The Novel Role of Arsenic (+3 oxidation state) Methyltransferase in Arsenic Genotoxicity

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Research

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Abstract

Background: Arsenic (+3 oxidation state) methyltransferase (*AS3MT*) is the key enzyme in methylation metabolism of arsenic. It is closely related to DNA methylation, but little is known about the novel molecular mechanisms.

Methods: 79 workers and 41 individuals in the control group were recruited. Arsenic, relative indexes, 28 relative RNAs, and base modifications of exon 5-8 of *p53* were detected. Enzyme linked immunosorbent assay (ELISA) was performed to detect the expression of *AS3MT* protein in all subjects. A series of methods were used to analyze the relationships between them. The *AS3MT* protein was detected in A549 and 16HBE cells after treated using sodium arsenite, MMA and DMA for 48 hours. Small interfering RNA (siRNA) transfection was used to investigate the role of *AS3MT* in arsenite-induced tumorigenesis. The cell proliferation and apoptosis were assessed with MTT assay, EdU assay, HO/PI double staining and *JC-1* assay. The real-time quantitative PCR (qRT-PCR) and Western Blot analyses were used to evaluate the expression of genes. The *p53* luciferase reporter gene assay and Co-immunoprecipitation (Co-IP) were used to identify the interactions of target proteins.

Results: *AS3MT* RNA is closely related to *p53*, a series of ncRNAs and mRNAs, and likely to have causal correlations. Base modifications of *p53*, *miR-548* and *miR-190* have significant distinctive effects, but arsenic may play limited roles. *AS3MT* is over expression in lung cancer patients who have not exposed to arsenic, human lung adenocarcinoma and bronchial epithelial cells with arsenic treatment for 48h. *AS3MT* protein is induced in arsenic exposed population. Down regulation of *AS3MT* inhibit proliferation and promotes apoptosis of cells. Mechanistically, *AS3MT* specifically bind with *c-Fos*, and block the binding ability between *c-Fos* and *c-Jun*. Additionally, knockdown of *AS3MT* mediated by siRNA enhance the phosphorylation level of *p53* Ser392 through activating *p38* MAPK. These probably lead to activation of *p53* signaling and up regulation in downstream targets, such as *p21*, *Fas*, *Puma* and *Bax*.

Discussion: Here showed that *AS3MT* RNA plays a great role in the genotoxicity and carcinogenesis which started by arsenic, but influenced by other factors. Up regulation of *AS3MT* can directly act on cell, and affect cell proliferation and apoptosis through activation of *p53* signaling and up regulation in downstream targets.

1. Background

Arsenic (+3 oxidation state) methyltransferase (*AS3MT*) is the key enzyme in the methylation metabolism of arsenic (1–3). It promotes methyl transfer to form methyl arsenate and dimethyl arsenate, which play an important role in the formation and development of toxicity (4). *AS3MT* is closely relative to DNA methylation. It probably disrupts DNA methylation in genome, especially in promoter regions of some genes which possess important functions (4, 5). Scientists used to believe that polymorphisms of *AS3MT* were associated with special carcinoma by affecting arsenic methylation capacity (6). Previous results indicate that *AS3MT* is not only involved in arsenic metabolism, but also could regulate the development of tumours (7). However, it is still unknown whether *AS3MT* could directly act on cells and promotes the growth of arsenic-induced tumours by regulating cell apoptosis and proliferation.

Lung tissue is considered to be the most sensitive site for arsenic toxicity, and long-term exposure to arsenic is closely associated with pulmonary malignant tumor(8). The preliminary investigation indicated chronic exposure to arsenic can increases the risk of lung cancer(9). Reports revealed NaAsO₂ can induce the oncogenic transformation of human lung bronchial epithelial cells via *STAT3/miR-21/PDCD4* signaling pathway (10). These results provide a basis for the study of arsenic carcinogenesis process.

The imbalance of cell proliferation and apoptosis is a crucial mechanism of tumorigenesis and progression. *P53*, as a typical tumor suppressor gene, play an important role in regulating this unbalance state. Studies have shown that *p53* mutation or deficiency has been found in about 50% of human malignant tumors (11). *P53* abnormal expression is influenced not only by phosphorylation, but also its transcriptional level. It is regulated by activator protein-1 (*AP-1*) that mainly composed of *Jun* and *Fos* (12, 13). Under normal circumstances, *p53* protein remains at very low level in cells. When external stimuli, DNA damage or the activation of proto-oncogenes, the expression of *p53* is increased and half-life is prolonged(14, 15). There is an increase

tendency of *p53* in human peripheral blood mononuclear with arsenic treatment and *AS3MT* knockdown(16). But, the molecular mechanism of how *AS3MT* regulate the expression of *p53* is entirely unknown.

According to the preliminary bioinformatics software analysis and function verification experiment in cell culture, combined with the comprehensive analysis of literature reports, it is found that miRNAs, such as *miR-548* and *miR-190*, play an important regulatory role in the process of *AS3MT*, and affect methylation metabolism of arsenic (17, 18). It may lead to genetic toxicity with great differences. The initial role of these RNAs is probably not to target arsenic, but become the main regulatory factor with human exposure to arsenic ceaselessly, and possess the potential to gradually evolve into the major regulatory factor in toxicity and methylation metabolism of arsenic. Such as the remarkable feature of *miR-548* is low conservation and high evolution (19). *MiR-548c-3p* may play an important role in the methylation metabolism and toxic effects of arsenic. It may through adaptive changes of key bases.

In this study, the expression pattern of *AS3MT* RNA and protein, and influencing factors were explored in vivo and in vitro, and plan to understand the processes and metabolism involved in carcinogenesis.

2. Materials And Methods

2.1 Study subjects

It is recruited that total of 76 workers in arsenic plants which were producing As_2O_3 and 23 farm laborers who resided in villages away from the arsenic plants more than 50 km and had similar living conditions to the arsenic exposure workers. Their demographic characteristics such as age, sex, education, years of service, chronic health problem, smoking and drinking and family medical history were collected by a questionnaire. In obtaining informed consent, 5.0mL venous blood were collected and placed in -80°C freezer for enzyme-linked immunosorbent assay, and used acid-washed tubes to collect 20.0 mL spot urine and immediately transferred to -20°C freezer for subsequent measurement. The project was permitted by the ethics committee of Yunnan center for disease control and prevention.

2.2 Reagents and standards

Arsenate ($\text{Na}_3\text{AsO}_4 \cdot 12\text{H}_2\text{O}$), arsenite (NaAsO_2), HCl, NaOH, and NaBH_4 are purchased from Shanghai Chemical Co. (Shanghai, China). All reagents used in this study are analytical grade and As free (<0.01 mg/L). It was used a mixed As standard of 1000 mg/L methylarsonic acid (MMA) and dimethylarsinic acid (DMA) (Tri Chemical Laboratories Inc., Yamanashi, Japan). Inorganic arsenic (iAs) standard of 1000 mg/L were acquired from the National Center for Standard Reference Materials (Beijing, China). Standard reference material of freeze-dried urine (SRM 2670) for toxic metals was obtained from the U.S. National Institute of Standards and Technology (NIST, Gaithersburg, MD, USA).

2.3 Sample collection

Written instructions regarding the hygienic conditions for collection of samples and polyethylene containers which treated with hydrochloric acid and rinsed with deionized water were provided to all participants. Subjects were asked to provide the first morning void urine. At the same time, blood samples were collected, and total DNA and RNA were extracted in 12 hours.

2.4 Determination of As metabolisms

1.0 mL of frozen urine sample was thawed at room temperature and digested with 2N-NaOH at 95°C for 3 h, followed by dilution with Milli-Q water. Next, the atomic absorption spectrophotometer (AA-6800) with an As speciation pretreatment system (ASA-2SP, Shimadzu Co. Kyoto, Japan) was performed to analyze the concentrations of iAs, MMA and DMA in urine. Arsenic speciation analysis was based on the well-established hydride generation of volatile arsines, followed by cryogenic separation in liquid nitrogen. The detection limit of this method was 2ng. Mg/g creatinine was used to indicate urinary arsenic concentration. Primary methylation index (PMI) = (MMA+DMA)/tAs, secondary methylation index (SMI) = DMA/(MMA+DMA).

2.5 Real-time quantitative PCR (qRT-PCR)

Relative RNAs and β -actin sequence (control fragment) are selected in this study. Total RNA (1 mg) was extracted using Trizol reagent (Invitrogen) following manufacturer's instruction, and then transcribed into cDNA with NCode™ VILO™ miRNA cDNA Synthesis Kit (Invitrogen). qRT-PCR was performed with the Platinum® SYBR® Green qPCR Super Mix-UDG (Invitrogen) in ABI7900(Applied Biosystems, America).

DNA was used to detect base modifications of exon 5-8 of *p53*. PCR primers were designed to amplify 4 exons of *p53*, and β -actin sequence (control fragment). High $\Delta\Delta Ct$ ($\Delta\Delta Ct = Ct_i - Cto$, Ct_i is one exon of *p53*, Cto is β -actin sequence) means high base modification, which related to severe DNA damages.

The total RNA from A549 and 16HBE cells was extracted using the TRIzol® Reagent (Vazyme, Nanjing, China). The cDNA was synthesized using the HiFiScript cDNA Synthesis Kit(CoWin Biotech,Beijing, China), and then analyzed by qRT-PCR in a LightCycler® 96 instrument Real-Time PCR system (Roche Molecular Systems, California,USA) using the SYBR Green PCR Master Mix (CoWin Biotech, Beijing, China). The sequences of primers are listed in Supplementary Table 1.

2.6 Enzyme-linked immunosorbent assay The level of *AS3MT* protein was quantitatively detected by ELISA method. The peripheral venous blood samples were collected from arsenic exposed population and control subjects, and tested by using commercial human *AS3MT* ELISA Kits (Qcheng, Shanghai, China) according to the manufacturer's instruction. The results were measured by the enzyme mark instrument at the absorbance of 450 nm.

2.7 Cell culture and treatment

A549 adenocarcinomic cells and 16HBE human bronchial epithelial cells were purchased from the Kunming Institute of Zoology (Kunming, China) in 2019. The 60T-16HBE cells, which were transformed by 16HBE cells exposed to 2.5 μ M NaAsO₂ for 60 passages, were provided by Dr. Che WJ. A549 cells were cultured in RPMI-1640/10% fetal bovine serum (FBS)/1% penicillin and streptomycin. 16HBE and 60T-16HBE cells were maintained MEM medium supplemented with 10%FBS and 1% penicillin and streptomycin. 2.5 $\times 10^5$ cells were resuspended and plated at 6-well plate. After 22h, A549 and 16HBE cells were exposed to different concentrations of NaAsO₂, MMA and DMA for 48 hours.

2.8 siRNA transfection

A549 and 16HBE cells were plated at 1 $\times 10^5$ per well in 6-well plate and cultured for another 19 hours, then transfect using RFect siRNA transfection reagent (Biogenerating Biotechnologies, Changzhou, China) according to the manufacturer's instruction. The transfection efficiency was measured by observing with fluorescence microscope and detecting the *AS3MT* using qRT-PCR and western blot after transfected for 72 hours. Small-interfering RNA duplexes (siRNA) against *AS3MT* (si*AS3MT*#1 and si*AS3MT*#2) and siRNA control (siCtrl) were purchased from GenePharma (Shanghai,China). The sequences of si*AS3MT* and siCtrl were listed in Supplementary Table 2.

2.9 Cell viability

For NaAsO₂ treatment, A549, 16HBE and 60T-16HBE cells were plated at 8000 per well in 96-well plate. After 22h, cells were treated with different concentrations of NaAsO₂ for 48h. For transient transfection, A549 and 16HBE cells were plated at 3000 per well in 96-well plate and transfected with si*AS3MT* for 72h. After that, 10 μ L of CCK-8 (Beyotime, Beijing, China) was added directly to per well. After 1-2h incubation, the results were measured by the enzyme mark instrument at the absorbance of 450 nm.

2.10 Cell proliferation assay

BeyoClick™ EdU Cell Proliferation Kit with Alexa Fluor 555 (Beyotime, Beijing, China) was used to assess the effect of *AS3MT* silencing on cell proliferation according to the manufacturer's protocol. In brief, A549 and 16HBE cells were plated at 0.75 $\times 10^5$ per well in 6-well plate. Cells transfected after 72 hours, the cells were gently rinsed twice with 1 $\times$ PBS, and incubated with EdU working solution for 2 h at 37°C in darkness. Then, the cells were gently rinsed by 1 $\times$ PBS and fixed with 4% paraformaldehyde for 30min at room temperature. After incubating with 0.3% TritonX-100 in PBS, and covered with Click reaction solution. The cell nuclear was stained with Hoechst 33342, and photographed by an inverted fluorescence microscope with excitation wavelength at 565 nm and 460 nm.

2.11 Cell apoptosis assay

A549 and 16HBE cells were plated at 1×10^5 per well in 6-well plate or 3000 per well in 96-well plate and transfected for another 72 hours. Next, cells apoptosis were measured by HO/PI double staining (BestBio, Shanghai, China) and mitochondrial membrane potential (*JC-1*) (Beyotime, Beijing, China) according to those manufacturer's instructions. For HO/PI double staining, the cells in 6-well plates were gently rinsed twice with $1 \times$ PBS, and stained with Hoechst 33342 (10 ng/ml) and PI (10 ng/ml) for 20 min at 26°C in darkness. Finally, the condensed or fragmented nuclei of apoptotic cells were observed by an inverted fluorescence microscope with excitation wavelength at 350 nm and 488 nm. For *JC-1* assay, cells in 96-well plates were gently rinsed twice with $1 \times$ PBS, and stained with *JC-1* staining solution (5 µg/ml) for 15 min at 37 °C in the darkness. The mitochondrial membrane potential was detected by a multifunctional microplate reader with excitation wavelength at 485 nm and 550 nm. Mitochondrial depolarization is described by an increase in the green/red fluorescence intensity ratio.

2.12 Luciferase reporter gene assay

The *p53* transcription activity was measured by the Firefly Luciferase Reporter Gene Assay Kit (Beyotime, Beijing, China) according to the manufacturer's protocols. A549 and 16HBE cells were plated at 1×10^5 per well in 6-well plate. After 18h, the cells were co-transfected with the luciferase reporter pp53-TA-luc (Beyotime, Beijing, China) and siAS3MT using Rfect siRNA transfection reagent and Rfect Plasmid DNA Transfection Reagent (Biogenerating Biotechnologies, Changzhou, China) respectively. Cells transfected after 72 hours, the cells were harvested and lysed, and protein concentration was normalized by Enhanced BCA Protein Assay Kit (Beyotime, Shanghai, China). Finally, the luciferase activity was measured by multifunctional microplate reader and described as a fold change compared to siCtrl+ pp53-TA-luc group. A549 and 16HBE cells were separately divided into three groups: siCtrl + pp53-TA-luc group, siAS3MT#1+ pp53-TA-luc group and siAS3MT#2+ pp53-TA-luc group.

2.13 Co-immunoprecipitation assays

A549 and 16HBE cells were harvested, and lysed using RIPA lysis buffer (Thermo Scientific, USA) containing protease inhibitors and phosphatase inhibitors (Beyotime, Beijing, China). Total protein (1mg) was incubated with 2 µg anti-*c-Jun* antibody (Hangzhou Huaan, ET1608-3) or 2 µg anti-*c-Fos* antibody (Proteintech, 66590-1-Ig) or rabbit IgG (Bclx) (Hangzhou Huaan, RE6009), or mouse IgG (Abclonal, AC011) on a rotating shaker at 4°C overnight. Then the protein A/G magnetic beads (Medchemexpress, HY-K0202) were added to the antigen/ antibody complex and shaken at 4°C for 6h. Subsequently, the beads were washed for three times and denatured in $1 \times$ loading buffer at 95°C for 5min. Finally, collected samples were measured using western blot.

2.14 Protein binding ability assays

The Co-immunoprecipitation assay was used to evaluate binding ability of *c-jun* to *c-Fos*. In brief, cells transfected after 72 hours, A549 and 16HBE cells were lysed and incubated with anti-*c-Jun* antibody or anti-*c-Fos* antibody. Finally, the *c-Fos* and *c-Jun* protein levels were assessed by western blot, and *c-Jun* and *c-Fos* were used as an internal control respectively.

2.15 Western blot analysis

Total protein was extracted from A549, 16HBE and 60T-16HBE cells using RIPA lysis buffer containing protease inhibitors and phosphatase inhibitors. The protein concentration was normalized using Enhanced BCA Protein Assay Kit. 30 µg of protein was subjected to 10% SDS-PAGE, and then electronically transferred to PVDF membranes (Roche, German) via semi-dry transfer method (BioRad, USA). The membranes were blocked with 5x Protein Free Rapid Blocking Buffer (EpiZyme, PS108, China) and incubated with primary antibodies overnight at 4°C. The membranes were washed four times using TBST and then incubated with the corresponding secondary antibodies at room temperature, but Mouse anti-Rabbit IgG light chain for immunoprecipitation was used. The bands were detected by Gel-Pro Analyzer software. Antibodies information is listed in Supplementary Table 3.

2.16 Statistical analysis

The concentrations of iAs, MMA and DMA are log-transformed to improve the normality of measures. After assessing the association among lncRNAs, miRNAs, mRNAs, base modifications of 4 fragments of *p53* and three arsenic species were

performed for the analysis under different levels of arsenic trioxide by correlation analysis, covariance and independent - samples *t*-test. Then the association between arsenic species, 4 fragments of *p53* and all lncRNAs, miRNAs and mRNAs were investigated. All statistical tests were two-side, with $p < 0.05$ is considered statistically significant. Statistical analyses were performed using SPSS software for above data (Version 23, USA).

Then data analyses were performed using ImageJ software, and GraphPad Prism 7.0 software. The difference between two groups and multiple group comparisons were analyzed by Student's *t*-test and one-way ANOVA, respectively. Wilcoxon rank sum test was used for the non-normal distribution of data.

Pearson correlation coefficient was performed to analyze associations between concentrations of different arsenic species and *AS3MT* expression. The data were described as the means $\pm$ standard error of the mean (SEM). Differences were considered statistically significant at $p < 0.05$.

3. Results

There are no essential differences in sex, smoking and alcohol consumption, and other factors between the exposed workers and control group in molecular epidemiological study. It did not been found that the number of years a person had worked in the plant affect the results. The workers had always worked and moved around the plant. Based on the characteristic of chemical components of ore and production techniques flow adopted, there are few other occupational hazard factors except for arsenic pollution in the selected plants.

3.1 Mean comparison for As species and relative indexes, base modifications of exon 5-8 of *p53*, RNA of *AS3MT*, relative lncRNAs, miRNAs and mRNAs between the workers and control group

Compared to control group, the increase of iAs, MMA and DMA are statistically significant in workers ($p \leq 0.05$). There exist statistically significant differences for PMI and SMI ($p \leq 0.05$). It is found significant changes for base modifications of exon 5-8 of *p53* and all selected RNAs in the peripheral blood of workers ($p \leq 0.05$). The expressions of most selected RNAs became higher, but *miR-548* and *miR-190* became lower ($p \leq 0.05$). Seen Figure 1.

3.2 Correlation analysis between RNA of *AS3MT* and arsenic compounds, or relative indexes, or lncRNAs, or mRNAs, or miRNAs, or base modifications of exon 5-8 of *p53*

As shown in table 1-2., Supplementary Figure 1-3, the log(DMA), all selected lncRNAs and mRNAs, base modifications of exon 7 and 8 of *p53* are positive correlation with the RNA of *AS3MT*, but *miR-548*, *miR-190* and base modification of exon 5 of *p53* are negative correlation with the RNA of *AS3MT* ($p \leq 0.05$).

3.3 Correlation analysis between log(iAs), or log(MMA), or log(DMA), or PMI, or SMI and relative lncRNAs, or mRNAs, or miRNAs, or base modifications of exon 5-8 of *p53* (r_{sp})

As shown in Table 1,3., the base modifications of exon 5 or 6 of *p53* is positive correlation with log(iAs) or log(MMA), but base modification of exon 8 of *p53* is negative correlation with log(iAs) or log(MMA) ($p \leq 0.05$). The *HAI1*, *MALAT1*, mRNAs of *trbp*, *dicer*, *ago2*, base modifications of exon 6 of *p53* are positive correlation with log(DMA) ($p \leq 0.05$). The mRNAs of *lin28b*, *dicer*, *ago2*, *mdm2*, *bcl2*, base modifications of exon 7 and 8 of *p53* are positive correlation with PMI, but *MiR-190* is negative correlation with PMI ($p \leq 0.05$). The *MALAT1*, mRNAs of *dicer*, *ago2*, *sox4*, *mdm2*, *noxa*, *bcl2*, base modifications of exon 7 and 8 of *p53* are positive correlation with PMI ($p \leq 0.05$).

Table 1
Correlation analysis between As indexes, AS3MT RNA, miRNAs and base modifications of p53 (*r*, *p*)

Variable	AS3MT RNA	<i>miR-548</i>	<i>miR-190</i>	Exon 5 of <i>p53</i>	Exon 6 of <i>p53</i>	Exon 7 of <i>p53</i>	Exon 8 of <i>p53</i>
Log(iAs)	-0.012,0.918	0.048,0.679	0.055,0.637	0.336,0.003*	0.247,0.030*	-0.136,0.240	-0.411,0.000*
Log(MMA)	0.057,0.623	0.061,0.600	-0.019,0.869	0.238,0.037*	0.261,0.022*	-0.006,0.959	-0.320,0.005*
Log(DMA)	0.232,0.043*	0.041,0.723	-0.063,0.588	0.186,0.106	0.244,0.033*	0.137,0.236	-0.066,0.574
PMI	0.11,0.922	-0.031,0.791	-0.262,0.022*	-0.190,0.098	-0.007,0.953	0.304,0.007*	0.294,0.010*
SMI	0.137,0.236	-0.112,0.337	-0.054,0.708	-0.146,0.205	-0.142,0.219	0.273,0.016*	0.476,0.000*
<i>miR-548</i>	-0.331,0.003*		0.434,0.000*	-0.002,0.988	0.047,0.686	0.022,0.850	-0.150,0.198
<i>miR-190</i>	-0.281,0.014*	0.434,0.000*		0.027,0.815	-0.005,0.967	-0.207,0.072	-0.357,0.002*
Exon 5 of <i>p53</i>	-0.263,0.021*	-0.002,0.988	0.027,0.815		0.527,0.000*	-0.196,0.088	-0.410,0.000*
Exon 6 of <i>p53</i>	-0.152,0.188	0.047,0.686	-0.005,0.967	0.527,0.000*		0.318,0.005*	-0.069,0.551
Exon 7 of <i>p53</i>	0.420,0.000*	0.022,0.850	-0.166,0.152	-0.196,0.088	0.318,0.005*		0.575,0.000*
Exon 8 of <i>p53</i>	0.376,0.001*	-0.150,0.198	-0.357,0.002*	-0.410,0.000*	-0.069 0.551	0.575,0.000*	
Stars (*) indicate that $p \leq 0.05$							

Table 2
Correlation analysis between relative RNAs and base modifications of p53 (r, p)

Variable	AS3MT RNA	miR-548	miR-190	Exon 5 of p53	Exon 6 of p53	Exon 7 of p53	Exon 8 of p53
<i>Lnc-p21</i>	0.485,0.000*	-0.318,0.005*	-0.102,0.386	-0.142,0.221	-0.231,0.045	0.242,0.035*	0.068,0.560
<i>MEG3</i>	0.502,0.000*	-0.362,0.001*	-0.227,0.051	-0.069,0.553	-0.217,0.060	0.161,0.164	0.060,0.609
<i>PANDA</i>	0.261,0.023*	-0.202,0.082	-0.124,0.291	-0.079,0.497	-0.237,0.039*	-0.080,0.491	-0.068,0.560
<i>HAIR</i>	0.471,0.000*	-0.242,0.036*	-0.067,0.569	-0.062,0.597	-0.221,0.055	0.039,0.738	-0.084,0.474
<i>HTTIP</i>	0.572,0.000*	-0.358,0.005*	-0.127,0.333	-0.088,0.502	-0.221,0.087	0.200,0.121	0.097,0.459
<i>TUG1</i>	0.469,0.000*	-0.075,0.524	-0.019,0.874	-0.228,0.047*	-0.210,0.069	0.260,0.023*	0.072,0.542
<i>MALAT1</i>	0.670,0.000*	-0.222,0.056	-0.198,0.088	-0.214,0.064	0.016,0.891	0.497,0.000*	0.436,0.000*
<i>lin28</i>	0.816,0.000*	-0.243,0.036*	-0.292,0.011*	-0.239,0.037*	-0.048,0.860	0.393,0.000*	0.351,0.002*
<i>lin28b</i>	0.731,0.000*	-0.015,0.914	-0.403,0.003*	-0.266,0.054	0.148,0.290	0.563,0.000*	0.414,0.002*
<i>dicer</i>	0.672,0.000*	-0.048,0.681	-0.278,0.016*	-0.247,0.031*	-0.003,0.982	0.453,0.000*	0.404,0.000*
<i>exportin-5</i>	0.829,0.000*	-0.249,0.031*	-0.343,0.003*	-0.259,0.024*	-0.052,0.655	0.426,0.000*	0.372,0.001*
<i>trbp</i>	0.647,0.000*	-0.273,0.031*	-0.389,0.002*	-0.119,0.352	-0.011,0.933	0.484,0.000*	0.337,0.007*
<i>ago2</i>	0.619,0.000*	-0.029,0.809	-0.387,0.001*	-0.286,0.014*	0.007,0.950	0.559,0.000*	0.521,0.000*
<i>hox10</i>	0.652,0.000*	-0.031,0.820	-0.233,0.081	-0.297,0.024*	-0.147,0.269	0.331,0.011*	0.298,0.024*
<i>sox4</i>	0.580,0.000*	-0.058,0.619	-0.349,0.002*	-0.294,0.010*	-0.051,0.664	0.348,0.002*	0.348,0.002*
<i>p15</i>	0.531,0.000*	-0.150,0.202	-0.128,0.277	-0.175,0.136	0.053,0.655	0.396,0.000*	0.074,0.535
<i>mdm2</i>	0.668,0.000*	-0.086,0.463	-0.241,0.037*	-0.271,0.018*	0.049,0.673	0.554,0.000*	0.422,0.000*
<i>Pcna</i>	0.330,0.004*	-0.120,0.307	-0.227,0.052	-0.114,0.333	0.015,0.901	0.104,0.376	-0.010,0.935
<i>Tgf-β</i>	0.532,0.000*	-0.029,0.800	-0.267,0.020*	-0.153,0.183	0.093,0.423	0.356,0.001*	0.256,0.026*
<i>Bax</i>	0.514,0.000*	0.036,0.760	-0.176,0.130	-0.240,0.037*	0.046,0.696	0.386,0.001*	0.242,0.036*
<i>Noxa</i>	0.663,0.000*	0.036,0.763	-0.162,0.168	-0.301,0.009*	-0.082,0.483	0.500,0.000*	0.363,0.001*
<i>Puma</i>	0.442,0.000*	-0.043,0.711	-0.144,0.216	-0.127,0.271	0.059,0.612	0.315,0.005*	0.081,0.488
<i>bcl2</i>	0.706,0.000*	-0.059,0.613	-0.148,0.202	-0.285,0.012*	-0.067,0.562	0.480,0.000*	0.484,0.000*
<i>Fas</i>	0.555,0.000*	-0.071,0.550	-0.307,0.008*	-0.299,0.009*	-0.129,0.272	0.330,0.004*	0.225,0.054
<i>p53</i>	0.577,0.000*	-0.036,0.759	-0.076,0.515	-0.190,0.098	0.212,0.064	0.360,0.001*	0.219,0.058
Stars (*) indicate that $p \leq 0.05$							

Table 3
Correlation analysis between relative RNAs and iAs indexes (r , p)

Variable	Log(iAs)	Log(MMA)	Log(DMA)	PMI	SMI
<i>Lnc-p21</i>	-0.109,0.347	-0.066,0.572	-0.016,0.893	-0.015,0.897	0.055,0.640
<i>MEG3</i>	-0.032,0.784	0.035,0.764	0.092,0.427	-0.010,0.934	0.028,0.810
<i>PANDA</i>	0.096,0.409	0.142,0.222	0.098,0.402	-0.064,0.580	-0.153,0.186
<i>HAIR</i>	0.088,0.449	0.185,0.110	0.240,0.036*	-0.033,0.778	-0.086,0.463
<i>HOTTIP</i>	-0.041,0.756	0.067,0.608	0.116,0.375	0.015,0.910	0.001,0.994
<i>TUG1</i>	-0.125,0.283	-0.077,0.509	-0.048,0.681	0.010,0.928	-0.027,0.816
<i>MALAT1</i>	-0.048,0.684	0.033,0.779	0.276,0.016*	0.23,0.844	0.328,0.004*
<i>lin28</i>	0.044,0.708	0.114,0.327	0.144,0.215	0.006,0.962	-0.016,0.894
<i>lin28b</i>	-0.015,0.912	0.093,0.510	0.176,0.208	0.287,0.037*	0.035,0.802
<i>dicer</i>	0.011,0.924	0.105,0.367	0.281,0.014*	0.246,0.032*	0.251,0.029*
<i>exportin-5</i>	0.075,0.522	0.150,0.195	0.221,0.055	0.018,0.878	-0.029,0.805
<i>trbp</i>	0.082,0.520	0.178,0.163	0.359,0.004*	0.038,0.770	-0.040,0.753
<i>ago2</i>	-0.103,0.383	0.015,0.897	0.274,0.018*	0.262,0.024*	0.303,0.009*
<i>hox10</i>	-0.114,0.393	-0.047,0.726	0.063,0.640	0.077,0.567	0.183,0.169
<i>sox4</i>	-0.065,0.575	0.007,0.953	0.084,0.470	0.075,0.520	0.308,0.007*
<i>p15</i>	0.071,0.548	0.159,0.175	0.202,0.084	0.047,0.689	-0.132,0.263
<i>mdm2</i>	-0.045,0.697	0.075,0.518	0.193,0.095	0.257,0.025*	0.267,0.020*
<i>pcna</i>	-0.132,0.263	-0.105,0.373	-0.062,0.598	0.081,0.492	0.013,0.913
<i>Tgf-β</i>	-0.007,0.951	0.028,0.811	0.070,0.546	-0.006,0.961	0.027,0.819
<i>bax</i>	-0.046,0.696	0.032,0.783	0.097,0.406	0.088,0.450	-0.019,0.872
<i>nox4</i>	-0.165,0.158	-0.077,0.510	0.072,0.540	0.112,0.340	0.327,0.004*
<i>puma</i>	-0.026,0.820	0.056,0.628	0.126,0.273	0.067,0.563	0.038,0.742
<i>bcl2</i>	-0.163,0.157	-0.038,0.744	0.138,0.231	0.252,0.027*	0.362,0.001*
<i>fas</i>	0.005,0.964	0.099,0.400	0.086,0.463	0.116,0.323	-0.065,0.579
<i>p53</i>	0.008,0.948	0.078,0.502	0.112,0.333	0.078,0.498	-0.044,0.702
Stars (*) indicate that $p \leq 0.05$					
https://www.editorialmanager.com/thelancetoncology/default.aspx?pg=mainpage.html					

3.4 Correlation analysis between miR-548, or miR-548 and relative lncRNAs, or mRNAs, miRNAs, or base modifications of exon 5-8 of p53(rnp)

As shown in table .1-2., the *miR-190* is positive correlation with *miR-548*, and the *Lnc-p21*, *MEG3*, *MAIR*, *HOTTIP*, mRNAs of *lin28*, *exportin-5*, *trip* are negative correlation with the *miR-548* ($p \leq 0.05$). The mRNAs of *lin28*, *lin28b*, *dicer*, *exportin-5*, *trip*, *ago2*, *sox4*, *mdm2*, *Tgf-β*, *fas*, base modifications of exon 8 of *p53* are negative correlation with the *miR-190* ($p \leq 0.05$).

3.5 Correlation analysis between base modifications of exon 5-8 of p53 and relative lncRNAs, or mRNAs, or miRNAs, or base modifications of exon 5-8 of p53 (r_p)

As shown in table .1-2., base modification of exon 6 of *p53* is positive correlation with base modification of exon 5 of *p53*, and the *TUG1*, mRNAs of *lin28*, *dicer*, *exportin-5*, *ago2*, *hox10*, *sox4*, *mdm2*, *bax*, *nox4*, *bcl2*, *fas*, base modifications of exon 8 of *p53* are negative correlation with base modification of exon 5 of *p53* ($p \leq 0.05$). Base modifications of exon 5 and 7 of *p53* are positive correlation with base modification of exon 6 of *p53*, and the *lnc-p21*, *PANDA* are negative correlation with base modification of exon 6 of *p53* ($p \leq 0.05$). The *TUG1*, *MALAT1*, all selected mRNAs but *pcna*, base modifications of exon 6 and 8 of *p53* are positive correlation with base modification of exon 7 of *p53* ($p \leq 0.05$). The *MALAT1*, all selected mRNAs but not *p15*, *pcna*, *puma*, *fas* and *p53*, base modification of exon 7 of *p53* are positive correlation with base modification of exon 8 of *p53* and the *miR-190*, base modification of exon 5 of *p53* are negative correlation with base modification of exon 8 of *p53* ($p \leq 0.05$).

3.6 The expression of AS3MT is correlated with arsenic exposure

Enzyme-linked immunosorbent assay was performed to assess the expression of *AS3MT* protein in peripheral blood samples from all subjects. The results reveal long-term exposure to arsenic significantly increased *AS3MT* protein compared with the control group (Supplementary Figure 4A). Furthermore, the inter relationship were evaluated between *AS3MT* expression and the urinary arsenic species levels. Pearson correlation coefficient shows *AS3MT* protein is positively linked to tAs, iAs, MMA and DMA (Supplementary Figure 4B-E). All subjects were divided into 2 groups based on PMI or SMI. Results show that the *AS3MT* protein was higher in high PMI group compared to low PMI group (Supplementary Figure 4F-G).

3.7 Arsenic and its metabolites increase AS3MT expression in 16HBE and A549 cells

A549 and 16HBE cells were treated with different concentrations of NaAsO₂, MMA and DMA according to MTT data to clarify the epidemiological results (Supplementary Figure 5A-B), and analyzed the level of *AS3MT* by western blot. For A549 cells, exposure to concentrations of NaAsO₂ (0, 20, 40 and 60 μmol/L), 60 μmol/L MMA and 60 μmol/L DMA significantly increased intracellular *AS3MT*, and show significant dose-dependent relationship (Supplementary Figure 5C). For 16HBE cells, *AS3MT* was significantly increased by NaAsO₂ (2, 4, and 6 μmol/L), 6 μmol/L MMA and 6 μmol/L DMA treatment compared to control group (Supplementary Figure 5D). *AS3MT* in both cell lines treated with NaAsO₂ was evidently higher than that of MMA and DMA at the same concentration.

3.8 Knockdown of AS3MT inhibit cell proliferation and promote cell apoptosis

The *AS3MT* was silenced by Rfect siRNA transfection reagent. qRT-PCR and western blot analysis results show si*AS3MT* successfully knocked down *AS3MT*, and the morphology of the cells were normal (Figure 2A-C). Results imply that these cells suitable for subsequent experiments.

After 72 hours of transfection, the data of CCK-8 assay demonstrated that the cell viability was lower in the si*AS3MT* groups than in the siCtrl group (Figure 3A), which imply *AS3MT* knockdown may inhibit cell proliferation and promote cell apoptosis. Furthermore, Edu staining, HO/PI double staining and *JC-1* assay were performed to verify the inferences. The results of Edu staining reveal that knockdown of *AS3MT* evidently inhibit cell proliferation in A549 and 16HBE cells (Figure 3B-E). Additionally, the number of apoptosis and necrosis significantly increase in si*AS3MT* cells compare to the siCtrl cells, but the late apoptotic cells were clearly observed in the 16HBE cells (Figure 3F-I). Moreover, the mitochondria transmembrane potentials was decreased in si*AS3MT* groups compared to the siCtrl group (Figure 3J). Taken together, the low *AS3MT* inhibit cell proliferation and promotes apoptosis significantly.

3.9 AS3MT inhibit p53 phosphorylation through p38 MAPK

The *p53* were examined by qRT-PCR and western blot in siCtrl and si*AS3MT* cells. Results show *p53* mRNA and protein was higher in si*AS3MT* cells (Figure 4A). Further experiments found that silenced *AS3MT* activate *p53* downstream genes, such as *p21*, *MDM2*, *Fas*, *Puma* and *Bax* (Figure 4C-D). Then, the phosphorylation state of *p53* Ser15 and Ser392 site was assessed.

Western blot show that only Ser392 site was changed in siAS3MT cells. The p38 MAPK of p53 protein kinase was remarkably increased after silencing of AS3MT (Figure 4B). It can be speculated that knockdown of AS3MT can promote p53 Ser392 site phosphorylation through activating p38 MAPK.

3.10 AS3MT inhibit p53 transcription through competitive binding with c-Fos

The Co-Immunoprecipitation assay was done to study the mechanism that AS3MT affect p53 transcription through regulating AP-1 related proteins, and verify the interaction of two proteins. Results show AS3MT, c-Jun and c-Fos were coprecipitated by each antibody in A549 and 16HBE cells (Figure 5A). Then, the interaction between AS3MT and c-Jun, c-Fos proteins were examined, which show AS3MT and c-Fos could be reciprocally co-immunoprecipitated in A549 and 16HBE cell lysates. It is almost impossible for AS3MT binding to c-Jun (Figure 5B). These results suggest AS3MT and c-Jun competitively bond to c-Fos, and inhibit the formation of AP-1 complex. Then, protein binding ability assay after AS3MT silencing were performed to identify whether knockdown of AS3MT increase the binding ability between c-Jun and c-Fos in A549 and 16HBE cells. As shown in Figure 5C, with c-Jun as the internal reference gene, c-Jun could combine more c-Fos in siAS3MT cells than in the siCtrl cells. c-Fos could combine more c-Jun in siAS3MT groups with c-Fos as the internal reference gene. Finally, the transcriptional activity of p53 was measured by the luciferase reporter gene assay, which demonstrat that AS3MT was able to inhibit p53 transcriptional activity (Figure 5D). In summary, AS3MT can affect the formation of AP1 dimer by competitively binding c-Fos, leading to reduce the transcriptional activity of p53.

3.11 AS3MT is over expressed in human lung cancer cell lines

The AS3MT was detected by western blot in different type cells. As shown in Supplementary Figure 6A, AS3MT has the highest level in A549 cell, middle level in 60T-16HBE cells, and relatively low level in 16HBE cell. There was an obvious contrast between the NaAsO₂-tolerance of A549, 16HBE and 60T-16HBE. The improved Karber methodanalysis show the half lethal Concentration (LC50) of A549, 60T-16HBE and 16HBE cells were 64.6μM, 10.7μM and 6.6Mm,respectively (Supplementary Figure 6B-D).

4. Discussion

There exist great individual differences for metabolism transformation and genotoxicity of arsenic (20). In this study, many methods are used to analyze the relationships along various arsenic species and a series of genotoxic markers. There are closely relationships between DMA or PMI or SMI and many genotoxic markers, but between iAs or MMA and genotoxic markers only for base modifications of p53. It suggests that iAs and MMA became DMA will related to many genotoxic changes. This probably related to AS3MT closely. This study show there may no dose-response relationships between arsenic exposure and most relative RNAs. The content and proportion of arsenic compounds may play limited roles.

In this study, the effect of arsenic on AS3MT protein was examined by western blot to identify the relationship between arsenic and AS3MT. A549 and16HBE cells treated with various doses of NaAsO₂ indicate that arsenic has increased AS3MT. It is consistent with previous epidemiological conclusion. AS3MT handled by MMA and DMA was evidently lower, compared with NaAsO₂. It suggests there exert different toxic effects. Results imply various arsenic species have special metabolic rate in human, and iAs, MMA, and DMA may induce different expression models of AS3MT.

Then, the relationship between the mRNA of AS3MT and relative indicators of genotoxicity were analyzed. The hypothesis is that arsenic and its metabolism compounds are the initial inducing factors for mRNA of AS3MT, but not the main influencing factors. There are some regulatory networks along p53, relative ncRNAs and mRNAs, which play special and important roles in arsenic inducing health hazards. A certain dose of inorganic arsenic can induce the change of AS3MT. This dose may vary greatly among individuals, and the vast majority of the selected population has reached this dose. The mRNA of AS3MT is mainly affected by factors other than arsenic, but such as the above RNAs regulatory networks, and changes in base modifications of particular fragments of p53.

It is found that mRNA of AS3MT was closely related to a series of genotoxic indexes. It is particularly noteworthy that the correlation coefficient *r* is greater than 0.3, even 0.7. There is a great possibility of causality. Combined with other reports, the

mRNA of *AS3MT* probably play a novel and very important role in arsenic inducing genotoxicity and tumorigenesis (3, 21).

The *miR-548* and *miR-190* may regulate the mRNA of *AS3MT* through base complementary pairing. They are significantly negatively correlated with mRNA of *AS3MT*. Adaptive changes of specific base in these genes need attention. The results show that *miR-190* is significantly negatively correlated with most selected mRNAs which related to miRNA production and maturation, and some selected mRNAs which related to *p53* and tumor formation and development. *MiR-548* is significantly negatively correlated with many selected lncRNAs and some selected mRNAs which related to miRNA production and maturation. Comprehensive analysis show that both of them are important factors in the regulation of *AS3MT* mRNA, but there are obvious differences in the regulation mechanism. The low conservation and high evolution for *miR-548* need paid great attention (19, 22).

In this study, most of the genes selected are related to *p53*, even belonged to *p53* family (20, 23, 24). It is found that the changes of base modification in different exons of *p53* are closely related to the genetic toxicity and health hazards in workers exposed to arsenic (25–27). In this study, there are significant correlations between the base modifications of *p53* and indexes of methylation metabolism of arsenic. The data tend to have an indirect correlation. The changes of base modification in different exons or sites of *p53* may play different roles by affecting the mRNA of *AS3MT*. The changes of base modifications in near regions seem to have similar effects.

Here it show *AS3MT* RNA play a great role in the genotoxicity and carcinogenesis which started by arsenic, but influenced by many other factors greatly, such as *p53*, relative ncRNAs and mRNAs.

In previous study, *AS3MT* mRNA was over expressed in lung cancer patients who have not exposed to arsenic(28). How *AS3MT* induced by arsenic could directly act on the cell and affect tumorigenesis and progression need to study. Then, the novel role of *AS3MT* was explored by knocking down *AS3MT* in A549 and 16HBE cells. Results indicate that the cell viability and proliferation rate of A549 and 16HBE was depressed after knockdown of *AS3MT*. Conversely, the number of karyopyknosis and/or late age apoptosis was increased significantly in si*AS3MT* cells. In addition, higher levels of cleaved-caspase3 and cleaved-caspase-7 were observed in si*AS3MT* cells. Research have shown that the deregulation of apoptosis and/or up-regulation of tumor cell proliferation play a key role in tumorigenesis and progression in various malignancies, including lung cancer. For example, Xue et al. demonstrated that NaAsO₂ accelerated the HaCaT cell cycle and induced malignant transformation by activating circ100284/ miR-217/ EZH2/ Cyclin D1 axis(29). Furthermore, the low dose of NaAsO₂ activated the ERKs signal pathways, which depressed *p53* activation and blocked apoptosis in human keratinocytes cells(30). Interestingly, the present results show that *p53* and its down-stream gene *p21*, *Bax*, *Puma*, and *Fas* were over expressed in A549and 16HBE cells after *AS3MT* silencing. *P21* is a negative regulator of the cell cycle, as *p53* binds to the *p21* promoter and activates its transcription, which inhibit DNA replication and impede entry into mitosis by binding to *PCNA*(31, 32). However, previous research revealed that *PCNA* and *CDKs* were significantly low expression in si*AS3MT* groups (28). So, it been speculated that *AS3MT* is involved in regulation *p53/p21* axis, and promote cell proliferation. Similarly, *p53* positively regulates the expression of *Bax*, *Puma* and *Fas*, and promote cells apoptosis(33). These results imply that *AS3MT*, as a new proto-oncogene, can promote cell proliferation and inhibit apoptosis by *p53* signaling pathway.

In mammalian cells, *p53*, as a “cellular gatekeeper”, is involved in the regulation of cell cycle arrest, metabolism, apoptosis, proliferation, or senescence(34). Studies indicated that more than 50% of malignant tumors patients have the *p53* mutation or deficiency, and the *p53* is regulated mainly through post-translational mechanisms and/or transcription regulation(11, 12). For instance, γ -irradiation and UV light enhance the phosphorylation levels of *p53* ser15 through *ATR*, which can activate *p53* and promote cell apoptosis(35); Homeodomain-interacting Protein Kinase 2 phosphorylated *p53* Ser46 and induced *p53* Lys382 acetylation(36). This study found that *p38* MAPK and *p53* Ser392 were obviously activated in si*AS3MT* cells. The up regulation of phosphorylated *p53*Ser392 require *p38*MAPK pathway activation, which increase its sequence-specific DNA-binding activity and regulate the balance between proliferation and apoptosis(37). It could be concluded from these results that *AS3MT* can activate *p53* Ser392 by *p38* MAPK and directly involving in cell proliferation and apoptosis regulation. In addition, the transcriptional activity of *p53* was regulated by a large number of transcription factors, including *YB1* (38), *RREB1*(39), *E2F1*(40) and *AP-1*(13). Among them, the expression level of *AP-1* was decreased in cervical cancer cells with As₂O₃ treatment(41). In

mammalian cells, *AP-1* is mainly composed of *Jun* and *Fos*. Based on this, the Co-Immunoprecipitation assay was done to verify the interaction of two proteins. The results showed that *AS3MT* can specifically bind with *c-Fos*, and knockdown of *AS3MT* facilitate the binding ability between *c-Fos* and *c-Jun*. These results indicate that *AS3MT* can specifically bind with *c-Fos* and suppress the activity of *AP-1*, which inhibit *p53* signaling pathway and involve in arsenic carcinogenesis.

Conclusions

AS3MT may serve as a novel and very important protooncogene, which may induced by arsenic, but influenced by *p53*, relative mRNAs and ncRNAs greatly. Further study found that *AS3MT* inhibit *p53* signaling pathway through inhibiting the activation of *p38* MAPK and competitive binding with *c-Fos*, which mean *AS3MT* can directly act on cell, and affect cell proliferation and apoptosis.

Abbreviations

AS3MT: arsenic (+3 oxidation state) methyltransferase

siRNA: small interfering RNA

qRT-PCR: real-time quantitative PCR

Co-IP: co-immunoprecipitation

AP-1: activator protein-1

iAs: inorganic arsenic

MMA: methylarsonic acid

DMA: dimethylarsinic acid

ELISA:enzyme-linked immunosorbent assay

siCtrl: siRNA control

Declarations

Availability of data and materials

All data generated or analysed during this study are included in this published article [and its supplementary information files].

Ethics approval and consent to participate

Ethical approval was given by the Medical Ethics Committee of Yunnan Center for Disease Control and Prevention.

Consent for publication

All authors are aware of and agree to the content of the paper and their being listed as a co-author of the paper.

Availability of data and material

The data and material used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

WW, MS, YH and HC initiated the work and designed the experiments. All authors performed the experiments and wrote the manuscript, contributed techniques and commented on the manuscript, analyzed data, contributed analytic tools, and provided clinical assistance. The authors read and approved the final manuscript.

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Figures

Figure 1

Mean comparison for arsenic species and relative indexes of methylation metabolism, base modification of exon 5-8 of p53, RNA of AS3MT, relative lncRNAs, miRNAs and mRNAs between the workers and control group.

Figure 2

SiAS3MT successfully inhibits the expression level of AS3MT in A549 and 16HBE cell lines. (A) Cells transfected after 72 hours, the transfection efficiency of reagent and the morphology of cells were assessed by fluorescence microscopy. (B) The silence efficiency of AS3MT mRNA was measured by qRT-PCR with β -actin as internal control. Data are presented as means $\pm$ SEM (n=4). Two-tailed Student's t test, compared with the siCtrl group * $p < 0.01$. (C) The silence efficiency of AS3MT protein was estimated by Western blot with β -tubulin as internal control. All experiments were repeated three times independently with similar results, and the data of one representative experiment were used to analyse.

Figure 3

Knockdown of AS3MT evidently inhibit proliferation and promotes apoptosis of A549 and 16HBE cells. (A)After 72 hours of transfection, the A549 and16HBE cells vitality were detected using MTT assay. Data are presented as means±SEM (n=4). Two-tailed Student's t test, compared with the siCtrl group *p< 0.05, * *p< 0.01. (B-C)EdU assay was conducted to measure the proliferation of A549 and 16HBE cells after AS3MT silencing. (D-E)Based on Fig.4b and 4c, the numbers of cells were counted by imageJ software, and the efficiency of cell proliferation was analyzed by Chisquare test. Data are presented as ratio. Compared with the siCtrl group *p < 0.05, * *p< 0.01. (F-G)HO/PI double staining was performed to assess the apoptosis of A549 and 16HBE cells with low expression of AS3MT gene. (H-I)The percentage of apoptotic was analyzed by Chisquare test. For A549 cells, the numbers of nuclear condensation cells were counted. For 16HBE cells, the numbers of late apoptotic cells were counted; Data are presented as ratio. Compared with the siCtrl group *p< 0.05, * *p< 0.01. (J)Cells transfected after 72 hours, the changes in Mitochondrial Membrane Potential were studied by JC-1. Data are presented as means±SEM (n=6). Two-tailed Student's t test, compared with the siCtrl group *p< 0.05, * *p < 0.01.

Figure 4

AS3MT regulates the p53 signaling pathway through p38MAPK and induces cells apoptosis. (A)After 72 hours of transfection, the p53 mRNA expression was measured by qRT-PCR with β -actin as internal control. Data are presented as means $\pm$ SEM (n=4). Two-tailed Student's t test, compared with the siCtrl group * $p < 0.01$. (B)The expression of p53, phospho-p53 and p38 MAPK was detected by western blot in A549 and 16HBE cells after AS3MT silencing. (C)After 72 hours of transfection, the p53 downstream genes MDM2, Fas, Puma, Bax and p21were measured by western blot.(D)The apoptosis implementing protein, Cleaved-Caspase3and Cleaved-Caspase7 were examined using western blot in A549 and 16HBE cells after AS3MT silencing.

Figure 5

AS3MT inhibit p53 transcription by directly targeting c-Fos and reduces the binding ability of c-Jun and c-Fos. (A) Co-immunoprecipitation and western blot assays were used to examine the availability of AS3MT, c-jun and c-Fos antibody for Co-immunoprecipitation. (B) When A549 and 16HBE cells fusion reached about 80%, Co-immunoprecipitation and western blot assays were used to confirm the target of AS3MT. (C) Based on Co-immunoprecipitation, the binding ability of c-Jun and c-Fos was analyzed by western blot in A549 and 16HBE cells after AS3MT silencing. (IP: c-Jun: c-Jun served as internal control; IP: c-Fos: c-Fos served as internal control). (D) 16HBE and A549 cells were transfected with siAS3MT for 72 hours, the transcriptional activity of p53 was assessed by luciferase reporter gene assay. Data are presented as means±SEM (n=10). Two-tailed Student's t test, compared with the siCtrl group **p< 0.01.

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