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Original Article

ODAM is a predictor for biomedical recurrence and inhibits the migration and invasion of prostate cancer

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Abstract: Odontogenic ameloblast associated protein (ODAM) is a protein contributed to cell adhesion and has been shown to express in normal prostate tissue, but the expression and significance of ODAM in prostate cancer remain unknown. In this study, we detected the protein expressions of ODAM in 88 prostate cancer tissues with immunohistochemical staining, and found that 53 cases (60.2%) was high expression of ODAM, which was shown in the cytoplasm and paranuclear regions. Furthermore, low expression of ODAM was significantly correlated with lymph node metastasis, preoperative PSA and Gleason score, but not with mean age, follow-up duration, PSM rate and distribution of pathological T stage. Additionally, our results of multivariate analysis showed that low ODAM expression was an independent predictor of biomedical recurrence, while the positive lymph node metastasis, Gleason score, and preoperative PSA were not the independent risks for biomedical recurrence. Overexpression of ODAM did not inhibit the growth of prostate cancer cells PC3, but significant suppressed their invasion and migration with decrease of the protein levels of MMP-2. These results suggest that ODAM is a predictor for biomedical recurrence and inhibits the migration and invasion of prostate cancer.

Keywords: ODAM, migration, invasion, prostate cancer

Introduction

Prostate cancer is one of the most common male tumors in the worldwide, and invasion and metastasis of prostate cancer is the main reason for death [1-3]. However, the mechanisms of invasion and metastasis of prostate cancer are complicated and remain to be further understood. In our previous study, we analyzed TLR9 signaling network in regulation of migration and invasion by whole-genome microarray and found that odontogenic ameloblast-associated protein (ODAM) might be associated with migration and invasion in prostate cancer cells [4]. ODAM is first identified as the protein constituent of calcifying epithelial odontogenic/Pindborg tumors and subsequent studies revealed that it was expressed in a wide range of normal odontogenic, glandular, and epithelial tissues [5-10] as well as in malignancies including odontogenic cancer, breast cancer, gastric cancer, and melanoma [11, 12]. The previous study have reported that ODAM was highly expressed in normal prostate tissue [13]. However, the expression and significance of ODAM in prostate cancer remain unknown.

In this study, we determined the expression of ODAM in the tissues of patients with prostate cancer and analyzed the correlation of ODAM expression with lymph node metastasis, biomedical recurrence, Gleason score, PSM, preoperative PSA, pathological T stage and Age. Furthermore, we overexpressed ODAM in human prostate cancer cells to gain further insight into the function of ODAM in regulating migration and invasion of prostate cancer.

Material and methods

Patients and specimens

A total of 88 formalin-fixed, paraffin-embedded specimens were collected from prostate cancer

patients who underwent radical prostatectomy without neoadjuvant hormonal therapy or transurethral resection of prostate at the Department of Urology, 3rd Affiliated Hospital of Sun Yat-Sen University, Guangzhou, China from July 2007 to June 2014. Patients with histologically confirmed prostate cancer with diagnostic prostate biopsies and no metastatic disease diagnosed by pelvic computed tomography and bone scan were eligible. The radical prostatectomy was performed with standard pelvic lymph node dissection as previously described [14, 15]. The clinical characteristics, preoperative serum PSA level, surgical margin state, the state of lymph node metastasis, pathological T stage, and Gleason score were obtained from patient records. The clinical follow-up data include biochemical recurrence (defined as PSA ≥ 0.2 ng/ml on 2 successive measurements in 3 months after radical prostatectomy), clinical recurrence (defined as identification of metastases or histologically confirmed local recurrence) and tumor specific death. Progression-free survival rates were calculated from the date of radical prostatectomy to either biochemical recurrence leading to second line treatments, clinical recurrence or the last day of follow-up. The study was conducted with the ethical approval of the hospital human ethics committee.

Immunohistochemical staining

Immunohistochemistry assay was performed using two micrometer thick formalin-fixed paraffin embedded archived tissue sections as previously described [16, 17]. Anti-ODAM antibodies were purchased from Proteintech Group, Inc., Chicago, USA. Staining was assessed by two independent investigators in a blind manner to reach a consensus. Staining intensity was recorded as negative (0), weakly positive (+1), positive (+2), and strongly positive (+3). Samples with +2 and +3 staining of ODAM were classified as 'high expression group', and those with 0 and +1 were assigned as 'low expression group'.

Cell culture and transfection

The human prostate cancer cell lines PC3 were obtained from the American Type Culture Collection and maintained in DMEM/F12 supplemented with 10% fetal bovine serum (FBS, Invitrogen), penicillin (100 units/mL), and streptomycin (100 μg/mL) at 37°C under 5% CO_2 in a humidified incubator. PC3 cells were transfected with pcDNA5T/O (Invitrogen) plasmid containing full-length human ODAM cDNA or the empty vector using Lipofectamine LTX reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Cell clones with stable ODAM overexpression were selected with hygromycin. ODAM overexpression and control cell clones were designated PC3-ODAM or PC3-CON, respectively.

Matrigel invasion assay

Matrigel invasion assay was performed as previously described [18]. Briefly, PC3-ODAM or PC3-CON cells were trypsinized, washed and suspended (1×10^5 cells/ml) in serum-free DMEM/F12 medium. One hundred μl cell suspension was added into the upper chamber of a Costar Transwell permeable support (8-μm pore size, Thermo-Fisher) coated with Matrigel at 5 mg/ml, and the lower chamber was filled with medium containing 10% FBS. After incubation for 24 h at 37°C, non-migrating cells were swabbed from the upper surface and those that passed through to thelower surface were fixed in 90% ethanol for 10 min, stained with 0.1% crystal violet, counted and photographed.

Western blotting

Western blotting was carried out according to previously reported [19, 20]. In brief, cells were harvested, lysed and centrifuged. Supernatants were collected, and protein concentration was quantified using with Bradford assay. Proteins (30 μg) were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Membranes were blocked and incubated with primary antibodies. Corresponding horseradish peroxidase-conjugated secondary antibodies were used against the primary antibody. GAPDH was used as an internal control (anti-GAPDH, 1:1000; Cell Signaling Technology, Inc., Beverly, MA, USA). Immunoreactive proteins were then visualized using the ECL western blotting system (Pierce Biotechnology, Rockford, IL, USA).

Wound healing assay

Wound Healing Assay was performed as previously reported [21, 22]. PC3-ODAM and PC3-

Table 1. Summary of clinical characteristics in 88 patients with differential expression of ODAM

Characteristics	ODAM expression			*P* value
	Total	Low expression (N=35)	High expression (N=52)	
Age	58.9±6.5	58.4±6.9	59.2±6.4	0.597
Preoperative PSA	15.3±6.3	20.2±5.2	12.1±4.7	<0.001
Gleason score	6.67±1.72	7.4±1.4	6.17±1.76	0.001
Follow up (M)	51.6±17.1	51.1±17.8	52.0±16.8	0.808

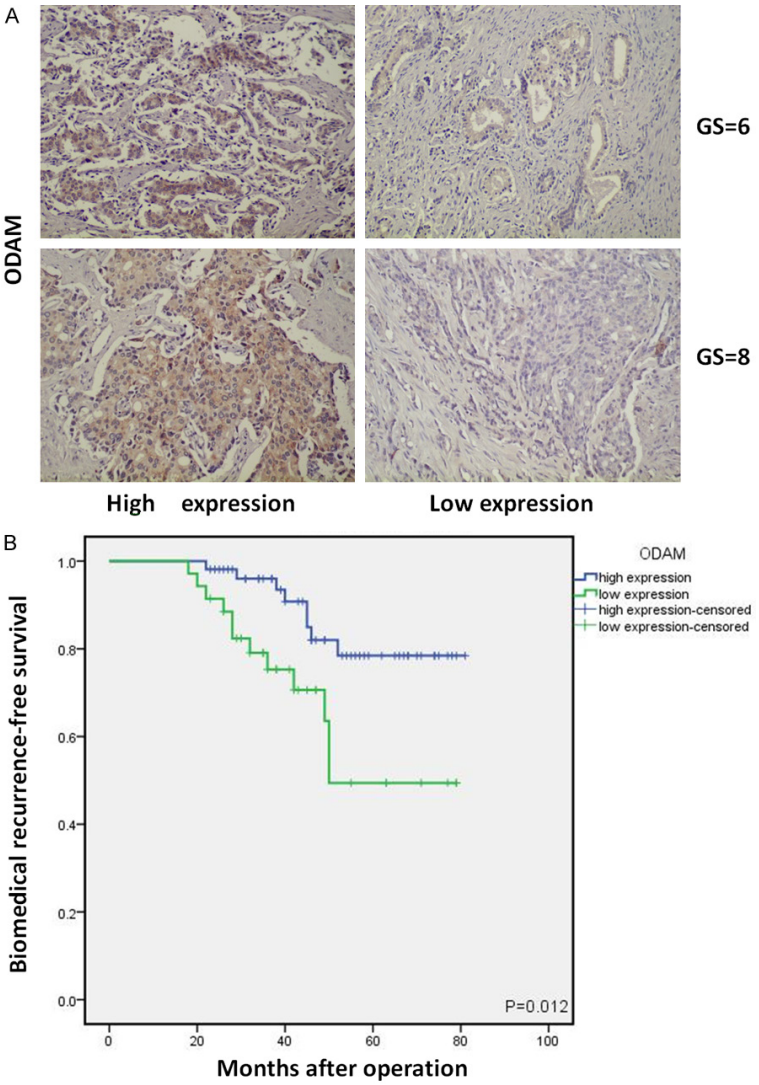

Figure 1. ODAM is an independent risk factor for biochemical recurrence. The expression of ODAM in human prostate cancer tissues were examined by immunohistochemistry staining. The representative samples (GS=6 and GS=8) with low and high expression of ODAM (A) and progression-free survival rate of patients with prostate cancer (B) were shown.

CON cells were seeded in the six-well plate until the cells reach a confluence of 80%. The cell monolayer was scratched using a sterile 10 μl pipette tip, and the detached cells were washed with PBS three times. Cells were allowed to migrate for 24 h and 48 h, and the scratches were carefully observed and photographed. The experiments were repeated 3 times independently.

Statistical analysis

All quantitative data are presented as the mean ± SD. Student's t test (two-tailed) was used to compare two groups. Correlation of ODAM expression and clinicopathological variables was analyzed using the χ^2 test or, in the case of low expected frequencies, by the Fisher's exact test. Correlation analysis was performed with spearman correlation analysis. Biomedical recurrence-free survival was analyzed using Kaplan-Meier method with the log-rank test for significance. Multivariate survival analysis was carried out using the Cox proportional hazards model. Hazard ratios (HRs) were assessed using Cox univariate analysis. $P<0.05$ was considered statistically significant.

Results

Low expression of ODAM is correlation with a higher probability of lymph node metastasis, preoperative PSA and Gleason score

To investigate the expression of ODAM in human prostate cancer tissues, a total of 88 prostate cancer specimens were collected to detect their ODAM expressions with immunohistochemical staining. The clinical characteristics of patient were summarized in **Table 1**. The mean age at surgery, pre-

Table 2. Correlation of ODAM expression with age, preoperative PSA, Gleason score, lymph node metastasis, biomedical recurrence, PSM and T stage

Characteristics	ODAM expression (No. patients)		*P* value
	Low expression	High expression	
Age			0.456
≤60	19	33	
>60	16	20	
Preoperative PSA			<0.001
≤10	2	21	
>10	33	32	
Gleason score			<0.001
≤7	11	38	
>7	24	15	
Lymph node metastasis			<0.001
Negative	22	48	
Positive	13	5	
Biomedical recurrence			0.036
No	23	45	
Yes	12	8	
PSM			0.535
Negative	31	49	
Positive	4	4	
Pathological T stage			0.217
≤T2	26	45	
>T2	9	8	

operative PSA value, Gleason score and follow-up duration was 58.9±6.5 years, 15.3±6.3 ng/ml, 6.67±1.72, and 51.6±17.1 months, respectively. Positive surgical margin (PSM) was observed in 8 cases (9.1%), and 18 cases (20.4%) were found to have lymph node metastasis after surgery. After a mean of 51.6 months of follow-up, biochemical recurrence occurred in 20 cases (22.7%), clinical recurrence was observed in no patients. The representative results of low and high expressions were shown in **Figure 1A**. The ODAM expression was found to reside in the cytoplasm and paranuclear regions. Thirty-five cases (39.8%) with low expression of ODAM were observed. As shown in **Tables 1** and **2**, there was no significant difference between patients with low and high ODAM expression for the mean age (58.4±6.9 VS 59.2±6.4 years, P=0.597), follow-up duration (51.1±17.8 VS 52.0±16.8, p=0.808), PSM rate (11.4% VS 7.5%, p>0.05) and distribution of pathological T stage (P=0.371), respectively. However, significant differences were observed in preoperative PSA values (20.2±5.2 VS 12.1±4.7 ng/ml, p<0.001) and Gleason score (7.4±1.4 VS 6.17±1.76, P=0.001) between low and high ODAM expression group. In patients with low and high ODAM expression, positive lymph node metastasis after surgery was observed in 13 (37.1%) and 5 (9.4%) patients, respectively. There was a statistically significant correlation between lymph node metastasis and low expression of ODAM (p<0.001). Biochemical recurrence occurred in 12 cases (34.3%) of patients with low ODAM expression, while in 8 cases (15.1%) of patients with high ODAM expression. Low level of ODAM expression was shown to be significantly correlated with biomedical recurrence (p=0.036) (**Table 2**).

ODAM is an independent risk factor for biochemical recurrence

To further determine whether ODAM expression and other clinic pathological characteristics are associated with prognosis of patients with prostate cancer, biomedical recurrence-free survival was compared in patients. As shown in **Figure 1B**, biomedical recurrence-free survival of patients with high ODAM expression was significantly larger than that of patients with low ODAM expression (P=0.012). No other characteristics showed statistical difference in biomedical recurrence-free survival of patients (**Figure 2**). Multivariate Cox regression analysis showed that low expression of ODAM (hazard ratio 3.902, 95% CI: 1.19-12.799, P=0.025) was the independent risk factor of biomedical recurrence during follow-up. No significant association was detected between biomedical recurrence and age, PSM, PSA level, Gleason score, T stage and lymph node metastasis (**Table 3**).

ODAM inhibits the invasion and migration of prostate cancer cells

To understand the role of ODAM in prostate cancer progression, we examined the expression of ODAM in high-invasive PC3 cancer cells. Western blot was performed to determine the expression of ODAM in PC3 cells, and the result showed that PC3 cell lines did not express detectable ODAM protein. Next, we generated

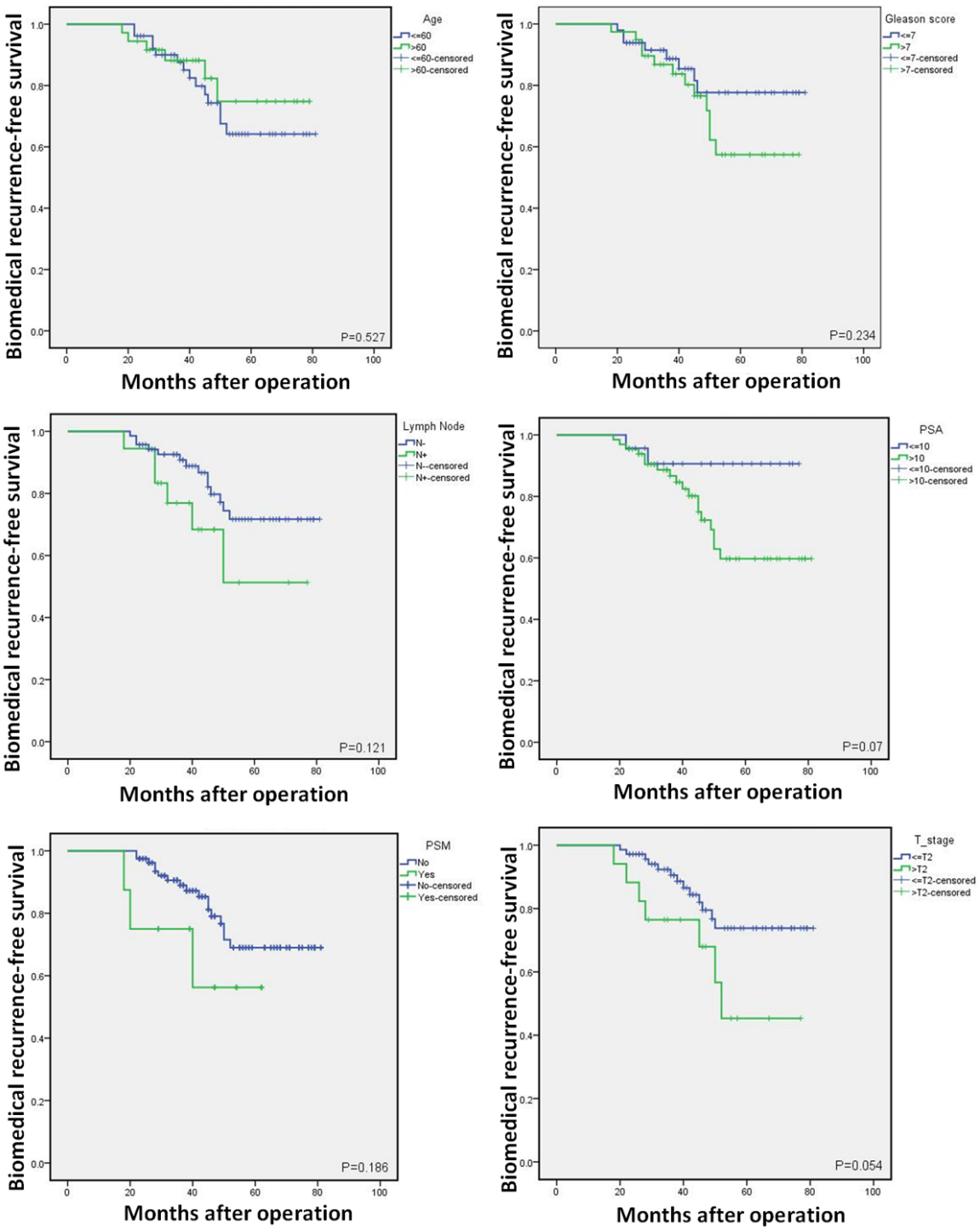

Figure 2. Kaplan-Meier analysis of biochemical recurrence-free survival in patients based on age, preoperative PSA, Gleason score, lymph node metastasis, PSM and T stage respectively.

PC3 cells with stable overexpression of ODAM (**Figure 3A**). The data of cell growth assays did not show significant growth inhibitory effect in PC3-ODAM cells compared to PC3-CON cells (data not shown). To further determine whether ODAM could affect PC3 cell migration, we conducted wound healing assay and the results revealed significant suppression of cell migration in PC3-ODAM cells compared to PC3-CON cells (**Figure 3B**). Consistently, the inhibitory

Table 3. Univariate and multivariate analyses of various prognostic parameters of biomedical recurrence.

		Univariate Analysis			Multivariate Analysis		
Variables	No. patients	P	HR	95% CI	P	HR	95% CI
Age		0.530	0.736	0.282-1.918			
≤60	52						
>60	36						
Preoperative PSA		0.091	3.524	0.817-15.208			
≤10	23						
>10	65						
Gleason score		0.242	1.707	0.697-4.179			
≤7	49						
>7	39						
Lymph node metastasis		0.13	2.104	0.803-5.514			
Negative	70						
Positive	18						
ODAM		0.017	2.987	1.215-7.346	0.025	3.902	1.19-12.799
High expression	53						
Low expression	35						
PSM		0.20	2.234	0.654-7.635			
Negative	80						
Positive	8						
T stage		0.063	2.395	0.955-6.007			
≤T2	71						
>T2	17						

effect of ODAM on cell invasion was confirmed by the results of Transwell invasion assay (**Figure 3C**). Furthermore, we found that the protein levels of MMP-2 were significantly inhibited after ODAM over-expression in PC3 cells (**Figure 3D**).

Discussion

Prior studies of breast cancer patient biopsies suggested that ODAM served as a favorable prognostic biomarker and these patients with nuclear ODAM expression had an survival benefit regardless of tumor stage [23, 24]. In the present study, we analyzed 88 specimens including a series of Gleason score from 4-10 and the result showed that 53 cases (60.2%) was high expression of ODAM, which was found in the cytoplasm and paranuclear regions. Furthermore, low expression of ODAM was significantly correlated with lymph node metastasis, biomedical recurrence and progression-free survival rate, but not with mean age, follow-up duration, PSM rate and distribution of pathological T stage. Additionally, our results of multivariate analysis showed that low ODAM expression was an independent predictor of biomedical recurrence, while the positive lymph node metastasis, Gleason score, and preoperative PSA were not the independent risks for biomedical recurrence. These data indicates ODAM may play a critical role in the metastasis and prognostic implications of prostate cancer.

Cell motility, which is essential for metastasis, is a complex, multistep process that integrates multiple proteins and cell signaling pathways. Tight junction proteins, such as VMP-1, JAM-A, PARD6B and Claudin-4, have been shown to take an important part on tumor invasion and metastasis [25, 26]. ODAM belongs to the family of tight junction proteins and have been shown to suppress the invasion and metastasis in breast cancer cells MCF-7 and MDA-MB-231, melanoma cells A375 and C8161 by activation of PTEN and inhibition of the phosphorylation of AKT [13, 27]. Moreover, ODAM expression could enhanced adhesion and apoptosis of the transfected breast cancer cells MDA-MB-231 and inhibited their growth rate, migratory and invasive activity *in vitro* and *in vivo* [23]. In our

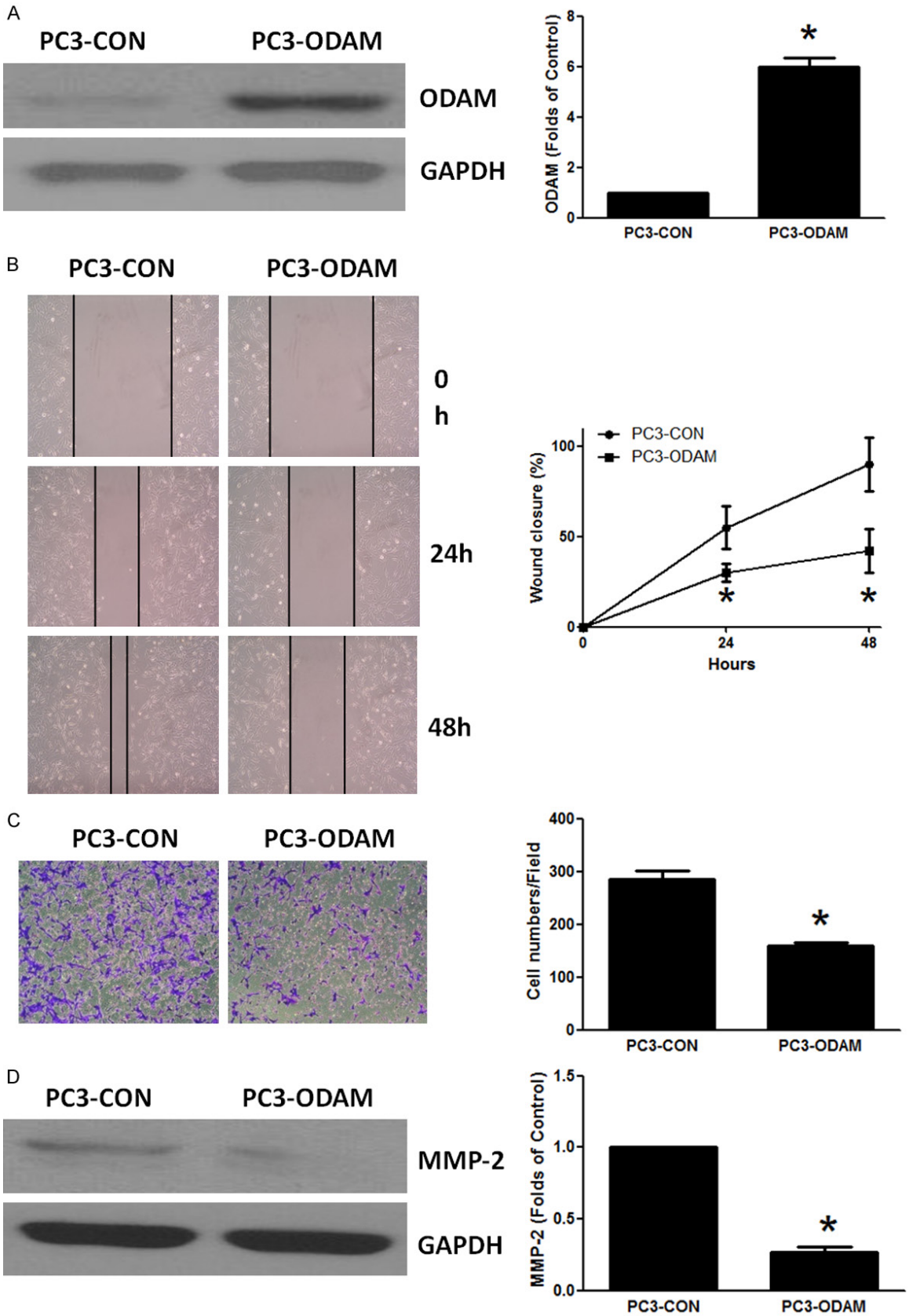

Figure 3. Overexpression of ODAM inhibits the migration and invasion of prostate cancer cells PC3. PC3 cells were transfected with the human ODAM pcDNA5T/O plasmid construct or the empty vector. After stably expressing cells

were generated, proteins were detected by Western blot with the indicated antibodies (A). Cell migration were analyzed by wound healing assays in PC3-CON and PC3-ODAM. The scratch-wounds were photographed at the indicated time points (0, 24 and 48 h) after cell scratching and the wound sizes were measured (B). Cells were seeded into the upper chamber of transwell with matrigel. Cell invasion were induced by FBS-containing media in the lower chamber. Invaded cells in the lower surface of the filters were stained and microphotographed after serum induction 24 h (C). Proteins were detected by Western blot with the indicated antibodies (D). The representative figures and quantified data from three individual experiments were shown. *Represents P<0.05.

case, overexpression of ODAM did not inhibit the growth of prostate cancer cells PC3, but significant suppress their invasion and migration. Additionally, the protein levels of MMP-2 were significantly inhibited after ODAM overexpression in PC3 cells. It is well known that tumor invasion and metastasis require increased expressions of matrix metalloproteinases (MMPs), where MMP-2 degrades type IV collagen in extracellular matrix and have been thought to be key enzymes [28-30]. Several groups have revealed that high expression of MMP-2 was correlated with lymph node metastasis of cancer [31, 32]. However, the molecular mechanisms of ODAM decreasing MMP-2 protein level and inhibiting the invasion and migration of prostate cancer need to be further investigated.

In summary, our study demonstrates that ODAM expression is significantly correlated with lymph node metastasis, preoperative PSA and Gleason score and serves as an independent risk for predicting biomedical recurrence in prostate cancer. Furthermore, our results also provide strong evidence for a functional role of ODAM in suppressing the migration and invasion of prostate cancer cells.

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Disclosure of conflict of interest

None.

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