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

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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 carcinogenesis. This study aims to clarify the correlation between arsenic pollution and functional defect of $p15^{\text{INK4b}}$ 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 $p15^{\text{INK4b}}$ 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 $p15^{\text{INK4b}}$ 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 $p15^{\text{INK4b}}$ through genetic alteration or epigenetic modification is a common event that is associated with arsenic exposure and the development of arsenicosis.

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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...

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aberration [7,8]. In addition, lines of evidence have shown that the epigenetic regulation such as promoter methylation of tumor suppressor gene is a critical event in the course of arsenic-induced carcinogenicity [9–12]. For example, it is supported by both epidemiological 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 CCND1 [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. p15INK4b is a critical tumor suppressor gene involved in control of cell proliferation [26,27]. Aberrant p15INK4b expression has been found in many kinds of human tumors including leukemia, lung and skin cancers [28]. The p15INK4b gene can be inactivated by point mutation, homozygous deletion, or DNA methylation in various human tumors [26,29,30]. The defect of p15INK4b 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 p15INK4b inactivation contributes to the development of arseniasis through detection of the expression of p15INK4b in arsenic-induced skin lesion tissues and examination of the p15INK4b deletion and promoter methylation in peripheral lymphocytes from arsenic-exposed villagers. Our results indicate that functional defect of p15INK4b 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 (HNO3). 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′-TGTGATGTTTGGATTTTG-3′; reverse: 5′-CCATAAACACACAC-3′) and methylation specific primers (forwards: 5′-GGTGTCGATTTTCTGGT-3′; reverse: 5′-CGTACATAACCCGACACGGA-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.SsI, 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\textsuperscript{INK4b} was analyzed by multiplex PCR. The primers used for amplification are as follows: forwards 5'-CCAGAACAGCAATCCAGGGCCG-3' and reverse: 5'-AATGCAACACCTGCC AACG-3' for exon 1 (532 bp); and forwards: 5'-CTTAAATGCTCCACTGC-3', and reverse: 5'-CTTGCGCCTCATC-3' for exon 2 (437 bp). A fragment (303 bp) of β-actin gene (forwards: 5'-GAAAATCTACCTCAACTTGC-3', and reverse: 5'-CTAGAACCTTGTGCGACAGTGGAGGGGCC-3') was used as an internal standard. All multiplex reactions were made in a total volume of 25 μl containing MgCl\textsubscript{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 Arsenicism 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\textsuperscript{INK4b} methylation and deletion, the trend χ\textsuperscript{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 3-6 months [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\textsuperscript{INK4b}

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

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

Data are expressed as mean ± SD or No. (%).

\textsuperscript{a} Two-tailed χ\textsuperscript{2} test.

\textsuperscript{b} Independent-samples t-test.
Table 2
Correlations between arsenic exposure and deletion or promoter methylation of p15\(^{INK4b}\).

| Urinary Arsenic (µg/L) | N | p15\(^{INK4b}\) deletion (%) | \(\chi^2\) | \(P\) | p15\(^{INK4b}\) methylation (%) | \(\chi^2\) | \(P\) |
|------------------------|---|-----------------------------|------|----|-------------------------------|------|----|
| 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 \(\chi^2\) test.

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

| N | p15\(^{INK4b}\) deletion (%) | \(\chi^2\) | \(P\) | p15\(^{INK4b}\) methylation (%) | \(\chi^2\) | \(P\) |
|---|-----------------------------|------|----|-------------------------------|------|----|
| 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)  |      |    | 13 (2.70) |      |    |
| Skin cancers                       | 17  | 8 (47.06)  | 0.04\(^a\) |      | 7 (41.18) |      |    |

Data are expressed as No. (%).

\(^a\) Two-tailed \(\chi^2\) 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 associated with the increasing levels of hair arsenic exposure (two-tailed \(\chi^2\) 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 \(\chi^2\) 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 \(\chi^2\) 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 \(\chi^2\) 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).

4. Discussion

Indoor coal use becomes one of the most severe 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 deletions in 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 p15NK4b whose inactivation might be involved in adverse health outcome induced by arsenic exposure.

The suppression or deletions of p15NK4b are found in numerous tumors such as hepatocellular carcinomas [42], basal cell carcinoma [43] and primary lymphoid malignancies [44]. It has been reported that p15NK4b 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 p15NK4b 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 mutlute tumors [46]. Arsenic has been found to cause changes in 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 p16INK4a [23,53]. In this study, we demonstrate a tendency of p15NK4b 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 p15NK4b exons in arsenicosis cases is correlated with the levels of arsenic exposure. Homozygous deletion of the p15NK4b gene has been proved as a common event in certain human primary tumors [30,56–59]. Here we find that the deletion of p15NK4b 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 p15NK4b and p16INK4a locate in INK4b/ARF/INK4a locus and function as redundant gene. Importantly, both p15INK4b and p16INK4a genes have been recognized as tumor suppression genes [66]. Our prior works have showed that arsenic exposure leads to a defect of p16INK4a 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 p15NK4b 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.

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