Original Article

Construction of a multigenic diagnostic, prognostic, and immune infiltration model with methylation-associated regulators in esophageal squamous cell carcinoma

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Background: Methylation-related regulators may be involved in the prognostic prediction of esophageal squamous cell carcinoma (ESCC). Our study aimed to apply bioinformatics to screen methylation-related regulators for the construction of a prognostic model for patients with ESCC and to assess their diagnostic value and correlation with immune infiltration.

Methods: Prognosis-related genes were identified from The Cancer Genome Atlas (TCGA) database. Methylation-associated genes were filtered using the GeneCards database. We used the least absolute shrinkage and selection operator (LASSO) and Cox proportional hazards regression to identify the prognostic indicators. We applied the single-sample gene set enrichment analysis (ssGSEA) to clarify the relationship between prognostic indicators and immune infiltration in patients with ESCC (n=82).

Results: We constructed a prognostic model using methylation-related regulators homocysteine-inducible ER protein with ubiquitin-like domain 1 (*HERPUD1*), trans-2,3-enoyl-CoA reductase (*TECR*), melanoma antigen gene A11 (*MAGEA11*), and NOP2/Sun RNA methyltransferase 6 (*NSUN6*) to evaluate the prognosis of patients with ESCC. A higher prognostic risk score was associated with shorter overall survival (OS) in patients with ESCC [hazard ratio (HR) =5.77, 95% confidence interval (CI): 2.13–15.58; P<0.001]. Time-dependent area under the curve (AUC) analysis revealed that *HERPUD1*, *TECR*, *MAGEA11*, and *NSUN6* had high prognostic predictive value at different time points. Furthermore, we found that the combined diagnostic model based on *HERPUD1*, *TECR*, *MAGEA11*, and *NSUN6* had excellent diagnostic efficacy for ESCC (AUC =0.911; 95% CI: 0.888–0.935). Finally, the ssGSEA algorithm showed that *HERPUD1* was significantly positively correlated with immune infiltration at both the cellular and genetic levels, while *TECR* showed a significant negative correlation with immune infiltration levels.

Conclusions: Our prognostic model, built with the methylation-related regulators *HERPUD1*, *TECR*, *MAGEA11*, and *NSUN6*, could effectively predict prognosis in patients with ESCC, enhance diagnostic efficacy, and reflect immune cell infiltration in their microenvironment. Our findings are hypothesis generating and larger confirmatory studies are needed to validate our results.

Keywords: Esophageal squamous cell carcinoma (ESCC); methylation; immune infiltration; prognosis; diagnosis

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Introduction

The latest epidemiological report on cancer reveals a global incidence of 510,716 cases of esophageal cancer, with 445,129 deaths, highlighting the exceptionally high mortality rate associated with this malignancy (1). In China, a recent report identified 224,000 cases of esophageal cancer, representing approximately 43.9% of the global total (2). In contrast to cases in higher-income, Western nations, approximately 90% of esophageal cancer cases in China are diagnosed as esophageal squamous cell carcinoma (ESCC) (3,4). Furthermore, significant variations in the incidence of ESCC have been observed across different genders and regions within China (4). The incidence of ESCC is influenced by a variety of factors, including nitrosamines, dietary habits, smoking, alcohol consumption, obesity, oral infections, human papilloma virus infection, and gastroesophageal diseases (4-8). The diverse pathogenic mechanisms associated with these factors present significant challenges for clinical treatment and the prediction of prognosis. Our study aimed to establish a highly practical prognostic and diagnostic model to provide a reliable and widely applicable framework for the clinical diagnosis and prognostic analysis of ESCC.

There is an urgent clinical need for reliable prediction models for ESCC to accurately assess patient prognosis and evaluate the efficacy of chemotherapy and immunotherapies. Various prognostic models for ESCC, which focus on ferroptosis, necroptosis, programmed cell death, immune modulators, immune checkpoint inhibitors, epithelial–mesenchymal transition molecular heterogeneity, and noncoding RNA, are currently in development (9-13). Furthermore, numerous methylation-related genes have been identified that are associated with the diagnosis and prognosis of patients with ESCC (14-17). For example, whole-genome methylation analysis has identified the methylated genes potassium voltage-gated channel subfamily A member 3 (*KCNA3*) and otopetrin 2 (*OTOP2*) as potential diagnostic markers for ESCC (14). In ESCC, protein arginine methyltransferase 5 (*PRMT5*) is upregulated, and its elevated levels are linked to metastasis, suggesting it as an independent prognostic factor for overall survival (OS) (16).

In this study, we investigated the association of methylation-related genes with the prognosis of patients with ESCC. Using The Cancer Genome Atlas (TCGA) database, we finally identified the diagnostic efficiency and immune infiltration associated with four genes in ESCC: homocysteine-inducible ER protein with ubiquitin-like domain 1 (*HERPUD1*), trans-2,3-enoyl-CoA reductase (*TECR*), melanoma antigen gene A11 (*MAGEA11*), and NOP2/Sun RNA methyltransferase 6 (*NSUN6*). We present this article in accordance with the TRIPOD reporting checklist (available at https://jtd.amegroups.com/article/view/10.21037/jtd-2025-341/rc).

Highlight box

Key findings

- We established a diagnostic and prognostic model for esophageal squamous cell carcinoma (ESCC) based on a panel of four methylation-related genes, which possesses significant diagnostic and prognostic merit.

What is known and what is new?

- Methylation-associated genes regulate ESCC advancement by influencing the expression of crucial genes throughout initiation and progression. Methylation-associated genes have been applied in various models for the diagnosis and prognosis of ESCC, yet the clinical outcomes differ.
- Our results indicated that four genes, including homocysteine-inducible ER protein with ubiquitin-like domain 1 (*HERPUD1*), trans-2,3-enoyl-CoA reductase (*TECR*), melanoma antigen gene A11 (*MAGEA11*), and NOP2/Sun RNA methyltransferase 6 (*NSUN6*), have good diagnostic and prognostic value for ESCC.

What is the implication, and what should change now?

- Our research findings suggest that four methylation-associated genes have prognostic implications in ESCC and for forecasting the efficacy of clinical treatment, including chemotherapy and immunotherapy.

Methods

Prognostic factor screening

Prognostic genes were selected using TCGA database (https://portal.gdc.cancer.gov), with 82 patients with ESCC being categorized into two groups: a good prognosis group (n=41) and a poor prognosis group (n=41). Good prognosis

and poor prognosis were classified according to the median of OS of ESCC patients. We identified 967 prognostic genes with a significance threshold of P<0.05. The "survival package" (version 3.3.1) in R software (version 4.2.1; The R Foundation for Statistical Computing, Vienna, Austria) was used for batch-fitting survival regression models. Nonzero coefficients were subsequently screened using least absolute shrinkage and selection operator (LASSO) regression. Sixteen nonzero coefficients were then used in a Cox proportional hazards model, with four genes ultimately being identified as the independent prognostic factors for patients with ESCC. *HERPUD1*, *TECR*, *MAGEA11*, and *NSUN6* were subsequently employed to construct both a nomogram model and a risk scale model. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

Gene expression analysis

The gene expression of *HERPUD1*, *TECR*, *MAGEA11*, and *NSUN6* were analyzed with TCGA database and three Gene Expression Omnibus (GEO) datasets (GSE161533, GSE45670, and GSE20347) via the "ggplot2" package (version 3.3.6), the "stats" package (version 4.2.1), and the "car" package (version 3.1-0) in R software (version 4.2.1).

Survival analysis in patients with ESCC

Risk scores of *HERPUD1*, *TECR*, *MAGEA11*, and *NSUN6* associated with OS, disease-specific survival (DSS), and progression-free survival (PFS) were used to evaluate the proportional risk hypotheses via the "survival" package (version 3.3.1) and to conduct fitted survival regression analyses. The outcomes of these analyses were subsequently visualized with the "survminer" (version 0.4.9) and "ggplot2" (version 3.3.6) packages in R software (version 4.2.1).

Diagnostic analysis

Using data from the TCGA (n=82) and the Genotype-Tissue Expression (GTEx) portal (n=653), we extracted the gene expression data for *HERPUD1*, *TECR*, *MAGEA11*, and *NSUN6* for the analysis of diagnostic efficacy in patients with ESCC. Receiver operator characteristic (ROC) curve analysis was performed on the data using the "pROC" package (version 1.18.0), and the results were visualized using "ggplot2" package (version 3.4.4) in R software (version 4.2.1).

Association between prognostic factors and immune infiltration

The single-sample gene set enrichment analysis (ssGSEA) algorithm was used to investigate the association between *HERPUD1*, *TECR*, *MAGEA11*, and *NSUN6* and immune infiltration based on markers for 24 immune cells in patients with ESCC. The algorithm was implemented via the "GSVA" package in R version 1.46.0.

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis

Firstly, the TCGA database was utilized to identify differentially expressed genes (DEGs) associated with *HERPUD1* and *TECR*. Subsequently, immune infiltration-related genes (score ≥10) were screened using the GeneCards database (https://www.genecards.org/). Finally, overlapping genes between the two gene sets were selected for further analysis. Enrichment analysis results were visualized using R (version 4.2.1) and the ggplot2 package (version 3.4.4).

Statistical analysis

The data are presented as the mean ± standard deviation. LASSO and Cox proportional hazards regression were employed to select prognostic indicators with the "glmnet" package (version 4.1.7), "survival" package (version 3.3.1), and "rms" package (version 6.3-0) in R software version 4.2.1. Spearman correlation coefficient analysis was used to assess correlations. The *t*-test or Wilcoxon rank-sum test was used for comparing the differences between groups via the "ggplot2" package (version 3.3.6), "stats" package (version 4.2.1), and "car" package (version 3.1-0) in R. The R "survival" package (version 3.3.1) was used to select the prognostic factors in patients with ESCC. Kaplan-Meier survival analysis was used to evaluate the association of prognostic indicators with OS, DSS, and PFS via the "survival" (version 3.3.1), "survminer" (version 0.4.9), and "ggplot2" (version 3.3.6) packages. Time-dependent area under the curve (AUC) analysis was used to evaluate the prognostic values of *HERPUD1*, *TECR*, *MAGEA11*, and *NSUN6* at various time points via the "timeROC" (version 0.4) and "ggplot2" (version 3.4.4) packages in R software (version 4.2.1). Nomogram and calibration related models were built and visualized using the R "rms" package (version 6.3-0). During data analysis, missing values were

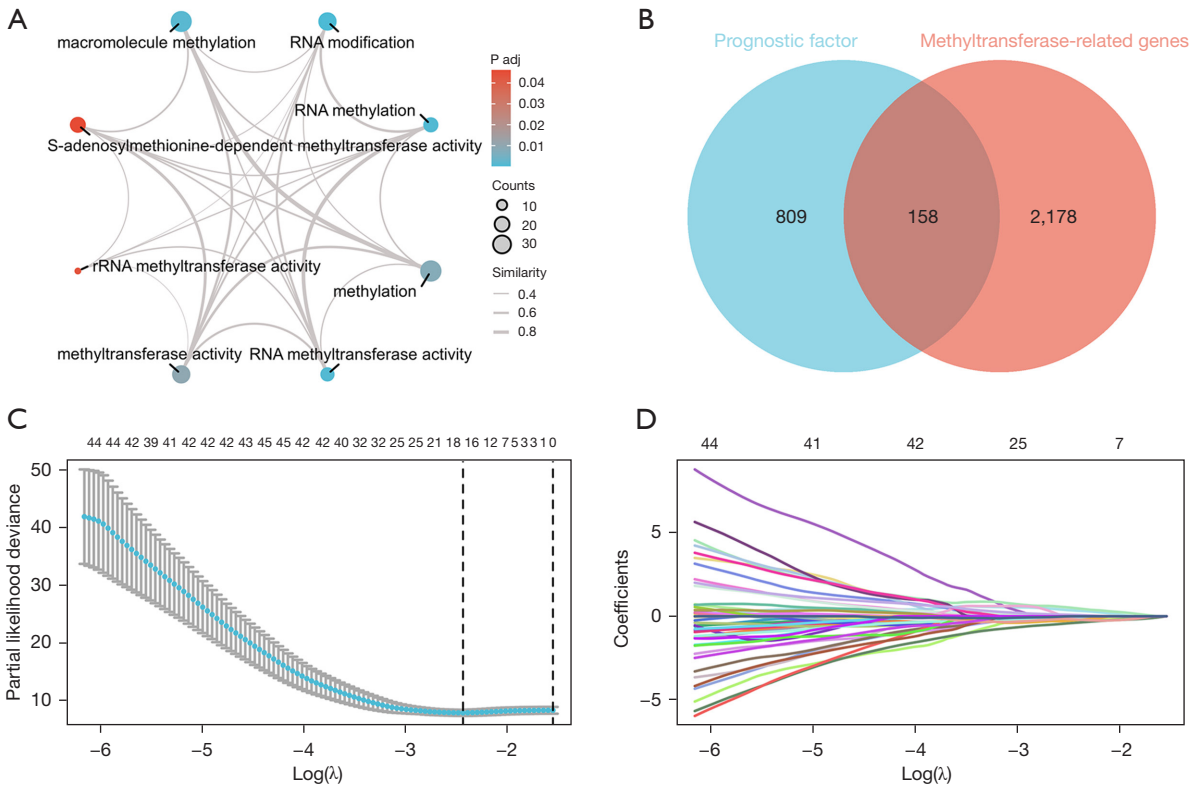

Figure 1 Screening of prognostic factors in ESCC. (A) ESCC-related prognostic factors were selected from TCGA database, and then functional enrichment analysis of prognostic factors was performed via GO analysis. (B) Methylation-related prognostic factors were screened with the GeneCards database. (C) LASSO cross-validation curves and (D) LASSO coefficient path diagrams were drawn to identify the nonzero coefficients. ESCC, esophageal squamous cell carcinoma; TCGA, The Cancer Genome Atlas; GO, Gene Ontology; LASSO, least absolute shrinkage and selection operator.

handled using imputation methods, and normalization was performed using the normalizeBetweenArrays function from the limma package (version 3.52.2). A P value <0.05 indicated statistical significance.

Results

Screening of prognostic factors in ESCC

We first analyzed the prognostic genes using TCGA database, categorizing 82 patients with ESCC into two groups: a good prognosis group (n=41) and a poor prognosis group (n=41). We identified 967 prognostic genes with a significance threshold of P<0.05. GO analysis identified methylation-associated signaling pathways, such as RNA methylation, RNA methyltransferase activity, S-adenosylmethionine-dependent methyltransferase activity, and macromolecule methylation as potentially linked to prognosis (*Figure 1A*). Based on these findings, we hypothesized that methylation-related genes could serve as prognostic indicators for patients with ESCC. We used GeneCards to screen 2,336 methylation-related genes and intersected them with prognostic factors for ESCC, identifying 158 methylation-related prognostic factors (*Figure 1B*). Finally, we performed LASSO regression analysis with cross-validation curves (*Figure 1C*) and LASSO coefficient path diagrams (*Figure 1D*) to identify nonzero coefficients. We ultimately identified 16 nonzero coefficients. Based on 16 nonzero coefficients, we screened four independent prognostic genes using Cox proportional hazards regression analyses: *HERPUD1* [hazard ratio (HR) =5.345, 95% confidence interval (CI): 1.297–22.024; P=0.02], *TECR* (HR =5.162, 95% CI: 1.379–19.325; P=0.02), *MAGEA11* (HR =0.037, 95% CI: 0.006–0.217; P<0.001), and *NSUN6* (HR =0.196, 95% CI: 0.054–0.705; P=0.01) (*Table 1*).

Table 1 Cox univariate and multivariate analyses were performed to evaluate the correlation of prognostic factors with OS

Genes	Univariate analysis		Multivariate analysis	
	HR (95% CI)	P value	HR (95% CI)	P value
TFB1M (*vs*. low)				
High	0.336 (0.144–0.786)	0.01	0.458 (0.132–1.582)	0.22
HERPUD1 (*vs*. low)				
High	3.270 (1.347–7.937)	0.009	5.345 (1.297–22.024)	0.02
DDX3Y (*vs*. low)				
High	2.404 (1.013–5.706)	0.047	1.103 (0.247–4.929)	0.90
TECR (*vs*. low)				
High	2.538 (1.112–5.790)	0.03	5.162 (1.379–19.325)	0.02
SLC25A15 (*vs*. low)				
High	0.407 (0.180–0.923)	0.03	0.846 (0.270–2.656)	0.78
PPP2CB (*vs*. low)				
High	0.325 (0.142–0.744)	0.008	2.556 (0.676–9.667)	0.17
HRH2 (*vs*. low)				
High	3.119 (1.211–8.033)	0.02	1.784 (0.499–6.377)	0.37
FAM98A (*vs*. low)				
High	0.319 (0.134–0.759)	0.01	1.159 (0.302–4.448)	0.83
NLRC5 (*vs*. low)				
High	2.662 (1.157–6.123)	0.02	1.602 (0.475–5.397)	0.45
MTA2 (*vs*. low)				
High	0.296 (0.127–0.691)	0.005	0.280 (0.065–1.218)	0.09
NOL6 (*vs*. low)				
High	0.362 (0.152–0.866)	0.02	0.581 (0.157–2.154)	0.42
MLLT3 (*vs*. low)				
High	0.341 (0.150–0.776)	0.01	0.350 (0.102–1.201)	0.10
NQO1 (*vs*. low)				
High	2.448 (1.068–5.610)	0.03	2.013 (0.495–8.181)	0.33
MAGEA11 (*vs*. low)				
High	0.285 (0.116–0.701)	0.006	0.037 (0.006–0.217)	<0.001
CSKMT (*vs*. low)				
High	0.262 (0.104–0.661)	0.005	0.482 (0.121–1.926)	0.30
NSUN6 (*vs*. low)				
High	0.192 (0.072–0.515)	0.001	0.196 (0.054–0.705)	0.01

N=41 in all low expression group; n=41 in all high expression group. OS, overall survival; HR, hazard ratio; CI, confidence interval; *TFB1M*, transcription factor B1, mitochondrial; *HERPUD1*, homocysteine inducible ER protein with ubiquitin like domain 1; *DDX3Y*, DEAD-box helicase 3 Y-linked; *TECR*, trans-2,3-enoyl-CoA reductase; *SLC25A15*, solute carrier family 25 member 15; *PPP2CB*, protein phosphatase 2 catalytic subunit beta; *HRH2*, histamine receptor H2; *FAM98A*, family with sequence similarity 98 member A; *NLRC5*, NLR family CARD domain containing 5; *MTA2*, metastasis-associated 1 family member 2; *NOL6*, nucleolar protein 6; *MLLT3*, MLLT3 super elongation complex subunit; *NQO1*, NAD(P)H quinone dehydrogenase 1; *MAGEA11*, melanoma antigen gene A11; *CSKMT*, citrate synthase lysine methyltransferase; *NSUN6*, NOP2/Sun RNA methyltransferase 6.

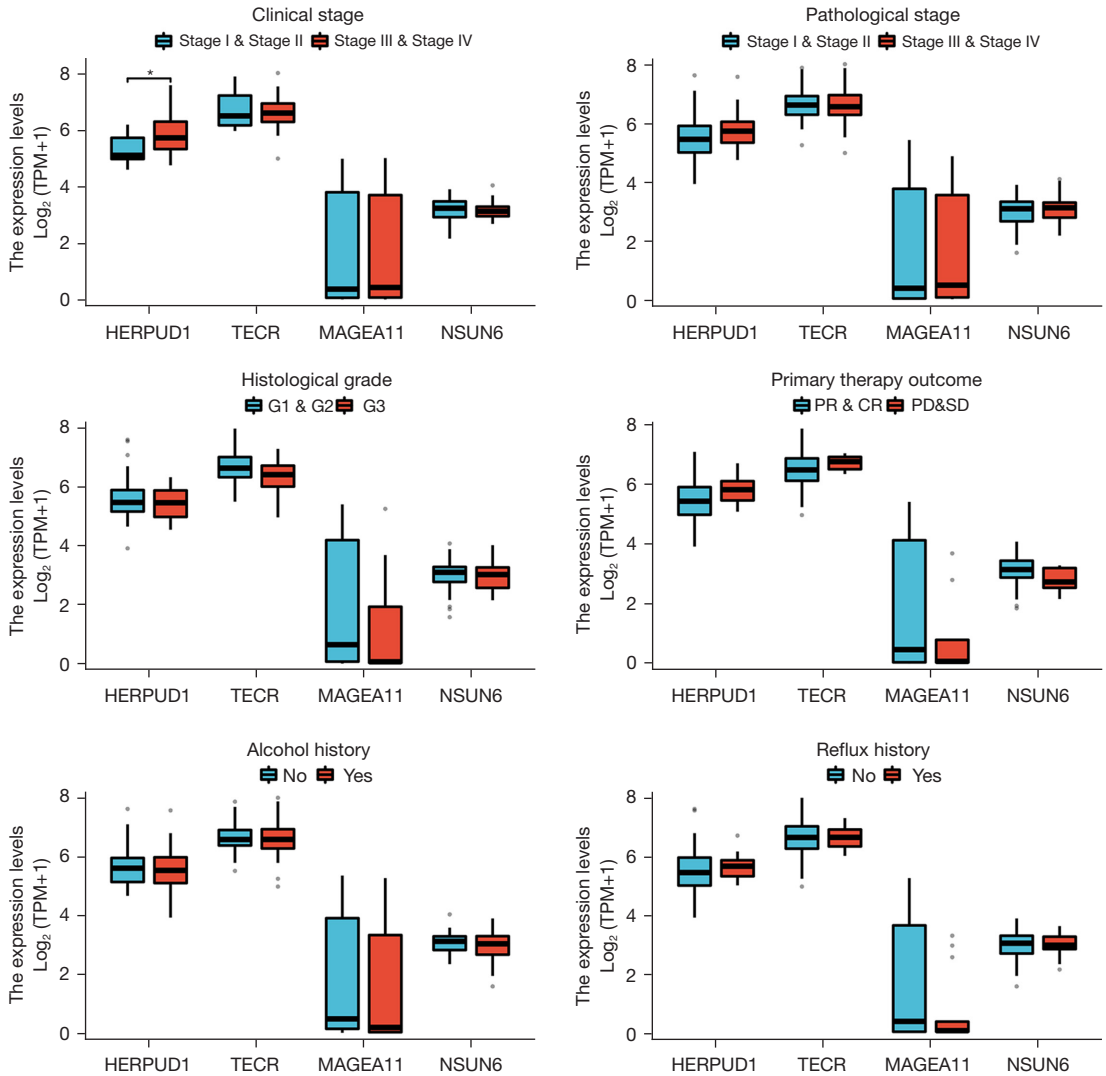

Figure 2 The association of *HERPUD1*, *TECR*, *MAGEA11*, and *NSUN6* with clinical parameters in patients with ESCC. The expression level of *HERPUD1*, *TECR*, *MAGEA11*, and *NSUN6* was used to analyze their correlation with clinical stage, pathological stage, histological grade, primary therapy outcomes, alcohol history, and reflux history. *, P<0.05. *HERPUD1*, homocysteine-inducible ER protein with ubiquitin-like domain 1; *TECR*, trans-2,3-enoyl-CoA reductase; *MAGEA11*, melanoma antigen gene A11; *NSUN6*, NOP2/Sun RNA methyltransferase 6; TPM, transcripts per million; ESCC, esophageal squamous cell carcinoma.

The association of HERPUD1, TECR, MAGEA11, and NSUN6 with clinical parameters in patients with ESCC

The expression level of *HERPUD1* was significantly higher in the high clinical stage (III–IV) group compared to the low clinical stage (I–II) group. Additionally, the expression levels of *TECR*, *MAGEA11*, and *NSUN6* were not significantly correlated with pathological stage, histological grade, primary therapy outcomes, alcohol history, or reflux history (*Figure 2*).

Verification of HERPUD1, TECR, MAGEA11, and NSUN6 expression in the GEO and TCGA databases in patients with ESCC

Our results showed that *HERPUD1*, *TECR*, and *MAGEA11* were significantly upregulated in ESCC tissues as compared to normal tissues. In contrast, *NSUN6* was significantly downregulated in ESCC tissues as compared to normal tissues (*Figure 3A*). We then used the GEO database to validate the expression levels of *HERPUD1*, *TECR*,

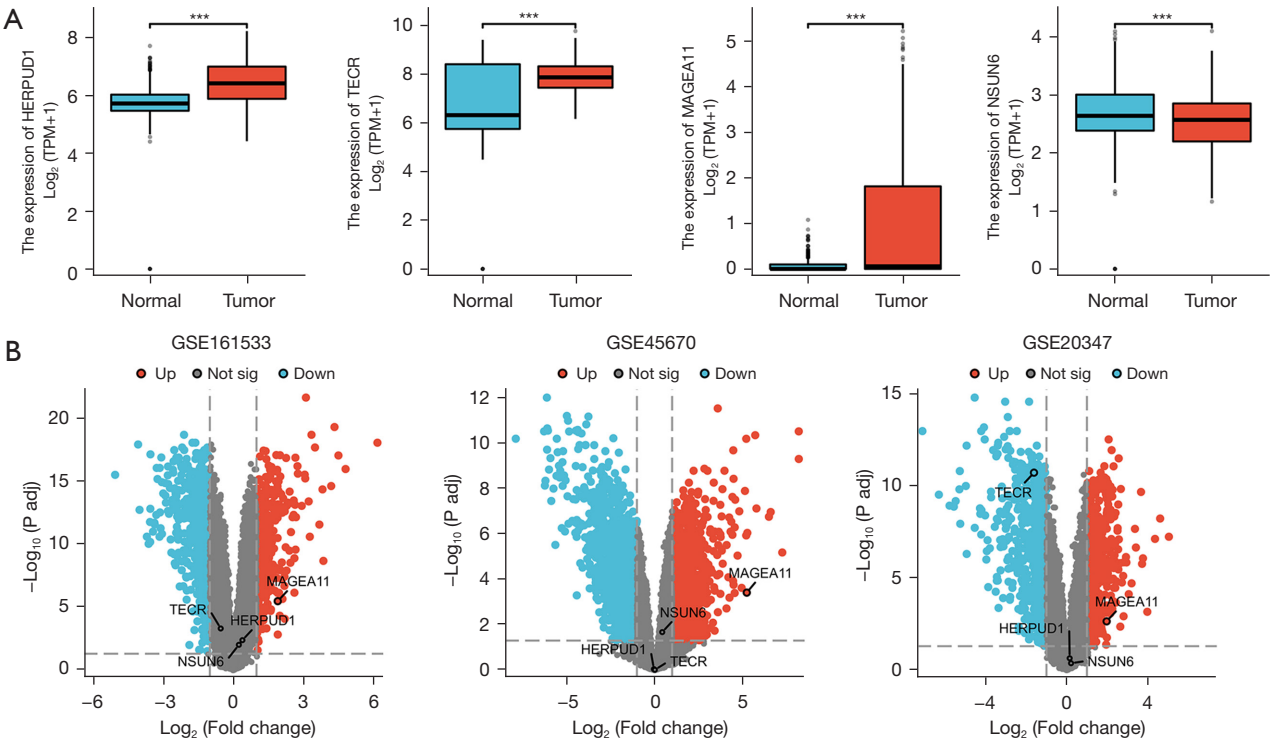

Figure 3 Verification *HERPUD1*, *TECR*, *MAGEA11*, and *NSUN6* expression with the GEO and the TCGA databases in patients with ESCC. (A) *HERPUD1*, *TECR*, *MAGEA11*, and *NSUN6* expression levels were evaluated with TCGA database. (B) GEO database was used to validate the expression levels of *HERPUD1*, *TECR*, *MAGEA11*, and *NSUN6* in ESCC tissues with three GEO datasets: GSE161533, GSE45670, and GSE20347. ***, P<0.001. TPM, transcripts per million; *HERPUD1*, homocysteine-inducible ER protein with ubiquitin-like domain 1; *TECR*, trans-2,3-enoyl-CoA reductase; *MAGEA11*, melanoma antigen gene A11; *NSUN6*, NOP2/Sun RNA methyltransferase 6; GEO, Gene Expression Omnibus; TCGA, The Cancer Genome Atlas; ESCC, esophageal squamous cell carcinoma.

MAGEA11, and *NSUN6* in ESCC tissues. Our findings indicated that MAGEA11 was significantly upregulated in ESCC tissues as compared to adjacent noncancerous tissues in three GEO datasets: GSE161533, GSE45670, and GSE20347. However, the expression levels of *HERPUD1*, *TECR*, and *NSUN6* did not differ significantly between ESCC tissues and adjacent noncancerous tissues in the three GEO datasets (*Figure 3B*).

The association of HERPUD1, TECR, MAGEA11, and NSUN6 with OS, DSS, and PFS in patients with ESCC based on the TCGA dataset

Patients with ESCC and high *HERPUD1* expression had a worse prognosis than did those with low expression, as indicated by shorter OS (HR =3.27, 95% CI: 1.35–7.94; P=0.009) and DSS (HR =6.05, 95% CI: 1.73–21.10; P=0.005). Similarly, patients with ESCC with high *TECR* expression experienced shorter OS (HR =2.54, 95% CI: 1.11–5.79; P=0.02) as compared to those with low expression. *TECR* expression did not correlate with DSS (HR =2.33, 95% CI: 0.89–6.09; P=0.08) or PFS (HR =1.65, 95% CI: 0.86–3.17; P=0.13). In contrast, patients with high *MAGEA11* expression had better OS (HR =0.29, 95% CI: 0.12–0.70; P=0.006), DSS (HR =0.27, 95% CI: 0.10–0.78; P=0.01), and PFS (HR =0.45, 95% CI: 0.23–0.87; P=0.01) than did those with low expression. Similarly, patients with high *NSUN6* expression showed better OS (HR =0.19, 95% CI: 0.07–0.52; P=0.001), DSS (HR =0.21, 95% CI: 0.07–0.65; P=0.006), and PFS (HR =0.43, 95% CI: 0.22–0.83; P=0.01) than did those with low expression (*Figure 4*).

The establishment of a prognostic nomogram model with HERPUD1, TECR, MAGEA11, and NSUN6 in patients with ESCC

First, we used time-dependent AUCs to evaluate the prognostic values of *HERPUD1*, *TECR*, *MAGEA11*, and

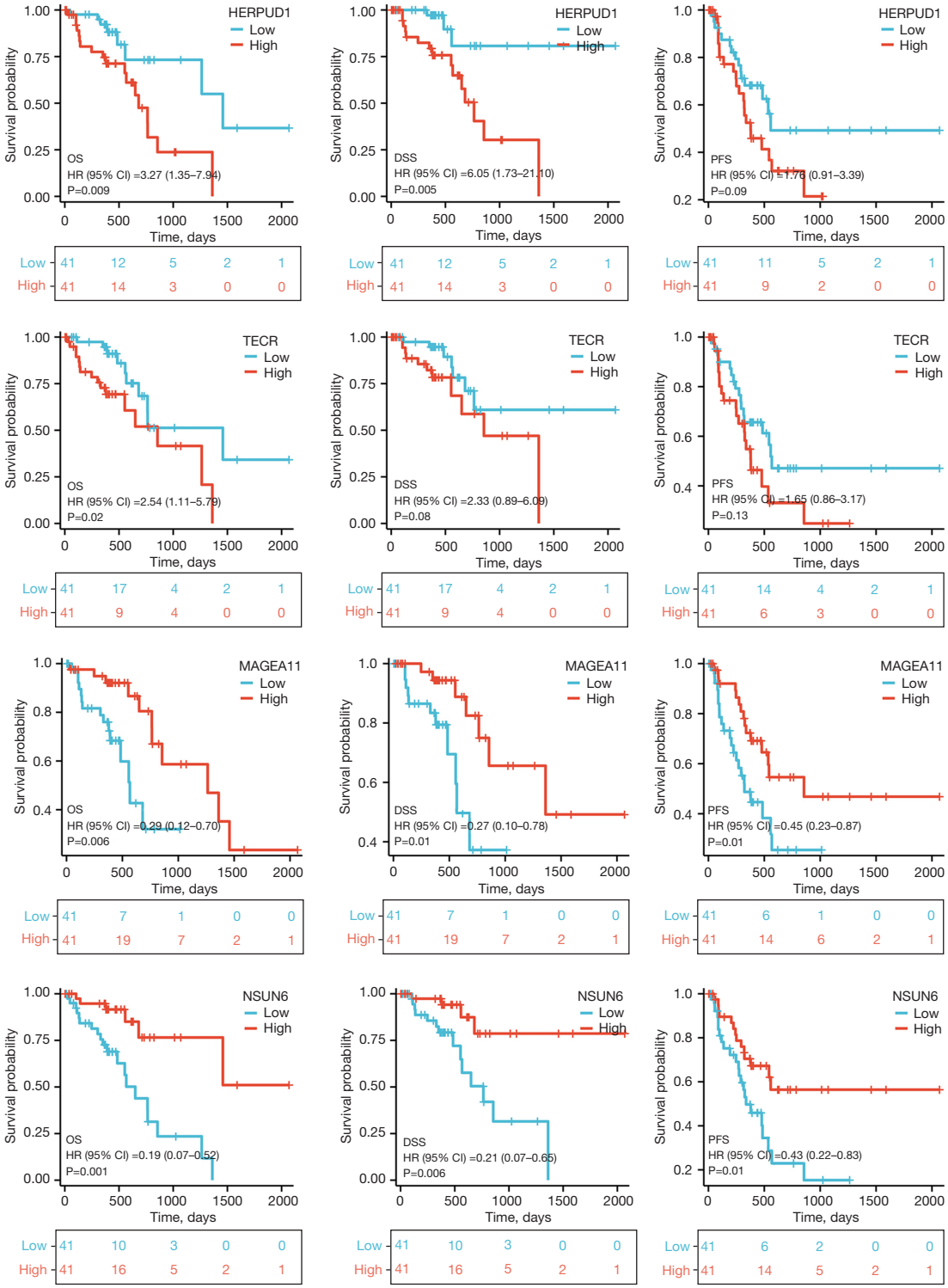

Figure 4 The association of *HERPUD1*, *TECR*, *MAGEA11*, and *NSUN6* with OS, DSS, and PFS in patients with ESCC. The correlation of *HERPUD1*, *TECR*, *MAGEA11*, and *NSUN6* with OS, DSS, and PFS in patients with ESCC was evaluated using Kaplan-Meier survival analysis. HR, hazard ratio; *HERPUD1*, homocysteine-inducible ER protein with ubiquitin-like domain 1; *TECR*, trans-2,3-enoyl-CoA reductase; *MAGEA11*, melanoma antigen gene A11; *NSUN6*, NOP2/Sun RNA methyltransferase 6; OS, overall survival; DSS, disease-specific survival; PFS, progression-free survival; ESCC, esophageal squamous cell carcinoma; CI, confidence interval.

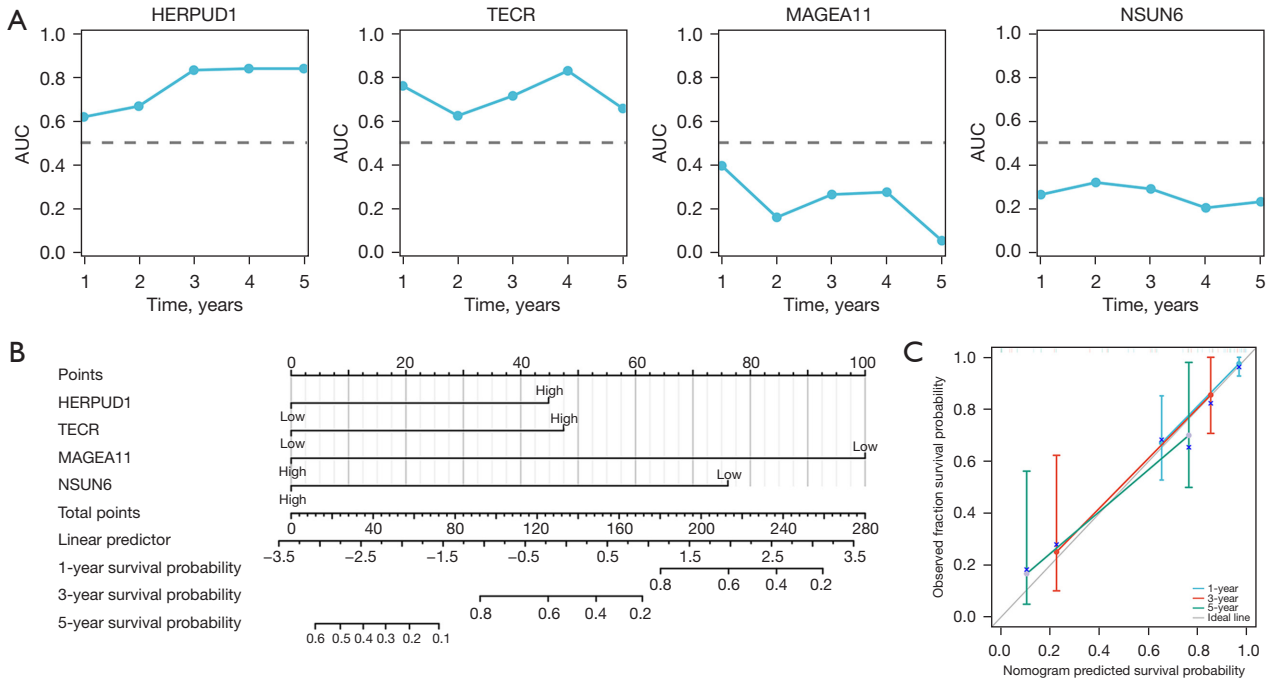

Figure 5 The establishment of a prognostic nomogram model with *HERPUD1*, *TECR*, *MAGEA11*, and *NSUN6* in patients with ESCC. (A) Time-dependent AUCs were used to evaluate the prognostic values of *HERPUD1*, *TECR*, *MAGEA11*, and *NSUN6* at various time points. *HERPUD1*, *TECR*, *MAGEA11*, and *NSUN6* were used to establish the (B) nomogram model and (C) calibration curves for patients with ESCC. *HERPUD1*, homocysteine-inducible ER protein with ubiquitin-like domain 1; *TECR*, trans-2,3-enoyl-CoA reductase; *MAGEA11*, melanoma antigen gene A11; *NSUN6*, NOP2/Sun RNA methyltransferase 6; AUC, area under the curve; ESCC, esophageal squamous cell carcinoma.

Table 2 The prognostic efficiency of combined model, including *HERPUD1*, *TECR*, *MAGEA11*, and *NSUN6* for patients with ESCC

Variable	Time (year)	AUC (95% CI)	Cut-off value	Sensitivity	Specificity
Combined model	1	0.881 (0.821–0.963)	0.000	0.749	0.864
	3	0.860 (0.807–0.941)	−2.091	1.000	0.600
	5	0.944 (0.901–0.989)	−2.091	0.888	1.000

HERPUD1, homocysteine-inducible ER protein with ubiquitin-like domain 1; *TECR*, trans-2,3-enoyl-CoA reductase; *MAGEA11*, melanoma antigen gene A11; *NSUN6*, NOP2/Sun RNA methyltransferase 6; ESCC, esophageal squamous cell carcinoma; AUC, area under the curve; CI, confidence interval.

NSUN6 at various time points. Our results showed that all four genes had strong predictive value at different time points. An AUC value greater than 0.5 indicates that the gene is a negative prognostic factor, while an AUC value less than 0.5 suggests that it is a positive prognostic factor. Consequently, our findings suggest that *HERPUD1* and *TECR* may serve as negative prognostic factors for patients with ESCC, whereas *MAGEA11* and *NSUN6* may act as positive prognostic indicators (*Figure 5A*). The AUC (95% CI) of combined model for predicting the OS for patients with ESCC was 0.881 (0.821–0.963) at 1-year, 0.860 (0.807–0.941) at 3-year, and 0.944 (0.901–0.989) at 5-year (*Table 2*). We created a prognostic nomogram based on four genes to predict the OS of patients with ESCC. We applied a weighted risk score derived from these four genes. This allowed us to calculate a total score to estimate survival probabilities at 1, 3, and 5 years (*Figure 5B*). The prognostic calibration curves showed the differences between the

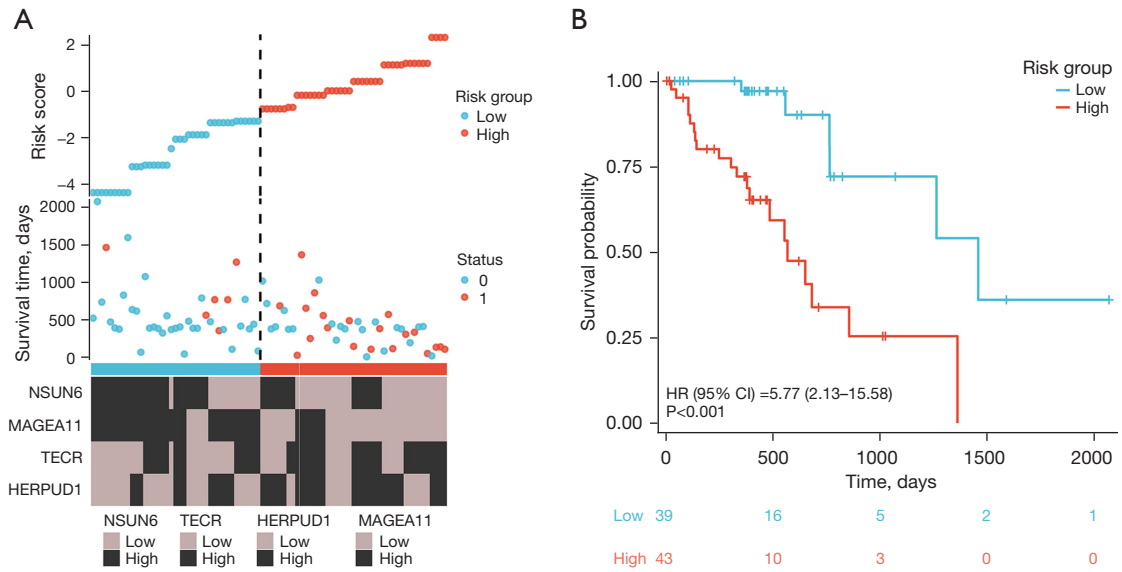

Figure 6 Risk scoring model of *HERPUD1*, *TECR*, *MAGEA11*, and *NSUN6* in patients with ESCC. (A) Risk factor graph of the trends of prognostic factors and risk scores. (B) The relationship of risk score with OS was analyzed using a Kaplan-Meier plotter. "0" represents survivor; "1" represents death. HR, hazard ratio; CI, confidence interval; *HERPUD1*, homocysteine-inducible ER protein with ubiquitin-like domain 1; *TECR*, trans-2,3-enoyl-CoA reductase; *MAGEA11*, melanoma antigen gene A11; *NSUN6*, NOP2/Sun RNA methyltransferase 6; ESCC, esophageal squamous cell carcinoma; OS, overall survival.

predicted probabilities from our nomogram for the OS of patients with ESCC at 1, 3, and 5 years and the observed probabilities. Our statistical analyses indicated that the prognostic model had a good fit, with a concordance index (C-index) of 0.839 (95% CI: 0.802–0.876), a likelihood ratio test value of 37.69 (P<0.001), and a Wald test result of 22.94 (P<0.001) (*Figure 5C*).

Risk score model of HERPUD1, TECR, MAGEA11, and NSUN6 for patients with ESCC

We used risk factor graphs to visually display the trends of prognostic factors and risk scores. We found that higher risk scores were associated with shorter survival times (*Figure 6A*). Subsequently, we quantitatively illustrated this relationship using the Kaplan-Meier plotter, and the results indicated that high risk scores were significantly correlated with shorter survival times (HR =5.77, 95% CI: 2.13–15.58; P<0.001) (*Figure 6B*).

The diagnostic efficacy of HERPUD1, TECR, MAGEA11, and NSUN6 in patients with ESCC

To evaluate the diagnostic performance of *HERPUD1*, *TECR*, *MAGEA11*, and *NSUN6*, we collected gene expression data from the TCGA and the GTEx datasets. Specifically, the GTEx datasets provided gene expression levels from normal tissues, while the TCGA datasets provided gene expression levels from tumor tissues. The biomarkers *HERPUD1* (*Figure 7A* and *Table 3*), *TECR* (*Figure 7B* and *Table 3*), *MAGEA11* (*Figure 7C* and *Table 3*), and *NSUN6* (*Figure 7D* and *Table 3*) were evaluated in terms of their ability to diagnosis ESCC, and their AUC values were 0.778 (95% CI: 0.734–0.821), 0.673 (95% CI: 0.638–0.708), 0.665 (95% CI: 0.618–0.712), and 0.584 (95% CI: 0.537–0.632), respectively. Although our results showed that individual biomarkers did not improve the diagnosis of ESCC, the combined analysis of all four biomarkers significantly improved diagnostic performance, achieving an AUC of 0.911 (95% CI: 0.888–0.935) (*Figure 7E* and *Table 3*).

Correlation of HERPUD1, TECR, MAGEA11, and NSUN6 with immune infiltration in patients with ESCC

To comprehensively determine the correlation of *HERPUD1*, *TECR*, *MAGEA11*, and *NSUN6* with immune infiltration, we used the ssGSEA algorithm to calculate their respective relationships with the enrichment scores of 24 types

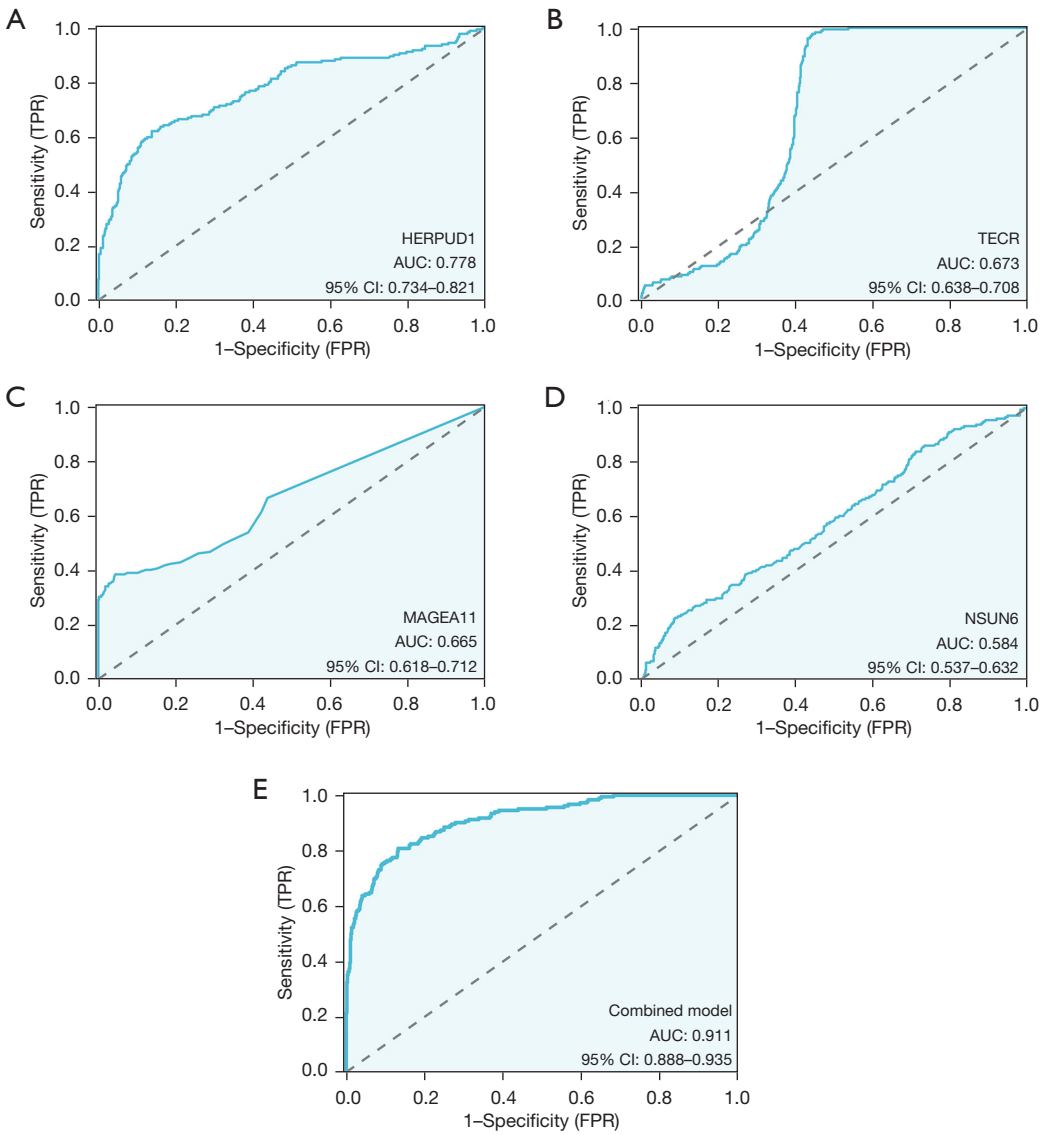

Figure 7 The diagnostic efficacy of *HERPUD1*, *TECR*, *MAGEA11*, and *NSUN6* in patients with ESCC. The ability of biomarkers (A) *HERPUD1*, (B) *TECR*, (C) *MAGEA11*, (D) *NSUN6*, and (E) a combined model in diagnosing ESCC as the ROC AUC. *HERPUD1*, homocysteine-inducible ER protein with ubiquitin-like domain 1; *TECR*, trans-2,3-enoyl-CoA reductase; *MAGEA11*, melanoma antigen gene A11; *NSUN6*, NOP2/Sun RNA methyltransferase 6; TPR, true positive rate; FPR, false positive rate; AUC, area under the curve; CI, confidence interval; ESCC, esophageal squamous cell carcinoma; ROC, receiver operator characteristic.

of immune cells (*Figure 8A*). Our results indicated that *HERPUD1* was significantly positively correlated with various immune cells, while *TECR* and *NSUN6* showed significant negative correlations with multiple immune cell enrichment scores. *MAGEA11* appeared to have a weak correlation with immune cell enrichment scores. Subsequently, the correlation between *HERPUD1* and *TECR* with immune cell enrichment scores was validated at the gene level. *HERPUD1* demonstrated a significant positive correlation with key immunomodulators, including immunoinhibitors (*Figure 8B*), major histocompatibility complex (MHC) molecules (*Figure 8C*), and immunostimulators (*Figure 8D*), whereas *TECR* was significantly negatively correlated with major immunomodulators. These results suggest that different prognostic factors may exhibit completely opposite correlations with immune infiltration.

Table 3 The diagnostic efficiency of *HERPUD1*, *TECR*, *MAGEA11*, *NSUN6*, and combined model for patients with ESCC

Variables	AUC (95% CI)	Cut-off value	Sensitivity	Specificity	Accuracy
HERPUD1	0.778 (0.734–0.821)	6.199	0.621	0.862	0.810
TECR	0.673 (0.638–0.708)	6.632	0.978	0.559	0.649
MAGEA11	0.665 (0.618–0.712)	0.305	0.385	0.956	0.834
NSUN6	0.584 (0.537–0.632)	2.109	0.225	0.913	0.765
Combined model	0.911 (0.888–0.935)	−1.203	0.808	0.868	0.855

HERPUD1, homocysteine-inducible ER protein with ubiquitin-like domain 1; *TECR*, trans-2,3-enoyl-CoA reductase; *MAGEA11*, melanoma antigen gene A11; *NSUN6*, NOP2/Sun RNA methyltransferase 6; ESCC, esophageal squamous cell carcinoma; AUC, area under the curve; CI, confidence interval.

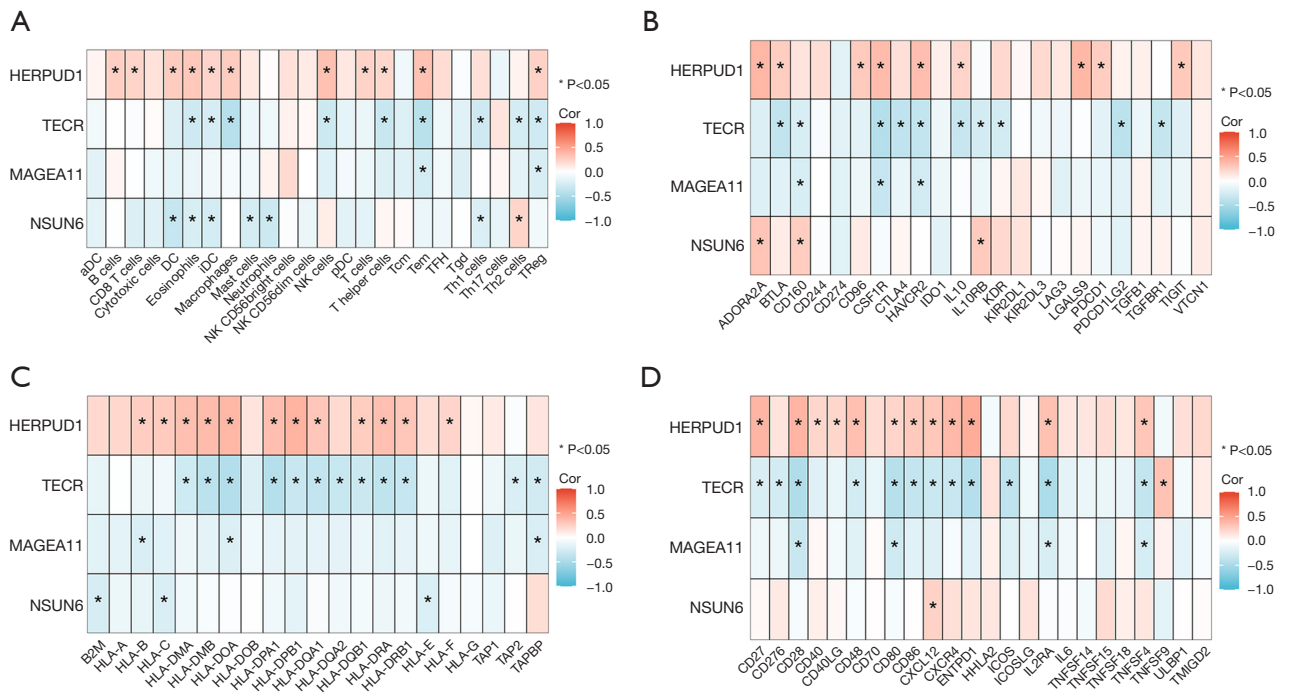

Figure 8 Correlation of *HERPUD1*, *TECR*, *MAGEA11*, and *NSUN6* with immune infiltration in patients with ESCC. (A) The ssGSEA algorithm was used to calculate the relationship of *HERPUD1*, *TECR*, *MAGEA11*, and *NSUN6* with the enrichment scores of 24 types of immune cells. The correlation of *HERPUD1*, *TECR*, *MAGEA11*, and *NSUN6* with (B) immunoinhibitors, (C) MHC molecules, and (D) immunostimulators was measured with Spearman correlation analysis. *, P<0.05. *HERPUD1*, homocysteine-inducible ER protein with ubiquitin-like domain 1; *TECR*, trans-2,3-enoyl-CoA reductase; *MAGEA11*, melanoma antigen gene A11; *NSUN6*, NOP2/Sun RNA methyltransferase 6; MHC, major histocompatibility complex; ESCC, esophageal squamous cell carcinoma; ssGSEA, single-sample gene set enrichment analysis; Cor, correlation.

GO and KEGG analysis of HERPUD1 and TECR-related immune infiltration pathway enrichment in patients with ESCC

Venn diagram represents the gene intersection (n=25) of immune infiltration-related genes and *HERPUD1*-related DEGs (*Figure 9A*). Subsequently, GO and KEGG analyses were employed to investigate the immune infiltration-associated signaling pathways mediated by *HERPUD1* (*Figure 9B*). *HERPUD1* may regulate immune infiltration through humoral immune response, mononuclear cell differentiation, and B cell activation. Venn diagram represents the gene intersection (n=48) of

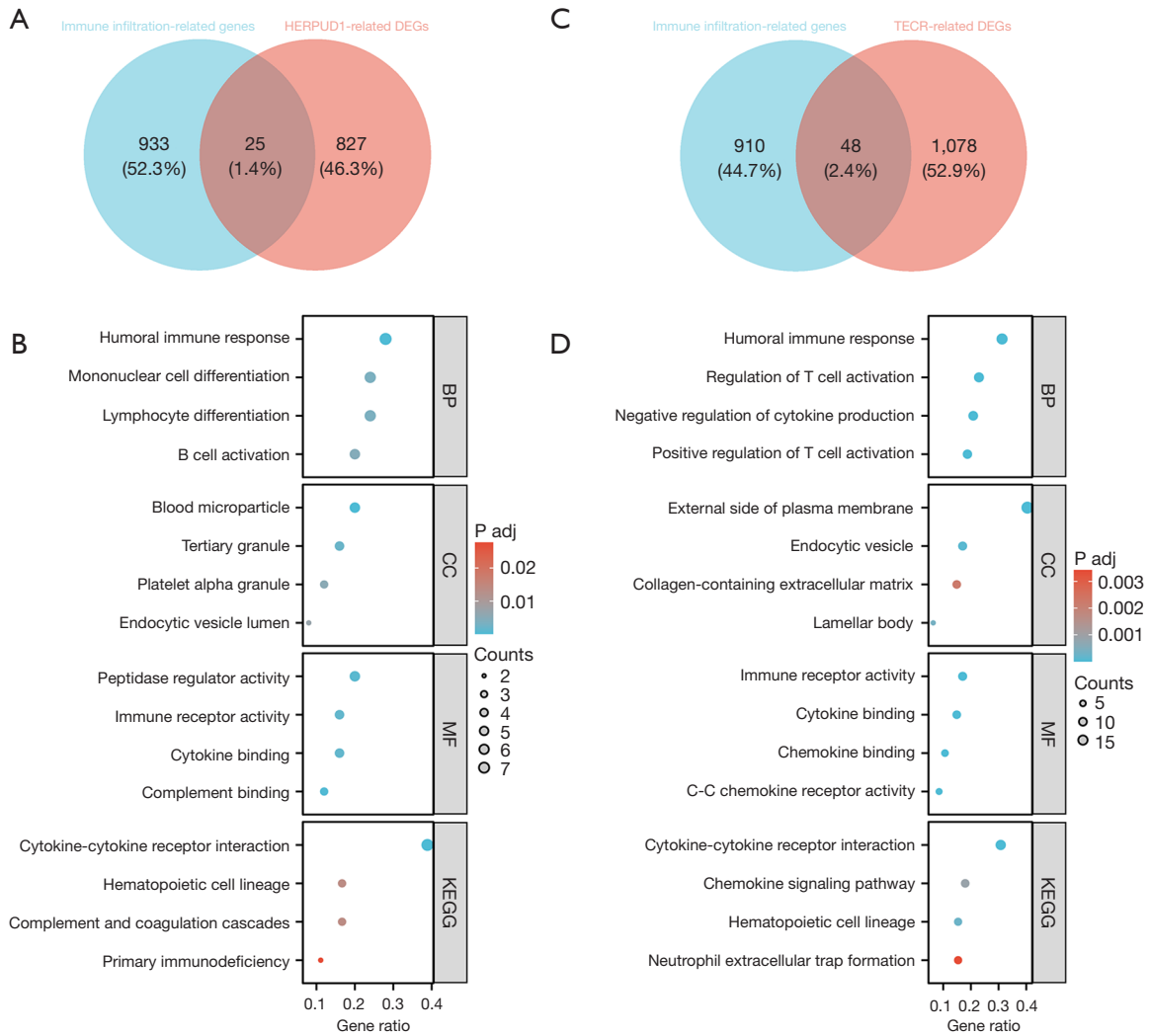

Figure 9 GO and KEGG analysis of *HERPUD1* and *TECR*-related immune infiltration pathway enrichment in patients with ESCC. (A) Venn diagram represents the gene intersection of immune infiltration-related genes and *HERPUD1*-related DEGs; (B) GO and KEGG analysis of the gene intersection of immune infiltration-related genes and *HERPUD1*-related DEGs; (C) Venn diagram represents the gene intersection of immune infiltration-related genes and *TECR*-related DEGs; (D) GO and KEGG analysis of the gene intersection of immune infiltration-related genes and *TECR*-related DEGs. GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; *HERPUD1*, homocysteine-inducible ER protein with ubiquitin-like domain 1; *TECR*, trans-2,3-enoyl-CoA reductase; DEGs, differentially expressed genes; BP, biological process; CC, cellular component; MF, molecular function; adj, adjusted.

immune infiltration-related genes and *TECR*-related DEGs (*Figure 9C*). On the other hand, *TECR* potentially influences immune infiltration via humoral immune response, positive regulation of T cell activation, and negative regulation of cytokine production (*Figure 9D*). These findings suggest that HERPUD1 and TECR may exhibit divergent signaling mechanisms in modulating immune infiltration, despite sharing some overlapping biological processes.

Discussion

In this study, we constructed a prognostic model using methylation-related regulators *HERPUD1*, *TECR*, *MAGEA11*, and *NSUN6* to evaluate the prognosis of patients with ESCC. A higher prognostic risk score was associated with shorter OS in patients with ESCC (HR =5.77, 95% CI: 2.13–15.58; P<0.001). Time-dependent AUC analysis revealed that *HERPUD1*, *TECR*, *MAGEA11*,

and *NSUN6* had high prognostic predictive value at different time points. Furthermore, our analysis revealed that combined diagnostic model based on *HERPUD1*, *TECR*, *MAGEA11*, and *NSUN6* demonstrated excellent diagnostic efficacy for ESCC (AUC =0.911; 95% CI: 0.888–0.935). Finally, through immune enrichment analysis, we discovered that *HERPUD1* was significantly positively correlated with immune infiltration at both the cellular and genetic levels, while *TECR* showed a significant negative correlation with immune infiltration levels. Overall, we found that the prognostic model constructed with the methylation-related regulators *HERPUD1*, *TECR*, *MAGEA11*, and *NSUN6* could effectively predict the prognosis of patients with ESCC, provide good diagnostic efficacy for ESCC, and reflect the immune cell infiltration of the immune microenvironment in patients with ESCC. These genes may serve as diagnostic and prognostic biomarkers as well as therapeutic targets for the diagnosis, treatment, and prognosis of ESCC.

Beyond the prognostic and diagnostic models we developed, our analysis of the TCGA and three GEO databases revealed that *MAGEA11* was consistently upregulated in patients with ESCC across all datasets. Furthermore, *MAGEA11* was identified as a protective factor for OS, DSS, and PFS in patients with ESCC. It was also found that *MAGEA11* had the best predictive efficacy for the 5-year OS of patients with ESCC.

The MAGE family is a well-known tumor-associated antigen composed of more than 60 genes. *MAGEA11* is a type I MAGE and is rarely expressed in normal adult tissues but is highly expressed in various cancers, including ESCC (18). In cancer cells, DNA methylation plays a primary role to mediate *MAGEA11* gene silencing (18). Xiao *et al.* (19) reported that the upregulation of *MAGEA11* in gastric cancer is significantly correlated with shorter survival and metastasis by immune infiltration. Mohsenzadegan *et al.* (20) found that a significant and positive correlation was found between both nuclear and cytoplasmic expressions of the MAGEA6 protein as well as expression of cytoplasmic MAGEA11 protein with histological grade, lamina propria invasion, and muscularis involvement in patients with bladder cancer; this suggested that the coexpression of cancer-testis antigens of MAGEA6 and MAGEA11 is associated with tumor aggressiveness. Kalvapudi *et al.* (21) indicated that the MAGE gene family, including MAGEA1, MAGEA3, MAGEA4, MAGEA1, and MAGEA12, exhibited the highest expression levels in gastroesophageal adenocarcinoma (GEAC) and may be associated with poor prognosis in GEAC, offering potential therapeutic targets for the treatment of GEAC. Given these aforementioned findings, *MAGEA11* may be a molecule associated with poor prognosis in bladder cancer and gastric cancer, which is completely contrary to our results. However, we also found that the expression of the *MAGEA11* gene is significantly upregulated in patients with ESCC. Furthermore, we did not find a significant association between *MAGEA11* and immune cell infiltration or immunomodulators. Therefore, we can infer that the prognostic significance of *MAGEA11* and its regulatory mechanisms may vary across different tumors.

Recent studies have shown that *HERPUD1* maintains mitochondrial function and energy metabolism, along with autophagy, while inhibiting apoptosis and ferroptosis to sustain the malignant characteristics of cancer cells (22,23). However, there are no relevant reports on the role and clinical significance of *HERPUD1* in regulating the tumor biology of ESCC. Based on bioinformatics results, we found that the high expression of *HERPUD1* is associated with poor prognosis in patients with ESCC and is related to the enrichment of various immune cells, including B cells, eosinophils, natural killer cells, and T effector memory (TEM) cells. The correlation of *HERPUD1* with immune cell infiltration and its regulatory mechanisms have not been validated by *in vivo* and *in vitro* experiments. Our findings also indicated that compared to the other three prognostic factors, *HERPUD1* (AUC =0.778; 95% CI: 0.734–0.821) had the strongest diagnostic efficacy for ESCC. Meanwhile, *HERPUD1* had a high predictive value for the 5-year OS of patients with ESCC.

In esophageal cancer, *TECR* has been identified as a differentially methylated gene, which may be associated with cancer stem-like cells (24). In our study, we found that the high expression of *TECR* was associated with shorter OS in patients with ESCC, despite its limited utility as a diagnostic marker. Our results also revealed that *TECR* was negatively correlated with the infiltration of various immune cells and immunomodulators. Notably, it was significantly negatively correlated with the enrichment scores of macrophages and TEM cells.

HERPUD1 and *TECR* exhibit divergent correlations with immune infiltration in our analyses. However, to date, no experimental evidence has been reported in the literature to elucidate the biological mechanisms underlying their association with immune infiltration. The observed correlations are currently supported only by bioinformatics algorithms (25,26), which suggest potential, but unproven, relationships. It is important

to note that genes directly linked to immune infiltration typically include immunoinhibitors, MHC molecules, and immunostimulators. While other genes (e.g., *HERPUD1* and *TECR*) may show associations with immune infiltration, these relationships could be indirect. In our study, we present these findings solely to hypothesize that the prognostic value of *HERPUD1* and *TECR* might involve interactions with immune-related molecules (e.g., via immunoinhibitory/stimulatory pathways or antigen presentation) to influence ESCC outcomes.

NSUN6 exhibits strong substrate specificity for messenger RNA (mRNA), primarily targeting the 3'UTR region of the common sequence motif CTCCA located within the stem-loop structure (27). The 5-methylcytosine (m5C) modification mediated by NSUN6 can enhance mRNA abundance and translation efficiency and promote the methylation of cytosine at position 72 (C72) in the 3' end receptor of tRNACys and tRNAThr, with target recognition dependent on the 3'-CCA tail (27). NSUN6 has also been shown to inactivate macrophage stimulating 1 (*MST1*) through m5C modification and to activate the target gene of yes-associated protein (YAP) in breast cancer, thereby triggering osteoclast differentiation and bone metastasis (28). NSUN6 mediates m5C modification of multiple genes to deteriorate the progression of cervical cancer, lung cancer, and colon adenocarcinoma (29-31). Fang *et al.* (32) found that *NSUN6* expression is significantly increased in colorectal cancer tissues and closely related to B cells and $CD8^+$ T cells. In our study, *NSUN6* was significantly downregulated in ESCC tissues, and its high expression was strongly associated with a favorable prognosis. However, its diagnostic value remained relatively limited. Moreover, *NSUN6* showed only a weak correlation with a few immune cells, and its correlation with immune cells was notably weaker than that of *HERPUD1* and *TECR* in patients with ESCC.

The prognostic model based on *HERPUD1*, *TECR*, *MAGEA11*, and *NSUN6* demonstrates high predictive performance, with AUC values no lower than 0.860 at various time points. For comparison, Cao *et al.* (9) constructed a combined cell death index (CCDI) model, which showed the highest reported accuracy in their study (AUC =0.834). Notably, our model achieves a maximum AUC of 0.944, markedly surpassing the predictive capability of the CCDI model. Based on single-cell RNA sequencing (scRNA-seq) and bulk RNA analysis of dendritic cells, Shi *et al.* (33) found a prognostic model that demonstrated good predictive performance with AUC values of 0.81, 0.76, and 0.74 for 1-, 3-, and 5-year survival predictions, respectively. Using the anoikis-related genes, Cao *et al.* (34) established a prognostic risk model for ESCC with AUC for 1- and 3-year OS of 0.684 and 0.627 respectively. Overall, our ESCC prognostic model, based on methylation-associated regulators, exhibits obviously enhanced predictive performance compared to existing models.

Although our study constructed a prognostic and diagnostic model based on *HERPUD1*, *TECR*, *MAGEA11*, and *NSUN6* for the diagnosis and prognostic prediction of ESCC, some limitations to the study should be noted. Firstly, the expression of *HERPUD1*, *TECR*, and *NSUN6* varies across different datasets, and we were able to account for this due to the different sample sizes of different datasets. Perhaps the expression of these genes needs to be validated in a study with a larger sample size. Secondly, there were differences in the correlation of *HERPUD1*, *TECR*, *MAGEA11*, and *NSUN6* with immune cells. We only predicted this relationship through bioinformatics without validating it in *in vivo* experiments. Thirdly, our study did not collect external clinical samples to validate the model. We plan to address this gap in future research by expanding the validation cohort. Specifically, we will collect peripheral blood samples and tumor tissue samples from additional ESCC patients to rigorously assess the robustness and generalizability of the model. Fourthly, the prognostic model comprising *HERPUD1*, *TECR*, *MAGEA11*, and *NSUN6* demonstrates significant predictive value in ESCC. However, translating theoretical findings into clinical practice remains challenging. Currently, evaluating these genes requires obtaining tumor tissue samples from ESCC patients, a process that is invasive, resource-intensive, and burdensome for patients, which limits its clinical feasibility. To address this, our future work will focus on non-invasive validation by investigating the consistency of *HERPUD1*, *TECR*, *MAGEA11*, and *NSUN6* expression between peripheral blood samples and tumor tissues, as well as validating the prognostic model's stability across these biospecimens. Developing an online platform to calculate prognostic probability using the following formula: Risk Score = Intercept + $\alpha \times$ [*HERPUD1* (low =0 or high =1)] + $\beta \times$ [*TECR* (low =0 or high =1)] + $\lambda \times$ [*MAGEA11* (low =0 or high =1)] + $\delta \times$ [*NSUN6* (low =0 or high =1)]; parameters: intercept =0; α=1.12; β=1.19; λ=–2.50; δ=–1.90.

Conclusions

We developed a robust prognostic model incorporating

HERPUD1, *TECR*, *MAGEA11*, and *NSUN6*, revealing that a higher risk score was significantly associated with worse OS. We have also shown that the model combining *HERPUD1*, *TECR*, *MAGEA11*, and *NSUN6* had high diagnostic efficacy for ESCC. Overall, the diagnostic and prognostic model based on *HERPUD1*, *TECR*, *MAGEA11*, and *NSUN6* may serve as a biomarker for improving the clinical outcomes of patients with ESCC. Our findings need to be validated in larger studies.

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None.

Footnote

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

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