Abstract. As a main active compound in the bark of waxberry (Myrica rubra), myricetin is a macrocyclic diarylheptanoid, and can trigger the apoptosis of HeLa and PC3 cells. The aim of the present study was to elucidate the anticancer effect of myricetin on human breast cancer MCF-7 cells and to explore the possible mechanisms of action. MCF-7 cells were treated with different concentrations of myricetin (0-80 µM) for 12, 24 and 48 h. In the present study, we found that myricetin suppressed the cell viability of the MCF-7 cells at least partly through the induction of apoptosis as determined by MTT assay and flow cytometry. Western blot analysis revealed that myricetin effectively suppressed the protein expression of p21-activated kinase 1 (PAK1), MEK and phosphorylated extracellular mitogen-activated protein kinase (ERK1/2). In addition, treatment of myricetin activated glycogen synthase kinase-3β (GSK3β) and Bax protein expression, and inhibited β-catenin/cyclin D1/proliferating cell nuclear antigen (PCNA)/survivin and promoted caspase-3 activity in the MCF-7 cells. These results demonstrated that myricetin suppressed the cell viability of human breast cancer MCF-7 cells through PAK1/MEK/ERK/GSK3β/β-catenin/cyclin D1/PCNA/survivin/Bax-caspase-3 signaling.

Introduction

Breast cancer is the most common malignant cancer among females. The highest morbidity occurs in northern America and northern European countries (1). Despite the fact that China is a country with low incidence, the morbidity is increasing year by year due to changes in dietary structures, living standards and life styles (2). According to new data, the morbidity of breast cancer is increasing yearly, and it is becoming a tumor with the highest death rates (3). Meanwhile, the age at onset is becoming increasingly younger (3).

p21-activated kinase 1 (PAK1) was first found as a member of the Pak family (4). Initially, it was cloned from cerebral tissues as p21 kinase (4). Members of the Pak family play an essential role in immune escape, motility, angiogenesis and genetic regulation (5). Therefore, the Pak family may constitute the critical node of signal transduction in the process of tumor progression. During the evolutionary process of colorectal malignant tumors, expression of Pak1 is increased. Recent studies have found that the activation of Pak1 is necessary for inducing lysophosphatidic acid and autotoxin in melanoma cells (6). In addition, Pak1 was found to be highly expressed in head and neck neoplasms (7).

As a key transmitter factor for the Wnt signal channel, β-catenin is expressed in many types of tumors (8). Its oncogenic potential in in vitro culture models and in vivo animal experiments have been extensively explored. The nuclear accumulation of β-catenin is generally considered as the symbol of Wnt/β-catenin signal routine. β-catenin accumulates and enters into the nucleus, which induces the expression of target genes (9).

Waxberry is a plant of the genus Myricaceae (10). Geographically, it is distributed between 18 and 33º north latitude while its economic cultivation is mainly distributed in southeast coast regions, such as Zhejiang, Jiangsu, Fujian, Guangdong and Jiangxi Provinces. Myricetin, found in the bark of waxberry, is bitter in taste and warm in property with antiviral, anti-inflammatory, antioxidant, free radical scavaging, immune adjustment, anti-androgenic and antiallergic functions (11-13). In the present study, we examined the anticancer effects of myricetin. We found that myricetin suppressed the cell viability of human breast cancer MCF-7 cells. The mechanisms involved in the effects of myricetin were also investigated.

Materials and methods

Cell culture and cell viability. Human breast cancer MCF-7 cells were maintained in RPMI-1640 medium supplemented with 10% US-qualified fetal bovine serum (FBS) (both from
Invitrogen, Grand Island, NY, USA) in a humidified incubator with 5% CO₂ at 37°C. MCF-7 cells (1x10⁴) were seeded into a 96-well plate, incubated at 37°C and then treated with different concentrations of myricetin (0-80 µM) for 12, 24 and 48 h. The medium was removed, and 50 µl MTT (5 mg/ml) was added to each well and then incubated at 37°C for 4 h. The supernatant was removed, and 200 µl of dimethyl sulfoxide (DMSO; Invitrogen) was dissolved for 20 min. Absorbance was measured at 490 nm.

**Flow cytometric analysis of the apoptotic rate.** MCF-7 cells (1x10⁶) were seeded into a 6-well plate, incubated at 37°C, and then treated with different concentrations of myricetin (0, 10, 20 and 40 µM) for 24 h. MCF-7 cells were washed with cold phosphate-buffered saline (PBS) twice and re-suspended in binding buffer. Subsequently, 5 µl of FITC Annexin V and 1 µl propidium iodide (PI) were added to the cells and incubated for 20 min at room temperature in the dark. The apoptotic rate was determined by flow cytometry (FACSCalibur system; BD Biosciences, San Jose, CA, USA).

**Western blot analysis.** MCF-7 cells (1x10⁶) were seeded into a 6-well plate, incubated at 37°C and then treated with different concentrations of myricetin (0, 10, 20 and 40 µM) for 24 h. MCF-7 cells were lysed in 100 µl mammalian protein extraction reagent (M-PER; Pierce, Rockford, IL, USA) and centrifuged at 7,500 x g for 15 min at 4°C. Total protein levels were determined by a BCA protein assay kit (Pierce). SDS-PAGE was performed using equivalent protein extracts (60 µg) from each sample, which were then blotted onto a nitrocellulose membrane using a Mini-Protean 3 system (Bio-Rad, Hercules, CA, USA). The blots were incubated in PBS containing 5% non-fat dry milk for 1 h. The membranes were incubated with the primary antibodies PAK1, MEK1/2, ERK1/2, GSK3β, β-catenin, cyclin D1, PCNA, survivin, Bax and β-actin at 4°C overnight. The membranes were then incubated with secondary antibody dilutions, washed with PBS containing 5% non-fat dry milk and visualized using enhanced chemiluminescence detection reagents (ECL Advance Western Blotting Detection kit; Amersham, UK).

**Enzyme-linked immunosorbent assay (ELISA).** The MCF-7 cells (1x10⁴) were seeded into a 96-well plate, incubated at 37°C and then treated with different concentrations of myricetin (0, 10, 20 and 40 µM) for 24 h. The caspase-3 assay kit (Ac-DEVD-pNA, 2 mM) was used to detect caspase-3 enzymatic activity in the MCF-7 cells. The absorbance was measured at 405 nm.

**Statistical analysis.** Data are expressed as mean ± standard error of the mean (SEM) and analyzed using ANOVA. The results were analyzed using a post hoc test (two-sided Dunnett's test) and one-way analysis of variance (ANOVA) to test differences between each treatment and the control. A p-value of <0.05 was considered to indicate a statistically significant result.

**Results**

**Myricetin suppresses the cell viability of human breast cancer MCF-7 cells.** The chemical structure of myricetin is shown in Fig. 1. MTT assay was performed to investigate the effect of myricetin on the viability of the MCF-7 cells following the treatment of myricetin. As shown in Fig. 2, myricetin suppressed the cell viability of the MCF-7 cells in a time- and dose-dependent manner. Particularly, the suppression of cell viability was evident after treatment with 80 µM of myricetin for 12 h, 20-80 µM of myricetin for 24 h and 10-80 µM of myricetin for 48 h (Fig. 2).

**Myricetin induces the cell apoptosis of human breast cancer MCF-7 cells.** Similarly, we examined the effect of myricetin on the cell apoptosis of MCF-7 cells using flow cytometric analysis. We observed that compared with the controls (myricetin 0 µM), 40 or 80 µM of myricetin significantly increased the apoptotic rate of the MCF-7 cells (Fig. 3).

**Myricetin affects the PAK1 pathway in human breast cancer MCF-7 cells.** In the MCF-7 models, we also examined whether myricetin affects the PAK1 pathway in MCF-7 cells. As shown in Fig. 4, myricetin (40 or 80 µM) significantly inhibited the
protein expression of PAK1 in the MCF-7 cells when compared with the controls (myricetin 0 µM).

Myricetin affects the MEK1/2 pathway in human breast cancer MCF-7 cells. To ascertain whether myricetin affects the MEK1/2 pathway in MCF-7 cells, MEK1/2 protein expression was analyzed using western blot analysis. However, compared to the controls (myricetin 0 µM), treatment with 40 or 80 µM of myricetin significantly suppressed the MEK1/2 protein expression in the MCF-7 cells (Fig. 5).

Myricetin affects the ERK1/2 pathway in human breast cancer MCF-7 cells. Next, the role of the ERK1/2 pathway in myricetin-induced apoptosis was determined. p-ERK1/2 protein expression was determined in the MCF-7 cells. Treatment with 40 or 80 µM of myricetin significantly reduced the protein expression of p-ERK1/2 in the MCF-7 cells when compared with the controls (myricetin 0 µM) (Fig. 6).

Myricetin affects the GSK3β pathway in human breast cancer MCF-7 cells. GSK3β, a tumor-suppressor protein, was measured using western blot analysis. Compared to the controls (myricetin 0 µM), treatment with 40 or 80 µM of myricetin significantly activated the protein expression of GSK3β in the MCF-7 cells (Fig. 7).

Myricetin affects the β-catenin pathway in human breast cancer MCF-7 cells. Western blot analysis was used to investigate the role of the β-catenin pathway on myricetin-induced apoptosis in human breast cancer MCF-7 cells. As shown in Fig. 8, treatment with 40 or 80 µM of myricetin significantly suppressed the β-catenin protein expression in the MCF-7 cells when compared with the controls (myricetin 0 µM).

Myricetin affects the cyclin D1 pathway in human breast cancer MCF-7 cells. Western blot analysis was used to investigate the role of the cyclin D1 pathway in the myricetin-induced...
apoptosis in human breast cancer MCF-7 cells. As shown in Fig. 9 treatment with 40 or 80 µM of myricetin significantly inhibited the protein expression of cyclin D1 in the MCF-7 cells when compared with the controls (myricetin 0 µM).

**Myricetin affects the PCNA pathway in human breast cancer MCF-7 cells.** To further examine the effect of myricetin on the PCNA pathway of human breast cancer MCF-7 cells, PCNA protein expression in MCF-7 cells was detected using western blot analysis. As shown in Fig. 10, treatment with 40 or 80 µM of myricetin significantly suppressed the PCNA protein expression in MCF-7 cells when compared with the controls (myricetin 0 µM).

**Myricetin affects the survivin pathway in human breast cancer MCF-7 cells.** The survivin pathway induces apoptosis in cancer cells. Thus, we aimed to ascertain whether myricetin affects the survivin pathway in human breast cancer MCF-7 cells. As shown in Fig. 11, pretreatment with 40 or 80 µM of myricetin significantly inhibited the protein expression of survivin in the MCF-7 cells when compared with the controls (myricetin 0 µM).

**Myricetin affects the Bax pathway in human breast cancer MCF-7 cells.** To investigate whether the anticancer effect of myricetin on human breast cancer was caused by the Bax pathway, Bax protein expression of MCF-7 cells following treatment with myricetin was analyzed by western blot analysis. As shown in Fig. 12, treatment with 40 or 80 µM of myricetin significantly activated the protein expression of Bax in the MCF-7 cells when compared with the controls (myricetin 0 µM).

**Myricetin affects the caspase-3 pathway in human breast cancer MCF-7 cells.** Caspase-3 assay kit was used to confirm the mechanism involved in the anticancer effects of myricetin on the apoptosis in human breast cancer MCF-7 cells. Compared to the controls (myricetin 0 µM), myricetin (40 or 80 µM) significantly increased the caspase-3 activity in the MCF-7 cells (Fig. 13).
Discussion

As one of the most common malignant cancers, the morbidity of breast cancer is increasing worldwide. In China, owing to changes in life styles and dietary structures, the morbidity of breast cancer is increasing rapidly and its age at onset is becoming increasingly younger (2,14). Due to rapid progress and the wide application of molecular biology, research on the pathogenesis of cancer and its therapy has made substantial achievements (15). Our results found that myricetin suppressed the cell viability of human breast cancer MCF-7 cells at least partly through the induction of apoptosis.

Pak1 is pivotal to physiological processes such as normal cell movement, mitosis, transcription and interpretation (7). In head and neck neoplasms and sarcoma, the activities of Pak1 have been found to be higher than that in normal tissues (6). In the evolutionary process of colorectal malignant tumors, expression of Pak1 is increased (16). Studies have confirmed that Pak1 is closely associated with the invasion and metastasis of breast cancer, human oophoroma and prostate cancer, indicating the Pak1 plays an important role in normal tissue development and tumor progression (17,18). Pak1 was found to be related to cellular orientation movement while motility is rather important for tumor metastasis. It has been confirmed that Pak1 has definite functions in invasion and metastasis of breast cancers induced by HER2 (18,19). Further studies suggest that expression of Pak1 in breast cancer and its activities are positively related with tumor grade, and expression levels in poorly differentiated ductal carcinoma were higher than levels in higher differentiated ductal carcinoma (6). These results indicate that inhibition of the viability of MCF-7 cells following treatment with myricetin is through the Pak1 pathway. Iyer et al provide striking evidence that myricetin induces the apoptosis of hepatocellular carcinoma through inhibition of Pak1 signaling (11).

The frequency of the overexpression of MEK noted in breast cancer is 30% and is related with the poor prognosis and resistance to therapy (20). The overexpression of MEK can realize autonomous activation under conditions without extracellular ligands. This results in the occurrence of malignant tumors through the blocking of apoptosis induced by TNF (20). ERK can facilitate the proliferation of tumor cells. As an important signaling transduction pathway of MAPK, ERK can be activated by growth factors, serum, ligands of G-protein-coupled receptors and transcription factors (21). Growth factors can activate ERK through the phosphorylation of the Ras-Raf-MEK pathway. Firstly, growth factors bind with corresponding receptors on the cell surface and induce the phosphorylation of tyrosine residues on endochylema of receptors, resulting in dipolymers (22). Phosphorylated tyrosine residues can provide binding sites for proteins with SH2 structural domain (23). Expression of ERK in pancreatic cancer cells is significantly increased. It is known that the MEK/ERK signaling pathway triggers the dissociation and motility of pancreatic cancer cells and improves the invasion and metastasis of pancreatic cancer cells (24). The activation of the ERK signaling pathway can regulate the migratory abilities of tumor cells and facilitate the dissemination of tumor cells. These results indicate that myricetin induced apoptosis
in the MCF-7 cells through the MEK/ERK signaling pathway. Lim et al. found that myricetin upregulated cyclooxygenase-2 expression in mouse epithelial cells through the MEK/ERK signaling pathway (25).

The Wnt signaling pathway plays a pivotal role in cell growth, progression and differentiation. Abrupt expression of the Wnt pathway is the origin of many diseases (8). In regards to the classical Wnt/β-catenin pathway, when the Wnt signal is lost, β-catenin in the cytoplasm is at low levels, which can be degraded continuously by axin compounds (26). Axin compounds include scaffolding protein, casein kinase 1 and GSK3β. GSK3β can continuously phosphorylate amino terminal of β-catenin, resulting in the degradation of β-catenin by ubiquitin (27). When cells are stimulated, Wnt signals are activated. Wnts proteins combine with FZD proteins and low density lipoprotein receptor-related protein 5/6. Dsh proteins are activated. GSK3β is phosphorylated, which can decrease the activity of GSK3β (24). Axin compound cannot trigger the phosphorylation and ubiquitylation of β-catenin, resulting in the accumulation of β-catenin. It combines with T cell transcription factor/lymphoid enhancer binding factors and activates the expression of cyclin D1, c-myc, MMP7, CD44, Bcl-2, VEGF and survivin (28). GSK3β can also activate the β-catenin signaling pathway and promote the occurrence of hyperplasia of the mammary glands (28). These results indicate that GSK3β/β-catenin/cyclin D1/PCNA/survivin-associated intrinsic pathways were, at least partly, involved in the myricetin-induced apoptosis of human breast cancer MCF-7 cells. Iyer et al. provide striking evidence that myricetin induces the apoptosis of hepatocellular carcinoma through inhibition of GSK3β/β-catenin/cyclin D1/PCNA/survivin signaling (11).

Bcl-2 and Bax play an essential role in cell apoptosis. The sensitivity of cells to apoptosis-stimulating factors largely depends on the ratio of bcl-2 proteins/bax proteins. The proportion of bcl-2/bax in normal tissues is constant, which creates a balance for cell division and proliferation (29). During cell apoptosis, many proteins in the bcl-2 family play an important role in cell apoptosis. Therefore, the comprehensive expression levels of bcl-2 and bax are valuable for the occurrence, progression and prognosis of tumors (30). Our results demonstrated that myricetin inhibited the cell growth of MCF-7 cells through induction of Bax and caspase-3. Kim et al. reported that myricetin induced apoptosis through the Bax/Bcl-2-dependent pathway in human colon cancer cells (10). In conclusion, our data demonstrated that myricetin suppressed the cell viability of human breast cancer MCF-7 cells at least partly through the induction of apoptosis. Our present results revealed that the anticancer effect of myricetin on human breast cancer involved PAK1/MEK/ERK/GSK3β/β-catenin/cyclin D1/PCNA/survivin/Bax-caspase-3 signaling (Fig. 14). Thus, myricetin may be a new drug for the treatment of human breast cancer.

References

16. DeSantiago J, Bare DJ, Xiao L, Ke Y, Solaro RJ and Banach K: NOTCH1 intracellular domain signaling (Fig. 14). Thus, myricetin may be a new drug for the treatment of human breast cancer.


