GASTROINTESTINAL, HEPATOBILIARY, AND PANCREATIC PATHOLOGY

LRRC8A Expression Influences Growth of Esophageal Squamous Cell Carcinoma

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The volume-regulated anion channel is composed of leucine-rich repeat-containing protein A (LRRC8A) and is activated by hypotonic conditions to implement the process of regulatory volume decrease. The role of LRRC8A in regulating genes related to progression of esophageal squamous cell carcinoma (ESCC) was investigated, as well as the prognostic significance of LRRC8A expression in this tumor. Knockdown experiments were conducted using ESCC cell lines and LRRC8A siRNA to assess the influence of this protein on tumor function. In addition, the gene expression profile of ESCC was determined by microarray analysis. Immunohistochemistry was performed on 64 primary tumor samples from ESCC patients receiving radical esophagectomy. It was found that depletion of LRRC8A decreased cell proliferation and migration and also promoted apoptosis. Microarray data demonstrated G₁/S checkpoint regulation and up-regulation or down-regulation of phosphatidylinositol 3-kinase/AKT signaling, matrix metalloproteinase, and integrin signaling-related genes (including p21, p27, *MMP1*, and *ITGAV*) in LRRC8A-depleted cells. Immunohistochemistry showed that LRRC8A expression was related to the pathologic N and T stage categories, and strong LRRC8A expression was correlated with a worse prognosis of ESCC. These findings indicate that LRRC8A modulates tumor progression by influencing cell cycle, apoptosis, and migration, providing new insights into its function as an effector or biomarker of ESCC. (*Am J Pathol* 2019, 189: 1973–1985; <https://doi.org/10.1016/j.ajpath.2019.06.006>)

Esophageal cancer is an extremely malignant tumor that is an important cause of cancer death.^{1–4} In Asia, the most frequent histologic finding of esophageal cancer is squamous cell carcinoma (ESCC). Despite advances in perioperative chemotherapy and/or radiotherapy, surgical procedures, and perioperative management, ESCC still has a poor prognosis because of its high invasiveness and frequent metastasis.^{1–3} To achieve better outcomes, more investigation of the detailed molecular mechanisms that regulate the development and progression of ESCC is needed.

Autoregulation of cell volume occurs when there is an imbalance between intracellular and extracellular osmolarity.^{5,6} Cell swelling results in activation of K⁺ and Cl[−] channels or KCl cotransporters, which is followed by release of KCl into the extracellular space and subsequent

release of water.^{5,6} The volume-regulated anion channel is a type of Cl[−] channel that may be essential for volume regulation, particularly the regulatory volume decrease (RVD) process.⁶ Leucine-rich repeat-containing protein A (LRRC8A) is a ubiquitous and integral component of the volume-regulated anion channel and has been reported to be required for regulation of cell volume.⁷ *LRRC8A* mRNA is expressed in the esophagus (35.7 reads per kb of exon per

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million mapped reads), the lung (27.9 reads per kb of exon per million mapped reads), and 29 other tissues according to the GTEx data set (The Human Protein Atlas, <https://www.proteinatlas.org/ENSG00000136802-LRRC8A/tissue>, last accessed December 12, 2018). LRRC8 proteins have also been detected in various other chordates, generally as five paralogues termed LRRC8A (the first protein described), B, C, D, and E.⁸ However, the role of LRRC8A in cancer remains unclear, including its impact on ESCC, and the clinicopathologic implications of LRRC8A expression in human ESCC have not been assessed.

Recent studies have shown an essential role of ion transporters in various cancers, suggesting that physiological intracellular factors may become new targets for cancer treatment.⁹ We previously reported on the importance of ion channels,^{10–13} water channels,^{14,15} and pH-regulating channels^{16–19} in gastrointestinal cancer. Our findings indicated that such physiological factors could be useful

biomarkers and that targeting them may lead to novel treatment strategies for ESCC.

This study investigated the influence of LRRC8A on tumorigenesis-related genes and its clinicopathologic importance in ESCC. Microarray analysis revealed that knockdown of LRRC8A with siRNA influenced the expression of various genes related to cellular growth and migration. LRRC8A expression was also investigated in human ESCC samples, analyzing relations with clinicopathologic findings and the prognosis. Our findings revealed an important role of LRRC8A in the progression of ESCC.

Materials and Methods

Cell Lines, Antibodies, and Other Reagents

Seven human ESCC cell lines (TE2, TE4, TE5, TE8, TE9, TE13, and TE15) and the PK59 human pancreatic cell line

Figure 1 Leucine-rich repeat-containing protein A (LRRC8A) expression, proliferation, and cell cycle progression of esophageal squamous cell carcinoma (ESCC) cells. **A:** LRRC8A protein expression was analyzed in 11 ESCC cell lines. Western blot analysis revealed that LRRC8A was strongly expressed by TE15 and KYSE170 cells. **B:** From Western blot analysis, LRRC8A siRNA effectively reduced LRRC8A protein levels in TE15 and KYSE170 cells. **C:** LRRC8A siRNA effectively reduced LRRC8A mRNA levels in TE15 and KYSE170 cells. **D:** Depletion of LRRC8A reduced proliferation of TE15 and KYSE170 cells. The number of cells was counted at 48 and 72 hours after transfection. **E:** Depletion of LRRC8A reduced cell cycle progression from the G₁ to S phase in TE15 and KYSE170 cells. Cells transfected with control or LRRC8A siRNA were stained with propidium iodide (PI) and analyzed by flow cytometry. Data are expressed as means $\pm$ SEM. $n = 3$ (C–E). * $P < 0.05$ versus control siRNA. ACTB, β -actin; Cont, control.

were obtained from the Riken Cell Bank (Tsukuba, Japan), and four other human ESCC cell lines (KYSE70, KYSE150, KYSE170, and KYSE790) were obtained from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). Two cell lines (TE15, KYSE170) were used for *in vitro* studies, with these cells being cultured in RPMI-1640 medium (Nacalai Tesque, Kyoto, Japan) supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, and 10% fetal bovine serum under a humidified 5% CO₂ atmosphere at 37°C.^{10–18}

For immunohistochemistry, a polyclonal anti-LRRC8A antibody (ab157489) and a monoclonal anti-cytokeratin 13 antibody (ab16112) were purchased from Abcam (Cambridge, MA), whereas the monoclonal anti-LRRC8A antibody (SAB1412855) for the protein assay was from Sigma-Aldrich (St. Louis, MO), and the mouse monoclonal antibody that targeted β-actin was also from Sigma-Aldrich. Horseradish peroxidase–conjugated anti-rabbit or mouse secondary antibodies were from Cell Signaling Technology (Beverly, MA).

Western Blot Analysis

M-PER lysis buffer (Pierce, Rockford, IL) supplemented with protease inhibitor (Pierce) was used to harvest the cells. The protein concentration was measured by a modification of the Bradford method (Bio-Rad, Hercules, CA). Then cell lysates that contained equal amounts of total protein were subjected to SDS-PAGE, followed by transfer of the proteins to polyvinylidene difluoride membranes (GE Healthcare, Piscataway, NJ). Membranes were probed with the relevant antibodies and proteins were detected by an ECL Plus Western Blotting Detection System (GE Healthcare). β-Actin was used as the loading control.^{10–18}

Transfection of siRNA

Cells were transfected with 20 nmol/L LRRC8A siRNA (Stealth RNAi siRNA number HSS125512; number 1: 327013F05; number 2: 327013G01; number 3: 327013B11; Invitrogen, Carlsbad, CA) by using Lipofectamine RNAi-MAX reagent (Invitrogen) according to the manufacturer's directions. After 24 hours, the siRNA-containing medium was removed and replaced with fresh medium. Control siRNA (20 nmol/L) provided by the manufacturer (Stealth RNAi siRNA Negative Control; Invitrogen) was the negative control.^{10–18}

Assessment of Overexpression

TE4 cells were transfected with either the control HaloTag plasmid (G6591; Promega, Madison, WI) or the LRRC8A HaloTag plasmid (FHC00834; Promega) using FuGENE HD transfection reagents (E2311; Promega) according to the instructions of the manufacturer. Vector transfection was confirmed by performing fluorescent microscopy to detect the HaloTag fusion protein stained by the TMR-conjugated HaloTag ligand (G8252; Promega) according to the manufacturer's protocol. Then the proliferation assay was performed using LRRC8A-expressing cells as described previously.^{13,18}

Proliferation Assay

After seeding these cells into 6-well plates at 0.4×10^5 cells/well, cells were cultured under a humidified 5% CO₂ atmosphere at 37°C. Transfection of control or LRRC8A siRNA was performed after 24 hours. Then the cells were removed from the flasks by using trypsin-EDTA 48 or 72 hours after transfection and were counted with a hemocytometer.^{10–18}

Cell Cycle Analysis

At 48 hours after transfection of siRNA, the cell cycle phase was evaluated by fluorescence-activated cell sorting. Briefly, after detachment from the flasks with trypsin-EDTA, the cells were treated with Triton X-100 and RNase, and nuclear staining was performed with propidium iodide (PI). Then the DNA content was measured by using a Becton-Dickinson Accuri C6 Flow Cytometer (BD Biosciences, Franklin Lakes, NJ), with analysis of at least 1.0×10^5 cells.^{10–18}

Detection of Apoptosis

At 48 hours after transfection with siRNA, cells were harvested and were stained with fluorescein isothiocyanate–conjugated annexin V and PI using an annexin V kit (Beckman Coulter, Brea, CA) as instructed by the manufacturer. Then apoptotic cells were detected by flow cytometry with an Accuri C6.^{10–18}

Migration and Invasion Assay

The migration assay was conducted by using a 24-well Transwell chamber, a Cell Culture Insert with 8-µm pores (BD Biosciences), and Biocoat Matrigel (BD Biosciences)

Figure 2 Leucine-rich repeat–containing protein A (LRRC8A) controlled survival, migration/invasion, and volume changes in esophageal squamous cell carcinoma (ESCC) cells. **A:** Depletion of LRRC8A induced early apoptosis of TE15 and KYSE170 cells. Apoptosis was assessed by flow cytometry using propidium iodide (PI)/annexin V double-staining. **B:** Depletion of LRRC8A inhibited migration and invasion of TE15 and KYSE170 cells. Cell migration and invasion were examined by using a Boyden chamber. **C:** Depletion of LRRC8A decreased the regulatory volume decrease (RVD) in TE15 cells. **Red and blue lines** indicate the lower and upper limits of the electronic volume threshold. Data are expressed as means ± SEM. $n = 3$ (A–C). * $P < 0.05$ versus control siRNA. Original magnification, $\times 100$ (B). Cont, control; FITC, fluorescein isothiocyanate.

Table 1 Cell Cycle G₁/S Checkpoint Regulation—Related Genes with Altered Expression Levels in KYSE170 Cells after the Depletion of Leucine-Rich Repeat—Containing Protein A

Gene symbol	Unigene ID	Cell cycle G ₁ /S checkpoint regulation pathway	Expression fold change
		Gene name	
<i>CDKN2A</i>	Hs.512599	Cyclin dependent kinase inhibitor 2A	2.141
<i>HDAC9</i>	Hs.196054	Histone deacetylase 9	1.937
<i>CDKN1A(p21)</i>	Hs.370771	Cyclin dependent kinase inhibitor 1A	1.707
<i>BTRC</i>	Hs.643802	β-Transducin repeat containing E3 ubiquitin protein ligase	1.662
<i>TGFB3</i>	Hs.592317	Transforming growth factor β 3	1.547
<i>E2F5</i>	Hs.445758	E2F transcription factor 5	1.537
<i>CDKN1B(p27)</i>	Hs.238990	Cyclin dependent kinase inhibitor 1B	1.536
<i>MYC</i>	Hs.202453	MYC proto-oncogene, bHLH transcription factor	−1.565
<i>E2F7</i>	Hs.416375	E2F transcription factor 7	−1.576
<i>MAX</i>	Hs.285354	MYC associated factor X	−1.584
<i>PAK1IP1</i>	Hs.310231	PAK1 interacting protein 1	−1.605
<i>MDM2</i>	Hs.484551	MDM2 proto-oncogene	−1.782
<i>HDAC1</i>	Hs.88556	Histone deacetylase 1	−1.817
<i>SUV39H1</i>	Hs.522639	Suppressor of variegation 3—9 homolog 1	−1.963

to evaluate invasion. At 24 hours after transfection with siRNA, cells (1.0×10^5 per well) were seeded into serum-free medium in the upper chamber, and the lower chamber was filled with medium that contained 10% fetal bovine serum. Cells were incubated for 48 hours at 37°C under 5% CO₂ after which time nonmigrating or non-invading cells were removed from the upper surface of the membrane by wiping with a cotton swab. The migrating or invading cells were fixed on the membrane, followed by staining with Diff-Quick staining reagents (Sysmex, Kobe, Japan). Then these cells were counted on the lower surface of each insert in four independent fields at a magnification of $\times 100$. Assays were performed in triplicate.^{10–18}

Isotonic and Hypotonic NaCl Solutions

Isotonic NaCl solution (NaCl buffer) contained 140 mmol/L NaCl, 5.0 mmol/L KCl, 1.0 mmol/L CaCl₂, 1.0 mmol/L MgCl₂, 5.0 mmol/L glucose, and 10 mmol/L HEPES. The pH was adjusted to 7.4 with NaOH, and the osmolarity was measured with a freezing point osmometer (model 110; Fiske Associates, Norwood, MA). To assess changes of cell volume due to hypotonic stress, NaCl buffer diluted twofold with distilled water was used.²⁰

Measurement of Volume Changes in ESCC Cells Subjected to Hypotonic Stress

Cell volume measurements were performed with a high-resolution flow cytometer, the Cell Lab Quanta (Beckman Coulter, Fullerton, CA), as described previously.²⁰ Data on the electronic volume of $>1.0 \times 10^4$ cells were collected and analyzed with Quanta control software version 1.0 (Beckman Coulter, Fullerton, CA). ESCC cells were detached from culture flasks using trypsin-EDTA and

centrifuged. Then the pellet (1.0×10^6 cells) was suspended in 1 mL of isotonic NaCl buffer or in hypotonic NaCl buffer diluted twofold with distilled water to induce hypotonic stress. The cell suspension was subsequently transferred to a Vi-CELLTM Sample Cup (Beckman Coulter), and the cell volume was measured after 1, 5, 10, and 20 minutes of hypotonic exposure. A cell suspension in isotonic NaCl buffer was the control without hypotonic stress (0 minute). To analyze changes of LRRC8A expression related to RVD, cells were compared at 48 hours after transfection with LRRC8A siRNA or control siRNA.

Real-Time RT-PCR

Total RNA was extracted by using an RNeasy kit (Qiagen, Valencia, CA) and mRNA expression was measured by performing real-time quantitative PCR (7300 Real-Time PCR System; Applied Biosystems, Foster City, CA) with a Taq-Man Gene Expression Assay (Applied Biosystems) according to the directions of the manufacturer. Expression of the following genes was measured: *LRRC8A* (Hs01555916_m1), *p21* (Hs00355782_m1), *p27* (Hs00153277_m1), *E2F7* (Hs00987777_m1), *AKT* (Hs00178289_m1), *BclII* (Hs00608023_m1), *CASP3* (Hs00234387_m1), *MMP1* (Hs00899658_m1), and *ITGAV* (Hs00233808_m1) (Applied Biosystems). Expression of each gene was normalized for that of the housekeeping gene *ACTB* (Hs01060665_g1) (Applied Biosystems). Each assay was performed in triplicate.^{10–18}

Preparation of Samples and Hybridization to Microarrays

The quality of RNA was assessed by using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). A Low Input Quick Amp Labeling Kit (Agilent Technologies) was used to prepare cyanine-3—labeled

cRNA from 0.1 µg of total RNA according to the protocol of the manufacturer. Samples were purified on RNeasy columns (Qiagen). Then 60 µg of cyanine-3–labeled cRNA was subjected to fragmentation, followed by hybridization to an Agilent SurePrint G3 Human Gene Expression 8 × 60 K Microarray for 17 hours. After hybridization, the slides were washed and immediately scanned with an Agilent DNA Microarray Scanner (G2565CA), using the one-color setting for 8 × 60 K array slides as described previously.^{10–18}

Microarray Data Processing

Scanned images of the microarray slides were analyzed with Feature Extraction Software version 10.10 (Agilent Technologies), using default parameters to obtain signal intensities with background subtraction and spatial detrending. Signaling networks were assessed by using Ingenuity Pathway Analysis (IPA) software version 2.3 (Ingenuity Systems, Inc., Redwood City, CA), as described previously.^{10–18}

Patients and Samples of Primary Tumors

Tumor samples were obtained from 64 patients with primary ESCC (histologically confirmed), who underwent esophagectomy at Kyoto Prefectural University of Medicine from 1999 to 2009. The eligibility criteria were no other synchronous or metachronous cancer and no preoperative chemotherapy or radiation therapy. Patients were excluded if they did not receive curative resection or if they had missing data. Samples of normal esophageal tissue and tumor tissue were obtained from six patients for mRNA analysis. All patients gave written informed consent to this study. Information on clinicopathologic factors and survival was obtained from the hospital medical records. Tumor staging was performed according to the International Union Against Cancer/TNM Classification of Malignant Tumors (eighth edition).²¹

Immunohistochemistry

With the use of paraffin sections of tumor tissues (4 µm thick), immunohistochemistry for LRRC8A protein was performed by the avidin-biotin-peroxidase method. After dewaxing paraffin sections with xylene and hydration in a graded alcohol series, endogenous peroxidases were blocked by incubation in 0.3% H₂O₂ for 30 minutes. Then an Avidin/Biotin Blocking Kit was used to achieve blockade of endogenous biotin, biotin receptors, and avidin binding sites (Vector Laboratories, Burlingame, CA). Subsequently, sections were treated with a protein blocker and incubated overnight at 4°C with anti-LRRC8A antibody, followed by visualization of avidin-biotin-peroxidase complex (Vectastain ABC Elite kit; Vector Laboratories) with diaminobenzidine tetrahydrochloride. Finally, sections were counterstained with hematoxylin

and subjected to dehydration in a graded alcohol series, clearing in xylene, and mounting.^{10–19}

LRRC8A expression was graded semiquantitatively by calculating the immunoreactive score (IRS) based on both staining intensity and the percentage of positive tumor cells.²² Staining of the cytoplasm was scored as follows: 0 (no staining), 1 (weak staining), 2 (moderate staining), and 3 (strong staining). In addition, the proportion of positive tumor cells was rated from 0 to 1.0. Then the score for each tumor sample was calculated as the maximum product of the above scores for staining intensity and the proportion of stained cells (range, 0 to 3.0). The IRS was used to define low or high expression based on a cutoff value.

Statistical Analysis

Differences between proportions were assessed with Fisher exact test, and *t*-test (for comparison between two groups) was used to assess continuous variables. Multiple comparisons were confirmed by the Tukey-Kramer test. Kaplan-Meier survival curves were generated, and differences between curves were investigated with the log-rank test. Multivariate analysis of prognostic factors was done with the Cox proportional hazards model. Analyses were performed with JMP statistical software version 12 (SAS Institute Inc., Cary, NC), and *P* < 0.05 was considered statistically significant.

Results

LRRC8A Expression by ESCC Cells

LRRC8A protein expression was examined in 11 ESCC cell lines: TE2, TE4, TE5, TE8, TE9, TE13, TE15, KYSE70, KYSE150, KYSE170, and KYSE790. Western blot analysis demonstrated strong expression of LRRC8A by TE15 and KYSE170 cells (Figure 1A), and quantitative RT-PCR showed strong *LRRC8A* mRNA expression by both cell lines (Supplemental Figure S1A). When comparison of *LRRC8A* expression between normal and cancerous esophageal tissues was performed, its expression was significantly higher in six ESCC tissue specimens than in the corresponding six normal specimens (*P* = 0.002) (Supplemental Figure S1B).

In TE15 and KYSE170 cells, knockdown experiments were performed using LRRC8A siRNA to examine the effect of depleting LRRC8A on cell proliferation. Transfection of these cell lines with LRRC8A siRNA reduced the LRRC8A protein level (Figure 1B) and the *LRRC8A* mRNA (Figure 1C). Considering these results for LRRC8A protein and mRNA expression, the following experiments were conducted using siRNA number 1. siRNA number 2 was also examined to exclude off-target effects (Supplemental Figure S2). At 48 and 72 hours after transfection with *LRRC8A* siRNA the count of LRRC8A-depleted cells was significantly lower for both cell lines than for cells transfected with control siRNA (Figure 1D). Thus, down-

A

B

regulation of LRRC8A partially inhibited progression of both cell lines from G₁ to S phase (Figure 1E).

Knockdown experiments were also conducted in TE4 cells with low LRRC8A expression, and similar results were obtained (Supplemental Figure S3). These results indicated that LRRC8A has an essential role in modulating cell cycle progression and proliferation of ESCC cells.

Second, to investigate whether 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB), which blocks the Cl⁻ channel, affected the proliferation of ESCC, cell counts were measured at 48 hours after transfection or exposure of cells to 200 μmol/L NPPB. After exposure of KYSE170 cells to NPPB, the count of these cells was significantly lower than those of control siRNA-transfected cells or LRRC8A-depleted cells (Supplemental Figure S4). These findings suggested that NPPB, a nonselective inhibitor of various Cl⁻ channels, had a significantly stronger influence on cell proliferation than LRRC8A siRNA.

An overexpression analysis was also performed. After transfection with the control HaloTag plasmid or LRRC8A HaloTag plasmid, cells were stained positive (Supplemental Figure S5A), revealing an increase of both the LRRC8A mRNA level and the LRRC8A HaloTag plasmid (Supplemental Figure S5B). Unlike the knockdown experiments, LRRC8A overexpression did not increase the growth of TE4 cells (Supplemental Figure S5C).

According to the Cancer Cell Line Encyclopedia, pancreatic cell lines showed similar LRRC8A expression as other cell lines (Supplemental Figure S6A). The knockdown experiment was also performed in PK59 cells, and similar results were obtained (Supplemental Figure S6, B–D).

LRRC8A Regulates ESCC Cell Survival

To investigate the influence of LRRC8A on ESCC cell survival, TE15 cells or KYSE170 cells were transfected with control siRNA or LRRC8A siRNA, followed by an assessment of apoptosis. Depletion of LRRC8A significantly increased the frequency of cells that displayed markers of early apoptosis (annexin V⁺/PI⁻) in both cell lines (Figure 2A). These findings suggested that LRRC8A expression has an influence on the viability of ESCC cells.

LRRC8A Regulates ESCC Cell Migration

Knockdown experiments were performed by transfecting ESCC cells with LRRC8A siRNA to study the effect of

LRRC8A depletion on cell migration and invasion. Transfection with LRRC8A siRNA significantly reduced migration and invasion of both cell lines after 24 and 48 hours (Supplemental Figure S7 and Figure 2B). These findings indicated that LRRC8A was important for controlling ESCC cell migration.

LRRC8A Regulates the Volume of ESCC Cells

To investigate serial changes in the volume of ESCC cells under hypotonic stress (hypotonic NaCl diluted twofold with distilled water), cell volumes and cell counts were simultaneously examined by using a Cell Lab Quanta. The effect of hypotonic stress on TE15 cells is shown in Figure 2C. The cells showed a unimodal distribution of cell volume under isotonic conditions. Cell volume increased after exposure to the hypotonic solution, indicating the occurrence of cell swelling. Although the cell volume initially increased during hypotonic stress, it then gradually returned to that before hypotonic stress despite the cells continuously remaining in hypotonic buffer, indicating the occurrence of RVD. Transfection with LRRC8A siRNA significantly suppressed the changes of cell volume by inhibiting RVD. These findings indicated a critical role of LRRC8A in regulating changes of ESCC cell volume.

Microarray Analysis of Cells Transfected with LRRC8A siRNA

Microarray analysis was used to assess the gene expression profile of KYSE170 cells transfected with LRRC8A siRNA. In cells with LRRC8A knockdown, expression of 2632 genes showed changes of >1.5-fold, with 1445 genes being up-regulated and 1187 being down-regulated. LRRC8A expression was reduced by 2.26-fold in transfected KYSE170 cells. Supplemental Table S1 lists the 20 genes with the strongest up-regulation or down-regulation of expression in transfected KYSE170 cells. According to IPA, cancer was one of the highest ranked diseases related to the affected genes in transfected KYSE170 cells, whereas cellular growth and proliferation was the highest ranked biological function (Supplemental Table S2). These results were consistent with our *in vitro* findings.

Figure 3 Signaling map of cell cycle G₁/S checkpoint regulation, the highest-ranked canonical pathway related to leucine-rich repeat-containing protein A (LRRC8A) depletion according to Ingenuity Pathway Analysis, and verification of gene expression by real-time quantitative RT-PCR. **A:** The signaling map of cell cycle G₁/S checkpoint regulation, the highest-ranked canonical pathway related to depletion of LRRC8A according to Ingenuity Pathway Analysis. Red and green indicate genes with higher or lower levels of expression compared with reference RNA, respectively. **B:** Expression of three selected G₁/S checkpoint regulation-related genes (p21, p27, and *E2F7*) was compared between LRRC8A-depleted TE15 cells or KYSE170 cells and control siRNA-transfected cells by real-time quantitative RT-PCR. Apoptosis-related genes, including *AKT*, *BCL2*, and *CASP3*, were regulated by LRRC8A in esophageal squamous cell carcinoma (ESCC) cells. Migration-related genes, including *MMP1* and *ITGA α* , were also regulated by LRRC8A. Depletion of LRRC8A affected the expression of *AKT*, *BCL2*, *CASP3*, *MMP1*, and *ITGA α* in TE15 cells and KYSE170 cells. Data are expressed as means $\pm$ SEM. *n* = 3. **P* < 0.05 versus control siRNA. ACTB, β -actin; Cont, control.

Figure 4 Leucine-rich repeat–containing protein A (LRRC8A) protein expression by human esophageal squamous cell carcinoma (ESCC) and survival after curative resection according to LRRC8A expression. **A:** Immunostaining of noncancerous esophageal epithelium with LRRC8A antibody. **B:** Immunostaining of primary human ESCC samples with LRRC8A antibody. **C:** Immunostaining of primary human ESCC samples revealed low expression (0 to 1) of LRRC8A. **D:** Immunostaining of primary human ESCC samples revealed high expression (2 to 3) of LRRC8A. **E:** Patients were classified into groups with low tumor expression of LRRC8A [immunoreactive score (IRS) <1.3] or high tumor expression of LRRC8A (IRS ≥1.3). $n = 25$ patients with low tumor expression (**E**); $n = 39$ patients with high tumor expression (**E**). * $P < 0.05$ (log-rank test). Scale bars: 50 μm (**A**); 25 μm (**B**); 250 μm (**C**); 100 μm (**D**). Original magnification: $\times 200$ (**A**); $\times 400$ (**B**); $\times 40$ (**C**); $\times 100$ (**D**).

Signaling Pathways and Molecular Mechanisms Regulated by LRRC8A in ESCC Cells

IPA revealed that the cell cycle G_1/S checkpoint regulation pathway was the highest ranked signaling pathway influenced by knockdown of LRRC8A (Supplemental Table S2). Further analysis of this pathway showed that *CKDN1A* (*p21*) pathway–related genes were strongly up-regulated by LRRC8A knockdown (Table 1).

To verify the microarray findings, some genes in the cell cycle G_1/S checkpoint regulation pathway map (Figure 3A) were analyzed by quantitative RT-PCR. It was found that the expression of *CKDN1A* (*p21*), *CKDN1B* (*p27*), and *E2F7* mRNA was increased or decreased in LRRC8A siRNA–transfected KYSE170 cells compared with cells

transfected with control siRNA (Figure 3B). Similar findings were obtained with TE15 cells (Figure 3B). Other genes influenced by depletion of LRRC8A were involved in apoptosis or adhesion, including *AKT*, *BclII*, *CASP3*, *MMP1*, and *ITGA α* (Supplemental Figure S8). From quantitative RT-PCR of both cell lines, expression of these mRNAs was consistent with the microarray data (Figure 3B).

Immunohistochemical Analysis of LRRC8A Expression by Human ESCC

Immunohistochemical analysis using the LRRC8A antibody revealed expression of LRRC8A in the upper layer of noncancerous esophageal mucosa (Figure 4A, Supplemental Figure S9A, and Supplemental Figure S10B). LRRC8A expression was also investigated in 64 samples of human ESCC, demonstrating that LRRC8A protein was chiefly present in the cell membranes or cytoplasm of the tumor cells (Figure 4B and Supplemental Figure S9B). The IRS of LRRC8A ranged widely among the tumors (Figure 4, C and D), with the median score being 1.4 (range = 0.5 to 2.2;

Table 2 Relationships between Clinicopathologic Features of Esophageal SCC and LRRC8A Expression

Variable	LRRC8A expression		<i>P</i> value
	Low group ($n = 25$)	High group ($n = 39$)	
Sex			
Male	20	34	0.4940
Female	5	5	
Age			
<65 years	13	21	0.8852
≥65 years	12	18	
Tumor length			
<50 mm	21	26	0.1553
≥50 mm	4	13	
Histologic type			
Well/moderately differentiated SCC	17	29	0.5809
Poorly differentiated SCC	8	10	
Lymphatic invasion			
Negative	13	17	0.5107
Positive	12	22	
Venous invasion			
Negative	16	20	0.3170
Positive	9	19	
pT			
pT1	16	15	0.0461*
pT2-3	9	24	
pN			
pN0	16	14	0.0279*
pN1-3	9	25	

Data are expressed as n unless otherwise indicated.

* $P < 0.05$ (Fisher exact test).

LRRC8A, leucine-rich repeat–containing protein A; pN, pathologic N stage; pT, pathologic T stage; SCC, squamous cell carcinoma.

Table 3 Prognostic Factors of Esophageal SCC according to Univariate and Multivariate Analyses

Variable	Univariate		Multivariate		
	5-year survival rate, %	P value	Hazard ratio	95% CI	P value
Sex					
Male	58.0	0.4477			
Female	70.0				
Age					
<65 years	58.4	0.8834			
≥65 years	61.7				
Tumor length					
<50 mm	61.0	0.4910			
≥50 mm	57.4				
Histologic type					
Well/moderately differentiated SCC	61.6	0.5782			
Poorly differentiated SCC	55.6				
Lymphatic invasion					
Negative	65.5	0.4250			
Positive	54.6				
Venous invasion					
Negative	71.5	0.0162*	Reference		0.0495 [†]
Positive	45.4		2.233	1.002–5.192	
pT					
pT1	67.2	0.1522			
pT2-4	53.2				
pN					
pN0	72.9	0.0486*	Reference		0.3417
pN1-3	48.6		1.514	0.652–3.821	
LRRC8A expression					
Low	83.4	0.0022*	Reference		0.0084 [†]
High	44.9		3.763	1.403–13.558	

* $P < 0.05$ (log-rank test).[†] $P < 0.05$ (Cox proportional hazard model).

LRRC8A, leucine-rich repeat-containing protein A; pN, pathologic N stage; pT, pathologic T stage; SCC, squamous cell carcinoma.

means $\pm$ SD = 1.47 ± 0.46). The ESCC patients were divided into two groups by the minimum P value approach,²³ which were a low expression group (IRS < 1.3 ; $n = 25$) and a high expression group (IRS ≥ 1.3 ; $n = 39$). Analysis of clinicopathologic features showed that LRRC8A expression was correlated with the pathologic T (pT) and N (pN) categories (Table 2). The expression of cytokeratin 13, a marker of esophageal-type differentiation expressed by various stratified epithelia, was also investigated. Cytokeratin 13 was expressed from the basal cell layer to the epithelial surface in keratinized tissues (Supplemental Figure S10A). In contrast, it was not expressed by tumor tissues, except in keratinized areas (Supplemental Figure S10, C and D). Therefore, LRRC8A expression in tumors was not restricted to cells expressing differentiation markers.

The prognostic impact of LRRC8A expression in ESCC patients was also investigated, revealing that the 5-year survival rate of the high LRRC8A expression group was significantly worse than that of the low LRRC8A expression group (44.9% versus 83.4%; $P = 0.0022$) (Figure 4E and Table 3).

Moreover, the prognostic influence of nine variables (sex, age, tumor length, histologic differentiation, lymphatic

invasion, venous invasion, pT category, pN category, and LRRC8A expression) was determined in patients receiving curative resection of ESCC. Univariate analysis showed that the prognosis was significantly correlated with venous invasion, the pN category, and LRRC8A expression ($P = 0.0162$, 0.0486 , and 0.0022 , respectively) (Table 3). Multivariate analysis with these three factors ($P < 0.05$ in the univariate analysis) confirmed venous invasion and LRRC8A expression as independent prognostic indicators ($P = 0.0495$ and 0.0084 , respectively) (Table 3). Thus, high LRRC8A expression was a poor prognostic factor for ESCC. The immunohistochemical findings were consistent with *in vitro* data, demonstrating an important role of LRRC8A in regulating the cell cycle, survival, and migration of ESCC.

Discussion

We investigated the function of LRRC8A in ESCC and the relationship with its expression. There have been several reports that LRRC8, including LRRC8A, is critical for

differentiation of adipocytes and lymphocytes.^{24–26} At the physiological level, it is an important protein in volume-regulated anion channel chloride channels, which are a major mechanism of RVD.²⁷ It has been unknown how RVD functions in tumor cells. In this study, it was confirmed that RVD is suppressed by LRRC8A siRNA (Figure 2C). *In vitro* experiments also demonstrated that LRRC8A acts as a tumor-promoting factor for ESCC because transfection of LRRC8A siRNA suppressed cell proliferation, promoted apoptosis, and inhibited cell migration. These findings suggested that suppression of RVD induced tumor inhibitory changes in ESCC cells. Conversely, recent research indicates that LRRC8A activation induces apoptosis and cell cycle arrest through promotion of cell shrinkage and increases expression of p21, p53, and *CASP9/3* in human ovarian carcinoma and alveolar carcinoma, whereas LRRC8A knockdown regulates the cell cycle by changing the expression of cyclins and cyclin-dependent kinases.^{28,29} From our microarray analysis, p21- and p27-related genes were up-regulated in cells transfected with *LRRC8A* siRNA, which had a marked influence on the cell cycle. The detailed molecular mechanisms underlying these effects of LRRC8A are still poorly understood. Although these data differed from previous findings, it is possible that loss of homeostasis influences tumor function.²⁸ Although further investigation is needed to reveal the detailed mechanism, RVD and volume changes may be critical for regulating tumor behavior through effects on the cell cycle. As far as we know, this is the first investigation into the influence of LRRC8A expression on RVD and cellular function in ESCC.

The *in vitro* experiments showed that cell migration was markedly suppressed in LRRC8A-depleted cells. Both transporters and ion channels are reported to be involved in cell migration, but the detailed mechanisms have not been clarified.³⁰ Expression of genes related to the epithelial–mesenchymal transition, including E-cadherin, vimentin, Snail, and ZEB, was almost unaffected by depletion of LRRC8A. However, expression of *MMP1* and *ITGA*v α was lower in cells transfected with *LRRC8A* siRNA than in the cells transfected with control siRNA. Thus, our findings may be explained by altered expression of *MMP* and integrin, rather than changes of epithelial–mesenchymal transition-related genes. The role of matrix metalloproteinases (MMPs) in cancer biology has been investigated, with various studies showing that MMPs are associated with tumor progression, prognosis, and cell migration.^{31–33} It was reported that *MMP1* knockdown decreased invasion and migration by prostate cancer cells,³⁴ and it was found that integrin expression was reduced by transfection with *LRRC8A* siRNA in this study. It was reported that integrins regulate growth factor signaling via integrin-growth factor receptor crosstalk and that close interaction between fibroblast growth factor and integrins is required for proliferation, migration, and angiogenesis of mammary epithelial cells.³⁵ LRRC8A may regulate the

behavior of ESCC via certain molecular changes. RVD was suppressed after depletion of LRRC8A, suggesting that cell migration was inhibited by a smaller change of volume in response to environmental changes.

Down-regulation of LRRC8A may be important for acquisition of cisplatin resistance by human alveolar cancer and ovarian cancer cells.^{28,36} Rubino et al²⁹ found that down-regulation of LRRC8A contributed to acquisition of resistance to anticancer drugs such as temozolomide and carmustine. Although it is controversial whether LRRC8A expression is associated with cisplatin resistance, it has been suggested that cisplatin may be more effective against tumors with high LRRC8A expression. It may be worth examining whether platinum agents are useful against tumors with high LRRC8A expression, suggesting the need for further investigation.

A few reports have indicated that LRRC8A is correlated with the prognosis, and high expression may be an indicator of a worse prognosis in patients with colon cancer.³⁷ LRRC8A expression was examined in ESCC to investigate its relationships with clinicopathologic findings and the prognosis, revealing a correlation between expression of LRRC8A and the pT or pN categories. In addition, it was found that the high expression of LRRC8A may be a poor prognostic factor for ESCC. As far as we know, this is the first investigation into the prognostic significance of LRRC8A in ESCC patients based on gene expression profiling of tumor cells with LRRC8A knockdown. Our findings indicated that suppression of LRRC8A decreases RVD in ESCC cells and promotes cell cycle arrest and induction of apoptosis through the cell cycle–related gene p21. Therefore, LRRC8A expression may be a prognostic indicator for ESCC.

This study had several limitations. First, it was performed retrospectively. Tumor length, lymphatic invasion, and pT category were not significantly correlated with the prognosis, possibly because of the small sample size. These days, adjuvant chemotherapy has been actively implemented for advanced ESCC in Japan,³⁸ so it has become difficult to obtain samples of advanced ESCC without preoperative therapy. Further, immunohistochemistry showed the LRRC8A was not only located on the cell membrane but also in the cytoplasm. Various research, including our previous studies, has indicated that changes in the intracellular localization of some membrane proteins can influence tumor progression.¹³ The detailed mechanisms involved in such changes of localization are unknown, so further investigations are required to clarify whether LRRC8A has a different role in the cytoplasm.

Conclusions

LRRC8A is important for the proliferation, survival, and migration of ESCC cell lines. The results of microarray analysis suggested that LRRC8A regulates the expression

of genes associated with the G₁/S checkpoint regulation pathway, including p21, p27, and *E2F7*, and cell migration-related genes such as *MMP1* and *ITGA α* . Furthermore, the results of immunohistochemistry clarified that strong LRRC8A expression is a poor prognostic indicator for ESCC. Further investigation of the functions of LRRC8A may help to define its potential as a biomarker and a novel target for the treatment of ESCC.

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Supplemental Data

Supplemental material for this article can be found at <http://doi.org/10.1016/j.ajpath.2019.06.006>.

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