RESEARCH COMMUNICATION

Genotype Frequencies of Cyclooxygenease 2 (COX2) Rare Polymorphisms for Japanese with and without Colorectal Cancer

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Abstract

Cyclooxygenase 2 (COX2) is an inducible enzyme synthesizing prostaglandins from arachidonic acid, which is thought to play an important role in colorectal carcinogenesis. Since the COX2 polymorphisms, if functional, may modify the carcinogenesis pathway, the associations between the reported polymorphisms and colorectal cancer risk were examined in a hospital-based case-control study. Six polymorphisms of the gene encoding COX2 were genotyped for 241 non-cancer individuals (controls) and 148 colorectal cancer patients (74 colon cancer, 73 rectal cancer, and 1 colorectal cancer date, 22 polymorphisms including the above six have been reported for COX2. The other polymorphisms remain to be examined.

Key Words: Colorectal cancer - COX2 - polymorphisms - PCR-CTPP

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Introduction

Cyclooxygenase (COX) is a rate-limiting enzyme to convert arachidonic acid to prostaglandins, which has two isoforms, COX1 and COX2 (Eberhart et al, 1995). While COX1 is constitutively expressed in a variety of tissues, COX2 is induced by several growth factors and cytokines, including tumor necrosis factor α, facapentaene-12, interleukin 1β, and platelet-activating factor (Kravchenko et al, 1995; Lukiw et al, 1998; Plummer et al, 1999). The overexpression of COX2 has been reported in various types of tumors, such as colorectal (Eberhart et al, 1994; Kargman et al, 1995), gastric (Ristimaki et al, 1997), lung (Hida et al, 1998), prostate (Liu et al, 1998), and breast cancers (Liu et al, 1996). Furthermore, it was found that COX2 induces BCL2 (Tsujii et al, 1995), which can inhibit apoptosis. The hypothesized role of COX2 in colorectal cancer carcinogenesis is also supported by epidemiologic findings that nonsteroidal anti-inflammatory drugs reduce the colorectal cancer risk (Giovannucci et al, 1995; Smalley et al, 1999). In an animal model, a specific COX2 inhibitior, celecoxib, prevented colon tumors remarkably (Kawamori et al, 1998), and recently the effectiveness of the inhibitor has been reported for patients with familial adenomatous polyposis (Steinbach et al, 2000). These findings indicate that COX2 plays a pivotal role in the carcinogenesis of colorectal cancer, and suggest that the inter-individual variations of the induced COX2 level may influence the risk of colorectal cancer. If there are functional polymorphisms in COX2, the susceptibility to colorectal cancer is likely to be affected by such polymorphisms.

To date, twenty-two polymorphisms have been reported for the COX2 gene with the average estimated heterozygosity ranging from 0.011 to 0.495 (Accession No. NT 001817.3). Five are on the promoter region, one on the 5' UTR (untranslated region), three missense mutations, five synonymous substitutions, six on the introns, and two on the 3' UTR. Since there were no reports on the function of the polymorphisms, we selected three polymorphisms at -765, -163, and -62 on the promoter region, one on 5' UTR

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at +10, and two missense mutations (Glu488Gly and Val511Ala), which can be genotyped by a newly developed simple technique, PCR-CTPP (polymerase chain reaction with confronting two-pair primers) at our laboratory (Hamajima et al, 2000).

Materials and Methods

The subjects were patients of Aichi Cancer Center Hospital, who consented to provide a 7ml of peripheral blood for genetic polymorphism genotyping. They were enrolled between March 1999 and July 2000. The cases were 74 colon cancer patients (42 males and 32 females), 73 rectal cancer patients (43 males and 30 females), and 1 male colorectal cancer patient with an unknown cancer site, who were diagnosed in and after 1995 except 5 cases. They were 24 to 78 years old at diagnosis. The controls were 241 patients (117 males and 124 females) aged 39 to 69 years without a history of cancer who underwent gastroscopy at Aichi Cancer Center Hospital.

Six single nucleotide polymorphisms (SNPs), G-765C, C-163G, C-62G, T10G, A5720G (Glu488Gly), and T5789C (Val511Ala), were genotyped by a new PCR method, PCR-CTPP. The method requires four primers for PCR. The amplified DNAs are allele-specific in their sizes, so that the DNA products can be applied directly for electrophoresis without the digestion by a restriction enzyme (Hamajima et al, 2000). The conditions of the PCR-CTPP and the primers are described in Table 1. Genomic DNA (30ng to 100ng) was used per 25μl of reaction with 0.15mM dNTPs, 25 pmol of each primer, 0.5 units of polymerase, and 2.5μl 10xPCR buffer including 15mM $MgCl_2$. The PCR products were visualized on a 2% agarose gel with ethidium bromide staining. The genotype was distinguished as shown in Table 1.

The allele and genotype frequencies were compared between the cases and controls by a chi-squared test. The 95% confidence intervals for the percentages were estimated using a binomial distribution. The calculations were performed by STATA (STATA Cooperation, College Station, TX).

Results

Figure 1 shows the gels for the genotypes for the six polymorphisms of COX2. Although no mutant alleles of polymorphisms at -62, +5720 (Glu488Gly), and +5789

Table 1. PCR Conditions and Primers for The PCR-CTPP Genotyping of Cyclooxygenase 2 PolymOrphisms

Position	Allele (products)	Polymerase	PCR condition
–765 (5'UTR)	G (520, 245bp)	AmpliTaq Gold	10 min at 95°C, 30 cycles of 1 min each at 95°C, 50°C, and 72°C,
	C (520, 314bp)	+10% Glycerol	and 5 min at 72°C
F1: 5'-ATC TCC TAT GAA GGG CTA GT-3'			R1: 5'-TGT TTC TTG GAA AGA GAG GC-3'
F2: 5'-AGG AGA ATT TAC CTT TCC CC-3'			R2: 5'-CTG GTC TGT ACG TCT TTA GA-3'
–163 (5'UTR)	G (394, 255bp)	AmpliTaq Gold	10 min at 95°C, 30 cycles of 1 min each at 95°C, 52°C, and 72°C,
	C (394, 179bp)		and 5 min at 72°C
F1: 5'-GGA AGC CAA GTG TCC TTC T-3'			R1: 5'-CAG GGT TTT TTA CCC ACG C-3'
F2: 5'-GGG TTT CCG ATT TTC TCA TTT C-3'			R2: 5'-TAA CCG AGA GAA CCT TCC TTT-3'
–62 (5'UTR)	G (483, 151bp)	TakaraTaq	5 min at 95°C, 30 cycles of 1 min each at 95°C, 55°C, and 72°C,
	C (483, 377bp)		and 5 min at 72°C
F1: 5'-CTT CCT GGG TTT CCG ATT TTC TC-3'			R1: 5'-AAC CAA GCC CAT GTG ACG AAA TC-3'
F2: 5'-CGA AAA GGC GGA AAG AAA CAG TC-3'			R2: 5'-TAT TCG GAG AGA AGT CGG AGT AC-3'
+10 (exon 1)	T (474, 223bp)	TakaraTaq	5 min at 95°C, 30 cycles of 1 min each at 95°C, 55°C, and 72°C,
	G (474, 292bp)		and 5 min at 72°C
F1: 5'-CAG CTT CCT GGG TTT CCG ATT-3'			R1: 5'-TGA CGC TCA CTG CAA GTC GTA-3'
F2: 5'-CGG TTA GCG ACC AAT TGT CAG-3'			R2: 5'-AGT CGG AGT ACT GGG ATA GAC-3'
+5720 (exon 10)	A (425, 216bp)	AmpliTaq Gold	10 min at 94°C, 30 cycles of 1min each at 94°C, 54°C, and 72°C,
	G (425, 244bp)	+10% glycerol	and 5 min at 72°C
F1: 5'-CCT GGC CCC TAA ACT TCT-3'			R1: 5'-GAA GGG CAG GAT ACA GCT-3'
F2: 5'-TGA CAT CGA TGC TGT GGG-3'			R2: 5'-AAA GGG ACA GCC CTT CAC-3'
+5789 (exon 10)	T (531, 361bp)	AmpliTaq Gold	10 min at 94°C, 30 cycles of 1min each at 94°C, 58°C, and 72°C,
	C (531, 209bp)	+10% glycerol	and 5 min at 72°C
F1: 5'-CTC AAA CTC CTG ACC TAG TG-3'			R1: 5'-CAA GGA GAA TGG TGC TCC AA-3'
F2: 5'-GGT GAA ACC ATG GTA GAA GC-3'			R2: 5'-ATG AGC TCT GGA TCT GGA AC-3'

* All PCRs were conducted by GeneAmp PCR System 9700 (Perkin Elmer Biosystems)

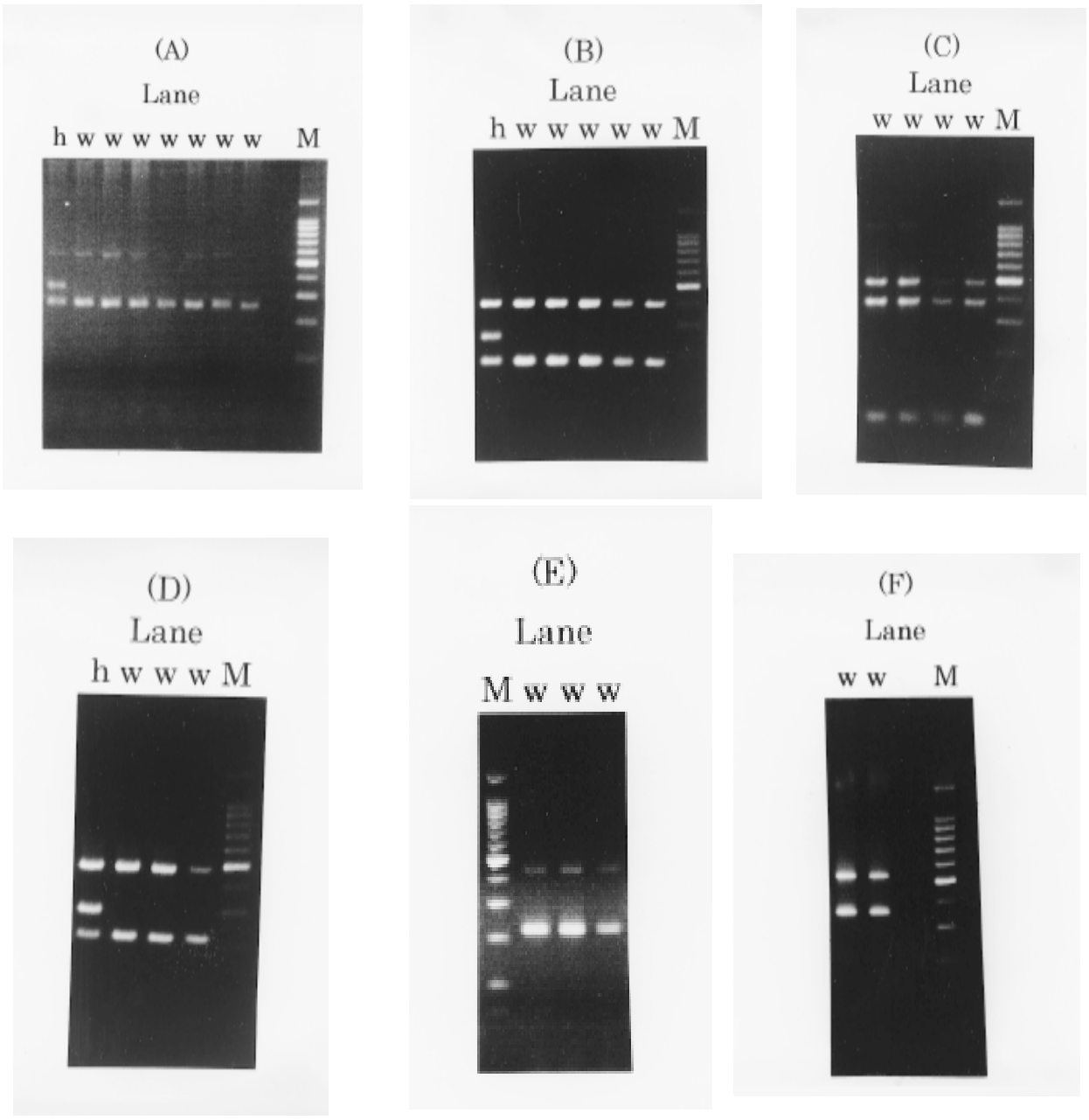

Figure 1. Gels Showing the Genotypes for the Single Nucleotide Polymorphisms at G-765C (A), C-163G (B), C-62G (C), T10G (D), A5720G (E), and T5789C (F); Lane M Contains a 100-bp DNA Ladder, Lane w for the Homozygous wild type, and Lane h for the Heterozygous Genotype.

(Val511Ala) were observed, the existence of the common bands indicated that the genotyping by the PCR-CTPP was succeeded for the three polymorphisms.

Since the first 50 controls and 50 cases all showed the homozygous genotype for these polymorphisms, we abandoned the genotyping for the rest of samples. The 95% binomial exact confidence interval is 0-0.036 for 0 out of 100 alleles and 0-0.018 for 0 out of 200 alleles. The average heterozygosity reported in GenBank NT 001817.3 was 0.011, 0.095, and 0.052, respectively. The allele frequency was not available for C-62G in NT 001817.3, but listed for A5720G with the A allele 0.950 and the G allele 0.050, and T5789C with the T allele 0.950 and the C allele 0.050.

As shown in Table 2, the controls harboring the C allele at –765 were found to be 11 (4.6%), and the allele frequency was 0.023 (95% CI, 0.011-0.040), giving a 0.045 (0.023x(1-

Table 2. Genotype Frequencies of COX2 Polymorphisms for Non-cancer Patients (Controls) and Colorectal Cancer Patients (Cases)

		Controls		Cases	
G-765C	GG	230	(95.4)	140	(94.6)
at 5'UTR	GC	11	(4.6)	8	(5.4)
	CC	0	(0.0)	0	(0.0)
	Total	241	(100)	148	(100)
C-163G	CC	230	(97.0)	NG	
at 5'UTR	CG	7	(3.0)	NG	
	GG	0	(0.0)	NG	
	Total	237	(100)	NG	
C-62G	CC	50	(100)	50	(100)
At 5'UTR	CG	0	(0.0)	0	(0.0)
	GG	0	(0.0)	0	(0.0)
	Total	50	(100)	50	(100)
T10G	TT	230	(97.0)	141	(96.6)
at exon 1	TG	7	(3.0)	5	(3.4)
	GG	0	(0.0)	0	(0.0)
	Total	237	(100)	146	(100)
A5720G	AA	50	(100)	50	(100)
(Glu488Gly)	AG	0	(0.0)	0	(0.0)
at exon 10	GG	0	(0.0)	0	(0.0)
	Total	50	(100)	50	(100)
T5789C	TT	50	(100)	50	(100)
(Val511Ala)	TC	0	(0.0)	0	(0.0)
	CC	0	(0.0)	0	(0.0)
	Total	50	(100)	50	(100)

% in parenthese. NG: not genotyped.

0.023)x2) of heterozygosity, which is slightly higher than the average heterozygosity reported in NT 001817.3 (0.011). There was no difference in the genotype frequency between the 241 controls and 148 cases (χ^2=0.1396, p=0.709). None out of 8 cases with the GC genotype aged 27 to 73 years had a family history of colorectal cancer among their first degree relatives (parents and siblings), while 13 out of 140 cases with the GG genotype had the family history.

A tight linkage was found between the polymorphisms at –163 and at +10. Seven controls with the CG genotypes of C-163G were all with the TG genotype of T10G, and the rest were with the CC of C-163G and the TT of T10G. At a pilot genotyping study, we found another two samples with the CG genotype. They were also with the TG genotype. Accordingly, we quit the C-163G genotyping for the case group. The TG genotype of the T10G polymorphism was 5 out of 146 cases, 0.034 (95%CI, 0.011-0.078), while the corresponding value was 0.030 (95%CI, 0.012-0.060) for 237 controls. The difference was not statistically significant (χ^2=0.0661, p=0.797). The 5 cases, aged 52 to 73 years, stated no family history of colorectal cancer among their first degree relatives. They were all with the GG genotype of G-765C. The reported allele frequency was 0.100 for the G allele at –163 (reported heteozygosity = 0.033) and 0.150 for the G allele at +10 (reported heterozygosity = 0.255).

Further analyses for the comparisons between the cases and controls seemed meaningless because of the low frequencies of the mutated alleles.

Discussion

The associations with the colorectal cancer risk have beenexamined for many genetic polymorphisms, including cytochrome p450 (CYP), Glutathione S-tranferases (GSTs), N-acetyltransferases (NATs), aldehyde dehydrogenase 2 (ALDH2), methylenetetrahydrofolate reductase (MTHFR), APC, and so on (Frayling et al, 1998; Potter, 1999; Kiyohara, 2000). However, to our knowledge, there are no reports on the association with the COX2 polymorphisms, notwithstanding the accumulated evidence indicating the importance of the colorectal carcinogenesis.

The promoter region of COX2 , which extends in the area between –1000 and +165 from transcription site, is rich in the sequences to combine the transcription factors such as activator proteins 1 (AP1) and 2 (AP2), NF-κB, gamma interferon activation site (GAS), TATA binding protein TFIID (transcription factor IID) and Egr1 (Kosaka et al, 1994; Lukiw et al, 1998). The four SNPs in that area, as well as the two SNPs causing a missense mutation were selected in this study because of their potential to be functional polymorphisms.

The results showed that the genotypes of three SNPs (C-62G, A5720G, and T5789C) were homozygous for all the study subjects. For the rest three SNPs, the mutated alleles were rare among the controls, two of which (C-163G and T10G) were tightly linked. The mutated alleles were also rare for the patients with colorectal cancer, and useless to predict the susceptibility to colorectal cancer. We conducted the T10G genotyping for 238 breast cancer patients and 82 lung cancer patients, but the TG genotype was similarly rare; 4 breast cancer patients (1.7%) and 4 lung cancer patients (4.9%). This is the first report concerning the genotype frequencies of these polymorphisms for Japanese. It indicates no associations with these polymorphisms because the frequencies of the mutated alleles were too low to detect them, or because they are not functional. For the other polymorphisms of COX2, there are no data available so far, remaining to be examined.

Of interest is the fact that the overexpression of COX2 is frequent among colorectal cancer patients, but not universal. It is less frequent in sporadic colorectal cancer with microsatellite instability resulting from defective DNA mismatch rapair, and in hereditary nonpolyposis colorectal cancer (Karnes et al, 1998; Sinicrope et al, 1999). It suggests other pathways of colorectal carcinogenesis, independent on the overexpression of COX2 (Toyota et al, 2000). The potential influence of the COX2 polymorphisms would be attenuated for the populations with a low frequency of COX2-overexpressed cases. There is no report on the percentage of the overexpressed colorectal cases for Japanese.

In conclusion, we found that the mutated alleles of COX2 polymorphisms examined in the present study were rare among Japanese, and were not associated with the risk of colorectal cancer in Japan. Although no associations were observed with the six polymorphisms, this study provided information useful to go on the next step of investigation, and this reporting has a meaning for the researchers to conduct the search for the same COX2 polymorphisms.

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Overleaf: Memorable moments of Dr Hamajima in Australia to take part in a Course - Christmas in the sun!