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*Original Article*

# Raloxifene suppress proliferation-promoting function of estrogen in CaSKi cervical cells

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**Abstract:** Raloxifene has demonstrated anti-estrogen activity in reproductive organs and tissues, but there are very few related studies in cervical cells. The aims of this study is to explore the function of raloxifene in CaSKi cervical cells. We examined the effects of raloxifene on cervical cancer cells exposed to estrogen. The effect of Raloxifene on cell growth, apoptosis was detected. The human papillomavirus (HPV) 16 E6E7 transcription in cervical cell line CaSki cells exposed to 17-estradiol was also examined. Apoptosis was measured by endonucleolytic degradation of DNA. HPV 16 E6E7 was measured by northern analysis. The results indicated that raloxifine inhibits estrogenic promotion activity on growth of CaSki cells. Raloxifene suppresses the proliferation promotion activity of estradiol in CaSki cells. Raloxifene suppresses the stimulation effect of estradiol on HPV 16 E6E7 transcription in CaSki cells. In conclusion, raloxifene inhibit the CaSki cells proliferation induced by estradiol, which suggests that raloxifine also has anti-estrogen activity in cervical cells.

**Keywords:** Cervical cells, raloxifene, estrogen, caski, human papillomavirus

## Introduction

Cervical cancer is the second most common cancer in women only secondary to the breast cancer [1]. Many possible mechanisms have been considered to be involved in cervical cancer. Much evidence suggests most cervical cancers may attribute to human papillomavirus (HPV) infection as the HPV infection rate of the cervical cancer patients is high up to 93% [2]. It has been demonstrated that steroid hormone estrogen is closely related with the occurrence and development, diagnosis and treatment, and prognosis of gynecological tumor. Estrogen can promote the expression of HPV gene and further induce carcinogenesis of cervical cells with High-risk HPVs infection [3]. Raloxifene is a selective estrogen receptor modulator (SERM), which can combine with the estrogen receptor with high affinity to adjust the gene expression. Raloxifene selectively acts as the agonist or antagonist in different tissues, particularly as antagonist in uterus and breast [4]. To disclose whether raloxifene also has anti-estrogen activity in cervical cells, we treated the CaSKi cells infected with HPV with raloxifene solution with/without estrogen and evaluate the growth and apoptosis level of CaSKi cells, and HPV expression level.

## Materials and methods

### *Cell lines and cell culture*

The cervical cancer cell line CaSki (containing multiple copies of integrated HPV16 DNA) were obtained from the American Type Culture Collection. Cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum. Fetal bovine serum was stripped with charocoal in some experiments.

### *Reagents*

17-estradiol (E2) were purchased from Sigma Chemicals (St. Louis, MO). Raloxifene was purchased from EliLilly and Company. Regular and charcoal-stripped fetal bovine serum (FBS) were purchased from Hyclone (Logan, UT). HPV 16 E6E7 expression vector plasmid pLXSN 16E6E7 was purchased from Biovector Science Lab, Inc.

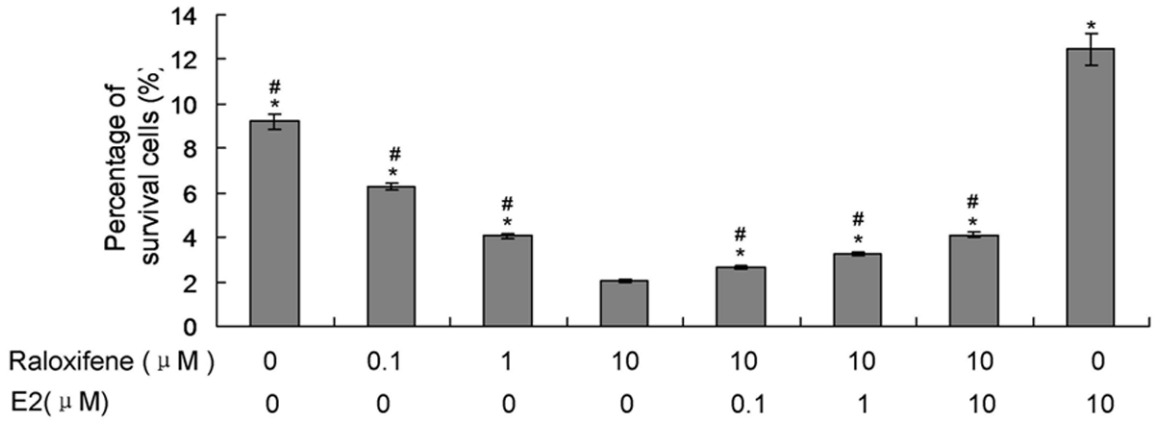


**Figure 1.** Effect of Raloxifene and E2 on the growth of CaSKi cells. *P < 0.05 represents the percentage of survival cells in Raloxifene (10 μM) and E2 (0 μM) complex group compared to other complex group (including both of Raloxifene and E2 0 μM, Raloxifene 0.1 μM and E2 0 μM, Raloxifene 1 μM and E2 0 μM, Raloxifene 10 μM and E2 0.1 μM, Raloxifene 10 μM and E2 1 μM, Raloxifene 10 μM and E2 10 μM, Raloxifene 0 μM and E2 10 μM ). #P < 0.05 represents the percentage of survival cells in Raloxifene (0 μM) and E2 (10 μM) complex group compared to the other complex group (including both of Raloxifene and E2 0 μM, Raloxifene 0.1 μM and E2 0 μM, Raloxifene 1 μM and E2 0 μM, Raloxifene 10 μM and E2 0.1 μM, Raloxifene 10 μM and E2 1 μM, Raloxifene 10 μM and E2 10 μM, Raloxifene 10 μM and E2 0 μM).

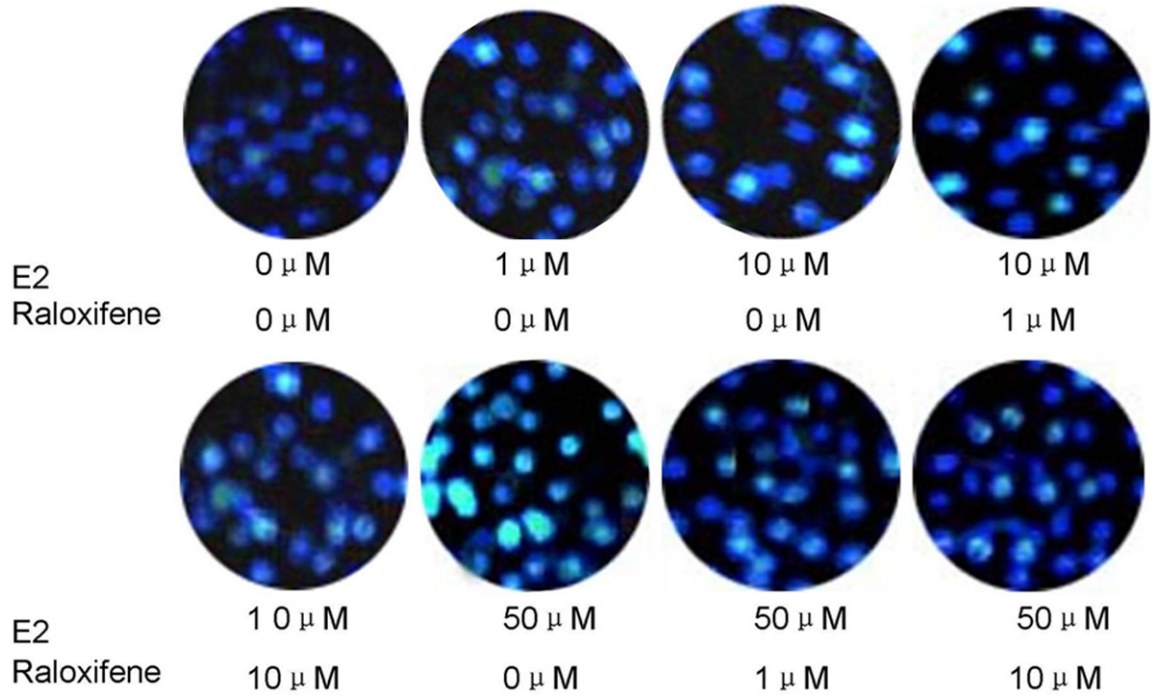


**Figure 2.** TUNEL assay for CaSki cells treated with varying concentration of Raloxifine and estradiol for 48 hours.

*Cervical cancer cell line growth assay*

CaSki cells grown overnight in 96 well plate seeded with 5 × $10^4$ cells per well. 200 μl culture solution prepared with charcoal-stripped FBS. The cells are treated with 0-10 μM Raloxifene with 0-10 μM E2. The culture solutions were renewed everyday. MIT method was employed to detect the light absorption value. At the 48$^{th}$ hour the cell growth was observed and numbers of the surviving cells were calculated.

*TUNEL assay*

TUNEL assay was performed used a kit (in situ cell death detection kit, POD, from Boehinger

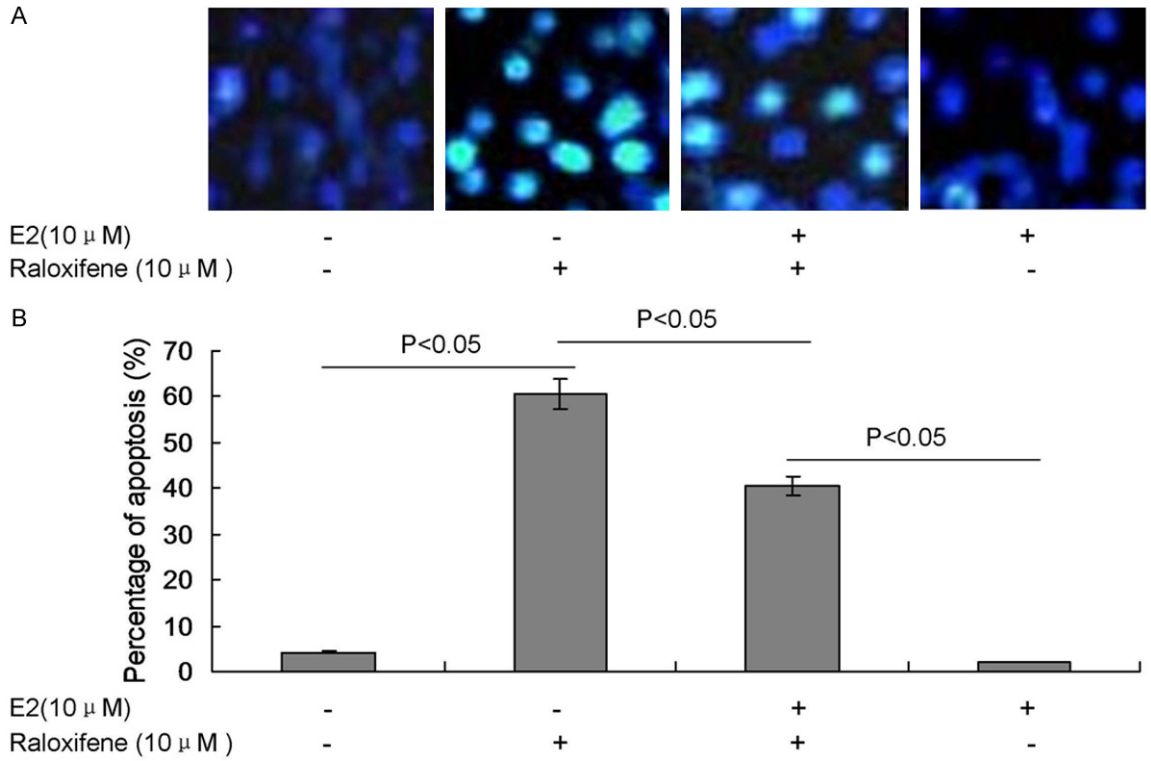


**Figure 3.** Effect of Raloxifene and E2 on DNA fragmentation in CaSki cells. A. TUNEL analysis for the effect of Raloxifene and E2. B. Statistical analysis for the TUNEL assay. CaSki cells in monolayer were treated with Raloxifene for 48 h with or without added E2 at various concentrations. DNA strand breaks were analyzed microscopically by TUNEL.

**Table 1.** CaSki cells were treated with varying concentration of Raloxifine and estradiol for 48 hours

| Raloxifine | Estradiol | % Apoptosis cells (± s.d.) | *P* value | |
|---|---|---|---|---|
| 0 | 0 | 5.6 ± 0.8% | | |
| 1 μM | 0 | 20.6 ± 3.6 | < 0.05 | |
| 10 μM | 0 | 60.2 ± 4.8% | | |
| 10 μM | 1 μM | 45.2 ± 2.1% | | < 0.05 |
| 10 μM | 10 μM | 41.3 ± 2.6% | | |
| 50 μM | 0 | 68.1 ± 4.3% | | |
| 50 μM | 1 | 46.2 ± 3.3% | < 0.05 | |
| 50 μM | 10 | 42.4 ± 4.1% | | |

Apoptosis was determined by TUNEL.

Mannheim Inc. Indianapolis, IN, USA). Cells were grown for 24 hours in 8-well chamber slides seeded with $10^5$ cells per well, treated and incubated of 37°C, for 48 hours. The slides were washed in PBS and fixed with 4% paraformaldehyde for 30 min at room temperature. Fixed cells were washed in PBS, permeabilized with sodium citrate buffer containing 0.1% Triton X-100 for 2 min on ice, and then incubated with terminal deoxynucleotidyl transferase for 1 h at 37°C. After rinsing with PBS, the slides were treated with converter-POD (conjugated with horseradish peroxidase) at 37°C for 30 min and mounted with a glass coverslip. At least 200 cells/well were evaluated for staining.

*RNA isolation*

Total RNA was isolated using RNA STAT-60 Kit (TelTest Inc, Friendswood, TX). Prior to analysis, RNA samples were treated with RNase-free DNase I to eliminate any contaminating DNA.

*Analysis of HPV16 E6/E7 transcription*

For northern analysis, 10 μg of RNA was separated by electrophoresis in formaldehyde-containing agarose gels, transferred by Southern and probed with 32P-labeled coding sequences for HPV 16 E6E7 (nt 56 to nt 875) isolated from the pLXSN16E6E7 plasmid and human beta-actin.

*Statistical analysis*

Statistical analysis was performed using SPSS 13.0 software. The data were analyzed by

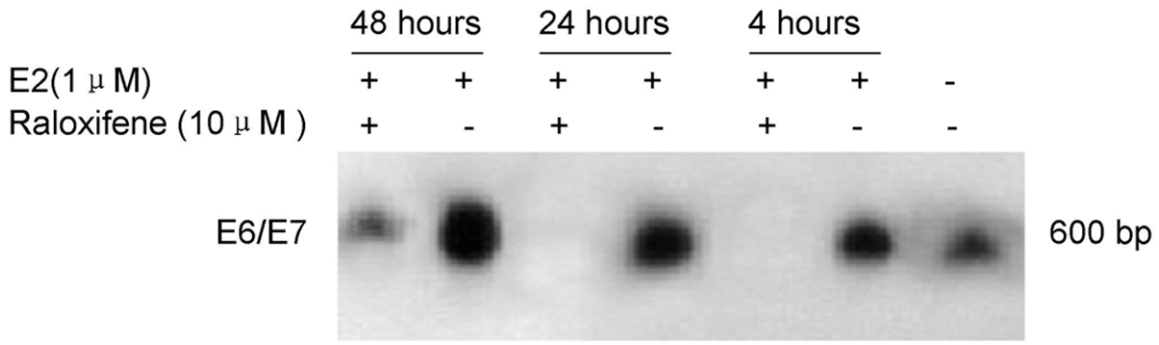


**Figure 4.** Raloxifene decrease transcription level of HPV16 E6/E7. Relative amounts of E6/E7 transcripts from CaSki cells treated or untreated with 1 μM E2 in the presence or absence of 10 μM Raloxifene were determined using Northern blot. The control (-) contained no E2 or Raloxifene and RNA was extracted at 48 hours.

Students *t* test. A *P* value of less than 0.05 was considered statistically significant.

## Results

*Raloxifine inhibits estrogenic promotion activity on growth of CaSki cells*

As shown in **Figure 1**, Raloxifine alone inhibit proliferation of CaSki cells with significantly efficient compared to the other complex group (all $P < 0.05$). The Results indicated that the higher concentrations of raloxifine, the more the inhibition effect of raloxifine is. On the contrary, estradiol alone promote proliferation of CaSki cells with significantly efficient compared to the other complex group (**Figure 1**, all $P < 0.05$). Thus, the higher concentration of estradiol, the less the inhibition effect of raloxifine is.

*Raloxifene suppresses proliferation activity induced by estradiol*

The results showed that raloxifene induces apoptosis of CaSki cells. Conversely, estradiol promotes their proliferation. Raloxifene suppresses the proliferation effects induced by estradiol. The higher concentrations of raloxifene, the more suppression effect it exerts (**Figures 2**, **3**; **Table 1**). On the contrary, the higher concentration of estradiol will significantly reverse the suppression effect induced by raloxifene (**Figures 2**, **3**; **Table 1**).

*Raloxifene suppresses stimulation effect of estradiol on HPV 16 E6/E7 transcription*

The northern experiments showed that raloxifene at 10 μM suppress the activity of 1 μM estradiol on HPV 16 E6E7 (**Figure 4**). The comparison with blank control demonstrated that Estradiol alone enhances E6/E7 expression (**Figure 4**). When raloxifene was added, the E6/E7 transcripts were barely detectable in 4 hours and 24 hours treatment.

## Discussion

The effect of Raloxifine on bones and cardiovascular system is very similar with that of estrogen, while the effects are opposite in reproductive system. Previous studies have demonstrated that raloxifine abrogate the effect of estrogen on breast cancer and endometrium tissues [5]. In the Postmenopausal women, raloxifine decreased the incidence of breast cancer up to 76% [6]. Raloxifine act as estrogen antagonist in granulose cells [7]. Furthermore high concentrations of raloxifine obviously inhibits proliferation of ovarian cancer cell line SKOV3 [8]. However there are very few studies on effect of raloxifine on cervical cells. In a recent study, Raloxifine has been shown to promote regression of high-grade dysplasia and cancer that arose in the cervix of K14E6/E7 transgenic mice treated long-term with estrogen [9]. In our study, raloxifine inhibit the CaSki cells proliferation induced by estradiol, which demonstrate that raloxifine also has anti-estrogen activity in cervical cells.

The mechanism with which raloxifine inhibit proliferation of cervical cells is not clear yet. However, raloxifine is estrogen analogue and competitively combine with estrogen receptor, which may suggest raloxifine and estradiol may affect the growth cervical cancer cells via their combination with estrogen receptor. Estrogen

receptor itself is significantly involved in the occurrence and development of cervical cancer. When the CaSki cervical cancer cells were transduced with dominant-negative estrogen receptor blocker gene, the cytotoxicity of anticancer drugs was augmented [10]. Furthermore, when the CaSki cervical cancer cells were transfected with adenovirus expressing a dominant negative estrogen receptor gene, the HPV E6 and E7 mRNA were reduced and cell proliferation was interrupted. All these together suggest Raloxifine may compete with estrogen to combine with the estrogen receptor, suppress the transcription of HPV 16 E6/E7 and further to inhibit the proliferation of cervical cancer cells. However this hypothesis is needed to be evidenced.

What's more, since E6 and E7 viral oncoproteins can promote cell cycle progression and prevent apoptosis through its inhibition activity on tumor suppressor proteins [11-14], raloxifine might be used to prevent or suppress cervical cancer. At present, it is mainly used as estrogen analogue to prevent osteoporosis induced by estrogen deficiency in menopausal women. However, some studies have proved that raloxifine can effectively reduce the incidence of breast cancer in menopausal women about 76% [6]. Again, in transgenic rats, Raloxifine has been shown to suppress cancer induced by estrogen [9]. However there are very few studies of raloxifine on cervical cancer and further investigation is very necessary.

## Disclosure of conflict of interest

None.

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