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Effects of arsenic exposure and genetic polymorphisms of p53, glutathione *S*-transferase M1, T1, and P1 on the risk of carotid atherosclerosis in Taiwan

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Abstract

To evaluate the joint effects between genetic polymorphisms of glutathione *S*-transferase M1, T1, P1, and p53, and arsenic exposure through drinking well water on the risk of carotid atherosclerosis, 605 residents including 289 men and 316 women were recruited from a northeastern area of Taiwan. Carotid atherosclerosis was diagnosed by either a carotid artery intima-media thickness (IMT) of >1.0 mm, a plaque score of ≥1, or stenosis of >50%. A significant age- and gender-adjusted odds ratio of 3.3 for the development of carotid atherosclerosis was observed among the high-arsenic exposure group who drank well water containing arsenic at levels >50 μg/L. The high-arsenic exposure group with GSTP1 variant genotypes of Ile/Val and Val/Val, and with the p53 variant genotypes of Arg/Pro and Pro/Pro had 6.0- and 3.1-fold higher risks of carotid atherosclerosis, respectively. In addition, the high-arsenic exposure group with one or two variant genotypes of GSTP1 and p53 had 2.8- and 6.1-fold higher risks of carotid atherosclerosis, respectively, and showed a dose-dependent relationship. A multivariate-adjusted odds ratio of 3.4 for the risk of carotid atherosclerosis among study subjects with the two variant genotypes of GSTP1 and p53 was also found. Our study showed the joint effects on the risk of carotid atherosclerosis between the genetic polymorphisms of GSTP1 and p53, and arsenic exposure.

Keywords: Arsenic; Glutathione *S*-transferase; p53; Atherosclerosis

1. Introduction

Arsenic is widely distributed in nature and mainly transported by water. Humans are exposed to arsenic through water, air, food, and medication, mainly by the way of ingestion and inhalation and rarely through skin absorption. In many parts of the world, including Taiwan, Argentina, Chile, Bangladesh, and China, it is a natural contaminant of ground water [1]. It can also be found in relatively high amounts in an organic form in seafoods, but this is easily excreted and is considered nontoxic [2]. The setting of the maximum contaminant level (MCL) that determines the concentration of arsenic in public water supplies has been an extraordinarily protracted process in the US. Recently, the MCL was lowered to 10 μg/L from the 50 μg/L standard established in 1942 [3]. Inorganic arsenic has been well documented as one of the major risk factors for blackfoot disease (BFD), a unique peripheral vascular disease identified in the endemic area of arseniasis located on the southwestern

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coast of Taiwan where residents used high-arsenic artesian well water for more than 50 years [4]. The pathological types of BFD include arteriosclerosis obliterans (70%) and thromboangiitis obliterans (30%) which develop from severe underlying systemic arteriosclerosis [5]. Current studies have also reported that long-term exposure to arsenic in drinking water is significantly associated with the risk of ischemic heart disease, cerebrovascular disease, and peripheral vascular disease in Taiwan [6–8]. In addition, associations of arsenic with hypertension and diabetes mellitus have also been reported in Taiwan and Bangladesh [9–11].

Carotid atherosclerosis consists of subclinical lesions of the superficial carotid artery, which has been recognized as an independent risk factor of arterial diseases that lead to cardiac and cerebral infarction. An increase in the intima-media thickness (IMT) of the carotid artery, which is caused by the accumulation of cells, lipids, and connective tissue, may reflect earlier changes. In addition, stenosis and plaque formation are relatively late manifestations of the atherosclerotic process [12,13]. Both human and animal studies have found that increased apoptosis occurs in atherosclerotic lesions, which mediate tissue turnover and lesion development. The tumor-suppressive gene, p53, has been found in vascular cells and may participate in the regulation of vascular apoptosis during the development of atherosclerosis [14,15].

The main arsenic species in artesian well water is arsenate. The mechanisms of arsenic metabolism and excretion in humans are not well elucidated. A substantial fraction of arsenate, which is readily absorbed from the gastrointestinal tract by humans, is reduced in the blood to arsenite. Arsenite is methylated mainly in the liver to monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA). The methylation is believed to occur via alternating reduction of pentavalent arsenic (As^V) to trivalent arsenic (As^{III}) and addition of a methyl group. *S*-Adenosylmethionine (SAM) is the main methyl donor in arsenic methylation. Because As^{III} is more toxic than As^V, the initial step in the biotransformation of arsenate may be regarded as a bioactivation. However, much of the formed As^{III} is distributed to the tissues where it is methylated to MMA and DMA [16–18]. Glutathione (GSH) and probably other thiols serve as reducing agents for As^V and MMA^V [19]. According to the proposed mechanism of arsenic methylation, the biomethylation of inorganic arsenic (InAs) generates mono- and dimethyl arsenic species, which are more readily excreted than InAs. Thus, the methylation of InAs has traditionally been considered to be a detoxification pathway. However, a growing literature of experimental studies indicates that the trivalent methylated arsenic intermediates (MMA^{III} and DMA^{III}) may be more toxic than $InAs^{III}$ or any of the pentavalent intermediates [20,21]. Glutathione *S*-transferases (GSTs) are a large family of phase II detoxification enzymes that catalyze the conjugation of GSH to a wide range of hydrophobic and electrophilic compounds [22]. Based on sequence homology and immunological crossreactivity, human cytosolic GSTs have been grouped into seven families, designated GST Alpha, Mu, Pi, Sigma, Omega, Theta, and Zeta [23]. Humans with null genotypes of GSTM1, T1 and the variant genotypes of GSTP1 are considered to be a high-risk group for cancers due to their deficiency in GSTs. The aim of this study was to evaluate the joint effects of arsenic exposure through drinking water and genetic polymorphisms of GSTM1, T1, P1, and p53 on the risk of carotid atherosclerosis.

2. Methods

2.1. Study area and subjects

Subjects were recruited from the Lanyang Basin of Ilan County in northeastern Taiwan. During the years of 1991–1994, a total of 8088 residents aged ≥40 years from 18 villages in four townships were interviewed and included as the study cohort [8]. In the year of 1996, we had completed home-visit follow-up interview and had sent invitation letter to 5146 subjects out of the original cohort for attending a health examination held in 1997–1998. Finally, a total of 1318 cohort members (≥40 years) who agreed to participate in this study and finished the carotid ultrasound assessment of extracranial carotid artery (ECCA) being conducted in Lotung Poh-Ai hospital. Case patients were diagnosed as having prevalent asymptomatic carotid atherosclerosis by a neurologist based on either a mean carotid IMT of >1.0 mm, plaque occurrence in at least two locations on one side (right or left) or the occurrence of stenosis of >50% in the left or right CCA. Among these examinees, a total of 605 subjects (including 279 cases and 326 controls) were randomly selected without any matched factors. These study subjects were not substantially different from the original cohort. This study was approved by the institutional review board for human subjects of Taipei Medical University, Taipei, Taiwan and each subject provided written informed consent.

2.2. Questionnaire interview and determination of arsenic in the well water

A standardized personal interview was conducted by well-trained public health nurses based on a structured questionnaire. Information of the personal interview included history of well water consumption, residential history, sociodemographic characteristics, levels of cigarette smoking and alcohol consumption, physical activities, as well as personal and familial history of hypertension, diabetes mellitus, cerebrovascular disease, heart disease, and cancer of various organs. Well water samples were collected during the home interview, immediately acidified with hydrochloric acid, and then stored at −20 °C until the subsequent assay. Hydride generation combined with flame atomic absorption spectrometry was used to determine the arsenic concentration in these samples. The standard calibration curve was established by serial dilution of standard arsenic solution as 1, 2, 4, 6, 8, 10, 20, 30, 40 and 50 ppb. The correlation coefficient of the standard calibration curve is between 0.996

and 0.999. Recovery rate ranged from 95% to 106%. The arsenic concentration in well water was found to range from undetectable (<0.15 μg/L) to 3.59×10^3 μg/L. The various exposure indices of arsenic through drinking water were described in a previous study [24].

2.3. Ultrasound measurements of carotid atherosclerosis

To evaluate the extent of carotid atherosclerosis, the ECCA was scanned bilaterally with a Hewlett-Packard Sono1000 ultrasound system, equipped with 7.5 MHz frequency in the B-mode and 5.6 MHz frequency in the pulsed-Doppler mode. The duplex scanning and evaluation of study subjects were described in a previous study [25]. Indications of carotid atherosclerosis were assessed mainly based on three indices: (1) the ECCA intima-media thickness (IMT); (2) the ECCA plaque score and (3) the maximal level of ECCA stenosis. The plaque score was also used as an index of atherosclerosis. The plaque score was counted if any plaque was observed in the areas of the external carotid artery (ECA), internal carotid artery (ICA), carotid bulb, or CCA. Study subjects were diagnosed as having prevalent asymptomatic carotid atherosclerosis by a neurologist based on a mean carotid IMT of >1.0 mm and either plaque occurrence in at least two locations on one side (right or left) or the occurrence of stenosis of >50% in the left or right CCA.

2.4. Biospecimen collection and laboratory measurements

Ten milliliters of whole-blood samples was collected from study subjects using a disposable vacuum syringe and needle, centrifuged to separate the buffy coat and plasma, and stored at −70 °C until the subsequent assay. DNA was purified from the buffy coat by the Viogene Blood and Tissue Genomic DNA Miniprep System (Viogene Inc., Taipei, Taiwan) resuspended in deionized distilled water, and stored at −20 °C until genotyping. The primers of GSTM1 were G5 (5′-GAA CTC CCT GAA AAG CTA AAG C-3′) and G6 (5′-GTT GGG CTC AAA TAT ACG GTG G-3′), and the PCR product showed a 215 bp band in the non-null genotype. The primers of GSTT1 were T1F (5′-TTC CTT ACT GGT CCT CAC ATC TC-3′) and T1R (5′-TCA CCG GAT CAT GGC CAG CA-3′), and the PCR product showed a 480 bp band in the non-null genotype. For GSTP1 and p53, PCR-RFLP (restriction fragment length polymorphism) was carried out with PCR primers P105F (5′-ACC CCA GGG CTC TAT GGG AA-3′) and P105R (5′-TGA GGG CAC AAG AAG CCC CT-3′) for GSTP1 and p53-1 (5′-ATC TAC AGT CCC CCT TGC CG-3′) and p53-2 (5′-GCA ACT GAC CGT GCA AGT CA-3′) for p53, respectively. The GSTP1 PCR product (176 bp) was subjected to *Bsm*AI digestion overnight at 55 °C, then loaded onto a 3% agarose gel and electrophoresed. The Ile/Ile genotype (wild type) showed a single band (176 bp), the Val/Val genotype (homozygote variant type) showed two bands (91 and 85 bp), and the Ile/Val genotype (heterozygote variant type) showed three bands (176, 91, and 85 bp). The p53 PCR product (296 bp) was subjected to *Bst*UI digestion overnight at 60 °C, then loaded onto a 3% agarose gel and electrophoresed. The Pro/Pro genotype (homozygote variant type) showed a single band (296 bp), the Arg/Arg genotype (wild type) showed two bands (169 and 127 bp), and the Arg/Pro genotype (heterozygote variant type) showed three bands (296, 169, and 127 bp). Beta-globulin and non-DNA template were used as internal positive and negative controls, respectively, in each experiment, and 10% of the total samples were randomly selected and reanalyzed with 100% of concordance.

2.5. Statistical analysis

Univariate analysis was conducted to test for risk factors including age, gender, body mass index (BMI), cigarette smoking, alcohol consumption, total cholesterol, triglyceride, hypertension, diabetes mellitus and arsenic concentration in well water. To evaluate the associations between these risk factors and the prevalence of carotid atherosclerosis, a multiple logistic regression analysis was used to estimate the age- and gender-adjusted odds ratio (OR) and 95% confidence interval (95% CI). The statistical significance of each multivariate-adjusted odds ratio was examined by significance testing of the regression coefficient based on the maximum likelihood method. Tests for Hardy–Weinberg equilibrium were conducted by comparing observed genotype frequencies with expected genotype frequencies among control subjects using a χ^2-test. Based on our initial results from univariate analysis, several variables such as age, gender, BMI, cigarette smoking, alcohol consumption, hypertension, diabetes mellitus, total cholesterol, triglyceride and arsenic concentration in well water were included in the subsequent analyses as shown in models I and II to control for potential confounding effect caused by these variables. Statistical Analysis Software (SAS Version 6.12; SAS Institute, Cary, NC) was applied to all statistical analyses. Values of $p < 0.05$ were considered statistically significant.

3. Results

In total, 605 study subjects, including 279 carotid atherosclerosis patients and 326 controls, were recruited in this study. Among them, there were 289 men and 316 women. As shown in Table 1, compared with the youngest age group, significant odds ratios of 5.8 and 2.3 were observed for the groups aged ≥65 and between 55 and 64.9 years old, respectively. Men had a higher risk of carotid atherosclerosis than women. A significant risk for development of carotid atherosclerosis was also observed among hypertensive patients with an age- and gender-adjusted odds ratio of 2.4. Compared with subjects whose BMI was ≤24 as a reference group, subjects with a BMI of >24 had a significant 1.7-fold higher risk of carotid atherosclerosis after adjusting for age and gender. Subjects with higher total cholesterol

Table 1
Characteristics of subjects with carotid atherosclerosis and of controls

	Cases[a] ($n=279$)	Controls ($n=326$)	OR (95% CI)[b]	OR (95% CI)[c]
Age (years)				
<55	25 (9.0%)	85 (26.1%)	1.0	
55–64.9	105 (37.6%)	153 (46.9%)	2.3 (1.4–3.9)**	
≥65	149 (53.4%)	88 (27.0%)	5.8 (3.4–9.7)***	
Gender				
Female	127 (45.5%)	190 (58.2%)	1.0	1.0
Male	152 (54.5%)	136 (41.8%)	1.7 (1.2–2.3)***	1.4 (1.0–1.9)
BMI (kg/m^2)				
≤24	174 (62.4%)	232 (71.2%)	1.0	1.0
>24	105 (37.6%)	94 (28.8%)	1.5 (1.1–2.1)*	1.7 (1.2–2.5)**
Cigarette smoking				
No	157 (56.3%)	223 (68.4%)	1.0	1.0
Yes	122 (43.7%)	103 (31.6%)	1.7 (1.2–2.4)**	1.1 (0.6–1.8)
Alcohol consumption				
No	214 (76.7%)	278 (85.3%)	1.0	1.0
Yes	65 (23.3%)	48 (14.7%)	1.7 (1.2–2.6)**	1.3 (0.8–2.1)
Hypertension[d]				
No	148 (76.3%)	244 (87.8%)	1.0	1.0
Yes	46 (23.7%)	34 (12.2%)	2.2 (1.4–3.6)**	2.4 (1.5–4.1)**
Diabetes mellitus[d]				
No	178 (91.8%)	256 (92.1%)	1.0	1.0
Yes	16 (8.2%)	22 (7.9%)	1.0 (0.5–2.0)	1.1 (0.5–2.2)
Total cholesterol (mg/dL)				
<200	166 (59.5%)	225 (69.1%)	1.0	1.0
≥200	113 (40.5%)	101 (30.9%)	1.5 (1.1–2.1)*	1.6 (1.1–2.3)**
Triglyceride (mg/dL)				
<200	247 (88.5%)	300 (92.0%)	1.0	1.0
≥200	32 (11.5%)	26 (8.0%)	1.5 (0.9–2.6)	1.4 (0.8–2.4)
Arsenic concentration in well water (μg/L)				
≤10	19 (6.8%)	48 (14.7%)	1.0	1.0
10.1–50.0	30 (10.8%)	68 (20.9%)	1.1 (0.6–2.2)	1.4 (0.7–2.8)
≥50.1	230 (82.4%)	210 (64.4%)	2.8 (1.6–4.9)***	3.3 (1.8–6.0)***

*$0.01<p<0.05$; **$0.001<p<0.01$; ***$p<0.001$.
[a] Cases: subjects with carotid atherosclerosis.
[b] Crude odds ratio.
[c] Age- and gender-adjusted odds ratio; 95% CI: 95% confidence interval.
[d] Some subjects with no information about the history of hypertension and diabetes mellitus.

(≥200 mg/dL) had a significant 1.6-fold higher risk of carotid atherosclerosis after adjustment for age and gender. In addition, smokers, alcohol drinkers, diabetes mellitus patients and subjects with higher triglyceride also had higher but not significant risks for the development of carotid atherosclerosis. A significant age- and gender-adjusted odds ratio of a 3.3-fold higher risk of carotid atherosclerosis was observed in the exposure group with an arsenic concentration in the well water of >50 μg/L. The genotype frequencies were in Hardy–Weinberg equilibrium in the control groups for both GSTP1 and p53 ($p=0.63$ and 0.87, respectively). As illustrated in Table 2, a significantly higher age- and gender-adjusted odds ratio of 2.0 for the development of carotid atherosclerosis was observed among study subjects with the Ile/Val and Val/Val genotypes of GSTP1 and of 1.9 for study subjects with the Arg/Pro and Pro/Pro genotypes of p53. In a gene–gene interaction analysis, compared with the reference group (wild genotypes of GSTP1 and p53), those who carried one and two variant genotypes of GSTP1 and p53 had significant age- and gender-adjusted odds ratios of 1.8 and 3.8, respectively, for the development of carotid atherosclerosis. In a further trend test, it also showed a dose–response relationship. However, study subjects with the null genotype for GSTM1 and T1 did not have a higher risk of carotid atherosclerosis.

Table 3 shows that study subjects with the Ile/Val and Val/Val genotypes of GSTP1 had significantly higher age-and gender-adjusted odds ratios of 2.7 and 6.0 for the development of carotid atherosclerosis among those who drank well water containing arsenic concentrations of >50 μg/L. Study subjects with the Arg/Pro and Pro/Pro genotypes of p53 had a significantly higher age- and gender-adjusted odds ratio of 3.1 for the risk of carotid atherosclerosis among those individuals drinking well water containing arsenic >50 μg/L. As shown

Table 2
Age- and gender-adjusted odds ratio and 95% CI of subjects with carotid atherosclerosis and of controls by the genotypes of GSTM1, T1, P1 and p53

	Cases[a] ($n=279$)	Controls ($n=325$)[b]	OR (95% CI)[c]	OR (95% CI)[d]
GSTM1				
Non-null	136 (48.7%)	139 (42.8%)	1.0	1.0
Null	143 (51.3%)	186 (57.2%)	0.8 (0.6–1.1)	0.9 (0.5–1.0)
GSTT1				
Non-null	134 (48.0%)	133 (40.9%)	1.0	1.0
Null	145 (52.0%)	192 (59.1%)	0.8 (0.5–1.0)	0.7 (0.5–1.0)
GSTP1				
Ile/Ile	178 (63.8%)	248 (76.4%)	1.0	1.0
Ile/Val and Val/Val	101 (36.2%)	77 (23.6%)	1.8 (1.3–2.6)***	2.0 (1.4–3.0)***
p53				
Arg/Arg	53 (19.0%)	91 (28.0%)	1.0	1.0
Arg/Pro and Pro/Pro	226 (81.0%)	234 (72.0%)	1.7 (1.1–2.4)*	1.9 (1.3–2.9)**
GSTP1and p53[e]				
0	36 (12.9%)	67 (20.6%)	1.0	1.0
1	159 (57.0%)	205 (63.1%)	1.4 (0.9–2.3)	1.8 (1.1–2.9)*
2	84 (30.1%)	53 (16.3%)	2.9 (1.7–5.0)***, P for trend <0.0001	3.8 (2.2–6.7)***, P for trend <0.0001

*$0.01<p<0.05$; **$0.001<p<0.01$; ***$p<0.001$.
[a] Case subjects with carotid atherosclerosis.
[b] For one control who lost the data of genotypes of GSTM1,T1,P1 and p53.
[c] Crude odds ratio.
[d] Age- and gender-adjusted odds ratio; 95%CI: 95% confidence interval.
[e] Total number of variant (homo- and hetero-zygote) genotypes of GSTP1 and p53.

in Table 4, significantly higher risks of 2.8 and 6.1 for the development of carotid atherosclerosis were observed among study subjects who carried one and two variant genotypes of GSTP1 and p53 and who had arsenic exposure through drinking well water with arsenic levels of >50 μg/L, thus showing a biological relationship.

Results of the multivariate models (models I and II) are shown in Table 5. Significant multivariate-adjusted risk factors for the development of carotid atherosclerosis were age, hypertension, variant genotypes of GSTP1 and p53, and arsenic concentration in well water in model I. As illustrated in model II, the joint effects of variant genotypes of GSTP1 and p53 on the risk of carotid atherosclerosis were observed as significantly independent risk factors after adjustment for those risk factors found in model I.

Table 3
Age- and gender-adjusted odds ratio and 95% CI of subjects with carotid atherosclerosis and of controls by the genotypes of GSTP1, p53 and arsenic exposure indices

	Arsenic concentration in well water (μg/L)			
	≤50		>50	
	OR[a]	95% CI	OR[a]	95% CI
GSTP1				
Ile/Ile	1.0		2.7	1.6–4.5**
Ile/Val and Val/Val	1.9	0.9–4.0	6.0	3.3–10.7***
p53				
Arg/Arg	1.0		1.4	0.7–3.0
Arg/Pro and Pro/Pro	0.9	0.5–1.9	3.1	1.7–5.7***

*$0.01<p<0.05$; **$0.001<p<0.01$; ***$p<0.001$.
[a] Age- and gender-adjusted odds ratio with arsenic exposure ≤50 μg/L and GSTP1 genotype of Ile/Ile or p53 genotype of Arg/Arg.

Table 4
Age- and gender-adjusted odds ratio and 95% CI of subjects with carotid atherosclerosis and of controls by the genotypes of GSTP1, p53 and arsenic exposure indices

Number of variant genotype of GSTP1 and p53[a]	Arsenic concentration in well water (μg/L)			
	≤50		>50	
	OR[b]	95% CI	OR[b]	95% CI
0	1.0		1.4	0.6–3.3
1	1.0	0.4–2.2	2.8	1.3–6.0**
2	1.9	0.7–5.3	6.1	2.7–13.9***

*$0.01<p<0.05$; **$0.001<p<0.01$; ***$p<0.001$.
[a] Total number of variant (homo- and hetero-zygote) genotypes of GSTP1 and p53.
[b] Age- and gender-adjusted odds ratio with arsenic exposure ≤50 μg/L and GSTP1 genotype of Ile/Ile or p53 genotype of Arg/Arg.

4. Discussion

The atherogenic effects of ingesting inorganic arsenic through drinking water have been well documented. Several studies in Taiwan have reported significant associations between long-term exposure to arsenic in drinking water and the risk for the development of atherosclerotic vascular diseases including ischemic heart disease, cerebrovascular disease, and peripheral vascular disease, all of which

Table 5
Multiple logistic regression analysis of related risk factors of subjects with carotid atherosclerosis and of controls

Variables	Model I (OR (95% CI)[a])	Model II (OR (95% CI)[a])
Age		
<55	1.0	1.0
55–64.9	2.2 (1.3–3.7)**	2.2 (1.3–3.7)**
≥65	5.8 (3.3–10.2)***	5.7 (3.3–10.0)***
Gender		
Female	1.0	1.0
Male	1.5 (0.9–2.7)	1.5 (0.9–2.7)
BMI (kg/m^2)		
≤24	1.0	1.0
>24	1.2 (0.8–1.8)	1.2 (0.8–1.8)
Cigarette smoking		
No	1.0	1.0
Yes	1.1 (0.6–1.8)	1.1 (0.6–1.8)
Alcohol consumption		
No	1.0	1.0
Yes	1.3 (0.7–2.1)	1.3 (0.7–2.1)
Hypertension		
No	1.0	1.0
Yes	1.8 (1.0–3.1)*	1.8 (1.0–3.0)*
Diabetes mellitus		
No	1.0	1.0
Yes	0.8 (0.4–1.6)	0.7 (0.4–1.6)
Total cholesterol (mg/dL)		
<200	1.0	1.0
≥200	1.2 (0.8–1.7)	1.2 (0.8–1.7)
Triglyceride (mg/dL)		
<200	1.0	1.0
≥200	1.0 (0.5–1.9)	1.0 (0.5–1.9)
Arsenic concentration in well water (μg/L)		
≤10	1.0	1.0
10.1–50.0	1.6 (0.7–3.3)	1.6 (0.7–3.3)
≥50.1	3.0 (1.6–5.9)**	2.9 (1.5–5.7)**
GSTP1		
Ile/Ile	1.0	
Ile/Val and Val/Val	2.1 (1.4–3.1)***	
p53		
Arg/Arg	1.0	
Arg/Pro and Pro/Pro	1.6 (1.1–2.5)*	
Number of variant genotype of GSTP1 and p53[b]		
0		1.0
1		1.6 (1.0–2.7)*
2		3.4 (1.9–6.2)***, P for trend < 0.0001

*0.01 < p < 0.05; **0.001 < p < 0.01; *** p < 0.001.
[a] Multiple related risk factors-adjusted odds ratio; 95% CI: 95% confidence interval.
[b] Total number of variant (homo- and hetero-zygote) genotypes of GSTP1 and p53.

showed dose–response relationships [25,26]. In addition, a significant dose–response relationship between long-term exposure to arsenic through drinking well water and risks of hypertension and diabetes mellitus was also found in Taiwan [10,11]. Atherosclerosis combined with hypertension and diabetes mellitus predisposed residents to the development of stroke and ischemic heart disease which are the leading causes of death in developed countries [27]. In our current study which was based on personal exposure data carried out in the Lanyang Basin of northeastern Taiwan, compared with the reference group of arsenic exposure in well water at ≤10 μg/L, there was a significant age- and gender-adjusted odds ratio of developing carotid atherosclerosis of 3.3 for the highest arsenic exposure group with arsenic concentrations in well water of >50 μg/L. Furthermore, an increased risk of atherosclerosis was also found among subjects with arsenic exposure through drinking well water containing arsenic concentrations which ranged between 10 and ≤50 μg/L.

Age was significantly associated with the risk of carotid atherosclerosis. This implies that age is a major risk factor for carotid atherosclerosis. Subjects with a BMI of >24 had an age- and gender-adjusted odds ratio of a 1.7-fold higher risk of carotid atherosclerosis, compared with the reference group with a BMI of ≤24. Our finding that hypertension was significantly associated with atherosclerosis risk is consistent with previous reports. However, a non-significant higher risk (OR = 1.1) of carotid atherosclerosis was observed for patients with diabetes mellitus. Besides, our results indicated that subjects with higher total cholesterol (≥200 mg/dL) had a significant age- and gender-adjusted odds ratio of 1.6. This result was concordant with the general concept that total cholesterol is a traditional risk factor of carotid atherosclerosis. However, cigarette smoking, alcohol consumption and higher triglyceride produced non-significant increased risks of carotid atherosclerosis, showing age- and gender-adjusted risks of 1.1, 1.3 and 1.4, respectively.

The concept that oxidized lipoproteins are involved in atherosclerotic lesion development was formulated from demonstrations that low-density lipoproteins can injure cells under certain conditions, which were later shown to be facilitated oxidation of lipoproteins [28]. Based on this evidence, arsenic might be involved in the formulation of atherosclerosis because it can induce oxidative stress which increases the level of accumulation of oxidized lipoproteins [29]. A recent study indicated that p53 accumulates in human atherosclerotic tissue in plaque areas with signs of chronic inflammation [30]. The effect of sodium arsenite on p53 protein levels may be relevant to the development of cancer, since its induction may reflect the presence of alterations in the cell proliferation 'machinery' [31]. Inorganic arsenic is metabolized through methylation using SAM as the methyl donor and a methyltransferase (MTase). Therefore, arsenic may be involved in alterations of MTase/SAM-dependent DNA methylation of the p53 tumor suppressor gene [32]. In this study, a significantly increased risk of carotid atherosclerosis was observed among study subjects with Arg/Pro and Pro/Pro genotypes of p53, with an age- and gender-adjusted odds ratio of 1.9. In addition, a highly significant synergistic effect on the risk of carotid atherosclerosis between these genotypes of p53 and

arsenic exposure of >50 μg/L was reported with an age- and gender-adjusted odds ratio of 3.1.

GSTs are necessary for arsenic methylation, perhaps through the formation of arsenite which is the major arsenic form for methylation, or through conjugation with arsenic [33]. Humans exposed to arsenic with null genotypes of GSTM1, T1 and the variant genotypes of GSTP1 have been considered to be a high-risk group for carotid atherosclerosis due to their deficiency in GSTs for efficiently conjugating arsenic with methyl groups to form hydrophilic metabolites. In this study, there were no significant differences in the risks of carotid atherosclerosis among study subjects with the null genotypes of GSTM1 and GSTT1. However, a significantly increased risk for the development of carotid atherosclerosis was observed among study subjects with the Ile/Val and Val/Val genotypes of GSTP1, with an age- and gender-adjusted odds ratio of 2.0. Previous studies suggested that genetic polymorphisms of GSTP1 exon 5 (Ile to Val) and exon 6 (Ala to Val) have functional relevance to the GST gene product resulting in reduced enzyme activity. Therefore, individuals with these variant genotypes of GSTP1 may be at greater risk for carotid atherosclerosis due to decreased detoxification of inorganic arsenic. A significant joint effect of the Ile/Val and Val/Val genotypes of GSTP1 and high-arsenic exposure (>50 μg/L) on the risk of developing carotid atherosclerosis was found in this study, with an age- and gender-adjusted odds ratio of 6.0.

Our results showed that those with one or two variant genotypes of GSTP1 and p53 had significant age- and gender-adjusted odds ratios of 1.8 and 3.8, respectively, compared with wild genotypes of GSTP1 and p53 as a reference group. The risk of carotid atherosclerosis increased as the number of variant genotypes of these two genes increased. Joint effects on the risk of carotid atherosclerosis among study subjects who drank well water containing arsenic at a concentration >50 μg/L and with one and two of variant genotypes of these two genes were 2.8 and 6.1, respectively. In addition, after adjustment for several traditional risk factors including age, gender, BMI, cigarette smoking, alcohol consumption, hypertension, diabetes mellitus, total cholesterol, triglyceride and arsenic exposure, significant multivariate-adjusted odds ratios of 2.1 ($p = 0.0002$) and 1.6 ($p = 0.028$) were observed among those with variant genotypes of GSTP1 and p53, respectively, in model I. Significant risks of carotid atherosclerosis were also found in model II for study subjects with two variant genotypes of GSTP1 and p53, with multivariate-adjusted odds ratios of 1.6 and 3.4. Based on the results in Tables 4 and 5, joint effects between arsenic exposure and variant genotypes of GSTP1 and p53 on the risk for development of carotid atherosclerosis were found in this study.

We did not include in our analysis the completed information about the familial history of stroke or cardiovascular disease and LDL, which might be a limitation to our results. Nonetheless, we collected the major risk factors including age gender, BMI, cigarette smoking, alcohol consumption, hypertension, diabetes mellitus, total cholesterol, triglyceride and arsenic concentration in well water. These important variables were further included in the subsequent models to control for the confounding effect. Regarding the IMT definition, the method of IMT assessment in the present study had been published [25]. It implied that the IMT definition used in our study to evaluate carotid atherosclerosis is appropriate. A detailed history of residential village water consumption, including water source and duration of consumption obtained from the questionnaire interview was used to estimate the arsenic exposure. Additionally, a total of 3901 well-water samples (one sample from each household) were collected during home interview and subsequently analyzed with a quality control procedure [8]. Compared with previous studies conducted in the BFD-endemic area [6,25], we can more exactly estimate the individual arsenic exposure for study subjects. To avoid selection bias, study subjects were invited to a representative hospital (Lotung Poh-Ai hospital) and the neurologist performed carotid ultrasound assessments with blindness to the individual arsenic exposure. In the future, more detailed information including dietary issues and familial history of stroke or cardiovascular diseases should be completely collected in a larger study.

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References

[1] Nordstrom DK. Public health. Worldwide occurrences of arsenic in ground water. Science 2002;296:2143–5.
[2] Campbell BG. Broadsheet number 48: mercury, cadmium and arsenic: toxicology and laboratory investigation. Pathology 1999;31:17–22.
[3] Smith AH, Lopipero PA, Bates MN, et al. Public health. Arsenic epidemiology and drinking water standards. Science 2002;296:2145–6.
[4] Chen CJ, Wu MM, Lee SS, et al. Atherogenicity and carcinogenicity of high-arsenic artesian well water. Multiple risk factors and related malignant neoplasms of blackfoot disease. Arteriosclerosis 1988;8:452–60.
[5] Yeh S, How SW. A pathological study on the blackfoot disease in Taiwan. Rep Inst Pathol Natl Taiwan Univ 1963;14:25–73.
[6] Hsueh YM, Wu WL, Huang YL, et al. Low serum carotene level and increased risk of ischemic heart disease related to long-term arsenic exposure. Atherosclerosis 1998;141:249–57.
[7] Chen CJ, Chiou HY, Chiang MH, et al. Dose–response relationship between ischemic heart disease mortality and long-term arsenic exposure. Arterioscler Thromb Vasc Biol 1996;16:504–10.
[8] Chiou HY, Huang WI, Su CL, et al. Dose–response relationship between prevalence of cerebrovascular disease and ingested inorganic arsenic. Stroke 1997;28:1717–23.
[9] Rahman M, Tondel M, Ahmad SA, et al. Diabetes mellitus associated with arsenic exposure in Bangladesh. Am J Epidemiol 1998;148:198–203.

[10] Tseng CH. The potential biological mechanisms of arsenic-induced diabetes mellitus. Toxicol Appl Pharmacol 2004;197:67–83.
[11] Chen CJ, Hsueh YM, Lai MS, et al. Increased prevalence of hypertension and long-term arsenic exposure. Hypertension 1995;25:53–60.
[12] Howard G, Sharrett AR, Heiss G, et al. Carotid artery intimal-medial thickness distribution in general populations as evaluated by B-mode ultrasound. ARIC investigators. Stroke 1993;24:1297–304.
[13] Salonen R, Salonen JT. Carotid atherosclerosis in relation to systolic and diastolic blood pressure: Kuopio Ischaemic heart disease risk factor study. Ann Med 1991;23:23–7.
[14] Geng YJ. Biologic effect and molecular regulation of vascular apoptosis in atherosclerosis. Curr Atheroscler Rep 2001;3:234–42.
[15] Geng YJ. Regulation of programmed cell death or apoptosis in atherosclerosis. Heart Vessels 1997;(Suppl. 12):76–80.
[16] Hughes MF. Arsenic toxicity and potential mechanisms of action. Toxicol Lett 2002;133:1–16.
[17] Kitchin KT. Recent advances in arsenic carcinogenesis: modes of action, animal model systems, and methylated arsenic metabolites. Toxicol Appl Pharmacol 2001;172:249–61.
[18] Aposhian HV, Zakharyan RA, Avram MD, et al. Oxidation and detoxification of trivalent arsenic species. Toxicol Appl Pharmacol 2003;193(1):1–8.
[19] Hayakawa T, Kobayashi Y, Cui X, et al. A new metabolic pathway of arsenite: arsenic–glutathione complexes are substrates for human arsenic methyltransferase Cyt19. Arch Toxicol 2005;79: 183–91.
[20] Nesnow S, Roop BC, Lambert G, et al. DNA damage induced by methylated trivalent arsenicals is mediated by reactive oxygen species. Chem Res Toxicol 2002;15(12):1627–34.
[21] Styblo M, Drobna Z, Jaspers I, et al. The role of biomethylation in toxicity and carcinogenicity of arsenic: a research update. Environ Health Perspect 2002;110(Suppl. 5):767–71.
[22] Strange RC, Jones PW, Fryer AA. Glutathione *S*-transferase: genetics and role in toxicology. Toxicol Lett 2000;112/113:357–63.
[23] Strange RC, Spiteri MA, Ramachandran S, et al. Glutathione *S*-transferase family of enzymes. Mutat Res 2001;482:21–6.
[24] Tseng CH, Huang YK, Huang YL, et al. Arsenic exposure, urinary arsenic speciation, and peripheral vascular disease in blackfoot disease-hyperendemic villages in Taiwan. Toxicol Appl Pharmacol 2005;206:299–308.
[25] Wang CH, Jeng JS, Yip PK, et al. Biological gradient between long-term arsenic exposure and carotid atherosclerosis. Circulation 2002;105:1804–9.
[26] Tseng CH, Chong CK, Chen CJ, et al. Dose–response relationship between peripheral vascular disease and ingested inorganic arsenic among residents in blackfoot disease endemic villages in Taiwan. Atherosclerosis 1996;120:125–33.
[27] Shantaram V. Pathogenesis of atherosclerosis in diabetes and hypertension. Clin Exp Hypertens 1999;21:69–77.
[28] Hessler JR, Morel DW, Lewis LJ, et al. Lipoprotein oxidation and lipoprotein-induced cytotoxicity. Arteriosclerosis 1983;3:215–22.
[29] Pi J, Yamauchi H, Kumagai Y, et al. Evidence for induction of oxidative stress caused by chronic exposure of Chinese residents to arsenic contained in drinking water. Environ Health Perspect 2002;110:331–6.
[30] Tabas I. p53 and atherosclerosis. Circ Res 2001;88:747–9.
[31] Salazar AM, Ostrosky-Wegman P, Menendez D, et al. Induction of p53 protein expression by sodium arsenite. Mutat Res 1997;381:259–65.
[32] Mass MJ, Wang L. Arsenic alters cytosine methylation patterns of the promoter of the tumor suppressor gene p53 in human lung cells: a model for a mechanism of carcinogenesis. Mutat Res 1997;386:263–77.
[33] Chiou HY, Hsueh YM, Hsieh LL, et al. Arsenic methylation capacity, body retention, and null genotypes of glutathione *S*-transferase M1 and T1 among current arsenic-exposed residents in Taiwan. Mutat Res 1997;386:197–207.