Published in final edited form as:

Adv Exp Med Biol. 2015 ; 815: 185–195. doi:10.1007/978-3-319-09614-8_11.

Alcohol consumption, Wnt/ β -catenin signaling and hepatocarcinogenesis

K. E. Mercer^{1,2}, L. Hennings³, and M. J. J. Ronis^{1,2}

¹Arkansas Children's Nutrition Center, University of Arkansas for Medical Sciences Little Rock, AR, USA

²Department of Pediatrics, University of Arkansas for Medical Sciences Little Rock, AR, USA

³Department of Pathology, University of Arkansas for Medical Sciences Little Rock, AR, USA

Abstract

Alcohol is a well-established risk factor for hepatocellular carcinoma, and the mechanisms by which alcohol liver cancer are complex. It has been suggested that ethanol (EtOH) metabolism may enhance tumor progression by increasing hepatocyte proliferation. To test this hypothesis, ethanol (EtOH) feeding of male mice began 7 weeks post-injection of the chemical carcinogen diethylnitrosamine (DEN), and continued for 16 weeks, with a final EtOH concentration of 28% of total calories. As expected, EtOH increased the total number of cancerous foci and liver tumors identified in *situ* fixed livers from the EtOH+DEN group compared to corresponding pair-fed (PF) +DEN and chow+DEN control groups. In the EtOH+DEN group, tumor multiplicity corresponded to a 3- to 4-fold increase proliferation and immunohistochemical staining of β -catenin in non-tumorigenic hepatocytes when compared to the PF+DEN and chow+DEN groups, $p < 0.05$. Analysis of EtOH-treated livers from a previously published rat model of chronic liver disease revealed increases in hepatocyte proliferation accompanied by a hepatic depletion of retinol and retinoic acid stores ($p < 0.05$), nuclear accumulation of β -catenin ($p < 0.05$), increased cytosolic expression p-GSK3 β ($p < 0.05$), significant up-regulation of soluble Wnts, Wnt2, and Wnt7a, and increased expression of several β -catenin targets involved in tumor promotion and progression, cyclin D1, c-myc, WISP1, and MMP7 ($p < 0.05$). These data suggest that chronic EtOH consumption activates the Wnt/ β -catenin signaling pathway, which increase hepatocyte proliferation thus promoting tumorigenesis following an initiating insult in the liver.

Keywords

DEN; hepatocarcinogenesis; alcohol; Wnt signaling; β -catenin; proliferation

Introduction

Cancer progression in an alcoholic liver occurs through the complex interaction of initiating and promoting mechanisms [1,2]. Experimentally, ethanol (EtOH) feeding induces the hepatic alcohol metabolizing enzyme, cytochrome P450 (CYP) CYP2E1. Increased CYP2E1 activity produces reactive oxygen species and lipid peroxidation products, as well as activates environmental pro-carcinogens, i.e. dietary nitrosamines and polycyclic hydrocarbons, all of which damage DNA, increase mutagenicity, and initiate

hepatocarcinogenesis in rodent models. EtOH metabolism by CYP2E1 and by alcohol dehydrogenase produce the reactive metabolite acetaldehyde, and reduces DNA methylation as a result of disruption of one-carbon metabolism, which may also contribute to tumor initiation [3]. In addition to these initiating mechanisms, chronic EtOH feeding also increases hepatocyte proliferation in animal models of alcoholic liver disease and HCC [4–9]. Several signaling pathways have been implicated in this process, one of which is decreased retinoic acid receptor (RAR) signaling resulting from vitamin A depletion in alcoholic livers [6]. Down-regulation of RAR signaling in EtOH-treated mice has been reported to increase expression of Wnt signaling targets, cyclin D1 and cJun [6]. Interestingly, a study using transgenic mice expressing a liver specific, dominant-negative form of RAR, the loss of RAR signaling also resulted in subsequent up regulation TCF-4/ β -catenin regulated targets such as cyclin D1, increased hepatocyte proliferation, and increased tumor incidence [10]. Clinically, a significant proportion of hepatocellular carcinomas (HCC) including those from alcoholics have been shown to be β -catenin positive [11]. In these tumors, aberrant activation occurs through mutations found either in the β -catenin gene, primarily in the phosphorylation site for GSK3 β , or in Axin1/2, a scaffolding protein necessary for targeting β -catenin for degradation [12,13]. However, studies using transgenic mice expressing mutant forms of β -catenin have shown that activation through mutations is not sufficient to initiate tumorigenesis, and suggest instead that Wnt/ β -catenin signaling participates in tumor promotion [12].

These data support a hypothesis linking EtOH-mediated loss of retinoic acids and RAR signaling, activation of Wnt/ β -catenin pathways and tumor promotion. In the present study we chronically fed a LieberDeCarli EtOH liquid diet to a diethylnitrosamine (DEN)-initiated HCC mouse model for 4 months to test if long-term EtOH ingestion increases tumor multiplicity through increased Wnt/ β -catenin signaling. At the same time, we utilized liver tissue from a previously published rat model of long-term, chronic alcoholic liver disease [8] to mechanistically validate the hypothesis that chronic EtOH ingestion alone increases hepatocyte proliferation through increased Wnt/ β -catenin signaling, resulting in increased expression of known progression markers of HCC.

Materials and Methods

In vivo model of EtOH promotion of DEN-induced hepatocarcinogenesis

C57Bl/6 male mice (n=51) received an i.p. injection 10 mg/kg DEN in saline on postnatal day (PND) 14. The mice were weaned on PND28 and maintained on rodent chow until PND53 at which the mice were randomly assigned to three weight-matched diet groups: a standard chow diet (n=17, chow+DEN), an EtOH-containing LieberDeCarli liquid diet (n=15, EtOH+DEN), and a corresponding pair-fed (PF) control LieberDeCarli diet (n=18, PF+DEN). PF and PF+DEN mice were fed the Lieber-DeCarli control diet (Dyets#710027), which were isocalorically matched to their corresponding EtOH group based on the diet consumptions of the previous day. At sacrifice, mice livers were perfused with 10% neutral buffered formalin for in situ fixation [14]. Liver samples acquired from a previously published rat model of alcoholic liver disease were also analyzed in this study [8]. Blood alcohol concentrations were analyzed using an Analox analyzer as previously reported [15].

Pathological evaluation

For each mouse, formalin fixed lobes were separated, embedded into paraffin, sectioned at 4 μ m, stained with H&E, and examined under a light microscope and scored by a veterinary pathologist. Within each lobe, lesions were counted at 40x magnification. Foci and tumors were defined as follows: basophilic focus, a non-compressive lesion less than the width of 4 hepatic lobules in which hepatocytes are smaller and stain more basophilic than normal; eosinophilic focus, a non-compressive lesion less than the width of 4 hepatic lobules in which hepatocytes are slightly larger than normal with more acidophilic cytoplasm; adenoma, a compressive lesion of any size without evidence of invasion or other criteria of malignancy; and hepatocellular carcinoma, a compressive and invasive lesion with criteria of malignancy[16].

Immunohistochemistry

β -catenin expression was assessed in paraffin-embedded mouse liver sections by immunohistochemistry as using a monoclonal β -catenin antibody detecting the active, dephosphorylated (Ser37 or Thr41) form (Anti-Active- β -Catenin, clone 8E7, EMD Millipore, Billerica, MA) as previously described[14]. Quantification of β -catenin staining was achieved by color deconvolution using Aperio Technologies Spectrum analysis algorithm package and ImageScope analysis software. Hepatocyte proliferation was also measured by immunohistochemical analysis of proliferating cell nuclear antigen (PCNA) staining as described previously [8]

Retinoid extraction and LC/MS/MS analysis

Retinoids were extracted from control and EtOH-treated rat livers as previously described[14]. Retinoid extracts were separated by HPLC using an Agilent 1100 HPLC system (Agilent Technologies, Santa Clara, CA) using Phenomenex Synergi 4 μ Max-RP 80A column (4 μ m, 3 $\times$ 150 mm) as previously described [6]. Retinol and retinoic acid were identified using a 4000 Q TRAP mass spectrometer (Applied Biosystems) coupled with the HPLC. The mass spectrometer was controlled using Analyst version 1.5.1 software and was operated in a MRM mode.

Protein isolation and Western blotting

Nuclear and cytosolic protein fractions were isolated from TEN control and EtOH-treated rat livers (Ronis et al., 2011) using NE-PER Nuclear and Cytoplasmic Extraction reagent kit (Thermo Fisher Scientific) as per manufacturer's instructions. Proteins (30 μ g) were separated by SDS-polyacrylamide gel electrophoresis using standard methods. Blotted cytosolic proteins were incubated with either an anti-active β -catenin antibody used in immunohistochemistry, or with a polyclonal antibody recognizing the phosphorylated form of GSK3 β , (Phospho-GSK-3 α/β (Ser21/9), Cell Signaling Technology, Beverly, MA). Total nuclear β -catenin expression was determined using standard procedures and the anti-active β -catenin antibody previously described [14]. Protein bands were quantified using a densitometer and band densities were corrected for total protein loaded by staining with 0.1% amido black.

Gene expression

RNA was isolated from livers from a previously published rat model of alcoholic liver disease [8]. Samples from the control and EtOH groups were combined separately into $n = 3$ pools/treatment, reversed transcribed using iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA), and then analyzed for gene expression using a WNT signaling pathway RT² profiler PCR array (#PARN 043Z, Sabiosciences, Qiagen, Valencia, CA) following manufacturer's instructions. Array gene expression was confirmed by real time RT-PCR of individual cDNA samples from each group ($n=9$) using SYBR green and an ABI 7500 sequence detection system (Applied Biosystems, Foster City, CA).

Data and statistical analysis

Data presented as means $\pm$ St.Err. Comparisons between two groups were accomplished using either Student's T-test or Mann-Whitney U rank-sum test. Number of lesions was compared across groups using negative binomial regression which generalizes Poisson regression to account for over-dispersion of the count data as previously described [14]. Continuous outcomes, such as PCNA and β -catenin were compared across groups using non-parametric Kruskal-Wallis rank test, and significant findings were followed by Bonferroni adjusted Wilcoxon/Mann-Whitney U rank-sum test for *post hoc* comparisons. Statistical analysis was performed using the SigmaPlot software package 11.0 (Systat Software, Inc., San Jose, CA) and Stata statistical software 13.1 (Stata Corporation, College Station, TX). Statistical significance was set at $P < 0.05$.

Results and Discussion

It is clear that alcohol consumption can result in the initiation and progression of HCC. In a recent case-control study involving U.S. HCC patients, heavy alcohol consumption (80g daily) was a primary contributing factor for one-third of the reported cases [17]. It was also reported that chronic drinking combined with other risk factors, particularly HCV/HBV infections or diabetes mellitus, increased risk an additional 2-fold. Although mechanisms underlying alcohol induced initiation have been well characterized, alcohol related signaling pathways involved in tumor promotion and progression are poorly described. In this study, we developed a mouse model of tumor promotion using DEN (i.p. 10mg/kg at PND13) as the initiating agent, followed by chronic EtOH feeding for 16 weeks. To eliminate any possible EtOH-associated initiating effects, primarily through increased CYP2E1 expression and activity in response to EtOH feeding, liquid diets were started 7 weeks post DEN injection. EtOH was added to the Lieber-DeCarli liquid diet by slowly substituting carbohydrate calories for EtOH calories in a stepwise manner until 28% total calories were reached, which constitutes a final EtOH concentration of 5.0% (v/v) respectively, and was maintained until sacrifice. On average, the EtOH+DEN group received 21g/kg/day of EtOH, which resulted in a blood alcohol concentration of 75 ± 29 mg/dL (range 13–393) at sacrifice. Histological analysis was performed using hematoxylin and eosin (H&E)-stained liver sections for each mouse receiving the chow+DEN, PF+DEN, and EtOH+DEN diets. Overall, EtOH-feeding significantly increased the total number of cancer foci, which encompassed basophilic and eosinophilic foci and adenomas, present in the EtOH+DEN group compared to the PF+DEN and chow+DEN group (Table 1). The number of

carcinomas identified the PF+DEN and EtOH+DEN groups were small and insufficient for statistical analysis. In the EtOH+DEN-treated mice, we also observed a 4-fold increase in hepatocyte proliferation in the non-tumor hepatic tissue as measured by PCNA immunohistochemistry when compared to the PF+DEN, and chow+DEN-treated mice, $p < 0.05$ (Figure 1a). These results are consistent with Yip-Schneider et al. who reported an increase in tumor multiplicity in male alcohol preferring rats receiving EtOH in their drinking water compared to pair-matched rats on water alone [9]. Brandon-Warner et al., also observed increased tumor burden in male DEN-treated mice receiving EtOH in the drinking water, and reported an association between tumor burden and hepatic PCNA and cyclinD1 expression in the EtOH-drinking DEN-treated mice, suggesting an EtOH-related promotional effect [5]. However, unlike these published models, we did not observe a significant increase in tumor incidence in the EtOH+DEN group compared to all other groups (Table 1). In contrast to EtOH feeding, the PF+DEN diet, composed of 47% carbohydrates, 35% fat and 18% protein, increased both adenoma incidence and multiplicity when compared to the DEN+chow fed mice (Table 1). These results are not surprising given that disease progression of alcoholic and non-alcoholic steatohepatitis share many signaling pathways [18;19]. Recent epidemiological studies have also shown that obesity-related diseases like metabolic syndrome, contribute to HCC risk in patient populations [17,20]. However, tumor promoting mechanisms between EtOH exposure and high fat feeding may be different. In this study, tumor incidence corresponded to increased TNF α signaling associated with high fat feeding [14]. In Figure 1a, we observed no significant differences in PCNA staining between the chow+DEN and PF+DEN controls, suggesting that high fat feeding does not increase proliferation as observed in the EtOH+DEN group.

Immunohistochemical staining of β -catenin was significantly increased in the non-tumor hepatic tissue of the EtOH+DEN treated mice in comparison to both the chow+DEN and PF+DEN controls (Figure 1b). Localization of β -catenin expression was different between diet groups. Previously we have reported that DEN alone increased membrane expression of β -catenin in the chow-fed mice, and similar to the chow+DEN group, β -catenin expression remains localized in the membrane of the PF+DEN-treated group [14]. Unlike the other groups, in the EtOH+DEN group we observed β -catenin staining in the membrane, cytosol, and in some areas, nuclear β -catenin accumulation, which is a staining pattern similar to what we observed in tumor tissue [14]. Equally as important, in a separate rodent model of alcohol liver disease [8], prolonged feeding of EtOH alone (TEN+EtOH) also increased nuclear β -catenin expression in rat hepatocytes compared to TEN controls (Figure 2a). For the most part, aberrant β -catenin activity in liver tumors corresponds to mutations in the *β -catenin* gene [13]. Interestingly, the use of Phenobarbital (Pb) as a promoting agent in the DEN-induced HCC mouse model produces a subset of β -catenin positive tumors which contains mutations in exon 3 of *β -catenin* [21,22]. Further analysis of this DEN/Pb model has shown that the chromosomal instability which produces these mutations occurs after 32 weeks of Pb treatment [22]. In our DEN/EtOH model, EtOH feeding occurs over 16 weeks, which suggests the possibility that early exposure to EtOH may activate β -catenin through a different mechanism. In addition to increased β -catenin accumulation in the TEN+EtOH group, we also observed increased cytosolic expression of the phosphorylated (Ser 21/9) GSK3 β , $p < 0.05$, when compared to the TEN controls (Figure 2b). This phosphorylated form

is unable to complex with β -catenin, APC, axin1, thus preventing the targeting β -catenin for degradation [13]. As expected in the TEN+EtOH rats, nuclear β -catenin expression also correlated with a 2-fold increase in mRNA expression of proliferative markers, Ki67 and cyclin D1, (Figure 3a) compared to TEN controls, $p<0.05$, and increased PCNA staining previously reported [8]. In addition, chronic EtOH feeding significantly reduced hepatic retinol and retinoic acid concentrations by 31% and 24% respectively, compared to the TEN control group (Figure 3b). Loss of hepatic retinoid storage is a well-established event in the progression of alcoholic liver disease and HCC development [23,24]. In rodents, Chung et al. reported that EtOH-mediated loss of hepatic retinoids increased proliferation through up-regulation of cyclin D1 [6]. Our current study provides a link between retinoid depletion, up-regulation of Wnt signaling, increased hepatocyte proliferation and tumor promotion in response to EtOH feeding. It also identifies other potential targets that may participate in the promoting effects observed in our DEN-treated mice receiving EtOH, which include mediators of the Wnt signaling pathway, soluble Wnt2 and Wnt7a, transcription factors associated with proliferative signaling pathways, c-fos and c-myc and known β -catenin targets like MMP7 and WISP1 (Table 2).

In conclusion, we have developed a new DEN-induced hepatocarcinogenesis mouse model designed to identify the EtOH-specific mechanisms involved in tumor promotion and progression. In response to EtOH feeding, DEN-treated mice have increased tumor burden compared to DEN-treated chow fed and PF controls, which corresponded to increased hepatocyte proliferation and increased β -catenin expression and nuclear localization in non tumor hepatic tissue. In a separate study, EtOH feeding alone reduced hepatic retinoid concentrations, increased hepatocyte proliferation and nuclear expression of β -catenin, and increased expression of established markers of HCC progression. Clinical administration of synthetic retinoids in primary liver cancer patients has been shown to prevent tumor occurrence, and may also have a chemopreventive effect through restoration of RXR α signaling [24]. We believe future studies using this novel animal model will provide valuable information on the molecular mechanisms whereby EtOH acts as a tumor promoter, and also potentially reveal additional targets in the hepatic Wnt/ β -catenin signaling system for cancer prevention studies.

Reference List

1. Morgan TR, Mandayam S, Jamal MM. Alcohol and hepatocellular carcinoma. *Gastroenterology*. 2004; 127:S87–S96. [PubMed: 15508108]
2. Poschl G, Seitz HK. Alcohol and cancer. *Alcohol Alcohol*. 2004; 39:155–165. [PubMed: 15082451]
3. Seitz HK, Stickel F. Molecular mechanisms of alcohol-mediated carcinogenesis. *Nat Rev Cancer*. 2007; 7:599–612. [PubMed: 17646865]
4. Baumgardner JN, Shankar K, Korourian S, Badger TM, Ronis MJ. Undernutrition enhances alcohol-induced hepatocyte proliferation in the liver of rats fed via total enteral nutrition. *Am J Physiol Gastrointest Liver Physiol*. 2007; 293:G355–G364. [PubMed: 17510198]
5. Brandon-Warner E, Walling TL, Schrum LW, McKillop IH. Chronic ethanol feeding accelerates hepatocellular carcinoma progression in a sex-dependent manner in a mouse model of hepatocarcinogenesis. *Alcohol Clin Exp Res*. 2012; 36:641–653. [PubMed: 22017344]
6. Chung J, Liu C, Smith DE, Seitz HK, Russell RM, Wang XD. Restoration of retinoic acid concentration suppresses ethanol-enhanced c-Jun expression and hepatocyte proliferation in rat liver. *Carcinogenesis*. 2001; 22:1213–1219. [PubMed: 11470752]

7. Isayama F, Froh M, Yin M, Conzelmann LO, Milton RJ, McKim SE, Wheeler MD. TNF alpha-induced Ras activation due to ethanol promotes hepatocyte proliferation independently of liver injury in the mouse. *Hepatology*. 2004; 39:721–731. [PubMed: 14999690]
8. Ronis MJ, Hennings L, Stewart B, Basnakian AG, Apostolov EO, Albano E, Badger TM, Petersen DR. Effects of long-term ethanol administration in a rat total enteral nutrition model of alcoholic liver disease. *Am J Physiol Gastrointest Liver Physiol*. 2011; 300:G109–G119. [PubMed: 21051528]
9. Yip-Schneider MT, Doyle CJ, McKillop IH, Wentz SC, Brandon-Warner E, Matos JM, Sandrasegaran K, Saxena R, Hennig ME, Wu H, Waters JA, Klein PJ, Froehlich JC, Schmidt CM. Alcohol induces liver neoplasia in a novel alcohol-preferring rat model. *Alcohol Clin Exp Res*. 2011; 35:2216–2225. [PubMed: 21790668]
10. Yanagitani A, Yamada S, Yasui S, Shimomura T, Murai R, Murawaki Y, Hashiguchi K, Kanbe T, Saeki T, Ichiba M, Tanabe Y, Yoshida Y, Morino S, Kurimasa A, Usuda N, Yamazaki H, Kunisada T, Ito H, Murawaki Y, Shiota G. Retinoic acid receptor alpha dominant negative form causes steatohepatitis and liver tumors in transgenic mice. *Hepatology*. 2004; 40:366–375. [PubMed: 15368441]
11. Edamoto Y, Hara A, Biernat W, Terracciano L, Cathomas G, Riehle HM, Matsuda M, Fujii H, Scoazec JY, Ohgaki H. Alterations of RB1, p53 and Wnt pathways in hepatocellular carcinomas associated with hepatitis C, hepatitis B and alcoholic liver cirrhosis. *Int J Cancer*. 2003; 106:334–341. [PubMed: 12845670]
12. Nejak-Bowen KN, Monga SP. Beta-catenin signaling, liver regeneration and hepatocellular cancer: sorting the good from the bad. *Semin Cancer Biol*. 2011; 21:44–58. [PubMed: 21182948]
13. Villanueva A, Newell P, Chiang DY, Friedman SL, Llovet JM. Genomics and signaling pathways in hepatocellular carcinoma. *Semin Liver Dis*. 2007; 27:55–76. [PubMed: 17295177]
14. Mercer KE, Hennings L, Sharma N, Lai K, Cleves MA, Wynne RA, Badger TM, Ronis MJ. Alcohol consumption promotes diethylnitrosamine-induced hepatocarcinogenesis in male mice through the activation of the Wnt/ β -catenin signaling pathway. *Cancer Prevention Research*. 2014 (submitted).
15. Mercer KE, Wynne RA, Lazarenko OP, Lumpkin CK, Hogue WR, Suva LJ, Chen JR, Mason AZ, Badger TM, Ronis MJ. Vitamin D supplementation protects against bone loss associated with chronic alcohol administration in female mice. *J Pharmacol Exp Ther*. 2012; 343:401–412. [PubMed: 22892342]
16. Cardiff RD, Anver MR, Boivin GP, Bosenberg MW, Maronpot RR, Molinolo AA, Nikitin AY, Reh J, Thomas GV, Russell RG, Ward JM. Precancer in mice: animal models used to understand, prevent, and treat human precancers. *Toxicol Pathol*. 2006; 34:699–707. [PubMed: 17074738]
17. Hassan MM, Hwang LY, Hatten CJ, Swaim M, Li D, Abbruzzese JL, Beasley P, Patt YZ. Risk factors for hepatocellular carcinoma: synergism of alcohol with viral hepatitis and diabetes mellitus. *Hepatology*. 2002; 36:1206–1213. [PubMed: 12395331]
18. Abdelmegeed MA, Banerjee A, Yoo SH, Jang S, Gonzalez FJ, Song BJ. Critical role of cytochrome P450 2E1 (CYP2E1) in the development of high fat-induced non-alcoholic steatohepatitis. *J Hepatol*. 2012; 57:860–866. [PubMed: 22668639]
19. Farrell GC, Larter CZ. Nonalcoholic fatty liver disease: from steatosis to cirrhosis. *Hepatology*. 2006; 43:S99–S112. [PubMed: 16447287]
20. Welzel TM, Graubard BI, Zeuzem S, El Serag HB, Davila JA, McGlynn KA. Metabolic syndrome increases the risk of primary liver cancer in the United States: a study in the SEER-Medicare database. *Hepatology*. 2011; 54:463–471. [PubMed: 21538440]
21. Aleksic K, Lackner C, Geigl JB, Schwarz M, Auer M, Ulz P, Fischer M, Trajanoski Z, Otte M, Speicher MR. Evolution of genomic instability in diethylnitrosamine-induced hepatocarcinogenesis in mice. *Hepatology*. 2011; 53:895–904. [PubMed: 21374661]
22. Stahl S, Ittrich C, Marx-Stoelting P, Kohle C, Altug-Teber O, Riess O, Bonin M, Jobst J, Kaiser S, Buchmann A, Schwarz M. Genotype-phenotype relationships in hepatocellular tumors from mice and man. *Hepatology*. 2005; 42:353–361. [PubMed: 15965925]

23. Leo MA, Lieber CS. Hepatic vitamin A depletion in alcoholic liver injury. *N Engl J Med.* 1982; 307:597–601. [PubMed: 7202119]
24. Shimizu M, Shirakami Y, Imai K, Takai K, Moriwaki H. Acyclic retinoid in chemoprevention of hepatocellular carcinoma: Targeting phosphorylated retinoid X receptor-alpha for prevention of liver carcinogenesis. *J Carcinog.* 2012; 11:11. [PubMed: 23230390]

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 1.

Quantification of immunohistochemical staining of a) PCNA and b) un-phosphorylated (Ser41/33) β -catenin in paraffin-embedded liver sections from chow+DEN (n=17), PF+DEN (n=18) and EtOH+DEN (n=15) diet groups. Data is expressed as means $\pm$ St. Err. Statistical significance was determined by using Bonferroni adjusted Wilcoxon/Mann-Whitney U rank-sum for post hoc analysis. Groups with different letters are significantly different from each other ($p < 0.05$) [14].

Figure 2.

Chronic alcohol consumption increased a) nuclear expression of β -catenin which corresponded to b) increased cytosolic expression of phosphorylated (Ser 21/9) GSK3 β in the TEN+EtOH-treated rats compared to TEN controls. Statistical analysis between the two groups was performed by Student's T-test, * $p < 0.05$) [14].

Figure 3. Biochemical analysis of hepatic retinoid concentrations, a) retinol and b) retinoic acid in TEN+EtOH-treated rats, and compared to TEN controls. Tissue extraction and quantification of retinol and retinoic acid was performed as previously described. Statistical analysis between the two groups was performed by Student's T-test, * $p < 0.05$ [14].

Table 1

Pathological assessment of tumor foci in Chow+DEN, PF+DEN and EtOH+DEN mice. % incidence = number of tumor-bearing animals/n $\times$ 100 (n = total number of animals per group); multiplicity = total number of lesions/n (n= total number of animals per group). Groups within a column with distinct letter subscripts are significantly different from each other (p<0.05) [14].

Group	Tumor foci		Adenoma	
	% incidence	multiplicity	% incidence	multiplicity
Chow+DEN (n=17)	16/17 = 94 ^a	100/17 = 5.8 ^a	1/17 = 6 ^a	2/17 = 0.1 ^a
PF+DEN (n=18)	18/18 = 100 ^a	209/18 = 11.6 ^b	10/18 = 55 ^b	15/18 = 0.8 ^b
EtOH+DEN (n=15)	16/15 = 100 ^a	266/15 = 17.7 ^c	9/15 = 60 ^b	33/15 = 2.2 ^c

Table 2

Relative expression of Wnt signaling components and β -catenin targets following chronic EtOH consumption in rats using the TEN feeding system. Gene expression was normalized to GAPDH; fold expression, relative to TEN controls, and reported as mean $\pm$ St.Err. Groups within a row with distinct letter subscripts are significantly different from each other. [14].

Gene	TEN control (fold change)	TEN+EtOH (fold change)
Wnt2	1.0 (0.12) ^a	2.6 (0.53) ^b
Wnt7a	1.0 (0.28) ^a	2.8 (0.49) ^b
cJun	1.0 (0.75) ^a	0.7 (0.13) ^a
cfos	1.0 (0.19) ^a	2.0 (0.46) ^b
Tcf3	1.0 (0.86) ^a	1.3 (0.08) ^b
cmyc	1.0 (0.14) ^a	1.5 (0.17) ^b
WISP-1	1.0 (0.38) ^a	3.3 (1.20) ^b
MMP7	1.0 (0.23) ^a	2.6 (0.67) ^b