Potential suppressive effects of theophylline on human rectal cancer SW480 cells *in vitro* by inhibiting YKL-40 expression

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Abstract. Chitinase-3-like-1 protein (YKL-40), a member of the mammalian chitinase-like glycoproteins, serves a key role in the pathogenesis of rectal cancer. The present study examined the antitumor effect of theophylline, a pan-chitinase inhibitor, in rectal cancer *in vitro* and investigated the mechanism by which it acted. SW480 cell lines were treated with varying theophylline concentrations (10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} mol/l). An MTT assay was used to observe cell proliferation and identify the optimal theophylline concentration. Western blotting was used to analyze YKL-40 expression. The cell cycle distribution of SW480 cell lines treated with theophylline was measured by flow cytometry. The angiopoietin-2 expression level was measured by ELISA. The expression levels of YKL-40 were evidently decreased in theophylline-treated SW480 cell lines. The proliferation ofSW480 cells was inhibited following theophylline treatment, which was associated with G_1 phase cell cycle arrest and a decrease in the expression of angiopoietin-2. The mechanism of theophylline action may involve the downregulation of YKL-40 expression, arrest of the cell cycle at G_1 phase and inhibition of angiopoietin-2 expression. These results provide a rationale for the potential use of anti-YKL-40 and anti-angiogenic strategies in treating rectal cancer.

Introduction

Colorectal cancer is one of the most prevalent malignant tumors in the Asia-Pacific region, with a high mortality rate (1). To the best of our knowledge, the cause of the disease remains unknown, and surgical resection is the main treatment modality for the therapy of rectal cancer; however, 30% of these patients develop disease recurrence and metastasis (2). Therefore, studies investigating novel therapeutic strategies, particularly molecular targeted agents, have become a topic of substantial interest.

Chitinase-3-like protein 1 (CHI3L1, also known as YKL-40) is a member of the mammalian chitinase-like protein family that is secreted by neutrophils, macrophages and tumor cells (3-5). YKL-40 serves a critical role in cellular proliferation, differentiation, angiogenesis, remodeling of the extracellular matrix and apoptosis (5-9), and the aforementioned processes are important for tumor growth and dissemination (10). Therefore, YKL-40 may represent a novel attractive therapeutic target.

Theophylline, a component of tea (*Camellia sinensis*), was identified as a pan-family 18 chitinase inhibitor, a family that includes YKL-40 and acidic mammalian chitinase. Recent evidence has shown that theophylline exhibits a potential anti-carcinogenic effect; however, to the best of our knowledge, the antitumor effect of theophylline on rectal cancer, and the precise etiology of disease, remains unknown. The aim of the present study was to evaluate the effect of theophylline on rectal cancer SW480 cells and investigate the mechanism by which this occurred.

Materials and methods

Cell culture and regents. Human rectal cancer SW480 cells were obtained from the Foundation Research Institute of Medical University of Chongqing (Chongqing, China) and were cultured in RPMI-1640 medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplied with 10% fetal bovine serum (Thermo Fisher Scientific, Inc.), 1% penicillin and 1% streptomycin (Thermo Fisher Scientific, Inc.) in a humidified atmosphere containing 5% CO_2 and 95% air at 37˚C. Theophylline ($C_7H_8N_4O_2$) was provided by Zhengzhou Shengyuan Industrial Co., Ltd. (Zhengzhou, China).

Cell proliferation. Rectal cancer SW480 cells ($1x10^5$) were seeded on a 96-well plate overnight and, when cells are 80% confluent, treated with varying theophylline concentrations (0, 10^{-2}, 10^{-3}, 10^{-4} or 10^{-5} mol/l) for 48 h. Following incubation of cells with 20 μl MTT (5 mg/ml), the medium was discarded after 4 hand formazan crystals were dissolved in dimethyl sulfoxide (150 μl). Next, the absorbance of each

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well was measured at 490 nm. Experiments were performed three times in duplicate and the SW480 cell growth inhibition rate was calculated. The optimal inhibitory concentration of theophylline was 10^{-4} mol/l at 48 h treatment times.

Western blotting. The rectal cancer SW480 cells ($2x10^5$) were seeded on a 6-well plate overnight and, when cells were 80% confluent, the treatment group was treated with 10^{-4} mol/l theophylline for 48 h, the negative group was treated with RPMI-1640 medium and the blank group was not treated for 48 h. The cells were lysed in Radioimmunoprecipitation assay lysis buffer on ice for 30 min and centrifuged for 10 min at 12,000 x g at 4˚C. Whole cell lysates (30 μg) were separated on 12% SDS polyacrylamide gels and were transferred to polyvinylidene difluoride membranes. Membranes were blocked in 5% non-fat milk in TBS and 0.1% Tween-20 at room temperature for 2 h. YKL-40 (rabbit anti-human; cat. no. Ab3427; Shanghai Yanjing Biotech Engineering Co., Ltd., Shanghai, China; dilution, 1:1,000) and β-actin (rabbit anti-human; cat. no. yb-0061R; Shanghai Yubo Biotech Engineering Co., Ltd., Shanghai, China; dilution, 1:1,000) antibodies were used in the western blotting analysis. Subsequent to washing with TBST (TBS containing 0.05% Tween-20, pH 7.6) three times, the membranes were incubated with goat anti-rabbit immunoglobulin G conjugated to horseradish peroxidase (cat. no. ab97051; 1:2,000; Abcam, Cambridge, MA, USA) for 1 h at room temperature. Densitometric analysis was performed using Quantity One software (version 4.0, Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Flow cytometry. The rectal cancer SW480 cells ($2x10^5$) were seeded on a 6-well plate overnight and when cells are 80% confluent. The treatment group was treated with 10^{-4} mol/l theophylline for 48 h. The negative group was treated with RPMI-1640 medium and the blank group was not treated for 48 h. Flow cytometry analysis was performed by BD FACSCalibur (BD Biosciences, Franklin Lakes, NJ, USA) and analyzed by MODFit LT version 3.3 software (BD Biosciences). The fraction of the total cell population present in the G_1, S and G_2/M phases was obtained.

ELISA. The rectal cancer SW480 cells ($2x10^5$) were seeded on a 24-well plate overnight and when 80% confluent, treated. The treatment group was treated with 10^{-4} mol/l theophylline for 48 h. The negative group was treated with RPMI-1640 medium and the blank group was not treated for 48 h. The level of angiopoietin-2 was measured using an ELISA kit (cat. no. MM-0309H1; Shenzhen Jingmei Biotech Engineering Co., Ltd., Shenzhen, China).

Statistical analysis. Qualitative variables were expressed as the count (percentage); quantitative variables were expressed as the mean ± standard deviation. Differences between two groups with a normal distribution were assessed using a Student's t-test for non-normal distributions, the Mann-Whitney rank-sum test was used. Analysis of variance was performed to evaluate the difference >2 groups followed by Tukey's post hoc test. All statistical analyses were performed using SPSS version 17 (SPSS, Inc., Chicago, IL, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Table I. Cell viability following culture with different theophylline concentration.

Group, mol/l	OD value	Cell viability, %
0	0.57±0.04	N/A
10^{-5}	0.50±0.04	87.71
10^{-4}	0.45±0.03	78.95
10^{-3}	0.44±0.03	77.20
10^{-2}	0.43±0.03	75.44

Chitinase-3-like protein 1expression was analyzed by western blotting. OD, optical density; N/A, not applicable.

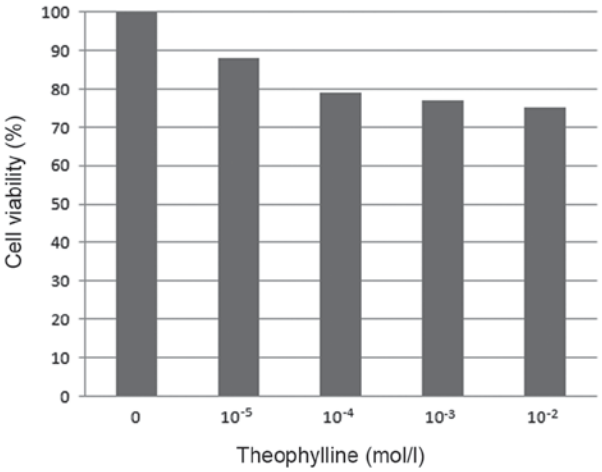

Figure 1. Effect of theophylline on SW480 cell viability.

Results

In vitro effects on cell proliferation. The viability of SW480 cells decreased markedly with increases in theophylline concentration (10^{-5}-10^{-2} mol/l); however, there was no significant difference in SW480 cell viability following culture with higher concentrations of theophylline (10^{-4}-10^{-2} mol/l), indicating that the optimal inhibitory concentration of theophylline was ~10^{-4} mol/l at 48 h treatment times. Therefore, a concentration of 10^{-4} mol/l was used for all subsequent investigations (Table I and Fig. 1).

Western blotting. Densitometry value analysis was used to assess YKL-40 expression and were repeated 10 times. The result revealed that Densitometry value in the treatment group, the negative group and the blank group were 11,848.7±92.2, 13,503.2±68.8 and 13,572.8±49.9, respectively, which indicated that YKL-40 protein expression decreased significantly in the treatment group compared with the negative and the blank groups ($F=1,816$; $P<0.05$). However, there was no significant difference in YKL-40 expression between the negative group and blank group (Fig. 2).

Flow cytometry assessment of cell cycle distribution. The SW480 cell cycle progression was assessed after 48 h culture with theophylline. The result indicated that the cell cycle was

Table II. Cell cycle distribution analysis.

Group	G_0/G_1 phase, %	S phase, %	G_2/M phase, %
Negative	67.72±3.65[a]	25.17±2.68	7.10±1.25
Blank	66.67±7.16[a]	24.96±7.96	8.35±1.82
Treatment	81.32±2.89	14.27±4.02	4.42±1.92

Data are presented as the mean ± standard deviation. [a]P<0.05 compared with the treatment group.

Table III. Angiopoietin-2 expression level.

Group	Angiopoietin-2, ng/ml
Negative	39.1±2.18[a]
Blank	40.6±2.07[a]
Treatment	29.2±2.04

[a]P<0.05 compared with the treatment group.

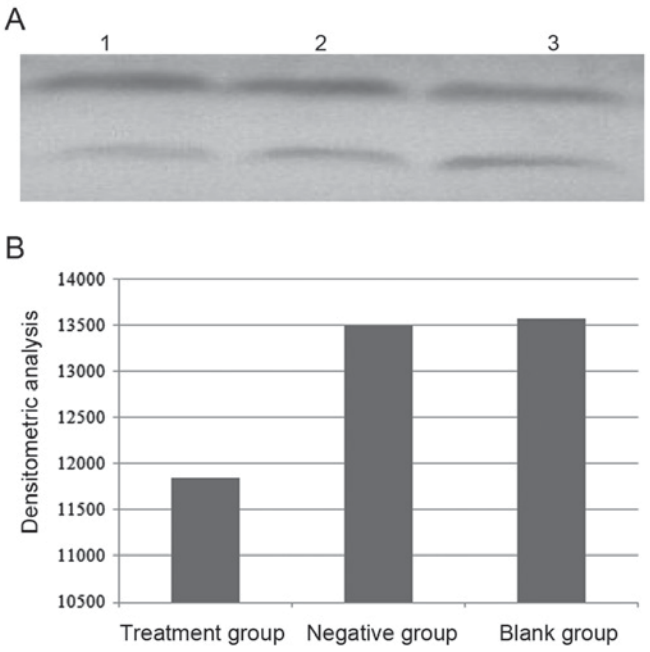

Figure 2. YKL-40 expression. (A) YKL-40 expression levels in different group. Lane 1, treatment group (10^{-4} mol/l theophylline); lane 2, negative (medium-treated) group; lane 3, blank (untreated) group. Bottom bands represents YKL-40; Top bands represents β-actin. (B) Densitometric analysis. YKL-40, Chitinase-3-like-1 protein.

arrested in the G_1 phase in 81.32% of treatment group cells, compared with 67.72% of negative group cells and 66.67% of blank group cells. Therefore, suppressing YKL-40 expression attenuated SW480 cell proliferation by arresting the cell cycle in G_1 phase (Table II and Fig. 3).

Angiopoietin-2 expression level in SW480 cells. In the treatment group, the angiopoietin-2 expression level decreased significantly compared with the negative group and the blank group (F=87.13; P<0.05). However, there was no significant difference in angiopoietin-2 expression between the negative group and blank group (Table III).

Discussion

Previous studies have demonstrated that YKL-40 is associated with oncogenesis (8,10). Elevated levels of serum YKL-40 were reported to be associated with several types of cancer, such as ovarian, esophageal, breast, lung, liver and kidney cancer (11-16). Furthermore, high expression of YKL-40 is associated with tumor grade, poor differentiation and shorter overall survival times, and may be a potential biomarker (5,11,13). Research by Johansen *et al* (17) revealed the presence of high levels of serum YKL-40 in patients with rectal cancer, meaning it may be a useful candidate biomarker for colorectal cancer risk assessment. Chen *et al* (18,19) confirmed that YKL-40 was highly expressed in the SW480 cell line, that treatment with exogenous YKL-40 could significantly promote SW480 proliferation and migration, and that YKL-40 serves a key role in inflammation-associated neoplastic changes. YKL-40 protein markedly enhanced nuclear factor-κB (NF-κB) signaling pathway activation, and NF-κB efficiently promoted proliferation of tumor cells (20). Shao *et al* (21) demonstrated that ectopic YKL-40 could stimulate colon cancer HCT-116 cell angiogenesis and tumor progression. In addition, YKL-40 restrained apoptosis by enhancing the activation of mitogen-activated protein kinase and phosphoinositide 3-kinase signaling cascades, and suppressing the expression of the pro-apoptotic S100A9 protein (22-24). Generally, YKL-40 serves a vital role in the pathogenesis of rectal cancer. Therefore modulating YKL-40 functions may represent an efficient strategy for rectal cancer prevention and treatment.

In recent years, certain compounds extracted from traditional herbal medicines and plants have indicated potential at preventing and curing tumors (25). Theophylline is the main constituent and characteristic component of the purine alkaloids found in tea plants; it is also a specific inhibitor of YKL-40 (26). Chang *et al* (27) suggested that theophylline exerts an antitumor effect; however, to the best of our knowledge, no research on theophylline in rectal cancer has been reported. The results of the present study revealed that theophylline could perform preventive actions for rectal cancer, demonstrating that *in vitro* the rectal cancer SW480 cells viability decreased significantly with as theophylline concentration increased (from 10^{-5} to 10^{-2} mol/l). One mechanism responsible for this protection were mediated via suppression of YKL-40 expression by theophylline, which was analyzed by western blotting in the present study. In addition, the result indicated the cell cycle was arrested in G_1 phase in 81.32% of SW480 cells cultured with theophylline. Chen *et al* (19) revealed YKL-40 could activate the Akt signaling pathway in colonic epithelial cells subsequent to phosphorylating the Thr 308 residue, and Sheng *et al* (28) demonstrated that expression of active Akt increased the expression of cyclin D1 and promoted S phase entry, and that the inhibition of the PI3k/Akt pathway resulted in accumulation of RIE cells in the G1 phase of the cell cycle. Eurich *et al* (29) demonstrated that a low dose of YKL-40 could be used to stimulate SW480 cell, could resulting in β-catenin nuclear translocation and subsequent activation of the transcription of target genes, including c-Myc and cyclin D1, then induce

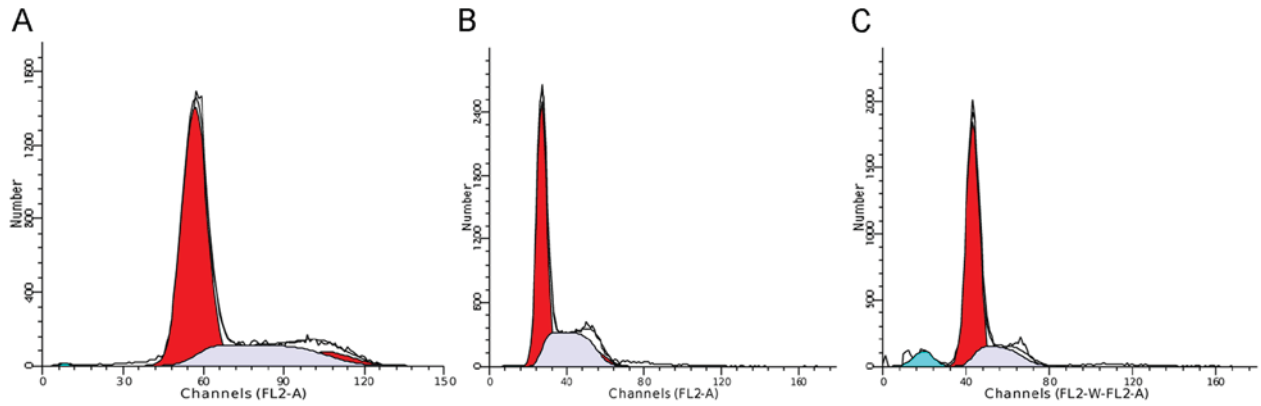

Figure 3. Cell cycle analysis. (A) The negative (medium-treated) group; (B) The blank (untreated) group; (C) The treatment (10^{-4} mol/l theophylline) group.

proliferation of SW480 cell through regulation of the cell cycle G_0/G_1 phase transition. Taken together, the results of the present study revealed that the pan-chitinase inhibitor theophylline suppressed rectal cancer SW480 cell proliferation by inhibiting YKL-40 expression, which may occur partly through arrest at the G_0/G_1 phase of the cell cycle.

Angiogenesis is necessary for the progression of colorectal cancer (30). Angiopoietin-2, a member of the angiopoietin family, binds to the endothelium-specific receptor TEK tyrosine kinase (Tie2) and serves a notable role in maintaining normal vasculature through the modulation of vessel stability (31). High expression of angiopoietin-2 has been associated with several cancer types, including lung, oral, nasopharyngeal and kidney cancer (32-35). The concentration of angiopoietin-2 was higher in patients with colon cancer and in colorectal mice models than in healthy patients or animals, and circulating angiopoietin-2 levels were reported to predict an unfavorable outcome in metastatic colorectal carcinoma (36,37). Angiopoietin-2 caninduce anarchical blood vessel organization during cancer progression and enhance the effect of vascular endothelial growth factor during active neovascularization (38,39). Additionally, angiopoietin-2 serves a notable role in the migration and invasion of a malignancy, and is partly responsible for inducing abnormal epithelial-mesenchymal transition (40). Upregulation of angiopoietin-2 expression was observed in diverse human malignant tumors, but notable expression does not occur in healthy individuals, meaning it may represent a novel mechanistically directed target for antitumor therapy. The blockade of angiopoietin/Tie2 signaling prevented the recruitment of myeloid cells in a colon cancer model, whereas blocking angiopoietin-2 resulted in limiting tumor metastasis (41,42). In addition, a peptibody that blocks angiopoietin-2 used to treat ovarian cancer is currently being evaluated in a phase III clinical trial (43).

YKL-40 has been demonstrated to be a novel proangiogenic factor (44). YKL-40 induces the migration of vascular smooth muscle cells (VSMCs) and promotes the attachment and spreading of VSMCs (45). Ngernyuang *et al* (46) demonstrated that YKL-40 could accelerate tumor cell proliferation by promoting angiogenesis. Zhang *et al* (47) reported that microvessel density was correlated with YKL-40 expression intensity (r=0.376, P=0.001) and the proportion of YKL-40 expression (r=0.364, P=0.002), and YKL-40 may serve a role in angiogenesis in clear cell renal cell carcinoma. Another study indicated that overexpression of YKL-40 could increase the levels of blood vasculature formed in rectal cancer SW480 cells, and also enhance the migration and tube formation of umbilical vein endothelial cells (48). In the present study, the expression level of angiopoietin-2 in SW480 cell was significantly decreased upon blockade of YKL-40. From these results, it can be deduced that YKL-40 expression is associated with angiopoietin-2 expression: Suppression of YKL-40 expression resulted in a decreased level of angiopoietin-2 in SW480 cells, which may be one reason for the pathogenesis of rectal cancer.

In summary, the findings of the present study support those of previous studies, which demonstrated that theophylline exerts an antitumor effect. The mechanism by which this occurs may involve the downregulation of YKL-40 expression; the cell cycle was arrested at the G_1 phase the expression of angiopoietin-2 protein was suppressed. Next, further research, such as an experimental animal study, may be required to understand better the functions of YKL-40 in rectal cancer development and progression.

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Availability of data and materials

All datasets generated and analyzed in the present study are included in this published article.

Authors' contributions

HP conceived and designed the study, and collected the data. HP, QS and ZCL conducted experiments. XHZ, MSP and ZBL analyzed the data. HP and QS wrote the manuscript.

Ethics and consent to participate

The study was approved by Nanchong City Central Hospital Ethical Committee, and written informed consent was gained from all participants.

Consent for publication

Written informed consent was gained from all participants.

Competing interests

The authors declare that they have no competing interests.

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