Mol Cell Biochem (2011) 347:1–11
DOI 10.1007/s11010-010-0606-3

Curcumin counteracts the proliferative effect of estradiol and induces apoptosis in cervical cancer cells

Mayank Singh · Neeta Singh

Received: 29 June 2010 / Accepted: 28 September 2010 / Published online: 13 October 2010

Abstract Cervical cancer is the most common cancer in Indian females and is associated with infection with high-risk Human papilloma viruses (HPVs) which encode viral oncoprotein E6 and E7. Estradiol has been established as a risk factor for cervical cancer and has been shown to play a synergistic role with viral oncoproteins. Curcumin (Diferuloyl methane), a chemopreventive agent, is a natural compound extracted from *Curcuma longa* that allows suppression and retardation of carcinogenesis in many types of cancer and is currently being tested in various human clinical trials as it has been found to be well tolerated at higher doses with a relatively well established safety profile. The objective of this study was to test the effect of curcumin on HPV-positive and negative cervical cancer cell lines HeLa, SiHa, CaSki, and C33A pretreated with estradiol. It was found that HPV-positive cells pretreated with estradiol show reduced apoptosis as compared to curcumin by itself. However, curcumin was able to counteract the proliferative response of estradiol, and induce apoptosis. There was no difference in percentage apoptosis as compared to estradiol pretreatment in HPV-negative cell line C33A. Molecular studies showed elevation of Telomerase, viral oncoproteins E6 and E7, PCNA, p16, Cyclin D1 in HPV-positive cell lines on treatment with estradiol but after treatment with curcumin the level of E7, PCNA, and Cyclin D1 was reduced but the level of E6, Telomerase, and p16 was unaltered. Furthermore, estradiol-pretreated HPV-negative cell line C33A showed reduction in level of Telomerase, PCNA, p16, and activation of both p53 and p73 tumor suppressor proteins, thus, demonstrating the importance of E6 in estradiol-mediated protective effect.

Keywords Cervical cancer · Estradiol · Curcumin · Telomerase · HPV

M. Singh · N. Singh (✉)
Department of Biochemistry, All India Institute of Medical Science, Room No 3027A, Ansari Nagar, New Delhi 110029, India
e-mail: singh_neeta26@rediffmail.com

Introduction

Cervical cancer is the second most common cancer in women worldwide. Epidemiologic and laboratory data support the conclusion that human papilloma virus (HPV) is the etiologic agent for the vast majority of premalignant and malignant epithelial lesions of the cervical mucosa, as HPV DNA can be detected in 95–100% of all cases [1]. HPV infections are usually transient and do not necessarily lead to clinically significant lesions of the cervical mucosa except persistent infection over a considerable period of time. Given the high incidence of HPV infection compared with the low prevalence of cervical cancer, other factors must be involved in the malignant transformation of the cervical mucosa. Cofactors may include smoking, oral contraceptive use, parity, infection with other sexually transmitted diseases such as Herpes simplex and host factors [2–4].

Sex hormones have been shown to increase gene expression of HPV-16 and -18, the two HPV genotypes frequently associated with cervical cancer. In addition, most cases of cervical cancer arise in the transformation zone, the most estrogen-sensitive region of the cervix [5, 6]. Furthermore, studies suggest that DNA damage by estrogen metabolites may be a factor in cervical

carcinogenesis [7]. Several studies have shown a link between hormonal exposure and risk of cervical cancer. One study has shown that women taking oral contraceptives are at an increased risk of developing cervical cancer and that their carcinomas possessed higher levels of estrogen receptor than women who have not taken estrogen-containing oral contraceptives [8, 9]. Furthermore, women whose mothers were prescribed the synthetic estrogen diethylstilbestrol during pregnancy are at high risk of developing cervical adenocarcinomas [10]. Similar lesions were observed in mice that were exposed to diethylstilbestrol perinatally. It is shown that direct hormonal activation by estradiol of the viral genome in K14 promoter-HPV transgenic mice induced multistage neoplastic progression in the squamous epithelium of cervix and vagina in 100% of transgenic mice [11]. These results highlight the role of estrogen in cervical carcinogenesis and show that estradiol enhances HPV persistence and contributes to subsequent neoplastic progression via enhanced expression of viral oncogenes.

Natural products offer an excellent alternative for therapeutic use as opposed to synthetic compounds because of their relatively well established safety profile. Curcumin is a natural compound extracted from *Curcuma Longa* that allows suppression, retardation, and inversion of carcinogenesis. Curcumin [1,7-*bis*-(4-Hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione] is a major constituent of turmeric powder extracted from the rhizome of the plant *Curcuma longa* found in South and Southeast tropical Asia. Curcumin has been shown to be a potent chemopreventive agent inhibiting tumor progression against skin, oral, intestinal, breast, colon, prostate, and cervical cancer [12].

This study is an attempt to assess the effect of curcumin on cervical carcinoma cells pretreated with estradiol and to study whether the proliferative effect of estradiol can be overcome by curcumin. Our findings reveal that curcumin was able to overcome the proliferation induced by estradiol and it in turn induced apoptosis. Estradiol-treated cells showed an increase in level of E6 and E7 oncoproteins in all HPV-positive cervical cancer cell lines. Curcumin treatment in estradiol-pretreated cell lines resulted in downregulation of cyclin D1, PCNA, and E7 but not E6. Telomerase and p16 remained unchanged. Involvement of E6 in estradiol-mediated proliferation in HPV-positive cell lines was further confirmed by the fact that estradiol pretreatment did not induce proliferation in HPV-negative cell line lacking E6 and the percentage apoptosis induced by curcumin in this cell line was similar to estradiol-pretreated and -untreated cells. This was accompanied by decrease in level of Telomerase, p16, PCNA, and Cyclin D1. The HPV-negative cell line undergoes apoptosis via p53-dependent pathway as reflected by increase in level of both p53 and p73 on treatment by curcumin.

Materials and methods

Cell culture and chemicals

HeLa, SiHa, CaSki, and C33A cells were obtained from National Centre for Cell Sciences, Pune, India and were maintained in Dulbecco's modified Eagle's medium and RPMI1640 (Sigma, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Hyclone), antibiotics, in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. Cells were treated with Estradiol (Sigma, USA) and Curcumin (Sigma, USA).

Antibodies against p16, PCNA, E6, E7, and Cyclin D1 as well as secondary AP conjugated antibodies were obtained from Santa Cruz, USA.

Flow cytometery

Cells (10,000 cells) were treated either with estradiol by itself for 24 h or followed by treatment with 25 and 50 μM Curcumin and then harvested. Cells were fixed in 70% ethanol and left overnight at −20 degree centigrade. Cells were then washed with PBS and incubated in staining solution (20 μg/ml propidium iodide, 50 μg/ml RNase, 0.1% Triton X-100, and 0.1 mM EDTA) for 2 h at 40°C in the dark. The DNA content of the cells was measured by flow cytometer (Becton Dickenson, USA) using Diva software [13].

MTT assay for cell viability/proliferation

The effect of Estradiol on the cell proliferation was determined using MTT assay. In brief, 5×10^3 cells/well were plated in 96-well culture plates. After overnight incubation, the cells were treated with varying concentrations of estradiol (0, 10, 20, 30, 40, 50 60, 70, and 80 nM) for 24 h. The cells were incubated with 5 mg/ml MTT, and the resulting formazan crystals were dissolved in dimethylsulfoxide (150 μl). Absorbance was recorded at 540 nm with a reference at 650 nm serving as the blank. The effect of Estradiol on cell viablilty was assessed as percent cell viability or stimulation index as compared to vehicle-treated control cells, which were arbitrarily assigned 100% viability. The dose of 20 nM Estradiol was selected for HeLa and CaSki while SiHa was treated with 30 nM Estradiol.

Assay of telomerase activity

This was measured using the PCR-ELISA kit (Roche Molecular Biochemicals, Germany). The samples were lysed, and an aliquot containing 2 μg protein was used for the assay. Telomerase-positive embryonic kidney cell line

(HEK-293) was used as positive control, whereas heat-inactivated HeLa extract was used as negative control. The telomerase activity detected was expressed as relative units (RUs) [14].

Immunocytochemistry

Protein expression of p16 and PCNA was assessed by immunocytochemistry (ICC) in control and treated cells grown on coverslips as described previously [15].

Western blot analysis

The expression of various proteins was determined in control and treated cells by Western blotting as described previously [16]. In brief, cells were washed twice in PBS and lysed in RIPA lysis buffer. Total protein was determined by the Bradford assay. Equal amount of protein was loaded and run on 10–15% SDS–polyacrylamide gel, and the proteins were transferred to a nitrocellulose membrane. The membrane was blocked with 5% BSA, followed by hybridization with respective primary and secondary antibody. Final detection was performed with BCIP/NBT substrate (Promega, USA). The bands were analyzed and quantified using $\acute{\alpha}$ image scanner densitometer (Alpha innotech, USA) and normalized with β actin control. The density of control was taken as 1, and results of treatment were expressed in relation to the control as RU.

Statistical analysis

Results are expressed as mean of three individual experiments, and standard deviation (SD) was calculated using Microsoft excel.

Results

Estradiol-induced proliferation in HPV-positive cell lines HeLa, SiHa, and CaSki but not in HPV-negative cell line C33A

To study the effect of estradiol on cervical cancer, we selected four cell lines, namely, HeLa which is HPV-18 positive, SiHa and CaSki which are HPV-16 positive, and C33A which is HPV-negative cell line. Our data shows that estradiol-induced proliferation in HPV-positive cell lines with SiHa showing maximum proliferation at a dose of 30 nM estradiol for 24 h, whereas HeLa and CaSki showed maximum proliferation at a dose of 20 nM estradiol. No proliferation was observed in C33A on treatment with varying concentration of estradiol (Fig. 1).

Curcumin counteracts the proliferative effect of estradiol in HPV-positive cell lines

HeLa cells showed 22 and 32.6% apoptosis on treatment with 25 and 50 μM curcumin alone for 24 h whereas in cells

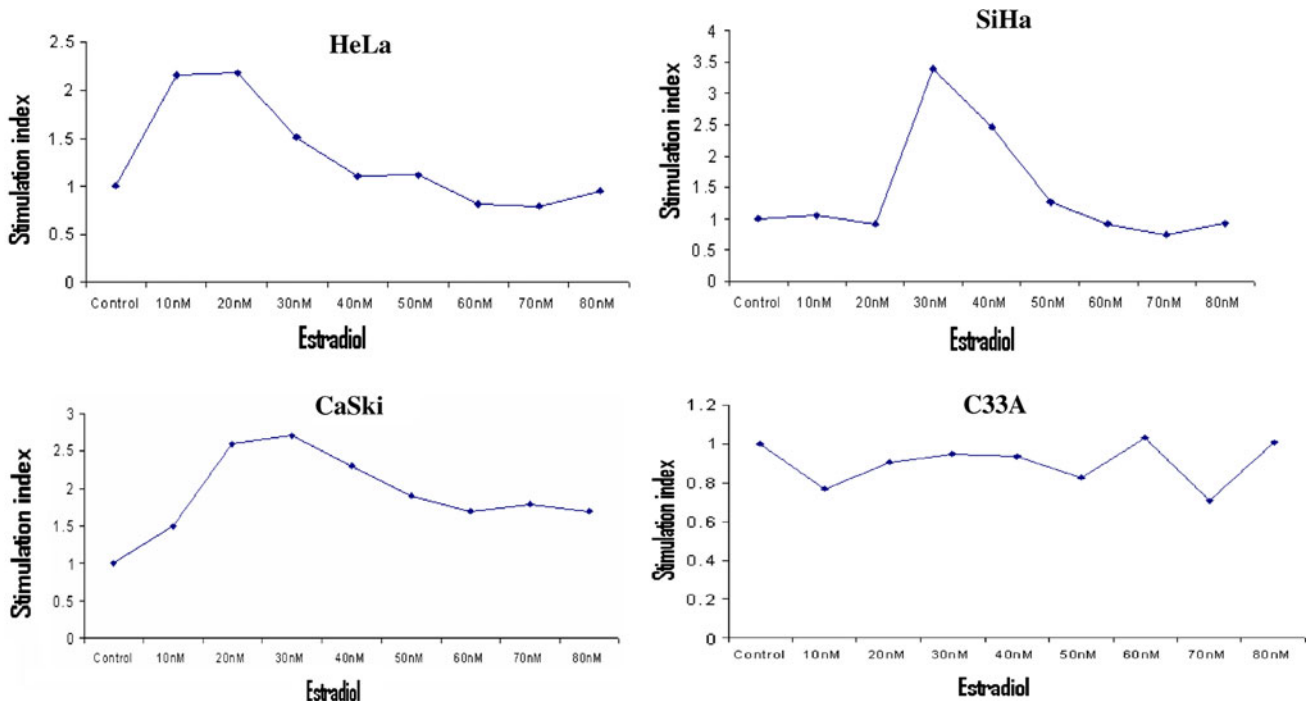

Fig. 1 Effect of estradiol on cervical cancer cells after 24 h as measured by MTT assay

pretreated with estradiol 11.2 and 13.2% apoptosis was observed. Similarly, SiHa cells showed 24.5 and 36% apoptosis, and CaSki showed 26.7 and 35% apoptosis on treatment with 25 and 50 μM curcumin alone for 24 h but in cell pretreated with estradiol, 10.2 and 15.4% apoptosis was seen in SiHa, and 8.3 and 16.2% apoptosis in CaSki cells was observed. HPV-negative cell line C33A showed 24.5 and 36% apoptosis on treatment with 25 and 50 μM curcumin alone and in estradiol-pretreated cells the apoptosis remained at 24.5 and 36%. These results show that curcumin overcomes the proliferative effect of estradiol in HPV-positive cervical cancer cell lines since no proliferation was observed on estradiol treatment in HPV-negative cell line C33A, hence the percentage apoptosis induced by curcumin in these cells with and without estradiol remains the same (Fig. 2).

Curcumin treatment in estradiol primed HPV-positive cells showed no reduction in telomerase activity whereas C33A showed reduction in telomerase activity

Telomerase has been identified as a marker of cervical dysplasia and cancer, in cervical biopsy specimens.

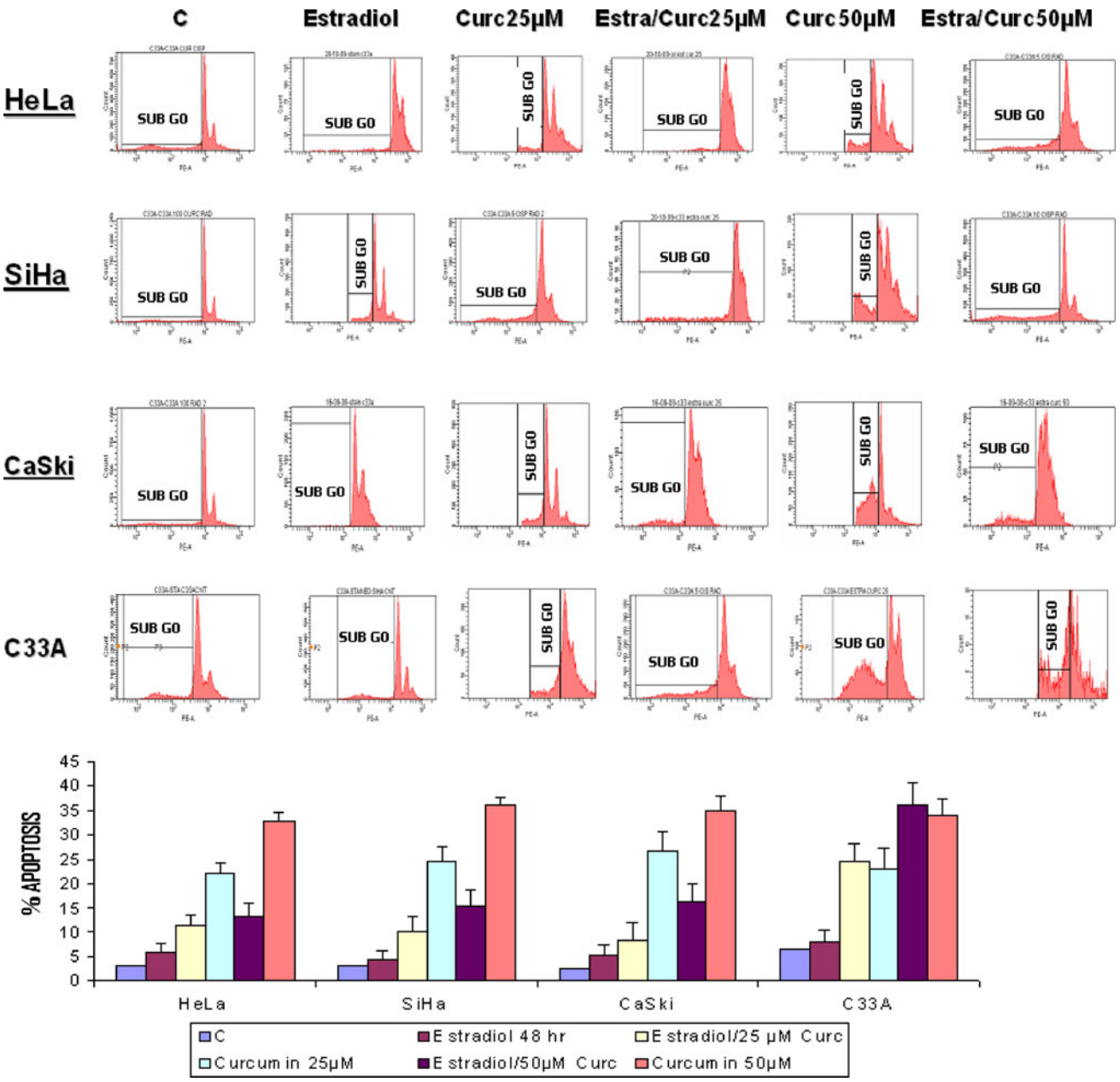

Fig. 2 Percentage apoptosis by flow cytometery in cervical cancer cells sensitized by estradiol for 24 h followed by treatment with 25 and 50 μM Curcumin. Results shown are mean of three individual experiments ± SD

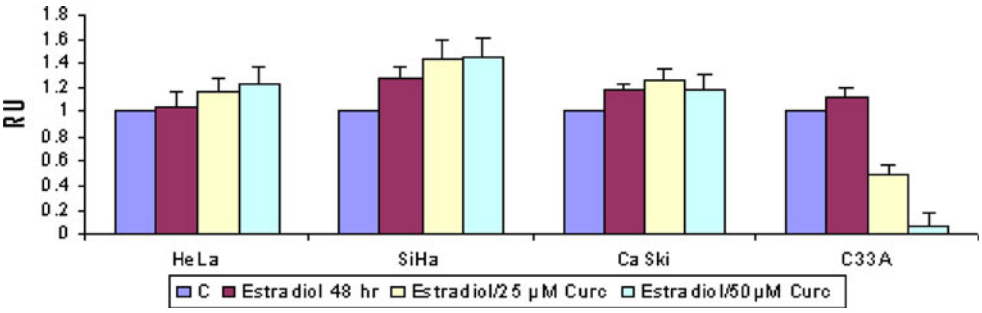

Fig. 3 Telomerase activity in cervical cancer cells sensitized by estradiol for 24 h followed by treatment with 25 and 50 µM Curcumin. Results shown are mean of three individual experiments ± SD

Telomerase activity was studied using PCR-ELISA. The HPV-positive cell lines showed increase in Telomerase activity on treatment with Estradiol as compared to HPV-negative cell line. However, the increase remained unaffected on challenging HPV-positive cells with curcumin but decreased significantly in HPV-negative C33A cells. Treatment of HeLa, SiHa, and CaSki cells for 48 h with estradiol showed a 5, 29, and 18% increase in telomerase activity, respectively, as compared to control. Pretreatment of these cell lines for 24 h with Estradiol followed by 25 or 50 µM curcumin treatment further increased telomerase activity to 16, 44, 26, and 23%, 45 and 19%, respectively. However, treatment of C33A cells for 48 h with estradiol showed increase of 12% in Telomerase activity. Pretreatment of these cells for 24 h with Estradiol followed by 25 and 50 µM Curcumin showed marked decrease in telomerase activity, i.e., by 51 and 93%, respectively (Fig. 3). Thus, curcumin did not affect telomerase activity in estradiol primed HPV-positive cells whereas it significantly decreased it in HPV-negative cells.

Curcumin causes reduction in p16 levels in HeLa and C33A but not in SiHa and CaSki cells

A wide range of studies have reported the immunocytochemical detection of p16 in cervical cytological specimens and that it can be a marker of cervical dysplasia and carcinoma. p16 is detected in a subset of cases that test positive for high-risk HPVs. We performed ICC to determine expression of p16 in the cervical carcinoma cell lines following curcumin treatment of estradiol-pretreated cells. Our results show localization of p16 in the nucleus in all the cell lines on exposure to estradiol. Following treatment with curcumin, p16 level remained unchanged in HPV-16-positive cell lines SiHa and CaSki but it was found to be reduced in HPV-18-positive cell line HeLa and the HPV-negative C33A cells (Fig. 4).

Curcumin reduces PCNA level

The PCNA has been established as an important proliferative marker in cervical cancer; hence, we studied the level of PCNA using ICC and Western blotting. ICC results show localization of PCNA in the nucleus in both control and estradiol-treated cell lines. Curcumin treatment resulted in disappearance of PCNA in all the cell lines indicating that curcumin caused reduction in its level. These finding were confirmed by western blotting. Treatment of SiHa, CaSki, and C33A cells with estradiol showed a 5, 18, and 15% increase in PCNA level as compared to control but remained unchanged in HeLa cells. Treatment with 25 µM Curcumin showed a marginal decrease in PCNA level. However, treatment with 50 µM Curcumin showed reduction of 75, 42, and 8% in HeLa, SiHa, and CaSki, respectively, but its level remained unchanged in C33A cells (Fig. 5).

Curcumin resulted in reduction of Cyclin D1 similarly in all the cell lines

Cyclin D1 plays an important role in transition of cells from G1 to S cell cycle phase, and its overexpression has been regarded as an important marker to monitor progress of cervical cancer. Increase in level of Cyclin D1 was found in HeLa, SiHa, CaSki, and C33A cells post estradiol exposure which increased to 42, 34, 39, and 19%, respectively. 25 µM Curcumin treatment showed reduction in Cyclin D1 with respect to estradiol-treated cells. However, treatment with 50 µM curcumin showed reduction of 52, 15, and 15% in HeLa, CaSki, and C33A cells (Fig. 6).

Curcumin reduced the estradiol mediated upregulation of E6 and E7 oncoprotein in HPV-positive cervical cancer cell lines

E6 is an oncoprotein encoded by HPV which causes ubiquitin-mediated degradation of p53. One of the hypothesis proposed by which estradiol has been proven as a risk factor in cervical cancer is that it causes elevation in level of E6 and E7 thereby acting as a potential carcinogen. We investigated whether there was any increase in level of E6 following treatment with estradiol. Our results show a marked elevation in E6 level in all the cell lines with HeLa, SiHa, and CaSki showing 60, 70, and 48% increase on

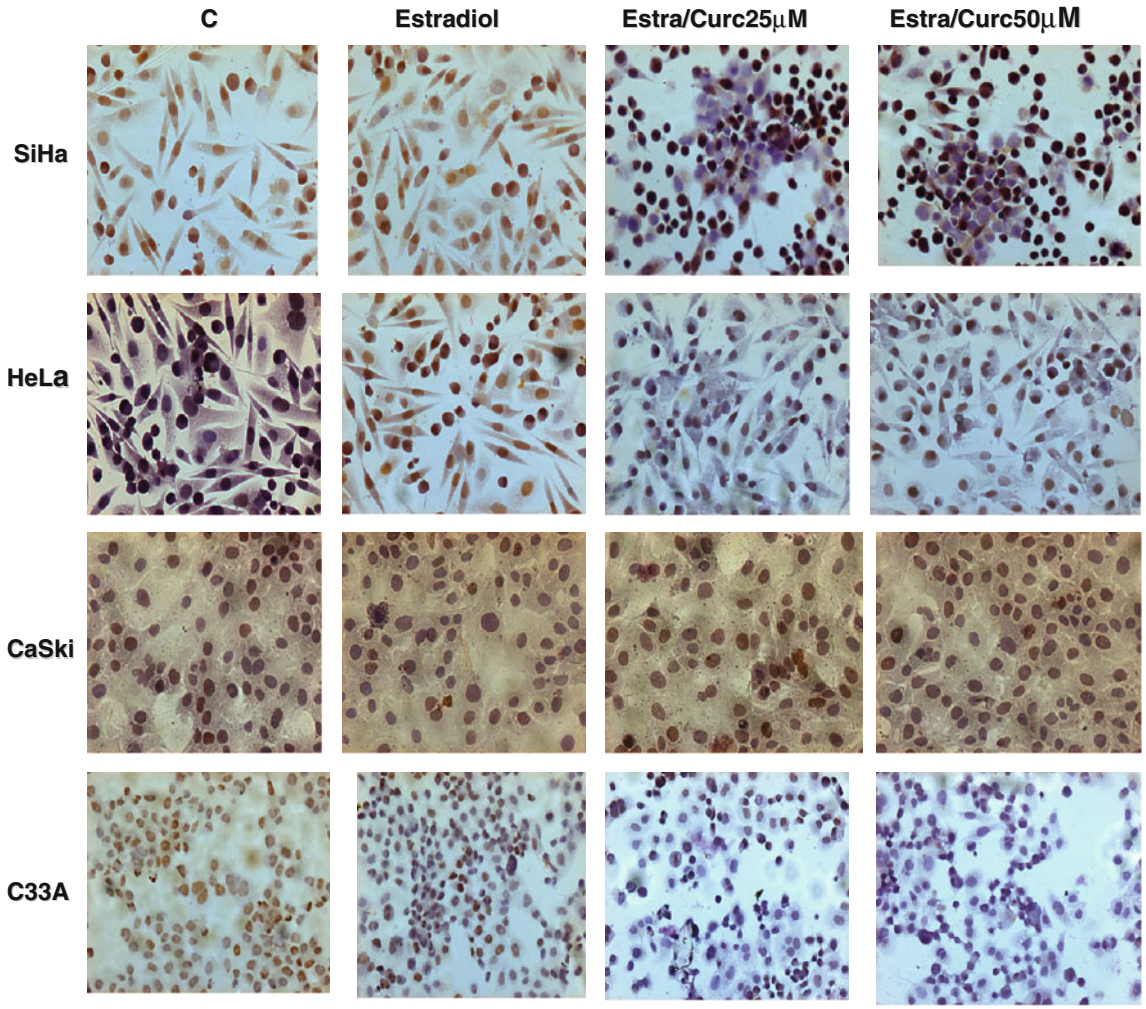

Fig. 4 Immuno cytochemistry to determine expression of p16 in SiHa, HeLa, CaSki, and C33A cervical cancer cells sensitized by estradiol for 24 h followed by treatment with 25 and 50 μM Curcumin

treatment with estradiol. Treatment with 25 and 50 μM Curcumin showed marginal reduction in level of E6 with respect to estradiol-treated cells implicating that pretreatment of estradiol nullified the effect of curcumin on E6. This might be the potential mechanism by which these cells survive curcumin-mediated cytotoxicity thereby showing reduced apoptosis as compared to curcumin itself, as curcumin has been shown to reduce E6 level in these cell lines (Fig. 7).

E7 is another oncoprotein encoded by HPV which interacts with various proteins most of which are important regulators of cell growth, especially retinoblastoma tumor suppressor family of protein. These interactions are the likely mechanism by which E7 deregulates cell cycle and hence leads to cell proliferation, immortalization, and finally transformation. We observed a marked elevation in E7 level in all the cell lines with HeLa, SiHa, and CaSki showing 30, 45, and 80% increase, respectively, on treatment with estradiol. Curcumin treatment showed distinct reduction in E7 with HeLa, SiHa, and CaSki showing 77, 66, and 32% reduction indicating that unlike E6, curcumin is causing the more downregulation of E7 protein (Fig. 7).

C33A cells undergo apoptosis via p53-dependent pathway

HPV-negative C33A cells have been shown to undergo apoptosis via p53-dependent pathway in response to curcumin treatment. We investigated whether the same mechanism operates in estradiol-pretreated cells. We observed an increase of 72 and 83% in level of both p53 and p73 in response to 25 μM curcumin treatment which was sustained even at a dose of 50 μM curcumin, suggesting that these cells are undergoing apoptosis via same mechanism as by treatment with curcumin alone (Fig. 7).

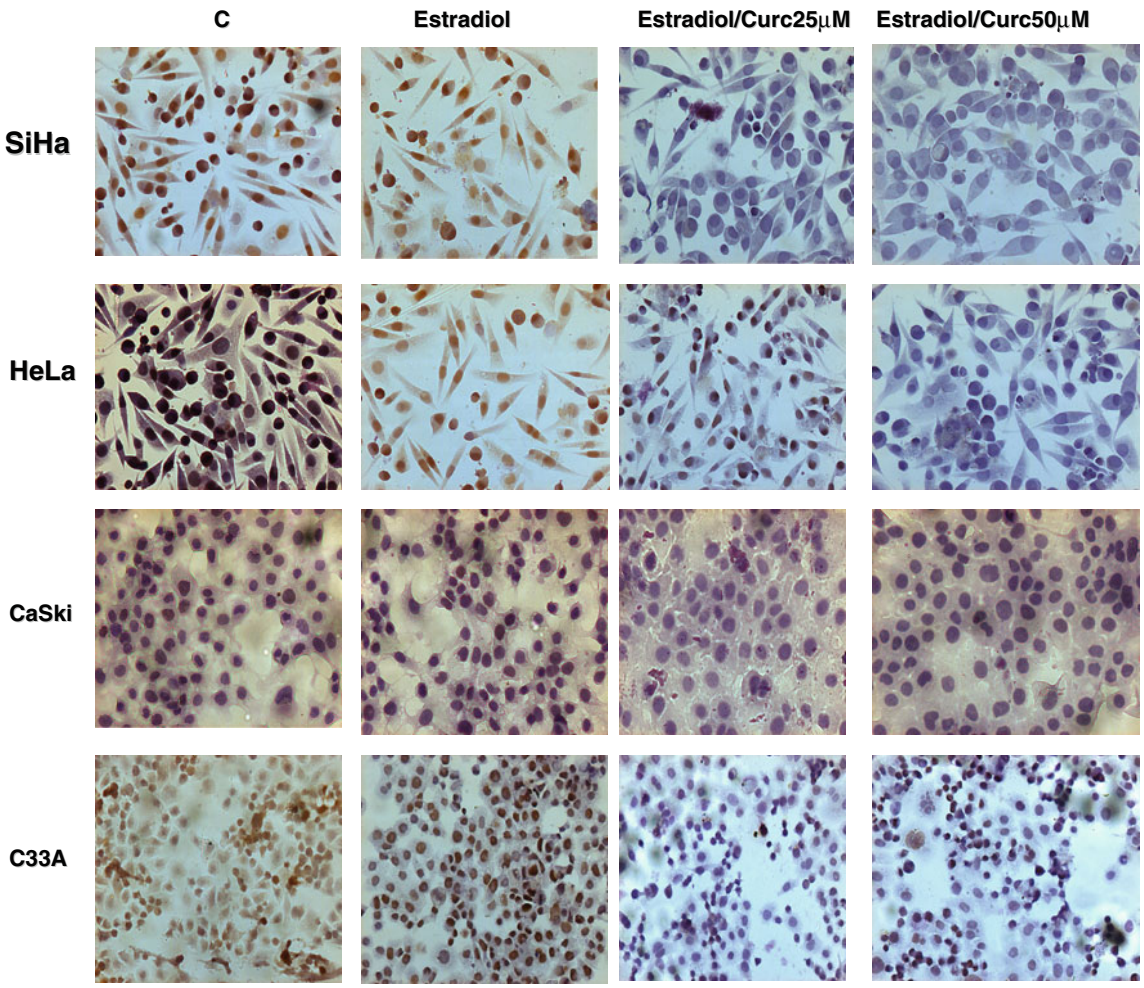

Fig. 5 Immuno cytochemistry to determine expression of PCNA in SiHa, HeLa, CaSki, and C33A cervical cancer cells sensitized by estradiol for 24 h followed by treatment with 25 and 50 μM Curcumin

Discussion

Curcumin exhibits great promise as a therapeutic agent, and is currently in human clinical trials for a variety of conditions, including multiple myeloma, pancreatic cancer, myelodysplastic syndromes, colon cancer, psoriasis, and Alzheimer's disease. The antitumor and antiviral properties of curcumin, in HPV-associated cervical cancer cells have been attributed to the inhibition of expression of viral oncogenes E6 and E7 which have been shown to be upregulated in response to estradiol treatment. The objective of this study was to investigate whether curcumin was able to inhibit estradiol-mediated upregulation of E6/E7 oncoproteins and subsequently induce apoptosis, in cervical cancer and to compare these effects with the HPV-negative cell line C33A.

The "high-risk" HPV types, such as HPV-16 and -18, are found in 70–80% of invasive cervical cancers. However, infection with HPVs in human cervical squamous epithelium does not always progress to in situ or invasive carcinomas implicating either environmental or genetic cofactors in those rare cases where progression occurs [17]. Another cofactor that has been repeatedly associated with HPV neoplasia is exposure to estrogen.

Pregnancy appears to be a permissive environment for persistent HPV infection while the prolonged use of oral contraceptives, most of which contain estrogen, has been shown to double the risk of HPV neoplasia and malignancy. The 1-kb enhancer/promoter of both HPV-16 and -18 has been shown to contain response elements for progesterone and glucocorticoids [18]. Further, estrogen itself has been shown to transactivate the viral genome in HPV-containing cell lines. Thus, estrogen contributes to HPV persistence and subsequent neoplastic progression by increasing viral gene expression. However, other mechanisms of estrogen action have been reported which suggest that estrogen and the HPV oncogenes cooperate at different levels to facilitate neoplastic progression [18]. In addition,

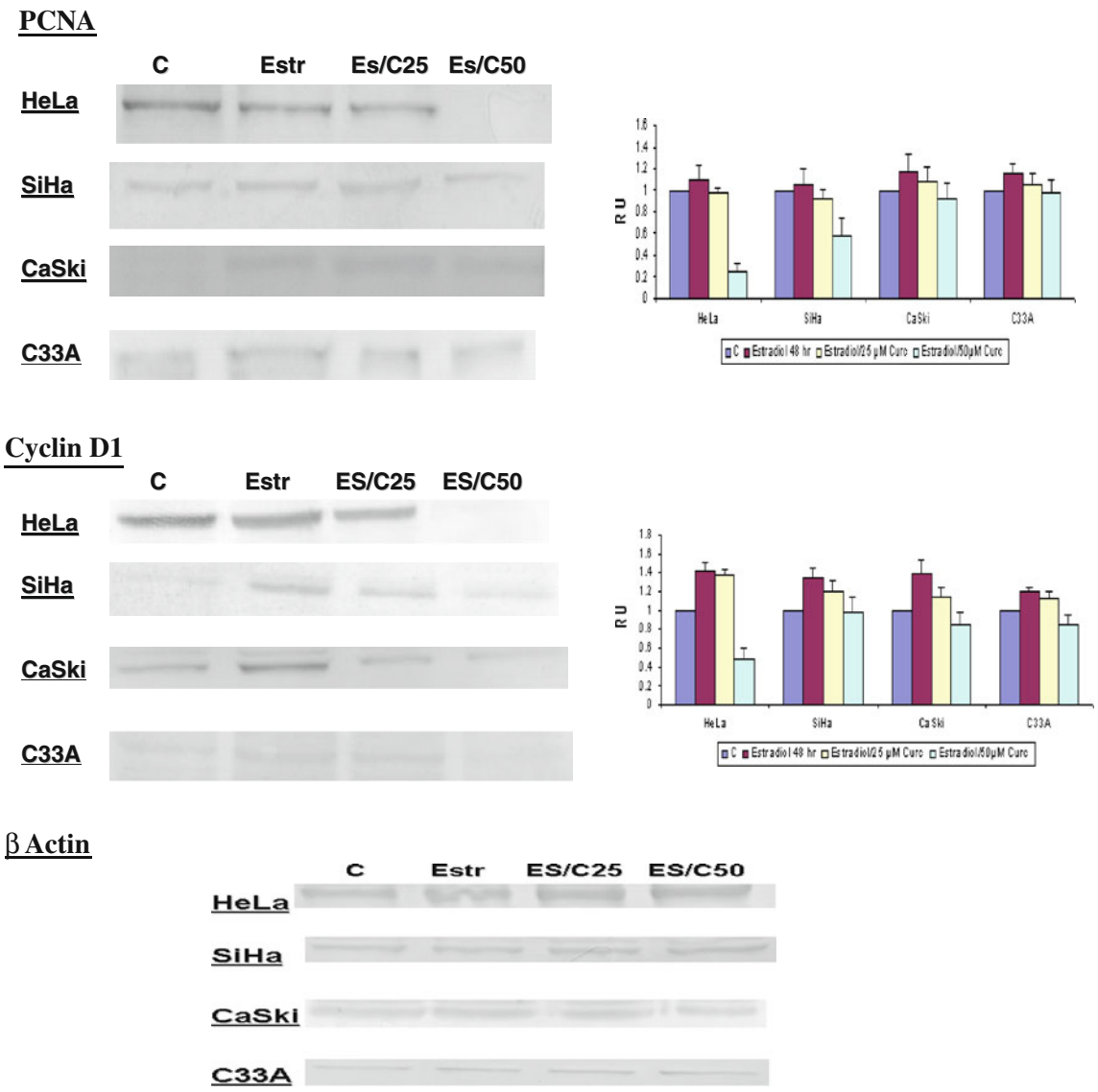

Fig. 6 Western blot analysis to demonstrate the effect of Estradiol and Estradiol/25/50 μM Curcumin on PCNA and Cyclin D1. Results shown are mean of three individual experiments ± SD

estrogen has been shown to be a direct carcinogen, an effect apparently linked to a specific pathway of oxidative hormone metabolism. HPV infection has also been shown to markedly increase the formation of potentially carcinogenic estrogen metabolites [19, 20].

Most human cancers have short telomeres and express high levels of telomerase activity as compared to normal tissue [21], cervical carcinoma cells have been shown to possess high level of telomerase protein [22]. Our data show that Telomerase activity was elevated in all the cell lines which indicate that Estradiol is causing upregulation of Telomerase activity. However, on treatment with curcumin further elevation in telomerase activity was seen in HPV-positive cell lines but a marked inhibition in HPV-negative cells. This partly explains the reduced apoptosis in HPV-positive cell lines as compared to curcumin alone and acute cytotoxicity in C33A as elevation of Telomerase activity has been shown to be sufficient to immortalize these cells.

The repression of p16 gene expression by hypermethylation or mutation is a common occurrence in a wide range of cancer cell lines and primary human tumors. However, in most cervical carcinomas, the functional inactivation of pRb by HPV E7 results in the reciprocal over expression of p16, because of a negative feedback loop between pRb and p16. Thus, p16 overexpression in cervical neoplasia is a surrogate marker of HPV E7-mediated pRb catabolism, reflecting disruption of mechanisms that control cell proliferation and indicating persistent infection with high risk of development of neoplasia [23, 24]. ICC results show

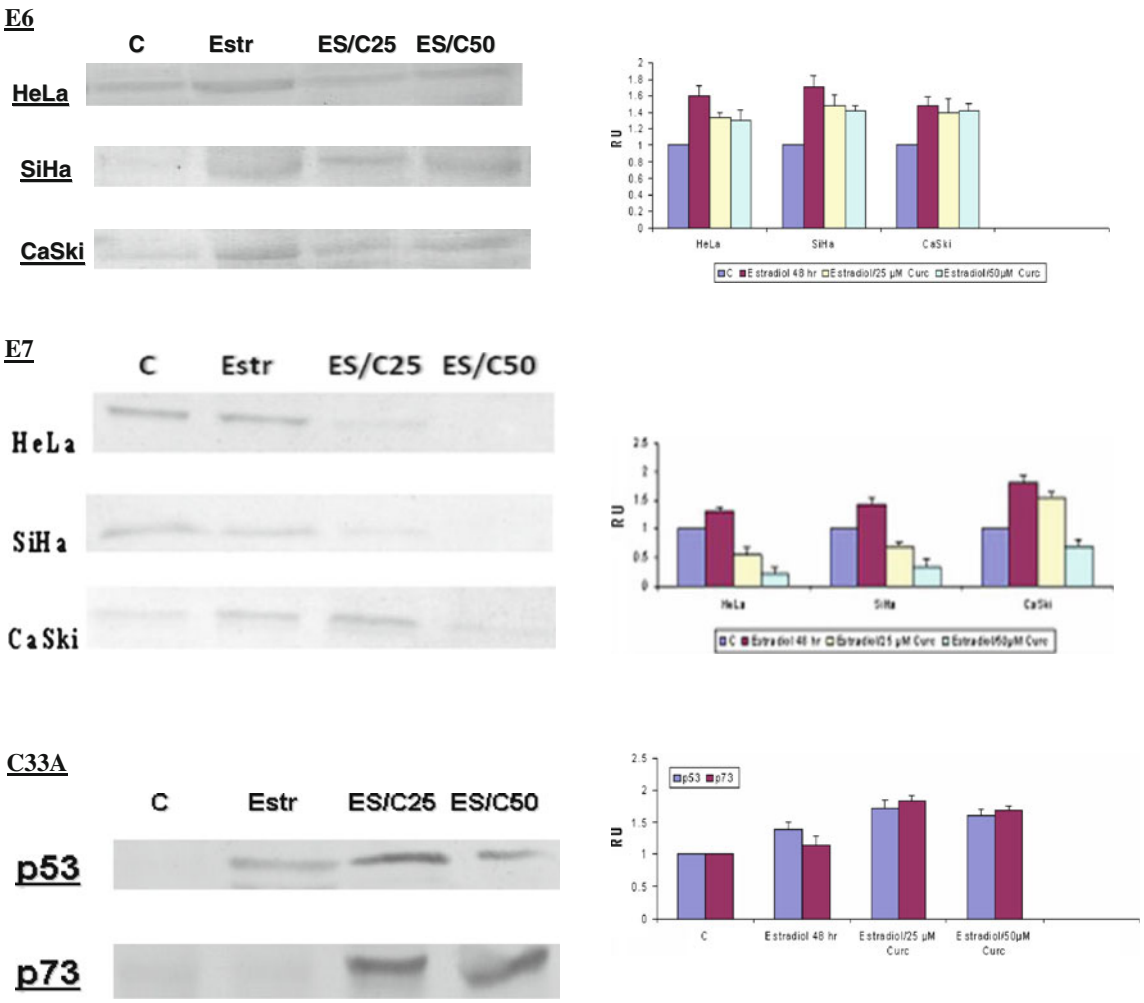

Fig. 7 Western blot analysis to demonstrate the effect of Estradiol and Estradiol/25/50 μM Curcumin Viral protein E6 and E7 in HPV-positive cell lines and activation of p53 and p73 on treatment of estradiol-pretreated C33A by curcumin. Results shown are mean of three individual experiments ± SD

strong nuclear staining in the HPV-16-positive cell lines on treatment with curcumin thereby demonstrating that p16 level remains unchanged even after curcumin challenge. However, p16 was reduced in case of HPV-18-positive cell line HeLa demonstrating that in this cell line there is reduction in level of p16. C33A showed reduced nuclear staining of p16 because of the absence of negative feedback loop and the absence of HPV. These results demonstrate that in HPV-16-positive cells estradiol stabilizes p16 and its level is not altered on curcumin treatment but in HeLa and C33A curcumin it was able to downregulate the level of p16.

The proliferating cell nuclear antigen (PCNA) is an essential component of DNA replication, cell cycle regulation, and epigenetic inheritance. High expression of PCNA is associated with poor prognosis in patients with breast cancer [25]. The 5′-region of the PCNA gene contains two computationally detected estrogen response element (ERE) sequences, one of which is evolutionarily conserved. Both of these sequences are of undocumented cis-regulatory function [26, 27]. It has been demonstrated that estradiol enhances PCNA mRNA expression in MCF7 breast cancer cells. Here, we report that in cervical cancer cell lines estradiol induces expression of PCNA which was found to be localized to the nucleus. Quantification by western blotting demonstrates increase in expression of PCNA on treatment with estradiol in all the cell lines and reduction in its levels after treatment with curcumin, but to varying extents thereby demonstrating cell type to cell type variation. This data demonstrate that curcumin causes reduction in level of PCNA. Thus, this might be one of the potential mechanism by which curcumin is contributing to growth arrest.

Cyclin D1 is important for development and progression of several cancers including those of breast, esophagus, bladder, and lung [28, 29]. Overexpression of cyclin D1

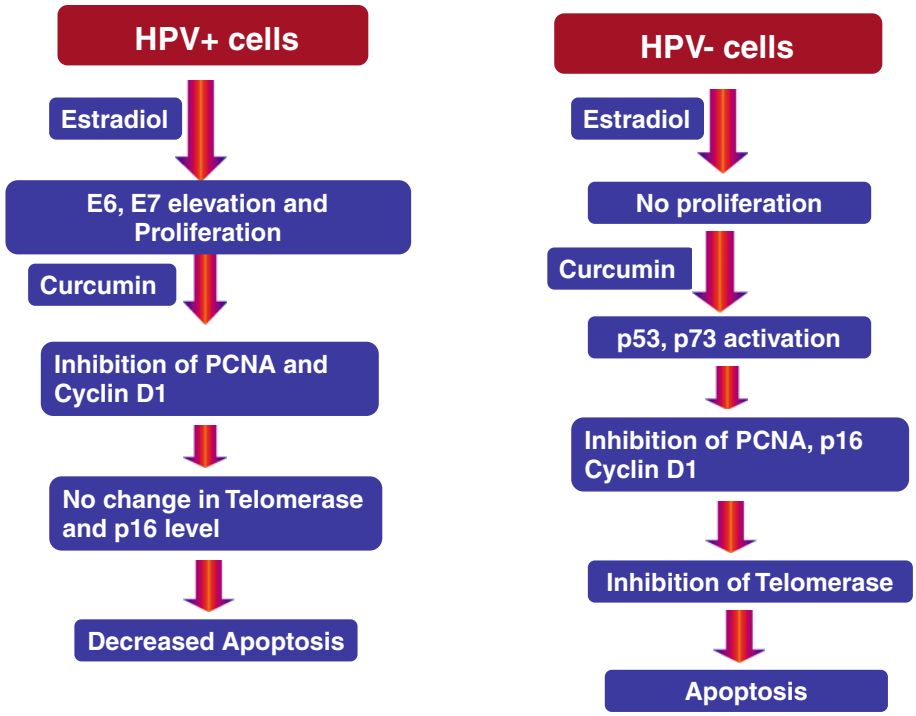

Fig. 8 Schematic diagram to demonstrate the effect of Estradiol in HPV-positive and negative cervical cancer cells against curcumin-induced cytotoxicity

has been linked to the development of endocrine resistance in breast cancer cells [30]. Curcumin has been shown to inhibit Cyclin D1 in prostate cancer cells and various breast cancer cell lines [29, 30]. We demonstrate that estradiol causes activation of Cyclin D1, thereby contributing to increased proliferation. However, Curcumin resulted in decrease of cyclin D1, thereby implicating that curcumin is causing decrease in level of Cyclin D1.

The E6 and E7 proteins of oncogenic HPVs can play critical roles in immortalization and malignant transformation of cervical epithelial cells. From previous epidemiologic data, it has been showed that long-term use of oral contraceptives may be a risk factor for cervical cancer. Investigation of the estrogenic and antiestrogenic effects on the proliferation of cervical cancer cells and the gene expression of HPV would help to explain the role of estrogen in the HPV-associated pathogenesis of cervical cancer. Data from previous studies show that estradiol induces increase in level of E6 and E7 HPV viral proteins. We found that estradiol results in increased expression of E6 and E7, but on treatment with curcumin it was unable to cause substantial decrease in level of E6. Curcumin was able to substantially inhibit expression of E7 in all the three HPV-positive cell lines. Thus, curcumin is partially able to overcome the effect of viral oncogenes. Data from previous studies have shown that E6 synergizes with estradiol to induce tumors and its enhanced expression, and pleiotropic effect is sufficient to immortalize cells in vitro. Though curcumin caused decrease in expression of E7 it was unable to decrease level of E6, This may be one of the major reason why curcumin is unable to induce apoptosis in estradiol-treated cells as it is able to do by itself. The observed increase in level of both p53 and p73 on treatment with curcumin implicates that C33A cells are undergoing apoptosis via p53 pathway.

In conclusion, our data provide the first insight into the therapeutic potential of curcumin in vitro in estradiol-pretreated HPV-positive and negative cervical cancer cell lines. Our findings show that curcumin treatment was partially able to overcome the proliferative effect of estradiol by causing decrease in level of PCNA, Cyclin D1, and viral oncoprotein E7, but was unable to inhibit the activity of telomerase, and E6 viral oncoprotein. We hypothesize that curcumin is having an overriding effect as reduced apoptosis was seen in estradiol-pretreated cells, but curcumin able to overcome the proliferative effect of estradiol and induce apoptosis. In contrast, HPV-negative cell line C33A showed good response to curcumin reflecting the importance of viral oncoproteins E6 in estradiol-mediated effect as we observed a good decrease in level of Telomerase level resulting in apoptosis which is mediated through p53 pathway in C33A. Based on our findings, we propose a hypothesis for effect of curcumin in these cervical cancer cell lines (Fig. 8). This data has vital importance in designing curcumin-based therapies for cervical cancer as estradiol is physiologically present at the tumor sites, and disrupting its effect on HPV may be an important aspect of anti cervical cancer therapy.

Acknowledgments This study was supported by a senior research fellowship from CSIR to Mayank Singh and by grant from DBT, Govt of India.

References

1. Dehn D, Torkko KC, Shroyer RK (2007) Human papillomavirus testing and molecular markers of cervical dysplasia and carcinoma. Cancer (Cancer Cytopathol) 111(1):1–14
2. Moreno V, Bosch FX, Munoz N, The International Agency for Research on Cancer Multicentric Cervical Cancer Study Group et al (2002) Effect of oral contraceptives on risk of cervical cancer in women with human papillomavirus infection: the IARC multicentric case-control study. Lancet 359:1085–1092
3. Munoz N, Franceschi S, Bosetti C, The International Agency for Research on Cancer Multicentric Cervical Cancer Study Group et al (2002) Role of parity and human papillomavirus in cervical cancer: the IARC multicentric case-control study. Lancet 359: 1093–1101
4. Castellsague X, Diaz M, de Sanjose S, The International Agency for Research on Cancer Multicenter Cervical Cancer Study Group et al (2006) Worldwide human papillomavirus etiology of cervical adenocarcinoma and its cofactors: implications for screening and prevention. J Natl Cancer Inst 98:303–315
5. Kim CJ, Um SJ, Kim TY et al (2000) Regulation of cell growth and HPV genes by exogenous estrogen in cervical cancer cells. Int J Gynecol Cancer 10:157–164
6. Kedzia W, Gozdzicka-Jozefiak A, Kwasniewska A, Schmidt M, Miturski R, Spaczynski M (2000) Relationship between HPV infection of the cervix and blood serum levels of steroid hormones among pre- and postmenopausal women. Eur J Gynaecol Oncol 21:177–179
7. Auborn KJ, Woodworth C, DiPaolo JA, Bradlow HL (1991) The interaction between HPV infection and estrogen metabolism in cervical carcinogenesis. Int J Cancer 49:867–869
8. Deligeoroglou E, Michailidis E, Creatsas G (2003) Oral contraceptives and reproductive system cancer. Ann N Y Acad Sci 997:199–208
9. Salazar EL, Sojo-Aranda I, Lopez R, Salcedo M (2001) The evidence for an etiological relationship between oral contraceptive use and dysplastic change in cervical tissue. Gynecol Endocrinol 15:23–28
10. Herbst AL, Ulfelder H, Poskanzer DC (1971) Adenocarcinoma of the vagina. Association of maternal stilbestrol therapy with tumor appearance in young women. N Engl J Med 284:878–881
11. Arbeit JM, Howley PM, Hanahan D (1996) Chronic estrogen-induced cervical and vaginal squamous carcinogenesis in human papillomavirus type 16 transgenic mice. Proc Natl Acad Sci USA 93:2930–2935
12. Anand P, Sundaram C, Jhurani S, Kunnumakkara AB, Aggarwal BB (2008) Curcumin and cancer: an "old-age" disease with an "age-old" solution. Cancer Lett 267:133–164
13. Singh M, Sharma H, Singh N (2007) Hydrogen peroxide induces apoptosis in HeLa cells through mitochondrial pathway. Mitochondrion 7:367–373
14. Khanna N, Sen S, Sharma H, Singh N (2003) S29 ribosomal protein induces apoptosis in H520 cells and sensitizes them to chemotherapy. Biochem Biophys Res Commun 304:26–35
15. Sen S, Sharma H, Singh N (2005) Curcumin enhances Vinorelbine mediated apoptosis in NSCLC cells by the mitochondrial pathway. Biochem Biophys Res Commun 331:1245–1252
16. Singh M, Singh N (2008) Induction of apoptosis in HPV 16 positive cervical cancer cell lines: involvement of mitochondrial pathway. Mol Cell Biochem 310:57–65
17. Castellsagué X (2008) Natural history and epidemiology of HPV infection and cervical cancer. Gynecol Oncol 110(3):4–7
18. Pater MM, Mittal R, Pater A (1994) Role of steroid hormones in potentiating transformation of cervical cells by human papillomaviruses. Trends Microbiol 2(7):229–234
19. Moodley M, Moodley J, Chetty R, Herrington CS (2003) The role of steroid contraceptive hormones in the pathogenesis of invasive cervical cancer: a review. Int J Gynecol Cancer 13(2):103–110
20. Divya CS, Pillai MR (2006) Antitumor action of curcumin in human papillomavirus associated cells involves downregulation of viral oncogenes, prevention of NFkB and AP-1 translocation, and modulation of apoptosis. Mol Carcinog 45(5):320–332
21. Shay JW, Wright WE (2006) Telomerase therapeutics for cancer; challenge and the new directions. Nat Rev Drug Discovery 5:577–584
22. Jaio MU, Wei XL (2002) Telomere and telomerase in oncology. Cell Res 12(1):1–7
23. Keating JT, Cviko A, Riethdorf S et al (2001) Ki-67, cyclin E, and p16INK4 are complementary surrogate biomarkers for human papilloma virus-related cervical neoplasia. Am J Surg Pathol 25:884–891
24. Klaes R, Friedrich T, Spitkovsky D et al (2001) Overexpression of p16(INK4A) as a specific marker for dysplastic and neoplastic epithelial cells of the cervix uteri. Int J Cancer 92:276–284
25. Aaltomaa S, Lipponen P, Syrjanen K (1993) Proliferating cell nuclear antigen (PCNA) immunolabeling as a prognostic factor in axillary lymph node negative breast cancer. Anticancer Res 13:533–538
26. Bourdeau V, Deschenes J, Metivier R, Nagai Y, Nguyen D et al (2004) Genome-wide identification of high-affinity estrogen response elements in human and mouse. Mol Endocrinol 18:1411–1427
27. Schultz-Norton JR, Gabisi VA, Ziegler YS, McLeod IX, Yates JR et al (2007) Interaction of estrogen receptor alpha with proliferating cell nuclear antigen. Nucleic Acids Res 35:5028–5038
28. Stacey DW (2003) Cyclin D1 serves as a cell regulatory switch in actively proliferating cells. Curr Opin Cell Biol 15(2):158–163
29. Alao JP (2007) The regulation of Cyclin D1 degradation; role in cancer development and potential for therapeutic intervention. Mol Cancer 6:24
30. Hui R, Finnay GL, Carroll JS, Lee CS, Mugrove EA, Sutherland RL (2002) Constitutive overexpression of Cyclin D1 but not Cyclin E confers acute resistance to anti estrogens in T-47 D breast cancer cells. Cancer Res 62(23):6916–6923