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Study on Apoptosis of Breast Cancer Cells Induced by Regulation of PI3K/Akt/mTOR Pathway by Syringin*

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Abstract **Objective** To study the anti-breast cancer effects and molecular mechanisms of syringin, and to provide a theoretical basis for the clinical application of syringin. **Methods** The inhibitory effect of syringin on the proliferation of breast cancer cells was measured with MTT assay. Trypan blue, TdT-mediated dUTP nick-end labeling (TUNEL), and Annexin V-FITC/PI staining were used to detect apoptosis. Caspase-3 activation was detected *via* Western blot to determine whether apoptosis occurred. The expression of apoptosis-associated protein B-cell lymphoma-2 (Bcl-2) was detected and the effect of syringin on the mitochondrial apoptosis pathway was investigated *via* JC-1 staining. The PI3K agonist Recilisib was used for comparison. qRT-PCR and Western blot were used to assess the role of syringin in regulating the PI3K/Akt/mTOR pathway and inducing the apoptosis of cancer cells. **Results** Syringin had a time- and dose-dependent inhibitory effect on the proliferation of breast cancer cells and induced their apoptosis. A further study showed that after syringin treatment, Caspase-3 was activated, Bcl-2 expression decreased, the mitochondrial membrane potential was significantly reduced, and the mRNA and protein expressions of PI3K, Akt, and mTOR were not significantly changed, but the protein phosphorylation levels were significantly decreased. Recilisib partially limits the effect of syringin on the apoptosis of breast cancer cells. **Conclusion** Syringin has a good inhibitory effect on MDA-MB-231 and MCF-7 breast cancer cells. It can inhibit cell proliferation and induce mitochondrial apoptosis by inhibiting the activation of the PI3K/Akt/mTOR signaling pathway. Syringin is a potential anti-breast cancer drug.

Key words syringin, breast cancer, apoptosis
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According to the latest data from the American Cancer Society, the incidence of breast cancer has been on the rise for nearly 4 decades, and is now one of the most common malignancies among women worldwide[1]. Additionally, breast cancer accounts for 15.5% of cancer deaths in women worldwide[1]. In China, breast cancer is one of the top 5 causes of cancer-related deaths among women, accounting for about 8.1%[2]. Chemotherapy is one of the main methods of treating breast cancer. However, chemotherapy drugs show extensive side effects, seriously affecting patients' physical and mental health[3]. Traditional Chinese medicine has many advantages in treating tumors with diverse targets and less toxic side effects. Natural plants can be used as a resource for discovering highly effective anti-tumor drugs. There is significant theoretical significance and application value in exploring the anti-tumor molecular mechanisms of compounds extracted from natural plants.

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Syringin (Syr), also known as eleutheroside B, is a bioactive component of many natural plants, such as acanthopanax senticoside, which can play an inhibiting role in some tumors. Syringin can effectively inhibit cervical cancer, prostate cancer and breast cancer[4]. In breast cancer, syringin can induce the cycle arrest of MCF-7 and MDA-MB-231 cells by increasing reactive oxygen species (ROS) production, and can also promote the activation of Caspase-3 and Caspase-9 to induce apoptosis[5]. Furthermore, syringin can act against breast cancer *via* the PI3K-Akt-PTGS2 and EGFR-RAS-RAF-MEK-ERK pathways[6]. In conclusion, syringin can have an inhibitory effect on breast cancer, but research on its anti-tumor molecular mechanism is not complete at present.

In this study, MDA-MB-231 and MCF-7 breast cancer cells were selected as the research objects, and the inhibitory effects of syringin on breast cancer cells were detected. This study determined the inhibitory effect of syringin on breast cancer cells and preliminarily explored its molecular mechanism. Our results provide a theoretical basis for applying syringin, and new ideas for the clinical treatment of breast cancer.

1 Materials and methods

1.1 Reagents and antibodies

Syringin (HY-N0824) was purchased from MCE, Shanghai, China. MTT (M8180) and trypan blue stain solution (C0040) were purchased from Solarbio, Beijing, China. The TUNEL (TdT-mediated dUTP nick-end labeling) apoptosis detection kit (KGA7072) was obtained from KeyGEN BioTECH, Jiangsu, China. Annexin V-FITC/PI kit (C1062L) and JC-1 (C2003S) were purchased from Beyotime Biotechnology, Shanghai, China. Antibodies against PI3K (#D55D5), mTOR (#7C10), p-mTOR (#D9C2), B-cell lymphoma-2 (Bcl-2, #15071), Bax (#5023) and Caspase-3 (#9662) were derived from Cell Signaling Technology, Shanghai, China. The p-PI3K (#AB4709N) antibody was sourced from Thermo Scientific, Shanghai, China. The Akt (#AF6261) and p-Akt (#AF3262) antibodies were from Affinity Biosciences, Jiangsu, China.

1.2 Cells lines and culture conditions

Breast cancer cells MDA-MB-231 (HTB-26) and MCF-7 (HTB-22) were purchased from American Type Culture Collection, Manassas, VA, USA. All cells were cultured in DMEM medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. The cells were grown in an incubator at 37℃ in a 5% CO_2 atmosphere.

1.3 Cytotoxicity assay

The cells were seeded in 96-well plates at a density of 3×10^3 per well and treated with different doses of syringin. The cells were cultured under various treatments in three groups containing 6 duplicate wells each: negative groups without any treatment, the blank wither group with only media (no cells) and the dosing group with many doses of syringin. After further cultivation for 24 , 48, or 72 h, MTT (5 g/L, 20 μl per well) was added into every well. Then, the breast cancer cells were subjected to light-avoidance incubation at 37℃ for 4 h, and a microplate reader measured the absorbance at 490 nm to calculate the ultimate cell proliferation activity. Drug-cell inhibition rate = [1 – (experiment – blank)/(negative – blank)]×100%.

1.4 Trypan blue staining

The breast cancer cells were collected after treatment with syringin (50 μmol/L) for 48 h. Next, the cells were resuspended with PBS, and their concentration was adjusted to $(1-2)\times10^9$/L. Following this, trypan blue staining solution (PBS：trypan blue=9：1) was added into the cell suspension for 3 min, with observing, photographing, and counting on cell count plates, and then the cell mortality was calculated. Cell mortality=dead cell number/total cell number×100%.

1.5 Apoptosis assay

TUNEL staining was used to observe the apoptotic morphology of the cells. Syringin (50 μmol/L) treatment for 48 h was followed by fixation, permeation, staining, DAPI redyeing, and tablet sealing. After interacting with the drugs for one day, each well's breast cancer cells were treated with a gradient concentration of syringin and digested with trypsin without EDTA. The cells were stained using an Annexin V-FITC/PI kit before being incubated under conditions of light avoidance for 10−15 min and finally detected using a flow cytometer.

1.6 Western blot

Cells were treated with different doses of

syringin, and then the total protein was extracted after 48 h. The protein content was quantified at 3 g/L using a BCA solution. The amounts of cellular protein were separated by 8%, 10%, or 12% SDS-PAGE, and the procedures were as follows: transposing to 0.22 μm PVDF membrane at the condition of 200−300 mA, 1.5−3 h, using 5% BSA for blocking at room temperature for 2 h, incubating with a primary antibody (1∶1 000) overnight (4°C, about 14−16 h), and then secondary antibody (1∶10 000) incubation for 1 h before imaging.

1.7 Detection of mitochondrial membrane potential（MMP）

The cells were cultured in 6-well plates at a density of 1.5×10^5/well, and syringin was used to treat the cells the next day. JC-1 staining was performed after 48 h: adherent cells were stained *in situ* on the reagent and photographed under a fluorescence microscope. At the same time, after the breast cancer cells from each parallel well were digested with trypsin, following the same steps applied in staining the suspended cells, the cells' mitochondrial membrane potential was detected using a flow cytometer.

1.8 Statistical analysis

The results are presented as the mean±standard error of at least three independent samples. Three or more datasets have been analyzed using one-way ANOVA analysis of variance followed by LSD testing for multiple comparison. $P<0.05$ was considered statistically significant. SPSS 25.0 was employed in the statistical analysis.

2 Results

2.1 Cytotoxicity of syringin on breast cancer cells

In order to detect the inhibitory effect of syringin on breast cancer, the breast cancer cells MDA-MB-231 and MCF-7 were selected as the research objects and treated with syringin at different concentrations (10, 25, 50, 75, and 100 μmol/L). The proliferation activity of each group was detected using an MTT assay at 24 h, 48 h and 72 h after addition. The results show that the proliferation activity of breast cancer cells was significantly decreased after treatment with syringin, and showed a significant time- and dose-dependent trend (Figure 1a). It was observed under a bright field microscope that the density and morphology of breast cancer cells treated with syringin decreased significantly (Figure 1b). These results indicate that syringin has a good inhibitory effect on breast cancer.

2.2 Syringin inhibits breast cancer cells by promoting apoptosis

Trypan blue staining is commonly used to detect living cells[7]. In order to verify the killing effect of syringin on breast cancer cells, 50 μmol/L and 48 h were selected as the representative treatment conditions, and trypan blue was used to detect cell death, because the cell proliferation is clearly inhibited and in a moderate state at this time, it is convenient for subsequent detection. The results show that, compared with the control group, the death rates of the two breast cancer cell groups significantly increased after syringin treatment (Figure 2a, b). In addition, applying the same processing conditions, we used TUNEL to detect cell apoptosis, and the results show that syringin significantly promoted cell apoptosis (Figure 2c). Syringin-treated cells showed bright green fluorescent particles, which is a specific apoptosis phenotype, while control cells did not show this phenotype. These results imply that promoting apoptosis may be an essential pathway by which syringin can inhibit breast cancer.

In order to further clarify the proapoptotic effect of syringin on breast cancer cells, 3 concentration gradients were set up in this study, and the apoptosis rate and apoptosis-related protein expression were detected 48 h after the addition of the drug. The flow cytometry results of Annexin V-FITC/PI double staining prove once again that syringin could promote the apoptosis of breast cancer cells (Figure 3b). After the administration of syringin, the apoptosis of breast cancer cells was significantly elevated in a dose-dependent manner. After the administration of syringin, the expressions of Caspase-3 and poly ADP-ribose polymerase (PARP) in MDA-MB-231 declined, and the expressions of cleaved-Caspase-3 and cleaved-PARP were increased considerably (Figure 3a). This demonstrates the activation of the Caspase-3 pathway in MDA-MB-231 cells. The above results show that promoting apoptosis is the main pathway by which syringin inhibits breast cancer cells.

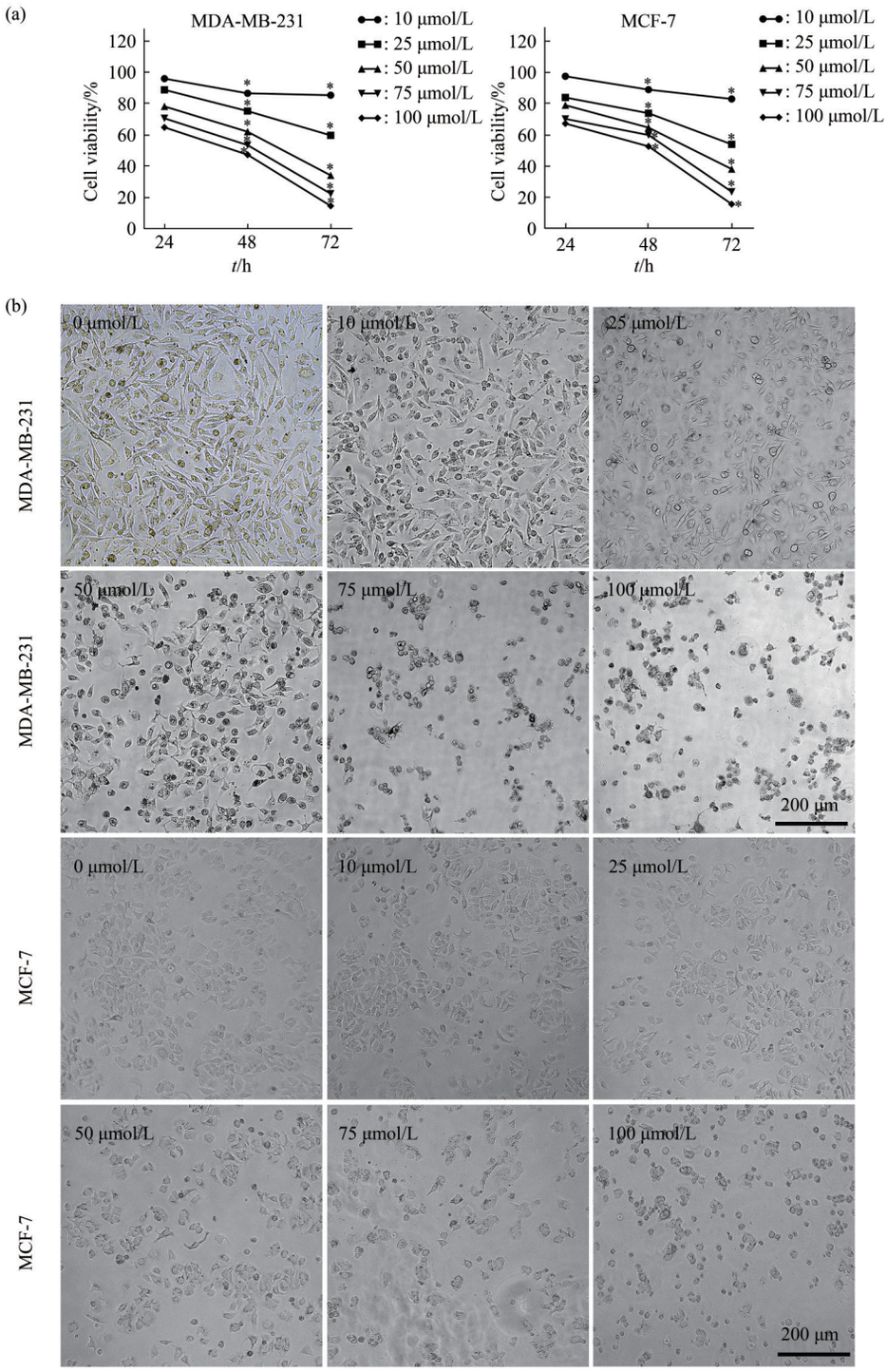

Fig. 1　Effect of syringin on the proliferation of breast cancer cells MDA−MB−231 and MCF−7

After treatment with different doses (10, 25, 50, 75, 100 μmol/L) of syringin for 24 h, 48 h or 72 h, MTT was used to detect the survival rate of each group of cells. (a) The results show that syringin significantly inhibited the proliferation activity of breast cancer cells MDA-MB-231 and MCF-7 in a time- and dose-dependent manner. (b) The growth of breast cancer cells treated with syringin was observed under light field microscope. The results show that the cell density decreased and the morphology was significantly abnormal after drug treatment. *P<0.05.

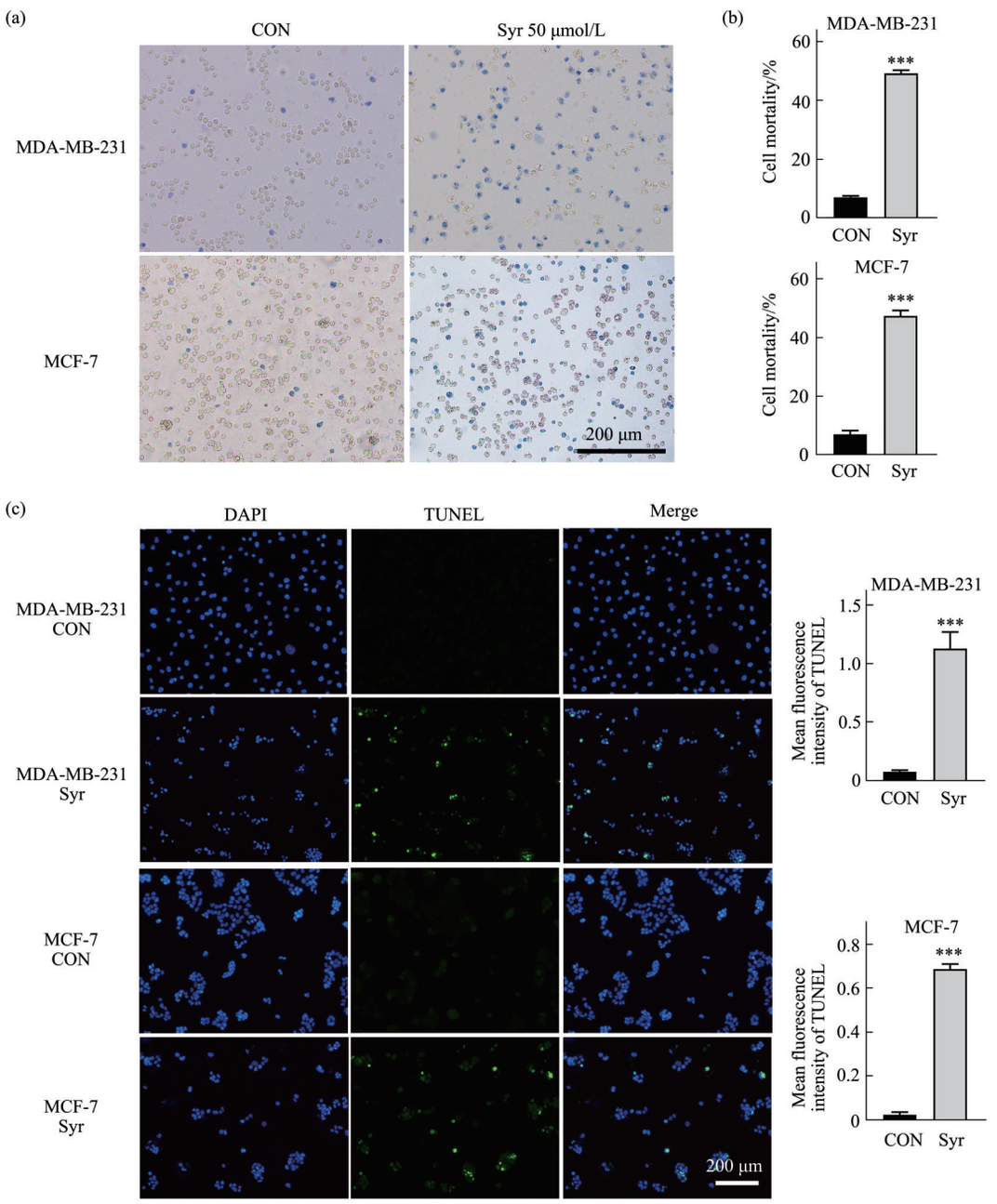

Fig. 2　Effect of syringin on apoptosis in breast cancer cells

After syringoside (50 μmol/L, 48 h) treatment, trypan blue staining was used to detect cell death, and TUNEL staining was used to detect apoptosis. The results show that compared with the normal group, the number of cells stained with trypan blue was significantly increased in the syringin group (a, b), and the number of green fluorescent particles after TUNEL staining was significantly increased (c). This indicates that syringin induced apoptosis in breast cancer cells. ***P<0.001.

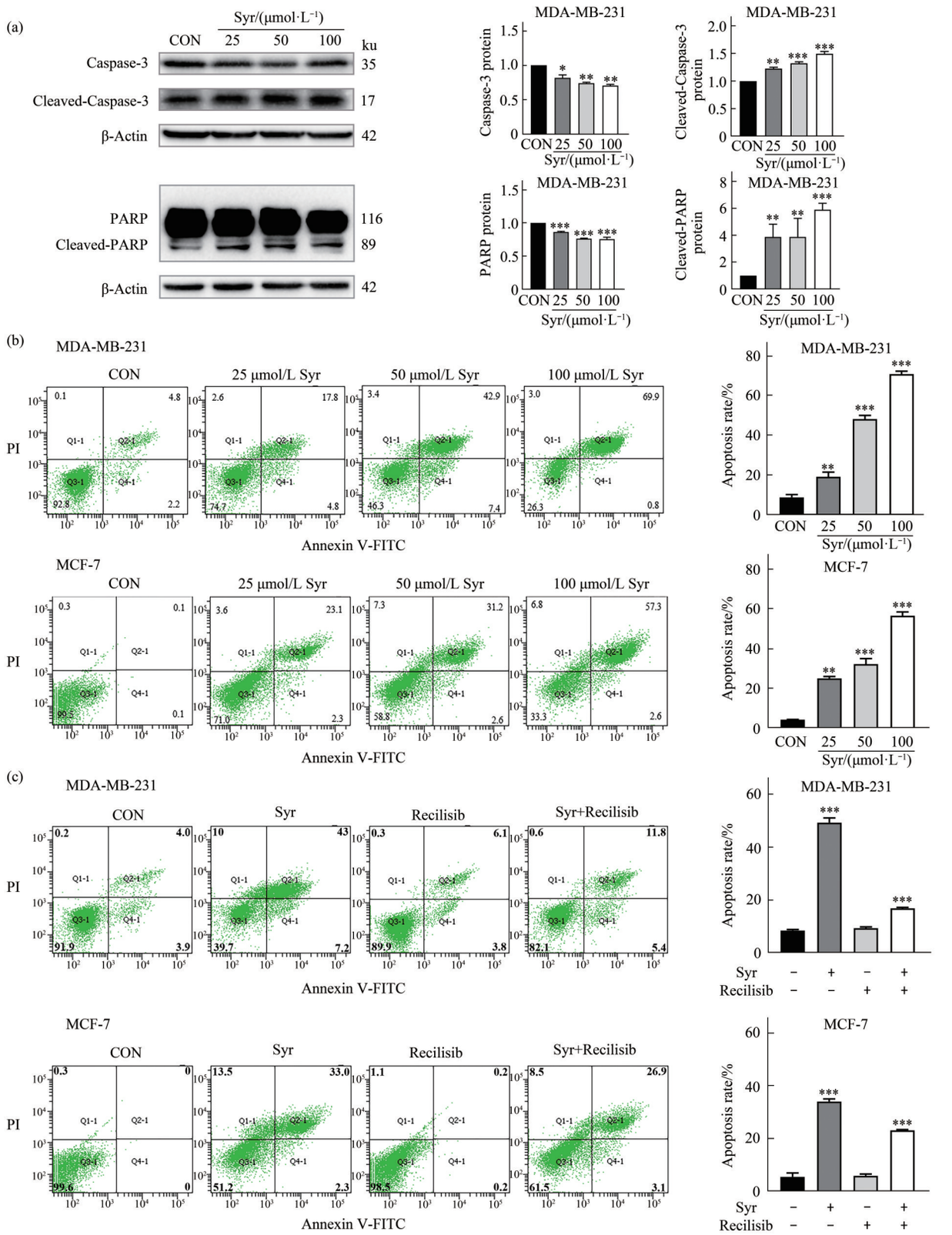

Fig. 3　Effect of syringin on apoptosis in breast cancer cells

The cells were treated with a concentration gradient of syringin for 48 h. Western blot was used to detect apoptosis-related protein expression, and flow cytometry was used to detect apoptosis rate after Annexin V-PI double staining. The results show that Caspase-3 and PARP contents decreased gradually and cleaved-Caspase-3 and cleaved-PARP contents increased gradually (a). The apoptosis rate increased significantly with increasing doses of administration (b). This suggests that syringin can induce apoptosis in breast cancer cells in a dose-dependent manner, and that apoptosis in MDA-MB-231 cells is associated with caspase-3 activation. Recilisib was introduced in (c). The results show that the apoptosis rate in the syringin and Recilisib combination group declined to a certain extent compared with that in the syringin group.*P< 0.05; **P< 0.01; ***P<0.001.

2.3 Syringin induces a reduction in mitochondrial membrane potential in breast cancer cells

A reduction in MMP is an irreversible factor of apoptosis and a landmark event in the early stage of apoptosis[8]. JC-1 is a fluorescent probe widely used to detect MMP[9]. Using JC-1 detection, we found that the MMP of breast cancer cells decreased significantly after 48 h of syringin (25, 50, and 100 μmol/L) treatment (Figure 4b, c), and the decrease in MMP was more significant with increases in syringin. The reduction in MMP is consistent with the reduction in the intracellular Bcl-2 expression of breast cancer (Figure 4a). This suggests that syringin activates the mitochondrial apoptotic pathway in breast cancer cells by causing a decrease in MMP through the downregulation of Bcl-2.

2.4 Syringin induces breast cancer cell apoptosis by inhibiting PI3K/Akt/mTOR signaling pathway

The over-activation of the PI3K/Akt/mTOR signaling pathway is an essential factor in the occurrence and development of breast cancer. It is also the main target of many drugs that exert anti-cancer effects[10]. The inhibition of the PI3K/Akt/mTOR signaling pathway is closely related to apoptosis[11]. To investigate the molecular mechanism of syringin-induced apoptosis in depth, this study further examined the expression of PI3K/Akt/mTOR pathway molecules at the mRNA and protein levels in cells after syringin treatment. The results show that although the mRNA and protein expression levels of PI3K, Akt and mTOR did not change significantly after the application of syringin, their phosphorylation was greatly reduced (Figure 5a−f), and this effect was dose-dependent. This suggests that syringin inhibited the activation of the PI3K/Akt/mTOR pathway in breast cancer cells. In addition, we applied Recilisib, an agonist of PI3K, as an interference group, and the results show that Recilisib (50 μmol/L, 48 h) could partially block the inhibitory effect of syringin (50 μmol/L, 48 h) on p-PI3K in MDA-MB-231 and MCF-7 cells (Figure 5g, h). The same treatment conditions were used, and apoptosis was detected by flow cytometry for each group of cells. The results show that Recilisib can partially offset the promotional effect of syringin on apoptosis (Figure 3c). The above results indicate that syringin promotes apoptosis in breast cancer cells by inhibiting PI3K/Akt/mTOR pathway activation.

3 Discussion

Breast cancer is a systemic disease. Current clinical treatments mainly include chemotherapy and surgery. Surgical therapy also requires adjuvant chemotherapy given before and after. Therefore, it is of great significance to discover and further study specific chemical drugs and explore their anti-tumor mechanisms. In this paper, we discussed the inhibitory effect of syringin on breast cancer. It was found that syringin could induce apoptosis in breast cancer cells by inhibiting the phosphorylation level of the PI3K/Akt/mTOR signaling pathway and down-regulating Bcl-2.

Tumor cells have 3 remarkable essential characteristics: immortality, migration, and loss of contact inhibition, the most important of which is immortality[12]. Therefore, finding drugs that specifically kill tumor cells is the primary strategy for tumor therapy. This study found that syringin has a specific targeted killing effect on breast cancer cells. The PI3K/Akt/mTOR signaling pathway is involved in cell proliferation, survival, invasion, migration, apoptosis, glucose metabolism, and DNA repair[9]. Many studies have revealed abnormal activation and mutation in breast cancer, and it is an important molecular target for breast cancer treatment[13]. The study by Du *et al*. [14] showed that Everolimus inhibited the growth, migration, and invasion of breast cancer cells by reducing the activity of the PI3K/Akt/mTOR signaling pathway. The results of this study show that syringin promoted the death of breast cancer cells and decreased the phosphorylation level of the PI3K/Akt/mTOR signaling pathway. PI3K agonists could partially block the pro-apoptotic effect of syringin in breast cancer. This indicates that PI3K/Akt/mTOR signaling is critical for syringin to inhibit the proliferation of breast cancer cells and promote their apoptosis (Figure 5, 3c).

Apoptosis is the primary mechanism of programmed cell death, and 3 induced pathways have been found: mitochondrial pathway, death receptor pathway, and endoplasmic reticulum pathway. Bcl-2 protein is the primary regulator of the mitochondrial pathway, and its main function is to regulate the permeability of the mitochondrial membrane. When the expression of Bcl-2 decreases, the integrity of the

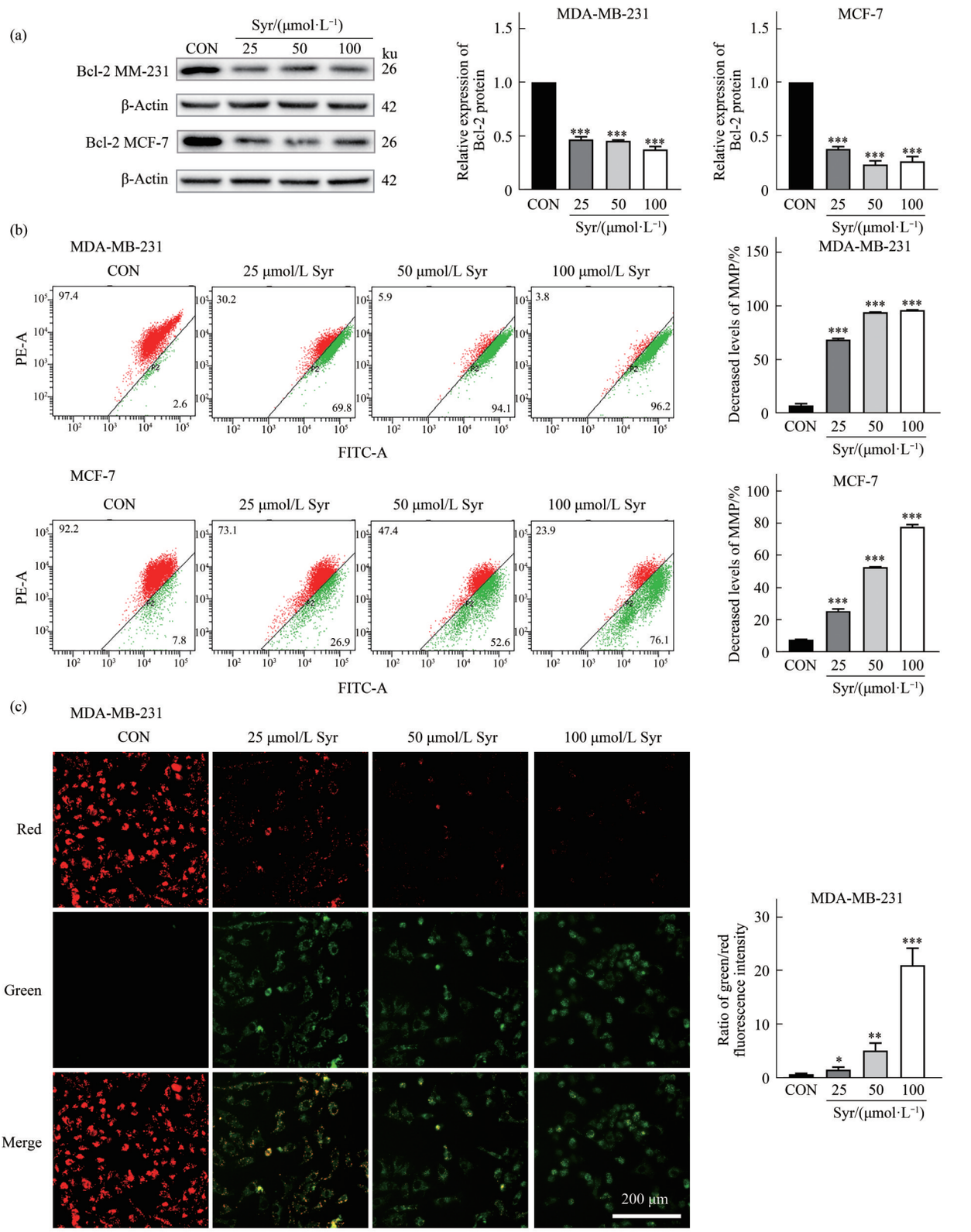

Fig. 4　Effect of syringin on Bcl−2 protein expression and mitochondrial membrane potential （MMP） in breast cancer cells

MDA-MB-231 cells were treated with different doses of syringin (25, 50, and 100 μmol/L) for 48 h. (a) Western blot was used to detect the expression of Bcl-2, and the results show that compared with the normal group, the expression abundance of Bcl-2 in the drug-treated group was significantly reduced. (b) JC-1 staining and flow cytometry were used to detect cell MMP, and the results show that with the increase in drug dose, MMP decreased more and more. (c) Taking MDA-MB-231 cells as an example, the fluorescence intensity of cells after JC-1 staining was observed under a fluorescence microscope. The results show that compared with the normal group, the red fluorescence of cells was weakened and the green fluorescence was enhanced after drug addition, indicating that MMP was decreased. **P*<0.05; ***P*<0.01; ****P*<0.001.

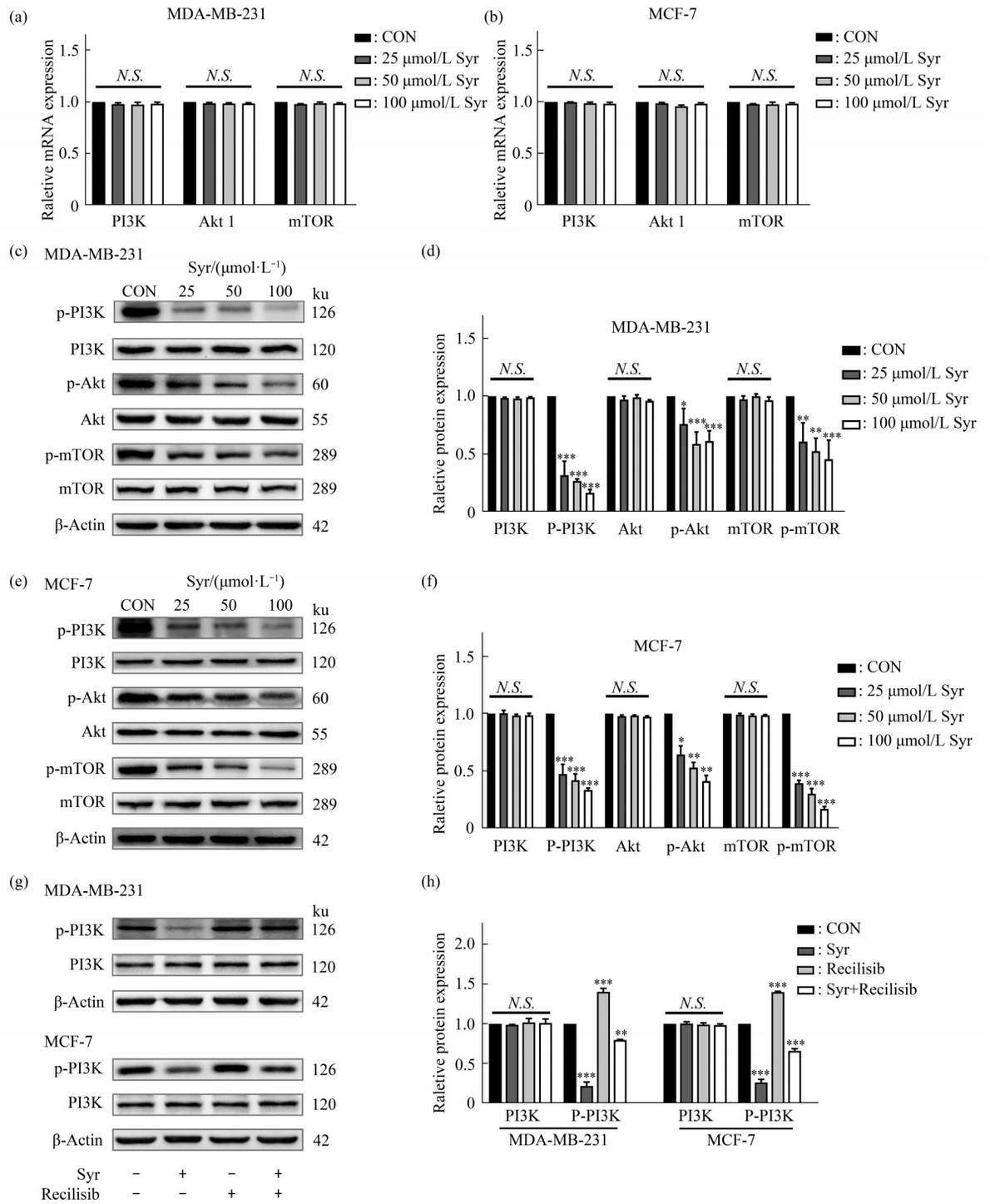

Fig. 5　Effect of syringin on PI3K/Akt/mTOR pathway in breast cancer cells

MDA-MB-231 cells were treated with different doses of syringin (25, 50, and 100 μmol/L) for 48 h. (a, b) qRT-PCR was used to detect the mRNA content of each molecule. The results show that there was no significant change in the mRNA content of the medicated group compared with the normal group, and the difference was not statistically significant. (c−f) Western blot was used to detect the protein expression and phosphorylation of PI3K, Akt and mTOR. The results show that compared with the normal group, the protein expression of the treated group underwent no significant change, but the phosphorylation level was significantly decreased. This suggests that syringin inhibits the activation of the PI3K/Akt/mTOR pathway. Recilisib was introduced in (g, h). The results show that the p-PI3K content in the syringin and Recilisib combination group increased to a certain extent compared with that in the syringin group. **P*<0.05; ***P*<0.01; ****P*<0.001; *N.S.*, no statistic difference.

mitochondrial membrane is destroyed, and cytochrome c (Cyt c) is released. Cyt c forms a complex with Apaf-1 and Caspase-9, which further activates Caspase-3 and causes apoptosis[15]. The results of this study show that after the administration of syringin, the expression of Bcl-2 decreased and the mitochondrial membrane potential decreased in breast cancer cells (Figure 4). TUNEL staining and flow cytometry also demonstrated the induction of apoptosis by syringin (Figure 2, 3). These results indicate that syringin induced apoptosis of the mitochondrial pathway in breast cancer cells. In addition, the expression levels of Caspase-3 and PARP precursor protein decreased, and the expressions of cleaved-Caspase-3 and cleaved-PARP increased in MDA-MB-231 cells (Figure 3a). However, the activation of Caspase-3 was not detected in MCF-7 cells, which is due to the deletion of 47 base pairs in the exon of the *CASP-3* gene in MCF-7 cells, resulting in the deletion of the protein[16-17]. Caspase-3 protein does play an important role in the apoptosis pathway, but this is not the only apoptotic pathway. Studies have shown that the complex formed by Cyt c, Apaf-1, and Caspase-9 can play an important role in Caspase-3 deficiency. The activation of Caspase-7 in MCF-7 cells induces apoptosis[18]. In addition, after the mitochondrial membrane potential drops, it can also cause the apoptosis-inducing factor (AIF) to be translocated from the mitochondria to the nucleus, resulting in the fragmentation of chromatin-condensed nuclear DNA, which eventually leads to apoptosis[19]. In this experiment, we demonstrated that syringin induced mitochondrial membrane potential reductions and apoptosis in MCF-7 cells without the involvement of Caspase-3. Therefore, we can speculate that the molecular mechanism of the syringin-induced apoptosis of MCF-7 may be related to Caspase-7 or AIF, which needs to be further studied.

4 Conclusion

This study has demonstrated that syringin has a potent inhibitory effect on breast cancer cells. This inhibitory effect promotes the apoptosis of breast cancer cells by inhibiting the PI3K/Akt/mTOR signaling pathway and inducing the mitochondrial apoptosis pathway. This study provides reliable experimental data for identifying potential anti-breast cancer drugs with few side effect.

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紫丁香苷调控PI3K/Akt/mTOR信号通路诱导乳腺癌细胞凋亡的研究*

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摘要　目的　研究紫丁香苷的抗乳腺癌作用及分子机制，为紫丁香苷的临床应用提供理论依据。**方法**　MTT检测紫丁香苷对乳腺癌细胞增殖的抑制作用；台盼蓝、TUNEL和Annexin V-FITC/PI染色检测细胞的凋亡状况，Western bolt检测Caspase-3的活化情况，判断细胞凋亡是否发生；检测凋亡相关蛋白B淋巴细胞瘤2（Bcl-2）的表达，结合JC-1染色探讨紫丁香苷对线粒体凋亡途径的影响；运用PI3K激动剂Recilisib做对比，qRT-PCR和Western bolt检测紫丁香苷调控PI3K/Akt/mTOR通路诱导癌细胞凋亡的作用。**结果**　紫丁香苷对乳腺癌细胞的增殖具有时间和剂量依赖的抑制作用，能诱导癌细胞发生凋亡。进一步研究发现，紫丁香苷处理后，细胞内Caspase-3被激活，Bcl-2表达下降，线粒体膜电位明显丧失，PI3K、Akt和mTOR的mRNA与蛋白质水平表达无明显变化，但蛋白质磷酸化水平明显下降；Recilisib处理后部分抵消了紫丁香苷对乳腺癌细胞凋亡的作用。**结论**　紫丁香苷对乳腺癌细胞MDA-MB-231和MCF-7具有良好的抑制作用，其通过抑制PI3K/Akt/mTOR信号通路的活化来抑制细胞增殖并诱导细胞发生线粒体途径的凋亡。紫丁香苷是具有开发潜力的抗乳腺癌药物。

关键词　紫丁香苷，乳腺癌，细胞凋亡

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