

RESEARCH ARTICLE

Targeted Efficacy of Dihydroartemisinin for Translationally Controlled Protein Expression in a Lung Cancer Model

Lian-Ke Liu¹, Heng-Fang Wu², Zhi-Rui Guo², Xiang-Jian Chen², Di Yang², Yong-Qian Shu¹, Ji-Nan Zhang^{2*}

Abstract

Objective: Lung cancer is one of the malignant tumors with greatest morbidity and mortality around the world. The keys to targeted therapy are discovery of lung cancer biomarkers to facilitate improvement of survival and quality of life for the patients with lung cancer. Translationally controlled tumor protein (TCTP) is one of the most overexpressed proteins in human lung cancer cells by comparison to the normal cells, suggesting that it might be a good biomarker for lung cancer. **Materials and Methods:** In the present study, the targeted efficacy of dihydroartemisinin (DHA) on TCTP expression in the A549 lung cancer cell model was explored. **Results and Conclusions:** DHA could inhibit A549 lung cancer cell proliferation, and simultaneously up-regulate the expression of TCTP mRNA, but down-regulate its protein expression in A549 cells. In addition, it promoted TCTP protein secretion. Therefore, TCTP might be used as a potential biomarker and therapeutic target for non-small cell lung cancers.

Keywords: Translationally controlled tumor protein - NCSLC - dihydroartemisinin - biomarker - targeted therapy

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Introduction

Lung cancer, one of the malignant tumors, has high morbidity and mortality around the world, and its 5-year survival rate is only 8%-15% (Molina et al., 2006; Al-Hashimi et al., 2014; Cui et al., 2014). In addition, the incidence of lung cancer has been rising steadily (Jemal et al., 2011; Fathallah et al., 2013; Liu et al., 2014). Non-small cell lung cancer (NSCLC) accounts for 80% of all pulmonary carcinomas. In spite of the development in diagnostic and therapeutic methods, the outcome after treatment remains poor mainly because of the potential of tumor cells to invade and metastasize (Rasheed et al., 2010; Deng et al., 2014; Huang et al., 2014). Targeted therapies are commonly used in combination with traditional chemotherapy currently (Liloglou et al., 2014), and targeted drugs are more effective and have less severe side effects than standard chemotherapy drugs (Lammers et al., 2012). Under these circumstances, discovery of novel and effective biomarkers for lung cancer diagnosis and prognosis as well as new therapeutic targets becomes imperative.

The translationally controlled tumor protein (TCTP), a highly conserved protein present in eukaryotic organisms, has been suggested as a tumor-associated antigen. TCTP, also called histamine releasing factor (HRF), tumor

protein translationally controlled 1 (Tpt1), p23 or fortilin, is over-expressed in various malignancies. It has been shown to play an important role not only in physiological events, such as cell proliferation, cell death and immune responses, but also in stress response and tumor reversion (Wang et al., 2013; Ma et al., 2010; Lee et al., 2008; Susini et al., 2008; Dong et al., 2009; Nagano-Ito et al., 2012; Miao et al., 2013). Many researches show that TCTP level in tumor is higher than that in the corresponding normal tissues, indicating its critical role in tumorigenesis (Amson et al., 2011; Amson et al., 2013). Among 20 screened proteins in lung cancer, TCTP is one of the most over-expressed proteins in human lung cancer cells compared with the normal cells, suggesting that TCTP can be a good biomarker for lung cancer (Lo et al., 2012; Baylot et al., 2012; Acunzo et al., 2014).

Dihydroartemisinin (DHA) is a kind of the sesquiterpene lactones isolated from the sweet worm wood *artemisia annua*. As a main active metabolite of artemisinin derivatives, it is also used as a first-line antimalarial drug in various countries with low toxicity (Keating, 2012). DHA is shown to have anticancer effects in a wide variety of cancer models in vitro and in vivo (Jiao et al., 2007; Sun et al., 2014). Many studies have shown that DHA inhibits cell proliferation, and induces apoptosis in various human cancer cell lines via down-regulating

¹Department of Oncology, The First Affiliated Hospital of Nanjing Medical University, Nanjing, ²Institute of Cardiovascular Disease, The First Affiliated Hospital of Nanjing Medical University, Nanjing, China *For correspondence: jnanzh506nj@sina.com

Table 1. Primer Pairs and Sizes for PCR

Human gene name	Forward (F) and reverse (R) primers	Size (bp)
TCTP-F	AGACCAGAAAGAGTAAAA	347
TCTP-R	TCCACTCCAAATAAATCACAG	
GAPDH-F	ACCACAGTCCATGCCATCAC	452
GAPDH-R	TCCACCACCCTGTTGCTGTA	

Table 2. Primer Pairs and Sizes for Real-time PCR

Human gene name	Forward (F) and reverse (R) primers	Size (bp)
TCTP-F	TGACTCGCTCATTGGTGAA	72
TCTP-R	GTGATTACTGTGCTTTCGGTACCTT	
TCTP-probe	CCTCCGCTGAAGGCCCCGA	
GAPDH-F	CCTCCGCTTCGCTCTCT	65
GAPDH-R	GCTGGCGACGAAAAGA	
GAPDH-probe	CCTCCTGTTTCGACAGTCAGCCGC	

cyclin (D1, E), Bcl-2, Bcl-xL, caspase-3, caspase-9 and VEGF, while up-regulating P21, P27 and Bax (Nam et al., 2007; Mu et al., 2008; Chen et al., 2009; Chen et al., 2010; Handrick et al., 2010). Therefore, the targeted efficacy of DHA on the TCTP expression of lung cancer cell was explored in the study.

Materials and Methods

Cell culture

Lung cancer cell line A549 was provided by Shanghai Cellular Institute of China Scientific Academy. Cells were cultured in RPMI 1640 medium containing 10% fetal calf serum (FCS) and with 100 µg/mL penicillin and 100 µg/mL streptomycin. The cells were incubated at 37°C in 5% CO₂ atmosphere and medium was replaced every other day.

Cell proliferation inhibitory study

The cells were plated at a density of 1×10⁴ cells/well in 96 well plates at 37°C in 5% CO₂ atmosphere. After 24 h of culture, the medium in the wells was replaced with the fresh medium containing 0-100 nmol/L DHA. And the final concentration of DMSO was 0.1% (v/v) for each treatment. After 24 h, 48 h and 72 h, the medium was removed and the cells were rinsed twice with fresh medium, then 20 µL of MTT (3,4,5-dimethylthiazol-yl-2,5-diphenyl tetrazolium, Sigma) dye solution (5 mg/mL in medium) was added to each well. After 4 h of incubation at 37°C, the medium was removed and Formazan crystals were dissolved in 150 µL dimethylsulphoxide (DMSO) and quantified by measuring the absorbance of the solution at 490/630 nm by a microplate reader (Model 680, Bio-RAD). The spectrophotometer was calibrated to zero absorbance, using culture medium without cells. The relative cell proliferation inhibitory rate (%) related to control wells containing culture medium and 0.1% DMSO without DHA was calculated by 1-[A]test/[A]control×100. Where [A]test was the absorbance of the test sample and [A]control is the absorbance of control sample. Half of the inhibition rate (IC₅₀) was calculated in accordance with the BLISS.

Polymerase chain reaction (PCR)

PCR was performed using a standard Taqman PCR kit (Takara, Dalian, China) protocol on the PTC-150 MiniCycler™ PCR amplification (Perkin Elmer, USA). Total RNA was prepared using TRIzol (Invitrogen, USA) and reversed transcribed using SuperScript reverse transcriptase (Takara, Dalian, China) following the instructions of the manufacturer. The Primers for PCR were showed in the Table 1. The 50 µL PCR reaction included 1 µL cDNA, 5µL 10×PCR buffer, 4 µL dNTP (2.5 mM), 0.5 µL Taq DNA polymerase, 1 µL Primer F (S) (10 pmol/µL), 1 µL Primer R (A) (10 pmol/µL) and 38.5 µL water. The reactions were incubated in a 96-well plate at 95°C for 5 min, 94°C for 30 s, 55°C for 30 s, followed by 35 cycles of 72°C for 1 min. A final extension of 5 min at 72°C was performed prior to storing the samples at 4°C. Following PCR, the products were analyzed by 1% agarose gel electrophoresis.

Real-time PCR

The RT reaction was carried out with THUNDERBIRD Probe qPCR Mix (TOYOBO) according to the manufacturer's instructions. Total RNA was prepared using TRIzol (Invitrogen, USA) and reversed transcribed using SuperScript reverse transcriptase (Takara, Dalian, China) following the protocol of the manufacturer. Primers for real-time PCR were showed in Table 2. Briefly, 20 µL of total RT-PCR reaction system was used including 1 µL DNA template, 6 pmol forward primer, 6 pmol reverse primer, 4 pmol Taqman probe, 0.4 µL 50×ROX reference dye and 7 µL RNase-free water. The reactions were incubated in a thermocycler at 94°C for 10 min, 94°C for 30 s, 60°C for 45 s and 72°C for 30 s. GAPDH was used as internal reference. The RT-PCR analysis was performed with LightCycler 480 (Roche, Switzerland). For relative quantification, the ΔΔCt method was employed, using GAPDH as the endogenous standard for each sample (Livak et al., 2001).

Western Blot Assay

Protein extracts were prepared from the cell lines using lysis buffer containing 50 mM Tris-HCl (pH 7.4), 1% NP-40 (Sigma, St. Louis, MO, USA), 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 1 µg/ml aprotinin, 1 µg/mL leupeptin and 1 µg/mL pepstatin. The protein concentrations were determined by Bradford assay. The protein lysate (35 µg) was separated on a 10% SDS-PAGE gel and transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA) for western blot analysis detection. The blot was blocked with 5% non-fat dry milk in a buffer containing 10 mM Tris (pH 7.5), 100 mM NaCl and 0.1% Tween-20 (Sigma) for 1 h at 37°C. The blot was washed and incubated with primary polyclonal anti-TCTP antibody (1:1 000 dilution, Abcam, UK) for 1 h and then incubated for 30 min with secondary goat anti-rabbit antibody conjugated with horseradish peroxidase (1:3000 dilution, Abcam, UK). Immunoreactive protein signals were visualized by an enhanced chemiluminescence kit (Pierce, USA). GAPDH was used as an internal reference. Quantitative evaluation of proteins was determined by

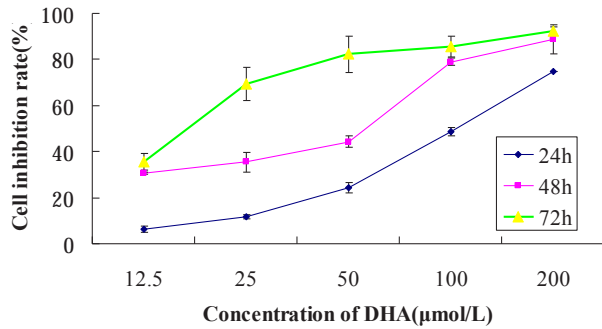


Figure 1. Cell Viability after Treatment with DHA (MTT Assay)

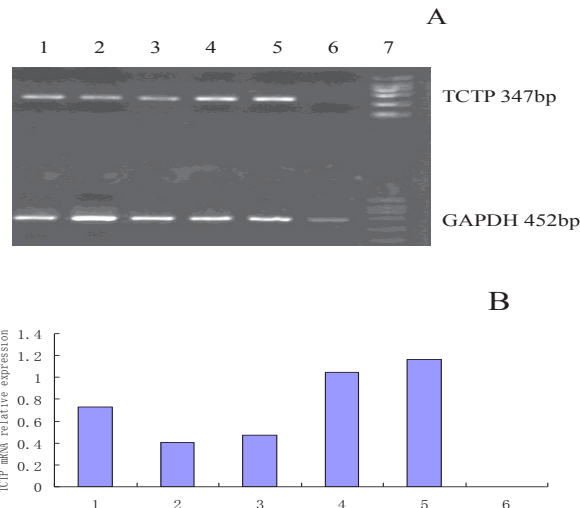


Figure 2. PCR Electrophoresis Plot after A549 Treated with DHA for 24 h (1: positive control; 2: 25 µmol/L 24 h; 3: 100 µmol/L 24 h; 4: 25 µmol/L 48 h; 5: 100 µmol/L 48 h; 6: negative control (mouse); M: Marker. (A) PCR electrophoresis plot; (B) The ratio of corresponding TCTP/GAPDH

UVI automatic image analysis system (Bio-Rad, USA) and Gel image analysis system (Vilber Lourmat, France).

Cell secretion study

In the cell secretion experiment, the cells were plated at a density of 1×10^4 cells/well in 6 well plates at 37°C in 5% CO₂ atmosphere. After 24 h of culture, the medium in the wells was replaced with the different concentrations of DHA in serum free medium for various incubation times. After indicated times, the cell culture supernatants were harvested and centrifuged (5 000 rpm, 10 min), then were transferred onto Amicon Ultra 10K (Millipore, Billerica, MA, USA) to centrifuge (7 500 rpm, 10 min) and recover the concentrated solutes. The concentrated solutes of cell secretion were re-suspended in SDS buffer, separated on SDS-PAGE and analyzed by Western blot assays. β-tubulin was used as an internal reference (Choi et al., 2009).

Statistical data analysis

Each experiment was repeated three times in duplicate. The results were presented as the mean ± standard deviation. Statistical significance was accepted at a level of $P < 0.05$.

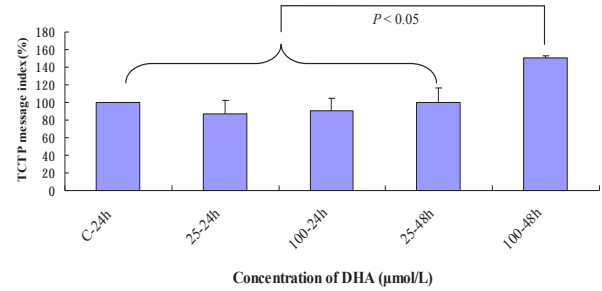


Figure 3. Expression of TCTP mRNA in A549 Pre- and Post-treatment with DHA (C-24 h: 24 h control; 25-24 h: DHA 25 µmol/L 24 h; 100-24 h: DHA 100 µmol/L 24 h; 25-48 h: DHA 25 µmol/L 48 h; 100-48 h: DHA 100 µmol/L 48 h. TCTP mRNA of trial groups were calculated by normalizing fortilin signals to corresponding GAPDH signals and expressing them as a percentage of the values at Control (n=3)

Results

Inhibition of DHA to A549 cell proliferation

The inhibition of DHA to A549 cell proliferation was investigated using MTT assay. The MTT assay relied on the mitochondrial activity of cells and represented a parameter for their metabolic activity. The cell inhibition rate was enhanced in dose and incubation time-dependent manners when the cells were treated with DHA. After incubated for 24 h, the concentration of DHA caused a minor increase in cell inhibition rate. With the increase of incubation time, the cell inhibition rate was also enhanced. The IC₅₀ of DHA was 100.93 µmol/L, 36.53 µmol/L and 15.57 µmol/L for 24 h, 48 h and 72 h, respectively (Figure 1).

Up-regulation of A549 cell TCTP mRNA

The expression of A549 cell TCTP mRNA was explored under the action of 25 µmol/L and 100 µmol/L DHA. PCR results showed that both higher and lower doses of DHA caused the up-regulation of A549 cell TCTP mRNA (Figure 2). Furthermore, the real-time PCR results showed that the expression of A549 cell TCTP mRNA was more accurate and consistent with the previous PCR results. TCTP mRNA was up regulated when 100 µmol/L DHA incubated with A549 cells for 48 h (Figure 3).

Down-regulation of A549 cell TCTP protein

A549 cells were treated with two groups of DHA concentration (25 µmol/L and 100 µmol/L) for 24 h and 48 h, respectively. The TCTP protein expression of A549 cells had no obvious difference between control groups at 24 h and 48 h. At the lower dose (25 µmol/L), the TCTP protein expression of A549 cells had no obvious change compared with the control group at 24 h, while the TCTP protein expression of A549 cells was suppressed with DHA at 48 h. When the DHA concentration was increased to 100 µmol/L, the TCTP protein expression of A549 cells was obviously suppressed both at 24 h and 48 h (Figure 4).

Regulation of A549 cell TCTP protein secretion

The lower concentration of DHA (25 µmol/L) was used

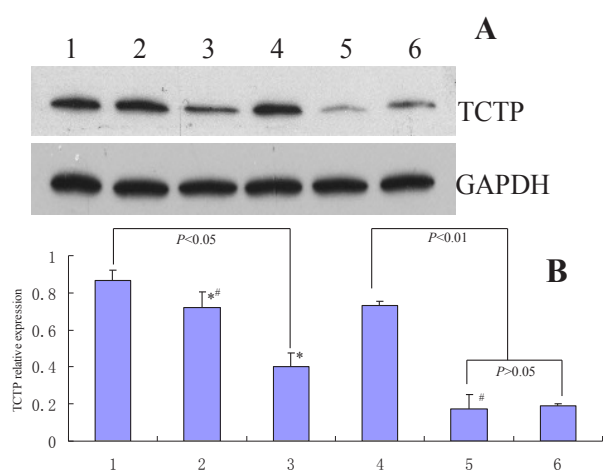


Figure 4. Decreased TCTP Expression in A549 Cells by DHA (1: 24 h control; 2: 24 h DHA 25 μmol/L; 3: 24 h DHA 100 μmol/L; 4: 48 h control; 5: 48 h DHA 25 μmol/L; 6: DHA 48 h 100 μmol/L. (A) TCTP protein expression by Western blot. GAPDH protein expression as the reference; (B) Relative expression of TCTP protein (Bar charts). *: Compared with 24 h DHA 100 μmol/L, $P < 0.05$; #: Compared with 48 h DHA 25 μmol/L, $P < 0.01$ ($n = 3$)

for exploring the effect on TCTP secretion in A549 cells. A time-dependent increase of TCTP protein expression in A549 cells was revealed (Figure 5). The cell secretion of TCTP was not detected when DHA was incubated with cells for 6 h and 12 h. However, as time went on, the cell secretion of TCTP in the culture supernatant was increased significantly.

Discussion

DHA, a semi-synthetic derivative of the herbal anti-malarial drug artemisinin, can obviously inhibit the growth of a variety of cancer cells, including breast cancer, leukemia, cervical cancer, ovarian cancer, lung cancer, glioma and oral cancer (Jiao et al., 2007; Nam et al., 2007; Mu et al., 2008; Chen et al., 2009; Handrick et al., 2010). Our studies indicated that DHA inhibited the growth of A549 lung cancer cells. The viability of A549 cells was measured by MTT assay after being cultured at different times. The IC_{50} of DHA was 100.93 μmol/L, 36.53 μmol/L and 15.57 μmol/L for 24 h, 48 h and 72 h, respectively.

TCTP is a highly conserved protein widely expressed in all eukaryotic organisms. Although its biological functions involved in many cell processes are not fully elucidated, in the study, the expression of A549 cell TCTP mRNA was obviously up-regulated at the higher concentration (100 μmol/L) of DHA when DHA incubated with the cells for 48 h. While at the lower concentration and less time, the expression of A549 cell TCTP mRNA showed the minor up-regulation, suggesting that the effect of DHA on the cell TCTP was slow. The mechanism of DHA up-regulating A549 cell TCTP mRNA maybe associated with its effect on the protein expression. Many factors may influence the expression of cell TCTP mRNA, including cell lines, source of DHA, cell culture environment and drug concentration. Three kinds of drugs (DHA, sertraline

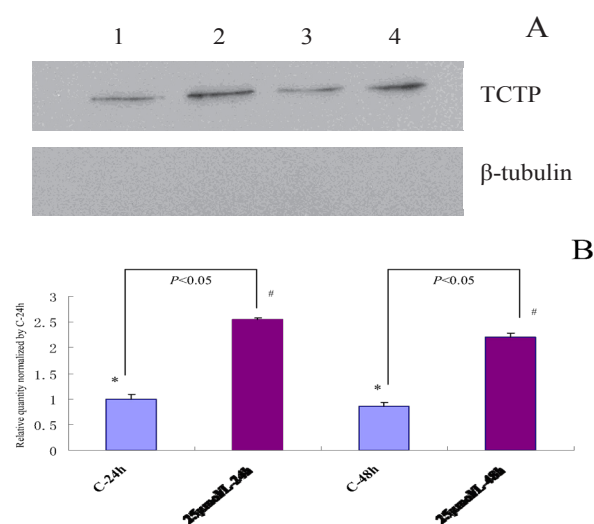


Figure 5. Increased TCTP Secretion of A549 Cells by DHA (1: 24 h supernatant; 2: 24 h DHA 25 μmol/L supernatant; 3: 48 h supernatant; 4: 48 h DHA 25 μmol/L supernatant. TCTP protein was detected by Western blot, and β-tubulin was used as control. *: 24 h control compared with 48 h control, $P > 0.05$; #: 24 h DHA 25 μmol/L compared with 48 h DHA 25 μmol/L (ANOVA analysis)

and thioridazine) were reported as the direct anti-cancer drug targets for TCTP. Fujita et al demonstrated that DHA could bind to human TCTP and decrease its cellular level through promoting ubiquitination and proteasome-dependent degradation (Fujita et al., 2008; Lucibello et al., 2011).

Secreted proteins are responsible for the cross talking among cells and understanding this language could largely increase our knowledge on the molecular mechanism of neoplasia (Amson et al., 2011). In addition, extracellular matrix components and other molecules secreted by tumor cells are rich sources of potential markers and drug targets for cancer treatment (Makridakis et al., 2010). The study found that DHA promoted the cell secretions of TCTP protein in the cell culture supernatant. Moreover, the cell secretions of TCTP protein were time-dependent. The higher concentration of DHA was not suitable for the cell secretion study since it could induce cell apoptosis. Kim et al analyzed the secretome of transformed bronchial epithelial cells (1198 and 1170-I), the parental immortalized normal cells (BEAS-2B) and non-transformed cells (1799) (Kim et al., 2008). The levels of TCTP were significantly increased in the conditioned media of both transformed cell lines when compared with those of BEAS-2B and 1799 cells. These proteins were also presented in significantly higher concentrations in plasma and tissue samples of patients with lung cancer by comparison to the controls.

In conclusion, DHA can inhibit the proliferation of A549 lung cancer cells, increase TCTP mRNA expression in A549 cells and reduce its protein level, which is consistent with previous reports. Besides, it can also facilitate A549 cells to secrete TCTP protein. Therefore, TCTP may be a potential target for NSCLC in cancer therapy, which is helpful to the diagnosis and treatment of lung cancer.

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