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YY1 is a novel potential therapeutic target for treatment of HPV-infection induced cervical cancer by arsenic trioxide

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Abstract

Objective: YY1 is a zinc-finger transcription factor involved in regulation of cell growth, development, and differentiation. Although YY1 can regulate human papillomavirus type (HPV) viral oncogene E6 and E7, it remains unknown if YY1 plays a key role in carcinoma progression of HPV infected cells. Here we sought to determine whether YY1 is up-regulated in the cervical

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Conflict of interest

The authors declare no conflict of interest.

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cancer tissues and YY1 inhibition contributes to apoptosis of cervical cancer cells which is at least partly p53 dependent. Therefore, YY1 can be a potential therapeutic target for cervical cancer treatment by arsenic trioxide (As₂O₃).

Methods/materials: YY1 expression level was examined and analyzed by Western blot in pathologically confirmed primary cervical cancer samples, the adjacent normal samples as well as normal cervix samples. The effects of YY1 inhibition by specific siRNA in HeLa cells were determined by Western blot analysis of p53 level, cell growth curve, colony formation assay, and apoptosis. The contribution of YY1 to As₂O₃-induced p53 activation and apoptosis were also examined by Western blot and cell cycle analysis.

Results: Here we report that the YY1 expression level is significantly elevated in the primary cancer tissues. In HPV-positive HeLa cells, siRNA-mediated YY1 inhibition induced apoptosis and increased the expression of p53. Treatment of HeLa cells with As₂O₃, a known anti-cervical cancer agent, reduced both protein and mRNA levels of YY1 in HeLa cells. YY1 knockdown significantly further enhanced As₂O₃-induced apoptosis.

Conclusions: These results demonstrated that YY1 expression is up-regulated in cervical carcinomas and YY1 plays a critical role in the progression of HPV-positive cervical cancer. In addition, YY1 inhibition induces p53 activation and apoptosis in HPV-infected HeLa cells. Thus, YY1 is an As₂O₃ target and could serve as a potential drug sensitizer for anti-cervical cancer therapy.

Keywords

YY1; therapeutic target; cervical cancer; arsenic trioxide

Introduction

Cervical cancer is a common gynecologic and life-threatening cancer for women worldwide. Accumulating clinical evidence indicates that about 90% of cervical carcinomas are associated with HPV infection [1]. It has been shown that HPV infection is able to promote tumorigenesis in cervical epithelial cells through the functions of HPV-encoded viral oncoproteins, E6 and E7, which inactivate two key tumor suppressors p53 and Rb in mammalian cells respectively [2, 3]. The HPV-encoded E6 oncoprotein is shown to directly bind to p53 and to cause its degradation via E6-AP-mediated ubiquitination pathway [2-4]. Ectopic expression of HPV18 E6 can initiate carcinogenesis in normal human keratinocytes by inhibiting p53 function [5-9]. These results suggest that most cervical cancers harbor a normal p53 gene [10] but its function is likely inhibited due to HPV infection.

HPV E6 expression can be regulated by the transcription factor C/EBP β -YY1 (Yin Yang 1) complex in HeLa cells [11, 12]. Importantly, YY1 is shown to bind to the HPV-18 upstream regulatory region (URR) to regulate the transcription of viral oncogenes E6 and E7 [13, 14]. Also, YY1 is a ubiquitous and multifunctional zinc-finger transcription factor [15-17]. It plays a regulatory role in cell growth, development, and differentiation by affecting the levels of its target proteins including Cyclin D1, c-Myc, Rb, MDM2 and p53 [18]. YY1 is identified to negatively regulate p53 by enhancing the MDM2-p53 interaction, consequently inducing p53 ubiquitination and degradation. Therefore, once highly expressed, YY1 promotes cell transformation. [19, 20]. Moreover, over-expression of YY1 has been reported in acute myeloid leukemia (AML) [21], breast cancer [18], osteosarcomas [22], and prostate cancer [23]. However, it is unknown if YY1 plays a role in cervical carcinogenesis and progression.

Arsenic trioxide (As_2O_3) was firstly used as a therapeutic agent for treatment of acute promyelocytic leukemia (APL) in China [24] and reported to be effective even in the patients who were resistant to conventional chemotherapy [25, 26]. Recent studies by several groups suggested that As_2O_3 inhibits cell growth in solid cancer cell lines including HeLa cells with HPV-18 infection by activating p53 [27]. It is not clear how this anti-cancer agent activates p53 in HPV-positive cancer cells. We wondered if YY1 may play an essential role in As_2O_3 -mediated tumor inhibition function in HPV-positive cancer.

We thus proposed to study YY1 expression in clinical cervical cancers and the potential mechanism underlying YY1 mediated tumor progression in HPV-positive cervical cancer. In this study, we firstly detected the YY1 level in primary cervical cancer samples from patients in China and found that the expression of YY1 in the cervical cancers increased compared with their adjacent non-cancerous normal tissues as well as normal cervix samples. siRNA-mediated YY1 knockdown in HeLa cells induced apoptosis and As_2O_3 treatment with siRNA-mediated YY1 inhibition further enhanced As_2O_3 -induced apoptosis, indicating that YY1 is a potential anti-cancer target for treatment of cervical carcinoma.

Materials and Methods

Clinical Samples

Fresh cervical cancer tissues (n=12) and adjacent normal tissues (n=12) with 1.5 cm away from tumor were provided by Obstetrics & Gynecology Hospital of Fudan University and the Cancer Hospital of Fudan University. The fresh samples were immediately frozen and stored in liquid nitrogen and optimum cutting temperature (OCT) embedded blocks were cut into 5- μm thick sections. The frozen sections were fixed in 4% polyformaldehyde for 30 minutes and stained with hematoxylin and eosin (H&E staining) for the pathological diagnosis.

Another set of samples on a tissues microarray was provided by Chaoying Biotechnology Co., Ltd (P. R. China), which includes cervical cancer tissues (n=24), adjacent normal tissues (n=24), and normal cervix tissues (n=24). For immunostaining YY1 on tissues microarray, endogenous peroxidases were destroyed by immersing the sections in 3 % H_2O_2 for 10 min, washed twice in phosphate-buffered saline (PBS), and blocked in 5 % bovine serum albumin (BSA) in PBS for 15 min. The YY1 antibody (H-10, Santa Cruz Biotech, 1: 50) was added and incubated overnight at 4°C. After washing in PBS twice, biotinylated secondary antibody was applied followed by streptavidin peroxidase. Signal was detected using 3, 3'-diaminobenzidine chromogen (DAB/ H_2O_2) and slides were counterstained with haematoxylin. The signal strength was scored manually from a presentation photograph under 200x microscope using the following scale: 0, no signal above background; 1, weak signal; 2, moderate signal; and 3, strong to very strong signal. H-score [28] was calculated using the following formula: H-score = (% cells not stained $\times$ 0) + (% cells stained weak $\times$ 1) + (% cells stained moderate $\times$ 2) + (% cells stained strong $\times$ 3). The statistical analyses of H-score results were completed by using Graphpad Prism software. The significance of the data was calculated by a student's t-test.

Reagents and Antibodies

Antibodies against YY1 (H-10, H-414), p53 (DO-1) and β -actin (C4) were purchased from Santa Cruz (San Diego, CA.). Etoposide was purchased from Sigma (Sigma Chemicals, St Louis, MO.) and prepared in dimethylsulfoxide (DMSO) to 20mM stock solution. Arsenic trioxide (As_2O_3) was purchased from Sigma (Sigma Chemicals, St Louis, MO.). Horseradish peroxidase-conjugated anti-mouse and anti-rabbit whole IgGs were purchased from CalbioChem (USA). The stock solution of As_2O_3 was dissolved in 1 N NaOH and was

diluted to different concentrations in PBS for its cellular effect assay. siRNA was synthesized by Shanghai Genechem Co., Ltd. P. R. China. Lipofectamine 2000 was purchased from Invitrogen Life Technologies.

Western blot

Samples were lysed with 1 x SDS cell lysis buffer [125 mM Tris-HCl (pH6.8), 4% SDS (W/V), 20% glycerol, 50mM DTT, and 0.01% bromophenol blue]. Equal amounts (about 30 ug) of protein from samples extract were subjected to electrophoresis separation and were transferred onto a nitrocellulose membrane (Millipore Corp., Bedford, MA). Blots were blocked at room temperature for 1 hour in 5 % skim milk made up in Tris-buffered saline with Tween 20 (0.05 %) (TTBS), washed 3 times with TTBS for 5 minutes, and incubated overnight at 4°C with primary antibodies. Membranes were washed three times in TTBS for 5 minutes and then incubated for 1 hour with the Horseradish Peroxidase (HRP) conjugated secondary antibodies at room temperature. Blots were developed with ECL substrate (Santa Cruz Biotechnology sc-2048). Quantitative results of Western blot were analyzed with OPTIQUANT software.

Cell culture

HeLa cells (American Type Culture Collection, ATCC) were maintained in Dulbecco essential medium (GIBCO, USA) supplemented with 10% fetal bovine serum (FBS) (GIBCO, USA) and cultured in a 37°C humidified incubator under an atmosphere of 5 % carbon dioxide (CO₂) in the air.

RT-PCR analysis

Total RNA was isolated from cells using Trizol reagents as recommended by the manufacturer's instruction (Invitrogen). cDNA was amplified from 1 µg total RNAs using a SuperScript II Reverse Transcriptase (Invitrogen). PCRs were conducted with forward and reverse primers specific to *YY1*, or *ACTB* (β-actin) using Taq polymerase (Takara, Kyoto, Japan). PCRs were performed under the following conditions: denaturation at 94°C for 5 min, followed by 24-36 PCR cycles (denaturation at 95 °C for 45s, primer annealing and extension at 60 °C for 1 m). PCR primers used for detection of *YY1* and *ACTB* cDNA were as follows: *YY1*-s, 5'-AGAAGTGGGAGCAGAAGCAG-3'; *YY1*-as, 5'-AAACGAGATTACAGAGCAAG-3'; *ACTB*-s, 5'-TACCACTGGCATCGTGATGGAC-3'; *ACTB*-as, 5'-GATCTCCTTCTGCATCCTGTCTG-3'.

Results

YY1 is over-expressed in cervical cancer tissues but not in adjacent normal tissues

First, we examined the YY1 and p53 levels in paired clinical fresh samples (cervical carcinoma samples (n=12) and adjacent normal samples (n=12)) by using Western blot analysis (Fig. 1A). Quantitative results of Western blot were analyzed with OPTIQUANT software. Compared with paired adjacent normal samples, the YY1 protein level was elevated in 8 out of 12 cervical cancer samples (Fig. 1B). Surprisingly, the reduced p53 level was shown only in 3 out of 12 pairs of cancer tissues compared to adjacent normal tissues. This might be due to the balance result among the multiple regulators of p53 in the adjacent normal tissues.

Furthermore, immunostaining of the clinical samples on the tissues microarray with anti-YY1 antibody showed a significant increase of YY1 levels in cervical carcinoma samples compared to adjacent normal samples and normal cervix tissues. Cancer tissues also displayed a strong nuclear and cytoplasmic YY1 staining while no or weak staining was

observed in adjacent normal tissues and normal cervix tissues, as shown in the representative tissue sections (Fig. 1C). Interestingly, the increase of cell numbers in cervical cancer cells (Fig. 1D, left panel) was significantly correlated with the increase of the YY1 level (Fig. 1D, right panel). In paired comparisons (cell numbers: T vs. A, and T vs. N. YY1 staining percentage: T vs. A, and T vs. N.), a significant difference was shown between all groups ($p < 0.001$). These results indicate that YY1 is highly expressed in primary cervical carcinomas.

Knockdown of YY1 by siRNA induces apoptosis in HeLa cells

The fact that YY1 is up-regulated in cervical cancer tissues suggests that this protein might play a potential oncogenic role in cervical carcinogenesis. To test this possibility, we analyzed the effect of YY1 depletion by siRNA on cervical cancer cell proliferation. Since YY1 has been shown to contribute to the reduction of p53 level by both MDM2 [29-33] and HPV18 E6 [11, 12], we wanted to test if knockdown of YY1 by siRNA may affect p53 level and cell growth in HeLa cells. As shown in Fig. 3A top panel, the p53 protein level was elevated in HeLa cells at 72, 96, and 120 hours post-transfection of YY1 specific siRNA compared to scramble siRNA, while over-expression of YY1 led to the reduction of the p53 level in HeLa cells (Fig. 2A, bottom panel). Furthermore, YY1 siRNA repressed cell growth (Fig. 2B) and induced apoptosis on a time-dependent way (Fig. 2C). These results demonstrate that YY1 is important for HeLa cell proliferation, as its reduction induced p53 level and inhibited cell growth.

As₂O₃ inhibits proliferation of HeLa cells and reduces the levels of YY1

A previous report showed that treatment of As₂O₃ induces p53 levels and cleavage of poly (ADP)-ribose polymerase in a dose-dependent manner in HeLa cells [27]. Here we detected cell viability by MTS. Cells were plated in 96-well plates and cultured overnight to allow cell attachment. Cells were then treated with increasing As₂O₃ concentrations (0-16 μ M) for 24 hours and treated with 2 μ M As₂O₃ for increasing time (0-72 hours) respectively. All assays were done in triplicate. The results were shown by percentage of cell viability relative to the medium control. Thus, cell growth was found to decrease in both dose- (48% by 16 μ M at 24 hours) and time-dependent (37% by 2 μ M at 72 hours) manners (Fig. 3A). Also, cell cycle arrest and apoptosis induced by As₂O₃ treatment in HeLa cells was assessed. HeLa cells were seeded in 6-well plates and cultured overnight for attachment, then treated with increasing concentrations (0-4 μ M) of As₂O₃ for 48 hours. Cells were then collected and single stained by PI or double stained with annexin V-FITC and PI and analyzed by flow cytometry. As₂O₃ induced HeLa cell cycle G2/M arrest (9.87%-47.3%) and apoptosis (1.31%-31.92%) both in a dose-dependent manner (Fig. 3B).

Next, we tested if As₂O₃ treatment induces apoptosis by repressing YY1 function. As shown in Fig. 3C, indeed YY1 transcriptional activity was negatively regulated by As₂O₃ treatment, as measured by dual-luciferase report assays. Correspondingly, As₂O₃ treatment led to the reduction of YY1 mRNA expression (Fig. 3D). Using the strategy of increasing PCR cycles to amplify the YY1 with 10 times diluted templates, YY1 product was seen in 32 cycles in the control cDNA while 4 cycles later in As₂O₃ treated cells' cDNA. In line with this result, As₂O₃ treatment reduced YY1 protein level (Fig. 3E). These results indicate that As₂O₃ treatment down-regulates YY1.

Knockdown of YY1 sensitizes HeLa cells to As₂O₃ treatment

Since YY1 was reduced both by As₂O₃ treatment and knockdown by specific siRNA in HeLa cells, we wanted to test if YY1 knock-down was synergized through further As₂O₃ treatment. As shown in Fig. 4A, YY1 depletion also sensitized HeLa cells to As₂O₃ treatment. Additionally, YY1 depletion enhanced G2/M arrest of HeLa cells as analyzed by

flow cytometry (Fig. 4B). These results suggest that reduction of YY1 levels in HeLa cells render cells more vulnerable to As₂O₃-induced apoptosis and growth arrest at G2/M phase, implying that YY1 may serve as a potential therapeutic target for cervical carcinomas.

Discussion

The tumor suppressor p53 has been regarded as “the Guardian of the Genome” because of its ability to trigger the cell cycle arrest and apoptosis by transactivating the expression of its target genes that encode proteins important for DNA damage repair, cell cycle and apoptosis [34, 35]. In unstressed cells, p53 is tightly controlled by MDM2-mediated ubiquitination and 26S proteasomal degradation [34-36]. It was previously shown that the transcription factor YY1 functions as an MDM2 partner to enhance the p53/MDM2 interaction and consequent p53 ubiquitination and degradation [37, 38]. YY1 regulates the expression of numerous genes that are mostly involved in cancers [19-20]. Consistently, over-expression of YY1 has been reported in human cancers including AML, osteosarcomas, breast cancer and prostate cancer [21-23]. YY1 over-expression is correlated with higher malignancy both in prostate cancer and osteosarcoma. In AML, cancer was reported with poorly differentiated. Because YY1 can regulate multiple targets and is involved in various signaling pathways, the underlying mechanisms for each cancer type might be distinctive. In fact, the mechanisms of YY1 over-expression in most cancers still remain unknown. Since more than 50% cancers were reported with p53 mutations or inactivated wild type p53, it is indicated that p53 plays a major role in cells' immortalization and tumor formation in most cancers. Therefore, cervical cancer with low level wild type p53 might be correlative with over-expression of p53 negative regulators.

In cervical cancer, p53 ubiquitination and degradation are mediated by an HPV-E6/E6-AP complex instead of MDM2 [1, 4, 10]. However, a recent report showed that MDM2 was re-activated when HPV-E6 was inhibited [29, 32], indicating a potential role of MDM2 in the ubiquitination-mediated degradation of p53 in cervical cancer cells, partially because MDM2 regulates acetylation and deacetylation of p53 [30, 31]. Therefore, YY1 might play a critical role in cervical cancer development and is a potential treatment target.

Indeed, our study confirmed that YY1 is up-regulated in cervical cancer since YY1 protein level increase in 8 out of 12 primary cervical cancers. YY1 was more significantly elevated in cervical cancer samples on tissues microarray which was analyzed by immunohistochemical staining. Our results are consistent with a recent study showing that YY1 is highly expressed in cervical cancers [39]. Also, inhibition of YY1 by siRNA induced p53 activation and apoptosis in HeLa cells, which indicates that YY1 induced transformation of cervical cancer cells is at least partly p53 dependent,

Furthermore, our study showed that HeLa cell proliferation was inhibited (Fig. 3A) and cell cycle G2/M arrest as well as apoptosis (Fig. 3B) was induced under As₂O₃ treatment. In addition, YY1 is the target of As₂O₃ treatment since both YY1 protein and mRNA level significantly decreased in HeLa cells with As₂O₃ treatment. More dramatically, combination of YY1 knockdown with As₂O₃ treatment synergized the HeLa cells growth arrest and apoptosis.

Our current data suggest that YY1 is an effective therapeutic target, specifically for HPV-positive cervical carcinoma. The results imply that YY1 may play as a crucial role in HPV-associated cervical epithelial cell transformation and/or consequent neoplasia formation. Inhibition of YY1 further enhanced As₂O₃-induced apoptosis, suggesting a potential therapeutic combination in HPV-positive cervical cancer.

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References

1. Fanta BE. The distribution of Human Papilloma Virus infection in women with cervical histological abnormalities from an area with high incidence of cervical cancer. *Ethiop Med J.* 2005; 43:151–8. [PubMed: 16370546]
2. Scheffner M, Huibregtse JM, Vierstra RD, Howley PM. The HPV-16 E6 and E6-AP complex functions as a ubiquitin-protein ligase in the ubiquitination of p53. *Cell.* 1993; 75:495–505. [PubMed: 8221889]
3. Scheffner M, Werness BA, Huibregtse JM, et al. The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. *Cell.* 1990; 63:1129–36. [PubMed: 2175676]
4. Hengstermann A, Linares LK, Ciechanover A, et al. Complete switch from Mdm2 to human papillomavirus E6-mediated degradation of p53 in cervical cancer cells. *Proc Natl Acad Sci U S A.* 2001; 98:1218–23. [PubMed: 11158620]
5. Chen X, Shen B, Xia L, et al. Activation of nuclear factor kappaB in radioresistance of TP53-inactive human keratinocytes. *Cancer Res.* 2002; 62:1213–21. [PubMed: 11861406]
6. Hawley-Nelson P, Vousden KH, Hubbert NL, et al. HPV16 E6 and E7 proteins cooperate to immortalize human foreskin keratinocytes. *EMBO J.* 1989; 8:3905–10. [PubMed: 2555178]
7. Li JJ, Cao Y, Young MR, Colburn NH. Induced expression of dominant-negative c-jun downregulates NFkappaB and AP-1 target genes and suppresses tumor phenotype in human keratinocytes. *Mol Carcinog.* 2000; 29:159–69. [PubMed: 11108661]
8. Li JJ, Rhim JS, Schlegel R, et al. Expression of dominant negative Jun inhibits elevated AP-1 and NF-kappaB transactivation and suppresses anchorage independent growth of HPV immortalized human keratinocytes. *Oncogene.* 1998; 16:2711–21. [PubMed: 9652737]
9. Pei XF, Meck JM, Greenhalgh D, Schlegel R. Cotransfection of HPV-18 and v-fos DNA induces tumorigenicity of primary human keratinocytes. *Virology.* 1993; 196:855–60. [PubMed: 8396813]
10. Butz K, Ristriani T, Hengstermann A, et al. siRNA targeting of the viral E6 oncogene efficiently kills human papillomavirus-positive cancer cells. *Oncogene.* 2003; 22:5938–45. [PubMed: 12955072]
11. Bauknecht T, Jundt F, Herr I, et al. A switch region determines the cell type-specific positive or negative action of YY1 on the activity of the human papillomavirus type 18 promoter. *J Virol.* 1995; 69:1–12. [PubMed: 7983700]
12. Bauknecht T, See RH, Shi Y. A novel C/EBP beta-YY1 complex controls the cell-type-specific activity of the human papillomavirus type 18 upstream regulatory region. *J Virol.* 1996; 70:7695–705. [PubMed: 8892890]
13. Bauknecht T, Shi Y. Overexpression of C/EBPbeta represses human papillomavirus type 18 upstream regulatory region activity in HeLa cells by interfering with the binding of TATA-binding protein. *J Virol.* 1998; 72:2113–24. [PubMed: 9499067]
14. Dong XP, Pfister H. Overlapping YY1- and aberrant SP1-binding sites proximal to the early promoter of human papillomavirus type 16. *J Gen Virol.* 1999; 80(Pt 8):2097–101. [PubMed: 10466808]
15. Flanagan JR, Becker KG, Ennist DL, et al. Cloning of a negative transcription factor that binds to the upstream conserved region of Moloney murine leukemia virus. *Mol Cell Biol.* 1992; 12:38–44. [PubMed: 1309593]

16. Hariharan N, Kelley DE, Perry RP. Delta, a transcription factor that binds to downstream elements in several polymerase II promoters, is a functionally versatile zinc finger protein. *Proc Natl Acad Sci U S A*. 1991; 88:9799–803. [PubMed: 1946404]
17. Shi Y, Lee JS, Galvin KM. Everything you have ever wanted to know about Yin Yang 1. *Biochim Biophys Acta*. 1997; 1332:F49–66. [PubMed: 9141463]
18. Begon DY, Delacroix L, Vernimmen D, et al. Yin Yang 1 cooperates with activator protein 2 to stimulate ERBB2 gene expression in mammary cancer cells. *J Biol Chem*. 2005; 280:24428–34. [PubMed: 15870067]
19. Park K, Atchison ML. Isolation of a candidate repressor/activator, NF-E1 (YY-1, delta), that binds to the immunoglobulin kappa 3' enhancer and the immunoglobulin heavy-chain mu E1 site. *Proc Natl Acad Sci U S A*. 1991; 88:9804–8. [PubMed: 1946405]
20. Shi Y, Seto E, Chang LS, Shenk T. Transcriptional repression by YY1, a human GLI-Kruppel-related protein, and relief of repression by adenovirus E1A protein. *Cell*. 1991; 67:377–88. [PubMed: 1655281]
21. Erkeland SJ, Valkhof M, Heijmans-Antonissen C, et al. The gene encoding the transcriptional regulator Yin Yang 1 (YY1) is a myeloid transforming gene interfering with neutrophilic differentiation. *Blood*. 2003; 101:1111–7. [PubMed: 12393438]
22. de Nigris F, Botti C, de Chiara A, et al. Expression of transcription factor Yin Yang 1 in human osteosarcomas. *Eur J Cancer*. 2006; 42:2420–4. [PubMed: 16962318]
23. Seligson D, Horvath S, Huerta-Yepez S, et al. Expression of transcription factor Yin Yang 1 in prostate cancer. *Int J Oncol*. 2005; 27:131–41. [PubMed: 15942652]
24. Shen ZX, Chen GQ, Ni JH, et al. Use of arsenic trioxide (As₂O₃) in the treatment of acute promyelocytic leukemia (APL): II. Clinical efficacy and pharmacokinetics in relapsed patients. *Blood*. 1997; 89:3354–60. [PubMed: 9129042]
25. Shen Y, Shen ZX, Yan H, et al. Studies on the clinical efficacy and pharmacokinetics of low-dose arsenic trioxide in the treatment of relapsed acute promyelocytic leukemia: a comparison with conventional dosage. *Leukemia*. 2001; 15:735–41. [PubMed: 11368433]
26. Soignet SL. Clinical experience of arsenic trioxide in relapsed acute promyelocytic leukemia. *Oncologist*. 2001; 6(Suppl 2):11–6. [PubMed: 11331435]
27. Um SJ, Lee SY, Kim EJ, et al. Down-regulation of human papillomavirus E6/E7 oncogene by arsenic trioxide in cervical carcinoma cells. *Cancer Lett*. 2002; 181:11–22. [PubMed: 12430174]
28. Kerfoot C, Huang W, Rotenberg SA. Immunohistochemical analysis of advanced human breast carcinomas reveals downregulation of protein kinase C alpha. *J Histochem Cytochem*. 2004; 52:419–22. [PubMed: 14966210]
29. Crinelli R, Bianchi M, Menotta M, et al. Ubiquitin over-expression promotes E6AP autodegradation and reactivation of the p53/MDM2 pathway in HeLa cells. *Mol Cell Biochem*. 2008; 318:129–45. [PubMed: 18612801]
30. Ito A, Kawaguchi Y, Lai CH, et al. MDM2-HDAC1-mediated deacetylation of p53 is required for its degradation. *EMBO J*. 2002; 21:6236–45. [PubMed: 12426395]
31. Kobet E, Zeng X, Zhu Y, et al. MDM2 inhibits p300-mediated p53 acetylation and activation by forming a ternary complex with the two proteins. *Proc Natl Acad Sci U S A*. 2000; 97:12547–52. [PubMed: 11070080]
32. Koivusalo R, Mialon A, Pitkanen H, et al. Activation of p53 in cervical cancer cells by human papillomavirus E6 RNA interference is transient, but can be sustained by inhibiting endogenous nuclear export-dependent p53 antagonists. *Cancer Res*. 2006; 66:11817–24. [PubMed: 17178878]
33. Li M, Luo J, Brooks CL, Gu W. Acetylation of p53 inhibits its ubiquitination by Mdm2. *J Biol Chem*. 2002; 277:50607–11. [PubMed: 12421820]
34. Teodoro JG, Evans SK, Green MR. Inhibition of tumor angiogenesis by p53: a new role for the guardian of the genome. *J Mol Med*. 2007; 85:1175–86. [PubMed: 17589818]
35. Wynford-Thomas D. p53: guardian of cellular senescence. *J Pathol*. 1996; 180:118–21. [PubMed: 8976867]
36. Honda R, Tanaka H, Yasuda H. Oncoprotein MDM2 is a ubiquitin ligase E3 for tumor suppressor p53. *FEBS Lett*. 1997; 420:25–7. [PubMed: 9450543]

37. Gronroos E, Terentiev AA, Punga T, Ericsson J. YY1 inhibits the activation of the p53 tumor suppressor in response to genotoxic stress. *Proc Natl Acad Sci U S A*. 2004; 101:12165–70. [PubMed: 15295102]
38. Sui G, Affar el B, Shi Y, et al. Yin Yang 1 is a negative regulator of p53. *Cell*. 2004; 117:859–72. [PubMed: 15210108]
39. Baritaki S, Sifakis S, Huerta-Yepez S, et al. Overexpression of VEGF and TGF-beta1 mRNA in Pap smears correlates with progression of cervical intraepithelial neoplasia to cancer: implication of YY1 in cervical tumorigenesis and HPV infection. *Int J Oncol*. 2007; 31:69–79. [PubMed: 17549406]

Fig. 1.

Up-regulation of YY1 protein level in cervical cancer tissues compared with adjacent normal tissues and normal tissues. (A) Western blot analysis of YY1 and p53 in 12 paired cervical cancer tissues (labeled: T) and adjacent normal tissues (labeled: A). Fresh tissues were homogenized in 1 x SDS cell lysis buffer for Western blot analysis. β -actin protein was used to justify equivalent sample loading. (B) The quantitative result of panel (A). YY1 and

p53 protein expression levels were normalized against the β -actin level. (C) Immunohistochemical YY1 staining results in clinical cervical samples on a tissue microarray including cervical cancer tissues (labeled: T), adjacent normal tissues (labeled: A) and normal tissues (labeled: N) (200x); a1, moderate to strong YY1 staining; b1, weak YY1 staining; c1, no YY1 staining. a2, b2, and c2 are enlarged image for a1, b1, and c1. (D) Mean values of cell numbers (left panel) and YY1 staining percentage (right panel).

Fig. 2.

YY1 knockdown induces p53 activation and apoptosis. (A) Top panel: Western blot analysis of YY1 and p53 protein expression at 72, 96, and 120 hours after transfection with scramble siRNA and YY1 specific siRNA. Lower Panel: Western blot analysis of YY1 and p53 protein expression in HeLa cells transfected with pcDNA3.0-YY1 and comparison of the chemotherapy drug etoposide treatment. (B) Top panel: For the colony assay, equal numbers (3,000) of siRNAs transfected HeLa cells were seeded in 24-well plates and cultured for 12 days. Cells forming colonies were then stained with Giemsa solution; Lower panel: siRNA transfected cell growth curve examined by MTS assay. Cells were seeded in 96-plates, and detected by MTS every 24 hours. (C) Apoptosis was analyzed by flow cytometry at different time points (72, 96, and 120 hours) after transfection of YY1 siRNA and scramble siRNA by double stained with annexin V-FITC and PI. *, $P < 0.05$; **, $P < 0.01$. Statistical significances as comparisons were made between YY1 siRNA and scramble siRNA transfected cells.

Fig.3.

As₂O₃ treatment inhibits HeLa cell growth and down-regulation of YY1. (A) Cell viability was detected by MTS. Left panel: HeLa cells were treated with increasing As₂O₃

concentrations (0-16 μM) for 24 hours; Right panel: HeLa cells were treated with 2 μM As_2O_3 for increasing time (0-72 hours). (B) Flow cytometry analysis of apoptosis and cell cycle. Cells were treated with different concentrations of As_2O_3 (0, 1, 2 and 4 μM) for 48 hours, then either double stained with annexin V-FITC and PI or single stained with PI for apoptosis and cell cycle analysis. **, $P < 0.01$, Statistical significances as comparisons were made between As_2O_3 treated cells and PBS treated control cells. (C) Luciferase activity of HeLa cells with increasing As_2O_3 concentration (0-4 μM) treatment. HeLa cells were transfected with YY1-Luc reporter plasmid. After 24 hours, the cells were treated with As_2O_3 for 12 hours. Cell extracts were then examined for luciferase activity. **, $P < 0.01$, Statistical significances as comparisons were made between As_2O_3 treated cells and PBS treated control cells. (D) RT-PCR of PBS (control) and As_2O_3 treated HeLa cells using the primers for YY1 and β -actin. cDNA was prepared from total RNA samples prepared from the HeLa cells treated with 4 μM As_2O_3 and PBS (control). RT-PCR was performed on treated or untreated samples with YY1 and β -actin primers. Lanes 1 & 5: 24 cycles; Lanes 2 & 6: 28 cycles; Lanes 3 & 7: 32 cycles; Lanes 4 & 8: 36 cycles. (E) Western blot analysis of YY1 protein expression in HeLa cells with 4 μM As_2O_3 treatment for different time points.

Fig.4.

Combination of YY1 knockdown with As_2O_3 treatment synergizes the HeLa cells growth arrest. (A) YY1 siRNA enhances the sensitivity of HeLa cells to As_2O_3 treatment. Left panel: Western blot analysis of HeLa cells transfected with YY1 specific siRNA and scramble siRNA for 24 hours and treated with 4 μ M As_2O_3 . Right panel: YY1 inhibition enhances the sensitivity of HeLa cells to As_2O_3 treatment. Cells transfected with YY1 siRNA and scramble siRNA were tested for cell viability by MTS assay after being treated with different concentrations of As_2O_3 . (B) Cell cycle analysis of HeLa cells transfected with YY1 siRNA and scramble siRNA plus As_2O_3 treatment. **, $P < 0.01$, Statistical significances as comparisons were made between YY1 siRNA transfected plus As_2O_3 treated and scramble siRNA transfected plus As_2O_3 treated control cells.