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Genetic polymorphisms of cytokine genes and risk for trichloroethylene-induced severe generalized dermatitis: A case-control study

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Trichloroethylene (TCE)-induced severe generalized dermatitis (SGD) is considered to be a contact allergic disease and is dependent on a cell-mediated immune response. Little is known about its pathogenesis. Several lines of evidence suggest that tumour necrosis factor (TNF) and interleukin 4 (IL-4) are involved in the immunological and inflammatory reactions. To investigate the relation between polymorphisms of TNF and the IL-4 gene and the risk of TCE-induced SGD, a case-control study was conducted consisting of 111 patients diagnosed with SGD and 152 TCE-exposed workers without SGD. Polymerase chain reaction-restriction fragment length polymorphism was used to detect the polymorphisms of TNF-α (G-238A, G-308A), TNF-β (intron 1) and IL-4 (C-590T). Logistic regression was applied to calculate the odds ratios (OR) and 95% confidence intervals. The results reveal that the frequency of TNF α-308 wild allele in cases was significantly higher than that in control subjects ($p = 0.049$). Individuals with a heterozygous genotype of TNF α-308 were associated with the decreased risk of TCE-induced SGD relative to the homozygous genotype (OR = 0.398, 95% CI = 0.164–0.967). No significant differences in the allele and genotype frequencies could be demonstrated at any other polymorphic loci among both groups. The finding of a possible contribution of a TNF-α genetic polymorphism is a primary result because the pathogenesis of TCE-induced SGD is complex and likely to involve the interaction of a number of genes. A further study should be conducted to illustrate the influence of a link between certain relevant alleles in the assessment of genetic susceptibility.

Keywords: trichloroethylene, severe generalized dermatitis, tumour necrosis factor, genetic polymorphism, interleukin 4.

Introduction

Trichloroethylene (TCE) is an industrial solvent mainly used in metal degreasing and dry cleaning. It is also present in household products, including typewriter correction fluid, paint removers, adhesives and spot removers. It causes acute and chronic toxicities and tumours involving multiple target organs in animals and humans (National Toxicology Programme 1976, 1990). The symptoms of TCE exposure include central nervous system depression, abnormal liver and kidney function, and irritating effects to the skin and mucous membranes of the respiratory tract (Reichert 1983).

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Recently, an increasing number of cases of TCE-induced severe generalized dermatitis (SGD) in TCE-exposed workers were reported in China (Li *et al*. 1998, Kuang *et al*. 1999, Zhang and Huang 2000). Since 1980, more than 200 cases of SGD were diagnosed according to the authors' survey in Guangdong Province, China. The clinic manifestations of the TCE-induced SGD include exfoliative dermatitis, multiform erythema and epidermolysis bullosa, accompanied by liver dysfunction and fever. These characters were clearly different from occupational TCE toxicity reported previously, which usually manifests as non-specific skin irritation and defatting as a result of exposure to TCE. The TCE-induced SGD has not been included in US Environmental Protection Agency (2001). Although TCE has been used in industry for nearly a century worldwide, only a few cases of TCE-induced SGD were reported in countries other than China. The dramatic ethnic difference in the TCE-induced SGD strongly suggests that genetic factors influence individual susceptibility to the disease. Furthermore, a significant number of cases have died because of a lack of an effective treatment method. The TCE-induced SGD appears to be a new occupational health issue that requires effective measures of control and prevention urgently. At present, no study of the mechanism for the TCE-induced SGD has been reported, except for some case reports.

Occupational epidemiological data on the TCE-induced SGD in Guangdong reveal that all cases occurred within 3 months of exposure to TCE, but no clear dose–response relationship was observed between TCE exposure and the incidence of the SGD. Although the epidemiological evidence indicates that occupational exposure to TCE is a major risk factor of SGD, only a small fraction of TCE-exposed workers developed the disease. A cross-sectional study shows that the incidence of the disease is less than 1% in Guangdong Province (Li *et al*. 1998). It suggests that genetic susceptibility and the interaction between genetic and environmental factors may play an important role in the aetiology of the TCE-induced SGD. Because of the clear exposure history, a consistent time frame of disease onset after exposure, severe clinical manifestations, an apparent idiosyncrasy of the TCE-induced SGD, and positive result in a patch test with TCE and its metabolites in fraction of patients, the disease was suggested to be an allergic contact dermatitis (ACD) (Nakayama *et al*. 1988, Yang 1999). In an animal study, Tang *et al*. (2003) also provided evidence that TCE exhibits contact allergenic capability by guinea pig maximization test.

Allergic contact dermatitis is one of the most common inflammatory diseases of the skin regarded as a prototype of T-cell-mediated delayed-type hypersensitivity reaction with sensitization and elicitation phases (Grabbe and Schwarz 1998). In the sensitization phase, contact of allergic compounds on the skin leads to the secretion of tumour necrosis factor alpha (TNF-α) and granulocyte/macrophage colony-stimulating factor (GM-CSF) by keratinocytes. Low-molecular-weight haptens stimulate the additional release of interleukin 1β (IL-1β), IL-10 and macrophage inflammatory protein 2 (MIP-2), these cytokines activate epidermal Langerhans cell (LC), dermal dendritic cells (DC) and endothelial cells, leading to an accumulation of even more DC at the site of antigen contact. LC or DC capture the haptens and migrate from the skin to regional lymph nodes where they can present to hapten-specific naive T-cells (Novak and Bieber 2000). TNF-α may also

represent an important stimulus for the movement of LCs from the epidermis following skin sensitization (Cumberbatch *et al*. 1994). Stimulation of hapten to CD4+ and CD8+ hapten-specific T-cells lead to proliferation and differentiation to effective T-cells and memory T-cells. Upon the second contact of the skin with a hapten, i.e. during the elicitation phase of ACD, allergen presenting cells (APC) may stimulate the antigen-specific memory T-cells and contribute to the initiation of the local inflammatory response, i.e. the dermatitis reaction (Novak and Bieber 2000). The CD4+ effective T-cells including Th1 and Th2 subtypes characterized by their typical cytokine pattern, Th1 cells secreting IL2, interferon γ and tumour necrosis factor β (TNF-β, lymphotoxin) is considered relative to contact allergy (cell-mediated immune processes), whereas Th2 cells secreting interleukins 4–6 and 10 are considered relative to humoral immune processes. Cytokines appear to play an important role in the initial generation of divergent Th cell subsets. IL-4 produced by mast cell (a cellular vector of the natural immune system) has been shown to favour the development of Th2 cells and depress dermal contact hypersensitivity reactions (Kimber 1994). Thus it can be seen that the process of allergic response is regulated by a complex network of numerous cytokines, of which the pro-inflammatory cytokine TNF-α and its effect in immunological inflammatory response has attracted particular attention.

The genetic bi-allelic polymorphisms have been described at positions −308 and −238 of the human TNF-α promoter (Labunski *et al*. 2001, Carole *et al*. 2003). It has been shown that the polymorphic variations within the promoter region exert influence on TNF-α production. The polymorphisms in the first intron of TNF-β gene and at position −590 in promoter region of IL-4 gene were also described (Majetschak *et al*. 1999, Soderhall *et al*. 2002). Polymorphisms of genes may result in inter-individual variation in transcriptional regulation and thus in altered expression. Genetic variants could therefore have phenotypic relevance and influence an individual's risk of disease. Accordingly, we have focused on polymorphisms of these genes, for which the functional relevance and the importance during contact allergy have been proposed, and have correlated genotyping data with risk of the TCE-induced SGD.

Materials and methods

Study subjects

From May 1999 to November 2003, 111 patients with SGD and 152 non-SGD TCE-exposed workers were recruited into this case-control study. Both cases and controls came from about 80 factories of electronic elements and metal-plating production in Guangdong Province, China. All subjects were employed in workshops where they cleaned and degreased metals with TCE. The TCE exposed levels in workshops ranged from 69 to 790 ppm based on Zhang and Huang (2000). The cases were diagnosed as occupational SGD by a panel of occupational disease physicians. All cases developed skin damage within 3 months of TCE exposure. These patients' dermatitis began on the hands and forearms, and then progressed to generalized eruption; clinical manifestation such as multiform erythema and exfoliative dermatitis were occurrence. Other potential risk factors, such as the medicine used and a history of previous skin disease, were excluded during diagnosis. The control subjects were selected from the same workshops of cases, but no skin abnormal manifestations were examined by the occupational physicians. The TCE-exposed duration of controls was more than 3 months (3–48 months). The study subjects were first surveyed by a brief questionnaire to determine their willingness to participate in research studies and to obtain personal information. All study subjects were interviewed to elicit data about their age, sex, ethnicity, and working and health history.

After informed consent was obtained, a 10 ml blood sample was obtained from all participants and stored at -70°C for genotype analysis.

The research project was approved by the authors' Institutional Review Board.

Genotyping

Genomic DNA was obtained from blood by the routine high salt method (Miller *et al*. 1988). DNA purity and concentration were determined by spectrophotometric measurement of absorbance at 260 and 280 nm.

The polymorphisms of TNF-α-308, TNF-α-238, IL-4-590 and bi-allelic restriction fragment legend polymorphism in the first intron of the TNF-β gene at positions 1064–1069 were detected by polymerase chain reaction (PCR) amplification and enzymatic digestion of the products according to previously described methods (Majetschak *et al*. 1999, Labunski *et al*. 2001, Soderhall *et al*. 2002, Carole *et al*. 2003). The -308 G→A nucleotide substitution of TNF-α will hereafter be termed '*TNF A II*' (the wild-type is *TNF A I*), whereas the -238 G→A nucleotide substitution is termed '*TNF A*' (the wild-type is *TNF G*), and bi-allelic polymorphism of TNF-β gene is termed '*TNFB1*' and '*TNFB2*', the -590C→T nucleotide substitution of IL-4 is termed '*IL-4*C*' and '*IL-4*T*'. PCR was performed in a 30 μl reaction mixture containing 50 ng genomic DNA, 1 ×PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 9.0 at 25°C and 1% Triton X-100), 2.0 mM $MgCl_2$, 0.20 mM each dATP, dTTP, dCTP and dGTP, 0.25 μM each primer and 1 U *Taq* polymerase (Shanghai Sangon Co., Shanghai, China).

The primer sequences for the -308 G→A polymorphism in the promoter region of the TNF-α gene were 5′-AGG CAA TAG GTT TTG AGG GCC AT-3′ and 5′-TCC TCC CTG CTC CGA TTC CG-3′. The 107-bp product was digested with *Nco*I (New England Biolabs, Inc., Beverly, MA, USA) and separated on 8% polyacrylamide gel stained with ethidium bromide solution (1 μg ml^{-1}). The wild-type allele (*TNF A I*) contains a restriction site for *Nco*I, which results in 87 and 20 bp fragments after digestion, whereas nucleotide substitution G-308A resulted in the loss of the *Nco*I site staying uncut.

The primer sequences for the -238 G→A polymorphism in the promoter region of the TNF-α gene were 5′-GGT CCT ACA CAC AAA TCA GT-3′ and 5′-CAC TCC CCA TCC TCC CTG GTC-3′. The 71-bp product was digested with *Ava*II (New England Biolabs) and separated on 12% polyacrylamide gel stained with ethidium bromide solution (1 μg ml^{-1}). The *TNF A* allele was not digested and gave a single 71 bp fragment, whereas the *TNF G* allele was completely digested (51 and 20 bp fragments).

The primer sequences for the TNF-β gene polymorphism were 5′-CCG TGC TTC GTG CTT TGG ACT A-3′ and 5′-AGA GGG GTG GAT GCT TGG GTT C-3′. The 782 bp fragment of the TNF-β gene including the first intron was amplified and digested with *Nco*I (New England Biolabs). Digestion products were analysed on 2% agarose gel and stained by ethidium bromide. The *TNFB1* allele includes a restriction site for *Nco*I, which results in 196 and 586 bp fragments after digestion. The *TNFB2* allele was not digested and gave a single 782 bp fragment.

The primer sequences for the -590 C→T polymorphism in the promoter region of the IL-4 gene were 5′-TAA ACT TGG GAG AAC ATG GT-3′ and 5′-TGG GGA AAG ATA GAG TAA TA-3′. The 195 bp product was digested with *Ava*II (New England Biolabs) and separated on 8% polyacrylamide gel stained with ethidium bromide solution (1 μg ml^{-1}). The *IL-4*T* allele was not digested and gave a single 195 bp fragment, whereas the *IL-4*C* allele was completely digested (177 and 18 bp fragments).

Statistical analysis

The distribution of allele frequencies between the cases and controls were compared using the χ^2-test. Because age and sex were well matched between cases and controls, the crude odds ratios (ORs) and their 95% confidence intervals (CIs) were calculated by logistic regression analysis to estimate the relative risk. A $p = 0.05$ was considered to be statistically significant. All statistic tests were two sided and performed using Statistical Analysis System software (version 6.01, SAS Institute, Inc., Cary, NC, USA).

Results

The case-control study consists of 111 cases, who were diagnosed by a panel of occupational physicians, and 152 control subjects, who were TCE-exposed workers without SGD. The TCE exposure duration of all control subjects was more than 3 months before examination. The cases and controls were frequency matched by gender and age with $p = 0.4632$ (χ^2-test) and 0.8061 (t-test), respectively. The demographic data of the cases and controls were summarized in table 1. Among the

Table 1. Demographic data of the case and control groups.

	Cases					
	Total	Exfoliative dermatitis	Multiform erythema	Epidermalysis Bullosa	Controls	*P* value
Age (mean ± SD) (years)	22.8 ± 5.49	22.6 ± 5.15	22.4 ± 5.61	32.3 ± 3.06	23.0 ± 4.69	0.806
Gender						
Male (*n*, %)	49 (44.1)	35 (46.7)	13 (39.4)	1 (33.3)	74 (48.7)	0.463
Female (*n*, %)	62 (55.9)	40 (53.3)	20 (60.6)	2 (66.7)	78 (51.3)	
Total (*n*)	111	75	33	3	152	
TCE exposure duration (month)	<3	<3	<3	<3	>3	

SGD cases, 75 patients exhibited exfoliative dermatitis, 33 patients multiform erythema and three patients epidermolysis bullosa. These clinical manifestations were different from most of the TCE toxicities previously reported, which includes non-specific skin irritation and defatting (McCunney 1988).

The distributions of genotype and allele frequencies of the cytokine gene polymorphisms in cases with SGD and controls were shown in table 2. The polymorphism frequencies in controls were in keeping with the Hardy–Weinberg equilibrium. For TNF-α-308 polymorphism site, the frequency of *TNF A I* allele was increased in cases compared with that in control subjects ($p = 0.049$). The frequencies of the *TNF A I/I* and *I/II* genotypes in cases were 93.7 and 6.3%, respectively, and in controls were 85.5 and 14.5%, respectively. The difference in

Table 2. Genotype and allele frequencies of cytokines in the case and control groups.

		Cases (*n* = 111)		Controls (*n* = 152)			
Gene	Genotype	*n*	%	*n*	%	Crude OR (95% CI)	*P* value
TNF-α −308 site	*TNF A I/I*	104	93.69	130	85.53	1	
	TNF A I/II	7	6.31	22	14.47	0.398 (0.164–0.967)	0.042
	I allele frequency		96.85		92.76	1	
	II allele frequency		3.15		7.24	0.417 (0.175–0.995)	0.049
TNF-α −238 site	*TNFG/G*	106	95.50	148	97.37	1	
	TNFG/A	5	4.50	4	2.63	1.745 (0.458–6.653)	0.415
	G allele frequency		97.75		98.68	1	
	A allele frequency		2.25		1.32	1.324 (0.351–4.994)	0.679
TNF-β	*TNFB1/1*	27	24.32	34	22.37	1	
	TNFB1/2	58	52.25	81	53.29	0.903 (0.484–1.684)	0.738
	TNFB2/2	26	23.42	37	24.34	0.905 (0.436–1.880)	0.736
	B1 allele frequency		50.4		49.35	1	
	B2 allele frequency		49.6		50.65	0.944 (0.668–1.335)	0.745
IL-4	*IL-4*T/T*	72	64.86	89	58.55	1	
	*IL-4*C/T + IL-4*C/C*	39	35.14	63	41.45	0.765 (0.461–1.269)	0.300
	T allele frequency		81.1		78.6	1	
	C allele frequency		18.9		21.4	0.858 (0.556–1.324)	0.489

genotype distribution of TNF-α-308 between two study groups was statistically significant ($p = 0.042$). The homozygous variant genotype *II*/*II* was not found in this population.

To evaluate the association between the TNF-α-308 polymorphism and the risk of TCE-induced SGD, crude OR and 95% CI were calculated. Using genotype *I*/*I* as a reference, the risk of SGD was significantly decreased in individuals with genotype *I*/*II* (OR = 0.398, 95% CI = 0.164–0.967) (table 2).

For TNF-α-238, TNF-β and IL-4-590, no statistical differences in the frequencies of allele and genotype between two groups were found (table 2).

Discussion

TNF-α is an important mediator of a wide variety of immunological and inflammatory reactions (Beutler and Cerami 1989, Strieter *et al*. 1993). It is likely to play a crucial role during contact allergic reactions (Kimber *et al*. 2000). It is primarily involved in amplifying inflammation by stimulating resident skin cells to synthesize chemokines that attract inflammatory cells and membrane molecules important for the retention and activation of T-cells. TNF-α gene lies in the class III region of the major histocompatibility complex (MHC) on the short arm of chromosome 6 (Carroll *et al*. 1987). The association between polymorphism of TNF-α promoter region and function has been reported in many studies with conflicted results. Higher transcriptional activity of the *TNF A II* allele was observed *in vitro* by using reporter gene constructs (Wilson *et al*. 1994). This result was challenged by Brinkman *et al*. (1995–96) and Stuber *et al*. (1996), who did not find a relation between the two TNF-α-308 alleles and the TNF-α production. *In vivo* studies have also shown conflicting results. Bouma *et al*. (1996) found individuals with homozygous *TNF A II* allele have higher levels of TNF-α than *TNF A I* homozygous, while Chen *et al*. (1996) showed high levels of TNF-α associated with the *TNF A 1* allele. In the present study, the frequency of *TNF A I* allele in patients was significantly higher than that in the control group, which suggested that the *TNF A I* allele might be a risk factor for TCE-induced SGD. Although the underlying molecular mechanism for the association between TNFα gene polymorphism and the risk of SGD is unclear, and the studies on the function change and potential biological significance of the −308 polymorphism do not give consistent results, it is hypothesized that the polymorphism of the TNF-α promoter region may effect the process of TCE-induced SGD, or the association observed here is the result of linkage disequilibrium of the polymorphism with other unknown alleles in the genome.

The result of an association between TNF-α polymorphism and allergic disease reported here is in agreement with Albuquerque *et al*. (1998), who found that homozygosis for allele *TNF A I* was associated with a five-fold increased risk of asthma in children. The result is contrary to Westphal *et al*. (2003), that carriers of the allele *TNF A II* tended to be more common among polysensitized individuals. In that study, the population was of Caucasian origin, polysensitized patients were defined as individuals with confirmed contact sensitization to *para*-substituted aryl compounds and at least one other structurally unrelated allergen, and the control

group consisted of healthy unrelated individuals without a history of eczema. For both groups, allergen exposure could not be investigated. The possibility could not be exclude that the control group includes individuals with genetic predisposition but low allergen exposure, while the polysensitized group may contain individuals characterized by high allergen exposure but a lack of prominent genetic risk factors. However, in the present study, the cases and controls were all TCE-exposed workers in the same workshop where TCE was a specific allergen. Thus, the discrepant results are possibly partly due to the difference in the selection of subjects, the sorts of allergen and the initial trigging event of the immunological reaction. Moreover, it cannot be excluded that the association observed here and in other studies is the result of a linkage disequilibrium of the polymorphism with other alleles in the HLA region (Albuquerque *et al*. 1998).

There was limited information about the association between IL-4 and ACD. Szepietowski *et al*. (1997) observed a significant increase in the mRNA expression for IL-4 after nickel challenge in subjects with previously proven contact allergy to nickel, and atopic patients and non-atopic subjects have a similar result in IL-4 expression. The polymorphism of IL-4 gene has been reported to be associated with atopic dermatitis susceptibility and severity (Kawashima *et al*. 1998, Soderhall *et al*. 2002). However, there was no data about the association between IL-4 gene polymorphism and ACD. The present study used a case-control design to address the possible role of IL-4 genetic polymorphism in TCE-induced SGD. The results showed that the frequencies of IL-4 genotype were similar in case and control groups, the association between IL-4-590 polymorphism and the SGD was not found.

The distributions of TNF-β and IL-4 alleles in controls were consistent with those described in the literature for the Chinese population (Wu *et al*. 2003, Fei *et al*. 2004), and the distributions of TNF-β genotype and allele were similar in case and control subjects, which suggested no selection bias for the subjects' enrolment in terms of genotypes. With regard to the difference in the TNF-α genotyping result in other literatures, ethnic and geographical differences should be considered, which might be just the reason for higher incidence of TCE-induced SGD in Guangdong.

In conclusion, the pathogenesis of TCE-induced SGD is complex and likely to involve the interaction of a number of genes. For example, the genetic polymorphisms of TCE-metabolizing enzymes might affect the risk of the disease (Nakajima *et al*. 2003). Huang *et al*. (2002) found that N-acetyltransferase 2 slow genotype significantly increased the risk of TCE-induced dermatitis. Therefore, the assessment of a single polymorphic genotype is thus generally not enough to evaluate individual susceptibility to the disease, but a combination of a variety of genetic susceptibility factors needs to be considered. The finding of a possible contribution of a TNF-α genetic polymorphism is a primary result. A further study should be conducted to illustrate the influence of linkage between certain relevant alleles in the assessment of genetic susceptibility and to address the molecular biological and immunological mechanism of the initiation and development of TCE-induced SGD.

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