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The Expression Pattern of Annexin A1 in Urinary Bladder Urothelial Carcinoma and Its Clinicopathologic Significance

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Background: Annexin A1 (ANXA1) is known to be involved in the progression and differentiation of various tumors. However, its significance and role in bladder carcinogenesis has not been fully elucidated. To determine the role ANXA1 plays in urothelial carcinoma (UC), we investigated the expression of ANXA1 protein in normal urothelial tissue, carcinoma *in situ* (CIS), and UC of the urinary bladder. **Methods:** Protein expression level of ANXA1 and its subcellular localization were analyzed in 88 cases of UCs and corresponding 24 normal tissues and 24 CISs by immunohistochemistry. **Results:** ANXA1 was significantly down-regulated at all subcellular localization in CIS and in the cytoplasm and membrane of cells of UC, compared to normal tissues. No significant correlation between ANXA1 expression level and tumor depth (pT), growth pattern, and recurrence was found. However, cytoplasmic and membranous ANXA1 were significantly up-regulated in high grade than in low grade UC ($p = 0.02$ in cytoplasm and $p = 0.03$ in membrane). **Conclusions:** These results suggest that ANXA1 dysregulation is involved in urothelial carcinogenesis and ANXA1 is potentially a marker for the pathologic differentiation of UC.

Key Words: Annexin A1; Urinary bladder; Urothelial carcinoma

Annexin A1 (ANXA1) is a 37-kDa protein with calcium and phospholipid binding properties. It is a member of the subfamily A of annexin (ANX), and presents in organisms from molds to mammals. ANX shares a conserved COOH-terminal with repeated domains and an N-terminal region, the variability of which confers specific annexin function.[1,2] Its anti-inflammatory property is attributed to ANXA1 binding to surface receptors of granulocytes and macrophages,[3] and its ability to inhibit phospholipase A_2.[4] It plays various roles in the regulation of apoptosis, intracellular signal transduction, differentiation, and proliferation.[5-8]

ANXA1 is also known to be involved in the carcinogenesis of many tumors. The expression of ANXA1 is increased in hairy cell leukemia, pancreatic cancers, and hepatocellular carcinoma.[9-11] However, it is decreased in cancers of esophagus, larynx, prostate and B-cell lymphoma.[12-15] ANX is known to be localized in the cytoplasm and moves to the membrane or the nucleus depending on calcium level and stimulation.[16,17] Recent studies have shown that specific subcellular localization of ANXA1 is associated with epithelial differentiation and may be a prognostic factor in oral squamous cell carcinoma (SCC).[18,19]

Urothelial carcinoma (UC) of the urinary bladder is the seventh most common cancer worldwide,[20] and comprises approximately 90% of all primary tumors of this organ. Development of UC is associated with chemical exposure, cigarette smoking, schistosomal infections, and genetic alterations.[21] UC is known to progress along two divergent pathways: one is characterized by low grade non-invasive papillary tumors preceded by hyperplasia. The other is that of high grade invasive tumors, which progress from carcinoma *in situ* (CIS) or arise *de novo*.[22] While low grade papillary tumors have frequent mutations of *HRAS* and fibroblast growth factor receptor 3 (*FGFR3*) genes activating the receptor tyrosine kinase-RAS pathway, high grade invasive tumors harbor defects in the p53 and/or the retinoblastoma protein (RB) pathways. Recently, ANXA1 emerges as a tumor-related protein in UC, however, the significance of ANXA1 in bladder carcinogenesis is not yet fully understood and study results remain inconsistent.[23,24] Cui *et al.*[23] showed that ANXA1 expression was down-regulated in higher histologic grade of UC, but Li *et al.*[24] reported contradictory outcome with their

study. Moreover, while subcellular localization of ANXA1 has been recognized as an important differentiation or prognostic factor in other cancers, there has been no study on the intracellular distribution of ANXA1 in UC.

The aim of this study was to investigate the immunohistochemical expression pattern of ANXA1 in normal urothelium, CIS, and UC of urinary bladder, and the association between subcellular ANXA1 expression and clinicopathologic features in order to determine if ANXA1 is involved in bladder carcinogenesis.

MATERIALS AND METHODS

Materials

Eighty eight UC patients whose tissue specimens obtained from transurethral resection of bladder tumor (77 cases) or radical cystectomy (11 cases) at the Eulji Medical Center, Eulji University School of Medicine between 2004 and 2008 were enrolled in this study. No patient was preoperatively treated with immunotherapy or chemotherapy. The pathology slides of each tumor were reviewed and 24 cases with normal adjacent bladder tissue were selected. For the evaluation of CIS, 18 cases associated with UC and 6 cases not associated with UC obtained from transurethral resection of bladder tumor were included, making a total of 24 CIS cases. The histologic grade of the tumor was determined according to the World Health Organization-International Society of Urologic Pathology (WHO-ISUP) classification.

Immunohistochemistry

Immunohistochemical stain was performed on tissue sections from formalin-fixed, paraffin embedded tissue blocks. For multiple tumors, sections containing the most invasive areas of each tumor were selected and 4 μm-thick tissue sections obtained us-

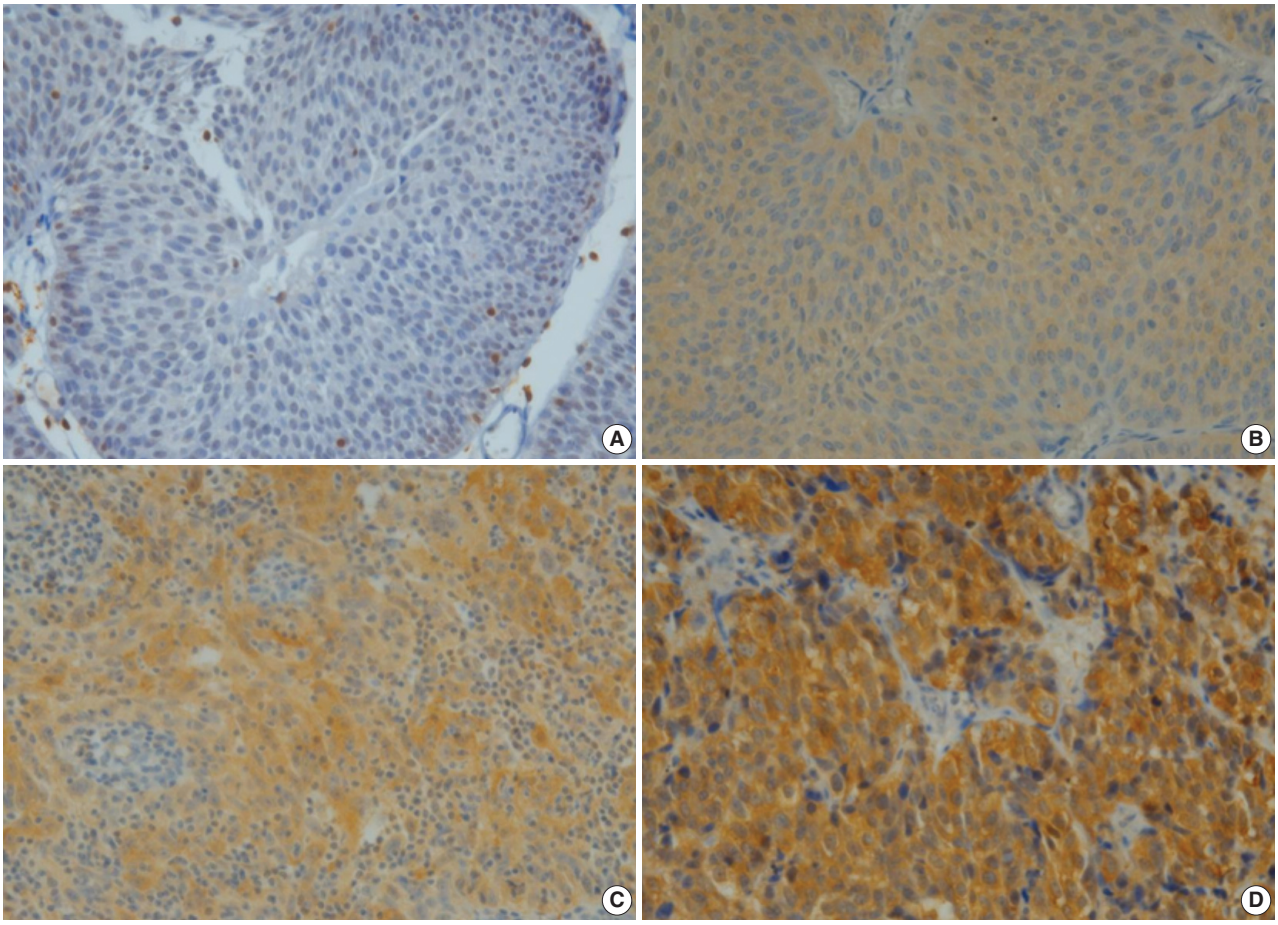

Fig. 1. Immunohistochemical staining intensity of cytoplasmic annexin A1. (A) 0, no staining in low grade papillary tumor. (B) 1+, weak intensity in low grade papillary tumor. (C) 2+, moderate intensity in high grade invasive tumor. (D) 3+, strong intensity in high grade invasive tumor.

ing microtome were transferred onto poly-L-lysine coated slides. After deparaffinization and rehydration, antigen retrieval was performed using 0.01 M citrate buffer (pH 6.0) in a pressure cooker. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 10 minutes, then the sections were incubated with primary rabbit anti-annexin A1 polyclonal antibody (1 : 250, Abcam, Cambridge, UK) and visualized using 3,3´-diaminobenzidine detection kit (Dako, Glostrup, Denmark). Finally, the slides were counterstained with hematoxylin. Normal urinary bladder mucosa was used as positive control, and cases not treated with primary antibody served as negative control for the interpretation of immunohistochemistry.

ANXA1 immunoexpression was evaluated in the nucleus, cytoplasm and membrane. The immunoreactivity of ANXA1 expression was determined semiquantitatively by assessing the percentage of stained cells and staining intensity. The percentage of positive cells was rated as follows: 0 point, no positive cells; 1 point, < 5%; 2 points, 5-50%; 3 points, > 50% positive cells. The staining intensity was rated as follows: 0, no staining; 1+, weak intensity; 2+, moderate intensity; 3+, strong intensity (Fig. 1). The overall ANXA1 expression was scored by adding the percentage of positive cells and staining intensity. Finally, an overall score between 0 and 3, and that between 4 and 6 were respectively considered negative and positive expression.

Statistical analysis

Comparison of subcellular ANXA1 immunoexpression between normal, CIS, and UC was performed using χ^2 test and Fisher's exact test. Correlation between ANXA1 expression and clinicopathologic features was evaluated by χ^2 test. Statistical significance was set at $p < 0.05$.

RESULTS

Expression of ANXA1 in normal urothelial tissue, CIS, and UC

In normal urothelial tissues, ANXA1 was expressed in the cytoplasm and membrane of all 24 cases (100%, each), and in the nuclei of 20 of 24 cases (83%) (Table 1). Immunoreactivity was seen in basal cells to intermediate layers sparing superficial umbrella cells (Fig. 2A). In CIS specimens, ANXA1 expression was markedly down-regulated compared to normal tissues, showing nuclear, cytoplasmic and membranous staining in 12 (50%), 12 (50%), and 11 (46%) of 24 cases, respectively (Fig. 2B). ANXA1 immunoreactive cases in UC were increased compared to those in CIS, although lower than those in normal urothelial tissue. Positive nuclear, cytoplasmic and membranous staining cases were detected in 66 (75%), 55 (62.5%), and 46

Table 1. Intracellular localization of ANXA1 in normal urothelium, CIS, and UC

Localization	ANXA1 expression		
	Normal bladder (n=24)	CIS (n=24)	UC (n=88)
Nucleus	20 (83)	12 (50)	66 (75)
Cytoplasm	24 (100)	12 (50)	55 (62.5)
Membrane	24 (100)	11 (46)	46 (52)

Values are presented as number (%).
ANXA1, annexin A1; CIS, carcinoma *in situ*; UC, urothelial carcinoma.

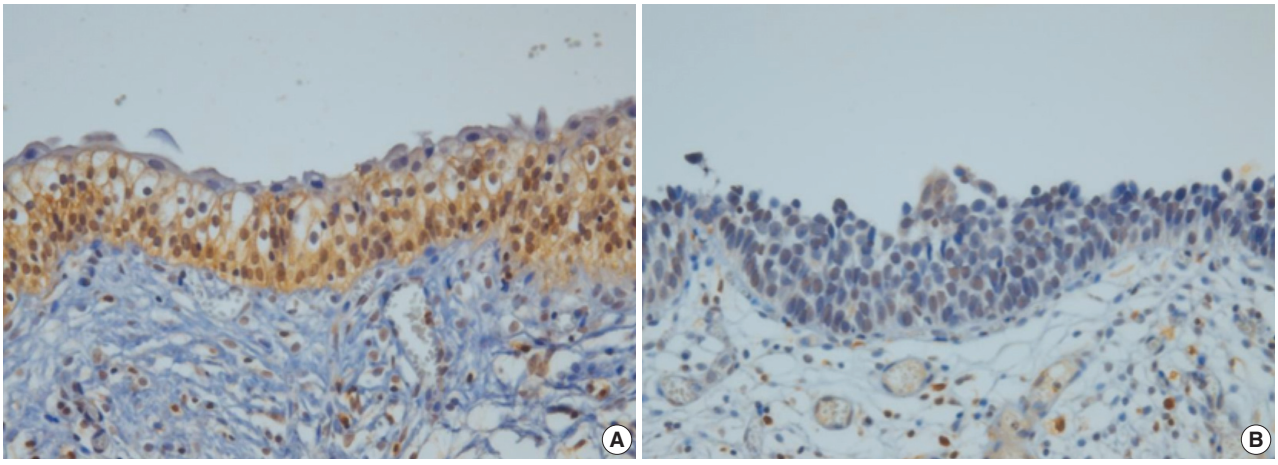

Fig. 2. Immunoreaction of annexin A1 (ANXA1) in normal urothelial tissue and carcinoma *in situ* (CIS). (A) Normal bladder mucosa cells strongly express ANXA1 in the nuclei, cytoplasm and membrane. ANXA1 expression is seen in basal cells to intermediate layers, except the superficial umbrella cells. (B) ANXA1 immunoreactivity is markedly reduced in urothelial CIS in all subcellular localization.

(52%) of 88 UC specimens, respectively. The percentage points and staining intensities of positive cells in UC are listed in Table 2. Regarding cytoplasmic expression, the case numbers of percentage point 1, 2, and 3 were 37, 12, and 6 out of 55 cases, respectively and those of staining intensity 1+, 2+, and 3+ were 29, 21, and 8 out of 58 cases, respectively. On 2×2 comparison using χ^2 test and Fisher's exact test, loss of ANXA1 was significant at all subcellular localization in CIS and in the cytoplasm and membrane of UC cells compared to normal tissues (Table 3).

Association between subcellular ANXA1 expression and clinicopathologic characteristics of UC

The clinicopathologic features of the 88 UC patients and their association with subcellular expression of ANXA1 are presented in Table 4. The median age of UC patients (73 males and 15 females) were 70 years (range, 38 to 97 years). No correlation was found between the level of ANXA1 expression and age, gender, and growth pattern of UC patients. ANXA1 positivity showed increasing tendency as the pathologic T stage advanced, albeit

Table 3. Two by two comparison of nuclear, cytoplasmic and membranous ANXA1 expression among normal urothelium, CIS and UC

Localization	p-value		
	Normal×CIS	CIS×UC	Normal×UC
Nucleus	0.050	0.062	0.693
Cytoplasm	<0.001	0.541	0.001
Membrane	<0.001	0.855	<0.001

ANXA1, annexin A1; CIS, carcinoma *in situ*; UC, urothelial carcinoma.

Table 2. The percentage points and staining intensities of ANXA1 positive cells in UC

Localization	Percentage point (n=88)				Staining intensity (n=88)			
	0	1	2	3	0	1+	2+	3+
Nucleus	22 (25.0)	47 (53.4)	10 (11.4)	9 (10.2)	20 (22.7)	31 (35.2)	30 (34.1)	7 (8.0)
Cytoplasm	33 (37.5)	37 (42.1)	12 (13.6)	6 (6.8)	30 (34.1)	29 (32.9)	21 (23.9)	8 (9.1)
Membrane	42 (47.7)	23 (26.2)	12 (13.6)	11 (12.5)	38 (43.2)	28 (31.8)	13 (14.8)	9 (10.2)

Values are presented as number (%).
ANXA1, annexin A1; UC, urothelial carcinoma.

Table 4. Correlation between subcellular expression of annexin A1 (ANXA1) and clinicopathologic characteristics of UC patients

		ANXA1 expression					
	No. of patients	Nucleus	p-value	Cytoplasm	p-value	Membrane	p-value
Total	88	66 (75.0)		55 (62.5)		46 (52.0)	
Age (yr)							
<70	42	31 (73.8)	1.00	28 (66.7)	0.58	22 (52.4)	1.00
≥70	46	35 (76.1)		27 (58.7)		24 (52.2)	
Gender							
Male	73	55 (75.3)	1.00	45 (61.6)	0.78	37 (50.7)	0.51
Female	15	11 (73.3)		10 (66.7)		9 (60.0)	
Growth pattern							
Papillary	70	54 (77.1)	0.36	43 (61.4)	0.68	35 (50.0)	0.40
Non-papillary	18	12 (66.7)		12 (66.7)		11 (61.1)	
Pathologic stage							
pTa	30	22 (73.3)	0.31	16 (53.3)	0.58	13 (43.3)	0.66
pT1	37	29 (78.4)		24 (64.9)		21 (56.8)	
pT2	13	11 (84.6)		9 (69.2)		7 (62.5)	
pT3	8	4 (50.0)		6 (75.0)		5 (62.5)	
Pathologic grade							
Low	50	35 (70.0)	0.21	26 (52.0)	0.02[a]	21 (42.0)	0.03[a]
High	38	31 (81.6)		29 (76.3)		25 (65.8)	
Total	77[b]	58 (75.3)		47 (61.0)		40 (51.9)	
Recurrence							
No	50	38 (76.0)	1.00	30 (60.0)	1.00	29 (58.0)	0.28
Yes	27	20 (74.1)		17 (63.0)		11 (40.7)	

Values are presented as number (%).
[a]Statistically significant; [b]Number of patients undergone transurethral resection of bladder.
UC, urothelial carcinoma.

without statistical significance. Significant association was seen between cytoplasmic and membranous expression of ANXA1 and tumor grade ($p=0.02$ and $p=0.03$, respectively). The immunoreactivity of ANXA1 was increased in high grade tumors compared to low grade tumors (Fig. 1). High grade tumors showed variable expression patterns of nuclear only, cytoplasmic only, or membranous only among each tumor and even within the same tumor (Fig. 3). Nuclear pleomorphism in high grade tumor was seen more in cytoplasmic and membranous ANXA1 positive tumor cells and ANXA1 was predominantly expressed at the invasive front of the tumors (Fig. 3B, C). Of 88 UC patients, 77 patients underwent transurethral resection of bladder tumor and 11 patients (2 low grade tumors and 9 high grade tumors) underwent radical cystectomy. In the evaluation of the relationship between ANXA1 expression and tumor recurrence, we excluded 11 cases of radical cystectomy, because tumor recurrence could not be evaluated precisely. In the remaining 77 patients, there was no correlation between subcellular ANXA1 expression and recurrence.

DISCUSSION

In this study, we observed significant reduction in ANXA1 immunoexpression in CIS and UC compared to normal urothelium. ANXA1 was expressed in the nuclei, cytoplasm, and membrane of the normal urothelium except for umbrella cells. ANXA1 expression in normal urothelium was previously reported by Dreier *et al.*[25] but its expression in specific cell layer and subcellular localization were not discussed.

To the best of our knowledge, this is the first study that describes the loss of ANXA1 in urothelial CIS. Down-regulation of ANXA1 expression in premalignant lesions was recently reported in oral and laryngeal SCC.[19,26] Alves *et al.*[26] showed ANXA1 was down-regulated in all cellular compartments in dysplastic laryngeal squamous epithelial cells compared to their normal counterparts. Nomura *et al.*[19] demonstrated ANXA1 down-regulation in the plasma membrane of premalignant and malignant lesions of the oral cavity. ANXA1 has been suggested to stimulate apoptosis and activate extracellular receptor kinase signaling cascade[15] that cause the disruption of the cyto-

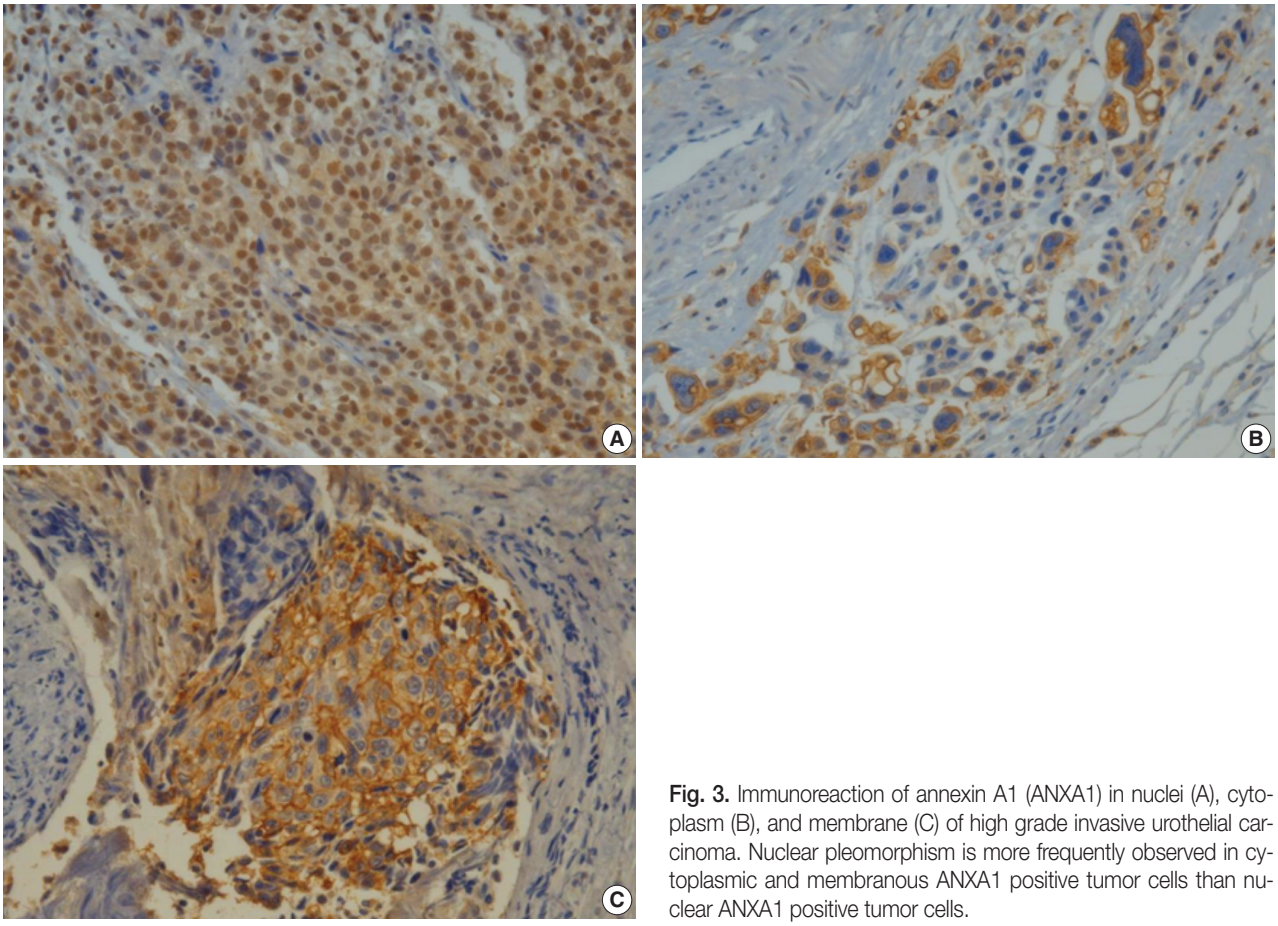

Fig. 3. Immunoreaction of annexin A1 (ANXA1) in nuclei (A), cytoplasm (B), and membrane (C) of high grade invasive urothelial carcinoma. Nuclear pleomorphism is more frequently observed in cytoplasmic and membranous ANXA1 positive tumor cells than nuclear ANXA1 positive tumor cells.

skeleton and the inhibition of cyclin D1.[27] Decreased expression of ANXA1 in premalignant and malignant lesions may be associated with the anti-apoptotic function of ANXA1 that renders normal cells to undergo cancerous progression. This study suggests that significant down-regulation of ANXA1 in CIS at all subcellular localization is an early event in bladder carcinogenesis.

There are two contradictory results on the expression of ANXA1 in UC. Cui *et al.*[23] demonstrated that ANXA1 expression was inversely related to the level of differentiation of UC in all subcellular location, however, their UC sample size was limited to 26 cases and there was no description of ANXA1 expression in CIS. On the contrary, Li *et al.*[24] reported ANXA1 up-regulation in higher pT status and histologic grade of UC, suggesting that ANXA1 might be related to tumor progression. Moreover, these authors showed that ANXA1 overexpression predicted disease-specific and metastasis-free survival in UC patients. They described absence of nuclear staining and ANXA1 reactivity seen only in the cytoplasm of tumor cells. We observed similar result that there was a significant positive correlation between tumor grade and cytoplasmic and membranous expression of ANXA1. Cytoplasmic and/or membranous ANXA1 positive tumor cells showed higher nuclear pleomorphism, suggesting that nuclear morphology is affected by cytoplasmic and/or membranous ANXA1 level. The relationship between ANXA1 expression and tumor recurrence was studied on 77 patients who underwent transurethral resection of bladder tumor, excluding 11 patients who underwent radical cystectomy, and there was no correlation between any subcellular ANXA1 expression and recurrence.

Wang *et al.*[28] studied ANXA1 cytoplasmic expression in esophageal cancer and found correlation between high level of cytoplasmic ANXA1 in esophageal and esophagogastric adenocarcinoma with high pathologic T stage and poor overall survival. They suggested that ANXA1 is a substrate for epidermal growth factor receptor (EGFR), and may exert its effect on esophageal adenocarcinoma by promoting the role of EGFR in ce llular proliferation and differentiation. Alves *et al*.[26] found progressive migration of ANXA1 from the nucleus towards the membrane during laryngeal tumorigenesis, and suggested translocation of ANXA1 is affected by impaired calcium levels and other factors dependent upon phosphorylation in tumor cells. In contrast, Lin *et al.*[18] reported cases of ANXA1 nuclear staining that were significantly increased in oral SCC compared to oral epithelial dysplasia, and suggested nuclear localization of ANXA1 would be a significant predictor of poor overall survival. They showed that oral cancer SAS cells treated with hepatocyte growth factor induced ANXA1 translocation from the cytoplasm to nucleus. These contradictory data suggest that the role of ANXA1 is complicated in the oncogenic process with organ or cell specific manner, and factors other than ANXA1 expression level may affect ANXA1 function. Further studies focused on investigating the association between ANXA1 and other oncogenic markers, such as EGFR, FGFR3, p53 and RB in UC are needed to elucidate its action mechanism.

In conclusion, this study reveals the initial loss of ANXA1 in CIS and increasing ANXA1 level as CIS progresses to UC, suggesting that ANXA1 dysregulation is associated with bladder carcinogenesis. Higher cytoplasmic and membranous ANXA1 expression is correlated with higher tumor grade in UC. As such ANXA1 may be a potential differentiation marker of UC.

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