Translational Oncology 22 (2022) 101462

Contents lists available at ScienceDirect

Translational Oncology

journal homepage: www.elsevier.com/locate/tranon

Original Research

A panel of necroptosis-related genes predicts the prognosis of pancreatic adenocarcinoma

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ARTICLE INFO

Keywords:
Pancreatic adenocarcinoma
Necroptosis
Prognosis
Exosomes

ABSTRACT

Pancreatic adenocarcinoma (PAAD) has become one of the deadliest malignancies in the world. Since necroptosis plays a crucial role in regulating the immune system, it is necessary to develop novel prognostic biomarkers associated with necroptosis and explore its potential role in PAAD. The transcriptome RNA-seq data of PAAD were downloaded from the TCGA and GTEx databases. A prognostic signature was constructed by the least absolute shrinkage and selection operator (LASSO) Cox regression, and its prognostic value was evaluated by nomogram and validated in an independent GEO cohort. We identified a total of 24 differentially expressed NRGs in PAAD, and constructed a prognostic signature with 5 NRGs, which showed good performance in predicting the prognosis of PAAD patients. The ROC curves for 1-, 3-, and 5-year survival rate were 0.652, 0.778, and 0.817, respectively. This prognostic signature showed consistent prognosis prediction in an independent patient cohort. Furthermore, the correlations between 5-NRGs signature and TMB, MSI, histopathological classification, immune infiltration, immune types, and immunomodulators were all significant. Notably, the expression profiles of the five NRGs in exosomes of serum were consistent with their expression in tumor tissues. These data suggested that the 5-NRGs signature is a promising biomarker for predicting the prognosis of PAAD.

Introduction

Pancreatic adenocarcinoma (PAAD) is one of the most lethal malignancies in the world, characterized with late diagnosis, poor therapeutic options, and inferior prognosis [1]. In 2020, there are 60,430 new cases of PAAD and 466,003 PAAD related deaths [2]. So far, surgery is the most commonly used treatment for PAAD, but it's limited for patients with advanced disease. On the other hand, the commonly used combination chemotherapy (such as FOLFIRINOX and gemcitabine/nab-paclitaxel) only increases the median survival by three months or so, and it causes more severe side effects [3,4]. Recently, immunotherapy has emerged as a novel strategy for treating PAAD. However, the genetic heterogeneity of patients and physical and chemical stimulation factors cause primary and acquired resistance during immunotherapy [5]. Moreover, the prognosis of patients is hard to predict regardless of the treatment strategy. Thus, it is an urgent need to identify novel biomarkers to improve the prognostic prediction of PAAD.

Necroptosis is a novel form of programmed cell death mediated by tumor necrosis factor α (TNF-α), RIP1/RIP3, and MLKL [6,7], which is different from apoptosis, pyroptosis, ferroptosis, autophagy, and necrosis [8]. Necroptosis can lead to cell swelling, cell membrane

Abbreviations: TCGA, The Cancer Genome Atlas; GTEx, Genotype-Tissue Expression; KEGG, Kyoto Encyclopedia of Genes and Genomes; GO, Gene Ontology; GEO, Gene Expression Omnibus; LASSO, Least absolute shrinkage and selection operator; PAAD, Pancreatic adenocarcinoma; TMB, Tumor mutational burden; MSI, Microsatellite instability; NRGs, Necroptosis-related genes; DAMPs, Danger-associated molecular patterns; TEM, Transmission electron microscope; NTA, Nanoparticle tracking analysis; LIF, Leukemia inhibitory factor; ICI, Immune checkpoint inhibitor; CAMK2B, Calmodulin-dependent protein kinase II beta; PLA2G4C, Phospholipase a2 group IV C; CHMP4C, Chromatin modification protein 4C; STAT4, Signal transduction and activator of transcription 4; TNFSF10, Tumor necrosis factor superfamily member 10.

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https://doi.org/10.1016/j.tranon.2022.101462
Received 8 April 2022; Received in revised form 20 May 2022; Accepted 22 May 2022

perforation, disintegration, and release of danger-associated molecular patterns (DAMPs) to induce innate and adaptive immune responses [9]. Increasing evidence has shown that necroptosis is widely involved in the progress of various tumors and plays a vital role in patient prognosis, cancer immune regulation, and therapeutic response [10,11]. Necroptosis could induce an immunosuppressive tumor microenvironment to promote the occurrence and metastasis of tumors. Studies have reported that the activation of necroptosis could make cancer cells attract specific tumor-associated macrophages and mediate the escape and metastasis of cancer cells [12]. Similarly, Ando et al. [13] revealed that necroptosis could promote pancreatic cancer cells migration and invasion by releasing CXCL5. Interestingly, some studies showed that necroptotic cells could be used as vaccines to specifically activate the immune system to eliminate tumor cells [14]. Moreover, necroptotic cells could trigger $BATF3^{+}cDC1^{-}$ and $CD8^{+}$ leukocyte-dependent antitumor immunity [15]. In addition, the construction of necroptosis-inducible polymeric nanobubbles promotes the maturation of dendritic cells and the activation of $CD8^{+}T$ cells, which enhances antitumor immunity [16]. Notably, some studies found that the necroptosis-related regulators might be prognostic biomarkers for some malignancies [17–19]. Taken together, necroptosis-related genes (NRGs) might be a promising prognostic signature for PAAD.

In this study, we comprehensively analyzed the expression profile of NRGs in PAAD and identified 5 NRGs associated with the prognosis of patients. Then, we constructed a prognostic signature based on 5 NRGs by LASSO regression and evaluated its prognostic value through time ROC, Cox regression, and an independent GEO cohort. We also analyzed the correlations between NRGs and tumor immune features. Finally, we examined the expression of five NRGs in serum samples using RT-qPCR, which provided a noninvasive method for predicting prognosis.

Materials and methods

Datasets collection and preprocessing

The transcriptomic FPKM data and clinical information were downloaded from The Cancer Genome Atlas (TCGA, https://portal.gdc.cancer.gov/), including 178 PAAD tissue samples and 4 adjacent tissue samples. Then, the transcriptomic TPM data of 167 normal pancreatic tissues were obtained from the Genotype-Tissue Expression (GTEx, https://www.gtexportal.org/). Meanwhile, the data of an independent PAAD patient cohort was downloaded from the Gene Expression Omnibus (GEO, GSE85916, https://www.ncbi.nlm.nih.gov/geo/). The patient data with missing overall survival (OS) values or smaller OS values (< a month) were excluded.

Identification and interaction analysis of the differentially expressed NRGs

A total of 133 NRGs were obtained from the Kyoto Encyclopedia of Genes and Genomes (KEGG, https://www.kegg.jp/) (Table S4). The r software package "limma" was used to analyze the differential expression of all NRGs in PAAD and normal tissues($|\log2FC| > 1$ and $p < 0.05$). Subsequently, a protein-protein interaction (PPI) network of NRGs was constructed using STRING (https://cn.string-db.org/). Moreover, we also analyzed NRGs mutations in PAAD with cBioPortal (http://www.cbioportal.org/).

Functional enrichment analysis

The r software package "ggplot2″ was used to perform functional enrichment analysis in Gene Ontology (GO), including biological process (BP), cellular component (CC) and molecular function (MF). Similarly, this package was used to perform the enrichment analysis in the Kyoto Encyclopedia of Genes and Genomes (KEGG).

Construction and verification of the NRGs related prognostic signature

A prognostic signature was constructed to investigate the relationship between NRGs and PAAD prognosis. In Kaplan-Meier survival analysis, log-rank was used to test the difference between low-risk and high-risk groups. ROC analysis was carried out to compare the accuracy and risk score of NRGs prediction. A nomogram was established for predicting the 1, 3 and 5-year recurrence rates. The p-value and hazard ratio (HR) with 95% confidence interval (CI) were obtained by log-rank test and univariate Cox proportional hazard regression. The least absolute shrinkage and selection operator (LASSO) was used for feature selection, and cross-validation was performed ten times. Similar methods were used for independent validation in the cohort downloaded from GEO. All the analyses were performed using R.4.1.1.

Analysis of the correlation between NRGs and immune features

The Tumor Immune Estimation Resource (TIMER, https://cistrome.shinyapps.io/timer/) was used to analyze the correlation between prognostic NRGs and immune infiltration and types. The "Gene" module of TIMER generated the correlation between NRG expression and the level of immune infiltration, and the "SCNA" module compared the level of tumor infiltration with different somatic copy number changes of a given NRG. We used the Tumor Immune System Interactions DataBase (TISIDB, http://cis.hku.hk/TISIDB/index.php) to analyze the relationship between NRGs and immunomodulators. In addition, Spearman correlation analysis was performed to calculate the correlation between NRG gene expression and TMB/MSI.

Samples collection and processing

A total of 20 pairs of tumor tissues and serum samples were obtained from patients who were histologically diagnosed with PAAD in Qilu Hospital of Shandong University from November 2018 to December 2020. This study was approved by the Ethics Committee of Qilu Hospital of Shandong University, and informed consent was obtained from each patient. Tissue samples treated with RNA Keeper Tissue Stabilizer (Vazyme, Nanjing, China) for 4 °C overnight, then stored at −80 °C. Serum samples were separated by two centrifugation (6000 g for 10 min followed by another centrifugation at 12,000 g for 10 min) and then stored at −80 °C.

Exosomes isolation and identification

The exosomes were extracted from the serum as previously described [20]. Firstly, 3 ml serum was mixed with 1 × phosphate-buffered saline (PBS) and centrifuged at 10,000 g for 20 min. Then, the supernatants were filtered through a 0.22 μm pore filter and centrifuged at 120,000 g for 60 min. Afterward, the pellets were resuspended in ice-cold 1 × PBS and centrifuged at 120,000 g for 60 min. Finally, the pellets(exosomes) were resuspended with 1 × PBS. Exosomes were mounted on a carbonaceous copper grid and imaged on a transmission electron microscope (TEM). Nanoparticle tracking analysis (NTA) was analyzed using Zeta-View PMX 110(Particle Metrix, Meerbusch, Germany). Western blot assay was used to detect the NRGs of exosomes, anti-CD63, anti-ALIX, and anti-TSG101 (1:1000; Abcam, Cambridge, MA, USA).

RNA extraction

The total RNA was extracted from tissue and serum samples using TRIzol and TRIzol LS Reagent (Invitrogen, Eugene, OR, USA), respectively. The concentration of RNA was measured using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

RT-qPCR

RNA was treated with gDNA wiper and then reverse transcribed into cDNA using HiScript III RT SuperMix for qPCR(Vazyme, Nanjing, China). qPCR was performed using ChamQ Universal SYBR qPCR Master Mix(Vazyme, Nanjing, China). The positive control contained the target gene and the negative template control contained all components except cDNA. qPCR reaction was performed under the following conditions: Stage1 95 °C for the 30 s to activate Taq DNA polymerase; Stage2 40 cycles with 95 °C for 10 s and 60 °C for 30 s; Stage3 melting curve acquisition with 95 °C for 15 s, 60 °C for 60 s and 95 °C for 15 s. Each reaction was performed in triplicate. The relative expression of target genes were normalized to GAPDH and calculated using the $2^{-\Delta Ct}$ method. The primer sequences are shown in Table S3.

Results

Differentially expressed NRGs in PAAD

The workflow for this study is shown in Fig. 1. First, the expression profiling data of NRGs were normalized according to [21] (Fig. S1A–D). The clinical information is shown in Table S1. A total of 24 NRGs were significantly differentially expressed between normal and tumor tissues (Fig. 2A, B). Next, a PPI network of NRGs was constructed (Fig. 2C), and we used the tumor gene public database (Oncomine, https://www.oncomine.org/resource/login.html) to examine the relationship between NRGs and histopathological classification of PAAD. We found that 12 NRGs were associated with clinical subtypes (Table S2). Finally, we analyzed the genetic changes of these genes and found 19 NRGs had a mutation rate of ≥1%, among which IFNA1 and IFNA14 genes had the highest mutation rate (7%). Missense mutation and truncated mutation were the two most common mutation types (Fig. 2D).

Functional enrichment analysis of NRGs

To further explore the function of NRGs, we performed GO and KEGG enrichment analyses. As expected, these NRGs were mainly involved in programmed necrotic-like cell death, necroptotic process, necrotic cell death, response to the virus, receptor signaling pathway via jak-stat, etc. (Fig. S2A, B). KEGG pathway analysis showed that these NRGs were associated with necroptosis, nod-like receptor signaling pathway, influenza A, hepatitis B, and inflammatory mediator regulation of trp channels (Fig. S2C, D).

Establishment of the prognostic signature of NRGs in PAAD

The prognostic significance of NRGs was first analyzed by Kaplan-Meier (KM) survival curve, and five of them were significantly associated with overall survival (OS): the PAAD patients with low expression of CAMK2B (Fig. S3A), PLA2G4C (Fig. S3C), STAT4 (Fig. S3D), and high expression of CHMP4C (Fig. S3B), TNFSF10 (Fig. S3E) had poor survival prevalence. Based on these findings, we constructed a prognostic signature from the five NRGs using LASSO Cox regression analysis (Fig. 3A, B): the risk score = (−0.3286) × PLA2G4C + (−0.0367) × CAMK2B + (0.4996) × TNFSF10 + (−0.1334) × STAT4 + (0.1341) × CHMP4C). Based on the risk score, the PAAD patients were divided into low-risk and high-risk groups. The survival status, risk score distribution, and the expression of five NRGs were shown in Fig. 3C. A high-risk score is positively correlated with patient's death risk and negatively correlated with the survival time. PAAD patients with high-risk scores had a significantly shorter survival time than those with low-risk scores (Fig. 3D), and the area under ROC curves of 1-, 3-, and 5-year survival were 0.652, 0.778, and 0.817, respectively (Fig. 3E). Univariate and multivariate analyses showed that TNFSF10, tumor grade, and radiation therapy were independent factors influencing the prognosis of PAAD

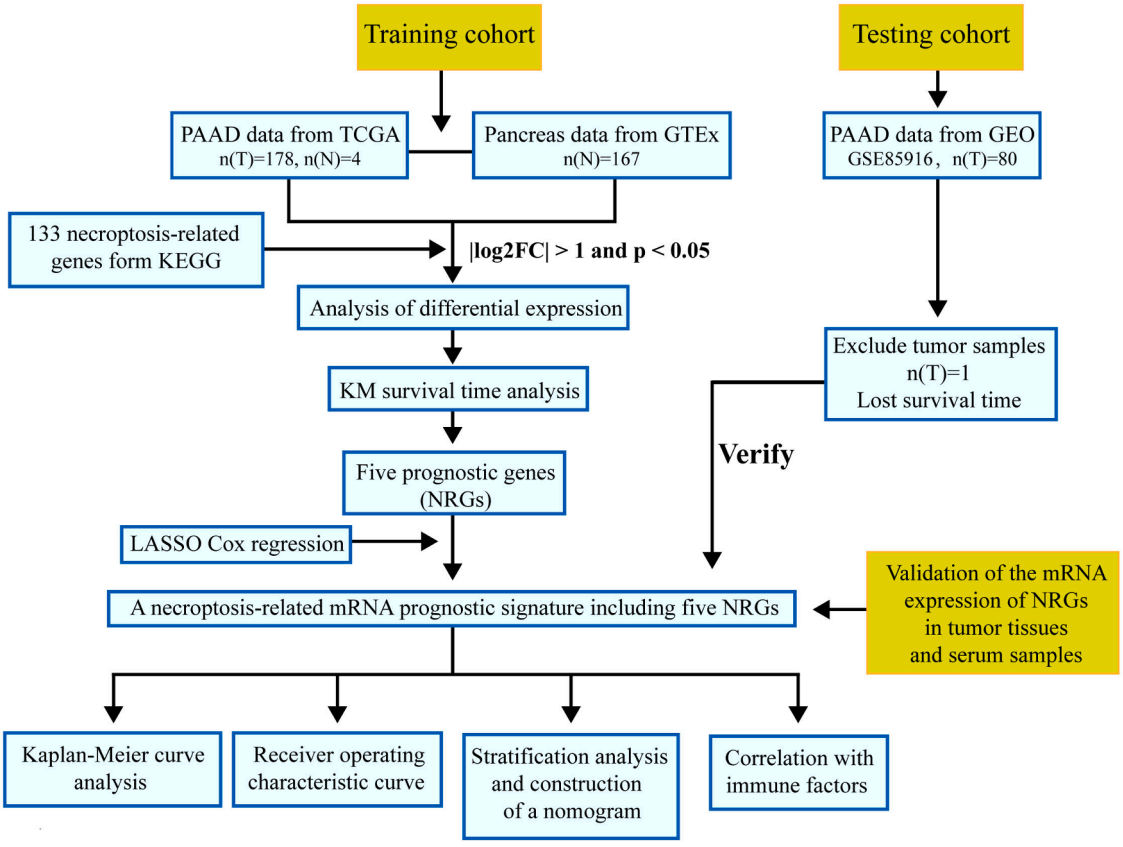

Fig. 1. Workflow based on analysis strategies of NRGs.

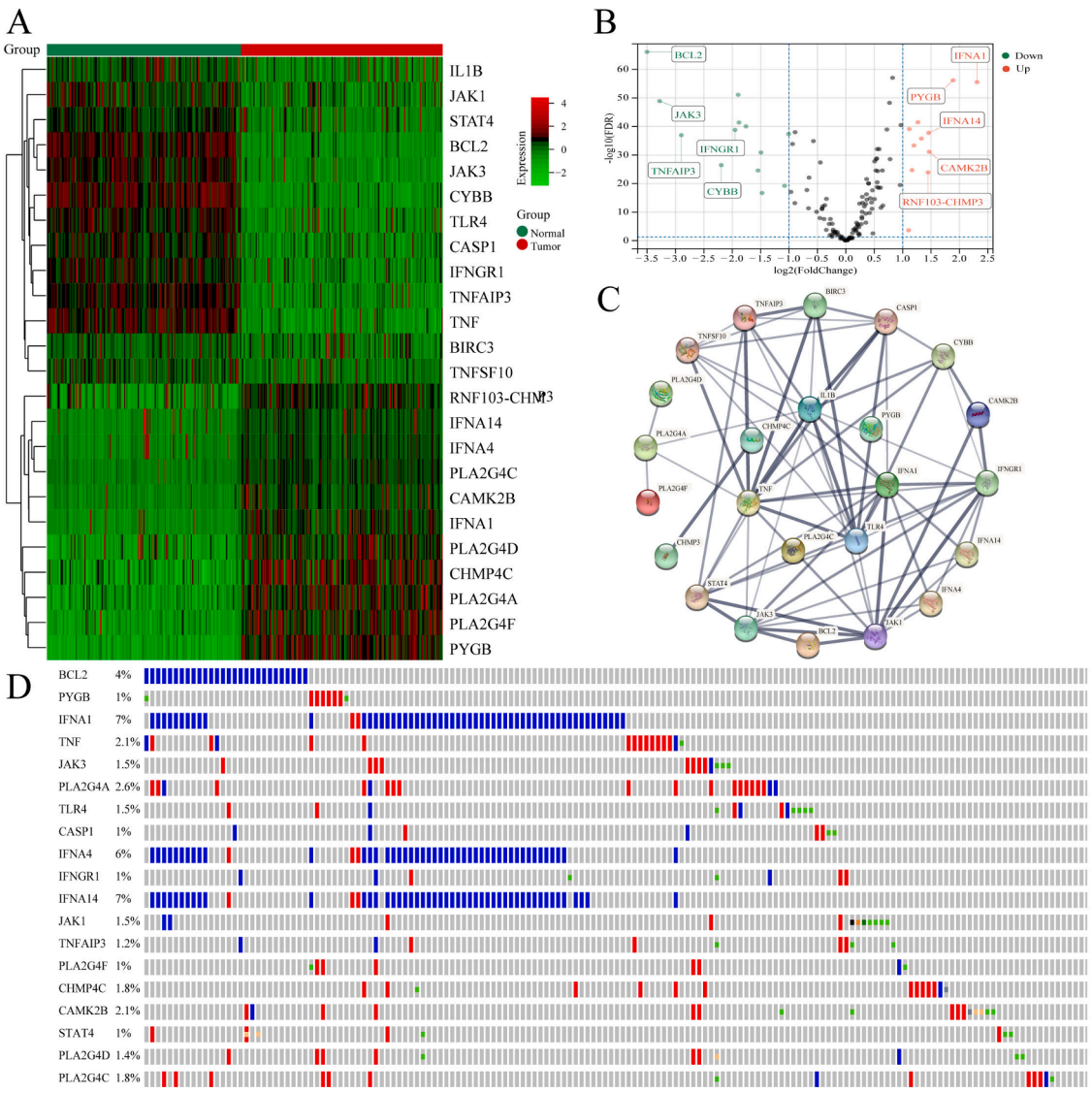

Fig. 2. The differentially expressed, interaction and mutant landscape analysis of NRGs in PAAD.
(A, B) The significant different expression levels of NRGs. (C) The PPI network showed protein-protein interactions of NRGs. (D) A total of 19 NRGs have a mutation rate≥1%.

(Fig. S4A, C). Finally, a nomogram was developed to predict the 1-, 3- and 5-year overall recurrence rate in PAAD patients (Fig. S4B).

Verification of the prognostic signature based on the geo cohort

To verify the accuracy of the prognostic signature, a total of 80 PAAD tissues from GSE85916 were used as the verification group. The ChIP data of these tissues were analyzed (Fig. S1E, F), and one unqualified tumor tissue sample was excluded. According to the median risk score, 39 patients in the GEO cohort were included into low-risk groups, and 40 patients were classified into high-risk groups. The survival status, risk score distribution, and NRGs expression of these patients are shown in Fig. 4A. The KM survival analysis showed a significant difference in the survival rate between low-risk and high-risk groups. The patients in low-risk group had longer survival time and lower mortality prevalence (Fig. 4C). Moreover, the ROC curve showed that our model had a good prediction performance (1y=0.72, 2y=0.62, 3y=0.75) (Fig. 4B).

The relationship between NRGs and tumor immune infiltration in PAAD

Necroptosis plays an important role in the development of the tumor immune microenvironment. The activation of necroptosis can induce autologous antitumor immune response [14,15]. In this study, we used the TIMER database to investigate the correlation between the prognostic NRGs and immune infiltration in PAAD. Our results showed that the expression of CAMK2B was positively associated with the abundance of CD4$^+$*T* cell (Cor = 0.276) and macrophages (Cor = 0.186)(Fig. 5A), and the expression of CHMP4C was negatively associated with B cell (Cor = 0.187) and CD8$^+$*T* cell (Cor = 0.283), which was the opposite of CD4$^+$*T* cell (Cor = –0.286)(Fig. 5B). Interestingly, STAT4 (Fig. 5C), TNFSF10 (Fig. 5D), and PLA2G4C (Fig. 5E) were positively associated with the abundance of six types of immune cells (B Cell, CD8$^+$*T* Cell, CD4$^+$*T* Cell, Macrophage, Neutrophil, and Dendritic Cell). Furthermore, we used bilateral Wilcoxon rank-sum test to compare the infiltration level of each SCNA category (arm-level deletion (−1), dipped/normal (0), arm-level gain (1), and high amplification (2)) with the normal level. The results showed that NRGs were highly correlated with the level of immune infiltration in tumors with different somatic copy number variations. In particular, compared with the normal levels, the infiltration levels of 5 prognostic NRGs showed statistical difference in Arm-level gain (1) mutation of CD4$^+$*T* Cell by a two-sided Wilcoxon rank-sum test (Fig. S5).

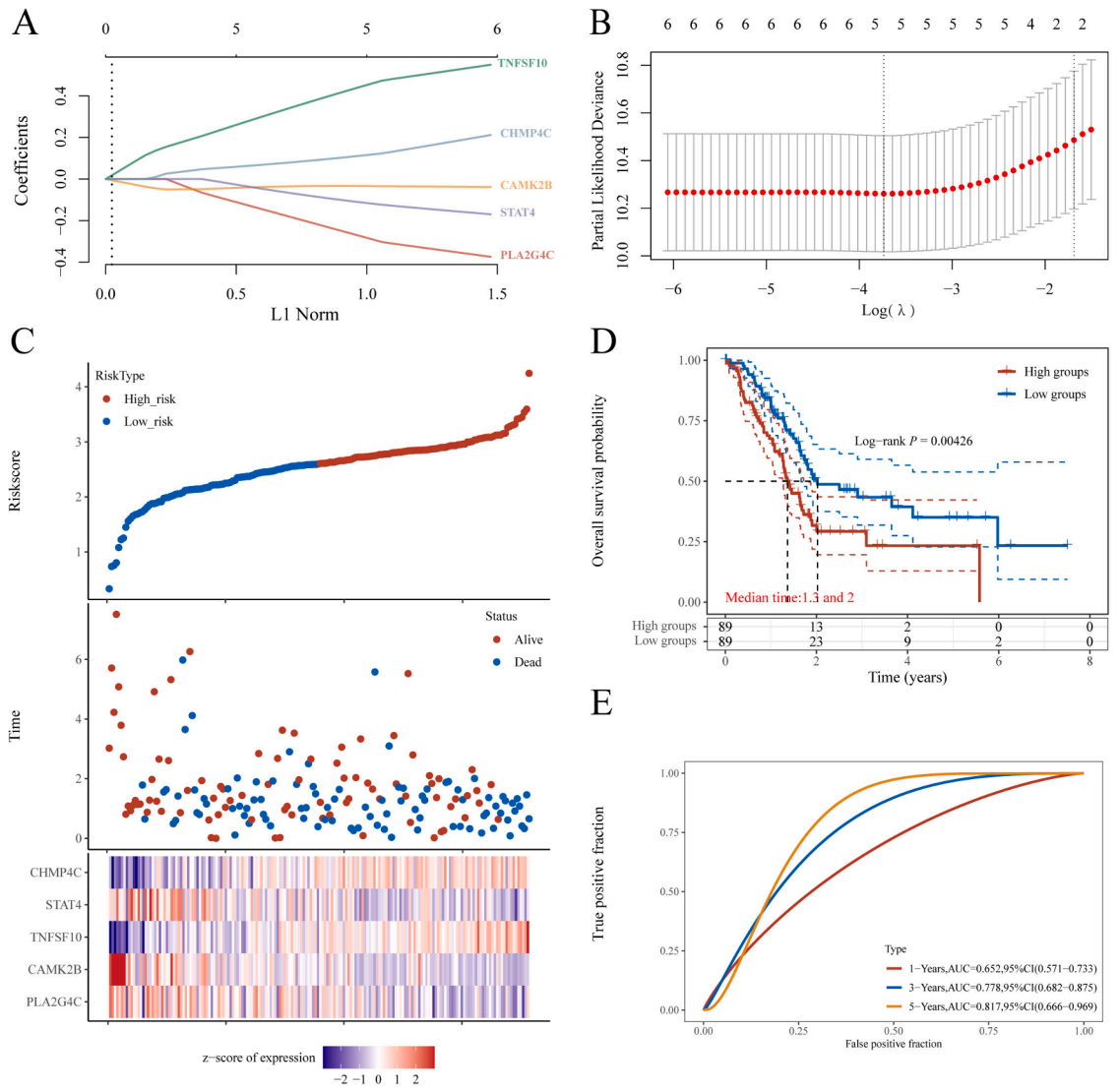

Fig. 3. The construction of prognostic signature based on NRGs.
(A) LASSO coefficient distribution of five NRGs. (B) 10-fold cross-validation error rate. (C) The risk score, survival status distribution, and expression of five prognostic NRGs in PAAD. (D, E)OS and ROC curve of predictive value for patients with high-risk/low-risk groups in PAAD.

NRGs were associated with immune subtypes and immunomodulators in PAAD

Immunotherapy is a novel treatment strategy for malignancies, which exerts an antitumor effect by activating the immune system. However, only a few patients respond well to immune checkpoint reagents in most cancers [22]. Therefore, we used the TISIDB database to analyze the correlation between NRGs and the immunomodulators, such as immuneinhibitor (CD160, CTLA4), immunostimulatory (CD27, CD28), and MHC molecule (HLA-DPA1, HLA-DRA). As a result, except CAMK2B, which was only associated with CD160 and CD28 (Fig. S6A), the other four NRGs, including CHMP4C (Fig. S6B), PLA2G4C (Fig. S6C), STAT4 (Fig. S6D), and TNFSF10 (Fig. S6E), were all significantly associated with immunomodulators and immune subtypes across all human cancers (Fig. S7).

The correlation analysis between NRGs and TMB, MSI

Studies have shown that tumor mutational burden (TMB) can predict immune response for many malignancies [23]. Also, microsatellite instability (MSI) is a predictive biomarker for tumor immunotherapy [24]. To determine whether NRGs could be used as response biomarkers for the prognosis of PAAD, we analyzed the association between NRGs and TMB/MSI. The results showed that TMB was positively correlated with CHMP4C (Fig. S8B), TNFSF10 (Fig. S8E), and negatively correlated with CAMK2B (Fig. S8A), PLA2G4C (Fig. S8C), and STAT4 (Fig. S8D), while MSI was negatively correlated with PLA2G4C (Fig. S8H) and TNFSF10 (Fig. S8J).

Identification of exosomes in serum and exo-nrgs associated with paad tissues

The exosomes extracted from the serum of PAAD patients showed a typical saucer-like shape under the transmission electron microscope (TEM) (Fig. 6A). The results of nanoscale tracking analysis (NTA) showed that the exosomal particle size was mainly distributed around 150 nm (Fig. 6B). In addition, Western blot assay showed that the classical markers of exosomes (CD63, ALIX, and TSG101) were highly expressed in the pellets we extracted (Fig. 6C). Next, we measured the expression levels of these 5 NRGs in serum exosomes to test if they could be effectively detected. As shown in Fig. 6D–H, the levels of 5 Exo-NRGs were significantly associated with their expression levels in tumor

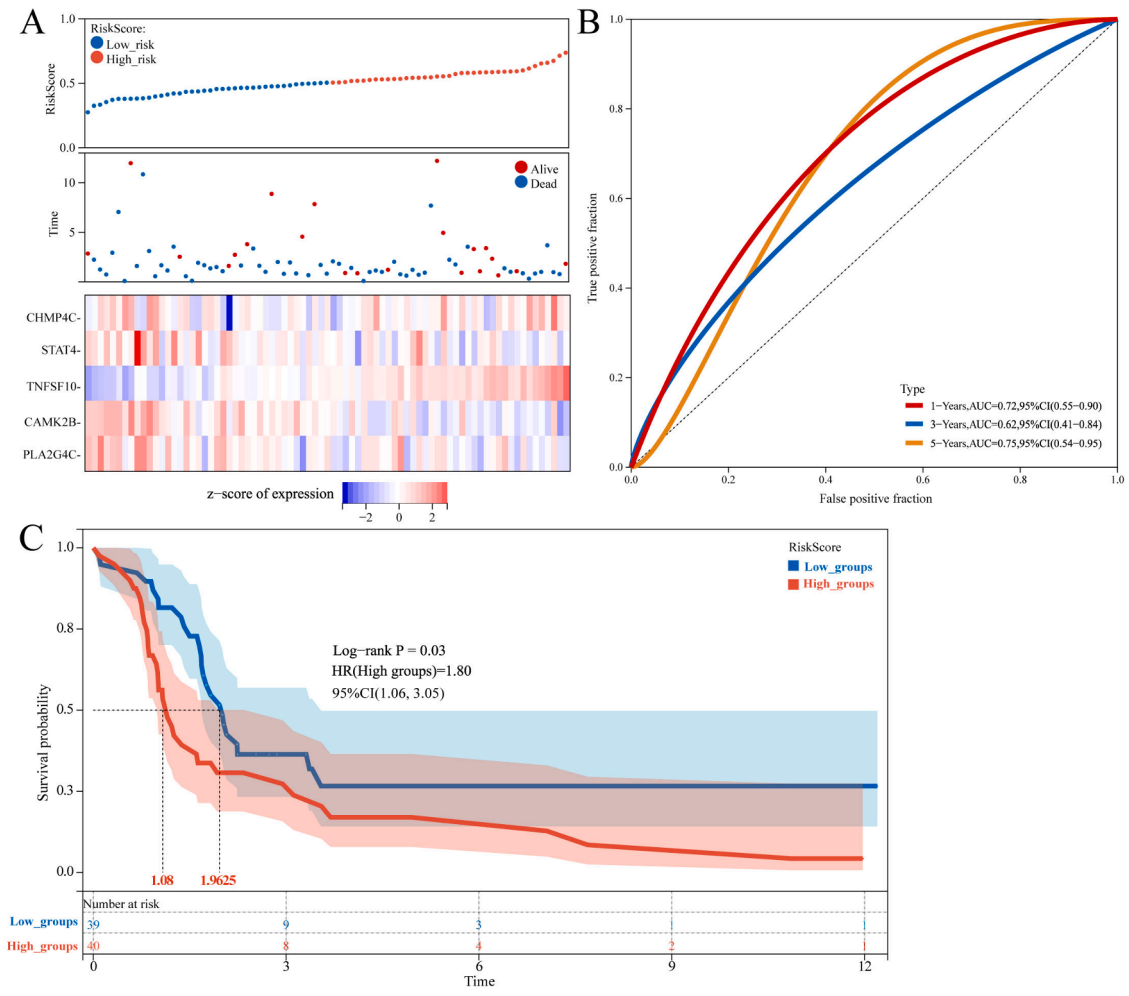

Fig. 4. The GEO cohort independent validation model.
(A) Distribution of patient risk score, survival status, and expression of five prognostic NRGs in GEO cohort. (B, C) ROC and OS curves for the predictive value of PAAD patients in the high-risk/low-risk groups in PAAD.

tissues ($r > 0.4$, $P < 0.05$). In addition, the relationships between risk scores and clinicopathological variables were analyzed in Table S5. We found that risk scores calculated from both tissue and serum samples were increased in TNM stage II compared with TNM stage I ($p = 0.015$, and $p<0.001$). And risk scores calculated from tissue while not serum were significant associated with local invasion ($p = 0.036$, and $p = 0.052$). No significant associations were observed between risk scores and age, gender, tumor size, differentiation, and lymph nodes metastasis.

Discussion

This study presents several new findings. First, a 5-NRGs panel was identified and validated for the prediction of PAAD prognosis. Second, we found significant associations between the 5 NRGs and TMB, MSI, histologic classification, and immune features, providing scientific references for clinical immunotherapy. Third, we were able to use the liquid biopsy method to noninvasively detect those NRGs in the exosomes of serum, suggesting that this method was suitable for dynamic monitoring of treatment response in clinical practice.

As a subtype of regulated necrosis, necroptosis plays a vital role in the occurrence and development of many diseases, especially cancers [25]. Increasing evidence has shown that the critical regulators in the necroptosis pathway could participate in the progress of malignancies [26–28], indicating that NRGs might serve as potential prognostic markers [29]. In this study, we identified 24 NRGs from differential expression analysis and investigated their mutation types and cellular functions. These regulators were mainly involved in inducing inflammation response and immune defense. KM survival analysis showed that 5 NRGs were significantly associated with the survival of PAAD patients. Among them, calmodulin-dependent protein kinase II beta (CAMK2B), phospholipase A2 group IVC (PLA2G4C), and signal transduction and activator of transcription 4 (STAT4) were protective factors, and chromatin modification protein 4C (CHMP4C) and tumor necrosis factor superfamily member 10 (TNFSF10) were risk factors. It has been reported that CHMP4C can be used to predict the prognosis of osteosarcoma [30]. Interestingly, CHMP4C disruption is beneficial to increase the radiosensitivity of human lung cancer cells, which provides a potential therapeutic strategy for non-small cell lung cancer [31]. Studies have found that leukemia inhibitory factor (LIF) could prevent STAT3-dependent Il 17a/Il 17f promoter activation by inducing STAT4 activation, which effectively inhibited Th17 accumulation and promoted the repair of damaged intestinal epithelium in colitis [32]. Additionally, activating STAT4 may help alleviate the lymphatic metastasis in HNSCC patients [33]. TNFSF10/TRAIL could transform cells and induce necrosis-like apoptosis in tumor cells through combining with TRAIL-R [34]. However, regardless of the progress on NRGs research, the detailed molecular mechanisms underlying the role of NRGs in cancer still need further investigation.

Although some mRNAs and ncRNAs have the potential to predict the prognosis of malignancies, the performance of a single marker is limited, and the combination of multiple markers can greatly enhance the

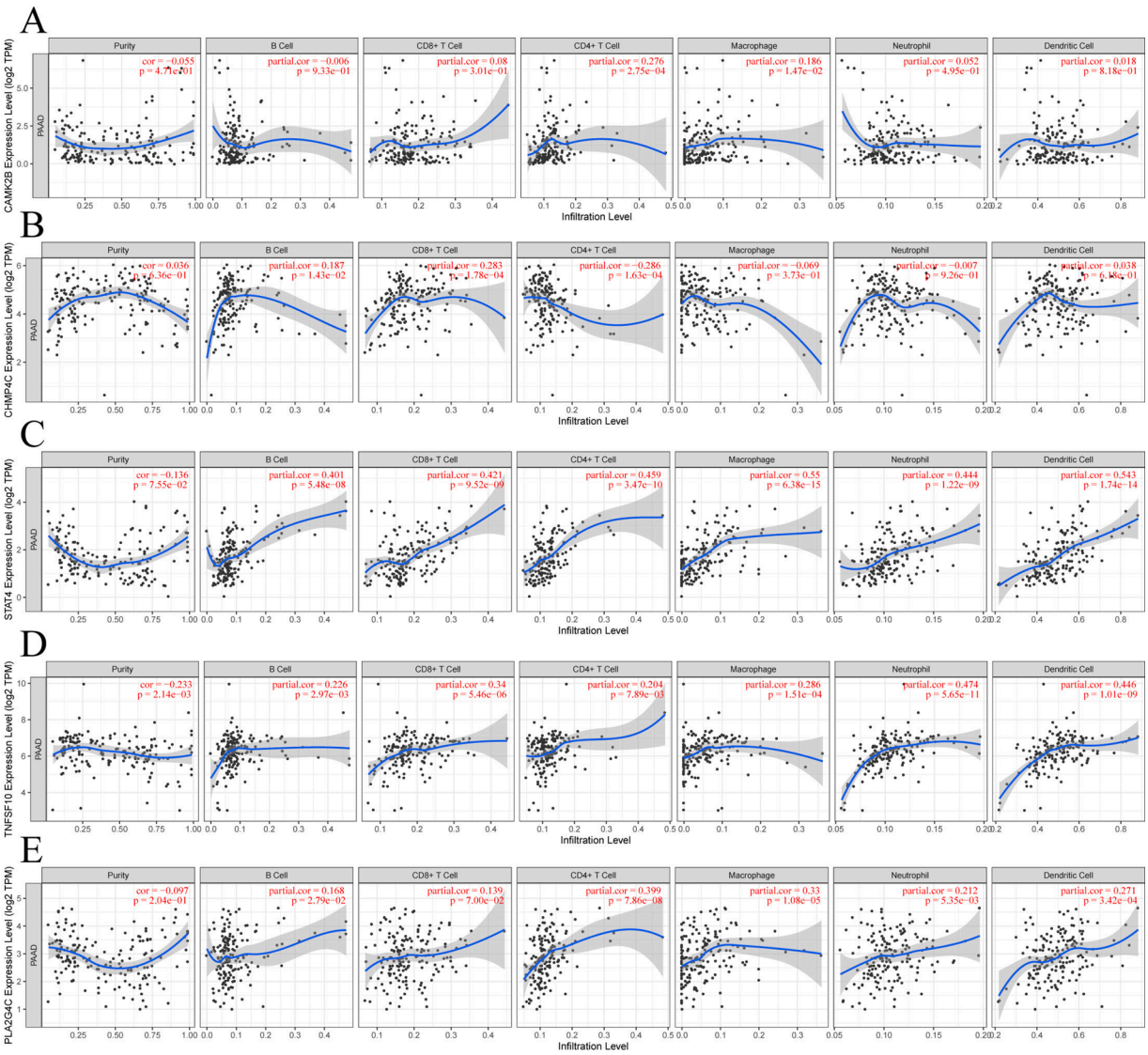

Fig. 5. Relationship between NRGs and immune infiltration.
The relationship between the abundance of six kinds of immune cells(B cells, CD8+*T* cell, CD4+*T* cell, Macrophage, Neutrophil, and Dendritic Cell) and the expression of (A) CAMK2B. (B) CHMP4C. (C) STAT4. (D) TNFSF10. (E) PLA2G4C in PAAD.

prediction value. Based on these 5 prognostic NRGs, we used LASSO Cox regression analysis to construct a necroptosis-related prognostic signature. Meanwhile, a nomogram and GEO cohort were used to evaluate the effectiveness of the prognostic signature. As respected, the results showed that this necroptosis-related prognostic signature had a good performance in predicting the prognosis of PAAD patients. Although other prognostic signatures of PAAD have been identified, such as m6A-related prognostic signature [35], immune-related prognostic signature [36], and ferroptosis-related prognostic signature [37], there is still a lack of effective signatures for evaluating the prognosis of PAAD. Wu et al. [38] reported that NRGs played important roles in the prognosis of PAAD. However, when identifying differential genes, they did not have enough paracancerous tissues, which might lead to data deviation. In contrast, we combined the datasets from TCGA and GTEx to overcome the problem and incorporated more NRGs. Moreover, we performed external validation using an independent cohort, and the results were further validated by RT-qPCR.

The development of immune checkpoint inhibitor (ICI) has provided a new powerful method for tumor treatment. Unfortunately, many cancer patients are resistant to this therapeutic strategy [39]. Recently, Shi et al. [40] construct a signature based on NRGs for prediction of pancreatic cancer prognosis, but whether it can reflect the immune infiltration has not been illuminated. In this study, we found that the 5 NRGs enrolled in our model were closely correlated with immune infiltration. It has been reported that CAMK2B may remodel the tumor microenvironment and affect the prognosis of kidney renal papillary cell carcinoma [41]. A previous study also found that the deficiency of STAT4 mediated immunosuppression promoted lymphatic metastasis of HNSCC [42]. Nevertheless, most pancreatic cancers are multi-resistant to TRAIL therapy, which limits its further clinical application [43]. In addition, the 5 NRGs were significantly correlated with immune subtypes and immunomodulators, suggesting that the 5-NRGs signature can serve as a candidate biomarker for dynamic monitoring of immunotherapy. Altogether, these results confirmed that NRGs played a vital role in the tumor immune microenvironment, which may provide a new strategy for monitoring the resistance of ICI treatment.

In previous study, we have reported an exosomal RNAs panel for predicting the prognosis in patients with colorectal cancer [44]. Although tissue biopsy is the gold standard for malignancy diagnosis, it is not suitable for longitudinal clinical monitoring because it is difficult to obtain clinical samples and the operation is complex [45]. Therefore, non-invasive sampling such as liquid biopsy is essential for early

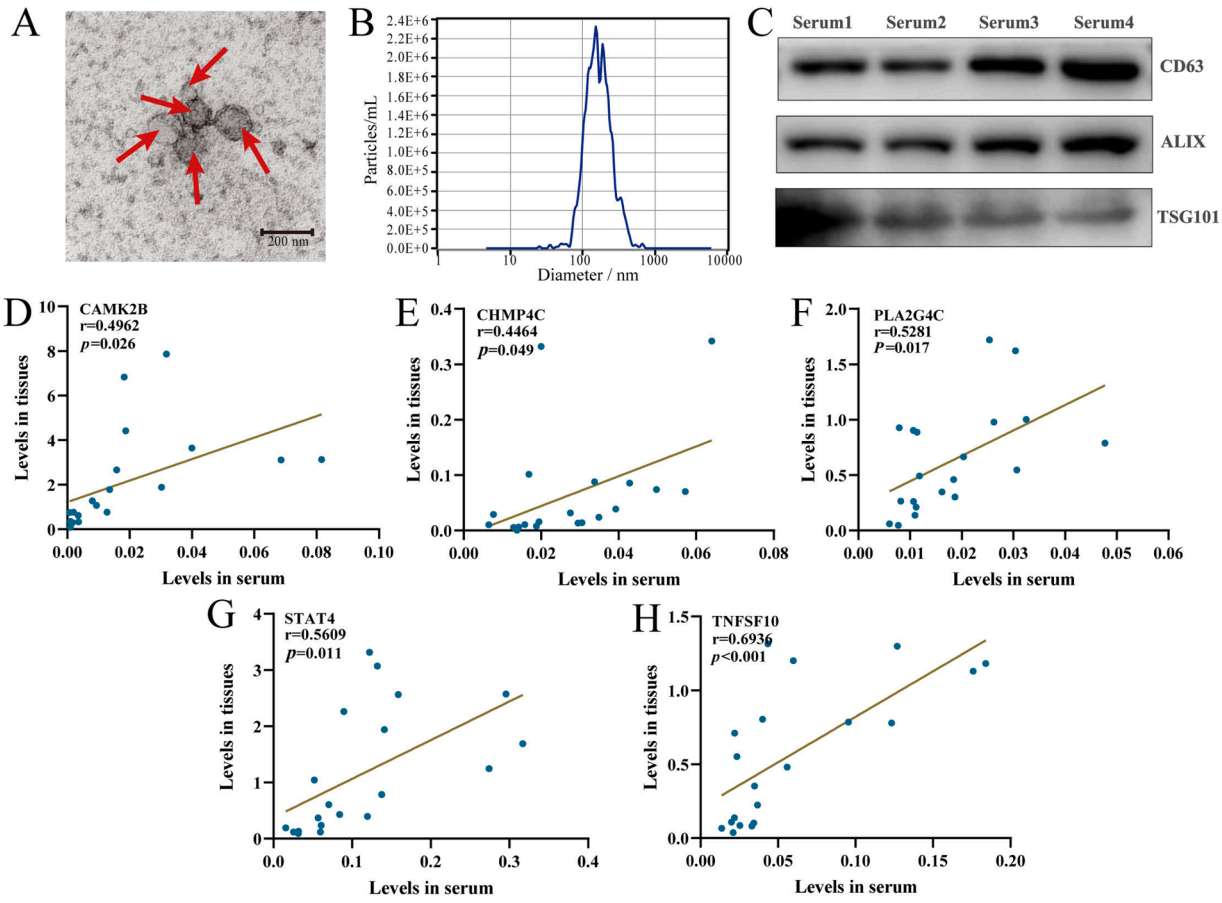

Fig. 6. Detection of NRGs in serum exosomes and PAAD tissues.
(A) Electron microscopy images of exosomes. (B) Nanoparticle tracking analysis of the size distributions of exosomes. (C) Western blotting analysis of the markers of exosomes (CD63, ALIX, and TSG101). (D–H) Correlation analyses between each lncRNA expression in tissues and matched serum exosomes. The relative expression levels were calculated using the 2ˆ −ΔCt method. *P*-value by Wilcoxon signed-rank test. $P < 0.05$ represents levels of significance.

detection, dynamic treatment management, and evaluation of clinical outcomes. Currently, exosomes have become a new focus of cancer liquid biopsy. The exosomes contain tumor-derived DNA, RNAs, lipids, proteins, metabolites, and so on, and they are stably present in human biofluids such as blood, saliva, urine, and cerebrospinal fluid [46]. Exosomes have become a platform with comprehensive cross-applications. Studies have found that extracellular vesicle-derived proteins can be used as reliable biomarkers for cancer detection and cancer type classification [47]. Krug et al. [48] also revealed the combination of exosomal RNAs and ctDNA could improve the sensitivity of EGFR mutation detection in plasma. To better understand whether circulating exosomal NRGs can represent their levels in tumor tissues, we examined the expression of 5 NRGs in matched tissue and serum samples from PAAD patients. The results showed that all 5 NRGs had consistent expression levels in serum exosomes and tumor tissues. Notably, CHMP4C was significantly over-expressed in paired tumor tissues and serum exosomes, which is consistent with previous studies [49]. These findings might provide a novel strategy to replace tissue biopsies with liquid biopsies.

Serveral studies have recently reported the association between NRGs and pancreatic cancer [38,40,50]. Consistent with these studies, we found that TNFAIP3, CHMP4C, IFNA1, BCL2 were mutation-prone necroptosis-related regulators, which provided a basis for targeting strategy of PAAD. Moreover, our study has some advantages. First, we have used the samples collected from our hospital to detect the expresstion of NRGs, while the current published papers only used bioinformatics analysis based on open databases. Second, we provided preliminary experimental data demonstrating the use of inexpensive RT-qPCR method instead of NGS to detect these genes. Moreover, we could detect them in exosomes of serum, which might be more suitable in clinical practice for preoperative assessment of the prognosis of PAAD. Some limitations should also be point out. First, our key findings were obtained by mostly open databases. These results should be verified by further studies using large case series and clinical samples. Second, the PAAD patients enrolled in this study had not been followed up enough, and the sample size was also small. Third, further experiments should be performed to explore the underlying molecular mechanism of NRGs in the tumorigenesis and development of PAAD.

Conclusions

We constructed a necroptosis-related signature, which was not only a promising biomarker for predicting the prognosis of PAAD, but also a possible therapeutic target. These findings provide useful insights for the prediction of clinical outcomes and therapeutic responses in PAAD.

Ethical approval statement

This study was reviewed and approved by the Ethics Committee of Qilu Hospital of Shandong University, and all the participants signed an informed consent form.

Declaration of Competing Interest

All authors declare no conflict of interest.

Acknowledgements

This work was supported by National Natural Science Foundation of China (grant numbers 82172339, 81972005), Natural Science Foundation of Shandong Province (grant numbers ZR2020MH238, ZR2021MH110), and Shandong Medical and Health Technology Development Project (grant number 2018WS327).

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.tranon.2022.101462.

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