

Increased Genetic Susceptibility to Intestinal-Type Gastric Cancer Is Associated With Increased Activity of the *RUNX3* Distal Promoter

Byungho Lim, PhD, Hyoungseok Ju, PhD, Minjin Kim, BS; and Changwon Kang, PhD

BACKGROUND: The runt-related transcription factor *RUNX3* plays essential roles in various types of tumors, including gastric cancer. Epigenetic changes in the methylation of the *RUNX3* proximal promoter, but not common genetic changes in *RUNX3*, have been associated with both changes in the gene expression and development of the cancer. **METHODS:** A case-control association study was conducted by genotyping 865 unrelated Korean subjects. Subsequent functional studies were performed to reveal functional implication of genetic association. **RESULTS:** Several single-nucleotide polymorphisms (SNPs) in *RUNX3* were significantly associated with susceptibility to intestinal-type gastric cancer ($.0028 \leq P \leq .022$) but not diffuse-type gastric cancer ($.70 \leq P \leq .96$). The risk-associated, minor variant of an intestinal-type gastric cancer-associated SNP in the *RUNX3* distal promoter (rs7528484) significantly increased promoter activity in a CREB1-dependent manner. The distal promoter-derived, 33 kDa isoform of *RUNX3* increased the activity of transcription factor nuclear factor kappa B (NF- κ B), which had been activated by *Helicobacter pylori* infection, a risk factor for intestinal-type gastric cancer, and the expression of the interleukin-1 β gene (*IL1B*), an NF- κ B target genetically and functionally associated with gastric cancer. In contrast, the proximal promoter-derived, 44 kDa isoform of *RUNX3* decreased both NF- κ B activity and *IL1B* expression. **CONCLUSIONS:** In addition to epigenetic changes in the *RUNX3* proximal promoter, genetic changes in the distal promoter may be associated with susceptibility to intestinal-type gastric cancer by increasing promoter activity. Functionally, 2 *RUNX3* isoforms may contribute differentially to intestinal-type gastric cancer susceptibility, at least in part through regulating NF- κ B activity and *IL1B* expression. *Cancer* 2011;117:5161-71. © 2011 American Cancer Society.

KEYWORDS: case-control association study, intestinal-type gastric cancer, promoter variations, *RUNX3* isoforms, single-nucleotide polymorphisms.

Gastric cancer, a complex disease caused by multiple genetic and environmental factors, is histopathologically categorized into 2 distinct subtypes—intestinal-type gastric cancer and diffuse-type gastric cancer—according to Lauren's classification.¹ Intestinal-type gastric cancer is characterized by the presence of well-differentiated adenocarcinoma and an expansive growth pattern, whereas diffuse-type gastric cancer is characterized by the presence of poorly differentiated adenocarcinoma and an infiltrative growth pattern.² The sequence of pathological events leading to gastric cancer is also different between the 2 subtypes. Whereas intestinal-type gastric cancer begins with *Helicobacter pylori* infection followed by chronic gastritis, atrophy, intestinal metaplasia, dysplasia, and adenocarcinoma, diffuse-type gastric cancer occurs through a shorter, less well-characterized sequence of events.^{2,3} To demonstrate the genetic backgrounds for aforementioned histopathological differences between intestinal-type gastric cancer and diffuse-type gastric cancer, it is necessary to identify the genetic factors specifically underlying each subtype, but such factors have not yet been fully elucidated.

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RUNX3 (runt-related transcription factor 3) is a member of the runt-domain transcription factor family that regulates the transcription of several genes involved in development, differentiation, immunity, and cancer.⁴⁻⁷ In vivo and in vitro evidence has demonstrated the tumor suppressive roles of RUNX3 in gastric cancer. *Runx3*-knockout mice showed hyperplasia of gastric epithelial cells because of reduced apoptosis and hyper-proliferation,⁷ or exhibited hyperplasia and gastric lesions accompanied by gastritis, metaplasia, and dysplastic foci in the gastric glandular mucosa.⁵ In addition, *RUNX3* expression is frequently down-regulated in gastric cancer cell lines and reduced in human intestinal metaplasia.⁷ Furthermore, unlike *Runx3*^{+/-} *p53*^{-/-} cells, *Runx3*^{-/-} *p53*^{-/-} gastric cells form tumors and differentiate into intestinal-type cells in nude mice,^{8,9} raising the possibility that *RUNX3* is related to intestinal-type gastric cancer pathogenesis.

RUNX3 itself is regulated by 2 alternative promoters: the proximal promoter, which produces the 44 kDa isoform (RUNX3/p44); and the distal promoter, which generates the 46 kDa (RUNX3/p46), 33 kDa (RUNX3/p33), and 27 kDa isoforms through alternative splicing.⁶ The proximal promoter is frequently inactivated by hypermethylation on CpG islands in gastric cancer tissues,^{7,10-12} revealing a mechanism for the down-regulation of *RUNX3* expression. In addition to epigenetic changes in the proximal promoter, genetic variations including rs11249206 at intron 1 and rs760805 at intron 3 showed association with susceptibility to gastric cancer in a Chinese population.¹³ However, functional genetic variations in *RUNX3* affecting gene expression and/or protein activity have not been reported to contribute to cancer development to date. Here, we report that a functionally relevant genetic variation located in the *RUNX3* distal promoter is associated with both promoter activity and intestinal-type gastric cancer subtype-specific susceptibility.

MATERIALS AND METHODS

Study Subjects

A total of 865 unrelated Korean participants including 434 cases with gastric cancer and 431 unaffected controls (Table 1) were recruited with their written informed consent at Seoul National University Hospital, Hanyang University Guri Hospital, Inje University Seoul Paik Hospital, Chungnam National University Hospital, or Eulji University Hospital in Korea. Gastric cancer was classified as diffuse (n = 255) or intestinal type (n = 179) according to Lauren's classification, and patients with mixed or uniden-

Table 1. Demographic Characteristics of the Study Subjects

Characteristic	Controls	IGC	DGC
Total, No. (%)	431 (100)	179 (100)	255 (100)
Male, No. (%)	303 (70)	132 (73)	160 (63)
Female, No. (%)	128 (30)	47 (27)	95 (37)
Age, y			
Mean $\pm$ SD	52.1 $\pm$ 8.4	59.5 $\pm$ 10.0	54.3 $\pm$ 13.7
Range	39-81	35-82	23-86
Tumor stage, No. (%)			
IA		27 (15)	15 (6)
IB		38 (21)	26 (10)
II		37 (21)	50 (20)
IIIA		43 (24)	65 (26)
IIIB		13 (7)	47 (18)
IV		21 (12)	52 (20)
Tumor depth, No. (%)			
Early		37 (20)	22 (9)
Advanced		142 (80)	233 (91)

Abbreviations: DGC, diffuse-type gastric cancer; IGC, intestinal-type gastric cancer; SD, standard deviation.

tified type were not included in the study. The control subjects were free of the disease by health examinations including endoscopy or an upper gastrointestinal series. This study was approved by the institutional review boards of Hanyang University Medical Center and Korea Advanced Institute of Science and Technology.

Sample Preparation and Genotyping

DNA samples were prepared and genotyped as described previously.¹⁴ Briefly, genomic DNA was extracted from peripheral blood samples or normal stomach tissues of each study subject using the Puregene DNA purification kit (Gentra, Minneapolis, Minn). Genotyping was performed using the MassARRAY iPLEX platform (Sequenom, San Diego, Calif) according to the manufacturer's instructions. Oligonucleotide primers for multiplex genotyping were designed using SpectroDESIGNER software (Sequenom).

Statistical Analysis

Allelic and genotypic associations of single-nucleotide polymorphisms (SNPs) were assessed by chi-square tests comparing the controls with the intestinal-type gastric cancer or diffuse-type gastric cancer cases. Odds ratio (OR) and 95% confidence interval values were calculated using SPSS 11.5 (SPSS Inc., Chicago, Ill). The Haploview 4.1 program was used to draw a linkage disequilibrium map for 75 SNPs (with minor allele frequency ≥ 0.2) located in and around the *RUNX3* region according to the genotype data for the Asian populations (Han Chinese in Beijing and Japanese in Tokyo [CHB+JPT]) of the International

HapMap Project and to measure the pairwise linkage disequilibrium values between SNPs.

All experiments were performed in triplicate, and the triplicate experiments were repeated once or twice. The results are presented as mean $\pm$ standard deviation. To compare means between different groups, Student *t* test was performed using SPSS 11.5, and a *P* value $< .05$ was considered statistically significant.

Cell Culture

Cell lines derived from 3 moderately differentiated gastric cancers (MKN28, SNU216, and SNU719), 2 poorly differentiated gastric cancers (MKN45 and SNU16), 2 monocytic cells (U937 and THP-1), and human embryonic kidney tissue (293T) were purchased from the Korean Cell Line Bank (Seoul, Korea), and maintained in RPMI-1640 medium (Gibco, Rockville, Md) supplemented with 10% fetal bovine serum (Hyclone, Logan, Utah) at 37°C in a humidified atmosphere with 5% CO₂, except for 293T, which was suspended in Dulbecco modified Eagle medium (DMEM; Gibco) supplemented with 10% fetal bovine serum. Cell culture experiments were performed without synchronization of cell cycle to measure average properties.

Plasmids

By using a previously described RUNX3/p44 cDNA¹⁵ as a template in a polymerase chain reaction, RUNX3/p46 cDNA was synthesized from a 99 nucleotide-long primer, a 5' part (57 nucleotides) of which corresponded to the N-terminal region of RUNX3/p46, and an additional primer of 21 nucleotides for the C-terminus. Two allelic variants of RUNX3/p46 cDNA bearing the nonrisk-associated and risk-associated alleles, respectively, at the position corresponding to rs6672420, as well as RUNX3/p44 cDNA, were cloned into the pCDNA3 expression vector (Invitrogen, Carlsbad, Calif). The 2 variants of the extreme N-terminal 19-amino acid domain cleaved from N-p46 (GBD-N) and R-p46 (GBD-R) were separately fused to a Gal4 DNA-binding domain in pM vector (Clontech Laboratories, Mountain View, Calif), and Gal4DNA binding activities were measured using pFR-luc vector (Stratagene, La Jolla, Calif). The CREB1 cDNA was amplified from MKN45 cell line and cloned into pCDNA3 vector (Invitrogen).

For luciferase assays, a *p21* (*CDKN1A*) promoter and a *RUNX3* distal promoter were cloned into pGL3-basic and pGL3-promoter vectors (Promega, Madison, Wis), respectively. pRL-CMV vector was used for normalization

of transfection efficiency. Luciferase reporter plasmids, pAP-1-luc,¹⁶ pCRE-luc,¹⁶ pSTAT3-luc,¹⁷ and pNF- κ B-luc¹⁸ had a response element for transcription factors AP-1 (activator protein 1), CREB1 (cAMP responsive element binding protein 1), STAT3 (signal transducer and activator of transcription 3), and NF- κ B (nuclear factor kappa-light-chain-enhancer of activated B cells), respectively.

To establish stable cell lines, eGFP, RUNX3/p44, and RUNX3/p33 cDNA were cloned into pLNHX vector (Clontech Laboratories), and pGAG-pol and pVSV-G were used for adenovirus transduction. Construction of all recombinant plasmids was confirmed by sequencing analysis.

Luciferase Reporter Assay

Cells (1×10^5) were transiently transfected with 0.45 μ g of promoter vector and 0.05 μ g of pRL-CMV control vector using Lipofectamine LTX reagent (Invitrogen) in a 24-well tissue-culture plate (SPL Life Sciences, Pocheon, Korea), and harvested 36 hours after transfection. Luciferase activities were measured using the Dual-Luciferase reporter assay system (Promega) and Victor plate reader (Perkin Elmer, Boston, Mass).

Establishment of Stable Cell Lines

The 293T cells were cotransfected with 3 vectors, pLNHX, pGAG-pol, and pVSV-G (Clontech Laboratories), for virus transduction. After 6 hours, the transfection medium was changed to DMEM supplemented with 2% fetal bovine serum. One day before transduction, MKN45 cells were placed on a 6-well tissue culture plate. On the next day, supernatant (containing adenovirus) was collected from the 293T cells, filtered, and applied to the MKN45 cells. After 8 hours, complete medium was placed on the cells, followed by selection in G-418 (Invitrogen) for 3 weeks. Stable expression was confirmed by fluorescence microscopy and Western blotting.

Antibodies and Western Blotting

A monoclonal RUNX3 antibody was purchased from MBL International (Woburn, Mass). THP-1 and 293T cell lines were used as positive¹⁹ and negative²⁰ controls for the expression of RUNX3 isoforms, respectively. Whole cell extracts were prepared using passive lysis buffer (Promega) and sonication. Cytoplasmic and nuclear fractions were prepared using Buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM ethylenediaminetetraacetic acid, 1 mM dithiothreitol, 0.5 mM phenylmethanesulfonyl fluoride) and Buffer B (20 mM HEPES, pH 7.9, 0.4 M NaCl,

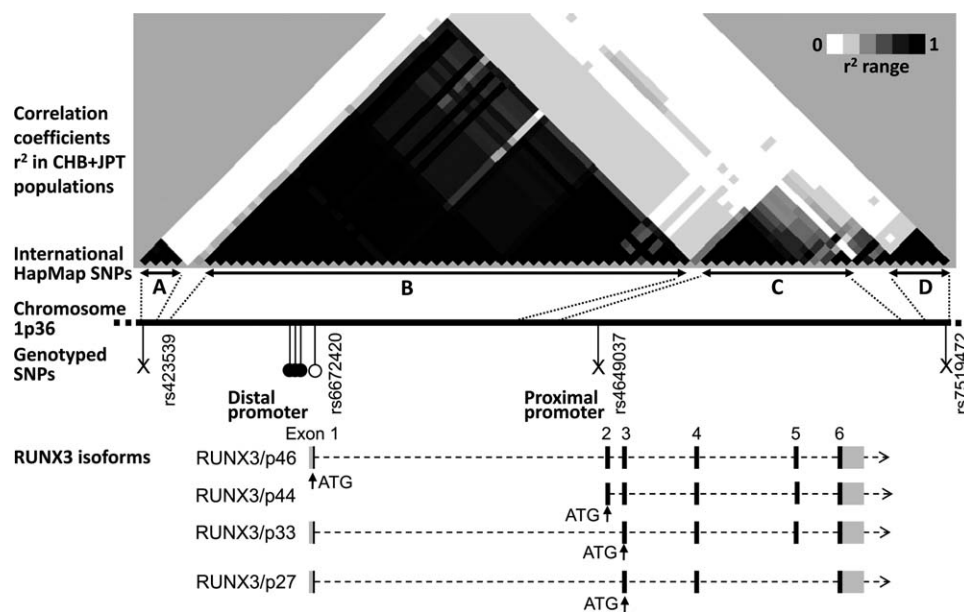


Figure 1. Genetic association of *RUNX3* with intestinal-type gastric cancer susceptibility is shown. In a 93 kb region surrounding *RUNX3*, 75 single-nucleotide polymorphisms (SNPs; minor allele frequency ≥ 0.2) could be divided into 4 linkage disequilibrium blocks denoted A, B, C, and D. Among the 7 genotyped SNPs, 3 HapMap SNPs (marked with Xs) were not associated with intestinal-type gastric cancer, but 1 HapMap SNP (marked with an open circle) and 3 non-HapMap SNPs (marked with filled circles) were associated with intestinal-type gastric cancer. The 46 kDa, 33 kDa, and 27 kDa isoforms of *RUNX3* are produced by transcription from the distal promoter and subsequent alternative splicing, whereas the 44 kDa isoform is produced by transcription from the proximal promoter located within intron 1. CHB indicates Han Chinese in Beijing; JPT, Japanese in Tokyo.

1 mM ethylenediaminetetraacetic acid, 1 mM dithiothreitol, 1 mM phenylmethanesulfonyl fluoride), respectively. Twenty micrograms of cell extracts was loaded into 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and subjected to Western blotting.

Quantitative Reverse-Transcription Polymerase Chain Reaction

Cellular mRNA levels were measured by quantitative reverse-transcription polymerase chain reaction using a Bio-Rad iCycler iQ5 and iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, Calif) after the isolation of total RNA using an RNeasy spin kit (Qiagen Biotechnology, Seongnam, Korea) and cDNA synthesis using an oligo-dT primer and Improm-II reverse transcriptase (Promega). The measurements were normalized against the glyceraldehyde-3-phosphate dehydrogenase level.

RESULTS

Intestinal-Type Gastric Cancer Susceptibility Association of *RUNX3* Distal Promoter and Exon 1

To identify the genetic variations in *RUNX3* that are associated with susceptibility to gastric cancer, we examined a

93 kb region encompassing *RUNX3* that included 4 linkage disequilibrium blocks (designated A, B, C, and D in Fig. 1). Four tag SNPs (rs423539, rs6672420, rs4649037, and rs7519472) were selected (1 from each block) to cover most of the 75 SNPs (minor allele frequency ≥ 0.2) in the region, according to the genotype data for the Asian populations provided by the International HapMap Project. Genetic association of the SNPs was examined by genotyping 865 unrelated Korean participants in this study.

Comparison of 179 intestinal-type gastric cancer or 255 diffuse-type gastric cancer cases with 431 controls showed that only the tag SNP in Block B (rs6672420) had a significant association with susceptibility to intestinal-type gastric cancer (OR, 1.49; $P = .0028$), but not diffuse-type gastric cancer ($P = .95$), whereas the other 3 tag SNPs showed no association with susceptibility to either intestinal-type or diffuse-type gastric cancer (Table 2). Block C, containing a nonassociated tag SNP, covered most of the gene ranging from the proximal promoter (located within intron 1) to the last exon (Fig. 1). In contrast, Block B, including the distal promoter, exon 1, and part of intron 1, was associated with intestinal-type gastric cancer susceptibility (Table 2). In a Chinese population, 2

Table 2. Allelic Association of SNPs in the *RUNX3* Locus With Gastric Cancer Subtype Susceptibility

SNP Major>Minor Allele (Position ^a / Location/LD Block)	Controls		IGC Cases		DGC Cases	
	AA/AB/BB ^b		AA/AB/BB ^b	OR (95% CI)	P	AA/AB/BB ^b
Tag SNPs in the 4 LD blocks in and around <i>RUNX3</i>						
rs423539 C>G (25182596/5' upstream/A)	129/133/47		48/43/11	0.81 (0.58-1.13)	.21	97/82/25
rs6672420 A>T (25163597/exon 1/B)	214/185/32		72/78/28	1.49 (1.15-1.93)	.0028	129/97/23
rs4649037 G>T (25130888/proximal promoter/C)	115/213/101		51/85/37	0.91 (0.71-1.17)	.45	86/113/56
rs7519472 A>C (25090713/3' downstream/D)	161/199/61		57/80/29	1.17 (0.89-1.53)	.28	77/137/36
SNPs in the distal promoter region of LD block B						
rs7414934 G>T (25165803/distal promoter/B)	215/178/37		77/73/29	1.39 (1.07-1.81)	.013	122/93/20
rs7536201 G>A (25165671/distal promoter/B)	210/185/30		74/83/22	1.36 (1.04-1.77)	.022	132/103/20
rs7528484 A>G (25165255/distal promoter/B)	185/154/31		65/65/24	1.41 (1.06-1.86)	.017	112/ 91/19

Abbreviations: DGC, diffuse-type gastric cancer; IGC, intestinal-type gastric cancer; LD, linkage disequilibrium; OR, odds ratio; CI, confidence interval; SNP, single-nucleotide polymorphism.

^a Nucleotide position refers to NT_004610.18 (National Center for Biotechnology Information build 36).^b The major and minor alleles in the control samples are referred to as A and B, respectively.

SNPs including rs11249206 at intron 1 (Block B) and rs760805 at intron 3 (Block C) previously showed significant association with susceptibility to gastric cancer.¹³ Taken together, several SNPs around *RUNX3* are possibly associated with gastric cancer susceptibility and, especially, several SNPs located in Block B exhibited intestinal-type gastric cancer association in a Korean population.

No Functional Effect of an Intestinal-Type Gastric Cancer-Associated Nonsynonymous SNP in Exon 1

To demonstrate the functional implication of genetic association, we subsequently performed functional studies. Because the intestinal-type gastric cancer-associated SNP (rs6672420) was nonsynonymous, we examined this SNP for possible functional effects on *RUNX3* activity. This SNP is located within exon 1 and codes for an asparagine-to-isoleucine change in the 18th residue of the largest isoform *RUNX3*/p46, but not for the other isoforms, the translation for which starts in exon 2 or 3 rather than exon 1 (Fig. 1).²¹ An N-terminal domain consisting of the extreme N-terminal 19 amino acid residues has an activity of transcriptional activation in isolation; it was demonstrated to be an additional domain that mediates transcriptional activation by *RUNX3*/p46 and is not shared by *RUNX3*/p44.²²

In the *RUNX3* activity assay, we used variants of a *RUNX3*/p46 cDNA expression vector harboring either the nonrisk-associated or the risk-associated allele at the position corresponding to the SNP (Fig. 2A) as well as a luciferase gene expression vector under the control of the *p21* (*CDKN1A*) promoter, which is directly regulated by *RUNX3*.¹⁵ The MKN45 gastric cancer cell line was cotransfected with 1 of the 2 *RUNX3*/p46 vectors (or *RUNX3*/p44 vector as an N-terminal domain-lacking control) and the *p21*-promoter luciferase vector (Fig. 2B). When the luciferase activity was measured, both the nonrisk-associated and the risk-associated variants of *RUNX3*/p46 exhibited increased transcriptional activity when compared with the control *RUNX3*/p44. However, there was no significant difference in stimulated *p21* promoter activity between cells expressing the 2 variants of *RUNX3*/p46.

The N-terminal domain was then separated from the rest of *RUNX3*/p46 and fused to the DNA-binding domain of the Gal4 transcription factor, and the transcriptional activity of the Gal4 DNA-binding domain-N-terminal domain fusion proteins was measured in a

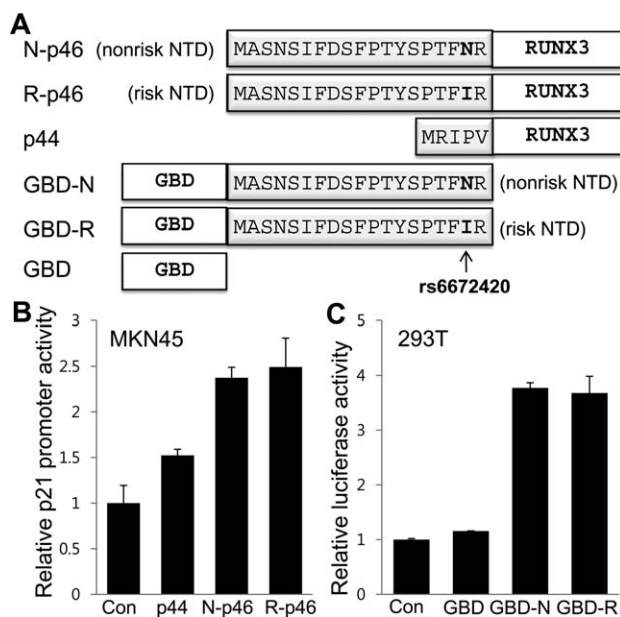


Figure 2. Assays for functional effects of a nonsynonymous single-nucleotide polymorphism in exon 1 are shown. (A) Two allelic variants of RUNX3/p46 cDNA bearing the nonrisk-associated and risk-associated alleles, respectively, at the position corresponding to rs6672420 (N-p46 and R-p46), as well as RUNX3/p44 (p44) cDNA, were cloned. The 2 variants of the extreme N-terminal 19-amino acid domain (NTD) cleaved from N-p46 (GBD-N) and R-p46 (GBD-R) were separately fused to a Gal4 DNA-binding domain (GBD). (B) At 36 hours after transfection with p21 promoter vector and N-p46 or R-p46, the luciferase activity dependent on p21 promoter activity was measured. (C) The activity of 2 GBD-NTD fusion constructs, GBD-N and GBD-R, was measured using the vector with a Gal4-responsive element. Mean values of 3 or more experiments are shown. Con, control.

manner that was similar to the luciferase assays noted above but used a Gal4-responsive luciferase vector (Fig. 2C). Both the nonrisk-associated and the risk-associated alleles of the N-terminal domain exhibited 3.8-fold enhanced activity compared with the Gal4 DNA-binding domain alone, which confirmed the independent transcriptional activation activity of the N-terminal domain. However, no significant difference was observed between the 2 variants of N-terminal domain. Accordingly, the nonsynonymous SNP in *RUNX3* exon 1 (rs6672420) did not affect the activity of the N-terminal transcriptional activation domain.

CREB1-Dependent Regulatory Effect of an Intestinal-Type Gastric Cancer-Associated SNP in the Distal Promoter

Next, we examined several SNPs located at 2.0 kb or less upstream of the transcription start site (position +1) of the *RUNX3* distal promoter for possible functional effects

on the promoter activity, among the SNPs located within intestinal-type gastric cancer-associated Block B. Three non-HapMap SNPs, rs7414934 (at position -1715), rs7536201 (at position -1583), and rs7528484 (at position -1167), were selected from the dbSNP database and genotyped (Table 2). All 3 showed significant association with intestinal-type gastric cancer susceptibility ($.013 \leq P \leq .022$), but none was associated with diffuse-type gastric cancer susceptibility ($.70 \leq P \leq .96$). All 3 highly correlated with rs6672420 ($0.88 \leq r^2 \leq 0.92$), supporting the intestinal-type gastric cancer association of the *RUNX3* distal promoter.

We then cloned a region of DNA ranging from position -1822 to position +53 from a case with the risk-associated allele for all 3 SNPs (this haplotype is designated here as RRR) and a control subject who had all 3 nonrisk-associated residues (haplotype NNN). Sequencing of the 2 clones revealed that the 2 subjects had different alleles at 2 additional non-HapMap SNPs, rs11249213 at position -1736 and rs11249212 at position -1700 (Fig. 3A), which were fairly close to the intestinal-type gastric cancer-associated rs7414934 at position -1715. These 2 SNPs may highly correlate with rs7414934 and hence possibly be associated with intestinal-type gastric cancer susceptibility.

The 2 DNA fragments were separately subcloned into the pGL3-promoter vector upstream of the luciferase gene under the SV40 promoter (Fig. 3A), and luciferase activity was measured. In these experiments, we used 2 gastric cancer cell lines: a moderately differentiated MKN28 and a poorly differentiated SNU16. A monocytic cell line U937 was in addition tested, because intestinal-type gastric cancer, an inflammation-related cancer, could be mediated from inflammatory cells such as monocytes. The risk-associated haplotype RRR exhibited a 2.0-, 2.2-, and 1.9-fold higher promoter activity than the nonrisk-associated haplotype NNN in MKN28, SNU16, and U937, respectively (Fig. 3B). In addition, RRR carriers had 1.5-fold higher intestinal-type gastric cancer risk than NNN carriers (Table 3). Accordingly, SNPs in the *RUNX3* distal promoter affected promoter activity as well as intestinal-type gastric cancer susceptibility.

Among the 5 SNPs in the distal promoter, 2 were predicted to affect transcription factor binding by the TESS and TFsearch programs.^{23,24} The transcription factor CREB1 was predicted to bind the CGTCA sequence carrying the risk-associated base G (underlined in the sequence above) of rs7528484 but not the sequence CATCA, which carries the nonrisk-associated base A.

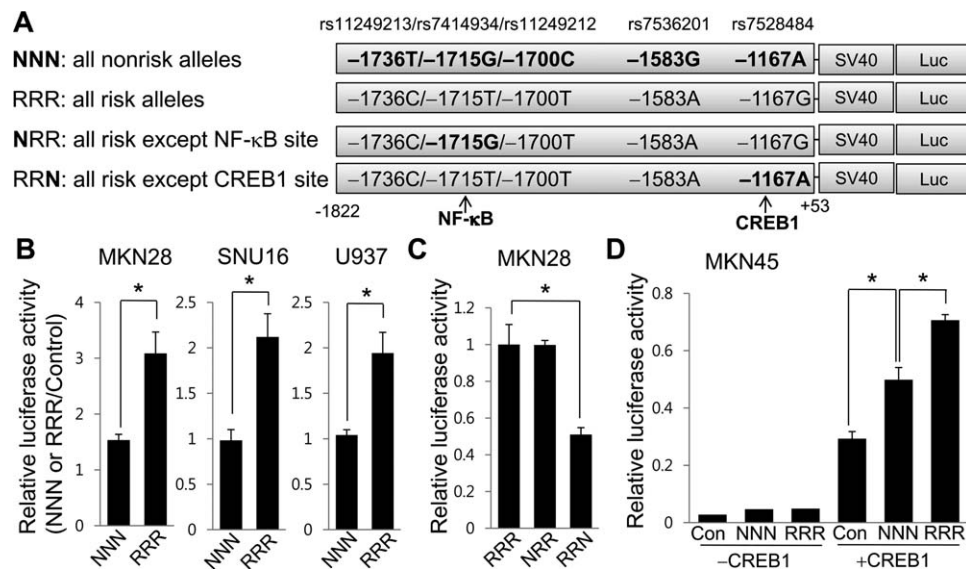


Figure 3. Assays for functional effects of several single-nucleotide polymorphisms in the distal promoter are shown. (A) Two distal promoter haplotypes cloned from an intestinal-type gastric cancer case (RRR) and a control (NNN), respectively, were subcloned into the pGL3-promoter vector. Two additional variants, NRR (with a change at the putative NF-κB binding site) and RRN (with a change at the putative CREB1 binding site), were generated by mutagenesis of RRR. (B) Relative luciferase activities of RRR and NNN measured in MKN28, SNU16, and U937 cells are shown. (C) Relative luciferase activities of RRN and NRR versus RRR measured in MKN28 cells are shown. (D) Relative luciferase activities of RRR and NNN measured in MKN45 cells in the absence and presence of exogenous CREB1 are shown. Mean values of 3 or more experiments are shown. * $P < .05$.

Table 3. Association of *RUNX3* Distal Promoter Haplotypes With IGC Susceptibility

Haplotype ^a	Controls, 2n = 844	IGC Cases, 2n = 340	OR [95% CI]	P
GGA, NNN	570 (0.67)	198 (0.58)	1, reference	—
TAG, RRR	225 (0.27)	113 (0.33)	1.45 [1.10-1.91]	.0091
GGG, NNR	21 (0.03)	10 (0.03)	1.32 [0.61-2.84]	.48
TAA, RRN	14 (0.02)	7 (0.02)	NC ^b	NC
GAA, NRN	7 (0.01)	6 (0.02)	NC	NC
TGG, RNR	3 (0.00)	3 (0.01)	NC	NC
TGA, RNN	2 (0.00)	2 (0.01)	NC	NC
GAG, NRR	2 (0.00)	1 (0.00)	NC	NC

Abbreviations: CI, confidence interval; IGC, intestinal-type gastric cancer; NC, not calculated; OR, odds ratio.

^aHaplotypes were constructed with 3 single-nucleotide polymorphisms in the distal promoter (rs7414934, rs7536201, and rs7528484). N and R refer to the nonrisk-associated and risk-associated alleles, respectively.

^bNC when a subgroup had <10 copies.

Likewise, NF-κB was predicted to bind the risk-associated but not the nonrisk-associated allele of rs7414934. Two SNP residues in the risk-associated haplotype (RRR) construct were separately mutated to the nonrisk-associated residues, generating 2 variant sequences, NRR and RRN (Fig. 3A). The change from the risk-associated to the non-risk-associated allele in the putative CREB1-binding site (from RRR to RRN) resulted in a 2.0-fold decrease in promoter activity, whereas an equivalent change in the putative NF-κB binding site (from RRR to NRR) changed the activity very little (Fig. 3C).

In contrast to these 3 cell lines, MKN45 (Fig. 3D) and SNU216 (data not shown) gastric cancer cell lines exhibited no such differential activity. One possible explanation for these effects is that CREB1 is active in the 3 cells showing differential activity but absent or inactive in the 2 cells with no differential activity. To test whether the addition of active CREB1 can induce the cells with no differential activity to exhibit differential activity, MKN45 cells were cotransfected with a *CREB1* expression vector and either the NNN or RRR promoter construct. In the presence of exogenous CREB1, the RRR activity was 1.4-

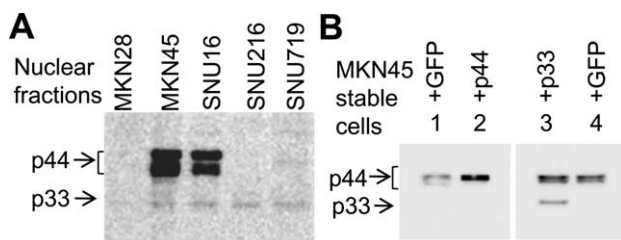


Figure 4. RUNX3 isoforms in gastric cancer cell lines are shown. (A) Nuclear fractions from 5 gastric cancer cell lines (MKN28, MKN45, SNU16, SNU216, and SNU719) were subjected to Western blotting using a monoclonal antibody recognizing RUNX3. In addition to the 2 previously reported RUNX3/p44 bands,¹⁹ 1 faint band corresponding to RUNX3/p33 is shown. (B) MKN45 cell lines stably overexpressing *RUNX3/p33*, *RUNX3/p44*, or *GFP* (as a control) were established and characterized by Western blotting with a RUNX3 monoclonal antibody. The p44 band was darker in the cells with RUNX3/p44 cDNA (lane 2) than those with GFP cDNA (lane 1), confirming increased production of RUNX3/p44. Overexpression of *RUNX3/p33* was confirmed by the p33 band visible in the cells with RUNX3/p33 cDNA (lane 3) but absent in the control (lane 4).

fold higher than the NNN activity in MKN45 cells (Fig. 3D).

The CGTCA sequence motif carrying the risk-associated allele of rs7528484 has been shown to be functional,²⁵ although it is part of the full consensus sequence (TGACGTCA) of the CREB1-binding site.²⁶ Accordingly, rs7528484 could be a *cis*-acting regulatory SNP that affects the *RUNX3* distal promoter activity in a CREB1-dependent manner, such that CREB1 favorably activates the risk-associated distal promoter over the non-risk-associated promoter.

***RUNX3/p33* Derived From the Distal Promoter Increases NF- κ B Activity and IL1B Expression**

Because the intestinal-type gastric cancer-associated SNP rs7528484 located in the *RUNX3* distal promoter changed the activity of the promoter, we examined the possibility that an isoform(s) of RUNX3 synthesized by transcription from the distal promoter might be involved in intestinal-type gastric cancer pathogenesis.³ To identify the distal promoter-derived isoforms, 5 gastric cancer cell lines (MKN28, MKN45, SNU16, SNU216, and SNU719) were subjected to Western blot analysis using a monoclonal antibody against RUNX3. This analysis revealed bands of 44 and 33 kDa, which represent RUNX3 isoforms derived from the proximal and distal promoters, respectively (Fig. 4A).

To evaluate the functional roles of these 2 isoforms in intestinal-type gastric cancer pathogenesis, stable cell

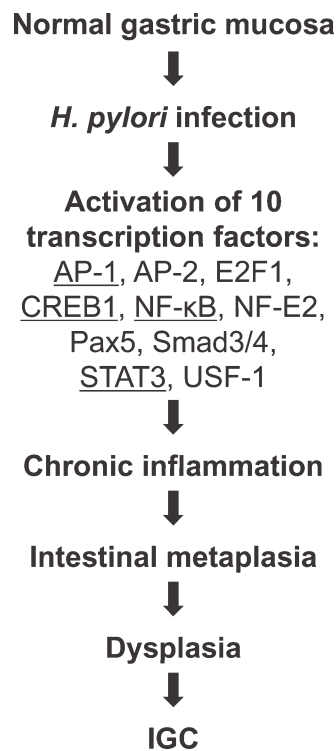


Figure 5. Activation of 10 transcription factors after *Helicobacter pylori* infection²⁹ in an intestinal-type gastric cancer pathogenesis model is shown.³ Among them the underlined 4 were monitored for activity change with RUNX3/p33 and RUNX3/p44 in this study. IGC, intestinal-type gastric cancer.

lines expressing RUNX3/p33 or RUNX3/p44 cDNA (or GFP cDNA as a background control) were generated using the MKN45 cell line, which had been used for in vitro regulation studies for chronic gastritis,²⁷ a predisposition to intestinal-type gastric cancer (Fig. 4B). *H. pylori* infection, a risk factor for intestinal-type gastric cancer,^{3,28} activates 10 transcription factors, including AP-1, AP-2, CREB1, E2F1, NF- κ B, NF-E2, Pax5, Smad3/4, STAT3, and USF, in the stomach tissues of Mongolian gerbils (Fig. 5).²⁹ Therefore, we examined the effects of the RUNX3/p33 isoform derived from the distal promoter in the regulation of 4 of these transcription factors, AP-1, CREB1, NF- κ B, and STAT3, and compared them with those of the RUNX3/p44 isoform synthesized from the proximal promoter.

The 4 transcription factor activity levels were separately measured using reporter genes with a promoter element optimized for response to each transcription factor. Whereas the levels of AP-1-dependent, CREB1-dependent, and STAT3-dependent reporters were not affected by *RUNX3/p33* or *RUNX3/p44* expression, the NF- κ B-

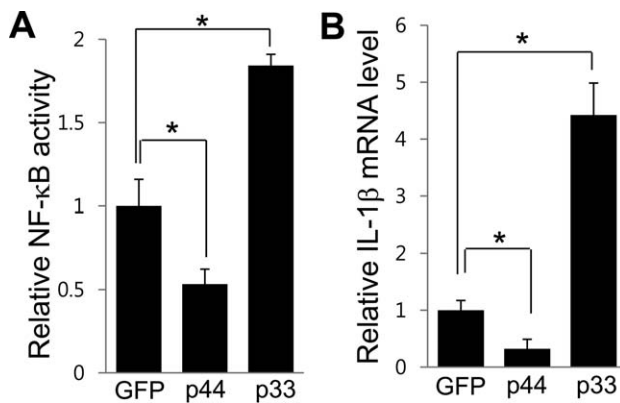


Figure 6. Opposing effects of RUNX3/p33 and RUNX3/p44 on NF-κB activity and *IL1B* expression are shown. (A) Relative activities of NF-κB measured in the presence of RUNX3/p44, RUNX3/p33, or GFP are shown. (B) Interleukin (IL)-1β mRNA levels measured by quantitative reverse-transcription polymerase chain reaction in the presence of RUNX3/p44, RUNX3/p33, or GFP were normalized against glyceraldehyde-3-phosphate dehydrogenase level. The mean values of 2 or more experiments are shown. * $P < .05$.

dependent reporter level was affected by *RUNX3* expression. Stable expression of *RUNX3/p33* elevated NF-κB transcriptional activity by 1.9-fold ($P = .00026$), although *RUNX3/p44* expression reduced it by 1.9-fold ($P = .019$), compared with *GFP* expression (Fig. 6A).

Next, we examined the expression levels of *IL1B*, because it is a target of NF-κB and because genetic variants of the *IL1B* promoter have previously been associated with gastric cancer risk and changes in gene expression.³⁰ Furthermore, transgenic mice overexpressing *Il1b* had intestinal-type gastric cancer-related pathophysiological phenotypes such as chronic inflammation, atrophy, and intestinal metaplasia.³¹ For these reasons, the mRNA level of interleukin (IL)-1β was measured in MKN45 cells overexpressing *RUNX3/p33*, *RUNX3/p44*, or *GFP* (Fig. 6B). Whereas *RUNX3/p44* expression reduced the mRNA level of IL-1β by 3.1-fold compared with the *GFP* control ($P = .0011$), *RUNX3/p33* expression increased it by 3.9-fold ($P = .048$), exhibiting a pattern that was consistent with the changes in NF-κB activity. These results suggest that the distal promoter-derived, 33 kDa isoform of RUNX3 may contribute to intestinal-type gastric cancer susceptibility, at least in part by regulating NF-κB activity and *IL1B* expression.

DISCUSSION

In this study we report genetic association and functional implication of the *RUNX3* distal promoter for intestinal-

type gastric cancer susceptibility. Especially, a distal-promoter SNP (rs7528484) acts as a CREB1-dependent *cis*-regulatory variation (Fig. 3). Furthermore, RUNX3/p33 derived from the distal promoter up-regulates NF-κB transcriptional activity and IL-1β mRNA levels (Fig. 6). Thus, we speculate that the risk-associated variant of the *RUNX3* distal promoter produces RUNX3/p33 at a higher level than the nonrisk-associated variant, increasing NF-κB activity and IL-1β mRNA levels.

Although more experiments are needed, the distal promoter may have cancer-enhancing activity rather than cancer-inhibitory activity, because intestinal-type gastric cancer risk-associated haplotypes of the distal promoter showed enhanced promoter activity (Fig. 3B) and increased intestinal-type gastric cancer risk (Table 3), and RUNX3/p33 derived from the distal promoter up-regulated oncogenic NF-κB activity and IL-1β mRNA levels (Fig. 6). These cancer-associated roles of the *RUNX3* distal promoter are supported by a previous finding that a retroviral insertion in the *Runx3* distal promoter cooperated with the *Myc* oncogene to promote the progression of T-cell lymphomas in Cd2-Myc mice.³²

The opposing regulatory actions of RUNX3/p33 and RUNX3/p44 observed in this study (Fig. 6) are consistent with recent findings showing that *CD36* expression is increased by p33 but suppressed by p44, and *CD11a* or *CD11c* promoter activity is suppressed by p33 but increased by p44.³³ Although it has been suggested that p33 could compete with p44 for binding to cofactors such as TLE1,³³ the detailed mechanism mediating these opposing effects needs to be further investigated.

Previously in a Chinese population, 2 SNPs (rs11249206 at intron 1 and rs760805 at intron 3) in *RUNX3* showed significant association with susceptibility to gastric cancer.¹³ The intron-1 SNP is located within the intestinal-type gastric cancer-associated Block B, consistent with our results. However, the intron-3 SNP is located in the nonassociated Block C, making it necessary to validate the association in larger case-control studies. It would also be necessary to confirm the intestinal-type gastric cancer-related functional roles of RUNX3 via NF-κB and its targets using animal models and to support clinical association by examining survival outcomes after intestinal-type gastric cancer subgrouping by the genotypes of the associated SNP or the expression patterns of RUNX3 isoforms.

In conclusion, multiple genetic variations in the *RUNX3* distal promoter are associated with intestinal-type gastric cancer susceptibility, and a risk-associated

allele in 1 of them (rs7528484) increases the distal promoter activity and, therefore, possibly the RUNX3/p33 level, which stimulates NF- κ B activity and IL-1 β levels. The effects could therefore lead to increased susceptibility to intestinal-type gastric cancer and can also be achieved via down-regulation of RUNX3/p44, the transcription for which may be decreased by hypermethylation of the RUNX3 proximal promoter.

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CONFLICT OF INTEREST DISCLOSURES

The authors made no disclosures.

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