COMMENTARY

Triplex Polymerase Chain Reactions with Confronting Two-Pair Primers (PCR-CTPP) for *NQO1* C609T, *GSTM1* and *GSTT1* Polymorphisms: a Convenient Genotyping Method

Haruya Kawase[1], Nobuyuki Hamajima[2], Akiko Tamakoshi[2], Kenji Wakai[2], Toshiko Saito[3], Kazuo Tajima[3]

Abstract

The polymerase chain reaction with confronting two-pair primers (PCR-CTPP) is a time-saving and inexpensive genotyping method, which is applicable for most single nucleotide polymorphisms (SNPs). To date, we have established PCR-CTPP conditions for tens of SNPs, including duplex genotyping. This paper introduces triplex PCR-CTPP to simultaneously genotype three functional polymorphisms of carcinogen-detoxifying enzymes, *NQO1* C609T, *GSTM1 null*, and *GSTT1 null*, all of which are reported to have a significant association with smoking-related cancers. We applied this method for 241 non-cancer patients to demonstrate the performance. Among the subjects, the genotype frequency of *NQO1* C609T was 35.7% for *CC*, 44.4% for *CT* and 19.9% for *TT*. The null type frequencies of *GSTM1* and *GSTT1* were 53.4% and 44.0%, respectively. Their distributions were similar to those reported for Japanese by other studies. This is the first paper reporting the success of triplex PCR-CTPP. The polymorphisms applied are useful examples, which could be adopted not only for research purposes, but also for risk assessment of individuals exposed to carcinogenic substances, such as smokers. This convenient genotyping approach has advantages for application in cancer prevention, especially in the Asian Pacific region.

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

Asian Pacific J Cancer Prev, **4**, 67-70

Polymorphisms relating to cancer susceptibility have the potential to be applied for cancer prevention, for example in the identification of individuals who might be highly sensitive to carcinogenic substances in the worksite, and for intervention against high risk behaviour such as smoking. Although genotyping is generally expensive, the costs are decreasing and the technology is improving. In addition, accumulated evidence in terms of both biology and epidemiology indicates rather constant associations between cancer risk and polymorphisms, including NAD(P)H:quinone oxidoreductase 1 gene (*NQO1*) C609T (Pro187Ser), glutathione *S*-transferase M1 (*GSTM1*), and glutathione *S*-transferase T1 (*GSTT1*).

Several studies have reported associations of the *NQO1 609TT* genotype with leukaemia (Larson et al., 1999; Naoe et al., 2000; Smith et al., 2001), lung cancer (Rosvold et al., 1995), colorectal cancer (Lafuente et al., 2000), urological malignancies (Schulz et al., 1997), and a possible interaction with smoking for cancers of the lung and esophagus (Hamajima et al., 2002a). On the other hand, there have been other studies reporting insignificant, no, or inverse associations for lung cancer (Wiencke et al., 1997; Chen et al., 1999; Lin et al., 1999; Xu et al., 2001; Yin et al., 2001) and renal cell carcinoma (Longuemaux et al., 1999). The enzyme encoded by *NQO1* is a flavoprotein involved in the detoxification of potentially mutagenic and carcinogenic

[1]Medical Student of Nagoya University School of Medicine, [2]Department of Preventive Medicine/Biostatistics and Medical Decision Making, Nagoya University Graduate School of Medicine, [3]Division of Epidemiology and Prevention, Aichi Cancer Center Research Institute

Correspondence to: Nobuyuki Hamajima, M.D., M.P.H., Ph.D., 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:nhamajim@med.nagoya-u.ac.jp

quinines, which are included in cigarette smoke. This enzyme catalyzes the two-electron reduction of potentially toxic quinoid compounds into their reduced forms like hydroquinones (Lafuente et al., 2000). While the enzyme encoded by the *CC* genotype has full activity, that encoded by the *TT* genotype has none. The enzyme activity of the *CT* genotype is intermediate between the two (Siegel et al., 1999).

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. Deletion variants lacking in enzyme activity exist for both genes. Individuals with homozygous deletions in the *GSTM1* or *GSTT1* genes may have less ability to metabolically eliminate carcinogenic compounds and may therefore be at increased cancer risk (Rebbeck et al., 1997). A meta-analysis of lung cancer showed a significantly increased risk for the *GSTM1* null type (Houlston, 1999).

This paper concerns a newly developed triplex polymerase chain reaction (PCR) to genotype simultaneously the above described three polymorphisms, using PCR with confronting two-pair primers (PCR-CTPP) (Hamajima et al., 2000, 2002b). Blood samples were collected from 241 non-cancer outpatients at the Aichi Cancer Center Hospital. Written informed consent was obtained from all subjects as described previously (Hamajima et al., 2002c). DNA was extracted from 200 µl of buffy coat preserved at -40°C with a QIAamp DNA Blood mini kit (Qiagen Inc., Valencia, CA). Triplex PCR was conducted to simultaneously genotype the three polymorphisms, *NQO1* C609T, *GSTM1*, and *GSTT1*, in one tube. Table 1 shows the primer sequences as well as the melting temperatures estimated by the base sequence algorithm (Breslauer et al, 1986). Two pairs of four primers were used for *NQO1* C609T genotyping by PCR-CTPP, and one pair of primers was used each for *GSTM1* and *GSTT1* by ordinary PCR.

Genomic DNA was applied in a volume of 25 µl with 0.18 mM dNTPs, 12.5 pmol of each primer, 0.5 units of AmpliTaq Gold (Perkin-Elmer Corp., Foster City, CA), and 2.5µl 10xPCR buffer including 15 mM MgCl2. PCR System 9700 (PE Biosystems, Foster City, CA) was used for the DNA amplification. The PCR was performed with initial denaturation at 95°C for 10 minutes, followed by 35 cycles of denaturation 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 separated by electrophoresis on a 2 % agarose gel containing 2 µl /100 ml of ethidium bromide.

Representative results of electrophoresis are shown in Fig. 1. The amplified DNA sequences are 161 base pairs (bp) for *NQO1 609C*, 283 bp for *609T*, 219 bp for *GSTM1*, and 507 bp for *GSTT1*, as well as a common band with 403 bp for *NQO1*. Bands were sufficiently clear for each sample to be genotyped correctly. All genotype combinations of three polymorphisms are demonstrated in Fig. 1. Table 2 shows the observed genotype frequencies for *NQO1* C609T, *GSTM1* and *GSTT1* among 241 non-cancer outpatients. The genotype frequency of NQO1 C609T was 35.7% for *CC*, 44.4% for *CT* and 19.9% for *TT*. They were in Hardy-Weinberg equilibrium with 57.9% of *C* allele and 42.1% of *T* allele. The value for the *GSTM1* null genotype was 53.4%, and that for the *GSTT1* null genotype was 44.0%. The genotype combinations of *NQO1* C609T, *GSTM1*, and *GSTT1* are also shown in Table 2. The largest population was 15.4% for those with the *NQO1* 609CT, *GSTM1* null type, and *GSTT1* present, as expected from the individual genotype frequencies.

The obtained genotype frequency for *NQO1* C609T in this study is consistent with the result with independent *NQO1* C609T PCR-CTPP for the same samples (Hamajima et al., 2002c). The distributions for *GSTM1* and *GSTT1* are also similar to those in another report for Japanese (Hamajima et al., 2002d). The findings thus partly support the conclusion that the triplex PCR-CTPP can genotype the samples correctly.

There is no substantial difference in the genotype frequency of *GSTM1* among Japanese, Koreans, Chinese, and Caucasians, while the *GSTT1* null genotype is more common in Japanese, Koreans and Chinese than in Caucasians (Hamajima et al., 2002d). The allele frequency of the *NQO1* C609T polymorphism determined in a study of 95 Chinese in Taiwan was 51% for the *T* allele (Lin et al., 1999), which was higher than that obtained in this study,

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

Primer	Sequence	Tm*
NQO1C609TF1	5' TAT CAG AGT GTC TTA CTG AGA	46.4 °C
NQO1C609TR1	5' AAT GCT ATA TGT CAG TTG AG<u>G</u>	51.6 °C
NQO1C609TF2	5' GTG GCT TCC AAG TCT TAG AA<u>T</u>	54.9 °C
NQO1C609TR2	5' TTT CTA GCT TTG ATC TGG TTG	54.5 °C
GSTM1F	5' GAA CTC CCT GAA AAG CTA AA	53.5 °C
GSTM1R	5' GTT GGG CTC AAA TAT ACG G	55.3 °C
GSTT1F	5' TTC CTT ACT GGT CCT CAC AT	54.2 °C
GSTT1R	5' AAT GCT TTG TGG ACT GCT	53.7 °C

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

Table 2. Genotype Frequencies of *NQO1 C609T, GSTM1,* and *GSTT1* among 241 Non-cancer Outpatients.

Genotype			N	%
NQ01	*GSTM1*	*GSTT1*		
CC			86	(35.7)
CT			107	(44.4)
TT			48	(19.9)
	+		109	(46.6)
	-		125	(53.4)
		+	131	(56.0)
		-	103	(44.0)
CC	+	+	18	(7.7)
CC	+	-	18	(7.7)
CC	-	+	26	(11.1)
CC	-	-	21	(9.0)
CT	+	+	23	(9.8)
CT	+	-	26	(11.1)
CT	-	+	36	(15.4)
CT	-	-	21	(9.0)
TT	+	+	16	(6.8)
TT	+	-	8	(3.4)
TT	-	+	12	(5.1)
TT	-	-	9	(3.8)

and Caucasians have been reported to have a lower *T* allele frequency (Hamajima et al., 2002d). Since the *609T* is presumably a sensitive allele with reference to carcinogenic substances, Japanese, Koreans and Chinese may have a higher risk to cancers than Caucasians in this polymorphism, under condition of exposure to carcinogens.

Only a few polymorphisms genotyped with multiplex PCR-CTPP have been described so far. 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 et al., 2001). This is the first report to describe a triplex PCR-CTPP. While a triplex PCR-RFLP (PCR- restriction fragment length polymorphism) for CYP1A1, *GSTM1* and *GSTT1* polymorphisms has been reported (Bailey et al., 1998), compared with PCR-RFLP, PCR-CTPP has the advantage of low cost and rapidity, because it allows genotyping of SNPs without incubation with a restriction enzyme for PCR product digestion. In addition, if we use PCR-RFLP, the sequences of *GSTM1* and *GSTT1* without restriction sites for HinfI, an endonuclease usually used for NQO1 genotyping, can be identified. When multiplex PCR-CTPP is applicable, there is no doubt that it is superior to multiplex PCR-RFLP. PCR-CTPP needs only half of the material input and time for PCR-RFLP, even for single polymorphism genotyping.

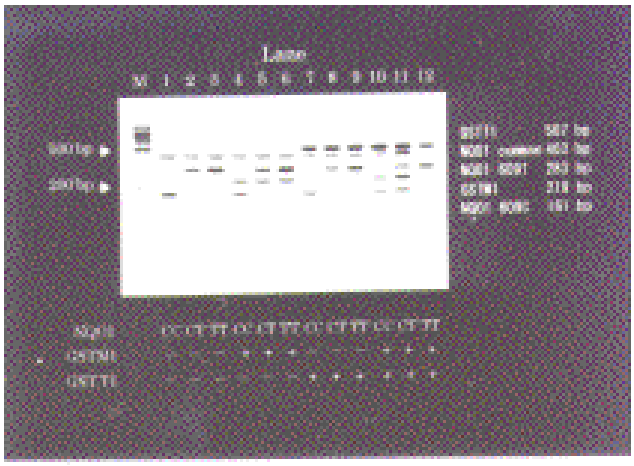

Figure 1. Representative Gel for *NQO1* C609T, *GSTM1*, and *GSTT1* by Triplex Polymerase Chain Reaction with Confronting Two-pair Primers (PCR-CTPP); Lane M for a 100-bp Marker.

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 could change the balance of the band strength. The balance is also sensitive to annealing temperature of PCR. The primers listed in Table 1 and annealing at 62°C provide one of the optimal combinations. The melting temperatures estimated for the primers are important, but the adjustment is unavoidable because of the limitation of the accuracy. General speaking, a similar melting temperature for all primers provides the best chance to find an optimal primer set. The conditions reported here were determined after several unsuccessful combinations were tried.

Cancer prevention is becoming more important in Asian Pacific regions, where smoking rates are still high, and carcinogenic substances is poorly regulated. PCR-CTPP, especially multiplex PCR-CTPP, is a useful tool for genotyping. Now, we are trying to design more multiplex PCR-CTPP for other polymorphisms, which should play an important role in the future.

References

Bailey LR, Roodi N, Verrier CS, et al (1998). Breast cancer and *CYP1A1*, *GSTM1*, and *GSTT1* polymorphisms: evidence of a lack of association in Caucasians and African Americans. *Cancer Res*, **58**, 65-70.

Breslauer KJ, Frank R, Blocker H, Marky LA (1986). Predicting DNA duplex stability from the base sequence. *Proc Natl Acad Sci USA*, **83**, 3746-50.

Chen H, Lum A, Seifried A, Wilkens LR, Le Marchand L (1999). Association of the NAD(P)H:quinone oxidoreductase 609 C→T polymorphism with a decreased lung cancer risk. *Cancer Res*, **59**, 3045-8.

Hamajima N, Saito T, Matsuo K, et al (2000). Polymerase chain reaction with confronting two-pair primers for polymorphism genotyping. *Jpn J Cancer Res*, **91**, 865-8.

Hamajima N (2001). PCR-CTPP: a new genotyping technique in the era of genetic epidemiology. *Expert Rev Mol Diagn*, **1**, 119-23.

Hamajima N, Matsuo K, Iwata H, et al (2002a). NAD(P)H:quinone oxidoreductase 1 (*NQO1*) C609T polymorphism and the risk of eight cancers for Japanese. *Int J Clin Oncol*, **7**, 103-8.

Hamajima N, Saito T, Matsuo K, Tajima K (2002b). Competitive amplification and unspecific amplification in polymerase chain reaction with confronting two-pair primers. *J Mol Diagn*, **4**, 103-7.

Hamajima N, Satio T, Matsuo K, et al (2002c). Genotype frequencies of 50 polymorphisms for 241 Japanese non-cancer patients. *J Epidemiol*, **12**, 229-36.

Hamajima N, Takezaki T, Tajima K (2002d). Allele frequencies of 25 polymorphisms pertaining to cancer risk for Japanese, Koreans and Chinese. *Asian Pacific J Cancer Prev*, **3**, 197-206.

Houlston RS (1999). Glutathione *S*-transferase M1 status and lung cancer risk: a meta-analysis. *Cancer Epidemiol Biomarkers Prev*, **8**, 675-82.

Lafuente MJ, Casterad X, Trias M, et al (2000). NAD(P)H:quinone oxidoreductase-dependent risk for colorectal cancer and its association with the presence of K-*ras* mutations in tumor. *Carcinogenesis*, **21**, 1813-9.

Larson RA, Wang Y, Banerjee M, et al (1999). Prevalence of the inactivating 609C→T polymorphism in the NAD(P)H:quinone oxidoreductase 1 (*NOQ1*) gene in patients with primary and therapy-related myeloid leukemia. *Blood*, **94**, 803-7.

Lin P, Wang HJ, Lee HS, et al (1999). The NAD(P)H:quinone oxidoreductase polymorphism and lung cancer in Taiwan. *J Toxicol Environ Health*, **58**, 187-97.

Longuemaux S, Delomenie C, Gallou C, et al (1999). Candidate genetic modifiers of individual susceptibility to renal cell carcinoma: a study of polymorphic human xenobiotic-metabolizing enzymes. *Cancer Res*, **59**, 2903-8.

Naoe T, Takeyama K, Yokozawa T, et al (2000). Analysis of genetic polymorphism in *NQO1*, *GST-M1*, *GST-T1*, and *CYP3A4* in 469 Japanese patients with therapy-related leukemia / myelodysplastic syndrome and de novo acute myeloid leukemia. *Clin Cancer Res*, **6**, 4091-5.

Rebbeck TR (1997). Molecular epidemiology of the human glutathione *S*-transferase genotypes *GSTM1* and *GSTT1* in cancer susceptibility. *Cancer Epidemiol Biomarkers Prev*, **6**, 733-43.

Rosvold EA, McGlynn KA, Lustbader ED, Buetow KH (1995). Identification of an NAD(P)H:quinone oxidoreductase polymorphism and its association with lung cancer and smoking. *Pharmacogenetics*, **5**, 199-206.

Schulz WA, Krummeck A, Rosinger I, et al (1997). Increased frequency of a null-allele for NAD(P)H:quinone oxidoreductase in patients with urological malignancies. *Pharmacogenetics*, **7**, 235-9.

Siegel D, McGuiness SM, Winski SL, Ross D (1999). Genotype-phenotype relationship in studies of a polymorphism in NAD(P)H:quinone oxidoreductase 1. *Pharmacogenetics*, **9**, 113-21.

Smith MT, Wang Y, Kane E, et al (2001). Low NAD(P)H:quinone oxidoreductase 1 activity is associated with increased risk of acute leukemia in adults. *Blood*, **97**, 1422-6.

Wiencke JK, Spitz MR, McMillan A, Kelsey KT (1997). Lung cancer in Mexican-Americans and African-Americans is associated with the wild type genotype of the NAD(P)H:quinone oxidoreductase polymorphism. *Cancer Epidemiol Biomarkers Prev*, **6**, 87-92.

Xu LL, Wain JC, Miller DP, et al (2001). The NAD(P)H:quinone oxidoreductase 1 gene polymorphism and lung cancer: differential susceptibility based on smoking behavior. *Cancer Epidemiol Biomarkers Prev*, **10**, 303-9.

Yin L, Pu Y, Liu TY, et al (2001). Genetic polymorphisms of NAD(P)H:quinone oxidoreductase, *CYP1A1* and microsomal epoxide hydrolase and lung cancer risk in Nanjing, China. *Lung Cancer*, **33**, 133-41.

Personal Profile: Haruya Kawase

Mr. Haruya Kawase was born in Nagoya in 1981. After graduating from Tokai High School, he entered Nagoya University School of Medicine in 2000. He has been investigating gene-environment interaction for cancers under Dr. Hamajima at the Department of Preventive Medicine/ Biostatistics and Medical Decision Making, Nagoya University Graduate School of Medicine, for about a half year. He has been not only studying hard as a medical student but also enjoying his life as a jazz pianist outside the school.