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Research Article

Genetic variations underlying trichloroethylene-induced occupational medicamentosa-like dermatitis identified through massive parallel sequencing

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Abstract

Trichloroethylene (TCE) induced occupational medicamentosa-like dermatitis (OMDT) is a systemic skin disorder accompanied by liver dysfunction in workers occupationally exposed to TCE. The present study employed massive parallel sequencing by AluScan developed by us to determine genetic variations associated with sensitivity to TCE exposure. With coverage of 14.54 Mb genome regions with at least four-fold depth across two OMDT patients and two control subjects, single-nucleotide polymorphism (SNP) rs941960 located in the CCL1 gene coding for cytokine conjugate-beta lyase, and copy number gains in CYP2C9 and CYP3A4 were identified as plausible causal factors of OMDT, all of which are components of TCE metabolic pathways. These results ascertained that variants of these genes could increase the concentrations of metabolic intermediates of TCE that may act as antigens to trigger abnormal immunological responses and give rise to liver dysfunction.

Introduction

1,1,2-Trichloroethylene is a colorless transparent liquid with odor like chloroform. It is widely used as a degreaser for metal parts for more than 70 years on account of its being an effective solvent for a variety of organic materials [1]. However, concern with the toxicity of TCE is increasing while the wide range of applications. Acute exposure to high concentrations TCE could cause headache, defatting dermatitis, lethargy, pulmonary edema, renal and hepatic damage, etc. In long-term exposure, patients experienced similar symptoms as in the acute exposure but with greater severity and persistence [2]. Risks of kidney carcinogenesis, liver carcinogenesis and non-Hodgkin lymphoma (NHL) were also enhanced with the long-term exposure [3]. In addition, a systemic skin disorder accompanied by liver dysfunction was reported in TCE occupational exposure cases. Differ from acute exposure cases, the severity of the disorder was not related to the concentrations or the duration of exposure; the period between onset and exposure was about one month, the mean age of patients was 29, and there was no bias between genders. Only a few cases were typically reported for any one work place [4]. The disorder has been reported in USA, England, Singapore, Japan, etc. In China, the number of cases increased significantly since the mid-1990. By 1998, there were at least 300 cases and several deaths reported, making this systemic skin disorder as one of the serious occupational health diseases [5]. In the diagnostic criteria of occupational medicamentosa-like dermatitis due to trichloroethylene (OMDT), this disorder is classified into four types: exfoliative dermatitis (ED), erythema multiform (EM), Stevens-Johnson syndrome (SJS), and toxic epidermal necrolysis (TEN) [6].

In skin sensitization test on guinea pig, IgG levels were found to be upgraded significantly suggesting that humoral immunity was involved in OMDT [7]. Immune tests in rats also showed that TCE can activate T lymphocytes, and stimulate B lymphocytes to secrete antigen-specific IgG antibodies [8]. Besides, several lines of evidence demonstrated the involvement of human leucocyte antigen DM (HLA-DM) [9], tumor necrosis factor (TNF) and interleukin 4 (IL-4) [10] in the immunological and inflammatory responses in OMDT. These studies suggested that OMDT might represent a type IV allergy. However, type IV allergies usually do not cause such serious illness with large areas of evident necrosis with peeling skin, and liver, kidney and other organ failures, raising the possibility that some metabolic products could be involved in the pathogenesis of OMDT. Accordingly, AluScan with high throughput DNA sequencing was applied in the present study to search for metabolic genes that can participate in OMDT etiology.

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Alu elements are a family of primate-specific short interspersed nucleotide elements (SINE) originated from 7SL RNA [11] that contains the three major subtypes of Alu J, Alu S and Alu Y. There are over 1 million copies of Alu-elements comprising 10% of the human genome [12]. They are enriched in gene-rich regions and enhance recombination rates in the regions [13]. Variations surrounding a human lineage-specific Alu Y located in the GABBR2 gene were found to be associated with schizophrenia and bipolar disorder [14-15]. This region also constitutes a focal point for positive evolutionary selection [16] and a recombination hotspot [17].

AluScan sequencing, which amplifies inter-Alu sequences using Alu consensus sequence-based PCR primers for next generation sequencing (NGS), combines the advantages of the wide distribution of Alu elements with that of NGS to permit an examination of the gene-rich inter-Alu regions of human genome for candidate genes of diseases. The method reduces not only sequencing costs and computation workload but also DNA sample requirement to the sub-microgram level. With the use of three Alu consensual primers, it captures ∼14 Mb amplicons between 0.2-6.0 kb in length if no mismatch in primers was allowed. Allowing single mismatches, coverage is increased to ∼106 Mb [18]. Application of AluScan to a single pair of control and glioma DNA generated over 10 Mb sequences covering more than 8,000 genes, resulting in the identification of 357 loss of heterozygositites, 341 somatic indels, 274 somatic SNPs, and 7 potential somatic SNP hotspots [18]. Moreover, CNV can be called efficiently from AluScan sequencing data with an AluScanCNV package. In the present study, SNP and CNV analysis based on AluScans have been employed to identify the genomic basis of patients' sensitivity to the TCE.

Materials and methods

Blood samples

All study participants gave their written consent in accordance with the Declaration of Helsinki. Four samples of peripheral white blood cells including two cases (Case 1 and 2) and two controls (Control 1 and 2) were supplied by Shenzhen Center for Disease Control (CDC) (refer to Table S1 for more information). The two controls were similar in age as the cases. Biochemical analyses of the two patients (Table S2) provided by CDC suggested the presence of liver damage: Case 1 was diagnosed as ED, and Case 2 was diagnosed as EM. All DNA samples were extracted from peripheral blood using the AllPrep kit (Qiagen).

Inter-Alu PCR and next-generation sequencing

Four samples including the two cases and two controls were subjected to AluScan analysis as described previously [18] using the four Alu-consensual primers AluY278T18, AluY66H21, R12A/267 and L12A/8 (Figure S1). Sequence reads between 200 bp to ∼6 kb in length were retrieved to build library and sequenced on the Illumina platform with 100 bp paired-end reads at BGI, Shenzhen China.

Reading mapping and variant analysis

Sequence reads from each sample were mapped to the hg19 reference human genome employing BWA (bwa-short algorithm version 0.6.1-r104) with default settings. The mapping results were transformed into sorted BAM format and indexed with SAMtools version 0.1.19, mark duplicated with Picard (version 1.115), recalibrated and locally realigned using the Genome Analysis Toolkit (GATK version 2.1-8). The UnifiedGenotyper module in GATK was used to call the initial SNPs with the parameter ‘-dco 50’. SNPs were filtered if more than one samples yielded a depth less than four-fold and genotyping quality was less than 10 at the site, and only SNPs with different genotypes between the cases and controls were retained for analysis. Indels were called by pindel (version 0.2.5a8) with default parameters. The results were also filtered to ensure at least eight folds coverage and indel lengths of less than 10 bp. The SNPs and indels obtained were annotated separately by SeattleSeq Annotation 138.

Pathway enrichment analysis

Genes bearing SNPs or indels were submitted to WebGestalt [19] for pathway enrichment analysis to determine the distribution within the KEGG database. All the genes submitted were classified according to the GO annotation prior to enrichment analysis. A hypergeometric test with Benjamini-Hochberg multiple comparison adjustment correction [20] was employed to assess the significance of gene enrichment, using the whole genome as the reference set. The top ten pathways enriched were selected to find differences between the cases and controls.

Variants verification

Potential SNPs on all four samples were selected for verification with two-step PCR. Following purification, the amplicons were loaded onto ABI 3130 Genetic Analyzer (PE Applied Biosystems) using the BigDyeTM Terminator Cycle Sequence kits with AmpliTaq DNA Polymerase to yield the DNA sequences for genotype determination.

CNV analysis

AluScanCNV package [21] was applied to the sequences data to reveal large extended CNVs. For this purpose, the reference genome was divided into contiguous 5 kb windows for the read-depth calculation. Neighboring windows with the same read-depth ratio were joined together by means of the circular binary segmentation algorithm. Genes located in regions where copy numbers were different between cases and controls were identified.

Results

An average of ∼14 M reads was obtained for each individual DNA sample (Table 1). Owing to higher duplication, the efficacious mapped reads of the cases were fewer than the controls. However, the coverage was still >20 Mb for the cases with a depth ≥ 4. The overlap regions across all four samples amount to ∼14.54 Mb with at least four-fold depth, containing 7.32 Mb genic regions. Figure 1 and Table S3 show the distributions of reads and genetic variations occurring in each sample.

A total of 6997 SNPs (Table S4) covering 1978 genes displayed different genotypes between the cases and controls. Among these SNPs, 2615 SNPs had been genotyped by dbSNP. Only 5 SNPs were nonsense and 3638 SNPs were located in introns (Figure 2). Furthermore, 398 indels (Table S5) covering 209 genes were called after

| Table 1. Statistics of AluScan mapping results of cases and controls. |
|---------------------------------------------------------------|
|                   | Case 1 | Case 2 | Control 1 | Control 2 |
| Total initial reads (million pairs) | 14.09 | 13.92 | 14.69 | 15.71 |
| Duplicates (million reads) | 7.86 | 7.06 | 2.62 | 4.20 |
| Mapped (properly paired) | 11.83 | 11.03 | 10.73 | 13.93 |
| Genomic regions mapped with coverage ≥ 1 (Mb) | 76.71 | 76.08 | 284.38 | 226.61 |
| Mean Coverage | 11.01 | 13.83 | 5.79 | 9.01 |
| Genomic regions mapped with coverage ≥ 4 (Mb) | 23.58 | 22.40 | 55.3 | 59.52 |
| Gene regions mapped with coverage ≥ 4 (Mb) | 12.14 | 11.51 | 28.91 | 31.3 |

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filtration; 243 of the indels were located in introns, whereas no indel was located in coding regions (Figure 2). Merging all the genes with sequence variations, altogether 2070 genes were subjected to pathway enrichment analysis. Based on GO Slim classification (Figure S2), most of the genes belonged to the metabolic process. According to KEGG pathway enrichment analysis (Table 2), 124 of the genes containing mutated SNPs and indels were significantly enriched in metabolic pathways ($P_{adj}=1.57E^{-17}$), suggesting the possible involvement of genes in the metabolic process group.

Based on the annotation results, two SNPs (rs941960 and rs11246020) were selected for verification by Sanger sequencing (Table 3).
In addition, with AluScanCNV analysis, 9 regions covering 4011 genes (Table 4) were found to be different in copy number between cases and controls. SNP rs941960 was found to be located in the copy number gain regions in the patients relative to controls. These two members of the cytochrome P450 family of oxidative enzymes therefore could also be significant factors underlying sensitivity to TCE.

**Discussion**

Genomic studies provide powerful tools in the investigation of the relationship between occupational diseases and genetic variations. Based on the microarrays, a recent study revealed distinct transcriptional profile changes in gene regulation of the MAPK/ERK signaling cascade and ubiquitin–proteasome system between mice and rats, in accord with species-specific influences in liver carcinogenesis, under exposure to TCE [26]. In this study, AluScan sequencing was employed to search for mutations in candidate genes that could play a role in OMDT pathogenesis through comparison of genomic sequences in OMDT patients and asymptomatic controls working in the same workplace. Although the number of available samples was limited, we found three genes that displayed genotypic differences between case and controls, all of which are known to participate in TCE metabolism.

There are two major pathways of TCE metabolism in the human body (Figure 3), viz. cytochrome P450 (CYP1/2)-dependent oxidation and conjugation with glutathione (GSH) catalyzed by glutathione S-transferases (GST) [27]. In the P450-dependent pathway, TCE is oxidized to chloral hydrate (CH) in the liver and lungs. CH undergoes oxidation by aldehyde dehydrogenase (ALDH) or reduction by alcohol dehydrogenase (ADH) to form trichloroacetate (TCA) or trichloroethanol (TCHO), respectively. A study on 108 OMDT patients and 145 healthy controls exposed to TCE in the same workplace found that the frequency of heterozygous ALDH2 in controls was significantly higher than in cases (43.4% vs 27.8%, P = 0.011), suggesting that ALDH2 produces the bioactive product TCA and its metabolite chloroacetic acid, which may form antigens upon combination with proteins [23]. Copy numbers of CYP3A4 and CYP2C9 could also affect individual susceptibility to OMDT. Results of human hepatic microsomal samples indicated that increase in CYP2E1 activity enhances individual susceptibility to TCE-induced toxicity [28]. Therefore, the copy number gains of CYP3A4 and CYP2C9 obtained in the present study could increase gene expression, thereby increasing transformation of TCE to chloral and TCA to result in the development of OMDT.

In a second metabolic pathway, TCE is conjugated with GSH to form S-(1,2-dichlorovinyl) glutathione (DCVG) in the liver and kidneys. Next, DCVG is hydrolyzed by renal γ-glutamyltransferase to form S-(1,2-dichlorovinyl)-L-cysteine (DCVC). DCVC is bioactivated by cysteine conjugate β-lyase (CCBL) to form the reactive intermediate S-(1,2-dichlorovinyl) thiol (DVT), or detoxificated by cysteine conjugate β-lyase (CCBL) to form reactive intermediate S-(1,2-dichlorovinyl) glutathione (DCVG) in the liver and kidneys. Both of these products of the detoxification processes are highly reactive and can cause direct damage to DNA and protein [29]. The detoxification process of TCE includes conjugation with glutathione (GSH), which is a major detoxification pathway for TCE, followed by possible activation of the activated compound to induce carcinogenesis. Subsequently, the activated compound could induce carcinogenesis and induce DNA damage.

Since the genetic alterations, we found in CCBL1 and cytochrome P450 all participate in TCE metabolism, these results suggest that TCE metabolites are important to the development of OMDT. In keeping with the results of the genetic studies, we found that the frequency of heterozygous ALDH2 in controls was significantly higher than in cases (43.4% vs 27.8%, P = 0.011), suggesting that ALDH2 produces the bioactive product TCA and its metabolite chloroacetic acid, which may form antigens upon combination with proteins [23]. Copy numbers of CYP3A4 and CYP2C9 could also affect individual susceptibility to OMDT. Results of human hepatic microsomal samples indicated that increase in CYP2E1 activity enhances individual susceptibility to TCE-induced toxicity [28]. Therefore, the copy number gains of CYP3A4 and CYP2C9 obtained in the present study could increase gene expression, thereby increasing transformation of TCE to chloral and TCA to result in the development of OMDT.
with these findings, pathway enrichment analysis also showed that the genomic differences between OMDT patients and controls were enriched in metabolic pathways. Therefore, the conclusion may be drawn that metabolites could lead to the production of active antigens to initiate the immune responses that are pivotal to the development of OMDT.

Author's contributions

HX and SBL conceived and initiated the study and helped draft the manuscript; ZGW, PL, JFY, SYT participated in data analysis and wrote the paper. PL, MKM, YBK, XYX, GHL, JHY, GHL developed the clinical approach and provided samples. All authors have read and approved the final manuscript.

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