Research Article

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Complete response of *Ctnnb1*-mutated tumours to β-catenin suppression by locked nucleic acid antisense in a mouse hepatocarcinogenesis model

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Background & Aims: Hepatocellular cancer (HCC) remains a disease of poor prognosis, highlighting the relevance of elucidating key molecular aberrations that may be targeted for novel therapies. Wnt signalling activation, chiefly due to mutations in *CTNNB1*, have been identified in a major subset of HCC patients. While several *in vitro* proof of concept studies show the relevance of suppressing Wnt/β-catenin signalling in HCC cells or tumour xenograft models, no study has addressed the impact of β-catenin inhibition in a relevant murine HCC model driven by *Ctnnb1* mutations.

Methods: We studied the *in vivo* impact of β-catenin suppression by locked nucleic acid (LNA) antisense treatment, after establishing *Ctnnb1* mutation-driven HCC by diethylnitrosamine and phenobarbital (DEN/PB) administration.

Results: The efficacy of LNA directed against β-catenin *vs.* scrambled on Wnt signalling was demonstrated *in vitro* in HCC cells and *in vivo* in normal mice. The DEN/PB model leads to HCC with *Ctnnb1* mutations. A complete therapeutic response in the form of abrogation of HCC was observed after ten treatments of tumour-bearing mice with β-catenin LNA every 48 h as compared to the scrambled control. A decrease in β-catenin activity, cell proliferation and increased cell death was evident after β-catenin suppression. No effect of β-catenin suppression was evident in non-*Ctnnb1* mutated HCC, observed after DEN-only administration.

Conclusions: Thus, we provide the *in vivo* proof of concept that β-catenin suppression in HCC will be of significant therapeutic benefit, provided the tumours display Wnt activation via mechanisms like *CTNNB1* mutations.

Keywords: Hepatocellular cancer; Wnt signalling; Molecular therapy; Oncogene; Mutations; Targeted therapy.

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Abbreviations: HCC, hepatocellular cancer; LNA, locked nucleic acid; DEN/PB, diethylnitrosamine and phenobarbital; GSK-3β, glycogen synthase kinase-3β; APC, Adenomatous polyposis coli; Fzd, Frizzled; LRP, low-density lipoprotein receptor related protein; CREB, cAMP response element-binding protein; CBP, CREB binding protein; TCF, T cell factor; LEF, lymphoid enhancing factor; GS, glutamine synthetase; FBS, foetal bovine serum; RIPA, radio immunoprecipitation assay; PVDF, polyvinylidene fluoride; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PBS, phosphate buffered saline; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labelling; ELISA, enzyme linked immunosorbent assay; IHC, immunohistochemistry.

Introduction

Hepatocellular cancer (HCC) usually occurs in cirrhotic livers associated with chronic liver diseases like hepatitis of viral, alcoholic or metabolic aetiology [12]. HCC is a major health burden as reflected by 748,000 new cases diagnosed in 2008 worldwide with corresponding 696,000 deaths [13,41]. HCC is now the seventh most common cancer in the world, as well as number three in cancer-related deaths. Current effective methods of treating primary HCC are surgical, including partial hepatectomy or orthotopic liver transplantation when possible, and do improve 5-year disease free survival. Loco-regional therapies are mostly palliative and gradually evolving. The only FDA-approved agent for HCC treatment consists of a multi-tyrosine kinase inhibitor sorafenib, which has shown some benefit, and does demonstrate effectiveness of targeted therapies in a heterogeneous disease like HCC [20].

Wnt/β-catenin signalling is an evolutionary conserved pathway with many important functions in hepatic development and homeostasis [25]. In the absence of Wnt, cytoplasmic β-catenin is phosphorylated at specific serine and threonine residues in exon-3 by a degradation complex composed of glycogen synthase kinase-3β (GSK-3β), adenomatous polyposis coli (APC) and Axin, targeting β-catenin for ubiquitin-proteasome degradation. β-Catenin activation occurs upon binding of Wnt to the receptor

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Frizzled (Fzd) and the co-receptor low-density lipoprotein receptor related protein (LRP) 5 or 6 via inactivation of the degradation complex, leading to its nuclear translocation. Here, it can interact with the T cell factor/lymphoid enhancing factor (TCF/LEF) family of transcription factors, to induce target genes, such as those encoding for cyclin-D1, c-Myc, glutamine synthetase (GS), and others in a tissue- or stage-specific manner.

In around 10–50% of primary HCCs, β-catenin gene (*CTNNB1*) mutations affecting serine, threonine or adjacent sites in exon-3 and mutations in its degradation components such as *AXIN1*, are associated with the Wnt autonomous nuclear localization and activation of β-catenin, which can have a multitude of effects on HCC biology, as various downstream targets are induced [7,19,23]. This makes β-catenin an attractive therapeutic target in a subset of HCCs. In the current study, we used a modified diethylnitrosamine (DEN) and phenobarbital (PB) model, in which 90% of HCCs display activating β-catenin gene mutations [1,22]. We administered locked nucleic acid (LNA) antisense against β-catenin to show a dramatic therapeutic benefit of β-catenin suppression in this model, but not in the DEN-only treatment where tumours occurred without β-catenin gene mutations. Thus, we show an *in vivo* efficacy of the therapeutic inhibition of β-catenin in HCC, paving the way for personalized medicine in HCC.

Materials and methods

Animals

All animal experiments were performed under the guidelines of the National Institutes of Health and the Institutional Animal Use and Care Committee at the University of Pittsburgh, School of Medicine.

For examining the *in vivo* efficacy of β-catenin inhibition by LNA, around 3-month old male C3H/He mice (Jackson Labs) were injected 5 times every 48 h (48 h) with 15 mg/kg of either EZN-3046 (scrambled control) (n = 2) and with EZN-3892 (directed against β-catenin) (n = 3) obtained under a Materials Transfer Agreement from Enzon Pharmaceuticals, New Jersey, USA.

For validation of the previously published chemical carcinogenesis model that utilizes *Ctnnb1* mutations to develop HCC [1,22], 6-week old C3H/He (Jackson Labs) male mice (n = 3) were injected intraperitoneally with 90 μg/g DEN (Sigma-Aldrich), followed 3 weeks later by initiation of a diet containing 0.05% PB (LabDiet) (DEN/PB group) for the duration of the experiment (∼30 weeks). Additional male mice (n = 3) were given DEN at the same dose and time but kept on regular mouse chow without PB (DEN-only group) for similar duration. Yet another group of mice (n = 3) were started on 0.05% PB (PB-only group) at 11 weeks of age for the duration of the experiment (around 30 weeks). Three additional male C3H/He mice without any intervention (control group) and on regular diet were killed when they were around 10 months old. Livers from all these mice were collected for analysis of HCC as described in the forthcoming sections.

For testing of the therapeutic efficacy of β-catenin suppression in the HCC in DEN/PB model, around 7 months after initiation of the PB diet, mice were stratified into two groups. Group 1 received EZN-3046 (n = 11) and group 2 received EZN-3892 (n = 6). LNAs were injected every 48 h intraperitoneally at 15 mg/kg for a total of 10 times. Livers and serum from mice after treatment were collected for further processing.

For testing any impact of β-catenin knockdown on HCC in a chemical carcinogenesis model that is not driven by *Ctnnb1* mutations, DEN-only treated mice at around 7 months received either EZN-3046 (n = 6) or EZN-3892 (n = 6). LNAs were injected every 48 h intraperitoneally at 15 mg/kg for a total of 6 times. Livers from mice after treatment were collected for further processing.

Cell culture and treatment

Hep3B HCC cells, stably transfected with mutated serine-33 to tyrosine (S33Y) β-catenin described recently, were grown to approximately 70% confluency and serum-starved for 24 h [8,9,19,26]. Cells were then transfected with 800 ng of TopFlash plasmid (Millipore), 200 ng of Renilla, and 3 μl of lipofectamine 2000 in 100 μl Opti-MEM media. After 4–6 h, 5 μM of EZN-3046 or EZN-3892 was added to the media containing 4% FBS. Cells were harvested 48 h or 72 h later and luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega) and normalized to Renilla levels. Statistical significance was calculated using a one-tailed Student's *t* test and $p < 0.05$ was considered significant.

Whole cell lysate preparation

At time of harvest, mice were anesthetized by isoflurane inhalation and subsequently killed by cervical dislocation. Livers were harvested, washed in PBS, and tissue was flash frozen in liquid nitrogen and stored at −80 °C until use. Part of the fresh or frozen tissue was homogenized in radioimmunoprecipitation assay (RIPA) buffer with protease/phosphatase inhibitor. Protein concentration was assessed by BCA protein assay (Pierce).

Western blots (WB)

Around 50 μg of whole cell lysate was run on a precast 7.5% or 4–14% gradient polyacrylamide gel (Bio-Rad). Gels were transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore). Membranes were blocked in 5% milk (Labscientific) in Blotto blocking buffer (0.15 M NaCl, 0.02 M Tris pH 7.5, 0.1% Tween in dH_2O) for 1 h at room temperature. Primary antibodies were diluted in 5% milk/Blotto and incubated on membranes overnight at 4 °C. Primary antibodies used were mouse monoclonal anti-β-catenin (BD Biosciences, 610154; 1:1000), rabbit polyclonal GS (Santa Cruz, SC-9067; 1:200) and rabbit glyceraldehyde-3-phosphate dehydrogenase or GAPDH antibody (Santa Cruz, SC-25778; 1:800). Membranes were washed in Blotto for 1 h at room temperature prior to the incubation of membranes with rabbit (1:10,000), mouse (1:25,000), or goat (1:10,000) secondary antibodies (Millipore) for 1 h. Membranes were again washed in Blotto for 1 h at room temperature prior to exposure with either SuperSignal West Pico or Femto Chemiluminescent Substrate (ThermoScientific) for 1–2 min at room temperature. The bands reflective of target proteins were viewed by autoradiography.

Histology and immunohistochemistry (IHC)

Tissue samples were embedded in paraffin and cut into 4 μm sections. Tissue sections were de-paraffinized in xylene and hydrated through graded alcohol rinses from 100% to 95% to dH_2O, and eventually washed in 1× phosphate buffered saline (PBS). Slides were next incubated in Eosin stain for 30 s followed by two washes in 95% ethanol and two washes of 100% ethanol. Slides were counterstained in Shandon's Haematoxylin solution for 1 min and gradually dehydrated in alcohol and xylene before mounting cover slips with DPX.

For IHC, deparaffinized sections were microwaved in citrate buffer for antigen retrieval. Endogenous peroxidases were quenched with 3% hydrogen peroxide. Slides were blocked with Super Block (UltraTek) for 10 min and additional antigen retrieval was conducted using antigen-unmasking solution (Vector Labs). Primary antibodies used were rabbit anti-β-catenin (Santa Cruz, SC-7199; 1:150) or goat anti-β-catenin (Santa Cruz, SC-1496; 1:150), rabbit anti-GS (Santa Cruz, SC-9067; 1:100) and rabbit anti-cyclin-D1 (Neomarkers, RB-9041; 1:100), diluted in PBS and sections were incubated for 1 h at room temperature. Sections were washed 3× in PBS, followed by horseradish-peroxidase-conjugated secondary anti-goat (1:200) or anti-rabbit (1:200) antibodies (Millipore) added to the slides for 30 min at room temperature. The secondary antibody signal was detected with DAB (Vector Labs) and the signal was quenched with dH_2O before counterstaining with Shandon solution (Sigma Aldrich). After dehydration in alcohol and xylene, slides were coverslipped with DPX (Fluka Labs). Negative controls were done without primary antibody. Images were taken on Axioskop 40 (Zeiss) inverted brightfield microscope.

For terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) IHC, slides were stained using the ApopTag peroxidase kit (Intergen Co., Purchase, NY) as per the manufacture's protocol.

Enzyme-linked immunosorbent assay (ELISA)-based serum Lect2 estimation

We recently reported serum LECT2 as a biomarker of β-catenin gene mutations in murine HCC [26]. Here, we used the mouse LECT2 ELISA kit (Medical & Biological Laboratories (MBL) Co, Ltd, Niigata, Japan) to measure serum levels, according to the manufacturer's protocol.