Original Article

EZH2-regulated immune risk score prognostic model predicts outcome of clear cell renal cell carcinoma

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Background: The enhancer of zeste homolog 2 (EZH2) plays an important role in the tumor microenvironment (TME), and EZH2 in shaping the epigenetic landscape of $CD8^+$ T cell fate and function, with a particular emphasis on cancer. Here, high EZH2 expression always leads to less $CD8^+$ T cell infiltration. However, clear cell renal cell carcinoma (ccRCC) is reportedly a "hot" tumor, with contradictory high EZH2 expression. Our goal was to construct a EZH2-regulated immune risk score prognostic model to predict ccRCC outcomes, and provide a prospect of clinical EZH2 inhibitors in fine-tuning T cell responses with immune therapy.

Methods: We downloaded and analyzed The Cancer Genome Atlas (TCGA), Cancer Cell Line Encyclopedia (CCLE), TISIDB database, and WebGestalt for ccRCC patients, EZH2-related tumor-infiltrating lymphocytes and immunomodulators. R packages "limma", "BiocManager", and "preprocessCore", etc. were downloaded to prepare CIBERSORT files, immune cells heatmap, multivariable Cox model and survival analysis. The EZH2-regulated immune risk model's prognostic ability was calculated by receiver operating characteristic (ROC) and area under the curve (AUC) analyses in R studio.

Results: EZH2 was highly expressed and related to poor outcome in ccRCC. However, high-expression EZH2 was not related to a "cool" tumor. Of the 49 immunomodulators significantly regulated by EZH2, forest plot showed 26 immunomodulators signatures independently associated with overall survival. The EZH2-regulated immune-risk score prognostic model was an independent prognostic factor (AUC =0.816), especially combined with clinicopathologic parameters in ccRCC overall survival prediction.

Conclusions: The EZH2-regulated immune-risk score prognostic model was an independent prognostic factor, with good accuracy and predictability, and could provide experimental data to the clinical area.

Keywords: Enhancer of zeste homolog 2 (EZH2); immunomodulators; overall survival (OS); renal cell carcinoma (RCC)

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Introduction

According to the 2022 cancer statistics, there were ≈431,288 new cases of kidney and renal pelvis cancer in 2020, and 179,368 estimated deaths (The Global Cancer Observatory). Approximately 85–90% of renal cell carcinoma (RCC) cases are the clear cell type (ccRCC) (1), and the prognosis of metastatic ccRCC is poor, with a 5-year survival rate <10% after diagnosis (2).

Enhancer of zeste homolog 2 (EZH2) has pleiotropic functions in both tumor and tumor microenvironment (TME) immune cells (3). In most cancer types, EZH2 has an important immunoediting function, leading to an immunosuppressive TME through a variety of mechanisms: (I) tumor-associated antigens or neoantigens loss of expression (4); (II) immunosuppressive molecules and changing the expression of proinflammatory cytokines (5); (III) aberrant expression of checkpoint pathway proteins [e.g., programmed cell death 1 ligand 1 (PD-L1)] (6). EZH2 is overexpressed in endometrial cancer, small-cell lung cancer, prostate cancer, breast cancer and melanoma. To date, EZH2 inhibitors have been evaluated in clinical trials targeting EZH2-driven epigenetic alterations alone or in combination with an immune checkpoint antibody, such as CPI-1205, CPI-0209, and DS-3201 (3,4,7). It is important to know if EZH2 inhibition will overcome its potential immunosuppressing effect in most cancers.

Sun *et al.* reported that EZH2 is highly expressed in *BAP1*-mutant ccRCC and is related to poor prognosis (8). Intrinsic or acquired resistance to receptor tyrosine kinase inhibitors is a major problem in ccRCC clinical treatment (9).

Highlight box

Key findings

- EZH2 regulated immune-risk score prognostic model was an independent prognostic factor, accuracy and good predictability in ccRCC. Our research also indicated immune-risk score prognostic model may guide EZH2-targeted therapies for ccRCC patients.

What is known and what is new?

- High EZH2 expression always leads to less $CD8^+$ T cell infiltration. However, ccRCC were "hot" tumor as reported, it is contradictory with high EZH2 expression in ccRCC;
- EZH2 regulated-immune risk score prognostic model to predict ccRCC patient outcome.

What is the implication, and what should change now?

- Assessment of the EZH2 effects on TEM will help inform the best utilization of EZH2 inhibitors in the clinic.

Adelaiye-Ogala *et al.* reported that EZH2 is a rational target for therapeutic intervention in sunitinib-resistant ccRCC (10). Furthermore, Eichenauer *et al.* (11) investigated the relationship between EZH2 expression and the density of $CD8^+$ T cells in 1,800 RCC patients' tumor tissues. They found that the density of $CD8^+$ T cells continuously increased with raising EZH2 levels. EZH2 was reported to decrease $CD8^+$ T cells infiltration though secretion of CXCL9/CXCL10 (6). There are contradictory phenomena (high EZH2 expression and "hot" TME) in ccRCC.

In this study based on the multiple immune effects of EZH2 on TME-forming immune cells, we aimed to establish a EZH2-regulated immunomodulators risk score prognostic model that could be applied to ccRCC diagnosis and treatment. In addition, this study demonstrated that the prognostic signature might show indispensable implications on modulating the TME and directing immunotherapy intervention in ccRCC. Pertaining to the limited data obtained from the TCGA database, these findings need to be further corroborated in a larger cohort or in cytological experiments. We present the following article in accordance with the TRIPOD reporting checklist (available at https://tau.amegroups.com/article/view/10.21037/tau-22-817/rc).

Methods

Datasets and samples

We downloaded data for 611 ccRCC patients from The Cancer Genome Atlas (TCGA), including transcriptome [count and fragments per kilobase of exon model per million mapped fragments (FPKM) value] and clinical information, and the 611 ccRCC patients' clinical information including: survival time, TNM, stage, age, and gender. This study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

Tumor-infiltrating lymphocytes (TILs)

The immune-related signatures of 28 TILs [activated B cell; activated $CD4^+$ T cell; activated $CD8^+$ T cell; central memory $CD4^+$ T cell; central memory $CD8^+$ T cell; effector memory $CD4^+$ T cell; effector memory $CD8^+$ T cell; gamma delta T cell; immature B cell; memory B cell; regulatory T cell; T follicular helper cell; type 1 T helper cell; type 17 T helper cell; type 2 T helper cell; activated dendritic cell; CD56bright natural killer (NK) cell; CD56dim NK cell; eosinophil; immature dendritic cell; macrophage; mast cell;

monocyte; myeloid-derived suppressor cell; NK cell; NK T cell; neutrophil; plasmacytoid dendritic cell] were analyzed in TISIDB website (12). The 28 TILs, gene expression, copy number, methylation, and mutation were viewed and downloaded. For each cancer type, the relative abundance of TILs was inferred from gene set variation analysis based on the gene expression profile.

Identification of EZH2-related immunomodulators

The relationship between two types of immunomodulator (immunoinhibitors and immunostimulators) and EZH2 expression was viewed and downloaded in TISIDB web site (12). The co-expression of immunoinhibitors and immunostimulators was analyzed with EZH2, and only immunoinhibitor or immunostimulator genes with a P value <±0.05 were regarded as EZH2-related immunogenes.

Enrichment analysis and protein-protein interaction (PPI) network of EZH2-related immune genes

WebGestalt (WEB-based Gene SeT AnaLysis Toolkit, http://www.webgestalt.org/) was utilized to generate a Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway of EZH2-related immune genes. PPI networks were generated on the STRING database (online), and the interactive source of minimum interaction score was set as 0.4.

Heatmap

We installed R packages: "e1071", "limma", "BiocManager", and "preprocessCore" to prepare CIBERSORT files. We used the R packages "pheatmap" and "vioplot", with P value <±0.05, to construct an immune cells heatmap in ccRCC.

Overall survival (OS)

Variance stabilizing transformation was used to normalize the 611 ccRCC patients, and then low-value genes were removed using heterogeneity analysis. According to EZH2 gene expression, Low and High groups were set up. Next, "BiocManager", "survival", and "survminer" packages were installed in R and used to calculate gene survival analysis.

Prognostic gene among EZH2-related immunogenes

EZH2-related immunogenes, and TCGA ccRCC clinical information were matched and merged using R package "limma". P value <±0.05 was considered as a prognostic immune-related gene.

Establishment of risk score prognostic model

A total of 26 related genes were analyzed to construct a predictive risk score prognostic model, using "glmnet", "survival", and "survminer" in a multivariable Cox model. According to the risk score prognostic model, ccRCC patients get a risk score, and divided into a high-risk group (risk score above the median value) and low-risk group (risk score below the median value) in R software. Furthermore, both groups were tested for OS, and prognostic model gene expression. Software package "survival ROC" was applied and the risk model prognostic ability was calculated by receiver operating characteristic (ROC) and area under the curve (AUC) analysis in R studio.

Statistical analysis

In this study, R software (version 4.0.3) was used for statistical analysis and visualization of results. Univariate and multivariate Cox regression analyses were applied for variable survival and OS. P<0.05 was considered as statistically significant.

Results

High-expression EZH2 not related to "Cool" TME in ccRCC

To understand the complexity and diversity of the role of EZH2 in the TME, we analyzed the relationships between the abundance of 28 TILs and EZH2 expression, copy number, and promoter methylation. As shown in *Figure 1A*, high mRNA EZH2 expression was negatively associated with almost all 28 TILs in various cancers (green arrowheads, blue small square in *Figure 1A* represents negative relationship), but not in thyroid carcinoma or ccRCC (indicated by arrowheads in *Figure 1A,1B*). In this study, we focused on ccRCC. We observed that immunostimulatory TILs (active $CD8^+$ T cells, P<0.05; effector $CD8^+$ T cells, P<0.05; active $CD4^+$ T cells, P<0.05) were all significantly positively correlated with high EZH2 expression. The increased number of immunostimulatory TILs indicated that EZH2 is a positive prognostic immunotherapy biomarker in ccRCC. We also observed immunosuppressive TILs: T regulatory cell (Treg)

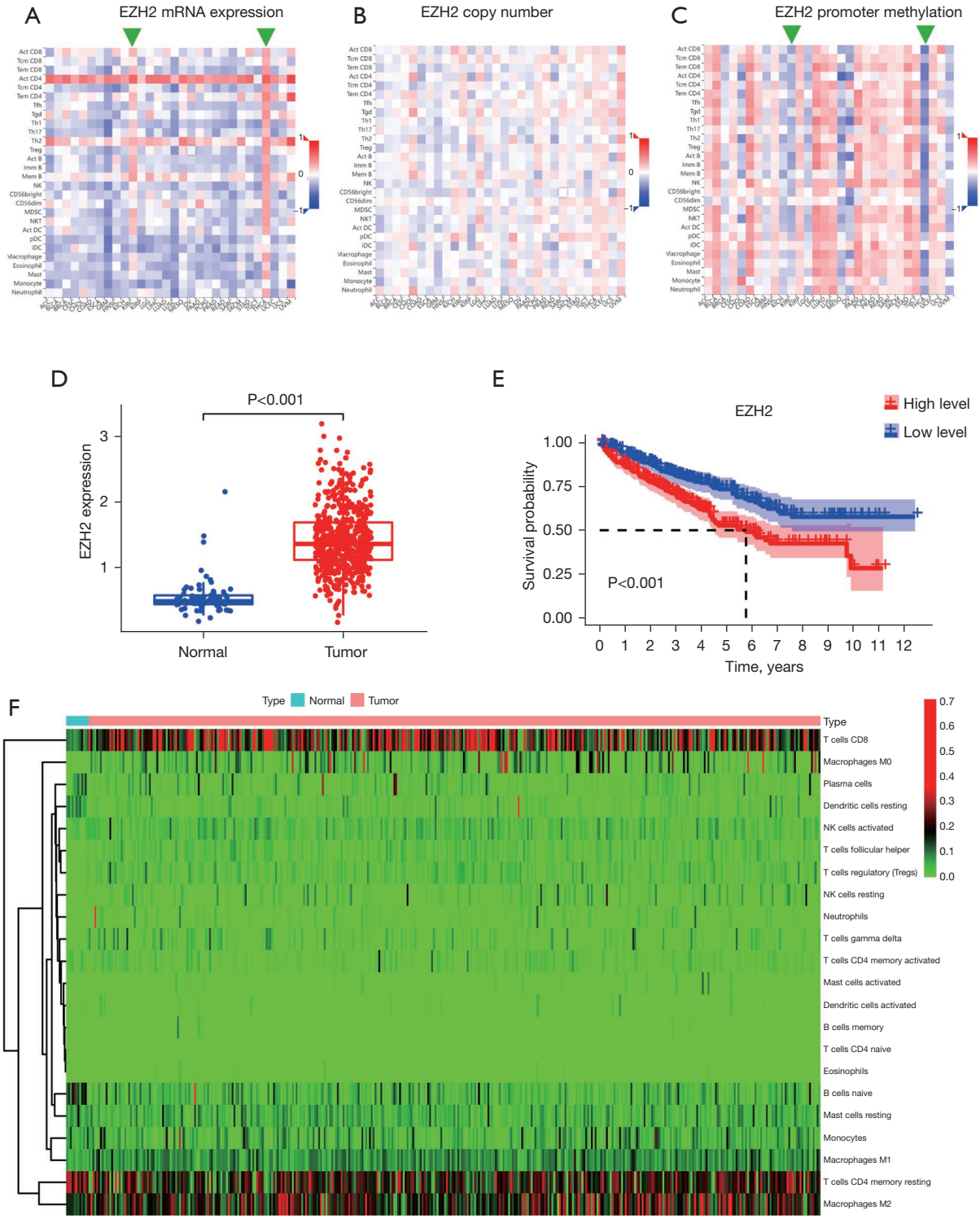

Figure 1 Negative prognostic predictor of EZH2 and "hot" TME in ccRCC. (A-C) Relationship of 28 TILs among EZH2 mRNA expression, copy number, and promoter methylation in TME. Green arrowheads pointed to the red column, that showed high EZH2 expression was positively correlated with 28 TILs in ccRCC and thyroid cancers (A), EZH2 promoter methylation was negatively correlated with 28 TILs in ccRCC and thyroid cancers (C). Red and blue small squares indicate positive and negative correlations, respectively. The color intensity is directly proportional to the strength of the correlation. (D) Relative expression of EZH2 in normal and individual ccRCC

tumor tissues (normal =12, tumor =530), P<0.05 is statistically significant. (E) High EZH2 expression predicts poor prognosis in ccRCC patients. Normal =265; tumor =265. P<0.05 is statistically significant. (F) Heatmap showing infiltration proportion of 22 immune cell types in individual ccRCC tumor tissues and normal tissues. EZH2, enhancer of zeste homolog 2; Act, activated; Tcm, central memory T cells; Tem, effector memory T cells; Tfh, follicular helper T cells; Tgd, gamma delta T cells; Th, T helper; Treg, regulatory T cells; Imm B, immature B cells; Mem B, memory B cells; NK, natural killer; MDSC, myeloid-derived suppressor cells; NKT, natural killer T cells; DC, dendritic cells; pDC, plasmacytoid dendritic cells; iDC, immature dendritic cells; ACC, adrenocortical carcinoma; BLCA, bladder urothelial carcinoma; BRCA, breast invasive carcinoma; CESC, cervical squamous cell carcinoma and endocervical adenocarcinoma; CHOL, cholangiocarcinoma; COAD, cholangiocarcinoma; ESCA, esophageal carcinoma; GBM, glioblastoma multiforme; HNSC, head and Neck squamous cell carcinoma; KICH, kidney chromophobe; KIRC, kidney renal clear cell carcinoma; KIRP, kidney renal papillary cell carcinoma; LGG, brain lower grade glioma; LIHC, liver hepatocellular carcinoma; LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma; MESO, mesothelioma; OV, ovarian serous cystadenocarcinoma; PAAD, pancreatic adenocarcinoma; PCPG, pheochromocytoma and paraganglioma; PRAD, prostate adenocarcinoma; READ, rectum adenocarcinoma; SARC, sarcoma; SKCM, skin cutaneous melanoma; STAD, stomach adenocarcinoma; TGCT, testicular germ cell tumors; THCA, thyroid carcinoma; UCEC, uterine corpus endometrial carcinoma; UCS, uterine carcinosarcoma; UVM, uveal melanoma; TME, tumor microenvironment; ccRCC, clear cell renal cell carcinoma; TILs, tumor-infiltrating lymphocytes.

infiltration increased with high EZH2 expression, which indicated that EZH2 is also a negative immunotherapy biomarker in ccRCC. As can be seen in *Figure 1B*,*1C*, contradictory immunostimulatory and immunosuppressive TILs are observed with EZH2 copy number and promoter methylation alteration in ccRCC (green arrowheads, blue small square in *Figure 1C* represents negative relationship).

We further explored EZH2 expression in tumor tissue and the effect on ccRCC OS. EZH2 mRNA expression was significantly increased in tumor tissues compared with normal tissues (*Figure 1D*), and was positively correlated with survival outcomes (*Figure 1E*, P<0.05). Using the CIBERSOFT algorithm, we estimated the infiltration proportion of 22 immune cell types from TCGA kidney renal clear cell carcinoma (KIRC) samples, divided into normal and tumor groups according to the clinical information. As shown in *Figure 1F*, $CD8^+$ T cells exhibited significantly higher infiltration levels in the tumor groups. In addition, we also observed that "T cells CD4 memory resting" were highly infiltrated in ccRCC tumors, which indicated ccRCC is not a "cool" tumor, but rather a "hot" tumor as reported.

Figure 1 shows that EZH2 was highly expressed and related to poor outcome. However, high-expression EZH2 is not related to a "cool" TME in ccRCC. It is urgent to investigate the complex effect of EZH2 on immunostimulatory and immunosuppressive TILs in ccRCC.

Immunostimulatory and immunosuppressive genes regulated by EZH2

To verify the complex effect of EZH2 on the immune TME of ccRCC, we further investigated the relationship between immunomodulators and EZH2 expression. As shown in *Figure 2A*, unlike other tumors, high EZH2 expression was positively correlated with most immunomodulators in ccRCC and thyroid carcinoma (purple arrowheads, red small square in *Figure 2A* represents positive relationship). We found immunostimulators such as lymphocyte activation gene 3 (LAG3), cytotoxic lymphocyte antigen 4 (CTLA4), CD96 (T-cell surface protein tactile, CD96 molecule), and T cell immunoreceptor with Ig and ITIM domains (TIGIT) were positively correlated with EZH2 expression. In *Figure 2B*, high EZH2 expression was also positively correlated with most immunosuppressors in ccRCC (purple arrowheads, red small square in *Figure 2A* represents positive relationship), such as: CD80, inducible T cell costimulator (ICOS), and TNF receptor superfamily member 9 (TNFRSF9). In *Figure 2A*,*2B*, it can be seen that 49 immunomodulator genes were significantly regulated by EZH2 (P>0.05 or P<0.05), 49 immunomodulator genes: *BTLA*, *CD96*, *CD160*, *CD244*, *CD274*, *CSF1R*, *CTLA4*, *HAVCR2*, *IL10*, *IL10RB*, *KDR*, *LAG3*, *LGALS9*, *PDCD1*, *PDCD1LG2*, *TIGIT*, *VTCN1*, *C10orf54*, *CD27*, *CD28*, *CD40LG*, *CD48*, *CD70*, *CD80*, *CD86*, *CXCR4*, *PVRL2*,

ICOS, *IL2RA*, *IL6*, *KLRC1*, *KLRK1*, *LTA*, *MICB*, *RAET1E*, *TNFRSF4*, *TNFRSF8*, *TNFRSF9*, *TNFRSF17*, *TNFRSF14*, *TNFRSF18*, *TNFRSF25*, *TNFSF4*, *TNFSF9*, *TNFSF13*, *TNFSF14*, *TNFSF13B*, *TNFSF15*, *ULBP1*. We submitted these 49 immunomodulators to WebGestalt for KEGG pathway enrichment and as shown in *Figure 2C*, intestinal immune network for IgA, T-cell receptor signaling pathway, and cytokine-cytokine receptor interaction were all influenced by these immunomodulators. *Figure 2C* shows the complex system immune effect, underlining the urgent need to construct a precise predictive immune risk score prognostic model for ccRCC.

Establishment of EZH2 immune risk score prognostic model

To explore the prognostic ability of EZH2-regulated immunomodulators, we analyzed the expression of the 49 immunomodulators and clinical information in TCGA ccRCC datasets. A forest plot showed 26 immunomodulator signatures independently associated with OS (*Figure 3A*): 5 low-risk genes and 21 high-risk genes (*CTLA4*, *HAVCR2*, *IL10RB*, *KDR*, *LAG3*, *LGALS9*, *PDCD1*, *TIGIT*, *CD80*, *IL2RA*, *IL6*, *KLRK1*, *LTA*, *MICB*, *RAET1E*, *TNFRSF8*, *TNFRSF9*, *TNFRSF17*, *TNFRSF18*, *TNFRSF25*, *TNFSF4*, *TNFSF13*, *TNFSF14*, *TNFSF13B*, *TNFSF15*, *ULBP1*). Next, based on the expression levels of the immunomodulators and their coefficients derived from the multivariable Cox model, we established an immune risk score prognostic model (*Figure 3B*), The computer risk score model = (HAVCR2 × −0.1057) + (IL10RB ×0.4372) + (KDR × −0.2385) + (TNFRSF25 ×0.2402) + (TNFSF13 × −0.1671) + (TNFSF14 ×0.1777) + (ULBP1 ×0.6268) + (TNFSF4 ×0.3271).

Next, to validate the immune risk score prognostic model, the high- and low-risk subgroups of ccRCC patients were evaluated by the risk score model. The OS results showed the low-risk group Kaplan-Meier OA curve was significantly higher than for the high-risk group (*Figure 4A*, P<0.001). According to the immune risk score, most of the deceased patients had been in the high immune risk score area (*Figure 4B*). The high-risk group patients had increasing risk score and death rate (*Figure 4C*). A heatmap showed the expression of 8 genes in both risk group patients (*Figure 4D*).

Based on the findings shown in *Figures 3*,*4*, the EZH2-regulated immune-risk score prognostic model was able to predict the OS of ccRCC patients. However, the question is whether the EZH2-regulated immune-risk score prognostic model is an independent predictor of ccRCC OS?

Assessment of the independence of the EZH2-regulated immune-risk score prognostic model

We conducted univariate and multivariate Cox regression analyses to evaluate whether our model can be used as an independent prognostic factor. As shown in *Figure 5A*,*5B*, immune risk score, age, grade, and stage were all significantly associated with OS in ccRCC. In both analyses the EZH2-regulated immune-risk score prognostic model correlated significantly with poor OS [hazard ratio (HR) =1.268, P<0.001; HR =1.142, P<0.001, respectively]. Furthermore, multivariate Cox regression analysis showed that age and clinicopathologic stage correlated with worse OS (HR =1.031, P<0.001; HR =1.608, P<0.001, respectively). Therefore, the EZH2-regulated immune-risk score prognostic model could be an independent prognostic factor of OS when adjusted by these variables.

Immune risk score prognostic model with clinicopathologic parameters to predict survival rate in ccRCC

To validate clinical diagnostic value of the immune risk score prognostic model with/without clinicopathologic parameters, ROC curves were obtained. As showed in *Figure 6A*, immune risk score prognostic model, grade, and stage were all had a ROC area. The bigger of the value of ROC area, the better of the clinical diagnostic value. The AUC of our immune risk score prognostic model was 0.708, especially, the immune risk score prognostic model plus clinicopathologic parameters (AUC =0.816). Next, the immune risk score prognostic model prediction and actual outcome in ccRCC were calculated. *Figure 6B* shows that the 5-year OS prediction fitted well in TCGA cohort validation, indicating the immune risk score prognostic model provided accuracy and good predictability. *Figure 6C* shows the nomogram combining meaningful clinical characteristics and immune risk score prognostic model. *Figure 6* indicates more reliable predictive ability of our immune risk score prognostic model.

Discussion

In 2019, the US Food and Drug Administration (FDA) approved programmed cell death 1 (PD-1)/PD-L1 inhibitors, or PD-1/PD-L1 inhibitors combined with

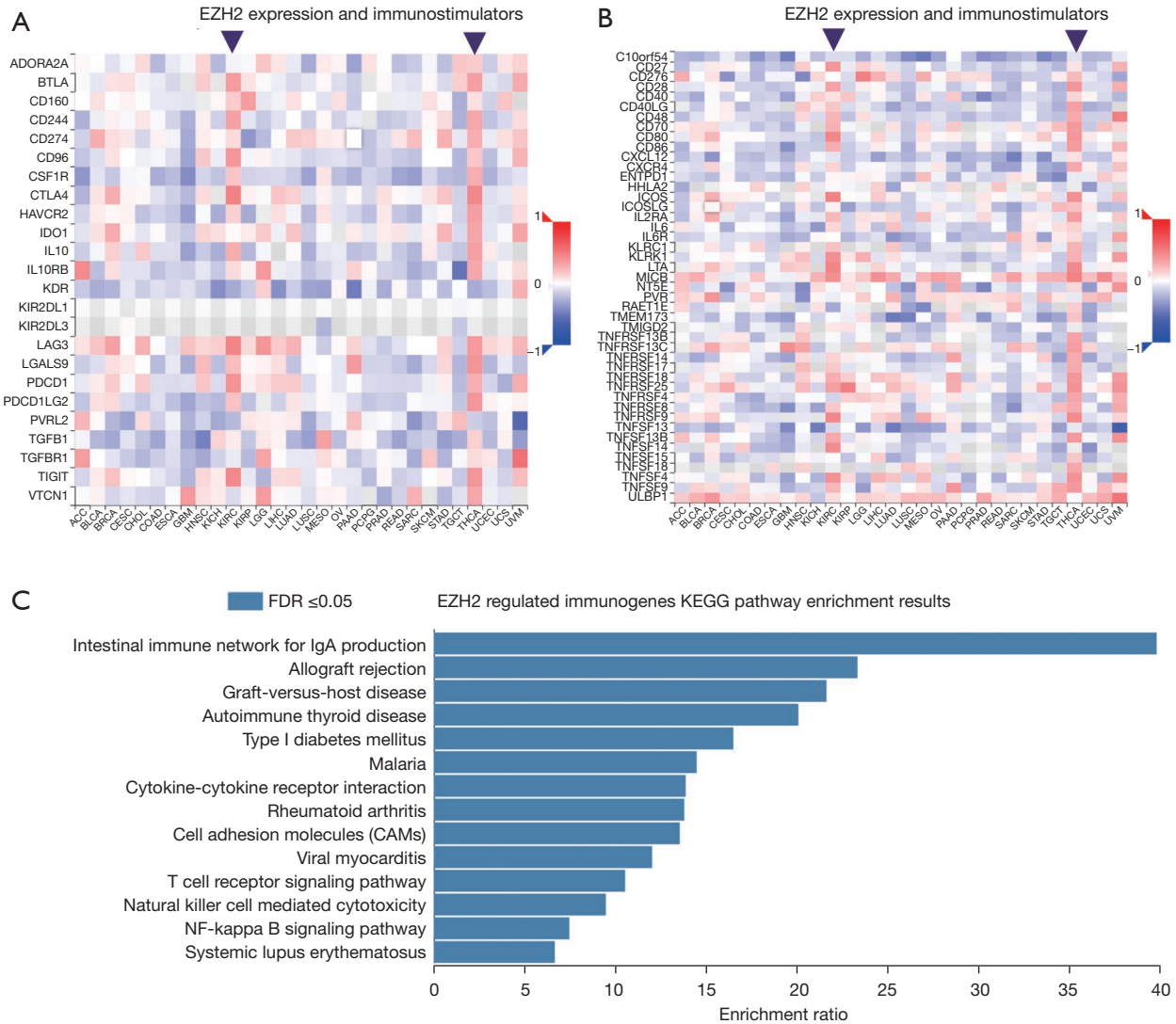

Figure 2 Interrelationship of EZH2 expression and immunomodulators. (A) Heatmap shows the positive relationship between EZH2 and immunostimulators. Purple arrowheads pointed to the red column, that showed high EZH2 expression was positively correlated with the most immunostimulators in ccRCC and thyroid cancers. (B) Heatmap shows the positive relationship between EZH2 and immunoinhibitors. Purple arrowheads pointed to the red column, that showed high EZH2 expression was positively correlated with the most immunoinhibitors in ccRCC and thyroid cancers. (C) EZH2-regulated immunomodulators KEGG pathway enrichment. P<0.05 is statistically significant. EZH2, enhancer of zeste homolog 2; ACC, adrenocortical carcinoma; BLCA, bladder urothelial carcinoma; BRCA, breast invasive carcinoma; CESC, cervical squamous cell carcinoma and endocervical adenocarcinoma; CHOL, cholangiocarcinoma; COAD, cholangiocarcinoma; ESCA, esophageal carcinoma; GBM, glioblastoma multiforme; HNSC, head and neck squamous cell carcinoma; KICH, kidney chromophobe; KIRC, kidney renal clear cell carcinoma; KIRP, kidney renal papillary cell carcinoma; LGG, brain lower grade glioma; LIHC, liver hepatocellular carcinoma; LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma; MESO, mesothelioma; OV, ovarian serous cystadenocarcinoma; PAAD, pancreatic adenocarcinoma; PCPG, pheochromocytoma and paraganglioma; PRAD, prostate adenocarcinoma; READ, rectum adenocarcinoma; SARC, sarcoma; SKCM, skin cutaneous melanoma; STAD, stomach adenocarcinoma; TGCT, testicular germ cell tumors; THCA, thyroid carcinoma; UCEC, uterine corpus endometrial carcinoma; UCS, uterine carcinosarcoma; UVM, uveal melanoma; KEGG, Kyoto Encyclopedia of Genes and Genomes; FDR, false discovery rate.

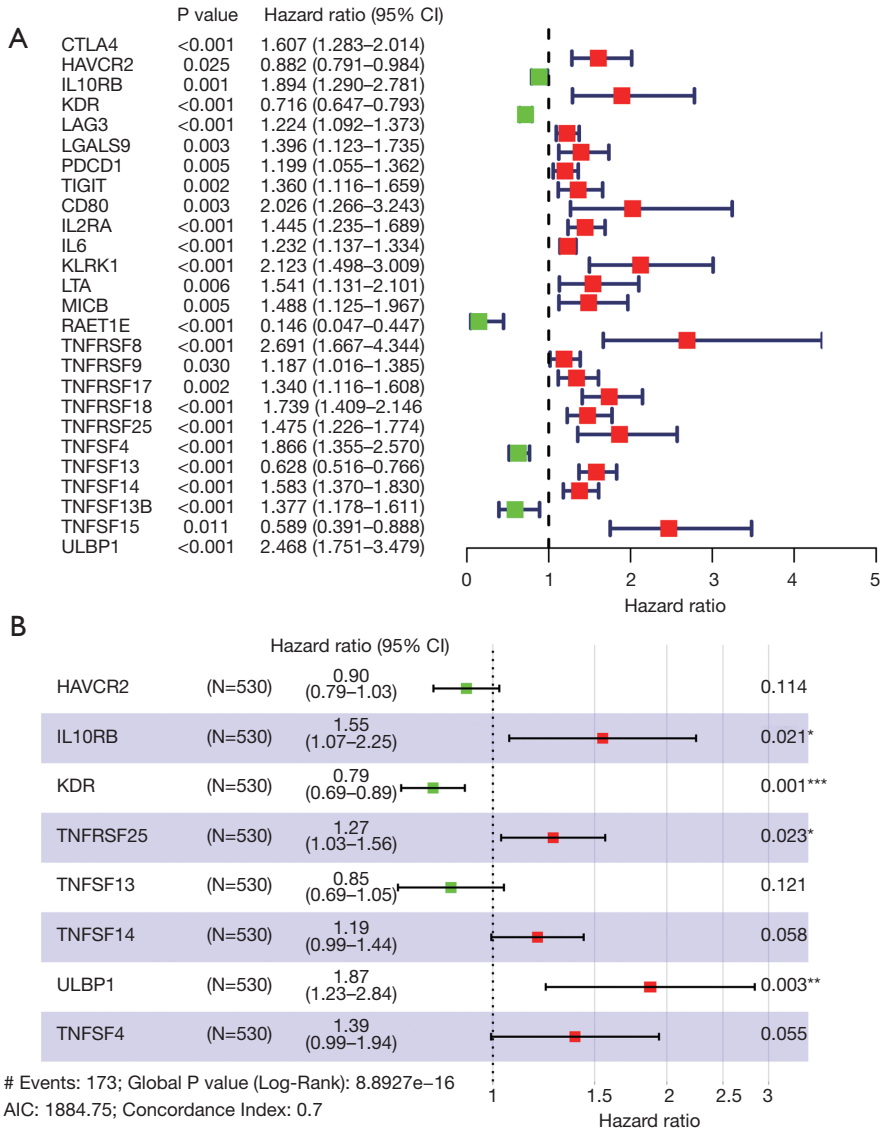

Figure 3 Construction of EZH2-regulated immune risk score prognostic model. (A) Forest plot showing 26 genes significantly regulated by EZH2 that are predictive of overall survival. P<0.05 is statistically significant. (B) Forest plot showing EZH2-regulated immune risk score prognostic model according to the corresponding coefficients derived from the multivariable Cox analysis. P<0.05 is statistically significant. *, P<0.05; **, P<0.01; ***, P<0.001. CI, confidence interval; EZH2, enhancer of zeste homolog 2; CTLA4, cytotoxic lymphocyte antigen 4; LAG3, lymphocyte activation gene 3; TIGIT, T cell immunoreceptor with Ig and ITIM domains; TNFRSF9, TNF receptor superfamily member 9; AIC, Akaike information criterion.

targeted drug therapy, to replace targeted drugs as first-line treatment of intermediate- and high-risk metastatic renal cancer (9,13-15). In nearly 2,000 kidney cancer patients in 151 countries, the objective response rate and progression-free survival was significantly better in the PD-1/PD-L1 inhibitor combined targeted drug therapy patient group, with respect to the targeted drug therapy patient group (16-20). However, up to 70% of renal cancer patient still do not respond to PD-1/PD-L1 inhibitors (21,22).

Substantial data have confirmed that EZH2 is involved in promoting metastasis, invasiveness (23,24), and maintenance of malignant phenotype (3,23,25). It influences

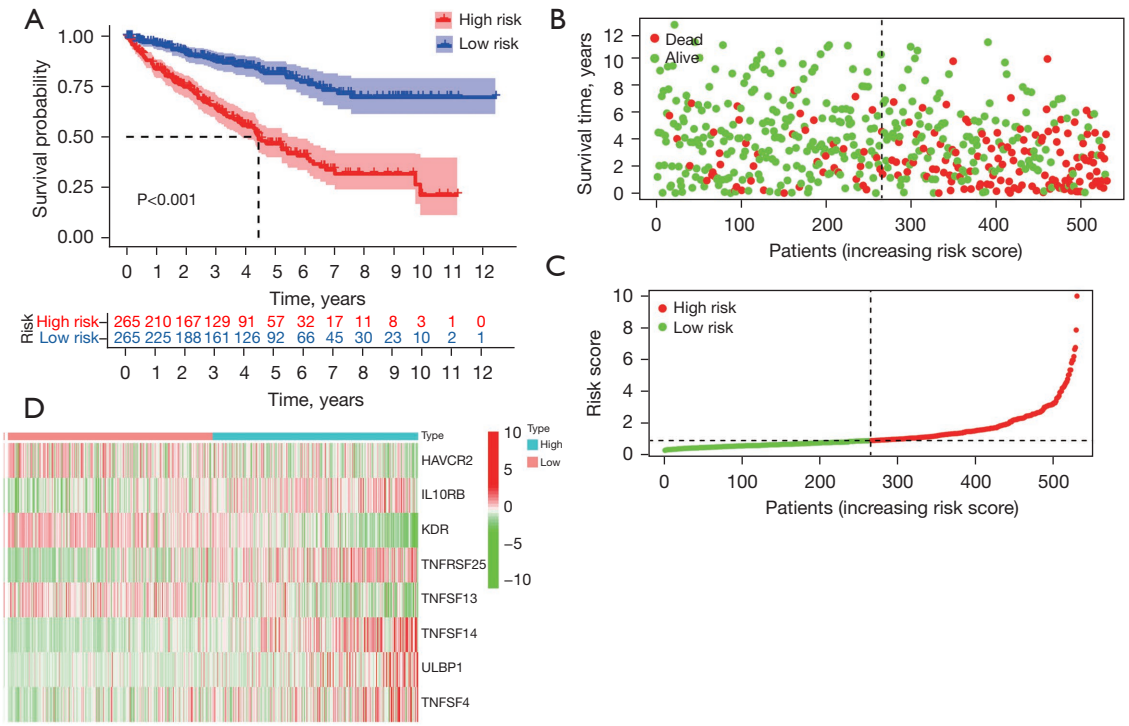

Figure 4 Validation of EZH2 regulated immune-risk score prognostic model. (A) Overall survival in high-risk and low-risk groups based on risk stratification and the Kaplan-Meier analysis. P<0.05 is statistically significant. (B) The scatterplot showed patients' status distribution. (C) The distribution of patients' risk score. (D) Heatmap showing the expression levels of genes in ccRCC patients. EZH2, enhancer of zeste homolog 2; ccRCC, clear cell renal cell carcinoma.

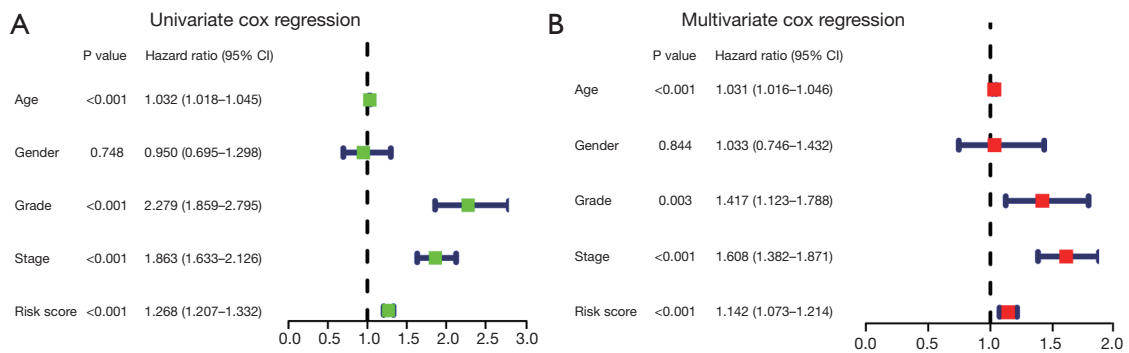

Figure 5 The immune risk score prognostic model is an independent prognostic factor. (A) Univariate analysis of the correlation of the immune risk score prognostic model to ccRCC patients' overall survival. (B) Multivariate analysis of the correlation of the immune risk score prognostic model to RCC patients' overall survival. P<0.05 is statistically significant. ccRCC, clear cell renal cell carcinoma; CI, confidence interval; RCC, renal cell carcinoma.

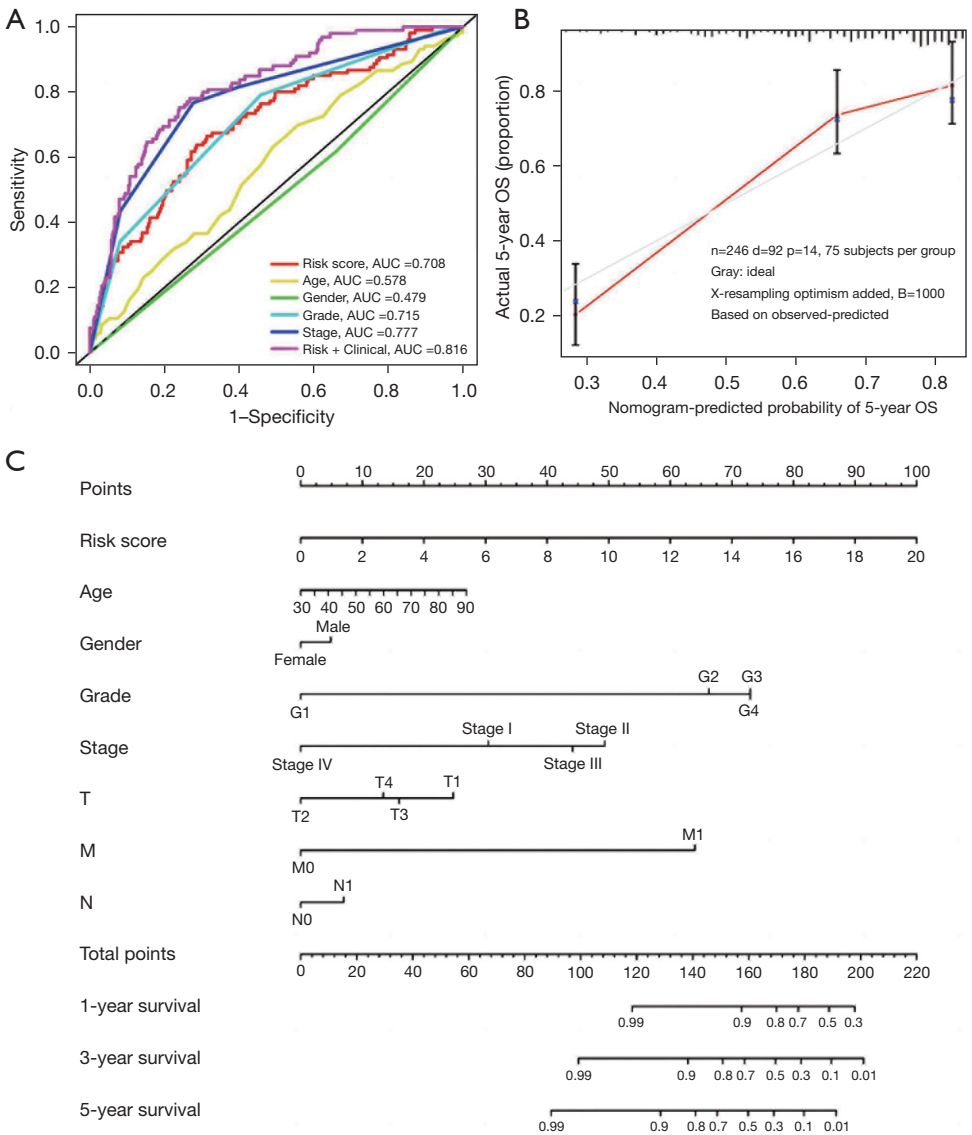

Figure 6 Immune risk model combined with clinicopathological features improves survival prediction. (A) Receiver operating characteristic curve analysis of sensitivity and specificity of the EZH2-regulated immune risk score prognostic model. (B) Calibration plot of the nomogram for 5-year overall survival prediction and actual outcome in ccRCC patients. (C) Nomogram constructed to evaluate the survival probability for individual ccRCC patients. AUC, area under the curve; n, data number; d, the number of outcome events; p, co-efficients in the cox model; OS, overall survival; EZH2, enhancer of zeste homolog 2; ccRCC, clear cell renal cell carcinoma.

key aspects of the TME, protects tumor cells from identification and elimination, and leads to tumor escape and immune resistance (3). For example, $CD8^+$ T cells play a key role in antitumor immunity, and EZH2 downregulates the chemokines (CCL28, CCL3L1, CXCL16, CXCL9, CXCL10) that inhibit $CD8^+$ T cell infiltration (6). EZH2 inhibitors could lead to increased Treg trafficking and impaired Treg capacity (4,5). It has been demonstrated that EZH2 stabilizes the functional phenotype of activated Tregs through CD28 co-stimulatory receptor (4). In mouse MC38 tumor models, breast cancer, and human colorectal cancer, EZH2 expression and associated H3K27me3 marking of tumor-infiltrating Tregs has been confirmed (4,7). NK cells are core cells of the natural immune system (4,7). EZH2 depresses NK cell development and cytolytic activity by regulating NKG2D and GzmB expression (4,7). In view

of these findings, studies suggest that targeting EZH2 expression could enhance antitumor immunity (3,6,11).

In ccRCC, the positive expression rate of EZH2 is as high as 80%, and the expression level of EZH2 in metastatic renal cancer is higher than in localized renal cancer (8,26). Furthermore, EZH2 positively correlates with ccRCC stage, grade and lymph node metastasis, and negatively correlates with patient prognosis (24,27). In our study, contradictory immunostimulatory and immunosuppressive TILs were observed in EZH2 copy number and promoter methylation alteration in ccRCC. We also observed that $CD8^+$ T cells and "T cells CD4 memory resting" exhibited significantly higher infiltration levels in tumor groups compared with normal groups, and high levels of infiltration of NK cells and Tregs were observed in ccRCC tumor groups, confirming that ccRCC is a "hot" tumor.

We found 26 EZH2 significantly regulated immune genes (*CTLA4*, *HAVCR2*, *IL10RB*, *KDR*, *LAG3*, *LGALS9*, *PDCD1*, *TIGIT*, *CD80*, *IL2RA*, *IL6*, *KLRK1*, *LTA*, *MICB*, *RAET1E*, *TNFRSF8*, *TNFRSF9*, *TNFRSF17*, *TNFRSF18*, *TNFRSF25*, *TNFSF4*, *TNFSF13*, *TNFSF14*, *TNFSF13B*, *TNFSF15*, *ULBP1*) closely related to ccRCC prognosis. Based on this, we established a EZH2-regulated immune-risk score prognostic model considered and filtered by TCGA database. Compared with ingle pathological features, our EZH2-regulated immune-risk score prognostic model combines pathological features showing profound ability to predict patients' prognosis (AUC =0.816). The calibration curve confirmed that the nomogram was more reliable for predicting the survival rate of patients at 3 years.

There are several advantages of our model Firstly, it can accurately predict prognostic survival status in ccRCC. Secondly, it evaluates the ccRCC TME immune status, and could be a model for evaluating EZH2 inhibitor sensitivity in ccRCC patients. However, our model does have some limitations. Firstly, the potent mechanisms among activated NK cells, more $CD8^+$ T cells, more Tregs and high expression of EZH2 are not yet clear in ccRCC and need further exploration. Secondly, whether the model is suitable for screening out ccRCC patients unsuitable for PD-1/PD-L1 inhibitor combined targeted drugs therapy still needs to explored and validated in the future.

Conclusions

We developed an immune risk score prognostic model based on EZH2-regulated immunogenes in ccRCC. This model can serve as an independent prognostic factor for ccRCC patients. The immune risk score prognostic model was based on all the immunostimulatory and immunosuppressive TILs within cancerous tissue. Our research also indicated model may be used to guide EZH2-targeted therapies for ccRCC patients. Further assessment of the effects of EZH2 on the TME will help inform the best utilization of EZH2 inhibitors in the clinic.

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Footnote

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://tau.amegroups.com/article/view/10.21037/tau-22-817/coif). The authors have no conflicts of interest to declare.

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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