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Arsenic trioxide inhibits cell proliferation and human papillomavirus oncogene expression in cervical cancer cells

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ABSTRACT

Arsenic trioxide (As_2O_3) has shown therapeutic effects in some leukemias and solid cancers. However, the molecular mechanisms of its anticancer efficacy have not been clearly elucidated, particularly in solid cancers. Our previous data showed that As_2O_3 induced apoptosis of human papillomavirus (HPV) 16 DNA-immortalized human cervical epithelial cells and cervical cancer cells and inhibited the expression of HPV oncogenes in these cells. In the present study, we systemically examined the effects of As_2O_3 on five human cervical cancer cell lines and explored the possible molecular mechanisms. MTT assay showed that HPV-negative C33A cells were more sensitive to growth inhibition induced by As_2O_3 than HPV-positive cervical cancer cells, and HPV 18-positive HeLa and C4-I cells were more sensitive to As_2O_3 than HPV 16-positive CaSki and SiHa cells. After As_2O_3 treatment, both mRNA and protein levels of HPV E6 and E7 obviously decreased in all HPV positive cell lines. In contrast, p53 and Rb protein levels increased in all tested cell lines. Transcription factor AP-1 protein expression decreased significantly in HeLa, CaSki and C33A cells with ELISA method. These results suggest that As_2O_3 is a potential anticancer drug for cervical cancer.

1. Introduction

Cervical cancer is the second most common cancer in women worldwide and human papillomavirus (HPV) as its cause is well recognized [1]. The carcinogenic risk associated with HPV infection is primarily due to the activity of two viral oncoproteins, E6 and E7 which can target tumor suppressor p53 and Rb family proteins, respectively, and interact with many other cellular proteins [2]. Therefore, any strategies that can inhibit the expression of E6 and E7 proteins would be desirable for use as a therapeutic tool to control HPV-associated cancers, including cervical cancer.

In approximately 90% of all cervical carcinomas, HPVs are found to be integrated into the host genome. Integration results in altered expression of *E6* and *E7* thereby providing the cells with a selective growth advantage. The expression of *E6* and *E7* genes is regulated by upstream regulatory region (URR) or long control region (LCR) containing *E6* and *E7* promoter and *cis*-regulatory elements. The URR region contains many binding sites of transcription factors, such as AP-1, SP-1, YY1, NF-1, OCT-1, E2 and so on, which can positively or negatively influence the transcription of viral oncogenes [3]. Several papers have shown that activator protein 1 (AP-1) is a principal factor for the expression of viral oncogenes in cervical cancer [4,5].

Arsenic trioxide (As_2O_3) has been widely studied and demonstrated to induce apoptosis in hematopoietic and non-hematopoietic tumors cells [6,7]. It has also been approved to treat acute promyelocytic leukemia (APL) by U.S. Food and Drug Administration (FDA). Although previous studies have shown that As_2O_3 can induce apoptosis in HPV-16 immortalized human cervical epithelial cells and cervical cancer cells by down-regulating HPV oncoprotein expression [4,8,9], they are individual study. In the present study, we systematically investigated the effects of As_2O_3 on the proliferation and expression of viral oncoproteins in HPV-positive and HPV-negative cervical cancer cells and explored the underlying mechanisms.

2. Materials and methods

2.1. Cell culture and reagent

Four human cervical cancer cell lines (HeLa, SiHa, CaSki and C33A) were obtained from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (Beijing). C4-I cells were obtained from Science Research Center of First Affiliated Hospital,

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Medical School, Xi'an Jiaotong University. HeLa and C4-I cells are HPV-18 positive. CaSki and SiHa cells are HPV-16 positive. C33A cells are HPV negative. All cells lines were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 IU/ml penicillin and 100 μg/ml streptomycin in a 5% CO_2 incubator at 37 °C.

As_2O_3 was purchased from Sigma–Aldrich Chemicals (St. Louis, MO, USA) and initially dissolved and diluted with distilled water to a concentration of 10^{-3} M and stored at 4 °C.

2.2. Cell viability assay

A total of 5×10^3 cells/well were seeded in 96-well plates and each group consisted of five parallel wells. After 24 h settlement, medium was replaced by fresh complete medium with different concentrations of As_2O_3 for 72 h culture. MTT (final 0.25 mg/ml) was added to each well and the plates were incubated for 4 h at 37 °C. The formazan product was dissolved by adding 100 μl dimethyl sulfoxide to each well. The MTT absorbance value was detected at 490 nm with a microplate reader (Bio-Rad, Hercules, California, USA). Cells viability (%) = [$OD_{(average\ dosing\ group)}$/$OD_{(control\ group\ mean)}$] × 100.

2.3. Preparation of RNA and quantitative polymerase chain reaction (qPCR)

In total, 2×10^5 cells/well were seeded in 6-well plates. After 24 h settlement, cells were treated with As_2O_3 for 72 h. Total RNA was isolated using Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Reverse transcription was performed in a reaction system containing 1 μg RNA, reverse transcriptase, RNase inhibitor and random primers. The obtained cDNAs were used to run qPCR with pairs of primers. The thermal cycling conditions were as follows: 20 s at 95 °C followed by the amplification reaction consisting of 40 cycles of denaturation for 3 s at 95 °C, annealing for 30 s at 60 °C. The resulting data were analyzed with the comparative Ct method for relative gene expression quantification against housekeeping gene, *GAPDH*. The primers were designed using the Oligo Primer Analysis 4.0 software and the sequences were blasted (http://www.ncbi.nlm.nih.gov/BLAST/) to check their specificity. All primer sequences are as follows: sense 5′-GACCCAGAAAGTTACCACAG-3′, antisense 5′-CATAA ATCCCGAAAAGCAAAG-3′ for HPV-16 *E6*; sense 5′-GAGGAGG AGGATGAAATA-3′, antisense 5′-ACAACCGAAGCGTAGAGT-3′ for HPV-16 *E7*; sense 5′-AAGATTTATTTGTGGTGT-3′, antisense 5′-GCT GGATTCAACGGTTTC-3′ for HPV-18 *E6*; sense 5′-CGACAGGAACGA CTCCAACGA-3′, antisense 5′-GCTGGTAAATGTTGATGATTAACT-3′ for HPV-18 *E7*; sense 5′-GTGCTCCATGAGGAGACAC-3′, antisense 5′-CCTCTTGGCAGCAGGATAG-3′ for *c-MYC*; sense 5′-AACGGATT TGGTCGTATTG-3′, antisense 5′-GGAAGATGGTGATGGGATT-3′ for *GAPDH*.

2.4. Western blot analysis

A total of 5×10^5 cells were seeded in a T-25 flask. After 24 h settlement, cells were treated with As_2O_3 for 72 h. Nuclear extracts from cells were prepared using a Nuclear Protein Extraction kit (BestBio, Shanghai, China). The protein concentrations were determined using an Enhanced BCA Protein Assay kit (Beyotime, Haimen, Jiangsu, China). 40 μg of nuclear proteins were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. The membranes were blocked in 1 × TBST (Tris-buffered saline with 0.5% of Triton X-100) containing 5% nonfat milk at room temperature for 1 h, and then incubated with the appropriate primary antibodies (anti-HPV16/18 E6, E7, p53, Rb or β-actin Abs) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4 °C. β-actin was used as an internal control. After incubation with the secondary antibody conjugated with horseradish peroxidase, membranes were extensively washed, and the specific immunoreactivity was visualized by enhanced chemiluminescence method according to the manufacturer's protocol (ECL kit, Santa Cruz Biotechnology).

2.5. Measurement of AP-1 levels

Cells were seeded in a T-25 flask, and each group was consisted of two parallel wells. After 24 h settlement, cells were treated with As_2O_3 for 72 h. The sample preparations of nuclear proteins were same as the method in Western blot analysis. The levels of AP-1 were measured with ELISA method (Catalog Number. CSB-E13403h, CUSABIO Biotech Co. Ltd. Wuhan, China).

2.6. Statistical analysis

All experiments were performed in duplicate or more. Data were expressed as mean value ± SD and statistical analysis was performed by using a standard Student's *t*-test analysis. $P < 0.05$ and $P < 0.01$ were considered to indicate statistically significant differences.

3. Results

3.1. As_2O_3 inhibits the proliferation of cervical cancer cells

As shown in Fig. 1, As_2O_3 inhibited the proliferation of cervical cancer cells and the inhibitory effect of 1 μM As_2O_3 was mild and increasing inhibition was started from 2 μM As_2O_3 treatment in a dose-dependent manner. HPV-negative C33A cells were more sensitive to As_2O_3 than other HPV-positive cells. After 2 μM or more concentrations of As_2O_3 treatment, C33A cells were showed more prominent cell death than other HPV-positive cells. Moreover, HPV-18 positive HeLa and C4-I cells were more sensitive to As_2O_3 than HPV-16 positive CaSki and SiHa cells. The inhibition in HeLa and C4-I cells was nearly 44.1% ($P < 0.05$) and 31.6% ($P < 0.05$), respectively, after 5 μM As_2O_3 treatment, and up to 70.7% ($P < 0.01$) and 49.6% ($P < 0.01$), respectively, after 10 μM As_2O_3 treatment. By contrast, the inhibition in CaSki and SiHa cells was 19.4% ($P < 0.05$) and 23.5% ($P < 0.05$), respectively, after 5 μM As_2O_3 treatment, and 42.1% ($P < 0.05$) and 26.1% ($P < 0.05$), respectively, after 10 μM As_2O_3 treatment.

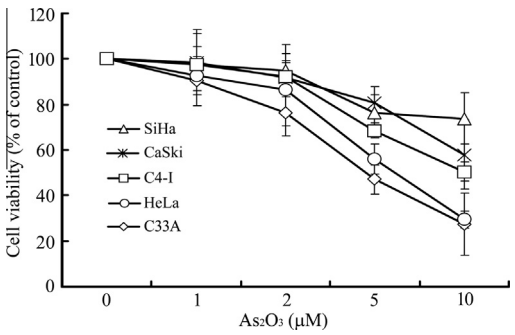

Fig. 1. Inhibitory effects of As_2O_3 on cell viability. Cervical cancer cells were treated with the indicated concentrations of As_2O_3 or vehicle for 72 h and the cells were harvested for MTT assay. The assay was repeated three times with similar results. The graph was the result of a single experiment and each point was the mean of pentaplicates ± standard deviation.

3.2. As_2O_3 inhibits the expression of HPV E6 and E7 in cervical cancer cells

The expression of HPV *E6* and *E7* is believed to be mainly responsible for the continuous growth of HPV-transformed cells. qPCR showed that As_2O_3 could effectively inhibit the expression of HPV E6 and E7 mRNA in cervical cancer cells (Fig. 2A and B). In general, the inhibitory effect of As_2O_3 on E6 and E7 mRNA was more obvious in HeLa and C4-I cells than CaSki and SiHa cells. After 1 μM As_2O_3 treatment, the inhibition of E6 and E7 mRNA was about 67.2% and 61.3% in HeLa cells, 92% and 77.8% in C4-I cells, respectively, and up to 94.8% and 98.7% in HeLa cells, 95.2% and 90.5% in C4-I cells, respectively, after 5 μM As_2O_3 treatment compared with control. However, when cells were treated with 1 μM As_2O_3, the inhibition of E6 and E7 mRNA was about 15.2% and 13.1% in SiHa cells, 33.7% and 63.6% in CaSki cells, respectively, compared with control. Since 2 μM As_2O_3 treatment, the expression of E6 and E7 mRNA was significantly inhibited in SiHa and CaSki cells, the inhibition of E6 and E7 mRNA were about 84.8% and 76.5% in SiHa cells, 63.4% and 78.5% in CaSki cells after 5 μM As_2O_3 treatment, respectively, compared with the control.

In addition, As_2O_3 could inhibit the transcription of c-*MYC* gene in cervical cancer cells. As shown in Fig. 2C, HeLa, C4-I and CaSki cells expressed higher levels of c-MYC mRNA than those in SiHa and C33A cells in steady-state. After 5 μM As_2O_3 treatment, c-MYC mRNA was significantly reduced by 88.7% (HeLa), 80.5% (C4-I) and 58.6% (CaSki) compared with the control. But the transcription of *c-MYC* was mildly changed in SiHa and C33A cells, there was no statistic difference compared with the control (Fig. 2D).

Western blot showed that As_2O_3 inhibited the expression of HPV E6 and E7 proteins, however, inhibitory effect was various (Fig. 3). Following 2 μM As_2O_3 treatment, E6 and E7 proteins were not inhibited in HeLa cells and the expression of E6 protein was not changed in SiHa cells compared with the control. However, the expression of E6 and E7 proteins was significantly down-regulated in all cervical cancer cells compared with the control after 5 μM As_2O_3 treatment. The results were not closely consistent with the qPCR results (Fig. 2) and might be resulted from alternatively splicing of E6 and E7 mRNAs [10] or the sensitivity of different detection methods, but the inhibitory trend of HPV E6 and E7 proteins in cervical cancer cells by As_2O_3 was clear.

3.3. As_2O_3 recovers the expression of p53 and pRb in cervical cancer cells

To determine the changes of the target proteins of E6 and E7, the expression of p53 and pRb were analyzed. After cells exposed to As_2O_3, the expression of p53 and pRb was significantly increased in HeLa and C4-I cells compared with the control except pRb in C4-I cells treated with 2 μM As_2O_3 (Fig. 4A), and the expression of p53 and pRb was also significantly increased in SiHa and CaSki cells compared with the control except p53 in SiHa and CaSki cells treated with 2 μM As_2O_3 (Fig. 4B). The upregulation of p53 and pRb was also found in C33A cells after As_2O_3 treatment as well (Fig. 4C).

3.4. As_2O_3 down-regulates the expression of AP-1 in cervical cancer cells

In order to study the molecular mechanism of inhibitory effect of As_2O_3 on HPV E6/E7 transcription, we measured AP-1 levels after As_2O_3 treatment. After cells exposed to As_2O_3, AP-1 was significantly reduced by 77.5% (HeLa), 24.1% (CaSki) and 34.5% (C33A) compared with the control, but AP-1 was mildly changed in C4-I and SiHa cells, reduced by 18.6% (C4-I) and 7.1% (SiHa), respectively, compared with the control (Fig. 4D).

4. Discussion

As_2O_3 as an anticancer drug has been widely studied [6,7]. In this study, we systematically studied the effects of As_2O_3 on human cervical cancer cells. Our results showed that As_2O_3 could inhibit the proliferation of cervical cancer cells and the expression of HPV oncogenes. AP-1 downregulation might contribute to downregulation of HPV oncogenes by As_2O_3 in HeLa and CaSki cells.

Our data showed that HPV negative C33A cells were more sensitive to As_2O_3 than HPV positive cancer cells. This result was different from Wen et al. [7], however, consistent with other reports [9,11]. In Wen's report, their data showed that C33A cells were less sensitive to As_2O_3 than HeLa and CaSki cells [7]. However, Yu et al. reported that C33A cells were more sensitive to As_2O_3 than HeLa, CaSki and SiHa cells [9]. In Padilla's report, they also found that CaSki cells exhibited much greater resistance to camptothecin and cisplatin than C33A cells [11]. The different sensitivity to As_2O_3 in cervical cancer cells may be associated with p53 status because p53 is mutant in C33A cells whereas p53 is wild type (wt) in the four HPV positive cervical cancer cell lines (Table 1). One paper has already shown that the p53 status influences chemosensitivity in cervical cancer cells [15]. Although many papers indicated that HPV positive cancers responded better to chemotherapy or radiation than HPV negative cancers, the reason might be reactive p53 in HPV positive cancers after treatment, and then p53 promotes apoptosis of cancer cells [16], some papers showed that HPV positive cancer cells are more resistant to

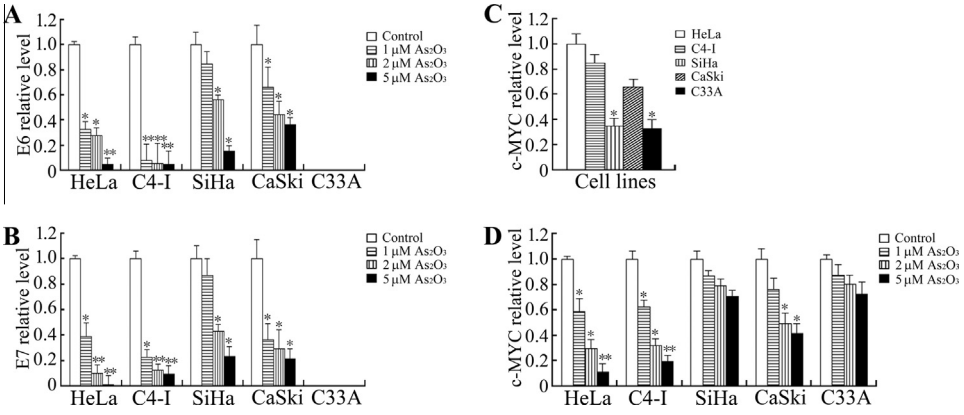

Fig. 2. Effects of As_2O_3 on the expression of HPV *E6* (A), *E7* (B) and *c-MYC* (D) mRNA in cervical cancer cell lines. Cells were treated with the indicated concentrations of As_2O_3 or vehicle for 72 h and the cells were harvested for qPCR analysis. As expected, no *E6* and *E7* mRNAs were detected in C33A cells. The relative levels of the *E6*, *E7* and c-*MYC* mRNA were normalized with *GAPDH*. The basic expression of *c-MYC* mRNA in cervical cancer cell lines was showed in (C). $^*P < 0.05$, $^{**}P < 0.01$.

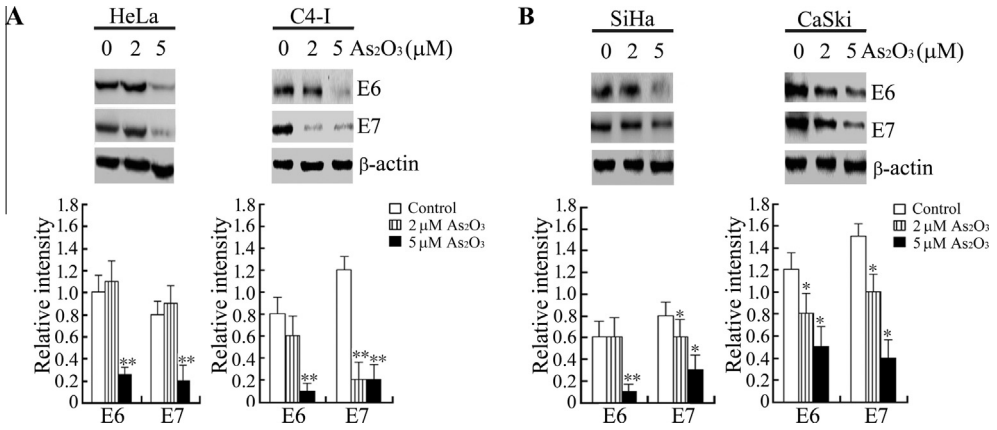

Fig. 3. Effects of As_2O_3 on the expression of HPV E6 and E7 proteins in HeLa and C4-I cells (A), SiHa and CaSki cells (B). Cells were treated with the indicated concentrations of As_2O_3 or vehicle for 72 h and the cells were harvested for Western blot analysis. The relative levels of E6 and E7 proteins were normalized with β-actin. $^{*}P < 0.05$, $^{**}P < 0.01$.

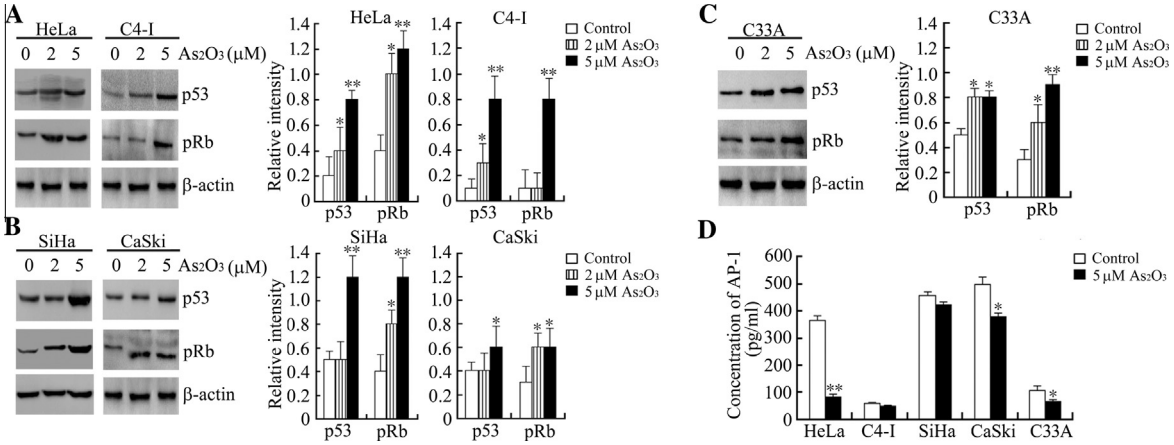

Fig. 4. Effects of As_2O_3 on the expression of p53 and pRb in HeLa and C4-I cells (A), SiHa and CaSki cells (B), C33A cells (C). Cells were treated with the indicated concentrations of As_2O_3 or vehicle for 72 h and the cells were harvested for Western blot analysis. The relative levels of p53 and pRb were normalized with β-actin. Effects of As_2O_3 on the expression of AP-1 in cervical cancer cell lines (D). Cells were treated with 5 μM As_2O_3 or vehicle for 72 h and the cells were harvested for AP-1 ELISA. The assay was repeated twice with similar results. The histogram represented the mean of duplicate samples. $^{*}P < 0.05$, $^{**}P < 0.01$.

Table 1
Basic information of cervical cancer cell lines used in this study.

Cell line	HPV type	Chromosomal location	Copy number	Methylation of the HPV DNA	p53/Rb status	Refs.
CaSki	HPV-16	Chr.[a] 2, 7, 8, 11, 11, 12, 20, 21, additional abnormal chromosomes	60–600	5′LCR, enhancer, E6 promoter	wt/wt	[12–14]
SiHa	HPV-16	Chr.13q21-q31	1–2	5′LCR, enhancer	wt/wt	[12–14]
HeLa	HPV-18	Chr.8q24.21	20–50	L1	wt/wt	[12–14]
C4-I	HPV-18	Chr.8q22.1	1	L1	wt/wt	[12,14]
C33A	ND				mt/mt	[14]

[a] Chr.: chromosome; LCR: long control region; mt: mutated; ND: no detected; wt: wild type.

cisplatin treatment than the HPV negative cancer cells [11,17]. Our data suggest that HPV positive cells show more resistant to As_2O_3 treatment than HPV negative cells and apoptosis of C33A cells by As_2O_3 is through p53-independent pathways.

On the other hand, our data showed that the HPV-18 positive cancer cells, HeLa and C4-I, were more sensitive to As_2O_3 than HPV-16 positive cancer cells, SiHa and CaSki. This difference might be associated with HPV types, HPV physical status or other mechanisms. HPV DNA integrates into the host genome which is an essential step for HPV induced cancer. Although sites of HPV DNA integration varies in different HPV associated cancer cells, proto-oncogene c-*MYC* (8q24) is a favor site for HPV DNA integration, particularly HPV18 DNA (Table 1) [18]. Therefore the transcription of c-*MYC* is activated by viral sequences or arranged host-viral sequences after HPV DNA integration. Our data showed that HeLa, C4-I and CaSki cells expressed higher levels of c-MYC mRNA than those in SiHa and C33A cells in steady-state (Fig. 2C), consistent with the previous data which the integration sites of HPV DNA are closely 5′ of the *c-MYC* locus in HeLa, C4-I and CaSki cells and *c-MYC* expression was elevated relative to SiHa cells which did not exhibit HPV integration on chromosome 8 [19]. As_2O_3 could inhibit the transcription of *c-MYC* in cervical cancer

cells, suggesting the different sensitive to As_2O_3 in the cells was related to downregulating of *c-MYC* gene expression.

Since the oncogenic activity of high-risk HPV types depends on the expression of E6 and E7 oncoproteins, they are idea targets for therapies of HPV associated cancers. Many papers have shown that knockdown of E6 and E7 expression induces growth inhibition of cervical cancer cells in vitro and in vivo [20,21]. By qPCR and Western blot analysis, our data showed that the downregulation of *E6* and *E7* was more obvious in HeLa and C4-I cells than SiHa and CaSki cells after As_2O_3 treatment. The different response might be related to the HPV DNA copy number, methylation status of viral URR and transcriptional activity (Table 1). AP-1 transcription factors are heterodimers composed of subunits of the JUN, FOS and ATF protein families. Many AP-1 proteins contain defined transcriptional activation domains except BATF. The dimer of JUN and BATF negatively regulates AP-1 activity [22]. So AP-1-mediated regulation depends on the composition of AP-1 dimers, the quality of stimulus, the cell type and the cellular environment [5]. There are three AP-1 binding sites in HPV16 and two AP-1 binding sites in HPV18. Several papers have shown that AP-1 plays a key role in the expression of HPV early genes [3,5], but the effects of As_2O_3 on AP-1 have different reports. Some studies showed that As_2O_3 achieve anti-tumor activity by inhibiting AP-1 [4,23]. However, there are different reports that As_2O_3 stimulated the activity of the AP-1 in cultured human fibroblasts [24] and acute promyelocytic leukemia [25]. Paradoxical results may be existed in various cells treated by As_2O_3 because many arsenic properties share in both anti-cancer and pro-cancer. In this study, we used JUN activation domain binding protein to detect the relative expression of AP-1 in cervical cancer cells after As_2O_3 treatment. Our data showed that As_2O_3 downregulated AP-1 levels in HeLa, CaSki and C33A cells, but the inhibition was mild in SiHa and C4-I cells, suggesting that As_2O_3 down-regulation of HPV oncogenes was partially due to the diminished AP-1 binding.

Tumor suppressor p53 and Rb proteins are targeted by E6 and E7 of high-risk HPV, respectively. Although our results showed that As_2O_3 induced the expression of *p53* and *Rb* in all tested cervical cancer cells, the mechanisms and meanings might be different. The upregulation of *p53* and *Rb* might be related to the downregulation of *E6* and *E7* and toxic response to As_2O_3 in HPV positive cancer cells with wt *p53* and *Rb*. Several papers have shown that As_2O_3 induces the expression of wt *p53* in tumor and non-tumor cells [26–28] except the gastric cancer cell line MGC803 in which As_2O_3 induced degradation of *p53* [29]. However, we found that As_2O_3 also induced the expression of mutant *p53* and *Rb* in C33A cells, making it difficult for explanation. Previous papers showed that effects of As_2O_3 on mutant *p53* have different results. Yan et al. found that mutant p53 protein was targeted by As_2O_3 for degradation and played a role in arsenic-mediated growth suppression [28]. However, some papers showed that As_2O_3 induced the expression of both mutant *p53* and wt *p53* in glioblastoma cell lines [30], in myeloma cell lines [27] and in human T-cell leukemia virus I infected T-cell lines [31]. We are not sure whether the upregulation of mutant *p53* and *Rb* in C33A cells is related to C33A cells more sensitive to As_2O_3 than other HPV positive cervical cancer cells.

Since As_2O_3 induces apoptosis of various cancer cell lines [6,23,25–31], including cervical cancer cell lines expressing or not expressing HPV [4,7–9], it is obvious that the mechanisms of As_2O_3 induced apoptosis are different in various cancer cell lines. In the study, only cervical cancer cell lines were used for investigation. We are not sure whether As_2O_3 also inhibits expression of viral oncogenes in non-cervical cancer cell lines expressing E6/E7 oncoproteins. Therefore more researches are needed to assess the effects of As_2O_3 on expression of HPV oncogenes.

Conflict of interest

The authors confirm that they have no conflict of interest.

Acknowledgments

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