Original Articles

Metronomic S-1 dosing and thymidylate synthase silencing have synergistic antitumor efficacy in a colorectal cancer xenograft model

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Metronomic chemotherapy is currently considered an emerging therapeutic option in clinical oncology. S-1, an oral formulation of Tegafur (TF), a prodrug of 5-fluorouracil (5-FU), is designed to improve the antitumor activity of 5-FU in tandem with reducing its toxicity. Clinically, metronomic S-1 dosing has been approved for the standard first- and second-line treatment of metastatic or advanced stage of colorectal (CRC). However, expression of intratumor thymidylate synthase (TS), a significant gene in cellular proliferation, is associated with poor outcome to 5-FU-based chemotherapeutic regimens. In this study, therefore, we examined the effect of a combination of TS silencing by an RNA interfering molecule, chemically synthesized short hairpin RNA against TS (shTS), and S-1 on the growth of human colorectal cancer cell (DLD-1) both in vitro and in vivo. The combined treatment of both shTS with 5-FU substantially inhibited cell proliferation in vitro. For in vivo treatments, the combined treatment of metronomic S-1 dosing with intravenously injected polyethylene glycol (PEG)-coated shTS-lipoplex significantly suppressed tumor growth, compared to a single treatment of either S-1 or PEG-coated shTS-lipoplex. In addition, the combined treatment increased the proportion of apoptotic cells in the DLD-1 tumor tissue. Our results suggest that metronomic S-1 dosing combined with TS silencing might represent an emerging therapeutic strategy for the treatment of patients with advanced CRC.

Introduction

Colorectal cancer (CRC) is one of the most common causes of malignancy-related deaths in the world [1]. Although the significant progress that has been attained in the chemotherapy of CRC over the past decade, chemotherapeutic agents have only improved the outcome of early stage CRC but not the outcome of advanced CRC [2]. Therefore, the introduction of novel therapeutic agents and/or options are urged to further improve the outcome of chemotherapy for advanced CRC.

The therapeutic concept of administering chemotherapeutic agents continuously at lower doses, relative to the maximum tolerated dose (MTD) without drug-free breaks over extended periods—known as “metronomic chemotherapy”—is a promising approach in cancer therapy [3,4]. Fluoropyrimidines (including 5-fluorouracil, 5-FU) are considered the mainstay for CRC and other gastrointestinal malignancies [5,6]. Recently, metronomic dosing of S-1, an oral 5-FU formulation consisting of tegafur (FT; a prodrug of 5-fluorouracil, 5-FU) [7], has been acknowledged for achieving sustained 5-FU plasma concentrations, mimicking the pharmacokinetics of a continuous infusion of 5-FU while eliminating the inconvenience associated with the continuous infusion of 5-FU [8] and the rapid degradation of 5-FU into its inactive form in the liver and tumors [2]. In addition, metronomic S-1 dosing has shown a superior efficacy than 5-FU in the treatment of advanced or metastatic gastric cancer and CRC [9]. Nonetheless, like other fluoropyrimidine derivatives, including 5-FU, S-1 showed a
limited efficacy as single agents for advanced CRC [10]. Instead, remarkable antitumor effects can only be realized in the clinical setting by combining the “metronomic therapy” with other therapeutic agents.

Many reports have emphasized the correlation between thymidylate synthase (TS; a folate-dependent enzyme that plays a crucial role in the biosynthesis of thymidylic acid, an essential precursor for DNA synthesis) expression level and the responsiveness in patients with advanced CRC treated with 5-FU-based chemotherapeutic [11–13]. High expression of TS has been associated with poor prognosis and/or disease refractory to fluoropyrimidine-based chemotherapy in several cancers including colorectal and head and neck cancer [14].

Many small-molecular weight therapeutics were applied to inhibit TS expression, however, treatment with such TS inhibitors failed to induce efficient down-regulation of TS expression [15]. Instead, silencing of TS by RNA interference (RNAi) may be considered as an efficient alternative approach to down-regulate TS expression. RNAi is considered as a promising regulatory process in which double-stranded RNA (dsRNA) induces specific degradation of its target mRNA [16]. A growing number of studies have used siRNAs/shRNAs as potential therapeutic agents for treating numerous diseases, including cancer and genetic or viral infections [17,18].

In this study, therefore, we investigated the efficacy of gene silencing using chemically synthesized short hairpin RNA targeting TS (shTS) in suppressing the TS expression, and thereby enhancing the cell growth inhibitory effect of 5-FU on colorectal tumor cell line, DLD-1, in vitro. Additionally, we evaluated the in vivo antitumor efficacy of a combination treatment with metronomic S-1 dosing (p.o.) and PEG-coated shTS-lipoplex (i.v.) in a subcutaneous xenograft mouse model.

Materials and methods

Materials

5-FU and S-1 were generously donated by Taiho Pharmaceutical (Tokyo, Japan). 1-palmitoyl-2-oleoyl phosphatidylcholine (POPC), dioleoylphosphatidylethanolamine (DOPE) and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(methoxy(polyethyleneglycol)-2000) (mPEG2000-DSPE) were generously donated by NOF (Tokyo, Japan). Cholesterol (Cho) was purchased from Wako Pure Chemical (Osaka, Japan). A cationic lipid, O,O’-ditetradecyl-N-(alpha-trimethyl ammonioacetylb) dithanolamine chloride (DC-Chol) was purchased from Sogo Pharmaceutical (Tokyo, Japan). Opti-MEM 1 and Lipofectamine RNAiMAX (Life Technologies, Carlsbad, CA, USA) were purchased from Invitrogen (San Diego, CA, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Nacalai Tesque (Kyoto, Japan). All other reagents were of analytical grade.

Animals and tumor cell line

5-week-old male BALB/c nu/nu mice were purchased from Japan SLIC (Shizuoka, Japan). The experimental animals were allowed free access to water and mouse chow, and were housed under controlled environmental conditions (constant temperature, humidity, and 12-h dark–light cycle). All animal experiments were evaluated and approved by the Animal and Ethics Review Committee of Tokushima University. A human colon carcinoma cell line, DLD-1 was maintained in RPMI-1640 medium (Wako Pure Chemical) supplemented with 10% heat-inactivated fetal bovine serum (Japan Bioserum, Hiroshima, Japan), 100 units/ml penicillin, and 100 μg/ml streptomycin (ICN Biomedicals, Irvine, CA, USA) in a 5% CO2 incubator at 37 °C.

Chemically synthesized shRNAs

All shRNAs, chemically synthesized and purified by high performance liquid chromatography, were purchased from Hokkaido System Science (Sapporo, Japan). Two shRNAs were designed; one to target thymidylate synthase (shTS) and the other was a nonspecific (shCont, not to target any gene in human and mouse genome). The sequence of shRNA against TS (shTS) was 5'-GUA-CAACAAUGCUAGAUCAGUUGCUCCUGGUGUCAUGAAGGUG-3' and for a nonspecific shRNA (shCont) was 5'-UCUAAUAGCCGUUAAUGGCUA-GUGCUCCUGGUGUCGCUUAAGGCUAAGAU-3'. shRNAs were dissolved in RNase free TE buffer at a final concentration of 100 μmol/l.

In vitro gene knockdown by shRNA transfection

DLD-1 cells were seeded at a density of 500,000 cells/dish in a 10-cm dish 24 h before shRNA transfection. The cells were transfected with 1, 5 or 10 nM shTS or 10 nM shCont in Opti-MEM I using LF RNAiMAX according to the manufacturer’s recommended protocol. Seventy-two hours later, gene silencing of TS was examined by Western blotting, as described below. Treated cells were washed with chilled phosphate buffered saline (37 mmol/l) NaCl, 2.7 mmol/l KCl, 81 mmol/l Na2HPO4 and 1.47 mmol/l KH2PO4, pH 7.4 and were lysed in an NP-40 lysis buffer (50 μmol/l Tris–HCl, pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mmol/l NaCl, protease inhibitor cocktail (Sigma–Aldrich, St Louis, MO, USA)). The lysate was collected into a 1.5 ml Eppendorf tube and then centrifuged at 4 °C for 15 min at 15,000 × g. The protein concentration in the supernatant was determined using the Bio-Rad DC Protein Assay kit (Bio-Rad Laboratories, Hercules, CA, USA) with bovine serum albumin (Sigma–Aldrich) as a standard according to the manufacturer’s recommended instruction. Equivalent amounts of protein (20 μg) from each cell lysate were separated on a 12% SDS-PAGE gel (ATTO Corp., Tokyo, Japan) and transferred electrophoretically onto Hybond-ECL nitrocellulose membrane (GE Healthcare, Cleveland, OH, USA). The membrane was blocked with Tris-buffered saline containing 0.05% Tween 20 and 5% BSA for 1 h at room temperature and then incubated overnight at 4 °C with primary antibodies at appropriate concentrations (1:500 for mouse monoclonal anti-TS antibody (AnaSpec, San Jose, CA, USA) and 1:5000 for mouse monoclonal anti-human β-actin antibody (BioVision, Mountain View, CA, USA), respectively). β-actin was used as a loading control. After three washes with Tris-buffered saline containing 0.05%. Tween 20, membranes were incubated with horseradish peroxidase-conjugated goat anti-mouse secondary antibody (MP Biomedicalals, Solon, OH, USA) for 1 h at room temperature. Finally, membranes were processed for enhanced chemiluminescence using the ECL Plus Chemiluminescence Reagent (GE Healthcare UK, Little Chalfont, UK), and the obtained images were analyzed using LAS-4000 EPUVmini and Multi Gauge v3.2 (FujiFilm, Tokyo, Japan).

Real time-polymerase chain reaction (RT-PCR)

RNA isolation and cDNA synthesis were performed according to the manufacturer’s instructions. Briefly, cells were seeded in 48-well plates at a density of 10,000 cells/well 24 h before shRNA transfection. The cells were transfected with 5 nM shTS or shCont for 24 h as described above. At 72 h post-transfection, the total RNA of the DLD-1 cells was isolated using an RNAqueous-micro kit (Ambion, Austin, TX, USA). To conduct the reverse transcription reaction, 2 μl of RNA was converted to cDNA with a total volume of 20 μl, including 500 ng of Oligo(dT)20, 500 μM dNTPs, 1 μl of RNase inhibitor, and 1 μl of ReverTra Ace (Toyobo, Osaka, Japan). Real-time PCR was performed on a StepOnePlus real-time PCR system (Applied Biosystems, CA, USA) with a FastStart TaqMan Probe Master (ROX) and Universal ProbeLibrary (Roche Diagnostics GmbH, Manheim, Germany) according to the manufacturer’s instructions. Briefly, the PCR mixture was applied to a 96-well plate in a total volume of 20 μl, including 250 nM of probe, 900 nM forward and reverse primers, 2 μl of the generated cDNA, and 10 μl of FastStart TaqMan Probe Master (ROX). The TS primers and a probe were from the Assay-on-Demand gene expression assay mix (TS assay ID Hs00426591_m1, PCR product size 87 bp) (Applied Biosystems). The GAPDH primers and a probe for real-time RT-PCR were designed using ProbeFinder software (Roche Diagnostics GmbH). The GAPDH primers and the probe were as follows: GAPDH primers and probe (forward 5’-GTCCTGTCCTGGTCC-3’ and reverse 5’-ACGGACATCCCTGGC-3’, probe #60). The amplification conditions were as follows: 10 min at 95 °C followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The quantity was determined from the experimental threshold cycle on a standard curve of the data from a series of serial dilutions of the mixture of generated cDNA. The TS mRNA level was normalized with GAPDH as an endogenous control.

Cell viability assay

To determine the cytotoxicity of single-drug treatment or combined treatment of shTS and 5-FU, DLD-1 cells were seeded in 96-well plates at a density of 2000 cells/well 24 h before shRNA transfection. The cells were treated with 5 nM shTS or shCont for 24 h as described above. After transfection, the culture medium was replaced with fresh medium containing 5-FU (0.1 μg/ml). Following different incubation time intervals (24, 48, 72 or 96 h) at 37 °C, the cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, as described previously [19]. Briefly, cells were incubated with 50 μl MTT reagent (5 mg/ml in PBS) for 4 h at 37 °C. Then, 150 μl of isopropanol (0.04 N HCl in isopropanol) was added to each well to dissolve formazan crystals. The absorbance of each well was read at 570 nm on a microplate reader, Sunrise-R (TEKAN Japan, Kanagawa, Japan). Results were expressed as follows: Cell viability (% of control) = (A570 (treated group)/A570 (non-treated control group)) × 100 (%), where A570 (treated group) is the absorbance of the untransfected or transfected cells exposed to treatment with 5-FU and A570 (control group) is the absorbance of the non-treated cells. Data shown are representative of three independent experiments.
Preparation of cationic liposomes

Cationic liposome composed of DOPE:POPC:CHOL:DC-6-14 (3:2:3:2, molar ratio) was prepared as described previously [20]. The mean diameter and zeta potential for the cationic liposomes were 110.3 ± 16.4 nm and 24.9 ± 1.1 mV (n = 3), respectively, as determined with a NICOMP 370 HPL submicron particle analyzer (Particle Sizing System, CA, USA). The concentration of phospholipids was determined by colorimetric assay [21].

Preparation of PEG-coated shRNA-lipoplexes

For the preparation of shRNA/cationic lipid complex (shRNA-lipoplex), shRNA and cationic lipid were mixed at a molar ratio of 2000/1 (lipid/shRNA, molar ratio), and the mixture was vigorously vortexed for 10 min at room temperature to form shRNA-lipoplex. The mean diameter and zeta potential of shRNA-lipoplex were 395.2 ± 29.0 nm and 17.4 ± 1.7 mV (n = 3), respectively. For in vivo application, shRNA-lipoplex was surface-modified by polyethylene glycol (PEG)-conjugated lipid (PEGylation) using a post-insertion technique [22]. Briefly, mPEG-SG-DOPE (5 mol% of total lipid) in 9% sucrose solution was added to the shRNA-lipoplex solution, and the mixture was gently shaken for 1 h at 37 °C. The mean diameter and zeta potential of PEG-coated shRNA-lipoplex were 406.3 ± 20.1 nm and 15.7 ± 1.5 mV (n = 3), respectively. To detect the free-shRNA in the prepared PEG-coated shRNA-lipoplex, electrophoresis was performed on 2% agarose gel in 40 mM Tris–acetate/1 mM EDTA buffer and shRNA visualization is carried out using a UV transilluminator. No bands relating free shRNA were detected, indicating that virtually 100% of the shRNA was associated with and/or encapsulated in the PEG-coated shRNA-lipoplex under our lipoplex preparation.

In vivo tumor growth suppression by the combined treatment with PEG-coated shTS lipoplex and metronomic S-1 dosing

5-week-old male BALB/c nu/nu mice were inoculated subcutaneously at the flank region with DLD-1 cells (2 × 10⁶) in a volume of 100 μl PBS. At day 5 post tumor cell inoculation, when the tumor volume reached 50–60 mm³, the animals were randomly grouped (n = 6) into four groups: a control group treated with sucrose and three groups treated with either PEG-coated shTS lipoplex, metronomic S-1 dosing or a combination of PEG-coated shTS lipoplex plus metronomic S-1 dosing. The tumor volume was measured every three days using a caliper, and tumor volume was calculated using the formula: tumor volume = (length) × (width)² / 2 [19].

Initial studies were conducted to determine the optimal shRNA concentration for efficient TS gene silencing. As shown in Fig. 1A, the suppression of the expression of TS by shTS was concentration-dependent. Cells transfected with a low shTS concentration (1 nM) failed to induce complete suppression of TS expression. However, higher shTS concentrations (5 nM and 10 nM) were efficient in suppressing TS protein expression in DLD-1 cells. Transfection with a non-specific control shRNA (shCont), even at a higher concentration (10 nM), had no effect on the expression level of TS protein. To further confirm the specific gene silencing effect of shTS, the TS gene expression at the mRNA level was also examined after the transfection of shTS and shCont (Fig. 1B). Approximately more than 90% reduction in TS mRNA levels were observed in cells transfected with either 5 nM or 10 nM shTS, compared to that transfected with 10 nM shCont, confirming that the reduction observed in TS protein levels was caused by TS mRNA degradation. Since there was no remarkable difference in gene silencing effect between the highest two concentrations of shRNA (5 nM and 10 nM), subsequent in vitro experiments were performed at a shTS concentration of 5 nM.

Effect of TS silencing on chemosensitivity of DLD-1 cells to 5-FU

The TS protein is the main target molecule of 5-FU [24]. It has been reported that the expression of TS protein is highly related with chemosensitivity of tumor cells to 5-FU [13,25]. Therefore, to reveal whether TS gene knockdown will improve the chemosensitivity of DLD-1 cells to 5-FU, the DLD-1 cells were transfected with either shTS or shCont, and then treated with 5-FU (0.1 μg/ml, a minimum toxic concentration of 5-FU) for different time intervals (24, 48, 72 and 96 h). As shown in Fig. 2, as single agents, both 5-FU and shRNA against TS (shTS) exerted a moderate cytotoxic effect against DLD-1 cells in a time-dependent manner (cell growth inhibition (%) were 41.3 ± 3.4% and 53.5 ± 2.8%, respectively, at 96 h post incubation). Control shRNA (shCont) showed no effect on the cell viability of DLD-1 cells at any time point examined. A combination of shCont and 5-FU seems to have no additive effect on the cytotoxicity of 5-FU against DLD-1 cells. On the other hand, combined treatment with 5-FU and shTS substantially decreased DLD-1 cell viability in a time-dependent manner; with a maximal cellular growth inhibition (72.8 ± 1.7%) at 96 h post-incubation. These results suggest that down-regulation of TS expression sensitizes the cells to the cytotoxic effect of 5-FU, and thereby, significantly improves the chemosensitivity of DLD-1 colorectal cancer cells to 5-FU.

In vivo tumor growth suppressive effect by the combined treatment with shTS and S-1

Next, to examine whether combination of shTS with 5-FU might be an effective strategy for the treatment of colorectal cancer, the tumor growth inhibitory effect of either metronomic S-1 dosing, an orally prodrug of 5-FU, or shTS alone or a combined treatment of shTS and metronomic S-1 dosing was evaluated. As shown in Fig. 3, a significant reduction in the tumor growth was observed in DLD-1 bearing nude mice treated with shTS alone or in combination with metronomic S-1 dosing compared with that in control group (Fig. 3A and B). When compared with shTS or S-1 alone, the combined treatment of shTS and S-1 strongly suppressed tumor growth in DLD-1 xenograft model; the tumor growth inhibition rate (TGI (%)) was 55.4% which was significantly higher than that of S-1 or shTS-treated groups (TGI (%)) were 33.5% and 33.9%, respectively). Throughout the therapeutic experiment, no body weight loss was observed in any of the treated groups (Fig. 3C). These results suggest that combined treatment with shTS and metronomic S-1 dosing significantly augmented the antitumor efficacy of 5-FU derived from S-1 in human colorectal tumor-bearing mice without causing remarkable toxicity.

Apoptosis induction in tumor tissue following combined treatment with PEG-coated shTS lipoplex and metronomic S-1 dosing

To verify whether the growth inhibition of implanted DLD-1 tumors by treatment with shTS alone or in combination with
metronomic S-1 dosing was related with apoptosis induction in DLD-1 tumor tissue, the percent of apoptotic cells within tumor tissues was identified by TUNEL assay following treatments (Fig. 4A and B). The number of TUNEL-positive cells in the tumor treated with the combination of shTS and S-1 was significantly higher than those in the tumor treated with either shTS or S-1 alone (apoptotic index was $35.2 \pm 4.7$ versus $21.3 \pm 2.2$ and $20.1 \pm 1.7$, respectively) (Fig. 4B). These results emphasize that apoptosis induced by the combined treatment substantially contributes to the augmented antitumor efficacy of the combined treatment in DLD-1 xenograft model.

Discussion

Thymidylate synthase (TS), a significant gene in cellular proliferation, is an important target in cancer chemotherapy due to its crucial role in DNA precursor synthesis and repair [26]. Not only is TS a mainstay target of many currently used chemotherapeutic agents such as the fluoropyrimidine including S-1 [27] and the antifolate drugs; raltitrexed and pemetrexed [28], but it is also the target for developing antisense therapies [29] to enhance the chemosensitivity to those drugs.

Fig. 1. TS gene silencing by chemically synthesized shTS in human colorectal DLD-1 cell line. Expression of TS protein (A) and mRNA (B) were detected by western blot and qRT-PCR analyses, respectively. DLD-1 cells were transfected with either 10 nM shCont or 1, 5 or 10 nM shTS using Li RNAiMAX. At 72 h post-transfection, protein or RNA was extracted from the cells and analyzed as described in the Materials and methods section. Data represent the mean ± S.D from three independent experiments. ***p < 0.005 compared with shCont-transfected cells.
In most human cancers including colorectal cancer, TS expression is reported to promote tumor growth [15]. In addition, TS mRNA and protein expression levels strongly predict the outcome with 5-FU-based therapeutic regimens [30,31]. Thus, the down-regulation of TS may be a promising approach to improve the therapeutic efficacy of TS targeting chemotherapeutic agents. In this study, using the RNAi strategy with TS-targeted chemically synthesized shRNA (shTS), we demonstrated that shTS could effectively inhibit the growth of implanted DLD-1 cells in nude mice (Fig. 3A and B). Such potent tumor growth inhibitory effect was strongly related to in vitro down-regulation of TS mRNA and protein expression (Fig. 1A and B) and induction of in vivo DLD-1 cell apoptosis (Fig. 4A and B). Interestingly, when combined with 5-FU, shTS sensitized the DLD-1 cells to the cytotoxic effect of 5-FU in vitro (Fig. 2) and augmented the antitumor efficacy of S-1, an oral produg of 5-FU, in xenograft model (Fig. 3A and B) via the induction of a potent apoptotic response in DLD-1 tumors (Fig. 4A and B). These results suggest that combination of shRNA targeting TS and S-1 may be a promising approach in the treatment of advanced colorectal cancer.

Effectively treating patients with advanced CRC is not an easy task. Conventional chemotherapy given at maximal tolerated doses (MTD) results in modest control in cancer progression [32,33]. Instead, metronomic chemotherapy, describes the close, regular administration of chemotherapy drugs at less-toxic doses over prolonged periods of time, has been recently emerged as an alternative therapeutic option to conventional chemotherapy [34,35]. Metronomic chemotherapy holds much promise to circumvent several of the major pitfalls of MTD regimens. A lower incidence of drug resistance, restoration of anti-tumor immunity, reduced toxicity, better quality of life, and/or a lower financial burden for patients, collectively exalts the therapeutic potential of metronomic chemotherapy compared with MTD-administered conventional chemotherapies [36–38]. It is unfortunate, however, that metronomic chemotherapy, as the sole therapeutic modality, has exhibited dismal therapeutic efficacy in many preclinical/clinical settings. Therefore, the introduction of new therapeutic strategies is potentially urged to further improve the therapeutic outcome of chemotherapy for colorectal cancer. Our strategy using RNAi would be an emerging therapeutic option to achieve good therapeutic outcome with metronomic 5-FU-based treapeutics in patients with advanced CRC.

RNAi technology has been developed as a potential therapeutic strategy for several diseases, including cancer [39]. Many chemically synthesized RNAi molecules [siRNA, oligodeoxynucleotides (ODNs), microRNA, and short-hairpin RNA] have emerged as agents for treating human disease [40]. RNAi can inhibit translation of specific mRNAs and/or target those mRNAs for destruction by sequence-specific cleavage and degradation [41]. Nevertheless, the potential role of RNAi molecules as therapeutic molecules has been limited due to issues relating to cellular uptake, long-term stability and specificity of in vivo delivery into target tissue [42]. Recently, Kadota et al. [25] have reported that the combined treatment with the adenoviral vector expressing shRNA targeting TS and 5-FU-derived agent S-1 showed a potent antitumor activity against 5-FU-resistant tumor xenografts upon the intratumor injection of the adenoviral vector. Besides the immunological problems and the limitations in the size of the carried therapeutic gene encountered with the use of viral vectors [42], systemic administration of adenoviral vector expressing shRNA targeting TS failed to exert any therapeutic benefit against 5-FU-resistant tumor xenografts probably through the inefficient in vivo delivery of genetic material to

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**Fig. 2.** Effect of combined treatment of shTS and 5-FU on the viability of DLD-1 cells in vitro. DLD-1 cells were transfected or not with 5 nM of shCont or shTS. At 24 h post-transfection, fresh medium containing 0.1 µg/ml 5-FU was added and the cells were further incubated for different time intervals (24, 48, 72 or 69 h). After each indicated incubation time, cell viability was measured by MTT assay. Data represent the mean ± S.D from three independent experiments. ***p < 0.005 versus shTS- or 5-FU-treated cells.
Fig. 3. Antitumor efficacy of combined treatment of PEG-coated shTS lipoplex and S-1 in human colorectal DLD-1 xenograft model. On days 5, 7, 9, 11, 13 and 15 post tumor inoculation, PEG-coated shTS lipoplex (80 μg shRNA/mouse per injection) was intravenously injected into mice bearing DLD-1 tumors. Metronomic S-1 dosing (tegafur 6.9 mg/kg) was orally administered daily from day 5–19 post tumor inoculation. For the control group, 9% sucrose solution was administered instead of S-1 or PEG-coated shTS lipoplex. (A) Antitumor activity as assessed by determining the tumor size. Data represents the average of tumor volume in each group (n = 6) ± S.D. *p < 0.05 versus PEG-coated shTS lipoplex- or S-1-treated group, ***p < 0.001 versus control. (B) A photograph of DLD-1 xenograft tumors taken 48 h after the end of treatment. A representative mouse per treatment group shows significant reduction of the tumor sizes that received a combined treatment of shTS and S-1 compared to single treatment with either shTS or S-1 alone. (C) Body weight change during the therapeutic experiments.
Fig. 4. Apoptosis in the tumor tissue induced by the combined treatment of PEG-coated shTS lipoplex and S-1. Mice bearing DLD-1 tumors were treated in the same way as described in legend of Fig. 3. At 48 h post the last treatment, mice were euthanized, and tumors were excised, snap frozen, and examined for apoptosis. (A) Representative immunofluorescent tumor sections (TUNEL: Red, Hoechst 33342: Blue). The bar indicates 100 μm. Original magnification, ×200. (B) Percent of TUNEL-positive cells in the section. Data represent the mean ± S.D. *p < 0.05.
tumor tissue. In this study, however, chemically synthesized shRNA conferred the specificity against the designated (TS) gene, indicated by the potent gene silencing effect (Fig. 1), while minimizing the off-target effect as indicated by the absence of remarkable toxicity or the loss of body weight during treatment (Fig. 3C). Furthermore, the non viral vector used, PEG-coated cationic liposome, was efficient in delivering the chemically synthesized shRNA to the tumor tissue following systemic administration as ascribed by the potent antitumor efficacy (Fig. 3A and B) and the remarkable apoptotic potential (Fig. 4A and B) induced by the combined treatment of S-1 and shTS-lipoplex. These lead an assumption that the combined treatment of shTS-lipoplex with S-1 has effective antitumor activity against 5-FU-resistant colorectal tumors. Further investigations are in progress to verify the assumption in our laboratory.

Tumor priming with metronomic chemotherapy have been reported to enhance the intratumor delivery and distribution of drugs and/or macromolecules including drug delivery nanocarriers. Chen and co-workers [43] have recently reported that tumor priming with metronomic paclitaxel could induce tumor vasculature normalization, as evidenced by the increased coverage of the pericytes and basement membranes of endothelial cells, by the enhancement of PO2 and vascular perfusion, and by decreases in the interstitial fluid pressure. Such tumor vasculature normalization efficiently enhanced systemic delivery and the resultant antitumor efficacy of a conventional chemotherapeutic agent, doxorubicin, administered during the induced normalization window. More recently, we have verified that tumor priming with metronomic S-1 dosing could induce a potent apoptotic response against both tumor endothelial cells and tumor cells adjacent to tumor angiogenic vessels as well, resulting in enhanced blood flow via transient normalization of tumor vasculature in tandem with alleviating the mechanical compression exerted on tumor blood vessels. Such alteration in the tumor microenvironment imparted by metronomic S-1 dosing was acknowledged for the enhanced intratumor delivery of PEGylated nanocarrier systems following their systemic administration [44]. Therefore, based on these recent studies, it is legitimate to account the superior antitumor effect of the combined treatment of shTS lipoplex and S-1 dosing to the metronomic S-1-mediated tumor priming effect which ensures the efficient in vivo delivery of PEG-coated shRNA lipoplexes to tumor tissue. Such enhanced intratumor delivery and/or accumulation of PEG-coated shTS lipoplexes is confirmed to sensitize the tumor cells to the cytotoxic effect of 5-FU derived from S-1 (Figs. 2 and 3) via down-regulating the expression of TS gene (Fig. 1), and consequently, resulted in an enhanced antitumor efficacy, as manifested by the significant reduction in tumor growth (Fig. 3) and the potent induction of apoptosis within tumor tissues (Fig. 4).

Conclusion

The present study demonstrated that RNAi targeted specifically against TS resulted in gene silencing with high efficacy in human DLD-1 colorectal cancer cells, leading to the inhibition of cell proliferation in vitro and in vivo, when combined with 5-FU and 5-FU prodrug S-1. Our results suggest that TS silencing may be a promising strategy to enhance the chemosensitivity of human CRC cells to 5-FU-based chemotherapeutic regimens and the combined treatment using shTS and metronomic S-1 dosing may constitute an effective therapeutic option for advanced human CRC.

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Abbreviations

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<td>3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
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Conflicts of interest

The authors report no conflicts of interest in this work.

References


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<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
<td></td>
</tr>
<tr>
<td>POPC</td>
<td>1-palmitoyl-2-oleoyl phosphatidylcholine</td>
<td></td>
</tr>
<tr>
<td>TS</td>
<td>thymidylate synthase</td>
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<tr>
<td>UFT</td>
<td>tegafur</td>
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<td>mPEG2000-DSE</td>
<td>1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-n-[methoxy(polyethylene glycol)-2000]</td>
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Conflicts of interest

The authors report no conflicts of interest in this work.

References


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