Arsenic levels in cutaneous appendicular organs are correlated with digitally evaluated hyperpigmented skin of the forehead but not the sole in Bangladesh residents

Ichiro Yajima[1,2,3], Nazmul Ahsan[4], Anwarul Azim Akhand[4], Aeorangajeb Al Hossain[1], Masafumi Yoshinaga[1], Nobutaka Ohgami[1,2,3], Machiko Iida[1,2,3], Reina Oshino[1,3], Mariko Naito[5], Kenji Wakai[5]
and Masashi Kato[1,2,3,6]

Departments of [1]Occupational and Environmental Health and [5]Preventive Medicine, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya, Aichi 466-8550, Japan. [2]Unit of Environmental Health Sciences, Department of Biomedical Sciences, College of Life and Health Sciences, Chubu University, 1200 Matsumoto, Kasugai, Aichi 487-8501, Japan. [3]Voluntary Body for International Health Care in Universities, 65 Tsurumai-cho, Showa-ku, Nagoya, Aichi 466-8550, Japan. [4]Department of Genetic Engineering and Biotechnology, University of Dhaka, Dhaka-1000, Bangladesh.

[6]Corresponding author: Masashi Kato MD, PhD,
Department of Occupational and Environmental Health, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya, Aichi 466-8550, Japan.
Tel: +81-52-744-2122, Fax: +81-52-744-2126, Email: katomasa@med.nagoya-u.ac.jp

ABSTRACT
There has been no report showing the effect of arsenic level on digitized skin pigmentation level, a typical diagnostic marker for arsenicosis. Correlations among history of drinking well water, arsenic levels in hair and toenails, and digitalized skin pigmentation levels (L*-value) in sunlight-exposed (forehead) and unexposed (sole) skin areas digitally evaluated by using a reflectance spectrophotometer were examined in 150 residents of Bangladesh. Univariate analysis showed that arsenic levels in hair and toenails of subjects with a history of drinking well water were 10.6-fold and 7.1-fold higher, respectively, than those in subjects without a history of drinking well water. The mean L*-value of foreheads, but not that of soles, in subjects with a history of drinking well water was 1.15-fold lower (more pigmented) than that in subjects without a history of drinking well water. Significant correlations were found between duration of drinking well water and arsenic concentrations in hair ($r=0.63$; $p<0.01$) and toenails ($r=0.60$; $p<0.01$). Multivariate analysis showed that the arsenic levels in hair and toenails and the duration of drinking well water were strongly correlated with the digitized pigmented level of the forehead but not that of the sole. An increase in the duration of drinking well water may increase hyperpigmentation in the forehead, but not that in the sole, through an increased arsenic level in the human body as shown in cutaneous appendicular organs (hair and toenails).

KEY WORDS: arsenic, skin pigmentation, arsenicosis, reflectance spectrophotometer

INTRODUCTION
Arsenic in drinking well water is a worldwide health risk for humans [1,2]. Millions of people worldwide suffer from arsenicosis [3-6]. Previous epidemiological studies have shown that chronic exposure to arsenic is associated with various diseases including hyperpigmentation and hyperkeratosis in the skin [7,8]. Skin hyperpigmentation, which is

often accompanied by hypopigmentation, develops in 100% of patients with arsenicosis, while skin hyperkeratosis develops in 58-80% of the patients [9-11]. The period from starting to drink arsenic-polluted well water to the development of skin hyperpigmentation is shorter than the period to the development of skin hyperkeratosis [12-14]. Results of these previous studies showed that skin hyperpigmentation is the most sensitive objective symptom for patients with arsenicosis [15]. Therefore, correct evaluation of skin hyperpigmentation based on an objective criterion is essential for correct diagnosis of arsenicosis. Accurate identification of patients with arsenicosis will contribute to identification of arsenic-polluted areas in the world.

Subjective scores have been used by dermatologists to evaluate skin pigmentation in most previous studies [15,16]. Previous studies using subjective scores showed that the prevalence rate of skin hyperpigmentation in residents drinking arsenic-polluted well water was significantly higher than that in residents drinking well water not polluted with arsenic [15,17]. However, it is possible that dermatologists have used different methods to obtain subjective scores for determining skin pigmentation level. Digitalization for a minimal difference in skin pigmentation levels in different skin areas is impossible by a subjective method. Therefore, it is difficult to identify sensitive and resistant skin areas for arsenic-mediated hyperpigmentation. To our knowledge, there has been no study showing a correlation between skin pigmentation level and arsenic level in the human body. There has also been no animal study showing that hyperpigmented skin was caused by drinking water with a high concentration of arsenic. Thus, direct evidence of arsenic-mediated development of hyperpigmented skin has not been obtained.

Skin pigmentation levels (L*-values) in humans and mice could be promptly digitalized by a reflectance spectrophotometer [18-21]. High and low L*-values indicate low and high levels of melanin density, respectively, in the skin [22]. In this study, we performed for the first time an epidemiological investigation to objectively evaluate the correlations among history of drinking well water, arsenic concentrations in hair and toenails, and digitalized skin pigmentation levels in sunlight-exposed (forehead) and unexposed (sole) skin areas by using a reflectance spectrophotometer in subjects in Bangladesh.

MATERIALS AND METHODS

Subjects

This study was performed for a random sample of 150 participants aged from 12 to 55 years (mean ± SD, 29.4 ± 10.9 years) who were drinking well water (n=111) or tap water (n=39, no history of drinking water) in Bangladesh. Arsenic levels in the drinking water from 4 wells were 20.6, 22.2, 53.8 and 221.0 μg/L. All of the participants agreed to participate in this study in writing after informed consent. A self-reporting questionnaire including questions on age, weight and height, duration of drinking well water and duration of working under the sun was given to each participant. Body mass index (BMI; mean ± SD = 21.9 ± 3.4) was calculated by the formula of [weight in kg/height in meters^2]. This study was ethically approved by Nagoya University International Bioethics Committee following the regulations of the Japanese government (approval number 2013-0070) and the Faculty of Biological Science, University of Dhaka (Ref. no. 5509/Bio.Sc).

Measurements of skin pigmentation levels and amounts of arsenic in hair and toenails

Skin pigmentation levels in a sunlight-exposed skin area (forehead) and unexposed skin area (sole) were estimated as L*-values by using a reflectance spectrophotometer

(RGB-1002, LUTRON ELECTRONIC ENTERPRISE CO., LTD.) according to a previously described method [20]. A reflectance spectrophotometer detects color levels as several types of color spaces including the L*a*b* system. Values of L*, a* and b* mean the lightness, green to red and blue to yellow, respectively. The probe of the reflectance spectrophotometer was applied to the skin of the forehead or sole to scan the color scale. Arsenic concentrations in hair and toenails were measured by a previously described method [23]. Briefly, human hair and toenail samples were incubated with 61% HNO3 at 80ºC for 48 hours and cooled at room temperature. Then 30% H2O2 was added to the samples and they were incubated at 80ºC for 3 hours. After filtration of treated samples with 45-μm filters, arsenic concentrations were measured by using an inductively coupled plasma mass spectrometer (ICP-MS) (7500cx, Agilent Technologies, Inc.).

Statistical analysis

Statistical analyses were performed according to methods previously described [2, 20]. The Mann-Whitney *U* test and Spearman's rank correlation coefficient were used for univariate analysis to determine a significant association between nonparametric variables, since the Shapiro–Wilks normality test showed that arsenic levels in biological samples and L*-values of skin were not normally distributed. Levene's test and Bartlett's test for equality of variances were conducted. A difference with $p<0.05$ was considered significant, and the actual P-value for each test is displayed except for *p*-values below 0.0001. For univariate and multivariate analyses, we carried out binary logistic regression analysis with medians of L*-values of the forehead and sole as dependent variables and duration of drinking well water (years) and arsenic concentrations in hair and toenail samples (μg/kg) as independent variables. For the binary logistic regression analysis, arsenic concentrations in hair and toenail samples were categorized into tertiles for comparison. All matching factors (age, sex, BMI and duration of working under direct sunlight in a day) were included in all multivariate logistic regression models. We evaluated the correlations among skin pigmentation levels in the forehead and sole, arsenic levels in hair and toenails, and duration of drinking well water by odds ratio (OR) with the corresponding 95% confidence interval (95% CI) and *p* value <0.05. All statistical analyses were performed using JMP Pro (version 11.0.0; SAS Institute Inc., Cary, NC, USA).

RESULTS

Baseline characteristics of participants in this study

The baseline characteristics of the 150 participants including age, sex, BMI, duration of drinking well water (year) and duration of working under direct sunlight per day (hours) are shown in Table 1.

Arsenic levels in hair and toenails and L*-values in participants

Arsenic concentrations in hair and toenails measured by the ICP-MS are shown in Table 1. As shown in a previous study [23], the concentration of arsenic in toenails was higher than that in hair in all of the participants in our study.

Mean concentrations of arsenic in hair and toenails of the participants without a history of drinking well water were 60.8 μg/kg and 246.8 μg/kg, respectively, while those in participants with a history of drinking well water were 642.7 μg/kg and 1,752.7 μg/kg, respectively. The arsenic concentrations in hair and toenails of participants with a history of drinking well water were 10.6-fold and 7.1-fold higher, respectively, than those in participants without a history of drinking well water.

L*-values of the foreheads and soles of participants were measured by a reflectance spectrophotometer to analyze the levels of skin pigmentation (Table 1). Higher L*-values indicate lower levels of skin pigmentation[22]. The mean L*-value of soles was 1.6-fold higher than that of foreheads, indicating that the skin of the forehead was darker (more pigmented) than that of the sole.

Univariate analysis for correlations of history of drinking well water with arsenic levels in hair and toenails and skin pigmentation level

We next compared arsenic concentrations (Figure 1) and L*-values (Figure 2) in participants with a history of drinking well water (n=111) and participants without a history of drinking well water (n=39). Mean concentrations of arsenic in hair and toenails of participants with a history of drinking well water were 10.6-fold and 7.1-fold higher, respectively, than those in participants without a history of drinking well water. The mean L*-value in foreheads of participants with a history of drinking well water was significantly lower than that in participants without a history of drinking well water, while there was no significant difference between L*-values in soles of participants with a history of drinking well water and participants without a history of drinking well water (Figure 2). Further univariate analysis using Spearman's rank correlation coefficient (Figure 3) showed significant correlations of duration of drinking well water with arsenic concentrations in hair ($r=0.63$, $p<0.01$) and toenails ($r=0.60$, $p<0.01$).

We also performed univariate analysis using binary logistic regression analysis with duration of drinking well water, arsenic concentrations in hair and toenails, age, sex, BMI and duration of working under direct sunlight in a day (Supplementary Table 1).

Multivariate analysis for correlations of history of drinking well water with arsenic levels in hair and toenails and skin pigmentation level

We finally performed multivariate analysis (Table 2) including duration of working under direct sunlight in a day in addition to age, sex and BMI as confounding factors because exposure to ultraviolet light from sunlight affects the level of skin pigmentation. There were significant correlations of L*-values of the forehead with duration of drinking well water (second, third, longest vs. shortest category: OR=5.43, 95%CI=1.16-27.67, OR=9.03, 95%CI=2.85-32.75, OR=11.48, 95%CI= 3.06-49.16), arsenic concentration in hair (second, highest vs. lowest tertile: OR=3.50, 95%CI= 1.31-9.96, OR=3.14, 95%CI=1.09-9.59) and arsenic concentration in toenails (second, highest vs. lowest tertile: OR=4.08, 95%CI=1.61-10.93, OR=3.45, 95%CI=1.27-9.91) with significant dose-dependent relationships of trend p=0.0008, 0.0158 and 0.0028, respectively. For example, the highest category of arsenic in hair (547.21-2598.56 μg/kg) shows higher levels of skin pigmentation of foreheads with an odds ratio of 3.14 than does the lowest category of arsenic in hair (13.48-162.95). However, there was no correlation between L*-values of the sole and other factors. We also performed multivariate analysis to clarify the correlation between arsenic exposure levels and L*-values of the sole with age, sex and BMI as confounding factors but without duration of working under direct sunlight in a day as a confounding factor because the sole is never exposed to ultraviolet light from sunlight. There was also no correlation of L*-values of the sole with duration of drinking well water (second, third, longest vs. shortest category: OR=1.03, 95%CI=0.23-4.29, OR=1.64, 95%CI=0.61-4.48, OR=2.02, 95%CI= 0.65-6.39), arsenic concentration in hair (second, highest vs. lowest tertile: OR=1.54, 95%CI= 0.62-3.90, OR=1.23, 95%CI=0.46-3.31) or arsenic concentration in toenails (second, highest vs. lowest tertile: OR=1.68, 95%CI=0.69-4.12, OR=1.36,

95%CI=0.54-3.43).

DISCUSSION

In this study, univariate analysis showed increased arsenic levels in hair and toenails and increased skin pigmentation level in participants with a history of drinking well water compared to those in participants without a history of drinking well water. The univariate analysis also showed a strong correlation between duration of drinking well water and arsenic concentrations in hair and toenails. Multivariate analysis further showed that arsenic levels in hair and toenails and duration of drinking well water were correlated with the L*-value of the forehead. Thus, our results uniformly provide evidence that a long duration of drinking well water may increase skin pigmentation level via increased arsenic levels in hair and toenails, which are developmentally organized from the skin.

Before undertaking this study, we assumed that arsenic affects hyperpigmentation in a skin area not exposed to sunlight (sole) rather than a sunlight-exposed skin area (forehead) because arsenic-mediated pigmentation in sunlight-exposed skin areas might be masked by sunlight-mediated pigmentation. Unexpectedly, however, multivariate analysis showed no correlation between arsenic levels in hair and toenails and L*-value of the sole or between duration of drinking well water and L*-value of the sole. This may be because melanocyte density in the sole is 5-fold lower than that in nonpalmoplantar sites in humans [24]. Another possible reason for the results showing no correlation is suppression of melanocyte growth and differentiation in the sole [24].

Levels of arsenic exposure were significantly correlated with skin pigmentation levels of the forehead, while levels of sunlight exposure showed no correlation with those pigmentation levels in multivariate analysis. The effect of arsenic exposure on skin pigmentation may be stronger than that of sunlight exposure in subjects routinely exposed to arsenic. Melanin production by melanocytes in sunlight-exposed skin areas including the forehead may be increased by arsenic exposure as well as by sunlight exposure. Skin keratinocytes around melanocytes secrete various melanocyte-stimulating factors such as Endothelin-1 (ET-1), Endothelin-2 (ET-2), Endothelin-3 (ET-3), Pro-opiomelanocortin (POMC) and stem cell factor (SCF) via production of Reactive oxygen species (ROS) induced by various stresses and activate melanin production in melanocytes [25]. Since the production of ROS is induced by arsenic exposure [6, 26], the surrounding environment including keratinocytes exposed to arsenic may increase melanin production from melanocytes.

Since skin hyperpigmentation is a hallmark of arsenicosis [9-11], accurate diagnosis is essential to identify patients with arsenicosis. This study suggests that evaluation by digitalized skin pigmentation level could be one way for accurate diagnosis of hyperpigmented skin after determining sensitive and resistant skin areas for arsenic-mediated hyperpigmentation. More importantly, this study demonstrated for the first time that the duration of drinking well water affects arsenic levels in appendicular organs of the skin (hair and toenails), resulting in an increased level of skin pigmentation. On the other hand, this study has not provided any results about a comparison between subjective scores of skin pigmentation level diagnosed by dermatologists and objective scores of skin pigmentation level evaluated by using a reflectance spectrophotometer. This study was performed in a special area in which drinking water was polluted by arsenic, and the number of participants was small. Therefore, the generalizability of our findings may be limited. Our novel digitized method to determine skin pigmentation levels has the potential to be generalized to residents of Bangladesh drinking water polluted with arsenic at concentrations in the

range of 20.6-221.0 μg/L. Further study with a larger number of participants, more detailed analysis including subjective scores by dermatologists and analysis of cumulative arsenic exposure (CAE) may enable our method to be generalized with possible clinical application for arsenicosis.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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Figure legends

Figure 1. ***Effect of history of drinking well water on arsenic levels in hair and toenails of participants.*** Concentrations (box plot) of arsenic in hair (A) and toenail (B) samples of participants with a history (n=111) or without a history (n=39) of drinking well water in Bangladesh are presented. The boxes contain 50% of all values (observations between the 25th and 75th percentiles). The horizontal lines inside the boxes represent medians. The bars extend from the boxes to the highest and lowest values. Significantly different (**, $p<0.01$) from arsenic concentrations in participants without a history of drinking well water by the Mann-Whitney *U* test.

Figure 2. ***Effect of history of drinking well water on skin pigmentation levels in the forehead and toenails of participants.*** Skin pigmentation levels (box plot) based on L*-values in the forehead (A) and sole (B) of participants with a history (n=111) or without a history (n=39) of drinking well water in Bangladesh are presented. The boxes contain 50% of all values (observations between the 25th and 75th percentiles). The horizontal lines inside the boxes represent medians. The bars extend from the boxes to the highest and lowest values. Significantly different (**, $p<0.01$) from skin pigmentation levels in participants without a history of drinking well water by the Mann-Whitney *U* test.

Figure 3. ***Correlations of duration of drinking well water with arsenic levels in hair and toenails of participants.*** Correlations of duration of drinking well water with arsenic levels in hair (A) and toenails (A) in participants analyzed by Spearman's rank correlation coefficient are presented.

Fig. 1

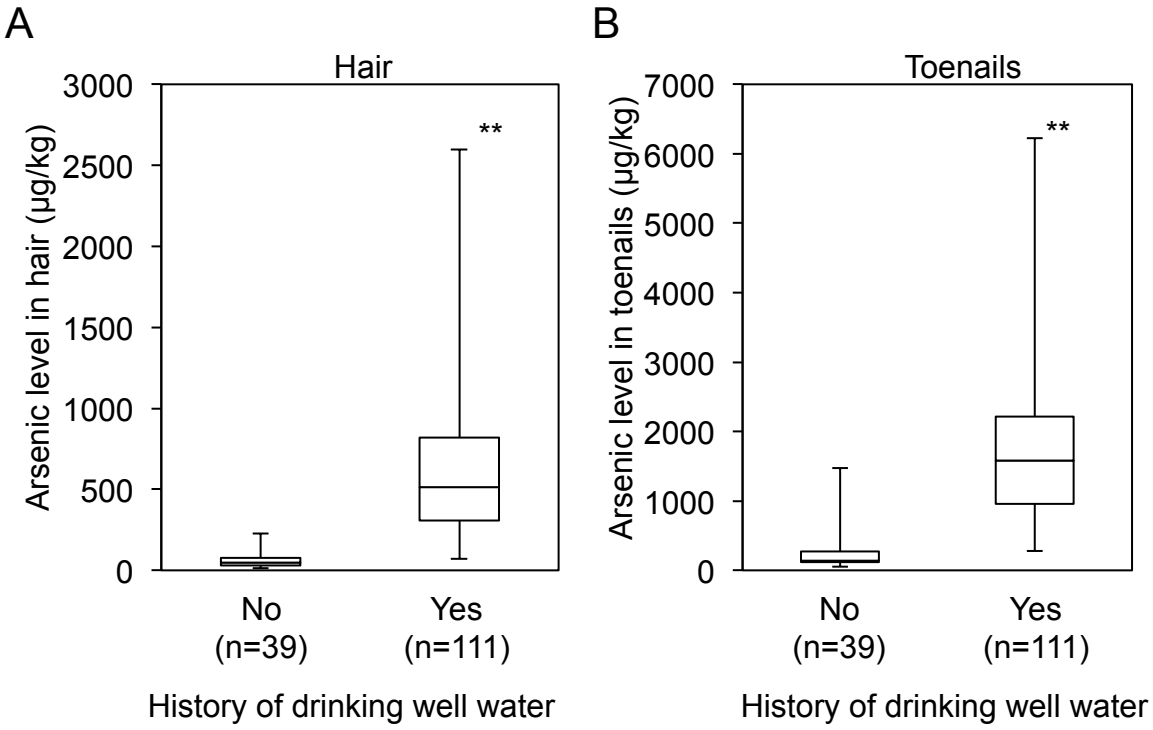
A
Hair
Arsenic level in hair (μg/kg)
3000
2500
2000
1500
1000
500
0
**
No
(n=39)
Yes
(n=111)
History of drinking well water
B
Toenails
Arsenic level in toenails (μg/kg)
7000
6000
5000
4000
3000
2000
1000
0
**
No
(n=39)
Yes
(n=111)
History of drinking well water

Fig. 2

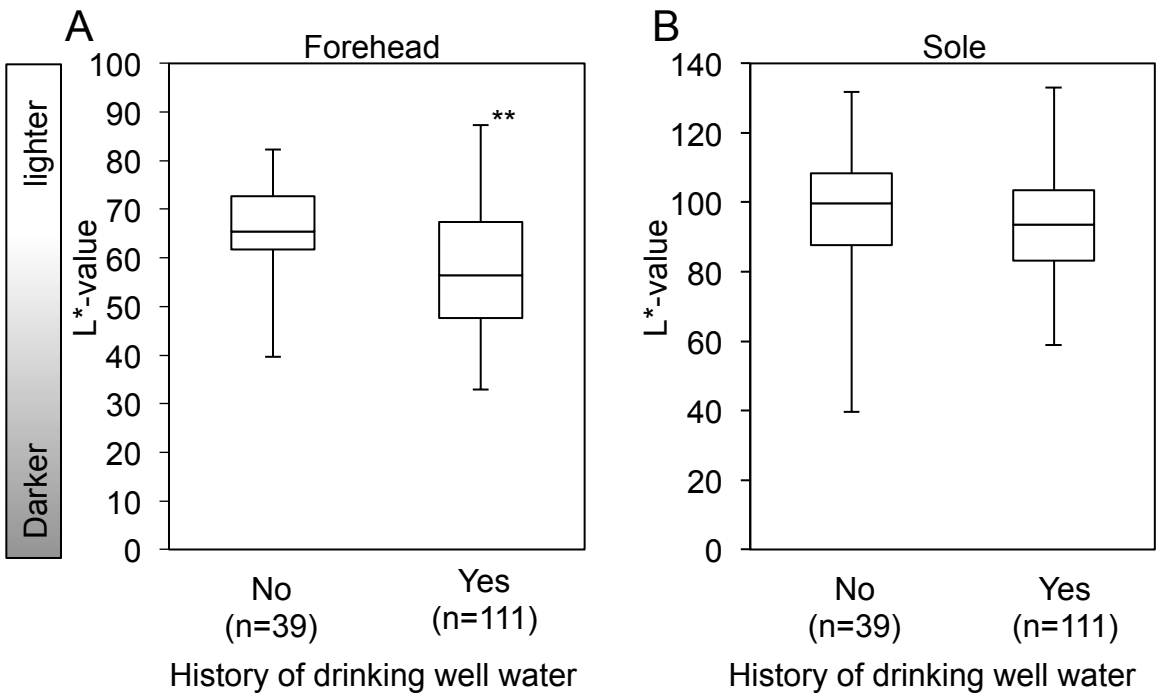
A
Forehead
Darker
lighter
L*-value
100
90
80
70
60
50
40
30
20
10
0
**
No
(n=39)
Yes
(n=111)
History of drinking well water
B
Sole
L*-value
140
120
100
80
60
40
20
0
No
(n=39)
Yes
(n=111)
History of drinking well water

Fig. 3

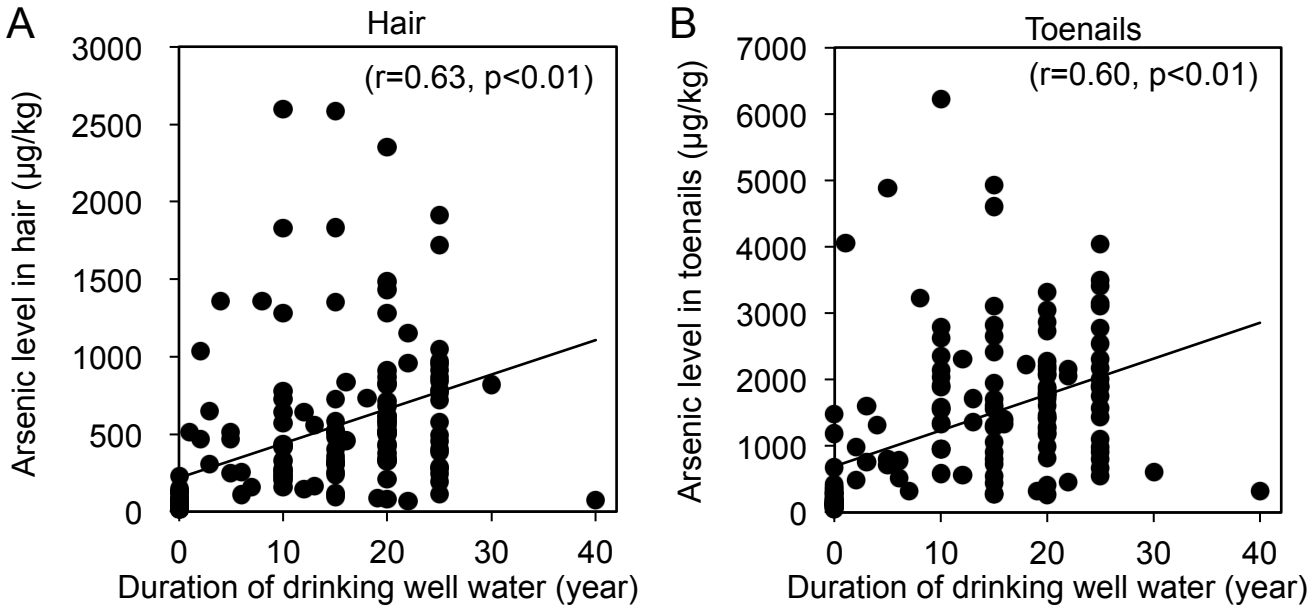
A
Hair
(r=0.63, p<0.01)
Arsenic level in hair (μg/kg)
3000
2500
2000
1500
1000
500
0
0
10
20
30
40
Duration of drinking well water (year)
B
Toenails
(r=0.60, p<0.01)
Arsenic level in toenails (μg/kg)
7000
6000
5000
4000
3000
2000
1000
0
0
10
20
30
40
Duration of drinking well water (year)

Table 1. Baseline characteristics of participants.

Total number		150
Age (year)	Mean	29.4
	SD	10.9
	Max	55
	Min	12
	Median	27
Sex	Female	80
	Male	70
BMI	Mean	21.9
	SD	3.4
	Max	31.9
	Min	13.8
	Median	21.6
Duration of drinking well water (years)		
	Mean	12.3
	SD	9.5
	Max	40
	Min	0[a]
	Median	15
Duration of working under direct sunlight per day (hrs)		
	< 0.5	53
	0.5≤ < 2	61
	2≤ < 6	18
	6≤ <10	10
	≥10	8
Arsenic levels in hair (μg/kg)		
	Mean[b]	264.56
	Max	2,598.56
	Min	13.48
	Median	350.98
Arsenic levels in toenails (μg/kg)		
	Mean[b]	829.83**
	Max	6,222.41
	Min	53.03
	Median	1,229.66
L*-values[c] of foreheads		
	Mean	59.3
	SD	12.18
	Max	87.27
	Min	32.86
	Median	60.67

L*-values[c] of sole		
	Mean	94.53##
	SD	15.40
	Max	133.06
	Min	39.64
	Median	94.31

[a]39 participants (26%) had no history of drinking well water.
[b]Mean values of arsenic levels in hair and toenails were shown as geometric means.
[c]Higher L*-values indicate lower levels of skin pigmentation.
** and ##, significantly different ($p<0.01$) as analyzed by the Mann-Whitney U test compared with arsenic in hair and L*-value of forehead, respectively.

Table 2. Multivariate analysis for association between arsenic exposure and L*-values of the forehead (<60.67) and sole (<94.31).

	L*-value[b]	
	forehead	sole
	OR (95% CI)[a]	OR (95% CI)
Duration of drinking well water (years)		
0	reference	reference
1-9	5.43 (1.16-27.67)*	0.69 (0.15-3.08)
10-19	9.03 (2.85-32.75)**	1.94 (0.67-5.86)
20≦	11.48 (3.06-49.16)**	2.62 (0.76-9.49)
p for trend	0.0008	0.1811
Arsenic in hair (μg/kg)		
13.48-162.95	reference	reference
162.96-547.20	3.50 (1.31-9.96)*	1.73 (0.64-4.80)
547.21-2598.56	3.14 (1.09-9.59)*	1.05 (0.35-3.14)
p for trend	0.0158	0.7405
Arsenic in toenails (μg/kg)		
53.03-566.72	reference	reference
566.73-1716.46	4.08 (1.61-10.93)**	2.05 (0.81-5.37)
1716.47-6222.41	3.45 (1.27-9.91)*	1.29 (0.46-3.65)
p for trend	0.0028	0.4849

Multivariate analysis included age, sex, BMI and duration of working under direct sunlight in a day as confounding factors.

[a]OR = odds ratio, 95%CI = confidence interval. *$p < 0.05$, **$p < 0.01$.

[b]Higher L*-values indicate lower levels of skin pigmentation. For example, a highest category of arsenic in hair (547.21-2598.56 μg/kg) shows higher levels of skin pigmentation of foreheads with odds ratio of 3.14 compared with a lowest category of arsenic in hair (13.48-162.95). [#1-Results-2]

Supplementary Table 1. Univariate analysis for association between arsenic exposure and L*-values of the forehead (<60.67) and sole (<94.31).

	L*-value[b]	
	forehead	sole
	OR (95% CI)[a]	OR (95% CI)
Duration of drinking well water (years)		
0	reference	reference
1-9	4.52 (1.20-18.09)*	1.12 (0.29-4.03)
10-19	5.30 (2.07-14.80)**	1.71 (0.72-4.17)
20≦	6.93 (2.76-19.06)**	3.20 (1.37-7.73)*
p for trend	0.0009	0.0271
Arsenic in hair (μg/kg)		
13.48-162.95	reference	reference
162.96-547.20	2.93 (1.20-18.09)*	1.76 (0.80-3.93)
547.21-2598.56	3.19 (1.42-7.38)**	1.91 (0.87-4.27)
p for trend	0.0053	0.561
Arsenic in toenails (μg/kg)		
53.03-566.72	reference	reference
566.73-1716.46	3.50 (1.55-8.19)**	1.76 (0.80-3.93)
1716.47-6222.41	3.50 (1.55-8.19)**	1.91 (0.87-4.27)
p for trend	0.0032	0.1043
Age	1.03 (1.00-1.06)*	1.04 (1.01-1.07)**
Sex		
female (vs. male)	1.24 (0.65-2.36)	0.90 (0.47-1.71)
BMI	0.96 (0.87-1.06)	0.95 (0.86-1.04)
Duration of working under direct sunlight in a day (hrs)		
<0.5	reference	reference
0.5-2	1.35 (0.64-2.84)	2.85 (1.34-6.25)**
2-6	2.04 (0.70-6.36)	2.65 (0.89-8.13)
6-10	1.30 (0.33-5.21)	8.47 (1.88-60.28)**
10<	2.17 (0.48-11.49)	3.53 (0.78-18.87)
p for trend	0.0766	0.0005

[a]OR = odds ratio, 95%CI = confidence interval. *$p < 0.05$, **$p < 0.01$.

[b]Higher L*-values indicate lower levels of skin pigmentation. For example, a highest category of arsenic in hair (547.21-2598.56 μg/kg) shows higher levels of skin pigmentation of foreheads with odds ratio of 3.19 compared with a lowest category of arsenic in hair (13.48-162.95). [#1-Results-2]