**LRRC3B, Encoding a Leucine-Rich Repeat-Containing Protein, Is a Putative Tumor Suppressor Gene in Gastric Cancer**

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**Abstract**

Leucine-rich repeat-containing 3B (LRRC3B) is an evolutionarily highly conserved leucine-rich repeat-containing protein, but its biological significance is unknown. Using restriction landmark genomic scanning and pyrosequencing, we found that the promoter region of LRRC3B was aberrantly methylated in gastric cancer. Gastric cancer cell lines displayed epigenetic silencing of LRRC3B, but treatment with the DNA methylation inhibitor 5-aza-2'-deoxycytidine and/or the histone deacetylase inhibitor trichostatin A increased LRRC3B expression in gastric cancer cell lines. Real-time reverse transcription-PCR analysis of 96 paired primary gastric tumors and normal adjacent tissues showed that LRRC3B expression was reduced in 88.5% of gastric tumors compared with normal adjacent tissues. Pyrosequencing analysis of the promoter region revealed that LRRC3B was significantly hypermethylated in gastric tumors. Stable transfection of LRRC3B in SNU-601 cells, a gastric cancer cell line, inhibited anchorage-dependent and anchorage-independent colony formation, and LRRC3B expression suppressed tumorogenesis in nude mice. Microarray analysis of LRRC3B-expressing xenograft tumors showed induction of immune response-related genes and IFN signaling genes. H&E-stained sections of LRRC3B-expressing xenograft tumors showed lymphocyte infiltration in the region. We suggest that LRRC3B is a putative tumor suppressor gene that is silenced in gastric cancers by epigenetic mechanisms and that LRRC3B silencing in cancer may play an important role in tumor escape from immune surveillance. [Cancer Res 2008;68(17):7147–55]

**Introduction**

Leucine-rich repeat (LRR)-containing 3B (LRRC3B) is a putative LRR-containing transmembrane protein found by the Secreted Protein Discovery Initiative undertaken to identify new secreted and transmembrane proteins (1). LRBs are protein interaction motifs of 20 to 29 amino acid residues characterized by repetition of hydrophobic residues, especially leucine, and that are separated by conserved distance (2–4). LRR-containing proteins, of which there are >2,000, participate in many important processes, including plant and animal immunity, hormone-receptor interactions, cell adhesion, signal transduction, regulation of gene expression, and apoptosis (5, 6). A number of microarray expression profiling studies on human cancers have shown that LRRC3B is down-regulated in gastric (7), breast (8), colon (9), testis (10), prostate (11), and brain cancers (12), suggesting LRRC3B involvement in carcinogenesis.

DNA methylation associated with histone modification is a key mechanism to inhibit the expression of tumor suppressor genes in cancer, and DNA methylation markers have been applied in cancer risk assessment, early detection, prognosis, and prediction of response to cancer therapy (13–15). We have used restriction landmark genomic scanning (RLGS) to identify methylation markers in gastric cancer on a whole-genome scale (16). We previously reported aberrant methylation of LMS2, a regulator of cell migration (17), DCDLD2, a cell growth repressor (18), and Protein kinase D1 (19) in gastric cancer by RLGS and pyrosequencing, a quantitative methylation analysis of multiple CpG sites.

Here, we report the identification of LRRC3B as a target of aberrant methylation in gastric cancer and show LRRC3B silencing by epigenetic mechanisms in gastric cancer cell lines and primary gastric tumor tissues. We also examined the function of LRRC3B as a tumor suppressor in vitro and in vivo and provide evidence that loss of LRRC3B function in gastric cancer likely promotes tumor escape from immune surveillance.

**Materials and Methods**

**Cell lines and tissue samples.** Ninety-six pairs of frozen gastric tumors and normal adjacent tissues were accessed from the Stomach Cancer Bank at Chungnam National University Hospital, Daejeon, Korea, as described (17–19). Specimens were originally obtained from tumors immediately after resection. Corresponding normal mucosa specimens were at least 5 cm away from the tumor edge. All samples were obtained with informed consent, and their use was approved by the Institutional Review Board of the Chungnam National University Hospital, Daejeon, Korea. Gastric cancer cell lines established from gastric cancer patients (20, 21) were obtained from the Chungnam National University Hospital, Daejeon, Korea, as described (17–19). Corresponding normal mucosa specimens were at least 5 cm away from the tumor edge. All samples were obtained with informed consent, and their use was approved by the Institutional Review Board of the Chungnam National University Hospital, Daejeon, Korea. Gastric cancer cell lines established from gastric cancer patients (20, 21) were obtained from the Korean Cell Line Bank 9 and were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic solution (Invitrogen).

**RLGS, spot cloning, and sequence analysis.** RLGS was performed as described (16, 22, 23). DNA fragments corresponding to a spot of interest on
two-dimensional gels were cloned as described (24). Briefly, 10 μg DNA from normal tissue was digested sequentially with NotI and EcoRV, and directly mixed with 2 μg labeled DNA for the first dimensional separation. This mixture was then subjected to RLGS. After the second-dimension separation, the gel was covered with plastic wrap and affixed to an imaging plate without drying. The overnight-exposed imaging plate was scanned using the BAS3000 system (FUJI Photo Film Co.), and printed on a transparent film. The film was overlaid on the original gel, and the portion of the gel corresponding to the spot was cut out and the DNA was purified. After DNA ligation with biotinylated NotI and HindII linkers (24), iron-coated streptavidin (Dynabeads M280 streptavidin; Dynal, Inc.) was added, and the DNAs trapped with the linkers were recovered and washed. A high-salt buffer was used to wash the beads, and the purified DNA was then amplified for 30 PCR cycles using 200 nmol/L each of primers I and primer II (24). The amplified DNA fragment was cloned into the Smal site of plasmid pUC19.

**Southern blot hybridization analysis.** Genomic DNA from paired normal tissue and lymph node tissue from one patient and from SNU-005 gastric cancer cell line was digested with NotI/EcoRV, NotI/HindII, or HindII alone (New England Biolabs) for 4 h. The digested DNAs were separated on a 0.8% agarose gel, transferred to a nylon membrane (Amersham Biosciences), and hybridized with a DNA probe (25). The probe was prepared by purifying HindIII/NotI-digested fragments from the plasmid containing the DNA that generated spot 3C43 and randomly priming the fragments with [α-32P]dATP with Prime-it II kit (Stratagene). A 700-bp fragment of LRRC3B (−238 to +462 with respect to the transcription start site) was obtained by PCR using the forward primer 5′-CGAGACGACGAGAGGAGT-3′ and the reverse primer 5′-GCTCTGGAAAACAGCACAG-3′ and inserted into the pGEM-T Easy vector (Promega). Then, the NcoI/Sacl fragment was inserted into the pgG3-Base vector (Promega) that contains a modified firefly luciferase gene to make pLRC8303BpG71-luc. pRL-CMV vector (Promega) was used to normalize the transfection efficiency. DNA (0.8 μg plLRC8303BPG71-luc DNA and 0.2 μg pRL-CMV DNA) was transfected into SNU-484 cells using Lipofectamine Plus reagent (Invitrogen) according to the manufacturer’s protocol. The pgG3-Base vector was used as a negative control. Firefly and Renilla luciferase activities were measured 48 h after transfection. Relative luciferase activities were calculated after normalization of the transfection efficiency by Renilla luciferase activity.

**Real-time reverse transcription-PCR analysis.** Real-time reverse transcription-PCR (RT-PCR) was performed as described (17–19). Total cellular RNA (5 μg) was reverse transcribed into cDNA using SuperScript II (Invitrogen). Real-time PCR was performed using the Exicyclic quantitative Thermal Block (BIOENEER). The RT reaction product (100 ng) was amplified in a 15-μL reaction volume with 2× SYBR Premix EX Taq (Takara). We used the Primer3 program10 to design the LRRC3B exon 2 forward (5′-TTCCTCTTCCATGTTCTCC-3′) and reverse (5′-CCAGATGTTCTAACAAC-3′) primers. Samples were heated to 95°C for 1 min and then amplified for 45 cycles with 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s, followed by a final extension step of 72°C for 10 min. PCR products were visualized on a 1% agarose gel by ethidium bromide staining, purified from the gel using the Qiagen Gel Extraction kit, and cloned using the pGEM-T Easy Vector (Promega). Ten clones were randomly chosen for sequencing. Complete bisulfite conversion was assured when <0.01% of the cytosines in non-CG dinucleotides in the final sequence had not converted.

**5-Aza-2′-deoxycytidine and trichostatin A treatment.** SNU-216, SNU-638, and SNU-638 cell lines were seeded at a density of 1 × 10⁵ cells per 10-cm dish and cultured for 1 d before drug treatment. The cells were treated with 1 μmol/L 5-aza-2′-deoxycytidine (5-aza-dC; Sigma) every 24 h for 3 d and then harvested. Another culture of cells was treated with 250 nmol/L trichostatin A (TSA; Sigma) for 1 d and then harvested. To test the synergistic effects of 5-aza-dC and TSA, the cells were first treated with 1 μmol/L 5-aza-dC for 3 d, followed by treatment with 250 nmol/L TSA for 1 d. Total RNA was prepared, and the effect on LRRC3B expression was assessed by real-time RT-PCR.

**Chromatin immunoprecipitation assay.** A chromatin immunoprecipitation (ChiP) assay was performed with a ChiP assay kit (Upstate Biotechnology) according to the manufacturer’s protocol with modifications (19). Briefly, proteins were crosslinked to DNA by addition of formaldehyde directly to the culture medium to a final concentration of 1% for 10 min at 37°C. The collected cells were washed twice with ice-cold PBS with protease inhibitors and resuspended in 200 μL SDS lysis buffer (Upstate Biotechnology) per 1 × 10⁶ cells. Lysates were sonicated 21 times for 5 s with 30-s intervals on ice, at power setting 3 (Fisher Sonicator D50, 100). These shearing conditions yielded DNA fragments ranging from 200 to 500 bp. The sheared samples were centrifuged at 12,000 × g for 10 min at 4°C, and the supernatants were diluted in 9 volumes of ChIP dilution buffer (Upstate Biotechnology). The cell supernatants were preclreated with salmon sperm DNA/protein A agarose beads (Upstate Biotechnology) then immunoprecipitated with 5 μL of antibody to either acetyl-histone H3 (Upstate Biotechnology) or acetyl-histone H4 (Upstate Biotechnology), or no antibody. Immunoprecipitated DNA was recovered using the QiAquick PCR Purification kit (Qiagen) and analyzed by real-time PCR using the primers 5′-GTCAGGTTATCCCT-TGCTG-3′ and 5′-TTGCGAGAGAGGACAGA-3′ (located in the LRRC3B CpG island; Fig. 1A). Samples were heated to 95°C for 1 min and then amplified for 45 cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 30 s. The amount of immunoprecipitated DNA was normalized to the input DNA.

**Pyrosequencing.** The promoter of LRRC3B was PCR amplified using forward primer 5′-GAGTATTTGTTGTTGTTG-3′ and biotinylated reverse primer 5′-AACCACTTCCTTCAAGC-3′ and amplified by PyroAssay Design (Biotage AB). The product size was 420 bp. Bisulfite-modified DNA (3 μL) was amplified in a 25-μL volume with the primers and 2× Premix EX Taq (Takara). Samples were heated to 95°C for 5 min and then amplified for 50 cycles of 95°C for 30 s, 60°C for 40 s, and 72°C for 30 s, followed by a final extension step at 72°C for 5 min. Pyrosequencing reactions were performed according to the manufacturer’s specifications with a sequencing primer 5′-GTGGTATGAGTTGTTTGGTA-3′ and run on the PSQ HS 96A System (Biotage AB). Six CpG sites were analyzed. The expected sequence was YGGTYGTTGATGAAGGGTTAGTGY (Y = T or C).

**Transfection and colony formation assay.** Because the LRRC3B coding region lies entirely within exon 2, the entire LRRC3B coding sequence was amplified from human genomic DNA by PCR using the primers BamHI LRRC3B_F 5′-GGATCCATGAATCTGGTAGACCTG-3′ and Xhol LRRC3B_B_5′-CTCGAGCTATACCACAGCTGCTAAT-3′ and cloned into the pGEM-T Easy vector. After confirming the sequences, the full-length LRRC3B cDNA was subcloned into pcDNA3.1 (Invitrogen) at the BamHI and Xhol sites. For a colony formation assay in a monolayer culture, SNU-601 cells were plated at 3 × 10⁵ cells per well in 6-well plates, and transfected with either pcDNA3.1-LRRC3B or empty vector control pcDNA3.1 using Lipofectamine Plus reagent. The cells were selected with G418 (500 μg/mL) for 4 wk. Two positive clones and one control clone were plated in 6-well plates at 200 or 400 cells per well. After 2 wk of incubation with G418, the cells were stained with crystal violet. To study colony formation in soft agar, the stable transfecants were suspended in RPMI 1640 containing 0.3% agarose, 10% FBS, and 500 μg/mL G418 and layered on RPMI 1640.

10 http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi
11 http://www.urogene.org/methprimer/index.html
containing 0.6% agarose, 10% FBS, and G418 in a 6-well plate. Colonies were photographed and counted after 2 wk of incubation at 37°C.

Subcellular localization of LRRC3B. To study LRRC3B subcellular localization, the entire LRRC3B coding sequence (without a stop codon) was amplified from human genomic DNA by PCR using the primers HindIII_LRRC3B_F 5'-CAAGCTTCGATGAATCTGGTAGACCTG-3' and BamHI_LRRC3B_R 5'-GGATCCTACCACAGTGCTAATATC-3', and cloned into the pGEM-T Easy vector. After confirming the sequences, the full-length LRRC3B cDNA was subcloned into pEGFP-N3 (Clontech) at the HindIII and BamHI sites to generate a COOH-terminal green fluorescent protein (GFP) fusion protein. SNU-601 cells were seeded on 25-mm diameter coverslips. pEGFP-N3-LRRC3B or control vector was transfected into the cells using Lipofectamine Plus. At 2 d posttransfection, the cells were observed under a Zeiss LSM 510 confocal laser scanning microscope (Zeiss).

Tumorigenesis assay in nude mice. We maintained 3-wk-old male nude mice (Japan SLC, Inc.) in accordance with the guidelines and under approval of the Institutional Review Committee for the Animal Care and Use, Korea Research Institute of Bioscience and Biotechnology. Two clones of SNU-601 cells stably expressing LRRC3B and vector control–transfected cells were used in a tumorigenesis assay. For the tumorigenesis assay, cells were collected by centrifugation, washed twice in PBS, and 3×10^6 cells were resuspended in 0.1 mL of PBS and injected s.c. into nude mice (7 mice per cell line). We measured tumor volume as described (27).

Microarray analysis. Microarray analysis was performed as described (28). Briefly, total cellular RNA (20 μg) from xenograft tumors and SNU-601 cells was used as template for the synthesis of Cy5- or Cy3-labeled (Genisphere, Inc.) cDNA using SuperScript II reverse transcriptase (Invitrogen) for 2 h at 42°C. The two labeled cDNAs were mixed together, filtered through Microcon YM-30 membrane (Millipore) to exclude unincorporated deoxynucleotide triphosphates, and hybridized to the cDNA microarray slide (containing 23,232 genes and 1,056 controls; Korea Research Institute of Bioscience and Biotechnology) at 50°C overnight using
a 3DNA Array 50 kit (Genisphere, Inc.). After hybridization, each microarray was washed twice with 2× SSC containing 0.2% SDS at room temperature for 5 min, and finally with 95% ethanol at room temperature for 1 min. The hybridized slide was scanned by a GenePix 4000B Scanner (Axon Instruments, Inc.) and analyzed using the GenePix Pro 4.0 program (Axon Instruments). Genes with significantly different expression levels according to Student's t test in xenograft tumors expressing LRRC3B compared with control xenograft tumors were selected (29). We deposited the raw data in Gene Expression Omnibus13 with an accession number GSE4003.

Immunohistochemistry. Immunohistochemical staining was done as described (30). Briefly, paraffin sections of xenograft tumors were dewaxed, rehydrated, and washed thrice with PBS. After treatment with proteinase K for 5 min at 37 °C, sections were treated with H2O2 for 10 min at room temperature, blocked in PBS containing 0.1% Tween 20 and 1% bovine serum albumin for 20 min, followed by reaction with anti-mouse NK-1.1 (diluted 1:100; BD Biosciences) for 1 h. Sections were incubated sequentially with peroxidase-conjugated secondary antibody and visualized with ChemMate EnVision detection kit (Dako). Sections without primary antibody were used as negative controls.

Statistical analysis. The Student’s t test was used to establish the statistical significance of differences in LRRC3B expression or LRRC3B promoter methylation between primary gastric tumors and adjacent normal tissues. The clinicopathologic factors in various groups of patients were compared using the χ² test or Student’s t test. Results with P values of <0.05 were considered statistically significant.

Figure 2. Expression and methylation analysis of LRRC3B in gastric cancer cell lines and gastric cancer tissues. A, real-time RT-PCR analysis of LRRC3B mRNA in 11 gastric cancer cell lines and three pairs of gastric tumor (T) and normal (N) tissues. B, bisulfite sequencing analysis of LRRC3B CpG sites in 11 gastric cancer cell lines and three pairs of gastric and normal tissues. The bisulfite sequencing covers 57 CpG sites in the LRRC3B CpG island (see Fig. 1A). Open squares, unmethylated CpG sites; filled squares, methylated CpG sites. Each row represents a single clone. The numbers on the right represent the mean percentages of CpG sites that were methylated for each cell line or tissue. +1, transcription start site. C, restoration of LRRC3B expression by treatment with 5-aza-DC and/or TSA in gastric cancer cell lines. SNU-216, SNU-601, and SNU-638 cells were treated with the inhibitors as described in the Materials and Methods. Total RNA was isolated, and LRRC3B expression was determined by real-time RT-PCR. Columns, mean of three independent experiments; bars, SD. D, ChIP assays of the LRRC3B CpG island. Chromatin DNA was immunoprecipitated with antibodies specific for acetylated histone H3 (AcH3) or acetylated histone H4 (AcH4). DNA fragments corresponding to the LRRC3B CpG island (see Fig. 1A) were amplified by PCR. The amount of immunoprecipitated DNA was normalized to the input DNA. Columns, mean from two independent ChIP experiments and a total of four independent PCR analyses; bars, SD.

Results

**LRRC3B is a target of aberrant methylation in gastric cancer.** RLGS assays were performed on gastric cancer cell lines and gastric cancer tissues to look for aberrant methylation of genomic DNA compared with normal gastric mucosal tissue (16). RLGS profiles of the gastric cancer cell lines showed that 82% (9 of 11) lacked a DNA spot named 3C43, and this spot was decreased in 73% (11 of 15) of the primary tumors tested. Figure 1B shows a decrease in the 3C43 spot in tumor and metastatic lymph node tissue from one patient and the gastric cancer cell line SNU-005. The DNA of the 3C43 spot was purified from the RLGS gel and was cloned. Sequence analysis of the cloned 3C43 spot DNA identified it as the sequence of a NotI/Hinfl fragment covering the 5' upstream region and first exon of LRRC3B at chromosome region 3p24.1. The 3C43 spot DNA sequence also partly overlapped with CpG island 71 predicted in the University of California, Santa Cruz (UCSC) genome browser14 (Fig. 1A).

To understand whether the decrease in the 3C43 spot intensity in the RLGS profiles of the gastric cancers was due to NotI site methylation or loss of heterozygosity, we performed Southern blot analysis using the 3C43 spot DNA fragment as a probe. We observed 1.1-kb HindI-digested bands in all tumor-related DNAs, as well as normal mucosal DNA, indicating that the decreased spot intensity was not due to loss of heterozygosity in this region. We also observed increased intensity of a high molecular weight band in the NotI/EcoRV-digested and NotI/Hinfl-digested DNA fragments from a gastric tumor, metastatic lymph node, and a gastric cancer cell line, indicating increased methylation at the NotI site in gastric cancer (Fig. 1C). This result suggested that the loss or decrease of 3C43 spot intensity in gastric cancer was due to hypermethylation.

To test whether the CpG island 71 is important for transcription of LRRC3B, we generated a luciferase reporter construct, named pLRRC3BCpG71-luc, containing a fragment of the entire CpG island 71 (−238 to +462). Construct pLRRC3BCpG71-luc displayed 8.6-fold higher luciferase activity than the control plasmid, pGL3-D, suggesting that the CpG island 71 of LRRC3B contains a functional promoter.

**LRRC3B is down-regulated by DNA methylation and histone deacetylation in gastric cancer.** To investigate a potential relationship between promoter methylation and down-regulation of LRRC3B expression in gastric cancer, we analyzed LRRC3B mRNA expression levels in 11 gastric cancer cell lines established from Korean gastric cancer patients by real-time RT-PCR. Most of the 11 gastric cancer cell lines showed very low levels of LRRC3B; only SNU-484 cells displayed a high level of LRRC3B mRNA. We also examined the expression level of LRRC3B in three paired gastric cancer and adjacent normal tissue samples. Tumor tissues expressed LRRC3B at lower levels than each of the respective paired normal tissues (Fig. 2A). Bisulfite sequencing analysis of the LRRC3B CpG island (−195 to +323, 57 CpG sites) showed that 44% to 96% of the CpG sites were methylated in 10 gastric cancer cell lines, but SNU-484 cells had methylation at only 2% of the CpG sites in the LRRC3B CpG island. Gastric tumor tissues were moderately methylated with methylation at 21% to 60% of the sites, whereas paired normal tissues contained only 0% to 3% methylated CpG sites (Fig. 2B), indicating that methylation correlated with reduced LRRC3B expression in gastric cancer cell lines and gastric cancer tissues.

DNA methylation is usually linked with histone deacetylation (31). To examine whether silencing of LRRC3B in gastric cancer cells could be restored by treatment with the DNA methylation inhibitor 5-aza-dC (32) and/or the histone deacetylase inhibitor TSA (33), we treated cell lines SNU-216, SNU-601, and SNU-638 (in which the LRRC3B CpG island is hypermethylated and transcriptionally silenced) with 5-aza-dC, TSA, or both. After treatment, we isolated RNA and measured LRRC3B expression by real-time RT-PCR (Fig. 2C). LRRC3B expression was restored in SNU-216 and SNU-638 cells by treatment with 5-aza-dC or TSA alone. TSA was more effective than 5-aza-dC at inducing LRRC3B expression in both cell lines, and the combination of 5-aza-dC and TSA resulted in synergistic reactivation of LRRC3B in all cell lines treated. These results provide evidence that both DNA methylation and histone deacetylation have a causal role in silencing of LRRC3B expression in gastric cancer cells. To examine local histone acetylation in the chromatin associated with the LRRC3B CpG island, we performed a ChIP assay in gastric cell lines having different expression levels of LRRC3B. The histone-associated DNAs, immunoprecipitated with antibodies against acetylated histone H3 (K9 and K14) or acetylated histone H4 (K5, K8, K12, and K16), were amplified with a primer set specific for the LRRC3B CpG

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14 http://genome.ucsc.edu/
island. Acetylation levels of histones H3 and H4 at the \textit{LRRC3B} CpG island were elevated in SNU-484 cells in which the \textit{LRRC3B} CpG island is unmethylated and transcriptionally active (Fig. 2D). These results clearly indicate that histone deacetylation is also a feasible mechanism for the observed transcriptional silencing of \textit{LRRC3B} in gastric cancer.

\textit{LRRC3B} is frequently silenced and aberrantly methylated in primary gastric cancers. To examine \textit{LRRC3B} expression during gastric carcinogenesis, we performed real-time RT-PCR in 96 paired gastric tumor and adjacent normal tissues. \textit{LRRC3B} expression was significantly reduced in tumors ($P < 0.0001$; Fig. 3A). A majority (88.5%; 85 of 96) of tumors expressed \textit{LRRC3B} at a level lower than

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\caption{LRRC3B has tumor suppressor activity. A, anchorage-dependent colony formation assay in monolayer culture. SNU-601 cells were stably transfected with either pcDNA3.1-LRRC3B or empty vector pcDNA3.1. Two positive clones (LRRC3B-1 and LRRC3B-2) and one control clone (pcDNA3.1) were plated in 6-well plates at 200 or 400 cells per well. After 2 wk of incubation, the cells were stained with crystal violet. RT-PCR analysis of LRRC3B mRNA in the three cell lines is shown. The graph shows the number of colonies formed by each stable transfecant. Columns, mean of three independent experiments, each performed in duplicate; bars, SD. B, anchorage-independent colony formation assay in soft agar. The stably transfected cells were plated in top medium with 0.3% agarose over a bottom medium containing 0.6% agarose. Colonies were counted after 2 wk of incubation. The graph shows the number of colonies formed by each stable transfecant. Columns, mean of three separate experiments, each performed in duplicate; bars, SD. C, photographs of tumors excised at 56 d after injection into nude mice of stably transfected cells—two LRRC3B-expressing SNU-601 cell lines and one control vector–transfected SNU-601 cell line. D, the left graph shows tumor volume as calculated on the indicated days, and the right graph shows tumor weight at the end of the experiment. Columns and points, mean; bars, SD.}
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their respective paired normal tissue, and 65.6% (63 of 96) of the tumors showed a >2-fold decrease. Supplementary Table S1 presents clinicopathologic characteristics of patients based on LRRC3B expression level. LRRC3B down-regulation in tumors was more frequent in tumor-node-metastasis (TNM) stage I than stages II to IV (P = 0.0351), suggesting that down-regulation may be an early event in multistep gastric carcinogenesis.

We next performed pyrosequencing to measure the extent of methylation of 6 CpG sites (Fig. 1A) in the LRRC3B CpG island in 78 paired normal and tumor tissues for which genomic DNA was available. LRRC3B methylation was significantly elevated in tumors compared with normal tissue at all CpG sites tested (P < 0.0001; Fig. 3B). LRRC3B methylation in gastric tumor tissues with respect to clinicopathologic characteristics is shown in Supplementary Table S2. Tumors of TNM stage I were methylated more frequently than tumors of stages II to IV (P = 0.0208). This methylation status corresponded well to low LRRC3B expression in stage I tumors. In addition, intestinal-type tumors were more frequently methylated than diffuse-type tumors (P = 0.0170).

LRRC3B is a membrane protein that is highly conserved among vertebrates. In silico analysis of the LRRC3B open reading frame predicted LRRC3B to be a ~29.3-kDa protein with a signal peptide, an LRR NH2-terminal domain, three internal LRRs, an LRR COOH-terminal domain, and a transmembrane domain (Supplementary Fig. S1A). The GFP-LRRC3B fusion protein localized to the cell membrane, as shown in Supplementary Fig. S1B. The amino acid sequence of human LRRC3B is 100% identical to chimpanzee and dog, differs by only one residue from LRRC3B of mouse and rat, and differs by two residues from LRRC3B of cow and pig (Supplementary Fig. S1C), indicating that LRRC3B has been highly conserved throughout vertebrate evolution.

LRRC3B has tumor suppressor activity in vitro and in vivo. To examine the possible activity of LRRC3B as a tumor suppressor, we performed colony formation assay and tumorigenesis assay. We established stably transfected LRRC3B-expressing SNU-601 cell lines. Among the gastric cancer cell lines in which LRRC3B is hypermethylated and transcriptionally silenced, our previous experiments established that SNU-601 cells are the most proficient at forming tumors in nude mice. SNU-216 cells also form tumors, but SNU-638 seldom do so in nude mice. Two independent stably transfected LRRC3B-expressing SNU-601 cell lines (LRRC3B-1 and LRRC3B-2) formed fewer colonies than the empty vector–transfected cells in the anchorage-dependent assay (Fig. 4A). Moreover, the LRRC3B-1 and LRRC3B-2 transfectants...
also formed fewer colonies than the control cells in an anchorage-independent assay on soft agar (Fig. 4B). These results suggest that LRRC3B suppresses cell proliferation signals in gastric cancer cells. We next examined the effect of LRRC3B overexpression by a tumorigenesis assay in nude mice. Nude mice were injected with the two stably transfected LRRC3B-expressing SNU-601 cell lines or empty vector–transfected cells, sacrificed 8 weeks after injection, and their tumors were dissected and weighed (Fig. 4C). The control cells formed rapidly growing tumors, whereas the LRRC3B-1 and LRRC3B-2 cells formed tumors that were much smaller (Fig. 4D), suggesting that LRRC3B has tumor suppressor activity and is a negative regulator of tumor growth both in vitro and in vivo.

Tumorigenicity of LRRC3B-transfected cells in nude mice is inhibited by immune responses. To understand how LRRC3B exerted tumor suppressor activity in xenografted mice, we compared the gene expression profiles of tumors derived from LRRC3B-2 cells and control vector–transfected cells using cDNA microarrays. We initially planned a transcriptome analysis using a microarray for the xenograft tumors derived from the LRRC3B-1 line as well as the LRRC3B-2 line; however, the tumors derived from LRRC3B-1 cell were too small to obtain enough RNA to be used in a microarray analysis. LRRC3B mRNA expression level in three xenograft tumors derived from LRRC3B-2 cells and three control tumors was confirmed by RT-PCR (Fig. 5A). We identified 140 genes with a >1.5-fold increase in expression in LRRC3B-expressing xenograft tumors and categorized those selected genes into functional groups (Fig. 5B). Seven categories potentially linked to the inhibition of tumor growth were identified, including mediators of immune responses, cell-cell signaling, cell adhesion, cell growth, cell death, signal transduction, and metabolism. Remarkably, 30 genes potentially promoting the immune responses showed increased expression in the LRRC3B-expressing xenograft tumors, and 11 genes among them were related to IFN response (Supplementary Table S3).

In H&E-stained sections, the LRRC3B-expressing xenograft tumor showed several lobular tumors and infiltrating lymphocytes, whereas the control cell–derived xenograft tumor showed a nonlobular form and few infiltrating lymphocytes (Fig. 5C). The presence of lymphocytes in the LRRC3B-expressing xenograft tumor was much more pronounced around each lobule. Compared with wild-type mice, athymic nude mice have higher levels of natural killer (NK) cells and fewer T cells that can contribute to immune surveillance (34, 35). To determine the infiltration of NK cells in each xenograft tumor, we visualized NK cells by immunostaining. A strong infiltration of positively stained NK cells was observed only in the LRRC3B-expressing tumors, whereas little or no infiltration of NK cell marker–positive cells was observed within control cell–derived xenograft tumors (Fig. 5D), indicating that the LRRC3B-expressing xenograft tumor was sensitized to lymphocytes such as NK cells. These results suggest that the LRRC3B-expressing xenograft tumor was sensitized to immune surveillance in nude mice.

Discussion

Our results show that LRRC3B expression is repressed in gastric cancer cells by epigenetic mechanisms and that its normal expression results tumor suppressor activity both in vitro and in vivo, suggesting that it is a new tumor suppressor gene in gastric cancer, as based on several lines of evidence. First, expression of LRRC3B was repressed in most of the gastric cancer cell lines (90.9%) and gastric tumor tissues (88.5%) we tested. Second, the chromosome 3p region, where human LRRC3B (3p24.1) is located, is one of the most frequently deleted chromosomal regions in gastric cancers (36, 37). For example, RASSF1A, at chromosome 3p21.3, is a tumor suppressor gene frequently silenced in gastric cancers by epigenetic modification and loss of heterozygosity (38). Third, restoration of LRRC3B expression in gastric cell lines strongly inhibited cell growth both in anchorage-dependent and anchorage-independent assays. Finally, expression of LRRC3B in xenografted mice greatly reduced tumor growth.

Epigenetic modification was the main mechanism of LRRC3B inactivation in the gastric cancer specimens we tested, with LRRC3B expression silenced by promoter hypermethylation. Sixty-seven percent of the primary gastric tumors showed >2-fold decreased expression levels of LRRC3B, and most of them had hypermethylated CpG sites in the LRRC3B promoter region. We also investigated the possibility of genetic mutations inactivating LRRC3B but found no nonsynonymous or frameshift mutations in the LRRC3B coding region (data not shown). Acetylation levels of histone H3 and H4 at the LRRC3B CpG island were decreased in the gastric cancer cell lines we tested, in which the LRRC3B CpG island is hypermethylated and transcriptionally silenced. Thus, DNA hypermethylation and histone deacetylation seem to be the main mechanisms of inactivation of LRRC3B in gastric cancer. Among subtypes of gastric tumors, LRRC3B expression was more frequently reduced in early-stage gastric cancer than in advanced-stage gastric cancer. Although epigenetic silencing of genes can occur at any time during tumor progression, it occurs most frequently during early stages (14, 39). The frequent loss of LRRC3B expression in early-stage gastric cancer suggests that epigenetic silencing of LRRC3B is an important event in tumor initiation.

To understand how LRRC3B exerts its tumor suppressive function, we performed microarray analysis with xenograft tumors derived from a stable LRRC3B-expressing cell line, as gene expression analysis of xenograft models can give useful information about in vivo tumorigenesis mechanisms involving secreted and cell surface proteins (40, 41). Our microarray analysis showed that the most significant gene expression change in the LRRC3B-expressing xenograft tumors was the induction of genes involved in the immune response and IFN pathways. CD59, CD74, and MHC class I antigens are important in recognition by the immune system. Down-regulation of MHC class I antigens and loss of tumor antigens is a major mechanism by which tumor cells escape immune surveillance (42). The induction of immune recognition genes in LRRC3B-expressing xenograft tumors suggests that one of the tumor suppressive mechanisms of LRRC3B is the induction of immune recognition against tumors and lymphocyte infiltration. In fact, we found many infiltrating lymphocytes in H&E-stained sections of an LRRC3B-expressing xenograft tumor. IFN pathway genes were also induced. The IFN signaling pathway is growth suppressive, and multiple IFN pathway genes are epigenetically silenced after cellular immortalization (43). Also, some IFN signaling genes such as RNaseL (44) and IRF-1 (45) show tumor suppressor activity. Additionally, the well-established tumor suppressor genes BRCA1 and DAP kinase have been identified as components in the IFN γ-regulated signaling pathway (46).

In summary, inactivation of LRRC3B, which encodes a membrane protein, may be a critical event in the early stages of gastric cancer tumorigenesis that induces cancer cells to escape immune
surveillance. We suggest that \textit{LRRC3B} is a tumor suppressor gene that triggers the innate immune response by lymphocyte infiltration and activation of the IFN signaling pathway.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References


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LRRC3B, Encoding a Leucine-Rich Repeat-Containing Protein, Is a Putative Tumor Suppressor Gene in Gastric Cancer

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