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Original Article

Characterization and verification of MMP family members as potential biomarkers in kidney clear cell renal carcinoma

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Abstract: Renal cell carcinoma can arise from lesions in the renal epithelium. This particular type of cancer is prevalent in the realm of renal cancers and is associated with an unfavorable prognosis. Among these cases, over 70% are classified as kidney renal clear cell carcinoma (KIRC). Since the underlying causes of KIRC haven't been fully understood, there is an urgent need for deeper investigation into its pathogenesis. Various tools, software, and molecular analysis was used, including Search Tool for the Retrieval of Interacting Genes/Proteins (STRING), Cytoscape, University of ALabama at Birmingham CANcer data analysis Portal (UALCAN), muTarget, Gene Expression Profiling Interactive Analysis (GEPIA), OncoDB, Human Protein Atlas (HPA), cBioPortal, Kaplan-Meier (KM) plotter, Gene Set Enrichment Analysis (GSEA), Tumor IMmune Estimation Resource (TIMER), Encyclopedia of RNA Interactomes (ENCORI), DrugBank, Encyclopedia of RNA Interactomes (RT-qPCR), targeted bisulfide sequencing (bisulfide-seq), and receiver operating curve (ROC) to matrix metallopeptidase (MMP) gene family constituents, with the precise objective of identifying a small set of hub genes. These hub genes hold the potential to be harnessed as molecular biomarkers for KIRC. By performing STRING and CytoHubba analyses of the 24 MMP gene family members, MMP2 (matrix metallopeptidase 2), MMP9 (matrix metallopeptidase 9), MMP14 (matrix metallopeptidase 14), and MMP16 (matrix metallopeptidase 16) were recognized as hub genes having highest degree scores. After conducting an in-depth expression analysis of MMP2, MMP9, MMP14, and MMP16 using various The Cancer Genome Atlas (TCGA) databases and RT-qPCR techniques, these displayed a significant increase in expression at both the mRNA and protein levels within KIRC samples when compared to control samples. The impact of the over expression of MMP2, MMP9, MMP14, and MMP16 also left a distinct mark on the worst overall survival (OS) rates of KIRC patients. Furthermore, a targeted bisulfide-seq investigation unveiled a correlation between promoter hypomethylation patterns and the up-regulation of these key genes in KIRC patients. Additionally, hub genes were involved in various diverse oncogenic pathways. In conclusion, four MMP gene family members, including MMP2, MMP9, MMP14, and MMP16 may serve as therapeutic target and molecular biomarker in KIRC.

Keywords: KIRC, MMP gene family, chemotherapy, overall survival

Introduction

Based on recent cancer statistics, kidney cancer holds the 9th position among the most commonly diagnosed cancers in males and the 14th position among females [1, 2]. In the year 2022, kidney cancer resulted in approximately 400,000 new cases and led to nearly 175,000 fatalities worldwide [3]. Recent research has also brought attention to the rapid rise in the global incidence of new kidney cancer cases [4, 5]. Out of all kidney cancers, kidney renal clear cell carcinoma (KIRC) stands out as the most frequently reported carcinoma [6]. Additionally, approximately 30% of patients with KIRC encounter metastasis as the disease advances [7]. Furthermore, the 5-year survival rate for this condition is below 10%, accompanied by a median survival of merely 13 months [8]. It is widely recognized that the prognosis for patients with KIRC is exceptionally bleak primarily because of late detection and resistance to currently available chemotherapy or radiotherapy treatments [9-11].

KIRC pathogenesis involves various genes like Von Hippel-Lindau disease (VHL), mesenchymal-epithelial transition factor (c-Met), BRCA1

associated protein-1 (BAP1), Polybromo-1 (PBRM1), and others [12]. Numerous targeted therapies have been employed, targeting different molecular signatures such as Platelet-derived growth factor (PDGF), Vascular endothelial growth factor (VEGF), Mesenchymal Epithelial Transition (MET), and immune checkpoints [13, 14]. However, due to the heterogeneity of KIRC, the effectiveness of targeted therapies significantly varies among patients. Consequently, selecting appropriate therapeutic agents for individual cases remains a considerable challenge in clinical practice. Therefore, the pursuit of novel biomarkers and predictive models for KIRC treatment is of utmost importance.

Matrix metalloproteinases (MMPs) are enzymes tasked with the degradation of various protein components within the extracellular matrix [15]. Within cancer cells, the MMP family plays a crucial role in tumor invasion and metastasis by damaging the extracellular matrix and basement membrane. It achieves this by activating growth factors and promoting angiogenesis [16]. The MMP family is linked to tumor proliferation, differentiation, and angiogenesis, rendering them promising targets for therapy and serving as molecular biomarkers for various cancers, including gastric and head and neck cancers [17-20]. Nevertheless, the comprehensive exploration of the MMP family's role in KIRC patients is yet to be undertaken.

This article aims to explore the expression, methylation, prognosis, mutation, and protein interactions of the MMP family in KIRC. Additionally, it seeks to investigate functional enrichment and related signaling pathways, as well as potential therapeutic drugs. Moreover, experimental validation will be conducted to assess the role of the MMP family in KIRC comprehensively. Through these investigations, the article seeks to identify key hub genes within the MMP family that could serve as valuable diagnostic and prognostic biomarkers for KIRC patients.

Methodology

Collection of KIRC and normal control tissue samples

Collection of KIRC and normal control tissue samples was carried out with the approval of the ethics committee at Gomal University, Dera Ismail Khan, Pakistan. A total of 20 pairs of KIRC tissues and their corresponding normal tissues were prospectively obtained from patients who visited Institute of Nuclear Medicine, Oncology, and Radiotherapy Hospital, and Ayub Medical Complex during the period from August 2022 to May 2023. Prior to their participation, all participants provided informed consent by signing consent forms. All patients enrolled in the study were diagnosed with KIRC and had not received any therapy prior to their surgical procedures.

Analysis of protein-protein interactions among MMP family members and identification of hub genes

The Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database is renowned for its extensive and current protein-protein interaction (PPI) information, making it a leading resource [21]. Researchers benefit from its integrated platform, enabling exploration of intricate protein interactions and functions in various biological systems, such as humans, yeast, and bacteria. The database also offers advanced visualization tools and interactive networks, providing an intuitive representation of protein interactions and functions [21]. In this investigation, we employed the STRING online resource with its default settings to build the PPI network for the MMP protein family.

Researchers often rely on Cytoscape software [22] to analyze protein-protein interaction networks effectively. This powerful tool provides a means to visualize intricate networks of protein interactions and identify essential components within the network. To identify hub genes from the constructed PPI using the degree method, we utilized the CytoHubba plugin application [23] within the Cytoscape platform.

Profiling mRNA and protein expression of hub genes in the cancer genome atlas datasets

University of ALabama at Birmingham CANcer data analysis Portal (UALCAN) is a publicly accessible and user-friendly database offering gene expression analysis of cancer data derived from The Cancer Genome Atlas (TCGA) [24]. Researchers can effortlessly explore gene expression levels, patient survival, and other clinical and molecular features across different

cancer types using this resource. UALCAN provides valuable information, including gene expression quantification, correlations between gene expression and clinical data, pathway analysis, and gene ontology analysis. In this study, we employed UALCAN to conduct messenger RNA (mRNA) and protein expression profiling of the hub genes in KIRC samples compared to controls.

Validation analysis of mRNA expression for hub genes using additional the cancer genome atlas datasets

muTarget [25] and Gene Expression Profiling Interactive Analysis (GEPIA) [26] are web-based platforms designed for analyzing gene expression patterns in cancer. These databases offer a wide array of analysis tools, including differential gene expression analysis, survival analysis, correlation analysis, and pathway analysis. In this study, we utilized the muTarget and GEPIA databases to validate the expression of hub genes in KIRC samples compared to controls. Furthermore, the GEPIA database facilitated the examination of hub gene expression across KIRC samples of different cancer stages.

Survival analysis and development of hub genes-based prognostic model

To assess the overall survival (OS) of the hub genes among KIRC patients, we conducted an analysis using the Kaplan-Meier (KM) plotter [27]. Additionally, we employed the "survival" package [28] in the R language to develop a prediction model using the least absolute shrinkage and selection operator (Lasso) and multivariate Cox proportional hazard regression analysis. For this analysis, we utilized the TCGA_KIRC dataset as the training dataset and the GSE22541, GSE167573, and E_MTAB_1980 datasets as validation datasets. The prognostic model for KIRC patients' prognosis was formulated as the risk score, which involved the summation of the multivariate Cox regression coefficient variation for each mRNA.

Subcellular localization and mutational analysis

The Human Protein Atlas (HPA) is an accessible database offering comprehensive expression and localization data for human proteins [29]. In this study, we utilized the HPA database to determine the subcellular localization of the proteins encoded by the hub genes.

cBioPortal is a freely accessible database that houses cancer genomics data derived from cancer patients [30]. It offers a user-friendly interface to explore genomic profiles of various cancers from diverse sources. In this study, we employed this tool for conducting mutational analysis of the hub genes in TCGA KIRC samples.

Analyzing promoter methylation level of hub genes using the cancer genome atlas dataset

In this study, we utilized the OncoDB [31] database to conduct the methylation analysis of the hub genes in KIRC samples compared to normal controls.

Functional enrichment analysis

Gene Set Enrichment Analysis (GSEA) is a computational method used in bioinformatics to interpret gene expression data [32]. It identifies gene sets that are significantly enriched in specific experimental conditions, offering valuable insights into biological pathways and processes. Herein, we performed Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses of the hub genes using the GSEA program.

miRNA network of hub genes

Encyclopedia of RNA Interactomes (ENCORI) is a comprehensive database that integrates various high-throughput data, including miRNA (MicroRNA), long non-coding RNAs (lncRNA), and mRNA expression profiles, along with clinical information from TCGA and other resources [33]. It offers researchers valuable tools to explore and analyze non-coding RNA function in cancer, enabling the discovery of potential biomarkers and therapeutic targets. For this study, the miRNA network of the hub genes was generated using the ENCORI database.

Immune cell infiltration analysis

Tumor IMmune Estimation Resource (TIMER) is a web-based resource that provides comprehensive and updated data on tumor-infiltrating immune cells. It offers researchers valuable

insights into the immune microenvironment of different cancer types, helping to better understand tumor-immune interactions and their clinical implications [34]. This study utilized the TIMER database to correlate levels of immune cell infiltration in KIRC with hub gene expression.

Hub genes' drug prediction analysis

DrugBank is a comprehensive online resource that provides valuable information on drugs and their targets [35]. It offers a vast collection of drug data, including chemical structures, pharmacological properties, and interactions with various biological targets. DrugBank serves as a valuable tool for researchers and clinicians in drug discovery and development. The DrugBank database was utilized to identify a diverse range of drugs linked to the identified hub genes.

Cell lines

A total of six human KIRC cell lines were used in this study: ACHN, Caki-3, and OS-RC-2 from the primary tumor site, and A-704 and 769-P from the metastatic tumor. Additionally, one normal renal tubular epithelial cell line (HKC-8) was obtained from the American Type Culture Collection (ATCC, USA) and cultured following the manufacturer's instructions.

DNA and RNA extraction

The extraction of total RNA from both clinical KIRC samples, cell lines, and normal control samples was carried out using the isopycnic centrifugation method as previously described [36]. DNA extraction was performed using the organic method [37]. The quality of the extracted RNA and DNA was assessed using a 2100 Bioanalyzer (Agilent Technologies, Germany).

Targeted bisulfite sequencing

DNA samples were sent to Beijing Genomics Institute (BGI) Company for bisulfite-seq analysis. After targeted bisulfite-seq analysis, the resulting methylation values were normalized as beta values. To identify variations in expression and methylation levels, the obtained beta values for the hub genes in KIRC samples, cell lines, and normal control samples were compared.

Reverse transcription quantitative real-time PCR (RT-qPCR) validation

The specific protocols employed in this study were as follows: Firstly, the PrimeScript™ RT reagent kit (Takara, Japan) was used for reverse transcription of the extracted RNA from clinical KIRC samples, cell lines, and normal control samples and cell lines to synthesize complementary DNA. Subsequently, RT-qPCR was conducted on an ABI ViiA 7 Real Time PCR System (Thermo Fisher, USA) using SuperReal SYBR Green Premix Plus (Tiangen Biotech, China) as the fluorescent dye. GAPDH was chosen as the internal reference in this study, and all experiments were performed independently in triplicate. The primer sequences for each hub gene are provided below. The $2^{-\Delta\Delta Ct}$ method was applied to assess the relative expression of each hub gene [38].

GAPDHF 5-ACCCACTCCTCCACCTTTGAC-3, GAPDHR 3-CTGTTGCTGTAGCCAAATTCG-5 [39].

MMP2F 5'-CTCAGATCCATGGTGAGATCT, MMP2R 5'-CTTTGGTTCTCCAGCTTCAGG-3' [40].

MMP9F 5'-GAGTGGCAGGGGGAAGATGC-3', MPP9R 5'-CCTCAGGGCACTGCAGGATG-3' [41].

MMP14F 5'-GGATACCCAATGCCCATTGGCCA-3', MPP14R 5'-CCTCGGTGCACCATGTTTGGC-3' [42].

MMP16F 5'-TCTGTCTCCCTTGAAATA-3', MMP16R 5'-ACCCTCATGACTTGATAACC-3' [43].

ROC curve generation

Based on the RT-qPCR and targeted bisulfite-seq expression and methylation data, receiver operating curve (ROC) curves of identified hub gene expression was generated using SRPLOT web source (https://bioinformatics.com.cn/srplot).

Hub gene survival analysis in Pakistani KIRC patients

The Kaplan-Meier (KM) method is a widely used statistical technique in survival analysis to estimate the probability of survival over time [44]. In this study, we conducted survival analysis of the hub genes on Pakistani KIRC patients using the KM method. For this purpose, hub gene expression levels were categorized as high or low, based on the standard cutoff value that

separates individuals with high expression from those with low expression of the gene. The survival curves provide valuable insights into the differences in survival rates between high and low hub gene expression groups.

Conventional PCR and Sanger sequencing

This study utilized conventional PCR to target Exon 13 of MMP2 and MMP14 genes to detect genetic mutations, if present. The primer pairs used for both genes were sourced from previous studies cited in the medical literature [45, 46]. An initial DNA amount of 100 ng obtained from 20 KIRC patients underwent PCR amplification using the optimized cyclic conditions mentioned in the cited studies. Thermo Scientific 2X PCR master mix (lot No. 00097068) was used for amplification. Subsequently, the PCR products were sent to Macrogen Company, Korea for bidirectional Sanger sequencing analysis.

Statistics details

For enrichment analysis, we used Fisher's Exact test for computing statistical difference [47]. Correlational analyses were carried out using Pearson method. For comparisons, a student t-test was adopted in the current study. All the analyses were carried out in R version 3.6.3 software.

Results

PPI network, hub genes, and profiling of mRNA and protein expression

Initially, a PPI network analysis of 24 MMP family members was performed using STRING, followed by visualization in Cytoscape (**Figure 1A**). Utilizing CytoHubba analysis, four key hub genes, including matrix metalloproteinase 2 (MMP2), matrix metalloproteinase 9 (MMP9), matrix metalloproteinase 14 (MMP14), and matrix metalloproteinase 16 (MMP16) were identified using the degree method (**Figure 1B**). Subsequently, the expression of these hub genes was analyzed at both mRNA and protein levels across the TCGA KIRC dataset through UALCAN. The findings demonstrated significant up-regulation ($P < 0.05$) of all hub genes in KIRC samples compared to controls (**Figure 1C**, **1D**), indicating their potential involvement in the pathogenesis of KIRC.

Additional validation of hub gene expression

Following the initial expression analysis of MMP2, MMP9, MMP14, and MMP16 genes in TCGA KIRC dataset through UALCAN (**Figure 1C**, **1D**), further validation was conducted using additional datasets from muTarget and GEPIA web sources. Remarkably, these independent datasets consistently revealed higher expression levels of the hub genes (MMP2, MMP9, MMP14, and MMP16) in KIRC samples compared to controls (**Figure 2A**, **2B**). The consistent up-regulation across multiple datasets strengthens the evidence supporting the pivotal role of MMP2, MMP9, MMP14, and MMP16 in KIRC. In the subsequent stage of the study, expression analysis of MMP2, MMP9, MMP14, and MMP16 genes was conducted across KIRC samples stratified into different cancer stages. The results revealed significant variations in the expression levels of these genes among KIRC patients at different cancer stages (**Figure 2C**). These findings suggest the potential utility of MMP2, MMP9, MMP14, and MMP16 genes as stage-specific biomarkers.

Survival analysis and development of hub genes-based prognostic model

In the next phase of the study, survival analysis of MMP2, MMP9, MMP14, and MMP16 genes was conducted using the KM plotter. The results demonstrated a significant association between higher expression levels of MMP2, MMP9, MMP14, and MMP16 genes and poor survival outcomes in KIRC patients (**Figure 3A**).

For constructing a prognostic model based on the MMP2, MMP9, MMP14, and MMP16 genes, we employed the TCGA_KIRC dataset as the training dataset, and the GSE22541, GSE167573, and E_MTAB_1980 datasets as validation datasets. Prognostic model was constructed using a stepwise Cox regression approach, which integrated hazard ratio, c-index, and risk score parameters. To assess the predictive performance of our model, we used the c-index and found it to be effective and robust in evaluating the prognosis of patients with KIRC (**Figure 3B**). These results suggest that MMP2, MMP9, MMP14, and MMP16 have the potential to serve as prognostic biomarkers for predicting patient survival in KIRC.

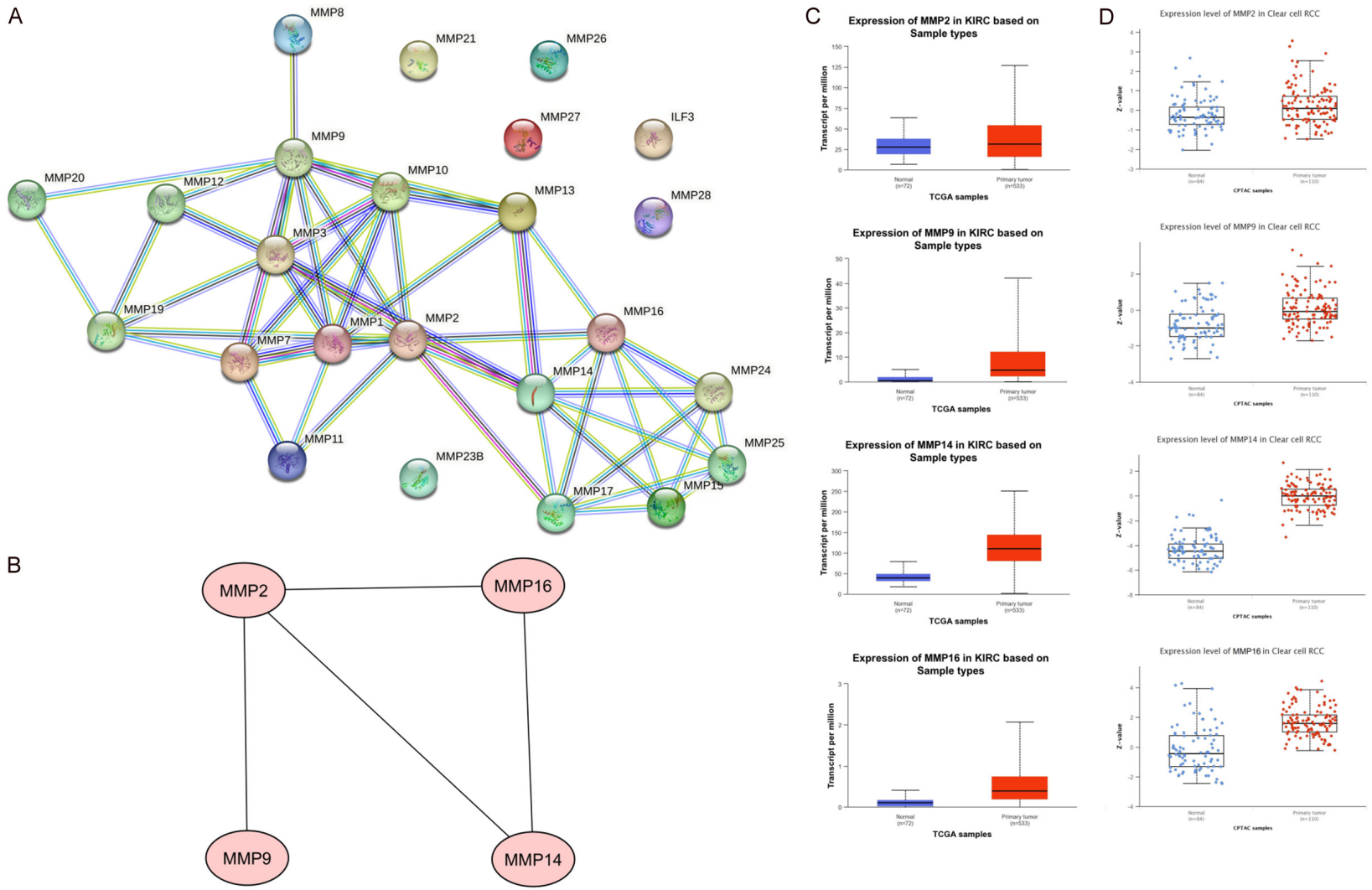

Figure 1. A PPI network of MMP family members, identification of hub genes based on the degree method, and expression profiling of hub genes in KIRC and normal control samples via UALCAN. (A) A PPI network of the MMP family members, (B) A PPI of the identified hub genes based on the degree method, (C) mRNA expression profiling of hub genes in KIRC and normal control samples via UALCAN, and (D) Protein expression profiling of hub genes in KIRC and normal control samples via UALCAN. A *p*-value < 0.05 was considered significant. PPI = Protein-protein interaction, MMP = Matrix metalloproteinase, KIRC = Kidney renal clear cell carcinoma.

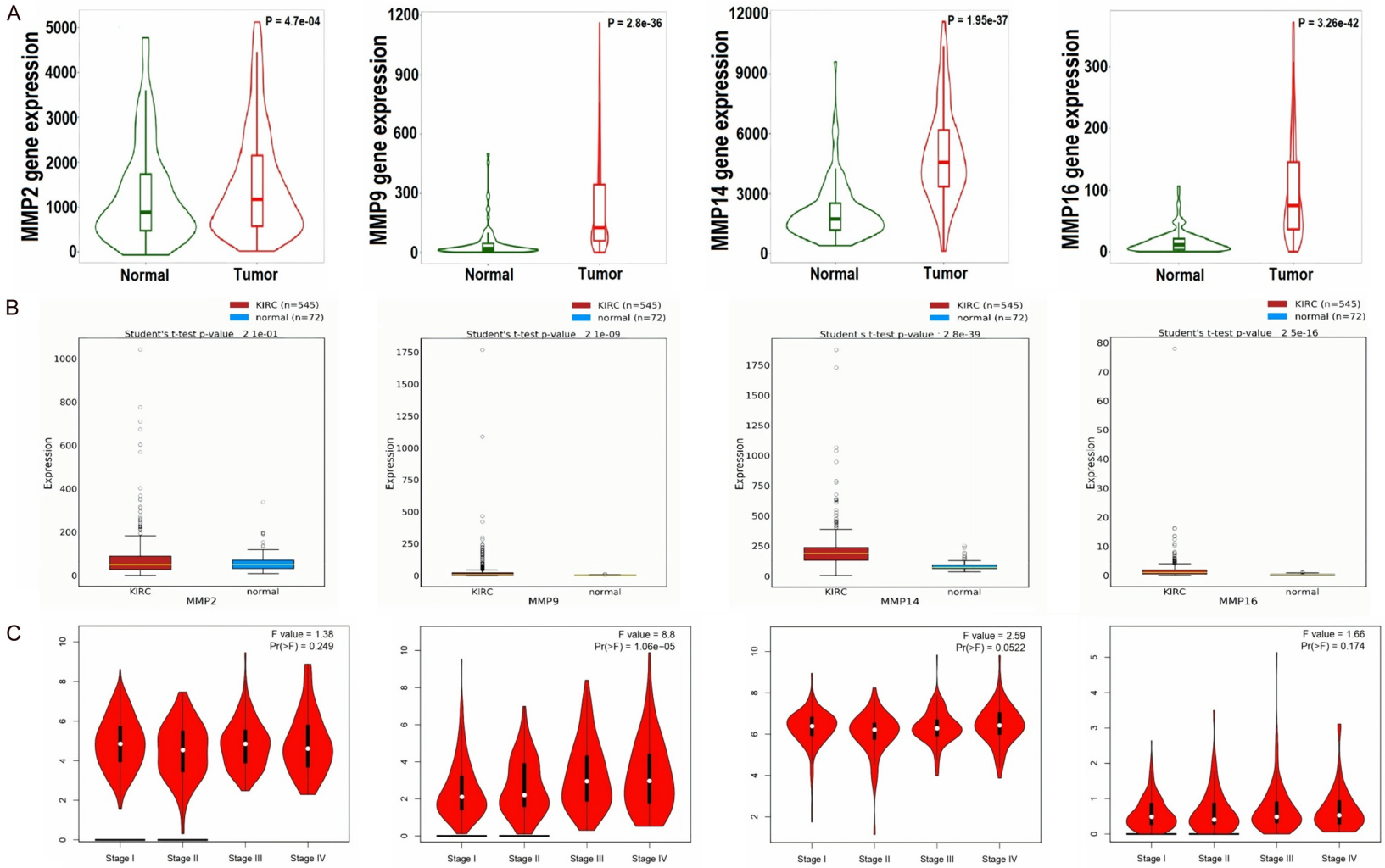

Figure 2. Expression analysis of hub genes on additional TCGA datasets using muTarget and GEPIA database. (A) Expression profiling of hub genes using muTarget database, (B) Expression profiling of hub genes using GEPIA database, and (C) Expression profiling of hub genes across KIRC samples stratified based on different cancer stages. A *p*-value < 0.05 was considered significant. TCGA = The Cancer Genome Atlas, KIRC = Kidney renal clear cell carcinoma.

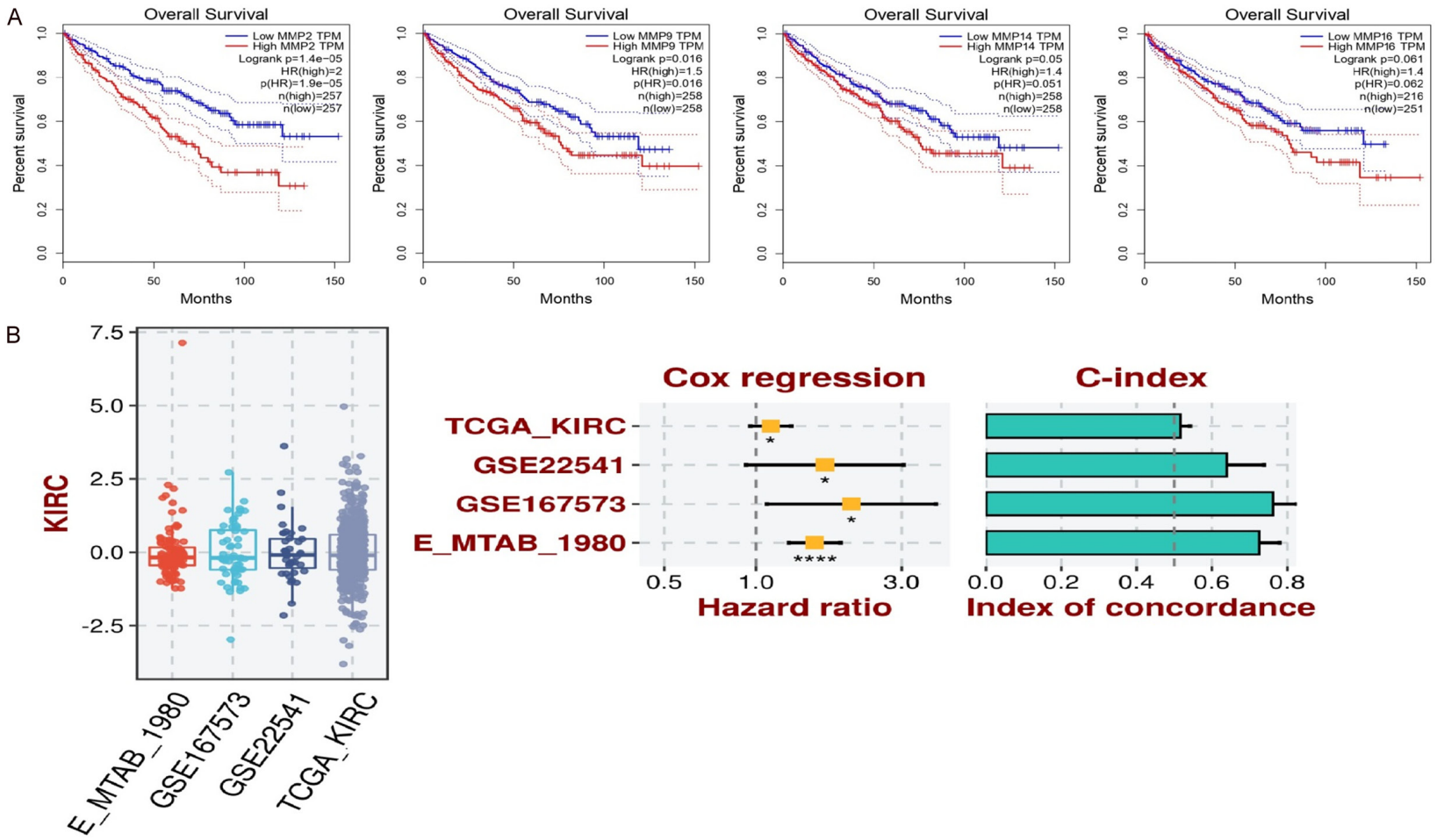

Figure 3. Survival analysis and construction of hub gene-based prognostic model for KIRC patients. (A) Survival analysis of hub genes via KM plotter tool, and (B) Univariate Cox regression analysis, c-index scores, and risk scores. A *p*-value < 0.05 was considered significant. KIRC = Kidney renal clear cell carcinoma.

Subcellular localization and mutational analysis

Using subcellular localization predicating feature of the HPA database, we predicted subcellular localization of MMP2, MMP9, MMP14, and MMP16 proteins. MMP2 protein was detected in vesicles, MMP9 protein was detected in cytosol, MMP14 protein was found in cytosol and intermediate filaments, and MMP16 protein was present in cytosol and vesicles (**Figure 4A**).

We conducted mutational analysis of the MMP2, MMP9, MMP14, and MMP16 genes using cBioPortal. The results revealed that among the hub genes, MMP2 and MMP14 were found to be mutated in a small subset of KIRC samples. Specifically, these mutations were observed in only 3 out of the 366 samples analyzed, accounting for approximately 0.89% of the total samples (**Figure 4B**). Notably, the most common type of mutation observed in both MMP2 and MMP14 was a missense mutation, where cytosine (C) was replaced by thymine (T) in the DNA sequence (**Figure 4C**).

Analyzing promoter methylation level of hub genes using TCGA dataset

A promoter methylation analysis of hub genes (MMP2, MMP9, MMP14, and MMP16) was conducted in KIRC and normal control samples using data from the OncoDB database. The results revealed a significant hypomethylation pattern in KIRC samples when compared to normal controls for all four genes (**Figure 5**). Hypomethylation of gene promoters often correlates with increased gene expression, potentially leading to altered cellular processes associated with cancer progression. These findings suggest that the deregulation of these matrix metalloproteinase genes through hypomethylation might play a role in KIRC development and progression.

Functional enrichment analysis

In this study functional enrichment of MMP2, MMP9, MMP14, and MMP16 genes was conducted with the help of GSEA program. GO analysis highlighted that hub genes were significantly involved in “pinosome, macropinosome, and Golgi lumen”, etc., CC terms (**Figure 6A**), “metallopeptidase activity, expopeptidase activity, and collagen binding”, etc., MF terms (**Figure 6B**), and “collagen metabolic process, endoderm development, cranial skeletal development system”, etc., MF term (**Figure 6C**). Moreover, KEGG analysis further revealed invlovment of these genes in “Matrix metalloprotienases, cell migration and invasion through p75NTR, and neural crest cell migration during development”, etc., cellular pathways (**Figure 6D**).

miRNA network and immune cell infiltration analyses

In this study, the role of miRNAs in regulating the expression of MMP2, MMP9, MMP14, and MMP16 hub genes was investigated using the ENCORI database. The analysis identified a total of 142 miRNAs that were predicted to target these hub genes (**Figure 7A**). Remarkably, one specific miRNA, hsa-mir-218-5P, emerged as the central hub miRNA, as it was found to target all four hub genes simultaneously (**Figure 7B**). This finding suggests that hsa-mir-218-5P may play a crucial role in the coordinated regulation of MMP2, MMP9, MMP14, and MMP16 expression.

Furthermore, the gene expression levels of MMP2, MMP9, MMP14, and MMP16 were investigated in relation to the infiltration levels of immune cells, including CD4+ T cells, CD8+ T cells, and Macrophages, using the TIMER database. The analysis revealed a significant positive correlation between the expression of the hub genes and the levels of infiltrating CD4+ T cells, CD8+ T cells, and Macrophages in the tumor microenvironment (**Figure 7C**). This suggests that higher expression of MMP2, MMP9, MMP14, and MMP16 in the tumor is associated with increased infiltration of these immune cells.

RT-qPCR-based validation of MMP2, MMP9, MMP14, and MMP16 gene expression across KIRC cell lines

To validate the expression of hub genes across KIRC cell lines, a total of three cell lines (ACHN, Caki-3, and OS-RC-2) derived from the primary tumor site, two cell lines (A-704 and 769-P) obtained from metastatic tumors, and one normal renal tubular epithelial cell line (HKC-8) were procured from ATCC and cultured following the manufacturer’s instructions. The cDNA

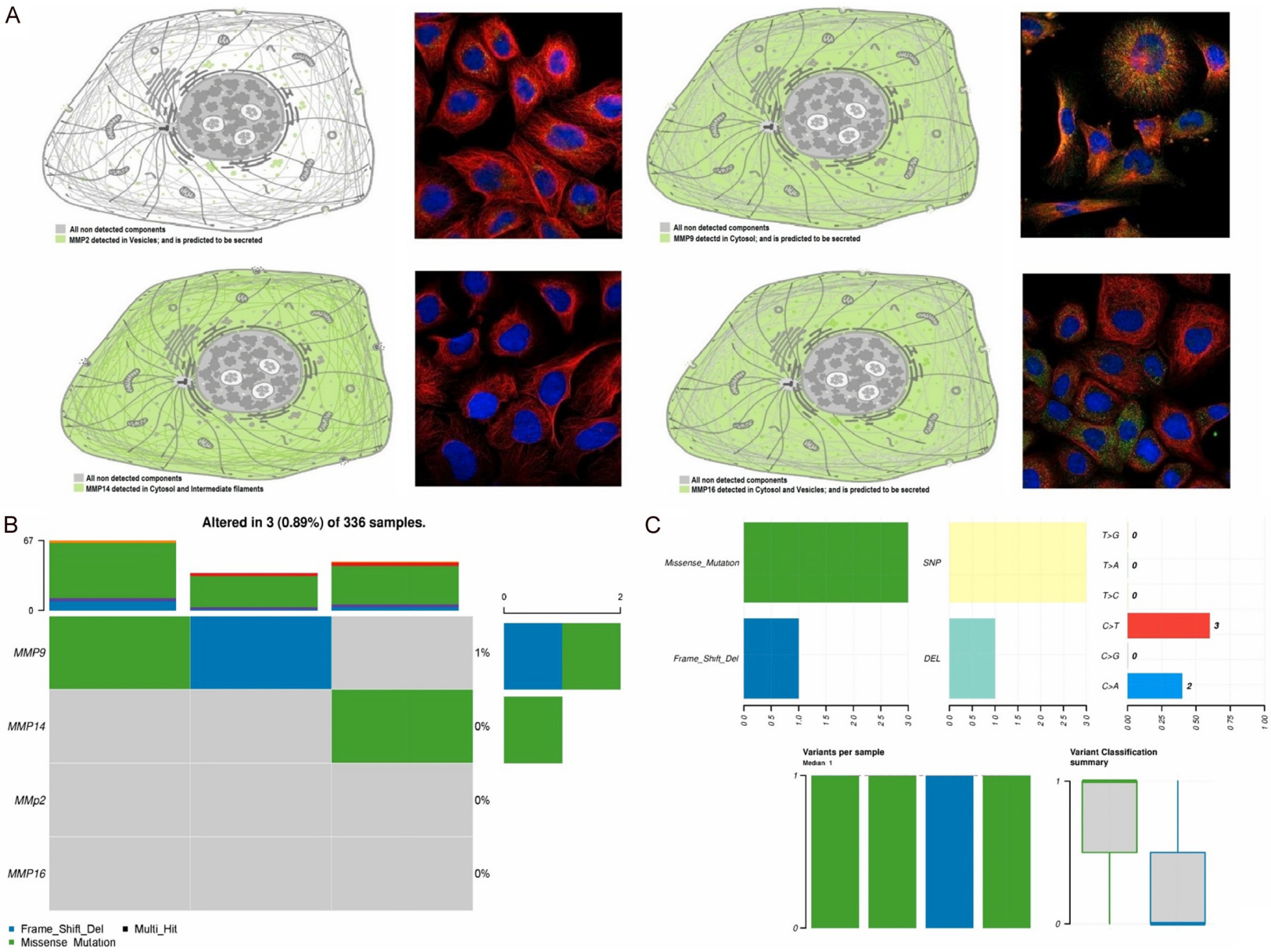

Figure 4. Subcellular localization and mutational profiling of hub genes in KIRC patients via cBioPortal. (A) Predicted subcellular localization of hub genes via HPA database, and (B, C) Summery and detail of the detected mutations in KIRC samples via cBioPortal. HPA = Human Protein Atlas, KIRC = Kidney renal clear cell carcinoma.

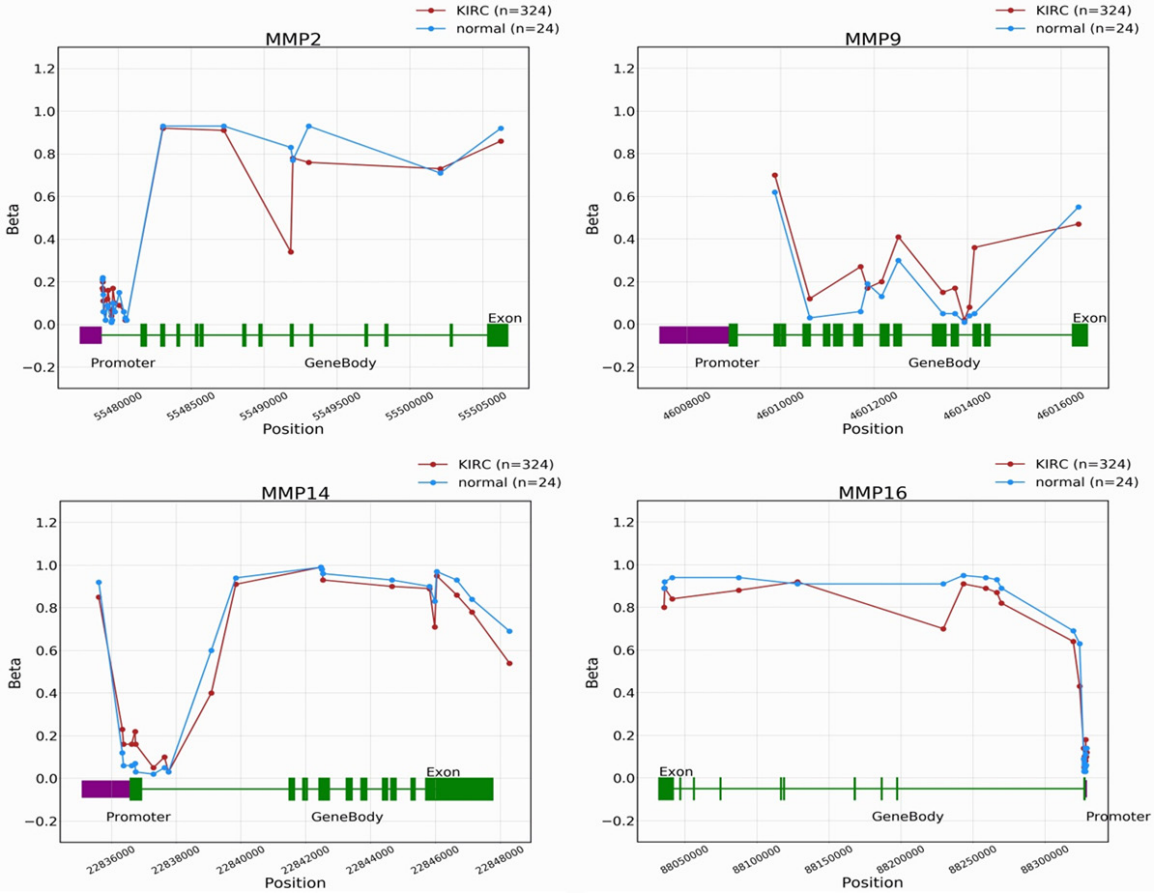

Figure 5. Promoter methylation analysis of hub genes in KIRC and normal control samples via OncoDB database. A *p*-value < 0.05 was considered significant. KIRC = Kidney renal clear cell carcinoma.

from these cell lines was subjected to RT-qPCR analysis to quantify the expression levels of MMP2, MMP9, MMP14, and MMP16 genes. The results indicated that the expression of MMP2, MMP9, MMP14, and MMP16 was significantly higher in KIRC cell lines compared to the control cell line (**Figure 8A**). Notably, the expression of these genes was particularly elevated in KIRC cell lines derived from metastatic tumors (A-704 and 769-P) compared to those obtained from the primary tumor site (ACHN, Caki-3, and OS-RC-2) (**Figure 8A**). These findings suggest that MMP2, MMP9, MMP14, and MMP16 may play a prominent role in KIRC progression, with their expression levels potentially associated with the metastatic potential of the tumor cells.

RT-qPCR-based validation of MMP2, MMP9, MMP14, and MMP16 gene expression across clinical KIRC samples via RT-qPCR

To further validate the expression of MMP2, MMP9, MMP14, and MMP16 genes, we conducted RT-qPCR analysis using cDNA from both KIRC tissue samples and control tissue samples. The results, presented in **Figure 8B**, revealed a remarkable up-regulation of MMP2, MMP9, MMP14, and MMP16 expression in the KIRC sample group (n = 20) compared to the control group (n = 20, *p*-value < 0.05). Furthermore, we generated ROC curves to assess the diagnostic potential of these genes. The ROC curves for MMP2 (AUC: 0.899, *p*-value < 0.05), MMP9 (AUC: 0.879, *p*-value < 0.05), MMP14 (AUC: 0.919, *p*-value < 0.05), and MMP16 (AUC: 0.990, *p*-value < 0.05) demonstrated excellent diagnostic accuracy, sensitivity, and specificity based on their expression levels, as depicted in **Figure 8C**.

Validation of the potential of MMP2, MMP9, MMP14, and MMP16 genes in predicting the OS of KIRC patients

Based on the KM survival curves, a notable association was observed between the expression levels of MMP2, MMP9, MMP14, and

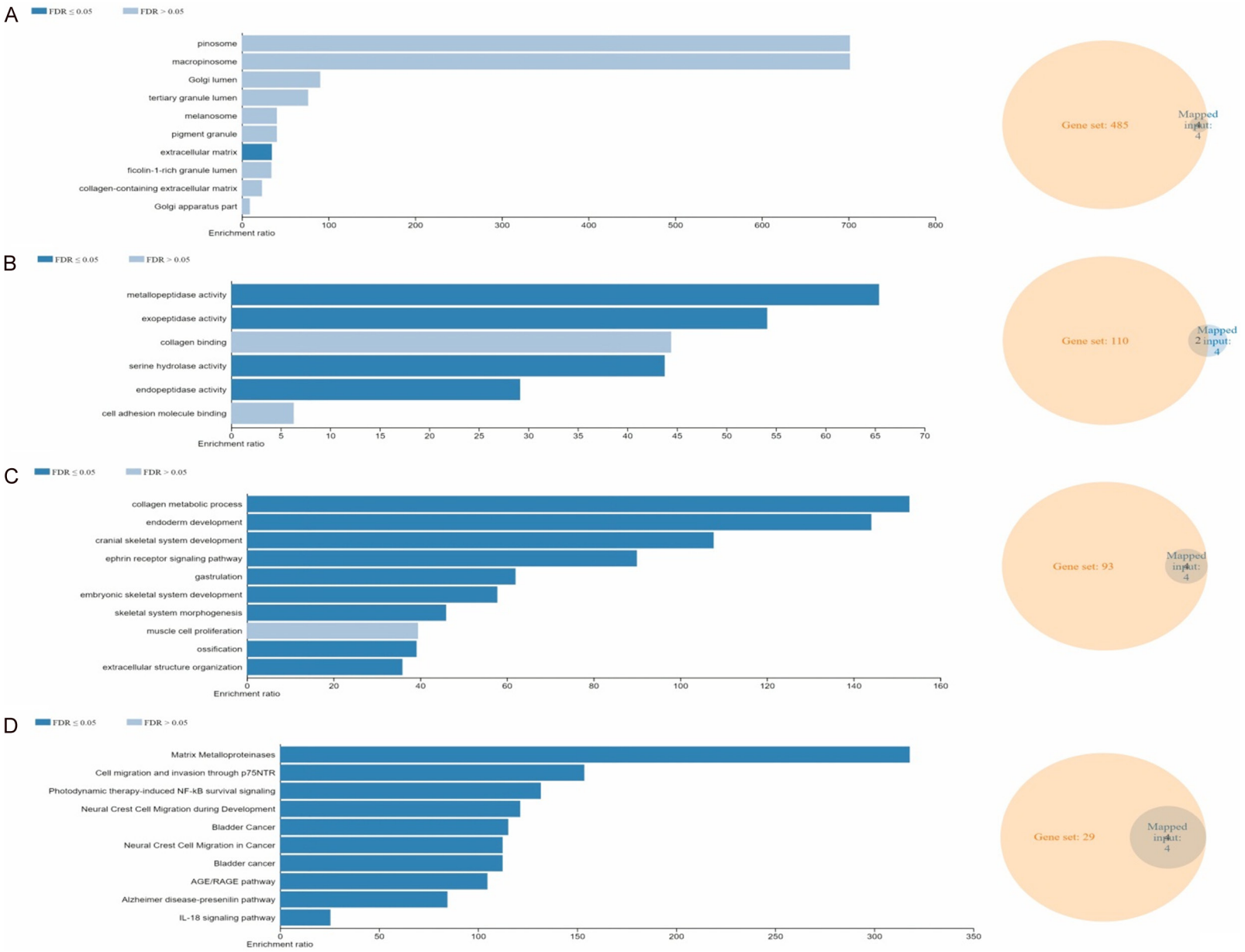

Figure 6. Gene enrichment analysis of hub genes via GSEA program. (A) Hub genes-associated CC terms, (B) Hub genes-associated BP terms, (C) Hub genes-associated MF terms, and (D) Hub gene-associated KEGG terms. A *p*-value < 0.05 was considered significant. CC = Cellular Component, BP = Biological Process, MF = Molecular Function, KEGG = Kyoto Encyclopedia of Genes and Genomes.

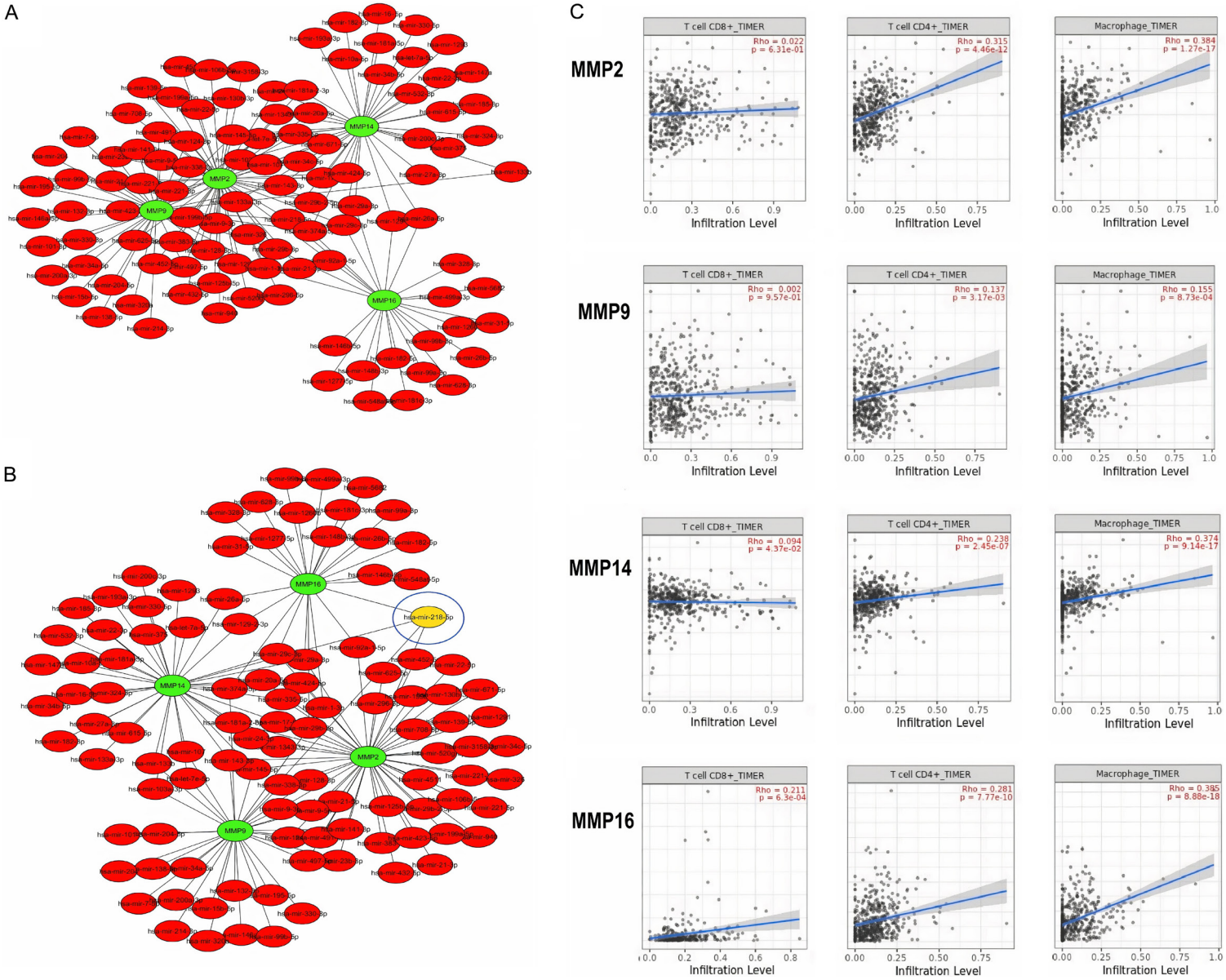

Figure 7. miRNA prediction and immune cell infiltration analyses of hub genes in KIRC samples. (A) Using ENCORI, a constructed PPI network of miRNAs targeting all hub genes, (B) A constructed PPI network of miRNAs and hub genes with a focus of highlighting hub miRNA (hsa-mir-218-5p), and (C) TIMER-based correlation analysis of hub genes expression and infiltration levels of CD8+ T cells, CD4+ T cells, and Macrophages across KIRC samples. A *p*-value < 0.05 was considered significant. miRNA = MicroRNA, PPI = Protein-protein interaction, KIRC = Kidney renal clear cell carcinoma.

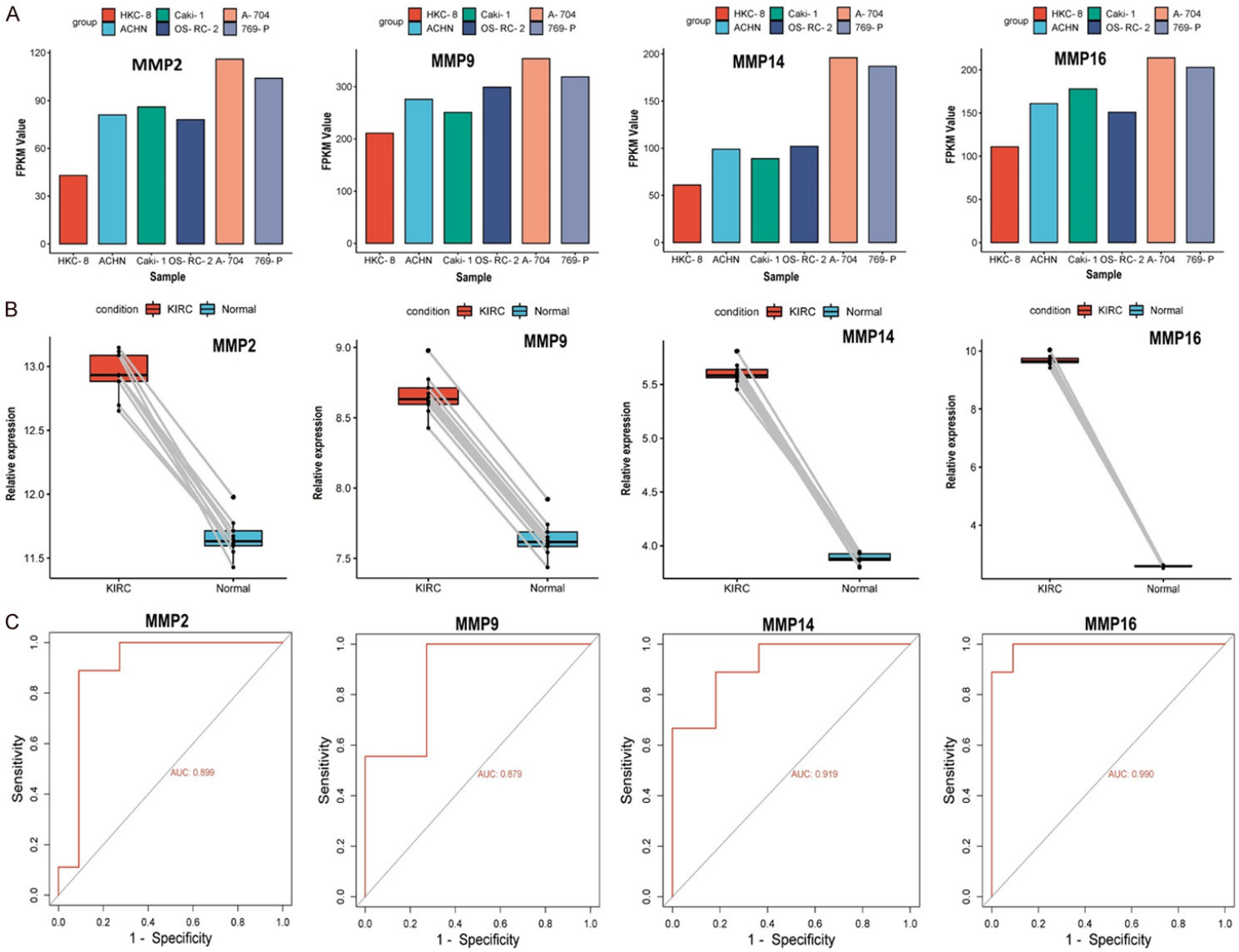

Figure 8. Expression profiling of hub genes using KIRC cell lines, clinical tissue samples paired with control samples, and ROC curve analysis. (A) Expression profiling of hub genes using KIRC cell lines purchased from the ATCC, (B) RT-qPCR-based expression profiling of hub genes using clinical tissue samples and control samples obtained from the Pakistani KIRC patients, and (C) RT-qPCR expression level-based ROC curves of hub genes. A *p*-value < 0.05 was considered significant. KIRC = Kidney renal clear cell carcinoma, ROC = receiver operating curve, RT-qPCR = reverse transcription quantitative real-time PCR.

MMP16 genes and the OS of the Pakistani KIRC patients (n = 20). The analysis revealed that KIRC patients with higher expression of MMP2, MMP9, MMP14, and MMP16 exhibited significantly lower OS compared to those with low expression levels of these genes (**Figure 9A**). The observed correlation between gene expression and OS highlights the clinical relevance of MMP2, MMP9, MMP14, and MMP16 as potential prognostic biomarkers for predicting KIRC patient survival.

Targeted bisulfite sequencing analysis to analyze promoter methylation levels of MMP2, MMP9, MMP14, and MMP16 in clinical KIRC samples

To evaluate the level of promoter methylation in the hub genes MMP2, MMP9, MMP14, and MMP16 among clinical KIRC samples, we recruited a cohort comprising 20 individuals diagnosed with KIRC, alongside 20 healthy individuals from the Pakistani population serving as controls. The bisulfite conversion efficiency, which is crucial for accurate methylation analysis, was remarkably high, exceeding 99.1%, in both the KIRC and control groups. Additionally, there were no significant differences in the read mapping rate between the two groups. Rigorous quality control measures were applied, and as a result, all 20 samples from the KIRC group and 20 samples from the control group were deemed suitable for further analysis.

Our analysis revealed a significant hypomethylation pattern in all candidate genes (MMP2, MMP9, MMP14, and MMP16) within the KIRC samples compared to the control group (**Figure 9B**). Moreover, the ROC curves generated for MMP2 (AUC: 0.789, p-value < 0.05), MMP9 (AUC: 0.802, p-value < 0.05), MMP14 (AUC: 0.806, p-value < 0.05), and MMP16 (AUC: 0.803, p-value < 0.05) based on their methylation levels (**Figure 9C**) displayed excellent discriminatory power. Additionally, these ROC curves exhibited remarkable sensitivity and specificity in effectively distinguishing between KIRC and control samples (**Figure 9C**).

Mutational analysis through conventional PCR and Sanger sequencing

Upon conducting a comprehensive mutational analysis of hub genes using TCGA datasets, a noteworthy revelation emerged. Specifically, among the samples examined, mutations were exclusively observed in MMP9 and MMP14 genes. Consequently, a subsequent inquiry was undertaken to scrutinize the mutation status of these genes within clinical samples obtained from KIRC patients. To achieve this, conventional PCR and Sanger sequencing techniques were employed to probe for mutations in 20 clinical KIRC samples. However, the findings yielded an unexpected outcome, as no mutations were detected in either of these genes across the scrutinized samples. This intriguing observation implies that the mutational profile of MMP9 and MMP14 genes may not exert a significant influence on the occurrence of KIRC.

Drug prediction analysis

This study employed the DrugBank database to investigate potential therapeutic drugs for KIRC, with a specific focus on the identified hub genes (MMP2, MMP9, MMP14, and MMP16) as promising targets for treatment. Significantly, our exploration led to the identification of seven noteworthy drugs, Calcitriol, Fluvastatin, Estradiol, Dronabinol, Heparin, Paclitaxel, and Vorinostat (**Table 1**), which hold promise as suitable treatment options for KIRC based on their potential interactions with the identified hub genes.

Discussion

KIRC exhibits biological heterogeneity and variable clinical outcomes, underscoring the importance of unraveling its molecular mechanisms for improved diagnosis, prognosis, and treatment. In this study, we focused on the MMP family's PPI network to identify key hub genes in KIRC patients. Among the MMP family, MMP2, MMP9, MMP14, and MMP16 emerged as significant hub genes in KIRC patients. Our expression analysis, utilizing TCGA datasets, cell lines, and clinical samples from KIRC patients, revealed a consistent up-regulation of MMP2, MMP9, MMP14, and MMP16 genes in KIRC samples compared to their normal counterparts. Furthermore, survival analysis demonstrated that KIRC patients with higher expression levels of MMP2, MMP9, MMP14, and MMP16 experienced worse overall survival outcomes.

The MMP2 gene, encoding matrix metalloproteinase 2 or gelatinase A, assumes a pivotal role in both physiological and pathological con-

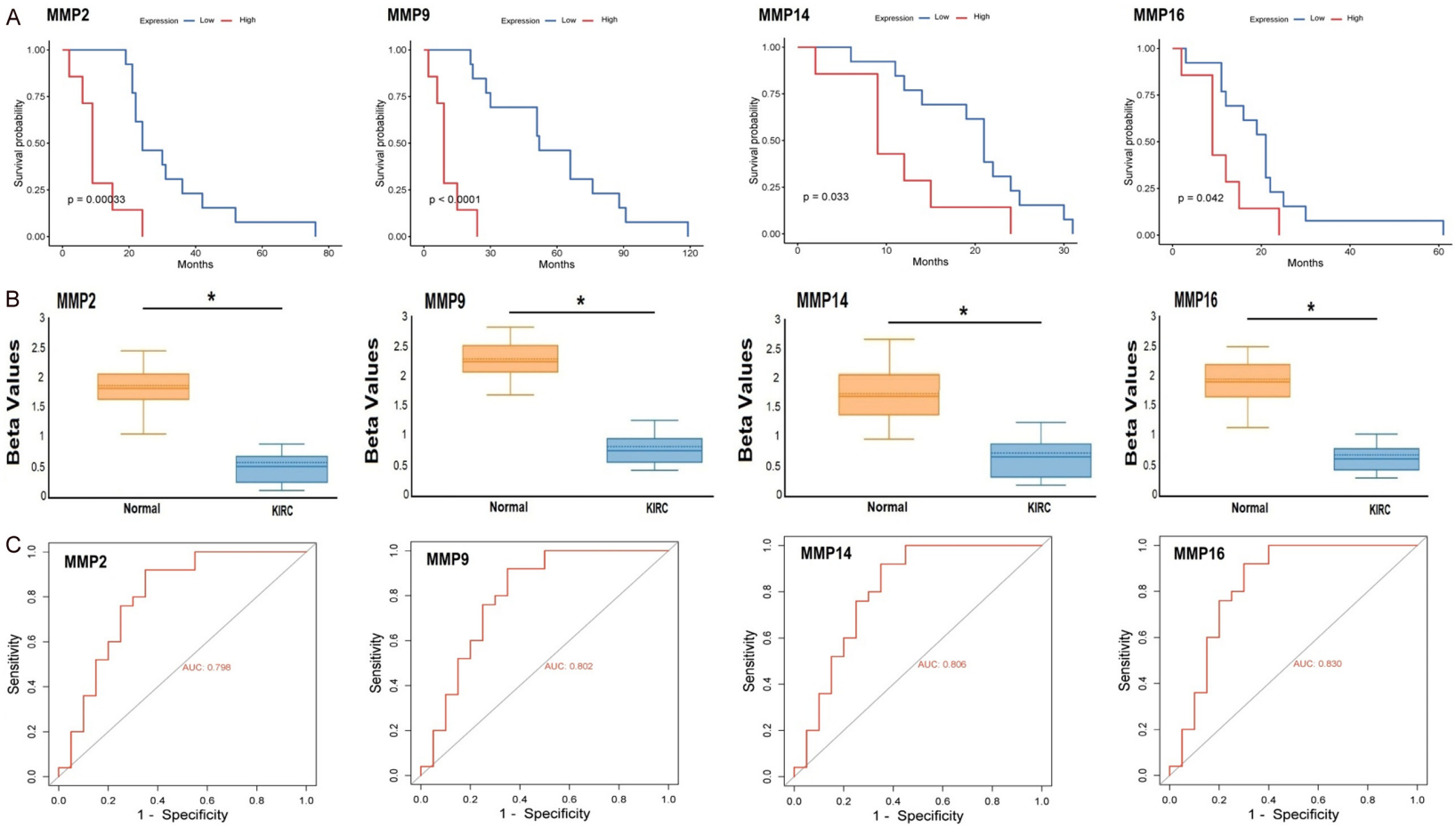

Figure 9. KM method based survival, targeted bisulfite-seq, and ROC curve analyses of hub genes. (A) KM curves based on the survival information of the Pakistani KIRC patients, (B) Targeted bisulfite-seq promoter methylation analysis of hub genes in clinical KIRC samples, and (C) Targeted bisulfite-seq promoter methylation level-based ROC curves of hub genes. A *p*-value < 0.05 was considered significant. KM = Kaplan-Meier, ROC = receiver operating curve, Targeted bisulfite-seq = Targeted bisulfite sequencing, KIRC = Kidney renal clear cell carcinoma.

Table 1. DrugBank-based MMP2, MMP9, MMP14, and hub genes-associated drugs

Sr. No	Hub gene	Drug name	Effect	Reference	Group
1	MMP2	Calcitriol	Decrease expression of MMP2 mRNA	A22300	Approved
		Fluvastatin		A22695	
2	MMP9	Estradiol	Decrease expression of MMP9 mRNA	A21329	Approved
		Dronabinol		A22085	
3	MMP14	Heparin	Decrease expression of MMP14 mRNA	A22885	Approved
4	MMP16	Paclitaxel	Decrease expression of MMP16 mRNA	A23553	Approved
		Vorinostat		A21035	

MMP2 = matrix metallopeptidase 2, MMP9 = matrix metallopeptidase 9, MMP14 = matrix metallopeptidase 14, MMP16 = matrix metallopeptidase 16, mRNA = Messenger RNA.

texts [48]. It belongs to the matrix metalloproteinase family, responsible for extracellular matrix component degradation. While MMP2 contributes to tissue remodeling, wound healing, and angiogenesis under normal conditions, its dysregulation has been prominently associated with human cancers [49]. Dysregulated MMP2 exerts a profound impact on cancer progression by fostering tumor invasion, metastasis, and angiogenesis [50]. Elevated MMP2 expression in tumor microenvironments leads to extracellular matrix degradation, enabling cancer cells to infiltrate surrounding tissues and metastasize to distant sites [51]. Additionally, MMP2's involvement in angiogenesis is crucial for tumor vascularization, as it breaks down barriers for new blood vessel formation, supporting the metabolic demands of proliferating cancer cells. Elevated MMP2 expression was notably found to be associated with tumor invasion, metastasis, and angiogenesis in various cancers such as breast [52], lung [53], colorectal [54], gastric [55], and ovarian cancer [56], influencing their aggressiveness and clinical outcomes.

Matrix metalloproteinase 9 (MMP9) is a crucial player in tissue remodeling and immune responses [57], yet its dysregulation has marked implications in human cancers. MMP9's primary function involves breaking down extracellular matrix components, a process crucial for tumor invasion, metastasis, and angiogenesis [58]. Its overexpression has been notably associated with various cancers including breast cancer [59, 60], lung cancer [53], colorectal cancer [61], gastric cancer [62], and brain tumors [63]. In these malignancies, heightened MMP9 levels often correlate with advanced disease stages, lymph node metastasis, and reduced survival rates. Clinical studies are exploring MMP9 inhibitors to counteract its influence on tumor aggressiveness and metastatic potential. However, the intricate balance of MMP-related functions necessitates careful consideration of potential side effects and complexities in therapeutic interventions.

Matrix metalloproteinase 14 (MMP14), also known as membrane-type 1 matrix metalloproteinase (MT1-MMP), is a critical enzyme in physiological tissue remodeling and pathological processes [64]. As a transmembrane protease, MMP14 primarily regulates pericellular matrix remodeling, facilitating tumor invasion and metastasis [65]. Its ability to activate other MMPs enhances extracellular matrix degradation and promotes angiogenesis, critical for tumor growth. Elevated MMP14 expression is associated with advanced disease stages, increased invasiveness, and metastasis in various cancers including breast cancer [66], lung cancer [67], colorectal cancer [68], and pancreatic cancer [69]. Furthermore, MMP14's role in creating a conducive microenvironment for cancer cell migration and invasion highlights its significance in the metastatic cascade [65].

Matrix metalloproteinase 16 (MMP16), also known as membrane-type 3 matrix metalloproteinase (MT3-MMP), plays a crucial role in both physiological and pathological contexts [70]. Its dysregulation has been associated with human cancer progression. MMP16 is a transmembrane enzyme that participates in pericellular matrix remodeling and influences tumor invasion and metastasis [71]. By degrading extracellular matrix components and modulating cell adhesion, MMP16 contributes to the invasive potential of cancer cells [72]. Elevated MMP16 expression has been linked to advanced stages of cancer, increased invasiveness, and metas-

tasis in several malignancies, including breast cancer [73], ovarian cancer [74], and lung cancer [43].

Earlier, mutations in MMP2 have been associated with increased tumor invasion and metastasis potential in breast cancer [75]. In MMP9, genetic alterations have been linked to enhanced angiogenesis and invasive properties in pancreatic and gastric cancers [76]. MMP14 mutations have been identified in various cancer types, leading to increased proteolytic activity and higher invasive behavior [77]. In MMP16, mutations have been found to influence cell adhesion and migration in glioblastoma and colorectal cancer [78]. In this investigation, it was observed that among the analyzed 336 KIRC samples, only three samples exhibited mutations in the MMP9 and MMP14 genes. This observation highlights a relatively low frequency of mutations in these hub genes within the context of KIRC samples.

The findings of this study, that hsa-mir-218-5p targets all four hub genes (MMP2, MMP9, MMP14, and MMP16) simultaneously suggests a pivotal role of this miRNA in modulating critical pathways involved in tumor progression. This regulatory convergence amplifies its impact, potentially influencing various stages of cancer development, including invasion, metastasis, and angiogenesis. Previous studies suggested that hsa-mir-218-5p down-regulated in various cancer types, such as breast cancer [79], lung cancer [80], and colorectal cancer [81], signifies its potential tumor-suppressive function. The perturbation in hsa-mir-218-5p levels could potentially contribute to the overexpression of MMP2, MMP9, MMP14, and MMP16 hub genes, thereby playing a role in the progression of KIRC.

As per the findings from TIMER analysis, a significant correlation emerges between the expression levels of MMP2, MMP9, MMP14, and MMP16 and the presence of key immune cells (CD8+ T cells, CD4+ T cells, and Macrophages) within the tumor microenvironment. These immune cell types, namely CD8+ T cells, CD4+ T cells, and Macrophages, hold pivotal roles in countering tumor growth by effectively targeting malignant cells. However, it is important to note that when these immune cells transition into a dysfunctional state, they might lose their capacity to respond to immunotherapeutic interventions [82, 83]. This intricate interplay suggests that the heightened expression of MMP2, MMP9, MMP14, and MMP16 could potentially contribute to the heightened aggressiveness of KIRC and foster resistance to immunotherapy. By potentially influencing the activity and performance of CD8+ T cells, CD4+ T cells, and Macrophages, the overexpression of these genes may foster an immunosuppressive milieu, thereby obstructing the efficacy of immune-mediated treatments.

Conclusion

In this current investigation, we identified four hub genes (MMP2, MMP9, MMP14, and MMP16) from the matrix metalloproteinase (MMP) family that displayed elevated expression levels, thus emerging as prospective candidates for diagnostic or prognostic biomarkers of KIRC. These genes had not been previously linked with KIRC, but our study offers compelling evidence highlighting their strong association with the disease. Nonetheless, additional experiments need to be conducted to verify the participation of the identified hub genes in KIRC.

Disclosure of conflict of interest

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