BIOMARKERS, 2002, VOL. 7, NO. 4, 337–346

Polymorphism of metabolic genes and susceptibility to occupational chronic manganism

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Received 12 November 2001, revised form accepted 12 April 2002

In this study we investigated genetic polymorphisms of five metabolizing genes and their association with occupational chronic manganism. We recruited 49 patients with chronic manganism and 50 unrelated healthy control subjects who were welders and ferromanganese smelters and occupationally exposed to manganese dust and fume in the same workshops from three metallurgical industries. The controls were matched to the cases by sex, age, cigarette and alcohol intake, as well as the manganese exposure duration. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was used to genotype the cytochrome P450 2D6L gene (*CYP2D6L*) and the NAD(P)H:quinone oxidoreductase gene (*NQO1*). Allele-specific PCR was used to detect the cytochrome P450 1A1 gene (*CYP1A1*), and the glutathione-S-transferase *mu* and *theta* genes (*GSTM* and *GSTT*). The frequency of polymorphic alleles, a mutation of *CYP2D6L*, was significantly lower in patients with chronic manganism (16.3%) than in controls (29.0%). Individuals with the homozygote polymorphism (L/L) of *CYP2D6* had a 90% decreased risk of chronic manganism compared with the wild-type (Wt/Wt) (odds ratio = 0.10, 95% confidence interval = 0.01–0.82). A significant association between the *CYP2D6* genotype subgroup and the latency of chronic manganese poisoning was also found. Patients who had homozygous (L/L) or heterozygous (Wt/L) mutant alleles developed manganism an average of 10 years later than those who were homozygous wild-type (Wt/Wt). However, the allele and genotype frequencies of *CYP1A1* and *NQO1* genes were distributed similarly in cases and controls. In addition, no difference in the frequencies of *GSTM1* and *GSTT1* null genotypes were observed between cases and controls. The results suggest that *CYP2D6L* gene polymorphism might influence susceptibility to manganese-induced neurotoxicity. However, because of limited sample size, our results should be validated in large-scale studies.

Keywords: manganism, manganese, genetic susceptibility, *CYP2D6L*, *CYP1A1*, *NQO1*, *GSTT1*, *GSTM1*.

Abbreviations: CI, confidence interval; *CYP1A1*, cytochrome P450 1A1 gene; *CYP2D6L*, cytochrome P450 2D6L gene; GSH, reduced glutathione; *GSTT1*, glutathione-S-transferase *theta* 1 gene; *GSTM1*, glutathione-S-transferase *mu* 1 gene; *NQO1*, NAD(P)H:quinone oxidoreductase gene; OR, odds ratio; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; ROS, reactive oxygen species.

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Biomarkers ISSN 1354–750X print/ISSN 1366–5804 online © 2002 Taylor &
http://www.tandf.co.uk/journals
DOI: 10.1080/13547500210146740

Introduction

Manganese (Mn) is a neurotoxicant whose critical target organ is the central nervous system. The globus pallidus is particularly vulnerable to manganese neurotoxicity (Newland *et al.* 1989, Chu *et al.* 1995). Chronic occupational manganism often occurs in workers exposed to manganese in mines, refineries and ferromanganese smelteries, as well as foundries and welders. Chronic manganism causes an extrapyramidal syndrome with features resembling those found in Parkinson's disease and postencephalitic parkinsonism. Patients with motor disturbances caused by manganese can live for many years after exposure has ceased (Chu *et al.* 1995). Several populations may be susceptible to the toxic effects of manganese, including children, elderly people and pregnant and nursing mothers (Frumkin and Solomon 1997). Occupational epidemiological studies have shown that manganese exposure is the major risk factor for manganism. The fact that only a fraction of workers exposed to manganese develop nervous system dysfunction suggests the presence of a genetic predisposition in the some individuals. The susceptibility factors for manganism may include genetically determined variations in metabolic enzymes such as the cytochrome P450s, glutathione-S-transferases and NAD(P)H:quinone oxidoreductase.

Manganese is a transition metal that can exist in multiple valences. The ability of manganese to enhance the formation of reactive oxygen species has been suggested as the underlying mechanism for manganese neurotoxicity (Graham 1984). Other mechanisms, such as a decrease in the tissue levels of protective thiols (Donaldson 1987), diminution of brain reduced glutathione (GSH) peroxidase and catalase content, and direct toxicity of high valency species of manganese (e.g. Mn^{3+}) on neuronal membranes (Archibald and Tyree 1987) have also been suggested. Manganese also increases cytochrome P450 enzymes, with superoxide radical formation (Liccione and Maines 1989), depletes cellular thiols and inhibits cellular antioxidant defences (Chu *et al.* 1995). Hence it is plausible to suggest that host factors involved in cellular oxidant and antioxidant defences may be a contributing factor to the aetiology of manganese neurotoxicity. Polymorphisms of cytochrome P450 genes, glutathione-S-transferase genes and the NAD(P)H:-quinone oxidoreductase gene could be candidate genetic factors influencing the susceptibility to manganese neurotoxicity in exposed workers.

Because inherited differences in metabolic enzymes play a critical role in the detoxification of manganese and in the process of manganese-induced neurotoxicity, we hypothesized that the polymorphism of metabolizing enzyme genes may modulate individual susceptibility to manganism. To test this hypothesis, we conducted a population-based case-control study on 49 patients with chronic occupational manganism and 50 'healthy' controls who worked in the same manganese-exposed workshops.

Materials and methods

Study subjects

Forty-nine patients with manganism and 50 healthy controls were recruited into this population-based case-control study. All subjects were unrelated Han Chinese. Both cases and controls were welders and ferromanganese smelters from three metallurgical factories in the Xinyu, Anshan and Benxi provinces, and had all been occupationally exposed to manganese dust and fumes for more than 6 years. All of the cases were found by annual routine medical surveillance over the previous 10 years, and were subsequently diagnosed as occupational chronic manganism by occupational disease panels, based on

the national diagnostic criteria issued by the Ministry of Public Health, China (1982). These patients developed neurological manifestations such as cogwheel-like muscle rigidity, dystonia, tremor and muscular weakness in limbs, as well as whispering speech, mask-like face and gait disturbance. Other causal factors, including drugs and neurological diseases, were excluded. The control subjects worked in the same workshops, but had no evidence of chronic manganism on annual medical surveillance. To confirm the diagnosis, both cases and controls were re-examined by occupational physicians.

According to the data from regular air monitoring of manganese dust, the workers' manganese exposure levels ranged between 0.06 mg m^{-3} and 7.8 mg m^{-3} (median = 3.9 mg m^{-3}). The subjects were interviewed to obtain detailed personal data, an occupational history and lifestyle information (cigarette smoking, alcohol intake). After informed consent was obtained, 3 ml of blood was collected from each subject, anticoagulated and stored in ethylene diamine tetra-acetic acid tubes. The research protocol was approved by the Research Ethic Committee of the Institute of Occupational Medicine, Chinese Academy of Preventive Medicine, China.

DNA isolation

DNA was isolated from peripheral blood samples using standard methods (Grimberg *et al*. 1989).

Genotyping

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was used to determine the polymorphism of the cytochrome P450 2D6L gene (*CYP2D6L*) according to the method previously described by Heim *et al*. (1990). The primers were 5-GCG GAG CGA GAG ACC GAG GA-3(2098-2117) and 5-CCG GCC CTG ACA CTC CTT CT-3 (3181-3200), which generate a 1102 bp fragment. The substitution of cytosine 2938 to thymine at exon 6 of the *CYP2D6* gene removes a cleavage site for the restriction enzyme *HhaI*. Aliquots of the PCR products were then digested with *HhaI* and electrophoresis was performed in a 2% agarose gel. The thermal cycle controller settings for PCR were 94°C for 5 min, then 32 cycles at 94°C for 1 min; 60°C for 1 min; 72°C for 1 min and 20 s, and a final extension at 72°C for 10 min.

Cytochrome P450 1A1 gene (*CYP1A1*) genotypes were determined by allele-specific PCR, as described by Hayashi *et al*. (1991). Each DNA sample was amplified in two separate reactions by using one of two 5 primers: 5′-GAA GTG TAT CGG TGA GAC CA-3′ or 5′-GAA GTG TAT CGG TGA GAC CG-3′. All reactions included the 3 primer 5-GTA GAC AGA TCT AGG CCT CA-3. PCR was preformed for 30 cycles, with denaturing at 95°C for 1 min, annealing at 65°C for 1 min and extension at 72°C for 1 min.

The genotypes of the glutathione-S-transferase *mu* and *theta* genes (*GSTM* and *GSTT*) were determined using a modified multiplex PCR method. The null genotypes resulting from a major homozygous deletion of the gene cause deficiencies in GSTM1 and GSTT1 activity, respectively. The primers have been described in previous studies (Shea *et al*. 1990, Pemble *et al*. 1994). The PCR product was not present for the null genotype, while both homozygote wild-type and heterozygotes gave rise to a product after amplification. Primers were used to amplify part of the β-globin gene in the same reaction as an internal positive control (Saiki *et al*. 1988). The thermal controller settings for PCR were 94°C for 5 min, then 30 cycles at 94°C for 1 min; 62°C for 1 min; 72°C for 1 min and a final extension at 72°C for 10 min.

Detection of the DT-diaphorase C-T609 mutation of the NAD(P)H:quinone oxidoreductase gene (*NQO1*) was conducted according to the method previously described by Chan *et al*. (1997). The 195 bp fragment containing part of exon 6 was amplified with the primers 5-AAG CCC AGA CCA ACT TCT-3 and 5-GCG TTT CTT CCA TCC TTC-3. The substitution of C to T at exon 6 (at the 609 bp position) creates a cleavage site for the restriction enzyme *HinfI* (GANTC). Therefore, restriction site polymorphism after digestion with *HinfI* results in three different combinations of bands: one 195 bp band, corresponding to the genotype of homozygotes for the wild-type allele (C/C); three bands with 195 bp, 119 bp and 76 bp, corresponding to the genotype of heterozygotes (C/T); and two bands with 119 bp and 76 bp, corresponding to the genotype of homozygotes for the mutant allele (T/T). The thermal controller settings for PCR were 95°C for 2 min, then 35 cycles at 94°C for 50 s; 52°C for 50 s; 72°C for 10 s and a final extension at 72°C for 5 min.

Statistical analysis

The distribution of genotypes and allelic frequencies between the cases and controls were compared using the χ^2 test. Subjects who had smoked more than 100 cigarettes in their lifetime (before the diagnosis for the cases) were defined as 'smokers'. Subjects who had drunk alcoholic beverages at least once a week for more than 1 year prior to the diagnosis were defined as 'drinkers'. The crude odds ratios (ORs) and their 95% confidence intervals (CIs) for each genotype were calculated by logistic regression analysis, with each genotype being recorded as a dummy variable. In addition, we evaluated the association between the latency of manganism and the genetic polymorphism of metabolizing enzymes by comparing the Kaplan–Meier survival curves of the different genotypes of the cases. The log-rank

test and Wilcoxon's test were used to evaluate the homogeneity of the survival curves by genotype. Cox's proportional hazard regression analysis was used to estimate the hazard ratios, and 95% CIs were applied to determine the strength of association between the latency of manganism and the genotypes. All statistical tests were two-sided ($\alpha = 0.05$) and performed using Statistical Analysis System software (version 6.01, SAS Institute Inc., Cary, North Carolina, USA).

Results

As shown in table 1, four potential confounders, i.e. age, gender, cigarette smoking and alcohol intake, were considered in this study. Since there was no statistically significant difference in the distribution of the matching variables (age, sex, smoking status and alcohol consumption) between the case group and the control group, it suggests that these variables were adequately matched ($p > 0.05$). Manganese exposure duration seemed to be shorter in the cases than in the controls, but the difference was not statistically significant ($p > 0.05$).

The frequencies of the five genotypes are shown in table 2. Both the L/L genotype and allele frequencies of *CYP2D6L* were significantly lower in patients with occupational manganism compared with the controls. The frequency of the homozygous genotype mutation L/L in the cases (2.0%) was significantly lower than in the controls (18.0%). Individuals with the homozygote polymorphism (L/L) of *CYP2D6* had a 90% decreased risk of chronic manganism compared with the wild-type (Wt/Wt) (OR = 0.10, 95% CI = 0.01–0.82). The frequency of the L allele of *CYP2D6L* was also significantly lower in the cases (16.3%) than in the controls (29.0%) ($p = 0.033$).

The frequencies of the *CYP1A1* allele and genotype distributions are also shown in table 2. The frequency of wild-type IIe/IIe in the cases was 57.1%, which was lower than in the controls (74%). Compared with the wild-type, heterozygotes

Table 1. Demographic data and manganese exposure duration in chronic manganism cases and controls.

	Cases ($n = 49$)		Controls ($n = 50$)			
	Number	%	Number	%	χ^2 value	p value
Age					0.8977	0.3
<43 years	32	65.3	28	56.0		
⩾43 years	17	34.7	22	44.0		
Gender					1.001	0.3
Female	1	2.0	3	6.0		
Male	48	98.0	47	94.0		
Smoking					4.626	0.1
Yes	21	42.8	30	60.0		
No	26	53.1	16	32.0		
Former	2	4.1	4	8.0		
Drinking habit					2.0987	0.4
Yes	31	63.3	30	60.0		
No	13	26.5	18	36.0		
Former	5	10.2	2	4.0		
Manganese exposure duration					1.7456	0.2
<20 years	30	61.2	24	48.0		
⩾20 years	19	38.8	26	52.0		

Table 2. Distribution of genotypes in manganism patients and controls.

Genotype	Cases ($n = 49$)		Controls ($n = 50$)		p value[a]	OR (95% CI)
	Number	%	Number	%		
CYP2D6L						
Wt/Wt	34	69.4	30	60.0	0.03	1.00
Wt/L	14	28.6	11	22.0		1.12 (0.44–2.84)
L/L	1	2.0	9	18.0		0.10 (0.01–0.82)
Wt/Wt or L/L	15	30.6	20	40.0	0.3	0.66 (0.29–1.52)
L allele frequency	0.163		0.290		0.03	0.48 (0.23–1.00)
CYP1A1						
Ile/Ile	28	57.1	37	74.0	0.2	1.00
Ile/Val	14	28.6	8	16.0		2.31 (0.85–6.27)
Val/Val	7	14.3	5	10.0		1.85 (0.53–6.45)
Ile/Val or Val/Val	21	42.9	13	26.0	0.08	2.14 (0.91–4.99)
Val allele frequency	0.286		0.180		0.07	1.82 (0.88–3.77)
NQO1						
CC	23	46.9	19	38.0	0.5	1.00
CT	17	34.7	17	34.0		0.83 (0.33–2.04)
TT	9	18.4	14	28.0		0.53 (0.19–1.49)
CT or TT	26	53.1	31	62.0	0.4	0.69 (0.31–1.54)
T allele frequency	0.357		0.450		0.2	0.68 (0.37–1.25)
GSTM1+	25	51.0	28	56.0	0.4	1.00
GSTM1−	24	49.0	22	44.0		1.22 (0.55–2.69)
GSTT1+	28	57.1	26	52.0	0.6	1.00
GSTT1−	21	42.9	24	48.0		1.44 (0.65–3.19)

[a] χ^2 test.

(Ile/Val) or homozygotes (Val/Val) had a borderline increased risk of developing manganism (OR = 2.14, 95% CI = 0.91–4.99). The OR for the Val allele was 1.82 (95% CI 0.88–3.77).

As shown in table 2, the frequency of the DT-diaphorase C-T609 mutation allele of *NQO1* was slightly lower in the cases than in the controls (35.7% versus 45.0%), although this difference was not statistically significant ($p = 0.183$), nor was the difference in the genotype distribution ($p = 0.482$). Compared with wild-type C/C, heterozygotes (C/T) or homozygotes (T/T) of *NQO1* did not have an increased risk of chronic manganism (OR = 0.69, 95% CI = 0.37–1.25).

The frequencies of the *GSTT1* null genotype in manganism cases and controls were 42.9% and 48.0%, respectively, but this difference was not statistically significant ($p = 0.627$). The frequency distribution of the *GSTM1* null genotype in the chronic manganism cases and the controls was also similar (49.0% versus 44.0%, $p = 0.619$). The results shown in table 2 indicate that there was no evident association between the *GSTT1* or *GSTM1* null genotypes and occupational manganism in the studied population.

The latencies of the manganism cases classified according to the different genotypes are shown in table 3. The median latencies were 13 years for subjects with the Wt/Wt genotype and 23 years for the Wt/L or L/L genotype of *CYP2D6L*. The difference in latency between these two genotype subgroups, either in terms of the median (10 years) or the mean (6 years), was statistically significant ($p = 0.012$). The age at which manganese exposure began was similarly distributed between these two *CYP2D6* genotype subgroups (median 21 versus

Table 3. The latency of manganism among different genotypes and a summary of survival analysis in manganism cases.

	Latency of manganism (years)				*p* values	
	n	Mean	SD	Median	*t*-test[a]	Log-rank test[b]
CYP2D6L					0.01	0.02
Wt/W/t	34	16.2	7.0	13.0		
Wt/L or L/L	15	22.5	9.0	23.0		
CYP1A1					0.6	0.7
Ile/Ile	28	18.8	8.6	17.5		
Ile/Val or Val/Val	21	17.3	7.9	15.0		
NQO1					0.7	0.8
C/C	23	18.6	8.4	19.0		
C/T or T/T	26	17.7	8.2	15.5		
GSTT1+	28	17.7	7.8	15.5	0.7	0.6
GSTT1−	21	18.7	9.0	17.0		
GSTM1+	24	17.1	6.6	16.5	0.4	0.2
GSTM1−	25	19.0	9.6	16.0		

[a] *t*-test for comparing means.
[b] Log-rank test for comparing medians.

22 years). There was no significant difference in the latencies of manganism among the genotype subgroups of *CYP1A1*, *NQO1*, *GSTT1* or *GSTM1*.

We also studied the latency of manganism in the cases for each gene polymorphism using Kaplan–Meier survival analysis. For *CYP2D6*, there was a significant difference in survival curve between the wild-type homozygote (Wt/Wt) and the other genotypes (figure 1). Individuals with the *CYP2D6* Wt/Wt genotype had a significantly shorter manganism latency than those with the other genotypes ($p = 0.023$, log-rank test), suggesting that this genotype may be a susceptibility marker for occupational manganism. Using the subjects with the *CYP2D6* wild-type homozygote as a reference, Cox's proportional hazards regression analysis gave a hazard ratio of 0.48, with a 95% CI of 0.25–0.94 ($p = 0.032$). The same analyses were done for the polymorphisms of *CYP1A1*, *NQO1*, *GSTT1* and *GSTM1* (data not shown); however, no significant statistical differences in the survival curves were found using the log-rank test and Cox's proportional hazards regression analysis.

Discussion

Individuals vary greatly in their likelihood of developing specific diseases and their response to environmental hazards, including manganese (WHO 1981, Huang *et al.* 1989). Many toxicant-induced impairments and diseases are affected by both the host and external environment factors (Smith *et al.* 1994, Manuel 1996). Recent findings suggest that inherited differences in metabolic capacity may play an important role in susceptibility to environmentally induced diseases. Genetic polymorphism exists in a number of phase I and phase II enzymes. It is conceivable that individuals with genotypes associated with a more efficient activating enzyme or a less efficient inactivating enzyme might be at particularly high risk of adverse effects if they are exposed to the same level of toxicant.

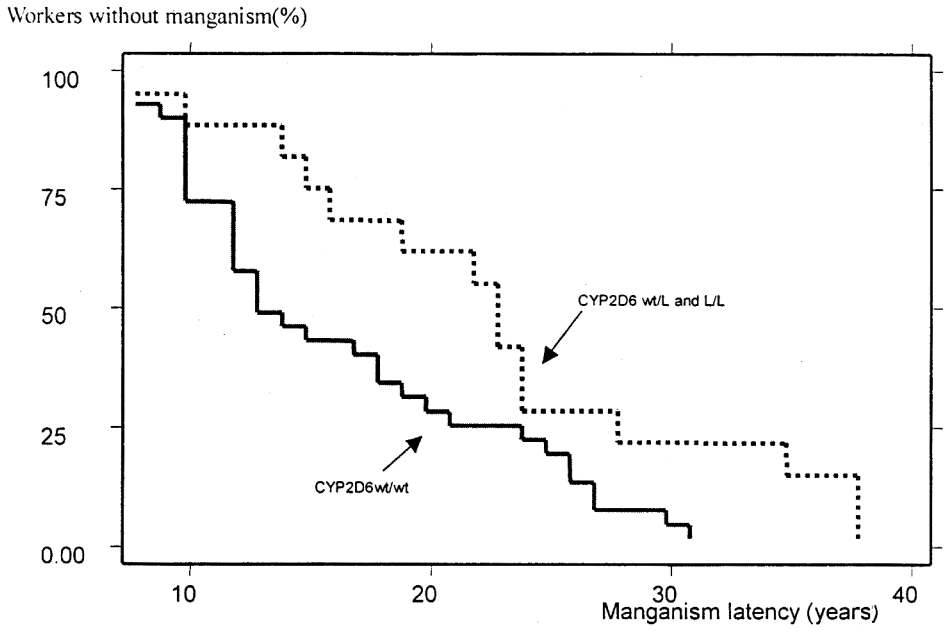

Figure 1. Kaplan–Meier survival estimates showing longer latency in cases of manganism with the *CYP2D6L* mutation allele. The *CPYP2D6L* wild-type genotype is shown relative to the Wt/L and L/L genotypes combined. $p = 0.023$, log-rank test.

Much of our knowledge about manganese intoxication comes from high exposures in the workplace. Its onset and development is insidious and its time course seems to be dependent on exposure level and duration, as well as individual susceptibility (WHO 1981, Huang *et al.* 1989). At present, manganism has been noted in thousands of cases worldwide; it is possible that host factors, such as genetic factors, may play a role in the development of manganism in exposed workers.

The microsomal cytochrome P450 isozyme that metabolizes environmental toxic chemicals and probably endogenous substances exhibits an enormous degree of inter- individual variability in its catalytic activity. In the present study, we found an association between the *CYP2D6L* mutation genotype and the development of manganism. Among workers exposed to the same levels of manganese, individuals with the L mutation allele of *CYP2D6* had a lower risk and longer latency of manganism. Our findings suggest that the *CYP2D6L* mutation is a protective factor in manganism.

The mechanism of the relationship between the *CYP2D6* gene and differences in susceptibility to manganese neurotoxicity is unclear. It may be due to a mutation of the *CYP2D6L* gene causing changes in the metabolism of manganese, or due to some endogenous substances resulting from manganese neurotoxicity. Alternatively, this gene polymorphism may represent a linkage to some other unknown genes. The evidence in our study implicates genetic variants of *CYP2D6* as the molecular mechanistic basis for inter-individual variability to manganese susceptibility. Manganese-induced neurological disorders are clinically similar to Parkinson's disease, and our data are consistent with the observations by Chan

(2000), who reported a similar gene polymorphism association with susceptibility to Parkinson's disease.

The glutathione-S-transferase isoenzymes are believed to play an important role in the cellular metabolism and detoxification of electrophilic compounds via their conjugation to glutathione (Mannervik *et al.* 1991). However, we did not find any association between manganism and the polymorphism of *GSTM1* and *GSTT1*. In our study, both of the alleles and genotype distributed very similarly in manganism cases and controls.

DT-diaphorase plays a role in protecting cells from some toxicant insults by virtue of its ability to catalyse the two-electron reduction of various quinones. These two electrons bypass the formation of semiquinones and therefore reduce the production of free radicals (Cadenas 1995). In this study, we did not find any association between mutation of the *NQO1* gene and the risk of manganism.

In addition, no association was found between *CYP1A1* gene polymorphism and susceptibility to manganism.

Using positron emission tomography, studies on patients suffering from chronic manganism (Wolters *et al.* 1989) have revealed that the main pathological change in manganism is due to degeneration of basal ganglia, with neural loss and gliosis. The lesions appear to be particularly located in the globus pallidus. Manganese was shown to be severely toxic to dopaminergic neurons, resulting in selective dopaminergic atrophy. In the 1980s it was postulated that the possible basis for manganese neurotoxicity may be the enhanced autoxidation of dopamine by a higher valence manganese ion, with increased generation of free radicals and cytotoxicity (Donaldson 1981). This hypothesis was supported by some later studies (Liccione *et al.* 1989). Williams *et al.* (2000) found that methylcyclopentadienyl manganese tricarbonyl-induced neurotoxicity may be mediated by oxidative stress, and protection can be afforded by antioxidants. Manganese is a powerful oxidant when present in a higher oxidation state. The Mn^{3+} ion favours oxidation of dopamine to the corresponding semiquinone, a reaction which would be followed by autoxidation (Donaldson *et al.* 1981, Graham 1984, Archibald and Tyree 1987). *In vitro*, exposure to either $MnCl_2$ (Mn^{2+}) or MnOAc (Mn^{3+}) produced dose-dependent increases in reactive oxygen species (ROS) in the striatum. The increase in reactive oxygen was 10-fold higher with Mn^{3+} compared with Mn^{2+}. *In vivo*, exposure to MnOAc produced significant increases in ROS in the striatum and hippocampus, whereas only the high dose of $MnCl_2$ produced significant effects on the hippocampus (Duhart *et al.* 1995). Oxidation of dopamine has been shown to result in the formation of dopamine semiquinones and dopamine orthoquinones, which are more cytotoxic than the oxidation products of other catecholamines. Unlike Mn^{3+}, Mn^{2+} is normally a scavenger of free radicals and thus a homeostatically controlled protector of cells (Barbeau 1984). Barbeau (1984) proposed a novel hypothesis suggesting that a variety of transient 'trigger factors' act at dopamine neurons to increase dopamine turnover. In turn, this increased synthesis of dopamine favours the production of large quantities of free radicals within the cells in the substantia nigra. Genetic factors may play a role in an individual's susceptibility to such triggers. Manganese itself may be one of the trigger factors.

In conclusion, we found that cytochrome P450 *CYP2D6* polymorphism was associated with susceptibility to chronic occupational manganism, suggesting that the *CYP2D6* gene may be a potential biomarker for identifying susceptible

individuals among workers exposed to manganese. Further study will help to understand the molecular mechanisms of environment–host interactions related to manganese neurotoxicity. However, because of the limited sample size, our results are preliminary and need to be validated in further studies with larger samples.

Acknowledgements

This work was supported by the National Natural Sciences Foundation of China (grants no. 39770640 and 39970633). We would like to thank Hongbing Shen (Department of Epidemiology, Nanjing Medical University, China) for his assistance in the preparation of this manuscript.

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