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Background— In previous studies, we observed an enhanced oxidative stress level in the plasma of arsenic-exposed individuals, and further profiled gene expression changes in inflammatory mediator genes in circulating lymphocytes from the study subjects. Inflammation molecule such as monocyte chemoattractant protein-1 plays a role in the initiation stage of atherosclerosis. Short allele of $(GT)_n$ length polymorphism in promoter region of Heme oxygenase-1 (HO-1) gene is found to be associated with its protective effect against oxidant stress. We perform this study (1) to investigate the relationship between MCP-1 and arsenic-associated atherosclersis and (2) to examine the modifying effect of HO-1 genetic variants on the disease risk.

Methods—A total of 436 and 500 cohort subjects were recruited from the BFD-endemic area and the Lanyang Basin areas, respectively. The study subjects included 174 and 240 patients, respectively, with an indication of atherosclerosis diagnosed in 1996-1997. For each study subject, serum samples were thawed out for the measurement of MCP-1 levels by the method of enzyme-linked immunoabsortent assay. Frozen samples of buffy coat were retrieved and extracted for genomic DNA, and length polymorphisn of HO-1 gene was sequenced and determined. Chi-square test and logistic regression analysis were used to examine the association and modification effects.

Results—Serum levels of MCP-1 have bben assayed for 75 patients and 122 controls recruited from the BFD-endemic area. The patients have a higher level of serum MCP-1 than controls 1.2 folds, but not significantly different (95% CI 0.6-2.3). Determination of the HO-1 (*GT*)n repeats polymorphism have been completed for a total of 173 patients and 170 control subjects, who were recruited from the Lanyang Basin area. Patients carried long alleles (number of repeats >27) more frequently than controls, but not statistically significant ($p>0.05$).

Conclusion— Further study with extended sample size for laboratory assay and more sophisticated statistical analysis are underway. Exploration of the other inflammatory factors and genetic biomarkers in the study subjects are also needed.

Key Words: arsenic ■ atherosclerosis ■ MCP-1 ■ heme oxygenase-1

Introduction

Arsenic has long been regarded as an environmental toxin, exposure to which may increase the risk of developing atherosclerotic vascular disease, such as ischemic heart disease, cerebrovascular infarction, and carotid atherosclerosis.[1-3] The pathogenic mechanisms underlying the arsenic-associated atherosclerosis remain largely unknown. In our previous studies, we observed an enhanced oxidative stress level in the plasma of arsenic-exposed individuals, and further profiled gene expression changes in inflammatory mediator genes in circulating lymphocytes from the study subjects.[4,5] In this project, a study was carried out for two specific aims: (1) to investigate the relationship between inflammation mediators and arsenic-associated atherosclersis; and (2) to examine the modifying effect of stress responsive genes. We chose first monocyte chemoattractant protein-1 (MCP-1) and heme oxygenase-1 (HO-1) as our study molecules to address our study questions.

Participation of inflammation is actually involved in all three stages of atheroscleosis: initiation, progression, and complication.[6] Specific molecules such as MCP-1 play a role in the initiation stage. Aberrant expression of inflammatory cytokines or growth factors have been consistently noted in both *in vitro* and *in vivo* arsenic studies, [7-11] including ours. Arsenic-associated vascular disease may involve an ongoing inflammatory response following prolonged exposure through induction of oxidative stress and redox-sensitive inflammatory gene expression in the vasculature of the exposed humans. Further, this hypothesized link between arsenic-mediators and vascular disease may be modified by the genetic make-up of individuals, such as stress proteins (SPs) response to the exposure. Several SPs are specifically related to arsenic exposure,[12] including heme oxygenase (HO). Heme oxygenase-1 (HO-1) has been reported to have association with vascular function or have inflammatory properties and could be involved in the progression of atherosclerosis.[13,14] Recently, long allele of $(GT)_n$ length polymorphism in the promoter of HO-1 gene is further found to be associated with susceptibility to coronary artery disease in type 2 diabetic patients.[15] We thus hypothesized that the genetic variants of HO-1 confer arsenic-exposed individuals for differential susceptibility to disease risk in the response to arsenic intoxication after an extended time.

Methods

Study Areas and Study Subjects

This research group has started a project of an epidemiologic cohort study on arsenic-related disease since the late 1980s.[16] The study area included the villages of Homei, Fuhsin, and Hsinming in Putai Township of southwestern Taiwan, where the BFD prevalence and mortality from major cancers were the highest in Taiwan.[17] Six follow-up examinations have been carried out since the initial recruitment. An ultrasonographic assessment of extracranial carotid artery (ECCA), as an indicator of atherosclerosis was conducted in the sixth examination in 1996.[3] A total of 436 (94%) cohort subjects completed the assessment of ECCA with their consent. Among these 436 study subjects, 174 (39.9%) individuals were diagnosed as with carotid atherosclerosis index (CAI). The CAI was defined as the presence of plaque or intima-media thickness (IMT) ≥ 1.0 mm. These 436 cohort subjects will form the basis of the target population for this study.

In addition to the BFD-endemic area, well water with a high arsenic level was also clustered in the Lanyang Basin area.[18] The variation of arsenic level in well water was more striking, and the concentration to which local residents have been exposed was much lower in the Lanyang Basin area than in the BFD-endemic area. A high prevalence of cerebrovascular diseases as well as bladder cancer associated with long-term arsenic exposure has recently been reported in the Lanyang Basin area.[19] Characterization of arsenic poisoning presenting typical skin lesions as observed in the BFD-endemic area, has however, not been observed in the Lanyang Basin area. In 1997, a total of 1,342 residents in the Lanyang Basin area were screened for CAI using the same criteria as the residents of the BFD-endemic area performed in 1996. Approximate 500 of the 1,342 residents were diagnosed as patients with CAI, assuming that both populations from different arseniasis areas have a similar prevalence rate of carotid atherosclerosis. 240 cases and their matched controls were selected according to the arsenic level in the household well water.

Detection of Serum MCP-1 Levels

MCP-1 levels in serum samples from each study subject were measured using enzyme-linked immunoabsorbent assay (Biotrak, Piscataway, NJ) according to the manufacturers' instructions.

Heme Oxygenase-1 Length Polymorphism

Venous blood samples from study subjects collected in 1996 and 1997 were thawed

out for the extraction of genomic DNA. The DNA was isolated with the use of a PUREGENE DNA purification kit (Gentra System) according to the manufacture's protocol. The HO-1 gene 5'-flanking region containing (GT)n repeats was amplified by polymerase chain reaction with a FAM-labeled sense primer, 5'-AGAGCCTGCAGCTTCTCAGA-3' and an antisense primer, 5'-ACAAAGTCTGGCCATAGGAC-3' according to the published sequence.[20] The PCR was performed over two stages with GenoTYPE *Tsp* DNA Polymerase (Invitrogen, -20°C) as 10 cycles of 30 s at 94°C, 30 s at 57°C, and 30 s at 72°C, and 25 cycles of 30 s at 89°C, 30 s at 57°C, and 30 s at 72°C. The PCR products were mixed together with the Gene Scan-500 LIZ size standard (PE Applied Biosystems) and analyzed on a laser-based automated DNA sequencer (ABI Prism 3700) by the National Genotyping Center, Academia Sinica. The respective sizes of the (GT)n repeats were each calculated by using the GeneMapper analysis software (PE Applied Biosystems). As a control DNA for the adjustment in each genotyping assay, the (GT)n repeats PCR products from four anonymous volunteers were also added throughout the assays. To confirm the number of the (GT)n repeats, control DNA as well as selected samples of the PCR products were cloned into pGEM-AT vector (Promega) and purified plasmid DNAs were subjected to sequence analysis.

Statistical analysis

Two sample-t-test or Chi-square test was used to compare the differences between case and control group. Likelihood ratio test in logistic regression was used to examine the adjusted effect of a risk factor after the adjustment of other confounding factors.

Results and Discussion

Characteristics of the study population

Table 1 shows the age and sex distributions, life style, and clinical characteristics of study subjects by the two study areas. A total of 240 and 344 study subjects, respectively, were completed for several laboratory assays. As shown in the Table, study subjects in the patients group tend to be older and contain more males. In addition, results consistent with previous findings also show increased risk of hypertension and diabetes mellitus in the BFD-endemic area, but not as high in the Lanyang Basin area. However, random variation may have occurred due to small sample size in these study population.

Association studies of MCP-1 with atherosclerosis in the BFD-endemic area

Although the difference between patients and controls in the serum MCP-1 level is small, the subjects with carotid atherosclerosis have a higher level of MCP-1 in serum than the controls do (Table 2). Further studies with increased sample size, as well as a statistical analysis using multivariate regression modeling for the adjustment of confounding factors will be needed to elucidate the relationship in this study.

Studies of HO-1 gene promoter polymorphism in the Lanyang Basin area

Figure 1 shows the allele frequencies of the (GT)n microsatellite in the HO-1 promoter region in the 343 study subjects. The proportions of alleles and genotypes frequencies are shown in Figure 1. $(GT)_{23}$ and $(GT)_{30}$ are the two most common alleles in our study subjects, which is consistent with the results of a previous study in Chinese.[15,21] Patients with carotid atherosclerosis carried long alleles (number of the repeats >27) more frequently than the control subjects, but it was not statistically significant (Table 3).

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(1) A larger samples size will be needed to make a clear conclusion. Actually, determination of the polymorphic locus in an additional 200 subjects and 450 subjects from BFD and Lanyany area, respectively, is underway fro the 2nd year project in our laboratory.
(2) Once more samples are added-in, a more sophisticated statistical analysis including the interaction effect between genetic and non-genetic risk factors will be examined in further analysis.

TABLE 1. Characteristics of Study Subjects by Arseniasis-endemic Area

Characteristics	BFD-endemic Area		Lanyang Basin Area	
	Controls (n=140)	Patients# (n=100)	Controls (n=169)	Patients (n=168)
Age, yr	53.0±9.3	62.2±8.0**	57.8±16.7	64.8±14.0**
Men, %	35	53**	38	54**
Cigarette smoker, %	10.7	26.0**	N.R.	N.R.
Alcohol intake, %	10.0	17.0	N.R.	N.R.
BMI, kg/m^2	24.4±3.3	24.5±3.4	24.1±3.3	23.8±3.5
TC, mg/dL	212±43	218±39	197±34	203±41
TG, mg/dL	109 (78-170)	129 (94-178)	101(69-140)	115(78-159)
HT history, %	25.0	51.0**	30.8	38.7
DM history, %	20.0	32.0*	9.5	6.6

TG values are given as median (Q1-Q3), and other values as mean ±SD. Data on Life style from the Lanyan Basin are not yet retrievable (N.R).

Study subjects with indication of atherosclerosis

* $P<0.05$, ** $P<0.01$ versus respective control.

TABLE 2. Plasma MCP-1 Level and Risk of Carotid Atherosclerosis in the 197 Study Subjects from the BFD-endemic Area

Characteristics	Controls N (%)	Patients[#] N (%)	Crude OR (95% CI)	Age-sex-adjusted OR (95% CI)
Plasma MCP-1 level (pg/ml)[*]				
< 806	61 (50.0)	31 (41.3)	1.0	1.0
≥ 806	61 (50.0)	44 (58.7)	1.4 (0.8-2.5)	1.2 (0.6-2.3)

Study subjects with indication of atherosclerosis

* Data are expressed in logarithmic scale.

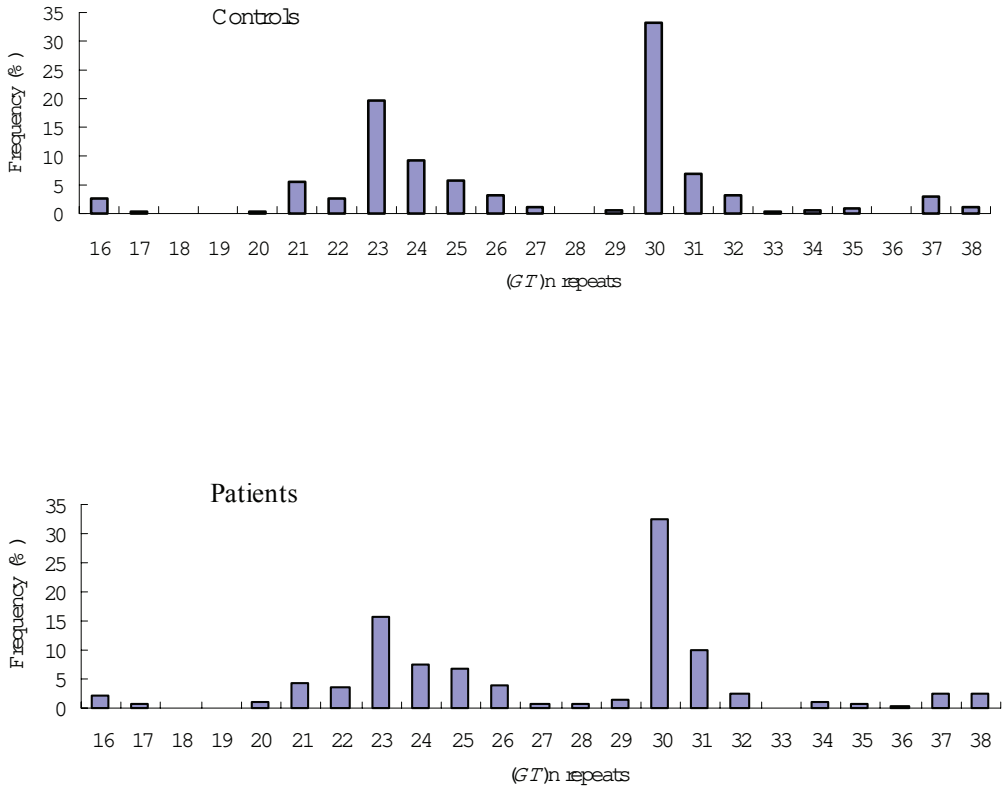

FIGURE 1. Frequency Distribution of the (GT)n Repeats in Controls Without Atherosclerosis (n=173) and Patients With Atherosclerosis (n=170), the Lanyang Basin Area.

TABLE 3. Distribution of HO-1 Promoter Allele and Genotype Frequencies of Study Subjects, the Lanyang Basin Area

	Controls	Patients*	
	(n=173)	(n=170)	*P*-value[#]
Alleles, n (%)			
S	174 (50.29)	156 (45.88)	
L	172 (49.71)	184 (54.12)	0.249
Genotypes, n (%)			
S/S	49 (28.32)	37 (21.76)	
L/S	76 (43.93)	82 (48.24)	
L/L	48 (27.75)	51 (30.00)	0.267

Derived from Chi-square test.

* Study subjects with indication of atherosclerosis

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