PD-L1 promotes malignant progression by inducing EMT and enhancing stemness in muscle-invasive bladder cancer

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Research

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Abstract

Objective: Despite radical treatment for aggressive muscle-invasive bladder cancer(MIBC), the prognosis remained poor. Programmed cell death ligand 1(PD-L1) plays an important role in suppressing immune responses.We investigate if PD-L1 and EMT synergistically contribute to MIBC progression.

Methods: In vitro experiments, we evaluated the effects of PD-L1 on proliferation, invasion, migration of MIBC cells and studied the relationship between PD-L1 and CSC/EMT markers by overexpressing and knocking down PD-L. The association of PD-L1 with EMT was detected in MIBC human specimens and the synergistic effect of PD-L1and EMT was assessed by analysis of overall survival (OS).

Results: Our data demonstrated that PD-L1 promotes proliferation, invasion and migration of MIBC cells. The positive correlation between PD-L1 and CSC/EMT markers was verified both in vitro experiments and in 130 MIBC specimens. Moreover, patients with positive PD-L1/positive EMT exhibited poorer overall survival than patients with other combinations.

Conclusion: The close relationship between PD-L1 and EMT may offer potential therapeutic strategy that co-targeting PD-L1 and EMT may improve the prognosis of MIBC patients.

Introduction

Bladder cancer, as the fifth most common cancer, is diagnosed in over 430 thousand patients worldwide per year. Muscle-invasive bladder cancer(MIBC) is more malignant and therefore calls for more powerful treatment compared with non-muscle-invasive disease, however, the prognosis of MIBC remained to be improved. MIBC is mainly composed of luminal and basal subtypes. The basal subtype, with increased markers of cancer stem cells(CSCs) and epithelial-to-mesenchymal transition(EMT), is prognostically unfavorable in comparision with the luminal subtype which highly express E-cadherin, analogous to normal urothelium cells[1-3].

Induced Pluripotent Stem (iPS) cells can be obtained from differentiated cells of distinct types, by transduction of four vital genes: Oct4, Sox2, c-Myc and Klf4, which are now famous as the Yamanaka factors and critical markers of CSCs as well. CSCs have been sucessfully isolated from bladder cancer[4]. Oct4 is ranked as core gene for inducing stemness[5] and Sox2 acts as direct or indirect regulator of Oct4. Intense Oct4 expression is relevant to progression of bladder cancer. EMT correlated with cancer is a reprogramming with comprehensiveness and complexity, through which cancer cells acquire functions of CSCs by reversion from differentiated epithelial phenotype towards undifferentiated mesenchymal state. A set of transcriptional genes including Snail, Twist and Slug contributes to inducing EMT. Snail is responsible for upregulating both Oct4 and Sox2. In the course of tumor progression, Oct4 expression can stimulate EMT by activating Stat3/Snail signal pathway and Sox2 expression can promote EMT by Wnt/β-catenin signal pathway. Collectively, EMT and CSC activate each other during the course of tumor progression. Considering the vital role of both EMT and CSC in cancer progression, this “axis of evil” poses a tough challenge for improving survival, but simultaneously provides strategic opportunities of

breaking through the treatment bottleneck as well, highlighting the necessity of clarifing the molecular regulation mechanism of CSC/EMT.

Programmed cell death protein 1(PD-1), combined with programmed cell death ligand 1(PD-L1) contributes greatly to immune inhibition and self-tolerance. James P. Allison and Tasuku Honjo were awarded with the 2018 Nobel Prize of Physiology or Medicine for disclosing the “brake” role of PD-1 and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) in immune function, which suggested that PD-1 or CTLA-4 suppression may efficiently reactivate T cells and eliminate cancer cells. Inhibition of PD-1/PD-L1 signal pathway has gained significant clinical response in urothelial cancer patients, leading to regulatory approval of five PD-1/PD-L1antibodies in USA[6-11]. However, the low response rate seriously limits its scope of application. Considering the vital role of EMT/CSC in therapeutic resistance and immune evasion[12,13], it is tempting to deduce that PD-L1 may promote progression and resistance to antitumor by upregulating EMT/CSC. If so, PD-L1 would be a vital regulatory gene of basal subtype MIBC. To the best of our knowledge, this is the first study to investigate the role of PD-L1 in tumor progression of MIBC from viewpoint of EMT/CSC.

In this study,we first detected PD-L1expression in MIBC human specimens and studied the relationship between PD-L1 with clinicopathological parameters. Then, we performed in vitro experiments to evaluate the effects of PD-L1 on tumor development and the association of PD-L1 with CSC/EMT markers. Furthermore, the synergistic effect of PD-L1and EMT was assessed by analysis of overall survival (OS). Our data indicated that PD-L1 positive regulation of CSC/EMT accounts for the promotive effect of PD-L1 on tumor progression, thus providing potential treatment strategies for MIBC patients.

Materials And Methods

Cell lines

We purchased urinary bladder cancer cell lines and human immortalized bladder epithelial cell line from Shanghai Institute of Biochemistry and Cell Biology. All cell lines were maintained in RPMI 1640 medium.

PD-L1-overexpressing cells and PD-L1-knockdown cells

We first cloned the human full-length PD-L1 cDNA into the PRK5-Flag expression vector. Then, we used DNA sequencing to confirm the PRK5-Flag-PD-L1 expression vector was successfully set up. Based on the manufacturer's specifications, we transfected PRK5-Flag-PD-L1 expression vector into UMUC-3 cells when confluence rate reached 50%. We took advantage of software on the Ambion website to design three siRNAs targeting PD-L1 mRNA as well as a scrambled siRNA served as negative control. We listed siRNA sequences in Supporting Table 1.

Real-time quantitative reverse transcription PCR

RT-PCR kit was obtained from Takara Corporation of Japan. The housekeeping gene β-actin was used as internal control. We carried out PCR reaction by an ABI PRISM 7300 PCR and detection system produced

by Applied Biosystems Corporation of Carlsbad,USA. We listed the primers of all studied genes in Supporting Table 2.

Westernblot

We first applied the Bradford assay to quantify the protein content of the cellular extracts. Then, after subjected to SDSPAGE and transferred onto polyvinylidene difluoride membranes, the extracted protein was immunoblotted by antibodies for PD-L1, E-cadherin, N-cadherin, Oct4, and C-myc. All antibodies were purchased from Abcam Corporation of USA.

Cell proliferation

We analyzed cell proliferation by a WST-8 cell counting kit-8 and measured absorbance at 450 nm by an ELX-800 spectrometer reader.

Transwell invasion and migration assay

We performed invasion and migration assay using a modified two-chamber plates with apore size being 8 mm. After traversing the membrane, the cells were fixed with methanol and stained by Trypan Blue. We counted the blue fixed cell under a microscope.

Patients and clinical specimens

We enrolled 130 patients(mean age, 59 years) with pathologically confirmed primary MIBC following radical cystectomy and collected follow-up data from January 2008 to December 2016. Patients who received either neoadjuvant or adjuvant platinum-based chemotherapy were excluded. We obtained five-year follow-up data from all patients and the follow-up deadline was March 2019. The Institutional Review Board of Huai'an Affiliated Hospital of Xuzhou Medical University authorized the use of specimens(reference no. HEYLL201609), which was also given informed consent by patients.

Immunohistochemistry

We detected protein expression in human MIBC specimens with antibodies for PD-L1, Oct4 and E-cadherin which were purchased from Abcam Corporation of USA. The expression of PD- L1 on tumor cells was evaluated based on report in recent trials[14,15].

Statistical analysis

We applied Pearson's $\chi 2$ test to study the relationship between different variables. Student t test was applied when appropriate. Univariate analysis of OS was evaluated by the Kaplane-Meier method, and the difference of survival rates was assessed by log-rank test. Multivariate analysis of OS was evaluated by Cox's proportional hazard regression model. A P value < 0.05 was regarded as being statistically significant. SPSS version 16.0 was used for data analysis.

Results

PD-L1upregulation is detected in MIBC

Westernblot was applied to investigate PD-L1 expression in the bladder cancer cell lines T24, SW780, UMUC-3 and the human immortalized bladder epithelial cell line SV-HUC-1. PD-L1 expression in invasive cell lines UMUC-3 and T24 was higher than SV-HUC-1 cells(Fig. 1A). UMUC-3 was chosen for follow-up experiment. The results from immunohistochemistry (IHC) analysis of 130 MIBC specimens demonstrated that PD-L1 expression was significantly increased in cancerous tissues in comparison with adjacent normal tissues(Fig. 1B). We detected positive expression of PD-L1 in 29.2%(38/130) of MIBC cases while in 6.3%(8/126) of adjacent normal tissues. Collectively, our data demonstrated that PD-L1 expression was increased in MIBC.

Association of PD-L1 expression with clinicopathologic parameters

Of 130 MIBC patients, positive and negative PD-L1 expression were 29.2% (38/130) and 70.8% (92/130), respectively(Table 1). The result from analysis of the relationship between PD-L1 expression and clinicopathological variables indicated increased PD-L1 expression was dramatically correlated with EMT(*P*=0.011, Fig. 2A) and basal subtype(*P*=0.001, Fig. 2B).We defined EMT when mesenchymal morphological changes and low E-cadherin expression were found at the invasive front of tumor. The upregulated PD-L1 expression is also dramatically related to regional lymph node metastases(*P*=0.01, Fig. 2C) and Oct4(*P*=0.038, Fig.2D). However, our data didn't indicate intimate association of PD-L1 expression with other clinicopathologic parameters, including sex, age, differentiation, invasion depth as well as tumor size(Table 1).

Table 1

The relationship between PD-L1 expression and clinicopathological factors in muscle-invasive bladder cancer

Clinicopathological factors	PD-L1 expression		
	Positive (%)	Negative (%)	*P*
Age			0.384
≥65	23(60.5)	48(52.2)	
<65	15(39.5)	44(47.8)	
Sex			0.639
Male	28(73.7)	64(69.6)	
Female	10(26.3)	28(30.4)	
Size			0.402
≥3cm	18(47.4)	51(55.4)	
<3cm	20(52.6)	41(42.6)	
Invasion depth			0.711
T2	16(42.1)	42(45.7)	
T3	22(57.9)	50(54.3)	
Local nodal metastasis			0.010
N0	12(31.6)	52(56.5)	
N1-3	26(68.4)	40(43.5)	
Differentiation			0.341
G2	20(52.6)	40(43.5)	
G3	18(47.4)	52(56.5)	
Subtype			0.001
Basal	25(65.8)	31(33.7)	
Luminal	13(34.2)	61(66.3)	
EMT			0.011
Positive	27(71.1)	43(46.7)	
Negative	11(28.9)	49(53.3)	
Oct4			0.038
Positive	22(57.9)	35(38)	
Negative	16(42.1)	57(62)	

PD-L1 promotes tumor progression by conferring stemness and inducing EMT

Based on real-time RT-PCR and westernblot results, PD-L1 expression in UMUC-3 cell line was dramatically upregulated in plasmid transfection (overexpression, OE) group in comparison with OE control (OEC) group, at both mRNA and protein levels(Fig.3A). PD-L1 expression was significantly inhibited by the siRNA3(knockdown, KD) and siRNA1(KD1) compared to KD control(KDC)(Fig.3B). Cell proliferation assay indicated that PD-L1 knockdown significantly suppressed cell proliferation, while PD-L1 overexpression drastically increased the ability of cell proliferation (Fig. 3C). We then studied the influence of PD-L1expression on ability of migration and invasion. Cell invasion assay indicated that invasive ability was downregulated by 41% and upregulated by 56% by PD-L1 knockdown and overexpression, respectively(Fig. 3D). Cell migration assay showed that migration ability was decreased by 35% and increased by 56% by PD-L1 knockdown and overexpression, respectively(Fig. 3E). To investigate whether PD-L1 promotes the MIBC progression by enhancing EMT/CSC, EMT-related genes such as E-cadherin, N-cadherin, Snail, Fibronectin and Vimentin as well as renowned Yamanaka factors including Oct4, Klf4, Sox2 and C-Myc, which confer stemming and give rise to iPS cells, were detected by real-time RT-PCR. Our data showed that the expression of Yamanaka factors(Oct4 and C-myc) and mesenchymal markers(N-cadherin, Snail and Vimentin) was significantly decreased($P < 0.001$), in contrast to remarkably increased expression of epithelial marker E-cadherin($P < 0.001$). The upregulation of E-cadherin as well as downregulation of N-cadherin, Oct4 and C-myc at protein level was verified by westernblot (Fig. 4A). On the contrary, PD-L1 overexpression reversed the changes of stemness genes(Oct4 and C-myc) and EMT-related genes(E-cadherin, N-cadherin, Snail and Vimentin) upon PD-L1 knowdown. The opposite changes of Oct4, C-myc, E-cadherin and N-cadherin at protein level were also verified by westernblot (Fig. 4B).

PD-L1 expression indicates poor prognosis in MIBC

Kaplane-Meier analysis demonstrated that positive PD-L1 expression was associated with poorer OS ($P< 0.001$, Fig. 5A), so is EMT($P=0.002$, Fig. 5B). In order to explore whether combined expression of PD-L1 and EMT presented with poorer prognosis, 130 MIBC patients were divided into four groups: one subgroup with both positive PD-L1 and EMT, either negative PD-L1 or negative EMT and both negative PD-L1 and EMT. Kaplane-Meier analysis indicated that OS of MIBC patients with positive PD-L1/positive EMT was poorer than that of other combinations ($P< 0.001$, Fig. 5C). Multivariate analysis demonstrated that PD-L1 expression was an independent prognostic factor ($P=0.031$), as was EMT($P= 0.026$), local nodal metastasis($P= 0.015$), subtype ($P= 0.002$).

Discussion

MIBC accounting for about 30-40% of total bladder tumors has a worse prognosis than non-muscle invasive disease. PD-1/PD-L1 pathway degenerates anti-tumor immune responses by suppressing T cell proliferation, activation and cytotoxic secretion, resulting in induction and maintenance of immune

tolerance within tumor microenvironment[16]. In other words, tumor cells take advantage of PD-L1 expression to escape anti-tumor responses, referred to as adaptive immune regulation[17]. NK cells can promote PD-L1 expression on tumor cells by secreting IFN-γ through the Janus kinase (JAK)1, JAK2 and transcription(STAT)1 pathways[18]. IFN-γ secreted by T cells can also regulate PD-L1 expression through JAK1/JAK2-STAT1/STAT2/STAT3-IRF1 signal pathway. Thus, both T and NK cells induce the expression of PD-L1 via secreting IFN-γ[19]. The increased PD-L1 expression on tumor cells contribute to solid tumors developing tolerance to immune regulation[20]. The resistance to bacille Calmette-Guérin (BCG) therapy in high-risk non-MIBC is, at least in part, attributable to PD-L1 expression[21]. Otherwise, PD-L1 serves as a pro-tumorigenic factor via binding to its receptors and activating proliferative and survival signaling pathways[22], indicating the crucial role of PD-L1 in subsequent tumor development. However, the mechanism accounting for PD-L1 being responsible for bladder cancer progression remains to be elucidated.

Strong evidence has demonstrated the close association of EMT with CSC phenotype[23]. The EMT inducer is an important driver of the stemness properties[24,25] On the other hand, tumors cells with CSC properties exhibited increased metastatic ability by promoting EMT[26]. Tumor cells undergoing EMT present various abilities in accordance with CSC[27].The famous Yamanaka factors Oct4, C-myc, Sox2 and Klf4 are vital markers of CSCs. Considering Oct4 can maintain cells in an undifferentiated state by binding to Sox2, intense Oct4 expression contributes a great deal to poor differentiation states of tumor cells. Oct4 can promote cervical cancer progression by increasing the expression of PD-L1 via a miR-18a-dependent signal pathway[28]. In addition, Sox2 can bind to the PD-L1 promoter to transactive PD-L1 expression, promoting proliferation of hepatocellular carcinoma cells[29]. Oct4 acts as a molecular marker of unfavorable prognosis in bladder cancer. Positive Oct4 expression was also closely related with intra-bladder tumor recurrence after operation. Nuclear C-myc plays a prominent role in EMT, contributing to tumor development. Nuclear expression of C-myc in tumor tissue was significantly correlated with poor prognostic factors including lymphovascular invasion, lymph node involvement, distant metastasis, sarcomatoid variant and advanced stage in MIBC. Several studies demonstrated that C-myc contributes to the origination and development of bladder cancer and there exists a close relationship between C-myc overexpression and high tumor grade as well as advanced tumor stage. High nuclear expression of C-myc in sarcomatoid bladder cancer and infiltrative pattern of conventional bladder cancer is attributed to the close relationship between C-myc and EMT. C-myc induces EMT by activating Snail transcription factor, which strongly suppressed E-cadherin. C-myc expression promotes human mammary epithelial cells to gain EMT characteristic. In cells expressing C-myc, E-cadherin expression is inhibited. cBioportal analysis indicated that PD-L1 is co-amplified along with Snail, N-cadherin, C-myc and Sox2 in both endometrial and ovarian cancer. All of these studies disclosed that PD-L1, EMT and three Yamanaka factors Oct4, Sox2, C-myc promote tumor progression in a cooperative manner. Our data showed that PD-L1 positively regulated Oct4,C-myc, N-cadherin expression and negatively regulated E-cadherin expression, indicating that PD-L1 promote the aggressiveness and poor prognosis of MIBC by enhancing stemness and EMT. Our data didn’t show intimate association of PD-L1 with Klf4 and Sox2. Altogether, PD-L1 contributes to MIBC progression by inducing EMT and upregulating stemness genes Oct4 and C-myc.

Our data also indicated that PD-L1 expression was dramatically correlated with CSC/EMT, regional lymph node involvement and basal subtype in clinical specimen of MIBC, which suggests that PD-L1expression increases as malignant transformation increases. Univariate analysis demonstrated that PD-L1expression predicted poor prognosis in MIBC. Given that a positive relationship was verified between PD-L1 expression and EMT both in vitro and in vivo, we investigated whether the combined expression of PD-L1 and EMT could predict the prognosis better. As expected, the prognosis of MIBC patients with both positive PD-L1 and EMT was the poorest. Thus, we drew a conclusion that coexpression of PD-L1/EMT played a more important role in predicting prognosis than PD-L1 alone. Altogether, PD-L1 and EMT are supposed to work in harmony and act cooperatively to promote MIBC development.

As immune inhibitor, PD-L1 expression stands for anti-tumor immune response as well[30-32]. Checkpoint inhibitors targeting the PD- 1/PD- L1 axis have gained unprecedented clinical benefit in multiple types of cancer. However, sustained responses take place in only a small minority of advanced cancer patients, making it necessary to identify biomarkers that predict outcomes of immune therapy. Enormous amount of research has demonstrated that checkpoint inhibitors produce a maximum effect in tumor microenvironment where there is coexistence between endogenous immune response and upregulation of immune checkpoints[33-35]. Thus, it is important to disclose biomarkers to identify patients who possess this coexistence in tumor microenvironment. Endogenous immune activation is characteristic of elevation of immune co-stimulatory molecules,such as IFN-γ and CXCL10. EMT is an essential mediator of inflammatory tumor microenvironment. The expression of EMT-related gene is negatively correlated with response rate and OS in metastatic urothelial cancer patients treated with nivolumab, a PD-1 inhibitor, suggesting immune resistance deriving from EMT and therefore providing potential treatment strategy of targeting both PD-1 and EMT[36]. Thus, the elevated immune checkpoints complicated with mesenchymal status indicate impaired rather than favourable immune response to immunotherapy, at least in part attributable to EMT-mediated inflammatory tumor microenvironment. For MIBC patients, especially for basal subtype, improvement of tumor microenvironment such as downregulating EMT-related genes is vital to improve outcome of immunotherapy.

In conclusion, Our data demonstrated the remarkable inhibitory effects of PD-L1 on MIBC and elucidated that the underlying mechanism is ascribed to PD-L1 upregulating both EMT and stemness genes Oct4 and c-Myc in vitro experiments. The intimate relationship between PD-L1 and EMT was further verified by an analysis of MIBC tissue specimens and OS. To the best of our knowledge, this is the first study to indicate significant association of PD-L1 with EMT and stemness genes in MIBC, which accounts for tumor progression and treatment resistance. We believe that co-targeting PD-L1 and EMT could provide a potentially efficient therapy for MIBC patients.

Declarations

Acknowledgements

Not applicable.

Authors' contributions

YZ designed the research. In vitro experiments were performed by FJ,YD and TC. XZ and SM collected survival data. FJ performed the statistical analysis and wrote the manuscript. YD and TC contributed to substantive revision of the manuscript. YZ was responsible for editing the manuscript. All authors provided valuable suggestions for further improvement of the final manuscript.

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Availability of data and materials

Data and materials are available on demand.

Ethics approval

This study was approved by The Institutional Review Board of Huai'an Affiliated Hospital of Xuzhou Medical University(reference no. HEYLL201609). The use of specimens was given informed consent by patients.

Consent for publication

All the patients involved in the study have consented to publication of the Data.

Competing interests

All authors have no conflicts of interest to declare.

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Figures

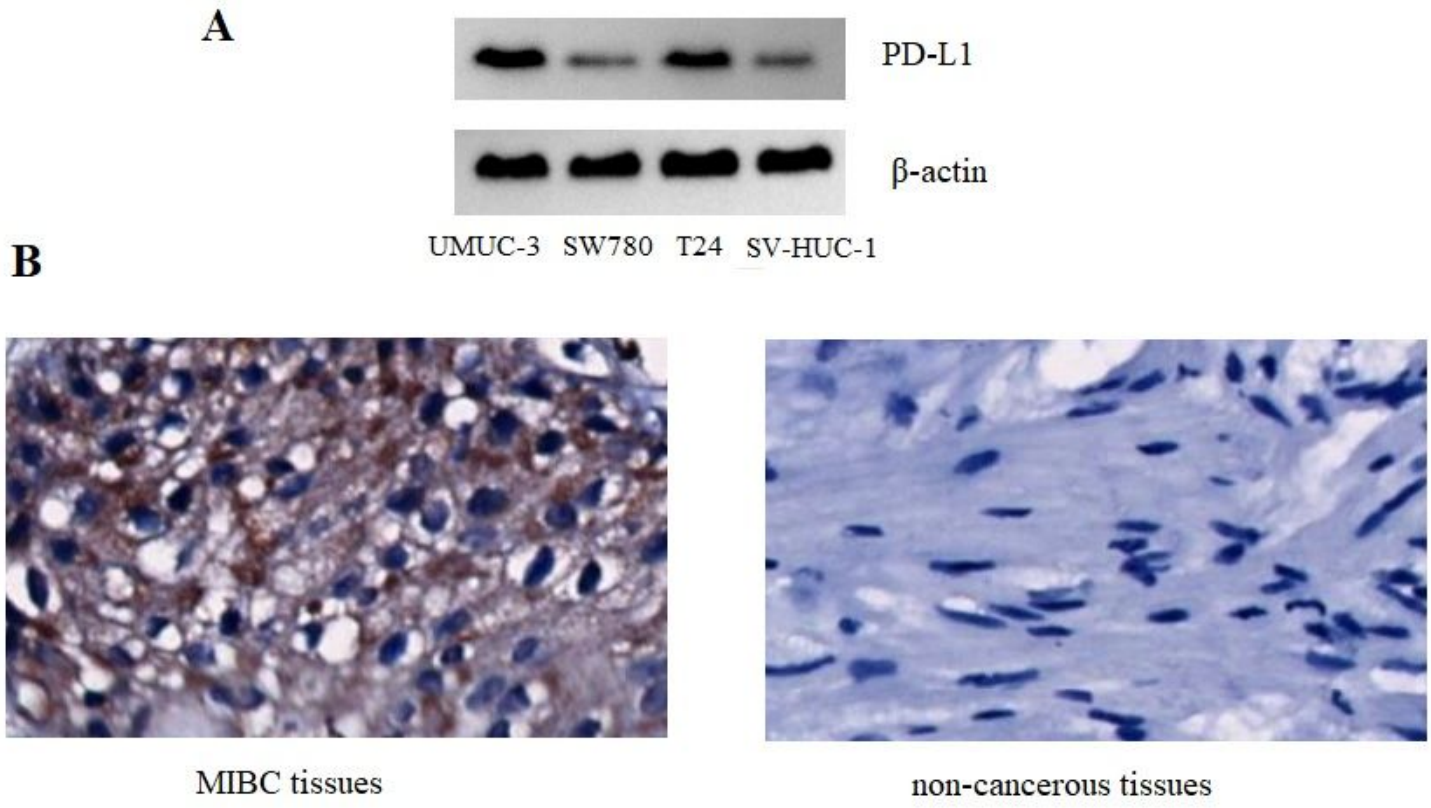

Fig. 1 Upregulation of PD-L1 in MIBC was confirmed by western blot and IHC. (A) Expression of PD-L1 in SV-HUC-1 and three bladder cancer cell lines was determined by westernblot. The PD-L1 expression in the MIBC cell lines T24 and UMUC-3 was upregulated compared with SV-HUC-1. β-actin was used as an internal control. (B) IHC confirmed that PD-L1 protein was upregulated in the MIBC tissue(left) compared to paired adjacent non-cancerous tissue(right).

Figure 1

(caption in figure image)

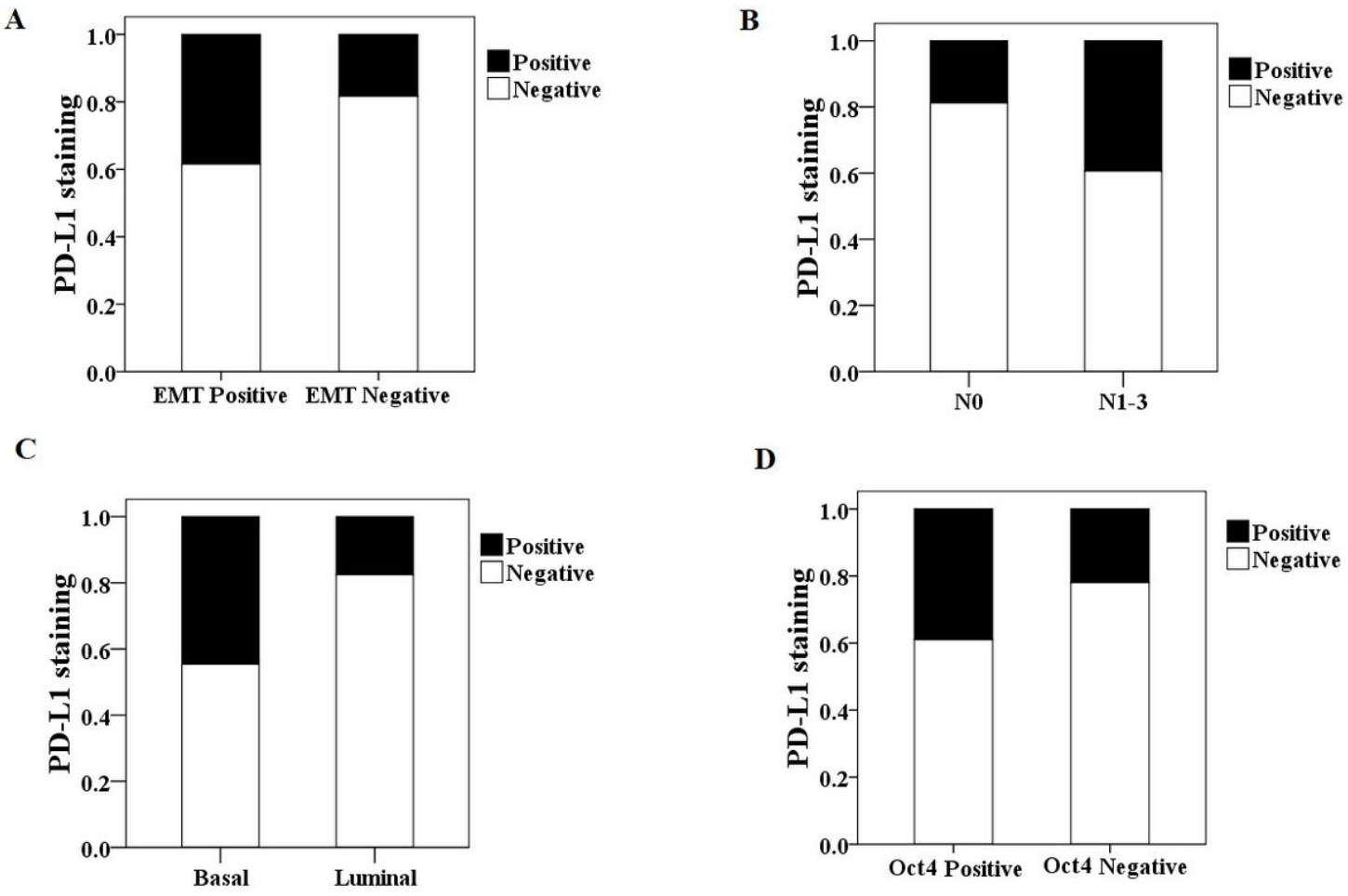

Fig. 2 Correlation between PD-L1 expression and clinicopathologic parameters in MIBC. (A) Increased PD-L1 expression is positively correlated with EMT (P =0.011, $\chi 2$ test). (B) Increased PD-L1 expression is positively correlated with basal subtype (P =0.001, $\chi 2$ test). (C) Increased PD-L1 expression is correlated with regional lymph node metastasis(P= 0.01, $\chi 2$ test). (D) Increased PD-L1 expression is correlated with positive Oct4 status(P =0.038, $\chi 2$ test).

Figure 2

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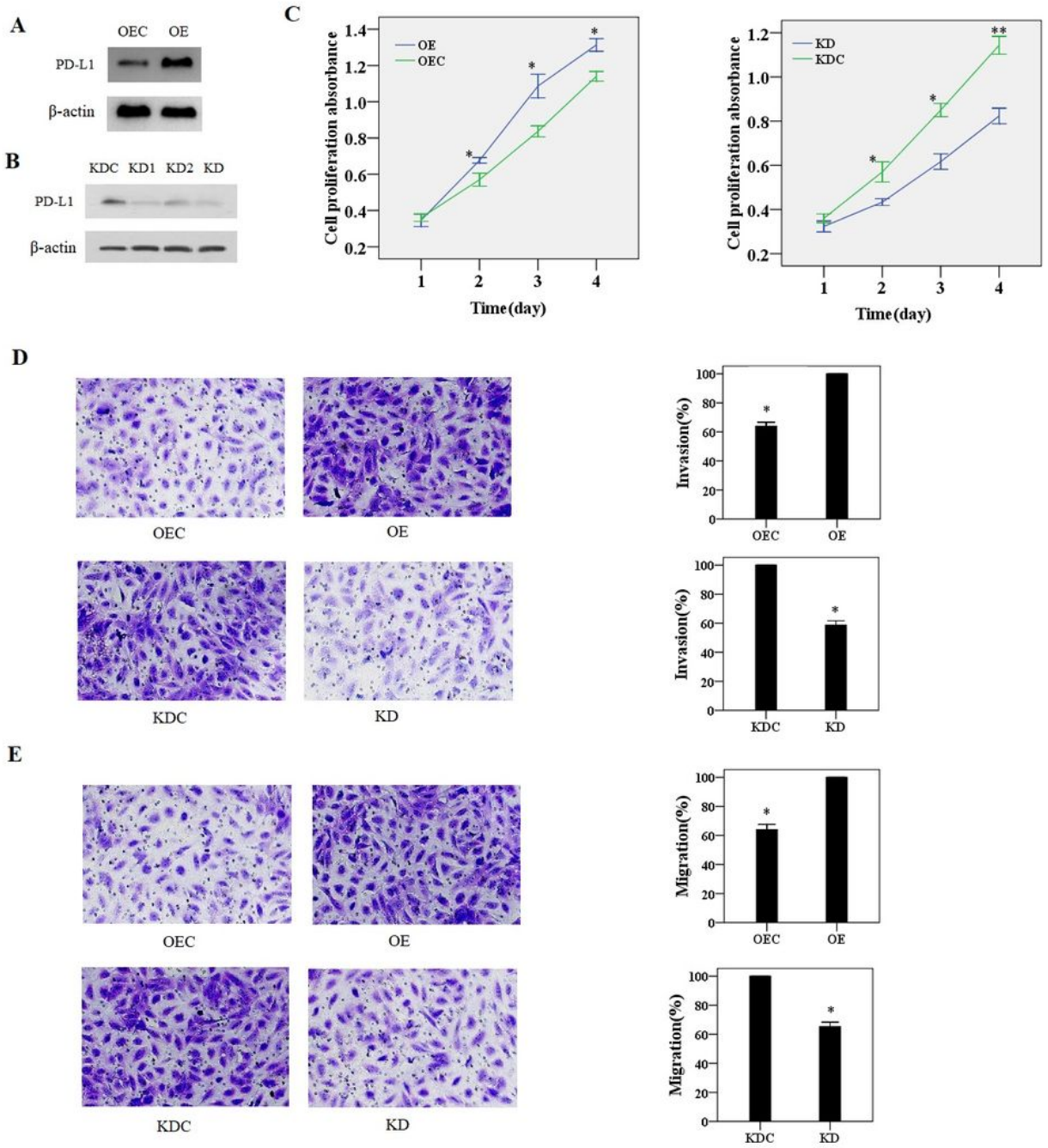

Fig. 3 PD-L1 promotes proliferation and metastasis of UMUC-3 cells by upregulating EMT and conferring stemness. (A) PD-L1expression was significantly upregulated in OE group compared with OEC group. (B) KD exhibited the best inhibition of PD-L1 at protein level. (C) CCK-8 cell proliferation assay after PD-L1 overexpression and knockdown. (D) A matrigel cell invasion assay after PD-L1 overexpression and knockdown. (E) Cell migration assay after PD-L1 overexpression and knockdown.

Figure 3

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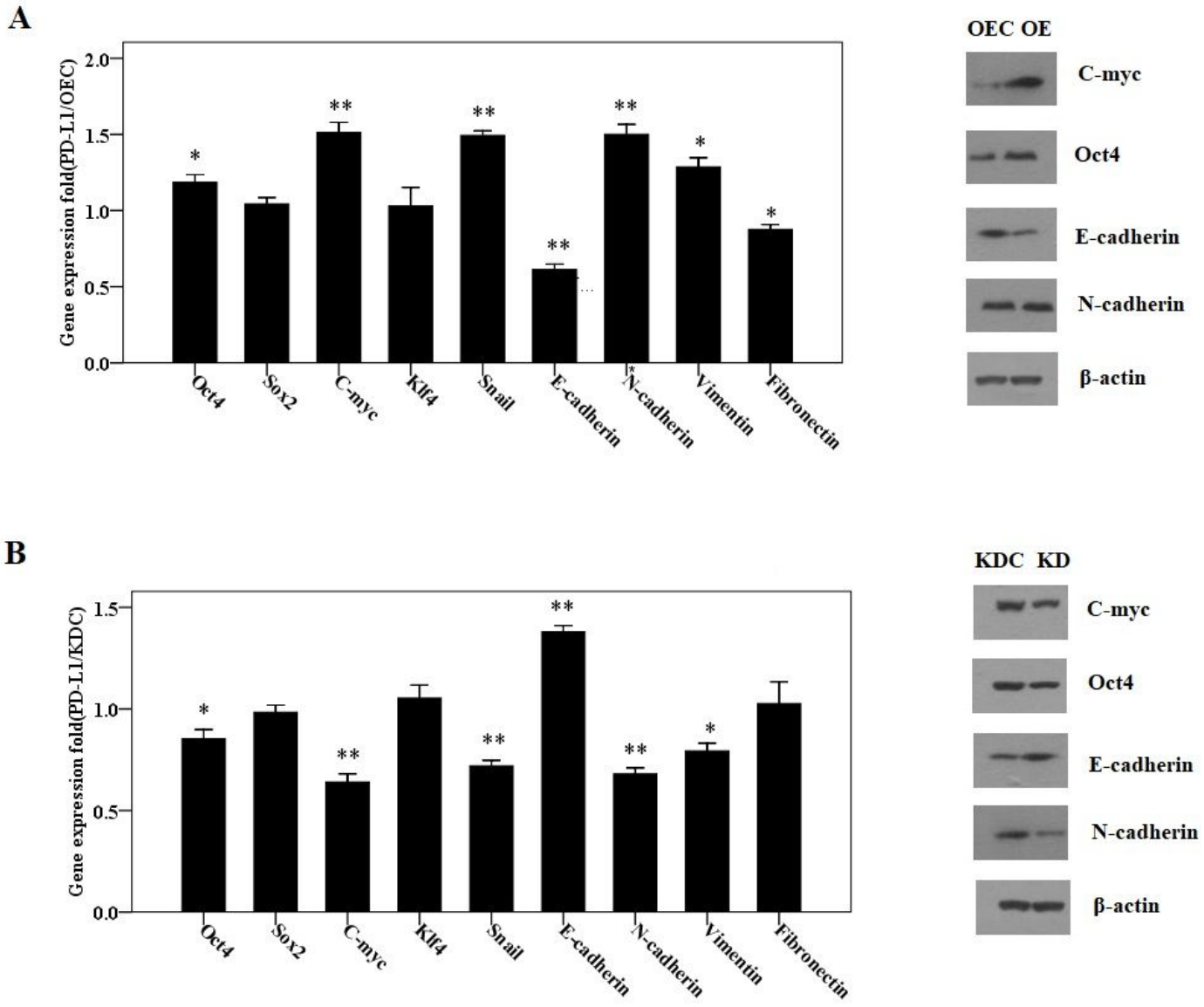

Fig. 4 PD-L1 promotes EMT and upregulates expression of stemness genes in UMUC-3 cells. (A) Significant upregulation of c-Myc, Oct4, Snail, N-cadherin, Fibronectin and Vimentin at the mRNA level was detected after PD-L1 overexpression, while the mRNA expression of E-cadherin was downregulated(left); Expression of c-Myc, Oct4 and N-cadherin at the protein level was remarkably upregulated and the protein level of E-cadherin was downregulated, as shown by westernblot in OE group compared with OEC group(right). (B) Significant downregulation of c-Myc, Oct4, Snail, N-cadherin, and Vimentin at mRNA level was detected after PD-L1 knockdown, while the mRNA expression of E-cadherin was upregulated(left); Expression of c-Myc, Oct4, N-cadherin at the protein level was remarkably downregulated and the protein level of E-cadherin was upregulated, as shown by westernblot in KD group compared with KDC group(right). The mRNA expression levels were normalized against β-actin. Each value represents the mean ±SD for triplicate samples. *$P < 0.01$, **$P < 0.001$(Student t test).

Figure 4

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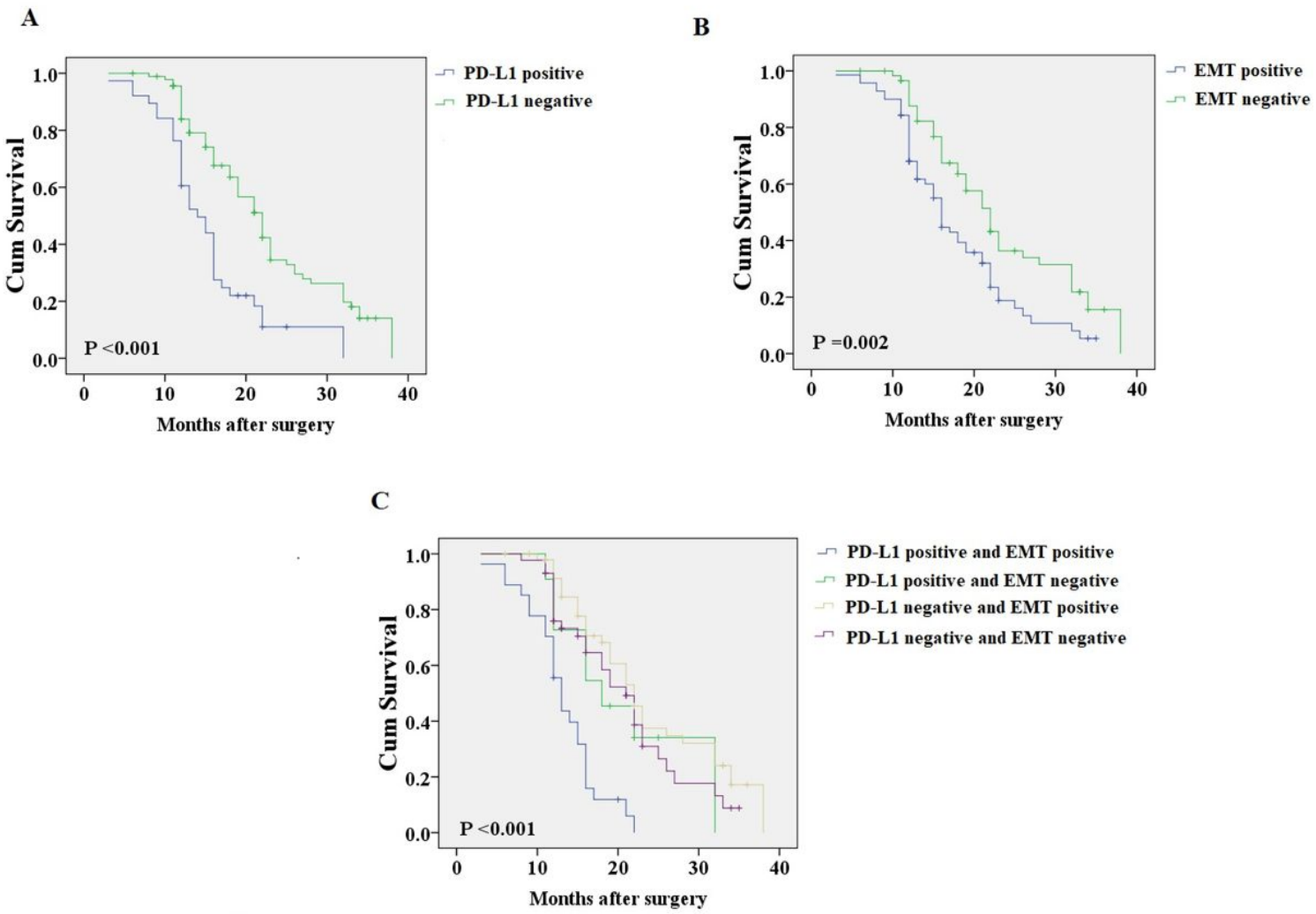

Fig. 5 Prognosis analysis. (A) Prognostic impact of PD-L1 expression in MIBC. (B) Prognostic impact of EMT in MIBC. (C) The overall survival curves stratified according to the combination of PD-L1expression and EMT. Overall survival rates were estimated from the time of surgery using Kaplan-Meier analyses. *P* values (2-sided) were calculated using the log-rank test.

Figure 5

(caption in figure image)

Supplementary Files

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- Supportingtable.doc