Increased damage of exon 5 of \( p53 \) gene in workers from an arsenic plant

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**A B S T R A C T**

Mutagenesis is a multistage process. Substitution mutations can be induced by base modified through alteration of pairing property. Mutations of exon 5 and 8 of \( p53 \) gene have been found in most arsenicosis patients with precarcinomas and carcinomas, but never in arsenicosis individuals without precarcinomas and carcinomas. This study investigates whether base modification exists in exon 5 and 8 of \( p53 \) gene, and explores the dose-effect relationship between damage of exon 5 of \( p53 \) gene and urinary arsenic. Concentrations of urinary 8-hydroxydeoxyguanine (8-OHdG) are analyzed to identify the occurrence of DNA damage. The real-time PCR developed by Sikorsky et al. is applied to detect base modification in exon 5 and 8 of \( p53 \) gene for apparently healthy participants. Our results show that the mean total arsenic concentrations of two exposed groups from an arsenic plant are significantly elevated compared with the control group, and the damage level of exon 5 of the high-exposed group is significantly higher than that of the control group, but which does not happen in exon 8. The closely correlation between the damage index of exon 5 and urinary organic arsenic concentration are found. Concentration of 8-OHdG of the high-exposed group is significantly higher than that of the control group. These results imply that base modification in exon 5 of \( p53 \) gene can be induced by arsenic. In addition, our study suggests that the damage level of exon 5 is a useful biomarker to assess adverse health effect levels caused by chronic exposure to arsenic.

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1. Introduction

Inorganic arsenic, a natural metalloid element, is a well recognized human carcinogen [1]. Epidemiological studies have indicated that long-term exposure to inorganic arsenic increases the risk of cancers of lung, skin, bladder, liver and probably other anatomic sites [2]. An appreciable portion of the population in the world is constantly exposed to arsenic through ingestion and inhalation [3]. Therefore, health risk due to arsenic exposure should be viewed as a global concern.

A number of studies have investigated the process by which the arsenic exposure increases the health hazard. Barchowsky et al. showed that, during its metabolism, arsenic favors the generation of reactive oxygen species (ROS) [4], which was suggested by Yamanaka et al. to be involved in the genotoxicity and tumorigenicity of arsenic [5]. ROS can attack base to cause oxidative base damage, which alters DNA secondary structure and increases mutational probability [6–10]. Previous studies have demonstrated the deleterious impacts of arsenic on DNA, including oxidative base damage [11], chromosome aberration [12], aneuploidy and micronuclei formation [13,14], DNA-protein cross-linking, sister chromatin exchange [15], and decrease DNA repair gene expression [16]. In addition, it has been suggested that these impacts are associated with inhibition of activity of \( p53 \) [17].

The human \( p53 \) tumor suppressor gene throughout cell cycle is consistently transcribed in the growing cells [18], which encode a nuclear phosphoprotein functioning in cell cycle arrest, apoptosis, inhibition of tumor growth, and preservation of genetic stability...
[19–22]. Genetic alterations in p53 gene have been shown to have a clear correlation with cancer development, occurring in nearly 50% of all cancers [23]. One intriguing fact is that mutations of p53 gene were frequently found in arsenic-exposure patients with precarcinomas and carcinomas, but were never observed in those without precarcinomas and carcinomas [24–26].

The understanding of the arsenic-associated process has been greatly enhanced during the past decade. However, the molecular mechanism by which arsenic exposure increases cancer risk remains unclear. Since cancer development is a stepwise process, different types of damage may be involved. In this study, we investigate whether other types of damage in exon 5 and 8 of p53, such as base modification, exists in its mutational hot-spot region for arsenic-exposure subjects without precarcinomas and carcinomas. Damage index of exon 5 and 8 of p53 gene is examined with the realtime PCR developed by Sikorsky et al. [27,28]. Relationship between the damage level of exon 5 of p53 gene and urinary arsenic concentration caused by arsenic is diagnosed. In addition, urinary 8-OHdG is analyzed to further identify the occurrence of DNA damage.

2. Materials and methods

2.1. Participating subjects

A total of 74 subjects participate in this study, including 51 arsenic-exposure workers from an arsenic plant, which produces arsenic trioxide by smelting arsenic ore, and 23 local residents who have no any known experience of arsenic exposure. A questionnaire was used to obtain information from each individual, including age, gender, type of work, service length, use of cigarettes and alcohols, and other potential chemical exposures (lead, carbon monoxide, sulfurred hydrogen, sulfur dioxide, silicon dioxide, and so on). Based on the type of work and the levels of environmental arsenic pollution, 51 arsenic-exposed individuals are divided into high-exposed group and low-exposed group. In the high-exposed group, 35 participants worked as smelter, collector, transporter and packer, and in the other, 16 participants worked as typist, seller, detectors, medical and logistical server. Participants of the high-exposed group have been exposed to arsenic by the way of inhalation and direct contact with arsenic production, while the subjects of the low-exposed group have been exposed to arsenic only through inhalation. All subjects participating in this study were surveyed in accordance with appropriate Chinese human subject protocols.

2.2. Collection and pretreatment of urine

Written instructions regarding the hygienic conditions for collection of samples and 500 ml polyethylene containers treated with hydrochloric acid and rinsed with deionized water were provided to all participants. Subjects were asked to provide the first morning void urine. After the collection, urine samples were immediately sent to the laboratory and stored at 4 °C. All analyses were completed within 2 days after the collection.

2.3. Measurement of total, inorganic and organic arsenic in urine

A spectrophotographic procedure using silver diethyldithiocarbamate (AgDDC) as the complexing agent was applied to determine total arsenic concentration. All measurements were performed strictly following the Chinese National Standards (GB) and the Technical Standard issued by Chinese Ministry of Health (WS/T).

Total arsenic contents in urine samples were determined according to the procedure specified in “Standard for determination of total arsenic in urine – silver diethyldithiocarbamate spectrophotometric method” (WS/T28–1996).

Inorganic arsenic concentration in urine was determined using the method of Hu et al., which includes the following steps [29]: (i) 15 ml 11% ammonium carbonate (NH₄HCO₃) solution was added into each sample of 25 ml urine in a taper bottle; (ii) the resulting mixture was stirred at a rate of 240 times per minute for one hour; (iii) with an addition of 1 ml strong sulphuric acid, the sample was incubated in a water bath at 80 °C for 10 min; and (iv) the sample was then filtrated and inorganic arsenic concentration in the filtrate was determined by AgDDC assay.

Both total and inorganic arsenic concentrations in urine were adjusted by urine specific gravity. The organic arsenic concentration in urine was taken as total arsenic minus inorganic arsenic.

2.4. Genomic DNA preparation

DNA was isolated using DNA Isolation Kit of Tiangen Biotech Company. Prior to its use, genomic DNA obtained from peripheral blood lymphocytes was stored at −20 °C in the dark.

2.5. Calculation of damage index of exon 5 and 8 of p53 gene

2.5.1. Template amplification using real-time PCR

PCR primers were designed to amplify exon 5 of p53 gene, exon 8 of p53 gene, and β-actin sequence (control fragment) with ABI primer Express Software v2.0. Our standard 25 μl PCR contained 12.5 μl TOYOBO 2× SYBR Green PCR Mater Mix, 150 ng genomic DNA, 1× 10⁻¹⁵ M PCR primer (5′-TACTCCCTCGTCTACAAAGA-3′), either 1× 10⁻¹⁰ M PCR primer (5′-CGCTTACGACGGCCATCAG-3′) for exon 5 of p53 gene, or 1× 10⁻¹⁰ M PCR primer (5′-GATGCTGTAATCTACGGAGA-3′) either 1× 10⁻¹⁰ M PCR primer (5′-CTGCTTGCTGCCCTGCTGGGCG-3′) for exon 8 of p53 gene, or 1× 10⁻¹⁰ M PCR primer (5′-GCTGCTACCTTCACGTCGCC-3′) for β-actin fragment. An additional amount of sterile deionized H₂O was added so that the total amount is 25 μl. A master Mix without template was prepared based on the total number of reactions. Amplifications were performed in 96-well plates capped with optical grade ABI PCR strip caps. The following are the conditions for the amplifications:

- exon 5 of p53 gene: 94 °C for 5 min followed by 40 cycles of 94 °C for 40 s, 58 °C for 40 s, 72 °C for 40 s, 91.5 °C for 5 s, and 72 °C for 5 min;
- exon 8 of p53 gene: 94 °C for 5 min followed by 40 cycles of 94 °C for 45 s, 58 °C for 45 s, 72 °C for 45 s, 89 °C for 5 s, and 72 °C for 5 min;
- β-actin: 94 °C for 5 min followed by 40 cycles of 94 °C for 40 s, 58 °C for 40 s, 72 °C for 40 s, 87 °C for 5 s, and 72 °C for 5 min.

Each sample was amplified in the same well. PCR amplification and detection were carried out in an ABI Model 7000 Sequence Detection System (SDS) [30]. Ct and delta Ct values were exported from SDS data files with comma delimited (.csv) format, from which RCT efficiencies were calculated with Microsoft Excel.

2.5.2. Determination of damage index

The efficiency of modified templates (Eₐ) was determined according to the method of Sikorsky et al. [28]. In this study, the levels of DNA damage were expressed using damage index (D), i.e. reciprocal of Eₐ.

2.6. Analysis of 8-OHdG

Levels of urinary 8-OHdG are determined by indirect competitive enzyme-linked immunosorbent assay (ELISA) [New 8-OHdG Check; Institute for the Control of Aging, Fukuroi, Shizuoka, Japan] following Chuang and Maeshima [31,32]. The following steps are taken sequentially to determine urinary 8-OHdG level:

- thaw urine samples and centrifuge them at 2000 rpm for 10 min to obtain the opaque layer;
- add a 50 μl aliquot of each sample and 50 μl of primary monoclonal antibodies to microtitre plates pre-coated with 8-OHdG;
- incubate the plates at 37 °C for 1 h with continuous mixing at 100 rpm;
- wash the binding of free 8-OHdG with 250 μl phosphate buffered saline;
- add Horseradish peroxidase (HRP) conjugated secondary antibodies (100 μl) to each well and incubate the plates for 1 h with mixing;
- wash out the unbound secondary antibody;
- add a 100 μl aliquot of chromatic substrate to each well and incubate in the dark at room temperature for 15 min; and
- add 100 μl of 1.0 M phosphoric acid to terminate the reaction.

The absorbance was measured at 450 nm with an ELISA reader (VERSAMAX, USA). The concentration of 8-OHdG of the urine samples was interpolated from a standard logarithmic curve with a concentration range from 0.5 to 2000 ng/ml. The results were expressed as μg/g creatinine.

2.7. Statistical analysis

Data is expressed as mean ± standard deviation. Differences on levels of urinary arsenic and 8-OHdG among the groups are analyzed by one-way analysis of variance. Linear correlation and regression are used for analyzing relationship among variables. The significance level of the statistics in demographic is determined using the Chi-square test. The statistics are calculated using SPSS (version 11.5). Results will be considered statistically significant for significance levels higher than 95% (P < 0.05).

3. Results

3.1. Effects of arsenic exposure on total and organic arsenic in urine

Arsenic contents in urine for all three groups are shown in Fig. 1. Concentrations of total arsenic for the high-exposure, low-exposure, and control groups were significantly different (P < 0.05), while the differences in urinary concentrations of 8-OHdG among the three groups were not significant (P > 0.05).
exposure, and control groups are 1106.9 ± 718.4, 436.3 ± 300.3, and 17.3 ± 6.2 µg/L, respectively. Corresponding values of organic arsenic are 478.7 ± 365.8, 143.8 ± 181.5, and 15.2 ± 5.8 µg/L. The increases of urinary total and organic arsenic in low- and high-exposure groups are statistically significant in comparison with the control group. These results indicate that urinary arsenic concentrations are correlated with arsenic exposure, and increase with the degree of arsenic exposure. Impacts of other factors have also been analyzed, but no significant differences are found in gender, age, smoking and alcohol consumption between the arsenic-exposed groups and the control group.

3.2. Effects of arsenic on damage index of exon 5 and 8 of p53 gene

Effects of arsenic on damage index of exon 5 and 8 of p53 gene are presented in Fig. 2. For exon 5, the damage index of the high-exposed group is 62.2% higher than that of the control group (P < 0.05). In addition, the difference on damage indexes are also significant between two exposed and the control groups. For exon 8, changes of damage index are not statistically significant in low- and high-exposed groups compared with the control.

3.3. Relationship between the damage index of exon 5 and urinary organic arsenic concentration

As shown in Fig. 3, the damage index of exon 5 is correlated with logarithm value of urinary organic arsenic concentration. Multiple linear regression shows that such a relationship for participants does not exist for other variables, such as age, gender, service length, smoking and total urinary arsenic concentration (see Table 1).

3.4. Effects of arsenic on urinary 8-OHdG contents

The mean 8-OHdG concentrations in urine increase with the degree of arsenic exposure (Fig. 4). The mean 8-OHdG concentrations of control, low-exposure, and high-exposure groups are 28.12 ± 16.45, 37.68 ± 21.83, and 43.18 ± 27.50 µg/mg creatinine, respectively. There is a significant difference in 8-OHdG between the high-exposed and control groups. In addition, urinary 8-OHdG concentrations are closely correlated with logarithm values of urinary organic arsenic concentrations with a Spearman correlation coefficient of 0.302 (P = 0.011).

4. Discussions

The plant chosen for our study is a factory producing arsenic trioxide and metal arsenic with arsenic ore by reverberator smelting and charcoal deoxidizing. Based on the characteristic of chemical components of ore and production techniques flow adopted, there are few other occupational hazard factors except for arsenic pol-
solution in the plant. The occupational protective measures of the plant are far from being sufficient and efficient. The arsenic concentrations in workplaces are several dozen times as high as the Chinese national standard. In addition, due to the lack of knowledge of health hazard of arsenic and awareness of self-protection, the occupational protective measures are ignored by most workers. Consistent with the severe arsenic exposure in workplaces, our results have shown that the concentrations of mean total urinary arsenic in high- and low-exposed groups are obviously higher than that of the local residents. This severe arsenic exposure is rare, and is far higher than that of villagers with arsenicosis caused by the burning of coal in Guizhou province, 76–145 $\mu$g/L [25], and than that of ACGIH’s recommended Biological Exposure Indices, $35 \mu$g/L [33]. The concentrations of urinary arsenic in workers are almost as high as that of patients with arsenic poisoning [10].

Based on data collected from 74 subjects including 51 arsenic-exposure workers from an arsenic plant and 23 local residents without known arsenic exposure, this study analyzes the levels of total, inorganic and organic arsenic, as well as 8-OHdG in urine, investigates the damage of exon 5 and 8 of $p53$ gene, and explores the relationship between urinary organic arsenic and damage of exon 5 and 8 of $p53$. For the first time, it is found that DNA damage exists in exon 5 of $p53$ gene in apparently healthy individuals who exposed to arsenic through inhalation.

8-OHdG, one of the products derived from oxidative modified guanine, has been widely used as a biomarker of oxidative DNA damage. If the lesion is repaired by the nucleotide excision repair pathway, mutations will be prevented, and 8-OHdG in urine will increase. If not repaired, the presence of 8-OHdG residues in DNA will lead to transversion of GC to TA. Our results show that the concentration of urinary 8-OHdG in high-exposed group is significantly higher than that of the control group. This is consistent with that of Yamauchi et al., who observed high urinary 8-OHdG concentrations in patients with arsenic poisoning [10]. It indicates that arsenic can induce base modification which may induce mutation finally.

$p53$ controls G1/S checkpoint and regulates DNA repair and apoptosis. Any damage to DNA will result in the activation of $p53$ functions, which prevent cells with damaged DNA from entering the S-phase of the cell cycle until the DNA damage is repaired. If DNA damage is overly severe, $p53$ can cause the cell to undergo apoptosis. Mutations of $p53$ disrupt these functions, leading to the accumulation of mutations and further development of malignant clones as cells enter S-phase with damaged DNA. The sites of $p53$ mutation are mainly exons 4–11. Some studies showed that, for different genotoxic compounds, base sites attacked is characteristic of themselves in $p53$ gene [34]. For arsenic, exon 5 and 8 of $p53$ gene are main regions attacked [35]. Several studies have shown that mutations of exons 5 and 8 exist in arsenicosis patients with precarcinomas and carcinomas, but never found in arsenicosis patients without precarcinomas and carcinomas [24–26,35].

Sikorsky et al. have shown that DNA lesions perturb PCR amplification to different degrees and that the reduced amplification efficiency of modified templates results in a delay during the initial cycles of amplification which in turn translates into an increase in the threshold cycle $C_T$ [27]. Based on this, small reduction in amplification efficiency can be detected by determining the mean modified efficiency (MME) of DNA samples [28]. Sikorsky et al. have proved that modified bases, such as a single 8-oxo-7, 8-dihydro-20-deoxyadenosine, abasic site, a cis-syn thymidine dimer, or two tandem 8-oxodG will change the template secondary structure lead to reduction in amplification efficiency in vitro [27]. With PCR-SSCP, only substituted bases can be observed in template DNA, but base modification cannot be detected. Using the method of Sikorsky et al., our study shows that some modified bases exists in exon 5 of $p53$ gene in individuals without precarcinomas and carcinomas but exposed to high concentration of arsenic trioxide. The damage index of exon 5 of subjects with high arsenic exposure is significantly higher than that of individuals without arsenic exposure, and the damage index increases with urinary arsenic concentration. In addition, the difference in damage index of exon 5 is statistically significant between two exposed groups and the control. These results suggest that $p53$ damage can be induced by arsenic, and the possible type of damage is base modification. This argument is consistent with the stepwise nature of mutation and cancer development. We speculate that modified bases alter base pairing properties, which lead to substitution mutations.

Experimental studies have suggested that arsenic increases the production of reactive oxygen species such as hydrogen peroxide and hydroxyl radicals [4,36], which lead to oxidative base damage and consequently reduce PCR amplification efficiency. If the bases modified are not repaired, DNA replication will induce mutations in the genome. Accumulation and maintenance of several mutations in $p53$ genes will lead to development of cancer [23]. It has been shown that metabolites of arsenic can induce DNA damage and affect sequent repair [37]. Chen et al. found that the longer the duration of arsenic exposure, the higher the mutation frequency of $p53$ [38]. Base modification is one of possible mechanisms of mutation and perhaps cancers in population exposed to arsenic. Interestingly, as shown in our study, arsenic exposure does not have clearly impacts on exon 8, implying that exon 5 of $p53$ gene is more sensitive to arsenic than exon 8. This suggests that exon 5 of $p53$ gene were easily attacked by arsenic and its metabolites. This accord with the results of Zhang et al. who has observed that mutation sites are only at codons 143, 146 and 151 for arsenicosis patients with precarcinomas and carcinomas in Guizhou province, which are all in exon 5 of $p53$ gene [35].

One interesting finding in this study is that there exists a positive correlation between damage index of exon 5 and urinary organic arsenic contents. It has been showed that metabolites resulted from inorganic arsenic, such as MMAIII and DMAIII, hold more severe genotoxic than inorganic arsenic [39,40]. Yamanaka et al. have shown that metabolites of arsenic play an important role in arsenic genotoxicity and carcinogenesis [10]. Our results are in agreement with these findings. However, further study is required to investigate the contribution of organic arsenic to damage of exon 5 of $p53$ gene.

To our knowledge, this is the first study showing that the DNA damage of $p53$ gene, possibly through base modifications, exists in high arsenic exposure individuals without precarcinomas and car-
cinomas. Mutations have never been found in similar population in previous studies. In addition, the levels of damage of exon 5 of p53 are found to be related to urinary organic arsenic concentrations. Our study provides a linkage between clinical symptom and DNA damage in arsenic exposure population. Because mutations have been found in exon 5 of p53 in arsenicosis patients with pre-carcinomas and carcinomas, one possibility is that what exists in arsenic exposed population without precarcinomas and carcinomas is base modification rather than mutation. This supports the argument that modified bases alter base pairing properties which lead to substitution mutations. Damage of exon 5 of p53 may be a useful biomarker to assess adverse health effect caused by chronic exposure to arsenic in human beings.

Conflict of interest

None.

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References

[14] H. Yamauchi, Y. Aminaka, K. Yoshida, G. Sun, J. Pi, M.P. Waalkes, Evaluation of active oxygen species are involved in the induction of DNA damage in arsenic exposure population. Because mutations have never been found in similar population with precarcinomas and carcinomas, one possibility is that what exists in arsenic exposed population without precarcinomas and carcinomas is base modification rather than mutation. This supports the argument that modified bases alter base pairing properties which lead to substitution mutations. Damage of exon 5 of p53 may be a useful biomarker to assess adverse health effect caused by chronic exposure to arsenic in human beings.

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References