BPB Reports

Regular Article

Elevated Expression of CELSR1 is Associated with Peritoneal Metastasis in Human Scirrhous Gastric Cancers

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Recommended by Masahiko Satoh; Received July 17, 2021; Accepted July 19, 2021

Cancer metastasis is the leading cause of death in patients with any type of solid cancer. To develop effective cancer treatments, it is essential to understand the molecular mechanisms underlying cancer metastasis. Previously we established peritoneal metastasis cell models derived from human scirrhous gastric cancer patients. In this article, we focus on the CELSR1 gene, which is involved in Wnt signaling but whose association with peritoneal metastasis is still unclear. We unveiled gene alterations and the prognostic relevance of the CELSR1 gene in cancer patients by analyzing public resources for cancer genomic and patient cohorts. RT-qPCR and immunoblot analyses revealed that CELSR1 expression was significantly elevated in our cell lines, which had high peritoneal metastatic property, compared with their parental cell lines, which had lower peritoneal metastatic property. Some of the gene alterations in the coding region of CELSR1 were observed in our metastatic cell lines, but they were not associated with the metastatic property or with patient prognosis. Knockdown of CELSR1 via the shRNA technique significantly decreased migration and invasion in the cell lines having high peritoneal metastatic property, whereas the knockdown did not significantly affect proliferation. These results show that CELSR1 plays an important role in peritoneal metastasis and that CELSR1 is a novel peritoneal metastasis-associated gene. The results also suggest that CELSR1 is a proper molecular target for therapy against peritoneal metastasis of scirrhous gastric cancers.

Key words scirrhous gastric cancer, peritoneal metastasis, invasion, short hairpin RNA, focal adhesion kinase

INTRODUCTION

Gastric cancer (GC) is one of the most common cancers in the world and the second most lethal.[1)] The recent development of a technique for the early detection of cancers has improved survival rates. Approximately 10% of GC is classified as scirrhous gastric cancer (SGC), defined as a diffusely infiltrating carcinoma, and is also known as Borrmann type 4.[2,3)] SGC is aggressive, typically infiltrating the gastric wall, and is frequently accompanied by peritoneal metastasis.[4)] Once some SGC patients start showing peritoneal metastasis, it is hard to treat them with either surgery or anticancer drugs.[5)] Therefore, the prognosis of SGC patients is markedly worse than that of patients with other types of GC. To improve the prognosis of SGC patients, effective therapeutic approaches, especially against peritoneal metastasis of SGC, are essential and urgently needed. However, the molecular mechanisms underlying peritoneal metastasis of SGC have not yet been fully elucidated. These mechanisms must be elucidated, and novel types of medicines to treat SGC patients must be developed.

Previously we established original peritoneal metastasis cell lines from SGC patients to analyze the molecular mechanism(s) underlying peritoneal metastasis by the repetition of orthotopic implantation with SGC patient-derived parental cell lines.[6,7)] Our previous studies showed enhanced expression of WNT3a, WNT5a, and β-catenin associated with the peritoneal metastatic property of the cells.[8)] To extend the significance of our previous findings, we further explored genes related to WNT3a, Wnt5a, and β-catenin using public resources on cancer patients, and we identified CELSR1 (cadherin EGF LAG 7-passed transmembrane G-type receptor) as a potential candidate for a peritoneal metastasis-associated gene in SGC.

Multiple functions of CELSR1 are known mainly in epithelial and nervous system development.[9,10)] In particular, CELSR1 is essential for the development of the nervous system in the brain through the regulation of planar cell polarity (PCP).[11)] In early postnatal development stage, CELSR1 is strongly expressed in neural stem cells.[12)] CELSR1-deficient mice lack normal organization of stereocilia bundles in inner ear hair cells and exhibit abnormal head-shaking behavior.[12)] The stereotypical orientation of ear hair bundles is accomplished by proper regulation of PCP core proteins such as Fzd3, Fzd6, Vangl2, and pk2.[13)] Recently, lines of evidence implicated the Wnt/PCP signaling pathway in cancer progression, including metastasis.[14,15)] However, the roles of CELSR1 in cancer metastasis have not been reported yet.

In the present study, we found significantly elevated expres-

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sion of CELSR1 in our peritoneal metastatic cell lines of SGC. The knockdown of CELSR1 in the peritoneal metastatic cell lines via specific shRNA significantly reduced cell proliferation, migration, and invasion. Interestingly, the alteration of the CELSR1 gene was widely found in various types of cancer patients, not only in those with SGC. Furthermore, elevated CELSR1 expression was associated with a poorer prognosis of cancer patients. Thus, our findings suggest that CELSR1 must play important roles in peritoneal metastasis of SGC.

MATERIALS AND METHODS

Cells We used our original human SGC parental cell lines (HSC-44PE and HSC-58) and their sub-cell lines (44As3 and 58As9), which exhibit higher metastatic properties to the peritoneum in nude mice upon orthotopic implantation. We reported these lines previously.[6] All of the cells were maintained in PRMI 1640 medium (Sigma) supplemented with 10% fetal bovine serum (FBS) at 37°C in a 5% CO_2 incubator. Negative status for mycoplasma infection was validated by PCR in all cell lines.

Short Hairpin RNA (shRNA)-Expressing Vector For CELSR1 knockdown, we employed the LT3GEPIR vector provided by Dr. Johannes Zuber.[16] To obtain shRNA candidate target sequences specific to CELSR1, we used the splashRNA website (http://splashrna.mskcc.org) and used selected sequences according to the instructions.[17] shRNA against Renilla luciferase was used as a negative control. After the cells were transfected with the LT3GEPIR vector, they were cultured in the presence of puromycin (4 μg/mL). To induce the expression of each shRNA, doxycycline (Dox, 200 ng/mL) was added.

Antibodies Anti-CELSR1 antibody (sc-514376) for immunoblot analysis and anti-CELSR1 antibody (ab225889) for immunostainings were purchased from Santa Cruz Biotechnology (Dallas, TX, USA)and Abcam (Cambrige, UK), respectively. Antibodies against β-actin (A5441; Sigma-Aldrich Japan, Tokyo, Japan), focal adhesion kinase, FAK (#3285; Cell Signaling Technology, Danvers, MA, USA), and phospho-FAK (#3281; Cell Signaling Technology) were used.

RT-qPCR Total RNAs were isolated using ISOGEN (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. Human normal stomach tissue RNAs were purchased from TaKaRa Bio (Shiga, Japan). First-strand cDNA was synthesized using iScript RT Supermix for RT-qPCR (Bio-Rad Laboratories, Hercules, CA, USA). Quantitative PCR was performed by the ΔΔCq method with the StepOne Real-time PCR System (Thermo Fisher Scientific, Tokyo, Japan), and the relative expression level was calculated using the level of a reference gene, SDHA (succinate dehydrogenase complex flavoprotein subunit A). The sequences of the primers are shown in Table 1.

Immunoblotting Total cell lysates were prepared using RIPA buffer (Thermo Fisher Scientific) in the presence of protease inhibitor mixtures (Sigma-Aldrich Japan). Protein concentration was determined using a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). The cell lysates were separated by SDS-PAGE and blotted onto Immobilon-P membranes (ATTO). The membranes were blocked with 5% skim milk in Tris-buffered saline (TBS) with 0.1% Tween 20 (TBS-T) and further incubated with specific antibodies. The membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology). Band intensity was detected using the ECL Prime Western Blotting Detection Reagent (GE Healthcare, Tokyo, Japan) with the ImageQuant LAS 4000 biomolecular imager (GE Healthcare).

Table 1. Nucleotide Sequences of Various Primers and shRNA for CELSR1

Primers	DNA sequences (5' - 3')
CELSR1 seq1 Fw	CCA ACT TCG CAA ACT TTC GC
CELSR1 seq1 Rv	GGA AAC TTC AGG CTC CCT CT
CELSR1 seq2 Fw	CGG AGA TCG ACC TCT GCT AC
CELSR1 seq2 Rv	GAA GTC TTC TGG CAG GTT GG
CELSR1 seq3 Fw	AGG ACA GCG TTC TGA TGG AG
CELSR1 seq3 Rv	CAC GGA AGG TCG AGT TTG TT
CELSR1 seq4 Fw	TTC GTC CTC CTT TTC CAC TG
CELSR1 seq4 Rv	CAA GAT GCC TTT CCT CTG CT
CELSR1 seq5 Fw	TTA CAT GGA GGG GCT GTT CG
CELSR1 seq5 Rv	GTG TCC ACC AGG CGA TAG TG
CELSR1 seq6 Fw	GCA CTA TCG CCT GGT GGA C
CELSR1 seq6 Rv	AGG AAC TGG GGT GCA TTG TC
CELSR1 seq7 Fw	ACC CTA GAG ATC CTC ATC CTC G
CELSR1 seq7 Rv	CAC AGA CAC CTC CAT GAG CG
CELSR1 seq8 Fw	ACC TGG ACA ACA ACC GGC
CELSR1 seq8 Rv	GAA GGT GAC GAA GGA CTG GG
CELSR1 seq9 Fw	GAA GTC CCT GGA TCT GAC CG
CELSR1 seq9 Rv	CAT CAC GGA CTC CAC ATC GG
CELSR1 seq10 Fw	GTG TGG GCC CAG TCA CTA C
CELSR1 seq10 Rv	ATC ATG TTG GCG GTG ACG AT
CELSR1 seq11 Fw	GAG GGC TAC TTC AGC AAC GTG
CELSR1 seq11 Rv	GAA ACG GGT TTT CCG TCT GG
CELSR1 seq12 Fw	CAG CAT TCA CAA GCA CCT CG
CELSR1 seq12 Rv	CAG CTC GCT ATC TGA GTC GG
CELSR1 seq13 Fw	CCT GGC TGA GAG TGA CAG TG
CELSR1 seq13 Rv	GTT GCA CCT CCT CAG AGT CC
CELSR1 qPCR Fw	CCTCACCGGCCCATCATTA
CELSR1 qPCR Rv	CTCCAGCAGGGCGAACTCCA
SDHA qPCR Fw	TGGGAACAAGAGGGCATCTG
SDHA qPCR Rv	CCACCACTGCATCAAATTCATG
M13 Forward (-20)	GTA AAA CGA CGG CCA G
CELSR1 shRNA	TGCTGTTGACAGTGAGCGCGAAGAGT CTACAATAAATTAATAGTGAAGCCACA GATGTATTAATTTATTGTAGACTCTTCT TGCCTACTGCCTCGGA

Sequencing of the CELSR1 Coding Region Total RNA isolated from the cells was reverse-transcribed into cDNA using the PrimeScript II first-strand cDNA Synthesis Kit (TaKaRa Bio) according to the manufacturer's instructions. cDNA (100 ng) was subjected to PCR with the primers specific to each part of the coding region of CELSR1. The fragments were inserted into the pCR-Blunt vector using a Zero Blunt PCR Cloning Kit (Thermo Fisher Scientific), and then DNA sequencing was performed with M13 Forward primer. Table 1 shows information about the primers.

Immunofluorescence Staining Immunofluorescence staining was performed as previously described.[8] In brief, the cells were cultured on round coverslips (Thermo Fisher Scientific) on a 12-well plate. After culturing for one day, the cells were fixed with 4% paraformaldehyde in PBS and then treated with 0.1% Triton X-100 in PBS for permeabilization. The fixed cells were treated with Blocking One Histo (Nacalai Tesque, Kyoto, Japan) and then incubated with anti-CELSR1 antibody for one hour at room temperature. Further, Cy3-conjugated anti-mouse IgG antibody (Abcam) was added to the sample. The nucle-

us was stained with the Cellstain DAPI solution (Dojindo, Kumamoto, Japan). Images of the stained cells were obtained with a confocal laser scanning fluorescence microscope, LSM 800 with Airyscan (Carl Zeiss, Jena, Germany).

Cell Proliferation Assay The rate of cell proliferation was determined using a Cell Counting Kit-8 (CCK-8; Dojindo), according to the manufacturer's instructions. In brief, the cells were seeded at 2×10^3 cells/well in a 96-well plate. CCK-8 solution was added to each well. After three hours of incubation, absorbance at 450 nm was measured with Spark (TECAN, Männedorf, Switzerland).

Wound-Healing Assay Wound-healing assay was performed using a culture insert (ibidi GmbH, Gräfelfing, Germany). The insert was placed onto a 24-well plate and cells (7×10^5/well) were seeded into each insert. After 24 h, the culture insert was removed from the 24-well plate. The wounded area was monitored by a microscopic picture taken every 6 h. The image of the picture was quantified and analyzed with ImageJ software.

Cell Invasion Assay The cells were cultured in the absence of FBS for 24 h prior to cell invasion assay. A trans-well insert (8 μm pore size) was coated with Matrigel (BD Biosciences, USA) for 2 h. The cells (2×10^5) were seeded onto the top chamber in the medium without FBS. The lower chamber was filled with 0.5 mL of medium containing 10% FBS as a chemoattractant. After 48 h, the cells that had not migrated or invaded through the pores were removed by a cotton swab. The cells on the lower surface of the membrane were fixed and stained with crystal violet. Pictures of the membrane were taken by a digital camera, and the invading cells were counted.

Public Database Resources For the functional enrichment analysis, the STRING database version 11.0 (https://string-db.org/) was used. To assess the prognostic relevance of the CELSR1 gene in GC patients, we used a Kaplan−Meier plotter (https://kmplot.com/analysis/). Open resources for multidimensional cancer genomics data were analyzed with the cBioPortal for Cancer Genomics (https://www.cbioportal.org).

Statistical Analysis All of the data in this article are presented as the mean ± standard deviation (SD) of at least three repeat experiments. Student's t-test (two-sided test) was used to analyze the variances between groups. Significant differences were considered when P values were less than 0.05.

RESULTS

Clinical Significance of CELSR1 Gene in GC Patients Our previous study found enhanced expression of WNT3a, WNT5a, and β-catenin associated with peritoneal metastasis human SGCs.[8] Wnt/PCP signaling is one of the major cellular signal transductions related to cancer metastasis. To gain new insights into the molecular mechanisms underlying peritoneal metastasis in SGCs, we examined functional proteins associated with Wnt/PCP signaling-related genes as analyzed by the STRING database. This revealed the CELSR1 gene, which shows functional enrichment to Wnt/PCP signaling-related genes (Fig. 1A). CELSR1 is involved in noncanonical Wnt/PCP signaling, but the functions of CELSR1 in cancer metastasis have not been reported. Thus, we attempt to analyze the genetic dynamics of the CELSR1 gene from public resources on cancer patients. As shown in Fig. 1B, GC patients with higher expression of CELSR1 indicated a poor prognosis compared with patients having lower expression. Next, we examined the alterations to the CELSR1 gene in various types of cancer patients from the public database. We found a high frequency of CELSR1 gene alterations, especially broad ranges of mutations over the coding region of the CELSR1 gene irrespective of cancer type (Fig. 1C and Fig. 1D)

Elevated Expression of CELSR1 is Associated with Metastatic Property RT-qPCR revealed that the CELSR1 mRNA level was significantly increased in our peritoneal metastatic cells (both 44As3 and 58As9 lines) compared with parental cell lines HSC-44PE and HSC-58 (Fig. 2A). It is notable that the CELSR1 mRNA levels in both parental cell lines were similar to that in normal human stomach tissues, suggesting that CELSR1 expression is increased accompanied by the acquisition of metastatic property. Consistent with the results of RT-qPCR, immunoblotting of CELSR1 showed increased protein levels in our peritoneal metastatic cells (both 44As3 and 58As9 cell lines) compared with their parental cell lines (Fig. 2B).

Genomic Alteration of CELSR1 Coding Sequences in Metastatic Cells Our analysis of public resources revealed frequent mutations on the CELSR1 gene locus in cancer patients. To understand the genomic status of the CELSR1 gene in our peritoneal metastatic cell models, we analyzed DNA sequences of CELSR1 coding sequences (CDS) based on cDNA generated from total RNA. We found that CELSR1 CDS was identical to NM_001378328.1 in the metastatic cell lines. It is notable that we found small fractions of single nucleotide variants (SNVs) in the CELSR1 coding region (Fig. 2C), whereas all of the SNVs found in our metastatic cell models have already been reported, as described in Table 2. We also found no difference in SNV between metastatic and parental cells. Although the public resources indicated a unique hotspot (G614Afs*54) for CELSR1 gene mutation (Fig. 1D), we did not observe it in our cell lines. Six SNVs were found in 44As3 and in 58As9, respectively. Among these, 4 SNVs, including synonymous SNV, were commonly found in 44As3 and 58As9 (Fig. 2C).

Intracellular Distribution of CELSR1 Protein in Metastatic Cells We investigated the intracellular distribution of CELSR1 protein by immunofluorescence staining and confocal microscope analysis. Consistent with the results of immunoblotting, abundant CELSR1 expression in metastatic cell lines 44As3 and 58As9 was found compared with parental cell lines HSC-44PE and HSC-58, respectively (Fig. 3A). CELSR1 protein was observed predominantly in plasma membranes rather than in nuclear and cytoplasmic regions. These distributions of CELSR1 are consistent with those in previous reports,[11] suggesting that CELSR1 functions as an oscillator in metastatic cancer cells.

DOX-Induced Knockdown of CELSR1 Expression Reduced Cell Proliferation, Migration, and Invasion in Metastatic Cells We aimed to decrease CELSR1 expression by doxycycline (DOX)-induced knockdown in both metastatic cell lines. Reduced CELSR1 expression was observed only in the presence of Dox (Fig. 4A and 4B) and led to a slight decrease in cell proliferation in 44As3 cells (Fig. 5A). In 58As9 cell lines, on the other hand, cell proliferation was significantly inhibited by CELSR1 knockdown (Fig. 5B). CELSR1 knockdown via DOX-induced shRNA vector significantly decreased migration activity (wound-healing assay) in 44As3 cells (Fig. 5C). Moreover, the knockdown significantly decreased invasion activity in 58As9 cells (Fig. 5D). Thus, CELSR1 expression has a strong impact on cell migration and

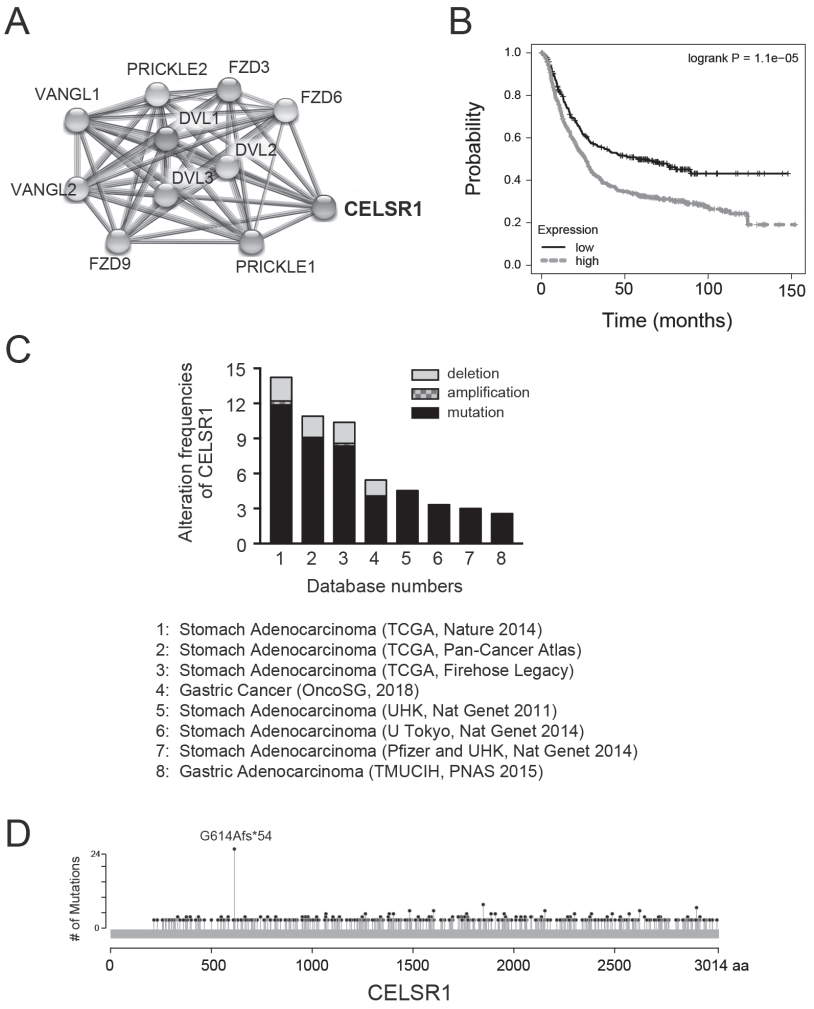

Fig. 1. Analysis of Public Resources for Genetic and Patient Information Implicated the CELSR1 Gene in Cancer Progression

A, Results of CELSR1 gene analysis by the functional enrichment of the protein association network. CELSR1 interacted with a series of proteins involved in Wnt/PCP signaling. B, The prognosis of GC patients in relation to CELSR1 expression was evaluated. Patients with high expression showed significantly poorer prognosis than those with low expression. C, Genetic alteration frequencies of the CELSR1 gene were analyzed in the public resources on GC patients. High frequencies of mutations in the CELSR1 gene were found. D. The number of each nucleotide alteration of the CELSR1 gene in GC patients was shown on the coding sequences of CELSR1. One unique hotspot (G614Afs*54) was observed.

invasion, but only a weak impact on cell proliferation.

Interestingly, we found that the 58As9 cell morphology changed upon CELSR1 knockdown (Fig. 3B). 58As9 cells exhibited the characteristic hummingbird shape (hummingbird phenotype), and CELSR1 knockdown significantly reduced the number of cells of this phenotype. The hummingbird shape is considered to correlate with a high metastatic property in human GC. Therefore, we examined the relationship between the hummingbird shape and CELSR1 knockdown. Because green fluorescent protein (GFP) was observed in the cells accompanied by shRNA vector expression plus Dox treatment, we counted the hummingbird-shaped GFP-expressing cells. A significantly lower proportion of GFP-positive cells had the hummingbird shape (Fig. 3B). These results indicate that high CELSR1 expression contributed to the formation of the hummingbird shape of the cells.

DOX-Induced Knockdown of CELSR1 Expression Reduced FAK Signaling in Metastatic Cells The hummingbird phenotype is generally regulated by cytoskeletal signaling. Other researchers have reported that RhoA, a major component of cytoskeletal signaling, was frequently mutated in human SGC. Although we did not observe the gene alteration of RhoA (data not shown), we planned to examine the cytoskeletal signaling. Phosphorylation of FAK in 44As3 cells was significantly decreased with CELSR1 knockdown (Fig. 4B and 4C). 58As9 with CELSR1 knockdown showed a similar decrease in FAK phosphorylation. On the other hand, Akt phosphorylation did not change (data not shown). These results indicated that CELSR1 knockdown suppressed FAK signaling, which is associated with the hummingbird phenotype, and finally inhibited the migration and invasion of the cells. Taken together, these results indicated that the aberrant expression of CELSR1 observed in our SGC metastatic cells plays one or more key roles in FAK signaling.

DISCUSSION

As a unique approach to analyzing the molecular mechanisms underlying peritoneal metastasis in SGC, we previously established peritoneal metastatic cell-based models from SGC patients. Using our previous findings and the analysis of some public resources, we focused here on CELSR1. CELSR1 is known as a regulator of PCP in the neural development of *Drosophila* as well as in heart and lung development. How-

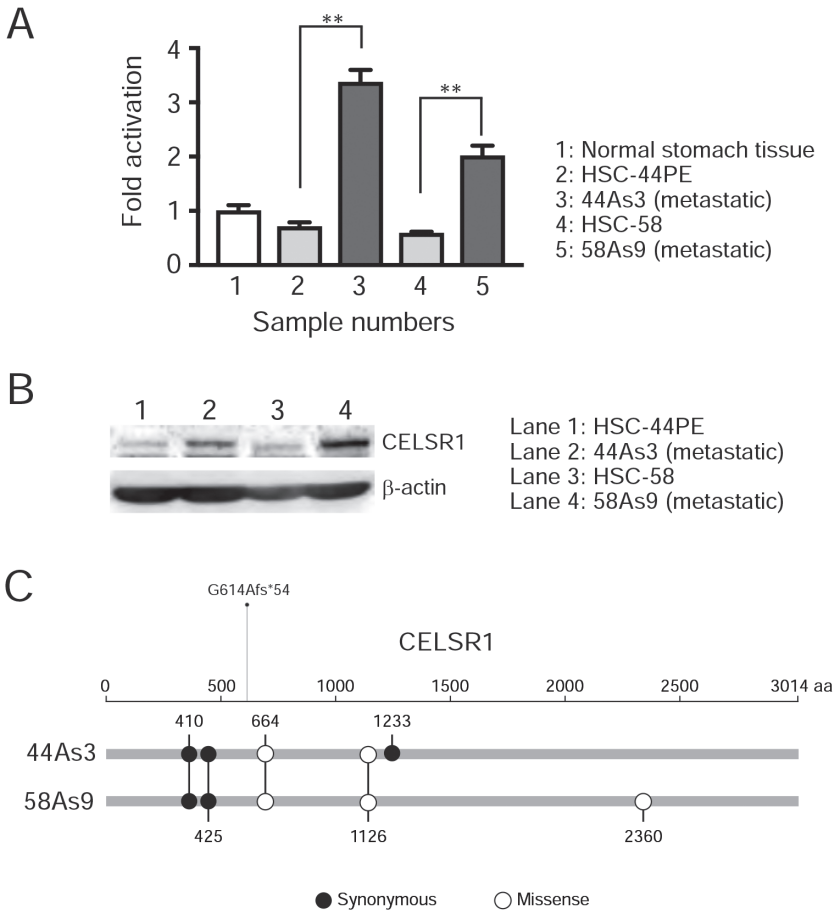

Fig. 2. Elevated Expression of CELSR1 was Observed in Our SGC Metastatic Cells

A, CELSR1 expression was examined by RT-qPCR in our SGC cells. Our metastatic cell lines 44As3 and 58As9 showed significantly higher expression compared with the parental cell lines HSC-44PE and HSC-58. CELSR1 expression in normal human stomach tissue is also shown. **, $p < 0.01$. B, Protein level of CELSR1 was analyzed by immunoblotting. Protein levels of CELSR1 in our metastatic cell lines were higher than those in the parental cell lines. C, Summary of the analysis of CELSR1 coding sequences. Four SNVs were commonly found in 44As3 and 58As9. Only 1 SNV was specific to the cell type.

Table 2. The List of Single Nucleotide Alterations Observed in the Coding Regions of the CELSR1 Gene in Our Cell Models

dbSNP #	Position (Reference)	Type (amino acid)	Alleles	Clinical significance	Variant type	HSC-44PE/ 44As3	HSC-58/ 58As9
rs1009155	chr22:46535941 (GRCh38.p12)	Synonymous (Ser410)	C > T	Not reported in ClinVar	SNV	O	O
rs1009154	chr22:46535896 (GRCh38.p12)	Synonymous (Leu425)	G > C	Not reported in ClinVar	SNV	O	O
rs4823850	chr22:46535180 (GRCh38.p12)	Missense (Ser664Trp)	G > C	Not reported in ClinVar	SNV	O	O
rs4823561	chr22:46533795 (GRCh38.p12)	Missense (Cys1126Arg)	A > G	Not reported in ClinVar	SNV	O	O
rs3747251	chr22:46464191 (GRCh38.p12)	Synonymous (Ala1233)	G > C	Not reported in ClinVar	SNV	O	N.D.
rs760047886	chr22:46381856 (GRCh38.p12)	Missense (Arg2360Cys)	G > A	Not reported in ClinVar	SNV	N.D.	O

N.D.: No detection of the nucleotide alteration in our analysis

ever, little is known about the functions of CELSR1, especially in cancer metastasis. According to our analysis of public resources on cancer patients (Fig. 1), CELSR1 is certainly significant not only in cancer patients but also in GC patients. Here we found that significantly elevated CELSR1 expression is strongly associated with the acquisition of a peritoneal metastatic property in SGC (Fig. 2). Interestingly, CELSR1 expression levels in the parental SGC cells are similar to those in normal human stomach tissues, suggesting that CELSR1 has an important role in the late stages of cancer progression and metastasis rather than in the early stages such as cancer carcinogenesis. On the other hand, CELSR1 expression was significantly elevated along with the acquisition of a metastatic property. Thus, the CELSR1 signaling pathway is always activated in both 44As3 and 58As9 cells. By means of the shRNA technique, CELSR1 knockdown significantly reduced the proliferation, migration, and invasion of 44As3 and 58As9 cells, leading to reduced phospho-FAK levels, the humming-

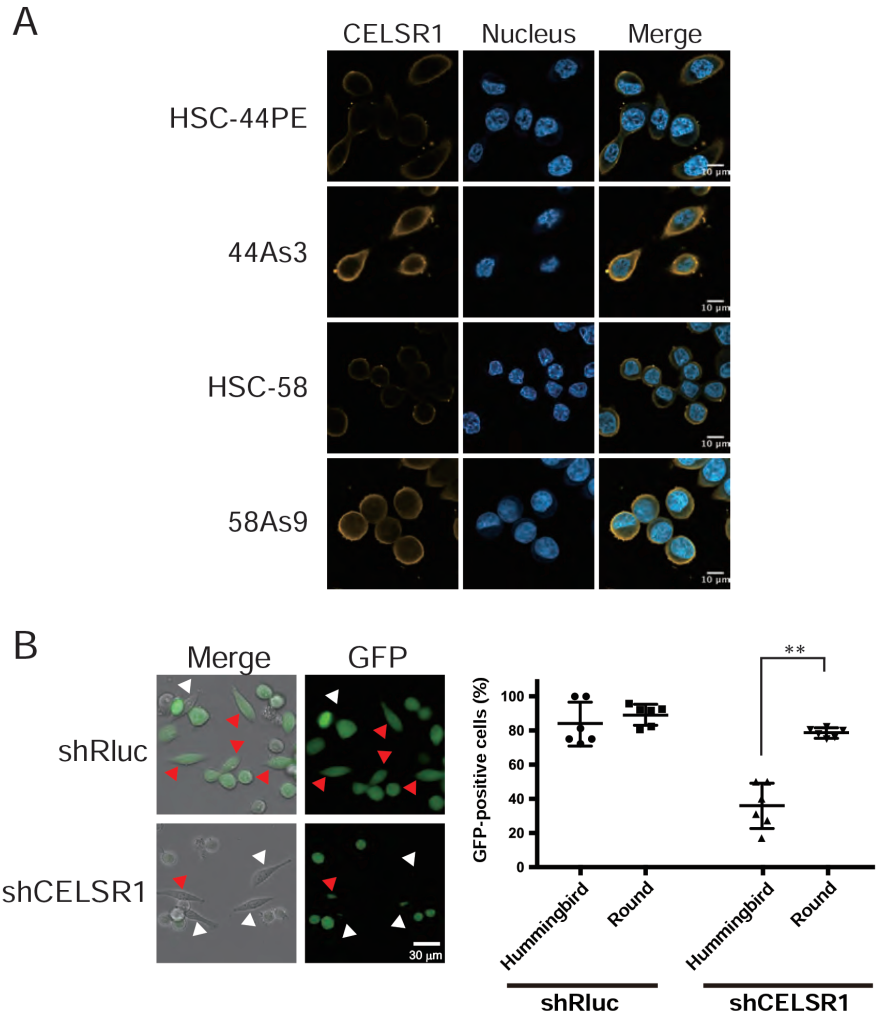

Fig. 3. Intracellular Distribution of CELSR1 Protein in Our SGC Cells

A, Immunostaining of CELSR1 with a specific antibody via confocal microscopy analysis. CELSR1 was expressed predominantly in plasma membranes. B, Morphological change in CELSR1 knockdown was examined. Arrowheads indicate the cells showing a hummingbird phenotype with GFP expression (red arrowheads) or without GFP expression (white arrowheads). **, $p < 0.01$.

bird phenotype, and finally metastatic property (Figs. 3–5).

In this study, some of the SNVs in the CELSR1 gene were identified in our metastatic cell lines, although we did not find any mutations in the coding region of the CELSR1 gene. Some SNVs were the 'missense' type, which causes the replacement of amino acid in the coding frame (Fig. 2). The detailed roles of SNVs in CELSR1 gene functions will be analyzed in our upcoming study.

Recent studies using high-resolution approaches to genetic alterations have unveiled the genome atlas of SGC patients. These studies commonly showed that RhoA mutations were the most frequent mutations in SGC patients.[18] In addition, RhoA/ROCK signaling is considered a potential therapeutic target for SGC.[19,20] On the other hand, our cell models do not carry RhoA mutations. Interestingly, our findings indicated that FAK, which is the downstream molecule of RhoA, was regulated by CELSR1. Thus, our results also support the importance of the RhoA/FAK axis in SGC progression. However, we could not address whether FAK is directly regulated by CELSR1 signaling. This will be determined in our future studies.

It is reported that metastasis-initiating cells (MICs) are a special subpopulation of cancer stem cells derived from primary tumors,[20–26] and they are thought to specialize in setting up cancer metastasis.[20–26] According to the theory, our metastatic cells (44As3 and 58As9) are a kind of MICs which positively set up peritoneal metastasis. In other words, these two cells are designated as peritoneal metastasis-initiating cells. Thus, our present study shows that CELSR1 can be a specific marker protein to capture MICs.

Conclusion We found that elevated CELSR1 expression plays a significant role in peritoneal metastasis in human SGC. The results also suggested that CELSR1 can be a promising molecular target to treat peritoneal metastasis of SGC. CELSR1 can be a specific marker protein to capture peritoneal metastasis-initiating cells.

Acknowledgments We thank Drs. Kazuo Kita, Takayuki Okubo, and Ayami Morita-Kondo for their helpful suggestions regarding the experiments. We also thank Ms. Naomi Maruyama and Ms. Sorami Kawamoto for their excellent technical assistance.

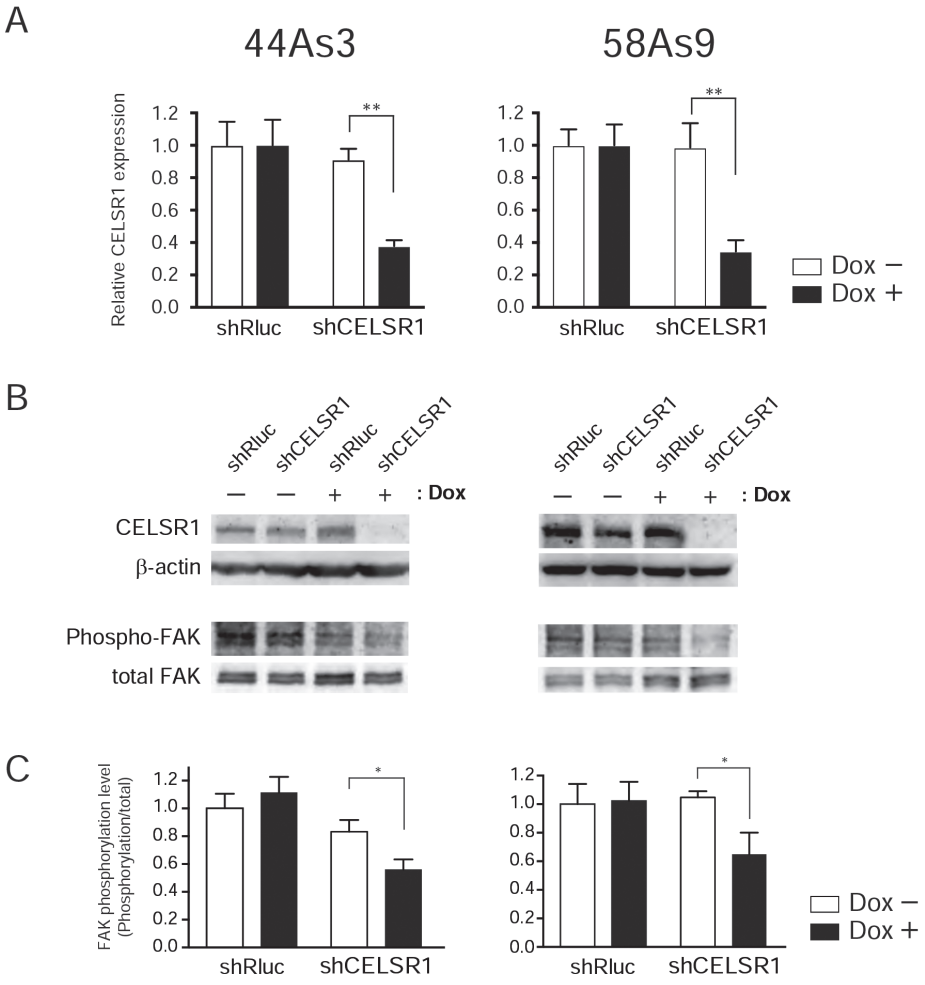

Fig. 4. Dox-Induced Knockdown of CELSR1 Reduced FAK Signaling in Our SGC Metastatic Cells

A, Dox-induced shRNA expression was examined by RT-qPCR in 44As3 and 58As9 cells. CELSR1 expression was significantly reduced only in the presence of Dox. shRNA against Renilla luciferase was used as a negative control. **, $p < 0.01$. B. Immunoblotting of CELSR1, β-actin, phospho-FAK, and total FAK. CELSR1 knockdown was observed only in the Dox-stimulated group. FAK phosphorylation was significantly decreased in CELSR1 knockdown cells. C, Densitometric analysis of phospho-FAK level normalized by total FAK. *, $p < 0.05$.

This work was supported in part by a Grant-in-Aid for Challenging Exploratory Research (15K15063), Grants-in-Aid for Scientific Research B (16H04697, and19H03149), and a Grant-in-Aid for Young Scientists B (17K14989) from Japan Society for the Promotion of Science.

Conflict of interest The authors declare no conflict of interest.

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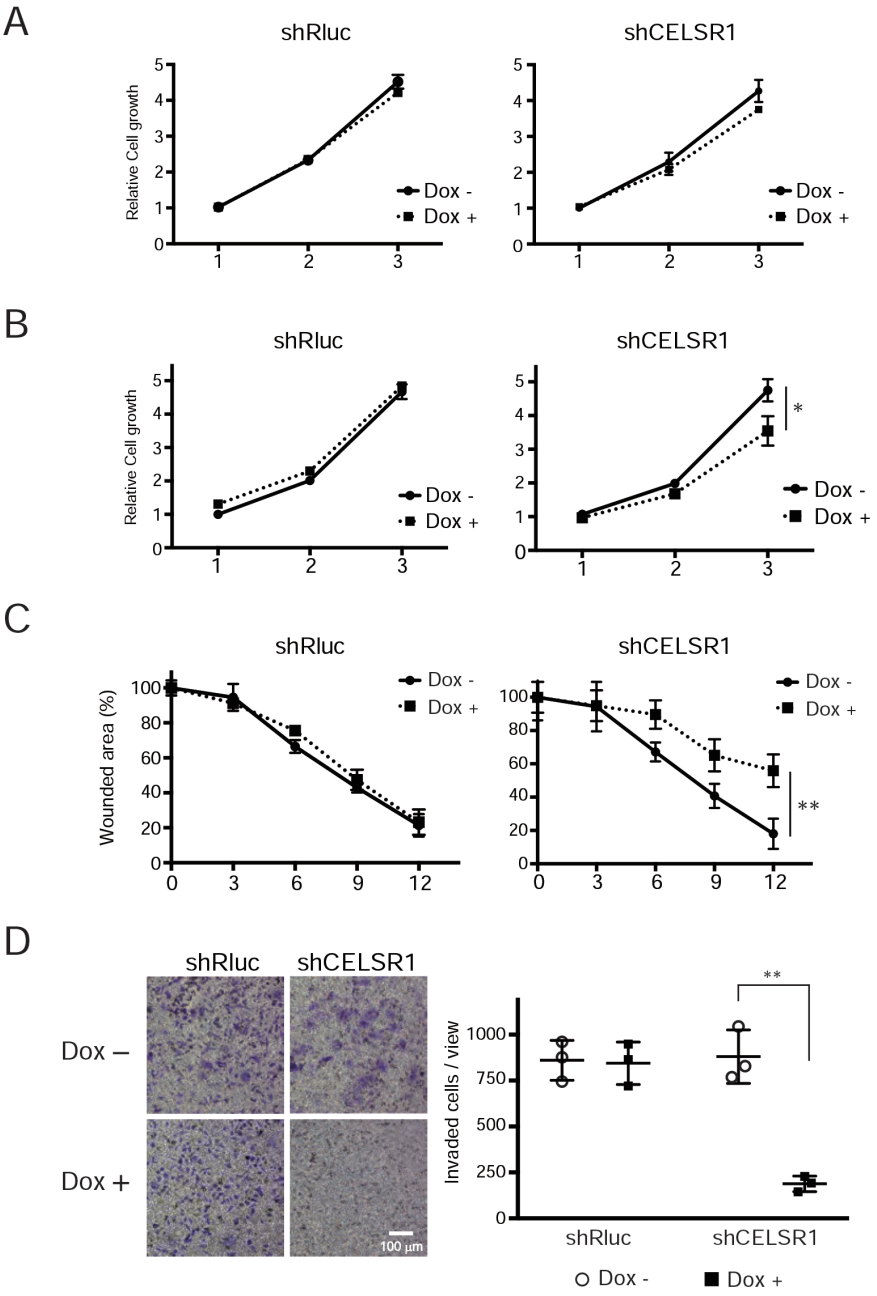

Fig. 5. CELSR1 Knockdown Reduced Cell Proliferation, Migration, and Invasion in Our SGC Metastatic Cells

A and B, Cell proliferation with or without CELSR1 knockdown was examined in 44As3 (A) and 58As9 (B) by CCK-8 assay. In 58As9 cells, CELSR1 knockdown significantly reduced cell proliferation. *, $p < 0.05$. C, Cell migration ability was determined in 44As3 by wound-healing assay. Cell migration was significantly inhibited by CELSR1 knockdown. **, $p < 0.01$. D, Cell invasion ability was evaluated in 58As9 by invasion assay. The number of invading cells was significantly decreased only in the CELSR1 knockdown 58As9 cells. **, $p < 0.01$.

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