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The chemosensory function of primary cilia regulates cholangiocyte migration, invasion and tumor growth

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Abstract

Cholangiocytes, the epithelial cells lining the biliary tree in the liver, express primary cilia that can detect several kinds of environmental signals and then transmit this information into the cell. We have previously reported that cilia are significantly reduced in cholangiocarcinoma (CCA) and that the experimental deciliation of normal cells induces a malignant-like phenotype with increased proliferation, anchorage independent growth, invasion and migration. Here, we tested the hypothesis that the chemosensory function of cholangiocyte primary cilia acts as a mechanism for tumor suppression. We found that in the presence of extracellular nucleotides, ciliary-dependent chemosensation of the nucleotides inhibited migration and invasion in normal ciliated cholangiocytes via a P2Y11 receptor and LKB1-PTEN-AKT dependent mechanism. In contrast, in normal deciliated cholangiocytes and CCA cells, the nucleotides induced the opposite effects, i.e. increased migration and invasion. As activation of LKB1 via a ciliary dependent mechanism was required for the nucleotide-mediated inhibitory effects on migration and invasion, we attempted to activate LKB1 directly, independent of ciliary expression, using the compound hesperidin methyl chalcone (HMC). We found that HMC induced activation of LKB1 in both ciliated and deciliated cells *in vitro*, resulting in the inhibition of migration and proliferation. Furthermore, using a rat syngeneic orthotopic CCA model, we found that HMC inhibited tumor growth *in vivo*. Conclusions: these findings highlight the importance of the chemosensory function of primary cilia for the control of migration and invasion, and suggest that by directly activating LKB1 and bypassing the need for primary cilia, it is possible to emulate this chemosensory function in CCA cells. These data warrant further studies for evaluating the possibility of using HMC as a novel therapy for CCA.

Keywords

Bile duct cancer; primary cilia; LKB1; purinergic receptors; hesperidin methyl chalcone

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INTRODUCTION

Cholangiocarcinoma (CCA) is a lethal and heterogeneous malignancy that can arise from different parts of the biliary tree, which can be classified as intrahepatic, perihilar, and distal CCA (1–3). Surgical treatment is the only possibility of cure for patients diagnosed with CCA. However, due to the absence of clinical manifestation in the early disease, most patients are diagnosed at later stages when tumors are not resectable and non-surgical therapeutic modalities are ineffective (4). Despite surgical treatment, the long-term outcomes may vary among patients depending on disease stage and the status of surgical margins, and recurrence is most likely (5). Therefore, it is imperative to find novel therapeutic targets for this devastating disease.

Cholangiocytes express primary cilia, organelles that function as mechano-, chemo-, and osmosensors (6). We previously described that the experimental deciliation of normal cholangiocytes induces a malignant-like phenotype characterized by increased migration, invasion, proliferation and anchorage independent growth. Moreover, we showed that CCA cells are devoid of primary cilia both *in vitro* and in human samples, suggesting that the loss of these organelles may be involved in CCA development and/or progression of the disease. (6–9).

Cholangiocytes are exposed to bile acids, nucleotides and other components of the bile (10). Nucleotides, in particular, are recognized by cholangiocytes purinergic receptors. Some of them localize in the primary cilia and are important for the chemosensory function of this organelle (11). ATP is one of the most important nucleotides released by both hepatocytes and cholangiocytes and functions as autocrine and paracrine stimuli for cholangiocytes bicarbonate secretion via purinergic receptors (P2) (12).

Herein we tested the hypothesis that the chemosensory function of cholangiocyte primary cilia, i.e. nucleotides detection, acts as a mechanism for tumor suppression by regulating cell growth, migration and invasion.

MATERIALS AND METHODS

Reagents.

Adenosine 5'-(T-thio)-triphosphate (ATPT), ADP- β -S, Adenosine-5'-O-(2-thiodiphosphate) and NKY80 (adenylyl cyclase 5 inhibitor) were purchased from Cayman Chemical (Ann Arbor, MI) and solubilized in water. H89 inhibitor was purchased from Selleckchem (Huston, TX). Hesperidin methyl chalcone purchased from Sigma (St. Louis, MO) and solubilized in water for *in vitro* or saline solution for *in vivo* experiments. Suramin sodium salt, ATP and Apyrase were purchased from Sigma.

Cells lines and cell culture.

Normal human ciliated cholangiocytes (NHC) and experimentally deciliated normal human cholangiocytes (NHC IFT88, shRNA-IFT88) cells (7, 8) were incubated at 37°C in a humidified atmosphere containing 5% CO₂. CCA cells (KMCH, OZ, EGI-1), the human intrahepatic CCA cell line (iCCA) HUCCT1, and the rat CCA cell line BDNeu, were

maintained in F-12 media supplemented with 10% FBS, penicillin (100 U/ml), streptomycin (100 µg/ml) and gentamicin (10 mg/ml) (7, 8).

Immunofluorescence.

Cells were seeded and grown on coverslips to confluence. Then, cells were incubated for 24 h in opti-MEM to induce ciliary expression, and finally washed and fixed with ice-cold methanol, and permeabilized with PBS Tween 0.1%. After blocking cells were incubated with primary antibodies rabbit anti LKB1 (1:100; Cell Signaling, Danvers, MA), anti-mouse acetylated alpha tubulin (1:200; Sigma), anti-mouse gamma tubulin (1:100; Sigma), and ARL13b (1:100; Santa Cruz, Dallas, TX) overnight at 4°C, washed and incubated with Alexa Fluor 594 conjugated goat anti-rabbit and Alexa Fluor 488 conjugated goat anti-mouse (1:100 Life Technologies, Carlsbad, CA) for 1 h. The nuclei were visualized with DAPI and the immunostainings were viewed and documented using a Zeiss Axio Observer inverted microscope. At least 200 cells were analyzed from each sample and the images were taken at a 630X magnification.

F-actin staining.

Cells were seeded at 150000 cells/ml and incubated overnight on coverslips. The following day, the media was changed to opti-MEM and cultured for 24 h, treated with or without ATP 100 µM for 30 min and then fixed with 3.7% ice cold paraformaldehyde in PBS for 10 min and permeabilized with 0.1% Triton-X100 in PBS. Afterwards, cells were stained with Alexa Fluor 488 Phalloidin (Thermo Scientific, Waltham, MA) for 30 min. Actin filaments were visualized and photographed with Zeiss Axio Observer inverted microscope. The number of filopodia per cell was determined by counting only filopodia crossing the cell edge and having fluorescence intensity 1.2× above background. Two researchers performed both measurements independently.

Stable cell lines.

Lentiviruses carrying specific shRNAs were used to infect cells that were then selected with puromycin. Experimental deciliated cholangiocytes were generated by specific shRNAs to IFT88 as previously described (7). To confirm the lack of cilia in NHC IFT88 and HUCCT1 cells, we stained cilia with acetylated α -tubulin as a ciliary marker and γ -tubulin as basal body marker. The results showed that NHC IFT88 and HUCCT1 do not express primary cilia as previously described by us (7) (Supplemental Figure 1).

Quantitative Real-Time PCR Assay.

Total RNA was performed by lysing cells in TRIzol reagent (Invitrogen, Carlsbad, CA), according to the supplier's instructions. cDNA was synthesized by a SuperScript III First-Strand using random hexamer primers and then, Quantitative real-time PCR was conducted using a CFX Connect Real-Time system (Bio-Rad, Hercules, CA). GAPDH was used as an internal control.

Cell proliferation assay.

CellTiter 96 AQueous One Solution Cell Proliferation Assay (MTS) was purchased from Promega (Madison, WI) and used to evaluate cell proliferation. Alternatively, cell proliferation was analyzed by live-cell imaging assays. A total of 5×10^3 cells in 200 μ L of media were seeded in 96-well plates respectively and incubated in an IncuCyte (Essen BioScience, Ann Arbor, MI). Images were captured every 4 h over 48 h to monitor proliferation.

Migration Assay.

Briefly, cells were cultured in 6-well plates and a 200 μ L pipet tip was used to scrape the cell monolayer in a straight line. At initial (0 h) and 24 h time points, scratches were observed and photographed using an inverted microscope. The migration distance between both sides of the scratch was measured. Alternatively, Real time cell migration assays using IncuCyte live imaging were performed. Briefly, 45,000 cells/well were cultured in a 96-well ImageLock Microplate (Essen BioScience) and then a scratch made using a 96-pin WoundMaker (Essen Biosciencer). Wound images were automatically acquired and registered by the IncuCyte software system. The data were analyzed using an integrated metric: relative wound density as previously described (13).

Matrigel invasion assay.

The invasion assay was performed using the Corning® BioCoat™ Matrigel® Invasion Chamber. Cells were suspended at 25,000 cells/well in its respective serum free media in the presence or absence of ATP 100 μ M or other compounds and seeded into the upper chamber well coated with Matrigel. Simultaneously, media supplemented with 10% FBS was placed in the lower well of the chamber. The cells were allowed to invade the Matrigel for 24 h at 37°C. After removal of the cells from the upper surface of the chamber, the membranes were stained with crystal violet and observed using an inverted microscope. The number of invading cells per field was assessed by counting 10 random fields at 200X magnification.

Apoptosis Assay.

In vitro apoptosis was assessed by flow cytometry. Cell death was detected by fluorescein isothiocyanate (FITC)-annexin V/propidium iodide (PI) staining. Fluorescence signals were detected on a FACScan (BD Bioscience, San Jose, CA). *In vivo* apoptosis was assessed by FragEL™ DNA Fragmentation Detection Kit, Fluorescent - TdT Enzyme (Calbiochem, San Diego, CA) according to manufacturer's instructions.

Western Blot Analysis.

Cells were washed with ice-cold PBS and lysed with RIPA lysis buffer. Proteins in the supernatants were separated by 4–20% SDS-polyacrylamide gel electrophoresis and transferred to 0.45 μ m nitrocellulose membranes. After blocking, the membranes were incubated with the appropriate primary antibodies against IFT88 (ProSci, Poway, CA), LKB1, p-LKB1(S428), AKT, p-AKT(S473), PTEN and p-PTEN(Ser380/Thr382/Thr383) (Cell Signaling), FAK (Cell Signaling) and GAPDH (ProSci) at 4°C overnight. After

washing, the blots were subsequently incubated with horseradish peroxidase- (HRP-) conjugated secondary antibodies. Protein signals were developed using the Clarity Western ECL Substrate Bio-Rad (Bio-Rad Laboratories, Hercules, CA, USA). All experiments were repeated at least three times.

Orthotopic model of Cholangiocarcinoma.

All animal experimentation was performed according to the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) using protocols approved by Institutional Animal Care and Use Committee (IACUC). Briefly, and as previously described by Sirica et al., (14) *in vivo* cell transplantation was carried out in young adult Fisher 344 male rats (Envigo, Madison, WI) with initial mean body weights typically ranging between 200–225 g. BDNeu cells (kindly provided by Dr. Alphonse E. Sirica, Department of Pathology, Virginia Commonwealth University-Medical College of Virginia, Richmond, VA) were inoculated at 1×10^6 suspended in 0.1 ml PBS under the capsule of the left hepatic lobe after ligation of the left bile duct. After 6 days, rats were treated for 8 days with daily IP injection of HMC 100 mg/Kg or saline solution.

Statistical Analysis.

Data are expressed as the mean $\pm$ standard error of the mean and statistically analyzed by Student's t-test. A level of * $p < 0.05$ and ** $p < 0.01$ were considered to be significant.

RESULTS

A chemosensory function of cholangiocytes primary cilia, nucleotide detection, regulates migration and invasion in a ciliary dependent manner.

We previously showed that cholangiocyte primary cilia can detect nucleotides (11). Therefore, we explored the effect of ATP on cell migration, invasion and proliferation. We evaluated the effect of ATP on cell migration by two different techniques. First, IncuCyte live cell imaging showed that ATP inhibited migration in normal ciliated cholangiocytes but, in contrast, induced migration in normal deciliated and iCCA cells (Supplemental Figure 2A,B). Then, migration was tested by wound healing assay. Consistently, treatment with ATP inhibited migration in ciliated cells while induced migration in deciliated and iCCA cells (Figure 1A). Moreover, to confirm that ATP is the molecule responsible for the observed effect on cell migration, we performed a series of experiments using ADP and Apyrase, an ATP-diphosphohydrolase that catalyzes the sequential hydrolysis of ATP to ADP and ADP to AMP releasing inorganic phosphate. The results showed that Apyrase abolished the effect of ATP on migration, while treatment with ADP did not have any significant effect (Figure 1B, and Supplemental Figure 2C). To assess the effect of ATP on invasion, cells were treated with vehicle or ATP for 24 h in invasion chambers coated with Matrigel. The results showed that ATP induced inhibition of invasion in normal ciliated cells, while ATP had no effect on normal deciliated cholangiocytes and induced invasion in iCCA (Figure 1C). Finally, we evaluated the effect of ATP on cell proliferation by both real time imaging by IncuCyte (Figure 1D) and by MTS (Figure 1E) in which no significant differences were observed.

Taken together, these data suggest that ATP inhibits migration and invasion in a ciliary dependent manner, with an inhibitory effect in the presence of primary cilia but a stimulatory effect in the absence of primary cilia.

The ciliary-dependent ATP-induced regulation of migration relies on the activation of LKB1 by a mechanism dependent on purinergic receptors and PKA.

To start exploring the mechanisms underlying the ciliary-dependent regulation of migration and invasion, we focused on LKB1, a serine/threonine kinase described to be involved in cell polarity, migration and invasion (15–17). Furthermore, LKB1 expression in primary cilia was reported in MDCK cells (18). However, its expression in cholangiocytes is unknown. Therefore, we evaluated the expression of LKB1 in normal cholangiocytes and four different CCA cell lines by western blotting (Figure 2A). Second, we evaluated the expression and localization of LKB1 by immunofluorescence in normal cholangiocytes and found that LKB1 is localized in the cytoplasm and heavily enriched in primary cilia (Figure 2B).

Additionally, we tested the effects of ATP on the phosphorylation of LKB1 at S428, which is required for activation of LKB1 (19–21). The results showed that ATP induces a significant increment of p-LKB1 only in ciliated cells (Figure 2C). Furthermore, treatment with suramin, a purinergic receptor antagonist, abolished the ATP-induced phosphorylation of LKB1 (Figure 2D). As ATP activates purinergic receptors, which are associated with different Gα proteins, we evaluated the possibility that ATP induces activation of Gαs which in turn may activate PKA. Thus, we pre-treated the cells with a selective cell permeable inhibitor of PKA, H89, which resulted in the abolishment of ATP-induced LKB1 phosphorylation (Figure 2D). In addition, we assessed the effect of H89 and suramin on cell migration and invasion in ciliated cells. Consistently, the results showed that both compounds abolished the inhibition of migration and invasion induced by ATP (Supplemental Figure 3A). Then, to confirm that LKB1 is required for inhibition of migration, we generated normal cholangiocytes stably transfected with specific shRNA, NHC LKB1 (NHC shRNA-LKB1) and assessed the effects of ATP on migration. The results showed that knockdown of LKB1 abolished the inhibition of migration normally observed in control cholangiocytes (Figure 2E and Supplemental Figure 3B). Taken together, these data suggest the ciliary-dependent ATP-induced regulation of migration progresses by a mechanism dependent on LKB1 activation via purinergic receptors and PKA.

The ciliary-dependent activation of LKB1 and migration regulation is mediated by ciliary P2Y11.

Since ATP induces activation of PKA, which is dependent on cAMP, we focused on human purinergic receptors previously described to be associated with the cAMP signaling pathway, i.e. P2Y11 and P2Y1 (22–24). Therefore, we generated the stable transfected normal cell lines NHC P2Y1 (NHC shRNA-P2Y1) and NHC P2Y11 (NHC shRNA-P2Y11) knockdowns (Figure 3A). We found that the ATP-induced activation of LKB1 was abolished by P2Y11 knockdown but not by P2Y1, suggesting that this regulation is mediated by P2Y11 (Figure 3B). Importantly, the downregulation of P2Y11 also inhibited the regulation of ATP-induced migration (Figure 3C and Supplemental Figure 4A). In addition, we evaluated the effect of P2Y11 knockdown on cell proliferation and no significant difference

was found compared with NHC SCR (Figure 3D). Finally, we assessed the expression of P2Y11 in NHC cells by immunofluorescence. using two different ciliary markers, acetylated- α -tubulin and ARL13b. We found that P2Y11 colocalizes with primary cilia in both independent experiments (Figure 3E). Taken together, these data indicate that the ciliary-dependent ATP-induced inhibition of migration depends on the ciliary purinergic receptor P2Y11.

Ciliary-dependent ATP detection induces PTEN stabilization and AKT inhibition.

To elucidate the mechanism by which the ciliary-dependent detection of ATP induces inhibition of migration and invasion in normal cholangiocytes, we evaluated the possibility that activated LKB1 inhibits AKT, which is a key regulator of several cellular pathways involved in metabolism, cell survival, migration and invasion among others. AKT phosphorylation at S473 is associated with increment in its activity and is required for the rearrangement of the cytoskeleton during migration. The results showed that treatment with ATP induced dephosphorylation of AKT only in ciliated cells (Figure 4A), suggesting that AKT is inhibited by the ciliary-dependent activation of LKB1. LKB1 has kinase activity and phosphorylates different downstream proteins and the principal inhibitor of the PI3K-AKT pathway is PTEN; therefore, we evaluated the possibility that activated LKB1 phosphorylates and activates PTEN. The results showed that treatment with ATP induced increment of p-PTEN only in ciliated cells. Moreover, phosphorylation of PTEN induced a significant increment of the total PTEN (Figure 4B). Taken together, these data suggest ciliary-dependent ATP-induced activation of LKB1 stimulates phosphorylation and stabilization of PTEN in ciliated cells, which in turn induces the inhibition of AKT by dephosphorylation.

ATP activates ciliary adenylyl cyclase 5 to inhibit migration in ciliated cholangiocytes.

Because cholangiocytes express adenylyl cyclase 5 (AC5), which participates in ATP response (25, 26), and P2Y11 is associated with Gas protein (27, 28), we evaluated the possibility that ATP stimulates AC5 to induce LKB1 phosphorylation through PKA activation. Thus, we evaluated the effect of NKY80, an AC5 inhibitor, on the ATP-induced LKB1 phosphorylation in normal ciliated cholangiocytes by western blotting. The results showed that treatment with NKY80 abolished LKB1 phosphorylation induced by ATP (Figure 5A). Moreover, we found that NKY80 abolished the inhibition of migration induced by ATP in normal ciliated cholangiocytes (Figure 5B).

The ciliary-dependent ATP detection inhibits actin polymerization and filopodia in ciliated cholangiocytes.

Enhanced cell migration and invasion is frequently associated with increased filopodia production, which occurs in an active AKT-dependent manner (29). Therefore, we evaluated the ability of the cells to reorganize the cytoplasm and protrusions when they are exposed to ATP. The results showed the incidence of filopodia was reduced in parallel to actin depolymerization in ciliated cells, while treatment with ATP was unable to induce inhibition of filopodia formation in both deciliated and iCCA cells (Figure 6A, B). Moreover, to determine the involvement of LKB1 in the inhibition of filopodia formation, we tested the effect of ATP on filopodia formation in NHC LKB1 cells. The results showed that treatment

with or without ATP on LKB1-downregulated cells had no significant difference in filopodia formation (Figure 6A, B). Thus, LKB1 is required for inhibition. Since focal adhesion kinase (FAK) is required for filopodia formation (30), we evaluated the effect of ATP treatment on the levels of this kinase. The results showed that treatment with ATP induced rapid degradation of FAK in ciliated cells, while ATP did not show any significant change in FAK levels in deciliated and iCCA cells (Figure 6C). Taken together, these data suggest that the ciliary-dependent detection of ATP inhibits cholangiocyte migration and invasion via a mechanism involving the regulation of actin polymerization, filopodia formation, and focal adhesion kinase activities.

HMC directly activates LKB1 in normal deciliated cholangiocytes and CCA cells, emulating the chemosensory function of primary cilia and tumor growth both *in vitro* and *in vivo*.

Chalcones have been described to activate LKB1 (31). Therefore, we evaluated the effect of hesperidin methyl chalcone (HMC), a semisynthetic water-soluble derivative obtained from hesperidin, *in vivo* and *in vitro*. To assess if HMC can activate LKB1 in cholangiocytes independent of ciliary expression, we treated normal ciliated, experimentally deciliated cells, and iCCA cells with HMC and evaluated phosphorylation of LKB1. We found treatment with HMC induced phosphorylation of LKB1 independent of ciliary expression (Figure 7A). Furthermore, HMC induced inhibition of proliferation and migration in normal and deciliated cholangiocytes and iCCA cells *in vitro* (Figure 7B,C and Supplemental Figure 4B), emulating the chemosensory function of primary cilia. Then, to confirm that LKB1 is required for HMC-induced inhibition of migration, we evaluated the effect of HMC in NHC LKB1 and no significant differences were found (Figure 7D and Supplemental Figure 4C). Finally, we also assessed the effect of HMC on apoptosis. The results showed that HMC induced apoptosis in normal and deciliated cholangiocytes and iCCA cells, but has no effect on LKB1-shRNA normal cholangiocytes (Figure 7E and Supplemental Figure 5). Importantly, to evaluate the potential use of HMC as a therapeutic approach, we assessed the effects on tumor progression *in vivo* utilizing an orthotopic syngeneic rat model of CCA (9). The results showed that treatment with 100 mg/kg HMC for 8 days induced a significant decrease in the tumor size compared to the control vehicle treated group (Figure 7F). Furthermore, consistently with the *in vitro* experiments, HMC induced significant apoptosis compared to controls (Figure 7G). Taken together, these data suggest that the activation of LKB1 by HMC emulates the chemosensory function of primary cilia and may be a potential therapeutic approach for CCA.

DISCUSSION

The key findings presented here relate to the novel ciliary-dependent regulation of cholangiocyte migration and invasion. We found that: (1) nucleotides inhibit migration and invasion in ciliated cells but induce them in experimentally deciliated cells and iCCA cell lines that do not express primary cilia; (2) the ciliary-dependent nucleotide-induced regulation of migration depends on the ciliary purinergic receptor P2Y11, PKA, and activation of LKB1 by phosphorylation; (3) the ciliary-dependent nucleotide-induced activation of LKB1 regulates the PTEN-PI3K-AKT signaling axis; (4) this chemosensory function of primary cilia regulates migration and invasion via F-actin and filopodia

formation inhibition; (5) the direct activation of LKB1 by HMC can emulate the ciliary chemosensory function and inhibit tumor growth both *in vitro* and *in vivo*.

In the present study, we demonstrated that extracellular nucleotides induce inhibition of migration and invasion in normal ciliated cholangiocytes, while induces the opposite effect in experimental deciliated cholangiocytes and iCCA. The inhibition of migration was associated with activation of LKB1 via a ciliary-dependent mechanism that involved the activation of P2Y11 and PKA. There are eight P2s that are expressed in mammalian cells, among them P2Y1, P2Y2 and P2Y11 are activated by ATP (32). These receptors are coupled to G α protein. P2Y1 is coupled to G α_q whose activation activates phospholipase C that results in the formation of the second messengers 2,3-diacylglycerol and inositol 1,45-triphosphate, which activate protein kinase C (PKC). P2Y2 is coupled to G α_q , G α_0 and G α_{12} . While the activation of G α_0 leads to activation of RhoA, activation of G α_{12} leads to activation of Rac, which participates in the cytoskeleton rearrangement involved in migration (33). Finally, P2Y11 is coupled to G α_q and G α_s . Activation of G α_s stimulate adenylyl cyclase (AC) to increase production of cAMP, resulting in activation of PKA (33, 34). Once activated, PKA regulates the activity of several targets and the role in migration and cytoskeletal rearrangements is dichotomic. PKA can phosphorylate the integrin α - and β_1 complex that blocks paxillin binding, which is required for cell migration and increases lamellipodial stability (35), and can also phosphorylate the cytoskeletal regulatory protein VASP, which is localized to focal adhesions, modifying its interaction with other proteins such as Vinculin and regulating adhesion. Even though our data show ATP inhibits migration by a ciliary dependent mechanism that involves PKA-LKB1, we cannot exclude other downstream targets of PKA.

Consistent with our observations, ATP has been reported to induce hepatocellular carcinoma cell migration via P2Y11 (36). Our results showing that ATP induces migration in deciliated cells and iCCA cells are in line with this data since hepatocellular carcinoma cells, like normal hepatocytes and CCA cells, do not express primary cilia. Our data highlight the importance of primary cilia expression for the regulation of migration and invasion. In particular, the nucleotide chemosensory function via ciliary associated P2Y receptors can activate ciliary associated protein targets such as PKA and adenylyl cyclase. Thus, the loss of the primary cilia in tumor cells abolishes these ciliary-dependent migration and invasion regulatory functions.

LKB1 is a tumor suppressor protein that regulates several signaling pathways. Most of the studies on migration and invasion in relation to LKB1 show that LKB1 expression is required to regulate these processes. However, to our knowledge, no study shows the importance of LKB1 activation for migration and invasion regulation. The fact that ciliated cholangiocytes with LKB1 shRNA knockdown migrate at the same rate as scramble controls when treated with ATP highlights the importance of LKB1 activation in this process. Recently, Zhang et al showed that LKB1 loss in melanoma cell lines does not promote migration and invasion, however, loss of LKB1 cooperates with other genetic alterations to induce migration and invasion (37). Thus, primary cilia may function as a tumor suppressor organelle, whose loss cooperates with other genetic events to promote progression of the disease.

LKB1 was suggested to antagonize the PI3K-AKT pathway via activation and stabilization of the tumor suppressor PTEN by phosphorylation. There is a cluster of PTEN phosphorylations (Ser380/Thr382/Thr383) reported to be phosphorylated by LKB1, which induce a conformational change that stabilizes the protein but decreases its functionality (38). Our data show that LKB1 phosphorylates PTEN at this cluster leading to its stabilization. However, LKB1-induced phosphorylation and stabilization of PTEN results in dephosphorylation of AKT, suggesting that phosphorylation in this cluster induces stabilization and activation of PTEN that leads to inhibition of the PI3K-AKT pathway in normal cholangiocytes. Consistently, Wong et al reported that activation of LKB1 induces phosphorylation and stabilization of PTEN to inhibit AKT in prostate cancer cells (21). In contrast, in normal experimentally deciliated cells and iCCA cells in which LKB1 is unable to be activated by the ciliary-dependent mechanism, PTEN is not activated. On the other hand, Tian et al reported that PTEN phosphatase activity is required to inhibit migration and invasion in hepatocellular carcinoma cells via inhibition of AKT and MMP-2 and MMP-9 expression (39).

Cell migration is important for cancer progression and is a prerequisite for the spread of cancer. Migration includes multistep cellular processes such as cell polarization, protrusion, adhesion and de-adhesion that relies on the coordinated function of actin filaments. It is known that filopodia originates from the lamellipodial actin meshwork and are formed by polymerized actin filaments, which are organized into parallel bundles (40), and increased filopodia formation promotes migration and invasion. Indeed, abundant filopodia is a marked characteristic of invasive carcinoma cells. In this study, we found that the ciliary dependent inhibition of migration induced by ATP in normal cholangiocytes was associated with inhibition of both F-actin and filopodia formation. Since filopodia is associated with the malignant phenotype of cancer cells and tumor cell transformation, migration and invasion, dysfunction of the cell chemosensory function caused by ciliary loss may contribute to increased filopodial activity and motility that is associated with increased metastatic and invasive potential of the cell (41).

Therefore, our data, showing that LKB1 is activated by a ciliary-dependent mechanism that regulates migration and invasion in normal cholangiocytes, suggest the loss of primary cilia in iCCA may contribute not only to cholangiocarcinoma development as we previously described (6, 8), but also to metastasis and progression of the disease. Thus, we propose a model where nucleotides inhibit migration and invasion, mechanisms that are lost in cholangiocarcinoma cells upon primary cilia resorption and lead to progression of the disease (Figure 8). In line with this, a recent publication shows that patients with iCCA and low levels of LKB1 protein in tumors have worse prognoses compared to patients with higher LKB1 expression, suggesting that LKB1 is involved, at least, in the progression of the disease (42); even more, the activation of LKB1 by phosphorylation at S428 mediates the cell cycle arrest induced by the combined lovastatin and gefitinib treatment in HuH-28 cells (43). Therefore, therapies based on activation of LKB1 via ciliary independent mechanism may be a feasible potential therapeutic approach for CCA treatment. In this study, we successfully used HMC as a therapeutic approach in a pre-clinical CCA rat model. Even though these experiments did not allow to assess the invasiveness of the tumor and metastasis, the net effect on tumor size suggested that LKB1 activation via HMC may be a

good therapeutic alternative. Flavonoids are natural compounds present in fruits and vegetables with interesting biological properties and possible medical applications such as anti-inflammatory, anti-hepatotoxic, anti-microbial, anti-oxidant and anti-tumoral among others (44, 45). The effect of flavonoids for cancer chemoprevention and therapy is very significant (46, 47). Hesperidin is a flavonone glycoside, a subclass of flavonoids that is found in almost all citrus fruits. Epidemiological studies and basic researches show the many benefits of hesperidin intake in daily diet on cancer (48, 49). Even though our data shows that HMC effects on migration, invasion, and apoptosis are dependent on the expression of LKB1, we cannot rule out the involvement of other molecular pathways in the *in vivo* and *in vitro* experiments because hesperidin and chalcones, in general, are molecules with broad mechanisms of action. Furthermore, our *in vivo* experiments are not able to

In summary, we described a novel ciliary-dependent mechanism regulating cholangiocyte migration and invasion and tumor growth (Figure 8), and it is possible to bypass the need of primary cilia by directly activating LKB1 with HMC, which may be a potential therapeutic strategy for CCA.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations.

CCA	Cholangiocarcinoma
HMC	hesperidin methyl chalcone
iCCA	intrahepatic cholangiocarcinoma
LKB1	Liver Kinase B1
FAK,	focal adhesion kinase

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Figure 1. Effect of ATP in migration, invasion, and proliferation.

(A) Effect of ATP in cell migration comparing normal ciliated cholangiocytes (NHC SCR), normal deciliated cholangiocytes (NHC IFT88) and the CCA cell line HUCCT1. Representative images obtained from the wound healing assay and bar graphs showing the distance migrated by the cells relative to control (vehicle) in 24 h are depicted (** $p < 0.01$, $n = 3$). (B) Cell migration analysis by wound healing assay showing the effects of ATP, ADP, Apyrase and their combinations. Bar graph shows the distance migrated by the cells relative to control (vehicle) in 24 h (* $p < 0.05$, ** $p < 0.01$, $n = 3$). (C) Invasion assay, representative

pictures and bar graph showing the percentage of invasion in 24 h (** $p < 0.01$, $n = 3$). **(D)** Proliferation rates were assessed in real time using IncuCyte or **(E)** by MTS assay. Results are expressed in % proliferation relative to control (vehicle).

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Figure 2. Primary cilia expression, LKB1 expression, and Effect of ATP on LKB1 phosphorylation.

(A) Expression of LKB1 was evaluated in normal cholangiocytes (NHC, H69), experimentally deciliated cholangiocytes (NHC IFT88, H69 IFT88) and CCA cell lines (KMCH, HUCCT1, OZ, EGI-1) by western blotting. (B) Presence of LKB1 in primary cilia using acetylated α -tubulin or ARL13b as ciliary markers. The expression was assessed by confocal immunofluorescence on scramble normal cholangiocytes (NHC SCR). (C) Western blot comparing the effect of ATP on LKB1 phosphorylation in normal cholangiocyte cell

line (NHC SCR), experimentally deciliated cholangiocytes (NHC IFT88) and iCCA cell line (HUCCT1) (** $p < 0.01$, $n = 3$). **(D)** Western blots for p-LKB1 and total LKB1 showing the effect of pre-treatment (30 min) with suramin 100 μM or H89 20 μM on the treatment with ATP for 30 min. **(E)** NHC cells transfected with shRNA-LKB1 (NHC LKB1) or shRNA-scramble (NHC SCR). Expression levels of LKB1 protein were evaluated by western blot (** $p < 0.01$, $n = 3$) and the effect of ATP on migration was evaluated by wound healing assay (** $p < 0.01$, $n = 3$).

Figure 3. ATP phosphorylates LKB1 by P2Y11 activation.

(A) NHC cells were transfected with shRNA-P2Y1 (NHC P2Y1), shRNA-P2Y11 (NHC P2Y11), or shRNA-scramble (NHC SCR) and the expression levels of P2Y1 and P2Y11 were evaluated by qPCR (** $p < 0.01$, $n = 3$). (B) Western blot showing the effect of ATP and ADP on LKB1 phosphorylation in NHC P2Y1 and NHC P2Y11 cells. (C) Wound healing assay showing the effect of ATP on migration in NHC P2Y11 cells compared to NHC SCR controls. (D) Proliferation rates comparing NHC SCR and NHC P2Y11 assessed in real time using IncuCyte. (E) P2Y11 expression was assessed by confocal immunofluorescence using ARL13b as ciliary marker.

Figure 4. Effect of ATP on PTEN and AKT.

(A,B) Western blots showing the effect of ATP for 30 minutes on AKT and PTEN phosphorylation in normal ciliated cholangiocytes (NHC SCR), experimentally deciliated cholangiocytes (NHC IFT88), and the iCCA cell line HUCCT1. Bar graph shows densitometry expressed as % of phosphorylated/total ratios in control conditions (* $p < 0.05$, $n = 3$) (** $p < 0.01$, $n = 3$).

Figure 5. Effect of AC5 inhibitor on ATP treatment.

(A) Western blot showing the effect of AC5 inhibitor on ATP treatment in normal ciliated cholangiocytes (NHC SCR). Bar graphs show densitometry expressed as % phosphorylated/total ratio compared to controls (**p<0.01, n=3). (B) Effect of AC5 inhibitor on cell migration evaluated in NHC SCR by wound healing assay, bar graph shows the distance migrated by the cells relative to control (vehicle) in 24 h (**p<0.01, n=3).

Figure 6. Effect of ATP on filopodia.

(A) F-actin and filopodia were evaluated by Phalloidin staining (red) in normal ciliated cholangiocytes (NHC SCR), experimentally deciliated cholangiocytes (NHC IFT88), the iCCA cell line (HUCCT1), and normal ciliated cholangiocytes with LKB1 knockdown (NHC LKB1), in the presence or absence of ATP. Nuclei were stained in blue with DAPI (Magnification X600). (B) Quantification of filopodia after treatment with ATP for 30–60 min is shown in bars representing the average number of filopodia per cell (** $p < 0.01$ $n = 67$).

(C) The effect of ATP for 60 min on FAK expression was evaluated by western blot (**p<0.01 n=3).

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Figure 7. Effect of HMC on LKB1 phosphorylation, proliferation, migration, and apoptosis. (A) Western blot analysis and graph bar showing the effect of HMC 1 mM for 30 min on LKB1 phosphorylation in normal cholangiocytes (NHC SCR), experimentally deciliated cholangiocytes (NHC IFT88 (shRNA-IFT88)) and iCCA cell lines (HUCCT1) (**p<0.01 n=3). (B) Effect of HMC 1 mM for 24–48 h on proliferation. Rates were assessed by MTS assay (**p<0.01, n=24). (C) Effect of HMC 1mM on migration. Bar graph shows the distance migrated by the cells relative to control (vehicle) in 48 h evaluated in NHC SCR, NHC IFT88 (shRNA-IFT88), and HUCCT1 cells assessed by wound healing assay

(** $p < 0.01$, $n = 3$). **(D)** Effect of HMC 1mM on NHC LKB1 (shRNA-LKB1) migration, bar graph shows the distance migrated by the cells relative to control (vehicle) in 24 h (** $p < 0.01$, $n = 3$). **(E)** Effect of HMC 1 mM on apoptosis evaluated in 24 h by flow cytometry using (FITC)-annexin V/propidium iodide (PI) staining (** $p < 0.01$, $n = 3$). **(F)** The anti-tumoral effect of HMC was assessed *in vivo* using a rat orthotopic syngeneic CCA model. Animals were treated for 8 days with 100mg/kg HMC or vehicle after 6 days of tumor initiation. Bar graph shows tumor/liver rate (%) comparing tumors treated with saline solution (control) and HMC (* $p < 0.05$, $n = 4$). **(G)** Apoptosis in tumor tissues were assessed by DNA fragmentation detection where green dots are cells in apoptosis. Bar graph shows % nuclei/field in control and treated tumors (* $p < 0.05$, $n = 4$).

Figure 8. Proposed model.

Nucleotides are detected by P2Y11 receptor localized in the primary cilium activating AC5, which induces increases in the levels of cAMP and activation of PKA. Once activated PKA phosphorylates and activates LKB1, LKB1 phosphorylates and stabilizes PTEN, leading to AKT inhibition. Inhibited AKT is unable to phosphorylate F-actin that is required to reorganize the cytoplasm and protrusions in migrating cells. HMC can activate LKB1 via a ciliary-independent mechanism emulating the chemosensory function of primary cilia.