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Antrodia camphorata extract induces replicative senescence in superficial TCC, and inhibits the absolute migration capability in invasive bladder carcinoma cells

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Abstract

The *Antrodia camphorata* crude extract (ACCE), an extract obtained from a precious traditional Chinese folkloric herbal medicine Zhan-Ku (a camphor tree mushroom) since the 18th century, has showed rather significant inhibitory effects on the growth and proliferation of the transitional cell carcinomas (TCC) cell lines RT4, TSGH-8301, and T24. On treatment with ACCE at 100 μg/mL, the p53-independent overexpression of p21 with simultaneous down alteration of pRb was observed in RT4, which was thus speculative of proceeding through a mechanism of replicative senescence. On the contrary treatment with ACCE, at 50 μg/mL, resulting in simultaneous down-regulations of Cdc2 and Cyclin B1, with suppression of the absolute migrating capability of the two cell lines TSGH-8301 and T24, and eventually the cell deaths. We conclude that ACCE can be rather effective and beneficial in suppression of both the superficial cancer cell line RT4 and the metastatic cell lines (TSGH-8301 and T24) through different mechanisms.
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Keywords: TCC; *Antrodia camphorata*; Replicative senescence; p53; p21; Rb

1. Introduction

1.1. Bladder cancers

Bladder cancer (BC) is a heterogeneous neoplasm, presenting as either primary superficial low grade tumors confined to the superficial mucosa (80%) or deeply muscle-invasive carcinomas (20%) (Knowles, 1999). Consisting of a broad spectrum of tumors including transitional cell carcinoma (TCC), BC has been identified to be a neoplastic lesion caused by environmental and/or occupational factors (Doll and Peto, 1981). Recently it has become the fourth and the eighth most common cancer in men and women in the United States (Jemal et al., 2003), as well as the seventh most common cancer in men with rising incidence and prevalence in Taiwan. In all genitourinary tumors, TCC is the second most common cause of death. Although of superficial low grade, yet with high recurrence rate (Skinner and Lieskovsky, 1988), only a small proportion of BC progresses to invasive diseases, while distant invasive metastases TCC only seldom develop, yet has caused the majority of deaths with TCC being resistant to chemotherapy. Many molecular and genetic changes in TCC of the bladder have exploded, which include (1) chromosomal alternations leading to carcinogenesis, e.g. mutations (point and insertional/deletional),

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translocation, and loss of alleles, each insult may effect the translated protein products; oncogenes such as c-*H-ras*, c-*myc*, and c-*erB-2* are believed to be categorized in this event, (2) loss of cell cycle regulation accounting for tumor cell proliferation, several tumor suppressor genes (TSGs) acting at the G_0/G_1 check point of the cell cycle are now recognized, and their protein products – p53, pRb, p16 and p14 – are vital for preventing cell cycle progression in bladder tumors: inactivation of the Rb gene or increased p53 immunoreactivity has been found in higher grade and stage bladder cancers, which is associated with the disease progression, and the overall and disease-specific survival rates, but these two proteins act in an independent yet synergistic manner in patients with bladder cancers, and (3) metastasis guided by events such as angiogenesis and loss of cell adhesion (Williams and Stein, 2004). This varied presentation results in widely divergent clinical outcomes. Detailed information, which identifies and characterizes the biological potential of various bladder cancers may be employed to dictate suitable treatments for TCC. One approach to control bladder cancers is through growth inhibition by which the disease can be prevented, slowed-down, or reversed substantially. This has been accomplished by the administration of one or more non-toxic naturally occurring or synthetic agents (Gee et al., 2002), among which naturally occurring compound *Antrodia camphorata*, formerly named as *Antrodia cinnamomea* Chang & Chou, sp. nov. Polyporaceae, commonly named “Zhan Ku,” or “Zhan Chi”, has come to practical applications (Tsai, 1982).

1.2. Origin of the fungus

Antrodia camphorata belongs to the following taxonomy: Kingdom, Mycoteae; Division, Amastigomycota; Subdivision, Basidiomytina; Class, Hymenomycetes; Order, Aphyllophorales; Family, Polyporaceae; Genus, *Taiwanofungus*; Species, *camphorautus* (Peng et al., in press).

Taiwanofungus camphoratus Sheng H. Wu, Z.H. Yu, Y.C. Dai & C.H. Su is proposed as a new genus of lignicolous family Polyporaceae (Wu et al., 2004). Originally, *Ganoderma comphoratum* M. Zang & CH. Su was once designated as its generic type (Zang and Su, 1990), in fact it did not belong to the genus of *Ganoderma* due to a careless misidentification from the contaminated specimen of *Ganoderma* spores, hence later renamed as *Antrodia cinnamomea* T.T. Chang & W.N. Chou, sp. nov. Polyporaceae (Chang and Chou, 1995). Recently, two new species of Genus *Taiwanofungus* have been identified, *viz*. *Taiwanofungus camphoratus* and *Taiwanofungus salmoneus* (T.T. Chang & W.N. Chou) Sheng H. Wu, Z.H. Yu, Y.C. Dai & C.H. Su. In folkloric remedies, it is popularly and commonly called “Zhan Ku”, “Zhan Chi”, “niu chang ku” or “jang-jy”, which in Chinese means “The mushroom from the camphor tree *Cinnamomum kanehirai* Hay Lauraceae in Taiwan” (Su, 2002), further renamed as *Antrodia camphorata* (Zang & Su) Sheng H. Wu, Ryvarden & T.T. Chang (Wu et al., 1997). Although the latter has been used for years and recently has been further assigned with a novel Latin name *Taiwanofungus camphoratus* (Wu et al., 2004), still it is not very popular (Peng et al., in press). In this manuscript, we are determined to use this traditional old name *Antrodia camphorata* in description of such a unique Formosan (Taiwanese) mushroom.

1.3. Historical medicinal uses

A. camphorata had long been popularly used as an amazing folkloric medicine by the Taiwan aborigine long before 1773 (Su, 2002) for the treatment of twisted tendon and muscle damages, terrified mental state, influenza and cold, headache, fever, and many internally affiliated diseases. In fact, since the eighteenth century, it has gradually become a more and more popular folkloric medicine with various effects as a carminative, a tonic, a laxative, an antidote, an anti-bacterial, a sedative, an analgesic, for the treatment of malignant tumors, viral infection, stomachitis, diabetes mellitus, nephritis, proteinuria, hepatoma, influenza, and motion-sickness (Su, 2002). Recently, it has been further investigated for its apoptotic (Song et al., 2005; Hsu et al., 2005), anti-oxidative (Hsiao et al., 2003), and anti-inflammatory (Shen et al., 2004) effects on human hepatocellular carcinomas or leukocytes. Yet, no documented effects of *A. camphorata* in treatment of bladder cancers have been described. In this study, we try to investigate the effect of the *A. camphorata* crude extract (ACCE) on three bladder cancer cell lines (RT4, TSGH-8301, and T24), which have been used as models of non-invasive (RT4), the moderately invasive (TSGH-8301), and the invasive (T24) bladder cancer, respectively. Through such an investigation, much clinical benefits can be expected in finding the molecular determinants of various bladder tumor cell sensitivity or resistance to *A. camphorata*.

2. Materials and methods

2.1. Antrodia camphorata crude extract (ACCE)

The *A. camphorata* used in this study was supplied by Well-Shine Biotechnology Development Co., Ltd. (Taipei, Taiwan), whereas ACCE was prepared as previously described (Hsiao et al., 2003). The ACCE was dissolved in 100% EtOH at a concentration of 80 mg/mL and stored at −20 °C. The concentrations of ACCE used were 10–200 μg/mL, respectively.

2.2. Compositional analysis of ACCE

2.2.1. Separation of fat fraction and ergosterol

The extraction method for fatty acids was according to Folch et al. (1957) and the extraction solvent used was a mixture of methanol and chloroform. Methods described by Pasanen et al. were followed to isolate ergosterol from the fat fraction of fruiting bodies supplied by Well-Shine Co. (Pasanen et al., 1999). Briefly, the sample obtained was subjected to further GC and GC/MS analysis. The lower layer (i.e. the non-saponifiable fraction) obtained in the above section after the ergosterol had been separated was further treated to obtain the fatty acid fraction, by blowing nitrogen to drive off the

residual solvent; the sample obtained was analyzed directly by GC.

2.2.2. GC and GC/MS analysis for ergosterol

An HPGC-5890 Plus was used to identify the constituent ergosterol as directed by the manufacturer HP, Palo-Alto (CA, USA). An MS: HP MSD-5972A was operated at an electron multiplier voltage (EMV) 1343 with a detection mode using a selective ion monitor and carrier gas helium at a flow rate of 1.0 mL/min.

2.2.3. GC analysis for fatty acids

An HPGC-5890 Plus was used for identification of the methylated fatty acids: an HP-CPWax58 CB capillary column (Chrompack, The Netherlands), a detector FID, and carrier gas helium at a flow rate of 1.6 mL/min were adopted.

2.3. Cell cultures

The human urinary bladder cancer cell lines, RT4, T24 (HTB-4, ATCC) and TSGH-8301, were purchased from the Food Industry Research and Development Institute (Hsinchu, Taiwan, ROC). RT4 was established from a well-differentiated papillary tumor of the bladder and has the wt *p53* and *Rb* gene (Rigby and Franks, 1970): TSGH-8301 cells, having wt *p53* but mutant *Rb* gene, were derived from a well-differentiated human TCC of the urinary bladder (Yeh et al., 1988), and T24 cells were derived from an invasive bladder tumor of grade 3, having *p53* non-sense mutation at codon 126 (TAC to TAG) (Bubenik et al., 1973). RT4 and T24, and TSGH-8301 were cultured in media McCoy's 5A, and RPMI 1640 with glutamine, respectively, and all were also supplemented with 10% fetal bovine serum (FBS) (GIBCO, USA). The cell line mouse embryonic fibroblast (MEF) used as normal cell control was kindly provided by Dr. Hung Li (Academia Sinica, Taipei, Taiwan) and cultured in DMEM medium (GIBCO, USA) supplemented with 10% FBS. All the culture media, after further additions of 100 IU/mL penicillin and 100 μg/mL streptomycin (GIBCO, USA), were used to cultivate cells at 37 °C in a humidified atmosphere containing 5% CO_2 in air.

2.4. Cell proliferation assay

Bladder carcinomas cells, RT4 (2×10^4), TSGH-8301 (5×10^3) and T24 (5×10^3), were plated onto each well of 24-well culture plates for 24 h and treated with ACCE at concentrations of 10, 50, 100, 150 and 200 μg/mL, respectively, or with vehicle alone for another 24–72 h. For MTT assay, the protocol was performed as previously described (Peng et al., 2006). From the results of MTT assay, the optimum dosages of ACCE were found to be 100, 50, and 50 μg/mL for the cell lines RT4, TSGH-8301, and T24, respectively. Hence these dosages were adopted to perform cell proliferation assay: thus cells (3×10^5 for cell lines RT4 and TSGH-8301; 1.5×10^5 for T24, respectively) were seeded to 6-cm culture plates then added with ACCE at doses as indicated and incubated for 24, 48, and 72 h, respectively.

2.5. Flow cytometry analysis

Cell cycle analysis was performed with a flow cytometer (FACS Calibur; BD Biosciences, CA, USA). Cells (2×10^4) were cultured in 6-cm culture plates and treated with ACCE at 50 μg/mL for cells TSGH-8301 and T24, and 100 μg/mL for RT4 cell, respectively; or by vehicle alone for another 24–72 h. The floating and adherent cells post trypsinization were collected and washed with ice-cold PBS, fixed and permeabilized with 70% ethanol at −20 °C overnight, then incubated with 30 μg/mL propidium iodide (PI) and 100 μg/mL RNase for 30 min at room temperature in the dark after washing with ice-cold PBS on the next day. Data acquisition and analysis were performed in the flow cytometer with the accompanying software (CellQuest; BD Biosciences, USA). Appropriate gating was used to select the easily distinguished single population. Ten thousand events per sample were counted and at least triplicate determinations were performed to assure each cell cycle distribution. The final percentages of cells in each phase were calculated from the triplicate experimentations.

2.6. Western blot analysis

The cells in 10-cm culture plates were harvested at indicated time following 50 μg/mL (T24, TSGH-8301), 100 μg/mL (RT4) of ACCE treatment or vehicle alone and washed with ice-cold PBS. The cells were incubated in ice-cold RIPA buffer [Tris, 1 M (pH 7.4), 5 M NaCl, 0.5 M EDTA (pH 8.0), 10% SDS, 10% DOS, 10% NP40] with freshly added protease inhibitor cocktail tablets (Roche, USA) over ice for 30 min. The cells were scraped and the lysate was collected in an Eppendorff tube and cleared by centrifugation at 13,000 rpm for 30 min at 4 °C. The supernatant was collected, aliquoted, and stored at −70 °C. The protein concentration in the lysates was measured by BCA protein assay kit (Pierce, USA) by following the manufacturer's protocol. Western immunoblotting protocol was conducted according to the protocol as advised by the manufacturer (Cell Signaling Technology Inc., USA), using proteins (30 μg) loaded over 5–15% SDS-PAGE gels. Then the proteins were added with the ECL Western blotting detection reagents (Amersham Biosciences, USA) and analyzed using the Fuji LAS-3000 imaging system (Japan). The antibodies used in this study included anti-p53 Ab (Santa Cruz, USA), anti-p21 Ab, anti-rabbit IgG HRP-linked Ab and anti-mouse IgG, HRP-linked Ab (Cell Signaling, USA), anti-pRB Ab (BD Pharmingen, USA), anti-Cyclin B1 mAb (Upstate, USA), anti-Cdc2 Ab (Calbiochem, Germany), and anti-Actin mAb (Chemicon, USA).

2.7. Wound scratch assay

Cells (2×10^4) were seeded into six-well tissue culture dishes and cultured in medium containing 10% FBS to confluent cell monolayers, which were then carefully wounded using a sterile 200 λ-pipette tip that was also used to remove any cell debris with PBS (Liu et al., 2005). The cells were then incubated in ACCE (50 μg/mL to TSGH-8301 and T24; 100 μg/mL to RT4) or vehicle alone for 24 h and photographed under a phase con-

trast microscope. Although the relative cell migration capability had been described by Liu et al. (2005), yet it has been modified in this paper into the absolute migration capability (MC_A) as

$$MC_A(\text{mm/h}) = \frac{G_0 - G_t}{2t} \quad (1)$$

where G_0 is the initial gap for each cell line at 0 h (mm), G_t the final gap for each cell line treated with ACCE at certain dose or vehicle alone for a time period of t (h),and t is the overall time period for incubation (h). Similar experiments were repeated in triplicates.

2.8. Transwell motility assay

Cells were plated at 10^5 cells/cm^2 in the upper compartment of 8 μm-pore size Transwell migration chamber (Corning Inc., USA) and cultured in medium containing 50 μg/mL (for TSGH-8301 and T24) or 100 μg/mL (for RT4) of ACCE or vehicle alone for 72 h. The cells on the upper surface were then removed by carefully wiping with a cotton swab and the filter was gently removed from the chamber and mounted on glass slides. The cells that invaded the filter and attached onto the lower surface of the filter were fixed, stained with hematoxylin, and counted in 10 randomly selected microscopic fields (400×) per filter. Similar experiments were repeated in triplicates.

2.9. Zymography

MMP activity in conditioned medium was demonstrated by gelatin zymography (7.5% zymogram gelatin gels). Briefly, samples were diluted in buffer (0.12 M Tris–HCl, 20% glycerol, 0.1% bromophenol blue, 10% SDS), and the total proteins (4 μg) were electrophoresed through gelatin-impregnated zymogram gels at 125 V for 90 min, which were then incubated at room temperature in a zymogram renaturing buffer (Invitrogen, CA) for 30 min and further washed in zymogram developing buffer (Invitrogen, CA) for 30 min. Thereafter, fresh zymogram developing buffer was added and the gels were incubated overnight at 37 °C. Zymograms were developed by staining with 0.5% Coomassie Blue (Bio-Rad, USA) for 90 min before destaining until clear bands of MMP activity appeared against a blue background.

2.10. Statistical analysis

Results were analyzed using a two-tailed Student's t-test to assess statistical significance. Values with $p < 0.05$ were considered statistically significant.

3. Results and discussion

3.1. ACCE is enriched in ergosterol and unsaturated fatty acids

The fatty acid content obtained in the extract of *A. camphorata* fruiting bodies (ACCE) was 1.46, 14.74, 6.20, 14.34, 10.78, and 12.69 mg/g for C14:0, C16:0, C17:0, C18:0, C18:1 and C18:2, respectively, whereas the ergosterol content was 0.71 ± 0.24 μg/g (Table 1).

Table 1
Fatty acid and ergosterol contents in fruiting bodies of ACCE

Fatty acids (mg/g)		Ergosterol (μg/g)
C14:0	1.46 ± 0.39	0.71 ± 0.24
C16:0	14.74 ± 2.76	
C17:0	6.20 ± 1.81	
C18:0	14.34 ± 3.51	
C18:1	10.78 ± 1.14	
C18:2	12.69 ± 2.20	

$n = 5$; data are expressed in mean ± S.D.

3.2. Cell growth characters affected by ACCE are related with the status of the cell differentiation

On addition of ACCE (50 μg/mL) to TSGH-8301 and T24, respectively, and to RT4, with ACCE at 100 μg/mL, the macroscopic growth appearances after incubated for 72 h were all distinctly affected. As can be seen in Fig. 1, for RT4, the cell shapes were apparently distorted and cell colonies more dispersed as compared with the appearance of the vehicle control (Fig. 1A and B); T24 also showed a distinct difference (Fig. 1E and F), although not as similarly dispersed as that found in the RT4 cells. For comparison, TSGH-8301 cells seemed to have behaved in between, the ACCE treated samples showed a less dense character than the control, however still appeared in clusters (Fig. 1C and D). Such responses can be attributed to the susceptibility of these three cell lines to ACCE, which revealed a well inversely correlated relationship between the status of cell differentiation and the growth inhibitory effect of ACCE. However, no similar inhibitory effect was observed in MEF cell lines (Fig. 2A; LM photo not shown).

3.3. Cell proliferation patterns affected by ACCE are depending on differentiation status of the bladder cancer cell lines

On exposure of the three bladder cancer cell lines to different levels of ACCE at 10, 50, 100, 150, and 200 μg/mL, respectively for 72 h, a dose-dependent response was observed, and surprisingly, the dose-responsive curves for both T24 and TSGH-8031 all showed distinct plateau at dosages over 50–200 μg/mL, whereas RT4 showed a normal declining proliferation in parallel to the increasing dosages of ACCE used, with an inflection point occurring at 100 μg/mL, implicating a maximum effect in linearity (Fig. 2A), a phenomenon quite similar to the cited response of bladder cancer cell line SCaBER to CDDP (Kawasaki et al., 1996). Apparently, the degree and status of differentiation can significantly influence the drug resistance, such as the case with ACCE. Furthermore, the proliferation of RT4 was seen halted by ACCE at 100 μg/mL in the initial 24 h (Fig. 2B) comparing to those of the other two treated with ACCE at 50 μg/mL (Fig. 2C and D), hence suspicious of the fact that

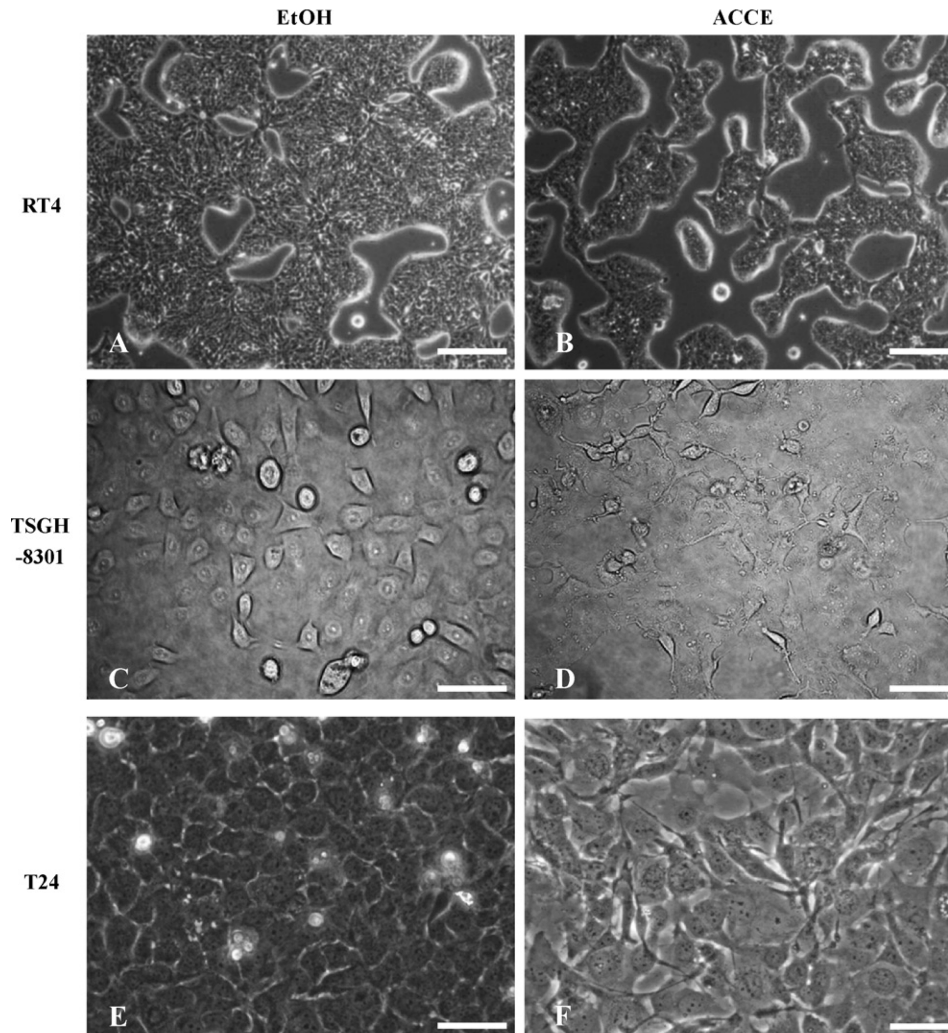

Fig. 1. Light microscopic images of the three cell lines. Cells were incubated with EtOH alone (A, C and E), ACCE at 100 μg/mL to RT4 (B), and 50 μg/mL to TSGH-8301 and T24 (D and F), respectively for 72 h. Magnification 100×, scale bar = 0.2 mm.

the latent period was associated with some growth signaling hampering by ACCE at least for the initial 24 h in RT4 cell lines.

The regressed cell proliferation rates were 2.27×10^4, 2.06×10^4 and 2.71×10^4 cells/h for the vehicle EtOH control, compared to the ACCE-treated 1.04×10^4, 1.126×10^4, and 1.72×10^4 cells/h, for RT4, TSGH-8301 and T24, respectively (Fig. 2B–D). Correspondingly, ACCE has inhibited the growth rates 54.0, 45.0, and 37.0%, respectively for the three cell lines; the most resistant character was again obviously evidenced for the most poorly differentiated cell line T24, supporting the fact that the inhibitory effect of ACCE can be closely dependent on the degree of cell differentiation. Deregulation of cell proliferation has been considered to be a hallmark of neoplastic transformation. Alteration in growth control pathways must translate into changes in the cell cycle regulatory machinery (Xiong et al., 1993). Alternatively, ACCE may contain various different constituents that could interact with different signal pathways associated with the cell differentiation and proliferation, a track left to be investigated in our future work.

3.4. *Cell cycle arrests at G_1 in RT4, at G_2M in TSGH-8301 and T24 cell lines*

Apparent cell cycle arrests were observed at 72 h post the treatment of ACCE at 50 μg/mL (TSGH-8301, T24) and 100 μg/mL (RT4). Significant ($p < 0.05$) arrests were observed for RT4 at phase G_1 (88.3% of the total cells arrested comparing to the control 80.4%) (Figs. 3 and 4A). In contrast, in TSGH-8301 (18.6% of total cells arrested compared to the control 13.1%) (Fig. 4B) and T24 cell lines, arrests were observed at phase G_2M (15.8% of total cell arrested compared to the control 7.1%) (Fig. 4C), respectively.

3.5. *Up-regulation of p21, and down-regulations of ppRB in RT4, of Cdc2 and Cyclin B1 in T24 and TSGH-8301 cell lines*

ACCE at 100 μg/mL has significantly down-regulated the expression of phospho-RB in cell line RT4 at 72 h post treatment (Fig. 5A), RT4 had no *p53* gene mutation, the key cell cycle regulatory protein p53 showed only faint images, while up-regulation

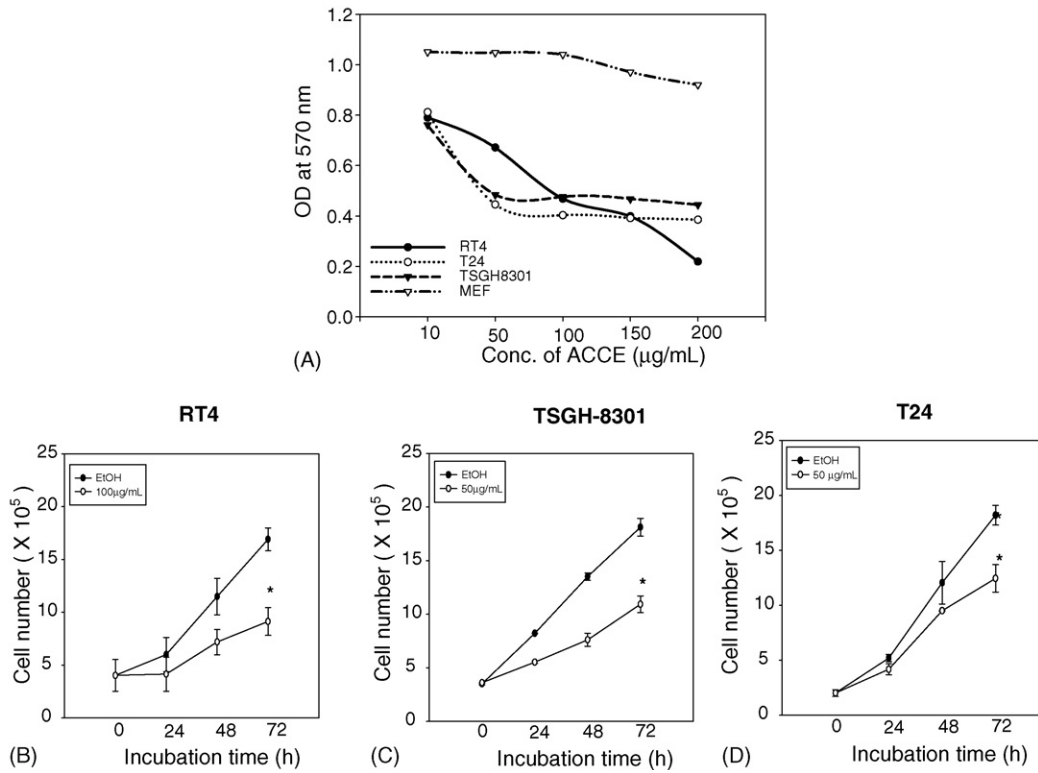

Fig. 2. Cell proliferation assay for the three cell lines: MTT assay for RT4, TSGH-8301 and T24 cells. ACCE was added with increasing doses 0, 10, 50, 100, 150 and 200 μg/mL, respectively, and incubated for 72 h (A). Cells were seeded with ACCE 100 μg/mL to RT4 (B), 50 μg/mL to TSGH-8301 (C) and T24 (D, also refer to Peng et al., 2006) for 24, 48 and 72 h, respectively and incubated at 37 °C. $n = 3$, $^{*}p < 0.05$.

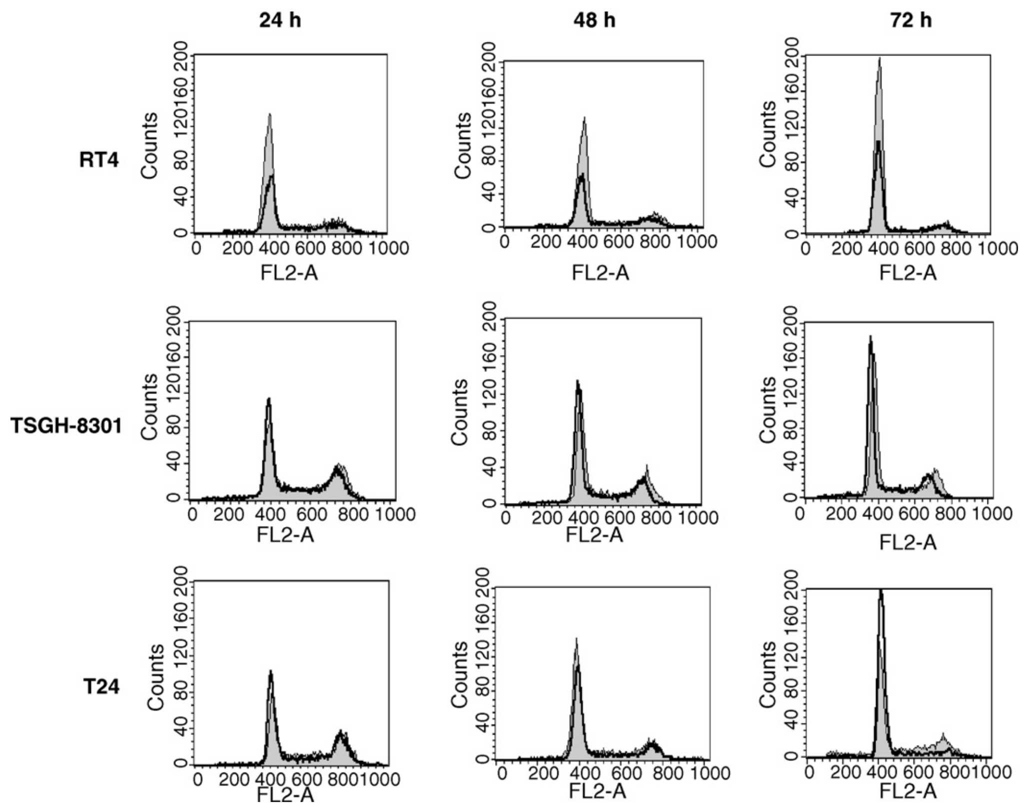

Fig. 3. Flow cytometric analysis for the phase arrest in the three cell lines on treatment with ACCE. The flow cytometric analysis of RT4, TSGH-8301 and T24 cells in the presence (100 μg/mL to RT4; 50 μg/mL to TSGH-8301 and T24, respectively) □, or the absence of ACCE for 72 h (▩).

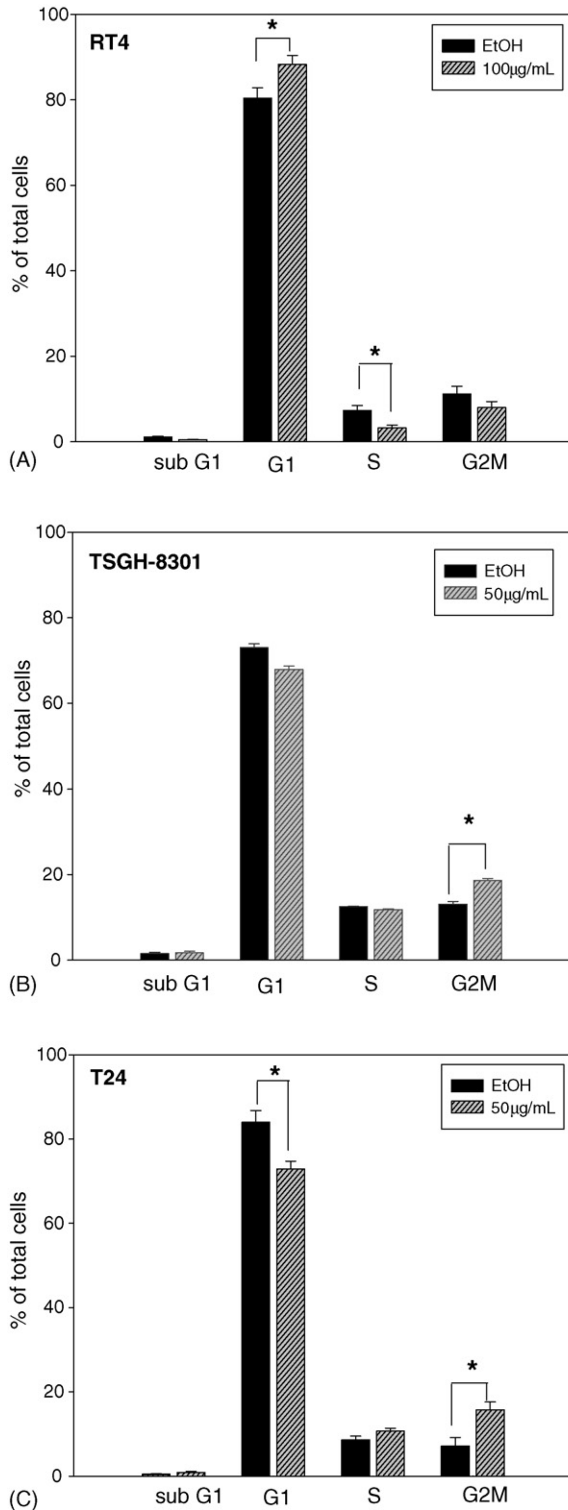

Fig. 4. Cell cycle distribution identified by flow cytometry. Cells were treated with ACCE for 72 h as indicated. Cell cycle distribution was quantified by CellQuest software. Results were averaged from triplicate experiments and expressed in mean ± S.D. $n = 3$, $^{*}p<0.05$.

of p21 was observed beginning at 24 h and proceeding to 48 h post treatment with ACCE at 100 μg/mL (Fig. 5A). Results were closely consistent with Kawasaki et al. (1994). p21 may be a universal inhibitor of cyclin kinases and inhibit the activity of each member of the cyclin/CDK family. Overexpression of p21 inhibits the proliferation of mammalian cells (Kawasaki et al., 1996). However, lacking any induction in p53 level (Fig. 5A), the p53-dependent mechanism is obviously unlikely. These results suggest the interesting possibility that the G_1 arrest of RT4 can be attributed to the so-called replicative senescence (Fang et al., 1999), an alternative pathway directly initiated by the activated $p21^{cip1}$, which in turn inhibited CDK2 with subsequent down-regulation of ppRB. P53-independent induction of p21 also occurs under a variety of conditions such as following serum or cytokine TGF-β stimulation (Datto et al., 1995), in response to chemicals such as mimosine or okadaic acid induced growth arrest (Alpan and Pardee, 1996), or in replicative senescence (Fang et al., 1999). Some similar results have been supported by Chatterjee et al. (2004). Thus, although tumor suppressor pathways involving p53, p21, and pRb overlap, and one determinant influences the others significantly, there are additional influences that alter the function of these proteins. There are two major classes of cell cycle checkpoints: DNA damage checkpoints and dependency checkpoints. DNA damage checkpoints delay cell cycle transition from G_1 to S and from G_2 to M, thereby providing more time for DNA repair. Essential components of the G_1 checkpoint include ATM, p53, RB, Chk2 and $p21^{WAF1}$ (Abraham, 2001). DNA damage activates ATM kinase, which phosporylates p53 and chk2, leading to the induction and activation of p53. In turn, p53 transactivates $p21^{WAF1}$, which inhibits the G_1 cyclin-dependent kinases that normally inactivate RB, and thereby represses the E2F transcription factors that initiate S phase (Sherr, 1996). Obviously, this was not with the case of RT4 post ACCE treatment. In human bladder TCC, alterations in tumor suppressor genes such as *p53* and *Rb* are known to be common events and have been reported to be associated with tumor progression (Chatterjee et al., 2004; Cote et al., 1998). Clinically, most bladder tumors are superficial (stage Ta or T1); among which 70% are papillary non-invasive (stage Ta) and the remaining 30% manifest with early stromal invasion (stage T1). Ta and T1 bladder urothelial tumors have important alterations of the cell cycle regulatory pathways of transition from G_1 to S phase of the cell cycle (the so-called pRb pathway), mainly characterized by overexpression of the upstream regulators cyclin D3 and cyclin D1, high tumor cell proliferation, and down-regulation of the CDK inhibitors $p21^{Waf1}$ and $p27^{Kip1}$ (Lopez-Beltran et al., 2004). Loss of $p21^{Waf1}$ expression is a strong predictor of reduced survival in primary superficial bladder cancers. The ACCE treated RT4 cells exhibited simultaneous up-regulation of p21, evidencing the fact that ACCE can exhibit a very effective therapeutic effect in treatment of superficial bladder cancers.

The simultaneous down-regulations of Cdc2 and Cyclin B1 for both cell lines TSGH-8301 and T24 as affected by ACCE at 50 μg/mL at 48–72 h (for Cyclin B1) and at 72 h (for Cdc2) (Fig. 5B and C) are speculative of resulting in insufficient amount of Cdc2–Cyclin B1 complexes that are normally required for the transition of G_2 to M phases (Clemens et al., 2003). Biologically, an efficient complex is needed for an effective signaling pathway, which in turn can be affected by change in either component qualitatively and quantitatively. Hence, the results shown

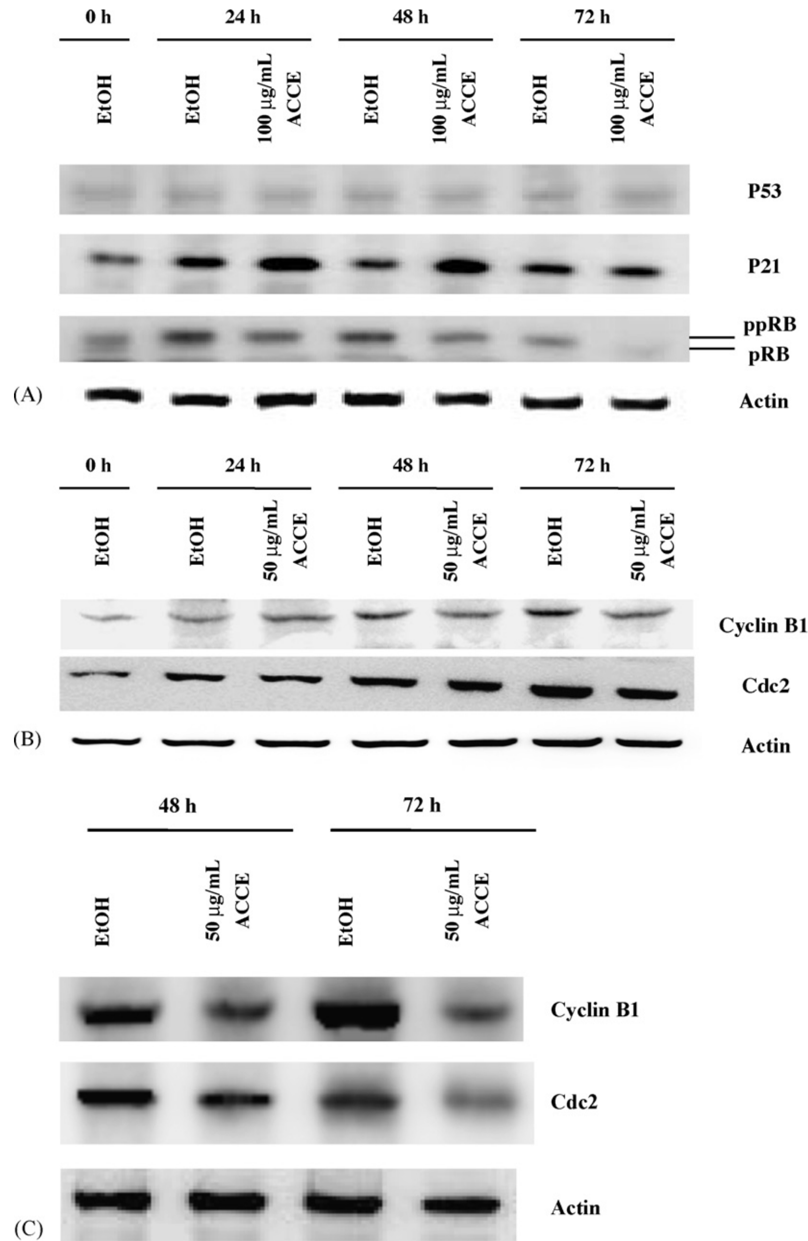

Fig. 5. Western blot analysis for the three TCC cell lines. ACCE (100 μg/mL added to RT4, 50 μg/mL added to TSGH-8301 and T24, respectively), after incubated for 24, 48 and 72 h, respectively, cell lysate proteins were loaded for investigating G_1 phase (for RT-4) (A); or G_2M phase TSGH-8301 (B) and T24 (C) (also refer to Peng et al., 2006), respectively. EtOH was used as the vehicle control.

in Fig. 5B and C could mean that Cdc2–Cyclin B1 complex had been significantly diminished, resulting in totally non-signaling, a fact further evidencing in parallel that the status of cell differentiation is associated with the drug resistance, e.g. a more significant degree of G_2M arrest was demonstrated by T24 than TSGH-8301 (Fig. 4B and C).

3.6. Migration capability is directly related to the invasive power as well as the status of differentiation

In comparison with the absolute migration capability (MC_A), T24 always showed the greatest capability. Thus, in plain vehicle EtOH, the MC_A values were 1.35×10^{-2}, 1.25×10^{-2}, and 0.17×10^{-2} mm/h, respectively for cell lines T24, TSGH-8301 and RT4, whereas the values were correspondingly reduced to 1.14×10^{-2}, 0.89×10^{-2}, and 0.11×10^{-2} mm/h at ACCE 50 μg/mL; and to 1.01×10^{-2}, 0.60×10^{-2}, and 0.03×10^{-2} mm/h at ACCE 100 μg/mL; respectively (Table 2, Fig. 6). Obviously, ACCE has shown very effective inhibitory power on the cell migration. Results again implicate the most invasive character of T24 among these three cell lines as often cited (Memon et al., 2005).

In the transwell assay, taking the vehicle cultivation as the control, the cell number ratio of ACCE/EtOH after incubated for 72 h at 50 μg/mL was 0.70 for T24, 0.38 for TSGH-8301 (Fig. 7; quantitative data for RT4 treated with ACCE at

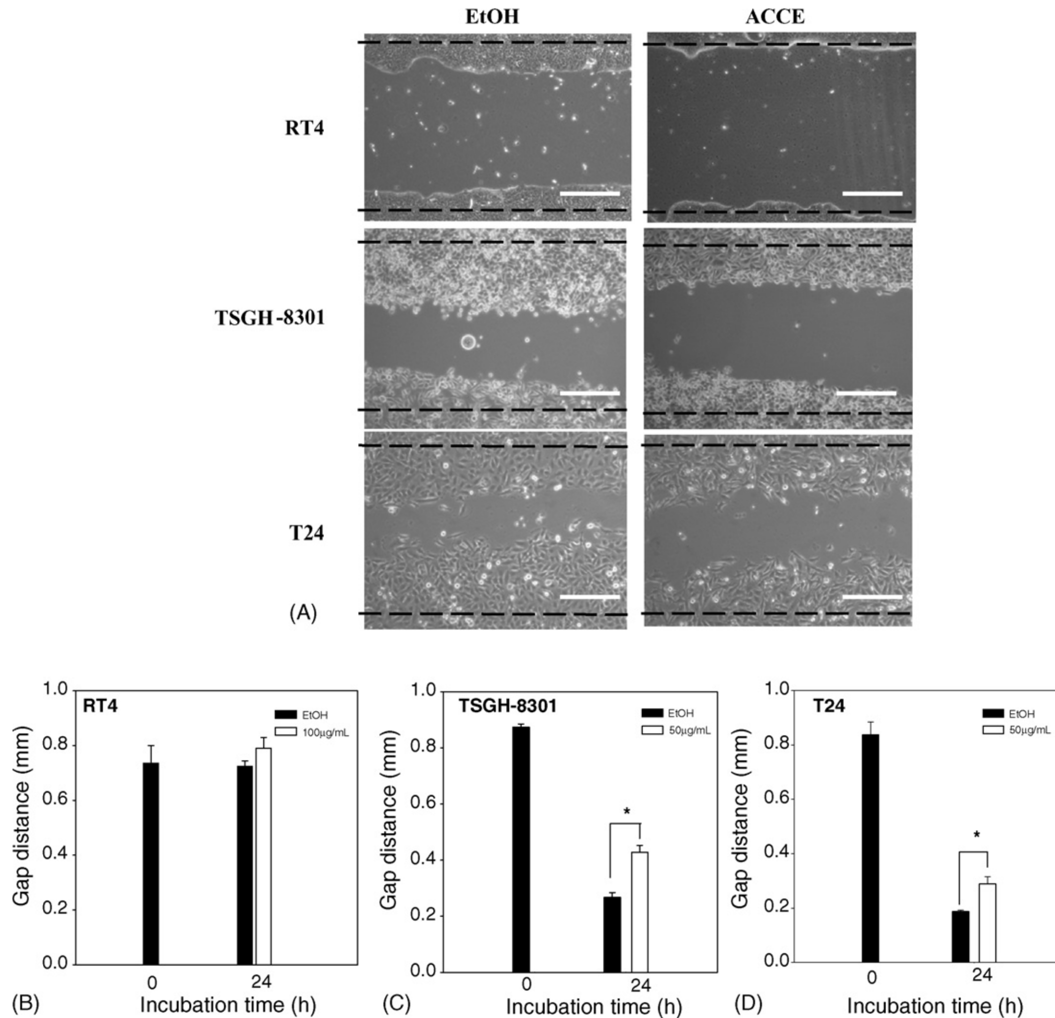

Fig. 6. Wound scratch assay on the three cell lines. The well surface with confluent cells was scratched by a 200 λ-pipette tip, then incubated with EtOH alone or with ACCE at 100 μg/mL (RT4), or 50 μg/mL (TSGH-8301 and T24) for 24 h (A), magnification 100×, scale bar = 0.2 mm. The migrating distances were measured and plotted for RT4 (B), TSGH-8301 (C), and T24 (D, also refer to Peng et al., 2006), respectively. $n = 3$, $^{*}p < 0.05$.

Table 2
The absolute migration capability (MC_A) determined by the wound scratch test on different cell lines as affected by the ACCE[a]

Incubation status[b]	Absolute migration capability (mm/h)		
	RT4	TSGH-8301	T24
EtOH control	0.17×10^{-2}	1.25×10^{-2}	1.35×10^{-2}
ACCE, 50 ng/mL	0.11×10^{-2}	0.89×10^{-2}	1.14×10^{-2}
ACCE, 100 ng/mL	0.03×10^{-2}	0.60×10^{-2}	1.01×10^{-2}

[a] MC_A: the absolute migration capability (mm/h) calculated by Eq. (1).
[b] Data obtained at 24 h of incubation.

100 μg/mL not shown, because of lacking motility). Together with the results shown in Fig. 6, it is conclusive that ACCE is an effective anti-migrating agent for cancer cells such as T24 and TSGH-8301, which can be extended to the consideration that the in vitro MC_A can be a good measure for evaluating the metastatic capability of a cancer cell line *in vivo*.

3.7. Active form of MMP-9 in T24 suppressed by ACCE

The results demonstrated by zymographic analysis revealed that ACCE failed to suppress both RT4 and TSGH-8031 with regard to both enzymes MMP-2 and MMP-9, whereas the expression of active MMP-9 (82 kDa) that was found for T24 cell line in the vehicle control was down-regulated at 72 h on treatment of ACCE (data not shown), implicitly ACCE can be therapeutically effective for the invasive bladder cancers such as T24. The pathway in which the active form of MMP-9 was down-regulated by ACCE remains for our further investigation.

The anti-tumor promoting effect of ACCE that can be ascribed to the enriched content of ergosterol in the ACCE (Table 1). Yazawa et al. (2000) has suggested that ergosterol present in aqueous extract of Polyporus could provide significant protection against the promotion of bladder tumor induced by many types of promoters. Recently, similar effect of ergosterol on breast cancer was also cited by Subbiah and Abplanalp (2003).

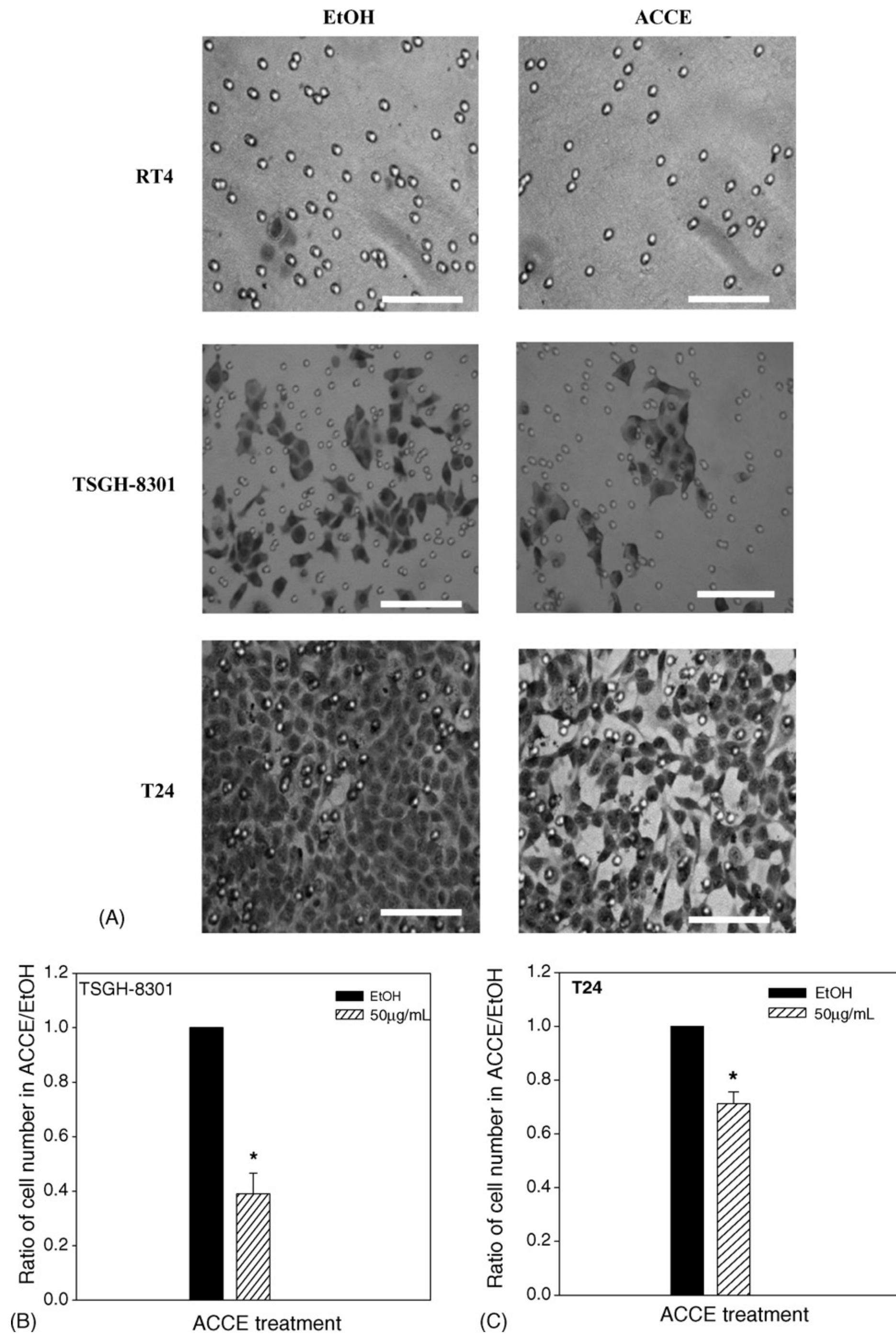

Fig. 7. Transwell assay on the three cell lines. Cells (10^5 cells/cm^2) were added with ACCE at 100 µg/mL (RT4), or 50 µg/mL (TSGH-8301 and T24, also refer to Peng et al., 2006), or with vehicle EtOH alone (A), respectively, and incubated for 72 h, magnification 400×, scale bar = 0.1 mm. The motility ratios calculated by dividing the cell numbers of ACCE-treated to that treated with EtOH alone are shown for TSGH-8301 (B), and T24 (C), respectively. $n = 3$, $^{*}p < 0.05$.

4. Conclusion

In conclusion, ACCE has showed rather different significant inhibitory effects on the growth and proliferation of TCC cell lines, RT4, TSGH-8301, and T24. In terms of the cell cycle regulatory protein expressions, RT4 proceeds most probably through the mechanism of replicative senescence, as evidenced by the p53-independent overexpression of p21 with simultaneous down alteration of pRb. On the contrary, growth inhibition of TSGH-8301 and T24 as affected by simultaneous down-regulations of Cdc2 and Cyclin B1 were attributed to the insufficient and destabilized Cdc2–Cyclin B1 complex formation. ACCE was shown to have effectively suppressed the most invasive T24 in terms of migration capability, an indicator of metastasis *in vivo*. Moreover, ACCE is also effective for the superficial TCC cell line RT4.

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References

Abraham, R.T., 2001. Cell cycle checkpoint signaling through the ATM and ATR kinases. Genes and Development 15, 2117–2196.

Alpan, R.S., Pardee, A.B., 1996. p21WAF1/CIP1/SDI1 is elevated through a p53-independent pathway by mimosine. Cell Growth and Differentiation 7, 893–901.

Bubenik, J., Baresova, M., Viklicky, V., Jakoubkova, J., Sainerova, H., Donner, J., 1973. Established cell line of urinary-bladder carcinoma (T24) containing tumor-specific antigen. International Journal of Cancer 11, 765–773.

Chang, T.T., Chou, W.N., 1995. *Antrodia cinnamomea* sp. nov. on Cinnamomum kanehirai in Taiwan. Mycological Research 99, 756–758.

Chatterjee, S.J., Datar, R., Youssefzadeh, D., George, B., Goebell, P.J., Stein, J.P., Young, L., Shi, S.R., Gee, C., Groshen, S., Skinner, D.G., Cote, R.J., 2004. Combined effects of p53, p21, and pRb expression in the progression of bladder transitional cell carcinoma. Journal of Clinical Oncology 22, 1007–1013.

Clemens, D.L., Calisto, L.E., Sorrell, M.F., Tuma, D.J., 2003. Ethanol metabolism results in G2/M cell-cycle arrest in recombinant HepG2 cells. Hepatology 38, 385–393.

Cote, R.J., Dunn, M.D., Chatterjee, S.J., Stein, J.P., Shi, S.R., Tran, Q.C., Hu, S.X., Xu, H.J., Groshen, S., Taylor, C.R., Skinner, D.G., Benedict, W.F., 1998. Elevated and absent pRb expression is associated with bladder cancer progression and has cooperative effects with p53. Cancer Research 58, 1090–1094.

Datto, M.B., Li, Y., Panus, J.F., Howe, D.J., Xiong, Y., Wang, X.F., 1995. Transforming growth factor beta induces the cyclin-dependent kinase inhibitor p21 through a p53-independent mechanism. Proceedings of the National Academy of Sciences of the United States of America 92, 5545–5549.

Doll, R., Peto, R., 1981. The causes of cancer: quantitative estimates of avoidable risks of cancer in the United States today. Journal of National Cancer Institute 66, 1191–1308.

Fang, L., Igarashi, M., Leung, J., Sugrue, M.M., Lee, S.W., Aaronson, S.A., 1999. $p21^{Waf1/Cip1/Sdi1}$ induces permanent growth arrest with markers of replicative senescence in human tumor cells lacking functional p53. Oncogene 18, 2789–2797.

Folch, J., Lees, M., Stanley, S.G.H., 1957. Simple method for the isolation and purification of total lipids from animal tissues. The Journal of Biological Chemistry 226, 497–509.

Gee, J., Sabichi, A.L., Grossman, H.B., 2002. Chemoprevention of superficial bladder cancer. Critical Reviews in Oncology/Hematology 43, 277–286.

Hsiao, G., Shen, M.Y., Lin, K.H., Lan, M.H., Wu, L.Y., Chou, D.S., Lin, C.H., Su, C.H., Sheu, J.R., 2003. Antioxidative and hepatoprotective effects of *Antrodia camphorata* extract. Journal of Agricultural and Food Chemistry 51, 3302–3308.

Hsu, Y.L., Kuo, Y.C., Kuo, P.L., Kuo, Y.H., N, L.T., Lin, C.C., 2005. Apoptotic effects of extract from *Antrodia camphorata* fruiting bodies in human hepatocellular carcinoma cell lines. Cancer Letters 221, 77–89.

Jemal, A., Murray, T., Samuels, A., Ghafoor, A., Ward, E., Thun, M.J., 2003. Cancer statistics. CA: A Cancer Journal for Clinicians 53, 5–26.

Kawasaki, T., Tomita, Y., Bilim, V., Takeda, M., Takahashi, K., Kumanishi, T., 1996. Abrogation of apoptosis induced b DNA-damaging agents in human bladder-cancer cell lines with *p21*/WAF1/CIP1 and/or *p53* gene alterations. International Journal of Cancer 68, 501–505.

Kawasaki, T., Tomita, Y., Watanabe, R., Tanikawa, T., Kumanishi, T., Sato, S., 1994. mRNA and protein expression of p53 mutations in human bladder cancer cell lines. Cancer Letters 82, 113–121.

Knowles, M.A., 1999. The genetics of transitional cell carcinoma: progress and potential clinical application. British Journal of Urology International 84, 412–427.

Liu, J., Zhang, X., Yang, F., Li, T., Wei, D., Ren, Y., 2005. Antimetastatic effect of a lipophilic ascorbic acid derivative with antioxidation through inhibition of tumor invasion. Cancer Chemotherapy and Pharmacology 2, 1–7, August 2 [Epub ahead of print].

Lopez-Beltran, A., Luque, R.J., Alvarez-Kindelan, J., Quintero, A., Merlo, F., Requena, M.J., Montironi, R., 2004. Prognostic factors in survival of patients with stage Ta and T1 bladder urothelial tumors: the role of G_1–S modulators (p53, $p21^{Waf1}$, $p27^{Kip1}$, cyclin D1, and cyclin D3), proliferation index, and clinicopathologic parameters. American Journal of Clinical Pathology 122, 444–452.

Memon, A.A., Chang, J.W., Oh, B.R., Yoo, Y.J., 2005. Identification of differentially expressed proteins during human urinary bladder cancer progression. Cancer Detection and Prevention 29, 249–255.

Pasanen, A.L., Yli-Pietila, K., Pasanen, P., Kalliokoski, P., Tarhanen, J., 1999. Ergosterol content in various fungal species and biocontaminated building materials. Applied and Environmental Microbiology 65, 138–142.

Peng, R.Y., Chyau, C.C., Su, C.H., Chen, C.C. (Eds.), in press. Review on the unique Formosan medicinal mushroom *Taiwanofungus camphoratus* (*Antrodia camphorata*), in: Govil, J.N. (Principal Scientist and Chief Editor), Recent Progress in Medicinal Plants, vol. 18, Division of Genetics, Indian Agricultural Research Institute, New Delhi 110 002, India, 2006.

Peng, C.C., Chen, K.C., Su, C. H., Hsieh-Li, H. M., 2006. Human urinary bladder cancer T24 cells are susceptible to the *Antrodia camphorata* extracts, Cancer Letters, 30 January, [Epub ahead of print].

Rigby, C.C., Franks, L.M., 1970. A human tissue culture cell line from a transitional cell tumor of the urinary bladder: growth, chromosome pattern and ultrastructure. British Journal of Cancer 24, 746–754.

Shen, Y.C., Wang, Y.H., Chou, Y.C., Chen, C.F., Lin, L.C., Chang, T.T., Tien, J.H., Chou, C.J., 2004. Evaluation of the anti-inflammatory activity of zhankuic acids isolated from the fruiting bodies of *Antrodia camphorata*. Planta Medica 70, 310–314.

Sherr, C.J., 1996. Cancer cell cycles. Science 274, 1672–1677.

Skinner, D.G., Lieskovsky, G., 1988. 16 years experience in the management of patients with deeply invasive bladder cancer. European Urology 14, 30–31.

Song, T.Y., Hsu, S.L., Yen, G.C., 2005. Induction of apoptosis in human hepatoma cells by mycelia of *Antrodia camphorata* in submerged culture. Journal of Ethnopharmacology 100, 158–167.

Su, C.H., 2002. Health Guardian Angel: *Antrodia camphorata*, 1st ed. EKS Book Publishing Co., Taipei.

Subbiah, M.T., Abplanalp, W., 2003. Ergosterol (major sterol of baker's and brewer's yeast extracts) inhibits the growth of human breast cancer cells in vitro and the potential role of its oxidation products. International Journal for Vitamin and Nutrition Research 73, 19–23.

Tsai, Z.T., 1982. In: Liaw, S.L. (Ed.), The Use and the Effect of Ganoderma, 1st ed. Sheng-Yun Publishers Inc., Taichung, Taiwan, pp. 116–117.

Williams, S.G., Stein, J.P., 2004. Molecular pathways in bladder cancer. Urological Research 32, 373–385.

Wu, S.H., Ryvarden, L., Chang, T.T., 1997. *Antrodia camphorata* ("niu-chang-chih"), new combination of a medicinal fungus in Taiwan. Botanical Bulletin of Academia Sinica 38, 273–275.

Wu, S.H., Yu, Z.-H., Dai, Y.-C., Dai, Y.-C., Chen, C.-T., Su, C.-H., Chen, L.-C., Hsu, W.-C., Hwang, G.-Y., 2004. *Taiwanofungus*, a polypore new genus. Fungal Science 19, 109–116.

Xiong, Y., Hannon, G.J., Zhang, H., Casso, D., Kobayashi, R., Beach, D., 1993. p21 is a universal inhibitor of cyclin kinases. Nature 366, 701–704.

Yazawa, Y., Yokota, M., Sugiyama, K., 2000. Antitumor promoting effect of an active component of Polyporus, ergosterol and related compounds on rat urinary bladder carcinogenesis in a short-term test with concanavalin A. Biological and Pharmaceutical Bulletin 23, 1298–1302.

Yeh, M.Y., Yu, D.S., Chen, S.C., Lin, M.S., Chang, S.Y., Ma, C.P., Han, S.H., 1988. Establishment and characterization of a human urinary bladder carcinoma cell line (TSGH-8301). Journal of Surgical Oncology 37, 177–184.

Zang, M., Su, CH., 1990. *Ganoderma comphoratum*, a new taxon in genus *Ganoderma* from Taiwan, China. Acta Botanica Yunnanica 12, 395–396.