

A Regulatory Polymorphism at Position –309 in *PTPRCAP* Is Associated with Susceptibility to Diffuse-type Gastric Cancer and Gene Expression¹

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Abstract

PTPRCAP (CD45-AP) is a positive regulator of protein tyrosine phosphatase PTPRC (CD45), which activates Src family kinases implicated in tumorigenesis. Single-nucleotide polymorphism (SNP) rs869736 located at position –309 of the *PTPRCAP* promoter was associated with susceptibility to diffuse-type gastric cancer in the current case-control study. The minor-allele homozygote was significantly associated with a 2.5-fold increased susceptibility to diffuse-type gastric cancer ($P = .0021$, $n = 252$), but not to intestinal-type ($P = .30$, $n = 178$), versus the major-allele homozygote, when comparing unrelated Korean patients with healthy controls ($n = 406$). Nine other SNPs were in nearly perfect linkage disequilibrium ($r^2 \geq 0.97$) with this SNP, exhibiting the same association, and spread out for 26 kb on chromosome 11q13.1 covering *RPS6KB2*, *PTPRCAP*, *CORO1B*, and *GPR152*. Among the four genes, however, only *PTPRCAP* expression was affected by haplotypes of the 10 SNPs. Endogenous transcript levels of *PTPRCAP* were linearly correlated with copy numbers (0, 1, and 2) of the risk-haplotype ($P = .0060$) in 12 lymphoblastoid cells derived from blood samples, but those of the other three genes were not. Furthermore, the cancer-risk, minor-allele T of rs869736 increased both promoter activity and specific nuclear protein-binding affinity than the nonrisk, major-allele G in luciferase reporter and electrophoretic mobility shift assays, respectively. Accordingly, the minor allele of rs869736 in the *PTPRCAP* promoter is associated with increased susceptibility to diffuse-type gastric cancer by increasing *PTPRCAP* expression, possibly leading to activation of the oncogenic Src family kinases.

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Abbreviations: CI, confidence interval; EMSA, electrophoretic mobility shift assay; LD, linkage disequilibrium; OR, odds ratio; PCR, polymerase chain reaction; SFK, Src family kinase; SNP, single-nucleotide polymorphism; UTR, untranslated region

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Introduction

Gastric cancer is a complex disease, and many environmental and genetic factors are involved in its development. *Helicobacter pylori* infection, smoking, and dietary habits are well-established environmental risk factors [1–4]. Human genetic polymorphisms of *IL1B*, *IL1R2*, *IL10*, *TLR4*, *TNF*, and *PSCA* have been associated with susceptibility to gastric cancer [5–8]. All of these, except for *PSCA*, are related to proinflammatory signals and may accelerate the cancerous changes such as inflammation, hypochlorhydria, and atrophic gastritis, which can explain the sequential events of intestinal-type gastric carcinogenesis. *PSCA* was identified as a diffuse-type gastric cancer gene in a genome-wide association study and is possibly involved in regulating gastric epithelial cell proliferation [8].

Notably, most genetic associations identified to date are specific for histologic subtypes of gastric cancer, and it reflects their distinct features in histologic, pathologic, and epidemiological aspects [9]. Intestinal-type gastric cancer develops through a series of precancerous changes known as Correa sequence, superficial gastritis, chronic atrophic gastritis, intestinal metaplasia, dysplasia, and adenocarcinoma [10]. In contrast, diffuse-type gastric cancer is not associated with atrophic gastritis or intestinal metaplasia, and lacks well-recognized precursor changes [11–13]. *H. pylori* gastritis is the only common precursor and a major risk factor in both types of gastric cancer [2,11].

Reversible tyrosine phosphorylation, which is regulated by the balanced yin-yang (complementary opposites within a greater whole) action of protein tyrosine kinases and protein tyrosine phosphatases, plays an important role in cell proliferation, adhesion, and migration [14]. The dynamic interactions between protein tyrosine kinases and phosphatases act as molecular switches for signal transduction pathways [15], and deregulated protein tyrosine phosphorylation is associated with tumorigenesis [14]. The protein tyrosine phosphatase receptor type C (PTPRC), also known as CD45, transmits important cellular signals by dephosphorylating the inhibitory tyrosine residue of Src family kinases (SFKs) [16], which are implicated in tumor progression and metastasis [17,18]. The phosphatase activity of PTPRC is enhanced by interaction with a transmembrane protein, PTPRC-associated protein (PTPRCAP), also known as CD45-AP [19–22]. However, little is known about the relation between PTPRCAP and gastric cancer.

In this study, the first evidence for genetic association of PTPRCAP with susceptibility to diffuse-type gastric cancer is presented. A promoter single-nucleotide polymorphism (SNP) G>T at position -309 of PTPRCAP affects the promoter activity and is associated with *in vivo* expression levels of PTPRCAP and susceptibility to diffuse-type gastric cancer.

Materials and Methods

Study Subjects

This study included 836 unrelated Korean subjects including 430 patients with gastric cancer and 406 healthy controls. The gastric cancers were classified either diffuse- ($n = 252$) or intestinal-type ($n = 178$) according to the Lauren system by two independent pathologists, and the mixed-type or ambiguous cases were not included in the study. The tumor stages at diagnosis were diverse: stage IA ($n = 42$), IB ($n = 66$), II ($n = 86$), IIIA ($n = 106$), IIIB ($n = 58$), and IV ($n = 72$) according to the AJCC Cancer Staging Manual, and 58 of them were early and 372 were advanced gastric carcinoma by

tumor depth. All the controls were confirmed free of the disease by health examinations including an endoscopy or an upper gastrointestinal track radiography. Patients were aged 57.3 ± 12.9 years (mean \pm SD), ranging from 23 to 86 years, whereas the controls were 52.1 ± 8.4 years old, ranging from 39 to 76 years. The male-to-female ratio was 2.0 in patients and 2.3 in controls. These subjects were recruited with written informed consent at Seoul National University Hospital, Hanyang University Guri Hospital, and Inje University Seoul Paik Hospital in the Seoul metropolitan area and at Chungnam National University Hospital and Eulji University Hospital in Daejeon city, with an approval from the institutional review board of Hanyang University Medical Center, and genotyped with an approval from the institutional review board of KAIST.

Genotyping and Sequencing

Genomic DNA was extracted from blood samples using the Puregene DNA purification kit (Gentra, Minneapolis, MN) or from frozen nontumorous gastric tissues using DNeasy tissue kit (Qiagen, Hilden, Germany), and quantified using the double-stranded DNA-specific fluorescent dye, PicoGreen (Molecular Probes, Eugene, OR). The final concentration of each DNA sample was adjusted to 2.5 to 10 ng/ μ l for genotyping assays. SNPs were genotyped using the MassARRAY system (Sequenom, San Diego, CA) according to the manufacturer's instructions. The overall genotyping success rate was 99.6% for the 23 SNPs, and genotype frequencies of the control subjects in all SNPs were under Hardy-Weinberg equilibrium.

Polymerase chain reaction (PCR) was performed using *Pfu* DNA polymerase (Solgent, Daejeon, Korea), and the products were purified from agarose gels using AccuPrep gel purification kit (Bioneer, Daejeon, Korea). Genomic DNA from two subjects, a younger (43 years old) patient with an advanced (stage IV) diffuse-type gastric cancer and an older (69 years old) healthy control were sequenced by the Solgent Corp. All primers for genotyping and sequencing analysis were synthesized by the Bioneer Corp.

Statistical Analyses

Allelic association of individual SNP with susceptibility to diffuse- or intestinal-type gastric cancer and Hardy-Weinberg equilibrium in the control subjects were assessed by χ^2 tests. Haplotypes were constructed using PHASE 2.1 and linkage disequilibrium (LD) maps using Haploview 4.1 [23–25]. In logistic regression analyses of genotype and diplotype associations, odds ratio (OR) and 95% confidence interval (CI) were adjusted for age and sex using SPSS 11.5 because the age distribution and sex ratio were different between the cases and controls.

Real-time Reverse Transcription-PCR

Genomic DNA was isolated from 12 lymphoblastoid cell lines and genotyped for rs1808279. Total RNA was extracted using NucleoSpin (Macherey-Nagel, Düren, Germany), and the first-strand complementary DNA was synthesized using an oligo(dT) primer and Improm-II reverse transcription (Promega, Madison, WI). Real-time PCR was performed using the Bio-Rad iQ SYBR Green Supermix and iCycler iQ5 machine. The first-strand complementary DNA and RNA (as a negative control) samples were amplified for four genes (*PTPRCAP*, *RPS6KB2*, *CORO1B*, and *GPR152*), and the amounts were normalized against that of *GAPDH*.

Luciferase Reporter Assays

DNA segments containing one of the two alleles in an SNP was amplified by PCR using *Pfu* DNA polymerase (Solgent) and cloned into the *KpnI* and *XhoI* sites of pGL3-Basic vector (Promega) or to the *XbaI* site of pGL3-Promoter vector. Especially, a *PTPRCAP* promoter region was amplified using previously reported primer sequences [26]. All the recombinant constructions were confirmed by sequencing analysis.

MKN28 cells (2×10^5), maintained in RPMI1640 (Gibco, Rockville, MD) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), were transfected with 1 μ g of plasmid and 50 ng of pRL-CMV control vector in 12-well tissue culture plates using Lipofectamine LTX reagent (Invitrogen, Carlsbad, CA). Cells were harvested at 36 hours after transfection, and luciferase activities were measured using the dual-luciferase reporter assay system (Promega). All experiments were performed in triplicate.

Electrophoretic Mobility Shift Assays

Double-stranded oligonucleotides for electrophoretic mobility shift assay (EMSA) were generated using complementary oligonucleotides 5'-AACCTGGAGACAGAGGATGTCACAGGAGT-3' and 5'-ACTCCTGTGACATCCTCTGTCTCCAGGTT-3' for the -309G carrier, and 5'-AACCTGGAGACAGATGATGTCACAGGAGT-3' and 5'-ACTCCTGTGACATCATCTGTCTCCAGGTT-3' for the -309T carrier (the SNP-corresponding position is underlined). Double-stranded probes were radioactively labeled with [γ - 32 P]dATP (Perkin Elmer, Wellesley, MA) at the 5' ends using the T4 polynucleotide kinase (Takara Bio, Shiga, Japan), and unincorporated [γ - 32 P]dATP was removed using the PROBER probe DNA purifying system (iNtRON Biotechnology, Seongnam, Korea).

A nuclear extract (8 μ g) from MKN28 cells and 0.6 μ g of poly(dI-dC) were preincubated with or without 200-fold molar excess of unlabeled competitor in 1 \times EMSA reaction buffer (10 mM HEPES-KOH, pH 7.9, 60 mM KCl, 1 mM EDTA, 10% glycerol, 10 mM MgCl₂, and 200 μ M dithiothreitol) for 20 minutes at room temperature. Incubation was continued with addition of a radioactively labeled probe (80 fmol) for another 20 minutes at room temperature. The whole 20 μ l of the reaction product was subjected to gel electrophoresis (6% polyacrylamide gel in 0.25 \times Tris/borate/EDTA running buffer at 180 V for 2.5 hours). Finally, the gel was scanned by BAS-3000 (Fujifilm, Tokyo, Japan).

Prediction for Transcription Factor Binding

Transcription factors that can bind to the *PTPRCAP* SNP rs869736 at position -309 in an allele-specific manner were predicted for the two 30-bp sequences, 5'-AACCTGGAGACAGA[G/T]GATGTCACAGGAGTC-3' using seven different programs: AliBaba 2.1 (BIOBASE, Wolfenbuettel, Germany), Match 1.0 (BIOBASE), MatInspector (Genomatix, Munich, Germany), Patch 1.0 (BIOBASE), TESS (<http://www.cbil.upenn.edu/cgi-bin/tess/tess>), TFSEARCH (<http://www.cbrc.jp/research/db/TFSEARCH.html>), and Tfsitescan (<http://www.ifti.org/cgi-bin/ifti/Tfsitescan.pl>). Their default settings were used, but target organisms were restricted to vertebrates or mammals.

Results

A 26-kb Locus on 11q13.1 Associated with Diffuse-type Gastric Cancer

Many SNPs around the *PTPRCAP* gene on human chromosome 11q13.1 were in high LD with one another according to the geno-

type data from the International HapMap Project (Figure 1A). From a 430-kb region surrounding *PTPRCAP*, 18 SNPs were selected and genotyped for 252 unrelated Korean patients with diffuse-type gastric cancer and 406 healthy controls. Among them, seven SNPs (rs1476792 and rs1790753 in *RPS6KB2*; rs869736 in *PTPRCAP*; rs872375, rs2302264, and rs1808279 in *CORO1B*; and rs1790761 in *GPR152*) showed significant allelic associations with susceptibility to diffuse-type gastric cancer (Table 1).

These seven associated SNPs were very tightly linked to each other ($r^2 \geq 0.97$), together constituting an LD block of 26 kb, and other 11 SNPs located outside this LD block were not associated (Figure 1A). Thus, a gastric cancer susceptibility locus was localized to a 26-kb block containing four genes, *RPS6KB2*, *PTPRCAP*, *CORO1B*, and *GPR152* by the seven SNPs very tightly linked together (Figure 1B).

Haplotypes Associated with Diffuse-type Gastric Cancer

To find more polymorphisms that are tightly linked to the seven associated SNPs and potentially affect protein activity or gene expression, all the exons, exon-intron junctions, and promoters (1-1.5 kb from the transcription start site) of the four genes in genomic DNA from two extreme individuals were sequenced. The two individuals were a younger patient with an advanced diffuse-type gastric cancer who was homozygous for the seven risk alleles and an older healthy control homozygous for the nonrisk alleles. Between the two individuals, the alleles were also different in five additional SNPs, all located in *RPS6KB2*: rs55987642, rs13859, rs4930427, rs10274, and rs1790752. Genotyping of these additional five SNPs disclosed that only three of them, rs4930427, rs1790752, and rs13859, were significantly associated with the diffuse-type gastric cancer (Table 1). From a total of 23 SNPs tested, 10 SNPs were significantly associated with diffuse-type gastric cancer and in nearly perfect LD with each other ($r^2 \geq 0.97$).

All the 10 SNPs passed a significance level of association adjusted for multiple testing. The adjusted significance level ($\alpha = 1 - (1 - 0.05)^{1/13.86} = 0.0037$) was calculated with the effective degree of freedom estimated for the set of SNP genotypes in LD, using the EDF software [27]. Moreover, four SNPs of these passed even the significance level of Bonferroni correction ($\alpha = 1 - (1 - 0.05)^{1/23} = 0.0022$) for multiple testing; however, the Bonferroni method could be an over-correction in cases with very tightly linked SNPs, similar to this case.

When haplotypes were constructed with the 10 significantly associated SNPs (their *P* values are underlined in Table 1), two haplotypes, TCGCCGGGAA (designated as H1) and CTATTTAAGG (H2), in the order of SNP nucleotide positions listed in Table 1, constituted 98.1% of entire haplotypes, and only a small portion of the subjects had other haplotypes (1.4% in controls and 2.8% in diffuse-type cancer patients). The H2 haplotype with the risk allele at every SNP was significantly associated with susceptibility to diffuse-type cancer (OR = 1.43, *P* = .00340) versus the H1 with the nonrisk alleles at all SNPs. Logistic regression analysis of diplotype association revealed 2.50- and 1.46-fold higher odds of having diffuse-type cancer in the H2/H2 homozygotes (*P* = .0021) and H1/H2 heterozygotes (*P* = .030), respectively, in comparison with the H1/H1 homozygotes (Table 2). Thus, the 10-SNP haplotypes were associated with susceptibility to diffuse-type gastric cancer.

Association of the Risk Haplotype with Increased In Vivo Transcript Levels of *PTPRCAP*

As explained in the previous section, a diffuse-type gastric cancer susceptibility locus was mapped to a 26-kb region, which contains four

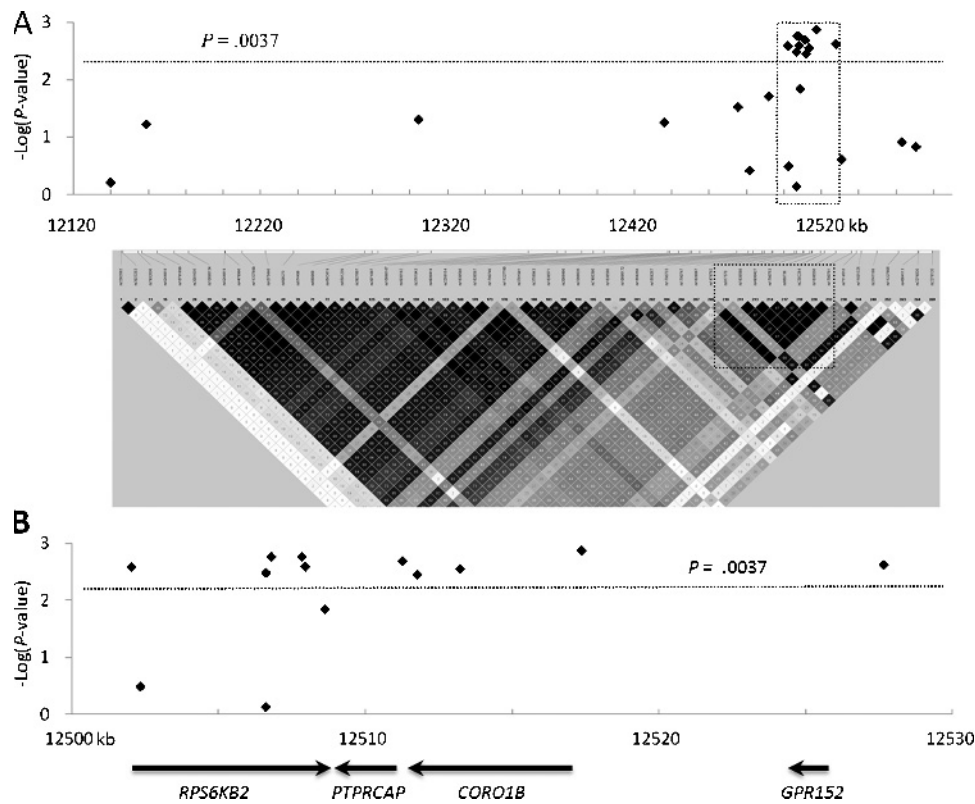


Figure 1. Association profile and LD map of all 23 tested SNPs. (A) *P* values in negative logarithm (y axis) for allelic associations with diffuse-type gastric cancer susceptibility are shown against chromosome positions (x axis) of 23 SNPs in the upper half. An LD map of 430-kb region generated by the genotype data of 56 SNPs with minor allele frequencies of 0.1 or higher in the International HapMap CHB + JPT population is shown in the lower half. (B) A detailed view of the 30-kb region boxed in A. The region contains four genes and the 10 SNPs significantly associated with the susceptibility to diffuse-type gastric cancer ($.0013 \leq P \leq .0036$).

Table 1. Allelic Association Tests of 23 SNPs with Diffuse-type Gastric Cancer.

SNP	Gene	Function	Allele*	MAF [†]	OR (95% CI) [‡]	<i>P</i> [‡]
rs2282502	<i>RHOD</i>	Asp88	C>T	0.42	0.95 (0.75-1.18)	.62
rs10791899		Intergenic	A>C	0.22	1.28 (0.99-1.66)	.060
rs3927807	<i>FBXL11</i>	Intron	G>A	0.23	1.29 (1-1.67)	.050
rs1790740		Intergenic	T>C	0.23	1.28 (0.99-1.65)	.056
rs7480390		Intergenic	C>G	0.23	1.33 (1.03-1.71)	.030
rs10896172	<i>TBC1D10C</i>	Intron	T>C	0.18	1.13 (0.85-1.5)	.39
rs1790733	<i>KIAA1394</i>	Intron	T>C	0.25	1.34 (1.05-1.72)	.020
rs1476792	<i>RPS6KB2</i>	Intron	T>C	0.29	1.44 (1.13-1.82)	.0026
rs917570	<i>RPS6KB2</i>	Intron	C>G	0.18	1.16 (0.87-1.54)	.32
rs55987642	<i>RPS6KB2</i>	Pro267Leu	C>T	0.07	1.08 (0.7-1.67)	.74
rs4930427	<i>RPS6KB2</i>	Phe269	C>T	0.29	1.43 (1.12-1.81)	.0033
rs1790753	<i>RPS6KB2</i>	Intron	G>A	0.29	1.46 (1.15-1.85)	.0017
rs1790752	<i>RPS6KB2</i>	Intron	C>T	0.29	1.46 (1.15-1.85)	.0017
rs13859	<i>RPS6KB2</i>	Ala420Val	C>T	0.29	1.44 (1.13-1.82)	.0026
rs10274	<i>RPS6KB2</i>	3' UTR	G>A	0.25	1.36 (1.06-1.75)	.014
rs869736	<i>PTPRCAP</i>	Promoter	G>T	0.29	1.45 (1.14-1.83)	.0020
rs872375	<i>CORO1B</i>	Intron	G>A	0.29	1.42 (1.12-1.8)	.0036
rs2302264	<i>CORO1B</i>	Intron	G>A	0.29	1.43 (1.13-1.81)	.0028
rs1808279	<i>CORO1B</i>	Promoter	A>G	0.29	1.47 (1.16-1.86)	.0013
rs1790761	<i>GPR152</i>	Promoter	A>G	0.29	1.44 (1.14-1.82)	.0024
rs17501521	<i>CABP4</i>	Intron	C>T	0.11	1.22 (0.87-1.72)	.25
rs4084113	<i>AIP</i>	Intron	G>A	0.18	1.24 (0.94-1.64)	.12
rs2276120	<i>PITPNM1</i>	Intron	C>T	0.18	1.23 (0.93-1.62)	.15

Significant values after correction for multiple testing ($P \leq .0037$) are underlined.

*Major allele > minor allele.

[†]Minor allele frequency in controls.

[‡]Crude OR, 95% CI, and *P* for allelic association test.

genes, *RPS6KB2*, *PTPRCAP*, *CORO1B*, and *GPR152* (Figure 1B). Because the strong LD of this locus did not allow for a further fine mapping by association analysis, functional analyses were then tried to identify the susceptibility gene(s). Abnormal protein activity or altered gene expression may cause an increased susceptibility to cancer. In this susceptibility locus, two coding SNPs in *RPS6KB2* showed significant association, but there was no amino acid change (Phe269) in rs4930427 or the change was too small (Ala420Val) in rs13859 to alter protein activity dramatically.

The next step involved investigating whether expression of any of the four genes was altered by the risk haplotype *in vivo*. Twelve

Table 2. Association of Haplotypes with Diffuse-type Gastric Cancer.

Category	Control	Diffuse-type	OR (95% CI)*	<i>P</i> [*]
Haplotype [†]	<i>n</i> = 812	<i>n</i> = 504		
H1 (TCGCCGGGAA)	571 (70.3%)	311 (61.7%)	1	
H2 (CTATT'TAAGG)	230 (28.3%)	179 (35.5%)	1.43 (1.12-1.82)	.0034
Others	11 (1.4%)	14 (2.8%)		
Diplotype	<i>n</i> = 406	<i>n</i> = 252		
H1/H1	196 (48.3%)	92 (36.5%)	1	
H1/H2	176 (43.3%)	118 (46.8%)	1.46 (1.04-2.07)	.030
H2/H2	27 (6.7%)	30 (11.9%)	2.50 (1.39-4.47)	.0021
Others	7 (1.7%)	12 (4.8%)		

*Crude OR, 95% CI, and *P* values calculated in χ^2 tests for haplotype association, and age/sex-adjusted OR, 95% CI, and *P* values calculated in logistic regression analysis for diplotype association.

[†]In each haplotype, the alleles are listed in the SNP order of rs1476792, rs4930427, rs1790753, rs1790752, rs13859, rs869736, rs872375, rs2302264, rs1808279, and rs1790761.

lymphoblastoid cell lines derived from healthy individuals were genotyped for rs1808279, which was almost perfectly linked to all the other haplotype-constituent SNPs ($r^2 \geq 0.97$) and was most significantly associated with the diffuse-type cancer ($P = .0013$). They were categorized into three groups according to their diplotypes that were estimated by their rs1808279 genotypes: five samples were homozygous for the nonrisk haplotype (H1/H1), five samples were heterozygous (H1/H2), and two samples were homozygous for the risk haplotype (H2/H2). Total RNA was isolated from each cell line and expression levels of the four endogenous genes were measured by real-time reverse transcription-PCR, except for those of *GPR152*, which were too low to make reliable measurements.

As shown in Figure 2, the risk-haplotype copy number was significantly correlated with the endogenous transcript levels of *PTPRCAP* ($P = .0060$) in linear regression analysis but not with those of *RPS6KB2* ($P = .29$) or *CORO1B* ($P = .53$). The linear correlation was positive, supporting for association of *PTPRCAP* expression with the risk haplotype in an additive genetic model (Figure 2). *PTPRCAP* transcript levels of the H2/H2 homozygotes and H1/H2 heterozygotes were 35% and 18% higher than those of the H1/H1 homozygotes, respectively. Thus, the diffuse-type gastric cancer risk haplotype was associated with increased endogenous transcript levels of *PTPRCAP* but not with those of *RPS6KB2*, *CORO1B*, or *GPR152*.

A Promoter SNP at Position -309 in *PTPRCAP* Affecting Promoter Activity

The alteration of *PTPRCAP* transcript levels depending on the risk-haplotype copy number could be attributed to one SNP, rs869736 G>T, which was cancer-associated and located at position -309 of the *PTPRCAP* promoter. Contribution of this SNP to the altered gene expression was evaluated using luciferase reporter assays. A 610-bp DNA segment (from position -580 to +30) with either risk or non-risk allele at the SNP was cloned upstream of the luciferase gene in the pGL3-Basic plasmid, and luciferase activities were measured in MKN28 gastric cancer cells (Figure 3). The luciferase levels relative to the empty vector were 55% higher in the construct of the risk allele T-carrying promoter than in the nonrisk G carrier ($P = .00039$ in one-way analysis of variance). Thus, the risk allele T at position -309 of *PTPRCAP* increased the promoter activity and gene expression.

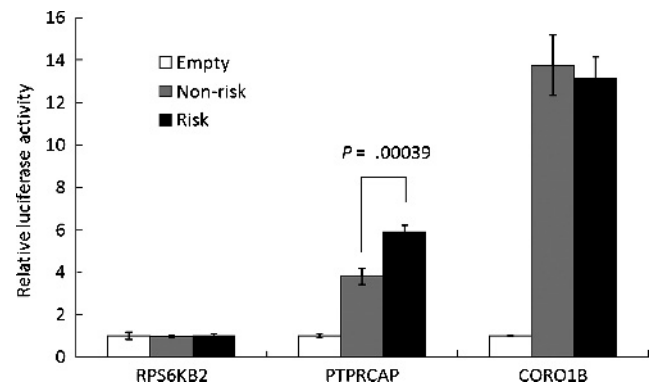


Figure 3. Luciferase reporter assays for evaluating allelic difference in regulatory activity of the associated SNPs. A promoter fragment with the risk or nonrisk allele in rs869736 (*PTPRCAP*) or rs1808279 (*CORO1B*) was cloned upstream of the luciferase gene in pGL3-Basic vector, and a 3'-UTR fragment with either allele in rs10274 (*RPS6KB2*) downstream of the gene in pGL3-Promoter vector. Relative luciferase activities were measured in the MKN28 gastric cancer cell line in triplicate. P values were calculated by one-way analysis of variance tests.

Three other cancer-associated SNPs, namely, rs10274, rs1808279, and rs1790761, were located in the 3' untranslated region (UTR) of *RPS6KB2* and in the promoter regions of *CORO1B* and *GPR152*, respectively, and were also tested for possibility of altering gene expression. The DNA segments containing a promoter SNP were cloned into the pGL3-Basic upstream of the luciferase gene, and those containing a 3'-UTR SNP were cloned into the pGL3-Promoter plasmid downstream of the luciferase gene. No allelic difference was detected in luciferase activity with respect to the UTR SNP in *RPS6KB2* or the promoter SNP in *CORO1B* (Figure 3), and no luciferase activity was observed with the constructs of the promoter SNP in *GPR152*. Thus, alternations of gene expression by the three SNPs were not observed.

Specific Nuclear Protein Binding to the -309 SNP in *PTPRCAP*

Because the SNP at position -309 of *PTPRCAP* affected the promoter activity, a possibility of its binding to a nuclear transcription

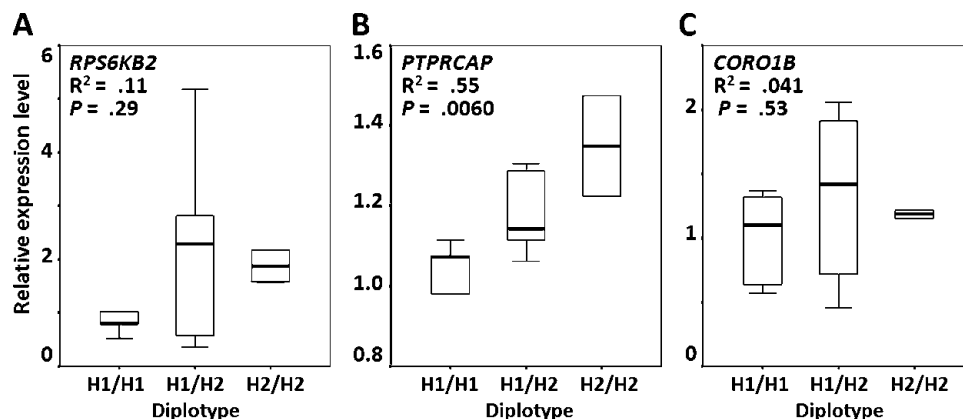


Figure 2. Relationship between the haplotypes and gene expression levels. Relative endogenous transcript levels of *RPS6KB2* (A), *PTPRCAP* (B), and *CORO1B* (C) were measured in five lymphoblastoid cell lines homozygous for the nonrisk haplotype (H1/H1), five heterozygotes (H1/H2), and two homozygotes with the risk haplotype (H2/H2) using real-time reverse transcription-PCR and displayed in box plots. The linear regression coefficient (R^2) and P values are shown. The haplotypes were estimated by the genotypes of rs1808279.

factor was tested using EMSA. Nuclear extracts from MKN28 cells and two radioactively labeled oligonucleotides of 29 bp carrying G or T in the SNP-corresponding position were used for the assays (Figure 4). A shifted band was observed with both variants, but the band intensity was much higher with the T-carrying variant (lane 6) than the G variant (lane 2). With 200-fold excess amounts of unlabeled probes of the two variants used in separate assays, the band intensities were markedly reduced for both variants (lanes 3, 4, 7, and 8), but the T variant showed a stronger competition than G variant (lanes 7 vs 8); this was consistent with the observation made earlier that the T variant had a stronger promoter activity than the G variant. Thus, the risk allele T at position -309 of PTPRCAP increased the specific affinity to a nuclear protein present in MKN28 cells versus the nonrisk allele G.

No Association with Intestinal-type Gastric Cancer

To test association of rs869736 at position -309 of PTPRCAP with susceptibility to intestinal-type gastric cancer, 178 unrelated patients with intestinal-type gastric cancer were additionally genotyped and compared with the 406 healthy controls. Either alleles or genotypes were not significantly associated with susceptibility to intestinal-type gastric cancer (Table 3). Although the minor allele T was more frequent in both diffuse- (37.1%) and intestinal-type (33.7%) cancers than in the controls (28.9%), the difference was significant only in diffuse-type cancer (diffuse-type, OR = 1.45, P = .0020, intestinal-type, OR = 1.25, P = .10). In logistic regression analysis, TT and GT genotypes showed 2.47- and 1.45-fold higher odds of having diffuse-type cancer than the GG genotype, respectively (P = .0021 and P = .031, respectively); however, the genotypes were not significantly associated with intestinal-type cancer risk (P = .30 and P = .87, respectively; Table 3). Thus, the association of rs869736 was specific for diffuse-type gastric cancer.

Discussion

This study is the first to find that PTPRCAP is a gastric cancer susceptibility gene specific to diffuse-type. The minor allele in SNP rs869736 (G>T) located at position -309 of the PTPRCAP promoter was significantly associated with a 1.45-fold increased susceptibility to diffuse-type gastric cancer versus the major allele (P = .0020). The minor-allele homozygote had a 2.47-fold higher odds than the major-allele homozygote (P = .0021). In contrast, either the alleles or genotypes were not significantly associated with susceptibility to intestinal-type gastric cancer. The results are consistent with a previous study that stated that the two types of gastric cancer develop through distinct pathways [11], although this type-specific association needs to be confirmed in other populations.

When compared with the nonrisk major allele G, the cancer-risk minor allele T at this SNP was observed to increase both promoter activity and the affinity to a nuclear protein, and as a consequence,

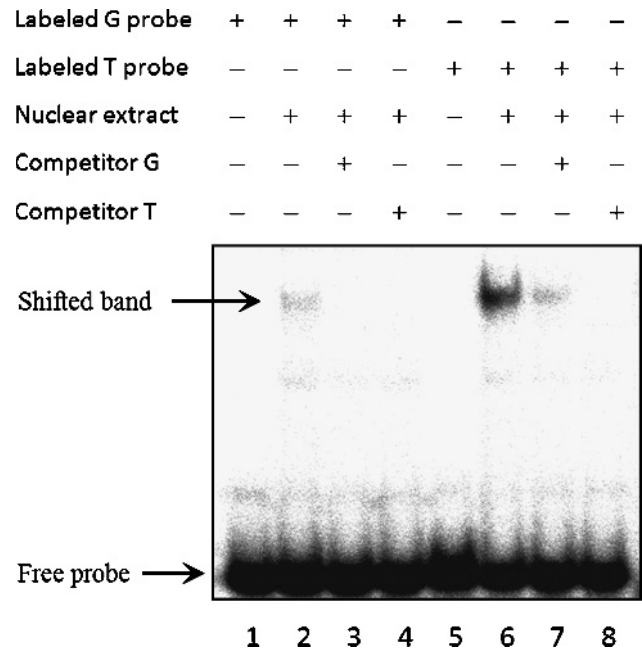


Figure 4. Electrophoretic mobility shift assays using DNA probes with the -309 SNP in PTPRCAP. A nuclear extract (8 μg) from MKN28 cells was incubated with a 29-bp [³²P]dATP-labeled DNA probe with either major (G) or minor (T) allele at the SNP-corresponding position (lanes 2 and 6). For competition assays, unlabeled DNA (200-fold excess) were pre-incubated with the nuclear extract before addition of the labeled probes (lanes 3, 4, 7, and 8).

the gene expression *in vitro* and *in vivo*. These results confirm that PTPRCAP expression is higher in human diffuse-type gastric cancer tissues compared with corresponding normal tissues [28]. The 1.55-fold difference in promoter activity between the two alleles observed in the luciferase assays conducted for this study using MKN28 gastric cancer cell lines is qualitatively consistent with the 2- and 1.3-fold differences in previous assays performed by other researchers using HEK297T and TE671 cell lines, respectively [26]. Several transcription activators such as AP-1, c-Jun, c-Fos, and CREB are predicted to prefer the T allele to G allele at this SNP based on the known sequence specificities of these transcription factors. Thus, the risk allele at position -309 of the PTPRCAP promoter could increase cancer susceptibility by enhancing PTPRCAP expression.

PTPRCAP (or CD45-AP) are functionally linked to the proto-oncogenic SFKs, which, in an activated state, transmit various cellular signals toward aberrant proliferation, increased motility and invasiveness, resistance to apoptosis, and increased angiogenesis [17]. SFK can be inhibited by phosphorylation of a carboxyl-terminal tyrosine residue, but this inhibitory phosphate group can be removed by the protein tyrosine phosphatase PTPRC (or CD45), leading to activation

Table 3. Type Specificity in Genotypic Association of rs869736 with Gastric Cancer Susceptibility.

Genotype	Control (n = 406)	Diffuse-type Gastric Cancer Patient			Intestinal-type Gastric Cancer Patient		
		(n = 252)	OR (95% CI)*	P	(n = 178)	OR (95% CI)*	P
GG	198 (48.8%)	96 (38.1%)	1		76 (42.7%)	1	
GT	181 (44.6%)	125 (49.6%)	1.45 (1.04-2.03)	.031	84 (47.2%)	1.03 (0.68-1.56)	.87
TT	27 (6.7%)	31 (12.3%)	2.47 (1.39-4.40)	.0021	18 (10.1%)	1.52 (0.72-3.20)	.30

*Age- and sex-adjusted OR and 95% CI in multivariate logistic regression.

of SFK [29–31]. The phosphatase activity of PTPRC is enhanced [32], and its interaction with SFK is stabilized when PTPRC dimer formation is inhibited by binding to PTPRCAP [22,33]. Thus, PTPRCAP binding to PTPRC can result in activation of SFK.

Overproduction or hyperactivation of SFK has been observed in a variety of human epithelial cancers [17] and is known to disrupt cell-cell adhesion [34,35], by inducing impairment in the membrane localization of E-cadherin (CDH1) [36], and to promote gain of invasive properties of epithelial cells [37]. Notably, down-regulation of *CDH1* is a recognized feature of diffuse-type gastric cancer [38], and mutations in *CDH1* have been found in hereditary diffuse-type gastric cancer [39]. Accordingly, a possible hypothesis is that enhanced levels of PTPRCAP facilitate activation of SFK and, consequently, impair E-cadherin function to increase susceptibility to diffuse-type gastric tumorigenesis.

In *H. pylori* infection, SFK can phosphorylate CagA of *H. pylori* to activate a cellular oncoprotein PTPN11 (or SHP2) [40], which is known to induce the sequential changes of intestinal-type gastric carcinogenesis [41]. Alternatively, PTPRCAP has been shown to enhance the sensitivity of T-cell receptor signaling through activation of SFK in hematopoietic cells [20,21,32,42], which may exacerbate inflammation and offer an attractive microenvironment for tumor growth, facilitate genomic instability, and promote angiogenesis [43,44]. However, these possibilities are yet unknown for diffuse-type gastric tumorigenesis.

Ten SNPs of the cancer-associated haplotype discovered in this study are dispersed in four genes, *RPS6KB2*, *PTPRCAP*, *CORO1B*, and *GPR152* (Figure 1B). Among them, *PTPRCAP* was identified to be functionally associated with the gastric cancer because 1) the endogenous transcript levels of *PTPRCAP* alone were linearly correlated with the risk haplotype copy number and 2) the promoter activity was increased by the risk allele at a promoter SNP of *PTPRCAP* compared with the nonrisk allele. Associations of the other nine SNPs, especially those showing no functional variation in this study, were probably because of their very high linkage disequilibria with the *PTPRCAP* promoter SNP. However, a possibility of their functional relevance in the cancer susceptibility should not be ruled out because not all of these SNPs were examined for every possible function.

In conclusion, a promoter SNP rs869736 at position –309 of *PTPRCAP* is associated with susceptibility to diffuse-type gastric cancer rather than to intestinal-type gastric cancer. Its minor allele T increases both promoter activity and promoter affinity to a nuclear protein (presumably a transcription factor) and, as a consequence, the gene expression. The resulting increase in cellular levels of PTPRCAP can increase the susceptibility to gastric cancer, as it might result in activation of the oncogenic SFKs and disruption of cell-cell contacts mediated by E-cadherin, among other possibilities.

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