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Original Article

# Pterostilbene suppresses human endometrial cancer cells *in vitro* by down-regulating miR-663b

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**Abstract**

Resveratrol has long been known as an antioxidant and a chemopreventive agent. Similar to resveratrol, pterostilbene (PT) is also a phenolic compound extracted from the Vitis species. However, there are few studies on the antitumor effect of PT. Thus, we investigated the effects of PT on the endometrial cancer (EC) cells *in vitro* and the related molecular mechanisms. Treatment of EC cell lines HTB-111 and Ishikawa with PT (25–100 μmol/L) dose-dependently suppressed the cell viability and induced apoptosis. Using miR microarrays, we examined the miR expression profile in Ishikawa cells with or without PT, and revealed that miR-663b was the most decreased in PT-treated Ishikawa cells. Furthermore, we predicted and verified that the pro-apoptosis factor BCL2L14 is the direct target of miR-663b. Over-expression of miR-663b and knock-down of BCL2L14 counteracted the suppressing effects of PT on HTB-111 and Ishikawa cells. In addition, we evaluated the miR-663b levels in EC tissues of 51 patients using an *in situ* hybridization technique. With the median of the score of miR-663b as a cut-off value, these EC patients were divided into two groups, and the patients with high miR-663b expression had significantly poor prognosis.

**Keywords:** pterostilbene; endometrial cancer; HTB-111 cells; Ishikawa cells; apoptosis; miR-663b; BCL2L14

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## Introduction

Endometrial cancer is not only one of the gynecologic malignancies with the highest attack ratio but is also the most common reason for hysterectomy[1, 2]. A typical symptom of EC is vaginal bleeding without pain. Unlike cervical cancer, there is no minimally invasive, simple but sensitive, special routine screening. Dilatation and curettage (D&C) is one of the only standard procedures available to evaluate patients with suspicious symptoms. Thus, with the increased popularity of Thinprep cytologic test (TCT), the incidence ratio of EC has exceeded that of cervical cancer. The age of onset is 50-60 years, but the age of occurrence has recently become younger[3]. Abuse of hormone replacement for menopause or breast cancer[4], adiposity with extreme BMI[5, 6], and poor dietary habits[7] are considered risk factors for EC. Filomeno *et al* reported that a human diet containing high amounts of fiber, phytochemicals, unsaturated fatty acids and antioxidants significantly reduces the risk of EC[7].

Pterostilbene (PT), a phytoalexin, is a well-known natural antioxidant that is extracted mainly from grapes[8]. PT belongs to the class of stilbenes, a type of small molecular weight (approximately 200–300 g/mol) compounds that are widely distributed in plant sources, aromatherapy products, and dietary supplements[9]. PT has been widely used for its antihyperlipidemic, antidiabetic, and antioxidant properties in the treatment of fatty liver, diabetes, cardia-cerebrovascular disease and so on[10-14]. Some reports have also shown that PT may have anticancer activity. Ko *et al*[15] demonstrated that PT increases autophagy and apoptosis in oral cancer cells through activating JNK1/2 but inhibiting Akt, ERK1/2, and p38. Dong *et al*[16] also found that PT significantly triggered apoptosis in ovarian cancer cells. However, compared with the analogue resveratrol (RV), for which a great deal of research has demonstrated an antitumor effect, PT requires more meticulous study. In reality, PT is as strong as RV in terms of antioxidant and antitumor activity. Moreover, Lee's results demonstrated that PT leads to more effective inhibition of lung cancer cell proliferation than RV[17]. Ours is the first study of the effects of PT on EC and presents thorough research on the molecular mechanism underlying its occurrence.

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## Materials and methods

### Cell proliferation assay

HTB-111 and Ishikawa cells were cultured in complete DMEM containing 10% FBS (Gibco, Grand Island, NY, USA) normally. PT was purchased from Sigma-Aldrich (USA) and used in the range from 25 to 100 μmol/L. For the cell liberation assay, a CCK8 kit (Dojindo, Kumamoto, Japan) was used according to manual. The inhibition ratio=1-[experimental group *OD* (optical density) value/control group *OD* (optical density) value] ×100%.

### Cell apoptosis assay

HTB-111 and Ishikawa cells were treated with PT as above for 24 h and then were stained using the Annexin V/PI kit (BioVision, Palo Alto, CA, USA). The concrete operations have been described previously[17].

### Caspase activation assay

The cascading activation of caspase triggered apoptosis. After treatment with PT for 24 h, the activities of caspase-3, -8, and -9 were detected using the Colorimetric Assay Kit (R&D, USA) in accordance with the protocol. Briefly, total cells from each group were collected and lysed with lysis buffer. The cell lysate was incubated on ice for 10 min and then centrifuged at 10 000×*g* for 1 min. A mixture of 5 μL caspase-3, -8, or -9 colorimetric substrates, 50 μL of 2× reaction buffer and 50 μL of cell lysate was incubated at 37 °C for 2 h. The enzymatic activities of the caspases were quantitated using a microplate reader with a wavelength of 405 nm.

### Microarray analysis of the microRNA profile

Ishikawa cells were treated with $IC_{50}$ of PT for 24 h, lysed with Trizol and sent to Bohao Biocompany for microarray analysis (Shanghai, China). The concrete performance was as described in a previous paper[18].

### Dual-luciferase system assay

Based on the miR profile, we focused on miR-663b. After predicting that BCL2L14 is the target of miR-663b, we constructed a BCL2L14 reporter plasmid using REPORT™ Luciferase (pMIR). The dual-luciferase activity assay was performed using the dual-luciferase assay kit (Promega; Madison, WI, USA) as previously reported[18].

### qRT-PCR assay

The PrimeScript miRNA RT-PCR kit (Takara, Dalian, China) was used to detect miR-663b and BCL214L levels as previously described[19]. The primers for BCL2L14 were forward, 5′-GCTCTGCTGTCTTCTCACCAAA-3′ and reverse, 5′-ATTTTCCTCCTTCTCTGCTACTCC-3′, and the β-actin primers were forward, 5′-TGAAGTGTGACGTGGACATC-3′ and reverse, 5′-GGAGGAGCAATGATCTTGAT-3′. The relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

### Western blot assay

Cells from each group were lysed on ice in RIPA buffer, and the protein concentrations were quantitated using BCA Protein Assay reagent (Pierce). After denaturing (10 min, 95 °C), the proteins were separated by SDS-PAGE and transferred to a membrane. The proteins were incubated with the antibodies of BCL2L14 (Invitrogen, USA) and horseradish peroxidase (HRP)-conjugated anti-mouse IgG in turn and were then imaged using an Image Quant LAS 500.

### Tissue *in situ* hybridization for miRNA detection

In our previous paper, we used ISH to demonstrate that miR-663 is overexpressed in breast cancer[19]. Here, we evaluated mIR-663 levels in 51 cases of EC using tissue paraffin sections. All EC patients were diagnosed at the Gynecology and Obstetrics Department of Oncology of the first affiliated Hospital of Jinan University from 2004 to 2009. This study was approved by the Ethics Committee of the First Affiliated Hospital of Jinan University on Nov 7, 2013. All patients signed the consent form. The operational processes and evaluation criteria were identical to those used in our previous study.

### Over-expression of miR-663b and knock-down of BCL2L14 in EC cells

The chemosynthetic miR-663b mimic was purchased from Jima Bio Co. BCL2L14 siRNA was purchased from Ambion (siRNA ID 120721). Before transfection with the miR-663b mimic or siRNA-BCL2L14, we starved the cells in DMEM without FBS for 24 h. Then, using the lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA, USA), the miR-663b mimic or siRNA-BCL2L14 were transfected into HTB-111 and Ishikawa cells, respectively, according to the manual. Six hours later, the medium was changed to fresh complete medium. Cells were treated with $IC_{50}$ of PT 24 h later.

### Statistical analysis

The results were described as the mean±SD and analyzed by one-way ANOVA using SPSS version 18.0 software. $P<0.05$ was considered statistically significant.

## Results

### PT-triggered activation of caspases induced apoptosis in EC cells

The EC cell lines HTB-111 and Ishikawa were exposed to different concentrations of PT (from 25 to 100 μmol/L) and detected using a CCK8 kit. Figure 1A shows that PT inhibited the viability of both EC cell lines in a dose-dependent manner, with an $IC_{50}$ of 71.64 nmol/L and 74.34 μmol/L. Figure 1B shows that the activity of caspase-3, -8, and -9 progressively heightened with the PT concentration enrichment ($P<0.001$ *vs* untreated control). Further, the apoptosis ratio had the same tendency, as shown in Figure 1C.

### PT down-regulated miR-663b but increased the expression of its target, BCL2L14

In recent decades, miRs have been found to be involved in the regulation of many types of cell processes. Here, using miR microarrays, we found 6 markedly down-regulated miRs

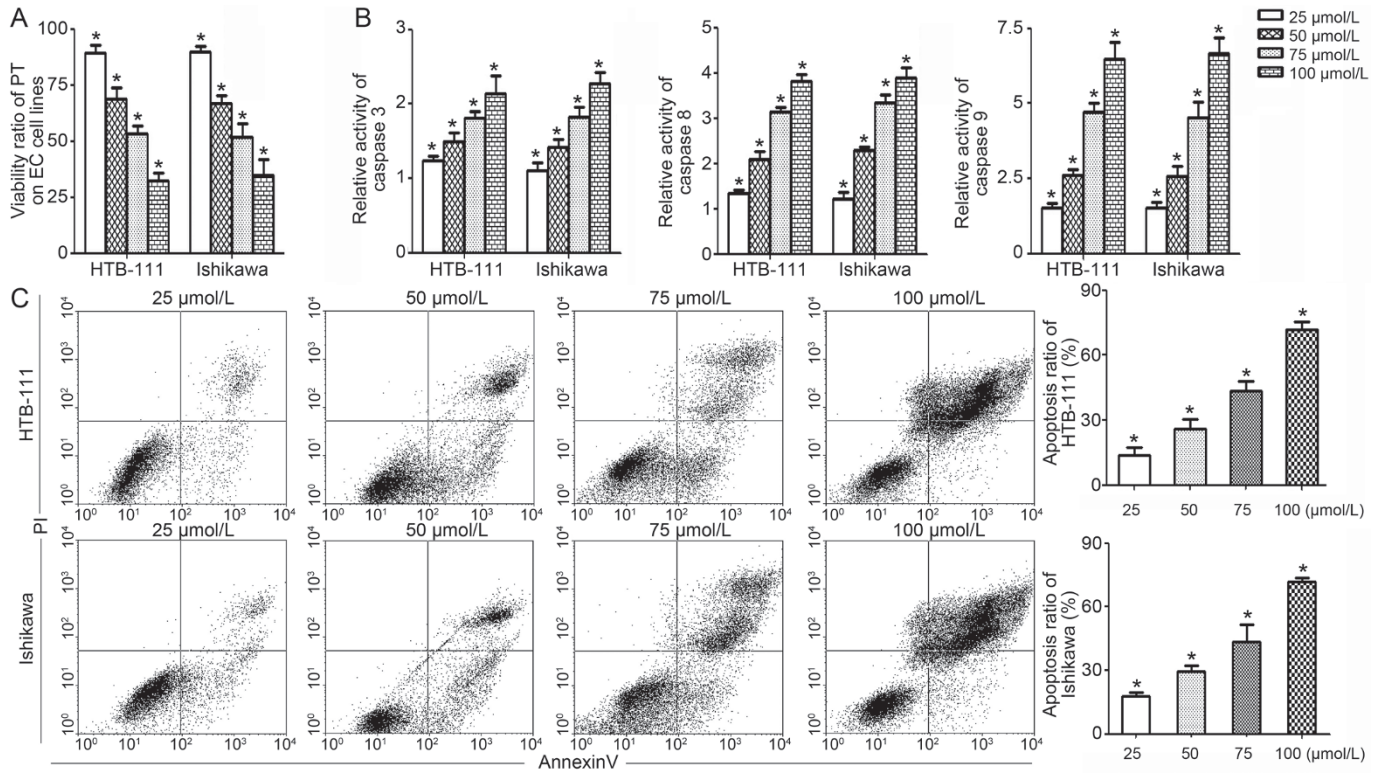


**Figure 1.** PT reduced cell proliferation and triggered caspase cascade apoptosis in EC cells. (A) The cytotoxicity of PT (from 25 to 100 mol/L) was detected by a CCK-8 assay; the proliferation ratio decreased in a dose-dependent manner (* means $P<0.001$ *vs* untreated control group 100%). (B) Caspase-3, -8, and -9 activities were detected by colorimetric assay. The relative activity of the caspases was measured as the ratio of optical density of the experimental group divided by the optical density of the control group, which was considered 1. Caspase-3, -8, and -9 were all activated by PT in a dose-dependent manner (* means $P<0.001$ *vs* untreated control group). (C) Annexin V/PI analysis showed that PT induces apoptosis in HTB-111 and Ishikawa cells. A higher concentration of PT was associated with an increased apoptosis ratio in EC cells.

in Ishikawa cells treated with $IC_{50}$ of PT (Figure 2A). Next, we amplified these 6 down-regulated miRs in both EC cell lines and then focused on the lowest steady one, miR-663b (Figure 2B). We also found a decrease in the miR-663b level accompanying the PT concentration in HTB-111 cells (Figure 2C). Furthermore, we predicted the targets of miR-663b using TargetScan (Figure 2D): miR-663b could complementarily bind with the 3'UTR of BCL2L14. A dual-luciferase report assay verified this interaction (Figure 2E). When the expression of miR-663b was regulated by its MIMIC or AMO in HTB-111 and Ishikawa cells, the BCL2L14 level changed accordingly. The $IC_{50}$ of PT had a similar effect on BCL2L14 expression to miR-663b-AMO.

### The effect of PT was counteracted by the miR-663b mimic or siRNA-BCL2L14

A rescue test was used to verify the pathway hypothesis. We overexpressed the miR-663b using its mimic and silenced BCL2L14 with siRNA. As expected, the proliferation rates of HTB-111 and Ishikawa cells significantly increased in the miR-663b mimic and siRNA-BCL2L14 group compared with the control group (Figure 3A). All caspase activity and apoptosis assays showed the same tendency, *ie*, that the miR-663b mimic and siRNA-BCL2L14 offset the effects of PT (Figure 3B and 3C).

### miR-663b is overexpressed in EC tissue

We analyzed the miR-663b expression in EC tissue using ISH to unveil the prognostic significance of miR-663b. The positive blue particles were scattered in the tumor cell plasma (Figure 4A). We evaluated the ISH score of miR-663b in these sections. The median fold change was used as the cut off value to divide the 51 patients into low- and high-expression groups. The clinical characteristics of these two groups are shown in Table 1. The high-expression miR-663b group is associated with increased distant metastasis ($P$=0.014), tumorous grading ($P$=0.010), and tumor stage ($P$=0.028). Because of the limited number of cases, we did not find a correlation between the miR-663b levels and lymph node metastasis ($P$=0.077) or the status of the vessels involved ($P$=0.054). Age, ER, PR, menopause and pathology were not correlated with the miR-663b level. A high expression of miR-663b was associated with poor overall survival, as shown by the Kaplan-Meier plot. Patients with high miR-663 levels had a significantly lower 5-year survival rate than did patients with low miR-663 expression (50% *vs* 73.5%, $P$=0.043; Figure 4B).

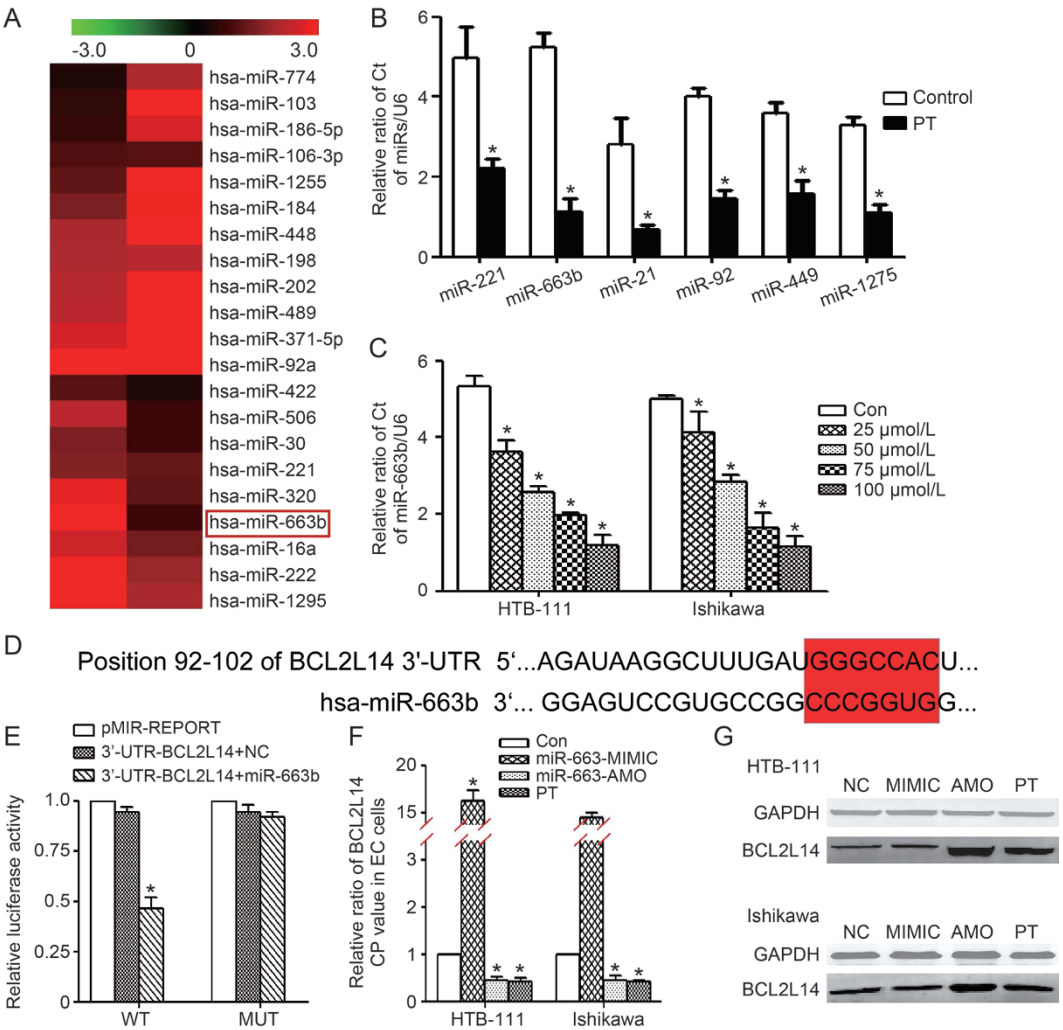


**Figure 2.** PT down-regulated miR-663b and indirectly up-regulated its target, BCL2L14. (A) Identification of the change in miR expression by microarray. The miR clusters are represented by red or green according to its score. The hierarchical clustering profile shows the variation of miRs in Ishikawa cells after treatment with $IC_{50}$ of PT. (B) We detected the 9 downregulated miRs by qRT-PCR. miR-663b was decreased the most significantly. (C) When exposed to different concentrations of PT, miR-663b levels in HTB-111 and Ishikawa cells, measured by qRT-PCR, increased gradually. (D) TargetScan predicted that miR-663b targets BCL2L14 by binding to its 3'UTR. (E) The luciferase activities of wild-type of pMIR-BCL2L14, but not mutant pMIR-BCL2L14 or a negative control of empty plasmid, were reduced by miR-663b mimics (* means $P<0.05$). (F-G) The BCL2L14 mRNA (F) and protein (G) levels in both HTB-111 and Ishikawa cells changed depending on miR-663b levels. We also treated the EC cells with $IC_{50}$ of PT and found that BCL2L14 increased in both mRNA and protein.

## Discussion

Primary, secondary, and tertiary prevention are all very important and effective strategies to eliminate the burden of cancer. Primary prevention is the most basic and essential method. However, it is the least used in developing countries because of widespread ignorance for such a long time. A rational diet, appropriate amounts of exercise, no smoking and limited alcohol consumption, as well as a psychological balance, are the four bases of health. Studies have found that a good diet significantly reduces the risk of tumors. RV was shown to be an effective antitumor agent but had the limitation of low bioavailability. PT, which is a natural small polyphenol that is structurally similar to RV and found in grapes, berries, peanuts, and red wine, has attracted our attention because of its strong antioxidant, anti-aging, and cancer chemopreventive properties[20]. Paul showed that PT not only inhibits the proliferation of colon cancer cells but also decreases the secretion of proinflammatory cytokines, such as TNFα, IL-4 and IL-1β, by reducing the expression of phospho-p65 in the nucleus. The results of *in vivo* tests also support these findings[21]. Xing reported that PT reduces brain metastasis of breast cancer *in vivo* and *in vitro*. PT can penetrate the blood-brain barrier and reduce c-Met signaling in breast cancer cells, thereby promoting metastasis by inducing the secretion of the inflammatory cytokines IL8 and CXCL1, and can trigger vascular reprogramming by activating tumor-associated astrocytes to secrete the c-Met ligand HGF[22]. PT also represents a potential drug for treating other cancers, including ovarian cancer, prostate can-

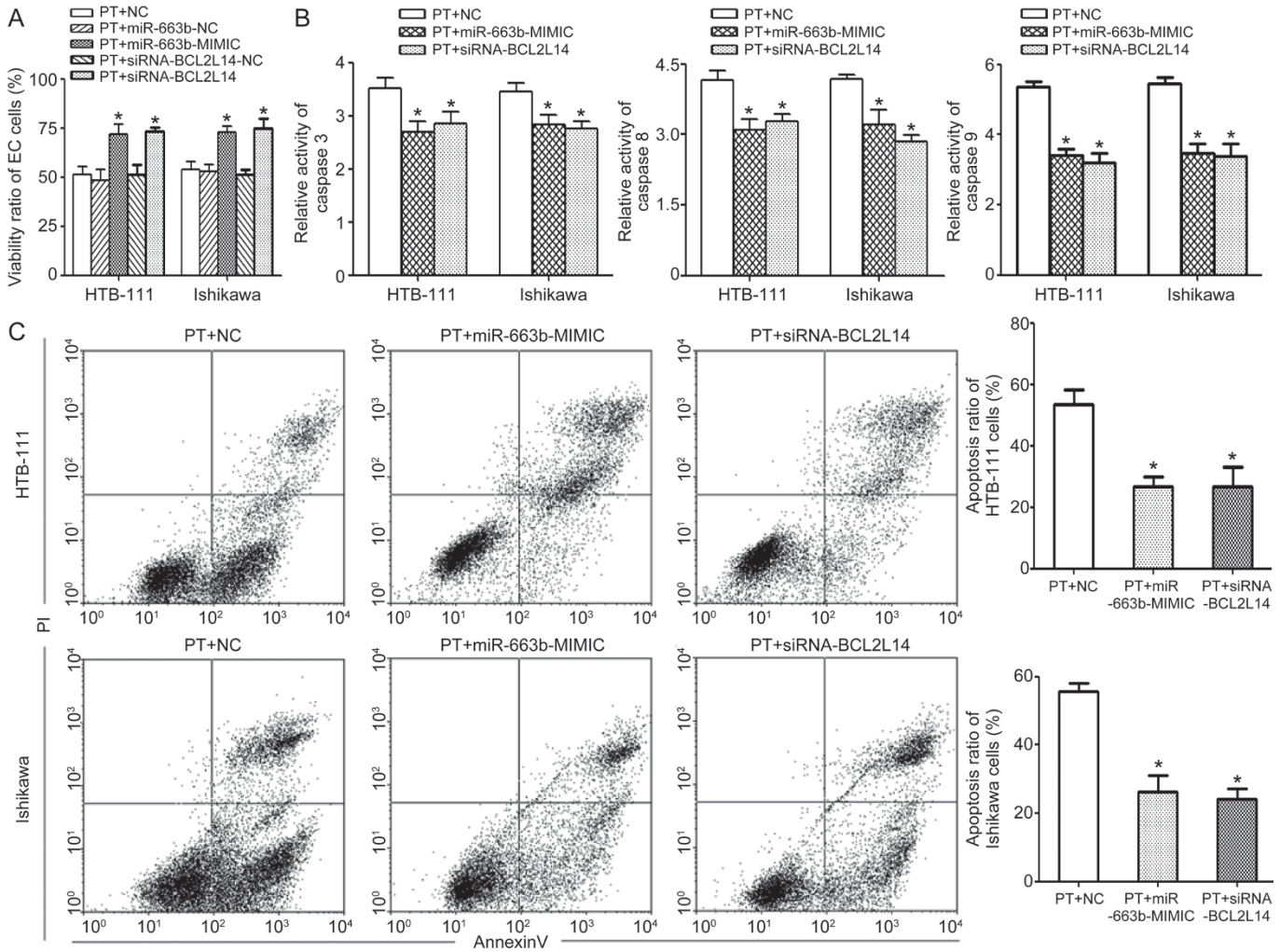


**Figure 3.** The miR-663b/BCL2L14 pathway plays an important role in PT effects in EC. (A) For the miR-663b mimic and siRNA-BCL2L14 groups, the cell proliferation of HTB-111 and Ishikawa cells was significantly higher than that of the NC group. (B) Data show that the miR-663b mimic and siRNA-BCL2L14 reduces the caspase activity induced by PT compared with the NC group (*$P$<0.05). (C) The apoptosis ratio showed the same tendency as the caspase activity. The miR-663b mimic and siRNA-BCL2L14 mediated decreased apoptosis in the two EC cell lines.

cer, oral cancer, and melanoma[23-26]. Here, we demonstrate the powerful cytotoxicity of PT on EC cells by triggering caspase-dependent apoptosis. Moreover, we found that miR-663b may be the key molecule in this process. Microarray and qRT-PCR results all showed that upon exposure to PT, miR-663b decreased dramatically in EC cells. We have previously described the oncogenic function of miR-663 in breast cancer[19]. Many studies reached the same conclusion. Yi found that miR-663b promotes cell proliferation and tumorigenesis in NPC cells[27]. However, it still works as a tumor suppressor. For glioblastoma (GBM), Shi found that miR-663 level was a good predictor of prognosis and negatively regulated the expression of CXCR4 and inhibited the proliferation and invasion of GBM cells[28]. Huang's report supported Shi's results. Over-expressed miR-663 induced apoptosis in hepatocellular carcinoma cells[29]. However, in different tumor types, miRs may have different effects. Here, we found that miR-663b played an oncogenic role in EC and that high miR-663b levels were associated with a lower survival ratio. The correlation analysis of miR-663b levels and clinical features of EC patients suggest that distant metastasis, advanced tumorous grading, and tumor stage were significantly associated with miR-663b levels. We also predicted that BCL2L14, also known as Bcl-G, is the direct target of miR-663b, which is located at chromosome 12p12 and was first identified by Guo[30] in 2001. Miled[31] generated a genome-wide map of p53 binding sites (p53BS) and found that the BCL-G/BCL2L14 gene binds to p53BS, thus contributing to p53-mediated apoptosis. BCL-G/BCL2L14 also binds to the anti-apoptotic Bcl-X(L) protein via its BH3 domain, triggering cascade apoptosis. Here, we found that miR-663b-AMO and PT upregulated the expression of BCL-G/BCL2L14. The rescue test supported our hypothesis that the over-expression of miR-663b by its mimic and the knock-down of BCL-G/BCL2L14 by its siRNA competed with

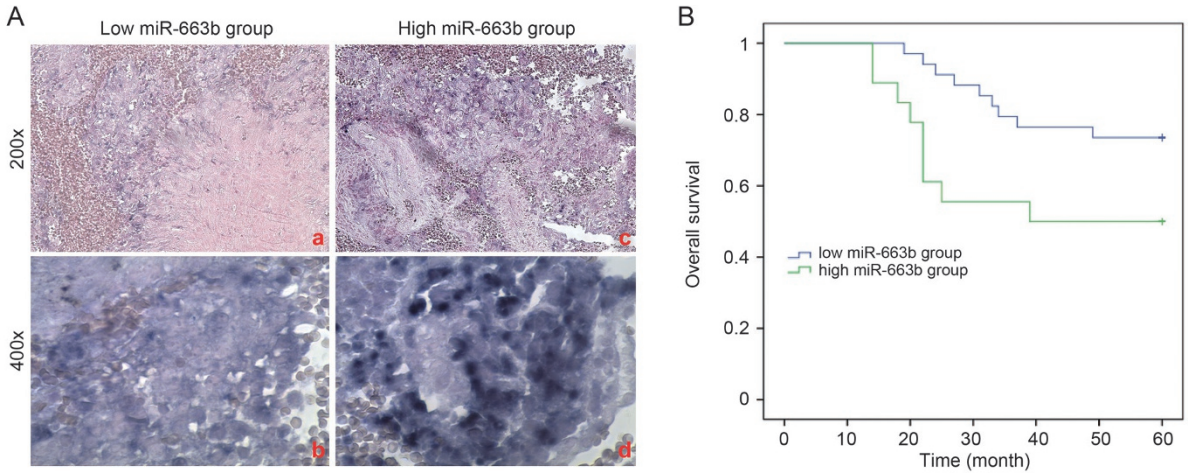


**Figure 4.** The expression of miR-663b in EC tissues and its correlation with survival of EC patients. (A) Representative images of miR-663b levels in EC tissues using the ISH method. a) and c) are extreme positive; b) and d) are weak positive. Magnification of a) and b) are ×200, c) and d) are ×400. (B) The difference in OS of EC patients with low and high miR-663b was presented by Kaplan-Meier survival analysis.

**Table 1.** Correlation between clinicopathologic features of EC and miR-663b level (*n*=52).

| Patients Character | | Low miR-663 group | High miR-663 group | *P* value |
|---|---|---|---|---|
| Age | <55 | 21 | 10 | 0.672 |
| | ≥55 | 13 | 8 | |
| Menopause status | negative | 20 | 10 | 0.825 |
| | positive | 14 | 8 | |
| Distant metastasis | negative | 28 | 9 | 0.014 |
| | positive | 6 | 9 | |
| Vessel invasive | negative | 26 | 9 | 0.054 |
| | positive | 8 | 9 | |
| Lymph node metastasis | yes | 22 | 11 | 0.077 |
| | no | 12 | 7 | |
| ER expression | negative | 13 | 6 | 0.733 |
| | positive | 21 | 12 | |
| PR expression | negative | 16 | 7 | 0.581 |
| | positive | 18 | 11 | |
| Pathology | adenocarcinoma | 28 | 14 | 0.697 |
| | Non- adenocarcinoma | 6 | 4 | |
| Stage | I | 16 | 6 | 0.028 |
| | II | 16 | 5 | |
| | III/IV | 2 | 7 | |
| Histological grade | G1 | 14 | 2 | 0.010 |
| | G2 | 17 | 11 | |
| | G3 | 3 | 5 | |

the effect of PT on EC cells.

Based on our results, the naturally occurring stilbene, PT, significantly induces apoptosis in EC cells via the miR-663b/BCL2L14 signaling pathway and could serve as a new and promising therapeutic agent for EC. Moreover, miR-663b is a predictor of poor prognosis in EC.

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