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Scientific Paper

DOES *GSTP1* POLYMORPHISM CONTRIBUTE TO GENETIC DAMAGE CAUSED BY AGEING AND OCCUPATIONAL EXPOSURE?

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The aim of our study was to see the effects of *GSTP1* polymorphism on biomarkers of ageing, including micronuclei (MN), comet tail length, and relative telomere length in automobile repair workers, who are exposed to a broad spectrum of potential mutagens. The analysis was performed on buccal cells collected from occupationally exposed and non-exposed (control) subjects. Samples were analysed using cytogenetic and molecular methods, including restriction fragment length polymorphism (RFLP), MN test, comet assay, and real-time PCR. The results confirmed the DNA damaging effects of substances used in the mechanical workshops, but did not confirm the influence of *GSTP1* gene polymorphism on DNA damage. However, further studies on both occupationally exposed and control populations are needed to understand the relationship between *GSTP1* polymorphism and genome damage.

KEY WORDS: *buccal cells, comet tail length, glutathione* S-*transferases, micronucleus frequency, PCR, RFLP, telomere length*

DNA damage resulting from exposure to mutagens is a major threat to the living cell (1). Ageing is an interaction between genetic and environmental factors, which takes place at the cellular level. Cellular ageing seems to be related to nuclear and mitochondrial DNA damage and diminished DNA repair (2). It is responsible for increased susceptibility to disease and increased death rate, which accompany advanced age (3). Aware of the association between advanced age and disease and death, researchers have continued to provide information about ways to support healthy life. The useful span of human life has been extended by slowing down the rate of ageing (4). According to Wojda (2), accumulation of cells at the end of their replicative life in the elderly contributes to the ageing of the tissue. How exactly this accumulation of senescent cells contributes to ageing is still unknown (5), but it is reasonable to assume that many organs become dysfunctional because their cells have lost or altered their function. Wojda (2) believes that multiple acting genes control replicative senescence.

Accumulated mutations resulting from DNA replication over a long period of time play an important role in ageing. These mutations are irreversible, which in many cases can be harmful. Unsafe mutations will become toxic to cells (6). Glutathione *S*-transferase (GST) detoxifies potentially mutagenic and toxic DNA-reactive electrophiles, including the metabolites of several chemotherapeutic agents (7). Its gene families include *GSTM1, GSTT1*, and *GSTP1*. The homozygous deletions of *GSTM1* and *GSTT1* (null genotypes) have no enzymatic activities (8). In contrast, *GSTP1*, located on chromosome 11q13, is involved in detoxifying environmental compounds.

However, its I105V polymorphism - with a switch from isoleucine to valine at codon 105 in exon 5 - seems to reduce the enzyme activity (9).

The aim of this study was to evaluate the effects of occupational exposure on DNA damage under the possible influence of *GSTP1* I105V polymorphism in exposed car repair workers and controls, as current data (10) suggest that the interaction between this gene polymorphism and genotoxic effects of exposure could speed up ageing.

MATERIALS AND METHODS

Sample collection

This study was approved by the ethics committee of the Faculty of Medicine and Health Sciences (Reference Number: UPM/FPSK/PADS/T7-MJKEtikaPer/F01 (JSB-Aug (08)05). Samples were collected from 120 exposed workers from car repair workshops and 120 unexposed controls. None of the occupationally exposed subjects wore gloves. None of the workshops had proper ventilation. The subjects were interviewed to evaluate their health status and lifestyles. They were asked to rinse their mouth with water before samples were collected. Epithelial cell samples were collected by scraping the inner part of both sides of the cheeks with a cytology brush. The cells were then gently mixed with 1.5 mL of 0.9 % sodium chloride and PBS in a micro-centrifuge tubes, taken to the laboratory and prepared for the micronucleus (MN) testing, comet assay, real-time polymerase chain reaction (PCR), and restriction fragment length polymorphism (RFLP).

Micronucleus test

The cells were smeared on slides, dried, and fixed with a cold solution of 1 % glutaraldehyde in 0.1 mol L^{-1} phosphate buffer (pH=7.5) for 20 min. The slides were stained with Feulgen reagent using a modified protocol by Beliën et al. (11). First the cells were hydrolysed in 5 mol L^{-1} HCl at 27 °C for 30 min. The slides were then washed in distilled water for 5 min and then stained with a fresh Schiff reagent (Sigma Chem, Japan) within 45 min. Finally, the slides were washed under tap water for 15 min, counterstained with 0.1 % naphtol-yellow (Sigma-Aldrich, India) within 20 s, and air-dried overnight. To determine MN frequency for each sample, 2000 cells were analysed under a light, dry lens microcope at 200x magnification (Nikon, Japan).

Comet assay

The comet assay was used to determine the extent of DNA damage in the cells. We used the Trevigen CometAssay™ kit protocol (Trevigen, USA). The assay was started immediately after the cells were prepared. As a precaution, all work must be done under dim light to prevent DNA damage from UV rays. The cells were combined with LM agarose at 37 °C at the ratio of 1:10, and 75 µL aliquots were immediately pipetted onto the slides. The slides were prepared in duplicate and placed flat in a dark place at 4 °C for 10 min. The slides were then immersed in the pre-chilled lysis solution (Trevigen, USA) for 60 min, followed by immersion in a freshly prepared alkaline solution, pH>13, at room temperature in the dark for 45 min. After that, the slides were placed flat on a gel tray. Electric power was set at 1 V cm^{-1} (measured from electrode to electrode) and applied for 10 min. The slides were then completely stained with 50 µL of diluted SYBR Green (Trevigen, USA) before viewing with a fluorescent microscope DM 2500 (Leica, Germany) at 200x magnification, and the images were captured. The cells were analysed using commercial TriTek Comet Score (version 1.5) software (TriTek Corp., Sumerduck, VA, USA). Tail lengths of the comets were determined from the centre of the head towards the last visible signs at the end of the tail and are expressed in micrometres.

DNA extraction

Genomic DNA was extracted from the cells using the QIAamp DNA blood miniKit (Qiagen, Courtaboeuf, France). The purity and the concentration of extracted DNA were quantified using a Nanodrop™1000 spectrophotometer (Thermo Scientific, USA) and the DNA was run on a 0.7 % to 1 % agarose gel. PCR reaction was performed to optimise the primers.

Real-time PCR

Real-time PCR was used to measure relative telomere length. The used primers were telomere and 36B4, as described earlier by Cawthon (12). The primer sequences were: tel1, GGTTTTT GAGGGTGAGGGTGAGGGTGAGGGT GAGGGT; tel2, TCCCGACT-ATCCCTATCCCTAT CCCTATCCCTA TCCCTA; 36B4u, CAGCAA GTGGGA AGGT-GTAATCC; 36B4d, and CCCA

TTCTATCA TCAACGGGTACAA. The telomere repeat copy number to single gene copy number (T/S) ratio was determined using the Corbett Rotor-Gene 6000 (Corbett Life Science, Sydney, Australia) with 36 wells. For PCR reaction, a 25 μL volume of solution was prepared in PCR tubes. In each run, both gene telomere and 36B4 were settled for one sample in separate tubes. Primers were obtained from Bioline (London, UK).

The solution for PCR reaction included 0.6 μL of each primer, 1 μL of Eva green, 1 μL of DNA, 5 μL of master mix Immomix (Bioline, London, UK), and 16.8 μL of pure water. The reaction consisted of 41 cycles; the first started with incubation at 94 °C for 5 min, followed by denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s, and extension at 72 °C for 50 s. Melting temperature was 70 °C to 95 °C. The data obtained from the samples were interpreted throughout the dataset to assess threshold cycle values. After the reaction, the product size was 76 bp for the telomere and 74 bp for 36B4.

PCR reaction

PCR reaction was performed to provide the product for RFLP. First it was used to optimise the applied primers. Forward and reverse primers have already been described by Harries et al. (13). Primer sequences were: sense, 5'-ACCCCAGGGCTCTATGGGAA-3' and anti-sense, 5'-TGAGGGCACAAGAAGCCC-CT-3'. The solution volume in the PCR tube was 25 μL. Immomix master mix (Bioline, London, UK) containing dNTPs, Taq polymerse, $MgCl_2$, and a buffer were applied to PCR reactions. To prepare the product, each tube received 5 μL of Immomix master mix, 0.6 μL of primer, 2 μL to 6 μL of genomic DNA, and 12.8 μL to 16.8 μL of pure water. The tubes were placed in a G-Storm Thermal Cycler (Gene technology Ltd, UK) for PCR reaction.

The PCR reaction was performed in 35 cycles. The first cycle started with incubation at 94 °C for 5 min, followed by denaturation at 94 °C for 30 s, annealing at 62 °C for 30 s, extension phase at 72 °C for 45 s, and the final extension at 72 °C for 10 min after the last cycle. Proper annealing temperature was obtained through gradient PCR analysis. After completing amplification, the samples were stored at 4 °C until use. A negative control without DNA template was carried out in every run. The specific PCR product was identified by running 1.8 % to 2 % agarose gel electrophoresis, and the gel was then viewed with an AlphaImager analysis system (Alpha Innotech, San Leandro, CA). The product size was 176 bp.

RFLP reaction of GSTP1

Fifteen microlitres of PCR reaction product was put aside for RFLP. First we added 7.2 μL of ddH_2O to the PCR tube, then 1.5 μL of restriction enzyme buffer, and then 6.0 μL of purified PCR product. Finally we added 0.3 μL of ALW26I restriction enzyme (10 units per μL; Fermentas, USA). The mixture was gently pipetted up and down to mix the solution well. The reaction mixture was incubated in a heating block at 37 °C for 16 h. After incubation, the enzyme was inactivated by incubating in the same heating block at 65 °C for 20 min.

Determination of products

Agarose gel electrophoresis identified PCR and RFLP products sizes. Gels used for PCR and RFLP were 2 % and 4 %, respectively. We also used DNA ladders of 50 bp and 100 bp (Bioline, London, UK) to identify product size. Finally the gel was viewed under UV light using the AlphaImager TM 2200 system.

Statistical analysis

The normality of variables was evaluated using the Kolmogorov-Smirnov test. The Mann-Whitney U-test, independent t-test, and ANOVA were used to compare the demographic characteristics of study populations. Differences in MN frequency and DNA damage measured by the comet assay were tested using the non-parametric Mann-Whitney U-test. Telomere length measurements were tested using the independent t-test. Gene frequencies were estimated by gene counting, and Hardy-Weinberg equilibrium was evaluated using the chi-square test for goodness of fit adjusted for samples. The critical level for rejection of the null hypothesis (two-tailed test) was the p value of 5 % ($p=0.05$). All analyses were performed using the Statistical Package for the Social Sciences (SPSS, Chicago, IL) software version 16.0.

RESULTS

Figures 1 and 2 are the images PCR and RFLP products of the *GSTP1* gene. The *GSTP1* gene was amplified by PCR reaction and the product was in the size of 176 bp (Figure 1). RFLP products of *GSTP1*

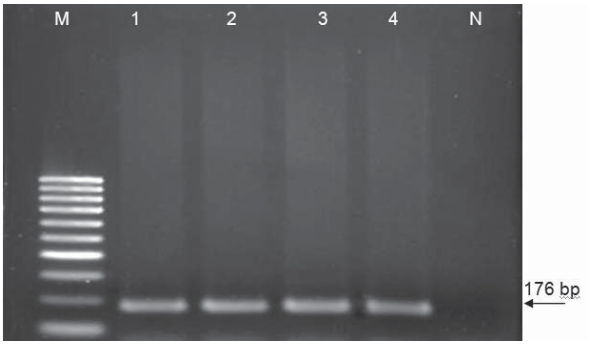

Figure 1 *PCR product of* GSTP1 *gene run on a 2-percent agarose gel in samples 1 to 4. M is a 100 bp DNA ladder. N is negative control in PCR reaction.*

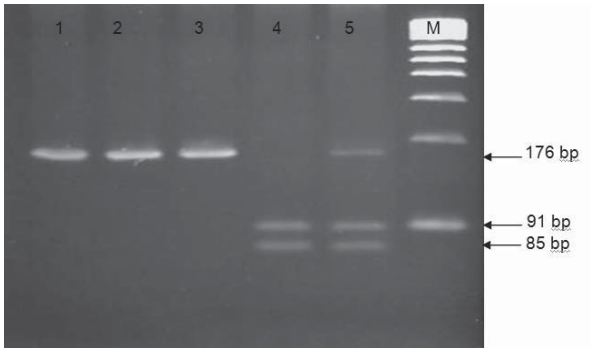

Figure 2 *Restriction fragments of* GSTP1 *gene run on a 4-percent agarose gel in samples of 1 to 5. M is a 100 bp DNA ladder. Lanes 1, 2, and 3 show that the enzyme has no restriction site (176 bp). Lane 4 shows that the enzyme produced two fragments of this gene (91 bp, 85 bp). Lane 5 shows that the enzyme produced three fragments of this gene (176 bp, 91 bp, and 85 bp).*

using ALW26I restriction enzyme were Ile-Ile (176 bp), Ile-Val (176 bp, 91 bp, and 85 bp) and Val-Val (91 bp and 85 bp) (Figure 2). The products were identified as wild and mutated. Ile-Ile genotype was placed in the wild group and Ile-Val and Val-Val were in the mutated group. The respective frequencies of Ile-Ile, Ile-Val, and Val-Val genotypes were 60.0 %, 30.4 %, and 9.6 % in all subjects. Gene polymorphism did not significantly influence ($p>0.05$) individual biomarkers (Table 1). Workers with the mutated genotype (Ile-Val, Val-Val) had a significantly higher MN frequency ($p<0.001$), shorter telomere length ($p=0.008$), and greater comet tail length ($p=0.025$) than controls. The same is true for workers with the wild genotype Ile-Ile, save for the comet tail length (Table 2). When workers and controls were divided by age into groups below 30 years and above, gene polymorphism did not significantly affect the studied biomarkers. However, the mutated genotype showed a significant effect on MN frequency ($p=0.021$) in the control group as a whole (see Table 3 control MN frequency, $p=0.021$).

Smoking, alcohol consumption, and educational level showed no statistically significant effect on MN frequency, comet tail length, and telomere length, but ethnicity had a significant effect on MN frequency

Table 1 *Effect of* GSTP1 *genotypes on the biomarkers in the individuals*

Biomarkers ***GSTP1*** **genotypes**	**N**	**MN**	**Comet tail length**	**Relative telomere length**
		p	**p**	**p**
Ile-Ile	144	0.481	0.984	0.763
(Ile-Val, Val-Val)	96			

Mann-Whitney U-test was used for DNA damage and MN-test and t-test for telomere length

Table 2 *Effect of* GSTP1 *genotypes on the biomarkers between the workers and controls*

Biomarkers ***GSTP1*** **genotypes**		**N**	**MN**		**Comet tail length**		**Relative telomere length**	
			Mean±SD	**p**	**Mean±SD**	**p**	**Mean±SD**	**p**
Ile-Ile	Workers	78	12.21±4.57	<0.001	25.21±9.52	0.120	0.30±0.67	0.019
	Controls	66	2.13±1.68*		16.70±7.62		2.65±7.94	
(Ile-Val, Val-Val)	Workers	42	12.45±3.92	<0.001	24.96±7.65	0.025	0.23±0.35	0.008
	Controls	54	2.82±1.92*		18.43±8.42		1.93±4.53	

Mann-Whitney U-test was used for DNA damage and MN test and t-test for telomere length
**Significant difference between controls with the wild and mutated genotype (p=0.021)*

Table 3 GSTP1 *polymorphism and age effects on the biomarkers between mutated and wild genotype groups in the workers and controls*

	Age	Geno-types	N	MN		Tail length		Telomere length	
				Mean±SD	p	Mean±SD	p	Mean±SD	p
Workers	≥30	W	34	15.95±3.70	0.259	32.51±7.73	0.160	0.03±0.02	0.518
		M	20	15.02±3.41		29.54±6.94		0.03±0.03	
	<30	W	44	9.32±2.70	0.220	19.58±6.47	0.442	0.51±0.84	0.598
		M	22	10.12±2.74		20.80±5.71		0.41±0.40	
Controls	≥30	W	3	6.78±2.70	0.921	25.79±9.41	1.00	2.62±3.93	0.527
		M	8	6.33±1.93		28.70±3.48		0.89±1.23	
	<30	W	63	1.91±1.27	0.104	16.27±7.33	0.861	2.66±8.10	0.687
		M	46	2.21±1.10		16.64±7.72		2.11±4.87	

Mann-Whitney U-test was used for DNA damage and MN test, and t-test for telomere length (p=0.05)
W = wild genotype; M = mutated genotype

(p=0.004) (Table 4). Duration of employment was divided in more than or less than 5 years and showed a significant effect on MN frequency (p<0.001), comet tail length (p<0.001), and telomere length shortening (p=0.001) (Tables 4, 5 and 6).

DISCUSSION

GSTP1 gene seems to play particular role in the detoxification of inhaled toxicants (14, 15). However, the mutated *GSTP1* variant seems to be less effective in detoxification than the wild genotype (16, 17). Some studies demonstrated that gene variants can influence the effect of occupational exposure. For instance, Heuser et al. (18) showed that the mutated genotype (Ile/Val or Val/Val) was associated with greater DNA damage in Brazilian footwear workers than the wild (Ile/Ile) genotype (19). These studies point to an interaction between the exposure and genotype.

In our study, *GSTP1* polymorphism was not associated with significantly higher MN frequency, comet tail length, and lower telomere length in the exposed workers and controls in the age groups, and we only found a significant difference in MN frequency of controls.

Previous studies (18, 20, 21) reported that the alteration of the transcriptional activation of enzyme played an important role in ageing. Even though age undeniably contributed to genome damage in our study, this can not be said for *GSTP1* polymorphism. However, the results of this study as well as those presented by Heuser et al. (18) must be interpreted with caution because of the relatively small number

Table 4 *Results of MN test in subgroups of workers and controls with different socio-demographic factors*

Group	Workers		Controls		All subjects		
	N	Mean±SD	N	Mean±SD	N	Mean±SD	p
All subjects	120	13.19±0.77[a]	120	3.11±0.96[b]	240	8.15±0.60	<0.001
Smokers	59	13.69±1.05	30	3.21±1.30	89	8.45±0.90	0.494
Non-smokers	61	12.70±0.89	90	3.01±1.02	151	7.85±0.72	
Educated	24	13.59±1.31	105	3.41±0.75	129	8.50±0.86	0.590
Non-educated	96	12.80±0.55	15	2.81±1.59	111	7.80±0.91	
Drinkers	12	13.51±1.13	3	3.42±1.25	3	8.47±1.00	0.520
Non-drinkers	108	12.88±0.65	117	2.79±0.90	117	7.83±0.46	
Malay	65	11.85±1.03	78	3.32±1.02	143	7.58±0.97[a]	0.004
Chinese	45	13.59±0.79	33	2.30±1.08	78	7.95±0.72[a]	
Indian	10	14.15±1.94	9	3.70±2.24	19	8.92±1.20[b]	
Working time >5 years	37	14.38±0.93[a]		-			<0.001
Working time <5 years	83	11.18±0.79[b]		-			

Means with different superscripts are significant at p<0.05

Table 5 *Result of comet assay (tail length) in subgroups of workers and controls with different socio-demographic factors*

Group	Workers		Controls		All subjects		
	N	Mean±S.D.	N	Mean±S.D.	N	Mean±S.D.	p
All subjects	120	26.10±2.08 [a]	120	19.97±2.59[b]	240	23.03±1.61	<0.001
Smokers	59	27.73±2.83	30	22.33±3.48	89	21.04±1.94	0.068
Non-smokers	61	24.47±2.40	90	17.61±2.76	151	25.03±2.43	
Educated	24	26.13±3.52	105	20.80±2.01	129	23.47±2.31	0.959
Non-educated	96	26.07±1.49	15	19.13±4.27	111	22.60±2.44	
Drinkers	12	27.17±3.03	3	21.04±3.35	3	24.11±2.70	0.667
Non-drinkers	108	25.02±1.76	117	18.90±2.41	117	21.96±1.24	
Malay	65	25.33±2.75	78	19.25±2.74	143	22.29±2.59	0.093
Chinese	45	26.11±2.12	33	17.63±2.90	78	21.87±1.93	
Indian	10	26.86±5.20	9	23.03±6.03	19	24.95±3.24	
Working time >5 years	37	32.05±1.80[a]		-			<0.001
Working time <5 years	83	21.47±1.53[b]		-			

Means with different superscripts are significant at p<0.05

Table 6 *Result of telomere length shortening biomarker in subgroups of workers and controls with different socio-demographic factors*

Group	Workers (N)	Controls (N)	All subjects (N)	p
All subjects	120	120	240	0.046
Smokers	59	30	89	0.377
Non-smokers	61	90	151	
Educated	24	105	129	0.437
Non-educated	96	15	111	
Drinkers	12	3	3	0.683
Non-drinkers	108	117	117	
Malay	65	78	143	0.814
Chinese	45	33	78	
Indian	10	9	19	
Working time>5 years	37			0.001
Working time<5 years	83			

of exposed and control subjects involved in each genotype and age subgroup.

GSTP1 is a major enzyme that is involved in the inactivation and detoxifying of cytotoxic environmental substances like benzo(a)pyrene diol epoxide and acrolein (9, 22). GSTP1 has a dual role in DNA damage in occupational and environmental exposure (23). There seems to be a controversy about its ability to alter the early effects of toxic exposure on DNA damage biomarkers (24). Our study confirms this controversy. Interpretation is further complicated by the socio-demographic factors (25, 26), as they influence the level of genome damage together with gene polymorphism.

While we were not able to isolate the influence of gene polymorphism, our results do not entirely exclude this possibility. The case in point are the significant ethnic differences in MN frequency regardless of occupational exposure (Table 4), which imply the influence of gene polymorphism on cell protection against genome damage along with the living/occupational environment (27).

CONCLUSION

Unlike earlier findings, our results suggest that *GSTP1* polymorphism does not contribute significantly to genome damage in the exposed workers, but do confirm the significant influence of the occupational setting and ageing. However, the interpretation of our results is limited by a relatively small number of

subjects involved, and further investigation is needed to elucidate the influence of the interaction between gene polymorphism and environmental factors on genome damage.

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Sažetak

PRIDONOSI LI POLIMORFIZAM GENA *GSTP1* OŠTEĆENJU GENOMA UZROKOVANOM STARENJEM I PROFESIONALNOM IZLOŽENOSTI?

Na populaciji radnika zaposlenih u radionicama za popravak automobila koji su svakodnevno izloženi različitim vrstama potencijalnih mutagena istražili smo utjecaj polimorfizma gena *GSTP1* na vrijednosti biomarkera starenja, uključujući pojavu mikronukleusa (MN), dužinu repa kometa te relativnu dužinu telomera. Analize su provedene na stanicama bukalne sluznice, skupljenim od izloženih ispitanika i odgovarajuće neizložene (kontrolne) populacije. Uzorci su analizirani primjenom citogenetičkih i molekularnobioloških metoda, uključujući polimorfizam restrikcijskih fragmenata na osnovi njihove duljine (engl. *restriction fragment length polymorphism*, RFLP), MN-test, komet-test, i lančanu reakciju polimerazom u stvarnom vremenu (engl. *real-time PCR*). Dobiveni nalazi potvrđuju da izloženost radnika mutagenima oštećuje njihovu DNA, ali nisu pokazali da polimorfizam gena *GSTP1* značajno utječe na razinu oštećenja DNA. Zbog malog broja ispitanika uključenog u ovo istraživanje za bolje razumijevanje odnosa između polimorfizma gena *GSTP1* i oštećenja DNA potrebna su daljnja istraživanja, i na profesionalno izloženim ispitanicima i na ispitanicima kontrolne populacije.

KLJUČNE RIJEČI: *dužina repa kometa, dužina telomere, glutation S-transferaze, PCR, RFLP, stanice bukalne sluznice, učestalost mikronukleusa*

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