



Research article

Curcumin enhances chemotherapeutic effects and suppresses ANGPTL4 in anoikis-resistant cholangiocarcinoma cells

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ABSTRACT

Anoikis resistance is a critical feature involved in tumor progression and chemoresistance. Finding approaches to improve the effect of chemotherapy on anoikis-resistant cancer cells is therefore critically important. In this study, we examined the effects of curcumin in anoikis-resistant cholangiocarcinoma (CCA) cells, including HuCCT1 and TFK-1 that were anchorage-independently cultured (AI-cells) using poly (2-hydroxyethyl methacrylate). The AI-CCA cells were treated with curcumin alone or in combination with anti-cancer agents and their responses to each treatment were determined by cell viability assay. Gene expression in AI-cells was determined by quantitative real-time PCR. The potential involvement of angiopoietin-like 4 (ANGPTL4) in anoikis resistance was examined by gene knockdown. It was found that AI-cells tended to resist anti-cancer agents tested, especially AI-HuCCT1, which significantly resisted gemcitabine and suberoylanilide hydroxamic acid (SAHA). Curcumin alone significantly inhibited viability and colony formation of AI-cells. Moreover, curcumin combination significantly enhanced the treatment effect of SAHA on AI-HuCCT1 and AI-TFK-1 cells. Gene expression analysis revealed that ANGPTL4 was markedly upregulated in AI-CCA cells and its knockdown tended to sensitize AI-cells to cell death and treatments. In addition, curcumin treatment decreased phosphorylated STAT3 and expression levels of Mcl-1, HDACs and ANGPTL4. Altogether, these findings reveal the beneficial property of curcumin to potentiate chemotherapeutic effects on anoikis-resistant CCA cells, which might suggest the potential use of curcumin for cancer treatment.

1. Introduction

Cholangiocarcinoma (CCA) is a primary tumor of the bile duct and the second most common hepatic malignancy after hepatocellular carcinoma. It is a deadly tumor with poor prognosis and increasing overall incidence worldwide [1]. Several risk factors have been associated with CCA development and the infection with liver fluke *Opisthorchis viverrini* is the key risk factor for CCA in Thailand [2]. Surgical resection is the curative treatment option eligible for patients with early-stage tumor. Systemic chemotherapy with gemcitabine and cisplatin is the first-line treatment option for patients with advanced or metastatic disease [1].

However, the effectiveness of these chemotherapies remains limited with the median overall survival of less than 1 year [3].

Cancer metastasis is a complex process that requires a series of sequential events including tumor cell survival in the bloodstream after detaching from their primary site [4]. Detached cells normally undergo anoikis, a programmed cell death triggered by the loss of cell-extracellular matrix (ECM) interaction [5, 6], to prevent improper anchorage-independent cell growth. Anoikis has thus been suggested to play a crucial role to prevent metastasis [7, 8]. Importantly, emerging evidence reveals that tumor cells can develop anoikis resistance through various dysregulations that protect cells against apoptosis and sustain

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pro-survival signals [8, 9]. The secreted protein angiopoietin-like 4 (ANGPTL4) has been implicated in anoikis resistance of various tumors such as hepatoma, scirrhous gastric cancer, and head and neck squamous cell carcinoma (HNSCC) [10, 11, 12, 13]. ANGPTL4 was shown to bind integrins to stimulate O_2^- production, resulting in activation of PI3K/PKB α and ERK pro-survival pathways in tumor cells [11]. In HNSCC, expression of ANGPTL4 induced by epidermal growth factor promotes anoikis resistance and metastasis via up-regulation of matrix metalloproteinase-1 [12]. However, the contribution of ANGPTL4 to anoikis resistance of CCA cells remains unclear.

Anoikis resistance was observed with resistance to chemotherapy. For instance, anoikis-resistant osteosarcoma cells were shown to significantly resist doxorubicin or cisplatin [14]. Likewise, CCA cells possessing anoikis resistance poorly responded to gemcitabine [15]. Therefore, seeking novel strategies to enhance treatment outcome in anoikis-resistant cells is of importance. Curcumin is a polyphenol and active component found in the *Curcuma longa*, which is also known as turmeric. Owing to its established safety and a variety of therapeutic potentials, curcumin has been widely studied in various diseases including cancer, in which diverse mechanisms responsible for its anti-cancer effects have been identified [16, 17]. Furthermore, anoikis-sensitizing effect of curcumin to induce anoikis of non-small cell lung cancer cells through the suppression of Bcl-2 was also reported [18]. Curcumin has also been used as a chemosensitizer in combination with conventional chemotherapies. A number of studies show that curcumin prominently enhances anti-cancer activity of the chemotherapeutic agents, which most likely suggests its chemosensitizing potential [19, 20]. In this study, we aimed to determine the effects of curcumin as single and combination treatment, the mechanisms underlying curcumin action, and the involvement of ANGPTL4 in anchorage-independently grown CCA cells.

2. Materials and methods

2.1. Cell lines and reagents

CCA cell lines, namely HuCCT1 and TFK-1, were obtained from the RIKEN BioResource Research Center (Tsukuba, Japan, <http://cell.brc.riken.jp/en/>). Cell lines were maintained in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μ g/mL streptomycin (Invitrogen, MA, USA) at 37 °C with 5% CO₂ in a humidified incubator. Murine fibroblast L929 was cultured in DMEM containing 10% FBS, 100 U/mL penicillin and 100 μ g/mL streptomycin. Curcumin, gemcitabine, cisplatin, SAHA, and poly (2-hydroxyethyl methacrylate) (poly-HEMA) were purchased from Sigma-Aldrich (MO, USA). RIPA buffer, protease inhibitor cocktail, phosphatase inhibitor, primary antibodies (STAT3, p-STAT3, Mcl-1, PARP, HDAC1, HDAC3, HDAC6, GAPDH), and secondary antibodies were purchased from Cell Signaling Technology (MA, USA). Anti-ANGPTL4 antibody was obtained from Invitrogen.

2.2. Anchorage-independent culture

CCA cell lines were cultured in anchorage-independent (AI) or suspension condition using poly-HEMA. As described previously, 2.5×10^5 cells/ml were seeded in plates coated with poly-HEMA (20 mg/mL) dissolved in ethanol [15]. Anchorage-independently grown cells (AI-cells) were examined for viability and death by trypan blue and collected for downstream assays. To determine gene expression, AI-cells were sub-cultured, re-plated in suspension condition, and harvested at indicated time points.

2.3. Colony formation assay

AI-cells grown in suspension for 48 h were harvested and seeded in regular 6-well plates at a density of 1×10^3 cells/well with curcumin (1.5

or 3 μ M) or 0.1% DMSO (control). Medium was replaced every 3 days and cells were incubated for ~2 weeks until colonies were visible. Colonies were fixed with methanol/acetic acid, stained with 0.5% crystal violet, and counted manually.

2.4. Cell viability assay

Cells cultured under adherent condition (AD-cells) and AI condition (AI-cells) for 48 h were collected for viability assay following curcumin and chemotherapeutic drug treatments. In brief, cells were seeded in 96-well plates at a density of 4×10^3 cells/well and incubated with the individual or combined agents. At the end of incubation intervals, cell viability was measured using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl) -(4-sulfophenyl)-2H-tetrazolium (MTS assay). MTS reagent (20 μ L) was added to each well and incubated for 2 h at 37 °C. Absorbance was then measured at 490 nm by using a microplate reader (Synergy/HTX multimode reader, BioTek, VT). ANGPTL4-knockdown cells and control cells were treated with gemcitabine, cisplatin, or SAHA for 48 h and their viability were measured as mentioned above. L929 (4×10^3 cells/well) was treated with varying doses of curcumin and subjected to MTS assay as described above.

2.5. Western blot assay

Cells were cultured in suspension and treated with different concentrations of curcumin. After incubation, cells were collected and lysed with RIPA buffer containing protease and phosphatase inhibitors. The protein concentration of each cell lysate was quantified using the Bradford assay (Bio-Rad). Samples containing an equal amount of total protein were resolved in 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. After blocking, membranes were incubated with relevant primary antibodies at 4 °C overnight, followed by horseradish peroxidase-conjugated secondary antibody. Protein targets were visualized by enhanced chemiluminescence (GE Healthcare, IL) and signals were read by X-ray film or ChemiDoc imaging System (Biorad Laboratories Inc., CA). Intensity of each HDAC was quantified by ImageJ software and was then normalized with the intensity of corresponding loading controls.

2.6. RNA isolation and quantitative real-time PCR

Total RNA was extracted from CCA cells using PureLink RNA Mini Kit (Thermo Fisher Scientific, MA) and RNA concentration was measured by using NanoDrop 2000 spectrophotometer, (Thermo Fisher Scientific). One microgram of total RNA was used to synthesize cDNA using iScript Reverse Transcription Supermix (Bio-Rad, CA) according to manufacturer protocol. Quantitative real-time PCR was performed using SsoFast EvaGreen Supermix (Bio-Rad, CA) and all reactions was processed in the Bio-rad CFX-96 real-time PCR instrument. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as housekeeping gene. The primers used to amplify the gene targets are listed in Table 1. Relative gene expression was normalized against respective controls and data were calculated by using the $2^{-\Delta\Delta Ct}$ method.

2.7. Knockdown of ANGPTL4 and trypan blue exclusion assay

Expression of ANGPTL4 was inhibited using the predesigned DsiRNAs (hs.Ri.ANGPTL4.13.3) (Integrated DNA Technologies, Inc., IL) and scrambled DsiRNA was used as a negative control. CCA cells grown in suspension were transfected with 30 pmol ANGPTL4 siRNA or scrambled siRNA with Lipofectamine RNAiMAX (Thermo Fisher Scientific, MA) according to the manufacturer's instructions. After 48 h, transfection efficiency was validated by real-time PCR and viability of siRNA-transfected cells was assessed by trypan blue exclusion assay. Briefly, transfected cells were collected and mixed with 0.4% trypan blue (Thermo Fisher Scientific, MA). The number of unstained (live) and

Table 1. List of primers used for quantitative real-time PCR.

| Gene | Forward (5'→ 3') | Reverse (5'→ 3') |
|----------------|--------------------------|--------------------------|
| E-Cadherin | GCAGTTGAGGATCCAATGGAGA | CTGAGGATGGTGAAGCGAT |
| EpCAM | GTGTACTTCAGTTGGTGCACAA | TTGCACTGCTTGGCCCTTAAAGA |
| N-Cadherin | TTGATGCTGACGATCCCAATG | GAAAGGCCATATGTGGGATTGC |
| Snail | GTGACAAGGAATATGTGAGCCT | TGCAGATGAGCCCTCAGATT |
| Vimentin | CAAAGCAGGAGTCCACTGAGTA | GAAGGTGACGAGCCATTTCCT |
| HIF-1 α | TGTGAGTTCGCATCTTGATAAGG | ATCATGTCCACCATCATCTGTGAG |
| ANGPTL4 | TGCGAATTCAGCATCTGCAAAG | GGATTTCAAATAGTCCACTCTGCC |
| Cyclin D1 | TTGCATGTTCTGGCCCTCTAA | AGTGTTCATGAAATCGTGCGG |
| p53 | CTCAGCATCTTATCCGAGTGGAA | CATGCGAGAACTGTTACACATGT |
| p21 | GACAGCAGAGGAAGACCATGT | GCTTCCTCTGGAGAAGATCAGC |
| HDAC-1 | GGTCATGACTGTGTCCTTTCAT | ACTAGGCTGGAACATCTCCAIT |
| HDAC-2 | GGTGATGGAGATGTATCAACCTAG | AGTCTCATATGTCCAACATCGAG |
| HDAC-3 | TTACAAGCACCTTTCCAGCC | CATTTCGGACAGTATAACCACCAC |
| HDAC-8 | AATGCCTGATTGACGGAATGTG | AGGTGAAACTGAATGCGTCTTC |
| GAPDH | CAAATCCATGGCACCGTCAAG | AGAGATGATGACCTTTTGGCT |

stained (dead) cells were counted using a hemacytometer under light microscope.

2.8. Statistical analysis

Data are presented as means \pm SE. Statistical analyses were performed using PASW Statistics 18 (SPSS Inc., IL). Statistical significance between two groups and multiple groups was determined by Student's t-tests and one-way analysis of variance followed by LSD post hoc test, respectively. P-value < 0.05 was considered to be statistically significant difference.

3. Results

3.1. Curcumin reduces viability and colony formation of anchorage-independently grown CCA cells

We initially examined resistance of HuCCT1 and TFK-1 to anoikis by anchorage-independent (AI) culture. After 24 and 48 h, the anchorage-independently grown cells (AI-cells) were subjected to trypan blue staining. More than 90% of viable AI-cells was observed, suggesting resistance of these cells to anoikis induced by AI culture (Figure 1A). To test toxicity of curcumin, we compared viability of non-malignant L929 and CCA cell lines after exposing to various doses of curcumin under adherent condition. As compared to CCA cell line HuCCT1 and TFK-1, less inhibitory effect of curcumin on L929 viability was noted, which suggests low cytotoxic effect of curcumin on non-malignant cells (Figure 1B).

Next, to determine the effect of curcumin on anoikis-resistant CCA cells, AI-HuCCT1 and AI-TFK-1 cells were treated with increasing concentrations of curcumin ranging from 5–40 μ M for 24, 48, and 72 h. Cell viability was subsequently assessed by MTS assay. As shown in Figure 2A, viability of curcumin-treated cells was largely inhibited compared to vehicle treated controls (0.1% DMSO). It was noted that AI-HuCCT1 and AI-TFK-1 cells consistently showed lower sensitivity to curcumin than adherent cells, suggesting resistance of AI-CCA cells to treatment. The effect of curcumin was further determined by colony formation assay, which demonstrated that colony sizes and numbers were significantly decreased in response to curcumin treatment (Figure 2B). Additionally, curcumin inhibited STAT3 pathway indicated by the decrease in phosphorylated STAT3 and anti-apoptotic protein Mcl-1, which is STAT3's downstream target. On the contrary, curcumin increased level of cleaved poly (ADP-ribose) polymerase or PARP, which serves as a marker for apoptosis [21], indicating activation of cell death (Figure 2C). These results indicate inhibitory effect of curcumin on survival of AI-CCA cells.

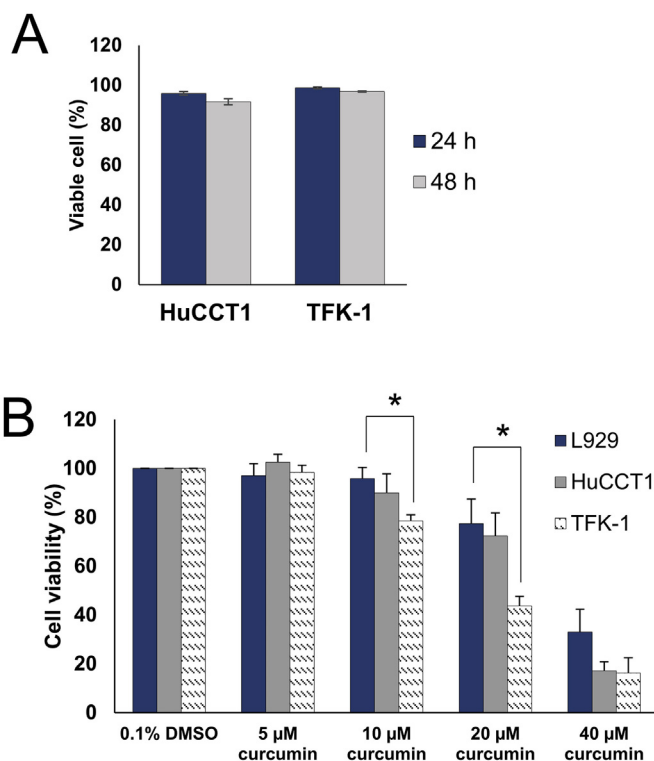


Figure 1. Anoikis induction of CCA cell lines and effect of curcumin on L929. (A) The percentage of viable HuCCT1 and TFK-1 cells cultured under anchorage-independent condition (AI-cells) for 24 h and 48 h. (B) Viability of non-malignant L929 and CCA cell lines after treatment with curcumin for 48 h. Percentage of cell viability was compared against control (0.1% DMSO). Data represent the mean \pm SE of three independent experiments. *P < 0.05.

3.2. Curcumin enhances effects of anti-cancer agents and inhibits expression of HDAC

It was previously revealed that AI-CCA cells tended to resist gemcitabine [15]. To better understand their response to chemotherapies, AI-CCA cells were separately treated with common anti-cancer agents, including gemcitabine and cisplatin, which are the first-line chemotherapy for CCA, and SAHA, which is histone deacetylase (HDAC) inhibitor that was recently shown to reduce anchorage-independent growth of human osteosarcoma cells [14]. After 48 h of treatments, MTS assay showed that AI-CCA cells were less sensitive to all individual agents as compared to respective adherent controls (Figure 3A). The significant differences were noted in HuCCT1 cells exposed to gemcitabine and SAHA as well as in cisplatin-treated TFK-1 cells. To determine whether curcumin improves treatment effects of the chemotherapeutic agents, AI-CCA cells were treated with gemcitabine, cisplatin, or SAHA in the presence of curcumin. The results showed that curcumin-SAHA combination effectively reduced HuCCT1 and TFK-1 cells' viability compared to either SAHA or curcumin alone (Figure 3B). The combination between curcumin and cisplatin also significantly inhibited TFK-1 cells' viability compared to treatment with the individual agents. Curcumin was previously shown to inhibit expression of HDACs, which are targets of SAHA [22]. It was therefore suspected that the improved treatment efficacy of curcumin-SAHA combination could be resulted from suppression of HDAC. To address this, we detected expression of class I and class II HDACs after curcumin treatment and found that expressions of HDAC1, 3, and 6 were inhibited at varying levels. The significant inhibition of HDAC3 and HDAC6 was observed in TFK-1 cells treated with 40 μ M curcumin for 48 and 24 h, respectively (Fig. 3C and D). Altogether, these results suggest chemosensitizing effect of curcumin on anoikis-resistant CCA cells.

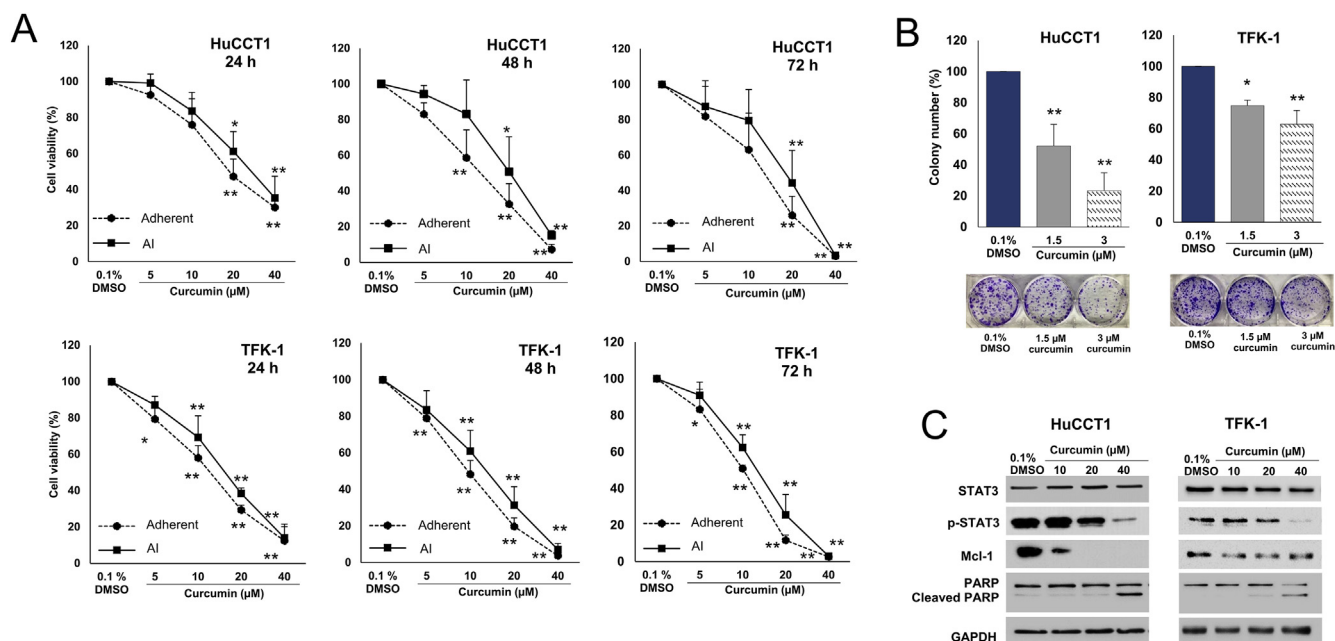


Figure 2. Effects of curcumin on viability and colony formation of AI-CCA cells. (A) MTS assay of adherent and AI-cells (HuCC1 and TFK-1) treated with curcumin. Percentage of viable cells was calculated against 0.1% DMSO-treated controls. (B) Colony formation assay of AI-HuCC1 and AI-TFK-1. Colony number of the cells treated with curcumin was analyzed versus 0.1% DMSO-treated cells. Data represent the mean \pm SE of three independent experiments. * $P < 0.05$ and ** $P < 0.01$ vs. control. (C) Western blot analysis showed the inhibition of p-STAT3 and Mcl-1 and the increase in cleaved PARP in AI-cells treated with curcumin for 48 h. GAPDH was detected as loading control for western blotting. Original images are shown in Sup Fig. 1.

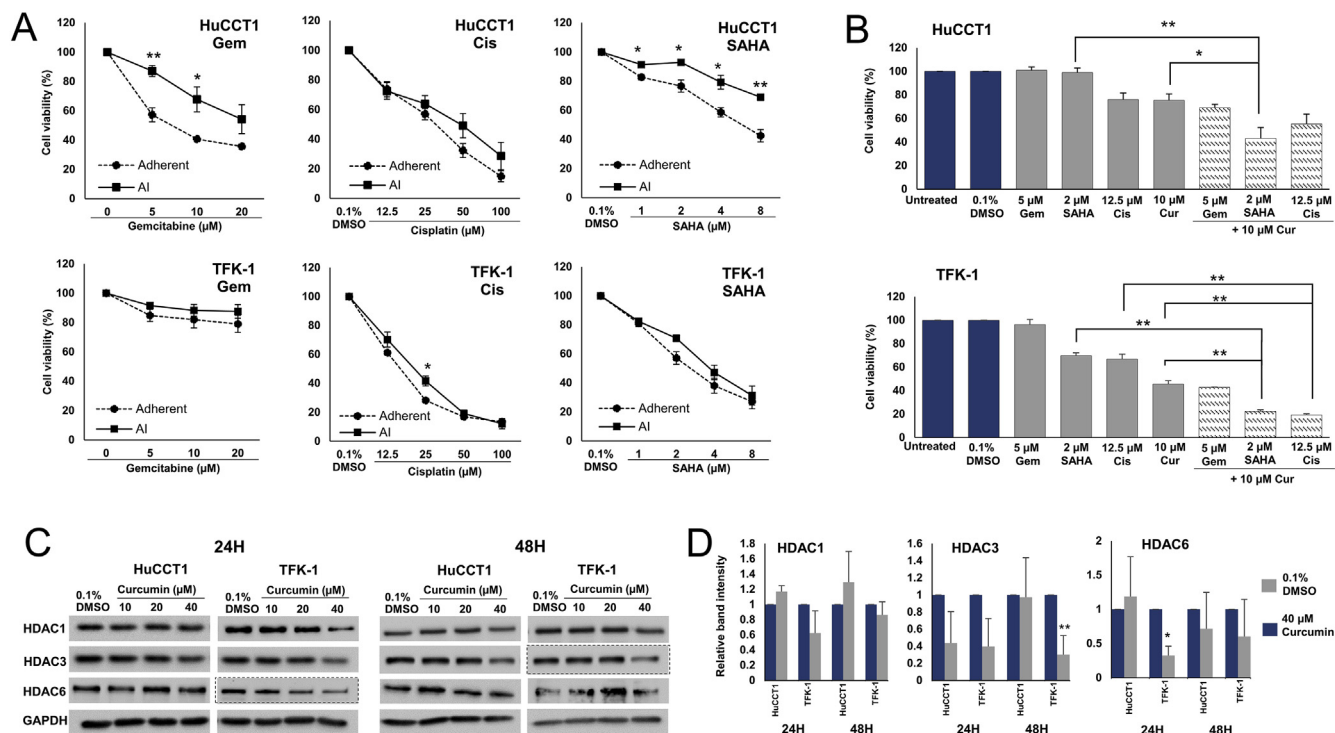


Figure 3. Effect of curcumin combined with gemcitabine, cisplatin, or SAHA. (A) Response of AI-HuCC1 and AI-TFK-1 cells to gemcitabine, cisplatin, and SAHA measured by MTS assay at 48 h after treatments. (B) Viability of AI-HuCC1 and AI-TFK-1 cells treated with individual agents or in combination with curcumin for 48 h. Untreated cells were used as control for gemcitabine and 0.1% DMSO-treated cells was used as control for cisplatin and SAHA. Data represent the mean \pm SE of three independent experiments. * $P < 0.05$ and ** $P < 0.01$ vs. controls. Gem, gemcitabine; Cis, cisplatin; Cur, curcumin. (C) Expression of HDACs was detected by western blotting. Original images are shown in Sup Fig. 2. (D) Relative band intensity of HDAC1, 3, and 6 in AI-cells treated with 40 μM curcumin for 24 and 48 h.

3.3. Upregulation of ANGPTL4 in CCA cells grown under anchorage-independent condition

To identify molecular targets that might play a role in anoikis resistance of CCA cells, expression of the genes representing several cellular processes such as epithelial-mesenchymal transition (EMT), epigenetic regulation, cell cycle, and hypoxia were determined in CCA cells suspended for 2 days and up to 9 days to compare gene expression pattern under short (2 days) and prolonged suspension (9 days) (Figure 4A). As shown in figure 4B-E, the expression of all selected genes was altered variedly in AI-CCA cells. Among the genes examined, the marked increase in ANGPTL4 expression was consistently observed in both HuCCT1 and TFK-1 cells (Figure 4E). This upregulation was predominantly observed at day 2 and remained at day 9 of AI culture. Relative fold-change of ANGPTL4 expression at day 2 was 11.5 and 4.9 in HuCCT1 and TFK-1, respectively.

3.4. Contribution of ANGPTL4 to survival of anchorage-independently grown CCA cells

We next investigated the contribution of ANGPTL4 to CCA cell survival in suspension condition. The expression of ANGPTL4 was silenced by siRNA and the efficiency of siRNA-mediated ANGPTL4 knockdown was validated by quantitative real-time PCR (Figure 5A). We found that suppression of ANGPTL4 enhanced cell death in suspension condition evidenced by the increased fraction of dead cells in ANGPTL4-knockdown cells compared to control (HuCCT1; 31.4% vs. 18.6% and TFK-1; 52.4% vs. 36.2%) (Figure 5B). ANGPTL4 knockdown also tended to sensitize both HuCCT1 and TFK-1 cells to SAHA treatment (Figure 5C) although not statistically significant. Then, we tested whether the expression of ANGPTL4 can be inhibited by curcumin. As shown in figure 5D and E, the level of ANGPTL4 was significantly decreased in AI-CCA

cells in response to curcumin treatment. Taken together, these results may suggest the potential involvement of ANGPTL4 in anoikis resistance of CCA and indicate inhibitory effect of curcumin on ANGPTL4 expression.

4. Discussion

The capability of cancer cells to escape cell death is one of the cancer hallmarks shared among various types of human malignancies [23]. Anoikis resistance has also been suggested as an important cancer hallmark because it allows tumor cells to overcome detachment-induced cell death. Our previous study showed that anoikis-resistant CCA cells resisted gemcitabine treatment [15]. Consistently, the present study elucidated chemoresistance of AI-CCA cells after treatments with the common anti-cancer agents (gemcitabine, cisplatin, and SAHA). Thus, these results emphasize the need to find approaches for sensitizing anoikis-resistant cells to treatment.

Chemosensitizing potential of curcumin was observed in various cancer models. For example, it sensitized head and neck squamous cell carcinoma to cisplatin [24] and sensitized pancreatic cancer cells to gemcitabine [20, 25]. This evidence prompted us to explore curcumin for improving the effects of chemotherapies on AI-CCA cells. We obviously found that curcumin potentiated the effectiveness of SAHA treatment on both CCA cells. A growing body of evidence describes curcumin as a HDAC inhibitor owing to its property to suppress expression and activity of HDAC in a wide range of human cancers [22]. For example, curcumin inhibited the expression of HDAC1, HDAC3, and HDAC8 in B cell non-Hodgkin lymphoma cell line [26] and down-regulated HDAC6 expression in leukemic cells [27]. Here, the suppression of HDAC1, 3, and 6 expressions in response to curcumin exposure was noted. Because HDACs play a crucial role in reversing chromatin acetylation, which regulates expression of a large number of genes, change in expression of

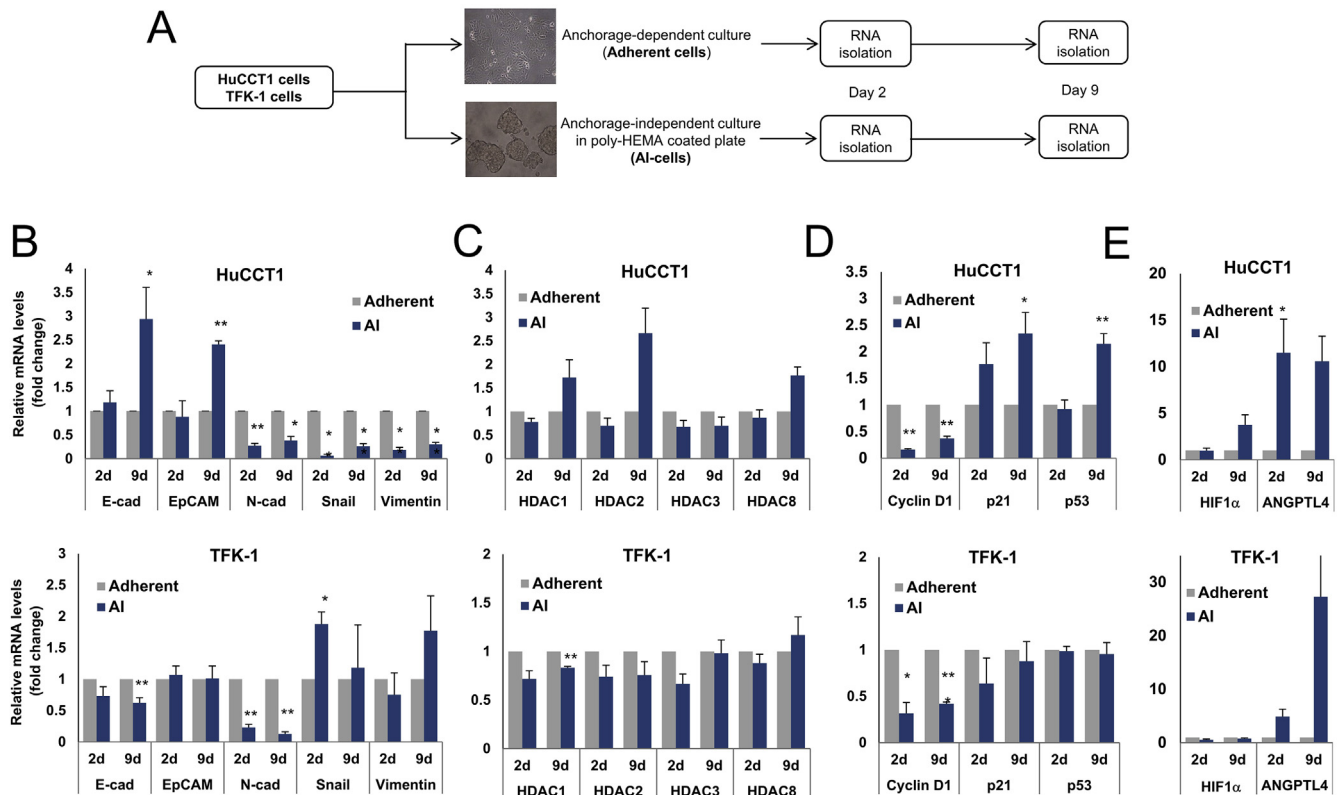


Figure 4. Gene expression in anchorage-independently grown HuCCT1 and TFK-1 cells. (A) Schematic representation of adherent and AI culture of HuCCT1 and TFK-1 cells for gene expression analysis. The relative expression levels of genes involved in EMT (B), epigenetic (C), cell cycle (D), and hypoxia (E) in AI-cells was compared against that of adherent cells. The mRNA levels were normalized to GAPDH reference gene. Data represent the mean \pm SE of three independent experiments. * $P < 0.05$ and ** $P < 0.01$ vs. controls.

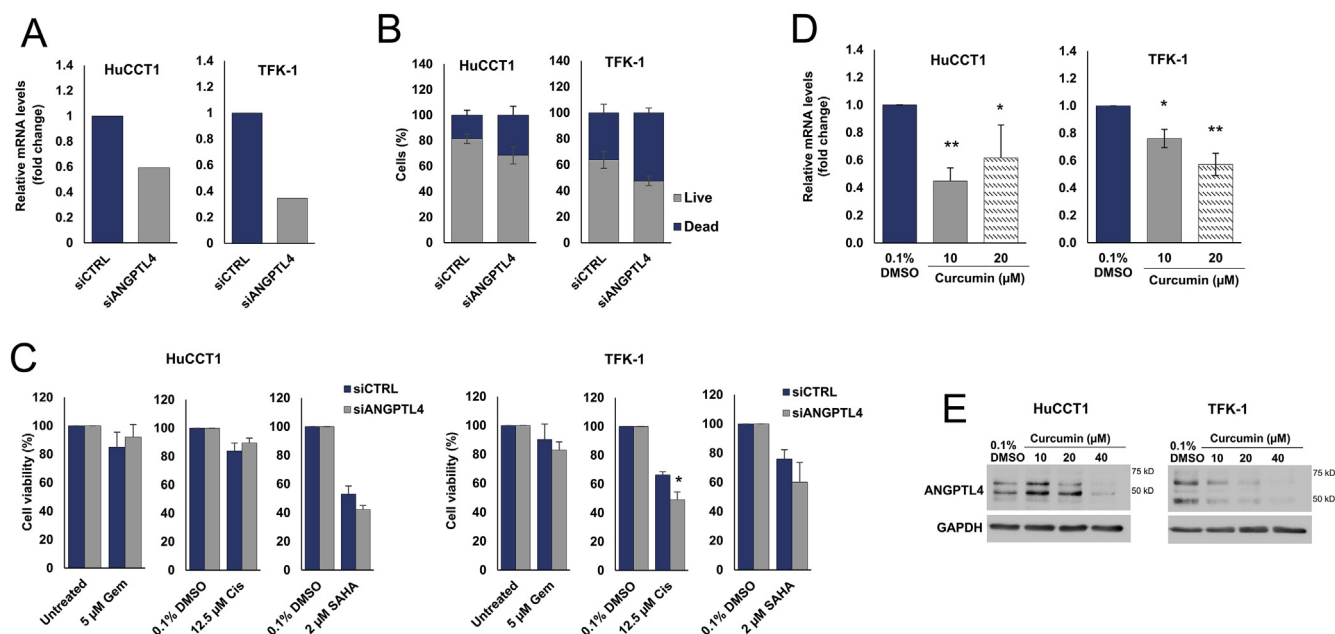


Figure 5. Inhibition of ANGPTL4 by siRNA and curcumin in AI-CCA cells. (A) ANGPTL4 expression in AI-HuCCT1 and AI-TFK-1 cells was decreased by siRNA. (B) Survival and (C) response to anti-cancer drugs of ANGPTL4-knockdown cells under suspension condition. Data represent the mean \pm SE of three independent experiments. * $P < 0.05$ and ** $P < 0.01$ vs. controls. Gem, gemcitabine; Cis, cisplatin. Inhibition of ANGPTL4 expression at mRNA (D) and protein (E) level after curcumin treatment. GAPDH was detected as loading control for western blotting. Original images are shown in Sup Fig. 3.

HDACs is associated with aberrant gene transcription [28]. HDAC inhibitors sensitized anoikis-resistant mammary carcinoma cells to therapies, increased toxic BH3 domain proteins, but reduced anti-apoptotic Mcl-1 [29]. It is speculated that inhibition of HDACs observed in this study may potentially disrupt the balance of gene transcription by increasing pro-apoptotic proteins and/or decreasing anti-apoptotic proteins, resulting in sensitization of AI-CCA cells to curcumin-SAHA treatment. However, precise mechanisms and involvement of HDACs in anoikis resistance of CCA still require comprehensive investigations. In addition, analysis of drug interaction by the Chou-Talalay method [30] will add to our understanding of the additive or synergistic effect of the combined drugs, which may be useful for further testing of the favorable drug combinations on cancer model.

Signal transducer and activator of transcription 3 (STAT3) is the transcription factor that has critical roles in cancer development and progression. STAT3 was found to enhance anoikis resistance of pancreatic cancer [31] and melanoma [32] and its silencing reduced capability of these tumor cells to resist anoikis. In CCA, phosphorylated STAT3 (p-STAT3) and Mcl-1 was highly increased in anoikis-resistant cells [15]. In this study, we found that curcumin reduced level of p-STAT3 and Mcl-1, which is a molecular target regulated by STAT3 signaling [33], in AI-CCA cells.

ANGPTL4 has been involved in cancer progression, metastasis and anoikis resistance [10, 11, 12, 13, 34]. Here, we found that the expression level of ANGPTL4 was strikingly increased in response to AI culture of CCA cells. Depletion of ANGPTL4 increased CCA cell death under AI condition, likely suggesting the potential contribution of ANGPTL4 to anoikis resistance of CCA. However, further investigation of ANGPTL4 effects using additional siRNAs or overexpression approach is needed to support the involvement of ANGPTL4 in the findings observed in the present study. Previous study showed that ANGPTL4 interacts with integrin $\beta 1/\beta 5$ and increases O_2^- levels, which subsequently activate Src, PI3K/PKB α , and ERK pathways. Activation of these survival pathways confer anoikis resistance of tumor cells [11]. Baba et al. also showed the increase in induction of apoptosis and decrease in phosphorylation of the FAK/Src/PI3K-Akt/ERK pathway in ANGPTL4-knockdown gastric cancer cells [10]. We therefore speculate that the FAK/Src/PI3K-Akt/ERK pathway may be activated by ANGPTL4 and provide survival signal to

CCA cells in AI culture. Suppression of ANGPTL4 may induce ANGPTL4-knockdown cells to undergo cell death through inactivation of these pro-survival signaling, however, these detailed mechanisms still require further investigation. Recently, a study in Sprague-Dawley rats reported the down-regulation of ANGPTL4 in curcumin-treated animals [35]. Consistently, we found that curcumin diminished the expression of ANGPTL4 in AI-CCA cells.

In summary, we have demonstrated the property of curcumin to increase efficacy of chemotherapeutic agents, especially SAHA as well as elucidate inhibitory effect of curcumin on ANGPTL4. Our findings further support the benefits of curcumin, which might be useful for cancer treatment.

Declarations

Author contribution statement

Tin Tin San: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Prasong Khaenam, Analyzed and interpreted the data; Wrote the paper.

Virapong Prachayasittikul: Analyzed and interpreted the data.

Banchob Sripa: Analyzed and interpreted the data.

Nawapol Kunkeaw: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Waraporn Chan-On: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

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