Traditional Chinese medicine formula T33 inhibits the proliferation of human colorectal cancer cells by inducing autophagy

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Abstract
Colorectal cancer (CRC) is a leading cause of cancer-related death globally. Although surgery is still the major method for CRC therapy, the adoption of alternative treatments, such as traditional Chinese medicine (TCM), for CRC treatment is increasing. Our previous study has indicated the anti-breast cancer activity of T33 (a TCM formula). Interestingly, a major ingredient in T33, Baishao (Paeoniae Radix Alba), was reported to have antiproliferative effects on CRC cells. Therefore, this study further validated the influences of T33 on HT-29 and Caco2 cells both in vitro and in vivo. Viability and migration assays were performed to analyze the influences of T33 on proliferation and migratory activity of HT-29 and Caco2 cells. Immunofluorescence (IF) staining and immunoblotting were performed to confirm T33-induced autophagy in HT-29 and Caco2 cells. Xenograft HT-29 tumors were generated to test the effects of T33 in vivo. Significantly reduced survival and migratory activity were observed in both HT-29 and Caco2 cells treated with T33 along with apparently increased LC3-II protein. Significantly decreased p62/SQSTM1 protein, increased LC3-II/LC3-I ratio, and elevated amounts of Atg7, Atg5, and Beclin-1 proteins were detected in both HT-29 and Caco2 cells treated with T33. Moreover, the volume of xenograft HT-29 tumors was significantly lower in mice receiving 200 or 600 mg/kg T33 than in control-treated mice. These findings indicate that T33 exerts anti-CRC activity by inducing autophagy and suggest the potential of T33 for CRC treatment.

Keywords
autophagy, colorectal cancer, T33, traditional Chinese medicine
1 | INTRODUCTION

Colorectal cancer (CRC) is a most common cancer among all ages and affects over 2 million people each year. Many risk factors have been associated with CRC incidence, including environmental, nutritional, genetic, and familial factors. Indeed, diets high in saturated fat and low in fiber and calcium, reduced physical activity, obesity, tobacco use, and alcohol consumption have been identified as factors that contribute to early-onset CRC. Although the success rates for CRC treatment can reach nearly 90% when the disease is localized, advanced CRC consistently ranks in the top three causes of cancer-related mortality. Moreover, many CRC patients who are diagnosed at an early stage and treated with surgery still develop synchronous or metachronous metastatic disease, which has a 5-year survival rate of 13%.

Ethnopharmacological approach and various traditional medicines, including traditional Chinese medicine (TCM), are recognized as the scientific investigations for verifying the underlying anthropological interpretation and the pharmacological foundation. For over 20 centuries, TCM is known as an ancient natural health care system that comprises various treatments such as acupuncture, herbal remedies, exercises (tai chi and qi gong), cupping, and moxibustion. TCM has also been used to ameliorate the side effects such as body weight loss, pain, fatigue, anemia, and dermatological issues during cancer therapy. Indeed, mounting studies have focused on the development and use of extracted products from natural herbal and traditional Chinese medicine (TCM) for cancer treatment. A previous study indicated that Radix Tetrastigma hemsleyani flavone (RTHF) extracted from T. hemsleyani inhibits colorectal tumor growth by decreasing Wnt/b-catenin pathway activity. The extract of Pegostemoncablin, an herb used in TCM, showed antitumor activity against HT-29 and CT26 cells, by inducing apoptosis. Caulis Spatholobi extracts suppressed the invasive capacity of MC38 cells in both cell and animal models. Moreover, a traditional Chinese medicine formula, FufangYiliu Yin (FYY), which contains eight herbs, was also used to induce apoptosis in both HCT116 cells and SW480 cells by modulating PI3K/Akt signaling.

T33, namely C2T20, contains five traditional Chinese herbs, including Gansui (Kansui Radix), Zhigancao (Glycyrrhizae Radix et Rhizoma Praeparata cum Melle), Baishao (Paeonieae Radix Alba), Jiangbanxia (Pinelliae Rhizoma Praeparatum Cum Zingibere et Alumine), and Dahuang (Rhei Radix et Rhizoma). Four ingredients of T33, specifically Gansui, Zhigancao, Baishao, and Jiangbanxia was mixed in a ratio of 3:2:1:2:6:9. The mixture was then put in a pot with 1000 ml RO water and simmered until reduced to 250 ml. The supernatant was sterilized by filtration through a 0.22-μm filter (Merck Millipore, Darmstadt, Germany), and the sterilized T33 solution was subsequently freeze-dried and stored at −80°C for further use.

2 | MATERIALS AND METHODS

2.1 | Traditional Chinese medicine formula T33

T33 is a traditional Chinese formula that consists of five traditional Chinese medicines including Gansui (Kansui Radix), Zhigancao (Glycyrrhize Radix et Rhizoma Praeparata cum Melle), Baishao (Paeonieae Radix Alba), Jiangbanxia (Pinelliae Rhizoma Praeparatum Cum Zingibere et Alumine), and Dahuang (Rhei Radix et Rhizoma). T33 were provided by DeYi Chinese Medicine Clinic (Changhua, Taiwan). Briefly, a total of 32 g mixture of Gansui, Dahuang, Baishao, Jiangbanxia, and Zhigancao was mixed in a ratio of 3:2:12:6:9. The mixture was then put in a pot with 1000 ml RO water and simmered until reduced to 250 ml. The supernatant was sterilized by filtration through a 0.22-μm filter (Merck Millipore, Darmstadt, Germany), and the sterilized T33 solution was subsequently freeze-dried and stored at −80°C for further use.

2.2 | Cell line

The human colorectal adenocarcinoma cell lines HT-29 (ATCC® HTB-38™) and Caco2 (ATCC® HTB-37™), and one normal cell line, CCD-18Co (ATCC® CRL-1459™) were obtained from ATCC (Rockville, MD). HT-29, Caco2, and CCD-18Co cells were cultured with Dulbecco's modified Eagle's medium (DMEM) in a 5% CO2 humidified incubator at 37°C.

2.3 | Cell viability

The survival of cells was measured as described elsewhere. A total of 5 × 10⁴ cells were seeded in each well of a 24-well culture plate overnight and different concentration of T33 was then added for another 24 or 48 h. To measure the viability of the cells, a total of 0.5 ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent was added for another 4 h. Crystals were dissolved by adding 0.2 ml DMSO, and the optical density was detected at 570 nm wavelength. Viability is presented as sample optical density value relative to the control value.

2.4 | Transwell assay

The cell migration was measured using 24-well Millicell Hanging Cell Culture inserts (EMD Millipore, Massachusetts) as described
elsewhere. Briefly, upper chamber was seeded with 2 × 10⁴ cells and T33 in serum-free medium, and lower chamber was loaded with medium containing 10% fetal bovine serum. Next, neutral-buffered formalin (10%) was added to fix the migrated cells, and 0.5% crystal violet was subsequently used to stain the migrated cells. The stained cells were then counted in five different microscopic fields per filter at ×200 magnification.

2.5 Immunofluorescence staining

A LC3B antibody kit (Invitrogen, MA) was used to detect the autophagy-specific marker LC3B (LC3-II). Briefly, HT-29 and Caco2 cells were maintained on Millicell EZ SLIDE 8-well glass slides and then fixed in 4% paraformaldehyde. After permeabilization with 0.2% Triton X-100 for 15 min, the slides were soaked in blocking solution and incubated with LC3-II antibodies (Invitrogen, MA). The slides were then incubated with Alexa Fluor®-conjugated antibodies (Invitrogen, MA) and mounted with ProLong™ Gold Antifade Mountant with DAPI (Thermo Fisher Scientific Inc.). Finally, a fluorescence microscope (Carl Zeiss, NY) was used to observe the cells.

2.6 Immunoblotting

Immunoblotting was performed as described elsewhere. Antibodies against LC3 (NB100-2220), Beclin-1, p62/SQSTM1 (Novus Biologicals, CO), Atg5 and Atg7 (Sigma-Aldrich, St. Louis, Missouri), and β-Actin (EMD Millipore Corporation, Temecula, CA) were used to detect autophagy-related molecules. Briefly, nitrocellulose (NC) membranes were soaked in PBS containing 2% BSA and appropriate antibodies for 2 h. Subsequently, a horseradish peroxidase (HRP)-conjugated antibody was hybridized with the NC membranes for 1 h. Finally, the complexes of antigen–antibody were detected with Immobilon Western HRP Chemiluminescent Substrate (EMD Millipore) and quantified by GE ImageQuant TL 8.1 (GE Healthcare Life Sciences).

2.7 Animals and tumor xenografts

The animal experiment was accolated by the Institutional Animal Care and Use Committee (IACUC) of Chung Shan Medical University (Approval number: 2272). Xenograft tumor was generated as described previously. Briefly, 15 male nude mice at 4-week age were purchased from National Center for Experimental Animals, Taiwan and breed in a specific pathogen-free facility. Animals were free access to food and water for a week to acclimate to the circumstance. Next, HT-29 cells were subcutaneously injected into the flank of mice to produce xenograft tumor. When the tumor volume was approximately 80 mm³, the mice were randomly separated into three groups and daily treated with 1X PBS, 200 mg/kg T33, or 600 mg/kg T33, respectively, by oral gavage. Tumor volume (V) was measured and calculated weekly according to the following formula, V = L × W²/2 (W: the widest tumor measurement; L: the longest tumor dimension). All animals were sacrificed with CO₂ asphyxiation at the end of experiments. The CO₂ flow rate was maintained at 50% of the chamber volume per minute during the euthanasia. After visually confirming that breathing of mice has stopped, CO₂ flow was maintained for 1 min to ensure the death of mice.

2.8 Statistics

The software G*Power version 3.1.9.2 (Franz, Universitat Kiel) was used to determine the sample size as five. Statistics were performed using GraphPad Prism 5 software. To compare the tumor size of mice among different groups, one-way ANOVA with Bonferroni post hoc tests followed by an unpaired two-tailed Student’s t-test was performed. Two-way ANOVA with Bonferroni’s post hoc test for multiple comparisons was used to calculate the effects of cell type and different treatments. The p-value less than .05 was regarded as significant. All values are shown as mean ± SEM.
3 | RESULTS

3.1 | T33 inhibits the proliferation and invasion of CRC cell lines

To test the influence of T33 on human colorectal adenocarcinoma, HT-29 and Caco2 cells were treated with different doses of T33, and cell viability and invasion were detected by MTT and Transwell tests. Significantly lower viability was observed for both CRC cell lines compared to CCD-18Co cells in the presence of 2.5, 5, or 10 mg/ml T33 at 24 h (Figure 1A). Similar results were also detected in both CRC cell lines at 48 h (Figure 1B). Compared to CCD-18Co cells, the survival rates of HT-29 and Caco2 cells were significantly lower in the presence of 2.5, 5, and 10 mg/ml T33. Therefore, the following experiments in this study were performed in HT-29 and Caco2 cells. Moreover, significantly reduced percentages of invaded HT-29 and Caco2 cells were detected in the presence of T33 at 24 h in a dose-dependent manner (Figure 2A,B). Quantified results for invaded cells are shown in the lower panels of Figure 2A,B, respectively.

3.2 | T33 induces autophagy in CRC cell lines

To determine whether autophagy is involved in T33-induced HT-29 and Caco2 cell death, antibodies against LC3-II (LC3B) were used to perform immunofluorescence staining. Obvious higher amount of LC3-II protein was detected in a dose-dependent manner in HT-29 cells receiving T33 for 24 h (Figure 3). Similar results were also observed in Caco2 cells in the presence of T33 for 24 h (Figure 4). To further confirm the T33-induced autophagy in HT-29 and Caco2 cells, immunoblotting was applied to detect the autophagy-related molecules. Significantly increased LC3-II/LC3-I ratio and significantly increased protein expression of Atg7, Atg5, and beclin-1 were detected in both CRC cell lines treated with T33 for 24 h, with the
increases occurring in a dose-dependent manner (Figures 5 and 6). Conversely, significant reductions in protein expression of P62 were observed to occur in a dose-dependent manner (Figures 5 and 6). Quantified results are shown in Figures 5B,C and 6B,C. Moreover, chloroquine (CQ), an autophagy inhibitor, was adopted to confirm the involvement of autophagy to T33-induced death in both CRC cell lines (Figures 7 and 8). After incubating with 25 μM CQ for an hour and subsequent 10 mg/ml T33 for 24 h, significantly decreased P62 and increased LC3-II were detected in both CRC cell lines (Figures 7 and 8). It indicated that CQ pretreatment prevents LC3-II and P62 breakdown by inhibiting lysosomal degradation and causing LC3-II and P62 accumulation.

### 3.3 T33 suppresses the growth of xenograft HT-29 tumor

To test the effects of T33 in vivo, tumor xenografts were generated by subcutaneous injection of HT-29 cells into nude mice. While tumor volume was approximately 80 mm$^3$, the mice were daily treated with
300 μl PBS, 200 mg/kg T33, or 600 mg/kg T33 by oral gavage. Significantly smaller HT-29 tumor volumes were detected in the mice treated with 200 mg/kg or 600 mg/kg T33 than those receiving PBS (Figure 9A). Notably, significantly lower HT-29 tumor volumes were detected in the mice treated with 600 mg/kg T33 than those receiving 200 mg/kg T33. Figure 9B shows representative images of xenograft HT-29 tumors excised from mice in the different groups at the experimental endpoint.

4 | DISCUSSION

TCM has been widely used for centuries in many Asian countries due to its anticancer potential. TCM has been known to reveal various advantages, such as suppression of tumor progression, reduced surgical complications, and attenuation of the damage caused by surgery, chemotherapy, or radiotherapy. Dahuang (Rhei Radix et Rhizoma) and Gan Sui Ban Xia Tang, which consists of Gansui (Kansui Radix), Zhigancao (Glycyrrhizae Radix et RhizomaPraeparata cum Melle), Baishao (Paeoniae Radix Alba), and Jiangbanxia (PinelliaeRhizomaPraeparatum Cum Zingibere et Alumine), are known as the ingredients of T33. A previous study indicated that Rheum palmatumL crude extract causes apoptosis via mitochondrial-dependent pathway in LS1034 human colorectal cancer cells. Another study indicated that Glycyrrhizaglabra and Paeoniae lactiflora extracts have moderate anti-proliferative effects on Caco-2 human colon cancer cells. Although these studies indicate that several T33 ingredients have anti-proliferative potential on various colon cancer cells, the precise mechanism of T33 on CRC are still unclear. For the first time, T33 was demonstrated to have significant cytotoxic activity against human CRC in both cell and animal models, providing an alternative approach for CRC treatment.
Although T33 formula exhibited a significant inhibitory effect on CRC cells, the anticancer activity for each herb of T33 as well as their potential functional ingredients were still unclear. Radix Kansui is the root of Euphorbia kansui T. P. Wang (Euphorbiaceae) and is commonly used for treating edema, ascites, and asthma. Evidence has reported that six methyl ester derivatives isolated from the root of Radix Kansui, including 11,13-eicosadienoic acid methyl ester, 12-octadecenoic acid methyl ester, (Z, Z)-methyl ester-9, 12-octadecadienoic acid, 10-methyl-heptadecanoic acid methyl ester, hexadecanoic acid methyl ester, and methyl ester-5-oxo-DL-proline, can inhibit the growth of human gastric cancer cell, SGC-7901, by inducing apoptosis. Notably, the major functional component of Radix Paeoniae Alba (Paeonia lactiflora), paeoniflorin, was found to have anticancer activity on various human cancers, including liver cancer, lung cancer, bladder cancer, pancreatic cancer, breast cancer, uterine cancer, and glioblastoma. For centuries, Pinelliae Rhizoma Praeparatum is also used for treating cough, phlegm, and cancer. Interestingly, N-oxalylglycine (NOG), known as a 2-oxoglutarate (2OG) inhibitor, is a natural derivate of Rheum rhabarbarum and has been found to reveal anticancer activity by silencing the over-expressed alkylation repair protein-B (AlkB) in cervical carcinoma and head and neck squamous cell carcinomas. Although very limited information about the direct anticancer activity of Glycyrrhizae Radix et Rhizoma is reported, the use of Glycyrrhizae Radix et Rhizoma for cancer treatment has been described. Glycyrrhizae Radix et Rhizoma, namely licorice root, is commonly used to treat HCV and chemotherapy-induced adverse effects such as nausea and vomiting. These findings did indicate the anticancer potentials for each herb of T33 formula and may provide a possible explanation for the anti-CRC activity of T33. However, more investigations are definitively required to verify the precise mechanism and the potential functional ingredients of T33 on CRC treatment.
Autophagy is a catabolic process for maintaining proper cellular homeostasis. The whole process of autophagy consists of several steps, including initiation, phagophore nucleation, elongation, lysosome fusion, and degradation. Autophagy initiates with the assembly of ULK1, ATG13, ATG101, and FIP200/RB1CC1 complex that leads to subsequent phagophore nucleation of VPS34, VPS15, Beclin-1, and ATG14 complex. During the elongation stage, ATG12-ATG7 complex is firstly transferred to ATG10 and interact with ATG5. Then, ATG12-ATG5 complex binds to ATG16 to generate the ATG12-ATG5-ATG16 complex. Eventually, LC3 is proteolysis by ATG4 and converted to LC3-I. Under the action of ATG3 and ATG7, LC3-I is reversibly bound with phosphatidylethanolamine (PE) to generate LC3-II-PE that leads to the formation of autophagosome and subsequent fusion with lysosome.49,50 Notably, the induction of autophagy is a common strategy for the treatment of cancer.51 How-ever, the induction of autophagy is a double-edged sword in cancer therapy.52 Administration of anticancer drugs to induce autophagy generally causes two general effects on cancer cells, excessive

**Figure 7** Autophagy involves in the response of HT-29 cells treated with T33 cells. HT-29 cells were pre-treated with 25 μM CQ (chloroquine) for an h before T33 treatment (10 mg/ml). (A) Cell lysates were harvested after 24 h, and western blot was used to detect P62 and LC3-II proteins. Bars indicate quantification of (B) P62 and (C) LC3-II relative to β-Actin. Experiments were repeated three times with similar results. The numbers 1, 2, and 3 represent a significant difference (P < .05) compared with control, CQ (25 μM), and T33 (10 mg/ml), respectively.

**Figure 8** Autophagy involves in the response of Caco2 cells treated with T33Caco2 cells were pre-treated with 25 μM CQ (chloroquine) for an h before T33 treatment (10 mg/ml). (A) Cell lysates were harvested after 24 h, and western blot was used to detect P62 and LC3-II proteins. Bars represent quantification of (B) P62 and (C) LC3-II relative to β-Actin. Experiments were repeated three times with similar results. The numbers 1, 2, and 3 indicate a significant difference (P < .05) compared with control, CQ (25 μM), and T33 (10 mg/ml), respectively.
autophagy leading to cancer cell death and cytoprotection, which is a drug resistance mechanism resulting in clinical treatment obstacles and a poor prognosis. In this study, autophagy-induced cell death in HT-29 and Caco2 cells was observed in the presence of T33, suggesting the therapeutic potential of T33 in CRC treatment is mediated by inducing excessive autophagy. Since autophagy is known to have different effects on cancers, more experiments are also required to verify the precise mechanism of T33-induced autophagy in CRC cells.

Mounting evidence have demonstrated that P62 involves in both autophagy and apoptosis. The P62 protein, known as sequestosome 1 (SQSTM1), is as an essential modulator in autophagosome formation through binding LC3. Accordingly, evidence further indicated that the LC3-interacting region (LIR) domain of P62 directly interact with LC3, leading to the generation of autophagosome. In addition, P62 is also known to induce apoptosis through activating caspase-8 that locates on autophagosomal membrane. Notably, this study only investigated whether T33 causes autophagy in HT-29 and Caco2 cells but did not explore whether T33 induces apoptosis in HT-29 and Caco2 cells. Since P62 plays dual roles in both autophagy and apoptosis, further experiments are required to verify the precise influences and mechanism of T33 on HT-29 and Caco2 cells.

Some concerns for the present study must be noted. Although HT-29 and Caco-2 cells are the most commonly used cell models for human CRC research, both of them are grade II CRC cell models. Therefore, further investigations are merited to verify the influence of T33 on other grade of CRC cell lines such as SW480 cells (grade III) and LoVo (grade IV) cells. Additionally, HT-29 cells were subcutaneously injected into the flank of nude mice, which created ectopic colonic tumors. This model cannot provide the microenvironment for colonic cancer cell growth or mimic the key features of human disease processes. Hence, an orthotopic animal model, such as inoculation of human colonic cancer cells into the cecal wall of mice, should be considered to further investigate the precise mechanism of T33 in CRC.

Moreover, converse effects achieved with combinational use of various traditional Chinese herbs should also be noted. Evidence has indicated that the combined use of Kansui and licorice in treating malignant pleural effusion may cause serious toxicity at a specific ratio. Notably, licorice, the most frequently used Chinese herb, is
known to play paradoxical roles in both detoxification and toxicity enhancement, leading it to be called a “two-faced” herb.6 These findings attract intensive attention to the diverse roles of specific Chinese herbs and their combinational use for cancer therapy. Therefore, more pharmacological studies are urgently required to assess the efficacy and combined use of the Chinese herbs in T33. For better utilization and development of T33 for the treatment of CRC, the mechanism of the T33 formula should be studied to determine whether synergistic or antagonistic effects exist.

5 | CONCLUSIONS

The current study reported a significant anti-cancer effect of T33 on both HT-29 and Caco2 cells by inducing robust autophagy and attenuating the growth of xenograft HT-29 tumor in nude mice (Figure 10), suggesting an alternative remedy for CRC treatment.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTIONS

Yu-Te Liu, Bor-Show Tzang, Chih-Yang Huang, and Tsai-Ching Hsu: Conceived and designed the studies. Yu-Te Liu, Jia Le Yow, Yi-Hsuan Chiang, and Tsai-Ching Hsu: Performed the experiments. Yu-Te Liu, Bor-Show Tzang, Chih-Yang Huang, and Tsai-Ching Hsu: Discussed the studies. Jia Le Yow and Bor-Show Tzang: Performed the data statistical analysis. Jia Le Yow, Bor-Show Tzang, and Tsai-Ching Hsu: Prepared the final figures. Yu-Te Liu, Bor-Show Tzang, and Tsai-Ching Hsu: wrote the paper. All authors read and approved the final manuscript.

DATA AVAILABILITY STATEMENT

Data is available on request from the authors.

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