

Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

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SUPPLEMENTAL MATERIAL

Generation of antisera and purification

To selected peptides for antiserum production DNASTAR Lasergene software

(<http://www.lasergene.com/products/lasergene.php>) was used. They were synthesized (Alpha Diagnostic International, San Antonio, TX; Genemed Synthesis, South San Francisco, CA) and conjugated to KLH. Rabbits were injected intramuscularly followed by subcutaneous booster injections (Genemed Synthesis, South San Francisco, CA; Cocalico Biologicals, Reamstown, PA). The antisera, designated R1AS-1 through R1AS-10, were purified using peptide-coupled column affinity chromatography (SulfoLink Kit, PIERCE Biotechnology, Rockford, IL). Briefly, the column was equilibrated to room temperature and washed with 6 ml of PBS. One milliliter of antiserum was allowed to enter the column followed by addition of 0.7 ml of sample buffer and incubation at room temperature for 1 hr. The column was washed with 12 ml of sample buffer. Bound antibody was eluted by applying 8 ml of elution buffer, aliquoted in 1 ml fractions, and neutralized by adding 100 μ l of 1 mol/L Tris, pH 7.5. The antibody was concentrated, 0.1% sodium azide was added, and stored at -20 °C.

Western blotting

Cells from permanent cultures of cell line NCI-H23, a subline transfected with human RRM1 out-of-frame (H23-Ct), and a subline transfected with RRM1 in-frame (H23-R1) were harvested in protein lysis buffer [20 mmol/L Tris-HCl (pH 7.6), 150 mmol/L NaCl, 1 mmol/L EDTA, 0.5% NP40, 1 mmol/L DTT, 5 μ mol/L trichostatin A, 1 mmol/L sodium orthovanadate, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L NaF, and complete protease inhibitors (Roche Applied Science, Penzberg, Germany)]. Lysates were centrifuged at 4°C for 15 min. at 13,000 x g, and the supernatant fraction was recovered. Protein extracts from whole tumor and normal lung specimens that had been immediately frozen after surgical resection were prepared using Trizol reagent (Invitrogen, Carlsbad, CA). Fractionation of cells into nuclear and cytoplasmic constituents was done using a commercially available kit (BioVision, Mountain View, CA). Protein extracts (50 μ g) were separated through 10% Novex tris-glycine gels (Invitrogen, Carlsbad, CA) and blotted onto pure nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA). The blots were incubated with primary antibody at 4°C overnight. The primary antisera/antibodies for RRM1 were R1AS-6 (1:1,000), R1AS-7 (1:1,000), R1AS-10 (1:1,000), and AD203 (1:1,000; Millipore, Billerica, MA). They were A2B1 (1:1,000, #sc-7974) for PTEN (Santa Cruz Biotechnology, Santa Cruz, CA), Ab-2 clone 8F1 (1:500, #MS-671-R7) for ERCC1 (Lab Vision Corp., Fremont, CA), Oct-1 antiserum (1:1,000, #3342-100) for Oct-1 (BioVision, Mountain View, CA), and GAPDH(V-8) (1:1,000, #sc-20357) for GAPDH (Santa Cruz). Protein bands were visualized with anti-rabbit, anti-mouse, or anti-goat IgG horseradish peroxidase secondary antibody (1:1,000; Santa Cruz) and SuperSignal West Pico chemiluminescence substrate (Pierce, Rockford, IL). The house-keeping genes GAPDH and α -actin were used as equal loading control. For blocking of the primary antisera, the peptide used to generate the respective antiserum was used in 50-fold excess.

Confocal microscopy

The lung adenocarcinoma cell line NCI-H23 was grown directly on Lab-Tek chamber slides. Adherent cells were washed in phosphate buffered saline (PBS), fixed by incubation for 20 min. in 4% paraformaldehyde in PBS, and washed in PBS. They were permeabilized for 1 hr. in 0.25% Triton-X100/PBS and washed in PBS. Affinity-purified R1AS-6 (1:300) and ERCC1 (1:500) or R1AS-6 (1:300) and PTEN (1:100) antibodies were diluted in binding buffer (1% BSA/0.1% NP40/PBS) and added to the chambers and incubated for 1 hr. After washing in PBS, the slides were incubated for 45 min. with 1:500 dilutions of Alexa Fluor 488 (anti-rabbit IgG) and Alexa Fluor 555 (anti-mouse IgG) (Molecular Probes, Invitrogen, Eugene, OR). The slides were washed with PBS and covered using ProLong Gold antifade reagent with DAPI (Molecular Probes, Invitrogen). As negative controls, the same procedure was performed without primary antibody and with primary antibody plus the peptide used to generate it in 10 μ M concentrations. Samples were viewed with an inverted Zeiss LSM 510 confocal microscope with a 63x /1.20NA water immersion objective. Argon and HeNe laser lines in conjunction with 488 and 555 band pass filters were applied to excite the samples using line switching to minimize crosstalk between fluorochromes. Nuclei were visualized with DAPI and imaged using widefield fluorescent light. Images were produced with dual photomultiplier detectors and the LSM 5 version 3.2.0.115 software suite.

Tissue microarray construction

Standard formalin-fixed and paraffin-embedded tissue blocks from the resected primary tumors were H & E stained and reviewed by two pathologists (ZZ, AS) to confirm the original diagnosis. Representative

tumor areas were marked, and tissue cores with a diameter of 0.6 mm were punched and arrayed into a recipient paraffin block using a tissue arrayer (Beecher Instrument, Silver Spring, MD). A total of 10 reference specimens were arrayed likewise to serve as controls. These included trachea, larynx, esophagus, stomach, liver, breast, lymph nodes, skin, and two normal lung specimens. Triplicate blocks were constructed. Sections of 5 μ m thickness were cut, transferred to 4x adhesive coated slides (Instrumedics, NJ), and exposed to UV light for 30 sec. to enhance adherence.

In situ detection and quantification of protein expression

Immunohistochemistry (IHC) based on immunofluorescence combined with a novel automated quantitative analysis (AQUA) was used to assess in situ expression of the target molecules.¹

Antigens were retrieved by microwave oven treatment for 15 min. in 0.01 mol/L of Na-citrate, Tris-HCl, or Tris-EDTA buffer at an optimized pH.² The slides were blocked for 30 min. with 0.3% BSA and then incubated overnight at 4°C in appropriately diluted primary antibody/antiserum (R1AS-6, 1:800 and R1AS-10, 1:400 for RRM1; A2B1, 1:100, #sc-7974, Santa Cruz, for PTEN; Ab-2 clone 8F1, 1:3, #MS-671-R7, Lab Vision Corp., for ERCC1). For identification of carcinomatous cells, antibody/antiserum to cytokeratin was used (murine anti-human pancytokeratin AE1/AE3, 1:200, #M3515, Dako Cytomation, Carpinteria, CA; rabbit anti-human pancytokeratin AE1/AE3, 1:200, #Z0622, Dako Cytomation). Slides were washed and incubated with two different secondary antibodies for 1 hr. (Envision® labeled polymer-HRP anti-rabbit, # K4011, or Envision® labeled polymer-HRP anti-mouse, # K4007, specific to the primary antibody/antiserum used for target protein detection, 1:200; Alexa 555 goat anti-mouse, #A21424, or goat anti-rabbit, #A21429, based on the source of the anti-cytokeratin antibody/antiserum, 1:200, Dako Cytomation). For fluorescence amplification, slides were exposed to Cy5-Tyramide (1:50) for 10 min. at room temperature. They were mounted with Prolong Gold antifade reagent with DAPI (4'-6-diamidino-2-phenylindole) mount solution. The final tissue microarray slides were scanned with SpotGrabber, and image data were analyzed with AQUA (PM-2000, HistoRx, New Haven, Connecticut). The lowest possible AQUA score is 0 and the highest is 255.

References

1. Camp RL, Chung GG, Rimm DL. Automated subcellular localization and quantification of protein expression in tissue microarrays. *Nat Med* 2002;8:1323-7.
2. Shi SR, Cote RJ, Taylor CR. Antigen retrieval immunohistochemistry: past, present, and future. *J Histochem Cytochem* 2001;45:327-44.