行政院國家科學委員會專題研究計畫　成果報告

膀胱移形細胞癌之個案,健康對照及一等親之慢性砷中毒相關酵素基因多型性之比較（子計畫五）

計畫類別： 整合型計畫
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行政院國家科學委員會補助專題研究計畫 ■ 成果報告
□ 期中進度報告

膀胱移形細胞癌之個案,健康對照及一等親之慢性砷中毒相關酵素基因多型性之比較

Comparison on Genetic Polymorphism of Arseniasis Related Enzymes among Bladder Transitional Cell Carcinoma Patients, Healthy Controls and First Degree Relatives

計畫類別：□ 個別型計畫 ■ 整合型計畫

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Abstract

Our study is to explore arsenic methylation capability and genetic polymorphism of arsenic methylation related enzyme cystathionine synthase (CBS), 5,10 methylene-tetrahydrofolate reductase (MTHFR) and methionine synthase (MS) of baldder/kidney urothelial carcinoma (UC) patients. A total of 1563 residents, aged 30 or older, were recruited from three arseniasis- hyperendemic villages from January to February 1993. By year 2000, cross-examination of household registration and cancer registry profile revealed 32 diagnosed UC cases and 873 controls (endemic area community based). Another, 37 UC cases from Chi-Mei Hospital and 80 community based controls (arseniasis area) as well as 113 UC cases and 28 hospital based controls from National Taiwan University Hospital (non-arseniasis area) were recruited from September, 2002 to April, 2003. Urine samples were measured for arsenic species level by HPLC and HGAAS. DNA was extracted from buffy coat to analyze *MTHFR*, *MS* and *CBS* genetic polymorphism utilizing PCR and RFLP. We found urinary arsenic species of 32 UC cases in arseniasis area (82.7±6.6 mg/L) was significantly different from 106 UC cases in non-arseniasis area (22.1±2.74 mg/L). In

multivariate models, the higher cumulative arsenic exposure was the higher UC risk. UC patients had higher MMA percentage and lower DMA percentage than healthy controls after age and gender adjustment. Hetero and variant genotype of methylation related enzyme MTHFR and MS had lower UC risk (OR=0.55 and 0.46, respectively) in arseniasis area, but it had inconsistency in non- arseniasis area. It needs further study.
Key words: Arsenic methylation capability, Urothelial carcinoma (UC), Arseniasis area

Introduction

Arsenic is a well-established human carcinogen of the skin and lung (1). Recent studies have well documented that the long-term exposure to inorganic arsenic through ingestion and inhalation is associated with an increased risk of urinary bladder and kidney cancers, especially transitional cell carcinoma (TCC) (2). In the southwestern arseniasis-endemic area in Taiwan, dose-response relationships between the exposure to arsenic in drinking water and the mortality from cancers of the urinary bladder and kidney have been consistently reported from ecological, case-control and cohort studies (3-5). Compatible findings have also been observed among vintners consuming arsenic-contaminated wine (2), patients treated with Fowler solution containing potassium arsenite (2), residents drinking high-arsenic water in Chile (6), Argentina (7) and Inner Mongolia (8), and copper smelter workers exposed to arsenic through inhalation (9). Our most recent study also reported a dose-response relationship between the arsenic level in drinking water and the TCC risk in the northwestern arseniasis-endemic area in Taiwan (10).

In vitro studies have documented that arsenic is weak to induce point mutations in a variety of mammalian cell systems. But it has various capabilities to potentiate the genotoxicity of several mutagens and carcinogens, to induce chromosomal aberration, sister chromatid exchanges, chromosome loss and micronulei, to increase gene amplification, to interfere DNA repair, to induce morphological cell transformation, and to disturb cell proliferation (2,11). However, the exact mechanism of arsenic-induced carcinogenicity remains to be elucidated. Several hypothesis including genetic and epigenetic effects of inorganic arsenic have been proposed. They include the free radicals and oxidative stress, DNA methylation, DNA repair, protein phosphorylation, cell cycle regulation, and signal transduction. It is essential to use high throughput technology to study these molecular mechanisms of arsenic to express its toxicity.

There were more than 200,000 residents lived in the arseniasis-endemic areas. Only a small fraction of them were affected with TCC. There seems to exist either

acquired or genetic susceptibility to the development of arsenic-induced TCC. Our previous studies have shown that poor capability of arsenic methylation is associated with an increased risk of arsenic-induced skin cancer (12). Null genotypes of glutathione S-transferase (GST) M_1 and T_1 and variant genotype of GST P_1 have been found to increase the risk of arsenic-induced skin cancer (13). Whether the genotypic and phenotypic polymorphisms of GSTs and other enzymes related to arsenic methylation might modify the risk of arsenic-induced TCC remain to be elucidated.

The biotransformation processes of inorganic arsenic in humans are very complicated. A substantial fraction of absorbed AsV is reduced in the blood to AsIII (14-16), that is then taken up by hepatocytes (17) and methylated to become monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA) (18). The methylation may be considered a detoxification mechanism, because the methylated metabolites, in comparison with inorganic arsenic, are less reactive with tissue constituents (19), less toxic, and more readily excreted in the urine (20-21). In general, inorganic arsenic and its metabolites in human urine contain 10%-15% inorganic arsenic, 10%-15% MMA, and 60%-80% DMA (22-23). However, recent studies suggest that methylated arsenic species, especially those in the trivalent state, may be more toxic than the present inorganic arsenic compounds (24-26). Properties that MMA (III) and DMA (III) are known to possess in various experimental systems include enzyme inhibition (24-25,) cell toxicology (26), genotoxicity, and clastogenicity (27). Complete carcinogenesis by DMA has been achieved in rat bladder (28); on the other hand, DMA might be as a promotor to develop rat bladder cancer (29). Recently, our study had shown that elevated proportion of MMA in total urinary arsenic level was associated with an increased risk of skin cancer in the arseniasis hyperendemic area in Taiwan (12). Unfortunately, this detoxification mechanism is typically incomplete and approximately 5-25% of inorganic arsenic is excreted unmethyated. This unmethylated form, which passes through the urinary tract and is briefly stored in the bladder that is thought to be responsible for the increased rates of bladder cancer seen in exposed populations. Therefore, which arsenic species is related to TCC needs further research.

The genetic and phenotypic polymorphism of various enzymes involved in the arsenic methylation and detoxification might be attributable to the discrepancy in individual susceptibility to arsenic-related TCC the same as arsenic-induced skin cancer (12). Because polymorphisms in the methyl group metabolism genes methylene-tetrahydrofolate reductase (MTHFR), methionine synthase (MS), and cystathione β-synthase (CBS) affect plasma homocysteine levels and intracellular concentrations of S-adenosylmethionine (SAM), they modify the susceptibility to colon cancer (30), prostate carcinoma (31), and acute lymphocytic leukemia (32).

Methyl group metabolism genotypes on their own could be relevant for susceptibility to those cancers in which DNA hypomethylation occurs regularly such as TCC of the urinary bladder (33-34). Recently Kimura et al reported the MTHFR, MS and CBS genotypes do not appear to act upon susceptibility to TCC or influence the extent of DNA hypomethylation in this cancer (35). It is very contradictory whether homocysteine metabolism related enzyme (CBS, MTHFR and MS) is related to the risk of TCC. It is worthy to verify whether the TCC in arseniasis endemic area be affected by the susceptibility of CBS, MTHFR and MS.

Study Purposes

1. To compare arsenic methylation capability profile between UC cases in arseniasis-endemic and non-endemic areas.
2. To explore the relationship between arsenic methylation capability and UC.
3. To elucidate the relationship between the genetic polymorphism of arsenic methylation related enzyme (cystathionine synthase, 5,10 methylene-tetrahydrofolate reductase and methionine synthase) and UC risk.
4. To examine gene-environment interaction on the risk of UC in arseniasis-endemic and non-endemic areas.

Material and Method

A total of 1563 residents aged 30 or older were recruited from three arseniasis-hyperendemic villages and collected their urine and blood sample from January to February 1993. By year 2000, cross-examination of household registration and cancer registry profile revealed 32 diagnosed UC cases and 873 controls (community based in endemic area). 37 UC patients from Chi-Mei Hospital and 80 community based controls (arsenasis area) as well as 113 UC patients and 28 hospital based controls from National Taiwan University Hospital (non-arsenasis area) were recruited from September, 2002 to April, 2003 and collected their plasma, buffy coat and urine. Well-trained research assistants will use structural risk factor questionnaire to collect information of water consumption, residence, life style, and personal disease history. The questionnaires will be revised, edited, coded and double-keyed in three local centers at Chi-Mei, Chiayi and health bureau of I-Lan Country. The raw data file will be delivered to central at Taipei.

Urine Arsenic Species Analysis: Urine samples will be thawed at room temperature, dispersed by ultrasonic wave, and filtered through Sep-Pak C18 column. Arsenic species in 200 mL urine will be separated by HPLC (Hitachi 7110, Naka, Japan) with column (Machey-Nagel, Nucleosil, 10m, 250´4.6 mm), and on line linked to HGAAS to quantify the levels of various species of inorganic arsenic and its

metabolites. Recover rates and coeffiencicy of variance cover to evaluated reliability. In addition, we will use Freeze-dried urine SRM 2670 obtained from the National Institute of Standards and Technology (NIST, Gaithersburg, MD, USA) and analyze together with test urine samples to control for the quality of the method. Chromatograms figure of speciation analysis is shown in Fig. 1Methylenetetrahydrofolate Reductase (MTHFR) Genotype: Twoprimers were designed from the cDNA sequence to generate a 198-bp fragment. The primer sequences are: 5'-TGAAGGAGAAGGTGTCTGCGGGA-3' and 5'-AGGACGGTGCGGTCAGAGTG-3'. Amplication was performed using initial denaturation at 95℃ for 2 min followed by 29 cycles of 94℃ for 30s, 60℃ for 30s, and 72℃ for 30s with a final extension at 72℃ for 10 min. Laboratory personnel were blinded to case-control status. To analyze for the presence of the HinfI site created by 677C ®Ttransition, 5ml of PCR product were digested with 2.5 U HinfI in the appropriate buffer for 2 hr at 37℃. Digested products were separated by electrophoresis on 6% polyacylamide gel.Methionine synthase (MS) genotyping assay: Twenty-five pmoles each of primers 5'-GAACTAGAAGACAGAAATTCTCTA-3' and 5'-CATGGAAGAATATCAAGATATT AGA-3' were used in a PCR reaction mix containing 10 mM Tris (pH 9.0), 50 mM KCl, 0.1% Txiton X-100, 4 mM MgCl2, 0.2 mM of each dNTP, and 1 U DNA polymerase. Amplification was performed using initial denaturation at 95℃ for 2 min, followed by 35 cycles of 94℃ for 30 sec, 53℃ for 30 sec, and 72℃ for 30 sec, with a final extension at 72℃ for 10 min. The PCR product was digested with HaeIII, followed by separation on 6% polyacrylamide gels and silver staining.

β-cystathione synthetase (CBS) genotyping assay: A 184-bp DNA fragment-containing exon 8 of the CBS gene was selectively amplified by PCR. 5'-CTGGCCTTGAGCCCTGAA-3', derived from intron 7; and antisense, 5'-GGCCGGGCTCTGGACTC-3', derived from intron 8), in a volume of 100ml. Afterdenaturation at 95℃ for 3 min, temperature was cycled 30 times (at 95℃ for 1 min, at 60℃ for 1 min, and at 72℃ for 2 min), followed by extension at 72℃ for 3 min to amplify the target DNA. The PCR products were electrophoresed on a 2% low-melting-point agarose gel containing ethidium bromide and were visualized on an UV transilluminator.

Result and Discussion

UC patients whose age ranged from 30-80 years old were recruited as study subjects if they were diagnosed as cancers of the bladder, urinary tract and pelvis on the basis of pathological examination. Our previous study, a total of 1563 residents, aged 30 or older, were recruited from three arseniasis- hyperendemic villages and

collected their urine and blood sample from January to February 1993. By year 2000, cross-examination of household registration and cancer registry profile revealed 32 diagnosed UC cases (community based). From 1998, March to 2002, July we recruited 426 UC patients and 222 healthy controls (hospital based) and collected blood samples from Chi-Mei hospital (endemic area) (Table 1), we will continue to collect urine when they return to the hospital for the routine physical examination during this year. First year of this component project, from 2002, September we cooperated with Dr. Pu (component project 3) and established a process to recruit new UC outpatients (hospital based) from National Taiwan University Hospital (NTUH) (non-endemic area), and collect their plasma, buffy coat, and urine (Table 2). In addition, we also cooperated with Dr. Wen-Hsiang Chiou to recruit Chi-Mei hospital (endemic area) study subjects and specimen as well as NTUH. We purchased a set of instruments included high performance liquid chromatography (HPLC), hydride generator (HG) and atomic absorption spectrometry (AAS), and set up urinary arsenic species analytical method. These instruments are paid in three installments over three years. Urinary arsenic species were examined by HPLC to specify AsIII, AsV, MMA, and DMA and then quantified by HG-AAS, chromatograms figure of speciation analysis is shown in Fig. 1. At present, we analyzed urinary arsenic species of 63 UC outpatients from NTU hospital and found their total arsenic (32.5±3.7μg/L) quite different from that of 32 UC patients from arseniasis-hyperendemic area (89.4±10.4 μg/L) (Fig.2). Arsenic methylation capability indices are measured as the inorganic arsenic percentage (arsenite and arsenate /total arsenic), monomethylarsonic acid (MMA) percentage (MMA/total arsenic), and dimethylarsinic acid (DMA) percentage (DMA/total arsenic). Chronic arsenic indices are measured as the duration of living in blackfoot disease endemic area, duration of drinking artesian well water, and cumulative arsenic exposure. UC risk of subjects from arseniasis-hyperendemic area was significantly associated with increasing cumulative arsenic exposure. After adjusting for age and gender, elevated proportion of urinary MMA percentage, and low level of DMA percentage were related to higher UC risk, but not statistically significant (Table 3). It suggested that UC patients might have poor arsenic methylation capability; more sample sizes are needed to identify arsenic methylation capability of UC patients between endemic area and non-endemic area (hospital based). Base on first year experience, we can collect the expected number of UC patients during second year of this component project, and analyze urinary arsenic species and compare arsenic methylation capability profile among UC patients from arseniasis-endemic and non-endemic areas and healthy controls. Recently, MMA^{III} and DMA^{III} are synthesized by component project 2, so we can use them as standard to set up urinary MMA^{III} and DMA^{III} analytical method and reanalyze arsenic species

(As^{III}, As^{V}, MMA^{III}, MMA^{V}, DMA^{III} and DMA^{V}) of all urine samples and explore their association with UC risk.

Genomic DNA was extracted from peripheral lymphocyte of 94 UC patients from NTU hospital and that of 37 UC patients from Chi-Mei hospital by proteinase K digestion and phenol/chloroform extraction. The extracted DNA was analyzed remethylation related enzymes included cystathionine synthase (CBS), 5,10 methylene-tetrahydrofolate reductase (MTHFR) and methionine synthase (MS) gene polymorphism.The distribution of genotype for MTHFR (C677T), MS (A2756G) and CBS from endemic area and non-endemic area UC patients were shown in Table 4. Hetero and variant genotype of methylation related enzyme MTHFR and MS had lower UC risk (OR=0.55 and 0.46, respectively) in arseniasis area, but it had inconsistency in non- arseniasis area . It needs further study.

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Tables and Figures

Table 1. Recruitment status of urothelial carcinoma patients from Chi-Mei hospital from 1994, March to 2002, July.

		Recruitment	Tumor tissue DNA	Questionnaire	Urine	Blood DNA
Chi-Mei hospital	Case	426	121	350	0	336
	Control	222	0	298	0	312
	Total	648	121	648	0	648

Table 2. Recruitment status of urothelial carcinoma from Chi-Mei and NTU hospitals from 2002, Sep.

	Case recruitment	Questionnaire	Urine	Blood
Chi-Mei hospital	37	37	16	37
NTU hospital	113	113	106	94

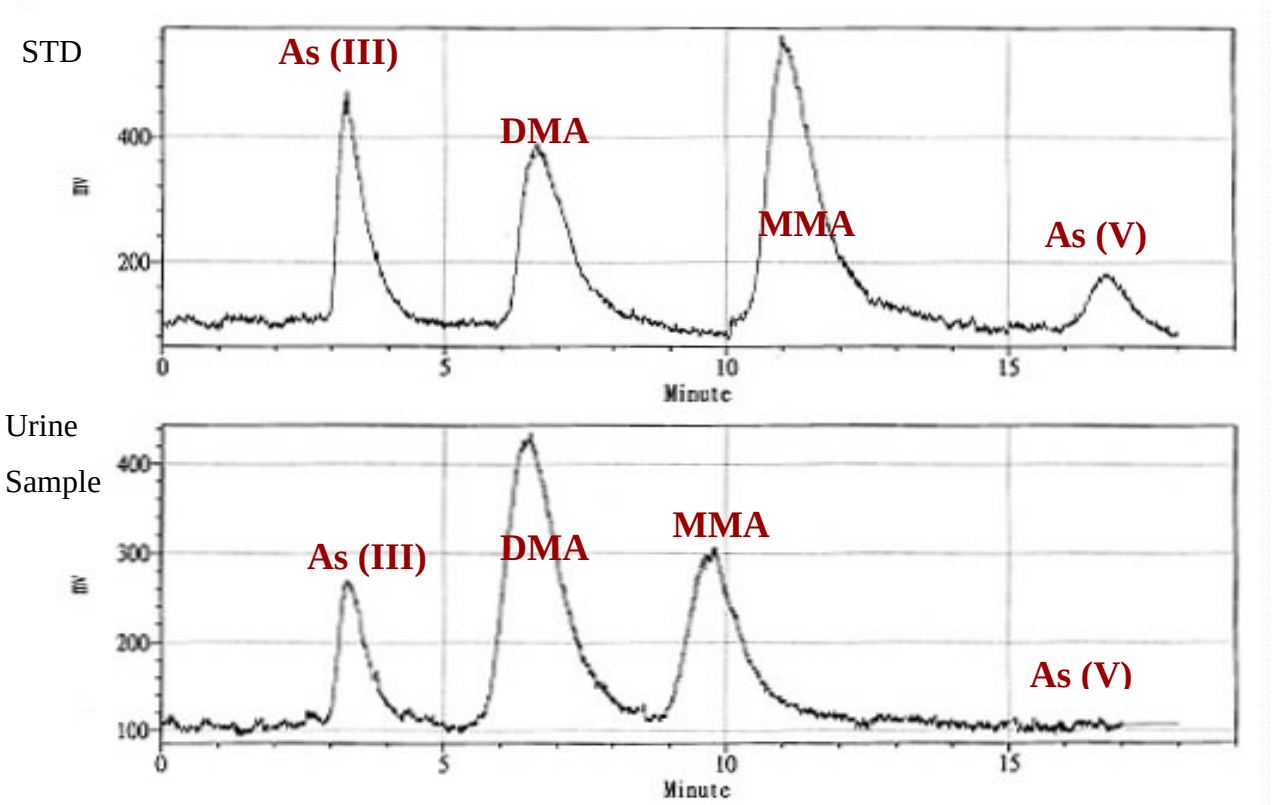

Fig. 1. Chromatograms Showing Speciation Analysis of Arsenite, DMA, MMA, and Arsenate in Standard Solution (STD) and Urine Sample (Analytic Method: HPLC-HG-AAS).

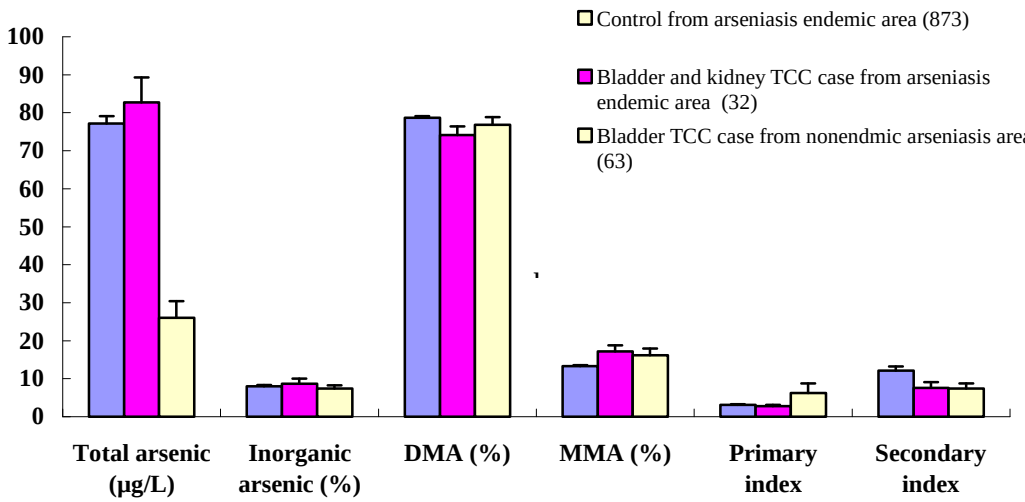

Fig. 2. Comparison of Total Arsenic and Percentage of Arsenic Species among Healthy Controls and Bladder and Kidney Transitional Cell Carcinoma (Urothelial Carcinoma; UC) patients from Arseniasis Endemic Area and Non-endemic Area.

Table 3. Multivariate analysis of risk factors from arseniasis-hyperendemic area 32 UC patients (community based).

	Case	Control	Model 1 OR (95% CI)	Model 2 OR (95% CI)
Cumulative arsenic exposure (mg/L×year)				
<13.4	2	332	1.0	1.0
≥13.4	22	310	7.1 (1.5-33.1)*	7.2 (1.5-33.6)*
MMA percentage				
<11.35	8	310	1.0	
≥11.35	16	332	1.5 (0.6-3.6)	
DMA percentage				
<81.05	17	318		1.0
≥81.05	7	324		0.5 (0.2-1.2)

8 UC patients, and 231 healthy controls had no cumulative arsenic exposure data.
Model 1 and 2 were adjusted for age and gender.

Table 4. Distribution of *MTHFR*, *MS* and *CBS* genetic polymorphisms among UC patients from arseniasis area and non- arseniasis area.

Variable	Arseniasis Area Subjects from Chi-Mei Hospital			Non- Arseniasis Area Subjects from NTU Hospital		
	Cases	Controls	OR (95 % CI)	Cases	Controls	OR (95 % CI)
MTHFR						
WW	22	42	1.00	44	14	1.00
WM	9	31	0.55 (0.24-1.29)	39	11	1.17 (0.49-2.80)
MM	2	7		5	1	
MS						
WW	31	66	1.00	71	21	1.00
WM	3	14	0.46 (0.12-1.70)	19	5	0.94 (0.33-2.64)
MM	0	0		0	1	
CBS						
DD	32	64		90	23	
DI or II	0	1		0	1	

Result Evaluation

The second year of this component project, we will continue to recruit UC patients and healthy controls from arseniasis-endemic and non-endemic areas, respectively to analyze gene polymorphism of remethylation related enzymes (CBS, MTHFR, and MS), oxidative enzyme NADPH oxidase and ROS related enzymes ($GSTM_1$, T_1, P_i, superoxide dismutase, nitric oxide synthase, glutathion peroxidase, glutathione reductase, heme oxygenase and catalase), and we will prepare for SNP

site of AR, HO-1, OATR2, MRP1, and MRP2 experiment next year.