

## Original Article

# Correlation of ANXA1 expression with drug resistance and relapse in bladder cancer

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**Abstract:** Objective: To investigate the expression of annexin a1 (ANXA1) in adriamycin-resistant human bladder cancer cell line (pumc-91/ADM) compared with the parental cell line (pumc-91) and its relevance to the drug resistance of bladder cancer, as well as explore the relevance of ANXA1 in recurrent bladder cancer tissues as pertinent to relapse. Methods: qRT-PCR and Western blot were implemented to research the level of ANXA1 in two cell lines (pumc-91/ADM and pumc-91). Immunohistochemistry was applied to explore ANXA1 expression in bladder cancer tissues of different intervals of relapse. The association of ANXA1 with clinicopathological parameters was analyzed. Results: The expression of ANXA1 was downregulated in drug-resistant cell line pumc-91/ADM compared to pumc-91. The bladder cancer tissues recurring two years later exhibited higher ANXA1 levels. ANXA1 expression level was positively correlated with T stage, while it was not connected with histological grade strongly. The expression level and influencing factors of ANXA1 in recurrent tissues of bladder cancer were clarified for the first time. Conclusion: ANXA1 may become a promising marker to predict the recurrence and drug resistance of bladder cancer and provide guidance for surveillance.

**Keywords:** Bladder cancer, annexin a1 (ANXA1), recurrence, drug resistance

## Introduction

Bladder cancer, as the most common urogenital system malignant tumor, is ranked fourth among the cancers related to males in the world [1]. Meanwhile, it is the ninth dominating cause of death from malignant tumor [2]. In accordance with the degree of invasion, bladder cancer can manifest as nonmuscle-invasive or muscle-invasive tumor, and about 75% of primary cases belong to the nonmuscle-invasive category [3]. Transitional cell carcinoma (TCC) is the main histological pattern, accounting for 90% of cases [4]. The transurethral resection of bladder tumor (TURBT) is essential for nonmuscle-invasive bladder cancer treatment. With regard to low-grade Ta and T1 tumor, intravesical chemotherapy or immunotherapy is necessary. As for muscle-invasive bladder cancer, radical cystectomy and lymph nodes dissection is the standard operation, which should also combine with adjuvant chemotherapy or radiation.

In recent years, many clinicians and researchers consider bladder cancer as a “costly dis-

ease” because of its continuous demands for surveillance and treatment, which is based on the characteristics of tendency for recurrence and drug resistance [5]. Conventional data shows that 50%-70% of nonmuscle-invasive bladder cancer will recur within 5 years and 10%-30% of cases may evolve into muscle-invasive diseases [6]. Chamie etc studied 7420 bladder cancer patients who were diagnosed between 1992 and 2002. Their most common features were nonmuscle-invasive urothelial, high-grade and nonmetastatic cancers. The results demonstrated that 2897 (39%) patients experienced recurrence and 2449 (33%) developed to muscle-invasive cancer. The recurrence rates 5-year and 10-year were 69%, 74% respectively [7]. Drug resistance is also a nearly intractable issue widespread in many kinds of tumor, including bladder cancer. Of course, it is a vital factor resulting in recurrence. In addition, bladder cancer patients’ physical and psychological quality of life is severely damaged by the necessity of repeated cystoscopic probes. However, there are very few effective biomarkers aimed at predicting and monitoring the relapse of bladder cancer. Moreover, we lack suffi-

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**Table 1.** Relationship between ANXA1 expression and clinicopathologic parameters

Parameters	n	ANXA1 expression		Percentage of high expression	P value (Scoring method)	P value (MOD method)
		Low	High			
Age					0.22	0.51
< 60	16	6	10	62.5%		
≥ 60	44	19	25	56.8%		
Gender					0.63	0.14
Male	46	19	27	58.7%		
Female	14	6	8	57.1%		
Interval of relapse					0.03*	0.004*
< 6 m	30	17	13	43.3%		
> 2 y	30	7	23	76.7%		
Differentiation					0.65	0.06
Low Grade	30	12	18	60.0%		
High Grade	30	15	15	50.0%		
Invasion depth					0.006*	0.02*
Tis	33	17	16	48.5%		
T1	17	7	10	58.9%		
T2	10	3	7	70.0%		
Total				60		

\*P < 0.05 means statistical significance.

cient strategies on how to estimate the recurrence or drug resistance in a noninvasive way.

ANXA1 is a member of the family of annexins, which belong to calcium ion-dependent phospholipid binding proteins. The elementary structure of ANXA1 contains a conservative C-region that can bind to phospholipids and calcium ions, as well as a variable N-domain, which possesses different kinds of kinase phosphorylation sites [8]. Therefore, ANXA1 participates in multiple biological activities in many physiological processes, such as inflammation, apoptosis, cell signal transduction and so on. Previous studies demonstrated that there was abnormal expression of ANXA1 in many types of cancer [9]. It implied a relationship between ANXA1 and tumor development.

In our present study, we explored the expression of ANXA1 in a human bladder cancer adriamycin-resistant cell line (pumc-91/ADM) and its parental cell line (pumc-91) from the cellular level as well as in 60 tissues with relapsing bladder cancer from the histological level. Furthermore, we examined the relationship between ANXA1 expression and clinicopathologic parameters.

### Material and methods

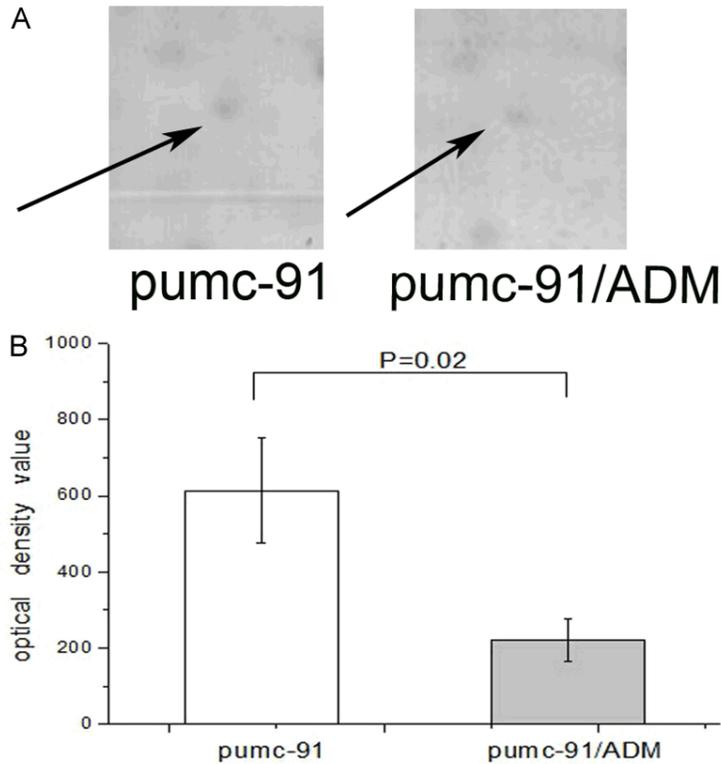
#### Cell lines

The human bladder cancer Pumc-91 cell line was kindly provided by Peking Union Medical College Hospital. Pumc-91/ADM was a drug-resistant cell line that was established by adding the dosage of adriamycin gradually. The final concentration of adriamycin was 1.0 µg/ml. The characteristics of drug-resistant cell line were verified by MTT and FCM measurement. Pumc-91 cells were grown in RPMI 1640 medium (Gibco, USA) supplemented with 10% fetal bovine serum (Dingguo Biotechnology, China) while pumc-91/ADM cells with 18% fetal bovine serum. Cells were cultured in 5% CO<sub>2</sub> humidified incubator at 37°C.

#### Sample collection

A total of 60 tissue sections of recurrent bladder cancer were collected from Peking University Health Science Center. All of the sections were reviewed by two pathologists in order to confirm the pathological related diagnosis. The criteria of T stage and histological differentiation were exactly in accordance with the latest World Health Organization (WHO) and

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**Figure 1.** ANXA1 was identified as differential expression protein between pumc-91 and pumc-91/ADM cell lines via 2-DE based high throughput proteomics methods. A. The spots of ANXA1 in 2-DE. B. The optical density value of the spots that were measured three times. The expression of ANXA1 was higher in pumc-91 cells and the difference had statistical significance ( $P < 0.05$ ).

American Joint Committee on Cancer (AJCC) criterion. This study was performed according to the standards of Institutional Committee for the Protection of Human Subjects and Declaration of Helsinki. Written consent informing the research nature was obtained from the patients. The patients associated information are listed in **Table 1**.

### Screening and identification of differential expression protein

Two-dimensional gel electrophoresis (2-DE) was carried out to screen the differential protein between pumc-91 and pumc-91/ADM. First-dimension isoelectric focusing was performed on PROTEAN IEF Cells (Bio-Rad). Second-dimension was 12% SDS-PAGE conducted on PROTEAN-II XI Electrophoresis Cell (Bio-Rad). The obtained 2-DE gels were analyzed by PDQuest software (Bio-Rad). After sta-

tistical analysis, the confirmed protein spots were excised, digested, extracted and then detected with ABI 4700 Proteomics Analyzer MALDI-TOF/TOF (Applied Biosystems) to acquire MS and MS/MS spectra. The spectra was compared and matched with IPI human database via MASCOT (Matrix Science) and GPS Explorer (Applied Biosystems).

### qRT-PCR

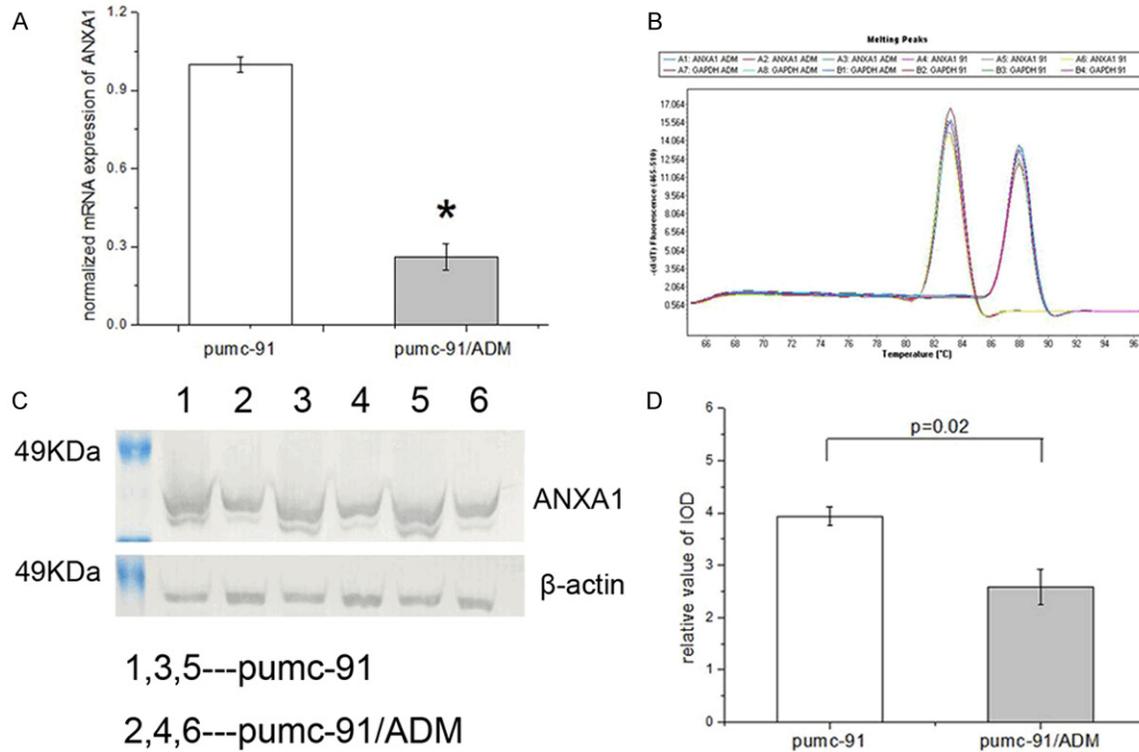
Total RNA was extracted from pumc-91 and pumc-91/ADM cells using TRIzol reagent (Ambion, life technologies, USA) according to manufacturer's instructions. 2  $\mu$ g of total RNA from each sample was obtained to be reverse transcribed and synthesized cDNA. The reaction was performed with oligo d(T) (Dingguo Biotech, China), dNTP (Genview, USA) under the action of M-MLV reverse transcriptase (Promega, USA). qRT-PCR was conducted with Roche Light-Cycler 480 Real Time PCR System with TransStart Top Green qPCR SuperMix (TransGen Biotech, China). Primers of ANXA1, GAPDH were as follows: ANXA1 forward 5'-TGTGAATGAAGACTTGGCTG-3' and reverse 5'-ACTCTGCGAAGTTGTGGATA-3'; GAPDH forward 5'-TTTGGTATCGTGG AAGGACT-3' and reverse 5'-AGTAGAGGCAGGGATGATGT-3'. The programme consisted of a pre-incubation step (5 min at 95°C), 45 cycles of amplification (10 s at 95°C, 10 s at 55°C, 10 s at 72°C). Then melting curve step was performed (5 s at 95°C, 1 min at 65°C, then melting at 0.11°C/s with continuous acquisition mode until 97°C) and cooling at 40°C for 30 s finally. GAPDH was used as endogenous reference. 2<sup>- $\Delta\Delta$ Ct</sup> method was applied for evaluated mRNA expression of the target gene. All of the experiments were repeated three times.

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### Western blot

The cells from pumc-91 and pumc-91/ADM cell lines were cultured to 90% confluence. Cells

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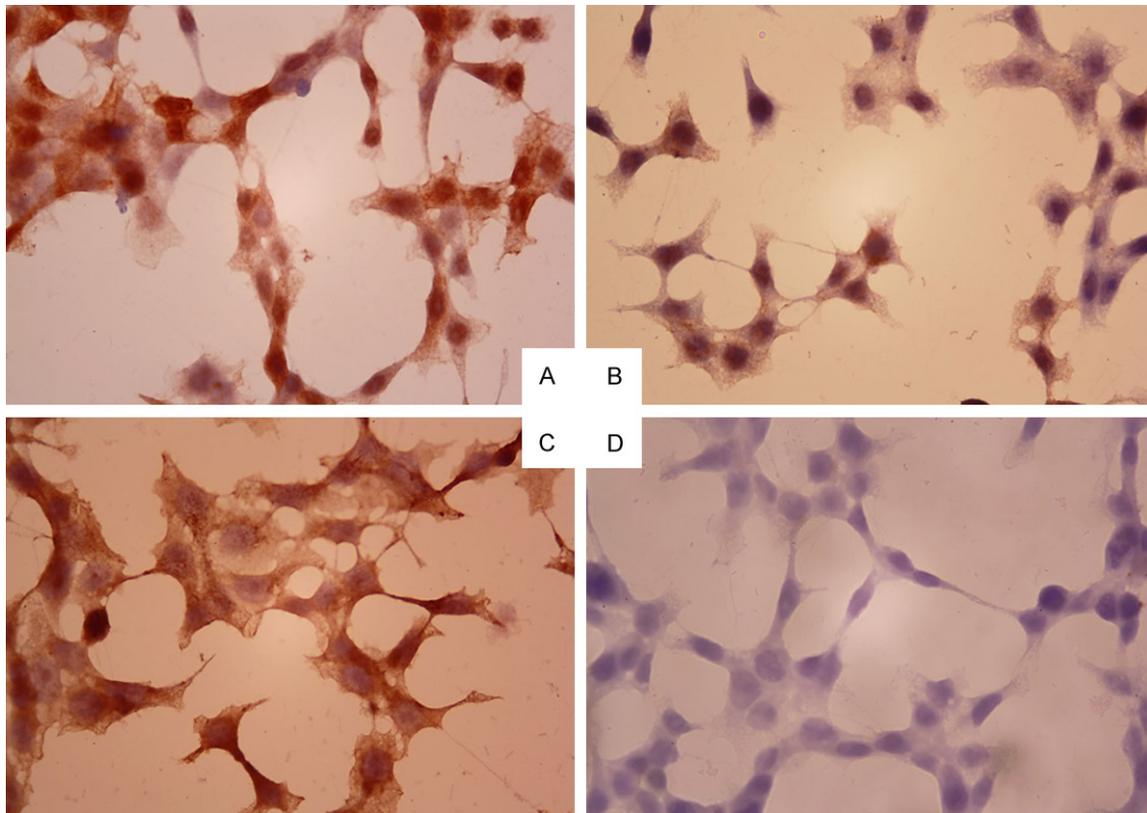
**Figure 2.** mRNA and protein expression of ANXA1 in pumc-91 and pumc-91/ADM cell lines. A, B. ANXA1 mRNA expression and corresponding melting curve in pumc-91 and pumc-91/ADM cell lines assessed by qRT-PCR.  $2^{-\Delta\Delta C_t}$  method was used to measure the relative level of ANXA1 in pumc-91/ADM compared to pumc-91 ( $*P < 0.05$ ). C, D. ANXA1 protein expression determined by Western blot. ANXA1 decreased in pumc-91/ADM significantly ( $*P < 0.05$ ). Each sample has three replicas.

were harvested and washed with cold PBS for three times, then lysed under the action of RIPA buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS). Total 60  $\mu$ g of protein per sample was separated by 12% SDS-PAGE and then was electrotransferred for 1.5 h to a PVDF membrane. Afterwards, the membrane was blocked in solution with 5% nonfat milk in PBST for 2 h at room temperature. The primary antibody annexin a1 (Abcam, USA, 1:800),  $\beta$ -actin (ZhongShan Co., China, 1:400) was diluted and incubated at 4°C over night. The PVDF membrane was washed with PBST three times for 20 min each and incubated together with horseradish-peroxidase-conjugated goat anti-mouse, goat anti-rabbit IgG as secondary antibodies for 1.5 h at room temperature. The PVDF membrane was washed with PBST three times for 15 min each. The bands were detected by enhanced HRP-DAB chromogenic kit. The integrated optical density (IOD) value of every

band was analyzed with Lane 1D gel analysis software (Sage Creation, China). All of the experiments were repeated three times.

### Immunohistochemistry

Two-step method was applied. Tissue sections were fully deparaffinized in xylene for 30 min and rehydrated under the action of graded ethanol. While the cells needed to be fixed with 4% paraformaldehyde. After washed in phosphate-buffered saline (PBS), the sections were blocked with hydrogen peroxide (Dingguo Biotechnology, China) for 15 min to deprive the endogenous peroxidase activity. Then sections were heated in sodium citrate buffer with microwave for antigen retrieval. The specimens were incubated with the anti-annexin a1 antibody (Abcam, USA, diluted 1:250 in PBS) at 37°C for 1 h. After rinsed in PBS, the specimens were incubated with strep-avidin-biotin marked second antibody (DAKO, USA) at 37°C for 30 min



**Figure 3.** Immunocytochemistry analysis of ANXA1 expression in pumc-91 and pumc-91/ADM cell lines ( $\times 100$  magnifications). A. ANXA1 in pumc-91 cells. B. ANXA1 in pumc-91/ADM cells. C.  $\beta$ -actin staining as positive control. D. PBS replaced the primary antibody as negative control. ANXA1 was localized in cytoplasm and nuclei of bladder cancer cells.

and washed in PBS again. DAB kit (DAKO, USA) and hematoxylin were used for dyed (3 min) and counterstained (1 min) respectively. The specimens were washed in running water at once and dehydrated with graded ethanol.

#### *Evaluation of immunohistochemistry*

Scoring method and optical density value assessment method were both used to evaluate the expression of ANXA1 to confirm the accuracy. The details of scoring method were as follows: The sections were assessed by two pathologists who were fully blinded to clinical information. The stained intensity of cells was scored as "0" negative; "1" weak; "2" moderate; "3" strong. The percentage of positive cells was divided into five categories: "0" no staining; "1" 1-10%; "2" 11-50%; "3" 51-80%; "4" 81-100%. The final total score was the multiplication of intensity and percentage. The total score  $< 4$  means low expression of ANXA1, yet total score  $\geq 4$  shows high level of expression. The approach of optical density value assessment was as follows: run the software of Image

Pro Plus 6.0 (IPP 6.0) on the basis of instructions. Measured the Integral Optical Density Sum (IOD sum) and the corresponding area. The index of Mean Optical density (MOD = IOD sum/area) was used to evaluate the expression of ANXA1.

#### *Statistical analysis*

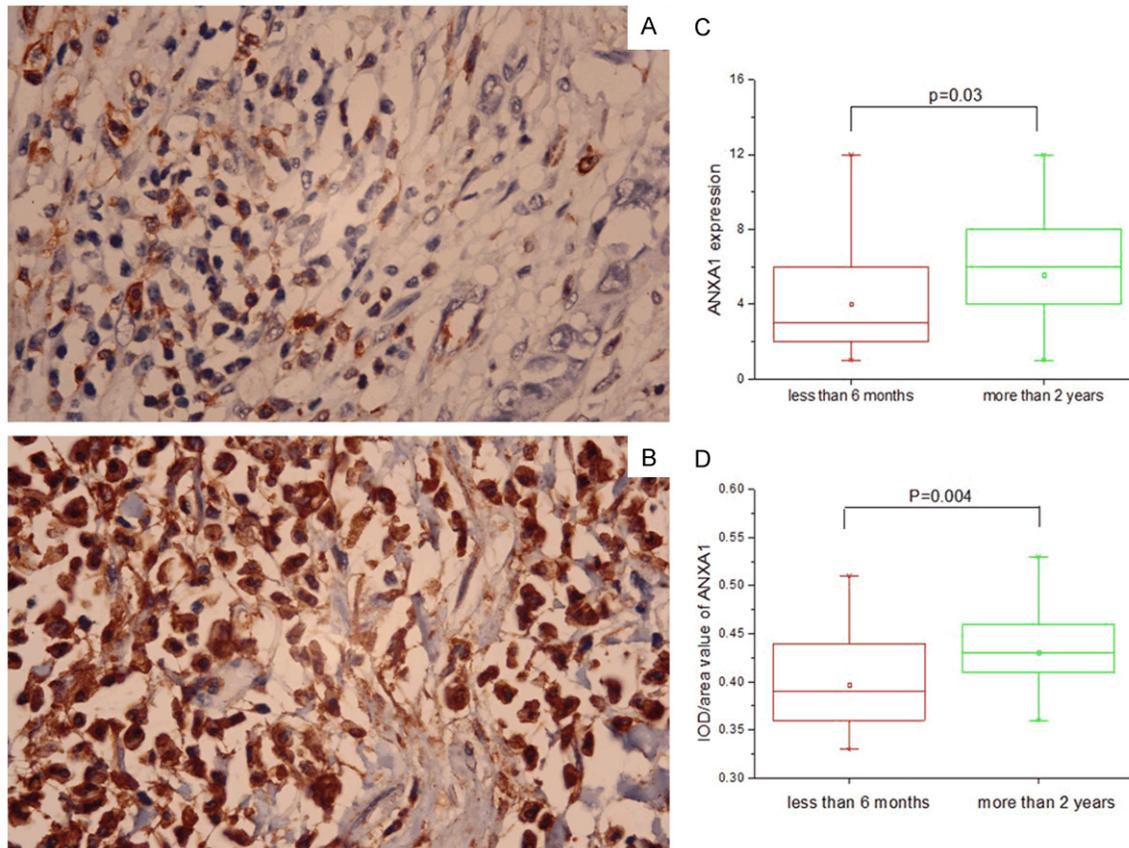
Student's T test (two-tailed) and one-way ANOVA were used to analyze the expression difference of ANXA1 in pumc-91, pumc-91/ADM cell lines and bladder cancer tissues. *P* value less than 0.05 was considered as statistically different. All of the statistical analysis was performed with SPSS 19.0.

#### **Results**

##### *The differential expression and verification of ANXA1 in pumc-91 and pumc-91/ADM cell lines*

In the past study, our laboratory had applied two-dimensional electrophoresis to screen the

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**Figure 4.** Immunohistochemistry of ANXA1 expression in bladder cancer sections of different intervals of relapse ( $\times 100$  magnifications). A. The bladder cancer tissues whose recurrent time  $< 6$  months. B. The bladder cancer tissues whose recurrent time  $> 2$  years. C, D. Analysis of ANXA1 expression of different intervals of relapse in bladder cancer with scoring method and MOD method. ANXA1 was associated with interval of relapse and bladder cancer recurred two years later exhibited higher expression of ANXA1 ( $P < 0.05$ ).

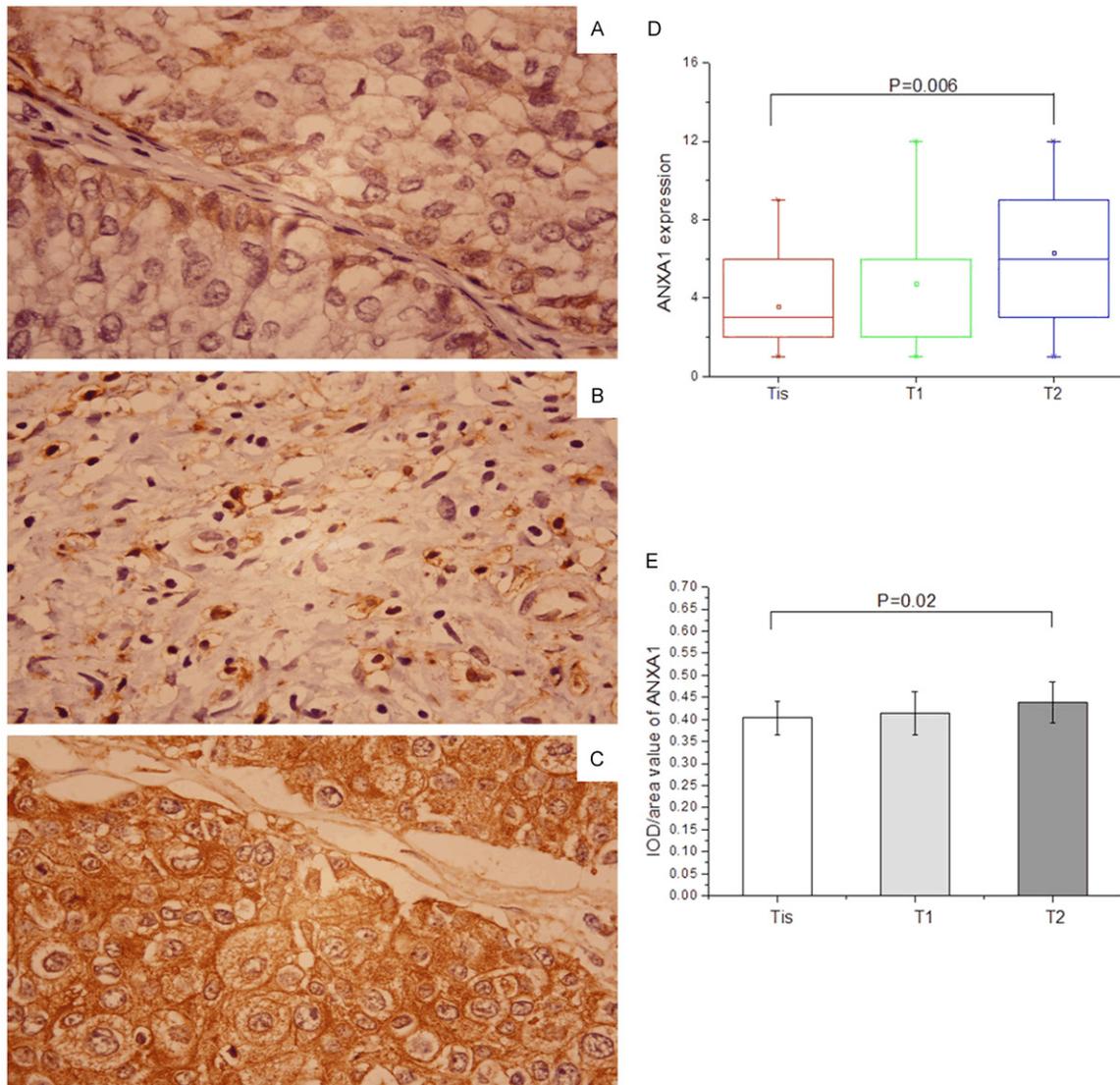
differentially expressed protein between pumc-91 and pumc-91/ADM cell lines. The acquired protein spots were then identified by MALDI-TOF/TOF mass spectrometry [10]. And the ANXA1 was just one of the protein spots with differential expression. The corresponding spots and the optical density value of three times were showed in **Figure 1**. Therefore, in the present study, we utilized the methods of qRT-PCR, Western Blot and immunocytochemistry to further verify and enrich the proteomics results.

The results of qRT-PCR demonstrated that the mRNA level of ANXA1 was obviously downregulated in pumc-91/ADM compared to pumc-91 cell lines (**Figure 2**). There was statistical difference between them ( $P < 0.05$ ).

Western Blot assay was conducted to confirm the protein level of ANXA1 in the two cell lines.

Consistent with the above result, the expression of ANXA1 protein was significantly decreased in pumc-91/ADM compared to pumc-91 cell lines (**Figure 2**). The difference has statistical significance ( $P < 0.05$ ). The bands of ANXA1 appeared as doublets (38-KDa and 34-KDa) in **Figure 2**. It is consistent with the previous studies that performed on the isoform of ANXA1 in urothelial carcinoma [11]. Moreover, the tendency of 34-KDa isoform bands was in accordance with 38-KDa bands.

Immunocytochemistry was adopted to explore the cellular location of ANXA1 and affirm its expression in various cell lines visually.  $\beta$ -actin served as positive control, while negative control was PBS instead of the primary antibody. All of the staining was processed in the same condition. From the results, we could see that the positive sites of ANXA1 were located in cytoplasm and nuclei. Compared to pumc-91/ADM



**Figure 5.** Immunohistochemistry of ANXA1 expression in bladder cancer sections of different invasion depth (T stage) ( $\times 100$  magnifications). A. Tis. B. T1. C. T2. D, E. Analysis of ANXA1 expression of different T stage in bladder cancer with scoring method and MOD method. The difference was tested by one-way ANOVA. The expression of ANXA1 between Tis and T2 showed statistical difference ( $P < 0.05$ ).

cell lines, the expression of ANXA1 in pumc-91 increased distinctly (**Figure 3**).

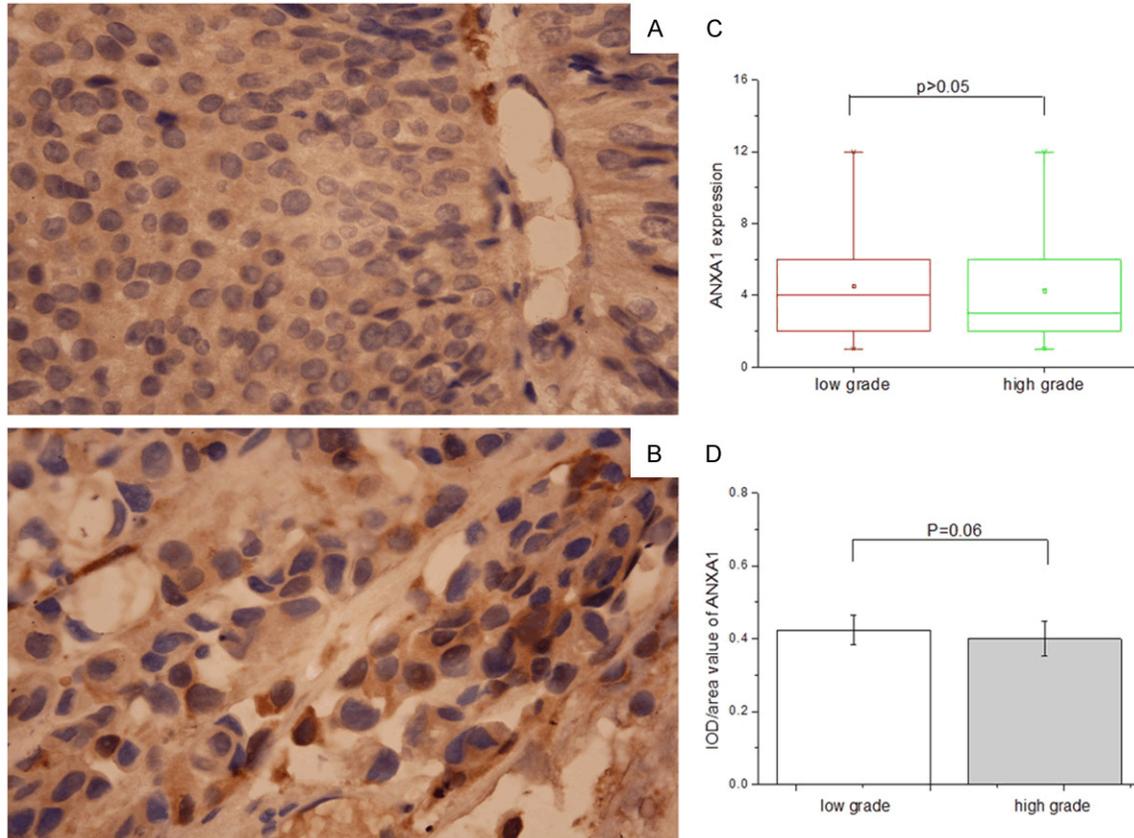
*Correlation between ANXA1 expression and clinicopathologic factors in recurrent tissues of bladder cancer*

In recurrent tissues of bladder cancer, ANXA1 was located in cytoplasm and nuclei. According to various intervals of recurrence, the tissues could be divided into a “less than six months” group and a “more than two years” group. From the **Figure 4**, it was evident that the expression level of ANXA1 was higher in the “more than two years” group in contrast to the “less than six months” group ( $P < 0.05$ ).

According to invasion depth of tumor (T stage), the tissues were classified into Tis, T1 and T2 group. Tis implies carcinoma in situ; T1 means tumor invade subepithelial connective tissue; T2 stands for tumor invade muscularis propria. The results (**Figure 5**) indicate that the expression of ANXA1 is notably increased in the T2 group compare to the Tis group ( $P < 0.05$ ). However, the differences between the Tis and the T1 groups, as well as the T1 and the T2 groups are not so distinct ( $P = 0.43$ ,  $P = 0.26$  respectively).

On the basis of the degree of histological differentiation, the tissues should be categorized into two groups: high-grade group and low-

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**Figure 6.** Immunohistochemistry of ANXA1 expression in bladder cancer sections of different histological differentiation ( $\times 100$  magnifications). A. Low grade. B. High grade. C, D. Analysis of ANXA1 expression in different histological grade of bladder cancer with scoring method and MOD method. There was no significant difference between low and high grade bladder cancer on the expression of ANXA1 ( $P > 0.05$ ).

grade group. But there is no definite difference between the two groups on the expression of ANXA1 (Figure 6,  $P > 0.05$ ).

### Discussion

In the present study we investigated the expression of ANXA1 in bladder cancer drug-resistant cell lines and bladder cancer tissues of different interval of relapse. The results demonstrate that ANXA1 was downregulated in pumc-91/ADM cell line compared to pumc-91. In addition, ANXA1 was also downregulated in bladder cancer tissues whose recurrent time was less than six months compared to the more than two years group. And ANXA1 expression level was associated with escalated T stage. All of these imply that ANXA1 might be involved in the process of tumor progression, drug-resistance and relapse.

ANXA1, as a kind of calcium ion dependent, phospholipid binding, cytomembrane protein,

exerts an effect on multifarious biological activities, for example, inflammation, cell proliferation and apoptosis, cellular signal transduction, cell aggregation, phagocytosis and so on [12]. In recent years, the relationship between ANXA1 and malignant tumor has become one of the research foci of Oncology. More intriguing is the role of ANXA1 in dissimilar types of cancer even opposite. The expression of ANXA1 is upregulated in cholangiocarcinoma, lung cancer, pancreatic cancer, colorectal cancer [13-16]; while ANXA1 is downregulated in esophageal cancer, breast cancer, oral and larynx cancer [17-19]; the expression trend in gastric cancer is still controversial [20]. It implies that ANXA1 may function either as a promoting factor or an inhibiting factor and that it depends on the combined effects of multiple regulatory mechanisms. Zhu F et al. [21] found that ANXA1 was downregulated in an adriamycin-resistant human erythroleukemia cell line (K562/ADR) with the method of MALDI-TOF/TOF and

Western Blot. They considered that the descending level of ANXA1 contributed to the drug resistance of the cell line. Our results were consistent with the above. However, the mechanism of how ANXA1 participates in the process of drug-resistance in bladder cancer is still not very clear. The classical mechanism of tumor associated drug-resistance mainly includes the expression of various resistant genes, proteins and enzymes, as well as the regulation of relevant signal transduction pathways [22]. Sabine Maschler et al. [23] considered that ANXA1 strengthened the invasiveness and migration ability of cancer cells via Jak/Stat3 and Erk1/2 pathways in breast cancer. Knockdown of ANXA1 by RNAi activated Jak/Stat3 and Erk1/2 signals and initiated the phosphorylation of tyrosine residues, translocation of STAT dimer and activation of transcription. The procedure caused the up-regulation of E-cadherin repressors and disrupted the polarity of tumor epithelium. All of these facilitated the epithelial-to-mesenchymal (EMT) and the status of drug-resistance to some extent. Beyond that, there were a number of non-classical mechanisms involved in tumor resistance. Li KN et al. [24] and Marjo de Graauw et al. [25] held the view that endocytosis mediated by ANXA1 with the help of actin was just one of these in chronic myeloid leukemia and breast cancer. ANXA1, which was located on the cell membrane widely, took part in multiple physiological and pathological activities through conjugation and conversion of conformation. The binding of ANXA1 with membranes could be divided into three modalities: monomer binding; two molecules of ANXA1 forming dimer in N-terminal; and two molecules of ANXA1 linking to S100A11 with N-terminal [26]. Actin was one type of cytoskeletal protein. It accelerated the invagination of cytomembranes and the formation of endocytotic vesicles. The previous study indicates that the dynamics of actin are decreased in ANXA1 knockdown cells, which might stiffen the cytomembrane [25]. Obviously, it is a disadvantage to the shape of vesicles and endocytosis. Finally, the knockdown of ANXA1 blocks the intake of chemotherapeutics and leads to drug resistance.

The crucial reason that drug resistance has turned into a hot spot of cancer research is resistance tends to result in progression and relapse of tumors. And tumor recurrence is still a huge challenge to be overcome. Kang WY et

al. [11] studied the expression of ANXA1 in urothelial carcinoma (196 cases) and normal urothelium (24 cases). The result was ANXA1 was overexpressed in urothelial carcinoma, and ANXA1 might be associated with oncogenesis. Li CF et al. [27] examined the expression of the ANXA1 protein in 81 primary localized bladder cancer tissues. They found that ANXA1 levels were related to ascending pT stage and a higher histological grade. ANXA1 could predict disease-specific survival (DSS) and metastasis-free survival (MFS). In our study, we explored for the first time the expression of ANXA1 in relapse tissues of bladder cancer and analyzed the relationship between ANXA1 and recurrence related factors. The results manifested the expression level of ANXA1 in the group with an interval of recurrence at less than six months was decreased compared to the group with an interval of recurrence at more than two years. With the elevated invasion depth (T stage) of relapse tissues, the expression of ANXA1 increased. However, there was no evident connection between ANXA1 and grade differentiation of relapse tissues. The mechanism involved in ANXA1 and relapse of bladder cancer is unknown. Zeng GQ et al. [28] found that the downregulation of ANXA1 was closely related to the radioresistance and relapse of nasopharyngeal carcinoma. They considered ANXA1 to be a tumor suppressor gene and reasoned that it participates in the process of tumor resistance and recurrence by means of p53-mediated response.

In conclusion, ANXA1 was downregulated in the bladder cancer drug-resistant cell line pumc-91/ADM. The expression level of ANXA1 decreased in the shorter relapse interval of bladder cancer tissues. This indicates the role of ANXA1 in the course of tumor resistance and recurrence. More importantly, it brings up valuable guidance for clinicians and patients. The level and variation of ANXA1 may become a significant reference point for the timing selection of follow-up. Therefore, the patients can reduce unnecessary examinations and the resulting suffering. ANXA1 can be considered a biomarker of recurrence, resistance, prediction, and surveillance in bladder cancer. The mechanism implied in it needs further investigation.

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**Disclosure of conflict of interest**

None.

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