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Correlation between *CYP1A1* polymorphisms and susceptibility to glyphosate-induced reduction of serum cholinesterase: A case-control study of a Chinese population

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ABSTRACT

Glyphosate (GLP) is one of the most common herbicides worldwide. The serum cholinesterase (ChE) may be affected when exposed to glyphosate. Reduction of serum ChE by herbicides is probably related to cytochrome P450 (*CYP450*) family polymorphisms. We suspect that the abnormal ChE caused by GLP could be correlated with the *CYP* family members. To determine whether *CYP1B1* (rs1056827 and rs1056836) and *CYP1A1* (rs1048943) gene polymorphisms and individual susceptibility to GLP-induced ChE abnormalities were interrelated in the Chinese Han population, we performed this genetic association study on a total of 230 workers previously exposed to GLP, including 115 cases with reduced serum ChE and 115 controls with normal serum ChE. Two even groups of cases and controls were enrolled. The *CYP1A1* and *CYP1B1* polymorphisms in both groups were genotyped using TaqMan. Subjects with the *CYP1A1 rs*619586 genotypes showed an increased risk of GLP-induced reduction of serum ChE, which was more evident in the following subgroups: female, > 35 years old, history of GLP exposure time < 10 years and > 10 years, nonsmoker and nondrinker. The results show that *CYP1A1* rs619586 was significantly associated with the GLP-induced reduction in serum ChE and could be a biomarker of susceptibility for Chinese GLP exposed workers. Because of a large number of people exposed to glyphosate, this study has a significance in protecting their health.

1. Introduction

Glyphosate [N-(phosphonomethyl), glycine, GLP] is one of the most common broad-spectrum herbicides used in agriculture worldwide and has been applied broadly since the 1970s (Szekacs, 2012). As an organophosphorus compound, GLP is the main active constituent in the Roundup® herbicides, which are manufactured by the Monsanto Company. GLP is not only the herbicide variety with the largest production and consumption worldwide but is also the most common herbicide exported by China at present.

Cholinesterase (ChE) is a closely related group of enzymes within the cholinesterase family. Inhibition of acetylcholinesterase increases the nervous system acetylcholine concentration(Saldanha, 2017). Serum ChE, which is also known as plasma ChE or butyrylcholinesterase (bChE), is found in the liver, muscle tissues, and in insignificant amounts in the plasma and serum. Acetylcholinesterase inhibition testing has been used as a biomarker for the effect of exposure to organophosphorus compounds, such as agricultural chemicals (Lionetto et al., 2013). As the most popular organophosphorus herbicide, many GLP poisoning cases have been reported with reduced ChE activity (Gallegos et al., 2018). GLP is a weak acetylcholinesterase inhibitor in rats; ChE activity in rats is stronger in the brain ($P < .05$) than in the kidney, liver, and plasma (Bradberry et al., 2004; Larsen et al., 2016). ChE activity can be inhibited by GLP. This inhibition was reported in the muscles and brain of *Rhinella arenarum* and several fish species exposed to a sublethal dose of GLP dissolved in their aquatic environment (Gholami-Seyedkolaei et al., 2013; Lajmanovich et al., 2011). Moreover, exposure to a high glyphosate concentration (0.25 to

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5 mM) can reduce ChE activity by approximately 13 to 20% in human erythrocytes (Kwiatkowska et al., 2014). Another study indicated that ChE could be a biomarker for the effect of GLP in the environment (Menendez-Helman et al., 2012). According to previous laboratory studies, a large amount of population-based data has shown that serum ChE levels in workers exposed to GLP are significantly different from those of control group workers (Pan and Zhu, 2016; Zhang et al., 2017).

Cytochrome P450 family 1 subfamily A member 1 (*CYP1A1*) and cytochrome P450 family 1 subfamily B member 1 (*CYP1B1*) are two key members of the Cytochrome P450 family. Following herbicide exposure, most herbicides are metabolized in the human body through the CYP1 isoenzymes(Nebert and Russell, 2002). Some studies have shown that GLP can suppress CYP450 enzyme activity. Studies have also shown that *CYP1A1* and *CYP1B1* are inhibited in both fish and mice after exposure to a certain glyphosate concentration (Larsen et al., 2014; Lopes et al., 2017; Richard et al., 2005).

Based on the above rationale, we suspect that the reduction in ChE caused by GLP is related to the CYP family members. Based on previous studies, HapMap and NCBI database, three SNPs in the *CYP1B1* and *CYP1A1* gene regions were selected, tested and analyzed in a case-control study to evaluate the association between *CYP1B1* and *CYP1A1* gene variants and susceptibility to GLP-induced serum ChE abnormalities. We hope that the study will provide an experimental basis for the damage caused by GLP in the occupational population or even the general population.

2. Methods and materials

2.1. Ethics principle

The local ethics committee of Jiangsu Provincial Center for Disease Control and Prevention approved this study (number 2012025) according to the principles of the Declaration of Helsinki. All participating subjects signed a written informed consent form before donating 4 mL of venous blood and 50 mL of urine for the study.

2.2. Study population

A total of 115 cases with reduced serum ChE and 115 controls with normal serum ChE were enrolled in our present study. These participants were selected from five herbicide production factories in Shandong and Jiangsu Provinces from January 2013 to January 2016. The selection principles of the participants are as follows: (1) All participants were workers in the production workshops where the herbicide glyphosate was produced. (2) The glyphosate workshop where participants work produces only raw materials or preparations of glyphosate. (3) Participants only worked in glyphosate production workshop for the past two years without exposure to other pesticides. All participants were genetically unrelated ethnic Han Chinese. Detailed information about the case group and the control group can be found in Table 1. Participants with a medical history and drug treatment now related to abnormal serum ChE or who were in touch with other factors associated with abnormal serum ChE were excluded. The exclusion criteria must be met to ensure that all detected abnormalities in serum ChE are mainly caused by GLP. The serum ChE reference standard used by the clinical laboratory of Jiangsu Provincial Center for Disease Control is 5410–13,000 U/L. (China, 2004) We defined individuals with serum ChE values below 5410 U/L as the case group. In addition, smokers referred to individuals who smoked every day for more than one year, and drinkers were defined as participants who drank three standard alcohol beverages per week for more than one year. (See Fig. 1.)

2.3. Determination of serum cholinesterase

We separated peripheral blood mononuclear cells from the participants' peripheral venous blood and determined the serum cholinesterase activity using the continuous monitoring method recommended by the German Society for Clinical Chemistry. The experiments were conducted on a biochemical analyzer (Beckman Coulter, Inc., USA) according to the instructions of the Cholinesterase FS test kit (DiaSys Diagnostic Systems, Shanghai, China).

Table 1
Demographic and selected variables in cases and controls.

Variables	Case (n = 115)		Control (n = 115)		P
	n	%	n	%	
Age, year[a]	34.1 ± 10.0		34.1 ± 10.1		0.903[b]
Exposure time(years)[c]	5.6 ± 6.3		5.8 ± 6.6		0.863[b]
< 10	98	85.2	96	82.6	0.856[d]
≥10	17	14.8	19	17.4	
Gender					
Male	54	47.0	54	47.0	1[d]
Female	61	53.0	61	53.0	
Smoking status					
Smoker	25	21.8	25	21.8	1[d]
Nonsmoker	90	28.2	90	28.2	
Drinking status					
Drinker	26	22.6	28	24.3	0.876[d]
Nondrinker	89	77.4	87	75.7	
Glyphosate concentration in urine(μg/mL)[e]	0.77	18.32	0.46	14.90	0.122[f]
Serum cholinesterase (U/L)[g]	4170	1200–4206	7390	5415–10,473	**0.001**[f]

The significance of bold means that the difference was considered statistically significant when $P < 0.05$.
[a] Use mean ± SD to describe the age.
[b] Two-sided t-test.
[c] Years of exposure to glyphosate.
[d] Two-sided $\chi 2$ test.
[e] Use median and range to describe the glyphosate concentration in urine.
[f] Mann-Whitney U test.
[g] Use median and range to describe the serum cholinesterase.

2.4. Determination of glyphosate in urine

Trifluoroacetic anhydride (TFA) and heptafluorobutanol (HFB) were mixed in a ratio of 2:1 to derivatize the urine sample and dried with nitrogen. The residue was dissolved in acetaldehyde ethyl acetate solution. The derivatized analytes were separated by gas chromatography using the GC 6890-MS5973 system equipped with a split/splitless injector (Agilent, USA) and an autosampler (Gerstel, German). The GC column was a capillary column (DB-5MS, 30 m × 0.25 mm × 0.25 μm). The injector temperature was 200 °C. The oven temperature was held at 80 °C for 1.5 min and then ramped to 260 °C at a rate of 30 °C per min and held for 1 min. Afterward, the samples were heated at 300 °C for 3.5 min to elute high-boiling compounds. Helium 4.5 was used as the carrier gas with a constant flow rate of 1.0 mL/min. Selective ion monitoring (SIM): the qualitative ions of the GLP derivatives are 611, 584, and 460. The quantitative ion of the GLP derivative is 612. Calculate the concentration of glyphosate (μg/mL) in the sample based on the ratio of the quantitative ion peak area of the sample to the quantitative ion peak area ratio of the internal standard derivative.

The samples were tested for stability at a concentration of 0.1, 0.25, and 0.5 μg/mL after pretreatment, and the samples were kept stable at room temperature (20 degrees) for 24 h.

2.5. SNP selection criteria

We chose SNPs according to former studies and the HapMap project and NCBI databases. We searched for target SNPs using the following criteria: a) located in the *CYP1A1* and *CYP1B1* gene regions and b) MAF (minor allele frequency) of CHB > 0.05. Based on these criteria, three

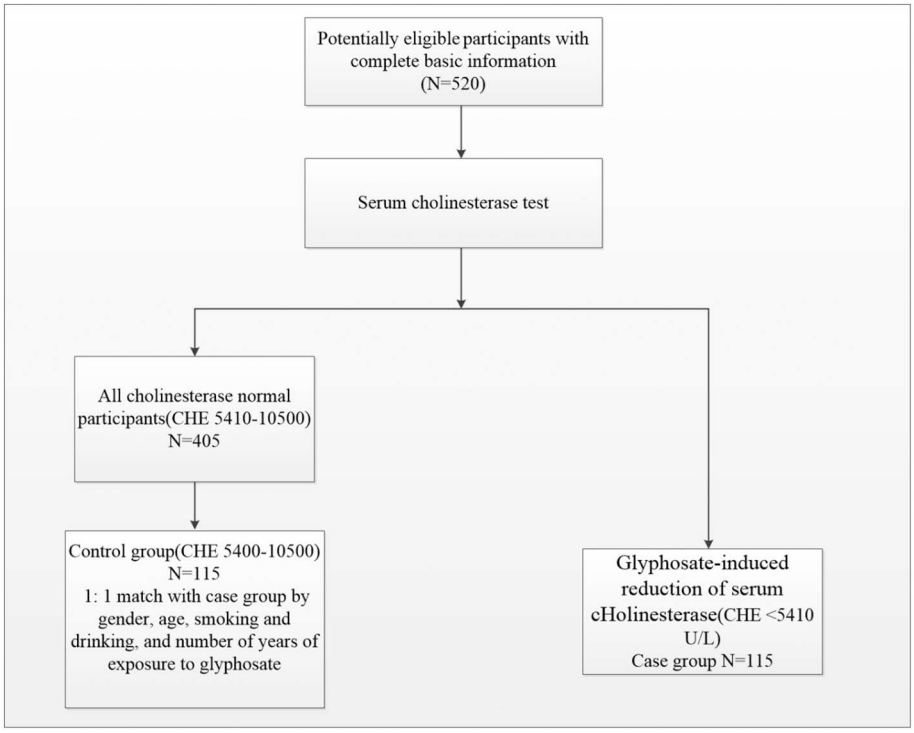

Fig. 1. STARD flowchart for the selection of subjects.

most commonly reported SNPs (rs1056827 and rs1056836 in CYP1B1 and rs1048943 in CYP1A1). In previous literatures related to many human diseases were finally confirmed for genotyping and further association analysis. The *CYP1B1* mutation rs1056827 changes Ala119 to Ser, rs1056836 changes Leu432 to Val, and the *CYP1A1* mutation rs1048943 changes Ile462 to Val.

2.6. DNA isolation and genotyping

We extracted genomic DNA from the participants' peripheral venous blood samples with QIAcube HT Plasticware and the QIAamp 96 DNA QIAcube HT Kit (Qiagen, Dusseldorf, Germany). The DNA was stored at −80 °C prior to use. All measurement operation steps were strictly followed. To prevent pollution with impurities, the A260/A280 purity of the DNA was between 1.8 and 2 based on the NanoDrop One (Thermo Fisher Scientific, USA) ultraviolet spectrophotometer.

Genotyping of the *CYP1B1* and *CYP1A1* polymorphisms was conducted with ABI TaqMan SNP genotyping assays using pre-designed commercial genotyping assays. According to the manufacturer's instructions, the extracted DNA and genotyping assay reagents were added to the TaqMan universal PCR master mix (Applied Biosystems, Foster City, CA, USA). We used a 96-well plate for genotyping with the ABI 7900HT real-time PCR system (Applied Biosystems, Foster City, CA, USA). The real-time PCR conditions were as follows: 50 °C, 2 min; 95 °C, 10 min; 95 °C, 15 s; and 60 °C, 1 min (40 cycles of the last two steps). The data were analyzed by the ABI 7900HT System sequence detection software 2.4. For further confirmation, 10% of the samples were randomly inspected with repeated tests, and the results were consistent.

2.7. Statistical analysis

The data were analyzed using SPSS 22.0. In this study, the goodness-of-fit χ^2 test was adopted to test for Hardy-Weinberg equilibrium (HWE). We used Student's *t*-test or the χ^2 test to demonstrate differences in the characteristics and frequencies of the genotypes between the cases and controls. The median and range were used to describe the GLP concentration in the urine and the ChE concentration because the two data sets did not conform to a normal distribution. Unconditional multivariate logistic regression analyses were performed to evaluate the importance of the *CYP1A1* and *CYP1B1* polymorphisms by estimating the adjusted odds ratios (ORs) and 95% confidence intervals (95% CIs).

3. Results

3.1. Characteristics of the study participants

The characteristics of all participants, including the 115 cases with reduced serum ChE and 115 controls with normal serum ChE, are provided in Table 1. No significant differences were found between the case and control groups in age (34.1 ± 10.0 *vs.* 34.1 ± 10.1, cases *vs.* controls, respectively, $P = .903$), years of exposure to GLP (5.6 ± 6.3 *vs.* 5.8 ± 6.6, cases *vs.* controls, respectively, $P = .863$), gender ($P = 1$), smoking ($P = 1$), drinking ($P = .876$) and the GLP concentration (0.77 *vs.* 0.43, cases *vs.* controls, respectively, $P = .122$). We used the median and range to describe the GLP concentrations in the urine and the ChE concentrations because the two data sets did not conform to a normal distribution.

3.2. Effects of the CYP1A1 and CYP1B1 polymorphisms on the risk of ChE abnormalities in the case-control study

The frequencies of the participants' genotypes confirmed the HWE ($\chi^2 = 3.78$, $P = .07$ for rs1048943, $\chi^2 = 0.88$, $P = .42$ for rs1056827, and $\chi^2 = 6.05$, $P = .02$ for rs1056836). Table 2 shows the allele frequencies of rs1056827 and rs1056836 in *CYP1B1* and rs1048943 in *CYP1A1* for the cases and controls. No significant effects for either SNP (rs1056827 or rs1056836) were found in any model.

Rs1048943 was the only one of the three SNPs that differed significantly between the case and control groups; the AA, AG and GG genotypes represented 45.2%, 50.4%, and 4.3% of the cases, and the AA, AG, and GG genotypes represented 65.2%, 34.7%, and 0% of the controls, respectively. The SNP rs1048943 displayed a protective ($P < .001$ for rs1048943) trend for the cases. Moreover, a similar trend of hazard was found in the comparison of G alleles between the cases

Table 2
Genotype and allele frequencies of *CYP1B1* and *CYP1A1* polymorphisms among Glyphosate-induced abnormal cholinesterase cases and controls.

Genotype	Cases (n = 115)		Control (n = 115)		P^a	OR (95%CI)
	n	%	n	%		
CYP1B1 rs1056827						
TT	12	10.4	8	7.0		
GT	21	18.3	20	17.4	0.518	
GG	82	71.3	87	75.7	0.332	
GT + GG	103	89.6	107	93.0	0.349	
G allele	185	80.4	194	84.3	0.366	
P trend					0.615	
CYP1B1 rs1056836						
GG	3	2.6	4	3.5		
CG	38	33	42	36.5	0.813	
CC	74	64.3	69	60	0.714	
CG + CC	112	97.3	111	96.5	0.701	
C allele	186	80.9	180	78.3	0.72	
P trend					0.772	
CYP1A1 rs1048943						
AA	52	45.2	75	65.2		1.00 (Ref.)
AG	58	50.4	40	34.7	**0.007**	**2.09(1.22–3.58)**
GG	5	4.3	0	0	**0.014**	/
AG + GG	63	54.8	40	34.8	**0.003**	**2.27 (1.33–3.86)**
G allele	68	29.6	40	17.4	**0.001**	**2.45(1.44–4.15)**
P trend					**0.001**	

The significance of bold means that the difference was considered statistically significant when $P < 0.05$.
[a] Two-side chi-square test.

and controls ($P < .001$ for rs1048943). Compared with the AA genotype in *CYP1A1* rs1048943, which was used as a control value, we found that the AG, GG, AG/GG and G allele genotypes increased the risk trend ($P = .007$, OR = 2.09, 95% CI = 1.22–3.58 for the AG genotype, $P = .014$ for the GG genotype, $P = .003$, OR = 2.27, 95% CI = 1.33–3.86 for the AG/GG genotype, and $P = .001$, OR = 2.45, 95% CI = 1.44–4.15 for the G allele genotype).

3.3. Stratification analyses between the CYP1A1 rs1048943 polymorphisms

Table 3 shows the stratification analysis results. We observed a higher risk in the female group ($P = .009$, OR = 2.85, 95% CI = 1.35–6.06), in the group aged > 35 years ($P < .001$, OR = 4.32, 95% CI = 1.76–10.52), in the group with < 10 years of exposure ($P = .022$, OR = 1.97, 95% CI = 1.11–3.50), in the group with ≥10 years of exposure ($P = .042$, OR = 5.13, 95% CI = 1.23–21.35), in the nonsmoker group ($P = .010$, OR = 2.24, 95% CI = 1.23–4.11) and in the nondrinker group ($P = .010$, OR = 2.25, 95% CI = 2.23–4.16).

Table 3
Stratification analysis of *CYP1A1* rs1048943 in a dominant model between glyphosate-induced abnormal acetylcholinesterase cases and controls.

Variable	Genotype (cases/controls)		P^a	OR (95%CI)
	AA	AG + GG		
Gender				
Male	23/31	31/23	0.178	
Female	29/44	32/17	**0.009**	2.85(1.35–6.06)
Age (years)				
≤35	36/44	34/27	0.236	
> 35	16/31	29/13	**0.001**	4.32(1.76–10.52)
Exposure time(years)				
< 10	46/61	52/35	**0.022**	1.97(1.11–3.50)
≥10	6/14	11/5	**0.042**	5.13(1.23–21.35)
Smoking status				
Smoker	9/14	16/11	0.256	
Nonsmoker	43/61	47/29	**0.010**	2.24(1.23–4.11)
Drinking status				
Drinker	9/16	17/12	0.111	
Nondrinker	43/59	46/28	**0.010**	2.25(1.23–4.16)

The significance of bold means that the difference was considered statistically significant when $P < 0.05$.
[a] Two-sided $\chi 2$ test of the frequency distributions of selected variables between the cases and controls.

4. Discussion

China occupies 70% of the global share of GLP production. In 2014, the yield of GLP in China was over 500 thousand tons, > 80% of which was exported. A prospective cohort study conducted by the US National Cancer Institute showed no apparent association between GLP and any solid tumors. The EU granted permission to continue to sell and use GLP for the next five years in 2017 (European Food Safety Authority, 2019). However, a large number of scholars are still worried about the safe use of GLP (Cai et al., 2017). A liver toxicity study in rats showed that metabolism and oxidative damage of the liver tissue was affected by GLP(Tang et al., 2017). A study based on a Chinese population showed that GLP affected hepatic and renal functions among the exposed occupational population and that an association existed between the effect and the exposure dose (Zhang et al., 2017).

In the clinic, determination of serum ChE activity is an important method to diagnose herbicide poisoning and evaluate the damage of liver parenchyma cells (Chen et al., 2009; Frappart et al., 2011). Serum ChE is an enzyme involved in hepatocyte synthesis. When the liver function is abnormal, ChE is reduced in liver cells, and the serum ChE activity is decreased. Serum ChE can be a very sensitive index for liver damage and reflects the function of hepatocyte synthesis due to its short half-life (Fernandez Prieto et al., 2011; Pohanka, 2013).

The CYP gene is susceptible to different pathologies because its polymorphisms mainly affect the metabolism of substances that are enzyme substrates, leading to changes in the response (Zhou et al., 2009). An epidemiological study based on the Han population indicated that *CYP450* enzyme polymorphisms were associated with anti-tuberculosis drug-induced liver injury (ADLI) (Feng et al., 2014). Subsequent biochemical testing on fish found that GLP had a significant effect on several biochemical indexes. Shiogiri et al. found that glyphosate exposure led to decreased liver function and severe liver injury in fish, leading to eventual death.(Mesnage et al., 2015; Shiogiri et al., 2012; Topal et al., 2015). Expression of the CYP family affects liver function. At low concentrations, CYP is mainly expressed in the liver. In contrast, at high levels, CYP expression in pulmonary cells and lymphocytes is more obvious (Schweikl et al., 1993). Procarcinogens, such as polycyclic aromatic hydrocarbons and 17 beta-estradiol are metabolized by *CYP1B1* (Li et al., 2014). *CYP1A2* is regulated by the aryl hydrocarbon receptor, and *CYP1B1* is expressed in hepatic cells (Lin and Lu, 2001). Therefore, we speculate that the abnormal liver function and serum ChE caused by GLP are closely related to the CYP genes. *CYP1A1* rs1048943, an A > G transition, leads to an amino acid substitution of Val for Ile in exon 7 significantly associated with *CYP1A1* inducibility. The SNP situated near heme-binding region of protein and regulates enzyme activity promoting the occurrence and development of cancer present at nucleotide 2455. The SNP m2 A2455G (rs1048943) in the *CYP1A1* have been studied as risk factors of cervical carcinogenesis, lung cancer and coronary artery disease (Li et al., 2016; Liu et al., 2016; Peng et al., 2017). Mustafa's study showed that the interaction between high organochlorine pesticides levels and *CYP1A1* rs1048943 may magnify the risk of preterm delivery (Mustafa et al., 2013).

In this study, we demonstrated that the SNP rs1048943 of *CYP1A1* was significantly associated with a GLP-induced reduction of serum ChE in a Chinese Han population. In addition, we found in the stratification analysis that the SNP rs1048943 was a risk factor between the cases and

controls in the following subgroups: female, age > 35 years, exposure time < 10 years, exposure time ≥ 10 years, nonsmoker and nondrinker. Therefore, these results revealed potential predictive and diagnostic values for the *CYP1A1* SNPs in the GLP-induced reduction of serum ChE and glyphosate poisoning. Based on the stratification analysis shown in Table 3, we propose the following discussion. In the current study, gender differences in the expression and function of CYP members were found in both rat and human liver tissues. In most cases, the metabolic capacity of male rats is higher than that of females. In humans, this difference is small and relatively controversial (Zanger and Schwab, 2013; Zaphiropoulos et al., 1989). An epidemiological survey of agricultural workers in Thailand showed that females had a higher prevalence of abnormal serum ChE levels (15.2%) than males (10.2%) (Guytingco et al., 2018). Table 3 shows that participants carrying the *CYP1A1* rs1048943 AG/GG genotypes in the female group had an increased risk of a GLP-induced reduction of serum ChE compared to that of subjects carrying the *CYP1A1* rs1048943 AG/GG genotypes. This finding is consistent with our previous population-based study results showing that the abnormal CHE rate in females is much higher than that of males ($P < .001$) (Zhang et al., 2017). Serum cholinesterase activity can be affected by many physiological and pathological conditions, including age, some drug therapies, and liver diseases. As people age, their C-reactive protein, interleukin 6 (IL-6) and tumor necrosis factor alpha (TNF-alpha) levels and butyryl cholinesterase activity decrease significantly(Mitrache et al., 2001). Classically, with an increase in the time or concentration of glyphosate exposure, the toxic effects of the accumulation in the body will also increase(Tarazona et al., 2017). In this study, we found that participants carrying the *CYP1A1* rs1048943 AG/GG genotypes in the age > 35 and the exposure time < 10 years and ≥ 10 years groups had an increased risk of GLP-induced reduction of serum ChE compared to those carrying the *CYP1A1* rs1048943 AA genotype. Therefore, we should pay more attention to serum ChE in female and older workers. In our study, we observed that the risk effect of the *CYP1A1* rs1048943 AG/GG genotypes was more obvious among workers who never smoked and never drank. Alcohol, which is the main trigger of alcoholic liver disease, is associated with changes in the serum ChE levels (Huang et al., 2017). Studies have demonstrated that some compounds present in cigarette smoke, such as nitric oxide and cyanide, are related to enhanced AChE activity and serum ChE (Jaques et al., 2012). However, the size of our research sample was small. These discrepancies may have caused the significant differences in the workers who did not smoke or drink.

This study explored whether polymorphisms in the *CYP1B1* (rs1056827 and rs1056836) and *CYP1A1* (rs1048943) genes were associated with the GLP-induced reduction in serum ChE for the first time. The cases and controls in this study worked in similar working conditions and were exactly matched based on their demographic characteristics. Various methods were used for quality control during the study. However, information bias and selection bias still exist, and the limitations of the study need to be noted. First, our results may not be extended to other populations due to the racial specificity. Second, environmental factors, such as diet and physical activity, have not been considered in our study due to the lack of detailed background information on the participants. Third, although the number of subjects fits the requirements of the analysis, the sample size is still small. Therefore, more samples and people of different races are required in the future to confirm the effects of *CYP1A1* polymorphisms on the glyphosate-induced reduction of serum ChE.

5. Conclusion

In conclusion, this study for the first time found the functionally significant rs1048943 in *CYP1A1* that might cause susceptibility to the GLP-induced reduction of serum ChE in a Chinese population. The *CYP1A1* rs1048943 AG/GG genotypes were associated with a significantly increased risk in the female, age > 35 years, exposure time < 10 years and ≥ 10 years, nonsmoker, and nondrinker groups. To determine the mechanism underlying this result, more functional studies between *CYP1A1* and glyphosate exposure should be performed.

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Declaration of Competing Interest

The authors have no conflicts of interest to declare.

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