A standardized extract of cultured *Lentinula edodes* mycelia downregulates cortactin in gemcitabine-resistant pancreatic cancer cells

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Abstract. AHCC®, a standardized extract of cultured *Lentinula edodes* mycelia, enhances the therapeutic effects and reduces the adverse effects of chemotherapy. Our previous study reported that treatment with AHCC® downregulated the expression levels of tumor-associated proteins in the gemcitabine-resistant pancreatic cancer cell line, KLM1-R. However, to the best of our knowledge, the role of AHCC® in the inhibition of cell migration remains unexplored. Cortactin (CTTN), an actin nucleation-promoting factor, has been reported to be upregulated and correlated with migration, invasion and metastasis in pancreatic cancer cells. The present study aimed to investigate the effects of AHCC® on cell migration and the protein expression level of CTTN in KLM1-R cells. The Gene Expression Profiling Interactive Analysis (GEPIA2), an online bioinformatics platform, was used to analyze CTTN mRNA expression levels in pancreatic cancer tissues compared with normal pancreatic tissues. CTTN mRNA expression and its association with clinicopathological characteristics were assessed by using the GEPIA2 platform. Next, the effects of AHCC® on KLM1-R cell migration were investigated by *in vitro* wound-healing assay. The KLM1-R cells were treated with AHCC® at a concentration of 10 mg/ml for 48 h. Western blotting was performed on of cell lysates with anti-CTTN or anti-actin antibodies to assess the protein expression levels of CTTN. Bioinformatics analysis indicated that the mRNA expression level of CTTN increased in pancreatic cancer tissues. The increased mRNA expression levels of CTTN were inversely associated with clinicopathological characteristics, including disease stages and prolonged patient survival times. The administration of 10 mg/ml AHCC® significantly inhibited KLM1-R cells migration compared with controls. The protein expression levels of CTTN were significantly reduced in AHCC®-treated KLM1-R cells, whereas actin expression was not affected. The downregulation of CTTN indicated the anti-metastatic potential of AHCC® in pancreatic cancer cells. Overall, AHCC® may have the potential to be a complementary and alternative therapeutic approach in treating pancreatic cancer.

Introduction

Pancreatic cancer is one of the most common human malignancies and a leading cause of cancer-related mortality worldwide. Patients with pancreatic cancer often have a poor prognosis. Despite advances in oncology, the overall 5-year survival rates of this cancer have not significantly improved for decades (1). The lack of adequate and credible interventions is the key cause of the high mortality rate in pancreatic cancer. Gemcitabine is the most effective and extensively used chemotherapeutic in pancreatic cancer thus far. However, the overall survival is dismal and is in part caused by gemcitabine resistance in pancreatic cancer (2). New therapeutic agents for the treatment of pancreatic cancer are thus urgently needed.

The metastatic progression of cancer cells requires the remodeling of the actin cytoskeleton. The altered expression of key regulatory proteins of the actin cytoskeleton, such as cortactin (CTTN), contributes to carcinogenesis (3). CTTN is involved in various cell functions, including actin polymerization, the formation of cell motility structures such

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as podosomes and invadopodia, and extracellular matrix (ECM)-protein deposition. These functions of CTTN can lead to deregulated cell migration, invasion, and metastasis (4).

AHCC® is a standardized extract of *Lentinula edodes* mycelia. AHCC® comprises polysaccharides, amino acids, minerals, and lipids enriched in α 1,4-glucans (5). The anti-oxidant, anti-tumor, and immunomodulatory potentials of AHCC® were described in several studies (6,7). Previously we reported that AHCC® downregulated tumor-associated proteins involved in pancreatic carcinogenesis (8-11). We demonstrated that following administration of AHCC®, the level of heat-shock protein 27 (HSP27), heat shock factor 1 (HSF1), sex-determining region Y-box 2 (SOX-2), and CUB domain-containing protein 1 (CDCP1) were significantly reduced in pancreatic cancer cells. Moreover, our proteomic analysis revealed that HSP27 expression was strongly related to gemcitabine resistance in pancreatic cancer (12). Similarly, SOX-2 and CDCP1 are highly expressed in malignant tissues and involved in tumor invasion and metastasis (8,11). Together, these findings suggested that AHCC® might suppress the proteins involved in chemoresistance and malignant progression of pancreatic cancer. Therefore, we hypothesized that AHCC® might inhibit CTTN expression and have an anti-metastatic potential in pancreatic carcinogenesis.

In the present study, we investigated CTTN mRNA expression levels in pancreatic cancer tissues from The Cancer Genome Atlas (TCGA) databases using an online bioinformatics platform. Next, we examined whether AHCC® suppressed cell migration and CTTN expression in KLM1-R cells using an *in-vitro* wound-healing assay and western blotting, respectively.

Materials and methods

mRNA expression analysis of CTTN in pancreatic cancer patients. The CTTN mRNA expression in pancreatic cancer tissues was analyzed from the TCGA and GTEx databases, using the Gene Expression Profiling Interactive Analysis (GEPIA2) platform (13). The GEPIA2 platform was also utilized to perform the association analyses of the CTTN expression level in pancreatic cancer with clinical characteristics, including pathological stages and Kaplan-Meier survival plots. The mRNA expression cut-off criteria were selected as follows: LogFC cut-off=1; P-value cut-off=0.05; datasets=pancreatic ductal adenocarcinoma; and matched normal data=match TCGA normal and GTEx. The quartile cut-off was selected for survival plot analysis. P<0.05 is considered to indicate a statistically significant difference.

Cancer cell line and conditions. The KLM1-R pancreatic cancer cell line is gemcitabine-resistant. It has been established at the Department of Surgery and Science, Kyushu University Graduate School of Medical Science, and derived from the gemcitabine-sensitive pancreatic cancer cell line KLM1, that was exposed to gemcitabine. In brief, the KLM1 cells cultured at an initial density of 1×10^6 cells on 6-well flat-bottomed plates containing 2 ml medium for 1 day were treated with 10 μg/ml gemcitabine for 1 week. Cells were then cultured in a gemcitabine-free medium for 2 weeks to recover cell density. After repeating the above treatment 4x,

a gemcitabine-resistant cell line, KLM1-R was established. KLM1-R exhibited stable characteristics with respect to growth rate, morphology, and drug resistance (14). The cells were then kept 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-hydroxyehylpiperazine-N'-2-ethanesulfonic acid (HEPES), and 1.0 mM sodium pyruvate, in a CO_2 incubator.

Preparation of AHCC®. AHCC® was kindly provided by Amino Up Co., Ltd. (Sapporo, Japan). AHCC® was dissolved in RPMI-1640 medium to a final concentration of 10 mg/ml, filter-sterilized, and stored at 4°C in aliquots. Fresh AHCC® solution was used in each experiment.

In vitro wound-healing assay. The inhibition of cell migration by AHCC® was investigated by *in vitro* wound-healing assay as described previously (15). Briefly, cells were allowed to grow to full confluence in 24-well plates, and then a vertical wound was created with a 10 μl pipette tip. After the cell debris was removed, a fresh RPMI-1640 complete medium supplemented 10% FBS with various concentrations of AHCC® [0 (control), 1, 5 and 10 mg/ml] was added. Cells were incubated at 37°C and 5% CO_2 for 24 h and images were captured using a microscope at three time points (3, 6, and 24 h). Wound healing was observed at different time points within the scraped line, and representative images of scraped lines were captured using a phase-contrast microscope at x40 magnification. The migration area was measured and analyzed with ImageJ software (version 1.48) (16), and the experiments were conducted in triplicate.

Western blot analysis. KLM1-R cells were treated with or without AHCC® (10 mg/ml) for 48 h *in vitro*. After treatment and washing three times, proteins were extracted from cells using lysis buffer (50 mM Tris HCl, pH 7.5; 10 mM EDTA, pH 7.5; 165 mM NaCl; 10 mM NaF; 1% Nonidet P-40; 1 mM PMSF; 1 mM NaVO₃; 10 μg/ml leupeptin; and 10 μg/ml aprotinin). The lysis reaction was carried out for 1 h at 4°C. The samples were centrifuged at 15,000 rpm for 30 min at 4°C, and the supernatant was used as a sample. Protein concentration was quantified by Lowry's protein assay. Fifteen micrograms of the protein samples were used for western blot analysis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in pre-cast gels (4-20% gradient of polyacrylamide; Mini-PROTEAN TGX Gels; Bio-Rad). After electrophoresis, gels were transferred electrophoretically onto polyvinylidene difluoride membranes (Immobilon-P; Millipore) and blocked for 1 h with Tris-buffered saline with 0.1% Tween-20 solution (TBS-T) containing 5% skimmed milk. Blocked membranes were washed twice with TBS-T.

The following primary antibodies were used: Rabbit monoclonal antibody against cortactin (dilution 1:1,000, #CST3502; Cell Signaling Technology, Beverly, MA, USA) and rabbit polyclonal antibody against actin (dilution 1:5,000, #sc1616R; Santa Cruz Biotechnology, Inc.). Membranes were incubated with the primary antibody overnight at 4°C, washed three times with TBS-T, and incubated with horseradish peroxidase-conjugated secondary antibody (dilution 1:10,000; Jackson Immuno-Research Laboratories Inc.) for 1 h at

room temperature. Bands of cortactin and actin were visualized by the enhanced chemiluminescence system (Clarity™ Western ECL Substrate; Bio-Rad) and LuminoGraph I (ATTO Corporation, Tokyo, Japan) and recorded using ImageSaver6 software (ATTO Corporation) (17). Cortactin and actin levels were quantified by analyzing each band intensity using CS Analyzer4 software (ATTO Corporation). Each experiment was performed in triplicate. Data are expressed as mean ± standard error (SE) of the target protein ratio to actin protein.

Literature review of the effects of AHCC® in different cancers. Two databases, namely PubMed and Scopus, were screened for relevant articles and were limited to articles published in English. Data were extracted from the databases on May 22, 2021, without applying any time restrictions. The formulated search strategy was used in the databases: (Active hexose correlated compound [MeSH Terms]) OR (AHCC [MeSH Terms]) AND (cancer [MeSH Terms]) OR (neoplasm [MeSH Terms]). After a comprehensive analysis, 17 studies were selected (8-11,18-30)

Statistical analysis. Statistical analysis was performed on a database using IBM SPSS Statistics v22 (IBM Corp.). All data are expressed as the mean ± SE. When ANOVA indicated differences among the groups, multiple comparisons among each experimental group were performed using Bonferroni's method. Mann-Whitney U test was performed between groups of AHCC®-treated and untreated samples. All statistical experiments were performed three times, and P<0.05 was considered to indicate a statistically significant difference.

Results

CTTN mRNA expression is inversely correlated with prolonged patient survival. The CTTN mRNA expression level in pancreatic cancer tissues was analyzed using the GEPIA2 platform. The results demonstrated that the CTTN mRNA expression level was significantly increased in pancreatic cancer tissues when compared to normal pancreatic tissues (Fig. 1A). Further analysis of TCGA pancreatic cancer data in GEPIA2 showed that CTTN expression was positively correlated with the pathological disease stages, underlying their prognostic value for pancreatic cancer (Fig. 1B; P=0.012). The Kaplan-Meier survival plots demonstrated a significant relation with elevated CTTN expression in pancreatic cancer. The high level of CTTN is inversely correlated with prolonged patient survival (Fig. 1C; P=0.032) and disease-free survival states in patients with pancreatic cancer (Fig. 1D; P=0.037).

AHCC® inhibited migration of KLM1-R cells. The effects of AHCC® on KLM1-R cell migration were assessed by wound healing assay. As shown in Fig. 2A, photomicrographs taken 24 h after wounding showed delayed wound closure by KLM1-R cells treated with AHCC® at concentrations ranging from 0 to 10 mg/ml. Quantification of the wound closure over time revealed a significant inhibitory effect of AHCC® on KLM1-R cell motility at the concentrations of 10 mg/ml (Fig. 2B; P=0.000). Beyond these dosages [0 (control), 1, and 5 mg/ml], adjacent cells migrated toward the scratched space on plates, and the gap was closed as time passed. These data

indicate that AHCC® effectively inhibits the migration of KLM1-R cells.

AHCC® treatment decreased CTTN protein levels in KLM1-R cells. To evaluate the effect of AHCC® on the CTTN expression, we analyzed the intracellular proteins from KLM1-R cells treated with or without AHCC® by western blot analysis with primary antibodies against CTTN and actin. The protein expression of CTTN was reduced by AHCC® treatment in KLM1-R cells, whereas actin was almost the same in all cells (Fig. 3A). In addition, the ratio of intensities of CTTN/actin in KLM1-R cells was measured. The CTTN/actin intensity ratio was significantly different between AHCC®-treated and untreated KLM1-R cells (Fig. 3B; P=0.049). These results suggested that CTTN was down-regulated by AHCC®-treatment *in vitro*.

AHCC® improve treatment outcome and reduces chemotherapeutic adverse effects. The literature review was performed to investigate the effects of AHCC® in different cancers. The search strategy resulted in 48 potentially eligible studies, with 16 in PubMed and 32 in Scopus. After removing the duplicates, the first screening resulted in 37 studies singled out for evaluation. After a comprehensive analysis, 17 studies were chosen based on the criteria which were set. The effects of AHCC® and its potential clinical relevance in different cancers were explored, as shown in Table I. In brief, AHCC® induces apoptosis, inhibits cellular proliferation and malignant progression in various cancer types. The apoptotic effects of AHCC® have been described to be mediated via both intrinsic and extrinsic apoptotic mechanisms. In addition, AHCC® was shown to decrease the levels of cellular reactive oxygen species (ROS). The inhibition of ROS via administration of AHCC® has been suggested to be involved in maintaining cellular integrity and preventing ROS-induced carcinogenesis. Meanwhile, it was also found that in combination with conventional anti-tumor agents, AHCC® improves the immune system and prevents immune invasion of cancer cells. These immunomodulatory effects of AHCC® are described to be involved in prolonging patient survival and reducing chemotherapeutic-related adverse effects.

Discussion

In the present study, we demonstrated that CTTN mRNA expression was significantly higher in pancreatic cancer than in normal tissues. The higher CTTN expression was significantly correlated with the pathological stages of pancreatic cancer. The Kaplan-Meier survival plots showed that elevated CTTN expression levels are inversely associated with prolonged patient survival. From our *in vitro* analysis, we showed that AHCC® significantly suppressed KLM1-R cell migration. A significant reduction of CTTN protein level was observed in cells treated with AHCC® compared to control. The down-regulation of CTTN possibly causes the anti-tumor potential of AHCC® in pancreatic cancer cells.

CTTN has been documented to play essential roles in regulating actin cytoskeletal dynamics (31). The basic structure of CTTN consists of four major domains: An N-terminal acidic (NTA), a central 6.5 tandems repeat, a proline-rich
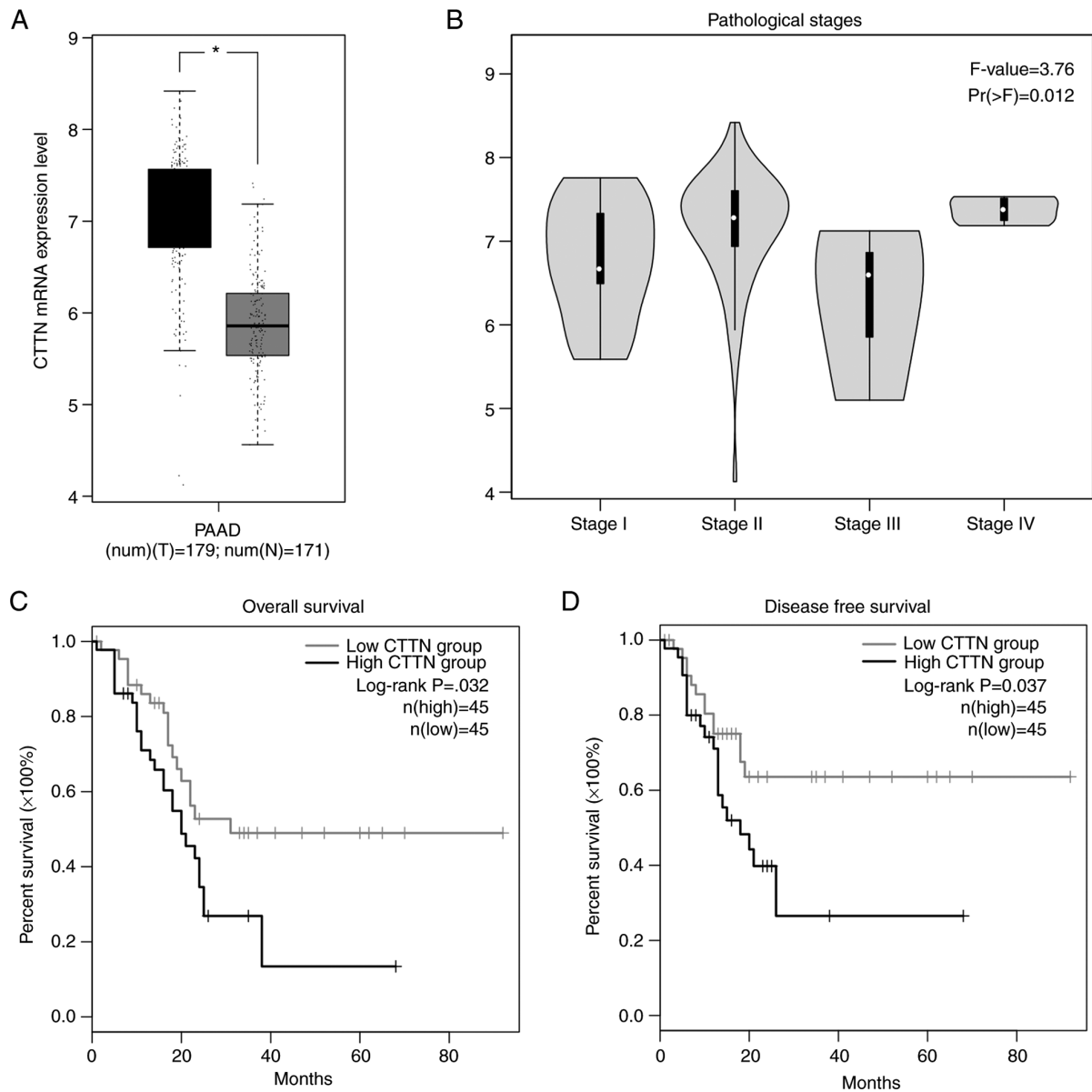
Figure 1. Expression level of CTTN mRNA and its clinical association with pancreatic cancer. (A) mRNA expression level of CTTN in pancreatic cancer tissues; boxplots were downloaded from the GEPIA2 platform. Black and gray box represent the expression levels in pancreatic cancer tissues and normal tissues, respectively. (B) Association of mRNA expression and pathological tumor stages in pancreatic cancer. Violin plots were created using GEPIA2 based on The Cancer Genome Atlas pancreatic cancer dataset. F-value indicates the statistical value of the F test; Pr (>F) indicates P-value. (C) Kaplan-Meier survival plot analysis. Overall survival analysis of CTTN performed using the GEPIA2 platform. Overall survival curve for CTTN expression in pancreatic cancer patients compared a high-risk group and a low-risk group. (D) Disease-free survival analysis of CTTN in pancreatic cancer patients comparing a high-risk group and a low-risk group. *P<0.05. CTTN, cortactin; GEPIA2, Gene Expression Profiling Interactive Analysis; PAAD, pancreatic ductal adenocarcinoma.

domain, and the C-terminal Src homology 3 domains (SH3 domains) (31,32). Those domains are modified and alter the interaction with binding partners to promote actin polymerization during cell motility. This then plays a central role in the formation of invadopodia, which are actin-driven protrusive structures in invasive cancer cells that degrade the ECM. The degraded ECM allows cancer cells to migrate towards the distant organs resulting in metastasis (33). The higher CTTN expression was previously described in several cancers and correlated with poor clinical outcomes in breast cancer, oral cancer, liver cancer, colon cancer, and melanoma (4,31,33).

However, little is known about CTTN involvement in the tumor progression of pancreatic cancer. A previous study has shown that elevated CTTN expression in pancreatic cancer is significantly involved with metastatic compared to primary tumors (34). In addition, it was demonstrated that inhibition of CTTN expression impaired the migration and invasion potential of pancreatic cancer (34). Based on these findings, it is imperative to regulate CTTN to treat pancreatic cancer. Our study observed a significant reduction in cell migration and CTTN levels in AHCC® treated cells compared to untreated cells. The downregulation of CTTN possibly caused the reduced cell migration observed in this study. It is, however, still unknown how AHCC® downregulated CTTN levels in pancreatic cancer cells.

In vitro, CTTN is overexpressed and activated by Src-mediated tyrosine phosphorylation, which leads to increased migration of fibroblasts and endothelial

Table I. Effects of AHCC® on different cancers.

Author (year)	Effects	Cancer types	(Refs.)
Kuhara *et al*, 2018	Downregulates CDCP1 levels; inhibits cell migration and malignant progression	Pancreatic cancer	(8)
Suenaga *et al*, 2014	Downregulates HSP27 levels; inhibits cancer cell proliferation	Pancreatic cancer	(9)
Tokunaga *et al*, 2015	Downregulates HSF1 levels	Pancreatic cancer	(10)
Nawata *et al*, 2014	Downregulates SOX-2 levels	Pancreatic cancer	(11)
Matsushita *et al*, 1998	Inhibits tumor metastasis and improves treatment outcome when combined with anti-tumor agents	Adenocarcinoma	(18)
Cowawintaweewat *et al*, 2006	Prolongs patient survival; decreases serum AST and ALT levels; increases IL-2 and neopterin levels	Hepatocellular carcinoma	(19)
Hunter *et al*, 2011	Inhibits tumor metastasis and improves treatment outcome when combined with anti-tumor agents	Ovarian cancer	(20)
Hangai *et al*, 2013	Inhibits chemotherapy-related adverse effects; inhibits cellular inflammation	Breast cancer	(21)
Ito *et al*, 2014	Decreases the salivary level of HHV-6 following chemo therapy; reduces chemotherapeutical-related adverse effects	Colon, pancreatic, lung and ovarian cancer	(23)
Ignacio *et al*, 2015	Maintains cellular ROS level and increases antioxidant production; inhibits tumor-related cytokine production	B6 melanoma murine model	(24)
Cao *et al*, 2015	Induces apoptosis and inhibition of cellular proliferation	Hepatoma tumor-bearing mice	(25)
Yanagimoto *et al*, 2016	Reduces chemotherapy-related adverse effects; Reduces CRP and albumin levels; Improves patient outcomes	Pancreatic cancer	(26)
Graham *et al*, 2017	Inhibits cellular proliferation and migration; upregulates tumor suppressor protein miR-335 and contributes to prevent immune invasion	Breast cancer	(27)
Fatehchand *et al*, 2017	Induces apoptosis through involvement with both extrinsic and intrinsic mechanisms	Acute myeloblastic leukemia	(28)
Choi *et al*, 2018	Induces apoptosis and proliferation; inhibits STAT3 phosphorylation; induces SHP-1 and inhibits cyclin D1, Bcl-2, Mcl-1, survivin and VEGF levels	Ovarian cancer	(29)
Suknikhom *et al*, 2017	Increases CD+T cell population and improves patient outcome	Ovarian cancer	(30)

AST, aspartate aminotransferase; ALT, alanine aminotransferase; ROS, reactive oxygen species; HSP27, heat shock protein 27; SOX-2, sex-determining region Y box 2; HHV-6, human herpesvirus 6; HSF1, heat shock factor 1; CRP, c-reactive protein; CDCP1, CUB domain-containing protein 1; STAT3, signal transducer and activator of transcription 3; Bcl-2, B cell lymphoma 2; VEGF, vascular endothelial growth factor; VE, vascular endothelial; miR, microRNA; SHP-1, tyrosine-protein phosphatase non-receptor type 6; Mcl-1, induced myeloid leukemia cell differentiation protein; CD+T, cluster of differentiation positive T cells.

cells (35,36). Phosphorylation of CTTN occurring primarily at tyrosine 421 (Tyr421) enhances the actin assembly during cytoskeletal remodeling (37,38). The tyrosine phosphorylation of CTTN correlates with the invadopodia activity necessary for matrix degradation, cell migration, and invasion (39). The protein-tyrosine phosphatase SHP-1 is a negative regulator of multiple signal transduction pathways and proposed to be a candidate tumor suppressor gene in various cancers (40). The substrates that are efficiently phosphorylated by Src kinase are, in turn, efficient substrates for SHP-1. Consequently, SHP-1 can negatively regulate Src-mediated autophosphorylation (41). Notably, a previous study has demonstrated that treatment with AHCC® significantly elevated SHP-1 in ovarian cancer, whereas elevated SHP-1 contributed to preventing the malignant progression of this cancer (29). Since Src-kinase-mediated phosphorylation is essential for the biological relevance of CTTN in cancer progression, the elevated SHP-1 level via AHCC® may control the expression and activation of CTTN in pancreatic cancer. The other possible mechanisms cannot be ruled out. Further studies are needed to clarify our speculation and elucidate the anti-tumor potential of AHCC® in pancreatic cancer.

Meanwhile, to explore potential anti-tumor effects of AHCC® in other cancers, we performed a literature review by using effective search engines. We found that AHCC® can inhibit malignant progression by inducing cellular apoptosis and inhibition of cellular proliferation. Moreover, AHCC® decreases the cellular ROS levels and maintains endothelial
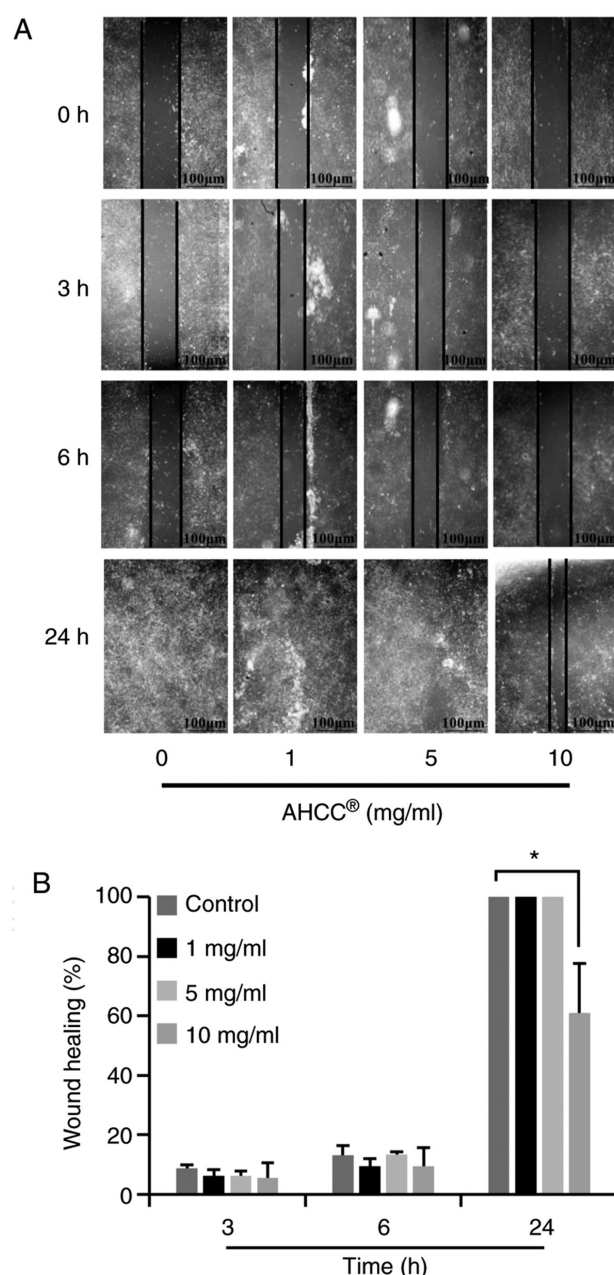
Figure 2. Effects of AHCC® on the migration capabilities in the gemcitabine-resistant pancreatic cancer cell, KLM-1R. (A) Wound healing assay was used to examine cellular migration. KLM-1R cells were allowed to grow to full confluence (80%) in 24-well plates, and then a wound was created with a sterile pipette tip. AHCC® was added to the well, and phase-contrast images of the wound at three different locations were captured at 0, 3, 6 and 24 h (scale bar, 100 μm). Black lines represent the width of the wound. A total of three independent experiments were examined, and representative images are presented. (B) Quantification of the average wound healing degree of KLM-1R cells. Wound healing was quantified as a percentage as the mean ± standard error. Each experiment was performed in triplicate. *P<0.05.

cellular plasticity, and prevents malignant transformation. Importantly, we observed that administration of AHCC® involved immune-modulatory functions, which contribute to improving overall patient survival and reducing the adverse effects of chemotherapeutics. Collectively, these findings indicated that AHCC® plays pleiotropic roles in preventing the onset of different cancers and may constitute an attractive therapeutic agent.
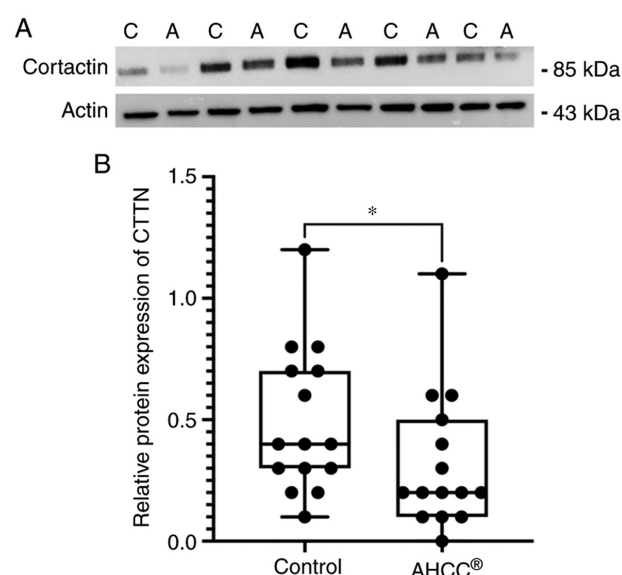

Figure 3. Effects of AHCC® on CTTN protein expression level. KLM-1R cells were treated with AHCC® at a concentration of 10 mg/ml. (A) CTTN expression was analyzed by western blotting (n=5). Protein expression level of CTTN was reduced in AHCC®-treated KLM1-R cells, whereas actin was almost the same in all cells. (B) Ratio of intensities of CTTN/actin in KLM-1R cells was measured by using densitometry. AHCC® significantly reduced the protein level of CTTN compared with that of the untreated control (*P=0.049). C, control; A, AHCC®. CTTN, cortactin.

In conclusion, our results suggested that AHCC® has anti-metastatic effects in pancreatic cancer cell lines via the downregulated CTTN level, and thus, this compound exhibits for the treatment of pancreatic cancer. However, a lack of adequate validation of the bioinformatics results in tissue samples is a limitation of this study. Therefore, further studies are needed to develop AHCC® as a complementary and alternative therapeutic approach to treating pancreatic cancer.

Acknowledgements

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

SI, TK and YK conceived and designed the study and performed the experiments. SI, BB, KK, HN, MK, IC and YK analyzed and interpreted the data. SI wrote the initial draft of the manuscript. BB, TK and YK contributed to critical revision of the manuscript. All authors read and approved the final

manuscript. SI and YK confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Not applicable.

Patient consent to participate

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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