Cardiac-Specific Gene TNNI3 as a Potential Oncogene for Kidney Cancer and Its Involvement in Wnt Signaling Pathway

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Research

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Abstract

Background

Cardiac troponin I (cTnI), encoded by TNNI3, is an inhibitory subunit of troponin complex expressed in cardiac muscle and is well known for its central role in the regulation of cardiac contraction. Although its aberrant expression has been reported in non-small cell lung cancer tissues and human carcinoma cells, its roles in cancers are still largely unknown.

Methods

In this work, the mRNA expression profiles of 289 Kidney renal papillary cell carcinoma (KIRP) samples were obtained from The Cancer Genome Atlas (TCGA) database. The tumor samples were classified into TNNI3-low and TNNI3-high groups. A total of 361 differentially expressed genes (DEgenes) were found correlated with TNNI3 expression. Univariate Cox analysis, log-rank test and the least absolute shrinkage and selection operator analysis were used to identify the candidate prognostic genes. The regulatory role of TNNI3 on Wnt signaling pathway was detected by western blot, wound healing assay and cell counting kit-8 assay.

Results

Multivariable Cox regression analysis was performed to establish a prognostic risk formula including genes PTPRH, LGR5 and DMRT3. The low-risk group had a better overall survival than the high-risk group ($p < 0.0001$), and the areas under the receiver operating characteristic curve for 3- and 5-year overall survival were 0.75 and 0.74, respectively. We thereafter constructed an target gene network to explore the potential functions of the three genes. The Gene Ontology and Kyoto Encyclopedia of Genes and Genomes pathway analysis identified their involvement with canonical Wnt signaling and several cancer-related pathways. In wound healing assay, the wound was closed obviously faster in the TNNI3 over-expression group as compared to control and blank groups. Significant increase in cell proliferation was seen at 72 h after transfection with TNNI3 as compared to the control and blank groups.The expressions of LGR5 and β-catenin increased in the TNNI3 over-expression group as compared to control and blank groups.

Conclusion

Collectively, our data provide evidence that TNNI3 could enhance cell migration and proliferation in 786-O cells and the three-gene signature related to TNNI3 could serve as an independent prognostic biomarker for KIRP. Insights are indeed provided into the potential oncogenic functions of TNNI3 in cancer research.

Background

Cardiac troponin I (cTnI), encoded by TNNI3, is a key and well-characterized phosphoprotein that binds to actin-tropomyosin and regulates cardiac muscle contraction and ATPase activity of actomyosin (1). It is

traditionally considered as a sarcomeric thin filament protein and has received extensive attention as a serum biomarker for myocardial tissue damage (2–4). Lots of linkage researches and animal experiments have proved that cTnI deficiency and point mutations in TNNI3 are associated with inherited cardiomyopathies (5–9). In these TNNI3 mutation caused cardiomyopathies, researchers speculate that cTnI, as an inhibitory subunit in troponin complex, may also play some regulation roles in the heart for the reason that the phenotypic variation caused by TNNI3 mutation could indicate the presence of alterations in multiple downstream genetic or epigenetic pathways (10). Recent studies have confirmed that cTnI do exist in nucleus. TnI has been found along with tropomyosins to regulate cell polarity and chromosomal stability in drosophila cells (11). Strong evidence has supported the presence of nuclear localization signal of cTnI in myocytes that differ from human embryonic stem cells and in rat mesenchymal stem cells during cardiac differentiation (12, 13). Although the function of intranuclear cTnI and the mechanism of its transportation from out of the nucleus are largely unknown, these studies could suggest other functions of cTnI besides serving as a contraction protein. We found TNNI3 expression in various cancers via analysis of TCGA datasets. Moreover, aberrant expression of cTnI have been reported in non-small cell lung cancer tissues and human cancer cells (14). However, the roles of cTnI in cancers are still largely unknown to date.

Kidney cancer has now gradually upgraded to be a commonly diagnosed cancer type worldwide without gender difference. There are nearly 380,000 diagnosed cases and over half died from the cancer per year across the world (15). Kidney renal papillary cell carcinoma (KIRP), stemming from the proximal nephron, comprises 15%-20% of kidney cancers (16, 17). It is a heterogeneous disease with two clinically histological subtypes that present significant variations in disease progression and survival outcomes (18). Despite the representation of several gene mutations in KIRP tissues, the underlying molecular mechanisms are not well understood (19). At present, no consensus has been reached on the optimal risk gene signature for prognostic evaluation of KIRP patients. Hence, there is an urgency to identify novel prognostic biomarkers of KIRP and new therapeutic targets for ameliorating the survival outcomes of its sufferers.

The Cancer Genome Atlas (TCGA), the largest publicly accessible cancer genomics database, profiled nearly 300 KIRP samples and provides a comprehensive genomic landscape with clinical annotations. We in this study explored expression profiling related to TNNI3 from 289 KIRP samples obtained from the TCGA database for better understanding of the roles of TNNI3 in KIRP. We also investigated the role of TNNI3 over-expression on cell migration and proliferation abilities in 786-O human renal cancer cells. Three potential prognostic genes related to TNNI3 in KIRP patients were identified and had an association with Wnt signaling pathway. We found TNNI3 over-expression could accelerate migration and proliferation of 786-O cells. In addition, TNNI3 may activate Wnt signaling pathway in 786-O cells. Our analyses suggest that TNNI3 seems a novel oncogene for KIRP in virtue of its correlation with signature genes, and its over-expression might be applied as an indicator for clinical unfavorable prognosis in KIRP patients.

Results

Differentially Expressed Genes are Correlated with TNNI3 in KIRP

The screening strategy of target genes and study workflow are displayed in Fig. 1. We first determined the mRNA expression of TNNI3 in KIRP samples from the TCGA database, and found that the transcripts of TNNI3 presented significantly higher expression in paired KIRP samples (n = 31) as opposed to adjacent normal tissues ($p < 0.002$) (Fig. 2A). TNNI3 also showed a trend of up-regulation when comparing unpaired tumor samples with normal samples ($p < 0.001$). Tumor tissues were divided into TNNI3-low and TNNI3-high KIRP tissues (Fig. 2C) for screening DEgenes. A total of 835 DEgenes, with a cutoff of fold change > |abs (logFold-Change) + 2*sd (abs (logFold-Change))| and adjusted $p < 0.05$, were gained using the R/Bioconductor package DESeq2. Among the DEgenes, 122 are up-regulated and 713 down-regulated, and 361 were correlated with TNNI3. The volcano plots were shown in Fig. 2D.

Recognition of Key Genes Correlated with Overall Survival

All 286 KIRP samples collected from TCGA database were taken as the training set. Univariate Cox regression analysis and log-rank test were performed to estimate the prognostic relationship between genes expression profiles and patient OS. Of the 361 TNNI3 related genes, 59 genes with a p-value < 0.05 from Cox regression analysis and 45 genes with a p-value < 0.05 from log-rank test were selected for the following study. The LASSO method was performed with the 59 genes from cox regression analysis and the minimize λ method screened out 17 genes. Collectively, 6 genes were selected out by virtue of the intersection of above three operations for further analysis (Fig. 3C). At last, via multivariable Cox regression analysis, genes PTPRH, LGR5 and DMRT3 were identified as integrated prognostic biomarkers for KIRP patients (Fig. 3D). The three genes were distinctly related with OS (Fig. 4A-C). The relationship between TNNI3 and the three genes are shown in Fig. 4D-F.

Establishment of Prognostic Risk Score Formula

We then established a prognostic risk score formula for the expression profiles of the genes PTPRH, LGR5 and DMRT3 and their regression coefficients. After calculation of the risk scores, all patients were randomized into high-risk (n = 141) and low-risk groups (n = 141), by applying the median risk score as the cutoff value. The distribution of risk scores and survival status of the included patients are shown in Fig. 5A. The expression profiles of the 3 genes including 282 patients were shown using a risk heatmap (Fig. 5A). These results suggested that patients with higher risk scores at the time had worse OS than those with lower risk scores. Subsequently, the prognostic value of the above risk formula was assessed by using Kaplan-Meier analysis. The low-risk group had a better OS as compared with the high-risk group ($p < 0.0001$) (Fig. 5B). Moreover, the prognostic capacity of the risk formula was evaluated via time-dependent ROC analysis. The areas under the ROC curve for 3- and 5-year OS were 0.75 and 0.74, respectively (Fig. 5C), presenting a fine prognostic value for predicting patient survival.

TNNI3 Over-expression is Associated with Canonical Wnt Signaling Pathway

We furthermore predicted the target relationships of the three genes respectively utilizing the STRING online analysis (www.string-db.org) (Fig. 6A). Target genes were chose for GO and KEGG pathway analysis to explore the potential mechanisms involved in the progression of KIRP, using the package clusterProfiler in the R software. As shown in Fig. 6B, the most enriched functional terms in GO analysis was canonical Wnt signaling pathway. Cell proliferation and metabolic process were also shown in GO analysis. In KEGG analysis, Wnt signaling pathway was also the highest enriched and most of the processes were related to cancers (Fig. 6C).

TNNI3 Induces 786-O Cell Migration and Proliferation

Considering the involvement of Wnt signaling pathway in multiple processes including cell migration and proliferation, assays for cell migration and proliferation were carried out for deeper exploration. Wound-healing assays were performed to assess the effect of TNNI3 on migration ability of 786-O cells. 786-O cells were scratched and assessed for wound closure after 48 h. As shown in Fig. 7A-B, the wound was closed obviously faster in the TNNI3 over-expression group as compared to control and blank groups. Moreover, CCK-8 assays were performed to examine whether TNNI3 is associated with cell proliferation. Significant increase in cell proliferation was seen at 72 h after transfection with TNNI3 as compared to the control and blank groups (Fig. 7C). These results indicated that the TNNI3 could induce the migration and proliferation capacities of 786-O cells.

TNNI3 Induces Wnt Signaling Pathway by Up-regulating LGR5 and β-catenin Expressions

Wnt signaling pathway is an important pathway involved in cell proliferation and migration. We sought to elucidate potential signaling pathways related to the migration and proliferation of 786-O cells over-expressing TNNI3, and as we figured, the changes in Wnt signaling pathway could explain the effects of TNNI3. Accordingly, western blot assay was performed to detect the activity of Wnt signaling pathway. We found that the expressions of LGR5 and β-catenin increased in the TNNI3 over-expression group as compared to control and blank groups. (Fig. 7D). These results suggest that TNNI3 may enhance the proliferation and migration abilities of 786-O cells by activating Wnt signaling Pathway.

Discussion

cTnI is a structural protein in the contractile apparatus of myocardial cells. It has been demonstrated to be one of the most specific and sensitive biomarkers for myocardial injury. Its measurement can also serve as a valid diagnostic tool for clinical screening, early identification, timely assessment and monitoring of cardiotoxicity induced by chemo-, radio-, or immuno-therapy for cancer sufferers (22–24).

This is the most reported association between cancer and cTnI. Although cTnI has been reported to present abnormal expression in non-small cell lung cancer tissues and human carcinoma cells (14), its roles in cancers are still unknown. Previously, we demonstrated that cTnI is located in nucleus in adult mouse and human fetal hearts, and found it is associated with the regulation of genes such as PDEs, PGC-1a, ND5 and ATP2A2 (20, 25, 26). This provides new directions for us to have a closer understanding of the role of gene TNNI3.

KIRP is the second most common kidney cancer following clear cell renal cell carcinoma, and ranks second in terms of morbidity rate. KIRP patients, however, are often excluded from molecular investigation due to limited number of cases (27). Therefore, there is indeed an urgency to identify effective diagnostic and prognostic biomarkers for improving survival outcomes of KIRP patients. In the present study, we revealed that the expression of TNNI3 extremely increased in KIRP samples as opposed to normal samples from TCGA datasets. As a result, the KIRP patients were classified into TNNI3-low and TNNI3-high groups. Although we did not found TNNI3 was directly related to survival outcomes in the KIRP patients, we demonstrated the expression of TNNI3-related genes were closely associated with tumor progression and survival outcomes in KIRP. We firstly selected out 835 DEgenes including TNNI3 itself among TNNI3-low and TNNI3-high KIRP patients. Of the DEgenes, 361 genes were positively or negatively related to TNNI3 in expression. Based on the results of univariate Cox regression analysis, log-rank test and LASSO analysis, we found six genes, including PTPRH, RYR2, PLA2G5, LGR5, DMRT3 and ACTG2, were associated with survival outcomes. PTPRH, LGR5 and DMRT3 were furthermore selected as the signature genes via multivariable Cox regression analysis. Hence, we constructed a prognostic risk score formula and stratified KIRP patients into high-risk and low-risk groups based on their risk scores. We demonstrated that the overall survival was shorter for the high-risk patients as compared to the low-risk ones. Collectively, these findings indicate the predictive value of PTPRH, LGR5 and DMRT3 for assessing the prognosis of KIRP patients.

To gain insight into the molecular mechanisms of PTPRH, LGR5 and DMRT3, the genes which potentially interacted with the three genes were predicted by STRING, and gene-gene interaction networks were obtained. A total of 31 genes in the network were taken into the subsequent GO and KEGG analysis. GO analysis indicated that the three genes were mostly associated with canonical Wnt signaling pathway. KEGG pathway analysis also indicated the association with Wnt signaling pathway. Moreover, KEGG analysis showed that these genes were potentially related to cancers such as breast cancer, gastric cancer, endometrial cancer, and prostate cancer etc. Wnt family is a group of proteins that can affect various cellular functions such as organogenesis, stem cell regeneration, and cell survival (28). Activation of Wnt signaling is essential for tumor progression and survival, even in the presence of anti-neoplastic agents or anti-tumoral immune response (29). To date, Wnt signaling pathway was reported to be involved in various cancers such as colorectal cancer (30), endometrial cancer (31), breast cancer (32), non-small cell lung cancer (33), and kidney cancer (34). These evidence could well support our GO and KEGG results in this study.

The key to activate Wnt pathway signaling is the accumulation of cytoplasmic β-catenin that presents in different forms and locations in the cell (29). Protein coding gene LGR5 potentiates canonical Wnt/β-catenin signaling and is a stem cell marker in intestinal epithelia, hair follicles, and hepatocytes. etc. (35–37). LGR5 is reportedly over-expressed in human colorectal adenomas and cancers (38–40), as well as in other solid tumors such as basal cell carcinoma, hepatocellular carcinoma and neuroblastoma (41, 42). LGR5 expression predicts adverse prognosis in patients with colorectal cancers (43). In our study, β-catenin and LGR5 showed increased expression in the presence of TNNI3 over-expression in 786-O cells. This evidence suggested that TNNI3 might activate Wnt signaling pathway. In addition, we found TNNI3 could accelerate the migration of 786-O cells and enhance their proliferation ability via wound-healing and CCK-8 assays. These results indicated that TNNI3 might be an adverse gene for kidney cancer.

Conclusion

Our data demonstrated that TNNI3 over-expressed in KIRP patients. We constructed an TNNI3-related gene (PTPRH, LGR5, and DMRT3) signature that was significantly associated with OS in KIRP patients which could differentiate patients with low prognostic risk from the opposite. We also found the three genes were associated with canonical Wnt signaling pathway. Furthermore, we detected enhanced abilities of cell migration and proliferation induced by TNNI3 in 786-O cells, suggesting that TNNI3 might activate Wnt signaling pathway via the inducement of LRG5 and β-catenin expressions. These results indicated that TNNI3 might potentially act as a prognostic gene in KIRP patients, and positively pointed out new directions for further research on the role of TNNI3 in various cancers.

Methods

Datasets

The detailed clinical information and transcriptomics of KIRP cohort were based on the TCGA KIRP dataset. The mRNA expression profiles (HTSeq–Counts) of 289 KIRP tissues and 32 non-tumor tissues were downloaded via the TCGA data portal (https:// portal.gdc.cancer.gov/). Genes were annotated by human gene annotation files (GRCh38.p12). The expression of gene TNNI3 was analyzed first in tumor and non-tumor tissues. Tumor tissues were then randomly divided into TNNI3-low group (n = 144) and TNNI3-high group (n = 142). Differential expression analysis of genes were performed using R/Bioconductor package DESeq2. Adjusted *p*-value less than 0.05, and the absolute value of $\log_2$ fold-change of gene expression ($|\log_2(\text{Fold-Change})|$) greater than the mean value of normalized counts (mean (abs (log(Fold-Change))) + 2*sd (abs (log(Fold-Change))), were applied for primary filtering of differentially expressed genes (DEgenes). Correlation analysis was conducted between cTnI and 835 DEgenes. A total of 361 correlated genes were selected out when a *p*-value of 0.05 was set as the cutoff.

Characterization of Genes Associated with Overall Survival

All the 289 KIRP patients were put into the training set. Univariate Cox analysis and log-rank test were performed to assess the association between the expression levels of the 361 positively correlated genes and overall survival (OS) of the patients. Intersections of genes with *p*-value < 0.05 in both analyses were selected for further analysis. The Least Absolute Shrinkage and Selection Operator (LASSO) analysis was then performed using glmnet package in R-software to find out vital genes from the prognostic genes.

Establishment of Prognostic Risk Score Formula

In terms of the expression levels of the genes, multivariable Cox regression analysis was performed for establishing a prognostic risk score formula. The random forest plot was developed using the R package survminer. Risk scores of each included patient were calculated via the formula as mentioned above. Afterwards, all patients were classified into high-risk and low-risk groups, by setting the median risk score as the cutoff value.

Assessment of the Prognostic Risk Score Model

Kaplan-Meier survival curve was used to compare the prognosis between the low-risk and high-risk groups. Additionally, a time-dependent receiver operating characteristic (ROC) curve, based on the risk scores for 3- and 5-year OS probability, was employed to assess the diagnostic accuracy. A *p*-value < 0.05 indicates statistical significance. Meanwhile, concordance probability (C-index) was calculated using the R package survcomp to assess the value of prognostic risk formula. Then risk heatmap was applied to plot the expression profiles of key genes in the low-risk and high-risk groups. The above analyses were processed using R software.

Gene-Set Enrichment Analysis and Functional Prediction

Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis was performed to annotate the potential functions of DEgenes using the clusterProfiler package in the R software.

Cell Culturing and Transfection

The human renal cancer cell line 786-O was used in this study. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, USA) supplemented with 100 U/ml of penicillin/streptomycin and 10% fetal bovine serum (FBS), at 37°C in an atmosphere containing 5% CO_2. Adenovirus-aided transfections were performed as per previous description (20). Briefly, the cells were transfected with empty vector or adenovirus over-expressing TNNI3. 48 hours after, transfected cells were collected for further analyses.

In-vitro Wound-Healing Assay

Transfected 786-O cells were removed using 0.5 mM ethylene diamine tetraacetic acid (EDTA) and plated on 6-well plates at a density of $1*10^6$ cells/ml for overnight incubation. Wounds were made with a 10 ml pipette tip, and images were taken immediately (time zero) and at 24 h after wounding respectively. The migrated distance of the cell monolayer to close the wounded area during each time period was

measured using ImageJ software. All assays were conducted in triplicate and repeated at least three times.

Cell Proliferation Assay

Cell Counting Kit-8 (CCK-8) assay (K1018, APExBIO) was used for the analysis of cell proliferation. The 786-O cells were plated on 96-well plates at a density of $1.5*10^3$ cells/well. Cell proliferation was detected at 24 h as per the manufacturer's protocol. Briefly, 10 ml of CCK-8 solution was added to each well and incubated at 37°C for 2 h. The optical density at 450 nm for each solution was then measured using a spectrophotometer.

Western Blot Assay

Western blotting was carried out as per previous description (21). Proteins bound to the polyvinylidene fluoride (PDVF) membrane were analyzed using primary antibodies against LGR5 (Abcam, USA), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Arigo, Taiwan), β-catenin (CST, USA) and DYKDDDK Tag (GenScript, China). Band intensity was quantified using a G:BOX imaging system (Syngene, UK).

Statistical Analysis

Both the expression profiles of genes and clinical information data were excavated from TCGA database by R software (Version 4.1). All statistical analyses were carried out using R software or Statistical Package for the Social Sciences (SPSS) software (IBM, USA). *p*-values less than 0.05 indicate statistical significance.

Abbreviations

KIRP: Kidney renal papillary cell carcinoma

TCGA: The Cancer Genome Atlas

cTnI: cardiac Troponin I

DEgenes: differentially expressed genes

KEGG: Kyoto Encyclopedia of Genes and Genomes

GO: Gene Ontology

Declarations

Data Availability Statement

The dataset analyzed during the current study was derived from the following public domain resources:https://portal.gdc.cancer.gov/repository

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Contributions

W.A.Z and L.Y conceived and designed the study and helped to draft the manuscript. X.X.C performed the statistical analysis. W.Q.H supervised and coordinated the study. All authors read and critically revised the manuscript for intellectual content and approved the final manuscript.

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Ethics declarations

Ethics approval and consent to participate

In vitro experimental protocols were approved by the Institutional Ethics Committee of the First Affiliated Hospital of Sun Yat-sen University, China (NO.2017-103).

Consent for publication

All participants have given informed consent.

Competing interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Figures

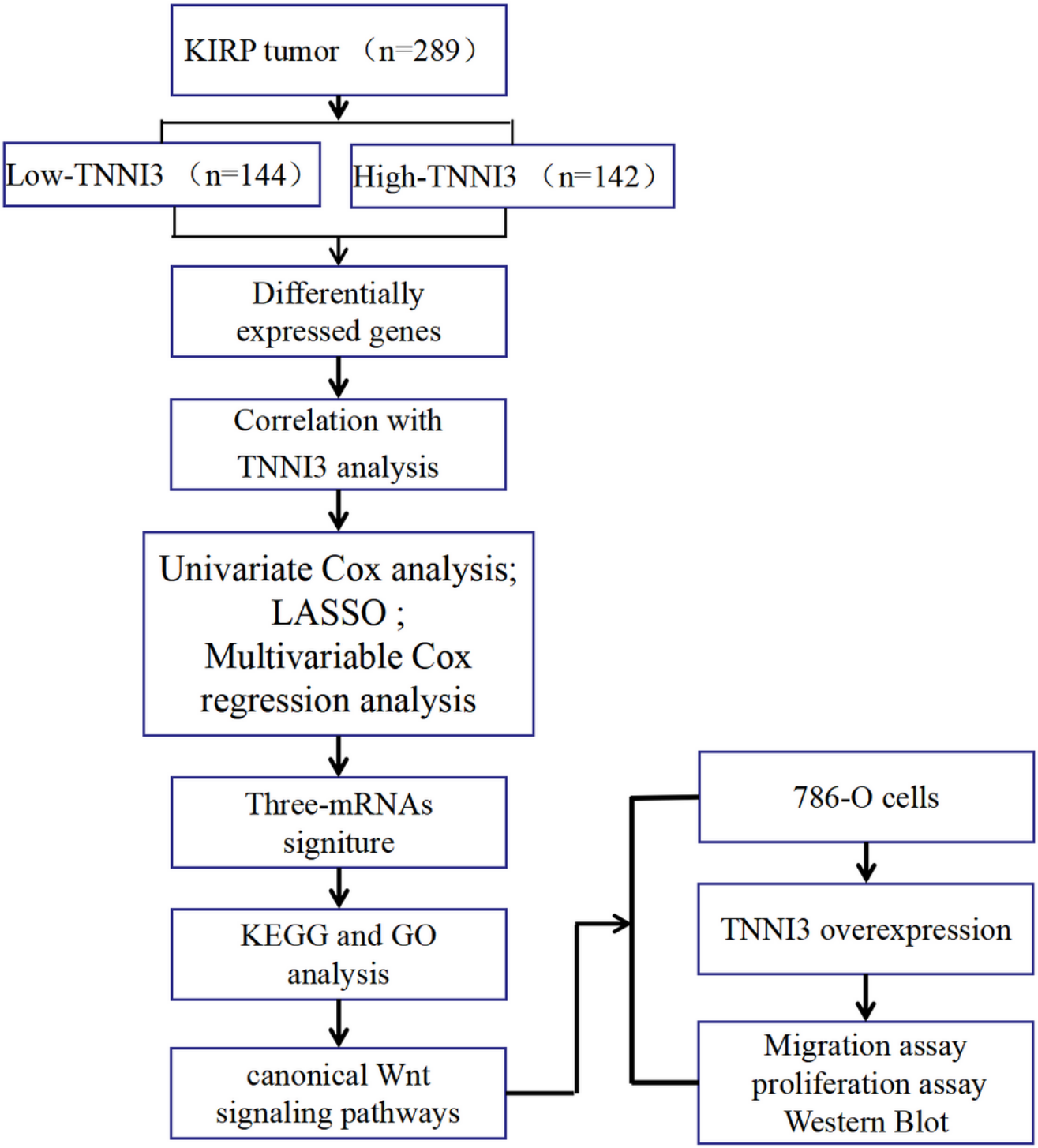

Figure 1

The screening strategy of target genes and study workflow. KIRP, kidney renal papillary cell carcinoma; LASSO, least absolute shrinkage and selection operator analysis; KEGG, Kyoto Encyclopedia of Genes and Genomes; GO, Gene Ontology

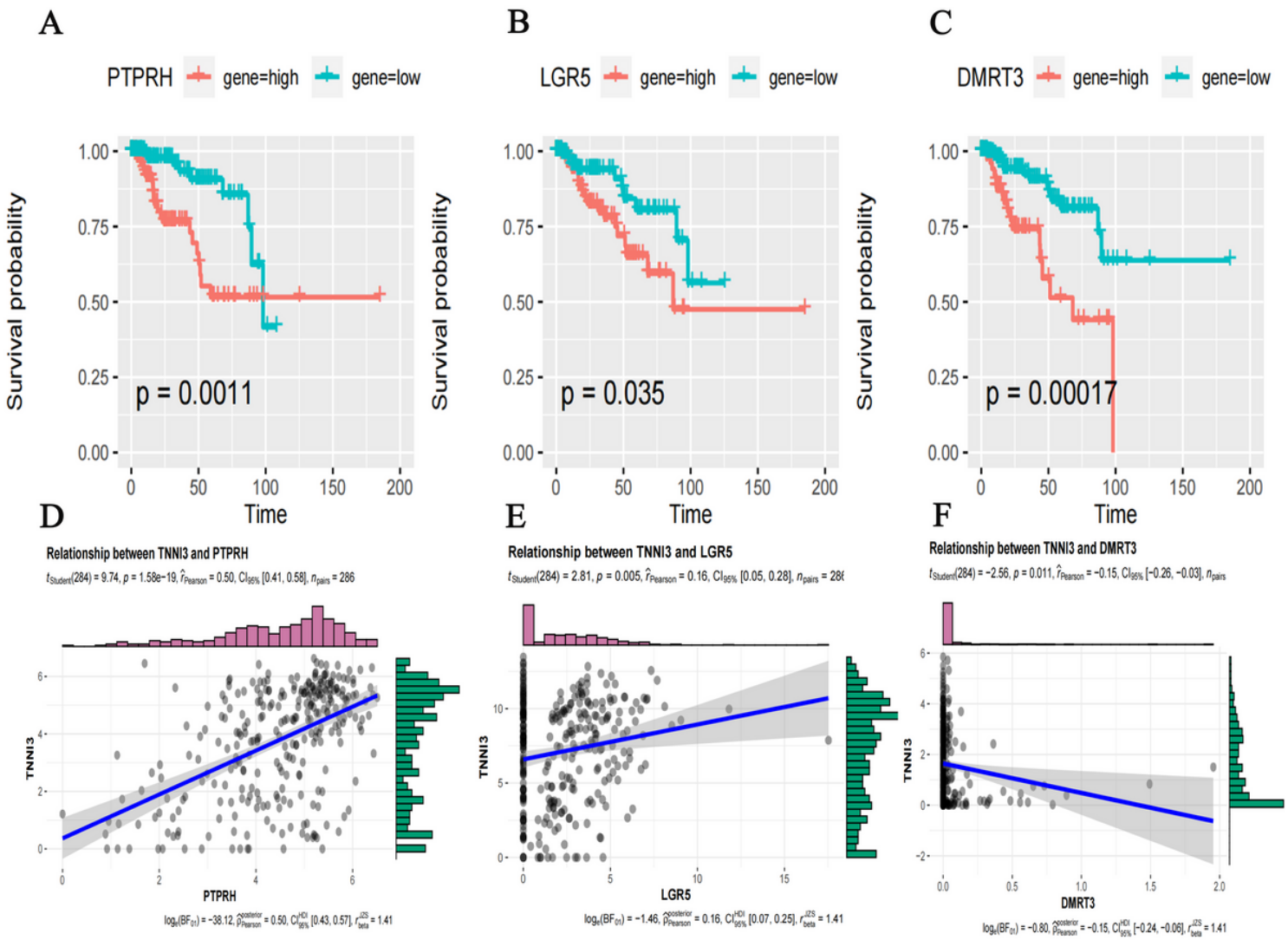

Figure 2

(A-B) Comparison of TNNI3 mRNA expression between tumor samples and normal samples from TCGA KIRP studies (C) Comparison of TNNI3 mRNA expression between TNNI3-low (n = 144) and TNNI3-high (n = 142) KIRP groups. (D) Volcano plot of TNNI3 related differentially expressed genes between TNNI3-low and TNNI3-high KIRP groups. TCGA, The Cancer Genome Atlas; KIRP, kidney renal papillary cell carcinoma.

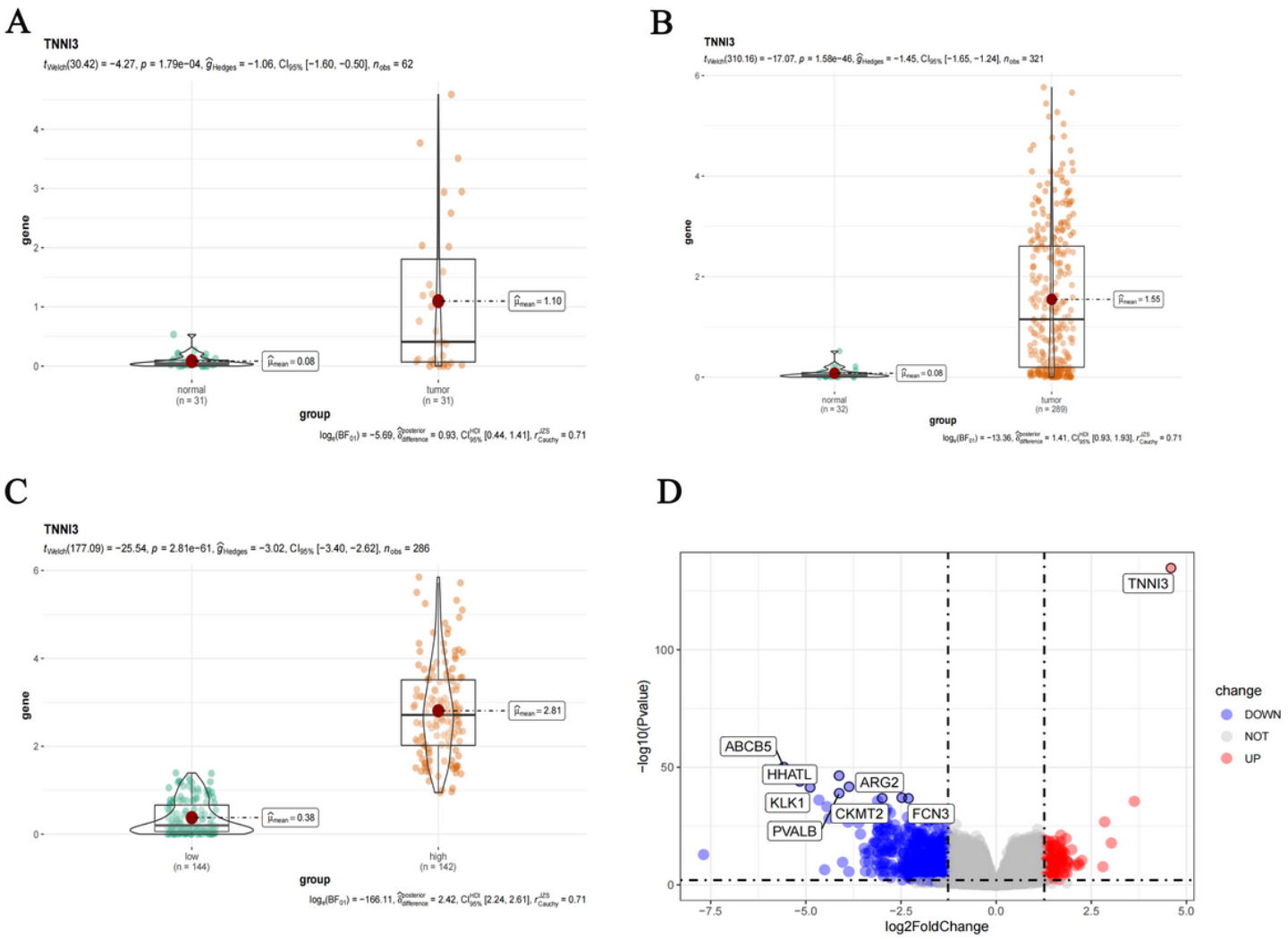

Figure 3

(A-B) λ and AUC values of LASSO model including 59 differentially expressed genes selected by univariate Cox regression analysis. (C) Numbers of genes or intersection of genes from univariate Cox, log-rank test and LASSO method. (D) Three genes (PTPRH, LGR5 and DMRT3) were identified as integrated prognostic biomarkers for KIRP patients using multivariable Cox regression analysis. AUC, area under the curve; LASSO, least absolute shrinkage and selection operator analysis; KIRP, kidney renal papillary cell carcinoma.

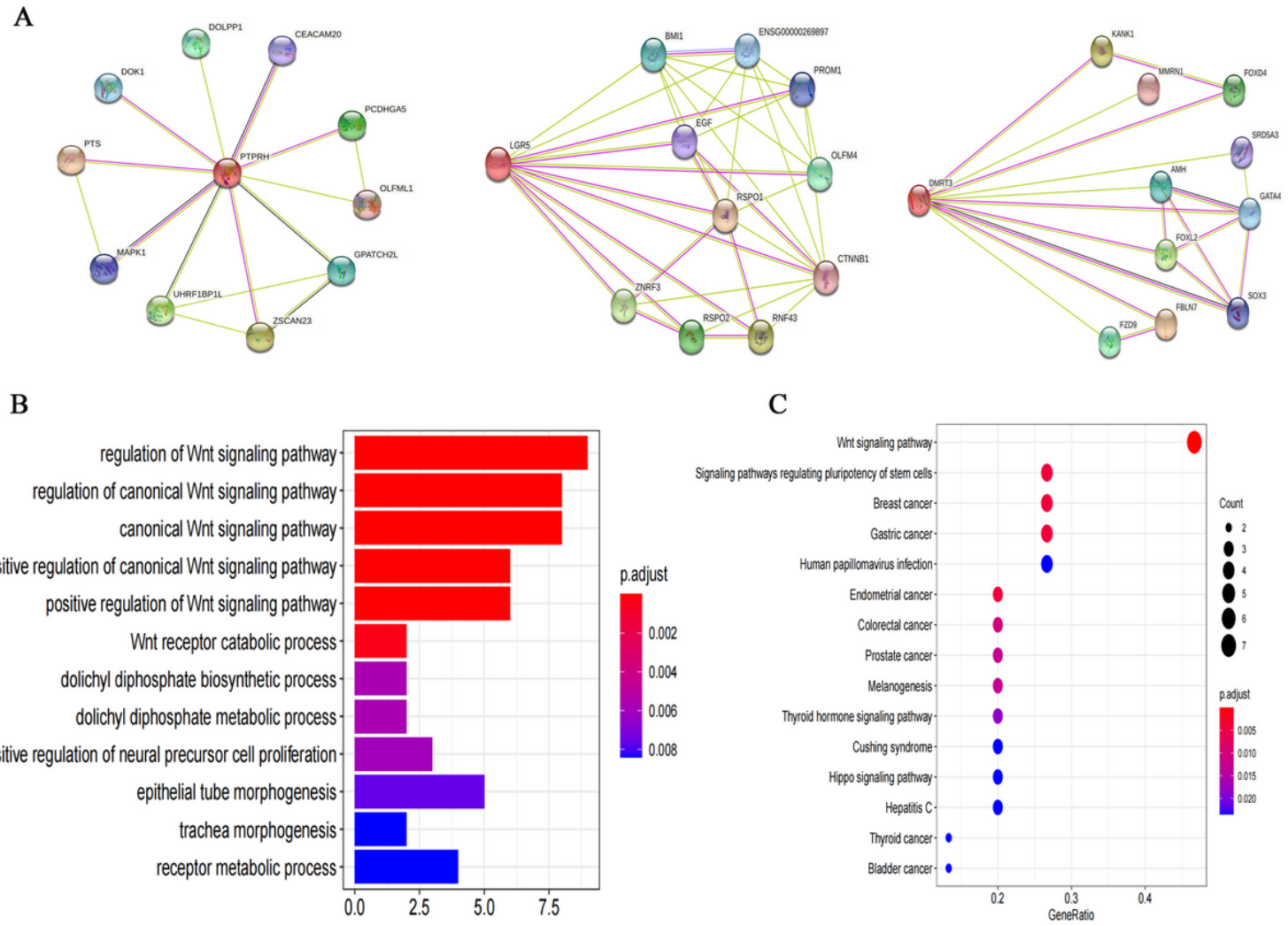

Figure 4

(A) Kaplan-Meier survival curves of genes PTPRH, LGR5 and DMRT3 in KIRP patients. (B) The expression relationships between TNNI3 and PTPRH, LGR5 and DMRT3. KIRP, kidney renal papillary cell carcinoma.

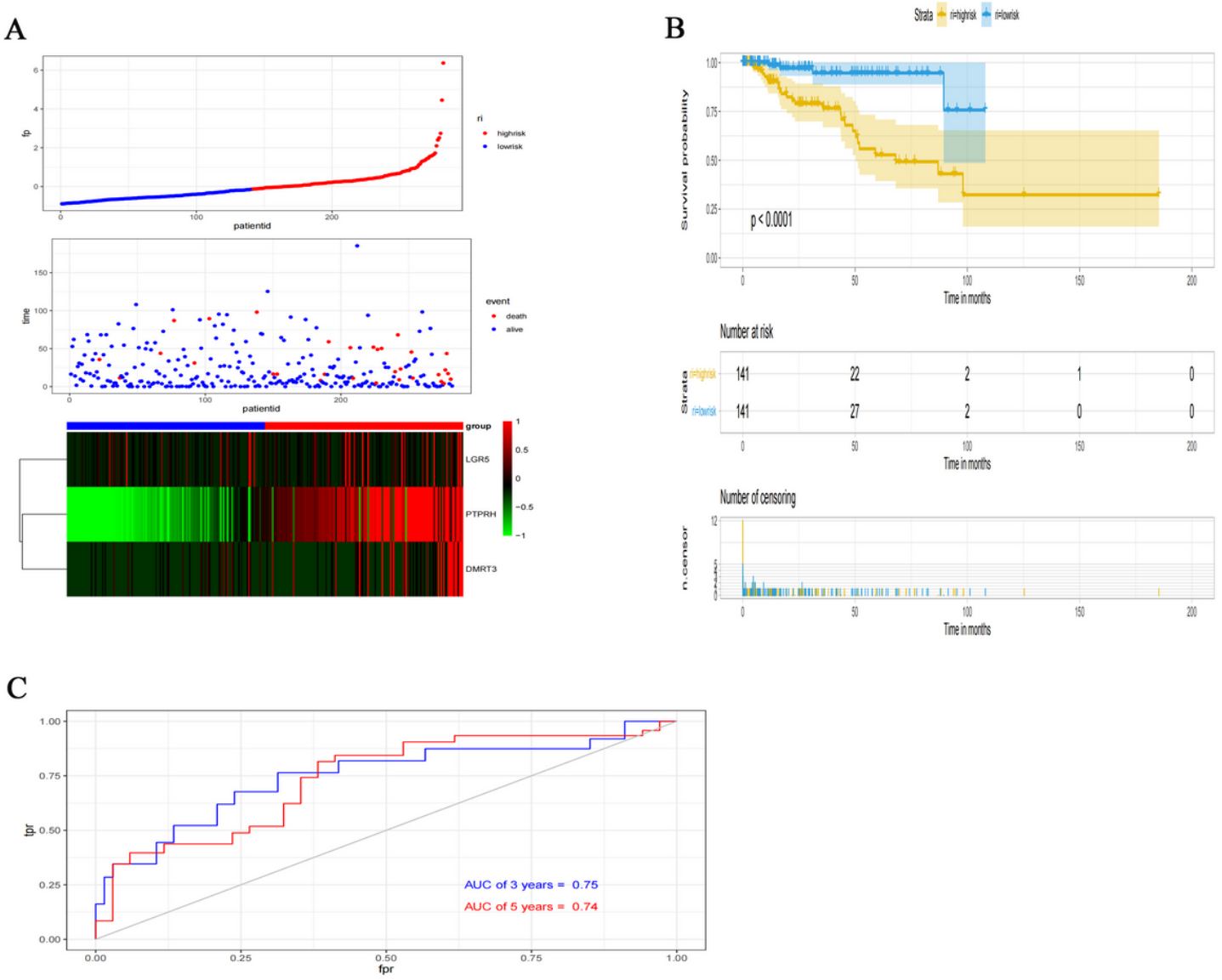

Figure 5

(A) Distribution of risk scores, survival status, and risk heatmap of three prognostic genes (PTPRH, LGR5 and DMRT3). (B) Kaplan–Meier survival curve for evaluating the prognosis between the low-risk and high-risk groups. (C) Time-dependent receiver operator characteristic curve analysis of the 3- and 5-year overall survival for evaluating the prognostic capacity of the risk formula.

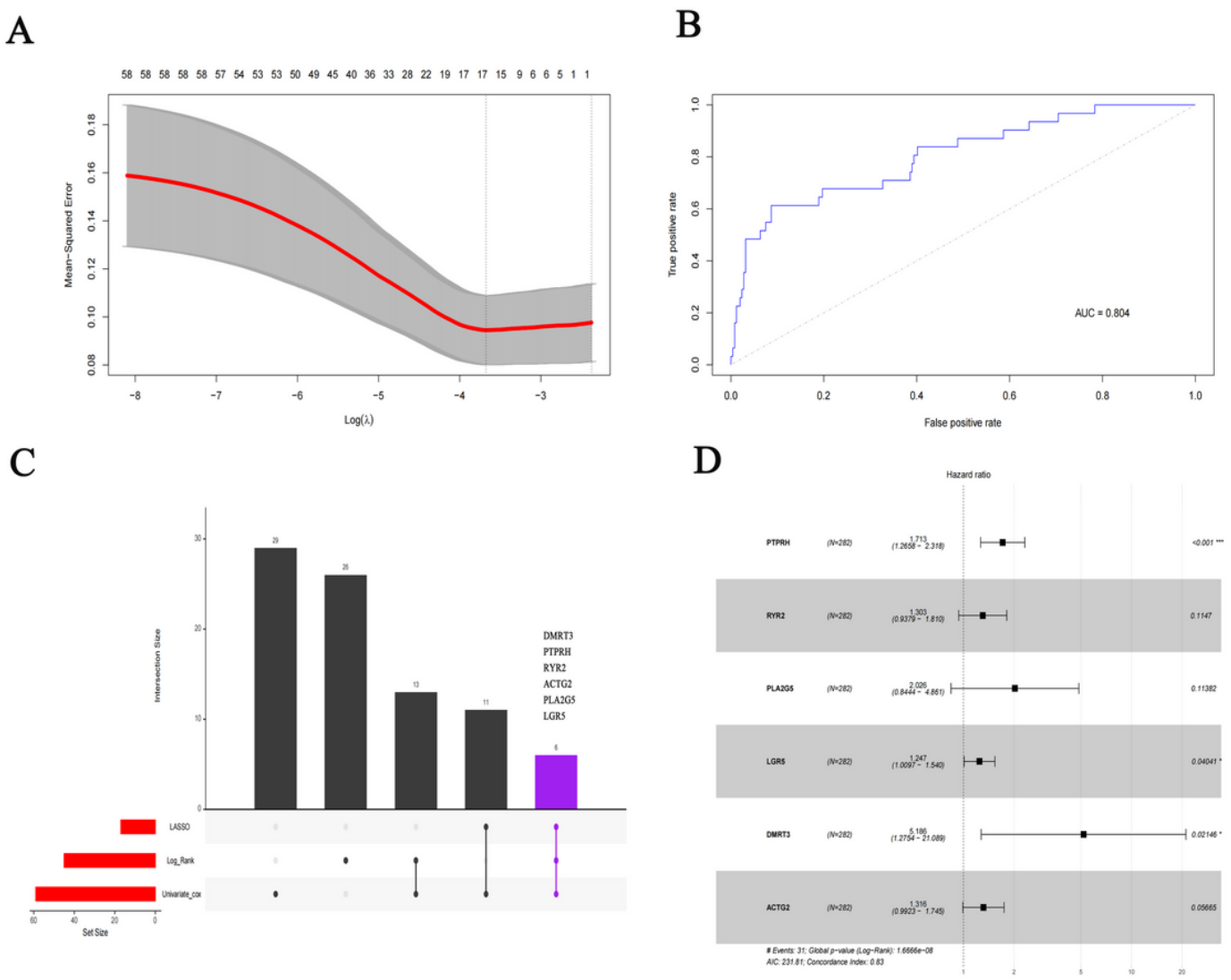

Figure 6

(A) The target genes associated with PTPRH, LGR5 and DMRT3. (B) Gene Ontology (GO) analyses for the target genes and the enriched functional terms. (C) Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses for the target genes.

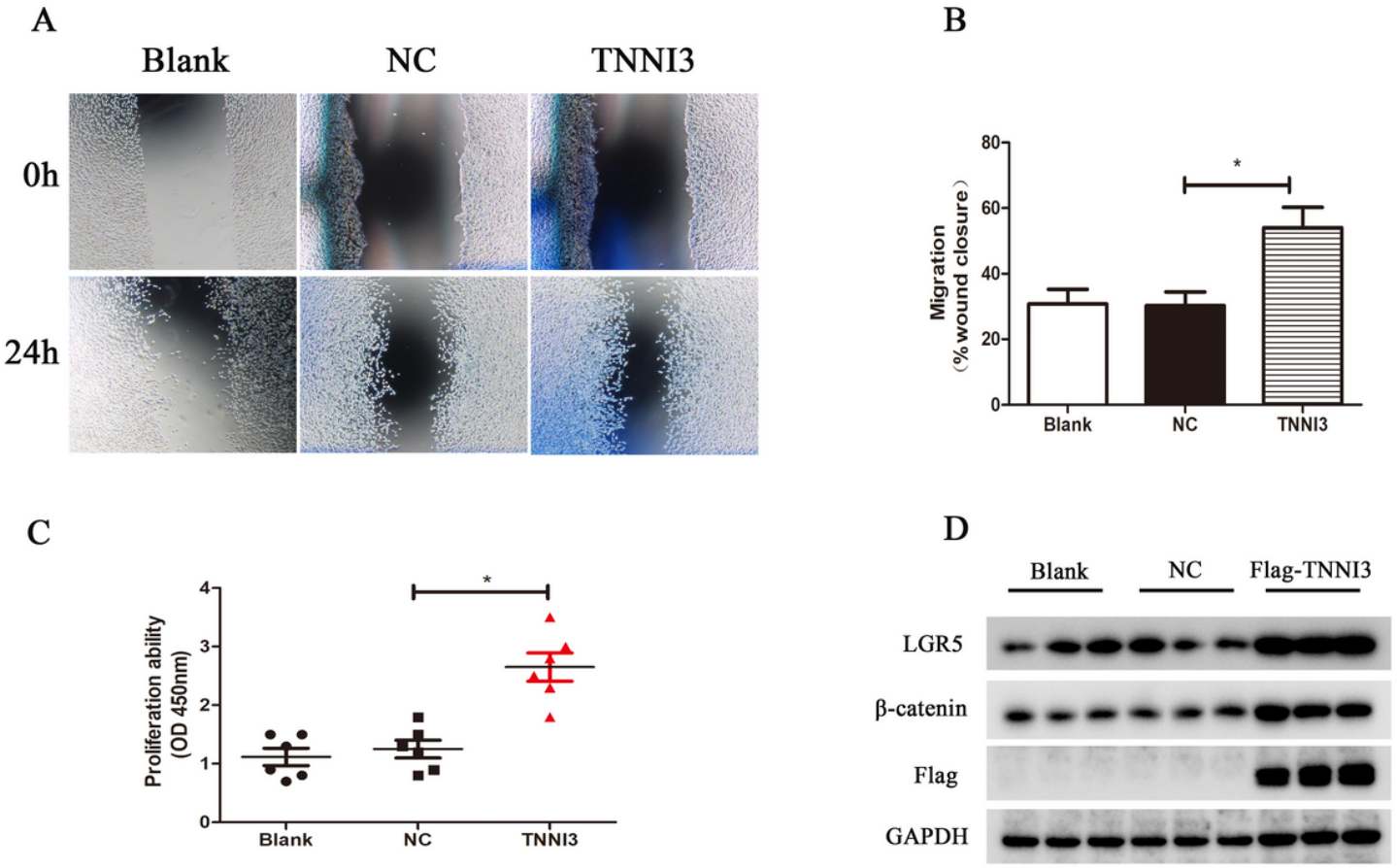

Figure 7

(A-B) 786-O cell migration as evaluated by wound healing assay showed that cells with TNNI3 over-expression migrate faster than NC (empty vector) and blank groups. (C) Cell Counting Kit-8 (CCK8) assay results showed that 786-O cell proliferation significantly increased in TNNI3 over-expression group as compared to NC and blank groups. (D) The expressions of LGR5 and β-catenin were determined by western blot. LGR5 and β-catenin were increased in TIIN3 over-expression group compared to NC and blank groups. The results are expressed as mean ± standard deviation. Statistical significance was determined by analysis of variance (ANOVA) followed by least significant difference (LSD) tests. *p < 0.05.