

**Tumor-suppressive effect of LRIG1, a negative regulator of ErbB, in non-small cell lung cancer harboring mutant EGFR**

Short title: Tumor-suppressive effect of LRIG1 in lung cancer

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## Abstract

Epidermal growth factor receptor (EGFR) is a member of the ErbB (HER) family that is known to play important roles in the pathogenesis of various human cancers. Mutations of the *EGFR* gene are commonly found as oncogenic driver mutations, and have been targeted for treatment in non-small cell lung cancer (NSCLC). Leucine-rich repeat and immunoglobulin-like domain protein-1 (LRIG1) is a cell-surface protein that is known as a negative regulator of the ErbB (HER) family. In this study, we first confirmed that the expression levels of LRIG1 were much lower in NSCLC than in non-malignant cells or tissues. Next, we focused on the effect of LRIG1 in NSCLC. For this purpose, we established clones stably overexpressing LRIG1, using *EGFR*-mutant (HCC827, HCC4011, and NCI-H1975) and wild-type (A549) cells. Transfection of LRIG1 was associated with a decrease in the expression and phosphorylation levels of EGFR in the HCC827, HCC4011, and NCI-H1975 cells. It was also associated with strong suppression of the cell proliferative, invasive, migratory and tumorigenic potential of the HCC827 cells. On the other hand, no such effects were observed in the A549 cells. In addition, LRIG1 also downregulated the expression and phosphorylation levels of other tyrosine kinase receptors, such as HER2, HER3, MET and IGF-1R, and prevented the epithelial-to-mesenchymal transition induced by TGF- $\beta$  in the HCC827 cells. These findings

suggest that LRIG1 exerts important tumor-suppressive effects in *EGFR*-mutant NSCLC, and has the potential to become a novel therapeutic target for *EGFR*-mutant NSCLC.

### Summary

5 The expression of LRIG1 was lower in NSCLC cells than in non-malignant cells. Transfection of LRIG1 into NSCLC cells harboring mutant EGFR led to decrease in the expression of mutant EGFR, suggesting that LRIG1 had the strong tumor-suppressive effect.

### Introduction

10 Lung cancer is the leading cause of cancer-related death worldwide (1). In approximately 10% of patients with non-small cell lung cancer (NSCLC) in the US and 35% in East Asia, the tumors are reported to harbor epidermal growth factor receptor (*EGFR*) mutations, well known as oncogenic alterations (2-4). These mutations occur within  
15 *EGFR* exons 18–21, which encodes a portion of the EGFR kinase domain, and increase the kinase activity of EGFR, leading to hyperactivation of downstream pro-survival signaling pathways (5). First-generation EGFR-tyrosine kinase inhibitors (TKIs), such as gefitinib and erlotinib, have been shown to exert significant anti-proliferative effects

against NSCLC harboring *EGFR* activating mutations in preclinical studies (2,3) and to prolong the disease-free survival in randomized phase III clinical studies (6-8). However, patients with *EGFR* activating mutations who initially respond to EGFR-TKIs often eventually acquire the resistance to them, which is a critical problem in the treatment of the patients with advanced *EGFR*-mutant NSCLC. Therefore, it is sought to develop new drugs with different mechanisms underlying the anti-tumor effects and with the potential to overcome the resistance to EGFR-TKIs in patients with NSCLC harboring mutant EGFR.

Leucine-rich repeats and immunoglobulin-like domain 1 (LRIG1) is a type 1 transmembrane protein whose extracellular domain contains 15 leucine-rich repeats (LRRs) and three immunoglobulin (Ig)-like domains (9). LRIG1 is known to negatively regulate the expression levels of members of the ErbB (HER) family [EGFR and ErbB (HER) 2-4] by accelerating the degradation of these receptors (10,11), and to exert tumor-suppressive effects in various cancers, including cancers of the breast (12), head and neck (13), and brain (14). In regard to its role in lung cancer, LRIG1 was identified, in a recent bioinformatics gene expression analysis of five cancers (breast, lung, bladder, glioma and melanoma) from eight independent studies (15), as one of the four genes whose decreased expression was best correlated with a poor survival. In addition, one study showed the

role of LRIG1 as a tumor suppressor gene in NSCLC cells without *EGFR* mutations, both *in vitro* and *in vivo* (16). In brain cancer, LRIG1 was reported to negatively regulate an oncogenic form of the EGFR (vIII), in which domain I and most of domain II are missing, more strongly than wild-type EGFR (14). However, to the best of our knowledge, there have been no reports investigating the role of LRIG1 in *EGFR*-mutant NSCLC. The hyperactivated EGFR signaling is required for tumor maintenance in NSCLC cells harboring *EGFR* mutations (17), which means that these cancer cells become addicted to the activated EGFR. Therefore, we hypothesized that LRIG1 had strong anti-tumor effect on them by negative regulation of the EGFR signaling.

In this study, we investigated the expression of LRIG1 in NSCLC cell lines and surgically resected primary NSCLC specimens with or without *EGFR* mutations. We also performed functional analyses, both *in vitro* and *in vivo*, to determine the tumor-suppressive effects of LRIG1.

## Materials and methods

### *Reagents and cell culture*

Seven NSCLC cell lines [HCC827, HCC4006, PC9, HCC4011, NCI-H1975 (H1975), A549, and NCI-H1299 (H1299)] and one normal human bronchial epithelial cell line

(BEAS-2B) were used in this study. All of the cell lines, with the exception of PC9 and HCC4011, were purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA). PC9 was purchased from RIKEN BRC cell bank (Ibaraki, Japan). HCC4011 was kindly provided by Dr. Adi F. Gazdar (The University of Texas Southwestern Medical Center, Dallas, TX). All of these cell lines except for HCC4011 were authenticated by ATCC or RIKEN BRC cell bank using short tandem repeat polymorphism (STRP) analysis, and used within 6 months of receipt. The identity of HCC4011 was routinely confirmed by STRP analysis. All the lung cancer cells were cultured in RPMI-1640 media (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% FBS, while the BEAS-2B cells were maintained in DMEM (Sigma-Aldrich) containing 10% FBS. The cells were cultured in a humidified incubator in the presence of 5% CO<sub>2</sub> at 37°C. Recombinant human TGF-β1 was purchased from R&D Systems (Minneapolis, MN, USA).

15 ***RNA extraction and mRNA expression analysis by quantitative reverse transcription-PCR***

Total RNAs were extracted from the cell lines or frozen tissues using the RNeasy Plus Mini Kit (Qiagen, Venlo, Netherlands). cDNAs were synthesized from the total RNAs

using High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Waltham, MA, USA), in accordance with the manufacturer's instructions.

Analysis of LRIG1 mRNA expression by quantitative reverse transcription PCR (qRT-PCR) was performed using the Power SYBR Green PCR Master Mix (Applied Biosystems). The primer sequences are shown in Supplementary Table S1. PCR amplification was conducted on an ABI Step One Real-Time PCR Instrument (Applied Biosystems), and the gene expression levels were calculated using the delta-delta-CT method. Three replicates per sample were assayed for each gene. The glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene was used as the endogenous control.

Informed consent was obtained from the patients along with the study protocol approved by the Institutional Review Board/Ethical Committee of Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences and Okayama University Hospital (permission number; Ken1703-004).

### 15 ***Gene expression analysis using the CCLE cell line panel***

Comprehensive gene expression data for 978 cancer cell lines were obtained from the Cancer Cell Line Encyclopedia (CCLE) (<http://www.broadinstitute.org/ccle/>) (18). A total of 101 NSCLC cell lines whose *EGFR* mutational status could be confirmed from

the literature (19-22) or from our in-house database were categorized into an *EGFR*-wild-type (*EGFR*-wt) group (n = 85) or an *EGFR*-mutant (*EGFR*-mt) group (n = 16). We classified cell lines in which more than 10% of the cells carried *EGFR* mutations into the *EGFR*-mt group. All cancer cell lines other than the NSCLC cell lines were categorized  
5 into the non-NSCLC group.

***Plasmid constructs and transfection.***

Human cDNA encoding full-length LRIG1 was designed to express a GFP fused form at the COOH-terminal side and inserted into the improved plasmid construct based on the  
10 pIDT-SMART (C-TSC) vector (23), aimed at stable expression of the delivered foreign genes.

HCC827, HCC4011, H1975 and A549 cells were transfected with the prepared constructs to insert either LRIG1-GFP or GFP alone in combination with the other vector containing puromycin-resistance gene, using Lipofectamine 3000 (Life Technologies,  
15 Carlsbad, CA, USA) and selected with 20 µg/ml puromycin. Puromycin-resistant clones were isolated and cultured. The established puromycin-resistant clones were then named HCC827-GFP, HCC4011-GFP, H1975-GFP and A549-GFP as the control clones showing stable GFP expression, and HCC827-LRIG1#1, HCC827-LRIG1#2, HCC4011-



LRIG1#1, HCC4011-LRIG1#2, H1975-LRIG1#1, H1975-LRIG1#2, and A549-LRIG1 as the clones showing stable expression of LRIG1-GFP.

***Western blot analysis.***

5 The total cell lysate was extracted with lysis buffer, consisting of a mixture of RIPA buffer, phosphatase inhibitor cocktails 2 and 3 (Sigma-Aldrich) and Complete Mini (Roche, Basel, Switzerland). Western blot analysis was carried out by conventional methods using the following primary antibodies: anti-EGFR, phospho- (p-) EGFR (Tyr1068), HER2, p-HER2 (Tyr1221/1222), HER3, p-HER3 (Tyr1289), MET, p-MET  
10 (Tyr1234/1235), LRIG1, AKT, p-AKT (Ser473), p44/p42 MAPK, p-p44/p42 MAPK, STAT3, p-STAT3 (Tyr705), E-cadherin, N-cadherin, vimentin, ZEB1 (Cell Signaling Technology, Danvers, MA, USA), IGF-1R, p-IGF-1R (Tyr1135/1136) (R&D Systems), and  $\beta$ -actin (used as the loading control) (Merck Millipore, Billerica, MA, USA). The secondary antibody was HRP-conjugated anti-mouse or anti-rabbit IgG (Santa Cruz  
15 Biotechnology, Dallas, TX, USA). To detect specific signals, the membranes were examined using the ECL Prime Western Blotting Detection System (GE Healthcare, Amersham, UK) and LAS-3000 (Fujifilm, Tokyo, Japan). The intensities of the bands were quantified using the imageJ software (developed by Wayne Rasband, National

Institutes of Health, Bethesda, MD, USA; <http://rsb.info.nih.gov/ij/>). The band intensity of p-EGFR and EGFR was standardized to that of  $\beta$ -actin.

### ***Cell proliferation assay***

5 The HCC827-GFP, HCC827-LRIG1#1, HCC827-LRIG1#2, HCC4011-GFP, HCC4011-LRIG1#1, HCC4011-LRIG1#2, H1975-LRIG1#1, H1975-LRIG1#2, A549-GFP and A549-LRIG1 cells were seeded (at  $2 \times 10^4$  cells per well) into 24-well plates (Sumilon; Sumitomo Bakelite, Tokyo, Japan) containing RPMI-1640 medium supplemented with 10% FBS. Growth curves were generated from bright-field images obtained using a label-  
10 free, high-content time-lapse assay system (IncuCyte Zoom; Essen Bioscience, Ann Arbor, MI, USA) that automatically expresses cell confluence as a percentage over a 3-day period. All experiments were carried out in quadruplicate.

### ***Invasion and Migration assays***

15 For the matrigel invasion assay, HCC827-GFP and HCC827-LRIG1#1 cells were plated (at  $2 \times 10^4$  per well lines) onto 6-well Matrigel invasion chambers (BD Biosciences, Bedford, MA, USA). After 48 hrs in the invasion chambers, the cells were fixed and stained in accordance with the manufacturer's instructions. The filters were air-dried and

photographed under the 100× objective lens. Five fields of the view for each well were counted, and the average results from three chambers for each cell line were used for the analysis.

For the migration assay, HCC827-GFP and HCC827-LRIG1#1 cells were plated (at  
5  $2 \times 10^4$  per well) onto 6-well Boyden chambers (Corning, Lowell, MA, USA) containing  
8-mm pore polycarbonate membranes in complete medium. After 48 hrs, the filters were  
fixed and stained in accordance with the manufacturer's instructions. The chambers were  
photographed and the quantification was performed as described above.

#### 10 ***Tumor cell implantation experiments***

HCC827-GFP, HCC827-LRIG1#1 A549-GFP and A549-LRIG1 cells were  
subcutaneously injected into nonobese diabetic/severe combined immunodeficiency  
(NOD/SCID) mice purchased from Charles River Laboratories Japan (Yokohama, Japan).

Groups of mice were inoculated with each cell line at  $5 \times 10^6$  cells per animal. Tumor  
15 growth was monitored and individual tumor volumes were measured using digital  
calipers and approximated according to the formula,  $V = 1/2ab^2$  (a, long diameter of the  
tumor; b, short diameter of the tumor). At the end of 6 weeks, the mice were sacrificed  
and the tumors were harvested, measured, and photographed. All of the animal

experiments were carried out in accordance with the protocols approved by the Animal Care and Use Committee, Okayama University (permission number; OKU-2017341).

### ***Phospho-receptor tyrosine kinase array and phosphokinase arrays***

5 The Human Phospho-Receptor Tyrosine Kinase (RTK) Array Kit (R&D Systems) was used to measure the relative level of tyrosine phosphorylation of 42 distinct RTKs. After HCC827-GFP and HCC827-LRIG1#1 cells were serum-starved overnight, the total cell lysate was extracted with lysis buffer in the same manner for the western blot analysis. This array experiment was conducted in accordance with the manufacturer's instructions.

10

### ***Epithelial-to-mesenchymal transition assay***

The experiment was performed using HCC827-GFP and HCC827-LRIG1#1 cells grown to 60% confluence in 60-mm dishes (BD Biosciences) containing RPMI-1640 supplemented with 10% FBS. After the cells were incubated in the presence or absence  
15 of TGF- $\beta$ 1 (10 ng/mL) for 72 hours in a humidified incubator under 5% CO<sub>2</sub> at 37°C, this assay was conducted in the same manner as the western blot analysis.

### ***Statistical analysis.***

All data were analyzed using the JMP v 9.0.0 software (SAS Institute Inc., Cary, NC, USA). Differences between groups were compared by the *t*-test.  $P < 0.05$  was considered as denoting statistically significant difference. All tests were two-sided.

5

## Results

### *LRIG1 expression was downregulated in NSCLC cells*

We examined the mRNA expressions of *LRIG1* in the HCC827, HCC4006, PC9, HCC4011, H1975, A549, H1299 (representing NSCLC cell lines) and BEAS-2B (normal human bronchial epithelial cell line) cell lines. Lower expression of *LRIG1* was observed in all the NSCLC cell lines, irrespective of whether they harbored wild-type EGFR [A549 (K-ras codon 12 serine mutation) and H1299 (large-cell carcinoma)] or mutant EGFR [HCC827 (exon19 del. E746-A750), HCC4006 (exon19 del. L747-E749), PC9 (exon19 del. E746-A750), HCC4011 (L858R), and H1975 (L858R/T790M)], as compared to that in the BEAS-2B cells (Figure 1A).

15 To investigate the *in vivo* expressions of *LRIG1*, we examined the expression levels of *LRIG1* in surgically resected primary NSCLC tissue specimens and compared them with those in the corresponding non-malignant tissues around the tumors. In 9 of 10 cases, including all three cases in which the tumor specimens harbored *EGFR* mutation [sample

#3013 (L858R), #3103 (exon19 del), and #3161 (L858R/ T790M)], the expression levels of *LRIG1* in the tumor tissues were significantly lower than those in the corresponding non-malignant tissues around the tumor (Figure 1B). These results suggest that the expression of *LRIG1* is suppressed in NSCLCs.

5        Next, we examined *LRIG1* promoter methylation status to investigate the mechanism that the expression of *LRIG1* is suppressed in NSCLC cells. Methylation-specific PCR (MSP) and bisulfite sequencing PCR (BSP) revealed that *LRIG1* promoter region was methylated in NSCLC cell lines (Supplementary Figure S1A and S1B). In addition, 5-aza-2-deoxycytidine (5-Aza) treatment increased the mRNA expression of  
10    *LRIG1* in NSCLC cell lines (Supplementary Figure S1C). These findings suggested that the promoter hypermethylation was strongly associated with downregulation of *LRIG1* expression in NSCLC cells. Detailed methods of methylation analysis are described in Supplementary Methods.

To investigate whether there were any differences in the expression levels of *LRIG1*  
15    in NSCLC cells depending on whether they carried wild-type or mutant *EGFR*, we compared the expression levels of *LRIG1* between *EGFR*-wt NSCLC cell lines and *EGFR*-mt NSCLC cell lines using the CCLE database. As shown in Figure 1C, the expressions of *LRIG1* in the *EGFR*-mt group were significantly lower than those in the

*EGFR*-wt group ( $P = 0.022$ ). The expression levels of *LRIG1* in the *EGFR*-wt group tended to be lower than those in the non-NSCLC group ( $P = 0.053$ ), whereas those in the *EGFR*-mt group were found to be significantly lower than those in the non-NSCLC group ( $P < 0.01$ ).

5

***LRIG1 downregulated mutant EGFR expression in NSCLC cells.***

To investigate whether *LRIG1* is capable of downregulating mutant *EGFR* expression in NSCLC tumors, we examined the effect of *LRIG1* transfection in HCC827 (*EGFR* exon 19 del.), HCC4011 (*EGFR* L858R), and H1975 (L858R/T790M) cells, which show high  
10 expression of mutant *EGFR* and are frequently used as a model of *EGFR*-mutant lung cancer (24). The mutant type of these cell lines is exon19 del. or L858R, respectively, which occupy approximately 90% of *EGFR* mutations (25). The *EGFR* T790M mutation is known as a major resistant mechanism to *EGFR*-TKI (26). The *EGFR*-wild-type lung cancer cell line A549 was used for comparison. These cell lines show low or undetectable  
15 endogenous *LRIG1* expression, making them an appropriate system for examining the effect of ectopic expression of *LRIG1*. Using these cell lines, we established clones stably expressing the transfectants (HCC827-GFP, HCC827-LRIG1#1, HCC827-LRIG1#2, HCC4011-GFP, HCC4011-LRIG1#1, HCC4011-LRIG1#2, H1975-GFP, H1975-

LRIG1#1, H1975-LRIG1#2, A549-GFP, and A549-LRIG1), and compared the expression and phosphorylation levels of EGFR and downstream signaling molecules between the clones stably expressing GFP and those stably expressing LRIG1.

As shown in Figure 2A, transfection of LRIG1 into HCC827, HCC4011, H1975 and  
5 A549 cells led to a decrease in the EGFR expression in these cells. Furthermore, in the HCC827, HCC4011, and H1975 cells, transfection of LRIG1 also led to a decrease in the expression of p-EGFR. Comparison of the band intensities showed that LRIG1 transfection was associated with stronger downregulation of p-EGFR than that of total EGFR in the HCC827, HCC4011, and H1975 cells (Figure 2B and C). In regard to the  
10 downstream signaling molecules, there was a marked decrease in the expression of p-STAT3, whereas the expressions of p-AKT and p-MAPK were rather increased in the HCC827-LRIG1 and HCC4011-LRIG1 cells. On the other hand, the expressions of p-AKT and p-MAPK were downregulated, whereas that of p-STAT3 was upregulated in H1975-LRIG1 cells. The A549 cells showed weak expression of p-EGFR even prior to  
15 the LRIG1 transfection, and no significant difference in p-EGFR expression was observed irrespective of LRIG1 transfection.

Half-life analysis using HCC827-GFP and HCC827-LRIG1#1 cells showed that the introduction of LRIG1 led to a marked decrease in half-life of mutant EGFR protein, from



16.9 to 8.7 h (Supplementary Figure S2A and S2B).

We also investigated the effect of LRIG1 knockdown by siRNA on BEAS-2B cells, whose LRIG1 expression was higher than those in NSCLC cells. Knockdown of LRIG1 upregulated the expression of EGFR (Supplementary Figure S3). This finding supported the suppressive effect of LRIG1 to EGFR.

***LRIG1 suppressed the cell-proliferative, migratory and invasive ability of NSCLC cells harboring mutant EGFR.***

To examine whether LRIG1 influences the proliferative activity of HCC827, HCC4011, H1975, and A549 cells, a cell proliferation assay was performed using IncuCyte Zoom. The automatically expressed growth curves are shown in Figure 3A. In *EGFR*-mutant cell lines, there was a significantly higher number of HCC827-GFP, HCC4011-GFP, or H1975-GFP cells as compared to the numbers of HCC827-LRIG1#1 and HCC827-LRIG1#2 cells, HCC4011-LRIG1#1 and HCC4011-LRIG1#2, or H1975-LRIG1#1 and H1975-LRIG1#2 cells at any time, indicating that LRIG1-expressing cells had a growth disadvantage. On the other hand, transfection of LRIG1 had no significant effect on the proliferative activity of the *EGFR*-wild-type A549 cells.

We next examined whether expression of LRIG1 attenuated the invasive and

migratory ability of the HCC827 cells. As shown in Figures 3B and C, LRIG1-expressing cells showed significantly lower invasive and migratory ability than the control cells. These results suggest that LRIG1 exerted strong tumor-suppressive effect in lung cancer cells harboring mutant EGFR *in vitro*.

5

#### ***Effect of LRIG1 in a xenograft mouse model.***

To examine the effect of LRIG1 on the tumorigenic potential of NSCLC cells *in vivo*, we subcutaneously implanted HCC827-GFP, HCC827-LRIG1#1, A549-GFP and A549-LRIG1 cells into immunocompromised mice. The findings revealed a significantly lower tumorigenic potential of HCC827-LRIG1#1 cells as compared to that of HCC827-GFP cells (Figure 4A and B); on the other hand, A549-LRIG1 cells exhibited the similar tumorigenic potential as the A549-GFP cells (Figure 4C and D). Thus, LRIG1 also showed strong tumor-suppressive effect in lung cancer cells harboring mutant EGFR *in vivo*.

15

#### ***LRIG1 also downregulated the expression of HER2, HER3, MET and IGF-1R***

To examine whether LRIG1 also affected the expression levels of other RTKs than EGFR in NSCLC cells harboring mutant EGFR, we conducted a phospho-RTK array analysis

and compared the expressions in the HCC827-LRIG1#1 with HCC827-GFP cells. As shown in Figures 5A, transfection of LRIG1 led to downregulation in the expressions of p-HER2, p-HER3 and p-MET. In addition, LRIG1 also downregulated the expression of p-IGF-1R (Insulin-like growth factor receptor 1) (Figure 5A).

5 To validate these results, we conducted western blot analysis to compare the expression of these RTKs of the HCC827-LRIG1#1 with that of HCC827-GFP cells, which showed the same results as that of the phospho-RTK array (Figure 5B). The expressions of the RTKs were downregulated, with the decrease in phosphorylation of these RTKs being more pronounced in the LRIG1-expressing cells, consistent with the results for the case of EGFR. These results suggest that in addition to suppressing the expression/phosphorylation of EGFR, LRIG1 also suppresses the expression/phosphorylation levels of HER2, HER3, MET and IGF-1R.

***LRIG1 prevented the epithelial-to-mesenchymal transition induced by TGF- $\beta$ .***

15 To investigate the ability of LRIG1 to prevent epithelial-to-mesenchymal transition (EMT), we induced EMT using TGF- $\beta$  in HCC827-GFP cells and HCC827-LRIG1#1 cells (27), and compared the epithelial and mesenchymal markers in the two cell lines by western blot analysis. As illustrated in Figure 5C, in the HCC827-GFP cells, the epithelial

marker E-cadherin was downregulated and the mesenchymal markers vimentin and ZEB1 appeared after the addition of TGF- $\beta$ , whereas no such changes were observed in the HCC827-LRIG1#1 cells. These results suggest that LRIG1 has the potential to prevent EMT.

5

### Discussion

Our study revealed that expression of LRIG1 is downregulated in NSCLC, as previously reported for the case of renal cell carcinoma (28), cervical cancer (29) and oropharyngeal cancer (30). Previous studies indicated that promoter hypermethylation contributed to the low LRIG1 expression in colorectal and cervical cancers (31,32). In our study, *LRIG1* promoter hypermethylation was detected in NSCLC cells, concordant with these reports. Expression levels of LRIG1 have been reported to differ among cancer subtypes (33). For example, Miller et al. reported that the expression of LRIG1 was downregulated, and that, therefore, its tumor suppressive effect was impaired, in HER2-positive breast cancer (34). In regard to the role of LRIG1 in NSCLC, because LRIG1 is known to negatively regulate the expression levels of ErbB (HER) family proteins including EGFR (10, 11), we focused on its relation to the EGFR mutational status of the tumor and performed gene expression analyses using a CCLE cell line panel. Our results indicated that the

expression of LRIG1 was significantly lower in NSCLC cell lines harboring *EGFR* mutations than in those without *EGFR* mutations. The growth of NSCLC cells harboring *EGFR* mutations is dependent on hyperactivated EGFR signaling (17). Therefore, LRIG1 may be more inconvenient for *EGFR*-mutant NSCLC cells to survive than for *EGFR*-wild-type NSCLC cells. Although LRIG1 has already been reported as a tumor suppressor in NSCLC cells without *EGFR* mutations (16), our findings suggest that the tumor-suppressive effects of LRIG1 were even stronger in NSCLCs harboring mutant EGFR.

Negative regulation of EGFR signaling by LRIG1 has been shown to result from a physical interaction between the extracellular domains of the two proteins, which induces recruitment of the E3 ubiquitin ligase Cbl to the cytosolic part of the LRIG1-EGFR complex, thereby promoting the ubiquitination and lysosomal degradation of the protein complex (10,11). In our study, the introduction of LRIG1 led to a marked decrease in half-life of mutant EGFR in NSCLC cells, concordant with these reports. Interestingly, the results of our western blot analysis showed that LRIG1 suppressed the expression of p-EGFR more strongly than that of total EGFR. Previously, Goldoni et al. reported that LRIG1 also had the ability to act as a monoclonal antibody preventing the intermolecular interactions that lead to dimerization and activation, that is, keeping the receptor in an

inactive state (35). The difference in the suppressive efficacy between p-EGFR and total EGFR observed in our experiment is thought to be associated with this secondary action of LRIG1 as a monoclonal antibody.

In the current study, the expression of p-STAT3 was downregulated, whereas the  
5 expressions of p-AKT and p-p44/p42 MAPK were upregulated in the HCC827-LRIG1  
and HCC4011-LRIG1 cells as compared to control cells. However, previous studies have  
reported that LRIG1 inhibited the EGFR/PI3K/AKT and EGFR/MEK/MAPK pathways  
in breast and brain tumors (12,14,36), as shown in H1975-LRIG cells. Although the  
reason for this discrepancy is unclear, the original features of the cell lines used may be  
10 associated with differences in the affected downstream molecules. Indeed, Nakamura et  
al. reported that LRIG1 inhibited STAT3-dependent inflammation in the cornea (37).

In the last decade, numerous studies have reported that LRIG1 interacts with many  
receptor kinases, including members of the GDNF/c-Ret (38), Met (12) and EGFR family  
(11,35) signaling systems. In fact, consistent with these reports, our experiments showed  
15 that LRIG1 downregulated not only EGFR, but also MET, HER2 and HER3. In addition,  
LRIG1 also downregulated IGF-1R, which has not been reported previously. IGF-1R is  
known to mediate anti-apoptotic signals and cell proliferation by the interaction with the  
ligands insulin-like growth factor I and II (IGF1 and IGF2) (39,40). IGF-1R plays

important role in the transformation and growth of malignant cells (41), and its overexpression is observed in various cancers, such as breast, prostate, and lung cancers (42). Therefore, the downregulation of IGF-1R is thought to contribute the strong tumor-suppressive effect of LRIG1. Although we could not identify the mechanism by which

5 LRIG1 downregulated IGF-1R, it has been reported that IGF-1R interacts and heterodimerizes with EGFR (43), suggesting that suppression of IGF-1R is also accompanied by downregulation of EGFR. Activation of MET, HER2 and IGF-1R are reported as novel mechanisms of acquired resistance to EGFR-TKIs in patients with NSCLC harboring mutant EGFR (44-46), suggesting that LRIG1 has the potential to

10 suppress the acquisition of drug resistance through activation of these bypass signaling pathways. In addition, our study revealed that LRIG1 prevented EMT, which is another major mechanism of development of resistance to EGFR-TKIs of NSCLCs harboring *EGFR* mutations (26,47); this finding was consistent with a previous report that LRIG1 inhibited hypoxia-induced EMT in glioma cells (36). Furthermore, we revealed that

15 LRIG1 transfection downregulated the expression of EGFR and cell growth in H1975 cells with the *EGFR* T790M mutation, which was a major resistant mechanism to EGFR-TKI (26). These findings suggest that in addition to exerting strong tumor-suppressive

effects, LRIG1 also has the potential to prevent the development of tumor resistance to EGFR-TKIs.

From the late 2000s, two studies reported that transfection of the soluble ectodomain of LRIG1 could suppress EGFR activation and inhibit cell growth *in vitro* (35,48). In addition, it was reported that when human glioblastomas expressing wild-type or VIII mutant EGFR were transplanted to the mouse brain, the tumor cell growth was inhibited by nearby encapsulated cells secreting the soluble ectodomain of LRIG1 (49). Although these reports suggest the possible therapeutic potential of LRIG1 against cancer, further research is required before clinical application can be considered.

In conclusion, our findings demonstrated that LRIG1 suppressed the expression and phosphorylation of mutant EGFR, and exerted strong tumor-suppressive effects in NSCLCs harboring *EGFR* mutations. LRIG1 also suppressed the expressions and phosphorylation of other RTKs, such as HER2, HER3, MET and IGF-1R, and abrogated TGF- $\beta$ -induced EMT, implying its potential ability to prevent the development of resistance to EGFR-TKIs. These findings suggest that LRIG1 plays important roles in *EGFR*-mutant NSCLC and has the potential to be used as a novel therapeutic target for *EGFR*-mutant NSCLC.



## **Supplementary materials**

Supplementary Table S1, Supplementary Figures S1-S3 and Supplementary Methods can be found at *Carcinogenesis* online.

5

### **Funding**

This study was supported by a Management Expenses Grants for National University Corporations in Japan.

### **Acknowledgment**

10 We thank Dr. Takehiro Matsubara (Biobank, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan) and Ms. Fumiko Isobe (Department of Thoracic, Breast and Endocrinological Surgery, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan) for their technical support.

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*Conflict of Interest Statement:* None declared.

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## Figure legends

**Figure 1.** mRNA expressions of LRIG1 (A) in non-small cell lung cancer (NSCLC) cell lines and BEAS-2B, (B) in surgically resected primary NSCLC tissue specimens and the corresponding non-malignant lung tissues around the tumors. (C) The mRNA expressions of LRIG1 were compared among the non-NSCLC, *EGFR*-wild-type (*EGFR*-wt) NSCLC, and *EGFR*-mutant (*EGFR*-mt) NSCLC cell lines, using the Cancer Cell Line Encyclopedia (CCLE) database.

**Figure 2.** Transfection of LRIG1 led to downregulation of the expression of mutant *EGFR* in NSCLC cells. (A) HCC827 (*EGFR* exon 19 del.), HCC4011 (*EGFR* L858R), H1975 (L858R/T790M), and A549 (no *EGFR* mutation) cells were stably transfected with either GFP alone or GFP-tagged LRIG1. The cells were serum-starved overnight and the lysates were subjected to western blot analysis with the indicated antibodies. (B) The band intensities of *EGFR* and phospho-*EGFR* in the HCC827, HCC4011, and H1975 cells were quantified using the imageJ software.

**Figure 3.** Transfection of LRIG1 suppressed the cell proliferative, invasive and migratory ability of lung cancer cells harboring mutant *EGFR*. (A) The cell proliferative

ability of HCC827-GFP, HCC827-LRIG1#1, HCC827-LRIG1#2, HCC4011-GFP, HCC4011-LRIG1#1, HCC4011-LRIG1#2, H1975-GFP, H1975-LRIG1#1, H1975-LRIG1#2, A549-GFP and A549-LRIG1 were measured using IncuCyte Zoom. (B, C) The cell invasive and migratory ability of the HCC827-GFP and HCC827-LRIG1#1 cells were measured using a Matrigel invasion chamber and Boyden chamber, respectively.

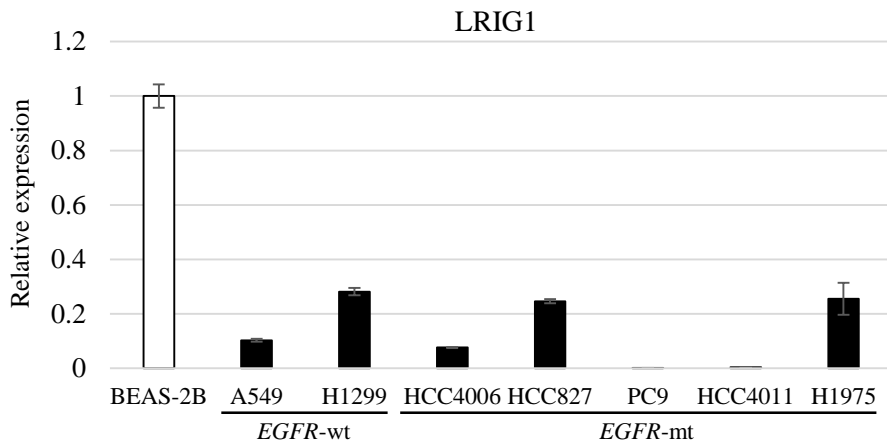
**Figure 4.** LRIG1 shows a strong suppressive effect on tumor growth in the mouse xenograft model of NSCLC harboring mutant EGFR. (A) Mice were subcutaneously implanted with HCC827-GFP or HCC827-LRIG1#1 cells. The tumor volumes were determined on the indicated days after implantation. Data represent means  $\pm$  SE (n = 8). (B) Appearance of the HCC827 tumors at 6 weeks after the implantation, when the mice were sacrificed. (C) Mice were subcutaneously implanted with A549-GFP or A549-LRIG1 cells. The tumor volumes were determined as in (A). Data represent means  $\pm$  SE (n = 8). (D) Appearance of the A549 tumors at 6 weeks after the implantation, when the mice were sacrificed.

**Figure 5.** Phospho-receptor tyrosine kinase array and epithelial-to-mesenchymal transition assay. (A) The phospho- (p-) receptor tyrosine kinase array revealed that

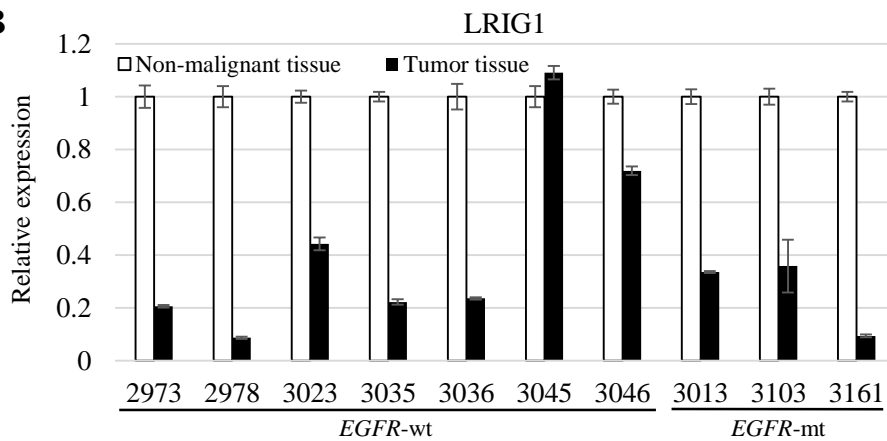
transfection of LRIG1 into the tumor cells downregulated the expressions of p-HER2, p-HER3, p-MET and p-IGF-1R. (B) The effect of LRIG1 on the expressions of HER2, HER3, MET and IGF-1R and on their phosphorylation levels were validated by western blot analysis. HCC827-GFP and HCC827-LRIG1#1 cells were serum-starved overnight and lysates were subjected to western blot analysis with the indicated antibodies. (C) The epithelial-to-mesenchymal transition (EMT) assay revealed that transfection of LRIG1 into the tumor cells prevented the changes in the expression levels of the epithelial marker E-cadherin and mesenchymal markers vimentin and ZEB induced by TGF- $\beta$  in the HCC827 cells.

# Figure 1

## A



## B



## C

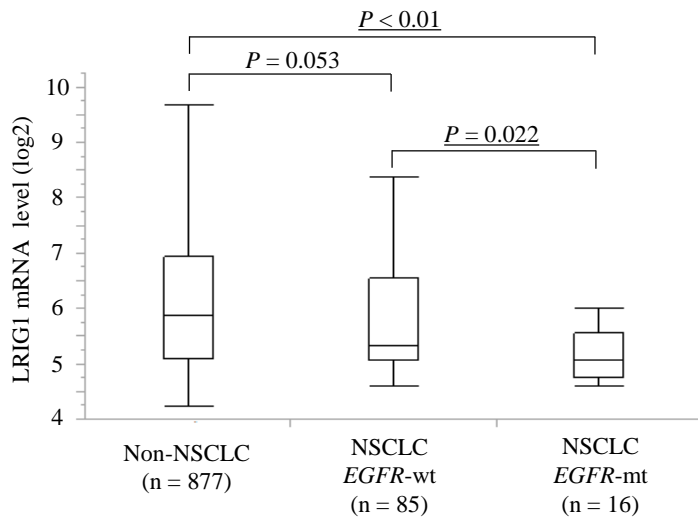
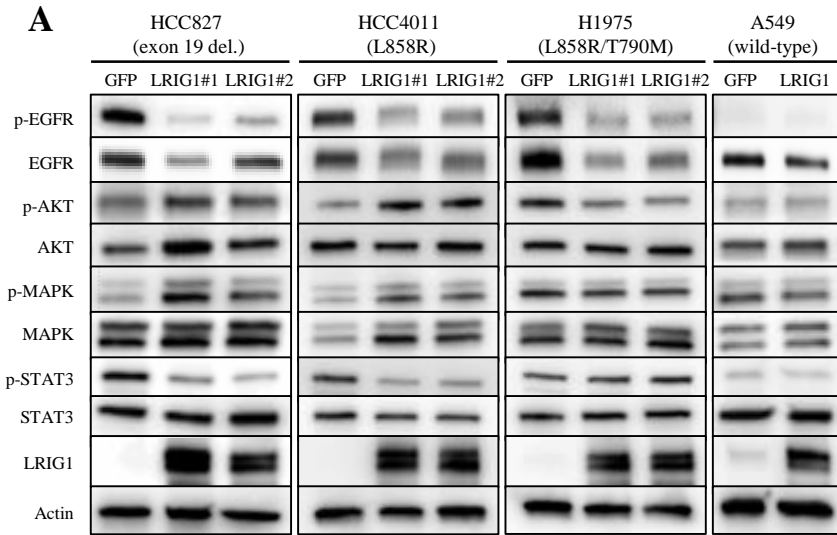
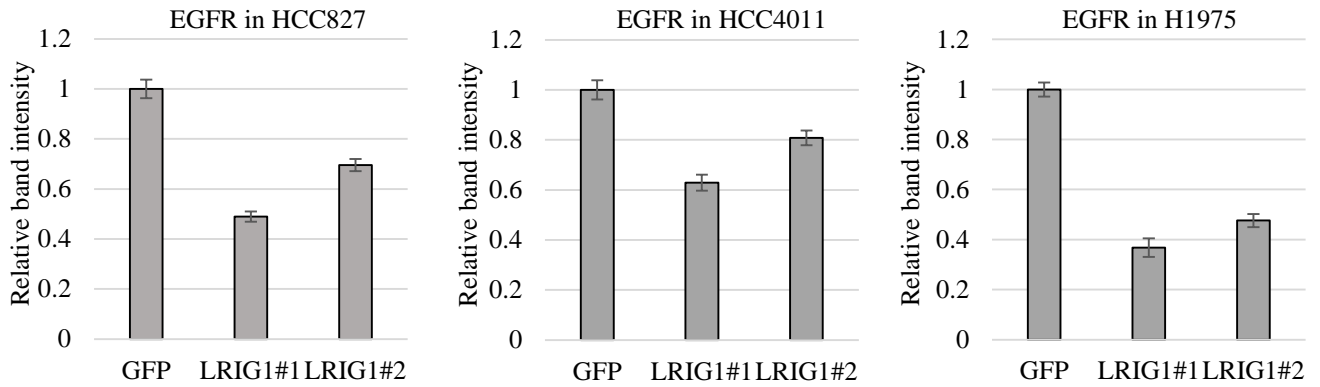


Figure 2



**B**



**C**

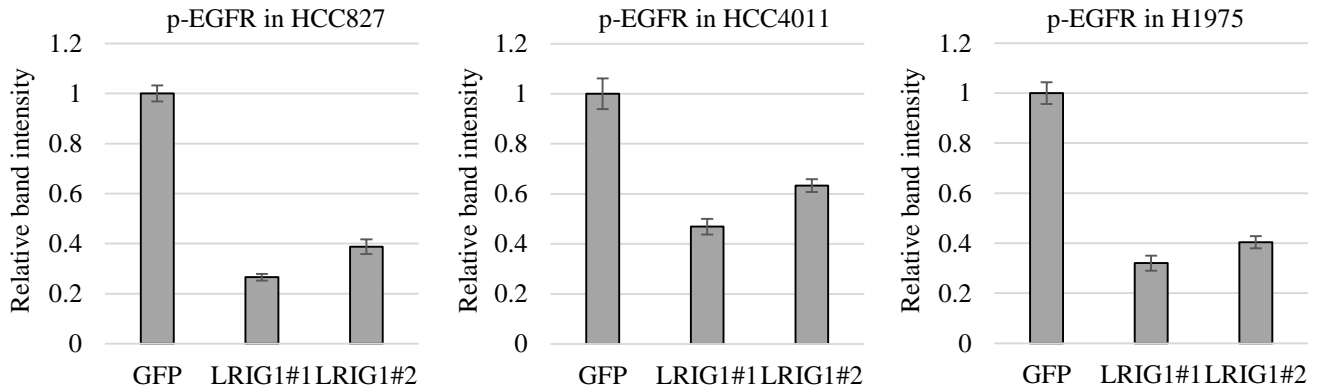
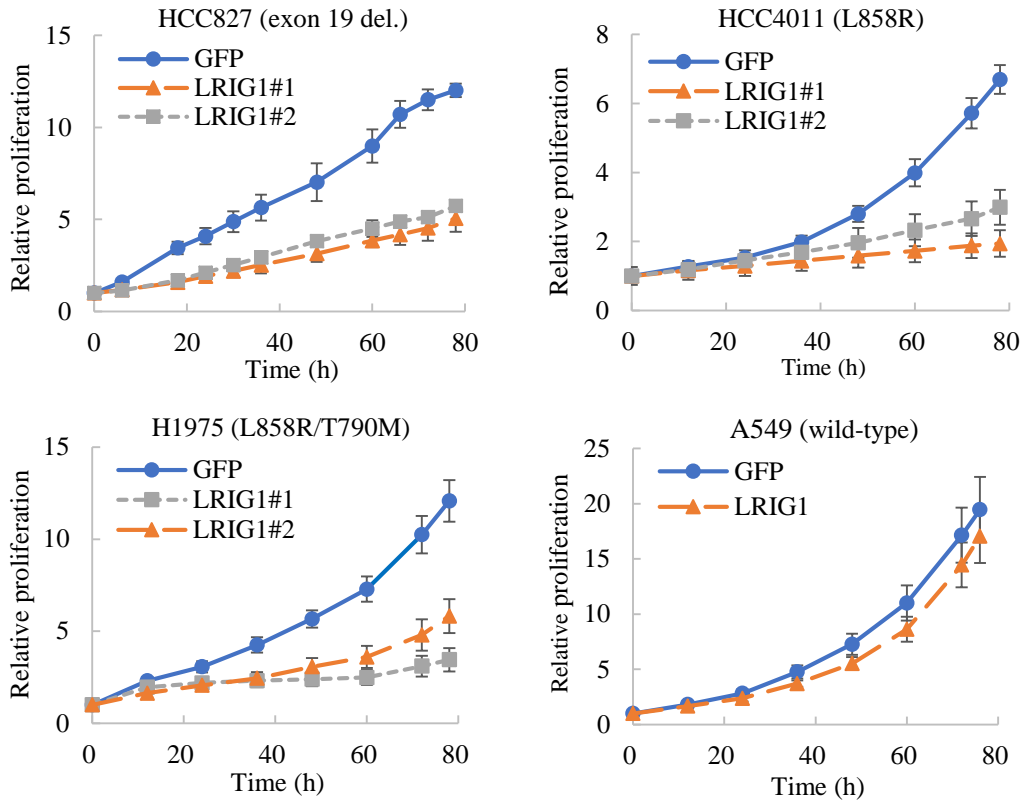
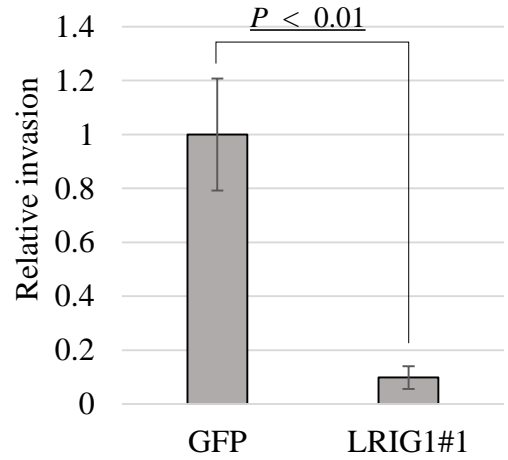
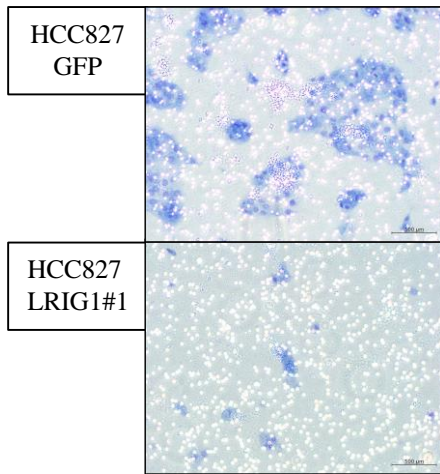


Figure 3

A



B



C

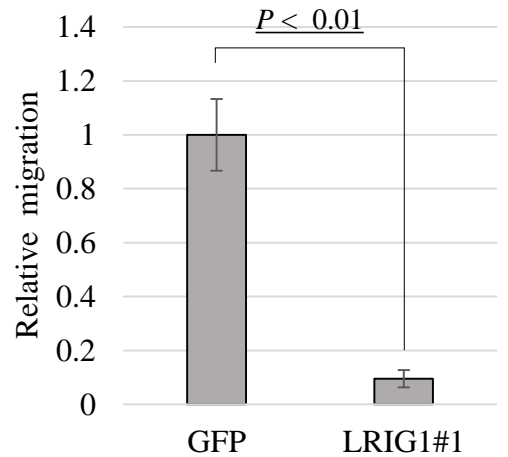
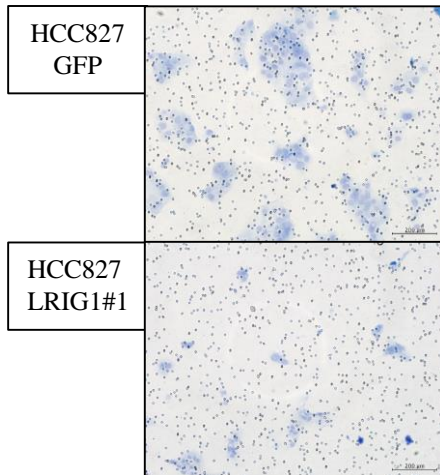
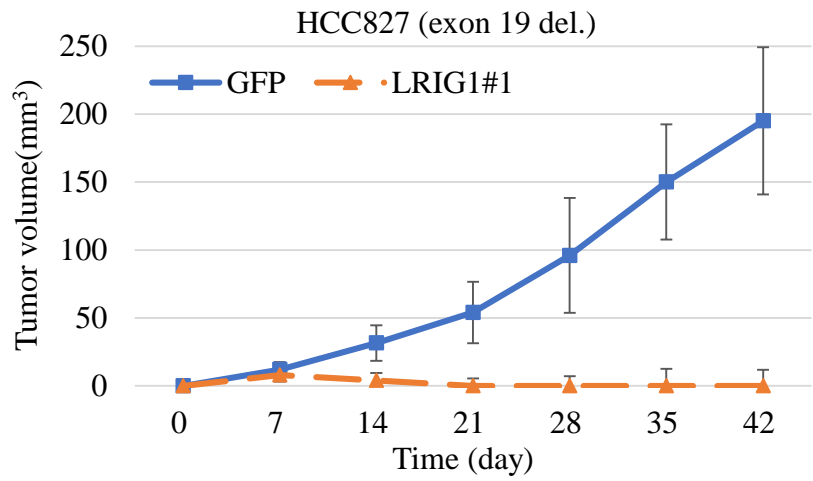
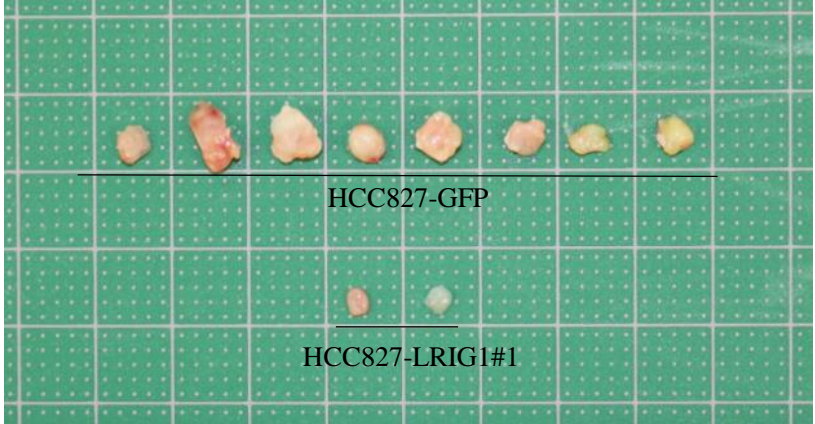


Figure 4

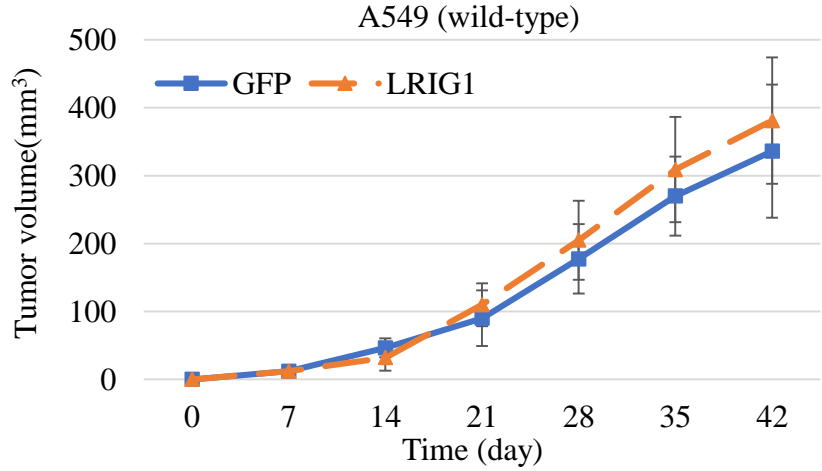
A



B



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D

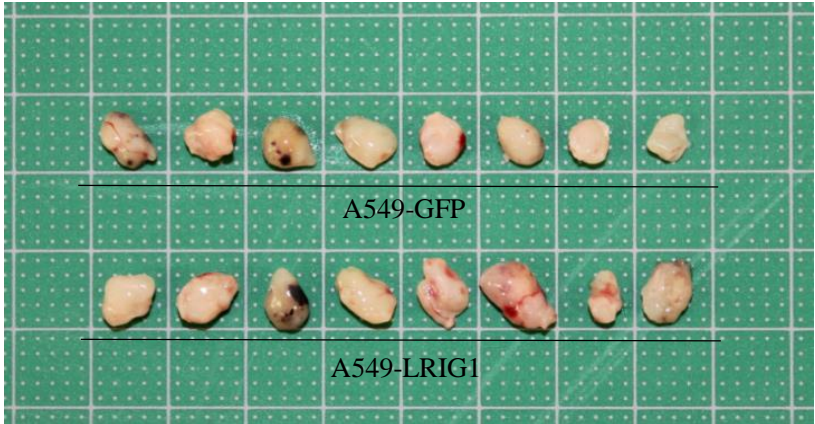
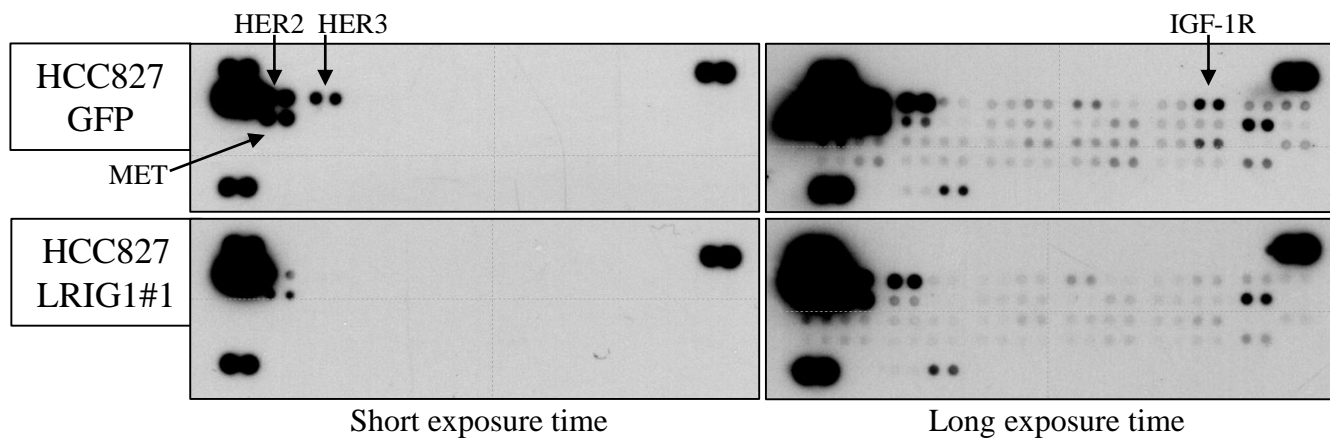
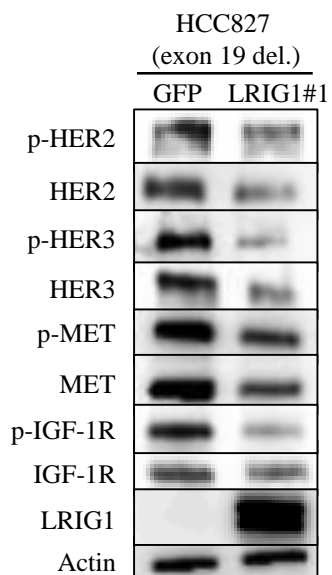


Figure 5

A



B



C

