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Urinary arsenic methylation capability and carotid atherosclerosis risk in subjects living in arsenicosis-hyperendemic areas in southwestern Taiwan☆

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ABSTRACT

Long-term exposure to inorganic arsenic from artesian drinking well water is associated with carotid atherosclerosis in the Blackfoot Disease (BFD)-hyperendemic area in Taiwan. The current study examined the arsenic methylation capacity and its risk on carotid atherosclerosis. A total of 304 adults (158 men and 146 women) residing in the BFD-hyperendemic area were included. The extent of carotid atherosclerosis was assessed by duplex ultrasonography. Chronic arsenic exposure was estimated by an index of cumulative arsenic exposure (CAE) and the duration of artesian well water consumption. Urinary levels of inorganic arsenite [As(III)], arsenate [As(V)], monomethylarsonic acid [MMA(V)] and dimethylarsinic acid [DMA(V)] were determined by high performance liquid chromatography linked on-line to a hydride generator and atomic absorption spectrometry (HPLC-HG-AAS). The percentage of arsenic species, primary methylation index [PMI=MMA(V)/(As(III)+As(V)] and secondary methylation index [SMI=DMA(V)/MMA(V)] were calculated and employed as indicators of arsenic methylation capacity. Results showed that women and younger subjects had a more efficient arsenic methylation capacity than did men and the elderly. Carotid atherosclerosis cases had a significantly greater percentage of MMA(V) [%MMA(V)] and a lower percentage of DMA [%DMA (V)] compared to controls. Subjects in the highest two tertiles of PMI with a median of CAE >0 mg/L-year had an odds ratio (OR) and a 95% confidence interval (CI) of carotid atherosclerosis of 2.61 and 0.98–6.90 compared to those in the highest two tertiles of PMI with a CAE=0 mg/L-year. We conclude that individuals with greater exposure to arsenic and lower capacity to methylate inorganic arsenic may be at a higher risk to carotid atherosclerosis.

1. Introduction

Arsenic is a ground water contaminant in various areas worldwide (Nordstrom, 2002). Blackfoot Disease (BFD), a rare peripheral vascular disease, was identified in the endemic area along the southwestern coast of Taiwan and has been correlated with the exposure to inorganic arsenic through drinking artesian well water (Tseng, 1989). Long-term exposure to arsenic through the ingestion of drinking artesian well water has also been found to be associated with an increased risk of diabetes mellitus (DM) (Lai et al., 1994), hypertension (Chen et al., 1995), ischemic heart disease (Chen et al., 1996), cerebral infarction (Chiou et al., 1997b) and carotid atherosclerosis (Wang et al., 2002) in a dose-dependent manner.

Arsenic in drinking water is usually found in the form of inorganic arsenate [As(V)] or arsenite [As(III)] (Shraim et al., 2002). The arsenic methylation pathway [As(V)→As(III)→MMA(V)→MMA(III)→DMA(V)→DMA(III)] was previously considered as a detoxification mechanism because monomethylarsonic acid [MMA(V)] and dimethylarsinic acid [DMA(V)], which are the endpoint products in humans, have relatively low toxicity (Yamauchi and Fowler, 1994) and are rapidly excreted in the urine (Gebel, 2002). The methylation of arsenic is catalyzed by an *S*-adenosylmethionine dependent arsenic methyltransferase, a *cyt19* (*AS3MT*) gene encoded in the mouse and human genome (Aposhian et al., 2006; Thomas et al., 2004), glutathione-*S*-transferase omega (Aposhian et al., 2004), and purine nucleoside phosphorylases (Radabaugh et al., 2002), but the mechanisms are not completely understood. Recent *in vivo* and *in vitro* studies, however, have confirmed the existence of trivalent intermediates and

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products of monomethylarsonous acid [MMA (III)] and dimethylarsinous acid [DMA(III)], which are more toxic than inorganic arsenite (Thomas et al., 2001; Kitchin, 2001; Styblo et al., 2002). Evaluation of arsenic methylation efficiency is primarily based on the relative amounts of the different metabolites present in urine. Our recent studies have documented that subjects with greater cumulative arsenic exposure (CAE) and a greater urinary MMA(V) percentage or lower urinary DMA(V) percentage suffered from a higher risk of peripheral vascular disease (Tseng et al., 2005) and hypertension (Huang et al., 2007). Whether the metabolism of arsenic could have an effect on the risk of developing carotid atherosclerosis is an interesting issue that has not been studied before in the BFD-hyperendemic area. In this area, a contaminated water supply was present that was corrected over a 30 year period. The present study evaluated the impact of the CAE and urinary arsenic species on the development of carotid atherosclerosis among residents in the BFD-hyperendemic area.

2. Materials and methods

2.1. Study area and subjects

The study area and subjects were previously described in detail (Chen et al., 1995). In brief, the study area included Homei, Fuhsing and Hsinming Villages in the Putai Township of Chiayi County located along the southwestern coast of Taiwan. A total of 2258 subjects, 30 years of age or older, registered in the household registration office in these villages were considered. Among them, 1571 (70%) were eligible and lived in these villages five or more days a week. A total of 1081 (69%) of the eligible subjects were interviewed from September to December 1988. All 1081 subjects were invited to participate in the first health examination during January and February 1989, and 941 (87%) subjects participated. Biannual health examinations were then conducted. An ultrasonographic assessment of the extracranial carotid artery (ECCA) atherosclerosis was performed during the sixth examination in 1996. All subjects provided informed consent forms before specimen collection and the questionnaire interview. During this examination, a total of 451 cohort members were invited, and 397 (88%) completed the ultrasonographic assessment of ECCA, and their urinary arsenic species were measured. The Institutional Review Board of National Taiwan University approved this study.

2.2. Questionnaire interview and arsenic exposure index

Two well-trained public health nurses carried out the standardized personal interview based on a structured questionnaire. Information obtained from the interview included the history of high-arsenic artesian well water consumption, residential history, socioeconomic and demographic characteristics, alcohol consumption, smoking behavior, consumption frequency of various dietary items, and personal and family histories of hypertension, diabetes and cardiovascular diseases.

Some subjects moved from one village to another, and the arsenic concentrations in the artesian well water of these villages were known to differ. As both the duration of exposure and the arsenic levels in the artesian well water may be associated with risk of CAI, an index of cumulative arsenic exposure (CAE) was derived to reflect the overall exposure to arsenic for each subject. The detailed residential history and duration of consuming high-arsenic artesian well water were used to derive the cumulative arsenic exposure (CAE) from consuming artesian well water for each study subject. The arsenic levels in the artesian well water of villages where subjects had lived were obtained from previous reports carried out in the 1960s. These water samples were collected from 155 wells located in the 42 villages where BFD is endemic (Kuo, 1964). The median arsenic concentration of artesian well water was between 0.70 and 0.93 mg/L in the early 1960s (Kuo, 1964). Arsenic levels of the artesian well water in the study area were found to be reasonably constant in two surveys carried out by the Taiwan Provincial Institute of Environmental Sanitation (Wu et al., 1961). Because these wells have been permanently sealed, it was impossible to determine whether the arsenic levels in the artesian well water has changed. One limitation of this study was that water consumption could not be estimated for each individual. We therefore employed the CAE index in mg/L-year for a given subject, defined by the following formula: $CAE = \Sigma(Ci \times Di)$, where Ci is the median arsenic concentration of artesian well water in mg/L in the village where the subject lived and Di is the duration of artesian well water consumption in years while residing in the village. The CAE for a given subject was considered to be unknown if the median arsenic concentration of artesian well water in any of the villages where the subject had lived was unknown. The CAE was not calculable for 93 of the 397 subjects (23.4%) in this study. The odds ratio for those without CAE data was between the odds ratio for the lowest and greatest arsenic exposure groups (data not shown). The unavailable of arsenic data for some study subjects did not appear to affect the association between arsenic exposure and carotid atherosclerosis risk.

2.3. Assessment of carotid atherosclerosis

An ultrasonographic assessment of ECCA atherosclerosis was performed with a Hewlett-Packard Sono 1000 equipped with a 7.5-MHz frequency in B-mode and 5.6-MHz frequency in pulsed Doppler mode. The duplex scanning was performed by a neurologist and a cardiologist from the Taiwan University Medical Center. Study subjects were in the supine position with the head slightly extended and rotated 45° away from the side being examined during the 6th health examination in 1996 (Wang et al., 2002). Transverse and longitudinal scans were performed to assess the common carotid artery, carotid bifurcation, and internal and external carotid arteries. Plaque of the carotid artery was defined as an irregular surface, luminal encroachment, significant wall thickening ≥50% of the adjacent intima media (IMT), and/or structural heterogeneity, such as an acoustic shadow. The IMT was measured in the far wall of three segments of bilateral ECCAs. Three segments were examined on each side: the distal 1.0 cm of the common carotid artery proximal to the bifurcation, the bifurcation itself and the proximal 1.0 cm of the internal carotid artery. The IMT was automatically estimated as the mean of these six measurements. The three carotid atherosclerosis indices (CAIs) were defined as follows: CAI-1, presence of plaque or IMT ≥ 1.0 mm; CAI-2, IMT ≥ 1.0 mm; and CAI-3, presence only of plaque.

2.4. Specimen collection and laboratory examinations

Fasting blood samples of 10 mL were collected from the subjects and centrifuged at 3000 rpm for 15 mins at room temperature. The obtained serum used for the determination of cholesterol and triglycerides. The total cholesterol and triglycerides concentrations were measured with an autoanalyzer (Hitachi 737, USA) with reagents obtained from Boehringer Mannheim Diagnostics (Indianapolis, IN, USA).

2.5. Urine collection and the determination of arsenic species present

Urinary samples were collected from 397 subjects. To collect samples, subjects were instructed to pass urine before 7:00 PM, which was discarded, and 7:00 PM to the first void urine upon awakening the next morning was collected. The samples were collected and drawn into a 1% nitric acid rinsed PE bottle and stored at −20 °C without any additive. The samples were retrieved for the determination of urinary arsenic species within six months after collection in 1989.

The urine sample was thawed at room temperature, mixed ultrasonically and filtered through a Sep-Pak C_{18} column. The analytical methods used for As(III), As(V), MMA(V), and DMA(V) were previously described (Hsueh et al., 1998). A total of 200 μL of the filtered urine

sample was used to separate As(III), As(V), MMA(V), and DMA(V) by high performance liquid chromatography (HPLC; Waters 501; Waters Associates, Milford, MA, USA) equipped with an anion column (Phenomenex, Nucleosil 10sB, Torrance, CA, USA) and linked on-line to a hydride generator and atomic absorption spectrometry to quantify the levels of various species of inorganic arsenic and its metabolites. Recovery rates of the four arsenic species were estimated according to the following calculation: [(sample containing a standard solution of known concentration)−sample concentration]/standard solution concentration×100. Recovery for As(III), DMA(V), MMA(V) and As(V) ranged between 93.8% and 102.2% with detection limits of 0.02, 0.06, 0.07 and 0.10 μg/L, respectively. The percentage of subjects below the limits of detection for As(III), AS(V), MMA(V) and DMA(V) were 7.0% (28/397), 3.5% (14/397), 0.3% (1/397) and 0%, respectively. The half level of the limit of detection for the three arsenic species was replaced in the data analysis. A standard arsenic reference material, SRM 2670, containing 480±100 μg/L arsenic was obtained from the National Institute of Standards and Technology (NIST, Gaithersburg, MD, USA) and used as a quality and internal quantity standard to check the procedural validity. The mean arsenic value of SRM 2670 determined by our system was 507±17 (SD) μg/L (n=4). The intra- and inter-day coefficients of variation for each of the arsenic species were used to test the reliability and was <5% for all experiments. A limitation in this study was that the creatinine levels of the urine samples did not determine the time for the measurement of urinary arsenic species. The percentage of arsenic species was therefore used to avoid being influenced by the dilution of urine volume.

The total arsenic level was calculated as the sum of As(III), As(V), MMA(V) and DMA(V). In addition to expressing as the percentage of arsenic species, the arsenic methylation efficiency index was also defined by two other indices: the primary methylation index (PMI), which was defined as the ratio between the MMA(V) level and InAs (inorganic arsenic, As(III)+As(V)) levels; the secondary methylation index (SMI) was defined as the ratio between the DMA(V) and MMA (V) levels.

2.6. Statistical analysis

Data was analyzed by SAS 8.2 software. A total of 304 subjects that had both cumulative arsenic exposure and urinary arsenic species data were analyzed. The indices of arsenic species are presented as the mean±standard error. Because the values were not normally

Table 1
Comparison of urinary arsenic profile based on gender, age, smoking behavior, alcohol drinking, lipid profile, hypertension and diabetes (DM) status

Variable	No.	Total urinary arsenic content (μg/L)	Inorganic arsenic percentage	MMA(V) percentage	DMA(V) percentage	PMI	SMI
Gender							
Male	158	89.1±5.4	7.3±0.5	15.5±0.7	77.1±0.9	3.0±0.2	7.9±0.7
Female	146	69.2±3.7	7.1±0.7	12.1±0.7	80.9±1.02	3.6±0.5	19.1±5.6
p value		0.012	0.115	<0.001	<0.001	0.063	<0.001
Age							
≤46	67	77.6±7.8	9.0±1.4	10.5±0.8[a]	80.6±1.7[a]	2.4±0.4[a,b]	28.2±12.0[a]
47–57	98	83.6±6.8	6.5±0.5	13.8±1.0	79.8±1.2	3.0±0.4[b]	10.1±1.4
>57	139	77.7±4.2	6.9±0.4	15.6±0.7[a]	77.6±0.9[a]	3.9±0.5[a]	8.3±0.8[a]
p value		0.560	0.371	<0.001	0.007	<0.001	<0.001
Smoking behavior							
No	238	77.9±3.6	7.3±0.5	13.6±0.6	79.1±0.8	3.4±0.3	14.7±3.5
Yes	66	85.5±8.8	6.9±0.5	14.8±1.0	78.4±1.3	2.9±0.3	8.0±0.8
p value		0.684	0.526	0.248	0.4536	0.568	0.307
Alcohol drinking							
No	259	75.2±3.1	7.3±0.5	13.7±0.5	78.9±0.7	3.4±0.3	14.08±3.2
Yes	45	104.5±13.9	6.5±0.6	14.6±1.3	79.0±1.7	2.8±0.3	9.2±1.2
p value		0.140	0.590	0.698	0.928	0.620	0.816
Total cholesterol (μg/dL)							
<200	126	74.7±3.8	7.0±0.5	13.1±0.7	79.9±0.9	2.9±0.3	13.6±2.5
≥200	178	83.0±5.1	7.4±0.6	14.4±0.7	78.3±0.9	3.6±0.4	13.0±4.3
p value		0.974	0.606	0.361	0.287	0.186	0.275
Triglyceride (μg/dL)							
<150	214	83.1±4.4	6.8±0.5	13.4±0.9	79.1±0.8	3.4±0.3	14.6±3.8
≥150	90	71.2±4.3	8.1±0.8	14.1±0.6	78.5±1.2	3.0±0.7	10.1±1.5
p value		0.318	0.089	0.581	0.690	0.091	0.720
Hypertension							
No	219	82.3±4.4	6.9±0.5	13.7±0.6	79.5±0.8	3.3±0.3	15.0±3.7
Yes	85	72.5±4.4	8.0±0.8	14.6±0.9	77.4±1.3	3.4±0.5	8.9±1.5
p value		0.598	0.474	0.156	0.100	0.523	0.113
DM							
No	192	83.4±4.4	7.1±0.5	13.4±0.6	79.5±0.8	3.0±0.2	15.8±4.3
Yes	65	66.7±4.7	7.6±0.8	15.0±1.0	77.4±1.3	3.9±0.6	7.2±0.6
p value		0.033	0.471	0.143	0.136	0.163	0.143

Data are mean±SE; Total arsenic=As(III)+As(V)+MMA(V)+DMA(V).
Inorganic arsenic percentage=((arsenite+arsenate)/total urinary arsenic)×100.
DMA (V) percentage=(DMA(V)/total urinary arsenic)×100.
MMA(V) percentage=(MMA(V)/total urinary arsenic)×100.
PMI=primary methylation index=MMA(V)/(arsenite+arsenate).
SMI=secondary methylation index=DMA(V)/MMA(V).
[a]Age≤46 compared with age >57, P<0.05; [b]age≤46 compared to age 47–57, P<0.05.

distributed, the Wilcoxon Mann–Whitney *U* test was used to analyze the differences in indices of urinary arsenic species between males and females, smokers and non-smokers, alcohol consumers and non-consumers, hypertension and normotension, diabetes (DM) and non-DM, normal and abnormal total cholesterol, normal and abnormal triglycerides, and presence or absence of carotid atherosclerosis. For comparing urinary arsenic indices among the three age strata, the urinary arsenic indices were ranked and analyzed by analysis of variance (ANOVA) followed by Scheffe's *post hoc* test. Linear regression was used to elucidate the relationship between internal arsenic exposure (urinary arsenic capability) and CAE, IMT and the duration of consuming artesian well water. The strength of association between urinary arsenic capability and CAE, IMT and the duration of consuming artesian well water is expressed by Spearmen correlation coefficient. The logistic regression analyses were further used to estimate the relative risk (in terms of odds ratio; OR) and their 95% confidence intervals (CIs) for any individual subgroup of subjects having a particular exposure dosage of arsenic and urinary arsenic profile in relation to the referent subgroup with an odds ratio of 1.00. The cut-off points for the PMI or SMI were the firs tertile of those indices. The cut-off point for the CAE was 0. When CAE was >0 in healthy residents, they were averagely separated into two strata.

3. Results

A comparison of the urinary arsenic profile among subgroups for gender, age, smoking behavior and alcohol drinking is presented in Table 1. A greater urinary total arsenic and %MMA(V) was observed for men and a greater %DMA(V) and SMI for women, indicating lower arsenic exposure or a more complete methylation capacity of arsenic to DMA(V) in women. The %MMA(V) and PMI increased with age and was accompanied by a lower %DMA(V) and SMI. Subjects with high triglyceride had borderline, yet significantly greater %InAs and lower PMI compared to those with low triglyceride. Subjects with DM had lower urinary total arsenic than non-DM subjects. The indices of arsenic species were not significantly different between cigarette smokers and non-smokers, alcohol drinkers and non-drinkers, hypertension and normal tension subjects, DM and non-DM subjects or high and low total cholesterol subjects.

Table 2
Comparison of urinary arsenic profile between patients to carotid atherosclerosis and controls defined by different carotid atherosclerosis indices (CAIs)

Variable	No.	Total urinary arsenic content (μg/L)	Inorganic arsenic percentage	MMA^V percentage	DMA^V percentage	PMI	SMI
CAI-1							
Controls	183	74.7±3.4	7.4±0.6	13.1±0.6	79.5±0.8	2.9±0.3	16.0±4.5
Patients	121	86.9±6.7	6.9±0.5	15.1±0.9	78.1±1.1	3.9±0.6	9.1±1.1
p value[a]		0.437	0.297	0.086	0.325	0.007	0.129
p value[b]		0.854	0.191	0.106	0.078	0.783	0.099
CAI-2							
Controls	183	74.7±3.4	7.4±0.6	13.1±0.6	79.5±0.8	2.9±0.3	16.1±4.5
Patients	69	76.0±7.9	7.4±0.7	15.4±1.0	77.2±1.4	3.6±0.6	7.8±0.8
p value[a]		0.608	0.883	0.05	0.126	0.037	0.060
p value[b]		0.375	0.594	0.147	0.233	0.746	0.172
CAI-3							
Controls	183	74.7±3.4	7.4±0.6	13.1±0.6	79.5±0.8	2.9±0.3	16.0±4.5
Patients	120	86.8±6.8	6.9±0.5	15.1±0.9	78.1±1.1	3.9±0.6	9.2±1.1
p value[a]		0.466	0.286	0.094	0.343	0.008	0.140
p value[b]		0.929	0.166	0.084	0.070	0.813	0.078

Data are mean±SE.
CAI-1 indicates presence of plaque or intima media thickness (IMT) ≥1.0 mm; CAI-2, IMT ≥1.0 mm; CAI-3, presence of plaque.
Inorganic arsenic percentage=((arsenite+arsenate)/total urinary arsenic)×100.
DMA(V) percentage=(DMA(V)/total urinary arsenic)×100.
MMA(V) percentage=(MMA(V)/total urinary arsenic)×100.
PMI=primary methylation index=MMA(V)/(arsenite+arsenate).
SMI=secondary methylation index=DMA(V)/MMA(V).
[a] Wilcoxon Mann–Whitney *U* test.
[b] Arsenic species indices were ranked and tested by a linear regression model and adjusted for age, gender, hypertension, diabetes, smoking behavior, total cholesterol and total urinary arsenic content.

Table 3
Multivariate analysis for carotid atherosclerosis by cumulative arsenic exposure and arsenic methylation capability

Variables	Odds ratio[a] (95% confidence interval)	Odds ratio[b] (95% confidence interval)
PMI≤1.28		
CAE=0	1.00	1.00
0<CAE≤12.1	1.45 (0.21–10.32)	0.65 (0.03–14.43)
CAE>12.1	0.80 (0.09–7.06)	0.50 (0.03–7.68)
PMI>1.28		
CAE=0	1.00[c,**]	1.00[c,*]
0<CAE≤12.1	0.90 (0.32–2.51)	0.77 (0.12–4.12)
CAE>12.1	3.61 (1.15–8.98)**	2.61 (0.98–6.90)[+]
SMI≤5.18		
CAE=0	1.00[c,+]	1.00
0<CAE≤12.1	1.07 (0.24–4.83)	0.71 (0.12–4.12)
CAE>12.1	2.80 (0.81–9.66)	2.11 (0.52–8.55)
SMI>5.18		
CAE=0	1.00[c,*]	1.00
0<CAE≤12.1	0.97 (0.32–3.00)	1.02 (0.27–3.84)
CAE>12.1	2.64 (0.90–7.74)[+]	2.17 (0.65–7.20)

CAE=cumulative arsenic exposure (mg/L-year).
PMI=primary methylation index=MMA(V)/(arsenite+arsenate).
SMI=secondary methylation index=DMA(V)/MMA(V).
[a] Age and gender were adjusted.
[b] Age, gender, hypertension, diabetes, smoking behavior, total cholesterol and total urinary arsenic content were adjusted.
[c] Trend test [+]0.05<*p*<0.1 *p<0.05 **p<0.01.

The comparison of urinary arsenic profiles between carotid atherosclerosis patients and controls defined by different CAIs is shown in Table 2. Carotid atherosclerosis patients, defined by CAI-1, CAI-2 or CAI-3, had a greater %MMA(V) and PMI than controls. Patients defined as CAI-2 had a lower SMI than controls. Furthermore, %DMA(V) was significantly lower, %MMA(V) was slightly but significantly higher and SMI was lower in CAI-1 and CAI-3 groups compared to controls after adjusting for age, gender, hypertension, DM, smoking behavior, total cholesterol and total urinary arsenic content. These results suggest that carotid atherosclerosis patients had insufficient arsenic methylation capacity.

There was a significant positive relationship between %MMA(V) and CAE and a negative relationship between %DMA(V), SMI and CAE after adjusting for age, gender, hypertension, DM, smoking behavior, total cholesterol and total urinary arsenic content (data not shown). These results are consistent with our previous data (Huang et al., 2007). The %MMA(V) was positively associated while the %DMA(V) and SMI were negatively associated with the amount of time spent consuming artesian well water and the IMT. After adjusting for age, gender, hypertension, DM, smoking behavior, total cholesterol and total urinary arsenic content, the significance disappeared in the IMT (data not shown). These results suggest that study subjects with longer arsenic exposure, showing arsenic accumulation or higher CAE, had a lower arsenic methylation capacity.

The multivariate-adjusted ORs for CAI are presented in Table 3. The association between the arsenic profile and the CAI risk was not significant (data not shown), indicating that methylation capability alone did not predict CAI risk. Compared to a PMI≤1.28, however, a significant dose–response relationship between CAE and CAI risk was observed in the subgroup of subjects who had a PMI>1.28 after

adjusting for DM, hypertension, smoking behavior, total cholesterol and total urinary arsenic content. Conversely, a CAI risk was related to an increase in CAE either in the subgroup of subjects who had an SMI>5.18 or an SMI≤5.18 after adjusting for DM, hypertension, smoking behavior, total cholesterol and total urinary arsenic content. These results suggested subjects with a low arsenic methylation capability (i.e., a PMI>1.28) and their CAI risk was significantly increased when they had a high cumulative arsenic exposure. The OR and 95% CI of the CAI in subjects with a PMI>1.28 and a CAE>12.1 mg/L-year was 2.61 (0.98–6.90) compared to subjects with a PMI>1.28 and a CAE=0 mg/L-year.

4. Discussion

Our previous study in the BFD hyperendemic area described a dose–response relationship between CAE and carotid atherosclerosis risk diagnosed by CAI-1, CAI-2 or CAI-3 (Wang et al., 2002). Using CAE as an exposure index may reflect the cumulative dosage of long term exposure to arsenic in individual subjects. A limitation for this study, however, was that the arsenic exposure assessment was unable to be based on the nearest well water to the place of residence instead of the median village levels and also could not be based on individual water consumption. The urinary arsenic species metabolically related to inorganic arsenic are better biomarkers of exposure to inorganic arsenic than total arsenic. This includes the high levels of organic arsenic derived from dietary intake of sea food (Buchet et al., 1980). An individuals' capacity to metabolize and detoxify the ingested inorganic arsenic is believed to have an important role on disease development (Tseng, 2007).

The capacity to metabolize inorganic arsenic differs among individuals and influences the biological effects on various organ systems. In general, 10–30% of inorganic arsenic, 10–20% of MMA(V) and 60–80% of DMA(V) are excreted in the urine, but the arsenic metabolic profile varies among different animal species (Vahter, 1999). The urinary arsenic species can provide useful insight into the arsenic methylation capacity in different animal species and humans (Francesconi and Kuehnelt, 2004). The detection of the transient metabolites of MMA(III) and DMA(III), however, depends on the conditions of sample storage and their concentration in the urine, which was beyond the analytical method at the time in 1989. Levels of trivalent methylated metabolites in the urine are expected to be very low, since these metabolites have short half-lives and are therefore considered not to be suitable markers for arsenic methylation at the present time (Del Razo et al., 2001; Francesconi et al., 2004). Further investigations that focus on the association between these highly toxic arsenic metabolic intermediates and clinical diseases would be potentially meaningful. In the arsenic methylation process, MMA(V) must be reduced to MMA(III) and methylated to DMA(V), which is the endpoint product in humans. The rat is the only species that excretes significant amounts of trimethylarsine oxide (TMAO) (Cohen et al., 2006). It cannot be excluded, however, that the higher MMA(V) in the urine is a reflection of higher MMA(III) in the blood or inside the cells, where the injury was induced by arsenic. The MMA(III) and DMA(III), however, are unstable, and they rapidly oxidize to MMA(V) and DMA (V), respectively (Gong et al., 2001).

Because of the high salinity of shallow well water, residents had drunk artesian well water for more than 50 years. Tap water was implemented in the early 1960s, but the coverage remained low until early 1970s. Artesian well water was no longer used for drinking after the mid-1970s. The arsenic concentration allowance in public water supplies in Taiwan was 50 μg/L, and a new standard of 10 μg/L was announced in 2000. The total urinary arsenic content of the residents in this area was higher than residents who lived in Taipei city (Hsueh et al., 2002). Arsenic may come from other potential sources, as at least 12 arsenic metabolites were biotransformed by arsenosugar ingestion in humans (Francesconi et al., 2002) and a novel thioarsenic metabolite was found in human urine after ingestion of an arsenosugar (Raml et al., 2005). The data for seafood ingestion was not available in the questionnaire during this study. Our previous study, however, showed the frequencies of fish, shellfish, and seaweed dietary intake were not significantly correlated with urinary arsenic species. In addition, the frequency of seaweed ingestion was not related to urinary DMA(V) (Hsueh et al., 2002), a result that appears contradictory to the observation from Japanese volunteers after consumption of seaweed (Ma and Le, 1998). Any variation of seaweed intake might be biased in the identification of urinary %DMA(V) in both cases of CAI and healthy residents, thereby causing a non-differential misclassification and displacement of the relative risk toward the null.

In this study, we demonstrated that patients with carotid atherosclerosis diagnosed by CAI-1 or CAI-3 had lower %InAs and %DMA(V) and greater %MMA(V) and PMI compared to normal subjects, indicating an efficient primary methylation capacity and an inefficient secondary methylation capacity related to CAI development. Our previous studies found that CAE was significantly related to the total urinary arsenic level and a lower capacity of methylate inorganic arsenic to DMA(V). These patients were found to have a greater risk of hypertension (Huang et al., 2007) and peripheral vascular disease (Tseng et al., 2005) in patients from the BFD hyperendemic area in Taiwan. Here, we also found that longer durations of consuming artesian well water, indicating increased arsenic accumulation, were associated with more inefficient arsenic methylation capacity. In addition, CAE was positively related to %MMA(V) and inversely related to %DMA(V) and SMI. These results suggest that individual variability for metabolizing inorganic arsenic is related to arsenic exposure.

Previous findings have demonstrated that traditional CAI risk factors, such as DM and hypertension (Hara et al., 2007; Taylor et al., 2007), may play an important role in the development of CAI. We, however, did not find that DM and hypertension has any influence in the impact of arsenic methylation capacity. By adjusted these traditional CAI risk factors, however, we found that in subjects with low arsenic methylation capability (PMI>1.28 or SMI≤5.18), the CAI risk was slightly significantly increased as the cumulative arsenic exposure increased.

The mechanisms of arsenic-induced atherosclerosis are not fully understood. The possible mechanisms of arsenic-induced atherosclerosis were proposed to induce cellular redox alteration (Pi et al., 2002), impair nitric oxide homeostasis (Pi et al., 2003), enhance coagulation activity (Simeonova and Luster, 2004), elevate homocysteinemia in individuals with high levels of MMA% in urine (Wu et al., 2006) and increase expression of inflammatory molecules (Wu et al., 2003). Studies that have explored the mechanism of methylated arsenic-induced atherosclerosis, however, are rare.

Several factors related to general health may affect arsenic methylation ability. This study found significantly greater total arsenic and %MMA(V) and lower %DMA(V) in males compared to females. These results suggest that females have lower arsenic exposure and/or have an influence of sex steroids, leading to a more efficient methylation capability compared to males as previously reported (Pu et al., 2007; Steinmaus et al., 2007; Lindberg et al., 2007). We found that the elderly had a less efficient arsenic methylation capacity compared to younger people, an observation consistent with our previous findings (Hsueh et al., 1998). An Inner Mongolia study found that children had a greater capacity for the secondary methylation of arsenic compared to adults exposed to the same arsenic levels in drinking water (Sun et al., 2007). These findings suggest that arsenic metabolism can be influenced by age. A more efficient arsenic methylation was observed in overweight or obese females compared to normal weight males (Lindberg et al., 2007). In this study, however, we did not find a relationship between the body mass index and arsenic methylation capacity (data not shown). Our previous study reported that high urinary selenium may reflect a greater dietary

intake of selenium and may also alter the arsenic methylation (Hsueh et al., 2003). Subjects were recruited from western Nevada and Kings County in California regions that had arsenic levels of nearly 100 μg/L in their drinking water. Subjects with lower dietary protein, iron, zinc and niacin intake excreted a greater amount of inorganic arsenic and had a greater %MMA and a lower %DMA compared to subjects with higher intakes of these nutrients (Steinmaus et al., 2005). Another study in Bangladesh adults showed that dietary intakes of cysteine, methionine, niacin, vitamin B-12 and choline modulated arsenic metabolism (Heck et al., 2007). A clinical trial demonstrated that folic acid supplementation in subjects with low plasma folate enhanced arsenic methylation (Gamble et al., 2006). Another study reported that fasting for a period of 12 hours resulted in a significant increase in the %MMA (Brima et al., 2007). The urine collection protocol required 8 hours of fasting in this study. The identification of urinary arsenic profile might therefore be biased in both cases and controls, resulting in non-differential misclassification and a displaced odds ratios toward the null.

Genetic polymorphisms may also contribute to individual variability in the biotransformation of arsenic. Subjects with the *GST M1* null genotype had an increased percentage of inorganic arsenic in their urine, while those with the *GST T1* null genotype had an elevated %DMA (Chiou et al., 1997a). Recently, a report from Central Europe found that the M287T polymorphism in the *AS3MT* gene and A222V polymorphism in the *MTHFR* gene influenced arsenic metabolism (Lindberg et al., 2007). The intronic polymorphisms in *AS3MT* (G12390C, C14215T, and G35991A) were associated with a lower % MMA and a greater %DMA in the urine of Argentinean subjects (Schlawicke et al., 2007), and that *GSTM1*, *GSTT1*, *MTR* and *MTHFR* polymorphisms are also responsible for inter-individual variability in arsenic metabolism. Subjects with the *MTHFR* 677TT and 1298AA variant genotypes excreted a significantly greater proportion of inorganic arsenic and a lower proportion of DMA. The null genotype of *GSTM1* excreted a significantly greater percentage of MMA compared to those with an active genotype (Steinmaus et al., 2007). In addition, due to individual variability in arsenic metabolism, there may be difference in disease susceptibility. The interaction between the *GSTT1* wild type and the secondary methylation index modified the risk of skin lesions among individuals exposed to arsenic in Bangladesh (McCarty et al., 2007). Another report from Bangladesh found a combined effect of either an *MTHFR* 677TT/1298AA and 677CT/1298AA diplotype or a *GSTω1* diplotype coupled with a greater %MMA increased the risk of skin lesions (Ahsan et al., 2007). It seems plausible to suggest that both genetic and acquired factors influence arsenic biotransformation and induce arsenic related health consequences.

Wu and colleagues found that either an increased efficiency of methylation from arsenite to MMA(V) or an increased methylation efficiency from MMA(V) to DMA(V) may be related to the influence of *S*-adenosylmethionine consumption and plasma homocysteine levels on the development of carotid atherosclerosis (Wu et al., 2006). Low arsenic exposure was positively related to high pulse pressure, especially among those with a low intake of nutrient-B vitamins (Chen et al., 2007). Another study demonstrated that genetic polymorphisms of *GSTP1* and *P53* modified the risk of carotid atherosclerosis when subjects were exposed to arsenic (Wang et al., 2007). It is possible that a trimethylated arsenic metabolite inhibits insulin-stimulated glucose uptake, impairs glucose tolerance (Paul et al., 2007) or generates reactive oxygen species and free radicals (Kitchin and Ahmad, 2003). Based on these studies, genetic susceptibility, homocysteine, *S*-adenosylmethionine consumption or low intakes of nutrients may influence the arsenic methylation capability. Inefficient methylation capability, however, may produce oxidative stress that induces carotid atherosclerosis. The precise mechanism for arsenic metabolite-related atherosclerosis requires further investigation. In conclusion, the findings of this study suggest that individuals with a greater arsenic exposure and a lower capacity to methylate inorganic arsenic to DMA(V) may have a higher risk of carotid atherosclerosis.

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