Published in final edited form as:

Curr Cancer Drug Targets. 2013 October ; 13(8): 867–878.

β-Catenin Knockdown in Liver Tumor Cells by a Cell Permeable Gamma Guanidine-based Peptide Nucleic Acid

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Abstract

Hepatocellular cancer (HCC) is the third cause of death by cancer worldwide. In the current study we target β-catenin, an oncogene mutated and constitutively active in 20–30% of HCCs, *via* a novel, cell permeable gamma guanidine-based peptide nucleic acid (γGPNA) antisense oligonucleotide designed against either the transcription or the translation start site of the human β-catenin gene. Using TOPflash, a luciferase reporter assay, we show that γGPNA targeting the transcription start site showed more robust activity against β-catenin activity in liver tumor cells that harbor β-catenin gene mutations (HepG2 & Snu-449). We identified concomitant suppression of β-catenin expression and of various Wnt targets including glutamine synthetase (GS) and cyclin-D1. Concurrently, γGPNA treatment reduced proliferation, survival and viability of HCC cells. Intriguingly, an angiogenesis quantitative Real-Time-PCR array identified decreased expression of several pro-angiogenic secreted factors such as EphrinA1, FGF-2, and VEGF-A upon β-catenin inhibition in liver tumor cells. Conversely, transfection of stabilized-β-catenin mutants enhanced the expression of angiogenic factors like VEGF-A. Conditioned media from HepG2 cells treated with β-catenin but not the mismatch γGPNA significantly diminished spheroid and tubule formation by SK-Hep1 cells, an HCC-associated endothelial cell line. Thus, we report a novel class of cell permeable and efficacious γGPNA that effectively targets β-catenin, a known oncogene in the liver. Our study also identifies a novel role of β-catenin in liver tumor angiogenesis through paracrine mechanisms in addition to its roles in proliferation, survival, metabolism and cancer stem cell biology, thus further strengthening its effectiveness as a therapeutic target in HCC.

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CONFLICT OF INTEREST

SPSM is a consultant for Bristol Myers Squibb, Merck Pharmaceuticals and PhaseRx. None of the authors however have any conflict of interests pertaining to the content of the current manuscript.

SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher's web site along with the published article.

Keywords

β -Catenin; Wnt signaling; liver cancer; angiogenesis; proliferation and antisense

INTRODUCTION

β -Catenin nuclear translocation and activation in a cell are controlled by upstream effectors comprised of Wnts. Wnts are secreted cysteine-rich glycoproteins from epithelial, mural, or endothelial cells, which bind to Frizzled (Fzd) receptors and low-density lipoprotein-receptor-related protein 5 and 6 (LRP5/6) co-receptors [1]. This initiates a cascade involving Disheveled (Dsh) to impact β -catenin degradation complex composed of glycogen synthase kinase-3 β (GSK-3 β), adenomatous polyposis coli (APC), and Axin to cause cytoplasmic accumulation of β -catenin and its nuclear translocation [2–7]. In the nucleus, β -catenin acts as a cofactor for the high-mobility group (HMG) box containing DNA binding protein T-cell factor 4 (TCF4), which in turn regulates the transcription of specific target genes. Several different factors have been shown to be subject to β -catenin regulation such as Cyclin D1, cmyc, glutamine synthetase (GS), and others that play a role in cell cycle, migration, survival and metabolism [8–10]. In the absence of Wnt signaling, GSK-3 β phosphorylates β -catenin at specific sites at the amino-terminus, enabling its targeting *via* proteasomal degradation [4]. β -Catenin activation has been reported in a significant subset of hepatocellular cancers (HCC). In around 30% of these cases, point mutations affecting serine/threonine residues in the exon-3 of *CTNNB1* gene render β -catenin stable and constitutively active [6, 11–13]. Aberrant β -catenin activation is associated with tumor cellular proliferation and survival, making it an effective target for treatment in a subset of HCC patients [14].

The process of angiogenesis is indispensable to tumor growth and progression including in HCC. Wnt signaling has been shown to be contributing to this process through mechanisms such as regulation of expression of vascular endothelial growth factor (VEGF) [15]. VEGF is a classic stimulator of angiogenesis, and has seven consensus binding sites on its promoter for the β -catenin/T-cell factor (TCF) complex [16]. Several studies also indicate the importance of VEGF in HCC progression and show overexpression of VEGF, and its respective receptors VEGFR-1 and VEGFR-2, in the tumors [17, 18]. However, a direct study that investigates β -catenin's impact on angiogenesis in HCC, both molecularly and functionally, is lacking.

Peptide nucleic acid (PNA) is a promising class of nucleic acid mimic developed in the last two decades in which the naturally occurring sugar phosphodiester backbone is replaced with *N*-(2-aminoethyl) glycine units [19]. PNA has many appealing features as compared to the natural counterparts including strong binding affinity and sequence selectivity towards DNA and RNA, and resistance to enzymatic degradation by proteases and nucleases. These properties along with the ease of synthesis make PNA an attractive molecular platform for regulating gene expression; however, a drawback is that it is not cell-permeable [20, 21]. Previously we have shown that installation of a guanidinium group at the α -backbone significantly improves the cellular uptake of PNA [22]. This new class of guanidine-based

PNA (GPNA) has been successfully employed in the selective knockdown of E-cadherin [23, 24]. More recently we reported the synthesis of a second-generation GPNA, in which the guanidinium group was incorporated at the γ -backbone (referred to as γ GPNA), with improved hybridization properties and economy of synthesis.

In the current study, we report the efficacy of γ GPNA to target β -catenin in HCC cells. As proof-of-concept, we show that targeting β -catenin impacts liver tumor cell proliferation and viability. In addition, β -catenin knockdown in these cells also affected expression of several factors involved in angiogenesis that led to decreased tubulogenesis and spheroid formation by the HCC-associated endothelial cells. Our results presented herein imply that β -catenin inhibition in HCC may impact multiple biological processes such as tumor cell survival, proliferation and angiogenesis and thus will have important therapeutic implications in HCC treatment.

MATERIALS AND METHODS

Oligomer Synthesis

All Boc/Z protected PNA monomers and Boc-^LArg-OH were purchased from Applied Biosystems and used without further purification. All γ GPNA monomers were synthesized by methods reported by Sahu and coworkers [25]. All commercial reagents were used without further purification. MALDI-TOF experiments were performed on a PerSeptive Biosystems Voyager STR MALDI-TOF mass spectrometer using a 10 mg/ml solution of α -hydroxycinnamic acid in ACN-water (1:1) with 0.1% TFA. UV-Vis measurements were taken on a Varian Cary 300 Bio spectrophotometer equipped with a thermoelectrically controlled multi-cell holder.

All γ GPNA oligomers were synthesized on solid-support according to standard protocol using standard Boc chemistry [26]. The oligomers were purified by reverse-phase HPLC and characterized by MALDI-TOF (online supplement; Fig. S1-S2). All γ GPNA stock solutions were prepared using nanopure water and the concentrations were determined at 90°C using the following extinction coefficients for GPNA monomers: 13,700 M⁻¹ cm⁻¹ (A), 6,600 M⁻¹ cm⁻¹ (C), 11,700 M⁻¹ cm⁻¹ (G), and 8,600 M⁻¹ cm⁻¹ (T).

Cell Culture

4×10^5 or 8×10^4 HepG2 cells (human hepatoblastoma cells) from American Type Culture Collection (ATCC) were plated in 6 well plates (BD Falcon) or 24 well plates (BD Falcon), respectively. Cells were cultured in EMEM (ATCC) supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals). 5×10^4 Snu-449 cells (HCC cells) also from ATCC were plated in 24 well plates in RPMI-1640 (ATCC) supplemented with 10% FBS. SK-Hep1 cells (ATCC) were maintained in EMEM with 10% FBS. Cells were treated with either mismatch γ GPNA (**MM**), γ GPNA directed against β -catenin transcription start-site (**T1**) or γ GPNA directed against β -catenin translation start-site (**T2**) for various studies as indicated in the forthcoming methods.

β-Catenin Luciferase Activity Assay

HepG2 or Snu-449 cells were transfected using FuGene (Roche) with both Renilla luciferase and TOPflash firefly luciferase plasmids together at the same time. 15 minutes after transfection, cells were treated with γGPNA for an additional 24 or 72 hours. Lysates were harvested using the Dual-Luciferase Reporter Assay System (Promega). Luciferase signals were normalized to Renilla as transfection controls. Student's t-test was used to determine the significance of the differences between treatments and $p < 0.05$ was considered significant and of $p < 0.01$ was considered highly significant.

Western Blot Analysis

HepG2 and Snu-449 whole cell lysates were prepared using a solution of 1% IgePAL CA-630, 0.5% Sodium Deoxycholate, 0.1% Sodium dodecyl sulfate in 1x Phosphate Buffered Saline (PBS) after 72 hour treatment with **T1** or **MM** γGPNA at 1 μM. Alternatively lysates obtained for luciferase assays were used. 60 μg of lysate was run on a 7.5% or 4–14% gradient polyacrylamide gel (Bio-Rad) at 60V for 1 hour, then 100V for 1.5 hours. Gels were then transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore) for 1 hour at 40°C. Membranes were blocked in 5% milk (LabScientific) in Blotto [0.15M NaCl, 0.02M Tris pH 7.5, 0.1% Tween in dH₂O] for 1 hour at room temperature. Primary antibodies for β-catenin (BD Transduction Laboratories), β-actin (Chemicon International), c-myc (Santa Cruz), Conductin/Axin-2 (Santa Cruz), Cyclin-D1 (ThermoScientific), GAPDH (Santa Cruz), Glutamine Synthetase (Santa Cruz), SMP30/Regucalcin (Santa Cruz), and VEGF-A (Santa Cruz) were diluted 1:1000, 1:2500, 1:200, 1:200, 1:200, 1:800, 1:200, 1:200, and 1:200 respectively in 5% milk/Blotto and incubated on membranes overnight at 4°C. Membranes were then washed in Blotto for 1 hour at room temperature prior to incubation of membranes with rabbit (1:10,000), mouse (1:25,000), or goat (1:10,000) secondary antibodies (Millipore) for one hour. Membranes were again washed in Blotto for 1 hour at room temperature prior to expose to SuperSignal West Pico Chemiluminescent Substrate (ThermoScientific) for 1–2 minutes at room temperature. The bands reflective of target proteins were viewed by autoradiography.

Transient Inhibition of β-catenin via siRNA

HepG2 cells cultured in 6 well plates were serum starved for 4 hours prior to Lipofectamine 2000 (Invitrogen) transfection using 50 nanomoles of either *CTNNB1* or negative control siRNA per well. After 4 hours at 37°C followed, EMEM containing 4% FBS was added and cells incubated overnight followed by replacement with EMEM containing 10% FBS. After 48 hours of transfection, cells were harvested.

RNA Isolation and qRT-PCR

RNA from HepG2 cells treated with 1 μM **MM** or **T1** for 72 hours or transfected with β-catenin or negative control siRNA for 48 hours was harvested using TRIzol (Invitrogen) and purified using a phenol-based method. RNA was DNase treated (Ambion), reverse-transcribed using SuperScript III (Invitrogen) cDNA synthesis kit, followed by RT-PCR for Fibroblast growth factor 2 (FGF2), VEGF-A and β-catenin. Primers used were: 5'-GGCTTCTAAATGTGTTACGGATG-3' and 5'-CCCAGGTCCTGTTTTGGAT-3' for

FGF2, 5'-AGGAGGAGGGCAGAATCATCA-3' and 5'-CTCGATT GGATGGCAGTAGCT-3' for VEGF-A, 5'-CTGGCCATAT CCACCAGAGT-3' and 5'-GAAACGGCTTTCAGTTGAGC-3' for β -catenin and 5'-TGCACCACCAACTGCTTAGC-3' and 5'-GGCATGGACTGTGGTCATGAG-3' for GAPDH. For identification of expression changes in genes involved in angiogenesis after GPNA treatment, RT² Profiler PCR Array System (SABiosciences) was used according to manufacturer's instructions. Data was analyzed using web based QIAGEN RT² Profiler PCR Array Data Analysis version 3.5 for DDCT and significance.

MTT Assay for Toxicity

HepG2 cells were plated 3×10^5 per well in 6 well plates for 24 hours. Cells were then treated for 72 hours with 1 μ M of either **MM** or **T1**. After incubation, cultures were changed into 1% MTT wt/v in PBS for 0.5 hours at 37°C. Cells are then lysed using room temperature isopropanol. Samples were read at 570 nm for colorimetric assessment.

Human HCC Cell Culture and Transfection with Stable β -catenin Mutants

Hep3B cells (Human HCC cells) from ATCC were plated in six-well plates and cultured in EMEM (ATCC) supplemented with 10% FBS (Atlanta Biologicals) at 37°C in a humidified 5% carbon dioxide atmosphere. Wild type β -catenin gene (WT) or β -catenin gene mutated at serine 33 to tyrosine (S33Y), which is constitutively active, were kindly provided by Dr. Jian Yu (Department of Pathology, Hillman Cancer Center, University of Pittsburgh, PA). The cells were grown to 90% confluence, 2 μ g of WT and S33Y β -catenin plasmid DNA was transfected with LipofectamineTM 2000 (Invitrogen), as the manufacturer's instructions. 48 hours after transfection, the cells were selected by multiple passaging using Geneticin (G418; Sigma; 500ug/ml) to generate stable transfected cell lines. Cells were harvested using lysis buffer as previously indicated for use in Western Blotting.

Thymidine Incorporation Proliferation Assay

HepG2 cells were plated in 6 well plates for 24 hours. Cells were then treated with 1 μ M **T1** for 72 hours and pulsed with 2.5 μ Ci of [5'-3H]-Thymidine for 48 hours. Media was aspirated and washed with 1x PBS and incubated with 5% Trichloroacetic acid for 15 minutes at 4°C. Plates were next washed in running water and placed to dry at 37°C. Cells were then lysed in 0.33 N NaOH for 20 minutes at room temperature. Samples were combined with scintillation fluid and measured in Beckman LS 6000 IC scintillation counter.

Live Cell Imaging

HepG2 cells plated in 6 well plates were maintained in media supplemented with tetramethylrhodamine (TAMRA)-labeled **T1** at 1 μ M with Hoescht dye for a period of 19 hours under live cell imaging. Imaging was done on Nikon Eclipse Ti live cell imager using DAPI and Cy3 filters.

Staining

2×10^3 HepG2 cells were plated in 4 well chamber slides for 24 hours prior to 1 μ M treatment with either **T1** or **MM**. For terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) immunohistochemistry, slides were fixed 72 hours after treatment in a 3:1 solution of methanol/glacial acetic acid for 5 min and allowed to air-dry. Detection of apoptotic nuclei was determined by TUNEL staining using the ApopTag peroxidase kit (Intergen Co., Purchase, NY).

For detection of cells in S-phase of cell cycle, immunofluorescence for Ki-67 was performed. Briefly, 72 hours after **T1**, **MM** or no control treatment, HepG2 cells grown on chamber slides were fixed in 4% paraformaldehyde. Wells were washed with 1x PBS and permeabilized with 0.1% Triton-X in PBS followed by washes in PBS alone and then in 0.5% bovine serum albumin (BSA) in PBS (PBB). Samples were blocked in 2% PBB for 1 hour, washed with 0.5% PBB and incubated in 0.5% PBB containing primary antibodies- β -catenin (BD Biosciences) and Ki-67 (Santa Cruz) each at 1:100 for 1 hour. After washing in 0.5% PBB, secondary antibodies-anti-Mouse-Alexa488 and anti-Goat-Alexa555 at 1:1000 dilution in 0.5% PBB, were applied to each well except negative control for 1 hour. Wells were washed with 0.5% PBB, then with 1x PBS wash and subsequently stained with Hoescht dye for 30 seconds. Samples were cover-slipped and imaged using Olympus Fluoview 500 Confocal Microscope.

In Vitro Endothelial Spheroid Assay

Serum free EMEM was used to create a 1.2% methocel stock solution using 4,000 centipoise methyl cellulose (Sigma). SK-Hep1 cells were trypsinized, counted and resuspended in a 20:80 ratio of 1.2% methocel stock:growth media. Cells were then seeded at approximately 3000 cells/well in non-adhesive, round bottom 96 well plates (Greiner bio-one) and grown for 6 days. Keeping everything on ice, rat-tail collagen and 10x MEM (Sigma-Aldrich) was mixed 9:1 respectively. Collagen mixture was then conditioned with Gentamycin (0.1% v/v), Insulin (1.0% v/v), and 3.5% NaOH (0.5% v/v). 250 μ l of collagen mixture was added per well to a 12 well plate and set at 37°C for 30 minutes for polymerization. After polymerization, residual media was removed *via* manual pipetting. SK-Hep1 spheroids were seeded on top of collagen gels and allowed to attach at 37°C for 30 minutes. Residual media was then removed again in order to lay a final 250 μ l of collagen on top of the attached spheroids and allowed to polymerize at 37°C for an additional 30 minutes. After polymerization, 100 μ l conditioned media from HepG2 cells treated with either 1 μ M **MM** or **T1** γ GPNA for 72 hours was mixed with 100 μ l serum free EMEM. Media was placed on top of the polymerized collagen sandwiches and incubated at 37°C under ambient oxygen, levels and 5.0% CO₂ for 24 hours. Images were taken using Nikon Eclipse Ti live cell imager. The number of tube sprouts and the length of the tubes from each spheroid were counted and averaged. Three spheroids from **MM** and four from **T1**-treated group were utilized for assessing tube sprout numbers and tube length. Using *Nikon Elements* software, a comparison of averages ($\pm$ SD) was made and significance assessed by student *t* test and $p < 0.05$ was considered significant and $p < 0.01$ was considered highly significant.

***In Vitro* Tubulogenesis Assay**

Tubulogenesis assay was conducted using *In Vitro* Angiogenesis Assay Kit (Millipore). 25 μ l of cold extracellular matrix was added to a 96 well plate (BD Falcon) and placed at 37°C for 1 hour. SK-Hep1 cells were seeded at 8×10^3 cells per well after being resuspended in respective conditioned media (HepG2 conditioned media collected after 72 hour treatment with either No Treatment, 1 μ M **MM**, or **T1**). Cells were incubated at 37°C for 3 hours prior to imaging under phase contrast using Nikon Eclipse *Ti* live cell imager. Tube length was measured in each group and averaged. Additionally, numbers of tubes measuring greater than 4 μ m in length were counted in each well for at least three wells per condition and averaged. Statistical comparison for each of the two parameters was done by comparing the averages in control and experimental group by student *t* test and $p < 0.05$ was considered significant.

To test for the presence of any remnant unused **T1** γ GPNA in the conditioned media used for spheroid and tubulogenesis assays, HepG2 cells transfected with TOPflash plasmid were cultured in it for 24 hours and harvested for luciferase assay as described earlier.

RESULTS

Design and Synthesis of Oligomers

Inspired by the effectiveness of α GPNA in knocking down E-cadherin in previous studies, we decided to target β -catenin, a well-recognized and imminent therapeutic target in human HCC. Here, we employed a second-generation γ GPNA with superior hybridization properties. We designed and synthesized two specific PNA's against the β -catenin gene, one against the transcription start site, called **T1**, and the other against the translation start site, labeled as **T2** (Fig. 1A–C). Both **T1** and **T2** contain regular PNA with inserted γ GPNA monomer units (5 units) to increase its cellular uptake properties. In order to demonstrate the selective binding of **T1**, a control PNA containing 4-basepair mismatch sites or **MM** was also synthesized (Fig. 1C). Finally, in order to examine the cellular uptake properties of designed γ GPNA, **T1** was also fluorescently labeled with TAMRA dye (Fig. 1C).

Unaided Uptake of T1-TAMRA by Hepatoma Cells and Selective Inhibition of CTNNB1 Expression

HepG2 cells were cultured in a media containing Hoescht nuclear dye and 1 μ M of **T1-TAMRA** γ GPNA for 19 hours under live cell imaging. Within 3 hours **T1-TAMRA** uptake was visible as red cytoplasmic inclusions in the cells (Fig. 1D). At 19 hours, **T1-TAMRA** was present as punctate structures, in cytoplasm and nuclei of the HepG2 cells. This result indicates a successful uptake of the γ GPNA by tumor cells without the aid of any transfection reagent or other manipulation.

γ GPNA Targeting Transcription Start Site of CTNNB1 More Effectively Impairs β -catenin Activity in Liver Tumor Cells

Next, we compared the β -catenin knockdown efficacy of γ GPNAs directed against the transcription and translation start sites of the β -catenin gene. Fifteen minutes after transfection with the TOPflash luciferase reporter to assess β -catenin-TCF activity, HepG2

cells were treated with either **T1** or **T2** at 5 μ M for 24 hours. A 90% and a 50% decrease in TOPflash reporter activity, was observed after **T1** and **T2** treatment, respectively (Fig. 2A). Further, **T1** treatment for 24 hours demonstrated a dose-dependent decrease in TOPflash reporter in HepG2 cells (Fig. 2B). This result showed that while both γ GPNA inhibited β -catenin, **T1** was more potent and hence used for all subsequent investigations. As a control and to address the specificity of **T1**, the **MM** γ GPNA was used alongside.

Prolonged β -Catenin Targeting by **T1** γ GPNA in HCC Cells

We utilized two liver tumor cell lines with distinct modes of β -catenin activation to address efficacious and prolonged inhibition by **T1** γ GPNA. HepG2 cells harbor a deletion in exon-3 in β -catenin, a common occurrence in hepatoblastomas, while Snu-449 cells have a point mutation in exon-3 of *CTNNB1*, a common occurrence in HCC [27, 28]. Both cell lines were treated with 1 μ M of **T1** for 72 hours to determine β -catenin inhibition over a prolonged duration. As compared to the **MM** control, **T1** treatment reduced TOPflash activity significantly in both tumor cell lines (Fig. 2C–D).

Successful β -catenin Targeting by γ GPNA Affects Downstream Signaling

We next assessed the effect of **T1** γ GPNA on *CTNNB1* expression and protein levels of β -catenin and its targets in HepG2 cells treated with 1 μ M **T1** or **MM** for 72 hours. Real-time PCR normalized to GAPDH showed a significant decrease in β -catenin mRNA as compared to **MM** control ($p < 0.05$) (Fig. 3A). Western blots showed that **T1** effectively decreased both the full-length and the truncated forms of β -catenin in HepG2 cells and the only β -catenin form existent in Snu-449 cells (Fig. 3B). Representative western blots shown also demonstrate decreased protein expression of GS, Cyclin-D1, c-Myc, Axin-2, Regucalcin, and VEGF-A in the **T1**-treated HepG2 and Snu-449 cells as compared to the **MM** controls (Fig. 3B). These findings further validate the anti- β -catenin efficacy of **T1** γ GPNA in liver tumor cells in culture.

Targeting β -catenin by γ GPNA Affects Liver Tumor Cell Biology

The impact of **T1** γ GPNA-mediated β -catenin suppression was next assessed on tumor cell proliferation and survival. [3 H]-Thymidine incorporation assay in 1 μ M **T1**-treated HepG2 cells for 72 hours showed a 20% decrease in DNA synthesis when compared to **MM** treatment indicating a significant decrease in cell proliferation ($p < 0.05$) (Fig. 3C). The numbers of cells in S-phase were also assessed. Immunofluorescence for Ki-67 (not shown), showed a reduction by around 75% ($p < 0.05$) after **T1** as compared to the **MM** treatment (Fig. 3D).

Mitochondrial activity as a measure of cell metabolism and viability was assessed next. MTT assay showed a notable decrease in the viability of HepG2 cells after 72-hour of **T1**-treatment (Fig. 3E). In addition, a significantly higher ($p < 0.01$) numbers of TUNEL-positive nuclei by immuno-histochemistry reflect an increase in oncotic or apoptotic cell death after 72 hours of **T1** treatment as compared to the **MM** (Fig. 3F). Thus, successful inhibition of β -catenin expression and activity by the γ GPNA notably affected liver tumor cell proliferation and viability.

Decreased Expression of Angiogenic Factors after β -catenin Inhibition in Liver Tumor Cells

Wnt/ β -catenin signaling is known to regulate VEGF expression in colorectal cancer [15, 16]. Indeed VEGF expression was downregulated in HepG2 cells after **T1** treatment (Fig. 3B). To investigate if β -catenin knockdown in hepatic tumor cells may be affecting the expression of any other angiogenic factors, we utilized a quantitative Real-Time PCR angiogenesis array. Interestingly, **T1** treatment of HepG2 cells for 72 hours led to a decrease in the expression of multiple angiogenic factors including CXCL5, Ephrin-A1, FGF2, IL-1b, Midkine, Placental Growth Factor (PGF), and SERPINF1 when compared to the **MM** control (Fig. 4A). In fact, fifteen secreted pro-angiogenic factors showed a significant decrease upon β -catenin knockdown with the exception of Heparan sulfate-cleaving enzyme heparanase (HPSE). Changes in β -catenin, VEGF-A, and FGF2 mRNA expression were also validated by a Real-time PCR using mRNA isolated from HepG2 cells treated with either 1 μ M **MM** or **T1** for 72 hours, or 50 nM of negative control or β -catenin siRNA for 48 hours. β -Catenin knockdown by any of these modalities led to a significant ($p < 0.01$) decrease in the expression of both VEGF-A and FGF2 (Fig. 4B–C).

Expression of Constitutively Active β -catenin Leads to an Upregulation of VEGF-A

To further validate if VEGF-A was indeed a relevant target of β -catenin in liver tumor cells, we assessed its expression in another HCC cell line Hep3B that harbors wild-type (WT) β -catenin gene. Stable transfection of Hep3B cells with serine-33 to tyrosine (S33Y)-mutated β -catenin led to a robust increase in the expression of VEGF-A as compared to the stable cells transfected with WT- β -catenin (Fig. 4D). Thus VEGF-A levels mirror β -catenin activity in the liver tumor cells in culture.

Inhibition of β -catenin in Liver Tumor Cells Impacts Secretion of Angiogenesis Factors that in Turn Impairs Growth of HCC-Associated Endothelial Cells

We next investigated if decreased expression of various angiogenic factors observed after β -catenin inhibition is functionally relevant and could hinder HCC-associated angiogenesis. We utilized SK-Hep1 cells, an endothelial cell line derived from a hepatic adenocarcinoma [29]. Endothelial spheroids generated after embedding SK-Hep1 cells in collagen gels, were grown for 24 hours in the conditioned media taken from HepG2 cells after either 72 hours of **MM** or **T1** γ GPNA treatment (Fig. 5A). A significant decrease in the numbers of tube sprouts ($p < 0.01$) from the spheroids was evident after culture in **T1**-conditioned media as compared to the **MM** (Fig. 5B). Likewise, the tube sprouts that emanated from the spheroids in the **T1**-conditioned media showed a significant blunting ($p < 0.05$) in their length as compared to the **MM** control (Fig. 5C).

SK-Hep1 cells were also cultured on an extracellular matrix that promoted tubule formation, another characteristic of endothelial cells. SK-Hep1 cells grown for 3 hours in the conditioned media from **T1**-treated HepG2 cells as described above, showed a dramatic decrease in tube formation as compared to those grown in the **MM** conditioned media (Fig. 5D). In fact, there was a statistically significant decrease in the average tube length in the **T1**

versus the **MM** group ($p<0.05$) (Fig. 5E). At the same time, the numbers of tubes 150 μm in length were significantly fewer in the **T1** than the **MM** group ($p<0.05$) (Fig. 5F).

To ensure that the conditioned media used in the spheroid and tubulogenesis assays does not contain any leftover γGPNA from the prior HepG2 cell-treatment, fresh HepG2 cells were transfected with the TOPflash plasmid and cultured for 24 hours in the conditioned media used in the above assays. Comparable TOPflash activity between the **T1** and the **MM** conditioned media verified absence of any residual **T1** γGPNA in the conditioned media (Fig. 5E). Thus, β -catenin suppression in the liver tumor cells impairs the growth and development of tumor-associated endothelial cells in a paracrine manner.

DISCUSSION

Heterogeneity in the signaling mechanisms among individuals afflicted with the same tumor type is the impetus behind personalized medicine. Several aberrantly active signaling pathways have been identified in HCC making them attractive therapeutic targets. The Wnt/ β -catenin signaling is one such pathway implicated in a subset of HCC patients [30]. While multiple means of β -catenin activation have been reported in HCC, the major mechanism is the stabilizing mutations in *CTNNB1*, which leads to constitutively active β -catenin protein, which in turn has been associated with tumor proliferation, invasion, growth and resistance to cell death [14, 31]. Active β -catenin signaling in the cancer stem cells within an HCC also makes its therapeutic inhibition an attractive means to suppress tumor resistance, recurrence and metastasis [32–34]. Recently, based on the regulation of GS expression by the Wnt signaling in the liver, β -catenin-mutated HCC cells have shown to be glutamine-addicted [35]. This provides an additional rationale for β -catenin inhibition to impair tumor cell metabolism in HCC. Thus, any modality to suppress β -catenin may be therapeutically beneficial in at least a major subset of HCC patients. Indeed anti- β -catenin therapies are increasingly discussed and in various stages of preclinical and clinical development [36, 37]. The role of β -catenin signaling however remains unexplored in HCC tumor angiogenesis. We now report regulation of several key angiogenic factors by the Wnt signaling thus broadening the advantages of targeting β -catenin therapy in this tumor type. In fact we use a novel and timely modality consisting of a new generation of γGPNA to inhibit β -catenin signaling and demonstrate its impact on tumor angiogenesis.

PNA is a particularly promising class of nucleic acid analogue, developed in early 1990's in which the natural sugar-phosphodiester backbone is substituted with achiral *N*-(2-aminoethyl) glycine units [19]. The neutral backbone contribute the outstanding features to PNA which include 1) strong hybridization with complementary DNA and RNA with high affinity (and sequence selectivity) through Watson-Crick base pairing and 2) resistance to enzymatic degradation by proteases and nucleases [38, 39]. These significant properties have made PNA an attractive reagent for many applications in biology and medicine. However, poor cell-permeability remains a challenge for PNA [20, 21]. Our group has reported a second-generation γGPNA , which can be prepared from a relatively cheap L-arginine and exhibits better hybridization and cellular uptake properties [25]. These γGPNA oligomers are already conformationally pre-organized into a right-handed helix and are capable of binding DNA and RNA with high affinity and sequence selectivity, and we have

already demonstrated that these can be taken-up by cells. Our studies indicate that within the first few hours of treatment, γ GPNA localizes to both nucleus and cytoplasm, where it can interact with mature mRNA.

Utilizing this new class of antisense molecules, we demonstrate an efficacy of β -catenin γ GPNA, directed against either the transcription (**T1**) or the translational (**T2**) start site of *CTNNB1*. The efficacy of **T1** was greater than **T2**, which was in agreement with a previously reported study [40]. Using two liver tumor cell lines that harbor either a β -catenin gene deletion or a point mutation, we demonstrate the effectiveness of **T1** in inhibiting β -catenin gene and protein expression, and its activity. The effect of γ GPNA treatment on tumor cells in culture was more robust at 24 hours than 72 hours. This is likely due to the lack of uptake of the γ GPNA by all cells in culture, giving such cells a growth and survival advantage over 72 hours. However, overall impact on tumor cell proliferation, viability and apoptosis was significant as has also been reported previously (Reviewed in [30]). The suppression of β -catenin activity after 72 hours of γ GPNA treatment on HepG2 cells was less robust as compared to Snu-449 cells. HepG2 HCC cells have a monoallelic, truncated β -catenin, which lacks exon-3 that contains all phosphorylation sites (serine 33, 27, 45 and threonine 41) required for degradation of β -catenin by the ubiquitin proteasome. Snu-449 cells have a single point mutation affecting serine 37, which also renders β -catenin stable. It is likely that the difference in the stability of the truncated versus the point-mutant form of β -catenin, may be accounting for the difference in the extent of decrease in the β -catenin activity in response to the γ GPNA mediated β -catenin knockdown. Thus, γ GPNA is a class of novel agents that could have a significant therapeutic efficacy in targeting any molecule.

β -Catenin knockdown in the liver tumor cells led to a decrease in the expression of several secreted molecules that were insufficient to sustain angiogenesis demonstrated by decreased growth and development of SK-Hep1 endothelial cells. Like other solid tumors, angiogenesis is also relevant in HCC and the growth and progression of hepatic tumors requires formation of new blood vessels [41]. One of the potential mechanisms of Sorafenib, the only FDA approved drug for stage IV unresectable HCC, is its impact on angiogenesis through inhibition of receptor tyrosine kinases like VEGFR2 (Flk1), platelet derived growth factor receptor (PDGFR) and others [42]. However, angiogenesis inhibitors by themselves have had a limited impact on clinical outcome of HCC, which has been attributed to the areas of hypovascularity and hypoxia within tumors, and also on the hypoxia induced by the anti-angiogenic therapy. Hypoxia indeed has been shown to be a major driver of HCC growth as well as in imparting chemoresistance. Intriguingly, judicious use of anti-angiogenesis therapy as adjunct has been shown to improve overall outcome in HCC patients. Similarly, agents such as Sorafenib, which impact multiple aspects of tumorigenesis including growth and viability of cancer cells and tumor angiogenesis, have shown some promise in the treatment of HCC. Our current study shows that therapeutic targeting of β -catenin may also have a broad impact on HCC cell biology including an effect on angiogenesis.

In the current study we show that β -catenin suppression in HCC cells impairs the expression of several secreted pro-angiogenic factors that may be released by the tumor cells to influence angiogenesis in a paracrine manner. We identified decreases in various factors

such as VEGF-A, Ephrin, FGF2, and CXCL5 after β -catenin suppression in liver tumor cells that are known to be relevant in HCC tumor angiogenesis [41]. VEGF-A has been noted to be a direct target of β -catenin in colorectal cancer [15]. It was interesting to note that FGF2 was decreased by both β -catenin-directed siRNA and γ GPNA to the same extent, although β -catenin suppression was much more robust in the former modality. This could be due to an autoregulation by FGF2 that has been described previously [43]. Intriguingly, we saw an increase in HPSE expression after β -catenin knockdown by **T1** PNA treatment for 72 hours. Since this enzyme cleaves heparan sulfate in extracellular matrix, it has been associated with increased metastasis and angiogenesis. However, several other pro-angiogenic secreted factors were decreased as a result of β -catenin inhibition. This demonstrates a paracrine mechanism by which β -catenin activation in HCC could be supporting tumor growth and progression. Indeed the conditioned media from HCC cells cultured in the presence of **T1** γ GPNA was insufficient to support the growth and development of HCC-associated endothelial cells. An earlier study has also shown adenoviral mediated inhibition of Wnt signaling by Wnt inhibitory factor 1 (WIF1) and secreted frizzled-related protein 1 (sFRP1) overexpression in endothelial cell progenitors impacted their differentiation and induced apoptosis [44]. Thus, we conclude that β -catenin inhibition may in fact have dual impact on angiogenesis by not only interfering with growth and differentiation of endothelial progenitor cells directly but also *via* inhibiting paracrine signaling emanating from HCC cells.

CONCLUSIONS

Our current study shows that therapeutic targeting of β -catenin will have a similar broader impact in select HCC patients where Wnt signaling is playing a key role in tumor pathogenesis such as those with activating mutations in *CTNNB1*. The therapeutic effect of β -catenin suppression by modalities like γ GPNA in HCC will be due to suppression of its target gene expression with a net negative impact on tumor cell proliferation, survival, metabolism, cancer stem cell renewal and last but not the least by inhibition of tumor angiogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This study was funded by NIH grants 1R01DK62277 and 1R01CA124414 to SPSM and Experimental Pathology Endowed Research Chair to SPSM. ED was a trainee on 1T32HL094295 (Angiopathy Training Grant).

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Fig. (1). Structure and sequence of antisense oligonucleotides and controls that show an unaided cellular uptake

(A) Chemical Structures of the PNA and the γGPNA (B) Human beta-catenin (*CTNNB1*) gene transcript and the location of targeted sequences and (C) Sequence of the γGPNA oligomers employed for the study. (D). HepG2 cells were cultured in the presence of a TAMRA-labeled T1 γGPNA for a period of 19 hours and the fluorescent dye was tracked by a live cell imaging microscope. Within 3 hours of the treatment, TAMRA-T1 notably increased within the cells (red) without the aid of any transfection reagent. Cells were also incubated with the Hoechst stain for nuclear visualization. Scale bars indicate 50 μm.

Fig. (2). γ GPNA designed against β -catenin decreases its reporter activity in the liver tumor cells with the T1's impact being more pronounced

(A) T1- and T2-targeted against the β -catenin gene transcription and translation start sites respectively, when incubated with HepG2 cells at 5 μ M for 24 hours, reduced TOPflash reporter activity, although T1 had a more robust impact than T2. (B) Twenty-four hour exposure of HepG2 cells to T1 ranging from 10 μ M to 25 nM exhibits a dose-response in reducing the TOPflash reporter activity represented as a ratio of the Firefly to Renilla luciferase that in turn was normalized to the no treatment control. Error bars represent standard deviation. (* $P < 0.05$, ** $P < 0.01$) (C) HepG2 cells incubated for 72 hours with 1 μ M MM or T1 led to a significant and sustained inhibition of TOPflash reporter activity after T1 treatment. (D) Snu-449 cells treated with 1 μ M MM and T1 for 72 hours also showed a reduction in the TOPflash activity only in the T1 treated samples.

Fig. (3). T1 γ GPNA affects expression of the β -catenin gene and the Wnt target expression to next impair HCC cell proliferation and survival

(A) When compared to the MM control, T1 treatment of HepG2 cells at 1 μ M for 72 hours resulted in a greater than 50% and significant (* p <0.05) decrease in β -catenin mRNA expression by the Real-Time PCR. (B) Western blots with the whole cell lysates of HepG2 and Snu-449 cells treated similarly with T1, showed a decrease in β -catenin protein along with the decreased expression of several of its targets, when compared to the MM control. (C) HepG2 cells incubated with 1 μ M T1 or MM for 72 hours and exposed to 2.5 μ Ci of

[5'-³H]-Thymidine for 48 hours, showed a significant decrease in the DNA synthesis in the T1 group. **(D)** HepG2 cells incubated with 1 μ M T1, MM or no γ GPNA for 72 hours and stained for Ki-67, showed a dramatic decrease in Ki-67 positive cells after T1 treatment only. **(E)** A significant decrease in HepG2 cell viability by the MTT assay was also evident after 72 hours of 1 μ M T1 treatment as compared to the MM. **(F)** Increased numbers of apoptotic nuclei were evident in the 1 μ M T1-treated HepG2 cells after 72 hours as compared to the MM group. All error bars represent a standard deviation. (* $P < 0.05$, ** $P < 0.01$). Abbreviation: KD-Kilo-Dalton.

Fig. (4). Modulation of β -catenin regulates expression of angiogenic factors in HCC cells
(A) Quantitative RT-PCR angiogenesis array on the mRNA isolated from HepG2 cells treated with 1 μ M MM or T1 for 72 hours revealed a notable decrease in the expression of several secreted pro-angiogenic factors **(B)**. Additional validation by the Real-Time PCR showed significant decreases in the expression of β -catenin and concomitantly of FGF2 and VEGF-A ($p < 0.01$) after T1 but not MM treatment of HepG2 cells. **(C)**. Results were also verified by Real-Time PCR on the mRNA from HepG2 cells transfected with β -catenin or control siRNA that also showed significant decreases in β -catenin, FGF2 and VEGF-A

expression ($p < 0.01$). **(D)**. Hep3B cells stably transfected with the S33Y- and not the WT- β -catenin showed a notable increase in β -catenin and VEGF-A expression.

Fig. (5). Conditioned media collected from 72 hours T1-treated HepG2 cells diminished angiogenesis in the SK-Hep1 cells
 (A) SK-Hep1 endothelial spheroids incubated with the conditioned media obtained from HepG2 cells cultured in the presence of T1 or MM for 72 hours, showed dramatically less branching in the T1 group. (B) The numbers of the tube sprouts from each spheroid were counted. The comparison of the averages (MM $n=3$; T1 $n=4$) (SD) showed a significant decrease in the number of sprouts in the T1 group (** $p < 0.01$). (C) Average tube length (SD) from SK-Hep1 endothelial spheroids (MM $n=3$; T1 $n=4$) also showed a significantly

less length in the T1 group (* $p < 0.05$). **(D)** Two representative phase contrast images each from SK-Hep1 cells incubated for 3 hours on the extracellular matrix while cultured in either the conditioned media from the HepG2 cells treated with MM γ GPNA or T1 γ GPNA for 72 hours, showed a noteworthy inhibition in the tube formation and connections in the presence of T1-conditioned media only. **(E)** Quantification of tube length from conditions described in D showed a significant decrease in the average length of the tubes formed by SK-Hep1 cells when cultured in the T1 conditioned media (* $p < 0.05$). **(F)** Number of tubes measuring 150 μ m were also significantly lower in the T1 as compared to the MM group (** $p < 0.01$). **(G)** The same conditioned media used for A and D, when utilized to culture HepG2 cells for 24 hours, showed comparable and low TOPflash activity, indicating lack of any unused T1 γ GPNA in the media that could have influenced endothelial cell function to confound angiogenesis assays.