Akebia trifoliate (Thunb.) Koidz Seed Extract Inhibits the Proliferation of Human Hepatocellular Carcinoma Cell Lines via Inducing Endoplasmic Reticulum Stress

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Received 30 April 2014; Revised 16 July 2014; Accepted 14 August 2014; Published 20 October 2014

1. Introduction

According to the World Cancer Report from IARC (International Agency for Research on Cancer), human hepatocellular carcinoma (HCC) is the sixth most common malignant cancer throughout the world and the third in China; the cases in China are more than half of the total worldwide [1, 2]. Surgical resection during early stages is still the dominant therapy for HCC. Unfortunately, most patients have already missed this opportunity by the time they see a doctor, and even patients who have been operated upon are at risk of recurrence, metastasis, and poor prognosis. On the other hand, most of the anticancer drugs may show severe side effects. Under these circumstances, the need of exploring new drugs in natural products, such as extracts of traditional Chinese herbs, becomes one promising alternative [3, 4]. In fact, in China, nearly 80% percent of patients suffering from HCC have taken herbs or herbal compounds to alleviate symptoms, relieve the side effects of radiotherapy or chemotherapy, improve the quality of life, and even extend life span [5–7].

Akebia Fructus is the near-mature dried fruit of plant Akebia and belongs to Lardizabalaceae family. The family is widely distributed over East Asia including China, Japan, and Korea. Akebia Fructus has long been used for hepatic carcinoma (HCC) in China, while the molecular mechanism remains obscure. Our recent work found that Akebia trifoliate (Thunb.) Koidz seed extract (ATSE) suppressed proliferation and induced endoplasmic reticulum (ER) stress in SMMC-7721. The present study aimed to throw more light on the mechanism. ER stress occurred after ATSE treatment in HepG2, HuH7, and SMMC-7721 cells, manifested as ER expansion, and SMMC-7721 was the most sensitive kind in terms of morphology. Cell viability assay showed that ATSE significantly inhibited cells proliferation. Flow cytometry analysis indicated that ATSE leads to an upward tendency of G0/G1 phase and a reduced trend of the continuous peak after G2/M phase in HepG2; ATSE promoted apoptosis in HuH7 and a notable reduction in G0/G1 phase; ATSE does not quite influence cell cycles of SMMC-7721. Western blot analysis showed an increased trend of the chosen ER stress-related proteins after different treatments but nonsignificantly; only HYOU1 and GRP78 were decreased notably by ATSE in HuH7. Affymetrix array indicated that lots of ER stress-related genes’ expressions were significantly altered, and downward is the main trend. These results suggest that ATSE have anticancer potency in HCC cells via partly inducing ER stress.
trifoliate (Thunb.) Koidz, and Akebia trifoliate (Thunb.) Koidz. var. australis (Diels.) Rehd, which are listed in the Chinese Pharmacopoeia [9]. In some experimental studies in vivo and vitro, the water extract of the whole fruit of Akebia trifoliate, the ethanol extract of Akebia quinata seed, or the separated ingredients showed antitumor activity [10–13], while the molecular mechanism remains obscure.

Akebia Fructus is always used as a whole fruit, which is composed of the pulp and the seed. In our recent research, extracts of different parts of Akebia trifoliate (Thunb.) Koidz, including the whole fruit, the pulp, and the seed, were used to treat SMMC-7721 HCC cells, respectively, and we unexpectedly found that seed part showed the strongest effect in inhibiting malignant proliferation and inducing endoplasmic reticulum (ER) stress [14–18], whilst no reference data on ER stress currently exist. In view of this, this further study aims to prepare an Akebia trifoliate (Thunb.) Koidz seed extract (ATSE) by n-butanol and throw more light on the molecular mechanism in a panel of three HCC cell lines, HepG2, HuH7, and SMMC-7721.

2. Material and Methods

2.1. Chemicals and Antibodies. Ethanol, Petroleum ether, ethyl acetate, and n-butanol are all AR grade and purchased from Sinophar Chemical Reagent Co., Ltd. (Shanghai, China). Trizol, G418, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Life Technologies (Carlsbad, CA, USA). Rabbit anti-SEC63 (1:800) polyclonal and rabbit anti-DNAJBI1 (1:500) polyclonal were purchased from Sigma-Aldrich (St. Louis, MO, USA). Rabbit anti-HSP90AA1 (1:1000) was purchased from Stressgen (Enzo Life Sciences, NY, USA). Rabbit antimonoclonal HYOU1 (1:5000) was purchased from Epitomics (Abcam, CA, USA). Mouse monoclonal anti-GRP78 (1:1000), mouse monoclonal anti-HSPA9 (1:1000), and mouse monoclonal anti-GAPDH (1:2500) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The secondary antibodies HRP Goat anti-mouse IgG and HRP Donkey anti-rabbit IgG were purchased from Biolegend (San Diego, CA, USA).

2.2. Plant Material. The whole fruit of Akebia trifoliate (Thunb.) Koidz was purchased from Kangqiao Herbal Pieces Co. Ltd. (Shanghai, China) in May 2013, which was produced in Zhejiang Province and authenticated by Lihong Wu, Institute of Chinese Materia Medica, Shanghai University of Traditional Chinese Medicine; a voucher specimen (number 130-319) was deposited in the standardization lab of this institute.

2.3. Preparation of Akebia trifoliate (Thunb.) Koidz Seed Extract (ATSE). The seed was peeled off from the whole fruit and dried at 60°C for 24 hours; dried seed (800 g) was smashed and soaked in 75% ethanol for 2 hours at room temperature and then extracted by 8 L 75% ethanol reflux at 80°C for 2 hours and filtered by gauze; the filtration residue was extracted again under the same condition; all resulting filtrations were combined and concentrated by Rotavapor (BÜCHI Labortechnik, Switzerland) under reduced pressure; 2700 mL concentrated extract was obtained, followed by successive extraction with the same volume of Petroleum ether, ethyl acetate, and water-saturated n-butanol, three times in each solvent; this procedure resulted in three extracts; the n-butanol soluble extract was further concentrated by Rotary Evaporator (IKA, Germany) at 60°C (20–40 rpm); 310 mL extractum was obtained and then freeze-dried to 33.26 g power, which is simply called ATSE in experiment. The extract yield was 4.26% (w/w). ATSE was diluted to 0.5 g/mL by distilled water and then dissolved in the RPMI 1640 culture medium to 10 mg/mL and finally filtered through a 0.45 μm filter for use.

2.4. Cell Lines and Culture. HepG2, HuH7, and SMMC-7721 cell lines were obtained from Chinese Academy of Sciences (Shanghai, China), cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin. RPMI 1640 medium, Trypsin, penicillin, and streptomycin were all purchased from HyClone (Thermo scientific, UT, USA). These cells were routinely cultured in humidified 95% air and 5% CO2 at 37°C.

2.5. Cell Morphological Assessment. Cells were split and seeded in 96-well and 6-well plates, 18–24 hours after seeding, and when the confluency was about 70%, G418 or ATSE was added to four different groups according to the requirement: untreated control; G418 group (0.45 mg/mL); ATSE group (0.625 mg/mL); the combination group was composed of half dose of each (0.225 mg/mL G418 plus 0.31 mg/mL ATSE). 72 hours after treatment, cells in 96-well plates were used for MTT assay, and the cell morphology of 6-well plates was firstly examined under an inverted phase contrast microscope (Olympus CKX41, Tokyo, Japan), then for the other different assays.

2.6. Cell Viability Assay. Cell survival was assessed using an MTT assay at 72 hours after treatment; the culture medium of 96-well plate was removed and replaced with 0.5 mg/mL MTT solution. After 4 h incubation at 37°C, this solution was removed and the resulting blue formazan was solubilized in 100 μL DMSO; then the optical density was read at 490 nm using a microplate reader (BioTek Synergy 2, BioTek Instruments, VT, USA). Cell viability was expressed as a percentage of the control.

2.7. Flow Cytometry Analysis of Cell Cycle. Treatments of cells were performed according to the protocol of the PI detection kit (KeyGEN Biotech, Shanghai, China). Briefly, cells were harvested and resuspended in cold PBS at a concentration of 1 × 10^6 cells/mL and then fixed by 3 times volume of cold anhydrous ethanol (final concentration is 75%) and kept at 4°C for 2 hours; ethanol was then removed and resuspended in 500 μL buffer A, followed by addition of RNase A (final concentration was 0.25 mg/mL) and 5 μL PI solution, and then incubated for 30 min at room temperature in the dark;
finally analysis was performed by using a Flow Cytometer (BD FACS Calibur, BD Biosciences, CA, USA).

### 2.8. Western Blot Analysis

Cell lysates were harvested in RIPA buffer (50 mM Tris (pH 7.4), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS). The whole cell lysates were centrifuged at 12,000 rpm for 15 minutes at 4°C; the supernatant was collected and protein concentration was measured by Pierce BCA Protein Assay Kit (Thermo scientific, UT, USA). Cell lysates (20 μg) were resolving on 6–8% SDS-PAGE followed by transfer onto 0.45 μm PVDF membrane (Merk millipore, MA, USA). Subsequently, membranes were incubated with 5% skimmed milk in TBST for 1 h, followed by probing with the primary antibody overnight at 4°C. After washing with TBST, the membranes were incubated for 1 h at room temperature with HRP-conjugated secondary antibodies; bands were visualized using BeyoECL Plus western blot detection system (Beyotime, Shanghai, China). Results of the Western blot assay reported here are representative of three experiments; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as internal control.

### 2.9. Affymetrix Array

Total RNA was isolated from cells with Trizol according to manufacturer’s protocol. The qualities of all RNA samples were monitored: the absorbance at 260 nm and 280 nm was measured by NanoDrop 1000 (Thermo Fisher Scientific, MA, USA) and acceptable A260/280 ratios were in the range of 1.7–2.1; the RNA 6000 Nano kit (Agilent Technologies, CA, USA) was used to detect RIN (RNA integrity number) and 28S/18S ratios by Agilent 2100 Bioanalyzer (Agilent Technologies, CA, USA). Then cDNA was synthesized using an Ambion High-capacity cDNA synthesis kit (Thermo Fisher Scientific, UT, USA). Cell lysates (20 μg) were resolving on 6–8% SDS-PAGE followed by transfer onto 0.45 μm PVDF membrane (Merk millipore, MA, USA). Subsequently, membranes were incubated with 5% skimmed milk in TBST for 1 h, followed by probing with the primary antibody overnight at 4°C. After washing with TBST, the membranes were incubated for 1 h at room temperature with HRP-conjugated secondary antibodies; bands were visualized using BeyoECL Plus western blot detection system (Beyotime, Shanghai, China). Results of the Western blot assay reported here are representative of three experiments; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as internal control.

### 3. Results

#### 3.1. ATSE Cause Different Morphological Changes in HCC Cell Lines

The morphological changes induced by ATSE were observed. HepG2 cells were not sensitive to 0.45 mg/mL G418 (Figure 1(b)), while mild endoplasmic reticulum stress (see the white arrowhead) was induced by 0.625 mg/mL ATSE (Figure 1(c)); the effect of the combination of half doses of each (Figure 1(d)) was similar to that in Figure 1(c). In contrast to HepG2 cells, 0.45 mg/mL G418 caused obvious morphological changes in HuH7 cells, including a decreased number of cells, increased blank area, and even emergence of the apoptotic body in a couple of cells (Figure 1(f)); 0.625 mg/mL ATSE caused mild ER stress (Figure 1(g)); the effect of the combination group (Figure 1(h)) was like the overlay of Figures 1(f) and 1(g). As to SMMC-7721 cells, 0.625 mg/mL ATSE resulted in remarkable ER stress, displayed as different degree of ER expansion (Figure 1(k)); in the combination group (Figure 1(l)), since the ATSE dose was cut by half, the ER stress was comparatively lighter.

In brief, ATSE induced ER stress in these three HCC cell lines but the degree varied and it seemed that SMMC-7721 cells were the most sensitive kind.

#### 3.2. ATSE Suppress Cell Viability in HCC Cell Lines

To assess the effect of ATSE on cell viability, MTT assay was performed. As shown in Figure 2, compared to the untreated control, cell viability decreased significantly but varied in degree: values of ATSE groups were reduced to 85.9% in HepG2, 19.1% in HuH7, and 91.8% in SMMC-7721 cells, respectively; besides, in G418 groups, values were reduced to 83.3% in HepG2, 3.8% in HuH7, and 29.6% in SMMC-7721 cells, respectively; in the combination groups, values were reduced to 70.3% in HepG2, 12.5% in HuH7, and 70.7% in SMMC-7721 cells, respectively. On the other hand, in HepG2 and HuH7 cells, ATSE and the combination groups exhibited no significant difference compared to G418, but in SMMC-7721 cells, the values of these two groups were notably higher than that of G418.

#### 3.3. Different Cell Cycle Influences of ATSE on HCC Cell Lines

To figure out whether the proliferation inhibition is due to cell cycle arrest, the cell cycle analysis by Flow cytometry was performed, and these HCC cell lines showed different features as shown below.

Firstly, in HepG2 cells, at 72 hours after G418 incubation, the percentage of cells in G0/G1 phase decreased significantly to 52.9% versus 59.7% of the control (Figures 3(b) and 3(m)), while the percentage of same phase for ATSE was 62.9% and slightly higher than the control, albeit nonsignificantly (Figures 3(c) and 3(m)), which possibly indicated various action modes between ATSE and G418 on HepG2 cells; furthermore, it seemed that the amount of the continuous peak after G2/M phase was reduced (see black arrowheads in Figure 3(c)); this could be another aspect of ATSE (Figures 3(a) and 3(c)). An apoptotic peak appeared in the combination group and the percentage reduced to 47.5% notably (Figures 3(d) and 3(m)).

In HuH7 cells (Figures 3(e) to 3(h)), 72 hours after cultivation, apoptosis occurred among all groups before G0/G1
Figure 1: ATSE causes different morphological changes in HCC cell lines. G418 (0.45 mg/mL), ATSE: Akebia trifoliate seed extract (0.625 mg/mL), and 1/2(G418 + ATSE): a combination of half dosage of each accordingly. (a–d) Images of HepG2 cells after different treatments, (e–h) images of HuH7 cells after different treatments, and (i–l) images of SMMC-7721 cells after different treatments. White arrowhead means different degree of ER expansion within cells. These images were taken under the 400x inverted phase contrast microscope.

Figure 2: ATSE suppress cell viability in HCC cell lines. Cell viability was tested by MTT. G418 (0.45 mg/mL), ATSE: Akebia trifoliate seed extract (0.625 mg/mL), and 1/2(G418 + ATSE): a combination of half dosage of each accordingly. The results are expressed as mean ± S.D. (n = 4). ** P < 0.01 and * P < 0.05 (versus control group); ## P < 0.01 and # P < 0.05 (versus G418 group).

3.4. Effect of ATSE on the Protein Expression of ER Stress-Related Biomarkers. To further clarify the molecular mechanism of ATSE induced ER stress in HepG2, HuH7, and SMMC-7721 cells, some ER stress-related proteins were chosen and examined by Western blot analysis. As indicated in Figure 4, compared with the untreated control, most of the proteins expressions displayed an upward tendency after different treatments, albeit nonsignificantly (P > 0.05). However, in HuH7 cells, ATSE decreased the protein expression of HYOU1 and GRP78 significantly (Figures 4(a) and 4(e)).
Figure 3: Different cell cycle influences of ATSE on HCC cell lines. Cell cycle analysis was performed by Flow Cytometry. G418 (0.45 mg/mL); ATSE: Akebia trifoliate seed extract (0.625 mg/mL); 1/2(G418 + ATSE): a combination of half dosage of each accordingly. (a–d) Images of HepG2 cells after different treatments, (e–h) images of HuH7 cells after different treatments, (i–l) images of SMMC-7721 cells after different treatments, (m) HepG2 cell cycle distribution, (n) HuH7 cell cycle distribution, and (o) SMMC-7721 cell cycle distribution. M1: G0/G1 phase, M2: G2/M phase, and M3: S phase. (↓↓↓↓) continuous peak after G2/M phase after ATSE treatment, (↓↓) apoptotic body peak, and (↓) apoptotic cell peak. The results are expressed as mean ± S.D. (n = 3). **P < 0.01 and *P < 0.05 (versus control group); ##P < 0.01 and #P < 0.05 (versus G418 group).
Figure 4: Continued.
3.5. Expression of ER Stress-Related Genes in HepG2 Cells. For full characterization of the molecular mechanism of ATSE, Affymetrix array was used. 82 ER stress-related genes were selected one by one manually from NCBI gene database. There were significant changes in the levels of gene expression in 8 of 82 genes (Table 1); HSP90AA1 was included and upregulated by ATSE significantly.

3.6. Expression of ER Stress-Related Genes in HuH7 Cells. There were significant changes in the levels of gene expression in 38 of 82 genes (Table 2); HSP90AA1 was included and upregulated by ATSE significantly. On the other hand, HSPA9, DNAJB11, SEC63, HYOU1, and GRP78 were all downregulated notably. Furthermore, most of the genes were downregulated.

3.7. Expression of ER Stress-Related Genes in SMMC-7721 Cells. There were significant changes in the levels of gene expression in 19 of 82 genes (Table 3). SEC63, DNAJB11, HSP90AA1, and GRP78 were all downregulated notably. It is similar to HuH7 that most of the genes were downregulated notably.

3.8. Expression of the KEGG Integrin-Mediated Cell Adhesion Pathway for HepG2 Cells. Affymetrix array data also presented genes expressions information of KEGG integrin-mediated cell adhesion pathway, which helps to explain the effect of ATSE on HepG2 cells. There were significant changes in 6 of 89 genes, 4 were upregulated and 2 were downregulated (Table 4).
### Table 1: List of ER stress-related genes with significant expression in HepG2 cells (fold change ≥ 1.5-fold).

| Probe set ID | Gene symbol | Control | G418 | ATSE | 1/2(G418 + ATSE) | ATSE/control |
|--------------|-------------|---------|------|------|------------------|--------------|
| 8152628      | DERL1       | 199     | 336  | 355  | 694              | 1.78         |
| 7954196      | MGST1       | 733     | 958  | 1305 | 1663             | 1.78         |
| 7995895      | HERPUD1     | 714     | 1239 | 1166 | 2351             | 1.63         |
| **7981335**  | HSP90AA1    | **1263**| **2402**| **1946**| **3732**          | **1.54**     |
| 7956593      | OS9         | 315     | 357  | 475  | 407              | 1.51         |
| 8156838      | SEC61B      | 528     | 426  | 348  | 631              | −1.52        |
| 8080084      | MANF        | 495     | 587  | 320  | 791              | −1.55        |
| 8075182      | XBP1        | 758     | 583  | 352  | 343              | −2.16        |

G418 (0.45 mg/mL); ATSE: Akebia trifoliate seed extract (0.625 mg/mL); 1/2(G418 + ATSE): a combination of half dose of each accordingly.

### Table 2: List of ER stress-related genes with significant expression in HuH7 cells (fold change ≥ 1.5-fold).

| Probe set ID | Gene symbol | Control | G418 | ATSE | 1/2(G418 + ATSE) | ATSE/control |
|--------------|-------------|---------|------|------|------------------|--------------|
| 7954196      | MGST1       | 479     | 815  | 1026 | 720              | 2.14         |
| 8024754      | CREB3L3     | 171     | 55   | 353  | 69               | 2.06         |
| 7967563      | UBC         | 2502    | 4281 | 4550 | 7132             | 1.82         |
| **7981335**  | HSP90AA1    | **1153**| **3916**| **1725**| **3257**          | **1.50**     |
| 8114555      | HSPA9       | 1116    | 1499 | 749  | 1115             | −1.50        |
| 7949383      | SYVN1       | 326     | 235  | 208  | 224              | −1.57        |
| 7946460      | DDIT3       | 239     | 137  | 151  | 332              | −1.58        |
| 8125295      | ATF6B       | 202     | 49   | 117  | 82               | −1.73        |
| 8080084      | MANF        | 268     | 490  | 152  | 351              | −1.77        |
| 8075182      | XBP1        | 820     | 705  | 448  | 596              | −1.83        |
| 8091458      | SERP1       | 561     | 1050 | 303  | 635              | −1.85        |
| 8137526      | INSG1       | 214     | 356  | 114  | 290              | −1.88        |
| 7916432      | DHCR24      | 2595    | 560  | 1278 | 280              | −2.03        |
| 8138108      | KDELRE2     | 1272    | 1329 | 622  | 605              | −2.04        |
| 8160914      | VCP         | 423     | 764  | 200  | 227              | −2.11        |
| 7989619      | PPIB        | 1939    | 2258 | 917  | 2377             | −2.11        |
| 8066889      | STAU1       | 398     | 248  | 187  | 98               | −2.12        |
| 8128811      | UBE2J1      | 474     | 344  | 199  | 183              | −2.38        |
| 7916120      | TXNDC12     | 817     | 404  | 330  | 172              | −2.48        |
| 7958644      | ATP2A2      | 492     | 570  | 186  | 206              | −2.65        |
| 8026106      | CALR        | 1329    | 1694 | 497  | 1060             | −2.67        |
| 8051998      | MCED2       | 411     | 507  | 149  | 213              | −2.76        |
| 7995895      | HERPUD1     | 915     | 870  | 297  | 960              | −3.08        |
| **8084634**  | DNAJB11     | **279** | **947**| **89**| **529**          | **−3.14**    |
| 7906819      | ATF6        | 268     | 361  | 80   | 174              | −3.33        |
| 8041967      | ERLEC1      | 275     | 363  | 77   | 109              | −3.57        |
| **8128650**  | SEC63       | **982** | **1218**| **265**| **504**          | **−3.71**    |
| 8135480      | DNAJB9      | 256     | 431  | 53   | 351              | −4.86        |
| **7952145**  | HYOU1       | **492** | **757**| **97**| **266**          | **−5.06**    |
| 7956593      | OS9         | 1008    | 857  | 195  | 383              | −5.17        |
| 8046759      | DNAJC10     | 452     | 202  | 77   | 27               | −5.87        |
| 8023561      | LMAN1       | 941     | 1298 | 159  | 448              | −5.93        |
| 7980547      | SELIL       | 333     | 295  | 46   | 68               | −7.27        |
| **8164165**  | GRP78       | **1834**| **2581**| **241**| **1442**         | **−7.62**    |
| 7904881      | PDIA3P      | 1192    | 1261 | 131  | 644              | −9.11        |
| 7958130      | HSP90B1     | 1813    | 2793 | 198  | 1396             | −9.17        |
| 7983274      | PDIA3       | 1181    | 1226 | 119  | 602              | −9.95        |
| 8095628      | ALB         | 3131    | 1860 | 63   | 435              | −49.59       |

G418 (0.45 mg/mL); ATSE: Akebia trifoliate seed extract (0.625 mg/mL); 1/2(G418 + ATSE): a combination of half dose of each accordingly.
Table 3: List of ER stress-related genes with significant expression in SMMC-7721 cells (fold change ≥ 1.5-fold).

| Probe set ID | Gene symbol | Control | G418 | ATSE | 1/2(G418 + ATSE) | ATSE/control |
|--------------|-------------|---------|------|------|-----------------|--------------|
| 7964460      | DDIT3       | 106     | 274  | 525  | 546             | 4.94         |
| 8076481      | CYB5R3      | 262     | 209  | 539  | 279             | 2.06         |
| 8139003      | HERPUD2     | 186     | 263  | 323  | 271             | 1.74         |
| 8042107      | VRK2        | 536     | 973  | 884  | 1075            | 1.65         |
| 8156838      | SEC61B      | 299     | 566  | 469  | 638             | 1.57         |
| 8128650      | SEC63       | 776     | 954  | 522  | 653             | −1.50        |
| 8062174      | ERGIC3      | 984     | 1042 | 744  | 2022            | −1.92        |
| 7958644      | ATP2A2      | 575     | 769  | 382  | 536             | −1.50        |
| 7989619      | PPIB        | 1595    | 2090 | 989  | 2036            | −1.61        |
| 8084634      | DNAJB1I     | 323     | 442  | 196  | 535             | −1.62        |
| 7981335      | HSP90AA1    | 1428    | 2906 | 831  | 2147            | −1.72        |
| 8178498      | HLA-B       | 327     | 268  | 190  | 246             | −1.72        |
| 7958130      | HSP90B1     | 1426    | 2138 | 744  | 2022            | −1.92        |
| 8091954      | GOLIM4      | 641     | 378  | 329  | 364             | −1.95        |
| 7983274      | PDIA3       | 855     | 1271 | 437  | 949             | −1.96        |
| 8023561      | LMAN1       | 735     | 816  | 372  | 690             | −1.98        |
| 7904881      | PDIA3P      | 889     | 1360 | 440  | 980             | −2.02        |
| 8164165      | GRP78       | 1317    | 2308 | 580  | 2346            | −2.27        |
| 8160914      | VCP         | 333     | 318  | 114  | 278             | −2.91        |

G418 (0.45 mg/mL); ATSE: Akebia trifoliate seed extract (0.625 mg/mL); 1/2(G418 + ATSE): a combination of half dose of each accordingly.

Table 4: List of genes with significant expression in the KEGG integrin-mediated cell adhesion pathway for HepG2 cells (fold change ≥ 1.5-fold).

| Probe set ID | Gene symbol | Control | G418 | ATSE | 1/2(G418 + ATSE) | ATSE/control |
|--------------|-------------|---------|------|------|-----------------|--------------|
| 7983763      | MAPK6       | 233     | 314  | 382  | 405             | 1.64         |
| 8046380      | ITGA6       | 216     | 323  | 385  | 220             | 1.78         |
| 8051670      | SOS1        | 291     | 386  | 520  | 515             | 1.79         |
| 8084963      | PAK2        | 173     | 252  | 360  | 338             | 2.08         |
| 8090162      | ITGB5       | 330     | 357  | 220  | 150             | −1.50        |
| 8119195      | SEPP1       | 3337    | 2847 | 1761 | 1131            | −1.87        |

G418 (0.45 mg/mL); ATSE: Akebia trifoliate seed extract (0.625 mg/mL); 1/2(G418 + ATSE): a combination of half dose of each accordingly.

3.9. Expression of the KEGG Apoptosis Pathway for HuH7 Cells. 89 genes in the KEGG apoptosis pathway were displayed in Affymetrix array data. There were significant changes in the levels of gene expression in 6 of 89 genes, 5 were upregulated and 1 was downregulated (Table 5).

4. Discussion

Akebia Fructus has been long used for HCC in TCM in China, in the manner of one member in herbal formula under most circumstances. The data from clinical epidemiological investigation on 2060 HCC cases [19], and retrospective analysis on the published papers containing the use of herbal formulas for HCC, indicated that the frequency for Akebiae Fructus is up to nearly 43% [20], which means that Akebiae Fructus is popular in herbal formula and plays an important role and also means that it is worthwhile to reveal the underlying molecular mechanism of how this herb works and a possibility of a new anticancer agent.

Therefore, in our recently work [14–18], we chose Akebia trifoliate (Thunb.) Koidz, one of the three medicinal species in China, to carry out research. Considering Akebia Fructus is always used as a whole fruit, we separated the fruit into two parts, the pulp and the seed, to observe the effect on SMMC-7721 cells, and the result was beyond expectation. We found that Akebia trifoliate seed extract (ATSE) could suppress the proliferation and caused notable ER stress in SMMC-7721 cells, which suggested ATSE could inhibit proliferation and probably via inducing ER stress. To further investigate this phenomenon, HepG2, HuH7, and SMMC-7721 cells were used and the dose of ATSE was according to our previous work [14–18]. In view of G418 being a commonly used cytotoxic antibiotic that can suppress proliferation of many cancer cells and presumable advantages of combined therapy, G418 and combination groups were also set up. The combination group is composed of half doses of each.

Our data exhibited that ATSE leads to different degrees of ER stress in these HCC cell lines and a decrease in cell
viability (Figures 1 and 2). In fact, a lot of previous work has been done to investigate the herbal medicine on HCC, and several mechanisms of action have been clarified [4], such as cytotoxic activities against cancer cell lines [21, 22], while the effect that related ER stress to HCC cells was relatively rarely mentioned. On the other hand, the endoplasmic reticulum has been posited as a potential anticancer target [23]. Indeed some anticancer chemotherapeutic agents have been shown to induce ER stress, such as the proteasome inhibitor bortezomib [24], as well as the cannabinoids [25]. However, most of these kinds of research did not provide visual morphological evidence of ER stress, while our data was presented intuitively and suggested that SMMC-7721 is the most sensitive in terms of morphology (Figure 1).

As shown in Figure 3, the cell cycle analysis by FCM was performed to find out the relevance of cell cycle and proliferation inhibition. Basically, not so many notable changes in statistical data were observed. On the other hand, in contrast to the uncertain cell cycle alteration, other information, just like cells which behaved differently in response to ATSE, cannot be ignored. Firstly, in HepG2 cells, a continuous peak can be seen after the G2/M phase (Figure 3(a)) and it seemed that the amount of peak was reduced by ATSE (Figure 3(c)), implying that ATSE may reduce the adhesion between HepG2 cells, which is meaningful for fewer tumor metastases. This speculation was based on the following: contact inhibition does not happen to HepG2, so cells could grow overlapped. It is difficult to separate cells to a single one in vitro, which is distinguished from the other two kinds. Therefore, when the PI stained HepG2 cells pass through FCM, the continuous peak after G2/M phase formed. Another reference is gene expression in the integrin-mediated cell adhesion pathway (Table 4). Further experiments such as the cell adhesion assay can be considered. Secondly, unlike the other two kinds, HuH7 cells proliferate very quickly. Apoptosis occurs spontaneously in the absence of ATSE (Figure 3(e)) and intensified in the presence of ATSE (Figure 3(g)). Thus, ER stress-mediated apoptosis is possibly involved; many genes expressions in the apoptosis pathway are upregulated by ATSE as well (Table 5). All these data reveal that the distinction exists in ATSE against different HCC cells. In addition, cell cycle regulatory proteins can be considered in further research.

Besides morphological changes and cell cycle alterations, ER stress is often accompanied with expression changes of a lot of genes and proteins, or called as biomarkers. Generally, this event started from three transmembrane sensors in UPR (unfolded protein response) pathway [26, 27], namely, ATF6, IRE1, and PERK, and a lot of downstream molecules, such as Grp78 and DDIT3. Some effective ingredients from herb, such as Tanshinone II A, have shown the effect of induction of ER stress on prostate cancer cells, and increased expression of Grp78 was identified [28]. ER stress was activated by Tubeimoside-1, a triterpenoid saponin extracted from Bolbos- temma paniculatum, with an increased expression of DDIT3 in human cervical carcinoma cells [29], while our previous data indicated that ATSE selectively suppressed mRNA expression of many ER stress-related genes in SMMC-7721 cells, especially of Hyou1, Hsp90aa1, Sec63, Dnajb11, Grp78, and Hspa9. To understand more about this, further proteins expressions were monitored by WB, genes’ profiles were assessed by Affeymetrix array as well and more cell lines were used besides SMMC-7721. First, in HepG2 and SMMC-7721 cells, ATSE led to an increased trend of these chosen proteins versus control, but not significantly, while in HuH7 cells, Hyou1 and Grp78 were notably downregulated (Figure 4).

| Probe set ID | Gene symbol | Control | G418 | ATSE | 1/2(G418 + ATSE) | ATSE/control |
|-------------|-------------|---------|------|------|-----------------|--------------|
| 8149733     | TNFRSF10B   | 408     | 688  | 794  | 805             | 1.95         |
| 7966746     | HRK         | 153     | 188  | 252  | 403             | 1.65         |
| 8065569     | BCL2L1      | 1172    | 1558 | 1822 | 1293            | 1.55         |
| 8012257     | TP53        | 212     | 209  | 320  | 66              | 1.51         |
| 7956989     | MDM2        | 766     | 1242 | 1138 | 680             | 1.50         |
| 7924733     | PARP1       | 102     | 90   | 67   | 56              | −1.50        |

G418 (0.45 mg/mL); ATSE: Akebia trifoliate seed extract (0.625 mg/mL); 1/2(G418 + ATSE): a combination of half dose of each accordingly.

Furthermore, even Affeymetrix array presented such a comprehensive gene expression profile; how ER stress was induced and how these genes or proteins are influenced by ATSE, active or passive, directly or indirectly remain unclear. Therefore, besides the continued analysis of these data, further investigations, such as cotreatment with some mechanism-known ER-stress-induced agents and such as
genes knocked down by RNA interference, are beneficial to know more about how ATSE works and the role of those significantly changed genes or proteins.

The phytochemistry of ATSE is not discussed in the present work, for the main ingredients are almost clear, which are triterpenoid saponins. Since the work of Ryuichi et al. in the last century [31, 32], there are around 90 triterpenoid saponins which have been discovered in Akebia Decne so far [33, 34], including Akebia trifoliate (Thunb.) Koidz and other medicinal species; medicinal parts involve fruit, seed, stem root, and other sections. However, it remains important to further characterize new biological activities of known ingredients.

The effect of drug combination is also worthy of attention. As shown in the MTT result (Figure 2), the cell viability of the combination group decreased significantly compared to the control, which was consistent in all cell lines, and the level was lower than G418 and the ATSE group in HepG2 cells as well as the apoptotic body peak before G0/G1 phase responded to this (Figure 3(d)). Overall though, we speculate that if ATSE combined with a cytotoxic antibiotic, it would make sense to affect a dosage reduction of cytotoxic antibiotics or herb preparation, for therapeutic efficacy to improve, for side effects to be reduced, and to create more opportunities for patients suffering from cancer.

In summary, our data demonstrated that the Akebia trifoliate (Thunb.) Koidz seed extract (ATSE) has a certain effect on proliferation inhibition in HepG2, HuH7, and SMMC-7721 cell lines and ER stress induction involved. It provides a promising candidate therapeutic agent anti-HCC, and further studies are required.

**Abbreviations**

ATSE: Akebia trifoliate seed extract  
HCC: Human hepatocellular carcinoma  
TCM: Traditional Chinese medicine  
MTT: 3-(4,5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide  
ER: Endoplasmic reticulum  
KEGG: Kyoto Encyclopedia of Genes and Genomes.

**Conflict of Interests**

The authors declare that there is no conflict of interests regarding the publication of this paper.

**Authors’ Contribution**

Wen-Li Lu and Hong-Yan Ren contributed equally to this work.

**Acknowledgment**

This work was supported by the National Natural Science Foundation of China (no. 30973703 and no. 81273641).

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