The biological importance of LRIG1 in lung cancer

Introduction

Lung cancer is the leading cause of cancer-related death worldwide, accounting for an estimated 23% of deaths (Jemal et al., 2011; Siegel et al., 2012). The disease is histologically subdivided into four major types — squamous cell carcinoma (SCC), adenocarcinoma, large cell carcinoma (LCC) and small cell lung carcinoma (SCLC). The first three diagnoses are often referred to as non-small cell lung cancer (NSCLC), and the disease spectrum is dominated by adenocarcinoma and SCC.

In Sweden, lung cancer accounts for approximately 3,500 deaths per year (16% of all cancer-related deaths) (Socialstyrelsen, 2011), making it the leading cause of cancer-related death here as well. Adenocarcinoma is the dominating subtype, accounting for 40% of all cases, followed by squamous cell carcinoma, accounting for 24% of all cases. Most patients are smokers and former smokers, although 6% of diagnosed men and 15% of diagnosed women in this group are never-smokers (Engholm et al.). During the last decades the incidence in men has decreased, but this is countered by a larger increase in women. Lung cancer is now the most common cause of cancer death among Swedish women (Socialstyrelsen, 2011).

Surgery is the first line of treatment in localized disease (i.e., stage I-II), with stereotactic body radiotherapy (SBRT) being the alternative for medically inoperable patients. In locally advanced disease (i.e., stage III) radiochemotherapy is given with curative intention to patients in good performance status. For metastatic disease (stage IV), palliative chemotherapy is the treatment of choice. Targeted therapy using epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) has been a second-line treatment for stage IV disease for several years, which has had considerable effect on a subset of tumors, mostly adenocarcinomas (Shepherd et al., 2005).

Recently, several mutations in the EGFR gene have been identified, of which in-frame deletions in exon 19 and a substitution for arginine with leucine at codon 858 (L858R) are the most clinically relevant in lung cancer, together accounting for more than 90% of all EGFR lung cancer mutations (Kumar et al., 2008). These mutations affect the kinase domain, destabilizing the inactive conformation and forcing the protein into a constantly active state. Due to this,
patients with these mutations have a high response rate to anti-EGFR therapy (Lynch et al., 2004; Paez et al., 2004). Therefore, EGFR-targeted TKIs are today used for first line treatment for the subset of patients with EGFR point mutations at diagnosis (Mok et al., 2009; Maemondo et al., 2010; Zhou et al., 2011).

LRIG proteins

The LRIG (leucine-rich and immunoglobulin-like domains) family of transmembrane glycoproteins consists in mammals of homologs LRIG1, 2 and 3. All LRIG proteins share the same basic structure, with an extracellular domain consisting of 15 leucine-rich repeats plus three immunoglobulin-like domains, a transmembrane domain and a cytoplasmic domain. Proteins in the LRIG family have been shown to form heterodimers with several receptor tyrosine kinases (RTKs) in the ErbB family, thereby regulating their activity.

The most studied LRIG-family member is LRIG1, which is known to interact with EGFR and Met, among others. Upon heterodimerization, LRIG1 recruits an E3 ligase which ubiquitinates the RTK, flagging it for proteolytic degradation (Gur et al., 2004), inhibiting downstream activity in their respective signaling pathways. Hence, LRIG1 is one of relatively few proteins known to negatively regulate growth factor signaling by regulating receptor trafficking. It has also been shown that LRIG1 expression can be induced by EGFR signaling in a feedback loop (Gur et al., 2004), indicating that feedback inhibition of growth factor receptors is an important component in the homeostasis of normal tissue (Segatto et al., 2011).

Considering that aberrant growth factor signaling is a key part in many human cancers, it would be reasonable to hypothesize that LRIG proteins play a significant role in tumorigenesis. This has also been shown to be the case for several tumor types, particularly for LRIG1 where correlation between expression and clinical outcome has been observed in skin (Tanemura et al., 2005), renal (Thomasson et al., 2003), cervix (Hedman et al., 2010) and prostate cancer (Thomasson et al., 2011), as well as glioma (Ye et al., 2009) and esophageal cancer (Wu et al., 2012). In addition, there is significant correlation between clinical outcome and different patterns of subcellular localization of LRIG proteins in malignant glioma (Holmlund et al., 2009), suggesting that defective trafficking of LRIG proteins within the cell can be a component in the dysfunctional signaling of malignant cells.

Recently, one study (Boelens et al., 2009) showed that LRIG1 is downregulated by smoking; a finding that is of particular interest when the importance of EGFR signaling in lung cancer is taken into consideration. Considering the correlation between LRIG1 and clinical outcome in other tumor types, this led us to investigate whether LRIG1 is of biological importance in lung cancer.
Methods

Tissue microarray
A tissue microarray (TMA) based on surgical material from 366 lung cancer patients that have undergone lobectomy or pulmectomy, was kindly provided by Dr Patrick Micke, at the Department of Pathology and Genetics at Uppsala University. The TMA consists of tumor material of all major lung cancer subtypes, with two cores per patient. The corresponding database contains data on patient age, gender, histological subtype, WHO classification and survival, as well as mutational status for certain genes (if available). Corresponding patient characteristics for the TMA is presented in table 1.

The TMA slides were immunohistochemically stained for LRIG1 using an automated system for immunohistochemical staining (Ventana, Tucson, AZ, USA) at the Department of Clinical Pathology at Umeå University Hospital. After staining, the material was digitized using a slide scanner (Aperio ScanScope, Aperio, Vista, CA, USA) at 200x magnification, into high-resolution uncompressed images.

Each TMA slide was manually quantified under the supervision of an experienced lung pathologist. For each core, the LRIG1 staining intensity was graded on a 0-3 scale, with 0 being completely negative, and scores 1 through 3 representing a range from weakly to strongly positive. Examples can be seen in figure 1A. The approximate amount of positive cells in the tumor epithelium (i.e., excluding leukocytes, stromal cells and normal epithelium), as assessed by us, was represented by a percentage ranging from 0 to 100% in steps of 5%. A score was then calculated for each patient, by multiplying the highest intensity value for the two cores with the mean amount of positive cells. This yields one score per patient, ranging from 0 to a theoretical maximum of 3.00. The quantification was performed jointly by two observers in a blinded fashion, with the patient ID being the only information available during assessment.

In vivo – mouse model
A mouse model carrying the L858R57 gene was kindly obtained from Dr. Katerina Politi at Harold Varmus’ research group at Memorial Sloan-Kettering Cancer Center. The gene consists of a human EFGR exon with the well-characterized L858R mutation, coupled to a pneumocyte-specific promotor with a Tet-On transcriptional activation system. Mice carrying both the L858R57 gene and the tetracycline reverse transcriptional activator (rtTA) gene will develop diffusely infiltrating EGFR-driven pulmonary carcinomas within approximately 6 weeks of doxycycline treatment (Politi et al., 2006). L858R57/rtTA-positive mice were cross-bred with heterozygous LRIG1 knockouts previously developed at the Oncology Research Lab. Offspring were put on a diet containing doxycycline at a concentration of 625 mg/kg (Teklad TD.05125, Harlan Laboratories, Indianapolis, IN, USA) directly at weaning and tail clippings were collected. Mouse genotype was determined using PCR. DNA was extracted from tail clippings using a ready-made extraction solution (Express Extract, KAPA Biosystems, Woburn, MA, USA)
according to the supplier’s protocol. PCR reactions were set up using 7.5 μl ReadyMix (HiFi HotStart ReadyMix, KAPA Biosystems, Woburn, MA, USA), 0.6 μl of forward primer at 5 μmol/l (primer sequences available upon request), reverse primer in the same amount and concentration, 1 μl of DNA extract and 5.3 μl PCR-grade water.

For L858R57 and rTA, reactions were run in a RoboCycler thermal cycle (RoboCycler Gradient 40, Stratagene, La Jolla, CA, USA) with 92°C initialization at 2 minutes and 30 cycles of denaturation at 94°C for 0:30, annealing at 60°C for 0:30 and elongation at 72°C for 0:30; followed by a final elongation at 72°C for 10 minutes. A similar protocol was used for LRIG1, but with annealing at 62°C for 1 minute. Amplified DNA was separated by electrophoresis in a 1% agarose gel stained with GelRed (GelRed, Biotium, Hayward, CA, USA). After genotyping, mice positive for both L858R57 and rTA, and with an LRIG1 knockout or wild-type genotype, were selected for the experiment.

Animals were housed under controlled conditions with a 12 hour day/night cycle and fed water and pellets ad libitum. All animal experiments were approved by the animal ethics committee at Umeå University (Dnr A42-10).

MRI

Respiration-gated T1-weighted MRI sequences were obtained using a 9.4 T Bruker Biospec 94/20 USR micro MRI scanner (Bruker, Billerica, MA) using a FLASH sequence with the following parameters: TR = 113 ms, TE = 2.8 ms, flip angle = 30 degrees, field of view = 30 mm, matrix = 256×256 and 16 averages. 15 axial and 17 sagittal slices were obtained with 0.50 mm slice thickness and 1.00 mm interslice spacing. More axial slices were added as needed in order to cover the lungs in their entirety on larger mice. A 40 mm quadrupolar volume coil was used in transmit/receive mode.

Images were obtained from each mouse at day 60 and day 95 of treatment. Due to MRI availability restrictions, we allowed for a deviation of ±3 days. For the imaging, mice were anesthetized with an induction dose of 3% isoflurane in oxygen and maintained at 2%. Body temperature was monitored by a rectal thermometer and held as closely as possible to 37°C by circulating water in a heating pad. A pressure sensor was used to monitor the breathing pattern. The mice were finally sacrificed immediately after the second MRI sequence. Lungs were extracted, photographed, weighed and subsequently fixated in 4% PFA solution for histopathological verification. Slides were H&E stained and scanned at 200x magnification.

Image quantification

MRI sequences were imported into the open source ImageJ scientific image analysis toolbox (Rasband, W.S., ImageJ, U.S. National Institutes of Health, Bethesda, MD, USA, http://rsb.info.nih.gov/ij/). Due to the difficulties of accurately measuring diffusely infiltrating lung tumors, a semiquantitative analysis was performed on each MRI sequence. The tumor burden was subjectively graded by two observers on a scale ranging from 0 to 3. A score of 0 was used
when no tumors could be seen in the MRI. Small but obvious tumors (streaks, scarce nodules) were given a score of 1, whereas tumors engaging large parts of the parenchyma resulted in a score of 2. The score 3 represents tumors engaging and solidifying entire lobes or large parts of the entire lung, with visible air bronchograms or widespread opacification of the parenchyma (the so-called “crazy paving” phenomenon). Examples can be seen in figure 2A-D.

Quantification of tumor volume was also performed as described by Krupnick et al. (Krupnick et al., 2012). Briefly, this was done by making a montage of all images in the sequence and drawing a region of interest (ROI) outlining the lung in each slice. In case of MRI artifacts in the lung parenchyma, affected slices were discarded. All ROIs were measured using the built-in measurement tool in ImageJ, resulting in an area and average intensity value for each ROI. To compensate for variations in signal intensity between animals and sequences, ROIs were defined for reasonably homogenous parts of the liver parenchyma (avoiding large vessels and obvious hypo-signaling regions) and measured in the same way as the lungs. In case of pronounced signal fall-off, which occurs when the imaged tissue (in this case, the liver) is too far from the center of the coil, only the most rostral parts of the liver were sampled.

The relative tumor burden for each animal was subsequently measured by multiplying the average intensity and the area of each lung ROI, adding the results together and dividing by the sum of all areas, which produces the average image intensity for the entire lung. The same was performed on the liver ROIs, and the lung image intensity was divided by this value, resulting in a single value representing the average lung image intensity as a fraction of the liver.

In vitro
Cell lines H460 (human large-cell lung carcinoma, EGFR wild-type) and H1975 (human lung adenocarcinoma, carrying EGFR mutations L858R and T790), were cultured in serum-supplemented RPMI medium (RPMI 1640, Invitrogen, NY, USA). Both cell lines were stably transduced with the LRIG1 gene, along with the GFP gene, using the lentiviral vector pLVX-LRIG1-IRES-ZsGreen1 (Clontech Lenti-X, Clontech Laboratories, Mountain View, CA, USA). This way, cells transduced with LRIG1 will turn fluorescent. For negative control, the same cell lines were transduced with the vector pLVX-IRES-ZsGreen1, which contains the GFP gene but not LRIG1.

Following a period of incubation, FACS analyses were performed on the transduced cells at regular intervals for 11 days, and the proportion of fluorescent cells in each cell population was determined at each time point.

Statistical methods
Multivariate survival analysis for the TMA data was performed using the Cox regression method where the risk for death was adjusted for possible correlation with the known clinical prognostic
factors: performance status (PS 0 vs 1-4), clinical stage (1 vs 2-4), sex (male vs female), histology (adenocarcinoma vs others) and age (<70 yr vs >70 yr). For the univariate survival analysis of TMA data, we used the Kaplan-Meier survival analysis and log rank test was used for hypothesis testing. For Kaplan-Meier analysis, patients were stratified as for the multivariate analysis. For comparison of medians in the in vitro and in vivo experiments, the non-parametric Mann-Whitney test was used for hypothesis testing since normal distribution of data could not be assumed. All statistical analysis was made using the SPSS statistical package (IBM SPSS, IBM, Armonk, NY, USA). A p value <0.05 was considered significant.

Results

LRIG1 is a positive prognostic factor in human lung cancer

When reviewing the patient database, we found that the median survival time in the cohort was 1,522 days. Patients below the median age of 67 years had significantly better outcome (median survival 1,353 and 1,764 days, p=0.009); the same applied to asymptomatic patients compared to symptomatic (2,333 and 1,155 days, p<0.001) and stage I patients compared to stages II-IV (2,092 and 925 days, p<0.001).

The TMA review revealed that a considerable portion of the TMA material was negative for LRIG1 (figure 1B). This places the median LRIG1 score for the entire cohort very close to zero (0.08), making it a suboptimal choice as cut-off for assessing the difference between high and low LRIG1 immunoreactivity. For further analysis, the LRIG1-negative patients (i.e., with an LRIG1 score of 0) were treated as one group and the positive cases (i.e., LRIG1 score > 0) were divided into two groups based on the median LRIG1 score of the positive cases (0.66).

The Cox regression analysis was based on the risk of death in the three patient groups, with the LRIG1-negative group as baseline. Results from the analysis are shown in table 2. As can be seen in the table, there is a statistically significant lower risk of death for patients with high LRIG1 score, whereas the low-scoring group exhibits no difference in outcome compared to the negative cases (HR 0.62; 95% CI 0.45 – 0.86). Importantly, the survival benefit for patients with high LRIG1 score persists even when adjusting for clinical prognostic factors, meaning that high levels of LRIG1 is an independent positive prognostic factor for lung cancer.

As shown in figure 1C, univariate Kaplan-Meier survival analysis shows that patients in the group with high LRIG1 expression had a significantly better clinical outcome than the other two groups (p=0.012). The difference in median survival between this group and the negative cases was 991 days. As can be seen in the figure, the survival curve is almost identical between low-expressing and negative cases; the survival analysis also shows that there was no significant difference in survival between those two groups (p=0.847). An analysis stratified for histologic subtype reveals that the largest difference in clinical outcome is seen among adenocarcinomas, with a significant (p=0.014) difference in median survival of 1,334 days between high-expressing...
and negative cases (fig. 1D). Squamous cell carcinomas exhibit a non-significant ($p=0.516$) difference in median survival of 486 days (fig. 1E).

Notably, no variation in LRIG1 staining type was observed. All tumor cells in the analyzed material that stained positively for LRIG1 had a nuclear stain.

**LRIG1 may inhibit experimental lung cancer growth in vivo**

38 animals were included in the experiment, which was run in two series of, respectively, 14 and 24 mice during a period of nine months. 36 mice were alive until the second MRI, when they were sacrificed as planned. A total of 2 mice died between the first and the second MRI. An additional 17 mice (8 from a previous pilot study and 9 from an ongoing experiment) had undergone the first MRI at the specified time point, and could be included in the analysis. Of these, 2 died after the first MRI. As might be expected, all the mice that died had a high tumor burden at the first MRI. Notably, very few mice exhibited any symptoms of disease, even with high tumor burden. Symptomatic animals were generally lethargic, which was more common in LRIG1 knockouts.

For the semiquantitative data, a Mann-Whitney analysis shows a significant difference in tumor score for the MRI sequences obtained at the first time point, with a median score of 2 in the knockout group, compared to 1 in the wild-type group ($p=0.025$). However, the tumor burden had evened out at the second time point, with a non-significant difference in score distribution. This is illustrated in figure 3A and 3B.

The quantitative analysis also shows a tendency towards higher tumor burden in LRIG1 knockouts at the first time point, but the Mann-Whitney analysis shows that this does not reach a significant level ($p=0.095$). Likewise, the data from the second MRI shows no significant difference in tumor burden between knockout and wild-type mice. Results are shown in figure 3C and 3D.

**LRIG1 inhibits EGFR-driven lung cancer cell proliferation in vitro**

In vitro, the EGFR mutated H1975 lung cancer cell line exhibited a trend towards decrease in the LRIG1+GFP-transduced population as compared to the control population at (figure 4A). This implies that the LRIG1-overexpressing cells were outcompeted by the non-transduced population. For the EGFR wt large cell carcinoma cell line H460, a less prominent change could be observed with regard to the percentage of positive cells. Results can be seen in figure 4.

**Discussion**

In this article, we have shown that LRIG1 expression, as measured by IHC, correlates to survival in lung cancer, and also serves as an independent positive prognostic marker. When comparing tumor progression in a EGFR L858R-driven mouse model, we also noted a trend towards higher tumor burden in LRIG1 knockouts, albeit with limited statistical significance. Finally, we
observed a lower proliferation rate in lung cancer cell lines when LRIG1 is overexpressed. Taken together, these findings implicate that LRIG1 is of biological importance in lung cancer.

Considering its biological role as an endogenous inhibitor of ErbB-family proteins, and that EGFR and other stimulatory members of that family are upregulated in lung cancer, it is not an unexpected finding that there is a correlation between higher levels of LRIG1 in tissue and a better clinical outcome. The strongest connection was seen in adenocarcinoma, which is a tumor type where EGFR amplification and upregulation is a well-known component of tumorigenesis (Moran, 2011). This supports the hypothesis that LRIG1 in non-small cell lung cancer may act as a tumor suppressor, and that part of the tumor-suppressive mechanism may be inhibition of EGFR driven tumor progression.

The results from our IHC analysis is also in line with findings in other tumor types with significant involvement of ErbB driven proliferation, such as renal cell carcinoma (Thomasson et al., 2003), skin cancer (Tanemura et al., 2005), breast cancer (Krig et al., 2011) and glioma (Ye et al., 2009). However, the prognostically significant variation in staining pattern (i.e., subcellular location) for LRIG1 that has been observed in glioma (Guo et al., 2006) and ependymoma (Yi et al., 2009), and the similar variations in staining pattern that have been observed for other LRIG proteins (Holmlund et al., 2009), does not seem to apply to lung cancer, as the immunostain is uniformly nuclear in the studied material. The latter finding is interesting and unexpected, considering that LRIG1 is primarily known as a transmembrane protein. It should also be noted that our samples of normal bronchial epithelium also exhibit a nuclear LRIG1 immunostain. This may suggest that LRIG1 interacts with a nuclear protein in both normal and malignant lung tissue; for instance, it would be reasonable to hypothesize that there is an interaction between LRIG1 and a transcription factor. Further studies will, hopefully, shed more light on this topic.

In the animal model, a non-significant difference in tumor progression was noted, seemingly favoring mice with wild-type LRIG1. Notably, the largest difference can be seen at 60 days, and the only statistically significant results are from the semiquantitative analysis. The difference between semi-quantitative and quantitative analysis may reflect that the tumor burden in this model is harder than others to accurately quantify, as the tumors are diffusely infiltrating the entire parenchyma, requiring that the signal level in the entire lung is measured. Lung MRI is frequently affected by artifacts, and since normal lung parenchyma has an extremely low signal level, this will result in false positives. Semi-quantitative analysis of tumor growth may be a more accurate way to assess tumor growth in this particular animal model.

Despite quantification issues, it remains clear that LRIG1 knockout only has a marginal effect on tumor progression in the mouse model. This was somewhat unexpected, considering the outcome from the TMA study. However, it should be pointed out that the L858R mutation results in a constitutive EGFR activity, making it a highly potent driver of tumorigenesis. One possible explanation is that LRIG1 is less potent as an inhibitor of mutated EGFR compared to wt EGFR. In the clinical situation, mutated EGFR is only seen in 10% of caucasian patients and
overexpression of wt EGFR constitutes the majority of clinical cases with aberrant EGFR signaling. This may reflect the discrepancy between the TMA data and the data obtained in the animal model. It is also not known whether, and to what extent, LRIG1 interacts with L858R-mutated EGFR. Other explanations to the less potent inhibition of EGFR L858R driven lung cancer in vivo may be that the constitutively active receptor signaling is too strong to be downregulated by LRIG1. Further studies are much needed, and a reasonable way to proceed would be to transduce lung cancer cell lines with LRIG1 in order to establish transplantable mouse models. That would make it possible to study the effect of LRIG1 overexpression in vivo on adenocarcinomas with wild-type EGFR. Studies of the effect of LRIG1 inhibition of L858R mutated EGFR tumor initiation may also be done by sampling tumors at an earlier time point than 60 days as done in the present study.

The in vitro data shows a difference in cell proliferation between controls and cells transduced to overexpress LRIG1, with a decreased proliferation rate in the latter population. A trend towards continuous decrease was seen in the adenocarcinoma-derived H1975 cell line, which harbors the L858R mutation in cis with the TKI resistance-conferring T790M mutation, whereas no clear trend could be observed for the large cell carcinoma-derived H460 line. These findings are well in line with earlier observations on human glioma (Ye et al., 2009) and breast cancer (Laederich, 2004; Miller et al., 2008) cell lines, which all showed decreased proliferation and increased apoptosis in LRIG1 over-expressing cells. As Laederich et al. show in their article, the effect is specifically related to inhibition of ErbB-driven proliferation, which is an important part in the growth signaling of human NSCLC as well.

Even though it falls outside the scope of this article, one question that may arise is whether these findings may have therapeutic implications. One unfortunate consequence of the increased clinical use of EGFR-targeting TKIs, such as gefitinib and erlotinib, is that the treatment causes selection pressure for TKI resistance mechanisms. As shown in the pooled analysis by Costa et al. (Costa et al., 2007), this is by no means a small problem, as the mean progression-free survival for EGFR-mutated patients on gefitinib therapy is as low as 7 to 12 months. The previously-mentioned T790M EGFR mutation is one important way for lung cancer cells to achieve resistance; another is a "kinase switch" mechanism, where the EGFR inhibition is countered by an acquired Met amplification (Bean et al., 2007; Engelman et al., 2007), resulting in decreased dependence on EGFR signaling. Interestingly, although the most studied mechanism of action of LRIG1 is an interaction in cis within the cell membrane, the inhibitory effect is not limited to the expressing cells themselves. The extracellular domain has been shown to interact in trans with EGFR on neighboring cells, also resulting in signal inhibition. As this domain is cleaved off into a soluble ectodomain as well, it inhibits cell growth in a paracrine fashion (Goldoni et al., 2006; Yi et al., 2011), making it a possible candidate for future therapy. Considering that LRIG1 is an endogenous inhibitor of both EGFR and Met, this could be of particular value in TKI-resistant lung cancer.
In summary, we have shown that high expression of LRIG1 in tissue correlates with improved survival and that LRIG1 inhibits growth of lung cancer cells in vitro. Also, in vivo data from an L858R-driven mouse model shows a tendency towards faster tumor progression in absence of LRIG1. Taken together, these findings lead us to conclude that LRIG1 is of biological significance in non-small cell lung cancer.

Acknowledgements

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References


Table 1. Basic characteristics for the TMA patient cohort.

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<td>I</td>
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<td>II</td>
<td>58</td>
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<td>51</td>
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<td>IV</td>
<td>11</td>
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### Table 2. Multivariate analysis of the TMA material. The risk of death \( \text{Exp}(B) \) is calculated for high and low LRIG1 score, with the negative cases as baseline, and adjusted for co-variability with known clinical prognostic factors.

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<th></th>
<th>B</th>
<th>SE</th>
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<td>16.466</td>
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<td>1.711</td>
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Legends to figures

**Figure 1:** Correlations between LRIG1 immunoreactivity and patient survival.
(A) Examples of LRIG1 staining intensity. From left to right, intensity score 0, 1, 2 and 3.
(B) Histogram showing the distribution of LRIG1 score in the studied material (median 0.08).
(C) Kaplan-Meier survival chart showing better survival in the group with high LRIG1 levels compared to the others (p=0.012).
(D) Survival charts showing that the survival benefit of high LRIG1 levels is the largest in adenocarcinoma (p=0.014).
(E) Similar chart showing a non-significant difference in squamous cell carcinoma.

**Figure 2:** Examples of different tumor burdens in the mouse model.
(A) Tumor-free lungs; score 0 in the semiquantitative analysis.
(B) Lungs with small tumors; score 1.
(C) Intermediate tumor burden; score 2.
(D) High tumor burden with solidification of large parts of the lung. Score 3.

**Figure 3:** Semiquantitative and quantitative analysis of tumor burden in the mouse model.
(A) Semiquantitative analysis results showing a significant (p=0.025) difference in tumor burden between LRIG1 knockouts and wild-types at day 60.
(B) Semiquantitative analysis of the tumor burden at day 95. No significant difference between knockouts and wild-type mice.
(C) Quantitative analysis showing a non-significant (p=0.095) difference in tumor burden at day 60.
(D) Quantitative analysis at day 95, with no difference in tumor burden between the two groups.

**Figure 4:** FACS analyses of LRIG1-overexpressing lung cancer cells.
(A) Chart showing progressive reduction in the percentage of LRIG1-transduced H1975 cells, as compared to the non-transduced population, from day 1 to 11. LRIG2-transduced cells included for comparison.
(B) Similar chart showing no obvious trend in H460 cells, from day 1 to day 11.
Figure 3

A

B

C

D

Genotype

Error Bars: 95% CI

Mean Score (MRI 1)

Genotype

Error Bars: 95% CI

Mean Score (MRI 2)

Genotype

Error Bars: 95% CI

Mean Score (MRI 1, 2)

Genotype

Error Bars: 95% CI

Mean Score (MRI 1, 2)
Figure 4

A

B