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ORIGINAL PAPER

The influence of CYP1A1 and GSTM1 polymorphism on the concentration of urinary 1-hydroxypyrene in cPAHs exposed Iranian anode plant workers

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Abstract Polycyclic aromatic hydrocarbons (PAHs) are a group of environmental pollutants in the occupational settings which are associated with an increased risk of cancer. CYP and GST enzymes have an important role in metabolism of PAHs. Genetic polymorphisms of these enzymes may affect their capacity for oxidation and detoxification of PAHs which will result in variation in PAHs metabolites. The aim of this study was to evaluate the association of urinary 1-hydroxypyrene levels with genetic polymorphisms of CYP1A1 and GSTM1 polymorphism in the Iranian anode plant workers.

The study population consisted of 42 workers working in carbon anode plant in an aluminum production industry and matched control group consisted of 43 office workers. Personal air sampling was performed to assess workers atmospheric exposure to carcinogenic PAHs. Blood samples were taken for DNA extraction and determination of GSTM1 and CYP1A1 polymorphism. Urine samples were taken immediately after the end of the working shift on the last working day for analysis of urinary 1-hydroxypyrene using High Performance Liquid Chromatography (HPLC). Statistical analysis was performed using SPSS ver. 16 software.

The mean concentration of occupational exposure to cPAHs in the exposed group was 11.42 μg/m^3 ranging from 3.6 to 31.5 μg/m^3. Mean level of urinary 1-hydroxypyrene in the exposed and control groups was 6.32±4.9 μmole/mole creatinine and 0.54±.48 μmole/mole creatinine respectively. According to the statistical analysis we found no influence of CYP1A1 MspI polymorphism on the urinary 1-hydroxypyrene levels of exposed and control subjects before and after stratification according to smoking habit. Our results also showed that, GSTM1 null genotype is associated with significantly higher levels of urinary 1-hydroxypyrene before stratification according to smoking habit in the exposed subjects (P=0.003) and after stratification the association remained statistically significant only in the non smoker subjects (P= 0.003).

Association of GSTM1 null genotype with higher levels of urinary 1-hydroxypyrene shows higher susceptibility of these genotype carriers to genotoxic effects of PAHs and employment of GSTM1 null carriers in the occupational settings with high levels of PAHs pollution should be reconsidered. Regarding the CYP1A1 polymorphism, it seems more studies, especially with large sample sizes, are necessary for achieving conclusive results.

Keywords CYP1A1, GSTM1, Polymorphism, 1-hydroxypyrene, CPAHs

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Polycyclic aromatic hydrocarbon (PAH) refers to a group of chemicals which consists of a few hundred compounds with two or more fused benzene rings[1]. PAHs can be generated naturally as a result of forest fires and volcanic eruptions or by human activities such as industry, heating, waste incineration, and traffic[2]. These compounds are associated with industrial processes in which materials such as coke, coal tar, and coal tar pitch are produced or used[3]. Epidemiological studies have demonstrated an association between PAHs exposure and increases in mortality and/or morbidity from respiratory diseases, cardiovascular diseases and cancer[4].

One of the work sites where workers are exposed to high concentrations of PAHs are anode plants where carbon anodes are fabricated to be used in the aluminum production process. Up to now, many researchers have addressed the problems of occupational PAHs exposure in the aluminum industries[5-9].

Among hundreds of PAHs identified, there has been no common international agreement on which compounds should be reported concerning human exposure in environmental or occupational settings, but for practical reasons, benzo(a)pyrene has been used as the surrogate marker of choice for measuring ambient exposure to PAH mixtures and measurement of carcinogenic polycyclic aromatic hydrocarbons (cPAHs) is a common appropach in PAHs genotoxicity studies[10-13]. PAHs internal exposure markers are urinary 1-hydroxypyrene, PAH-DNA and PAH-protein adducts and response markers include DNA damage, chromosomal aberrations, sister chromatid exchanges and micronuclei[9,14-16,41,42].

In the human body, PAHs endure metabolic activation by phase I enzymes, such as cytochrome P4501A1 (CYP1A1) and transform to diol epoxides which are capable of interacting with DNA, potentially starting a carcinogenic process. Phase II enzymes, such as uridine diphosphoglucuronosyltransferase (UDP glucuronyl-transferase) and glutathione S-transferases (GSTs) which catalyze conjugative reactions of oxidative products can detoxify the activated PAH metabolites and facilitate their excretion into the urine[17,18].

Inter-individual differences in terms of genetic polymorphism is associated with individuals' susceptibility to cancer and it is one of the factors which may determine the response of human body in the case of exposure to PAHs[19-23]. It has been suggested that Polymorphism of CYP (such as CYP1A1) and GST genes (such as GSTM1) can affect the PAHs metabolism and effects. However, the study results regarding the influence of CYP and GST genes polymorphism on PAHs exposure and effect biomarkers are conflicting[17,24-29].

Up to our knowledge, no study has been conducted in Iran to address the effect of metabolic gene polymorphism on the urinary 1-hydroxypyrene in cPAHs exposed workers. Hence, this study was designed to evaluate the influence of CYP1A1 and GSTM1 polymorphism on the concentration of urinary 1-hydroxypyrene in Iranian workers in a carbon anode plant towards the evaluation of gene-environment interactions and clarification of PAHs related risks. The results of this study will also help to recognize genetically susceptible individuals in the case of exposure to PAHs.

Characteristics of the studied population

General characteristics of the studied population are shown in Table 1. Subjects with urinary cotinine level of more than 500 ng/mg creatinine have been considered as smokers[4].

Exposure monitoring and urinary 1-hydroxypyrene levels

The mean concentration of occupational exposure to cPAHs in the exposed group was 11.42 μg/m^3 ranging from 3.6 to 31.5 μg/m^3 and the mean level of occupational exposure to Benzo(a)pyrene in this group was 1.41 μg/m^3 ranging from 0.5 to 5.2 μg/m^3. Total cPAHs exposure profile in the exposed group according to the genotype is presented in Table 2. Internal exposure dose to cPAHs was evaluated by measuring urinary 1-hydroxypyrene. Mean level of urinary 1-hydroxypyrene in the exposed and control groups was 6.32± 4.9 μmol/mol creatinine and 0.54±.48 μmole/mole creatinine respectively. According to the statistical

Table 1. General characteristics of the studied population.

Factors	Exposed workers	Controls
Number of subjects	42	43
Agea (years)	30.4±4.5	32.5±5.7
Number of smoker subjects	12	12
Number of non smoker subjects	30	31

[a]: Mean±SD

Table 2. Comparison of total cPAHs exposure in the exposed group according to the genotype status.

Genotypes	Total cPAHs[a] (μg/m^3)	*P*-value
CYP1A1		
TT	10.6±5.3	0.73
TC+CC	11.4±8.6	
GSTM1		
Positive	10.7±8.2	0.55
Null	12.1±7.0	

[a]: Mean±SD

Table 3. Genotypes status and its relation to the urinary 1-hydroxypyrene.

Genotypes	Number of subjects (%)		Urinary 1-hydroxpyrene[a] (μmol/mol creatinine)			
	Exposed group	Control group	Exposed group	*P*-value	Control group	*P*-value
CYP1A1						
TT	16 (38.1%)	23 (53.5%)	7.42±4.55	0.25	0.73±0.56	0.005
TC+CC	24+2 (61.9%)	14+6 (46.5%)	5.64±5.09		0.33±0.26	
GSTM1						
Positive	20 (47.62%)	18 (41.9%)	4.05±3.66	0.003	0.50±0.43	0.64
Null	22 (52.38%)	25 (58.1%)	8.38±5.05		0.57±0.53	

[a]: Mean±SD

Table 4. Genotypes status and its relation to the urinary 1-hydroxypyrene stratified by smoking habit.

Genotypes	Urinary 1-hydroxpyrene[a] (μmol/mol creatinine)							
	Exposed group				Control group			
	Smokers	*P*-value	Non smokers	*P*-value	Smokers	*P*-value	Non smokers	*P*-value
CYP1A1								
TT	3.41±1.41	0.67	8.00±4.57	0.76	0.87±0.62	0.42	0.63±0.51	0.06
TC+CC	2.81±1.84		7.41±5.69		0.48±0.38		0.31±.25	
GSTM1								
Positive	2.20±1.81	0.16	4.84±4.01	0.003	0.46±0.44	0.78	0.70±0.42	0.73
Null	3.62±1.47		10.17±4.75		0.42±0.38		0.84±0.66	

[a]: Mean±SD

analysis the level of 1-hydroxypyrene in the exposed group was significantly higher than the control group ($P<0.001$). In addition, there was a significant difference in urinary 1-hydroxypyrene between smokers and non smokers in the both exposed ($P<0.001$) and control ($P=0.027$) groups.

Statistical analysis of atmospheric exposure to cPAHs and excretion of 1-hydroxypyrene in the urine using Pearson correlation test showed that, there is a strong and significant correlation between total cPAHs exposure and urinary 1-hydroxypyrene ($r=0.79$, $P<0.001$). Moreover, a direct positive and significant correlation was found between benzo(a)pyrene exposure and urinary 1-hydroxypyrene ($r=0.69$, $P<0.001$).

Genotyping

Regarding the CYP1A1 MspI polymorphism, 45.9%, 44.7%, and 9.4% of the all 85 studied cases were respectively TT, TC, and CC carriers. In the case of GSTM1, 44.7% and 55.3% of the studied cases were respectively GSTM1 positive and null carries. Genotypes and its relation to the urinary 1-hydroxypyrene in the exposed and control groups is presented in Table 3. According to these results, urinary 1-hydroxypyrene is significantly higher in GSTM1 null carriers in the PAHs exposed group but not in the control group. In addition, a relationship was found between CYP1A1 polymorphism and urinary 1-hydroxypyrene in the control group. However, no relationship was found in the exposed group. As cigarette smoking can have a confounding effect in our study, further analysis was performed regarding the smoking habit and the results are presented in Table 4. According to these results, urinary 1-hydroxypyrene has been significantly affected by GSTM1 polymorphism only in the non smoker exposed subjects ($P=0.003$).

Discussion

Aluminum industry workers may be exposed to high levels of PAHs especially during the anode production. In this study the anode plant workers' exposure to cPAHs was evaluated and the results showed significant personal exposure to cPAHs ranging from 3.6 to 31.5 μg/m^3. In the body, PAHs undergo metabolic activation by phase I enzymes such as CYP1A1 to diol epoxides which may covalently bind to DNA and initiate a carcinogenic process. An alternative path for activated PAHs is to be detoxified by phase II enzymes such as GSTM1 which catalyzes conjugation of PAHs reactive metabolites to GSH, making them more water

soluble and excretable. Urinary 1-hydroxypyrene is a commonly used biomarker of exposure to PAHs and an indicator for internal dose of activated PAHs[17,30]. Our results revealed a significant correlation between urinary 1-hydroxypyrene and personal exposure to cPAHs (r=0.79) and benzo a pyrene (r=0.69). These results are in agreement with studies which confirm the applicability of 1-hydroxypyrene as a biomarker of exposure to PAHs and carcinogenic PAHs and suggest 1-hydroxypyrene as a good biomarker for monitoring the cPAHs exposure and internal dose in the studied population[9,17,31,32]. As 1-hydroxypyrene formation and excretion is catalyzed by CYP1A1 and GSTM1 enzymes, polymorphism of related genes is expected to affect urinary 1-hydroxypyrene concentration[31].

According to our results, after controlling related confounders including BMI and occupational exposure level, CYP1A1 MspI polymorphism had no significant effect on urinary 1-hydroxypyrene levels in the cPAHs exposed subjects before and after stratifying the subjects according to the smoking habit. However, it had a statistically significant effect on urinary 1-hydroxypyrene levels in the control subjects before stratification according to the smoking habit (P=0.005) and after the stratification the significant effect disappeared emphasizing on the role of smoking as a confounding factor (P=0.06). In general, no influence of CYP1A1 MspI polymorphism on the urinary 1-hydroxypyrene levels of exposed and control subjects was found. This finding is in agreement with Bosso *et al*. who found no influence of CYP1A1 MspI polymorphism on 1-hydroxypyrene excretion in sugarcane workers[17]. Likewise, Apostoli *et al*. reported no effect of CYP1A1 polymorphism on urinary 1-hydroxypyrene levels in workers of an iron foundry and two electric steel plants who were exposed to significant levels of PAHs[28]. Hemminki *et al*. also reported no influence of CYP1A1 polymorphism on urinary 1-hydroxypyrene concentrations in PAHs exposed foundry workers[33]. In contrast, Alexandrie *et al*. and Petchpoung *et al*. studies revealed a relationship between CYP1A1 MspI polymorphism and urinary 1-hydroxypyrene concentrations[29,31]. An explanation for not finding any relationship between CYP1A1 polymorphism and urinary 1-hydroxypyrene is that, heterozygote genotype (TC) capacity for catalyzing the oxidation of PAHs may not be that much lower than wild homozygote genotype (TT) to be reflected in the urinary 1-hydroxypyrene concentrations and the homozygote CC genotype is too rare to have a significant effect in the statistical calculations (in our study 2 (4.7%) of the exposed subjects were CC carriers).

Regarding the GSTM1, our results showed that GSTM1 null genotype is associated with significantly higher levels of urinary 1-hydroxypyrene before stratification according to smoking habit in the exposed subjects (P=0.003), but not in the control subjects (P=0.64). After stratification according to the smoking habit, GSTM1 null genotype was still significantly associated with higher urinary 1-hydroxypyrene levels in the exposed non smokers (P=0.003). Compared to the GSTM1 positive carriers, urinary 1-hydroxypyrene level was higher in the GSTM1 null carriers in the exposed smoker subjects. However, the difference was not statistically significant (P=0.16). In agreement with the results obtained in this study, Alexandrie *et al*. reported an association between GSTM1 null genotype and increased urinary 1-hydroxypyrene in aluminum smelters[29]. In contrast, Petchpong *et al*. study on Thai bus drivers showed decreased urinary 1-hydroxypyrene concentrations in GSTM1 null carries[31]. Nan *et al*. study showed no relationship between urinary 1-hydroxypyrene and GSTM1 polymorphism in coke oven workers[34]. Apostoli *et al*. and Chen *et al*. studies also found no association between GSTM1 polymorphism and urinary 1-hydroxypyrene levels in the PAHs exposed workers[28,35].

Regarding the GSTM1 polymorphism, beside all discrepancies in the results presented by many researchers, our results are well supported by Vaury *et al*. in vitro study which showed high inducibility of CYP1A1 in the presence of homozygous GSTM1 null genotype which will result in accumulation of PAH metabolites such as 1-hydroxypyrene produced by CYP1A1 catalyzed oxidation of PAHs[36]. Accumulation of oxidized PAHs inside the body cells will bring about an opportunity for PAH-DNA interactions which may result in higher genotoxic effects. On the other hand, the deficiency of GSTM1 in catalyzing the PAH derivatives glutathione conjugation may increase the glucoronidation pathway which will finally lead to the higher levels of urinary PAH metabolites including 1-hydroxypyrene in GSTM1 null carriers[28].

In conclusion, this study found a significant relationship between the GSTM1 polymorphism and urinary 1-hydroxypyrene concentrations in the cPAHs exposed Iranian anode plant workers. Association of GSTM1 null genotype with higher levels of urinary 1-hydroxypyrene shows the higher susceptibility of these genotype carriers to genotoxic effects of PAHs. Therefore, employment of the GSTM1 null carriers in the occupational settings with high levels of PAHs pollution should be reconsidered. Regarding the CYP1A1 polymorphism, it seems that, more studies, especially with large sample sizes, are necessary for achieving conclusive results.

Materials & Methods

Study subjects

The exposed population consisted of 42 workers working in carbon anode plant in an aluminum production industry. The age, BMI (body mass index), economic status, and smoking habit matched control group consisted of 43 volunteered office workers. The inclusion criteria was not having medical treatment, radiography, or vaccination up to 3 months before sampling. Each participant completed a questionnaire on personal information and lifestyle. All participants signed an informed consent form and could exit from the study at any time during the study. In addition the study was approved by Tehran University of medical sciences ethical committee.

Chemicals and reagents

1-hydroxypyrene was purchased from Sigma-Aldrich (Germany); indeno (1,2,3 cd) pyrene from Supelco (USA); all other c-PAHs from Dr. Ehrenstorfer GmbH (Germany); β-glucoronidase-arylsulphatase from Roche (Germany); primers for polymerase chain reaction from Tib Molboil (Germany), dNTP from Qiagen (UK); $MgCl_2$,Taq polymerase, Tris-HCl and KCl from Sinagen (Iran); MspI from Fermentas (Germany); actonitrile, benzene, cyclohexane, methanol, methylene chloride and toluene from Merck (Germany), C18 cartridge from Macherey-Nagel (Germany); PTFE Filters and washed XAD-2 sorbent tube from SKC (USA).

Personal exposure monitoring

Air sampling and analysis was performed to assess workers' atmospheric exposure to cPAHs including benzo[a]pyrene (B[a]P), benz[a]anthracene (B[a]A), benzo[b]fluoranthene (B[b]F), benzo[k]fluoranthene (B[k]F), benzo[ghi]perylene (B[ghi]Pe), chrysene (CHRY), dibenz[ah]anthracene (DB[ah]A), and indeno[cd]pyrene (I[cd]P) according to the NIOSH 5515[37]. In this regard, air samples were taken with a flow rate of 2 L/min using a personal air sampling pump, PTFE Filter (2-μm pore size, 37-mm diameter) and washed XAD-2 sorbent tube. Analysis of the air samples was performed using a GC-FID with a capillary column (30 m × 0.32-mm ID, fused silica, 1-μm DB-5).

Determination of GSTM1 polymorphism

QIAamp DNA Blood Mini kit (from Qiagen) was used to extract Genomic DNA from blood samples. Polymorphism of GSTM1 was determined by polymerase chain reaction (PCR)[38]. The forward primer is 5′-GAACTCCCTGAAAAGCTAAAGC-3′ and the reverse primer is 5′-GTTGGGCTCAAATATACGG TGG-3′. The amplification was performed in 30 μL final volume containing 0.5 mM of each primer, 0.2 mM dNTP, 1.5 mM $MgCl_2$, 1 unit Taq polymerase, 20 mmol/L Tris-HCl (pH 8.4), 50 mM KCl and 100 ng of genomic DNA. After denaturation at 94°C for 5 min samples were subjected to 35 cycles of 94°C for 20 s, 57°C for 25 s and 72°C for 50. The thermal cycles finished by a final extension step of 72°C for 7 min. 10 μL of the PCR products were then resolved on a 2.1% agarose gel containing ethidium bromide. Amplification of β-Globin with the primers 5′-CAACTT CATCCACGTTCACC-3′ and 5′-GAAGAGCCAA GGACAGGTAC-3′ was used as an internal control and produced a 268 bp product. The presence and absence of the 215-bp band reveals respectively the GSTM1-positive and GSTM1-null genotype.

Determination of the CYP1A1 MspI (T6235C) polymorphism

PCR-RFLP was used to determine the MspI polymorphism of CYP1A1 gene[39]. The forward primer is P1: 5′-CAGTGAAGAGGTGTAGCCGCT-3′; the reverse primer is P2: 5′-TAGGAGTCTTGTCTGATGCCT-3′. The amplification was performed in 25 μL final volume containing 20 pmol of each primer, 2.0 mmol/L $MgCl_2$, 25 μmol/L dNTP, 50 mmol/L KCl, 5 mmol/L Tris-HCl (pH 8.3), 1 U Taq polymerase and 100 ng genomic DNA. After denaturation at 95°C for 1 min, samples were subjected to 30 cycles of 95°C for 30 s, 57 and 72°C for 1 min. The thermal cycles finished by a final extension step of 72°C for 5 min. The PCR products were then digested by MspI restriction enzyme at 37°C overnight and resolved on a 2.1% agarose gel containing ethidium bromide. The presence of 200 and 140 bp bands indicates the mutant homozygote (CC), the presence of three bands (340, 200 and 140 bp) indicates heterozygosity (TC) and the non-digested band of 340 bp represents the wild type TT genotype.

1-hydroxypyrene analysis

Urine samples were collected after the workshift on the last working day and stored at −20° C until analysis. Urinary 1-hydroxypyrene was measured according to the Jongeneelen *et al.* method[40]. In brief, 10 mL of urine of each participant was adjusted to a pH of 5 using 1 N HCl. Then, 0.1 M acetate buffer (pH 5) was added to urine to a final volume of 30 mL. This solution was incubated overnight (16 hours) with 15 μL glucoronidase-arylsulphatase in a shaking bath at 37 °C. A C18 reversed-phase cartridge was used for the

extraction of the metabolites. The cartridge was activated with 5 mL of methanol followed by 10 mL of distilled water. The urine sample was passed through the cartridge at a flow rate of 10 mL/min. The cartridge was washed with 3 mL of distilled water followed by 3 mL of 50% methanol in water. Thereafter, 1-hydroxypyrene was eluted with 8 mL methanol. The solution was completely evaporated and reconstituted with 1 mL of methanol. The concentration of 1-hydroxypyrene in this extract was determined by high performance liquid chromatography (HPLC) with a C18 reversed phase column and fluorescence detector.

Creatinine concentration of urine was determined by a qualified laboratory and urinary 1-hydroxypyrene was calculated in μmol/mol creatinine.

Analysis of urinary cotinine

For determination of urinary cotinine level, a commercially available direct ELISA kit (from Abnova, Taiwan) was used. The concentration of cotinine was measured according to the manufacturer's instructions.

Statistical analysis

Statistical analysis was performed using SPSS ver. 16 software. Kolmogorov-Smirnov Test was used to test the normality of data. Independent samples t-test was used for comparison of variables between two groups and pearson correlation test was used to evaluate the relationship between quantitative variables.

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