ORIGINAL ARTICLE

ROLE OF THE *CYP1A2* GENE POLYMORPHISM ON EARLY AGEING FROM OCCUPATIONAL EXPOSURE

Eshkoor SA[1,2,*], Ismail P[1], Rahman SA[2], Moin S[2], Adon MY[3]

*Corresponding Author: Dr. Sima A. Eshkoor, Institute of Gerontology, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia (UPM), 43400, Malaysia; Tel.: +60-129-797-812; Fax: +60-389-472-744; E-mail: simaataolahi@yahoo.com

ABSTRACT

The ageing process is influenced by many internal and external factors. The toxic substances in the environment can cause genomic damages to cells, which increase the risk of early ageing. Furthermore, the cytochrome P450 1A2 (*CYP1A2*) gene polymorphism is a susceptibility factor and may enhance the risk of DNA damage in cells. The current study was carried out to show whether occupational exposure could cause genotoxicity in cells carrying the *CYP1A2* gene polymorphism, thus enhancing the likelihood of early ageing. This study was conducted on mechanical workshop workers and a control group by collecting buccal cells from their mouths. Restriction fragment length polymorphism-polymerase chain reaction (RFLP-PCR) was used to identify the *CYP1A2* gene polymorphism in the cells. In addition, three extra methods including micronuclei (MN) test, comet assay and real-time PCR (RT-PCR) were applied to determine the effects of gene polymorphisms on DNA damage and ageing from occupational exposure. The results showed that DNA damage in the cells carrying the mutated genotype was higher than the wild geno-type. In addition, the difference in MN frequency (p = 0.001) and relative telomere length (p = 0.002) between workers and controls was significant (p <0.05) in the mutated genotype. The findings indicated a possible protective effect of gene polymorphism against early ageing, which was characterized by lack of a significant influence of *CYP1A2* gene polymorphism on genetic material in the subjects (p >0.05). It was concluded that the *CYP1A2* gene could be a contributing factor to prevent early ageing from occupational exposure.

Keywords: Ageing; Buccal Cells; Cytochrome P450 (CYP), Cytochrome P450 1A2 (*CYP1A2*) gene polymorphism; Micronuclei (MN) test; Occupational exposure

INTRODUCTION

Ageing happens due to the accumulation of mutations in the genome of somatic cells. It results in tissue atrophy, development of neoplasia and decreased functions of cells and tissues [1]. A combination of both genetic and environmental factors can affect the process at the cellular level [2]. As ageing affects resistance against diseases and speeds up the end, new data and investigations suggest that the ageing process can be slowed down at the molecular and cellular level, thereby increasing ones' life-span [3].

Cytochrome P450 (CYP) enzymes are involved in phase I of xenobiotic metabolism to oxidize these compounds. Such enzymes can metabolically produce

[1] Institute of Gerontology, Universiti Putra Malaysia (UPM), Serdang, Selangor, Malaysia

[2] Department of Biomedical Sciences, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia (UPM), Serdang, Selangor, Malaysia

[3] Institute for Medical Research, Ministry of Health Malaysia, Kuala Lumpur, Malaysia

activated substances from chemicals that may act as highly reactive mutagenic metabolites [4]. There are specific forms of CYP450 enzymes that are major susceptibility biomarkers to activate mutagens and their activities associated with a variety of socio-demographic factors and genetic characteristics [5].

The cytochrome P450 1A2 (*CYP1A2*) gene polymorphism is one of CYP450 enzymes family involved in metabolic activation of many of chemicals. The reactive oxygen species (ROS) generated by the CYP1A2 enzyme activity can lead to the oxidative DNA damage and mutagenesis in cells. Apparently, the enzyme activity is an important tool to assess the risk of mutagenesis from chemical exposure. The polymorphism of the *CYP1A2* gene can affect the levels of enzyme activity. The polymorphism type can cause a higher activity of enzyme from exposure to different environmental factors such as smoking and caffeine [6].

Using different molecular and cytogenetic techniques in various studies of toxicology helps to reflect the risk of exposure to mutagenic agents such as environmental and occupational exposures. In addition, biomarkers are used in the studies to evaluate exposure, effect and susceptibility in individuals. As a susceptibility biomarker, the CYP genetic polymorphism can affect the activation or inactivation of xenobiotics and determine the risk of DNA damage at the exposure to genotoxic agents. Such biomarkers, and the biomarkers of early biological effects, help to identify the risk of genome damage in cells [7]. These data can serve as an early warning to show the potential risk of health damage from long term chemical exposure. Thus, using biological parameters increased our ability to study the effects of exposure and determine the spectrum of DNA damage [8]. This study was carried out to identify whether occupational exposure had any effect on DNA damage in the cells carrying the *CYP1A2* gene polymorphism that enhanced the risk of early ageing.

MATERIALS AND METHODS

Permission and approval for the study were obtained from the ethical committee of the Medical and Health Sciences Faculty, University Putra Malaysia (UPM), Ser-dang, Selangor, Malaysia [Reference Number: UPM/ FPSK/PADS/T7-MJKEtikaPer/F01 (JSB-Aug (08)05]. The samples were epithelial cells of buccal mucosa. For this project 120 mechanical workshop workers were selected. The exposed group included males aged 18 years and above. The considered duration time of working in the workshops was at least 1 year or more. Furthermore, 120 people who were not exposed to petrochemical products such as fruit sellers, textile shop keepers, sellers in mobile phone shops, restaurant workers, sundry shops workers, bank staff, photography shop workers, supermarket staff, workers in computer centers, electronic centers and optical examination centers, were selected as a control group. Subjects were interviewed about their health status, educational level, smoking habits, alcohol consumption, work history, duration of working at one occupation and other aspects relevant to the study. In addition, workers were divided into two groups, those with 5 or more years in one group and those with less than 5 years in another group.

Respondents were asked to rinse their mouth with water before collection of the buccal samples. The cells were collected by scraping the inner part of the cheeks both sides with a cytology brush. Then, the cells were gently mixed with 0.9% sodium chloride and phosphate buffered saline (PBS) in separate micro-centrifuge tubes and brought to the laboratory. The cells were treated for micronuclei (MN) test, comet assay, real-time polymerase chain reaction (RT-PCR) and restriction fragment length polymorphism (RFLP). The effect of polymorphism on the samples was assessed by MN formation, comet tail length and telomere length shortening as the biological parameters. The methods of the MN test and comet assay were performed according to a pattern described in [9]. In the current study, genomic DNA was extracted from the cells using QIAamp DNA blood MiniKit (Qiagen, Courtaboeuf, France) and then was quantified by Nanodrop™ 1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Meanwhile, the extracted DNA was run on a 0.7-1.0% agarose gel. Genomic DNA was used to run RT-PCR and RFLP.

Real-Time Polymerase Chain Reaction. In RT-PCR, the reaction was performed to optimize primers and determine a suitable annealing temperature. The primers for telomere and 36B4 were those described in a prior study [10]. The appropriate annealing temperature for both primers was 56°C. The primer sequences were: tel1 (5'-GGT TTT TGA GGG TGA GGG TGA GGG TGA GGG TGA GGG T-3'); tel2 (5'-TCC CGA CTA TCC CTA TCC CTA TCC CTA TCC CTA TCC

CTA-3'); 36B4u (5'-CAG CAA GTG GGA AGG TGT AAT CC-3'); 36B4d (5'-CCC ATT CTA TCA TCA ACG GGT ACA A-3'). Real-time PCR improved the assessment of relative telomere length measurement. The telomere repeat copy number to single gene copy number (T:S) ratio was detected by using Corbett Rotor-Gene 6000 (Corbett Life Science, Sydney, New South Wales, Australia) in a 36-well format. The extracted DNA from buccal cells was used in the procedure. During the reaction, each sample was evaluated concurrently for both telomere and the housekeeping gene *36B4*. The primers were obtained from Bioline (London, UK). For each PCR reaction, a 25 μL volume of solution was prepared in the PCR tube. The solution for the PCR reaction included 0.6 μL of each primer, 1 μL of Eva green (Biotium, Hayward, CA, USA), 1 μL of DNA, 5 μL of master mix Immomix (Bioline), and 16.8 μL of pure water. The reaction proceeded one cycle of denaturation at 94°C for 5 min., followed by 40 cycles at 94°C for 30 seconds, 56°C for 30 seconds, and 72°C for 50 seconds. The melting temperature was arranged between 70°C and 95°C. Finally, the products were resolved in 2.0% gel to ensure amplification of the specific products. All samples were run in triplicate for both genes, and the threshold value was assessed. In addition, a serial dilution of genomic DNA derived from one sample was run to ensure having a good view of the efficiency of the PCR reaction standard curve. The obtained data from the samples were interpreted throughout the data set to assess the threshold cycle (Ct) values. The T:S ratio was calculated based on the fractional number between the average *36B4* Ct value and the average telomere Ct value for each sample. One sample was used as reference in each run in triplicate for comparing the results. The formula $2^{-\Delta\Delta}$ was used to calculate the T/S ratio. For calculating, $\Delta C_T = C_t$ (telomere) $- C_t$ (*36B4*) formula was applied. Final calculation was based on the results of ΔC_t (target) $- \Delta C_t$ (reference) formula to compare the measurements and take proper T:S ratio. Furthermore, the RFLP method was applied to identify the *CYP1A2* gene polymorphism.

Polymerase Chain Reaction and Restriction Fragment Length Polymorphism CYP1A2. Polymerase chain reaction was performed to optimize primers and obtain proper annealing temperature by gradient PCR analysis. Primer sequences for CYP1A2 were: sense (5'-GCT ACA CAT GAT CGA GCT ATA C-3') and anti-sense (5'-CAG TCT CTT CAC TGT AAA GTT A-3'). The forward and reverse primers were selected from the published article [11]. The solution volume in the PCR tube was 25 μL. Immomix master mix (Bioline) containing dNTPs, Taq polymerase, $MgCl_2$ and a buffer were used for PCR reactions. To prepare the product, each tube received 5 μL of immomix master mix, 0.6 μL of primer, 2 to 6 μL of genomic DNA and 12.8 to 16.8 μL of pure water. The tubes were placed in a G-Storm Thermal Cycler (Gene Technology Ltd, Braintree, Essex, UK) for PCR reaction. The PCR was performed in 35 cycles. The first cycle initiated by an incubation time at 94°C for 5 min. The reaction was then followed by a denaturation step at 94°C for 30 seconds, annealing at 62°C for 30 seconds, extension phase at 72°C for 45 seconds and a final extension at 72°C for 10 min. after the last cycle. After completing amplification, the samples were stored at 4°C until used. A negative control without a DNA template was carried out in every run. The specific PCR product was identified by running 1.8 to 2.0% agarose gel electrophoresis, and the gel was then viewed with an AlphaImager analysis system (Alpha Innotech, San Leandro, CA, USA). The product size was 596 bp.

Fifteen microlitres of PCR reaction product was put aside for RFLP. First, 7.3 μL of double-distilled water (ddH$_2$O) was added to the PCR tube, then 1.5 μL of restriction enzyme (RE) buffer, and then 6.0 μL of amplified PCR product. Finally, 0.2 μL of each RE including Fast Digest *Bsl*I and *Dde*I 10 U/μL was added to the tube. The mixture was mixed well by gently pipetting. The reaction mixture for each enzyme was prepared in the discrete tubes and then incubated on a heating block at 37°C for 16 hours. After incubation, *Dde*I enzyme was inactivated by incubating at 65°C for 20 min. on a heating block. Fast Digest *Bsl*I enzyme inactivation could be done by phenol or chloroform extraction. The size of the products was then identified using agarose gel electrophoresis. The gels used for PCR and RFLP products were 2.0 and 4.0%, respectively. Meanwhile, DNA ladders of 50 bp and 100 bp (Bioline) were used to identify the size of the products. Finally, the gel was viewed under UV light using the AlphaImager™ 2200 (Alpha Innotech) system.

Statistical Analyses. All data were analyzed using the Statistical Package for the Social Sciences (SPSS, Chicago, IL, USA) software version 16.0. The statistical tests were independent *t*-test and the

Mann-Whitney U-test. Micronuclei frequency and DNA damage tail length were tested using the non parametric Mann-Whitney U-test. The analysis method of the relative telomere length was the independent *t*-test. The Hardy-Weinberg equilibrium was evaluated by using the χ^2 to test the rightness of the fit between adjusted samples. The critical level for rejection of the null hypothesis (two-tailed test) was the *p* value of 5.0% ($p = 0.05$).

RESULTS

The *CYP1A2* gene polymorphism was amplified by PCR followed by the RFLP method using *Dde*I and *Bsl*I restriction enzymes. Using both restriction enzymes helped to fix the determination of the *CYP1A2* gene polymor-phisms. Figures 1, 2 and 3 show the images of PCR and RFLP products of the *CYP1A2* gene. The *CYP1A2* gene polymorphism was amplified by PCR and the product was 596 bp (Figure 1).

The RFLP products resulting from the *Dde*I enzyme digestion were the wild (WW) and mutant (WM, MM) genotypes. The size of the WW genotype was 596 bp; the fragment sizes for the heterozygous mutant (WM) genotypes were 596, 464 and 132 bp, and for
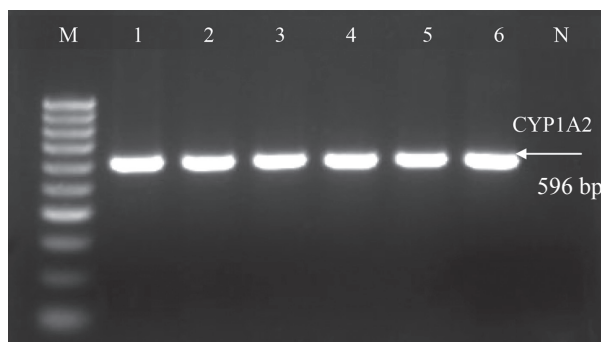
Figure 1. Amplification of the CYP1A2 PCR in 2.0% agarose gel electrophoresis in lanes 1 through 6; M represents the 100 bp DNA ladder; N is a negative control during the PCR reaction.

homozygous mutant (MM) genotypes were 464 and 132 bp (Figure 2). The RFLP products after digestion with the *Bsl*I enzymes were MM (475 and 121 bp), MW (475, 343, 132 and 121 bp) and WW (343, 132 and 121 bp) genotypes (Figure 3). The RFLP products of CY-P1A2 using the *Dde*I enzyme digestion were referred as the wild and mutated genotypes. The WW genotype was placed in the wild group and WM and MM were in the mutated group. The respective frequencies of the WW, WM and MM genotypes were 49.2, 46.3 and 4.6%

**Table 1.** Effect of the CYP1A2 genotypes on the biomarkers in the individuals.

Biomarkers			MN	Comet Tail Length	Relative Telomere Length
CYP1A2 Genotypes	*n*		*p* Value	*p* Value	*p* Value
WW	118		0.272	0.122	0.287
MW, MM	122		0.272	0.122	0.287

MN: Micronuclei; WW: wild genotype; MW: heterozygous mutant genotype; MM: homozygous mutant genotype.
The Mann-Whitney U-test was used for DNA damage and MN and the independent *t*-test for telomere length ($p = 0.05$).

Table 2. The CYP1A2 genotype effects on the biomarkers between the workers and controls.

Genotypes		*n*	MN		Comet Tail Length		Relative Telomere Length	
			Mean ± SD	*p* Value	Mean ± SD	*p* Value	Mean ± SD	*p* Value
WW	Workers	58	11.66 ± 4.31		23.70 ± 8.59		0.23 ± 0.37	
	Controls	60	2.38 ± 4.31	0.001	17.14 ± 7.81	0.089	1.68 ± 7.02	0.114
MW, MM	Workers	62	12.89 ± 4.38		26.46 ± 9.01		0.32 ± 0.72	
	Controls	60	2.50 ± 1.85	0.001	17.82 ± 8.24	0.062	2.98 ± 6.17	0.002

MN: Micronuclei; WW: wild genotype; MW: heterozygous mutant genotype; MM: homozygous mutant genotype.
The Mann-Whitney U-test was used for DNA damage and MN and the independent *t*-test for telomere length ($p = 0.05$).
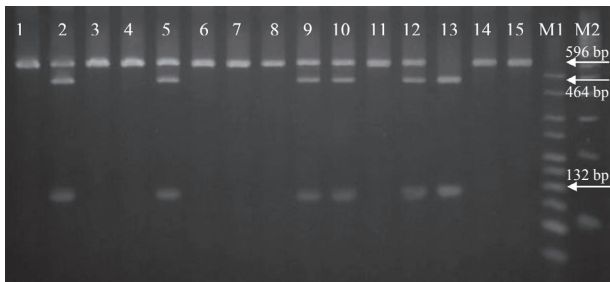
Figure 2. Restriction fragments of the *CYP1A2* gene with the *Dde*I enzyme resolved in 4.0% agarose gel electrophoresis in lanes 1 through 15; M1 and M2 represent the 50 and 100 bp DNA ladders, respectively.
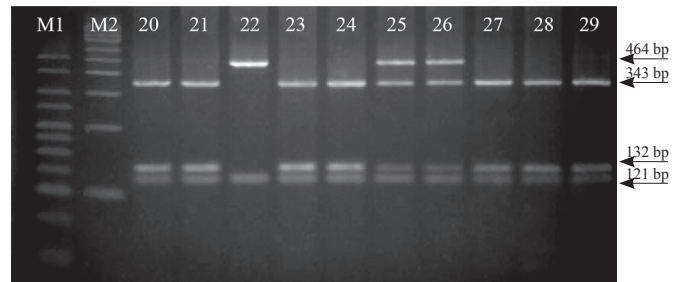
Figure 3. Restriction fragments of the *CYP1A2* gene with the *Bsl*I enzyme resolved in 4.0% agarose gel electrophoresis in lanes 20 through 29; M1 and M2 represent the 50 and 100 bp DNA ladders, respectively.

in all individuals. Furthermore, the results showed that the gene polymorphism did not significantly influence ($p >0.05$) the individual biomarkers (Table 1).

Differences in biomarkers between workers and controls was evaluated in wild (WW) and mutated (WM and MM) genotypes. It was found that difference in MN frequency between workers and controls was statistically significant in both wild ($p = 0.001$) and mutated ($p = 0.001$) genotypes. In addition, the results showed that the mutated genotype significantly affected the relative telo-mere lengths ($p = 0.002$) in workers. No statistically significant effect on comet tail length ($p >0.05$) was found in wild or mutated genotypes (Table 2).

The results showed that the gene polymorphism had no significant effects on the biomarkers in workers and control groups below 30 years old or above. However, the wild genotype significantly affected comet tail length in workers below 30 years of age ($p = 0.047$) (Table 3). The findings of socio-demographic factors indicated that ethnicity had a significant effect on MN frequency ($p = 0.004$). Furthermore, duration time of 5 years or more significantly affected MN frequency ($p = 0.001$), comet tail length ($p = 0.001$) and relative telomere length ($p = 0.001$). It was found that smoking, alcohol consumption and educational levels showed no statistically significant effect on each of the biomarkers ($p >0.05$).

DISCUSSION

DNA damage can occur due to the effects of the *CYP1A2* gene and environmental factors [12]. The subjects showed a shorter telomere length on the mu-

tated genotype yet a higher MN frequency in both wild and mutated genotypes. Such effects suggest the possible influence of gene polymorphism on DNA damage mediation in the cells [13], which could be due to the increased enzyme activity as well as sensitivity of cells to genotoxic effects [12]. Despite the paradoxical reports, our result confirmed a correlation between the different genotypes and MN frequency [14]. Such correlation is probably due to more sensitivity of MN than the other biomarkers to express the effects of genotypes on genetic material damage from occupational exposure. Contrary to previous reports indicating DNA damage in the cells [5,6,15], no statistically significant influence was observed on comet tail length in our research either in wild or mutated genotypes.

In the current study, age did not contribute to enhance the influence of the *CYP1A2* gene presenting in higher MN, shorter telomere length and greater comet tail length in workers and controls, except greater comet tail length in the younger group of workers. Lack of age effects suggest a protection effect of gene against ageing. Apparently, the *CYP1A2* gene prevents the modulation of DNA damage [16-18] by transcriptional activation and resistance to changes, which can be interfered with lifestyle factors [19,20], different genotypes and genes interactions [21].

Among all lifestyle factors, only ethnicity significantly affected MN frequency regardless of occupational exposure, which indicates the possible influence of gene polymorphism on the cell protection against genome damage [22]. Meanwhile, this research was a confirmation of the studies [23,24] indicating the association of DNA damage with working duration time. It seems that good interpretation of results

Table 3. The *CYP1A2* gene polymorphism and age effects on the biomarkers between the mutated and wild genotypes in the worker and control groups.

Groups				MN		Comet Tail Length		Relative Telomere Length	
	Age	Genotypes	*n*	Mean ± SD	*p* Value	Mean ± SD	*p* Value	Mean ± SD	*p* Value
Workers	>30 years	WW	23	15.77 ± 3.33		31.86 ± 6.04		0.03 ± 0.03	
		MW, MM	31	15.48 ± 3.82	0.909	31.07 ± 8.54	0.535	0.03 ± 0.02	0.939
Workers	<30 years	WW	35	8.95 ± 2.23		18.34 ± 5.03		0.36 ± 0.43	
		MW, MM	31	10.30 ± 3.06	0.074	21.85 ± 6.94	0.047	0.62 ± 0.93	0.166
Total Workers	All ages	WW	58	11.66 ± 4.31		23.70 ± 8.59		0.23 ± 0.37	
		MW, MM	62	12.89 ± 4.32	0.116	26.46 ± 9.01	0.102	0.32 ± 0.72	0.373
Controls	>30 years	WW	5	6.07 ± 1.90		29.29 ± 3.96		1.39 ± 1.36	
		MW, MM	6	6.78 ± 2.25	0.537	26.76 ± 6.33	0.537	1.35 ± 2.85	0.976
Controls	<30 years	WW	55	2.04 ± 1.38		16.03 ± 7.11		1.71 ± 7.33	
		MW, MM	54	2.04 ± 1.01	0.583	16.83 ± 7.86	0.565	3.16 ± 6.43	0.275
Total Controls	All ages	WW	60	2.38 ± 1.80		17.14 ± 7.81		1.68 ± 7.02	
		MW, MM	60	2.50 ± 1.85	0.543	17.82 ± 8.24	0.607	2.98 ± 6.17	0.286

MN: Micronuclei; WW: wild genotype; MW: heterozygous mutant genotype; MM: homozygous mutant genotype.
The Mann-Whitney U-test was used for DNA damage and MN and the independent *t*-test for telomere length ($p = 0.05$).

depends on a suitable sample size in each group of various genotypes and age. However, the study was cross-sectional, therefore, finding effective and non effective factors were difficult. Another limitation of the study was the difficulty of determining and isolating the exact effects of gene polymorphisms. Despite the above limitations, this study can serve as a base to address the effects of these genotypes and surrounding risk factors on early ageing. Accordingly, CYP1A2 genotypes contributed to DNA damage from occupational exposure, hence, further investigations are needed to evaluate the exact effects of different genotypes on a subject's premature aging.

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