Increased expression of *CD81* is associated with poor prognosis of prostate cancer and increases the progression of prostate cancer cells *in vitro*

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Abstract. *CD81*, a member of the tetraspanin family, has been revealed to be upregulated and associated with prognosis in several types of cancer; however, this relationship has not been explored in prostate cancer. The present study aimed to investigate the prognostic significance and functional role of *CD81* in prostate cancer. The expression of *CD81* in prostate cancer tissues and cell lines was evaluated using qRT-PCR analysis. Kaplan-Meier survival analysis and Cox regression analysis were conducted to explore the prognostic significance of *CD81*. Cell experiments were used to explore the effects of *CD81* on cell proliferation, migration, and invasion in prostate cell lines *in vitro*. The expression of *CD81* was increased in both prostate cancer tissues and cell lines. Upregulation of *CD81* was significantly associated with lymph node metastasis and TNM stage. Moreover, patients with high *CD81* levels had poorer overall survival than those with lower levels. Additionally, tumor cell proliferation, migration, and invasion were inhibited by knockdown of *CD81*. The present results indicated that *CD81* plays an oncogenic role in prostate cancer. Overexpression of *CD81* may serve as a prognostic biomarker and therapeutic target and is involved in the progression of prostate cancer.

Introduction

Prostate cancer is ranked as the most common genitourinary malignancy among males worldwide, with a high morbidity rate in most developed countries (1). Although the incidence of prostate cancer is lower in China than that in the USA, the survival outcomes are generally lower than those in the USA (1-3). Currently, widespread screening for prostate-specific antigen (PSA), as a tumor marker, has greatly helped us to diagnose prostate cancer patients at an early stage (4). However, this method still has some shortcomings in the metastatic setting (5). Due to the strong metastatic ability of prostate cancer, the quality of life and prognosis of patients at advanced stages are still unfavorable (6). Thus, there is an ongoing need for more effective gene therapy programs for prostate cancer.

The tetraspanin family, a large evolutionarily conserved family of proteins, is widely expressed in most cells in multicellular organisms; these proteins contain four transmembrane domains, short N- and C-terminal cytoplasmic domains, two extracellular loops and a small intracellular loop (7). Tetraspanins form tetraspanin-enriched microdomains (TEMs) in the cell membrane with their partner proteins, such as integrins and immunoglobulins, to regulate diverse cellular functions and play a role in tumor progression (8,9). *CD81* is a member of the tetraspanin family that was originally identified as a target of the antiproliferative antibody *TAPA-1* (10). In addition to its important role in the immune system, *CD81* has been revealed to be involved in the progression of most types of cancer (11,12). *CD81* expression was revealed to be increased in breast cancer and promoted cell migration and proliferation in breast cancer cell lines (13). In prostate cancer, a gene expression profiling study evaluated several differentially expressed prostate cancer-associated genes, including *CD81*, in two prostate cancer cell lines (14). However, the expression of *CD81* in prostate cancer tissues and other prostate cancer cell lines, as well as its potential role, are still unclear.

The aim of the present study was to identify whether *CD81* is upregulated in prostate cancer tissues and linked to poor prognosis. In addition, the effects of *CD81* on prostate cancer cell proliferation, migration, and invasion were explored.

Materials and methods

Patients and tissue specimen collection. The study was approved by the Research Ethics Committee of Tongren Hospital, Shanghai Jiao Tong University School of Medicine (Shanghai, China). All the patients signed written informed consent. All specimens were handled and anonymized according to ethical and legal standards.

Paired prostate cancer tissue specimens and adjacent normal tissue specimens were obtained from 114 prostate cancer patients who received the same radical prostatectomy treatment at the hospital from February 2011 to January 2013. None of the enrolled patients had received any

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androgen-deprivation treatment, chemotherapy, or radiotherapy prior to sampling. The prostate cancer tissues and adjacent normal tissues were snap frozen in liquid nitrogen after collection for further usage. Moreover, the clinicopathological information of the prostate cancer patients was collected and summarized in Table I. After surgery, a 5-year follow-up survey was collected and recorded for the subsequent survival analysis.

Cell lines and transfection. PC3, DU145, LNCaP, and 22RV1 prostate cancer cell lines and normal prostate epithelial RWPE-1 cells were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). PC3 and DU145 cells were cultured in Ham's F-12K medium (HyClone; GE Healthcare Life Sciences) supplemented with 10% fetal bovine serum (FBS; Invitrogen; Thermo Fisher Scientific, Inc.). LNCap, 22RV1, and RWPE-1 cells were cultured in RPMI-1640 medium (Hyclone; GE Healthcare Life Sciences) supplemented with 10% FBS. All cells were cultured in a humidified atmosphere containing 5% CO_2 at 37˚C.

Cell transfection was conducted by using Lipofectamine RNAiMax (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. *CD81* small interfering RNA (siRNA; 5'-CACGTCGCCTTCAACTGTA-3') and scrambled-siRNA control (5'-AATTCTCCGAACGGTCACGT-3') were purchased from Guangzhou RiboBio Co., Ltd., which was used to inhibit *CD81* expression or as the negative control of CD81 siRNA, respectively. The transfection efficiency was detected using quantitative real-time polymerase chain reaction (qRT-PCR). Untreated cells were used as a control.

RNA extraction and qRT-PCR. Total RNA was isolated from prostate cancer tissues and cell lines using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The concentration and quality of RNA were confirmed using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Inc.). Then, complementary DNA (cDNA) synthesis was performed using a PrimeScript RT Reagent Kit (Takara Biotechnology Co., Ltd.). qRT-PCR was performed using SYBR Green I Master Mix kit (Invitrogen; Thermo Fisher Scientific, Inc.) and a 7300 Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.). The primer sequences were as follows: *CD81* forward, 5'-GGGAGTGGAGGGCTGCACCAAGTGC-3' and reverse, 5'-GATGCCACAGCACAGCACCATGCTC-3'; GADPH forward, 5'-CCAAAATCAGATGGGGCAATGCTG G-3' and reverse, 5'-TGATGGCATGGACTGTGGTCATTC A-3'. The relative mRNA levels of *CD81* were calculated using the $2^{-\Delta\Delta Cq}$ method (15) and normalized to *GAPDH*.

Cell proliferation assay. Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc.) assays were used to detect the effects of *CD81* on the cell proliferation of prostate cancer cells. Briefly, ~$4x10^3$ transfected cells/well were seeded in 96-well plates. Cell proliferation assays were assessed at 0, 24, 48, and 72 h. CCK-8 reagent (10 μl) was added to the wells at each time-point and the absorbance value of each sample was measured at 450 nm with a microplate reader (Bio-Rad Laboratories, Inc.).

Cell migration and invasion assays. Transwell analysis with a 24-well Transwell chamber (Corning Life Sciences) was used to assess the effects of *CD81* on the migration and invasion capacities of prostate cancer cells. Cells transfected with *CD81* siRNA or control vectors ($3x10^4$ cells/well) were seeded and incubated in serum-free culture medium in the upper chamber. The lower compartment was filled with 500 μl complete medium containing 10% FBS. For invasion assays, the upper chambers were pre-coated with Matrigel (BD Biosciences). After incubation for 24 h at 37˚C with 5% CO_2, the cells remaining on the upper membranes were removed, and migratory or invasive cells on the lower chamber membranes were fixed with 4% paraformaldehyde for 20 min at room temperature and stained with 0.1% crystal violet for 30 min at room temperature. Five random fields from each membrane were counted with a light microscope (magnification, x200).

Statistical analysis. All the aforementioned experiments were performed at least three times. All of the data are presented as the mean ± SD. Statistical analyses were performed using SPSS 20.0 (IBM Corp.) and GraphPad 5.0 (GraphPad Software, Inc.). A Student's t-test was used to analyze differences between the tumor and normal groups. A χ^2 test was used to analyze the association of CD81 expression and clinical characteristics of prostate cancer patients. In addition, one-way ANOVA followed by Tukey's post hoc test was used to compare differences in more than three groups. The Kaplan-Meier method and Cox regression analyses were used to perform survival analysis and determine the prognostic performance of *CD81* for prostate cancer. P-values <0.05 were considered to indicate a statistically significant difference.

Results

CD81 expression in tissue specimens and cell lines. To investigate the expression pattern of *CD81* in prostate cancer tissues and cell lines, qRT-PCR was performed. The analysis results revealed that *CD81* expression was significantly higher in prostate cancer tissues than in adjacent normal tissues (P<0.001, Fig. 1A). In addition, *CD81* was markedly upregulated in all prostate cancer cell lines compared with that in RWPE-1 cells (P<0.01, Fig. 1B). Thus, it was speculated that overexpression of *CD81* may play an oncogenic role in prostate cancer. Considering that the expression levels of *CD81* were relatively higher in PC3, DU145 and LNCaP cells, these three cell lines were selected for subsequent experiments to verify the potential role of *CD81 in vitro*.

In addition, the relative mean value of the *CD81* expression level (2.851) in all prostate cancer tissues was used as the cutoff point for the grouping the patients. All prostate cancer patients were divided into a low-*CD81* expression group (n=55, based on the relative expression levels <2.851) and high-*CD81* expression group (n=59, based on the relative expression levels >2.851).

Increased expression of CD81 in prostate cancer tissues is associated with the clinicopathological features of prostate cancer patients. The associations between *CD81* expression and the clinicopathological features of prostate cancer patients were analyzed by χ^2 test. As summarized in Table I, high

Table I. Relationship between *CD81* expression and clinical characteristics of prostate cancer patients.

Parameters	No. of cases (n=114)	*CD81* expression		P-value
		Low (n=55)	High (n=59)	
Age				0.731
<65	52	26	26	
≥65	62	29	33	
PSA (ng/ml)				0.660
<10	66	33	33	
≥10	48	22	26	
Differentiation				0.207
Well-Moderate	69	30	39	
Poor	45	25	20	
Gleason score				0.190
<7	57	31	26	
≥7	57	24	33	
Lymph node metastasis				0.038[a]
Negative	59	34	25	
Positive	55	21	34	
TNM stage				0.008[a]
I-II	62	37	25	
III-IV	52	18	34	

[a]P<0.05. PSA, prostate-specific antigen.

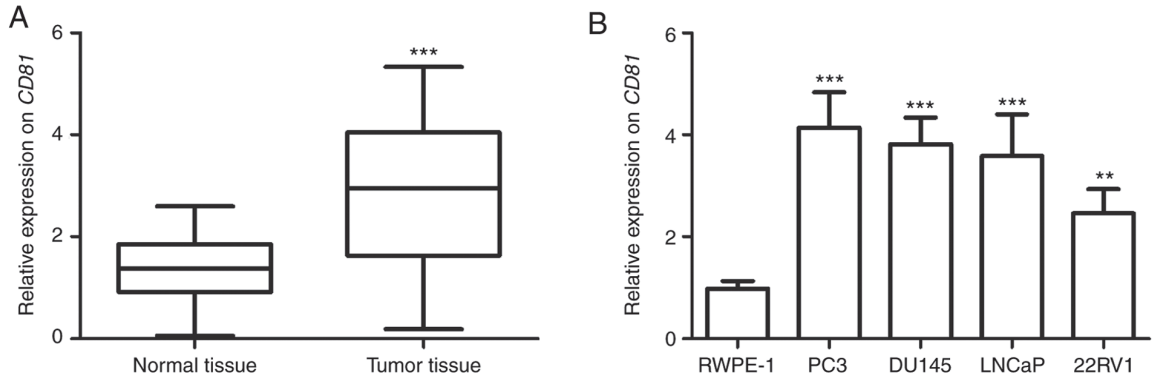

Figure 1. Relative mRNA levels of *CD81* are detected in prostate cancer tissues and cell lines by qRT-PCR. (A) The expression of *CD81* was higher in prostate cancer tissues than in matched adjacent normal tissues. (B) The expression of *CD81* was higher in prostate cancer cell lines than in normal cells (**P<0.01, ***P<0.001). qRT-PCR, reverse transcription-quantitative PCR.

CD81 expression in prostate cancer tissues was significantly associated with positive lymph node metastasis (P=0.038) and advanced TNM stage (P=0.008). However, the expression status of *CD81* was not associated with patient age, PSA, differentiation, or Gleason score.

Increased expression of CD81 in prostate cancer tissues predicts poor prognosis. To assess the potential prognostic value of *CD81* as a biomarker in prostate cancer, the 5-year survival information of prostate cancer patients was analyzed using the Kaplan-Meier method. The Kaplan-Meier curve indicated that prostate cancer patients with high-*CD81* expression levels exhibited a significantly shorter survival time than those with low-*CD81* expression levels (P=0.028, Fig. 2). Furthermore, the multivariate survival analysis with the Cox proportional hazards model demonstrated that *CD81* was closely correlated with poor overall survival and could be used as an independent prognostic factor for prostate cancer patients (HR=2.350, 95% CI=1.038-5.318, P=0.040, Table II).

Effects of silencing CD81 on cell proliferation, migration, and invasion in prostate cancer cells. To investigate the biological functions of *CD81* in prostate cancer, cell viability, migratory, and invasive capacities were determined in PC3, DU145, and

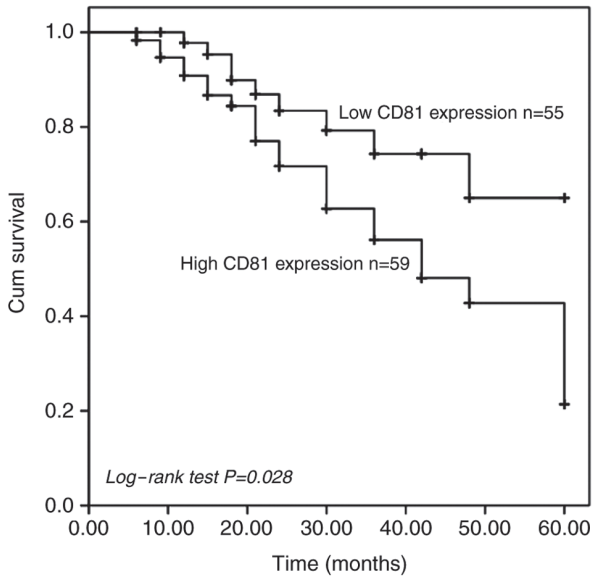

Figure 2. Kaplan-Meier survival curves for prostate cancer patients based on the expression of *CD81*.

Table II. Multivariate Cox analysis for *CD81* in prostate cancer patients.

	Multivariate analysis		
Characteristics	HR	95% CI	P-value
CD81	2.350	1.038-5.318	0.040[a]
Age	1.640	0.801-3.359	0.176
PSA	0.853	0.398-1.830	0.684
Differentiation	0.692	0.336-1.426	0.319
Gleason score	1.378	0.621-3.057	0.430
Lymph node metastasis	1.246	0.548-2.837	0.600
TNM stage	0.445	0.189-1.051	0.065

[a]P<0.05. HR, hazards ratio; CI, confidence interval; PSA, prostate-specific antigen.

LNCaP cells. These cell lines were transfected with *CD81* siRNA to regulate the expression of *CD81* in cancer cells. The results of qRT-PCR revealed that the expression of *CD81* in prostate cancer cells transfected with *CD81* siRNA was significantly downregulated compared with that in control cells (P<0.001, Fig. 3A). The results of the CCK-8 assay revealed that prostate cancer cell proliferation was suppressed in *CD81* siRNA-transfected cells compared with that in control cells (P<0.05, Fig. 3B). In addition, Transwell migration and invasion assays were used to examine whether *CD81* is involved in metastasis. The results presented in Fig. 4 indicated that downregulation of *CD81* significantly inhibited the migratory and invasive properties of prostate cancer cells compared with the control (P<0.001).

Discussion

Prostate cancer is a markedly heterogeneous tumor. Since it is the most prevalent cancer occurring in men, it has received great attention, and the prognosis has significantly improved in developed countries (1). However, the overall survival of prostate cancer patients is significantly lower in China than in some developed countries. Although marked therapeutic method advancements have led to efficacy improvements in patients with prostate cancer, the prognosis of some cancer patients at advanced stages remains unideal, and ~27-53% of patients experience biochemical recurrence after local therapy (16,17). Therefore, prognosis improvement is essential for prostate cancer patients. Recently, molecular biomarkers have received considerable attention for their diagnostic and prognostic abilities, and for their involvement in tumor formation and progression. In the present study, the expression pattern of *CD81* was detected and it was revealed that *CD81* was higher in prostate cancer tissues than in adjacent normal tissues and higher in prostate cancer cells than in normal prostate epithelial RWPE-1 cells. Overexpression of *CD81* in prostate cancer tissues was identified to be significantly associated with positive lymph node metastasis, advanced TNM stages, and poor prognosis in prostate cancer patients. Furthermore, cell functional analysis of PC3, DU145, and LNCaP human prostate cancer cells revealed that *CD81* functions as an oncogene in prostate cancer by promoting cell proliferation, migration, and invasion. These findings indicated that *CD81* functions as an oncogene in prostate cancer and may be a potential prognostic biomarker for human prostate cancer.

In recent years, an increasing number of studies have focused on the development of accurate molecular biomarkers for better detection, diagnosis, prognosis, and treatment (18-20). For instance, forkhead transcription factor (*FoxM1*) functions as an oncogene in the initiation, development, and progression of cancer, and its neoplastic functions can be used as a strong biomarker for the diagnosis and treatment of cancer (21). In prostate cancer, numerous prognostic biomarkers were also investigated (22-24). For instance, minichromosome maintenance 10 replication initiation factor (*MCM10*) was revealed to be significantly upregulated in prostate cancer patients, and overexpression of *MCM10* promoted cell proliferation and predicted poor prognosis in prostate cancer (25). Another study revealed that ribosome binding protein 1 (*RRBP1*) was upregulated in prostate cancer tissues and significantly associated with T stage, lymph node metastasis, PSA, Gleason score, and shorter survival time in prostate cancer patients, thus, *RRBP1* may serve as a potential biomarker in prostate cancer (26). The aforementioned studies revealed the pivotal role of cancer-related molecules in cancer diagnosis, prognostication, and treatment.

The present findings as well as those from other studies indicate that *CD81* is an oncogene in various cancers, including prostate cancer. In the present study, *CD81* was upregulated in prostate cancer tissues and cell lines. Moreover, high expression of *CD81* was revealed to be significantly associated with lymph node metastasis and TNM stage. *CD81* was considered to function as an oncogene in prostate cancer. Previous studies have revealed differential expression of *CD81* in various types of cancers, such as classic vs. variant hairy cell leukemia, breast cancer, and gastric cancer (13,27,28). A study by Vences-Catalan *et al* indicated that *CD81*, which is a

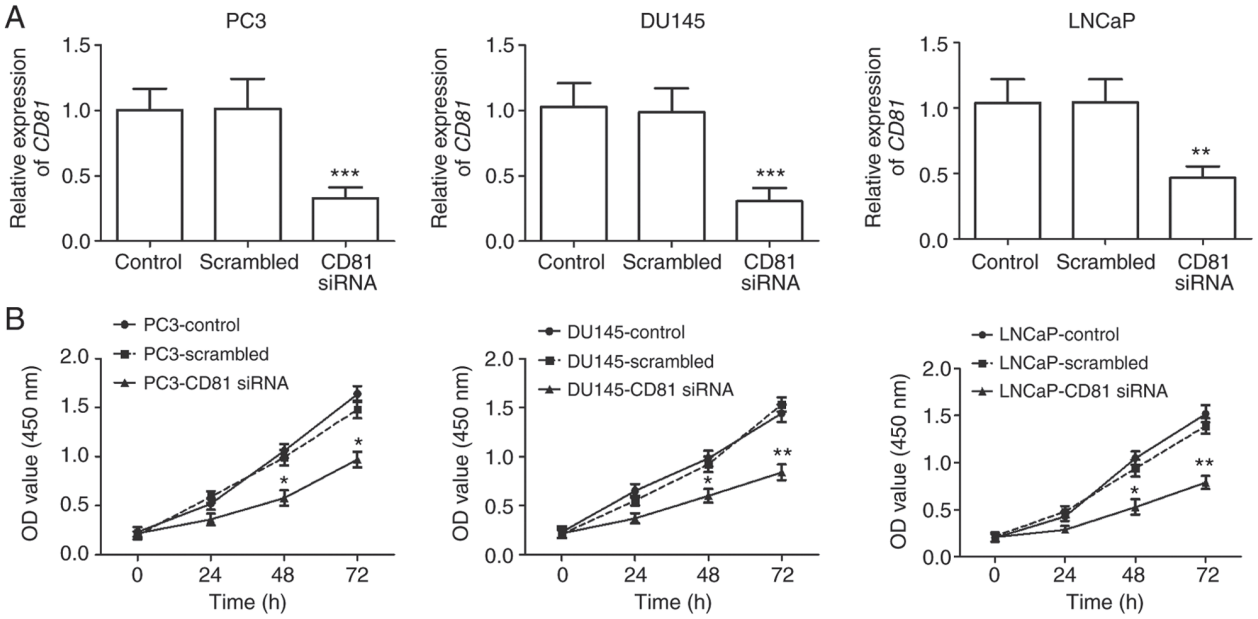

Figure 3. Downregulation of *CD81* suppresses the proliferation of PC3, DU145, and LNCaP prostate cancer cells *in vitro*. (A) qRT-PCR analysis confirmed the efficacy of the *CD81* siRNA. (B) Growth curves of PC3, DU145, and LNCaP cells are presented (*P<0.05, **P<0.01, ***P<0.001). qRT-PCR, reverse transcription-quantitative PCR.

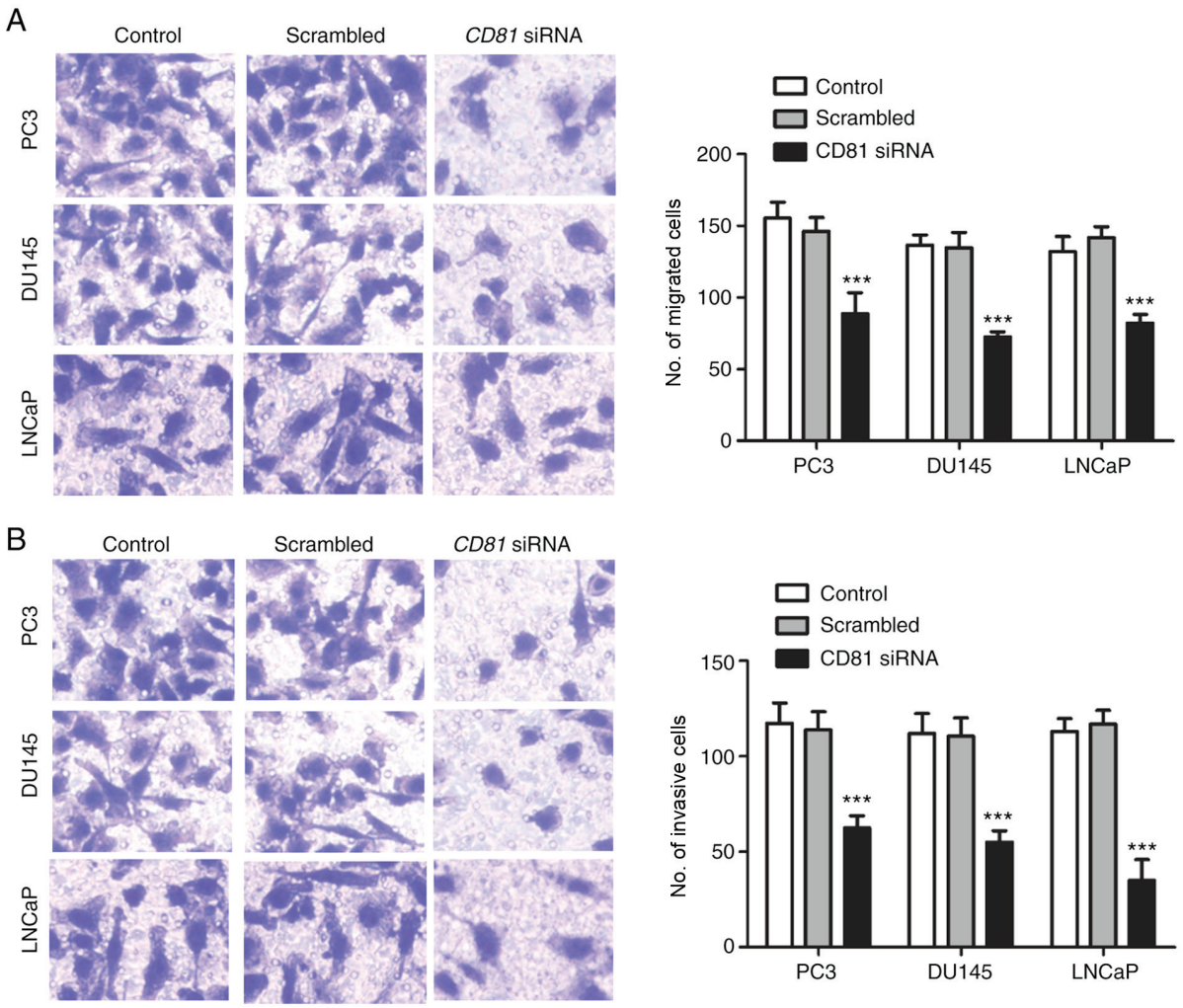

Figure 4. Downregulation of *CD81* inhibits the migration and invasion of PC3, DU145, and LNCaP cells *in vitro*. (A) Migration analysis of prostate cancer cells (magnification, x200). (B) Invasion analysis of prostate cancer cells (magnification, x200) (***P<0.001).

promoter of tumor growth and metastasis, is widely expressed in most tissues and on the majority of tumor cells (11). In breast cancer, *CD81* was also revealed to be upregulated in tumor tissues, associated with poor overall survival, and promoted tumor cell proliferation and migration (13). In plasma cell myeloma, it was revealed that *CD81* was an independent factor affecting the overall survival and progression-free survival of patients, and *CD81* positivity predicted poor prognosis (29). The aforementioned studies demonstrated that *CD81* also has prognostic value in cancers. In the present study, the clinical significance of *CD81* in prostate cancer was also investigated. Kaplan-Meier survival curves revealed that patients with high-*CD81* expression levels had shorter survival times than those with low-*CD81* expression levels, indicating that increased *CD81* expression was correlated with poor overall survival. The multivariate Cox analysis results indicated that *CD81* expression was an independent prognostic factor for prostate cancer patients.

Several tetraspanins have been studied for their essential role in tumor cell growth, migration, invasion, and metastasis, including *CD81* (12,30,31). In the present study, downregulation of *CD81* significantly inhibited cell proliferation, migration, and invasion in transfected PC3, DU145, and LNCaP metastatic cell lines. Vences-Catalan *et al* revealed that tetraspanin *CD81* promotes tumor growth and metastasis by modulating the functions of T regulatory and myeloid-derived suppressor cells (32). In melanoma, upregulation of *CD81* was revealed to increase melanoma cell motility by upregulating metalloproteinase *MT1-MMP* expression through the AKT-dependent Sp1 activation signaling pathway, leading to increased cell invasion and metastasis (33). Overexpression of *CD81* in different types of cancers could potentially induce greater susceptibility to antibody binding and may thus represent a promising tumor target for immunotherapy due to its unique properties (33,34). Although the exact mechanism of action has not been clarified, it is clear that *CD81* has an important function in cancer. Further studies are required to assess the precise molecular mechanisms underlying the role of *CD81* in prostate cancer.

Collectively, the data in the present study revealed that *CD81*, a member of the tetraspanin family, was significantly upregulated in prostate cancer tissues and cell lines compared with that in controls, respectively. To the best of our knowledge, these findings provide the first evidence that *CD81* may be a potential prognostic biomarker and therapeutic target for prostate cancer and correlated with the progression of prostate cancer cells.

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Availability of data and materials

All data generated or analyzed during the present study are included in this published article.

Authors' contributions

YZ conducted the experiments, analyzed the data, and wrote the manuscript. HQ conceived the study, and revised the manuscript critically for important intellectual content. AX and GY made substantial contributions to patient and tissue specimen collection and data interpretation. All authors read and approved the final version and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

The present study was approved by the Research Ethics Committee of Tongren Hospital, Shanghai Jiao Tong University School of Medicine (Shanghai, China). All the patients signed written informed consent. All specimens were handled and anonymized according to ethical and legal standards.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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