Chemico-Biological Interactions 183 (2010) 172–180

Contents lists available at ScienceDirect

Chemico-Biological Interactions

journal homepage: www.elsevier.com/locate/chembioint

The suppressive effect of Rho kinase inhibitor, Y-27632, on oncogenic Ras/RhoA induced invasion/migration of human bladder cancer TSGH cells

Horng-Rong Chang[a,b,1], Hui-Pei Huang[c,1], Yu-Lin Kao[d], Sung-Lang Chen[d], Sheng-Wen Wu[b], Tung-Wei Hung[b], Jong-Da Lian[b], Chau-Jong Wang[c,*]

[a] Institute of Medicine, Chung Shan Medical University, Taichung 402, Taiwan
[b] Department of Nephrology, Chung Shan Medical University Hospital, Taichung 402, Taiwan
[c] Institute of Biochemistry and Biotechnology, Chung Shan Medical University, 110, Section 1, Jianguo North Road, Taichung 402, Taiwan
[d] Department of Urology, Chung Shan Medical University Hospital, Taichung 402, Taiwan

ARTICLE INFO

Article history:
Received 27 August 2009
Received in revised form 24 October 2009
Accepted 28 October 2009
Available online 5 November 2009

Keywords:
Bladder cancer
Ras
RhoA
RhoA kinase inhibitor

ABSTRACT

Urothelial cell carcinoma is the most common type of malignancy found in long-term dialysis patients and kidney transplant recipients in Taiwan. Surgical specimens of tumorous and non-tumorous bladder tissues were collected from 12 patients with bladder cancer. Increased expressions of Ras, RhoA, Akt, PI-3K were demonstrated in the tumors as compared to adjacent control tissues. To understand the impact of Ras over-expression on bladder cancer progression, human bladder cancer TSGH 8301 cells were transfected with Ras DNA. The Ras-transfected cells were then treated with either a PI-3K inhibitor (wortmannin) or Rho kinase inhibitor (Y-27632) and the expressions of Ras, PI-3K, Akt, NF-κB, and RhoA were analyzed. Fluorescent phalloidin staining demonstrated more intense F-actin staining in the Ras over-expressed cells than in the control cells, and the intensity of F-actin was inhibited by Y-27632. A gelatin zymography study demonstrated that the MMP-2 and MMP-9 expressions of the Ras-transfected cells were enhanced, and Y-27632 treatment reduced the levels of MMP-2 and MMP-9. Similarly, a wound healing assay revealed that the ability of cell migration was markedly increased by Ras transfection and the healing rate after treatment of Y-27632 was delayed. Our results provide evidence that Ras-induced RhoA and NF-κB activation was involved in the invasion/migration of bladder cancer. Through Ras and/or RhoA inhibition, there might be an opportunity for new therapeutic interventions in bladder cancer.

1. Introduction

Bladder carcinoma is the most common malignancy of the urinary tract, and 90% of bladder carcinomas are urothelial cell carcinomas (UCCs). At the initial diagnosis of bladder cancer, 70% of cases are diagnosed as non-muscle-invasive disease and 30% as muscle-invasive disease. The 2004 WHO grading system classifies papillary urothelial carcinoma into only two grades: (1) low-grade papillary urothelial carcinoma exhibiting an overall orderly appearance, but with minimal variability in architecture and/or cytologic features, which are easily recognizable at scanning magnification. (2) High-grade papillary urothelial carcinomas which are characterized by a disorderly appearance from marked architectural and cytologic abnormalities, recognizable at low magnification [1]. The risk factors for bladder cancer include tobacco smoking and occupational exposure which are the most well-established factors [2]. The substances most commonly involved in occupational exposure are polycyclic aromatic hydrocarbons and arylamines. Professions in which this exposure occurs include those that use dyes, rubbers, textiles, paints, leathers and chemicals [3]. Other risk factors include elevated arsenic levels in drinking water [4], phenacetin, radiation therapy, dietary factors and chronic urinary tract infections. The cancer prevalence rates in patients with end-stage renal disease reported by the United States Renal Data System (USRDS, 2005) involves the digestive system (13.6%), reproductive system (9%), kidneys (4.2%), skeletal cancer (2.7%), skin cancer (2.4%), lymphoid tissue (2.1%) and respiratory system (1.7%) [5]. However, UCC is the most common malignancy found in long-term dialysis patients in Taiwan, and chronic tubulointerstitial nephritis is the most likely underlying renal disease [6]. UCC is also the most common type of cancer in kidney transplant recipients in Taiwan, with an incidence of 4.1% [7] and a high percentage of 43.6 among all post-transplant cancers [8]. The incidence of UCC in the general population was 2.76% according to the 2005 annual report of the Taiwan cancer registry. The risk factors predisposing to UCC in Taiwan include being of older age, underground water intake, being female, compound analgesics use as well as Chinese herbs

* Corresponding author. Tel.: +886 4 24730022x11670; fax: +886 4 23248167.
E-mail address: wcj@csmu.edu.tw (C.-J. Wang).
[1] Contributed equally to the results of this study.

0009-2797/$ – see front matter.
doi:10.1016/j.cbi.2009.10.018

Table 1
Patient demographics.

Patient No.	Group	Age	Sex	TMN stage	Grade	Invasive (I) or non-invasive (NI)
1	General	82	F	T1	Low	NI
2	General	82	F	T1	High	NI
3	Dialysis	51	M	T1	High	NI
4	Transplant	21	M	T3	High	I
5	Transplant	62	F	T2	Low	I
6	General	74	F	T2	Low	I
7	General	77	F	T2	High	I
8	Dialysis	63	M	T1	Low	NI
9	General	76	M	T1	Low	NI
10	Dialysis	77	F	T1	Low	NI
11	General	70	M	T1	Low	NI
12	General	78	F	T1	Low	NI

use [6,7,9,10]. Therefore, in regard to developing chronic tubulointerstitial nephropathy and UCC, chronic usage of analgesics and Chinese herbs are particular and important risk factors in Taiwan. Chronic usage of analgesics is associated with those of a lower social-economic status, and the use of aristolochic acid was not banned until 2003 in Taiwan.

Although bladder cancer is a chemosensitive neoplasm, metastatic disease is related with a poor prognosis and short-term survival. For two decades, the treatment of choice for metastatic bladder cancer has been cisplatin-based chemotherapy. More recently, non-platinum regimes have been tested, such as taxanes and gemcitabine, which are considered attractive alternatives. However, owing to treatment-related toxicities and short-response durations, identification of more molecular prognostic factors and application of targeted therapies have been sought with considerable interest.

Ras oncogenes are considered to play a key role in the carcinogenesis and progression in several cancers. Activation of Ras proteins has been reported to induce the constitutive activation of downstream kinase cascades, which results in continuous mitogenic signaling and transformation of immortalized cells in human bladder cancer [11]. Additionally, Rho is important for the ability of tumor cells to metastasize [12]. Kamai et al. demonstrated that RhoA, RhoC, and Rho kinase (ROCK), but not RhoB protein expressions were greater in bladder tumors [13]. Although both Ras and RhoA have been reported to be associated with bladder tumors, to the best of our knowledge, a relationship between Ras, RhoA signaling and the invasion/migration process of human bladder cancer has not been described. Thus, the aim of this study was to clarify the association between Ras and RhoA and their impact on the invasion/migration of human bladder cancer.

2. Materials and methods

2.1. Patients

Bladder cancer surgical specimens were obtained between 2004 and 2006 from 12 patients (5 men and 7 women; age 66.3 ± 16.5 years) including 3 dialysis patients (samples 3, 8, and 10) and 2 kidney transplant recipients (samples 4 and 5) with newly diagnosed primary UCC of the bladder (Table 1). The patients underwent surgical intervention before receiving any other therapy. Transurethral resection was performed for superficial bladder tumors and two non-tumorous epithelia. The non-tumorous epithelia were at least 5 cm apart from the tumor lesions. Similarly, in all the partial or total cystectomy cases, one tumor site and one portion of adjacent non-neoplastic bladder tissue, which was also at least 5 cm from the tumor, were resected for study. Three patients had metastatic diseases (samples 2, 4 and 5). Each subject provided written informed consent and the protocol of the study was reviewed and approved by our Institutional Review Board.

2.2. Chemicals

Tris-base, EDTA, SDS, phenylmethylsulfonyl fluoride, bovine serum albumin (BSA), leupeptin, Nonidet P-40, deoxycholic acid, sodium orthovanadate and phalloidin-FITC were purchased from Sigma–Aldrich (St. Louis, MO). Phosphate buffer solution (PBS), trypsin–EDTA, and powdered RPMI 1640 medium, fetal bovine serum, 100 U/ml penicillin G and 100 mg/ml streptomycin were purchased from Gibco/BRL (Gaithersburg, MD). Antibody against Akt and MAPK/ERK1/2, phosphorylated proteins were purchased from Cell Signaling Tech. (Beverly, MA). PI-3K (p85), NF-κB (p65), Ras, RhoA, and RhoB antibodies were purchased from BD Transduction Laboratories (San Diego, CA).

2.3. Cell culture and transfection

TSGH 8301 cells, established from a well-differentiated human UCC of the urinary bladder (grade II, stage A), were cultured in RPMI 1640 medium containing 10% (v/v) fetal bovine serum, 100 U/ml penicillin G and 100 mg/ml streptomycin at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. The medium was changed twice a week, and the cells were subcultured when confluence was achieved. Ras DNA was a gift from Dr. J.L. Ko. The cells were transfected with the Ras DNA using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. When necessary, an appropriate amount of empty vector was added as the control group.

2.4. Electrophoresis and immunoblotting

Analysis of Ras, PI-3K, Akt, NF-κB, and RhoA was performed using SDS-PAGE and immunoblotting. Before the analysis of the expression of proteins, the Ras DNA was transfected into the TSGH 8301 cells for 12 h. Various durations of transfection were tested, the best being 12 h (data not shown). The medium was removed and washed with PBS. Then 0.5 ml of cold RIPA buffer (1% NP-40, 50 mM Tris-base, 0.1% SDS, 0.5% deoxycholic acid, 150 mM NaCl, pH 7.5) with fresh leupeptin (17 μg/ml) and sodium orthovanadate (10 μg/ml) were added. Scraping of cells and transferal of the lysate into an Eppendorf were performed prior to incubation for 30 min on ice with the addition of 5 μl of 10 mg/ml PMSF stock. The cell lysate was centrifuged ($10{,}000 \times g$) for 10 min at 4 °C. Cell lysate (50 μg purified protein) was mixed with an equal volume of electrophoresis sample buffer and then boiled for 10 min, followed by analysis using SDS-PAGE and transfer of protein from the gel to nitrocellulose membranes (Millipore, Bedford, MA) using electroblotting apparatus. Nonspecific binding was

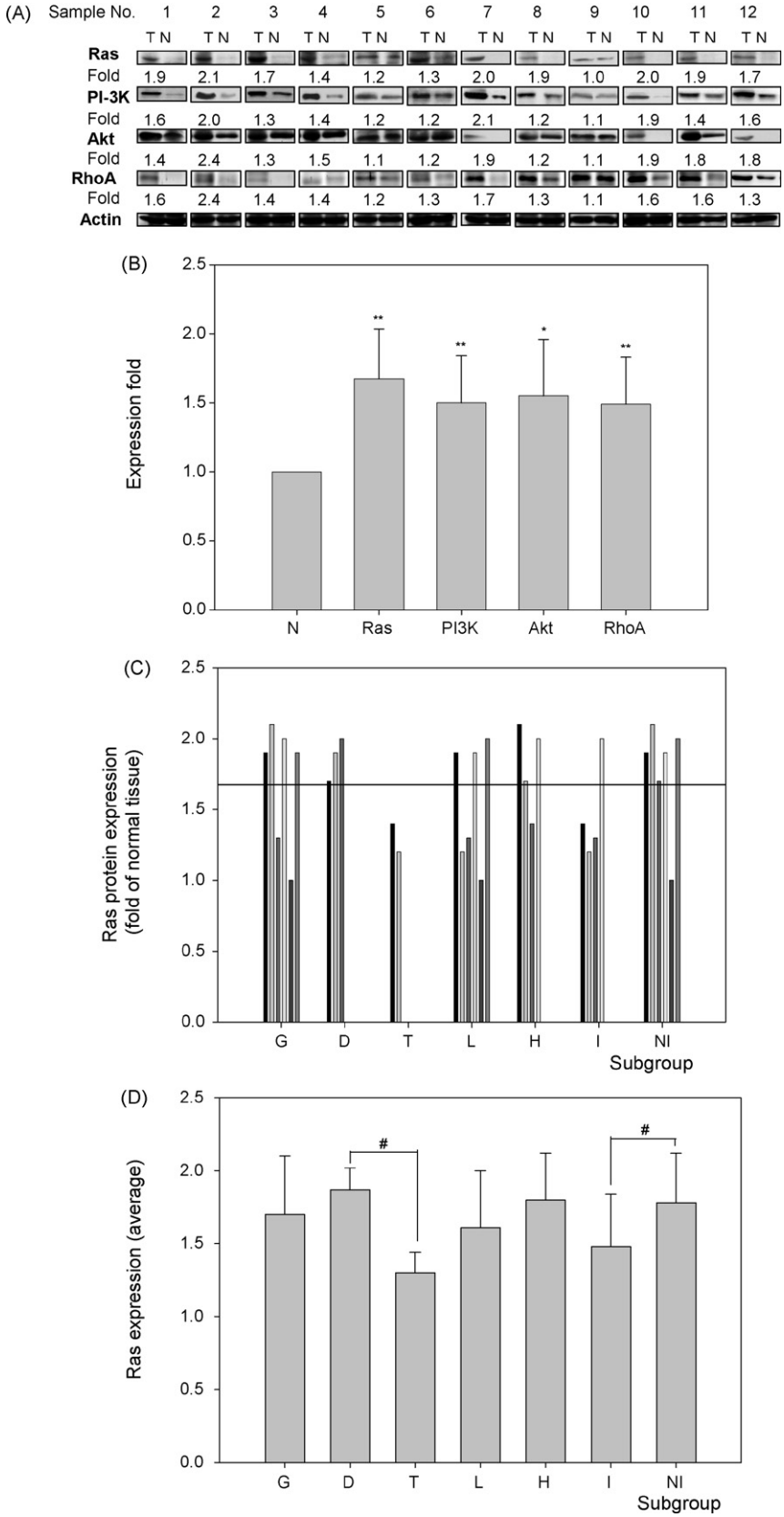

Fig. 1. (A) Levels of Ras, RhoA, PI-3K and Akt proteins in the bladder cancer patients. The proteins of the tumor (T) and normal (N) tissues obtained from the patients were analyzed by immunoblotting. Actin was the loading control. (B) Expression folds of Ras, RhoA, PI-3K and Akt of the bladder cancer tissues compared with the normal bladder tissues. Expression folds of Ras (C) and the average of Ras (D) in different patient subgroups were compared. Quantitative assessment of the expression levels of proteins in the denuded zone is expressed as the mean ± SD of three independent experiments. $^{\#}p < 0.01$, $^{*}p < 0.001$ and $^{**}p < 0.0001$, compared with the normal tissues. G: general ($n = 5$); D: dialysis ($n = 3$); T: transplant ($n = 2$); L: low grade ($n = 6$); H: high grade ($n = 4$); I: invasive ($n = 4$); NI: non-invasive ($n = 6$).

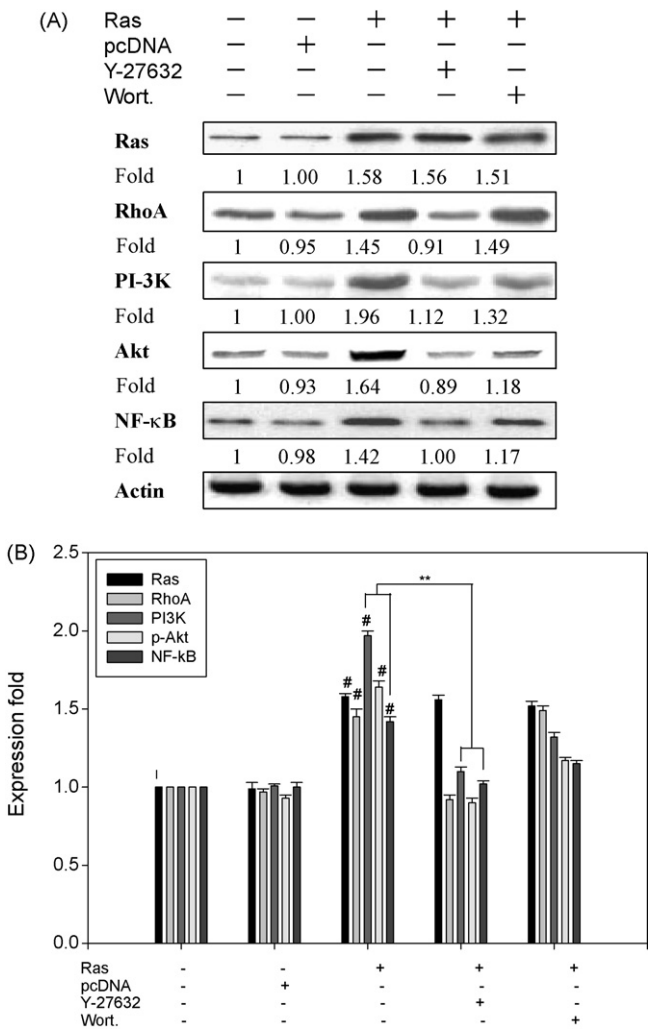

Fig. 2. The expression of signal transduction proteins by the TSGH 8301 cells transfected with Ras DNA. (A) Total cell lysate was extracted and the expression of Ras, RhoA, PI-3K, NF-κB, and β-actin were assayed by Western blotting. Actin was the loading control. (B) The average of three independent expressions ± SD. $^{\#}p < 0.0001$ compared with the untreated group. $^{**}p < 0.0001$ expression levels of PI-3K, Akt, and NF-κB of the Y-27632 treatment group compared with the protein levels of the Ras transfection alone group.

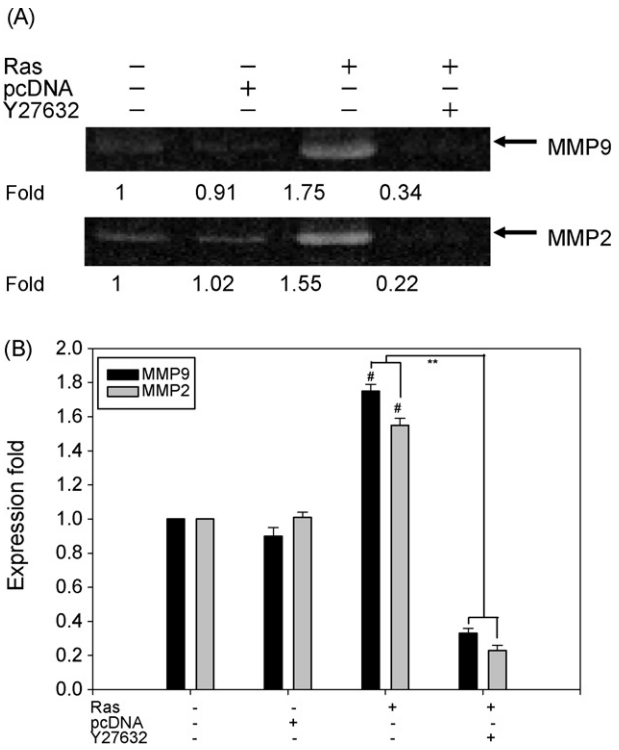

Fig. 4. Gelatin zymography assay of the TSGH 8301 cells. (A) Ras-transfected cells were treated with 20 μM Y-27632 or not for 1 h. The incubated medium was removed, then fresh serum free medium added and allowed to incubate for 24 h. Finally, the conditioned medium was collected and MMP-2/-9 levels were detected by gelatin zymography. (B) MMP-2/-9 activities were quantified by densitometer analysis. The densitometric data are expressed as the mean ± SD of three independent experiments. $^{\#}p < 0.0001$ compared with the untreated group. $^{**}p < 0.0001$ the activities of MMP-2/-9 treated with Y-27632 compared with the MMP-2/-9 expressions of the Ras treatment alone group.

blocked by incubation of the membrane with Tris-buffered saline (TBS) containing 1% (w/v) nonfat dry milk and 0.1% (v/v) Tween-20 (TBST) for more than 2 h. Membranes were washed with TBST three times for 10 min and incubated with an appropriate dilution of primary antibody in TBST for 2 h. Membranes were then extensively washed with TBST before being incubated with an appropriate amount of horseradish peroxidase-conjugated secondary antibody for 1 h. After washing the membrane three times for 10 min in TBST, detection was performed using ECL reagents for 1 min, and exposed ECL hyperfilm in a darkroom. Protein expression was determined by quantitative densitometry using AlphaImager Series 2200 software.

2.5. Fluorescent phalloidin staining

Phalloidin staining is a useful tool for investigating the distribution of F-actin in cells [14]. Briefly, a 0.2 mg/ml stock solution of phalloidin-FITC was prepared in methanol. The cells were washed with PBS, fixed for 1 h with 3.7% formaldehyde in PBS, and washed with PBS again. Next, the cells were dehydrated in acetone, rendered permeable with 0.1% Triton X-100 in PBS, and

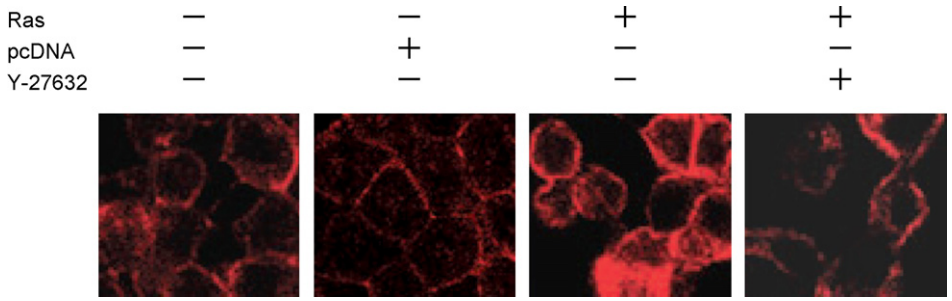

Fig. 3. The increased phalloidin binding and cellular F-actin levels by over-expression of Ras DNA in the TSGH 8301 cells. The Ras-transfected cells (1×10^5 cells/ml) were seeded in a six-well plate and treated with 20 μM Y-27632 or not for 1 h. F-actin was stained with rhodamine phalloidin (red). The more intense F-actin staining in the Ras over-expression groups than control cells and the amount of F-actin was inhibited by the Rho kinase inhibitor.

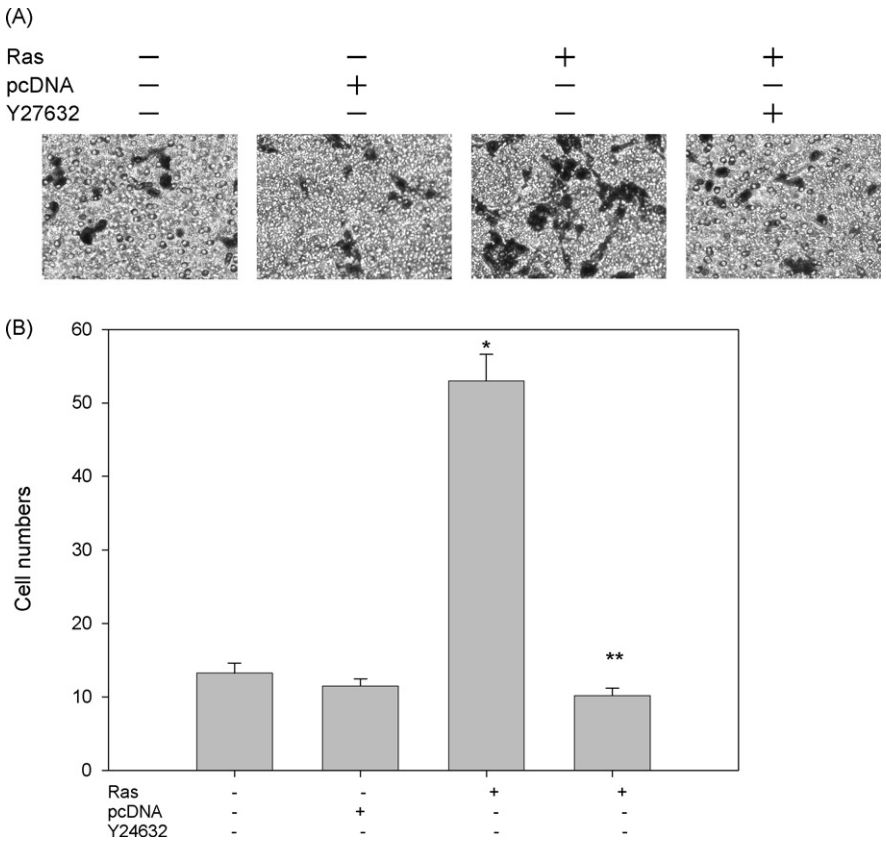

Fig. 5. Effects of over-expression of Ras DNA on the migration of TSGH 8301 cells. (A) The Ras-transfected cells (1×10^5 cells/ml) were treated with 20 μM Y-27632 or not for 1 h and migrated cells were analyzed using a modified Boyden chamber. Cells in serum-free RPMI medium were added to the upper chamber and allowed to migrate for 5 h through an 8-mm porous membrane toward the lower chamber to conditioned medium. Motility was quantified by counting the number of cells that migrated to the undersides of the membrane under microscopy (100×). (B) The results are shown as means ± SD of eight independent experiments. *$p < 0.001$ compared with the normal control. **$p < 0.0001$ migration analysis of the cell numbers compared with the migrated cells of the Ras-transfected alone group.

washed again with PBS. Finally, the cells were stained with a 50 μg/ml fluorescent phalloidin conjugate solution in PBS (containing 1% DMSO from the original stock solution) for 1 h at room temperature. After at least four 1-min washes with PBS to remove the unbound phalloidin conjugate, the samples were then mounted in 50% PBS/50% glycerol (v/v) and examined in an Olympus fluorescence microscope with a 100× oil immersion objective.

2.6. Determination of MMP-2/-9 by zymography

The levels of MMP-2/-9 released in the cultured medium were detected by gelatin zymography assays as previously described [15]. First, serum free conditioned medium was prepared with a 5× loading buffer containing 0.01% SDS without β-mercaptoethanol. The prepared samples were subjected to electrophoresis with 8% SDS polyacrylamide gels containing 0.1% gelatin. Electrophoresis was performed at 140 V for 3 h in an ATTO apparatus. Gels were washed twice with 50 ml distilled water containing 2% Triton X-100 on a gyratory shaker for 30 min at room temperature to remove the SDS after electrophoresis. The gel was then incubated in 50 ml reaction buffer (40 mM Tris–HCl, pH 8.0, 10 mM $CaCl_2$, 0.02% NaN_3) overnight at 37 °C, stained with Coomassie brilliant blue R-250 and destained with methanol–acetic acid–water (5%, 7.5%, and 87.5%, v/v/v).

2.7. Cell migration assays

After transfection, the cells were trypsinized, and the in vitro migration was tested in a Boyden chamber assay [16]. The Ras-transfected cells were seeded in the Boyden chamber (Neuro Probe, Cabin John, MD). In the upper chamber, a density of 1×10^4 cells/well in 50 μl of serum free medium was incubated for 5 h at 37 °C. The bottom chamber also contained standard medium with 10% FBS. The cells that migrated to the lower surface of the membranes were fixed with methanol and acetate (3:1), stained with Giemsa and counted under a light microscope.

2.8. Wound healing assay

The TSGH 8301 cells were cultured in one well of a six-well culture dish. A line was drawn on the underside of the well with a yellow P200 pipette tip. These lines served as fiducial marks for the wound areas to be analyzed. The growth medium was replaced by calcium-free PBS then the PBS was removed and fresh medium added. The cells were observed using phase contrast microscopy on an inverted microscope.

2.9. Preparation of nuclear fractions

Harvested cells were lysed with buffer A (10 mM HEPES, 10 mM KCl, 0.1 mM EDTA, 1.5 mM $MgCl_2$, 0.2% NP-40, 1 mM DTT and 0.5 mM phenylmethylsulfonyl fluoride), and then centrifuged to shear the cytoplasmic membranes. Nuclei were pelleted at 800 × *g* for 30 s at 4 °C in a microcentrifuge; nuclear proteins were extracted with high-salt buffer B (20 mM HEPES, 25% glycerol, 1.5 mM $MgCl_2$, 0.1 mM EDTA, 420 mM NaCl, 1 mM DTT and 0.5 mM phenylmethylsulfonyl fluoride).

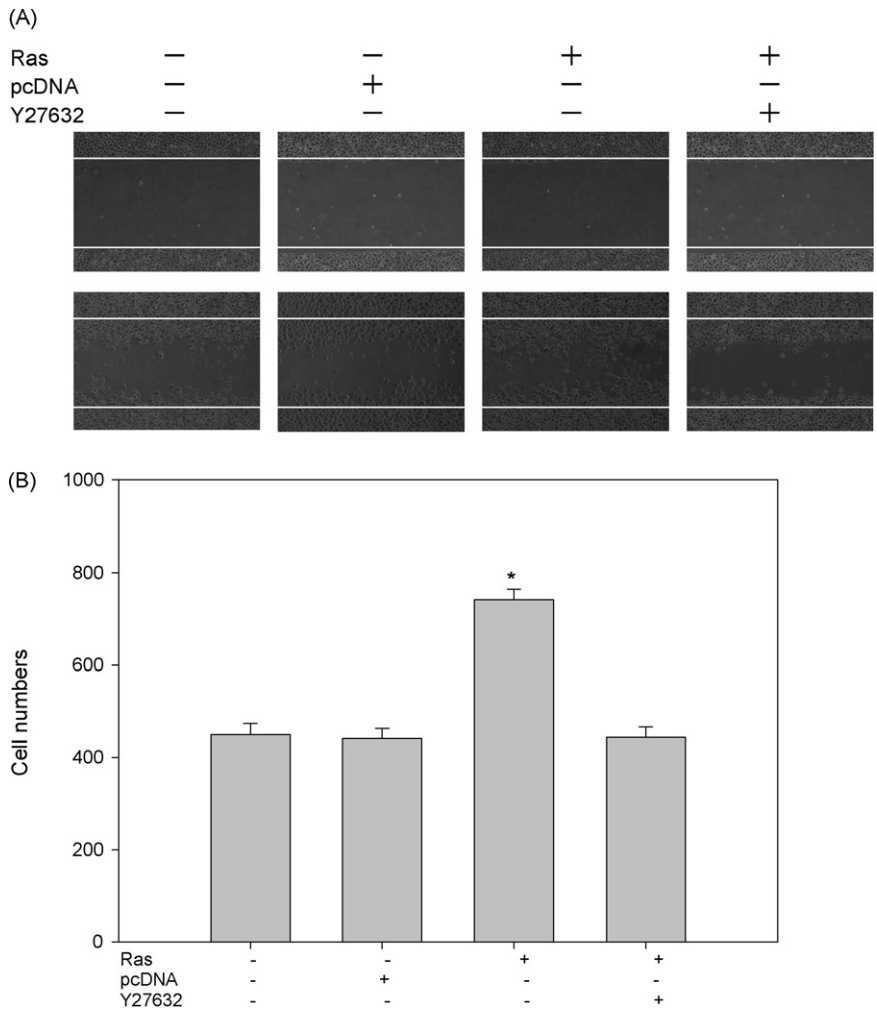

Fig. 6. Wound healing analysis of the TSGH 8301 cells transfected with Ras DNA. (A) The treated cells were cultured in a six-well plate after monolayer wounding with a yellow P200 pipette tip. The healing ability was observed using phase contrast microscopy on an inverted microscope after 48 h. (B) Quantitative assessment of the average number of cells in the denuded zone is expressed as the mean ± SD of three independent experiments. *$p < 0.001$ compared with the untreated controls.

2.10. Electrophoretic mobility shift assay (EMSA)

AP-1 and NF-κB binding assays in nuclear extracts were performed with biotin-labeled double-stranded AP-1 or NF-κB oligonucleotides (Promega, Madison, WI, USA). EMSA was carried out with a LightShift EMSA Optimization and Control Kit and Chemiluminescent Nucleic Acid Detection Modules (Pierce, Rockford, IL, USA). Binding reactions containing 10 μg of nuclear protein, 2 μl of 10× binding buffer, 1 μg poly-(dI·dC), 12.5 μg poly-L-lysine, and 2 pmol of oligonucleotide probe were incubated for 20 min at room temperature. Protein DNA complex was separated by electrophoresis on a 6% non-denaturing acrylamide gel, transferred to positively charged nylon membranes, and then cross-linked in a Stratagene cross-linker. Gel shifts were visualized by streptavidin–horseradish peroxidase followed by chemiluminescent detection.

2.11. Statistics

The results of the cell migration assays and the wound healing analysis were shown as the mean ± SD. Statistical comparisons were performed using Student's *t*-tests. *p* values <0.05 were considered to be statistically significant.

3. Results

3.1. Increased expressions of Ras, RhoA, PI-3K and Akt in bladder cancer tissues

Cancer metastasis has been linked to Ras overexpression. The important role that Ras plays in the regulation of cell growth and differentiation has been verified by the fact that approximately 70% of neoplasms display mutations in this gene [17]. We assayed the expressions of Ras and related protein levels among the samples from the patients with bladder cancer. The proteins in tumorous (T) and non-tumorous (N) tissues obtained from the same patient were analyzed by immunoblotting. The expression of Ras protein was most marked among the bladder cancer tissues (Fig. 1A) with a 1.675-fold increase compared with normal tissues ($p < 0.0001$) (Fig. 1B). In addition, the levels of RhoA ($p < 0.0001$), PI-3K ($p < 0.0001$) and Akt ($p < 0.001$) were also significantly elevated in the bladder cancer tissues. These data suggest that the formation of bladder cancer might be coupled to the Ras pathway.

The patients were divided into subgroups as follows: general (G), dialysis (D), transplant (T), low grade (L), high grade (H), invasive (I) and non-invasive (NI). The Ras expression in these subgroups was also compared. Fig. 1C shows the average level of Ras

(1.675-fold, higher compared with normal tissue). The levels of Ras in the three dialysis patients (1.7-, 1.9-, and 2.0-fold) were all higher than the average level, but this was not found in other groups. The average levels of Ras expression in the different subgroups were then further analyzed (Fig. 1D). The average level of Ras in the dialysis group was significantly increased when compared with the transplant group, whereas there was no significance when compared with the general group. In addition, there was no statistical significance between low-grade and high-grade patients, although Ras over-expression is associated with low-grade lesions rather than high-grade lesions. The Ras expression of patients with non-invasive tumor was higher than that of patients with invasive lesion ($p < 0.01$). A reasonable explanation why our results were different from previous studies might be the limited number of patients. Collecting a larger and homogenous series of patients to reconfirm the results should be our next task.

3.2. Over-expression of Ras DNA induced the activation of RhoA and PI-3K-related proteins

Many Ras effectors are promoted after Ras has been stimulated [18]. We investigated whether the interaction of Ras affected the expression of downstream Ras-dependent signaling. As shown in Fig. 2, the Ras-transfected cells displayed significantly higher PI-3K, RhoA, Akt, and NF-κB levels than control cells, suggesting that the over-expression of Ras promoted both the PI-3K and RhoA pathways. Moreover, the addition of Y-27632, a Rho kinase inhibitor caused a significant reduction in the levels of RhoA, PI-3K and PI-3K-related downstream proteins. This suggests that RhoA might regulate PI-3K and its related protein expressions.

3.3. Over-expression of Ras DNA stimulated the actin cytoskeleton organization

RhoA promotion of actin–myosin contractility and actin cytoskeleton reorganization is required for cell shape modification and cell migration. We examined the effect on intracellular distribution of F-actin in Ras-transfected cells. The results revealed that the expression of Ras-induced dense accumulation of cytoskeletal filaments along the plasma membrane using phalloidin staining (Fig. 3). Nevertheless, such dense phalloidin accumulations in Ras-transfected cells diminished following treatment with Y-27632, suggesting that the formation of F-actin activated by Ras was via the RhoA signaling pathway.

3.4. Inhibition of RhoA prevented Ras-induced migration

In addition to facilitating tumor invasion, extracellular products such as MMPs can modulate migration, cancer cell proliferation and metastasis [18,19]. We investigated the levels of MMP-2 and MMP-9 by gelatin zymography. When TSGH cells were transfected with Ras DNA, the MMP-2 and MMP-9 expressions of the cells were enhanced (Fig. 4). In contrast, the inhibition of the RhoA expression by Y-27632 reduced the levels of MMP-2 and MMP-9.

Cancer cell migration can be viewed as a process regulated by matrix degrading proteinases, integrins, other cell adhesion molecules and the healing of a wound [20,21]. Thus, the impact of Ras over-expression on cell migration and wound healing was studied. The ability of cell migration was markedly increased when the cells were transfected with Ras DNA (Fig. 5). In addition, the curing rate after treatment of Y-27632 with Ras-transfected cells was delayed (Fig. 6). Therefore, we suggest that Rho kinase inhibitors can hold back cell–cell matrix adhesion and the capability of wound healing.

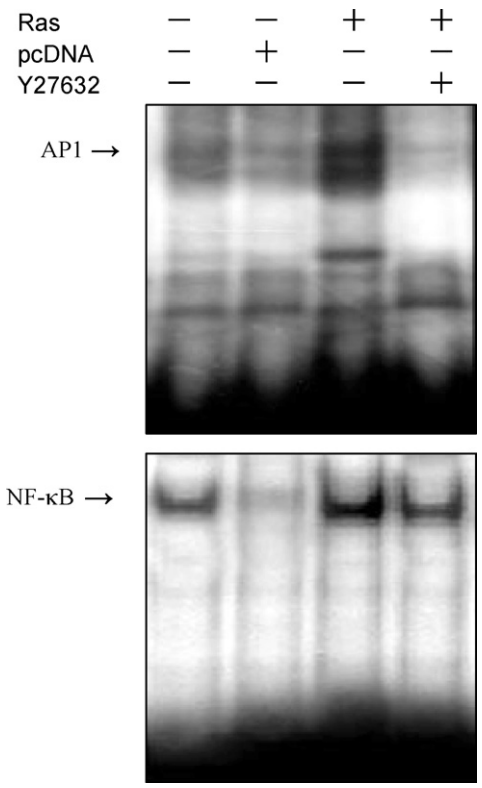

Fig. 7. The DNA binding ability of AP-1 and NF-κB. Nuclear extracts were analyzed for AP-1 and NF-κB DNA binding ability using biotin-labeled AP-1 and NF-κB specific oligonucleotide by EMSA. Three independent experiments were conducted, all showing similar patterns of change.

3.5. Rho kinase inhibitor mediated Ras-induced activation of AP-1 and NF-κB

MMP gene expression is chiefly regulated by transcriptional factors (for example, NF-κB and AP-1) via the PI-3K/Akt or ERK pathways [22,23]. Active NF-κB consists of a dimer of a Rel family/p65 subunit and a p50 or p52 subunit. NF-κB is maintained in the cytoplasm through interactions with an inhibitor of NF-κB (IκB), but upon dissociation, moves into the nucleus and promotes cancer cell proliferation, angiogenesis, and metastasis. c-Fos and c-jun also are important transcription factors and oncogenes, which form a heterodimer (AP-1 complex). This is associated with the invasion and metastasis of cancer cells [24].

Over-expression of Ras DNA induced the migration of the TSGH 8301 cells and was associated with increased levels of RhoA/NF-κB (Fig. 2). Recently we described that AP-1 and NF-κB mediate downstream of RhoA activated by the transfection of Ras DNA [25]. To confirm the DNA binding ability of AP-1 and NF-κB, the EMSA method was used. Ras expression increased DNA binding, and thereby, inhibition of RhoA impeded the AP-1 DNA binding activity (Fig. 7). Similarly, when compared with the Ras-transfected cells, the DNA binding ability of NF-κB of the cells treated with Y-27632 decreased. A proposed model for the Ras and RhoA mediated invasion/migration of human bladder cancer cells is summarized in Fig. 8.

4. Discussion

Our results demonstrated that human bladder cancer showed increased expressions of Ras, RhoA, PI-3K/Akt and NF-κB, and that over-expression of Ras had an invasion/migration-promoting effect on the bladder cancer TSGH 8301 cells. Previously, Ras and

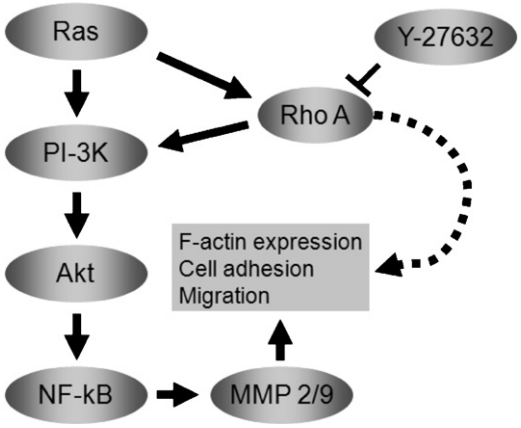

Fig. 8. A proposed model for the Ras and RhoA mediated invasion/migration of human bladder cancer cells. The dotted arrow represents the pathway which has been published in the literature and the solid arrows represent the findings in this present study.

RhoA have been reported to be involved in the carcinogenesis and progression of bladder cancer individually [13,26]. However, the linkage and downstream relationship between Ras and RhoA have not been reported in the literature.

Aristolochic acid-containing Chinese herbs have been known to be a potential risk factor for development of urothelial carcinoma [27,28]. Accumulating evidence has demonstrated that the carcinogenic properties of aristolochic acid are based on the formation of DNA and mutations in codon 61 of H-ras triggering tumorigenesis [29]. A possible inference about aristolochic acid inducing the activation of the Ras pathway is that the free radicals from the metabolites of aristolochic acid trigger the Ras signaling. In addition, oxidative stress and Ras activation result in the production of reactive oxygen species, which then leads to the tumorigenesis [30]. Recently we reported that 12-week intragastric feeding with aristolochic acid induced urothelial proliferation in rats, and that this phenomenon was through cell cycle progression via activation of cyclin D1/cdk4 and cyclin E/cdk2 [31]. In addition, exposure to aristolochic acid has been reported to be associated with urothelial dysplasia in rats [32], and urothelial carcinoma in humans [33,34]. Because of the high association between H-ras and development of aristolochic acid-related UCC, we conducted this study to evaluate the expression of the Ras signaling pathway and the impact of Ras over-expression on RhoA expression in urothelial cancer cell lines.

The carcinogenesis of human bladder cancer is a multistep process. As a result of a better understanding of the molecular biology of bladder cancers, various signaling pathways are known to be involved in both carcinogenesis and tumor progression. Among these, Ras oncogenes are considered to play a key role in the carcinogenesis of human bladder cancer [35,36]. Locally advanced, and/or metastatic bladder cancer is characterized by mutations of the p53 and retinoblastoma (Rb) genes, which interact with the Ras-mitogen activated protein kinase (MPAK) transduction pathway. Activation of the PI-3K pathway is involved in tumor invasion and inhibition of apoptosis. Over-expression of tyrosine kinase receptors, including EGFR, VEFGR and HER2/neu, is correlated with tumor progression [37].

It is not yet clear whether Ras triggers PI-3K/Akt downstream proteins, even though the role of Ras oncogenes leading to bladder cancer initiation and progression is well understood. Przybojewska et al. investigated 19 bladder cancer specimens. H-ras activation was found in 15 (about 84%) and N-ras gene mutations were observed in all cases except one in which H-ras gene mutations were detected. Their results suggest a strong relationship between H-ras and N-ras gene activation in bladder cancer, while changes in the K-ras gene in bladder cancers seem to be rare events [38]. Shinohara and Koyanagi reviewed the mechanisms of Ras activation in human bladder cancer and found the incidence rate of point mutation of the Ras gene was 6–84%, over-expression of Ras protein 40–50%, but an unknown incidence of the functional activation of the Ras signaling pathway [11]. Although the results remain controversial, activation of the Ras oncogene by point mutation or over-expression might be important in the carcinogenesis and progression of human bladder cancer. Surprisingly, recent studies have confirmed that NF-κB is mediated by adaptor p62 to promote lung cancer formation through the Ras/JNK pathway [39]. Our recently published results suggested that melanoma metastasis promoted by Ras/PI-3K/Akt/NF-κB activation was inhibited after treatment with mulberry anthocyanins (MACs) in vitro and in vivo [25]. In this study, our results demonstrated that bladder cancer tissues had increased expressions of Ras, RhoA, PI-3K/Akt and NF-κB, and that over-expression of Ras and RhoA had invasion/migration-promoting effects on the bladder cancer TSGH 8301 cells. Ras triggers a myriad of signaling pathways and results in different cancers, implying the importance of clearing Ras downstream signaling in cancer therapy.

A specific inhibitor of the Rho kinase, Y-27632 [40,41] has been reported to block both Rho-mediated activation of actomyosin and the invasive activity of cultured rat MM1 hepatoma cells [42]. Continuous treatment with this inhibitor reduced dissemination of MM1 cells implanted into the peritoneal cavity of syngeneic rats [43]. These reports suggest that Rho kinase inhibition may represent a way to prevent cancer invasion and metastasis by inhibiting cell migration and morphological alterations. Our results also demonstrated that inhibition by Y-27632 prevented Ras-induced migration and the curing rate was also delayed. Therefore, we suggest that Rho kinase inhibition could hold back cell–cell matrix adhesion and the capability of wound healing. These results may offer hope with regards to the possibility of developing new treatment strategies.

Because only a minority of patients agreed to provide informed consent, the limitations of this study are the small patient number and heterogeneous underlying diseases of the patients. For example, two patients were kidney transplant recipients and three were dialysis patients. A larger and homogenous series of patients would be better to reconfirm the results.

In summary, our study demonstrated that human bladder cancer tissue had increased expressions of Ras, PI-3K, Akt, NF-κB as well as RhoA, and that over-expression of Ras had an invasion/migration-promoting effect. These results provide additional evidence that through Ras and/or RhoA inhibition, opportunities for therapeutic interventions in bladder cancer exist.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

References

[1] Z. Kirkali, T. Chan, M. Manoharan, F. Algaba, C. Busch, L. Cheng, L. Kiemeney, M. Kriegmair, R. Montironi, W.M. Murphy, I.A. Sesterhenn, M. Tachibana, J. Weider, Bladder cancer: epidemiology, staging and grading, and diagnosis, Urology 66 (Suppl. 1 (6)) (2005) 4–34.
[2] A Stenzl, N.C. Cowan, M. De Santis, G. Jakse, M.A. Kuczyk, A.S. Merseburger, M.J. Ribal, A. Sherif, J.A. Witjes, The updated EAU guidelines on muscle-invasive and metastatic bladder cancer, Eur. Urol. 55 (2009) 815–825.
[3] C.L. Pashos, M.F. Botteman, B.L. Laskin, A. Redaelli, Bladder cancer: epidemiology, diagnosis, and management, Cancer Pract. 10 (2002) 311–322.
[4] C.H Chen, H.Y. Chiou, Y.M. Hsueh, C.J. Chen, H.J. Yu, Y.S. Pu, Clinicopathological characteristics and survival outcome of arsenic related bladder cancer in Taiwan, J. Urol. 181 (2009) 547–552.
[5] J.L. Xue, F. Dalleska, A.M. Murray, A.J. Collins, Cancer Prevalence in Patients with End-Stage Renal Disease, United States Renal Data System (USRDS) Annual Data Report, 2005.

[6] C.H. Chang, C.M. Yang, A.H. Yang, Renal diagnosis of chronic hemodialysis patients with urinary tract transitional cell carcinoma in Taiwan, Cancer 109 (2007) 1487–1492.
[7] M.J. Wu, J.D. Lian, C.R. Yang, C.H. Cheng, C.H. Chen, W.C. Lee, K.H. Shu, M.J. Tang, High cumulative incidence of urinary tract transitional cell carcinoma after kidney transplantation in Taiwan, Am. J. Kidney Dis. 43 (2004) 1091–1097.
[8] Y.L. Kao, Y.C. Ou, C.R. Yang, H.C. Ho, C.K. Su, K.H. Shu, Transitional cell carcinoma in renal transplant recipients, World J. Surg. 27 (2003) 912–916.
[9] Y.M. Hung, K.J. Chou, S.Y. Hung, H.M. Chung, J.C. Chang, De novo malignancies after kidney transplantation, Urology 69 (2007) 1041–1044.
[10] M.H. Yang, K.K. Chen, C.C. Yen, W.S. Wang, Y.H. Chang, W.J. Huang, F.S. Fan, T.J. Chiou, J.H. Liu, P.M. Chen, Unusually high incidence of upper urinary tract urothelial carcinoma in Taiwan, Urology 59 (2002) 681–687.
[11] N. Shinohara, T. Koyanagi, Ras signal transduction in carcinogenesis and progression of bladder cancer: molecular target for treatment? Urol. Res. 30 (2002) 273–281.
[12] L. del Peso, R. Hernandez-Alcoceba, N. Embade, A. Carnero, P. Esteve, C. Paje, J.C. Lacal, Rho proteins induce metastatic properties in vivo, Oncogene 15 (1997) 3047–3057.
[13] T. Kamai, T. Tsujii, K. Arai, K. Takagi, H. Asami, Y. Ito, H. Oshima, Significant association of Rho/ROCK pathway with invasion and metastasis of bladder cancer, Clin. Cancer Res. 9 (2003) 2632–2641.
[14] E. Wulf, A. Deboben, F.A. Bautz, H. Faulstich, T. Wieland, Fluorescent phallotoxin, a tool for the visualization of cellular actin, Proc. Natl. Acad. Sci. U.S.A. 76 (1979) 4498–4502.
[15] Y.S Hsieh, S.C. Chu, S.F. Yang, P.N. Chen, Y.C. Liu, K.H. Lu, Silibinin suppresses human osteosarcoma MG-63 cell invasion by inhibiting the ERK-dependent c-Jun/AP-1 induction of MMP-2, Carcinogenesis 28 (2007) 977–987.
[16] S.C. Chu, H.L. Chiou, P.N. Chen, S.F. Yang, Y.S. Hsieh, Silibinin inhibits the invasion of human lung cancer cells via decreased productions of urokinase-plasminogen activator and matrix metalloproteinase-2, Mol. Carcinog. 40 (2004) 143–149.
[17] J. Fujita, O. Yoshida, Y. Yuasa, J.S. Rhim, M. Hatanaka, S.A. Aaronson, Ha-ras oncogenes are activated by somatic alterations in human urinary tract tumours, Nature 309 (1984) 464–466.
[18] M. Macaluso, G. Russo, C. Cinti, V. Bazan, N. Gebbia, A. Russo, Ras family genes: an interesting link between cell cycle and cancer, J. Cell Physiol. 192 (2002) 125–130.
[19] C.M. Overall, O. Kleifeld, Tumour microenvironment—opinion: validating matrix metalloproteinases as drug targets and anti-targets for cancer therapy, Nat. Rev. Cancer 6 (2006) 227–239.
[20] H.A. Chapman, Plasminogen activators, integrins, and the coordinated regulation of cell adhesion and migration, Curr. Opin. Cell Biol. 9 (1997) 714–724.
[21] V Falanga, Wound healing and its impairment in the diabetic foot, Lancet 366 (2005) 1736–1743.
[22] S.O. Yoon, S. Shin, H.J. Lee, H.K. Chun, A.S. Chung, Isoginkgetin inhibits tumor cell invasion by regulating phosphatidylinositol 3-kinase/Akt-dependent matrix metalloproteinase-9 expression, Mol. Cancer Ther. 5 (2006) 2666–2675.
[23] T.W. Chung, Y.C. Lee, C.H. Kim, Hepatitis B viral HBx induces matrix metalloproteinase-9 gene expression through activation of ERK and PI-3K/Akt pathways: involvement of invasive potential, FASEB J 18 (2004) 1123–1125.
[24] S.O. Lee, Y.J. Jeong, H.G. Im, C.H. Kim, Y.C. Chang, I.S. Lee, Silibinin suppresses PMA-induced MMP-9 expression by blocking the AP-1 activation via MAPK signaling pathways in MCF-7 human breast carcinoma cells, Biochem. Biophys. Res. Commun. 354 (2007) 165–171.
[25] H.P. Huang, Y.W. Shih, Y.C. Chang, C.N. Hung, C.J. Wang, Chemoinhibitory effect of mulberry anthocyanins on melanoma metastasis involved in the Ras/PI-3K pathway, J. Agric. Food Chem. 56 (2008) 9286–9293.
[26] G. Oxford, D. Theodorescu, The role of Ras superfamily proteins in bladder cancer progression, J. Urol. 170 (2003) 1987–1993.
[27] A. Lemy, K.M. Wissing, S. Rorive, A. Zlotta, T. Roumeguere, M.C. Muniz Martinez, C. Decaestecker, I. Salmon, D. Abramowicz, J.L. Vanherweghem, J. Nortier, Late onset of bladder urothelial carcinoma after kidney transplantation for end-stage aristolochic acid nephropathy: a case series with 15-year follow-up, Am. J. Kidney Dis. 51 (2008) 471–477.
[28] J.P. Cosyns, M. Jadoul, J.P. Squifflet, F.X. Wese, C. van Ypersele de Strihou, Urothelial lesions in Chinese-herb nephropathy, Am. J. Kidney Dis. 33 (1999) 1011–1017.
[29] V.M. Arlt, M. Stiborova, H.H. Schmeiser, Aristolochic acid as a probable human cancer hazard in herbal remedies: a review, Mutagenesis 17 (2002) 265–277.
[30] J.S. Pan, M.Z. Hong, J.L. Ren, Reactive oxygen species: a double-edged sword in oncogenesis, World J. Gastroenterol. 15 (2009) 1702–1707.
[31] H.R. Chang, J.D. Lian, C.W. Lo, Y.C. Chang, M.Y. Yang, C.J. Wang, Induction of urothelial proliferation in rats by aristolochic acid through cell cycle progression via activation of cyclin D1/cdk4 and cyclin E/cdk2, Food Chem. Toxicol. 44 (2006) 28–35.
[32] F.D. Debelle, J.L. Nortier, E.G. De Prez, C.H. Garbar, A.R. Vienne, I.J. Salmon, M.M. Deschodt-Lanckman, J.L. Vanherweghem, Aristolochic acids induce chronic renal failure with interstitial fibrosis in salt-depleted rats, J. Am. Soc. Nephrol. 13 (2002) 431–436.
[33] J.L. Nortier, M.C. Martinez, H.H. Schmeiser, V.M. Arlt, C.A. Bieler, M. Petein, M.F. Depierreux, L. De Pauw, D. Abramowicz, P. Vereerstraeten, J.L. Vanherweghem, Urothelial carcinoma associated with the use of a Chinese herb (Aristolochia fangchi), N. Engl. J. Med. 342 (2000) 1686–1692.
[34] G.M. Lord, T. Cook, V.M. Arlt, H.H. Schmeiser, G. Williams, C.D. Pusey, Urothelial malignant disease and Chinese herbal nephropathy, Lancet 358 (2001) 1515–1516.
[35] B. Czerniak, G.L. Cohen, P. Etkind, D. Deitch, H. Simmons, F. Herz, L.G. Koss, Concurrent mutations of coding and regulatory sequences of the Ha-ras gene in urinary bladder carcinomas, Hum. Pathol. 23 (1992) 1199–1204.
[36] S.H. Kroft, R. Oyasu, Urinary bladder cancer: mechanisms of development and progression, Lab Invest. 71 (1994) 158–174.
[37] H. Wallerand, G. Robert, J.C. Bernhard, A. Ravaud, J.M. Ferriere, Targeted therapy for locally advanced and/or metastatic bladder cancer, Prog. Urol. 18 (2008) 407–417.
[38] B. Przybojewska, A. Jagiello, P. Jalmuzna, H-ras, K-ras, and N-ras gene activation in human bladder cancers, Cancer Genet. Cytogenet. 121 (2000) 73–77.
[39] A. Duran, J.F. Linares, A.S. Galvez, K. Wikenheiser, J.M. Flores, M.T. Diaz-Meco, J. Moscat, The signaling adaptor p62 is an important NF-κB mediator in tumorigenesis, Cancer Cell 13 (2008) 343–354.
[40] M. Uehata, T. Ishizaki, H. Satoh, T. Ono, T. Kawahara, T. Morishita, H. Tamakawa, K. Yamagami, J. Inui, M. Maekawa, S. Narumiya, Calcium sensitization of smooth muscle mediated by a Rho-associated protein kinase in hypertension, Nature 389 (1997) 990–994.
[41] T.T. Ishizaki, M. Uehata, I. Tamechika, J. Keel, K. Nonomura, M. Maekawa, S. Narumiya, Pharmacological properties of Y-27632, a specific inhibitor of Rho-associated kinases, Mol. Pharmacol. 57 (2000) 976–983.
[42] F. Imamura, M. Mukai, M. Ayaki, H. Akedo, Y-27632, an inhibitor of Rho-associated protein kinase, suppresses tumor cell invasion via regulation of focal adhesion and focal adhesion kinase, Jpn. J. Cancer Res. 91 (2000) 811–816.
[43] K. Itoh, K. Yoshioka, H. Akedo, M. Uehata, T. Ishizaki, S. Narumiya, An essential part for Rho-associated kinase in the transcellular invasion of tumor cells, Nat. Med. 5 (1999) 221–225.