LRIG1 Affects Clonogenicity but Not Metastatic Potential in Non–small Cell Lung Cancer Cell Lines

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Research article

Keywords: leucine-rich repeats and immunoglobulin-like domains protein 1 (LRIG1), chemosensitivity, radiosensitivity, clonogenicity

DOI: https://doi.org/10.21203/rs.3.rs-98526/v1

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Abstract

Background. High levels of the leucine-rich repeats and immunoglobulin-like domains protein 1 (LRIG1) in tumor tissue are associated with a survival benefit in early-stage non–small cell lung cancer (NSCLC) due to presently unknown mechanisms.

Methods. A panel of NSCLC cell lines was transduced with LRIG1 expression vectors. Cell proliferation, chemosensitivity, radiosensitivity, clonogenicity, and migration were measured in vitro. Mice were implanted with mixed-cell populations, and the fraction of LRIG1-overexpressing cells was compared among metastatic sites, primary tumors, and injected cell populations.

Results. Clonogenicity was reduced in LRIG1-overexpressing cell lines. Minor or no changes were observed in the other analyzed functions. LRIG1 was neither enriched nor depleted in tumor cell populations at different metastatic sites.

Conclusion. LRIG1 reduced clonogenicity in vitro, but no other single underlying mechanism for LRIG1 tumor suppression in NSCLC was identified. Cell lines established from advanced NSCLC might not be a suitable model for mechanistic studies of early-stage disease.

Background

Lung cancer is the most common cause of cancer-related deaths worldwide (1, 2). Current lung cancer treatment is mainly based on clinical stage. For advanced disease, predictive biomarkers are used, but so far no prognostic or predictive markers are in routine clinical use for early-stage disease (3–6). Currently, postoperative adjuvant chemotherapy is recommended to patients based on stage only. New molecular markers to guide clinical decision making in early-stage disease are therefore needed. Leucine-rich repeats and immunoglobulin-like domains protein 1 (LRIG1) is a newly described prognostic marker in early-stage non–small cell lung cancer (NSCLC) (7). However, it is not known if and how LRIG1 influences the course of NSCLC or if LRIG1 expression is only a reflection of other important traits of the disease.

The LRIG family of proteins consists of three paralogs: LRIG1, LRIG2, and LRIG3. LRIG1 is the most well-characterized of the three family members, and it acts as an endogenous inhibitor of multiple receptor tyrosine kinases (RTKs). Inhibitory effects from LRIG1 have been observed on ErbBs 1–4 (including EGFR) (8, 9), RET (10), MET (11), PDGFRα (12), and AXL (13). The expression of LRIG1 in tumor tissue is associated with better survival in several human cancers, including carcinoma of the breast (14), uterine cervix (15, 16), bladder (17), prostate (18), melanoma (17), oligodendroglioma (17), cutaneous squamous cell carcinoma (19), and NSCLC. In NSCLC, high levels of LRIG1 in tumor tissue confer a large survival benefit in early-stage lung adenocarcinoma (7, 20), especially in tumors with low levels of the LRIG1-interacting protein LIM domain only protein 7 (LMO7) (21). The survival benefit of LRIG1 in NSCLC appears to be limited to early clinical stages, for which adjuvant chemotherapy is offered in order to prevent the development of metastatic disease. Therefore, it is reasonable to hypothesize that LRIG1 sensitizes cells to chemotherapy, which is in line with previous findings showing that ectopic LRIG1 expression confers increased sensitivity to cisplatin and vinorelbine in cells (22–25). Alternatively, LRIG1 might exert an inhibitory effect on the steps involved in the metastatic process. The metastatic process is incompletely characterized and involves multiple steps, from epithelial-to-mesenchymal transition, invasion into surrounding tissue and out into blood vessels, survival in circulation, extravasation, and establishment of a metastatic niche in a target organ (26–28). In this respect, in cancer cells in vitro LRIG1 has been shown to suppress both the epithelial-to-mesenchymal transition and invasion (11, 25, 29, 30).

In the present study, we analyzed possible effects of LRIG1 overexpression on hallmark features in NSCLC cells using in vitro assays of cell proliferation, chemosensitivity, radiosensitivity, clonogenicity, and migration and by developing an in vivo mouse model of hematogenous micrometastatic disease.

Methods

Expression vectors

The bicistronic lentiviral expression vectors pLVX-IRES-ZsGreen1 and pLVX-IRES-mCherry were obtained from Takara Bio (Kyoto, Japan) and used to assemble vectors pLVX-LRIG1-IRES-ZsGreen1 and pLVX-LRIG1-IRES-mCherry. A human full-length LRIG1 cDNA (Gene bank accession no. AF381545) was PCR amplified using CloneAmp HiFi premix (Takara Bio) with forward and reverse primers, both containing 15 bases that are complementary with the p3xFLAG-CMV-13 vector (Sigma-Aldrich, St. Louis, MO, USA) at their 5' ends. All PCR primer and probe sequences are listed in supplemental Table 1. The p3xFLAG-CMV-13 vector was linearized using FastDigest HindIII and EcoRV restriction enzymes (Fermentas Sweden AB, Fisher Scientific, Gothenburg, Sweden) run on agarose gel, excised and eluted from the gel using an EZNA Gel Extraction Kit (Omega Bio-Tek, Norcross, GA, USA). The LRIG1 amplicon was cloned into the linearized p3xFLAG-CMV-13 vector in a seamless Golden Gate–type assembly, using an In-Fusion HD EcoDry kit (Takara Bio) as per the manufacturer's instructions, using a 2:1 molar ratio for the insert and vector, followed by transformation of NEB 5-alpha competent E. coli (Takara Bio) and plasmid preparation. From this plasmid, the FLAG-tagged full-length human LRIG1 was PCR amplified using forward and reverse primers that contained 15 base sequences that were identical to the intended fusion sites in the final bicistronic recipient plasmids pLVX-IRES-ZsGreen1 and pLVX-IRES-mCherry. Using the same methods as above, the plasmids were linearized using NdeI and BamHI restriction enzymes (Fermentas Sweden AB), then purified and co-incubated with the LRIG1-containing amplicon in separate In-Fusion reactions. Sequencing primers were designed to cover the reference sequence of LRIG1 and 200 bp 5’ and 3’ of the predicted insertion sites of both expression vectors (here referred to as pLVX-LRIG1-IRES-ZsGreen1 and pLVX-LRIG1-IRES-mCherry), and the purified vectors were sequenced to verify that the sequences and insertion sites were correct.

Cell culture and lentiviral transduction

The NSCLC cell lines H1975, H1299, HCC827, H520, and A549 were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and 50 µg/ml gentamicin. H1975DoxLRIG1, HCC827DoxLRIG1, H520DoxLRIG1, and
A549\textsuperscript{DoxLRIG1} cells with doxycycline-inducible LRIG1 expression were generated by transducing the parental cell lines with pLVX-LRIG1-TRE3G and pLVX-Tet3G, as previously described (31).

Lentiviral particles carrying pLVX-LRIG1-IREZsGreen1, pLVX-IREZsGreen1, pLVX-LRIG1-IREZmCherry, and pLVX-IREZmCherry were generated by transfecting Lenti-X cells (Takara Bio) with the vectors as per the manufacturer's instructions. H1299 and H1975 cells were stably transduced with all four of the above-mentioned lentiviral particles by incubation with 700 µl of fresh culture medium, 1 µl of 6 mg/ml polybrene solution, and 300 µl of viral supernatant in 12-well culture plates, followed by centrifugation at 800 x g for 1 hour at room temperature. To control for artifacts due to clonal variation, all transductions were carried out in duplicates and kept separate from each other, resulting in two separate cell line variants for each combination of cell line and viral vector. Transduced cells were isolated by two sequential fluorescence-activated cell sortings on a FACS Aria cell sorter (BD Biosciences, East Rutherford, NJ, USA) using a 488 nm laser with a 510/21 filter (for ZsGreen1) or a 550 nm laser with a 585/42 filter (for mCherry). The 16 resulting cell line variants were verified to be pathogen-free at IDEXX BioAnalytics (Ludwigsburg, Germany). The H1975\textsuperscript{DoxLRIG1}, HCC829\textsuperscript{DoxLRIG1}, HS20\textsuperscript{DoxLRIG1}, and A549\textsuperscript{DoxLRIG1} cell lines as well as one of the H1975-LRIG1-IREZmCherry cell lines and one of the H1299-LRIG1-IREZmCherry cell lines were authenticated with regard to their original parental cell lines through short tandem repeat profiling via IDEXX BioAnalytics.

**Western blotting**

Cells were cultured to 90% confluency in 30 mm wells and then lysed using 250 µl of cell extraction buffer (Thermo Fisher, Waltham, MA, USA) with added Complete EDTA-free protease inhibitor cocktail (Roche, Basel, Switzerland) for 30 minutes at 4 °C, then centrifuged at 20,000x g for 10 minutes. Protein concentrations in the cleared lysates were determined using a Pierce BCA assay kit (Thermo Fisher) by measuring linear absorbance at 562 nm on a NanoDrop spectrophotometer (Thermo Fisher) and comparing to a pre-diluted albumin standard as per the manufacturer's instructions. Prior to analysis, lysates were diluted in lysis buffer to the same protein concentrations, then denatured through the addition of LDS buffer and reducing agent (Thermo Fisher), followed by incubation at 70 °C for 10 minutes. The proteins were separated via gel electrophoresis using a 3–8% Tris-Acetate PAGE gel (Thermo Fisher), followed by semi-dry transfer to an LF PVDF membrane (Bio-Rad, Berkeley, CA, USA) using a TurboTransfer machine (Bio-Rad). Membranes were blocked using Odyssey PBS blocking buffer (Li-Cor, Lincoln, NE, USA). The primary antibodies used, and their dilutions, were mouse anti-actin, 1:7,500 (Abcam, Cambridge, UK), and rabbit anti-LRIG1, 1:1,000 (Agnisera, Vännäs, Sweden). The secondary antibodies used, and their dilutions, were goat anti-rabbit IgG IRDye 680 and goat anti-mouse IRDye 800 (Li-Cor), both at 1:15,000. Membranes were incubated with the respective antibodies for 1 hour at room temperature and washed using TBST (20 mM Tris, 150 mM NaCl and 0.1% Tween 20) in a Freedom Rocker BlotBot (Next Advance, Troy, NY, USA), then scanned at 700 and 900 nm using an Odyssey imager (Li-Cor) at 84 µm resolution.

**Live-cell proliferation and migration assays**

For all live-cell assays, cells were seeded at a density of 10,000 cells per cm² in either standard 6-well or Sarstedt Lumox 24-well plates. To induce LRIG1 expression in LRIG1-inducible cells, 1 µg/ml of doxycycline was added. Plates were incubated overnight. Prior to recording, wells were washed once in PBS to remove debris, resupplemented with standard cell culture medium, covered with HoloLids (Phase Holographic, Lund, Sweden), and inserted in a HoloMonitor holographic microscope (Phase Holographic) mounted inside a standard cell culture incubator. Live-cell imaging was performed on 16 predefined 0.52 × 0.52 mm areas per well, which were continuously recorded at 8-minute intervals for 48 hours. Proliferation curves were created by measuring the cell count in each area at 1-hour intervals, and doubling time was calculated by comparing cell counts at \( t = 0\) and \( t = 48\) hours. Cell migration, measured as average cell motility in µm/hour, was calculated by tracking individual cell movements on a frame by frame basis.

**Competitive cell proliferation assay**

For competitive cell proliferation, H1299 control - mCherry and H1299 LRIG1 - mCherry cells with H1299 control - ZsGreen1, both in a 1:1 fashion, using three separately transduced clones of each vector. Tubes containing cell suspensions were subsequently labeled in a blinded fashion, and cells were plated onto 6-well tissue culture plates (Sarstedt AG, Nümbrecht, Germany) supplied with a standard medium containing 10% FBS. The plates were incubated for up to 18 days, with replacement of the growth medium when needed. Cells were lysed at 0, 3, 8, 11, 15, and 18 days using a DNEasy blood and tissue kit (Qiagen, Venlo, Netherlands), and the ratio of LRIG1 to control vector gene copies was determined using digital droplet PCR (ddPCR) as described in the section below.

**Chemosensitivity assay**

To analyze chemosensitivity, four separately transduced LRIG1 over-expressing H1299 or H1975 cell lines and four separately transduced vector control H1299 or H1975 cell lines were plated in triplicates onto 96-well plates with 4,000 cells per well and incubated overnight. A cell culture medium was prepared followed by semi-dry transfer to an LF PVDF membrane (Bio-Rad, Berkeley, CA, USA) using a TurboTransfer machine (Bio-Rad). Membranes were blocked using Odyssey PBS blocking buffer (Li-Cor, Lincoln, NE, USA). The primary antibodies used, and their dilutions, were mouse anti-actin, 1:7,500 (Abcam, Cambridge, UK), and rabbit anti-LRIG1, 1:1,000 (Agnisera, Vännäs, Sweden). The secondary antibodies used, and their dilutions, were goat anti-rabbit IgG IRDye 680 and goat anti-mouse IRDye 800 (Li-Cor), both at 1:15,000. Membranes were incubated with the respective antibodies for 1 hour at room temperature and washed using TBST (20 mM Tris, 150 mM NaCl and 0.1% Tween 20) in a Freedom Rocker BlotBot (Next Advance, Troy, NY, USA), then scanned at 700 and 900 nm using an Odyssey imager (Li-Cor) at 84 µm resolution.

**Clonogenic assay**

To analyze clonogenicity, cells were detached using trypsin, resuspended in culture medium, then counted, diluted, and plated onto 6-well tissue culture plates at a density of 100 cells/cm². Stably transduced cells were plated in triplicates. LRIG1-inducible cells were plated onto all six wells, with 1 µg/ml doxycycline added to three wells. Once macroscopically visible colonies had formed, cells were fixed in methanol at −20 °C for 10 minutes and then stained using 0.5%...
crystal violet solution. Plates were dried and scanned in a GelDoc imager (Bio-Rad) in transillumination mode. Colonies were automatically counted in a blinded fashion using an ImageJ (34) macro [Additional file 1].

**Radiosensitivity assay**

To analyze radiosensitivity, cells were plated in 6-well tissue culture plates and grown to approximately 75% confluency. The plates were then irradiated to an absorbed dose of 1, 2, 4, 6, 8, or 10 Gy using a Clinac IX photon beam linear accelerator (Varian, Palo Alto, CA, USA). In order to avoid beam dispersion due to air gaps, each cell culture well was filled to the brim with culture medium and encased in rigid Perspex bolus blocks during treatment. Clonogenic assays were performed immediately after the radiation treatment. A clonogenic assay was performed on the treated cells using the same method as above, but with 300 cells per cm² seeded in triplicates for each radiation dose.

**Transwell and wound closure assays**

For the transwell migration assay, cells were plated and serum-starved in 0.1% FBS for 24 hours. PET membrane tissue culture inserts with a 4 µm pore size (Sarstedt AG) were placed on a 24-well plate. The cell culture wells were filled with medium with 10% FBS, while the inserts were filled with serum-free medium along with 5,000 cells. Each of the four cell line variants was plated in triplicates. Plates with inserts were put in a cell culture incubator for 12 hours, after which the inside of each membrane was swabbed and washed to remove stationary cells. Membranes with adherent migrated cells were subsequently fixated with methanol for 10 minutes at ~20 °C and stained using Meyer’s hematoxylin and labeled in a blinded fashion. Cells were counted manually.

For the wound closure assay, the cells were plated into 6-well tissue culture plates and grown to a 75% confluent monolayer, after which a cross-shaped wound was made by scraping a plastic pipette tip across the surface. Wells were washed twice in PBS to remove detached cells. Fresh medium with 10% FBS was added, and the plates were kept in a cell culture incubator. At predefined time intervals, the wound was imaged using a Zeiss AxioCam ICC1 and an Axio A1 phase contrast microscope (Carl Zeiss AG, Oberkochen, Germany) with a 10x lens. The wound area was quantified in an automated blinded fashion using an ImageJ script (34) [Additional file 2]. Wound closure rates were calculated for each well using linear regression, and a ratio was calculated by dividing the closure rate of LRIG1 over-expressing cells with the closure rate of the vector controls.

**Mouse strains and animal husbandry**

Female CIEA NOG mice (Taconic, Ejby, Denmark) were kept in individually filtered ventilated racks in a controlled environment with a constant 27 °C temperature and a 12-hour light cycle. Mice were fed standard chow and watched daily for symptoms. All animal experiments were performed according to the Swedish animal welfare law and with ethical approval from the regional animal welfare board (DNR A 28–15).

**Orthotopic implantation and tissue analysis**

For an initial investigation of the metastatic potential of a series of NSCLC cell lines, A549, H1299, H1975, and HCC827 cells were trypsinized and counted, then pelleted and resuspended on ice in RPMI 1640 medium with 20% added soluble matrix (Geltrex, Thermo Fisher) to a concentration of 2 x 10⁶ cells/ml (A549 and H1299) or 1 x 10⁷ cells/ml (H1975 and HCC827), of which individual aliquots of 50 µl (1 x 10⁵ cells or 5 x 10⁵ cells, respectively) were prepared for each animal. Cell suspensions were kept on ice until implantation to avoid matrix solidification. Animals were anesthetized using 5% isoflurane (Baxter, Deerfield, IL) in pure oxygen, and an ultrasound probe (Fujifilm VisualSonics, Toronto, ON, Canada) was used to identify the dorsal intercostal spaces. Cell suspensions were gently vortexed and stereotaxically injected into the left lung using a 16G needle through the sixth left intercostal space to a depth of 2 mm, with ultrasonographic confirmation that the injected suspension was inside the parenchyma. After 4 weeks, animals were anesthetized using 5% isoflurane, and a Bruker Bio-Spec 94/20 USR small animal MRI was used to obtain respiration-gated T1 FLASH sequences with the following parameters: TR = 113 ms, TE = 2.8 ms, flip angle = 30 degrees, field of view = 30 mm, matrix = 256 x 256 and 16 averages. Fifteen axial slices were obtained with 0.50 mm slice thickness and 1.00 mm interslice distance. A 40 mm quadrupolar volume coil was used in transmit/receive mode. Mice were euthanized immediately after imaging. For each animal, the left and right lung, brain, liver, and any other visceral organ with macroscopically visible metastases were fixated in 4% paraformaldehyde, paraffin-embedded, sectioned, and H&E stained.

For the second experiment, pooled cell suspensions were prepared in triplicates in a 1:1 fashion, with separate H1299 LRIG1-ZsGreen clones pooled with H1299 control-mCherry cells, and inversely, with H1299 LRIG1-mCherry cells pooled with H1299 control-ZsGreen cells. Cell suspensions were then injected as a primary tumor in the left lung using the method described above, in a double-blinded fashion, with 0.5 x 10⁶ cells per animal. A 500 µl sample of each injected cell suspension was kept at −20 °C in order to compare the composition of the tumors with that of the injected cells. Animals were euthanized at the first sign of symptoms, or after 4 weeks if they did not develop symptoms. For each animal, the left lung was longitudinally cut through the middle of the primary tumor, and the four lobes of the right lung were separated. Representative samples were taken from the liver and mediastinal lymph nodes if macroscopic metastases were visible. Genomic DNA was isolated from the stored cell suspensions, half of the left lung, two of the right lobes (middle and postcaval), and representative samples of micrometastases, using a DNEasy blood and tissue kit (Qiagen). The other half of the left lung, the superior lobe of the right lung, and representative samples of micrometastases were fixated in 4% paraformaldehyde, paraffin-embedded, sectioned, and H&E stained.

**Digital droplet PCR**

To quantify the numbers of integrated ZsGreen1 and mCherry gene copies or Lrig1, used as a mouse genomic reference, ddPCR was used. The primer and probe sequences used are summarized in supplemental Table 1. Genomic DNA samples were restriction digested using KpnI with added 10x FastDigest buffer (Fermentas Sweden AB) at 37 °C for 60 minutes, followed by heat inactivation at 80 °C for 3 minutes. PCR reactions were prepared using 900 nM of forward and reverse primers for gene 1 (mCherry or ZsGreen1) and gene 2 (ZsGreen1 or Lrig1), 250 nM of FAM probe for gene 1, 250 nM of HEX probe for gene 2, 10 µl ddPCR supermix without dUTP (Bio-Rad), 240 ng of sample DNA, and nuclease-free water, up to a total volume of 20 µl. Droplets were generated by adding 20 µl of PCR reaction and 70 µl droplet-generation oil into the corresponding wells in a droplet-generation cartridge (Bio-Rad), which was then processed in a QX1000 droplet generator (Bio-Rad) and transferred onto a PCR plate. Each plate was run for 10 min at 95 °C, followed by 40 cycles of 95 °C for 30 sec and
56 °C for 60 sec, then a final step at 98 °C for 10 min, then analyzed in a ddPCR droplet analyzer (Bio-Rad), with automatic identification of positive droplets in the FAM and HEX channels if possible, or else using manual thresholding at a signal amplitude of 1500. For each sample, a ratio was calculated by dividing the number of positive droplets corresponding to the LRIG1 overexpressing vector (in either the FAM or HEX channel) by the number of positive droplets corresponding to the control vector. For the animal tissue DNA samples from the left lung containing a primary tumor, the obtained ratio was normalized against the corresponding ratio in the injected cell suspension. For samples corresponding to metastatic sites, the LRIG1-to-vector control ratio was normalized against the primary tumor.

**Data analysis and statistics**

Statistical significance of paired populations at single time points, drug concentrations, or radiation doses was analyzed using the independent samples $t$-test. Comparisons against fixed values were done using the one-sample $t$-test. Comparisons between multiple populations or analysis of multiple repeated measurements were performed using the Wilcoxon signed-rank sum test. IC$50$ values were determined using logistic regression. For comparisons of data not normally distributed, the Mann-Whitney $U$-test was used. All statistical analyses were performed in Jamovi (35, 36). A $p$-value of < 0.05 was considered significant.

**Results**

**LRIG1 overexpression did not affect cell proliferation in most NSCLC cell lines in vitro**

To investigate whether LRIG1 affects the proliferation rates of NSCLC cells, LRIG1 was ectopically overexpressed in a series of NSCLC cell lines, followed by analyses of the proliferation rates of the wild-type and LRIG1-overexpressing cells. At no time point did the proliferation rates of the LRIG1 overexpressing H1299LRIG1-mCherry and H1975LRIG1-mCherry cell lines differ significantly from their vector control counterparts, H1299control-mCherry and H1975control-mCherry, respectively (Fig. 1A, Student's t-test, $p = 0.373$ and $p = 0.056$, respectively). Also, no significant differences could be observed between the cell populations based on fluorescent reporter ($p = 0.227$ for H1299 and $p = 0.547$ for H1975; data not shown), indicating that the fluorescent reporters themselves had no effect on the cell proliferation rates. In our additional panel of LRIG1-inducible NSCLC cells, the only significant difference in proliferation rate was observed for HCC827, in which LRIG1 overexpression was associated with a reduced proliferation rate (i.e., an increased population doubling time) ($p = 0.042$). Again, a non-significant trend towards a slower proliferation rate for LRIG1-overexpressing H1975 cells was observed ($p = 0.057$). The proliferation rates in A549 and H520 were unaffected by the induction of LRIG1 expression ($p = 0.875$ and $p = 0.267$, respectively).

In a competitive proliferation assay, no statistically significant changes were observed in the relative proportion of LRIG1 overexpressing cells when co-cultured with control and cells grown past confluency in a dense monolayer. At 18 days, the relative proportion of LRIG1-overexpressing cells had seemingly decreased, but not significantly so (Fig. 1B, Student's t-test, $p = 0.352$).

Taken together, the cell proliferation experiments showed that changes in proliferation rate in LRIG1-overexpressing cells were only minor, and overexpressing cells were not outcompeted by vector controls in a mixed-cell population.

**NSCLC chemosensitivity was unaffected by LRIG1 overexpression in vitro**

To see if ectopic overexpression of LRIG1 affected the chemosensitivity in NSCLC cell lines, cytotoxic assays were performed on H1299 and H1975 cells treated with cisplatin or vinorelbine. Cisplatin-induced cytostasis and cell death occurred at similar concentrations for H1299 and H1975, with no significant differences between vector controls and LRIG1 overexpressing cells. For H1299, mean IC$_{50}$ was 34.9 µmol/l for vector controls and 35.8 µmol/l for LRIG1-overexpressing cells. The difference was not significant (Fig. 2A, Mann-Whitney U-test, $p = 0.984$). Mean IC$_{50}$ for H1975 vector controls was 24.0 µmol/l, compared to 34.1 for LRIG1-overexpressing cells, but the difference was not significant ($p = 0.060$).

Vinorelbine potently induced cytostasis for both H1299 and H1975 cells at extremely low concentrations but failed to induce cytotoxicity, as cells remained alive but non-replicating, even at high drug concentrations; therefore, IC$_{50}$ could not be reliably determined. When comparing vector controls with LRIG1-overexpressing cells, no significant curve separation was seen for H1299 (Fig. 2B, Wilcoxon rank-sum test, $p = 0.298$) or H1975 ($p = 0.152$).

Thus, no changes in cisplatin or vinorelbine chemosensitivity were observed when LRIG1 was overexpressed in H1299 and H1975 cells.

**LRIG1 overexpression did not affect radiosensitivity but affected clonogenicity**

To determine whether ectopic LRIG1 overexpression affected the radiosensitivity of H1299 and H1975 cells, a clonogenic assay was performed. H1299 vector control cells had significantly reduced clonogenicity after treatment with radiation doses of 4 Gy or higher (paired samples $t$-test, $p = 0.041$, Fig. 2C), whereas H1975 cells had significantly reduced clonogenicity from 2 Gy onwards ($p = 0.003$, Fig. 2D). For both H1299 and H1975, LRIG1-overexpressing cells did not exhibit any significant differences in clonogenicity compared to vector control cells at any radiation dose, indicating that LRIG1 overexpression did not significantly affect the radiosensitivity in these cells.

A separate experiment was performed to measure the effects of LRIG1 overexpression on the baseline colony-forming ability of more sparsely plated non-irradiated NSCLC cells. This experiment showed a general decrease in clonogenicity in LRIG1-overexpressing cells (Fig. 2E). Significant decreases in the number of colonies were observed for LRIG1-overexpressing H1299 (Student's t-test, $p < 0.001$), H1975 ($p = 0.033$), and HCC827 ($p = 0.041$), but not for A549 ($p = 0.872$) or H520 ($p = 0.467$).

**Cell migration was mostly unaffected by LRIG1 overexpression**
To investigate the effects of ectopic LRIG1 overexpression on cell migration, we performed long-term holographic live-cell imaging, as well as wound closure assays and standard transwell migration assays, using a panel of NSCLC cell lines. Under unstimulated conditions, with live-imaged cells, a significant increase in motility rate was observed for LRIG1-overexpressing H1299, A549\(\text{TetLRIG1}\), H520\(\text{TetLRIG1}\), and HCC827\(\text{TetLRIG1}\) cells, while a significant decrease in motility rate was observed for LRIG1-overexpressing H1975\(\text{TetLRIG1}\) cells. However, the differences in motility were, in general, only minor (Fig. 3A).

In the wound closure assay, LRIG1-overexpressing H1299 cells closed the wound area slightly faster than control cells, a difference that was nevertheless significant overall and with significant differences at all time points (Fig. 3B and C). When calculating the relative wound closure rate, the LRIG1-overexpressing cells bridged the area at a rate of 4.16% per hour, compared to 3.57% per hour for control cells (Mann-Whitney \(U\) test, \(p = 0.029\)). For H1975, the LRIG1-overexpressing cells bridged the gap at a rate similar to that of the control cells, with a relative closure rate 2.28% vs. 2.21% per hour (Mann-Whitney \(U\) test, \(p = 0.857\)).

In the transwell migration experiments, no statistically significant difference in migrated cells could be observed between LRIG1-overexpressing H1299 cells or H1975 cells compared to their respective vector control cells (Student's \(t\) test, \(p = 0.264\) and \(p = 0.356\), respectively) (Fig. 3D). No significant differences in cell migration were observed when evaluating the effect of fluorescent reporters on either cell line (\(p = 0.109\) and 0.694, respectively).

Taken together, LRIG1 overexpression affected the cell motility of all cell lines except one of the H1975 cell lines. However, the effects of LRIG1 on cell motility and migration were not consistent—both positive and negative—and were generally small.

**Orthotopically implanted H1299 cells gave rise to hematogenous micrometastases**

To be able to assess the influence of LRIG1 on NSCLC metastases in vivo, we first screened four cell lines (A549, H1975, HCC827, and H1299) for metastatic potential when implanted orthotopically in immunodeficient mice (Supplemental Table 2). Mice injected with A549 cells remained asymptomatic, and no tumor cells could be observed in tissue sections of either the implantation site of the left lung or in any other organ. For HCC827, mice remained asymptomatic, with normal behavior throughout the observation period; however, extensive tumor growth was observed at the implantation site. No metastases were observed in any other parenchymal organ. Mice injected with H1975 became symptomatic starting at week 3 of observation, with tachypnoea and visible cyanosis in the tail. MRI sequences showed extensive bilateral pleural effusion and pathologically enlarged mediastinal lymph nodes. Histopathologic evaluation showed tumor growth at the implantation site and bilateral nodes on the visceral pleura. One mouse had a macroscopically visible metastasis in the tail of the pancreas. For mice injected with H1299, symptomatology and development over time were identical to the H1975 mice, and the MRI evaluation also showed pleural effusion, as well as enlarged mediastinal lymph nodes (Fig. 4A). Injection sites showed tumor growth, and both lungs on most animals exhibited hematogenous micrometastases in the form of numerous spindle-shaped cell clusters embedded in the alveolar walls, deep in the lung parenchyma (Fig. 4B). Additionally, one mouse had a macroscopically visible subcutaneous metastasis on the thoracic wall, contralateral to the injection site.

**LRIG1-overexpressing H1299 cells had a competitive advantage when orthotopically implanted**

Finally, we sought to investigate whether LRIG1 overexpression conferred a selective disadvantage or advantage on H1299 cells at the primary tumor implantation site or in the metastatic process in vivo. This was assessed by injecting a pooled population of H1299 cells, with a 1:1 ratio between LRIG1-overexpressing cells and vector control cells, orthotopically into the left lung of immunocompromised mice and then comparing the ratio between LRIG1 vectors and control vectors in tumor DNA prepared from the injected cell populations, primary tumors, and metastatic sites. After implantation, 21 of 24 injected mice became symptomatic starting at week 3 of observation. Symptoms were the same as the initial experiment, except for one mouse that presented with flaccid paresis of the hind legs, later found to be due to spinal cord overgrowth from the primary tumor at the mid-thoracic level. In total, 18 left lungs with macroscopically visible primary tumors, 18 right lungs with detectable micrometastases, 5 livers with macroscopically visible metastases, and 3 intact mediastinal lymph node metastases were included in our analysis.

When comparing the ratio of LRIG1 to control vector, we found an enrichment of LRIG1 in primary tumors compared to the ratio in the injected cell suspension, indicating that the cell population with LRIG1 overexpression had a competitive advantage over the control population in establishing a primary tumor after injection. Regarding our primary outcome, the composition of hematogenous micrometastases in the right lung, no significant trends could be observed when comparing the LRIG1-to-control vector ratio between the micrometastases and their corresponding primary tumors (Fig. 4C).

**Discussion**

In this study we investigated possible mechanisms that could account for the observed association between high LRIG1 expression and increased survival among early-stage NSCLC patients. To this end, we overexpressed LRIG1 in a series of NSCLC cell lines and monitored possible effects on cell proliferation, sensitivity to chemotherapy and radiotherapy, and colony formation and migration, as well as the cellular competitiveness in an in vivo model of NSCLC metastasis. Table 1 provides an overview of the results for all cell lines. Overall, the only consistent effect of LRIG1 that we could detect was reduced colony-formation efficiency among the LRIG1-overexpressing cells. This could imply that LRIG1-expressing circulating tumor cells have a decreased ability to form viable metastases (colonies) in the metastatic niche, which clearly warrants further investigation.
In summary, although our results did not reveal a clear mechanistic link between high LRIG1 expression and favorable survival in early-stage NSCLC, the observed association between high LRIG1 expression and poor NSCLC cell clonogenicity may be worth exploring further. It may also be desirable to establish new cell lines from early-stage disease by using tumor tissue from percutaneous transthoracic biopsies in order to more accurately study the mechanisms of early-stage disease.

Conclusions

In contrast to other reports, we did not consistently observe reduced migration rates or increased sensitivity to chemotherapy or radiotherapy among LRIG1-overexpressing cells. Regarding cell motility and migration, our results were contradictory. Four LRIG1-overexpressing cell lines showed increased motility, and one showed decreased motility. The LRIG1-overexpressing H1299 and H1975 cell lines showed an increased migration rate in the wound healing assay, while they showed a decreased migration rate in the transwell assay. Previous studies have shown that LRIG1 decreases in vitro migration in melanoma and glioma cell lines through pathways downstream of EGFR (37, 38). However, that does not seem to be a consistent finding among the NSCLC cell lines tested in this study. Similarly, in contrast to previous studies in other cancer cell types, our LRIG1-overexpressing NSCLC cell lines did not show increased sensitivity to cisplatin, vinorelbine, or radiation. An LRIG1-mediated increase in radiosensitivity, attributed to down-regulation of EGFR signaling, has previously been described for U-251 glioma cells (39). Despite the fact that one of the NSCLC cell lines in our study harbored an EGFR driver mutation (HCC827), this cell line's radiosensitivity remained unaffected by LRIG1 overexpression as well. One possible clinical implication of our in vitro treatment results is that LRIG1 expression may not be a candidate predictive marker for chemotherapy or radiotherapy in NSCLC.

Regrettably, only one of our tested NSCLC cell lines, H1299, formed hematogenous micrometastases in mice. Therefore, we were restricted to H1299 for our experiments to directly address the role of LRIG1 in the metastatic spread of orthotopically implanted NSCLC. In vitro, LRIG1-overexpressing H1299 cells showed, on one hand, increased motility and migration rates, and on the other hand, decreased colony-formation capacity. Nevertheless, in vivo, overexpression of LRIG1 in H1299 cells appeared to confer a competitive advantage during establishment of the primary tumor but did not affect the metastatic potential of the cells. These results are interesting but do not explain increased survival among patients with high LRIG1 expression. Despite the inconclusive results of the mouse experiments, the in vivo model might be useful for studies of other mechanisms in NSCLC metastasis. As far as we know, this is the first time ddPCR on whole organ lysates has been used to quantify differences in cell populations between primary tumors and micrometastatic sites. Orthotopic implantation is an efficient model of hematogenous metastasis that often gives rise to a high metastatic burden (40), and our model provides an unbiased way of detecting and quantifying micrometastases with high throughput.

Notably, whereas the previously observed survival benefit of high LRIG1 expression is limited to early-stage NSCLC cases, all of the cell lines used in the present study were established from individuals harboring stage III or IV disease. Thus, the cell lines used may not be representative of the NSCLC types where LRIG1 has the strongest impact. Regrettably, there is a shortage of available NSCLC cell lines that are established from early-stage disease. Therefore, it would be desirable to establish new cell lines from early-stage disease by using tumor tissue from percutaneous transthoracic biopsies in order to more accurately study the mechanisms of early-stage disease.

Conclusions

In summary, although our results did not reveal a clear mechanistic link between high LRIG1 expression and favorable survival in early-stage NSCLC, the observed association between high LRIG1 expression and poor NSCLC cell clonogenicity may be worth exploring further. It may also be desirable to establish improved experimental models that better recapitulate the key features of early-stage NSCLC.

Abbreviations

ddPCR Digital droplet PCR
FBS Fetal bovine serum
GR(c) Concentration-dependent growth rate
IC50 50% inhibitory growth concentration
MRI Magnetic resonance imaging
NSCLC Non–small cell lung cancer
PBS Phosphate buffered saline
RTK Receptor tyrosine kinase
TBST Tris-buffered saline with Tween 20 detergent

Declarations

Acknowledgments
We thank Magnus Karlsson for performing cell irradiation.

Funding
This investigation was supported by grants from the Cancer Research Foundation in Northern Sweden, the Swedish Cancer Society, and the Västerbotten Regional Council.

Availability of data and materials
All the experimental data analyzed and displayed in the present manuscript are available from the corresponding author upon reasonable request.

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Contributions
SK performed the experiments with technical guidance from HH, performed data collection and analysis, contributed to the design of the study and wrote the manuscript. MJ and HH conceived the idea, designed the study, co-wrote and edited the manuscript. All authors have read and approved the manuscript.

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Ethics approval
All animal experiments were performed according to the Swedish animal welfare law and with ethical approval from the regional animal welfare board (DNR A 28–15).

Consent for publication
Not applicable.

Competing interests
The authors have no competing interests to declare.

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