Aqueous Extracts of Fructus Ligustri Lucidi Enhance the Sensitivity of Human Colorectal Carcinoma DLD-1 Cells to Doxorubicin-Induced Apoptosis via Tbx3 Suppression

Jin-fang Zhang, PhD1,2,3, Ming-liang He, PhD3, Qi Dong, PhD3, Wei-dong Xie, PhD2, Yang-chao Chen, PhD3, Marie C. M. Lin, PhD4, Ping-chung Leung, PhD3, Ya-ou Zhang, PhD2, and Hsiang-fu Kung, PhD2,3

Abstract
Chemoresistance has imposed a great challenge for cancer therapy. Fructus Ligustri Lucidi (FLL) is one of the commonest Chinese herbs that has been used for thousand years. This study shows that the aqueous extract of FLL (AFLL) enhanced the sensitivity of DLD-1 colon cancer cells to doxorubicin-induced apoptosis. Furthermore, Tbx3 expression was found to be suppressed by AFLL when the expression of tumor suppressor genes p14 and p53 were activated. Therefore, reduction of Tbx3 rescued the dysregulated P14ARF-P53 signaling, which in turn contributed to the sensitivity of DLD-1 cells to doxorubicin-induced apoptosis. As a conclusion, the findings suggest that FLL has a potential of being an appealing agent for auxiliary chemotherapy in treatment of human colorectal carcinoma.

Keywords
sensitivity, Fructus Ligustri Lucidi, doxorubicin, apoptosis

Introduction
Colorectal cancer is one of the commonest cancers in Western countries.1 The average 5-year survival, which is about 50%, has only improved modestly over the past 40 years.2 Surgery is the most prevalent treatment for colon cancer, and chemotherapy was used to reduce recurrence after surgery. However, chemoresistance is a major problem in cancer chemotherapy. There is an increasing need for the discovery of a new medicine for auxiliary chemotherapy to strengthen the clinical effects of the conventional cancer therapies.

Fructus Ligustri Lucidi (FLL) is the ripened fruit of Ligustrum lucidum Ait. (family Oleaceae), which has been used in traditional Chinese medicine (TCM) for more than a thousand years. As a commonly used TCM herb, FLL has shown the therapeutic effects on nourishing Liver and Kidney; stimulating Heart; enhancing organism immunization; anti-inflammation; and so on.3 A variety of physiologically active compounds (including salidroside, nuzhenide, oleanolic acid, ursolic acid, and quercetin) have been found in FLL.4 Oleanolic acid and ursolic acid, the main triterpenoid saponin of Ligustrum lucidum, can induce apoptosis of many kinds of tumors.5-7

Recent studies have shown that the aqueous extract of FLL (AFLL) regulated the differentiation of human mesenchymal stem cell8 and enhanced calcium balance.9 In this study, we investigated the role of aqueous extract of FLL as a potential treatment for human colon cancer, and examined the combining action of AFLL and chemotherapy. Furthermore, the underlying anticancer mechanism will also be investigated.

1Tsinghua University, Beijing, People’s Republic of China
2Tsinghua University, Shenzhen, People’s Republic of China
3The Chinese University of Hong Kong, Hong Kong SAR, People’s Republic of China
4The University of Hong Kong, Hong Kong SAR, People’s Republic of China

Corresponding Author:
Hsiang-fu Kung, Room 511A, Ming Li Basic Medicine Science Building, Stanley Ho Centre for Emerging Infectious Diseases, The Chinese University of Hong Kong, Shatin, Hong Kong, People’s Republic of China
Email: b110473@mailserv.cuhk.edu.hk
Material and Method

Aqueous Extract of FLL

Fructus Ligustri Lucidi was purchased from Anguo Mayway Herb Company Ltd (Anguo City, Heibei Province, People’s Republic of China). Aqueous extract of FLL was prepared by extracting 50 g of FLL with 1.5 L distilled deionized water for 2 hours, and the extraction process was repeated twice. The solution obtained was then filtrated through the small absorbent gauze. The eluate was concentrated under a reduced pressure at 50°C, and lyophilized by the FreezeMobile Freeze dryer (Virtis, Gardiner, NY). The dried aqueous extract was obtained and stored at –20°C. The extract was finally redissolved in phosphate-buffered saline (PBS) with a stock concentration of 100 mg/mL.

Cell Culture

Human colorectal carcinoma DLD-1 cells were cultured in Dulbecco’s modified Eagle’s medium (GIBCO, Carlsbad, CA) supplemented with 10% fetal bovine serum (GIBCO, Carlsbad, CA), 100 U/mL penicillin, and 100 μg/mL streptomycin, and incubated in a humidified atmosphere of 5% CO₂ at 37°C.

Cell Proliferation

Cell proliferation was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells pretreated or nonpretreated were plated at a density of 5 × 10³ cells per well in 96 flat-bottomed well plates. After incubating for 24 hours, the culture medium was replaced by medium containing AFL. The cells were incubated at 37°C for successively 1, 2, and 3 days. A solution of 20 μl MTT (0.5 mg/mL) was added to the medium and incubated for 4 hours. Then the medium was decanted, the formazan salts were dissolved with 200 μl dimethyl sulfoxide (DMSO) for 30 minutes at 37°C, and the absorbance was determined at 550 nm using a VICTOR3 V Multilabel Counter (PerkinElmer, Wellesley, MA).

Cell Cycle Analysis

DLD-1 cells were incubated with 50 μg/mL AFL for 3 days and were harvested. The total RNA was extracted using TRIZOL (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. Reverse transcription was carried out by using the SuperScript III Reverse Transcriptase kit (Invitrogen, Carlsbad, CA) and the cDNA fragments were amplified by PCR using GoTaq DNA Polymerase (Promega, Madison, WI). Thermocycling was performed with a gradient thermocycler (Takara, Shiga, Japan) using GoTaq Flexi DNA Polymerase (Promega, Madison, WI). Primer sequences for each gene are listed in Table 1.

Apoptosis Assayed by Double Staining

Samples with different treatments were washed with PBS and mixed with 10 μl Hoechst and 5 μl PI, then incubated for 10 to 15 minutes at 37°C. After the incubation, the stained cells were observed under fluorescence microscopy. Apoptotic cells and dead cells were counted to obtain the apoptotic and dead percentage.

Reverse Transcriptase–Polymerase Chain Reaction (RT-PCR)

DLD-1 cells were treated with AFL for 3 days and were harvested. GAPDH was used as an internal control.

Transfection of DLD-1 Cells With Small Interfering Ribonucleic Acid (SiRNA)

Tbx3-specific siRNA and one negative control siRNA (NC) were synthesized by Shanghai GenePharma Co. (Shanghai, China). The sequences of the sense strands are listed in the following: siRNA of Tbx3, sense: 5’GAGGAUGUACAUUCACCAG3’; NC, sense: 5’UUCUCCGAACGUGUCAC3’.
DLD-1 cells were seeded into 96- or 12-well plates and transfected with siRNA/NC at a concentration of 5 pmol/well for 96-well plates, and 50 pmol/well for 12-well plates using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) within 24 hours according to manufacturer’s instructions. The samples were collected and examined for MTT and apoptosis assay.

### Western Blotting

Cell extracts were separated by SDS-PAGE (12%) and transferred to PVDF membranes. After that, the membranes were blocked with 5% nonfat dry milk for 1 hour. The membranes were then incubated with Tbx3 (A-20) antibody (Santa Cruz), with a dilution of 1:500 overnight. Secondary antibody donkey-anti-goat was used at a dilution of 1:1000 for one hour. The ECL detection system (GE Healthcare, Buckinghamshire, UK) was used to visualize the results.

### Luciferase Activity Assays

A 2 kb human genomic DNA sequence upstream of the Tbx3 transcriptional start site was amplified by high fidelity DNA polymerase (Invitrogen, Carlsbad, CA) and the target sequence was cloned into a pGL3-basic vector (Promega, Madison, WI).

DLD-1 cells were seeded in a 24-well plate at the density of $1 \times 10^5$ and cultured overnight. The cells were transfected with 800 ng of the reporter plasmid using Lipofectamine 2000. AFLL was added to final concentration of 50 μg/mL. The empty pGL3 vector was used as a control. Cell lysates were collected 30 hours post-transfection. Firefly luciferase activities were measured using the Luciferase Reporter Assay System (Promega, Madison, WI). Total protein concentrations of cell lysates were determined by using the Bradford assay (Bio-Rad, Hercules, CA) at 595 nm on a spectrophotometer (TECAN, Grödig, Austria). The luciferase activity was normalized according to the total protein contents of the cell lysates.

### Statistical Analysis

Results were statistically analyzed by using the independent-samples Student’s t-test (SPSS Inc, Chicago, IL). Data were expressed as mean ± standard deviation (mean ± SD). $P < .05$ was regarded as being statistically significant.

### Results

#### AFLL Inhibited Proliferation of Human Colorectal Cancer Cells

To examine the anti-proliferation effect of the aqueous extract of FLL on colorectal cancer cells, the proliferation of DLD-1 cells was examined by using the MTT assay. Results showed that AFLL moderately inhibited the proliferation of DLD-1 cells in a dose-dependent manner (Figure 1A) without showing obvious cytotoxicity even at very high concentrations (200 μg/mL). Morphological investigation also showed the proliferation was inhibited (Figure 1B). Furthermore, we assayed the cell cycle and found that G0/G1 cell cycle arrest occurred in the AFLL group (Figure 1C).

#### AFLL Enhanced Apoptosis Induced by Doxorubicin Treatment

After treating the DLD-1 cells with AFLL for 3 days, doxorubicin was added in the culture media. MTT assays were employed to detect the combined effects of AFLL and doxorubicin on cell proliferation. Compared with nonpretreated cells, the proliferation of treated cells was markedly inhibited by 500 ng/mL doxorubicin when they were pretreated with AFLL or siRNA of Tbx3 (Figures 2A and 2B). This suggested that doxorubicin, a topoisomerase inhibitor, could trigger apoptosis in DLD-1 human colorectal carcinoma cells. To further investigate the mechanisms of the enhanced inhibition of cell growth by AFLL, we carried out an apoptotic assay. As shown in Figure 2C, doxorubicin significantly induced apoptosis in cancerous cells when the cells were pretreated with AFLL. As shown experimentally by flow cytometry, 40% of cells pretreated with AFLL and 42.6% of cells pretreated with siRNA of Tbx3 exhibited apoptosis; whereas only 20% of cells treated with doxorubicin alone could demonstrate apoptotic symptoms. Results of Hoechst and PI double staining showed that compared with nonpretreated DLD-1 cells, doxorubicin induced about 40% apoptotic cells at day 3 and about 15% apoptotic cells and about 70% dead cells at day 6 (Figures 2D and 2E).

### Table 1. Polymerase Chain Reaction Primers and Conditions for the Specific Amplification of Human mRNA (in Alphabetic Order)

| Gene   | Forward (5'→3')             | Reverse (5'→3')             | Annealing Temperature (°C) |
|--------|-----------------------------|-----------------------------|---------------------------|
| GAPDH  | tccatgacaacttggatcg         | tgtagccaaattctggca          | 56                        |
| SPRAC  | cctcactctctcgctct         | gcttgccagacacttcgca         | 55                        |
| P14ARF | gttggctccagcttcgtaag       | gatgtcagctctctctcttg        | 53                        |
| P53    | gaaattttgctggtagatattg     | gttccgtcccagtagattacca      | 52                        |
| Tbx3   | gttggactccggctctgaacctc    | aagttggggcacagggct         | 53                        |
AFLL Enhanced Sensitivity of DLD-1 Cells to Doxorubicin-Induced Apoptosis by Suppressing Tbx3 Expression In Vitro

Tbx3 downregulation was reported to render sensitivity of breast cancer cells to doxorubicin-induced apoptosis. To address the role of Tbx3 in doxorubicin-induced apoptosis, we checked both the mRNA and protein levels of Tbx3 in DLD-1 cells. Results showed that both the mRNA (Figure 3A) and protein (Figure 3B) levels were significantly reduced when the DLD-1 cells were treated with AFLL. As a direct target gene of Tbx3, P14ARF becomes involved in the doxorubicin-induced apoptosis via ARF-Mdm2-P53 signaling in tumorigenesis. Consistent with these reports, the downstream p14ARF and P53 expressions were upregulated when the cells were treated with AFLL (Figures 3A and 3C).

To evaluate whether Tbx3 could directly respond to AFLL, we cloned the Tbx3 promoter upstream of the luciferase reporter gene and determined its activities. As shown in Figure 3D, the luciferase activity of the treated cells was markedly reduced after AFLL treatment. Further, based on our results, we proposed a working cascade illustrating the interaction between the AFLL and p14-Mdm2-p53 signaling pathways (Figure 4).

Discussion

Chemotherapy is commonly used to treat cancer after surgery or radiation therapy to prevent recurrence of the tumors. Chemotherapeutic drugs can destroy cancer cells, keep them from multiplying, reduce the size of a tumor, and relieve cancer symptoms. However, resistance often occurs during chemotherapy, causing a major challenge for the treatment of malignant tumors for decades. In light of this, it is of significant importance to discover combination protocols that overcome resistance during chemotherapy. An effective therapeutic strategy would not only reduce the economic burden of patients but also improve the anticancer effects in addition to reducing the side effects of the therapeutics. In this study, we demonstrated that the aqueous extract of FLL could enhance the sensitivity of the cancer cells to...
Tbx3 was shown to decrease the apoptotic sensitivity of cancer cells to doxorubicin treatment.\textsuperscript{16} Tbx3 and the closely related gene Tbx2 are members of the T-box gene family, playing an important role in development. Missense mutations of Tbx3 contribute to the ulnar-mammary syndrome, an autosomal dominant disorder with variable clinical features.\textsuperscript{17} Recent studies rendered Tbx3 a high potential of being an attractive candidate for preventing senescence and immortalizing cells.\textsuperscript{10,16} These findings revealed the role of Tbx3 as an important player in tumorigenesis. We found that the aqueous extract of FLL increased the doxorubicin-induced apoptosis in malignant cells, and downregulated the Tbx3 expression (Figures 3A and 3B). These findings were consistent with the previous reports in other cancer cells.\textsuperscript{16} To demonstrate whether there were direct interactions between the effect of apoptosis and the downregulation of Tbx3 expression, we performed reporter assays. Our results showed that FLL directly suppressed the Tbx3 promoter activity (Figure 3C), demonstrating that FLL promoted doxorubicin-induced apoptosis via suppressing the Tbx3 expression in cancer cells.

The underlying mechanism for the action of Tbx3 appears to be through transcriptional repression of the Cdkn2a (p19\textsuperscript{ARF}) gene.\textsuperscript{11,18} The murine tumor suppressor p19\textsuperscript{ARF} (p14\textsuperscript{ARF} in humans), acting through the p53 pathway, is thought to fulfill an important protective role in preventing primary cells from oncogenic transformation.\textsuperscript{19} It is known that Tbx3 directly binds on the p19\textsuperscript{ARF} promoter and suppresses p19\textsuperscript{ARF} expression.\textsuperscript{20} As a consequence, the suppression of P14\textsuperscript{ARF} by the overexpression of Tbx3 in a variety of cancer cells may dysregulate the P14\textsuperscript{ARF}-Mdm2–p53 pathway, thereby causing cell senescence and carcinogenesis.\textsuperscript{18,21}

In addition, the reduction of Tbx3 proteins in cancer cells was also shown to rescue the dysregulated P14\textsuperscript{ARF}-P53 signaling, which in turn contributed to the sensitization of doxorubicin-induced apoptosis in DLD-1 cells (Figures 3A, 3B, and 3C). Taking all these into consideration, we proposed that Tbx3 was a direct target of the aqueous extract of doxorubicin-induced apoptosis. This would guarantee more extensive applications of FLL for cancer treatment in the future.

Doxorubicin, a topoisomerase II inhibitor, is commonly used in the treatment of sarcomas; however, its cardiotoxicity and resistance are some of its main clinical limitations.\textsuperscript{12} Doxorubicin triggers apoptosis by eliciting direct DNA damage, at least in part in a p53-dependent manner.\textsuperscript{13-15} It is urgent to find some agents to strengthen the effects of doxorubicin and to rescue its side effects. FLL, one common traditional Chinese medicine, not only can inhibit proliferation of DLD-1 cells and induce G0/G1 cell arrest (Figure 1) but can also enhance doxorubicin-induced apoptosis in colon cancer cells (Figure 2). Therefore, FLL is an ideal agent for auxiliary chemotherapy in treatment of human colorectal carcinoma.

Figure 2. Aqueous extract of Fructus Ligustri Lucidi (FLL) enhanced the sensitivity of DLD1 cells to doxorubicin-induced apoptosis
Dox significantly inhibited proliferation of both FLL-pretreated DLD-1 cells (A) and siRNA of Tbx3-pretreated DLD-1 cells (B) (*P < .01 vs control). (C) Apoptosis analysis by flow cytometry. (C) DLD-1 cells; FLL, DLD-1 cells were treated with FLL at a concentration of 50 μg/mL for 3 days; Dox, DLD-1 cells were treated with doxorubicin alone for 3 days. FLL + Dox, DLD-1 cells were pretreated with FLL for 3 days and then treated with doxorubicin for another 3 days. Si-Tbx3: DLD-1 cells were transfected with siRNA of Tbx3 for 3 days; Si-Tbx3 + Dox, DLD-1 cells were transfected with siRNA of Tbx3 for 3 days and then treated with doxorubicin for another 3 days. (D). Apoptosis analysis by Hoechst and PI double staining. (E) Apoptotic and dead percentage. A, DLD-1 cells were treated only with FLL; A + D-3, DLD-1 cells were pretreated with FLL for 3 days and then treated with doxorubicin for another 3 days; A + D-6, DLD-1 cells were pretreated with FLL for 3 days and then treated with doxorubicin for another 6 days.
As a summary, our study demonstrated that the aqueous extract from Fructus Ligustri Lucidi enhanced the anti-cancer activity of doxorubicin. Considering its low cost and strong auxiliary anticancer effect, FLL may have a potential of being further developed into a complementary and alternative medicine for auxiliary chemotherapy of colorectal cancer.

**Declaration of Conflicting Interests**

The author(s) declared no conflicts of interest with respect to the authorship and/or publication of this article.

**Funding**

The author(s) received no financial support for the research and/or authorship of this article.

**References**

1. Benson AB. Epidemiology, disease progression, and economic burden of colorectal cancer. *J Manag Care Pharm.* 2007;13: S5-S18.

2. Hawk ET, Levin B. Colorectal cancer prevention. *J Clin Oncol.* 2005;23:378-391.

3. Committee of National Pharmacopoeia. *Pharmacopoeia of P. R. China*. Beijing, China: Committee of National Pharmacopoeia; 2005:34.
4. Chen G, Zhang LY, Wu XL, Ye JN. Determination of mannitol and three sugars in *Ligustrum lucidum* Ait by capillary electrophoresis with electrochemical detection. *Analytica*. 2005;530:15-31.

5. Manu KA, Kuttan G. Ursolic acid induces apoptosis by activating p53 and caspase-3 gene expressions and suppressing NF-kappaB mediated activation of bel-2 in B16F-10 melanoma cells. *Int Immunopharmacol*. 2008;8:974-981.

6. Yamai H, Sawada N, Yoshida T, et al. Triterpenes augment the inhibitory effects of anticancer drugs on growth of human esophageal carcinoma cells in vitro and suppress experimental metastasis in vivo. *Int J Cancer*. 2009;125:952-960.

7. Bonaccorsi I, Altieri F, Sciamanna I, et al. Endogenous reverse transcriptase as a mediator of ursolic acid’s anti-proliferative and differentiating effects in human cancer cell lines. *Cancer Lett*. 2008;263:130-139.

8. Li G, Chan CY, Zhang JF, et al. the aqueous extract of Fructus Ligustri Lucidi regulates the differentiation of human mesenchymal stem cell. *Int J Integr Biol*. 2008;3:182-190.

9. Zhang Y, Lai WP, Leung PC, Che CT, Wong MS. Improvement of Ca balance by Fructus Ligustri Lucidi extract in aged female rats. *Osteoporos Int*. 2008;19:235-242.

10. Carlson H, Ota S, Song Y, et al. Tbx3 impinges on the P53 pathway to suppress apoptosis, facilitate cell transformation and block myogenic differentiation. *Oncogene*. 2002;21:3827-3835.

11. Jacobs JJ, Keblusek P, Robanus-Maandag E, et al. Senescence bypass screen identifies TBX2, which represses Cdkn2a (p19ARF) and is amplified in a subset of human breast cancers. *Nat Genet*. 2000;26:291-299.

12. Temple HT. Clinical evaluation and treatment of soft tissue tumors. *Semin Musculoskelet Radiol*. 1999;3:5-14.

13. Lowe S, Ruley H, Jacks T, Housman D. p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. *Cell*. 1993;74:957-967.

14. Lowe S, Bodis S, McClatchey A, et al. p53 status and the efficacy of cancer therapy in vivo. *Science*. 1994;266:807-810.

15. Blagosklonny M, El-Deiry W. Acute overexpression of wt p53 facilitates anticancer drug-induced death of cancer and normal cells. *Int J Cancer*. 1998;75:933-940.

16. Renard C, Labalette C, Armengol C, et al. Tbx3 is a downstream target of the wnt-B catenin pathway and a critical mediator of B-catenin survival functions in liver cancer. *Cancer Res*. 2007;67:901-910.

17. Banshad M, Lin RC, Law DJ, et al. Mutation in human TBX3 alter limb, apocrine and gential development in ulnar-mammary syndrome. *Nat Genet*. 1997;16:311-315.

18. Brummelkamp TR, Kortlever RM, Lingbeek M, et al. TBX-3, the gene mutated in ulnar-mammary syndrome, is a negative regulator of p19ARF and inhibits senescence. *J Biol Chem*. 2002;277:6567-6572.

19. Humbey O, Pimkina J, Zilfou JT, et al. The ARF tumor suppressor can promote the progression of some tumors. *Cancer Res*. 2008;68:9608-9613.

20. Lingbeek ME, Jacobs JJ, van Lohuizen M. The T-box repressors TBX2 and TBX3 specifically regulate the tumor suppressor gene p14ARF via a variant T-site in the initiator. *J Biol Chem*. 2002;277:26120-26127.

21. Yarosh W, Barrientos T, Esmailpour T, et al. TBX3 is overexpressed in breast cancer and represses p14 ARF by interacting with histone deacetylases. *Cancer Res*. 2008;68:693-699.