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Lymphocytes, DNA adducts and genetic polymorphism for metabolic enzymes in low dose cigarette smokers

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The aim of this study was to investigate the relationship between genetic polymorphism of metabolic enzymes and DNA adduct levels in lymphocytes of low dose cigarette smokers (less than 20 cigarettes per day). We previously reported the effects of cytochrome P4501A1 (CYP1A1) and glutathione S-transferase M1 (GSTM1) on lymphocyte DNA adducts. This time we considered not only CYP1A1 and GSTM1 but also cytochrome P4502E1 (CYP2E1) and glutathione S-transferase T1 (GSTT1). DNA adducts in lymphocytes obtained from low dose cigarette smokers ($n = 41$) and non-smokers ($n = 56$) were measured by the ^{32}P-postlabelling method. The adduct levels were compared regarding smoking status and polymorphic genotypes of these four enzymes. The mean ± SD of DNA adduct levels in all low dose cigarette smokers and non-smokers was 1·05 ± 0·83 per 10^8 nucleotides and 0·85 ± 0·35 per 10^8 nucleotides, respectively. In low dose cigarette smokers, adduct levels were higher in the rare homozygous (MM) for CYP1A1-exon 7 polymorphism compared with the other types such as common homozygous (WW) and heterozygous (WM). CYP1A1-WM, MM in combination with GSTM1 null showed highest adduct level among low smokers. The low smokers with rare homozygous for CYP2E1 Dra1 polymorphism tended to have lower adduct levels than wild types. Low dose cigarette smokers with combined GSTM1 null and T1 null had a higher tendency for adduct levels than others. However none of the differences reached statistical significance.

Keywords: smoking, DNA adducts, lymphocyte, polymorphism.

Introduction

Most environmental carcinogens require metabolic activation by Phase I enzymes; cytochrome P-450 (CYP). Activated metabolites of carcinogens are detoxificated by Phase II enzymes; glutathione S-transferase (GST). CYP and GST have been known as polymorphic enzymes. Susceptibility markers such as genetic polymorphism of metabolic enzymes related to higher risk of developing cancer have attracted much attention in recent years. Several epidemiological studies reported that the combination of genotypes of a homozygous rare allele of the CYP1A1 gene and the GSTM1 null was related to high risk of lung cancer (Hayashi *et al.* 1992, Alexandrie *et al.* 1994). In particular, in low dose cigarette smokers, the genetic difference in lung cancer susceptibility derived from combined polymorphism of GSTM1 and CYP1A1 was remarkably large, although the difference was decreased in high dose smokers (Nakachi *et al.* 1993).

Recently cytochrome P4502E1 (CYP2E1) or glutathione S-transferase T1 (GSTT1) has been noticed in experimental or epidemiological studies of

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carcinogens. Uematsu *et al.* (1994) reported that in low smoking exposure, the Dra 1 polymorphism of the CYP2E1 gene is associated with susceptibility to lung cancer. Schröder *et al.* (1995) reported that GSTT1 polymorphism influences background SCE rate. Deakin *et al.* (1996) indicated that GSTT1 null genotypes did not increase in the lung, oral or gastric cancer cases compared with controls, but the frequency of this genotype significantly increased in the colorectal cancer cases.

Hypothetically, the genetic difference of metabolic enzymes occurring as genetic polymorphism has been thought to lead to the individual variation of DNA adduct formation. Studies on the genetic polymorphism are currently extended to DNA adduct analysis in lung tissue, considering the effects of CYP1A1 and GSTM1 (Shields *et al.* 1993, Bartsch *et al.* 1995), CYP1A1, 2D6, 2E1 and GSTM1 (Kato *et al.* 1995), and in peripheral blood cells, considering the effects of GSTM1 (Grinberg-Funes *et al.* 1994), CYP1A1 and GSTM1 (Ichiba *et al.* 1994, 1996, Rothman *et al.* 1995, Hemminki *et al.* 1997, Mooney *et al.* 1997), GSTM1 and NAT2 (Hou *et al.* 1995, Nielsen *et al.* 1996a, b). However, the results concerning the influence of genetic polymorphism on DNA adduct levels have not been consistent.

In this study, we analysed the relationship between DNA adducts and the genetic polymorphism for not only CYP1A1 and GSTM1 but also other gene mutations, such as CYP2E1 and GSTT1, particularly in low dose cigarette smokers. This is the first report dealing with the effect of CYP2E1 and GSTT1 polymorphism on DNA adducts in lymphocytes.

Subjects and methods

The subjects were 97 adult men, 41 low dose cigarette smokers (36 ± 15 years old, less than 20 cigarettes per day) and 56 non-smokers (38 ± 14 years old), who were selected from our previous study (Ichiba *et al.* 1996). Heparinized venous blood and urine samples were obtained from them. DNA was isolated from lymphocytes by the method using RNAase, protease digestion and ethanol precipitation (Kendall *et al.* 1991). DNA samples thus prepared were stored at –80 °C until analysis.

The nuclease P1 modification of the ^{32}P-postlabelling method (Reddy and Randerath 1986, Ichiba *et al.* 1996) was used for the measurement of DNA adducts. For this measurement, 5 μg of DNA was digested with micrococcal nuclease, spleen phosphodiesterase and nuclease P1. The digested material was dried and taken up in a total of 2 μl of T4 polynucleotide kinase labelling mixture containing [γ-^{32}P]ATP. The labelled samples were spotted and developed on polyethyleneimine-cellulose thin layer chromatography (TLC) plates (Macherry-Nagel, Germany) using three solvent systems: D1, 1 M sodium phosphate, pH 6·0; D3, 3·6 M lithium formate, 8·5 M urea, pH 3·5; D4, 0·8 M lithium chloride, 0·5 M Tris, 8·5 M urea, pH 8·0. DNA adducts were detected using a Bio-Image Analyzer (BAS2000; Fuji Photo Film Co., Tokyo, Japan) after exposing TLC plates to the Fuji imaging plate. A diagonal radioactive area on the TLC plates was counted and a background level obtained from the same plate was subtracted. The measurements were performed in duplicate or triplicate for each sample. The results were given as a total number of adducts per 10^8 normal nucleotides.

Restriction fragment length polymorphism of CYP1A1 gene was performed by the methods of Oyama *et al.* (1995). For CYP1A1, the Ile-Val polymorphism (exon 7) results in population subgroups corresponding to genotype Ile/Ile (W/W), Ile/Val (WM) and Val/Val (MM). We missed one subject for CYP1A1 analysis. Restriction endnuclease Dra1 polymorphism of the 2E1 gene (intron 6) was performed by the method of Uematsu *et al.* (1994). Subjects were divided into three groups, homozygous wild type (DD), heterozygous (CD) and homozygous mutant (CC). We missed three subjects for CYP2E1 analysis. The analysis of the GSTM1 gene was performed according to the method of Groppi *et al.* (1991). GSTT1 gene was performed according to the method of Nelson *et al.* (1995). Polymorphism results in population subgroups corresponding to genotype GSTM1 or T1 present (+) and null (–).

Differences in frequency for genetic polymorphism were assessed by the χ^2 test. DNA adducts data were log transformed before statistical analysis. Difference in adduct levels were assessed by ANOVA among three groups and Student's or Welch's *t*-test for two groups.

Results

Table 1 shows the distribution frequencies for four genotypes in low dose cigarette smokers and non-smokers. There was no significant difference in the frequencies for genotypes between low dose cigarette smokers and non-smokers, although CYP1A1 showed a difference which approached statistical significance ($p = 0{\cdot}07$).

The mean ± SD of DNA adduct levels in all low dose cigarette smokers and non-smokers was $1{\cdot}05 \pm 0{\cdot}83$ per 10^8 nucleotides and $0{\cdot}85 \pm 0{\cdot}35$ per 10^8 nucleotides, respectively (figure 1). The adduct levels were compared among four separate genotypes in low dose cigarette smokers and non-smokers (table 2). The DNA adduct levels for each genotype were higher, although not significantly, in low dose cigarette smokers than in non-smokers, in particular, CYP2E1-DD types ($p = 0{\cdot}16$).

Table 1. Genotype frequencies in low dose cigarette smokers and non-smokers.

		Low smoker	Non-smoker	All	*p*
CYP1A1	WW	29 (71 %)	31 (56 %)	60 (63 %)	0·07
	WM	9 (22 %)	23 (42 %)	32 (33 %)	
	MM	3 (7 %)	1 (2 %)	4 (4 %)	
CYP2E1	DD	22 (58 %)	28 (50 %)	50 (53 %)	> 0·5
	CD	15 (39 %)	25 (45 %)	40 (43 %)	
	CC	1 (3 %)	3 (5 %)	4 (4 %)	
GSTM1	+	20 (49 %)	33 (59 %)	53 (55 %)	0·32
	–	21 (51 %)	23 (41 %)	44 (45 %)	
GSTT1	+	23 (56 %)	33 (59 %)	56 (58 %)	> 0·5
	–	18 (44 %)	23 (41 %)	41 (42 %)	
All		41 (100 %)	56 (100 %)	97 (100 %)	

One subject was unknown for CYP1A1 and three were unknown for CYP2E1.

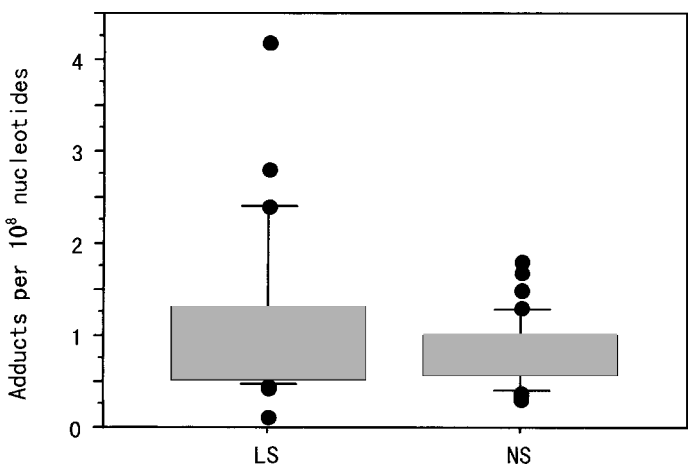

Figure 1. Lymphocyte DNA adducts in low dose cigarette smokers (LS, $n = 41$) and non-smokers (NS, $n = 56$). Boxes indicate 25th and 75th percentiles with median levels indicated with horizontal line. Outer horizontal lines indicate 10th and 90th percentiles. Small circles indicate data over 10th and 90th percentiles.

Table 2. DNA adducts (mean ± SD per 10^8 nucleotides (numbers)) with respect to smoking status and genotypes.

		Low smoker	Non-smoker	*p*
CYP1A1	WW	1·01 ± 0·85 (29)	0·88 ± 0·26 (31)	> 0·5
	WM	0·85 ± 0·54 (9)	0·83 ± 0·44 (33)	> 0·5
	MM	2·01 ± 1·04 (3)	0·60 (1)	
	p	0·13	0·41	
CYP2E1	DD	1·23 ± 0·81 (22)	0·80 ± 0·26 (28)	0·16
	CD	0·90 ± 0·95 (15)	0·92 ± 0·41 (25)	0·32
	CC	0·53 (1)	0·65 ± 0·48 (3)	
	p	0·32	0·29	
GSTM1	+	0·90 ± 0·61 (20)	0·86 ± 0·36 (33)	> 0·5
	–	1·20 ± 1·00 (21)	0·82 ± 0·34 (23)	0·36
	p	0·39	> 0·5	
GSTT1	+	0·88 ± 0·62 (23)	0·87 ± 0·35 (33)	> 0·5
	–	1·27 ± 1·02 (18)	0·82 ± 0·36 (23)	0·26
	p	0·23	> 0·5	
All		1·05 ± 0·83 (41)	0·85 ± 0·35 (56)	> 0·5

In the low dose cigarette smokers, CYP1A1-MM showed the highest level among CYP1A1 genotypes ($p = 0·13$). GSTM1 or T1 null had higher adducts than GSTM1 or T1 positive subjects but the differences were not statistically significant. The data obtained from non-smokers showed no significant difference in the DNA adduct levels among each genotype.

DNA adduct levels were compared with respect to the combination of CYPs and GSTs (table 3). In low dose cigarette smokers, the subjects with the combination of CYP1A1-WM, MM and GSTM1 null had the highest adduct levels. The combination of CYP1A1-WM, MM and GSTT1 null was also highest in low dose cigarette smokers. But these differences were not statistically significant. In subjects with the combination of CYP2E1-DD and GSTT1 null adduct levels were higher than those with CD, CC and null but these were not statistically significant ($p = 0·09$). In non-smokers, subjects with the combination of CYP1A1-WW and GSTM1 null had higher adduct levels than those with WM, MM and M1 positive but the differences did not reach statistical significance ($p = 0·09$). From other comparisons we could observe no significant difference.

DNA adduct levels for the combination of CYP1A1 and 2E1 genotypes are shown in table 4. In low dose cigarette smokers, subjects with the combination of CYP1A1-WM, MM and 2E1-DD showed the highest adduct levels and higher than WM, MM and CD, CC although this difference did not reach statistical significance ($p = 0·09$). There was no significant difference among non-smokers.

DNA adduct levels for the combination of GSTM1 and T1 genotypes are shown in table 5. In low dose cigarette smokers, subjects with the combination of GSTM1 null and T1 null showed the highest adduct levels and higher than those of GSTT1 positives although not statistically significantly so ($p = 0·09$). There was no significant difference among non-smokers.

Table 3. DNA adducts (mean ± SD per 10^8 nucleotides (numbers)) with respect to smoking status and combined genotypes.

Low smoker		CYP1A1			CYP2E1		
		WW	WM, MM	*p*	DD	CD, CC	*p*
GSTM1	+	0·90 ± 0·66 (12)	0·90 ± 0·56 (8)	> 0·5	1·10 ± 0·73 (11)	0·66 ± 0·31 (9)	0·11
	–	1·09 ± 0·97 (17)	1·64 ± 1·13 (4)	0·3	1·35 ± 0·89 (11)	1·16 ± 1·35 (7)	> 0·5
	p	> 0·5	0·26		> 0·5	0·31	
GSTT1	+	0·91 ± 0·70 (17)	0·80 ± 0·37 (6)	> 0·5	1·01 ± 0·76 (14)	0·69 ± 0·25 (8)	0·43
	–	1·16 ± 1·04 (12)	1·48 ± 1·04 (6)	> 0·5	1·60 ± 0·79 (8)	1·07 ± 1·29 (8)	0·1
	p	0·34	> 0·5		0·09	> 0·5	

Non-smoker		CYP1A1			CYP2E1		
		WW	WM, MM	*p*	DD	CD, CC	*p*
GSTM1	+	0·86 ± 0·28 (21)	0·92 ± 0·47 (11)	> 0·5	0·81 ± 0·30 (16)	0·91 ± 0·41 (17)	> 0·5
	–	0·93 ± 0·23 (10)	0·74 ± 0·40 (13)	0·09	0·78 ± 0·21 (12)	0·87 ± 0·46 (11)	> 0·5
	p	0·44	0·39		> 0·5	> 0·5	
GSTT1	+	0·95 ± 0·28 (19)	0·79 ± 0·41 (13)	0·11	0·79 ± 0·32 (17)	0·94 ± 0·37 (16)	0·31
	–	0·77 ± 0·19 (12)	0·86 ± 0·48 (11)	> 0·5	0·81 ± 0·14 (11)	0·82 ± 0·48 (12)	> 0·5
	p	0·15		> 0·5	0·49	0·37	

Table 4. DNA adducts (mean ± SD per 10^8 nucleotides (numbers)) with respect to smoking status and combined CYP genotypes.

Low smoker		CYP1A1		
		WW	WM, MM	*p*
CYP2E1	DD	1·09 ± 0·75 (15)	1·51 ± 0·90 (7)	0·28
	CD, CC	0·98 ± 1·04 (12)	0·56 ± 0·33 (4)	0·23
	p	> 0·5	0·07	

Non-smoker		CYP1A1		
		WW	WM, MM	*p*
CYP2E1	DD	0·86 ± 0·24 (15)	0·76 ± 0·26 (12)	0·28
	CD, CC	0·90 ± 0·29 (16)	0·89 ± 0·56 (12)	0·44
	p	> 0·5	> 0·5	

Table 5. DNA adducts (mean ± SD per 10^8 nucleotides (numbers) with respect to smoking status and combined GST genotypes.

Low smoker		GSTM1		
		+	−	*p*
GSTT1	+	0·92 ± 0·65 (13)	0·84 ± 0·62 (10)	> 0·5
	−	0·88 ± 0·58 (7)	1·52 ± 1·18 (11)	0·16
	p	> 0·5	0·09	

Non-smoker		GSTM1		
		+	−	*p*
GSTT1	+	0·87 ± 0·38 (21)	0·86 ± 0·30 (12)	> 0·5
	−	0·84 ± 0·33 (12)	0·78 ± 0·40 (11)	> 0·5
	p	> 0·5	0·47	

Discussion

The studies on the genetic polymorphism of some metabolic enzymes are currently extended to DNA adduct analysis (Shields *et al.* 1993, Grinberg-Funes *et al.* 1994, Ichiba *et al.* 1994, 1996, Bartsch *et al.* 1995, Hou *et al.* 1995, Kato *et al.* 1995, Rothman *et al.* 1995, Nielsen *et al.* 1996a, b, Hemminki *et al.* 1997, Mooney *et al.* 1997). Although DNA adduct levels were expected to be higher in CYP mutant or GST null genotypes, the results of these studies have not been consistent. We previously reported the data concerning the effect of CYP1A1 and GSTM1 on lymphocyte DNA adducts from smokers and non-smokers (Ichiba *et al.* 1996). In that study, we indicated that CYP1A1 genotype had no significant effect on adduct levels and unexpectedly GSTM1 null genotypes had lower adduct levels than GSTM1 positives in smokers. This time we considered not only CYP1A1 and GSTM1 but also CYP2E1 and GSTT1. There have been no reports

on the effects of CYP2E1 and GSTT1 polymorphisms on DNA adducts in lymphocytes. Another point of the present study was to consider the smoking status of subjects. In our previous study (Ichiba *et al.* 1996) smoking subjects mainly consisted of heavy smokers. On the other hand, in the present study, low dose cigarette smokers (less than 20 cigarettes per day) were selected as subjects. We thought that the genetic effect might be stronger in the group of low dose cigarette smokers. The past epidemiological studies indicated that, particularly in low level cigarette smokers, the genetic difference in lung cancer susceptibility derived from combined polymorphism for GSTM1 and CYP1A1 was remarkably large, although the difference was decreased in high dose smokers (Nakachi *et al.* 1993). Uematsu *et al.* (1994) reported that in low smoking exposure the Dra 1 polymorphism of the CYP2E1 gene was associated with susceptibility to lung cancer. Kato *et al.* (1995) also reported that in low serum cotinine subjects there was significant difference in the adduct levels between different genotypes for CYP2E1 or CYP2D6.

In the present study the results suggested that the DNA adduct level in CYP1A1-MM was higher than those in WW among low dose cigarette smokers, although this difference was not significant because of small sample numbers ($n = 3$). In addition, the adduct levels in GSTM1 null tended to be higher than in positives, although in our previous study with relatively high dose smokers, the adduct levels in GST nulls were significantly lower than those in positives (Ichiba *et al.* 1996).

For CYP2E1, DNA adduct levels of CD were slightly lower than those of DD among low dose cigarette smokers. This result is not consistent with that of a recent epidemiological study (Uematsu *et al.* 1994) which indicated that the frequency of CD in lung cancer was higher than that in the control group. Kato *et al.* (1995) also found higher 7-methyl-dGMP adduct levels in lung tissue of CD subjects.

In the Japanese population only one report on the frequency of GSTT1 genotype has been published (Katoh *et al.* 1996). They showed that the percentage of GSTT1 null genotype was 44 %. Our result (42 %) was similar to that of Katoh *et al.* (1996). Subjects with the combination of GSTM1 null and T1 null had higher adduct levels than others in low dose cigarette smokers. Recently Popp *et al.* (1977) found a negative correlation between DNA adducts of mononuclear cells and GSTT1 activity in erythrocytes of coke oven workers.

In conclusion, we found weak effects of genetic polymorphism on lymphocyte DNA adducts. Though the nuclease P1 method was used for DNA adduct analysis in this study, in future studies we will use the analysis using the butanol extraction method (Gupta 1985). In addition we should consider not only the balance between the Phase I and II enzymes but also the additive effects of different enzymes, such as DNA repair systems. Further studies are necessary to explain interindividual variations of DNA adduct formation.

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