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Repression of HPV E6-activated RSV promoter activity by anti-cancer agents

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Abstract

Human papillomavirus E6 forms a complex with p53 tumor suppressor and E6-associated protein, leading to the degradation of p53 via the ubiquitination pathway, resulting in the oncogenesis of cervical carcinomas. Several viral and cellular gene promoters were shown to be transactivated by E6 oncogene. In this study, to understand the role of transcription activity of E6 related to cervical carcinogenesis, the effect of cervical cancer drugs on E6 induced transcription activity has been elucidated. Several viral promoter (RSV, CMV, SV40, and HIV)—luciferase reporter gene constructs, and eukaryotic E6 expression vector were prepared as an E6 transcription analysis system and an exogenous E6 protein source, respectively. It was shown that the promoters of RSV, SV40, and HIV, but not CMV, were transactivated by HPV 16 E6 in cervical cancer cell line. Several known cervical cancer drugs were investigated for their effects on transcription activity of E6 in SiHa stably transfected with E6-responsive promoters. Cervical cancer drugs consistently reduced luciferase activity, in transfectants with RSV-luc (SiHa/pRSV-luc, KCTC 0427BP) E6 mRNA also. Thus, in this study, we have demonstrated that the promoters of RSV, HIV, and SV40 were transactivated by E6 in cervical cancer cells. Three cervical cancer drugs downregulated RSV-luc transcription and E6 expression by a p53 independent pathway. RSV-luc promoter analysis system could be useful for understanding the role of transcription activity of E6 related to cervical cancer and also for screening drugs against cervical cancers caused by HPV infection.

Keywords: HPV 16 E6; Transcription activity; Viral promoter; Cervical cancer drugs

1. Introduction

Human papillomaviruses (HPVs) are small DNA viruses that have been etiologically linked to cervical cancer. HPVs are divided into two groups: low risk HPVs (HPV 6 and HPV 11), which cause benign squamous epithelial tumors such as warts and papillomas, and high risk HPVs (HPV 16 and HPV 18), which associate with malignant tumors (de Villiers, 1989; Desaintes et al., 1992; zur Hausen, 1988). Cervical cancer tissues that harbor HPV DNAs express two of the viral early genes, E6 and E7. Both E6 and E7 proteins of high risk HPV 16 and HPV 18 have transforming activities. The continuous expression of E6 and E7 is necessary for maintaining transformed status (Baker et al., 1987). Therefore, E6 and E7 could be targets for cervical cancer therapy. E6 protein of HPV 16 is responsible for cellular immortalization and transformation by functionally inactivating p53 tumor suppressor protein (Baker et al., 1990). The E6 protein by itself or in cooperation with HPV 16 E7 gene product can immortalize primary keratinocytes, fibroblasts, and epithelial cells (Halbert et al., 1991; Hawley-Nelson et al., 1989; Munger et al., 1989). The transcription activation of E6 is also implicated in its oncogenic functions. The HPV 16 E6 protein transactivates heterologous viral promoters, such as adenovirus E2 (AdE2), herpes simplex virus thymidine kinase gene (HSV-TK), human immunodeficiency virus long terminal repeat (HIV), adenovirus major late (Adml), and simian virus 40 (SV40) early promoters (Sedman et al., 1991; Shirasawa et al., 1994; Crook et al., 1991; Desaintes et al., 1992; Akutsu et al., 1996). In addition, E6 oncogene induced transactivation was observed from cellular promoters such as c-*fos* promoter, transforming

Abbreviations: HPV 16 E6, E6 of human papillomavirus type 16; RSV-luc, Rous sarcoma virus promoter-luciferase reporter gene

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growth factor-β 1 promoter, c-*myc* promoter, and the mouse fibronectin gene (Morosov et al., 1994; Desaintes et al., 1992; Dey et al., 1997; Kinoshita et al., 1997; Shino et al., 1997). The transcriptional repression activity of HPV 16 E6 has been reported for several promoters including the moloney murine leukemia virus LTR and cytomegalovirus immediate early promoter (Etscheid et al., 1994). However, most of this information was obtained from non-cervical cells giving no concrete information of a direct relationship of transcription activity with E6 and oncogenesis. In our study, we investigated transcription activity of several viral promoter–reporter by HPV 16 E6 and their applications for viral promoter–reporter gene systems could be available for the development of potential drugs against HPV infection.

2. Materials and methods

2.1. Preparation of mammalial expression vector for HPV 16 E6

E6 was prepared by PCR amplification from total RNA isolated from CaSki cell lines using following primer pairs: 5′-GCG GCC GCC ACC ATG TTT CAG GAC CAC AG-3′ (sense) and 5′-CTG CGG CCG CGA TTA CAG CTG GGT TTT CTC T-3′ (antisense). The PCR products were inserted into pBluescript KS(+) (Stratagene, La Jolla, CA) as described (Marchuk et al., 1990). E6 insert excised from pBluescript KS(+)/E6 was inserted into *Not*I site of pRc/CMV (Invitrogen, Carlsbad, CA). Then this vector was named as pRc/CMV 16 E6.

2.2. Construction of viral promoter–reporter analysis system

As promoter analysis systems, promoter–reporter gene constructs of luciferase and viral promoters such as simian virus 40 promoter, rouise sarcoma virus promoter, cytomegalo virus promoter, and human immunodeficiency virus promoter were constructed. SV40 promoter from pGVC vector (TOYO Inc., Japan) was inserted into *Xho*I–*Hin*dIII site of pGVB (TOYO Inc.). RSV promoter from pOP13CAT (Clonetech, Palo Alto, CA) was inserted into *Sma*I–*Bgl*II site of pGVB vector. CMV promoter from pBluescript KS(+)/CMV (Invitrogen) was inserted into *Sma*I–*Nhe*I site of pGVB vector. HIV promoter from pU3R III (Clonetech) was inserted into *Xho*I–*Hin*dIII site of pGVB vector. These vectors were named pSV40-luc, pRSV-luc, pCMV-luc and pHIV-luc.

2.3. Cervical cancer cell culture

Human cervical cancer cell lines, C-33A, CaSki and SiHa were from American Type Culture Collection (ATCC) (Rockville, MD). These cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum (FBS) (Gibco BRL, GrandIsland, NY). The cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air.

2.4. Transient transfection

Cells were transfected with calcium phosphate precipitated DNA. Briefly, 5 μg pRcCMV-16 E6 plasmid or control salmon sperm DNA (ssDNA) (POSCOCHEM R&D Center, South Korea) with 2 μg of the β-galactosidase expression plasmids (HIV-luc, SV40-luc, RSV-luc, and CMV-luc) were added to 250 ml of 0.25 M $CaCl_2$ and mixed with 250 ml of HBS-buffer. The precipitate was allowed to form for 30 min at room temperature, then 0.5 ml of precipitate was added dropwise to each monolayer. The cells were incubated for 16 h with the DNA, washed twice with 5 ml of Dulbecco's phosphate-buffered saline (D-PBS), and 5 ml of culture medium was added. After incubation for 24 h, the cells were harvested for measurement of luciferase and β-galactosidase activity in extracts of cells using ONPG from molecular cloning 16.66.

2.5. Establishment of stable transfectant

Stable cell lines expressing viral promoter transactivated by HPV 16 E6 were established. Transfection was performed using SuperFect™ (Qiagen, Germany) according to the manufacturer's instructions. Briefly, SiHa cells were seeded in 6 cm dishes to reach 70% confluence at 24 h. Medium was then removed, and complex of 30 μl SuperFect and 5 μg DNA (pRSV-luc plasmid) was directly transferred to the cells. After the plate was incubated for 2 h at 37 °C and 5% CO_2, medium containing the remaining complexes was removed, and replaced with fresh cell growth medium. Cells were allowed to incubate for 48 h followed by growing in selective DMEM medium containing G-418 (800 μg/ml) (Gibco BRL) for 2 weeks. G-418 resistant colonies were pooled for each cell population.

2.6. Treatment of anti-cancer agents

SiHa cells, stably transfected with viral promoter-luc expression plasmid, were treated with 10 μg/ml of anti-cancer agents such as carboplatin, mitomycin *c*, and cisplatin (Sigma, USA) for 18 h. Cell extracts were prepared from luciferase assay system according to the manufacture's instruction and luciferase activity was determined (Promega, Madison, MI) using Lumat LB 9501 (Berthoid).

2.7. Observation of cell morphology and cell proliferation assay for cell viability

Stable transfectant SiHa/pRSV-luc cells were plated in microtiter plates (tissue culture grade, 96 wells, flat bottomed) in a final volume of 100 μl/well culture medium in

a humidified atmosphere (e.g. 37 °C, 5% CO_2). The cells were treated with 10 μg/ml of anti-cancer agents such as carboplatin, mitomycin *c*, and cisplatin for 18 h. Cell morphology was observed using an inverted microscope (Zeiss Axiovert 25) and cell viability was determined using cell proliferation reagent WST-1 (Roche, Germany) according to the manufacture's instruction.

2.8. Northern blot analysis

Northern probe for E6 was labeled with random priming. Total cellular RNA was extracted from SiHa containing pRSV-luc plasmid (SiHa/pRSV-luc, Korea Culture Type Collection (KCTC; Yuseong, Taejon, South Korea), stock number 0427BP) after the treatment of anti-cancer drugs using the RNAzol B reagent (Tel-Test, Friendswood, TX, USA) according to the manufacturer's instruction. RNA samples (20 μg) were separated on 1.2% agarose/formaldehyde gels and transferred to nylon membranes. The filter was hybridized with radiolabeled E6 probe, washed and autoradiographed at −70 °C (Sambrook et al., 1989).

2.9. Western blot analysis

The level of expressed p53 protein was determined by SDS-polyacrylamide gel electrophoresis (PAGE; 12% acrylamide) and Western blot analysis. Gels were transferred to an Immobilon-P membrane (Millipore, Bedford, MA) at 50 V for 1.5 h at room temperature and were blocked by soaking into methanol for 5 min and drying at room temperature. The membrane was probed with goat anti-p53 monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:1000 in 3% skimmed milk, followed by an alkaline phosphatase-conjugated anti-goat antibody (Sigma), and visualization was achieved with NBT/BCIP substrate kit (Bio-Rad, Hercules, CA).

2.10. Statistical analysis

All data were expressed as mean ± S.E.M.. These data were analyzed by one-way ANOVA using SPSS/PC(+). Post hoc comparisons were assessed using Tukey's method (Grafen and Hails, 2002).

3. Results

Promoter–reporter gene constructs of luciferase and viral promoters such as pSV40-luc, pRSV-luc, pCMV-luc, and pHIV-luc plasmid were constructed (Fig. 1A–D). SV40 promoter with *Xho*I and *Hin*dIII site from pGVC vector was inserted into *Xho*I–*Hin*dIII site of pGVB. RSV promoter with *Bst*XI and *Bgl*II site from pOP13CAT was inserted into *Sma*I–*Bgl*II site of pGVB vector. CMV promoter with *Nsi*I and *Nhe*I site from pBluescript KS(+)/CMV was inserted into *Sma*I–*Nhe*I site of pGVB vector. HIV promoter

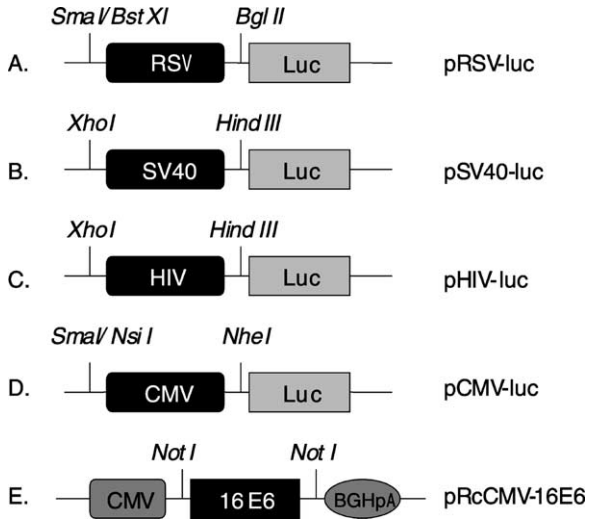

Fig. 1. Construction schemes for expression vectors of viral promoter–reporter gene and HPV 16 E6. (A) RSV promoter was inserted into *Sma*I–*Bgl*II site of luciferase gene expression vector, pGVB. (B) SV40 promoter was inserted into *Xho*I–*Hin*dIII site of pGVB vector. (C) HIV promoter was inserted into *Xho*I–*Hin*dIII site of pGVB vector. (D) CMV promoter was inserted into *Sma*I–*Nhe*I site of pGVB vector. (E) Amplified 16 E6 was inserted into pBluescript KS(+). E6 excised from pBluescript KS(+)/E6 was inserted into *Not*I site of pRc/CMV.

with *Xho*I and *Hin*dIII site from pU3R III was inserted into *Xho*I–*Hin*dIII site of pGVB vector. As shown in Fig. 1E, pRcCMV-16 E6 eukaryotic expression plasmid was constructed for exogenous E6 protein source.

3.1. The promoters of RSV, HIV, and SV40 but not CMV were transactivated by E6

C-33A cells, which do not harbor HPV genome, were cotransfected with several promoter-luciferase plasmids and the E6 expression plasmid. Five micrograms of cell extracts were used to monitor luciferase activity. The transcription activity of RSV promoter was only 18% upregulated after pRc/CMV-16 E6 cotransfection compared with salmon sperm DNA (ssDNA) cotransfection. The transcription activity of SV40 promoter and HIV promoter increased up to 70 and 87% after E6 cotransfection compared with ssDNA cotransfection, respectively. However, the transcription activity of CMV promoter was not altered after E6 cotransfection. These results indicated that RSV, HIV, and SV40 promoters but not CMV promoter were transactivated by E6 in cervical cancer cell line C-33A (Fig. 2).

3.2. The cell viability of SiHa/pRSV-luc cells was not affected after treatment with anti-cancer agents

Stable SiHa cells transfected with RSV-luc plasmid were prepared and named SiHa/pRSV-luc cells. These cells were used to monitor E6-mediated transcription activity of RSV promoter after treatment of anti-cancer drugs such as carboplatin, mitomycin *c*, and cisplatin. Before luciferase assay,

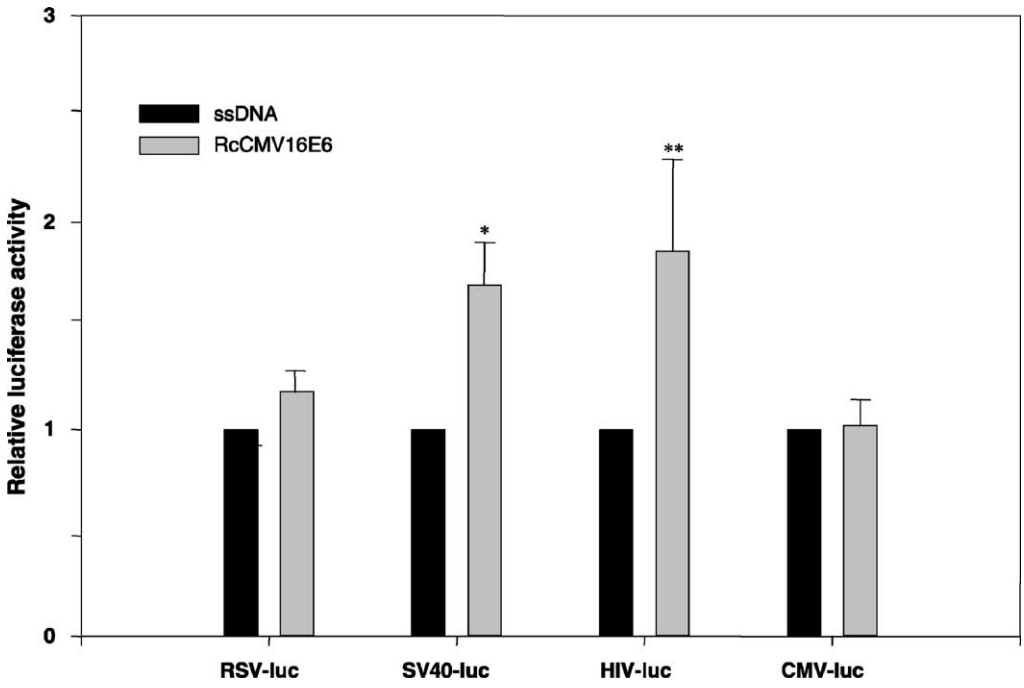

Fig. 2. Transcriptional activation of several heterologous promoters by the HPV 16 E6 protein in C-33A cells. C-33A cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 mg/ml streptomycin. The cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. On day 0, cells were transfected with calcium phosphate precipitated DNA. Briefly, 6 μg of pRcCMV-16 E6 plasmid or salmon sperm DNA with control, 2 μg of β-galactosidase expression plasmid (pCMVβ, Clonetech), each 2 μg of pHIV-luc, pSV40-luc, pRSV-luc, and pCMV-luc were added to 250 ml of 0.25 M $CaCl_2$ and mixed with 250 ml of HBS-buffer. The precipitate was allowed to form for 30 min at room temperature, after which 0.5 ml was added dropwise to each monolayer. The cells were incubated for 16 h with the DNA, then washed twice with 5 ml of (DPBS), and refilled with 5 ml of culture medium. After incubation for 24 h, the cells were harvested for measurement of luciferase and β-galactosidase activity. The relative transcriptional activities are mean values of at least three independent experiments. At least, data were represented as mean ± S.E.M. of three independent experiments performed in duplicate; $^{*}P < 0.05$ vs. untreated control and; $^{**}P < 0.005$ vs. untreated control (ANOVA followed by Tukey's test).

the cell viability using WST-1 reagent was elucidated. As shown in Fig. 3, the morphology and the viability of cells were not altered compared with untreated control after treatment of anti-cancer drugs.

3.3. E6-activated RSV promoter was repressed in SiHa/RSV-luc cells by anti-cancer agents

We analyzed the effect of anti-cancer drugs on luciferase gene expression in E6-activated RSV promoter in SiHa/RSV-luc cells. As shown in Fig. 4, the transcription activity of RSV promoter was reduced to about 35, 46, and 50%, respectively, after treatment of carboplatin, mitomycin *c*, and cisplatin compared to control (RLU degree of not treated control was equilibrated as 1.00). The transcription activities of both HIV promoter and CMV promoter were strongly reduced by mitomycin *c* and cisplatin whereas they were not affected by carboplatin compared with control (data not shown). These data indicated that putative cervical cancer drugs such as carboplatin, mitomycin *c*, and cisplatin showed different responses on the promoters of RSV, HIV and SV40. Our results suggest that all three anti-cancer drugs down-regulated the transcription activity of E6-activated RSV promoter.

3.4. E6 expression was down regulated in SiHa/pRSV-luc cells by anti-cancer agents

To investigate the effects of anti-cancer drugs on E6 expression of high-risk HPV type 16, the stable SiHa/pRSV-luc cells were exposed to the alkylating agent mitomycin *c*, DNA cross-linking agents cisplatin and carboplatin and the expressed E6 mRNA was analyzed by Northern blotting. As controls, total mRNAs were presented (Fig. 5A). As depicted in Fig. 5B, treatment with carboplatin and cisplatin resulted in a weak reduction of E6 expression whereas mitomycin *c* strongly reduced E6 transcript level (Fig. 5B). However, p53 expression was not significantly altered by treatment with these agents (Fig. 5C). These results indicate that repression of HPV 16 E6 expression is not necessary for the induction of the *p53* gene following genotoxic treatment of SiHa/pRSV-luc cells.

3.5. The p53 protein level was not changed in SiHa/pRSV-luc cells after treatment with anti-cancer agents

As shown in Fig. 5, p53 transcription levels after treatment with anti-cancer agents were not affected. The p53

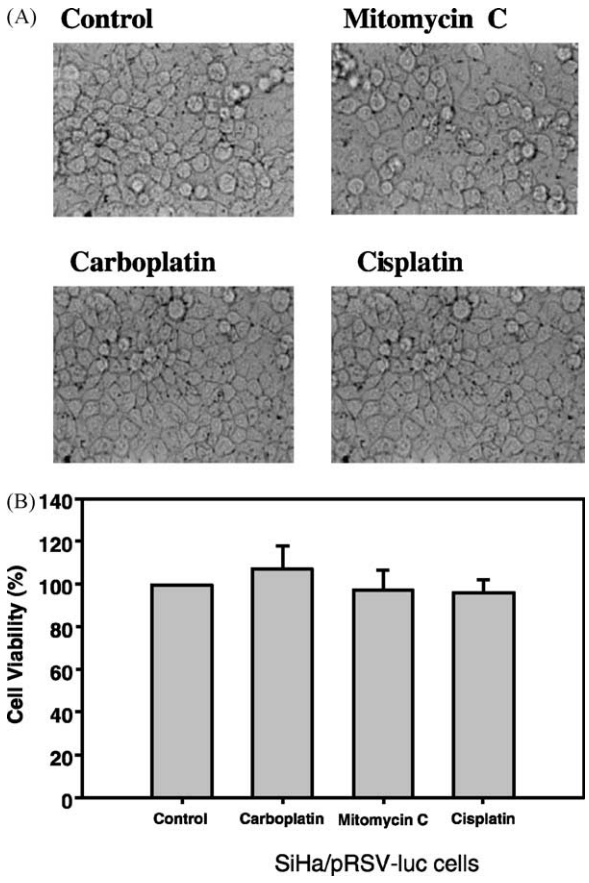

Fig. 3. Cell viability of SiHa/pRSV-luc cells after treatment of anti-cancer agents such as carboplatin, cisplatin, and mitomycin *c* for 18 h. (A) Cell morphology was observed using inverted microscope (200×). (B) Cell proliferation assay using cell proliferation reagent WST-1.

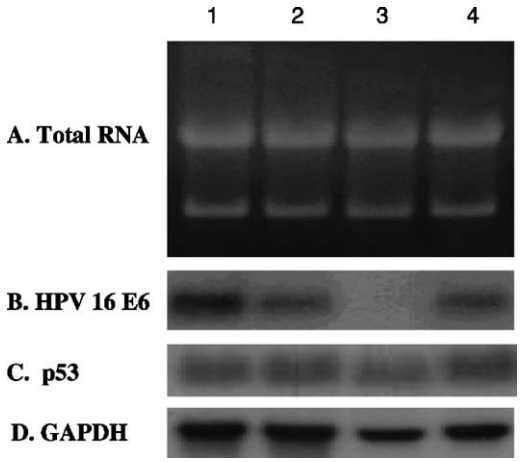

Fig. 5. Northern blot analysis of E6 mRNA expression. (A) Total mRNAs stained with EtBr. (B) The effects of anti-cancer drugs such as carboplatin, mitomycin *c*, and cisplatin on E6 mRNA expression. (C) p53 mRNA. (D) GAPDH. Lane 1, control; lane 2, treated with carboplatin; lane 3, treated with mitomycin *c*; lane 4, treated with cisplatin.

protein expression levels were detected using Western blot analysis. As control, C-33A cells were used in which mutated, but not functional p53 protein is expressed. As shown in Fig. 6B, p53 protein expression levels in SiHa/pRSV-luc cells were very low. However, p53 protein levels after treatment with the anti-cancer agents were not changed compared to untreated control cells. In these results, we suggest that the pathway on repression of E6-activated RSV promoter activity by anti-cancer drugs would be p53 independent.

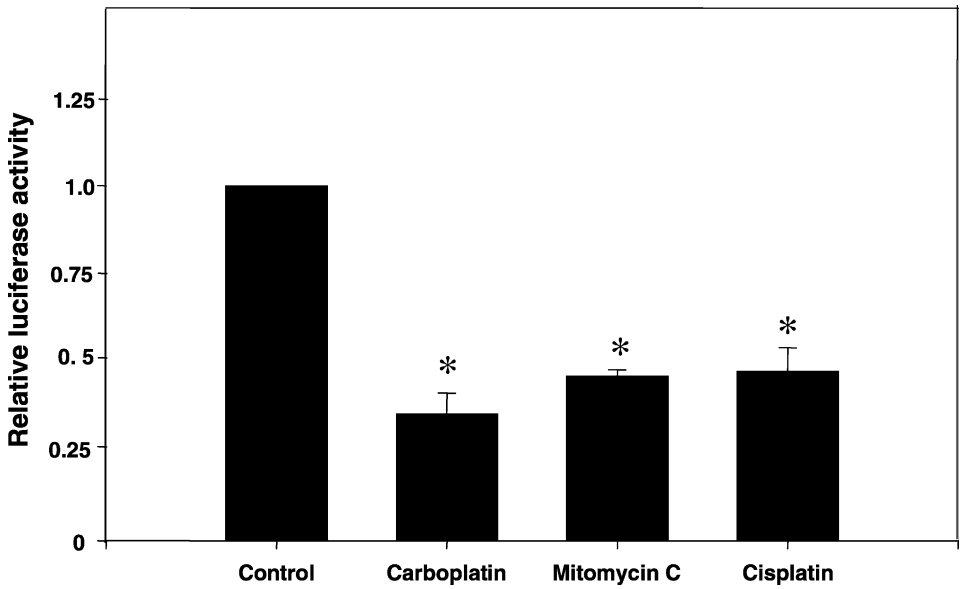

Fig. 4. Effects of anti-cancer agents on expression of luciferase gene under the E6-activated RSV promoter. Transfectant, SiHa cells containing pRSV-luc expression plasmid, were treated with 10 μg/ml of anti-cancer agents such as carboplatin, mitomycin *c*, and cisplatin for 18 h. Cell extracts were prepared and luciferase activity was performed with luciferase assay system (Promega). At least, data were represented as mean ± S.E.M. of three independent experiments performed in duplicate; $^{*}P < 0.001$ vs. untreated control (ANOVA followed by Tukey's test).

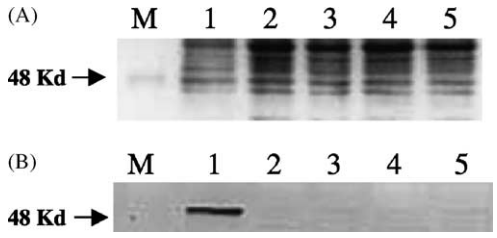

Fig. 6. Western blot analysis of p53 protein. The cell lysates were separated into pellets and supernatants by centrifugation at 12,000 rpm for 30 min. The supernatants were used for SDS-PAGE. (A) The Coomassie brilliant blue staining. (B) Western blotting of p53 protein. M, size marker; lane 1, C-33A cells harboring mutated p53 which is expressed but is not functional; lane 2, SiHa/pRSV-luc cells as control; lane 3, SiHa/pRSV-luc cells after treatment of carboplatin; lane 4, SiHa/pRSV-luc cells after treatment of mitomycin *c*; lane 5, SiHa/pRSV-luc cells after treatment of cisplatin.

4. Discussion

Many oncoproteins, including Jun, Fos, and Myc, mediate their growth promoting functions through transcription activation. Although E6 does not appear to be a sequence specific DNA-binding protein (Kanda et al., 1991; Mallon et al., 1987; Grossman et al., 1989), several reports have demonstrated that E6 from both high-risk HPVs and BPV-1 exhibits transcriptional activation. The low-risk viruses HPV 6 and HPV 11 E6 also have transactivation activity, indicating that transactivation by E6 is not sufficient for its oncogenic functions (Sedman et al., 1991; Crook et al., 1991; Morosov et al., 1994). In addition, transcriptional repression activity has also been reported for HPV 16 E6 (Shirasawa et al., 1994; Etscheid et al., 1994). In the present study, we showed that HPV 16 E6 activated transcription from RSV, HIV, and SV40 promoter, but not altered transcription from CMV promoter (Fig. 2). It has been reported that transcription activity of the cytomegalovirus mediates early promoter repression by HPV 16 E6 (Etscheid et al., 1994). Conflicting results exist regarding the regulation of p97 promoter by HPV 16 E6; Desaintes et al. reported activation of this promoter by HPV 16 E6 while Shirasawa et al. showed repression (Desaintes et al., 1992; Shirasawa et al., 1994). These differing activities appear to be due to experimental differences. The mechanism by which E6 modulates transcription is not well known. Some of these activities may not reflect the intrinsic transcriptional activation but rather an indirect effect. Modulation of the transcriptional activity of p53 could certainly account for some of these activities. Consistent with this notion, E6 from high-risk HPVs was shown to abrogate the transcriptional inhibitory effect of p53 on various promoters (Elbel et al., 1997; Sherman et al., 1997; Mietz et al., 1992; Hoppe-Seyler and Butz, 1993; Pim et al., 1994; Mansur et al., 1995; Thomas et al., 1995). E6 from high-risk HPVs was also shown to abrogate transcriptional activation by p53 (Kiyono et al., 1994; Mietz et al., 1992). Modulation of p53 transcriptional activity may be a component of the transcriptional regulatory activity of E6. However, p53-independent E6 transcriptional functions have been observed. In addition, several studies demonstrated E6-mediated transcriptional activation on p53-deficient cell lines (Akutsu et al., 1996; Kinoshita et al., 1997; Shino et al., 1997). It has been suggested that this is promoter specific: the AdE2 promoter was transactivated in both p53 positive and p53 negative cell lines, while the Rous sarcoma virus LTR was only transactivated in p53 containing cells (Akutsu et al., 1996). E6-mediated repression was also shown to be independent of p53 (Etscheid et al., 1994). As shown in Fig. 2, Rous sarcoma virus promoter was transactivated by HPV 16 E6 in C-33A cells. In this study, we suggest that RSV promoter is transactivated by HPV 16 E6 in mutant p53 containing cells. Our data showed that E6 mediated transactivation of RSV promoter was repressed by anti-cancer drugs such as carboplatin, mitomycin *c*, and cisplatin in p53 independent manner (Figs. 4–6). These data confirmed that E6-mediated repression was p53 independent (Etscheid et al., 1994). In this study, to understand the role of transcription activity of E6 related to cervical cancer oncogenesis and to utilize the obtained information for cancer therapy, several viral promoter-luciferase reporter gene and eukaryotic E6 or p53 expression plasmids were constructed and the regulation of E6 transcription activity by cancer drugs has been analyzed. The promoters of RSV, SV40, and HIV, but not CMV were transactivated by HPV 16 E6 in cervical cancer cell line, C-33A, in a p53 independent manner. Cancer drugs such as carboplatin, cisplatin and mitomycin *c* were tested for their effects on E6 transcription activity in cervical cancer cell line, SiHa, stably transfected with E6-responsive promoter-carrying plasmids. Treatment with three agents resulted in the repression of luciferase activity only in RSV-luc transfected cells. In this SiHa/RSV-luc cell line, E6 down regulation by anti-cancer drugs was also confirmed by Northern blot. This repression was also shown to be independent of p53. The RSV-luc promoter analysis system prepared in this study could be useful not only for understanding the role of transcription activity of E6 related to cervical cancer oncogenesis but also for the development of cervical cancer drugs based on the molecular oncogenesis studies.

References

Akutsu, N., Shirasawa, H., Asano, T., Isono, K., Simizu, B., 1996. p53-Dependent and p53-independent transactivation by the E6 protein of human papillomavirus type 16. J. Gen. Virol. 77, 459–463.

Baker, C.C., Phelps, W.C., Lindgren, V., Braun, M.J., Gonda, M.A., Howley, P.M., 1987. Structural and transcriptional analysis of human papillomavirus type 16 sequences in cervical carcioma cell lines. J. Virol. 61, 962–971.

Baker, S.J., Markowitz, S., Fearon, E.R., Willson, J.K.V., Vogelstein, B., 1990. Suppression of human colorectal carcinoma cell growth by wild type p53. Science 249, 912–915.

Crook, T., Tidy, J., Vousden, K., 1991. Degradation of p53 can be targeted by HPV E6 sequences distinct from those required for p53 binding and trans-activation. Cell 67, 547–556.

de Villiers, E.M., 1989. Heterogeneity of the human papillomavirus group. J. Virol. 63, 4898–4903.

Desaintes, C., Hallez, S., van Alpphen, P., Burny, A., 1992. Transcriptional activation of several heterologous promoters by the E6 protein of human papillomavirus type 16. J. Virol. 66 (1), 325–333.

Dey, A., Atcha, I.A., Bagchi, S., 1997. HPV 16 E6 onoprotein stimulates the transforming growth factor-beta 1 promoter in fibroblsts through a specific GC-rich sequence. Virology 228, 190–199.

Elbel, M., Carl, S., Spaderna, S., Iftner, T., 1997. A comparative analysis of the interactions of the E6 proteins from cutaneous and genital papillomaviruses with p53 and E6AP in correlation to their transforming potential. Virology 239, 132–149.

Etscheid, B.G., Foster, S.A., Galloway, D.A., 1994. The E6 in of human papillomavirus type 16 functions as a transcriptional repressor in a mechanism independent of tumor suppressor protein, p53. Virology 205, 583–585.

Grafen, A., Hails, R., 2002. Modern Statistics for the Life Sciences, 1st ed. Oxford University Press, New York, pp. 322–329.

Grossman, S.R., Mora, R., Laimins, L.A., 1989. Intracellular localization and DNA-binding properties of human papillomavirus type 16 E6 protein expressed with a baculovirus vector. J. Virol. 63, 366–374.

Halbert, C.L., Demers, G.W., Galloway, D.A., 1991. The E7 gene of human papillomavirus type 16 is sufficient for immortalization of human epithelial cells. J. Virol. 65, 473–478.

Hawley-Nelson, P., Vousden, K.H., Hubbert, N.I., Lowy, D.R., Schiller, J.T., 1989. HPV 16 E6 and E7 cooperate to immortalize human foreskin keratinocytes. EMBO J. 8, 3905–3910.

Hoppe-Seyler, F., Butz, K., 1993. Repression of endogenous p53 transactivation function in HeLa cervical carcinoma cells by human papillomavirus type 16 E6, human mdm-2 and mutant p53. J. Virol. 67, 3111–3117.

Kanda, T., Wantanabe, S., Zanma, S., Sato, H., Furuno, A., Yoshiike, K., 1991. Human papillomavirus type 16 E6 proteins with glycine substitution for cysteine in the metal-binding motif. Virology 185, 536–543.

Kinoshita, T., Shirasawa, H., Shino, Y., Moriya, H., Desbarats, L., Eilers, M., Simuzu, B., 1997. Transactivation of prothymosin alpha and c-*myc* promoters by human papillomavirus type 16 E6 protein. Virology 232, 53–61.

Kiyono, T., Hiraiwa, A., Shunsuke, I.T.T., Ishibashi, M., 1994. Inhibition of p53-mediated transactivation by E6 of type 1, but not type 5, 8, or 47, human papillomavirus of cutaneous origin. J. Virol. 68, 4656–4661.

Mallon, R.G., Wojciechowicz, D., Defendi, V., 1987. DNA-binding activity of papillomavirus proteins. J. Virol. 61, 1655–1660.

Mansur, C.P., Marcus, B., Dalal, S., Androphy, E.J., 1995. The domain of p53 required for binding HPV 16 E6 is separable from the degradation domain. Oncogene 10, 457–465.

Marchuk, D., Drumm, M., Saulino, A., Collins, F.S., 1990. Construction of T-vectors, a rapid and general system for direct cloning of unmodified PCR products. Nucleic Acids Res. 19, 1154.

Mietz, J.A., Unger, T., Huibregtse, J.M., Howly, P.M., 1992. The transcriptional transactivation function of wild-type p53 is inhibited by SV 40 large T-antigen and by HPV 16 E6 oncoprotein. EMBO J. 11, 5013–5020.

Morosov, A., Phelps, W.C., Raychaudhuri, P., 1994. Activation of the c-*fos* gene by the HPV 16 oncoproteins depends upon the cAMP-response element at $-60\ ^{\circ}$C. J. Biol. Chem. 269, 18434–18440.

Munger, K., Phelps, W.C., Bubb, V., Howley, P.M., Schlegel, R., 1989. The E6 and E7 genes of human papillomavirus type 16 together are necessary and sufficient for transformation of primary human keratinocytes. J. Virol. 63, 4417–4421.

Pim, D., Storey, A., Thomas, M., Massimi, P., Banks, L., 1994. Mutational analysis of HPV-18 E6 identifies domains required for p53 degradation in vitro, abolition of p53 transactivation in vivo and immortalization of primary BMK cells. Oncogene 9, 1869–1876.

Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular Cloning: A Laboratory Manual, 2nd ed.

Sedman, S.A., Barbosa, M.S., Vass, W.C., Hubbert, N.L., Hass, J.A., Lowy, D.R., Schiller, J.T., 1991. The full-length E6 protein of human papillomavirus type 16 has transforming and trans-activating activities and cooperates with E7 to immortalize keratinocytes in culture. J. Virol. 65, 4860–4866.

Sherman, L., Jackman, A., Itzhaki, H., Stoppler, M.C., Koval, D., Schlegel, R., 1997. Inhibition of serum- and calcium-induced differentiation of human keratinocytes by HPV 16 E6 oncoprotein: role of p53 inactivation. Virology 237, 296–306.

Shino, Y., Shirasawa, H., Kinoshita, T., Simuzu, B., 1997. Human papillomavirus type 16 E6 protein transcriptionally modulates fibronectin gene expression by induction of protein complexes binding to the cyclic AMP response element. J. Virol. 71, 4310–4318.

Shirasawa, H., Jin, M., Shimizu, K., Akutsu, N., Shino, Y., Simizu, B., 1994. Transcription-modulatory activity of full-length E6 and E6*I proteins of human papillomavirus type 16. Virology 203, 36–42.

Thomas, M., Massimi, P., Jenkins, J., Banks, L., 1995. HPV-18 E6 mediated inhibition of p53 DNA binding activity is independent of E6 induced degradation. Oncogene 10, 261–268.

zur Hausen, H., 1988. Papillomavirues in human cancers. Mol. Carcinogen. 1, 147–150.