



Rhubarb inhibits hepatocellular carcinoma cell metastasis via GSK-3- β activation to enhance protein degradation and attenuate nuclear translocation of β -catenin

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ABSTRACT

The aim of our study was to investigate the mechanisms by which rhubarb regulates β -catenin as well as metastasis of hepatocellular carcinomas. Our results revealed that rhubarb extract inhibited HA22T cell migration ability in wound healing, migration and invasion assays in a dose-dependent manner. Rhubarb also reduced β -catenin protein level, downregulated its downstream proteins, cyclin D, Tbx3 and c-Myc, and attenuated the expression of MMP9 and contactin-1 metastatic factors. Additionally, rhubarb inhibited β -catenin nuclear accumulation and induced its degradation *via* proteasome-mediated pathway. Furthermore, we found that rhubarb suppressed the p-ser⁹ GSK-3- β protein level to inactivate Wnt signalling and reduce β -catenin protein level. Taken together; we found that rhubarb blocked the metastatic process of HA22T hepatocellular carcinoma cells mediated through GSK-3- β activation, and enhancement of protein degradation as well as reduction of the nuclear accumulation of β -catenin.

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1. Introduction

Hepatocellular carcinoma (HCC) is the fifth most frequent neoplasm worldwide, and, in some regions, it represents the primary cause of cancer-related deaths. Years ago, HCC was considered a major health problem in Asia and Africa, with a minor prevalence in Europe and America. However, current data indicate that its incidence is steadily increasing in the West (Bruix, Boix, Sala, & Llovet, 2004).

As observed with other carcinomas, HCC has been attributed to accumulation of genetic alterations; including activation of oncogenes N-ras, H-ras, K-ras (Choudhury, Krishna, & Bhattacharya, 1996), c-erbA (Arbuthnot, Kew, Parker, & Fitschen, 1989), c-met, and c-myc (Huber & Thorgeirsson, 1987), in addition to transcriptional activation of c-jun and nuclear factor-kB by hepatitis B virus factors (Twu, Lai, Chen, & Robinson, 1993). Repression or loss of function of the tumour suppressor p53 is also involved. The expression of these

genes can be used as an index for evaluating differentiation and proliferation of liver malignancy, as well as liver premalignant status (Kondoh *et al.*, 1999). Recently, β -catenin mutations were also found in 19–23% of aetiologically unspecified HCCs, suggesting a role of Wnt signalling pathway in the development of HCCs (Huang *et al.*, 1999).

The therapeutic strategy for HCC patients has to take into account that in the vast majority of individuals, the tumour develops in an already diseased liver with a variable degree of functional impairment. Thereby, treatment should have a limited impact on liver functions. On the other hand, if the degree of liver function impairment is severe, it will determine by itself a very dismal outcome for the patient, irrespective of the treatment success, which would be absolutely irrelevant. Unfortunately, there are no definitive molecular tools to characterise the biology of HCC, and most treatment decisions are still based on parameters such as tumour size and number, as defined by imaging techniques. Patients diagnosed at an early stage can benefit from effective treatments, such as surgical resection, transplantation, percutaneous ablation and palliative therapy.

The metastatic process consists of a series of steps, all of which must be successfully completed to give rise to a secondary tumour.

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As the primary tumour grows, it requires the development of a blood supply to support its metabolic needs, a process known as angiogenesis. These new blood vessels can also provide an escape route by which cells can leave the primary tumour site and enter into the body's circulatory blood system known as intravasation. Tumour cells might also enter the blood circulatory system indirectly via the lymphatic system. Cells need to survive in the circulation until they can reach a new organ; here, they might extravasate from the circulation into the surrounding tissues. Once in the new site, cells must initiate and maintain growth to form pre-angiogenic micrometastases. This growth must be sustained by the development of new blood vessels for a macroscopic tumour to form (Chambers, Groom, & MacDonald, 2002).

The process of metastasis appears to be regulated by a variety of gene products including cell–cell and cell–extracellular matrix receptors (Juliano & Varner, 1993), proteolytic enzymes that facilitate breakdown and invasion of the basement membrane, vascular channels and organs (Matrisian, 1992), motility factors which allow migration through tissues, receptors mediating organ-specific invasion, growth factors necessary for the maintenance of the tumour microcolonies in the secondary organ, and angiogenic factors that result in neovascularisation of the metastasis, allowing the supply of nutrients, removal of metabolites and haematogenous spread of metastatic cells (Folkman, 1995). Weakening of cell–cell adhesion is obviously imperative for tumour cells to metastasise.

The canonical Wnt/Wingless signalling pathway plays a pivotal role in regulating growth as well as cell fate in early and late stages of development (Cadigan & Nusse, 1997). These effects are achieved through stabilisation of β -catenin and its translocation to the nucleus as a coactivator for high mobility group-box proteins of the Tcf/Lef family (Behrens et al., 1996). β -Catenin may exist in three different subcellular forms: membrane-bound (as part of the adherens complex), cytosolic, and nuclear form (Morin, 1999).

For cell–cell adhesion, β -catenin binds the cytoplasmic domain of cadherin adhesion receptors along with the actin binding protein β -catenin, to bridge the extracellular adhesive activity of cadherins with the underlying actin cytoskeleton (Rimm, Koslov, Kebriaei, Cianci, & Morrow, 1995). This cadherin-bound pool of β -catenin ultimately serves to link the cytoskeletal networks of adjacent cells, which are considered essential for normal tissue architecture and morphogenesis (Gottardi & Gumbiner, 2004). However, binding of the protein to other members of the adherens complex; i.e. E-cadherin and β -catenin, is thought to be regulated by tyrosine phosphorylation. Indeed, physical association of the complex with tyrosine kinases and phosphatases is kept under tight regulation (Muller, Choidas, Reichmann, & Ullrich, 1999). Tyrosine phosphorylation of β -catenin leads to its dissociation from the adherens complex and probable transfer of the protein to the cytosol, where it exists in a soluble monomeric state. Cytosolic β -catenin may subsequently be degraded or translocated into the nucleus. The degradation of β -catenin (in the absence of Wnt) involves binding of the protein to a complex involving APC protein, as well as two other proteins, AXIN and GSK-3 β . The latter serves to phosphorylate serine and threonine residues on β -catenin, a crucial step required to target the protein for ubiquitination and proteosomal degradation. Both APC and AXIN enhance this phosphorylation and are, therefore, promoters for β -catenin degradation. For APC protein to have this promoting effect, it must bind to β -catenin via two possible binding regions, one of which contains tandem repeats of 20 amino acids (Polakis, Hart, & Rubinfeld, 1999).

Phosphorylation of β -catenin is needed for binding to the F box protein β -TrCP and hence ubiquitin-mediated proteolysis (Liu et al., 2001). However, it has been recently shown that β -catenin may also be targeted for such degradation independent of GSK-3 β -mediated phosphorylation. This putative alternative pathway requires interaction between β -catenin, APC, and a complex of

proteins including the p53-inducible protein and Siah-1. Activation of the canonical Wnt pathway includes the inhibition of GSK-3 β -mediated phosphorylation of β -catenin that blocks its subsequent proteasomal degradation, leading to nuclear accumulation of β -catenin and transcriptional activation of Tcf/Lef responsive target gene (Levina, Oren, & Ben-Ze'ev, 2004).

A number of downstream target genes of Wnt signalling have been identified in colorectal cancer. These genes play important roles in neoplastic transformation, by affecting growth control and cell cycling (c-Myc, cyclin D1, c-Jun, fra-1, gastrin, WISP-1, and ITF-2), cell survival (Id2, MDR1, and COX2), or invasion and tumour dissemination (matrilysin, laminin γ 2, and VEGF) (Gottardi & Gumbiner, 2004).

Rhubarb root (rhubarb) is one of the oldest and best-known Chinese herbal medicines, appearing in the classic *Materia Medica*, *Shen Nong Ben Cao Jing*1 of the Han dynasty, where it was classified as a top medicinal plant. The most commonly used species is *Rheum palmatum* or *Rheum officinale* Baill of the Polygonaceae family. It has been used traditionally as a laxative, and for treatment of constipation, jaundice, gastro-intestinal haemorrhage, and ulcers (Huang, Lu, Shen, Chung, & Ong, 2007). Currently, many Chinese herbal preparations also contain rhubarb, and can be used for treating liver diseases and inflammation (Li et al., 2009), in addition to prevention of liver fibrosis. Moreover, many papers have reported that rhubarb has an anticancer activity; the water extract of rhubarb can induce apoptosis in human lung adenocarcinoma A549 and human breast cancer MCF-7 cell lines (Li et al., 2009). Emodin is a bioactive constituent of Rhubarb, which can induce apoptosis of human hepatoma cells through a p53-dependent pathway. It can also inhibit prostate cancer cells growth via downregulation of androgen receptors (Cha, Qiu, Chen, Wen, & Hung, 2005).

In this study, we investigated the mechanisms by which rhubarb regulates β -catenin as well as metastasis in HCCs.

2. Methods and materials

2.1. Rhubarb extract

The roots were collected and washed briskly with water and prepared as mentioned by Yang et al. (2010). The samples were freeze dried and preserved at -20°C until use.

2.2. Cell culture

HA22T cells were cultured in Dulbecco's modified Eagle's medium (DMEM). Medium was supplemented with 10% foetal bovine serum (FBS), 1% penicillin, and 0.1 mM non-essential amino acids, 1.5 g/L sodium bicarbonate and 1.0 mM sodium pyruvate and incubated at 37°C in 5% CO_2 .

2.3. Whole cells extraction

Cells were extracted in a cell lysis buffer (50 mM Tris base, 0.5 M NaCl, 1.0 mM EDTA, 1% NP40, 1% glycerol, 1 mM mercaptoethanol, proteinase k inhibitor). The extracts were then clarified by centrifugation.

2.4. Cytoplasmic and nuclear fractionation

HA22T cells cytoplasmic and nuclear fractionation was performed with the extraction reagent, lysis buffer A (50 mM Tris-base, 0.5 M NaCl, 1.0 mM EDTA, 1% NP40, 1% glycerol, 1 mM mercaptoethanol, proteinase k inhibitor) and lysis buffer B (50 mM Tris base, 0.5 M NaCl, 1.0 mM EDTA, 1% glycerol, proteinase k inhibitor). In brief, 5×10^6 cells were trypsinised (0.05% trypsin/0.53 mM

EDTA) and resuspended in 100 μ l lysis buffer B. After a 10-min ice-cold incubation, the suspension was centrifuged at 3000g to pellet the nuclei. The supernatant was then stored for use as the cytoplasmic fraction and the nuclei were lysed with 100 μ l of lysis buffer A.

2.5. Western blotting

Equal concentrations of proteins were resolved on 8–12% SDS-PAGE, and then transferred onto PVDF membranes using electrophoresis. Transferred proteins were blocked in Tween/Tris-buffer saline (TBS) containing 5% skim milk. Membranes were washed and incubated with primary antibodies α -tubulin (Neomarkers Inc., Fremont, CA), ER α , β -catenin, c-myc (all Santa Cruz Biotechnology Inc., Santa Cruz, CA) in TBS plus 2.5% skim milk at recommended concentrations at 4 °C overnight. Subsequently, the membranes were washed and incubated with secondary antibodies for 1 h at room temperature. Antibody reaction was visualised with enhanced chemiluminescence (ECL) reagent for Western blotting.

2.6. Migration assay

Migration assay was performed as mentioned by Shih, Wu, Lee, Shi, and Chiang (2009) using a Boyden chamber and polyvinylpyrrolidone-free polycarbonate membranes with 8- μ m pores (Neuro Probe, Inc., Gaithersburg, MD). Medium containing 10% FBS was applied to the lower chamber and then cells were seeded on the upper chamber in serum free medium. The Boyden chamber was incubated for 4 h at 37 °C; at the end of incubation membranes were carefully removed and stained using 5% Giemsa solution. Migrated cells were counted using a counting grid fitted into an eyepiece of a phase contrast microscope.

2.7. Wound healing assay

Cells were initially seeded uniformly onto 60-mm culture plates with an artificial “wound” carefully created at 0 h, using a P-200 pipette tip to scratch on the subconfluent cell monolayer. After 48 h of culturing in 1% serum-supplemented DMEM, cell migration was observed using a phase contrast microscope.

2.8. Invasion assay

In vitro invasion assay was performed using a Boyden chamber. The surfaces of filter (0.8- μ m pore size) were coated with 50 μ g Matrigel of uniform thickness for 4 h at room temperature. The uniformity of the coating was checked by Coomassie blue staining and low-power microscope observation. The lower chamber was filled with 10% FBS DMEM medium. Cells were re-suspended in serum free DMEM medium onto the upper surface of the filters in the chamber. After 4 h incubation at 37 °C, the filter was gently removed from the chamber. The cells on the upper surface were removed with a cotton swab; cells that had passed through the Matrigel and attached themselves to the lower surface of the filter were fixed with methanol and then stained with Giemsa stain. Cells that migrated through the membrane were counted using a counting grid fitted into the eyepiece of a phase contrast microscope.

2.9. MTT assay

Cells were seeded in 24-well plates and incubated to allow adherence. A solution of 5 mg/ml MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] dissolved in PBS was prepared and filtered. Five hundred microliters of MTT solution were added to each well, and then the plate was incubated in a CO₂ incubator at 37 °C for 4 h. Medium was removed with pipetter, and 500 μ l

isopropanol were added to each well and pipetted to dissolve crystals, followed by transfer to the reading plate, and then analysed at 570 nm.

3. Results

3.1. Rhubarb inhibits the growth of HA22T cells

HA22T cells were treated with rhubarb at 0, 100, 200, 300, 400 and 500 μ g/ml for 24 h. A significant inhibitory effect was noted, compared to the control group (Fig. 1A and B).

3.2. Rhubarb inhibits HA22T cells metastasis in a dose-dependent manner

After 4-h serum free incubation, HA22T cells were treated with rhubarb at 0, 50, 100, 150 μ g/ml for 24 h. Cell migration was then assessed by wound healing, migration (Fig. 2A), and invasion assays (Fig. 2B). Treatment with rhubarb resulted in a substantial inhibition of HA22T cell migration ability, which was reduced by approximately 34%, 56%, and 90% following rhubarb treatment at concentrations of 50, 100, and 150 μ g/ml, respectively. Similar results were found in the invasion assay, which was reduced by approximately 35%, 74%, and 85% after rhubarb treatment at concentrations 50, 100 and 150 μ g/ml, respectively.

3.3. Rhubarb inhibits the β -catenin protein level in HA22T cells

After 4-h serum free incubation, HA22T cells were treated by rhubarb at 0, 50, 100, and 150 μ g/ml for 24 h. β -Catenin protein level was then analysed by Western blotting assay. Moreover, we treated HA22T cells with 100 μ g/ml rhubarb for different times. Rhubarb downregulated β -catenin protein level in both a dose-dependent (Fig. 3) and time-dependent manner (Fig. 4).

3.4. Rhubarb inhibits the nuclear accumulation effects of β -catenin

After 4-h serum free incubation, HA22T cells were treated with rhubarb at concentrations of 0, 50, 100, 150 μ g/ml for 24 h. The cytoplasmic and nuclear fractions of HA22T cancer cells were subjected to SDS-PAGE and then immunoblotted with anti β -catenin, α -tubulin, and HDAC-1. β -Actin was used as a cytoplasmic protein loading control, and HDAC-1 was used as a nuclear protein loading control. The indirect immunofluorescence was performed on HA22T cells using anti β -catenin antibody (1:200, green), followed by DAPI nuclear counterstaining (blue). The merge of β -catenin (green) with DAPI (blue) is also shown. Results showed significant cytosolic decrease of β -catenin in response to rhubarb treatment (Fig. 3A). The same results were obtained by immunofluorescence (Fig. 3B).

3.5. Rhubarb downregulates the downstream proteins of β -catenin

We evaluated the inhibitory effects of rhubarb on Cyclin D, Tbx3 and c-Myc protein levels. HA22T cells were treated with rhubarb at 0, 50, 100, and 150 μ g/ml for 24 h. Cyclin D, Tbx3, c-Myc, and APC protein levels were analysed by Western blotting assay. β -Catenin protein has been shown to produce aberrant transactivation of downstream proto-oncogenes, such as cyclin D1, c-Myc and Tbx3. Our results showed that rhubarb downregulated the downstream proteins of β -catenin (c-Myc, Tbx3 and Cyclin D) in a dose-dependent manner (Fig. 4A and 4B).

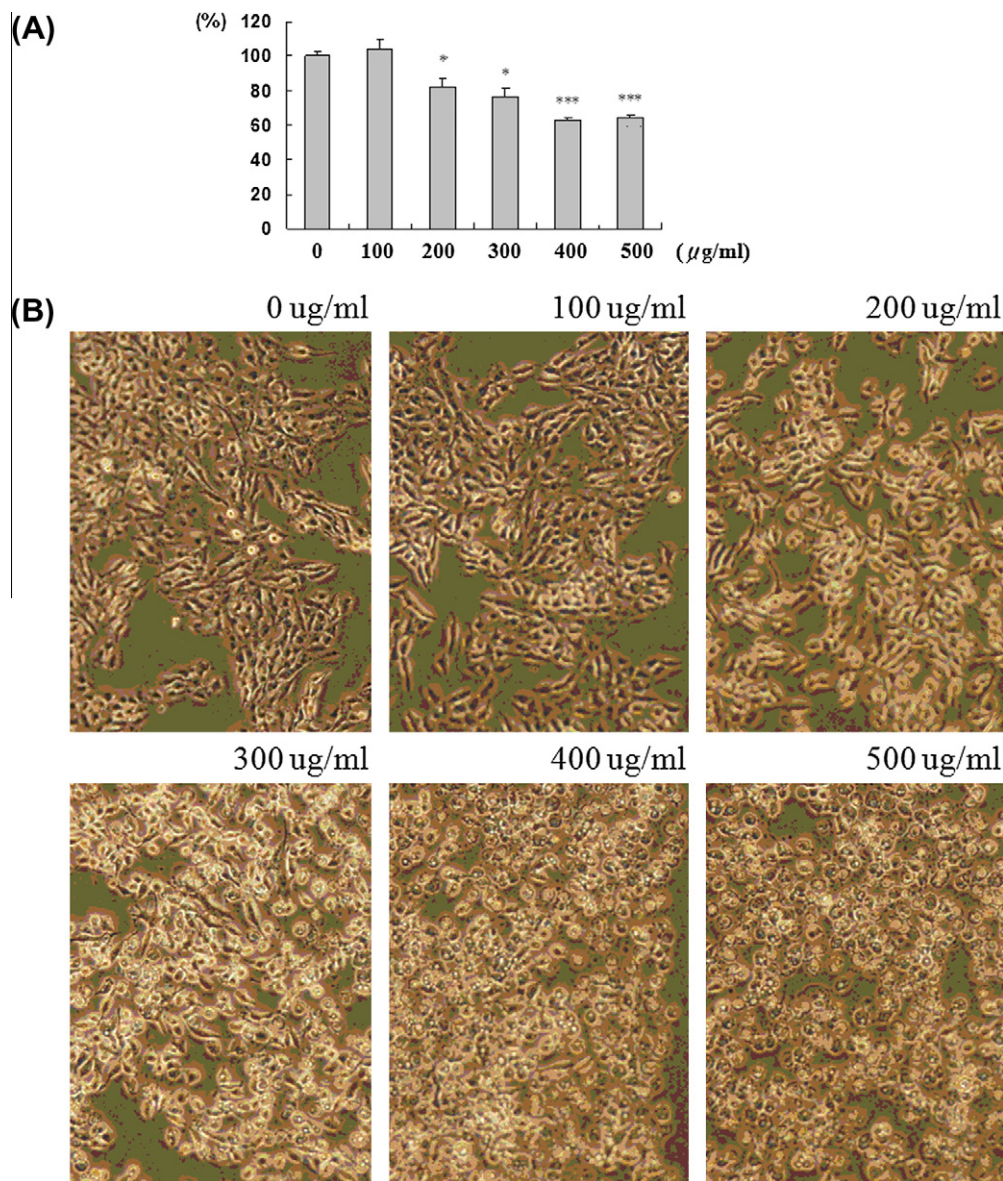


Fig. 1. Growth inhibitory effect of rhubarb on HA22T cells. (A) Cells were treated with rhubarb at 0, 100, 200, 300, 400 and 500 $\mu\text{g/ml}$ for 24 h. Data are expressed as cell survival percentages as mean \pm S.E.M., $n = 3$. * $p < 0.05$ and *** $p < 0.001$ represent significant differences when compared to the control group. (B) Representative photos showing the morphology of rhubarb HA22T cells after treatment with rhubarb water extract at 0 and 500 $\mu\text{g/ml}$ for 24 h using bright field microscopy (200 \times). Treated cells demonstrated rounding and shrinkage at the higher dose.

3.6. Rhubarb down-regulates metastasis factors

Inhibitory effects of rhubarb on MMP9 and Contactin-1 protein levels were observed. HA22T cells were treated by rhubarb at concentrations of 0, 50, 100, and 150 $\mu\text{g/ml}$ for 24 h. The metastasis factors MMP9 and contactin-1 protein levels were analysed by Western blotting assay (Fig. 4C). Result showed that rhubarb downregulated the metastasis factors, MMP9 and contactin-1 protein levels in a dose-dependent manner.

3.7. Rhubarb decreases β -catenin protein level and inactivates Wnt signalling via GSK-3 β activation

After 4-h serum free incubation, HA22T cells were treated with rhubarb at concentrations 0, 50, 100 and 150 $\mu\text{g/ml}$ for 24 h. GSK-3- β and P-Ser⁹ GSK-3- β protein levels were analysed by Western blotting assay. HA22T cells were treated with 100 $\mu\text{g/ml}$ rhubarb for different times. Results displayed that rhubarb downregulated

the P-Ser⁹ GSK-3- β protein levels in both dose (Fig. 5A) and time-dependent manners (Fig. 5B). HA22T cells were pre-treated with GSK-3- β inhibitor at 10 μM for 1 h, and rhubarb at 100 $\mu\text{g/ml}$ was added into the cells for 24 h. β -Catenin protein levels were analysed by Western blotting assay. Results showed that rhubarb enhanced β -catenin degradation. This effect was blocked by pre-treatment with GSK-3- β inhibitor (Fig. 5C).

3.8. Rhubarb enhances β -catenin degradation through proteasome-mediated pathway

After 4-h serum free incubation, HA22T cells were pre-treated with proteasome inhibitor at 0, 1, 5, 10 μM for 1 h, followed by addition of rhubarb (100 $\mu\text{g/ml}$) for 24 h. GSK-3- β and P-Ser⁹ GSK-3- β protein levels were analysed by Western blotting assay. Rhubarb enhanced β -catenin degradation. This effect was blocked by pre-treatment with proteasome inhibitor (Fig. 6).

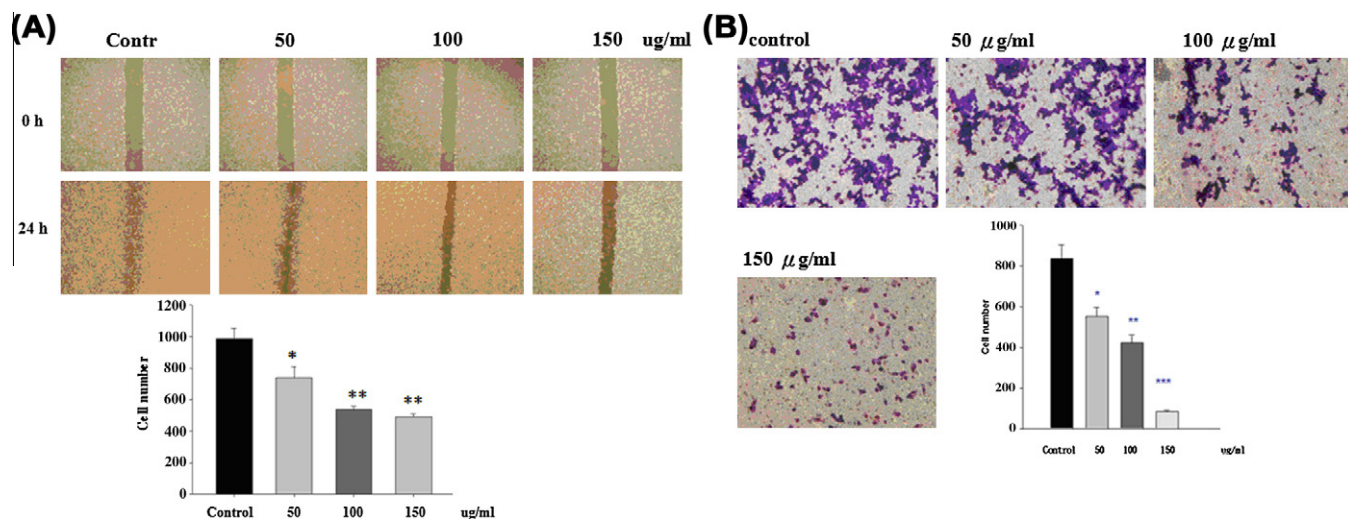


Fig. 2. Effect of rhubarb on the invasion of HA22T cells. (A) HA22T cells were treated with rhubarb at 0, 50, 100, and 150 µg/ml for 24 h. Cell migration was assessed by wound healing assay. Overall mean values of cells migration from triplicate experiments is shown in the bar graph compared with control group. *indicates statistically significant difference ($p < 0.05$). (B) HA22T cells were treated with rhubarb at 0, 50, 100, and 150 µg/ml for 24 h. Cell migration was then assessed by Boyden chambers. Invasion assay was performed as described in "Methods". Overall mean values of cells invasion from triplicate experiments is shown in the bar graph compared with control group. *indicates a significant difference ($p < 0.05$), and ***indicates a significant difference ($p < 0.001$).

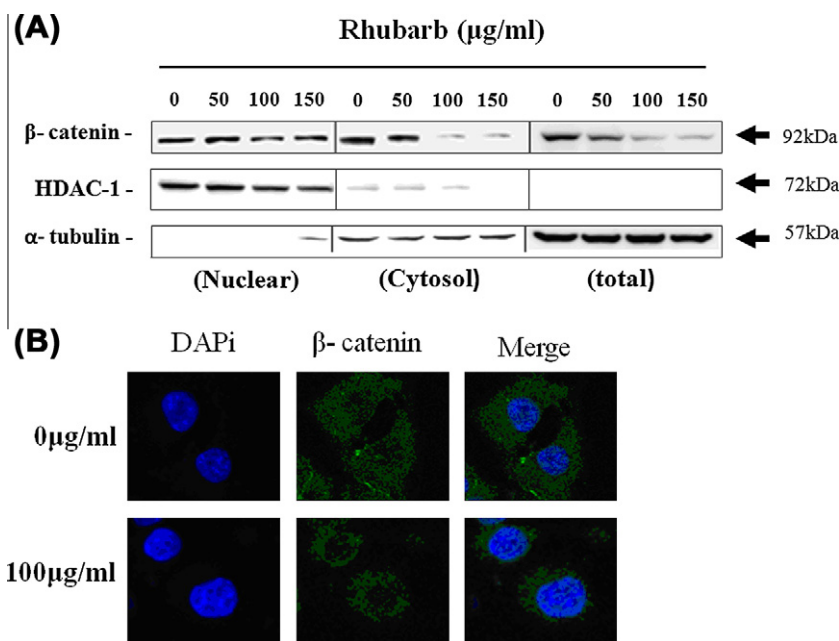


Fig. 3. HA22T cells were treated with at 0, 50, 100, and 150 µg/ml rhubarb for 24 h. (A) Cytoplasmic and nuclear fractions of liver cancer cells were subjected to SDS-PAGE and then immunoblotted with anti β-catenin, α-tubulin, and HDAC-1. α-Tubulin was used as a cytoplasmic protein loading control, and HDAC-1 was used for nuclear protein loading control. (B) Indirect immunofluorescence was performed on HA22T cells using anti β-catenin antibody (1:200, green), followed by DAPI nuclear counterstaining (blue). The merge of β-catenin (green) with DAPI (blue) is also shown (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

4. Discussion

The activation of Wnt pathway is involved in the carcinogenesis of HCC (Tian et al., 2012). Activation of the Wnt pathway in colon cancer and melanomas may be attributed to either β-catenin mutations involving the GSK-3 β phosphorylation sites or inactivation of APC, in addition to other related factors. Loss of heterozygosity at the APC locus on chromosome 5 has been detected only at low frequency in human HCC, suggesting that inactivation of APC may be infrequent. Mutation of exon 3 of β-catenin gene is probably one of the most important factors for activating Wnt pathway with

subsequent cytoplasmic accumulation of β-catenin protein HCC (Cieply, Zeng, Proverbs-Singh, Geller & Monga, 2009). Abnormal expression of β-catenin protein, especially the accumulated type, is closely related to the invasiveness of HCC among Chinese people.

Rhubarb has been used for treating liver diseases and inflammation (Li et al., 2009), and is reported to have anticancer activity (Cha et al., 2005). Rhubarb was reported to attenuate TGF-β1-mediated migration of hepatic stellate cells, possibly by interfering with Smad2/3 phosphorylation, the MAPK pathway, and MMP-2 activity (Lin, Wu, & Huang, 2009). Despite the fact that rhubarb biological activities have been investigated extensively, the

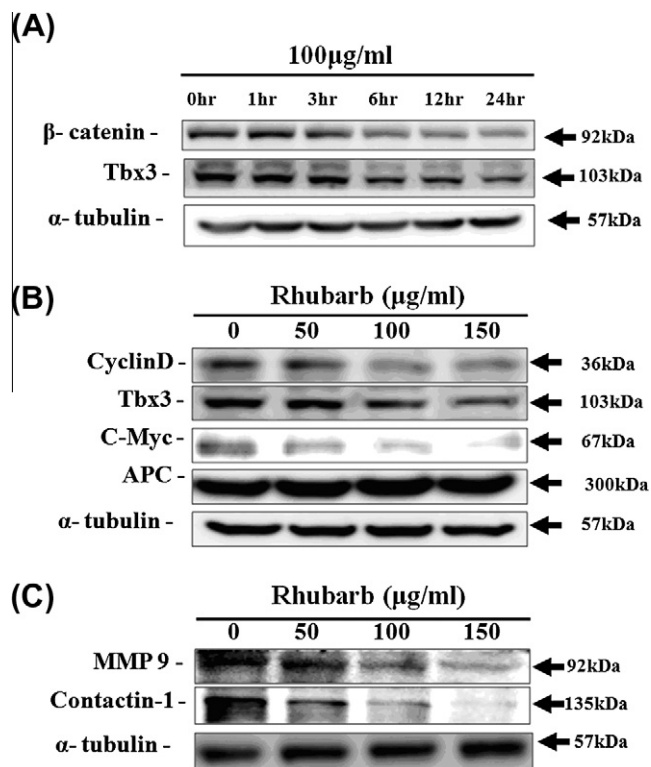


Fig. 4. Effect of rhubarb on β -catenin and its downstream protein Tbx3 expression. (A) and (B) Effect of rhubarb on Wnt β -catenin downstream proteins. Cells were treated with rhubarb at 0, 50, 100, and 150 $\mu\text{g/ml}$ for 24 h. Downregulation of Cyclin D, Tbx3, c-Myc, and APC proteins levels in a dose-dependent manner. (C) Dose-dependent downregulation of the metastasis factors MMP9 and contactin-1 protein levels.

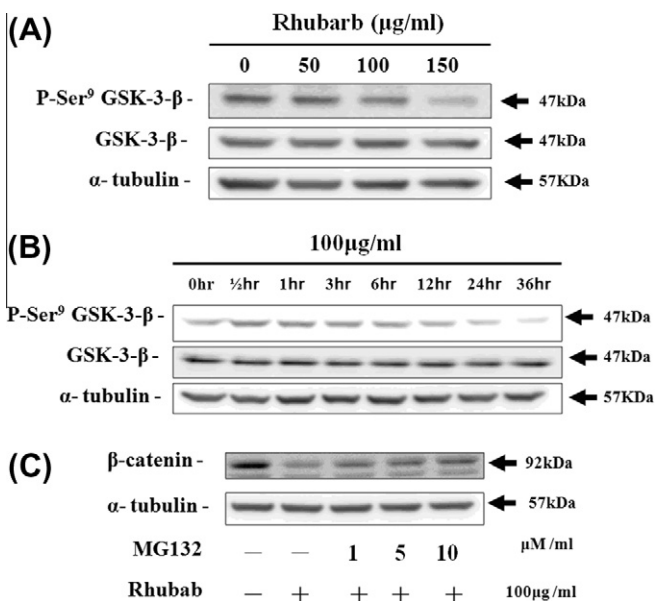


Fig. 5. Effect of rhubarb on GSK-3- β and P-Ser⁹ GSK-3- β expression. (A) HA22T cells were treated with rhubarb at 0, 50, 100, and 150 $\mu\text{g/ml}$ for 24 h. Downregulation of GSK-3- β and P-Ser⁹ GSK-3- β protein levels revealed by Western blotting. (B) HA22T cells were treated with 100 $\mu\text{g/ml}$ rhubarb for various time durations. Western blotting showed time dependent down-regulation of GSK-3- β and P-Ser⁹ GSK-3- β protein levels.

relationship between rhubarb and HCC metastasis was rarely discussed. Therefore, we designed this study to determine whether rhubarb can inhibit the β -catenin positive HA22T liver cancer cell

metastasis. Our results showed that rhubarb induced a substantial inhibition of HA22T cells migration and invasion assays in a dose-dependent manner, suggesting an inhibitory effect on HA22T metastasis.

An association between expression, localisation and gene mutation of β -catenin and various cancers has been reported. Moreover, β -catenin over-expression is suggested to be related to tumour progression and cell proliferation (Huber & Thorgeirsson, 1987). Our previous results indicated that β -catenin plays a key role in HCC metastasis as well. We treated HA22T cells with 100 $\mu\text{g/ml}$ rhubarb for different times and analysed β -catenin protein level by Western blotting. Our results revealed that rhubarb downregulated β -catenin protein levels in both dose and time-dependent manners. Additionally, our results showed a significant cytosolic decrease of β -catenin, as well as a decreased nuclear translocation of β -catenin, which was further confirmed by immunofluorescence assay.

The serine/threonine kinase GSK3- β binds to and phosphorylates several proteins in the Wnt pathway and induces downregulation of β -catenin. As a negative regulator of Wnt signalling, GSK3- β can be considered as a potential tumour suppressor (Sparks, Morin, Vogelstein, & Kinzler, 1998). Our results revealed that rhubarb downregulated P-Ser⁹ GSK-3- β protein levels in both dose and time-dependent manners, suggesting that rhubarb might activate GSK-3- β to reduce β -catenin protein levels.

Prior to discussing the potential role for LEF/TCF transcription factors in cancer, it is important to outline the mechanism by which they have been proposed to operate. Although LEF/TCFs bind directly to DNA through their HMG domains, they are incapable of activating gene transcription independently (Eastman & Grosschedl, 1999). A number of downstream target genes of Wnt signalling have been identified in colorectal cancer. These genes play important roles in neoplastic transformation through the effects on growth control and cell cycling (c-Myc, cyclin D1, c-Jun, fra-1, gastrin, WISP-1, ITF-2), cell survival (Id2, MDR1, COX2), invasion and tumour dissemination (matrilysin, laminin γ 2, VEGF) (Gottardi & Gumbiner, 2004). Tbx3 is also reported to be a critical mediator of β -catenin survival functions in liver cancer (Renard et al., 2007). In our experiments, rhubarb suppressed β -catenin expression and inactivated Wnt signalling leading to inhibition of the downstream proteins (c-Myc, Tbx3, and Cyclin D).

The 26S proteasome is a highly organised multi-subunit complex, which is the primary protease of the ubiquitin-mediated proteolytic system with a molecular mass of \sim 2500 kDa that catalyses the ATP-dependent degradation of ubiquitinated proteins (Coux, Tanaka, & Goldberg, 1996). The ubiquitin-proteasome pathway consists of several components that act sequentially in a hierarchical mode: a concerted two-step reaction that results in a high-energy thioester linkage between ubiquitin and a single conserved ubiquitin-activating enzyme (E1) and ubiquitin transfer through transacylation to one of several ubiquitin-conjugating enzymes (E2). The latter collaborates with a large series of ubiquitin ligases (E3) in attaching ubiquitin molecules to the ϵ -amino group of the substrate's lysine residues, thus forming a reversible isopeptide bond. For proteolysis-associated ubiquitination, polymerisation of a ubiquitin chain, which is facilitated by the same E2-E3 pair that attaches the first ubiquitin molecule to the substrate is required for the next catalytic step. The polyubiquitin chain then serves as a recognition marker for the substrate degrading 26S proteasome (Bachmair, Novatchkova, Potuschak, & Eisenhaber, 2001). This system is highly conserved in all eukaryotes and plays an important role in a broad array of cellular and developmental processes (Weissman, 2001). In our experiment, we blocked the 26S proteasome activities by a proteasome inhibitor MG-132. We pre-treated with proteasome inhibitor at 0, 1, 5 and 10 μM for 1 h before rhubarb addition; we observed that in pre-treated proteasome inhibitor plus rhubarb, the β -catenin protein level is rescued compared to rhubarb-only treatment at 100 $\mu\text{g/ml}$.

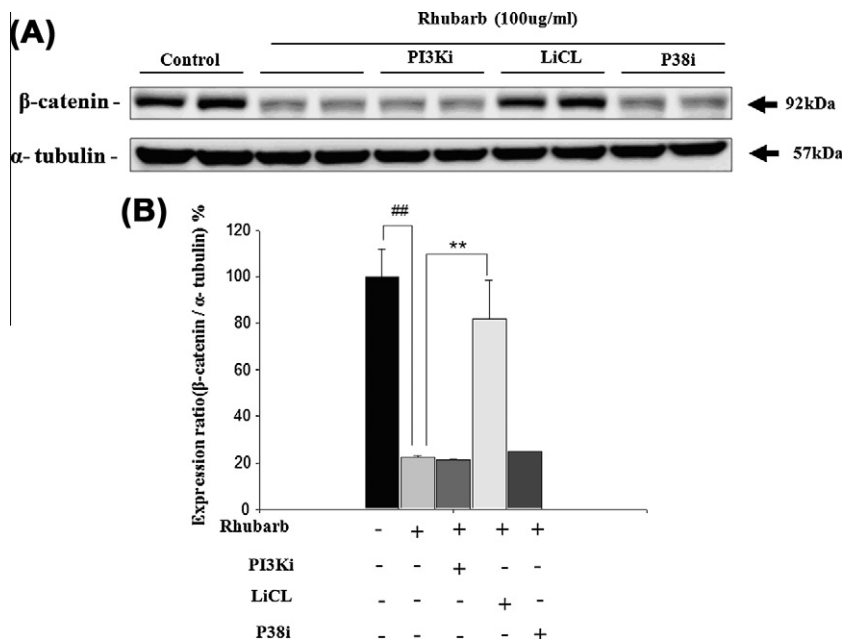


Fig. 6. Rhubarb can increase the degradation of β -catenin through the proteasome. (A) Rhubarb can increase the degradation of β -catenin via active the GSK-3- β . After 4-h serum free incubation, HA22T cells were pre-treated with PI3Ki P38 inhibitor and LiCl for 1 h, then rhubarb (100 μ g/ml) was added for 24 h. β -Catenin protein level was analysed by Western blotting. (B) β -Catenin/ α -tubulin protein level expressions in (A) were analysed and shown in a bar graph compared with control group. **indicates a significant difference ($p < 0.01$) compared with rhubarb treatment group. ##; indicates a significant difference ($p < 0.01$) compared with control group.

In summary, we found that rhubarb decreased β -catenin nuclear translocation and β -catenin degradation through a proteasome-mediated pathway, which is dependent on GSK-3- β activities, with subsequent inhibition of HA22T HCC cell metastasis function.

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Reference

Arbuthnot, P., Kew, M., Parker, I., & Fitschen, W. (1989). Expression of c-erbA in human hepatocellular carcinomas. *Anticancer Research*, 9(4), 885–887.

Bachmair, A., Novatchkova, M., Potuschak, T., & Eisenhaber, F. (2001). Ubiquitylation in plants: A post-genomic look at a post-translational modification. *Trends in Plant Science*, 6(10), 463–470.

Behrens, J., von Kries, J. P., Kuhl, M., Bruhn, L., Wedlich, D., Grosschedl, R., & Birchmeier, W. (1996). Functional interaction of beta-catenin with the transcription factor LEF-1. *Nature*, 382(6592), 638–642.

Bruix, J., Boix, L., Sala, M., & Llovet, J. M. (2004). Focus on hepatocellular carcinoma. *Cancer Cell*, 5(3), 15–219.

Cadigan, K. M., & Nusse, R. (1997). Wnt signaling: A common theme in animal development. *Genes & Development*, 11(24), 3286–3305.

Cha, T. L., Qiu, L., Chen, C. T., Wen, Y., & Hung, M. C. (2005). Emodin down-regulates androgen receptor and inhibits prostate cancer cell growth. *Cancer Research*, 65(6), 2287–2295.

Chambers, A. F., Groom, A. C., & MacDonald, I. C. (2002). Dissemination and growth of cancer cells in metastatic sites. *Nature Reviews Cancer*, 2(8), 563–572.

Choudhury, S., Krishna, M., & Bhattacharya, R. K. (1996). Activation of ras oncogenes during hepatocarcinogenesis induced by N-nitrosodiethylamine: possible involvement of PKC and GAP. *Cancer Letters*, 109(1–2), 149–154.

Coux, O., Tanaka, K., & Goldberg, A. L. (1996). Structure and functions of the 20S and 26S proteasomes. *Annual Review of Biochemistry*, 65, 801–847.

Eastman, Q., & Grosschedl, R. (1999). Regulation of LEF-1/TCF transcription factors by Wnt and other signals. *Current Opinion in Cell Biology*, 11(2), 233–240.

Folkman, J. (1995). Angiogenesis in cancer, vascular, rheumatoid and other disease. *Nature Medicine*, 1(1), 27–31.

Gottardi, C. J., & Gumbiner, B. M. (2004). Distinct molecular forms of beta-catenin are targeted to adhesive or transcriptional complexes. *Journal of Cell Biology*, 167(2), 339–349.

Huang, H., Fujii, H., Sankila, A., Mahler-Araujo, B. M., Matsuda, M., Cathomas, G., & Ohgaki, H. (1999). Beta-catenin mutations are frequent in human hepatocellular carcinomas associated with hepatitis C virus infection. *American Journal of Pathology*, 155(6), 1795–1801.

Huang, Q., Lu, G., Shen, H. M., Chung, M. C., & Ong, C. N. (2007). Anti-cancer properties of anthraquinones from rhubarb. *Medicinal Research Reviews*, 27(5), 609–630.

Huber, B. E., & Thorgeirsson, S. S. (1987). Analysis of c-myc expression in a human hepatoma cell line. *Cancer Research*, 47(13), 3414–3420.

Juliano, R. L., & Varnier, J. A. (1993). Adhesion molecules in cancer: the role of integrins. *Current Opinion in Cell Biology*, 5(5), 812–818.

Kondoh, N., Wakatsuki, T., Ryo, A., Hada, A., Aihara, T., Horiuchi, S., Goseki, N., Matsubara, O., Takenaka, K., Shichita, M., Tanaka, K., Shuda, M., & Yamamoto, M. (1999). Identification and characterization of genes associated with human hepatocellular carcinogenesis. *Cancer Research*, 59(19), 4990–4996.

Levina, E., Oren, M., & Ben-Ze'ev, A. (2004). Downregulation of beta-catenin by p53 involves changes in the rate of beta-catenin phosphorylation and Axin dynamics. *Oncogene*, 23(25), 4444–4453.

Li, W. Y., Chan, S. W., Guo, D. J., Chung, M. K., Leung, T. Y., & Yu, P. H. (2009). Water extract of *Rheum officinale* Baill. Induces apoptosis in human lung adenocarcinoma A549 and human breast cancer MCF-7 cell lines. *Journal of Ethnopharmacology*, 124(2), 251–256.

Lin, Y. L., Wu, C. F., & Huang, Y. T. (2009). Effects of rhubarb on migration of rat hepatic stellate cells. *Journal of Gastroenterology and Hepatology*, 24(3), 453–461.

Liu, J., Stevens, J., Rote, C. A., Yost, H. J., Hu, Y., Neufeld, K. L., White, R. L., & Matsunami, N. (2001). Siah-1 mediates a novel beta-catenin degradation pathway linking p53 to the adenomatous polyposis coli protein. *Molecular Cell*, 7(5), 927–936.

Matrisian, L. M. (1992). The matrix-degrading metalloproteinases. *Bioessays*, 14(7), 455–463.

Morin, P. J. (1999). Beta-catenin signaling and cancer. *Bioessays*, 21(12), 1021–1030.

Muller, T., Choidas, A., Reichmann, E., & Ullrich, A. (1999). Phosphorylation and free pool of beta-catenin are regulated by tyrosine kinases and tyrosine phosphatases during epithelial cell migration. *The Journal of Biological Chemistry*, 274(15), 10173–10183.

Polakis, P., Hart, M., & Rubinfeld, B. (1999). Defects in the regulation of beta-catenin in colorectal cancer. *Advances in Experimental Medicine and Biology*, 470, 23–32.

Renard, C. A., Labalette, C., Armengol, C., Cougot, D., Wei, Y., Cairo, S., Pineau, P., Neuveut, C., de Reyniès, A., Dejean, A., Perret, C., & Buendia, M. A. (2007). Tbx3 is a downstream target of the Wnt/beta-catenin pathway and a critical mediator of beta-catenin survival functions in liver cancer. *Cancer Research*, 67(3), 901–910.

Rimm, D. L., Koslov, E. R., Kebriaei, P., Cianci, C. D., & Morrow, J. S. (1995). Alpha 1(E)-catenin is an actin-binding and-bundling protein mediating the attachment of F-actin to the membrane adhesion complex. *Proceedings of the National Academy of Sciences of the United States of America*, 92(19), 8813–8817.

Shih, Y. W., Wu, P. F., Lee, Y. C., Shi, M. D., & Chiang, T. A. (2009). Myricetin suppresses invasion and migration of human lung adenocarcinoma A549 cells:

- Possible mediation by blocking the ERK signaling pathway. *Journal of Agricultural Food Chemistry*, 57(9), 3490–3499.
- Sparks, A. B., Morin, P. J., Vogelstein, B., & Kinzler, K. W. (1998). Mutational analysis of the APC/beta-catenin/Tcf pathway in colorectal cancer. *Cancer Research*, 58(6), 1130–1134.
- Twu, J. S., Lai, M. Y., Chen, D. S., & Robinson, W. S. (1993). Activation of protooncogene c-jun by the X protein of hepatitis B virus. *Virology*, 192(1), 346–350.
- Weissman, A. M. (2001). Themes and variations on ubiquitylation. *Nature Reviews Molecular Cell Biology*, 2(3), 169–178.
- Yang, C. J., Huang, Y. J., Wang, C. Y., Wang, P. H., Hsu, H. K., Tsai, M. J., Chen, Y. C., Kumar, V. B., Huang, M. S., & Weng, C. F. (2010). Antiproliferative effect of *Toona sinensis* leaf extract on non-small-cell lung cancer. *Translational Research*, 155(6), 305–314.