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IER3 is a crucial mediator of TAp73 β -induced apoptosis in cervical cancer and confers etoposide sensitivity

SUBJECT AREAS:

CELL DEATH

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Infection with high-risk human papillomaviruses (HPVs) causes cervical cancer. E6 oncoprotein, an HPV gene product, inactivates the major gatekeeper p53. In contrast, its isoform, TAp73 β , has become increasingly important, as it is resistant to E6. However, the intracellular signaling mechanisms that account for TAp73 β tumor suppressor activity in cervix are poorly understood. Here, we identified that *IER3* is a novel target gene of TAp73 β . In particular, TAp73 β exclusively transactivated *IER3* in cervical cancer cells, whereas p53 and TAp63 failed to do. *IER3* efficiently induced apoptosis, and its knockdown promoted survival of HeLa cells. In addition, TAp73 β -induced cell death, but not p53-induced cell death, was inhibited upon *IER3* silencing. Moreover, etoposide, a DNA-damaging chemotherapeutics, upregulated TAp73 β and *IER3* in a c-Abl tyrosine kinase-dependent manner, and the etoposide chemosensitivity of HeLa cells was largely determined by TAp73 β -induced *IER3*. Of interest, cervical carcinomas from patients express no observable levels of two proteins. Thus, our findings suggest that *IER3* is a putative tumor suppressor in the cervix, and the c-Abl/p73 β /*IER3* axis is a novel and crucial signaling pathway that confers etoposide chemosensitivity. Therefore, TAp73 β and *IER3* induction would be a valuable checkpoint for successful therapeutic intervention of cervical carcinoma patients.

Cervical cancer is one of the most common cancers and the second leading cause of cancer-related death in women worldwide¹. More than 99% of cervical cancer develops upon infection with human papilloma viruses (HPVs). Among over 120 HPVs, 15 of them are thought to cause cervical cancer, with HPV 16 and 18 being the two major types that account for more than 70% of all cases^{2,3}. Viral E6 and E7 are two critical oncoproteins responsible for cervical cancer development from high-risk HPVs and dysregulate cell proliferation, apoptosis, and genome instability⁴.

TP73 (p73) and p63 are homologues of the tumor suppressor p53, and they exhibit overlapping and unique roles⁵. Although p53 is the major cellular “gatekeeper” that inhibits tumor development, p53 is not functional in most of cervical cancers because E6 oncoprotein prevents p53 function by targeting p53 for degradation^{6,7}. Unlike with p53, HPV E6 protein does not physically interact with p73⁸, and the ectopic expression of TAp73 isoform efficiently inhibits the growth of E6-expressing HPV-positive cervical cancer cells^{9–11}. Two isoforms of p73, α and β , contain transactivation (TA) domains required for the transcriptional regulation of their target genes, which induce apoptosis and cell cycle arrest⁵.

Immediate early response gene 3 (IER3), also known as *IEX-1*, *Dif-2*, *gly96*, or *p22/PRG-1*, is an early response gene rapidly induced by a wide range of stimuli, including growth factors, cytokines, DNA-damage, and viral infection¹². *IER3* is an evolutionally conserved gene present in diverse species, including *Caenorhabditis elegans* and *Drosophila melanogaster*. In the human genome, *IER3* does not have other close homologues, and its sequence is extremely well conserved among mammals. *IER3* mRNA is widely expressed in most human tissues, with more abundant expression in epithelial tissues with high cell turnover¹³. Its transcription is regulated by p53, Sp1, c-Myc, and NF- κ B in a synergistic or opposing manner in different cells^{14–16}. *IER3* regulates multiple cellular processes, including apoptosis^{17–20}, proliferation^{21,22}, differentiation^{21,23}, and DNA repair²⁴, and its response varies depending on cellular context. Although the mechanism underlying the *IER3*-mediated induction of apoptosis is not clearly understood at present, its involvement with the BCL-2 family, which is the pivotal regulator of cell



survival and death, has been demonstrated; the presence of functional BIM is required for IER3-induced apoptosis. IER3 interacts with MCL-1, and IER3 inhibits the expression of BCL-2 and BCL-xL, respectively^{17,25}.

In the present study, we identified *IER3* as a specific transcriptional target gene of TAp73, but not p53, in cervical cancer cells. In addition, we demonstrated that IER3 is a critical mediator of TAp73 β -induced cell death in cervical carcinoma cells, and etoposide chemosensitivity of HeLa cells was largely governed by TAp73 β -induced IER3. Furthermore, we found that IER3 and TAp73 β expression levels were undetectable in cervical carcinoma tumors, implying that downregulation of these two proteins could be implicated in the development of cervical cancer.

Results

***IER3* is a specific transcriptional target gene of TAp73 β in cervical cancer cells.** To investigate transcriptional activities of the p53 family proteins p53, p63, and p73 on *IER3*, we generated a human *IER3* promoter construct (-1384 bp) possessing a previously known p53-binding element¹⁶ and performed luciferase reporter assays in different cell lines. Overexpressed TAp73 β specifically activated *IER3* gene transcription in a dose-dependent manner in human cervical cancer cells, including the HeLa, KB, Caski and SiHa cell lines, which express E6 or E7 oncoproteins from high-risk HPV types 18 or 16, whereas neither p53 nor TAp63 were able to stimulate *IER3* promoter activation (Figure 1A). Similar results were confirmed at the mRNA level of *IER3* as determined by a real-time PCR analysis (Supplementary Figure 1A). In contrast, we did not observe this specific regulation in other cell lines, including human embryo kidney (293T), colorectal carcinoma (HCT116 and SW480), and ovarian adenocarcinoma (SK-OV-3) cells (Figure 1A and Supplementary Figure S1B). In addition, knockdown of TAp73 β by small-interfering RNA (siRNA) resulted in 50% decreased *IER3* promoter activity and its mRNA level of the controls (Figure 1B and Supplementary Figure S1C). Endogenous expression of TAp73 α was not readily detected in HeLa cells by western blot analysis (Figure 1B), implying that TAp73 β but not TAp73 α may play a significant role in cervical carcinoma cells. In order to identify the TAp73 β -binding element in the *IER3* promoter, we constructed serially truncated *IER3* reporter plasmids as shown in Figure 1C. Luciferase reporter assay results showed that TAp73 β retained transcriptional activity with truncated forms (-754, -561, and -283 bp) of *IER3*, but no transactivation was observed using the shorter construct (-69 bp) or the construct in which the p53-binding element was deleted (Δ -246--218) (Figure 1C), indicating that TAp73 β binds to the p53 consensus motif upstream of the *IER3* promoter.

TAp73 β binds to the p53 consensus motif of the *IER3* promoter. To confirm the sequences of *IER3* required for TAp73 β binding, nuclear extracts of HeLa cells transfected with HA-tagged TAp73 β or p53 were prepared for EMSA. As shown in Figure 2A, incubation of the TAp73 β -overexpressing nuclear fraction with radiolabeled oligonucleotides corresponding to the p53 consensus element (-246--218) yielded a clear complex formation that disappeared upon the addition of extra cold probe (lanes 6 vs 7). The complex formation was also detectable without the TAp73 β overexpression (lanes 2 and 4), indicating that this DNA binding association is physiologically relevant. In contrast, binding of p53 to the *IER3* promoter sequences was not observed, in agreement with the luciferase reporter assays (Figure 2A). These differential binding patterns were further confirmed *in vivo* by ChIP assay. TAp73 β was strongly recruited of *IER3* promoter, whereas p53 did not show any significant enrichment of *IER3* DNA in HeLa cells (Figure 2B). On the contrary, 293T cells showed the opposite results, with p53 protein strongly recruited to the *IER3* promoter

compared to TAp73 β (Figure 2B), which supports the differential *IER3* promoter activations by TAp73 β and p53 observed in Figure 1A. Furthermore, the increased expression of IER3 protein and its mRNA induced by TAp73 β but not by p53 was also confirmed (Figure 2C and Supplementary Figure S1D), and depletion of TAp73 β indeed decreased the level of endogenous IER3 protein in HeLa cells (Figure 2D). Thus, these results indicate that TAp73 β specifically binds to the p53 binding element of the *IER3* promoter and modulates its transcription, but this does not happen with p53 in cervical cancer cells.

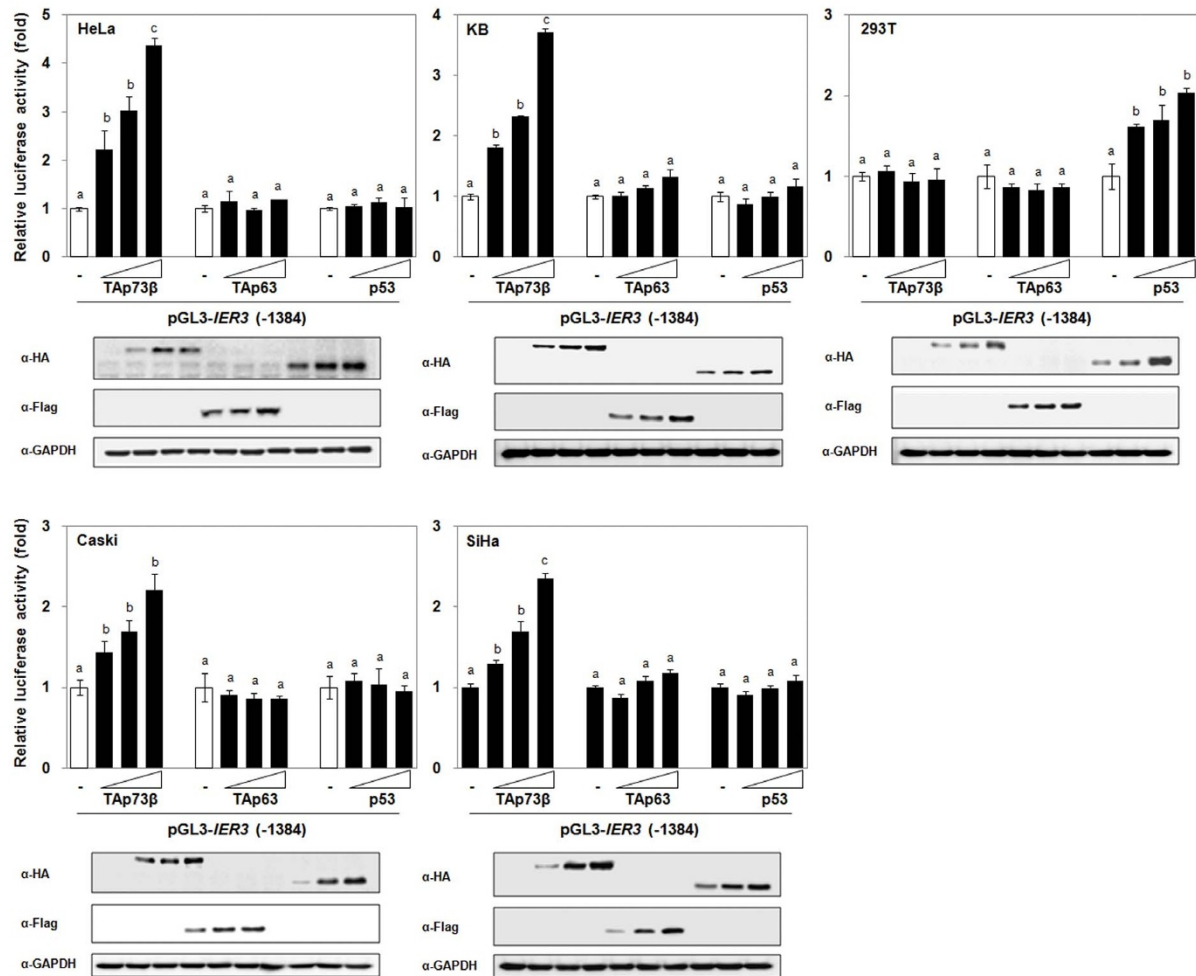
IER3 mediates TAp73 β -induced cell death. In order to determine the functional role of IER3 in cervical cancer cells, we assessed cell viability. Ectopic expression of IER3 promoted cell death (Figure 3A), while its knockdown enhanced survival of HeLa cells (Figure 3B). A similar trend was also observed in other HPV-positive cell lines including KB, Caski, and SiHa cells (Supplementary Figures S2A and S2B). Ectopic expression of TAp73 β or p53 efficiently induced death of HeLa cells to a similar extent (Figure 3C). However, TAp73 β -induced cell death was significantly compromised in IER3-silenced cells, while IER3 knockdown did not affect p53-mediated cell death (Figure 3C), suggesting that IER3 could be a specific mediator of cell death induced by TAp73 β , but not p53. In addition, the population of TAp73 β -induced Annexin V-positive apoptotic cells (Figure 3D) and TAp73 β -induced activation of Caspase 9, 7, and 3 (Figure 3E) were significantly reduced in IER3-depleted HeLa cells. In contrast, activation of Caspase 8 is not likely involved in this apoptotic signaling (Figure 3E).

TAp73 β -mediated IER3 upregulation is necessary for etoposide-induced cell death. Moreover, we treated HeLa cells using agents that may promote DNA damage-induced apoptosis and assessed the change in TAp73 β and p53 levels. In general, these agents, including UV, nocodazole, camptothecin, doxorubicin, etoposide, and cisplatin, upregulated TAp73 β or p53. However, only limited agents simultaneously upregulated both TAp73 β and IER3 in HeLa cells (Supplementary Figure S3A). In particular, well-known DNA damaging agents etoposide (200 μ M) and doxorubicin (2 μ M) significantly induced TAp73 β protein expression by 7–9-fold, but only etoposide concomitantly increased the level of IER3 (Figure 4A), implying a functional role of IER3 in mediating etoposide signaling. Indeed, the IER3 knockdown cells were significantly resistant to etoposide-induced cell death, while they remained sensitive to doxorubicin-induced killing (Figure 4B). Furthermore, concentration-dependent cell death in response to etoposide was greatly inhibited by silencing of either TAp73 β or IER3, whereas the silencing did not affect doxorubicin-induced cell death at all (Figures 4C and D). Etoposide-induced Annexin V-positive apoptotic cells were also decreased by silencing of either p73 β or IER3 (Supplementary Figure S3B), while the knockdown did not affect doxorubicin-induced apoptosis (Supplementary Figure S3C). IER3 induction after etoposide treatment is suggested to be mediated by TAp73 β because etoposide failed to increase IER3 expression in TAp73 β -depleted cells (Figure 4E). In addition, IER3 is likely a key downstream mediator that is required for etoposide-induced cell death, as the partially inhibited etoposide activity after TAp73 β depletion was fully recovered upon the knock-in of IER3 in the TAp73 β -silenced cells (Figure 4F). Consistent results were observed by flow cytometry analysis of Annexin V-positive apoptotic cells (Supplementary Figure S3D).

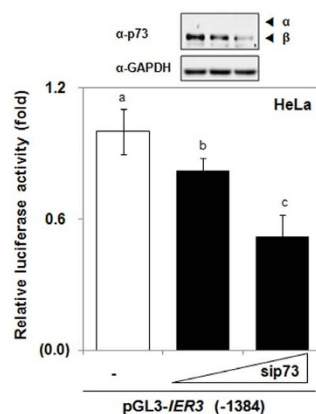
c-Abl-mediated stimulation of TAp73 β is critical for etoposide-mediated cell death. In order to elucidate how etoposide upregulates TAp73 β in cervical carcinoma cells, we assessed the change in TAp73 β mRNA level and found that its transcript level was not altered by etoposide treatment (Supplementary Figure S4A). Thus, next, we investigated whether etoposide-induced upregulation of



A



B



C

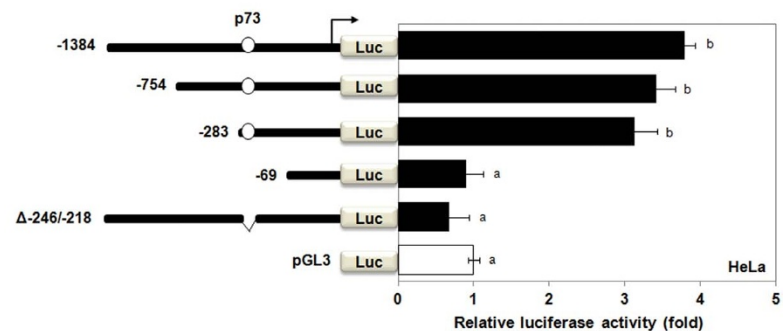


Figure 1 | *IER3* is a novel target gene of TAp73 β . (A) Promoter activation of *IER3* by p53 family proteins was determined by luciferase reporter assay after transfection with increasing amounts of plasmids (50, 100, or 200 ng) encoding HA-tagged TAp73 β , Flag-tagged TAp63, and HA-tagged p53 in HeLa, KB, Caski, SiHa and 293T cells. The luciferase activity was analyzed 24 h after transfection. Overexpression of TAp73 β , TAp63, p53 was confirmed by immunoblot analysis using anti-HA or anti-Flag antibodies. The full-blots membrane was cut into pieces according to estimated molecular weight of proteins of interest and probed with indicated antibodies. All cropped bolts have been run under the same experimental condition. (B) Reduced transcriptional activity of *IER3* in TAp73 β knockdown (100 and 200 nM) HeLa cells is presented. Efficient silencing of TAp73 β using its specific siRNA (100 or 200 nM) was confirmed by western blot analysis. GAPDH was used as a loading control. (C) The indicated truncated and deletion mutants of the *IER3* promoter constructs were cotransfected with HA-TAp73 β expression plasmid (200 ng) in HeLa cells. Cells were harvested for luciferase assays 24 h after transfection. All of results are expressed as the mean \pm SEM of three independent experiments performed in triplicate. Different letters denote statistically significant values ($p < 0.05$).

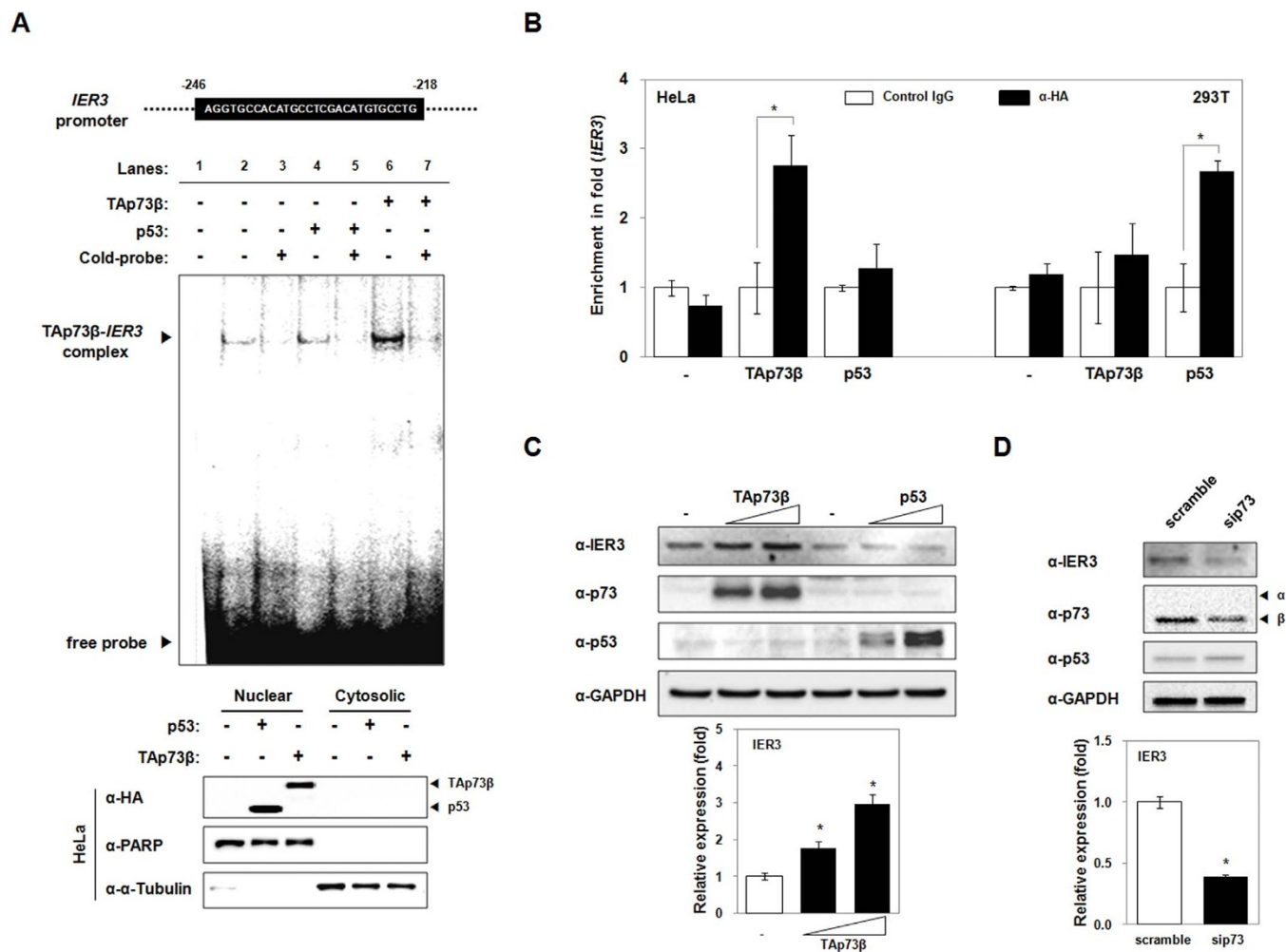


Figure 2 | TAp73 β binds to the *IER3* promoter and regulates *IER3* expression. (A) The sequences of the *IER3* probe encompassing the p53-binding element used to generate radiolabeled double-stranded oligonucleotides are shown. EMSA was performed using nuclear extracts (5 μ g) isolated from HeLa cells overexpressing HA-TAp73 β or -p53. No nuclear extract was incubated in lane 1. For cold probe, a 200 times excess of unlabeled oligonucleotides were used. Overexpression of HA-TAp73 β or -p53 proteins and efficient nuclear subcellular fractionation were determined by immunoblotting using the indicated antibodies. (B) Both HeLa and 293T cells were transfected with plasmids encoding HA-TAp73 β or -p53. The ChIP assay was performed using *IER3*-specific primers that targeted the p53-binding element, and quantitative real-time RT-PCR results are shown as enrichment in fold. As a negative control, control IgG was used for immunoprecipitation. Asterisks indicate significant values compared with the control, and the results are from three independent experiments run in duplicate ($p < 0.05$). HeLa cells were transfected with plasmids encoding HA-TAp73 β or -p53 (C) and sip73 (D) for 24 h, and cell lysates were prepared and immunoblotted with indicated antibodies. Quantitative analyses of the *IER3* protein levels induced by TAp73 β were shown as the means \pm SEM of three independent experiments (bottom panel). GAPDH was used as an equal loading control. (A, C, and D) These full-blots membrane was cut into pieces according to estimated molecular weight of proteins of interest and probed with indicated antibodies. All cropped bolts have been run under the same experimental condition.

TAp73 β involved tyrosine kinase c-Abl, which is known to stimulate p73 activity by increasing stability²⁶. Using a specific inhibitor of c-Abl, imatinib (STI571; Gleevec), alteration of etoposide-mediated TAp73 β protein level was determined. As shown in the Figure 5A, etoposide increased the phosphorylation of Tyr 99 residue of etoposide failed to increase the level of TAp73 β and *IER3* in the presence of imatinib, while imatinib had no effect on doxorubicin-mediated regulation of TAp73 β . This failure of etoposide to upregulate TAp73 β and *IER3* was also confirmed by knockdown of c-Abl using a siRNA specific to c-Abl (Supplementary Figure S4B). In addition, TAp73 β -mediated transcriptional activation of *IER3* was diminished upon c-Abl inhibition (Figure 5B). Similarly, etoposide-induced cell death was significantly inhibited by imatinib (Figure 5C) and c-Abl knockdown (Figure 5D), and the inhibitory action of imatinib on etoposide-mediated cell death induced was overcome by ectopic expression of either TAp73 β or *IER3* (Figure 5E and Supplementary Figure S4C). Thus, together these

results suggest that the activation of TAp73 β by c-Abl tyrosine kinase leads to the upregulation of *IER3* and is an important signaling axis in etoposide-induced apoptosis of HeLa cells.

Expression of TAp73 β and *IER3* is downregulated in cervical cancer patients. Since our molecular and cellular experimental results suggested that *IER3* induction by upregulated TAp73 β is crucial for apoptosis of cervical cancer cells, we examined the expression profiles of TAp73 β and *IER3* in HPV-infected cervical cancer patients. Western blot analysis of the epithelium isolated from cervical tissues of women free of cancer showed clear expression of both TAp73 β and *IER3* proteins with correlation coefficient of 0.83 (Figure 6A). In sharp contrast, the expression levels of p73 β and *IER3* proteins were extremely low or undetectable in cervical cancer tissues (Figure 6A). The expression of their mRNAs was also significantly downregulated in cervical cancer tissues (Figure 6B). In accordance, immunohistochemical analysis of normal and cervical carcinoma

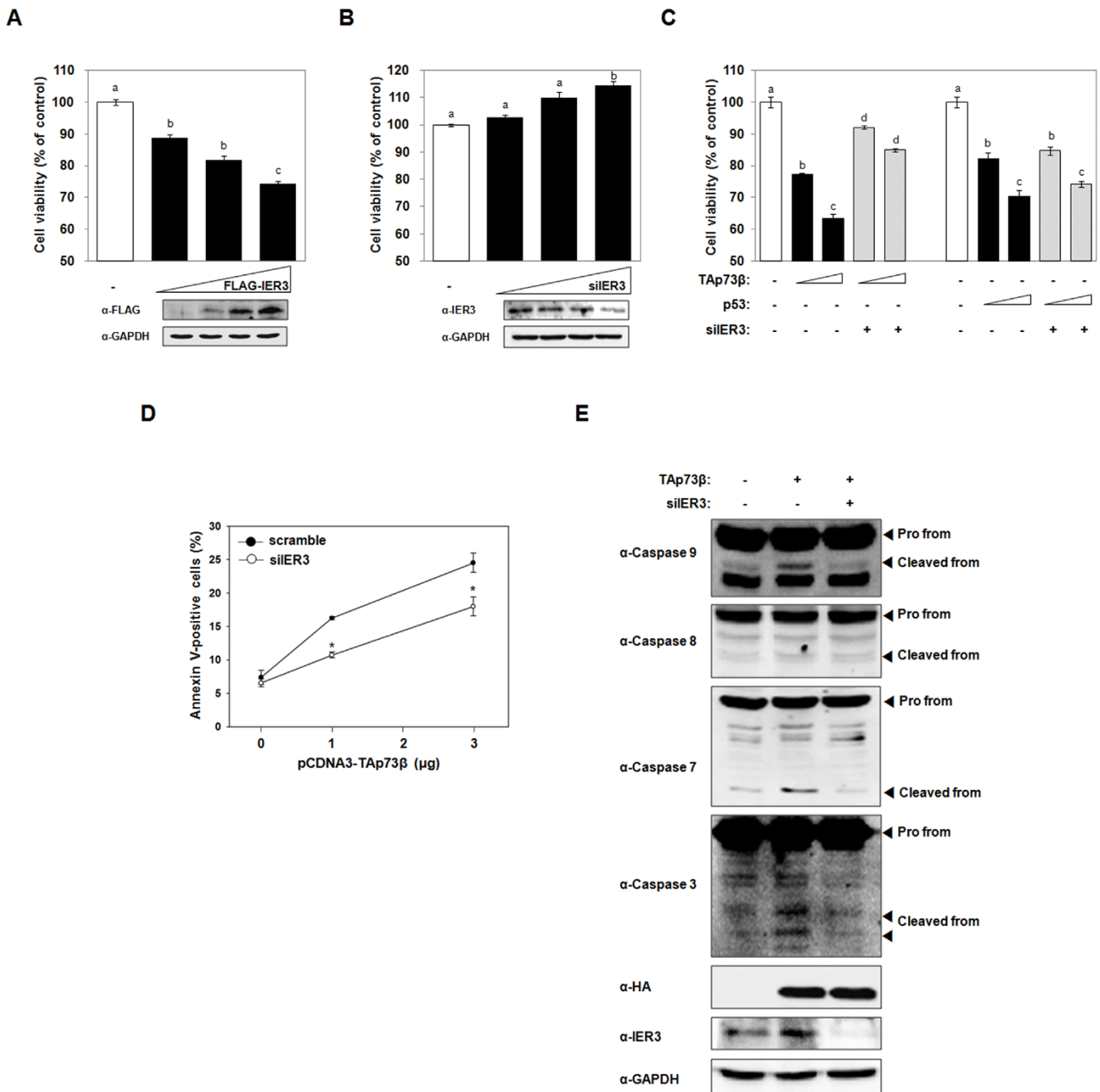


Figure 3 | IER3 is a mediator of TAp73β-induced apoptosis. HeLa cells were transfected with plasmids encoding FLAG-IER3 (10, 30, and 50 ng) (A), siRNA for IER3 (50, 100, and 200 nM) (B), and TAp73β- or p53-expression construct in the presence or absence of siIER3 (C), and cell viability was measured at 24 h after transfection. (D) The proportion of Annexin V-positive apoptotic cells was analyzed by flow cytometry after cotransfection with TAp73β and scrambled or IER3-specific siRNA. Results are expressed as the means \pm SEM of three independent experiments performed in triplicate. The statistically significant values are indicated with different letters or asterisks ($p < 0.05$). (E) HeLa cells were cotransfected with TAp73β and scrambled or IER3-specific siRNA and harvested 24 h after transfection. Activation of Caspase 3, 7, 8, and 9 was determined by western blot analysis using respective antibodies. (A, B, and E) These full-blots membrane was cut into pieces according to estimated molecular weight of proteins of interest and probed with indicated antibodies. All cropped bolts have been run under the same experimental condition.

tissues supported the western blot results, in which both proteins showed positive nuclear staining in normal cervical epithelium but not in cancer tissues (Figure 6C).

Discussion

The accumulation of p53 after DNA-damage has been considered an important step in inducing apoptosis and cell cycle arrest of various cancers upon chemotherapy and radiotherapy²⁷. However,

p53-based gene therapy to restore its expression often fails to inhibit cervical cancer¹⁰ because E6 oncoprotein, produced by high-risk HPVs, recruits E6-associated protein (E6AP), an E3 ligase, and forms a trimeric complex with p53, resulting in ubiquitination and proteosomal degradation of p53^{7,9,28}. In contrast, because p73 and E6 protein do not interact⁸, p73 is an important and promising molecule that may inhibit the growth of cervical cancer^{9–11,29–32}.

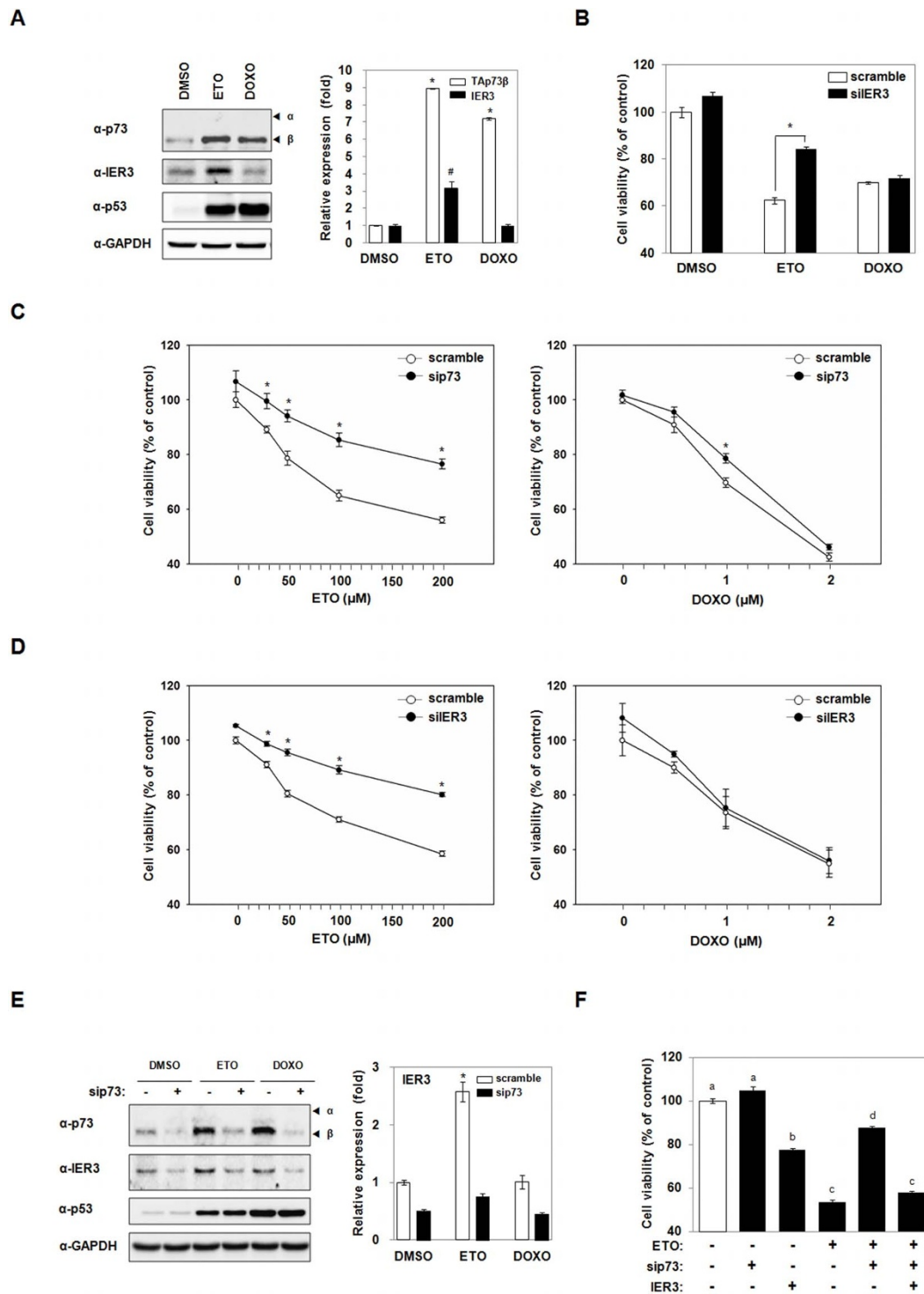


Figure 4 | Etoposide-induced apoptosis of cervical cancer cells is mediated by TAp73 β and IER3. (A) HeLa cells were incubated with 0.1% dimethylsulfoxide (DMSO), etoposide (ETO, 200 μ M), or doxorubicin (DOXO, 2 μ M) for 24 h. Representative immunoblot (left panel) and quantitative analysis of the TAp73 β and IER3 levels are shown (right panel). Symbols (* and #) indicate significant values compared with the respective solvent controls, and the results represent three independent experiments run in triplicate ($p < 0.05$). (B) The HeLa cells were transfected with scrambled or IER3-specific siRNAs, cells were treated with DMSO, ETO, or DOXO for 24 h, and their viability was measured. The HeLa cells were transfected with scrambled, p73-specific (C), or IER3-specific (D) siRNAs, they were incubated with increasing concentrations of ETO (left panel) or DOXO (right panel) for 24 h, and cell viability was measured. (E) HeLa cells were transfected with scrambled or p73-specific siRNA and then exposed to DMSO, ETO, or DOXO for 24 h. Using cell lysates, changes in the expression level of IER3 were determined by immunoblot analysis. Quantitative analysis of the IER3 levels is shown in the right panel ($n = 3$). (F) HeLa cells were transfected with the IER3-expressing plasmid and p73-specific siRNA as indicated and treated with DMSO or ETO, and their cellular viability was measured. All results are expressed as the mean \pm SEM of three independent experiments performed in triplicate. Statistically significant values are indicated with different letters or asterisks ($p < 0.05$). (A and E) These full-blots membrane was cut into pieces according to estimated molecular weight of proteins of interest and probed with indicated antibodies. All cropped bolts have been run under the same experimental condition.

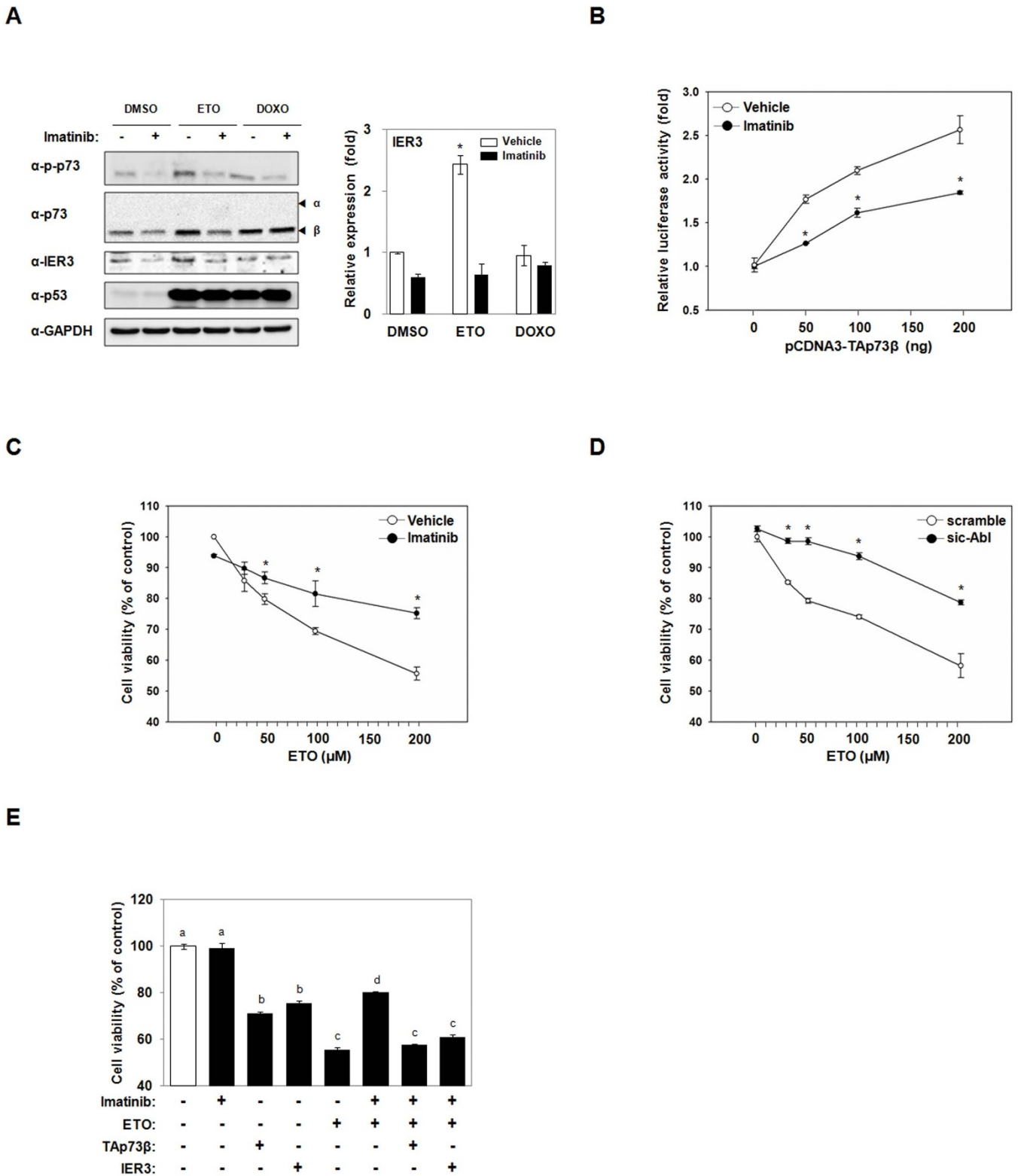
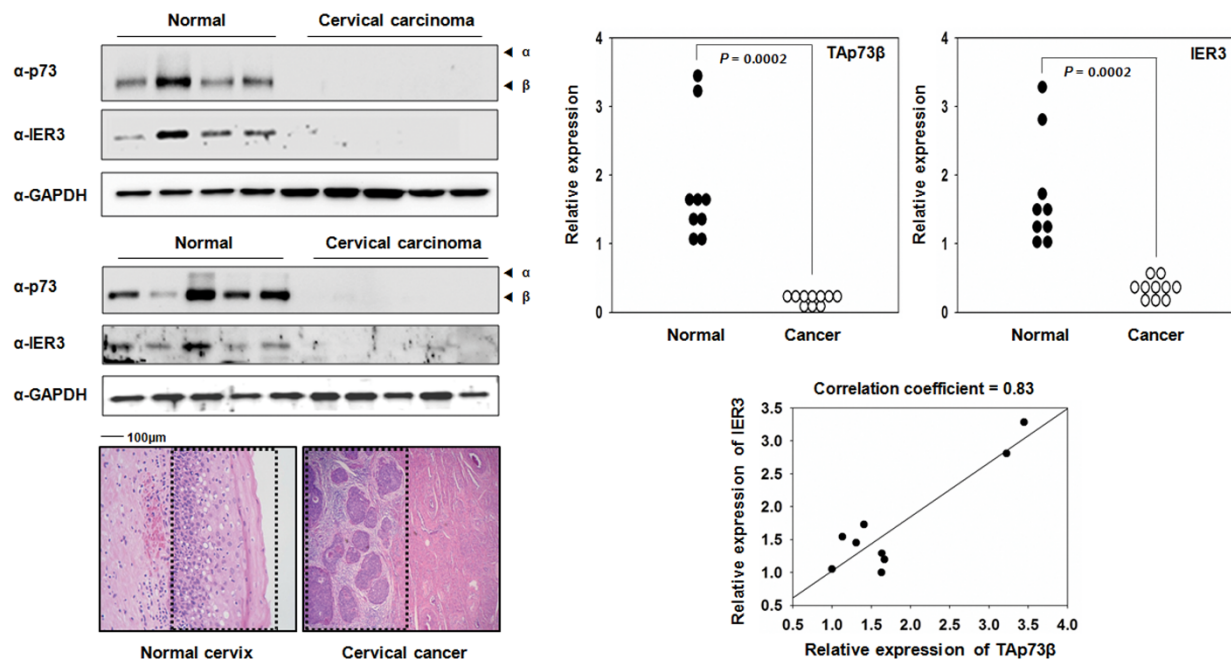


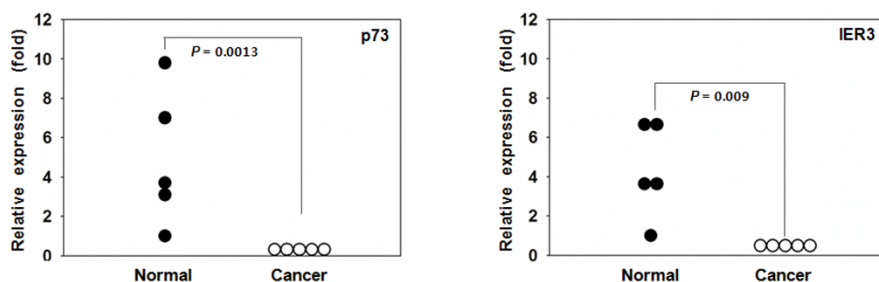
Figure 5 | Etoposide-induced upregulation of TAp73 β and IER3 is mediated by c-Abl. (A) HeLa cells were incubated with DMSO, ETO, or DOXO for 24 h in the presence or absence of imatinib (10 μ M). The changes in the level of TAp73 β and IER3 were shown by immunoblotting (left panel), and quantified results are presented in the right panel. The full-blots membrane was cut into pieces according to estimated molecular weight of proteins of interest and probed with indicated antibodies. All cropped bolts have been run under the same experimental condition. (B) HeLa cells were cotransfected with TAp73 β -encoding plasmid and IER3 promoter construct and incubated with or without imatinib for 24 h. Then, luciferase activity was measured using the cell lysates. (C) HeLa cells were incubated with increasing concentrations of ETO in the presence or absence of imatinib for 24 h, and cell viability was measured. (D) HeLa cells were transfected with scrambled or c-Abl siRNAs (200 nM). Following transfection for 12 h, the cells were incubated with ETO for 24 h, and cell viability was measured. (E) HeLa cells were transfected with IER3- and/or TAp73 β -expression plasmids with or without ETO and/or imatinib, and cell viability was measured after 24 h incubation. All results are expressed as the means \pm SEM of three independent experiments performed in triplicate. Statistically significant values are indicated with different letters or asterisks ($p < 0.05$).



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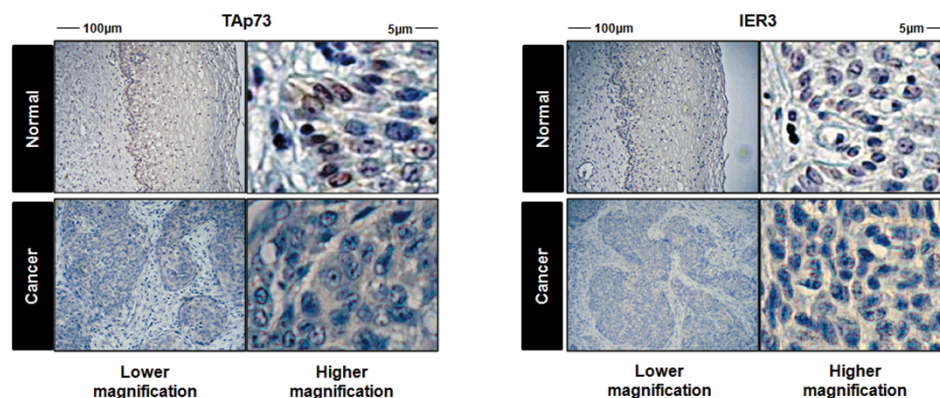


Figure 6 | Lack of the expression of TAp73 β and IER3 was observed in cervical carcinoma tissues. (A) Equal amounts of extracted protein from FFPE tissues of normal human cervical epithelium ($n = 9$) and cervical carcinoma tissues ($n = 10$) were subjected to SDS-PAGE for immunoblot analysis using indicated antibodies (upper panel). The full-blots membrane was cut into pieces according to estimated molecular weight of proteins of interest and probed with indicated antibodies. All cropped bolts have been run under the same experimental condition. Areas of cervical tissues scrapped for protein extraction are indicated with dotted lines (lower panel). The relative quantified expression of both p73 β and IER3 proteins between the control cervix and cervical cancer were compared. Estimated regression line superimposed on scatter plot of levels of p73 β and IER3 proteins in normal cervix is also presented with a correlation coefficient. (B) The total mRNAs from FFPE tissues of control human cervix ($n = 5$) and cervical carcinoma ($n = 5$) were extracted and used for a quantitative real-time PCR analysis of *p73* and *IER3*. (C) Representative immunohistochemical analyses of p73 and IER3 expression in control cervix and cervical cancer are shown.



Here, we identified IER3, for the first time, as a critical and specific mediator of TAp73 β function in HPV-positive human cervical cancer. TAp73 β associates with the *IER3* promoter at the known p53 consensus sequence and stimulates *IER3* transcription in cervical carcinoma cells (Figures 1 and 2). However, p53 exhibited no transcriptional activity on *IER3* in HPV-infected cervical cancer cells, including HeLa, KB, Caski and SiHa cells (Figures 1 and 2) even though the present and previous studies showed that p53 can transcriptionally regulate human *IER3* in other types of cells, such as the observed activation in human embryonic kidney (293T) and hepatoma (Hep3B) cell lines¹⁴ and repression in human colorectal carcinoma (HCT116 and SW480) and fibroblast cell lines (HaCaT)¹⁶ (Supplementary Figure S1B). The distinctive lack of p53 activity in cervical cancer cells reflects its failure to associate with the *IER3* promoter, as evident by EMSA and ChIP (Figures 2A and B). Understanding the mechanism underscoring this discriminative effect by TAp73 β and p53 in different cellular contexts requires further studies. However, other factors that associate with p53 in HPV-infected cervical cancer cells may possibly hinder its binding to the p53-responsive element in *IER3* sequences.

Differential transcriptional activities of the p73 protein on its target genes such as BAX, NOXA, and NIS have been observed in different cell types by others^{33–35}. In the present study, we have also found that TAp73 β distinctively regulates *IER3* in cervical cancer cells. This discriminatory effect of TAp73 β on *IER3* regulation in different cellular contexts that we have found in this study is likely a consequence of TAp73 β 's differential ability to associate with the *IER3* promoter, as TAp73 β failed to associate with the *IER3* promoter and was unable to stimulate transcription of *IER3* in 293T cells (Figures 1A and 2B). Although a detailed understanding of the underlying regulatory molecular mechanism responsible for the differential effects exerted by TAp73 β has yet to be achieved, we can speculate the potential involvement of distinct cellular factors, such as transcriptional co-activators of TAp73 that allow TAp73 access to its binding element in the *IER3* promoter.

At present, little information is available about how TAp73 β inhibits the growth of cervical cancer cells. In this study, we discovered that functional *IER3* is necessary and integral to TAp73 β -mediated apoptotic activity in cervical cancer cells (Figure 3). In addition, we obtained a striking result, that the expression levels of both TAp73 β and *IER3* proteins were undetectable in cervical carcinomas, whereas the two proteins were more abundantly expressed in the epithelium of normal human cervix (Figure 6A). To our knowledge, this is the first report that showed this striking difference in the level of TAp73 β and *IER3* proteins between cervical carcinoma and normal cervical epithelium by quantitative western blot analysis. Additionally, TAp73 β and *IER3* expression correlated, supporting the idea that *IER3* is probably a pathophysiologically relevant downstream target that acts as a mediator of TAp73 β -induced apoptosis in human cervix. In addition, the undetectable expression levels of TAp73 β and *IER3* proteins in cervical cancer patients implies that the two proteins likely act as tumor suppressors by preventing aberrant proliferation and can also serve as molecular markers to discriminate cervical carcinoma from other cancers. At present, the role of *IER3* in tumorigenesis is not clearly understood, and only limited studies have been performed that examine the expression levels of *IER3* in different types of cancer. The proposed role of *IER3* is even contradictory. It is increased in multiple myeloma and colorectal carcinoma, while decreased in ovarian carcinoma, and positive *IER3* expression is associated with better prognosis of pancreatic adenocarcinoma patients^{36–40}. This variable profile of *IER3* expression in cancer is likely reflective of contrasting functions of *IER3* in different cellular contexts, acting as a pro-apoptotic or an anti-apoptotic molecule.

DNA damage-induced upregulation of p73 and subsequent apoptosis of cancer cells in response to radiotherapy and chemotherapeu-

tics, including etoposide, doxorubicin, cisplatin, camptothecin, nocodazole, and taxol has been reported^{11,30,41–44}. In this study, we, for the first time, identified that TAp73 β -induced *IER3* expression is a crucial mediator of etoposide-induced death in cervical cancer cells (Figure 4). Etoposide is a widely used chemotherapeutic for many cancers, including cervical carcinoma, because of its ability to break DNA strands by forming a ternary complex with topoisomerase 2 and DNA, leading to the apoptosis of cancer cells^{45–49}. The TAp73 β -induced *IER3* expression specifically correlated with chemosensitivity to etoposide (Figure 4), suggesting that the ability of cervical cancer cells to upregulate TAp73 β and *IER3* proteins is a critical factor for responsiveness to etoposide. In addition, etoposide-induced apoptotic cell death was regulated by c-Abl tyrosine kinase signaling, as etoposide-mediated apoptosis and upregulation of TAp73 β protein was effectively inhibited by the c-Abl inhibitor imatinib or the c-Abl knockdown (Figure 5 and Supplementary Figure S5B). c-Abl is an important kinase that mediates apoptotic cell death and cell cycle arrest⁵⁰. When DNA damage has occurred, c-Abl is activated by its phosphorylation and phosphorylates p73 isoforms at Tyr 99²⁶, resulting in the stabilization of p73 proteins⁵¹. Therefore, c-Abl-mediated upregulation of TAp73 β and subsequent transactivation of *IER3* confers chemosensitivity of cervical cancer cells to etoposide. Of interest, our findings also suggest that the use of imatinib, which is also used to treat multiple cancers, should be avoided for cervical cancer patients who undergo etoposide chemotherapy, as it prevents etoposide-induced upregulation of TAp73 β and *IER3*.

In summary, we identified that, in cervical carcinoma cells, *IER3* was distinctively regulated by TAp73 β , unlike in other types of cells where p53 is the main regulator, and the c-Abl/TAp73/*IER3* signaling axis controlled etoposide-induced death of cervical cancer cells.

Methods

Cells culture and reagents. HeLa, SiHa and 293T cells were cultured in Dulbecco's modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. RPMI1640 was used to culture CaSki, SK-OV-3, HCT116, and SW480 cells. KB cells were cultured in RPMI1640 media that contain 25 mM HEPES and sodium bicarbonate. Cells were grown in an incubator at 37°C with 5% CO₂. Reagents used for cell culture were purchased from Caisson (Caisson, North Logan, UT, USA). The anti-p73 (558787, BD Biosciences, San Jose, CA, USA), anti-HA (H6908, Sigma-Aldrich, St. Louis, MO, USA), and anti- α -tubulin (LF-PA0146, AbFrontier Seoul, Korea) were purchased for experiments. The anti-p53 (sc-126), anti-*IER3* (sc-33171), anti-PARP (sc-74469), and anti-GAPDH (sc-25778) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The anti-Caspase 3 (9662), 8 (9746), 9 (9508), anti-FLAG (2368) and anti-p-p73 (4665) antibodies were purchased from Cell Signaling (Danvers, MA, USA). Caspase 7 antibody was purchased from NOVUS Biologicals (NB100-56529, Littleton, CO, USA). Etoposide, camptothecin, and cisplatin were purchased from Calbiochem (San Diego, CA, USA), and imatinib was obtained from Santa Cruz Biotechnology. Doxorubicin, nocodazole, sodium vanadate (Na₃VO₄), and sodium fluoride (NaF) were purchased from Sigma-Aldrich. Padoxol (taxol) was obtained from Shin Poong Pharm. Co (Seoul, Korea). The protease inhibitor cocktail was purchased from GenDEPOT (Barker, TX, USA).

Plasmid constructs. The human *IER3* promoter (-1384 – +74) was generated by recombinant PCR using extracted blood genomic DNA as a template and the following primers: p(-1384)-F (5'-GGGACGGCTCTCCTGAGCTCAAGT) with p(+75)-R (5'-GATGGTCATGGTCGGGTGGCA) and p(+75)-F (5'-TGCCACCCGACCATGACCATCATGGAAGACGCCAAAAACATA) with SphI-R (5'-ATCTCTGGCATGCGAGAATCT). The truncated p(-754), p(-283), p(-69) promoters of *IER3* were PCR amplified using following primers: -754-F (5'-GGGACGGCTGGGTGAGTATTGCAGCAGGAT) with SphI-R, -283-F (5'-GGGACGGCTCTGTGAGGGATCCTGTGGCT) with SphI-R, and -69-F (5'-GGGACGGCTTGGCGGAGGAGGATTAGAAG) with SphI-R. The deletion construct of the *IER3* promoter was generated by recombinant PCR using following primers: p(-1384)-F with p(-1384mut)-R (5'-CCGGGCTGCAGACCTGAG) and p(-1384mut)-F (5'-CCTCTCCAGGTCTGCAGC). The PCR products were digested with *MluI* and *SphI* (Enzymomics, Seoul, Korea) and ligated into the pGL3 basic vector (Clontech, Mountain View, CA, USA). The FLAG-tagged *IER3* expression plasmid was produced by PCR amplification using the following primers: *IER3*-F (5'-GCCTCCGGATCCATGGACTACAAAGACGACGACGACAAATGTCACTCTCGCAGC) and *IER3*-R (5'-GAAGCCGAATCTTAGAAGGCGCGCGGT). The PCR products were digested with *Bam*HI and *Xho*I (Enzymomics) and ligated into pcDNA3 (Invitrogen, Carlsbad, CA, USA). The pcDNA3 HA-tagged TAp73 β and p53 plasmids were generous gifts from Dr. Kyung Hee Choi (Chung-Ang



University, Seoul, Korea). Human TAp63 cDNA was purchased from Thermo Scientific (Rockford, IL, USA) and amplified using following primers: TAp63-F (5'-GACGGATCCATGGACTACAAGACGACGACGACAAAAATTTTGAACCTT-CACG) with TAp63-R (5'-GCTCGAGCGCGCTCACTCCCTCTCTTT-GA). The product was digested with *Bam*HI and *Not*I (Enzymomics) and cloned into pcDNA3.

Luciferase assay. 293T, KB and SiHa cells (5×10^4) and other cell lines (2×10^4) were transfected with 100 ng of *IER3*-luciferase reporter, 50 ng of pCMV β -galactosidase (Clontech), and indicated amounts of plasmids encoding TAp73 β , TAp63, or p53 using Neon transfection system (Invitrogen) or Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Cells were then incubated in 12 or 48-well plates containing medium for 24 h. Luciferase activity was assessed as previously described⁵². Absorbances were measured with the FlexStation3 Microplate Reader (Molecular Devices, Sunnyvale, CA, USA).

Subcellular fractionation. HeLa cells (1×10^6) were transfected with expression plasmids, and fractionation of nuclear and cytosolic compartments was performed as reported previously⁵³.

Electrophoretic mobility shift assay (EMSA). EMSA was performed as previously described⁵². Double-stranded oligonucleotides of human IER3 sense (5'-AGGTGCCACATGCCTCGACATGTGCCTG) and antisense (5'-CAGGCACATGTCGAGGCATGTGGCACCT) were annealed before use.

Chromatin immunoprecipitation (ChIP) analysis. ChIP assays were performed as previously described⁵². DNA was amplified using the following primer set flanking the TAp73 β binding element in the *IER3* promoter: forward (5'-CCTGTGAGG-GATCCTGTGGC) and reverse (5'-AGTGGGTGGAGACTTGACAT). Products were analyzed by quantitative real-time PCR.

Cell viability assay. HeLa cells (2×10^4) were transfected using the Neon system (Invitrogen). Cell viability was measured by CellTiter-Glo Assay (Promega, Madison, WI, USA), according to the manufacturer's instructions.

Flow cytometry analysis. Annexin V-positive apoptotic cells were detected as previously reported⁵⁴.

Immunoblot analysis. HeLa cells (1×10^6) were transfected with the indicated plasmids as well as small interference nucleotides (siRNAs). After a 24-h transfection, cell lysates were prepared and subjected to SDS-PAGE for immunoblotting with respective antibodies. The membranes were detected using a ChemiDocTM XRS+ System Imager (Bio-Rad Laboratories, Hercules, CA, USA), and the intensity of each band was quantified using Quantity One software (Bio-Rad Laboratories).

RNA interference. Small-interfering RNA (siRNA) target sequences against p73 and IER3 were 5'-CGGAUCCAGCAUGGACGU and 5'-CCAGCCAAAAGGC-UUCUCUUU, respectively. The control siRNA sequence used was 5'-CCUACGC-CACCAAUUUCGU. The sense and antisense oligonucleotides were annealed in the presence of Annealing Buffer (Bioneer, Daejeon, South Korea). A siRNA specific to c-Abl was purchased from Santa Cruz Biotechnology.

Human subjects and cervical tissues. Formalin-fixed paraffin-embedded (FFPE) block sections (15 μ m thick) of cervical tumors from 10 patients (mean age = 47.7) and control cervical tissues from 9 women (mean age = 49.2) diagnosed with uterine myoma were examined. The sections were reviewed by pathologists and obtained from the Bio-Resource Center at the Seoul Asan Medical Center. The present study was reviewed and approved by the Seoul Asan Medical Center Institutional Review Boards. Informed consent was obtained from all subjects participated in this study. The methods were carried out in accordance with the approved guidelines.

Protein extraction from human cervical tissues. Total proteins were extracted from the epithelial layer of normal cervical tissue and cervical cancer sections of FFPE tissue sections. The FFPE tissues were deparaffinized with xylene (Duksan, Ansan, Korea) at room temperature for 10 min three total times. Then, tissue protein extracts were retrieved and pelleted by centrifugation at $14,000 \times g$ for 2 min, and the supernatant was carefully removed. The deparaffinized tissue pellets were then rehydrated with a graded series of ethanol (EMD Millipore Corp, Billerica, MA, USA), 100% for 5 min and then two repeated minutes with 95%, 90%, 80%, and 70%. The rehydrated tissue sections were resuspended in extraction buffer (RIPA buffer with 2% SDS, 1 mM Na_3VO_4 , 10 mM NaF, and protease inhibitor cocktail). The samples were mixed by vortexing and boiled at 100°C for 20 min, followed by the incubation at 80°C in a heat block for 2 h. During the incubation, samples were briefly vortexed every 20 min. At the end of the incubation, protein extracts were collected by centrifugation ($14,000 \times g$) for 30 min at 4°C and then quantitated using a BCA protein assay kit (Pierce Chemicals Co, Rockford, IL, USA).

RNA extraction and real-time PCR analysis. Total RNAs from cervical tissues and other cell lines were isolated by the PureLinkTM FFPE total RNA isolation kit (Invitrogen) and TRIzol reagent (Invitrogen), following the manufacturer's instructions. The concentration and quality of RNA were determined with an ND-1000 spectrophotometer (NanoDrop, Waltham, MA, USA). Reverse-transcription to

cDNA was performed using the SuperScriptIII first-strand synthesis kit (Invitrogen). All cDNAs used in real-time PCR were normalized with GAPDH. Quantitative real-time PCRs were performed using an iQTM SYBR Green Supermix (Bio-Rad Laboratories). Gene expression was quantified by the delta-delta-CT method, and real-time PCRs were performed in a CFX-96TM thermal cycler and detection system (Bio-Rad Laboratories). The nucleotide sequences of primers used for real-time PCR (Bioneer) are: p73-F (5'-GACGAGACACGCTACTACCT) and p73-R (5'-CTGCCGATAGGAGTTCGACCA); IER3-F (5'-CAGCCGACGGTTCTCTAC) and IER3-R (5'-GATCTGGCAGAAGACGATGGT); GAPDH-F (5'-AGGGGCC-ATCCACAGTCTT) and GAPDH-R (5'-AGCCAAAAGGGTCATCATCTCT).

Haematoxylin and eosin staining and immunohistochemistry.

Immunohistochemistry was performed according to our previous study⁵⁵. The sections were incubated with anti-human p73 (1:50) or anti-human IER3 (1:50) in antibody diluent (Dako, Carpinteria, CA, USA) for 24 h at 4°C .

Statistical analysis. Multiple comparison analyses of values were performed with the Student-Newman-Keuls test using SAS version 9.2 (SAS Institute, Cary, NC, USA), and Student's *t*-test was used for comparisons with control. The data are presented as means \pm SEM, and $p < 0.05$ was considered to be statistically significant.

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Author contributions

H.J., D.S., T.K. and J.Y. prepared figures and analyzed the data. K.L. and J.B. supervised the project, analyzed the data, and wrote the paper.

Additional information

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