

Active Hexose-correlated Compound Down-regulates HSP27 of Pancreatic Cancer Cells, and Helps the Cytotoxic Effect of Gemcitabine

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Abstract. *Background/Aim: Active hexose-correlated compound (AHCC), an extract of basidiomycete mushroom, is used as health food to enhance the therapeutic effects and reduce the adverse effects of chemotherapy. Our previous proteomic analysis revealed that up-regulation of heat-shock protein 27 (HSP27) was responsible for gemcitabine resistance of pancreatic cancer cells. The aim of the present study was to investigate the effect of AHCC on the expression of HSP27 and the effect of combinatorial treatment of AHCC and gemcitabine on the gemcitabine-resistant pancreatic cancer cell line KLM1-R. Materials and Methods: KLM1-R cells were treated with AHCC, and the expression of HSP27 as well as the cytotoxic effects of combinatorial treatment of AHCC and gemcitabine were investigated with western blotting and MTS assay, respectively. Results: AHCC down-regulated HSP27 and exhibited a cytotoxic effect on KLM1-R cells. Furthermore, the cytotoxic effect of the combinatorial treatment of AHCC and gemcitabine was synergistic. Conclusion: This study supports the potential therapeutic benefits of combinatorial treatment of AHCC and gemcitabine for patients with pancreatic cancer.*

Pancreatic cancer is associated with poor prognosis and a 5-year survival rate of less than 5%. Pancreatic cancer is the fifth most common cause of cancer deaths worldwide (1). Surgical resection is the only curative treatment, but most patients are diagnosed with disease at an advanced,

unresectable stage. In addition, the lack of effective systemic treatment associated with a dismal prognosis (2-4).

Gemcitabine (2'-deoxy-2',2'-difluorocytidine monohydrochloride; Gemzar[®]) is a deoxycytidine analog with structural and metabolic similarities to cytarabine. Gemcitabine has long been used as the therapeutic standard drug for many patients with pancreatic cancer, since gemcitabine improved quality of life in a subset of patients and moderately extended survival (5-8). However, intrinsic or acquired resistance of pancreatic cancer disturbs the therapeutic effect of gemcitabine (9, 10).

Our previous studies investigated proteomic expression in gemcitabine-resistant and -sensitive human pancreatic adenocarcinoma cell lines. Expressions of many proteins were different between gemcitabine-resistant and -sensitive cell lines, and liquid chromatography-tandem mass spectrometry and western blotting identified heat-shock protein 27 (HSP27) as being up-regulated in the gemcitabine-resistant cell lines (11, 12). Further experiments showed that HSP27 plays an important role in gemcitabine resistance, because knock-down or down-regulation of HSP27 in gemcitabine-resistant cells increased the cytotoxic effect of gemcitabine (13-15).

Active hexose-correlated compound (AHCC) is a mixture of polysaccharides, amino acids, lipids, and minerals derived from the culture of the basidiomycete mushroom *Lentinula edodes*. The predominant components of AHCC are oligosaccharides, of which major portions are α 1,4-glucans with a molecular weight of around 5,000 Daltons. AHCC has been reported to have many health benefits, including both immunomodulatory and antitumor effects (16, 17). Several clinical studies showed that AHCC improved the prognosis and quality of life, and reduced adverse effects of chemotherapy of patients with head and neck cancer, advanced liver cancer and lung cancer (18-20).

There is no literature regarding the effect of AHCC on HSP expression or its effects on pancreatic cancer cells *in vitro*. In the present study, we investigated whether AHCC

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had an impact on expression of HSP families and the effect of combinatorial treatment of AHCC and gemcitabine on gemcitabine-resistant pancreatic cancer KLM1-R cells.

Materials and Methods

Cancer cell line and culture conditions. KLM1-R, gemcitabine-resistant pancreatic cancer cell line, was kindly provided by the Department of Surgery and Science, Kyushu University Graduate School of Medical Science. KLM1-R was established by exposing gemcitabine-sensitive KLM1 cells to gemcitabine, as previously described (21). This gemcitabine-resistant cell line did not exhibit any morphological changes, including spindle-shaped morphology and appearance of pseudopodia such as in epithelial-to-mesenchymal transition, compared to the parental cells (data not shown). The cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (inactivated at 56°C for 30 min), 2 mM L-glutamine, 1.5 g/l sodium bicarbonate, 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), and 1.0 mM sodium pyruvate, and maintained in a humidified 5% carbon dioxide-95% air mixture at 37°C.

Agents. AHCC and cyclodextrin were kindly provided by Amino Up Chemical Co., Ltd. (Sapporo, Japan). Cyclodextrin is a diluting agent for AHCC, and it was used as a control. Gemcitabine was obtained from Eli Lilly Japan K. K. (Kobe, Japan).

Sample preparation. KLM1-R cells were treated with AHCC (0, 1, 5, 10 mg/ml) for 48 h. Cells were then homogenized in lysis buffer [50 mM Tris-HCl, pH 7.5, 165 mM sodium chloride, 10 mM sodium fluoride, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM ethylenediaminetetra-acetic acid (EDTA), 10 µg/ml aprotinin, 10 µg/ml leupeptin, and 1% nonyl phenoxypolyethoxyethanol-40 (NP-40)] on ice. Suspensions were incubated for 1 h at 4°C and centrifuged at 15,000 × g for 30 min at 4°C. The supernatants were collected and used for western blotting after protein concentrations were measured by Lowry method (22). The samples from KLM1-R cells were prepared three times independently.

Western blot analysis. Twenty micrograms of protein samples were used for western blot analysis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in pre-cast gels (12% acrylamide; Mini-PROTEAN TGX Gels, Bio-Rad, Hercules, CA, USA). After electrophoresis, gels were transferred electrophoretically onto polyvinylidene difluoride membranes (Immobilon-P; Millipore, Bedford, MA, USA) and blocked overnight at 4°C with Tris-buffered saline (TBS) containing 5% skimmed milk. Primary antibodies were: mouse monoclonal antibody against HSP27 (dilution 1:200, #sc-13132 (F-4); Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit polyclonal antibody against HSP60 (dilution 1:5000, ab46798; Abcam, Cambridge, MA, USA), goat polyclonal antibody against HSP70 (dilution 1:200, #sc-1060 (K-20); Santa Cruz Biotechnology), mouse monoclonal antibody against heat shock cognate 71 kDa protein (HSC70) (dilution 1:200, #sc-7298 (B-6); Santa Cruz Biotechnology), goat polyclonal antibody against 78 kDa glucose-regulated protein (GRP78) (dilution 1:200, #sc-1050 (N-20); Santa Cruz Biotechnology), and goat polyclonal antibody against actin (dilution 1:200, #sc-1616 (I-19); Santa Cruz Biotechnology). Membranes were incubated with the primary antibody overnight at

4°C, washed three times with TBS containing 0.05% Tween-20 and once with TBS, and then incubated with a horseradish peroxidase-conjugated secondary antibody (dilution 1:10,000; Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) for 1 h at room temperature. Bands of HSP27, HSP60, HSP70, HSC70, GRP78 and actin were visualized by enhanced chemiluminescence system (ImmunoStar Long Detection; Wako, Osaka, Japan), and recorded by using Image Reader LAS-1000 Pro (Fujifilm Corporation, Tokyo, Japan) (23-27). Expression levels of HSP27 and actin with and without AHCC treatment in KLM1-R cells were quantified by analyzing the intensity of each band with the Multi Gauge ver3.0 software (Fujifilm Corporation, Tokyo, Japan). Statistical significance of differences in expression levels of HSP27 with and without AHCC treatment in KLM1-R cells was calculated by one-way analysis of variance (ANOVA). A value of $p < 0.05$ was considered to be statistically significant. The software application used was JMP 9 (SAS Institute Inc., Cary, NC, USA).

Cytotoxic effect of AHCC. Cells (2×10^3 cells per well) were seeded in complete medium in 96-well plates, and cultured for 24 h, and then exposed to different concentrations (0, 2, 4, 6, 8, 10 mg/ml) of AHCC or cyclodextrin for 72 h. After incubation, 20 µl of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) (Promega Co., Madison, WI, USA) solution was added to each well. After 2 h, the optical density of the dissolved material was measured at 490 nm with a microtiter plate reader (Model 550 Microplate Reader; Bio-Rad, Hercules, CA, USA). Results were derived from at least three independent sets of triplicate experiments. Statistical significance of differences in proliferation rate of KLM1-R cells treated with AHCC or cyclodextrin were calculated by Student's *t*-test. A value of $p < 0.05$ was considered to be statistically significant.

Synergetic cytotoxic effect of AHCC and gemcitabine on KLM1-R cells. Cells (2×10^3 cells per well) were seeded in complete medium in 96-well plates, and cultured for 24 h, and then exposed to different concentrations (0 or 2 mg/ml) of AHCC for 48 h, and then treated with gemcitabine (0 or 25 ng/ml) for 72 h. After incubation, 20 µl of MTS solution was added to each well. After 2 h, the optical density of the dissolved material was measured at 490 nm with microtiter plate reader. Results were derived from at least three independent sets of triplicate experiments. Statistical analysis of the differences in the percentage of control cell growth with and without AHCC and gemcitabine treatment was performed using one-way or two-way ANOVA. A value of $p < 0.05$ was considered to be statistically significant.

Results

The effect of AHCC on the HSP27 expression levels in KLM1-R cells. The intracellular proteins from KLM1-R cells were analyzed by western blotting with a primary antibody against HSP27, HSP60, HSP70, HSC70, GRP78 and actin. The protein expression of HSP27 was reduced by AHCC treatment in KLM1-R cells. On the other hand, the protein expression of HSP60, HSP70, HSC70, GRP78 and actin were the same in all cells (Figure 1).

The ratio of intensities of HSP27 to actin (HSP27/actin) in KLM1-R cells without AHCC treatment was considered

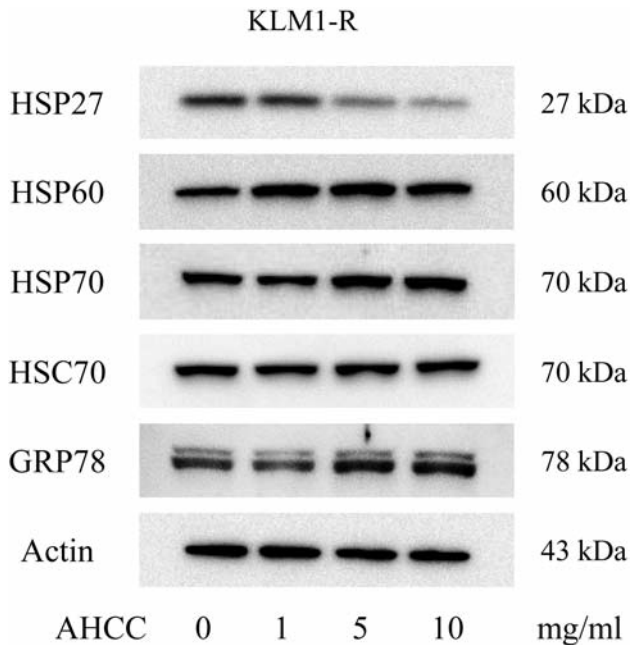


Figure 1. Expression levels of heat-shock protein (HSP) family proteins in KLM1-R cells treated with active hexose correlated compound (AHCC). Western blot analysis of HSP family proteins and actin in gemcitabine-resistant pancreatic cancer KLM1-R cells treated with AHCC (0, 1, 5, 10 mg/ml). The protein expression of HSP27 (bands of 27 kDa) was reduced by AHCC treatment in KLM1-R cells. On the other hand, protein expression of HSP60, HSP70, HSC70, GRP78 and actin were similar in all cells.

100%. HSP27/actin in KLM1-R cells treated with 1, 5 and 10 mg/ml AHCC was $88.7 \pm 6.6\%$, $71.5 \pm 0.9\%$, $48.6 \pm 1.6\%$, respectively. The results show that the HSP27 protein levels were reduced by AHCC in KLM1-R cells in a dose-dependent manner (Figure 2).

Cytotoxic effect of AHCC on KLM1-R cells. To investigate whether AHCC had a cytotoxic effect on KLM1-R cells, we added AHCC or cyclodextrin to the growth media and calculated the proliferation rate by the MTS assay. The proliferation rate of KLM1-R cells without AHCC or cyclodextrin as a control treatment was taken as 100%. The proliferation rate of KLM1-R cells treated with 2 and 4 mg/ml AHCC were $77.5 \pm 2.9\%$ and $6.3 \pm 3.4\%$, respectively, and of cells treated with 6, 8 and 10 mg/ml AHCC was 0%. The proliferation rate of KLM1-R cells treated with 2, 4, 6, 8 and 10 mg/ml cyclodextrin were $97.9 \pm 8.4\%$, $95.6 \pm 7.3\%$, $85.4 \pm 7.3\%$, $90.2 \pm 15.7\%$, $87.5 \pm 11.3\%$, respectively. These results show that cyclodextrin did not affect the growth of the KLM1-R cells. In contrast, AHCC had a cytotoxic effect on KLM1-R cells (Figure 3).

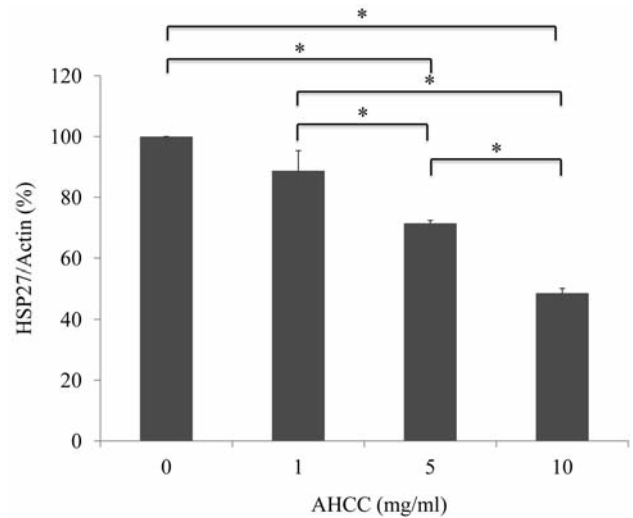


Figure 2. The intensity of the heat-shock protein-27 (HSP27)/actin bands in KLM1-R cells treated with active hexose correlated compound (AHCC). This graph shows the ratio of the intensities of HSP27 protein to actin protein bands in KLM1-R cells treated with AHCC. The intensities of HSP27/actin were significantly reduced by AHCC in KLM1-R cells in a dose-dependent manner (* $p < 0.01$ by one-way ANOVA). A value of $p < 0.05$ was considered statistically significant ($n = 3$).

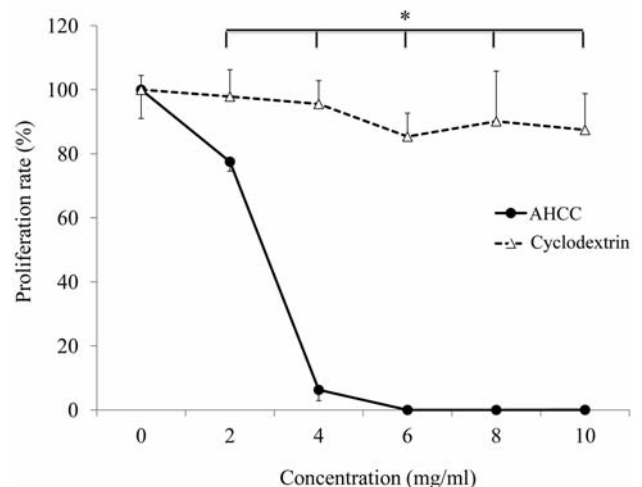


Figure 3. Cytotoxic effect of active hexose correlated compound (AHCC) on KLM1-R cells. The proliferation rates of KLM1-R cells treated with different concentrations of AHCC or cyclodextrin, a diluting agent, were calculated by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assay. This graph shows that cyclodextrin as a control did not affect the growth of KLM1-R cells. In contrast, AHCC exhibits a cytotoxic effect on KLM1-R cells (* $p < 0.01$ by Student's *t*-test). *p*-Values indicate between AHCC-treatment and cyclodextrin-treatment at each concentration. A value of $p < 0.05$ was considered to be statistically significant ($n = 6$).

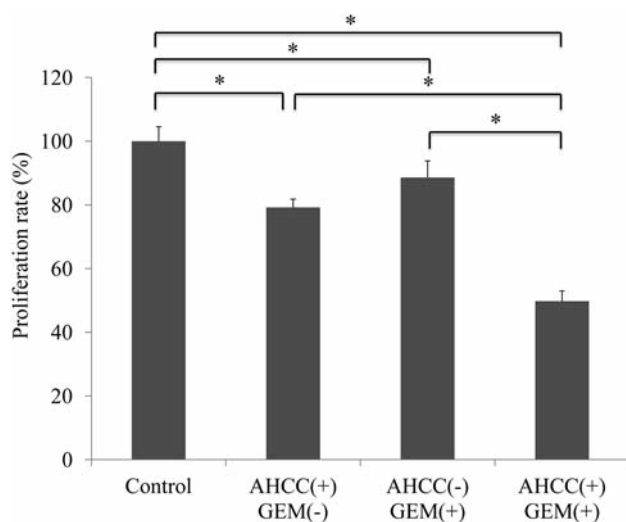


Figure 4. The cytotoxic effect of the combinatorial treatment of active hexose correlated compound (AHCC) and gemcitabine (GEM) on KLM1-R cells. This graph shows the proliferation rates of gemcitabine-resistant KLM1-R cells treated with gemcitabine alone (25 ng/ml), AHCC (2 mg/ml) alone, combination of AHCC and gemcitabine or no-treatment. The cytotoxic effect of the combinatorial treatment of AHCC and gemcitabine was significantly higher compared to treatments with AHCC or gemcitabine-alone (* $p < 0.01$ by one-way ANOVA). A value of $p < 0.05$ was considered statistically significant.

The cytotoxic effect of AHCC and gemcitabine on KLM1-R cells. We examined the effect of combinatorial treatment with AHCC and gemcitabine on gemcitabine-resistant KLM1-R cells by the MTS assay. As shown in Figure 4, we calculated the proliferation rate of the cells treated with gemcitabine-alone, AHCC-alone, combination of AHCC and gemcitabine, or no treatment. The cytotoxic effect of the combinatorial treatment of AHCC and gemcitabine was significantly higher compared to the treatment with AHCC alone or gemcitabine alone (one-way ANOVA). Furthermore, two-way ANOVA showed that the effect of the combinatorial treatment was synergistic ($p = 0.0008$).

Discussion

HSP27 is a member of the small heat-shock protein family and possesses chaperon-like activity, preventing aggregation of improperly-folded or partially-denatured proteins (28-30). HSP27 also regulates client proteins that are involved in the apoptotic pathway including protein kinase B (Akt), p53 and nuclear factor-kappa B (NF- κ B) (31). Furthermore, many groups have reported that overexpression of HSP27 is associated with promoting drug resistance and poor prognosis in many types of cancer (32-40). Our previous studies revealed that the expression of HSP27 was up-regulated in a

gemcitabine-resistant pancreatic cancer cell lines compared to gemcitabine-sensitive ones (11, 12). Hsu *et al.* reported that quercetin inhibited the expression of HSP27, and reduced the viability of lung cancer cells when used in a combinatorial treatment with either cisplatin or gemcitabine (41). Heinrich *et al.* reported that RP101 (bromovinyldeoxyuridine), inhibitor of HSP27 function, prevented resistance of rat sarcoma cells to cisplatin (42). These reports show that reduction of HSP27 might increase sensitivity to chemotherapy. In fact, our previous studies showed that interferon- γ or *N*-formyl-3,4-methylenedioxy-benzylidene- γ -butyrolactam (KNK437) down-regulated the expression of HSP27, and increased gemcitabine sensitivity in gemcitabine-resistant pancreatic cancer KLM1-R cells (13, 14).

AHCC has been extensively studied for safety in both patients with cancer and healthy volunteers (43-45). Several studies have investigated the alleviating effects of AHCC for chemotherapy-related side-effect. Nakamoto *et al.* reported that AHCC reduced hematological toxicity of gemcitabine in non-tumor-bearing mice (46). Sun *et al.* reported that AHCC reduced cytosine arabinoside-induced hair loss, and 6-mercaptopurin and methotrexate-induced liver injury in mice (47). Furthermore, AHCC also enhanced the chemotherapeutic effects of UFT (tegafur and uracil in a 4:1 molar concentration) for mammary adenocarcinoma SST-2 cells in rats (48), and cisplatin for Colon-26 tumor cells in mice (49).

The present study showed that AHCC down-regulated the expression of HSP27, and combinatorial treatment of AHCC and gemcitabine synergistically increased the cytotoxic effect on gemcitabine-resistant pancreatic cancer cells. Although the molecular mechanism of AHCC for down-regulation of HSP27 is unknown at this time, AHCC can be considered a possible candidate for combinatorial therapy in anticancer drug regimens. It is clear that further studies are needed in order to evaluate AHCC functions. This study supports the potential therapeutic benefits of combinatorial treatment of AHCC and gemcitabine for patients with pancreatic cancer.

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