STK11 pathway *in vitro* validation

Janne Lehtiö (✉ janne.lehtio@ki.se)

Department of Oncology and Pathology, Karolinska Institutet, Science for Life Laboratory, Solna, SE-17165, Sweden

Taner Arslan

Department of Oncology and Pathology, Karolinska Institutet, Science for Life Laboratory, Solna, SE-17165, Sweden

Ioannis Siavelis

Department of Oncology and Pathology, Karolinska Institutet, Science for Life Laboratory, Solna, SE-17165, Sweden

Yanbo Pan

Department of Oncology and Pathology, Karolinska Institutet, Science for Life Laboratory, Solna, SE-17165, Sweden

Fabio Socciarelli

Department of Oncology and Pathology, Karolinska Institutet, Science for Life Laboratory, Solna, SE-17165, Sweden

Olena Berkovska

Department of Oncology and Pathology, Karolinska Institutet, Science for Life Laboratory, Solna, SE-17165, Sweden <https://orcid.org/0000-0002-8811-0591>

Husen M. Umer

Department of Oncology and Pathology, Karolinska Institutet, Science for Life Laboratory, Solna, SE-17165, Sweden

Georgios Mermelekas

Department of Oncology and Pathology, Karolinska Institutet, Science for Life Laboratory, Solna, SE-17165, Sweden

Mohammad Pirmoradian

Department of Oncology and Pathology, Karolinska Institutet, Science for Life Laboratory, Solna, SE-17165, Sweden

Mats Jönsson

Division of Oncology, Department of Clinical Sciences, Lund and CREATE Health Strategic Center for Translational Cancer Research, Lund University, Lund, Sweden

Hans Brunnström

Department of Pathology, Laboratory Medicine Region Skåne, Lund, Sweden, Division of Pathology, Department of Clinical Sciences, Lund, Lund University, Lund, Sweden

Odd Terje Brustugun

Section of Oncology, Drammen Hospital, Vestre Viken Health Trust, Drammen, Norway, Department of Cancer Genetics, Institute for Cancer Research, Oslo University Hospital, Oslo, Norway

Krishna Pinganksha Purohit

University of Edinburgh Centre for Inflammation Research, Institute for Regeneration and Repair, Queen's Medical Research Institute, Edinburgh bioQuarter, 47 Little France Crescent, Edinburgh EH16 4TJ, UK, MRC Centre for Regenerative Medicine, Institute for Regeneration and Repair, University of Edinburgh, Edinburgh bioQuarter, 5 Little France Drive, Edinburgh EH16 4UU, UK

Richard Cunningham

University of Edinburgh Centre for Inflammation Research, Institute for Regeneration and Repair, Queen's Medical Research Institute, Edinburgh bioQuarter, 47 Little France Crescent, Edinburgh EH16 4TJ, UK, MRC Centre for Regenerative Medicine, Institute for Regeneration and Repair, University of Edinburgh, Edinburgh bioQuarter, 5 Little France Drive, Edinburgh EH16 4UU, UK

Hassan Foroughi Asl

Genomic Medicine Center, Karolinska University Hospital, Stockholm, Sweden. Clinical Genomics Facility, Department of Microbiology, Tumour and Cell Biology, Karolinska Institutet, Stockholm, Sweden.

Sofi Isaksson

Division of Oncology, Department of Clinical Sciences, Lund and CREATE Health Strategic Center for Translational Cancer Research, Lund University, Lund, Sweden

Elsa Arbajian

Division of Oncology, Department of Clinical Sciences, Lund and CREATE Health Strategic Center for Translational Cancer Research, Lund University, Lund, Sweden

Mattias Aine

Division of Oncology, Department of Clinical Sciences, Lund and CREATE Health Strategic Center for Translational Cancer Research, Lund University, Lund, Sweden

Anna Karlsson

Division of Oncology, Department of Clinical Sciences, Lund and CREATE Health Strategic Center for Translational Cancer Research, Lund University, Lund, Sweden

Marija Kotevska

Division of Oncology, Department of Clinical Sciences, Lund and CREATE Health Strategic Center for Translational Cancer Research, Lund University, Lund, Sweden, Department of Respiratory Medicine and Allergology, Skåne University Hospital, Lund, Sweden

Carsten Gram Hanson

University of Edinburgh Centre for Inflammation Research, Institute for Regeneration and Repair, Queen's Medical Research Institute, Edinburgh bioQuarter, 47 Little France Crescent, Edinburgh EH16 4TJ, UK, MRC Centre for Regenerative Medicine, Institute for Regeneration and Repair, University of Edinburgh, Edinburgh bioQuarter, 5 Little France Drive, Edinburgh EH16 4UU, UK

Vilde Drageset Haakensen

Department of Cancer Genetics, Institute for Cancer Research, Oslo University Hospital, Oslo, Norway, Department of Oncology, Oslo University Hospital, Oslo, Norway

Åslaug Helland

Department of Cancer Genetics, Institute for Cancer Research, Oslo University Hospital, Oslo, Norway,
Department of Oncology, Oslo University Hospital, Oslo, Norway, Faculty of Medicine, University of Oslo,
Norway

David Tamborero

Department of Oncology and Pathology, Karolinska Institutet, Science for Life Laboratory, Solna, SE-17165, Sweden

Henrik J. Johansson

Department of Oncology and Pathology, Karolinska Institutet, Science for Life Laboratory, Solna, SE-17165, Sweden

Rui M. Branca

Department of Oncology and Pathology, Karolinska Institutet, Science for Life Laboratory, Solna, SE-17165, Sweden

Johan Staaf

Division of Oncology, Department of Clinical Sciences, Lund and CREATE Health Strategic Center for Translational Cancer Research, Lund University, Lund, Sweden

Lukas M. Orre

Department of Oncology and Pathology, Karolinska Institutet, Science for Life Laboratory, Solna, SE-17165, Sweden

Method Article

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Abstract

The associated publication reports proteogenomic analysis of non-small cell lung cancer (NSCLC), where we identified molecular subtypes with distinct immune evasion mechanisms and therapeutic targets, and validated our classification method in separate clinical cohorts. The in-depth proteomic analysis pointed to a potential role of STK11 inactivation in liver-specific signaling and subsequent cancer growth and immune evasion mechanisms. This protocol describes *in vitro* validation of the downstream effects of STK11-AMPK signaling on HNF1A and FGL1 in a liver and two lung cancer cell lines.

Introduction

Reagents

List of antibodies:

- Anti-FGL1 (mouse monoclonal antibody A-8, cat. No. sc-514057, Lot J0520, Santa Cruz Biotechnology).
- Anti-LKB1 (STK11) (mouse monoclonal antibody Ley 37D/G6, cat. No. ab15095, Lot GR260328-12, Abcam).
- Anti-HNF1A (rabbit monoclonal antibody D7Z2Q, cat. No. 89670S, Lot 1, Cell Signaling Technology).
- Anti-beta-actin (mouse monoclonal antibody C4, cat. No. sc-47778, Lot K1617 Santa Cruz Biotechnology).
- Anti-alpha-tubulin (mouse monoclonal antibody B7, cat. No. sc-5286, Lot E2010, Santa Cruz Biotechnology).
- Anti-GAPDH (rabbit polyclonal antibody FL-335, cat. No. sc-25778, Santa Cruz Biotechnology)
- Anti-HSP90 (monoclonal purified mouse antibody clone 68/Hsp90, cat. No. 610418, BD Biosciences)
- Mouse anti-rabbit IgG-HRP (cat. No. sc-2357, Lot E0819, Santa Cruz Biotechnology)
- Sheep anti-mouse IgG-HRP (cat. No. NA931V, Lot 9471754, GE Healthcare UK)

Equipment

Procedure

Cell lines

NCI-H1944 (ATCC CRL-5907), NCI-H1395 (ATCC CRL-5868), and HepG2 (ATCC HB-8065) cells were purchased from LGC Standards (ATCC). Lung cancer cell lines were cultured in RPMI-1640 (Sigma-

Aldrich, cat. No. R2405) containing 10% fetal bovine serum (FBS, Sigma-Aldrich, cat. No. F7524) and 1% penicillin-streptomycin (P/S, Sigma-Aldrich, cat. No. P4333). HepG2 cells were cultured in low glucose DMEM (Sigma-Aldrich, cat. No. D6046) containing 10% FBS and 1% P/S. The cells were tested for *Mycoplasma* contamination using MycoAlert detection kit purchased from BioNordika (Lonza, cat. No. LT07-118).

AMPK activation

NCI-H1944, NCI-H1395, and HepG2 cells were expanded and reseeded into 6-well plates at seeding densities that were estimated to result in 80% confluence after 36 and 60 hours. After an initial 12-hour incubation, the standard culture media were changed to media containing an AMPK activator (250 μ M A-769662, Tocris, cat. No. 3336). The cells were harvested by trypsination after 24 and 48 h, washed with PBS, and pelleted by centrifugation (300 $\times g$, 5 min). Each of the experiments was performed in triplicates.

Introduction of STK11wt

Stable NCI-H1944 cells expressing FLAG-LKB1 (LKB1 is STK11), or vector control were generated by retroviral transduction. pBABE FLAG-LKB1 (Addgene plasmid # 8592) virus was harvested, filtered and added to polybrene treated cells. Following a 6–8 h incubation, the cell medium was changed. Stable cell lines were generated following puromycin selection. Virus was generated in HEK293T cells. Biological triplicates were performed for the experiment.

Western blot and antibodies

For the AMPK activation experiments, each of the cell pellet samples was lysed by adding 25–100 μ L of lysis buffer, heating on a shaker (95 $^{\circ}$ C, 500 rpm, 5 min, Thermomixer comfort, Eppendorf), and sonicating (50% amplitude, 1 s pulse, 30 s, Bandelin Sonopuls with a microtip, Heco Laboratorietstyr AS). The lysis buffer was prepared fresh and contained 4% (w/v) sodium dodecyl sulfate (SDS, Fluka BioChemika), 25 mM HEPES pH 7.6 (Sigma-Aldrich), and 1 mM DL-dithiothreitol (DTT). The lysates were then centrifuged at 14,000 $\times g$ for 15 min. The total protein concentration in cell lysates was measured using a standard protocol for the Bio-Rad DC protein assay kit (with bovine serum albumin (BSA, BioRad, cat. No. 500-0007) for a five-point standard curve. Lysates containing 100 μ g of protein were used for western blot analysis of each of the samples, with the exception of samples derived from NCI-H1395 cells, in which case 50 μ g of protein were loaded. Western blot analyses were performed according to standard suppliers' protocols using NuPAGE 4–12% Bis-Tris gels (Invitrogen, Thermo Fisher Scientific) and iBlot 2 Dry Blotting System with PVDF iBlot Transfer Stacks (Invitrogen). Anti-FGL1 (sc-514057, 1:300 dilution), anti- β -actin (sc-47778, 1:2,000 dilution), anti- α -tubulin (sc-5286, 1:1,000 dilution), and mouse anti-rabbit IgG-HRP (sc-2357, 1:10,000 dilution) antibodies were purchased from Santa Cruz Biotechnology, anti-LKB1 (ab15095, 1:1,000 dilution) from Abcam, anti-HNF1A (D7Z2Q, 1:1,000 dilution) from Cell Signaling Technology, and sheep anti-mouse IgG-HRP (NA931V, 1:10,000 dilution) from GE Healthcare UK. All antibodies were diluted in 5% skimmed milk powder in TBS with Tween-20 buffer (TBS-

T, Thermo Scientific) with dilution ratios in accordance with the suppliers' recommendations. Clarity Western ECL substrate (Bio-Rad) and iBright CL1500 imaging system (Invitrogen) were used for imaging of the western blots. ImageJ (v. 1.53f) software was used for densitometric analysis of the western blots. Signal intensity was normalized against the β -actin or α -tubulin loading control in each lane, thereafter the signal intensity was normalized against the mean signal of experimental control (48 h) samples in each blot. Densitometric results were reported as mean $\pm$ standard deviation and compared using two-tailed Welch test. For the introduction of STK11wt experiment, the cells were lysed in β -mercaptoethanol containing sample buffer and samples were boiled for ten minutes. Cell lysates were run on home-cast gels and wet-transferred onto PVDF membrane. Membranes were incubated in blocking solution (5% dry milk containing TBS-T) for 30 min, followed by three one-minute washes in TBS-T, and incubation with primary antibody (as described above) in TBS-T supplemented with 5% BSA overnight, and washes with TBS-T. The membrane was then incubated in 5% milk containing TBS-T with the appropriate HRP-conjugated antibodies for 1 h, washed and developed using Millipore Immobilon Western Chemiluminescent HRP mix (WBKLS0500) onto autoradiography films (SLS). Antibodies specifically used in this part were anti-GAPDH (sc-25778) from Santa Cruz Biotechnology and anti-HSP90 (Cat# 610418) from BD Biosciences.