Published in final edited form as:
Cancer Lett. 2014 February 1; 343(1): . doi:10.1016/j.canlet.2013.09.020.

Beta-catenin signaling in hepatocellular cancer: Implications in inflammation, fibrosis, and proliferation

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Abstract

β-Catenin signaling is implicated in hepatocellular carcinoma (HCC), although its role in inflammation, fibrosis, and proliferation is unclear. Commercially available HCC tissue microarray (TMA) of 89 cases was assessed for β-catenin, **one of its** transcriptional targets glutamine synthetase (GS), proliferation (PCNA), inflammation (CD45), and fibrosis (Sirius Red). HCC cells transfected with wild-type (WT) or mutant-β-catenin were evaluated for β-catenin-T cell factor transactivation by TOPFlash reporter activity and expression of certain targets. Hepatocyte-specific-serine-45-mutated β-catenin transgenic mice (TG) and controls (Con) were used to study thioacetamide (TAA)-induced hepatic fibrosis and tumorigenesis. Sustained β-catenin activation was only observed in mutant-, not WT-β-catenin transfected HCC cells. Aberrant intratumoral β-catenin stabilization was evident in 33% cases with 9% showing predominant nuclear with some cytoplasmic (N/C) localization and 24% displaying predominant cytoplasmic with occasional nuclear (C/N) localization. N/C β-catenin was associated with reduced fibrosis ($p=0.017$) and tumor-wide GS staining ($p<0.001$) while C/N correlated with increased intratumoral inflammation ($p=0.064$) and proliferation ($p=0.029$). A small subset of HCC patients (15.5%) lacked β-catenin staining and exhibited low inflammation and fibrosis ($p<0.05$). TG and Con mice exposed to TAA showed comparable development of fibrosis and progression to cirrhosis and HCC. Taken together the data suggests a complex relationship of β-catenin, inflammation, fibrosis and HCC. GS staining is highly sensitive in identifying HCC with nuclear β-catenin, which may in turn represent β-catenin mutations, and does so with high negative predictive value. Also, β-catenin mutations and cirrhosis do not appear to cooperate in HCC pathogenesis in mice and men.

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Conflict of interest: SPSM is a consultant for PhaseRx and Merck.

Keywords

β-catenin; glutamine synthetase; inflammation; fibrosis; proliferation; hepatocellular carcinoma

1. INTRODUCTION

Hepatocellular cancer (HCC) is a major health burden worldwide [1]. It is the fifth most common cancer in men, and its annual incidence reaches more than half a million worldwide [2]. There is a need for an improved treatment since less than 50% patients survive more than a year [3]. Approximately 90% of HCCs in patients with underlying liver injury or infection are associated with chronic inflammation. The most well known causes of chronic inflammation in the liver are hepatitis B and C, nonalcoholic steatohepatitis (NASH), and chronic alcohol consumption. These agents cause repeated cycles of injury, hepatocyte necrosis and oxidative stress followed by hepatocyte proliferation and wound healing response mediated by stellate cells that generates hepatic fibrosis and eventually cirrhosis [4; 5]. Various molecular pathways are implicated in the HCC pathogenesis including β-catenin, p53, EGF, HGF, TGFβ and others [6].

The Wnt/β-catenin pathway implicated in hepatic tumorigenesis, also plays indispensible roles in hepatic development and regeneration [7]. Aberrant activation of Wnt/β-catenin signaling allows β-catenin to resist degradation and enter the nucleus where it acts as a co-factor for the T cell factor (TCF) family of transcription factors to regulate the expression of several genes relevant to cell proliferation and apoptosis, including *c-myc*, *cyclin-D1*, and *survivin*.

Abnormal accumulation of β-catenin has been demonstrated in 17–40% of HCCs [8]. This occurs due to a diverse set of mechanisms such as methylation of Wnt inhibitors, inactivation of GSK3β, Wnt3/Fz7 upregulation, mutations in β-catenin gene (19–44%), axin-1 (5–14%) and axin–2 (3–10%) and others [7; 8; 9; 10]. Mutations in β-catenin gene appear to be functionally most relevant in HCC since they prevent its degradation thus eliminating any negative feedback brought about by some of its downstream targets, although it has not been directly tested. One such mutation that is commonly described in HCC affects serine-45 [11]. Transgenic mice (TG) expressing this mutation in hepatocytes showed enhanced HCC in response to chemical carcinogenesis as compared to wild-type controls (WT), although no increase in spontaneous HCC was observed [12].

In the current study, we used an *in vitro* assay and an HCC tissue microarray (TMA) to investigate the relationship of intratumoral β-catenin localization and activation to inflammation, fibrosis and proliferation. We identify distinct association of predominant nuclear versus predominant cytoplasmic β-catenin localization to intratumoral cell proliferation, inflammation and fibrosis. We also address the role of β-catenin mutations in development and progression of hepatic fibrosis, cirrhosis and HCC by exposing TG and WT mice to thioacetamide [12; 13]. We eventually discuss some of the implications of these observations that may shed light on the complex role of β-catenin in HCC pathogenesis.

2. MATERIALS AND METHODS

Cell culture

Human HCC cell line Hep3B (ATCC) were plated in six-well plates and cultured in EMEM (ATCC) supplemented with 10% FBS (Atlanta Biologicals) at 37°C in 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) and S45Y was kindly provided by Dr. Sabine Colnot, Inserm, France. The cells were grown to 90% confluence, 2 μg of β-catenin plasmid DNAs were transfected with Lipofectamine™ 2000 (Invitrogen), as per the manufacturer's instructions. 48 hours after transfection, the cells were selected by multiple passages using Geneticin (G418; Sigma; 500ug/ml) to generate stable transfected cell lines. Cells at 60% to 80% confluence were serum starved for 4–16 hours and transiently transfected with the reporter construct TOP-flash (Upstate, Lake Placid, New York, USA), which has three copies of TCF sites upstream of a thymidine kinase (TK) promoter and the firefly luciferase gene. Cells were co-transfected with Renilla Luciferase to control for transfection efficiency for Luciferase assays were performed using Dual Luciferase Reporter Assay System (Promega, Madison, WI, USA). Average relative light units (RLU) from triplicate experiments were compared for statistical significance by Student's t-test.

Tissue microarray slides

For immunohistochemistry (IHC), commercially available tissue microarray (TMA) slides of human HCC (LV2082, US Biomax, Inc, Rockville, MD) were used. They are provided in duplicate cores per patient with clinical information including sex, age, tumor grade, and tumor staging. The tumor grade was scored as well, moderately, or poorly differentiated. Staging was scored according to the American Joint Committee on Cancer TNM staging. Out of 94 cases of HCC included, 5 cases were excluded because of lack of clinical information, and 89 cases were analyzed.

Immunohistochemistry and Sirius Red Staining

TMA were analyzed by IHC for β-catenin, glutamine synthetase (GS), PCNA, CD45, and Sirius Red to determine their expression and localization using the indirect immunoperoxidase technique as described previously [12]. Briefly, the slides were passed through xylene, graded alcohol, and rinsed in phosphate-buffered saline. Endogenous peroxide was inactivated using 3% hydrogen peroxide. Slides were microwaved in zinc sulfate for PCNA or in citrate buffer for β-catenin, GS, CD45, and Sirius Red, then were immersed in Ultra V Block (Lab Vision Products, Fremont, CA) followed by a 1 hour incubation at room temperature with the primary antibody. After washes, the sections were incubated in the appropriate biotin-conjugated secondary antibody (Chemicon, Temecula, CA), for 30 minutes at room temperature. Signal was detected using the Vectastain ABC Elite kit (Vector Laboratories, Inc., Burlingame, CA) and developed using DAB (Vector Laboratories, Inc., Burlingame, CA). Sections were then counterstained with Shandon hematoxylin solution (Thermo Fisher Scientific, Pittsburgh, PA) and passed through the dehydration process and covered. For negative control, the sections were incubated with secondary antibodies only.

For measurement of fibrosis, Sirius red staining was performed. Briefly, sections were rehydrated and placed in Sirius red staining solution for 1 hour at room temperature. Sections were washed with acidified water, dehydrated, and covered.

Image capture

Images were captured as described elsewhere [14]. Automated, whole slide image capture was performed on an Aperio XT slide scanner (Aperio Technologies, Vista CA) outfitted with a Nikon Plan Fluor 20 ×, 0.7 Numerical Aperture Objective Lens and Basler L301 "trilinear array" line scan camera. Images were compressed during the capture process in a multi-layered JPG2000 format using a Matrox Morphis compression board (Matrox Incorporated, Montreal Canada) with a quality setting of 30 resulting in file sizes ranging

between 10 MB to 300 MB. Aperio Image Server software version 5.6 was used for image capture.

Analysis and quantification of staining

The staining was assessed, quantified and averaged for various markers in the duplicate cores of HCC samples by a single individual (J.M.L.). The β-catenin staining was categorized as predominantly nuclear with or without significant cytoplasmic (N/C) representing its sustained activation; predominantly cytoplasmic with only occasional cells displaying nuclear localization (C/N) representing transient β-catenin activation; or membranous with or without some cytoplasmic (M/C) staining representing normal β-catenin. No β-catenin staining in the tissue cores was also noted. The staining of CD45 as a marker of inflammation was categorized into absent, mild, moderate, or severe based on presence of 0%, 0~10%, 10~30%, or over 30%, positive cells respectively. The staining for Sirius Red as an indicator of fibrosis was also categorized into being absent, mild, moderate, or severe, if fibrosis occupied 0%, 0~10%, 10~30%, or over 30% of the field, respectively. PCNA as a marker of cell proliferation was categorized as absent, mild (<30% PCNA+), moderate (30–60% PCNA+) and high (>60% PCNA+). For quantification, all stains were assessed at 260X magnification and at least 3 fields from each of the two cores were counted.

Statistical analysis

Data were expressed as median (range) or mean ± standard deviation. Continuous data were compared using Student's t-test or analysis of variance as appropriate. Categorical data were compared using chi-square test or linear by linear association as appropriate. All statistical analyses were performed using SPSS 17.0 (SPSS Inc., Chicago, IL), and p value < 0.05 was considered statistically significant.

Animals

All animal usage was within NIH and Institutional guidelines and approved by the IACUC at Mount Sinai School of Medicine. Hepatocyte-specific serine-45 mutated β-catenin expressing transgenic (TG) mice and wild-type (WT) controls (FVB background) have been described previously [12]. TG and WT FVB mice were treated for up to 12 months with thioacetamide (TAA) [13]. TAA is a fibrogenic agent with some tumorigenic activity, and was added to the drinking water of the treatment group [15]. Control animals received normal drinking water without TAA. At the start of treatment, mice were exposed to 50 mg/L TAA, which was increased weekly by 50 mg/L up to a dose of 300 mg/L. Mice were individually weighed every week to monitor toxic side effects of TAA treatment, and dosage was adjusted weekly based on the individual mouse weight compared to average weight of the control group to minimize toxicity. The average lifetime TAA dosage in WT and TG was 162 mg/L and 167 mg/L, respectively. Livers were harvested at 6, 9 and 12 months and assessed macroscopically for number and size of tumor nodules and compared for statistical significant by student *t*-test. Representative livers from each group were fixed in 10% formalin and paraffin embedded. Paraffin blocks were sectioned and 4 μm sections were stained for Hematoxylin and Eosin (H&E) and reviewed by a single blinded pathologist (Dr. Swan Thung, Mount Sinai School of Medicine) for microscopic assessment of disease process in all groups of animals.

3. RESULTS

Patient characteristics

Online supplement (OS) Table 1 summarizes patient characteristics. Median age of patients included on the TMA was 51. 78 patients (87.6%) were male. Numbers of patients with tumor grade 1, 2, and 3 were 8 (9.0%), 63 (70.8%), and 18 (20.2%), respectively. Similarly, the number of patients with TNM stage 1, 2, 3, and 4 was 8 (9.0%), 68 (76.4%), 12 (13.5%), and 1 (1.1%), respectively.

Immunohistochemical characterization

Representative IHC on TMA is shown (Fig. 1). The information is summarized in OS Table 1. For β-catenin IHC, 8 patients (9%) showed predominant nuclear with some cytoplasmic (N/C) localization; 21 (23.6%) showed predominant cytoplasmic with a few cells showing nuclear (C/N); 47 patients (52.8%) showed mostly membranous and some cytoplasmic (M/C); and 13 patients (15.5%) lacked any β-catenin staining. 29/89 HCCs (32.6%) displayed tumor-wide GS staining.

IHC for proliferating cell nuclear antigen (PCNA) was utilized to detect cells in S-phase as a marker of proliferation (Fig. 1). Staining was categorized as either absent, mild, moderate and high as described in methods. 3 HCCs lacked PCNA, 31 showed mild, 26 showed moderate and 29 showed high PCNA-positivity (OS Table 1). CD45 was used as a marker of inflammation (Fig. 1). While 6 patients showed no CD45-positive cells, 36 showed mild, 24 showed moderate and 23 showed severe inflammation (OS Table 1). Hepatic fibrosis as assessed by Sirius Red staining (Fig. 1), showed 6 patients lacking any fibrosis, 23 displaying mild, 21 showing moderate and 39 showing severe fibrosis (OS Table 1).

Association between inflammation, fibrosis, and proliferation

We next wanted to assess any correlation between inflammation, fibrosis and proliferation in our HCC dataset. A significant positive correlation was observed between intratumoral inflammation, proliferation ($p<0.001$) and fibrosis ($p=0.004$) and between intratumoral hepatic fibrosis and proliferation ($p<0.001$) (OS Table 2). These observations affirm a positive relationship between these three processes that represent an important cellular basis of majority of HCCs.

β-Catenin mislocalization in HCC is associated with increased proliferation and modestly increased inflammation

β-Catenin is normally M/C in localization. In TMA, 29 patients (32.6%) showed either N/C or C/N β-catenin representing aberrant localization (OS Table 3). These HCC showed significant correlation with tumor-wide GS-positivity (p=0.007) and showed significantly greater proliferation (p=0.031) and modestly enhanced CD45 infiltration (p=0.082). There was no change in hepatic fibrosis. Aberrant β-catenin localization also correlated with better tumor grade and only 7% HCCs in this category belonged to grade 3 in contrast to 27% in the other group (OS Table 3).

Comparison of HCCs displaying nuclear versus cytoplasmic β-catenin localization

Within the groups of aberrant β-catenin localization in HCC, two clear patterns were apparent. One group showed notable N/C β-catenin while the other showed predominant C/N β-catenin. In absence of tissue availability from these tumors to address mutations in β-catenin gene or other components of the β-catenin degradation complex, we first validated if these β-catenin mislocalization groups were indeed different in molecular and cellular attributes. Indeed significant differences were evident in GS staining as a marker of β-

catenin activity such that 87.5% of N/C β-catenin-positive HCCs versus 38% of C/N-β-catenin-positive HCCs were GS-positive (p=0.035) (OS Table 4). No differences were evident between these two groups in proliferation or inflammation, however, N/C-β-catenin group showed significantly lower fibrosis (p=0.016) (OS Table 4).

GS staining to predict β-catenin mislocalization and HCC phenotype

Since GS has been shown to be a surrogate target of β-catenin signaling, and others and us have reported a positive correlation between GS expression and β-catenin mutations [10; 16; 17], we next stratified patients based on GS staining. Around 33% of all HCC samples were GS-positive (OS Table 5). Interestingly, while several GS-positive tumors showed N/C or C/N β-catenin, around 13/29 GS-positive tumors showed predominantly M/C β-catenin. Only 1/29 tumors was GS-positive and β-catenin-negative simultaneously. Conversely, only 1/60 GS-negative tumor was nuclear β-catenin-positive.

GS-positive staining of the tumors by itself however, was not associated with any changes in intratumoral proliferation, fibrosis or inflammation as compared to the GS-negative group of tumors indicating only limited biological implications of this marker by itself (OS Table 5).

Sustained Wnt activation apparent with mutant- but not WT-β-catenin overexpression in HCC cells

Knowing the heterogeneity in the mechanisms leading to β-catenin activation in HCC, we hypothesized that N/C may represent sustained (possibly due to mutations in β-catenin gene or genes encoding for the components of its degradation complex) and C/N may represent transient β-catenin stabilization (due to increased Wnt ligands or increased Frizzled expression). To determine the extent and sustainability of β-catenin activation due to mutations versus other reasons, we transfected Hep3B cells that harbor normal full-length β-catenin gene, with control plasmid or plasmid expressing WT-β-catenin, S33Y-β-catenin or S45Y-β-catenin. A comparable increase in TOPFlash reporter activity in all β-catenin-transfected Hep3B cells was evident over the control plasmid at 24 hours (24h) and 48h after transfection (Fig. 2A). However at 72h only S45Y and S33Y show increased reporter activity while WT-β-catenin reached steady state and compared to the control plasmid-transfected cells (Fig. 2A). Corresponding lysates show increase in β-catenin targets cyclin-D1 and GS prominently at 72h in only S33Y- and S45Y-transfected Hep3B cells (Fig. 2B). These findings indicate that WT β-catenin overexpression leads to transient and mutant-β-catenin to sustained Wnt signaling in HCC cells.

Nuclear β-catenin localization is associated with increased GS immunoreactivity and decreased intratumoral fibrosis in HCC patients

Based on the preceding findings, we next compared HCCs exhibiting N/C, which may represent sustained β-catenin localization, to all remaining HCCs. HCCs with N/C β-catenin showed significant correlation to GS-positivity (p=0.001) (Table 1). Additionally, these tumors showed significantly less intratumoral fibrosis as compared to the rest (p=0.017). However no significant differences in inflammation or proliferation within the duplicate tumor cores were observed in this group when compared to the remainder of cases.

Predominant cytoplasmic β-catenin localization is associated with increased proliferation and inflammation in the HCC core

Next HCC cases with C/N-β-catenin (n=21) that may represent transient β-catenin activation were compared with the remaining cases (Table 2). Indeed these cases did not show consistent GS staining (p=0.538). These tumors however, showed significantly higher cell

proliferation (p=0.029) and marginally higher inflammation (p=0.064) than the remaining cases. Intriguingly, no association with hepatic fibrosis was observed in this group.

A subset of HCC lacking β-catenin immunoreactivity show reduced inflammation and fibrosis

A novel group of HCC was identified that lacked β-catenin staining all together in the tumor cores. This group of 13 patients (15%) was also GS-negative except one case (p=0.053) (Table 3). β-Catenin-negative HCCs showed significantly lower fibrosis (*p*=0.045) and inflammation (*p*=0.007), but unremarkable differences in proliferation.

β-Catenin mutation does not predispose TG mice to thioacetamide induced hepatic fibrosis and HCC

Since HCCs with N/C β-catenin showed significantly less fibrosis, we wondered if β-catenin mutations expedite HCC development by bypassing the natural course of progressive fibrosis, cirrhosis and HCC. To address this, we utilized a previously characterized WT and TG mouse harboring serine-45 mutated-β-catenin in hepatocytes [12] and exposed it to TAA, a profibrogenic and carcinogenic agent as described in methods [11]. At 6 months, no tumors were observed in either WT or TG mice although histological changes reminiscent of mild inflammation and fibrosis were comparably evident (Fig. 3A). At 9 months also, no tumors were detected in either treatment group, however inflammation and fibrosis had comparably progressed to cirrhosis in both WT and TG mice (Fig. 3A). At 12 months, macroscopic tumors and nodules were detected in about 50% of animals in both the TG and WT groups (not shown and OS Table 6). Interestingly, no differences in number or size of tumors and nodules were evident between the two groups (Fig. 3B-D). H&E staining and blinded review by a pathologist revealed that 29% of the WT, and 31% of the TG animals had developed HCC (OS Table 6). WT and TG not exposed to TAA had normal liver structure, without any fibrosis or tumors (OS Table 6). Based on these results, we conclude that β-catenin mutation does not cooperate with fibrosis to alter its progression to cirrhosis and development of HCC.

4. DISCUSSION

β-catenin is central to liver biology and is the major downstream effector of canonical Wnt pathway. In normal liver, β-catenin is expressed at hepatocyte membrane. In pericentral hepatocytes, β-catenin is also evident in the cytoplasm and nucleus to regulate centrizonal expression of GS and specific cytochrome P450s [18]. β-Catenin mislocalization in HCC can occur due to mutations in *CTNNB1, AXIN1/2,* methylation of Wnt inhibitors such as WIF and FRP, or increased expression of certain Wnt/Frizzled genes (reviewed in [7]). Suffice to say that β-catenin activation in a subset of HCCs is deemed oncogenic. Our current analysis on a TMA representing duplicate cores from 89 HCC cases, demonstrates aberrant β-catenin localization in around 33% of HCCs. These patients, all males, showed predominantly grade 1 or 2 disease, were GS-positive in 52% cases, exhibited higher proliferative index, and showed marginally higher inflammation (p=0.08). Indeed β-catenin activation is linked to greater cell proliferation and cyclin-D1 expression in liver regeneration and HCC [19; 20].

However, two subgroups of β-catenin mislocalization were evident in our dataset-one with predominant nuclear and the other with mostly cytoplasmic staining. To see if these two categories had distinct molecular and biological attributes especially since we did not have access to tissues to assess mutations in β-catenin gene, we asked if staining for GS, a surrogate for β-catenin mutation could predict aberrant β-catenin localization [16]. Previously, others and we have reported an increase in GS specifically in HCCs with

CTNNB1 mutations based on frozen tissue analysis [10; 17; 21]. When IHC for GS is used to determine sensitivity and specificity in detecting all β-catenin mislocalization cases (nuclear and cytoplasmic; n=29), its sensitivity was only 24% while specificity was 77%. While the positive predictive value of GS IHC in identifying aberrant β-catenin localization was 52%, its negative predictive value was around 77%. However, when IHC for GS was used to identify patients displaying predominantly nuclear β-catenin localization (8 of the 89 cases), the sensitivity went up to 87.5% while specificity remained at 73%. Intriguingly, the positive predictive value decreased to around 24% while negative predictive value climbed to 98%. Thus, GS-positive tumors may identify nuclear β-catenin or its mutations with great sensitivity, although false-positive cases may be high. Similarly lack of GS staining in a tumor may be valuable in ruling out β-catenin mutations. GS staining by itself yielded no biological information pertaining to the tumors. This lack of any distinct tumor phenotype was attributable to a subset of HCCs that are GS-positive in duplicate cores, despite β-catenin being predominantly membranous. One possibility is that GS changes in some of these tumors may reflect metabolic disturbances such as hyperammonemia, which are known to cause changes in the GS expression [22].

The two subgroups based on β-catenin staining did show differences in tumor biology since C/N correlated with increased proliferation and intratumoral inflammation while N/C β-catenin group showed lesser intratumoral fibrosis. The group of HCCs with predominant cytoplasmic β-catenin localization did not show correlation with GS staining. This may suggest that in the C/N group somehow β-catenin activity is curbed and may represent transient upregulation. Such a model was also supported by the *in vitro* studies where only HCC cells transfected with mutant-β-catenin showed sustained Wnt activation. While the mechanisms behind these observations may be complex, it is conceivable that some known downstream targets such as *Axin2, Dickkopf (Dkk)* and *β-transducing repeat-containing protein (βTrCP)* that induce β-catenin degradation may be able to downregulate β-catenin signaling especially when β-catenin is not mutated and hence degradable. Thus cytoplasmic with some nuclear β-catenin localization may represent transient ‘bursts’ of activation that may be kept in check by simultaneous expression of feedback inhibitory targets.

Inflammation is a well-known driver of HCC [5] and in our dataset correlated with greater tumor cell proliferation and hepatic fibrosis. Intriguingly, enhanced tumoral inflammation in C/N β-catenin was associated with notable proliferation but not fibrosis. The small number of tumors with N/C β-catenin staining did not demonstrate increased inflammation; in fact these HCCs exhibited significantly less intratumoral fibrosis. This reveals a complex relationship between β-catenin, inflammation and fibrosis in HCC. Since N/C β-catenin represents its sustained transcriptional activation, we believe that Wnt signaling is not promoting inflammation. In fact leukocyte cell-derived chemotaxin 2 (*Lect2*), a known β-catenin target [23], which is known to inhibit inflammation, has also been reported to be upregulated in HCCs with *CTNNB1* mutations [24]. Similarly, murine models that develop HCC via Wnt signaling do not display enhanced inflammation, injury or fibrosis including hepatocyte-specific APC deletion, diethylnitrosamine (DEN)-challenged S45D transgenic mice, Met-overexpressing mice, combined H-Ras and β-catenin exon-3 deletion, and C3H mice with DEN/PB exposure [12; 25; 26; 27; 28]. It is likely that C/N-β-catenin may represent β-catenin stabilization due to Wnt secretion by inflammatory cells [29] that may in turn be contributing to overall tumor cell proliferation and survival. In this context, β-catenin is known to counteract oxidative stress and promote cell survival through interactions with transcription factors such as FoxO1 or HIF1α [30; 31; 32].

HCC with N/C-β-catenin showed significantly less fibrosis without any impact on inflammation or proliferation. Others and we have reported β-catenin mutations to be less frequent in HCCs associated with cirrhosis [17; 21; 33]. These observations are further

supported by paradoxical increase in HCC in β-catenin-conditional null mice after chemical carcinogen exposure, which was associated with fibrosis [34; 35; 36]. Similarly, hepatic adenomas that progress to HCC, lack fibrosis and harbor CTNNB1 mutations [37]. In our dataset no information on preexisting adenomas is available. We however investigate the possibility that β-catenin mutations may either accelerate tumor development during progression of hepatic fibrosis or that β-catenin mutations and hepatic fibrosis are two mostly independent paths to HCC and don't necessarily cooperate with one another in hepatic tumorigenesis. To address these, we utilize a TG mouse expressing serine45-mutant-β-catenin in hepatocytes, which only displays enhanced hepatocarcinogenesis after exposure to a chemical carcinogen as compared to the WT [12]. When exposed to a well-known fibrogenic chemical TAA for 6–12 months, there were no differences in either development, or progression, of fibrosis to HCC, when compared to the controls. These data suggest that β-catenin mutations do not alter the course of hepatic fibrosis or of HCC development in a cirrhotic milieu. Thus *CTNNB1* mutations and fibrosis may be independent risk factors, which may not be cooperating in HCC pathogenesis.

Intriguingly, 15.5% of HCCs lacked β-catenin. To our knowledge, this specific group has never been reported previously and displayed significantly lesser inflammation and fibrosis although proliferation was unaltered. Equally enigmatic is the mechanism of β-catenin loss in these tumors. Additional studies will need to determine if this could be an epigenetic event since *CTNNB1* promoter methylation has been reported in breast cancer [38]. Similarly, miRNA aberrations that have been identified in HCC may suppress β-catenin expression [39] and may be responsible for observed loss in a subset of tumors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

FUNDING SUPPORT: This study was funded by NIH grants 1R01DK62277, 1R01DK100287 and Endowed Chair for Experimental Pathology to SPSM. This study was also supported in part by 1R01DK56621 to SLF.

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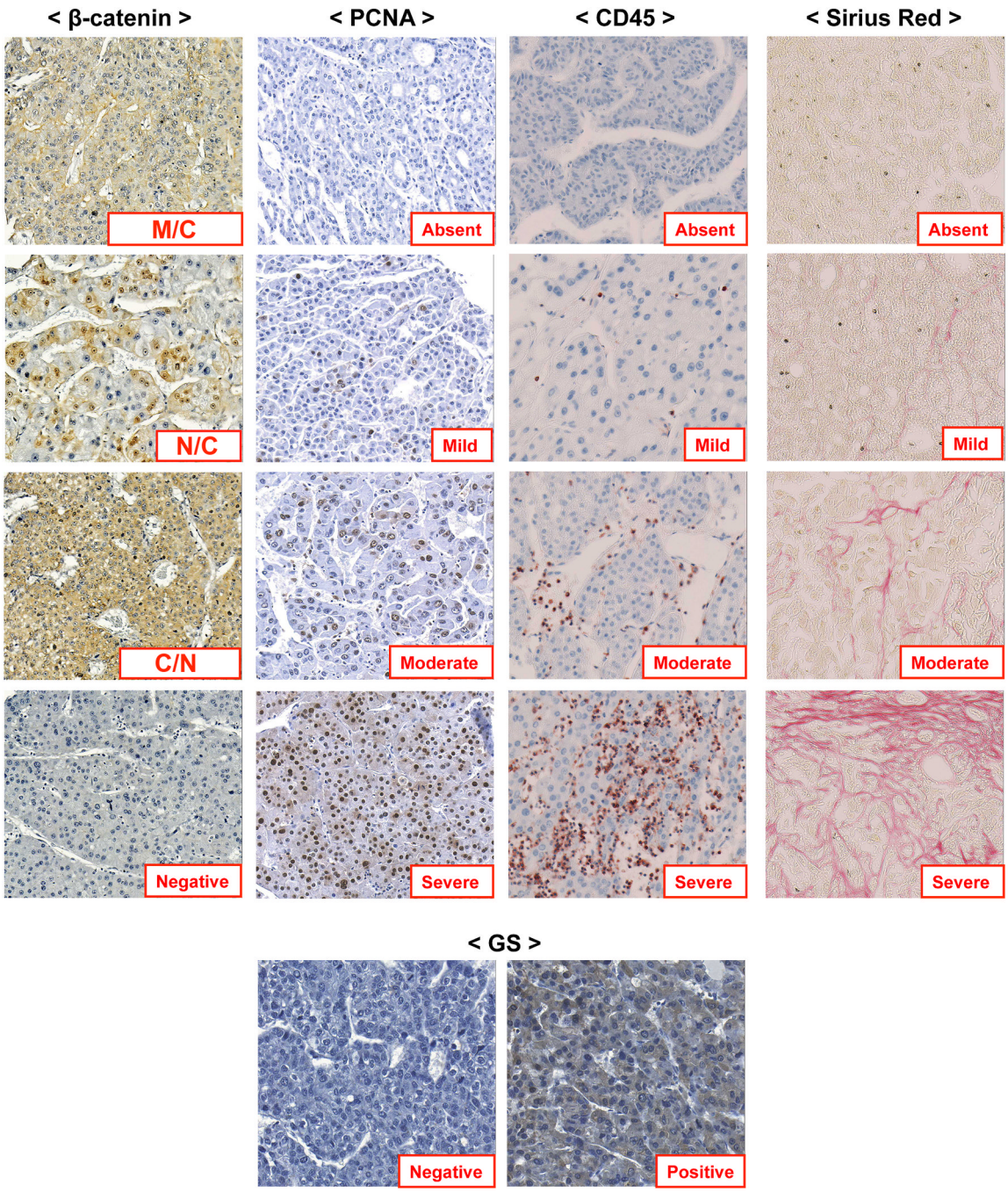

Figure 1. Sample of staining on the HCC tissue array

β-Catenin was classified as being N/C, C/N, M/C or negative. PCNA, CD45 and Sirius Red as markers of proliferation, inflammation and fibrosis, respectively, were categorized as absent, mild, moderate and severe. IHC for GS was labeled as positive or negative.

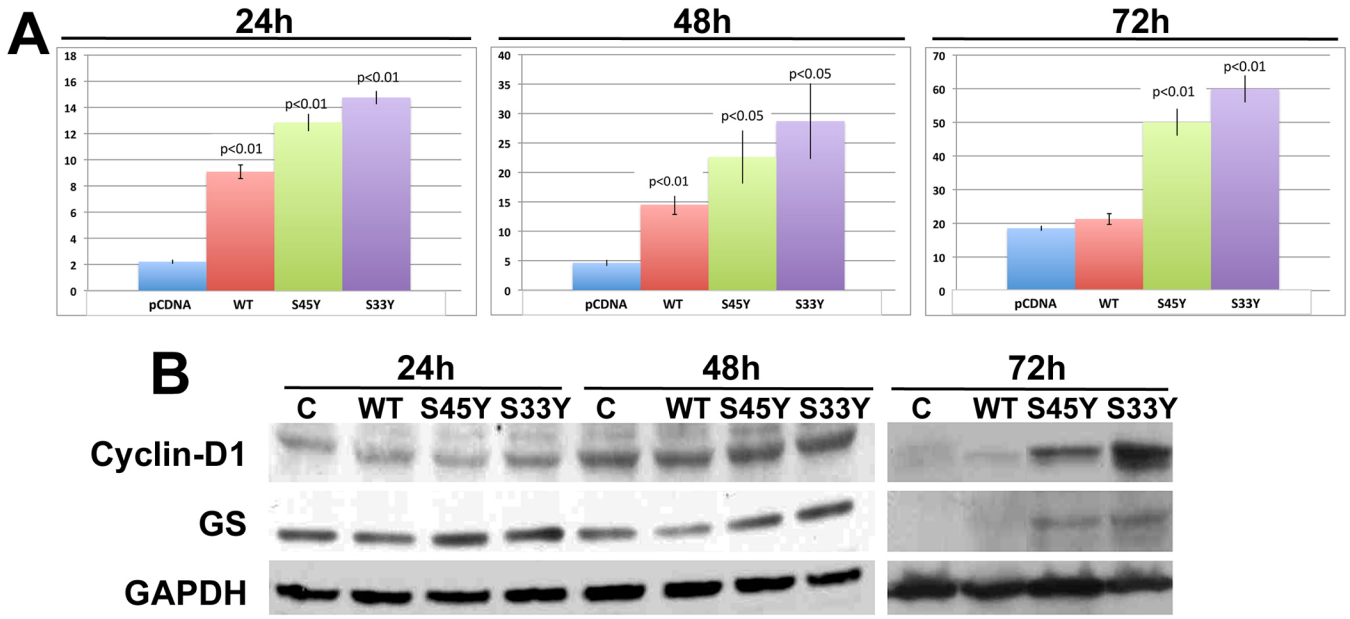

Figure 2. Sustained β-catenin activation following mutant not wild-type β-catenin expression in hepatoma cells

A. Increased TOPFlash reporter activity (Firefly/Renilla value) over pcDNA plasmid is evident in Hep3B cells transfected with WT, S45Y and S33Y at 24h and 48h after transfection, however at 72h pcDNA and WT show comparable low activity, while S33Y and S45Y show significantly higher luciferase activity.

B. Western blots from corresponding lysates show comparable low level increases in GS and cyclin-D1 at 24h and 48h while a notable increase is visible at 72h in S33Y and S45Y versus others.

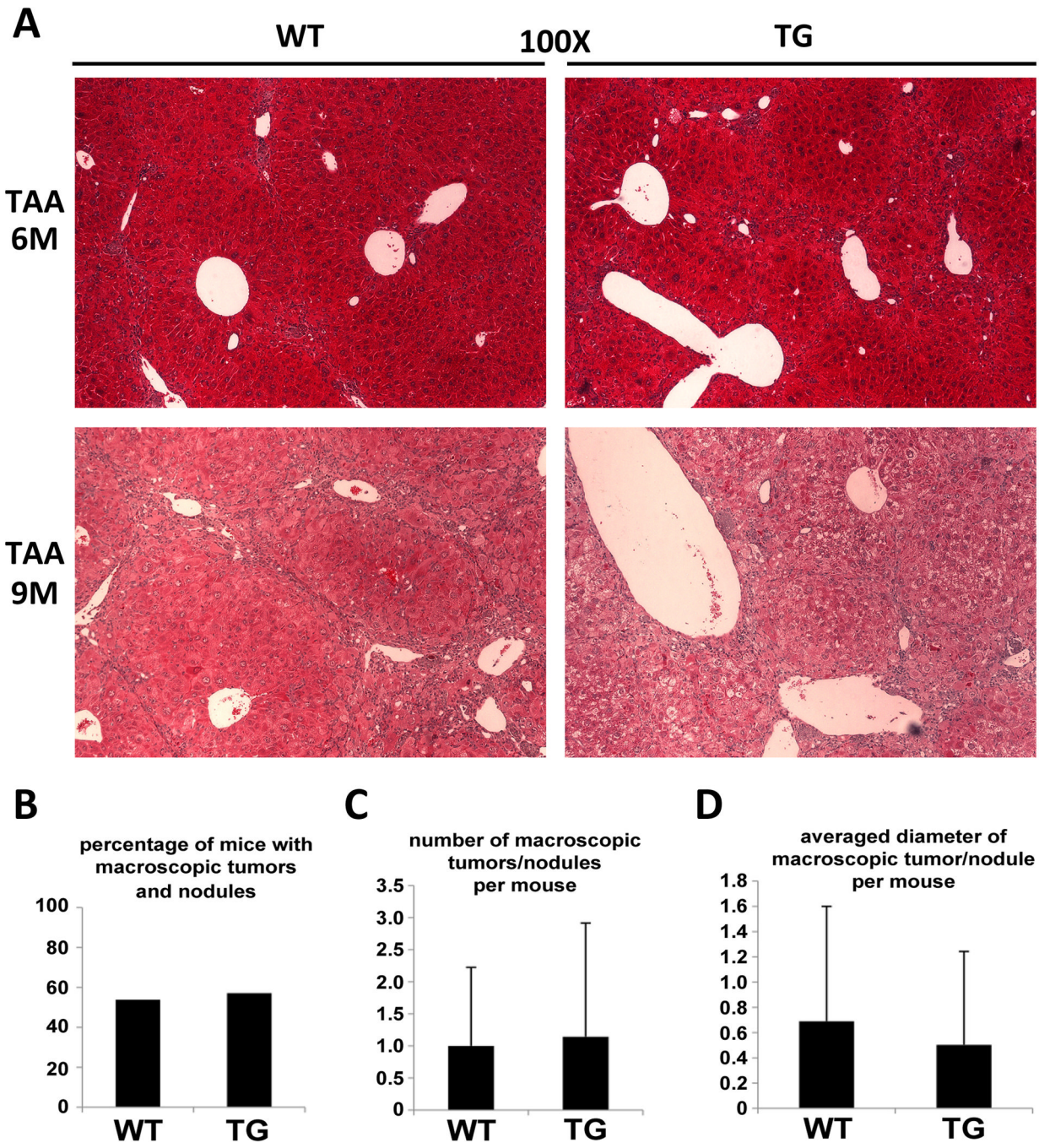

Figure 3. Histological changes and tumorigenesis in WT controls and Serine45-mutated-β-catenin transgenic (TG) mice after chronic exposure to Thioacetamide

A. Comparable and progressive inflammation and fibrosis in WT and TG livers at 6 months (6M) and 9M after TAA-exposure as seen in representative H&E staining (100x magnification).

B. No change in percentage of WT and TG mice with macroscopic disease.

C. Insignificant difference in the numbers of macroscopic tumors and nodules evident per TG or WT mice after TAA exposure.

D. Comparable size of macroscopic tumors and nodules in WT and TG mice after TAA exposure.

Table 1

Comparison of HCCs displaying N/C β-catenin to remaining HCCs.

β-Catenin		**N/C (*n*=8)**	**Others (*n*=81)**	***p*-value**
Age, yr, mean ± SD		50.8 ± 8.3	49.9 ± 11.0	0.825
Male sex, *n* (%)		8 (100.0)	70 (86.4)	0.589
Grade, *n* (%)				0.531
	1	1 (12.5)	7 (8.6)	
	2	6 (75.0)	57 (70.4)	
	3	1 (12.5)	17 (21.0)	
Stage, *n* %)				0.271
	1	1 (12.5)	7 (8.6)	
	2	7 (82.5)	61 (75.3)	
	3	0 (0.0)	12 (14.8)	
	4	0 (0.0)	1 (1.2)	
GS (+), *n* %)		7 (87.5)	22 (27.2)	0.001
PCNA (+) cells, *n* %)				0.767
	Absent	0 (0.0)	3 (3.7)	
	Mild	3 (37.5)	28 (34.6)	
	Moderate	2 (25.0)	24 (29.6)	
	High	3 (37.5)	26 (32.1)	
Sirius Red, *n* %)				0.017
	Absent	1 (12.5)	5 (6.2)	
	Mild	5 (62.5)	18 (22.2)	
	Moderate	1 (12.5)	20 (24.7)	
	Severe	1 (12.5)	38 (46.9)	
CD45, *n* %)				0.921
	Absent	0 (0.0)	6 (7.4)	
	Mild	4 (50.0)	32 (39.5)	
	Moderate	2 (25.0)	22 (27.2)	
	Severe	2 (25.0)	21 (25.9)	

Abbreviations: GS-Glutamine synthetase; PCNA-Proliferating cell nuclear antigen

Table 2

Comparison of HCCs showing C/N β-catenin to remaining HCCs.

β-Catenin		**C/N (*n*=21)**	**Others (*n*=68)**	***p*-value**
Age, yr, mean ± SD		46.4 ± 12.9	51.0 ± 9.8	0.081
Male sex, *n* (%)		21 (100.0)	57 (83.8)	0.060
Grade, *n* (%)				0.115
	1	2 (9.5)	6 (8.8)	
	2	18 (85.7)	45 (66.2)	
	3	1 (4.8)	17 (25.0)	
Stage, *n* (%)				0.445
	1	1 (4.8)	7 (10.3)	
	2	16 (76.2)	52 (76.5)	
	3	4 (19.0)	8 (11.8)	
	4	0 (0.0)	1 (1.5)	
GS† (+), *n* (%)		8 (38.1)	21 (30.9)	0.538
PCNA (+) cells, *n* (%)				0.029
	Absent	0 (0.0)	3 (4.4)	
	Mild	4 (19.0)	27 (39.7)	
	Moderate	7 (33.3)	19 (27.9)	
	High	10 (47.6)	19 (27.9)	
Sirius Red, *n* (%)				0.201
	Absent	1 (4.8)	5 (7.4)	
	Mild	4 (19.0)	19 (27.9)	
	Moderate	4 (19.0)	17 (25.0)	
	Severe	12 (57.1)	27 (39.7)	
CD45, *n* (%)				0.064
	Absent	0 (0.0)	6 (8.8)	
	Mild	7 (33.3)	29 (42.6)	
	Moderate	6 (28.6)	18 (26.5)	
	Severe	8 (38.1)	15 (22.1)	

Abbreviations: GS-Glutamine synthetase; PCNA-Proliferating cell nuclear antigen

Table 3

Comparison between β-catenin negative vs. remaining HCCs.

β-catenin		**Neg (*n*=13)**	**Others (*n*=76)**	***p*-value**
Age, yr, mean ± SD		53 ± 8	49 ± 11	0.244
Male sex, *n* (%)		13 (100)	65 (85.5)	0.356
Grade, *n* (%)				0.410
	1	3 (23.1)	5 (6.6)	
	2	7 (53.8)	56 (73.7)	
	3	3 (23.1)	15 (19.7)	
Stage, *n* (%)				0.515
	1	1 (7.7)	7 (9.2)	
	2	9 (69.2)	59 (77.6)	
	3	3 (23.1)	9 (11.8)	
	4	0 (0.0)	1 (1.3)	
GS (+), *n* (%)		1 (7.7)	28 (36.8)	0.053
PCNA (+) cells, *n* (%)				0.201
	Absent	1 (7.7)	2 (2.6)	
	Mild	6 (46.2)	25 (32.9)	
	Moderate	3 (23.1)	23 (30.3)	
	High	3 (23.1)	26 (34.2)	
Sirius Red, *n* (%)				0.045
	Absent	2 (15.4)	4 (5.3)	
	Mild	4 (30.8)	19 (25.0)	
	Moderate	5 (38.5)	16 (21.1)	
	Severe	2 (15.4)	37 (48.7)	
CD45, *n* (%)				0.007
	Absent	2 (15.4)	4 (5.3)	
	Mild	8 (61.5)	28 (36.8)	
	Moderate	3 (23.1)	21 (27.6)	
	Severe	0 (0.0)	23 (30.3)	

Abbreviations: GS-Glutamine synthetase; PCNA-Proliferating cell nuclear antigen