Virology Journal

RESEARCH

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Activating transcription factor 3 induces oxidative stress and genotoxicity, transcriptionally modulating metastasis-related gene expression in human papillomavirus-infected cervical cancer

Elham Naderzadeh[1], Mohammad Kargar[2], Mohammad Javad Mokhtari[3] and Ali Farhadi[4,5*]

Abstract

Background Activating Transcription Factor 3 (ATF3) is known for its tumor-suppressive properties in cervical cancer, particularly through its role in stress response and interactions with human papillomavirus (HPV) oncogenes. This study investigates ATF3's regulatory impact on metastasis-related genes, oxidative stress, and DNA damage in HPV-positive cervical cancer cells.

Methods HeLa and Ca Ski cell lines were transfected with ATF3-expressing vectors. Western blotting and quantitative reverse transcription polymerase chain reaction (RT-qPCR) were used to confirm ATF3 overexpression following transfection. ROS assays and Comet assays assessed the impact of ATF3 on oxidative stress and DNA damage, while RT-qPCR was used to evaluate changes in HPV E6/E7, SHARP1, and MMP1 gene expression.

Results ATF3 overexpression led to elevated ROS levels ($p < 0.02$), resulting in oxidative DNA damage. These results demonstrate ATF3's cytotoxic impact on cervical cancer cells through oxidative stress and DNA damage. Additionally, ATF3 overexpression significantly decreased MMP1 expression ($p < 0.03$), indicating a potential anti-metastatic effect, while SHARP1 and HPV E6/E7 expression levels were not significantly altered, indicating selective gene modulation by ATF3.

Conclusion These findings reveal that ATF3 contributes to tumor suppression in cervical cancer by modulating oxidative stress and DNA damage, selectively targeting genes involved in metastasis. These findings supports ATF3's role in regulating key pathways in HPV-positive cervical cancer cells, providing a basis for further exploration of ATF3 as a target in therapeutic strategies aimed at improving outcomes in cervical cancer.

Keywords ATF3, HPV, MMP1, SHARP1, ROS, DSBs, Cervical cancer

*Correspondence:
Ali Farhadi
farhadi_a@sums.ac.ir
[1]Department of Microbiology, College of Science, Agriculture and Modern Technology, Shiraz Branch, Islamic Azad University, Shiraz, Iran
[2]Department of Biology, Zand Institute of Higher Education, Shiraz, Iran
[3]Department of Biology, Zarghan Branch, Islamic Azad University, Zarghan, Iran
[4]Department of Medical Laboratory Sciences, School of Paramedical Sciences, Shiraz University of Medical Sciences, Shiraz 7143918596, Iran
[5]Diagnostic Laboratory Sciences and Technology Research Center, School of Paramedical Sciences, Shiraz University of Medical Sciences, Shiraz, Iran

Background

Activating Transcription Factor 3 (ATF3) is a crucial transcription factor implicated in various human cancers, particularly in response to cellular stress. It plays a significant role in regulating adaptive responses, influencing processes such as cell survival, metabolism, and immune responses [1]. ATF3 is increasingly recognized as a tumor suppressor in cervical cancer, particularly in the context of human papillomavirus (HPV) infection. Research indicates that ATF3 overexpression in HPV-positive cervical cancer cells, such as HeLa and Ca Ski cells [2, 3], leads to significant apoptosis and cell cycle arrest, primarily through the inhibition of the NF-κB p65 signaling pathway [3]. The role of ATF3 in cervical cancer is further emphasized by its interactions with other signaling pathways, such as the Wnt/β-catenin pathway, which is implicated in cancer progression [4]. Moreover, ATF3 is crucial in activating p53 by preventing the binding of E6-associated protein (E6AP) to the E6 oncoprotein of HPV. The E6 protein, particularly from HPV-16, interacts with E6AP to form a heterodimer that ubiquitinates p53, leading to its degradation and loss of function [5]. By inhibiting this interaction, ATF3 stabilizes p53, allowing it to exert its tumor suppressor functions [6]. Thus, the ability of ATF3 to disrupt the E6/E6AP complex not only prevents p53 degradation but also mitigates the feedback loop that would otherwise enhance the inhibitory effects of E6 on p53 [7]. While this mechanism highlights the intricate interplay between viral oncogenes and host tumor suppressor pathways in cervical carcinogenesis, the effect of the transcriptional activity of ATF3 on the expression levels of HPV16 and 18 E6/E7 oncotranscripts remains unclear.

SHARP1, a basic helix-loop-helix transcription repressor, has been implicated in regulating malignant behaviors in various cancers, including cervical cancer. Although specific studies on SHARP1 in cervical cancer are limited, its role in other malignancies suggests potential functions in this context. For example, SHARP1 has been shown to suppress angiogenesis by decreasing hypoxia-inducible factor-1α (HIF-1α) levels, which is crucial for tumor growth and metastasis [8]. This mechanism may also be relevant in cervical cancer, where angiogenesis is a key factor in tumor progression. Additionally, the regulation of epithelial-mesenchymal transition (EMT) by transcription factors like Twist1 suggests that SHARP1 could similarly influence cellular migration and invasion in cervical cancer [9]. While it has been shown that ATF3 promotes the expression of the metastasis suppressor SHARP1 in mutant p53-expressing cells by preventing p63 inactivation and suppressing cancer cell migration [2, 10], the direct relationship between SHARP1 and ATF3 in p53-null cervical cancer cells is still under investigation.

Matrix metalloproteinase-1 (MMP1) plays a significant role in the progression of cervical cancer, primarily through its involvement in the degradation of the extracellular matrix (ECM), which facilitates tumor invasion and metastasis. MMP1 is regulated by the tumor suppressor p53, and its dysregulation can lead to enhanced tumor metastasis due to increased ECM degradation [11]. While specific studies on MMP1 in cervical cancer are limited, its overexpression has been linked to poor outcomes in other cancers, such as head and neck carcinoma, where it correlates with advanced tumor stages and lower survival rates [12]. Although the relationship between MMP1 and ATF3 in cervical cancer cells is not directly established in the provided studies, there are significant correlations between ATF3 overexpression and the expression and activation of MMPs in glioblastoma cells [13]. Therefore, further investigation is needed to clarify the specific relationship between MMP1 and ATF3 in cervical cancer cells.

Reactive oxygen species (ROS) are chemically reactive molecules containing oxygen, can lead to oxidative stress, resulting in DNA damage and apoptosis in cervical cancer cells [14]. ATF3 plays a key role in mitigating mitochondrial oxidative stress, particularly in the context of cardiomyopathy, where its expression is upregulated in response to stressors like angiotensin II (Ang II) [15]. This regulation of oxidative stress is crucial, as excessive ROS can lead to cellular damage and inflammation, processes in which ATF3 is also implicated [16]. Additionally, ATF3 has been shown to influence ferroptosis and inflammatory responses in various cell types, including human periodontal stem cells, by modulating ROS levels and related signaling pathways such as Nrf2/HO-1 [17]. Although the direct interaction between ATF3 and ROS in cervical cancer is not explicitly detailed in the abstracts, the evidence suggests that ATF3 may influence cellular responses to oxidative stress, potentially affecting cancer progression and treatment efficacy. Furthermore, ATF3 is involved in the DNA damage response, as demonstrated by its activation in response to the topoisomerase II inhibitor dexrazoxane, which induces DNA double-strand breaks (DSBs) through ATM signaling [18]. This suggests that ATF3 plays a critical role in mediating cellular responses to DNA damage, potentially influencing therapeutic outcomes in cervical cancer. Consequently, further investigation is required to elucidate the precise mechanisms by which ATF3 interacts with ROS, its dual roles in different cellular contexts, and its implications in the DNA damage response. This study aims to investigate the role of ATF3 in cervical cancer, with a particular focus on its transcriptional effect on the expression levels of HPV16 and 18 E6/E7 oncotranscripts, the metastasis suppressor SHARP1 and MMP1. Additionally, the study will explore how ATF3 influences

ROS levels and the DNA damage response in cervical cancer cells. By elucidating these mechanisms, the research seeks to clarify the tumor suppressive functions of ATF3 and its potential therapeutic implications in cervical carcinogenesis.

Methods

Cell culture and pCMV6-ATF3-GFP transfection

HeLa and Ca Ski cell lines were acquired from the National Cell Bank of Iran (NCBI Code: C115, Pasteur Institute, Tehran) and the American Type Culture Collection (ATCC; Manassas, VA), respectively. HeLa cells were maintained in RPMI-1640 medium, while Ca Ski cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM). Both cell lines were grown in complete media containing 10% fetal bovine serum (FBS) and 1% Penicillin/Streptomycin (all from Sigma-Aldrich), and were kept at 37 °C with 5% CO2. After 24 h of growth, the cells were transfected with pCMV6-AC-IRES-GFP plasmids carrying the ATF3 gene (pCMV6-ATF3-GFP), obtained from a prior study [2]. For transfection, cells were plated at a density of 8 × 10^5 per well in 6-well plates. Once they reached 60–70% confluency, HeLa cells were treated with 2 µg of pCMV6-ATF3 plasmid, and Ca Ski cells with 2.5 µg, based on the previously determined inhibitory concentration [2, 3]. Transfection was performed using Lipofectamine 3000 (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's protocol.

Quantitative and qualitative transfection efficiency assays

To evaluate transfection efficiency, both fluorescence and transmission light microscopy were used. Observations were made using an Olympus microscope (Olympus, Tokyo, Japan) with a 20x objective lens at 24, 48, and 72 h after transfection. Fluorescent images were captured with the GFP filter cube. Transfection efficiency was determined by counting the total number of cells and those expressing GFP. At each time point, five separate images were taken and analyzed. Additionally, flow cytometry was performed at 48 and 72 h post-transfection. Cells were first washed with PBS, then detached from the culture plates using trypsin-EDTA. Following detachment, cells were centrifuged at 350 g for 5 min, resuspended in PBS, and analyzed for fluorescent intensity using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA). Each experiment analyzed 10,000 cells, and the results were processed using FlowJo software version 10.0 (FlowJo LLC, USA).

SDS-PAGE and Western blotting analysis for ATF3 expression

HeLa and Ca Ski cells were seeded into 6-well plates and incubated overnight at 37 °C with 5% CO2. The cells were then transfected following previously established protocols [2, 3]. After 72 h, the cells were collected, washed with cold PBS, and sonicated (Amplitude: 80–100, two 30-second cycles with 30-second cooling intervals) in RIPA buffer (1% Triton X-100, 0.1% SDS, 150 mM NaCl, 50 mM Tris-HCl, pH 8.0) containing 25 µL of a protease inhibitor cocktail (Sigma-Aldrich). The samples were centrifuged at 15,000 g for 20 min at 4 °C, and the supernatants were collected. Protein concentrations from the test and control cells were determined using the Bradford protein assay (Bio-Rad), creating an absorbance standard curve.

The cell lysates were then mixed with 5× sample buffer (0.25 M Tris-HCl pH 6.8, 50% glycerol, 10% SDS, 0.1% bromophenol blue, and 5% β-mercaptoethanol) and heated at 95 °C for 10 min. Equal protein amounts from each sample were separated on a 12% SDS-polyacrylamide gel and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, UK) using a Bio-Rad Mini Trans-Blot® system at 300 mA for 3 h. The membranes were blocked with a solution of 5% skim milk and 0.05% Tween 20 for 16 h, followed by a 15-minute wash with TBST buffer (150 mM NaCl, 50 mM Tris-base, and 0.05% Tween 20). ATF3 protein levels were detected using a monoclonal anti-ATF3 antibody (1:500, Santa Cruz Biotechnologies). β-actin (1:5000, Santa Cruz Biotechnologies) was used as a loading control. After three washes with TBST, the membranes were incubated with a horseradish peroxidase-conjugated secondary antibody (1:10,000, Sigma-Aldrich) at room temperature for 2 h, followed by two washes with TBS buffer (50 mM Tris-base and 150 mM NaCl). The membranes were then treated with 10 mL of 3,3′-Diaminobenzidine (DAB) substrate (5 mg DAB in 10 mL DDW) and 5 µL H2O2. The reaction was stopped by rinsing the membranes with DDW.

Reverse transcription quantitative PCR analysis

The primer sequences for RT-qPCR were designed using Gene Runner 3.0 software (available at http://www.generunner.net/) and are detailed in Table 1. Total RNA was extracted from cultured cells using the RiboEx TM kit (GeneAll, Korea), and cDNA synthesis was performed at 37 °C for 15 min with the PrimeScript RT reagent kit (TaKaRa, Japan). RT-qPCR was conducted using the ABI Prism 7000 Sequence Detection System (Applied Biosystems) and RealQ Plus 2x Master Mix Green (Ampliqon, Denmark). To validate the primers, standard curves were generated for each primer set. The relative expression levels of target genes were determined using the Pfaffl method [19], with GAPDH serving as the reference gene (Forward: GGCCTCCAAGGAGTAAGACC, Reverse: AGGGGTCTACATGGCAACTG) [13]. The thermocycling conditions included an initial denaturation step at 95 °C for 15 min, followed by 40 cycles of 95 °C for 15 s,

Table 1 Oligonucleotide primer sequences used for reverse-transcription real-time PCR assay

Gene	Sequence (5′ to 3′)	PCR product size (bp)	Annealing temperature (˚C)
ATF3-Forward	GAGTGCCTGCAGAAAGAGT	117	58
ATF3-Reverse	CCGATGAAGGTTGAGCATG		
HPV-16-E6/E7 Forward	GAGAAACCCAGCTGTAATCATGC	174	55
HPV-16-E6/E7 Reverse	GTAATGGGCTCTGTCCGGTTC		
HPV-18-E6/E7 Forward	GACATTGTATTGCATTTAGAGCC	155	56
HPV-18-E6/E7 Reverse	CATTGTGTGACGTTGTGGTTC		
MMP-1-Forward	GAAAAGCGGAGAAATAGTG	153	51
MMP-1-Reverse	GAGGACAAACTGAGCCAC		
SHARP1-Forward	GAGACGACACCAAGGATAC	140	56
SHARP1-Reverse	CTCCAGATGTCCCAGAGTTG		

primer-specific annealing for 30 s, and a final extension at 72 °C for 20 s.

Detection of reactive oxygen species (ROS)

To measure intracellular ROS levels, 2×10^4 cells were plated in culture medium and incubated at 37°C for 24 hours, followed by transfection as previously described. After 48 hours post-transfection, the culture medium was discarded, and the cells were washed twice with PBS. The cells were then detached using Trypsin/EDTA, collected, and resuspended in PBS. Following this, the cells were incubated with 2'-7'dichlorofluorescin diacetate (DCFH-DA, SigmaAldrich) at 37 °C for 30 min. Upon cellular uptake, DCFH-DA is deacetylated, forming non-fluorescent DCFH, which can be oxidized by ROS into a fluorescent compound. The fluorescence intensity, reflecting ROS levels, was measured using a FACSCalibur flow cytometer, and the results were analyzed with FlowJo software, version 10.0.

Comet assay

The comet assay was performed under alkaline conditions, adapting the protocols by Singh et al. [20] and Tice et al. [21] with some modifications. Following transfection cells were harvested 48 h post-transfection. A 1 mL aliquot, containing 1×10^5 cells, was centrifuged at 350 g for 5 min, and the resulting pellet was resuspended in 50 µL of PBS. The cells were mixed with 100 µL of low-melting-point agarose (0.5%, Cleaver Scientific Ltd, Rugby, UK) in PBS and spread onto microscope slides pre-coated with normal-melting-point agarose (1.5%). After covering with a coverslip, the slides were incubated at 4 °C for 20 min to solidify. Next, the slides were submerged in a freshly prepared cold lysis solution (2.5 M NaCl, 100 mM EDTA, 1% Triton X-100, 10 mM Tris, pH 10) and incubated at 4 °C in the dark for 60 min. After lysis, the slides were placed in a cold horizontal electrophoresis chamber filled with an alkaline electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH > 13) and subjected to electrophoresis at 1 V/cm (25 V, 300 mA) for 30 min to allow DNA unwinding and reveal alkali-labile sites. The slides were then washed with a neutralization buffer (0.4 M Tris-HCl, pH 7.5) for 20 min, rinsed with water, and dehydrated in 75% ethanol for 5 min. Once dried, the slides were stained with 50 µL of diluted SYBR® Green I (Fermentas, Vilnius, Lithuania) and immediately examined under a fluorescence microscope to visualize the DNA of individual cells. All procedures were performed under dark or dim light conditions to minimize additional DNA damage.

Statistical analysis

The data analysis was performed using SPSS software version 22. Results were presented as mean ± standard deviation (SD). Due to the non-normal distribution of data, the Kruskal-Wallis test was utilized to determine significant differences between groups. Statistical significance was set at a p-value of less than 0.05.

Results

Transfection of recombinant vectors into HeLa and Ca ski cells

Images captured by a fluorescence microscope confirmed that over 80% of HeLa and Ca Ski cells were successfully transfected with the pCMV6-ATF3 plasmid 72 h post-transfection, as shown in Fig. 1A. Flow cytometry further quantified the transfection efficiency, showing an average of 86.41% in HeLa cells and 88.34% in Ca Ski cells at the same time point (Fig. 1B).

Western blot analysis confirmed the overexpression of ATF3 in the transfected cells. The results showed a substantial increase in ATF3 protein levels in the pCMV6-ATF3-transfected cells compared to both untreated and mock-transfected controls, reaching statistical significance ($p < 0.001$) (Fig. 1C and D).

Impact of ATF3 overexpression on HPV-16 and HPV-18 E6/E7, MMP1, and SHARP1 gene expression

In addition to western blot analysis, the overexpression of ATF3 mRNA in transfected HeLa and Ca Ski cells was confirmed using RT-qPCR. The expression levels of HPV-16-E6/E7, HPV-18-E6/E7, MMP1, SHARP1, and GAPDH

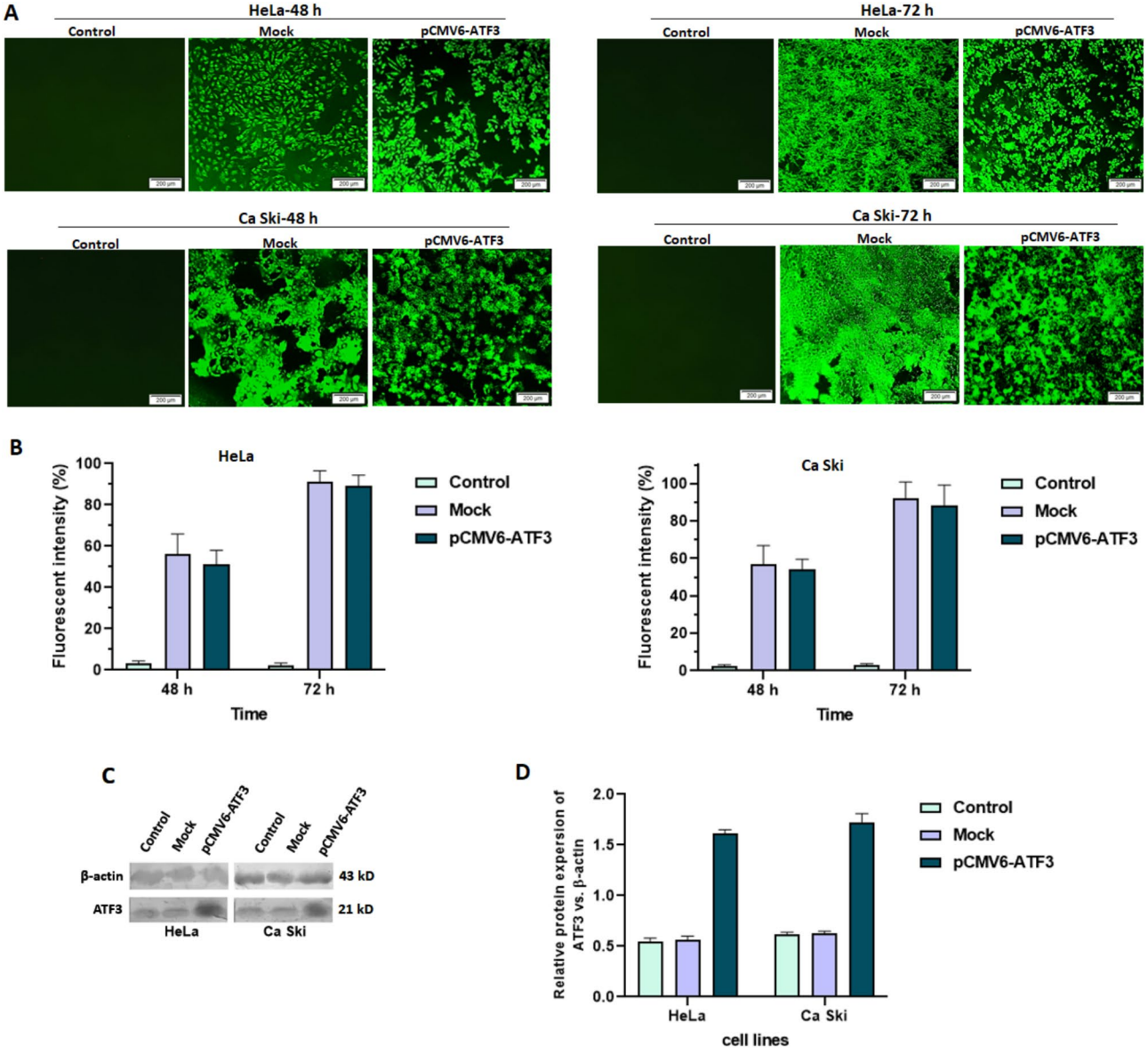

Fig. 1 Analysis of fluorescence intensity in HeLa and Ca Ski cells transfected with pCMV6-ATF3, a mock vector, or left untreated as controls, utilizing fluorescence microscopy and flow cytometry at 48 and 72 h post-transfection. (**A**) Fluorescent imaging of HeLa and Ca Ski cells was performed at 20x magnification, comparing cells transfected with pCMV6-ATF3 to mock and control groups. (**B**) Mean fluorescence intensity levels were measured across pCMV6-ATF3, mock, and control samples. Flow cytometry results showed transfection efficiencies of 89.41% in HeLa and 88.13% in Ca Ski cells at 72 h post-transfection, significantly higher than the mock and control groups ($p < 0.0001$). (**C**) Western blot analysis was conducted to assess ATF3 protein expression in HeLa and Ca Ski cell lysates, using anti-ATF3 and anti-β-actin antibodies. (**D**) Densitometry of ATF3 expression levels showed a marked increase in cells transfected with pCMV6-ATF3 compared to control and mock groups ($p < 0.001$). Results are presented as the mean ± SD from three independent experiments

mRNAs were measured in untreated, mock-transfected, and pCMV6-ATF3-transfected groups. RT-qPCR analysis conducted 72 h post-transfection indicated that elevated ATF3 expression ($p < 0.001$) in HeLa cells led to a significant reduction in MMP1 expression ($p < 0.02$) compared to the control and mock groups. Similarly, in Ca Ski cells, increased ATF3 expression ($p < 0.001$) resulted in a significant decrease in MMP1 expression ($p < 0.03$). However, ATF3 overexpression did not significantly affect SHARP1 or E6/E7 expression in HeLa cells ($p > 0.05$) nor in Ca Ski cells ($p > 0.05$), compared to the control and mock-transfected groups. Figure 2A and B summarizes the gene expression changes observed in the pCMV6-ATF3-transfected, mock-transfected, and control groups for both HeLa and Ca Ski cells.

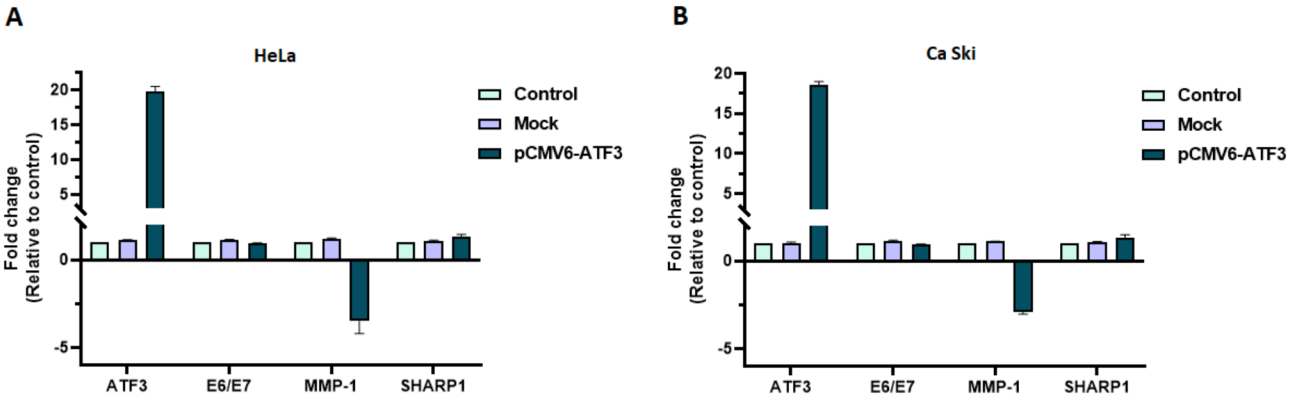

Fig. 2 Effect of ATF3 overexpression on the expression of HPV-16 and HPV-18 E6/E7, MMP1, and SHARP1 genes in HeLa (**A**) and Ca Ski (**B**) cells, analyzed by RT-qPCR. (**A**) In HeLa cells, overexpression of ATF3 resulted in a significant decrease in MMP1 gene expression ($p < 0.01$), while SHARP1 and HPV-18 E6/E7 mRNA levels remained largely unaffected ($p > 0.05$) compared to mock and control groups. (**B**) Similarly, in Ca Ski cells, ATF3 overexpression significantly reduced MMP1 expression ($p < 0.02$), but SHARP1 and HPV-18 E6/E7 expression levels showed no significant difference ($p > 0.05$) relative to mock and control groups. Both HeLa and Ca Ski cells transfected with pCMV6-ATF3 demonstrated markedly higher ATF3 mRNA expression compared to mock and control groups ($p < 0.001$). Data are presented as the mean ± SD from three independent experiments

Effect of ATF3 overexpression on intracellular ROS levels

To further elucidate the mechanism behind the inhibition of cell proliferation, we assessed ROS levels in HeLa and Ca Ski cells using DCFDA staining followed by flow cytometric analysis (Fig. 3A and B). The results demonstrated that ATF3 overexpression in HeLa cells significantly increased ROS levels ($p < 0.01$) compared to the mock-transfected and control groups. Similarly, as shown in Fig. 3, ATF3 overexpression in Ca Ski cells led to a significant increase in basal ROS levels ($p < 0.02$). These findings suggest that the elevated ROS levels associated with ATF3 overexpression are correlated with its cytotoxic effects in cancer cells.

DNA damage lesions following ATF3 overexpression

Given that ROS can induce oxidative DNA damage, we investigated whether ATF3 overexpression increased the occurrence of DNA breaks in HeLa and Ca Ski cells. The Comet assay was employed to assess the presence of DSBs, which are indicative of oxidative DNA damage. Figure 4A and B illustrate that cells transfected with pCMV6-ATF3 showed an increase in long-tail cells, indicating a higher occurrence of DSBs ($p < 0.05$) compared to the mock-transfected and control groups. These data suggest that ATF3 overexpression in HeLa and Ca Ski cells, which increases ROS production and clusters oxidative DNA damage, is associated with the formation of DNA DSBs in these cells.

Discussion

Although much is known about the effects of ATF3, the present study uniquely investigates its role in genotoxicity, oxidative stress, and modulation of metastasis-related gene expression, specifically in the context of HeLa and Ca Ski cells. Matrix metalloproteinase-1 is closely associated with cervical cancer metastasis and prognosis [22], significantly influencing tumor progression and patient outcomes. Notably, MMP-1 is persistently overexpressed in cervical cancer, especially in lymph node-positive cases, suggesting its potential as a biomarker for metastasis [23]. This overexpression contributes to the degradation of the extracellular matrix (ECM), facilitating tumor invasion and metastasis. MMP-1 is regulated by the p53 tumor suppressor, and its dysregulation by the E6 oncoprotein of high-risk HPVs (hr-HPVs) enhances tumor aggressiveness in HPV-infected cervical cancer cells [11]. ATF3, a transcriptional repressor, significantly influences MMP-1 expression, particularly in inflammatory and cancerous settings. Ho et al. demonstrated that IFN-γ increases ATF3 expression in human monocytes, leading to the suppression of MMP-1 by recruiting ATF3 to the MMP-1 promoter and inhibiting AP-1 transcription factors, which normally promote MMP expression [24]. while ATF3 generally suppresses MMP-1 in inflammatory contexts, its role in cancer may be more complex, potentially promoting invasiveness through other MMPs, indicating a dual role depending on the cellular context. Zugowski et al. demonstrated that aberrant STAT3 activation, which can interact with AP-1, leads to increased MMP-1 expression. ATF3 may counteract this effect, although its specific role in this context requires further exploration [25]. Additionally, Ma et al. found that elevated ATF3 levels correlate with increased MMP-2 expression, which may promote tumor invasiveness, although the direct impact on MMP-1 remains less clear [26]. In the present study, we explored the impact of ATF3 overexpression on MMP-1 expression in HeLa and Ca Ski cells. Our results demonstrated that ectopic ATF3 overexpression in HPV16- and HPV18-positive cervical cancer cells led to a significant reduction in MMP-1

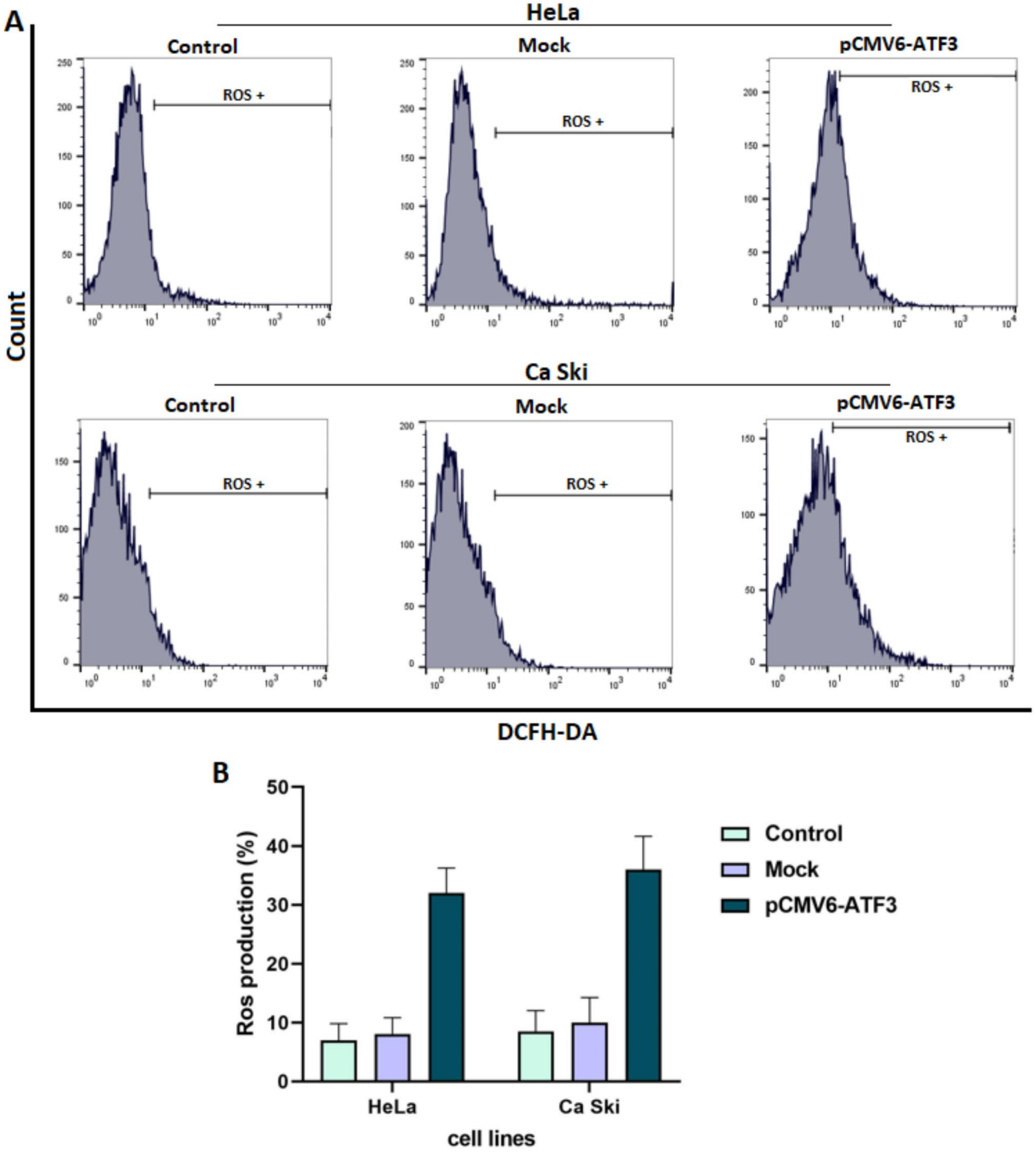

Fig. 3 Evaluation of ROS levels in HeLa and Ca Ski cells following pCMV6-ATF3 transfection. (**A**) Flow cytometry histograms illustrating ROS levels in HeLa and Ca Ski cells, measured with the DCHF-DA assay. (**B**) Quantitative comparison shows a significant rise in ROS levels in pCMV6-ATF3-transfected cells relative to mock and control groups ($p < 0.01$). Results are shown as the mean ± SD from three separate experiments

mRNA levels. This suggests that ATF3 may modulate metastasis-related factors, underscoring its potential role as an anti-metastatic tumor suppressor in cervical cancer cells.

While the expression levels of SHARP1 in cervical cancer tissues have yet to be fully characterized, its role as a tumor suppressor in other cancers suggests it may similarly influence CC. SHARP1 expression is regulated through multiple cellular pathways, primarily involving

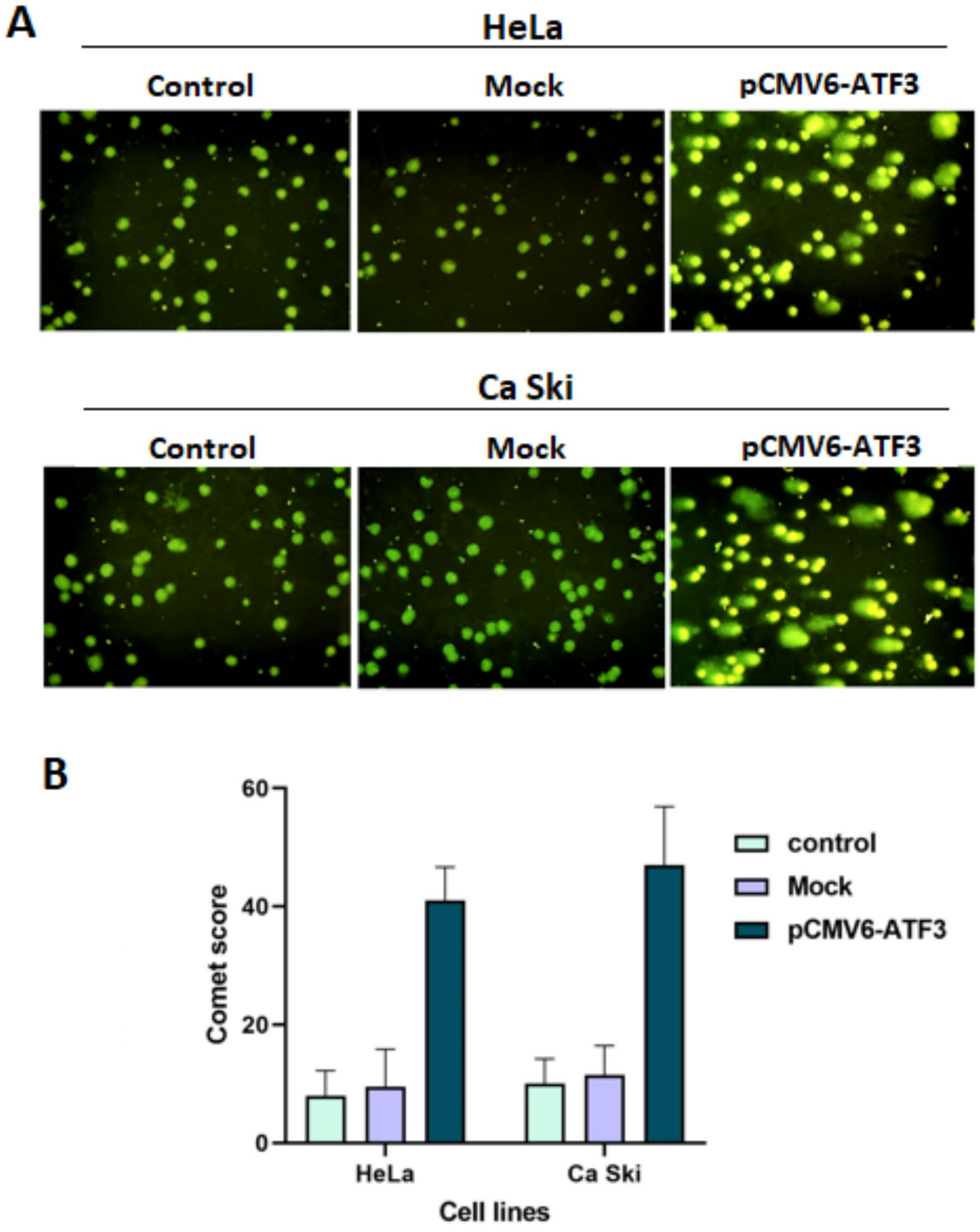

Fig. 4 DNA damage analysis by comet assay using alkaline single-cell gel electrophoresis. (**A**) Representative images from the comet assay display DNA migration patterns in HeLa and Ca Ski cells post-transfection with pCMV6-ATF3. In these images, intact DNA forms **a** distinct head, while fragmented DNA trails behind, forming a comet tail—longer tails signify greater DNA damage. (**B**) Quantification of comet assay results for HeLa and Ca Ski cells across pCMV6-ATF3-transfected, mock-transfected, and control groups. Minimal DNA damage was observed in both control and mock-transfected cells. Data are expressed as the mean ± SD from three independent replicates

insulin signaling and the NOTCH1 pathway [27], where its overexpression suppresses EMT and metastasis in endometrial cancer [28]. ATF3 has been identified as a co-regulator that binds alongside p53 at cis-regulatory elements (CREs) and can either promote or inhibit the expression of target genes, including SHARP1 [29]. Our findings demonstrated that, although normalized fold change levels of SHARP1 expression were higher in ATF3-overexpressing cervical cancer cells compared to control and mock-transfected groups, the increase was not statistically significant. Since ATF3's specific role in CC is not extensively documented, its involvement in regulating malignant behaviors in other cancers suggests potential implications for CC progression. However, the present study does not support a direct role for ATF3 in enhancing SHARP1 mRNA expression levels in cervical cancer cells. The impact of ATF3 on SHARP1 expression may vary across cell types, depending on the cellular environment and stimuli, as seen in its role in skeletal muscle stem cells, where it prevents premature activation [30]. While ATF3 is a critical regulator in many contexts, its influence on SHARP1 expression may not be universally applicable, as other transcription factors and regulatory mechanisms could also modulate SHARP1 levels in different biological scenarios.

The ability of ATF3 to interact with viral elements suggests it may serve as a transcriptional regulator that can either suppress or promote viral gene expression depending on the context [31]. Evidence shows that ATF3 binds to genomic regions rich in transcriptional coactivators, such as p300 and H3K27ac, supporting its involvement in transcriptional regulation [32]. For instance, in ZIKV-infected cells, ATF3 restricts viral gene expression by modulating innate immune responses and autophagy pathways [33]. Similarly, during herpes simplex virus (HSV) infections, ATF3 promotes the buildup of latency-associated transcripts, which are essential for maintaining viral latency [34]. However, in HPV infections—especially within cervical cancer cells—ATF3's role appears complex. While ATF3 seems to indirectly suppress HPV gene expression, likely through interactions with the NF-κB signaling pathway [3] and other transcription factors involved in HPV regulation [35], its direct regulatory effect on the E6 and E7 oncotranscripts remains unclear. In this study, ATF3 overexpression did not significantly alter HPV-18-E6/E7 expression in HeLa cells or HPV-16-E6/E7 in Ca Ski cells compared to control and mock-transfected groups. These findings suggest that ATF3 does not play a direct role in upregulating HPV-E6/E7 mRNA expression in cervical cancer cells. The observed lack of direct impact of ATF3 overexpression on HPV-E6/E7 mRNA expression in cervical cancer cells may rely on specific co-factors or chromatin states that are absent or inactive in HeLa and Ca Ski cells, limiting its direct influence on E6/E7 transcription. These findings could also indicate a cell-type-specific regulatory mechanism where ATF3's role in viral gene expression varies depending on cellular context and epigenetic modifications.

Studies suggest that oxidative stress biomarkers can predict outcomes in cervical cancer, underscoring the critical role of ROS in disease prognosis [36]. Excessive ROS levels cause oxidative stress, leading to DNA damage and apoptosis in cervical cancer cells [14], a phenomenon also observed in the present study, suggesting ROS as potential therapeutic targets. Given the dual role of ROS in cancer biology, where moderate to high levels are essential for activating survival signaling pathways that may promote tumor progression, increased ROS levels can downregulate MMP-1 expression in cancer cells through several molecular mechanisms, inhibiting metastasis [37]. These mechanisms primarily involve the activation of specific signaling pathways that modulate MMP-1 expression in response to oxidative stress. Elevated ROS levels, particularly hydrogen peroxide (H2O2), lead to sustained activation of c-Jun N-terminal kinase (JNK), which in turn recruits c-jun to the MMP-1 promoter, influencing MMP-1 transcription [38]. Additionally, mitochondrial ROS (mtROS) play a role in regulating MMP levels, including MMP-1, by affecting mRNA stability and expression. The molecular adaptor HIC-5 and NADPH oxidase 4 (NOX4) are involved in this regulation: HIC-5 suppresses NOX4, reducing mtROS and destabilizing MMP mRNA [39]. It is important to note that the redox state of the cell influences the expression of various MMPs, including MMP-1, through different redox mechanisms. Alterations in intracellular redox conditions can downregulate MMP-1 and other MMPs, suggesting that ROS levels critically determine MMP expression profiles in cancer cells [40]. The ROS-MMP-1 pathway has significant therapeutic implications for cancer treatment, particularly in relation to oxidative stress and tumor progression. Conversely, ATF3 plays a dual role in regulating ROS production, depending on the context. It upregulates GPX4, a ferroptosis-responsive gene, reducing Fe^{2+} accumulation, ROS production, and malondialdehyde (MDA) release. Conversely, in erastin- and RSL3-treated cardiomyocytes, ATF3 overexpression decreases Fe^{2+} levels, ROS, and MDA associated with ferroptosis, ultimately reducing cell death [41]. These examples underscore the importance of examining the direct relationship between ATF3 and ROS in cervical cancer cells. However, the data presented in this study primarily reflect gene expression levels, which may not fully represent changes in protein expression. While these results provide valuable insights, the lack of proteomic validation may limit the interpretation of protein-level alterations.

In this study, we showed that ATF3 overexpression in cervical cancer cells significantly increases ROS levels. This accumulation of ROS leads to oxidative DNA damage, contributing to the formation of DSBs in these cells. These DSBs can activate ataxia telangiectasia mutated (ATM) and Rad-3–related (ATR) kinases, triggering cellular stress responses that lead to cell cycle arrest, apoptosis, and increased oxidative stress [42]. ROS production is also linked to the homologous recombination (HR) and non-homologous end joining (NHEJ) pathways [43]. Our previous findings have shown that ATF3 overexpression impairs cell growth by inducing significant apoptosis and cell cycle arrest in cervical cancer cells, particularly in HPV-infected HeLa and Ca Ski cells, through the downregulation of the NF-κB signaling pathway [2, 3]. Taken together, this suggests that the cell cycle arrest and apoptosis observed with ATF3 overexpression may be partly due to ROS accumulation caused by ATF3 expression—an alternative pathway alongside the downregulation of the NF-κB signaling pathway. Studies have shown that combination therapies using ROS-inducing agents with other treatments, including polyphenolic compounds [44, 45], and biodegradable polymers, are being explored to modulate ROS levels specifically at tumor sites [46]. These strategies enhance chemotherapy efficacy while minimizing side effects and improve treatment outcomes by enhancing drug delivery.

Conclusions

ATF3 contributes to tumor suppression in cervical cancer by modulating oxidative stress and DNA damage, selectively targeting genes involved in metastasis. Our results suggest that inducing ATF3 expression may be a potential therapeutic strategy to maintain excessive ROS levels, leading to oxidative stress, DNA damage, and apoptosis in cervical cancer cells. While ROS play a significant role in cervical cancer pathogenesis and treatment, their management remains complex. Future research should focus on optimizing ROS modulation strategies to enhance therapeutic efficacy while minimizing adverse effects.

Abbreviations

ATF3	Activating Transcription Factor 3
ATM	Ataxia Telangiectasia Mutated
ATR	ATM and Rad3-Related
DCFDA	2′,7′-Dichlorofluorescein Diacetate
DCFH-DA	2′,7′-Dichlorodihydrofluorescein Diacetate
GPX4	Glutathione Peroxidase 4
HPV	Human Papillomavirus
HR	Homologous Recombination
MDA	Malondialdehyde
MMP1	Matrix Metalloproteinase-1
NHEJ	Non-Homologous End Joining
ROS	Reactive Oxygen Species
SHARP1	Basic Helix-Loop-Helix Transcription Repressor

Acknowledgements

The authors would like to acknowledge Haniyeh Abouei for formal analysis and data validation from division of medical biotechnology, department of medical laboratory sciences, school of paramedical sciences, Shiraz university of medical sciences, Shiraz, Iran.

Author contributions

E.N. performed the methodology and prepared the original draft, M.K. supervised the project, M.J.M. provided resources and data interpretation and A.F. conceptualized, carried out the statistical analysis and reviewed and edited the manuscript. All authors reviewed the manuscript.

Funding

No funds, grants, or other support were received during the preparation of this manuscript.

Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethical approval

In this study, all the experiments were approved by the Institutional Ethics Committee (approval number: (IR.IAU.SIRAZ.REC.1402.278), Islamic Azad University, Shiraz, Iran. All methods and experiments were carried out in accordance with relevant guidelines and regulations.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

Received: 23 January 2025 / Accepted: 18 February 2025
Published online: 24 February 2025

References

1. Wu D, Liang J. Activating transcription factor 4: a regulator of stress response in human cancers. Front Cell Dev Biol. 2024;27:12:1370012.
2. Kooti A, Abuei H, Farhadi A, Behzad-Behbahani A, Zarrabi M. Activating transcription factor 3 mediates apoptotic functions through a p53-independent pathway in human papillomavirus 18 infected HeLa cells. Virus Genes. 2022;58(2):88–97.
3. Akbarpour Arsanjani A, Abuei H, Behzad-Behbahani A, Bagheri Z, Arabsolghar R, Farhadi A. Activating transcription factor 3 inhibits NF–κB p65 signaling pathway and mediates apoptosis and cell cycle arrest in cervical cancer cells. Infect Agent Cancer. 2022;15(1):62.
4. Yu X, Li Z, Bai R, Tang F. Transcriptional factor 3 binds to Sirtuin 1 to activate the Wnt/β-catenin signaling in cervical cancer. Bioengineered. 2022;2(5):12516–31.
5. Ye X, Zhang P, Tao J, Wang JCK, Mafi A, Grob NM, Quartararo AJ, Baddock HT, Chan LJG, McAllister FE. Discovery of reactive peptide inhibitors of human papillomavirus oncoprotein E6. Chem Sci. 2023;14(44):12484–97.
6. Shai A, Pitot HC, Lambert PF. E6-associated protein is required for human papillomavirus type 16 E6 to cause cervical cancer in mice. Cancer Res. 2010;15(12):5064–73.
7. Vats A, Skrabar N, Del Sal G, Banks L. Loss of the E6AP ubiquitin ligase induces p53-dependent phosphorylation of human papillomavirus 18 E6 in cells derived from cervical cancer. J Virol. 2022;23(6):e01503–21.
8. Liao Y, Lu W, Che Q, Yang T, Qiu H, Zhang H, He X, Wang J, Qiu M, Zou Y. SHARP1 suppresses angiogenesis of endometrial cancer by decreasing hypoxia-inducible factor-1α level. PLoS ONE. 2014;11(6):e99907.
9. Wang D, Li Q, Li K, Xiao P, Yin R. Twist-related protein 1-mediated regulation of mesenchymal change contributes to the migration and invasion of cervical cancer cells. Oncol Lett. 2015;1;10(5):3107-12.
10. Wei S, Wang H, Lu C, Malmut S, Zhang J, Ren S, Yu G, Wang W, Tang DD, Yan C. The activating transcription factor 3 protein suppresses the oncogenic function of mutant p53 proteins. J Biol Chem. 2014;8;289(13):8947-59.

11. Kurnia I, Rauf S, Hatta M, Arifuddin S, Hidayat YM, Natzir R, Kaelan C, Bukhari A, Pelupessy NU, Patelonggi IJ. Molecular patho-mechanisms of cervical cancer (MMP1). Ann Med Surg. 2022;1;77:103415.
12. Zhang W, Huang X, Huang R, Zhu H, Ye P, Lin X, Zhang S, Wu M, Jiang F. MMP1 overexpression promotes cancer progression and associates with poor outcome in head and neck carcinoma. Comput Math Methods Med. 2022;2022(1):3058342.
13. Guenzle J, Wolf LJ, Garrelfs NWC, Goeldner JM, Osterberg N, Schindler CR, Saavedra JE, Weyerbrock A. ATF3 reduces migration capacity by regulation of matrix metalloproteinases via NFκB and STAT3 inhibition in glioblastoma. Cell Death Discov. 2017;27(1):1–2.
14. Abdullah H, Sazeli S, Mamat N, Hapidin H, Sulong S. ROS modulate cell death mechanism in cervical cancer cells treated with the combination of polyphenolic compounds and anticancer drug cisplatin: a review. Curr Cancer Ther Rev. 2023;1;19(4):334–48.
15. Ke H, Chen Z, Zhao X, Yang C, Luo T, Ou W, Wang L, Liu H. Research progress on activation transcription factor 3: a promising cardioprotective molecule. Life Sci. 2023;1;328:121869.
16. Liu S, Li Z, Lan S, Hao H, Baz AA, Yan X, Gao P, Chen S, Chu Y. The dual roles of activating transcription factor 3 (ATF3) in inflammation, apoptosis, ferroptosis, and pathogen infection responses. Int J Mol Sci. 2024;9;25(2):824.
17. Lu H, Zheng Y, Wang D. ATF3 affects osteogenic differentiation in inflammatory hPDLSCs by mediating ferroptosis via regulating the Nrf2/HO-1 signaling pathway. Tissue Cell. 2024;15:102447.
18. Deng S, Yan T, Nikolova T, Fuhrmann D, Nemecek A, Gödtel-Armbrust U, Kaina B, Wojnowski L. The catalytic topoisomerase II inhibitor dexrazoxane induces DNA breaks, ATF 3 and the DNA damage response in cancer cells. Br J Pharmacol. 2015;172(9):2246–57.
19. Pfaffl MW. A new mathematical model for relative quantification in real-time RT–PCR. Nucleic Acids Res. 2001;29:2002–7.
20. Singh NP, McCoy MT, Tice RR, Schneider EL. A simple technique for quantitation of low levels of DNA damage in individual cells. Exp Cell Res. 1988;1;175(1):184–91.
21. Rr T. The single cell Gel/comet assay. Guidelines for in vitro and in vivo genetic toxicology testing. Environ Mol Mutagen. 2000;35:206–21.
22. Chen W, Huang S, Shi K, Yi L, Liu Y, Liu W. Prognostic role of matrix metalloproteinases in cervical cancer: a meta-analysis. Cancer Control. 2021;28(2):10732748211033743.
23. Tian R, Li X, Gao Ye, Li Y, Yang P, Wang K. Identification and validation of the role of matrix metalloproteinase-1 in cervical cancer. Int J Oncol. 2018;1;52(4):1198–208.
24. Ho HH, Antoniv TT, Ji J-D, Ivashkiv LB. Lipopolysaccharide-induced expression of matrix metalloproteinases in human monocytes is suppressed by IFN-γ via superinduction of ATF-3 and suppression of AP-1. J Immunol. 2008;1;181(7):5089-97.
25. Zugowski C, Lieder F, Müller A, Gasch J, Corvinus FM, Moriggl R, Friedrich K. STAT3 controls matrix metalloproteinase-1 expression in colon carcinoma cells by both direct and AP-1-mediated interaction with the MMP-1 promoter. Biol Chem. 2011;392(5):449–59.
26. Ma S, Pang C, Song L, Guo F, Sun H. Activating transcription factor 3 is overexpressed in human glioma and its knockdown in glioblastoma cells causes growth inhibition both in vitro and in vivo. Int J Mol Med. 2015;1;35(6):1561–73.
27. Takagi K, Asano K, Haneishi A, Ono M, Komatsu Y, Yamamoto T, Tanaka T, Ueno H, Ogawa W, Tomita K. Insulin stimulates the expression of the SHARP-1 gene via multiple signaling pathways. Horm Metab Res. 2014;46(06):397–403.
28. Liao Y, He X, Qiu H, Che Q, Wang F, Lu W, Chen Z, Qiu M, Wang J, Wang H. Suppression of the epithelial-mesenchymal transition by SHARP1 is linked to the NOTCH1 signaling pathway in metastasis of endometrial cancer. BMC Cancer. 2014;14:1–0.
29. Catizone AN, Uzunbas GK, Celadova P, Kuang S, Bose D, Sammons MA. Locally acting transcription factors regulate p53-dependent cis-regulatory element activity. Nucleic Acids Res. 2020;7;48(8):4195–213.
30. Zhang S, Huang Y, Yang F, He L, Li Y, Esther Wan YC, Ding Y, Chan KM, Xie T, Sun H. ATF3 preserves skeletal muscle stem cell quiescence by preventing precocious activation. bioRxiv. 2022;12:2022-08.
31. Liu J, Wang B, Wang W, Sun M, Li Y, Jia X, Zhai S, Dang S. Computational networks of activating transcription factor 3 gene in Huh7 cell lines and hepatitis C virus-infected Huh7 cell lines. Mol Med Rep. 2015;1;12(1):1239-46.
32. Zhao J, Li X, Guo M, Yu J, Yan C. The common stress responsive transcription factor ATF3 binds genomic sites enriched with p300 and H3K27ac for transcriptional regulation. BMC Genomics. 2016;17:1–4.
33. Badu P, Baniulyte G, Sammons MA, Pager CT. Activation of ATF3 via the integrated stress response pathway regulates innate immune response to restrict Zika virus. J Virol. 2024;22;98(10):e01055-24.
34. Shu M, Du T, Zhou G, Roizman B. Role of activating transcription factor 3 in the synthesis of latency-associated transcript and maintenance of herpes simplex virus 1 in latent state in ganglia. Proc Natl Acad Sci. 2015;29(39):E5420–6.
35. Sichero L, Sobrinho JS, Villa LL. Identification of novel cellular transcription factors that regulate early promoters of human papillomavirus types 18 and 16. J Infect Dis. 2012;15(6):867–74.
36. Jelic M, Mandic A, Maricic S, Bozin B, Kladar N, Sudji J, Conic BS. Predictive power of oxidative stress biomarkers in recurrence and survival in advanced cervical cancer. Exp Oncol. 2023;11(452):231–41.
37. Aggarwal V, Tuli HS, Varol A, Thakral F, Yerer MB, Sak K, Varol M, Jain A, Khan MA, Sethi G. Role of reactive oxygen species in cancer progression: molecular mechanisms and recent advancements. Biomolecules. 2019;9(11):735.
38. Dasgupta J, Kar S, Liu R, Joseph J, Kalyanaraman B, Remington SJ, Chen C, Melendez JA. Reactive oxygen species control senescence-associated matrix metalloproteinase-1 through c-Jun-N-terminal kinase. J Cell Physiol. 2010;225(1):52–62.
39. Mori K, Uchida T, Yoshie T, Mizote Y, Ishikawa F, Katsuyama M, Shibanuma M. A mitochondrial ROS pathway controls matrix metalloproteinase 9 levels and invasive properties in RAS-activated cancer cells. FEBS J. 2019;286(3):459–78.
40. Savaraj N, Wei Y, Unate H, Liu PM, Wu CJ, Wangpaichitr M, Xia D, Xu HJ, Hu SX, Tien Kuo M. Redox regulation of matrix metalloproteinase gene family in small cell lung cancer cells. Free Radic Res. 2005;1(4):373–81.
41. Liu H, Mo H, Yang C, Mei X, Song X, Lu W, Xiao H, Yan J, Wang X, Yan J. A novel function of ATF3 in suppression of ferroptosis in mouse heart suffered ischemia/reperfusion. Free Radic Biol Med. 2022;20:189–122.
42. Cuadrado M, Martinez-Pastor B, Murga M, Toledo LI, Gutierrez-Martinez P, Lopez E, Fernandez-Capetillo O. ATM regulates ATR chromatin loading in response to DNA double-strand breaks. J Exp Med. 2006;20;203(2):297–303.
43. Li X, Yang C, Wu H, Chen H, Gao X, Zhou S, Zhang T-C, Ma W. DSB-induced oxidative stress: uncovering crosstalk between DNA damage response and cellular metabolism. DNA Rep. 2024;1:141–103730.
44. Li C, Xue Y, Wu J, Zhang L, Yang T, Ai M, Han J, Zheng X, Wang R, Boldogh I. MTH1 inhibition synergizes with ROS-inducing agents to trigger cervical cancer cells undergoing parthanatos. BBA-Mol Basis Dis. 2024;1(5):167190.
45. Gareev I, Jiang J, Beylerli O, Beilerli A, Ilyasova T, Shumadalova A, Bai Y, Du W, Yang B. Adjuvant anti-tumor therapy with polyphenolic compounds: a review. Curr Med Chem. 2024. https://doi.org/10.2174/0109298673284605240301035057
46. Debnath SK, Singh B, Agrawal N, Srivastava R. EPR-selective biodegradable polymer-based nanoparticles for modulating ROS in the management of cervical cancer. In: Chakraborti, S. editors. Handbook of oxidative stress in cancer: therapeutic aspects. edn.: Springer; 2022. pp. 2863–89.

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