

Differential apoptotic response in HPV-infected cancer cells of the uterine cervix after doxorubicin treatment

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Abstract. This study aimed to evaluate differential apoptotic response in uterine cervical cancer cells with and without HPV infection after chemotherapy. CaSki (HPV 16-positive) and C33A (mutant p53 and HPV-negative) cells were used. Cell viability was assessed by trypan blue cell exclusion test. Apoptosis was evaluated by DNA fragmentation analysis and flow cytometric analysis. Differential apoptotic responses were evaluated using Western blot analysis after chemotherapy. Decreased cell viability and apoptosis were displayed in CaSki and C33A cells after chemotherapy using doxorubicin (DOX). Regarding apoptosis-related molecules, the appearance of cleaved PARP expression was more prominent in CaSki cells. p53 and p21 expression in CaSki cells were increased. On the other hand, JNK expression was different in C33A cells (increased) and CaSki cells (decreased). CaSki cells and C33A cells showed differential apoptotic responses after chemotherapy using DOX. CaSki cells seem to be related with p53/p21 expression, but C33A cells seem to be related with PARP-JNK expression, which is involved in the mitochondrial pathway.

Introduction

Cervical cancer-related apoptosis has been widely studied, but the precise mechanism and regulation of apoptosis remain unclear (1-3). It was reported that the growth of uterine cervical squamous cell carcinoma (SCC) is also regulated by apoptosis. Fragmentation of DNA is one of the major hallmarks of apoptosis (4). The enzyme poly-(ADP-ribose)

polymerase (PARP) is localized within the cell nucleus and catalyzes DNA-repair. During the early phase of apoptosis, PARP is enzymatically cleaved and results in a 89-kDa molecule. Cleavage products are characteristic of apoptosis (5).

Among the genes regulating apoptosis, p53 performs multiple cellular functions including transcription of genes, DNA synthesis and repair, apoptosis and angiogenesis. Mutation of p53 gene has been well documented in ovarian, endometrial and cervical cancers (6). Especially in cervical cancer, human papilloma virus (HPV) E6 protein from high risk HPV infection inactivates p53, which is a well-known carcinogenesis pathway of uterine cervical cancer (7). p21 is an important inhibitor of G1-specific cyclin dependent kinases (CDKs) and its transcriptional activation is under the control of p53 (8). Bax, Bcl-2-associated X protein, promotes apoptosis and is up-regulated by p53 whereas Bcl-2 regulated anti-apoptosis (9-11). c-Jun kinases (JNKN-terminals) relevant mitogen-activated protein (MAP) kinases and is involved in apoptosis (12).

Platinum-based chemotherapy and chemoradiotherapy has proved to be an effective regimen for cervical cancer (13). Doxorubicin (DOX) has been used to manage a variety of human malignancies (14). The DNA double strand breaks and DNA oxidative damage caused by DOX result in cell cycle arrest and induction of apoptosis (15). The signal transduction pathway leading to DOX-induced apoptosis may involve p53, nuclear factor (NF)- κ B, p38 MAP kinase, JNKs, and/or PARP. DOX triggers apoptosis in cancer cells via both death-receptor and the mitochondrial pathways, and has been studied in chemotherapy-naive SCC of the uterine cervix. However, the exact mechanism of DOX activity in cervical cancer remains controversial (16-18). In this study we aimed to evaluate differential apoptotic response in uterine cervical cancer cells, CaSki (HPV 16-positive) and C33A (mutant p53 and HPV-negative), after chemotherapy using DOX.

Materials and methods

Cell lines and cell culture. The cervical cancer cell lines, CaSki (HPV-16-positive; ATCC, Manassas, VA, USA) and

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C33A (mutant p53 and HPV-negative; ATCC, Manassas, VA, USA) were used. Cell culture procedures were performed in DMEM supplemented with 10% fetal bovine serum (FBS, Life Technologies, Grand Island, NY, USA), 0.37% NaHCO₃, non-essential amino acids, L-glutamine (2 mM), penicillin (100 U/ml) and streptomycin (100 µg/ml) at 37°C in 5% CO₂ humidified incubator.

Chemotherapeutic agents. Cisplatin (Platinol, Bristol-Myers Squibb, NJ, USA), paclitaxel (Taxol, Bristol-Myers Squibb), gemcitabine (Gemzar, Eli Lilly and Company, IN, USA) and doxorubicin hydrochloride (Doxorubicin HCL Korea United, Korea United Pharm, Seoul, Korea) were preliminarily used for our experiment. We tested these chemotherapeutic agents for the comparison of their effect to cytotoxicity and apoptotic responses in cervical cancer cells using trypan blue cell exclusion test, DNA fragmentation, flow cytometric analysis, Western blot analysis and JNK assay. All chemotherapeutic agents were used four times to compare their effect. DNA fragmentation and apoptotic responses in the cancer cells were the most prominent after using DOX (data not shown). So, we decided to use DOX as a chemotherapeutic agent for evaluating the apoptotic responses in different cervical cancer cell lines.

Cell viability assay: trypan blue cell exclusion test. At 16, 24, and 48 h after treating cells with 1.4 µM DOX, the cells were harvested by trypsinization, centrifuged at 200 g for 5 min at 4°C, and resuspended in culture medium. A small volume of 0.4% trypan blue (Sigma-Aldrich, St. Louis, MO, USA) in phosphate-buffered saline (PBS) was added to an equal volume of trypsinized cell suspension and incubated for 10 min at room temperature. Cell death was determined by the presence of cytoplasmic trypan blue. Dose (1.4 µM DOX) and time (48 h) were determined after confirming the IC₅₀ values in both cervical cancer cell lines. Total cell number and the number of non-viable cells per culture dish were counted in a hemocytometer under light microscopy (Olympus BX, Tokyo, Japan).

Analysis of DNA fragmentation. For visual observation of apoptosis, DNA fragmentation was assessed directly using 2% agarose gel electrophoresis. Low molecular weight genomic DNA was extracted in a routine manner. DNA fragments identified and photographed with Gel-Doc 1000 (Bio-Rad, Richmond, CA, USA).

Flow cytometric analysis. The DNA content and cell cycle phase distribution of cells were evaluated by flow cytometry. Subconfluent cells were serum-starved for 24 h. Medium was then replaced with DMEM/10% FBS, supplemented with 1.4 µM DOX. After 16, 24, and 48 h, cells were harvested and fixed in 70% ethanol containing 0.05% Tween-20. Then cells were washed twice with PBS, resuspended in RNase (200 µg/ml) and incubated for 30 min at 37°C. Propidium iodide (final concentration 100 µg/ml) was added and cells were stained for 15-30 min in the dark at 4°C. Cell cycle phase distribution of nuclear DNA was analyzed by a FACScan flow cytometer (Becton-Dickinson, San Jose, CA, USA) using CellQuest software (Becton-Dickinson).

Western blot analysis. Cells treated with 1.4 µM DOX were harvested and lysed at -20°C in PRO-PREP protein extraction solution (iNtRON Biotechnology, Sunnam, Korea). Lysates were centrifuged at 13,000 x g for 10 min. The protein extract in the supernatant was collected, and the concentration was determined according to Bradford (Bio-Rad). Equivalent amounts (40 µg) of protein were loaded onto 12% SDS/polyacrylamide gels. After electrophoresis at 100 V for 1 h at room temperature, protein bands were transferred to nitrocellulose membranes (Bio-Rad) by electroblotting 1 h in Transfer Buffer (25 mM Tris, pH 8.3, 192 mM glycine, and 20% methanol). Equal loading was confirmed by staining the membrane with Ponceau S (Sigma-Aldrich). Before incubation with primary antibodies, the membranes were blocked with 5% non-fat dry milk in TBST (20 mM Tris-HCl, pH 7.6, 137 mM NaCl, and 0.1% Tween-20) for 1 h at room temperature. Proteins were detected by incubating overnight at 4°C with the following antibodies at 1:1,000 dilution: p53 (Oncogene Research Products, MA, USA), p21^{WAF/CIP1} (Transduction Laboratories, Lexington, KY, USA), PARP (Cell Signaling Technology, Beverly, MA, USA), Bcl-2 (Calbiochem, Darmstadt, Germany) and Bax (Calbiochem). After washing in TBST, the filters were incubated with HRP-conjugated anti-mouse or anti-rabbit IgG antibodies (Amersham Pharmacia Biotechnology, Buckinghamshire, UK) at 1:1,000 dilution for 1 h at room temperature. Antibodies bound to proteins were detected using the enhanced chemiluminescence (Amersham Pharmacia Biotechnology).

JNK assay. Cells treated with 1.4 µM DOX were lysed at 4°C in lysis buffer (135 mM NaCl, 25 mM β-glycerophosphate, 20 mM Tris, 2 mM EDTA, 2 mM sodium pyrophosphate, 2 mM DTT, 1 mM Na₃VO₄, 10%(v/v) glycerol, 1%(v/v) Triton X-100 detergent, and 1.5 µg/ml aprotinin, pH 7.5). Lysates were centrifuged at 13,000 x g for 10 min at 4°C, equalized for protein. For JNK/SAPK assay, the supernatants were incubated with an JNK-specific antibody (1:1,000, Calbiochem), and then protein A sepharose beads (Calbiochem) for immunoprecipitating JNK from cell lysates. The immune complexes were pelleted and washed twice with lysis buffer and then once with kinase buffer (25 mM Tris, 5 mM β-glycerophosphate, 12 mM MgCl₂, 2 mM DTT, 100 µM Na₃VO₄). The kinase reaction for JNK/SAPK assay was carried out in kinase buffer containing c-Jun protein/ATP mixture, and c-Jun phosphorylation was measured using a phospho-c-Jun-specific antibody (1:1,000, Calbiochem). The antigen-antibody complexes were visualized by enhanced chemiluminescence (Amersham Pharmacia Biotechnology).

Statistical analysis. The data from the trypan blue cell exclusion test were statistically estimated by one-way analysis of variance (ANOVA). The density ratios of cleaved PARP product to intact PARP protein were also compared between CaSki and C33A cells, and then statistically analyzed by the non-parametric Mann-Whitney U test. Statistical analyses were carried out using SPSS 14.0 (SPSS Inc., Chicago, IL). P-value <0.05 was regarded as statistically significant.

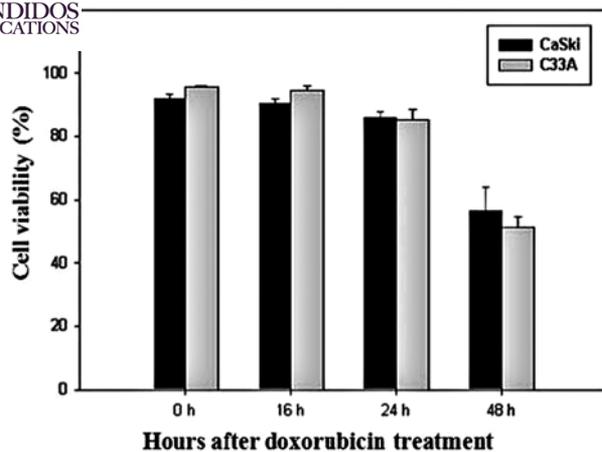


Figure 1. The results of the trypan blue cell exclusion test in both cervical cancer cell lines after chemotherapy using doxorubicin. h; hours after DOX treatment.

Results

Cell viability assay: trypan blue cell exclusion test. Cell viabilities of CaSki and C33A cells were decreased (Fig. 1). There were no significant differences of cell viability between CaSki and C33A cells. At 48 h after chemotherapy using DOX, we were able to obtain IC_{50} values in both cell lines.

Analysis of DNA fragmentation. After chemotherapy using DOX, DNA fragmentations were observed in both CaSki and C33A cells (Fig. 2). Nucleosomal 'laddering' of cellular DNA, characteristic of apoptosis, was more clearly observed in C33A cell than in CaSki cell.

Changes in the cell cycle in cervical cancer cells after chemotherapy using DOX. Cell cycle arrests in CaSki and C33A cells were analyzed using flow cytometry. CaSki and C33A cells exhibited a subG0 peak with G1 arrest, except for CaSki cell at 16 h. With a time-dependent manner, the greater tendency of subG0 peak was expressed and a maximal peak was noted at 48 h (Fig. 3). As a whole, subG0 peak was much more prominent in C33A cells.

Changes of apoptosis-related molecules in cervical cancer cell lines after chemotherapy using DOX by Western blot analysis and JNK assay. Apoptosis-related molecules, including PARP, p53, p21, Bax/bcl-2 and JNK, in CaSki and C33A cells after chemotherapy using DOX, were evaluated by Western blot analysis and JNK assay. The changes of these molecules in both cell lines are summarized in Table I.

PARP expression. In the present study, PARP cleavage products were distinctively expressed in both cervical cancer cell lines (Table I, Fig. 4). With densitometry (LabWorks software 4.0; Ultra-Violet Products Ltd., Nuffield Road, Cambridge, UK), the density ratios of cleaved PARP product to intact PARP protein were calculated. The ratio (mean \pm standard deviation) of CaSki cell measured 4.53 ± 2.96 and that of C33A cell 1.47 ± 1.29 . A higher tendency for the

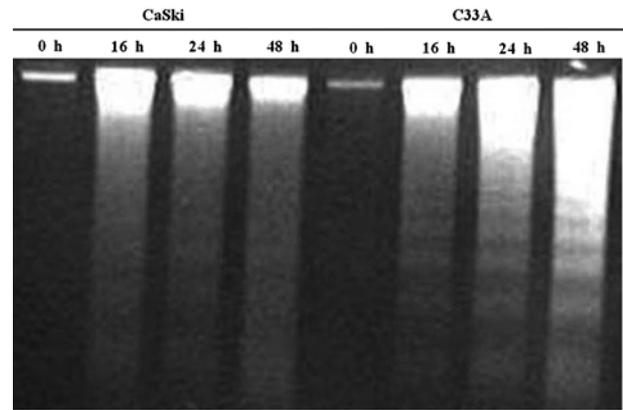


Figure 2. DNA fragmentation after chemotherapy using doxorubicin was analyzed by 2% agarose gel electrophoresis.

Table I. Differential expression of apoptosis-related molecules in Caski and C33A cells after chemotherapy using doxorubicin.

Molecules	CaSki cell	C33A cell
Cleaved PARP	Stronger ^a	Weaker ^a
p53	Increased	Constant
p21	Increased	Not expressed
bcl-2	Slightly decreased	Slightly decreased
JNK	Decreased	Increased

^aExpressed as relative density ratios of PARP bands.

mean density ratio was noted in CaSki cell, but there was no statistical significance ($p=0.083$).

p53/p21 expression. p53/p21 showed different expression during apoptosis between CaSki and C33A cells (Table I, Fig. 4). In the CaSki cell, p53 and p21 expression increased continuously. In the C33A cell, however, p53 showed constant expression, and p21 expression never emerged.

Bax/bcl-2 expression. The expression of Bax protein, an apoptosis agonist, was not detected in either of the cancer cell lines, although repeated experiments were attempted (data not shown). The expression of bcl-2 protein showed a slight decrease in both cervical cancer cell lines but there was no difference over time (Table I, Fig. 4).

JNK expression. The expression of JNK protein, probably an important negative regulator of cell growth, was remarkably decreased in CaSki cells whereas its expression was increased in the C33A cell (Table I, Fig. 4).

Discussion

Cervical cancer is the second most common cancer in women, with an estimated worldwide incidence of about

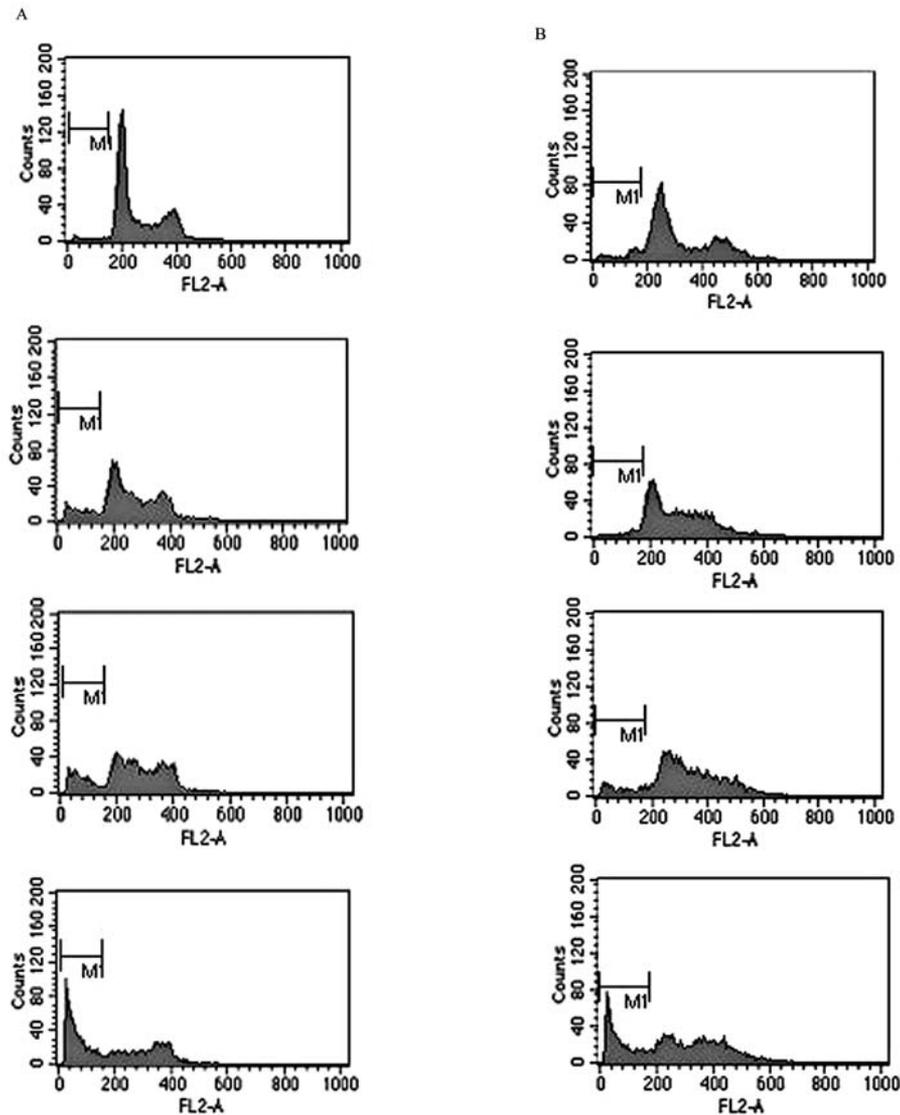


Figure 3. Flow cytometric analysis of cell cycle in CaSki (A) and C33A cells (B) after chemotherapy using doxorubicin. From top to bottom 0, 16, 24 and 48 h.

493,000 new cases and 274,000 deaths per year (19). HPV infection is the strongest epidemiological factor for carcinogenesis. Among viral serotypes, HPV 16 is the most prevalent HPV type among women with cervical cancer followed by HPV 18. In SCC of the uterine cervix, the genomes of HPV 16 and 18 are integrated in host genome up to 60% of the cases (20).

With viral integration, the HPV genome breaks in the E2 region, resulting in loss of its suppressive function on E6 and E7 genes. The E6 gene product binds with high affinity to p53, inactivating it by undergoing p53-mediated control, promoting uncontrolled cell proliferation and eventual development of cervical cancer (21). On the other hand, the HPV E7 protein binds to the Rb-E2F complexes, releasing E2F protein which activates the transcription of genes for cell cycle S-phase and induces apoptosis in the presence of functional p53 protein. Thus, during HPV infection, the proapoptotic signals generated by E7 may be totally or partially counter-balanced by E6 protein. E7 also activates cyclins E and A, and blocks the cellular proliferation-

inhibiting activities of cyclin-dependent kinase (CDK) inhibitors such as p21 and p27 (22,23).

Apoptosis (programmed cell death) is a function to eliminate abnormal cells, and dysregulation of apoptotic cell death has been implicated in the premature death of cells, aging, states of disease, and neoplastic transformation (24). Recently, many investigators have reported that the growth of uterine cervical SCC cells is also regulated by apoptosis, but the precise mechanism and regulation of the apoptosis are still unknown. With regard to the initiation of apoptotic cascade, the breaks of double-stranded DNA are the well-known biologic signal. Fragmentation of DNA is one of the major hallmarks of apoptosis (4).

Although DOX is not widely used in cervical cancer because of limited activity and considerable side effects including cardiac toxicity (25,26), the present study found that decreased cell viability and different apoptotic responses were observed in both cervical cancer cell lines after chemotherapy using DOX. As a characteristic of apoptosis, nucleosomal 'laddering' of DNA was more clearly showed

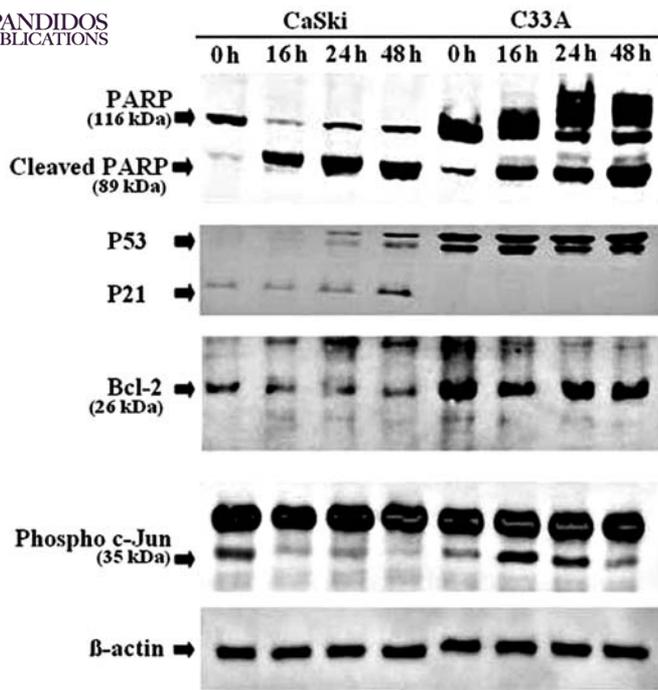


Figure 4. Western blot analysis of PARP, p53, p21, bcl-2 and JNK assay in CaSki and C33A cells after chemotherapy using doxorubicin.

in C33A (mutant p53 and HPV-negative) cells after DNA fragmentation analysis.

There was a subG0 peak in both CaSki (HPV-16-positive) and C33A (mutant p53 and HPV-negative) cells. But the subG0 peak was more prominent in C33A cells from flow cytometric analysis. These results suggest that HPV might inhibit apoptotic responses in cervical cancer cells consistent with previous studies that HPV E6 inhibits the function of p53. Thus, HPV might be a cause of chemoresistance in cervical cancer (27,28).

The process of apoptosis usually involves activation of caspases and the subsequent cleavage of several cellular substrates such as PARP, actin, fodrin and lamins (29). Mizukawa *et al* reported that cleaved PARP was a proapoptotic factor in etoposide-induced apoptosis in human glioblastoma cell lines (30). In the present study, PARP cleavage products were distinctively expressed in both cervical cancer cell lines. This result suggests that cleaved PARP may be related with apoptosis in cervical cancer.

Lipponen and Aaltomaa reported a meaningful increase of apoptosis in a p53 positive group in bladder cancer (31). However, other reports showed decreased apoptosis with increased p53 expression in Wilms' tumor and small cell lung cancer (32,33). In this study, CaSki cells showed increased expression of p53 but in C33A cells did not show increased p53 expression. Thus, it seems that the function of p53 may be cell type-dependent.

p21 may also inhibit proapoptotic factors such as JNK/SAPKs, E2F and has been considered as an important p53 target in response to DNA damage (34). Watanabe *et al* reported that cisplatin induced p53 and p21 expression and resulted in sequential activation of JNK in a time-dependent manner in urogenital cancer cell lines (35). In this

study, CaSki cells showed increased expression of p53 and p21 genes while JNK expression decreased, but C33A cells showed constant p53 expression without p21 expression and increased JNK expression. These findings suggest that apoptosis in CaSki cells (HPV-16-positive) is related with p53 and p21 expression after chemotherapy using DOX. It has been described that PARP and JNK activation are related with mitochondrial apoptotic pathway (36,37). Mitochondrial signaling apoptotic pathway may be the major mechanism of DOX activity (38). Thus, according to their presumption and our results, C33A cells (mutant p53 and HPV-negative) seems to undergo cell death related with PARP-JNK expression which is related with mitochondrial apoptotic pathway.

In conclusion, our results show that apoptosis in CaSki (HPV-16-positive) cells may be related with the expression of p53 and p21 genes after chemotherapy using DOX. On the other hand, apoptosis in C33A (mutant p53 and HPV-negative) cells may follow PARP/JNK related mitochondrial apoptotic pathway. So, it seems that there are differential apoptosis-inducing pathways according to cervical cancer cells after chemotherapy, and also HPV might affect differential apoptotic responses in cervical cancer cells. However, the clarification of more exact apoptotic pathways related with HPV will require further studies, coupled with molecular biological approaches.

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