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RESEARCH ARTICLE

Arsenic exposure increased expression of HOTAIR and LincRNA-p21 in vivo and vitro

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Abstract

Arsenic is an environmental contaminant, its multiple effects on human tend to increase the rate of disease, cancer and other health problems. Some of long non-coding RNAs (lncRNAs) can be induced in major cellular processes such as necrosis, proliferation, and mutation. While the toxicity of arsenic is well established, the association between arsenic exposure and long non-coding RNAs has not been studied enough. This study investigated the association between arsenic and the expression of HOTAIR and LincRNA-p21 in vivo and vitro. In epidemiological studies, the expression of HOTAIR and LincRNA-p21 was increased after long-term arsenic exposure. HOTAIR and LincRNA-p21 expression were positively linked to monomethylarsenic acid (MMA), dimethylarsenic acid (DMA), inorganic arsenic (iAs), total arsenic (tAs), and MMA% and negatively linked to secondary methylation index (SMI). In A549 cells, arsenic exposure resulted in enhanced HOTAIR and LincRNA-p21 expression dose-dependently. The expression of HOTAIR was considerably high in the presence of $NaAsO_2$ and MMA but showed no difference in DMA compared with control group. And LincRNA-p21 expression was increased in the presence of $NaAsO_2$, MMA, and DMA. The expression of HOTAIR and LincRNA-p21 induced by iAs was much higher than that induced by MMA and DMA. Compared with the control group, treatment of A549 cells with $NaAsO_2$/S-adenosylmethionine (SAM) and $NaAsO_2$/glutathione (GSH) combination increased HOTAIR and LincRNA-p21 expression. The expression of LincRNA-p21 in combination of $NaAsO_2$/GSH was significantly decreased compared with $NaAsO_2$ alone. Besides, in the presence of arsenic, both of HOTAIR and LincRNA-p21 were upregulated significantly when P53 was knocked down. We revealed that inorganic arsenic, its methylated metabolites, and arsenic metabolism efficiency affect the expression of HOTAIR and LincRNA-p21.

Keywords Arsenic · MMA · DMA · GSH · SAM · HOTAIR · LincRNA-p21

Introduction

Arsenic, a nonmetallic element, is widely distributed in the air, rock, soil, and water. It has been classified as one of the carcinogens known to human. Millions of people worldwide are exposed to arsenic with varying degrees via water, air, food, metal contact, and so on. So, arsenic poisoning is also a major global environmental health concern. Several epidemiological studies have established a strong association between arsenic exposure and a variety of human malignancies in the liver, lung, and bladder (Sankpal et al. 2012). Because of the long-term accumulation in the human body, a series of the oxidation methylation reactions have been founded after inorganic arsenic (iAs) enters human bodies. In the human arsenic metabolic pathway, iAs is reduced by arsenate reductase in erythrocytes. Glutathione (GSH) is the reductant to reduced pentavalent arsenic to trivalent arsenic. After being absorbed by hepatocytes, trivalent arsenic produced MMA^{5+} under the catalysis of methyltransferase, with S-adenosylmethionine (SAM) as the methyl-donating cofactor (Drobna et al. 2005). With the role of MMA^{5+} reductase, MMA^{5+} is converted to MMA^{3+}. Then, a methylation reaction occurs to generate DMA^{5+}. Finally, methylated products are excreted in urine as end products. Methylation plays an important part in

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conversion of inorganic to organic arsenic. There are various studies that indicate that the monomethylarsenic acid (MMA) and the dimethylarsenic acid (DMA) were more effectively discharged than inorganic arsenic (Mass et al. 2001). In some extent, methylation is a process of detoxification (Rosen 2002). MMA%, DMA%, primary methylation index (PMI), and secondary methylation index (SMI) are regarded as the good indicator of methylation ability (Kuo et al. 2017).

Several studies have shown that differential lncRNA expression can be a hallmark of cancer, and lncRNAs can function as potential oncogenes or tumor-suppressor genes influencing tumor development, progression, and therapy (Tahira et al. 2011). In general, there are more than 90% transcripts in human genome, but only about 2% of them are protein-coding genes. This means that non-coding RNAs (ncRNAs) are important components of mammalian transcriptome (Niland et al. 2012), and among these non-coding RNAs, microRNAs (miRNAs) with length of 21–23nt have been proved to play an important role in many biological and pathological processes. Long non-coding RNAs (LncRNAs) and their subtypes, long inter gene non-coding RNAs (lincRNAs), which are more than 200 nt long, have also been proved to be important components of non-coding RNAs, which can play biological functions in recent years (Schlackow et al. 2017). Multiple studies reveal that lncRNAs play a majority role in the development of tumor.

One of the lncRNAs, LincRNA-p21, was first identified in 2010. As a target gene upstream of transcription factor p53, its transcription is positively regulated by p53 (Huarte et al. 2010). LincRNA-p21, as an important component of p53 pathway, can participate in the regulation of p53 to upstream genes through physical binding of p53 coactivator. Several studies have reported that LincRNA-p21 is classified as a known cause for the gene regulation by directly binding with the target gene mRNA sequence (Jin et al. 2019). HOX antisense RNA, HOTAIR, is the first gene found to have trans-acting function. Although HOTAIR is located in the antisense chain of HOXC gene cluster, it regulates HOXD gene cluster (Rinn et al. 2007). It is noteworthy that the HOTAIR gene sequence, except for specific regions, is generally less conservative, and its evolution speed is faster than that of the HOXC gene cluster which is near it (Tang et al. 2013). Studies confirm that HOTAIR expression has significant association in a variety of common malignant tumors; HOTAIR inhibited PTEN expression, activated Akt pathway, inhibited p53 protein activity and further promoted Bcl-2, and inhibited BAX expression (Rinn et al. 2007).

Until now, the two genes in the field of tumor research have been studied extensively, but these studies are not sufficient to evaluate the potential influence of inorganic arsenic (iAs) to lncRNAs. Therefore, in our research, we investigated whether inorganic arsenic and its metabolites affected HOTAIR and LincRNA-p21 expression in vivo and vitro.

Methods and materials

Study population

The arsenic-exposed study participants were selected from the arsenic smelting plant, which was producing arsenic trioxide, and all of individuals were exposed to arsenic trioxide environment at least 3 months. Based on the production techniques in the arsenic smelting plant, there are few other occupational hazard factors except for arsenic pollution in the plant. And if laborers had peripheral vascular disorder and renal disease and had eaten fish and shellfish in a week, they would be excluded. As for the control group, we had chosen 25 people within similar age and live far away 10 km from the arsenic smelter, which had no history of arsenic exposure. Two groups have similar living habits and environment. With the written consent of the study participants, blood and urine samples were collected for further analysis. Their demographic characteristics such as age, sex, smoking, and drinking habits (subjects who smoke more than 10 cigarettes each day, any consumption of alcohol) were collected by questionnaires. The research design was approved by the Theory Committee of Kunming Medical University.

Measurement of urinary arsenic metabolites

The middle stream of the first morning urine (15 ml) was collected with polypropylene tube and stored in the ice box. For each participant, 1-ml urine was taken separately for the determination of creatinine (CR). The collected urine samples were stored in the environment of − 20 °C, to ensure that the urine arsenic metabolites were measured within 3 months, thawed at room temperature before detection, and digested at 100 °C for 3 h with 2N-NAOH solution. The morphology of iAs, MMA, and DMA did not change after digestion. The arsenic speciation was based on the well-established hydride generation of volatile arsines, followed by separation at low temperature in liquid nitrogen. The absorbance of arsenic in urine samples was determined at 193.7 nm. SRM 2670 (National Institute of Standards and Technology (NIST), Gaithersburg, MD, USA), which contain 480 ± 100 μg/L arsenic, was used as the standard reference material for the quality control. Finally, we determined arsenic species (iAs, MMA, and DMA) in urine using atomic absorption spectrophotometer (AA-6800) with an arsenic speciation pretreatment system (ASA-2SP; Shimadzu Co., Kyoto, Japan). (Li et al. 2015; Zhang et al. 2014). The value measured by our system was 474 ± 20 μg/L. The detection limit of the hydride generation and atomic absorption spectrometry (HG-AAS) was 1 ng, and RSD < 5%. Spiking urine samples with 10 μg/L of iAs, MMA, and DMA resulted in recoveries of 81–92%, 88–98%, and 89–103%, respectively. Urinary creatinine was determined using a creatinine assay kit (Hangzhou Deanqi Biological Engineering Co. Ltd, Hangzhou, China)

to remove the influence of urine dilution in urine sample. All the experiments were performed 3 times independently.

$$tAs = iAs + MMA + DMA$$

$$iAs\% = 100 \times iAs/tAs$$

$$MMA\% = 100 \times MMA/tAs$$

$$DMA\% = 100 \times DMA/tAs$$

$$\text{Primary methylation index (PMI)} = MMA/iAs$$

$$\text{Secondary methylation index (SMI)} = DMA/MMA$$

Cell culture and grouping

A549 cells were cultured in RPMI 1640 medium containing 10% FBS (QuaCell Biotechnology, Co., Ltd., China) at 37 °C, 5% CO_2 incubator. Cells in the logarithmic growth phase were collected, seeded in a 6-well plate at 0.8×10^5 cells/well. After incubating for 22 h, fresh medium containing $NaAsO_2$ (CAS 7784-46-5; purity ≥ 90.0%) with different concentration (0, 20, 40, 60 μmol/L) was added. The cells were cultured for another 48 h and labeled as control group, 20 μmol/L group, 40 μmol/L group, and 60 μmol/L group. We cultured the same number of cells in 6-well plate. Replace the culture medium with 40 μmol/L $NaAsO_2$, MMA (MF: CH_5AsO_3; CAS 124-58-3; purity≥ 99.0%), and DMA (MF: $C_2H_7AsO_2$; CAS 75-60-5; purity ≥ 99.0%); this group was incubating for 72 h and named as control group, MMA group, and DMA group. As for the $NaAsO_2$ group, the $NaAsO_2$ + SAM group, and the $NaAsO_2$ + GSH group, which were firstly incubated with 40 μmol/L $NaAsO_2$ for 24 h, and then added SAM (MF: $C_{15}H_{12}N_6O_5S$; CAS 29908-03-0; purity≥ 98.0%) (200 μM) and GSH (MF: $C_{10}H_{17}N_3O_6S$; CAS 70-18-8; purity≥98.0%) (500μM) separately to incubate 48 h (Cheng et al. 2010; Du et al. 2012).

Si-RNA preparation

When A549 cells were grown to a confluency of about 45%, the cells were transfected with NC and p53 siRNA (German, A08007; A06001) and continuously cultured for 24 h according to the instructions of RFect transfection reagent (Changzhou Biogenerating Biotechnologies Co, Ltd). Cells were divided into NC group and si-p53 group. Then, the culture medium was replaced by fresh medium containing 40 μmol/L $NaAsO_2$ for 72 h. Cells were divided into si-p53 group and $NaAsO_2$ + si-p53 group.

Protein isolation and Western blotting

Total protein was extracted from A549 cells that had been transfected with si-p53 for 72 h, cells were washed twice with ice-cold phosphate-buffered saline (PBS) and lysed in RIPA buffer (Thermo Fisher Scientific lnc. USA) containing protease inhibitors. Protein concentration was measured using the BCA Protein Assay Kit (Beyotime Biotechnology Co., Ltd., China). The protein was subjected to electrophoresis (30 μg per well) and electronically transferred to PVDF membranes (Roche, German). After blocking with 1× Western Block Buffer in PBST with BSA (Dalian Meilun Biotechnology Co., Ltd., China), the membranes were incubated with p53 (Santa-Cruz, clone DO-1, 1 : 200) or beta actin (Proteintech, USA, 1:10000) antibody 2 h at room temperature, and then goat anti-mouse IgG, HRP-conjugated antibody (Proteintech, USA) at 1:2000 dilution was incubated for 2 h at room temperature. BeyoECL Plus chromogenic substrate (Beyotime Biotechnology Co., Ltd., China) was used to visualize the bands, and the intensity of the bands was quantified by Gel-Pro Analyzer software (Media Cybernetics).

cDNA preparation and Q-PCR

Peripheral blood was stored in the centrifuge tube with ethylenediaminetetraacetic acid (EDTA), and lymphocytes were separated by lymphocyte separation medium (Solarbio Biotech Company, China). Cells were collected and fully lysed. Total RNA was extracted by Trizol method to detect RNA purity and concentration. The purity was determined using a spectrophotometer, obtaining 260/280 ratios between 1.9 and 2.1 in all the samples. The RNA was reverse transcribed into cDNA using the Roche reverse transcription kit (Roche, German). Finally, the expression levels of genes were measured by LightCycler®96 real-time PCR instrument (Roche, German). The β-actin was used as an internal reference. All experiments were performed three times, and the experimental results were analyzed using the $2^{-\triangle\triangle CT}$ method. HOTAIR primers: Forward: GCTGCTCC GGAATTTGAGAG Reverse: TGCTGCCAGTTAGA AAAGCG; LincRNA-p21 primers: Forward: CAGGGTGC CAGAAGAGTGAG Reverse: AGACTAAAGCTCCT ACTTCAGCAG; p53 primers: Forward: TTCAGATC CGTGGGCGTGAG Reverse: ATGGCGGGAGGTAG ACTGAC; β-actin primers: Forward: GCCGAGGACTTTGA TTGCAC Reverse: TGGACTTGGGAGAGGACTGG.

Statistical analysis

The experimental data were expressed as mean ± SEM and analyzed by SPSS 22.0. In epidemiological analysis, HOTAIR and LincRNA-p21 expression and three arsenic species levels were transformed by logarithm. Spearman's correlation coefficient, Pearson's correlation coefficient, and Pearson's product moment correlation were used in data analysis. The data were compared between the two groups by t test. The data of multiple groups were compared by one-way analysis of variance. $P < 0.05$ was considered statistically significant.

Table 1 General characteristics of subjects and urinary arsenic levels

Variables	Control group $n = 20$	Exposure group $n = 75$
Age (year, mean ± SD)	35.21 ± 3.19	36.24 ± 5.71
Gender (male/female)	12/6	46/29
Smokers (yes/no)	11/7	45/30
Alcohol users (yes/no)	9/11	37/38
iAs(mean ± SD)	0.40 ± 0.03	2.00 ± 0.05**
MMA (mean ± SD)	0.35 ± 0.03	2.13 ± 0.05**
DMA (mean ± SD)	1.09 ± 0.07	2.65 ± 0.06**
iAs%(mean ± SD)	13.66 ± 2.74	16.69 ± 1.26
MMA%(mean ± SD)	8.15 ± 0.48	19.23 ± 0.48**
DMA%(mean ± SD)	78.18 ± 2.70	64.07 ± 1.57**
PMI(mean ± SD)	1.23 ± 0.31	1.63 ± 0.14
SMI(mean ± SD)	10.40 ± 0.84	3.83 ± 0.25**

**The difference from control group is statistically significant ($P < 0.01$).

Results

Baseline characteristics of the study population

We examined the levels of arsenic in urine. The general information and urinary arsenic species of the subjects are listed in Table 1. No significant difference between control and exposure group in age, sex, smoking, and drinking habits. Inorganic arsenic, MMA, DMA, iAs%, MMA%, and PMI were increased in the exposed group, whereas DMA% and SMI were lower than the control group.

The interrelationship between arsenic exposure and expression of HOTAIR and LincRNA-p21

To clarify the effect of arsenic exposure on HOTAIR and LincRNA-p21 expression in vivo, the levels of genes were evaluated by Q-PCR, respectively. Our results showed that the expression of HOTAIR induced a 5.24-fold increase after a long-term arsenic exposure compared with control group (Fig. 1a, left panels). Similar results were seen with the steady level of LincRNA-p21 (11.3-fold, $P < 0.001$) (Fig. 1a, right panels). In the presence of inorganic arsenic, the expression of HOTAIR was positively correlated with LincRNA-p21. Furthermore, we used to evaluate the effect of total arsenic on gene expression. And the levels of HOTAIR and LincRNA-p21 were positively correlated with total arsenic in individual subjects used in the study (Fig. 1b, c).

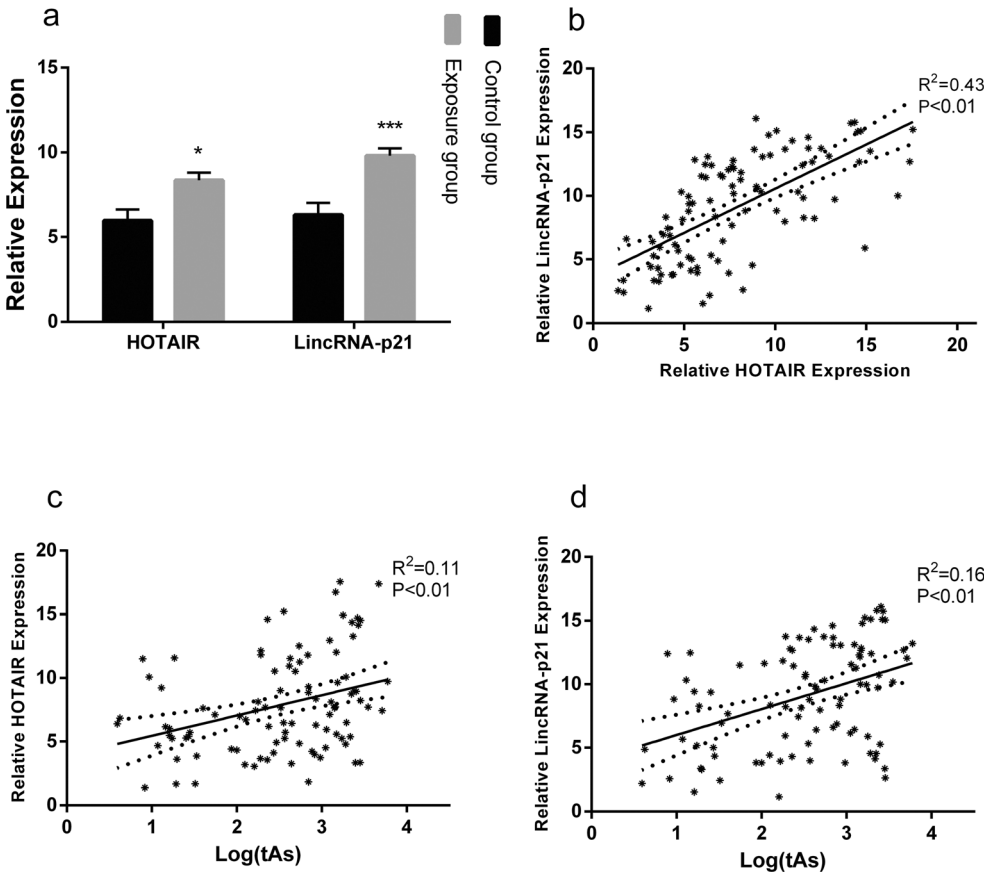

Fig. 1 Arsenic exposure promotes HOTAIR and LincRNA-p21 expression. **a** Total RNA was purified and analyzed for HOTAIR and LincRNA-p21 expression by Q-PCR. Data were represented as mean ± SEM of two independent experiments. Student's *t* test was used, *$P<0.05$, ***$P<0.001$ compared with control group. **b** Relationship between HOTAIR and LincRNA-p21 expression in all subjects. **c** Relationship between total arsenic and HOTAIR expression in all subjects. **d** Relationship between total arsenic and LincRNA-p21 expression in all subjects. Data analyses were performed by applying Pearson correlation to tAs with gene expression.

Table 2 Association between arsenic species and levels of HOTAIR and LincRNA-p21 expression in all subjects

Variables	Log (MMA)		Log (DMA)		Log (iAs)	
	R	*P* value	*R*	*P* value	*R*	*P* Value
HOTAIR expression	0.35	0.00	0.34	0.01	0.33	0.01
LincRNA-p21 expression	0.40	0.00	0.42	0.00	0.36	0.00

MMA monomethylarsonic acid, *DMA* dimethylarsenic acid, iAs inorganic arsenic

Association of arsenic metabolites and methylation metabolic index with expression of HOTAIR and LincRNA-p21

Because of the diverse methylation process of arsenic, we further clarified whether arsenic induced the expression of HOTAIR, and LincRNA-p21 is associated with its metabolites or metabolic transformation process. Arsenic metabolism index was measured and made linear correlation analysis with gene expression. The results are summarized in Table 2 and 3. HOTAIR and LincRNA-p21 expressions were positively linked to MMA, DMA, iAs, total arsenic, and MMA% and negatively linked to SMI.

Inorganic arsenic increased HOTAIR and LincRNA-p21 expression in A549 cells

Previous studies reported that the exposure of arsenic promotes A549 cell apoptosis in vitro and in vivo. To clarify whether inorganic arsenic affects HOTAIR and LincRNA-p21 expression in A549 cells, we dissected the expression of two genes by Q-PCR. The expression of two genes was significantly increased by $NaAsO_2$ treatment (20, 40, and 60 μM) compared with in the control group. (HOTAIR 1.3-fold, 2.0-fold, and 2.5-fold higher, respectively; LincRNA-p21 2.15-fold, 5.18-fold, and 4.62-fold higher, respectively) (Fig. 2a, b), implying that inorganic arsenic has increased the HOTAIR and LincRNA-p21 expression in a dose-dependent manner.

MMA, DMA, and arsenic in combination with GSH and SMA affect HOTAIR and LincRNA-p21 expression in A549 cells

A549 cells were treated with MMA, DMA, and $NaAsO_2$ (60μM) for 72 h and then the expression of gene was investigated. Figure 3 a showed that the HOTAIR expression levels exhibited a remarkable 3.8-fold increase in the $NaAsO_2$ group, whereas in the MMA group exhibited a modest 1.2-fold increase compared with the control group. Furthermore, compared with the $NaAsO_2$ group, HOTAIR expression in MMA and DMA was decreased significantly (68% in MMA, $P < 0.001$; 70% in DMA, $P < 0.001$) (Fig. 3a). On the other hand, LincRNA-p21 expression in MMA, DMA, and $NaAsO_2$ group was increased compared with the control group (3.21-fold in MMA, $P < 0.05$; 2.5-fold in DMA, $P < 0.001$; 4.15-fold in $NaAsO_2$, $P < 0.001$). And LincRNA-p21 expression in DMA exhibited a 40% decrease compared with the $NaAsO_2$ group (Fig. 3c).

We then examined whether the effect of inorganic arsenic on HOTAIR and LincRNA-p21 expression was mediated by the combination with GSH and SAM. When tested by Q-PCR, the expression levels of two genes were enhanced and treated with GSH + $NaAsO_2$, SAM + $NaAsO_2$, and $NaAsO_2$ cells (HOTAIR: 3.14-fold, 6.45-fold, and 5.56-fold higher, respectively; LincRNA-p21: 4.67-fold, 5.48-fold, and 7.38-fold higher, respectively) (Fig. 3b). Although the expression of HOTAIR was increased in the combination of SAM, GSH, and $NaAsO_2$, the changes were not significantly different with $NaAsO_2$ alone ($P > 0.05$). Moreover, the expression of LincRNA-p21 exhibited a 37% decrease in combination of GSH (500 μM) with $NaAsO_2$ over that in $NaAsO_2$ alone. (Fig. 3d).

The regulation of inorganic arsenic on HOTAIR and LincRNA-p21 expression after knockdown p53

First, we examined the effect of p53 on two gene expressions. HOTAIR and LincRNA-p21 expressions in infected A549 cells were determined by Q-PCR, which are shown in Fig. 4. The efficiency of siRNA is shown in Fig. 5. LincRNA-p21 was downregulated significantly when p53 was knocked

Table 3 Association between the percentages of urinary arsenic, PMI, SMI, and levels of HOTAIR and LincRNA-p21 expression in all subjects

Variables	iAs%		MMA%		DMA%		PMI		SMI	
	R	*P* value	*R*	*P* value	*R*	*P* value	*R*	*P* value	*R*	*P* value
HOTAIR expression	− 0.08	0.40	0.252	0.01	− 0.04	0.64	0.04	0.65	− 0.23	0.02
LincRNA-p21 expression	− 0.16	0.10	0.24	0.01	0.02	0.84	0.10	0.31	− 0.21	0.03

iAs inorganic arsenic, *MMA* monomethylarsonic acid, *DMA* dimethylarsenic acid, *PMI* primary methylation index, *SMI* secondary methylation index

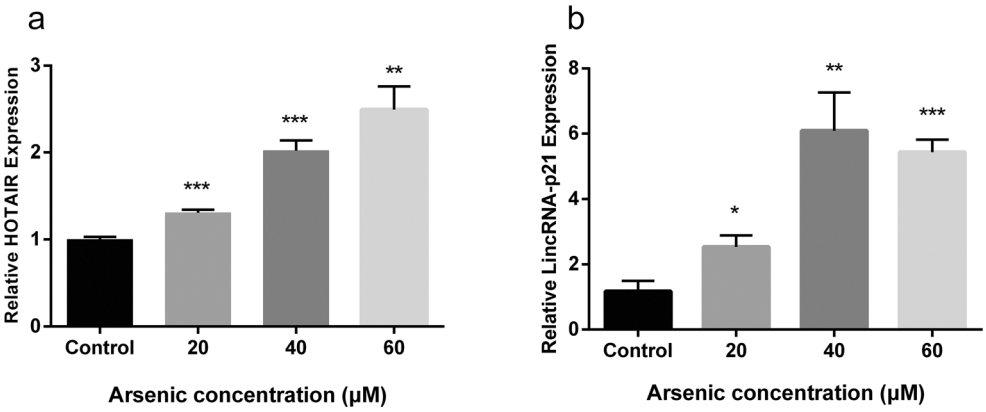

Fig. 2 Enhancement of HOTAIR and LincRNA-p21 expression in A549 cells treated with $NaAsO_2$. **a** A549 cells were treated with indicated concentration of $NaAsO_2$ for 72 h, and expression of HOTAIR was examined by Q-PCR. **b** Expression of LincRNA-p21 was examined by Q-PCR. β-actin was used as a loading control. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ compared with control group

down (54%, $P < 0.05$) (Fig. 4d). However, in the presence of inorganic arsenic, HOTAIR and LincRNA-p21 expressions were 5.44- and 19.14-fold higher than that in the control group when p53 was knocked down (Fig. 4b, d).

Discussion

Long-term exposure of arsenic in association with a wide range of adverse health effects and inorganic arsenic is a human carcinogen targeting mainly the urinary bladder, lung, and skin (Spratlen et al. 2018; Spratlen et al. 2019). Alteration in genome, signal transduction, and cell mutation apoptosis are involved in arsenic carcinogenesis (Rager et al. 2014; Shimoda et al. 2018; Suzuki et al. 2013). Several studies have reported that arsenic acts on the change of lncRNA expression. By contrast, the differential expression of lncRNAs and target genes at early stage of arsenite exposure may contribute to arsenic-induced carcinogenesis (Jiang et al. 2014; Mao et al. 2018). Relatively, arsenic exposure is a direct and

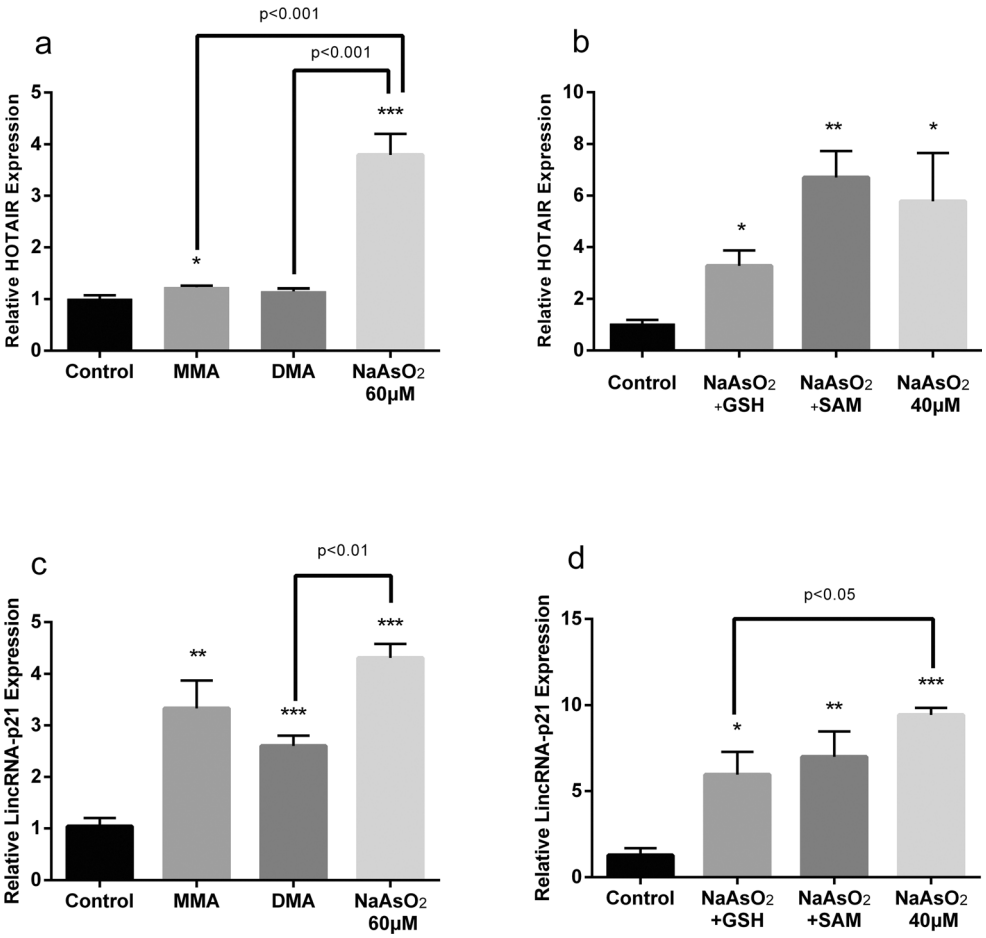

Fig. 3 Effects of arsenic metabolism and $NaAsO_2$/SAM, $NaAsO_2$/GSH combination on HOTAIR and LincRNA-p21 expression in A549 cells. **a**, **c** A549 cells were treated with MMA, DMA, and $NaAsO_2$ for 72 h, and RNA was extracted to evaluate HOTAIR and LincRNA-p21 expression. **b**, **d** A549 cells were treated with GSH/$NaAsO_2$ and SAM/$NaAsO_2$ combination. Expression of genes were examined by Q-PCR. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ compared with control group. β-actin was used as a loading control.

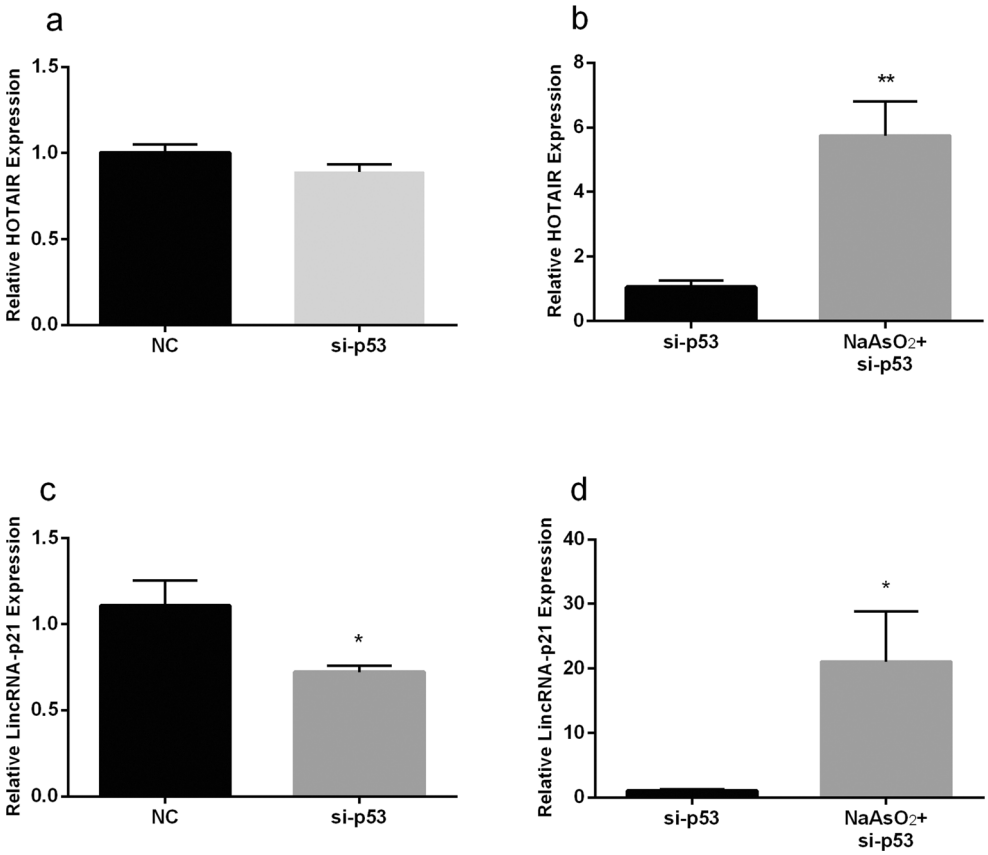

Fig. 4 The regulation of arsenic on HOTAIR and LincRNA-p21 expression after si-p53. **a**, **c** A549 cells were treated with si-p53 for 72 h, and RNA was extracted to evaluate HOTAIR and LincRNA-p21 expression. **b**, **d** A549 cells were treated with si-p53 and $NaAsO_2$. Expression of genes were examined by Q-PCR. $^*P < 0.05$; $^{**}P < 0.01$ compared with control group. β-actin was used as a loading control.

significant factor affecting gene expression (Wen et al. 2016). To identify the association of arsenic exposure with HOTAIR and LincRNA-p21, we examined the effect of inorganic arsenic on HOTAIR and LincRNA-p21 expression by Q-PCR. A549 cells treated with $NaAsO_2$ showed that inorganic arsenic dose-dependently has increased HOTAIR and LincRNA-p21 expression. Our epidemiologic studies also support this conclusion that the levels of two genes were higher in the exposure group than in the control group. The expression of HOTAIR was positively correlated with LincRNA-p21 in the presence of arsenic exposure, which is in agreement with other studies (Wen et al. 2016). HOTAIR and LincRNA-p21 were positively correlated with total arsenic in individual subjects. Our results suggest that arsenic exposure induced the expression of HOTAIR and LincRNA-p21 in vivo and vitro.

After arsenic enters human bodies, it is mainly metabolized by the liver (Mahalanobish et al. 2019). MMA and DMA are generated in a series of oxidative methylation processes. In addition, several epidemiological studies have linked MMA and DMA to a variety of human malignancies (He et al. 2018; Zhang et al. 2014). And various forms of arsenic have different metabolic rates in human bodies, and their effects on gene expression have shown inconsistent results (Wei et al. 2017). Our epidemiological evidence regarding the DMA and MMA was positively linked to HOTAIR and LincRNA-p21, which are in agreement with other studies (Wen et al. 2016). Moreover, different results were observed in A549 cells that the LincRNA-p21 expression was induced by iAs, MMA, and DMA and alteration of HOTAIR induced by iAs and MMA. And the gene expression levels in DMA and MMA group were significantly lower than the arsenic group, suggesting that iAs, MMA, and DMA exert different toxic effects. Our results gave significantly imply that inorganic arsenic and its metabolites are likely to be responsible for the increased of HOTAIR and LincRNA-p21 expression, and inorganic arsenic was the major influence that induced HOTAIR and LincRNA-p21 expression.

The methylation of arsenic is a recognized mechanism of arsenic metabolism, in which SAM and GSH participate in the regulation and promote the metabolism of arsenic and its by-products (Xu et al. 2019). GSH and SAM are the indicator of methylation metabolism process. The biotransformation of iAs and its metabolite conjugates play a crucial role in arsenic carcinogenicity at both genetic and epigenetic levels (Bakir et al. 2019; Deshpande et al. 2017). We hypothesized that the metabolism efficiency of arsenic would modulate gene expression. We calculated the index of arsenic methylation metabolic in individual subjects. It is estimated that the DMA% and SMI of exposure group were reduced compared with the control group, while MMA% was increased in the exposure group, suggesting that metabolism of arsenic methylation was lower in the group of people in long-term arsenic

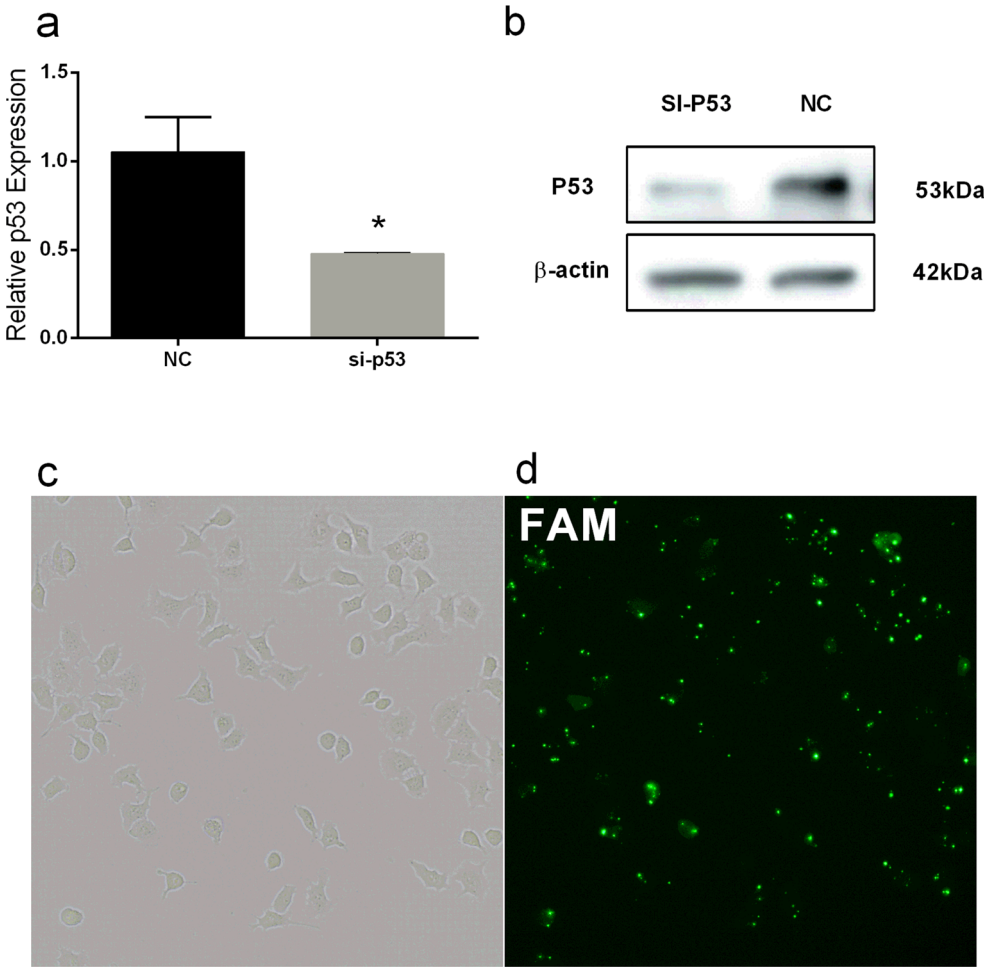

Fig. 5 Knockdown of p53. **a** The mRNA expression of p53 was measured after transfected si-p53. **b** P53 protein expression in cells compared with the si-P53 group. **c**, **d** A549 cells transfected FAM to identify the transfection efficiency. *$P < 0.05$ compared with the control group.

exposure. And MMA% was positively linked to HOTAIR and LincRNA-p21, while HOTAIR and LincRNA-p21 were negatively linked to SMI. The lower ability of arsenic methylation metabolism comes with high accumulation of inorganic arsenic and MMA, and thereby induced HOTAIR and LincRNA-p21 expression. Previous studies have shown that individuals having relatively lower capacity to methylate arsenic to DMA or higher MMA% are at greater risk for cancer (Wang et al. 2013; Wei et al. 2017). Then, we treated A549 cells with GSH/$NaAsO_2$ and SAM/$NaAsO_2$ combinations respectively; the study showed that the HOTAIR expression increased by GSH + $NaAsO_2$ and SAM + $NaAsO_2$ treatment. The expression of LincRNA-p21 in GSH/$NaAsO_2$ combinations decreased significantly compared with the $NaAsO_2$ group, which has statistical significance; the possible reason was that the GSH accelerated metabolism in vivo and weakened the effect of inorganic arsenic on gene expression (Cheng et al. 2010). Our results clearly support that the metabolism efficiency of arsenic altered HOTAIR and LincRNA-p21 expression, but further work is needed to understand the underlying mechanisms and to clarify the effect of altered gene expression on arsenic-induced toxicity and carcinogenesis.

It is notable that arsenic can induce apoptosis in a variety of cells, for which several mechanisms have been accounted for. The signal transduction pathway mediated by p53 plays an important role in regulating the normal life activities of cells. The relationship between p53 and other signal transduction pathways is very complicated. There are more than 160 genes involved in the regulation of p53, because p53 pathways are known as critical regulators of the expression of a variety of genes, including apoptosis, growth inhibition, inhibition of cell cycle progression, differentiation, acceleration of DNA repair, genotoxicity, and cell senescence after stress (Stracquadanio et al. 2016). Like other tumor suppressors, the p53 gene normally slows down or monitors cell division. In addition, p53 is independent of its activity and acts as a transcription factor to induce apoptosis. The function of p53 in promoting apoptosis has been further studied. P53 can regulate apoptosis through Bax/Bcl2, Fas/Apo1, IGF-BP3, and other proteins (el-Deiry 1998; Roos et al. 2004; Shabnam et al. 2004). LincRNA-p21, as a p53-dependent transcription target gene and potential diagnostic marker, is involved in cell proliferation, cycle, metabolism, and reprogramming (Huarte et al. 2010). And there is a report that HOTAIR activates the

Akt pathway and inhibits the activity of p53 protein, thus producing anti-apoptotic effects on tumor cells (Sun et al. 2018).

Because p53 pathways are known as critical regulators of cell proliferation, differentiation, and apoptosis (Behera et al. 2018; Ter Huurne et al. 2020), and are also known as key regulators of HOTAIR and LincRNA-p21 gene (Jin et al. 2019; Yu et al. 2017), we examined whether inorganic arsenic caused the gene alteration by activation or suppression of p53. To primarily confirm that overexpressed expression is regulated by p53, the RNA interference–mediated silence of p53 expression was performed. After knocked down p53, LincRNA-p21 expression levels were greatly reduced as compared with the control group. This is consistent with the Giovanni et al.'s (Huarte et al. 2010). And we next suggested that inorganic arsenic exposure might activate HOTAIR and LincRNA-p21 expression by regulation of p53. Conversely, our research indicates that iAs exposure leads to higher levels of HOTAIR and LincRNA-p21 while p53 was knocked down. Compared with our previous studies, this activation that is mediated through p53 could not be proved. Inhibition of p53 by siRNA remarkably attenuates P53 expression. Arsenic exposure may result in the increase of p53, which masks the siRNA effect. Therefore, HOTAIR and LincRNA-p21 expressions were still increasing. The relative contribution of inorganic arsenic induced HOTAIR and LincRNA-p21 expression, and p53 could not be assessed from the available data.

Taken together, HOTAIR and LincRNA-p21 expressions were induced by arsenic exposure and its metabolism in vivo and vitro. Arsenic's metabolic capacity also affects their expression. It remains unclear what the role of p53 in arsenic-induced HOTAIR and LincRNA-p21 gene expression is. Further studies are necessary to extend and elucidate the mechanism and function of arsenic.

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Compliance with ethical standards

Conflict of Interest The authors declare that they have no conflicts of interest.

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