RESEARCH COMMUNICATION

Multiplex PCR with Confronting Two-pair Primers for *CYP1A1* Ile462Val, *GSTM1*, *GSTT1*, and *NQO1* C609T

Sayo Kawai[1], Kazuko Nishio[1], Sakurako Nakamura[2], Yoshitaka Sekido[3], Toshimitsu Niwa[2], Nobuyuki Hamajima[1]

Abstract

Polymerase chain reaction with confronting two-pair primers (PCR-CTPP) is an effective genotyping method for single nucleotide polymorphisms (SNPs) in aspects of reducing time and costs for analysis. So far we have established PCR-CTPP conditions for tens of SNPs, including a triplex genotyping (Kawase et al., 2003). In the present study we report a quadruplex PCR-CTPP to genotype simultaneously four functional polymorphisms of carcinogen-metabolizing enzymes, *CYP1A1* Ile462Val, *GSTM1 null*, *GSTT1 null* and *NQO1* C609T, which were reported that they have significant associations with smoking-related cancers. We applied this method for 475 health check-up examinees to demonstrate the performance. Among the subjects, the genotype frequency of *CYP1A1* Ile462Val was 56.8% for *Ile/Ile*, 38.1% for *Ile/Val* and 5.1% for *Val/Val*. The *null* type frequencies of *GSTM1* and *GSTT1* were 52.8% and 49.9%, respectively. And the genotype frequency of *NQO1* C609T was 41.9% for *C/C*, 41.3% for *C/T* and 16.8% for *T/T*. Their distributions were similar to those reported for Japanese by other studies. To the best of our awareness, this is the first paper that reports the success in quadruplex PCR-CTPP. The applied polymorphisms are useful ones, which would be adopted not only for research purposes, but also for risk assessment of individuals exposed to carcinogenic substances. This convenient genotyping would be applied for cancer prevention especially in Asian Pacific regions, where expensive genotyping methods are hardly available.

Key Words: Polymerase chain reaction confronting two-pair primers (PCR-CTPP) - polymorphisms - *NAD(P)H:quinone oxidoreductase 1 (NQO1) - glutathione S-transferase M1 (GSTM1) - glutathione S-transferase T1 (GSTT1) - cytochrome P450 1A1 (CYP1A1)*

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Introduction

Polymorphisms relating to cancer susceptibility have a potential of being applied for cancer prevention. For instance, identification of individuals' highly sensitiveness to carcinogenic substances could be one of the most helpful information for intervention against their high-risk behaviours such as smoking. Although genotyping in general is yet expensive, accumulated evidence especially in biology and epidemiology has enabled the confirmation of the relation between cancer risk and polymorphisms including *NAD(P)H:quinone oxidoreductase 1 (NQO1)* C609T (Pro187Ser), *glutathione S-transferase M1 (GSTM1)*, *glutathione S-transferase T1 (GSTT1)* and *cytochrome P450 1A1 (CYP1A1)* A4889G (Ile462Val). The roles of the enzymes are depicted in Figure 1.

The enzyme encoded by *NQO1* is a flavoprotein involved in the detoxification of potentially mutagenic and carcinogenic quinones, which are included in cigarette smoke. This enzyme catalyzes the two-electron reduction of potentially toxic quinoid compounds into their reduced form like hydroquinones (Lafuente et al., 2000). While the

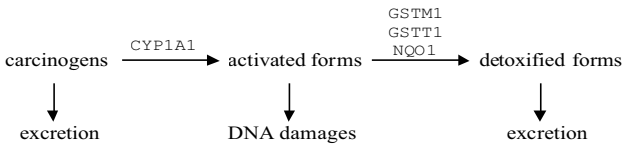

Figure 1. Roles of *CYP1A1*, *GSTM1*, *GSTT1* and *NQO1* in Carcinogen Metabolism

[1] Department of Preventive Medicine / Biostatistics and Medical Decision Making, Nagoya University Graduate School of Medicine, Nagoya, Japan [2] Department of Clinical Preventive Medicine, Nagoya University Hospital, Nagoya, Japan [3] Division of Molecular Oncology, Aichi Cancer Center Research Institute Correspondence to: Sayo Kawai, Department of Preventive Medicine / Biostatistics and Medical Decision Making, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8550 Japan, TEL:+81-52-744-2132, FAX:+81-52-744-2971, e-mail: ksayo@med.nagoya-u.ac.jp

enzyme encoded by the *C/C* genotype has a full activity of this function, that encoded by the *T/T* genotype has no activity. The enzyme activity for the *C/T* genotype might be intermediate between those for the two genotypes (Siegel et al., 1999). Several studies have reported the associations of the *T/T* genotype with leukaemia (Wiemels et al., 1999; Larson et al., 1999; Naoe et al., 2000; Smith et al., 2001), lung cancer (Rosvold et at., 1995; Xu et al., 2001), colorectal cancer (Lafuente et al., 2000), urological malignancies (Schulz et al., 1997) and a possible interaction with smoking which is suspected of causing cancer of lung and esophagus (Hamajima et al., 2002a).

Glutathione S-transferase M1 (*GSTM1*) and *glutathione S-transferase T1* (*GSTT1*) are cancer susceptibility genes because of their ability to regulate the conjugation of carcinogenic compounds to excretable hydrophilic metabolites. The *null* type variants have no enzyme activity in both genes. One study reported that combined *GSTM1 null* genotype and high-dose environmental tobacco smoke (ETS) exposure conferring a significantly higher risk compared to the *GSTM1 present* genotype and low-dose ETS exposure (Kiyohara et al., 2003). On the other hand, there is a report that deletion of the *GSTT1* gene is associated with protection against the risk of developing head and neck cancer, especially in the female population (Evans et al., 2003).

Cytochrome P450 1A1 (CYP1A1) is involved in the metabolic activation of polycyclic aromatic hydrocarbons into mutagenic and carcinogenic derivatives that bind to DNA and may subsequently initiate the neoplastic transformation, besides that involved in the oxidative metabolism of estrogens. Several SNPs in the *CYP1A1* have been reported and associations of these SNPs and smoking-related cancers, such as lung, oral, esophageal, bladder and breast cancers, have been well investigated (Bartsch et al., 2000). According to former investigation, A4889G transition in exon 7 (Ile462Val) might be more frequent in the Japanese population than in the Caucasian population (Alexandrie et al., 1994; Hirvonen et al., 1992) and this polymorphism seems to be associated with susceptibility to cancer in Asians since Ile462Val polymorphism has been shown to be associated with breast cancer in Chinese women in Taiwan (Huang et al., 1999), and ovarian cancer in a Turkish population (Aktas et al., 2002).

This paper reports a newly developed quadruplex polymerase chain reaction (PCR), called PCR with confronting two-pair primers (PCR-CTPP), which makes possible to genotype polymorphisms of four genes simultaneously.

Materials and Methods

Blood samples were collected from persons consented to participate in the study, who attended the health check-up examination at Nagoya University Hospital in 2003-2004. The subjects comprised 476 examinees (291 males and 185 females) with no prior or present diagnosis of any cancers. We obtained informed consent from each participant for providing information and serum to our epidemiological study, and provided some free genotype announcement on eight polymorphisms (*ADH2* His47Arg, *ALDH2* Glu487Lys, *GSTM1*, *GSTT1*, *NQO1* C609T, *IL-1B* C-31T, *TNF-A* T-1031C and *ACE* Ins/Del) for the participants if they wished (Nishio et al., 2004). Excluding one sample with degraded DNA, 475 samples were applied for the present study. This study was approved by the Ethics Committee of Nagoya University Graduate School of Medicine.

DNA was extracted from 200μl of buffy coat preserved at-40˚C by BioRobot EZI (Qiagen Inc., Valencia, CA). Present quadruplex PCR-CTPP was conducted to genotype four polymorphisms, *NQO1* C609T, *GSTM1*, *GSTT1* and *CYP1A1* Ile462Val simultaneously in one tube. Table 1 shows the primer sequences as well as the melting temperatures estimated by base sequence algorithm (Breslauer et al, 1986). The *NQO1* and *CYP1A1* polymorphisms were conducted using each two sets of four primers for PCR-CTPP, and the *GSTM1* and *GSTT1* polymorphism were conducted using each one set of primers for ordinary PCR.

These sets of primers were used for amplification of genomic DNA as a template. The PCR reaction volume was 25μl with mixtures containing 50-100ng of genomic DNA,

Table 1. Primers of Multiplex PCR-CTPP for *CYP1A1* Ile462Val, *GSTM1*, *GSTT1* and *NQO1* C609T

Primer	Sequence	Tm*
CYP1A1IleValF1	5' CCT ACC TGA ACG GTT TCT CAC C	62.0˚C
CYP1A1IleValR1	5' CCT CCC AGC GGG CAA C	63.8˚C
CYP1A1IleValF2	5' CGG AAG TGT ATC GGT GAG ACC A	64.5˚C
CYP1A1IleValR2	5' CAG CCT GCT GGT CTG GCT	62.6˚C
GSTM1F	5' GAA CTC CCT GAA AAG CTA AAG	54.8˚C
GSTM1R	5' TGG AGG TCA AGG ACA TCA	55.2˚C
GSTT1F	5' TTC CTT ACT GGT CCT CAC AT	54.2˚C
GSTT1R	5' AAT GCT TTG TGG ACT GCT	53.7˚C
NQO1C609TF1	5' TAT CAG AGT GTC TTA CTG AGA	46.4˚C
NQO1C609TR1	5' AAT GCT ATA TGT CAG TTG AGG	51.6˚C
NQO1C609TF2	5' GTG GCT TCC AAG TCT TAG AAT	54.9˚C
NQO1C609TR2	5' TTT CTA GCT TTG ATC TGG TTG	54.5˚C

Tm*: Melting temperature estimated by base sequence algorithm (Breslauer et al, 1986). Polymorphic bases are underlined.

Table 2. Distribution of *CYP1A1* Ile462Val, *GSTM1*, *GSTT1* and *NQO1* C609T Genotypes in Japanese Non-cancer Examinees

Gene	*N*[a] (n=*475*)	*Genotype frequencies (%)*		*Allele frequencies*	
CYP1A1	270	*Ile/Ile*	56.8	*Ile(4889A)*	0.759
	181	*Ile/Val*	38.1	*Val(4889G)*	0.241
	24	*Val/Val*	5.1		
GSTM1	224	*Present*	47.2		
	251	*Null*	52.8		
GSTT1	238	*Present*	50.1		
	237	*Null*	49.9		
NQO1	199	*C/C*	41.9	*609C*	0.625
	196	*C/T*	41.3	*609T*	0.375
	80	*T/T*	16.8		

[a] N is the number of subjects.

0.18mM dNTPs, 12.5pmol each of the primers, 0.5 units of AmpliTaq Gold (Perkin-Elmer Corp., Foster City, CA), and 2.5µl 10x of PCR buffer including 15mM $MgCl_2$.The PCR System 9700 (PE Biosystems, Foster City, CA) was used for the DNA amplification. PCR was performed at 95˚C for 10 minutes for the initial denaturing step, followed by 35 cycles of denaturing at 95˚C for 1 minute, annealing at 62˚C for 1 minute and extension at 72˚C for 1 minute. The final extension was at 72˚C for 5 minutes. All PCR products were resolved directly by electrophoresis on a piece of 2% agarose-TBE gel containing ethidium bromide, and the DNA bands were then visualized by use of ultraviolet transillumination.

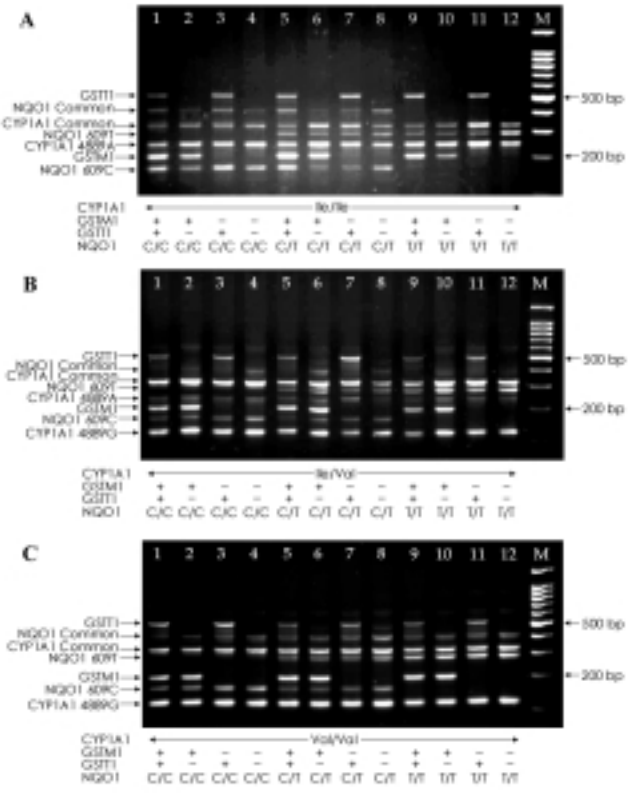

Figure 2. Representative Gel Appearances for *CYP1A1*, *GSTM1*, *GSTT1* and *NQO1* by Quadraplex Polymerase Chain Reaction with Confronting Two-pair Primers (PCR-CTPP); Lane M for a 100-bp Marker. Figure A is for *CYP1A1 Ile/Ile*, *GSTM1*, *GSTT1* and *NQO1* C609T, B is for *CYP1A1 Ile/Val*, *GSTM1*, *GSTT1* and *NQO1* C609T, and C is for *CYP1A1 Val/Val*, *GSTM1*, *GSTT1* and *NQO1* C609T.

Results

The amplified DNA are 119 base pair (bp) for *CYP1A1 4889G (Val)* allele, 245 bp for *4889A (Ile)* allele, 200 bp for *GSTM1*, 507 bp for *GSTT1*, 161 bp for *NQO1 609C* allele, 283 bp for *609T* allele, as well as common bands with 327 bp for *CYP1A1* and 403 bp for *NQO1*. Accordingly, Genotyping was distinguished as follows; with *CYP1A1* polymorphisms, 327, 245 bp for *A/A(Ile/Ile)*, 327, 245 and 119 bp for *A/G(Ile/Val)*, and 327, 119 bp for *G/G(Val/Val)*, and with *NQO1* polymorphisms, 403, 161 bp for *C/C(Pro/Pro)*, 403, 283 and 161 bp for *C/T(Pro/Ser)*, and 403, 283 bp for *T/T(Ser/Ser)*. Bands were sufficiently clear for each sample to be genotyped correctly. All 36 genotype combinations of four polymorphisms are demonstrated in Figure 2.

Table 2 shows the observed genotype frequencies for *CYP1A1*, *GSTM1*, *GSTT1* and *NQO1* among 475 health check-up examinees. The genotype frequencies of *CYP1A1* were 56.8% for *A/A(Ile/Ile)*, 38.1% for *A/G(Ile/Val)* and 5.1% for *G/G(Val/Val)*. Those of *NQO1* were 41.9% for *C/C(Pro/Pro)*, 41.3% for *C/T(Pro/Ser)* and 16.8% for *T/T(Ser/Ser)*. The value for the *GSTM1 null* genotype was 52.8% and the *GSTT1 null* genotype was 49.9%. Combined genotype frequencies of *CYP1A1* Ile462Val, *GSTM1*, *GSTT1* and *NQO1* C609T are showed in Table 3. The *CYP1A1* Ile462Val genotype frequency was in Hardy-Weinberg equation (p=0.367), while the *NQO1* C609T genotype frequency was not in Hardy-Weinberg equation (p=0.009). The expected values for the observed *NQO1* C609T genotype frequency were 185.7 for 199 *C/C* carriers, 222.6 for 196 *C/T* carriers and 66.7 for 80 *T/T* carriers.

Table 3. Combined Genotype Frequencies of *CYP1A1* Ile462Val, *GSTM1*, *GSTT1* and *NQO1* C609T

CYP1A1	*NQO1*	*GSTM1*	*GSTT1*	*N*[a)] (n=*475*)	*Frequencies (%)*
Ile/Ile	*C/C*	+	+	31	6.53
		+	-	21	4.42
		-	+	34	7.16
		-	-	29	6.11
	C/T	+	+	23	4.84
		+	-	32	6.74
		-	+	24	5.05
		-	-	31	6.53
	T/T	+	+	10	2.11
		+	-	11	2.32
		-	+	12	2.53
		-	-	12	2.53
Ile/Val	*C/C*	+	+	15	3.16
		+	-	19	4.00
		-	+	18	3.79
		-	-	19	4.00
	C/T	+	+	16	3.37
		+	-	23	4.84
		-	+	24	5.05
		-	-	16	3.37
	T/T	+	+	6	1.26
		+	-	4	0.84
		-	+	15	3.16
		-	-	6	1.26
Val/Val	*C/C*	+	+	1	0.21
		+	-	7	1.47
		-	+	2	0.42
		-	-	3	0.63
	C/T	+	+	2	0.42
		+	-	1	0.21
		-	+	2	0.42
		-	-	2	0.42
	T/T	+	+	2	0.42
		+	-	0	0.00
		-	+	1	0.21
		-	-	1	0.21

[a)] N is the number of subjects.

Discussion

The allele frequencies of *NQO1* C609T and *CYP1A1* Ile462Val, and the genotype frequencies of *GSTM1* and *GSTT1* in this study are also similar to those in other reports for Japanese (Table 4). *GSTM1 null* allele is common and there is no difference in the genotype frequencies among Japanese, Koreans, Chinese, Arabs and Caucasians, with exception of Indians and Thais having a slightly lower *null* frequency. *GSTT1 null* allele is more common in Japanese, Koreans, Chinese and Thais, comparison with in Indians, Arabs and Caucasians. The distributions of the allele frequency of *NQO1* in Japanese, Koreans and Chinese are mostly the same, except for one report (Lin et al., 1999), while Arabs and Caucasians have a lower *609T* allele frequency. *CYP1A1 462Val* minor allele of Japanese, Koreans and Chinese is more frequent than in Indians except in 76 subjects from a study of Sobt et al. Moreover, the *Val/Val* genotype is rare (Abbas et al., 2004) in Caucasians.

Only a few polymorphisms genotyped with multiplex PCR-CTPP have been described. To date, we have reported duplex PCR-CTPP for *interleukin 1B* (*IL-1B*) C-31T and *interleukin 1RN* (*IL-1RN*) VNTR (variable number of tandem repeats), and for the *secretor* (*fucosyltransferase 2*) gene with complex alleles, *Se*, *sej*, and *se5* (Hamajima, 2001), and triplex PCR-CTPP for *GSTM1*, *GSTT1* and *NQO1* (Kawase et al., 2003). Although triplex PCR-RFLP (PCR-restriction fragment length polymorphism) has been reported (Renee et al., 1998), yet quadruplex PCR-RFLP has rarely reported.

The main difference between PCR-CTPP and PCR-RFLP is that PCR-CTPP allows genotyping of SNPs without incubation with restriction enzymes for PCR product digestion. Therefore, PCR-CTPP has an advantage of low cost and rapidity. PCR-CTPP doesn't meet any problems of restriction enzymes and allows us to go to the next step immediately after PCR is finished. PCR-CTPP needs only half of the time even for single polymorphism genotyping compared with PCR-RFLP, and much shorten time for multiplex PCR-CTPP.

However, technical problems should be noted for PCR-CTPP (Hamajima et al., 2002b). The strength of bands is dependent on the balance in melting temperature of each primer. Addition of one base to a primer changes its melting temperature and causes distraction of the balance of the bands strength. The balance is also sensitive to annealing temperature of PCR. The primers listed in Table 1 should anneal at 62˚C, though the estimated melting temperatures in Table 1 vary in a relatively wide range. According to former studies, the best way to find an optimal primer set might be adjusting primers' lengths to select the similar melting temperature for each primer. The condition reported here was determined after several unsuccessful combinations were tried. Among the bands of the quadruplex PCR-CTPP, *NQO1* 609C band was relatively weak, requiring repeated genotyping to obtain a clear band. Since the *NQO1* primers in solution more than several months tended to make a less clear band, newly synthesized/ solubilized primers are to be used.

In the present data, the obtained genotypes of *CYP1A1*, *GSTM1*, *GSTT1* and *NQO1* were the same as those genotyped with a single PCR-CTPP for *CYP1A1* and a triplex PCR-CTPP for *GSTM1*, *GSTT1* and *NQO1*. Although the genotype frequency was not in Hardy-Weinberg equation, the difference in the observed and the expected were 5.6% at maximum. The genotypes errors seemed unlikely, so that this phenomenon might be due to an unknown cause for subject selection, or by chance.

In conclusion, a quadruplex PCR-CTPP for *CYP1A1* Ile462Val, *GSTM1*, *GSTT1* and *NQO1* C609T was developed. The applied polymorphisms are useful ones, which would be adopted not for research purposes to assess risk of individuals exposed to carcinogenic substances. With awareness of technical problems, this convenient genotyping

Table 4. Allele Frequencies of *CYP1A1* Ile462Val, *GSTM1*, *GSTT1* and *NQO1* C609T according to Ethnic Group

Ethnic group	CYP1A1 *(Ile462Val)* N *Ile* allele/*Val* allele	*GSTM1* N *Null* genotype	*GSTT1* N *Null* genotype	*NQO1 (C609T)* N *C* allele / *T* allele
Japanese	622 0.777 / 0.223 (Oyama et al., 1997) 200 0.785 / 0.215 (Murata et al., 2001) 334 0.786 / 0.214 (Yoshimura et al., 2003)	201 0.453 (Kihara et al., 1994) 622 0.513 (Oyama et al., 1997) 220 0.559 (Inoue et al., 2000)	126 0.444 (Katoh et al., 1996) 200 0.520 (Murata et al., 2001)	150 0.617 / 0.383 (Naoe et al., 2000) 241 0.579/ 0.421 (Hamajima et al., 2002) 336 0.622 / 0.378 (Yoshimura et al., 2003)
Koreans	63 0.508 / 0.492 (Hong et al., 1998) 48 0.750 / 0.250 (Kim et al., 1999)	63 0.524 (Hong et al., 1998) 181 0.525 (Park et al., 2000) 220 0.599 (Kim et al., 2000)	181 0.420 (Park et al., 2000) 220 0.459 (Kim et al., 2000)	106 0.585 / 0.415 (Pae et al., 2004)
Chinese	404 0.744 / 0.256 (Song et al., 2001[M]) 106 0.816 / 0.184 (Chen et al., 2001[M])	106 0. 368 (Chen et al.,2001[M]) 417 0.508 (Setiawan et al., 2001[M]) 187 0.636 (Zhao et al., 2001[SN])	417 0.456 (Setiawan et al., 2001[M]) 187 0.545 (Zhao et al., 2001[SN]) 333 0.462 (Wong et al., 2002[T])	95 0.490 / 0.510 (Lin et al., 1999[T]) 141 0.589 / 0.411 (Zhang et al., 2003[M])
Thais		53 0.302 (Kietthubthew et al., 2001)	53 0.472 (Kietthubthew et al., 2001)	
Indians	76 0.454 / 0.546 (Sobt et al., 2003) 227 0.881 / 0.119 (Sikdar et al., 2003) 118 0.890 / 0.110 (Joseph et al., 2004c)	76 0.316 (Sobt et al., 2003) 215 0.298 (Badu et al., 2004 f)	76 0.145 (Sobt et al., 2003) 215 0.158 (Badu et al., 2004 f)	
Arabs		513 0.546 (Bu et al., 2004)	513 0.258 (Bu et al., 2004)	504 0.761 / 0.239 (Bu et al., 2004)
Caucasians	4790 0.948 / 0.052 (Garte et al., 2001[MA]) 107 0.972 / 0.028 (Abbas et al., 2004[FR])	1949 0.546 (Slattery et al., 1998[US] f) 10514 0.531 (Garte et al., 2001[MA]) 120 0.492 (Abbas et al., 2004[FR]) 939 0.550 (Wang et al., 2004[US])	5577 0.197 (Garte et al., 2001[MA]) 115 0.261 (Abbas et al., 2004[FR]) 939 0.198 (Wang et al., 2004[US])	838 0.812 / 0.188 (Smith et al., 2001[UK]) 323 0.822 / 0.178 (Krajinovic et al., 2002[CA])

[M] Conducted in main land of China, [T] in Taiwan, [SN] in Singapore, [US] in the United States, [CA] in Canada, [UK] in the United Kingdom, [FR] in France, and [MA] for meta-analysis. 'f' signifies females and 'c' children

could be applied for cancer prevention especially in Asian Pacific regions, where expensive genotyping methods are hardly available.

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