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Original Article

Lp-PLA2 variants associated with delayed encephalopathy after acute carbon monoxide poisoning

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Abstract: Background: This study aimed to aim to investigate the association between LP-PLA2 rs1805017/rs1051931 gene polymorphism and acute carbon monoxide (CO) poisoning. Methods: Two Lp-PLA2 single nucleotide polymorphisms (SNPs), rs1805017 and rs1051931, selected from DNA pooling base genome-wide association study, were genotyped by in 921 acute CO poisoning patients using polymerase chain reaction restriction fragment length polymorphisms (PCR-RFLPs). The patient group consisted of 408 patients with delayed encephalopathy after acute carbon monoxide poisoning (DEACMP) and 513 patients with no signs of lasting neurological damage (control population). Results: The frequency of the rs1805017 A allele was significantly higher in the DEACMP population (OR=1.58, 95% CI: 1.25-1.98), as was the GG vs. AG genotype (OR=1.42, 95% CI: 1.06-1.91) and the GG vs. AA frequency (OR=2.83, 95% CI: 1.52-5.23) compared to controls. Association analysis revealed a significant association between DEACMP and rs1805017 (P<0.01) and rs1051931 (P<0.05). However, C.I. values consisted of 1. Conclusion: These data suggest that the allelic variant of rs1805017 is a risk factor for DEACMP. The Lp-PLA2 protein may modulate the susceptibility to DEACMP, which attaches importance of examining the relationship between the LP-PLA2 polymorphisms and clinical outcome following acute CO poisoning.

Keywords: Delayed encephalopathy, Acute carbon monoxide poisoning, Lp-PLA2, SNP

Introduction

CO poisoning is one of the most common causes of poisoning in China Zhou, et al. [1]. The incidence of CO poisoning has decreased year by year in China, but a major fraction of acute poisoning patients recover from the acute stage of CO intoxication, and some patients exhibit a recurrence of neuropsychiatric symptoms after a latent period (usually 3 to 60 days) of normal or near normal neurological function termed the lucid interval. These symptoms are termed delayed encephalopathy after acute carbon monoxide poisoning (DEACMP) [2, 3]. It is higher in patients over 40 years of age and increases progressively with age, while it is rare in children under 10 years and shows no significant gender differences [4, 5]. The signs and symptoms of DEACMP include dementia, amnestic syndromes, Parkinsonism, aphasia, apraxia, tardive dyskinesia, cognitive deterioration, urinary incontinence, gait disturbance, mood disorders, memory deficits and personality changes [6].

The mechanism of this disease is not yet fully known, and not only, no specific treatment is available for DEACMP [7], but also poor prognosis is a characteristic of it. About 25% of DEACMP cases result in permanent neuropsychological deficits. Identification of the most vulnerable patient groups combined with early diagnosis may improve the quality of care and reduce permanent disability following acute CO toxication [8]. Up to now there is still a lack of early prognostic indicators. Secondary cerebral circulation disorder and a variety of biochemical changes may be the key factors in the attack of DEACMP. The immune and inflammatory responses may be the main factors involved in the process.

Lp-PLA2, a member of the phospholipase superfamily, is an enzyme produced by mono-

cytes and macrophages, T cells, and mast cells. Oxidized low-density lipoprotein (LDL) within the subendothelial space is converted by Lp-PLA2 into oxidized free fatty acids and lysophoshatidyl choline [9, 10]. These products trigger an inflammatory cascade by stimulating the expression of adhesion molecules and the release of cytokines by endothelial cells and plaque-based macrophages, which recruit more monocytes into the subendothelial space where they become activated and differentiate into macrophages. The fact that Lp-PLA2 is produced locally within atherosclerotic lesions itself likely accounts for its high specificity for vascular as opposed to systematic inflammation. Plasma Lp-PLA2 level is not affected by systemic inflammatory diseases [11, 12].

Plasma Lp-PLA2 is bound mainly to LDL. Epidemiological studies have found there are consistent and significant positive associations between Lp-PLA2 mass or activity and stroke [13, 14].

We have found that Lp-PLA2 is a novel independent risk factor and new treatment target in acute ischemic stroke and plasma Lp-PLA2 was increased in acute ischemic cerebral stroke. Lp-PLA2 polymorphism may be associated with plasma concentration of Lp-PLA2 and the occurrence of stroke (unpublished data). A recent study showed that patients with ischemic stroke history had a higher risk for DEACMP [15]. So we' d like to know if Lp-PLA2 plays a role in pro-inflammatory effects associated with DEACMP and mutations in the LP-PLA2 gene are associated with DEACMP.

In the present case control study, we compared the variant frequencies of two LP-PLA2 gene polymorphisms, rs1805017 and rs1051931 (G/A), in 921 Chinese Han individuals from Northern-west Jiangsu Province, to determine if these particular LP-PLA2 variants influence DEACMP susceptibility.

Materials and methods

Study design, setting, population and selection of participants

From January 1998 to August 2014, data was retrospectively collected from 921 patients with acute CO poisoning at the affiliated hospital of Xuzhou Medical college in the north-western Jiangsu province, People's Republic of China. The acute CO poisoning patients were chosen if they had documented exposure to carbon monoxide (elevated carbon monoxide hemoglobin (CO_{Hb}) level or ambient carbon monoxide concentration) or obvious exposure to carbon monoxide, and if they had any of the following symptoms: loss of consciousness, confusion, headache, malaise, fatigue, forgetfulness, dizziness, visual disturbances, nausea, vomiting, and cardiac ischemia. If the CO_{Hb} level was below 10 percent, the patient was eligible only if acute CO poisoning was the only plausible diagnosis. Patients were excluded if they had a history of neurological disease or psychiatric disorders. We defined an age variable of ">35 years" based on a study [16] which reported that being >35 years old was a risk factor for neuropsychiatric sequelae. All patients were monitored or followed-up for 90 days. The DEACMP patients were diagnosed according to the following criteria [17, 18]: (1) acute CO poisoning leading to coma in the previous 1 to 2 months, (2) an intervening "lucid interval" prior to the appearance of delayed symptoms, (3) delayed acute dementia indicating widespread cortical dysfunction as the main clinical manifestation, (4) EEG, CT, and/or MRI abnormalities.

The enrolled patients were divided into two groups: (i) without DEACMP ($DEACMP^-$ Group: 513 cases, 233 men, 280 women, ranging from 37 to 86 years, means 56.33 ± 8.63 years) and (ii) with DEACMP ($DEACMP^+$ Group: 408 cases, 182 men, 226 women, ranging from 39 to 87 years, means 58.32 ± 10.35 years). All the study variables were used for comparisons between groups.

Data collection and definition of variables

All the patients were given routine therapy at the time that acute CO poisoning was suspected. Peripheral blood samples from each patient were extracted and stored in -80 degrees. This study was approved by our institutional Clinical Research Ethics Board and written informed consent was obtained from each patient involved in the study, the reviewers were blinded to the patients' hospital course and outcomes. Information for a number of variables for each patient was recorded. Any variable not present or equivocal in the

Table 1. Genotype and allele frequencies of rs1805017 in samples

Genotype	DEACMP-		DEACMP+		OR (95% C.I.)[a]	*P*-value
	n	%	n	%		
GG	364	70.9	249	61.0	1.00 (reference)	-
GA	133	26.0	128	31.4	1.41 (1.06-1.91)	0.026
AA	16	3.1	31	7.6	2.83 (1.52-5.23)	0.001
G	861	83.9	626	76.7	1.00 (reference)	-
A	165	16.1	190	23.3	1.58 (1.25-1.98)	0.0001
Ptrend						0.0002[b]

Notes: [a]adjusted by appropriate confounders; [b]Chi-square test for linear trend in proportions.

Table 2. Genotype and allele frequencies of rs1051931 in samples

Genotype	DEACMP-		DEACMP+		OR (95% C.I.)[a]	*P*-value
	n	%	n	%		
GG	351	68.4	240	58.9	1.00 (reference)	-
GA	150	29.3	138	33.8	1.34 (0.94-1.72)	0.04
AA	12	2.3	30	7.3	3.65 (0.83-7.18)	0.0002
G	852	83.0	618	75.7	1.00 (reference)	-
A	174	17.0	198	24.3	1.57 (0.99-1.83)	0.0001

Notes: [a]adjusted by appropriate confounders.

patient's medical history or physical exam was considered absent.

Definition of endpoint

We used DEACMP as the primary endpoint.

Genotyping

Genomic DNA was extracted from peripheral blood samples from each participant using the RelaxGene Blood DNA System (Tiangen Biotech, Beijing, China). The LP-PLA2 gene polymorphisms rs1805017 and rs1051931 were chosen. For rs1805017, PCR amplification was performed using TCTTCAATCACCACAGCAGC and TCTGGAGAGTTTGATGGCTT as the forward and reverse primer pairs respectively. For rs1051931, the forward primer ATACTGCTTTGTTCCATTGT and the reverse primer ATCAAGATACCAAGCAAGAAC were used for PCR reaction. Each 20 μL PCR reaction mixture consisted of 1 μL of genomic DNA, 0.5 μL of each primer (10 pmol/L), 10 μL of 2× Taq PCR Mastermix (20 mM Tris-HCl, pH 8.3, 100 mM KCl, 3 mM $MgCl_2$, 0.1 U Taq Polymerase/μL, 500 μM dNTP each; Fermentas, USA), and 8 μL of ddH_2O (DNase/RNase-free). After initial denaturizing at 95°C for 5 min, the reaction mixture was subjected to 33 cycles of 45 s denaturation at 95°C, 30 s annealing at 53°C and extension 50 s at 72°C, followed by a final 10 min extension at 72°C. Then 1 U of Bcl*I* (rs1805017) or Cla*I* (rs1051931) restriction enzyme was added directly to the PCR products (10 μL) and digested at 37°C overnight. After restriction enzyme digestion of the amplified DNA, genotypes were identified by electrophoresis on 1.5% agarose gels and visualized with ethidium bromide staining ultraviolet illumination. 10% samples detected by the PCR-RFLP were also confirmed by direct sequencing (Invitrogen). The three genotypes resulting from digestion with Bcl*I* were GG (191 bp, 499 bp), AG (690 bp, 191 bp, 499 bp) and AA (690 bp) for 1805017. Similarly, the three genotypes yielded by digestion with Cla*I* were GG (522 bp), AG (173 bp, 349 bp, 522 bp) and AA (173 bp, 349 bp) for rs1051931.

Statistical analyses

All genetic analysis was performed using the SNPStats, a web tool. Genotype and allele frequency between two groups were compared using *Chi*-square test. Hardy-Weinberg equilibrium (HWE) was assessed using the chi-square test with one degree of freedom. Odds ratios (ORs) and 95% confidence intervals (95% C.I.) were calculated to evaluate the effects of alleles and genotypes. To evaluate interactions between SNP and sex, a global test for interaction was performed, in addition to a test for the interaction in the linear trend of the nested variable. Taking into account a possible effect of age adjusts their models by age. A two-tailed $P \leq 0.05$ was considered statistically significant.

Results

We compared variants of the LP-PLA2 gene SNPs rs1805017 (G/A) and rs1051931 (G/A) between patients with DEACMP (n=408) and without DEACMP (n=513). Of the total cases (921), all cases were genotyped for rs1805017 and rs1051931. The genotype and allele fre-

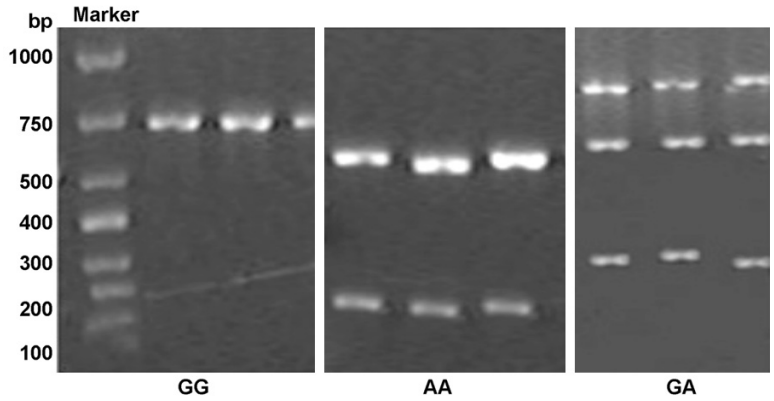

Figure 1. Results of rs1805017 electrophoresis and direct sequencing.

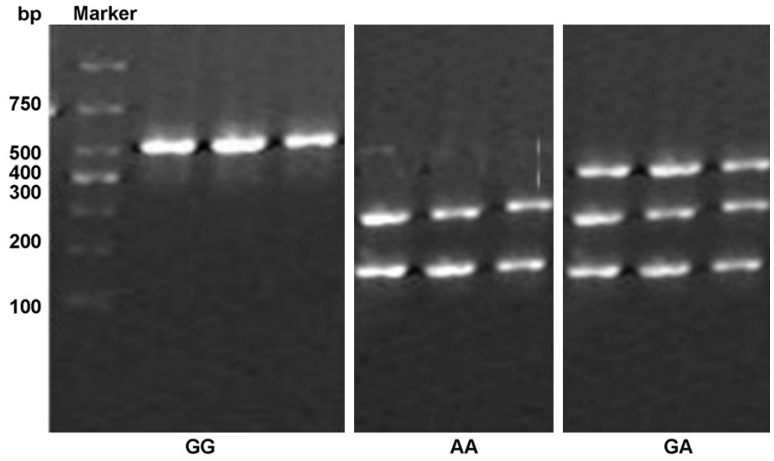

Figure 2. Results of rs1051931 electrophoresis and direct sequencing.

quencies of both polymorphisms are presented in **Tables 1** and **2**. There was a significant difference in rs1805017 allele frequencies between DEACMP$^-$ and DEACMP$^+$ group (*OR*=1.41; *OR*=2.83; *P*<0.05). There was significant difference in rs1805017 allele frequencies between both DEACMP$^-$ and DEACMP$^+$ group (*OR*=1.34; *OR*=3.65; *P*<0.05), however, the C.I. value showed it as invalid data. Electrophoresis and direct sequencing are consistent in **Figures 1** and **2**.

Discussion

The incidence of CO poisoning is still high in China, especially in some colder areas such as northwestern Jiangsu province of China, A high proportion of patients still suffer severe poisoning due to DEACMP, which has a great harm to the humans, because there were no accurate time and exact causes, no gender distinction of the incidence, There's no predictive likelihood of occurrence so that we could not timely prevent it. Even if the general prevention still did not obviously reduce the occurrence rate. The treatment is no specific and only symptomatic treatment. Although patients with severe DEACMP receive the full course of hyperbaric oxygen therapy (HBOT), many are still left with serious sequelae. Moreover, there is a lack of early prognostic indicators [19].

The mechanism involved in the development of DEACMP is unclear. Demyelination and destruction of cerebral white matter (WM) induced by CO are considered to be the main pathologic features of delayed encephalopathy. Choi et al. [20]. found that cerebral white matter lesions were more commonly associated with neurological sequelae than globus pallidus lesions. DEACMP can result from demyelination of the cerebral WM. WM rarefaction may be present with axonal damage and gliosis. To date, the pathogenesis of demyelinating leukoencephalopathy has been shown to include apoptosis, lipid peroxidation, and inhibition of the mitochondrial electron transfer enzyme system, mitochondrial oxidative stress and adaptive immunological response [21-23].

Through clinical research, we found that a significant association between the LP-PLA2 rs1805017 polymorphism and DEACMP in the Han population of Northwestern Jiangsu Province. That is to say, allelic variants of the LP-PLA2 gene may influence the susceptibility to DEACMP. As we know, Lipoprotein-associated phospholipase A2 (Lp-PLA2) belongs to the phospholpase A2 superfamily. Plasma Lp-PLA2 is an enzyme mostly produced by inflammatory cells including macrophages. About 80% of Lp-PLA2 is bound to low-density lipoprotein (LDL) through apolipoprotein B (apo B), in circu-

lation. Lp-PLA2 hydrolyzes oxidized LDL, Forming lysophosphatidylcholine and oxidized nonesterified fatty acids, which are inflammatory molecules [9]. However, Lp-PLA2, also known as platelet-activating factor acetylhydrolase, can also hydrolyze platelet activating factor, which is involved in activating platelets, monocytes, and macrophages [4]. These products encourage expression of inflammatory cells and their consequent apoptosis to foam cells. Higher Lp-PLA2 is associated with the stimulation of several pro-inflammatory cytokines, which leads to necrosis of sclerotic plaque core, The affected sclerotic plaques is prone to rupture, and this kind of unstable plaque is clinically associated with risk of coronary heart disease and stroke independent of traditional cerebrovascular risk factors [24]. A meta-analysis involving over 79,000 individuals reported modest increases up to 15% of coronary heart disease, stroke and vascular mortality which is related to 1 SD higher Lp-PLA2 mass and activity [25]. Lp-PLA2 may also be associated with risk of developing dementia.

So we initially hypothesized that LP-PLA2 may play a role in pro-inflammatory effects associated with DEACMP and Mutations in the LP-PLA2 gene which may be associated with DEACMP. Our research suggests that testing the genotype and allele frequencies of LP-PLA2 may help physicians to identify those which would develop DEACMP at a high risk. Furthermore, this may help physicians make decisions about treatment strategies for patients with acute CO poisoning. In patients with a higher risk for DEACMP, earlier treatment and more appropriate utilization of healthcare services, including hyper baric oxygen and close follow up, should be considered.

Conclusions

In conclusion, we demonstrated a significant association between the LP-PLA2 rs1805017 polymorphism and DEACMP among the Han population from North-western Jiangsu Province. Thus, allelic variants of the LP-PLA2 gene may influence the susceptibility to DEACMP. However, it is still unclear whether other SNPs within LP-PLA2 also influence the susceptibility to DEACMP. More efforts should be made to treat patients with such characteristics. These studies may help reveal the functional mechanisms of DEACMP and the role of LP-PLA2 mutations in neurodegeneration. It is vital for clinician to recognize and properly treat the patients who have the susceptibility to DEACMP.

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Disclosure of conflict of interest

None.

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