A New Single Nucleotide Polymorphism in the *Insulin-Like Growth Factor I* Regulatory Region Associates with Colorectal Cancer Risk in Singapore Chinese

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Abstract

Elevated levels of plasma insulin-like growth factor I (IGF-I) are a potential risk factor for several cancers, including colorectal cancer. Physiologic levels of plasma IGF-I vary greatly; this variation may be in part genetically determined. We identified two single nucleotide polymorphisms (SNP) in perfect linkage disequilibrium with each other and in partial linkage disequilibrium with a previously studied cytosine-adenine microsatellite [*-969(CA)$_n$*]. We investigated one of the SNPs, *-533T/C,* and the *969(CA)$_n$* in relation to the risk of colorectal cancer in a case-control study nested within a cohort of Singapore Chinese (cases/controls = 290:873). The *(CA)$_{21}$* allele, rather than the previously implicated *(CA)$_{19}$* allele, was associated with a reduced risk of colorectal cancer (odds ratio for 21/21 versus all other genotypes, 0.48; 95% confidence interval, 0.28-0.84). For the *-533C/T* SNP, persons carrying one or more copies of the C allele had a decreased in risk of colorectal cancer compared with noncarriers (odds ratio for *CC/CT* versus *TT*, 0.58; 95% confidence interval, 0.41-0.82). This association was specific for colon, as opposed to rectal cancer and was modified by age. We also examined a functional insulin-like growth factor binding protein (IGFBP3) promoter SNP, *-202 A/C*, previously reported to predict serum IGFBP3 levels. Although we were able to confirm this genotype-phenotype association, the *-202A/C IGFBP3* SNP was not significantly associated with colorectal cancer risk. In conclusion, we report a novel SNP in the *IGF-I* regulatory region that is associated with colorectal cancer risk. (Cancer Epidemiol Biomarkers Prev 2005;14(1):144–51)

Introduction

Insulin-like growth factor I (IGF-I) is a cellular survival factor implicated in various neoplasia (reviewed in refs. 1-3), including colorectal cancer (reviewed in refs. 4, 5). IGF-I is strongly mitogenic in colon cancer cell lines (6-8) as well as antiapoptotic in colonic epithelial cells (9) and cancer cell lines (10, 11). These potential carcinogenic IGF-I effects may be, in part, exerted through hepatic-derived circulating levels (reviewed in ref. 12). More than 75% of serum IGF-I circulates as complexes with its predominant binding protein, IGFBP3 (13). The sequestering of IGF-I by IGFBP3 modulates IGF-I bioavailability. In addition, many *in vitro* studies indicate that independent of IGF-I, IGFBP3 inhibits replication and promotes apoptosis (14, 15).

Circulating IGF-I promotes colonic carcinogenesis, as evidenced by animal models and human studies. In mice, circulating levels of IGF-I regulate colon cancer growth and metastasis (16). Among human prospective studies, although two studies failed to find an association, (17, 18), two studies (19, 20) found a clear association between elevated plasma IGF-I (adjusted for IGFBP3 levels) and increased colorectal or colon cancer risk, and two found positive associations of borderline significance (21, 22).

Twin studies suggest that circulating IGF-I and IGFBP3 levels are, in part, genetically determined (23, 24). The only identified candidate polymorphism for IGF-I levels is a cytosine-adenine (CA) microsatellite polymorphism, 969 kbp upstream from the *IGF-I* transcription start site (25, 26), having 15 to 23 CA repeats in the Caucasian population. An initial small study (n = 116) reported lower circulating IGF-I levels among individuals homozygous for the *(CA)$_{19}$* allele versus individuals with all other genotypes (129 versus 154 ng/mL, P = 0.03; ref. 27). For IGFBP3, alleles of an *IGFBP3* promoter region single nucleotide polymorphisms (SNP, *-202A/C)* differ in transcriptional capacities (28) and plasma IGFBP3 levels consistently correlate with genotype in the predicted direction (28-30).

In this study, we examine polymorphisms in the *IGF-I* and *IGFBP3* genes in relation to plasma levels of the respective gene products and in relation to colorectal cancer risk in a case-control study nested within a prospective cohort of 63,257 Singapore Chinese.

Materials and Methods

Study Population. The subjects were participants of the Singapore Chinese Health Study, a population-based, prospective investigation of diet and cancer risk. Between April 1993 and December 1998, we recruited 63,257 Chinese men and women from two major dialect groups in Singapore (Hokkien and Cantonese) who originated from geographically contiguous regions in Southern China: the Hokkiens from the southern part of Fujian Province and the Cantonese from the central region of Guangdong. Subjects were between the ages of 45 and 74 years and resided in government housing estates. Eighty-six percent of the Singapore population lived in such facilities. Eighty-five percent of eligible subjects were enrolled. The gender-dialect breakdown is as follows: 15,617 (25%) Hokkien men, 18,356 (29%) Hokkien women, 12,342 (19%) Cantonese men, and 16,942 (27%) Cantonese women. Cohort members were comparable with the general

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Chinese population in Singapore with respect to two factors, level of education and smoking status, for which governmental data were available. According to a 1992 national survey, 31% of Chinese men ages 45 to 69 years were current smokers, and they smoked an average of 17 cigarettes per day. The corresponding figures based on male cohort members are 32%, and 16, respectively. The 1990 Singapore Census data show that 74% of Chinese ages 45 to 74 years achieved no more than a primary school education. The corresponding figure from our cohort is 72%.

Each subject completed a structured questionnaire given in person by a trained interviewer. Current diet was assessed using a validated 165-item, semiquantitative food frequency questionnaire. Personal intakes of 96 nutritive/nonnutritive dietary components were computed using the Singapore Food Composition Table (31). Apart from dietary history, the questionnaire also elicited information on lifetime tobacco use, usual physical activity, medical history, family history of cancer, and menstrual and reproductive history (women only).

A 3% random sample of study participants and all incident colorectal cancer cases were contacted for biospecimens (blood or buccal cells and single-void urine specimens) collection as previously detailed (32). Briefly, between April 1994 and July 1999, of an estimated 1898 cohort participants contacted, blood (n = 908) or buccal cells (n = 286) were collected from 1,194 subjects, representing a participation rate of 63%. Additionally, beginning in January 2000, biospecimen collection was extended to all surviving members of the cohort and is expected to be complete by May 2004.

Controls. Of the 1,194 randomly sampled subjects who donated biospecimens (908 blood and 286 buccal samples), 13 had developed incident colorectal cancer by April 30, 2002. The 895 (908 minus 13) cohort subjects for whom blood was drawn and who were free of a history of colorectal cancer on April 30, 2002 comprised the comparison group for this case-control analysis.

Cases. Incident colorectal cancer cases were identified by record linkage with the population-based Singapore Cancer Registry (33). The Singapore Cancer Registry was established in 1968 and since then, has been continuously included in the ''Cancer Incidence in Five Continents'' serial publications by the IARC in Lyon, France. Migration out of Singapore, especially among housing estate residents, has been negligible since inception of the cohort (Department of Statistics, Singapore Ministry of Trade and Industry, 2001).

As of April 30, 2002, 592 cases of incident colorectal cancer (International Classification of Diseases for Oncology C18-C20) had developed among cohort members. Blood (n = 228) or buccal (n = 84) specimens were available on 53% (312 of 592) of the colorectal cancer cases. Of the 312 available biospecimens, 50 were collected prediagnostically (13 were from the 3% random sample and 37 were from the expanded biospecimen collection after January 2000, described above). Of the 262 (312 minus 50) cases that were collected postdiagnostically, median time from diagnosis to blood draw was 9.5 months.

Participants who donated biospecimens were comparable to nondonors with respect to body mass index [body mass index expressed as weight (kg)/height (m^2)], family history of colorectal cancer, smoking history (never, ex-smoker, and current smoker) and physical activity (moderate activity: 0, 0.5-3, >4 hours/wk). Compared with those who had no formal education, a higher proportion of subjects who had primary school or higher education donated a blood or buccal cell specimen (56% versus 46%). More male cases donated specimens (56%) compared with females (49%), and more Cantonese (57%) donated specimens compared with Hokkiens (50%). The average age at diagnosis of cancer was comparable between cases with and without specimens (65 versus 66 years).

Histologic information on each colorectal cancer diagnosis was confirmed by reviewing the pathology report. The cases included one carcinoid tumor, two *in situ* cases, and three with unknown histologies but ascertained by death records and clinical evidence. Because these cases are unlikely to differ etiologically from carcinomas and because excluding these cases did not alter the results, these six cases were retained in our analyses.

The study protocol was approved by the Institutional Review Boards of the University of Southern California and the National University of Singapore. All participants gave written, informed consent at the time of recruitment and at collection of blood (or buccal cells) and urine specimens.

Laboratory Methods. DNA was purified from buffy coats of peripheral blood and from buccal cell samples using standard, published methods (34). All three genotype assays described below were done with case-control status blinded to the laboratory technician. Six percent of the samples were replicated as blind duplicates distributed across all genotyping batches. At least three negative controls (water blanks) were included on each PCR plate. Genotyping failure rate was <2% for each of the two *IGF-I* loci and 6% for the *IGFBP3* SNP. Samples with genotyping failure for one or both of the *IGF-I* loci were excluded, leaving 290 cases and 873 controls. In addition, in Table 6 and Fig. 2, an additional 18 cases and 52 controls having missing *IGFBP3* genotypes were excluded.

IGF-I Promoter Region Sequencing. To identify additional common polymorphisms in the *IGF-I* 5′ untranslated region, the 1 kb region spanning the (CA) repeats and the IGF-I translation start site was resequenced for 60 Singapore Chinese and 96 non-Hispanic White, Black, and Hispanic White subjects (192 total chromosomes) from the Hawaii/Los Angeles Multiethnic Cohort (35). Two overlapping segments were amplified using two sets of primers: 5′-AATTGTTTGCCCCCCA-3′ and 5′-GAACCCTGTCAC-3′ and 5′-CCCATCCCCCATATTCCT-3′ and 5′-GTGCTG-CTTTTGTGATTTC-3′. Sequencing was carried out using an ABI Prism 3700 DNA Sequencer (PE Biosystems, Foster City, CA).

Genotyping

IGF-I-969(CA)$_n$. The genomic region containing the CA repeat was PCR amplified using previously described oligonucleotides (27). The ^{33}P-labeled PCR products were separated on 6% denaturing polyacrylamide gels and visualized by autoradiography. Genotypes were scored by comparison with controls that had genotype confirmed by sequencing. Genotypes were independently scored by two investigators, and samples for which there were discrepant readings were reassayed.

IGF-I-533T/C. Alleles for the C → T polymorphism at position -533 upstream of the transcription start site of the *IGF-I* gene (Genbank accession no. S85346) were identified by the fluorogenic 5′-nuclease assay (Taqman Assay; ref. 36) using the Taqman PCR Core Reagent Kit (Applied Biosystems, Foster City, CA) according to manufacturers' instructions. The oligonucleotide primers for amplification of the polymorphic region were GC029 for (5′-gcccctccataggttctagga-3′) and GC029 rev (5′-cgggtgacccctgtcc-3′). Fluorogenic oligonucleotide probes used to detect each of the alleles were GC029F (5′-agatcacacccctcacttggcaac-3′) labeled with 6-FAM and GC029 C (5′-agatcacacctctcacttggcaac-3′) labeled with CY3 (BioSearch Technologies, Novato, CA). PCR amplification was done in a thermal cycler (MWG Biotech, High Point, NC) with an initial step of 95°C for 10 minutes followed by 50 cycles of 95°C/25 seconds and 63°C/1 minutes. The fluorescence profile of each well was measured in an ABI 7900 HT Sequence Detection System (Applied Biosystems) and the results analyzed with Sequence Detection Software (Applied

Biosystems). Any samples that were outside the variables defined by the controls were identified as noninformative.

IGFBP3-202A/C. Alleles for the A → C polymorphism at position. -202 of the *IGFBP3* gene were identified using direct sequencing of the polymorphic region. The region of the gene containing the polymorphism was amplified by PCR using primers GC082 for (5′-GAGTTGGCCAGGAGTGACTG-3′) and GC082 rev (5′-GCGTGCAGCTCGAGACTC-3′). PCR reaction mix was prepared using HotStart Taq Polymerase (Qiagen, Valencia, CA) according to manufacturers' instructions using 20 ng of genomic DNA, 2 mmol/L $MgCl_2$, and 300 mol/L of each primer. PCR amplification was done in a thermal cycler (MWG Biotech) using a touchdown protocol with an initial step of 95°C for 15 minutes finishing with 35 cycles of 95°C/25 seconds, 57°C/1 minute, and 72°C/1 minute. DNA sequencing was done using primer GC082S (5′-CCAGGAGTGACTGGGGTGA-3′) using ~10 to 20 ng of purified PCR product using fluorescently labeled dideoxynucleotide triphosphates (ABI Dye Terminator Sequencing Kit, Applied Biosystems) by cycle sequencing for 50 rounds of 95°C/15 seconds and 58°C/3.5 minutes. The sequencing reactions were run on an ABI3700 Capillary DNA Analyzer.

Serum Assays. Total serum IGF-I and IGFBP3 levels were measured as previously described (17). Briefly, measurements of serum IGF-I and IGFBP3 concentrations were carried out using immunoradiometric assay kits (Diagnostic Systems Laboratories, Inc., Webster, TX), following the instructions of the manufacturer.

Statistical Analysis

Genotype-Colorectal Cancer Risk Association. Although we sampled our controls from the whole cohort, this study is more case-control than case-cohort in design because the time period of follow-up was comparable between the cases and subcohort, with only 13 subjects in the latter group developing colorectal cancer during the observation period. Nonetheless, parallel analyses were conducted using standard case-control and case-cohort methods and did not materially differ. The data presented in this manuscript are based on case-control analysis.

Specifically, to assess the extent of cancer risk associated with genotypes, unconditional multiple logistic regression models (37) were fitted and odds ratio (OR) and their corresponding 95% confidence intervals (95% CI) were reported. All logistic regression models included age at recruitment (continuous), year of recruitment, gender, and dialect group (Cantonese, Hokkien) as covariates. Colorectal cancer risk factors which were considered as potential confounders were body mass index, height, education levels, alcohol intake, physical activity, and smoking history. None were included in the final model because inclusion did not substantially alter (>5%) the variable estimates for the exposures (genotypes).

Colorectal cancer was coded by anatomic subsites per the *International Classification of Disease Oncology* (2nd ed.): colon (C18.0-C18.9) and rectal (C19.0-C20.0) cancers. To test for heterogeneity of odds ratios across anatomic subsite as well as age at diagnosis for cases (young cases: <60 years; old cases: ≥60 years), polychotomous logistic regression models were fitted and likelihood ratio tests were conducted.

To investigate the possible interaction of the IGF-I genotype with gender and factors associated with serum IGF-I in this population (body mass index, calcium intake, and physical activity; ref. 38), the respective multiplicative interaction terms were included in the regression models and likelihood ratio tests were conducted for significance of the interaction variables.

Haplotype Inference and Allelic Cosegregation/Association. Allele frequencies were determined by gene counting (39). The observed allele frequencies among controls were used to calculate the expected genotype frequencies under Hardy-Weinberg equilibrium. Departures from Hardy-Weinberg equilibrium was assessed by testing the difference between the observed (sampled) and expected (under Hardy-Weinberg equilibrium) genotype frequencies in controls using a χ^2 test (40). Linkage disequilibrium (LD) between *IGF-I* polymorphisms was assessed by using a χ^2 test of allelic association (41).

To estimate haplotype frequencies from genotype information within our population of unrelated individuals, we used the expectation-maximization algorithm, as implemented in the STATA command hapipf, to resolve phase uncertainties (42–44). To estimate ORs for haplotype combinations, each individual in the sample was replicated for all possible haplotype configurations that are compatible with their genotypes and weighed by the estimated haplotype frequencies in logistic regression models (45).

Genotype-Phenotype Association. Of the 895 controls in this study, 628 had serum IGF-I and 595 had serum IGFBP3 measurements available (described previously by Probst-Hensch et al. 38). Kruskal-Wallis test statistics were used to compare distributions of the serum markers by genotype categories. Multiple regression models were also fitted with age, sex, body mass index, dialect group, and year of recruitment as covariates but were not reported as none of these nongenetic risk factors acted as confounders.

All *P*s are two sided and statistical analyses were done using STATA 8.0 (Stata Co., College Station, TX).

Results

Study Characteristics. The baseline characteristics of the Singapore Chinese Health Study cohort have been described (32). Briefly, the mean age of cohort subjects at enrollment was 56.5 years. Fifty-six percent of cohort subjects were women and 54% belonged to the Hokkien dialect group. Most were married (83%) at the time of recruitment. Eighty-eight percent of cohort subjects were born in Singapore or Malaysia (Singapore and Malaysia are neighboring countries with similar sociocultural groups), whereas virtually all of the remaining 12% were born in China. The cohort was relatively uneducated; 27% of its members had no formal education and 44% received only a primary school education.

The control group for this study was comparable to the whole cohort with respect to demographic variables and colon cancer risk factors. Table 1 summarizes the demographic characteristics among cases and controls. Cases were heavier than controls, marginally taller, less educated, and more likely to be male. Controls and cases did not differ significantly in terms of physical activity, dietary calories, fat, fiber, or calcium. In addition, they did not differ by dialect group, family history of colon or rectal cancer, smoking, alcohol consumption, age at menarche, age at menopause, parity, or age at first birth (data not shown). The age at diagnosis for cases ranged from 47 to 82 years (median, 66 years).

IGF-I -969(CA)$_n$ Genotypes. Eleven alleles, having 12 to 23 repeats, were observed in the Singapore Chinese population (Table 2). The $(CA)_{19}$ allele, at 35.5%, the most frequent among both cases and controls, is the most common allele in previously reported Caucasian (62.1-67.9%, refs. 46, 47), Japanese (40.8%, ref. 48), Indian Pakistani (56%) and African American (37.8%, ref. 30) populations. Although alleles $(CA)_{18}$ and $(CA)_{19}$ seemed to be slightly more common among cases and allele $(CA)_{21}$ was more common among controls, there was no overall significant difference in the genotype distributions between cases and controls ($P = 0.12$).

Genotype distributions did not deviate from Hardy-Weinberg expectations. Table 3 shows the ORs for allele 19, the allele previously associated with lower levels of

Table 1. Selected characteristics of colorectal cancer cases and controls, Singapore Chinese Health Study

Characteristics	Controls	Cases	*P*
Total no.	873 (100)*	290 (100)*	
Sex			
Female	492 (56.4)	122 (42.0)	<0.01
Male	381 (43.6)	168 (58.0)	
Body mass index (kg/m^2)			
≤20	144 (16.4)*	44 (15.2)*	0.04
20 to <24	485 (55.6)*	143 (49.3)*	
24 to <28	200 (23.0)*	83 (28.6)*	
>28	44 (5.0)*	20 (6.9)*	
Height	160 (147,170)†	160 (147,175)†	0.01
Moderate physical activity (h/wk)			
0	653 (74.8)	217 (74.8)	0.69
0.5-3	139 (15.9)	42 (14.5)	
>4	81 (9.3)	31 (10.7)	
Education level			
High school	604 (69.2)*	193 (66.6)*	<0.01
None (formal)	207 (23.7)*	87 (30)*	
Post-high school	41 (4.7)*	6 (2.0)*	
University	21 (2.4)*	4 (1.4)*	
Total calcium intake (mg/d)	388.7 (160.1, 865.0)†	364.8 (149.3, 781.8)†	0.20
Total calories (kcal/d)	1,494.5 (835.6, 2,480.2)†	1,497.4 (814.4, 2,583.0)†	0.91
Total fiber intake (g/d)	12.3 (5.3, 23.7)	11.9 (4.2, 22.7)	0.54
Total fat (g/d)	41.4 (19.7, 82.3)†	39.5 (18.3, 77.2)†	0.20

*Number of subjects (%).
†Median (5th, 95th percentile).

circulating IGF-I (27, 30), and for the next most common allele, *(CA)$_{21}$*. There was no decrease in risk among those who carried one or two copies of the *(CA)$_{19}$* allele. Given the previously reported lower levels of circulating IGF-I only among homozygous carriers of the *(CA)$_{19}$* allele, we also combined heterozygotes and noncarriers. No decreased risk was observed for genotype *19/19* versus others. For the second most common allele, *(CA)$_{21}$*, possession of two copies was associated with approximately half of the risk for colorectal cancer compared with all other genotypes (Table 3).

***IGF-I -533T/C* Genotypes.** Resequencing revealed two previously unreported SNPs (*-533T/C* and *-484T/A*) in perfect LD with each other (Fig. 1). We genotyped one of the SNPs, *-533T/C*, for all cases and controls. Genotype frequencies were in agreement with Hardy-Weinberg equilibrium.

Table 4 summarizes the effect of the *−533T/C* genotype on colorectal cancer risk. An ∼30% decrease in risk was associated with possession of one or two copies of the *C* allele as compared with genotype *TT*. This association was confined to risk of colon cancer.

The effect of the *C* allele seemed to be modified by age, a strong predictor of circulating IGF-I levels in adulthood (49-51). It was primarily among young participants (below age 60 years) that the protective effect of the *C* allele was observed. Young carriers of the C allele had a 54% reduction in risk of colorectal cancer (Table 4). In addition, the effect of the *-533T/C* genotype on colorectal cancer risk was stronger in overweight persons (i.e., body mass index = 24 kg/m^2; *TT* versus *CT/CC:* OR, 0.48; 95% CI, 0.29-0.79) as contrasted to lean persons (i.e., body mass index < 24 kg/m^2; *TT* versus *CT/CC:* OR, 0.83; 95% CI, 0.58-1.17) with a nearly significant formal test for interaction (P = 0.08). There was no evidence of interaction by gender, calcium intake, or physical activity.

***IGF-I* Haplotypes.** The *-969(CA)$_n$* and *-533T/C* loci were not independently distributed (P < 0.001). The frequency of the *(CA)$_{21}$- C* haplotype was higher than expected under the hypothesis of no LD (25.1% versus 17.5%; Table 5). Seventy-five percent (439 of 584) of the C alleles were observed to be linked to the *(CA)$_{21}$* allele. The T allele, on the other hand, was more often associated with alleles *(CA)$_{17}$* to *(CA)$_{19}$*. Only 8% (97 of 1,162) of *T* alleles were linked to *(CA)$_{21}$*.

We estimated ORs for the *(CA)$_{21}$− C* haplotype, the haplotype carrying the two alleles, *(CA)$_{21}$* and *-533 C*, that

Table 2. Distribution of the *-969(CA)$_n$* allele frequencies in Singapore Chinese

Cytosine-adenine repeats	Controls (%)	Cases (%)
12	7 (0.4)	3 (0.5)
14	1 (0.1)	0 (0)
15	2 (0.2)	0 (0)
16	6 (0.3)	0 (0)
17	133 (7.6)	50 (8.6)
18	279 (16.0)	112 (19.3)
19	620 (35.5)	215 (37.1)
20	100 (5.7)	38 (6.6)
21	536 (30.7)	151 (26.0)
22	56 (3.2)	10 (1.7)
23	6 (0.3)	1 (0.2)

Table 3. ORs and 95% CIs for the *-969(CA)$_n$* polymorphism and colorectal cancer, Singapore Chinese Health Study

Genotype	Controls/cases (n = 873/n = 290)	OR (95% CI)*
Repeat 19		
Others	374/110	1.00 (reference)
(CA) 19/others	378/145	1.33 (0.98-1.80)
(CA)19/(CA)$_{19}$	121/35	1.03 (0.64-1.63)
(CA)$_{19}$/(CA)$_{19}$ versus all other genotypes		0.88 (0.57-1.36)
Repeat 21		
Others	426/157	1.00 (referent)†
(CA)$_{21}$/others	358/115	0.90 (0.67,1.23)†
(CA)$_{21}$/(CA)$_{21}$	89/18	0.46 (0.26,0.81)†
(CA)$_{21}$/(CA)$_{21}$ versus all other genotype		0.48 (0.28,0.84)

*Adjusted for age at recruitment (continuous), sex, dialect group (Cantonese or Hokkien), and year of recruitment.
†P for trend = 0.02.

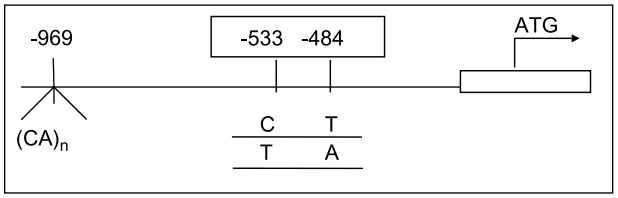

Figure 1. Insulin-like growth factor I promotes polymorphisms.

were univariately associated with lower risk. Compared with those carrying no copies of the $(CA)_{21}$– *C* haplotype, ORs for those carrying one or two copies were 1.00 (95% CI, 0.74-1.36) and 0.63 (95% CI, 0.34-1.15), respectively.

IGFBP3 *-202 A/C.* Allele frequencies among control subjects for the *-202A/C* polymorphism were 77% and 23%, respectively, for the *A* and *C* alleles. Genotype frequencies did not deviate from Hardy-Weinberg expectations. Overall, the genotypes were not associated with colorectal cancer risk (Table 6). However, there was evidence of heterogeneity by anatomic site ($P = 0.04$). This result seemed to be driven by the relatively small number of subjects with genotype *CC*. Compared with persons carrying at least one copy of the *A* allele, persons homozygous for the *C* allele had a nonstatistically significantly increased risk of colon cancer and a nonstatistically significantly decreased risk of rectal cancer. There was no evidence of heterogeneity by age or interaction with body mass index, gender, calcium intake, or physical inactivity.

Gene-gene Interaction. There was no evidence that the relationship between *IGF-I* genotypes [*-969*$(CA)_{19}$, *-969*$(CA)_{21}$, and *-533T/C)* and colorectal cancer risk was modified by *IGFBP3* genotype (*-202A/C*).

Genotype-Phenotype Analyses

IGF-I Genotypes and Serum IGF-I Levels. In the *IGF-I* gene, the −969$(CA)_n$, and in particular the two most common alleles $(CA)_{19}$ and $(CA)_{21}$, did not predict serum IGF-I among the 628 controls with serum levels available. Median values for $(CA)_{19/19}$, $(CA)_{19/others}$, $(CA)_{others/others}$ were 125, 132, and 127 ng/mL, respectively ($P = 0.56$). Median values for $(CA)_{21/21}$, $(CA)_{21}$, $(CA)_{others/others}$ were 127, 130, and 127 ng/mL, respectively ($P = 0.87$).

Neither did the *-533T/C* SNP predict serum IGF-I levels. Median values for the *CC*, *CT*, and *TT* genotypes were 130, 133, and 122 ng/mL, respectively ($P = 0.35$).

IGFBP3 Genotype and Serum IGFBP3 Levels. The *-202A/C* SNP in the *IGFBP3* gene was associated with serum IGFBP3 levels in the predicted direction (Fig. 2). Median serum IGFBP3 levels were 3,994, 3,785, and 3,307 ng/mL for genotypes *AA*, *AC*, and *CC*, respectively ($P < 0.001$).

Discussion

In this study, we examined polymorphisms in the *IGF-I* and *IGFBP3* genes in a cohort of Singapore Chinese. We identified two new IGF-I promoter region SNPs, in LD with a CA microsatellite that, in previous studies, has been inconsistently associated with cancer risk and other phenotypes. We report here that the new polymorphisms are associated with risk of colorectal cancer, specifically in the colon. We also report that IGFBP3 genotype, whereas not related to risk of colorectal cancer, is a predictor of serum IGFBP3 levels.

IGF-I Genotype. Previous studies do not support a direct functional effect of the CA microsatellite polymorphism. Although lower serum IGF-I levels among men with the $(CA)_{19/19}$ genotype were initially reported in a small study of men with idiopathic osteoporosis ($n = 116$; ref. 27), three prospective studies, the United Kingdom component of EPIC ($n = 660$, ref. 46), the Nurses' Health Study ($n = 202$ controls refs. 52, 53), and the Hawaii/Los Angeles Multiethnic Cohort ($n = 230$ ref. 35), found no association between CA genotype and serum levels. Two other large studies reported an association between genotype and serum levels; however, results were in opposing directions. Reduced circulating IGF-I levels were associated with the absence of the $(CA)_{19}$ ($n = 900$, $P = 0.003$; ref. 54) in a Dutch population and with the presence of the $(CA)_{19}$ allele ($n = 640$, $P_{trend} = 0.01$; ref. 47) in a study in South Wales. Whereas the results of these latter two studies suggest that polymorphism at this locus influences serum IGF-I levels, the conflicting direction of the results suggest that it is not the CA microsatellite polymorphism that is responsible (47).

To explore the possibility that the CA microsatellite is a marker of a functional polymorphism, we resequenced the promoter region of the *IGF-I* gene from the CA microsatellite to the translation start site. We identified two new SNPs *(-533T/C* and *-483A/T)* that are in partial LD with the *-969*$(CA)_n$. Because the two new SNPs are in perfect LD, only one of the SNPs (i.e., *-533T/C*) was examined in the current study. The *C* allele of the *-533T/C* SNP was partially linked with the $(CA)_{21}$ allele and both were associated with colorectal cancer risk in this study. In addition, the $(CA)_{21}$- *C* haplotype also predicted lower risk but was not more informative than either of the single markers. Possible scenarios are that either the new SNP is causal or it is in tighter LD with the putative causal SNP than is the haplotype marker. Indeed, due to the hypermutable nature of

Table 4. ORs and 95% CIs for the IGF-I *-533T/C* genotype and colorectal cancer, Singapore Chinese Health Study

Genotype	Controls (%)	Cases (%)	OR (95% CI)*
TT	390 (44.7)	156 (53.8)	0.68 (0.50,0.93)
CT	382 (43.8)	101 (34.8)	0.68 (0.50,0.93)
CC	101 (11.5)	33 (11.4)	0.71 (0.44,1.13)
CC/CT vs. TT			0.69 (0.52,0.92)
Subsite			
Colon			
TT	390 (44.7)	99 (58.9)	1.00 (reference)
CT	382 (43.8)	54 (32.2)	0.59 (0.40-0.86)
CC	101 (11.5)	15 (8.9)	0.54 (0.29-0.99)
CC/CT versus *TT*			0.58 (0.41-0.82)
Rectal			
TT	390 (44.7)	57 (46.7)	1.00 (reference)
CT	382 (43.8)	47 (38.5)	0.91 (0.59-1.37)
CC	101 (11.5)	18 (14.8)	1.08 (0.60-2.00)
CC/CT versus *TT*			0.94 (0.63-1.40)
P for heterogeneity = 0.04†			
Age at diagnosis			
<60 y			
TT	390 (44.7)	45 (62.5)	1.00 (reference)
CT	382 (43.8)	20 (27.8)	0.42 (0.24-0.74)
CC	101 (11.5)	7 (9.7)	0.61 (0.28-1.43)
CC/CT versus *TT*			0.46 (0.28-0.76)
≥60 years			
TT	390(44.7)	111(50.9)	1.00 (referent)
CT	382(43.8)	81(37.2)	0.84 (0.58,1.22)
CC	101(11.5)	26(11.9)	0.74 (0.42,1.29)
CC/CT versus *TT*			0.81 (0.57-1.14)
P for heterogeneity = 0.06‡			

*OR from unconditional logistic regression; adjusted for age at recruitment, gender, dialect groups (Cantonese or Hokkien), and year of recruitment (continuous).

†The odds of the cases carrying the *IGF1* genotype *TT* compared to the combined genotypes of *CT/CC* were contrasted between colon cancer and rectal cancer cases.

‡Test of heterogeneity comparing odds ratio of carriers of *TT* versus *CT/CC* among younger and older cancer cases.

Table 5. Estimated haplotype frequencies for *IGF-I* -969(CA)$_n$ and *-533T/C* polymorphisms, Singapore Chinese Health Study

Haplotype	Frequencies (%)		Haplotype	Frequencies (%)	
	Estimated*	Expected†		Estimated*	Expected†
$(CA)_{17}$− *C*	8 (0.5)	26 (1.5)	$(CA)_{17}$− *T*	125 (7.2)	106 (6.1)
$(CA)_{18}$− *C*	18 (1.0)	64 (3.7)	$(CA)_{18}$− *T*	262 (15.0)	215 (12.3)
$(CA)_{19}$− *C*	48 (2.7)	123 (7.0)	$(CA)_{19}$− *T*	572 (32.8)	496 (28.4)
$(CA)_{20}$− *C*	16 (0.9)	24 (1.4)	$(CA)_{20}$− *T*	84 (4.8)	77 (4.4)
$(CA)_{21}$− *C*	439 (25.1)	306 (17.5)	$(CA)_{21}$− *T*	97 (5.6)	230 (13.2)
$(CA)_{22}$− *C*	50 (2.9)	35 (2.0)	$(CA)_{22}$− *T*	6 (0.3)	22 (1.3)
Other, *C*	5 (0.3)	6 (0.3)	Other, *T*	16 (0.9)	16 (0.9)
Total	584	584	Total	1,162	1,162

*Estimated using the expectation-maximization algorithm to resolve phase uncertainty.
†Expected frequencies under the null hypothesis of no linkage disequilibrium.

microsatellites (55, 56), the haplotype marker may contain more measurement error.

Although it is possible that the *-533T/C* SNP is directly responsible for the observed association, there is currently no evidence that it has a functional effect. In fact, we found no association between genotype and serum levels. Whereas measurement of IGF-I serum levels can be problematic due to variable cleavage products in stored specimens (57), IGF-I serum levels have been associated with colorectal cancer risk in some previous studies. The reason for the lack of association between the SNP and IGF-I serum levels in this population remains unresolved.

The *-533T/C* SNP was primarily associated with risk of colon but not rectal cancer. Consistent with our findings, elevated serum IGF-I levels have been associated with risk of colon but not rectal cancer in a cohort of Hawaiian Japanese (20). Colon and rectal cancer incidence differ in distribution by geography, ethnicity, age, and gender (58), suggesting differences in etiology between the cancers (reviewed in ref. 59). However, heterogeneity by subsite was not observed in the Physicians' Health Study (19), or in the two negative prospective studies [i.e. the Shanghai Chinese Male Cohort (17) or the New York Women's Study (18).

We observed effect-modification by age. Levels of circulating IGF-I decline with age (49-51) and the growth hormone axis is thought to be responsible. Only among younger persons (<60 years) was the genotypic effect evident. Among older people, who have presumably already undergone a significant age-related decline in serum IGF-I levels, no effect of genotype on cancer risk was observed. A similar pattern was seen for serum IGF-I levels in a Hawaiian Japanese cohort (20). Furthermore, a previous study reported an interaction between age and the *-961(CA)$_n$* genotype: the age-related decrease in circulating levels of IGF-I was stronger among homozygotes for *-961(CA)*$_{19}$ (60).

Table 6. ORs and 95% CIs for the -202 *IGFBP3* and colorectal cancer, Singapore Chinese Health Study

Genotype	Controls/Cases (n = 821/n = 272)	OR (95% CI)*
AA	480/166	1.00 (reference)
AC	306/90	0.90 (0.66-1.23)
CC	35/16	1.27 (0.67-2.45)
CC versus *AA/AC*		1.32 (0.69-2.52)
Subsite		
Colon		
AA	480/93	1.00 (reference)
AC	306/51	0.90 (0.61-1.32)
CC	35/13	1.82 (0.89-3.74)
CC versus *AA/AC*		1.89 (0.94-3.82)†
Rectum		
AA	480/73	1.00 (reference)
AC	306/39	0.88 (0.57-1.35)
CC	35/3	0.54 (0.56-1.35)
CC versus *AA/AC*		0.56 (0.17-1.91)†
P for heterogeneity=0.04†		

*Adjusted for age at recruitment (continuous), sex, dialect group (Cantonese or Hokkien), and year of recruitment.
†The odds of the cases carrying the *IGFBP3* genotype CC compared with the combined genotypes of AA/AC were contrasted between colon cancer and rectal cancer cases.

IGFBP3 Genotype. We confirmed that the IGFBP3 genotype predicts serum IGFBP3 levels. Consistent with previous studies (28-30) and with *in vitro* assays (28), there was a trend for decreasing serum IGFBP3 levels with increasing copies of the *C* allele. Whereas there was no significant association between genotype and cancer risk, there was evidence of heterogeneity by anatomic site, with a nonsignificantly increased risk of colon cancer among subjects with genotype *CC*. Larger sample sizes are needed to confirm these results.

Gene-gene Interaction. Whereas a main effect of the *IGFBP3* genotype on colorectal cancer risk in our population was not observed, *IGFBP3* genotype might plausibly influence the effect of *IGF-I* genotype on cancer risk. IGFBP3 potentially influences the effects of IGF-I on cellular growth and proliferation through stabilizing and increasing IGF-I half-life, modulating IGF-I transportation and cellular localization, extending metabolic clearance and regulating IGF-I/IGF-I-receptor binding. Although we found no evidence of gene-gene interaction, we had very low power to conduct a formal test of interaction.

Conclusion. Our finding of an association between genetic polymorphism in the IGF-I promoter region and colorectal cancer risk is unlikely to be an artifact of population

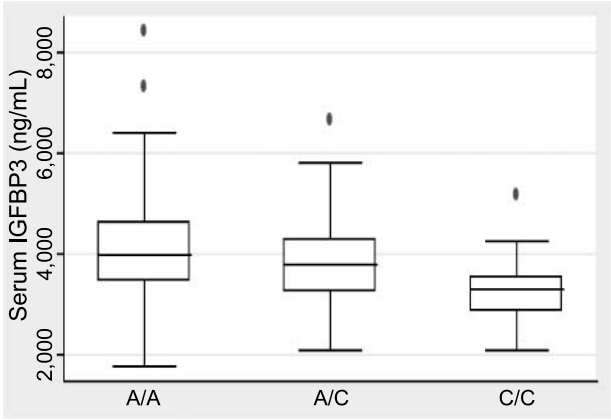

Figure 2. Circulating levels of IGF-I by -202IGFBP3 genotype.

stratification and admixture (61) because the Singapore Chinese Health Study is a population-based cohort investigation involving subjects drawn from an ethnically homogeneous southern Chinese population. This population originates from the contiguous coastal provinces, Fujian and Guangdong, and forms a tight genetically homogeneous subcluster within the relatively genetically similar Southern Chinese population (ref. 62 and references therein). Neither is selection bias likely to explain our results since participation rate was high (85%), participants seemed to be similar to the general population, and biospecimen donors and nondonors differed only by dialect group, gender, and education, none of which were related to genotype. Furthermore, the validity of this finding is supported by the observation that a strong predictor of serum IGF-I (age) modifies the effect of IGF-I genotype on colorectal cancer occurrence.

However, the identity of the polymorphism causally responsible for this association has not been definitively determined. All three makers, the $(CA)_{21}$-*C* haplotype, the -*969*$(CA)_{21}$ allele, and the -*533 C* allele, predicted lower risk. Whereas none of these three markers was clearly most informative, the -*533 T/C* SNP has the advantages of being less prone to measurement error (compared with a microsatellite marker), and of producing more stable (less sparse) data. Our finding of an association between the -*533 T/C* SNP and colorectal cancer risk supports the utility of this newly identified *IGF-I* promoter region SNP for IGF-I association studies.

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