Active Hexose-correlated Compound Down-regulates Sex-determining Region Y-box 2 of Pancreatic Cancer Cells

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Abstract. *Background/Aim: Active hexose-correlated compound (AHCC) is an extract of basidiomycete mushroom. It has been used as health food due to its efficacy of enhancing antitumor effects and reducing adverse effects of chemotherapy. Our previous research showed that AHCC down-regulated heat-shock protein (HSP)-27 and exhibited cytotoxic effects against gemcitabine-resistant pancreatic cancer cells. Sex-determining region Y-box 2 (SOX2) is reported to be up-regulated in other kinds of cancer cells and involved in carcinogenesis and malignancy. The aim of this study was to investigate the effects of AHCC on protein expression of SOX2 in the gemcitabine-resistant pancreatic cancer cell line KLM1-R. Materials and Methods: AHCC was applied to KLM1-R cells and expression of SOX2 was analyzed by western blotting. Results: AHCC down-regulated SOX2 in KLM1-R cells. Nanog and Oct4, co-workers of SOX2 in maintaining pluripotency, did not exhibit any significant change in protein expression. Conclusion: We showed the potential of AHCC to be a candidate for combinatorial therapy in anticancer drug regimens. This result suggests that the target of AHCC in expressing therapeutic efficacy was not the pluripotent cells such as cancer stem cells (CSCs) but SOX2-specific.*

Pancreatic cancer is known for its miserable course and poor prognosis. The 5-year survival rate is no more than 7% (1). The only treatment that can bring complete cure is surgical resection, but it is difficult to diagnose pancreatic cancer at poorly-advanced and resectable stage. What is left for the patients with unresectable pancreatic cancer is chemotherapy but gemcitabine, the standard drug used for the patients with pancreatic cancer, has only restrictive efficacy on expanding life span and soon is inhibited by intrinsic or acquired resistance against gemcitabine.

Active hexose-correlated compound (AHCC), an extract of basidiomycete mushroom called *Lentinula edodes*, is composed of polysaccharides, amino acids, lipids and minerals. The predominant components of AHCC are oligosaccharides and their major portions are 5-kDa molecules named α1,4-glucans. AHCC is used as a health food to support the therapeutic effects and alleviate adverse effect of chemotherapy owing to immunomodulatory and antitumor effects of AHCC. Our previous study showed that AHCC down-regulated heat-shock protein 27 (HSP 27) and exhibited a cytotoxic effect on gemcitabine-resistant pancreatic cancer cells while the cytotoxic effect of the combinatorial treatment of AHCC and gemcitabine were synergistic (2). HSP 27 is a 27-kDa chaperon protein induced by environmental or pathophysiological stressor and protects cells from cell death. Many groups have reported its involvement towards malignancy, prognosis and resistance against chemotherapy in many kinds of cancer. (3-7). As shown above, there are many molecules reported to be overexpressed in cancer cells other than HSP27. In the present study, we focused on the Sex-determining region Y-box 2 (SOX2), a transcription factor which belongs to SOX family and have abundantly reported to be overexpressed in various cancer cells (8). SOX2 plays important roles in maintaining the pluripotency of embryonic stem cell properties and differentiation restriction. SOX2 has been shown to encourage carcinogenesis. Lou *et al*. reported that SOX2 promoted migration, invasion and clonal formation of ovarian cancer cells (9). Chou *et al*. reported that SOX2-

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overexpression promoted oncogenic phenotypes and on the other hand, *SOX2*-silensing attenuated proliferation in lung cancer cells (10). Therefore, in the present study we attempted to treat a pancreatic cancer cell line to observe the effect of AHCC on the expression of SOX2.

Materials and Methods

Cancer cell line and culture conditions. The gemcitabine-resistant human pancreatic cancer cell line KLM1-R was kindly provided by the Department of Surgery and Science, Kyusyu University Graduate School of Medical Science. KLM1-R was established by applying gemcitabine to gemcitabine-sensitive KLM1 cells, as previously described (11). The cells were cultured in Roswell Park Memorial Institute (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, following our routine procedure.

Agents. AHCC and cyclodextrin were kindly provided by the Amino Up Chemical Co., Ltd. (Sapporo, Japan). Cyclodextrin is a diluting agent for AHCC and used as a control in the present study.

Sample preparation. AHCC (0, 10 mg/ml) or cyclodextrin (10 mg/ml) was applied to KLM1-R cells. 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 vandate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM ethylendiaminetetra-acetic acid (EDTA), 10 μg/ml aprotinin, 10 μg/ml leupeptin and 1% nonylphenoxypolyethoxylethnol-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 the standard Lowry method. The samples from KLM1-R were prepared five times independently.

Western blot analysis. Fifteen micrograms of protein samples were used for Western blotting analysis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was done in pre-cast gels (4-20% gradient 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 1 h at room temperature with Tris-buffered saline (TBS) containing 5% skimmed milk. Primary antibodies were: goat polyclonal antibody against SOX2 (1:300, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), rabbit monoclonal antibody against Oct4 (1:1000, Cell Signaling Technology, Inc., Danvers, MA, USA), rabbit polyclonal antibody against Nanog (1:500, CHEMICON International, Inc., Billerica, MA, USA), goat polyclonal antibody against actin (1:200, 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 antibodies (anti-goat for SOX2 and actin, anti-rabbit for Oct4 and Nanog, dilution 1:10,000; Jackson Immuno Research Laboratories Inc., West Grove, PA, USA) for 1 h at room temperature. Bands of SOX2, Oct4, Nanog and actin were visualized by enhanced chemiluminescence system (ImmunoStar Long Dectection; Wako, Osaka, Japan) and recorded by Image Reader Las-1000 Pro (Fujifilm Corporation, Tokyo, Japan). Expression levels of SOX2 and actin with or without AHCC treatment in KLM1-R cells were quantified by analyzing the intensity of each band with Multi Gauge ver3.0 software (Fujifilm Corporation, Tokyo, Japan). Statistical significance of differences in expression levels of SOX2 with or without AHCC treatment in KLM1-R were calculated by Student's *t*-test. A value of $p<0.05$ was considered statistically significant.

Results

The intracellular proteins from KLM1-R cells were analyzed by western blotting with a primary antibody against SOX2. The protein expression of SOX2 in KLM1-R cells were reduced by AHCC-treatment (10 mg/ml), while the protein expression of actin was practically the same in all cells (Figure 1). The ratio of intensities of SOX2 to actin (SOX2/Actin) in KLM1-R cells was measured. SOX2/Actin in KLM1-R cells treated with or without 10 mg/ml AHCC were 25.4% and 110.0%, respectively (Figure 2). The pluripotency of embryonic stem cell properties and differentiation restriction is mediated not by SOX2-alone. Oct4 and Nanog are indispensable co-workers of SOX2 and they form a crucial network in regulating pluripotency (12). Here we analyzed the change in expression of Oct4 and Nanog in addition to SOX2. However, no significant change in expression of Oct4 and Nanog was observed (Figure 3). These findings suggest that the target of AHCC in expressing therapeutic efficacy was not the pluripotent cells such as cancer stem cells (CSCs) but SOX2-specific.

Discussion

As previously discussed, SOX2 is a transcription factor which belongs to the SOX family and plays important roles in maintaining the pluripotency of embryonic stem cell properties and differentiation restriction. It is involved in CSC maintenance and then impairs cell growth and tumorigenicity. Berezovsky *et al*. showed that *SOX2*-knockdown in the differentiated state abolished de-differentiation and acquisition of CSC phenotype in glioblastoma (13). Herreros-Villanueva *et al*. reported that SOX2 was aberrantly expressed in pancreatic cancer and contributed to cell proliferation and stemness/ de-differentiation through the regulation of a set of genes controlling G_1/S transition and epithelial-to-mesenchymal transition (EMT) phenotype (14). On the other hand, we could not observe any remarkable change in the expression of Oct4 and Nanog, the crucial coworkers of SOX2 in maintaining pluripotency. Herein we showed that AHCC induced the down-regulation of SOX2, not influencing Oct4 and Nanog. These findings suggest that AHCC down-regulates SOX2 not by suppressing CSCs.

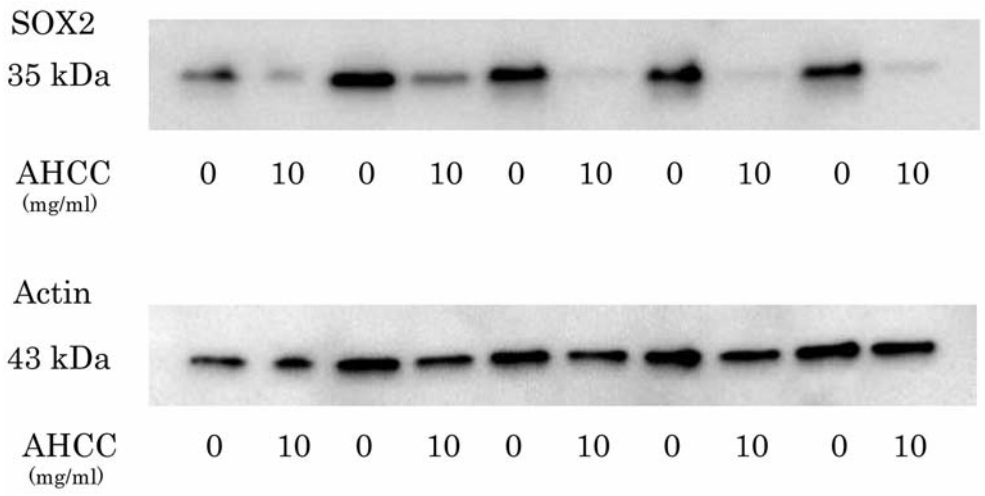

Figure 1. *Exprression levels of Sex-determining region Y-box 2 (SOX 2) in KLM-1 R cells treated with active hexose correlated compound (AHCC). Western blotting of SOX2 and actin in gemcitabine-resistant pancreatic cancer KLM1-R cells treated with or without AHCC (10 mg/ml). The protein expression of SOX2 (bands of 35 kDa) was reduced by AHCC treatment in KLM1-R cells while those of actin were all similar.*

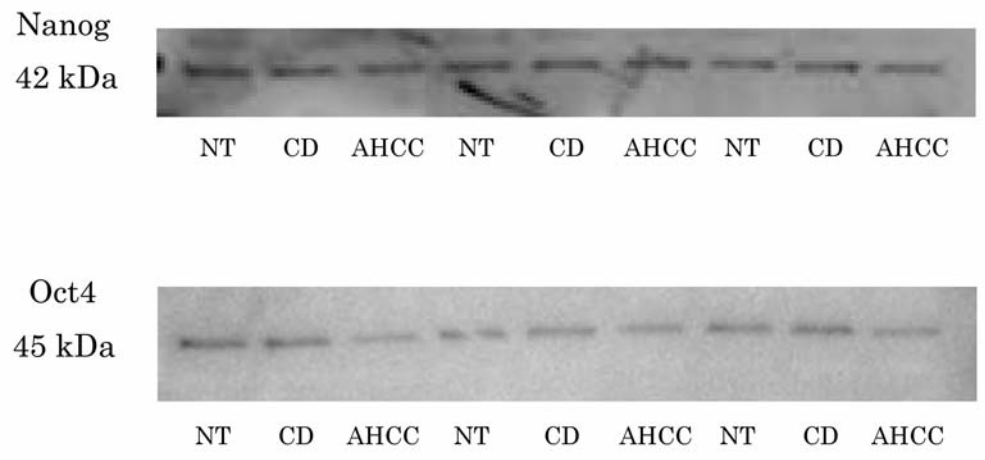

Figure 2. *Expression levels of Nanog and Oct4 in KLM-1 R cells treated with active hexose correlated compound (AHCC). Western blotting analysis of Nanog and Oct4 in gemcitabine-resistant pancreatic cancer KLM1-R cells treated with AHCC (10 mg/ml), cyclodextrin (10 mg/ml), a diluting agent for AHCC. The protein expression of Nanog (bands of 42 kDa) and Oct4 (bands of 45kDa) in KLM1-R cells were all similar regardless of the presence of AHCC treatment. NT, Non-treatment; CD, cyclodextrin; AHCC, active hexose correlated compound.*

Several groups have reported the AHCC's efficacy of alleviating the toxic side-effect of anticancer drugs and enhancing the chemotherapeutic effects. Ito *et al*. reported that administration of AHCC significantly decreased the levels of human herpesvirus type-6 (HHV-6) in saliva during chemotherapy and improved quality of life (QOL) scores in the EORTC QLQ-C30 questionnaire but also hematotoxicity and hepatotoxicity (15). Hangai *et al*. reported that the AHCC group had significantly fewer neutropenia induced by breast cancer chemotherapy and significantly lower use of granulocyte colony-stimulating factor (G-CSF) compared to the control group (16). Sun *et al*. reported the effect of AHCC in modulating cytosine arabinoside-induced hair loss, and 6-mercaptopurine- and methotrexate-induced liver injury in rodents (17). Our previous study showed that combinatorial therapy of AHCC plus tegafur and uracil (UFT) significantly reduced the metastasis of rat mammary adenocarcinoma (18), while AHCC down-regulated the HSP27 of pancreatic cancer cells and helped the cytotoxic effect of gemcitabine (2).

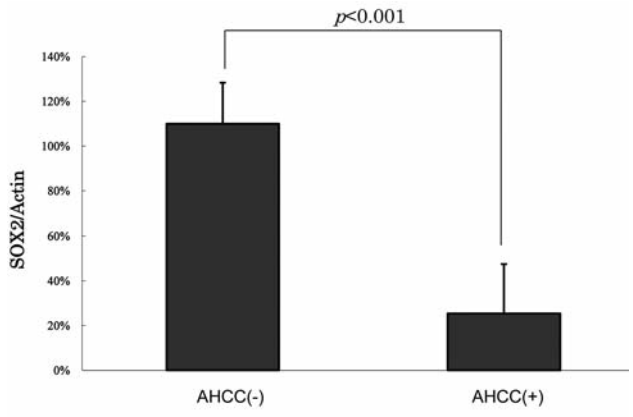

Figure 3. *The intensity of the Sex-determining region Y-box 2 (SOX2)/Actin bands in KLM-1 R cells treated with active hexose correlated compound (AHCC). This graph shows the ratio of the intensities of SOX2 protein to actin protein bands in KLM1-R cells treated with or without AHCC. The intensities of SOX2/Actin were significantly reduced in AHCC treated cells. ($p<0.001$ by Student's t-test). A value of $p<0.05$ was considered statistically significant.*

As we showed in the present study, AHCC has the potential to enhance the effect of chemotherapy and reduce the adverse effects. Still the molecular mechanism of AHCC for down-regulating SOX2 is unknown. In the present study, we showed the potential benefit of AHCC for treatment of pancreatic cancer.

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Conflicts of Interest

The Authors declare no conflicts of interest.

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