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Published OnlineFirst on February 8, 2011; DOI:10.1158/0008-5472.CAN-10-2733

Prevention and Epidemiology

Cancer Research

Replication and Functional Genomic Analyses of the Breast Cancer Susceptibility Locus at 6q25.1 Generalize Its Importance in Women of Chinese, Japanese, and European Ancestry

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Abstract

We evaluated the generalizability of a single nucleotide polymorphism (SNP), rs2046210 (A/G allele), associated with breast cancer risk that was initially identified at 6q25.1 in a genome-wide association study conducted among Chinese women. In a pooled analysis of more than 31,000 women of East-Asian, European, and African ancestry, we found a positive association for rs2046210 and breast cancer risk in Chinese women [ORs (95% CI) = 1.30 (1.22–1.38) and 1.64 (1.50–1.80) for the AG and AA genotypes, respectively, P for trend = 1.54×10^{-30}], Japanese women [ORs (95% CI) = 1.31 (1.13–1.52) and 1.37 (1.06–1.76), P for trend = 2.51×10^{-4}], and European-ancestry American women [ORs (95% CI) = 1.07 (0.99–1.16) and 1.18 (1.04–1.34), P for trend = 0.0069]. No association with this SNP, however, was observed in African American women [ORs (95% CI) = 0.81 (0.63–1.06) and 0.85 (0.65–1.11) for the AG and AA genotypes, respectively, P for trend = 0.4027]. *In vitro* functional genomic studies identified a putative functional variant, rs6913578. This SNP is 1,440 bp downstream of rs2046210 and is in high linkage disequilibrium with rs2046210 in Chinese ($r^2 = 0.91$) and European-ancestry ($r^2 = 0.83$) populations, but not in Africans ($r^2 = 0.57$). SNP rs6913578 was found to be associated with breast cancer risk in Chinese and European-ancestry American women. After adjusting for rs2046210, the association of rs6913578 with breast cancer risk in African Americans approached borderline significance. Results from this large consortium study confirmed the association of rs2046210 with breast cancer risk among women of Chinese, Japanese, and European ancestry. This association may be explained in part by a putatively functional variant (rs6913578) identified in the region.

Cancer Res; 71(4); 1344–55. ©2011 AACR.

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Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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doi: 10.1158/0008-5472.CAN-10-2733

Published OnlineFirst on February 8, 2011; DOI:10.1158/0008-5472.CAN-10-2733

Introduction

Breast cancer, one of the most common malignancies among women worldwide, is a complex polygenic disorder for which genetic factors play a significant role in disease etiology (1, 2). We recently identified a novel genetic susceptibility locus at 6q25.1 for breast cancer risk in a genome-wide association study (GWAS) conducted among Chinese women living in Shanghai (3). A nearly 60% elevated risk for breast cancer was found among women homozygous for the variant A allele in rs2046210, a single nucleotide polymorphism (SNP) located approximately 29 kb upstream of the *ESR1* gene. It has yet to be determined whether this SNP is associated with breast cancer risk in other populations. Investigation of the association in other racial and ethnic groups is needed to determine the generalizability of this finding and to identify causal variants for the association. In this article, we report a pooled analysis of the association between rs2046210 and breast cancer risk in a consortium of 14 studies including more than 31,000 women of East-Asian, European, and African ancestry. We also conducted functional genomic studies to identify possible causal variants at this locus.

Materials and Methods

Study population

Fourteen studies contributing a total of 17,188 breast cancer cases and 14,660 controls participated in this consortium. Detailed descriptions of participating studies are included in the Supplement. Briefly, the consortium included 18,414 Chinese women from 7 studies conducted in Shanghai [$n = 10,373$: Shanghai Breast Cancer Study (SBCS)-I (3, 4), SBCS-II (3), and Shanghai Breast Cancer Survival Study (SBCSS)/Shanghai Endometrial Cancer Study (SECS; ref. 3)], Tianjin [$n = 3,115$; Tianjin Study (5)], Nanjing [$n = 2,084$; Nanjing Study (6, 7)], Taiwan [$n = 2,014$; Taiwan Study (8, 9)], and Hong Kong [$n = 828$; Hong Kong Study (10)]; 3,142 Japanese women from 3 studies conducted in Nagoya [$n = 1,288$; Hospital-based Epidemiologic Research Program at Aichi Cancer Center (HERPACC-II; ref. 11)], Hawaii [$n = 1,048$; Multiethnic Cohort Study (MEC; refs. 12, 13)], and Nagano [$n = 806$, Nagano Breast Cancer Study (14)]; 8,258 European-ancestry Americans from 3 studies conducted in WI/MA/NH [$n = 3,266$; Collaborative Breast Cancer Study (CBCS; refs. 15, 16)], TN [$n = 3,060$; Nashville Breast Health Study (NBHS; ref. 3), and NY [$n = 1,932$; Long Island Breast Cancer Study Project (LIBCSP; ref. 17)]; and 2,034 African Americans from 2 studies conducted in 12 southern U.S. states [$n = 1,568$: Southern Community Cohort Study (SCCS; ref. 18) and TN ($n = 466$; NBHS) (Table 1)].

Genotyping

Genotyping assays were done at 6 different centers. The genotyping assay protocol was developed and validated at the Vanderbilt Molecular Epidemiology Laboratory, and TaqMan genotyping assay reagents were provided to investigators of the Tianjin study (Tianjin Cancer Institute and Hospital), Nanjing study (Nanjing Medical University), LIBCSP (Columbia University), MEC (University of Southern California), and Nagano Breast Cancer study (Japan National Cancer Center), who conducted the genotyping assays at their own laboratories. Samples from the other 8 studies were genotyped at Vanderbilt using TaqMan and Affymetrix SNP arrays or at Proactive Genomics using the iPlex Sequenom MassArray platform. The Shanghai study samples were genotyped with Affymetrix Genome-Wide Human SNP Array 5.0 or 6.0 (Stage 1 of the initial GWAS) and Sequenom (Stages 2 and 3) as described previously (3). The SCCS samples were genotyped with Sequenom. All other samples were genotyped with the TaqMan assay.

Genotyping quality controls

Quality control (QC) procedures for samples from the Shanghai studies have been described previously (3). The consistency rate was 99.7% based on 2,572 comparisons with blinded QC samples and 99.2% based on 1,751 comparisons with HapMap DNA samples. For the SCCS samples genotyped with the Sequenom platform, 2 negative controls, 2 blinded duplicates, and 2 samples from the HapMap project were included in each 96-well plate. The QC consistency rate was 100% for blinded duplicates and 100% for the HapMap samples comparing genotyping data obtained from the current study with data obtained from the HapMap project. For TaqMan genotyping assays conducted at the Vanderbilt Molecular Epidemiology Laboratory, 2 negative controls and 2 blinded duplicates were included in each 96-well plate, along with 30 unrelated European and 45 Chinese samples from the HapMap project for QC purposes. The consistency rate was 98.8% for the blinded duplicates and 100% for the HapMap samples comparing genotyping data obtained from the current study with data obtained from the HapMap project. Each of the non-Vanderbilt laboratories was asked to genotype a trial plate containing DNA from 46 unrelated European-ancestry and 70 Chinese-ancestry samples before the main study genotyping was conducted. The consistency rate across all centers for these trial samples was 100% compared with genotypes previously determined at Vanderbilt. In addition, replicate samples comparing 3% to 7% of all study samples were dispersed among the genotyping plates at all centers. The genotype distribution for rs2046210 was in Hardy–Weinberg equilibrium among controls for all participating studies, with the exception of control samples from the Taiwan study ($P = 0.003$). The genotype distributions for rs6929137, rs3734804, rs6913578, and rs7763637 were in Hardy–Weinberg equilibrium among controls for all participating studies.

Imputation

To evaluate the association of breast cancer risk with SNPs that were not directly genotyped in the initial GWA scan, we imputed the genotypes of these SNPs using the program MACH (19). MACH determines the probability distribution of missing genotypes conditional on a set of known haplotypes, while simultaneously estimating the fine-scale recombination map. For the Shanghai studies, the imputation was based on 660,118 autosomal SNPs genotyped using Affymetrix Genome-Wide Human SNP Array 6.0 with a minor allele frequency (MAF) greater than 1% that passed the QC check

Published OnlineFirst on February 8, 2011; DOI:10.1158/0008-5472.CAN-10-2733

Table 1. Characteristics of studies participating in the breast cancer consortium

Study (reference)	Ethnicity	Study design[a]	Study period	N[b]	Age (mean)	Menopause (%)	ER (+)[c] (%)
SBCS-I[d] (3, 4)	Chinese	Population	1996–1998	1,105/1,213	47.5/47.3	32.7/36.3	63.8
SBCS-II[d] (3)	Chinese	Population	2002–2005	1,915/1,836	50.9/51.7[e]	43.6/49.4[e]	63.6
SBCSS[d]/SECS[d] (3)	Chinese	Population	2002–2006	3,405/899	55.0/54.9	53.7/61.7[e]	64.5
Tianjin (5)	Chinese	Hospital	2004–2008	1,532/1,583	51.7/51.9	51.7/55.4[f]	44.3
Nanjing (6, 7)	Chinese	Hospital	2004–2008	1,050/1,034	51.6/52.0	53.7/55.2	55.4
Taiwan (8, 9)	Chinese	Hospital	2004–2007	1,001/1,013	51.6/47.4[e]	52.7/39.8[e]	65.9
Hong Kong (10)	Chinese	Hospital	2003–2004	407/421	45.5/45.4	52.2/41.4[e]	72.0
Nagoya, Japan[d] (11)	Japanese	Hospital	2000–2005	644/644	51.4/51.1	48.5/48.5	72.8
MEC[d] (12, 13)	Japanese	Population	1993–2008	541/507	65.1/60.3[e]	86.4/83.3	86.2
Nagano, Japan[d] (14)	Japanese	Hospital	2001–2005	403/403	53.7/53.9	54.6/65.0[e]	74.8
NBHS-White[d] (3)	European	Population	2001–2008	1,592/1,468	54.8/52.2[e]	65.5/58.9[e]	74.9
CBCS[d] (15, 16)	European	Population	1998–2001	1,828/1,438	53.7/53.4	59.0/61.0	NA[g]
LIBCSP[d] (17)	European	Population	1996–1997	953/979	58.8/56.7[e]	67.6/66.5	76.7
CGEMS	European	Population	NA[g]	1,145/1,142	NA[g]	NA[g]	NA[g]
SCCS-Black[d] (18)	African	Population	2002–2008	522/1,046	48.1/56.6[e]	59.5/76.7[e]	NA[g]
NBHS-Black (3)	African	Population	2001–2008	290/176	54.5/52.1[f]	70.7/61.9	NA[g]

[a]With the exception of the MEC and SCCS, all other studies used the case–control study design using either a population-based or hospital-based approach.

[b]Cases/controls.

[c]Among cases with ER data

[d]SBCS-I: Shanghai Breast Cancer Study-I; SBCS-II: Shanghai Breast Cancer Study-II; SBCSS: Shanghai Breast Cancer Survival Study; SECS: Shanghai Endometrial Cancer Study; Nagoya, Japan: Hospital-based Epidemiologic Research Program at Aichi Cancer Center; Nagano, Japan: Nagano Breast Cancer study; MEC: Multiethnic Cohort Study; LIBCSP: Long Island Breast Cancer Study Project; CBCS: Collaborative Breast Cancer Study; NBHS: Nashville Breast Health Study; SCCS: Southern Community Cohort Study.

[e]Significant at $\alpha = 0.01$ level (*t* test for continuous variables, Chi-square test for categorical variables).

[f]Significant at $\alpha = 0.05$ level (*t* test for continuous variables, Chi-square test for categorical variables).

[g]Data not available.

and using phased HCB/JPT data from HapMap Phase II (release 22). For the National Cancer Institute Cancer Genetic Markers of Susceptibility (CGEMS) study (20), genotypes were imputed on the basis of 513,602 autosomal SNPs genotyped using Illumina HumanHap550 BeadChip with a MAF greater than 1% and phased CEU data from HapMap Phase II (release 22). Logistic regression was used to estimate the association of imputed SNPs of interest with breast cancer risk taking into account the degree of uncertainty of genotype imputation.

Plasmid constructs and luciferase assays

DNA fragments carrying the minor alleles of study SNPs were amplified by using PCR and cloned upstream of a luciferase reporter vector, pGL3 promoter or pGL3 basic (Promega). The major alleles were generated by using a QuickChange Site-Directed Mutagenesis Kit (Strategene). Details on PCR primers and site-specific mutagenesis oligonucleotides are provided in the Supplement. All DNA constructs were verified by sequencing analysis. Enhancer and promoter activities were determined by transient transfection followed by an *in vitro* luciferase assay in HEK293 cells. Transfection was done with the use of FuGene 6 Transfection Reagent (Roche Diagnostics) in triplicate for each of the constructs. Briefly, 2×10^5 cells were seeded in 24-well plates and cotransfected with pGL4.73, a Renilla-expressing vector, which served as a reference for transfection efficiency. Thirty-six to 48 hours later, the cells were lysed with Passive Lysis Buffer and luminescence (relative light units) was measured using the Dual-Luciferase Assay System (Promega). Regulatory activity was measured as a ratio of firefly luciferase activity to *Renilla* luciferase activity, and the mean from at least 3 independent experiments are presented.

Electrophoretic mobility shift assay

Biotin-labeled, double-stranded oligonucleotide probes (details in Supplement) containing either the major or minor allele sequence were synthesized. The probes were incubated with nuclear protein extracts from HEK293 and MCF7 cells, in the presence or absence of competitors (that is, unlabeled probes). Protein-DNA complexes were resolved by polyacrylamide gel electrophoresis and detected using a LightShift Chemiluminescent EMSA kit (Pierce Biotechnology).

Statistical analysis

Individual data were obtained from each study for a pooled analysis. Case–control differences in selected demographic

Published OnlineFirst on February 8, 2011; DOI:10.1158/0008-5472.CAN-10-2733

characteristics and major risk factors were evaluated using *t* tests (for continuous variables) and Chi-square tests (for categorical variables). Associations between SNPs and breast cancer risk were determined using odds ratios (OR) and 95% CIs derived from logistic regression models. ORs were estimated for heterozygotes and homozygotes for the variant allele compared with homozygotes for the common allele. ORs were also estimated for the variant allele on the basis of a log-additive model and adjusted for age, study site, and ethnicity, when appropriate. Adjusting for nongenetic risk factors, including age at first live birth, age at menarche, age at menopause, body mass index, participation in exercise, family history of breast cancer and history of benign breast diseases, did not alter the observed association, and thus only age-adjusted and study site-adjusted results are presented. Heterogeneity across studies and between ethnicities was assessed with likelihood ratio tests. Stratified analyses by ethnicity, menopausal status, and estrogen receptor (ER) status were carried out.

Results

The distributions of age and menopausal status for participating studies are shown in Table 1. Higher risk of breast cancer was consistently observed for all known major breast cancer risk factors, including a family history of breast cancer, a prior history of benign breast disease, physical inactivity, early onset of menarche, late onset of menopause, and late age at first live birth (data not shown). Except for the CBCS and SCCS, data on ER status were available from all studies.

Generalizability of the association of rs2046210 with breast cancer risk

Table 2 presents associations between rs2046210 genotypes and breast cancer risk by study site and ethnicity. The variant A allele, which was the minor allele in all groups except African Americans, was associated with increased breast cancer risk in all Chinese studies. Pooled analyses of samples from all studies conducted among Chinese women (SBCS-I, SBCS-II, SBCS/SECS, Tianjin, Nanjing, Taiwan, and Hong Kong) produced ORs of 1.30 (95% CI: 1.22–1.38) and 1.64 (95% CI: 1.50–1.80) for the AG and AA genotypes, respectively (P for trend = 1.54×10^{-30}). After excluding from the analysis the Shanghai data from which the original association was derived, the association with breast cancer was stronger; ORs were 1.26 (95% CI: 1.14–1.39) and 1.77 (95% CI: 1.55–2.02), respectively, for the AG and AA genotypes (P for trend = 2.82×10^{-17}). SNP rs2046210 was also associated with increased breast cancer risk in all 3 studies conducted among Japanese women (Nagoya, MEC, and Nagano), with pooled ORs of 1.31 (95% CI: 1.13–1.52) and 1.37 (95% CI: 1.06–1.76) for the AG and AA genotypes, respectively (P for trend = 2.51×10^{-4}). The homogeneity test for results between the Chinese and Japanese studies was not statistically significant ($P = 0.42$); therefore, all studies conducted among Chinese and Japanese women were combined into an "East-Asians" group for subsequent pooled analyses.

Among women of European ancestry, a positive association between the A allele of the rs2046210 variant and breast cancer risk was found in all 3 studies (NBHS, CBCS, and LIBCSP) with directly genotyped data, although the trend test was statistically significant only in the NBHS (Table 2). SNP rs2046210 was not directly genotyped in the CGEMS study. Genotype data for this SNP among 1,145 breast cancer cases and 1,142 controls were imputed (MACH score = 1.00). An association with breast cancer risk was found with ORs of 1.10 (95% CI: 0.92–1.31) and 1.26 (95% CI: 0.96–1.66) for the AG and AA genotypes, respectively, which is consistent with the data from the 3 studies conducted among women of European ancestry included in the current analysis. In pooled analyses of all samples (5,518 cases/5,027 controls) from women of European ancestry (NBHS-White, CBCS, LIBCSP, and CGEMS), ORs were 1.07 (95% CI: 0.99–1.16) and 1.18 (95% CI: 1.04–1.34) for the AG and AA genotypes, respectively (P for trend = 0.0069; Table 2).

SNP rs2046210 was not associated with breast cancer risk among African Americans (Table 2). In the SCCS analysis of prevalent breast cancer cases, the case and control distributions of alleles were nearly identical, whereas among NBHS African Americans the ORs for the AG and AA genotypes were below 1.0. In pooled analyses of African American samples (812 cases/1,222 controls) from the 2 studies (SCCS and NBHS-Black), ORs were 0.81 (95% CI: 0.63–1.06) and 0.85 (95% CI: 0.65–1.11) for the AG and AA genotypes, respectively (P for trend = 0.40). The sample size for African Americans included in this study, however, was small and the frequency of the A allele in the African American population (62.0%) was considerably higher than that in the East-Asian (34.8%) and European-ancestry (35.5%) populations. Figure 1 presents a forest plot summarizing the results of these studies. We also did analyses stratified by menopausal and ER status and found that the association with rs2046210 is more evident for ER(−) breast cancer compared with ER(+) breast cancer ($P = 0.0004$) in East-Asian women but not in women of European ancestry (Table 3).

Functional genomic studies of the chr 6q25.1 locus

There are 2 nonsynonymous SNPs (rs6929137 and rs3734804) in the *C6orf97* gene, which is in the 6q25.1 locus. These 2 SNPs are in strong linkage disequilibrium (LD) with rs2046210 ($r^2 = 0.91$ in Chinese, 0.87 in Europeans, and 0.001 in Africans for rs6929137; $r^2 = 0.91$ in Chinese, 0.56 in Europeans, and 0.42 in Africans for rs3734804). In an attempt to identify SNPs that may be more strongly associated with breast cancer risk in women of European ancestry than the originally reported SNP (rs2046210), we genotyped these 2 SNPs in 1,592 European-ancestry American cases and 1,468 controls from the NBHS (NBHS-White). The variant alleles of the 2 SNPs were also associated with breast cancer risk [per variant allele OR = 1.11 (95% CI: 0.99–1.24) for rs6929137 and 1.12 (95% CI: 1.01–1.24) for rs3734804]. The associations, however, were not stronger than the initially reported SNP rs2046210 in the NBHS-White group (OR per variant allele = 1.15, 95% CI: 1.04–1.28). These 2 SNPs are not included in Affymetrix Genome-Wide Human SNP Array 6.0 and thus we imputed genotype data for these 2 SNPs. Again, these SNPs showed a significant association with breast cancer risk in the Shanghai

Published OnlineFirst on February 8, 2011; DOI:10.1158/0008-5472.CAN-10-2733

Table 2. Association of SNP rs2046210 with breast cancer risk by study site and ethnicity

	Frequency of the A allele (%)	GG		AG		AA		
		N[a]	OR (95% CI)	N[a]	OR (95% CI)	N[a]	OR (95% CI)	*P* for trend
By study site[b]								
—Shanghai	41.9/36.4	2,144/1,611	1.00[d]	3,183/1,802	1.33 (1.22–1.45)	1,098/535	1.54 (1.37–1.74)	4.67×10^{-15}
—Tianjin	42.1/35.5	512/655	1.00	750/732	1.31 (1.12–1.53)	270/196	1.76 (1.42–2.19)	9.60×10^{-8}
—Nanjing	43.2/36.8	341/415	1.00	510/477	1.30 (1.08–1.57)	199/142	1.71 (1.32–2.21)	2.66×10^{-5}
—Taiwan	42.0/36.7	334/384	1.00	494/514	1.11 (0.91–1.34)	173/115	1.73 (1.31–2.28)	5.02×10^{-4}
—Hong Kong	45.0/36.4	129/256	1.00	190/278	1.36 (1.02–1.80)	88/87	2.01 (1.40–2.89)	1.63×10^{-4}
—Nagoya, Japan	34.0/26.9	273/349	1.00	295/236	1.60 (1.27–2.02)	69/54	1.63 (1.11–2.41)	1.28×10^{-4}
—MEC-Japanese	28.7/26.3	280/277	1.00	211/193	1.08 (0.84–1.40)	50/37	1.34 (0.85–2.11)	0.2251
—Nagano, Japan	30.3/28.0	195/214	1.00	172/152	1.24 (0.93–1.66)	36/37	1.07 (0.65–1.76)	0.3310
—NBHS-White	37.6/34.4	613/618	1.00	761/691	1.11 (0.95–1.29)	218/159	1.38 (1.10–1.75)	0.0077
—CBCS-White	37.3/36.6	706/567	1.00	882/690	1.03 (0.89–1.19)	240/181	1.07 (0.85–1.33)	0.5684
—LIBCSP-White	37.4/36.8	370/391	1.00	454/455	1.05 (0.87–1.28)	129/133	1.02 (0.77–1.36)	0.7309
—CGEMS-White	36.9/34.5	446/478	1.00	554/541	1.10 (0.92–1.31)	145/123	1.26 (0.96–1.66)	0.0838
—SCCS-Black	62.6/62.1	74/149	1.00	242/494	0.99 (0.72–1.35)	206/403	1.03 (0.74–1.43)	0.7848
—NBHS-Black	57.2/61.4	62/26	1.00	124/84	0.62 (0.36–1.06)	104/66	0.66 (0.38–1.15)	0.2332
By ethnic group[c]								
—Chinese	42.2/36.3	3,460/3,321	1.00	5,127/3,803	1.30 (1.22–1.38)	1,828/1,075	1.64 (1.50–1.80)	1.54×10^{-30}
—Chinese (excl. Shanghai)	42.7/36.2	1,316/1,710	1.00	1,944/2,001	1.26 (1.14–1.39)	730/540	1.77 (1.55–2.02)	2.82×10^{-17}
—Japanese	31.3/27.0	748/840	1.00	678/581	1.31 (1.13–1.52)	155/128	1.37 (1.06–1.76)	2.51×10^{-4}
—East-Asians	40.7/34.8	4,208/4,161	1.00	5,805/4,384	1.30 (1.22–1.38)	1,983/1,203	1.61 (1.48–1.76)	2.47×10^{-33}
—European ancestry	37.3/35.5	2,135/2,054	1.00	2,651/2,377	1.07 (0.99–1.16)	732/596	1.18 (1.04–1.34)	0.0069
—African Americans	60.7/62.0	136/175	1.00	366/578	0.81 (0.63–1.06)	310/469	0.85 (0.65–1.11)	0.4027
All women[c]	40.6/38.0	6,479/6,469	1.00	8,822/7,605	1.20 (1.15–1.26)	3,025/2,482	1.41 (1.32–1.50)	3.64×10^{-27}

[a]Cases/controls.
[b]Adjusted for age and study site.
[c]Adjusted for age, study site, and ethnicity.
[d]Reference group.
[e]*P* values derived from homogeneity tests were 0.7615 for 7 studies conducted among Chinese women, 0.4222 between Chinese and Japanese studies, 0.3529 across 4 studies conducted among women of European ancestry, and 3.18×10^{-6} between East-Asian and European-ancestry women.

Published OnlineFirst on February 8, 2011; DOI:10.1158/0008-5472.CAN-10-2733

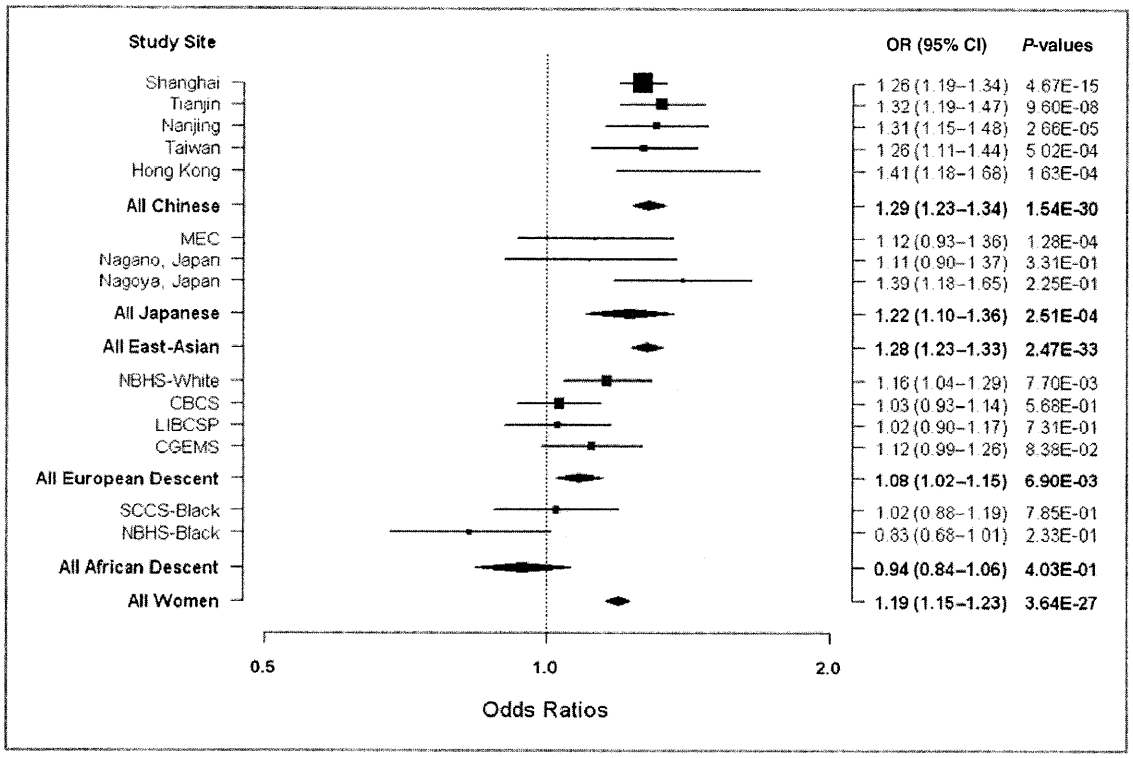

Figure 1. ORs (95%) per risk allele for breast cancer by study site and ethnicity. The size of the boxes is proportional to the sample size of each study. The width of the diamonds represents the range of confident intervals of combined ORs derived from meta-analyses.

samples [2,073 cases and 2,084 controls; ORs per variant allele, 1.26 (95% CI: 1.15–1.39) for rs6929137 and 1.27 (95% CI: 1.16–1.39) for rs3734804]. The associations with these 2 SNPs were slightly stronger than with the initially reported SNP rs2046210 identified in the GWAS [OR per variant allele = 1.25 (95% CI: 1.14–1.36)]. However, rs6929137 was not associated with breast cancer risk in African American (21). Thus, further evaluations of these 2 SNPs were not conducted.

To evaluate whether SNP rs2046210 has any regulatory function, we conducted luciferase reporter assays. The reporter construct containing the major G allele and the construct containing the minor A allele produced similar levels of luciferase activity. The results of a search for transcription factor binding sites [TFBS; the "TFBS Conserved" track of the UCSC Genome Browser (22)] showed that rs2046210 does not alter putative transcription factor binding.

To identify potential causal SNPs, we conducted a series of heterologous promoter and enhancer assays, focusing on the 36-kb region between the *C6orf97* and *ESR1* genes. We divided the 36-kb region (chromosome 6:151,983,304–152,019,420) into 4 parts and used long-range PCR to amplify 4 DNA fragments [a, 8.6 kb (harboring the rs2046210 polymorphic site); b, 9.2 kb; c, 9.3 kb; and d, 8.9 kb], then cloned the 4 fragments into both pGL3 basic and pGL3 promoter vectors (Fig. 2A). The templates for PCR were DNA carrying the minor or major alleles of SNP rs2046210. The SNP rs2046210A construct carried the minor alleles of SNP rs2046210 and other SNPs in close proximity and strong LD with rs2046210, whereas the rs2046210G construct carried the major alleles of rs2046210 and other SNPs in close proximity and strong LD with rs2046210. Luciferase activity derived from the fragment "a" construct in the pGL3 promoter vector with rs2046210A was significantly different from the fragment "a" construct with rs2046210G (data not shown). To refine the location of potential causal SNPs, we subdivided fragment "a" into 3 smaller DNA fragments (e, 2.2 kb; f, 4.1 kb; and g, 2.3 kb) containing either rs2046210A or rs2046210G into the pGL3 promoter vector and carried out luciferase assays. Luciferase activity derived from fragment "g" with rs2046210A was significantly different from that of fragment "g" with rs2046210G (data not shown). Six SNPs, including rs2046210, in fragment "g" were associated with breast cancer risk in Stage 1 of the initial SBCS GWAS. After excluding SNPs that showed no evidence of alteration of putative transcription factor binding in the database search, we found 3 candidate SNPs (rs7740686, rs7763637, and rs6913578) in this region (Fig 2A). We then generated major allele constructs for each of these 3 SNPs by using site-directed mutagenesis by using the 2.3-kb fragment "g" with the rs2046210A construct as the template. Luciferase activity was significantly higher in constructs harboring the major alleles of rs6913578 (rs6913578-A in Fig. 2B) or rs7763637 (rs7763537-G in Fig. 2B) compared with the corresponding minor alleles (Minor Alleles in Fig. 2B).

Published OnlineFirst on February 8, 2011; DOI:10.1158/0008-5472.CAN-10-2733

Table 3. Association of SNP rs2046210 with breast cancer risk by ethnicity, menopausal status, and ER status[a]

	East-Asians			European-ancestry Americans[b]		
	Cases	Controls	OR (95% CI)	Cases	Controls	OR (95% CI)
All women						
—GG	4,208	4,161	1.00 (reference)	1,689	1,576	1.00 (reference)
—AG	5,805	4,384	1.30 (1.22–1.38)	2,097	1,836	1.06 (0.97–1.17)
—AA	1,983	1,203	1.61 (1.48–1.76)	587	473	1.16 (1.01–1.33)
—Per A allele			1.28 (1.23–1.33)			1.07 (1.01–1.14)
—*P* for trend			2.47×10^{-33}			0.0321
Premenopausal women						
—GG	2,040	1,867	1.00 (reference)	600	585	1.00 (reference)
—AG	2,827	2,075	1.23 (1.13–1.34)	727	651	1.08 (0.93–1.27)
—AA	956	572	1.50 (1.33–1.69)	198	176	1.10 (0.87–1.39)
—Per A allele			1.23 (1.16–1.30)			1.16 (0.95–1.18)
—*P* for trend			4.35×10^{-12}			0.2998
Postmenopausal women						
—GG	2,127	2,126	1.00 (reference)	1,031	908	1.00 (reference)
—AG	2,910	2,121	1.36 (1.25–1.48)	1,249	1,081	1.02 (0.90–1.15)
—AA	1,002	577	1.71 (1.52–1.93)	359	272	1.17 (0.97–1.40)
—Per A allele			1.32 (1.25–1.40)			1.06 (0.98–1.16)
—*P* for trend			4.48×10^{-22}			0.1562
	P for interaction with menopause: 0.0850			*P* for interaction with menopause: 0.9816		
ER (+)[c]						
—GG	2,418	4,161	1.00 (reference)	425	1,009	1.00 (reference)
—AG	3,142	4,384	1.24 (1.16–1.33)	522	1,146	1.08 (0.93–1.26)
—AA	1,043	1,203	1.52 (1.37–1.68)	133	292	1.07 (0.85–1.36)
—Per A allele			1.23 (1.18–1.29)			1.05 (0.94–1.17)
—*P* for trend			1.06×10^{-18}			0.3847
ER (−)[c]						
—GG	1,295	4,161	1.00 (reference)	138	1,009	1.00 (reference)
—AG	1,930	4,384	1.36 (1.25–1.48)	167	1,146	1.07 (0.84–1.37)
—AA	695	1,203	1.77 (1.58–1.98)	42	292	1.05 (0.73–1.52)
—Per A allele			1.34 (1.27–1.41)			1.04 (0.88–1.23)
—*P* for trend			3.64×10^{-25}			0.6745
	P for association with ER status[d]: 0.0004			*P* for association with ER status[d]: 0.9883		

[a]Adjusted for age and study site.
[b]Includes NBHS-White, CBCS, and LIBCSP.
[c]No ER information was available in the CBCS, and thus this study was not included in the analysis.
[d]Derived from the Chi-squared test to examine the association between ER status and rs2046210 genotypes in the case group only.

To investigate whether the DNA sequences containing rs6913578 or rs7763637 interact with nuclear proteins and, if so, whether these SNP alter protein-DNA interactions, we conducted electrophoretic mobility shift assays (EMSA). We found that the minor allele (C) of rs6913578 significantly altered DNA-protein complex (II) intensity in both HEK293 and MCF7 cells (Fig. 2C), whereas there was no detectable interaction of rs7763637 with nuclear proteins (data not shown).

Evaluation of putative functional variants with breast cancer risk

SNP rs6913578 is located 1,440 bp downstream of rs2046210. SNP rs2046210 is in strong LD with rs6913578 and rs7763637 in Chinese populations (r^2 = 0.91 and 0.901, respectively) and European-ancestry populations (r^2 = 0.83 and 0.87, respectively), but is not in African populations (r^2 = 0.57 for both). Both rs6913578 and rs7763637 are associated with breast cancer risk in Chinese women and European-ancestry

Published OnlineFirst on February 8, 2011; DOI:10.1158/0008-5472.CAN-10-2733

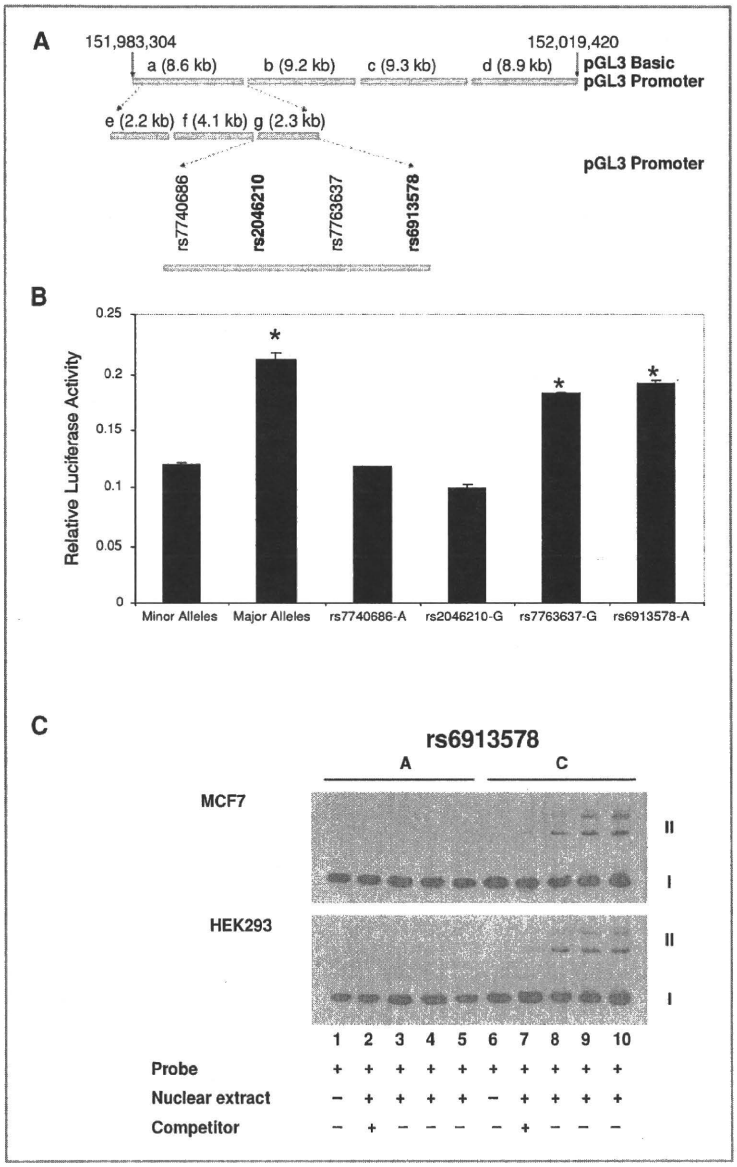

Figure 2. *In vitro* functional characterization of SNP rs2046210 and other potential functional SNPs at 6q25.1. A, diagram of cloning strategy. A 36-kb region (chromosome 6:151,983,304–152,019,420) between the *C6orf97* and *ESR1* genes was divided into 4 DNA fragments (a–d), which were separately cloned into pGL3 basic and pGL3 promoter vectors. The 8.6-kb "a" fragment was further divided to 3 DNA fragments (e, f, and g) and subcloned into a pGL3 promoter vector. The "g" fragment harbored 4 SNPs (rs7740686, rs2046210, rs7763637, and rs6913578). B, luciferase reporter activity assays: HEK293 cells were transiently transfected with pGL3 promoter/luciferase reporter constructs containing the 2.3-kb "g" fragment. 1. Minor alleles construct: contained the minor alleles for all 4 SNPs (rs7740686-T, rs2046210-A, rs7763637-A, and rs6913578 C); 2. Major alleles construct: contained the major alleles for all 4 SNPs (rs7740686-A, rs2046210-G, rs7763637-G, and rs6913578-A); 3. rs7740686-A construct: contained the rs7740686 major allele A and the minor alleles for the other 3 SNPs; 4. rs2046210-G construct: contained the rs2046210 major allele G and the minor alleles for the other 3 SNPs; 5. rs7763637-G construct: contained rs7763637 major allele G and the minor alleles for the other 3 SNPs; 6. rs6913578-A construct: contained rs6913578 major allele A and the minor alleles for the other 3 SNPs. Relative luciferase activity is shown as the mean ± SD of 3 experiments conducted in triplicate (relative to the Minor Allele construct). Statistical analysis was conducted by using Student's *t* test to compare the minor and major alleles (*, $P < 0.01$ when compared with the minor alleles, $n = 9$). C, EMSA. Nuclear protein extracts from MCF-7 (top) and HEK293 (bottom) cells were incubated with biotin-labeled probes corresponding to reference allele (lanes 1–5) or the risk allele (lanes 6–10) of rs6913578 in the absence or presence of competitors. Lanes 1 and 6, no nuclear extracts; lanes 2 and 7, unlabeled competitor in 200-fold molar excess; lanes 3 and 8 (5 mmol/L $MgCl_2$), lanes 4 and 9 (2.5 mmol/L $MgCl_2$), and lanes 5 and 10 (1.25 mmol/L $MgCl_2$), no competitor. I: free biotin-labeled probes. II: specific DNA-protein complex bands.

Published OnlineFirst on February 8, 2011; DOI:10.1158/0008-5472.CAN-10-2733

Americans, and the association was stronger than with rs2046210 in European-ancestry Americans (Table 4). The positive associations of these SNPs with breast cancer risk diminished (Table 4) after adjusting for rs2046210, which is not surprising given the high LD with rs2046210. These 2 SNPs, rs6913578 and rs7763637, showed weak associations with breast cancer risk in African Americans (Table 4). After adjusting for rs2046210, however, the associations in African Americans approached borderline significance (P for trend = 0.096 and 0.077, respectively).

Discussion

In this pooled analysis of 17,188 cases and 14,660 controls, we confirmed the association of rs2046210 at 6q25.1 with breast cancer risk among women with Chinese, Japanese, and European ancestry. *In vitro* functional genomic studies identified a putatively functional variant, rs6913578, a SNP 1,440 bp downstream of rs2046210, which is in high LD with rs2046210 in Chinese and European-ancestry populations, but is not in Africans. SNP rs6913578 had a stronger association with breast cancer risk in European-ancestry Americans than rs2046210, the SNP originally associated with breast cancer risk in a GWAS conducted in a Chinese population. In African Americans, the association of rs6913578 with breast cancer risk approached borderline significance after adjusting for rs2046210.

Genes that are located in the 1-Mb region centered around rs2046210 include *PLEKHG1, MTHFD1L, AKAP12, ZBTB2, RMND1, C6orf211, C6orf97, ESR1, C6orf98, SYNE1*, and *NANOGP11*. SNP rs2046210 is located 29 kb upstream of the first untranslated region of the *ESR1* gene, 180 kb upstream of the transcription start site of its first exon (3, 23), and 6 kb downstream of the *C6orf97* gene. Because of its relative proximity to the *ESR1* gene and the biological function of ER-α, it is possible that SNP rs2046210, or SNPs in LD with it, may alter *ESR1* gene expression and thereby affect susceptibility to breast cancer. A search of predicted TFBS using the "TFBS Conserved" track of the UCSC Genome Browser (22) indicated that there is no TFBS on this SNP. We further scanned for noncoding RNA (Evofold) and miRNA/snoRNA/scaRNA (sno/miRNA) in this region by using the UCSC Genome Browser and found that this SNP is not in the coding region for any noncoding RNA or miRNA/snoRNA/scaRNA. Our functional genomic analyses also provided no support for the potential functionality of rs2046210.

Our *in vitro* functional genomic experiments indicated that the location of the potential functional SNPs may be in a 2.3-kb region. Specifically, SNPs rs6913578, which is 1,440 bp downstream of rs2046210, and rs7763637, which is 947 bp downstream of rs2046210, altered luciferase reporter activity. These results suggest that these 2 common SNPs may influence DNA binding protein interactions and affect the expression of neighboring genes. We conducted EMSA to examine this hypothesis and confirmed that the C allele of rs6913578 significantly altered DNA-nuclear protein interaction. Thus, it is possible that nuclear protein(s) selectively and differently bind to specific alleles of the rs6913578 polymorphic site resulting in modification of the transcription of neighboring genes. However, there has been no confirmation to date that the putative transcription factors or their associated proteins are involved in the regulation of *ESR1, C6orf97*, or nearby genes. Interestingly, both rs6913578 and rs7763637 were associated with breast cancer risk among Chinese women and European-ancestry Americans (and the associations were stronger than rs2046210 in European-ancestry Americans), but not among African American women. However, after adjusting for rs2046210, the association of rs6913578 and rs7763637 with breast cancer risk in African Americans approached a borderline significance level. Our data show and highlight the importance of conducting interracial genetic association studies in populations with different LD structures to identify potential causal genetic variants for breast cancer and other complex diseases. Further studies will be required to determine causal SNPs related to breast cancer risk at the 6q25.1 locus.

In a recent study, Stacey and colleagues (24) reported an association of rs9397435 at the 6q25.1 locus with breast cancer risk in European, Chinese, and African populations. This SNP is located 2,854 bp downstream of rs2046210 and 1,414 bp downstream of rs6913578 and is only weakly correlated with rs2046210 in European ($r^2 = 0.087$) and African ($r^2 = 0.039$) populations. The risk allele frequency of this SNP in Asians is approximately 32%, comparable to that for rs2046210, but it is very low, only about 6.3% among European and African populations. Very recently, Turnbull and colleagues (25) evaluated the 6q25.1 locus with breast cancer risk in a GWAS conducted among 3,659 European-ancestry cases and 4,897 similar controls and identified SNP rs3757318 (MAF = 7%) to have the most significant association with breast cancer risk. This SNP is located approximately 200 kb upstream of *ESR1* in an intron of the *C6orf97* gene and 34,253 bp upstream of rs2046210. SNP rs3757318 is only weakly correlated with rs2046210 in Europeans ($r^2 = 0.088$), whereas the correlation is stronger in Chinese populations ($r^2 = 0.48$). Similarly, SNP rs3757318 is only weakly correlated with rs6913578 in European ($r^2 = 0.038$) and Chinese populations ($r^2 = 0.181$). Using imputed data from our GWAS, we showed that rs3757318 was associated with breast cancer risk with a per variant allele OR of 1.21 (P for trend = 5.4×10^{-4}), an association that is not as strong as rs2046210. It is unclear, however, whether this SNP is functional or is related to breast cancer risk in women of African ancestry.

Results reported to date from GWAS have clearly shown that GWAS results cannot be applied uniformly across all ethnic groups. Several SNPs identified in GWAS conducted among women of European ancestry could not be replicated in Asian-ancestry women (26–30). In our study, we have shown that the strength of the association with rs2046210 varies considerably across ethnic groups. This is not surprising given that most, if not all SNPs identified in GWAS are tagging SNPs, and there exists considerable differences in genetic architecture across ethnic groups. Fine-mapping studies are needed to identify additional genetic risk variants and/or causal variants for breast cancer.

Published OnlineFirst on February 8, 2011; DOI:10.1158/0008-5472.CAN-10-2733

Table 4. Association of SNPs rs6913578 and rs7763637 with breast cancer risk

SNP	Study[a]	Allele[b]	Genotyped or imputed	Adjusted SNP	No. of cases	No. of controls	Frequency (%)[c]	OR (95% CI)[d]		P for trend
								Heterozygous	Homozygous	
rs6913578		C/A								
	Chinese		Imputed	None	2,069	2,080	39.6/34.4	1.26 (1.10–1.44)	1.54 (1.27–1.87)	1.44×10^{-6}
				rs2046210	2,064	2,077	39.6/34.4	1.15 (0.85–1.55)	1.27 (0.72–2.26)	0.3852
	European-ancestry Americans		Imputed + genotyped	None	2,691	2,571	33.7/31.4	1.06 (0.95–1.19)	1.31 (1.08–1.60)	0.0128
				rs2046210	2,653	2,532	33.6/31.3	0.89 (0.68–1.17)	0.92 (0.57–1.47)	0.5349
	African Americans		Genotyped	None	799	1,736	47.9/47.5	1.04 (0.84–1.28)	1.13 (0.88–1.45)	0.3363
				rs2046210	795	1,727	48.1/47.5	1.16 (0.89–1.49)	1.38 (0.94–2.03)	0.0964
rs7763637		A/G								
	SBCS-GWAS		Genotyped	None	1,927	1,936	39.5/33.9	1.28 (1.11–1.46)	1.62 (1.33–1.98)	3.49×10^{-7}
				rs2046210	1,924	1,933	39.6/33.9	1.26 (0.92–1.72)	1.58 (0.87–2.86)	0.1294
	European-ancestry Americans		Imputed + genotyped	None	2,693	2,571	34.8/32.5	1.08 (0.96–1.21)	1.29 (1.07–1.55)	0.0104
				rs2046210	2,654	2,528	34.7/32.3	0.94 (0.71–1.24)	0.85 (0.53–1.36)	0.4950
	African Americans		Genotyped	None	790	1,749	47.8/47.8	1.01 (0.82–1.25)	1.12 (0.87–1.43)	0.3974
				rs2046210	786	1,741	48.0/47.8	1.15 (0.89–1.49)	1.42 (0.97–2.09)	0.0768

[a]Chinese: SBCS-GWAS; European-ancestry Americans: NBHS-White and CGEMS; African Americans: SCCS and NBHS-black.
[b]Risk allele/reference allele.
[c]Test allele frequency in cases/controls.
[d]Adjusted for age.

Published OnlineFirst on February 8, 2011; DOI:10.1158/0008-5472.CAN-10-2733

Major strengths of our study are its large sample size and its ability to evaluate the consistency of the findings across multiple studies conducted in different locations and in populations with different ethnic ancestry. In addition, we conducted functional genomic studies of this locus to identify possible functional variants. Ancestry informative markers, however, were not adjusted for in this study. In addition, our *in vitro* functional experiments were conducted only on a 36-kb region and then were narrowed down to 4 common polymorphisms in a 2.3-kb region. It is possible that other functional SNPs, both common and rare, exist at this locus.

In summary, results from this large consortium study confirmed the association of rs2046210 with breast cancer risk among Chinese women, Japanese women, and European-ancestry Americans. SNP rs6913578 may be a functional SNP responsible for the observed association with breast cancer risk of SNPs at the 6q25.1 locus. Additional fine-scale mapping studies are needed to identify causal variants at this locus.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

The authors thank study participants and research staff for their contributions and commitment to this project.

Grant Support

This research was supported by US NIH grant R01CA124558. The genotyping assays conducted at Vanderbilt University were done at the Survey and Biospecimen Core, which is supported in part by the Vanderbilt-Ingram Cancer Center (P30CA68485). Participating studies (Principal Investigator, grant support) of the consortium are as follows: the Shanghai Breast Cancer Study (W. Zheng, R01CA64277), the Nashville Breast Health Study (W. Zheng, R01CA100374), the Shanghai Breast Cancer Survival Study (X.O. Shu, R01CA118229), the Shanghai Endometrial Cancer Study (X.O. Shu, R01CA92585, contributed only controls to the consortium), the Tianjin Study (K. Chen, the National Natural Science Foundation of China Grant No. 30771844), the Nanjing Study (H. Shen, IRT0631, China), the Taiwan Biobank Study (C.-Y. Shen, DOH97-01), the Hong Kong Study (U.S. Khoo, Research Grant Council, Hong Kong SAR, China, HKU 7520/05M and 76730M), the Nagoya study (K. Tajima, Grants-in-Aid for Scientific Research on Priority Areas (17015052) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan; H. Tanaka, Grants-in-Aid for the Third Term Comprehensive Ten-Year Strategy for Cancer Control from the Ministry of Health, Labor and Welfare of Japan, H20-002), the Multiethnic Cohort Study (B. E. Henderson, CA63464; L. Kolonel, CA54281; and C.A. Haiman, CA132839), the Nagano Breast Cancer Study [S. Tsugane, Grants-in-Aid for the Third Term Comprehensive Ten-Year Strategy for Cancer Control from the Ministry of Health, Labor and Welfare of Japan, and for Scientific Research on Priority Areas (17015049) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan], the Collaborative Breast Cancer Study including Massachusetts (K.M. Egan, R01CA47305), Wisconsin (P.A. Newcomb, R01 CA47147) and New Hampshire (L. Titus-Ernstoff, R01CA69664) centers, the Long Island Breast Cancer Study Project (M.D. Gammon, U01CA/ES66572; R.M. Santella, P30ES009089; J.A. Swenberg, P30ES010126), and the Southern Community Cohort Study (W.J. Blot, R01CA092447). The content is solely the responsibility of the authors and does not necessarily represent the official views of the funding agencies.

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Received July 26, 2010; revised November 3, 2010; accepted December 4, 2010; published OnlineFirst February 8, 2011.

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Published OnlineFirst on February 8, 2011; DOI:10.1158/0008-5472.CAN-10-2733

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Prevention and Epidemiology

Cancer Research

Interaction between Adiponectin and Leptin Influences the Risk of Colorectal Adenoma

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Abstract

Obesity has been associated with an increased risk of colorectal neoplasia, but the mechanisms of this potential association have not been elucidated. We hypothesized that the adipokines adiponectin, leptin, and tumor necrosis factor-α (TNF-α) may mediate an association between obesity and colorectal cancer. We measured plasma concentrations of total and high-molecular-weight (HMW) adiponectin, leptin, and TNF-α in healthy volunteer examinees who underwent total colonoscopy between February 2004 and February 2005, and conducted a case-control study consisting of 778 cases and 735 controls. An inverse association of total and HMW adiponectin was observed with colorectal adenoma (P trend < 0.001 and 0.03, respectively). Further, total adiponectin interacted with leptin, but not TNF-α, in relation to colorectal adenoma (P interaction = 0.007). An inverse association of total adiponectin with colorectal adenoma was apparent in the highest two tertiles of leptin, particularly the middle (P trend < 0.001), whereas a positive association of leptin was obvious in the lowest tertile of total adiponectin (P trend = 0.01) after adjusting for potential confounders and body mass index, which is a major determinant of insulin resistance. Adiponectin may exert an anticarcinogenic effect on the large intestine by interfering with leptin, whereas leptin could conversely exert a carcinogenic effect under conditions of a lower abundance of adiponectin. Our findings provide the first epidemiologic evidence for interactive effects of adiponectin and leptin in the early stage of colorectal tumorigenesis, distinct from their involvement in insulin resistance. *Cancer Res; 70(13); 5430–7. ©2010 AACR.*

Introduction

Overweight and obesity have been consistently associated with an increase in the risk of colorectal cancer and adenoma, a well-established precursor lesion of colorectal cancer (1). However, the mechanisms of this potential association between adiposity and colorectal neoplasia have not been fully elucidated. Adipose tissue, long considered an inert energy storage depot, is now recognized as an active endocrine organ, and in fact releases a wide variety of biologically functional molecules, collectively referred to as adipokines (2). Importantly, accumulating evidence suggests that several adipokines, namely adiponectin, leptin, and tumor necrosis factor-α (TNF-α), have the potential to mediate the association between adiposity and colorectal neoplasia (1). These adipokines are in fact all related to insulin resistance (2), which has been suggested to be an early and fundamental disorder in the path to several obesity-related malignancies, including colorectal cancer (3).

Adiponectin, an insulin-sensitizing hormone, is secreted exclusively by adipocytes, and circulates in plasma in three forms of oligomeric complex: a simple complex of a trimer, a low-molecular-weight complex of two trimers, and a high-molecular-weight (HMW) complex of up to six trimers (3). Although HMW adiponectin is now considered the active form of the hormone, different forms have shown distinct biological effects through differential activation of downstream signaling cascades (3). Besides its well-known effect on insulin resistance, adiponectin seems to directly modulate several intracellular signaling pathways involved in colorectal carcinogenesis (4, 5), probably through the two isoforms of its receptors, adiponectin receptor 1 and 2, which are expressed in normal colon epithelium and colon cancer tissue (6, 7). Further, recent basic research has found that adiponectin inhibits leptin- and TNF-α–induced signaling cascades, both of which lead to cell proliferation and survival (8–11). However, few epidemiologic studies have examined the association of circulating levels of adiponectin with colorectal adenoma (12) and cancer (13–15), and no epidemiologic study has evaluated the interaction of adiponectin with leptin and TNF-α in relation to the risk of colorectal neoplasia.

Here, we measured plasma concentrations of total and HMW adiponectin, leptin, and TNF-α among middle-aged and elderly Japanese men and women, and investigated not only the association of circulating levels of these adipokines with colorectal adenoma but also the interaction of total and

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Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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doi: 10.1158/0008-5472.CAN-10-0178

HMW adiponectin with leptin and TNF-α in relation to the risk of colorectal adenoma.

Materials and Methods

Study population

The Research Center for Cancer Prevention and Screening was established in 2004 as a branch of the National Cancer Center of Japan with the goal of developing preventive methods for various types of cancers. Among its efforts, the Research Center conducted the Colorectal Adenoma Study in Tokyo (16, 17), a case-control study specifically designed to investigate environmental and genetic factors related to the early stage of colorectal carcinogenesis among healthy volunteer examinees of a colorectal cancer screening. All examinees gave written informed consent to allow their data and materials collected through the screening to be used for medical research. The study protocol was approved by the institutional review board of the National Cancer Center.

Eligible subjects were defined in advance as men ages 50 to 79 years and women ages 40 to 79 years who underwent total colonoscopy from the anus to the cecum and who were without a history of colorectal adenoma, any malignant neoplasia, ulcerative colitis, Crohn's disease, familial adenomatous polyposis, carcinoid tumor, or colectomy. Of a consecutive series of 3,212 examinees undergoing magnifying colonoscopy with indigo carmine dye spraying between February 2004 and February 2005, 2,234 met these conditions. Based on the pit pattern of colorectal lesions, namely the characteristics of mucosal crypts, 526 men and 256 women were determined to have at least one adenoma and were thus included as adenoma cases. Pit-pattern classification based on magnifying chromo-endoscopy has been detailed elsewhere (18). Of the remaining 1,452 examinees, we identified 482 men and 721 women as potential controls who were also free from other benign lesions (e.g., hyperplastic polyps, inflammatory polyps, and diverticula). For efficiency, 256 of the potential female controls were frequency-matched to the female cases in five age categories (40–49, 50–54, 55–59, 60–64, and ≥65 years of age) and two screening periods (first and second halves). Because there were fewer potential male controls than male cases, all potential male controls were included in the study. Finally, the study enrolled 782 cases and 738 controls. Cases with adenomas of ≥5 mm in diameter were referred to clinical hospitals for definitive diagnosis and treatment.

Blood collection and laboratory procedures

Examinees were scheduled for blood collection before any cancer screening procedures on the first day of screening. Fasting venous blood was drawn into a vacutainer tube with EDTA. Almost three-quarters of examinees had fasted since the day before the screening day. The blood sample was centrifuged to obtain blood plasma and buffy coat, and these specimens were preserved at −80°C until analysis.

Plasma concentrations of total and HMW adiponectin were measured at Mitsubishi Chemical Medience, Tokyo, Japan, and those of leptin and TNF-α at GeneticLab, Hokkaido, Japan. All laboratory personnel were blinded with respect to case and control status. Plasma concentrations of total and HMW adiponectin were simultaneously analyzed using a Human Adiponectin ELISA Kit for Total and Multimers (Sekisui Medical) by the enzyme-linked immunosorbent assay method. Minimum detection level was 0.39 μg/mL for both total and HMW adiponectin. The kit manufacturer has reported that intra-assay coefficients of variation for total and HMW adiponectin are 5.4% and 5.0%, respectively. Plasma concentrations of leptin and TNF-α were simultaneously assayed using a Human Serum Adipokine (Panel B) LINCO*plex* Kit (Millipore) based on the xMAP Technology (Luminex). Minimum detection levels of leptin and TNF-α were 85.4 and 0.14 pg/mL, respectively. According to the manufacturer, the intra-assay coefficients of variation were reported to be 1.4% to 7.9%.

Self-administered questionnaire and anthropometric measurements

Before cancer screening, all examinees were encouraged to complete a self-administered questionnaire concerning lifestyle and socioeconomic characteristics as well as personal and family medical history. Details of the questionnaire have been described elsewhere (16, 17). In brief, the questionnaire inquired about smoking habits by first determining smoking status (current, past, and never) and then expressing lifetime exposure to cigarette smoking among ever smokers (i.e., past and current smokers) by pack-years, with 1 pack-year defined as the smoking of 20 cigarettes every day for 1 year. The questionnaire also inquired about drinking habits by first determining drinking status (current, past, and never) and then calculating the amount of alcohol consumed per week among current drinkers on the basis of the frequency of alcohol drinking and the number of standard units consumed per occasion for five different alcoholic beverages (sake, *shochu/awamori*, beer, whisky, and wine).

At the beginning of cancer screening, body weight and height were measured by medical personnel, and body mass index (BMI) was calculated as the weight in kilograms divided by the height in meters squared.

Statistical analysis

An unconditional logistic regression model was used to estimate odds ratios (OR) and their 95% confidence intervals (95% CI) of colorectal adenoma according to sex-specific tertiles of total and HMW adiponectin, leptin, and TNF-α, with the lowest tertile for each adipokine used as the reference. Statistical adjustment was made in three models. Model 1 controlled for matching variables (i.e., age categories and screening periods) and the duration of fasting (from the day before the screening day, from the day of screening), whereas model 2 additionally adjusted for the following covariates: cigarette smoking (never, ≤20, 21–40, and >40 pack-years), alcohol drinking (never, past, <150, 150–299, ≥300 g/wk), family history of colorectal cancer (yes or no), and nonsteroidal anti-inflammatory drug use (yes or no). These covariates were suggested to be potential confounders in previous reports from the Colorectal Adenoma Study in Tokyo (16, 17). Model 3 further adjusted model 2 for BMI (<21.0, 21.0–22.9, 23.0–24.9, and ≥25.0 kg/m^2). Spearman's

Table 1. Selected characteristics of cases and controls by sex

Characteristic	Men			Women		
	Cases (*n* = 523)	Controls (*n* = 480)	*P* difference*	Cases (*n* = 255)	Controls (*n* = 255)	*P* difference*
Categorical variables, *n* (%)						
≥65 y of age	172 (33)	123 (26)	0.04	61 (24)	61 (24)	0.99
>40 pack-years	136 (26)	68 (14)	<0.001	6 (2)	2 (1)	0.03
≥300 g of alcohol/wk	153 (29)	98 (20)	0.004	6 (2)	8 (3)	0.14
Family history of CRC	72 (14)	65 (14)	0.91	55 (22)	26 (10)	<0.001
NSAID use	21 (4)	40 (8)	0.004	12 (5)	15 (6)	0.55
Overweight and obesity	188 (36)	124 (26)	0.002	46 (18)	37 (15)	0.31
Continuous variables, median (IQR)						
Total adiponectin (μg/mL)	3.98 (3.08–5.21)	4.37 (3.13–5.95)	0.002	6.81 (4.93–8.65)	7.36 (5.07–9.22)	0.21
HMW adiponectin (μg/mL)	1.20 (0.71–1.95)	1.33 (0.77–2.29)	0.02	2.78 (1.76–4.08)	3.01 (1.78–4.26)	0.28
Leptin (pg/mL)	3,333 (1,747–5,357)	2,671 (1,417–4,670)	<0.001	6,237 (3,789–10,739)	5,667 (3,138–9,260)	0.13
TNF-α (pg/mL)	2.70 (2.29–3.20)	2.67 (2.24–3.13)	0.42	2.45 (2.06–2.89)	2.50 (2.08–2.93)	0.42

Abbreviations: CRC, colorectal cancer; NSAID, nonsteroidal anti-inflammatory drug; IQR, interquartile range.
*Based on the χ^2 test for percentage difference and the Wilcoxon rank-sum test for median difference.

correlation coefficients of BMI with total and HMW adiponectin, leptin, and TNF-α were −0.24, −0.23, 0.59, and 0.06, respectively, for male controls, and −0.21, −0.22, 0.64, and 0.18, respectively, for female controls. Linear trends in the ORs of colorectal adenoma were also assessed by assigning ordinal values to tertiles of respective adipokines. Finally, we combined men and women according to sex-specific tertiles of total and HMW adiponectin, leptin, and TNF-α, and examined whether the association between these adipokines and colorectal adenoma was modified by sex. Interaction terms were created between indicator variables representing categories of each adipokine and of sex, and their significance was statistically evaluated based on the likelihood ratio test with two degrees of freedom.

We then examined whether adiponectin interacted with leptin or TNF-α to modify its association with colorectal adenoma. We obtained ORs and 95% CIs of colorectal adenoma for nine combinations of tertiles of adiponectin and of leptin/TNF-α, with reference to the combination of the lowest tertile of adiponectin and the highest tertile of leptin/TNF-α. Finally, we statistically evaluated these interactions based on the likelihood ratio test with four degrees of freedom. Interaction terms were created between indicator variables representing tertiles of adiponectin and of leptin/TNF-α.

Of 1,520 study subjects, 7 had missing information, namely 3 with regard to cigarette smoking and 4 for BMI. These were then excluded, and the current analysis was conducted in 1,003 men (523 cases, 480 controls) and 510 women (255 cases, 255 controls). Of these, 121 and 57 had plasma concentrations of HMW adiponectin and leptin below the minimum detection levels, respectively, and were assigned the putative values of 0.30 μg/mL and 50.0 pg/mL, respectively. Two-sided *P* values <0.05 were regarded as statistically significant. All statistical analyses were carried out using Statistical Analysis System (SAS), version 9.1 (SAS Institute).

Results

Selected characteristics of cases and controls by sex

Table 1 summarizes selected characteristics of cases and controls by sex. Male cases were more likely to be old and overweight, and tended to consume more cigarettes and alcohol, whereas male controls tended to use more nonsteroidal anti-inflammatory drugs. Female controls were more likely to be never smokers and tended to have less family history of colorectal cancer than female cases. Table 1 also shows plasma concentrations of total and HMW adiponectin, leptin, and TNF-α among cases and controls by sex. Male cases had lower plasma concentrations of total and HMW adiponectin and higher plasma concentrations of leptin than male controls. Of note, we observed substantial sex difference in plasma concentrations of total and HMW adiponectin and leptin. Correlations between total and HMW adiponectin, leptin, and TNF-α are presented in Supplementary Table S1. Total and HMW adiponectin were weakly inversely correlated with leptin, whereas leptin was weakly positively correlated with TNF-α.

Association of total and HMW adiponectin with colorectal adenoma

Table 2 shows the ORs of colorectal adenoma according to sex-specific tertiles of total and HMW adiponectin. In men, we observed a statistically significant trend of decreasing

adjusted ORs for colorectal adenoma across tertiles of total adiponectin (P trend = 0.002), and a marginally significant trend for HMW adiponectin (P trend = 0.08). A significantly reduced OR was also seen among men in the highest tertile of total adiponectin. Adjusted ORs of colorectal adenoma for the highest compared with the lowest tertile were 0.60 (95% CI, 0.44–0.83) and 0.75 (95% CI, 0.54–1.03) for total and HMW adiponectin, respectively. On further adjustment for BMI, the inverse association between total adiponectin and colorectal adenoma was still evident (P trend = 0.01). In women, neither total nor HMW adiponectin was measurably associated with colorectal adenoma, although adjusted ORs of colorectal adenoma for the highest tertile were below unity for both forms of adiponectin. When men and women were combined according to sex-specific tertiles, a significant trend of decreasing adjusted ORs across tertiles was observed for both total and HMW adiponectin (P trend < 0.001 and 0.03, respectively). Although additional adjustment for BMI attenuated the inverse association between both forms of adiponectin and colorectal adenoma, a significant trend across tertiles remained for total adiponectin (P trend = 0.01). The inverse association of total adiponectin remained significant after further adjustment for indicators of energy balance (i.e., total energy intake, physical activity, and height), dietary factors (i.e., intakes of meat; fruits and vegetables; dairy products; folate; vitamins B_2, B_6, and B_{12}; vitamin D; calcium; and total isoflavones), and metabolic factors (i.e., serum concentrations of triglycerides, total cholesterol, and glucose; P trend = 0.02; data not shown). When total and HMW adiponectin levels were treated as a continuous variable in model 2, adjusted ORs of colorectal adenoma for a 1 μg/mL increase were 0.95 (95% CI, 0.92–0.99) and 0.94 (95% CI, 0.88–1.01) for total and HMW adiponectin, respectively (data not shown). In this analysis of HMW adiponectin, 121 subjects

Table 2. Association of total and HMW adiponectin with colorectal adenoma

Measurement	Tertile			*P* trend*
	Lowest	Middle	Highest	
	OR (95% CI)	OR (95% CI)	OR (95% CI)	
Total adiponectin				
Men, range (μg/mL)	–3.64	3.65–5.26	5.27–	
Model 1†	1.00 (reference)	0.79 (0.59–1.07)	0.55 (0.40–0.76)	<0.001
Model 2‡	1.00 (reference)	0.83 (0.61–1.13)	0.60 (0.44–0.83)	0.002
Model 3§	1.00 (reference)	0.85 (0.62–1.15)	0.66 (0.47–0.92)	0.01
Women, range (μg/mL)	–5.76	5.77–8.49	8.50–	
Model 1†	1.00 (reference)	1.01 (0.66–1.53)	0.69 (0.44–1.08)	0.11
Model 2‡	1.00 (reference)	1.05 (0.68–1.61)	0.80 (0.50–1.27)	0.36
Model 3§	1.00 (reference)	1.07 (0.69–1.65)	0.88 (0.54–1.41)	0.61
Men and women combined				0.68‖
Model 1† ¶	1.00 (reference)	0.86 (0.67–1.09)	0.60 (0.46–0.77)	<0.001
Model 2‡ ¶	1.00 (reference)	0.87 (0.68–1.11)	0.64 (0.49–0.83)	<0.001
Model 3§ ¶	1.00 (reference)	0.89 (0.69–1.14)	0.70 (0.53–0.91)	0.01
HMW adiponectin				
Men, range (μg/mL)	–0.88	0.89–1.91	1.92–	
Model 1†	1.00 (reference)	1.04 (0.77–1.41)	0.71 (0.52–0.98)	0.04
Model 2‡	1.00 (reference)	1.05 (0.78–1.43)	0.75 (0.54–1.03)	0.08
Model 3§	1.00 (reference)	1.08 (0.79–1.47)	0.82 (0.59–1.15)	0.28
Women, range (μg/mL)	–2.19	2.20–3.90	3.91–	
Model 1†	1.00 (reference)	1.13 (0.74–1.71)	0.75 (0.48–1.18)	0.22
Model 2‡	1.00 (reference)	1.17 (0.76–1.80)	0.85 (0.54–1.36)	0.52
Model 3§	1.00 (reference)	1.20 (0.78–1.87)	0.94 (0.58–1.53)	0.85
Men and women combined				0.93‖
Model 1† ¶	1.00 (reference)	1.07 (0.84–1.36)	0.73 (0.56–0.94)	0.01
Model 2‡ ¶	1.00 (reference)	1.07 (0.83–1.36)	0.75 (0.58–0.97)	0.03
Model 3§ ¶	1.00 (reference)	1.10 (0.85–1.40)	0.83 (0.63–1.08)	0.19

*Statistical tests for trend (two-sided) were assessed by assigning ordinal values to tertiles of each measurement.
†Adjusted for age, screening period, and duration of fasting.
‡Model 1 + cigarette smoking, alcohol drinking, family history of colorectal cancer, and nonsteroidal anti-inflammatory drug use.
§Model 2 + BMI.
‖Values are P interaction instead of P trend.
¶Further adjusted for sex.

below the minimum detection levels were excluded. Despite the sex differences in plasma concentrations of adiponectin, a significant effect modification by sex was not seen for either total or HMW adiponectin (P interaction = 0.68 and 0.93, respectively).

Association of leptin and TNF-α with colorectal adenoma

We also investigated the association of leptin and TNF-α with colorectal adenoma (Table 3). When men and women were combined according to sex-specific tertiles of leptin, a significant trend of increasing adjusted ORs across tertiles was observed (P trend < 0.001) with a significantly elevated OR for the highest tertile (OR, 1.57; 95% CI, 1.21–2.02). On additional adjustment for BMI, the positive association between leptin and colorectal adenoma was considerably attenuated (P trend = 0.10). In contrast, no material association was seen between TNF-α and colorectal adenoma. When leptin and TNF-α levels were treated as a continuous variable in model 2, adjusted ORs of colorectal adenoma for a 1 ng/mL increase in leptin and a 1 pg/mL increase in TNF-α were 1.03 (95% CI, 1.01–1.05) and 0.99 (95% CI, 0.96–1.02), respectively (data not shown). In this analysis of leptin, 57 subjects below the minimum detection levels were excluded. Again, effect modification by sex was not observed for either leptin or TNF-α (P interaction = 0.53 and 0.42, respectively).

Association of total and HMW adiponectin with colorectal adenoma according to tertiles of leptin and TNF-α

We then examined whether adiponectin interacted with leptin or TNF-α to modify its association with colorectal

Table 3. Association of leptin and TNF-α with colorectal adenoma

Measurement	Tertile			P trend*
	Lowest	Middle	Highest	
	OR (95% CI)	OR (95% CI)	OR (95% CI)	
Leptin				
Men, range (pg/mL)	–1,756	1,757–3,842	3,843–	
Model 1†	1.00 (reference)	1.29 (0.94–1.78)	1.69 (1.24–2.30)	0.001
Model 2‡	1.00 (reference)	1.30 (0.94–1.80)	1.73 (1.26–2.38)	<0.001
Model 3§	1.00 (reference)	1.18 (0.84–1.67)	1.44 (0.99–2.08)	0.05
Women, range (pg/mL)	–3,856	3,857–7,908	7,909–	
Model 1†	1.00 (reference)	1.31 (0.85–2.03)	1.36 (0.88–2.10)	0.18
Model 2‡	1.00 (reference)	1.23 (0.78–1.93)	1.36 (0.87–2.13)	0.18
Model 3§	1.00 (reference)	1.15 (0.71–1.86)	1.11 (0.65–1.92)	0.70
Men and women combined				0.53‖
Model 1† ¶	1.00 (reference)	1.30 (1.00–1.67)	1.55 (1.21–2.00)	<0.001
Model 2‡ ¶	1.00 (reference)	1.28 (0.99–1.66)	1.57 (1.21–2.02)	<0.001
Model 3§ ¶	1.00 (reference)	1.17 (0.89–1.54)	1.29 (0.95–1.74)	0.10
TNF-α				
Men, range (pg/mL)	–2.38	2.39–2.97	2.98–	
Model 1†	1.00 (reference)	1.19 (0.87–1.62)	1.01 (0.74–1.38)	0.97
Model 2‡	1.00 (reference)	1.24 (0.90–1.69)	0.97 (0.70–1.34)	0.85
Model 3§	1.00 (reference)	1.24 (0.90–1.70)	0.94 (0.68–1.30)	0.70
Women, range (pg/mL)	–2.22	2.23–2.79	2.80–	
Model 1†	1.00 (reference)	0.98 (0.64–1.49)	0.74 (0.47–1.15)	0.18
Model 2‡	1.00 (reference)	0.88 (0.56–1.37)	0.69 (0.43–1.10)	0.11
Model 3§	1.00 (reference)	0.85 (0.54–1.33)	0.65 (0.41–1.05)	0.07
Men and women combined				0.42‖
Model 1† ¶	1.00 (reference)	1.11 (0.87–1.42)	0.91 (0.71–1.18)	0.47
Model 2‡ ¶	1.00 (reference)	1.15 (0.89–1.48)	0.88 (0.68–1.14)	0.34
Model 3§ ¶	1.00 (reference)	1.13 (0.88–1.46)	0.85 (0.65–1.10)	0.21

*Statistical tests for trend (two-sided) were assessed by assigning ordinal values to tertiles of each measurement.
†Adjusted for age, screening period, and duration of fasting.
‡Model 1 + cigarette smoking, alcohol drinking, family history of colorectal cancer, and nonsteroidal anti-inflammatory drug use.
§Model 2 + BMI.
‖Values are P interaction instead of P trend.
¶Further adjusted for sex.

Table 4. Association of total adiponectin with colorectal adenoma according to tertiles of leptin and TNF-α

Measurement	Tertiles for total adiponectin*			*P* trend†
	Lowest	Middle	Highest	
	OR (95% CI)	OR (95% CI)	OR (95% CI)	
Leptin‡ §				0.007‖
Highest tertile	1.00 (reference)	0.78 (0.52–1.15)	0.70 (0.44–1.09)	0.05
Middle tertile	1.02 (0.68–1.53)	0.85 (0.57–1.28)	0.40 (0.25–0.64)	<0.001
Lowest tertile	0.52 (0.32–0.84)	0.69 (0.43–1.09)	0.71 (0.45–1.10)	0.21
TNF-α§ ¶				0.20‖
Highest tertile	1.00 (reference)	1.03 (0.68–1.58)	0.57 (0.35–0.93)	0.04
Middle tertile	1.33 (0.87–2.02)	1.00 (0.65–1.55)	1.19 (0.76–1.86)	0.96
Lowest tertile	1.24 (0.80–1.93)	1.14 (0.74–1.76)	0.77 (0.49–1.21)	0.01

*Cutoff points were 3.64 and 5.26 μg/mL for men and 5.76 and 8.49 μg/mL for women.

†Statistical tests for trend (two-sided) were assessed by assigning ordinal values to tertiles of each measurement.

‡Cutoff points were 1,756 and 3,842 pg/mL for men and 3,856 and 7,908 pg/mL for women.

§Adjusted for age, screening period, duration of fasting, sex, cigarette smoking, alcohol drinking, family history of colorectal cancer, nonsteroidal anti-inflammatory drug use, and BMI.

‖Values are *P* interaction instead of *P* trend.

¶Cutoff points were 2.38 and 2.97 pg/mL for men and 2.22 and 2.79 pg/mL for women.

adenoma. In this analysis, men and women were combined according to sex-specific tertiles of adiponectin, and stratified by leptin and TNF-α, respectively, based on sex-specific tertiles for controls. We observed a statistically significant interaction of total adiponectin with leptin (*P* interaction = 0.007), but not with TNF-α (*P* interaction = 0.20; Table 4). Compared with those in the lowest tertile of total adiponectin and highest tertile of leptin, those in the lowest tertiles of total adiponectin and leptin showed a statistically significant decrease in OR for colorectal adenoma (OR, 0.52; 95% CI, 0.32–0.84). However, a further decrease in ORs was not seen with increasing levels of total adiponectin among those in the lowest tertile of leptin (*P* trend = 0.21). In contrast, those in the middle and highest tertiles of leptin showed an inverse association between total adiponectin and colorectal adenoma. An inverse association was more prominent among those in the middle tertile of leptin (*P* trend < 0.001), with a significantly reduced OR of colorectal adenoma for the highest tertile of total adiponectin (OR, 0.40; 95% CI, 0.25–0.64). Of note, increasing levels of leptin were associated with elevated ORs of colorectal adenoma only among those in the lowest tertile of total adiponectin (*P* trend = 0.01; data not shown). After adjustment for BMI and other potential confounders, ORs for the lowest, middle, and highest tertiles of leptin were 1.00 (reference), 1.96 (95% CI, 1.21-3.17), and 1.92 (95% CI, 1.19-3.11), respectively, in the lowest tertile of total adiponectin (data not shown). If the above analysis of total adiponectin and leptin was repeated without interaction terms, mutually adjusted ORs of colorectal adenoma for the lowest, middle, and highest tertiles were 1.00 (reference), 0.90 (95% CI, 0.70–1.15), and 0.71 (95% CI, 0.54–0.93), respectively, for total adiponectin, whereas the corresponding values were 1.00 (reference), 1.13 (95% CI, 0.86–1.49), and 1.25 (95% CI, 0.92–1.69), respectively, for leptin (data not shown). In accordance with the above results, we observed a marginally significant interaction of HMW adiponectin with leptin (*P* interaction = 0.07), but not with TNF-α (*P* interaction = 0.21; Table 5). Again, these results were not essentially changed by additional adjustment for indicators of energy balance, dietary factors, and metabolic factors (*P* interaction with leptin = 0.006 and 0.07 for total and HMW adiponectin, respectively; data not shown). Results were essentially the same when the above analysis was conducted for men and women separately (*P* interaction of total adiponectin with leptin = 0.04 and 0.01 for men and women, respectively; data not shown).

Discussion

In this study, we observed an inverse association between total adiponectin and colorectal adenoma with statistical significance. This association remained significant, albeit considerably attenuated, after further adjustment for BMI, a major determinant of insulin resistance (2), suggesting that adiponectin may decrease the risk of colorectal neoplasia through mechanisms other than the indirect mechanism through insulin resistance. We also observed an inverse association of HMW adiponectin with colorectal adenoma, although significance was lost with additional adjustment for BMI. HMW adiponectin has a potent insulin-sensitizing effect, whereas circulating levels of HMW adiponectin and the degree of insulin sensitivity are determined mainly by the amount of adipose tissue (2, 3). Given that improved insulin sensitivity has been related to a decreased risk of

colorectal neoplasia (1), the HMW form of adiponectin may mediate the association between adiposity and colorectal neoplasia through its well-recognized influence on insulin resistance.

Our observations for colorectal adenoma agree with those for colorectal cancer from a case-control study nested in the Health Professionals Follow-up Study (13), in which a statistically significant inverse association was seen between plasma adiponectin level and the risk of colorectal cancer. Several clinical studies have also provided supportive evidence that patients with colorectal neoplasia had lower circulating levels of adiponectin than controls, although these studies were small (19–21). However, circulating adiponectin levels were not associated with risk in a case-control study of colorectal adenoma in a Japanese population (12) or in nested case-control studies of colorectal cancer in Norwegian and Swedish populations (14, 15). In contrast, the only epidemiologic investigation of HMW adiponectin in relation to the risk of colorectal neoplasia reported results inconsistent with ours (12). To date, epidemiologic evidence for the association of total and HMW adiponectin with colorectal neoplasm is both sparse and controversial, and further studies to corroborate our results are needed.

To our knowledge, this is the first study to provide epidemiologic evidence that adiponectin and leptin interact to modify the risk of colorectal adenoma separate to their profound involvement in insulin resistance. After adjusting for BMI and other potential confounders, an inverse association of adiponectin with colorectal adenoma was apparent in the highest two tertiles of leptin, particularly the middle, whereas a positive association of leptin was obvious in the lowest tertile of adiponectin. A recent basic research study in a model of preneoplastic colon epithelial cells analogously showed that adiponectin inhibited multiple signaling cascades associated with leptin-induced cell proliferation (8). These findings lead to the hypotheses that adiponectin may exert an anticarcinogenic effect on the large intestine by interfering with leptin, and that leptin could conversely exert a carcinogenic effect under conditions of a lower abundance of adiponectin. This interaction would be independent to their well-documented influences on insulin resistance. These hypotheses require further interdisciplinary examination.

Among the strengths of the present study, the provision of total colonoscopy to all study subjects likely decreased the possibility of misclassification between cases and controls. Also, the number of subjects was considerably larger than in previous studies of the association between circulating levels of adiponectin and colorectal neoplasia (12–15).

A major limitation of this study is its cross-sectional nature, and the observed associations might be due to reverse causality. In contrast to colorectal cancer, however, it is unlikely that colorectal adenoma affects the amount of adipose tissue, a major determinant of circulating adiponectin levels (3), because colorectal adenoma is an asymptomatic benign tumor. A second limitation is the relatively small body size of the study population: Given that median BMI for male and female controls was 23.4 and 21.8 kg/m^2, respectively, and the prevalence of overweight and obesity was 26% and 15%, respectively, our observations may not be directly applicable to severely obese populations, often found in North American and European countries, where more than half of adults are overweight or obese (22).

Table 5. Association of HMW adiponectin with colorectal adenoma according to tertiles of leptin and TNF-α

Measurement	Tertiles for HMW adiponectin*			*P* trend†
	Lowest	Middle	Highest	
	OR (95% CI)	OR (95% CI)	OR (95% CI)	
Leptin‡ §				0.07‖
Highest tertile	1.00 (reference)	1.03 (0.70–1.53)	0.73 (0.47–1.15)	0.16
Middle tertile	1.00 (0.66–1.51)	0.93 (0.62–1.39)	0.60 (0.38–0.94)	0.05
Lowest tertile	0.49 (0.30–0.82)	0.87 (0.55–1.37)	0.77 (0.50–1.20)	0.13
TNF-α§ ¶				0.21‖
Highest tertile	1.00 (reference)	1.47 (0.96–2.26)	0.76 (0.47–1.22)	0.36
Middle tertile	1.46 (0.95–2.23)	1.39 (0.90–2.13)	1.46 (0.93–2.31)	0.62
Lowest tertile	1.45 (0.92–2.29)	1.37 (0.89–2.11)	1.03 (0.65–1.61)	0.06

*Cutoff points were 0.88 and 1.91 μg/mL for men and 2.19 and 3.90 μg/mL for women.
†Statistical tests for trend (two-sided) were assessed by assigning ordinal values to tertiles of each measurement.
‡Cutoff points were 1,756 and 3,842 pg/mL for men and 3,856 and 7,908 pg/mL for women.
§Adjusted for age, screening period, duration of fasting, sex, cigarette smoking, alcohol drinking, family history of colorectal cancer, nonsteroidal anti-inflammatory drug use, and BMI.
‖Values are *P* interaction instead of *P* trend.
¶Cutoff points were 2.38 and 2.97 pg/mL for men and 2.22 and 2.79 pg/mL for women.