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The HPV16 *E6/E7* oncogene sensitizes human ovarian surface epithelial cells to low-dose but not high-dose 5-FU and 5-FUdR ☆

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Abstract

To evaluate the effect of HPV16 E6/E7 on drug sensitivity, primary human OSE cells were infected with HPV16 E6/E7 expressing retrovirus and then exposed to chemotherapeutic agents. Apoptosis induced by mitomycin C was dose-dependent in both primary OSE and E6E7/OSE cells. E6E7/OSE cells were more sensitive to mitomycin C than parental OSE cells. HPV16 E6/E7 also sensitized OSE cells to 5-FU and its derivative 5-FUdR, but only at low doses. This phenomenon was also observed in cervical cancer cells and was independent of thymidylate synthase, a target of thymine and thymidine analogues. We conclude that HPV16 E6/E7 specifically modulates the activity of 5-FU and 5-FUdR, and confers OSE cells hypersensitivity to low-dose but not high-dose 5-FU and 5-FUdR. Molecular analysis indicates that induction of p53 and p21, and suppression of pRB are associated with apoptosis induced by 5-FUdR and may partly explain the hypersensitivity of E6E7/OSE cells to low-dose 5-FUdR.

Keywords: HPV16 E6/E7; OSE; 5-FU; 5-FUdR; Apoptosis

High-risk human papillomavirus (HPV) infections are tightly associated with cervical and other anogenital cancers [1]. Inactivation of tumor suppressor genes plays an important role in cell transformation and tumorigenesis. High-risk HPV E6 oncoprotein has been shown to enhance degradation of the p53 tumor suppressor protein through a ubiquitin-dependent pathway, whereas E7 oncoprotein inactivates retinoblastoma tumor suppressor pRB and its related proteins [2]. Immortalization is a crucial step towards cell transformation. The *E6* and *E7* oncogenes from high-risk HPV16 and HPV18, but not low-risk HPV6, efficiently immortalize normal epithelial cells [1,2]. Although HPV may not be involved in the etiology of other types of cancers, the HPV *E6/E7* gene provides a good tool to extend the lifespan of a variety of normal epithelial cells including keratinocytes [1], mammary epithelial cells [3], and ovarian surface epithelial cells [4]. Since *p53* and *RB* are the most frequently mutated tumor suppressor genes in human cancers, these cells are very useful for in vitro studies of the effect of p53 and pRB inactivation on cell transformation and drug sensitivity.

Cells with functional p53 respond to DNA-damaging agents by increasing protein levels of p53 and its downstream mediators (e.g., p21 and Bax) and arrest in G1 or undergo apoptosis [5]. E6 inactivates p53 and is expected to be anti-apoptotic, and confer resistance to DNA-damaging agents [1,2]. Indeed, it has been shown that apoptosis can be abolished or delayed by HPV E6 in some cell types [6,7]. However, exogenous expression of HPV16 E6 sensitizes human mammary epithelial cells to apoptosis induced by DNA damage [8]. Ectopic expression of HPV16 E6, E7, or E6/E7 from heterologous promoters also sensitizes human fibroblasts and

☆ *Abbreviations:* HPV, human papillomavirus; OSE cells, ovarian surface epithelial cells; 5-FU, 5-fluorouracil; 5-FUdR, 5-fluorodeoxyuridine; MMC, mitomycin C; TFT, trifluorothymidine.

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keratinocytes to apoptosis induced by a variety of chemotherapeutic agents or radiation [9–11]. Consistent with these findings, several HPV16 or 18-positive cervical cancer cell lines, such as HeLa, C-4II, and CaSki, are also sensitive to anti-cancer agents [12]. These HPV-positive cancer cell lines carry integrated HPV viral sequence and express E6/E7 from the viral promoter. Both p53 and p21 are induced by DNA-damaging anti-cancer agents in these cell lines.

Tumor suppressor pRB is a negative regulator of G1/S cell cycle progression. E7 oncoprotein interacts with and inactivates pRB and other members of the RB family by interfering with pRB/E2F interaction and/or by targeting pRB for protein degradation, which in turn results in uncontrolled entry from G1 to S phase, and eventually stimulates cell growth or leads to apoptosis via a p53-dependent pathway. Thus, either induction of p53 or suppression of pRB is associated with enhanced apoptosis [1,2,13].

We introduced a retrovirus, which expressed high-risk HPV16 E6/E7, into primary human ovarian surface epithelial (OSE) cells and evaluated the effect of E6/E7 on the sensitivity of OSE cells to apoptosis induced by anti-cancer agents. We found that E6/E7 sensitized OSE cells to mitomycin C (MMC). Surprisingly, E6/E7 also sensitized OSE cells to low-dose but not high-dose 5-fluorodeoxyuridine (5-FUdR) and 5-fluorouracil (5-FU). Similar effect was observed in HPV-positive cervical cancer cells.

5-FU, one of the most frequently used anti-cancer agents, exerts cytotoxic effects through incorporation of its metabolites into DNA and RNA, and inhibition of thymidylate synthase, the target of thymine or thymidine analogues. 5-FU can be reversibly converted to 5-FUdR by thymidine phosphorylase. Apoptosis induced by 5-FU and 5-FUdR can be mediated through p53-dependent up-regulation of pro-apoptotic genes, such as *Fas* and *Bax*, and down-regulation of anti-apoptotic gene *Bcl-2* [14].

We show here that hypersensitivity of E6E7/OSE cells to low-dose 5-FU and 5-FUdR is independent of thymidylate synthase. Expression of HPV16E6/E7 in OSE cells resulted in increased p53, p21 protein levels, and decreased pRB in response to low-dose 5-FUdR. These molecular changes may underlie the hypersensitivity of E6E7/OSE cells to apoptosis induced by low-dose 5-FU and 5-FUdR. Furthermore, our studies also indicate that E6/E7 or E7 but not E6 confers upon OSE cells the hypersensitivity to low-dose 5-FUdR.

Materials and methods

Cell culture and retroviral infection. Primary OSE cells were cultured in medium 199/MCDB105 (1:1) supplemented with 15% fetal bovine serum, penicillin, and streptomycin as described [15]. OSE cells were infected with retrovirus overnight in the presence of 8 μg/ml of polybrene. G418 selection was carried out for several weeks at a concentration of 50–100 μg/ml. Two primary OSE cultures were used in this study: OSE18 and OSE21. Passage one OSE18 cells and primary outgrowth of OSE21 were infected with LXSN16E6E7 retrovirus. OSE18 cells were also infected with LXSN16E6 and LXSN16E7. Cervical cancer cell lines C-4II and C-33 A were obtained from ATCC and cultured in minimal essential medium supplemented with 10% fetal bovine serum.

Immunofluorescence staining and microscopy. Cells were plated in chamber slides. Near confluent cells were fixed in 2% neutral paraformaldehyde and permeabilized in 0.2% Triton X-100 as described previously [16,17]. Primary antibody used was mouse monoclonal anti-cytokeratin clone K8.13 (ICN). Mouse IgG was used as a negative control. Secondary antibody was FITC-conjugated goat anti-mouse IgG.

Cytotoxicity assay. MMC and 5-FU were purchased from Sigma, 5-FUdR and trifluorothymidine (TFT) were purchased from ICN. Exponentially growing cells in 6-well plates were treated with 0–20 μg/ml MMC, or 0–100 μM of 5-FUdR, 5-FU, or TFT in culture medium for 3–5 days. Floating and adherent cells were collected. Live and dead cells were distinguished by trypan blue staining and counted using a hemocytometer. Alternatively, pictures of cells were taken after drug treatment using a polaroid camera.

DNA fragmentation analysis. Floating cells were collected by centrifugation and resuspended in DNA digestion buffer (100 mM NaCl, 10 mM Tris–Cl, pH 8.0, 0.5% SDS, and 25 mM EDTA) with proteinase K (100 μg/ml). Incubation was carried out overnight at 50 °C. Genomic DNA was extracted by phenol/chloroform and analyzed using a 1.5% agarose gel with ethidium bromide.

Western analysis. Floating and adherent OSE18 and E6E7/OSE18 cells were harvested after treated with 5-FUdR for 3 days and lysed in RIPA buffer supplemented with protease inhibitors with brief sonication [16]. Fifty micrograms of protein was separated on a 4–15% SDS–PAGE gradient gel and transferred to nitrocellulose membrane. Western analysis was performed as described before [16,17]. Primary antibodies used were p53 (Calbiochem Ab-6, 1:200 dilution), p21 (Calbiochem Ab-1, 1:100 dilution), pRB (Pharmingen clone G3-245, 1:300 dilution), and α-tubulin (ICN clone DM1A, 1:200 dilution).

Results

OSE culture

Primary OSE cells were cultured from ovarian surface scrapings as described [15]. After 6–7 passages, they became enlarged and eventually underwent senescence. In contrast, LXSN16E6E7-infected OSE cells, E6E7/OSE18 and E6E7/OSE21, had an extended lifespan and survived at least 15–20 passages. The epithelial origin of OSE and E6E7/OSE cells was confirmed by morphology and by expression of cytokeratins in early passage cells. An example is shown in Fig. 1. As reported by Auersperg et al. [18] OSE cells underwent epithelial–mesenchymal conversion over time in culture and became more and more like stromal cells morphologically (see Figs. 3 and 5).

E6/E7 sensitizes OSE cells to low-dose but not high-dose 5-FUdR

To measure cytotoxicity, OSE and E6E7/OSE cells were treated with various doses of 5-FUdR for 3–5 days.

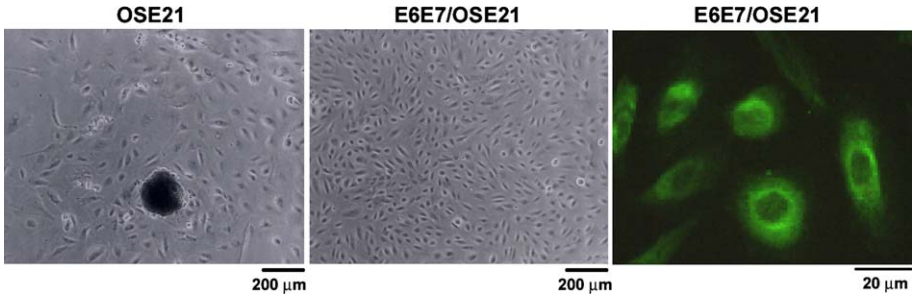

Fig. 1. Morphology and cytokeratin expression in primary OSE and E6E7/OSE cells. The left and middle panels show the morphology of primary OSE21 and E6E7/OSE21 cells, respectively. Right panel shows E6E7/OSE21 cells stained with a cytokeratin antibody. All cells expressed cytokeratins.

As illustrated in Fig. 2A, 5-FUdR induced moderate elevation of cell death with the increase of doses from 0 to 100 μM in OSE18 cells. Approximately 20% of OSE18 cells died at 100 μM of 5-FUdR. Surprisingly, low-dose (5–10 μM) 5-FUdR induced the maximal cell death (~50%) in two LXSN16E6E7-infected OSE cultures, E6E7/OSE18 and E6E7/OSE21. Cell death declined with the increase of 5-FUdR concentrations from 25 to 100 μM in E6E7/OSE cells. There was no obvious difference in the induction of cell death between OSE and E6E7/OSE cells at 50 and 100 μM of 5-FUdR (Fig. 2A). Therefore, HPV16E6/E7 only sensitized OSE cells to low-dose, but not high-dose 5-FUdR. In contrast, MMC-induced cell death in a dose-dependent manner (0–20 μg/ml) in both OSE and E6E7/OSE cells with a maximal cell killing effect (~100% cell death) at

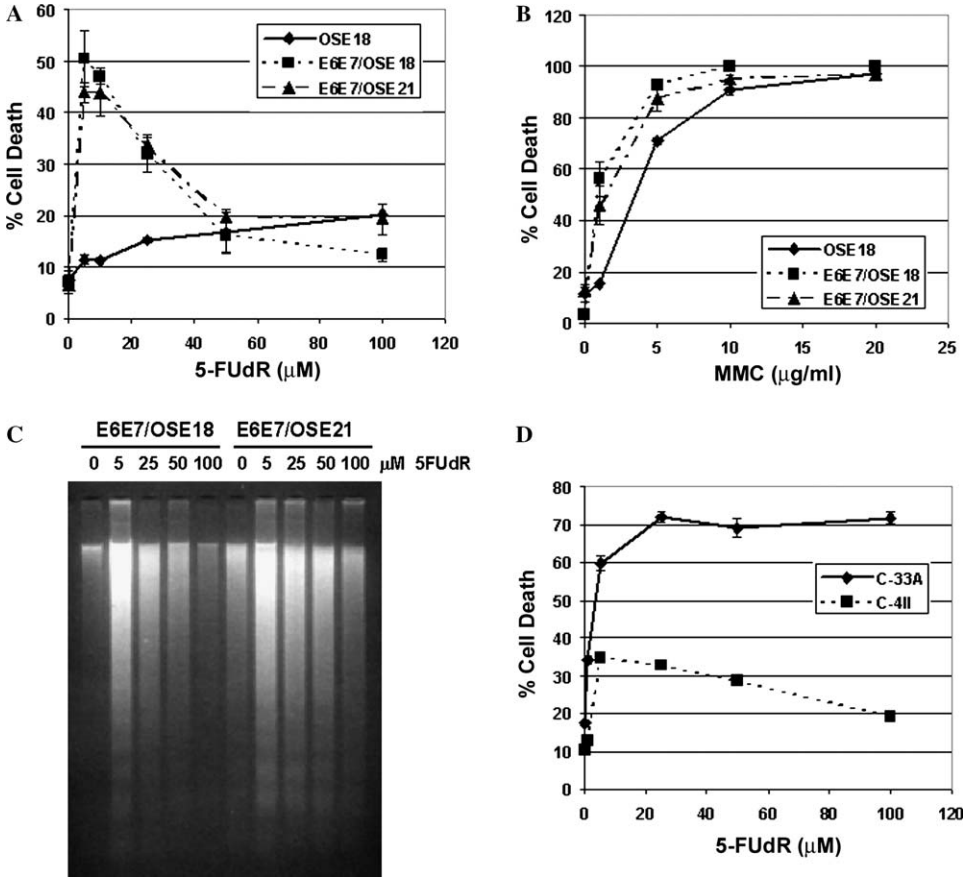

Fig. 2. HPV E6/E7 sensitizes cells to low-dose 5-FUdR. (A) Dose–response curves for OSE18, E6E7/OSE18, and E6E7/OSE21 treated with 0, 5, 10, 25, 50, and 100 μM of 5-FUdR for 5 days (OSE) or 4 days (E6E7/OSE). (B) Dose–response curves for OSE18, E6E7/OSE18, and E6E7/OSE21 treated with 0, 1, 5, 10, and 20 μg/ml MMC for 3 days. (C) DNA fragmentation analysis of E6E7/OSE cells treated with various concentrations of 5-FUdR for 3 days. (D) Dose–response curves for cervical cancer cells C-33 A and C-4II treated with 0, 1, 5, 25, 50, and 100 μM of 5-FUdR for 3–5 days. Data in A, B, and D are presented as means ± SD. Similar results were obtained in 2–4 independent experiments.

the highest dose, 20 μg/ml. Similar to E6/E7-infected human keratinocytes [10], E6E7/OSE18 and E6E7/OSE21 were hypersensitive to MMC treatment compared to OSE18. Both E6E7/OSE18 and E6E7/OSE21 showed approximately 50% cell death, whereas, OSE18 only displayed ~15% cell death, in response to 1 μg/ml MMC (Fig. 2B).

To confirm that cell death induced by 5-FUdR was due to apoptosis, DNA fragmentation analysis of E6E7/OSE18 and E6E7/OSE21 cells was performed after cells were treated with 0, 5, 25, 50, and 100 μM of 5-FUdR for 3 days. 5-FUdR-induced apoptosis in E6E7/OSE cells (Fig. 2C). There was a good correlation between DNA fragmentation (Fig. 2C) and cytotoxicity results (Fig. 2A). The intensity of fragmented DNA was highest at 5 μM of 5-FUdR, the dose inducing the highest cytotoxicity in E6E7/OSE cells.

Although HPV16E6/E7 increased the sensitivity of OSE cells to MMC in various doses, both cytotoxicity assay and DNA fragmentation analysis indicated that HPV16E6/E7 sensitized OSE cells to apoptosis induced by low-dose but not high-dose 5-FUdR.

HPV infection is a major cause of cervical cancer and E6/E7 expression has been detected in many cervical cancer cell lines and cancer biopsies [1]. We then tested whether E6/E7-induced hypersensitivity to low-dose 5-FUdR in HPV-positive cervical cancer cells. Although not as profound as the response in E6E7/OSE cells probably due to lower expression of E6/E7, HPV18-positive cervical cancer cells C-4II did show the highest sensitivity to 5 μM of 5-FUdR (35% cell death). Cytotoxicity declined as the concentration increased from 25 to 100 μM (less than 20% cell death at 100 μM of 5-FUdR) (Fig. 2D). In contrast, HPV-negative cervical cancer cells C-33 A were more sensitive to high-dose 5-FUdR (Fig. 2D). Thus, E6/E7-induced hypersensitivity to low-dose 5-FUdR is not cell type specific.

Hypersensitivity of E6E7/OSE cells to low-dose 5-FU and 5-FUdR is independent of thymidylate synthase

5-FUdR is a derivative of 5-FU, one of the thymine analogues which incorporate into RNA/DNA and cause RNA/DNA damage. Thymine analogues also act as thymidylate synthase inhibitors, which arrest cells in early S phase and induce thymineless cell death [14]. To test whether E6/E7-induced hypersensitivity to low-dose 5-FUdR was thymidylate synthase-dependent, E6E7/OSE18 cells were treated with 5-FUdR, 5-FU, and another thymine analogue, TFT, at 0, 5, 25, and 100 μM for 4 days. Without drug treatment, the cells grew almost to confluence (Fig. 3a). With drug treatment, cell growth was inhibited (Figs. 3b–d). In addition, treated cells underwent apoptosis, became rounded-up, and eventually detached from the culture dish to various degrees (Figs. 3b–d) and therefore were much less confluent compared to the 0 μM controls (Fig. 3a). Like 5-FUdR, 5-FU induced the maximal cell death at 5 μM as indicated by the appearance of apoptotic cells and low cell density, whereas TFT was more potent at 100 μM (Fig. 3). These results indicate that HPV16E6/E7-mediated sensitization of OSE cells to low-dose \5-FU and 5-FUdR is independent of thymidylate synthase.

Expression of HPV16E6/E7 in OSE cells results in increased p53, p21 protein levels, and decreased pRB in response to low-dose 5-FUdR

Expression of E6 induces ubiquitin-mediated p53 protein degradation [2]. As expected, protein levels of

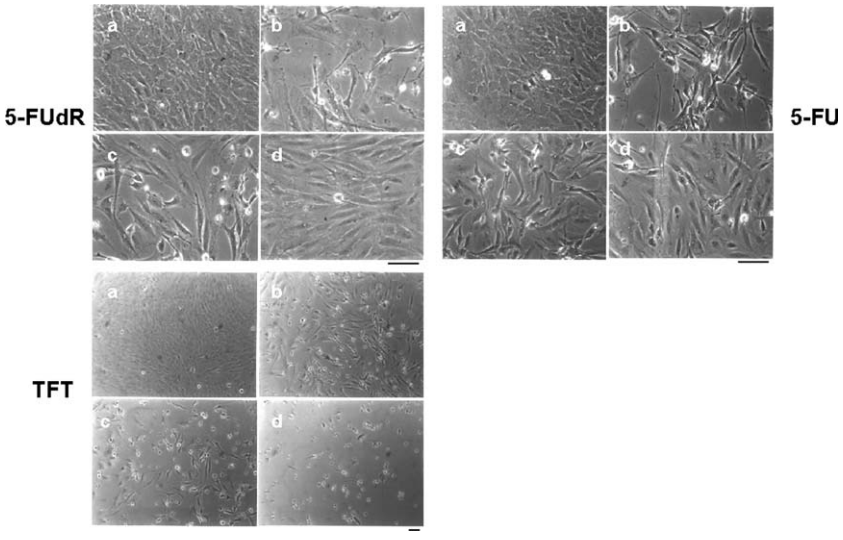

Fig. 3. Morphological changes of E6E7/OSE18 cells treated with 0 μM (a), 5 μM (b), 25 μM (c), and 100 μM (d) of 5-FUdR, 5-FU or TFT. Scale bar: 100 μm.

p53 and its downstream effector p21 were dramatically decreased in E6E7/OSE18 and E6E7/OSE21 cells compared to OSE18 cells (Fig. 4A), indicating that the transduced E6/E7 gene was expressed and E6 protein enhanced degradation of p53 in OSE cells. The change of pRB protein levels in E6E7/OSE cells was not as obvious (Fig. 4) because E7 may inactivate pRB through binding to pRB and/or inducing degradation of pRB [2]. 5-FUdR-induced p53 and p21 proteins in a dose-dependent manner, but suppressed pRB at high doses in OSE18 cells (Fig. 4B). Although p53 and p21 proteins were markedly reduced in E6E7/OSE18 cells, they were induced when cells were treated with low-dose 5-FUdR, with the highest induction at 5 μM. As in OSE18 cells, the profile of pRB protein levels was opposite to those of p53 and p21 in 5-FUdR-treated E6E7/OSE18 cells. The protein level of pRB was suppressed when E6E7/OSE18 cells were treated with low-dose 5-FUdR, and went up at higher doses (Fig. 4C). Thus, induction of p53, p21, and suppression of pRB are associated with increased apoptosis in OSE and E6E7/OSE cells.

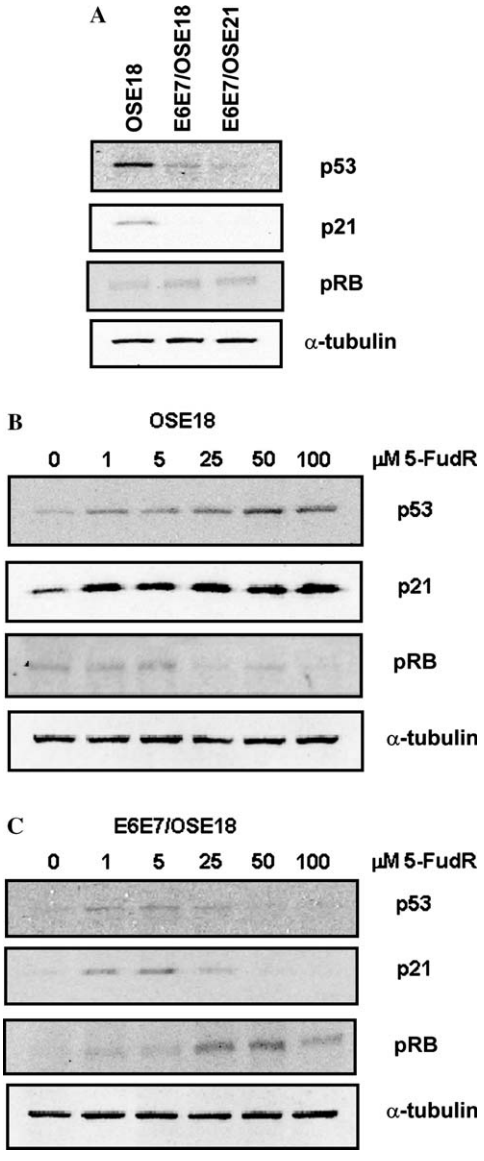

Fig. 4. Induction of p53, p21, and suppression of pRB are associated with E6/E7-induced hypersensitivity to low-dose 5-FUdR in OSE cells. Western analysis of p53, p21, and pRB in exponentially growing OSE18, E6E7/OSE18, and E6E7/OSE21 cells (A); 5-FUdR-treated OSE18 cells (B); and 5-FUdR-treated E6E7/OSE18 (C). α-Tubulin was used as a loading control.

E6/E7 or E7 but not E6 confers OSE cells hypersensitivity to low-dose 5-FUdR

To determine whether E6 or E7 conferred OSE cells hypersensitivity to low-dose 5-FUdR, we also infected OSE18 cells with LXSN16E6 and LXSN16E7 retroviruses and tested their sensitivity to 5-FUdR. Similar to E6E7/OSE18, E7/OSE18 cells were more sensitive to 5 μM than 100 μM of 5-FUdR, while E6/OSE18 cells were more sensitive to 100 μM of 5-FUdR (Fig. 5). E7/OSE18 cells underwent more extensive cell death than E6E7/OSE 18 in response to 5 μM of 5-FUdR probably due to higher p53 and p21 protein levels in the absence of E6.

Discussion

We report here a novel finding that HPV16E6/E7 specifically modulates the activity of 5-FU and 5-FUdR, and confers OSE cells hypersensitivity to apoptosis induced by low-dose but not high-dose 5-FU and 5-FUdR. This unusual phenomenon has not been observed before and is not specific for OSE cells. The HPV18-positive cervical cancer cells C-4II also showed a similar trend and had a better response to 5-FUdR at 5 μM compared with higher doses. In contrast, this phenomenon was not seen in HPV-negative cervical cancer cells C-33 A.

The molecular mechanism underlying this intriguing effect is unclear. We have excluded the possibility that it is dependent on thymidylate synthase, a well-known target of 5-FU and 5-FUdR. Molecular analysis indicates that 5-FUdR-induced cell death seems to be correlated with induction of p53 and p21, and suppression of pRB in both OSE and E6E7/OSE cells.

Compared to parental OSE cells, p53 protein levels were dramatically decreased in E6E7/OSE cells. 5-FUdR treatment induced p53 and p21 in parental OSE cells in a dose-dependent manner. However, only low-dose 5-FUdR moderately induced p53 and p21 in E6E7/OSE cells (Fig. 4). One may argue the significance of the moderate induction of p53 and p21 in E6E7/OSE cells and its correlation with apoptosis. However, Liu et al. [10] have demonstrated that p53 induction in HPV16 E6/E7 expressing human keratinocytes after exposure to

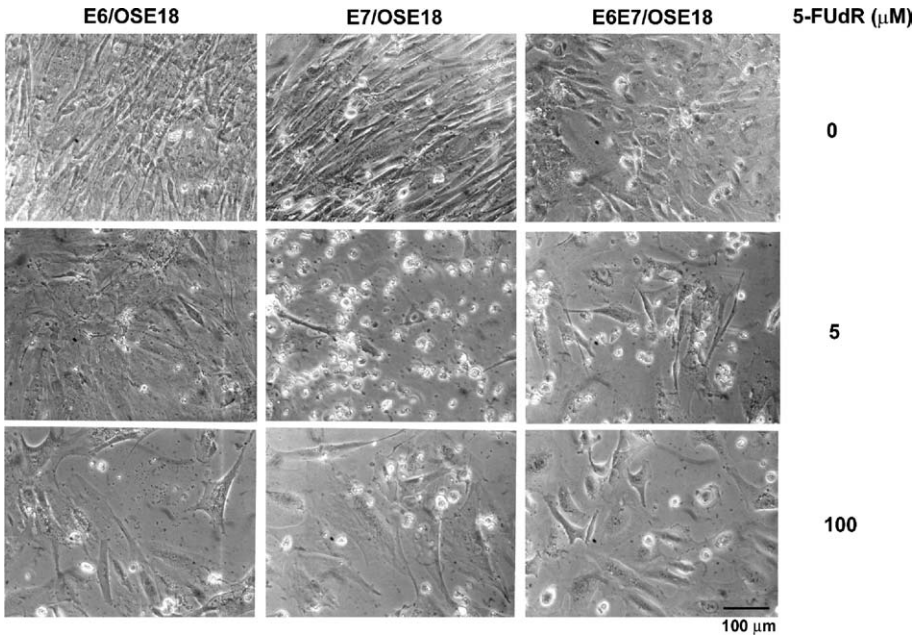

Fig. 5. HPV16 E6/E7 or E7 but not E6 sensitizes OSE cells to low-dose 5-FUdR.

chemotherapeutic agents is required for E6/E7-induced sensitization to apoptosis.

Low-dose 5-FUdR treatment resulted in suppression of pRB in E6E7/OSE cells, whereas, high-dose 5-FUdR had a similar effect on parental OSE cells. Thus, suppression of pRB is correlated with the induction of apoptosis in E6E7/OSE and OSE cells but to a different extent (50% vs. 20% cell death, see Fig. 2). It has been reported that destabilization of pRB and stabilization of p53 are associated with HPV16 E7-induced apoptosis [13]. Indeed, we found that expression of HPV16 E7 alone also sensitized OSE cells to low-dose 5-FUdR. The induction of p53 and p21 in E6E7/OSE cells may be partly due to the expression of E7 [1,2,13]. Furthermore, expression of E6/E7 may alter dose response of OSE cells to 5-FUdR and trigger extensive apoptosis at much lower p53 and p21 protein levels in the low-dose range.

5-FU is used topically for the treatment of cervical and vaginal HPV infections [19,20] and also commonly used as an anti-neoplastic agent in cancers including cervical cancer [14]. Therefore, our observation that low-dose 5-FU is better than high-dose may have clinical significance. Although HPVE6/E7 confers cell transformation, it may also enhance the cellular sensitivity to apoptosis induced by low-dose 5-FU. The hypersensitivity of E6/E7 expressing cells to low-dose but not high-dose 5-FU argues that a lower and continuous dose of 5-FU, which is clinically achievable by either continuous 5-FU infusion or oral administration of capecitabine (a pro-drug of 5-FU), could be a better approach for cancer that is HPV-positive or defective in p53 or pRB. Likewise, topical use of low-dose 5-FU gel might work better in the treatment of HPV infections. High-dose 5-FU may not be the right choice for an optimal effect. Further examination with clinical trials would thus be warranted.

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