Abstract. Lymphatic metastasis plays a critical role in ovarian cancer, indicates poor prognoses and correlates to the majority of cancer deaths. Camptothecin derivatives exhibit promising activity for the treatment of solid tumors because of its specific inhibition of eukaryotic DNA topoisomerase I. Yet, its application is hindered due to extreme water insolubility and severe side effects. It is essential to establish an efficient and safe protocol for the administration of camptothecin versus tumor metastasis and growth. In the current research, we encapsulated camptothecin with N-trimethyl chitosan (CPT-TMC) to increase its water-solubility and lower its side effects, and tested it on a high potential lymphogenous metastatic model of human ovarian cancer. In the prophase study, we successfully transfected SKOV3 cells with VEGF-D recombinant plasmid DNA (pcDNA3.1(+)/VEGF-D) to construct a cell line named SKOV3/VEGF-D and establish a feasible lymphogenous metastatic model of human ovarian cancer. In the prophase study, we successfully transfected SKOV3 cells with VEGF-D recombinant plasmid DNA (pcDNA3.1(+)/VEGF-D) to construct a cell line named SKOV3/VEGF-D and establish a feasible lymphogenous metastatic model. The antitumor and antimetastatic activities of CPT-TMC were evaluated in nude mice subcutaneously inoculated with SKOV3/VEGF-D cells at the left hindlimb claw pad. The tumor-bearing mice were divided randomly into four groups and treated twice per week for three weeks. Evan’s Blue Dye was used to delineate functional lymphatic vessels. Lymphatic metastasis rates were detected by hematoxylin and eosin (HE) staining. Expression of VEGF-D and MMP-9 were investigated by immunohistochemistry. In contrast to controls, administration of CPT-TMC achieved effective inhibition in primary tumor volume and lymphogenous metastasis, yet without apparent systemic toxic effects. These effects were associated with simultaneously down-regulated VEGF-D and MMP-9 expression, significantly decreased tumor-associated lymphatic and blood sprouts, tremendously reduced systemic toxic effects, dramatically increased tumor apoptotic index. Our data indicate that CPT-TMC is superior to CPT by maximizing its anticancer and antimetastatic activities with minimal toxicity on hosts. CPT-TMC may become a potentially therapeutic strategy against human advanced ovarian cancer.

Introduction
Ovarian cancer accounts for the highest mortality rate among gynecological malignancies, making it the greatest threat to the health of women. Tumor cells spread to local lymph nodes, as an important indicator of tumor aggressiveness, is often an early event in metastatic disease (1). Metastasis via the lymphatic vessels plays a critical role for dissemination of tumor cells to sentinel lymph nodes in ovarian cancer, and indicates poor prognoses (2).

Although most primary tumors can be surgically removed, high incidence of lymphogenous metastasis and recurrence are still considerable aspects of ovarian cancer patients. Recent experimental evidence recognized that lymphangiogenic growth factor, especially the vascular endothelial growth factor-D (VEGF-D), which is thought to be involved in inducing tumor-associated lymphangiogenesis (3), could promote cancer cells spread to regional lymph nodes (4-7). In the prophase of the study, we successfully transfected the human ovarian serous cystadenocarcinoma SKOV3 cells with recombinant plasmid pcDNA3.1(+)/ VEGF-D (SKOV3/VEGF-D) to establish a feasible high lymphogenous metastatic mouse model, and aimed to introduce it to experimental therapy research.

Even in advanced stages, ovarian cancer is still sensitive to a variety of chemotherapeutics. This highlights the need to
develop reliable and efficacious chemotherapies that can inhibit metastasis and prolong survival time with minimal toxicity to patients.

Camptothecin, a pentacyclic cytotoxic alkaloid, and a novel effective anticancer drug originally isolated from Camptotheca acuminate Decne in 1966 (8), is believed to act by stabilizing the topoisomerase I-induced DNA single strand break, so as to prevent religation (9). As a strong inhibitor of the DNA-replicating enzyme topoisomerase I (10), camptothecin has many novel activities, such as anticancer, antiproliferative effects and induces cell apoptosis in a large amount of cancer cell lines, especially in human xenografts of colon, lung, breast, ovarian, and melanoma cancers (11-14). Additionally, camptothecin also showed promising potential of antimetastasis in a broad spectrum of cancers, such as melanoma, ovarian cancer, colon, pancreatic cancer (15-18). These inspiring data suggested that camptothecin could be developed as a promising therapeutic impact on cancer progression and metastasis.

However, the extreme water insolubility and severe side effects hampered the application of camptothecin. In order to conquer the problems, our laboratory used N-trimethyl chitosan, a promising drug vector, to encapsulate camptothecin and named it as CPT-TMC. The present study was aimed to evaluate whether camptothecin could overcome tumor progression and lymphogenous metastasis in ovarian cancer while we addressed its disadvantages by encapsulating it in a suitable nanocarrier named N-trimethyl chitosan, which increased the drug solubility and minimized its systemic toxic effects.

In this study, CPT-TMC provided tremendous advantages in antitumor and anti-lymphatic metastasis in terms of significantly enhanced the inhibition in tumor growths and lymphatic metastases, down-regulated the expression of VEGF-D and reduced toxic side effects. These data demonstrate that CPT-TMC may be an efficient and safe protocol for human advanced ovarian cancer.

Materials and methods

Preparation of SKOV3/VEGF-D ovarian cancer cell line. The SKOV3 cells, obtained from the American Type Culture Collection (ATCC, Manassas, VA), were seeded in 6-well plates (1x10⁵/well) and incubated at 37°C in 5% CO₂ overnight. Then cells were transfected with recombinant plasmid pcDNA3.1(+)/VEGF-D (obtained from the Division of Cancer Biotherapy in our laboratory) by lipofectamin 2000 reagent (Invitrogen). Transfected cells were then maintained in cell culture medium RPMI-1640 (Gibco) containing 10% fetal bovine serum and 400 μg/ml G418 for three weeks, followed by G418 antibiotic resistance selection. After screening, the stably transfected cell clones (SKOV3/VEGF-D) were obtained.

RT-PCR. RT-PCR was employed to verify the stable expression of VEGF-D in tumor cells. Total RNA was extracted from 1x10⁶ tumor cells (SKOV3 cells, SKOV3 cells transfected with empty plasmid, SKOV3 cells transfected with recombinant plasmid pcDNA3.1(+)/VEGF-D, which named as SKOV3, SKOV3/pcDNA, and SKOV3/VEGF-D, respectively), performed as the introduction of TRizol (Invitrogen) and detected by spectrophotometric analysis. The following specific primer pairs were used: 5'-GC AAG CTT ATG TAT GGA GGA TGG GGA ATG C-3' (VEGFDFOR) and 5'-GC AAG CTT ATG TAT GGA GGA TGG GGA ATG-3' (VEGFDFREV) (amplifies nucleotides 283-1359 of mouse VEGF-D, GenBank accession number NM-010216 GI: 6753873).

Tumor models. The research protocol was reviewed and approved by the Institutional Animal Care and Treatment Committee of Sichuan University. Female athymic BALB/c nude mice, 6-8-week old, were maintained in pathogen-free conditions and fed sterile chow. Before inoculation, mice were randomly assigned into three groups (six per group). Three kinds of cells (SKOV3, SKOV3/pcDNA, SKOV3/VEGF-D) were inoculated subcutaneously into the hind left footpad of recipients (2x10⁶ cells resuspended in 50 μl of PBS), respectively. Tumor growth and mice behavior or physical appearances were evaluated consecutively for two months.

Preparation of CPT-TMC. CPT-TMC was prepared by combination of microprecipitation and sonication. Briefly, 6 mg/ml of camptothecin was first prepared by dissolving 30 mg camptothecin into 5 ml dimethyl sulfoxide (DMSO) solution. Then TMC was dissolved in water solution at the concentration of 5 mg/ml. Subsequently, 0.1 ml of camptothecin solution was added dropwisely into 2 ml of TMC solution at 4°C. The obtained colloid solution was ultrasonicated for 10 min keeping the temperature at 4°C. Finally, the colloid solution was dialyzed against water using a membrane with a molecular weight cut-off of 8000-14000 (Solarbio, China) for 3 days, and then the solution was centrifuged at 10000 x g for 10 min to remove insoluble camptothecin.

Cytotoxicity assay in vitro. The cytotoxicity of CPT-TMC on SKOV3/VEGF-D cells was determined by MTT assay; and the absorbance at 490 nm was measured by using a multi-well plate reader (BioTek Instruments, Winooski, VT, USA). Effects of CPT-TMC, CPT and TMC on SKOV3/VEGF-D cell viability were assessed as percent cell viability in terms of media-only treated control cells (% of control).

Treatment for high lymphogenous metastasis human ovarian cancer model. SKOV3/VEGF-D cells (2x10⁶ cells resuspended in 50 μl of PBS) were inoculated subcutaneously into the hind left footpad of recipients, which would later receive the following treatments. Before treatment, the tumor-bearing mice were randomly divided into 4 groups (5 per group) when tumors were palpable: i) CPT-TMC (2.5 mg/kg); ii) CPT (2.5 mg/kg); iii) TMC (25 mg/kg); and iv) 0.9% NS. Treatment started 7 days after tumor cell challenged and administration via i.v. performed twice per week for 3 weeks. Tumor growth was monitored every 4 days and tumor volume was calculated as 0.52 x length x width². Lymphangiography and lymphogenous metastasis. When tumors grew to >1 cm in diameter, Evan’s blue dye (5 mg/ml in PBS) was infused intradermally into the tumor area after anesthesia to visualize the functional lymphatic vessels inside
and around the tumor masses (19). The lymphatic vessels specifically take up Evan's blue dye, whereas the blood vessels do not (20,21). Multistep metastasis to LNs were evaluated with multilevel serial sections by hematoxylin and eosin (HE) staining and examined under microscope 8 weeks post tumor inoculation. The metastasis rates of LNs were assessed as the number of LNs with metastases out of the total number of LNs.

**Immunohistochemistry staining.** Sections of 3-5 μm thick were first deparaffinized and rehydrated. Then antigen retrieval was carried out by heating at 120˚C in 10 mM citrate buffer solution (pH 6.0). Endogenous peroxidase activity was blocked by 3% H2O2 and non-specific binding of reagents was quenched by 10% normal goat or rabbit serum. Then sections were incubated with primary antibodies, followed by incubating with biotinylated rabbit anti-goat/rat IgG and then streptavidin-biotin-horseradish peroxidase complex successively.

**Quantification of lymphangiogenic and angiogenic micro-vessel density**. To determine the lymphatic microvessel density (LMVD) and angiogenic microvessel density (MVD), sections were first scanned under a light microscope with low power magnification to identify the areas with the highest number of lymphatic or angiogenic vessels. Vessels within five high-power fields of these areas, with a clearly defined lumen or well defined linear vessel shape, were taken into account for lymphatic or blood microvessel and an average count was recorded.

**Apoptotic analysis.** Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) was performed to analyse the inducible effect on apoptosis by CPT-TMC according to the manufacturer’s protocol (Promega). Pyknotic nuclei stained with dark green, as viewed by fluorescence microscopy, were thought to be TUNEL-positive nuclei. Percent apoptosis was determined by counting the number of TUNEL-positive cells and dividing by the total number of cells in the field (five high-power fields/slide).

**Toxicology analysis.** Relevant indexes such as weight loss, anorexia, diarrhea, skin ulceration, ruffled fur or toxic death were observed consecutively for >2 months to evaluate the potential side effects and toxicity of CPT-TMC. Sections of various organs (heart, liver, spleen, lung, and kidney) were detected by HE staining under microscope.

**Statistical analysis.** Numerical values are described as means ± one standard error (SE). One-way analysis of variance (ANOVA) was used to determine statistical significance in comparisons of experimental data among different groups. Differences were considered significant at P<0.05.

**Results**

**Stable expression of VEGF-D in SKOV3/VEGF-D cell lines.** Expression of VEGF-D in SKOV3, SKOV3/pcDNA, and SKOV3/VEGF-D cells was expanded and analysed by RT-PCR, respectively. Strong positive expression of VEGF-D was detected in SKOV3/VEGF-D cells versus the two controls (SKOV3, 25.2±10.7%; SKOV3/pcDNA, 23.7±10.9%; SKOV3/VEGF-D, 86.1±13.7%) two months after tumor cell challenge.

**VEGF-D promoted tumor growth and lymphangiogenesis.** Significant differences in the size of primary tumor were observed in SKOV3/VEGF-D group versus control groups (P<0.05) from day 25. LMVD was evaluated by LYVE-1 staining. SKOV3/VEGF-D group displayed significant increase in LMVD (104.92±17.95), compared with SKOV3/pcDNA (33.71±10.29) and SKOV3 (27.64±8.37) groups.

**Anti-proliferation by CPT-TMC in vitro.** MTT assay revealed that CPT-TMC and CPT resulted in significant reduction in cell viability of SKOV3/VEGF-D cells compared with TMC-treated and media-only treated cells (P<0.05). No significant difference was observed between CPT-TMC and CPT, or
between TMC-treated and media-only treated cells (P>0.05 Fig. 1B).

**CPT-TMC inhibited tumor growth in vivo.** Treatment with CPT-TMC significantly inhibited the primary tumor growth in comparison with controls (P<0.05, Fig. 2). Complete tumor regression occurred in one of the five mice administrated with CPT-TMC. CPT-TMC resulted in >70% inhibition in tumor volume compared with NS and TMC groups two months after tumor inoculation. No significant difference was observed between TMC and NS groups (P>0.05). Statistical significance was observed between CPT-TMC and CPT groups in the ultimate stage of this study.

**Lymphangiography and reduction of lymphogenous metastasis.** The intravital peritumoral lymphangiogram...
exhibited abundant dilated lymphatic vessels with sprout clumps observed in NS, TMC and CPT groups, yet seldom in CPT-TMC group. Consecutive histopathological analysis of LNs showed that metastasis were found in all group two months after the tumor cell challenge, but CPT-TMC significantly suppressed the progression of metastases into sentinel lymph nodes versus NS and TMC controls (P<0.05, Fig. 3A and B). However, no statistical significance was found between CPT-TMC and CPT groups.

CPT-TMC inhibited VEGF-D expression and lymphangiogenesis. Immunohistochemistry showed that distinct clusters of VEGF-D-positive cells presented in the tumor tissues of different controls, which exhibited extensive invasion and metastases to remote multistep LNs and organs, yet only a few could be identified in CPT-TMC group (Fig. 3A). LYVE-1 staining exhibited that plentiful nascent lymphatic vessels were identified in NS and TMC groups, yet seldom in CPT-TMC group (Fig. 3A). CPT suppressed the expression of VEGF-D and LYVE-1 to some extent. The most significant reduction in VEGF-D expression and LMVD occurred in the CPT-TMC group compared with the other three groups (P<0.05, Fig. 3C). No significant difference was found between TMC and NS groups (P>0.05).

CPT-TMC suppressed angiogenesis and down-regulated MMP-9 expression. MVD was examined by CD31 staining. Numerous newborn blood capillaries could be observed in

Figure 4. (A) Top panel, CD31 staining revealed plentiful nascent microvessels in control groups, but few in CPT-TMC group. Upper-middle panel, MMP-9-positive cells were rich in NS and TMC groups, yet not in CPT-TMC group. CPT reduced the expression to some extent. Lower-middle panel, many KI-67-positive cells were identified in NS and TMC groups compared to CPT and CPT-TMC groups. Bottom panel, TUNEL assay showed only a few positive nuclei in tumor tissues of controls, but plentiful in CPT-TMC group. (B) CPT-TMC significantly reduced CD31-positive microvessels compared with the controls. (C) Percentages of KI-67-positive nuclei showed tremendous reduction occurred in CPT-TMC group. (D) CPT-TMC markedly increased the percent apoptosis versus controls. Data are presented as mean ± SE. (*P<0.05; **P<0.01).
control groups, yet only a few CD31-positive staining appeared in CPT-TMC group (Fig. 4A and B). Matrix metalloproteinase-9 (MMP-9)-positive cells which were originally implicated in cancer progression, invasion and metastasis, were identified in the tumor tissues of different controls, yet only a few in CPT-TMC group (Fig. 4A).

**CPT-TMC inhibited proliferation and increased apoptosis in vivo.** Ki-67 staining indicated that dramatically reduction occurred in CPT-TMC-treated group compared with controls (P<0.05, Fig. 4A and C). The number of Ki-67-positive cancer cell nuclei was counted as a ratio of immunoreactive positive cells to the total number of cells counted. TUNEL assay revealed many strongly TUNEL-positive nuclei in CPT-TMC-treated tumor tissues, yet rarely in control groups (Fig. 4A). Apoptotic indexes showed only CPT-TMC therapy resulted in a significant increment of apoptotic index versus controls (P<0.01, Fig. 4D).

**Toxicity observation.** Weight of mice was monitored every 4 days and considered as a parameter for evaluation of physical status, anorexia, or cachexia, and there was no evidence of toxicity in terms of body weight loss in CPT-TMC group. Furthermore, HE staining of the heart, liver, spleen, lung, kidney, intestine and bone marrow did not reveal any significant difference among CPT-TMC-treated group and control groups.

**Discussion**

Ovarian cancer causes the most cancer-related death in gynecological malignancies (22), owing to its high incidence of lymphogenous metastasis and recurrence, as well as the severe toxic side effects of available chemotherapeutic agents. Besides the well-informed blood vessels, lymphatic vascular system provides another suitable pathway for the spread of cancer cells (2). Multiple lines of evidence have demonstrated that cancer cells spread to local lymph nodes, as a novel prognostic parameter for the metastatic risk, occurring in the early stage of the progression in many types of human neoplasms (23).

Although lymphatic vessels are important for tumor dissemination, little is known on the major molecular mechanisms of how lymphangiogenesis facilitates metastasis. Recent research indicates that VEGF-D, as one of the strongest agents promoting lymphangiogenesis and metastasis spreading of tumor cells to lymph node (24), is thought to be an important prognostic factor predicting short survival (25,26). Many studies showed that cancer cells may exploit the vascular systems by spontaneously expressing protein growth factors, such as VEGF-C and VEGF-D, the best characterized vascular growth factors which can alter the normal pattern of angiogenesis, increase vascular leakage and drive lymphangiogenesis thus creating conduits for facilitating tumor metastasis (27). In the prophase of the current study, we successfully transfected SKOV3 cells with recombinant plasmid pcDNA3.1(+) /VEGF-D and injected them subcutaneously into the hind left footpad of recipients to establish a high lymphogenous metastasis mouse model of human ovarian cancer. We observed that VEGF-D could dramatically promote tumor growth, induce lymphangiogenesis, and increase lymphatic metastasis. Because of these encouraging data, we used this promising high lymphatic metastatic mouse model of human ovarian cancer in our research.

Lymphogenous metastasis, recurrence and severe side effects of chemotherapy lead to low 5-year survival (28) and became the major obstacle to clinical treatment in ovarian cancer. However, even in advanced stages, ovarian cancer is still sensitive to a variety of chemotherapeutics. This highlights the need to develop reliable, low toxicity and efficacious chemotherapies against lymphatic metastasis to prolong survival.

We selected camptothecin, a specific inhibitor of the DNA-replicating enzyme topoisomerase I (10), as the model drug for this study. It has multiple biological activities as described above. However, the extreme water insolubility and unpredictable side effects, as the major drawbacks of camptothecin, hindered its application in cancer therapy. The pharmacologically important lactone ring of camptothecin and its analogs is apt to bind albumin when in the presence of human serum albumin, resulting in conversion of the active drug to the inactive carboxylate form (29). These disadvantages impose severe pharmacokinetic limitation on the systemic use of camptothecin and related compounds. To solve these problems, an advisable drug delivery system is urgently needed to optimize its beneficial therapeutic efficacy and limit the disadvantages.

Chitosan, known as a natural aminopolysaccharide obtained by hydrolysis of chitin, has attracted much attention as a potential pharmaceutical excipient for drug delivery. It has favorable biological properties, such as safe, effective, biocompatibility, biodegradability, homogeneity, positive charge, non-toxicity, and bioadhesivity (30,31). However, this polymer is only soluble in acidic environments with pH value lower than 6.0. Therefore, we chose a water-soluble quaternized cationic chitosan derivative, N-trimethyl chitosan chloride (TMC), which maintained all the advantages of chitosan and can be soluble in the entire pH range. Many studies proved that TMC could facilitate the paracellular transport of drugs by effectively opening the intercellular tight junctions (32). These attributes have so far been beyond the reach of any other type of cell vehicles. This non-absorbable and non-toxic polymer represents an attractive technology platform for favoring the uptake and retention of camptothecin in cancer. We used this feasible drug carrier to encapsulate camptothecin and named it as CPT-TMC, aimed to pave the way to improve the security of practical application of camptothecin.

In the current study, we tested CPT-TMC on a high potential lymphatic metastasis model of ovarian cancer. CPT-TMC showed tremendous inhibition of advanced tumor growth and metastasis to multistep LNs yet without systemic toxic effects, by contrast, CPT, TMC and NS groups lack these attributes. Complete tumor regression occurred in one of the five mice administered with CPT-TMC. Emerging evidence shows that MMP-9 was associated with cancer invasion and progression (33,34). In this study, quantitative histomorphometric analysis showed both VEGF-D-positive and MMP-9-positive expression in tumor tissues were
remarkably down-regulated by CPT-TMC. Immunohistochemistry staining of tumor tissues with LYVE-1, CD31 and Ki-67 revealed that LMVD, MVD and Ki-67 positive cells were significantly reduced in CPT-TMC-treated group in contrast to other groups (P<0.05). These delectable results indicated that overexpression of VEGF-D increased the lymphatic sprouts and the potential of lymphogenous metastasis, yet down-regulated its expression could remarkably inhibit lymphangiogenesis and tumor growth. TUNEL assay showed significant increment of apoptotic index by CPT-TMC (P<0.05). With the drug administered in this dose and schedule, the histopathology of animal tissues revealed no obvious systemic toxicity. These results strongly suggest that CPT-TMC may successfully overcome lymphogenous metastasis in ovarian cancer.

MITT assay was carried out to confirm the toxicity effect of CPT-TMC on SKOV3/VEGF-D cells in vitro. The results showed that both CPT-TMC and CPT could significantly induce growth inhibition in SKOV3/VEGF-D cells, since the TMC itself (without camptothecin) produced no cytoxicity according to the MITT assay. Our findings were in line with the anti-proliferative ability of camptothecin reported before (12).

In conclusion, our investigation suggested that CPT-TMC had displayed not only local anti-tumor ability but also strongly anti-lymphangiogenesis and anti-angiogenesis effects with low toxicity on the high lymphogenous metastatic model of ovarian cancer. CPT-TMC should be further investigated to pave the way for a new powerful therapeutic weapon against advanced human ovarian malignancies, particularly for patients with lymphogenous metastasis.

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