BIOMEDICAL AND ENVIRONMENTAL SCIENCES **20**, 284-290 (2007)

Carboxylic Esterase and Its Associations With Long-term Effects of Organophosphorus Pesticides[1]

ZHI-JUN ZHOU[2], JIE ZHENG, QIANG-EN WU, AND FANG XIE

Department of Occupational Health and Toxicology, School of Public Health, WHO Collaborating Center for Occupational Health (Shanghai) / Key Laboratory of Public Health and Safety, Ministry of Education, Fudan University, Shanghai 200032, China

Objective To examine a) the effect of organophosphorus pesticide exposure on activity of carboxylic esterases, namely butyrylcholinesterase (BChE), carboxylesterase (CarbE) and paraoxonase (PonE); and b) the association of polymorphisms of BChE and PonE with individual genetic susceptibility to organophosphorus pesticide exposure. **Methods** A cross-sectional study was conducted in 75 workers exposed to organophosphorus pesticides and 100 non-exposed controls. The serum activity of these enzymes was measured. Variant forms of *BCHE-K*, *PON-192*, and *PON-55* were detected. A symptom score was developed as a proxy measure of clinical outcomes. **Results** Activities of both BChE and CarbE were lower in exposed workers (27.3±21.65 nmol·h^{-1}·mL^{-1} and 235.6±104.03 nmol·min^{-1}·mL^{-1}) than in non-exposed workers (78.313±30.354 nmol·h^{-1}·mL^{-1} and 362.681±194.997 nmol·min^{-1}·mL^{-1}). The activity of PonE was not associated with exposure status. The AChE activity in the exposed workers with *BCHE-K* genotype UU (61 cases), genotype UK (12 cases) and genotype KK (2 cases) was 105.05, 84.42 and 79.00 mmol·h^{-1}·mL^{-1}, respectively and the accumulative symptom scores were 3.74, 9.17, and 12.50 accordingly. The AChE activity in the exposed workers with *PON-192* genotype BB (37), genotype AB (27) and genotype AA (11) was 116.8, 91.2, and 72.3 mmol·h^{-1}·mL^{-1}, respectively and the symptom scores were 2.00, 6.74, and 9.73 accordingly. The AChE activity in those with *PON-55* genotype LL (70) and genotype LM (5) was 102.4 and 82.8 mmol·h^{-1}·mL^{-1} and the symptom scores were 4.53 and 9.20. The symptom score was the highest in individuals with abnormal homozygote for each of the three gene loci. **Conclusions** Long-term exposure to organophosphorus pesticides can inhibit BChE and CarbE activity, but exerts no inhibitory effect on PonE activity. Different genotypes of *BCHE-K, PON-192,* and *PON-55* may be related to the severity of adverse health effects of organophosphorus pesticide exposure. Implications of potentially higher susceptibility of workers with mutant homozygotes should be evaluated to reduce health risks.

Key words: Carboxylic esterases; Organophosphorus pesticides; Polymorphism; Susceptibility

INTRODUCTION

Butyrylcholinesterase (BChE EC 3.1.1.8), paroxonase (PonE EC 3.1.8.1) and carboxylesterase (CarbE EC3.1.1.1) are critical enzymes for the degradation of organophosphate toxicants. It is well known that butyrylcholinesterase and paroxonase have polymorphisms[1].

The gene of BChE is located at q21-25 of the 3rd chromosome, with a length of 73 kb at least, involving 4 extrons and 3 introns. There are several single nucleotide polymorphisms (SNP), and variation K derived from the point mutation of nucleotide at 1615 loci (G to A) leads to the change of amino acid at 539 position from Ala to Thr, resulting in a decline in activity of enzyme by 33%[2]. Previous studies on BChE are limited to: (1) the time course of BChE change after organophosphorus pesticide poisoning which is a potential marker for the severity of poisoning and recovery[3-4]; and (2) the correlation between its polymorphism and respiratory paralysis as a result of injection of anesthetic drugs[5-9].

PonE gene is located at q 21.3-22.1 of the 7th chromosome, with a length of 26 kb, including 9 extrons. Two polymorphic sites have been reported: one is at codon 55 mutated T to A, leading to the change of amino acid from Leu to Met; the other is at codon 192 mutated A to G, resulting in Gln to Arg[10]. Studies of PonE polymorphism have mostly aimed to clarify its association with vulnerability to cardiovascular diseases[11-15] and diabetes[16], but few

[1]This work was supported by the grant from National 973 Project (2002CB512902) and the grant from Shanghai Shuguang Program.
[2]Correspondence should be addressed to Zhi-Jun ZHOU, Tel: 86-21-54237675. E-mail:zjzhou@shmu.edu.cn
Biographical note of the first author: Zhi-Jun ZHOU, male, born in 1964, professor in occupational health and toxicology.

0895-3988/2007
CN 11-2816/Q

reports are available on the polymorphism of these enzymes pertaining to health effects due to long-term exposures[17].

Carboxylesterase is a member of the serine hydrolase super-family of esterases and is abundantly expressed in mammalian liver. It can catalyze the hydrolysis of esters, amides and thioesters, and is important for the detoxification of a number of organophosphorus insecticides[18]. Traditionally, it is classified into B esterase, and the inhibition of CarbEs is thought to be of no physiological significance in human beings, but carboxylesterases plays an important role in the metabolism of some drugs[19].

This study is designed to explore the serum activity of 3 enzymes, polymorphisms of BChE and PonE and their associations with health outcomes in workers who have been exposed to organophosphorus pesticides.

MATERIALS AND METHOD

Subjects

A total of 75 employees engaged in packing dimethoate and methamidophos in a pesticide factory in Shanghai were enrolled (36 males and 39 females) at the age of 29-55 years (mean age of 42.0±6.0 years). All of them were healthy and had no history of cardiovascular diseases, type I diabetes, or genetic illness. One hundred non-exposed workers engaged in the mechanical process were enrolled from another factory (82 males and 18 females) with a mean age of 40.0±7.8 years and had no history of occupational hazardous exposure, cardiovascular diseases, type I diabetes or genetic illness.

Field Survey and Physical Examination

Standard questionnaires were used to collect data on age, education background, economic status, smoking, drinking, and previous disease history as part of the annual occupational health surveillance in the factory. The records including job title and time period, type of exposed chemicals, and duration of exposure, and some data of environmental monitoring, *etc*., were abstracted from factory documentation. Current health conditions of workers such as health complaints and symptom were recorded and medical signs were checked. Blood samples were taken, and serum and coagulated clot were separated for further laboratory experiments.

Symptom and Sign Score Assignment

A score reflecting the severity of clinical features due to long-term exposures to organophosphorus pesticides was generated using a weighted method. The assignment of symptom scores was based on the typical symptoms of organophosphorus pesticide intoxication[17]. One point was assigned to the presence of headache, head lightness, fatigue or weakness of lower extremity. Two points were assigned to loss of appetite, nausea, vomiting, abdominal pain, diarrhea, insomnia, dream, decreasing memory, and drowsy. Three points were assigned to palpitation, chest tightness and blurred vision. Four points were assigned to perspiration, especially on extremities, numbness, muscular fasciculation, tremor, excess salivation, weakness, and stiffness of neck muscle. The typical acute symptom of miosis was not included since it was not noted in workers exposed to long-term organophosphorus pesticides.

Measurement of Enzymes Activity

Serum AChE activity was measured based on the principle of Ellman's method[20]. Serum CarbE activity was measured with a method established in our laboratory[21]. Measurement of PonE activity was done as previously described[22].

Genotyping

DNA was extracted from peripheral blood using the phenol-chloroform method. Two separate PCR assays were performed to detect the genotypings of *BCHE-K*, *PON-192*, and *PON-55.* The primers used in the two PCR assays are listed in Table 1.

TABLE 1

BCHE-K, PON-192, 55 loci Primer Series Used in the PCR Assay

Inducer Code	Inducer Series (5'-3')
BCHE-K Forward Primer	**ATATTTTACAGGAAATATTGATGTA**
BCHE-K Reverse Primer	**ATTAGAGACCCACACAACTT**[23-24]
PON-192 Forward Primer	**TTGAATGATATTGTTGCTGTGGGACCTGAG**[25]
PON-192 Reverse Primer	**CGACCACGCTAAACCCAAATACATCTCCCAGAA**
PON-55 Forward Primer	**GAGTGATGTATAGCCCCAGTTTC**
PON-55 Reverse Primer	**AGTCCATTAGGCAGTATCTCCG**

PCRs of *BCHE-K* were carried out in a PCR buffer containing 50 ng DNA gene, 8 pmol primers, 200 μmol/L of each dNTP, and 1.5 unit of Taq polymerase in a final volume of 25 μL. Thirty cycles were performed at 94℃ for 3 min, at 94℃ for 1 min, at 55℃ for 1.5 min, at 72℃ for 1.5 min and a final extension at 72℃ for 10 min. The products were then digested with Mae III to produce diagnostic fragments of the gene (Fig. 1).

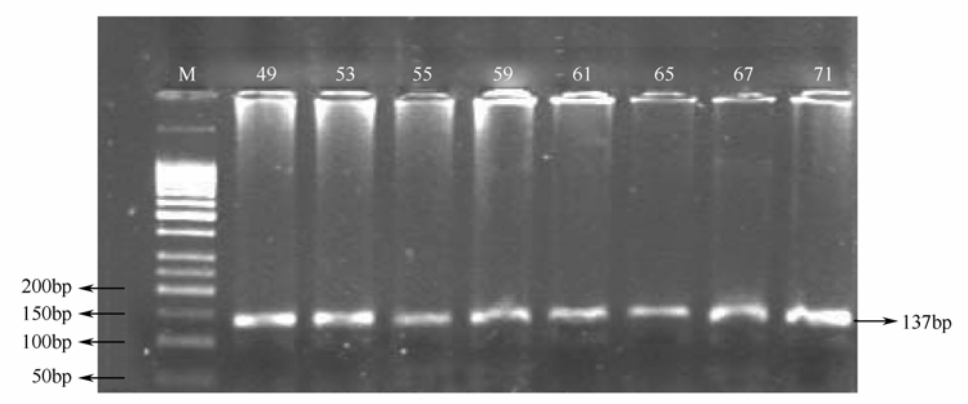

FIG. 1. Mae III digestion for polymorphism of BChE (Variation K).

For *PON-192* and *PON-55*, the PCR buffer contained 100 ng DNA gene, 3 units TagDNA polymerase, 400 μmol/L dNTPs, and 8 pmol of each primer with a final volume of 50 μL. Forty PCR cycles were performed at 94℃ for 5 min, at 94℃ for 1 min, at 61℃ for 45 s, at 72℃ for 45 s, and a final extension at 72℃ for 10 min. The products of PON were then digested with Hinf I to produce diagnostic fragments of the gene (Fig. 2).

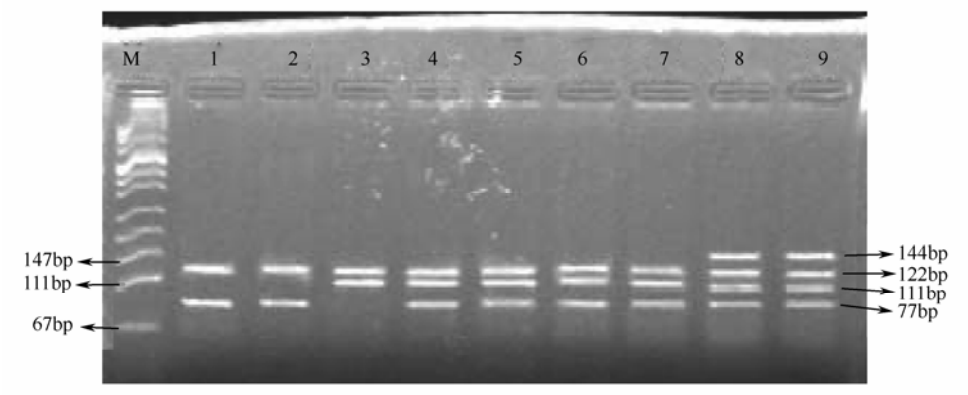

FIG. 2. Hinf Ⅰ digestion for polymorphism of paraoxonase.

Both *BCHE-K* and *PON* fragments were separated by electrophoresis on a polyacrylamide gel containing 3% agarose. The gels were stained with ethidium bromide and transilluminated with ultraviolet light. The representative fragments corresponding to each allele, including Ala/Ala, Ala/Thr, and Thr/Thr for *BChE-K*, Glu/Glu, Arg/Glu, and Arg/Arg for *PON-192* and Met/Met, Leu/Met, and Leu/Leu for *PON-55*, were cloned and sequenced to confirm the specificity of PCR.

Statistical Analysis

The original data were input into a database, and analyzed using the SPSS statistical software. The sum of symptom scores and carboxylic esterase activity in different groups of people with *BCHE-K*, *PON-192*, *PON-55* genotypes in homozygote (wild type and mutant) and heterozygote were compared. This was followed by multivariate analysis of symptom score and its association with various combinations of genotype, attempting to analyze the joint effects and interactions of the three polymorphisms. In a multivariate linear regression analysis, the symptom score was a dependent variable Y and each genotype of polymorphism was used as an independent variable: genotype of *BCHE* (X_1), genotype of *PON-192* (X_2) and genotype of *PON-55* (X_3). For each genotype, three levels of polymorphism were

assigned, with wild homozygote being level 1, heterozygote level 2, and mutant homozygote level 3. Gender, age, and duration of exposure were taken into account in this analysis.

RESULTS

Organophosphorus Pesticide Exposure

The subjects exposed to organophosphorus pesticides were engaged in packing dimethoate and/or methamindophos. According to occupational health monitoring records over the past 15 years, the average air concentration at the worksite was 2.60 mg/m^3 of dimethoate and 0.2 mg/m^3 of methamindophos. The hand contaminations found at the end of work-shift were 7.86 mg of dimethoate and 4.52 mg of methamindophos.

Genotyping

There was no difference in gene distribution between workers exposed and not exposed to organophosphorus pesticides (Table 2).

TABLE 2

Genotypes of *BCHE-K*, *PON-192*, and *PON-55*

Gene Location	Genotype	Not-exposed Case (%)	Exposed Case (%)	Statistics
BCHE-K	Ala/Ala (UU)	74 (74.0)	61 (81.3)	$\chi^2=2.64$ $P=0.2678$
	Ala/Thr (UK)	25 (25.0)	12 (16.0)	
	Thr/Thr (KK)	1 (1.0)	2 (2.7)	
PON-192	Arg/Arg (BB)	50 (50.0)	37 (49.3)	$\chi^2=0.02$ $P=0.9915$
	Arg/Glu (BA)	36 (36.0)	27 (36.0)	
	Glu/Glu (AA)	14 (14.0)	11 (14.7)	
PON-55	Leu/Leu (LL)	93 (93)	70 (93.3)	$\chi^2=0.01$ $P=0.9314$
	Leu/Met (LM)	7 (7)	5 (6.7)	
	Met/Met (MM)	0 (0)	0 (0)	

Carboxylic Esterases Activity

The mean BChE activity in the non-exposed workers was 78.3±30.35 $nmol \cdot h^{-1} \cdot mL^{-1}$ (range 8.5-168.6 $nmol \cdot h^{-1} \cdot mL^{-1}$). For CarbE activity, the mean was 362.7±195.00 $nmol \cdot min^{-1} \cdot mL^{-1}$(range 63.3-807.8 $nmol \cdot min^{-1} \cdot mL^{-1}$). The range of PonE activity was 114.1-524.4 $nmol \cdot min^{-1} \cdot mL^{-1}$ and the mean value was 332.6±96.16 $nmol \cdot min^{-1} \cdot mL^{-1}$. The activity of the three enzymes was normal and there was no difference in gender and age (Figs. 3 and 4).

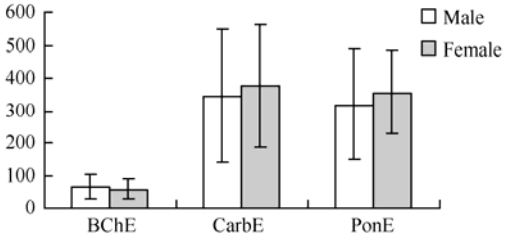

FIG. 3. BChE, CarbE, and PonE activities in two genders of the reference group.

Among workers exposed to organophosphorus pesticides, the BChE and CarbE activity was lower, and the mean value was 27.3±21.65 $mmol \cdot h^{-1} \cdot mL^{-1}$ and 235.6±104.03 $nmol \cdot min^{-1} \cdot mL^{-1}$ respectively. PonE activity was 307.8±107.00 $nmol \cdot min^{-1} \cdot mL^{-1}$, similar to that in the non-exposed workers (Fig. 5).

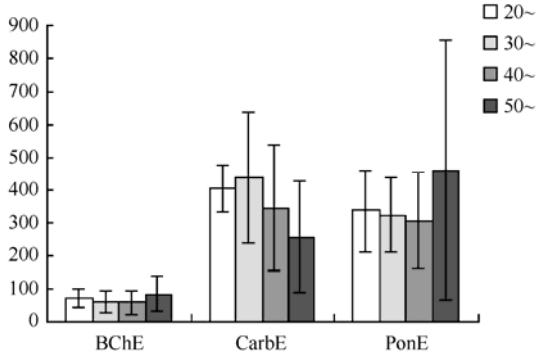

FIG. 4. BChE, CarbE, and PonE activities in different ages of the eeference group.

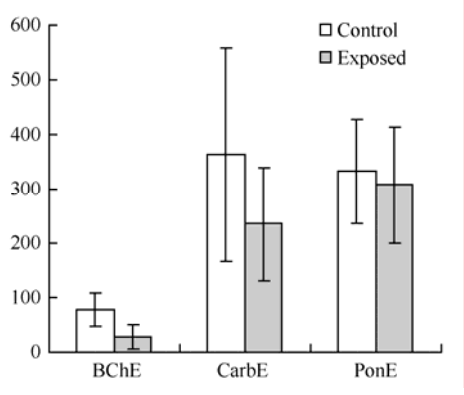

FIG. 5. Effect of organophosphorus pesticide exposure on the activity of carboxylic esterases.

Symptom Scores

Most common complaints were non-specific symptoms of nervous system, such as headache, light dizziness, insomnia, loss of memory, perspiration on extremities, numbness of extremities, *etc.*

Association of Genotypes With Enzyme Activity

The BChE activity in the exposed individuals with different genotypes of UU, UK, and KK was 38.1, 21.4 and 4.7 $mmol \cdot h^{-1} \cdot mL^{-1}$ respectively. The differences in various genotypes were significant ($P<0.05$). Individuals had the highest value for wild type homozygote, moderate value for heterozygote and low value for mutant homozygote.

The PonE activity in the exposed workers with genotypes of BB, BA, and AA of *PON-192* was 470.1, 277.9, and 190.9 $nmol \cdot min^{-1} \cdot mL^{-1}$, respectively ($P<0.05$). Similarly, the activity of wild type homozygote, heterozygote and mutant homozygote was the strongest, moderate and mild respectively.

Effect of Different Genotypes on the AChE Level and Accumulative Symptom Scores

The activity of blood AChE was the strongest in wild type homozygote, moderate in the heterozygote, and mild in mutant homozygote. The sequence was just the reverse of the series of accumulative symptom scores (Table 3). The univariate analysis showed that the individuals with abnormal homozygote had the highest accumulative symptom scores, those with heterozygote and normal homozygote had the second highest scores and the lowest scores, suggesting that the health status of individuals with abnormal homazygote was the worst.

TABLE 3

Genotype of Carboxylic Esterases versus Symptom Scores and AChE Level in Exposed Workers

Gene Site	Geno-type	No. of Case	Symptom Scores ($\bar{x} \pm s$)	AChE Activity ($mmol \cdot h^{-1} \cdot mL^{-1}$) ($\bar{x} \pm s$)	Difference Between Matched Pair		
					Comparison of Genotypes	Difference of Score	Difference of AchE
	UU	61	3.74±3.75	105.05±23.00	UK-UU	5.43*	20.6*
BCHE-K	UK	12	9.17±2.98	84.42±16.36	KK-UU	8.76*	26.1
	KK	2	12.50±0.71	79.00±9.90	KK-UK	3.33	5.4
	BB	37	2.00±3.25	116.81±15.11	AB-BB	4.74*	25.6*
PON-192	AB	27	6.74±3.31	91.19±15.61	AA-BB	7.73*	44.5*
	AA	11	9.73±1.79	72.27±21.39	AA-AB	2.99*	18.9*
	LL	70	4.53±4.18	102.36±22.95	LM-LL	4.67*	19.6
PON-55	LM	5	9.20±3.56	82.80±22.03			
	MM	0					

Note. *$P<0.05$.

Multivariate Analysis

Multivariate analysis revealed that BChE, *PON-192*, and *PON-55* significantly affected the symptom score in the exposed employees. However, no interaction was found (Table 4).

Multiple linear regression revealed that the polymorphism of carboxylic enzymes (X_1, X_2, and X_3) was the only independent factor for symptom score ($P<0.05$). No such association was found with age, gender or exposure duration ($P>0.05$). The equation was derived as follows:

$$Y = -13.840 + 6.608X_1 + 5.013X_2 + 3.131X_3$$

Table 5 lists the comparison of accumulative symptom scores by combining polymorphism of these

TABLE 4

Multivariate Analysis of Genotypes and Accumulative Symptom Scores

Source of Variance	Variance	*F*	Prob>*F*
Whole model	1022.992	22.18	0.0000*
BCHE	64.360	12.56	0.0007*
PON-192	73.869	14.42	0.0003*
PON-55	25.994	5.07	0.0277*
*BCHE*PON-192*	20.932	4.08	0.062
*BCHE*PON-55*	1.581	0.31	0.584
*PON-192*PON-55*	11.445	2.23	0.140
*BCHE*PON-192*PON-55*	0.00		
Residual	333.088		
Total	1356.08		

Note. *$P<0.05$.

enzymes. The wild genotype of enzymes was marked (-), whereas heterozygote and mutant homozygote were marked (+). The influence of polymorphism of carboxylic enzymes on the symptom scores was presented much more directly and clearly.

TABLE 5

Genotyping Polymorphism and Accumulative Symptom Scores

BCHE-K	*PON-192*	*PON-55*	Number of Cases	Accumulative Symptom Scores
−	−	−	27	0.296
−	+	−	26	6.462
+	−	−	10	8.700
+	+	−	4	10.250
−	−	+	4	5.250
−	+	+	3	8.330
+	−	+	0	N. A.
+	+	+	1	13.000

DISCUSSION

BChE and CarbE belong to the B esterase found in serum, liver, and other organs. The inhibition of CarbE activity is an important mechanism by which insecticides kill the target organism. BChE and CarbE can conjugate with organophosphates as a scavenger, and retard the process of intoxication by declining the concentration of ionic organophosphate targeting the functional AChE. The degree of inhibition of AChE and attenuation of the clinical feature of organophosphate poisoning could be reduced[26]. When exposed to low and long-term organophophorus pesticides, the inhibition of AChE is not obvious and so it cannot be used as a good biomarker. Efforts should be made to find other more sensitive biomarkers. Previous studies have failed to pay sufficient attention to the inhibition of BChE and CarbE, which were thought to have no physiological function. However, a recent study has shown that the inhibition of BChE and CarbE is closely related with exposure to organophophorus pesticides[27]. Our study has verified this finding. However, more work should be done before the biomarker is established.

Paraoxonase (PonE) belongs to esterase A. It is nominated by its capability of hydrolyzing (degrading) the potent toxic paraoxon into p-nitrophenol and di-ethylphosphate. The latter is nearly non-toxic to the nervous system. PonE can also swiftly decompose many organophosphorus esters and carbamate insecticides and serve as a direct detoxifying agent[28]. Individuals with extensive activity of PonE have a higher ability to detoxify organophosphorus pesticides and therefore are less likely to experience adverse effects of exposure to such pesticides. At least, screening for PonE could exclude those at high risk of exposing to organophosphorus pesticides.

Since the incidence of major clinical events is low after exposure to occupational organophosphorus pesticides, the composite score of symptoms and signs, and blood AChE activity has been studied. Results of our study have shown that symptom score is not an objective indicator for health status, but it could reflect the early change of symptom development. Usually the extent of exposure is an important factor for health status and can be viewed as the concentration of toxicant and duration of exposure. In our study, as the concentration is almost identical in a given packing workshop, the exposure duration is the dominant determining factor. Based on this consideration, duration of exposure is the only factor which is taken into consideration in our analysis. However, this parameter is not incorporated into the multiple linear regression analysis.

Similar results have been shown by analyzing the correlation between the polymorphism and symptom score. *BCHE-K*, *PON-192*, or *PON-55* present the same pattern of effect, or the activity of wild type homozygote, heterozygote and mutant homozygote and heterozygote is categorized as the strongest, moderate and mild, respectively. Compared with the wild type homozygote, heterozygote and mutant homozygote are more vulnerable to organophosphorus pesticides.

The results of multivariate regression analyses of the health effect of three genotypes have indicated that in the presence of these three mutants, the effects are all statistically significant. There is no interaction between these three mutants possibly due to the relatively small sample size. The possible explanations may be as follows: the gene of *BCHE* is located at q21-25 of chromosome 3, and the *PON-192* and *PON-55* are located at q 21.3-22.1 of chromosome 7.

When the genotype shifts from the wild homozygote to heterozygote, the symptom score is increasing. When further mutation brings the genotype into mutant homozygote, the symptom score will further increase. When the three loci are completely occupied by mutant homozygote, the symptom score becomes the highest, i.e., the health status would be the worst after exposure to organophosphorus pesticides. It has been reported that the health status of individuals after exposure to organophosphorus pesticides is related to the polymorphism of PonE[29].

Although the sample size and the number of subjects are small in our study, our results indicate that polymorphism of *BCHE and PON* can influence

the health status of employees exposed to organophosphorus pesticides. However, further study is required to confirm the role of polymorphism of *BCHE and PON* in the long-term exposure to organophosphours pesticides.

ACKNOWLEDGEMENTS

The authors are grateful to Dr. Wei DONG, Professor Shou-Zheng XUE and Dr. Yun-Guang SUN for their assistance in polishing English.

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(*Received October* 10, 2006 *Accepted May* 14, 2007)