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Original Article

Effect of *Marsdenia tenacissima* extract on G2/M cell cycle arrest by upregulating 14-3-3σ and downregulating c-myc *in vitro* and *in vivo*

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ABSTRACT

Objective: *Marsdenia tenacissima* extract (MTE) is a traditional Chinese herbal medicine with anti-cancer activity. In some previous studies, different mechanism actions of the anti-cancer effect of MTE have been revealed. In this study, we first observed that MTE exhibited G2/M cell cycle arrest on two different human breast cancer cell lines, MDA-MB-231 and MCF-7 by mediating 14-3-3σ and c-myc.

Methods: The effect of MTE on G2/M cell cycle arrest was evaluated in MDA-MB-231 and MCF-7 cell lines. MTT assay was done for evaluation of cell viability. Flow cytometry was employed for cell cycle analysis. Western blotting analysis and immunohistochemistry were performed to analyze the expression of G2/M cell cycle-related key protein in cells and tissue samples. Animal studies have been conducted to elucidate the anti-tumor effect of MTE.

Results: Cell cycle is the backbone for developing cancer. Cell cycle proteins play a major role in the progression of cell cycle and cell proliferation. However, some key protein directly or indirectly modulate the action of cell cycle protein that highly affect cell cycle regulation. In order to investigate cellular proliferation of cancer, we observed that MTE induced the upregulation of 14-3-3σ and downregulation of c-myc, and then reduced the expression of G2/M cell cycle associated key protein, leading to the inhibition of cellular entry into mitosis phase. We also confirmed that MTE exerted a significant antitumor effect on the MDA-MB-231 xenograft model *in vivo*.

Conclusion: G2/M cell cycle arrest occurred by the action of MTE, mediated by the upregulation of 14-3-3σ as well as downregulation of c-myc in MDA-MB-231 and MCF-7 cell lines.

1. Introduction

Chinese materia medica (CMM) is widely used not only in China, but also in the world. About 90% of oncologist prescribed CMM in China without adverse effects was observed (McQuade et al., 2012). Numerous herbal medicines are available in China for breast cancer treatment and it is reported that Chinese patients use CMM in combination with other medical treatments in all stages of breast cancer according to one business survey (Cohen, Tagliaferri, & Tripathy, 2002; Cui et al., 2004). The widespread use of CMM is related to a belief that it can recover adverse effects associated with other treatments and develop an immune system (McQuade et al., 2012).

Marsdenia tenacissima (Roxb.) Wight et Arn. is a very common herbal plant in Asian countries including China, India, and Japan, and especially it is widely available in the southwestern regions of China (Lei et al., 2008). This plant is mostly seen in the moist and semi-deciduous forest. It belongs to the family of Asclepiadaceae. Stem and roots of *Marsdenia tenacissima* extract (MTE) have been used as CMM for many decades, and it is widely used as herbal medicine in some Asian countries for the treatment of tonsillitis, asthma, pneumonia, and pharyngitis (Huang et al., 2013). Major active components of MTE are C21 steroidal glycosides and polyoxypregnane esters and it has been previously hypothesized that these active components are responsible for therapeutic effects of MTE (Sheng, Qiu, Luo, Lin, & Geoffrey, 1996; Zhang et al., 2010). However, Luo and his coworkers evaluated the cytotoxic effects of C21 steroidal glycosides on K.B cancer cell lines and they proved that C21 steroidal glycosides are responsible for anticancer effects of MTE (Si-Qi, Lin, Geoffrey, Xue, & Michael, 1993).

This study was designed to evaluate the effect of MTE on G2/M cell cycle arrest by mediating 14-3-3σ and c-myc. 14-3-3 is a vital protein which has numerous roles to inhibit cell cycle progression and cell proliferation and 14-3-3 has different iso-

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forms. Among them, 14-3-3σ is a key molecule that directly acts to attenuate G2/M cell cycle progression through phosphorylation of cdc25c (Lodygin & Hermeking, 2006; Peng et al., 1997). c-myc is an oncogenic factor which enhances G2/M cell cycle progression by promoting the action of cyclin B1-CDK1 and upregulation of cdc25 genes (Born, Frost, Schönthal, Prendergast, & Feramisco, 1994). Furthermore, cdc25c potentiates the action of cyclin B1-CDK1, and leads to G2/M cell cycle progression (Gautier, Solomon, Booher, Bazan, & Kirschner, 1991). Distinct studies reported that c-myc alters many tightly regulated biological pathways, related to cell growth and tumor initiation by mediating numerous target genes. In this study, it has been explored that 14-3-3σ induces G2/M cell cycle arrest also through downregulation of c-myc, by the effect of MTE. We observed and confirmed through Western blotting analysis that MTE upregulated the action of 14-3-3σ which elicited downregulation of c-myc. In this study, we have treated MTE on two different breast cancer cell lines, MDA-MB-231 and MCF-7, for 48 h and observed the effect of G2/M cell cycle arrest through flow cytometry analysis.

2. Materials and methods

2.1. Chemical and reagents

MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide was obtained from Nanjing Sunshine Biotechnology Ltd., DMSO (dimethyl sulfoxide). PI (propidium iodide) was obtained from BD Biosciences (Sun Jose, CA, USA). Primary and secondary antibodies were purchased from Cell Signaling Technology (CST) and ABclonal Technology. We obtained MTE as a trade name of "Xiao-Ai-Ping" from Sanhome Co., Ltd. Paclitaxel (TAX) was obtained from Chongqing Taiji Industry (Group) Co., Ltd. (Nanjing, China) and Topotecan (TPT) was obtained from Hengrui Medicine Co., Ltd. (Jiangsu, China).

2.2. Cell culture

Human breast cancer cells MDA-MB-231 and MCF-7 were obtained from the Cell Bank of the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). MDA-MB-231 and MCF-7 cells were cultured in L-15 medium (Gibco, Grand Island, NY, USA) and RPMI 1640 medium (Gibco, Grand Island, NY, USA), respectively, containing 10% fetal bovine serum (FBS) (Hyclone, Logan, UT, USA), 100 U/mL streptomycin, and 100 U/mL penicillin (Gibco, Grand Island, NY, USA), and cells were incubated at humidified incubator (Thermo Fisher Scientific Inc. Waltham, MA, USA) at 37 °C with 5% CO_2.

2.3. Cell viability

MDA-MB-231 and MCF-7 cells were subcultured into 96 well plates and treated with MTE (0, 20, 40, 60, 80, 100, and 120 mg/mL) for 48 h. Each concentration of MTE was added repeatedly into three different wells. MTT was added in every treated well and then incubated for 4 h at 37 °C. Then after 4 h, 100 µL DMSO was added into treated well to dissolve formazan, and the absorbance was measured at 570 nm wavelength using spectrophotometric plate reader (Tecan, Durham, NC, USA). The cell viability assay was performed three times ($n = 3$). Cell viability was calculated as mean value of O.D. of drug-treated sample/mean value of O.D. of Control × 100%.

2.4. Cell cycle analysis

MDA-MB-231 and MCF-7 cells were plated into small dishes. MTE was treated at doses of 40, 60, and 80 mg/mL respectively on MDA-MB-231, and 30, 60, and 120 mg/mL respectively on MCF-7. Both cell lines were treated for 48 h. After that, cells were fixed gently and carefully with 70% ethanol and re-suspended with PI solution, containing 500 µL stain buffer, RNase 10 µL and PI 25 µL for each sample. Samples were incubated for 30 min at 37 °C and then samples were analyzed by using flow cytometer (BD Biosciences).

2.5. Western blotting

MDA-MB-231 and MCF-7 cells were plated into small dishes. MTE was treated at doses of 0, 40, 60, and 80 mg/mL respectively on MDA-MB-231, and 0, 30, 60, and 120 mg/mL respectively on MCF-7 for 48 h. Then protein was extracted from the cells using RIPA lysis buffer contains protease and phosphatase inhibitor (Roche Diagnostics, 4906845001). The supernatants were collected after centrifugation at 12 000 r/min, 4 °C, for 20 min. A total of 20 µg of protein of each sample was applied onto polyacrylamide gel (SDS-PAGE), and transferred to Polyvinylidene Fluoride Membrane (PVDF; Millipore). The blots were incubated at 4 °C for 12 h with an appropriate dilution of monoclonal antibodies, cdc2, cyclin B1, cdc25c, p-cdc25c (Cell Signaling Technology), 14-3-3σ and c-myc (ABclonal). Blots were washed with Tris buffered saline with Tween 20 (TBST) and then incubated with secondary antibody for 1 h. Immune complexes were visualized by chemiluminescence kits (Millipore) and exposed using an Image analyzer (Gel, 2000, Bio-Rad Laboratories Inc. Richmond, CA, USA).

2.6. Animal model

Animal protocols were approved by preclinical medicine and clinical pharmacy college, and rules and regulations were followed during processing of entire experiments on animals. MDA-MB-231 cells (2×10^6 cells/mice) were injected subcutaneously to female Balb/c nude mice. When the average tumor volume was more than 100 mm^3, mice have been sacrificed. Thereafter, tumors were harvested and tumor pieces were transplanted under the skin of female Balb/c nude mice. Two weeks later, the mice were divided into five different groups including negative control (treated with saline), positive control (treated with 0.01 g/kg paclitaxel), and MTE treated group (2.5 g/kg, 5 g/kg, and 10 g/kg) when the tumor volume reached more than 100 mm^3. Saline (10 µL/g), TAX, and MTE were administered intravenously through a tail vein to mice in each group once a week up to three weeks. After three weeks, mice were sacrificed and tumors were harvested, weighed, and measured the volume of each tumor of the respective group.

2.7. Calculation of tumor volume

Tumor volume (TV): $TV = 1/2 \times a \times b2$, a was the length and b was the width of the tumor. Relative tumor volume (RTV) = TVt/TV0, where TVt was tumor volume, after treatment of MTE, and TV0 was initial tumor volume before treatment of MTE.

2.8. Immunohistochemistry

Breast tissue samples from MDA-MB-231 treated nude mice were harvested after sacrificing the mice. The immunohistochemistry (IHC) was conducted on formalin fixed and paraffin embedded slides of breast cancer tissue. Thereafter, this paraffin-embedded tissue sections were de-paraffinized, rehydrated, rinsed, and immersed by using 10 mmol/L sodium citrate (pH 6.0). Then, after treatment with methanol containing 3% hydrogen peroxide, tissue sections were blocked with a primary antibody, cdc2, cyclin B1, p-cdc25c, c-myc, and 14-3-3σ respectively, followed by blocking with secondary antibody after washing with TBST.

2.9. Statistical analysis

The experimental data were analyzed as the means of three replications ($n = 3$) with standard deviation (mean ± SD). The *t*-test was done for single variable comparison. Data were analyzed by Graph Pad Prism software and measured by one way and two-way Analysis of Variance (ANOVA) for multiple variable comparisons. $P < 0.05$ indicates that all results were significant.

3. Results

3.1. Cytotoxic effects of MTE on MDA-MB-231 and MCF-7 cells in vitro

Cell viability of MTE (0, 20, 40, 60, 80, 100, 120 mg/mL) treated human breast cancer cells was determined by MTT assay for 48 h, as shown in Fig. 1. The degree of cytotoxicity varied with the different concentrations of MTE on these two cell lines. Results showed that 60, 80, 100, and 120 mg/mL MTE had the most significant cytotoxic effect on both cell lines. Cell viability was reduced in a dose-dependent manner. It has been shown that MTE exerted a significant cytotoxic effect on both cell lines. The IC_{50} of MDA-MB-231 and MCF-7 for 48 h was 51.86 mg/mL and 59.53 mg/mL, respectively.

3.2. Effect of MTE on cell cycle transition

Flow cytometry analysis was performed to determine cell cycle transition on MCF-7 and MDA-MB-231 cell lines. It has been observed that MTE exhibited G2/M cell cycle arrest on both cell lines (Fig. 2 and Fig. 3). Cell population has been elevated significantly on the G2 phase. The proportion of cells at G2 phase was increased markedly in a dose-dependent manner. However, it has been identified that the effect of G2/M arrest varied on these selected lines and it was found to be most potent on the MDA-MB-231 cell line, all concentration exerted significant response but the highest concentration (80 mg/mL) showed the most significant effect compared to control group (Fig. 3B). Whereas, on MCF-7 cell line, all concentrations of MTE showed effective response but only the highest concentration exerted significant effect (Fig. 2B).

3.3. Effects of MTE on G2/M checkpoint associated protein expression

Key protein at the G2/M checkpoint plays a major role in G2/M transition. We investigated that the levels of key protein have changed by the action of MTE (Figs. 4 and 5). G2/M checkpoint protein that facilitates G2/M cell cycle progression was downregulated and this phosphorylated form of cdc25c was upregulated in both cell lines. We also observed that the expression of 14-3-3σ was upregulated, which phosphorylates cdc25c and inhibits the action of c-myc. The c-myc, is an oncogenic factor, which enhances G2/M progression. The results showed that the level of c-myc was downregulated.

3.4. Antitumor effect of MTE in vivo

2×10^6 MDA-MB-231 cells were implanted into female nude mice, after formation of tumor, mice were divided into five different groups and treated with saline, MTE, and TAX for once in a week, up to three weeks. Three weeks later, the tumor weight and volume were measured after sacrificing them. We observed that the tumor inhibition rate of high (10 000 mg/kg) and low concentration (2500 mg/kg) of MTE were 2.83% and 20.19% respectively, and median concentration (5000 mg/kg) did not exhibit any antitumor effect (Fig. 6). We also observed that relative tumor volume (RTV) and weight of the tumor were markedly lower in the low concentration of MTE (2.5 g/kg) group compared with the control group. The weight of the mice in the different groups has been changed with increasing time and it has been shown in Fig. 6D. Tumor xenograft model has been shown in Table 1.

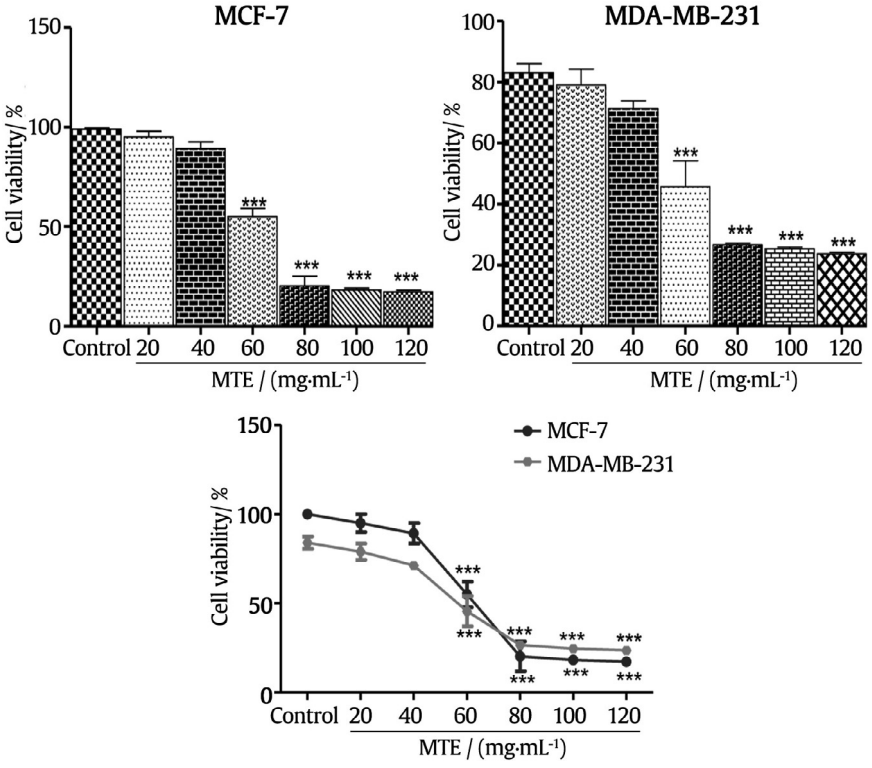
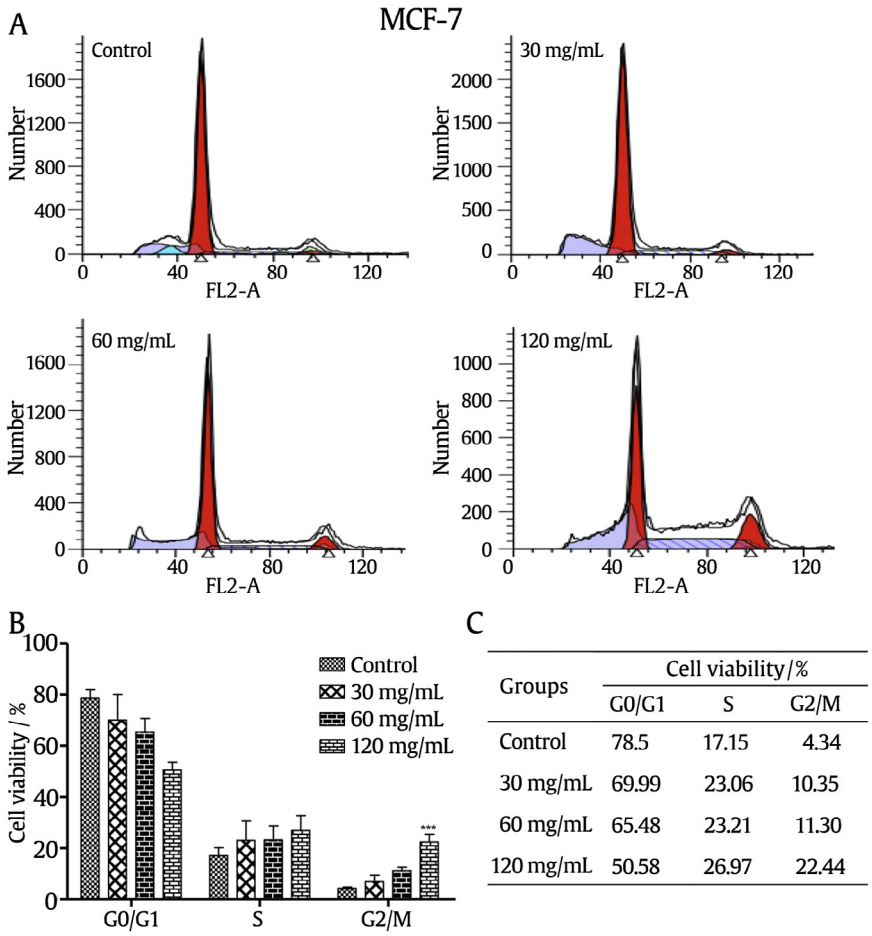


Fig. 1. Cytotoxic effect of MTE on breast cancer cell lines MCF-7 and MDA-MB-231 after 48 h treatment (mean ± SD, $n = 3$). ***$P < 0.001$ *vs* control group.



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Groups	Cell viability/%		
	G0/G1	S	G2/M
Control	78.5	17.15	4.34
30 mg/mL	69.99	23.06	10.35
60 mg/mL	65.48	23.21	11.30
120 mg/mL	50.58	26.97	22.44

Fig. 2. Cell cycle inhibitory effect of MTE on MCF-7 cells.
(A) MCF-7 cells were treated with different concentrations of MTE (0, 30, 60, and 120 mg/mL) for 48 h. (B) Cell cycle experiments were performed in triplicate (mean ± SD, $n = 3$). Two different concentrations of MTE (30 and 60 mg/mL) showed G2/M arrest effect but highest concentration of MTE (120 mg/mL) had significant effect on G2/M arrest compared with control group (***$P < 0.001$). (C) Percentages of cells in different phases of cell cycle were shown, regarding to different MTE concentrations.

3.5. Immunohistochemistry (IHC)

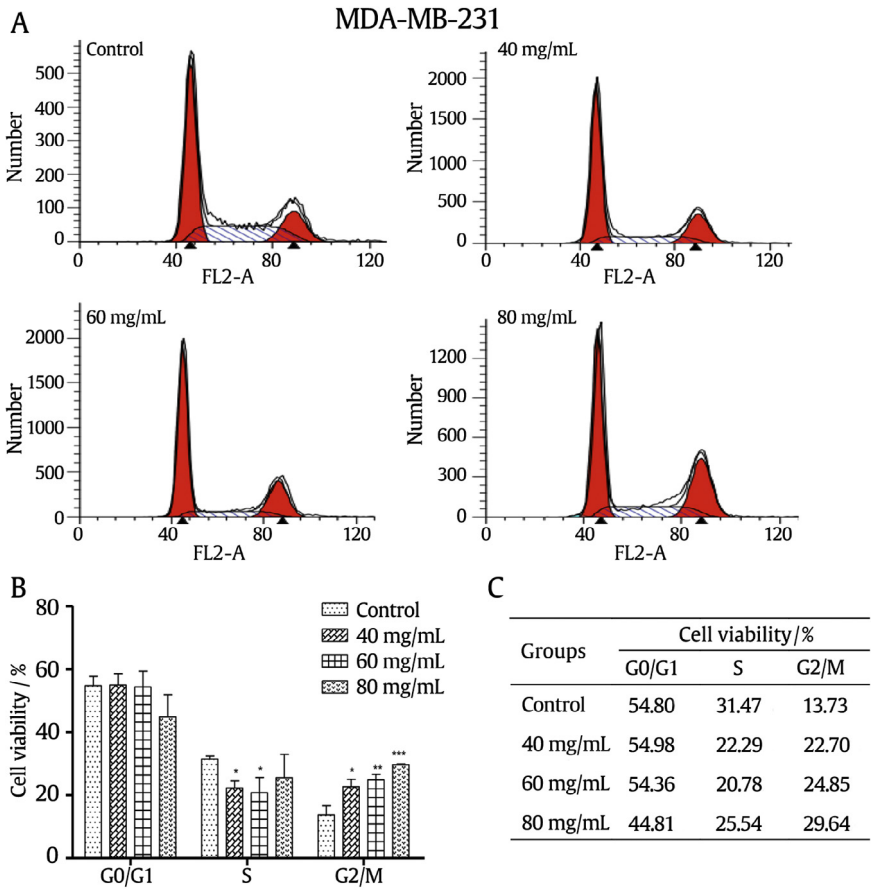
Immunohistochemistry on animal tissue sample was performed to evaluate the expression of G2/M checkpoint associated key protein of the animal tissue sample (Fig. 7). IHC results showed that the expression of cdc2, Cyclin B1, and c-myc were lower compared to the control group, and the expression of p-cdc25c and 14-3-3σ were higher compared to control group.

4. Discussion

We have harvested MDA-MB-231 and MCF-7 cell lines separately, and the cells were then treated with control and MTE of different concentrations. The protein extracted from the control group and MTE treated cells was determined by Western blot. After analyzing the data, we observed that c-myc and cdc25c were overexpressed in the control group. We plotted the graph using different band intensities. As per the graph, the band intensity of 14-3-3σ was decreased and the band intensities of c-myc and cdc25c were increased in the control group. Hence, we concluded that 14-3-3σ was downregulated and c-myc was upregulated in cancer cells, which causes overexpression of cdc25c. Progression of G2/M cell cycle occured due to higher expression of cdc25c. Conversely, 14-3-3σ downregulated c-myc, but 14-3-3σ was downregulated in cancer cells, followed by upregulation of c-myc, which upregulates cdc25c and leads to G2/M cell cycle progression. Furthermore, 14-3-3σ was upregulated in MTE treated cells, which causes downregulation of c-myc followed by lower expression of cdc25c, as a result, the G2/M cell cycle progression was inhibited.

Previous studies reported that MTE has an antiangiogenic effect (Huang et al., 2013). It can synergistically potentiate the anti-growth effect of cisplatin through enhancing infiltration and function of CD_{8+} *T* lymphocytes (Li et al., 2013). MTE also potentiates gefitinib induced antigrowth effect through mediating the action of EGFR in non-small cell lung cancer cells (Han, Hui, Wei, Fei, & Ping, 2014). Previous studies elucidated that more than 40 glycosides were isolated from MTE, but the active compounds for the anticancer action are still unknown (Deng, Zhi, & Dao, 2005; Miyakawa, Kimiko, Koji, Koh, & Hiroshi, 1986; Singhal, Maheshwari, & Anakshi, 1980; Wang et al., 2006; Xia et al., 2004). It has been proved clinically by Nanjing Sanhome Pharmaceutical Co., Ltd., that MTE exerts anticancer effect against esophageal cancer, lung cancer, gastric cancer, and hepatocellular carcinoma (Zhang et al., 2010). As an herbal medicine, MTE extract has been widely used in China as well as many Asian countries for many decades. It is also reported that MTE is commonly used in numerous diseases, such as urinary disease, arthritis, heart disease, skin disease, vomiting, and intermittent fever (Tripathi, Deepti, Aakanksha, Pawan, & Sourabh, 2014).

14-3-3σ is a p53 responsive gene, its expression is decreased in human tumors including breast and prostate cancers, and loss



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Groups	Cell viability/%		
	G0/G1	S	G2/M
Control	54.80	31.47	13.73
40 mg/mL	54.98	22.29	22.70
60 mg/mL	54.36	20.78	24.85
80 mg/mL	44.81	25.54	29.64

Fig. 3. Cell cycle inhibitory effect of MTE on MDA-MB-231 cells.
(A) MDA-MB-231 were treated with different concentrations of MTE (0, 40, 60, and 80 mg/mL) for 48 h. Then cells were harvested for determination of cell cycle inhibitory effect by flow cytometry, data showed that MTE exhibited G2/M arrest. (B) Experiment was carried out in triplicate ($n = 3$). MTE at doses of 40, 60, and 80 mg/mL had significant effect on G2/M arrest compared to control group ($^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$). (C) Percentages of cells in different phases of cell cycle were shown, regarding to different MTE concentrations.

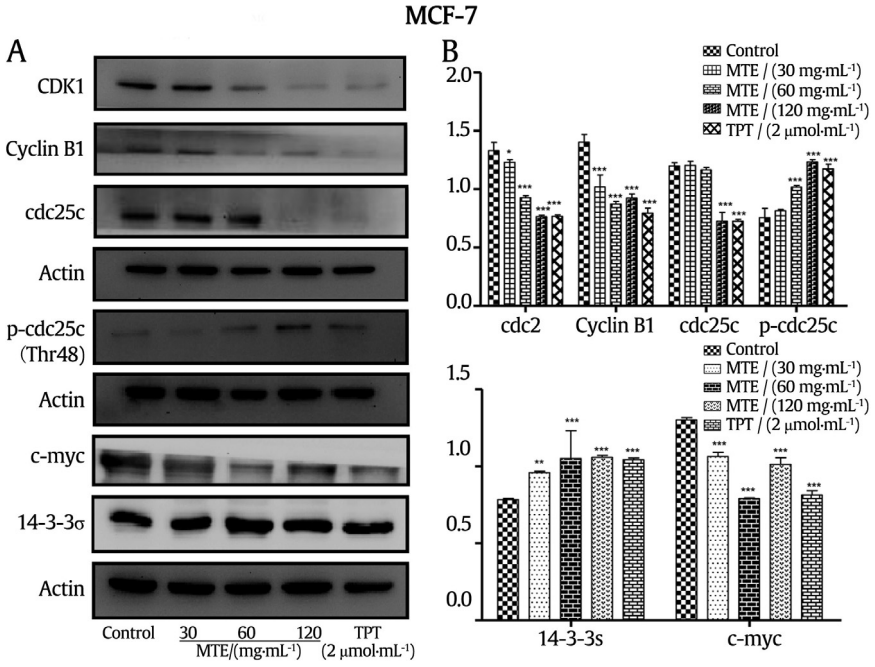

Fig. 4. Cell cycle inhibitory effect of MTE on MCF-7 cells by inducing G2/M associated protein and mediated by 14-3-3σ and c-myc.
(A) MCF-7 cells were treated with different concentrations of MTE (0, 30, 60, and 120 mg/mL) for 48 h, and Western blot analysis was then performed to determine expression of 14-3-3σ, c-myc, and G2/M associated proteins, including cdc2, cyclin B1, cdc25c, and p-cdc25c. (B) Experiment was performed in triplicate ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ *vs* control group.

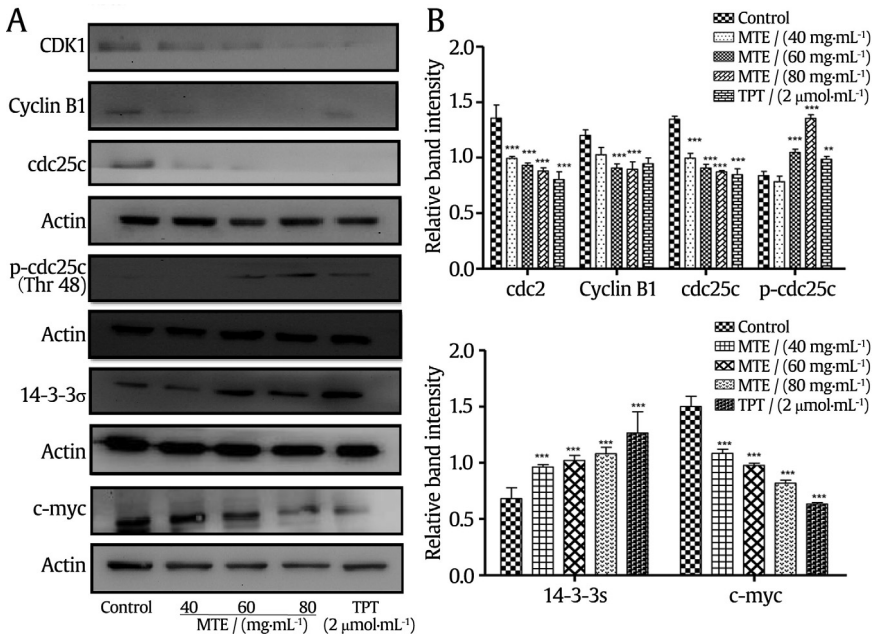

Fig. 5. Cell cycle inhibitory effect of MTE on MDA-MB-231 cells by inducing G2/M associated protein and mediated by 14-3-3σ and c-myc.
(A) MDA-MB-231 cells were treated with different concentrations of MTE (0, 40, 60, and 80 mg/mL) for 48 h, then determined the expression of 14-3-3σ, c-myc, and G2/M associated proteins by using Western blotting. (B) This experiment was performed in triplicate ($n = 3$). $^{**}P < 0.01$ and $^{***}P < 0.001$ *vs* control group.

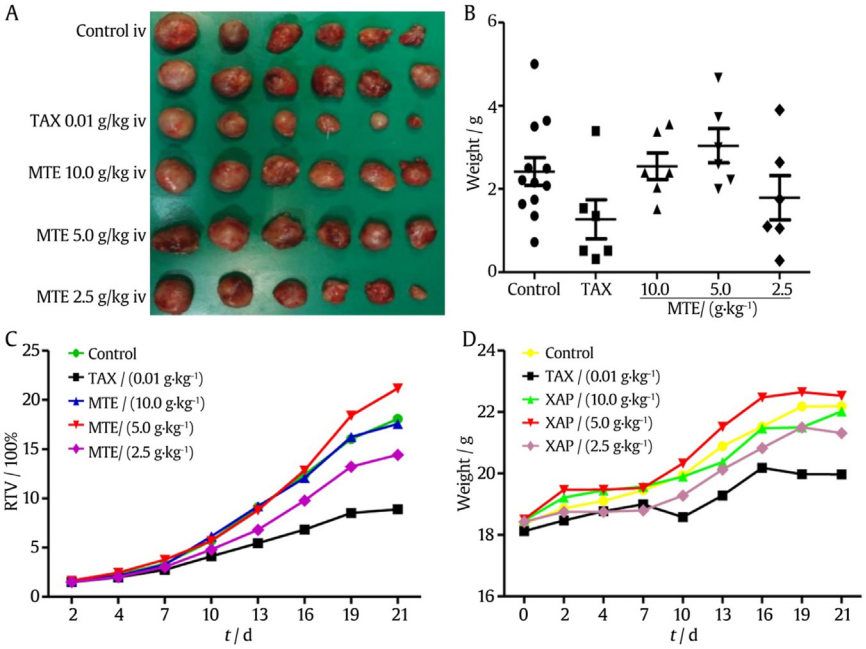

Fig. 6. Therapeutic effect of MTE on animal breast tumor induced by MDA-MB-231 cells *in vivo*.
(A) Therapeutic effects of MTE were evaluated in breast tumor model via tumor volume. (B) Weight of tumor mass in per mice after administrated. (C) Relative tumor volume (RTV) of different groups. (D) Weight of mice in different groups.

Table 1
Effect of MTE on animal tumor induced by human breast cancer MDA-MB-231 cells.

Groups	Dose / (g · kg^{-1})	Number of mice	Weight / g		TV / mm^3		RTV	T/C /%	Inhibition rate /%
			D0	D21	D0	D21			
Control	–	12	18.4 ± 0.88	22.2 ± 1.68	192 ± 62	3469 ± 1268	18.08	–	–
TAX	0.01	6	18.1 ± 1.16	20.0 ± 0.67	194 ± 68	1747 ± 1122	8.89	49.17**	50.83
MTE-H	10.0	6	18.5 ± 1.39	22.0 ± 1.51	189 ± 61	2977 ± 610	17.57	97.17*	2.83
MTE-M	5.0	6	18.5 ± 0.86	22.5 ± 1.75	191 ± 56	3676 ± 1144	21.18	117.15	–.
MTE-L	2.5	6	18.4 ± 1.1	21.3 ± 1.43	191 ± 47	2756 ± 1381	14.43	79.81	20.19

D0 was first day when mice were divided into different groups and taken their initial body weight, and D21 was 21st day after MTE treatment and took their final body weight. T/C denoted RTV of MTE treated group/ RTV of control group. $^{**}P < 0.05$ and $^{*}P < 0.5$ *vs* control group.

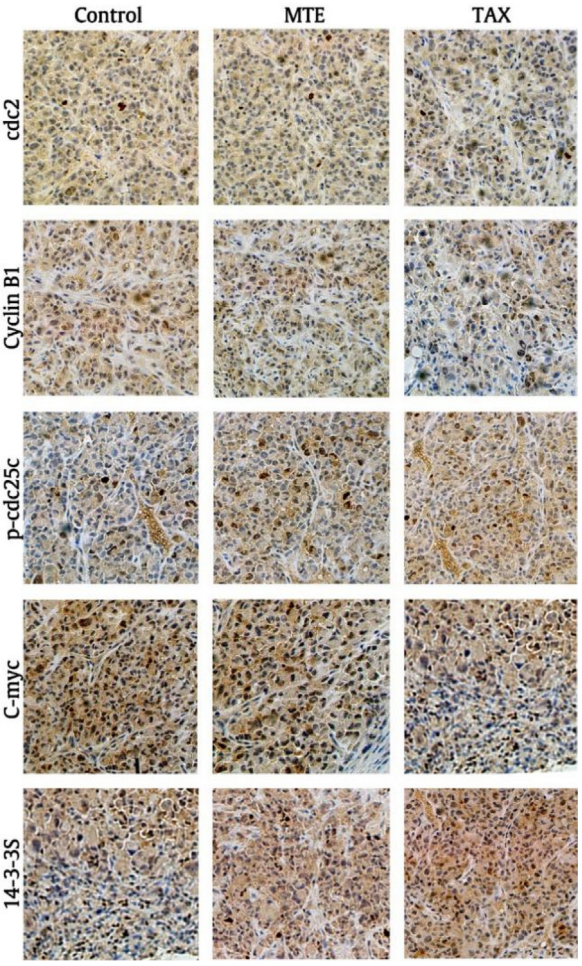

Fig. 7. Expression of G2/M checkpoint associated protein, *i.e.* cdc2, Cyclin B1, p-cdc25c, c-myc, and 14-3-3σ of animal tissue sample.
IHC result showed protein expression of control, MTE and TAX treated group. Expression of cdc2, Cyclin B1, and c-myc were lower compared to control group, and expression of p-cdc25c and 14-3-3σ were higher compared to control group.

of 14-3-3σ occur not only in the tumorous tissues but also in the surrounding tissues (Wilker et al., 2007). The cdc25c dephosphorylates Thr-14 and Thr-15 residues, which is the cause of cdc2 activation and initiates G2/M transition (Liu, Jeffrey, Zhiqi, & Helen, 1997). 14-3-3σ is a key protein which inhibits G2/M progression by sequestering cdc2-cyclin B1 complex and by downregulation of c-myc (Chan, Heiko, Christoph, Kenneth, & Bert, 1999; Wen et al., 2013).

However, we analyzed that MTE also induced cell cycle arrest on G2/M transition on breast cancer cell lines. In this present study, we have shown that MTE exhibited a significant inhibitory effect on G2/M transition by the mechanism of 14-3-3σ/c-myc pathway. By this pathway, all G2/M cell cycle-related protein, Cyclin B1, CDK1, cdc25c, and p-cdc25c are regulated. The c-myc potentiates the action of all these protein and facilitates G2/M progression (Song et al., 2013; Ushmorov, Klaus, & Christian, 2005). However, 14-3-3σ is the backbone of this pathway, which markedly inhibits the action of c-myc and downregulates the effect of Cyclin B1, CDK1, and cdc25c, and upregulates phosphorylation of cdc25c (Hermeking, 2003; Hermeking & Benzinger, 2006). Furthermore, we have performed all experiments simultaneously on two different cell lines, MDA-MB-231 and MCF-7. IC_{50} of both of this cell lines were the same. Based on *in vitro* studies on this cell lines, it was proved that MTE significantly reduces the action of c-myc and side by side markedly, enhances the action of

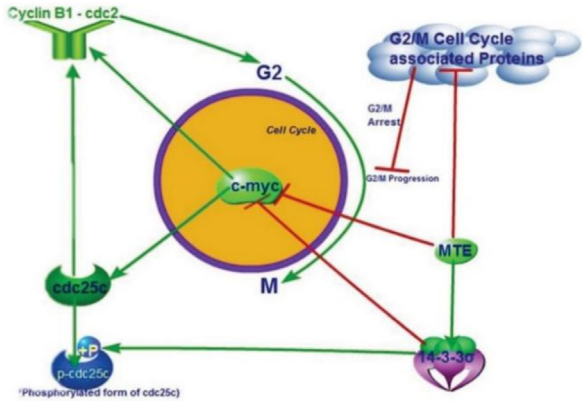

Fig. 8. Proposed mechanism of MTE by modulating G2/M progression mediated by 14-3-3σ and c-myc.

14-3-3σ that regulate checkpoint protein of G2/M phase, and leads to inhibition of cellular entry into mitosis phase (Fig. 8).

5. Conclusion

These findings confirmed that MTE regresses the entry of the cell into the mitosis by accelerating the action of 14-3-3σ which elicits downregulation of c-myc. Generally, the expression of 14-3-3σ is reduced in many cancer types including breast cancer, but we found that MTE enhances the expression of 14-3-3σ. Moreover, our study showed that 14-3-3σ initiated the phosphorylation of cdc25c, which due to the suppression of cdc2 on the G2/M checkpoint. Also, 14-3-3σ was responsible for sequestering of cdc2-cyclin B1, resulting in G2/M cell cycle arrest. We also proved that MTE exploited antitumor action *in vivo*.

Conflict of interest

The authors declare that they have no competing interests.

Acknowledgments

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