

Prognostic Effects and Regulation of Activin A, Maspin, and the Androgen Receptor in Upper Urinary Tract Urothelial Carcinoma

KUN-MING RAU^{1,2}, YI-JU CHEN³, MING-TSE SUN⁴ and HONG-YO KANG^{1,5}

¹Graduate Institute of Clinical Medical Sciences, College of Medicine, Chang Gung University, Taiwan, R.O.C.;

²Division of Hematology and Oncology, Department of Internal Medicine,

⁴Department of Pathology, and ⁵Center for Menopause and Reproductive Research, Chang Gung Memorial Hospital-Kaohsiung Medical Center,

Chang Gung University College of Medicine, Kaohsiung, Taiwan, R.O.C.;

³Department of Pathology, I-Da Hospital, Kaohsiung, Taiwan, R.O.C.

Abstract. *Background:* Molecular mechanisms responsible for carcinogenesis in upper urinary tract urothelial carcinoma (UUTUC) are not yet clear. This study aimed to examine and correlate the subcellular localization of activin A, maspin, and the androgen receptor (AR) with demographic characteristics, pathological grade, and stage of UUTUC in a Taiwanese population, and to investigate the regulatory mechanisms for activin A, maspin, and AR. *Material and Methods:* Sections of stage I and II of UUTUCs from 93 patients were examined, with immunohistochemical detection of activin A, maspin, and AR. Patients were divided into four groups according to stage, grade, and disease-free interval (DFI). Pathologic characteristics and the subcellular localization of these markers were correlated with DFI. The urothelial carcinoma cell line HT1197 was stimulated with activin A at different time-points, and the mRNA expression of maspin before and after activin A stimulation was analyzed by reverse transcription-polymerase chain reaction (RT-PCR). *Results:* Expression of AR was observed to be stronger in stage II than in stage I of UUTUC. Expression of cytosolic activin A correlated with longer DFI for early-stage UUTUC (=0.048). Cellular and molecular localization examination revealed that a high level of activin A in the cytosol positively

correlated with a high level of maspin in the cytosol (=0.038), and with increased AR expression in the cytosol (=0.044). By RT-PCR, mRNA expression of maspin was significantly induced after administering activin A to HT1197 cancer cells. *Conclusion:* Activin A can induce maspin expression in urothelial carcinoma cells. The expression level and localization of activin A, maspin and AR may be exploited and used as predictive markers for UUTUCs.

Upper urinary tract urothelial carcinomas (UUTUCs) are relatively rare tumors in Western countries, especially carcinomas of the renal pelvis, which account for only 5% of all kidney tumors, and occur more frequently in men than in women (2:1 to 3:1) (1). The major histological type of UUTUC is transitional cell carcinoma (TCC), which accounts for 90% of the tumors of the renal pelvis and ureter. Although UUTUCs represent only a small fraction of urinary tract neoplasms, evidence suggests that the frequency of UUTUC is increasing (2). Risk factors for developing UUTUCs include smoking, the use of analgesics, chronic inflammation, exposure to toxins or heavy metals, and previous history of bladder cancer or other urinary tract cancer (3). In Taiwan, cancer of the renal pelvis and ureter accounts for 30.48% of all urinary tract malignancies, with greater incidence in women than in men; however, the incidence of each of these malignancies is quite different in Western countries. The incidence of UUTUCs in the endemic area for 'blackfoot disease' in southern Taiwan was noted to be significantly higher than anywhere else in the world, which is believed to be related to arsenic exposure (4). Unlike other types of cancer, the formation of UUTUC is a multifocal process. The probability of multifocal occurrence is greater in patients with larger lesions and in those with carcinoma *in situ*. Close follow-up of patients with UUTUCs is necessary because they

Correspondence to: Hong-Yo Kang, Graduate Institute of Clinical Medical Sciences and Center for Menopause and Reproductive Research, Chang Gung Memorial Hospital-Kaohsiung Medical Center, Chang Gung University College of Medicine, Kaohsiung, Taiwan, R.O.C. Tel: +886 77317123 ext 8898, Fax: +886 77336970, e-mail: hkang3@mail.cgu.edu.tw

Key Words: Maspin, activin A, androgen receptor, upper urinary tract urothelial carcinoma, prognosis.

have a 30% to 50% chance of developing concomitant bladder tumors and 2% to 4% of patients with a UUTUC develop bilateral tumors of the renal pelvis (5).

UUTUCs are divided by the TNM classification into four stages. Radical nephroureterectomy with excision of an ipsilateral bladder cuff is the gold-standard therapy (6). Pathological stage and grade determine the clinical outcome, the 5-year actuarial survival rates were >90% for pathologic stage pTa, pTis, and pT1 lesions, but was below 50% for pT3 lesions, and under 5% for pT4 tumors (1). Chemotherapy with platinum-based regimen is the standard for advanced urothelial carcinoma (7).

Mechanisms responsible for carcinogenesis in UUTUCs are not yet clear. While several molecules have been reported to be related to the prognosis of urothelial carcinoma, including androgen receptor (AR), vascular endothelial growth factor (VEGF), p53, matrix metalloprotease, survivin, and maspin (mammary serine protease inhibitor) (8), there is no definite predictive marker for the evaluation of recurrence risk of UUTUC.

Maspin was identified by subtractive hybridization analysis of normal mammary tissue and breast cancer cell lines (9). It was first demonstrated to be a tumor suppressor in breast cancer (10), and later in squamous cell carcinomas (SCC) (11). In other reports, maspin functioned as an oncogene, the overexpression of which was associated with a worse prognosis (12, 13).

Maspin is predominantly cytoplasmic with some membrane association, partially secreted and nuclear localization (14). The partitioning of maspin into different subcellular locations is indicative of its different functions and thus distinct binding partners. Both intercellular and extracellular maspin have several distinct functions including promoting cell adhesion and apoptosis and inhibiting cell motility, invasion, and angiogenesis.

In breast cancer, nuclear staining of maspin is significantly associated with good prognostic factors, while cytoplasmic staining is associated with markers of poor prognosis (15). Nuclear expression of maspin was also associated with a lower recurrence rate and a longer disease-free interval (DFI) after surgery for SCC of the larynx (16). The correlation between prognosis and the subcellular localization of maspin has not been discussed previously for urinary tract cancer.

Activin A is known to be a member of the transforming growth factor- β (TGF- β) superfamily (17). It can act as a tumor suppressor, inhibiting telomerase activity and in turn, reducing cancer cell proliferation (18). On the other hand, activin A can also act as an oncogenic factor, promoting prostate cancer cell migration through the activation of the AR (19). Activin A also has been reported to correlate with tumor burden (20). Whether the subcellular localization of activin A correlates to the prognosis of UUTUCs remains to be further investigated.

Table I. *The clinical and pathological features of patients and specimens of this study.*

	No of patients	%
Gender		
Male	38	40.9
Female	55	59.1
Stage		
I	47	50.5
II	46	49.5
Primary site		
Renal pelvis	25	28.1
Ureter	64	71.9
Grade		
High	55	59.2
Low	38	40.8
Surgery for primary tumor		
Radical nephroureterectomy	60	64.5
Simple nephrectomy	12	12.9
Ureterectomy	21	22.6
Disease-free interval		
≤ 2 years	39	41.9
> 2 years	54	58.1
Recurrence site		
Bladder	31	67.4
Ureter	5	10.9
Renal pelvis	5	10.9
Distal	5	10.9

Men have a substantially higher risk of bladder cancer than women, and it has been suggested that the AR is a potential mediator of this sex-specific difference (21). The AR, a member of the nuclear receptor superfamily, is a ligand-dependent transcriptional factor that mediates the biologic effects of androgens (22, 23). Expression of the AR has been detected in normal bladder epithelium (24) and in bladder carcinomas from both male and female patients (25). In addition, AR signaling has been demonstrated to promote the development and progression of bladder tumors in mice (21); however, little is known about AR expression and function in the urothelial carcinoma.

In our current study, clinical data and tissue were collected from patients with UUTUCs. Patients were divided into different groups by TNM stage, grade, and DFI. In addition, histological grade was reviewed. Our goal was to investigate the relationship between subcellular localization of activin A, maspin and AR, and tumor grade and recurrence risk. A better understanding of the significance of subcellular location and regulation of these molecules may help clinicians to predict the prognosis more accurately, and accordingly, may help investigators to design more appropriate interventions for UUTUC.

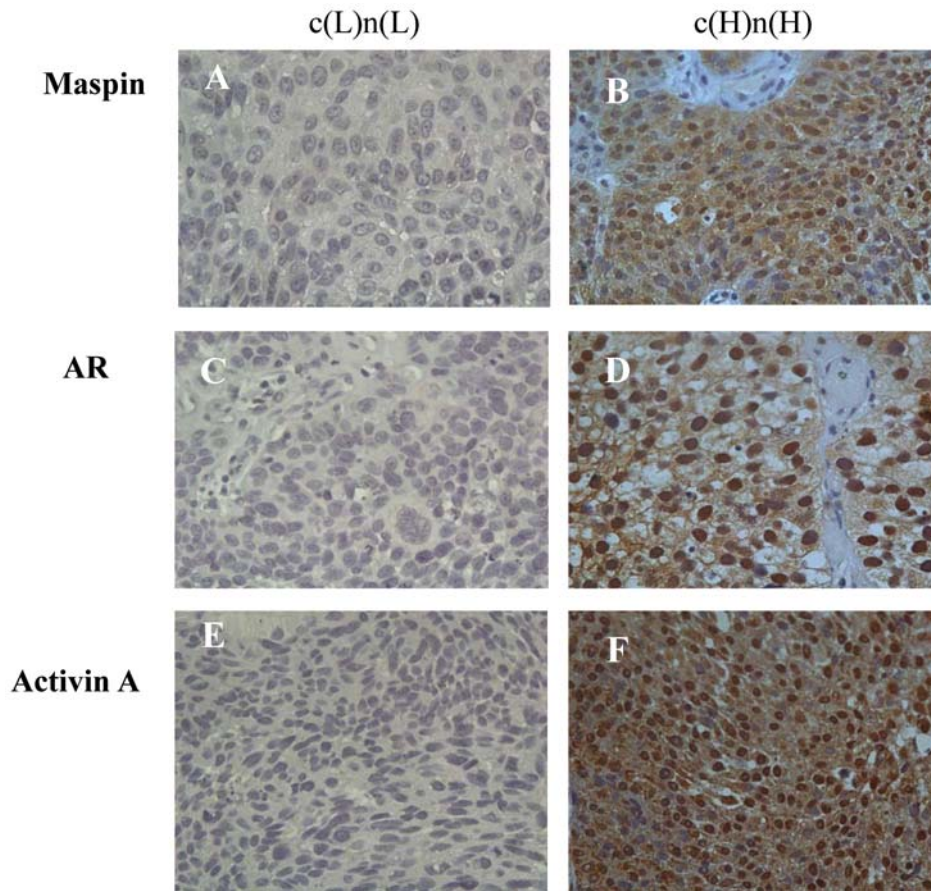


Figure 1. Scoring and illustrations of expression of maspin (A, B), AR (C, D), and activin A (E, F) in the nucleus (n) and cytosol (c). When fewer than 10% of cells were stained either in the nucleus or cytosol, staining was scored as low (L); when more than 10% of cells were stained, staining was scored as high (H) ($\times 400$).

Materials and Methods

Materials. Under the permission from the institutional Review Boards, we collected clinical data and pathologic specimens from patients with UUTUCs, including tumors of the renal pelvis and ureter, diagnosed from 1999 to 2003.

Immunohistochemical staining. Paraffin-embedded specimens from these patients were collected. Two-micrometer serial sections were cut from each paraffin-embedded specimen on adhesive-coated glass slides and dried overnight at 37°C. The slides were deparaffined in xylene and rehydrated through graded alcohols to water. For antigen retrieval, slides were heated in 10 mM citrate buffer (pH 6.0) for 14 minutes. The slides were subsequently washed using TBS buffer with 0.1% Tween 20 for this and subsequent washes. Endogenous peroxidase activity was quenched by 3% H₂O₂ treatment. After washing, the slides were incubated overnight at 4°C with primary antibodies targeting maspin (H-130; Santa Cruz, CA, USA), AR (06-680; Millipore, Temecula, CA, USA), activin A (A1594; SIGMA, St. Louis, MO, USA). Primary antibodies were then detected using the Dako EnVision™ Detection Systems (K5007;

DAKO, Glostrup, Denmark). UUTUC slides were counterstained with hematoxylin and then coverslipped by using mounting medium, Entellan® new (Merck, Darmstadt, Germany). Incubation without the primary antibody was used as a negative control. The subcellular localization and intensity of these markers were determined by two independent pathologists and were classified into low and high at different subcellular localizations. A score of low was given if fewer than 10% of the cells were stained either in the nucleus or cytosol; if more than 10% of cells were stained, this was scored as high. The intensity of protein expression was tested for correlation to the tumor stage, grade and prognosis of the disease. Fisher's exact test was used to test the hypothesis of independence between categorical variables.

Cell lines and culture conditions. Urothelial carcinoma cell line HT1197 was provided by Bioresource Collection and Research Center (BCRC), Taiwan. Cells were maintained in minimal essential growth medium (Eagle's; Sigma, St. Louis, MO, USA) with 10% fetal bovine serum (FBS; Invitrogen, NY, USA). To investigate the effects of activin A, 1×10^6 cells were incubated overnight and pretreated with 1% charcoal-dextran stripped fetal

bovine serum (CD-FBS; Invitrogen, NY, USA) medium for 3 h. After starvation, cells were stimulated with 0, 50 or 100 ng/ml activin A (338-AC; R&D, Minneapolis, MN, USA) in 1% CD-FBS medium for 24, 48, and 72 h for real-time RT-PCR analysis of maspin RNA expression.

Reverse transcription-polymerase chain reaction (RT-PCR). For RNA isolation and real-time RT-PCR analysis, total RNA was isolated using TRIzol reagent (Life Technologies, Frederick, MD, USA) and was reversely transcribed. Real-time RT-PCR was performed by using a Syber green PCR master mix kit (PE Applied Biosystems, Foster City, CA, USA). Sequence analysis was performed using an ABI Prism 7500 sequence detection system (PE Applied Biosystems). Primer sequences used for RT-PCR were as followed: maspin: forward, GAAGAGACCGTATGCAAAGGAATT and reverse, TAGGCAGCATTAACCACAAGGA; 18S: forward, GTAACCCGTTGAACCCCAT and reverse, CCATCCAATCGG TAGTAGCG. Results were reported as maspin expression relative to that of 18S.

Statistics. DFI was defined as the time interval between the curative surgery and the first evidence of disease recurrence, whether local recurrence or distant metastasis. Statistics software (version 10.0; SPSS, USA) was used for all calculations. The χ^2 test and Spearman rank-order correlation were used to test the correlation between immunohistochemical findings and conventional clinical features such as pathologic stage, histological grade and DFI. *P*-value <0.05 was considered to reflect significant differences between groups.

Results

In total, 93 specimens from the 93 patients were included in this study. Primary sites included renal pelvis (28%) and ureter (72%); most of the patients (64.5%) received radical nephroureterectomy; 41.9% of patients had DFI <2 years and 58.1% had DFI \geq 2 years. The most frequent site of recurrence was the bladder (67.4%) (Table I). Histological grade of the tumors were determined on hematoxylin-eosin (HE) stained slides, 55 (59.2%) were high grade and 38(40.8%) were low grade.

Activin A, maspin and AR were detected by immunohistochemical methods. The subcellular localization of these markers was identified, and intensity was classified as low or high at different subcellular localizations. Distribution of maspin, AR and activin A was seen in the cytosol and the nucleus (Figure 1). Importantly, the relationship between the subcellular localization of activin A, maspin, and AR, with tumor grade and recurrence risk were analyzed. We found that cytosolic activin A expression significantly correlated to better DFI ($p=0.048$) greater expression of AR in the nucleus was detected in stage II than stage I disease ($p=0.001$); and the expression of maspin in the cytosol correlated to lower grade cancer ($p<0.001$). Tumors which had high cytosolic activin A expression also showed a trend for lower grade ($p=0.061$) and early stage ($p=0.072$, Figure 2).

The correlation of subcellular localization and expression individually of activin A, maspin, and AR were also analyzed. AR is a nuclear hormone receptor that acts as a transcriptional factor to regulate the expression of a variety of genes. This regulation includes suppression of maspin expression (26). We found when nuclear AR expression was high, the cytosolic expression of maspin was low ($p=0.044$); when cytosolic expression of AR was high, the frequency of tumors with high maspin expression was greater than in tumors with low cytosolic AR expression ($p=0.039$) (Figure 3). While our previous study revealed that activin A expression was highly correlated with AR expression in metastatic prostate cancer (19), the correlation between AR and activin A expression was not significant in this study of UUTUC (data not shown). Unexpectedly, we discovered that high cytosolic but not nuclear activin A expression significantly correlated with high expression of maspin in the cytosol (Figure 4, $p=0.038$).

In order to examine whether activin A can regulate maspin expression, we treated urothelial carcinoma cell line HT-1197 with two different concentrations of activin A. mRNA of maspin was collected at 24, 48 and 72 hours separately after the start of treatment. RT-PCR of maspin mRNA was performed. We found the expression of mRNA of maspin increased significantly after 24 hours of activin A treatment. This increase in activity was not observed for examination at either 48 or 72 hours after treatment (Figure 5).

Discussion

Urothelial cell carcinoma accounts for more than 90% of all carcinomas derived from the renal pelvis, ureter, and bladder. Although urothelial carcinomas from different sites are histopathologically similar, these tumors, including carcinomas of the renal pelvis and ureter, have different incidence in different parts of the world. In Western countries, bladder tumors account for about 95% of all urothelial tumors, and UUTUCs constitute the remaining 5%. Moreover, 80% of all UUTUCs arise in the renal pelvis, and 20% are ureteral tumors (27). Although bladder cancer is still the major cancer of the urinary tract in Taiwan, the ratio of bladder cancer to UUTUC is only 2:1, and the incidence of UUTUCs is higher in women than in men (28). Since the incidence of UUTUCs in the endemic area for 'blackfoot disease' in southern Taiwan, whether the incidence and sex ratio differing from anywhere else in the world is related to arsenic exposure remains to be further investigated.

UUTUC and bladder cancer share a common histology and similar risk factors. These kinds of tumors are usually multi-focal, tumors can occur metachronously or synchronously, as a single lesion or as multiple lesions, especially when the first tumor is a UUTUC. It is important to learn which group of patients has a high risk of recurrence

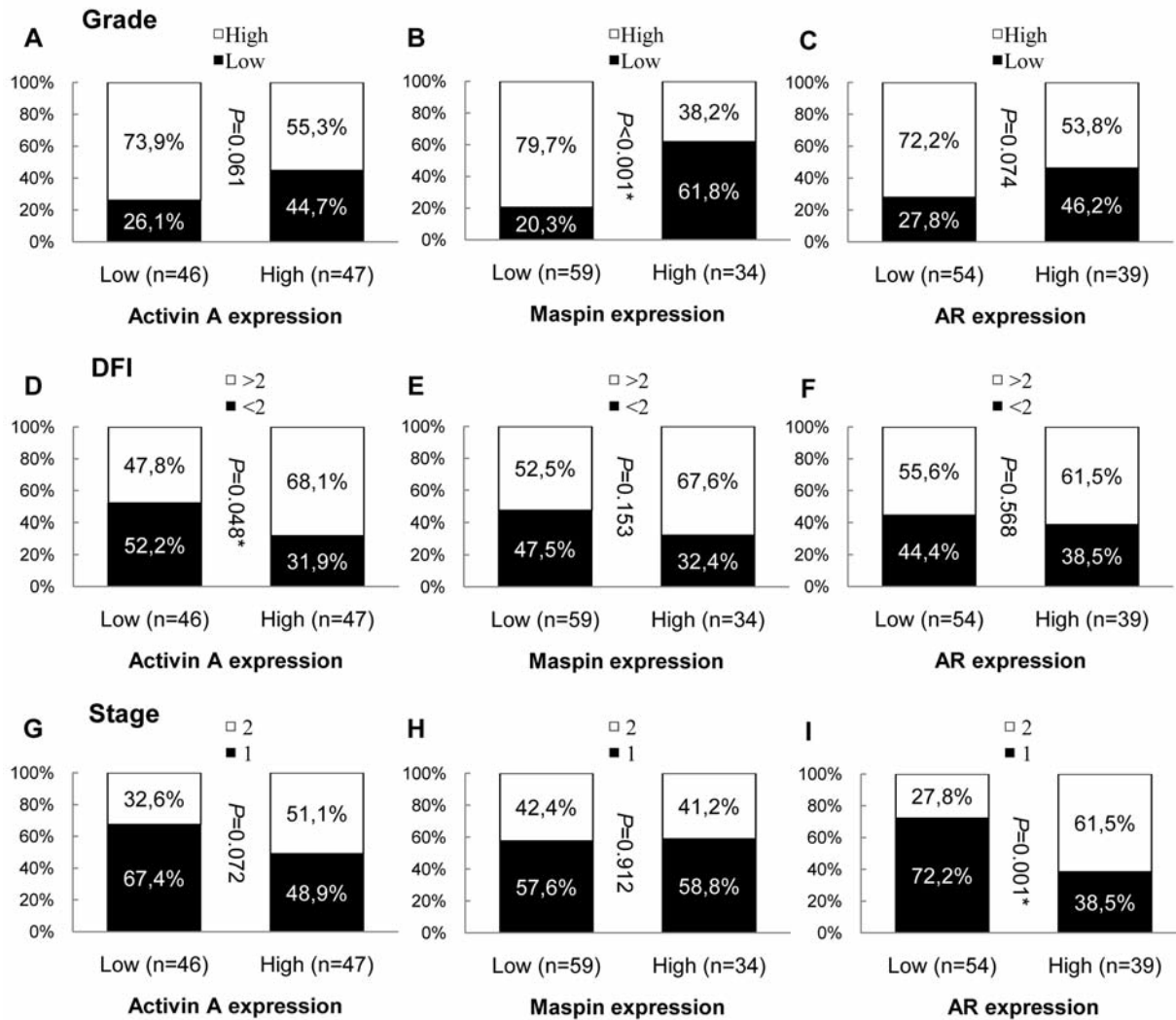


Figure 2. Correlation between grade, disease-free interval (DFI), stage, and cytosolic expression of maspin, activin A and AR. High cytosolic maspin expression correlated significantly with lower tumor grade ($p < 0.0001$, A-C); high activin A expression correlated significantly with better DFI ($p = 0.048$, D-F); and high AR expression correlated significantly with higher stage at diagnosis ($p = 0.001$, G-I). * $p < 0.05$.

of cancer in order to arrange close follow-up and close surveillance for patients of this group. Maspin is a tumor suppressor that may be a useful marker for the prognosis of several types of cancer, including invasive bladder cancer (8), yet the prognostic value differs between cancer types (29, 30). In breast cancer, prostate cancer, and head and neck cancer, maspin was reported to be a good prognostic factor (31). In contrast, in ovarian cancer (12), and non-small cell lung cancer (13) maspin overexpression was reported to be an indicator of poor prognosis. It is now established that maspin is epigenetically regulated with its tissue-specific expression closely associated with DNA methylation (32). Epigenetic changes of maspin expression occur in the 5' regulatory region of the maspin gene, and involve cytosine

methylation, histone de-acetylation, and chromatin accessibility. Tumor suppressor gene *p53* regulates maspin expression through cytosine methylation of the maspin gene promoter (33, 34). This suggests that the role of regulatory factors on maspin expression is quite important in the prognosis of cancer patients.

In our current study, we found that cytosol-predominant localization of activin A related to longer DFI for UUTUCs. We also found a statistical trend for cells of lower grade UUTUCs to have more AR in the cytosol than did those of higher grade UUTUCs. In addition, we found that high activin A expression in the cytosol correlated significantly with high maspin expression in the cytosol and low AR expression in the nucleus. Patients who had tumors with these characteristics had

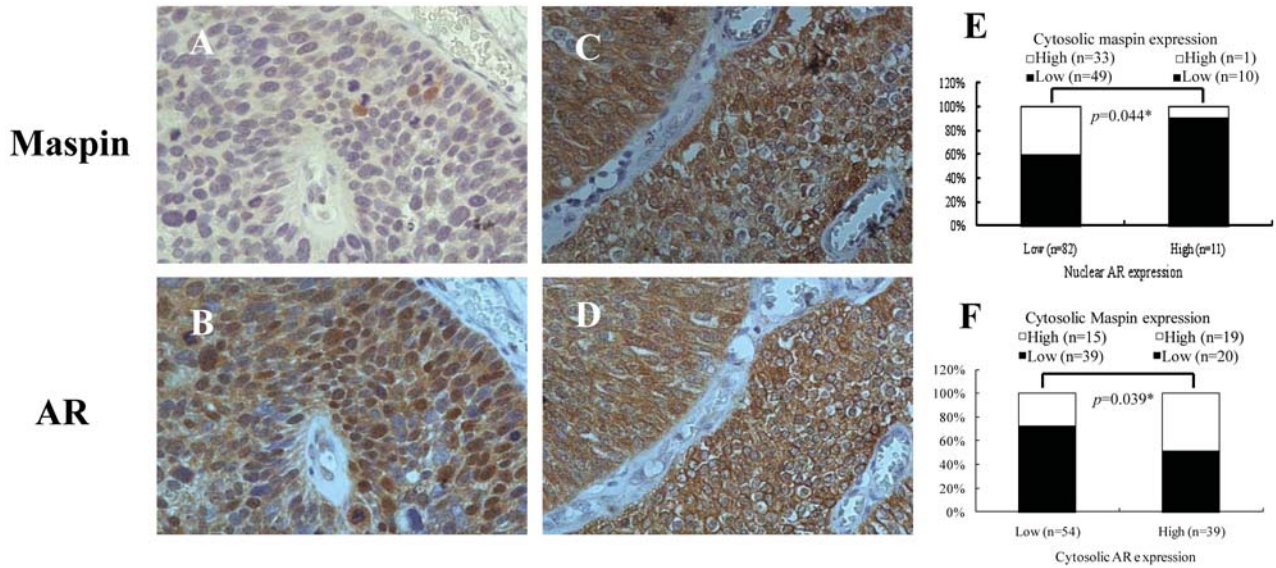


Figure 3. Cytosolic maspin expression was inversely correlated with nuclear AR expression in human UUTUC specimens. Samples with low maspin expression, especially in the cytosol (A), had high AR expression in the nucleus (B). Samples with high maspin expression in the cytosol (C) had high AR expression in the cytosol (D). Thus, low expression of maspin in the cytosol correlated significantly with high expression of AR in the nucleus (E); high expression of maspin in the cytosol correlated significantly with high expression of AR in the cytosol (F) ($\times 400$). $*p < 0.05$.

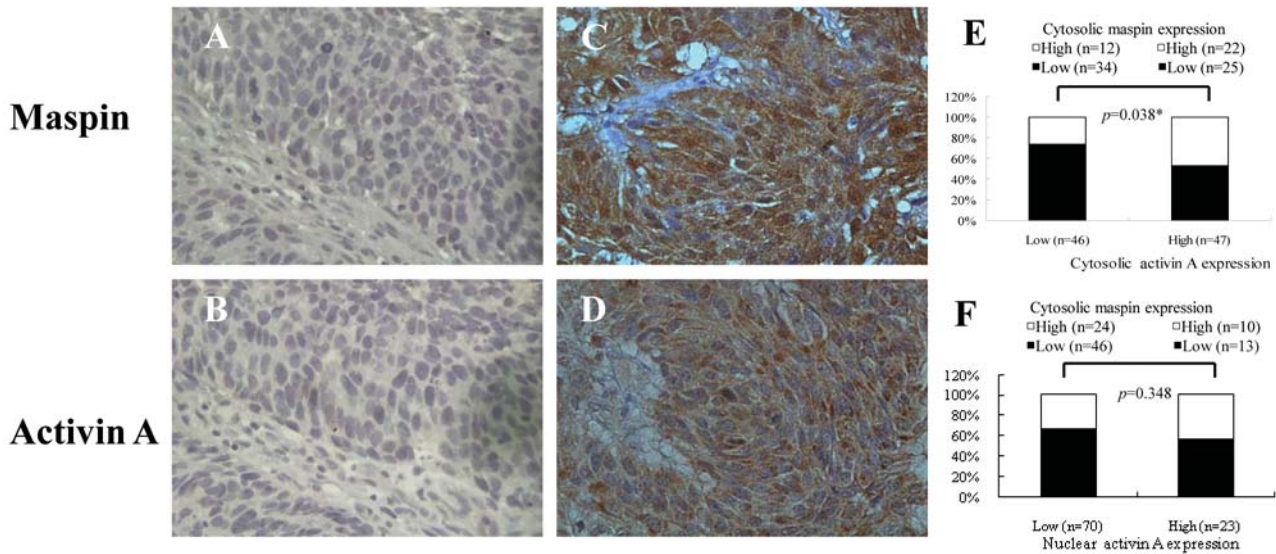


Figure 4. Cytosolic expression of maspin positively correlated with cytosolic activin A expression in human UUTUC specimens. When maspin expression was low in the cytosol (A), there was significantly less activin accumulated in the cytosol (B). When maspin expression was high in the cytosol (C), there was significantly high activin A expression in the cytosol (D). Maspin expression in the cytosol correlated significantly with activin A in the cytosol (E, F) ($\times 400$). $*p < 0.05$.

longer DFI. It is possible that maspin may prevent AR from entering into the nucleus to operate as a transcription factor and thus from inducing several oncogenic pathways in UUTUCs. The detailed mechanism of how maspin may prevent AR from entering the nucleus remains to be elucidated.

These observations raise the question of how cytosolic expression of activin A is related to synchronous expression of maspin in the cytosol, and whether activin A regulates maspin expression in urothelial carcinomas. Using RT-PCR, we found that activin A treatment, indeed

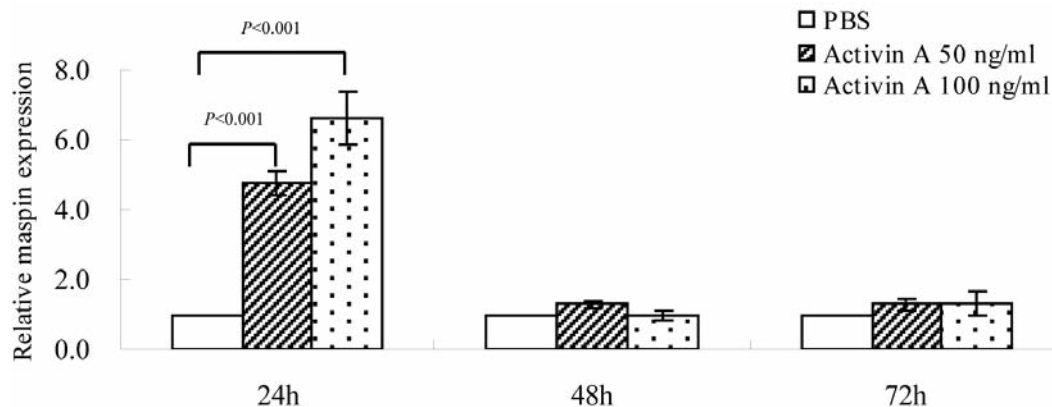


Figure 5. HT-1197 cells were treated with PBS, and 50 ng/ml or 100 ng/ml activin A. mRNA of maspin was collected at 24, 48, and 72 hours after the start of treatment. RT-PCR of maspin mRNA performed after 24 hours of treatment with activin A showed that expression of mRNA of maspin was significantly increased. This increase had disappeared at 48 and 72 hours after treatment. Two different concentrations of activin A yielded similar results ($n=3$, $*p<0.05$, ANOVA). Data are represented as the mean \pm standard deviation.

appeared to up-regulate maspin mRNA expression. This phenomenon was time-dependent but not dose-related. The expression of maspin surged in the first 24 hours, but dropped soon afterwards. The reason for this is not clear. Molecular and cellular studies of the interaction between activin A and maspin may provide further insight into the etiology and mechanisms of progression and metastasis of this disease. To our knowledge, this is the first time that activin A has been demonstrated to be involved in regulating maspin expression.

In summary, our study found that cytosolic predominance of activin A is highly predictive of longer DFI in patients with UUTUCs. In comparison, nuclear AR expression in advanced-stage disease is associated with poor prognosis in UUTUCs. Activin A can up-regulate maspin expression, and greater expression of activin A and maspin in the cytosol is negatively correlated with nuclear localization of AR. These markers may be used as a panel of molecular markers to predict DFI. Taken together, these results highlight the need for more accurate molecular prognostic tools (and combinations of tools) to guide follow-up of patients with UUTUCs. Those with low cytosolic expression of maspin and activin A, and high nuclear AR expression, may require a different follow-up schedule and corresponding treatment.

Acknowledgements

We thank J. K. Hsiao for his technical support. This work was supported by grants CMRPD 870841-3, CMRPG 890501, and CMRPG 860261-3 from Chang Gung Memorial Hospital and NMRPD 170341-3 (NSC97-2321-B-182-004-MY3) from the National Science Council to Dr. Hong-Yo Kang.

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Received February 24, 2011

Revised April 1, 2011

Accepted April 4, 2011