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Association between metabolic gene polymorphisms and susceptibility to peripheral nerve damage in workers exposed to *n*-hexane: A preliminary study

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Abstract

Chronic exposure to *n*-hexane may result in peripheral neuropathy. 2,5-Hexanedione (2,5-HD) has been identified as a toxic metabolite of *n*-hexane. The *CYP2E1*, *CYP1A1* and *GST* genes are involved in the formation of 2,5-hexanedione from *n*-hexane as well as the elimination of 2,5-HD-formed electrophile, and these genes are highly polymorphic in the general population. A nested case-control study in an industrial cohort was conducted to evaluate the associations between polymorphisms in these metabolic genes and *n*-hexane-induced peripheral nerve damage. The study subjects included 22 cases, who worked in a printing factory with symptoms of peripheral nerve damage, and 163 controls, who came from the same factory of cases. DNA was extracted from blood samples and genotyping was conducted for *CYP2E1 Pst*, *CYP2E1 Dra*, *CYP2E1 Ins96*, *CYP1A1 Msp*, *GSTT1 null*, *GSTM1 null* and *GSTP1 105V*. Unconditional logistic regression was applied to estimate the odds ratio and 95% confidence intervals. There were no significant differences between the two groups regarding age, sex, smoking and alcohol status. A significant association between Dra polymorphism and peripheral nerve damage was found. The frequency of *CYP2E1 Dra* homozygous mutation in the case group (18.2%) was higher than that in the control group (3.7%, $p = 0.015$). Individuals with homozygote genotype (CC) of *CYP2E1 Dra* had a significantly higher risk of peripheral nerve damage compared with those with DD genotype (adjusted OR = 5.58, 95% CI = 1.32–23.65) after *n*-hexane exposure duration, sex, age, smoking and alcohol status were adjusted. No significant association was found that *CYP2E1* Pst, *CYP2E1* Ins96, *CYP1A1* Msp, *GSTT1*, *GSTM1*, *GSTP* gene polymorphisms associated with the susceptibility of peripheral nerve damage. These findings suggested that *CYP2E1* gene might increase the susceptibility to *n*-hexane-induced peripheral damage.

Keywords: n-*Hexane, peripheral nerve damage,* CYP2E1, CYP1A1, GSTT1, GSTM1, GSTP1, *gene polymorphism*

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Introduction

n-Hexane is a solvent, thinner and cleaning agent currently used in a variety of industrial and commercial processes, including rubber, adhesive, ink and paint manufacturing (He et al. 1999, 2000). Chronic exposure to *n*-hexane may result in various signs and symptoms of peripheral neuropathy such as acral paresthesia, weakness in the lower limbs and compromised tendon reflexes. These motor and sensory deficits are symmetrical and usually limited to the feet and hands. Electrophysiological findings include prolonged nerve conduction velocities (NCV) in the median and ulnar nerves and decreased muscle-resting potential amplitudes (Wang et al. 1986, Chang et al. 1993, Oge et al. 1994, Pastore et al. 2002).

Numerous studies have provided evidence that 2,5-hexanedione (2,5-HD) is the ultimate neurotoxic agent that causes peripheral neuropathy (Couri et al. 1978, Spencer et al. 1978). Cytochrome (CYP) P450 enzymes are involved in several steps of *n*-hexane biotransformation. 2,5-HD is formed via sequential hydroxylation at the 2- and 5-positions by P4502E1 and 2B (Toftgard et al. 1986, Crosbie et al. 1997, Jenner 1998), followed by oxidation of the hydroxyl function by alcohol dehydrogenase (Frommer et al. 1974). Iba et al. (2000) found that continued daily *n*-hexane treatment resulted in an increased urinary level of 2,5-HD in wild-type mice but not in CYP2e1 $-/-$ mice. Pretreatment of rats with the inducer of CYP2E1 increased 2,5-HD formation from rats administered *n*-hexane (Robertson et al. 1989, Raunio et al. 1990), suggesting that CYP2E1 is the predominant P450 isozymes responsible for the biotransformation of *n*-hexane to 2,5-HD.

n-Hexane is also hydroxylated at other positions of carbon atoms by P450 isozymes (such as *CYP1A1* and *CYP1A2*), and is metabolized to 1-hexanol, 3-hexanol, 4,5-dihydroxy-2-hexanone and other compounds. However, this leads to detoxification (Morohashi et al. 1983, Toftgard et al. 1986).

2,5-HD could disturb energy metabolism processes and then alter neurofilament transport and axonal degeneration (Spencer et al. 1979). In addition, decreased adenosine triphosphate production and inhibition of state 3 respiration also occurred in rat brain mitochondria exposed to 2,5-HD (Medrano & LoPachin 1989). Studies also showed that 2,5-HD could react directly with axonal protein by the formation of *n*-substituted 2,5-dimethylpyrrole adducts at ε-amine nitrogen of the lysine residues of neurofilaments (Pyle et al. 1992, Graham et al. 1995, Decaprio et al. 1997). Secondary oxidation of the pyrrole ring to an electrophile reacted with neurofilament nucleophiles resulting in intra- and intermolecular protein cross-linking, and this was considered to be the determinant event in 2,5-HD neuropathy (Graham et al. 1982, Zhu et al. 1994). The electrophiles formed by 2,5-HD can be eliminated through the glutathione conjugation reaction. This reaction can be catalysed by GSTs-produced enzymes including mu (M), pi (P), theta (T) and then by preventing the electrophile from conjugating with DNA protein (Mannervik et al. 1992).

Recently, there has been an increased number of reported cases of *n*-hexane-induced peripheral neuropathy in China (He et al. 2002a,b) since the first case was reported in 1994 in Guangdong province (He et al. 1999). *n*-Hexane neuropathy has become a relatively new occupational disease in China. Although epidemiological studies have shown that occupational exposure is a major risk factor for *n*-hexane neuropathy (Wang et al. 1986), only a small fraction of workers exposed to *n*-hexane develop neuropathy. There is a possibility that genetic factors such as the variants of

metabolic genes for *n*-hexane are involved in the procession of *n*-hexane neuropathy (Chang et al. 1993, Shao et al. 1999).

The present authors hypothesized that workers with variant alleles for *CYP2E1*, *CYP1A1* and *GST* genes may be different in their ability to metabolize *n*-hexane and, thus, may alter the risk of peripheral nerve damage. A nested case-control study was conducted to test whether *CYP2E1*, *CYP1A1* and *GST* genes' polymorphisms could modulate the susceptibility of peripheral nerve damage induced by *n*-hexane.

Material and methods

Study population

The case-control study was conducted based on a study cohort in an offset printing factory in Shenzhen, China. There were 266 workers (whose job title was 'printers') recruited in the follow up study. Twenty-two cases were found during a 1-year occupation health surveillance. The cases had at least one of the following symptoms: (1) diminution of the tendon reflex in the limbs, (2) a decreased tactile sensibility in the lower extremities, (3) a reduced vibratory sensibility below the knee or ankle, and (4) decreased muscle power. All cases were validated by an occupational physician according to the *Diagnostic Criteria of Occupational Acute Neurotoxic Disorders Caused by Chemicals* (GBZ76 2002a). They were then re-diagnosed by specialists on a panel of a diagnostic group responsible for the project of nerve growth factor (NGF) treatment on *n*-hexane-induced neuropathy patients.

One hundred and sixty-three controls were recruited from the other workers working in the same workshops as cases. The controls had to have the following characters during the health surveillance: (1) no relative signs and symptoms of cases found during occupational health surveillance, (2) working in the same kind of job position as the cases, (3) having the same *n*-hexane exposure duration as the cases, and (4) no relative disease with peripheral nerve damage such as diabetes and neurasthenic.

The demographic data for all subjects including gender, age, nationality, smoking habit, alcohol intake, occupational exposure duration and the recent disease history were collected by a questionnaire completed by trained interviewers.

All subjects wore general protective masks and gloves for the prevention of possible inhalation and skin penetration during working time. Data from environmental measurements of *n*-hexane were available from the historical records of the workshops over 2000–03. Based on these results, the median *n*-hexane concentrations in the air of the work sites were 49, 126, 48 and 57.5 mg m^{-3} in 2000, 2001, 2002 and 2003, respectively.

The study was approved by the Research Ethic Committee of the National Institute for Occupational Health and Poison Control, Chinese Center for Disease Control and Prevention.

Genotyping

After an informed consent was obtained, venous blood was collected from each subject and then coded. Ethylenediaminetetraacetic acid disodium salt (EDTA-2Na) was used as an anticoagulant. DNA was isolated from whole blood using the standard method (Miller et al. 1988). Two single nucleotide polymorphisms (SNPs) of

CYP2E1, including T7668A Dra site in intron 6, G1259C Pst site in the promoter region were detected by using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) (Kato et al. 1992, Stephens et al. 1994). The Ins96 in the 5′-flanking region of *CYP2E1* gene was measured by using the PCR method (Cao et al. 2001). The Msp site of the 3′-flanking region of *CYP1A1* was genotyped using the methods of Tefre et al. (1991), and analysis of *GSTP1* gene polymorphism resulting in an Ile/Val substitution at the residual 105 at exon 5 was done as described by Saarikoski et al. (1998). The genotypes of *GSTM1* and *GSTT1* were detected by using a modified multiplex PCR method with globins as the positive control (Zhong et al. 1993, Katoh et al. 1996). All genotypes were evaluated by at least two persons independently. Ten per cent of DNA samples randomly chosen from all the samples were genotyped a second time and the concordance rate was 100%.

Statistical methods

Individuals who had smoked more than 100 cigarettes in their lifetime were considered as smokers. The subjects who drank at least twice every week during the past 6 months were defined as alcohol users. The effect of each gene variant on the peripheral nerve system dysfunction, using the wild genotype as the reference, was estimated by using unconditional logistic regression to calculate crude and adjusted odds ratios (ORs) and 95% confidence intervals (CIs). Potential confounders, such as age, sex, exposure history, the status of smoking and alcohol, were adjusted in unconditional logistic regression models. All statistical tests were two-sided ($\alpha = 0.05$) and performed using Statistical Analysis System software (version 6.12; SAS Institute, Inc., Cary, NC, USA).

Results

The general characteristics of the study population are summarized in Table I. All cases were of Han nationality with an average age of 25.23 years and average *n*-hexane exposure duration of 6.54 years. Fifty per cent of cases were current smokers and 48% were alcohol users. The average age of controls was 27.24 years and the average length of occupational exposure was 5.97 years. Of them, 42.9% were smokers and 39.3% were alcohol users. Confounding factors such as age, gender, exposure duration, smoking and alcohol statue were also comparable, and there were no significant difference between cases and controls. Although *n*-hexane exposure duration in the

Table I. Demographic data and *n*-hexane exposure history in cases and controls.

Variables	Cases ($n = 22$)	Controls ($n = 163$)	p
Age (years, mean ± SD)	25.23 ± 5.24	27.24 ± 5.86	0.128[1]
Gender (male/female)	20/2	146/17	0.846[2]
Exposure history (years, mean ± SD)	6.54 ± 4.81	5.97 ± 3.89	0.719[3]
Current smoker (yes/no,%)	11/11 (50.00)	70/93 (42.94)	0.563[2]
Alcohol user (yes/no,%)	10/12 (48.00)	64/99 (39.26)	0.609[2]

[1]Student's *t*-test for differences between cases and controls.
[2]χ^2-test for differences between cases and controls.
[3]Wilcoxon *U*-tests for differences between cases and controls.

case group tended to be longer than that of the control group, the difference was also not statistically significant.

The frequencies for variant alleles of Dra, Pst and Ins96 polymorphisms of *CYP2E1* were 0.249, 0.273 and 0.216, respectively, and those of Msp polymorphisms of *CYP1A1* and Ile105 alleles of *GSTP1* gene were 0.551 and 0.205 in the studied subjects. The distributions of all five genotypes distributions were in a Hardy–Weinberg equilibrium, indicating that study subjects were sufficiently random and representative. The ratios of the non-null of *GSTT1* gene and *GSTM1* gene were 0.454 and 0.476, respectively.

The frequencies of *CYP2E1*, *CYP1A1*, *GSTP1*, *GSTM1* and *GSTT1* genotype distributions in cases and controls are shown in Table II. There was a statistic difference among the Dra polymorphism. The frequency of the homozygous genotype mutation C/C in the case group was 18.2%, which was significantly higher than that in the control group (3.7%). Individuals with homozygous genotype (C/C) had a significantly higher risk of *n*-hexane-induced peripheral nerve damage in comparison

Table II. Frequencies of *CYP2E1*, *CYP1A1*, *GSTP1*, *GSTM1* and *GSTT1* genotype distributions in cases and controls.

Candidate gene	Genotype	Number of cases	Number of controls	Crude OR (95% CI)	Adjusted OR (95% CI)
*CYP*2E1Dra					
	DD	12	91	1.00 (reference)	1.00 (reference)
	CD	6	66	0.69 (0.25–1.93)	0.69 (0.24–1.94)
	CC	4	6	5.06 (1.25–20.5)*	5.58 (1.32–23.65)*
*CYP*2E1 Pst					
	c1c1	15	109	1.00 (reference)	1.00 (reference)
	c1c2	6	52	0.84 (0.31–2.29)	0.83 (0.29–2.34)
	c2c2	1	2	3.63 (0.31–42.54)	3.39 (0.25–45.12)
*CYP*2E1Ins96					
	−/−	13	99	1.00 (reference)	1.00 (reference)
	−/+ or +/+	9	64	1.12 (0.44–2.87)	1.14 (0.45–2.94)
*CYP1A1*Msp					
	m1m1	6	49	1.00 (reference)	1.00 (reference)
	m1m2	13	81	1.31 (0.47–3.67)	1.49 (0.51–4.33)
	m2m2	3	33	0.74 (0.17–3.18)	0.93 (0.21–4.24)
GSTP1					
	Ile/Ile	15	102	1.00 (reference)	1.00 (reference)
	Ile/Val	6	54	0.74 (0.25–2.11)	0.76 (0.28–2.06)
	Val/Val	1	7	1.14 (0.12–10.65)	0.97 (0.11–8.46)
GSTT1					
	null	10	74	1.00 (reference)	1.00 (reference)
	non-null	12	89	0.99 (0.41–2.44)	1.22 (0.48–3.11)
GSTM1					
	null	12	76	1.00 (reference)	1.00 (reference)
	non-null	10	87	0.73(0.30–1.78)	0.77 (0.30–1.96)

Odds ratios are adjusted for age gender, exposure history, smoking and alcohol status.

with those with wild-type genotype (D/D) (adjusted OR = 5.58, 95% CI = 1.32–23.65) after adjusting for exposure history, gender, age, smoking and drinking status.

Other sites of *CYP2E1* were Pst and Ins96bp polymorphism in the regulatory region. The ratios of c2c2 (*Pst*I homozygous mutation) and Ins96 (+/+ or +/−) genotype were 1/22, 9/22 in cases and 2/163, 64/163 in controls, respectively, which were consistent with the result of De Roos et al. (2003). Nevertheless, no association was found between Pst I and Ins96 polymorphisms of the *CYP2E1* gene and susceptibility to peripheral nerve damage. The frequency of *CYP1A1* homozygous mutation was not different between cases and controls (13.6 versus 20.26%).

The distributions of *GSTP1 GSTT1* and *GSTM1* genotypes in case group were similar to the control group. The data also showed no associations of *GSTs* polymorphism with peripheral nerve damage.

Discussion

Both the host and external environment factors affect many toxicant-induced health impairments and diseases. The differences in individual expressions of toxicity have become a hot point in toxicology. The possible impact of metabolic enzyme gene polymorphisms on individual susceptibility to *n*-hexane-induced peripheral nerve damage needs to be investigated. Up to now the authors are aware of no previous study that has investigated the possible impact of *n*-hexane metabolic enzyme gene polymorphisms on individual susceptibility to peripheral nerve damage. The results showed that workers with homozygote mutation (C/C) of *CYP2E1* Dra had a greater risk of peripheral nerve damage compared with the D/D genotype, suggesting that the CYP2E1 Dra polymorphism may be a susceptibility gene to *n*-hexane-induced peripheral nerve damage.

The *CYP2E1* gene lies on chromosome 10 (Umeno et al. 1988) and has been identified as the main CYP450 isoform responsible for the metabolism of *n*-hexane (Robertson et al. 1989, Iba et al. 2000). Since chronic exposure to alcohol is an inducer well known to enhance CYP2E1 activity (Koop & Tierney 1990, Daiker et al. 2000), the ratio of alcohol users between cases and controls was compared and there was no difference in this study.

Polymorphisms of the *CYP2E1* gene have been identified in humans, of which the Dra polymorphism particularly is located in intron 6 (C/D). The relationship of the Dra polymorphism and gene expression has been postulated. Uematsu et al. (1994) suggested that the Dra polymorphism might be associated with the gene expression of CYP2E1 at the mRNA level. Studies have showed that the frequency of the C allele is different in different races: 0.11 in Spanish and Finnish people (Savolainen et al. 1997, Vidal et al. 2004) and 0.26 in the Japanese population (Uematsu et al. 1991). In the current study, the frequency of the C allele was 0.25, which is similar to a previous report of Chinese people (Zhai et al. 1998). The present study found that individuals with homozygote genotype (CC) of *CYP2E1 Dra* had a significantly higher risk of peripheral nerve damage in comparison with those with DD genotype (OR = 5.06, 95% CI = 1.25–20.5). This might be due to an increase of 2,5-HD formation in workers with CC genotype than in ones with DD genotype because of elevated *CYP2E1* activities. Similar results were obtained after *n*-hexane exposure duration, gender, age, smoking and alcohol status were adjusted (OR = 5.58, 95% CI = 1.32–23.65). Another two polymorphism sites were *Pst*, Ins 96 of *CYP2E1* located in the

regulatory 5′-flanking region (c1/c2, Ins 96 +/−) that may affect its transcriptional regulation (Hayashi et al. 1991) and increased activity (McCarver et al. 1998); the present data did not show the distribution difference between cases and controls.

CYP1A1 is also an important phase I enzyme in metabolizing *n*-hexane to 2,5-HD, which can be hydroxylated at the 1- or 3-position carbon atoms of *n*-hexane and form detoxification compounds. Two major relevant genetic polymorphisms have been demonstrated in the *CYP1A1* gene. One is a T→C substitution in the 3′-flanking region altering protein folding. In this study, the frequency of *CYP1A1 Msp I* was consistent with those described in the literature for the Chinese population (Wang et al. 2003) and no significant association was found between *CYP1A1 Msp I* polymorphism and peripheral nerve damage.

Glutathione S-transferases (GSTs) are phase II enzymes responsible for catalysing the biotransformation of a variety of electrophiles. They have a central role in the detoxification of activated metabolites. In the present study, *GSTs* gene variants played no significant role in the risk of peripheral nerve system dysfunction.

In the present study, the workers with peripheral nerve damage who were defined as observation subjects according to the *Diagnostic Criteria of Occupational Chronic* n-*Hexane Poisoning* (GBZ84 2002b) were selected as our cases, whose symptoms were slighter than in *n*-hexane-induced neuropathy patients. There are some disadvantages in the study of hospitalized patients since these patients usually present due to an exposure to a high level of *n*-hexane (223.7–17107.5 mg m^{-3}) in a relatively short period. However, in the present study, the levels of *n*-hexane in workshop air were mostly below 100 mg m^{-3}, therefore it is convenient to explore the genetic effect on impairment induced by *n*-hexane at a relatively low exposure level. The disadvantage of our study, however, is that a small number of subjects with *CYP2E1 Dra* variant genotypes would make estimates imprecise. A small sample size may explain the lack of associations of *Pst*, 96 Ins of *CYP2E1*, *CYP1A1*, *GSTT1*, *GSTM1* and *GSTP1* with peripheral nerve damage in this study. The misclassification may have occurred only in controls with slight damage, although the cases were defined by an occupational physician and then re-diagnosed and validated by a diagnostic panel.

In conclusion, it was found that Dra of *CYP2E1* polymorphism may be a susceptibility gene to *n*-hexane-induced peripheral nerve system dysfunction. A larger study is needed to validate these findings.

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