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SHORT COMMUNICATION

Effect of genetic polymorphism for metabolic enzymes on the relationship between smoking dose and DNA adducts in lymphocytes

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The genetic polymorphism of metabolic enzymes on the relationship between smoking dose and DNA adduct levels in lymphocytes were evaluated in 51 smokers. The genetic polymorphisms of cytochrome P4501A1 (CYP1A1) and glutathione S-transferase M1 (GSTM1) were analysed by a PCR method. Lymphocyte DNA adducts were measured by two analytical versions of a ^{32}P-postlabelling method; nuclease P1 digested method and butanol extracted method. Mean adduct levels obtained with the nuclease P1 method ($1{\cdot}21 \pm 0{\cdot}74$ per 10^8 nucleotides) were higher than those obtained with the butanol extracted method ($0{\cdot}82 \pm 0{\cdot}47$, $p < 0{\cdot}01$). There was a significant correlation between adduct levels by the nuclease P1 method and those by the butanol extracted method ($r = 0{\cdot}49$, $p < 0{\cdot}01$). A significant correlation was not found between smoking dose and DNA adduct levels obtained using both methods in lymphocytes of all subjects. When subjects were divided into two groups by CYP1A1 genotypes, significant correlations between smoking indices, such as number of cigarettes per day ×years or tar intake per day ×years, and DNA adduct levels measured by the butanol extracted method was found in heterozygous or miner homozygous for CYP1A1 exon 7 polymorphism. We could not get a significant effect of GSTM1 on the relationship between smoking dose and DNA adducts.

Keywords: smoking, DNA adducts, lymphocyte, polymorphism.

Introduction

Environmental carcinogens are enzymatically activated to form intermediates that can react with cellular DNA and form DNA adducts. The measurement of DNA adducts is a useful indicator for environmental carcinogen exposure monitoring in humans (Hemminki 1995). The ^{32}P-postlabelling analysis has been known as the most sensitive method for DNA adduct analysis. There are two enrichment procedures for the ^{32}P-postlabelling analysis; the nuclease P1 digested method (Reddy and Randerath 1986) and the butanol extracted method (Gupta 1985). There are several studies in which smoking-related DNA adducts in peripheral blood samples were measured by the ^{32}P-postlabelling method. In their cases, the nuclease P1 digested method was mainly used (Holz *et al.* 1990, Jahnke *et al.* 1990, Phillips *et al.* 1990, Savela and Hemminki 1991, van Schooten *et al.* 1992, Popp *et al.* 1993, Szyfter *et al.* 1994, Wiencke *et al.* 1995). However, most of the studies have shown a poor correlation between DNA adduct levels and smoking dose, because the interindividual variation of adduct levels was very large. The

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genetic difference of metabolic enzymes occurring as genetic polymorphism has been thought to lead the individual variation of DNA adduct formation. Polycyclic aromatic compounds contained in cigarette smoke are activated by cytochrome P450s, such as cytochrome P4501A1 (CYP1A1), and detoxificated by conjugated enzymes, such as glutathione S-transferase M1 (GSTM1). CYP1A1 and GSTM1 are polymorphic enzymes. It has been indicated that increased cancer risk is related to polymorphism for CYP1A1 or GSTM1 (D'Errico *et al.* 1996). The studies on genetic polymorphism to cancer susceptibility are currently extended to DNA adduct analysis in peripheral blood cells with the ^{32}P-postlabelling method (Ichiba *et al.* 1994, 1996, 1998, Hou *et al.* 1995, Neilsen *et al.* 1996a, b, Hemminki *et al.* 1997, Wang *et al.* 1997) or the enzyme-linked immunosorbent assay (Grinberg-Funes *et al.* 1994, Rothman *et al.* 1995, Mooney *et al.* 1997). Their results are summarized in table 1. However, the effects of genetic polymorphism on DNA adduct levels have not been consistent.

In this study, we measured smoking-related DNA adduct levels in lymphocytes from smokers using two different versions of the ^{32}P-postlabelling method; the nuclease P1 digested method and the butanol extracted method. Then we compared the two results thus obtained to evaluate the effect of genetic polymorphism on the relationship between smoking dose and DNA adducts.

Subjects and methods

Subjects were 51 healthy adult men, who were selected for our previous study (Ichiba *et al.* 1996). Mean age was 40 ± 14 years old. We asked them their smoking history (number of cigarettes per day, smoking years and brand names of cigarette). Their daily tar and nicotine intake were calculated from the data of Tobacco catalog (Japan Tobacco Inc., August 1994). Number of cigarettes per day ×years and daily tar intake per day ×years were also calculated. Heparinized venous blood and urine samples were obtained from them after getting informed consent. Urinary cotinine was determined according to the HPLC method (Yamano *et al.* 1990). Data obtained were adjusted with the urinary creatinine concentration. Creatinine was measured by the Jaffe method. Lymphocytes were separated by Ficoll-Paque (Pharmacia LKB Biotechnology). DNA was isolated from lymphocytes by the method using RNAase, protease digestion and ethanol precipitation (Kendall *et al.* 1991). DNA samples thus prepared were coded and all analyses were blindly performed.

The nuclease P1 digested version (Reddy and Randerath 1986) and the butanol extracted version (Gupta 1985) of the ^{32}P-postlabelling method were used for the measurement of DNA adducts. For this measurement, 5 μg of DNA was digested with micrococcal nuclease and spleen phosphodiesterase. This hydrolysate was digested by nuclease P1 for the nuclease P1 method. On the other hand, adducted nucleotides were extracted from the hydrolysate by butanol for the butanol extracted method. The digested or extracted 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 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 thin layer plates to the Fuji imaging plate. A diagonal radioactive area on the thin layer 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.

The analysis of Ile–Val polymorphism in the CYP1A1 gene (exon 7) was performed according to the method of Oyama *et al.* (1995). The Ile–Val polymorphism results in population subgroups corresponding to genotypes Ile/Ile, Ile/Val and Val/Val. The analysis of the GSTM1 gene was performed using the method of Groppi *et al.* (1991). Polymorphism results in population subgroups corresponding to genotype GSTM1 present (+) and null (–).

Spearman's rank correlation coefficient was used for the correlation analysis.

Table 1. Studies about genetic polymorphism on DNA adduct levels in peripheral blood cells.

Subjects	Sample	Genotypes	Method	Result	Reference
Chimney sweepers $n = 69$ Control $n = 35$	WBC	CYP1A1, GSTM1	P1	CYP1A1 Wild and GSTM1(–) were high level	Ichiba *et al.* 1994
Bus maintenance workers (NS) $n = 47$ Control $n = 22$	Lymphocyte	GSTM1, NAT2	P1	GSTM(–) and NAT slow were high	Hou *et al.* 1995
SM $n = 76$, EX, NS $n = 81$	Lymphocyte	CYP1A1, GSTM1	P1	GSTM(–) were low	Ichiba *et al.* 1996
Low SM $n = 41$, NS $n = 56$	Lymphocyte	CYP1A1, 2E1, GSTM1, T1	P1	No clear	Ichiba *et al.* 1998
Foundry workers (SM, NS) $n = 95$	WBC	CYP1A1, GSTM1	P1	No clear	Hemminki *et al.* 1997
SM $n = 52$, NS $n = 52$	Lymphocyte	CYP1A1, GSTM1	P1	No clear	Wang *et al.* 1997
Urban (NS) $n = 91$ Rural (NS) $n = 17$	Lymphocyte	GSTM1	Butanol	No clear	Neilsen *et al.* 1996a
Bus drivers (NS) $n = 90$, Control (SM, NS) $n = 60$	Lymphocyte	GSTM1, NAT2	Butanol	No clear, non-significant high in GSTM(–)	Neilsen *et al.* 1996b
SM $n = 63$	Lymphocyte	GSTM1	ELISA	Highest subject in GSTM(–)	Grinberg-Funes *et al.* 1994
Fire fighter (NS) $n = 47$	WBC	CYP1A1, GSTM1	ELISA	No clear	Rothman *et al.* 1995
Heavy SM $n = 159$	WBC	CYP1A1, GSTM1	ELISA	CYP1A1 Val were high	Mooney *et al.* 1997

SM: smoker, NS: non-smoker, WBC: Total white blood cells.

Results

Table 2 shows questionnaire data about smoking and analytical results from 51 subjects. They were moderate smokers. Mean adduct levels obtained with the nuclease P1 method was higher than those obtained with the butanol extracted method ($p < 0{\cdot}01$). Interindividual variation of adduct levels obtained with both methods were comparatively large. There was a significant correlation on adduct levels between the nuclease P1 method and the butanol extracted method (figure 1).

Table 3 shows DNA adduct levels with respect to genotypes. For CYP1A1 genotypes, subjects were divided into two groups according to Ile/Ile and Ile/Val, Val/Val. Because subject number of Val/Val was only 3 there were no significant differences between each genotype.

Table 4 shows Spearman's rank correlation coefficients between DNA adduct levels and smoking indices with respect to analytical method and CYP1A1 genotypes. In all subjects ($n = 51$), we could not find a significant correlation between smoking dose and DNA adducts obtained with the two different methods. When the butanol extracted method was used for CYP1A1 Ile/Val, Val/Val types ($n = 22$), significant correlations were found between DNA adduct levels and two smoking indices, number of cigarettes per day ×years and tar intake per day ×years. When subjects were divided by GSTM1 genotypes, there was not significant correlation between smoking indices and DNA adducts.

Discussion

Our main purpose of this study was to evaluate the effect of genetic polymorphism on the relationship between smoking dose and DNA adducts. Though smoking is one of the important origins of carcinogen exposure, most of the studies did not show a significant correlation between smoking dose and DNA adducts in peripheral blood cells (Jahnke *et al.* 1990, Phillips *et al.* 1990, van Schooten *et al.* 1992, Popp *et al.* 1993), except for a few studies (Savela and Hemminki 1991, Wiencke *et al.* 1995). Thereby some researchers considered the effect of genetic polymorphism of metabolic enzymes on DNA adduct levels. Their studies are summarized in table 1. But their results were not consistent and there were few studies to evaluate the effects of genetic polymorphism on the relationship between smoking dose and DNA adducts.

Table 2. Questionnaire data about smoking, lymphocyte DNA adduct levels (mean ± SD, range) and numbers of genotypes (number, %) in 51 subjects.

Numbers of cigarettes per day	21 ± 9	(2–50)
Smoking (years)	18 ± 12	(1–35)
Tar intake per day (mg)	229 ± 116	(18–600)
Nicotine intake per day (mg)	18 ± 9	(1·6–45)
Urinary cotinine (μg per gCr)	970 ± 1001	(0–5387)
DNA adducts (per 10^8 nucleotides)		
Nuclease P1	1·21 ± 0·74	(0·10–4·0)
Butanol extraction	0·82 ± 0.47	(0·32–2·8)
CYP1A1 Ile/Ile	29 (57 %)	
Ile/Val	19 (37 %)	
Val/Val	3 (6 %)	
GSTM1 +	29 (57 %)	
–	22 (43 %)	

Table 3. DNA adducts (mean ± SD per 10^8 (number)) with respect to genotypes.

	CYP1A1		
	Ile/Ile	Ile/Val, Val/Val	All
P1			
GSTM1 +	1·39 ± 0·63 (17)	1·18 ± 0·90 (15)	1·29 ± 0·76 (32)
–	1·00 ± 0·61 (12)	1·20 ± 0·84 (7)	1·07 ± 0·69 (19)
All	1·23 ± 0·64 (29)	1·19 ± 0·86 (22)	1·21 ± 0·74 (51)
Butanol			
GSTM1 +	0·94 ± 0·51 (17)	0·70 ± 0·29 (15)	0·83 ± 0·43 (32)
–	0·73 ± 0·25 (12)	0·93 ± 0·83 (7)	0·80 ± 0·53 (19)
All	0·86 ± 0·43 (29)	0·77 ± 0·52 (22)	0·82 ± 0·46 (51)

Table 4. Spearman's rank correlation coefficients between DNA adducts and smoking indices with respect to analytical method and CYP1A1 genotypes.

	Nuclease P1 method			Butanol extracted method		
	All (n = 51)	II (n = 29)	IV, VV (n = 22)	All (n = 51)	II (n = 29)	IV, VV (n = 22)
Number of cigarettes per day	0·075	0·147	–0·101	0·224	0·208	0·296
Smoking (years)	0·065	0·161	–0·132	0·103	–0·059	0·383
Number of cigarettes per day ×years	0·169	0·308	–0·127	0·174	–0·017	0·544*
Tar intake per day	0·022	0·187	–0·166	0·187	0·091	0·380
Tar intake per day ×years	0·171	0·328	–0·073	0·111	–0·048	0·458*
Urinary cotinine	–0·007	0·237	–0·383	0·030	–0·083	0·110

* $p < 0·05$.

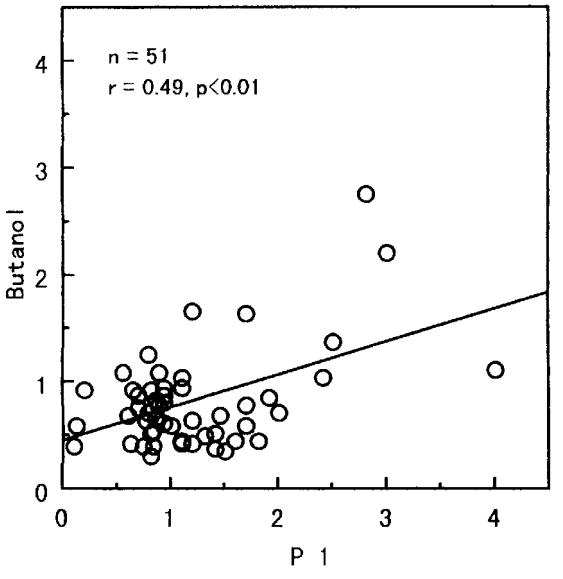

Figure 1. Comparisons between DNA adduct levels obtained with the nuclease P1 (P1) and butanol extracted methods (Butanol). Adduct levels were expressed as per 10^8 nor

In this study, we measured DNA adducts by two different methods; the nuclease P1 digested method and the butanol extracted method, and compared with the results obtained by these two methods. It is generally considered that PAH adducts were measured by these two methods and show similar recovery in both methods while some PAH adducts are better recovered by the nuclease P1 method. The butanol extracted method is also useful for the analysis of aromatic amine adducts (Beach and Gupta 1992). Segerbäck and Vodicka (1993) compared the nuclease P1 method with the butanol extracted method, indicating that both methods show a similar pattern and level of major adducts. Widlak *et al.* (1996) reported that the butanol extracted method gave adducts radioactivity similar to the nuclease P1 method. But there was a quantitative difference that fewer adducts were seen after the butanol extracted method. In our study DNA adduct levels obtained with the nuclease P1 method was higher than those obtained with the butanol method. The quantitative difference was not found.

We could show significant correlations between DNA adducts and smoking indices, such as number of cigarettes per day ×years or tar intake per day ×years, when we used the butanol extracted method and considered genetic polymorphism of metabolic enzyme CYP1A1. We did not get significant correlation between DNA adducts and number of cigarettes per day or urinary cotinine, such as short time exposure indices. Because lymphocytes are long life cells (Savela and Hemminki 1995), they must reflect long time smoking exposure, such as number of cigarettes per day ×years or tar intake per day ×years. Though we could not find a significant effect of GSTM1 genotype on the dose–response relationship, the highest adduct levels were found in GSTM1 null and CYP1A1 Val/Val genotypes (2·80 per 10^8 for P1, 2·77 per 10^8 for butanol). We have not found significant results when using the nuclease P1 method. We could not show the reason for this result. There may be some smoking-related aromatic adducts which were enriched only by the butanol extracted method.

Further studies are needed to decide whether the butanol extracted method is a better method of analysis than the nuclease P1 digested method for carcinogen exposure assessment.

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