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Cordyceps cicadae induces G2/M cell cycle arrest in MHCC97H human hepatocellular carcinoma cells: a proteomic study

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Abstract

Background: *Cordyceps cicadae* is a medicinal fungus that is often used for treating cancer. However, the anticancer mechanisms of *C. cicadae* are largely unknown. This study aims to investigate the anticancer mechanisms of *C. cicadae* against hepatocellular carcinoma cells *in vitro* using a proteomic approach.

Methods: Human hepatocellular carcinoma MHCC97H cells were treated with a water extract of *C. cicadae* (0, 100, 250, 500, and 1000 µg/mL) for 48 h and harvested for cell viability assays. The significant differences in protein expression between control and *C. cicadae*-treated cells were analyzed by two-dimensional gel-based proteomics coupled with matrix-assisted laser desorption ionization-time of flight mass spectrometry. Flow cytometry analysis was employed to investigate the cell cycle and cell death. The anticancer molecular mechanism was analyzed by whole proteome mapping.

Results: The water extract of *C. cicadae* (0, 100, 250, 500, and 1000 µg/mL) inhibited the growth of MHCC97H cells in a dose-dependent manner *via* G2/M phase cell cycle arrest with no evidence of apoptosis. Among the identified proteins with upregulated expression were dynactin subunit 2, N-myc downstream-regulated gene 1, heat shock protein beta-1, alpha-enolase isoform 1, phosphatidylinositol transfer protein, and WD repeat-containing protein 1. Meanwhile, the proteins with downregulated expression were 14-3-3 gamma, BUB3, microtubule-associated protein RP/EB family member 1, thioredoxin-like protein, chloride intracellular channel protein 1, ectonucleoside triphosphate diphosphohydrolase 5, xaa-Pro dipeptidase, enoyl-CoA delta isomerase 1, protein-disulfide isomerase-related chaperone Erp29, hnRNP 2H9B, peroxiredoxin 1, WD-40 repeat protein, and serine/threonine kinase receptor-associated protein.

Conclusion: The water extract of *C. cicadae* reduced the growth of human hepatocellular carcinoma MHCC97H cells *via* G2/M cell cycle arrest.

Background

Primary liver cancer accounted for 6% of the total cancer cases worldwide in 2008 [1]. The highest incidences of liver cancer were in East Asia (Japan, Korea, and China) [2,3]. In China, liver cancer has the third highest estimated age-standardized cancer incidence rate in men and the fourth in women, and the second and third highest cancer mortality rates in men and women, respectively [1]. The high incidence of liver cancer in China is attributed to consumption of aflatoxin-contaminated grains, liver virus infection, and alcohol drinking [3]. Hepatitis B vaccination can

effectively prevent liver cancer, but the treatment of liver cancer is still difficult [3,4].

Chinese medicine (CM) has been widely used in conjunction with chemotherapeutic drugs for liver cancer treatment in China with positive outcomes [5]. *Cordyceps* is a genus of ascomycete fungi belonging to the Clavicipitaceae family. All *Cordyceps* species are endoparasitoids, and most of them parasitize insects and other arthropods. The *Cordyceps* genus includes nearly 400 species, and some of them have potential anticancer effects. *Cordyceps sinensis* is a medicinal fungus that has been used for cancer treatment in CM and Traditional Tibetan medicine since the 15th century [6,7]. The anticancer properties of *C. sinensis* *via* cancer cell apoptosis induction, proliferation inhibition, or both in various

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types of cancers, including leukemia, melanoma, Leydig tumor, breast cancer, and human hepatocellular carcinoma (HCC) have been investigated [8-10]. *C. sinensis* inhibited tumor metastasis *in vivo* [11,12]. Another well-known species, *Cordyceps militaris*, showed anticancer effects on leukemia, lung cancer, and breast cancer *in vitro* and *in vivo* [13-17], and its bioactive compound, cordycepin, exhibited cytotoxic and reactive oxygen species-generating activity in relation to cancer proliferation inhibition [18-22].

Cordyceps cicadae, another well-known medicinal mushroom that grows on larvae of *Cicadae flammata*, has been used in CM in China for the prevention and treatment of various diseases, including kidney disease, immune disease, and cancer [23-25]. *C. cicadae* exhibited immunoregulatory effects on human T lymphocytes and modulated the growth of mononuclear cells [26-29]. *C. cicadae* inhibited the growth of lung adenocarcinoma and melanoma *in vivo* and *in vitro* [30,31]. However, the anticancer mechanism of *C. cicadae* for liver cancer is still unknown.

In the last decade, proteomics has been widely used in medical studies for clinical biomarker identification, pathogenesis investigation, new drug discovery, pharmacological research, toxicological examination, and so on [32]. Most biological functions are transmitted via proteins such as enzymes, receptors, and structural components. Therefore, comprehensive proteomic analyses help us to understand the molecular modifications in physiological conditions [33]. This study aims to investigate the anticancer mechanisms of *C. cicadae* against HCC *in vitro* by two-dimensional gel-based proteomics coupled with matrix-assisted laser desorption ionization-time of flight (MALDI-TOF/TOF) mass spectrometry (MS), flow cytometry analysis, and proteome mapping.

Materials and methods

Cell culture and reagents

The MHCC97H cell line was purchased from the Liver Cancer Institute of Fudan University (China). MHCC97H cells were cultured in DMEM (Gibco BRL, USA) supplemented with 10% fetal bovine serum (Gibco BRL) in a humidified incubator containing 5% CO₂ in air at 37°C, and subcultured with 0.25% trypsin-0.02% EDTA (Gibco BRL). A lyophilized hot water extract of wild-type *C. cicadae* (BioAsia Co., China) was dissolved in phosphate-buffered saline (PBS) and adjusted to a final concentration of 10 mg/mL.

Cell proliferation assay

The dose-dependent effect of *C. cicadae* on cell viability was assessed by the MTT assay. Briefly, suspended MHCC97H cells (1×10^5 cells/mL; 100 μ L) were dispersed into the wells of 96-well microtiter plates. After 24 h of incubation, various concentrations of *C. cicadae* were added to each well and incubated for 48 or 72 h. Next, 10 μ L of

MTT solution (5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide dissolved in c-DMEM) (USB Corporation, USA) was added to each well, and incubated for 3 h at 37°C. The MTT solution was then removed and the insoluble purple formazan crystals formed were dissolved in 50 μ L of isopropanol in 0.1 M HCl (MERCK, Germany). The optical density (OD) of each well was measured using a Bio-Rad 550 Microplate Reader (Bio-Rad, USA) at 595 nm with a reference wavelength of 655 nm. The percentage of cell viability was expressed as $(A_{\text{treatment}} / A_{\text{control}}) \times 100\%$.

Cell cycle analysis

The dose-dependent effect of *C. cicadae* on the cell cycle distribution was assessed by flow cytometry as described in our previous report [34]. Briefly, MHCC97H cells (1×10^5 cells/mL) were treated with various concentrations of *C. cicadae* (0, 100, 250, 500, and 1000 μ g/mL) for 48 h, and the cells were then harvested, fixed in 70% ethanol (MERCK, Germany), and stored at -20°C for 24 h until further analysis. Next, the cells were washed twice with ice-cold PBS, and incubated with RNase and propidium iodide (PI) (Sigma-Aldrich, USA) for 30 min. The PI-stained cells were excited at a wavelength of 488 nm and emitted at a maximum wavelength of 617 nm. Acquisition of 10,000 events was chosen for measurement of the DNA cell cycle distribution using a COULTER XL Flow Cytometer (Beckman and Coulter, USA). The distribution of cells in the different cell cycle phases shown in the DNA histograms was analyzed using Becton Dickinson Cell Fit Software (BD, USA).

Sample preparation for proteomic analysis

MHCC97H cells were seeded in 100-mm culture dishes at 1×10^6 cells/dish (1×10^5 cells/mL), incubated overnight, and then treated with or without 500 μ g/mL *C. cicadae* for 48 h. The cells were harvested by trypsinization, washed three times with PBS, and centrifuged in a Beckman Spinchron DLX (Beckman and Coulter) at $400 \times g$ for 5 min. The cell pellet was resuspended in lysis buffer [1% Triton X-100 (USB Corporation), 25 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; Sigma-Aldrich), 150 mmol/L NaCl (UNIVAR, USA), 1 mmol/L EDTA disodium salt (Sigma-Aldrich), 1 mmol/L dithiothreitol (DTT; USB Corporation)] with Protease Inhibitor Cocktail Set III (AEBSF, aprotinin, bestatin, E-64, leupeptin hemisulfate, pepstatin A; Bio-Rad). The superfluous salt in the extract was removed by incubation with trichloroacetic acid (TCA)-acetone solution [20% TCA (MERCK, Germany), 20 mmol/L DTT in acetone (MERCK, Germany)] for 4 h at -40°C. The protein pellet was obtained by centrifugation at $15,800 \times g$ for 30 min at 4°C. Excess TCA was removed by three washes with acetone containing 20 mmol/L DTT. The air-dried protein pellet was

resuspended in buffer [7 mol/L urea (USB Corporation), 2 mol/L thiourea (Sigma-Aldrich), 4% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS; Sigma-Aldrich), 100 mmol/L DTT, 5% glycerol (USB Corporation)], and the protein solution was stored at -80°C until 2-DE analysis. The protein concentration was determined by the Bradford assay (Bio-Rad).

Two-dimensional electrofocusing and polyacrylamide gel electrophoresis

The 2-DE procedures were carried as described in our previous report with modifications [35]. The samples were examined in duplicate and 12 gels (6 for control cells and 6 for *C. cicadae*-treated cells) were used in total. For the first-dimensional electrophoresis of proteins, protein samples (150 μg) were mixed with 350 μL of rehydration buffer [9.5 mol/L urea, 2% CHAPS, 0.28% DTT, 1% immobilized pH gradient buffer with pH 3–10 (Bio-Rad), 0.002% bromophenol blue (Sigma-Aldrich)] and then rehydrated for 10 h before isoelectric focusing *via* the following program: (a) linear increase up to 500 V in 1 h; (b) held at 500 V for 2 h; (c) linear increase up to 10,000 V in 4 h; (d) linear increase up to 10,000 V in 3 h; (e) held at 10,000 V to reach a total of 90,000 V \times h. Focused immobilized pH gradient (IPG) gel strips were equilibrated for 15 min in a solution [50 mmol/L Tris-HCl, pH 8.8 (Bio-Rad), 6 mol/L urea, 30% glycerol, 2% sodium dodecyl sulfate (SDS; USB Corporation), containing 20 mmol/L DTT], followed by incubation with the same buffer containing 20 mmol/L iodoacetamide (Sigma-Aldrich) for another 15 min. The second-dimensional separation was performed in 1-mm-thick 12.5% polyacrylamide gels by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) at 15-mA current for 30 min followed by 30-mA current for the rest of the analysis.

Image acquisition and analysis

After the electrophoresis, the gels were stained with SYPRORuby Protein Stain (Bio-Rad), scanned with a Molecular Imager PharoFX Plus System (Bio-Rad), and analyzed using PDQuest 8.0 (Bio-Rad) according to the following procedures: background subtraction, spot detection, and spot matching. The expression levels, expressed as the percentage volume (% vol), were exported. The relative intensities of spots were used for comparisons between the two groups and only those spots with a significant difference (>1.5 -fold increase or decrease, $P < 0.05$) were selected for protein identification.

Protein identification

Spots with differential expression ($P < 0.05$) after 500 $\mu\text{g}/\text{mL}$ *C. cicadae* treatment were sent to the Genome Research Centre (The University of Hong Kong, Hong Kong) for protein identification by MALDI-TOF/TOF MS (4800 MALDI

TOF/TOF Analyzer; Applied Biosystems, USA) analysis after trypsin digestion. The identification was performed by Mascot peptide mass fingerprinting, which can identify proteins from the NCBI database with taxonomy limited to *Homo sapiens*. Mascot reported the molecular weight search (MOWSE) score, which was calculated as $-10 \times \log_{10}(P)$, where P is the probability that the observed match is a random event. P was limited by the size of the sequence database being searched (limited by taxonomy), with conditions such as peptide fixed modification and variable modification and the settings for trypsin digestion. Each calculated value that fell within a given mass tolerance of an experimental value counted as a match. The threshold was accepted if an event occurred at random with a frequency of $< 5\%$. In this study, a protein match with a score of > 71 was regarded as significant.

Western blot analysis

Western blot analysis was performed with Mighty Small II SE250/260 Cell (Hoefer, USA) and TE 77 PWR Transfer (GE Healthcare, USA) electrophoresis units. Briefly, MHCC97H cells treated with or without various concentrations of *C. cicadae* (0, 100, 250, and 500 $\mu\text{g}/\text{mL}$) for 48 h were harvested, washed twice with PBS, and lysed in the lysis buffer for sample preparation described above. The lysates were centrifuged at $11000 \times g$ for 30 min. The total protein concentration in the supernatant was determined by the Bradford assay (Bio-Rad). For immunoblotting, each protein extract was mixed with sample buffer [62.5 mmol/L Tris, pH 6.8 (Bio-Rad), 25% glycerol, 2% SDS, 350 mmol/L DTT, 0.01% bromophenol blue] at a ratio of 1:1 (v/v) and then kept in boiling water for 5 min. An aliquot (30 mg protein) of each sample was applied for electrophoresis in 12.5% SDS-PAGE gels with constant voltage (120 V) and transferred to a polyvinylidene difluoride membrane (GE Healthcare). The membranes were blocked with 5% skimmed milk in PBST [PBS, pH 7.4, 0.1% Tween-20 (USB Corporation)] for 3 h at room temperature, followed by incubation for 2 h with the following primary antibodies (ABCAM, UK): anti-CDK1 (ab32384; 1:2000); anti-cyclin B1 (ab32053; 1:2000); anti-HSP27 (ab2790; 1:2000); anti-PRDX1 (ab16805; 1:2000); anti-STRAP (ab46784; 1:2000). The membranes were washed three times with PBST, and incubated with HRP-conjugated goat anti-rabbit or anti-mouse IgG (Bio-Rad; 1:10 000) for 1 h at 37°C . After three washes with PBST, the membranes were developed using Immobilon-Star HRP peroxide buffer (Bio-Rad). The relative molecular weight of each protein band was estimated using molecular markers (Precision Plus Protein Standards Dual Color; Bio-Rad). Each sample was measured three times.

Statistical analysis

In cell proliferation assay, experiments were repeated three times, measurements within an experiment were done in

six duplicate, and in cell cycle analysis, experiments were repeated three times. Data from three independent experiments were expressed as mean \pm standard deviation (SD). The significance of differences in data was determined by Kruskal-Wallis one-way analysis of variance (ANOVA) followed by Dunnett's *post hoc* test for determination of dose-response dependence. In proteomic analysis, six individual samples were prepared and run for 2D gel in each group (*i.e.*, $n = 6$) the significance of differences in data was determined by a two-tailed Student's *t*-test with Levene's test for equality of variances. All analyses were performed using SPSS 17.0 (IBM, USA) with $P < 0.05$ as the significance level.

Results

Effects of *C. cicadae* on MHCC97H cell proliferation

MHCC97H cells were grown in medium containing various concentrations of *C. cicadae* (0, 100, 250, 500, and 1000 $\mu\text{g/mL}$) for 48 or 72 h to investigate the effects of *C. cicadae* on cell proliferation. Dose-dependent inhibition of cell growth was observed with treatment of *C. cicadae* ($P < 0.05$) (Figure 1).

Effect of *C. cicadae* on the cell cycle distribution of MHCC97H cells

Figure 2A shows the effects of *C. cicadae* (0, 100, 250, 500, and 1000 $\mu\text{g/mL}$) treatment for 48 h on the cell cycle phase distribution of MHCC97H cells. As shown in Figure 2B, treatment with *C. cicadae* (100, 250, and 500 $\mu\text{g/mL}$) for 48 h resulted in cell accumulation in G2/M phase at 23.8%, 30.2%, and 38.6%, respectively, compared with the control cells (19.8%), and decreased the cell percentages in G0/G1 phase by 48.2%, 43.9%, and 35.6%, respectively, compared with the control cells (53.3%). Treatment with *C. cicadae* at

100, 250, and 500 $\mu\text{g/mL}$ for 48 h did not affect the cell population in S phase (27.9%, 25.8%, and 25.9%, respectively, compared with the control cells (26.91%). Furthermore, most cells (64.74%) were arrested in G2/M phase with the highest dose of 1000 $\mu\text{g/mL}$ *C. cicadae*. The sub-G1 apoptotic fraction of cells showed no significant changes when *C. cicadae* (100–500 $\mu\text{g/mL}$) was added, suggesting that no apoptosis occurred during the period of examination.

CDK1/cyclin B complex is involved in the G2/M phase checkpoint by regulating the process of M phase [36]. Figure 3 shows that while the CDK1 protein level was decreased in MHCC97H cells after *C. cicadae* treatment, the cyclin B1 protein level was unaffected.

Differential expression of proteins in *C. cicadae*-treated MHCC97H cells

2-DE analyses were performed for each treated sample and repeated six times. Figure 4 shows representative gel images in which more than 1000 spots were detected on each gel. Proteins within the range of 15–225 kDa and having isoelectric points between 3 and 10 were well separated. Table 1 presents the differential expression levels of the identified proteins in MHCC97H cells without (control) and with *C. cicadae* treatment. Twenty-eight proteins with significant ($P < 0.05$) changes of > 1.5 -fold in volume intensity were selected and further analyzed by MALDI-TOF-MS/MS for peptide identification after trypsin digestion. Among these proteins, the nine upregulated proteins were identified as tubulin beta 2C, dynactin subunit 2 (DCTN2), keratin type II cytoskeletal 7, keratin type I cytoskeletal 10, N-myc downstream-regulated gene 1 (NDRG1), heat shock protein beta-1 (HSPB1, Hsp27), phosphatidylinositol transfer protein beta isoform (PITPNB), alpha-enolase isoform 1 (ENO1), and WD repeat-containing protein 1 isoform 1 (WDR1). The 13 downregulated proteins were identified as 14-3-3 gamma (YWHA), microtubule-associated protein RP/EB family member 1 (MAPRE1), chloride intracellular channel protein 1 (CLIC1), WD-40 repeat protein (STRAP), thioredoxin-like protein (GLRX3), ACTB protein, xaa-Pro dipeptidase isoform 3 (PEPD), enoyl-CoA delta isomerase 1 mitochondrial (EC11), protein-disulfide isomerase-related chaperone Erp29 (ERP29), ectonucleoside triphosphate diphosphohydrolase 5 isoform (ENTPD5), hnRNP 2H9B (HNRNPH3), peroxiredoxin 1 (PRDX1), and mitotic checkpoint protein BUB3 isoform A (BUB3). The main properties of the identified proteins were summarized in Table 2.

Western blot analyses of a few randomly selected proteins were performed for proteomic data validation. In agreement with the proteome changes identified by the proteomic analysis, the expression levels of Hsp27, peroxiredoxin 1, and WD 40 were upregulated in the cancer cells with *C. cicadae* treatment at 500 $\mu\text{g/mL}$

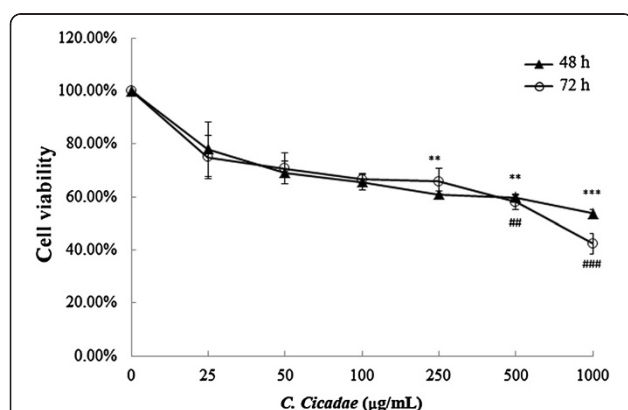


Figure 1 The effects of *C. cicadae* on MHCC97H cell viability.

The cell viability was tested by MTT assay. Data were expressed as mean \pm SD ($n = 3$ separate experiments). **,***, $P < 0.01$, 0.001 vs. control in 48 h treatment group, and ##, ###, $P < 0.01$, 0.001 vs. control in 72 h treatment group, respectively.

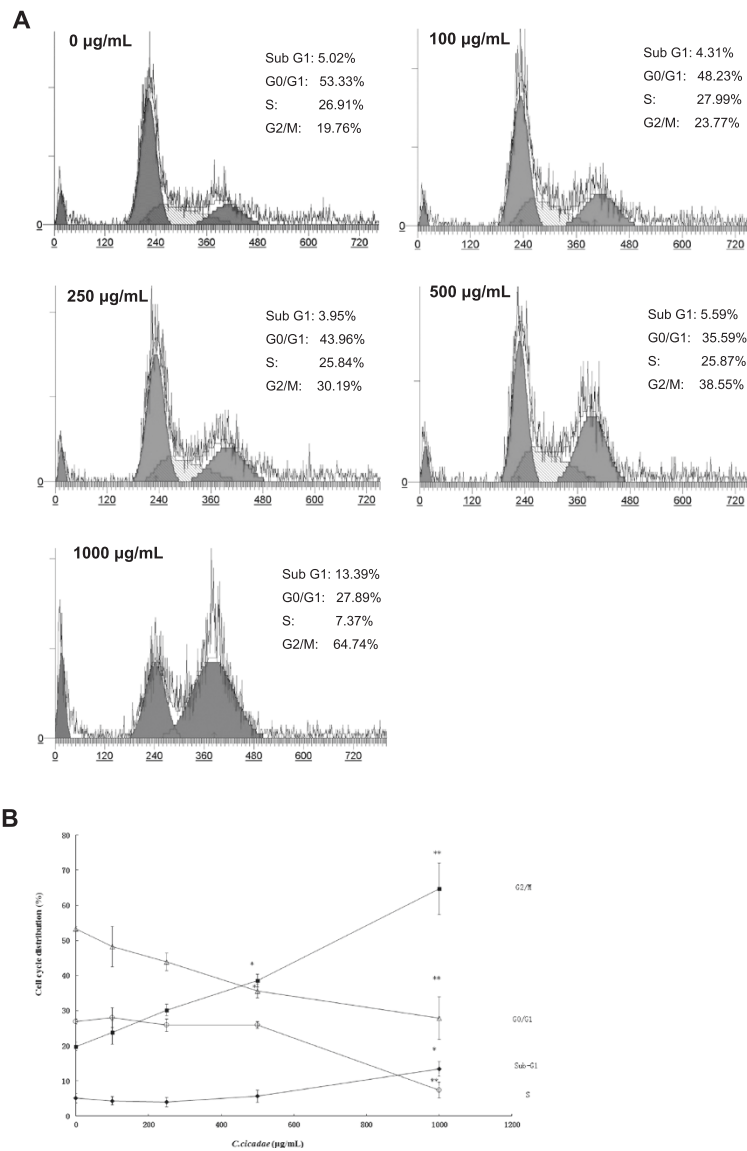


Figure 2 The effects of *C. cicadae* on MHCC97H cell cycle. **(A)** Cellular DNA contents in *C. cicadae*-treated and control cells were monitored by flow cytometry. The cell cycle distribution of sub-G1, G1, S and G2/M phase cells are presented as DNA histogram. **(B)** The percentage of cells in each cell cycle phase of MHCC97H after *C. cicadae* treatment were shown. The data were expressed as mean \pm SD and were representative of three independent experiments. *, **, $P < 0.05, 0.01$, vs. control, respectively.

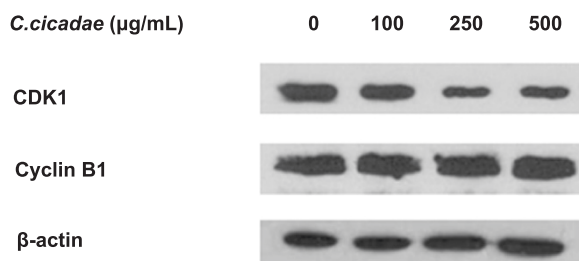


Figure 3 The effects of *C. cicadae* on cell cycle checkpoint related proteins. *C. cicadae* treatment decreased the expression of CDK1 in MHCC97H cells.

compared with the control cells (Figure 5). These results were similar to those obtained in the proteomic analysis.

Discussion

Species in the genus *Cordyceps* can reduce cancer cell proliferation [14,15,55-58], but only limited studies on liver cancer are available. Several polysaccharides isolated from *C. cicadae* exhibited antitumor and immunomodulatory properties [14,15,55-58] but the mechanisms have not been fully explored. In the present study, we showed that *C. cicadae* inhibited the growth of the human HCC cell line MHCC97H via G2/M cell cycle arrest. In support

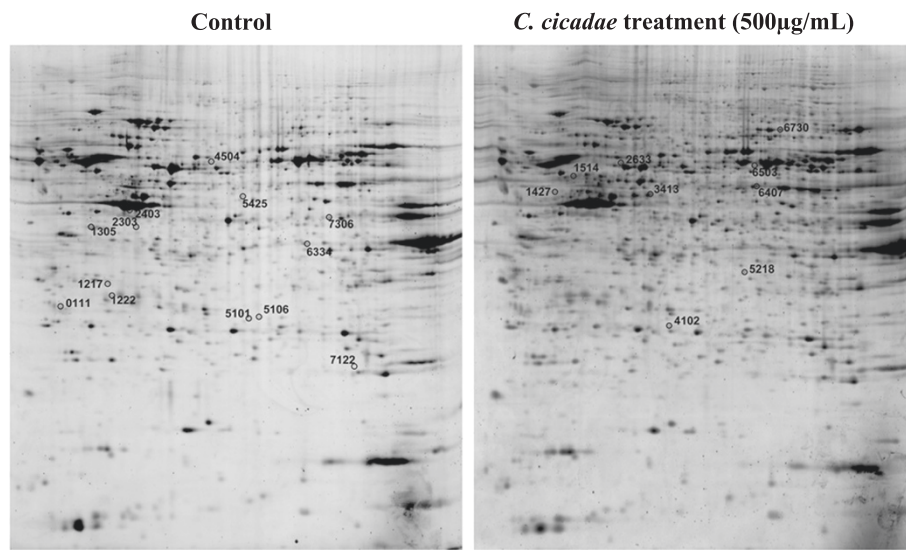


Figure 4 Representative of 2-DE gel proteomic maps of MHCC97H cells with (left) or without (right) *C. cicadae* treatment. The labelled spots were representative of proteins with significant change.

of the cell cycle measurements, several differentially expressed proteins for cell cycle regulation, mitosis, and protein synthesis were identified by the proteomic analysis.

Western blot analyses revealed a decrease in the level of CDK1, which is a G2/M checkpoint cyclin. The cell cycle measurements also indicated accumulation of cells in G2/M phase rather than induction of apoptosis with *C. cicadae*. To our knowledge, this is the first study to demonstrate that the anticancer mechanisms of *C. cicadae* involve G2/M arrest. Other studies showed that *C. militaris* could induce G2/M cell cycle arrest in human colon cancer HT-29 cells [37].

Figure 6 summarizes the magnitude changes in expression of the identified proteins with reference to the control cells. 14-3-3 gamma, BUB3, DCTN2, MAPRE1, GLRX3, and CLIC1 are thought to be involved in cell cycle regulation, G2/M checkpoint control, microtubule polymerization, and spindle organization during mitosis *etc.* Although their mechanisms related to the G2/M cell cycle arrest induced by *C. cicadae* were not clear, 14-3-3 gamma is important for cell cycle regulation and 14-3-3 gamma expression change-induced cell cycle deregulation has been implicated in cancer formation [37]. 14-3-3 gamma blocked premature mitotic entry into G2/M phase after DNA damage [59,60], while 14-3-3 gamma-knockdown cells showed inhibition of cdc25C function, which induced overriding of both the incomplete S phase and the G2 DNA damage checkpoint [61]. Overexpression of 14-3-3 gamma in HCC patients was associated with extrahepatic metastasis and worse survival [62,63]. Our proteomic data showed that *C. cicadae* treatment reduced the expression of 14-3-3 gamma in the human HCC cell

line MHCC97H, suggesting that the anticancer and G2/M phase arrest effects of *C. cicadae* could be attributed to 14-3-3 gamma expression inhibition.

BUB3, DCTN2, and MAPRE1 are involved in spindle checkpoint and mitosis regulation. BUB3 is a mitosis checkpoint protein for early stages of mitosis to prevent premature sister chromatid separation, missegregation, and aneuploidy *via* regulation of anaphase-promoting complex/cyclosome (APC/C) [38,64]. DCTN2 is involved in functions for the microtubule anchor dynactin complex, inactivation of the spindle checkpoint through kinetochore disassembly, and recruitment of cell cycle regulators to centrosomes [40,65]. MAPRE1 is involved in microtubule polymerization, microtubule dynamics control [39], and mitotic spindle function [66], and is dominantly expressed in poorly differentiated HCCs [67]. The expression changes of BUB3, DCTN2, and MAPRE1 in *C. cicadae*-treated MHCC97H cells indicate a mitosis-regulatory effect of *C. cicadae*, which may contribute to G2/M phase arrest.

GLRX3 maintains redox homeostasis in living cells [41]. In GLRX3-knockout mice, embryonic fibroblasts were arrested at G2/M phase [68]. CLIC1 is a nuclear chloride ion channel and involved in G2/M cell cycle regulation [42]. These findings may suggest a reason why the expression levels of GLRX3 and CLIC1 were changed in *C. cicadae*-treated MHCC97H cells.

ENTPD5 is an endoplasmic reticulum (ER) enzyme that hydrolyzes purine nucleoside diphosphates and is essential for liver functions, and ENTPD5-deficiency resulted in hepatocellular neoplasia [69]. Overexpression of ENTPD5 was observed in prostate cancer [43,70]. NDRG1 is a member of the NDRG gene family, and exhibited anticancer and metastasis-suppression effects in pancreatic cancer, colon

Table 1 Differentially expressed proteins between *C. cicadae*-treated and non-treated (control) MHCC97H cells

Spot No. ¹	Protein name	GenInfo identifier ²	Protein score ³	Expression quantity (×10 ⁴) control ⁴	Expression quantity (×10 ⁴) <i>C. cicadae</i>	Expression change (<i>C. Cicadae</i> /control)	<i>P</i>	<i>pI</i> ⁵	<i>Mr</i> (kDa) ⁵
0111	14-3-3 Gamma In Complex With A Phosphoserine Peptide	gi 82407948	76	33.0 ± 7.2	19.0 ± 5.3	0.58	0.0004	4.80	28.3
1217	microtubule-associated protein RP/EB family member 1	gi 6912494	219	10.0 ± 3.7	5.7 ± 2.2	0.57	0.026	5.02	30.1
1222	Chloride intracellular channel protein 1	gi 4588526	307	24.8 ± 8.4	9.3 ± 3.3	0.38	0.004112	5.02	27.2
1305	WD-40 repeat protein	gi 4519417	221	42.3 ± 10.2	12.0 ± 4.1	0.28	0.001183	4.93	38.8
1427	Tubulin, beta 2C	gi 23958133	188	3.6 ± 1.7	7.8 ± 4.2	2.17	0.025345	4.83	50.2
1514	Dynactin subunit 2	gi 22096346	436	9.8 ± 2.4	19.2 ± 5.8	1.97	0.000491	5.10	44.3
2303	thioredoxin-like protein	gi 3646128	123	9.3 ± 5.4	3.4 ± 1.9	0.37	0.038567	5.25	37.8
2403	ACTB protein	gi 15277503	444	101.7 ± 33.4	60.3 ± 18.3	0.59	0.001814	5.55	40.5
3413	N-myc downstream regulated gene 1	gi 119612570	105	9.0 ± 3.3	30.8 ± 11.8	3.44	0.00106	5.98	34.0
4102	heat shock protein beta-1	gi 4504517	368	18.1 ± 7.6	27.7 ± 10.6	1.53	0.026641	5.98	22.8
4504	xaa-Pro dipeptidase isoform 3	gi 260593665	193	8.7 ± 2.7	3.3 ± 1.2	0.38	0.027093	5.7	48.0
5101	Enoyl-CoA delta isomerase 1, mitochondrial	gi 60593479	67	11.8 ± 7.8	5.8 ± 2.9	0.49	0.040511	6.00	28.9
5106	The Protein-Disulfide Isomerase Related Chaperone Erp29	gi 192987144	247	16.3 ± 6.8	9.3 ± 4.3	0.57	0.033331	7.07	27.2
5218	phosphatidylinositol transfer protein beta isoform	gi 6912594	181	5.5 ± 1.9	8.5 ± 2.6	1.56	0.004165	6.41	31.8
5425	ectonucleoside triphosphate diphosphohydrolase 5, isoform	gi 119601555	181	5.9 ± 2.1	2.3 ± 0.8	0.39	0.007848	5.74	45.7
6334	hnRNP 2H9B	gi 7739445	127	31.1 ± 12.6	17.4 ± 6.5	0.56	0.023	6.76	31.5
6407	alpha-enolase isoform 1	gi 4503571	349	21.4 ± 7.5	34.8 ± 12.6	1.63	0.028265	7.01	47.5
6730	WD repeat-containing protein 1 isoform 1	gi 9257257	217	9.0 ± 4.6	13.9 ± 6.3	1.54	0.01346	6.17	66.8
7122	peroxiredoxin 1	gi 55959887	79	2.7 ± 1.0	0.5 ± 0.1	0.20	0.024662	6.41	19.1
7306	mitotic checkpoint protein BUB3 isoform a	gi 4757880	147	37.1 ± 17.4	22.1 ± 10.1	0.60	0.045568	6.36	37.6

¹Spot no.: automatically assigned by the PDQuest software.²GenInfo identifier: sequence identification number assigned by GenBank.

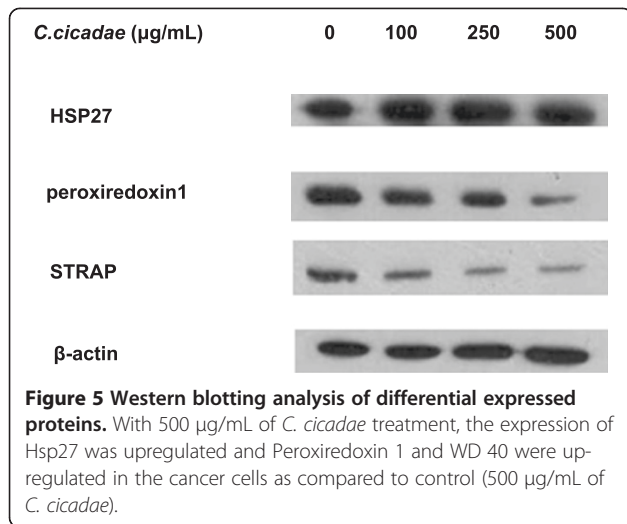
³Protein score: generated by the MS identification system.

⁴Expression Quantity were calculated by the PDQuest software, data expressed as mean ± SD.

⁵*Mr* and *pI*: relative molecular mass (*Mr*) and isoelectric point (*pI*) generated by the MS system.

Table 2 The major bio-functions of identified proteins

Protein name	Protein symbol	Subcellular location	Protein functions	Up/down regulation
Cell growth and cell cycle regulation				
14-3-3 Gamma, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, gamma	14-3-3γ	Cytoplasm	G2 DNA damage checkpoint [37]	↓
mitotic checkpoint protein BUB3 isoform A	BUB3	Nucleus	Spindle-assembly checkpoint signalling and establishment of correct kinetochore-microtubule attachments [38]	↓
microtubule-associated protein RP/EB family member 1	MAPRE1	Cytoplasm	Microtubule polymerization [39]	↓
Dynactin subunit 2	DCTN2	Cytoplasm	Modulates cytoplasmic dynein binding to an organelle, and prometaphase chromosome alignment and spindle organization during mitosis [40]	↑
thioredoxin-like protein	GLRX3	Cytoplasm	Human cancer cell growth and metastasis regulation [41]	↓
Chloride intracellular channel protein 1	CLIC1	Nucleus membrane, cell membrane	Formation of chloride ion channels, and regulation of the cell cycle [42]	↓
Anti-cancer effects				
ectonucleoside triphosphate diphosphohydrolase 5, isoform	ENTPD5	Endoplasmic reticulum lumen	Promoting glycolysis in proliferating cells in response to phosphoinositide 3-kinase signalling in the AKT1-PTEN signalling pathway [43]	↓
N-myc downstream regulated gene 1	NDRG1	Cytoplasm, nucleus, cell membrane	Metastasis suppression [44]	↑
heat shock protein beta-1	HSPB1	Cytoplasm, nucleus	Stress resistance and actin organization [45]	↑
alpha-enolase isoform 1	ENO1	Cytoplasm, cell membrane	Multifunctional enzyme in various processes such as growth control, hypoxia tolerance and allergic responses [46]	↑
Other functions				
xaa-Pro dipeptidase isoform 3	PEPD	Cytoplasm	Collagen and GSH metabolism [47]	↓
Enoyl-CoA delta isomerase 1, mitochondrial	ECI1	Mitochondrion matrix		↓
The Protein-Disulfide Isomerase Related Chaperone Erp29	ERP29	Endoplasmic reticulum lumen	Processing of secretory proteins within the endoplasmic reticulum, possibly by participating in the folding of proteins in the ER [48]	↓
phosphatidylinositol transfer protein beta isoform	PITPNB	Cytoplasm, Golgi apparatus	Catalyzes the transfer of PtdIns and phosphatidylcholine between membranes [49]	↑
hnRNP 2H9B	HNRNPH3	Nucleus	Involved in the splicing process and participates in early heat shock-induced splicing arrest [50]	↓
WD repeat-containing protein 1 isoform 1	WDR1	Cytoplasm	Induces disassembly of actin filaments in conjunction with ADF/cofilin family proteins [51]	↑
peroxiredoxin 1	PRDX1	Cytoplasm	Involved in redox regulation of the cell. Reduces peroxides with reducing equivalents provided through the thioredoxin system but not from glutaredoxin [52]	↓
WD-40 repeat protein, serine/threonine kinase receptor associated protein	STRAP	Cytoplasm, nucleus	The SMN complex plays an essential role in spliceosomal snRNP assembly in the cytoplasm and is required for pre-mRNA splicing in the nucleus [53,54]	↓



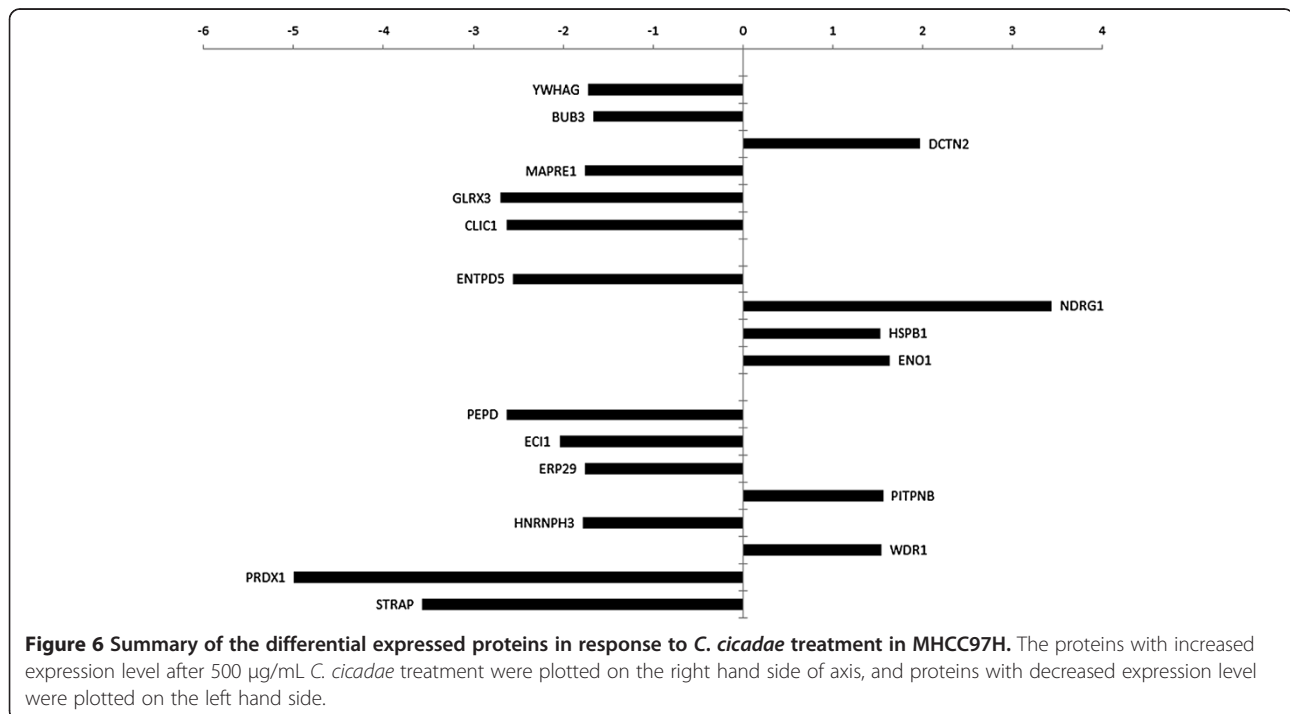
cancer, cervical and ovarian cancer, prostate cancer, and breast cancer [44,71-73]. MHCC97H cells exhibited an 80% pulmonary metastatic rate when injected into nude mice [74]. HSPB1 is a subunit of HSP27, and suppressed cell apoptosis in MHCC97H cells *via* an NF-kappaB pathway [45]. The elevation of this protein indicated some resistance of the cancer cells, and could be responsible for the absence of apoptosis in the *C. cicadae*-treated cancer cells. ENO1, a glycolysis module protein, was overexpressed in HCC cells [46,75], and its increased level suggests that *C. cicadae* treatment increased the glycolytic activity of the cancer cells.

A reduction in PEPD is involved in collagen and GSH metabolism of *C. cicadae*. PEPD is a homodimeric iminodipeptidase that releases carboxy-terminal proline or hydroxyproline residues from oligopeptides, and is used as a marker of non-alcoholic liver fibrosis [47,76]. ERP29 works in the early secretory pathway in the ER, and overexpression of ERP29 resulted in higher expression of HSP27 in breast cancer cells [48]. The reduced expression of ERP29 is likely to be attributed to reduced ER stress with treatment of *C. cicadae*.

STRAP is involved in spliceosomal mRNP assembly in the cytoplasm and pre-mRNA splicing in the nucleus [53]. Overexpression of STRAP was reported in several cancers [53,77]. A STRAP-activated p53-related apoptosis-induction effect and higher expression of STRAP in drug-treated HCC cells were observed [54,78]. In the present study, the expression of STRAP was reduced, which may be related to the non-apoptotic effect of *C. cicadae*.

PRDX1 is a member of the peroxiredoxin family involved in redox regulation in cells, regulation of hydrogen peroxide signaling through its peroxidase activity, and a protein chaperone function. The aspect of whether the reduced expression of PRDX1 contributes to reduced survival of MHCC97H cells *via* failure in redox regulation against reactive oxygen species remains to be determined. Elevated expression of PRDX1 was found in various cancers [52,79,80].

The present study has identified novel molecular signatures in *C. cicadae*-induced cell cycle arrest and cell death in MHCC97H cells using proteomics. Among the



differentially expressed proteins identified, 14-3-3 gamma, BUB3, DCTN2, MAPRE1, GLRX3, and CLIC1 may play some significant roles in the *C. cicadae*-induced G2/M phase arrest in MHCC97H cells. The identified proteins will further enhance a molecular explanation for the cell cycle arrest process induced by *C. cicadae*. Further validation of these markers in gene knockout studies would greatly improve our understanding of the molecular mechanisms behind the liver cancer therapy potential of *C. cicadae*.

Conclusions

The water extract of *C. cicadae* reduced the growth of the human HCC cell line MHCC97H via G2/M cell cycle arrest.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

HW and JMFV conceived and designed the study. HLW, ZJ and WHS performed experiments. HLW performed data analysis. HW, CYJL and JMFV wrote the manuscript. All authors read and approved the final version of the manuscript.

Acknowledgments

This work was supported by the Chinese Medicine Research Fund funded by BioAsia (Institute of Life Sciences Shanghai Pan Asia, Shanghai, China) and partially funded by Seed Funding Programme for Basic Research funded by the University of Hong Kong.

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Received: 18 November 2013 Accepted: 30 April 2014

Published: 8 May 2014

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doi:10.1186/1749-8546-9-15

Cite this article as: Wang *et al.*: Cordyceps cicadae induces G2/M cell cycle arrest in MHCC97H human hepatocellular carcinoma cells: a proteomic study. *Chinese Medicine* 2014 **9**:15.

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