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Chronic administration of ethanol leads to an increased incidence of hepatocellular adenoma by promoting *H-ras*-mutated cells

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Abstract

This study used tissue samples from male B6C3F1 mice treated with ethanol in drinking water (0, 2.5, or 5%) for 4 or 104 weeks. We tested whether chronic alcohol drinking promotes oxidative stress in the liver and characterized the mutation profile of spontaneous and ethanol-induced tumors. We show that ethanol does not cause detectable oxidative stress in the liver at any time point and acts by promoting *H-ras* mutated cells.

Keywords

alcohol; liver tumors; H-ras; β-catenin; HNF1α

2. Introduction

Excessive consumption of alcoholic beverages is a major public health issue around the world. According to the World Health Organization Global Burden of Disease Project, alcohol drinking accounts for approximately 3.2% of all deaths and 3.6% of all cancers [1]. Since 1988, alcohol (ethanol) drinking has been classified as *carcinogenic to humans* (Group 1) by the International Agency for Research on Cancer [2]. Evidence accumulated in the past decade, especially the data on the carcinogenicity of ethanol in animals [3], prompted the International Agency for Research on Cancer to re-evaluate alcoholic beverages and classify “ethanol in alcoholic beverages” as *carcinogenic to humans* (Group 1) in 2007 [4].

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Conflicts of Interest Statement

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Alcohol consumption has been causally linked to the occurrence of malignant tumors of the oral cavity, pharynx, larynx, esophagus, liver, colorectum, and female breast [4]. The addition of breast cancer and colorectal cancer, two of the most common cancers worldwide, to the list of ethanol-related cancers (4) suggests that the proportion of cancers attributable to alcohol consumption is even higher than previously estimated. Liver is one of key target organs for alcohol-associated disease in humans [5;6]. Liver pathological states that have been causally linked to alcohol consumption include steato-hepatitis (characterized by early steatosis, inflammation, and necrosis), fibrosis and cirrhosis, and hepatocellular carcinoma [7]. With regard to liver tumors, ethanol was long considered to be a co-carcinogen as well as a tumor promoter [8–11] since no experimental evidence was available to demonstrate that ethanol administration alone was sufficient to induce tumors. Only recently have several life-time studies with ethanol in drinking water showed an increased incidence of tumors in the liver in both rats and mice [12–14].

The metabolism of ethanol plays a major role in tumor formation at multiple sites [7]. Ethanol is metabolized to acetaldehyde mainly by alcohol dehydrogenase, catalase and cytochrome P450 2E1 (CYP2E1), followed by oxidation of acetaldehyde to acetate by acetaldehyde dehydrogenase [15]. One of the major negative side-effects in this metabolic pathway is associated with the ability of ethanol to induce CYP2E1, which is known to generate reactive oxygen species [16] that may damage DNA, lipids, and proteins [7;17;18]. Additionally, acetaldehyde, recently classified as carcinogenic to humans when “associated with consumption of alcoholic beverages” [19], reacts with DNA causing the formation of N^2-ethyl-2′-deoxyguanosine (after reduction of the Shiff’s base resulting from acetaldehyde) and 1, N^2-propano-2′-deoxyguanosine DNA adducts [20]. Only limited evidence exists from human studies to confirm these or other modes of ethanol’s carcinogenic action [21;22]. Thus, animal studies where chronic administration of ethanol in drinking-water caused a dose-related increase in the incidence of hepatocellular adenomas in male mice [3;12] provides an important opportunity to understand better the mechanisms of alcohol-induced liver carcinogenesis.

In this study we used normal liver tissue and, when available, liver tumors from male B6C3F1 mice treated with ethanol in drinking water (0, 2.5, or 5%) for 4 or 104 weeks to test whether alcohol drinking promotes oxidative stress in the liver and to characterize better the genetic changes in spontaneous and ethanol-induced tumors. We demonstrate that ethanol does not cause detectable oxidative stress in the liver at either time point. Most of the adenomas and carcinomas observed in the mice from the 2-year study (regardless of treatment with ethanol) were positive for *H-ras* and all were negative for *Hnf1α* mutations. While most of the spontaneous adenomas and carcinomas exhibited β-catenin activation, only a few adenomas (and all carcinomas) in ethanol-treated groups were positive. This suggests that in addition to *H-ras* mutations, mechanisms other than β-catenin activation may be involved in preferential formation of liver adenomas in ethanol-treated mice.

3. Material and Methods

3.1. Samples and study design

Formalin-fixed liver tissues (5 μm sections) were obtained from the archives of the National Toxicology Program 2-year carcinogenicity and toxicity studies with ethanol [3]. Details on animal treatment, necropsy, and histopathological findings were published elsewhere [3;12]. Tissue samples from male B6C3F1 mice that received 0%, 2.5%, or 5% ethanol in the drinking water for 4 or 104 weeks (starting at 28 days of age) were used in this study. All experimental protocols for the *in life* portion of this study were reviewed and approved by the Animal Care and Use Committee at the National Center for Toxicological Research.

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3.2. Immunohistochemistry

Cell proliferation was determined by immunodetection of proliferating cell nuclear antigen (PCNA). Formalin-fixed paraffin-embedded sections of liver tissues were deparaffinized in xylene and rehydrated in graded alcohol series, and placed in phosphate -buffered saline (PBS) with 0.1% Tween 20. Endogenous peroxidase activity was quenched for 10 min with Peroxidase Blocking Reagent (Dako, Carpinteria, CA). Immunostaining was performed using EnVision System HRP (Dako) with a primary monoclonal anti-PCNA antibody (Dako, M0879) diluted (1:200) in PBS containing 1% bovine serum albumin and an incubation overnight at 4°C. Slides were counterstained for 5 min with hematoxylin. Quantitative analysis of immune-stained liver sections was performed by determining the ratio of positive stained nuclei to total nuclei within 10 random fields at 400×.

The extent of oxidative DNA damage was evaluated using goat anti-8-oxodeoxyguanosine (8-OH-dG) antibody (1:200, RDI division of Fitzgerald Industries International, Concord, MA) as previously described [23]. Quantitative analysis of immune-stained liver sections was performed by determining the ratio of positive stained nuclei to total nuclei within 5 random fields at 400×.

The expression of F4/80, tissue macrophage-specific receptor, in liver was evaluated using immunostaining on deparaffinized and rehydrated liver sections incubated in proteinase K (20 μg/ml, Roche Diagnostics, Mannheim, Germany) for 5 min and in Peroxidase Blocking Reagent (Dako) for 10 min at room temperature. Primary rat anti-mouse F4/80 antibody (1:200 dilution, 30 minutes at room temperature, Serotec, Raleigh, NC) and Vectastain Elite ABC-Peroxidase Kit (Vector Labs, Burlingame, CA) were used following manufacturer's instructions. Slides were counterstained for 5 min with hematoxylin. Quantitative analysis of the stained liver sections was performed using Image-Pro® Plus (Media Cybernetics, Silver Spring, MD) software by determining the ratio of positively stained area to total area within 10 random fields at 200×.

3.3. Screening for H-ras codon 61 mutation

Tumors were identified in the formalin fixed and paraffin-embedded liver sections from 0% and 5% ethanol groups and genomic DNA was isolated by digestion with proteinase K, followed by phenol–chloroform extraction and ethanol precipitation as detailed elsewhere [24]. *H-ras* codon 61 mutations were screened using enriched PCR analysis as reported by Mitchell and Warshawsky [25]. Briefly, a primary PCR amplification of tumor DNA was performed and the PCR products were digested with Bcl I restriction endonuclease. Digested PCR products were subject to a secondary PCR amplification, followed by digestion with Bcl I restriction endonuclease. The digested products were separated on an agarose gel by electrophoresis and the mutant bands were sequenced.

3.4. Screening for β-catenin activation and Hnf1α inactivation mutations

Immunostaining was performed on 5 μm sections of formalin fixed, paraffin-embedded liver. Sections were deparaffinized, rehydrated and antigen retrieval was conducted using Target Retrieval Solution (Dako). Slides were microwaved (high power, 5 min, 2 cycles) followed by cooling at room temperature for 20 min. Glutamate-ammonia ligase (glutamine synthetase, Glul) immunodetection was performed using Vector M.O.M. Immunodetection Peroxidase Kit (Vector Labs, Burlingame, CA), following manufacturer's instructions and mouse anti-glutamine synthetase antibody (BD Transduction Lab, BD, Franklin Lakes, NJ) was diluted (1:200) in PBS containing 1% bovine serum albumin and incubation 30 min at room temperature. Liver fatty acid binding protein 1 (Fabp1) immunodetection was performed using a rabbit anti-Fabp1 antibody (Abcam, Cambridge, MA) as previously described [26]. Tumors with homogeneous or heterogeneous glutamine synthetase-positive

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staining were considered to be β-catenin activated, while a lack of Fabp1 staining in tumor tissues signified Hnf1α inactivation.

3.5. Statistical analyses

Statistical analyses were performed using GraphPad Prism5 software (GraphPad Software, San Diego, CA). Qualitative variables were compared with each other in contingency tables by using a Chi-square or Fisher's exact test. Quantitative variables were expressed as mean and standard deviation. The differences between quantitative variances were evaluated by one-way analysis of variances since the variances were equal. Differences in mutation frequency were evaluated using the methodology of Cariello et al [27].

4. Results

4.1. Ethanol effect on cell proliferation in mouse liver

It has been shown previously that sub-chronic (4 weeks) treatment of male B6C3F1 mice with ethanol in drinking water had little effect on cell proliferation and apoptosis in the liver [12]. We also assessed cell proliferation in animals treated with ethanol for 2 years and observed no effect on PCNA staining in non-tumoral liver tissues (Table 1). However, as expected, the number of positive-stained nuclei was markedly elevated in the tumor tissues (data not shown).

4.2. Ethanol effect on liver oxidative DNA damage and inflammation

Treatment with large amounts of ethanol has been shown to cause oxidative stress in liver in rodent models. Traditional models for acute or sub-chronic administration of ethanol in mice or rats, such as liquid diet [28], intragastric intubation [29], oral gavage binges [30], or drinking water [31], have been shown to promote oxidant production in the liver, albeit at very high doses of ethanol. Conversely, sub-chronic administration of low ethanol doses (3% v/v in drinking water) to rats has shown little effect on oxidative stress [32]. The exocyclic DNA adducts, etheno-deoxyadenosine and etheno-deoxycytidine, known to arise from alcohol-induced oxidative stress and lipid peroxidation [33], were not increased in animals treated with up to 5% of ethanol in drinking water for 4 weeks [12]. To evaluate further whether chronic ingestion of ethanol at low dose can lead to oxidative stress and inflammation, we assessed the extent of oxidative DNA damage using 8-OH-dG immunohistochemistry. Ethanol had no effect on the level of 8-OH-dG in nuclear DNA in mice treated for either 4 or 104 weeks (Table 1). In addition, the number of mature macrophages was assessed using F4/80, which indicated no significant differences in the liver macrophage populations between the groups (Table 1).

4.3. Chronic ingestion of ethanol in drinking water leads to a dose-dependent increase in the incidence of liver adenomas in male B6C3F1 mice

Histopathological evaluation and body weights of male B6C3F1 mice treated with ethanol in drinking water for 104 weeks have been reported previously [3;12]. Ethanol had no significant effect on the incidence of non-neoplastic liver lesions or body weight in male mice; however, a significant dose-related increasing trend in liver neoplastic lesions (combined hepatocellular adenomas and carcinomas) was observed [12]. Upon further examination of the individual trends for adenomas and carcinomas (Table 2), we determined that ethanol led to a significant dose-dependent increase in the incidence of hepatocellular adenomas, while carcinoma incidence was not affected, as previously reported [3]. Importantly, the incidence of hepatocellular adenomas in the 5% ethanol group (39.6%) was significantly elevated ($p=0.02$, Fisher's exact test) by more than 2.5-fold compared to incidence of adenomas arising spontaneously in the control group (15.2%).

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4.4. Molecular characterization of the mutation profile of mouse liver tumors

The evaluation of the frequency and spectra of mutations in tumor-related genes in chemically-induced neoplasms is useful for determining whether the carcinogenic effect is due to the chemical or a spontaneous event [34]. We examined several genes that have been reported to contain mutations in liver tumors in mice and humans. *H-ras* and *Catnb* (β-catenin) are frequently mutated in mouse liver tumors. Interestingly, we observed a high frequency of *H-ras* mutations at codon 61 in both spontaneous (control group) and ethanol-induced (5% ethanol) tumors (Table 3), with little difference between frequency of these mutations in adenomas or carcinomas. Next, we compared the spectrum of codon 61 *H-ras* mutations in spontaneous tumors and 5% ethanol-induced tumors. We identified the same 3 substitutions at codon 61 in each group (Figure 1). There was no significant difference (p=0.87) between both spectra; however, we observed a trend for an increase in the number of CAA to CTA transversions and a decrease of CAA to AAA transversions in the tumors from the 5% ethanol group.

The activation of β-catenin in formalin-fixed tissue was assessed by immunostaining for one of its target genes, Glul, (Figure 2, top panel). Almost all of the spontaneous tumors (both adenomas and carcinomas) and ethanol-group carcinomas were positive for Glul; however, the frequency of positive staining was lower in ethanol-treated animals when either all tumors or adenomas were considered (Table 3).

In humans, hepatocellular adenomas frequently exhibit *HNF1A* inactivation mutations [35]. Since ethanol caused a significant increase in the incidence of adenomas, we also tested for this mutation. All tested tumors, as well as non-tumoral liver tissue, were uniformly positively stained for Fabp1 (Figure 2, bottom panel), a target of Hnf1α, suggesting there was no loss of Hnf1α function (Table 3).

5. Discussion

The mode of carcinogenic action of ethanol in the liver is complex and includes multiple molecular events that may lead to tumor initiation, promotion, and progression [7]. Ethanol may act as a co-carcinogen and a tumor promoter; however, several recent experiments performed in rodents that received alcohol in their drinking water for 2 years or longer indicated that ethanol is a complete carcinogen [4;12–14]. While these studies provide evidence for the carcinogenic potential of ethanol, additional research is needed to determine what mechanisms of ethanol-induced liver injury, which have been gleaned largely from studies with high doses and short-term exposures, may also be applicable to the outcomes of chronic low-level experiments. In this study, we performed additional experiments by using formalin-fixed tissues available from male B6C3F1 mice chronically exposed to ethanol in drinking water [12].

While ethanol metabolism is known to induce oxidative stress, we observed little evidence for oxidative stress in the liver. The level of 8-OH-dG adducts, a marker of DNA damage, was not affected by 4 weeks or 2 years of exposure to ethanol, while it has been previously reported to be increased in other studies in both rats or mice [36–39]. When enteral ethanol was administered for 28 days to rats, using a much higher concentration of ethanol as compared to this study, 8-OH-dG adducts were significantly elevated after ethanol [18]. Assunçao *et al.* [40] also observed an increase in the level of 8-OH-dG adducts in rats exposed to ethanol in drinking water, but also with a much higher concentration of ethanol (20%). Similarly to our observation, we reported previously that there was no increase in the level of 1, N^6-ethenodeoxyadenosine or N^2-ethyldeoxguanosine (13), DNA adducts from either acetaldehyde (i.e., N^2-ethyldeoxguanosine) or lipid peroxidation (i.e, 1, N^6-ethenodeoxyadenosine) formed during the metabolism of ethanol. It is possible that the

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methods that were used in this study for detection of oxidative stress may not be sensitive enough to discern a small, yet biologically important effect. A similar challenge exists with other liver carcinogens [41]. Thus, a limitation of this study is in the availability of only formalin-fixed tissue and it may be difficult to find a biomarker or assay with sufficient sensitivity.

The induction of CYP2E1 and activation of Kupffer cells have been suggested as primary sources of oxidants in the liver after ethanol exposure [5;6]. It was previously shown that neither the total cytochrome P450 content, nor the activity of CYP2E1 was affected after 4 weeks of treatment with up to 5% of ethanol [12]. In the present study, there was no effect of ethanol on the number of Kupffer cells after either 4 or 104 weeks of treatment, suggesting that there is no liver inflammation in this model of ethanol carcinogenesis. Finally, since there was no evidence for increased cell proliferation in non-tumoral liver tissues, we posit that ethanol may act by promoting spontaneously initiated cells.

Lifetime carcinogenicity assays in experimental animals also provide an invaluable opportunity to compare rodent tumor's molecular profiles with human malignancies, such as the mutation spectra for known tumor-related genes, thus allowing for a more complete understanding of carcinogenesis in rodents and the potential for extrapolation to human risk. In this regard, it has been reported that the frequency and spectra of *ras* mutations in spontaneous and chemically induced neoplasms extend the knowledge base for understanding the mechanisms of carcinogenesis [34]. Thus, we evaluated genetic alterations in both spontaneous and ethanol-induced tumors to identify whether the spectrum and/or frequency of the mutations can serve as indicators of a genotoxic or non-genotoxic mechanism.

It has been demonstrated that genotoxic hepatocarcinogens frequently increase the incidence of codon 61 of *H-ra*s mutations in tumors, while the frequency is the same or decreased with non-genotoxic hepatocarcinogens [42–44]. In our study, we observed a very high incidence of codon 61 of *H-ra*s mutations in both spontaneous and ethanol-induced tumors while the percentage of mice with tumors, mainly hepatocellular adenomas, significantly increased among mice exposed to ethanol. This observation is similar to that reported with methylene chloride where chemical-induced liver tumors had an *H-ras* mutation profile at codon 61 similar to that of spontaneous tumors [45]. Moreover, it also has been shown in studies with mice that genotoxic compounds have an effect on the distribution of base changes in codon 61 of *H-ra*s mutations, while non-genotoxic compounds have no effect [46–48]. In the present study, there was no significant shift in the frequency of different *H-ras* mutations in ethanol-induced tumors compared to spontaneous tumors, suggesting that ethanol acted as a promoter agent. Thus, the similarities in mutation profile for the *H-ras* gene between spontaneous liver tumors and ethanol-induced ones suggest that ethanol may act in liver by promoting cells with spontaneous DNA lesions.

We also observed a decrease of the incidence of Glul-positive (β-catenin activated) hepatocellular adenomas but not carcinomas in ethanol-treated animals. Since incidence of spontaneous ethanol-induced hepatocellular carcinomas was the same, this result indicates that β-catenin activation is not required for ethanol tumorigenesis. These data further support the effect of ethanol on preferential promotion of *H-ras* mutated hepatocellular adenomas and that ethanol is acting through a non-genotoxic mechanism.

Interestingly, all tumors were positive for Fabp1 staining, suggesting that Hnf1α was still functional and ethanol does not induce *Hnf1α*-mutated adenomas. Thus far, HNF1 inactivation has been found only in human hepatocellular adenomas and not in mice [35;49;50]. In humans, hepatocellular adenomas are relatively rare and preferentially occur

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in women in association with use of oral contraceptives [51]. In addition, a recent analysis of *HNF1A* mutations in human adenomas suggested that occurrence of *HNF1A*-mutated adenomas may be the consequence of a genotoxic damage [52]. Data on *H-ras* mutations in codon 61 and the lack of evidence for Hnf1a inactivation provide additional support to non-genotoxic effect of ethanol.

In conclusion, while chronic exposure to up 5% ethanol in drinking water significantly increased incidence of hepatocellular adenomas in male B6C3F1 mice, oxidative stress and inflammation, pathological features associated with high-dose exposures to ethanol, were not increased. We observed that ethanol exposure was significantly associated with *H-ras*-mutated hepatocellular adenomas and decreased frequency of β-catenin activation mutations, suggesting that increased incidence of tumors in ethanol-treated groups is most likely due to a promotion of *H-ras*-mutated cells by mechanisms other than β-catenin activation.

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Abbreviations

Catnb	β-catenin
CYP2E1	cytochrome P450 2E1
Fabp1	liver fatty acid binding protein 1
Glul	glutamine synthetase
Hnf1α	hepatic nuclear factor 1 alpha
PCNA	proliferating cell nuclear antigen

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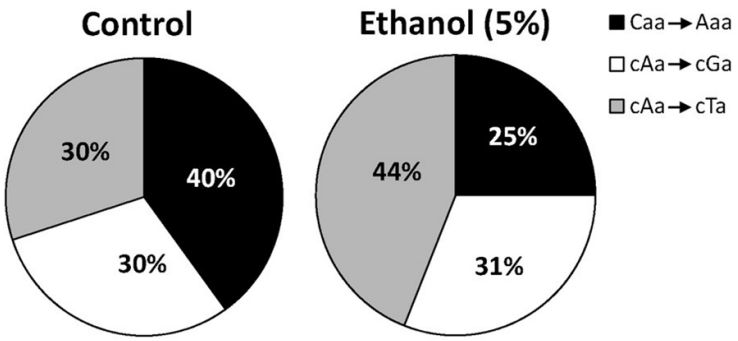

Figure 1. Spectrum of codon 61 *H-ras* mutations in hepatic tumors from male B6C3F1 mice

Sub-types of codon 61 *H-ras* mutations were quantitated in spontaneous tumors arising in animals receiving 0% (left panel) or 5% ethanol (right panel) for 2 years.

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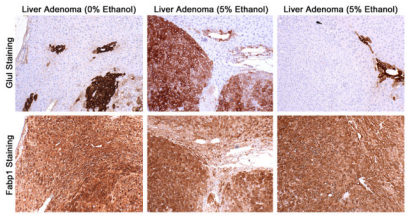

Figure 2. Glutamine synthetase (Glul) and liver-fatty acid binding protein 1 (Fabp1) staining in adenomas and non-tumorous liver tissue from male B6C3F1 mice

Top panel: Liver adenomas (large masses) were positively stained for Glul in many, but not all (top right) animals. In non-tumoral tissues the positive staining is restricted to pericentral hepatocytes. Original magnification: 100×. *Bottom panel*: Serial sections were stained for Fabp1. Uniform staining is evident in both tumorous and non-tumorous tissues.

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Table 1

Sub-chronic and chronic exposure to ethanol in drinking water has no effect on cell proliferation or oxidative stress markers in livers from male B6C3F1 mice.

Treatment Duration	4 Weeks			104 Weeks		
Ethanol dose[a]	**0% (n=4)**	**2.5% (n=4)**	**5% (n=4)**	**0% (n=9–11)**	**2.5% (n=10–13)**	**5% (n=12–15)**
PCNA Staining[b]				3.1±1.6	4.2±1.8	5.0±2.9
8-OH-dG Staining[c]	55.2±7.0	60.0±6.2	58.6±6.1	58.7±5.9	62.2±3.7	61.4±5.6
F4/80 Staining[d]	0.80±0.29	0.46±0.12	0.66±0.34	0.50±0.46	0.57±0.57	0.74±0.47

[a] Concentration of ethanol in drinking water. Numbers of animals in each group/endpoints are shown.

[b] Average number (±S.D.) of positively stained nuclei in 10 microscopic fields (400× magnification).

[c] Average percent (±S.D.) of positively stained nuclei in 5 microscopic fields (400× magnification).

[d] Average area (±S.D.) occupied by positive staining in 10 microscopic fields (200× magnification).

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Table 2

Incidence of liver tumors (hepatocellular adenomas or carcinomas) in male B6C3F1 mice treated with ethanol in drinking water for 104 weeks.

Ethanol dose	Animals with adenomas or carcinomas (%)	Animals with adenomas (%)	Animals with carcinomas (%)	Total number of animals
0%	12 (26.1)†	7 (15.2)†	7 (15.2)	46
2.5%	16 (34.0)†	12 (25.5)†	6 (10.6)	47
5%	25 (52.1)*†	19 (39.6) ‡†	7 (14.6)	48

*An asterisk indicates a significant (p=0.01) difference compared to spontaneous incidence of any tumors in control (0% ethanol) group.

†A dagger indicates a significant (p=0.03) dose-dependent increasing trend.

‡A double dagger indicates a significant (p=0.01) difference as compared to incidence of hepatocellular adenomas in control (0% ethanol) group.

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Table 3

Mutation profile of liver tumors in male B6C3F1 mice treated with ethanol in drinking water for 104 weeks.

	Ethanol dose	All tumors	Hepatocellular adenomas	Hepatocellular carcinomas
H-ras-mutated tumors	0%	10/11 (91%)	5/5 (100%)	3/4 (75%)
	5%	16/20 (80%)	11/13 (85%)	4/5 (80%)
Glul-positive (*β-catenin* activation) tumors	0%	13/14 (93%)	5/6 (83%)	8/8 (100%)
	2.5%	5/11 (45%)*	0/6 (0%)*	5/5 (100%)
	5%	10/21 (48%)*	5/15 (33%)†	5/6 (83%)
L-Fabp-negative (*Hnf1α* inactivation) tumors	0%	0/14 (0%)	0/6 (0%)	0/6 (0%)
	2.5%	0/11 (0%)	0/6 (0%)	0/5 (0%)
	5%	0/21 (0%)	0/15 (0%)	0/6 (0%)

*An asterisk indicates a significant difference from 0% ethanol group ($p<0.05$, Fisher's exact test) when the numbers of animals with/without positive staining were compared within "all tumors" or "hepatocellular adenomas" categories.

†A dagger indicates a marginally significant ($p=0.055$) difference from the corresponding 0% ethanol group.