Aus der Klinik für Urologie
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DISSERTATION

The function of MMP-14 in bladder cancer

Die Funktion von MMP-14 bei Blasenkrebs

zur Erlangung des akademischen Grades
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von

Shuai Zhu

aus Hunan, China

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List of Abbreviations

Abbreviation	Full name
ACC	Adrenocortical carcinoma
Ag	Antigen
APC	Antigen-presenting cell
APS	Ammonium persulfate
BCG	Bacillus Calmette-Guérin
BH	Benjamini and Hochberg
BMI	Body mass index
BLCA	Bladder urothelial carcinoma
BRCA	Breast cancer
BXXX	Banxia xiexin decoction
CAR-T	Chimeric antigen receptor T cell
CDK5	Cyclin-dependent kinase 5
CESC	Cervical cancer
CHOL	Cholangiocarcinoma
CI	Confidence interval
COAD	Colon cancer
CR	Complete response
CT	Computed tomography
DDR1	Discoidin Domain Receptor 1
DEGs	Differentially expressed genes
DLBC	Diffuse large B lymphoma
ECM	Extracellular matrix
EGF	Epidermal growth factor

ERGs	expression-related genes
ESCA	Esophageal cancer
FBS	Fetal bovine serum
FC	Fold change
FDA	Food and Drug Administration
FDR	False discovery rate
FGF	Fibroblast growth factor
GBM	Glioblastoma multiforme
GEPIA	Gene Expression Profiling Interactive Analysis
GO	Gene Ontology
GSEA	Gene Set Enrichment Analysis
HIFs	Hypoxia-inducible factors
HNSC	Head, and neck squamous cell carcinoma
HR	Hazard ratios
IC50	half-inhibitory concentrations
IFN	Interferon
IL	Interleukin
JAK	Janus kinase
KD	Knockdown
KEGG	Kyoto Encyclopedia of Genes and Genomes
KICH	Chromophobe renal cell carcinoma
KIRC	Clear renal cell carcinoma
KIRP	Papillary renal cell carcinoma
LAML	Acute myeloid leukemia
LGG	Low-grade glioma
LIHC	Liver hepatocellular carcinoma

LUAD	Lung adenocarcinoma
LUSC	Lung squamous cell carcinoma
MCODE	Molecular Complex Detection
MESO	Mesothelioma
MHC	Major histocompatibility complex
MIBC	Muscle-invasive bladder cancer
MMP	Matrix metalloproteinase
MRI	Magnetic resonance imaging
NEAA	Non-Essential Amino Acids
NK	Natural killer
NMIBC	Non-muscle invasive bladder cancer
NSCLC	Non-Small Cell Lung Cancer
OV	Ovarian cancer
PAAD	Pancreatic adenocarcinoma
PD-1	Programmed Cell Death Protein 1
PD-L1	Programmed Cell Death 1 Ligand 1
PCPG	Pheochromocytoma and paraganglioma
PD	Progressive disease
PR	Partial response
PRAD	Prostate adenocarcinoma
READ	Rectal adenocarcinoma
RTK	Receptor tyrosine kinase
SARC	Sarcoma
SD	Stable disease
SH2	Src-homology-2 domain
SKCM	Skin cutaneous melanoma

ssGSEA	Single sample gene set enrichment analysis
STAD	Stomach adenocarcinoma
STAT	Signal transducers and activators of transcription
TCGA	The Cancer Genome Atlas
TCR	T cell receptor
TGCT	Testicular germ cell tumor
TGF-β	Transforming growth factor beta
THCA	Thyroid carcinoma
THYM	Thymoma
TIMER	Tumor IMmune Estimation Resource
TLR	Toll-like receptor
TNM	Tumor, Node, Metastasis
TNF-α	Tumor necrosis factor alpha
Tregs	T regulatory cells
Tris-HCl	Tris(hydroxymethyl)-aminomethane-hydrochloride
TURBT	Transurethral resection of bladder tumor
UCEC	Uterine corpus endometrial carcinoma
UCS	Uterine carcinosarcoma
UVM	Uveal melanoma
3'-UTR	Three prime untranslated region

Abstract

Background: As one of the matrix metalloproteinases, MMP-14 is involved in the degradation of the extracellular matrix. In this study, we aim to explore the function of MMP-14 in bladder cancer.

Methods: We analyzed MMP-14 expression in various cancers and detected its relationship with the patients' prognosis with urological cancer from the TCGA database. Additionally, we explored the correlation between MMP-14 expression and the corresponding patients' clinicopathological characteristics in bladder cancer. Next, functional enrichment analysis was used to study the biological function and signaling pathways of MMP-14 in bladder cancer. In addition, we used the ssGSEA algorithm and two databases (GEPIA and TIMER) to investigate the effect of MMP-14 on immune infiltration in bladder cancer. Furthermore, Western blotting or immunofluorescence was used to detect the expression of MMP-14, STAT3, p-STAT3 and PD-L1 in bladder cancer cells. The Knock TF and hTFtarget databases were used to predict the transcription factors of MMP-14. We also evaluated the effect of NSC405020 and HO-3867 on bladder cancer cells by proliferation assay, MMP-14 activity assay, cell death detection assay and cell cytotoxicity assay. Moreover, we used CRISPR-Cas9 technology to knockdown MMP-14 expression in bladder cancer cells.

Results: The expression level of MMP-14 was significantly up-regulated in bladder cancer, and bladder cancer patients with a high expression level of MMP-14 had shorter overall survival. MMP-14 expression was significantly correlated with some clinicopathological characteristics in bladder cancer. MMP-14 was involved in the negative regulation of the immune response process. Moreover, MMP-14 expression was moderately correlated with Treg cell enrichment, and weakly correlated with $CD8^+$ T cells. The high MMP-14 expression showed more enrichment of Treg cells compared with the low expression. Additionally, MMP-14 expression was significantly positively correlated with Treg cell markers and T cell exhaustion markers in bladder cancer. In addition, MMP-14 was positively correlated with PD-L1 expression in bladder cancer cells, and down-regulation of MMP-14 reduced PD-L1 expression in bladder cancer cells. STAT3 was predicted to be the transcription factor of MMP-14 and had a significant correlation with MMP-14 expression. Furthermore, the treatment of NSC405020 and HO-3867 decreased the cell proliferation and MMP-14 activity, and they also increased cell apoptosis and cytotoxicity in bladder cancer cells. Inhibiting MMP-14 expression resulted in the decreased expression

level and phosphorylation of STAT3. Applying Colivelin resulted in altered expression levels of MMP-14 and PD-L1 in bladder cancer cells. Colivelin upregulated PD-L1 expression in MMP-14 knockdown cells.

Conclusion: MMP-14 plays a crucial role in the progress of bladder cancer, and is promised to be a novel target of bladder cancer in the future.

Zusammenfassung

Hintergrund: Als Matrix-Metalloproteinase ist MMP-14 am Abbau der extrazellulären Matrix beteiligt. In dieser Studie wollen wir die Funktion von MMP-14 bei Blasenkrebs untersuchen.

Methoden: Wir analysierten die Expression von MMP-14 bei Krebs und ermittelten den Zusammenhang zwischen der MMP-14-Expression und der Prognose von Patienten mit urologischem Krebs aus der TCGA-Datenbank bzw. den entsprechenden klinisch-pathologischen Merkmalen der Patienten mit Blasenkrebs. Funktionelle Anreicherungsanalysen wurden durchgeführt, um die biologische Funktion von MMP-14 bei Blasenkrebs zu untersuchen. Mit Hilfe des ssGSEA-Algorithmus und zweier Datenbanken (GEPIA und TIMER) untersuchten wir die Wirkung von MMP-14 auf die Immuninfiltration bei Blasenkrebs. Mittels Western Blotting oder Immunfluoreszenz wurde die Expression von MMP-14, STAT3, p-STAT3 und PD-L1 in Blasenkrebszellen nachgewiesen. Knock TF und die hTFtarget-Datenbank wurden verwendet, um Transkriptionsfaktoren von MMP-14 vorherzusagen. Die Wirkung NSC405020 und HO-3867 auf Blasenkrebszellen wurden in Bezug auf Proliferation, einem MMP-14-Aktivität, einem Zelltod und Zellzytotoxizität untersucht. Wir vverwendeten die CRISPR-Cas9-Technologie, um die Expression von MMP-14 in Blasenkrebszellen zu unterdrücken.

Ergebnisse: Die Expression von MMP-14 war bei Blasenkrebs signifikant erhöht und ging mit einer kürzeren Gesamtüberlebenszeit einher. Das Expressionsniveau von MMP-14 korrelierte signifikant mit einigen klinisch-pathologischen Merkmalen bei Blasenkrebs. MMP-14 war an der negativen Regulierung des Immunantwortprozesses beteiligt. Die Expressionshöhe von MMP-14 korrelierte mäßig mit der Anreicherung von Treg-Zellen und schwach mit CD8+ T-Zellen. Eine hohe MMP-14-Expression zeigte eine stärkere Anreicherung von Treg-Zellen im Vergleich zu einer niedrigen. Die Expressionshöhe von MMP-14 korrelierte signifikant positiv mit Treg-Zell-Markern und T-Zell-Erschöpfungsmarkern bei Blasenkrebs. Darüber hinaus war MMP-14 positiv mit der Expression von PD-L1 in Blasenkrebszellen korreliert, und die Herunterregulierung der MMP-14-Expression reduzierte die Expression von PD-L1. STAT3 wurde als Transkriptionsfaktor von MMP-14 vorhergesagt und wies eine signifikante Korrelation mit der MMP-14-Expression auf. Darüber hinaus verringerte die Behandlung mit NSC405020 und HO-3867 die Zell-

proliferation und MMP-14-Aktivität und erhöhte die Zellapoptose und Zytotoxizität in Blasenkrebszellen. Die Hemmung der Expression von MMP-14 führte zu einem Rückgang der Expression und Phosphorylierung von STAT3. Der Einsatz des STAT3-Aktivators Colivelin führte zu veränderten Expressionsniveaus von MMP-14 und PD-L1 in Blasenkrebszellen. Colivelin regulierte die Expression von PD-L1 in MMP-14-Knock-down-Zellen hoch.

Schlussfolgerung: MMP-14 spielt eine entscheidende Rolle beim Fortschreiten von Blasenkrebs und könnte in Zukunft ein neues Zielmolekül in der Behandlung von Blasenkrebs sein.

1 Introduction

1.1Research progress on bladder cancer

1.1.1Epidemiological characteristics in bladder cancer

Bladder cancer has become the most common malignancy of the genitourinary system, and it is the fourth leading cause of cancer-related death in men worldwide[1]. Bladder cancer is the ninth most common cancer, with an incidence of more than 1.3 million worldwide[1]. Studies have found that North America and Western Europe have exceptionally high rates, while Eastern European and Asian countries have the lowest rates. According to one study, from 1998 to 2006, the age-specific incidence of papillary non-invasive bladder cancer rose from 5.52 to 9.09 per 100,000 persons[2]. The analysis of the most recent global epidemiological profile of bladder cancer by Richters et al. [3] revealed that Southern Europe, Western Europe, North America, and Western Asia have the highest incidence rates. Furthermore, when it comes to death rates for men, Western Asia, Northern Africa, and Central and Eastern Europe had the highest. Furthermore, Northern Europe have higher rates of female mortality than South-East Asia and Central Asia (Figure. 1).

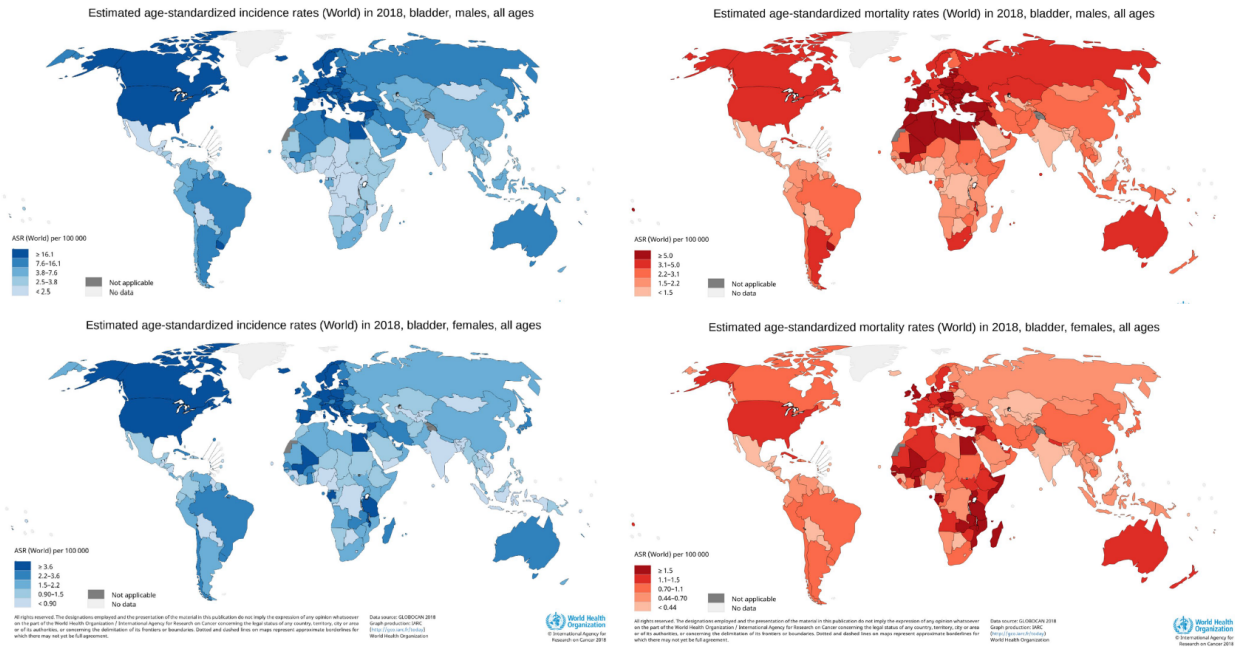


Figure 1 Incidence (left part) and mortality (right part) of urinary bladder cancer worldwide in 2018 according to different sex[3].

1.1.2 Risk factors of bladder cancer

Age, ethnicity, weight, way of life, and family history are all risk factors for bladder cancer. Age raises the risk of bladder cancer. Currently, urothelial bladder cancer patients mainly range in age from 65 to 70[4], and men have a 3–4 times greater incidence of bladder cancer compared to women[5]. Race may be a key determinant in disparities in bladder cancer incidence among males. White men are twice as likely to get bladder cancer as yellow or black men, while there is no statistically significant difference in incidence among women [4]. Obesity is related to a higher chance of getting bladder cancer [6]. Recent research has demonstrated that smoking and obesity dramatically raise the risk of bladder cancer death and recurrence in people with muscle-invasive bladder cancer (MIBC), in addition to raising the chance of developing bladder cancer[7]. Bladder cancer risk factors were also connected to low-quality drinking water that contains impurities or chemicals[8]. In addition, the risk of bladder cancer is elevated by several workplace dangers, including prolonged exposure to diesel exhaust [9], polycyclic aromatic hydrocarbons[10], and certain pesticides[11] and herbicides[12]. It has been reported that patients treated with Cytoxan may develop bladder cancer, which is one of the possible adverse effects of chemotherapy[13]. Studies have indicated that family members of bladder cancer patients have a 2-fold greater risk of developing bladder cancer, and that familial bladder cancer is sporadic[14].

1.1.3 Diagnosis of bladder cancer

The most common symptom of bladder cancer is gross hematuria[15]. The diagnosis of bladder cancer can rely on various invasive and non-invasive tests[16]. Non-invasive tests include urine exfoliation cytology, and the exfoliation of malignant tumor cells can be observed in bladder cancer patients' urine. Urine samples can detect bladder cancer biomarkers, but this test lacks high specificity and sensitivity and has a high false-positive rate. Cystoscopy biopsy is the gold standard for the diagnosis of bladder cancer[17].

Other imaging technology is also used for bladder cancer diagnosis, such as computed tomography, magnetic resonance imaging, and ultrasound [18]. Computed tomography (CT) for bladder cancer has the advantage of being less invasive than cystoscopy. The sensitivity of CT can reach 95%-99%, but CT has poor specificity (83%) and is prone to false positives in the detection of bladder cancer [18]. CT combined with cystoscopy can

improve the ability to diagnose bladder cancer (the sensitivity can be up to 100%, with a specificity of 94%). Magnetic resonance imaging (MRI) is not commonly used in the diagnosis of bladder cancer, but is beneficial for the staging of bladder cancer[19], increasing the staging accuracy to 85%[20]. The sensitivity of ultrasound in diagnosing bladder cancer is 72%, and the sensitivity of contrast-enhanced ultrasound is 88%, which can further improve the detection rate of tumors of less than 5 mm in diameter[21].

Biopsy specimens are evaluated histologically after cystoscopy, combined with imaging studies to determine the grade and stage of bladder cancer[22]. According to the depth of tumor invasion, bladder cancer is clinically divided into non-muscle invasive bladder cancer (NMIBC) and MIBC (Figure. 2). About 75% of patients initially diagnosed with bladder cancer belong to NMIBC, and the remaining 25% belong to MIBC[23].

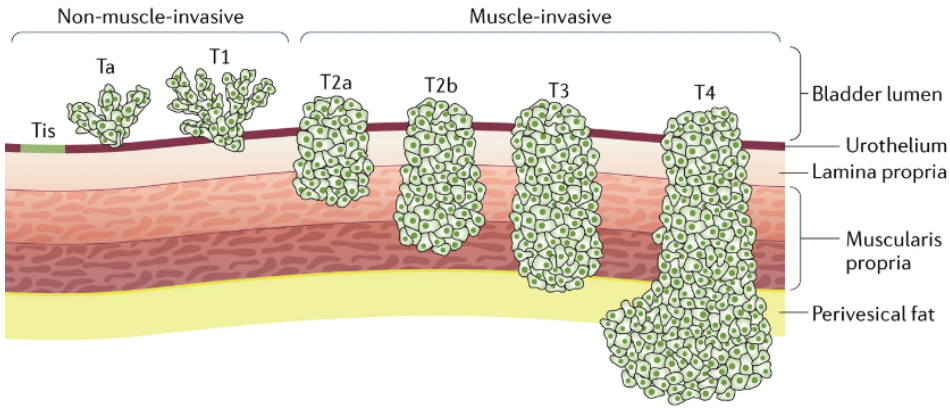


Figure 2 Different T stages and clinical stages of bladder cancer are shown according to the Tumor, Node, Metastasis system[24].

Table 1 Definition of invasive tumor status in different T stages[25]

T stage	Tumor condition
TX	Primary tumor cannot be assessed
T0	No evidence of primary tumor
Tis	Carcinoma *in situ* is a flat, poorly differentiated tumor confined to mucosa
Ta	Papillary tumors that are confined to mucosa
T1	Tumors have invaded the lamina propria (submucosa)
T2a	Tumors have invaded the muscle layer superficially

T2b	Tumors have invaded the the deep muscle
T3	Tumors have invaded beyond the muscularis propria into perivesical fat
T4a	Tumors have invaded the prostate, uterus, vagina and/or bowel
T4b	Tumors have invaded the pelvic or abdominal walls.

1.1.4 Main treatments of bladder cancer

In terms of treatment, patients with NMIBC mainly undergo transurethral resection of bladder tumor, with subsequent intravesical infusion therapy of chemotherapy drugs based on risk stratification scores being performed, and there are some chemotherapy drugs for intravesical instillation, such as mitomycin and epirubicin or pirarubicin[26-28]. Postoperative infusion chemotherapy can reduce the recurrence rate of bladder cancer, which is 14% lower than that of Transurethral resection of bladder tumor (TURBT) alone, but there is no apparent inhibitory effect for tumor progression[29]. Postoperative infusion of Bacillus Calmette-Guérin for bladder cancer can inhibit tumor progression, and the progression rate is reduced by 4% compared with TURBT alone or intravesical infusion chemotherapy[30]. Patients with NMIBC have an 80% response rate to TURBT and intravesical infusion therapy, but 50% of patients relapse after subsequent treatment, and 25% of relapsed patients progress to MIBC, due to incomplete tumor resection[31]. For patients with MIBC, radical cystectomy usually needs to be performed. Surgery typically removes adjacent organs, including lymph nodes, prostate, and seminal vesicles in men, and the uterus, ovaries, and part of the vagina in women[32]. Adjuvant radiotherapy and chemotherapy are carried out after surgery, and platinum-based chemotherapy is the standard first-line treatment. With the continuous improvement of chemotherapy, the overall response rate of patients can reach 60%. Regarding the patients' prognosis, the 5-year survival rate of MIBC patients is about 50%. Up to 50% of patients will relapse, and most die of metastatic disease within 3 years[32].

A growing body of research has focused on the immune system's role in treating bladder cancer. In 1976, Morales reported that intravesical infusion of Bacillus Calmette-Guérin (BCG) has a significant effect on the prevention of postoperative recurrence of superficial

bladder cancer[33], and intravesical infusion of BCG has been the standard treatment for NMIBC since 1990[34]. As mentioned previously, the patients' survival rate for bladder cancer has not been significantly improved, despite the diagnosis and treatment of bladder cancer having been continuously optimized in the past three decades[35]. The tumor mutation rate of bladder cancer ranks third among all tumors, representing its very high antigenicity[36, 37]. This feature undoubtedly provides favorable conditions for the application of immunotherapy in the treatment of bladder cancer. The emergence of new immunotherapies will bring new hope to patients with bladder cancer.

1.2 Tumor immunity

1.2.1 Tumor immune resistance

The immune system is generally balanced by both stimulating and inhibitory signals, thereby precisely regulating the function of the immune system. The membrane receptor molecules that play a regulatory role are called immune checkpoints. Under normal conditions, T regulatory cells (Tregs) can induce apoptosis of T cells by expressing some inhibitory signal receptors, which avoids damage to the body caused by excessive activation of the immune system. Tumor cells evade immune surveillance by abnormally expressing immunoregulatory-related proteins or secreting immunosuppressive cytokines and suppressing the immune response to survive (Figure. 3); this process is called tumor cell immune escape[38]. Studies have shown that tumor cells can directly induce T cell apoptosis by down-regulating antigen expression (such as Major histocompatibility complex (MHC)), up-regulating anti-apoptotic signals (like BCL-XL, FLIP), or expressing cell surface proteins such as PD-L1[39]. In addition, tumor cells can form an immunosuppressive microenvironment by releasing cytokines (like TGF-β, IL-10, VEGF, LXR-L, IDO, gangliosides, or soluble MICA) that inhibit immune cell function, or by recruiting Tregs to secrete IL-4, IL-13, GM-CSF, IL-1β, VEGF, or PGE2[39]. IL-4 and IL-13 can induce the formation of M2 macrophages, and in addition secrete transforming TGF-β and IL-10, inhibiting the maturation of dendritic cells, and simultaneously inhibiting T cell proliferation and cytotoxicity[40]. A decrease in $CD8^+$ T cells and an increase in Tregs would also further increase immunosuppression in the tumor microenvironment[41].

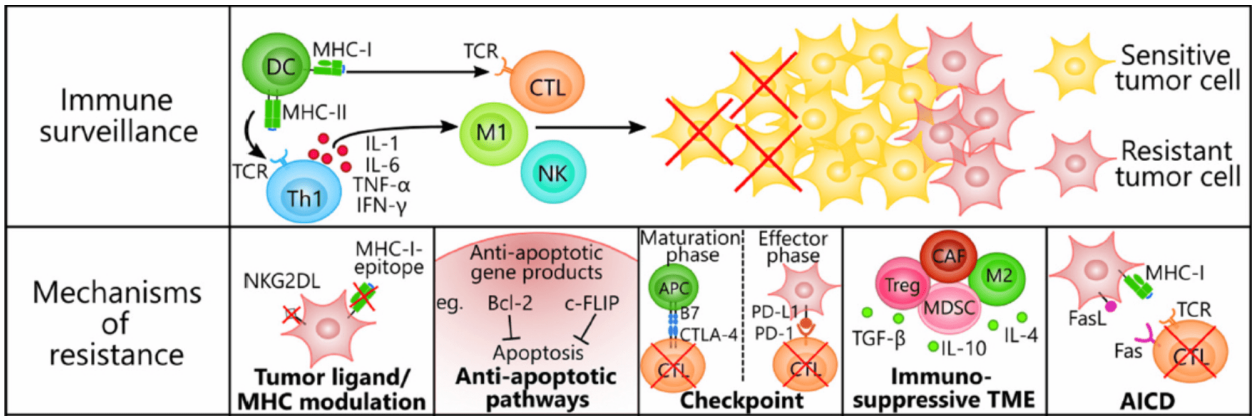


Figure 3 Mechanisms of tumor cell immune resistance [42].

1.2.2 Tumor immunotherapy

Cancer immunotherapy induces anti-tumor effects by enhancing or changing the body's immune system, and mainly includes vaccines, adoptive cell therapy, and immune checkpoint inhibitor therapy. Tumor vaccines mainly use tumor cell antigens to activate the patient's immune system and induce the body to produce tumor antigen-specific immune responses to eliminate tumor cells and control tumor progression. In 2011, the first tumor vaccine, Sipuleucel-T (dendritic cell-dependent vaccine), was approved by the U.S. Food and Drug Administration (FDA) for the treatment of advanced prostate cancer, and survival was extended by 4.1 months[43].

Adoptive cellular immunotherapy mainly uses immune cells, isolated from blood or tumor tissue, to be transformed and expanded *in vitro* and then reinfused into the patient to kill tumor cells. Adoptive cellular immunotherapy is divided into an autologous lymphokine-activated killer, autologous tumor-infiltrating lymphocytes, natural killer cells, cytokine-induced killer cells, cytotoxic T lymphocytes, and genetically modified T cells due to different development processes[44]. In clinical trials, chimeric antigen receptor T cell therapy has shown good targeting and durability, showing excellent development potential and application prospects. Studies have shown that the adoptive transfer of Chimeric Antigen Receptor T cells (CAR-T cells) to treat $CD19^{+}$ hematological malignancies has achieved good therapeutic effects[45]. People have also tried using CAR-T methods to treat solid tumors in recent years. 50% of melanoma patients showed apparent clinical effects [46]. But the rapid expansion of T cells releases many cytokines in CAR-T cell therapy, causing cytokine release syndrome[47].

In recent years, immune checkpoint inhibitor therapy has significantly progressed in various types of tumors, e.g., MIBC and metastatic renal cell carcinoma[48, 49]. In 2011, the first CTLA4 monoclonal antibody, ipilimumab, was approved by the FDA as a first-line drug for treating advanced melanoma[50]. The anti-Programmed Cell Death Protein 1 (PD-1) monoclonal antibodies Nivolumab and Pembrolizumab have been approved by the FDA for treating melanoma and Non-Small Cell Lung Cancer (NSCLC) since 2014[51, 52]. The first study found that the average adequate response time of ipilimumab monoclonal antibody was two years, and about 15-20% of patients had an adequate immune response time of more than 2.5 years, while the average adequate time of traditional chemotherapy to control tumor growth was only 4-8 months[53]. Numerous clinical trials have also shown that PD-1/Programmed Cell Death 1 Ligand 1 (PD-L1) monoclonal antibodies have made significant progress in the treatment of various malignant tumors, such as bladder cancer[48], NSCLC [54], breast cancer[55], follicular lymphoma[56], head and neck squamous cell carcinoma[55], prostate cancer[57] and renal cell carcinoma[55]. Patients' durable response rates to the PD-1/PD-L1 antibodies range from 20-50% in different types of tumors[58]. Therefore, immunotherapy targeting immune checkpoints can achieve more durable and practical anti-tumor effects by activating the patients' tumor-specific T cells.

1.3 The molecular structure of PD-L1 and its function

1.3.1 PD-L1/PD-1 structure and expression

PD-L1 (CD274, B7-H1) is a B7 superfamily member encoded by the CD274 gene, located at chromosome 9p24.2, and the full-length DNA coding region of PD-L1 was cloned from the DNA library after splicing with homologous expressed sequence tags created by bioinformatics. PD-L1 is a type I transmembrane glycoprotein with a total length of 290 amino acids[59]. PD-L1 contains an Ig V domain, an Ig C domain, a transmembrane hydrophobic domain, and an intracellular domain consisting of 30 amino acids[59]. Figure. 4 shows that human PD-L1 mRNA has 4 different transcripts. Transcripts 1, 2, and 4 encode PD-L1 protein, while transcript 3 (NR 052005.1) lacks a sequence between exons 4 and 5,

which makes it unable to be translated into protein and it becomes a long non-coding RNA sequence (Figure. 4).

Figure 4 The 4 different transcription versions of PD-L1 [60].

The mRNA of PD-L1 is expressed in various tissues, such as lungs, heart, liver, and other solid organs, and is also weakly expressed in some antigen-presenting cells. However, PD-L1 mRNA is highly expressed on epithelial cells and epithelial-derived tumor cells[61]. Furthermore, as a co-stimulatory or co-suppressive molecule, PD-L1 protein is an inducible protein, which is widely expressed on the surface of various cells, and highly expressed on activated T cells, B cells, monocytes, and tumor cells[62].

The PD-L1 receptor PD-1 (CD279) is an essential immunosuppressive molecule that belongs to the CD28 superfamily. It is a transmembrane protein encoded by the PDCD1 gene with a full-length of 288 amino acids, which consists of an extracellular Ig V segment, a transmembrane segment, and an intracellular segment[63]. The tyrosine residue at the N-terminus in the cytoplasm and the adjacent amino acid residues collectively form an immunoreceptor tyrosine-based inhibitory motif, which antagonizes the stimulatory signals of antigen receptors through tyrosine phosphorylation and plays a negative regulatory role in the immune response[64]. After phosphorylation of the immunoreceptor tyrosine switching motif at the C-terminus of the cytoplasm, it recruits downstream signal transduction molecules such as SHP-1 and SHP-2 to inhibit further activation of immune cells[65]. PD-1 is mainly expressed on the surface of activated T cells as well as activated B cells, NK cells, and monocytes.

1.3.2 PD-L1/PD-1 function

T cells are activated by T cell receptor (TCR)-mediated antigen recognition signals when the body's immune system is activated. Additionally, numerous costimulatory and co-inhibitory signals finely regulate the intensity and quality of T cell responses to complete the immune response[66]. As shown in Figure. 5, the activation of T cells requires the co-

stimulation of dual signals. The specific binding of MHC-Ag (Antigen) complexes transmits the first signal on Antigen-presenting cells (APCs) to TCRs. The second signal comes from binding APCs' co-stimulatory molecules and T cells' corresponding receptors. There are numerous co-stimulatory molecules, such as CD28/CD80, and co-inhibitory molecules, such as PD-1/PD-L1, on the surface of T cells, which precisely regulate the activation of T cells together[67]. To study the mechanism of the inhibitory effect of PD-L1/PD-1 signaling, after stimulating T cell activation *in vitro*, it was found that the interaction between PD-L1 and PD-1 does not immediately induce T cell apoptosis, but it inhibits the proliferation of T cells due to cell cycle arrest at the G0/G1 phase[68].

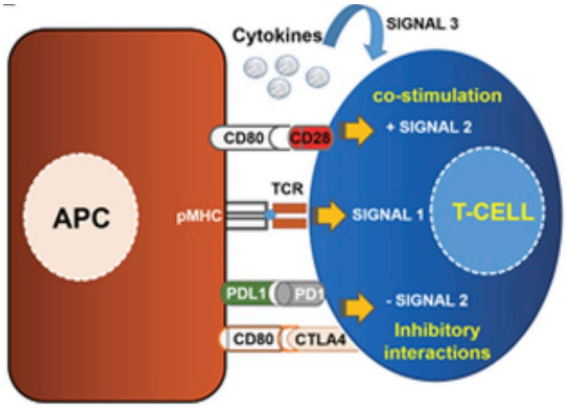


Figure 5 Activation of T cells requires antigen recognition and co-stimulatory or co-inhibitory signals[66].

The binding of PD-1/PD-L1 plays a vital role in regulating T-cell activation and maintaining peripheral immune tolerance. Studies have found that PD-1 knockout (PDCD1-/-) mice with different genetic backgrounds will spontaneously develop different types of autoimmune diseases with increasing age. PDCD1-/-C57BL/6 mice develop spontaneous lupus glomerulonephritis and arthritis, with excessive deposition of complement C3 and IgG3 in the glomeruli; PDCD1-/-BALB/C mice died of dilated heart failure at 10 weeks after birth, and histological examination revealed the accumulation of complement C3 and IgG in the cardiomyocytes of the dilated ventricle[69]. Studies by Nishimura et al. and Ansari et al.[64, 70] showed that PD-1/PD-L1 binding involves peripheral immune tolerance rather than central immune tolerance. In the autoimmune diabetes model, pancreatic islet cells upregulated the expression of PD-L1, and enhanced PD-1 inhibitory signaling significantly reduced the release of inflammatory factors from islet-specific T cells. Therefore, the PD-1/PD-L1 inhibitory pathway not only plays a role in the early stage of autoreactive T cell activation progression but also participates in the immune response to self-antigens in the effector stage of autoreactive T cells.

Negative co-stimulatory signals are critical in regulating organ transplantation rejection from allogeneic tissue. In the heart transplantation model, it was found that PD-L1 was up-regulated one day after transplantation[71], which suggests that PD-L1/PD-1 plays a vital role in the acute rejection of the graft and may be an essential part of graft tolerance, and enhancing the PD-L1/PD-1 signaling pathway contributes to long-term graft survival. The PD-1/PD-L1 inhibitory pathway also plays a vital role in the body's clearance of viral infections. A study found[72] that, compared with wild-type mice, PDCD1-/- mice cleared adenovirus infection faster, suggesting that the virus may evade the immune response through the PD-1/PD-L1 inhibitory signaling pathway and achieve persistent infection. Additionally, the expression of PD-L1 is closely related to the degree of inflammatory injury and inflammatory cell infiltration in liver tissue[73], suggesting that the PD-1/PD-L1 inhibitory pathway may play a dual role in the upregulation of chronic hepatitis B infection in the liver. On the one hand, it protects liver tissue from pathological immune damage. On the other hand, it also leads to the down-regulation of specific immune responses and the continued inability to clear the virus.

1.3.3 Expression regulation mechanism of PD-L1/PD-1

In recent years, monoclonal antibodies that inhibit PD-1/PD-L1 have significantly progressed in treating various malignant tumors. Clinical trials have shown that the expression of PD-L1 is closely related to the efficiency of treatment response. Therefore, it is crucial to understand the regulation mechanism of PD-L1 expression on the cell surface.

1. Genome-level regulation for PD-L1 expression

Some scholars performed RNA-Seq on the transcriptome of the Hodgkin's lymphoma cell line KMH2. The results showed that the MHCII transcriptional activator gene CIITA has a gene fusion, and this gene fusion downregulates HLA II and up-regulates the expression of PD-L1 and PD-L2 [74]. In addition, the study found an increase in the copy number of 9p24.1 in Hodgkin's lymphoma and primary mediastinal large B-cell lymphoma cell lines, followed by abnormal amplification of PD-L1, PD-L2, and Janus kinase (JAK) 2 genes, and abnormal amplification of JAK2 also activated transcription of PD-L1. A study by Kataoka et al.[75] found that deletion of the PD-L1 three prime untranslated region (3'-

UTR) caused upregulation of PD-L1 expression in various tumors. PD-L1 3'-UTR-deficient cell lines were treated with Actinomycin D, and PD-L1 3'-UTR deletion delayed the clearance of PD-L1 mRNA compared with WT cells, suggesting that PD-L1 3'-UTR may be a negative regulator of PD-L1 mRNA stability. The above studies have shown that changes at the genome level could upregulate PD-L1 expression and enhance the PD-L1/PD-1 inhibitory signaling pathway. Changes at the genome level may serve as potential biomarkers.

2. Inflammatory factor signaling pathway regulates PD-L1 expression

In response to anti-tumor cytokines in the microenvironment, tumor cells inducibly express PD-L1 in the adaptive immune response. IFNγ is a pro-inflammatory cytokine secreted mainly by activated T cells and Natural killer (NK) cells, and it has been shown to upregulate PD-L1 expression in various tumors. In melanoma[76], IFNγ upregulates PD-L1 on tumor cells and inhibits PD-1^{+} T cell function. The study by Garcia-Diaz et al.[77] further revealed the molecular mechanism by which IFNγ upregulates PD-L1 in melanoma. IFNγ can activate the expression of transcription factor IRF1 through JAK1/JAK2-STAT1/STAT2/STAT3, and IRF1 can directly bind to the promoter region of PD-L1 to upregulate the expression of PD-L1. In addition, other cytokines, such as IL-17, TNFα, etc.[78, 79], are also involved in regulating PD-L1 expression in tumor cells, endothelial cells, monocytes, and other cells. In Table 2, the cytokines currently known to regulate PD-L1 expression are summarized. The above studies show that various inflammatory factors are directly or indirectly involved in regulating the expression of PD-L1, but the specific regulatory mechanism remains to be elucidated.

Table 2 Cytokines regulating PD-L1 expression

Cytokines	Cellular or pathological type
IFN-γ	Endothelial cells, gliomas, renal tubular epithelial cells, colon cancer, monocytes, DCs and macrophages, neutrophils[80-85]

IFN-α/IFN-β	Endothelial cells, monocytes, melanoma[77, 86, 87]
IL-1β	Dendritic cells[88]
IL-4	Renal cell carcinoma[89]
IL-6	Dendritic cells[88]
IL-10	Dendritic cells，monocytes[90, 91]
IL-12	Endothelial cells[86]
IL-17	Monocytes，prostate and colon cancer[78, 90]
TGF-β	Dendritic cells, T cells[92, 93]
TNF-α	Endothelial cells, dendritic cells, monocytes, renal cell carcinoma, breast cancer[79, 88, 89, 94]
TLR3	Dendritic cells，endothelial cells[95, 96]
TLR4	Macrophages, bladder cancer, monocytes, dendritic cells[95, 97-99]

3. Tumor signaling pathway regulates PD-L1 expression

Various signaling pathways, like cell proliferation, apoptosis, and differentiation, regulate the development of tumors[100]. Studies have shown that some related genes from tumor signaling pathways can change the expression of PD-L1 and can be used as clinical therapeutic targets in combination with immunotherapy. Tumor signaling pathway genes regulate PD-L1 expression in various ways, including transcription factors, effector components, and upstream receptors that can affect PD-L1 expression.

Recent studies have pointed out that some transcription factors of oncogenes can directly regulate the transcription of PD-L1. Wang et al.[101] showed that the inactivation of MYC downregulates PD-L1 expression, which is present in tumors such as NSCLC, melanoma,

and hepatocellular carcinoma, and the chromatin immunoprecipitation assay demonstrated that MYC could directly bind to the promoter sequence of PD-L1, suggesting that MYC directly regulates the transcription level of PD-L1[102]. Hypoxia exists during the growth of most tumors, and the lack of oxygen activates a series of HIFs, which induce vascular survival and metastasis in tumors. Mechanistically, HIF-1α and HIF-2α upregulate PD-L1 expression by binding to the HRE sequence on the PD-L1 promoter sequence[103]. In addition, studies have shown that other transcription factors such as NF-κB bind to PD-L1 promoter sequence[104], and that AP-1 binds to the first intron sequence of PD-L1 101 and upregulates the transcription level of PD-L1[105]. Signal transducers and activators of transcriptions 3 (STAT3) promotes the proliferation, metastasis, and survival of tumor cells. Many studies have shown that STAT3 can directly bind to the promoter region of PD-L1 and promote the transcription of PD-L1[106].

Studies have shown that the inactivation of JUN and STAT3 in the MEK signaling pathway inhibits the MEK signaling pathway and then downregulates the expression of PD-L1[107]. CDK5 regulates the expression of PD-L1 by affecting the IFNγ signaling pathway. As a serine-threonine kinase, CDK5 post-transcriptionally modifies IRF2BP2 to degrade IFRF2, promotes IRF1 expression, and then upregulates PD-L1[108]. The PI3K signaling pathway regulates the downstream interferon-related mRNA translation by activating the downstream AKT-mTOR cascade and then participates in interferon-mediated PD-L1 expression[109]. KRAS, EGFR, and ALK mutations can induce related signaling pathways to stimulate the expression of PD-L1[110-112].

4. The research progress on PD-L1/PD-1 in immunotherapy

Immunotherapy for bladder cancer has focused on immune checkpoints recently. PD-1 and PD-L1 monoclonal antibodies block the combination of PD-L1 on tumor cells and PD-1 on T cells, cut off tumor cell immune escape, and enhance the killing ability of T cells, resulting in the outstanding achievement of relieving or even curing tumors. Figure. 6 shows that TCR recognizes surface antigens of MHC molecules in the tumor microenvironment. Then, T cells are induced to stimulate tumor cells to overexpress PD-L1 and bind to PD-1, ultimately inhibiting T cell activation. When PD-1 or PD-L1 monoclonal antibody is used, PD-L1 on the tumor surface cannot bind to PD-1 on the surface of T cells,

and T cells are subsequently activated to carry out the function of infiltrating and killing tumor cells[113].

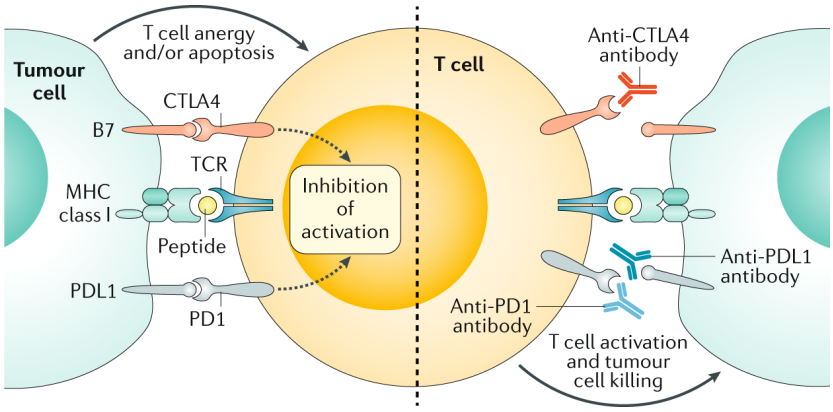


Figure 6 Anti-tumor mechanism of PD-1/PD-L1 antibody in immunotherapy[113].

Since 2016 (Figure.7), the FDA has approved five PD-1/PD-L1 inhibitors for first-line and second-line treatment of locally advanced or metastatic urothelial carcinoma[114]. Nivolumab and Pembrolizumab are monoclonal antibodies that bind to PD-1, while Atezolizumab, Durvalumab, and Avelumab are monoclonal antibodies against PD-L1. PD-1/PD-L1 immune checkpoint inhibitors offer a compelling alternative treatment option for patients with locally advanced or metastatic bladder cancer who have disease progression during or after adjuvant (or neoadjuvant) platinum-based chemotherapy[115].

Figure 7 Timeline for FDA approval of PD-1/PD-L1 inhibitors for the treatment of advanced bladder urothelial carcinoma[114].

1.4 Research progress on STAT3

1.4.1 The structure of STAT3

Signal transducers and activators of transcriptions (STATs) are a class of cytoplasmic transcription factors activated by cytokines, growth factors, and other peptide ligands, which have the dual functions of transmitting cytoplasmic signals and initiating intranuclear gene transcription[116]. They are essential in regulating normal cell proliferation, differentiation, and apoptosis. The human STAT family consists of seven members (STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, STAT6), whose coding genes belong to different chromosomal regions[117].

STAT3 was originally identified as an acute transmission factor in the study of the interleukin-6 signaling pathway in 1994[118]. In terms of functional structure, STAT3 can be divided into six functional regions (Figure.8), including the conserved amino acid-terminal sequence at the N terminus, the coiled-coil helix region, the DNA binding domain, the linker structural region, the SH2 structural domain, and the carboxy-terminal transcriptional activation domain[119]. Notably, the SH2 domain is a conserved part of the STAT family, which promotes the formation of complexes between STAT3 molecules and activated receptors, mediates the interaction between STAT3 and activated JAK, and causes STAT3 to form a dimer, finally leading to the nucleation of STAT3 proteins and regulation of gene expression[120].

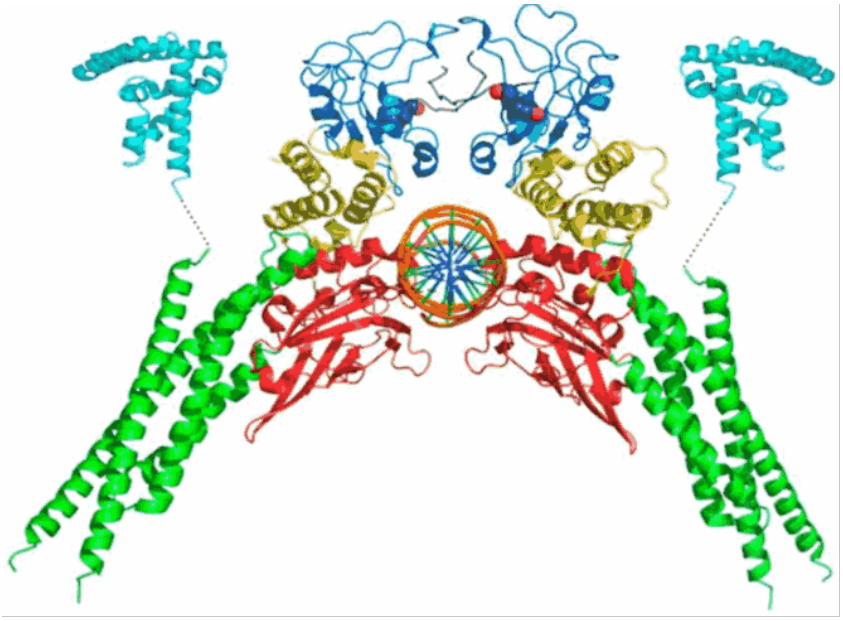


Figure 8 The structure model of STAT3 protein.

cyan = amino acid-terminal region; green = coiled-coil helix region; red = DNA binding domain; yellow = linker structural region; blue = SH2 structural domain; violet = carboxy-terminal transcriptional activation domain; orange = DNA.[121]

1.4.2 Activation of STAT3

Normally, the STAT3 protein is dormant in the plasma of cells. Some extracellular stimuli can activate STAT3, including cytokines, epidermal growth factor (EGF), and fibroblast growth factor (FGF)[122]. By binding to their respective receptors on the cell surface, these cytokines activate the Janus kinase (JAK) family of kinases. STAT3 docks to the receptor cytoplasmic domain after JAKs phosphorylate it. In addition to being recruited to the receptor, STAT3 is additionally activated by JAKs after being phosphorylated on a certain tyrosine residue (Y705). Receptor tyrosine kinase (RTK) engagement is a significant method of STAT3 activation[122]. When RTKs, like the EGF receptor, activate the Ras-Raf-MEK-ERK pathway, JAKs and STAT3 are also stimulated[123]. In addition to tyrosine phosphorylation, STAT3 can potentially be triggered by serine phosphorylation[122]. Numerous stimuli, like growth factors, and some stress-related signals might drive this phosphorylation to occur on a particular serine residue (S727). Serine phosphorylation is hypothesized to be involved in the regulation of particular target genes as it promotes the transcriptional activity of STAT3[122].

1.4.3 The function of STAT3 in cancer

STAT3 encourages the expression of genes like cyclin D1 and c-myc, which manage cell cycle progression[122, 124]. Furthermore, it enhances cell growth by suppressing the production of CDKIs (cyclin-dependent kinase inhibitors) including p21 and p27 [125]. By blocking the pro-apoptotic proteins Bax and Bak as well as facilitating the anti-apoptotic proteins Bcl-2 and BCL-XL, STAT3 activation discourages apoptosis and induces survival[126]. In addition, vascular endothelial growth factor, a powerful angiogenic agent that encourages the development of new blood vessels, is increased when STAT3 is activated, which promotes tumor growth and metastasis[122]. Moreover, STAT3 elevates the expression of matrix metalloproteinases (MMPs), which dissolve the extracellular matrix and enhance tumor invasion and metastasis[127-129]. Besides this, it provokes the epithelial-mesenchymal transition, which facilitates the migration and invasion in cancer cells[122]. Interestingly, STAT3 encourages the expression of immunosuppressive molecules, including PD-L1, IL-10, and TGF-β, which block the activity of immune cells like

T cells, NK cells, and dendritic cells, allowing cancer cells to escape immune surveillance[130, 131].

Persistent activation of STAT3 signaling is associated with the development of many tumors and represents a poor prognosis, and excessive activation of STAT3 has been found in 70% of solid and hematologic tumors[132]. Therefore, STAT3 protein has gained increasing attention in oncology research.

1.5 Molecular function and regulatory mechanism of MMP-14

1.5.1 Introduction to the MMP protein family

MMPs were first discovered in the early 1960s and identified as having collagenolytic activity, which can lead to the degradation of the extracellular matrix (ECM)[133]. MMPs are part of the endopeptidase or chromatin family, and are produced by multiple tissues and cells, including connective tissue, vascular smooth muscle, fibroblasts, endothelial cells, osteoblasts, pro-inflammatory cells, macrophages, and lymphocytes[134]. The MMPs family contains multiple metalloprotease members, such as secreted proteases (MMP-1, -2, -3, -7, -8, -9, -10, -11, -12, -13, -19, -20, -21, -22, -27, -28) and membrane-anchored proteases (MMP-14, -15, -16, -17, -23, -24, -25)[135]. As is shown in Figure.9, MMPs have a heme-like domain consisting of four looping sequences and contain a disulfide bond (S-S) between the head and tail subdomains, which are linked to the catalytic domain through their flexible hinge domains[136]. MMPs generally consist of three parts: a plasma peptide of about 80 amino acids, a domain of about 170 amino acids for catalytic metalloproteinases, and a variable-length linker peptide (hinge region)[134]. MMPs play essential roles in physiological processes, including tissue remodeling and organ development[137, 138], and they are regulated by multiple signaling pathways in diseases like inflammation and cancer [139, 140]. As matrix metalloproteinases, the central role of MMPs is to assist in the degradation of various proteins in the ECM. Fibers, proteoglycans, and polysaccharides are the three main components of the ECM. MMPs maintain the structural integrity of the vascular wall by facilitating the transport of various ECM proteins, including gelatin, elastin, collagen, and other matrix glycoproteins and proteoglycans, and play an essential role in tissue remodeling[141]. MMPs induce the degradation of the

ECM to promote the invasion and metastasis of cancers, which has always been a hot topic in MMP research.

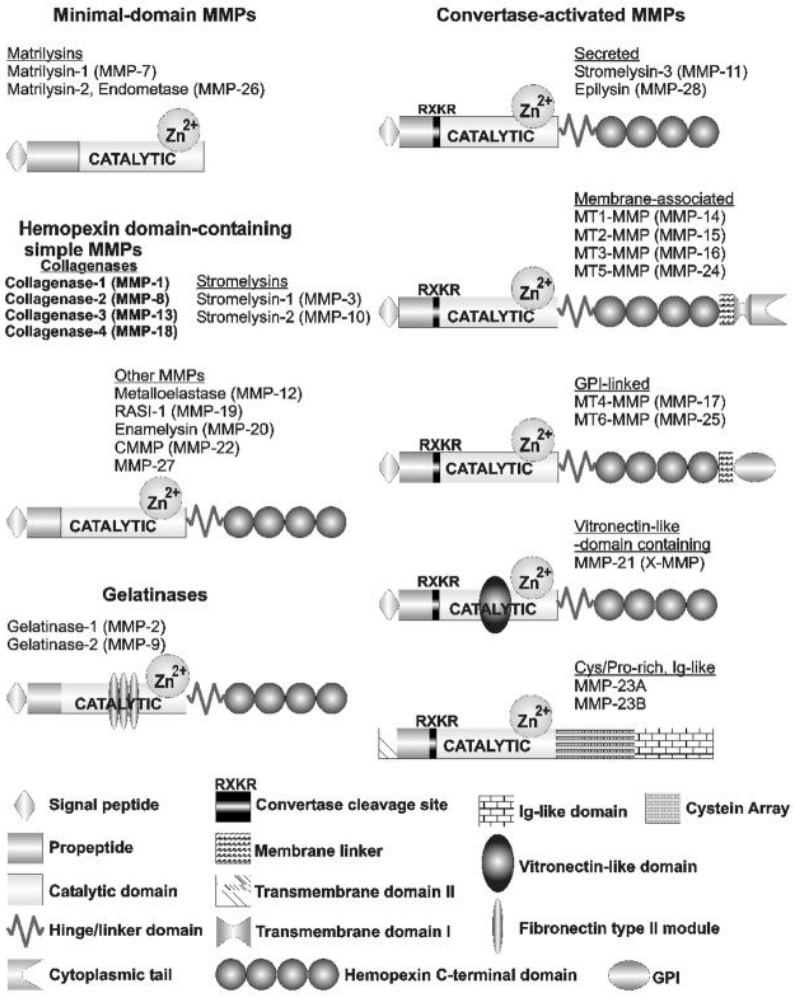


Figure 9 Composition of MMP family protein domains [142].

1.5.2 The structure and essential functions of MMP-14

Matrix metalloproteinase MMP-14, also known as MT1-MMP, is the first member of the membrane-type matrix metalloproteinase family, and was first cloned and identified by Sato in 1994[143]. MMP-14 has the basic structure of the MMPs family: signal peptide region, propeptide region, catalytic region, hinge region, hemopexin region, stalk (linker-2) region, and transmembrane domain[144]. In addition, it has a unique region (RXKR) containing a furin recognition site between the propeptide region and the catalytic region[144]. Most of the MMPs are clustered on chromosome 11, while MMP-14 is located on 14ql[145]. MMP-14 is synthesized in the form of the zymogen, which is recognized by

a subtilisin-live proteolytic enzyme and activated by enzymatic hydrolysis, and then secreted to the outside of the cell and exposed to the cell membrane[146]. The most important biological feature of MMP-14 is that it can activate MMP-2 and MMP-9[143], so the MTs-MMP/MMP enzyme chain activation system becomes a new way to regulate ECM degradation. However, its activation mechanism still needs to be determined. The theory of molecular complexes has received particular attention[147]. MMP-14 expressed on the cell membrane is activated by proteolytic enzymes and binds to the N-terminus of TIMP-2 like a cell membrane receptor. Then, the complex binds MMP-2 to form the MMP-14/TIMP-2/MMP-2 trimolecular complex.

1.5.3 Molecular mechanisms regulating MMP-14

1. Gene transcription regulation level

MMP-14 is overexpressed in various cancer cells and is associated with poor patient prognosis[148]. The MMP-14 genome contains 10 exons and 9 introns, but unlike other MMP family proteins, the promoter of MMP-14 lacks the conserved TATA box at the -25 binding site and AP-1 at the -80 binding site[149]. Previous studies have reported that various transcription factors regulate the transcriptional activity of MMP-14[149-151]. Sp1 and EGR1 can dramatically affect the transcriptional effects of MMP-14 in endothelial cells[152]. The decreased expression of NF-κB also down-regulated MMP-14 transcriptional levels in glioblastoma cells[150]. In fibroblasts, the presence of Snail-1 upregulates cellular protein expression levels of MMP-14. Sp1, AKT, JNK, and ERK jointly regulate the expression level of MMP-14[151]. CD81 induces the expression of MMP-14 by promoting the phosphorylation of Sp1 in melanoma cells[153]. In renal cell carcinoma with genetic deletion of the ubiquitin ligase VHL, Sp1 can bind to hypoxia-inducible factor 2α (HIF-2α) to upregulate the transcription level of MMP-14[154]. Vinculin knockdown in squamous carcinoma cells promotes the transcription of MMP-14 and enhances the invasive ability of cells[155]. Moreover, some researchers have found that PROX1 can inhibit the transcriptional activity of MMP-14, and that PROX1 is located in the transcriptional repression regulatory region of MMP-14 and can bind to the promoter[156]. In addition, various transcription factors, including AP-4, c-Myc, CARG Box, E-box, E2F, Lyf1, and Nkx-2, have also been reported to regulate the transcriptional efficiency of MMP-14[152].

2. Activation of pro-MMP-14

MMP-14 is initially synthesized in the form of a proenzyme (pro-MMP-14). During secretion, the signal peptide contained in the MMP-14 molecule is removed by the signal peptidase in the rough endoplasmic reticulum. The 108 RRKR sequences at the C-terminus of the MMP-14 molecule can be recognized by furin in the trans-Golgi apparatus to help it remove the propeptide[143]. Finally, MMP-14 is expressed on the surface of the cell membrane as an active enzyme.

3. Inhibition of endogenous physiology

Endogenous inhibitors play an important role as an essential process regulating cell-matrix degradation. MMP-14 has been reported to be degraded by TIMP-2, -3, and -4, while TIMP-1 has no inhibitory effect on MMP-14[157]. Recently, it has been found that different TIMPs have different inhibitory mechanisms on MMP-14. In the extracellular matrix of TIMP-2-null mice, the researchers did not find an increase in MMP-14 activity[158]. Nevertheless, it was unexpectedly found that pro-MMP-2 was over-activated, suggesting that TIMP-2 inhibits MMP-14 activation of MMP-2 to regulate its function[158]. In the extracellular matrix structure of TIMP-3-deficient mice, the activities of various MMPs, including MMP-14 and MMP-2, were significantly increased, and the ECM structure was severely degraded[159]. In addition, MMP-14 can also be inhibited by other proteins. Oh et al. [160] found that RECK protein affects the integrity of the extracellular matrix and regulates angiogenesis by regulating MMP-14[161]. Our previous study also showed that RECK affected the aggressiveness of prostate cancer through MMP-14. In addition, the tight junction protein claudin can also promote the activation of pro-MMP2 by MMP-14 independently of TIMP2[162].

1.5.4 The function of MMP-14 in tumors

1. The effect of MMP-14 on tumor cell migration and invasion

The migration and invasion of tumor cells are affected by various mechanisms, such as cell motility, adhesion strength, and degradation of the basement membrane and extracellular matrix. In the initial stage of tumor cell infiltration and metastasis, MMP-14 can be

aggregated at the cellular pseudopodia, which are special cell membrane protrusions rich in scaffold proteins, muscle actin, and various enzymes to promote the movement of tumor cells[163]. In addition, MMP-14 can degrade collagen types I, II, IV as well as fibronectin, participate in the degradation process of the extracellular matrix, including basement membrane, and activate pro-MMP-2[164]. MMP-2 further enhances its degradative effect on the extracellular matrix. Laminin-5 is a significant basement membrane component, supporting tumor cell migration. Studies have shown that MMP-14 regulates laminin-5 through an enzymatic reaction to promote the migration of mammary epithelial cells, and antisense oligonucleotides targeting the MMP-14 gene inhibit the processing of the laminin-5 γ2 chain and thereby affect the signal pathway of cell migration[165, 166]. CD44 is another target of MMP-14 in the process of cell migration[163]. It can bind to and be cleaved by MMP-14. The cleaved CD44 can activate extracellular signal-regulated kinase to promote cell migration[167]. Additionally, it can promote cell migration through transcriptional activation of target genes like the transcription factor Notch[168]. Integrins are common protein molecules in cell migration and adhesion. Vitronectin-binding integrin αvβ3, which is highly expressed in aggressive tumor cells, is initially a single polypeptide, and is converted to a double-stranded form by proprotein convertase and processed into a functional form by MMP-14[169]. αvβ3 and MMP-14 co-stimulate cell migration on the stroma and promote the phosphorylation of focal adhesion kinase in breast cancer cells[170]. Moreover, MMP-14 can also hydrolyze cell surface transglutaminase bound to integrin β1 or β3 chains, thereby inhibiting the adhesion of fibronectin to tumor cells[171].

Furthermore, each domain of the MMP-14 protein plays a critical role in cell invasion. Using a DNA recombination approach, Cao et al. [172] demonstrated that the catalytic region and the hemopexin protein region are the significant components of MMP-14-mediated cell migration: the catalytic region primarily mediates ECM degradation activity, and the catalytic region and the hemopexin region jointly regulate cell migration. The PEX domain of MMP-14 is also essential for Matrigel invasion[173]. The hydrophobic transmembrane region at the C-terminus is also an essential part of MMP-14 in promoting cell invasion. Previous studies have shown that cells stably transfected with wild-type MMP-14 lacking the C-terminal region failed to invade 3D collagen gels[174]. The cytoplasmic tail of MMP-14 also plays a vital role in the invasion-promoting activity in Matrigel, and its mutation significantly slows the speed of cell invasion in Matrigel[175].

2. The effect of MMP-14 on tumor angiogenesis

Tumor angiogenesis is closely related to cell interaction and extracellular matrix interaction. Angiogenesis is mainly divided into two stages: the activation stage and the generation stage[176]. During the activation phase, the permeability of blood vessels is increased, extravascular cellulose is formed, the vessel wall and basement membrane are degraded, cells migrate and invade the extracellular matrix, and endothelial cells proliferate and form the capillary lumen. The generative phase includes inhibition of endothelial cell proliferation, cessation of cell migration, basement membrane remodeling, and formation of the vessel wall by recruitment and differentiation of smooth muscle cells and pericytes. Previous studies have shown that the early activation stage of neovascularization is related to MMP-14; it can activate MMP-2 and amplify the degradation process of the basement membrane[177].

Additionally, endothelial cells migrate through the degradation of the basement membrane by MMP-14 during the generation stage[178]. Furthermore, MMP-14 induces tumor angiogenesis by upregulating VEGF expression during thrombin-induced vascular responses[179]. MMP-14 can enhance the bioavailability of TGFβ1 and the variability of collagen fibers, thereby regulating the stability of blood vessels[180]. In the process of blood vessel formation, vascular smooth muscle cells play a specific role in the composition and function of the blood vessel wall. MMP-14 can activate the PDGFRβ receptor by binding the LDL receptor-related protein LRP1 to promote the dedifferentiation of vascular smooth muscle, and the dedifferentiation of vascular smooth muscle cells is significantly attenuated in the absence of MMP-14 expression[181].

3. The effect of MMP-14 on cell apoptosis

Resistance to apoptosis is an essential feature of tumor cells. Type I collagen triggers apoptosis in luminal breast cancer cells by inducing the cell-killing effect of the pro-apoptotic factor Bcl-2 interacting killer (BIK). At the same time, MMP-14 can inhibit its apoptosis by hydrolyzing type I collagen[182]. In addition, MMP-14 inhibits apoptosis by directly inhibiting collagen-induced up-regulation of BIK (a pro-apoptotic tumor suppressor) expression. In addition, it has been reported that MMP-14 cleaves DDR1 by activating the DDR1-BIK signaling axis. DDR1, a collagen-specific activated receptor tyrosine kinase,

can cause severe apoptosis[183]. Furthermore, MMP-14 protects tumor cells from apoptosis induced by serum starvation by binding to TIMP-2 and activating ERK1/2 and AKT signaling pathways[184].

4. The effect of MMP-14 on cell proliferation

MMP-14 can decompose the cell matrix components outside normal tissues and promote the unlimited proliferation of tumor cells. The study by Moss et al. showed that the proliferation rate of tumor cells in three-dimensional collagen gel accelerated with the increase of MMP-14 expression[185]. MMP-14 affected the proliferation ability of cells through the TNF pathway in cervical cancer cells[186]. Gene silencing of MMP-14 induced the expression of E-cadherin and decreased the expression of vimentin and tumor cell proliferation in gastric cancer cells[187]. MMP-14 promotes aggressive tumor growth in ovarian cancer cells by proteolytically activating heparin-binding EGF-like growth factor and further stimulating the EGFR signaling pathway[188]. Collagen XIV is a fiber-associated collagen that promotes fiber assembly and restricts lateral cell growth, and the MMP-14 of fibroblasts relieves its restriction on tumor cell growth in melanoma by hydrolyzing collagen XIV[189]. Meanwhile, MMP-14 can also cleave Notch1 and thereby promote the growth of tumor cells in melanoma[190].

As mentioned above, immune escape is an essential mechanism for tumor cells to escape endogenous killing, and immune checkpoints play an essential role in tumor immunity. Our previous bioinformatics analysis showed that MMP-14 is closely related to the immunosuppression of tumor cells in bladder cancer. Only a few pieces of literature have reported the role of MMP-14 in the immune system, but the relationship between MMP-14 and tumor immunity is largely unknown. Considering that PD-L1 is an essential immune checkpoint, we hypothesized that MMP-14 could affect tumor immune escape by regulating the expression of PD-L1.

1.6 Objectives

1. To explore the expression of MMP-14 in bladder cancer and its correlation with clinicopathological characteristics of patients.

2. To explore the effect of MMP-14 on bladder cancer cells.

3. To explore the relationship of MMP-14 with PD-L1.

2 Materials and Methods

2.1 Materials

2.1.1 Oncomine Database

The Oncomine database is one of the largest tumor data platforms, which can comprehensively mine the expression of differential genes in common cancer subtypes and normal tissues[191]. In this part of the study, the research condition of MMP-14 in different tumor types was mined by collecting the database information from Oncomine.

2.1.2 TCGA tumor database

The Cancer Genome Atlas (TCGA) is a cancer genome project that has characterized more than 20,000 primary cancers by molecular features and matched normal samples across 33 cancer types[192]. In this study, the collected data are based on RNAseq data in the level 3 HTSeq-FPKM format from the TCGA, ALL (pan-cancer) project (http://cancergenome.nih.gov). The RNAseq data in the FPKM (Fragments Per Kilobase per Million) format was converted to the TPM (transcripts per Million reads) format and then transformed with log2. In addition, the corresponding clinical data were downloaded from the TCGA database. In total, 33 types of cancers were analyzed, including adrenocortical carcinoma (ACC); bladder urothelial carcinoma (BLCA); breast cancer (BRCA); cervical cancer (CESC); cholangiocarcinoma (CHOL); colon cancer (COAD); diffuse large B lymphoma (DLBC); esophageal cancer (ESCA); glioblastoma multiforme (GBM); head and neck squamous cell carcinoma (HNSC); renal chromophobe carcinoma (KICH); renal clear cell carcinoma (KIRC); renal papillary carcinoma (KIRP); acute myeloid leukemia (LAML); brain low-grade glioma (LGG); liver hepatocellular carcinoma (LIHC); lung adenocarcinoma (LUAD); lung squamous cell carcinoma (LUSC); mesothelioma (MESO); ovarian cancer (OV); pancreatic adenocarcinoma (PAAD); pheochromocytoma and paraganglioma (PCPG); prostate adenocarcinoma (PRAD); rectal adenocarcinoma (READ); sarcoma (SARC); skin cutaneous melanoma (SKCM); stomach adenocarcinoma (STAD); testicular germ cell tumor (TGCT); thyroid carcinoma (THCA); thymoma (THYM); uterine corpus endometrial carcinoma (UCEC); uterine carcinosarcoma (UCS); and uveal melanoma (UVM).

2.1.3 Knock TF database

The knock TF database (http://www.licpathway.net/KnockTF/) is based on expression profile data from human transcription factor knockdown/knockout-related genes[193]. Currently, it contains 308 transcription factors from 570 RNA-seq and microarray datasets in various tissues and cells. In addition, KnockTF also provides detailed information about the binding of transcription factors to promoters and enhancers. In this study, we used the Knock TF database to predict the potential transcription factors of MMP-14 in bladder cancer.

2.1.4 hTFtarget database

hTFtarget[194] (http://bioinfo.life.hust.edu.cn/hTFtarget/#!/) is one of the most comprehensive databases for studying human transcription factor-target regulation, integrating large-scale data on transcription factors and the corresponding target genes (including 7190 ChIP-seq samples for 659 transcription factors and high-confidence binding sites for 699 transcription factors, as well as comprehensively considering the effects of transcription factor binding sites and genomic epigenetic modification on transcription factor binding. It is an open-source database between transcription factors and target genes, which analyzes the cell line-specific regulation of transcription factors and cooperative regulation between transcription factors, providing various solutions for studying transcription factor-target regulation. In this study, we used the hTFtarget database to predict the potential transcription factors of MMP-14 in bladder cancer.

2.1.5 GEPIA database

The GEPIA database[195] (Gene Expression Profiling Interactive Analysis) (http://gepia.cancer-pku.cn/) is based on TCGA and GTEx, and contains RNA sequencing data of 9,736 tumor samples and 8,587 normal samples. Functionally, it is an online tool for differential expression analysis of genes, and it can also be used to carry out survival prognosis analysis and gene correlation analysis[31]. This study used the GEPIA database to explore the correlation between MMP-14 and markers of immune infiltration in bladder cancer tissue and normal bladder urothelial tissue.

2.1.6 TIMER database

The TIMER database[196] (Tumor IMmune Estimation Resource) (http://cistrome.org/TIMER/) is a web database based on various algorithm systems, which can analyze the infiltration degree of 6 immune cell types (B cells, $CD4^+$ T cells, $CD8^+$ T cells, neutrophils, macrophages, and dendritic cells) in different tumors. It can analyze gene expression differences between tumor tissues and normal tissues, the correlation of co-expressed genes, and genetic variation. In this study, we used the TIMER database to analyze the correlation of MMP-14 with markers of immune infiltration in bladder cancer.

2.1.7 Cell lines and plasmids

Four bladder cancer cell lines (UM-UC-3, RT-4, RT-112, HT-1376) and one normal urothelial cell line (HCV-29) were obtained from American Type Culture Collection. The pCRISPR-CG04 vector plasmid with MMP-14 knockdown (cat. No. HCP302442-CG04-3-10-GC, GeneCopoeia) and control (CCPCTR-CG04-10-GC, GeneCopoeia) were purchased from BioCAT (Germany). The interference sequences and blank sequences with CRISPR/Cas9 technology are shown in Table 3.

Table 3 The interference sequence

Knockdown target	Sequence（5′-3′）
Control	GGCTTCGCGCCGTAGTCTTA
MMP-14-knockdown (KD)1	ACAACGGGGGGGTCTTGGGG
MMP-14-KD2	CTGCTCCCCCTGCTCACGCT
MMP-14-KD3	UTGCTGCTTTGGGCCGAGCCG

2.1.8 Main reagents

Reagents	Manufacturer
Ultra pure water	Biochrom, Germany
Trypan Blue Stain (0.4%)	Gibco, U.S.A.
MEM medium	Bio-Sell, Germany
RPMI medium (1640)	Gibco, U.S.A.
Opti-MEM medium	Gibco, U.S.A.

Reagents	Manufacturer
DMSO	Sigma, U.S.A.
Methanol	Merck, Germany
Ethanol	Serva, Germany
Fetal bovine serum	Bio-sell, Germany
Penicillin-Streptomycin-Mix	Bio-sell, Germany
Non Essential Amino Acids	Bio-sell, Germany
Natrium Pyruvate	Bio-sell, Germany
Geneticin Selective Antibiotic (G418)	Thermo Scientific, U.S.A.
EndoFectin™ Max transfection reagent	GeneCopoeia, Germany
Phosphate Buffered Saline	Gibco, U.S.A.
TrypLE™ Express	Gibco, U.S.A.
Cytotoxicity Detection Kit Plus (LDH)	Roche, Germany
Cell Death Detection ELISA Plus	Roche, Germany
Human MMP-14 activity assay	QuickZyme, Netherlands
Colorimetric Cell Viability Kit (CCK-8)	PromoKine, Germany
TBS	Roth, Germany
Tris-aminomethane-hydrochloride	MERCK, Germany
Tris-base	Sigma, U.S.A.
Tween 20	Serva, Germany
Glycine	Roth, Germany
Ammonium Persulfate (APS)	Bio-Rad, Japan
Roti-Block 10X	Roth, Germany
ECL advance blocking agent	Merck, U.K.
Western Blot Stripping Buffer	Thermo Scientific, U.S.A.
TEMED	Roth, Germany
40% Acrylamide/Bis-acrylamide	Roth, Germany
Protease Inhibitor Cocktail	Cell Signaling Technology, U.S.A.
PhosSTOP	Roche, Germany
Paraformaldehyde	Sigma, U.S.A.
BSA	Sigma, U.S.A.
Bisbenzimide	Sigma, U.S.A.
Mounting medium	Thermo Scientific, U.S.A.

Reagents	Manufacturer
Human Phospho-Kinase Array	R&D systems, U.S.A.
Pierce BCA Protein Assay Kit	Thermo Scientific, U.S.A.
Rabbit anti-MMP-14 antibody (ab51074)	Abcam, U.S.A.
Rabbit anti-PD-L1 antibody (#13684)	Cell Signaling Technology, U.S.A.
Mouse anti-STAT3 antibody (#9139)	Cell Signaling Technology, U.S.A.
Rabbit anti-Phospho-Stat3 antibody (Tyr705) (#9145)	Cell Signaling Technology, U.S.A.
Rabbit anti-GAPDH antibody (#5174)	Cell Signaling Technology, U.S.A.
Mouse anti-β-actin antibody (A5441)	Sigma, U.S.A.
Goat anti-rabbit HRP antibody (P0448)	Dako, Germany
Rabbit anti-mouse HRP antibody (sc-2357)	Dako, Germany
Goat anti-rabbit Alexa Fluor 594 antibody (A-21207)	Invitrogen, U.S.A.
Colivelin	Tocris, U.S.A.
HO-3867	Selleckchem, U.S.A.
NSC405020	Merck, Germany

2.1.9 Main supplies

Supplies	Manufacturer
Tips (0.1-10, 10-100, 100-1000µl)	Eppendorf, Germany
Tubes (0.5, 1.5, 2.0ml)	Eppendorf, Germany
Plastic tips (1, 2, 5, 10, 25, 50ml)	Corning, U.S.A.
Microscope glass slides	Carl Roth, Germany
Wet boxes	Servicebio, China
RS glass slides (8 well)	Thermo Scientific, U.S.A.
Beakers (1, 2, 5, 10, 100, 500, 1000, 2000ml)	Cole-Parmer, Germany
Graduated cylinders (10, 50, 100, 500, 1000ml)	Stonylab, Germany
Glass bottles (50, 100, 500ml)	Sigma, Germany
Cell culture flasks (25, 75, 175 cm^2)	Corning, Germany
Multi-well plates (6, 12, 24, 96 wells)	Corning, Germany

Supplies	Manufacturer
Centrifugal tubes (15, 50ml)	Corning, Germany
Frozen tubes (1.0, 1.5ml)	Thermo Scientific, China
Syringes (25, 50, 200μl)	Hamilton, Switzerland
PVDF membranes (0.2, 0.45μm)	Merck, Germany
CASSETTES (1.0mm)	Novex, U.S.A.
Thick blot paper	BIO-RAD, U.S.A.
Cell counting chamber slides	Invitrogen, Germany

2.1.10 Main equipment

Equipment	Manufacturer
Micropipettes (0.1-10, 10-100, 100-1000μl)	Eppendorf, Germany
Electric pipettes	Greiner bio-one, Germany
Horizontal shaker	LTF Labortechnik, Germany
Vortex Oscillator	Scientific Industries, Germany
Thermomixer	Eppendorf, Germany
Precision electronic scale	Sartorius, Germany
-80℃ refrigerator	Thermo Scientific, U.S.A.
-20℃ and 4℃ refrigerator	Liebherr, Germany
Constant temperature oven	Biometra, Germany
Low-speed centrifuge	Eppendorf, Germany
High-speed centrifuge	Eppendorf, Germany
Ice maker	Ziegra, Germany
Western blot Electrophoresis instrument	Novex, U.S.A.
Western blot Transfer Unit	Amersham Pharmacia Biotech, U.S.A.
Western blot Power Supply	Bromma, Germany
Ultra-clean workbench	Thermo Scientific, U.S.A.
Ultrapure water Purification Filter	Millipore, U.S.A.
Cell culture incubator	Binder, Germany
Western blot HRP-chemiluminescence imager	Peqlab, Germany

Equipment	Manufacturer
Sonicator	Bandelin, Germany
Cell counter	Life technologies, Germany
pH Reader	Bench Meter, Germany
Horizontal rotary mixer	Intelli, Germany
Constant temperature water bath	Julabo, Germany
Multimode Microplate Reader	Berthold technologies, Germany
Fluorescence Optical Microscopy	Olympus, Japan
Normal Optical Microscope	Olympus, Japan

2.1.11 Main solutions and buffers

1. 1M Tris-buffer pH 6.8

Reagent	Amount/Volume
Tris-base	12.114g
5N HCL	18ml
double distilled water	Made up to 100ml (adjusted pH= 6.8)

After being fully dissolved and mixed, it was stored at room temperature.

2. 1M Tris-buffer pH 8.8

Reagent	Amount/Volume
Tris-base	30.29g
5N HCL	7ml
double distilled water	Made up to 250ml (adjusted pH= 8.8)

After being fully dissolved and mixed, it was stored at room temperature.

3. 500mM EDTA pH 8

Reagent	Amount/Volume
EDTA	9.3g
5N NaOH	4ml
double distilled water	Made up to 50ml (adjusted pH= 8)

After being fully dissolved and mixed, it was stored at room temperature.

4. 10% SDS solution

Reagent	Amount/Volume
SDS	25g
double distilled water	Made up to 250ml

After being fully mixed, it was stored at room temperature.

5. 50× PhosSTOP solution

Reagent	Amount/Volume
PhosSTOP	1 tablet
double distilled water	Made up to 200 μL

After being fully dissolved, it was stored at -20℃.

6. Protein lysis buffer

Reagent	Amount/Volume
1M Tris-buffer pH 6.8	25μL
10% SDS	50μL
500mM EDTA pH 8	1μL
Protease inhibitor cocktail	5μL
50× PhosSTOP solution	10 μL
double distilled water	Made up to 500 μL

After being fully mixed, it was stored at room temperature.

7. 1×TBS buffer

Reagent	Amount/Volume
TBS	10 tablets
double distilled water	Made up to 5000ml

After being fully dissolved, it was stored at room temperature.

8. 1×TBST buffer

Reagent	Amount/Volume
1×TBS buffer	5000ml
0.1% Tween-20	5ml

After being fully mixed, it was stored at room temperature.

9. 4×SDS-PAGE sample buffer

Reagent	Amount/Volume
SDS	2g
Glycerin	5g
1M Tris-buffer pH 6.8	5ml
Bromophenol Blue	8mg
DTT	77.1mg
double distilled water	20ml

After being fully dissolved and mixed, it was stored at -20℃.

10. 10% Ammonium persulfate

Reagent	Amount/Volume
Ammonium persulfate	100mg
double distilled water	Made up to 1ml

After being fully dissolved, it was stored at -20℃.

11. 10×Western blotting buffer

Reagent	Amount/Volume
Tris-base	30.28g
Glycine	144.13g
double distilled water	Made up to 1000ml

After being fully dissolved, it was stored at 4℃.

12. 1× Electrophoresis buffer

Reagent	Amount/Volume
10×Western blotting buffer	80ml
10% SDS	8ml
double distilled water	Made up to 800ml

After being fully mixed, it was placed at room temperature and prepared for immediate use.

13. 1× Transfer buffer

Reagent	Amount/Volume
10×Western blotting buffer	10ml
Anhydrous methanol	10ml
double distilled water	Made up to 100ml

After being fully mixed, it was placed at room temperature and prepared for immediate use.

14. Roti Blocking Buffer

Reagent	Amount/Volume
10×Roti blocking reagent	1ml
double distilled water	Made up to 10ml

After being fully mixed, it was placed at room temperature and prepared for immediate use.

15. ECL advanced Blocking Buffer

Reagent	Amount/Volume
ECL advanced Blocking Powder	0.2g
1×TBST buffer	Made up to 10ml

After being fully dissolved, it was placed at room temperature and prepared for immediate use.

16. 3% Paraformaldehyde solution

Reagent	Amount/Volume
16% Paraformaldehyde	1400μL
PBS	Made up to 7.5ml

After being fully mixed, it was placed at room temperature and prepared for immediate use.

17. 3% BSA solution

Reagent	Amount/Volume
BSA	440mg
PBS	Made up to 14.7ml

After being fully dissolved, it was placed at room temperature and prepared for immediate use.

18. Complete cell culture medium (500ml)

Reagent	Amount/Volume
Penicillin-streptomycin solution	5ml
fetal bovine serum(10%/20%)	50ml/100ml
Incomplete medium (DMEM/1640)	Made up to 500ml

After being fully mixed, it was stored at 4°C.

19. Freezing medium for HCV-29, RT-4 and RT-112 (10ml)

Reagent	Amount/Volume
complete cell culture medium	7ml
fetal bovine serum	2ml
DMSO	1ml

After being fully mixed, it was stored at 4°C in the dark.

20. Freezing medium for UM-UC-3 (10ml)

Reagent	Amount/Volume
complete cell culture medium	9.5ml
DMSO	0.5ml

After being fully mixed, it was stored at 4°C in the dark.

21. Freezing medium for HT-1376 (10ml)

Reagent	Amount/Volume
complete cell culture medium	4.25ml
ProFreeze™-CDM,NAO, Chemically Defined Freeze Medium (2X)	4.25ml
DMSO	1.5ml

After being fully mixed, it was stored at 4°C in the dark.

22. HO-3867 stock solution (10mM)

Reagent	Amount/Volume
HO-3867	10mg
DMSO	2.15ml

After being fully dissolved (warmed with 50°C water bath), it was stored at -80°C.

23. Colivelin stock solution (1mg/ml)

Reagent	Amount/Volume
Colivelin	1mg
20% Ethanol	1ml

After being fully dissolved, it was stored at -20°C.

24. NSC405020 stock solution (50mM)

Reagent	Amount/Volume
NSC405020	25mg
DMSO	1.92ml

After being fully dissolved, it was stored at -80°C.

2.1.12 Main software

Software	Manufacturer
Adobe Illustrator 2020	Adobe, U.S.A.
Adobe Photoshop 2020	Adobe, U.S.A.
Image J 1.53	National Institutes of Health, U.S.A.
Microsoft Office 2019	Microsoft, U.S.A.
Microsoft Windows 10	Microsoft, U.S.A.
SPSS 21.0	SPSS, U.S.A.
CorelDRAW 2019	Corel, Canada
EndNote X9®	Thomson Reuters, U.S.A.
GraphPad Prism 9.0	GraphPad, U.S.A.
RStudio 1.4	RStudio, U.S.A.
R 3.6.3	R foundation, U.S.A.

2.2 Methods

2.2.1 Research condition of MMP-14 in various tumors

The Oncomine web server was used to assess the research condition of MMP-14 expression in various human cancer tissues and the corresponding normal tissues. After logging into the Oncomine database, we inputted the following search criteria: (1) Gene:

MMP-14, (2) Data type: mRNA, (3) screening criteria: p value < 0.01, fold change > 2, gene ranks = all ranks.

2.2.2 Expression analysis of MMP-14 mRNA in various tumors

R (version 3.6.3) was used to analyze the expression level of MMP-14 in 33 tumor tissues and their relative unpaired normal tissues in the TCGA database. The differences between the two tissues were analyzed using the Wilcoxon rank-sum test. P <0.05 indicates statistical significance. Furthermore, we analyzed the expression level of MMP-14 in 18 tumor tissues and the corresponding adjacent normal tissue in the TCGA database. We used the Wilcoxon rank-sum test to examine the difference between the two, with p<0.05 representing statistical significance. ggplot2 [version 3.3.3][197] was used for data visualization.

2.2.3 The relationship between the expression of MMP-14 and patients' prognosis with urological tumors

We extracted the expression datasets of urological tumors (BLCA, KICH, KIRC, KIRP, PRAD) and the corresponding patients' prognosis data from the TCGA database using R (version 3.6.3). We divided them into two groups based on the median expression of MMP-14 (MMP-14 high expression group and MMP-14 low expression group). The samples with MMP-14 expression lower than the median value were included in the MMP-14 low expression group (the sample with median value was included in the MMP-14 low expression group), and the samples with MMP-14 expression higher than the median value were included in the MMP-14 high expression group. We used the Kaplan-Meier method to draw the survival curves, and the log-rank test was used to compare survival between the two groups. p<0.05 represented statistical significance. ggplot2 [version 3.3.3] was used for data visualization.

2.2.4 The relationship between the expression of MMP-14 and the patients' clinicopathological features in bladder cancer

We extracted the expression dataset of bladder cancer and the corresponding clinicopathological data from the TCGA database with R (version 3.6.3). We divided them into

two groups based on the median expression of MMP-14 (MMP-14 high expression group and MMP-14 low expression group). The samples with MMP-14 expression lower than the median value were included in the MMP-14 low expression group (the sample with median value was included in MMP-14 low expression group), and the samples with MMP-14 expression higher than the median value were included in the MMP-14 high expression group. Additionally, the Chi-square test or Fisher's exact test was used to analyze the correlation between clinicopathological factors (age, gender, race, height, weight, BMI, smoker, T stage, N stage, M stage, histological grade, pathological stage, lymphovascular invasion, subtype, radiation therapy, primary therapy outcome, PFI event, and overall survival event) and MMP-14 expression. In addition, the above clinicopathological features which correlated with the expression of MMP-14 were screened out. The expression levels of MMP-14 with different clinicopathological characteristics were compared using the t-test or one-way ANOVA test. A p value <0.05 represented statistical significance. ggplot2 [version 3.3.3] was used for data visualization.

2.2.5 Selection of differentially expressed genes according to the expression level of MMP-14 and screening of MMP-14 expression-related genes

We extracted the expression dataset of bladder cancer from the TCGA database using R (version 3.6.3). We divided it into two groups based on the median expression of MMP-14 (MMP-14 high expression group and MMP-14 low expression group). The samples with MMP-14 expression lower than the median value were included in the MMP-14 low expression group (the sample with median value was included in the MMP-14 low expression group), and the samples with MMP-14 expression higher than the median value were included in the MMP-14 high expression group. DESeq2[198] was used to screen DEGs between the two groups. The screening criteria were |log2(fold change)| > 1 and adjusted p value < 0.05. In addition, Pearson correlation analysis was used to screen the genes significantly correlated with MMP-14 expression in bladder cancer, and the screening condition was: |the correlation coefficient|≥0.3, p value < 0.05. Furthermore, we used Venn diagrams to identify the common genes of MMP-14 correlated genes and DEGs.

2.2.6 Gene functional enrichment analysis

We used the clusterProfiler package [version 3.14.3][199] to perform Gene Ontology (GO) analysis[200] of the shared genes of MMP-14 expression-related genes and DEGs (including cellular component, molecular function, biological process), along with the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis[201], and p values for multiple hypothesis tests were corrected using the Benjamini and Hochberg method, with a q value < 0.2. The org.Hs.eg.db package [version 3.10.0][202] was used for ID conversion, and the GOplot package [version 1.0.2][203] was used to calculate Z-scores. GSEA [204] was performed using the molecular tag database MSigDB Collections (https://www.gsea-msigdb.org/gsea/msigdb/collections. jsp#C2), and the feature gene dataset c2.cp.v7.2.symbols. The Gmt [Curated] dataset was analyzed, and the enrichment was defined as significant if there was a False discovery rate (FDR) < 0.05 and a p adjust value < 0.05.

2.2.7 Prediction of MMP-14 transcription factors

In this study, we used the Knock TF database and hTFtarget database to predict transcription factors of MMP-14 and used Venn diagrams to filter out the public transcription factors in the two databases. Furthermore, Spearman correlation analysis was used to analyze the expression correlation of MMP-14 with public transcription factors in bladder cancer from the TCGA database.

2.2.8 Immunoinfiltration analysis of MMP-14

ssGSEA was used for immune infiltration analysis of MMP-14. Single sample gene set enrichment analysis [205] is designed for single samples, and is an algorithm used for immune deconvolution analysis to estimate the abundance of immune cell types, T-cell infiltration score, immune infiltration score, and proportion of immune cells. In this study, we used the GSVA software package [version 1.34.0][206] to detect the correlation between MMP-14 expression and the abundance of CD8+ T cells and Treg cells in bladder cancer.

2.2.9 Correlation of MMP-14 with immune cell markers

With the TIMER database, we analyzed the correlation of MMP-14 with $CD8^+$ T cell markers (CD8A and CD8B), Treg cell markers (FOXP3, CD25 (IL2RA), CCR8), and T cell exhaustion markers (PD-1 (PDCD1), CTLA4, LAG3, TIM-3 (HAVCR2)) in bladder cancer, and correlations were corrected using tumor purity. In addition, the GEPIA database was used to analyze the association of MMP-14 with $CD8^+$ T cell markers (CD8A and CD8B), Treg cell markers (FOXP3, CD25(IL2RA), CCR8), and T cell exhaustion markers (PD-1 (PDCD1), CTLA4, LAG3, TIM-3 (HAVCR2)) in bladder cancer tissue and normal bladder tissue, and correlation analysis was performed by Pearson correlation analysis[199].

2.2.10 Cell culture

Normal bladder epithelial cells (HCV-29) and bladder cancer cells (RT-4, RT-112) were cultured in RPMI-1640 cell complete medium with 10% fetal bovine serum and 1% Penicillin-Streptomycin, UM-UC-3 bladder cancer cells were cultured in DMEM cell complete medium with 10% fetal bovine serum and 1% Penicillin-Streptomycin, and HT-1376 bladder cancer cells were cultured in DMEM cell complete medium with 20% fetal bovine serum and 1% Penicillin-Streptomycin. All cells were incubated at 37℃ and 5% CO_2, and the medium was changed every 24 - 72h regularly.

2.2.11 Cell passage

1. When cell fusion reached 70-80%, the old medium was aspirated and cells were washed 1-2 times with PBS.
2. 3 ml Tryple Express was added and the cells were digested in the incubator for the appropriate time. When cells were observed as rounded under a microscope and detached from the flask wall, 5 ml PBS was added to neutralize their digestion, it was pipetted repeatedly, and the cell suspension was transferred to a 15 ml centrifuge tube and centrifuged at 300 g for 4 min.
3. The supernatant was aspirated, 5ml of complete culture medium was added, it was pipetted repeatedly to prevent the cells from clumping together, and the cell suspension was aspirated into new 75mm cell culture flasks (each flask was pre-filled with 13ml of complete culture medium) according to the appropriate dilution ratio, the culture flask was

gently shaken to make the cells evenly distributed, the cell name was recorded as well as the passaging date, and the incubation was continued in the cell culture incubator.
4. We observed the condition of the cells under the microscope the next day.

2.2.12 Cell freezing

1. The cells were digested with Tryple and centrifuged.
2. The supernatant was discarded after centrifugation, 1-1.2 ml of cell freezing solution was added, and it was pipetted repeatedly to prevent the cells from forming clumps.
3. The cell suspension was transferred to a sterile lyophilization tube, the name of the frozen cells was recorded and their freezing time, they were stored in a freezer box at -80°C for 24h, and the cells were subsequently stored in liquid nitrogen for long-term storage.

2.2.13 Cell thawing

1. The frozen cells were removed from the liquid nitrogen tank and quickly placed in a water bath at 37°C for 2-3 min.
2. After complete thawing, the cell suspension was transferred to a 15 ml sterile centrifuge tube and 2-3 ml of pre-warm complete medium was added, and it was pipetted repeatedly and centrifuged at 300 rpm for 4 min.
3. The supernatant was discarded after centrifugation, 3ml of complete medium was added, it was pipetted repeatedly, and the cell suspension was transferred to a 75mm cell culture flask (the flask was pre-filled with 10ml of complete medium), the names of the thawed cells and the thawing time were recorded, and they were placed in the cell culture incubator.
4. We observed the cell status and changed the cell culture medium the next day.

2.2.14 Determination of half maximal inhibitory concentration of NSC405020 and HO-3867 in bladder cancer cells

1. Bladder cancer cells in the logarithmic growth phase were seeded into 96-well plates at appropriate cell numbers (UM-UC-3:3000, RT-4, RT-112, HT-1376:4000).

2. After 24 h, the normal medium was replaced by the corresponding medium containing different concentrations of NSC405020 (0, 30, 60, 80, 100, 120, 150, 200, 400, 600 μmol/L) and HO-3867 (0, 0.5, 1, 2, 5, 10, 15, 20 μmol/L), 5 replicate sample wells were set for each concentration group, and PBS was added into the control groups.
3. After incubation for 48 h in the cell incubator, 10 μL of CCK-8 reagents were added to each well and incubated at 37°C for 2 h. The absorbance value of each well was read at 450 nm using a Multimode Microplate Reader. Each cell's IC50 values for NSC405020 and HO-3867 were calculated, taking the IC50 values as the treatment concentration in the subsequent experiment.

2.2.15 Treatment with Colivelin in bladder cancer cells

1. The bladder cancer cells were seeded into T75 cell culture flasks at the appropriate cell density (50-60%).
2. After 6-8h, when the cells were fully plastered, the medium was replaced with a serum-free medium for cell culture.
3. After 24h of culturing cells in serum-free medium, the cells were treated with serum-free medium containing Colivelin (2μmol/L) for 2h.

2.2.16 Establishment of stably transfected bladder cancer cell lines

1. HT-1376 bladder cancer cells with a relatively high level of MMP-14 expression were selected for knockdown plasmid transfection.
2. When the cells were adjusted to reach 50-60% fusion, the cells were transfected with different fluorescent plasmids according to the corresponding transfection protocol.
3. After 24 hours of transfection, fluorescence microscopy cells were observed for fluorescence intensity in different cells to understand the status of plasmid transfection in individual cells.
4. Transfected cells were screened using a medium containing Geneticin (500μg/mL), and growing clones of cells resistant to geneticin were selected for further cell culture.
5. The efficiency of MMP-14 knockdown was detected by Western blot, and the cell clones with the highest knockdown efficiency were selected for subsequent experiments.
6. Stably transfected cell lines were maintained in a medium containing geneticin (500μg/mL) for cell culture.

2.2.17 Cell proliferation assay

1. Bladder cancer cells were seeded into 96-well plates at the appropriate cell number (UM-UC-3:1500 /per well, RT-4, RT-112, HT-1376: 2000 /per well).
2. After 24h, cells were treated with HO-3867 or NSC405020 at the corresponding IC50 concentration in the treatment group, and PBS was added to the control group. 5 replicate wells were set up for each group.
3. The cell viability was measured at 0h, 24h, 48h, and 72h after treatment. Before each measurement of cell viability, 10ul CCK-8 reagent was added to each well and incubated for 2h at 37°C.
4. The absorbance was measured at 450 nm with a Multimode Microplate Reader, and the values were recorded.
5. Cell growth curves were plotted based on the cell activity values at 3 different time points.

2.2.18 Extraction of cellular proteins

1. We prepared a protein lysate containing protease inhibitors and phosphatase inhibitors.
2. When the cell density reached 70%~80%, the old culture medium was discarded, the cells were washed with cold PBS 3 times, the PBS was aspirated, 200μL of protein lysate was added for each T75 cell culture flask, the cells were scraped using cell scrapers, and they were collected in 1.5ml Protein LoBind® tubes.
3. The lysate was sonicated and we subsequently put the lysis on ice for 30 minutes; after centrifugation at 14,000g for 10 minutes at 4°C, the supernatant was transferred to a new 1.5ml Protein LoBind® tube.
4. We diluted 2 mg/mL of BSA protein standard into 0, 0.125, 0.25, 0.5, 0.75, 1, 1.5, and 2 mg/mL for making the standard curve, and the tested samples were also diluted 5 times with lysis buffer, then the standard samples and test samples were added into the 96-well plate.
5. We prepared a compatibility reagent solution using reconstitution buffer, added 4μL of compatibility reagent solution to each well, and incubated them for 15 min at 37°C.
6. We prepared the appropriate amount of BCA working solution (BCA Reagent A: BCA Reagent B = 50:1) according to the number of samples, and mixed them thoroughly. We

added 260 µL of BCA working solution to each well, and incubated them for 30 min at 37°C.

7. We measured the absorbance of each well at A560 nm with a Multimode Microplate Reader, and calculated the protein concentration of the samples based on the standard curve.

8. We stored the sample in a -80℃ refrigerator.

2.2.19 Western blotting

1. The 10% SDS-PAGE gel, electrophoresis solution, transfer solution, and TBST buffer were configured according to the protocols.

Table 4 Protocol for 10% separating gel (1gel)

Component	Volume
double distilled water	2.7 ml
1M Tris pH8.8	2.8 ml
10% SDS	75 µL
40% Acrylamide/Bis-acrylamide	1.88 ml
10% APS	75 µL
TEMED	4 µL

Table 5 Protocol for 5% stacking gel (1gel)

Component	Volume
double distilled water	1.8 ml
1M Tris pH6.8	313 µL
10% SDS	25 µL
40% Acrylamide/Bis-acrylamide	350 µL
10% APS	25 µL
TEMED	3 µL

2. We thawed the protein samples, and adjusted the protein concentration by adding lysis buffer to ensure that each sample had the same loading protein amount (15µg). Then, for

each sample, we added the appropriate volume of 4X SDS loading buffer, heated them at 95°C for 5 minutes, and placed them on ice for further use.

3. We added the protein samples with an equal volume into each well and set up the marker.

4. We used 60V constant voltage electrophoresis for 30min and then switched to 120V constant voltage electrophoresis for 150min.

5. We activated the PVDF membrane with methanol for 1min.

6. We transferred the protein to the PVDF membrane with a constant current of 45mA/per gel for 90min.

7. We blocked the PVDF membrane for 1h at room temperature using a suitable blocking solution (Roti Blocking Buffer or ECL Advanced Blocking Buffer).

8. We washed the PVDF membrane 3 times with TBST for 5 min each time.

9. We incubated the PVDF membrane overnight at 4°C with the diluted primary antibody.

10. We washed the PVDF membrane 3 times with TBST for 5 min each time.

11. Incubation was carried out with the corresponding secondary antibody labeled with HRP for 1h at room temperature.

12. We washed the PVDF membrane 3 times with TBST for 5 min each time.

13. We incubated the membrane with an ECL developer and used UVP exposer for photography.

14. Quantitative analysis of the bands in the photographs was performed using ImageJ.

Table 6 Dilution ratio of antibodies for Western blotting

Antibody	Diluted ratio
MMP-14	1:5000
STAT-3	1:2000
p-STAT3	1:1000
PD-L1	1:2000
β-actin	1:10000
GAPDH	1:2000
Goat anti-rabbit HRP antibody	1:2000
Rabbit anti-Mouse HRP antibody	1:2000

2.2.20 MMP-14 activity assay

1. Cells were grown in 24-well plates, and when cell density reached 50%, the experimental group was treated with medium containing HO-3867 or NSC405020 at the IC50 concentration for 24h or 48h, while the control group was cultured with normal medium.
2. After 24h or 48h of treatment, we removed the medium and replaced it with 500 µl extract buffer per well. It was incubated at 4 ºC for 15 minutes and slight shaking was performed.
3. We transferred the extract to a 1.5ml tube, and centrifuged the extract at 12,000g for 5 minutes at 4 ºC。
4. We transferred the supernatant into a new tube, diluted it 10 times with extract buffer, and put it on the ice.
5. We planned for enough wells to run all zeros (blanks), standards, and samples as needed on the microplate.
6. We added the MMP-14 antibody into the desired wells and incubated the microplate in a humidified room at 37°C for 2 hours.
7. We removed the coated antibody and pipetted the blocking buffer into the wells, and incubated the microplate at 25 °C for 2 hours.
8. We washed the wells 4 times with washing buffer and placed the microplate on ice.
9. We made different concentrations of standard samples according to the protocol, added the standard sample and the tested sample to the wells, and incubated the microplate overnight in a humidified room at 4 °C.
10. We washed the wells four times with washing buffer and pipetted the assay buffer into the wells, then incubated them at 37°C for 2 hours in a humid environment.
11. Near the end of the incubation time, we prepared the detection reagent according to protocol and pipetted the detection reagent into the wells.
12. We read the microplate at 405 nm to obtain values at t = 0h.
13. We incubated the microplate for 24 hours at 37°C in a humid environment.
14. We read the microplate at 405 nm to obtain values at t = 24h.
15. We drew standard curves with the standard sample, and then the A405 value of the tested sample could be calculated as a MMP-14 concentration.

2.2.21 Immunofluorescence

1. We seeded the cells into 8-well plates on RS glass slides and incubated them for 48h to reach 50-70% of cell density.
2. We washed the cells gently twice using pre-cooled PBS.
3. We fixed the cells using 3% paraformaldehyde at room temperature for 20 min.
4. We washed the cells gently with PBS once.
5. We blocked the cells with 3% BSA at room temperature for 60 min.
6. We washed the cells gently with PBS once.
7. We diluted the primary antibody according to a reasonable ratio and incubated the cells overnight at 4 °C with the diluted primary antibody.
8. We washed the cells gently with PBS 3 times.
9. We incubated the fluorescently labeled secondary antibody for 1h at room temperature and protected the slides from light.
10. We washed the cells gently with PBS 2 times.
11. We added bisbenzimide into the cells and incubated the cells for 5min.
12. We mounted the cells with a fluorescence mounting medium.
13. We observed the fluorescence intensity under a fluorescence microscope and took photographs.

Table 7 Dilution ratio of antibodies for immunofluorescence

Antibody	Diluted ratio
MMP-14	1:100
PD-L1	1:50
Goat anti-rabbit Alexa Fluor 594 antibody	1:500

2.2.22 Human phospho-kinase antibody array assay

1. We seeded HT-1376 cells into a T75 cell culture flask at the appropriate cell density (60-70%). After cell apposition, we treated the cells with the corresponding IC50 concentration of NSC405020 in the treatment group and added PBS for the control group.
2. After 24h of treatment, we extracted cellular protein and measured protein concentration.

3. We placed each membrane into separate compartments, added blocking buffer into each compartment and incubated them for 1h at room temperature.
4. We added the protein extracts (amount: 400 µg volume: 1 ml) from the treatment group and control group into the different compartments containing the membranes, and incubated them overnight at 4 °C on a rocking platform shaker.
5. We washed each membrane 3 times with 10 ml of washing buffer; each lasted 10 min.
6. We added 1 ml of detection antibody cocktail to the membrane compartment and incubated them for 2 hours at room temperature on a rocking platform.
7. We washed each membrane 3 times with 10 ml of washing buffer; each lasted 10 min.
8. We pipetted 1.0 mL of streptavidin-HRP into each well and incubated them for 30 minutes at room temperature on a rocking platform.
9. We washed each membrane 3 times with 10 ml of washing buffer; each lasted 10 min.
10. We applied the Chemi Reagent Mix and exposed it to film.
11. We performed quantitative analysis of the images using ImageJ.

2.2.23 Cell death detection assay

1. We seeded the cells into 96-well plates, and when cell density reached 50%, the experimental group was treated with medium containing an IC50 concentration of HO-3867 or NSC405020 for 24h or 48h, while the control group was cultured with normal medium.
2. After treatment, we centrifuged the 96-well plate at 200 x g for 10 min and removed the cell culture medium.
3. We incubated the cells for 30 min on a rocking platform at room temperature using 200 µl of cell lysate.
4. We centrifuged the cell lysate at 200 x g for 10min and transferred the supernatant to a new tube.
5. We planned enough wells on the microtiter plate to run positive control, background control, and samples under test measurements as needed, and prepared immune reagents according to the protocol.
6. We carefully transferred 20 µl of positive control, background control, and sample under test to the streptavidin-coated microtiter plate.
7. We added 80 µl of immune reagent to each well, shook them gently on a shaker, and incubated them for 2 hours at room temperature.
8. We washed each well with incubation buffer 3 times for 30 seconds each.

9. We transferred 100 µl ABTS solution into each well and incubated them on a plate shaker at 250rpm for 15 minutes.
10. We aspirated 100 µl ABTS termination solution into each well.
11. We read the microtiter plate at 405nm to obtain the values.
12. We calculated the apoptotic enrichment of the test samples (apoptotic enrichment = absorbance of the treated group/absorbance of the corresponding control group).

2.2.24 Cell cytotoxicity assay

1. We planned enough wells to run the high control wells, low control wells, and test samples as needed on the microplate.
2. We seeded the cells into 96-well plates at 50% cell density.
3. For the experimental group, we treated the cells with a medium containing an IC50 concentration of HO-3867 or NSC405020 for 6h, 24h, or 48h, and added a normal medium for the control group.
3. For each high control well, we added 5 µl of lysis buffer and incubated them for 15 min at 37°C until the end of the incubation time.
4. We prepared the reaction mixture, added 100µl of reaction solution into each well, incubated for them 15min at room temperature, and protected them from light.
5. We added 50µl of stop solution into each well to terminate the reaction and shook the plate on the shaker for 10 seconds.
6. We read the microplate at 492 nm to obtain the value of each well.
7. We calculated the percentage of cytotoxicity of each sample according to the following formula: cytotoxicity (%)=(sample. value- the corresponding low control. value) / (the corresponding high control. value- the corresponding low control. value)*100.

2.2.25 Statistical analysis

All experimental data represent the mean of 3 independent experiments, and the error line represents the standard deviation of the mean. The data were statistically analyzed using SPSS 21.0 or GraphPad Prism 9.0 software and tested for normality by the Shapiro-Wilk test. The Mann-Whitney U test or Wilcoxon signed-rank test was used for nonparametric analyses, and parametric tests were performed using the Student test. Comparison among multiple groups was performed using one-way ANOVA, and Bonferroni or

Dunnett post hoc tests for correction. Association analysis of categorical variables was carried out by Chi-square test or Fisher's exact test. Correlation of continuous variables was analyzed by Pearson or Spearman correlation analysis. We defined a p value < 0.05 (*), < 0.01 (**) and < 0.001 (***) as significant differences, while n.s. indicated a non-significant p-value.

3. Results

3.1 Expression of MMP-14 in bladder cancer and its clinical significance

3.1.1 Expression of MMP-14 in a variety of tumor tissues

We used the Oncomine database to query MMP-14 mRNA expression in various tumor studies (Figure 10A). The results showed that MMP-14 showed significantly high expression in all studies of tumors (including bladder cancer, colorectal cancer, esophageal cancer, gastric cancer, head and neck cancer, leukemia, lung cancer, lymphoma, melanoma, ovarian cancer, pancreatic cancer, and sarcoma). However, MMP-14 showed relatively low expression in liver cancer. In addition, for brain and CNS cancer, breast cancer, cervical cancer, kidney cancer, and prostate cancer, MMP-14 showed different expression profiles in different studies. In brain and CNS cancer, MMP-14 showed relatively high expression in 6 studies and relatively low expression in 1 case. In breast cancer, 9 cases had relatively high expression of MMP-14, and 6 cases had relatively low expression of MMP-14. In cervical cancer and prostate cancer, high and low expression of MMP-14 accounted for 1 case, respectively. In kidney cancer, high and low expression of MMP-14 accounted for 5 cases, respectively. Next, we explored the expression of MMP-14 in various tumors using the TCGA database (Figure 10B). The results showed that for unpaired normal tissues, MMP-14 was significantly overexpressed in BLCA, BRCA, CHOL, COAD, ESCA, GBM, HNSC, KIRC, KIRP, LIHC, LUAD, LUSC, READ, STAD and THCA ($p<0.05$), while significantly downregulated in KICH, PRAD, and UCEC ($p<0.05$), and there was no significant difference between normal and tumor tissues in CESC, PAAD, and PCPG. Furthermore, for paired normal tissues (Figure 10C), the expression levels of MMP-14 were also significantly increased in BLCA, BRCA, CHOL, COAD, ESCA, HNSC, KIRC, KIRP, LIHC, LUAD, LUSC, READ, STAD and THCA ($p<0.05$), while significantly downregulated in KICH and PRAD ($p<0.05$), and they showed no significant difference in PAAD and UCEC.

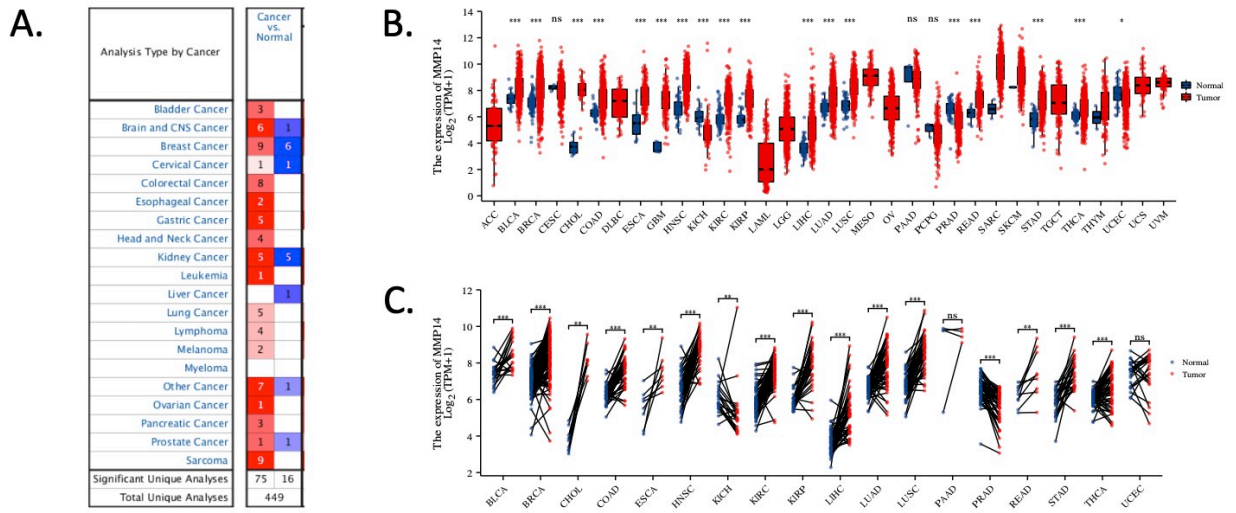


Figure 10 The expression levels of MMP-14 in various tumors.

A. Study conditions of MMP-14 in different tumors from the Oncomine database. Red represents studies with relatively high expression of MMP-14 in tumors, and blue represents studies with relatively low expression of MMP-14 in tumors compared to corresponding normal tissue. Differences were assessed according to the following screening criteria: p-value <0.01, abs|Fold Change| >2, including all gene ranks (100%). B. Expression levels of MMP-14 in multiple tumor tissues versus corresponding normal tissues (unpaired) in the TCGA database, red for tumor tissues and blue for normal tissues, significant results were determined by Mann-Whitney U test. C. Expression levels of MMP-14 in different tumor tissues versus paired normal tissues in the TCGA database, significant results were determined by Wilcoxon signed-rank test. Red represents tumor tissues, and blue represents normal tissues. *p<0.05, **p<0.01, ***p<0.001, ns: no significance.

3.1.2 Relationship between MMP-14 expression and prognosis of urological tumors

To analyze the relationship between MMP-14 expression and the prognosis of urological tumors, we analyzed the prognostic information of patients collected in the TCGA database and plotted Kaplan-Meier survival curves based on MMP-14 expression information (Figure 11). The results showed that for overall survival, bladder cancer patients with high MMP-14 expression had shorter overall survival time (Hazard ratios (HR)= 1.39, 95% Confidence interval (CI): 1.01-1.82, p=0.014), although different MMP-14 expression statuses did not show significant differences in overall survival for KICH (HR=0.49, 95%CI: 0.13-1.82, p=0.28), KIRC (HR=1.18, 95%CI: 0.87-1.58, p=0.40), KIRP (HR=1.24, 95%CI: 0.69-2.24, p=0.44) and PRAD (HR=0.50, 95%CI: 0.14-1.77, p=0.55). Next, we also examined the effect of MMP-14 expression on the patients' progression-free survival. The results showed that the differential expression statuses of MMP-14 did not show significantly different progression-free survival rates in BLCA (HR=1.19, 95%CI: 0.89-1.60,

p=0.11), KICH (HR=0.54, 95%CI: 0.17-1.78, p=0.39), KIRC (HR=1.30, 95%CI: 0.95-1.78, p=0.10), KIRP (HR=1.44, 95%CI: 0.85-2.43, p=0.18) and PRAD (HR=1.05, 95%CI: 0.70-1.57, p=0.76). Since bladder cancer patients with different MMP-14 expression statuses had associated different overall survival, we chose bladder cancer as our study subject.

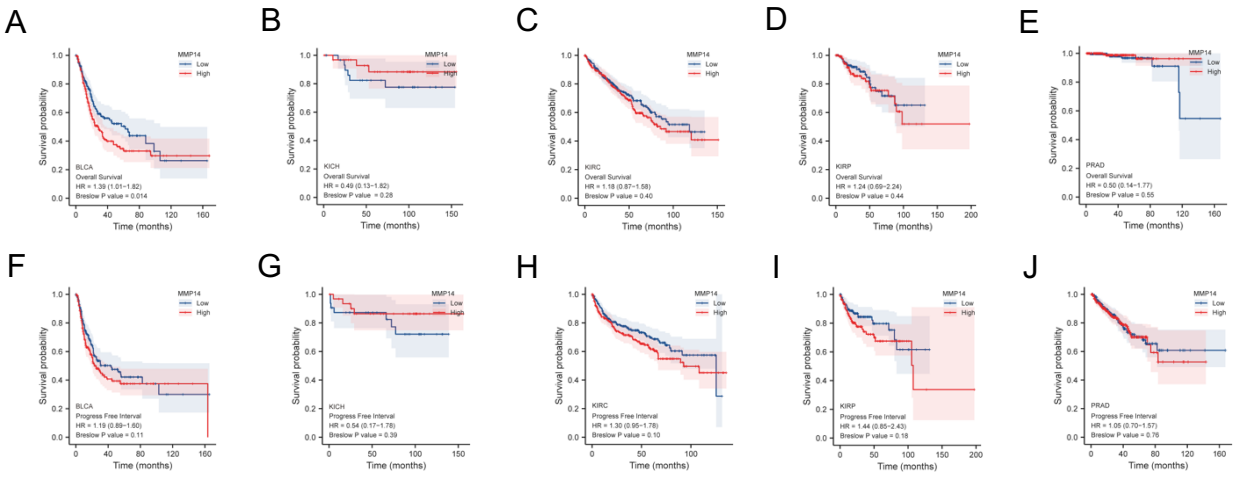


Figure 11 Effect of MMP-14 expression level on the prognosis of major urological tumors.
The relationship between MMP-14 expression level and overall survival of BLCA (A), KICH(B), KIRC(C), KIRP(D), PRAD(E); the relationship between MMP-14 expression level and progression-free survival of BLCA(F), KICH(G), KIRC(H), KIRP(I), PRAD(J). Significant results were determined by Gehan-Breslow-Wilcoxon test.

3.1.3 Correlation of MMP-14 expression with clinicopathological characteristics in bladder cancer

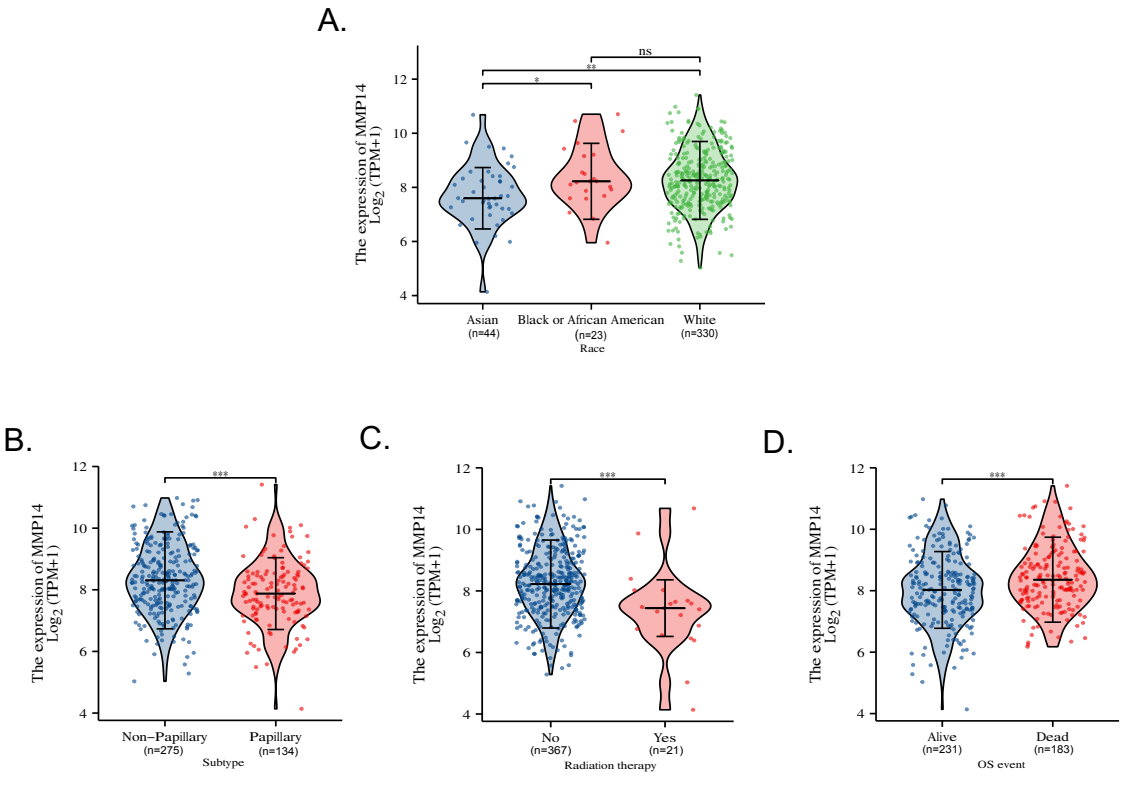
To clarify the clinical significance of MMP-14 in bladder cancer, we downloaded the clinicopathological data of bladder cancer from the TCGA database. We analyzed the correlation between MMP-14 expression levels and the clinicopathological characteristics of bladder cancer (Table 8). The results showed that there were significant correlations between MMP-14 expression levels and patients' race (p=0.03), T stage (p=0.049), pathological stage (p=0.048), subtype (p=0.005), radiation therapy (p=0.002), primary therapy outcome (p=0.039), and overall survival events (p=0.018). However, MMP-14 expression levels were not significantly associated with patients' age (p=0.372), gender (p=0.264), height (p=0.975), weight (p=1.000), Body mass index (BMI) (p=0.377), smoking (p=0.676), N stage (p=0.092), M stage (p=0.362), histological grade (p=0.071), as well as lymphovascular invasion (p=0.892) and progression-free interval events (p=0.372).

Table 8 Correlation between MMP-14 expression and patients' clinicopathological characteristics in bladder cancer

Characteristic	Levels	Number (n)	Low expression of MMP-14 (n=207)	High expression of MMP-14 (n=207)	p value
Age, n (%)	<=70	234(56.6%)	122 (29.5%)	112 (27.1%)	0.372
	>70	180(43.4%)	85 (20.5%)	95 (22.9%)	
Gender, n (%)	Female	109(26.3%)	49 (11.8%)	60 (14.5%)	0.264
	Male	305(73.7%)	158 (38.2%)	147 (35.5%)	
Race, n (%)	Asian	44(11.1%)	30 (7.6%)	14 (3.5%)	0.030
	Black or African American	23(5.8%)	11 (2.8%)	12 (3%)	
	White	330(83.1%)	155 (39%)	175 (44.1%)	
Height, n (%)	<=170	158(43.2%)	79 (21.6%)	79 (21.6%)	0.975
	>170	207(56.7%)	105 (28.8%)	102 (27.9%)	
Weight, n (%)	<=80	205(55.2%)	104 (28%)	101 (27.2%)	1.000
	>80	166(44.7%)	84 (22.6%)	82 (22.1%)	
BMI, n (%)	<=25	153(42.1%)	82 (22.5%)	71 (19.5%)	0.377
	>25	211(57.9%)	102 (28%)	109 (29.9%)	
Smoker, n (%)	No	109(27.2%)	57 (14.2%)	52 (13%)	0.676
	Yes	292(72.8%)	144 (35.9%)	148 (36.9%)	
T stage, n (%)	T1	5(1.3%)	2 (0.5%)	3 (0.8%)	0.049
	T2	119(31.3%)	66 (17.4%)	53 (13.9%)	
	T3	196(51.6%)	82 (21.6%)	114 (30%)	
	T4	60(15.7%)	34 (8.9%)	26 (6.8%)	
N stage, n (%)	N0	239(64.6%)	124 (33.5%)	115 (31.1%)	0.092
	N1	46(12.5%)	15 (4.1%)	31 (8.4%)	
	N2	77(20.8%)	38 (10.3%)	39 (10.5%)	
	N3	8(2.2%)	5 (1.4%)	3 (0.8%)	

Characteristic	Levels	Number (n)	Low expression of MMP-14 (n=207)	High expression of MMP-14 (n=207)	p value
M stage, n (%)	M0	202(94.8%)	121 (56.8%)	81 (38%)	0.362
	M1	11(5.2%)	5 (2.3%)	6 (2.8%)	
Pathologic stage, n (%)	Stage I	4(0.9%)	1 (0.2%)	3 (0.7%)	0.048
	Stage II	130(31.6%)	77 (18.7%)	53 (12.9%)	
	Stage III	142(34.5%)	68 (16.5%)	74 (18%)	
	Stage IV	136(33%)	60 (14.6%)	76 (18.4%)	
Histologic grade, n (%)	High Grade	390(94.9%)	190 (46.2%)	200 (48.7%)	0.071
	Low Grade	21(5.1%)	15 (3.6%)	6 (1.5%)	
Lymphovascular invasion, n (%)	No	130(45.9%)	66 (23.3%)	64 (22.6%)	0.892
	Yes	153(54.1%)	80 (28.3%)	73 (25.8%)	
Subtype, n (%)	Non-Papillary	275(67.2%)	124 (30.3%)	151 (36.9%)	0.005
	Papillary	134(32.8%)	81 (19.8%)	53 (13%)	
Radiation therapy, n(%)	No	367(94.6%)	177 (45.6%)	190 (49%)	0.002
	Yes	21(5.4%)	18 (4.6%)	3 (0.8%)	
Primary therapy out-come, n (%)	PD	70(19.6%)	29 (8.1%)	41 (11.5%)	0.039
	SD	31(8.7%)	20 (5.6%)	11 (3.1%)	
	PR	22(6.2%)	7 (2%)	15 (4.2%)	
	CR	234(65.5%)	124 (34.7%)	110 (30.8%)	
PFI event, n (%)	Non-Progres-sion	234(56.6%)	122 (29.5%)	112 (27.1%)	0.372
	Progression	180(43.4%)	85 (20.5%)	95 (22.9%)	
OS event, n (%)	Alive	231(55.8%)	128 (30.9%)	103 (24.9%)	0.018
	Dead	183(44.2%)	79 (19.1%)	104 (25.1%)	

In addition, we selected the previous significantly correlated clinicopathological features and further analyzed the expression levels of MMP-14 under different clinicopathological features. In our observations, Asians showed significantly lower expression of MMP-14 ($p<0.05$) compared to Black and African Americans in different racial classifications, while no significant difference in MMP-14 expression was seen between Black and African Americans (Figure 12A). Among subtypes, papillary carcinomas had higher MMP-14 expression than non-papillary carcinomas ($p<0.001$, Figure 12B). In addition, patients who received preoperative radiotherapy had lower MMP-14 expression ($p<0.001$, Figure 12C). In terms of overall survival events, patients who died had higher MMP-14 expression than those who survived ($p<0.001$, Figure 12D). For different T-stages, patients with T3 had higher MMP-14 expression than those with T2 ($p<0.01$), but no significant differences were seen among the remaining T stages (Figure 12E). For pathological stages, MMP-14 expression was significantly lower in stage 2 than in stages 3 and 4 ($p<0.01$), but no significant differences were seen between stages 3 and 4 (Figure 12F). PR patients had significantly higher MMP-14 expression in the primary therapy outcome than SD patients ($p<0.05$), but no significant differences were seen among the remaining outcomes (Figure 12G).



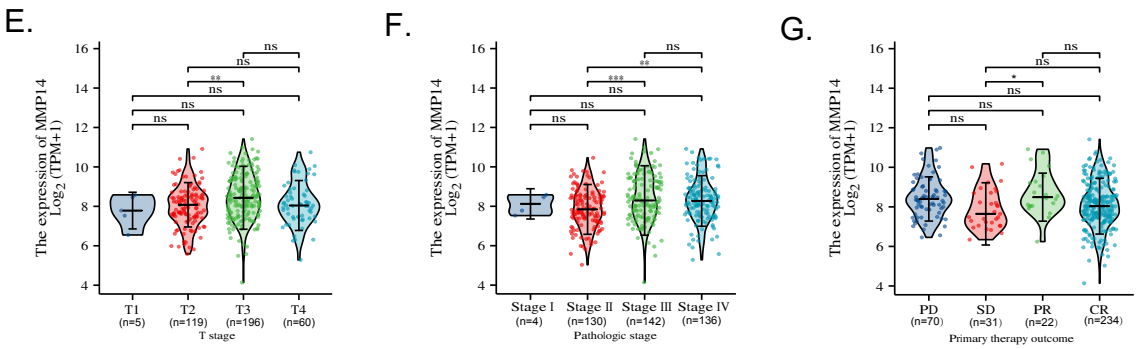


Figure 12 Expression condition of MMP-14 with different clinicopathological features in bladder cancer.

MMP-14 expression levels were compared in subgroups of bladder cancer with different clinicopathological features (race(A), subtype(B), radiation therapy(C), overall survival(D), T stage(E), pathological stage(F), and primary therapy outcome(G)). Student tests or one-way ANOVA were used to evaluate significant results, and Bonferroni's multiple comparison procedure was used. * p <0.05, **p <0.01, *** p<0.001, ns: no significance.

3.2 Functional enrichment analysis of MMP-14 in bladder cancer

3.2.1 Screening of Differentially expressed genes (DEGs) and expression-related genes (ERGs) of MMP-14 in bladder cancer

We screened differentially expressed genes according to the expression level of MMP-14 using volcano plot (absolute value (log_2 Fold change (FC))>1, adjusted p value<0.05), and 1798 DEGs were selected (Figure 13A), among which there were 983 up-regulated genes and 815 down-regulated genes. Meanwhile, we screened out 3955 genes associated with MMP-14 expression by correlation analysis (absolute value (Pearson correlation coefficient)>0.3, p value<0.05), among which there were 3600 genes with positive expression correlation (Pearson correlation coefficient= 0.3 - 0.71) and 355 genes with negative expression correlation (Pearson correlation coefficient= -0.46 - (-0.3)). Next, a Venn diagram was used to screen common genes between DEGs and ERGs, and finally, 767 common genes were shown (Figure 13B).

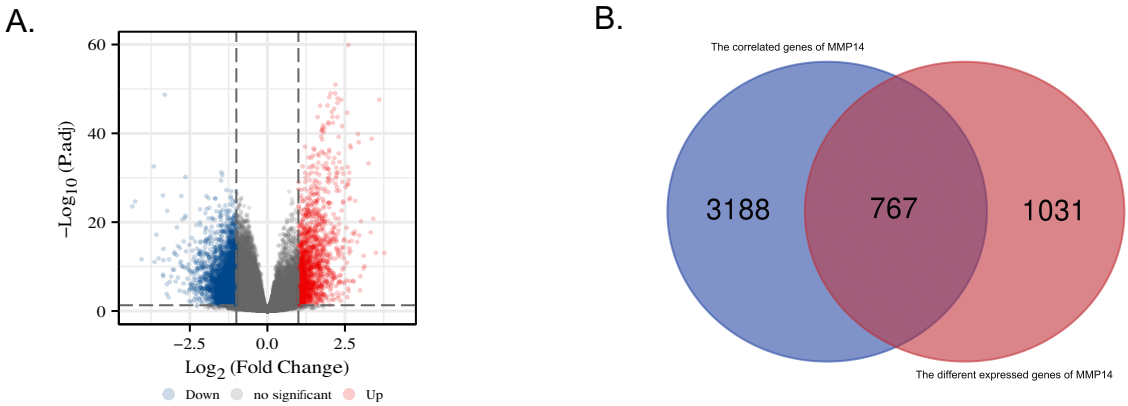
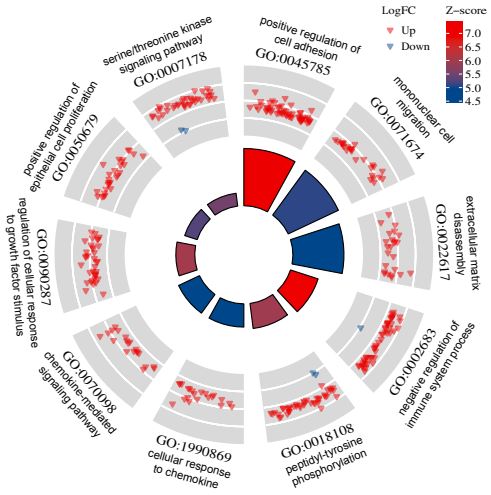


Figure 13 Selection of DEGs and expression-related genes according to the MMP-14 expression level in bladder cancer. A. A volcano plot was used to screen the DEGs between the high MMP-14 expression group and the low MMP-14 expression group in bladder cancer. Screening conditions: abs|$\log_2$(Fold Change)| > 1 and adjusted p value < 0.05. Blue dots represent significantly down-regulated genes, and red dots represent significantly up-regulated genes. B. A Venn diagram screened out public genes between DEGs and expression-related genes.

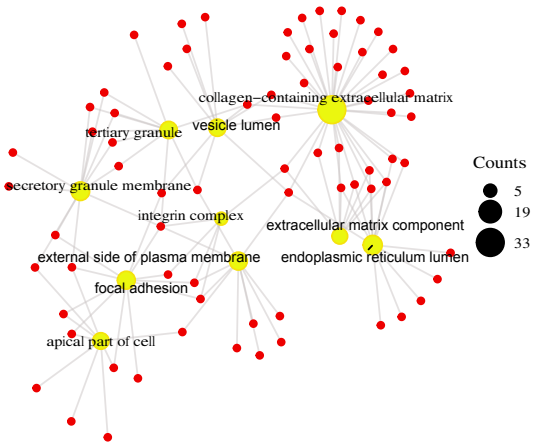
3.2.2 GO functional enrichment analysis of the public genes between DEGs and ERGs according to the MMP-14 expression level in bladder cancer

We performed a GO enrichment analysis of the previously screened public genes. The results showed that, for the biological process (Figure 14A), these genes were mainly enriched in positive regulation of cell adhesion, mononuclear cell migration, extracellular matrix disassembly, negative regulation of immune system process, peptidyl-tyrosine phosphorylation, cellular response to chemokines, chemokine-mediated signaling pathways, regulation of cellular response to growth factor stimulation, positive regulation of epithelial cell proliferation, and the serine/threonine kinase signaling pathway. In terms of cell structure (Figure 14B), these genes were mainly enriched in the extracellular matrix containing collagen, extracellular matrix components, tertiary granules, integrin complexes, endoplasmic reticulum lumen, secretory granule membrane, the external side of plasma membrane, vesicle lumen, focal adhesion and the apical part of the cell. In addition, from the point of molecular function (Figure 14C), these genes were significantly enriched in growth factor binding, chemokine activity, cytokine receptor binding, extracellular matrix binding, sulfur compound binding, integrin binding, receptor ligand activity, cytokine activity, heparin binding and extracellular matrix construction for tensile strength.

A.



B.



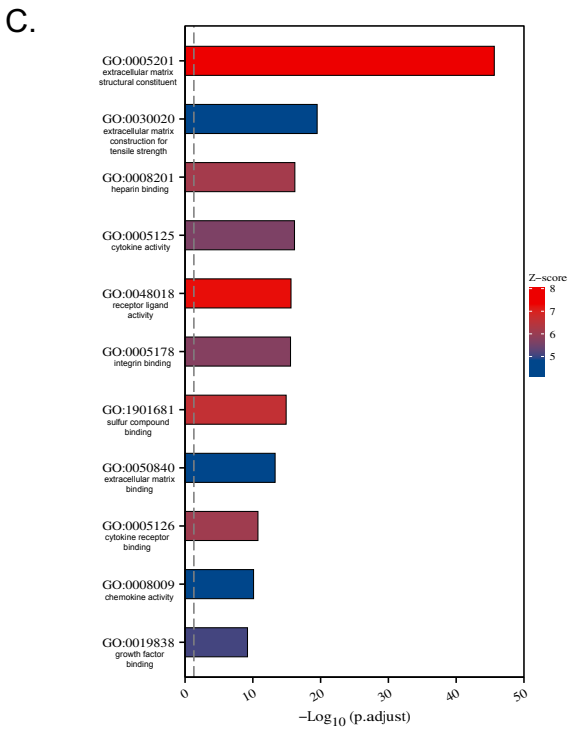


Figure 14 GO functional enrichment analysis of the public genes between DEGs and ERGs according to MMP-14 expression level in bladder cancer.

According to the previous public genes, A. GO enrichment analysis was used to explore the main biological processes that MMP-14 may be involved in. B. GO enrichment analysis was performed to investigate the possible localization of MMP-14 to the cellular structure according to the previous public genes. C. GO enrichment analysis was used to detect the main molecular functions in which MMP-14 may be involved according to the previous public genes.

3.2.5 GSEA functional enrichment analysis of the public genes between DEGs and ERGs according to the MMP-14 expression level in bladder cancer

Next, we used GSEA analysis to perform another functional enrichment analysis of the public genes, which showed that these genes were significantly enriched in the MMP-cytokine connection, cell-extracellular matrix interactions, IL6 pathway, PD-1 signaling, neural crest cell migration in cancer, and T cell receptor signaling pathway (Figure 15).

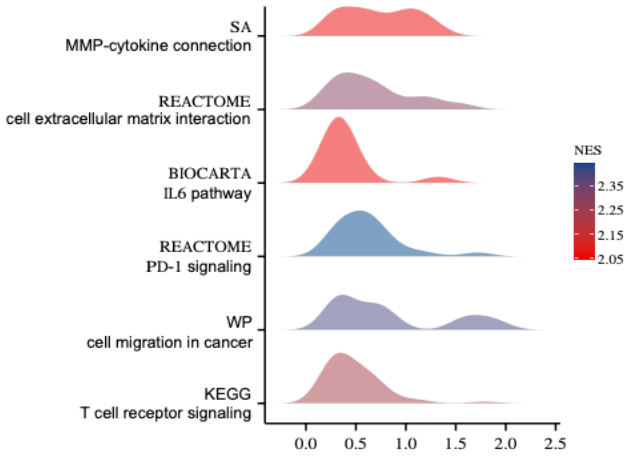


Figure 15 GSEA functional enrichment analysis of the public genes between DEGs and ERGs according to the MMP-14 expression level in bladder cancer.

3.2.5 Immunosuppressive effects of MMP-14 may involve Treg cells

Considering the vital role of MMP-14 in tumor immunity, we explored the correlation between the enrichment degree of $CD8^+$ T cells (the central effector cells in tumor immunity) and MMP-14 expression using the ssGSEA algorithm. The results showed a weak correlation between the two ($p=0.001$, Figure 16A). According to our previous functional enrichment results, MMP-14 was involved in tumor immunosuppression. Therefore, we also examined the correlation between the enrichment degree of Treg cells (the central immunosuppressive cell) and MMP-14 expression. The results showed a moderate correlation between the two ($p<0.001$, Figure 16B). Furthermore, we compared the enrichment degree of $CD8^+$ T cells and Treg cells with different MMP-14 expression levels. The results showed that there was no significant difference in the enrichment degree of $CD8^+$ T cells between the high and low expression levels of MMP-14 (Figure 16C). But the high MMP-14 expression group had a significantly higher enrichment of Treg cells than the low group ($p< 0.001$, Figure 16D). Additionally, we explored the correlation of MMP-14 expression with some immune markers using the TIMER database and GEPIA database (Table 9). We found that no significant correlations with MMP-14 expression were seen for CD8A and CD8B (the markers of $CD8^+$ T cells) after purification for tumor abundance in the TIMER database. Only CD8A showed a weak correlation with MMP-14 in bladder cancer from the GEPIA database ($p<0.01$). However, the expression levels of MMP-14 were significantly positively correlated with FOXP3, CD25(IL2RA), and CCR8 (Treg cell markers) in bladder cancer from the Timer database and the GEPIA database ($p<0.05$). We also analyzed the relationship between the markers of T-cell exhaustion (PD-1 (PDCD1), CTLA4, LAG3, TIM-3 (HAVCR2)) and MMP-14 expression levels, and the results showed significant positive correlations ($p<0.05$) between the expression levels of MMP-14 and the markers of T-cell exhaustion in bladder cancer from the Timer database and the GEPIA database. In contrast, there were no significant differences between the expression levels of MMP-14 and the markers of T-cell exhaustion in normal tissues from the GEPIA database.

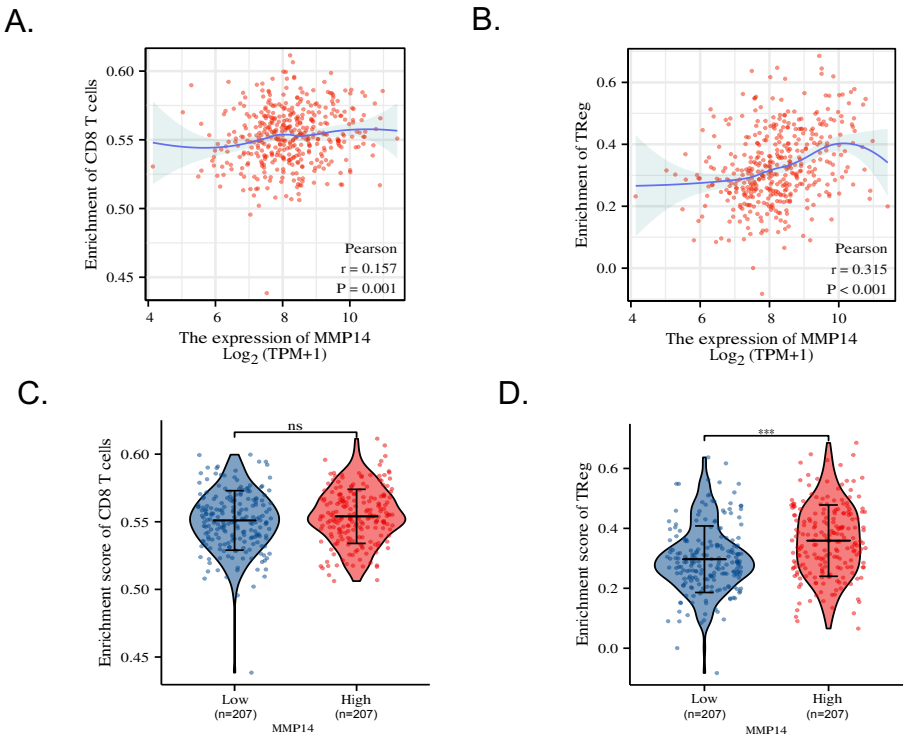


Figure 16 Effect of MMP-14 expression on immune infiltration in bladder cancer.

A. Correlation analysis of MMP-14 expression with $CD8^+$ T cell infiltration in bladder cancer. B. Correlation analysis of MMP-14 expression with Treg cell infiltration in bladder cancer. C. Comparison of the enrichment degree of $CD8^+$ T cells between high and low expression of MMP-14 in bladder cancer; significant results were determined by Mann-Whitney U test. D. Comparison of the enrichment degree of Treg cell between high and low expression of MMP-14 in bladder cancer; significant results were determined by Mann-Whitney U test. ***p<0.001, ns: no significance.

Table 9 Correlation analysis between MMP-14 expression and markers of CD8+ T cell, Treg cell, and T cell exhaustion from the TIMER and GEPIA databases in bladder tissues

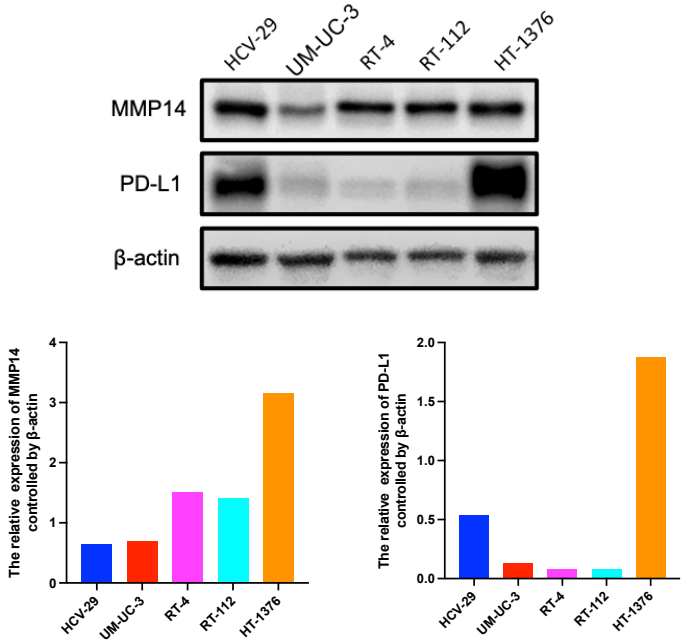
Cell type	Gene marker	TIMER database				GEPIA database			
		None		Adjusted by Purity		Tumor tissues		Normal tissues	
		Cor	p value	Cor	p value	Cor	p value	Cor	p value
$CD8^+$ T cell	CD8A	0.262	<0.001	0.068	0.195	0.17	<0.001	0.36	0.062
	CD8B	0.13	0.0087	-0.026	0.614	0.0043	0.93	0.24	0.23
Treg	FOXP3	0.394	<0.001	0.258	<0.001	0.34	<0.001	0.18	0.37
	CD25 (IL2RA)	0.49	<0.001	0.341	<0.001	0.31	<0.001	-0.15	0.45
	CCR8	0.408	<0.001	0.291	<0.001	0.31	<0.001	0.39	0.042
T cell exhaustion	PD-1 (PDCD1)	0.319	<0.001	0.124	0.017	0.11	0.031	0.055	0.78
	CTLA4	0.346	<0.001	0.171	<0.001	0.22	<0.001	-0.049	0.81
	LAG3	0.329	<0.001	0.149	0.004	0.23	<0.001	-0.021	0.92
	TIM-3 (HAVCR2)	0.497	<0.001	0.326	<0.001	0.38	<0.001	0.27	0.16

3.3 Function of MMP-14 in bladder cancer cells

3.3.1 The basic expression level of MMP-14 and PD-L1 in bladder cancer cells

From the previous GO functional enrichment analysis, it was clear that MMP-14 might be involved in immune escape in bladder cancer. In addition, GSEA analysis also showed that overexpression of MMP-14 activates the PD-1 pathway. As a ligand molecule for PD-1, the immune checkpoint PD-L1 is expressed on the surface of tumor cells and plays a vital role in the immunosuppression of tumor cells. Therefore, we speculated whether PD-L1 expression was also associated with MMP-14. Next, we used Western blotting to detect the expression of MMP-14 and PD-L1 in four bladder cancer cell lines (UM-UC-3, RT-4, RT-112, HT-1376) and one bladder-transformed epithelial cell line (HCV-29). The results showed that MMP-14 had the highest expression level in HT-1376 cells and the remaining four cells had relatively low expression levels (Figure 17A). Meanwhile, PD-L1 was expressed at high levels in HT-1376 and HCV-29 cells; the remaining three cells had relatively low PD-L1 expression. In addition, we also examined the surface expression levels of MMP-14 and PD-L1 in the four bladder cancer cell lines by immunofluorescence (Figure 17B). The results showed that at the cell surface, MMP-14 and PD-L1 were relatively highly expressed in HT1376 cells and had a low expression in UM-UC-3 cells, RT-4 cells, and RT-112 cells.

A.



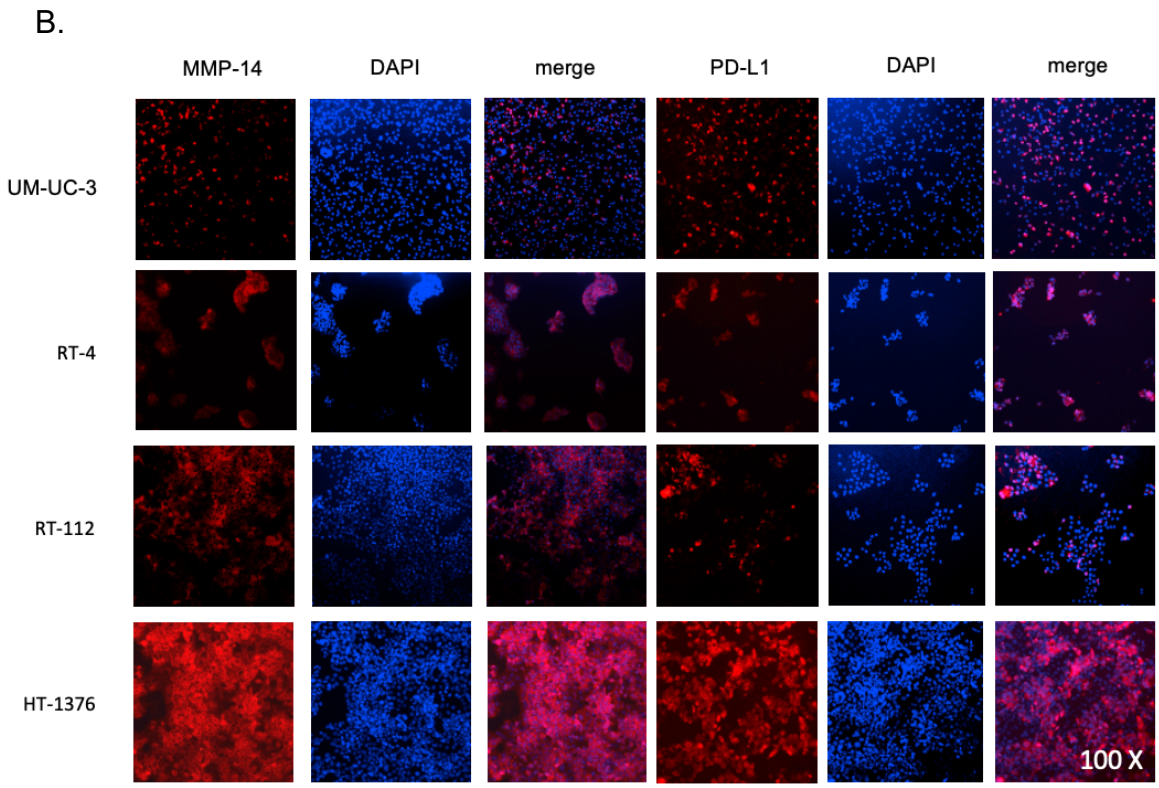


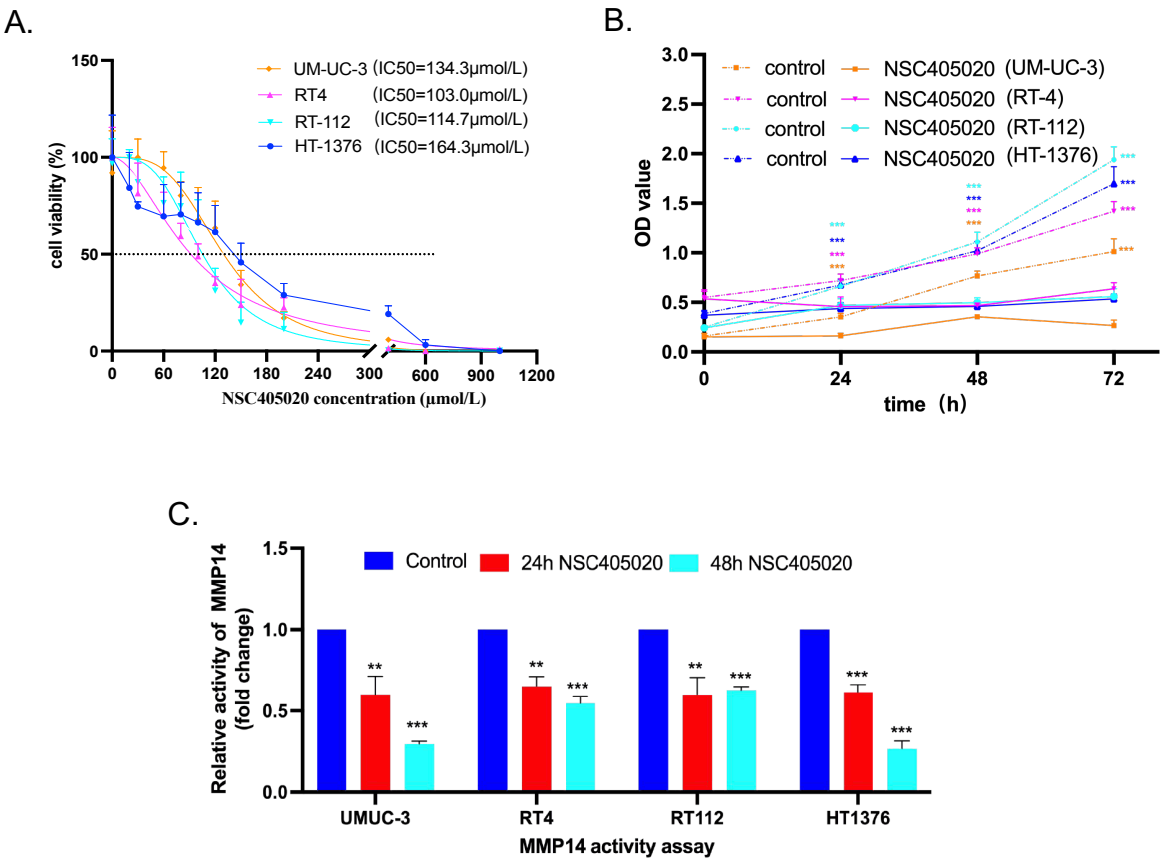
Figure 17 Expression levels of MMP-14 and PD-L1 in various bladder cancer cells and bladder transformed epithelial cells.

A. Western blotting was used to detect protein expression levels of MMP-14 and PD-L1 in normal bladder cells (HCV-29) and bladder cancer cells (UMU-UC-3, RT-4, RT-112, HT-1376). B. Immunofluorescence detected MMP-14 and PD-L1 surface expression in different bladder cancer cells. (Magnification:100X)

3.3.2 Effect of NSC405020 (MMP-14-specific inhibitor) on bladder cancer cells

To explore the effect of MMP-14 on bladder cancer cells, we applied the MMP-14-specific inhibitor NSC405020 to down-regulate the expression level of MMP-14. We first applied different concentrations of NSC405020 to treat bladder cancer cells for 48 hours, then calculated the respective half-inhibitory concentrations (IC50), which showed that HT-1376 cells had the highest IC50 value (IC50=164.3 μmol/L), and the IC50 values of the remaining three cells were 134.4 μmol/L (UM-UC-3), 103 μmol/L (RT-4), and 114.7 μmol/L (RT-112) (Figure 18A). Furthermore, after treatment with the respective IC50 concentrations of NSC405020 for 24h, 48h and 72 h, cell growth was significantly inhibited in the NSC405020-treated group for all 4 cells compared to the control group ($p<0.001$, Figure 18B). We next examined the effect of NSC405020 on cellular MMP-14 activity using the MMP-14 activity assay. The results showed that the 24h and 48h NSC405020-

treated groups of all 4 cells showed significant decreases in MMP-14 activity compared to the control groups ($p<0.05$, Figure 18C). We then performed cell death assays to assess the effect of NSC405020 on apoptosis in bladder cancer cells. The results showed that the 24h NSC405020-treated group had no significant apoptosis indexes compared with the control group in 4 cells. However, when the treatment duration of NSC405020 reached 48h, the apoptosis indexes of all 4 cells in the treated group were significantly increased ($p<0.05$, Figure 18D). Meanwhile, LDH release assays were used to assess the cytotoxicity of NSC405020 on bladder cancer cells, measured at three time points (6h, 24h, 48h) of NSC405020 treatment. It was observed that the cytotoxicity of the NSC405020-treated group was significantly higher compared to the corresponding control groups at all time points ($p<0.05$, Figure 18E). We collected proteins from the 24h NSC405020-treated group and the corresponding control group. We detected the expression levels of MMP-14 and PD-L1 in each group by Western blotting (Figure 18F), and we saw that the protein expression levels of MMP-14 were significantly downregulated in all cells after NSC405020 treatment ($p<0.05$). Although the expression level of PD-L1 in the NSC405020-treated group of RT4 cells was not significantly different compared to the control group, the expression of PD-L1 was significantly down-regulated in the NSC405020-treated groups of the UM-UC-3, RT-112 and HT-1376 cells ($p<0.05$).



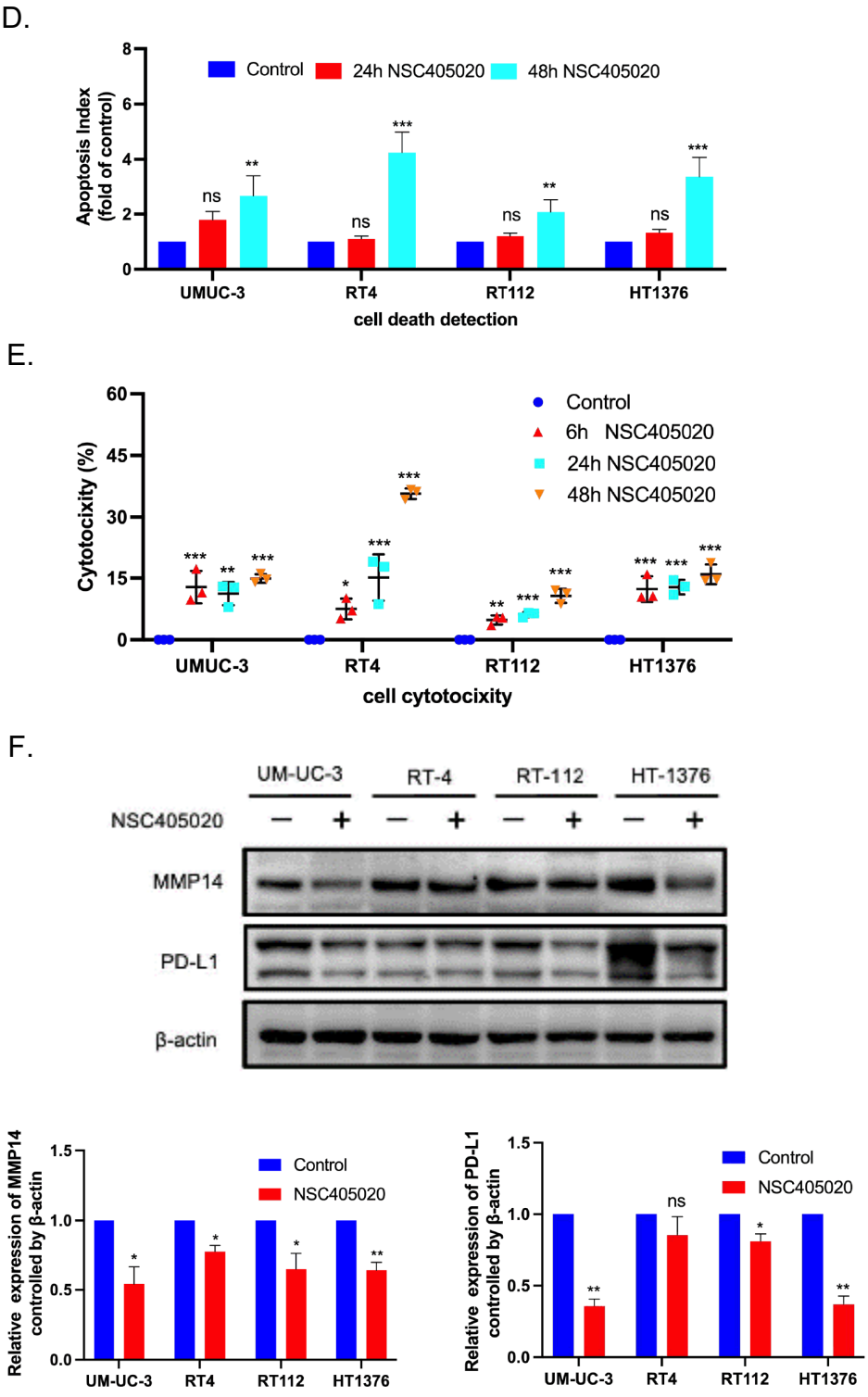


Figure 18 Effect of NSC405020 (MMP-14-specific inhibitor) on bladder cancer cells.

A. Cells were treated with different concentrations of NSC405020 (0, 30, 60, 80, 100, 120, 150, 200, 400, 600 µmol/L) for 48h, and the IC50 was determined by nonlinear regression; (n=3). B. 4 bladder cancer cell lines were treated with the corresponding IC50 of NSC405020, PBS was added to the controls, CCK-8 was applied to analyze cell viability at 24h, 48h, and 72h of treatment with NSC405020, two-way ANOVA was employed to detect significant results, and Bonferroni's multiple comparison approach was used; (n=3). C. 4 bladder cancer cells were treated with NSC405020

at the corresponding IC50, PBS was added to the controls, and the MMP-14 activity assay detected MMP-14 activity at 24h and 48h of treatment with NSC405020. One-way ANOVA was used to identify significant results, and Dunnett's procedure was used for multiple comparisons; (n=3). D. 4 bladder cancer cells were treated with the corresponding IC50 of NSC405020, the controls were treated with PBS, cell death detection was performed to test the cell apoptosis at 24h and 48h of NSC405020 treatment, one-way ANOVA was used to identify significant results, and Dunnett's procedure was used for multiple comparisons; (n=3). E. 4 bladder cancer cells were treated with NSC405020 at the corresponding IC50 concentrations, and the control group was treated with PBS. The cytotoxicity induced by NSC405020 was measured by LDH release assay at 6h, 24h, and 48h of treatment, one-way ANOVA was used to identify significant results, and Dunnett's procedure was used for multiple comparisons; (n=3). F. 4 bladder cancer cells were treated with NSC405020 at the corresponding IC50, and the controls were added to PBS. Cellular proteins were collected at 24 h treatment, and the protein expression of MMP-14 and PD-L1 was detected by Western blotting. Significant results were determined by independent t-test; (n=3). *p <0.05, **p <0.01, ***p<0.001, ns: no significance.

3.3.3 MMP-14 knockdown reduced PD-L1 expression in bladder cancer HT-1376 cells

Additionally, to clarify the regulatory relationship of MMP-14 on PD-L1, we used CRISPR/Cas9 technology to knockdown MMP-14 in bladder cancer HT-1376 cells, and constructed stable knockdown of MMP-14 in HT-1376 cells. We detected the knockdown efficiency of MMP-14 by Western blotting, and the results showed that the expression level of MMP-14 was significantly decreased in each knockdown clone compared with the control group ($p<0.001$, Figure 19). In addition, we also examined the expression level of PD-L1, and it was observed that except for a slight decrease in the MMP-14-KD5 clone, the expression of PD-L1 in the rest of the knockdown clones had significant decreases compared to the control group ($p<0.01$, Figure 19).

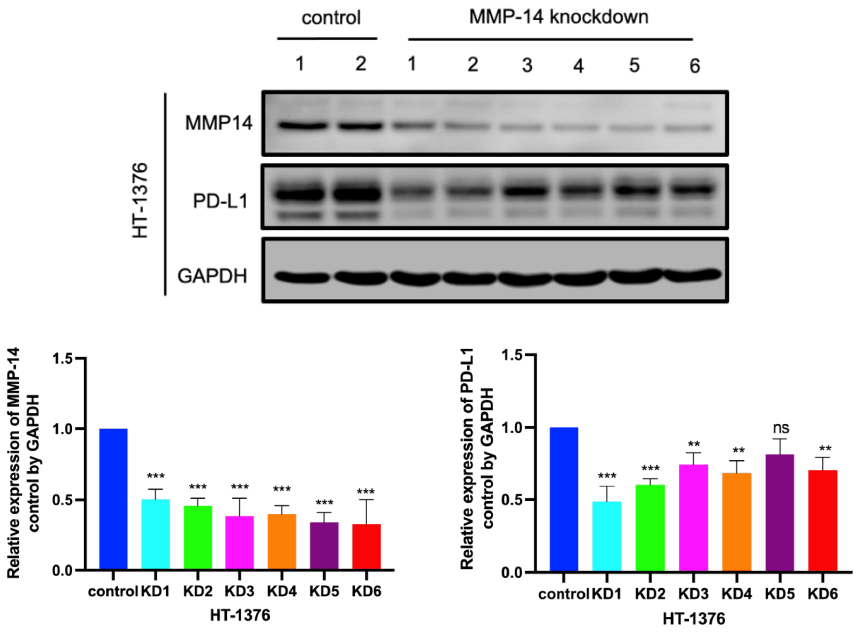


Figure 19 Expression levels of PD-L1 decreased by knockdown of MMP-14 in HT-1376 cells.

After establishing stable knockdown of MMP-14 in HT-1376 cells, we collected proteins from the corresponding control groups and knockdown groups, and the expression levels of MMP-14 and PD-L1 were detected by Western blotting. One-way ANOVA was used to identify significant results, and Dunnett's procedure was used for multiple comparisons. (n=3), **p <0.01, *** p<0.001, ns: no significance.

3.4 Role of STAT3 in bladder cancer

3.4.1 STAT3 is a potential transcription factor for MMP-14 and PD-L1 in bladder cancer

Next, we explored the upstream regulatory mechanisms of MMP-14. We first used the KnockTF and the hTFtarget databases to predict potential transcription factors bound to the promoter sequence of the MMP-14 transcription region. The KnockTF database predicted 34 potential transcription factors of MMP-14, and the hTFtarget database showed that there might be 119 transcription factors potentially binding to the promoter of the MMP-14 transcription region. Furthermore, we screened the common transcription factors of both databases using a Venn diagram, and finally 11 public transcription factors were screened (BRD4, EWSR1, JUND, MYC, JUN, STAT3, GATA1, TFAP4, USF1, FOXM1) (Figure 20A). Moreover, we analyzed the co-expression correlation of these public transcription factors with MMP-14 using transcriptomic expression data from the TCGA database in bladder cancer, which showed that STAT3 ($R=0.422$, $p<0.001$), MYC ($R=0.360$, $p<0.001$), JUN ($R=0.354$, $p<0.001$), FOXM1 ($R=0.242$, $p<0.001$), GATA1 ($R=0.179$, $p<0.001$), and BRD4 ($R=0.170$, $p<0.001$) showed significant positive correlation with MMP-14 expression in bladder cancer, while EWSR1 ($R=0.087$, $p>0.05$), JUND ($R=-0.045$, $p>0.05$), TFAP4 ($R=-0.055$, $p>0.05$), MYB ($R=-0.064$, $p>0.05$), and USFB1 ($R=-0.067$, $p>0.05$) did not show significant correlation with MMP-14 expression (Figure 20B). In addition, STAT3, a common transcription factor, has been reported to regulate the transcriptional process of various MMPs [128, 207-210] and the immune checkpoint gene PD-L1[106]. Therefore, we examined the correlation between the expression of MMP-14, STAT3, and PD-L1 (CD274), and the results showed that there were significant positive correlations between MMP-14 and STAT3 ($R=0.422$, $p<0.001$), MMP-14 and PD-L1 ($R=0.346$, $p<0.001$), and STAT3 and PD-L1 ($R=0.428$, $p< 0.001$) (Figure 20C) in bladder cancer.

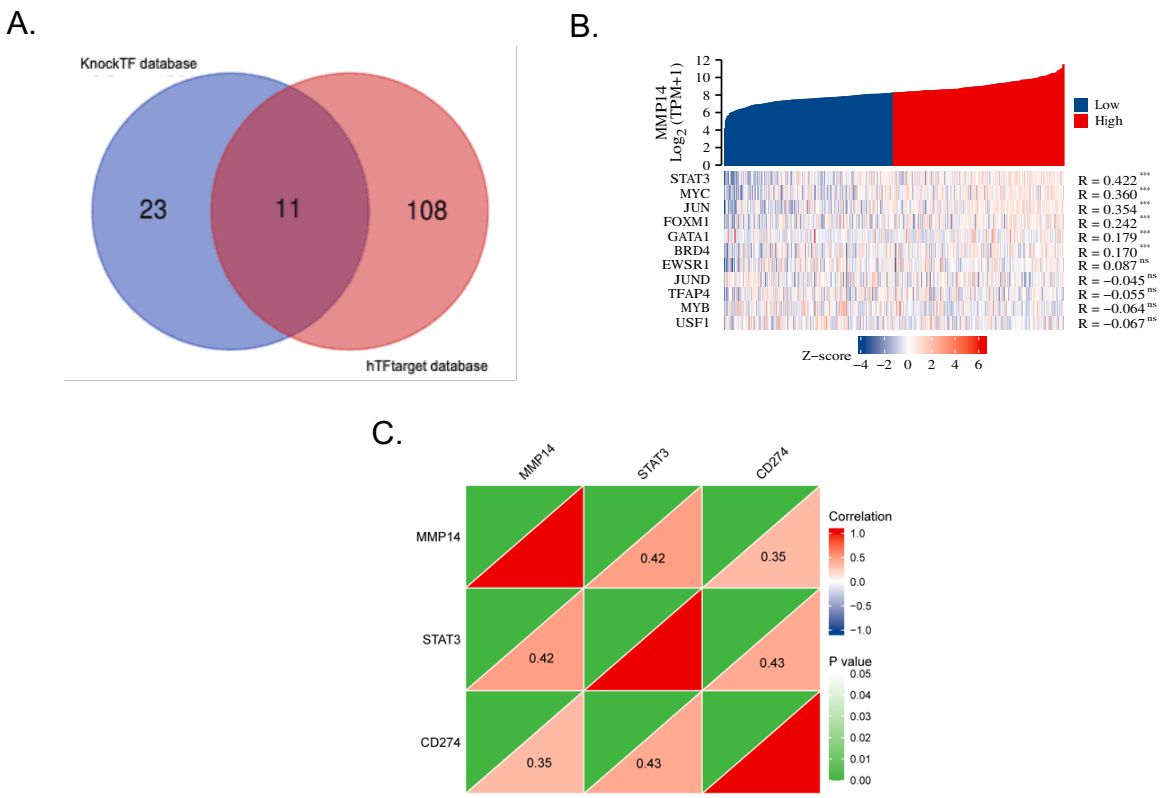


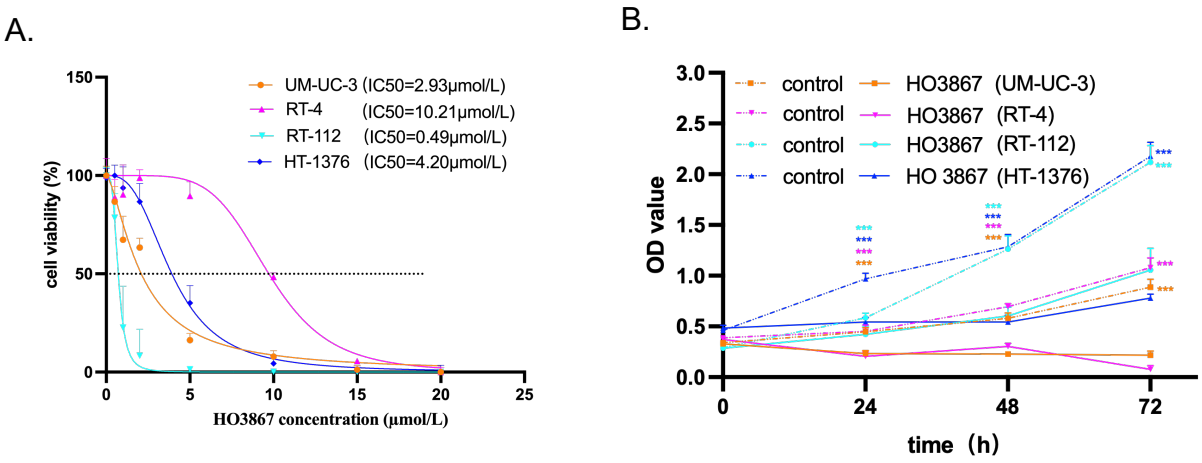
Figure 20 Prediction of MMP-14 transcription factors in bladder cancer.

A. A Venn diagram was used to screen the public transcription factors of MMP-14 in both databases, KnockTF and hTFtarget. B. A heat map was used to show the correlation of public transcription factors with MMP-14 expression. C. A correlation heat map showed the expression correlation among MMP-14, STAT3, and PD-L1. ***p<0.001, ns: no significance.

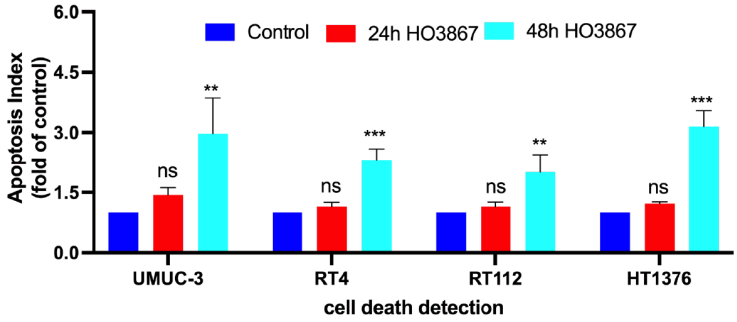
3.4.2 Effect of HO-3867 (STAT3-specific inhibitor) on bladder cancer cells

To investigate the effect of STAT3 on bladder cancer cells, we applied the STAT3-specific inhibitor HO-3867 to inhibit STAT3 expression and reduce its phosphorylation level. First, different concentrations of HO-3867 (0, 0.5, 1, 2, 5, 10, 15, 20 μmol/L) were used to treat bladder cancer cells for 48 h. The corresponding IC50 of HO-3867 was calculated for each cell line, and the results showed that RT-4 cells had the highest IC50 value (IC50=10.21 μmol/L). The IC50 of the other three cells was 2.93 μmol/L (UM-UC-3), 0.49 μmol/L (RT-112), and 4.20 μmol/L (HT-1376), respectively (Figure 21A). We next examined cell proliferation using the CCK8 assay. After treating bladder cancer cells with the corresponding IC50 of HO-3867 for 24h, 48h and 72 h, cell viability was significantly lower in the HO-3867-treated group compared to the control group for all 4 bladder cancer cells (p<0.001, Figure 21B). We also applied cell death assays to explore the effect of HO-

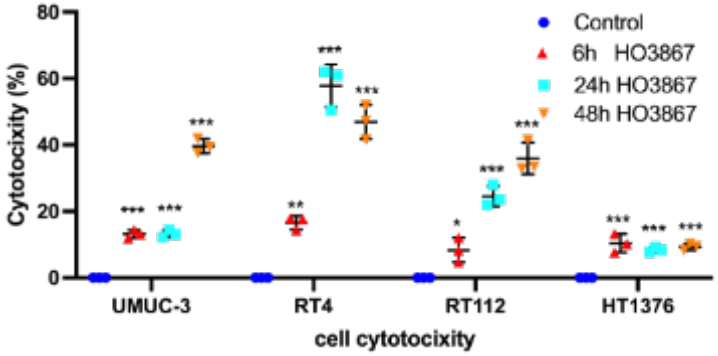
3867 on the apoptosis of bladder cancer cells. It was observed that after 24h of treatment with HO3867, there was no significant apoptosis index between the treatment groups and the corresponding control group in 4 cell lines. However, the apoptotic index of all 4 bladder cancer cells was significantly higher in the treated group compared to the control group when the treatment duration reached 48h ($p<0.05$, Figure 21C). Furthermore, cytotoxicity assays at three time points (6h, 24h, 48h) showed that the HO-3867-treated groups had significantly increased cytotoxicity compared to the control group ($p<0.001$, Figure 21D). Furthermore, to verify that STAT3 has a regulatory role on MMP-14 in bladder cancer, MMP-14 activity assays were used to detect the effect of HO-3867 on MMP-14 activity. The results showed that UM-UC-3, RT-4 and HT-1376 cells had decreased MMP-14 activities after 24h of treatment of HO-3867. Furthermore, when the treatment duration reached 48h, the treatment group of all 4 cells had significantly reduced MMP-14 activity compared to the respective control groups ($p<0.01$, Figure 21E). Additionally, we collected the cell proteins after bladder cancer cells were treated with IC50 HO-3867 for 24h as well as the cell proteins from the control. Western blotting detected the protein expression levels of STAT3, p-STAT3, MMP-14, and PD-L1. In fact, p-STAT3 has two isoforms (full-length p-STAT3α (86KD) and the shortened p-STAT3β (79KD)), p-STAT3α, as a oncogene, and is the main executor of STAT3 function, while p-STAT3β is a tumor suppressor gene, which can feedback regulate the expression of p-STAT3α[211]. We mainly focused on the expression of p-STAT3α in this study. We observed that the expression levels of p-STAT3 and MMP-14 proteins were significantly downregulated in all 4 cells after 24h of HO-3867 treatment ($p< 0.05$). For STAT3, significant decreases in STAT3 expression were seen in UM-UC-3 cells, RT-4 cells, and HT-1376 cells ($p<0.05$). Additionally, significant decreases in PD-L1 expression were seen in RT-4 cells, RT-112 cells, and HT-1376 cells ($p<0.05$, Figure 21F).



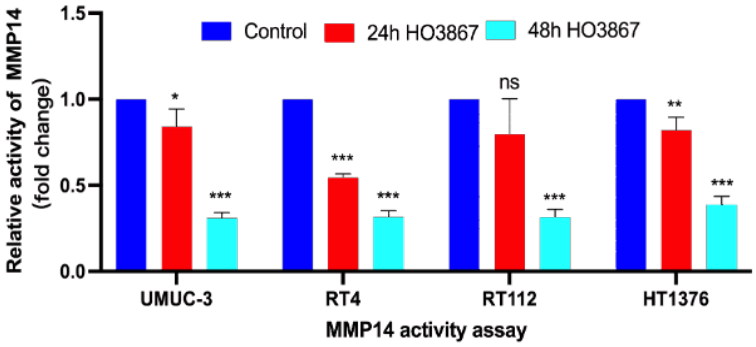
C.


Control
24h HO3867
48h HO3867
Apoptosis Index (fold of control)
6.0
4.5
3.0
1.5
0.0
ns
**
ns
***
ns
**
ns
***
UMUC-3
RT4
RT112
HT1376
cell death detection

D.


Control
6h HO3867
24h HO3867
48h HO3867
Cytotocixity (%)
80
60
40
20
0
UMUC-3
RT4
RT112
HT1376
cell cytotocixity

E.


Control
24h HO3867
48h HO3867
Relative activity of MMP14 (fold change)
1.5
1.0
0.5
0.0
*
***
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ns
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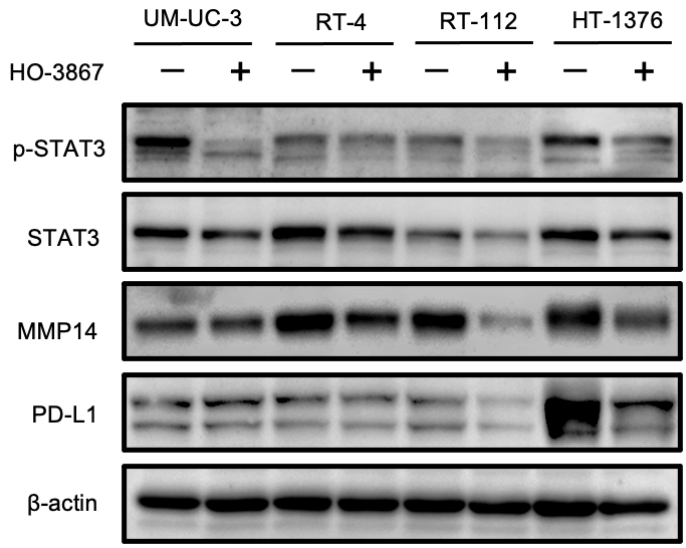
UMUC-3
RT4
RT112
HT1376
MMP14 activity assay

F.


UM-UC-3
RT-4
RT-112
HT-1376
HO-3867
— + — + — + — +
p-STAT3
STAT3
MMP14
PD-L1
β-actin

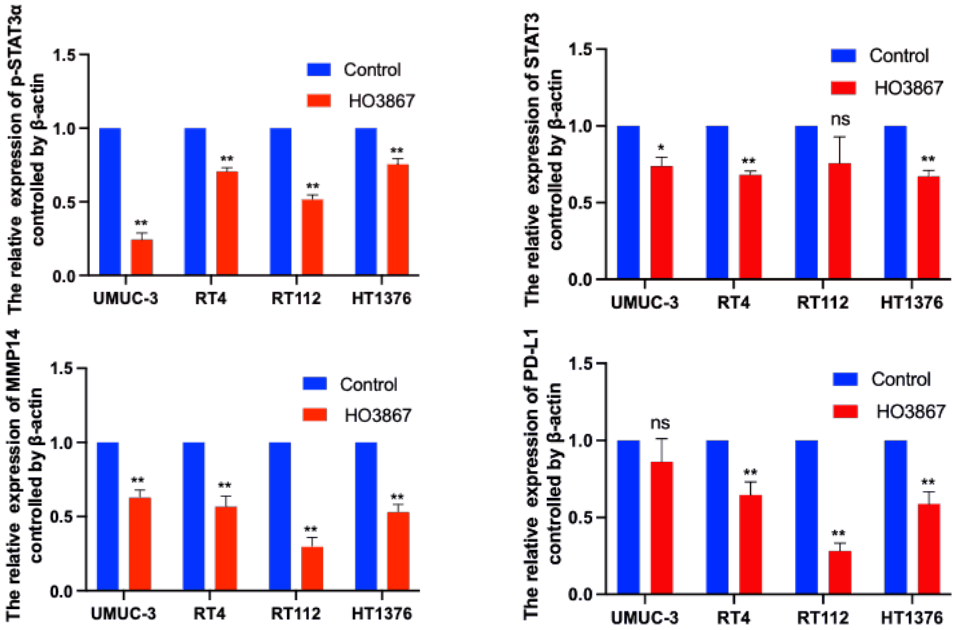


Figure 21 Effect of HO-3867 (STAT3-specific inhibitor) on bladder cancer cells.

A. Cells were treated with different concentrations of HO-3867 (0, 0.5, 1, 2, 5, 10, 15, 20 μmol/L) for 48h, and the IC50 was determined by nonlinear regression; (n=3). B. 4 bladder cancer cells were treated with the corresponding IC50 of HO-3867, the controls were added to PBS, CCK-8 was implemented to quantify cell viability at 24h, 48h, and 72h treatment of HO-3867, significant results were determined by two-way ANOVA, and multiple comparison was done by Bonferroni's method; (n=3). C. 4 bladder cancer cells were treated with HO-3867 at the corresponding IC50, the controls were added to PBS, the MMP-14 activity assay detected MMP-14 activity at 24h and 48h treatment of HO-3867, one-way ANOVA was used to identify significant results, and Dunnett's procedure was used for multiple comparisons; (n=3). D. 4 bladder cancer cells were treated with the corresponding IC50 of HO-3867, the controls were treated with PBS, cell death detection was performed to test the cell apoptosis at 24h and 48h of HO-3867 treatment, one-way ANOVA was used to identify significant results, and Dunnett's procedure was used for multiple comparisons; (n=3). E. 4 bladder cancer cells were treated with HO-3867 at the corresponding IC50 concentrations, and the control group was treated with PBS. The cytotoxicity induced by HO-3867 was measured by LDH release assay at 6h, 24h, and 48h of treatment, one-way ANOVA was used to identify significant results, and Dunnett's procedure was used for multiple comparisons; (n=3). F. 4 bladder cancer cells were treated with HO-3867 at the corresponding IC50, and the controls were added to PBS. Cellular proteins were collected at 24 h treatment, the protein expression of STAT3, p-STAT3, MMP-14, and PD-L1 was detected by Western blotting, and significant results were determined by independent t-test; (n=3). *p <0.05, **p <0.01, *** p<0.001, ns: no significance.

3.4.3 Effect of Colivelin (STAT3-specific activator) on bladder cancer cells

Additionally, Colivelin (STAT3-specific activator) was used to elevate the phosphorylation level of STAT3. After culturing the cells with serum-free medium for 24 h, the cells were treated with 0.5 μmol/L Colivelin for 2 h in each bladder cancer cell treatment group. Their

cell proteins were collected to detect protein expression assay by Western blotting (Figure 22). The results showed that Colivelin significantly increased the phosphorylation of STAT3 in UM-UC-3, RT-4, and RT-112 cells ($p<0.05$), and the expression level of STAT3 was significantly decreased in UM-UC-3 cells compared to the control group ($p<0.05$). In addition, the treatment of Colivelin significantly upregulated the expression levels of MMP-14 and PD-L1 in RT-4, RT-112 and HT-1376 cells ($p<0.05$).

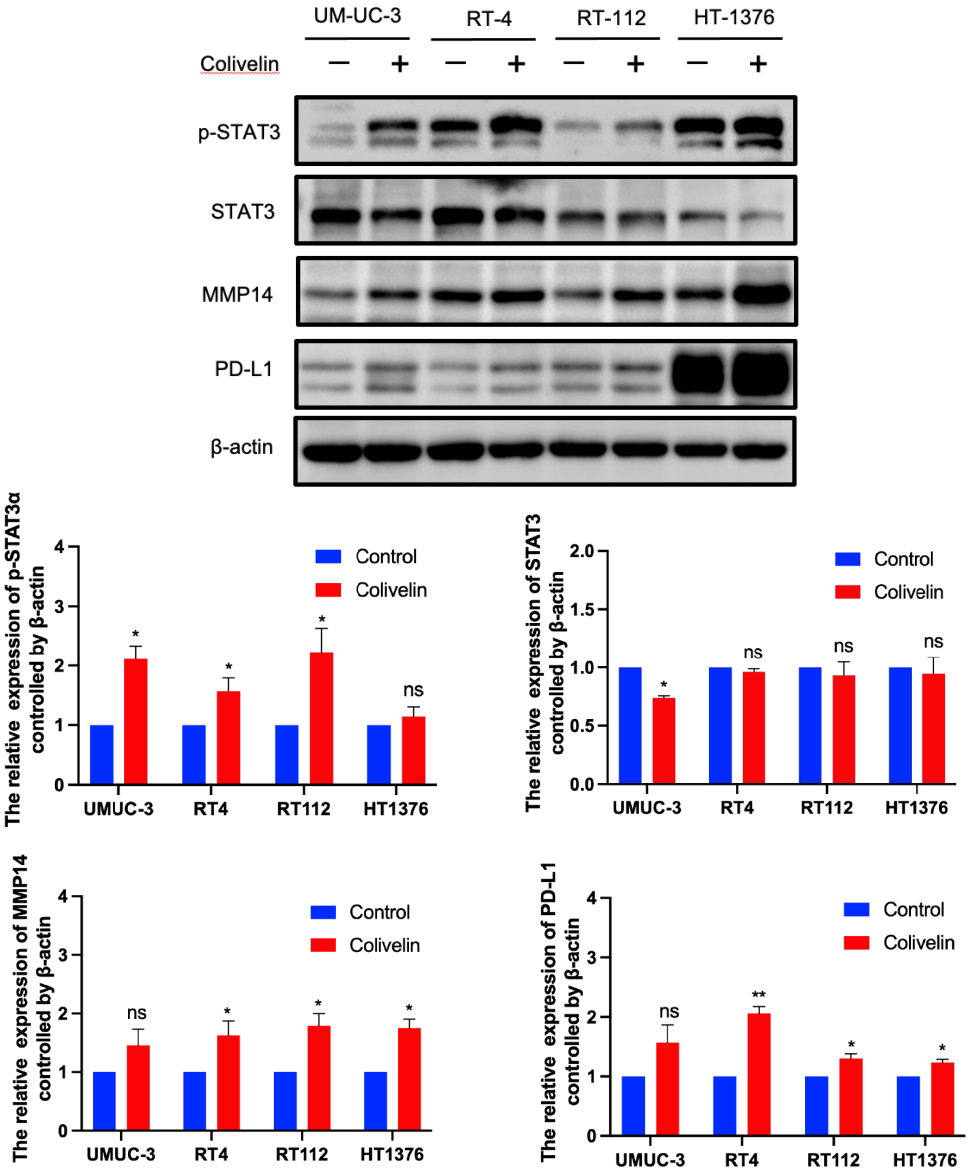


Figure 22 Effect of Colivelin (STAT3-specific activator) on bladder cancer cells.

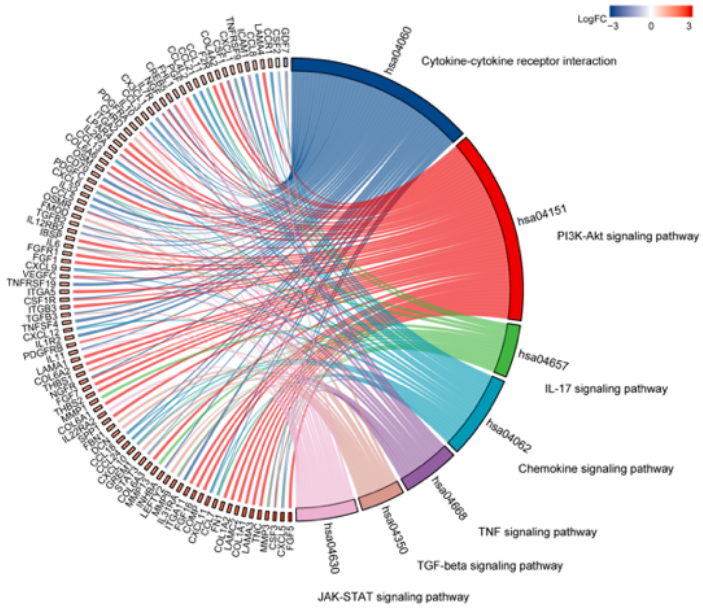
The cells were treated with serum-free medium for 24 h, and then 0.5 μmol/L Colivelin was used to treat the cells in the treatment group, and for the control group, PBS was added. Cell proteins were collected after 2h of treatment, and the protein expression of STAT3, p-STAT3, MMP-14, and PD-L1 was detected by Western blotting. Significant results were determined by independent t-test. (n=3), *p <0.05, **p <0.01, *** p<0.001, ns: no significance.

3.5 Interaction of MMP-14 with STAT3 in bladder cancer

3.5.1 Exploration of signaling pathways involved in MMP-14 function in bladder cancer

After detecting the regulatory relationship of STAT3 on MMP-14, we investigated the downstream pathways regulated by MMP-14. First, we performed KEGG pathway enrichment of previously screened public genes to explore the immune-related signaling pathways involved in MMP-14. The results showed that MMP-14 might be involved in cytokine-cytokine receptor interaction in bladder cancer, as well as the PI3K-Akt signaling pathway, IL-17 signaling pathway, Chemokine signaling pathway, TNF signaling pathway, TGF-beta signaling pathway, and JAK-STAT signaling pathway ($p<0.001$, Figure 23A). Next, we used human phosphorylated kinase antibody microarrays to detect the effects of NSC405020 on 43 phosphorylated kinases in bladder cancer. After treating HT-1376 cells with IC50 NSC405020 for 24h, we found that many of the kinases were significantly downregulated. Specifically, the downregulated kinase sites included CREB(S133), PRAS40(T246), WNK1(T60), RSK1/2(S221/S227), GSK-3β(S9), p70 S6 Kinase (T421/S424), GSK-3α/β (S21/S9), c-Jun (S63), p53 (S392), STAT3 (Y727), p70 S6 Kinase (T389), p53 (S15), RSK1/2/3 (S380/S386/S377), Akt 1/2/3 (S473), Lck (Y394), STAT5a/b (Y699), Chk-2 (T68), STAT1 (Y701), p53 (S46), Yes (Y426), ERK1/2 (T202/Y204, T185/ Y187), Fgr (Y412), Akt 1/2/3 (T308), Src (Y419), STAT6 (Y641), PYK2 (Y402), p38α (T180/Y182), HSP27 (S78/S82), PDGF Rβ(Y751), STAT3 (Y705), STAT2 (Y689), Lyn (Y397), MSK1/2 (S376/S360), β-Catenin, JNK 1/2/3 (T183/Y185, T221/Y223) and HSP60. In contrast, a few phosphorylation sites, such as EGF R (Y1086), PLC-γ1 (Y783), and eNOS (S1177), showed upregulated expression (Figure 23B).

A.



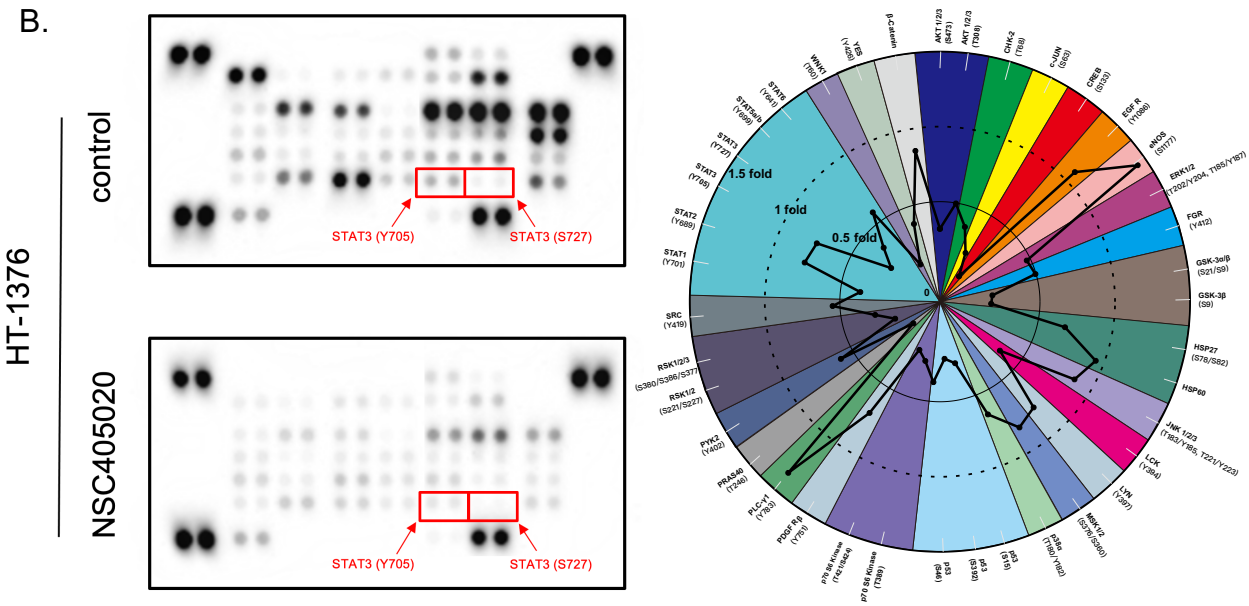


Figure 23 Exploration of signaling pathways involved in MMP-14 function in bladder cancer.

A. KEGG pathway enrichment analysis of previous public genes was used to find the immune-related signaling pathways involved in MMP-14. B. HT-1376 cells were treated with IC50 NSC405020 in the treatment group, and PBS was added in the control group; after 24h of treatment, the protein lysates were collected, and human phosphorylated kinase antibody microarrays were performed to detect the effects of NSC405020 on 43 phosphorylated kinases in bladder cancer.

3.5.2 Inhibition of MMP-14 could down-regulate STAT3 expression levels and its phosphorylation in bladder cancer cells

Considering the critical role of STAT3 in immunosuppression and combining the previous results of the KEGG pathway enrichment analysis and human phosphorylated antibody microarray, we hypothesized that MMP-14 could regulate STAT3 and thus perform immunosuppressive effects. Therefore, with this hypothesis, we treated bladder cancer cells with IC50 NSC405020 for 24h and detected the expression levels of STAT3 and p-STAT3 by Western blotting; the results showed that compared to the control group, the expression levels of p-STAT3 were significantly decreased in the NSC405020-treated group for all 4 cells ($p<0.01$), while for STAT3, significant lower expression could be seen in RT-4, RT-112 and HT-1376 cells ($p<0.01$, Figure 24A). In addition, after MMP-14 was knocked down in HT-1376 cells, the expressions of p-STAT3 and STAT3 were also significantly decreased ($p<0.001$, Figure 24B).

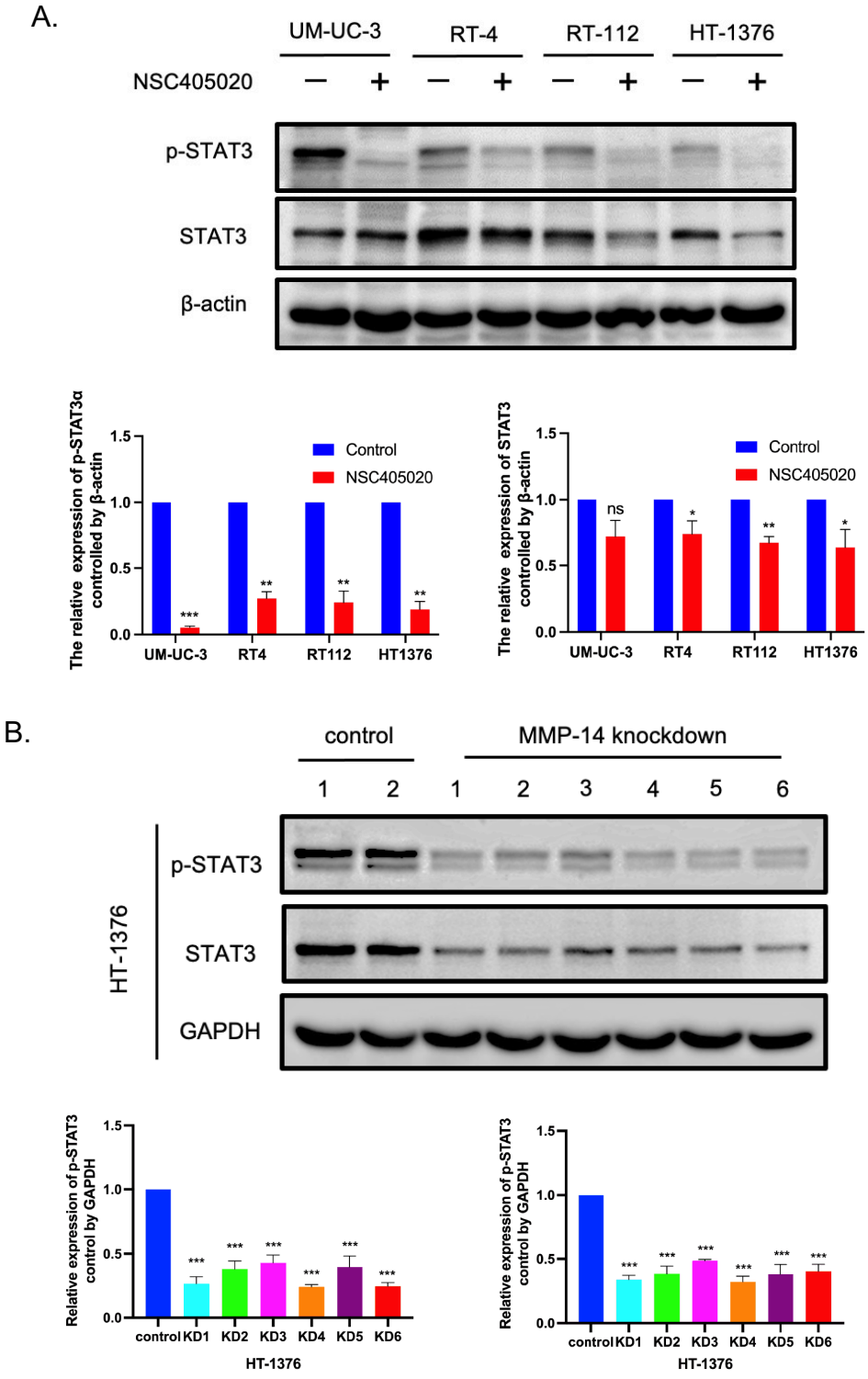


Figure 24 Inhibition of MMP-14 could down-regulate STAT3 expression levels and its phosphorylation in bladder cancer cells.

A. 4 bladder cancer cells were treated with the corresponding IC50 NSC405020 in the treatment group, and PBS was added to the control group. After 24 h of treatment, proteins were collected, the protein expression levels of STAT3 and p-STAT3 were detected by Western blotting, and significant results were determined by independent t-test (n=3).

B. The protein expression levels of STAT3 and p-STAT3 were detected by Western blotting after MMP-14 was knocked down in HT-1376 cells, one-way ANOVA was used to identify significant results, and Dunnett's procedure was used for multiple comparisons. (n=3), *p <0.05, **p <0.01, *** p<0.001, ns: no significance.

3.5.3 Colivelin could upregulate the expression of PD-L1 in MMP-14 knockdown cells

In the previous experiments, we confirmed that MMP-14 could regulate the expression levels of STAT3 and PD-L1, while STAT3, as a transcription factor, has also been shown to regulate the expression levels of MMP-14 and PD-L1, but no study has yet shown the molecular mechanism by which MMP-14 regulates PD-L1. We speculated that MMP-14 could affect the expression level of PD-L1 by regulating STAT3. Therefore, after culturing the HT-1376 control cells and HT-1376 MMP-14 knockdown cells with serum-free medium for 24 h, the cells were treated with 0.5 μmol/L Colivelin for 2 h in each treatment group, and the cell proteins were collected to detect protein expression by Western blotting. The results showed that the knockdown of MMP-14 decreased the expression of STAT3, p-STAT3 and PD-L1($p<0.001$), but then again the application of Colivelin upregulated the expression of p-STAT3 and PD-L1 in MMP-14 knockdown cells ($p<0.001$, Figure 25), suggesting that MMP-14 might promote the expression of PD-L1 by regulating the phosphorylation of STAT3.

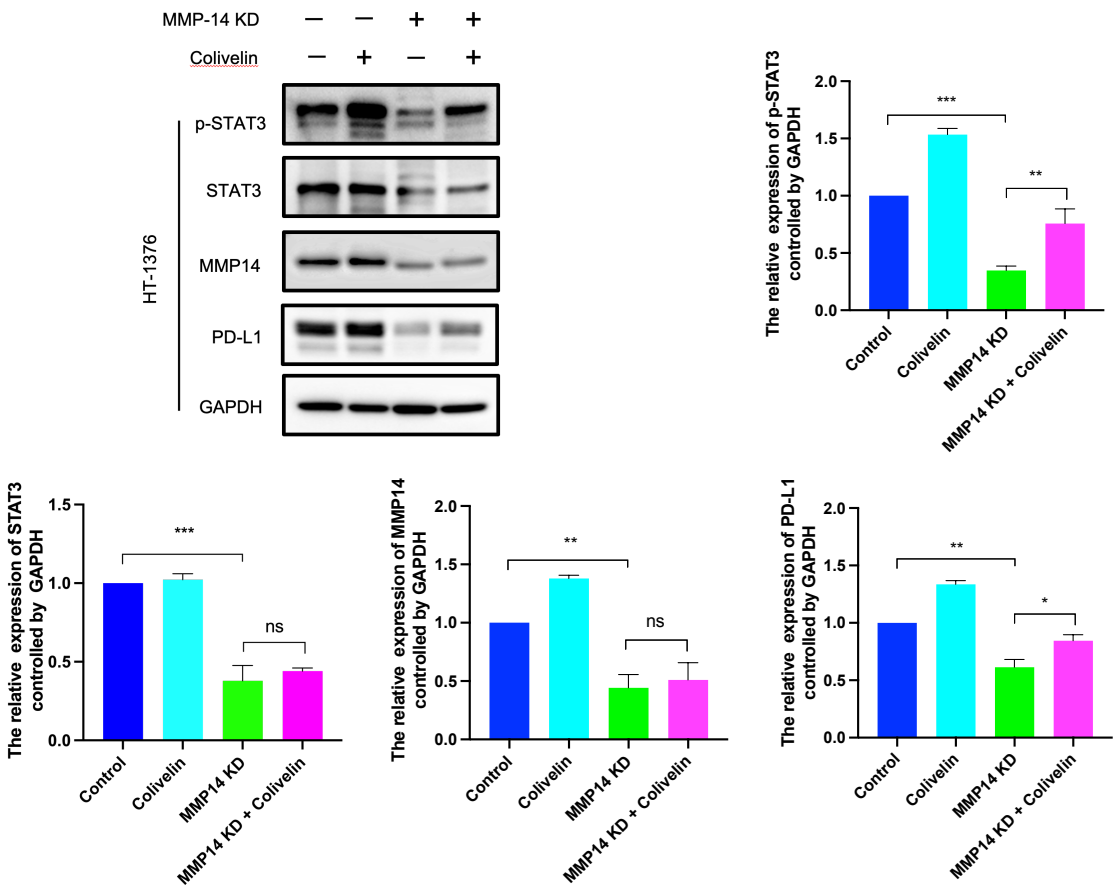


Figure 25 Colivelin rescued the expression of PD-L1 in MMP-14 knockdown HT-1376 cells.

The control HT-1376 cells and MMP-14 knockdown HT-1376 cells were treated with serum-free medium for 24 h, and then 0.5 μmol/L Colivelin was used to treat the cells in the treatment group, and for the control group, PBS was added. Cell proteins were collected after 2h of treatment, and the protein expression of STAT3, p-STAT3, MMP-14, and PD-L1 was detected by Western blotting. one-way ANOVA was used to identify significant results, and Bonferroni's procedure was used for multiple comparisons. (n=3), *p <0.05, **p <0.01, *** p<0.001, ns: no significance.

4. Discussion

4.1 Expression and clinical significance of MMP-14 in bladder cancer

As a member of the matrix metalloproteinase family, MMP-14 degrades the extracellular matrix by activating pro-MMP2 and pro-MMP9. Mohammad et al. carried out Western blotting and found that MMP-14 was significantly overexpressed in bladder cancer[212]. ELISA experiments by Kudelski et al. showed that the activity of MMP-14 was increased in high grade bladder cancer compared with normal bladder tissue[213]. Moreover, Wang et al. used the TCGA database, Western blot and immunohistochemistry and found that MMP-14 was significantly highly expressed in muscle-invasive bladder cancer compared with normal tissues[214]. In our study, the TCGA database was used to show that MMP-14 was significantly upregulated in bladder cancer compared to the paired and unpaired normal bladder tissues. Interestingly, a study used quantitative real-time polymerase chain reaction to detect the mRNA expression level of MMP-14, and the results showed that MMP-14 had relatively low expression in 40 bladder cancer tissues compared to 5 control bladder tissues[215].

The poor prognosis of tumor patients has been a challenging clinical problem. It has been shown that bladder cancer patients with over median MMP-14 expression had shorter 5-year disease-free survival and overall survival than those with low MMP-14 expression, and multivariate Cox regression tests showed that MMP-14 expression level was an independent predictor of disease-free survival[216]. Sagara et al. found that MMP-14 expression was not significantly associated with metastasis-free survival and cause-specific survival in bladder cancer patients[217]. A study showed that patients with high MMP-14 expression in muscle-invasive bladder cancer had poor short-term prognoses[214]. In our study, MMP-14 expression levels were significantly associated with patients' overall survival in bladder cancer, and MMP-14 expression levels were significantly lower in the survivor group compared to the death group, but MMP-14 expression levels were not significantly associated with disease-free progression in bladder cancer, suggesting that high MMP-14 expression is not strongly associated with patient disease progression. Nevertheless, it poses a significant challenge for prolonging overall survival in bladder cancer.

Racial factors play an essential role in the development of bladder cancer[218]. In the pathogenesis of gallbladder cancer, Vinay et al. investigated the genetic variation of the MMP-14 promoter in different Indian races. The results showed that the allele carriers of rs1003349 and rs1004030 had higher MMP-14 expression levels and were significantly associated with the development of gallbladder cancer[219]. MMP-14 expression was correlated with race in diffuse large B-cell lymphoma, and MMP-14 expression was significantly higher in White patients than in Black or African patients and the Asian patients[220]. Our study suggested that the White patients had higher MMP-14 expression levels than the Black and African patients as well as Asian patients, which may be due to specific genetic traits carried by the White patients that promote MMP-14 expression levels and contribute to the development and progression of bladder cancer.

TNM stage is an important indicator used by the Union for International Cancer Control to evaluate the condition of tumor patients based on the primary tumor status, lymph node metastasis, and distant metastasis and to guide clinical treatment and assess prognosis. Generally speaking, the higher the TNM stage, the worse the patient's prognosis. Our study also found that patients with stages III and IV had higher MMP-14 expression levels than those with stage II, suggesting that MMP-14 drives the progression of bladder cancer.

Furthermore, in terms of T stage, Yuji et al. found a significant increase in MMP-14 expression levels in patients with T2 stage bladder cancer compared to low T-stage (Ta, T1)[217]. In the present study, we found that patients with the T3 stage had significantly higher MMP-14 expression levels than those with the T2 stage. For the N stage, Mohammad et al. found that metastases of lymph node and MMP14 expression were not connected[212], and our findings also showed no significant correlation between MMP-14 expression levels and lymph node status. Wang et al. found that MMP-14 expression was closely related to tumor metastasis in MIBC[214], while our study showed that there was no significant correlation between MMP-14 expression and tumor metastasis in bladder cancer, which indicates that MMP14 may play an important role in the metastasis of MIBC, while the probability of metastasis in NMIBC is relatively low, which results in no significant correlation of MMP14 with tumor metastasis in all stages of bladder cancer. However, this also might be related to the small number of samples that included tumor metastases in our study, which ultimately led to sample bias.

Uroepithelial carcinomas can be divided into two groups according to the pathological type: papillary and non-papillary tumors (basal infiltrative tumors). Papillary tumors originate from peribasal cells and have highly differentiated histological features. They are genetically stable and mostly superficially tubulointerstitial. Patients with papillary tumors have frequent recurrences but are at low risk of tumor progression on clinical prognosis. The non-papillary tumors are poorly differentiated, and originate from basal cells. Morphologically, they are solid and infiltrative tumors with many genetic alterations in the tumor cells, and the prognosis for patients with non-papillary tumors is poor[221]. Non-papillary tumors had higher MMP-14 expression levels in our study than papillary tumors. Alterations of some pathways, including STAT3, EGFR, and HIF-1, promote MMP-14 transcriptional activity and increase MMP-14 expression levels in non-papillary carcinomas compared with papillary carcinomas. In addition, the cell surface receptor CD44, a component of the invasive pseudopod of tumor cells, is often overexpressed in non-papillary carcinomas. CD44 can bind to MMP-14 to form β-actin, promoting distant metastasis of tumor cells, which elevates the activity of MMP-14 in this process[222].

Radiation therapy is vital in treating bladder cancer as an effective modality in oncologic therapy. Radiation therapy was first used to treat bladder cancer as a part of bladder preservation protocols, in which urologists perform a maximal transurethral resection of the tumor to preserve the bladder in patients with muscle-invasive bladder cancer, then administer concurrent chemotherapy and radiation therapy. Radiation therapy also lowers the chance of local or incisional recurrence in individuals who have had partial cystectomy. Recent studies have shown that radiation therapy can be a palliative treatment for locally advanced or metastatic bladder cancer patients. It relieves bleeding and pain from local progression or tumor metastasis[223]. Our results showed that specimens from patients treated with radiation therapy showed lower MMP-14 expression levels than those treated without radiation therapy, suggesting that radiation therapy exerts a pernicious effect on tumor cells and reduces their aggressiveness to some extent.

The Response evaluation criteria in solid tumors (RECIST) guidelines are often used clinically to assess the effectiveness of oncologic therapy, including Complete response (CR), Partial response (PR), Stable disease (SD), and Progressive disease (PD)[224]. CR is defined as the disappearance of all tumor target lesions, and the absence of new lesions and regular tumor markers, maintained for at least 4 weeks; PR is defined as a ≥30%

decrease in the sum of the maximum diameter of the tumor target lesions, maintained for at least 4 weeks; SD represents a decrease in the sum of the maximum diameter of the tumor target lesions that do not reach PR or an increase that does not reach PD; PD indicates an increase in the sum of the maximum diameter of the tumor target lesions by at least ≥20%, or the appearance of new lesions. The data from our study showed that MMP-14 had relatively higher expression levels in PR than in SD, while no significant differences were seen with other disease states. MMP-14, as an indicator of tumor aggressiveness, decreases when tumor viability decreases. PR has a lower tumor load than SD, representing a better treatment outcome. Nevertheless, to our surprise, the PR with lower tumor activity had higher MMP-14 expression, which may be related to the tumor metastasis that occurred during the treatment and may also be related to sample bias because of the small sample size associated with the inclusion of the SD versus PR group in this study.

4.2 Roles of MMP-14 in bladder cancer

MMP-14 is an essential member of the MMPs and was the first membrane matrix metalloproteinase to be identified on the membranes of malignant cells. MMP-14 can further activate MMP-2 and MMP-9 to degrade the extracellular matrix on the surface of cells, which plays a vital role in malignant cells [225].

NSC405020 is a specific inhibitor of MMP-14 that binds to the hemopexin domain of MMP-14 and affects the homodimerization of hemopexin [226]. It has been shown that NSC405020 reduced the movement and invasion of the human trophoblast cell line HTR-8/SVneo by inhibiting the expression of MMP-14[227]. In addition, NSC405020 reduced Notch3 activity in lymphatic vessel endothelial cells and led to restricted invasive germination of human WM852 melanoma cells in 3D culture, ultimately reducing the metastasis and invasion of melanoma[228], while Tang et al. treated human squamous cell carcinoma HSC5 cells with NSC405020 and found that it reduced the invasive ability and intracellular phosphorylation of extracellular signal-regulated kinase expression in HSC5 cells[229]. In our study, we treated four different bladder cancer cell lines with NSC405020. We verified the effect of NSC405020 on MMP-14 expression and activity using Western blotting and MMP-14 activity assays. The results showed that NSC405020 decreased MMP-14 expression levels and inhibited the activity of MMP-14 in bladder

cancer cells. Furthermore, a series of functional assays indicated that NSC405020 inhibited the proliferation of bladder cancer cells, accelerated the apoptosis of bladder cancer cells, and induced remarkable cytotoxicity to bladder cancer, which indicated that NSC405020 has a good prospect for oncologic therapy.

Novelly, we found that MMP-14 has an immunosuppressive function in bladder cancer using GO functional enrichment analysis. Immunosuppression is an essential mechanism by which tumor cells evade immune killing[230]. As is known, tumor cells can express some immune checkpoints to prevent antigen-presenting cells, T cells, and NK cells from recognizing them[231]. On the other hand, tumor cells can secrete a series of cytokines to recruit Treg cells and prevent activation of tumor-killing T cells, inducing an immunosuppressive tumor microenvironment. Previous studies have shown that MMP-14 can negatively regulate the inflammatory response by activating MMP-2 to regulate the concentration of alarm protein S100A9[232]. In colorectal cancer, upregulation of MMP-14 expression due to PROX1 deficiency is associated with the activation of cytotoxic T cells[233]. In addition, MMP-14 has been reported to be involved in the immune infiltration of various tumor tissues[234]. As a member of the degrading extracellular matrix enzyme, MMP-14 might contribute to the development of tumor immunosuppression by remodeling the tumor microenvironment. Our study analyzed the relationship between two central immune cells ($CD8^+$ T cells and Treg cells) and MMP-14 mRNA expression levels using the ssGSEA algorithm. We showed that MMP-14 was weakly correlated with $CD8^+$ T cells and moderately correlated with Treg cells. In contrast, in the subgroups with different MMP-14 expressions, the group with high MMP-14 expression had a higher Treg cell enrichment, suggesting that MMP-14 may be involved in the immunosuppression of tumor cells through Treg cells in bladder cancer.

Furthermore, our GSEA analysis revealed that MMP-14 was associated with the PD-1 signaling pathway. PD-1 is an immunosuppressive molecule expressed mainly on the surface of T cells and plays an essential role in regulating T cell activation[235]. In addition, as a ligand molecule of PD-1, the immune checkpoint PD-L1 is expressed on the cell surface of tumor cells and induces the production of tumor immunosuppression[236]. Therefore, we speculated whether MMP-14 exerts an immunosuppressive function via PD-L1 in bladder cancer. We first detected the primary expression of MMP-14 and PD-L1 in different bladder cancer cell lines using Western blotting and immunofluorescence,

and the results proved that MMP-14 was positively correlated with PD-L1 expression. Next, we treated bladder cancer cells with NSC405020, and the expression level of PD-L1 in the treated group decreased with the downregulation of MMP-14 expression. Furthermore, we showed a consistent change in PD-L1 expression after the knockdown of MMP-14 in HT-1376 cells, suggesting that MMP-14 can regulate PD-L1 expression to be involved in tumor immunosuppression. To our knowledge, our study is the first to demonstrate the relationship between MMP-14 and PD-L1, which has not been reported in previous literature.

4.3 Roles of STAT3 in bladder cancer

STAT3 plays a critical role in cell proliferation and apoptosis. STAT3 activation is transient and tightly regulated in normal tissues[237, 238]. However, persistently activated STAT3 proteins can upregulate various gene expressions for cell proliferation, apoptosis, and the cell cycle in tumor cells, including c-Myc, Cyclin D1/D2, p19INK4D, Pim-1, c-Fos, MCL1, Survivin and Bcl-xL[239-243]. Kijima et al. showed that inhibition of STAT3 activation effectively reduced intracellular expression of Cyclin D1 and Bcl-2 in head and neck squamous cell carcinoma, which significantly inhibited cell proliferation and caused significant apoptosis[244]. Similarly, decreased STAT3 activity inhibited cell proliferation and induced apoptosis in nasopharyngeal carcinoma and breast cancer[245, 246]. Our study used the STAT3-specific inhibitor HO-3867 to treat bladder cancer cells. HO-3867 is a novel STAT3-selective inhibitor whose main component is diarylipenylpiperiden-4-one [247]. Previous studies have shown that HO-3867 has a higher bioavailability in tumor tissues than in normal tissues and a dose-dependent inhibitory effect on tumor growth[248]. At the same time, HO-3867 can selectively kill cancer cells by converting mutant p53 proteins into transcriptionally active wild-type p53 and had a significant cytotoxic effect on tumor cells[248-250]. In human oral squamous cell carcinoma and human osteosarcoma cells, HO-3867 induced apoptosis by activating caspase-3, caspase-8, and caspase-9 through the JNK1/2 pathway[251, 252]. HO-3867 mediated G2/M cell cycle arrest and mitochondrial damage, and promoted apoptosis through intracellular reactive oxygen species-dependent endoplasmic reticulum stress in human pancreatic cancer cells[253, 254]. Moreover, HO-3867 significantly inhibited the growth of ovarian xenograft tumors in a dose-dependent manner and exhibited significant cytotoxicity against ovarian

cancer cells by inhibiting the JAK/STAT3 signaling pathway[255]. Similarly, our results showed that HO-3867 down-regulated STAT3 and p-STAT3 expression in bladder cancer cells. Additionally, HO-3867 inhibited cell proliferation dose-dependently and significantly induced apoptosis. Lactate dehydrogenase release assay also illustrated that HO-3867 induced significant cytotoxic effects on bladder cancer cells at IC50.

STAT3 is closely related to tumor invasion and metastasis[256]. One of the critical reasons for failed treatment in malignant tumors is that the malignant tumor breaks down the basement membrane and extracellular matrix from the in situ tumor site, and therefore the tumor spreads externally[257]. Histologically, the hallmark of tumor invasion is the destruction of the basement membrane. Metastasis is the formation of tumor cells elsewhere via lymphatic vessels or vascular processions based on the invasion of the tumor. Matrix metalloproteinases (MMPs) play a prominent role in tumor invasion and metastasis as critical enzymes for degrading basement membrane and extracellular matrix components[258]. MMPs can degrade most proteins in the basement membrane and extracellular matrices, such as collagen and fibronectin, and reshape the tumor microenvironment[259, 260]. They are also critical pro-angiogenic factors that promote tumor angiogenesis to influence tumor invasion and metastasis[135]. Therefore, an increase in the expression of MMPs will facilitate tumor invasion and metastasis. Under normal physiological conditions, MMPs and their inhibitors (tissue inhibitors of metalloproteinases, TIMP) can work together to achieve a state of equilibrium[261]. Numerous studies have shown that blocking the STAT3 signaling pathway downregulates the expression and activity of various MMPs, including MMP-1, 2, 3, 7, and 9[128, 207-210]. Therefore, the highly activated STAT3 in tumor cells may promote tumor cell invasion and metastasis by upregulating the expression and activity of MMPs. In the present study, HO-3867 treatment resulted in the downregulation of MMP-14 expression and the significant inhibition of MMP-14 activity in bladder cancer cells. In a previous study, HO-3867 significantly reduced the migration and invasion of ovarian cancer cells by inhibiting STAT3 expression and thereby downregulating fatty acid synthase as well as adherent spot kinase expression[262, 263]. HO-3867 treatment also slowed the invasion rate in human osteosarcoma cells[264]. In addition, we treated starved bladder cancer cells with Colivelin and found that it transiently increased STAT3 phosphorylation and promoted the upregulation of MMP-14 expression. Colivelin, a heterodimeric peptide composed of activity-depend-

ent neurotrophic factor and AGA-(C8R)HNG17, was initially used to treat Alzheimer's disease. It was influential in protecting neurons and maintaining their activity, but later mechanistic studies showed that Colivelin could activate STAT3 phosphorylation specifically[265, 266]. In a study of proliferative vitreoretinopathy, Colivelin treatment increased STAT3 phosphorylation and upregulated MMP-2 activity, increasing retinal pigment epithelial cell proliferation and migration[267]. The addition of Colivelin upregulated p-STAT3 expression levels in colon cancer cells[268]. In cervical cancer, Colivelin reduced apoptosis and enhanced cancer cells' invasion, metastasis, and stemness[269]. Colivelin enhanced tumor cell survival and inhibited apoptosis in nasopharyngeal cancer[270]. This suggests that STAT3 can mediate the invasion and metastasis of bladder cancer cells through MMP-14.

Interestingly, STAT3 is a transcription factor that mediates tumor-induced immunosuppression. Some studies have suggested that activated STAT3 in tumors could upregulate IL-6, IL-10 and VEGF to build a tumor immunosuppressive state and reduce the tumor-killing effect of the immune system by downregulating IFNγ, IL-5, CXCL10, and CCL5 as well as co-stimulatory factors CD80 and CD86[130, 131, 271]. Furthermore, the level of STAT3 activation in tumor cells was closely related to the infiltration of lymphocytes and the migration of immune cells[272, 273]. In addition to activating tumor cells to produce a variety of negative immune regulators, activation of the STAT3 pathway in immune cells directly produces immunosuppressive effects. It has been shown that activated STAT3 signaling could inhibit the activation of dendritic cells and reduce the antigen-presenting ability of immune cells[130]. Secondly, activated STAT3 signaling positively regulates the proliferation and recruitment of immature myeloid cells, including Myeloid-derived suppressor cells and Tumor-associated macrophages, thereby suppressing the effects of $CD4^+$ and $CD8^+$ effector T cells[271, 274]. Thirdly, activated STAT3 signaling led to the proliferation of tumor-infiltrating Tregs [271]. At the same time, STAT3 could specifically bind to the transcriptional promoter sequences of IL-10 and TGFβ, promoting the secretion of IL-10 and TGFβ, thereby efficiently suppressing the natural and acquired immune systems and inhibiting the killing function of $CD8^+$ T cells and the maturation of DC cells[131]. It has been observed that STAT3-$CD8^+$ T cells derived from the mouse blood system produced more antigen-specific IFN-γ in response to vaccine or tumor stimulation. IFN-γ played a critical role in attenuating the immunosuppression produced by Treg cells[275]. Therefore, STAT3 can promote the immune escape of tumors in multiple ways.

In the present study, we treated bladder cancer cells with HO-3867 to inhibit the STAT3 signaling pathways and, interestingly, we found that PD-L1 expression was also down-regulated. As mentioned earlier, many tumor cells overexpress PD-L1, which can bind to PD-1 on the surface of cytotoxic T cells leading to impaired T cell function, and bind to PD-1 on the surface of Treg cells leading to proliferation and activation of Treg cells[276, 277]. Moreover, PD-L1 can also act synergistically with other immunosuppressive signals to form a suppressive immune microenvironment[278]. In addition, after using Colivelin to activate STAT3 phosphorylation in bladder cancer cells, we observed an upregulation of PD-L1 expression in bladder cancer cells. Similarly, in another study, colivelin treatment increased PD-L1 expression levels by activating STAT3 phosphorylation in osteosarcoma[279]. This suggests that phosphorylated STAT3 (p-STAT3α) regulates PD-L1 expression in bladder cancer, promoting immunosuppression of bladder cancer.

4.4 Relationship of MMP-14 with STAT3 in bladder cancer

We explored the pathways through which MMP-14 regulated PD-L1 expression and promoted immune escape in bladder cancer. Using the KEGG database, we first performed a pathway enrichment analysis of the previous common genes between DEGs and expression-related genes according to the MMP-14 expression level in bladder cancer. We screened out some immune-related pathways, and it was shown that MMP-14 might be involved in the JAK/STAT3 signaling pathway. Antibody microarray and Western blotting also demonstrated that MMP-14 could regulate STAT3 expression and affect its phosphorylation in bladder cancer cells. In previous studies, STAT3 was reported to be activated mainly by tyrosine phosphorylation, and STAT3 monomers could interact with phosphorylated tyrosine residues of another STAT3 molecule through the SH2 structural domain to form a dimer to enter the nucleus and regulate the transcription of target genes[237]. Additionally, various peptides were reported to activate STAT3, like growth factors (epidermal growth factor, platelet-derived growth factor) and cytokines (interleukin-6, interleukin-11, oncotoxin, ciliary neurotrophic factor, leukemia inhibitory factor, IFN-γ, G-CSF and leptin)[280-282]. Our study clarified the regulatory relationship of MMP-14 on STAT3 in bladder cancer cell lines. Similarly, Concanavalin-A (an MMP-14 activator) induced STAT3 phosphorylation[283], whereas silencing the MMP-14 gene significantly inhibited STAT3 phosphorylation[284]. Additionally, Djedial et al. used Concanavalin-A to

induce transient activation of STAT3 in U87 glial cells[285]. Furthermore, we explored the mechanism of STAT3 regulation by MMP-14, and our results verified that MMP-14 promoted STAT3 being phosphorylated. Pratt and Annabi demonstrated that MMP-14 could regulate the STAT3 signaling pathway, and that the cellular domain structure of MMP-14 was required for STAT3 phosphorylation[286]. In addition, the knockdown of MMP-14 inhibited STAT3 phosphorylation induced by Concanavalin-A in mesenchymal stem cells[284].

4.5 MMP-14 is suggested to regulate PD-L1 expression through phosphorylation of STAT3 in bladder cancer

Feng et al. found that the traditional Chinese medicine Banxia xiexin decoction (BXXX) significantly inhibited the proliferation of drug-resistant gastric cancer cells and inhibited the expression levels of IL-6, IFN-γ, JAK/STAT3, and PD-L1, but Colivelin treatment reverted the effect of BXXX on drug-resistant gastric cancer cells and significantly reversed the inhibitory effect of BXXX on PD-L1 expression[287]. Thymosin α1 significantly inhibited migration and invasion by inhibiting STAT3-MMP2 signaling pathway transduction in PD-L1 high-expressing non-small cell lung cancer cells. However, colivelin partially reversed Thymosin α1 inhibition of MMP-2 expression and cell migration phenotype[288]. In addition, He et al. found that water extract of sporoderm-broken spores of G. lucidum could downregulate PD-L1 expression by inhibiting p-STAT3 phosphorylation in osteosarcoma, while colivelin treatment reactivated STAT3 phosphorylation and thus upregulated PD-L1 expression[279]. In our study, we found that colivelin significantly reversed the inhibitory effect of MMP-14 on PD-L1 expression in MMP-14 knockdown HT-1376 cells, which suggests that MMP-14 may regulate PD-L1 through STAT3 in bladder cancer cells.

Of course, there are some limitations in our study. First, for the correlation analysis between the expression level of MMP-14 and the clinicopathological characteristics of bladder cancer patients, we only used transcriptomic data from the TCGA database, and too few clinical samples were included in some parts of the group with specific clinicopathological features, which may have led to sample bias. Second, we used CRISPR-Cas9 technology for knocking out MMP-14 in this study, but the experimental results only

reached the level of MMP-14 knockdown, and the knockdown efficiency was low, which may have had some impact on the study of relevant regulatory mechanisms. Third, this study still needs to fully clarify the regulatory mechanisms of MMP-14, STAT3 and PD-L1 in bladder cancer.

In a follow-up study, we will collect clinical specimens of bladder cancer tissues and their corresponding clinicopathological features data, detect the protein expression level of MMP-14 using immunohistochemistry, and analyze the correlation between MMP-14 expression and clinicopathological features in bladder cancer. Additionally, we will construct bladder cancer cell lines with stable overexpression of MMP-14 and use MMP-14 knockdown and MMP-14 overexpression cell lines to further clarify the effects of MMP-14 on cell proliferation, apoptosis and invasion in bladder cancer. Furthermore, we will use protein immunoprecipitation to explore the protein interaction of MMP-14, STAT3 and PD-L1 in bladder cancer.

5. Conclusions

Overall, we confirmed that MMP-14 was upregulated in bladder cancer and correlated with clinicopathological factors (race, T stage, pathological stage, cell subtype, radiation therapy, initial treatment outcome, and overall survival events) in bladder cancer patients. Furthermore, we observed that NSC40520 and HO-3867 inhibited cell proliferation, promoted cell apoptosis, and induced cytotoxicity in bladder cancer cells. Mechanistically, STAT3 can regulate the protein expression of MMP-14, and MMP-14 can also positively regulate the protein expression of STAT3 and regulate the expression level of PD-L1 possibly with STAT3. Given the critical role of MMP-14 in tumor immunity, it is expected to become a novel target in bladder cancer immunotherapy.

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Statutory Declaration

“I, Shuai Zhu, by personally signing this document in lieu of an oath, hereby affirm that I prepared the submitted dissertation on the topic the function of MMP-14 in bladder cancer (Die Funktion von MMP-14 bei Blasenkrebs), independently and without the support of third parties, and that I used no other sources and aids than those stated.

All parts which are based on the publications or presentations of other authors, either in letter or in spirit, are specified as such in accordance with the citing guidelines. The sections on methodology (in particular regarding practical work, laboratory regulations, statistical processing) and results (in particular regarding figures, charts and tables) are exclusively my responsibility.

Furthermore, I declare that I have correctly marked all of the data, the analyses, and the conclusions generated from data obtained in collaboration with other persons, and that I have correctly marked my own contribution and the contributions of other persons (cf. declaration of contribution). I have correctly marked all texts or parts of texts that were generated in collaboration with other persons.

My contributions to any publications to this dissertation correspond to those stated in the below joint declaration made together with the supervisor. All publications created within the scope of the dissertation comply with the guidelines of the ICMJE (International Committee of Medical Journal Editors; www.icmje.org) on authorship. In addition, I declare that I shall comply with the regulations of Charité – Universitätsmedizin Berlin on ensuring good scientific practice.

I declare that I have not yet submitted this dissertation in identical or similar form to another Faculty.

The significance of this statutory declaration and the consequences of a false statutory declaration under criminal law (Sections 156, 161 of the German Criminal Code) are known to me.”

Date	Signature

Curriculum Vitae

My curriculum vitae does not appear in the electronic version of my paper for reasons of data protection.

List of publications

1. Xie Y, **Zhu S,** Zang J, Wu G, Wen Y, Liang Y, Long Y, Guo W, Zang C, Hu X, Fan G, Xiang S, Zhang J. ADNP prompts the cisplatin-resistance of bladder cancer via TGF-β-mediated epithelial-mesenchymal transition (EMT) pathway. J Cancer. 2021 Jun 22;12(17):5114-5124.

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3. **Zhu S,** Xu Z, Zeng Y, Long Y, Fan G, Ding Q, Wen Y, Cao J, Dai T, Han W, Xie Y. ADNP Upregulation Promotes Bladder Cancer Cell Proliferation *via* the AKT Pathway. Front Oncol. 2020 Nov 9;10:491129.

4. Jian C, **Zhu S**, Mingji Y, Kan L, Zhizhong L, Weiqing H, Yu X. Evaluation of Three-Dimensional Printing-Assisted Laparoscopic Cryoablation of Small Renal Tumors: A Preliminary Report. Urol J. 2020 Apr 19;18(2):171-175.

5. Cao J, **Zhu S,** Ye M, Liu K, Liu Z, Han W, Xie Y. Comparison of Renal Artery *vs* Renal Artery-Vein Clamping During Partial Nephrectomy: A System Review and Meta-Analysis. J Endourol. 2020 Apr;34(4):523-530.

6. Fan G, Meng Y, **Zhu S,** Ye M, Li M, Li F, Ye Y, Liu Z, Weiqin H, Xie Y. Three-dimensional printing for laparoscopic partial nephrectomy in patients with renal tumors. J Int Med Res. 2019 Sep;47(9):4324-4332.

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Certificate of an accredited statistician

CHARITÉ

CharitéCentrum für Human- und Gesundheitswissenschaften

Charité | Campus Charité Mitte | 10117 Berlin

Institut für Biometrie und klinische Epidemiologie (iBikE)

Direktor: Prof. Dr. Frank Konietschke

Postanschrift:
Charitéplatz 1 | 10117 Berlin
Besucheranschrift:
Reinhardtstr. 58 | 10117 Berlin

Tel. +49 (0)30 450 562171
frank.konietschke@charite.de
https://biometrie.charite.de/

Name, Vorname: Zhu, Shuai
Email addresse: shuai.zhu@charite.de
Matrikelnummer: 229902
PromotionsbetreuerIn: PD Dr. Anja Rabien
Promotionsinstitution / Klinik: Klinik für Urologie

Bescheinigung

Hiermit bescheinige ich, dass Herr *Shuai Zhu* innerhalb der Service Unit Biometrie des Instituts für Biometrie und klinische Epidemiologie (iBikE) bei mir eine statistische Beratung zu einem Promotionsvorhaben wahrgenommen hat. Folgende Beratungstermine wurden wahrgenommen:

- Termin 1: 03.01.2023

Folgende wesentliche Ratschläge hinsichtlich einer sinnvollen Auswertung und Interpretation der Daten wurden während der Beratung erteilt:

- One-way und Two-way ANOVA
- Survival analysis
- Der t-Test für unabhängige Stichproben

Diese Bescheinigung garantiert nicht die richtige Umsetzung der in der Beratung gemachten Vorschläge, die korrekte Durchführung der empfohlenen statistischen Verfahren und die richtige Darstellung und Interpretation der Ergebnisse. Die Verantwortung hierfür obliegt allein dem Promovierenden. Das Institut für Biometrie und klinische Epidemiologie übernimmt hierfür keine Haftung.

Datum: 13.01.2023 Name der Beraterin: Pimrapat Gebert

[redacted]

Unterschrift BeraterIn, Institutsstempel

CHARITÉ
UNIVERSITÄTSMEDIZIN BERLIN
Institut für Biometrie und
Klinische Epidemiologie
Campus Charité Mitte
Charitéplatz 1 | D-10117 Berlin
Sitz: Reinhardtstr. 58