Polymethoxylated flavone sudachitin is a safe anticancer adjuvant that targets glycolysis in cancer-associated fibroblasts

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Abstract. Sudachitin is a polymethoxylated flavone found in the peel of *Citrus sudachi*, a unique specialty citrus fruit in Tokushima Prefecture, Japan. Previous reports have demonstrated that sudachitin has anti-inflammatory and metabolic regulatory activities. However, to the best of our knowledge, no studies have explored whether sudachitin can act as an antitumor therapeutic agent by regulating metabolic functions in the tumor microenvironment. In the present study, cell proliferation and cytotoxicity assays were used to determine whether sudachitin inhibited the *in vitro* growth of liver cancer and pancreatic carcinoma, cholangiocarcinoma and colorectal cancer cells and to compare its toxicity against normal fibroblasts and induced cancer-associated fibroblasts (CAFs). Using lactate assays and reverse transcription-quantitative PCR, the effects of sudachitin on glycolysis in CAFs were investigated. The effects of CAFs on malignant tumor cells were evaluated *in vitro* using cell proliferation, wound healing and invasion assays. As result, sudachitin inhibited various types of tumor cells with different half-maximal inhibitory concentrations. Treatment with 50 μM sudachitin for 48 h suppressed tumor and CAFs proliferation but was not cytotoxic against normal fibroblasts. This dose also inhibited glycolysis in CAFs and, thus, diminished their pro-tumorigenic activities. Overall, the present study revealed that sudachitin has promise as a safe and widely available natural antitumor adjuvant.

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Abbreviations: CAF, cancer-associated fibroblast; CCK-8, Cell Counting Kit-8; CM, conditioned medium; IC50, half-maximal inhibitory concentrations; MCT4, monocarboxylate transporter 4; PFK, phosphofructokinase; TME, tumor microenvironment

Key words: cancer-associated fibroblasts, sudachitin, glycolysis, reverse Warburg effect, antitumor

Introduction

Current therapeutic strategies for cancer have shifted from focusing on the tumor cells themselves to targeting the tumor microenvironment (TME) (1). Multiple cellular components and signaling pathway networks create a TME that surrounds the tumor cells and supports tumor growth (2). Among these components, cancer-associated fibroblasts (CAFs) are important for promoting tumor progression through various mechanisms, including remodeling of extracellular matrix, maintenance of stemness and angiogenesis (3,4). Previous reports confirmed that CAFs underwent metabolic reprogramming to enhance glycolysis and form metabolic couplings with colon cancer cells, thereby promoting malignant tumor behaviors *in vitro* (5). The tumor-promoting effects of CAFs could be attenuated by hindering glycolysis (6).

Sudachitin is a polymethoxylated flavone derived from the peel of the citrus fruit *Citrus sudachi*, a specialty food in Tokushima Prefecture, Japan. Sudachitin has been reported to have anti-inflammatory activities (7) and induce mitochondrial biogenesis, which protects against metabolic disorders (8). Liu N *et al* have reviewed that flavonoids derived from citrus peels, which are normally wasted, may prevent cancer through various mechanisms and may be health-promoting food components (9). Therefore, in our study, we investigated the effects of sudachitin on tumor cells and the TME, with a specific focus on CAFs. The aim of this study was to update knowledge regarding the biological activities of sudachitin and determine its usefulness as a safe anticancer adjuvant.

Materials and methods

Cell culture and reagents. Human colorectal cancer cell lines HCT-116 (ECACC 91091005) and HT-29 (ECACC 91072201) were purchased from The European Collection of Authenticated Cell Cultures (ECACC) and cultured in McCoy's 5A medium (Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS, Thermo Fisher Scientific, Inc.) and 1% penicillin-streptomycin (Thermo Fisher Scientific, Inc.). Human cholangiocarcinoma cell lines HuCCT1 (RRID: CVCL_0324) and RBE (RRID: CVCL_4896) were purchased from Cell Bank, RIKEN BioResource Research Center and

maintained in RPMI-1640 (Thermo Fisher Scientific, Inc.) supplemented with 10% FBS and 1% penicillin-streptomycin. The human pancreatic cancer cell lines PANC-1 and MIA PaCa-2 and human liver cancer cell lines Huh-7 and HepG2 were from storage in our laboratory and cultured in Dulbecco's Modified Eagle Medium [DMEM high glucose (4.5 g/l), pyruvate (110 mg/l) (cat. no. 11995065; Thermo Fisher Scientific, Inc.)] supplemented with 10% FBS and 1% penicillin-streptomycin. Human intestinal fibroblasts (HIFs) were obtained from ScienCell Research Laboratories (Cat. 2920) and cultured in a complete fibroblast medium (Cat. 2301; ScienCell Research Laboratories). All cell lines used in the present study from a commercial source were aliquoted and frozen in liquid nitrogen immediately upon receipt, and used for the present experiments within 6 months of thawing. Cells were cultured at 37˚C in 5% CO_2 with normal oxygen saturation and were selected for experiments during the logarithmic growth phase under mycoplasma-free conditions. Sudachitin was supplied by Ikeda Yakusou Co., Ltd.

Cell co-culture and collection of conditioned medium (CM). To generate CAFs, HIFs were co-cultured for 3 days with HCT-116 or HT-29 cells at a ratio of 1:3 using Falcon permeable supports for six-well plates with 0.4-μm pores (353090; Corning, Inc.), which provided indirect contact between cell types but used the same DMEM medium containing 10% FBS as we described in our previous study (10). For generating CAF (sudachitin), CAFs were further treated with 50 μM sudachitin for 24 h. Total RNA samples for quantitative real-time reverse transcription PCR (qRT-PCR) in each group were extracted in this step. To generate CM, the medium was further changed by fresh serum-free DMEM and continued to incubate cells for 48 h. Then the supernatants were collected, centrifuged (500 x g) at room temperature for 20 min, and filtered through 0.2-μm filter membranes to remove cellular debris. The supernatants were named as CAF-CM in CAFs group, CAF (sudachitin)-CM in CAFs (sudachitin) group and HIF-CM in HIFs group, respectively. As shown via flow charts (Fig. S1A-C). The CM from each of the above groups was stored at -80˚C and avoid repeated freezing and thawing. For treatment of cancer cells in the subsequent experiments, the CM was warmed to room temperature and added to fresh DMEM medium at a ratio of 1:1, which will include the soluble factors in the CM and supply enough nutrients to support the cells for the next experiment. The final FBS concentration were varies according to the different experiments and details were mentioned in each of the experiments.

Cell proliferation and cytotoxicity assay. Cancer cells were seeded at a density of $0.7x10^4$ cells/well in 96-well plates. After cells attachment, increasing concentrations of sudachitin ranged from 1 to 500 μM were added to treat the cells for 48 h. The medium in each well was replaced with fresh medium containing a 10% (v/v) Cell Counting Kit-8 (CCK-8) solution (Dojindo Molecular Technologies), and the cells were incubated further for 2 h. Cell proliferation was analyzed by measuring the absorbance values at 450 nm with a microplate reader (SpectraMax i3; Molecular Devices, LLC) in accordance with the manufacturer's instructions. To determine the effects of HIFs, CAFs and CAFs (sudachitin) on proliferation, HCT-116 and HT-29 cells were incubated with the indicated CM with 10% final FBS concentration for 48 h after adhesion, and cell proliferation was analyzed using the above method. To determine cytotoxicity of sudachitin against non-tumor cells, HIFs were seeded in 96-well plates at a density of $0.5x10^4$ cells/well and treated with increasing concentrations of sudachitin ranged from 1 to 300 μM. The proliferative status of the cells was measured every 24 h between days 1 and 3 using the CCK-8 solution as described above. To further compare the sensitivity of CAFs and HIFs on sudachitin. HIFs after they were co-cultured with HIFs, HCT-116 cells and HT-29 cells were seeded in 96-well plates at a density of $0.5x10^4$ cells/well and 10, 30, 50, 75, 100 μM sudachitin were used to treat the cells for 24 and 48 h. And the proliferative status of the cells was measured using the CCK-8 solution in the same manner. The cell proliferation rate was measured by comparing with control group.

Wound healing assay. HCT-116 or HT-29 cells were seeded at a density of $6x10^5$ cells/well in six-well plates and incubated overnight to form a 90% confluent monolayer. A 200-μl pipette tip was used to scratch a wound through the entire center of each well. After washing with PBS, the cells in each group were cultured with the indicated CM in the absence of FBS for 48 h. The areas of the wounds were observed at 0 and 48 h after scratching, and images were captured using a light microscope (x40 magnification) equipped with a DP22-CU digital camera (Olympus). The cell migration rates were calculated using ImageJ v1.46r software (National Institutes of Health) using the following equation: relative migration rate=[width (0 h)-width (48 h)]/width (0 h) x100%.

Migration assay. A 24-well Transwell system with 8.0-μm pores (Corning) was used for the migration and invasion assays. Serum-starved cancer cells ($7x10^4$/well) were seeded in the upper chamber in a 100-μl suspension. After cell attachment, the culture medium was replaced with the indicated CM in each group. The final FBS concentration was 5% in the upper chambers and 10% in the lower chambers. After 36 h of incubation, the unattached cells in the upper chambers were cleaned using cotton swabs. Then, inserts were fixed by methanol for 20 min and stained with 1% crystal violet (031-04852; Fujifilm Wako Pure Chemical Corporation) for 20 min. Images were captured and the numbers of cells at the bottom of the membrane in each chamber were calculated.

qRT-PCR. Total RNA from each group of cells was extracted with an RNeasy Mini kit (Qiagen GmbH) in the stage mentioned above, and concentrations were measured using a NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific, Inc.). Subsequently, 2.5 μg RNA was reverse transcribed into cDNA in a total volume of 50 μl using a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems/Thermo Fisher) in accordance with the manufacturer's instructions. A StepOnePlus™ Real-Time PCR System (Applied Biosystems) was used to perform TaqMan qPCR with the following reaction conditions: initial denaturation at 95˚C for 3 min followed by 40 cycles of denaturation (95˚C, 30 sec), annealing (58˚C, 30 sec), and extension (72˚C, 45 sec) and a final extension at 72˚C for 10 min. The following TaqMan gene

expression assays were used: *PFKP* (assay ID, Hs00737347_m1, catalog, 4331182, FAM-labeled, Thermo Fisher Scientific), and *SLC16A3* (*MCT4*) (assay ID, Hs00358829_m1, catalog, 4331182, FAM-labeled, Thermo Fisher Scientific). *GAPDH* (assay ID, Hs99999905_m1, catalog, 4326317E, VIC-labeled, Thermo Fisher Scientific) was used as the internal control to normalize the raw data. The $2^{-\Delta\Delta Ct}$ method was used for data analysis, and the results were presented as the fold changes in the relative mRNA expression for each experimental group compared with that in the control group.

Lactate assay. To collect the sample for lactate assay, fresh serum-free DMEM was replaced in all groups of cells. After culturing cells for another 6 or 24 h, cell supernatants were collected using the method described above for CM and cells were fully digested and counted using a cell counter (model R1, Olympus) after trypan blue staining. A lactate assay kit (MAK064; Sigma-Aldrich) was used to detect the concentrations of lactate in the supernatants. Briefly, supernatant and standard samples were diluted with lactate assay buffer following the manufacturer's instructions and added to a 96-well plate. After a 30 min incubation with 50 μl master reaction mix at room temperature, the absorbance values were measured at 570 nm with a microplate reader. The lactate concentrations for each sample were calculated according to a standard curve constructed using the standard samples. The relative secretion of lactate was normalized to the cell number.

Statistical analysis. All data are presented as the mean ± SD. GraphPad Prism v7.0 software (GraphPad Software) and ImageJ v1.46r software were used for the statistical analysis and construction of graphs. The unpaired Student's t-test or Mann-Whitney U test was used for comparisons between two groups. Differences between multiple groups were analyzed using one-way analysis of variance followed by Tukey's post hoc test. More than three biological replicates were included for each experiment, and $P<0.05$ (two-sided) was considered statistically significant.

Results

Sudachitin directly inhibited tumor proliferation in vitro. As a type of polymethoxylated flavone, the chemical structure of sudachitin was shown in Fig. 1A (7). After 48 h treatments with different concentrations of sudachitin, a dose-dependent inhibition of proliferation was observed in four types of cancer cells (Fig. 1B-E). Our data demonstrated that the efficacy of sudachitin-mediated antiproliferative activity varied among different cell lines. For liver cancer lines, the half-maximal inhibitory concentrations (IC50) of sudachitin were 82.04 μM for Huh-7 and 49.32 μM for HepG2 cells (Fig. 1B). For cholangiocarcinoma lines, the IC50 values were 53.21 and 24.1 μM for HuCCT1 and RBE cells, respectively (Fig. 1C). For pancreatic cancer, the IC50 values were 43.35 μM for MIA PaCa-2 cells and 32.73 μM for PANC-1 (Fig. 1D). The IC50 values of sudachitin were 56.23 and 37.07 μM for the colorectal cancer lines HCT-116 and HT-29, respectively (Fig. 1E). We also investigated the direct effects of sudachitin on the survival of HIFs to determine cytotoxicity against normal cells. We demonstrated that a 48-h treatment with up to 50 μM sudachitin did not significantly inhibit the proliferation of HIFs (Fig. 1F and G). Next, we further compared the sensitivity of CAFs and HIFs on sudachitin. Although 24-h treatment with up to 50 μM sudachitin did not significantly affect the growth in both HIFs and CAFs (Fig. 1H). When treatment time was extended to 48 h, CAFs became more sensitive to sudachitin compared with HIFs (Fig. 1I).

Sudachitin suppressed the tumor-promoting capabilities of CAFs. To investigate the effect of sudachitin on the metabolic couplings between CAFs and tumor cells, we treated colorectal cancer cells with CM collected from HIFs, CAFs and CAFs (sudachitin). Proliferation assays illustrated that after pretreatment with 50 μM sudachitin for 24 h, CAF-induced stimulation of HT-29 colorectal cancer cell proliferation was decreased, while no significant effect was observed for HCT-116 cells (Fig. 2A and B). As shown in Fig. 2C-E, the ability of CAFs to promote the migration and invasion of HCT-116 and HT-29 cells was also suppressed after 50 μM sudachitin pretreatment for 24 h.

Sudachitin treatment inhibited the glycolytic activity of CAFs. Next, we determined the glycolytic activity in CAFs after treatment with 50 μM sudachitin for 24 h. CAFs presented higher lactate productions and mRNA expressions of phosphofructokinase (*PFK*) and monocarboxylate transporter 4 (*MCT4*) compared with HIFs as shown in Fig. 3A-C. However, 50 μM sudachitin treatment for 24 h decreased lactate production by CAFs (Fig. 3A) as well as the mRNA expression of *PFK* and *MCT4* (Fig. 3B and C). These data indicated that glycolytic activity of CAFs was inhibited after treatment with sudachitin.

Discussion

In this study, we demonstrated that sudachitin broadly and directly inhibited the proliferation of a wide range of tumor cells *in vitro*. Furthermore, sudachitin reduced the glycolytic activity of CAFs, thereby hindering the metabolic coupling to colorectal cancer cells and reducing CAF-induced tumor progression. Therefore, a relatively small dose of sudachitin can exert antitumor effects by targeting the TME.

A previous study showed that polymethoxyflavones induced apoptosis of gastric cancer cells by upregulating retinoic acid receptor β both *in vitro* and *in vivo* (11). Nobiletin, a polymethoxyflavone similar to sudachitin, was able to inhibit tumor cell proliferation through various mechanisms, such as classical autophagy, mitochondrial autophagy (mitophagy), apoptosis, and pyroptosis (12). Moreover, sudachitin was shown to induce apoptosis by regulating the MAPK pathway (13). Given that much has been published regarding polymethoxyflavone-induced inhibition of tumor cell proliferation, a direct mechanism of action of sudachitin on tumor cells is theoretically supported. Because of its role in metabolism, especially mitochondrial and glucose metabolism, we investigated the effects of sudachitin on glucose metabolism in CAFs (8,14). To illustrate the safety of the sudachitin's treatment, we treated CAFs with a concentration of 50 μM and a treatment time of 24 h. This dose did not show significant inhibition to the proliferation of HIFs according to our result. As shown in Fig. 3, sudachitin inhibited mRNA expression of

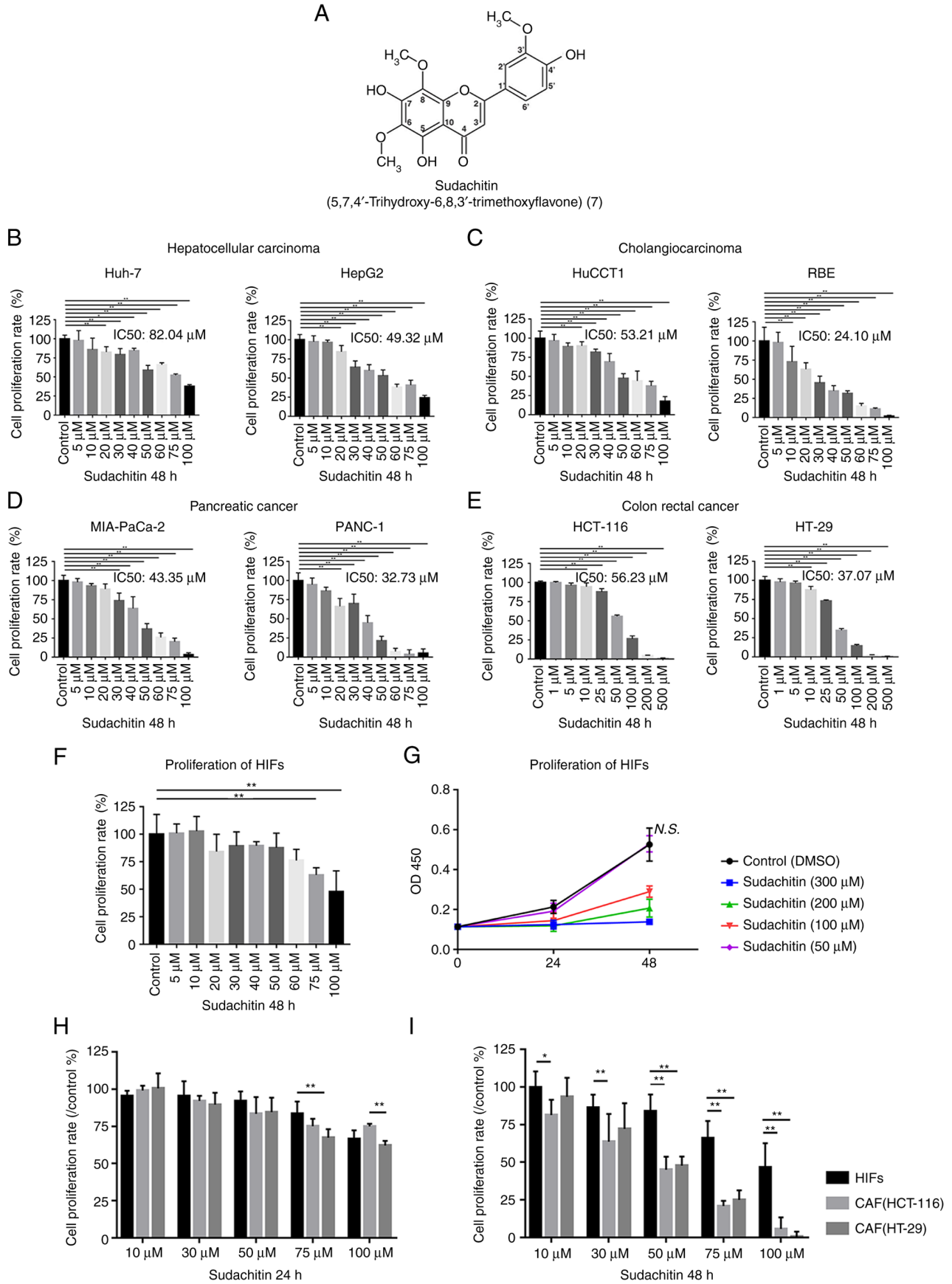

Figure 1. Effect of sudachitin treatment on the proliferation of tumor, CAFs and normal cells. (A) Chemical structure of Sudachitin. The effect of different concentrations of sudachitin on the proliferation of (B) liver cancer cell lines (Huh-7 and HepG2), (C) cholangiocarcinoma cell lines (HuCCT1 and RBE), (D) pancreatic cancer cell lines (MIA PaCa-2 and PANC-1) and (E) colorectal cancer cell lines (HCT-116 and HT-29). The effect of sudachitin treatment on the proliferation of HIFs, presented as a (F) bar chart and (G) line chart. Comparison of growth on HIFs and CAFs after (H) 24 h and (I) 48 h treatment of sudachitin. *P<0.05, **P<0.01. IC50, half-maximal inhibitory concentration; HIFs, human intestinal fibroblasts; N.S., not significant; CAFs, cancer-associated fibroblasts.

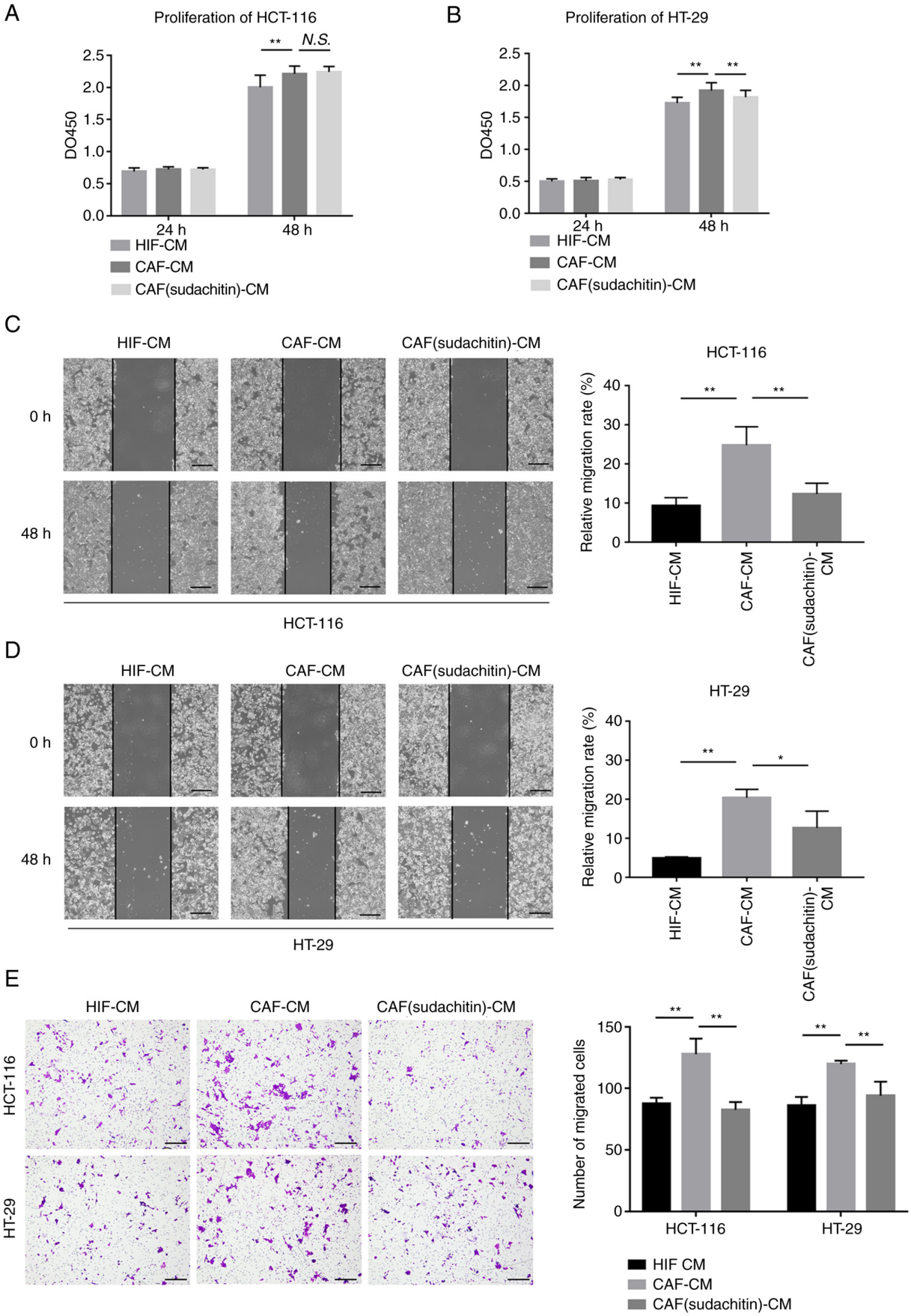

Figure 2. Sudachitin treatment suppresses the tumor-promoting ability of CAFs. The Cell Counting Kit-8 assay demonstrated that CAF pretreatment with 50 μM sudachitin suppressed the tumor-promoting effect of CAF-CM on the proliferation of (A) HCT-116 and (B) HT-29 cells after a 48 h culture. Wound healing assay for (C) HCT-116 and (D) HT-29 cells (scale bar, 400 μm) and (E) Transwell migration assay (scale bar, 200 μm) indicated that sudachitin treatment suppressed the tumor-promoting effects of CAFs on the migration and invasion of these cells. *P<0.05, **P<0.01. HIFs, human intestinal fibroblasts; CAFs, cancer-associated fibroblasts; CM, conditioned medium; N.S., not significant.

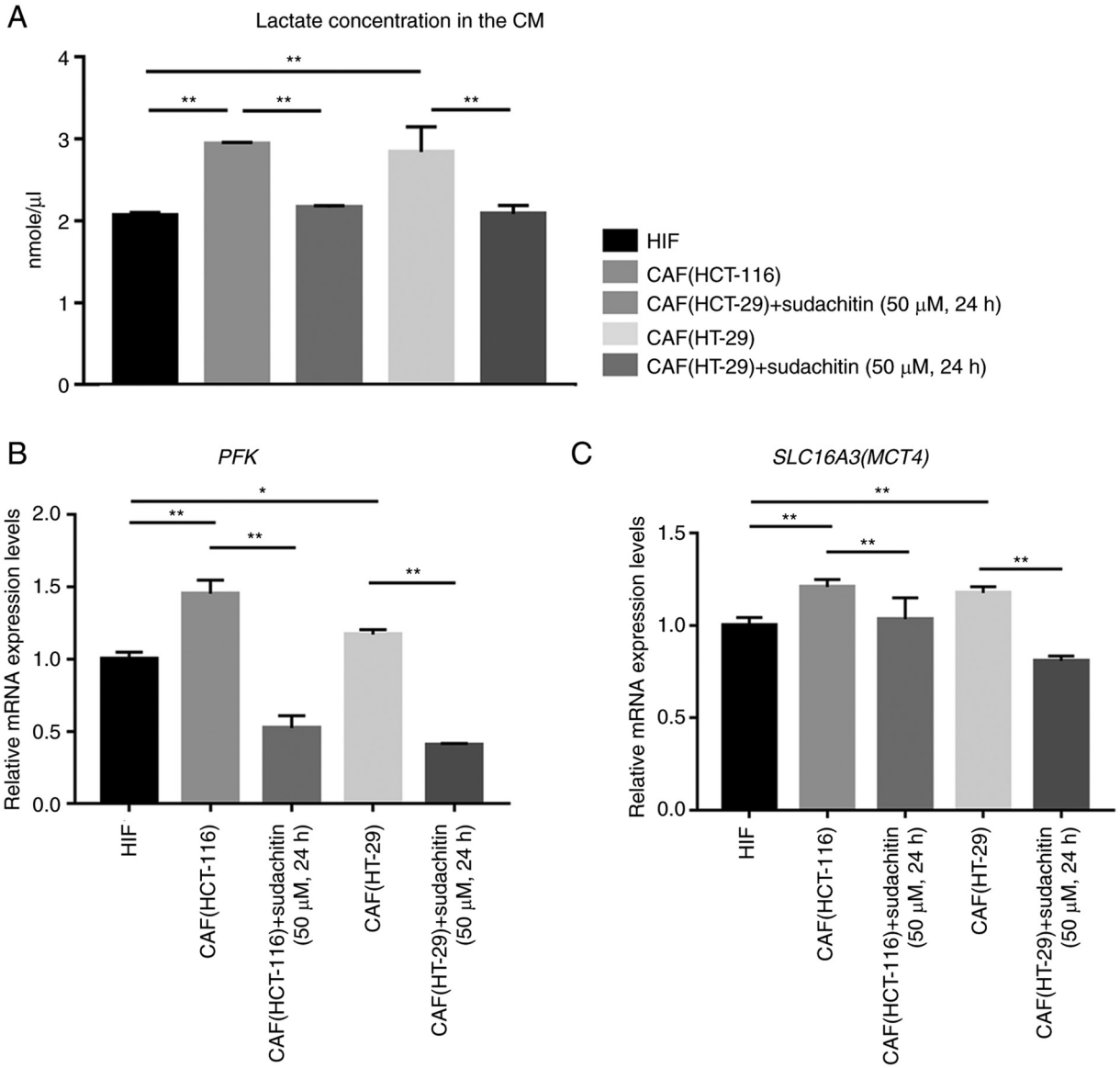

Figure 3. Sudachitin treatment inhibits the glycolytic activity in CAFs. (A) Lactate assay results demonstrated that sudachitin treatment decreased lactate production in the CM from CAFs after a 6 h culture. Gene expression levels of (B) *PFK* and (C) *MCT4* were detected in HIFs, CAFs and sudachitin-treated CAFs by reverse transcription-quantitative PCR analysis. *P<0.05, **P<0.01. HIFs, human intestinal fibroblasts; CAFs, cancer-associated fibroblasts; CM, conditioned medium; PFK, phosphofructokinase; MCT4, monocarboxylate transporter 4.

PFK, which encodes a key enzyme in glycolysis, and *MCT4*, which encodes a cellular membrane transporter of lactate, thereby inhibiting glycolysis and lactate export in CAFs. Recently, 'the reverse Warburg effect' has been proposed as a new theory that CAFs also undergo a metabolic transition similar to that in tumor cells. It makes the hypothesis that in the normal oxygen saturation, CAFs undergo glycolysis and produce a significant amount of lactate in the TME and support the tumor malignancy (15,16). Inhibition of *PFK* and *MCT4* may diminish the promotion of tumor malignancy *in vitro* by CAFs. As evidenced by our findings, sudachitin not only acted directly against tumor cells, it also exerted antitumor effects by targeting the glycolytic pathway of CAFs, which are present in the TME. Moreover, the concentration required for these antitumor effects was relatively low and safe for normal cells. Interestingly, CAFs were more sensitive to sudachitin. We inferred that the altered glucose metabolic profile might account for the improved sensitivity to sudachitin of CAFs, which further suggested that sudachitin was expected to play a unique antitumor role by targeting the TME.

Currently, Ikeda Yakusou Co., Ltd. has achieved the preparation of powdered extracts from *Citrus sudachi* peels and further obtained high purity sudachitin powder. This allows sudachitin to be easily taken by individuals via capsules. The results of a 12-week randomized, double-blind, controlled trial by Shikishima Y *et al* showed that the intake of *Citrus sudachi* peel extract powder, containing a dose of 4.9 mg/day sudachitin, significantly reduced the ratio of visceral fat to subcutaneous fat and moderately reduced waist circumference, a marker of metabolic syndrome, compared to placebo (17). According to information from Ikeda Yakusou Co., Ltd., ~3.5 kg of *Citrus sudachi* peel extract powder can be obtained from 100 kg of peel, which contains ~1.4% of sudachitin. So it is estimated that the daily intake in the

above experiment is equivalent to ~10 g of *Citrus sudachi* peel. In Japan, *Citrus sudachi* produces ~8,000 tons per year, approximately half of which is extruded and processed into juice and produces ~200 tons of peel residue, which is a very abundant resource (8). Although we performed the *in vitro* experiments with sudachitin, a limitation is a lack of *in vivo* data, such as the bioavailability, maximum blood concentration and metabolism characteristics. Therefore, more studies in the future should focus on *in vivo* studies involving the blood levels and safety of sudachitin. In our study of sudachitin impaired glycolysis in CAFs, the lack of measurements on pyruvate in conjunction with lactate was another limitation. And although we found that sudachitin reduced the expression of *PFK* and *MCT4*, the detailed mechanism remains further explored. In conclusion, we investigated for the first time the effects of sudachitin on tumor cells and CAFs. Sudachitin directly inhibited tumor growth and indirectly blocked CAF-mediated support of tumor cells by targeting glycolysis in CAFs. This study extends the understanding of the biological function of sudachitin and suggests that sudachitin is a cost-effective, safe, and widely available anticancer agent.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

SC, YM and MN designed the study. SC, KY, AN, TS and TT performed the experiments. SC, HK, MS and CT collected and analyzed the data. YW and TY interpreted the data. SC and MN wrote the original draft. YM, AN, TS, and MS reviewed and edited the manuscript. SC and MN confirm the authenticity of all the raw data. SC and MN agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All the authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

This study was approved by The Ethics Committee of Tokushima University Hospital (TOCMS approval no. 2901-2).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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