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BASIC AND TRANSLATIONAL—PANCREAS

Ribonucleoprotein HNRNPA2B1 Interacts With and Regulates Oncogenic KRAS in Pancreatic Ductal Adenocarcinoma Cells

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BACKGROUND & AIMS: Development of pancreatic ductal adenocarcinoma (PDAC) involves activation of c-Ki-ras2 Kirsten rat sarcoma oncogene homolog (KRAS) signaling, but little is known about the roles of proteins that regulate the activity of oncogenic KRAS. We investigated the activities of proteins that interact with KRAS in PDAC cells. **METHODS:** We used mass spectrometry to demonstrate that heterogeneous nuclear ribonucleoproteins (HNRNP) A2 and B1 (encoded by the gene *HNRNPA2B1*) interact with KRAS G12V. We used co-immunoprecipitation analyses to study interactions between HNRNPA2B1 and KRAS in KRAS-dependent and KRAS-independent PDAC cell lines. We knocked down HNRNPA2B1 using small hairpin RNAs and measured viability, anchorage-independent proliferation, and growth of xenograft tumors in mice. We studied KRAS phosphorylation using the Phos-tag system. **RESULTS:** We found that interactions between HRNPA2B1 and KRAS correlated with KRAS-dependency of some human PDAC cell lines. Knock down of HNRNPA2B1 significantly reduced viability, anchorage-independent proliferation, and formation of xenograft tumors by KRAS-dependent PDAC cells. HNRNPA2B1 knock down also increased apoptosis of KRAS-dependent PDAC cells, inactivated c-akt murine thymoma oncogene homolog 1 signaling via mammalian target of rapamycin, and reduced interaction between KRAS and phosphatidylinositide 3-kinase. Interaction between HNRNPA2B1 and KRAS required KRAS phosphorylation at serine 181. **CONCLUSIONS:** In KRAS-dependent PDAC cell lines, HNRNPA2B1 interacts with and regulates the activity of KRAS G12V and G12D. HNRNPA2B1 is required for KRAS activation of c-akt murine thymoma oncogene homolog 1–mammalian target of rapamycin signaling, interaction with phosphatidylinositide 3-kinase, and PDAC cell survival and tumor formation in mice. HNRNPA2B1 might be a target for treatment of pancreatic cancer.

Keywords: Signal Transduction; Carcinogenesis; Oncogene; Mouse Model.

RAS proteins are well-known small GTPases involved in the regulation of key signal transduction pathways. Cycling from the inactive (guanosine diphosphate-bound) to the active (guanosine triphosphate [GTP]-bound) state faithfully responds to extracellular signals due to its tight regulation by GTP-exchange factors and GTPase activating proteins. Activating point mutations that render RAS proteins insensitive to the extracellular signals are crucial steps in the development of the vast majority of cancers.[1–3] Three different genes code for a total of 4 different Ras isoforms named c-H-ras Harvey rat sarcoma oncogene, c-N-ras, neuroblastoma rat sarcoma oncogene homolog, KRAS4A, and KRAS4B. KRAS4B (referred to here as KRAS) is the most frequently mutated oncogene in solid tumors and its oncogenic mutations occur mostly in pancreatic (95%), colon (40%), and adenocarcinomas of the lung (35%).[3–5] Mutational activation of KRAS in these tissues is sufficient to initiate neoplasia in mice.[6–8] The most prevalent oncogenic mutation in KRAS is at codon 12 (66% KRAS mutations)[4] and preserves the GTP-bound active state by inhibiting intrinsic GTPase activity or interfering with the action of GTPase activating proteins. In the GTP-bound form, KRAS is able to interact with different effector proteins and consequently activates signal transduction pathways. Among those, the best characterized are the c-Raf rapidly accelerated fibrosarcoma oncogene/extracellular signal-regulated kinase and the phosphatidylinositol-3-kinase (PI3K)/AKT.[9,10] Because oncogenic mutations of KRAS give rise to an always GTP-bound protein that constitutively binds to effectors to activate them, positive or negative physiologic regulation of oncogenic KRAS was not initially

Abbreviations used in this paper: **AKT, c-akt murine thymoma oncogene homolog 1; FCS, fetal calf serum; GFP, green fluorescent protein; GTP, guanosine triphosphate; HA, hemagglutinin; hnRNP, heterogeneous nuclear ribonucleoprotein; KRAS, c-Ki-ras2 Kirsten rat sarcoma oncogene homolog; mTOR, mammalian target of rapamycin; PDAC, pancreatic ductal adenocarcinoma; PI3K, phosphatidylinositide 3-kinase; PKC, protein kinase C; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; shRNA, small hairpin RNA.**

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expected. In recent years, emergence of new regulators, either by direct interaction or by reversible post-translational modifications, proved to be crucial to fully display KRAS oncogenic phenotype. Interaction of KRAS with Galectin-3,[11] calmodulin,[12] and phosphodiesterase δ,[13,14] or with the initially defined nuclear protein Nucleophosmin and Nucleolin,[15] proved to be necessary for correct activation of c-Raf rapidly accelerated fibrosarcoma oncogene/extracellular signal-regulated kinase signaling pathway.

As human cancers usually evolve through a multistage process in which tumors accumulate multiple oncogenic lesions, the presence of an oncogene could often be negligible for tumorigenesis, despite this complexity, tumor growth and survival are often impaired by the inactivation of a single oncogene. This phenomenon, called "oncogene addiction," provides a rationale to identify novel therapeutic molecular targets.[16,17] "KRAS addiction" has been deeply investigated in murine and human cancer models or in clinical studies.[18–25]

Pancreatic ductal adenocarcinoma (PDAC) is a nearly uniformly fatal disease, despite intensive treatment, with <1%–5% of patients surviving 5 years.[26,27] PDAC is one of the best-defined KRAS-driven human malignancies, with a wealth of molecular studies identifying mutant KRAS as the initiating event in the vast majority of cases.[28–32] Expression of KrasG12D or KrasG12V in the murine pancreas induces acinar cell de-differentiation that progresses to metastatic PDAC,[29,30,33–35] and is associated with activation of the PI3K/PDK1/AKT signaling pathway.[36,37] The identification of KRAS-dependent (KRAS oncogenic "addicted") vs KRAS-independent PDAC cell lines[18,38] allows the identification of KRAS-driven PDAC subtype-specific mechanisms of oncogenesis that can be exploited for therapeutic benefit.[13,39]

In a search for specific regulators of KRAS oncogenic activity in PDAC, we identified heterogeneous nuclear ribonucleoprotein (HNRNP) A2B1 as a novel oncogenic KRAS interactor, which was highly specific for previously validated KRAS-dependent human PDAC cell lines.[18,38] By down-regulating HNRNPA2B1 in these cell lines, we found a KRAS-dependent specific requirement of HNRNPA2B1 for certain oncogenic features. Our data indicate that the interaction between HNRNPA2B1 and KRAS is important for PI3K/AKT activation in KRAS-dependent PDAC cells. In addition, this interaction is dependent on KRAS Ser181-phosphorylation status, suggesting that this post-translational modification could play a pivotal role in KRAS-dependent PDAC.

Material and Methods

Cells

HeLa cells were obtained from American Tissue and Cell Collection (Manassas, VA) and routinely verified according to the specifications outlined in the American Tissue and Cell Collection Technical Bulletin. PDAC cell lines (MPanc-96, HPAF-II, pa-tu-8902, SW-1990, 8988-T, and PANC-1) were kindly provided by Prof Dr A. Kimmelman (Harvard Medical School, Boston, MA).

HeLa and the PDAC cell lines (MPanc-96, HPAF-II, pa-tu-8902, SW-1990, 8988-T, and PANC-1) were grown in Dulbecco's modified Eagle's medium, supplemented with 10% fetal calf serum (FCS; Biological Industries, Kibbutz Beit-Haemek, Israel), penicillin, streptomycin, and nonessential aminoacids.

Statistics

All analyses were performed with GraphPad Prism software, version 5.0 (GraphPad, La Jolla, CA). Data are presented mean ± SEM. Mann-Whitney test was used to analyze significance levels. Specific significance levels are found in figure legends. $P < .05$ was considered significant.

See Supplementary Material for additional information.

Results

Identification of HNRNPA2B1 as a Novel Oncogenic KRAS Interactor

To identify novel regulators of oncogenic KRAS, HeLa cells were transfected with a plasmid expressing the constitutively active hemagglutinin (HA)-KRASG12V and immunoprecipitated with an anti-HA antibody using non-transfected cells as a control. A mild elution with HA peptide was performed to increase the specificity of the bound fraction. The bound fraction was resolved in a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Flamingo (Bio-Rad Laboratories, Inc, Hercules, CA). The main bands reproducibly specific for HA-KRASG12V transfected cells were excised, digested with trypsin, and analyzed by matrix-assisted laser desorption/ionization time of flight. We identified 6 specific proteins (Supplementary Figure 1). We focused on HNRPNA2/B1 (Mowse score = 3.89e+005; sequence coverage = 35%; peptides matched = 10/108) given the described involvement of its cytoplasmic accumulation in malignancies harboring oncogenic KRAS.[40–43]

To confirm and further characterize the interaction, co-immunoprecipitation with the active form of the major RAS isoforms was studied. We observed a specific interaction with the K- and N-RAS isoforms being excluded from the H-RAS (Figure 1*A*). To determine whether the activation status of KRAS was important for the interaction with HNRNPA2B1, we compared the co-immunoprecipitation with either the fully active mutant (V12) or the wild-type form of KRAS. We observed a specific interaction with the active oncogenic mutant (V12) (Figure 1*B*), which was confirmed by the reverse co-immunoprecipitation (Figure 1*C*).

HNRNPA2B1 is a ribonucleoprotein that is involved in messenger RNA processing that is mainly located in the cell nucleus.[44,45] Although a membrane-bound fraction was identified previously,[44,46] the interaction with RAS proteins has not been described previously. Using cell fractionation and Western blotting, we found a substantial amount of HNRNPA2B1 protein in the membrane fraction (P100) (Supplementary Figure 2*A*). In addition, using immunofluorescence in fully intact cells, despite an extensive nuclear localization, HNRNPA2B1 was also found scattered throughout the cytoplasm and decorated plasma membrane