Effects of genetic polymorphisms of *MDR1*, *FMO3* and *CYP1A2* on susceptibility to colorectal cancer in Koreans

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The aim of the present study was to evaluate the effects on the susceptibility to colorectal cancer (CRC) of genetic polymorphisms in P-glycoprotein (PGP) and the metabolic enzymes cytochrome P450 1A2 (CYP1A2) and flavin-containing monooxygenase 3 (FMO3). We analyzed five single-nucleotide polymorphisms (SNP) in 93 cancer-free volunteers and 111 patients with CRC: one common genetic variant of the PGP-encoding *MDR1* gene and four SNP in genes for metabolic enzymes (two SNP in *FMO3* and two SNP in *CYP1A2*). The genotypes and allele frequencies of the *MDR1*/C3435T, *FMO3*/G488A, *FMO3*/A923G and *CYP1A2*/G-3860 A polymorphisms were not significantly different in cancer-free subjects and CRC patients. However, a significant association was found between the *CYP1A2*/A-163C polymorphism and the risk of CRC, particularly in elderly (>55 years) subjects and smokers. A phenotyping study in normal smokers showed that the CYP1A2 activity of subjects with the *CYP1A2*/–163 AA genotype was significantly lower than that of subjects carrying the *CYP1A2*/–163C allele. Combined results show that the *CYP1A2*/–163C allele is significantly associated with an increase in CYP1A2 activity and a consequent increased risk of CRC in Koreans, particularly in elderly people and smokers. (*Cancer Sci* 2006; 97: 774–779)

Colorectal cancer (CRC) is one of the most common malignancies in developed countries. It occurs via an interaction between an individual genetic background and environmental parameters such as dietary factors. A number of studies have suggested that dietary procarcinogens, such as heterocyclic amines, *N*-nitroso compounds and polycyclic aromatic hydrocarbons, might be related to the carcinogenesis of CRC and prostate cancer.[1–6] The carcinogens that cause the development of CRC enter the body as (pro)carcinogens via transporters,[7–9] and are activated to carcinogens or eliminated by various enzymes.[10] These toxicokinetic-related proteins are also controlled by our genetic background (e.g. by genetic polymorphisms). In considering the effects of genetic polymorphisms on the toxicokinetic profiles of xenobiotics, particularly (pro) carcinogens, we must evaluate the combined effects of genetic polymorphisms in both transporters and metabolic enzymes.

To date, numerous studies have been conducted to correlate the genetic polymorphisms of single proteins and the risk of disease development or progression. In particular, genetic polymorphisms in drug metabolizing enzymes, such as the cytochrome P450 1 (CYP1A) family,[11,12] glutathione S-transferases,[13] *N*-acetyltransferases[14] and UDP-glucuronosyltransferases,[15] are the most well-known and important genetic factors in the development of CRC. Flavin-containing monooxygenase 3 (FMO3) is one of the major hepatic metabolic enzymes that catalyze the NADPH-dependent attachment of molecular oxygen to endogenous and foreign chemicals containing nucleophilic N, S and P heteroatoms. There are few studies of the relationship between FMO3 activity and carcinogenesis. A drug transporter, P-glycoprotein (PGP), is a recognized gatekeeper that limits the accumulation of xenobiotics in the body by facilitating ATP-consuming efflux. Recently, studies of the relationship between *MDR1* polymorphisms and disease susceptibility have been conducted.[16,17] However, there has been no report of the relationship between the combined genetic polymorphisms of drug transporters and metabolic enzymes and CRC development.

Against this background, we analyzed the common genetic polymorphisms in the genes for the drug transporter *MDR1* and the metabolic enzymes *CYP1A2* and *FMO3*, and attempted to elucidate the association between these polymorphisms and sporadic CRC in Koreans. We also compared the inducibility of CYP1A2 activity according to *CYP1A2* genotype by *in vivo* measurement of the urinary caffeine metabolite ratio.

Materials and Methods

Subjects

A total of 204 unrelated Koreans participated voluntarily in the genotype–CRC association study. They consisted of 111 patients (mean age 62.5 ± 10.6 years, M : F = 60 : 51) who had been diagnosed with primary CRC by pathology tests at the Inha University Hospital in Incheon (Republic of Korea) and 93 control (cancer-free) volunteers (mean age

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Table 1. Polymerase chain reaction (PCR) conditions, sequences of primers and restriction enzymes used for PCR–restriction fragment length polymorphism (RFLP)

Gene and SNP	Primer	Sequence (5′→3′)	PCR conditions	Band size (bp)	Restriction enzyme	Fragments†
MDR1/C3435T	Forward	TGCTGGTCCTGAAGTTGATCTGTGAAC	30 × (1 min 94°C, 1 min 55°C, 2 min 72°C)	252	*Mbo*I	188, 64
	Reverse	ACATTAGGCAGTGACTCGATGAAGGCA				252
FMO3/G472A	Forward	GCTAGCATAGAAAAGAGGGA	30 × (1 min 94°C, 1 min 55°C, 2 min 72°C)	615	*Hinf*I	217, 66‡
	Reverse	CGAGAGTCACCCGAGTACCCG				283
FMO3/A923G	Forward	GTCTCTGTTTTCCATAGAG	30 × (1 min 94°C, 1 min 55°C, 2 min 72°C)	408	*Dra*II	408
	Reverse	CTTCGCAATCCATGAGCCTC				293, 115
CYP1A2/G-3860A	Forward	GCTACACATGATCGAGCTATAC	30 × (1 min 94°C, 1 min 53°C, 3 min 72°C)	536	*Dde*I	598
	Reverse	CAGGTCTCTTCACTGTAAAGTTA				466, 132
CYP1A2/A-163C	Forward	CAACCCTGCCAATCTCAAGCAC	30 × (1 min 94°C, 1 min 60°C, 3 min 72°C)	921	*Apa*I	921
	Reverse	CCTTCTCGGCCACAGAGCTTCT				715, 206

†Upper line, fragments of wild type allele; lower line, fragments of mutant allele. ‡PCR products (615 bp) were digested with *Hinf*I, cleaving the wild-type product into four fragments (217, 205, 127 and 66 bp) but cleaving the mutant type into three fragments (283, 205 and 127 bp). SNP, single nucleotide polymorphism.

49.2 ± 12.8 years, M : F = 55 : 38) who visited Inha University Hospital for other problems unrelated to colorectal disease, between February 2000 and July 2003. CRC was ruled out by colonoscopy in the CRC-free volunteers. Among the CRC patients, the site of CRC development in 31 patients was the colon and in the remainders was the rectum. Unfortunately, we did not determine the presence of somatic mutations in CRC-related genes such as *APC*, *β-catenin* or *Tcf*. The study protocol was approved by the Institutional Review Board of Inha University Hospital, and all volunteers provided their written informed consent.

Fifty-five young male native Korean smokers (mean age 23.3 ± 2.1 years) participated in the phenotype–genotype association study, having also provided their written informed consent. They were judged to be healthy by medical history, physical examination and routine laboratory analyses.

Genotyping

To genotype the five target single nucleotide polymorphisms (SNP), we used a polymerase chain reaction (PCR)–restriction fragment length polymorphism (RFLP) method, as described previously.[18–20] The conditions for each PCR amplification and the restriction enzymes used are shown in Table 1. Based on the results of genotype frequencies, we designated the *CYP1A2*/−163A allele as wild type, contrary to the proposed International CYP Allele Nomenclature (http://www.imm.ki.se/CYPalleles/cyp1a2.htm), because the frequency of the *CYP1A2*/−163A allele is higher in both Caucasian and Asian populations.[19,21]

Phenotyping using the urinary caffeine challenge test

We recruited 55 normal volunteers who were habitual smokers and analyzed their *CYP1A2*/A-163C genotypes and CYP1A2 activity using the urinary caffeine challenge test, as described previously.[22] Briefly, we calculated the CYP1A2 activity of individuals as the urinary molar ratio of (paraxanthine + 1,7-dimethyluric acid) : caffeine in a 1-h urine sample between 4 and 5 h after caffeine consumption (110 mg). After an overnight fast (10 h), each volunteer was given a cup of coffee (200 mL) prepared from two packets of instant coffee (Taster's choice, 12 g; Nestle, Vevey, Switzerland) that contained 110 mg of caffeine, immediately after voiding the control urine. All volunteers were asked not to consume any methylxanthine-containing drinks, foods or drugs for 2 days before and during the study period. Because the baseline control urine contained no measurable amounts of caffeine metabolites, participants were believed to have complied with the request to abstain from methylxanthine-containing foods and drugs. Immediately after the collection of the 1-h urine sample, the pH of the sample was adjusted to 3.5 with hydrochloric acid and a 10 mL aliquot was stored at −80°C for high-performance liquid chromatography (HPLC) analysis. Urinary caffeine metabolites were determined using HPLC, as described previously.[22,23]

Statistical analysis

To assess the effect of each genotype on CRC risk, the genotype and allele frequencies for each SNP were compared between the normal subjects and patients with CRC using a χ^2-test. We used unconditional logistic regression analyses to obtain odds ratios (OR) minimally adjusted for age, smoking status and current number of cigarettes smoked per day. The final parameters used to assess the influence on CRC risk were selected by multiple regression analysis with stepwise selection. To compare the ages of normal and CRC subjects and the enzymatic activities of enzymes among genotypes, the Mann–Whitney *U*-test or the Kruskal–Wallis test was used.

Results

Among the five SNP analyzed, the genotype and allele frequencies for the *MDR1* and *FMO3* polymorphisms did not differ significantly between the normal and patient groups, as shown in Tables 2 and 3. The frequency of the *MDR1*/3435T mutant allele was higher than that reported in previous data on Koreans,[24] but the difference was not significant. No

Table 2. Genotype frequencies of five single nucleotide polymorphisms in normal subjects and colorectal cancer patients

Gene	Site	Genotype	Normal (n = 93)		Patient (n = 111)		P-value
			n	%	n	%	
MDR1		CC	22	23.7	32	28.8	0.378
	3435	CT	55	59.1	63	56.8	
		TT	16	17.2	16	14.4	
FMO3		GG	62	66.7	79	71.2	0.648
	472	GA	29	31.2	31	27.9	
		AA	2	2.1	1	0.9	
		AA	68	73.1	80	72.1	0.705
	923	AG	24	25.8	28	25.2	
		GG	1	1.1	3	2.7	
CYP1A2		GG	48	51.6	68	61.3	0.304
	–3860	GA	39	41.9	35	31.5	
		AA	6	6.5	8	7.2	
		AA	44	47.3	24	21.6	0.003
	–163	CA	37	39.8	71	64.0	
		CC	12	12.9	16	14.4	

Table 3. Frequencies of five alleles in normal subjects and colorectal cancer patients

Gene	Site	Allele	Normal (n = 93)		Patient (n = 111)		P-value
			n	%	n	%	
MDR1	3435	C	99	53.2	127	57.2	0.420
		T	87	46.8	95	42.8	
FMO3	472	G	153	82.3	189	85.1	0.432
		A	33	17.7	33	14.9	
	923	A	160	86.0	188	84.7	0.704
		G	26	14.0	34	15.3	
CYP1A2	–3860	G	135	72.6	171	77.0	0.302
		A	51	27.4	51	23.0	
	–163	A	125	67.2	119	53.6	0.005
		C	61	32.8	103	46.4	

allele or haplotype frequency analyzed for the *FMO3* gene was significantly different between the two groups. The metabolic activity of FMO3 was influenced by the two SNP studied, particularly in the double mutant for the two SNP, as described previously.[20] Although the frequencies of the homozygous *FMO3*/G472A and *FMO3*/A923G variants were low, the genotype frequency of the homozygous *FMO3*/472 A variant in the CRC group was lower (0.9%) than in the control group (2.1%), whereas the frequency of the homozygous *FMO3*/923G variant was higher in the CRC group (2.7%) than in the control group (1.1%). However, because only one person carrying the double mutant was detected in both groups, we failed to detect significant differences in these frequencies between the two groups (data not shown).

Genotype analysis of the *CYP1A2* gene polymorphisms revealed that the genotype frequency of the A-163C variant, but not that of the G-3860 A variant, was significantly different between the control and CRC groups (Table 2). In allele frequency analysis, the *CYP1A2*/–163A allele was found more frequently in control subjects than in CRC patients (Table 3; $P = 0.0053$). Subjects with a non-AA genotype for the A-163C polymorphism had a significantly higher risk of developing CRC than did subjects with the AA genotype (OR = 3.26; 95% confidence interval [CI] = 1.772–5.980; $P = 0.0001$). There was a significant difference in the mean ages of the two groups (Student's t-test, $P < 0.0001$), although the age distributions were not significantly different (Mantel–Haenszel test, $P = 0.330$) when subjects were grouped by age in 10-year intervals. The frequency of smokers was higher in the patient group (57/111, 51.4%) than in the control group (35/93, 37.6%), as expected (χ^2-test, $P = 0.0315$). Therefore, we reanalyzed the effect of the *CYP1A2*/A-163C SNP on CRC development after adjustments were made for age and smoking habits. After these adjustments, there was no significant difference in the risk of developing CRC in subjects with a non-AA genotype for the A-163C polymorphism who were less than 55 years old ($P = 0.123$), whereas the OR was significantly higher in subjects with a non-AA genotype who were more than 55 years old (OR = 4.11; 95% CI = 1.619–10.475; $P = 0.003$). By χ^2 analysis of genotype frequencies after stratification according to smoking status, there was no significant difference in the risk of CRC between genotypes in non-smokers ($P = 0.097$). However, among smokers, the risk of CRC in subjects with a non-AA genotype was significantly higher than that in subjects with the AA genotype (OR = 6.49; 95% CI = 2.388–17.611; $P = 0.0001$). Multiple regression analysis with stepwise selection identified the *CYP1A2*/A-163C SNP and age as significant factors contributing to the risk of CRC development. In subsequent logistic analysis, the *CYP1A2* genotype (OR of the non-AA genotype

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Table 4. Results of logistic regression analysis

Independent variable	OR	95% C.I	*P*-value
CYP1A2 (non-AA genotypes)	3.113	1.547–6.265	0.0015
Age (≥55 years)	8.142	4.189–15.826	<0.0001

CI, confidence interval; OR, odds ratio.

= 3.113; 95% CI = 1.547–6.265) and age (OR for subjects over 55 years = 8.142; 95% CI = 4.189–15.826) were associated with an increased risk of CRC (Table 4).

The effect of the *CYP1A2* genotype on the inducibility of CYP1A2 activity by tobacco smoking, an important factor in the induction of CYP1A2, is controversial.[25–27] In contrast to the data presented here, a recent study has suggested that the *CYP1A2*/–163A allele is associated with a higher inducibility of CYP1A2 activity and a risk of CRC.[28] To investigate this issue, we measured the *CYP1A2* genotype–phenotype association in smokers with normal hepatic function. Genotype analysis indicated that the frequencies of the CC, CA and AA genotypes were 10.9%, 43.6% and 45.5%, respectively. As shown in Fig. 1, the CYP1A2 activity of the subjects with each genotype differed marginally but not significantly (Kruskal–Wallis test; $P = 0.0839$). However, the CYP1A2 activity of subjects with AA genotype was significantly lower than that of subjects carrying the *CYP1A2*/–163C allele (CC and CA genotypes) (Mann–Whitney *U*-test; $P = 0.0299$).

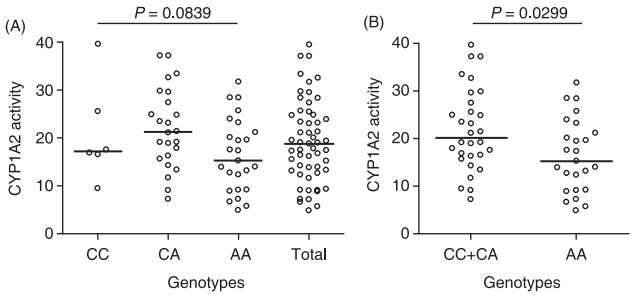

Fig. 1 Cytochrome P450 1A2 (CYP1A2) activity, measured by urinary caffeine metabolite ratios, according to *CYP1A2*/A-163C genotypes in 55 smokers. (A) Kruskal–Wallis test, (B) Mann–Whitney *U*-test.

Discussion

Not only multidrug-resistant cancer cells, but also normal human tissues constitutively express the *MDR1*-encoded transporter PGP, which contributes to the absorption and distribution of xenobiotics, including environmental toxins and drugs. The *MDR1*/C3435T polymorphism is one of the well-known functional SNP affecting drug absorption and distribution.[29] FMO3, one of the major hepatic metabolic enzymes, is associated with the activation of procarcinogens,[30] and displays genetic polymorphisms in humans.[20] Therefore, if procarcinogens and/or carcinogens that cause CRC development are substrates for PGP and/or FMO3, it is necessary to evaluate the relationship between the genetic polymorphisms of these proteins and the risk of CRC. The SNP analyzed in these proteins were not associated with the risk of CRC, as shown in Tables 2 and 3. These results suggest that the environmental or dietary procarcinogens and/or carcinogens that cause CRC are not substrates of these proteins. Further study of the carcinogenic substrate specificities of these proteins is necessary to address this issue.

We analyzed the relationship between two SNP in the *CYP1A2* gene and the risk of CRC. Although it has been reported that the *CYP1A2*/G-3860A polymorphism is related to CYP1A2 inducibility,[25] we failed to detect a correlation between this SNP and the risk of CRC. However, another SNP, *CYP1A2*/A-163C, was closely associated with the risk of CRC. Therefore, the higher CYP1A2 activity caused by both the non-AA genotypes and environmental factor (such as cigarette smoking), if any, may be risk factors activating the procarcinogen-to-carcinogen transition that develops into CRC. Our results suggest that the *CYP1A2*/A-163C polymorphism is a useful marker for the assessment of CRC risk, particularly in elderly subjects.

To our knowledge, there is no data on the phenotype–genotype association for the *CYP1A2*/A-163C polymorphism in Koreans. In some studies, *CYP1A2*/C-163A (designated *CYP1A2*1F*) was associated with increased CYP1A2 inducibility in Caucasian smokers but not in non-smokers.[25,31] However, our present data suggest that subjects with non-AA genotypes for the *CYP1A2*/A-163C polymorphism have a higher risk of CRC development. Therefore, it is possible that subjects with the *CYP1A2*/–163C allele have higher CYP1A2 activity, which is contrary to the results of the previous reports. In a previous genotype–CRC association study, Sachse and colleagues argued that CRC patients have unexpectedly low CYP1A2 activity relative to that of controls.[26] They explained these unexpected data by suggesting that CRC patients have low general hepatic metabolic function, including CYP1A2 activity. Furthermore, they failed to detect a significantly different frequency for any allele between normal volunteers and CRC patients.

Cigarette smoking is a well-known inducer of CYP1A2 activity. In a recent study, the *CYP1A2*1F* polymorphism was associated with increased CYP1A2 activity when extreme values were omitted, and with the risk of CRC.[28] Therefore, we conducted an additional genotype–phenotype association study of smokers with normal hepatic function ($n = 55$) to clarify the effects of the A-163C SNP on induced CYP1A2 activity. Based on our results, we concluded that the increase in CYP1A2 activity induced by smoking was significant in subjects with non-AA genotypes, but not in subjects with the AA genotype. The mean CYP1A2 activity in subjects with the AA genotype (16.5 ± 7.7) was similar to the CYP1A2 activity of young non-smokers (13.5 ± 5.9) reported in our previous study.[22] However, the CYP1A2 activity of smokers with non-AA genotypes (21.9 ± 8.6) was 1.6 times higher than that of non-smokers.

Contrary to previous results obtained from a Caucasian population,[28,31] our current study demonstrates that the AA genotype of the *CYP1A2*/A-163C polymorphism is associated with decreased CYP1A2 inducibility. This discrepancy may be attributable to several causes. First, the frequencies of other SNP, such as *CYP1A2*/T-2467delT and *CYP1A2*/G-3860A, in Caucasians were quite different from those in Koreans or other Asians.[21,26,32] Furthermore, the linkage disequilibrium status of these SNP might differ among these

ethnic groups. Second, the groups of subjects analyzed in the present study differed from those in the previous study. In fact, Moonen and colleagues detected a significant difference only when they omitted extreme values, and their subjects in the high-risk group ($n = 38$) had pathologically confirmed adenoma or CRC.[28] However, we analyzed the genotype–phenotype association only in volunteers with no colorectal disease, including CRC. This issue may be resolved by increasing the sample size of normal smokers and non-smokers. Finally, different methods for measuring CYP1A2 activity were used in these studies. Some investigators measured CYP1A2 activity using the plasma metabolite ratio (17X : caffeine),[25,31] whereas others measured it using urinary metabolite ratios (e.g. [17X + 17 U] : caffeine or 5′-acetylamino-6-formylamino-3-methyluracil (AFMU) + 1X + 1 U) : 17 U).[22,27,28,31] These issues should be resolved by an additional study that uses identical methods for the measurement of CYP1A2 activity in different ethnic groups.

The limit of our study is a lack of genotype analysis due to a relatively small sample size for other genes encoding metabolic enzymes, such as glutathione S-transferases, *N*-acetyltransferase 2 (NAT2), CYP1A1 and methylenetetrahydrofolate reductase, related to an increase in CRC risk.[33] This limitation may be solved by another study recruiting a large population, as we focused on evaluating the combined effects of genotypes in transporter (*MDR1*) and metabolic enzymes (*FMO3* and *CYP1A2*) on CRC risk and on elucidating the relationship between CYP1A2 genotype and phenotype particularly in Korean smokers. The cancer risk–genotype association studies conducted in other Asians suggest that the influence of CYP1A2 activity on the risk of variable cancers is clearly affected by other polymorphic enzymes (e.g. NAT2) or environmental factors (e.g. diet habit or smoking status).[34,35] Therefore, an additional study containing more abundant data for genetic and environmental confounding factors in a large Korean population should be carried out to compare with other populations.

In summary, our results suggest that the *CYP1A2*/A-163C SNP is a useful marker for risk assessment of CRC development and that the other genotypes examined in the present study are not related to CRC development in Koreans. However, paradoxical differences of a genotype–phenotype correlation in the present study as compared with other studies should be further evaluated, as a similar phenomenon was observed in another polymorphic enzyme.[36] Further studies of the substrate specificity of transporters and other metabolic enzymes for various (pro)carcinogens are necessary to identify the genetic factors that contribute to the development of CRC.

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