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Novel Functional Single Nucleotide Polymorphisms in the Latent Transforming Growth Factor-β Binding Protein-1L Promoter

Effect on Latent Transforming Growth Factor-β Binding Protein-1L Expression Level and Possible Prognostic Significance in Ovarian Cancer

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Latent transforming growth factor (TGF)-β binding proteins (LTBPs) play important roles in the secretion and activation of TGF-β. We previously reported that LTBP-1L is overexpressed in some patients with ovarian cancer. To clarify the molecular mechanism of LTBP-1L regulation, we analyzed DNA sequences in the promoter region of *LTBP-1L* and identified two novel single nucleotide polymorphisms, −202G/C and +20A/C. While the alleles with −202C and +20C were initially reported, our data demonstrated that −202G and +20A are common in both ovarian cancer patients and healthy patients in the Japanese population. Luciferase reporter assays revealed that the G-A haplotype induced transcriptional activation in a Sp1-dependent manner. Electrophoretic mobility shift assays showed that increased binding affinity of Sp1 to the promoter with −202G and +20A. Interestingly, ovarian cancer patients (n = 42) with G-A/G-A homozygous genotype had increased expression of LTBP-1 and apparently poorer survival than those with other genotypes (P = 0.02). These findings suggest that the single nucleotide polymorphisms −202G/C and +20A/C on the *LTBP-1L* promoter may affect the clinical outcome of ovarian cancer patients, probably via up-regulating protein expression. Further studies using a larger number of samples will definitively determine the correlation between LTBP-1 haplotype and clinical behavior of ovarian cancer. *(J Mol Diagn 2006, 8:342–350; DOI: 10.2353/jmoldx.2006.050133)*

Transforming growth factor (TGF)-β is a potent growth inhibitor for most cell types, including epithelial cells.[1,2] The secretion of TGF-β by tumor cells may contribute to tumor suppression by autocrine growth inhibition, but on the other hand, it may also promote tumor progression by stimulating tumor invasion and angiogenesis, and inhibiting immune response.[3–5] TGF-β is synthesized as latent high-molecular weight complexes, composed of TGF-β, the NH_2-terminal part of the TGF-β precursor, and the latent TGF-β binding protein (LTBP).[6–9] LTBP is a glycoprotein with a molecular weight of more than 190 kd; it possesses 16 to 18 epidermal growth factor (EGF)-like domains and several repeats of a unique motif containing eight cysteine residues.[10–12] Four isoforms of LTBP have been found in mammalian species (LTBP-1 to LTBP-4).[13–17] LTBP-1 binds to TGF-β1 through one of the eight cysteine motifs and facilitates assembly and secretion[18,19] and activation[20–22] of TGF-β1. Immunoelectron-microscopic observations indicate that LTBP-1 is one of the extracellular microfibrillar components[23]; it targets TGF-β1 to extracellular structures and participates in the activation of latent TGF-β1, perhaps by concentrating latent TGF-β1 on the cell surface where activation occurs.[9] LTBP-1S (short) and LTBP-1L (long) are derived from independent promoters and alternative splicing between codons 145 and 146 of LTBP-1S.[24] LTBP-1L has a N-terminal extension of 346 amino acids that is not found in the LTBP-1S.[25] The N-terminal extension contains an EGF-like domain that facilitates matrix incorporation, and LTBP-1L has been confirmed to associate more efficiently than LTBP-1S with the extracellular matrix.[25]

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We previously demonstrated overexpression of LTBP-1 and TGF-β1 proteins in some ovarian cancer patients, predominantly because of up-regulation of LTBP-1L mRNA rather than LTBP-1S mRNA.[26] This overexpression is thought to affect the function of TGF-β1 and its signaling, possibly contributing to ovarian carcinogenesis. However, little information is available concerning the transcriptional regulation of these genes. The present study therefore aimed to clarify the molecular mechanisms of LTBP-1L overexpression in ovarian cancers and to investigate the clinical significance of the overexpression. We found that two novel single nucleotide polymorphisms in the 5′-regulatory region of the *LTBP-1L* gene critically contribute to the overexpression of this gene.

Materials and Methods

Samples and DNA Preparation

Tissue specimens were surgically obtained from a consecutive series of 42 patients aged 23 to 77 with ovarian cancer in Kanazawa University Hospital and stored at −80°C for DNA analysis or fixed with formaldehyde for immunohistochemical analysis. Patients gave written informed consent for the use of their tissues in this study. All of the patients were newly diagnosed, previously untreated (chemotherapy or radiotherapy), and histologically confirmed without family history of ovarian cancer. Healthy control DNA samples were obtained from buccal epithelial cells of 156 Japanese individuals (80 males and 76 females) age 20 to 60 years, including staff and students in Kanazawa University, with written informed consent. Buccal epithelial cells were collected by modifying the mouthwash method described previously.[27,28] Briefly, at least 3 hours after brushing their teeth, participants swished 10 ml of mouthwash, Mondahmin (Earth Chemical, Tokyo, Japan), vigorously in their mouths for 30 seconds and then spat it into a conical tube. The buccal cells were harvested by centrifugation at 3000 × *g* for 10 minutes. Total genomic DNA was purified using a Wizard genomic DNA purification kit (Promega, Madison, WI).

Sequencing of LTBP-1L Promoter

The *LTBP-1L* promoter region spanning −2164 and +130 was analyzed by direct sequencing. The polymerase chain reaction (PCR) primer sequences were F(forward)-1: 5′-CGTCGACTCGATCTCAAAGTGTTGC-3′ and R(reverse)-1: 5′-GAGGATTGAGGTGAGTCACAAGG-3′, F-2: 5′-GTAGAACAAGGAATTGGATCCGT-3′ and R-2: 5′-TTGATTTGGCAGGCAGGGCCTC-3′, F-3: 5′-GTTCTCACAAGCAGCTAGTGCT-3′ and R-3: 5′-GAAAGTCCACAGTCATAGCAGTC-3′, F-4: 5′-CAAAGCCTTGGAAACACACCATC-3′ and R-4: 5′-TTAGGGTAGGACTAGAGTTCA-3′, F-5: 5′-GTCGGATTACGGTCCCGTGA-3′ and R-5: 5′-TTACTGAACGATCCTGTCCTTTC-3′, F-6: 5′-AGTATCACAGCAAACACGGAT-3′; and R-6: 5′-GGTGCACCACGTAGGTGATCCTCC-3′. All PCR products were purified and subsequently directly sequenced from both ends. All sequencing reactions were performed using dye-terminator chemistry (Applied Biosystems, Foster City, CA) in an ABI 310 DNA analyzer.

Haplotype Analysis

The haplotypes of −202G/C and +20A/C were analyzed by PCR-based restriction fragment length polymorphism (RFLP) methods. The *LTBP-1L* promoter region spanning −392 and +154 was amplified using F-7: 5′-TTGGCTGCTCAGGTCTGACA-3′ and R-6 primers. The PCR cycling conditions were 2 minutes at 94°C followed by 31 cycles of 1 minute at 94°C, 1 minute at 56°C, and 1 minute at 72°C, with a final step at 72°C for 10 minutes to allow for the complete extension of all PCR fragments. The number of cycles was within the exponential region of the PCR reaction. The 546-bp PCR products were subjected to *Eco*RII (Toyobo, Osaka, Japan) and *Csp*I (Toyobo) digestion, and the DNA fragments were separated on a 3% agarose gel. The haplotypes were determined as follows: three fragments of 187, 269, and 90 bp represent the G-A haplotype; three fragments of 407, 49, and 90 bp represent the C-C haplotype; four fragments of 187, 220, 49, and 90 bp represent the G-C haplotype; and two fragments of 456 and 90 bp represent the C-A haplotype. Hetero-duplex fragments of 456 bp, which could not be digested by the restriction enzymes, were also produced because of mismatched annealing of heterogeneous alleles. We confirmed that such 456-bp products detected in patients with heterozygous genotypes were not due to the C-A allele by means of DNA sequencing of the purified bands.

Cell Culture

The RMUG-S cell line was derived from ovarian mucinous adenocarcinoma (JCRB, Osaka, Japan). The cells were maintained in Ham's F12 medium (Sigma-Aldrich Corp., St. Louis, MO) containing 10% fetal bovine serum (JRH Bioscience, Lenexa, KS), 100 U/ml penicillin, and 100 μg/ml streptomycin.

Preparation of the Plasmids

The *LTBP-1L* promoter-luciferase reporter plasmid was a generous gift from Dr. Jorma Keski-Oja (University of Helsinki, Helsinki, Finland).[24] It contains the promoter fragment spanning −2211 to +54 upstream of the initiating ATG codon, inserted into luciferase reporter vector pGL3-Basic (Promega). The mutated plasmids were constructed using the GeneTailor site-directed mutagenesis system (Invitrogen Life Technologies, Carlsbad, CA). The forward and reverse mutagenic primers for −202C to G were 5′-TGCGCGGCCCGCTCCCCT*G*GCCCCTCCCCGCTCCC-3′ and 5′-AGGGGAGCGGGCCGCGCAAGGTGAGGGTCC-3′, respectively. The forward and reverse mutagenic primers for +20C to A were 5′-GGCCGGGGGAGGGGGCCGGAC*A*GCGCGCGACC-3′ and 5′-GTCCGGCCCCCTCCCCCGGCCGTGCGGCTCGCCT-3′, respectively (mutated sites are underlined). Nucleotide sequences were checked by DNA sequencing analyses. The plasmid con-

structs pM, pM-Sp1, and pCMV-DNSp3 were kind gifts from Dr. Yoshihiro Sowa (Kyoto Prefectural University of Medicine, Kyoto, Japan). pM-Sp1 is an expression vector for Sp1 protein. pCMV-DNSp3 is an expression vector for the dominant-negative form of Sp3, in which the function of Sp1 is abrogated.[29,30]

Luciferase Assays

RMUG-S cells (5×10^4) were seeded on 24-well plates 1 day before transfection. The cells were transfected with 0.4 μg of each reporter plasmid alone or together with the same amount of effector plasmid using FuGENE6 transfection reagent (Roche Molecular Biochemicals, Mannheim, Germany). *Renilla* luciferase plasmid phRL-tk was co-transfected to normalize the transfection efficiency. After 48 hours, the cells were lysed using a dual-luciferase reporter assay system (Promega) and the firefly luciferase and *Renilla* luciferase activities were measured with a Lumat LB9507 (Berthold Technologies, Tokyo, Japan). All experiments were performed at least three times for each reporter plasmid and the relative luciferase activity was calculated. Results are mean ± SD ($n = 3$).

Electrophoretic Mobility Shift Assay (EMSA)

EMSA was performed as described previously.[31] Briefly, synthetic double-stranded oligonucleotides 5′-GAGAATGCGGACCCTCCTGGGAGT-3′ and 5′-GAGAATGCGGAC*T*CTCCTGGGAGT-3′ corresponding to the −202C and −202G sequences of the *LTBP-1L* promoter region were labeled with [γ-^{32}P]ATP (Amersham Pharmacia Biotech, Piscataway, NJ) using the MEGALABEL kit (Takara, Shiga, Japan). One μg of recombinant human Sp1 protein (Promega) was incubated with 1 μg of poly(dI-dC) (Amersham Biosciences) in the presence or absence of a 100-fold molar excess of unlabeled competitor DNA on ice for 20 minutes in a 25-μl reaction mixture containing 10% glycerol, 25 mmol/L HEPES (pH 7.9), 50 mmol/L KCl, 0.5 mmol/L phenylmethyl sulfonyl fluoride, and 1 mmol/L dithiothreitol. For supershift assay, specific antibodies against Sp1 (Santa Cruz Biotechnologies, Santa Cruz, CA) or estrogen receptor (Santa Cruz Biotechnologies) were preincubated with recombinant Sp1 proteins (Promega) on ice for 60 minutes. After incubation, an aliquot of labeled oligonucleotide (>30,000 cpm) was added, and the reaction mixture was incubated at room temperature for an additional 20 minutes. The DNA-protein complexes were then separated from free probes by electrophoresis on a 5% polyacrylamide gel. The gel was dried and subjected to autoradiography using a Fuji BAS-III bioimaging analyzer (Fuji Photo Film, Kanagawa, Japan). Consensus oligonucleotides for Sp1 (5′-ATTCGATCGGGGCGGGGCGAGC-3′; Promega), or AP2 (5′-GATCGAACTGACCGCCCGCGGCCCGT-3′; Promega) were also used as competitors to confirm the specific binding of Sp1.

Immunohistochemistry

Immunohistochemical assays for LTBP-1 were performed on formalin-fixed, paraffin-embedded specimens of ovary tissues, using the Vectastain ABC Elite kit (Vector Laboratories, Burlingame, CA) as described previously.[32] Antigen retrieval was performed for 10 minutes in 1× antigen retrieval solution (Biogenex, San Ramon, CA), and endogenous peroxidase was quenched in methanol with 3% H_2O_2. Then, the tissue sections were incubated for 16 hours at 4°C with mouse monoclonal anti-LTBP-1 antibody (R& D Systems, Minneapolis, MN) at a dilution of 1:1000 or with nonimmune whole mouse serum. After washing the sections were incubated with a biotinylated secondary antibody, followed by detection by sequential reaction with a streptavidin-biotin-horseradish peroxidase complex, biotinylated tyramide, streptavidin peroxidase, and 3,3′-diaminobenzidine (Dako Cytomation, Carpinteria, CA). Sections were lightly counterstained with Mayer's hematoxylin, and then mounted. Staining was evaluated as negative 0 (no staining), low 1+ (positive in 5 to 25% cells or staining intensity was extremely low), intermediate 2+ (positive in 25 to 75% cells, intensely positive), high 3+ (positive in >75% cells and staining intensity was extremely high). Two independent pathologists judged the results and cases with discordant results were excluded from the analyses.

Statistical Analysis

Deviations of the genotype frequencies in the cancer cases and controls from those expected under Hardy-Weinberg equilibrium were assessed with the χ^2 test. The luciferase assay data were analyzed by analysis of variance followed by Fisher's protected least significant difference (PLSD) test. Immunohistochemical staining was analyzed by Fisher's exact probability test. Overall survival was measured from primary surgery to death or the date of last follow-up. Patient follow-up was updated through December 1, 2004. Survival curves were plotted by using the Kaplan-Meier method,[33] and compared using the log-rank test.[34]

Results

Identification of Novel SNPs in the LTBP-1L Upstream Region

We compared the *LTBP-1L* promoter sequences in patients with ovarian cancers and in healthy controls with those registered in GenBank (accession no. AF171934) and identified two novel SNPs, −202G/C and +20A/C (Figure 1, A and B). Because −202G generates an *Eco*RII site and +20C generates a *Csp*I site (Figure 1B), the haplotypes between −202G/C and +20A/C were distinguished by RFLP analysis (Figure 1C). Of four predicted haplotypes, only two types, G-A and C-C were identified in both ovarian cancer patients and healthy controls and therefore three genotypes, G-A/G-A, G-A/C-C, and C-C/C-C, were detected.

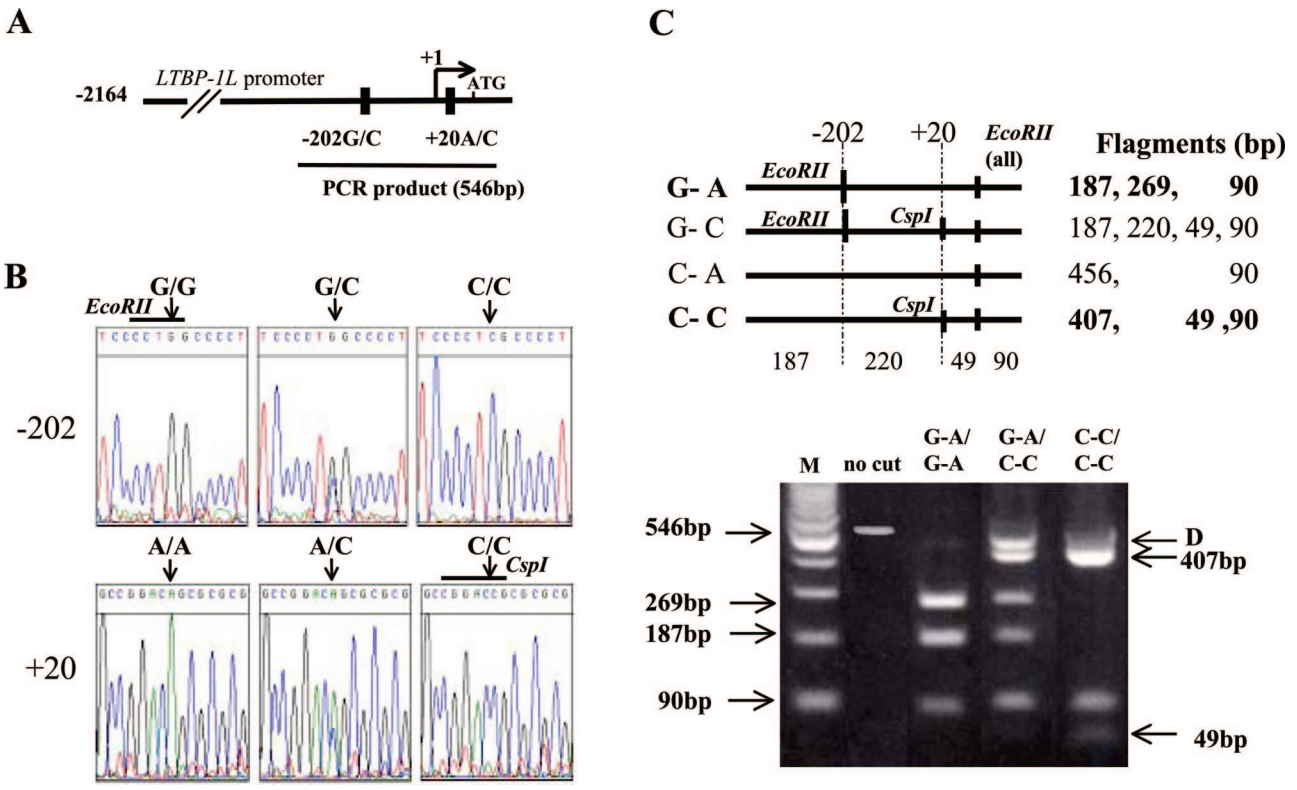

Figure 1. Screening for the *LTBP-1L* genotypes. **A:** Schematic representation of the two novel SNPs in the *LTBP-1L* promoter. The transcriptional start site is indicated as +1. **B:** Representative results of nucleotide sequence analysis. **Arrows** indicate the location of SNPs. Restriction enzymes used in PCR-restriction fragment length polymorphism (RFLP) analysis are indicated above the sequences. **C:** Representative results of PCR-RFLP analysis. Numbers in parentheses are the size of each fragment (bp). **Arrow D** indicates the heteroduplex complexes produced by mismatched annealing of heterogeneous alleles (see Materials and Methods). M, 100-bp DNA ladder marker.

The −202C and +20C, which were originally registered in GenBank, were rather rare in both ovarian cancer patients and healthy Japanese controls (Table 1). The allele frequency in the *LTBP-1L* promoter SNPs in ovarian cancer patients was not significantly different from that in healthy controls (Table 1). Thus, we newly identified the G-A allele in the *LTBP-1L* promoter, and showed that it is frequently detected in the Japanese population.

Impact of LTBP-1L SNPs on the Transcriptional Activity of the Promoter

To examine the effect of the SNPs at −202 and +20 on the transcriptional activity of the *LTBP-1L* promoter, luciferase reporter plasmids containing the *LTBP-1L* promoter region spanning −2211 to +54 with four haplotypes (G-A, G-C, C-A, C-C) were prepared (Figure 2A) and transfected into ovarian cancer RMUG-S cells, and luciferase assays were performed. Transcriptional activity of the G-A haplotype was the highest, being 3.6-fold higher than that of the C-C haplotype (Figure 2B) (25.7 ± 11.7 versus 7.17 ± 3.77, $P < 0.001$), whereas the G-C and C-A haplotypes exhibited intermediate activity.

The *LTBP-1L* promoter contains GC-boxes, which are reminiscent of a Sp1 binding motif. Computer-assisted homology search identified five putative Sp1 motifs in the *LTBP-1L* promoter (Figure 2A), and two of them overlapped with, or were adjacent to, SNP sites. We therefore tested whether Sp1 regulates the *LTBP-1L* promoter. Co-transfection of reporter plasmids having the G-A haplotype with Sp1 expression vectors markedly increased the transcriptional activity (Figure 2C). In contrast, co-transfection with a dominant-negative Sp3 expression vector, which is known to inhibit the function of Sp1, significantly abrogated the transcriptional activity. Similar effects of Sp1 were observed with promoter having the C-C haplotype as well, but with lower efficiency. These data suggest a potential role of Sp1 in activating the *LTBP-1L*

Table 1. Genotyping of *LTBP-1L* Promoter in Ovarian Cancers and Healthy Controls

	Genotyping of *LTBP-1L* promoter			Allele frequency	
	G-A/G-A	G-A/C-C	C-C/C-C	G-A	C-C
Ovarian cancer (n = 42)	15 (35.7%)	18 (42.9%)	9 (21.4%)	0.571	0.429
Healthy control (n = 156)	49 (31.4%)	84 (53.9%)	23 (14.7%)	0.583	0.417

Hardy-Weinberg equilibrium test indicates that both populations were in equilibrium.
$P = 0.391$ by χ^2 test

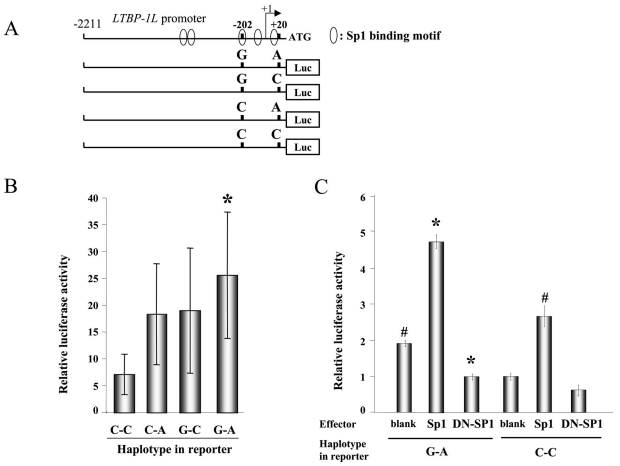

Figure 2. Luciferase assays with *LTBP-1L* promoter reporter constructs having the four haplotypes, using ovarian cancer RMUG-S cells. **A:** Schematic representation of luciferase reporter constructs containing a −2211 to +54 promoter region with different haplotypes at the SNP sites. **B:** The relative luciferase activities of reporter plasmids with the different haplotypes. Each column represents the mean of three independent experiments, each done in triplicate; bars, ±SD ($^{*}P < 0.01$ compared with the C-C construct). **C:** Luciferase assays to examine the role of Sp1 in *LTBP-1L* promoter. Luciferase plasmids with G-A or C-C haplotype were co-transfected with blank vector, Sp1, or dominant-negative Sp3 expression vector to inhibit Sp1 activity. The relative luciferase activities in each reporter plasmid are shown, based on the luciferase activity in C-C reporter plasmid transfected with blank vector, normalized to 1. Each column represents the mean of three independent experiments, each done in triplicate; bars, ±SD ($^{*}P < 0.0001$ compared with the G-A blank, $^{\#}P < 0.0001$ compared with the C-C blank).

promoter, especially in the case of the G-A haplotype. To further clarify the functional impact of these SNPs on the promoter activity, EMSAs were performed. Recombinant Sp1 proteins were incubated with promoter fragments spanning −217 to −189, with G or C at −202, respectively (Figure 3A). No band or only faint bands were

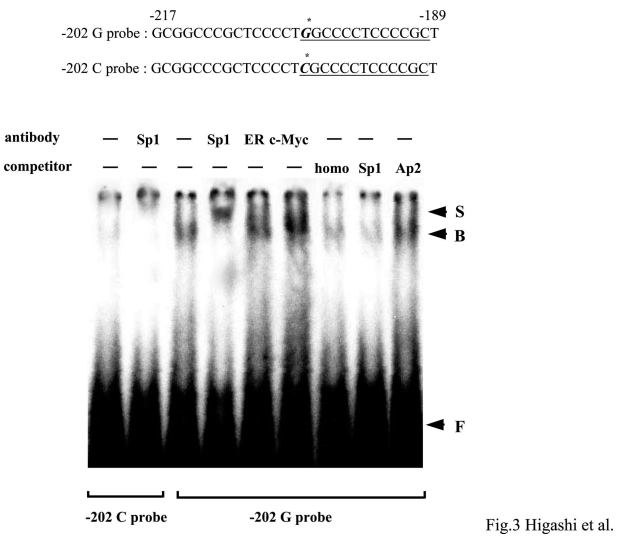

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Figure 3. EMSAs for Sp1 binding to the *LTBP-1L* promoter. The nucleotide sequences of the probes with SNP at −202 are shown. **Underlining** indicates the Sp1 binding motif. The **asterisk** shows the SNP site. Recombinant Sp1 proteins were incubated with ^{32}P-labeled probes with G or C at −202, respectively, and separated by electrophoresis. Antibodies and competitors used are shown. Homo, homologous competitors of −202G probe. **Arrow B** represents specific binding complexes of Sp1 and **arrow S** exhibits the supershifted bands. **Arrow F** shows free probes.

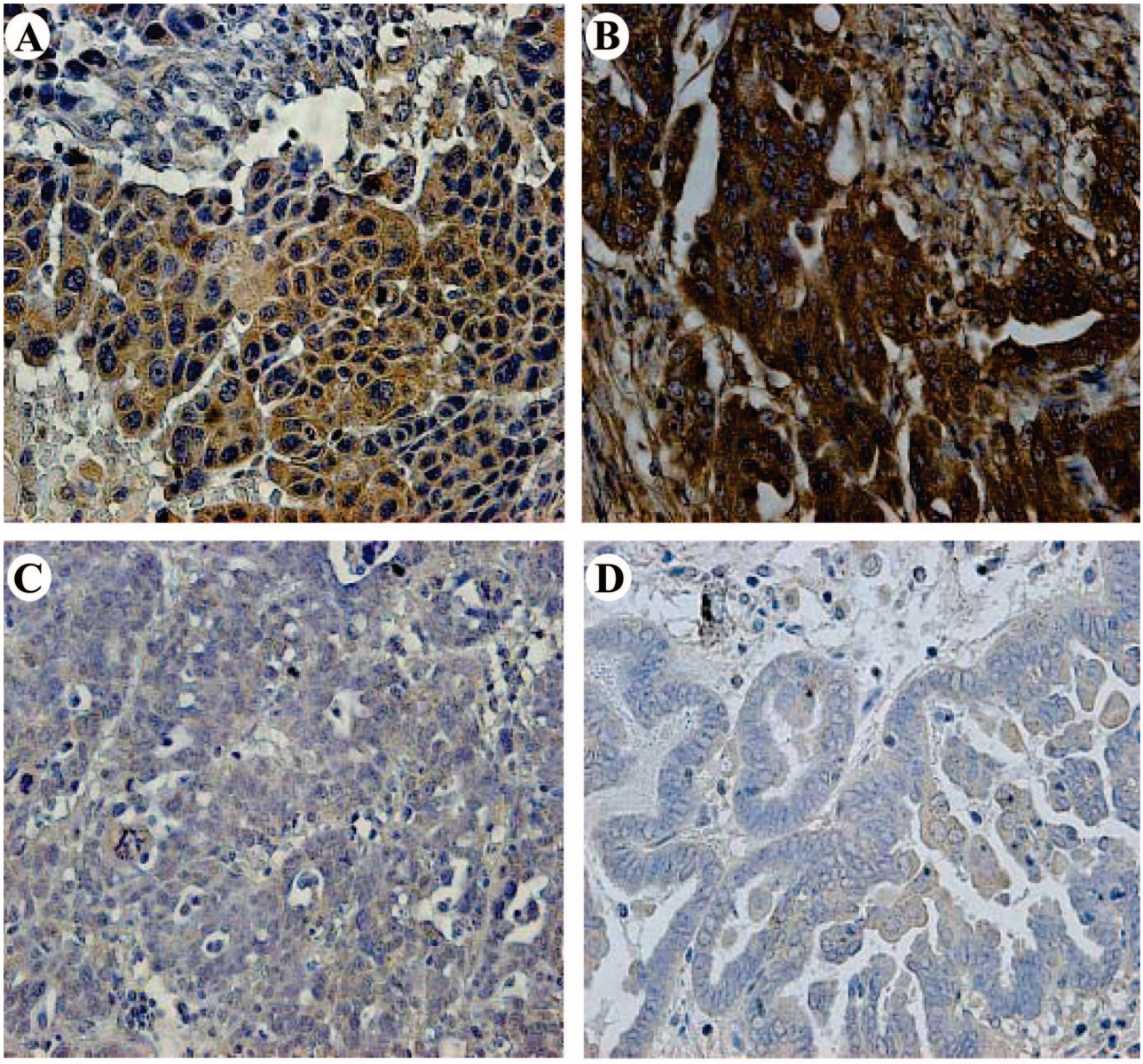
formed with probes with −202C, whereas probes with −202G clearly generated binding complexes. To confirm the binding specificity, supershift assays were performed. The binding complexes were supershifted by the addition with Sp1 antibody, but not by unrelated estrogen receptor or c-*myc* antibody. Furthermore, these bands were eliminated by the addition of homologous competitors, as well as Sp1 consensus oligonucleotides, but not by unrelated Ap2 oligonucleotides. Similar findings were observed using probes containing the SNP site at +20: probes with +20A formed definite binding complexes, whereas those with C did not. However, the binding affinity of Sp1 with the probe containing +20 was weaker than that with the probe containing −202G (data not shown). Taken together, these results clearly demonstrate that the SNPs at −202 and +20 in the *LTBP-1L* promoter contribute to increased binding affinity of Sp1, probably leading to transcriptional activation of this promoter.

Immunohistochemical Analyses of LTBP-1 Expression in Ovarian Cancers

The effect of these two SNPs on the LTBP-1 protein expression level was further examined by immunohistochemical analysis using surgical specimens obtained from 36 patients with different genotypes. Immunostaining of LTBP-1 was detected in both cancer and stromal cells in all tissue samples examined (Figure 4). Of 13 ovarian cancers with G-A homozygous alleles, 4 exhibited high and 6 showed intermediate expression (Figure 4, A and B). Of 14 cancer samples with heterozygous alleles, 4 exhibited high expression and 2 showed intermediate expression, and 8 had low expression (Figure 4C). Of nine cancers with C-C homozygous alleles, one exhibited high expression and two showed intermediate expression, and five had low and one had no expression (Figure 4D). When patients with intermediate or high expression were categorized as having increased expression, G-A homozygous alleles were significantly associated with increased expression of LTBP-1, compared with heterozygous and C-C homozygous alleles ($P < 0.05$).

Impact of LTBP-1L SNPs on Clinical Features and Outcomes of Patients with Ovarian Cancer

We next examined the relationship between genotype of *LTBP-1L* and clinicopathological characteristics of ovarian cancers, including age, histological types, tumor grading, and FIGO clinical staging. There was no significant correlation between *LTBP-1L* genotype and such parameters. Survival analyses were then performed. In univariate analyses, we found that the 5-year overall survival rate was significantly lower in patients with G-A homozygous alleles than in patients with the heterozygous or C-C homozygous alleles ($P = 0.021$) (Figure 5).



Correlation between genotype of *LTBP-1L* promoter and LTBP-1 expression in ovarian cancer

Genotype	n	Staining intensity				
		0	1+	2+	3+	
G-A/G-A	13	1	2	6	4	#
G-A/C-C	14	0	8	2	4	
C-C /C-C	9	1	5	2	1	

# $P<0.05$, G-A/G-A vs. G-A/C-C and C-C/C-C, by Fisher's exact probability test.
2+ or 3+ were categorized into increased expression compared to 0 or 1+.

Figure 4. Immunohistochemical staining of LTBP-1 protein in ovarian cancer tissues. Representative results of immunohistochemistry of LTBP-1 are shown in ovarian cancers with different genotypes of *LTBP-1L*. Tissues from patients with G-A/G-A homozygous (**A**, **B**), those with G-A/C-C heterozygous (**C**) and C-C/C-C homozygous (**D**) *LTBP-1L* promoter are shown. Cancer tissues with G-A/G-A genotypes exhibited high levels of LTBP-1 staining. Original magnifications, ×200.

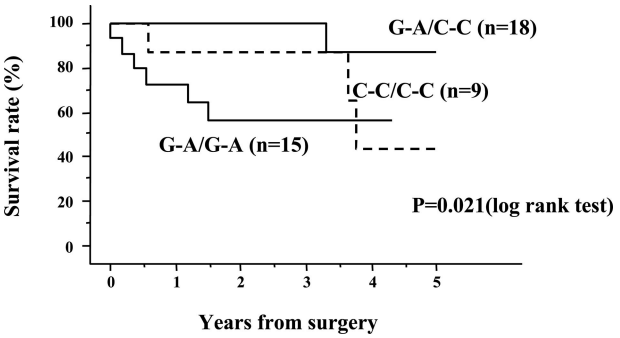


Figure 5. Survival curves according to *LTBP-1L* promoter genotype in ovarian cancer patients. Patients with G-A/G-A homozygous genotype had a significantly lower survival rate than those with G-A/C-C heterozygous or C-C/C-C homozygous genotype. The *P* value was determined using the log-rank test.

Discussion

We identified two novel SNPs, −202G/C and +20A/C, in the *LTBP-1L* promoter and 5′-UTR regions. The *LTBP-1L* promoter was initially cloned from a human placental library,[24] and −202C and +20C were registered in GenBank. However, we found that G at −202 and A at +20 are commonly observed in the Japanese population. Haplotype analyses between −202G/C and +20A/C revealed that there are only G-A and C-C haplotypes in the *LTBP-1* promoter, so −202G is tightly linked to +20A. No difference of allele frequency was observed between male and female healthy controls (data not shown). At present, we do not know the ethnic differences in allele frequencies or haplotypes of these SNPs, and this would be a worthwhile direction for future investigation. There was no difference in the allele frequency between patients and healthy controls. These findings indicate that the SNPs are not involved in susceptibility to ovarian cancer.

It has been reported that SNPs in promoter regions can modulate gene transcription by affecting DNA binding of *trans*-acting elements.[35–39] Luciferase reporter assays revealed that the G-A haplotype caused transcriptional activation more efficiently than other haplotypes. The *LTBP-1L* promoter has a high GC content, but lacks a TATA box. Such promoters are often activated by Sp family proteins.[40,41] In fact, there are five putative Sp1 motifs in the *LTBP-1L* promoter, and interestingly, one overlaps with the SNP site at −202, while another is adjacent to the SNP site at +20. Luciferase reporter assays revealed that overexpression of Sp1 significantly activated the *LTBP-1L* promoter, with higher efficiency in G-A haplotype than C-C haplotype. EMSAs showed increased binding affinity of Sp1 to the promoter with −202G and +20A. These findings suggest that a change in Sp1 binding affinity by SNPs leads to increased transcriptional activity. It is well known that Sp1 is constitutively overexpressed in a variety of human cancers.[42–45] In ovarian cancers, such overexpression may facilitate LTBP-1 expression in patients with G-A alleles, as was observed in the present immunohistochemical analyses. Tightly linked SNPs within or adjacent to Sp1 binding sites are also known in the promoters of other cancer-related genes, such as EGFR, MMP-2, and vascular endothelial growth factor.[37,38,41] Most of them affect the transcriptional activity of the promoters by interacting with Sp1. The SNPs we identified in the LTBP-1 promoter may be categorized as such functional SNPs. Thus, the Sp1 site may be a hot spot of functional SNPs in GC-rich promoters, and it might be interesting to screen these regions in a variety of cancer-related genes. We conclude that the newly identified SNPs in the *LTBP-1L* promoter may play roles in up-regulation of *LTBP-1L*.

One interesting finding in the present study is that ovarian cancer patients with G-A/G-A alleles had significantly poorer survival although analysis using a larger number of samples is required to obtain a definite conclusion. How can we explain such a correlation? TGF-β can act both as a tumor suppressor and as a stimulator of tumor progression. At an early stage of carcinogenesis, when growth-inhibitory responses to TGF-β are maintained in cells, TGF-β directly suppresses tumor growth.[46,47] However, after cancer cells become resistant to TGF-β growth inhibition at the later stages of carcinogenesis, TGF-β contributes to tumor invasion and metastasis by stimulating tumor cell motility, angiogenesis, remodeling of the extracellular matrix protein by stromal cells, and even by suppressing the immune system.[48–53] Because LTBP-1 may be an important molecule for activation of TGF-β1,[18–22] one possible explanation of the poor prognosis of patients with G-A/G-A alleles may be that increased expression of LTBP-1L enhances TGF-β1 activity and facilitates tumor progression. We are currently investigating the possible correlation between elevated levels of TGF-β1 activity and LTBP-1L expression both *in vitro* and *in vivo* to test this hypothesis.

In summary, we have identified novel SNPs in the *LTBP-1L* promoter, and shown that they contribute to transcriptional activation, leading to up-regulation of LTBP-1L expression and poorer survival in ovarian cancer patients. Further studies using a larger number of samples will definitively determine the correlation between LTBP-1 haplotype and clinical behavior of ovarian cancer. Analysis of the roles and mechanisms of LTBP-1L expression in ovarian cancer may improve our understanding of the molecular mechanisms of ovarian carcinogenesis, as well as assisting in the development of novel molecular-based therapies.

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