ARTICLE

Whole-genome sequencing of 508 patients identifies key molecular features associated with poor prognosis in esophageal squamous cell carcinoma

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Esophageal squamous cell carcinoma (ESCC) is a poor-prognosis cancer type with limited understanding of its molecular etiology. Using 508 ESCC genomes, we identified five novel significantly mutated genes and uncovered mutational signature clusters associated with metastasis and patients’ outcomes. Several functional assays implicated that NFE2L2 may act as a tumor suppressor in ESCC and that mutations in NFE2L2 probably impaired its tumor-suppressive function, or even conferred oncogenic activities. Additionally, we found that the NFE2L2 mutations were significantly associated with worse prognosis of ESCC. We also identified potential noncoding driver mutations including hotspot mutations in the promoter region of SLC35E2 that were correlated with worse survival. Approximately 5.9% and 15.2% of patients had high tumor mutation burden or coding and noncoding genomic alterations and revealed three major subtypes that robustly predicted patients’ outcomes. Collectively, we report the largest dataset of genomic profiling of ESCC useful for developing ESCC-specific biomarkers for diagnosis and treatment.

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INTRODUCTION

Esophageal cancer (EC) remains the seventh most common cancer and the sixth leading cause of cancer deaths globally, with over 477,900 new cases and 375,000 annual deaths occurring in China. Esophageal squamous cell carcinomas (ESCC), the predominant histological subtype, is characterized by its aggressive clinical course and constitutes a poor-prognosis subgroup within EC. Epidemiologically, the incidence rate of ESCC shows marked geographical variation across China’s regions. It predominates in Taihang Mountain, Xinjiang province and Chaoshan district compared to the rest of China, and rapidly increases in other regions in China. ESCC is a highly heterogeneous disease with unclear molecular classifications and with variable clinical outcomes, and no prognostic biomarkers are available. The precise molecular events underlying ESCC etiology are only partially understood, leading to limited targeted therapies and insufficient clinical management in ESCC patients.

Recent whole-genome and -exome analyses in ESCC revealed a complex mutational landscape and identified significantly mutated genes (SMGs) including TP53, ZNF750, NOTCH1, FAT1, NFE2L2, recurrent copy number amplifications occurring in SOX2, TERT, FGFR1, MDM2, and common deletions of RB1. However, previous genomic studies in ESCC had several limitations including small sample sizes for the identification of rare driver genes, limited patient outcome information and the focus of targeted protein-coding sequence with little characterization of noncoding regions across ESCC genome.

Despite potential therapeutic targets, few genes altered in ESCC are clinically actionable and a very limited number of inhibitors are currently approved for treatment of advanced
ESCC. Although the classification of ESCC has been recently revised, the prognostic value of the molecular classification has not been fully elucidated.\(^{11}\) Hence, there is a clear need to identify novel cancer driver genes based on whole-genome sequencing (WGS) data and to explore additional prognostic biomarkers in a larger set of ESCC patients with available clinical data.

To overcome these challenges, we performed deep WGS in microneedle-punctured formalin-fixed paraffin-embedded (FFPE) tumor tissues and matched adjacent noncancerous specimens from 508 ESCC patients with clinical follow-up data. Herein, we classify ESCC into three main subtypes based on clinically relevant genomic alterations observed across 508 genomic profiles and implicate the association of these subtypes with patients’ outcomes. Our genomic study uncovers an extensive landscape of driver genetic alterations across coding and noncoding regions, and defines their potential molecular pathology in ESCC.

**RESULTS**

Genomic profiling of ESCC

Two clinical centers (Shanxi Cancer Hospital and Tumor Hospital affiliated to Xinjiang Medical University) in Shanxi and Xinjiang provinces, the districts with higher incidence of ESCC in China, participated in this study. WGS was performed on 508 pairs of ESCC tumors and matched adjacent noncancerous esophagus tissues (referred to as 508-WGS cohort). The cohort contains tumor samples collected at the time of diagnosis and includes 437 cases from Han population of Shanxi cohort and 71 cases from Kazak population of Xinjiang cohort (Supplementary information, Tables S1 – S3). All tumors were therapy naive.

The mean sequencing coverages for WGS were 98x and 44x for tumor tissues and matched nontumor samples, respectively. Sequence coverage exceeding 20x was 98.0% for tumors and 92.6% for normal samples. We detected 7,630,294 somatic mutations including single nucleotide variants (SNVs) and small insertions and deletions (InDels) with a median of 12,877 mutations per patient (Supplementary information, Tables S4, S5). We selected 153 mutations for further validation and 78/79 (98.7%) selected coding mutations and 69/74 (93.2%) selected noncoding mutations were confirmed by Sanger sequencing (Supplementary information, Tables S6, S7). A total of 66,260 (0.87% of total mutations) candidate somatic mutations occurred within coding regions linked to 14,971 genes of which, 61.57% were missense mutations and 5.82% were InDels (Supplementary information, Fig. S1a). The median number of coding mutations was 105.5 (range 1–1217). Among the 13,801 genes with amino acid altering changes (nonsynonymous/truncation), 65 showed mutation rates of > 5% in our cohort (Supplementary information, Fig. S1b).

Mutational signatures and association of APOBEC signature with patient stage

To better understand the contribution of these mutations for ESCC etiology, we investigate mutational signatures. Using a modified nonnegative matrix factorization (NNMF) algorithm,\(^{15,16}\) we identified 11 mutational signatures (S1 – S11) in the 508-WGS cohort (Fig. 1a; Supplementary information, Fig. S2a). Other than S7 and S10, all other signatures corresponded to mutation signatures in the COSMIC (Catalogue of Somatic Mutations in Cancer) database\(^{14}\) (Supplementary information, Fig. S2b and Table S8a). Comparing with the COSMIC signatures, we found that S1 and S2 were related with APOBEC (apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like) activity, S3 with DNA mismatch repair deficiency (dMMR), S4 with age, S8 with aristocratic acid, S9 with alcoholic consumption, and S11 with homologous recombination deficiency. S6 was similar to COSMIC signature S17 and recent studies implicated its association with gastric acid reflux.\(^{15,16}\)

Next, we correlated the proportions of different mutational signatures with patients’ clinical characteristics. Patients at advanced stages of ESCC had significantly higher proportions of S1, S2, and S11 than those at earlier stages (Fig. 1b). Conversely, the proportions of S3, S4, S9 and S10 were significantly larger in patients at earlier stages (Fig. 1b). These data suggest that multiple factors may contribute to the initiation of ESCC, whereas APOBEC activity may continuously contribute to mutagenesis during tumor progression as illustrated in a recent study using cancer cell lines.\(^{17}\) Moreover, we observed a strong correlation of S9, a signature similar to the drinking-related COSMIC-Signature 16, with both drinking (Cochran–Armitage-trend-test, \(P = 0.0005\)) and smoking (\(P = 0.00049\)) in 508-WGS cohort (Supplementary information, Fig. S2c, d). Drinking and smoking status were significantly interrelated in our cohort (Fisher Exact Test, \(P = 2.2e^{-16}\)), indicating that the significant association of S9 with smoking may be partially attributed to the correlation between smoking and drinking.

Interestingly, clustering analysis of NMF signatures displayed three distinct subtypes across 508 ESCC patients, denoted NMF-cluster 1–3 (Fig. 1c). Cluster 1 (100 patients) was dominated by APOBEC-associated signatures (S1 and S2) while cluster 3 (312 patients) was correlated with mismatch repair deficiency (S3) and spontaneous deamination of 5-methylcytosine (S4), and cluster 2 (96 patients) shared features with deficient homologous recombination repair signatures (S11 and S6). To further investigate APOBEC-associated signatures across 508 ESCC genomes, we examined the occurrence of C>T transition in TpCpW motifs.\(^{18}\)

Notably, all samples in cluster 1 were APOBEC signature-enriched whereas only a small proportion of samples in cluster 2 and cluster 3 were APOBEC signature-enriched (Supplementary information, Fig. S2e). The mean APOBEC signature enrichment score of cluster 1 was dramatically larger than that of cluster 2 and cluster 3 (2.83 vs. 1.60, Student’s t-test, \(P = 2.2e^{-16}\)). In addition, we found that ZNF750 mutations were highly enriched in cluster 1 (Fisher Exact Test, \(P = 2.42e^{-09}\), FDR = 4.35e-08) whereas mutations of FAT2 (Fisher Exact Test, \(P = 0.0117\), FDR = 0.21) and CASP8 (Fisher Exact Test, \(P = 0.041\), FDR = 0.39) showed a weak depletion in cluster 2 (Fig. 1d).

The overall survival (OS) rates were significantly different among three clusters, with APOBEC signature-enriched cluster 1 exhibiting worse prognosis (Kaplan–Meier analysis, log rank \(P = 0.0029\), FDR = 0.087, hazard ratio (HR) = 0.71, 95% confidence interval (CI), 1.007–1.746, Fig. 1e). Consistent with our findings, overexpression of APOBEC8 was associated with poor prognosis or treatment resistance in ER\(^+\) breast cancer.\(^{19,20}\) Moreover, NMF-cluster 1 was significantly associated with lymph node metastasis and advanced stages (Fisher Exact Test, \(P = 0.004\), 0.001, respectively, Table 1). Therefore, our results suggest that cluster 1 may be a reliable clinical predictor of metastasis and poor outcome in ESCC.

Hypermutations and its clinical correlation

Recently, tumor mutational burden (TMB) was proved to be an effective biomarker for immunotherapy. Patients with high TMB (TMB-H) generally had a better response to immunotherapy than those with low TMB.\(^{21}\) Of 508 ESCC patients, we found that 30 (5.9%) were hypermutated (TMB-H, > 10 mutations per Mb). In contrast, only 11 (2.2%) were hypermutated (TMB-H). The TMB-H was significantly associated with lymph node metastasis (M1 stage) (Fisher Exact Test, \(P = 3.72e^{-5}\)) or patients with high microsatellite instability (MSI-H) (Fisher Exact Test, \(P = 0.00102\)) (Fig. 2a; Supplementary information, Table S8b). In addition, TMB-H patients also exhibited a significantly higher proportion of the APOBEC-associated S2 mutations (Wilcoxon Test, \(P = 3.999e^{-05}\), Fig. 2b). Furthermore, the TMB was significantly larger in patients enriched with APOBEC-associated mutations within the TpCpW motif (Wilcoxon Test, \(P = 2.2e^{-16}\), Supplementary information, Fig. S2f), supporting that the APOBEC activity may be closely
related to the hypermutation phenotype. Among 30 TMB-H patients, 24 exhibited either MSI-H/dMMR mutation or APOBEC enrichment. Logistic regression coefficient analyses indicated that the hypermutation phenotype was related to MSI ($P = 1.34 \times 10^{-05}$) and APOBEC ($P = 5.30 \times 10^{-08}$) activity in ESCC (Supplementary information, Table S8c). Importantly, the TMB-H patients had significantly worse OS than other patients (Kaplan–Meier analysis, log rank $P = 0.011$, HR = 1.87, 95% CI, 1.14–3.07, Fig. 2c).

Significantly mutated genes (SMGs) in ESCC

Analysis of the 508-WGS data using the MutSigCV algorithm and oncodriveFML identified 22 candidate driver genes ($q < 0.1$, Fig. 3a). Analysis of six independent ESCC expression datasets showed that all of these 22 genes had moderate to high expression in ESCC (Supplementary information, Table S9). Of these 22 genes, 17 were reported as SMGs in at least one previous ESCC genome study, including TP53 (74.80%), FAT1 (15.94%), NOTCH1 (15.35%), KMT2D (14.76%), CDKN2A (10.04%), FBXW7 (8.66%), ZNF750 (8.27%), FAT2 (7.68%), PIK3CA (7.48%), EP300 (6.69%), NFE2L2 (6.10%), AJUBA (4.53%), RBP1 (4.53%), KMT2C (3.94%), CREBBP (3.54%), KMD6A (3.54%), and TGFBR2 (3.15%). NFE2L2 (also known as NRF2) was predicted to have a total of 31 missense mutations, with the most frequent hotspot at p.R34P and R34Q, followed by p.E79K and E79Q within the DLG and ETGE protein domains, respectively, that bind to KEAP1 (Fig. 3b). Although the oncogenic role of NFE2L2 mutations that inactivate KEAP1-binding site was well established in several squamous cell carcinomas, the biological function of NFE2L2 and its mutations in ESCC have not been systematically determined.

Among the 22 SMGs, we found that patients with NFE2L2 mutations had a much worse prognosis (Kaplan–Meier analysis, log rank $P = 0.00035$, FDR = 0.0081; Cox regression, $P = 0.001$, HR = 2.21, 95% CI, 1.34–3.25, Fig. 3c), which was further supported by a joint multivariate regression analysis (Cox regression, $P = 1.70 \times 10^{-5}$, HR = 2.76, 95% CI, 1.74–4.38, Supplementary information, Table S10). Immunohistochemistry (IHC) analysis based on tissue microarray (TMA) that included sequenced tumors and matched normal tissues...
showed lower expression of NFE2L2 in tumors compared to normal tissues (paired t-test, \( P = 2.0e-06 \), Fig. 3d; Supplementary information, Fig. S3a). Cell proliferation assay showed that a reduction of endogenous wild-type NFE2L2 (NFE2L2-wt) level in KYSE410 and KYSE450 cells led to increased cell proliferation whereas ablation of mutant NFE2L2 (NFE2L2-mut) in KYSE70 and KYSE180 cells caused decreased cell proliferation (Supplementary information, Fig. S3b–e). Further studies verified the promotion of cell proliferation by stable NFE2L2 knockdown in KYSE450 cells (Supplementary information, Fig. S3f). In contrast, overexpression of exogenous NFE2L2-wt in KYSE150 cells with low-expression of NFE2L2-wt significantly attenuated cell proliferation. Notably, NFE2L2 mutants (p.R34Q, p.E79K, p.K438E, p.R569H) completely interfered with the suppressive activity of NFE2L2, and some NFE2L2 mutants (p.R34Q, p.E79K) even exerted an oncogenic role (Supplementary information, Fig. S3g). Consistently, xenograft mouse model showed that NFE2L2-wt ablation promoted tumor growth whereas NFE2L2-wt overexpression suppressed tumor growth. Some NFE2L2 mutants (p.R34Q, p.E79K) significantly promoted tumor growth, while other NFE2L2 mutants (p.K438E, p.R569H) had no significant effect on tumor growth compared with the vector control (Fig. 3e, f). Taken with our genetic observations, these functional data indicate that NFE2L2 may act as a tumor suppressor in ESCC; mutations in NFE2L2, which serve as a poor prognosis biomarker, probably impaired its tumor-suppressive function, or even conferred oncogenic activities.

Beyond the known SMGs, we identified five novel SMGs including KRT5, CD1H10, LILRB3, YEAT52 and CASP8 in ESCC (Supplementary information, Table S11). Among them, CASP8 was listed as a cancer consensus gene in the COSMIC database. The other genes including CD1H10, and YEAT52 have been reported as drivers in other cancer types.27–28

| Table 1. The association between clusters of mutational signatures and clinical phenotypes of ESCC patients. |
|---------------------------------------------------------------|
| Phenotype                              | NMF Cluster 1 | NMF Cluster 2 | P value | FDR  |
|----------------------------------------|---------------|---------------|---------|------|
| Population (Han)                       | 88 (88.0%)    | 349 (85.5%)   | 0.630   | 1    |
| Population (Kazak)                     | 12 (12.0%)    | 59 (14.5%)    |         |      |
| Gender (Male)                          | 72 (72.0%)    | 263 (64.5%)   | 0.160   | 0.96 |
| Gender (Female)                        | 28 (28.0%)    | 145 (35.5%)   |         |      |
| Tumor location lower                   | 32 (32.0%)    | 128 (31.4%)   | 0.904   | 1    |
| Tumor location middle                  | 63 (63.0%)    | 259 (63.5%)   |         |      |
| Tumor location upper                   | 5 (5.0%)      | 21 (5.1%)     |         |      |
| Never smoking                          | 41 (41.0%)    | 213 (52.2%)   | 0.759   | 1    |
| Light smoking                          | 4 (4.0%)      | 17 (4.2%)     |         |      |
| Medium smoking                         | 41 (41.0%)    | 111 (27.2%)   |         |      |
| Heavy smoking                          | 14 (14.0%)    | 67 (16.4%)    |         |      |
| Never drinking                         | 65 (65.0%)    | 282 (69.1%)   | 0.517   | 1    |
| Light drinking                         | 16 (16.0%)    | 57 (14.0%)    |         |      |
| Medium drinking                        | 16 (16.0%)    | 51 (12.5%)    |         |      |
| Heavy drinking                         | 3 (3.0%)      | 18 (4.4%)     |         |      |
| Invasion degree 1                      | 5 (5.0%)      | 38 (9.3%)     | 0.626   | 1    |
| Invasion degree 2                      | 22 (22.0%)    | 112 (27.5%)   |         |      |
| Invasion degree 3                      | 73 (73.0%)    | 258 (63.2%)   |         |      |
| Not lymphatic metastasis               | 47 (47.0%)    | 258 (63.2%)   | 0.004   | 0.028|
| Lymphatic metastasis                   | 53 (53.0%)    | 150 (36.8%)   |         |      |
| Stage I and Stage II                   | 48 (48.0%)    | 273 (66.9%)   | 0.001   | 0.008|
| Stage III                              | 52 (52.0%)    | 133 (33.1%)   |         |      |

Copy number alterations (CNAs)
Analysis of somatic CNAs (SCNAs) identified 8 recurrent arm-level amplifications, 4 arm-level deletions, 24 focal amplifications and 28 focal deletions (Supplementary information, Fig. S4a). In addition to known focal events, such as the 11q13.3 amplification containing CCND1 and the 9q21.3 deletion containing CDKN2A/B,8 we identified two novel focal deletions at 11p15.5 and 22q13.33, encompassing the interferon regulatory factor IRF729 and a component of the MOZ/MORF acetyltransferase complex BRD1,30 respectively. Putative SCNAs were validated in 22 cases within CCND1, CTTN, MYEOV, CNTNS, TRPC6 and PGR genes using HBB as an internal control and 95.5% were confirmed by quantitative PCR (Supplementary information, Table S12). Amplification of 11q13.3 was significantly associated with patients’ poor outcomes (Kaplan–Meier analysis, log rank \( P = 0.016 \), HR = 1.38, 95% CI, 1.058–1.805, Fig. 4a). Intriguingly, we identified, for the first time, that losses of 13q12.11, 13q14.2, 17q25.3 and 22q13.33 were significantly associated with patients’ poor outcomes (log rank \( P \) in Supplementary information, Table S13). Moreover, four amplified and ten deleted regions were associated with tumor stage and lymph node metastasis (Supplementary information, Table S13).

Finally, we performed hierarchical clustering based on the SCNAs and identified three CNA clusters. CNA-cluster 3 had the highest level of SCNA, followed by CNA-cluster 2 and 1 (Fig. 4b) and 11q13.3 amplification was more enriched in CNA-cluster 3 (Fisher Exact Test, \( P < 2.2e-16 \)). Patients in CNA-cluster 2 and CNA-cluster 3 tended to have a poorer prognosis than CNA-cluster 1 (Kaplan–Meier analysis, log rank \( P = 0.08 \), Supplementary information, Fig. S4b).

 Genome-wide analysis of noncoding regulatory mutations
We examined noncoding somatic mutations located at gene promoter regions, 5’ and 3’ untranslated regions (UTRs), long noncoding RNAs (lncRNAs), introns, and intergenic regions. As expected, intergenic and intron regions carried the highest mutational burden (Supplementary information, Fig. S4c). Recurrent noncoding hotspot mutation analysis identified 13 hotspots including hotspots in the promoter of WDR74 that were previously reported in multiple human cancer types31 (Fig. 4c upper panel; Supplementary information, Table S14). Survival analysis revealed that the hotspot mutation at the promoter of SLC35E2 was correlated with a worse prognosis (Kaplan–Meier analysis, log rank \( P = 0.0025 \), FDR = 0.0058, HR = 3.24, 95% CI, 0.984–3.507, Fig. 4d). Meanwhile, 5.9% (20 of 508) ESCC samples had the A30G hotspot mutation at the transcript lncRNA RP11-69182.2 that has not been previously linked to cancer (Supplementary information, Fig. S4d). We found that inhibition of RP11-69182.2 may significantly suppressed cell proliferation whereas its overexpression enhanced cell proliferation (Supplementary information, Fig. S4e). These data suggest that the hotspot mutation of RP11-69182.2 may be a gain-of-function mutation and its mutant may exert a tumor-promoting property.

Since the recurrent hotspot analysis was conservative, we next searched for recurrently mutated noncoding elements by investigating their mutation frequencies. We identified 112 IncRNAs, 225 3’-UTRs, 34 5’-UTRs, and 627 promoters that were significantly frequently mutated than expected (FDR < 0.05, Fig. 4c lower panel; Supplementary information, Table S15). NEAT1 and MALAT1 were the most frequently mutated IncRNAs. We identified 107 NEAT1 mutations in 94 patients (FDR = 7.3e–16) and 67 MALAT1 mutations in 54 patients (FDR = 7.3e–16). Both IncRNAs were found to be recurrently mutated in multiple tumor types and play important roles in tumorigenesis.23,29 Further study needs to be done to explore the role of NEAT1 and MALAT1 in ESCC tumorigenesis. Among the recurrently mutated noncoding elements, one lncRNA, one 3’-UTR and eight promoters

**Table 1. The association between clusters of mutational signatures and clinical phenotypes of ESCC patients.**
were significantly correlated with survival, such as the lncRNA LINC00966 and the promoter of CCND1 (FDR < 0.05, Fig. 4e).

Potential actionable targets and associated pathways in ESCC. ESCC patients are diagnosed at advanced stages of the disease and only a small group of patients will benefit from standard of care therapies. Significant efforts have been made to identify molecular-targeted therapies for ESCC patients. Here, we found that 77 out of 508 (15.2%) patients had at least one genomic alteration among the 40 targetable alterations in the curated precision oncology knowledge base (oncoKB34) (Supplementary information, Table S16a). Amplification of the MYC pathway was also significantly correlated with patient's survival (Kaplan–Meier analysis, log rank $P = 0.017$, HR = 1.40, 95% CI, 1.058–1.847, Fig. 5b middle panel). For cell cycle pathway, CDKN2A/B were mostly altered by deletions whereas CCND1, located in 11q13.3, was frequently amplified. Interestingly, CCND1 also harbored promoter mutations in 17 samples and patients with promoter mutations showed much worse prognosis than those with amplifications (Supplementary information, Fig. 5c). Additionally, within the NFR2 pathway, NFE2L2 was mutated in an exclusive manner with Cul3 and KAAP1 as previously reported.37 HES1 and NOV ranked the two most amplified genes within the NOTCH pathway.38 For the Wnt pathway, LRPS, GSK3B, LRPS, SFRP1, and DKK4 were amplified while mutations were not frequently detected.

Integrative analysis of clinically relevant genomic alterations across 508 ESCC genomes. To build a robust predictive model, we used the stability to perform feature selection in the Cox regression and found that NFE2L2 mutation and the RTK-RAS-related amplification were the two most stable molecular features, with selection probabilities of 0.90 and 0.83, respectively (Supplementary information, Table S16a). Although the MYC pathway-related amplification was significantly correlated with survival, the selection probability of MYC pathway was only 0.53, partly due to its high co-occurrence with RTK-RAS amplification (Fisher Exact Test, all $P < 1 \times 10^{-7}$). Amplification of the RTK-RAS pathway tended to co-occur with deletion of cell cycle pathway (Fisher Exact Test, all $P < 1 \times 10^{-7}$). Amplification of the MYC pathway was also significantly correlated with patient's survival (Kaplan–Meier analysis, log rank $P = 0.017$, HR = 1.40, 95% CI, 1.058–1.847, Fig. 5b middle panel). For cell cycle pathway, CDKN2A/B were mostly altered by deletions whereas CCND1, located in 11q13.3, was frequently amplified. Interestingly, CCND1 also harbored promoter mutations in 17 samples and patients with promoter mutations showed much worse prognosis than those with amplifications (Supplementary information, Fig. 5c). Additionally, within the NFR2 pathway, NFE2L2 was mutated in an exclusive manner with Cul3 and KAAP1 as previously reported.37 HES1 and NOV ranked the two most amplified genes within the NOTCH pathway.38 For the Wnt pathway, LRPS, GSK3B, LRPS, SFRP1, and DKK4 were amplified while mutations were not frequently detected.

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log rank \( P = 0.00018, \text{HR} = 1.66, 95\% \text{CI} = 1.27–2.17 \). Replacing the RTK-RAS amplification with the RTK-RAS-MYC amplification in the Cox regression model controlling clinical features led to an increase of the \( R^2 \)-square from 0.165 to 0.173, and both \( NFE2L2 \) mutation and RTK-RAS-MYC amplification were highly significant (\( P = 1.25 \times 10^{-05} \) and \( 5.11 \times 10^{-04} \), respectively, Supplementary information, Table S16b).

We classified the tumors into three distinct subtypes, \( NFE2L2 \)-mutated, RTK-RAS-MYC-amplified, and double-negative subtypes (Fig. 5c). The \( NFE2L2 \)-mutated subtype consisted of tumors with \( NFE2L2 \) mutations, the RTK-RAS-MYC amplification included tumors with the RTK-RAS-MYC amplifications but without \( NFE2L2 \) mutations, and the double-negative subtype was all other tumors. The survival of these three subtypes was significantly different from each other, even after controlling for other covariates (Supplementary information, Table S16b). The \( NFE2L2 \)-mutated subtype had the worst survival, followed by the RTK-RAS-MYC-amplified subtype (Kaplan–Meier analysis, log rank \( P = 9.1 \times 10^{-07} \), Fig. 5d; Supplementary information, Table S16c).

**DISCUSSION**

This study provides the most comprehensive characterization of ESCC genomes, to date, using deep WGS in a large Chinese patient cohort with clinical follow-up data. In addition to potential driver mutations reported in other studies, this massive amount of WGS data allowed us to identify novel driver-coding (e.g., \( KRT5, YEATS2 \)) and noncoding mutations (e.g., \( SLC35E2 \) and \( RP11-69I8.2-003 \)). A set of functional assays confirmed \( NFE2L2 \) and \( RP11-69I8.2-003 \) mutations as oncogenic drivers for ESCC progression. We also uncovered previously unexplored features in ESCC genomes, including the three mutation signature clusters, the discovery of TMB-H/MSI-H ESCC genomes, and the three CNV clusters. We found that a significant portion of ESCC patients had actionable mutations and may potentially benefit from targeted therapies. Most importantly, available clinical data allowed us to identify potential prognostic biomarkers such as \( NFE2L2 \) mutations, \( SLC35E2 \) promoter mutations, clusters of mutation signatures, TMB-H, and RTK-RAS-MYC amplification. Integrative analysis
of these clinical relevant features revealed three subtypes of ESCC (\(NFE2L2\)-mutated, RTK-RAS-MYC-amplified, and double-negative) that may robustly predict patients’ outcomes. We provide a public genotype/phenotype-coupled resource that represents a further step toward targeting the ESCC genome for clinical purposes.

Compared with prior ESCC studies, our larger number of microneedle-punctured tumor samples provided additional power to identify cancer driver genes, leading to the discovery of five novel driver genes (\(KRT5\), \(CDH10\), \(LILRB3\), \(YEATS2\), and \(CASP8\)), as well as recurrent CNAs. Previous studies mostly used whole-exome...
sequencing, leaving the nonprotein-coding part of ESCC genomes remains widely unexplored. Our WGS analysis identified many novel recurrent- and prognostic-related noncoding mutations (e.g., SLC35E2 and CCND1 promoter mutations). Functional analysis clearly demonstrated that RP11-69I8.2-003 hotspot mutation exerted gain-of-function properties. These noncoding mutations and rare driver-coding mutations may improve the understanding of ESCC tumorigenesis and can be used for genetic counseling, to

Fig. 5 Genomic alterations in actionable targets and cancer pathways, and an integrative model of the alterations. a Copy number alterations and nonsilent somatic mutations of key genes in five cancer pathways. Genes are ordered by alteration frequency. Wide bars represent the amplifications (red) and deletions (blue) while narrow bars represent various types of somatic mutations. b Kaplan–Meier curves of RTK-RAS amplification (upper), MYC amplification (middle) and RTK-RAS-MYC amplification (lower). c Integrative profiling of multiple genomic alterations in 508 patients. d Kaplan–Meier curve of the NFE2L2-mutated, RTK-RAS-MYC-amplified and double-negative ESCC subtypes.
predict patients’ outcome and to determine the most optimal treatment for ESCC patients.

APOBEC cytidine deaminase activity is an endogenous mutagen and APOBEC signature mutations can accumulate by the ongoing APOBEC activity, consistent with our observation that late-stage tumor tended to have more APOBEC signature mutations. Paradoxically, therapies targeting APOBEC may be achieved by either enhancing the mutagenic effect of APOBEC or inhibiting APOBEC activity. In fact, recent studies implicated that inhibition of DNA damage response (e.g., inhibition of PARP and ATR) in cells with high APOBEC expression promoted apoptosis and cell death. Meanwhile, inhibiting APOBEC expression led to prolonged tamoxifen responses for ER+ breast cancer in murine xenografts. On the other hand, APOBEC signature was associated with high mutation burden and a large number of neoantigens may be generated due to APOBEC mutagenic effect. Hence, immune-therapy may be a better therapeutic option for patients enriched with APOBEC signature mutations.

Alterations of genes involved in the RTK-RAS pathway were largely mutually exclusive, as observed in previous pan-cancer studies. This may be explained by (1) the second hit within the same pathway provides no further survival advantages or (2) the second hit results in survival disadvantage and even synthetic lethality. Forced expression of mutant EGFR and KRAS can be synthetic lethal, implying that the second explanation may be more likely for the mutual exclusivity of RTK-RAS-related alterations. The co-occurring alteration pattern of the RTK-RAS pathway with cell cycle-related signaling and the MYC pathway may have important therapeutic implications in ESCC. In lung cancer, co-occurring cell cycle alterations were significantly associated with the lack of response to osimertinib treatment in EGFR-mutated patients. In breast cancer, JQ1, a BET bromodomain inhibitor that decreases MYC expression, sensitized ERBB2-amplified breast cancer cells to lapatinib. These mutual exclusive and co-occurring patterns in ESCC may provide new treatment options for ESCC patients.

Although recent efforts have focused on characterizing ESCC genomic alterations, the number of clinically relevant biomarkers is still limited. In this study, we uncover NFE2L2 mutation and the RTK-RAS-MYC pathway amplification as better molecular features in predicting ESCC patients’ poor survival. In line with our finding, the RTK-RAS amplification has been associated with adverse prognosis in gastric cancer. After further validation of its prognostic value and therapeutic implication, our molecular subtypes may help in selection for administration of therapeutic trials.

Recent research systematically characterized the genomic landscape of 551 esophageal adenocarcinomas (EAC) and defined genomic biomarkers to be pursued in the clinic. Although EAC and ESCC share a number of recurrent driver alterations, such as TP53 mutation and CDKN2A deletion, their genomic landscapes are substantially different. NFE2L2 mutations were only present in ESCC. GATA4/6 and SMAD4 alterations were much more frequent in EAC. EGFR and FGFR1 were the most often amplified RTK-RAS-related genes in EAC, but KRAS and ERBB2 amplifications were more common in EAC. Interestingly, clinical biomarkers were also different, such as NFE2L2 mutation and RTK-RAS-MYC amplification in ESCC, and GATA4 amplification and SMAD4 alteration in EAC. These data show that ESCC and EAC were distinct diseases and their effective therapies may be totally different.

Our study has a few limitations. Firstly, the relationship between genomic landscape of ESCC and epidemiological and transcriptomic backgrounds remains unruaveled. Complementary to this study, sequencing approaches that comprehensively determine transcriptome, epigenome and the omics-interactions will be an important next step to provide further insight into the molecular changes underlying the pathogenesis in patients with ESCC. Secondly, hypotheses generated from this study will require clinical validation. Going forward, it would be important to evaluate the candidate predictive biomarkers identified from this study in clinical trials. In summary, this in-depth analysis of all classes of somatic mutations demonstrates a unique molecular profile and provides clues to the etiology of ESCC. Our study also sets the stage for improved prognosis prediction of ESCC based on their unique genetic alterations with potential clinical relevance.

**MATERIALS AND METHODS**

Sample collection and clinicopathological features of ESCC patients

Two clinical centers from Shanxi and Xinjiang provinces participated in this study. A total of 508 patients diagnosed with ESCC who had received no prior treatment were recruited. Informed consent was obtained from all subjects, and this study was approved by the ethical committees of the Shanxi Medical University. Each tumor specimen had a companion normal tissue specimen. All cases were classified according to WHO criteria. Hematoxylin and eosin (H&E)-stained sections from each sample were subjected to review by at least three independent pathologists with no clinical or molecular information to confirm that the tumor specimen was histologically consistent with ESCC and that the adjacent tissue specimen contained no tumor cells. The clinical backgrounds for this cohort are shown in Supplementary information, Tables S1–S3.

DNA extraction and whole-genome sequencing

Experimental DNA samples were obtained from microneedle-punctured FFPE tissues which have high quality to ensure tumor purity. Briefly, our pathologist examined the pathological section of all tumors by H&E staining first, then selected and marked the area with the most abundant tumor cells and the least stromal components on the slide, avoiding necrosis and inflammatory cells. Then our pathologist marked the same area on the wax block according to the slide, punctured at the marked area of wax block with a hollow core punch needle. The inner diameter of hollow core puncture needle was about 2 mm, and the thickness of wax block was about 2–3 mm. Tumor purity of all samples was evaluated by pathologist based on H&E staining (Supplementary information, Table S1). After microneedle-punctured procedure, high-quality total DNA from 10 mg tissues was extracted by Maxwell 16 Tissue DNA Purification Kit (Promega) according to the manufacturer’s instructions. Approximately 300 ng high-quality DNA sample (OD260/280 = 1.8–2.0) was sheared with Covaris S220 Sonicator (Covaris) to ∼350 bp. Fragmented DNA was purified using Sample Preparation Beads (Illumina). Adapter-ligated libraries were prepared with the TruSeq Nano DNA Sample Prep Kits (Illumina) according to Illumina’s protocol. Sequencing was performed using an Illumina HiSeq system for 2 × 150 paired-end sequencing in WuXi NextCODE at Shanghai, China. For variant calls, please see Supplementary information, Data S1.

Somatic mutation calling and SMG identification

High-quality reads were aligned to the UCSC human reference genome (hg19) using Burrows–Wheeler Aligner (BWA v0.7.12) with default parameters. For each paired sample, somatic SNVs and InDels were detected by Sentieon TNseq. Mutations in low complexity regions such as tandem repeat regions and highly homologous regions in the genome were filtered out. Low confidence variants were removed if any one of the following criteria is not satisfied: total depth > 10, alternative allele depth > 3 and mutation frequency > 0.01. All high-confident mutations were then annotated with ANNOVAR (Version 2016-02-01). SMGs were detected using MutSigCV (v1.4) and OncodriveFML (v2.0.2) with default settings. Genes with FDR < 0.1 were considered to be significantly mutated.
Mutation signature and mutation signature cluster analysis

SomaticSignatures\textsuperscript{12,13} was used to identify de novo mutation signatures. Number of signatures was determined by choosing the reflection point in the curves of explained variance and residual sum of squares (RSS) (Supplementary information, Fig. S2a). De novo mutational signatures were then compared to curated signatures in COSMIC using cosine similarity. The Cochran–Armitage trend test was used to examine the mutation signature contribution among various groups. For each tumor, we calculated the frequencies of the 96 mutation subtypes and obtained a 96 × 508 mutation subtype frequency matrix. Then NMF (https://cran.r-project.org/web/packages/NMF/index.html) in combination of the consensus clustering is applied to cluster the tumors.

APOBEC enrichment analysis

We used the method as previously described\textsuperscript{51} to examine the APOBEC enrichment. This method was implemented in Maftools\textsuperscript{52} and we used this tool for our APOBEC enrichment analysis. Tumors with enrichment scores > 2.5 were considered as APOBEC enriched.

Noncoding elements mutation analysis

We followed the previously described method\textsuperscript{53} to identify hotspot in noncoding regions. Noncoding elements regions were downloaded from the git repository OncodriveFML (v2.0.2). Promoter regions were defined as 1000 bp upstream and downstream around transcription start site. For IncRNAs, only exonic mutations were retained for downstream analysis. We categorized these mutations following the priority hierarchy, promoter > 5′ UTR > 3′UTR > IncRNA. To evaluate the significance of mutation frequency in noncoding elements, we first fitted a Poisson regression of mutation frequency against the length of noncoding elements. More specifically, let \( Y_i \) be the number of mutations detected on the \( i \)th noncoding element and \( L_i \) be its length. We modeled \( Y_i \) following a Poisson distribution with a mean parameter \( \lambda_i \). We assumed that \( E(Y_i | L_i) = \lambda_i = \beta_0 + \beta_1 \log L_i \). After obtaining the parameter estimates \( \beta_0 \) and \( \beta_1 \), we could calculate the expected number of mutations \( E_i = \exp(\beta_0 + \beta_1 \log L_i) \). Finally, we determined the \( P \) value of the \( \text{th} \) noncoding element as \( P(Y_i > E_i) \) using the Poisson distribution with parameter \( E_i \). This significance analysis was performed separately for promoters, 5′UTRs, 3′UTR and IncRNAs. In survival analysis, the genes having both coding and noncoding mutations were excluded.

Somatic copy number variation calling and profiling

We used BIC-seq\textsuperscript{23} to analyze SCNA s. SCNA s were called by BIC-seq2-seg by comparing the normalized tumor and normal data. Regions with absolute log2 copy number ratios at least 0.58 (= log2(1.5)) were viewed as losses or gains. For actionable amplification identification, we used a more stringent cutoff with log2 copy number ratio at least 1 (log2(2)). Hierarchical clustering was performed to cluster tumors’ SCNA profiles. GISTIC2.0\textsuperscript{54} was used to identify recurrent arm-level and focal SCNA segments.

Cox regression and survival analysis

The OS distributions were described by the Kaplan–Meier curves, and statistical significance was calculated using the log rank test. Univariate and multivariate analyses with the Cox proportional hazards model were used to examine the association between overall survival and clinical phenotypes. For multivariate analysis, in addition to the covariates with univariate \( P \) values of < 0.05, stages and lymphatic metastasis were adjusted for the analysis of the prognosis. Cox regression and survival analysis were performed with R package survival (https://github.com/therneau/survival) and survminer (https://cran.r-project.org/web/packages/survminer/index.html).

Integrative analysis of molecular features

We used lasso regularized Cox regression model coupled with the stability feature selection procedure\textsuperscript{40} to select the most stable features that could predict the patient’s outcome. In the Cox regression model, we considered molecular and clinical phenotypic features. The molecular features included the 22 SMGs, the 5 prognosis-related focal deletions/amplifications, TMB-H status, MSI-H status, somatic mutations at dMMR-related genes, APOBEC enrichment status, the NMF cluster, the CNV cluster and mutation status, amplification status and deletion status of the seven key cancer pathways (Nrf2, RTK-RAS, Myc, Cell cycle, Notch, Wnt, Chromatin remodeling). The clinical phenotypic features included TNM stages, lymphatic metastasis, tumor grade, gender, age, drinking, smoking and population. We varied the lasso penalty parameter \( \lambda \) from 0.03 to 0.2 stepped by 0.01 and defined the selection probability of each feature as its maximum of selection probabilities for different penalty \( \lambda \).

TMA and immunohistochemical staining

TMA blocks were made using the TMA builder (TC IV, Chloe, China) with 508 sequenced tumors and matched normal tissues. Immunohistochemical analysis was performed as previously described.\textsuperscript{29} The images were captured by Aperio Scan Scope (Leica, Nussloch, Germany). The immunoreactive H score was determined by Aperio ImageScope Cytoplasm 2.0 software.

Sanger sequencing

The somatic mutation was detected by PCR amplification and Sanger sequencing. We randomly selected 100 coding mutations and 100 noncoding mutations for validation. Considering the sensitivity of Sanger sequencing, we further filtered 175 mutations with a frequency of over 10% for validation. Among them, PCR amplification failed in 22 cases and PCR amplicon sequencing succeeded in 153 cases. Mutations of NFE2L2 gene in cell lines were detected by Sanger sequencing. Sequences of primers are available in Supplementary information, Tables S6, S7, S17.

Cell culture

Immortalized human normal esophageal epithelial cell line Het-1A was purchased from ATCC (Manassas, VA). Immortalized esophageal epithelial NE-2 cells were gifted from Dr. Enmin Li. ESCC cell lines (KYSE series)\textsuperscript{55} were provided by Dr. Y. Shimada. ZEC014 and ZEC134 cells were gifted from Dr. Dan Su. Het-1A cells were cultured in bronchial epithelial basal medium with growth supplements (Clonetics, USA). NE-2 cells were cultured in a 1:1 mixture of Epilife and dKSFM (Gibco). KYSE series cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and antibiotics. And ZEC014 and ZEC134 cells were cultured in DMEM-F12 medium containing 10% FBS, 1% nonessential amino acid (NEAA) and antibiotics. All cell lines were maintained at 37 °C in humidified atmosphere containing 5% CO\textsubscript{2}. All of the cells were authenticated by short tandem repeat (STR) analysis and regularly tested for mycoplasma contamination.

siRNA transfection

siRNAs were purchased from GE Dharmacon. Cells were transfected with 40 nM of siRNA using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Sequences of siRNAs are available in Supplementary information, Table S17.

Vector construction

The cDNA was cloned into the pLVX-IRES-Neo vector. Site-directed mutagenesis was performed based on overlap extension by PCR. shRNA oligos were inserted into lentiviral vector pSIH1-H1. Sequences of primers and shRNAs were listed in Supplementary information, Table S17. All constructs were verified by DNA sequencing.
Lentiviral transduction
The lentiviral vector together with packaging vectors was transfected into HEK293T cells according to the manufacturer’s instructions. Lentivirus infected cells in media containing polybrene (8 μg/mL).

Cell proliferation assay
Cells were plated on 96-well plates. As the indicated time points, the viability of cells was determined by Cell Counting Kit 8 (Roche) and measured at OD 450 nm with the BioTek Gen5 system (BioTeck, USA). The experiments were repeated three times with the representative experiment shown here.

Western blotting
Cells were lysed in RIPA buffer supplemented with protease inhibitors and subjected to Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis. Proteins were transferred onto polyvinylidene fluoride membranes (ImmobilonP). Blots were blocked and incubated overnight at 4 ℃ with the primary antibodies listed in Supplementary information, Table S17. Secondary antibodies were incubated and then washes were done. Blots were developed with chemiluminescent reagents from Pierce.

Quantitative real-time PCR (qRT-PCR) and qPCR copy number analysis
QRT-PCR analysis was performed and analyzed as previously described. For copy number analysis, copy number was assessed in paired sequenced tumors and adjacent noncancerous tissues. DNA (10 ng) was amplified by real-time PCR with Power SYBRs Green. HBB was used as a diploid control. Data were analyzed via the comparative (delta-Delta Ct) Ct method. The primers are listed in Supplementary information, Table S17.

Animal studies
All animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee of Chinese Academy of Medical Sciences Cancer Hospital. Cells were subcutaneously injected into the flanks of nude mice. Tumor growth was monitored weekly by caliper measurements of the tumor length and width and calculated individually as the formula: volume = a x b x h/2 (a represents length and b represents width).

Experimental statistical analyses
Data are presented as mean ± SD. Statistical analyses were performed with Graphpad Prism 7 software. Student’s t-test, one-way ANOVA or two-way ANOVA (two-sided) were used. Statistical significance was assessed at *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

Accession codes
FASTQ files will be uploaded to Genome Sequence Archive (GSA) in BIG Data Center (http://bigd.big.ac.cn/gsa), Beijing Institute of Genomics (BIG), Chinese Academy of Sciences, with an accession number HRA000021.

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AUTHOR CONTRIBUTIONS
Z.L., Yanhong L. and Q.Z. supervised the study, designed the experiments, and edited the manuscript. Y.C. supervised all data analyses and experimental studies, and wrote the manuscript. H. Chen, Y. Zhao and F.H. conceived the experimental studies and performed laboratory analyses. H. Chen interpreted the functional results and reviewed the manuscript. Y. Zhao, F.H., Yang L., X.Z. and X.Y. performed the functional experiments. R.X. oversaw the bioinformatics and statistics analyses, interpreted the results and wrote and reviewed the manuscript. H. Cui, Jiawei W., Y. Zhou, W.Z., Jie W. and B.W. provided medical records and survival data, performed bioinformatics analyses, validation of variations, and statistics analyses. T.Y., E.X., X.L., Y.M., S.G., L.Z., Y.X., R.S., B.Y. and X.C. provided clinical samples and data. T.Y., P.K., Y.M., J.L. and B.S. reviewed the histopathology. T.Y., E.X., P.K., Y.M., Y. Liu, F.W., J.Y., H.L., Y.W. and Y. Zhang prepared microneedle-punctured FFPE tissues. Y. Zhai and C.C. performed the immunohistochemistry analyses.

ADDITIONAL INFORMATION
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