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Association between Variant Alleles of the X-Ray Cross Complementing Gene (XRCC1) with Benzo[a]Pyrene Levels in Iraqi Workers

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Abstract

Objectives: Assessment of a single nucleotide polymorphism of the XRCC1 (codon Arg399Gln) gene and its association with levels of benzo[a]pyrene in blood of car repairers and control group.

Methods: The current study included (111) participants, (37) of the Iraqi car repairers, (37) sellers of spare part, and (37) healthy (control), with the same age range. Benzo[a]pyrene was determined by using HPLC technique. Alleles frequency of single nucleotide polymorphisms of XRCC1 gene was determined by Restriction fragment length polymorphisms (PCR-RFLP).

Results: The results of current study indicated that there is a highly significant increase of B[a]P level ($P = 0.0001$) in the repairers group compared to the groups of spare part sellers and control together. The genotype frequencies studies of XRCC1 gene of car repairers group and controls. The effect of rs25487 genotypes on exposure to PAHs include higher levels of PAHs and PAH-DNA adduct in TT genotype followed by CT genotype and CC showed lower levels.

Conclusion: As results of prolonged exposure to the higher levels of PAHs that Iraqi car repairs suffer from, they have high levels B[a]P. Especially those who have the TT genotypes, as results of the inefficiency of their DNA repair system. Compared to the other genotypes.

Keywords: Polycyclic aromatic hydrocarbons (PAHs), B[a]P, XRCC1 gene, car repairers, spar part seller

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a large group of aromatic organic compounds comprising two or more fused benzene rings, the main sources of these global pollutants are industrial processes and incomplete combustion of organic materials, its produced as complex mixture contain more than a hundred compounds.[1] Among all the compounds, B[a]p is considered a representative of all PAHs compounds, and it is often applied in many studies as a positive control in biological assays for other PAHs individuals.[2,3] Also Benzo[a]Pyrene, among all individuals of the PAHs, represent one of the global studied pollutants and its found in Car exhaust fumes. Numerous studies have confirmed that its a potent immunosuppressive, proinflammatory and carcinogenic agent.[3,4] Fuel-powered cars and machines are protable sources that emit PAHs and thus increasing pollution in areas. Auto repairers are a group of the population are exposed to PAHs emitted from car exhaust on a daily basis, the main routes exposure to PAHs via inhalation or skin contact. This group have a high chance of exposure to PAHs, because they are in direct contact with fume emitted by cars containing PAHs, in addition to other reasons related to the personal hygiene and wear occupational safety equipments during work.[4-6] Garage workers are exposed to complex mixture of PAHs, because of their occupation, Also automobile mechanics are at an increased risk skin, lung, urinary tract cancer.[7] Several recent studies indicate that car repair shops are considered as anthropological sources of PAHs and heavy metals in some cities of the world.[8] Generally, the carcinogencity of PAHs espically B[a]P is begins after phase I biotransformation by inducible P450 superfamily (CYP1). And later the reactive speices like (Benzo[a]pyrene-7,8-diol-9,10-epoxides) (BPDE) are formed and mediated by the CYP2E1. The interaction between environmental and genetics factors play an effective role in the development of most cancers in humans.[6] Bulky PAHs-DNA adducts are repaired by two pathways, the first is base excision repair pathway (BER) and second is single strand breaks (SSB), where the X-ray repair cross-complementing gene (XRCC1) is implicated in both pathways, and it is play vitol role in the base excision repair pathway (BER).[8] Protein XRCC1 acts as a scaffolding protein in BER, via interaction with the ADP-ribose polymerase, DNA polymerase β, and DNA ligase III.14 A polymorphism of the XRCC1 gene at codon results from substitution of amino acidarginine in the place of glutamine, resulting in an ineffective repair pathway.[9] We conducted the present study to investigate the relationship between PAHs exposure in car repaires, and DNA repairing gene (*XRCC1*) polymorphisms in detecting workers at risk. Especially, the reports on occupational exposure and related health risks are almost non-existent, and this reflects paucity of availability of survey data and criteria for estimating whether unsafe exposure has occurred.

Subjects, Materials and Methods

The current cross-sectional study included 111 male volunteers with age range (25–45 years). Volunteers were divided according to their exposure to vehicle exhaust emissions into three groups: (i) (No. = 37) car repairers (as highly exposed group), (No. = 37) spare part sellers, and (iii) (No. = 37) non-exposed volunteers. All exposed subjects were matched in age, smoking status with unexposed group.

Five ml of venous blood sample was taken from each volunteers and divided into two tubes:

A. One ml was placed into disposable EDTA containing tubes and stored at –20°C until it was used in the genotyping study after DNA extraction. Quantity and quality of extracted DNA was determined by nano-drop, UK, using the scanning power of the diode assembly, within the wavelength 200–320 nm. The quantity and quality of

extracted DNA was determined by calculating the (260/280) and (260/230) ratios. Where samples that were (260/280) ratio less than 1.8 and/or (260/280) ratio 2, were re-extracted. The integrity and molecular weight of extracted DNA was determined by agarose gel electrophoresis according Samboork and Russell.

B. Four milliliters of blood sample pushed slowly into disposable gel containing tubes, and was allowed to clots 20 minutes at room temperature. After coagulation, the sera were separated by centrifugation at 3000 rpm for 10 min and stored at –20°C until it was used in the estimation of B[a]P.

Quantification of Benzo[a]pyrene

PAHs Extraction

Five ml of aqueous ethanolic and 2N of sodium hydroxide [9:1] was added to 1 ml of serum, and solution was ultrasound for 2 hours at 42°C. The sample was extracted by mixing it well with 5 ml of n-hexane and ultrasoned at 42°C for 2 hours, and the organic layer has been withdrawn to another container. Anhydrous sodium sulphate was added to clear supernatant to remove excess water, this sample was extracted two times with 10 ml of n-hexane. The extracts were evaporated to dryness at 40°C, and suspended with 0.25 ml of acetonitrile as shown in Figure 1 and PAH contents in the samples were determined by HPLC.[10,11]

HPLC Analysis

Pyrene and benzo[a]pyrene in serum were analysed by using C18 reverse phase column. The rate of flow was 1 ml min^{-1}, 10 µL of samples were injected into Knauer- HPLC (with System components listed in Table 1) and monitored at wavelength 254 nm. The peaks of individual PAH (Pyrene and benzo[a]pyrene) were recognized by comparing with the retention times of authenic standards as in Figure 2.

A gradient of mobile phase was prepared from water as (A) component, and acetonitrile as (B) component, and a gradient details is give in Table 2.

A serial dilution of standard of PAHs mixture was prepared by acetonitrile to achieve a concentration from 0.02–200 ng/ml.

PCR-RFLP Analysis Design

According to,[12] the genotyping technique was selected, while the primers are designed according to protocol of[13] briefly as follows: The primers were designed by the aid of NCBI-primer BLAST online software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome),

AR87f TAAGCAGGCTTCACAGAGCC

AR87r TGGCATCTTCACTTCTGCCC.

and the produced primers were checked for specificity of their target sequence by performing a BLAST against the human genome, then the primers pair was selected according to the demand criteria such as: product length, the similarity of melting temperature, primers length, specificity, etc. Then the mutations was interred according to the design demands. The primer ability to form secondary structure was checked by the aid of Oligo Calc online software (http://www.basic.northwestern.edu/biotools/oligocalc.html), the primer would be rejected if it had 5 bases or more able to form self-dimerization and/or it had 4 bases able to form hairpin. Each primers pair was checked for dimer formation by the aid of "Multiple Primer Analyzer" online software from Thermo Fisher Scientific Inc.©, the sensitivity of the software was adjusted to the value 2, the primer pair would be rejected if it made any dimers in this degree of sensitivity.

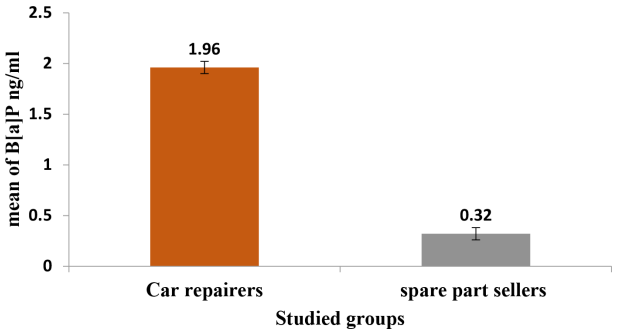

Fig. 1 **Comparison of levels of B[a]P in studied groups (car repairers and spare part sellers).**

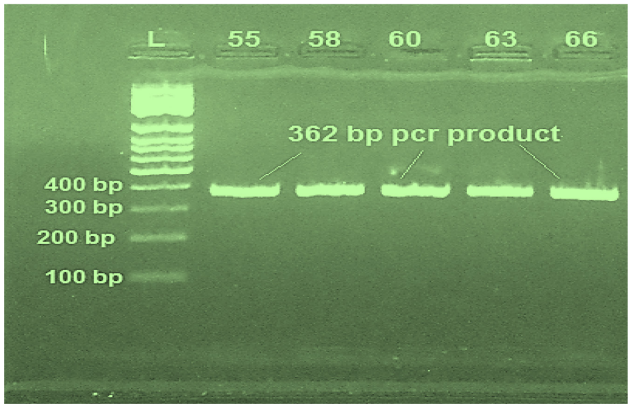

Fig. 2 **PCR-reaction products at different annealing temperatures (55, 58, 60, 63, 66 °C), under same optimized PCR conditions of Arg399Gln (rs25487).**

Table 1. **System components of HPLC, Knuaer, Germany**

No	Component	Model or version	Company and origin
1	Binary high pressure gradient pump	P6.1L	Knuaer, Germany
2	Diode array detector	DAD 2.1L	Knuaer, Germany
3	Sample loop (20 µl) and injector	D1357	Knuaer, Germany
4	Analyses and system control software	Claritychrom, V 7.4.2.107	Dataapex, Czech Republic

Table 2. **The gradient of mobile phase**

Time (min)	Mobile A (water) concentration%	Mobile B (acetonitrile) concentration%	Flow rate (ml/min)
Initial	40	60	0.7
10	0	100	0.7
35	0	100	0.7

Restriction Enzyme Selection

The selection of the suitable restriction enzyme (AsuC2I CC^SGG sib) was performed by the aid of WatCut online software (http://watcut.uwaterloo.ca/template), we selected the restriction enzyme according to several criteria such as: the lesser primer mutations needed, the distance of mutation from the variant, compatibility of the produced primers, cost and availability.

Optimization of PCR Condition of Arg399Gln (rs25487)

The mixture shown in Table 3. Was used as a preliminary mixture in the PCR reaction.

Then, different annealing temperatures were used to obtain a specialized and efficient product. The temperatures and optimized PCR condition used are shown in Table 4.

Arg399Gln (rs25487) Genotyping

Genotyping of XRCC1 (rs25487) polymorphisms was conducted by PCR-RFLP technique. And the restriction digestion of amplicon was digested by (AsuC2I), and the reaction mixture whose components were used: one unit of enzyme 0.25 µl, 5 µl of PCR product, 1.5 µl of buffer, and volume was completed to 15 µl by molecular graded water. The reaction mixture accubated in 37°C overnight. Then the reaction product resolved in 2% of agarose gel.

Statistical Analysis

The statistical calculations included in this study were carried out using SPSS software (IBM Corp. Released 2012. IBM SPSS statistics for windows, Version 21.0. Armonk, NY: IBM. Corp. USA) and Microsoft Excel (2010 Microsoft Corp. USA). The results expressed as mean ± SEM, and $P < 0.05$ was considered statistically significant. To evaluate the presence of significant differences, ONE WAY ANOVA, and unpaired-sample T-Test were employed. Also regression analysis used to asses presence of correlations, and the logistic regression was performed to adjust odd ratio.

Table 3. **Optimized reaction mixture for PCR**

No	Composition	Concentration	Volume
1	Master mix	2.5X	8 µl
2	Forward primer	10 pmol/µl	1 µl
3	Reverse primer	10 pmol/µl	1 µl
4	DNA sample	10-20 ng/µl	2 µl
5	Nucleases free water		7.5 µl
6	$MgCl_2$	25 mM	0.5 µl
	Total volume		20 µl

Table 4. **Optimized PCR condition of Arg399Gln (rs25487)**

Stage	Step	Temperature °C	Time	No. of cycles
1	Initial denaturation	94	5 min	1
2	DNA denaturation	94	30 sec	35
	Primer annealing	55–67	30 sec	
	Extension	72	30 sec	
3	Final Extension	72	5 min	1

Results and Discussion

The results demonstrate that the highest level of B[a]P was recorded in the group of car repairers, followed by the group of workers whose work in the same area. Where the results showed highest level of B[a]P in the group of Car repairers (1.97 ± 0.06), followed by the group of spare part sellers (0.32 ± 0.04). Where levels of B[a]P were significantly higher ($P < 0.001$) in group of car repairers compared to the group of spare part sellers (Table 5, and Figure 1).

B[a]P is considered the first chemical carcinogen that was discovered among all the PAHs individuals, and it was observed in the car exhaust fume.[13] Its carcinogenic pathway depends on the enzyme metabolize it, starting from the first stepandendingwiththemutagenicmetabolitebenzo[a]pyrene-7,8-diol-9,10-epoxide (BPDE).[14-15] Also the recent studies indicated that the automobile mechanics are at an increased risk of skin and lung cancers as a result of exposure to PAHs.[16] Annealing temperatures range (55–67°C) were used in optimization PCR conditions of Arg399Gln (rs25487) to obtain efficient product. The results obtained are shown in Figure 2. The best PCR product was obtained at a temperature of 63°C .

The results obtained from PCR-RFLP of (RS 25487) genotyping shown in the Figure 3, where the CC allele revealed 221bp and 141bp fragments, whiles TT allele was not digested, and visualized at 362bp single product. CT (hetrozoyget) allele revelaed three bands of 362bp, 221bp, and 141bp.

The results of RFLP-PCR for SNP (rs25487) genotyping of XRCC1 gene are listed in Table 6 for the car repaires, spar part sellers and control subjects. Where the results of genotype

Table 5. **Comparison of B[a]P levels among studied groups based on t-student test**

Group	Control mean ± SD	Car repaires mean ± SD	Spare part sellers mean ± SD	*P*-value
B[a]P	–	1.97 ± 0.06	0.32 ± 0.045	<0.0001

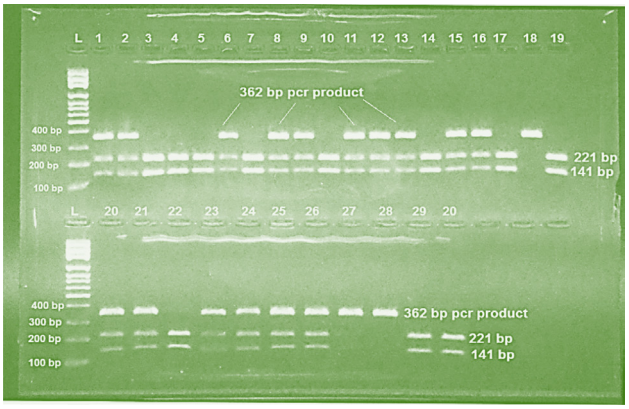

Fig. 3 **PCR-RFLP of (rs25487) genotyping; Lane L = 100bp DNA ladder; Lane (362bp) = TT genotype; Lanes (221bp + 141bp) = CC genotype; and Lanes (362bp + 221bp + 141bp) = TT genotype.**

frequencies of car repaires specimens revealed that CC genotype were higher in sample of auto repairs (45.94%) compared to the control group (16.21%), this is showed a significant differences (<0.0001) with an odd ratio equal to (17.8). The heterozygous genotype CT had lower frequency in samples of car repaires (29.72%) than controls (32.34%) and this combined with non-significant ($P = 0.08$) and low odd ratio (0.421). The frequency of TT genotype was higher in control samples (51.35%) compared to the samples of car repaires (24.32%) with odd ratio (P <0.13) differences was significant ($P = 0.001$).

The results of genotypes of XRCC1 gene of are listed in Table 7 for the spare part sellers and control subjects. Where the results of genotype frequencies of spare part sellers specimens revealed that CC genotype were higher in spare part sellers (45.94%) compared to the control group (16.21%), this is showed a significant differences ($P < 0.0001$) with an odd ratio equal to (17.8). The heterozygous genotype CT had lower frequency in samples of spare part sellers (29.72%) than controls (32.34%) and this combined with non-significant ($P = 0.08$) and low odd ratio (0.421). The frequency of TT genotype was higher in control samples (51.35%) compared to the samples of spare part sellers (24.32%) with odd ratio ($P = 0.13$) differences was significant ($P = 0.001$).

Effect of rs25487 genotypes on B[a]P levels

The effects of SNP (rs25487) genotyping of XRCC1 gene on B[a]P level are illustrated on the Figures (3 and 4), whereas the highest level of B[a]P had been detected in the car repaires subjects who possess the TT genotype (1.76 ± 0.08) followed by heterozygouse genotype CT (1.607 ± 0.06) and then CC genotypes (0.424 ± 0.08). And also the spare part sellers, where the highest level of B[a]P was in the subjects who carriers TT genotype (0.963 ± 0.04), followed by heterozygouse genotype CT (0.681 ± 0.08) and then CC genotypes showed the lowest level (0.096 ± 0.04).

The result of our study showed the CC dominant homozygous genotype of the XRCC1 (DNA repairing gene) showed significantly lower levels of measured PAHs comparison to the other genotypes, followed by heterozygous genotype CT, and than TT shown higher levels (Figure 4). Our results are agreement with many previous study[17] found that the CC genotype has lower levels of PAHs. It was also found in another study[18] that the Chinese coke oven workers carrying the TT genotype had higher levels of BPDE-DNA adduct. The variations in the levels of PAHs among the studied genotypes are due to the variation in the activity of xenobiotics

Table 6. **Frequencies association between genotyping of XRCC1 gene of car repairs group samples and controls**

Genotype	Control ($N = 37$)	Car repairers ($N = 37$)	*P*-value	Odds ratio	95% C.I.
CC Wild	4 (10.81%)	25 (67.56%)	<0.0001	17.18	4.49 to 9.702
CT Heterozygous	16 (43.24%)	9 (24.32%)	0.088	0.421	0.156 to 1.139
TT Mutant	17 (45.94%)	3 (8.10%)	0.001	0.103	0.027 to 0.398
Allele frequency %					
Allele	**Control ($N = 37$)**	**Car repairers ($N = 37$)**	***P*-value**	**Odds ratio**	**95% C.I.**
C	24	59	<0.0001	8.1944	3.88 to 17.29

Table 7. **Frequencies association between genotyping of XRCC1 gene of spare part sellers group samples and controls**

Genotype	Control ($N = 37$)	Spare part sellers ($N = 37$)	*P*-value	Odds ratio	95% C.I.
CC Wild	4(10.81%)	21(56.75%)	0.0001	10.828	3.181to36.849
CT Heterozygous	16(43.24%)	9(24.32%)	0.088	0.421	0.156 to 1.139
TT Mutant	17(45.94%)	7(18.91%)	0.0155	0.274	0.096 to 0.781
Allele frequency %					
Allele	**Control ($N = 37$)**	**Worker ($N = 37$)**	***P*-value**	**Odds ratio**	**95% C.I.**
C	24	51	<0.0001	5.503	2.059 to 10.22
T	50	21			

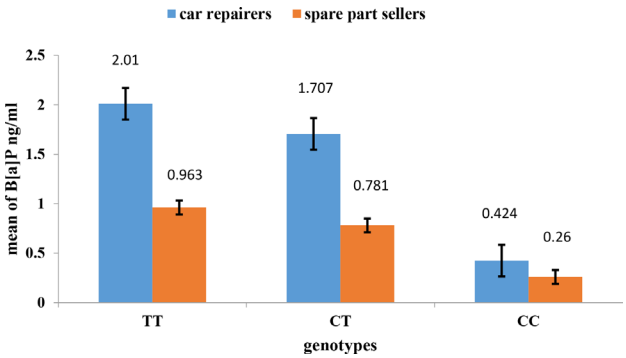

Fig. 4 **Effects of SNP (rs25487) genotyping of XRCC1 gene on B[a]P level.**

metabolizing enzymes.[19] Many previous studies reported that the TT genotype had a decrease in activity of xenobiotics metabolizing enzymes (especially CYP2E1), as result, they have high levels of PAHs. This resulting in long-term adverse effects, promoting cytotoxicity and genotoxicity, making individuals more susceptible to different types of cancers.[19] And considering that B[a]P is a complete carcinogen, that act as an initiator and a promoter of carcinogenesis according to EPA, 2017.[20] At the same time, it was observed that the CC genotypes had low levels of BPDE-DNA adduct levels this is supports findings of[21] Matullo et al., who stated that the carriers of CC showed a relatively lower levels of bulky DNA adduct in lymphocytes, which may be association with reduced capacity of DNA repair system. Also in meta-analysis conducted by Kiyohara et al.[22] TT genotype of the XRCC1 rs 25487 (Arg399Gln) polymorphism found that was increased PAHs levels and it may be association with low DNA repair capacity. The results obtained in this study were agreement with other previous study stating that CT and TT genotype is represent a risk of various types of cancer like lung cancer caused by the environment.[23]

Conclusion

As results of prolonged exposure to the higher levels of PAHs that Iraqi car repairs suffer from, they have high levels B[a]P. especially those who have the TT genotypes, as results of the inefficiency of their DNA repair system. Compared to the other genotypes.

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Conflicts of Interest

None.

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