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An intron 4 VNTR polymorphism of the endothelial nitric oxide synthase gene is associated with early-onset colorectal cancer

Chih-Ching Yeh[1,2,*], **Regina M. Santella**[2], **Ling-Ling Hsieh**[3], **Fung-Chang Sung**[4], and **Reiping Tang**[5]

[1]Department of Health Risk Management, China Medical University College of Public Health, Taichung 404, Taiwan

[2]Department of Environmental Health Sciences, Mailman School of Public Health, Columbia University, New York NY 10032

[3]Department of Public Health, Chang Gung University, Tao-Yuan 333, Taiwan

[4]Institute of Environment Health, China Medical University College of Public Health, Taichung 404, Taiwan

[5]Colorectal Section, Chang Gung Memorial Hospital, Taipei 105, Taiwan

Abstract

Endothelial derived nitric oxide, which is produced by endothelial nitric oxide synthase (eNOS), may play an important role in colorectal carcinogenesis. However, the putative contribution of common *eNOS* genetic polymorphisms to colorectal cancer risk remains unknown. We genotyped 3 polymorphisms of *eNOS* (T-786C, G894T, and intron4b/a) in 727 colorectal adenocarcinoma cases and 736 age- and sex-matched healthy controls in Taiwan. Genotypes of the T-786C and G894T polymorphisms were determined by fluorescence polarization assays and the 27-bp variable number of tandem repeat (VNTR) polymorphism in intron 4 (intron4b/a) was analyzed by PCR. Logistic regression was used to calculate odds ratios (ORs) and 95% confidence intervals (CIs). Among younger participants (≤ 60 yrs), the intron4a variant genotype was associated with a significantly increased risk of colorectal cancer, compared with the intron4bb genotype (OR = 1.60, 95% CI = 1.04-2.46). In addition, those young individuals bearing a greater number of high-risk genotypes (OR > 1, i.e. CT+TT for T-786C, ba+aa for intron4b/a, and GG for G894T) of *eNOS* had a higher colorectal cancer risk ($P_{trend} = 0.039$). Compared with younger individuals without any putative high-risk genotypes, those with three high-risk genotypes had a significantly greater cancer risk (OR = 1.89, 95% CI = 1.04-3.43). Our results suggest that the *eNOS* intron4b/a polymorphism may contribute to early-onset colorectal cancer risk in the Taiwanese population.

Keywords

colorectal cancer; endothelial nitric oxide synthase; polymorphisms; early-onset

[*]Requests for reprints and correspondence: Chih-Ching Yeh, Ph.D. Department of Health Risk Management, China Medical University 91 Hsueh-Shih Road, Taichung, Taiwan 40402 TEL: 886-4-2205-3366 ext 6505 FAX: 886-4-2207-0429 d88844003@ntu.edu.tw ccyeh@mail.cmu.edu.tw.

Novelty and impact of our paper: This study is the first to evaluate *eNOS* polymorphisms in relation to colorectal cancer risk in a Chinese population. Our results suggest that an intron 4 VNTR polymorphism of *eNOS* may contribute to risk of colorectal cancer among subjects younger than 60 years of age.

Introduction

The free radical nitric oxide (NO) is synthesized during the conversion of L-arginine to L-citrulline by nitric oxide synthase (NOS) isoforms, i.e., endothelial (e), inducible (i), and neuronal (n) NOS.1 NO has an impact on multiple physiologic and pathophysiologic processes, including vasodilatation, neuronal transmission, smooth muscle relaxation, immunity, and carcinogenesis.2–4 Data with respect to the role of NO in tumor promotion or inhibition are conflicting.4 Studies suggest that overproduction of NO can cause DNA damage and inhibit DNA repair5 and that NO also promotes tumor angiogenesis and metastasis.6 However, other studies suggest that NO may protect cells from DNA damage by upregulating p53,7 poly(ADP-ribose) polymerase (PARP)8 and DNA-dependent protein kinase (DNA-PK).9. Moreover, NO may exert antitumor effects with the reduction of tumor cell adhesion to endothelium,10 activation of antioxidant defenses11 and induction of apoptosis.12

Studies have demonstrated elevated NOS expression and activity in ovarian cancer,13 breast cancer14 and central nervous system tumors15. However, expression of NOS in colorectal cancer has not been extensively investigated and results are controversial. Chhatwal et al.16 found aberrant expression of NOS activity in colonic polyps and carcinomas compared to normal mucosa. Three studies also found diminished expression of eNOS in colorectal tumor tissues,17–19 whereas one study showed enhanced expression of eNOS in tumor microvessels.17 This discrepancy may be due to genetic variation in *eNOS*.

The gene encoding eNOS is located on chromosome 7q36 in humans.20 A single nucleotide polymorphism (SNP), T-786C, was identified in the 5' flanking region involving a substitution of thymine (T) to cytosine (C) at a locus 786 base pairs upstream.21 Another common variant of *eNOS* with a G to T transversion at nucleotide position 894 (G894T) leading to a change in amino acid at 298 (Glu298Asp) has been reported,22 as well as a 27-bp variable number of tandem repeats (VNTR) polymorphism in intron 4 (intron4b/a).23 Several epidemiological studies evaluated the *eNOS* polymorphisms in human cancers, but the results are conflicting.24–29 Therefore, in the present study, we evaluated the relationship between three *eNOS* polymorphisms and the development of colorectal cancer in a Chinese population.

Materials and methods

Subjects

A detailed description of the specific characteristics of the study participants have been published previously.30 In brief, participants were recruited from one teaching hospital in Linkou, Taiwan (Chang Gung Memorial Hospital), between January 1995 and January 1999. Ninety-five percent of eligible participants agreed to participate in the study. The colorectal adenocarcinoma cancer cases (n=776) were newly diagnosed and histologically confirmed. Patients suffering from familial adenomatous polyposis, hereditary nonpolyposis colorectal cancer, other colorectal diseases, or other related malignancies or with a family history of colorectal cancer were excluded. Seven hundred and twenty-seven of the original 776 cases were finally included in this study. Seven hundred and forty-seven age- (same age) and sex-matched controls were recruited from the Physical Check-Up Department during the same period. All participating controls received comprehensive health examinations including colonoscopies. After excluding individuals diagnosed with other colorectal diseases, a history of other cancers or the existence of a family history of colorectal cancer, 736 controls were finally included in this study. More than 80% of participants were living in north Taiwan including Keelung city, Taipei city, Taipei county, Taoyuan county and Hsinchu county.

Data collection

With informed consent, professional nurses interviewed all participants in the hospital prior to the surgery for cases and colonoscopy for controls. A standardized interview was conducted using a structured questionnaire covering socio-demographic characteristics, lifestyle factors (including physical activities, cigarette and alcohol use, and coffee intake), and dietary factors. The reliability of lifestyle and dietary variables was 0.92 (standardized Cronbach's alpha). Dietary consumption was ascertained by frequency categorized into six levels ranging from never, less than once a month, 1-3 times a month, once a week, 2-3 times a week to almost everyday. Cigarette and alcohol use were evaluated by both the amount and duration. Total cigarette consumption in pack-years was estimated using daily consumption multiplied by years of use.

Laboratory analysis

Following collection of questionnaire data, 10ml of venous blood was collected and shipped to Chang Gung University the same or next day for laboratory analyses. The buffy coat from whole blood was isolated the same day and kept at -80°C until DNA isolation. All sample processing and laboratory assays were performed by laboratory personnel blinded to the subject's disease and exposure status.

The -786 T→C (rs2070744) and 894 G→T (rs1799983) SNPs in *eNOS* were determined using template-directed primer extension with detection of incorporated nucleotides by fluorescence polarization in a 96 microwell-based format essentially as described.31, 32 Master DNA 96 well plates containing 10 ng/μl were used to make replica plates containing 25 ng DNA/well. First, genomic DNA was amplified by PCR using the appropriate primers (forward 5'-GTG TAC CCC ACC TGC ATT CT-3' and reverse 5'-GGG ACA CAA AAG AGC AGG AA-3' for the T-786C SNP; forward 5'-AAG GCA GGA GAC AGT GGA TG-3' and reverse 5'-CAG TCA ATC CCT TTG GTG CT-3' for the G894T SNP). Conditions for amplification were 0.3 μl (3 pmol/μl) forward and reverse primers, 1 μl 10× PCR buffer, 0.05 μl (0.25 unit) Taq polymerase (Roche Molecular Biochemicals, Indianapolis, IN), 0.15 μl (1.5 mM) dNTPs (Roche) and 5.7 μl water. Specific thermocyclic conditions were used for amplifying the T-786C SNP (denaturation at 94°C for 5 min was followed by 35 cycles of 94°C for 30 s, 66°C for 45 s and 72°C for 30 s, followed by 5 min at 72°C for final extension) and the G894T SNP (denaturation at 94°C for 5 min was followed by 35 cycles of 94°C for 30 s, 67.5°C for 45 s and 72°C for 30 s, followed by 5 min at 72° for final extension). After PCR amplification (200 bp for T-786C and 246 bp for G894T), the primers and dNTPs were digested with 1 unit of shrimp alkaline phosphatase (1 μ/μl, Roche) after addition of 1 μl of 10× buffer and 1 unit E. coli exonuclease I (10 u/μl, United States Biochemical, Cleveland, OH) and 7.9 μl of water for 45 min at 37°C followed by heating at 95°C for 15 min. Then, single nucleotide extension was carried out in the presence of the appropriate allele specific ddNTPs differentially fluorescence-labeled with either R110 or TAMRA (Acycloprime FP SNP Detection kits purchased from Perkin Elmer Life Sciences, Boston, MA) (G/A for T-786C and G/T for G894T). Two μl of reaction mixture was added 0.025 μl Acycloprime enzyme, 0.5 μl Terminator mix, 2 μl 10× reaction buffer, 0.9 μl extension primer (9 pmol/μl) and 14.575 μl water. Extension was carried out by heating at 95°C for 2 min followed by 30 cycles of 95°C for 15 s and 52°C for 30 s for T-786C and by 50 cycles of 95°C for 15 s and 55°C for 30 s for G894T. For the single nucleotide extension reaction, both forward and reverse probes were tested to select the optimum based on clear signal differences. The optimums chosen were the reverse extension probe: 5'-GCT GAG GCA GGG TCA GCC-3' for T-786C and the forward extension probe: 5'-GCT GCA GGC CCC AGA TGA-3' for G894T. Finally, the plates were read on a Perkin Elmer Victor instrument. The 27-bp intron 4 VNTR polymorphism was analyzed using primers (forward, 5'-AGG CCC TAT GGT AGT GCC TT-3'; reverse, 5'-TCT CTT AGT GCT GTG GTC

AC-3') as published.23 A 20 μl reaction volume was used for each PCR, including 2 μl (4 pmol/μl) of each primer, 0.2 μl (2.0 mM) dNTPs (Roche), 0.6 U of Taq polymerase (Roche), and 2 μl 10× PCR buffer together with 25 ng DNA. The thermocycling procedure consisted of initial denaturation at 94°C for 5 min, 35 cycles of denaturation at 94°C for 30 s, annealing at 63°C for 45 s, extension at 72°C for 1 min, and a final extension at 72°C for 5 min. The PCR products were separated by 2% agarose gel electrophoresis and visualized by ethidium bromide staining. In addition to the wild-type allele (4b), which has five 27-bp repeats, we detected two variants for the intron4b/a polymorphism. The variants 4a and 4c, which correspond to four and six 27-bp repeats, respectively, have been observed previously.33

Statistical analysis

The consumption of each food item was scored from 1 for “almost everyday” to 6 for “never”. Dietary factors were stratified into “Low”, “Medium” and “High” using the tertile of the score distribution among the controls. Alcohol intake and total cigarette consumption were also categorized into three strata. The non-users of alcohol and cigarette were treated as references and ever-users were divided into two groups according to the distribution in the controls. The detailed description for dietary factors for the participants were described previously.30

Hardy-Weinberg equilibrium and LD (D') of the SNPs were assessed using THESIAS.34 Haplotypes were inferred using THESIAS excluding individuals with missing values. Haplotype effects were tested for all possible haplotypes in an additive model and are shown as difference from the most common haplotype. Distributions of the *eNOS* genotypes and other exposures and covariates were compared between cases and controls using Chi-Square tests. The participants in this study were classified as carriers of the variant alleles vs. non-carriers of the variant alleles for all *eNOS* SNPs. The association between genotypes and risk for colorectal cancer was examined by odds ratios (ORs) and associated 95% confidence intervals (CIs) estimated through maximum likelihood estimates from multiple unconditional logistic regression models with the matching factors (age and gender) included in the model.35 The potential confounders (such as physical activity, cigarette smoking, alcohol use, coffee intake, and consumption of meat, vegetable/fruit and fish/shrimp) included in the model were based on the initial bivariate assessments to determine if they had an impact >10% on the effects estimates. However, none of these factors met the inclusion criteria. Because exposure to tobacco smoke causes an irreversible inhibition of eNOS activity and a reduction in eNOS protein and mRNA levels,36 cigarette smoking (pack-year) was forced into the multiple logistic regression models. Combined effects of the high-risk genotypes (genotypes with ORs more than 1, i.e. GG for G894T, TC+TT for T-786C and ba+aa for intron4b/a) were estimated using indicator variables to denote different combinations of joint exposures. Based on the multiplicative scale, the likelihood ratio test was used to evaluate the interaction between these genotypes on the risk for colorectal cancer. ORs for cancer risk differences by tumor sites (colon and rectum), age groups (≤ 60 years old and > 60 years old) and environmental factors (such as physical activity, cigarette smoking, alcohol use, coffee intake, and consumption of meat, vegetable/fruit and fish/shrimp) were estimated using stratified analyses with the low-risk genotypes as the reference in each stratum. All analyses were performed using the SAS statistical package (version 8.1 for windows; SAS Institute, Inc., Cary, NC, USA) and all tests were two-sided.

Results

More men (56%) than women participated in this study (Table 1). The mean age for both groups was 60 years. Colorectal cancer cases had higher alcohol and meat consumption but lower vegetable/fruit and fish/shrimp consumption than their matched controls ($p<0.05$).

Detailed analyses of the sociodemographic characteristics and potential risk factors associated with colorectal cancer amongst the study population have been published previously.30

The genotypic distributions of the three *eNOS* SNPs for both cancer cases and controls are shown in Table 2. The frequencies for the T-786C C allele, intron4b/a 4a allele and 4c allele, and G894T T allele amongst the controls were 8.82%, 8.46%, 0.68% and 11.2%, respectively. These genotype frequencies are in Hardy-Weinberg equilibrium. None of the associations between *eNOS* genotypes and colorectal cancer risk were significant. Because of the low frequency of the 4c variant and its protective role in risk of colorectal cancer (OR = 0.85; 95% CI = 0.33-2.18), we grouped it with the wild-type 4b allele for further data analyses.

Of 8 possible haplotypes defined by the three SNPs, 6 were observed in our study population. Of these haplotypes, three occurred at frequencies >5% and accounted for 98% of the observed haplotypes (Table 3). The most common haplotype (T4bG), which served as reference haplotype in our analyses, was present in 80% of our study population. None of the haplotypes was associated with colorectal cancer risk. Linkage analysis between each pair-wise combination showed significant associations (all $p<0.001$). The D' values for the -786C and 4a, 4a and 894G, and -786C and 894G pairs were 0.91, -1, and -0.79, respectively.

Stratified analyses by tumor sites and age groups for risk of colorectal cancer associated with the *eNOS* polymorphisms are shown in Table 4. Data stratified by age at diagnosis showed that the variant genotypes in intron4b/a (ba+aa) were associated with a 69 % increase in colorectal cancer risk among subjects less than 60 years old (multivariate OR = 1.69, 95% CI = 1.08-2.64), compared with the bb+bc genotypes. However, no significant associations between the T-786C and G894T polymorphisms and risk of colorectal cancer were found. In addition, none of the *eNOS* polymorphisms was significantly associated with colon cancer or rectal cancer, respectively. Furthermore, we investigated gender differences in the impact of *eNOS* polymorphisms on colorectal cancer risk, but no significant associations were found. Nevertheless, we did find that the increased risk of intron4a variant genotypes among younger participants was much higher in men (OR = 2.16, 95% CI = 1.21-3.85) than in women (OR = 1.26, 95% CI = 0.61-2.61).

Table 5 presents results on adjusted risk estimates for the combined effect of the three *eNOS* SNPs on colorectal cancer. As compared to non-carriers of any high-risk genotypes, the risk for carriers of three high-risk genotypes for these SNPs was 1.89-fold among subjects ≤ 60 years of age (95% CI = 1.04-3.43, $P_{trend} = 0.039$). This risk was similar to that of the combination of subjects carrying two and more high-risk genotypes (OR = 1.88; 95% CI = 1.07-3.31, $P_{trend} = 0.028$)(data not shown). Risks for colorectal cancer were not significantly different by tumor site.

Furthermore, we assessed possible interactions between *eNOS* polymorphisms and environmental factors on colorectal cancer risk. There was no significant interaction between *eNOS* genotypes and selected risk factors (data not shown).

Discussion

In this large hospital-based, case-control study in Taiwan, we found an increased risk for colorectal cancer among carriers of variant genotypes of *eNOS* intron4b/a (ba+aa) among younger subjects ≤ 60 years of age. More importantly, this direct association with colorectal cancer was apparent for carriers of three high-risk genotypes (i.e. GG for G894T, CT+TT for T-786C, and ba+aa genotype for intron4b/a).

To our knowledge, our study is the first to evaluate the *eNOS* polymorphisms in relation to colorectal cancer risk in a Chinese population. Although no association between *eNOS* polymorphisms and colorectal cancer risk was found in a study of a Spanish population, it is plausible that the enzyme could play a role in colorectal cancer etiology.37 A recent study found that high expression of eNOS in peritumoral microvessels protects against colorectal tumor metastasis38 and increased *in situ* expression of eNOS in colorectal tumor tissue.39 Moreover, studies have shown that *eNOS* genotypes may be associated with the development of lung,29 prostate,25 and breast cancer.26, 40, 41

A smoking-dependent increase in coronary risk in *eNOS* intron 4aa homozygotes was first reported in Australia, and this genotype was actually associated with basal NO production in blood vessels.23, 42 Several studies have demonstrated positive correlations between the presence of the *eNOS* intron4a allele and the development of prostate cancer,25 with the metastasis of prostate cancer43 and ovarian cancer,27 and with worse prognosis of vulva cancer.28 However, a negative correlation with lung cancer was also observed,29 while others have not found *eNOS* intron4b/a polymorphism to be a predisposing factor to cancer risk.26, 40 Recently, Zhang et al.44 have shown that cells containing five or ten 27-bp repeats produced higher levels of 27-bp sir-RNA and lower levels of eNOS mRNA than cells with four 27-bp repeats. These results support our data showing that the *eNOS* intron4a allele is associated with a predisposition to early-onset colorectal cancer among a Chinese population in Taiwan.

Although the mechanism responsible for the association of the *eNOS* G894T polymorphism to endothelial dysfunction is still largely unknown,45 various groups have determined correlations of genotypes with disease. While one study showed the T allele associated with breast cancer risk,26 others found the G allele to be a predisposing factor to invasiveness and radiotoxicity for breast cancer,46, 47 as well as occurrence and metastasis of prostate cancer.48, 49 However, no association has been found in patients with non-Hodgkin's lymphoma,50, 51 prostate cancer,25, 43 breast cancer,24, 40, 41, 52–55 colorectal cancer,37 ovarian cancer,27 and vulvar cancer.28 In the present study, the lack of an association between the *eNOS* G894T polymorphism and risk of colorectal cancer is consistent with most studies.

The T-786C polymorphism in the promoter region of *eNOS* was initially observed in patients with coronary vasospasm, and this substitution results in the inhibition of eNOS promoter activity,21 leading to reduced NO production in blood vessels and endothelial dysfunction. In this study, we observed a lack of correlation between the *eNOS* T-786C polymorphism and colorectal cancer risk, which is consistent with several previous studies.24, 37, 47, 51, 54 In contrast, one study found that the -786C variant genotype was a predisposing factor for breast cancer in non-Hispanic white women age younger than 55 years.40 The discrepancy between studies for *eNOS* polymorphisms and cancer risks could be the result of differences in disease, ethnicity, and sample size. Distinct biological pathways during disease pathogenesis or ethnic differences in allele frequency for the polymorphisms may partly explain these discrepancies. However, study power should also be considered when no association is present. According to the case/control ratio, sample size and the allele frequencies, we have 80% power to detect ORs > 1.61 for T-786C, > 1.59 for Intron4b/a and > 1.54 for G894.

After stratification by age at diagnosis, the effects of potential at-risk genotypes were higher in the younger than in the older group, particularly in men. Consistent with the present study, our previous studies with the same subjects found that genetic associations with colorectal cancer risk were significantly elevated in younger subjects.56, 57 Most studies of the association between polymorphisms of biotransformation and DNA-repair genes and

cancer risk found that younger subjects had higher susceptibility.[58-61] It is possible that individuals with insufficient capacity for biotransformation, DNA repair, and antioxidant activity may develop tumors at a younger age than those with efficient metabolic capacity. Among younger men the increased colorectal cancer risk of intron4a variant genotypes may be related to the beneficial effect of estrogen which stimulates expression of endothelial NO synthase,[62, 63] and regulates basal endothelial NO release.[64] Therefore, increased risk of colorectal cancer among men is biologically plausible.

We also found that elevated cancer risk was associated with the three high-risk genotypes of *eNOS*. Although the exact molecular mechanism by which multiple *eNOS* variants work together to affect the risk of colorectal cancer is unknown, a recent study showed that the circulating concentration of NO products was associated with the presence of some *eNOS* haplotypes in healthy nonsmoking men.[65] It remains to be determined whether these polymorphic variants have a joint effect on gene expression or mRNA stability and whether they are in LD with other untyped functional variants of other colorectal cancer-related susceptibility genes.

In conclusion, in this hospital-based case-control study of patients with sporadic colorectal cancer, we found a significant association between the *eNOS* intron4b/a polymorphism and risk of colorectal cancer in Taiwanese younger than 60 years of age, especially in those individuals carrying three high-risk *eNOS* genotypes. Our results suggest that variant genotypes of *eNOS* may contribute to risk of sporadic colorectal cancer, particularly in young subjects but replicated studies are needed to verify the findings.

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TABLE I

COMPARISON OF LIFESTYLE, DIET AND GENOTYPES BETWEEN CASES AND CONTROLS

	Cases (n=727)	Controls (n=736)	
Variable	**No. (%)**	**No. (%)**	***P*[4]**
Male	410(56.4)	409(55.6)	0.75
Mean age (SD), years	60.3(12.8)	60.7(13.0)	0.64
Cigarette (SD), pack-year	9.81(17.7)	9.54(16.6)	0.77
Alcohol (SD), year	9.07(15.9)	8.33(15.1)	0.37
Meat consumption [1]			<0.001
Low	230(31.6)	341(46.3)	
Medium	147(20.2)	202(27.5)	
High	350(48.1)	193(26.2)	
Vegetable/fruit consumption [2]			<0.001
Low	179(24.6)	103(14.0)	
Medium	175(24.1)	170(23.1)	
High	373(51.3)	463(62.9)	
Fish/shrimp consumption [3]			<0.001
Low	148(22.2)	137(18.6)	
Medium	221(33.1)	176(23.9)	
High	299(44.8)	423(57.5)	

[1] The cut-point of score for meat was ≤3, 4, ≥5

[2] The cut-point of score for vegetable/fruit was ≤2, 3, ≥4

[3] The cut-point of score for fish/shrimp was 1, 2, ≥3

[4] Continuous variables were tested by t-test, categorical variables were tested by Chi-square test

TABLE II

eNOS POLYMORPHISMS AND RISK OF COLORECTAL CANCER

	Cases (n=727)	Controls (n=736)		
Genotypes	**No. (%)**	**No. (%)**	**OR (95%CI)**	**OR (95%CI)** [1]
T-786C				
TT	566(82.9)	604(83.2)	1.00	1.00
TC	110(16.1)	116(16.0)	1.01(0.76-1.35)	1.01(0.75-1.35)
CC	7(1.0)	6(0.8)	1.25(0.42-3.73)	1.63(0.51-5.17)
P_{trend}			0.81	0.69
TC+CC	117(17.1)	122(16.8)	1.02(0.78-1.35)	1.03(0.78-1.37)
Intron4b/a				
bb	591(82.0)	605(82.5)	1.00	1.00
ba	115(16.0)	112(15.3)	1.05(0.79-1.40)	1.06(0.80-1.41)
aa	7(1.0)	6(0.8)	1.19(0.40-3.57)	1.55(0.49-4.93)
bc	8(1.1)	10(1.4)	0.82(0.32-2.09)	0.85(0.33-2.18)
P_{trend}			0.94	0.76
bb+bc	599(83.1)	615(83.9)	1.00	1.00
ba+aa	122(16.9)	118(16.1)	1.06(0.81-1.40)	1.08(0.82-1.44)
G894T				
GG	568(80.9)	575(79.0)	1.00	1.00
GT	124(17.7)	143(19.6)	0.88(0.67-1.15)	0.88(0.67-1.15)
TT	10(1.4)	10(1.4)	1.01(0.42-2.45)	1.08(0.44-2.61)
P_{trend}			0.42	0.47
GT+TT	134(19.1)	153(21.0)	0.89(0.68-1.15)	0.89(0.69-1.16)

[1] Adjusted for age, sex and smoking

TABLE III

ESTIMATED *eNOS* HAPLOTYPE FREQUENCIES IN THE CASES AND CONTROLS AND THEIR EFFECTS ON RISK OF COLORECTAL CANCER

Haplotype [1]			Cases (1454 alleles)	Controls (1472 alleles)	
T-786C	Intron4b/a [2]	G894T	No. (%)	No. (%)	OR (95%CI) [3]
T	4b	G	1164(80.11)	1157(79.55)	1.00
T	4a	G	2(0.15)	6(0.42)	0.37(0.07-1.91)
C	4b	G	12(0.80)	9(0.54)	1.47(0.56-3.87)
C	4a	G	126(8.69)	117(8.05)	1.04(0.80-1.36)
T	4b	T	145(9.96)	159(10.96)	0.90(0.71-1.14)
C	4b	T	4(0.28)	3(0.22)	1.33(0.26-6.88)

[1] The LD (D') values for the -786C and 4a, 4a and 894G, and -786C and 894G pairs were 0.91, -1, and -0.79, respectively.

[2] 4b allele included 4c allele

[3] Adjusted for age, sex and smoking

TABLE IV

eNOS POLYMORPHISMS AND RISK OF COLORECTAL CANCER BY AGE AT DIAGNOSIS AND TUMOR SITE

	T-786C		Intron4b/a		G894T	
	TT	**TC+CC**	**bb+bc**	**ba+aa**	**GT+TT**	**GG**
Total population						
Cases/Controls	566/604	117/122	599/615	122/118	134/153	568/575
OR (95%CI) [1]	1.00	1.03(0.78-1.37)	1.00	1.08(0.82-1.44)	1.00	1.12(0.86-1.46)
Age at diagnosis						
≤60 years						
Cases/Controls	250/273	56/42	263/279	59/39	54/69	258/248
OR (95%CI) [1]	1.00	1.45(0.93-2.27)	1.00	1.69(1.08-2.64) [2]	1.00	1.31(0.87-1.96)
>60 years						
Cases/Controls	316/331	61/80	336/336	63/79	80/84	310/327
OR (95%CI) [1]	1.00	0.81(0.56-1.18)	1.00	0.80(0.55-1.16)	1.00	1.00(0.71-1.42)
Tumor site						
Colon						
Cases/Controls	272/604	58/122	291/615	57/118	57/153	282/575
OR (95%CI) [1]	1.00	1.02(0.72-1.46)	1.00	1.03(0.73-1.46)	1.00	1.36(0.96-1.92)
Rectum						
Cases/Controls	294/604	59/122	308/615	65/118	77/153	286/575
OR (95%CI) [1]	1.00	1.05(0.74-1.48)	1.00	1.14(0.81-1.60)	1.00	0.96(0.71-1.32)

[1] ORs and 95% CIs were estimated from multivariate unconditional logistic regressions controlling for age, sex and smoking

[2] $p<0.05$

TABLE V

NUMBER OF HIGH-RISK GENOTYPES OF *eNOS* POLYMORPHISMS AND COLORECTAL CANCER RISK BY AGE AT DIAGNOSIS AND TUMOR SITE

	Number of high-risk genotypes [2]				
	0	**1**	**2**	**3**	**P trend**
Total population					
Cases/Controls	116/135	447/465	22/27	92/97	
OR (95% CI) [1]	1.00	1.11(0.84-1.48)	0.86(0.46-1.63)	1.13(0.77-1.66)	0.70
Age at diagnosis					
≤60 years					
Cases/Controls	44/64	202/208	13/8	43/34	
OR (95% CI) [1]	1.00	1.37(0.89-2.11)	1.86(0.68-5.08)	1.89(1.04-3.43) [3]	0.035
>60 years					
Cases/Controls	72/71	245/257	9/19	49/63	
OR (95% CI) [1]	1.00	0.95(0.65-1.39)	0.50(0.21-1.19)	0.78(0.47-1.29)	0.19
Tumor site					
Colon					
Cases/Controls	51/135	221/465	9/27	46/97	
OR (95% CI) [1]	1.00	1.29(0.89-1.86)	0.61(0.24-1.58)	1.31(0.81-2.12)	0.55
Rectum					
Cases/Controls	65/135	226/465	13/27	46/97	
OR (95% CI) [1]	1.00	0.99(0.70-1.39)	1.06(0.51-2.19)	1.01(0.63-1.62)	0.92

[1] ORs and 95% CIs were estimated from unconditional logistic regressions, controlling for age, sex and smoking

[2] High-risk genotypes were CT+TT for T-786C, ba+aa for intron4b/a, and GG for G894T

[3] $p<0.05$