Research Article

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Non-canonical Hedgehog signaling contributes to chemotaxis in cholangiocarcinoma

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Background & Aims: The Hedgehog signaling pathway contributes to cholangiocarcinoma biology. However, canonical Hedgehog signaling requires cilia, and cholangiocarcinoma cells often do not express cilia. To resolve this paradox, we examined non-canonical (G-protein coupled, pertussis toxin sensitive) Hedgehog signaling in cholangiocarcinoma cells.

Methods: Human [non-malignant (H69), malignant (HuCC-T1 and Mz-ChA-1)] and rat [non-malignant (BDE1 and NRC), and malignant (BDEneu)] cell lines were employed for this study. A $BDE^{\Delta Loop2}$ cell line with the dominant-negative receptor Patched-1 was generated with the *Sleeping Beauty* transposon transfection system.

Results: Cilia expression was readily identified in non-malignant, but not in malignant cholangiocarcinoma cell lines. Although the canonical Hh signaling pathway was markedly attenuated in cholangiocarcinoma cells, they were chemotactic to purmorphamine, a small-molecule direct Smoothened agonist. Purmorphamine also induced remodeling of the actin cytoskeleton with formation of filopodia and lamellipodia-like protrusions. All these biological features of cell migration were pertussis toxin sensitive, a feature of G-protein coupled (G_is) receptors. To further test the role of Hedgehog signaling *in vivo*, we employed a syngeneic orthotopic rat model of cholangiocarcinoma. *In vivo*, genetic inhibition of the Hedgehog signaling pathway employing $BDE^{\Delta Loop2}$ cells or pharmacological inhibition with a small-molecule antagonist of Smoothened, vismodegib, was tumor and metastasis suppressive.

Conclusions: Cholangiocarcinoma cells exhibit non-canonical Hedgehog signaling with chemotaxis despite impaired cilia expression. This non-canonical Hedgehog signaling pathway appears to contribute to cholangiocarcinoma progression, thereby, supporting a role for Hedgehog pathway inhibition in human cholangiocarcinoma.

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Keywords: Dominant-negative Ptch1; Biliary tract cancer; G-protein coupled receptor; Patched-1; Smoothened.

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Abbreviations: CAFs, cancer-associated fibroblasts; CCA, cholangiocarcinoma; DAPI, 4′-6-diamidino-2-phenylindole; FBS, fetal bovine serum; Hh, Hedgehog; Gli, glioma-associated transcriptional factor; Ptch1, Patched-1; PTX, pertussis toxin; qRT-PCR, quantitative reverse transcription polymerase chain reaction; PBS, phosphate-buffered saline; Shh, Sonic Hh; Smo, Smoothened.

Introduction

Cholangiocarcinoma (CCA) is a lethal primary hepatobiliary malignancy [1]. Therapeutic advances for CCA will require a deeper understanding of the molecular pathways driving progression of this neoplasm. However, knowledge regarding the pivotal signaling pathways responsible for CCA biology is incomplete. Several studies suggest activation of the Hedgehog (Hh) signaling pathway as a key feature of CCA progression [2,3].

In mammalian cells, Hh signaling comprises complex relationships between the Hh ligands [Sonic Hh (Shh), Indian Hh, and Desert Hh] and two plasma membrane proteins, Patched-1 (Ptch1) and Smoothened (Smo) [4] Canonically, binding of the Hh ligand to Ptch1 leads to derepression and translocation of Smo to cilia resulting in activation of the glioma-associated transcriptional factors (Gli1, Gli2, and Gli3) [4]. Smo translocation into the cilia membrane appears to be requisite for Gli activation [5–9]. This facet of Hh signaling results in a conundrum. Aberrant activation of Hh pathway signaling is a characteristic of many malignancies [10], and tumor progression is often Hh signaling responsive despite the lack of cilia expression by many cancers. This puzzle is particularly relevant to CCA cell biology. For example, Gli activation can be identified in the KMCH CCA cell line, which variably forms rudimentary or incomplete cilia and robustly expresses Gli1 [11,12]. However, most CCA cell lines appear to be Hh responsive despite the absence of cilia and minimal Gli expression.

Recently, a non-canonical pathway for Hh signaling was identified in drosophila cells lacking cilia, namely, a non-Gli, inhibitory G-protein (G_is) coupled pathway [7]. The identified downstream effect of this non-canonical Hh signaling activation is cytoskeleton remodeling and cell migration via involvement

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of the small Rho GTPases Rac1 and RhoA [13]. This pathway is pertussis toxin (PTX) sensitive, a feature of G-protein coupled receptors involving G_is. A role for this non-canonical Hh signaling pathway in mammalian, especially CCA, cell biology remains plausible, but has yet to be examined.

Herein, we identify Hh signaling in CCA cells despite failure to express cilia. The pathway is inhibitable by the small-molecule Smo inhibitor vismodegib, is PTX sensitive, blocked by forced expression of a dominant-negative Ptch1 construct, and is not associated with Gli activation. The pathway appears to be critical for CCA migration *in vivo*. These observations provide further insight regarding Hh signaling in cancer biology, and support a role for Hh signaling inhibition in the treatment of human CCA.

Materials and methods

Cell lines and cell culture

H69 is a non-malignant SV40-immortalized human cholangiocyte cell line [14]. Malignant, patient-derived CCA cell lines were: KMCH, HuCC-T1, and Mz-ChA-1 [15,16]. The BDE1 and NRC are immortalized non-tumorigenic rat cholangiocyte cell lines [14,17]. The BDEneu cell line was generated by genetic transformation of BDE1 cells with the mutationally activated rat *neu* oncogene [18]. All cell lines were cultured as previously described by us in detail [11,19].

Immunofluorescence

Cells were cultured and incubated at 37 °C in an atmosphere containing 5% CO_2 at 100% confluency for 5 days with media exchange daily to stimulate cilia expression. In an experiment examining Smo translocation from the cell interior to the plasma membrane, cells were cultured and treated with either vehicle, recombinant mouse Shh ligand (6 μM; rm-Shh-N; R&D Systems, Minneapolis, MN), or a direct small-molecule agonist of Smo, purmorphamine (2 μM; Calbiochem, Billerica, MA, USA) with and without PTX (200 μg/ml; Sigma-Aldrich) for 16 h. In an experiment examining Gli2 translocation to the cell nuclei, cells were cultured and treated with either vehicle or purmorphamine (2 μM; Calbiochem) for 8 h. For immunofluorescence, cells were washed with phosphate-buffered saline (PBS) and fixed with either ice cold methanol (5 min) or 4% paraformaldehyde (10 min) for cilia and Gli2 or Smo immunofluorescence, respectively. All subsequent washes were performed using PBS with (cilia and Gli2 immunofluorescence) or without (Smo immunofluorescence) 0.1% Triton X-100 (Fisher Scientific, Pittsburg, PA, USA). Cells were incubated for 1 h at room temperature in blocking serum [5% fetal bovine serum (FBS) with 1% bovine serum albumin in PBS for cilia and Gli2 immunofluorescence; and 1% bovine serum albumin (BSA) with 10% goat serum, and 0.3 M glycine in PBS for Smo immunofluorescence], and then with primary antiserum (Supplementary Table 1) at 4 °C overnight. Cells were washed, incubated for 1 h with secondary antiserum (Supplementary Table 1) at room temperature, washed again, and mounted using Prolong Gold Antifade with DAPI (Invitrogen, Carlsbad, CA, USA). Cells were examined with confocal microscopy (LSM 510, Carl Zeiss, Jena, Germany) in at least 5 high power fields for Gli2 translocation to the cell nuclei, for percent of ciliated cells, or cells with Smo translocation to the plasma membrane.

To study actin cytoskeleton remodeling and expression of paxillin [22], we treated cultured cells either with vehicle or purmorphamine (2 μM; Calbiochem) with and without PTX (200 μg/ml; Sigma-Aldrich). Cell were washed with PBS, fixed with 4% paraformaldehyde, permeabilized with the 0.1% of Triton X-100 (Fisher Scientific), incubated in blocking serum (5% goat serum and 5% glycerol in PBS) and then with primary antibodies (Supplementary Table 1) for 2 h at 37 °C. Cells were subsequently washed with PBS and incubated with secondary antibodies (Supplementary Table 1) and phalloidin-FITC (Sigma-Aldrich; dilution of 1:300) for 1 h at 37 °C. Slides were mounted with Prolong Gold Antifade with DAPI (Invitrogen) and examined with fluorescence microscopy (Carl Zeiss).

Cell migration assay

The lower well of the modified Boyden chamber (Neuro Probe, Gaithersburg, MD, USA) was filled with the growth medium containing either vehicle, rm-Shh-N (6 μM, R&D), purmorphamine (2 μM, Calbiochem) with or without PTX (200 μg/ml; Sigma-Aldrich), or GANT61 (20 μM; Selleck, Randor, PA, USA) with or without purmorphamine (2 μM; Calbiochem). The polycarbonate membrane with 10 μm pores (Neuro Probe) covered by 0.01% collagen was placed on top of the lower well, and cells (at 10^5 density) suspended in a reduced-serum media (5% FBS) were added to the upper well. After incubation for the desired time period, the chamber was carefully disassembled; the membrane was washed with PBS; fixed with 4% paraformaldehyde; washed again; and mounted with Prolong Antifade with DAPI (Invitrogen). Both sides of the membrane were examined via fluorescence microscopy using excitation and emission wavelengths of 358 and 461 nm, respectively, to identify labeled cells. At least 5 high power fields per experimental condition were examined, and migrated cells were expressed as a percentage of total cells.

Supplementary materials and methods

Details regarding the assays for generation of the BDEneu cell line expressing dominant-negative Ptch1, quantitative real time PCR (qRT-PCR), cell proliferation assay, cell surface protein biotinylation, animal experiments, and statistical analysis are described in detail within the Supplementary materials and methods section.

Results

CCA cells have impaired cilium expression

We initially examined cellular cilia expression using immunocytochemistry for acetylated α-tubulin [11]. Human CCA cells minimally expressed cilia (0.4% of HuCC-T1, and none of the Mz-ChA-1 cells) as compared to non-malignant cholangiocytes which abundantly expressed cilia (40.3% of H69 cells; p <0.001; Fig. 1A). Similar findings were observed in rat cell lines where BDEneu cells completely lacked cilia expression unlike non-malignant cholangiocytes in which 18.5% of BDE1 and 15.6% of NRC cells expressed cilia (p <0.001; Fig. 1B). Thus, we confirmed prior findings that human and rat CCA cells minimally express cilia [11]. As cilia expression is highly cell-cycle dependent [23], cilia expression by cultured proliferating non-malignant cholangiocytes was less than 50% as expected.

CCA cells do not display canonical Hh signaling pathway activation

Increase in *Gli1* mRNA expression and translocation of the transcriptional factor Gli2 from the cell cytoplasm to the nuclei are indicators of canonical Hh signaling [4]. Therefore, we initially measured basal expression of *Gli1* mRNA in non-malignant and malignant cholangiocytes (Supplementary Fig. 1A). We observed that in the human cell lines relative basal expression of *Gli1* is increased in the non-malignant H69 cells and malignant KMCH cells expressing cilia as compared to the malignant HuCC-T1 and MzChA-1 cells. Among the rat cell lines, non-malignant NRC and BDE1 cell lines had lower expression of *Gli1* as compared to the malignant BDEneu cells where overexpression of *neu* oncogene likely upregulates *Gli1* in a non-canonical manner (Supplementary Fig. 1A). We next examined BDE1, HuCC-T1, Mz-ChA-1, and BDEneu cells for *Gli1* mRNA expression and translocation of Gli2 into the cell nuclei following stimulation with the direct Smo agonist, purmorphamine [7]. We observed that all studied human and rat CCA cell lines (e.g., Mz-ChA-1, HuCC-T1, and BDEneu) treated with purmorphamine failed to increase *Gli1* mRNA expression or translocate Gli2 protein into the cell nuclei. However, the non-malignant cholangiocyte cells, BDE1, demonstrated a 1.7-fold increase in *Gli1* mRNA expression (p <0.05; Fig. 1C) and efficient nuclear translocation of Gli2 protein (Supplementary Fig. 1B). Thus, canonical Hh signaling

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