Toxicology Reports 1 (2014) 692–698

Contents lists available at ScienceDirect

Toxicology Reports

journal homepage: www.elsevier.com/locate/toxrep

Inactivation of $p15^{INK4b}$ in chronic arsenic poisoning cases

Aihua Zhang[a,*,1], Chen Gao[b,1], Xue Han[a], Lifang Wang[a], Chun Yu[a], Xiaowen Zeng[b], Liping Chen[b], Daochuan Li[b], Wen Chen[b]

[a] Department of Toxicology, School of Public Health, Guiyang Medical University, Guiyang 550004, China
[b] Department of Toxicology, School of Public Health, Sun Yat-sen University, Guangzhou 510080, China

ARTICLE INFO

Article history:
Received 4 May 2014
Received in revised form 30 July 2014
Accepted 13 August 2014
Available online 17 September 2014

Keywords:
Arsenic
Domestic coal use
Skin lesions
$p15^{INK4b}$
DNA methylation
Homozygous deletion

Chemical compounds studied in this article:
Arsenic (PubChem CID: 5359596)

ABSTRACT

Arsenic exposure from burning high arsenic-containing coal has been associated with human skin lesion and cancer. However, the mechanisms of arsenic-related carcinogenesis are not fully understood. Inactivation of critical tumor suppression genes by epigenetic regulation or genetic modification might contribute to arsenic-induced carcinogenicity. This study aims to clarify the correlation between arsenic pollution and functional defect of $p15^{INK4b}$ gene in arsenic exposure residents from a region of Guizhou Province, China. To this end, 103 arsenic exposure residents and 105 control subjects were recruited in this study. The results showed that the exposure group exhibited higher levels of urinary and hair arsenic compared with the control group (55.28 *vs* 28.87 μg/L, 5.16 *vs* 1.36 μg/g). Subjects with higher arsenic concentrations are more likely to have $p15^{INK4b}$ methylation and gene deletion ($\chi^2 = 4.28$, $P = 0.04$ and $\chi^2 = 4.31$, $P = 0.04$). We also found that the degree of $p15^{INK4b}$ hypermethylation and gene deletion occurred at higher incidence in the poisoning cases with skin cancer (3.7% and 14.81% in non-skin cancer group, 41.18% and 47.06 in skin cancer group), and were significantly associated with the stage of skin lesions ($\chi^2 = 12.82$, $P < 0.01$ and $\chi^2 = 7.835$, $P = 0.005$). These observations indicate that inactivation of $p15^{INK4b}$ through genetic alteration or epigenetic modification is a common event that is associated with arsenic exposure and the development of arsenicosis.

1. Introduction

The inorganic arsenic exposure is a worldwide health problem. The usage of coal containing high concentrations of arsenic was reported as a serious environmental pollution that causes adverse health effects in China [1–4]. The unique cooking style of villagers in Guizhou Province using high arsenic content coal in the indoor stove without sufficient ventilation. More than 200,000 residents exposed to arsenic through diet, inhalation and direct skin contact [2]. The high incidence of skin lesion and cancer development was reported in this region recognized as one of the most serious endemic arseniasis areas in China [5].

Arsenic is a well-known chemical carcinogen and classified as Group 1 carcinogen, which is carcinogenic to humans, by International Agency for Research on Cancer (IARC) [6]. Although it is evident that chronic arsenic exposure is associated with human cancers, the mechanism of arsenic-induced carcinogenicity is still not clear. The arsenic is considered as a poor mutagen in both bacterial and mammalian mutagenicity assays [7]. However, arsenic perturbs transcriptional activity and cause aberrant gene expression *via* generation of reactive oxygen species (ROS), inhibition of DNA repair, and chromosomal

* Corresponding author. Tel.: +86 8516908908; fax: +86 8516908908.
E-mail addresses: aihuagy@aliyun.com, 940879123@qq.com (A. Zhang).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.toxrep.2014.08.007

aberration [7,8]. In addition, lines of evidence have shown that the epigenetic regulation such as promoter methylation of tumor suppresser gene is a critical event in the course of arsenic-induced carcinogenicity [9–12]. For example, it is supported by both epidemiology and *in vivo* studies that chronic inorganic arsenic exposure induced urinary bladder cancer through epigenetic modifications such as DNA methylation [13,14]. Moreover, a number of studies have reported that arsenic exposure results in alteration of the expression of many important genes such as ERCC2 and CCND 1 [15–17]. Our prior works have linked human exposure of arsenic with skin lesions and cancer [18–21]. We found that arsenic exposure affected the DNA methylation in gene promoters including *TP53* [22] and *p16* [23], leading to the changes in gene expression in the chronic arsenic exposure patients [24,25]. Taken together, these findings support the notion that the disruption of tumor suppression genes may contribute to chronic arsenic exposure induced carcinogenesis.$p15^{INK4b}$ is a critical tumor suppressor gene involved in control of cell proliferation [26,27]. Aberrant $p15^{INK4b}$ expression has been found in many kinds of human tumors including leukemia, lung and skin cancers [28]. The $p15^{INK4b}$ gene can be inactivated by point mutation, homozygous deletion, or DNA methylation in various human tumors [26,29,30]. The defect of $p15^{INK4b}$ is also correlated with tumorigenicity induced by chemical carcinogens such as benzo(a)pyrene [31] and benzene [32,33]. In this study, we devote to address whether $p15^{INK4b}$ inactivation contributes to the development of arseniasis through detection of the expression of $p15^{INK4b}$ in arsenic-induced skin lesion tissues and examination of the $p15^{INK4b}$ deletion and promoter methylation in peripheral lymphocytes from arsenic-exposed villagers. Our results indicate that functional defect of $p15^{INK4b}$ gene is associated with the development and progression of chronic arsenic poisoning.

2. Materials and methods

2.1. Study population

The study areas and subjects recruitment have been described previously in details [23]. Briefly, the residents use coal containing high content of arsenic in cooking and expose to arsenic *via* polluted food and air. The 103 arsenic exposure subjects from Jiao Le village in Xinren county, Guizhou Province were recruited. The average age of arsenic-exposed group and control group was 49 and 43 years old, respectively. The diagnosis of arsenism was made according to the *Chinese National Standard of Arsenicosis Diagnosis* [34] by medical professionals working in the forty-fourth Hospital of Chinese People's Liberation Army. Among those 103 arsenic exposure subjects, 44 arsenism patients were enrolled in the surgical treatment and their skin lesions were collected. Served as a control group, 105 cases were randomly selected in the neighboring Ma Jiatun Township village where residents cooked using coal containing low arsenic levels and had no signs of arseniasis. The study proposal was reviewed and approved by the Ethical Committee of the Guiyang Medical University. Prior to data collection, written informed consent was obtained from each participant.

2.2. Interviews and sample collection

A structured questionnaire was used for recording the data on demographic factors, lifestyle and residential history of participants. A physical examination was taken by the medical professionals. The urine, hair, and peripheral blood samples were collected. The urinary samples were stored at −20 °C immediately after collection. Scalp hair (length, 1 cm) was kept in plastic zip lock bags. Fasting peripheral blood was collected in an EDTA-coated vacuum tube and lymphocytes were isolated as soon as the blood was drawn. For the patients who received skin surgery, the biopsy skin tissues were collected and subjected to pathological and immunohistochemistry examinations.

2.3. Arsenic concentrations in hair and urine

Total arsenic content of urine and hair samples were used for assessing the level of arsenic exposure. All the urine and hair samples were analyzed within 1 month after sample collection. The details of the analytical method have been described previously [20]. Briefly, the hair samples were first washed sequentially with high-purity-deionized water, then with ethanol and acetone to remove external contaminants. The hair samples were then digested with concentrated nitric acid (HNO_3). Total arsenic content in samples was determined by hydride generation, inductively coupled plasma atomic emission spectrometry (HG-ICP-AES) (Varian Australia, Mulgrave, Australia). Urinary arsenic concentrations were adjusted by specific gravity to equilibrate the differences in urine dilution [35].

2.4. DNA extraction and methylation-specific PCR (MSP)

Genomic DNA is isolated from the lymphocytes of the subjects. To perform MSP, 2 μg DNA was denatured by freshly prepared 2 mol/L NaOH, mixed with 30 μl of 10 mmol/L freshly prepared hydroquinone and 500 μl of 3 mol/L freshly prepared sodium bisulfate (pH5.0) and then incubated at 55 °C for 16 h in dark. The DNA was purified by DNA Wizard clean-up resin, and desulfonated by adding NaOH. Finally, DNA was precipitated by ethanol and dissolved in sterile distilled water. This modified DNA was used for PCR amplification by unmethylation specific primers (forwards: 5′-TGTGATGTGTTTGTATTTTGTGGTT-3′; reverse: 5′-CCATACAAT AACCAAACA ACCAA-3′) and methylation specific primers (forwards: 5′-GCGTTC GTATTTTGCGGTT-3′; reverse: 5′-CGTACAATAACCGAACGACCGA-3′). Two fragments, 148 bp and 154 bp that represents unmethylated and methylated signaling, respectively was expected to appear after amplification. The CpG methyltransferase, M.SssI, treated human placental DNA served as a positive control.

2.5. Homozygous deletion detection by multiplex PCR

Homozygous deletion at the first and second exons of $p15^{INK4b}$ was analyzed by multiplex PCR. The primers used for amplification are as follow: forwards 5′-CCAGAAGCAATCCAGGCGCG-3′ and reverse: 5′-AATGCACACCTCGCC AACG-3′ for exon 1 (532 bp); and forwards: 5′-CTTTAAATGGCTCCACCTGC-3′, and reverse: 5′-CGTTGGCAGCCTTCATCG-3′ for exon 2 (437 bp). A fragment (303 bp) of β-actin gene (forwards: 5′-GAAACTACCTTCAACTCCATC-3′, and reverse: 5′-CTAGAAGCTTTTGCGGACGATGGAGGGGCC-3′) was used as an internal standard. All multiplex reactions were made in a total volume of 25 μl containing $MgCl_2$ (2 mM), dNTP (200 μM), 1.25 U Taq DNA polymerase (Takara, Dalian, China) and specific reverse primers (0.2 μM each) in the polymerase buffer. PCR cycles were preceded by an initial denaturation at 94 °C for 5 min, then reactions were run for 35 cycles of 95 °C for 45 s, 60 °C (exon 1) or 57 °C (exon 2) for 45 s and 72 °C for 1 min, and completed by a final elongation at 72 °C for 7 min.

2.6. Statistical analysis

The Statistical Package for the SPSS version 16.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. The concentrations of the urinary and hair arsenic were used as continuous measures and also categorized into tertiles. The arsenism patients were classified as "mild," "intermediate" and "severe" based on the degree of skin lesions according to the Chinese National Standard of Arsenicosis Diagnosis [34].

Independent-samples *t*-test was used to compare the age, urinary and hair arsenic levels between arsenism and the control group. The bivariate correlations were used to assess the relationships between the concentrations of arsenic and potentially important covariates, such as age and smoking status. To analyze the associations between tertiles of urinary and hair arsenic and trichotomous skin lesions and the frequencies of $p15^{INK4b}$ methylation and deletion, the trend χ^2 tests were performed.

3. Results

3.1. Epidemiological characteristics

The characteristics of the study population are described in Table 1, providing the information regarding age, sex, smoking and alcohol use in the controls and arsenic-exposed group. No significant differences were found in all these characteristics between two group people after statistical analysis.

3.2. Case-control study in arsenic exposure

The presence of arsenic in urine is an indication of recent exposure (approximately within days), while arsenic in hair or fingernails is of exposure within 3-6months [36]. As shown in Table 1, the total arsenic contents of both urine and hair samples were significantly higher in arsenic exposure group than that in controls. The average content of urinary arsenic in exposure group is 1.92 fold higher that in control group ($P < 0.01$, independent-samples *t*-tests). In parallel, the average content of hair arsenic in exposure group is 3.79 fold higher than that in control group ($P < 0.01$, independent-samples *t*-tests). These observations confirm the high level of exposure in arseniasis subjects.

3.3. Arsenic exposure is associated with hypermylation of $p15^{INK4b}$

DNA methylation of the $p15^{INK4b}$ genes is detected in 14 (13.33%) of arseniasis patients and 3 (2.91%) of control, respectively (Table 1). The degrees of $p15^{INK4b}$

Table 1
Demographic data of the subjects and biomarkers analyzed.

Variables	Controls No. (%) or Mean ± SD	Arsenic exposure No. (%) or Mean ± SD	*P*
Epidemiological characteristics			
Number	103	105	
Age			0.38[a]
≥40	45(43.69)	38(36.19)	
<40	58(56.31)	67(63.81)	
Sex			0.50[a]
Male	58(56.31)	64(60.95)	
Female	45(43.69)	41(39.05)	
Smoking			0.56 [a]
No	41(39.8)	46(43.81)	
Yes	62(60.2)	59(56.19)	
Alcohol use			0.77 [a]
No	56(54.36)	55(52.38)	
Yes	47(45.64)	50(47.62)	
Arsenic exposure and $p15^{INK4b}$			
$p15^{INK4b}$ deletion (%)	16(15.53)	31(29.52)	0.02[a]
$p15^{INK4b}$ methylation (%)	3(2.91)	14(13.33)	0.04[a]
Urinary Arsenic (μg/L)	28.8 ± 1.64	55.28 ± 1.89	<0.01[b]
Hair Arsenic (μg/g)	1.36 ± 0.20	5.16 ± 0.62	<0.01[b]

Data are expressed as mean ± SD or No. (%).
[a] Two-tailed χ^2 test.
[b] Independent-samples *t*-test.

Table 2
Correlations between arsenic exposure and deletion or promoter methylation of $p15^{INK4b}$.

	N	$p15^{INK4b}$ deletion (%)	χ^2	P	$p15^{INK4b}$ methylation (%)	χ^2	P
Urinary Arsenic (μg/L)							
0–<40	26	5(19.23)	4.90	0.03[a]	2(7.69)	2.08	0.15[a]
40–<80	36	12(33.33)			6(16.67)		
80–	27	13(48.15)			6(22.22)		
Hair Arsenic (μg/g)							
0–<3	34	7(20.59)	4.31	0.04[a]	2(2.88)	4.28	0.04[a]
3–<6	30	9(30.00)			4(13.33)		
6–	34	15(44.12)			8(23.53)		

Data are expressed as No. (%).
[a] Two-tailed χ^2 test.

Table 3
Correlations between the severity of the arsenic poisoning and deletion or promoter methylation of $p15^{INK4b}$.

	N	$p15^{INK4b}$ deletion (%)	χ^2	P	$p15^{INK4b}$ methylation (%)	χ^2	P
Control	103	16(15.53)	7.84	0.01[a]	3(2.91)	12.82	0.00[a]
Mild	35	8(22.88)			2(5.71)		
Intermediate	44	13(29.55)			6(13.64)		
Severe	26	10(38.46)			6(23.08)		
Histology							
Non-skin cancers	27	4(14.81)		0.04[a]	1(3.70)		0.00[a]
Skin cancers	17	8(47.06)			7(41.18)		

Data are expressed as No. (%).
[a] Two-tailed χ^2 test.

methylation were not significantly different in terms of sexes or age between two groups (data not show). The exposure subjects were divided into three subgroups according to the content of urinary arsenic (<40 μg/L, <80 μg/L and ≥80 μg/L, respectively) and to the content of hair arsenic (<3 μg/L, <6 μg/L and ≥6 μg/L, respectively). The rates of methylation of $p15^{INK4b}$ promoter are positively correlated with the increasing levels of hair arsenic exposure (two-tailed χ^2 test, $P<0.05$), but not correlated with the urinary arsenic exposure. These results revealed that higher levels of arsenic exposure might be associated with hypermethylation of $p15^{INK4b}$.

3.4. Arsenic exposure is associated with $p15^{INK4b}$ gene deletions

As shown in Table 1, homozygous deletions of the $p15^{INK4b}$ genes in at least one exon are detected in 31 (29.52%) of arseniasis patients and 16 (15.53%) of control. The degree of $p15^{INK4b}$ homozygous deletion was not significantly different in terms of sex or age between two groups (data not shown). The frequency of $p15^{INK4b}$ gene deletion (at least one exon) is much higher in the highest urinary arsenic subgroup (48.15%) compared to the low urinary arsenic subgroup (9.23%) (two-tailed χ^2 test, $P<0.05$). Similar results were also found when the comparison performed between the frequency of $p15^{INK4b}$ deletion and the amounts of hair arsenic exposure (two-tailed χ^2 test, $P<0.05$) (Table 2).

3.5. $p15^{INK4b}$ methylation and homozygous deletion are correlated with the severity of arsenic poisoning

Next we determine whether functional defect of $p15^{INK4b}$ resulting from promoter methylation or homozygous deletion is associated with signs of clinical arsenic poisoning. Based on the Chinese National Standard of Arsenicosis Diagnosis, we divided the subjects into four groups based on the severity of the poisoning, control, mild, intermediate, and severe. We also divided the subjects into two groups, non-skin cancers and skin cancers. Factors such as gender, age, smoking or drinking status had no impact on $p15^{INK4b}$ methylation and gene deletion (data not shown). As a result, we found that the stage of skin lesions was positively correlated with the rates of promoter methylation and the deletions of $p15^{INK4b}$ (two-tailed χ^2 test, $P<0.05$). Moreover, the extent of $p15^{INK4b}$ methylation and deletion is significantly higher in the patients with skin cancers than non-skin cancers ($P<0.05$) (Table 3). Taken together, these observations reveal that the inactivation of $p15^{INK4b}$ is relative with the stage of the arsenicosis.

4. Discussion

Indoor coal use becomes one of the most serious environmental problems in China [1,37–39]. Previously, we reported a severe arsenic pollution village named Jiao Le in Guizhou Province, where numerous arsenicosis cases appeared with the skin lesion or skin cancers [40,41]. To explore the mechanisms of arsenic poisoning and related carcinogenicity, here we conduct a case-control study and demonstrate that arsenic exposure is associated with $p15^{INK4b}$ inactivation *via* DNA methylation and gene deletionsin Guizhou residents. These findings provide new insight into the role of $p15^{INK4b}$ in arsenicosis.

The disruption of tumor suppression genes is a pivotal mechanism of arsenic carcinogenesis in both human and experimental studies. Our previous works have linked human exposure to arsenic coal fire with the skin lesions

and cancers. The alterations in gene expression of two important tumor suppressor genes, *p16* [23] and *p53* [22] have been identified in samples of arsenic exposure residents. Adding to the previous findings, here we identify a novel gene $p15^{INK4b}$ whose inactivation might be involved in adverse health outcome induced by arsenic exposure.

The suppression or deletions of $p15^{INK4b}$ are found in numerous tumors such as hepatocellular carcinomas [42], basal cell carcinoma [43] and primary lymphoid malignancies [44]. It has been reported that $p15^{INK4b}$ exhibits an antitumor effect in basal cell carcinoma by inducing growth arrest and apoptosis in both *in vitro* and *in vivo* studies [26,27]. Recent studies have shown that both DNA methylation status and deletions of $p15^{INK4b}$ affect the expression of genes [31,45]. DNA hypermethylation occurs often in the promoter region of gene and leads to a transcriptional silencing of the genes found in mutilate tumors [46]. Arsenic has been found to cause changes in globe genome DNA methylation status in animal experiments and humans [47–49]. With respect to tumor suppressor genes, prior studies showed that inorganic arsenic exposure induced significant DNA hypermethylation in tumor suppressor gene *p53* [50–52] and $p16^{INK2a}$ [23,53]. In this study, we demonstrate a tendency of $p15^{INK4b}$ hypermethylation in arsenic exposed population in a dose-dependent manner. This tendency also appears in the skin lesion tissues, correlating with the stage of illness. It indicates that alteration of the DNA methylation of critical genes may be a plausible mechanism of arsenic-induced malignancy. Although the mechanism of DNA hypermethylation after arsenic exposure remains elusive, prior studies revealed that arsenic exposure could directly affect DNA cytosine methyltransferases and result in a change in whole genomic DNA methylation [54,55]. Further studies are required to address the critical pathways that trigger arsenic exposure-related epigenetic modification.

The deletion in $p15^{INK4b}$ exons in arsenicosis cases is correlated with the levels of arsenic exposure. Homozygous deletion of the $p15^{INK4b}$ gene has been proved as a common event in certain human primary tumors [30,56–59]. Here we find that the deletion of $p15^{INK4b}$ gene existing in exposed group is nearly twice as much as those in the control subjects. Although prior studies do not provide strong evidence that arsenic is a direct mutagen [7,8], we speculate that the mechanisms including oxidative damage [60,61], DNA repair inhibition [62] and genome instability [60] might contribute to arsenic-induced genotoxicity. Furthermore, we could not eliminate the possibility that genetic mutations detected in our study subjects might be due to the existence of other environmental hazards as demonstrated in previous studies [63–65].

Adding to our previous findings, these observations indicate that the inactivation of INK4b/ARF/INK4a locus is likely associated with chronic arsenic poisoning. It is known that $p15^{INK4b}$ and $p16^{INK2a}$ locate in INK4b/ARF/INK4a locus and function as redundant gene. Importantly, both $p15^{INK4b}$ and $p16^{INK2a}$ genes have been recognized as tumor suppression genes [66]. Our prior works have showed that arsenic exposure leads to a defect of $p16^{INK2a}$ gene [23]. Taken together, we speculate that the locus of INK4b/ARF/INK4a is the target of arsenic-induced genetic or epigenetic modifications. The functional defects of INK4b/ARF/INK4a might be attributable to arsenic-induced adverse health effects.

5. Conclusions

In conclusion, this study demonstrates that inactivation of $p15^{INK4b}$ gene *via* DNA methylation or genetic mutation may result from human arsenic exposure and contribute to the development of arsenicosis.

Conflict of interest

The authors declare that there are no conflicts of interest.

Transparency document

The Transparency document associated with this article can be found in the online version.

Acknowledgments

This work was supported by the National Key Basic Research and Development Program (2010CB912803); the National Natural Science Foundation of China (NSFC) (81172603 and 30760225).

References

[1] R.B. Finkelman, H.E. Belkin, B. Zheng, Health impacts of domestic coal use in China, Proc. Natl. Acad. Sci. U. S. A. 96 (1999) 3427–3431.
[2] J. Liu, B. Zheng, H.V. Aposhian, Y. Zhou, M.L. Chen, A. Zhang, M.P. Waalkes, Chronic arsenic poisoning from burning high-arsenic-containing coal in Guizhou, China, Environ. Health Perspect. 110 (2002) 119–122.
[3] G. Yu, D. Sun, Y. Zheng, Health effects of exposure to natural arsenic in groundwater and coal in China: an overview of occurrence, Environ. Health Perspect. 115 (2007) 636–642.
[4] D.X. Zhou, [Investigation of chronic arsenic poisoning caused by high arsenic coal pollution], Zhonghua yu fang yi xue za zhi [Chin. J. Prev. Med.] 27 (1993) 147–150.
[5] D. An, D. Li, Y. Liang, Z. Jing, Unventilated indoor coal-fired stoves in Guizhou province, China: reduction of arsenic exposure through behavior changes resulting from mitigation and health education in populations with arsenicosis, Environ. Health Perspect. 115 (2007) 659–662.
[6] IARC, IARC monographs on the evaluation of carcinogenic risk of chemicals to humans: some drinking water disinfectants and contaminants, including arsenic. IARC Monographs, vol. 84, 2004, pp. 1–477.
[7] M.F. Hughes, B.D. Beck, Y. Chen, A.S. Lewis, D.J. Thomas, Arsenic exposure and toxicology: a historical perspective, Toxicol. Sci. 123 (2011) 305–332.
[8] P. Bhattacharjee, M. Banerjee, A.K. Giri, Role of genomic instability in arsenic-induced carcinogenicity: a review, Environ. Int. 53 (2013) 29–40.
[9] M.M. Niedzwiecki, M.N. Hall, X. Liu, J. Oka, K.N. Harper, V. Slavkovich, V. Ilievski, D. Levy, A. van Geen, J.L. Mey, S. Alam, A.B. Siddique, F. Parvez, J.H. Graziano, M.V. Gamble, A dose-response study of arsenic exposure and global methylation of peripheral blood mononuclear cell DNA in Bangladeshi adults, Environ. Health Perspect. 121 (2013) 1306–1312.
[10] X. Ren, C.M. McHale, C.F. Skibola, A.H. Smith, M.T. Smith, L. Zhang, An emerging role for epigenetic dysregulation in arsenic toxicity and carcinogenesis, Environ. Health Perspect. 119 (2011) 11–19.
[11] U. Schuhmacher-Wolz, H.H. Dieter, D. Klein, K. Schneider, Oral exposure to inorganic arsenic: evaluation of its carcinogenic and non-carcinogenic effects, Crit. Rev. Toxicol. 39 (2009) 271–298.

[12] T.Y. Yang, L.I. Hsu, A.W. Chiu, Y.S. Pu, S.H. Wang, Y.T. Liao, M.M. Wu, Y.H. Wang, C.H. Chang, T.C. Lee, C.J. Chen, Comparison of genome-wide DNA methylation in urothelial carcinomas of patients with and without arsenic exposure, Environ. Res. 128 (2014) 57–63.
[13] S.M. Cohen, L.L. Arnold, B.D. Beck, A.S. Lewis, M. Eldan, Evaluation of the carcinogenicity of inorganic arsenic, Crit. Rev. Toxicol. 43 (2013) 711–752.
[14] S.M. Cohen, T. Ohnishi, L.L. Arnold, X.C. Le, Arsenic-induced bladder cancer in an animal model, Toxicol. Appl. Pharmacol. 222 (2007) 258–263.
[15] M. Argos, M.G. Kibriya, F. Parvez, F. Jasmine, M. Rakibuz-Zaman, H. Ahsan, Gene expression profiles in peripheral lymphocytes by arsenic exposure and skin lesion status in a Bangladeshi population, Cancer Epidemiol. Biomark. Prev. 15 (2006) 1367–1375.
[16] P. Ghosh, M. Banerjee, A.K. Giri, K. Ray, Toxicogenomics of arsenic: classical ideas and recent advances, Mutat. Res. 659 (2008) 293–301.
[17] L. Smeester, J.E. Rager, K.A. Bailey, X. Guan, N. Smith, G. Garcia-Vargas, L.M. Del Razo, Z. Drobna, H. Kelkar, M. Styblo, R.C. Fry, Epigenetic changes in individuals with arsenicosis, Chem. Res. Toxicol. 24 (2011) 165–167.
[18] Y. Hu, A.H. Zhang, M.L. Yao, X.D. Tang, X.X. Huang, [The regulation mechanism of protein kinase Cdelta on arsenic liver injury caused by coal-burning], Zhonghua yu fang yi xue za zhi [Chin. J. Prev. Med.] 47 (2013) 777–782.
[19] J. Li, A.H. Zhang, Y.J. Ren, Z.Y. Liu, X.X. Huang, D.P. Yang, [Regulating effects of rosa roxburghii tratt preparation on immune function in arseniasis patients caused by coal burning], Zhonghua yu fang yi xue za zhi [Chin. J. Prev. Med.] 47 (2013) 783–787.
[20] P. Luo, A.H. Zhang, Y. Xiao, X.L. Pan, X.X. Dong, X.X. Huang, [The mRNA expression of mitogen-activated protein kinase signal pathway related genes in the blood of arseniasis patients caused by burning coal], Zhonghua yu fang yi xue za zhi [Chin. J. Prev. Med.] 47 (2013) 788–793.
[21] M.L. Yao, A.H. Zhang, C. Yu, Y.Y. Xu, Y. Hu, T.T. Xiao, L. Wang, [The establishment of the arsenic poisoning rats model caused by corn flour baked by high-arsenic coal], Zhonghua yu fang yi xue za zhi [Chin. J. Prev. Med.] 47 (2013) 799–805.
[22] A.H. Zhang, X.L. Pan, Y.J. Xia, Q. Xiao, X.X. Huang, [Relationship between the methylation and mutation of p53 gene and endemic arsenism caused by coal-burning], Zhonghua Yu Fang Yi Xue Za Zhi 45 (2011) 393–398.
[23] A.H. Zhang, H.H. Bin, X.L. Pan, X.G. Xi, Analysis of p16 gene mutation, deletion and methylation in patients with arseniasis produced by indoor unventilated-stove coal usage in Guizhou, China, J. Toxicol. Environ. Health: A 70 (2007) 970–975.
[24] C.J. Hu, A.H. Zhang, H.H. Huang, Molecular pathology of skin carcinogenesis due to arsenicalism from coal-burning, Arch. Environ. Health 58 (2003) 92–96.
[25] A.H. Zhang, [Strengthen application oriented arsenic toxic mechanisms research], Zhonghua yu fang yi xue za zhi [Chin. J. Prev. Med.] 47 (2013) 774–776.
[26] J. Gil, G. Peters, Regulation of the INK4b-ARF-INK4a tumour suppressor locus: all for one or one for all, Nat. Rev. Mol. Cell Biol. 7 (2006) 667–677.
[27] W.Y. Kim, N.E. Sharpless, The regulation of INK4/ARF in cancer and aging, Cell 127 (2006) 265–275.
[28] S. Ortega, M. Malumbres, M. Barbacid, Cyclin D-dependent kinases, INK4 inhibitors and cancer, Biochim. Biophys. Acta 1602 (2002) 73–87.
[29] N. Popov, J. Gil, Epigenetic regulation of the INK4b-ARF-INK4a locus: in sickness and in health, Epigenetics 5 (2010) 685–690.
[30] D.A. Solomon, J.S. Kim, W. Jean, T. Waldman, Conspirators in a capital crime: co-deletion of p18INK4c and p16INK4a/p14ARF/p15INK4b in glioblastoma multiforme, Cancer Res. 68 (2008) 8657–8660.
[31] H. Yasaei, E. Gilham, J.C. Pickles, T.P. Roberts, M. O'Donovan, R.F. Newbold, Carcinogen-specific mutational and epigenetic alterations in INK4A, INK4B and p53 tumour-suppressor genes drive induced senescence bypass in normal diploid mammalian cells, Oncogene 32 (2013) 171–179.
[32] C. Xing, Q.F. Wang, B. Li, H. Tian, Y. Ni, S. Yin, G. Li, Methylation and expression analysis of tumor suppressor genes p15 and p16 in benzene poisoning, Chem. Biol. Interact. 184 (2010) 306–309.
[33] C. Xing, L. Zhang, M. Zheng, H. Tian, G. Zhou, G. Li, Q. Wang, [Correlation of the down-regulation of tumor suppressor gene p15 and hematotoxicity from benzene exposure], Wei sheng yan jiu [J. Hyg. Res.] 41 (2012) 489–492.
[34] CN-WS, 2001. Chinese National Standard of Arsenicosis Diagnosis. vol. WS/T 211-2001, China.
[35] B. Nermell, A.L. Lindberg, M. Rahman, M. Berglund, L.A. Persson, S. El Arifeen, M. Vahter, Urinary arsenic concentration adjustment factors and malnutrition, Environ. Res. 106 (2008) 212–218.
[36] ATSDR, Toxicological Profile for Arsenic, 2005.
[37] G. He, B. Ying, J. Liu, S. Gao, S. Shen, K. Balakrishnan, Y. Jin, F. Liu, N. Tang, K. Shi, E. Baris, M. Ezzati, Patterns of household concentrations of multiple indoor air pollutants in China, Environ. Sci. Technol. 39 (2005) 991–998.
[38] D. Li, D. An, Y. Zhou, J. Liu, M.P. Waalkes, Current status and prevention strategy for coal-arsenic poisoning in Guizhou, China, J. Health Popul. Nutr. 24 (2006) 273–276.
[39] A. Shraim, X. Cui, S. Li, J.C. Ng, J. Wang, Y. Jin, Y. Liu, L. Guo, D. Li, S. Wang, R. Zhang, S. Hirano, Arsenic speciation in the urine and hair of individuals exposed to airborne arsenic through coal-burning in Guizhou, PR China, Toxicol. Lett. 137 (2003) 35–48.
[40] C.X. Wu, K.R. Shan, Y.S. Zhou, Q. Yang, C.X. Li, Y. Zhao, M.L. Cheng, [Research into methylation of p16 gene in the patients suffering from coal-burnt arsenism in Xingren County Guizhou Province], Zhonghua yu fang yi xue za zhi [Chin. J. Prev. Med.] 41 (2007) 29–32.
[41] A. Zhang, H. Feng, G. Yang, X. Pan, X. Jiang, X. Huang, X. Dong, D. Yang, Y. Xie, L. Peng, L. Jun, C. Hu, L. Jian, X. Wang, Unventilated indoor coal-fired stoves in Guizhou province, China: cellular and genetic damage in villagers exposed to arsenic in food and air, Environ. Health Perspect. 115 (2007) 653–658.
[42] Y. Edamoto, A. Hara, W. Biernat, L. Terracciano, G. Cathomas, H.M. Riehle, M. Matsuda, H. Fujii, J.Y. Scoazec, H. Ohgaki, Alterations of RB1, p53 and Wnt pathways in hepatocellular carcinomas associated with hepatitis C, hepatitis B and alcoholic liver cirrhosis, Int. J. Cancer 106 (2003) 334–341.
[43] P. Kanellou, A. Zaravinos, M. Zioga, D.A. Spandidos, Deregulation of the tumour suppressor genes p14(ARF), p15(INK4b), p16(INK4a) and p53 in basal cell carcinoma, Br. J. Dermatol. 160 (2009) 1215–1221.
[44] A. Hangaishi, S. Ogawa, N. Imamura, S. Miyawaki, Y. Miura, N. Uike, C. Shimazaki, N. Emi, K. Takeyama, S. Hirosawa, N. Kamada, Y. Kobayashi, Y. Takemoto, T. Kitani, K. Toyama, S. Ohtake, Y. Yazaki, R. Ueda, H. Hirai, Inactivation of multiple tumor-suppressor genes involved in negative regulation of the cell cycle, MTS1/p16INK4A/CDKN2, MTS2/p15INK4B, p53, and Rb genes in primary lymphoid malignancies, Blood 87 (1996) 4949–4958.
[45] C.R. Geyer, Strategies to re-express epigenetically silenced p15(INK4b) and p21(WAF1) genes in acute myeloid leukemia, Epigenetics 5 (2010) 696–703.
[46] D.I. Martin, J.E. Cropley, C.M. Suter, Epigenetics in disease: leader or follower? Epigenetics 6 (2011) 843–848.
[47] S. Chanda, U.B. Dasgupta, D.G. Mazumder, J. Saha, B. Gupta, Human GMDS gene fragment hypermethylation in chronic high level of arsenic exposure with and without arsenic induced cancer, SpringerPlus 2 (2013) 557.
[48] J.F. Reichard, A. Puga, Effects of arsenic exposure on DNA methylation and epigenetic gene regulation, Epigenomics 2 (2010) 87–104.
[49] T. Suzuki, S. Yamashita, T. Ushijima, S. Takumi, T. Sano, T. Michikawa, K. Nohara, Genome-wide analysis of DNA methylation changes induced by gestational arsenic exposure in liver tumors, Cancer Sci. 104 (2013) 1575–1585.
[50] P. Intarasunanont, P. Navasumrit, S. Waraprasit, K. Chaisatra, W.A. Suk, C. Mahidol, M. Ruchirawat, Effects of arsenic exposure on DNA methylation in cord blood samples from newborn babies and in a human lymphoblast cell line, Environ. Health 11 (2012) 31.
[51] M.L. Kile, A. Baccarelli, E. Hoffman, L. Tarantini, Q. Quamruzzaman, M. Rahman, G. Mahiuddin, G. Mostofa, Y.M. Hsueh, R.O. Wright, D.C. Christiani, Prenatal arsenic exposure and DNA methylation in maternal and umbilical cord blood leukocytes, Environ. Health Perspect. 120 (2012) 1061–1066.
[52] M.J. Mass, L. Wang, Arsenic alters cytosine methylation patterns of the promoter of the tumor suppressor gene p53 in human lung cells: a model for a mechanism of carcinogenesis, Mutat. Res. 386 (1997) 263–277.
[53] N. Banerjee, S. Paul, T.J. Sau, J.K. Das, A. Bandyopadhyay, S. Banerjee, A.K. Giri, Epigenetic modifications of DAPK and p16 genes contribute to arsenic-induced skin lesions and nondermatological health effects, Toxicol. Sci. 135 (2013) 300–308.
[54] X. Cui, T. Wakai, Y. Shirai, N. Yokoyama, K. Hatakeyama, S. Hirano, Arsenic trioxide inhibits DNA methyltransferase and restores methylation-silenced genes in human liver cancer cells, Hum. Pathol. 37 (2006) 298–311.
[55] Z.J. Han, G. Song, Y. Cui, H.F. Xia, X. Ma, Oxidative stress is implicated in arsenic-induced neural tube defects in chick embryos, Int. J. Dev. Neurosci. 29 (2011) 673–680.

[56] E. Grafstrom, S. Egyhazi, U. Ringborg, J. Hansson, A. Platz, Biallelic deletions in INK4 in cutaneous melanoma are common and associated with decreased survival, Clin. Cancer Res. 11 (2005) 2991–2997.
[57] M. Kim, S.H. Yim, N.S. Cho, S.H. Kang, D.H. Ko, B. Oh, T.Y. Kim, H.J. Min, C.J. She, H.J. Kang, H.Y. Shin, H.S. Ahn, S.S. Yoon, B.K. Kim, H.R. Shin, K.S. Han, H.I. Cho, D.S. Lee, Homozygous deletion of CDKN2A (p16, p14) and CDKN2B (p15) genes is a poor prognostic factor in adult but not in childhood B-lineage acute lymphoblastic leukemia: a comparative deletion and hypermethylation study, Cancer Genet. Cytogenet. 195 (2009) 59–65.
[58] P. Krimpenfort, A. Ijpenberg, J.Y. Song, M. van der Valk, M. Nawijn, J. Zevenhoven, A. Berns, p15Ink4b is a critical tumour suppressor in the absence of p16Ink4a, Nature 448 (2007) 943–946.
[59] E. Laharanne, E. Chevret, Y. Idrissi, C. Gentil, M. Longy, J. Ferrer, P. Dubus, T. Jouary, B. Vergier, M. Beylot-Barry, J.P. Merlio, CDKN2A-CDKN2B deletion defines an aggressive subset of cutaneous T-cell lymphoma, Mod. Pathol. 23 (2010) 547–558.
[60] D. Beyersmann, A. Hartwig, Carcinogenic metal compounds: recent insight into molecular and cellular mechanisms, Arch. Toxicol. 82 (2008) 493–512.
[61] S.J. Flora, Arsenic-induced oxidative stress and its reversibility, Free Radic. Biol. Med. 51 (2011) 257–281.
[62] A.S. Andrew, J.L. Burgess, M.M. Meza, E. Demidenko, M.G. Waugh, J.W. Hamilton, M.R. Karagas, Arsenic exposure is associated with decreased DNA repair in vitro and in individuals exposed to drinking water arsenic, Environ. Health Perspect. 114 (2006) 1193–1198.
[63] R. Colognato, F. Coppede, J. Ponti, E. Sabbioni, L. Migliore, Genotoxicity induced by arsenic compounds in peripheral human lymphocytes analysed by cytokinesis-block micronucleus assay, Mutagenesis 22 (2007) 255–261.
[64] A. Sampayo-Reyes, A. Hernandez, N. El-Yamani, C. Lopez-Campos, E. Mayet-Machado, C.B. Rincon-Castaneda, L. Limones-Aguilar Mde, J.E. Lopez-Campos, M.B. de Leon, S. Gonzalez-Hernandez, D. Hinojosa-Garza, R. Marcos, Arsenic induces DNA damage in environmentally exposed Mexican children and adults. Influence of GSTO1 and AS3MT polymorphisms, Toxicol. Sci. 117 (2010) 63–71.
[65] S.B. Vuyyuri, M. Ishaq, D. Kuppala, P. Grover, Y.R. Ahuja, Evaluation of micronucleus frequencies and DNA damage in glass workers exposed to arsenic, Environ. Mol. Mutagen. 47 (2006) 562–570.
[66] J. Li, M.J. Poi, M.D. Tsai, Regulatory mechanisms of tumor suppressor P16(INK4A) and their relevance to cancer, Biochemistry 50 (2011) 5566–5582.