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Inhibition of Apoptosis in Human Laryngeal Cancer Cells by E6 and E7 Oncoproteins of Human Papillomavirus 16

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Abstract The carcinogenesis of human papillomaviruses type 16 (HPV-16) is mainly due to its two oncoproteins, E6 and E7. Their carcinogenic features in term of their relationship with Bcl-2 family are still unclear. We thus aimed to analyze the expression of Bcl-2 family members, Bcl-2, Bax, and Bak in laryngeal cancer cells transfected with the E6 or E7 and to determine the sensitivity of these cells to apoptotic stimuli. We employed two human laryngeal cancer cell lines, UMSCC12 and UMSCC11A in this study. These two cell lines were stably transfected with HPV16 E6, E7 or empty vector, pcDNA3.1. We found that E6 and E7 inhibited apoptosis induced by TNF-α/CHX in both UMSCC11A and UMSCC12 cells, enhanced the stability of Bcl-2 protein and increased the degradation of Bak protein. Furthermore, it was found that HPV-16 E7 statistically enhanced the expression of Bcl-2 in laryngeal cancer. The alteration of Bak by E6 and E7 was not through the influence on the Bak promoter, as the luciferase assay showed that neither E6 nor E7 changed the Bak promoter activity. We conclude that the evasion of apoptosis mediated by HPV-16 E6 and E7 is associated with increased Bcl-2 and decreased Bak in laryngeal carcinogenesis and that the decreased level of Bak by E6 and E7 is not caused by the regulation of the Bak promoter but by reducing its protein stability. J. Cell. Biochem. 103: 1125–1143, 2008.

Key words: HPV-16; laryngeal cancer; Bcl-2; Bak; apoptosis

Laryngeal cancer is the sixth commonest cancer and the second commonest respiratory tract cancer worldwide [Cattaruzza et al., 1996; Hirvikoski et al., 1997], with an incidence of about two million new cases and a mortality of about one million deaths per year [Parkin et al., 2005]. In addition to the well-known risk factors for laryngeal cancer such as tobacco and alcohol abuse, there is increasing evidence to suggest that human papillomavirus type 16 (HPV-16) is also associated with laryngeal cancer [Brandwein et al., 1993; Franceschi et al., 1996]. Carcinogenesis by HPV-16 is mainly mediated by its two viral oncoproteins E6 and E7. The inactivation of p53 by E6 and the degradation of pRb by E7 are thought to result in cellular resistance to apoptosis and cancer formation [Iglesias et al., 1998; Patel et al., 1999; Huang and McCance, 2002].

Bcl-2 family members are important mediators in the regulation of apoptosis [Pignataro et al., 1998; Jackel et al., 2000; Friedman et al., 2001; Georgiou et al., 2001; Trask et al.,

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2002]. They are divided into two subgroups: pro-apoptotic family members are Bak, Bax, Bok, Bid, Bad, Bim, and Bok; and anti-apoptotic family members are Bcl-2, Bcl-x_L, Mcl-1, Bcl-w, and A1. The balance between the two subgroups determines whether cells survive or die under physiological and pathological conditions [Kroemer, 1997].

Although the oncogenic role of HPV-16 in laryngeal cancer has been established, the relationship between HPV-16 viral oncoproteins E6 and E7 and Bcl-2 family members remains uncertain. The impact of E6 and E7 on the regulation of Bcl-2 family members needs further investigation. In this study, we aim to analyze the expression of three Bcl-2 family members, Bak, Bax, and Bcl-2 in human laryngeal cancer cells transfected with E6 or E7, and to investigate the role of Bcl-2 family members in the regulation of apoptosis in these cells.

MATERIALS AND METHODS

Transfection and Establishment of a Stable Cell Line

Two laryngeal squamous cell cancer cell lines, UMSCC11A and UMSCC12—kindly provided by Carey of the University of Michigan [Carey, 1994], were used in this study. Complimentary DNA HPV-16 E6 and E7 templates were generated from extracts of Caski cells which are known to contain an integrated HPV-16 genome of about 600 copies per cell [Pater and Pater, 1985; Yee et al., 1985; Baker et al., 1987]. RNA extraction and cDNA synthesis were performed according to the manuals of the RNeasy Mini kit (Promega, Madison, WI) and the Reverse Transcription System kit (Promega). Primers E6_F1 (5′-GCGGTACCATGCACCAAAAGAGA) and E6_R1 (5′-GG-CGAATTCTTACAGCTGGGTT) were used in the amplification of HPV-16 E6 while primers E7_F (5′-CGGGATCCATGCATG-GAGATACA) and E7_R (5′-GCGGGCCCTT-ATGGTTTCTGA-GA) were used in the amplification of HPV-16 E7. The E6 and E7 genes were subcloned into a neomarker-containing vector, pcDNA3.1 (Invitrogen, Carlsbad, CA), forming pcDNA3.1_E6 and pcDNA3.1_E7 plasmids. The authenticity of the inserts was confirmed by DNA sequencing.

5×10^5 UMSCC11A cells per well or 3×10^5 UMSCC12 cells per well were seeded in six-well plates for 24 h. The following was then prepared for each well: 8 μl of LipofectAMINE, 2 μg of plasmid DNA (the empty vector pcDNA3.1, the cDNA3.1_E6, or the pcDNA3.1_E7), and 200 μl serum-free MEM medium. After the incubation for 45 min to form DNA–liposome complexes, 800 μl of serum-free minimal essential medium (MEM) was gently mixed with the 208 μl complex solution for another 45 min. The diluted complex solution was then added to the cells in the wells and the cells incubated for 24 h at 37°C. After 24 h, the diluted complex solution was replaced by fresh MEM containing 10% BSA. UMSCC11A_pcDNA3.1, UMSCC11A_E6, UMSCC11A_E7, UMSCC12_pcDNA3.1, UMSCC12_E6, and UMSCC12_E7 cells were, respectively, established after G418 (400 μg/ml) selection. The transfected cell lines were checked for the expression of HPV-16 E6 and E7 by RT-PCR with primers E6_F2 (5′-ATG-ACTTTG CTTTTCGGGATT), E6_R2 (5′-GCAA-CAAGACATACATCGACC), and E7_F, E7_R. Primers of the vector pcDNA3.1, p3.1_F (836–882, sequence: 5′- TAATAC GACTCACTATA-GGG-3′), and p3.1_R (1022–1039, sequence: 5′-TAGAAGGCACA GTCGAGG-3′) were also used to confirm the stability of the transfection insert.

All of these cell lines were grown in MEM supplemented with 10% fetal bovine serum (FBS), penicillin (100,000 U/L), streptomycin (100 mg/ml), and G418 (400 mg/ml) in a humidified incubator at 37°C.

Cytotoxicity Assay

Cells were seeded at a density of 10^4/200 μl/well in 96-well microtitration plates. Twenty-four hours after plating, cells were incubated with 0.5 μg/ml of cycloheximide (CHX) and different concentrations (0, 5, 10, 20, and 40 ng/ml) of tumor necrosis factor alpha (TNF-α) for 24 and 48 h. After aspirating the medium, 200 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) solution diluted with MEM medium (5 mg/ml of MTT in MEM) was added to each well and incubated for 4 h at 37°C to allow MTT to be metabolized. The medium was aspired and the formazan crystals dissolved by adding 100 μl of dimethylsulphoxide (DMSO) to each well. A microplate reader was used to measure the absorbance at 570 nm using a reference wavelength of 630 nm. Cell viability was measured as a percentage of the control culture. MTT is designed to indicate viable cells. The number of

viable cells present after certain treatments for certain periods of time represent cellular proliferation and growth in a specific environment.

Apoptosis Detection

Annexin V staining. The annexin V fluorescein kit (Molecular Probes, Eugene, OR) was used to detect spontaneous apoptosis. Transfected UMSCC11A or UMSCC12 cells were seeded at $5 \times 10^5/2$ and $3 \times 10^5/2$ ml/well, respectively, in six-well plates and incubated overnight to allow cell attachment to the plate. Forty-eight hours after seeding, the cells were collected to detect spontaneous apoptosis. The cells were detached from the culture plates by trypsin and washed three times with PBS. After washing, the cells were incubated with 100 μl of binding buffer (10 mM HEPES/NaOH, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4), 5 μl annexin V and 1 μl PI (100 μg/ml) for 15 min in the dark. The cells were finally suspended in 400 μl binding buffer and analyzed by flow cytometry (FACScalibur, Becton Dickinson, CA) using Cell Quest software (Becton Dickinson).

Tunel assay. The instruction of the APO-DIRECT™ apoptosis assay kit (Chemicon International, San Diego, CA) was followed for the Tunel assay to detect apoptotic cells after stimulation with CHX and TNF-α. Transfected UMSCC11A or UMSCC12 cells were seeded at $5 \times 10^5/2$ and $3 \times 10^5/2$ ml/well, respectively, in six-well plates and incubated overnight to allow cells to attach to the plates. The cells were exposed to CHX 0.5 μg/ml and TNF-α 10 ng/ml for 0, 12, 24, and 48 h. The cells were then detached from the culture plates by trypsin, washed three times with PBS and suspended in 1% (w/v) paraformaldehyde in PBS and at a concentration of $1–2 \times 10^6$ cells/ml. The cell suspension was then placed on ice for 30–60 min. After centrifuging the suspension for 5 min at 300*g*, the supernatant was discarded and the cells washed in 5 ml of PBS and the cell pellet resuspended in PBS by a gentle vortex in a tube. The cell concentration was adjusted to $1–2 \times 10^6$ cells/ml in ice-cold 70% (v/v) ethanol and allowed to stand on ice for 30 min prior to staining for apoptosis. Apoptosis was measured according to the protocol provided by the kit.

Western Blotting

Thirty micrograms of sample proteins were electrotransfered onto nitrocellulose membranes and blocked in PBS with 0.1% Tween and 5% nonfat dried milk at room temperature for 1 h. Blots were incubated overnight at 4°C with primary antibody in PBS with 0.1% Tween, anti-Bak (1:1000; Cell Signaling, Danvers, MA), anti-Bax (1:1000; Cell Signaling) or anti-Bcl-2 (1:500; Santa Cruz Biotechnology, Santa Cruz, CA) and then incubated with the corresponding secondary antibody in PBS with 0.1% Tween and 2% nonfat dried milk. Immunoreactive proteins were detected with the enhanced chemiluminescence Western blotting detection system (ECL Western Blotting detection, Amersham Biosciences, Little Chalfont Buckinghamshire, England). Anti-α-Tubulin (1:2000; Santa Cruz Biotechnology) or Anti-β-Actin (1:10000; Calbiochem, San Diego, CA) was used to control protein loading.

Protein Stability Assay

Transfected UMSCC11A (2×10^7) or UMSCC12 cells (10^7) were seeded in 100 mm plates for 24 h. After the cells were attached to the plates, they were incubated with 0.5 μg/ml of CHX for 48 h and then the total protein extracted. Thirty micrograms of protein was subjected to Western blotting to determine the levels of Bak and Bcl-2 in transfected cells.

Plasmid Construction

Different lengths of Bak fragments were amplified according to the genomic DNA from normal volunteers. Primers listed in Table I were used in PCR amplification. Eleven forward primers were paired with the same reverse primer, Bak_3278_R, to amplify different lengths of Bak fragments (Fig. 1). The pGEM-T Easy Vectors (Promega) was used in the construction of plasmids containing different lengths of Bak fragments. Following the steps in the user's manual, blunt-ended PCR products of Bak fragments were generated and cloned into pGEM-T Easy vectors, resulting in eleven pGEM-T-Easy_Bak plasmids with different lengths of Bak fragments. The 11 pGEM-T-Easy_Bak plasmids were then, respectively, subcloned into a pGL3-Basic vector (Promega) by digestion with *Mlu* I and *Xho* I to generate plasmids pGL3-Basic_Bak108, pGL3-Basic_Bak942, pGL3-Basic_Bak1313, pGL3-Basic_Bak1533, pGL3-Basic_Bak1749, pGL3-Basic_

TABLE I. Primers Used in Amplification of Bak Fragments

Primer	Sequence	Position
Bak_108_F[a]	5′-CCG ACG CGT GGT ATT TCA GAA TCT CTG AG	108–128
Bak_1313_F	5′-CCG ACG CGT GTA GAG ACG GGA TTT TGC CAT GT	1313–1336
Bak_1533_F	5′-CCG ACG CGT AGA GAC AGA TTC TCG CAA TGT T	1533–1555
Bak_1749_F	5′-CCG ACG CGT ACA AAG AGG ATC ATG GTC GTG A	1749–1771
Bak_1998_F	5′- CCG ACG CGT GAG ACG GGT GGA TCA CGA A	1998–2017
Bak_2169_F	5′-CCG ACG CGT TTG CAG TAA GCC AAG GT	2169–2186
Bak_2397_F	5′-CCG ACG CGT AAA ATA GCC AGG TGT GGT GG	2397–2417
Bak_2572_F	5′-CCG ACG CGT AAG GTG AGG GTG AAA CTT CTC T	2572–2594
Bak_2797_F	5′-CCG ACG CGT TCA AAT GTT ATA GAA CTG AAA AAG A	2797–2822
Bak_2991_F	5′-CCG ACG CGT TGC TAA GAA CGT AGA TAC TGA	2991–3012
Bak_3097_F	5′-CCG ACG CGT AAA GGC TAC ATC CAG ATG CT	3097–3117
Bak_3278_R[a]	5′-CCG CTC GAG TTT TCA GGT CTC AGT GGA GGA	3257–3278

[a]F stands for forward primer while R stands for reverse primer.

Bak1998, pGL3Basic_Bak2169, pGL3-Basic_Bak2397, pGL3-Basic_Bak2572, pGL3-Basic_Bak2797, pGL3-Basic_Bak2991 and pGL3-Basic_Bak3097. The authenticity of these inserts was confirmed by enzyme digestion and DNA sequencing.

Luciferase Assay

Transfected UMSCC11A (5×10^5/well) or UMSCC12 cells (3×10^5/well) were seeded into six-well plates the day prior to transfection. Cells were transiently transfected by lipofectamine with 2 μg of the plasmid per well (Invitrogen). Two micrograms of pGL3-Basic-Bak plasmids (with different lengths of Bak inserts), pGL3-Promter (Promega), or pGL3-Control vector (Promega) was transfected to serve as a negative or positive control. A Luciferase assay system (Promega) was used in this study. Cells were collected 72 h after transfection. After washing the cells with PBS twice, 200 μl lysis buffer was added to each well and the cells were extracted from the plate by a scraper. The lysates were transferred into a microcentrifuge tube and incubated on ice. After centrifuging at 14,000 rpm for 2 min at 4°C, the supernatants were aspirated and transferred into a fresh tube. Twenty micrograms of cell lysate together with 65 μl of Luciferase assay reagent was transferred into a well of a 96-well plate. The light intensity of each well was measured by a luminometer (Molecular Derices, Fullerton, CA). All experiments were performed at least three times.

Statistical Analysis

All values are expressed as the mean ± SD. The statistical analyses (ANOVA, *t*-test, or paired *t*-test) were performed by SPSS10.0 for Windows. A *P*-value less than 0.05 was taken as statistically significant.

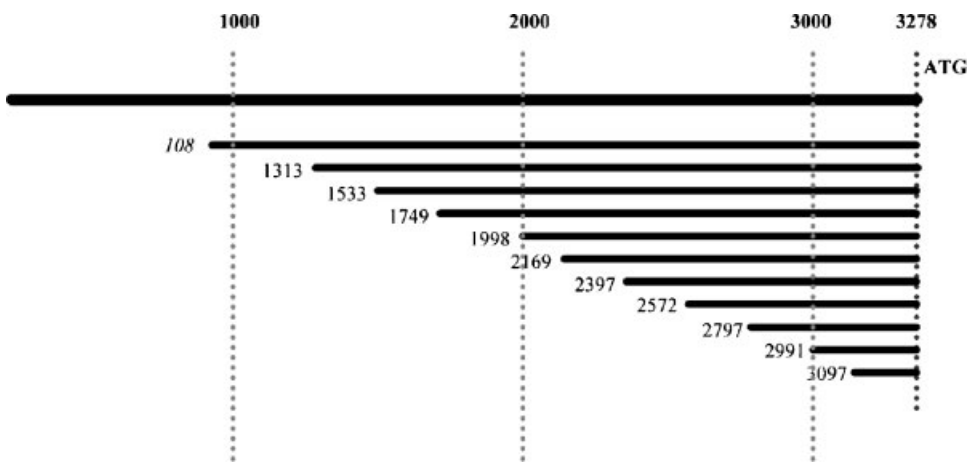

Fig. 1. The positions of synthetic oligomers of Bak fragments used in PCR.

RESULTS

Establishment of HPV-16 E6/E7-Expression in Human Laryngeal Cancer Cell Lines

We first confirmed the expression of HPV-16 E6 and E7 in transfected UMSCC11A and UMSCC12 cells by RT-PCR with primers E6_F2, E6_R2, and E7_F1, E7_R1, respectively (Fig. 2A,B). Primers pc3.1_F and pc3.1_R were also used to check the accuracy of the transfected insert. UMSCC11A and UMSCC12 cells without the transfection, which did not contain HPV-16, were used as negative controls while Caski cells which are positive for HPV-16 were used as the positive control.

In Figure 2A, UMSCC11A cells transfected with HPV-16 E6 showed a single clear band of 293 bp on PCR by using primers E6_F2 and E6_R2 (a, Lane 1) which was very similar to the positive control (a, Lane 5), while there was no amplification band detected in UMSCC11A_E7 cells (a, Lane 2), UMSCC11A_pcDNA3.1 cells (a, Lane 3) and UMSCC11A cells (a, Lane 4) which was the negative control. On the other hand, using primers E7_F1 and E7_R1 showed a single clear amplification band of 197 bp in the positive control which was Caski cells (b, Lane 4), and UMSCC11A_E7 cell lines (b, Lane 2). There was no band formed in UMSCC11A_E6 (Fig. 2Ab, Lane 1) UMSCC11A_pcDNA3.1 (b, Lane 3) and UMSCC11A cells (b, Lane 4). Using primers pc3.1_F and pc3.1_R, fragments of 625, 402, and 177 bp were shown in UMSCC11A_ E6 (c, Lane1) UMSCC11A_E7 (c, Lane2) and UMSCC11A_ pcDNA3.1 cells (c, Lane 3), respectively. Caski cells were used as a positive control (Lane 5) whereas a reaction containing no template as a PCR negative control (Lane 6).

A similar result was shown in transfected UMSCC12 cells (Fig. 2B). UMSCC12_E6 (a, Lane 1) and Caski cells (a, Lane 4) showed clear single bands of 293 bp on PCR by using primers E6_F2 and E6_R2, while there was no amplification band from UMSCC12_E7 (a, Lane2), UMSCC12_pcDNA3.1 cells (a, Lane 3) and UMSCC12 cells (a, Lane 5) which was the negative control. Furthermore, with primers E7_F1 and E7_R1, Caski (b, Lane 4) and UMSCC11A_E7 cells (b, Lane 2) showed a clear single amplification band of 197 bp. No band was seen in UMSCC12_E6 (b, Lane 1), UMSCC12_pcDNA3.1 (b, Lane 3) and

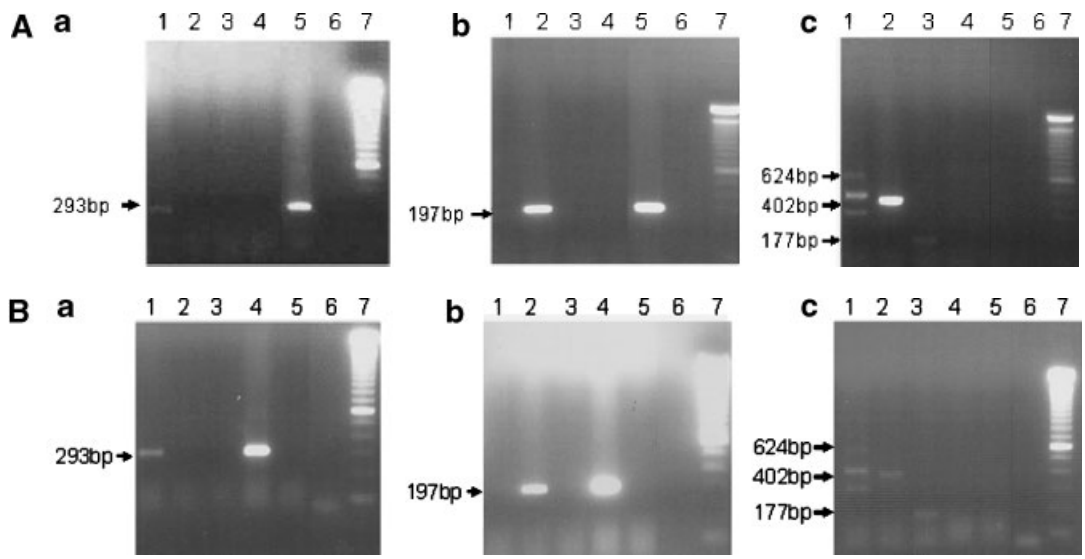

Fig. 2. RT-PCR of transfected (**a**) UMSCC11A and (**b**) UMSCC12 cells. After transfected (A) UMSCC11A and (B) UMSCC12 cell lines were successfully established, they were checked for their expression of E6 and E7 by RT-PCR. **a**: A 293 bp fragment of HPV-16 E6 was amplified with primers E6_F2 and E6_R2 in cells transfected with E6 and in Caski cells, the positive control. **b**: A 197 bp fragment of HPV-16 E7 was shown in cells transfected with E7 and Caski cells by using the primers E7_F1 and E7_R1. **c**: pc3.1_F and pc3.1_R were used to check the size of the inserts. Fragments of about 625, 402 and 177 bp were shown, respectively, in E6-, E7- and pcDNA3.1-transfected cells. A—**Lane 1**: UMSCC11A_E6 cells; **Lane 2**: UMSCC11A_ E7 cells; **Lane 3**: UMSCC11A_pcDNA3.1 cells; **Lane 4**: UMSCC11A cells (negative control); **Lane 5**: Caski cells (positive control); **Lane 6**: PCR negative control; **Lane 7**: PCR 100 bp marker. B—**Lane1**: UMSCC12_E6 cells; **Lane 2**: UMSCC12_E7 cells; **Lane 3**: UMSCC12_pcDNA3.1 cells; **Lane 4**): Caski cells (positive control); **Lane 5**: UMSCC12 cells (negative control); **Lane 6**: PCR negative control; **Lane 7**: PCR 100 bp marker.

UMSCC12 cells (b, Lane 5). Similar to transfected UMSCC11A cells, UMSCC12_E6 cells (c, Lane1), UMSCC12_E7 cells (c, Lane2), and UMSCC12_pcDNA3.1 cells (c, Lane 3) showed fragments of 625, 402, and 177 bp, respectively. A reaction containing no template was used as a PCR negative control (Lane 6).

The Expression of Bcl-2 Family Members in Either HPV-16 E6 or E7 Transfected Cell Lines

Since the Bcl-2 family is known to be a prognostic marker for laryngeal cancer, we determined whether the expression of Bcl-2 family proteins was altered in the presence of the E6 or E7 gene. We examined both the pro-apoptotic (Bax and Bak) and anti-apoptotic (Bcl-2) members in transfected UMSCC11A and UMSCC12 cell lines. Figure 3A compares the expression of Bax in UMSCC11A_E7 and UMSCC11A_E6 cells using UMSCC11A_pcDNA3.1 cells as a control and shows no difference (relative density:UMSCC11A_pcDNA3.1:UMSCC11A_E7:UMSCC11A_E6 = 1:0.98:0.95). However, the level of Bak expression in either E7- ($P = 0.007 < 0.01$) or E6- ($P = 0.04 < 0.05$) transfected UMSCC11A cells was significantly less than in the control. Furthermore, the level of Bcl-2 was significantly more in UMSCC11A cells with either HPV-16 E7 or E6 transfection compared to the control (pcDNA3.1; the P values of E7 vs. pcDNA3.1 and E6 vs. pcDNA3.1 were 0.04 and 0.03, respectively, both <0.05). In transfected UMSCC12 cells, there was only a significant increase in Bcl-2 protein in UMSCC12_E6 cells (Fig. 3B, $P = 0.05$), and no significant alteration in the levels of other Bcl-2 family members was found.

We further determined the spontaneous apoptotic rate of the six cell lines by annexin V flow

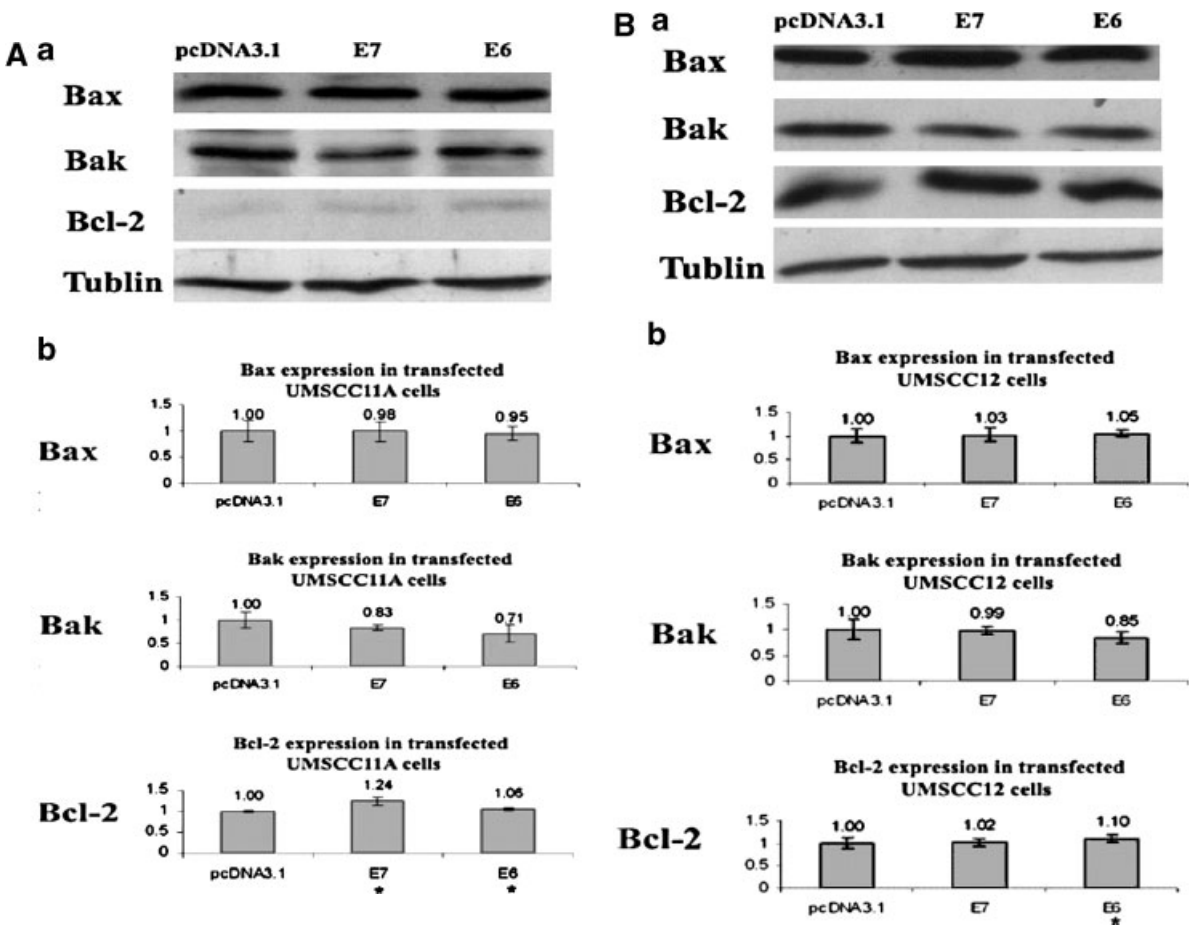

Fig. 3. The expression of Bax, Bak, and Bcl-2 in transfected (**A**) UMSCC11A and (**B**) UMSCC12 cells. **a**: The Western blots are representative of three independent experiments. **b**: The diagrams show the average density of each protein. Statistically significant differences between (A) UMSCC11A_pcDNA3.1 and (B) UMSCC12_pcDNA3.1 cells were labeled as $^{*}P < 0.05$ and $^{**}P < 0.01$.

cytometry analysis. Although there was no statistical significance, the trend of apoptosis in the three cell lines transfected with pcDNA3.1, HPV-16 E6 or E7, respectively, is shown in Figure 4A. UMSCC11A_pcDNA3.1 showed the highest average apoptotic rate of 4.55% while the apoptotic rates in UMSCC11A_E6 and UMSCC11A_E7 were only 2.79% and 3.85%, respectively. A similar trend was seen in transfected UMSCC12 cells (Fig. 4B). The apoptotic rates in the three transfected UMSCC12 cell lines were 3.07%, 2.39%, and 2.98%, respectively.

E6 and E7 Prevented the Growth Arrest Induced by CHX and TNF-α

We performed a MTT assay to detect cell proliferation after exposure to CHX and TNF-α. Cell proliferation of UMSCC11A-pcDNA3.1

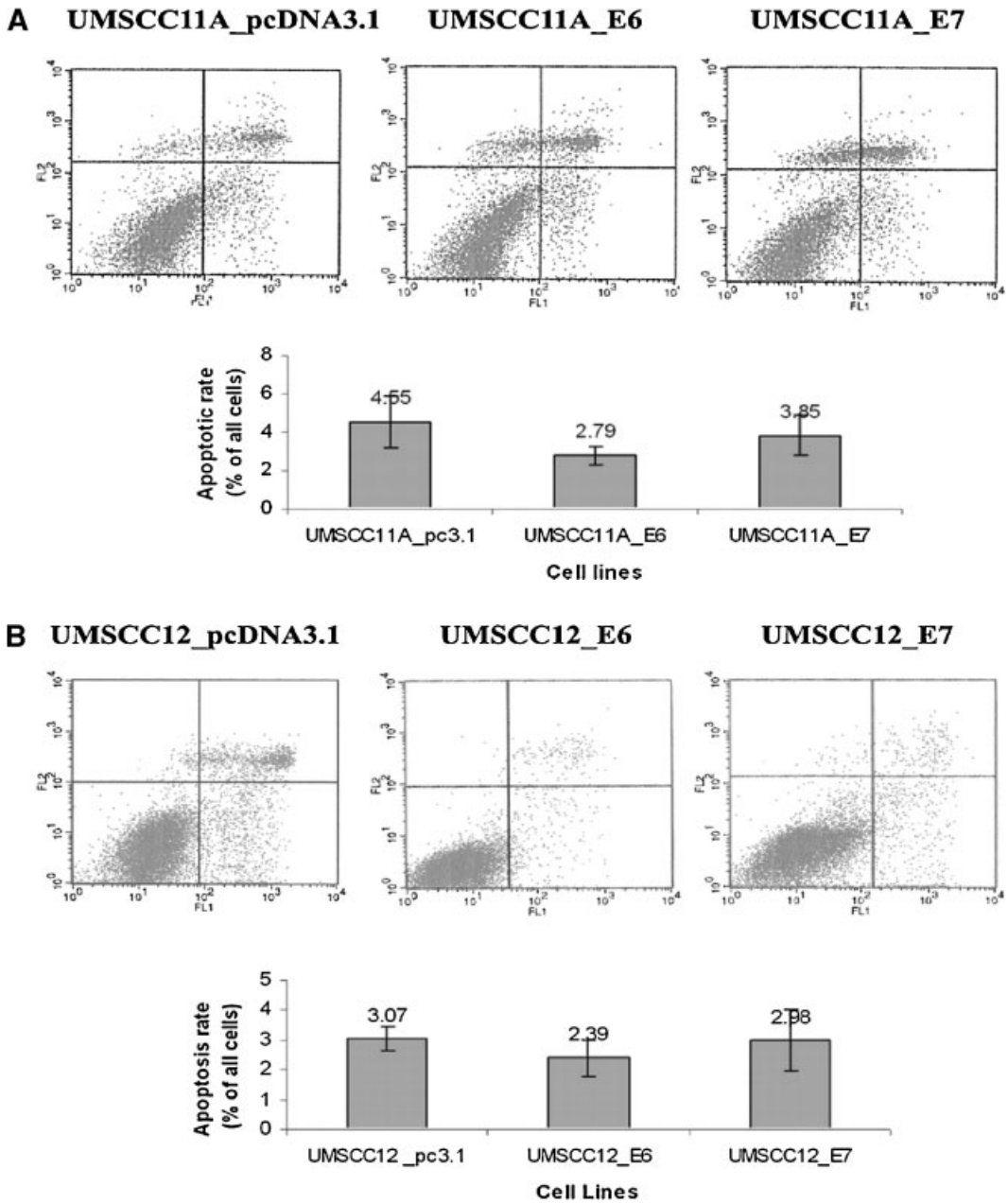

Fig. 4. Spontaneous apoptosis in transfected (**A**) UMSCC11A and (**B**) UMSCC12 cells. The number of cells undergoing apoptosis and necrosis was determined by flow cytometry after staining with annexin V and PI. In each annexin V plot, the lower left quadrant shows viable cells, the lower right quadrant shows early apoptotic cells and the upper left and right quadrants show late apoptotic and necrotic cells. The diagram shows the average apoptotic rate calculated from three experiments for each cell line.

cells was significantly inhibited by CHX and TNF-α in a time- and dose-dependent manner (Fig. 5A). As shown in Figure 5A, TNF-α 10 ng/ml with CHX 0.5 μg/ml effectively inhibited the growth of UMSCC11A_pcDNA3.1, UMSCC11A_E6, and UMSCC11A_E7 cells by 60.4%, 35.3% and 36.5%, respectively, at 24 h after the treatment, and by 70.5%, 43.7%, and 61.1% at 48 h after the treatment. It is obvious that the proliferation of both HPV-16 E6- and E7-transfected-UMSCC11A cells was higher than of cells without E6 or E7 transfection

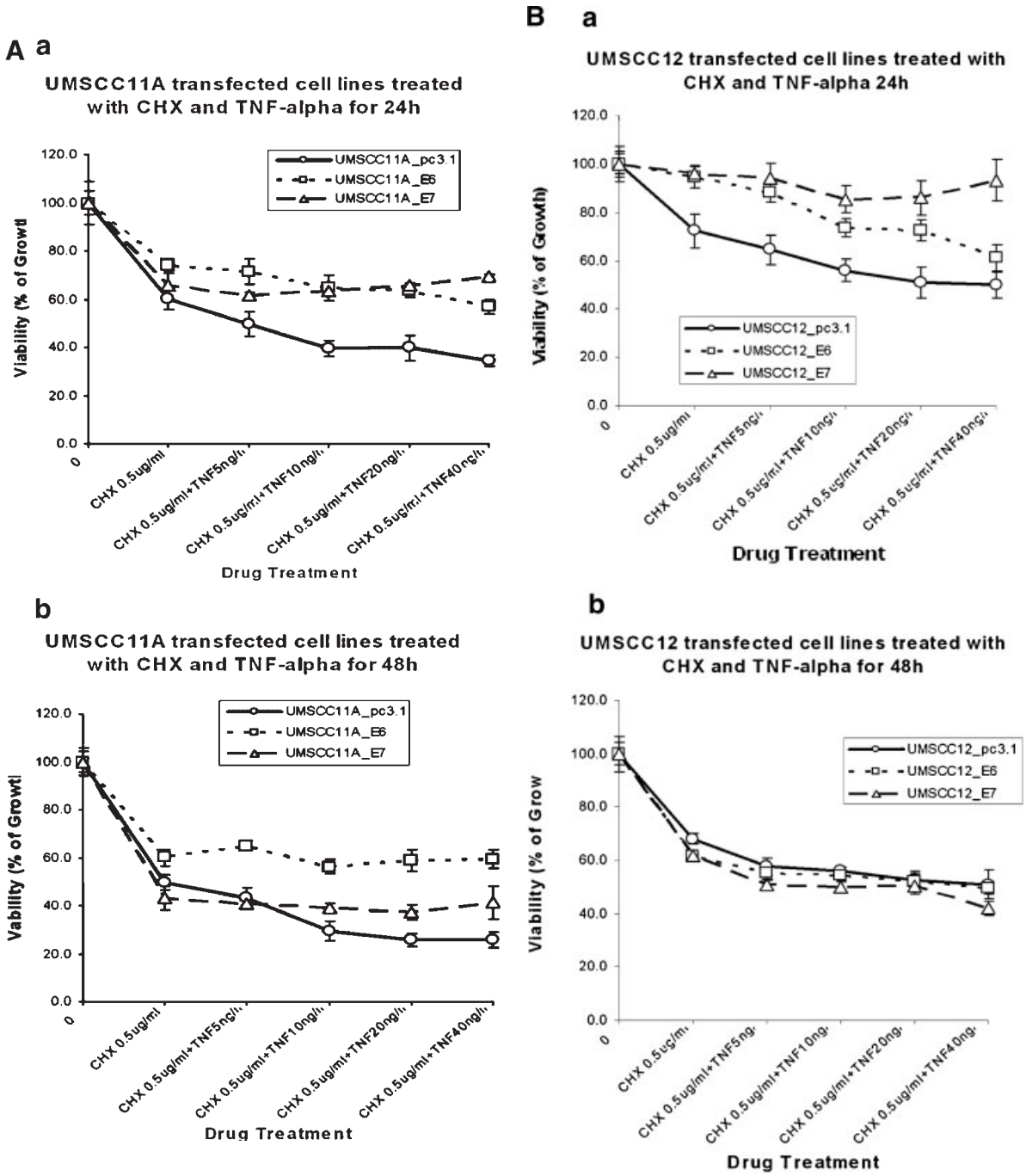

Fig. 5. Antiproliferation effects of CHX and TNF-α on transfected (**A**) UMSCC11A cells and (**B**) UMSCC12 cells. Cells were incubated with increasing doses of TNF-α (0, 5, 10, 20, and 40 ng/ml) and CHX 0.5 μg/ml for 24 h [A (**a**),B (**a**)] and for 48 h [A (**b**),B (**b**)]. The cytotoxicity assay was performed as described in Materials and Methods. Values shown represent the mean of three independent experiments.

(pcDNA3.1-transfected cells; $P < 0.05$) following CHX and TNF-α treatment, especially when the concentration of TNF-α was higher than 10 ng/ml (Fig. 5A). The results suggest that HPV-16 E6 and E7 have the potential to prevent the growth arrest induced by CHX and TNF-α.

Similar to UMSCC11A_pcDNA3.1 cells, UMSCC12_pcDNA3.1 cells showed a time- and dose-dependent decrease after exposure to CHX and TNF-α (Fig. 5B). After treatment for 24 h (Fig. 5Ba), the reduction in cell growth of UMSCC12_E6 and UMSCC12_E7 cells was less obvious than in UMSCC12_pcDNA3.1 cells following stimulation with TNF-α. The inhibitory rates were 44.1% for UMSCC12_pcDNA3.1 cells, 26.3% for UMSCC12_E6 cells, and 14.6% for UMSCC12_E7 cells following 10 ng/ml TNFα and 0.5 μg/ml CHX treatment for 24 h, indicating that the inhibition is higher in cells without E6 or E7 transfection ($P < 0.05$). After CHX and TNF-α treatment for 48 h, the significant difference in cell proliferation between cells with E6- ($P = 0.630$) or E7-transfection ($P = 0.101$) and those without disappeared and was equally inhibited in the three transfected UMSCC12 cell lines studied. Ultimately, CHX and TNF-α have a less inhibitory effect on the cell proliferation of HPV-16 E6- and E7-transfected cells than on pcDNA3.1-transfected cells.

E6 or E7 Protected TNF-α-Induced Apoptosis in Laryngeal Cancer Cells

To further analyze the antiproliferation effect of TNF-α on both UMSCC11A and UMSCC12 cell lines transfected with pcDNA3.1, HPV-16 E6, and HPV-16 E7, we performed Tunel flow cytometry analysis to detect apoptosis. Based on the MTT results, we performed the assay after the transfected UMSCC11A and UMSCC12 cells were treated with 0.5 μg/ml CHX and 10 ng/ml TNF-α for 0, 12, and 24 h. The results are shown in Figure 6.

Coinciding with the MTT assay, TNF-α treatment resulted in an increase in apoptotic rates in both UMSCC11A_pcDNA3.1 (Fig. 6A,Ca) and UMSCC12_pcDNA3.1 cells (Fig. 6B,Cb). We noted a direct relationship between the exposure time and the apoptotic rate in UMSCC11A_pcDNA3.1 cells. However, in UMSCC12_pcDNA3.1 cells, the highest apoptotic rate occurred 12 h after treatment. When compared to untreated cells, the percentage of apoptosis of UMSCC11A_pcDNA3.1 cells increased by 39.79 ($P < 0.01$) and 41.00 folds ($P < 0.01$), respectively, after 12- and 24-hours of exposure to TNF-α and CHX (Fig. 6Ca). On the other hand, treatment with TNF-α and CHX for 12 and 24 h resulted in 41.78 ($P < 0.01$) and 39.37-fold increases ($P < 0.01$) in apoptosis of UMSCC12_pcDNA3.1 cells, respectively, compared to untreated cells.

TNF-α-Induced Apoptosis in E6- and E7-Transfected Cell Lines and the Alteration of Bcl-2 Family Proteins

In order to find out the possible mechanism by which E6 and E7 prevent TNF-α-inducing apoptosis, we performed Western blotting to detect the expression of Bcl-2 family proteins. From the previous experiment, we know that the induction of apoptosis by TNF-α in the cell lines occurred mostly within 24 h. Therefore we focused on detecting Bak, Bax, and Bcl-2 at 0, 12, and 24 h after treatment with CHX and TNF-α. In UMSCC11A_pcDNA3.1 cells, the CHX and TNF-α-mediated induction of pro-apoptotic proteins Bak and Bax was time-dependent (Fig. 7A). Treatment with CHX and TNF-α significantly increased the expression of Bak ($P = 0.05$) and Bax ($P = 0.02$) by 1.6- and 1.42-fold at 24 h (Fig. 7Ab) compared to untreated cells. However, treatment with CHX and TNF-α decreased the expression of Bak and Bax in UMSCC11A_E6 and UMSCC11A_E7 cells. There was a 60% ($P = 0.002$) and 48% ($P = 0.01$) reduction of Bak at 12 and 24 h, respectively, and an 11% ($P = 0.03$) and 20% reduction ($P = 0.02$) of Bax at 12 and 24 h, respectively, in UMSCC11A_E6 cells. The expression of Bak and Bax showed statistical differences between UMSCC11A_pcDNA3.1 and UMSCC11A_E6 (for Bak, $P = 0.01$; for Bax, $P = 0.008$) or UMSCC11A_E7 cell lines (for Bak, $P = 0.024$; for Bax, $P = 0.017$) at 24 h after the treatment. Furthermore, CHX and TNF-α did not reduce the expression of the anti-apoptotic member, Bcl-2 in UMSCC11A_pcDNA3.1 cells (Fig. 7A), but its expression in both UMSCC11A_E6 and UMSCC11A_E7 was slightly increased after the stimuli (Fig. 7A). The induction of Bcl-2 expression was obvious in UMSCC11A_E7 cells, with 2.13-fold ($P = 0.002$) and 3.13-fold ($P = 0.000$) increases in the expression after 12 and 24 h of treatment, respectively (Fig. 7Ab). There was no significant change in the expression of Bcl-2 in either UMSCC11A_pcDNA3.1 or in UMSCC12_E6 cells. However, the expression of Bcl-2 in UMSCC12_E7 cells was significantly higher

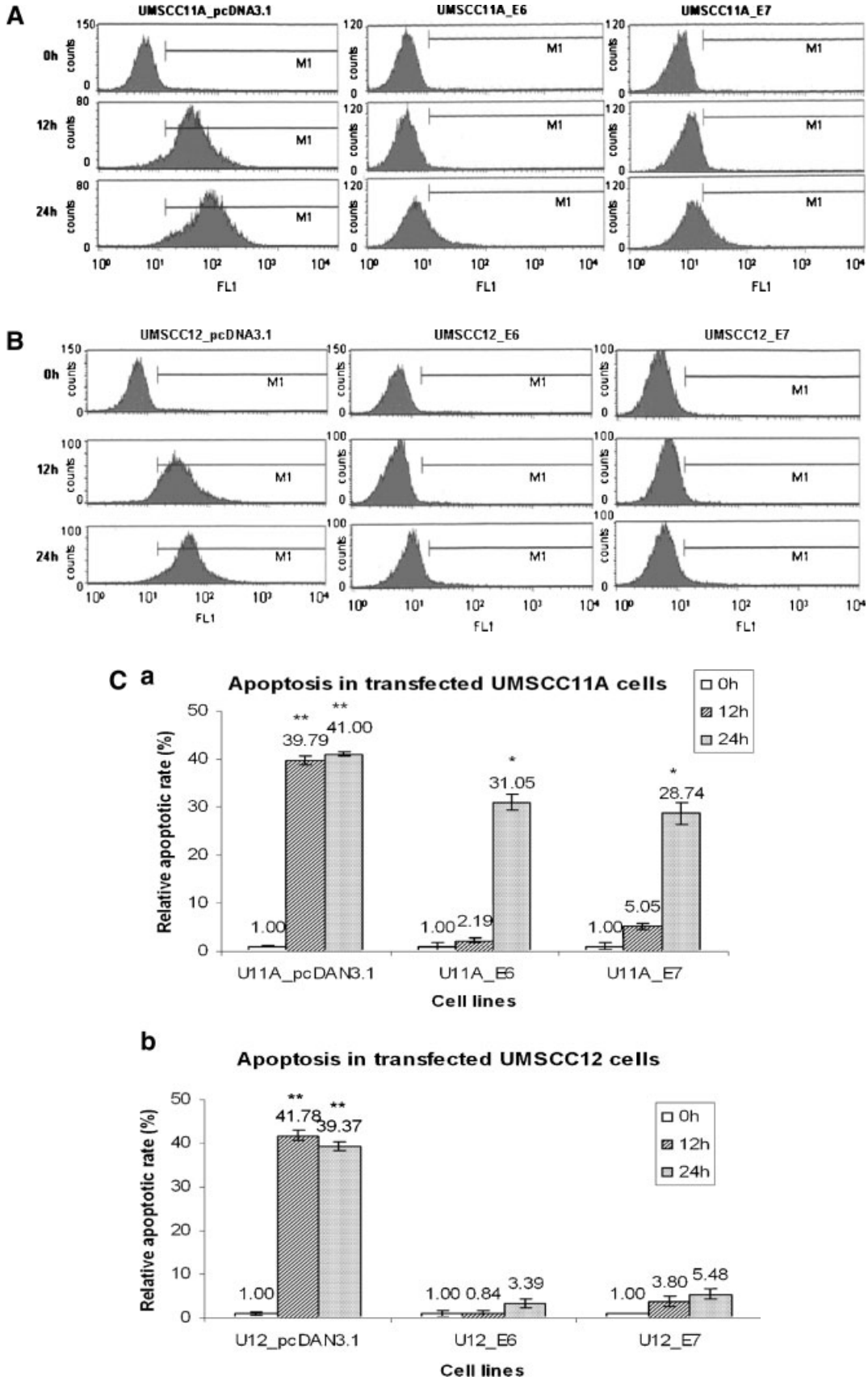
A
UMSCC11A_pcDNA3.1
UMSCC11A_E6
UMSCC11A_E7
0h
12h
24h
counts
M1
FL1
B
UMSCC12_pcDNA3.1
UMSCC12_E6
UMSCC12_E7
C a
Apoptosis in transfected UMSCC11A cells
0h
12h
24h
Relative apoptotic rate (%)
39.79
41.00
31.05
28.74
1.00
2.19
5.05
U11A_pcDAN3.1
U11A_E6
U11A_E7
Cell lines
b
Apoptosis in transfected UMSCC12 cells
41.78
39.37
0.84
3.39
3.80
5.48
U12_pcDAN3.1
U12_E6
U12_E7
Cell lines

Fig. 6.

than in UMSCC12_pcDNA3.1 cells at 12 ($P = 0.002$) and 24 h ($P = 0.001$) after the treatment.

A similar trend in the expression of pro-apoptotic Bcl-2 family members was seen in the transfected UMSCC12 cell lines (Fig. 7B). There was a significant induction of pro-apoptotic members in UMSCC12_pcDNA3.1 cells. Compared with untreated cells, the expression of Bak was 1.32-fold higher ($P < 0.01$) and of Bax 1.46-fold higher ($P = 0.01$). The decrease in pro-apoptotic factors was more obvious in UMSCC12_E6 cells than in UMSCC12_E7 cells. There was a 35% reduction in Bak ($P = 0.002$) and a 39% reduction in Bax ($P = 0.002$) at 24 h after the treatment in UMSCC12_E6 cells whereas there were only a 19% reduction in Bak ($P < 0.01$) and a 6% reduction in Bax ($P = 0.018$) at the same time point in UMSCC12_E7 cells. There was a significant difference in the expression of pro-apoptotic proteins Bak ($P = 0.002$) and Bax ($P < 0.01$) between UMSCC12_pcDNA3.1 and UMSCC12_E6 at 24 h and in the expression Bak ($P = 0.002$) and Bax ($P = 0.009$) between UMSCC12_pcDNA3.1 and UMSCC12_E7 at 24 h.

The inhibition of Bcl-2 expression was clearly detected in UMSCC12_pcDNA3.1 cells, which caused a 30% ($P = 0.001$) and a 53% ($P = 0.006$) reduction in the level of Bcl-2 protein after treatment for 12 and 24 h, respectively (Fig. 7Bb). A change in the expression of Bcl-2 was not obvious in either UMSCC12_E6 or UMSCC12_E7 cells. However, there was a significant difference of Bcl-2 between UMSCC12_pcDNA3.1 and UMSCC12_E6 cells after 12- ($P = 0.017$) or 24-h ($P = 0.022$) treatments. The difference was also observed between UMSCC12_ pcENA3.1 and UMSCC12_E7 cells after 12- ($P = 0.001$) or 24-h treatments ($P = 0.047$).

E6 and E7 Altered the Degradation of Pro-Apoptotic Bcl-2 Family Proteins

To determine whether the HPV-16 E6 or E7 viral protein could independently affect the stability of the Bcl-2 protein, the rate of Bak or Bcl-2 protein degradation in transfected UMSCC11A and UMSCC12 cells after exposure to 0.5 μg/ml CHX was determined. Representative Western blotting analysis of the protein levels at various time points is shown in Figure 8A,B. In transfected UMSCC11A cells (Fig. 8A), stable transfection with E6 or E7 viral genes was associated with an accelerated decline in Bak expression. There was a 27.03% and 33.97% reduction compared with a 3.42% reduction in UMSCC11A_E6, UMSCC11A_E7, and UMSCC11A_pcDNA3.1 cells, respectively, after 12 h of CHX exposure. Similar results were obtained in transfected UMSCC12 cells (Fig. 8B). The decline in Bak levels in UMSCC12_pcDNA3.1 cells (5.39% reduction) was smaller than that in UMSCC12_E6 cells (31.89% reduction) and UMSCC12_E7 cells (23.38% reduction) after 12 h of CHX exposure. However, the degradation of Bcl-2 protein was different to that of Bak and Bax. The degradation of Bcl-2 in UMSCC12_pcDNA3.1 cells (33.01% reduction) and UMSCC12_E7 cells (33.27% reduction) was more obvious than in UMSCC12_E6 cells (4.97% reduction) after 12-h treatment.

E6 and E7 Did Not Alter the Transcriptional Activity of Bak

To characterize the transcriptional regulation of Bak by HPV-16 E6 and E7, we conducted a luciferase reporter assay of the Bak promoter. UMSCC11A and UMSCC12 cells that have a stable transfection of HPV-16 E6 and/or E7 were transiently cotransfected with Bak promoter DNA. The basal promoter and nearby upstream regulatory elements of the gene are thought to be located at 550 bp upstream from the ATG [Trinklein et al., 2003]. Thus, we used the same reverse primer with different forward primers to clone different lengths of the upstream ATG region (−3178–0). The luciferase assay indicated that pGL3-promoter and pGL3-control activated luciferase expression by 11.82–16.13 and 73.1–85.33-fold, respectively, above the pGL3-basic control (Fig. 9A,B). However, when compared to pcDNA3.1-transfected cells, neither E6- nor E7-transfected UMSCC11A or UMSCC12

Fig. 6. Effect of CHX and TNF-α on apoptosis in transfected (**A**) UMSCC11A cells and (**B**) UMSCC12 cells. Cells were treated with CHX 0.5 μg/ml and TNF-α 10 ng/ml for 12 and 24 h. After the treatment, apoptosis was assayed by the Tunel method using an APO-DIRECT™ apoptosis assay kit. A,B: Typical results obtained by flow cytometry. **C**: summary of data for (**a**) UMSCC11A cells and (**b**) UMSCC12 cells at 0 (blank), 12 (stripes), and 72 h (spots). The results are expressed as the relative variation in the apoptotic cell fraction compared to untreated controls. Experiments were repeated three times and statistically significant differences to untreated cells were labeled as $^{*}P < 0.05$ and $^{**}P < 0.01$.

cells showed significant differences in luciferase expression when transfected with different pGL3-Basic_Bak plasmids, suggesting that HPV-16 E6 or E7 might not alter the expression of Bak by regulating its promoter.

DISCUSSION

In this study, we demonstrated the effect of two oncogenic HPV-16 proteins, E6 and E7, on spontaneous apoptosis and TNF-α-induced apoptosis in human laryngeal cancer cells. We first performed Western blotting to investigate the expression of Bcl-2 family members in HPV-16 E6, E7, and pcDNA3.1-transfected cells. Using pcDNA3.1-transfected cells as a baseline, while there was no obvious difference in the expression level of pro-apoptotic protein Bax in either E6- or E7-transfected UMSCC11A or UMSCC12 cells, the expression of Bak was

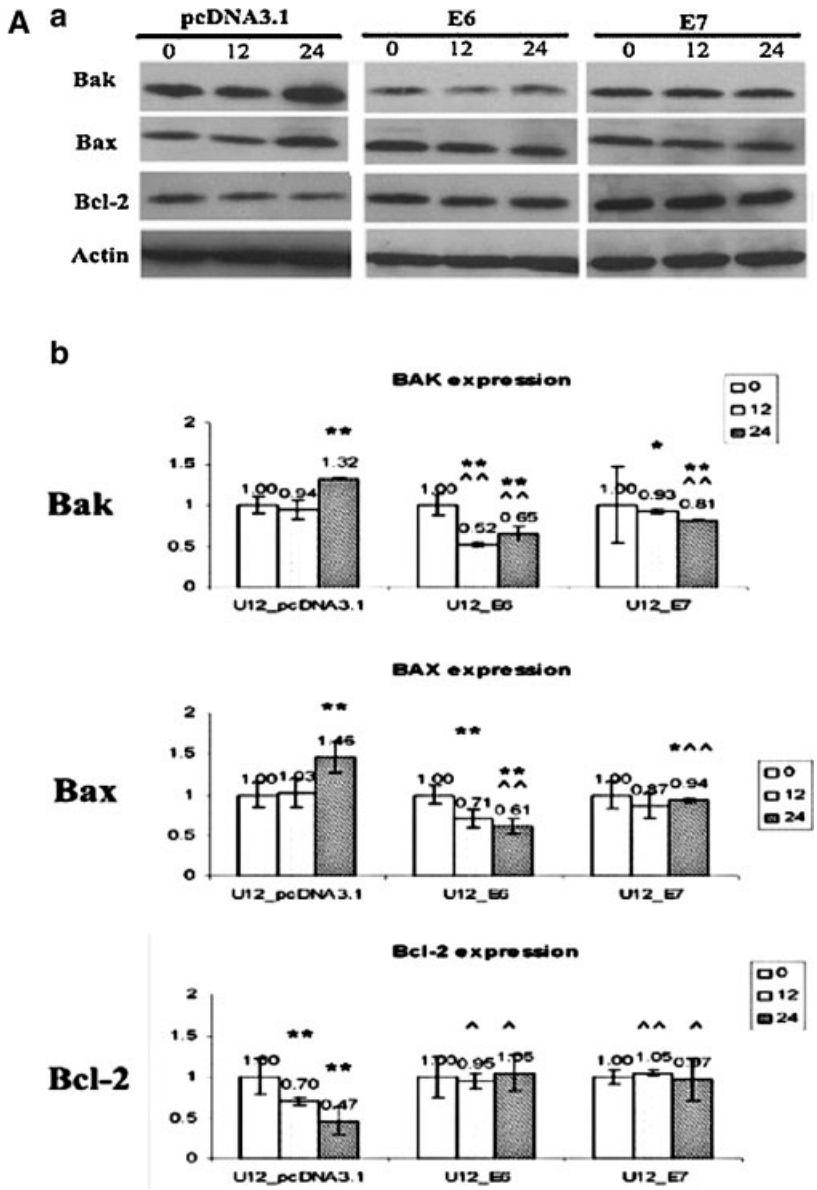

Fig. 7. CHX and TNF-α altered the expression of Bcl-2 family in transfected (**A**) UMSCC11A cells and (**B**) UMSCC12 cells. Cells were treated with CHX 0.5 μg/ml and TNF-μ 10 ng/ml for 0, 12, and 24 h and cell lysates were prepared for protein determination. **a**: Typical Western blot results. **b**: Summary of Western blot data. $*P < 0.05$ and $**P < 0.01$ compared with untreated controls. The difference between E6- or E7- transfected cells and pcDNA3.1-transfected cells at the same time point is presented as $\hat{}P < 0.05$ and $\hat{}\hat{}P < 0.01$.

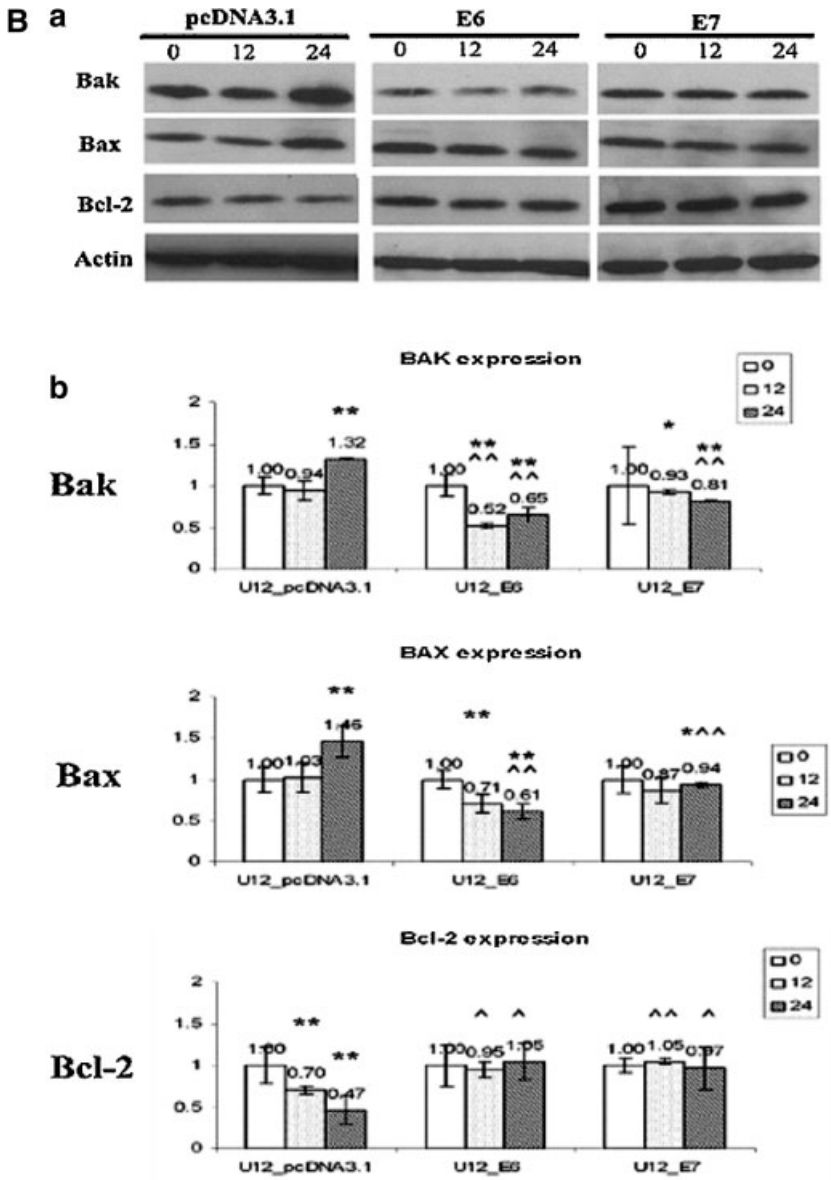

Fig. 7. (*Continued*)

significantly inhibited in UMSCC11A_E6 and UMSCC11A_E7 cells. The level of anti-apoptotic protein Bcl-2 was significantly raised in UMSCC11A_E6, UMSCC11A_E7, and UMSCC12_E6 cells. The inhibition of Bak and the increased level of Bcl-2 agree with the pathological features of HPV-16 E6 and E7 in human cancer. Although the interaction between Bcl-2 family members and HPV-16 E6 or E7 oncogenes in relation to spontaneous apoptosis in laryngeal cancer has not been defined, the altered expression of Bcl-2 members in different cancers has been widely discussed. Some papers have tried to correlate Bcl-2 expression with prognosis in laryngeal cancer [Vlachtsis et al., 2005]. However, there are limited reports that discuss the expression of Bcl-2 in HPV-16-associated laryngeal cancers. The findings of our study suggest that the elevated levels of Bcl-2 protein induced by HPV-16 E6 and E7 may play a role in protecting laryngeal cancer cells from apoptosis.

We induced apoptosis with TNF-α and CHX [Gupta, 2002; Deng et al., 2003]. While an obvious reduction in cell proliferation in the control pcDNA3.1-transfected cells was found, there was no inhibition in cell proliferation in all HPV-16 E6- and E7-transfected laryngeal

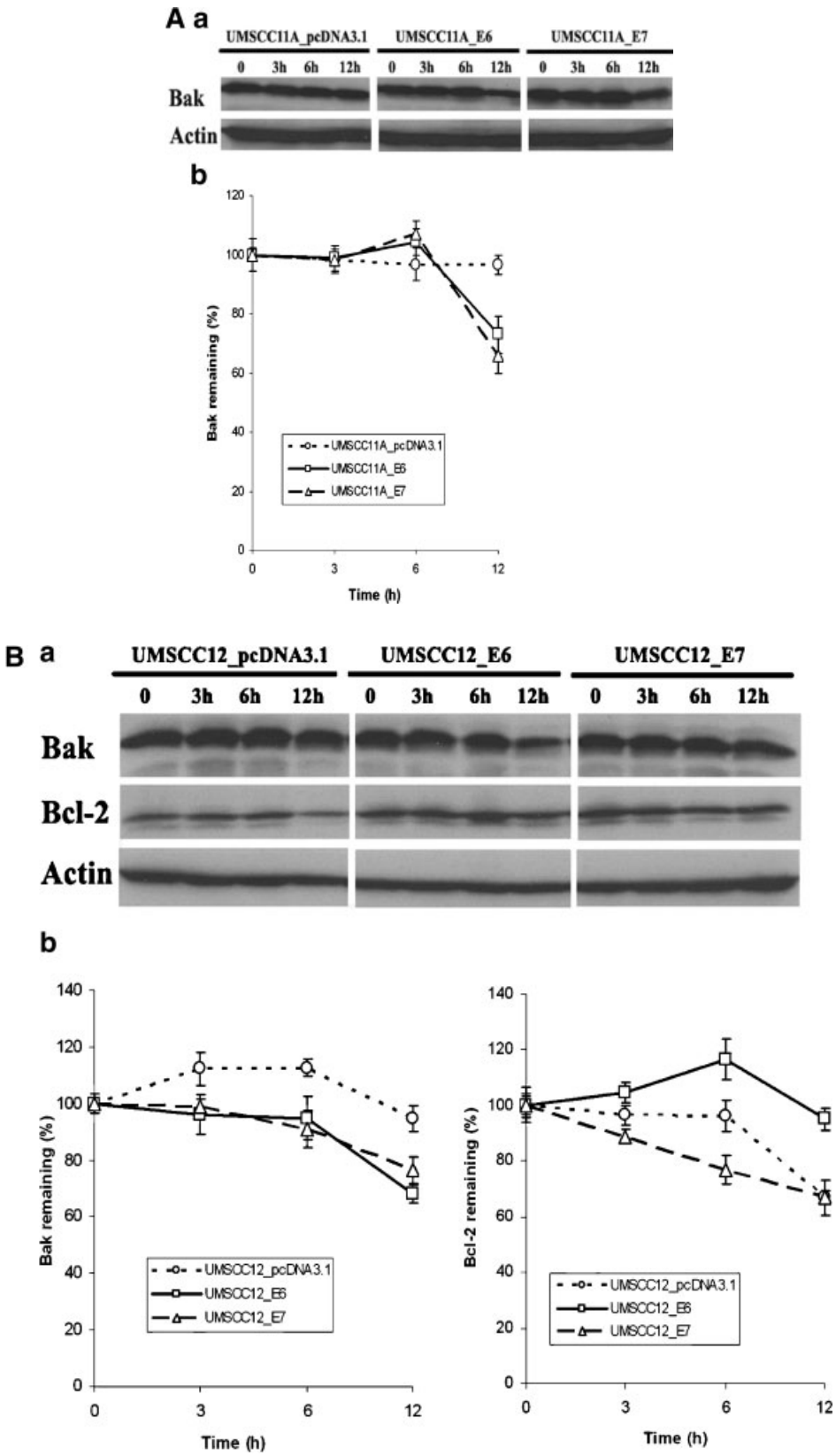

Fig. 8. Degradation of Bak protein in HPV-16 E6-, E7- or vector-transfected (**A**) UMSCC11A and (**B**) UMSCC12 cells. After cells were treated with CHX 0.5 μg/ml for the indicated periods of time, they were lysed in RIPA buffer and equal protein samples (30 μg) were analyzed by SDS–PAGE and immunoblotting. **a**: Representative blots. **b**: Relative levels of residual Bak (percentage of time 0).

cancer cell lines. Under apoptotic stimuli, low apoptotic rates were detected in both HPV-16 E6- and E7-transfected cells by TUNEL analysis. These results demonstrate that UMSCC11A and UMSCC12 cells transfected with HPV-16 E6 or E7 are less sensitive to apoptotic stimuli than control cells, suggesting that HPV-16 E6 and E7 are capable of protecting the cells from apoptosis. This escape mechanism is considered to be an important characteristic of cancer development [Hanahan and Weinberg 2000].

The change in the expression of Bcl-2 family members after treatment with TNF-α and CHX revealed the possible mechanism by which HPV-16 E6 and HPV-16 E7 prevent apoptosis. While there was an increase in the expression of Bak and Bax in pcDNA3.1-transfected UMSCC11A and UMSCC12 cells, the expression of these two proteins was decreased in E6- and E7-transfected cells. The expression of Bcl-2 protein increased significantly in both E6- and E7-transfected cells. These results suggest that HPV-16 E6 and E7 oncoproteins may contribute to carcinogenesis by decreasing pro-apoptotic proteins Bak and Bax and by increasing anti-apoptotic protein Bcl-2. These changes result in an imbalance between pro-apoptotic and anti-apoptotic Bcl-2 members, which may lead to the promotion of cancer [Ayhan et al., 1994; Harada et al., 1997; Hanahan and Weinberg, 2000; Gobe et al., 2002; Martin et al., 2003; Thomadaki et al., 2006]. Bcl-2 can interfere with the regulation of apoptosis by forming Bcl-2/Bax heterodimers [Oltvai et al., 1993; Haldar et al., 1997] or by loosely binding with Bak [Chittenden et al., 1995]. Therefore, overexpression of Bcl-2 in

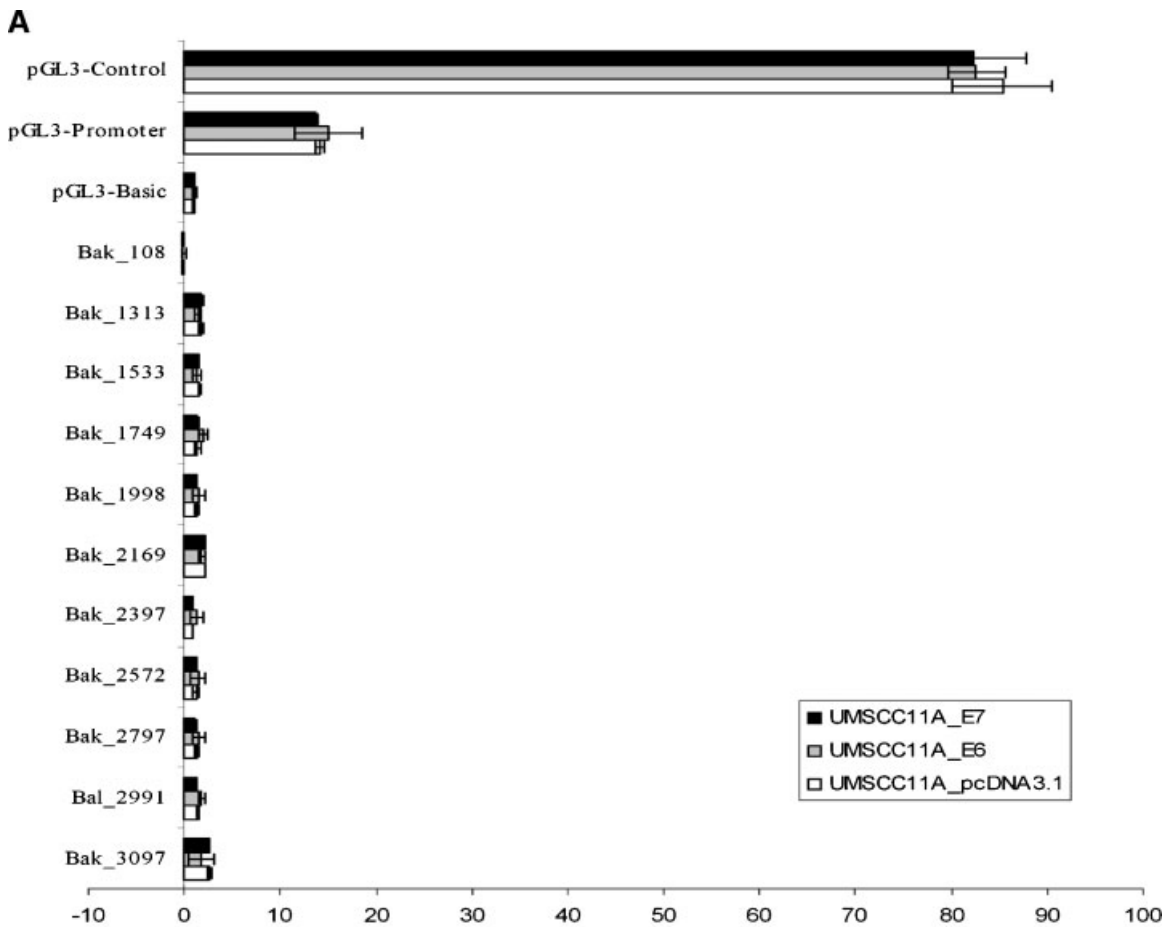

Fig. 9. Relative luciferase activity in transfected (**A**) UMSCC11A cells and (**B**) UMSCC12 cells. 5×10^5 cells/well in six-well plates were transfected with 2 μg of Bak promoter controlled-luciferase construct (−3178~0), pGL3-Basic, pGL3-Promoter, or pGL3-Control. Luciferase activity is shown in relative luciferase units (fold over pGL3-Basic).

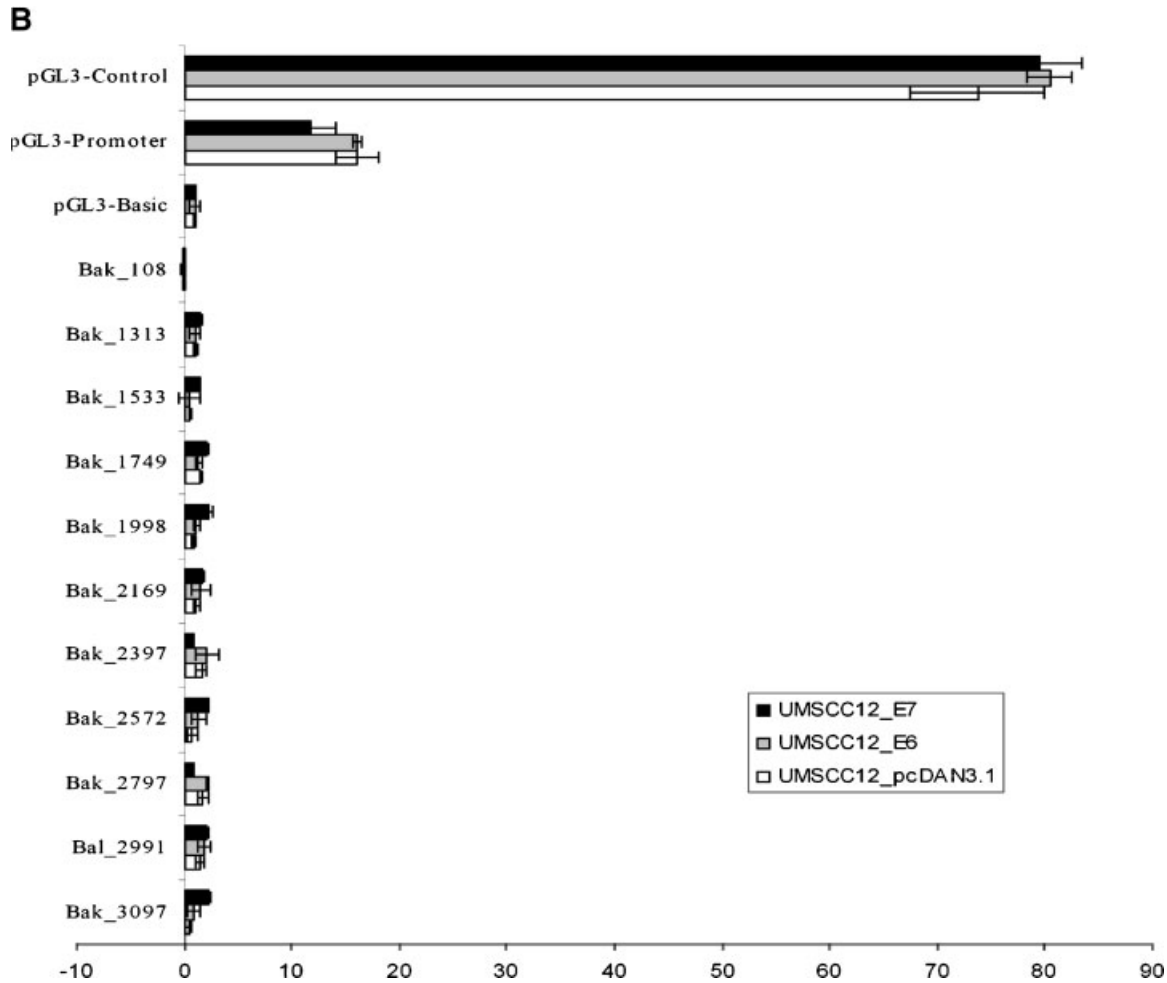

Fig. 9. (*Continued*)

E6- and E7-transfected cells may increase the level of the Bcl-2/Bax heterodimer, prohibit the activity of Bak, which ultimately protects E6- and E7-transfected laryngeal cancer cells from apoptosis [Haldar et al., 1997].

To further investigate whether HPV-16 E6 and E7 affects the stability of Bak and Bcl-2 proteins, we performed a protein stability assay and found that transfection with E6 or E7 stimulated Bak degradation in both transfected UMSCC11A and UMSCC12 cells, while the degradation of Bcl-2 protein was inhibited by the transfection of HPV-16 E6 in UMSCC12 cells, suggesting that HPV-16 E6 and/or E7 play a direct role in the modulation of the Bak and Bcl-2 proteins.

The mechanism responsible for the overexpression of Bcl-2 and the down-regulation of Bak and Bax in HPV-16-infected laryngeal cancer is still unclear. Few papers have investigated the relationship between HPV-16 E6 and Bak [Thomas and Banks, 1999; Du et al., 2004] or Bax [Magal et al., 2005]. There are two possible pathways responsible for the reduction of these pro-apoptotic factors. Firstly, HPV-16 E6 may degrade Bak [Thomas and Banks, 1999] or Bax [Magal et al., 2005] by direct binding. However, the mechanism by which the viral E6 interferes with the expression of Bak or Bax is still unknown. It has been hypothesized that E6 negatively controls the promoter region of Bak [Du et al., 2004] by directly blocking it or by interacting with other transcription factors, such as p300 and CCAAT/enhancer binding protein (C/REB) [Patel et al., 1999; Huang and McCance, 2002], c-IAP2 [Yuan et al., 2005] or nuclear factor-κB (NF-κB) [Nees et al., 2001]. However, the result of the Bak promoter luciferase assay performed in transfected UMSCC11A and UMSCC12 cells disproved

the hypothesis that we proposed in an earlier study [Du et al., 2004], suggesting that the degradation of HPV-16 E6 or/and E7 by Bak may be through a mechanism other than by interfering with the Bak promoter. Secondly, HPV-16 E6 causes p53 ubiquitination and degradation through the association with ubiquitin ligase E6AP, thereby abolishing p53-mediated apoptosis. Since Bax is identified as a p53 early response gene [Selvakumaran et al., 1994] and a unique p53-regulated gene [Zhan et al., 1994], abrogation of Bax may thus be caused by the prompt ubiquitination of p53 by HPV-16 E6. In addition, p53 may regulate apoptosis by directly promoting Bak activity [Moll et al., 2005]. Different statuses of p53 were observed in the two cell lines employed in our research. UMSCC12 cells have truncated p53 [Hauser et al., 2002; Bradford et al., 2003] with a non-sense mutation (condon 104, cag → tag), resulting in an early stop codon in exon 4 of the p53 gene while UMSCC11A cells have mutant but expressional p53 with a mis-sense mutation (condon 242, tgc → tcc; data not shown). Thus, it is highly unlikely that the abolishment of p53 by HPV-16 E6 is the main mechanism by which Bak or Bax protein is degraded in the model used in this study. Further experiments are needed to investigate whether HPV-16 E6 and E7 display different biological or molecular characteristics in laryngeal cancers with different p53 statuses.

To our knowledge, the relationship between HPV-16 E7 and Bcl-2 family members has not been studied before. This study demonstrated that the expression of Bcl-2 was enhanced by the transfection of HPV-16 E7 into laryngeal cancer cells, which indicates that the E7 viral oncoprotein may play an important role in the regulation of Bcl-2 expression. There are several possible mechanisms to explain how HPV-16 E7 leads to an increase in the level of Bcl-2. Firstly, an increase in Bcl-2 expression may be due to HPV-16 E7-mediated inactivation of pRb. This possibility is supported by the finding that the expression of Bcl-2 is suppressed by the activation of Rb protein [Huang et al., 2004]. It is also been shown that siRNA-mediated reduction of Rb expression inversely correlates with an increase in Bcl-2 expression [Huang et al., 2004]. Secondly, the presence of HPV-16 E7 in cancer cells tends to exert an anti-apoptotic effect [Puthenveettil et al., 1996; Iglesias et al., 1998; Stoppler et al., 1998]. The facilitation of p53 mutants by HPV-16 E7 may also contribute to the imbalance of Bcl-2 family members. Immortalization of human cells can be achieved by either HPV-16 E6 or HPV-16 E7 oncogenes, but occurs more efficiently when both oncogenes are present [zur Hausen, 2000]. The findings of the present study also support this concept since both HPV-16 E6 and E7 can decrease pro-apoptotic Bcl-2 family members and increase anti-apoptotic ones. Furthermore, in the present study, it appears that HPV-16 E6 is more powerful than HPV-16 E7 in down-regulating Bak and Bax expression. However, HPV-16 E7 displays a stronger ability to activate the expression of Bcl-2 protein.

Two human laryngeal cancer cell lines, UMSCC11A and UMSCC12 were used in the study. We have noted that the response of these two cell lines to apoptotic stimulation is different to some degree. The percentage of cell death in UMSCC11A measured by either MTT assay or annexin V/PI staining is higher than UMSCC12. Both of UMSCC11A and UMSCC12 cells were derived from male patients with laryngeal cancer though the former was at stage IV and the latter at stage III [Carey, 1994]. Except p53 (please refer above), other laboratory marker information is unavailable for these two cells. Therefore, it is difficult to speculate the mechanism responsible for the different sensitivity to cell death stimulation, which is also not the major focus of this study. Nevertheless, by comparing the apoptotic proteins tested in this study, we found that the expression of Bcl-2 was hardly detectable in UMSCC11A but it was clearly present in UMSCC12. Whether such difference in anti-apoptotic Bcl-2 protein level can contribute to the different sensitivity observed needs further investigation.

We have shown in this study that laryngeal cancer cells which express HPV-16 E6 or E7 are less apoptotic than cells which do not express HPV-16 E6 or E7. The reduction in apoptosis caused by HPV-16 E6 and E7 is associated with an overexpression of Bcl-2 protein and a reduction of Bak and Bax. Furthermore, laryngeal cancer cells infected with HPV-16 E6 or E7 genes show lower sensitivity to apoptotic stimuli than those without HPV-16 infection. The decrease of Bak or Bax and the increase of Bcl-2 may contribute to the inhibition of apoptosis. We have also shown that the inhibition of Bcl-2 degradation requires

HPV-16 E6 while the enhancement of Bcl-2 expression is mediated by HPV-16 E7. In addition, HPV-16 E6 and/or E7 does not alter the transcriptional activity of Bak, indicating that the degradation of Bak by E6 or E7 may be mediated through other mechanisms.

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