Enhanced RASGEF1A Expression Is Involved in the Growth and Migration of Intrahepatic Cholangiocarcinoma

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Abstract

Purpose and Experimental Design: To identify novel molecular targets for the treatment of intrahepatic cholangiocarcinoma (ICC), the second most common type of primary hepatobiliary cancer, we earlier analyzed genome-wide expression profiles of genes in 25 ICCs. Among the genes whose expression levels were commonly elevated in the tumors, we identified a novel gene termed RASGEF1A that encodes a putative Ras guanine nucleotide exchange factor domain-containing protein.

Results: We showed in this article that RASGEF1A protein has a guanine nucleotide exchange activity to K-RAS, H-RAS, and N-RAS proteins in vitro. Consistently, exogenous RASGEF1A expression increased the activity of Ras. In addition, suppression of RASGEF1A by small interfering RNA retarded the growth of cholangiocarcinoma cells. Interestingly, COS7 cells expressing exogenous RASGEF1A showed enhanced cellular motility in Transwell and wound-healing assays.

Conclusions: These data suggest that elevated expression of RASGEF1A may play an essential role for proliferation and progression of ICC. Our data indicate that RASGEF1A may be a promising therapeutic target for the majority of ICCs.

Intrahepatic cholangiocarcinoma (ICC) is the second most common type of primary hepatobiliary cancer. The incidence of ICC reveals wide geographic variations; the highest incidence has been reported in Southeast Asia, such as Laos and northeastern Thailand (1, 2). Notably, the incidence of ICC is now increasing in Japan and Western countries, especially in the United Kingdom (3–5). Early detection of this disease is very difficult due to the lack of sensitive tumor markers or information of high-risk population. Surgical resection of detectable tumors improves patients’ prognosis (6–8), but complete removal of cancer cells in patients at an advanced stage is barely possible. Chemotherapy to ICCs showed no survival benefits, although 5-fluorouracil and gemcitabine-based regimens revealed partial response rates of 20% to 30% (9). Therefore, prognosis of patients with ICC remains poor. The development of novel diagnostic and/or therapeutic strategies and identification of high-risk population to this disease are matters of pressing concern.

Molecular studies on ICC have disclosed involvement of multiple genetic alterations in cholangiocarcinogenesis. Mutations in K-RAS and p53 were detected in 20% to 50% and 20% to 37% of ICCs, respectively (10–12). The Ras family of small GTPases plays a key role in integrating and transmitting signals from cell surface receptors to downstream effector molecules. Mutation in K-RAS results in constitutive activation of its downstream signaling pathway. Activation of Ras proteins promotes oncogenesis by disturbing a multitude of cellular processes, such as gene expression, cell cycle progression, and cell proliferation as well as cell survival and cell migration (13).

Ras proteins are interconvertible between an active GTP-bound form and an inactive GDP-bound form (14). Their activities are modulated by regulatory proteins that stimulate GDP/GTP exchange [guanine nucleotide exchange factor (GEF) proteins] and GTP hydrolysis (GTPase-activating proteins). On activation by extracellular stimuli, GEFs promote the release of GDP from Ras, permitting GTP to associate with Ras. This GTP-bound Ras forms an active conformation and mediates signals to downstream effector proteins, such as Raf, phosphatidylinositol 3-kinase (PI3K), and RalGDS (15, 16). Although several Ras family GTPases have been identified, a limited number of Ras GEFs have been studied [e.g., SOS1, SOS2, GRF, and GRP for Ras; SmgGDS, C3G, CalDAG1, and Epac for Rap1; and RalGDS family members for Ral (16–21)].

To uncover precise mechanisms of ICC and identify novel molecular targets for the treatment, we earlier analyzed genome-wide expression profiles of genes in 25 ICCs (22). Among the genes with elevated expression in ICC, we focused in this study on a gene, RASGEF1A (RasGEF domain family, member 1A), encoding a putative guanyl nucleotide exchange factor for Ras because Ras activation is involved in a wide range of human tumors. We report here that RASGEF1A has a guanine...
nucleotide exchange activity to K-RAS, H-RAS, and N-RAS and that RASGEF1A may serve for a novel therapeutic target for ICCs.

Materials and Methods

Cell lines and tissue specimens. A monkey kidney cell line, COS7, a mouse fibroblast cell line, NIH3T3, and a primary human embryonic kidney cell line, HEK293, were obtained from the American Type Culture Collection (Rockville, MD). A human cholangiocarcinoma cell line, SSP25, was obtained from the Japanese Collection of Research Bioresources (Osaka, Japan). All cells were grown in monolayers in appropriate medium as follows: RPMI 1640 (Sigma, St. Louis, MO) for SSP25 and DMEM (Sigma-Aldrich Corp., St. Louis, MO) for COS7, NIH3T3, and HEK293. Clinical tissues were obtained with informed consents from surgical specimens of patients who underwent hepatectomy. All ICC tumors were clinically diagnosed as peripheral type and had no association with known etiology, such as primary sclerosing cholangitis, cholelithiasis, intrahepatic cholangitis, or infection with liver flukes. Clinicopathological data of the tumors were shown in our earlier report (22).

RNA preparation and semiquantitative reverse transcription-PCR. We used laser microbeam microdissection technology to collect pure populations of cholangiocarcinoma cells as well as noncancerous bile duct epithelia from surgical specimens. Preparation of sections, laser microdissection, extraction of total RNA, and T7-based amplification were done according to the manufacturer’s recommendations (23). A mixture of normal intrahepatic biliary epithelial cells in liver tissues from 10 patients with metastatic liver tumor was prepared as a universal control. Total RNA was extracted from cultured cells using Trizol reagent according to the manufacturer’s protocol (Invitrogen, Carlsbad, CA). Extracted RNA was reversely transcribed for single-stranded cDNAs using poly(dI)-dC (primer (Amersham Biosciences, Buckinghamshire, United Kingdom) with SuperScript II reverse transcriptase. Each single-stranded cDNA was diluted for subsequent PCR amplification. Standard reverse transcription-PCR (RT-PCR) was carried out in a 12 µL volume of PCR buffer (Takara, Tokyo, Japan) and amplified for 5 minutes at 94°C for denaturing followed by 25 cycles for (ACTB) or 30 cycles for (RASGEF1A) for 94°C of 20 seconds, 53°C (for ACTB) or 60°C (for RASGEF1A) for 30 seconds, and 72°C for 30 seconds in the GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA). Primer sequences were as follows: ACTB, 5′-TCCGGGATCATGCTGCTTC-3′ and 5′-TCCCTTCTATTCCTTGGCTCC-5′ and RASGEF1A, 5′-TTGCACTTTGAGTCTGAC-3′ and 5′-GTCCTGGAATTCCAGCCGTCG-5′.

Northern blot analysis. Human multiple-tissue Northern blots (BD Biosciences, Palo Alto, CA) were hybridized with a 32P-labeled RASGEF1A cDNA as a probe. Prehybridization, hybridization, and washing were done according to the supplier’s recommendations. The blots were autoradiographed with intensifying screens at −80°C for 120 hours.

Ras nucleotide dissociation assay. The entire coding regions of K-RAS (amino acids 1-189), Ha-RAS (amino acids 1-189), N-RAS (amino acids 1-189), RASGEF1A, or the catalytic domain of SOS (amino acids 1-189) were cloned into an appropriate cloning site (pCAGGS-n3Fc-RASGEF1A (RASGEF1A) or pCAGGS-n3Fc-Mock (Mock) at 4°C for 45 minutes. Proteins bound to RAS were precipitated, separated on SDS-PAGE, and subsequently analyzed by Western blotting with anti-Ras antibody. Cell growth was analyzed in triplicate using cell-counting kit 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Roche Diagnostics, Mannheim, Germany). The cells were maintained in culture medium containing 10% fetal bovine serum supplemented with 0.9 mg/mL genetin for 2 weeks. The viable cells were measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay using a cell-counting kit (Dojindo, Kumamoto, Japan). To analyze the knockdown effect on RASGEF1A, we extracted total RNA from the cells at 24 hours after transfection and carried out semiquantitative RT-PCR analysis.

Cell viability assay. SSP25 cells plated on 10-cm dish (1 × 10^6 per dish) were transfected with plasmids expressing siRNA using either Nucleofector (Amaxa, Gaithersburg, MD) or Fugene 6 reagent (Roche Diagnostics, Mannheim, Germany). The cells were maintained in culture medium containing 10% fetal bovine serum supplemented with 0.9 mg/mL genetin for 2 weeks. The viable cells were measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay using a cell-counting kit (Dojindo, Kumamoto, Japan). To analyze the knockdown effect on RASGEF1A, we extracted total RNA from the cells at 24 hours after transfection and carried out semiquantitative RT-PCR analysis.

Wound-healing and Matrigel invasion assays. The entire coding region of RASGEF1A (Genbank accession no. AK095136) was amplified by RT-PCR using a set of primers, 5′-TTGCACTTTGAGTCTGAC-3′ and 5′-AAATGAGATCATGCTGCTTC-5′, for RASGEF1A; and 5′-TCCGGGATCATGCTGCTTC-5′ and 5′-AAATGAGATCATGCTGCTTC-5′, for Mock. The paired oligonucleotides were phosphorylated with T4 polynucleotide kinase and subsequently annealed to produce double-stranded oligonucleotides by boiling for 5 minutes and cooling down slowly. Expression of RASGEF1A was examined by semiquantitative RT-PCR 24 hours after transfection.
2 days, and a scrape in the form of a cross was made through the confluent monolayers with a plastic pipette tip. Several wounded areas were marked for orientation, observed, and then photographed by phase-contact microscopy at indicated times after the scratch. We further investigated invasion of cells using BD BioCoat Matrigel invasion chambers with 8-μm pores (BD Biosciences, San Jose, CA). The number of COS7-RASGEF1A and COS7-Mock cells that migrated through the chamber was counted in five independent visual fields under a microscope at 36 hours of incubation.

Statistical analysis. Statistical analyses were done by ANOVA and Scheffe’s F tests. A P value of <0.05 was considered to be statistically significant.

Results

Elevated expression of RASGEF1A in the majority of ICCs. Using genome-wide cDNA microarray containing 27,648...
genes, we analyzed expression profiles of 25 ICCs and identified genes whose expression was frequently up-regulated in the tumors (22). Among the up-regulated genes, we in this study focused on a gene corresponding to an expressed sequence tag (EST) of Hs. 125293 in the UniGene database (Build 183) in National Center for Biotechnology Information. Semiquantitative RT-PCR analysis of this gene revealed the enhanced expression in 9 of 13 ICCs that were subjected to the microarray analysis (Fig. 1A). To evaluate its expression levels in human adult normal tissues, we did multiple-tissue Northern blot analysis using cDNA as a probe and detected a transcript of ~3.3 kb that was moderately expressed in the brain and spinal cord and weakly expressed in the lymph node and adrenal gland but not in any of 19 other tissues examined (Fig. 1B). The gene was recently termed as RASGEF1A because it contained a Ras GEF domain. A homology search with the deduced RASGEF1A amino acid sequence showed 30% identity with PDZ-GEF2 and 29% with PDZ-GEF1.

**Ras nucleotide dissociation activity of RASGEF1A.** Because RASGEF1A contained a putative Ras GEF domain, we investigated whether it has GDP dissociation activity. We prepared recombinant K-RAS, Ha-RAS, and N-RAS as substrates and incubated each of the proteins with [3H]-labeled GDP. We subsequently added the reaction mixture with/without recombinant RASGEF1A protein and measured GDP-associated Ras by scintillation counter. We used SOS, one of the known RASGEF proteins, as a positive control. SOS decreased the GDP-bound form of K-RAS by ~40%. Similarly, RASGEF1A reduced GDP-bound K-RAS by ~50% (Fig. 2A). It also dissociated GDP bound to H-RAS and N-RAS by approximately 40% and 80%, respectively (Fig. 2B and C). These data indicate that RASGEF1A has GDP dissociation activity to all of the three Ras proteins examined.

**Activation of Ras by RASGEF1A.** We additionally examined activation of Ras by RASGEF1A in COS7 cells expressing exogenous RASGEF1A (Fig. 2D, top). Because an active form of Ras was known to have higher affinity to Raf-1, one of the major effectors of Ras, than its inactive form, we did a pull-down assay using recombinant Raf-1 and extracts from the COS7 cells transfected with RASGEF1A or mock vector. Consequently, the amount of Raf-1-interacting Ras was significantly increased by expression of RASGEF1A (Fig. 2D), indicating that RASGEF1A activated Ras.

**RASGEF1A expression is essential for the growth of cancer cells.** We carried out colony formation assay to examine oncogenic activity of RASGEF1A in COS7 cells and found no difference in the number of colonies between the cells with or without RASGEF1A overexpression (data not shown). To further evaluate its potential role in cell growth, we prepared two forms of plasmids expressing RASGEF1A-specific siRNAs (RASGEF1A-siRNA-B and RASGEF1A-siRNA-E) together with neomycin-resistant gene (psiH1BX-RASGEF1A-B and psiH1BX-RASGEF1A-E). As control, a three-nucleotide substitution was introduced into siRNA-B (psiH1BX-Bmis3). We transfected each of three plasmids, psiH1BX-RASGEF1A-B, psiH1BX-RASGEF1A-E, or psiH1BX-RASGEF1A-Bmis3, into SSP25 cholangiocarcinoma cells that expressed RASGEF1A at a high level. Semiquantitative RT-PCR showed that RASGEF1A expression was suppressed by psiH1BX-RASGEF1A-B or psiH1BX-RASGEF1A-E compared with psiH1BX-Bmis3, psiH1BX-EGFP, or psiH1BX-ACTB.
psiH1BX-Mock (Fig. 3A). In concordant to the expression level of RASGEF1A, the cells transfected with psiH1BX-RASGEF1A-B or psiH1BX-RASGEF1A-E started to show apparent growth retardation at day 6 and revealed marked growth retardation at day 14 compared with those transfected with the other plasmids (Fig. 3B). These data suggest that expression of RASGEF1A is crucial for the survival of SSP25 cholangiocarcinoma cells and that continuous inhibition of RASGEF1A is necessary to inhibit the growth of cancer cells.

**Effect of RASGEF1A on migration of cancer cells.** Because Ras family proteins have been reported to play an important role in cell migration as well as cell growth, we tested a possible involvement of RASGEF1A in migration and invasion of cancer cells. We established COS7-RASGEF1A cells expressing exogenous RASGEF1A and control cells (COS7-Mock) and carried out wound-healing assay. As shown in Fig. 4A, COS7-RASGEF1A cells migrated rapidly and filled in the wound significantly faster than COS7-Mock cells. The growth of COS7-RASGEF1A cells was similar to that of control cells in another experiment (data not shown). These data implied that RASGEF1A plays a role in cellular migration. To investigate the effect of RASGEF1A on invasion, we additionally carried out a Transwell assay using Matrigel. Consistent with the data of wound-healing assay, we observed augmented number of COS7-RASGEF1A cells through the pores of chambers compared with COS7-Mock cells (Fig. 4B). These data suggested that RASGEF1A is involved in cellular migration.

**Discussion**

We have reported in the present study that RASGEF1A expression is elevated in the majority of human ICCs and that its suppression results in the growth retardation of ICC cell. In addition, we have clarified that RASGEF1A has a guanine nucleotide exchange activity to K-RAS, H-RAS, and N-RAS.

From the structural point of view, RASGEF1A contained five structurally conserved regions that are found in other Ras GEF members (26, 27). It also had a Ras exchange motif or RasGEF domain between codons 49 and 178, which is supposed to stabilize the large helical hairpin structure that pries open the GTP-binding pocket. However, RASGEF1A does not have any additional conserved domains, such as Dbl homology domain, pleckstrin homology domain, the EF hands, cysteine-rich C1 domain, or PDZ domain shown in SOS, GRF1, RasGRP, or PDZ-GEF (28). Therefore, RASGEF1A should exert different function from these Ras GEFs. Members of other Ras GEFs have different spectrums of substrates; for example, SOS1 modulates K-RAS, H-RAS, N-RAS, R-RAS2, R-RAS3, and Rac1 but not R-RAS, Ras-GRP2 stimulates K-RAS, H-RAS, N-RAS, R-RAS, R-RAS2, Rap1A, Rap2A but not H-RAS (29). Hence, RASGEF1A may also activate its specific substrates that have not been examined in our study.

Recent studies indicated that Ras signaling is involved not only in cell proliferation and survival but also in cell motility and cell adhesion. Active Ras interacts and regulates multiple downstream effectors that stimulate diverse signaling pathways. These include Raf/mitogen-activated protein (MAP)/extracellular signal-regulated kinase (ERK) kinase/ERK, PI3K/Akt, Raf/GDS/Ral, and Tiam1/Rac1 pathways. Accumulating evidences suggest that Raf/MAP/ERK kinase/ERK is involved in Ras-mediated cell proliferation. Because our Raf association assay showed an increase of activated Ras in the presence of RASGEF1A, downstream Raf/MAP/ERK kinase/ERK is supposed to be enhanced in cells expressing RASGEF1A. In accordance with this view, we have revealed that elevated expression of RASGEF1A is essential for the growth of ICC because suppression of RASGEF1A by introduction of specific siRNA decreased the growth of ICC cells. Although we tested oncogenic activity of RASGEF1A by colony formation assays using COS7 and NIH3T3 fibroblast cells, the number of colonies was unchanged between cells expressing exogenous RASGEF1A and controls. Introduction of RASGEF1A alone may not be sufficient to cause transformation in COS7 or NIH3T3 fibroblast cells. Alternatively, oncogenic activity of RASGEF1A may be tissue and/or cell type dependent. Because RASGEF1A has GEF activity to K-RAS and activated Ras transduces growth signaling, inhibition of GEF activity of RASGEF1A should be a promising therapeutic option for ICCs.

We additionally evidenced that exogenous expression of RASGEF1A enhanced cellular motility. This observation is in good agreement with the view that Ras activation plays a role in cell motility (13). GTP-bound form of Ras activates its downstream effectors, including Raf and PI3K (30, 31). Enhanced PI3K activity resulted in increased invasion of breast cancer cells, which was mediated through β3 integrin in a Rac1-dependent manner (32). PI3K also activates Rac GEFs and induces actin reorganization and membrane ruffling, which are associated with cell motility (33). Additionally, Ras enhances Tiam1, which results in activated form of Rac1, a key player for cellular migration and remodeling of actin cytoskeleton (34). Although further investigations on the mechanisms of the increased motility by RASGEF1A are necessary, elevated RASGEF1A expression is likely to be involved not only in development of ICC but also in its progression.

We investigated expression levels of RASGEF1A and mutation of K-RAS in ICC tissues, but we failed to detect any correlation between the expression and mutation (data not shown). Because RASGEF1A activates H-RAS and N-RAS in addition to K-RAS, enhanced RASGEF1A expression may have an additional role(s) in the carcinogenesis of ICC compared with mutation in K-RAS. Therefore, inhibitors of RASGEF1A may be effective anticancer drugs, although inhibitors of K-RAS failed to bring successful results in clinical studies.

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