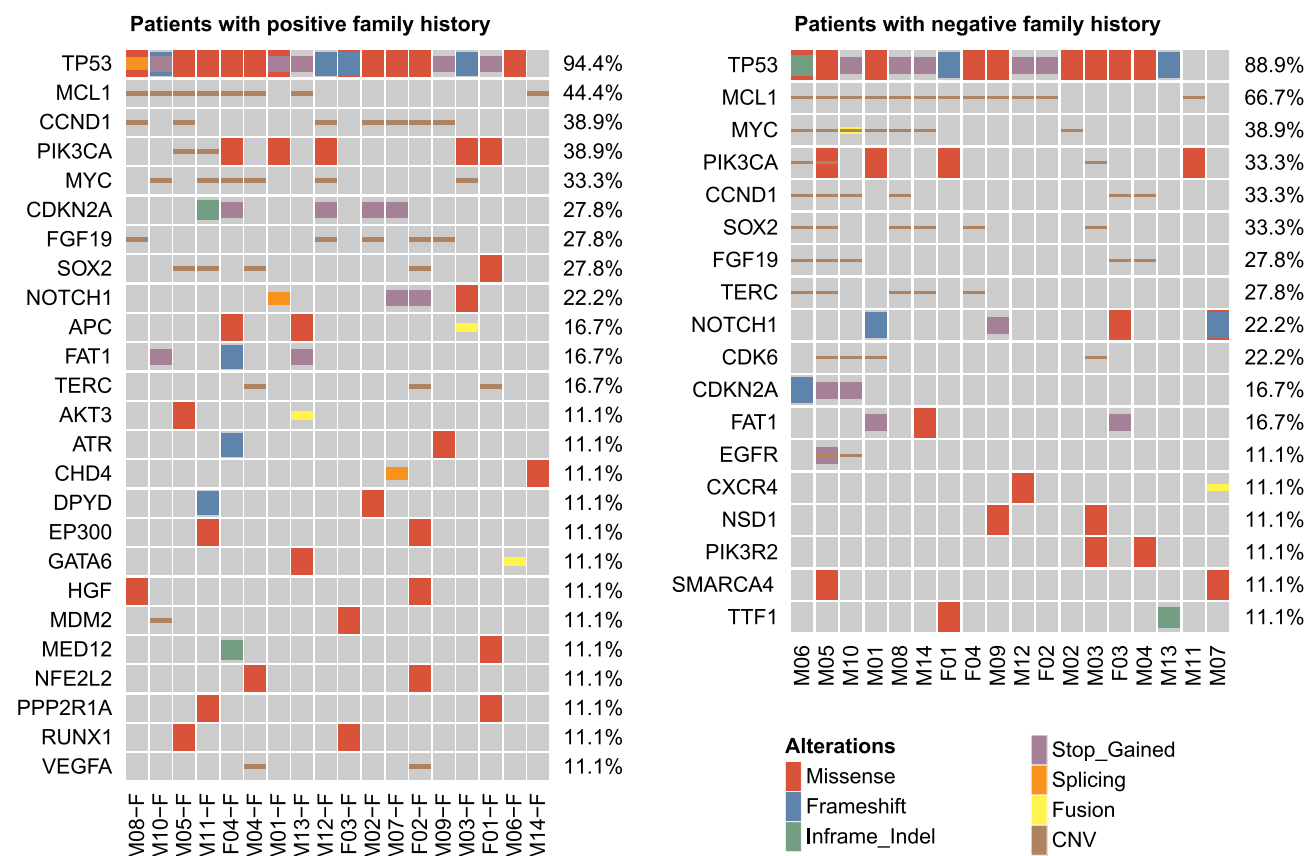
### Patient Characteristics

A total of 40 ESCC patients were included in the study. One cancer tissue specimen and one adjacent non-tumor tissue specimen were obtained from each patient for sequencing. Four patients were excluded because of unqualified sequencing and quality control. Sequencing information was obtained and analyzed from 36 patients, including 18 cases with family history and 18 cases without. Patient characteristics are presented in Table 1. Statistical differences were observed in age, gender, stage, tumor location,

and smoking status between the two cohorts ( $p < 0.05$  for each).

### Germline Mutations Detected in Patients with Positive Family History

Of the 36 patients with ESCC, four patients with family history (11.1%, 4/36) harbored germline mutations. The *CHEK1* mutation in patient M10-F was in an intron, while the remaining three germline mutations identified, *BAX* E41G in patient M09-F, *TP53* E286K in patient M14-F, and *CDKN2A* D125E in patient F02-F fell within exons (Table 2). We did not detect germline mutation in patients without a family history of ESCC. The parents of three of these four patients suffered from ESCC and the sibling of one patient had ESCC. Fisher exact tests were used to evaluate the correlation between pathogenic germline



**Figure 1** Tumor-specific mutation landscape in esophageal squamous cell carcinoma. The oncoprint presents the most frequent tumor-specific mutations accounting for more than 10% of patients with familial and non-familial ESCC.

mutation and family history of ESCC risk. The odds ratio (OR) of co-occurring germline mutation and low risk of familial ESCC was 0 (95% confidence interval (CI), 0–1.4,  $P = 0.104$ ). This implies that germline mutation may result in a greater risk of familial ESCC. However, this observation was not statistically significant, possibly because of the limited number of patients included in the study.

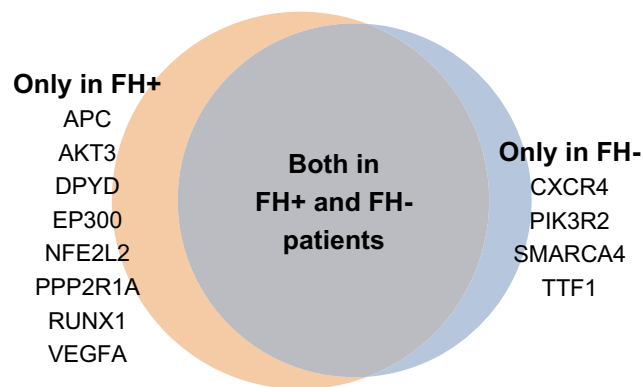
## ESCC Tumor-Specific Mutation Gene Profile

NGS results revealed that patients with familial and non-familial ESCC had similar high-frequency mutation gene profiles. The most frequent mutations observed were missense mutations (Figure 1). Patients with familial ESCC had mutation frequencies more than 20% in *TP53* (94.4%), *MCL1* (44.4%), *CCND1* (38.9%), *PIK3CA* (38.9%), *MYC* (33.3%), *CDKN2A* (27.8%), *FGF19* (27.8%), *SOX2* (27.8%), and *NOTCH1* (22.2%). Patients with non-familial ESCC had mutation frequencies more than 20% in *TP53* (88.9%), *MCL1* (66.7%), *MYC* (38.9%), *PIK3CA* (33.3%), *CCND1* (33.3%), *SOX2* (33.3%), *FGF19* (27.8%), *TERC* (27.8%),

*NOTCH1* (22.2%), and *CDK6* (22.2%). The most commonly mutated gene observed in both cohorts was *TP53*. In both cohorts, mutated genes were mainly observed in the *NOTCH1* and *PI3K* pathways. In patients with familial ESCC, the special high-frequency mutated genes, with a mutation burden more than 10%, were *APC*, *AKT3*, *DPYD*, *EP300*, *NFE2L2*, *PPP2R1A*, *RUNX1*, and *VEGFA*, and in patients with non-familial ESCC these genes were *CXCR4*, *PIK3R2*, *SMARCA4*, and *TTF1* (Figure 2). Most of these genes had missense mutations. These two groups of genes are mainly involved in the *WNT* and *VEGF* signaling pathways.

## Patients with Familial ESCC Had Higher Tumor Mutational Burden (TMB)

Comparisons of TMB values between patients with familial and non-familial ESCC are shown in Figure 3. The TMB values in patients with familial ESCC (TMB:  $7.8 \pm 4.1$  Muts/Mb) were significantly higher than those of patients with non-familial (TMB:  $5.0 \pm 2.4$  Muts/Mb,  $P = 0.038$ ). Therefore, patients with familial ESCC harbored more mutations in gene coding regions within ESCC tumors.

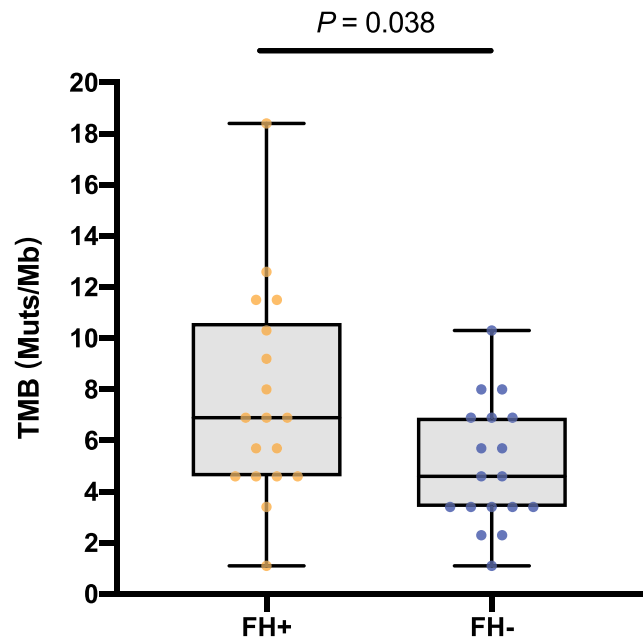


**Figure 2** Mutually exclusive tumor-specific gene mutations in patients with familial (FH+) and non-familial (FH-). The summary of mutual exclusive genes only includes genes with mutation frequencies higher than 10% in patients with positive family history or negative family history of ESCC.

## Discussion

ESCC seriously endangers human health. Numerous studies have shown that the incidence of ESCC varies geographically and is observed in family groups. In our study, we used NGS technology to explore germline mutations, gene mutation profiles, and their frequencies in patients with familial and non-familial ESCC. Additionally, TMB values for the two cohorts revealed that those with familial TMB had a higher tumor mutation burden. To our knowledge, this is the first study to explore mutations in patients with ESCC in western China. These results may reveal the molecular mechanisms of ESCC, and how familial and non-familial ESCC differ at the molecular level.

We identified four genes with germline mutations in patients with familial ESCC. The protein encoded by the *BAX* gene belongs to the BCL2 protein family, whose expression is regulated by the TP53 tumor suppressor. *CDKN2A* contains an alternate open reading frame (ARF) whose product functions as a stabilizer of TP53. One combined analysis of TP53, BAX, and CDKN2A showed that patients with ESCC and high levels of BAX and CDKN2A expression had a good prognosis. In our study, we identified mutations in *BAX* (c.121dupG: p.E41G fs\*33) and *CDKN2A* (c.374dupA: p.D125E fs\*17). However, no published studies about these alterations indicate how they could influence disease development in patients with ESCC at the mechanistic level. Interestingly, Clemons et al detected *TP53* mutation (c.856G>A: p.E286K) in a novel esophageal adenocarcinoma cell line, ONAC1, which was derived from a Barrett's-associated EA.<sup>11</sup> Creemers et al also found a *TP53* mutation (c.856G>A: p.E286K) in a series of adrenocortical



**Figure 3** TMB distribution by family history status in esophageal squamous cell carcinoma. The top and bottom of the boxes show the upper and lower and quartiles and the middle line is median. Mann-Whitney test was used for inter-group comparison and the P value calculation was two-tailed.

**Abbreviations:** FH+, patients with positive family history; FH-, patients with negative family history.

carcinoma case reports.<sup>12</sup> Of note, we identified a novel donor splice site mutation (c.923+1G>A) in *CHEK1*, which might affect the canonical splice sequence and result in abnormal CHEK1 proteins.

Our study highlights the high-frequency gene mutation profiles of patients with familial and non-familial ESCC, and highlights the differences between the two cohorts. Similar mutation profiles suggest that ESCC related mutations are more frequent in the NOTCH1 and PI3K pathways in both cohorts, which is consistent with previous reports.<sup>8,9,13</sup> This implies that additional factors, such as the environment, also play a major role in the development of ESCC. A strong cumulative effect of genetic risk factors has been described in familial ESCC, and the identification of the same SNPs in familial and non-familial ESCC can be explained by environmental factors.<sup>10</sup>

Herein, we observed some special high-frequency mutated genes in patients with familial ESCC, including *APC*, *AKT3*, *DPYD*, *EP300*, *NFE2L2*, *PPP2R1A*, *RUNX1*, and *VEGFA*. Deng et al showed that *EP300* and *NFE2L2* had high mutation rates in Asian patients with ESCC.<sup>14</sup> *EP300* encodes the adenovirus E1A-associated cellular p300 transcriptional co-activator protein, which plays an important role in cell proliferation and differentiation.



Previous studies have demonstrated that *EP300* mutation predicted a shorter survival time in patients with ESCC.<sup>15</sup> *NFE2L2* (also known as *NRF2*) encodes a transcription factor that plays a key role in cellular defense against oxidative stress and can be induced by radiotherapy through the production of reactive oxygen species in cancer cells. *NRF2* mutations caused patients with ESCC to respond poorly to chemoradiation therapy, resulting in a poor prognosis.<sup>16</sup>

We also observed mutations in the WNT (*APC*, *EP300*, and *PPP2R1A*) and VEGF (*AKT3* and *VEGFA*) signaling pathways. These molecular alterations that may contribute to the development of familial ESCC may represent the basis of novel drug development. However, Salem et al showed that gene mutations in the WNT pathway (*APC* and *RNF43*) were present in low frequencies in patients with ESCC.<sup>13</sup> This inconsistency may be due to methodological differences in the studies. *PPP2R1A* encodes the subunit of protein phosphatase 2A (PP2A), which is implicated in the negative control of cell growth and division. In a previous study, *PPP2R1A* was shown to promote the growth of malignant cells in uterine cancer,<sup>17</sup> but no studies have described the role of *PPP2R1A* in ESCC. Our study suggests that *PPP2R1A* may be a new candidate gene for facilitating our understanding of ESCC and establishing therapeutic targets.

The RUNX1 transcription factor is a differentiation regulator in hematopoietic cells, and *RUNX1* mutation often results in the development of leukemia. Jiang et al found that RUNX1 can promote cell proliferation in ESCC.<sup>18</sup> Additionally, DPYD is a rate-limiting enzyme in the catabolism of 5-fluorouracil (5-fu) and can transform 5-fu into inactive metabolites and reduce its side effects.<sup>19</sup> Our findings suggest that RUNX1 and DPYD might be further used as therapeutic targets to guide chemoradiotherapy in patients with familial ESCC, and could improve the safety of treatment.

The special high-frequency mutated genes, *CXCR4*, *PIK3R2*, *SMARCA4*, and *TTF1* were observed in patients with non-familial ESCC. CXCR4 is a chemokine receptor which plays a pivotal role in the regulation of cell migration. Lu et al reported that CXCR4-positive ESCC cells showed strong migration ability, especially to lymph nodes.<sup>20</sup> In a recent study by Song et al, the activation of the CXCL12/CXCR4 axis in vascular endothelial cells was shown to stimulate angiogenesis by upregulating MAPK/ERK, PI3K/AKT, and Wnt/ $\beta$ -catenin pathways.<sup>21</sup> *PIK3R2* is also involved in the MAPK/ERK and PI3K/AKT pathways. Additionally, in the present study, we found that *TTF1* mutation frequency was higher in patients with non-familial ESCC. Previously, *TTF1*

was associated with esophageal small cell carcinoma, which is more aggressive than ESCC and differs in response to therapy and prognosis.<sup>22</sup>

Finally, we analyzed TMB values in the two cohorts (Figure 3). TMB refers to the total amount of somatic non-synonymous mutations in the coding region of tumor genes. The higher the TMB value is, the more mutation-related new antigens are produced, leading to greater T lymphocytes infiltration, and a stronger anti-tumor immune response.<sup>23</sup> Previous studies have shown that high TMB, in combination with PD-L1 and MSI-H/dMMR, can be used to select patients who will benefit from immunotherapy.<sup>24,25</sup> Song et al reported that patients with ESCC and high TMB can benefit from PD-1 inhibitor treatment, but TMB, as a single predictor, may not accurately estimate the efficacy of immunotherapy.<sup>26</sup> In our study, patients with familial ESCC generally had higher TMB values than did those with non-familial ESCC, which could imply the former may benefit more from immunotherapy. Of note, Parikh et al reported that the TMB value in patients with frameshift mutations was significantly higher than that in patients with any other mutation type.<sup>27</sup> Our findings were consistent with this research. In the two cohorts, frameshift mutations were observed in *FAT1* and *ATR* in patient F04-F, *DPYD* in patient M11-F, *CDKN2A* in patient M06, and *NOTCH1* in patient M01 and these patients had higher TMB values. Interestingly, these mutations were identified to have durable immunotherapy benefits.<sup>28–31</sup> Therefore, it is of interest to further explore the relationship between frameshift mutations in specific genes and the conferred immunotherapy benefits.

Our study has some limitations. First, because of the strict enrollment conditions and funding constraints, we are unable to include more patients. Further studies with larger cohorts should be performed to validate our results and elaborate upon them. Secondly, TMB value may be related to smoking status. Due to small sample size in this study, subgroup analyses were not performed to investigate the relationship between TMB values and smoking status.

## Conclusion

Our results show that patients with familial and non-familial ESCC had similar high-frequency mutation gene profiles, but different harbored tumor-specific mutated genes. Moreover, we detected special germline mutations and higher TMB values in patients with familial ESCC, which suggest that they may have a greater benefit from immunotherapy. More importantly, these findings might provide novel insights into the molecular alterations in ESCC and assist in the identification of potential patients

in families with familial ESCC. These results might facilitate the development of precision targeted therapies for ESCC.

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## Disclosure

Yang Shao and Xue Li are employees of Nanjing Geneseeq Technology, Inc. The authors report no other potential conflicts of interest in this work.

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