Biomarkers and Genomic Medicine (2015) 7, 159–164

Available online at www.sciencedirect.com

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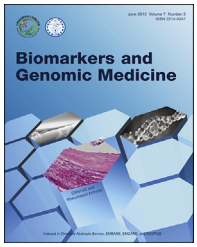


ORIGINAL ARTICLE

Association of *CYP2E1* and *CYP1A1m2* (*BsrD1*) polymorphisms with cytogenetic biomarkers in petrol pump workers

Kanupriya [a], Anita Yadav [a,*], Neeraj Kumar [a], Sachin Gulati [a], Neeraj Aggarwal [c], Ranjan Gupta [b]

[a] *Department of Biotechnology, Kurukshetra University, Kurukshetra, Haryana, India*
[b] *Department of Biochemistry, Kurukshetra University, Kurukshetra, Haryana, India*
[c] *Department of Microbiology, Kurukshetra University, Kurukshetra, Haryana, India*

Received 16 July 2015; received in revised form 18 September 2015; accepted 18 November 2015
Available online 8 December 2015

KEYWORDS
CYP2E1;
CYP1A1m2;
petrol pump workers;
polymorphism;
cytogenetic biomarkers

Abstract Petrol pump workers are occupationally exposed to benzene through their contact with gasoline vapors. The toxicity of benzene has been related to its metabolism. This study investigated the association of *CYP2E1* and *CYP1A1m2* with sister chromatid exchanges (SCE) and tail moment (TM) value in workers occupationally exposed to gasoline fumes. Blood and urine samples were collected from 50 petrol pump workers and 50 control individuals matched with respect to age and other confounding factors except for exposure to benzene through gasoline vapors. To determine the benzene exposure, hydroquinone level was analyzed in urinary samples of exposed and control individuals. Urinary mean hydroquinone level was found to be significantly high ($p < 0.05$) in exposed workers. Our results showed that mean SCE frequency and TM value were significantly higher ($p < 0.05$) in exposed workers (5.56 ± 0.80 and 19.50 ± 2.16 μm, respectively) than control individuals (2.83 ± 0.39 and 1.00 ± 0.00 μm, respectively). Regarding the effect of *CYP2E1* polymorphism, it was found that mutant genotypes (homozygous and heterozygous) showed significant high mean frequency of SCE (6.11 ± 0.51 and 5.98 ± 0.54, respectively) and TM (16.13 ± 4.36 μm and 13.24 ± 2.24 μm, respectively) value in exposed individuals ($p < 0.05$). With regard to the *CYP1A1m2* polymorphism, it was observed that mutant genotypes (homozygous and heterozygous) had higher but nonsignificant mean value of SCE frequency (5.86 ± 1.07 and 5.86 ± 1.07, respectively) and significantly higher TM value (14.97 ± 3.74 μm and 13.93 ± 2.23 μm, respectively) in exposed individuals.

* Corresponding author. Department of Biotechnology, Kurukshetra University, Kurukshetra, Haryana (Hr.)-136119, India.
E-mail address: ayadav@kuk.ac.in (A. Yadav).

http://dx.doi.org/10.1016/j.bgm.2015.11.003

Introduction

Air pollution caused by traffic transportation, fuel combustions and volatile organic compounds from gasoline, has progressed to be a serious health problem, especially among occupational workers such as petrol pump workers as they are routinely exposed to gasoline fumes. Exposure to gasoline vapors is classified by the International Agency for Research on Cancer as possibly carcinogenic to humans, mainly on the basis of the established carcinogenicity of some component chemicals such as benzene,[1] which has also been shown to be genotoxic (causing damage to DNA) in experimental animal studies.[2] Evaluation of genetic damage in populations exposed in work places is most valid for predicting heath risk. An increased level of cytogenetic damage in peripheral blood lymphocytes of workers exposed to gasoline has been demonstrated using different biomarkers, such as sister chromatid exchange (SCE) or DNA strand break.[3,4] Alkaline single cell gel electrophoresis technique, also known as the comet assay, is a sensitive, simple, and rapid technique for detecting DNA single and double strand breaks, alkali labile sites, incomplete excision repair sites, and genomic structural discontinuities.[5] Due to its sensitivity in detecting genetic damage at the individual cell level and its potential application to different cells, the assay has been adopted as a useful tool in short-term genotoxicity and human biomonitoring studies.[6,7] Cytogenetic alterations in cultured peripheral blood lymphocytes such as SCE have been applied for many years as biomarkers of genotoxic exposure and early effects of genotoxic carcinogens.[8] SCE are interchanges of DNA replication products between sister chromatids at apparently homologous loci, suggested to represent homologous recombination repair of DNA double strand breaks.[9] They are analyzed from second division metaphases using a staining method based on sister chromatid differentiation by 5-bromo-2-deoxyuridine labeling.[10] The toxicity of benzene has been related to its metabolism.[11] *CYP2E1* and *CYP2A1* are the two major Phase 1 genes involved in metabolism of benzene.

CYP1A1 and *CYP2E1* have been most extensively evaluated as potential contributors to cancer susceptibility. *CYP2E1* polymorphism has been reported to be associated with increased risk of lung cancer and lymphomas.[12,13] *CYP2E1* gene forms 2 alleles c1 and c2. The genotype c1/c1 is defined as type A, genotype c1/c2 as type B, and homozygote c2/c2 as type C or *CYP2E1*5B*.[14] The *CYP2E1* genetic polymorphism varies significantly among different ethnic groups.[15,16] Studies have suggested an enhanced transcription of the *CYP2E1* gene associated with the variant c2 allele. Among several polymorphisms identified in the *CYP1A1* gene, two closely linked mutations have been extensively studied in relation to cancer risk. The *CYP1A1* Ile/Val (m2) mutation in the heme-binding region doubles the microsomal enzyme activity and it is in linkage disequilibrium in Caucasians with the *CYP1A1 MspI* (*m1*) mutation that has also been associated experimentally with increased catalytic activity.[17]

The objective of this study was to determine the effect of genetic polymorphisms of *CYP2E1* and *CYP1A1m2* genes on genetic damage in petrol pump workers using SCE and comet assay [in terms of tail moment (TM) value] as biomarkers.

Materials and methods

Study population

The study was carried out on petrol pump workers ($n = 50$) and control group consisting of healthy individuals ($n = 50$) matched with respect to age and other confounding factor except for exposure to benzene through gasoline vapors. Participants were informed about the objectives of the study. They were asked to sign an informed consent form and each participant was personally interviewed using a standard questionnaire having information related to age, sex, personal medical history, and occupational history of exposure to gasoline fumes. The research protocol was approved by the Ethical Committee of Kurukshetra University, Kurukshetra, Haryana, India.

Sample collection

A total of 5-mL venous blood was collected from each participant in two separate vacutainer tubes containing sodium heparin or dipotassium EDTA for lymphocyte culture set up or DNA extraction, respectively. The samples were brought to laboratory in a well-insulated ice box. All exposed individuals had at least 5 years of working exposure. The urine and blood samples of individuals were collected after 8 hours of their working time.

Urinary hydroquinone analysis for internal benzene exposure

To assess the benzene exposure in exposed population, the level of hydroquinone in random urine samples of exposed and control individuals was analyzed by standard method using gas chromatography—mass spectrometry.[18]

Culture set up

Short-term peripheral blood lymphocytes cultures were set up using earlier studied techniques of Moorhead et al[19] with minor modifications. Cultures were set up in duplicate from whole blood of exposed and control groups.

SCE in peripheral blood lymphocytes

For SCE analysis, whole heparinized blood (0.4 mL) was added to 5 mL of RPMI 1640 culture medium containing 1% L-glutamine, 20% fetal bovine serum, penicillin (100 UI/mL), streptomycin (100 μg/mL), and 2% phytohaemagglutinin. 5-Bromo-2-deoxyuridine at a final of concentration of 10 μg/mL culture was added after 24 hours of incubation. The cultures were incubated for another 48 hours at 37°C and ± 5% CO_2. Two drops of colchicines in a final concentration of 0.2 μg/mL was added 45 minutes prior to the harvesting. The cells were harvested by centrifugation and then treated with hypotonic solution (0.075M KCl) and fixed in methanol: acetic acid (3:1). From a suspension of fixed cells, slides were prepared by the air drying method and stained with Hoechst 33258 and 4% Giemsa stain solution following the method

of Perry and Wolff.[20] To calculate the frequency of SCE per cell, 50 metaphases were analyzed.

DNA damage analysis by comet assay

Alkaline comet assay was performed according to the method of Singh et al[5] and Tice et al[21] with minor modifications. Slides were prepared in duplicate for each sample. Dust free, plain slides was covered with a layer of 150 μL of 1% normal melting agarose and allowed to dry for 10 minutes in hot air oven. This layer served as an anchor for additional layers to prevent slippage. The blood sample (5—10 μL) was mixed with 90 μL of warm 0.5% low melting agarose and this mixture was layered as second additional layer and gelled at 4°C for 15 minutes. A third additional layer of 150 μL of 0.5% low melting agarose was added on top and gelled again at 4°C for 15 minutes. The slides were treated for 2 hours at 4°C in freshly prepared, chilled lysis buffer solution (25mM NaCl, 100mM sodium EDTA, 10mM Tris, 1% Triton X-100, 10% DMSO) added before use and pH adjusted to 10. They were removed from the lysis solution, incubated in alkaline electrophoresis buffer (10N NaCl, 200mM EDTA, pH adjusted to 13) for 20 minutes followed by electrophoresis (25 V and 300 mA) for 30 minutes, in the same buffer. The slides were then neutralized with Tris buffer (0.4M Tris, pH adjusted to 7.5), rinsed with distilled water and stained with 100 μL ethidium bromide (20 μg/mL) for 5 minutes under dim or dark condition.

Comet scoring

A total of 50 cells from each of the duplicate slides were examined randomly under a fluorescence microscope. The extent of DNA damage was measured quantitatively as TM using Lucia Comet assay software (Laboratory Imaging, s.r.o., Prague, Czech). The TM is defined by the percentage of DNA in the tail multiplied by the length between the center of the head and tail.[22]

*CYP2E1**5B genotyping

The 5′-flanking polymorphic site of the *CYP2E1* gene was analyzed according to the protocol of Hayashi et al.[14] The primers F 5′-CAG TCG AGT CTA CAT TGT C-3′ and R 5′ TTC ATT CTG TCT TCT AAC TG-3′ generated a 410 bp band. Polymerase chain reaction products were digested with *Pst*1 restriction enzyme and then subjected to electrophoresis in 2.5% agarose. The genotypes of *CYP2E1* were classified as homozygous wild (c1/c1: 410 bp), heterozygote (c1/c2: 410 bp, 290 bp, 120 bp), and homozygous mutant (c2/c2: 290 bp, 120 bp). The digested polymerase chain reaction products were separated by 2.5% agarose gel electrophoresis and detected by ethidium bromide staining.

CYP1A1m2 genotyping

The primers for the m2 sites were 5′-TTC CAC CCG TTG CAG CAG GAT AGC C-3′ and 5′-CTG TCT CCC TCT GGT TAC AGG AAG-3′.[23]

These generated a 204 bp fragment. The restriction enzyme *BsrD*I was used to identify mutant genotypes as later generate 149-bp and 55-bp (for the m2 site) fragments from a single 204-bp fragment. The restricted products were analyzed by electrophoresis in 3% agarose gel containing ethidium bromide.

Statistical analysis

Statistical analysis was performed using SPSS software package (version 16.0 for Windows; SPSS Inc., Chicago, IL, USA). All tests were performed in duplicate and results expressed as means ± standard deviation. Multivariate analysis of variance test was used with *post hoc* analysis for the comparison of frequencies in multiple subgroups among studied population. A p-value < 0.05 was considered significant for the statistical analyses.

Results

There was no significant difference between control and exposed group except for exposure to benzene through gasoline fumes. The exposed workers had an average age of 36.20 ± 9.87 years while mean age of control individuals was 36.04 ± 8.02 years. We categorized the participants into three age groups: < 30 years; 30—40 years; and > 40 years. According to exposure duration, individuals were divided into three groups: > 10 years; 10—20 years; and > 20 years (Table 1).

Urinary hydroquinone assessment

The mean concentration of hydroquinone in random urine samples of control and exposed individuals were found to be 0.026 ± 0.012 μg/mL and 0.60 ± 0.015 μg/mL, respectively. The level of hydroquinone in urine of exposed individuals was found significantly to be higher than that of control individuals ($p < 0.05$).

SCE frequency and TM value in control and exposed population

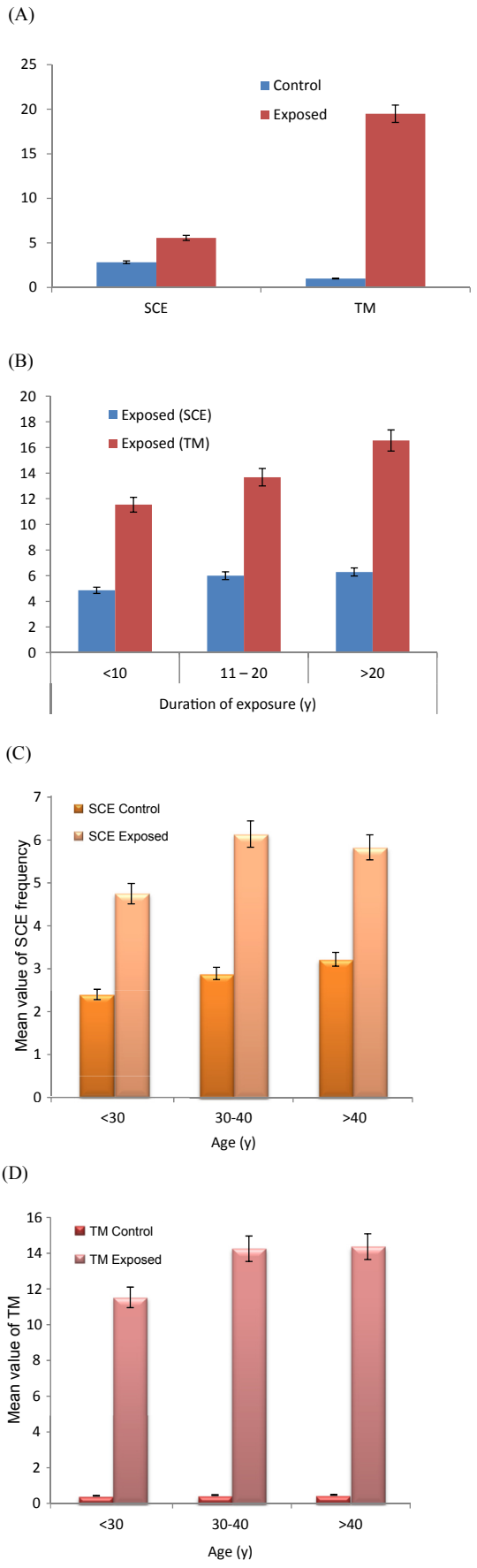
We analyzed SCE frequency and TM value in exposed and control individuals by age and exposure duration. The results showed significantly higher mean value of SCE

Table 1 Demographic characteristics of control and exposed populations.[a]

Variable	Control	Exposed	p
All (n)	50	50	
Age (y)	36.04 ± 8.025	36.20 ± 9.879	0.929
Duration of exposure (y)			
<10		19 (38)	
10—20		20 (40)	<0.05
>20		11 (22)	

Data are presented as n (%) or mean ± standard deviation, unless otherwise indicated.

[a] Student t test was applied to compare mean value of age among control and exposed groups. Chi-square test was applied for difference in exposure history among studied population. Level of significance was set at $p < 0.05$.



(5.56 ± 0.80) and TM (19.50 ± 2.16 μm) in the exposed population when compared to the control group ($p < 0.05$). There was an increase in SCE frequency and TM value with age. In the exposed population, high SCE frequency was observed in age group of 30–40 years (6.14 ± 0.30) while there was an increase in TM frequency with increase in age in both the groups, i.e., < 30 years (0.43 ± 0.26 μm and 11.53 ± 0.75 μm, respectively), 30–40 years (0.47 ± 0.33 μm and 14.25 ± 2.23 μm, respectively), > 40 years (0.48 ± 0.34 μm and 14.37 ± 2.58 μm, respectively) of control and exposed populations, respectively. With regard to exposure duration, it was observed that there was a gradual increase in SCE frequency and TM value with increase in exposure duration with significant high mean values of SCE and TM found in workers having > 20 years of exposure (6.29 ± 0.26 and 16.54 ± 1.86 μm, respectively; $p < 0.05$). Figure 1 shows the overall mean SCE and TM values (Figure 1A), effect of duration of exposure (Figure 1B) and effect of age on SCE frequency and TM value in both the exposed and control populations (Figure 1C and 1D).

Association of CYP genotypes with SCE and TM

Influence of *CYP2E1* and *CYP1A1m2* genotypes on SCE and TM frequency is shown in Table 2. Mutant genotypes (homozygous and heterozygous) of *CYP2E1* and *CYP1A1m2* were more susceptible with higher mean value of SCE and TM value in the exposed population. *CYP1A1m2* homozygous and heterozygous mutant genotypes were found to have high mean value of TM and SCE, respectively, while a significantly high frequency of SCE was observed for *CYP2E1* heterozygous and homozygous mutant genotypes in the control population. Homozygous mutant of *CYP1A1m2* showed increased TM frequency. In the case of *CYP2E1*, homozygous mutant showed a high nonsignificant TM frequency in the exposed group while in controls, wild type and heterozygous group were the same. Association of CYP genotypes with SCE and TM value by linear regression is shown in Table 3.

Discussion

In present study we found that the exposed population had significantly higher mean SCE frequency and TM value than that of control. Similar to our findings, Edward and Priestly[24] found significant increase in sister chromatid exchange frequency in petroleum workers employed in suburban petroleum retail outlets. Sul et al[25] studied DNA damage in T- and B-lymphocytes using single strand DNA breaks in workers exposed to benzene and found that in T-lymphocytes, DNA damage was slightly higher in exposed workers than in controls, while DNA damage of B-lymphocytes in the two groups showed the most significant difference among the

Figure 1 (A) Sister chromatid exchanges (SCE) frequency and tail moment (TM) value (μm) in control and exposed population; (B) effect of exposure duration on SCE frequency and TM value in exposed population; (C) effect of age on SCE frequency; and (D) TM value in exposed and control populations.

Table 2 Influence of *CYP* genotypes on tail moment (TM) value and sister chromatid exchange (SCE) frequency.

Biomarkers	Control group			Exposed group		
	N	TM value (μm)	SCE frequency	N	TM value (μm)	SCE frequency
CYP2E1						
wt/wt	27	0.53 ± 0.34	2.71 ± 0.37	22	13.27 ± 2.35	5.05 ± 0.79
wt/mt	18	0.45 ± 0.27	2.97 ± 0.35[a]	26	13.24 ± 2.24	5.98 ± 0.54[a]
mt/mt	5	0.25 ± 0.12	3.50 ± 0.00[a]	2	16.13 ± 4.36	6.11 ± 0.51[a]
CYP1A1m2						
wt/wt	29	0.48 ± 0.29	2.70 ± 0.37	23	12.57 ± 2.18	5.32 ± 0.90
wt/mt	20	0.43 ± 0.29	3.00 ± 0.39[a]	24	13.93 ± 2.23[a]	5.74 ± 0.68
mt/mt	1	1.23 ± 0.00	2.93 ± 0.22[a]	3	14.97 ± 3.74[a]	5.86 ± 1.07

Data are presented as mean ± standard deviation.
mt = mutant; wt = wild type.
[a] Multivariate analysis of variance test was used for the differences in SCE and TM frequency adjusted with age and exposure duration among control and exposed group.

Table 3 Association of *CYP* genotypes with on sister chromatid exchange (SCE) value and tail moment (TM) value as analyzed by linear regression.

Genotype	SCE frequency			TM value		
Control	β[a]	R^2	*p*[b]	β[a]	R^2	*p*[b]
CYP2E1	0.293	0.164	<0.05	−0.005	0.000	0.972
CYP1A1m2	0.183	0.100	<0.05	0.013	0.000	0.918
Exposed						
CYP2E1	0.742	0.310	<0.05	0.296	0.103	0.083
CYP1A1m2	0.370	0.068	0.067	0.447	0.224	<0.05

[a] Unstandardized coefficient.
[b] Model P value. Regression analysis was used for the differences in SCE and TM frequency adjusted for age and exposure duration.

three cell types. Celi and Akbaş[26] worked on gasoline station individuals and revealed that SCE frequency and chromosomal aberrations were elevated in gasoline station attendants. Roma-Torres et al[27] found a significant increase in comet assay in a group of workers from a petroleum refinery aromatics plant. Keretetse et al[28] worked on African petrol attendants and found significant higher DNA damage with the exposed group compared to the unexposed group, they also found that period of exposure influenced the level of DNA damage; smoking and age had a significant influence on the level of DNA damage. Rekhadevi et al[29] investigated the genotoxicity risk among 200 fuel filling station attendants and 200 matched controls and the results of the present study suggest that there was a statistically significant increase in mean comet tail length. Moohammadaree et al[30] found a significant excess of DNA damage in workers who were exposed occupationally to gasoline vapor and air pollutants, when compared to the matched controls. These findings confirm the potent genotoxic effect of gasoline and air pollutants as an inducer of DNA damage.

In our study, individuals in the 30—40 years age group showed a significant increase in SCE frequency. Contrary to our studies, Khalil et al[31] found a nonsignificant elevation of SCE frequency in workers exposed to petrochemicals and reported that SCE frequencies were not influenced by age.

We studied the influence of *CYP2E1* and *CYP1A1m2* genotypes on SCE and TM and found that homozygous and heterozygous mutant genotypes of *CYP2E1* and *CYP1A1m2* were more susceptible with higher mean value of SCE and TM value in exposed population. To the best of our knowledge, there is no such study in case of Indian petrol workers. However, a few reports have been found in other occupations related to organic solvents. Heuser et al[32] found a significant association of *CYP2E1* polymorphism with DNA damage in Brazilian footwear workers occupationally exposed to solvent-based adhesive and solutions containing organic solvents, mainly toluene. Contradictory to our results, Laffon et al[33] found no effect of CYP2E1 variants in the biomarkers analyzed such as SCE, micronucleus, and comet assay on exposed population in production of rubber types.

It is recommended that occupational workers are required to train regularly and always be given appropriate personal protective equipment. The determination of *CYP2E1* and *CYP1A1m2* genotypes status could also be helpful in providing baseline data as an individual marker of susceptibility in gasoline pump exposed population.

Conflicts of interest

All authors have no conflicts of interest to declare.

Acknowledgments

We are thankful to Kurukshetra University for grant of University Research Fellowship to the first author, and to all blood donors. We are also grateful to Mr Rajesh, Statistical Officer, Department of Forensic Sciences, Post Graduate Institute of Medical Education and Research, Chandigarh, Haryana (India) for statistical analysis.

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