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LMO7 and LIMCH1 interact with LRIG proteins in lung cancer, with prognostic implications for early-stage disease

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ABSTRACT

Objectives: The human leucine-rich repeats and immunoglobulin-like domains (LRIG) protein family comprises the integral membrane proteins LRIG1, LRIG2 and LRIG3. LRIG1 is frequently down-regulated in human cancer, and high levels of LRIG1 in tumor tissue are associated with favorable clinical outcomes in several tumor types including non-small cell lung cancer (NSCLC). Mechanistically, LRIG1 negatively regulates receptor tyrosine kinases and functions as a tumor suppressor. However, the details of the molecular mechanisms involved are poorly understood, and even less is known about the functions of LRIG2 and LRIG3. The aim of this study was to further elucidate the functions and molecular interactions of the LRIG proteins.

Materials and methods: A yeast two-hybrid screen was performed using a cytosolic LRIG3 peptide as bait. In transfected human cells, co-immunoprecipitation and co-localization experiments were performed. Proximity ligation assay was performed to investigate interactions between endogenously expressed proteins. Expression levels of *LMO7* and *LIMCH1* in normal and malignant lung tissue were investigated using qRT-PCR and through in silico analyses of public data sets. Finally, a clinical cohort comprising 355 surgically treated NSCLC cases was immunostained for LMO7.

Results: In the yeast two-hybrid screen, the two paralogous proteins LMO7 and LIMCH1 were identified as interaction partners to LRIG3. LMO7 and LIMCH1 co-localized and co-immunoprecipitated with both LRIG1 and LRIG3. Endogenously expressed LMO7 was in close proximity of both LRIG1 and LRIG3. *LMO7* and *LIMCH1* were highly expressed in normal lung tissue and down-regulated in malignant lung tissue. LMO7 immunoreactivity was shown to be a negative prognostic factor in LRIG1 positive tumors, predicting poor patient survival.

Conclusion: These findings suggest that LMO7 and LIMCH1 physically interact with LRIG proteins and that expression of LMO7 is of clinical importance in NSCLC.

1. Introduction

The leucine-rich repeats and immunoglobulin-like domains (LRIG) proteins 1, 2 and 3 are integral membrane proteins that play important roles in human cancer [1]. The mRNA expression of *LRIG1* is down-regulated in squamous cell carcinoma of the lung [2] as well as several other cancer types [3–5]. High levels of LRIG1 in tumor tissue have been associated with better patient survival in several different tumor types such as squamous cell carcinoma and adenocarcinoma of the uterine cervix [4,6], breast cancer [7–9] and lung cancer [7,10]. *Lrig1* functions as a tumor suppressor in the mouse intestine [11,12] and brain [13]. Additionally, *Lrig1*-deficient mice show skin [14,15] and airway hyperplasia [16]. In contrast, high LRIG2 expression correlates with poor survival for patients harboring oligodendroglioma [17] or early-stage squamous cell carcinoma of the uterine cervix [18], and *Lrig2*-deficient mice are protected against glioma [19]. In non-small cell lung cancer (NSCLC), LRIG2 expression has been reported both to correlate with poor survival [20] and to have no effect on survival [10]. LRIG3 has been identified as a positive prognostic factor in astrocytic gliomas and is a proposed tumor suppressor in glioma [21,22]. LRIG3 has also been suggested to be a serum biomarker for NSCLC [23].

Mechanistically, LRIG1 is a negative regulator of receptor tyrosine kinase (RTK) signaling, and has been shown to inhibit all four ErbB family members (EGFR, ERBB2, ERBB3 and ERBB4) [24,25], MET [26],

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RET [27], PDGFRA [28] and NTRK2 [29]. Several mechanisms have been proposed for the negative regulation of RTKs by LRIG1 (reviewed in [30,31]). These mechanisms include the down-regulation of RTK expression levels, inhibition of ligand binding and exclusion of RTKs from signaling complexes. Additionally, LRIG1 can be proteolytically cleaved at the ectodomain; the soluble LRIG1 that is shed then negatively regulates EGFR signaling in a paracrine fashion [32]. LRIG1 also regulates epidermal stem cell quiescence [33] and cadherin-dependent contact inhibition [16]. The knowledge about LRIG2 and LRIG3 function is comparatively poor. Membrane-bound LRIG2, as well as its soluble ectodomain, have been shown to interact with and enhance the activation of EGFR, suggesting a possible role as a therapeutic target [34]. LRIG3 has been shown to directly interact with and oppose the action of LRIG1 [35].

Lung cancer is the leading cause of cancer-related death worldwide with 1.59 million deaths in 2012 [36]. Lung cancer is divided into small cell lung cancer and NSCLC which is further divided into adenocarcinoma, squamous cell carcinoma and large cell carcinoma. Smoking is a leading cause of lung cancer, and it has been shown that LRIG1 is down-regulated in the bronchial epithelium of smokers [2].

To further elucidate the molecular functions of the LRIG proteins we performed a yeast two-hybrid (YTH) screen with a cytosolic LRIG peptide as bait. We identified LIM domain 7 (LMO7) and LIM and calponin homology domains 1 (LIMCH1) as interaction partners of the LRIG proteins, and we then investigated the clinical importance of this interaction in a cohort of surgically treated lung cancer patients.

2. Materials and methods

2.1. Yeast two-hybrid screen

The YTH screen, using a cytosolic peptide of LRIG3 as bait, was carried out by Dualsystems Biotech AG, Zürich, Switzerland. The bait construct for our YTH screening was made by subcloning a cDNA encoding amino acids 831 to 881 of LRIG3 (amino acids numbering according to NP_700356) into the vector *pLexA-DIR* (Dualsystems Biotech AG). The bait construct was transformed into the strain DSY-1 (MATa his3Δ200 trp1-901 leu2-3,112 ade2 LYS2::(lexAop)4-HIS3 URA3::(lexAop)8-lacZ GAL4) using standard procedures [37]. Correct expression of the bait was verified by Western blotting of cell extracts using a mouse monoclonal antibody directed against the LexA domain (Santa Cruz Biotechnology, Santa Cruz, CA). The absence of self-activation was verified by co-transformation of the bait together with a control prey and selection on minimal medium lacking the amino acids trypthophan, leucine, and histidine (selective medium). For the YTH screen, the bait was co-transformed together with a human brain cDNA library into DSY-1. Two hundred thousand transformants were screened, yielding 32 transformants that grew on selective medium. Positive transformants were tested for β-galactosidase activity using a filter assay [38] and transformants lacking β-galactosidase activity were discarded as false positives. Library plasmids were isolated from positive clones and assayed in a bait dependency test with [1] the bait plasmid and [2] a control bait encoding a LexA- laminC fusion using a mating strategy [39]. Of the 32 positives, 24 showed β-galactosidase activity when co-expressed with the bait but not when co-expressed with the control bait; these were considered to be bait-dependent positive interactors. The identity of positive interactors was determined by nucleotide sequencing.

2.2. RNA and quantitative RT-PCR

Total RNA was obtained from the indicated suppliers: ovary, adrenal gland, bladder, small intestine, testicle, thymus, stomach, colon and spleen (Thermo Fisher, Waltham, MA, USA); kidney, placenta, heart, liver, and brain (BD Biosciences, San Jose, CA, USA); thyroid and lung (Agilent, Santa Clara, CA, USA). Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed with the following primer/probe sets; *LMO7*: Hs01009229_m1 (Thermo Fisher); *LIMCH1*: Hs00971627_m1 (Thermo Fisher) and *RN18S* (18S ribosomal RNA) as previously described [40], using the ThermoScript™ RT-PCR System (Thermo Fisher) as previously described [40]. Standard curves for *LMO7* and *LIMCH1* were prepared using in vitro transcribed RNA. Standard curve for *RN18S* was prepared using total RNA from HEL2 cells. Expression levels of *LMO7* and *LIMCH1* were normalized to *RN18S*. All samples were run in triplicates.

2.3. Development of antibodies

Polyclonal antibodies against LMO7 were generated by immunizing rabbits with the synthetic peptide CTQSPTPRSHSPSAS (LMO7-1250). The antisera were affinity purified by using the corresponding synthetic peptide coupled to UltraLink columns (Pierce Chemical, Rockford, IL, USA). Antibodies were eluted from the column at pH 7.0 with an ActiSep elution medium (Sterogene Bioseparations, Carlsbad, CA, USA). Specificity was assessed using HA tagged overexpression constructs in a Western blot assay. The antibodies were produced by AgriSera (Vännäs, Sweden).

2.4. Cloning and vector constructs

LMO7 was cloned from the Human Lung Marathon-Ready cDNA library (Clontech, Mountain View, CA, USA) using forward primer, 5′ – AGTTCAATATATGGGTACGGTC – 3′ and reverse primer, 5′ – GCTTTC GTATGGAGGCTTAC – 3′. *LIMCH1* was cloned from the human Lung Marathon-Ready cDNA library (Clontech) and the human Brain Marathon-Ready cDNA libraries (Clontech) using forward primer, 5 – TGCGCAAATGGCTTGTC – 3′ and reverse primer, 5′ −CCTGAGCTTG GGATTTTGTT-3′. The PCR program used was 1 cycle 94 °C 60 s, 30 cycles 94 °C 15 s, 55 °C 30 s (*LMO7*) or 58 °C 30 s (*LIMCH1*), 68 °C 300 s, and 1 cycle 68 °C 360 s. PCR products were purified and cloned into 4-TOPO vector (Invitrogen) according to the manufacturer's instructions. Thereafter, the cloned *LMO7* and *LIMCH1* cDNAs were PCR amplified and subcloned into mammalian expression vectors. To this end, the following PCR primers were used: *LMO7* forward primer, 5′ – AAGGT ACCAGTTCAATATATGGGTACGGTC – 3′; *LMO7* reverse primer, 5′ – GAGGTACCCATGGCGGTTGGC – 3′; *LIMCH1* forward primer, 5′ – AAGGTACCATGGCTTGTCCCGCTCT – 3′; and *LIMCH1* reverse primer, 5′ – GGAGGTACCCAATGTTGTAGGCTGCCC – 3′. The PCR program used was: 1 cycle 94 °C 60 s, 20 cycles 94 °C 60 s, 58 °C 30 s, 68 °C 300 s. PCR products were purified and cloned into the expression vectors pMH and pcDNA3.1 using the KpnI restriction sites. DNA sequencing confirmed that LMO7 and LIMCH1 had been successfully cloned into the pMH and pcDNA3.1 vectors.

2.5. Cell culture and transfection

COS-7 and HEK293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 50 μg/ml gentamicin. H460 (ATCC, Manassas, VA, USA) cells were cultured in RPMI medium 1640 supplemented with 10% FBS and 50 μg/ml gentamicin. For transient overexpression, cells were transfected using X-tremeGENE 9 (Roche, Basel, Switzerland) transfection reagent, then fixed or lysed 48 h post-transfection. For siRNA experiments, 9000 cells per well were transfected in a 12-well plate using *LMO7* 15 nM siRNA ID# 149889 (Thermo Fisher) or 15 nM silencer negative control siRNA vector (Thermo Fisher) using the siPORT NeoFX transfection reagent (Thermo Fisher), according to the manufacturer's instructions. Cells were fixed or lysed 48 h post-transfection.

2.6. Western blotting

Cells were lysed in Cell Extraction Buffer (Thermo Fisher) with