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Original Research Article

Evaluation of Benzo (a) Pyrene DNA Adducts as a Biomarker of Genotoxicity and its Association with Genetic Polymorphism of Liver Detoxification Enzymes GSTM1 and GSTT1 in a Sample of Lebanese Population

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ABSTRACT

Humans are exposed exogenously to chemical carcinogen from occupational, environmental, life style or dietary source, and that such exposure can cause a number of different human cancers. Genotoxic biomarkers are used to measure specific occupational and environmental exposure and to predict the risk of disease in subjects exposed to chemical carcinogens. To our knowledge, there is no study about the effect of the Polycyclic aromatic hydrocarbons (PAH) pollution on the Lebanese population, notably the effect of the procarcinogen Benzo{a}pyrene (B{a}P) and its ultimate mutagen Benzo{a} pyrene diol epoxide (BPDE) on human's DNA. BPDE-DNA adduct genotoxic biomarker was studied on subjects from different regions of the North District of Lebanon of various age and gender. BPDE-DNA adducts were detected by the ELISA method. An alarming result was observed for Al-Tal Area residents, a crowded area of Tripoli city where the highest percentage of 63.2%, for positive BPDE-DNA adducts was obtained followed by 52.9% for Chekka region, and a low percentage was observed for Al-Mina region (42.9 %). The highest levels of BPDE-DNA concentrations adducts were notably observed in Al-Tal region with 31.6% of the residents of this area showed higher than 125 ng/mL. The association of BPDE-DNA adducts with the genetic polymorphism of the glutathione S-transferase (GST) genes: GSTM1, and GSTT1 which have been shown to play an important role in the metabolic detoxification of B{a}P showed that GSTM1 has little or no effect on the occurrence of BPDE-DNA adducts, while the GSTT1 null phenotypes showed higher percentage of positive BPDE-DNA adducts.

Keywords: Benzo(a)pyrene, DNA adducts, pollution, gene-environment, GSTM1, GSTT1, genetic polymorphism.

INTRODUCTION

Over the last decade, molecular epidemiology studies using biomarkers of exposures have provided evidence of genotoxic effect of pollutants particularly for the atmospheric Polycyclic Aromatic Hydrocarbons (PAHs). [1,2] The most potent PAH carcinogens include benzo[a]anthracene, benzo[a]pyrene, and dibenz[a,h]anthracene. [3] These carcinogens products share the common feature of reacting with DNA to form covalently modified DNA bases or DNA adducts that can be detected in various tissues including

blood.[4] Benzo[a]pyrene Diol epoxide DNA adduct (BPDE-DNA adduct) is considered as the most significant genotoxic biomarker, its half-life in lymphocyte is in the order of months. The repair of aromatic DNA adduct vary between individuals according to their genetic susceptibility.[5] They play a significant role in the causation of certain types of cancer and represent a measure for exposure as well as risk. Biological monitoring of BPDE-DNA adducts in WBC account for all exposure route (inhalation, ingestion and dermal absorption). [6] Studies on the levels of BPDE-DNA adduct and chromosome damage proved in many cases an increased risk of genetic damage in population occupationally exposed to high levels of B{a}P generated either from industrial or urban sources. [7,8] Variation in the susceptibility to chemical- induced mutagenic or carcinogenic activity in different individuals may partly be explained by differences in the activity or gene polymorphisms of enzymes participating in the metabolism of B{a}P and their detoxification such as glutathione S-transferases or GSTs. [9,10] GSTs are super family of multifunctional detoxification enzymes found in all eukaryotic organisms. They are classified into three main families: cytosolic, mitochondrial, and membrane-bound microsomal. [11] The cytosolic GST (cGSTs) is the most important family found predominantly in the liver but is also distributed into various tissues and has a number of isoenzymes. GST has many functions involved in the biotransformation of exogenous substances including mutagens, carcinogens, and other poisonous chemicals. [12] Seven classes of GSTs are recognized in mammals designated as Alpha, Mu, Pi, Sigma, omega, theta and Zeta and are encoded by GSTA, GSTM,GSTK, GSTP, GSTS, GSTO, GSTT and GSTZ gene. They are grouped according to amino acid sequence, substrate specificity and immunological cross-reactivity. [11] The best known substrate for GSTs enzymes are the metabolite derived from the bioactivation of PAHs, specifically PAH diol epoxide. The GSTM1, GSTT1 and GSTP1 polymorphisms are the most common polymorphisms of GSTs enzymes in the human population, with major ethnic differences. They have been studied most extensively due to their important detoxification function and their association with cancer. [13] The aim of the study is to effect of Benzo(a) pyrene on Lebanese human health and the potential associated risks. The study evaluates the frequency of the BPDE-DNA adducts genotoxic biomarker among the subjects recruited from three different regions of the North District of Lebanon (Chekka, Tripoli, El Mina). The study aims to analyse correlation of BPDE-DNA adducts with different demographic variants. The purpose of the study was to investigate the association of BPDE-DNA adducts with the genetic polymorphism of GSTM1 & GSTT1 genes involved in Benzo{a}pyrene metabolism.

MATERIALS AND METHODS

Subjects' recruitment

The study group consisted of fifty unrelated Lebanese volunteers aging 20-43 years old from three different regions in North Lebanon. Participants were recruited from Dagher Medical Laboratories in Chekka, Hamidi Dispensary in Zehriyeh-Al Tal, Habib Fallah Dispensary in El Mina. A signed informed consent was obtained. The volunteers were asked to complete a questionnaire concerning their age, gender, residential area, smoking status, occupational exposure, general health conditions and drugs. Peripheral blood sample were withdrawn by venipuncture into tubes containing EDTA and were collected between March and June of the year the study was conducted. Samples were coded, placed on ice, transported immediately to the laboratory, and frozen at -20 ° C.

DNA extraction and analysis

Leukocytes in whole blood from all the subjects were obtained by venipuncture

and separated after centrifugation at 1000 rpm. Extraction of genomic DNA was performed using the FlexiGene DNA kit (QIAGEN) according to the manufacturer's instructions. In brief, 1250 µl of lysis buffer was added to 500 µl of blood sample, followed by centrifugation at 10000 x g for 20 seconds in order to remove the cell nuclei and mitochondria. The supernatant was discarded and the pellet was resuspended and incubated in 250 µl denaturation buffer containing protease for the removal of proteins. DNA was precipitated by addition of 250 µl isopropanol, recovered by centrifugation, washed in 70% ethanol, dried and resuspended in 200 µl of hydration buffer (10 mMTris-Cl, pH 8.5). The concentration of DNA was determined by measuring the UV absorbance at 260 nm and the purity was ascertained by the ratio at 260/280 nm. Genomic DNA samples were examined by using 1% agarose gel electrophoresis.

Detection of BPDE-DNA adducts

Then genomic DNA samples were analyzed by the highly sensitive Benzo{a} pyrene 7,8diol9,10 epoxide (BPDE)-DNA enzyme linked immune-sorbent assay (ELISA). In brief, the extracted DNA samples were diluted to 2µg/ml in cold PBS. 100µl of the DNA samples and of BPDE-DNA standards were loaded in duplicate into the DNA high binding plate and left at 4°C overnight. The DNA solutions are removed and washed twice with PBS. A 200 µl of assay diluents was added to each well and blocked for 1 hour at room temperature. 100µl of the primary antibody anti-BPDE I was added to the wells after removing the assay diluents and the plate is incubated for 1 hour at room temperature on an orbital shaker. Later the wells were washed with washing buffer and blocked for 60 min with the blocking reagent before being probed with 100 µl of secondary antibody, HRP conjugate and incubated again. Finally a substrate solution was added to the plate and an incubation period of 2-30 min took place according to the change of color and the enzymatic reaction was stopped by the addition of a stop solution. The absorbance of BPDE adducts was immediately read on a microplate reader at 450 nm at the chamber of Commerce, Industry and Agriculture of Tripoli.

PCR amplification

The analysis of the GSTM1 and the GSTT1 genotypes was performed by polymerase chain reaction (PCR) using specific primers. The exon 7 of CYP1A1 gene was amplified and used as internal control. DNA samples were amplified by PCR using 100-200 ng genomic DNA as an initial template. The PCR assay was carried out using a REDTaqReadyMix PCR reaction mix containing MgCl2. The PCR reaction mix final volume was 40µl and included 1µM of each primer.

Acrylamide gel electrophoresis for PCR products

The PCR products from coamplification of GSTM1, GSTT1 andCYP1A1 genes were then analyzed using 5% acrylamide gel electrophoresis. A volume of 2.6bml of 30% acrylamide was mixed with 13.2ml water, 2 ml of 10X TBE buffer and of 80 % glycerol, 100 µl of APS and 25µl TEMED. Poor the solution between the 2 glass plates and allow the solution to polymerize. A volume of 10 µl of each of the PCR products was mixed with 5µl loading dye then loaded into the acrylamide gel. The electrophoresis was run in 1x TBE buffer for 2 hrs at 65V, then stained with ethidium bromide. DNA bands were then visualized by UV-transilluminator. The presence or absence of GSTM1 and GSTT1 genes was detected by the presence or absence of a band at 215 bp and 480 bp respectively. A band at 312 bp (corresponding to CYP1A1 gene) was always present and was used as internal control to document a successful PCR amplification.

Statistical analysis

Data was analyzed using the statistical package SPSS (version 18). All values are represented as mean ± S.D. Genotype and allelic frequencies are presented as percentages and were

computed and tested by student's t-test e. The differences in genotype and allele frequencies between the samples were tested using the t-test. At p-value < 0.05, differences were considered statistically significant.

RESULTS

Demographic characteristics of recruited subjects

Table 1: Demographic and physical characteristics of subjects recruited for the study

Gender (male/female)	**Male**	**18**
	Female	32
Age (years)	20-25	18
	25-30	9
	30-35	9
	35-40	12
	>40	4
Geographic location[1]	Chekka	17
	Tripoli- Al Tal	19
	AL Mina	14

Fifty subjects were initially enrolled in this study. The recruited volunteers were randomly selected and unrelated. All subjects have been recruited without any history of chronic diseases from residential and rural areas. Demographic characteristics are represented in Table 1.

Subjects were considered to match a region if their house residence and their work are both located within the region.

The subjects were distributed into 32 females and 18 males, the age group ranged between 20-43 years old. Subjects were asked for their residence geographic location and their work location, only volunteers with both their work and house residence falls within the corresponding geographic location were considered resident of the region or if they spend more than 16 hours per day within the region.

Table 2: Frequency of BPDE-DNA adducts in the North region

	BPDE-DNA adducts		**p-value**
	Negative	**Positive**	
Total (n=50)	23 (46%)	27 (54%)	0.509
Tripoli-Al Tal (n=19)	7 (36.8%)	12 (63.2%)	
El Mina(n=14)	8 (57.1%)	(42.9%)	
Chekka (n=17)	8 (47.1%)	9 (52.9%)	

The volunteers were randomly selected from three different regions in North; Al-Tal is a highly populated area at the centre of the city and a crowded area with buses and public transport. Al Mina region is a less populated city than Tripoli and is open to the sea (Figure 1). Chekkais an industrialized area where two cement factories Holcim (HC) and Cimenterie du Liban (CLi) are usually found in addition to the presence of quarries. The study was designed to collect blood samples between March and June of the same year where the study was conducted. This period was characterized over the last few years by high levels of air born particulate matter pollution PM10 according to Tripoli Environment and Development Observatory where the PM10 of 103 µg/m3 exceed the Daily National Standard of 80 µg/m3.

Detection of BPDE-DNA adduct in the North region

Whole genomic DNA of recruited subjects was extracted from blood samples and levels of BPDE-DNA adducts were assessed by the ELISA method. All the concentrations of BPDE higher than15.6 ng/mL were considered to be positive for BPDE-DNA adduct. Results showed that 54% of the recruited subjects were positive for BPDE-DNA adducts compared to 46% of them, which were negative for the BPDE-DNA adducts (Table 2).

To determine whether the BPDE-DNA adducts would be correlated to the geographic location of the recruited subjects, the distribution of the frequency of BPDE adducts was analyzed for three different regions of the North district. Results showed that the center of Tripoli – Al Tal showed the highest BPDE-DNA adducts frequency with a 63.2 % of subjects which are positive to BPDE-DNA adducts compared to 52.9% and 42.9% of DNA adducts in Chekka and Mina respectively; but this result was not statistically significant when the three areas are compared (p=0.509).The recruited subjects of the three different areas were then stratified according to BPDE-DNA adducts concentrations (Table 3). Results showed that low concentration of BPDE-DNA adducts, below 15.6ng/ml, was the most

frequent in El Mina residents with 57.1% followed by Chekka and Tripoli with 47.1% and 36.8% of DNA adducts respectively. Medium range of BPDE-DNA adducts concentration between15.6 ng/ml and 125 ng/ml was equally observed in El Mina and Chekka with 42.9% and 41.2% of DNA adducts and Tripoli residents showed the lowest frequency of adducts with 31.6%. While at high range of BPDE-DNA adducts concentration, above 125ng/ml, Tripoli residents showed the highest frequency, 31.6%, compared to Chekka with 11.8%. It is interesting to note that none of the volunteers who lived in El Mina region had a high concentration of BPDE-DNA adducts.

Figure 1: Aerial photo of geographical studied regions represented on the Map of Lebanon: Chekka ,Tripoli–EL Tal, EL Mina. Figure was generated using Google maps applications.

Table 3: Frequency of BPDE-DNA adducts concentration according to region

	BPDE-DNA adduct concentration			
	[BPDE]<15.6 ng/ml	**15.6<[BPDE]<125ng/ml**	**[BPDE]>125ng/ml**	**p-value**
Tripoli-Al Tal (n=19)	7 (36.8%)	6 (31.6%)	6 (31.6%)	
El Mina (n=14)	8 (57.1%)	6 (42.9%)	0 (0%)	
Chekka (n=17)	8 (47.1%)	7 (41.2%)	2 (11.8%)	0.189

Table 4: Genotype frequency of GSTM1 and GSTT1 among Lebanese North population

	Genotype Frequency			
	GSTM1 (n=50)		**GSTT1(n=50)**	
	+/+ (active)	**Null 0/0 (Inactive)**	**+/+ (active)**	**Null 0/0 (Inactive)**
Total (n=50)	34 (68%)	16 (32%)	36 (72%)	14 (28%)
Male (n=18)	13 (72.2%)	5 (27.5%)	15 (83.3%)	3 (16.7%)
Females (n=32)	21 (65.5%)	11 (34.4%)	21 (65.6%)	11 (34.4%)
p-value	0.631		0.181	

Detection of GSTM1 and GSTT1 genetic polymorphism by multiplex PCR

The polymorphism of both GSTMl and GSTTl genes was detected using multiplex PCR analysis. Primers were designed to detect the null alleles of both genes (GSTT1 0/0, GSTM1 0/0), the null allele of one of the genes (GSTT1 +/+, GSTM1 0/0 or GSTT1 0/0, GSTM1 +/+) or the presence of both of the genes (GSTM1+/+, GSTT1+/+). Results of the multiplex PCR were also confirmed by running samples with a conventional PCR, and multiplex PCR results were in complete agreement with that observed using independent analysis of both genes.

As shown in Figure 2, after PCR reactions on genomic DNA using specific primers and separation on 5% polyacrylamide gels, several bands were

observed: a band of 215 bp which corresponds to the presence of an active GSTM1, a band of 480bp which corresponds to the presence of an active GSTT1. All the PCR reactions contained an amplified product of 312 bp for the internal control of the CYP1A1 gene fragment used for multiplex PCR reactions.

Frequency of GSTM1 and GSTT1 genotypes in the recruited subjects

The frequency of GSTM1 and GSTT1 genotypes was analyzed among the recruited subjects; and the prevalence of the deleted genotypes among the recruited subjects is given in Table 4.

Results showed that the null GSTM1 0/0 genotype was 32% among North Lebanese population compared to 68% for the GSTM1+/+ genotype. The deleted GSTT1 0/0 null genotype was similar to the GSTM1 frequency with 28% for the null genotype and 72% for the GSTT1+/+ genotype indicating that most of the north Lebanese population possesses an active GSTM1 and GSTT1 genes.

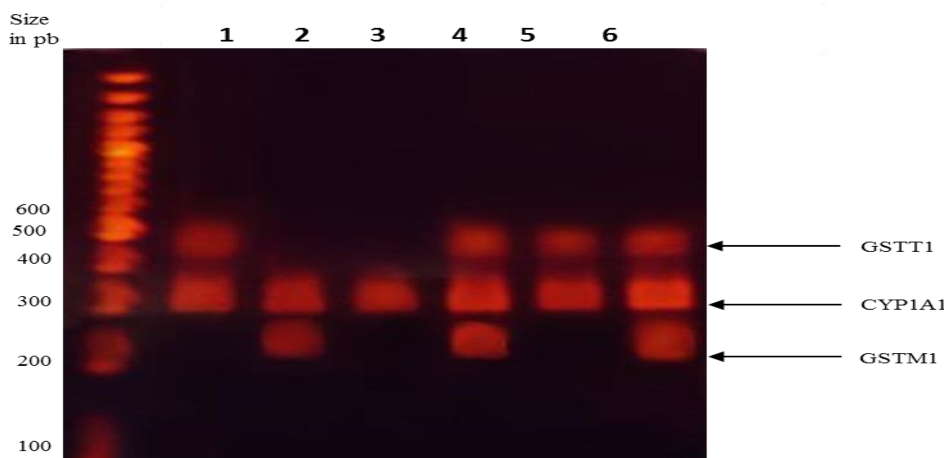

Figure 2: Multiplex PCR amplification of GSTM1 and GSTT1 gene. Multiplex PCR products analyzed on 5% (w/v) polyacrylamide gel stained with ethidium Bromide.
Lane 1,5: individuals harboring GSTTl +/+ and GSTMl 0/0 alleles
Lane 2: individual harboring GSTTl 0/0 and GSTMl +/+ allele
Lane 3: individual with null allele for both GSTMl and GSTTl genes (GSTMl 0/0 and GSTTl 0/0)
Lane 4,6: individuals with GSTTI +/+ and GSTMI +/+ alleles
All lanes show an intermediate band at 312 bp corresponding to the internal control (CYPlAl gene fragment)

Table 5: Association of BPDE-DNA adduct with GSTM1 and GSTT1 genetic polymorphism among Lebanese North population

		Genotype Frequency			
		GSTM1 (n=50)		GSTT1 (n=50)	
		+/+ (active)	Null 0/0 (Inactive)	+/+ (active)	Null 0/0 (Inactive)
BPDE-DNA adduct	Negative (n=23)	16 (47.1%)	7 (43.8%)	18 (50%)	5 (35.7%)
	Positive (n=27)	18 (52.9%)	9 (56.2%)	18 (50%)	9 (64.3%)
p-value		0.827		0.363	

Table 6: Association of BPDE- adduct with combined GSTM1 and GSTT1 genetic polymorphism among Lebanese North population

		Genotype Frequency			
		GSTM1+/GSTT1- (n=8)	GSTM1-/GSTT1+ (n=10)	GSTM1+/GSTT1+ (n=26)	GSTM1-/GSTT1- (n=6)
BPDE-DNA adduct	Negative (n=23)	3 (37.5%)	5 (50%)	13 (50%)	2 (33.3%)
	Positive (n=27)	5 (62.5%)	5 (50%)	13 (50%)	4 (66.7%)
p-value		0.837			

Effect of GSTM1 and GSTT1 on Benzo (a) Pyrene DNA adducts

The effect of GSTM1 and GSTT1 genetic polymorphism on BPDE-DNA adducts was also investigated. The genetic polymorphisms of GSTM1, GSTT1each alone or the combination of both genes were examined (Table 5,6).

The results of GSTM1 genetic polymorphism, showed that 56.2% of the Null GSTM1 0/0 carrier were positive for BPDE-DNA adducts. While 52.9% of the GSTM1+/+ carriers were positive for DNA adducts indicating that the GSTM1 is not significantly associated with BPDE-DNA adducts (P= 0.823).

Analysis of GSTT1 polymorphism, showed that 64.3% of the recruited subjects with the Null GSTT1 0/0 were positive for BPDE-DNA adducts compared to 50% of the GSTT1 +/+ carriers who were also positive to BPDE-DNA adducts. Although this result was not significantly associated with BPDE-DNA adducts (P=0.363), but it showed that GSTT1 gene may play an important role in the formation of BPDE-DNA adducts.

To examine the effect of the presence or absence of both genes GSTM1 and GSTT1, the recruited subjects were compared for the occurrence of both polymorphism with the BPDE-DNA adducts formation. An equal frequency (50%) was observed when subjects have only the inactive GSTM1 (GSTM1-/GSTT1+) or both genes were active (GSTM1+/GSTT1). This result indicate that the Null GSTM1 alone may not play a significant role in the formation of adducts. In contrast, a high frequency of adducts (62.5%) was observed when subjects have only inactive GSTT1 (GSTM1+/GSTT1-), indicating the significant role of GSTT1 gene. In addition, the combination of both Null genes (GSTM1-/GSTT1-) reveal a high frequency of adducts with 66.7 %. Thus, the association of both Null genes is a major contributor in the formation of BPDE-DNA adducts.

DISCUSSION

Genotoxic biomarkers, particularly DNA adducts have been shown to be a promising biomarker to measure the environmental exposure to chemical carcinogen like the metabolite benzo {a} pyrene diol epoxide. This study shed the light on the effect of benzo{a}pyrene pollution in the North district of Lebanon. Results show that BPDE-DNA adducts were significantly higher in city residents explaining the fact that exposure to pollutants (PAHs compounds including Benzo {a} pyrene) generated from gasoline vapors, increased the formation of DNA adduct in exposed population. [14] Overall, our data are in agreement with a number of studies as in Cotonou and Poland which reported that population exposed to environmental pollution show increased levels of DNA adducts. [15,16] For example a study carried out in Cotonou showed that city residents are exposed to significantly higher levels of adducts in traffic jammed area of Cotonou and are of elevated risk for development of chronic lung diseases and cancer related to Benzene, B{a}p and other PAH. [17] Similar results were shown in a highly polluted area in Silesia (Poland) where the adduct level was 2-3 times higher than the suburbs. [18,19]

Concerning the association of BPDE-DNA adducts with the genetic polymorphism of GSTM1 and GSTT1 genes, the existence of a Null allele is associated with the lack of expression of a functional protein, that could result in increased concentration of epoxide intermediate and hence higher DNA adducts. [20,21] Several studies have reported conflicting results regarding the influence of genetic polymorphisms in DNA adduct formation. For example Garte et al found an association between glutathione S-transferase (GSTs) genes inactivation and DNA adducts formation by PAHs[16]. Another positive association between the GSTM1 null genotype and PAH-DNA adducts levels were found in human lung tissue and mononuclear white blood cells [22,23]

In contrast, other studies did not report differences in PAH-DNA adduct levels between subjects with null or active GST genotypes when analysing occupationally or environmentally PAH exposed subjects and smokers. [17,24 -26] Our data confirmed that genetic polymorphisms

has an important role in the formation of adducts and increasing cancer risks, suggesting that GSTT1 gene is a potential candidate for cancer risk.

CONCLUSION

The present study is a pilot study reflecting the effect of environmental pollutions on human health and the relation between gene-environment. An alarming situation of air pollution was detected in al Tal region of Tripoli city for Benzo{a}pyrene mutagenic effect on Lebanese population, this study should increase the awareness of the Lebanese society to environmental pollution in the overpopulated cities like Tripoli. The study highlights the necessity to put an urgent plan to monitor this pollutant in air and to minimize its dangerous effect.

Author Disclosure Statement

No competing financial interests exist.

Ethics Statement

This study was approved by the Ethics in Research Committee at the University and was conducted according to the Ethical Principles for Medical Research Involving Human Subjects of the World Medical Association Declaration of Helsinki. Written informed consent forms were obtained from all participants.

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