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Chemo-radio Resistance in Cervical Cancer Induced by HPV16 E7

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ABSTRACT: Alteration of the apoptosis pathway, as well as the presence of human papilloma virus (HPV), has been linked to the proliferative capacity and drug resistant phenotype of SiHa cervical cancer. We investigated the roles of E6 and E7 HPV oncoproteins in the expression of apoptosis regulating genes in cervical cancer cells that contain the characteristics of apoptosis resistance, and also their correlation with resistance to various apoptosis inducing agents. The expression of the sets of apoptosis regulating genes in both extrinsic (receptor) and intrinsic (non-receptor) pathways were monitored in parental SiHa and multi-drug resistant SiHa (SiHaR) cell lines by RNase protection assay and RT-PCR. An increase in gene expression of intrinsic pathway anti-apoptotic protein Bcl-X_L was seen, both at the mRNA and protein levels, in SiHaR compared with SiHa cells, whereas the expression of the genes involved in the extrinsic pathway remained unchanged. SiHaR cells also expressed higher levels of E6 and E7 than did SiHa. Caspase 3 activity was lower in SiHaR compared with that in SiHa cells. A colony formation assay demonstrated enhanced resistance of SiHaR cells to several types of apoptosis inducing agents, including etoposide, doxorubicin, cisplatin, and γ-radiation. Transfection of HPV-negative C33a cells with HPV oncogenes, E7 in particular, induced transcription of *Bcl-X_L*, supporting the role of HPV oncoproteins in affording chemo-radio resistance in cervical cancer.

KEYWORDS: HPV16 oncoproteins, *Bcl-X_L*, cross resistance.

INTRODUCTION

Persistent infection of human papilloma virus (HPV) is the main cause of cervical cancer. Although an HPV vaccine has recently emerged, it has not been proven to be fully protective for life nor for those previously infected with the virus[1]. The presence of HPV, particularly HPV16, in cervical cancer cells has been implicated in 33–70% of cases[2-4]. HPV16 produces 2 major transforming proteins, E6 and E7, which can alter the cellular growth regulation mainly by inactivating the products of the tumor suppressor P53 and Rb genes respectively[5]. The levels of viral proteins have been shown to be directly linked to the proliferation capacity of cervical carcinoma cells[6]. In addition, a number of studies have indicated roles of HPV in evasion of cell death[7-9]. It has been shown that HPV16-immortalized and transformed cells increase their resistance to apoptosis induced by various chemotherapeutic drugs. Apoptosis inhibitory effects of HPV oncoproteins are believed to play an important role in cellular immortalization and transformation. The alteration of apoptotic molecules conferring drug resistance has been investigated in several cell lines, including cervical carcinoma cell lines. Apoptosis regulating proteins involved in chemotherapeutic resistance have previously been observed, both in intrinsic (non-receptor mediated) and extrinsic (receptor mediated) apoptosis pathways[10-13]. Up-regulation of anti-apoptotic molecules in the intrinsic pathway, such as Bcl-2 and Bcl-X_L[14-16], deficiency of pro-apoptotic p53[17] or inactivation of caspase-3[18] can significantly increase drug resistance. On the other hand, down-regulation or loss function of CD95/Fas in the extrinsic pathway contributes to chemo-resistance[19]. Up-regulation of cellular inhibitor of apoptosis protein 2 (c-IAP2) in human oral keratinocytes[20], as well as AP-1 proteins in cervical carcinoma cell lines[21], has been associated with the expression of the *E6* oncogene. However, the expression of HPV18 *E6* and *E7* in the head and neck KB carcinoma cell line could not prevent apoptosis induced by 5-fluoruracil[22]. It is not known what events occur in naturally HPV16-infected cells that have been induced to have a drug resistant phenotype and how the HPV oncoproteins are involved. We used an HPV16-containing cervical cell line, SiHa, which was derived from a high grade squamous intraepithelial lesion and

had acquired a few copies of HPV16 by natural infection[23], to address this issue. A multi-drug resistant SiHa cell line (SiHaR) selected from SiHa parental cells[24] was compared to its parental line on patterns of anti-apoptotic gene expression and on HPV oncogene expression. The roles of HPV oncoproteins in both chemo- and radio- resistance were also investigated.

Materials and Methods

Cell Lines and Culture Conditions

SiHa, a squamous cell carcinoma of the cervix that contained a few copies of the HPV16 genomes was cultured under standard conditions (5% CO_2 at 37° C) in MEM containing Earle's salts (Gibco BRL, Invitrogen, Carlsbad, CA, USA.) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 mg/ml streptomycin and 0.25 mg/ml amphotericin B (Gibco BRL). SiHaR was obtained by stepwise selection in increasing concentrations of etoposide in MEM, as previously described, and had a multi-drug resistant phenotype against various chemotherapeutic drugs (24). SiHaR was maintained in MEM containing 10 μM etoposide until use. Cells were then subcultured in etoposide-free medium for two passages prior to experiments.

RNase Protection Assay (RPA)

An RNase protection assay was used to monitor the expression pattern of apoptotic regulating genes, in both extrinsic and intrinsic pathways. After 4 days of culture (mid-exponential phase), total RNA was extracted from SiHa and SiHaR cells using the High Pure RNA Isolation Kit (Roche,Germany). RPA was performed on samples using RiboQuant™RPA kits (Pharmingen, USA). In brief, ^{32}P-labeled nucleotide probes were *in vitro* synthesized from the multi-probe template sets which included, hStress-1, representing P53 and Bcl-2 family in the intrinsic pathway and the hAPO-3 set, representing Fas/CD 95 of the extrinsic pathway, according to manufacturer's instructions. An aliquot of 50 μg of total RNA was hybridized with ^{32}P-labeled probes for 16 hrs and digested with RNase A (80 ng/μl) and RNase T1 (250 U/μl). The RNase-protected probes were separated in a denaturing 5% polyacrylamide gel. The ^{32}P-labeled bands in the gels were exposed to X-ray film and the bands quantitated with a phosphoimager (FujiX Bas 2000).

Semi-quantitative RT-PCR

mRNAs from both SiHa and SiHaR cells were isolated using the mRNA Isolation Kit (Roche). cDNAs were obtained using SuperScript II reverse transcriptase, (Gibco BRL) and used in RT-PCR. *HPRT* and *β-actin* genes were employed as internal controls for the *Bcl-X_L* and HPV *E6* and *E7* genes, respectively. For PCR amplification, the following primers were employed: *Bcl-X_L*, sense primer 5' TGG TGG TTG ACT TTC TCT CC 3' and anti-sense primer 5'GAG TTC ATT CAC TAC CTG TTC 3'; *HPRT*, sense primer 5' TGT GAT GAA GGA GAT GGG AGG 3' and anti-sense primer 5' AAG CTT GCG ACC TTG ACC ATC T 3'; HPV *E6*, sense primer 5' ATG CAC CAA AAG AGA ACT GC 3' and anti-sense primer 5' TTA CAG CTG GGT TTC TCT AC 3'; HPV *E7*, sense primer 5' ATG CAT GGA GAT ACA CCT ACA 3' and anti-sense primer 5' T TA TGG TTT CTG AGA ACA GAT 3'; and *β-actin*, sense primer 5' GAC CTT CAA CAC CCC AGC CA 3' and anti-sense primer 5' AGG CTG GAA GAG TGC CTC AG 3'. PCR amplification of *Bcl-X_L* and *HPRT* genes was performed in a Minicycler™ PCR system (MJ Research, Beverly, MA, USA) using the cycling parameters: for *Bcl-X_L*, 10 min at 94°C, followed by 30 cycles of 1 min at 94°C, 1 min at 60°C, 1 min at 72°C, and a final cycle at 72°C for 10 min; for the *E6, E7* and *β-actin* genes, 10 min at 94°C, followed by 25 cycles of 1 min at 94°C, 1 min at 60°C, 1 min at 72°C, and a final cycle at 72°C for 10 min. The PCR products of *Bcl-X_L* (368 bp), *E6* (403 bp) and *E7* (297 bp) genes were separated and visualized in 1.5% agarose gel stained with ethidium bromide. Quantitative measurements of the PCR products were performed using the Quantity One program in a Gel Doc 2000 (Bio-Rad, Hercules, CA, USA) and normalized with products from *HPRT* (333 bp) or *β-actin* (418 bp) genes.

Western Blot Analysis

Approximately 1×10^6 cells were harvested and washed twice with cold PBS. Cells were lysed in radio-immunoprecipitation assay (RIPA) buffer containing 10 mM Tris-HCL, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.1% SDS, 0.5% deoxycholate, 10% glycerol, 1% NP-40, 25 mM sodium pyrophosphate, 1 mM sodium fluoride, 10 mM β-glycerophosphate, 2 mM sodium orthovanadate, 1 mM DTT, and protease inhibitor cocktail (Roche). The lysate was clarified by centrifugation and the total protein concentration was determined using the Bio-Rad Protein Assay kit (Bio-Rad). One hundred mg of protein were separated by 15% sodium dodecyl sulfate-polyacrylamide gel-electrophoresis (SDS-PAGE) and subsequently transferred onto a polyvinyl difluoride (PVDF) membrane (Bio-Rad). Primary mouse monoclonal antibody specific for Bcl-X (Catalog no. 610746, BD. Pharmingen) and anti-mouse secondary antibody conjugated to horseradish peroxidase (Zymed Laboratories, Inc., South San Francisco, CA, USA) were used to detect the desired product. Signals were developed using the BM Chemiluminescence (POD)

procedure (Roche) and immunoreactive bands were visualized by exposing the membrane to Hyperfilm ECL (Amersham Bioscience, Buckinghamshire, UK).

Transient Transfection of C33a Cells with HPV Oncogenes

The effect of HPV oncoproteins on transcriptional activation of the anti-apoptotic *Bcl-X*$_L$ gene was studied in C33a cervical cancer cells that contained no HPV DNA. One microgram each of pcDNA3 plasmid containing HPV16 E6 and pcDNA3 plasmid containing HPV16 E7 or a combination of both plasmids were transiently transfected into C33a cells using Lipofectamine™2000 Reagent (LF2000) under the conditions recommended by the manufacturer (Invitrogen). Controls of the transfection reaction and efficiency were performed with 1 μg of the pcDNA3 vector and β-galactosidase reporter gene plasmid, pCH110, respectively. Twenty four hours after transfection, mRNA of the transfected cells were isolated and the levels of *Bcl-X*$_L$ gene expression were measured as described above.

Assay of Caspase 3 Activation

Caspase-3 activity was measured by using Caspase-3 Colorimetric Activity Assay Kit (Chemicon Int., USA). In brief, equal numbers of SiHa and SiHaR cells were plated onto 100 mm dishes. After treatment with 100 μM of etoposide for 72 hrs, cells were harvested and lysed in ice-cold lysis buffer (150 mM NaCl, 20 mM Tris HCl, pH 7.2, 1% Triton X-100, and 1 mM DTT) for 10 min. After removal of cellular debris by centrifugation, protein concentrations in the lysates were measured by the Bio-Rad Protein Assay Reagent and adjusted to obtain 300 μg of cytosolic extract protein per sample. Samples were incubated with 200 μM caspase-3 substrate *N*-acetyl-Asp-Glu-Val-Asp (DEVD)-*p*-nitroanilide at 37°C for 3 hrs. Optical densities of the samples were measured at 414 nm in a Multiskan EX microtiter plate reader (Thermo Lab Systems, Finland). Data are expressed as fold increase or decrease in caspase-3 activity compared with control cells.

Colony Formation Assay

A colony formation assay (CFA) was conducted to test drug resistance, as previously described[25]. Three-day-old cultures of SiHa and SiHaR were separately treated for 24 hrs with etoposide (10, 50,100 and 200 μM), doxorubicin (0.1, 0.5, 1, and 2 μM) and cisplatin (1, 2, 3, and 4 μM). For γ-radiation treatment, SiHa and SiHaR were irradiated with Co-60 γ-rays using a teletherapy machine (Theratron 780C) with doses of 2, 4, 6 and 8 Gy in a field size 10 x 10 cm. Cells were positioned at the central axis of the machine that delivered a dose rate of 1 Gy/min. After drug or irradiation treatment, 500 cells were re-plated onto 10 cm plates and were grown under normal culture conditions for 10 days. Surviving colonies were stained with 5% methylene blue in 50% ethanol for 10 min and counted. Only colonies larger than 50 cells were counted and percent viability was calculated.

Statistical Analysis

A student's *t* test was used to compare mean values and statistical significance was accepted when $P < 0.05$.

Results

Differential Expression of Apoptosis Regulatory Genes

The mRNA levels of the apoptotic regulating molecules in the intrinsic pathway, as detected by multi-probe hStress-1 and in the extrinsic pathway as detected by multi-probe hAPO-3, were measured in SiHa and SiHaR cells by RNase protection assay (Fig 1). Analysis by phosphoimager revealed significant increases of *P21*^{WAF1} (3.1 fold) and *Bcl-X*$_L$ (2.7 fold), both involved in

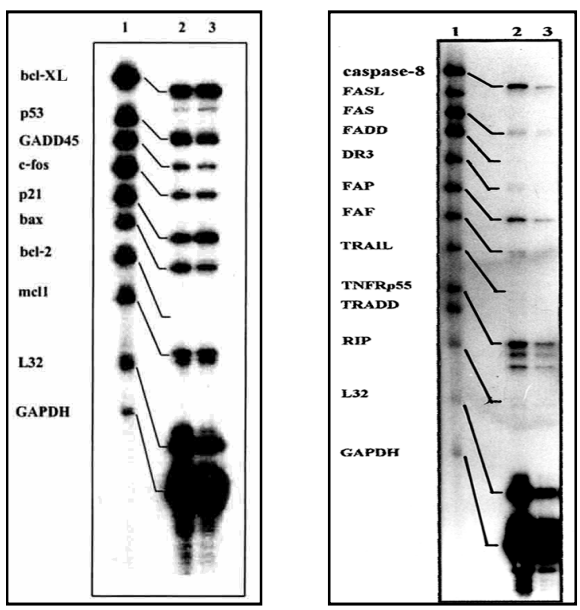

Fig 1. Ribonuclease protection assay (RPA) analysis for Bcl-X_L, p53, GADD45, c-fos, p21, bax, bcl-2 and mcl-1 molecules by multi probe hStress-1(Panel A) and for caspase-8, FASL, FAS, FADD, DR3, FAP, FAF, TRAIL, TNFRp55, TRADD, and RIP by multi probe hAPO-3 (Panel B) in SiHa (2) and SiHaR (3). L32 and GAPDH are controls used to normalize sample load in each well. The probe lengths (1) are larger than the protected fragment lengths (2 and 3).

the intrinsic pathway of apoptosis, in SiHaR compared with levels in SiHa parental cells. There was also a small increase of the *P53* transcript (1.8 fold). This increase may result from the drug treatment used in cell line maintenance. However, no differences of the transcripts in the extrinsic FAS/CD95 pathway, including caspase 8, FasL, Fas, FADD, FAP, FAF, TRAIL and TRADD, were observed between SiHa and SiHaR.

Analysis of Bcl-X_L, E6 and E7 Gene Expression

The increase in the mRNA level of *Bcl-X_L* in SiHaR was confirmed by RT-PCR. Using this semi-quantitative technique, the level of *Bcl-X_L* transcript of SiHaR was 2.9 ± 0.4 fold (p-value<0.01) of that of SiHa (Fig 2A). In addition, RT-PCR demonstrated that the levels of *E6* and *E7* gene expression in SiHaR were 2.35 ± 0.16 fold

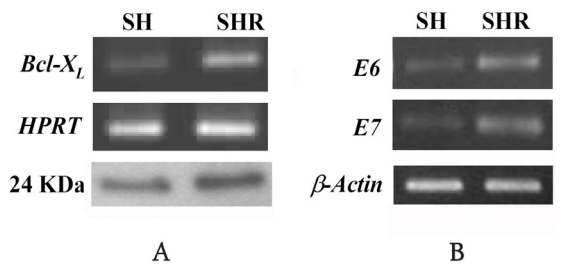

Fig 2. Expression of *Bcl-X_L* gene (A), *E6* and *E7* (B) analyzed by RT-PCR and western blot. *Bcl-X_L* mRNA levels were normalized relative to *HPRT* gene product whereas *E6* and *E7* mRNA levels were normalized relative to that of *the β-actin* gene. Immunoblotting for Bcl-X_L protein detected 24 kDa band of Bcl-X_L protein (A) in both SiHa (SH) and SiHaR (SHR). PonceauS-stained protein blots are performed to allow comparison of relative amounts of protein lysates for each sample (data not shown).

and 2.55 ± 0.27 fold of those seen in SiHa respectively, (p value< 0.01) (Fig 2B). Western blot analysis using a monoclonal antibody specific to Bcl-X_L revealed an elevated level of the 24 KDa Bcl-X_L protein in SiHaR cells when compared with that of SiHa (Fig 2A), confirming the results of the RT-PCR experiments.

Analysis of Caspase-3 Activity

A commonly used index in assessing apoptosis is caspase-3 activity. Both SiHa and SiHaR cell lines were exposed to 100 mM etoposide for 72 hrs before the caspase 3 activity was measured. Drug-treated SiHa parental cells exhibited 3.25 ± 0.42 fold increases in caspase-3 activity compared to untreated cells, whereas SiHaR cells showed comparable levels of caspase-3 activity between untreated and drug-treated conditions. These data were consistent with the observation that the SiHaR cell line exhibited higher drug resistance to apoptosis than its parental SiHa line.

Apoptosis Resistance Properties Assessed by CFA

We investigated whether the higher level of *Bcl-X_L* in SiHaR resulted in resistance to other apoptosis inducing agents or not. We used the CFA method to evaluate percent cell viability after exposure to apoptosis inducing agents. Both SiHa and SiHaR were

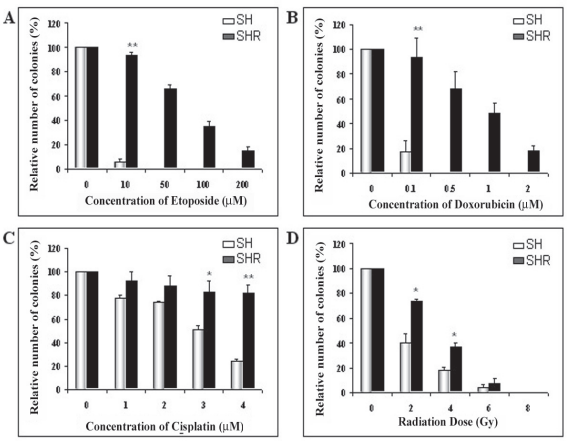

Fig 3. Effect of apoptotic inducing agents: etoposide (A), doxorubicin (B), cisplatin (C) or γ-radiation (D), on SiHa (SH) and SiHaR (SHR), as measured by colony forming assay. Cell viability is expressed as percent colonies formed at 10 days after treatment and normalized versus controls of untreated cells. * and ** indicate significantly different values at p<0.01 and p<0.05, respectively. The mean values are the average of at least two independent experiments.

treated with 3 different drugs (etoposide, doxorubicin and cisplatin) and with γ- radiation. Cell viability was expressed as percent treated colonies surviving after 10 days compared with control untreated cells (Fig 3). These results showed increased resistance of SiHaR compare with parental SiHa to both drugs and γ-radiation.

Bcl-X_L Transcript Following Transfection with HPV Oncogenes

As both *Bcl-X_L* and HPV oncogene levels were increased in multidrug resistant SiHaR cells, we further investigated the role of HPV oncogenes on the *Bcl-X_L* gene transcription by transiently transfecting the HPV-negative C33a cell line with the HPV16 *E6* and *E7* oncogenes. As shown in Fig 4, the transcriptional activation of *Bcl-X_L* gene was observed in HPV16 *E7* transfected cells (1.92 ± 0.08 fold over control), as well as in HPV16 *E6* and *E7* co-transfected cells (1.76 ± 0.16 fold over control), but not in cells transfected with HPV16 *E6* gene alone (1.13 ± 0.11).

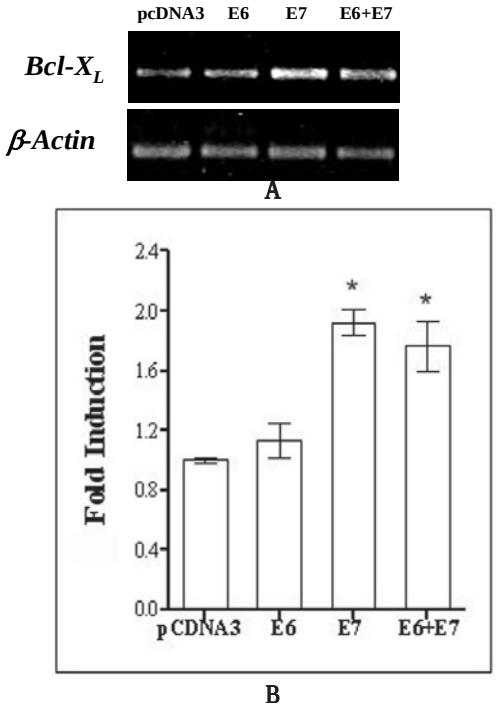

Fig 4. Levels of *Bcl-X_L* transcripts were monitored by RT-PCR (A) after transient transfection with HPV oncogenes: E6, E7 and the combination of E7 and E6. Their quantification is shown in (B). * indicates the significant values at $p<0.01$. The mean values are the average of at least two independent experiments.

Discussion

In this study, we have examined the anti-apoptotic properties of a multi-drug resistant cervical cancer cell line, SiHaR, in relation to its HPV oncoprotein expression. Investigation of stress genes and a series of genes involved in apoptosis, both of intrinsic and extrinsic pathways, revealed increased *Bcl-X_L* expression, both at the mRNA and protein levels, in SiHaR compared to its parental SiHa cells. No differences in gene expression involved in the external apoptosis pathway were detected.

The precise mechanism of how drugs activate the *Bcl-X_L* expression is largely unknown. Besides *Bcl-X_L* gene products, *P21* and *P53* transcripts were also elevated in SiHaR. Although both *P21* and *Bcl-X_L* have been previously shown to be associated with drug resistance in many cell lines[16, 26], up-regulation of these two genes have never been reported in the same cell. However, an increase of *P21*, *GADD45* and *Bax* transcripts by *P53* gene activation could not be excluded as they are *P53* targets. The level of *P53* transcript, as well as those of genes controlled by *P53*, might be affected by DNA damage caused by drug exposure. We have followed the level of *P53* transcripts in cells maintained in drug-free medium for a period of time and found that p53 gene expression between the SiHa and SiHaR cell lines was similar (data not shown). Therefore, it seems likely that the small increase of *P21* transcripts might result from the increase in *P53* level in SiHaR. The absence of detectable alterations in expression of genes in the extrinsic pathway emphasized the role of *Bcl-X_L* in chemoresistance. It was previously shown that lower levels of *Bcl-X_L* rendered cells more sensitive to chemotherapeutic drugs[14] and γ radiation[27]. On the other hand, overexpression of *Bcl-X_L* confers resistance to various anti-neoplastic drugs in murine haematopoietic cell (FL5.12)[14], squamous cell carcinoma (MIT) [16], and cervical cell carcinoma[18] and also to γ radiation in pancreatic cancer cells[27]. Besides having a multidrug resistance phenotype, SiHaR cells also were resistant to γ-radiation, exhibiting approximately 2 fold the level of resistance of its parental SiHa at all doses tested. These characteristics of chemo-radio cross resistance have previously been observed in several cell lines of different tissue origins, viz. a C6 rat glioma cell line selected by doxorubicin[28], a J82 neoplastic bladder cell line induced by cisplatin[29] and an HL-60 leukemia cell line selected by camptothecin and etoposide[30]. All these data indicate the central role played by *Bcl-X_L* in conferring both chemo- and radio-resistance. Interestingly, the two HPV oncoproteins, E6 and E7, were both elevated in the multi-drug resistant cervical cancer SiHaR cells, which contained a few copies of the HPV16 genome. Previous evidence has shown a link between apoptosis resistance and HPV16 E6 and E7 in laryngeal squamous carcinoma cells, in which HPV16 E6 or E7 oncoproteins are capable of inhibiting apoptosis[15]. Nevertheless, its resistance to apoptosis was shown to be associated with the increased *Bcl-2* expression. Apart from HPV oncoproteins, other viral proteins, such as Hepatitis C core protein[31], HTLV-I and HTLV-II proteins,[32] could also inhibit apoptosis by enhancing *Bcl-X_L* expression. In addition, direct evidence linking HPV16 oncoproteins and anti-apoptosis was observed by Santin *et al*., who showed that exposure to high doses of γ-irradiation enhanced E6/E7 expression in SiHa cells that exhibit radioresistance[33]. This effect was shown to be long lasting. However, no data on anti-apoptotic molecules were reported. Studies by Saxena *et al*. also emphasized the role of HPV oncoproteins in response to both chemotherapy and radiation by demonstrating that cervical cancer cell lines containing HPV were more resistant to both treatments than the cell lines without HPV[34]. Though the finding that radiation could enhance the expression of E6 and E7 HPV oncogenes in cervical carcinoma cell lines, our study demonstrated

that increase of HPV oncogene expression in drug-treated cells could enhance radiation resistance, and this activity was mediated through the elevation of *Bcl-X_L*. Transfection of HPV-negative C33a cervical cancer cells with E7 or the combination of E6 and E7 HPV oncoproteins resulted in a rise in *Bcl-X_L* gene expression, demonstrating a direct effect of viral proteins on the expression of the anti-apoptotic gene *Bcl-X_L*. In this regard, E7 showed a much clearer effect than E6 when each protein was usedseparately. This evidence supports the notion that over-expression of HPV16 E7 oncoprotein might be a crucial factor causing chemo-radio cross resistance in cervical cancer cells and therefore is an important etiology in aggressive malignant phenotype.

Nevertheless, there are HPV-negative cell types that exhibit the multi-drug resistant phenotype but remain sensitive to radiation[35] indicating that another anti-apoptosis mechanism exists.

In summary, despite the therapeutic efficacy of chemotherapy in cervical cancers, the up-regulation of HPV E7 oncogene in drug-treated cancer cells might promote acquisition of both multi-drug resistance and radiation resistance through the activation of the *Bcl-X_L* gene, and thereby compromise this cancer cure.

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