Overexpression of annexin 1 in the development and differentiation of urothelial carcinoma

Wan-Yi Kang a, Wan-Tzu Chen b, Ya-Chun Huang c,d, Yue-Chiu Su e, Chee-Yin Chai b,d,*

a Department of Pathology, Kuo General Hospital, Tainan, Taiwan
b Department of Pathology, Kaohsiung Medical University Hospital, Kaohsiung Medical University, Kaohsiung, Taiwan
c Graduate Institute of Medicine, College stained with Hematoxylin-eosin of Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan
d Department of Pathology, School of Medicine, College of Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan
e Department of Pathology, Foo-Yin University Hospital, Ping-Tong Hsien, Taiwan

Received 28 December 2010; accepted 18 April 2011
Available online 18 January 2012

KEYWORDS
Annexin 1; Immunohistochemical staining; Tissue microarray; Urothelial carcinoma

Abstract This study investigates the expression of annexin 1 in urothelial carcinoma (UC) and its relation with clinicopathologic factors, and evaluates its potential clinical significance. Annexin 1 expression was analyzed by immunohistochemical staining with manual tissue microarrays and Western blot in UC. Immunohistochemical analysis of UC in tissue microarrays showed that annexin 1 protein was 76.5% (150/196) positive, which was markedly increased compared with that in the normal urothelium 20.8% (5/24) (p < 0.01). In addition, the positive expression rate of annexin 1 was higher in the high-grade UC (81.7%; 143/175) than in the low-grade UC (33.3%; 7/21). Western blot revealed that the expression of annexin 1 was low in low-grade UC, and markedly increased in high-grade UC. In conclusion, annexin 1 overexpression is observed in UC, which suggests it may be associated with tumorigenesis and its expression correlates with the differentiation of UC.

Introduction

Significant improvements in gene and protein expression analysis mean that most researchers have greater opportunities to study and understand events responsible for human cancer development. It is important that useful molecular information from relevant tissue samples and
from efficient analysis of vast genomic and proteomic databases is obtained. With the development of molecular profiling techniques, numerous key gene and protein expressions associated with cancer proliferation and progression are identified, enabling researchers and clinicians to find better ways to treat disease.

Urinary bladder cancer is the most prevalent type of cancer worldwide to occur in the urinary tract and an estimated 290,000 new cases are diagnosed each year in men and 88,000 in women [1]. In Taiwan, cancer of the urinary bladder accounted for approximately 735 deaths in 2010 [2]. Urothelial carcinoma (UC) of renal pelvis and ureter, also known as upper UC, is uncommon throughout most of the world but the incidence is unusually high in Taiwan [3,4]. It has also been reported that approximately 42% of patients with upper UC had associated bladder cancer [5].

Annexin 1, also known as lipocortin-1 or calpactin II, is a 38 kDa protein that is one of the 20 members of the annexin family of calcium and phospholipid-binding proteins. These members share a common core domain harboring the \( \text{Ca}^{2+} \)- and phospholipid-binding sites but each has a unique N-terminal tail that imparts its functional specificity [6]. It is thought that phosphorylation of this region regulates specific biological actions of annexin 1. It has been suggested to have a biological role in inhibiting the activity of phospholipase A2 and as a substrate for epidermal growth factor receptor (EGFR) [6]. In addition, it may be a crucial mediator of apoptosis, intracellular calcium release and membrane trafficking [7,8]. Structurally, annexin 1 is a component of the cornified envelope and may play an important role in keratinization [9]. Other functions of annexin 1 include regulation of hepatocyte growth factor receptor signaling, facilitation of epidermal growth factor degradation, and control of intracellular calcium release [10,11]. Overexpression of annexin 1 was found in some epithelial malignancies including gastric and breast cancer [12]. Downregulation of annexin 1 protein expression is a common finding in high-grade prostatic intraepithelial neoplasm and prostate cancer [13]. Loss of annexin 1 protein expression may be involved in the development of esophageal squamous cell carcinoma [14]. These findings suggested that dysregulation of annexin 1 protein may be associated with the development of human cancer.

In this investigation, we demonstrate annexin 1 protein expression in UC compared with the normal urothelium using immunohistochemical (IHC) staining and Western blot.

Materials and methods

Tissue samples

A total of 196 cases were collected from routinely processed paraffin-embedded material from renal pelvis (77), ureter (56) and urinary bladder (63) including infiltrating UC and noninvasive UC, and each group was divided into a low- and high-grade subgroup. The criteria of low- and high-grade and TNM stage of UC were defined according to the most recent World Health Organization criteria [15].

All tissue materials were selected after histological review from the archives of the Department of Pathology, Kaohsiung Medical University Chung-Ho Memorial Hospital between 1991 and 2000. The median age of these patients at diagnosis was 63.5 years (range, 21–87 years). This study was approved by the Institutional Review Board of Kaohsiung Medical University Hospital (reference number KMUH-IRB-960028). The tissue microarray (TMA) was constructed using formalin-fixed paraffin-embedded UC tissue samples. The original slides were reviewed by two pathologists (W.-Y. Kang and C.-Y. Chai) using the latest World Health Organization classification. Slides containing the representative area of the tumor were circled in color ink. For each case, one core of the tumor (2.0 mm in diameter) was carefully transferred with forceps from the selected areas to the recipient metal paraffin block box. Four-micrometer sections of the TMA block were cut and stained with hematoxylin-eosin to verify that the cores adequately represented diagnostic areas.

In addition, 24 urothelial epithelia were obtained from patients who did not suffer from urothelial tumor including seven from the renal pelvis, 12 from the ureter and five from the bladder.

Fresh tissue samples of five high-grade and five low-grade UCs were obtained after resection during 2001 to 2006. Tumors were sampled and stored at −140 °C and the residual parts of specimens were fixed with 10% formalin for histological diagnosis. Histological diagnosis of these samples was all UC. None had received preoperative radiotherapy or chemotherapy.

TMA construction and IHC analysis

The hematoxylin-eosin slides were examined under a light microscope. The representative area of each donor block was circled in color ink. A biopsy needle was used to acquire cylindrical core tissue biopsies with a diameter of 2 mm from a histologically representative area of each donor block. The cylindrical core tissues were carefully transferred with forceps to a recipient metal paraffin block box. One recipient box could accommodate 56 \((8 \times 7)\) cylinders. One normal tissue core was arranged at one corner of the block for the purpose of internal control and proper orientation of the tissue matrix. After all the cylinders were aligned in the box, the box was covered with a plastic cassette, and then liquid wax was gently poured into the box until it was full. Then the box was cooled to room temperature slowly. Before sectioning, the tissue array paraffin block was chilled to −4 °C and removed from the box. Four-micrometer sections were cut using an ordinary microtome, and the sections were mounted on plain slides for hematoxylin-eosin staining, and then they were placed on silane-coated slides for IHC.

IHC analysis using the streptavidin-biotin method was utilized to detect annexin 1. In brief, sections were deparaffinized and autoclave-treated at 121 °C for 8 minutes in 0.1 M citrate buffer (pH 6.0). Endogenous peroxidase in the section was blocked by incubation in 3% hydrogen peroxide for 5 minutes at room temperature. After washing with Tris buffer solution and incubation with 5% bovine serum albumin for 2 hours, the sections were
incubated with the primary antibodies annexin 1 (BD-610066, 1:1000; BD Transduction Lab) and were applied at room temperature for 50 minutes. Biotinylated second antibody and peroxidase-conjugated streptavidin from the DAKO Universal LSAB kit (DAKO, Denmark) were applied for 20 minutes each. Finally, sections were incubated in 3,3′-diaminobenzidine for 5 minutes, followed by hematoxylin counterstaining and mounting. Negative controls were obtained by replacing the primary antibody with nonimmune serum while known immunostaining-positive slides were used as positive controls.

The level of annexin 1 expression was calculated by combining an estimate of the percentage of immunoreactive cells with the staining intensity (staining intensity score) as previously described [16]. No staining was scored as 0, 1–10% as 1, 11–50% as 2, 51–70% as 3, and 71–100% as 4. Staining intensity was evaluated as follows: 0 = negative (no color), 1 = weak (weak brown), 2 = moderate (brown), and 3 = strong (dense brown). The IHC score was obtained by multiplying the percentage of positive cells and staining intensity. The scores ranged from 0 to 12. An IHC score greater than 3 was considered to be a positive expression. Two pathologists (W.-Y. Kang and C.-Y. Chai) blind to the clinical outcome independently evaluated the immunostaining patterns. If a discrepancy was present, the pathologists reanalyzed the slides together and reached a consensus regarding the final score.

Western blot analysis

Ten micrograms of protein extracts of UC tissues from 10 different patients including five high-grade and five low-grade UCs were prepared for Western blot analysis. All fresh samples were prepared on an extraction buffer containing 50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 10 g/L Triton-100, 1 g/L SDS, 1 mmol/L EDTA, 1 mmol/L AEBSF, and 20 μg/ml leupeptin. After centrifugation at 12000 rpm for 20 minutes at 4°C, the supernatant was collected. Equal amounts of total protein from each sample were loaded and separated by SDS-polyacrylamide gel electrophoresis, and then transferred to polyvinylidenedifluoride membrane. After being blocked with 5% nonfat dried milk in Tris buffer solution (pH 7.4) with Tween-20, the membrane was probed with the anti-annexin 1 mouse monoclonal primary antibody (1:5000 dilution, BD Biosciences Pharmingen, Chicago, IL). Secondary antibody was conjugated with horseradish peroxidase (Cell Signaling Technology, USA). Annexin 1 was visualized by enhanced chemiluminescence (Amersham, Buckinghamshire, England); this antibody could detect specific bands migrating at 38-kDa. Parallel Western blot was probed with the anti-glyceraldehyde-3-phosphate dehydrogenase monoclonal antibody (GAPDH) (Cell Signaling Technology, USA) as an internal control.

Statistical analysis

Statistical analysis was performed using the SPSS 8.0 software program (Chicago, IL, USA). The annexin 1 expression in different groups was analyzed using the Mann–Whitney U test whereas the correlation between annexin 1 and each clinicopathologic factor was assessed with Spearman rank correlation test. A p value of less than 0.05 was considered statistically significant.

Results

Expression of annexin 1 in normal urothelia and UC

Of 24 normal urothelia and 196 UCs, annexin 1 was found positive in five normal (20.8%) and in 150 UCs (76.5%). All 196 carcinomas were divided into two groups, infiltrating urothelial carcinomas and noninvasive carcinomas: 120 (81.6%) cases of infiltrating carcinomas and 30 (61.2%) cases of noninvasive carcinomas showed positive expression of annexin 1. A low positive expression rate of annexin 1 in normal urothelia was found; in contrast, positive expression of annexin 1 in UCs was upregulated significantly (p < 0.01). Expression of annexin 1 also showed no difference between infiltrating UC and noninvasive UC.

Correlation of annexin 1 expression with clinicopathologic factors of UC

Histological differentiation

Table 1 shows that 143 out of 175 high-grade UCs revealed positive expression of annexin 1, whereas 7 out of 21 low-grade UCs had positive results. Fig. 1 demonstrates that positive annexin 1 expression was statistically higher in high-grade UC than in low-grade UC.

T stage, Lymph nodes status and upper versus lower UC

There were no statistically significant correlations between annexin 1 expression and T stage, the depth of tumor invasion or the status of lymph nodes metastases. Also, there was no difference of expression of annexin 1 in upper UC versus lower UC (Table 1).

<table>
<thead>
<tr>
<th>Clinicopathologic factors</th>
<th>n</th>
<th>Positive expression rate of annexin 1(%)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Histological grade</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>175</td>
<td>81.7 (143/175)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Low</td>
<td>21</td>
<td>33.3 (7/21)</td>
<td></td>
</tr>
<tr>
<td><strong>T stage</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ta</td>
<td>49</td>
<td>61.2 (30/49)</td>
<td>0.272</td>
</tr>
<tr>
<td>T1</td>
<td>25</td>
<td>76.0 (19/25)</td>
<td></td>
</tr>
<tr>
<td>T2</td>
<td>40</td>
<td>82.5 (33/40)</td>
<td></td>
</tr>
<tr>
<td>T3</td>
<td>77</td>
<td>84.4 (65/77)</td>
<td></td>
</tr>
<tr>
<td>T4</td>
<td>5</td>
<td>60.0 (3/5)</td>
<td></td>
</tr>
<tr>
<td><strong>Lymph node metastasis</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>7</td>
<td>57.1 (4/7)</td>
<td>0.120</td>
</tr>
<tr>
<td>No</td>
<td>189</td>
<td>77.2 (146/189)</td>
<td></td>
</tr>
<tr>
<td><strong>Location</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Upper urinary tract</td>
<td>133</td>
<td>74.4 (99/133)</td>
<td>0.175</td>
</tr>
<tr>
<td>Lower urinary tract</td>
<td>63</td>
<td>80.9 (51/63)</td>
<td></td>
</tr>
</tbody>
</table>
Western blot analysis of annexin 1 expression

Western blot revealed that the expression of annexin 1 was low in low-grade UC whereas the level of annexin 1 expression markedly increased in high-grade UC. The intensity of annexin 1 expression was measured by densimeter as 453.517 in low-grade UC and 1050.040 in high-grade carcinoma. A high-grade UC also appeared as a doublet (38 and 34-kDa bands) (Fig. 2), and such findings are consistent with a previous study showing annexin 1 isoform expression [17].

Discussion

The expression of annexin 1 was evaluated in tissue samples derived from patients with UC by using the immunohistochemistry with a manual TMA as well as Western blot. We showed that high annexin 1 expression in UC was associated with tumor grade but there was no difference between tumor stage and the presence of lymph node metastasis. Previous studies have shown both upregulation and downregulation of annexin 1 in a variety of malignancies; however, as yet there is no consensus on the mechanism of how annexin 1 influences tumor initiation and/or progression. Annexin 1 may have a tissue-specific effect in tumor carcinogenesis. Upregulated annexin 1 was also found in a drug-resistant stomach cancer cell line, pituitary carcinoma, hairy cell leukemia, hepatocellular carcinoma, and breast cancer [12,18–21].

The annexin family is composed of a group of structurally related calcium-binding proteins. All these proteins have a conserved domain (so-called annexin domain) in common that binds to phospholipids. Every annexin has a unique amino terminal domain that determines specificity and functional diversity [22]. Many different ligands were found to be bound to the annexin family and this associates them with numerous mechanisms by which annexin 1 potentially can be linked to malignancy. A tissue-specific

![Figure 1. Immunohistochemical staining demonstrating negative expression of annexin 1 in low-grade urothelial carcinoma (A, B) whereas positive expression was observed in high-grade urothelial carcinoma (C, D). Original magnification: A, C: 40×; B, D: 200×.](image)

![Figure 2. (A) Annexin 1 expression is high in high-grade urothelial carcinoma (lanes 1, 2) and low in low-grade urothelial carcinoma (lanes 3, 4). Lane 2 also shows 38 and 34 kDa isoforms; (B) densitometric analysis for annexin 1 in high-grade and low-grade urothelial carcinoma.](image)
A manner of annexin 1 was found in human tissue. Very low or absent expression of annexin 1 was found in brain, muscle, and liver tissue; moderate presence was noted in submaxillary glands, prostate, thymus, and spleen; and the highest expression were in lung and placenta \[23\]. Overexpression and phosphorylation of annexin 1 were found both during liver degeneration and transformation in antithrombin III SV40 T large antigen transgenic mice, and in the transcriptional and translational levels in tumors and non-tumorous areas of hepatocellular carcinoma \[21,24\].

Upregulation of annexin 1 was found to be associated with increased synthesis of epidermal growth factor and consequently with increased phosphorylation of EGF receptor. Annexin 1 may also modulate the extracellular signal-regulated kinase at an upstream site possibly by the key signal components including the adaptor protein Grb2 \[25\]. Overexpression of annexin 1 might result in constitutive activation of ERK1/2 kinase in macrophage \[26\]. The level of annexin 1 expression was found to increase by 3 to 4 times when quiescent human diploid foreskin fibroblast cells were stimulated to proliferate \[27\]. Such findings imply that annexin 1 may participate in mitogenic signal transduction and regulate cell growth, and they indicate that annexin 1 may participate in cellular proliferation directly or indirectly.

In bladder UC, some studies showed that the expression of EGFR increased with T stage and tumor grade. These studies found a marked upregulation in UC *in situ* rather than in superficial noninvasive carcinoma but there was no difference between UC *in situ* and infiltrating carcinoma, nor was there such a difference when comparing primary tumors and corresponding metastasis \[28,29\]. Similar findings were found in our study. Because annexin 1 is a substrate protein of EGFR, we might make the hypothesis that the activated EGFR pathway promotes the annexin 1 upregulation and it is consequently associated with urothelial cell malignancy transformation. When analyzing the relation between annexin 1 and clinicopathological factors, we found that high-grade types of UC were correlated with urothelial cell malignancy transformation. In addition, we also noticed that annexin 1 positivity varied in the tumor stroma of both low- and high-grade UCs. However, the significance of this positivity is still unclear and requires further investigation.

In Taiwan, the incidence of upper UC is unusually high \[3\]. Unlike bladder UC, few studies have discussed the molecular indicators, such as the p53 mutation, EGFR or erbB2, and MIB-1 \[30–33\]. Due to limited case numbers, the conclusions of such studies were controversial. Annexin 1 expression was analyzed by comparing 133 upper UCs and 66 bladder UCs in our study and there was no statistical difference between them. These results suggest that the role of annexin 1 in carcinogenesis is similar in all urinary tract diseases.

The present study shows that overexpression is frequent in UC, and it may be one of the factors associated with malignant transformation and histological differentiation. However, the molecular mechanism needs to be investigated further.

### Acknowledgments

This work was supported by a research grant from the National Sun Yat-Sen University-Kaohsiung Medical University Joint Research Center, Kaohsiung, Taiwan.

### References


